Elucidating how HIV-1 infection influences CD4, CD8 and regulatory T cell phenotype and function

By

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Abstract

Human Immunodeficiency Virus (HIV) infection is still a global health issue. Although the advent of highly active antiretroviral therapies (HAART) to suppress viral replication has transformed HIV infection from a lethal disease to a chronic and manageable infection, it does not cure HIV. CD8 T cells are the crucial cells in mediating immune responses against viral infections; however, in the setting of chronic conditions such as HIV infection, their function is compromised. One potential mechanism associated with CD8 T cell impairment is the upregulation of several co-inhibitory receptors on their surface. Our group has previously shown that Galectin-9 (Gal-9) on regulatory T cells (Tregs) interacts with TIM-3 on CD8 T cells to render them dysfunctional. Thus, we decided to further study the role of Gal-9 on CD8 T cells in HIV-infected individuals. As a comparison, we also investigated the expression of other co-inhibitory receptors. Our data confirmed a higher proportion of CD8 T cells expressing a wide range of co-inhibitory receptors in HIV-infected individuals. In addition, for the very first time, we observed significant abundance of Gal-9 and VISTA, a recently discovered co-inhibitory receptor, expressing CD4 and CD8 T cells in HIV-infected individuals. We further showed that Gal-9 and VISTA expression on CD8 T cells was associated with a dysfunctional phenotype characterized by lower production of inflammatory cytokines and cytotoxic molecules. In contrast, we found a lower proportion of CD73 expressing CD8 T cells in HIV-infected patients, which was due to a decrease in the CD73 gene expression. Our further investigations to determine the mechanism(s) that result in the downregulation of CD73 revealed that the high plasma ATP in HIV-infected individuals downregulates CD73 through the up-regulation of miR-30b,30c, and 30e in CD8 T cells. CD73 is an ectoenzyme that works in tandem with CD39 to convert ATP to adenosine that has immunomodulatory properties. It also functions as a co-signalling and adhesion molecule on T cells, leading to lymphocyte homing to different tissues, such as the brain and lymph nodes. Since CD4 T cells in the gut and lymph nodes constitute major reservoirs of HIV, the loss of CD73 on CD8 T cells may contribute to HIV persistence due to the limited of CD8 T cell access to the viral

reservoirs. Therefore, our results demonstrate that HIV infection via mechanisms intrinsic to T cells impairs their effector functions. Moreover, to determine the role of regulatory T cells (Tregs) as the most important extrinsic regulatory element of T cells, we investigated transcription profile and functional properties of Tregs in different subpopulations of HIV-infected individuals (e.g.either receiving antiretroviral therapy (ART) or Long-term non-progressors (LTNPs)). Our RNAseq analysis revealed that Tregs exhibit different transcription profiles in HIV-infected individuals compared to healthy controls. While Tregs from patients on ART upregulate pathways associated with a more suppressive (activated) phenotype, Tregs in LTNPs exhibit upregulation of pathways associated with impaired suppressive properties. These observations may identify Tregs as one potential mechanism for the impaired functionality of CD8 T cells in HIV-infected individuals on ART. Thus, we believe that understanding the extrinsic and intrinsic mechanisms that lead to CD8 T cells dysfunction may pave the way for finding potential therapeutic strategies in HIV-infected individuals.

Preface

Research Ethics Approval

This thesis is an original work by Shima Shahbaz. This research project, of which this thesis is a part, received the two ethics approvals indicated below by the Institutional Health Research Ethics Board at the University of Alberta:

1. Investigating Immune Correlates of Protection in HIV infection, Protocol #Pro000046064, valid until 2022.
2. Human Galectin-9 as a Novel Weapon to Reactivate latent HIV, Protocol #Pro000070528, valid until 2022.

In addition, some samples were obtained from the Center for AIDS Research (CFAR)-the University of Washington /Fred Hutch Centre for AIDS Research through an already established Material transfer agreement (MTA).

Contribution of others in this study

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Chapter 1: Introduction

1.1 Human Immunodeficiency Virus (HIV) infection

1.1.1 Epidemiology

Human Immunodeficiency Virus (HIV) infection is still a major global health issue. According to the World Health Organization (WHO) data, around 38 million people were infected with HIV at the end of 2019 (1). However, the rate of infection is different between countries and regions. Africa constituted the most severely affected region, where two-thirds of HIV-infected patients live (1). HIV infection and transmission are also an ongoing public health concern in the western world, such as Canada. According to data published by the Public Health Agency of Canada (PHAC), approximately 62,050 people were infected with HIV by the end of 2018, a 3% increase in the prevalence of HIV infection from 58,291 at the end of 2016 (2). PHAC estimates that 2,242 people were newly diagnosed with HIV in Canada in 2018, representative of an incidence rate of 6 per 100,000 people. This incidence rate is greater compared to similar statistics obtained in 2016, where only 1960 new cases were identified (2).

1.1.2 Transmission

HIV is transmitted through contact with infected body fluids such as blood, semen, rectal and vaginal fluids, and breast milk. It is most commonly spread through three main routes:

- Sexual contact with infected individuals
- Sharing needles with HIV-infected individuals
- Mother to child transmission during pregnancy, childbirth, or breastfeeding (3).

1.1.3 Virology

It is believed that HIV crossed from chimpanzees to humans around 1920 in an area called Kinshasa in the Democratic Republic of Congo (4). In 1982, CDC, for the first time, used the term acquired immunodeficiency syndrome (AIDS) to describe a disorder characterized by severe immune dysfunction

observed primarily in homosexual men (5). In 1984, the cause of AIDS was found to be a retrovirus, which was officially called HIV in 1986 (5, 6).

HIV is a Lentivirus, which belongs to the Retroviridae family (7). The most characteristic features of lentiviral infections are a long duration of the infection and prolonged incubation period (8). HIV viral genome consists of two copies of single-stranded RNA enclosed by a capsid (9). More specifically, the RNA genome is composed of nine genes (pol, nef, gag, tat, rev vif, vpr, vpu, and env) that encode the material necessary for making a new virus (9). The gene env gives rise to glycoproteins gp120 and gp41 that are capsid and matrix proteins, respectively, making up the virus's outer shell (7, 9). The gag gene is involved in creating several structural proteins such as capsid protein, matrix protein, and nucleoprotein (9). The pol gene encodes several enzymes such as reverse transcriptase, integrase, and RNAase that are critical for generating a new virus. The remaining genes (nef, vif, vpr, vpu, tat, and rev) regulate the expression of proteins that control the infectivity, replication, and release of the virus (10).

1.1.4 Mechanism(s) of HIV infection

After HIV enters the body, it mainly infects CD4 T cells because both CD4 and the chemokine receptors CXCR4 and CCR5 are required for the virus to enter the cell (7, 11). Although other CD4 expressing cells such as monocytes, macrophages, and dendritic cells (DCs) are also susceptible to HIV infection (12, 13), these cells do not support HIV replication since there is a lower rate of viral production in these cells compared to CD4 T cells (14, 15). However, these cells have a major role in viral dissemination throughout the body (14, 15). The primary infection of CD4 cells is followed by a period of rapid proliferation during which the levels of viral particles may reach several million per millilitre of blood (16). As the result of viral replication, the number of circulating CD4 T cells drops significantly.

After this primary phase of viremia, CD8 T cells become activated to kill HIV-infected cells. This is followed by the production of antibodies against the virus or seroconversion. CD8 T cells are the most crucial cells in controlling viral replication, as an efficient CD8 T cell response is associated with slower

progression of the disease and better prognosis in HIV-infected individuals (17). Although CD8 T cells' function results in the control of viral replication and subsequent partial recovery of CD4 T cell count, the virus will not be eliminated (17). Eventually, in the absence of antiretroviral therapy (ART), after a latent period (average eight years) which can be different between individuals, the number of CD4 T cells decline substantially. Since CD4 T cells have crucial roles as helper T cells in the recruitment and activation of a variety of immune cells (18, 19) and antibody production (20), their loss results in the development of opportunistic infections and cancers in HIV infected patients.

1.1.5 Clinical manifestations of HIV infection

There are three main stages of HIV infection: Acute (primary) HIV infection, Clinical latent infection and AIDS.

- Acute (primary) HIV infection: Most HIV-infected people develop a flu-like syndrome within 1-2 month after the HIV enters the body, manifested by fever, headache, rash, muscle and joint pain, sore throat, and swollen lymph nodes (LNs) (21).
- Chronic (clinical latent) infection: during this stage, patients have no specific signs and symptoms. However, the virus stays in the body, mainly in white blood cells. This stage usually lasts about ten years in the absence of treatment and can last forever in patients who are receiving treatment (21).
- AIDS: the immune system is seriously impaired at this stage. Patients develop signs and symptoms collectively characterized as AIDS. The hallmarks of this stage are the development of opportunistic infections and cancers (21).

1.1.6 HIV tests

- Diagnosis
  - Anti-HIV antibodies

    The presumptive diagnosis of HIV infection is made by ELISA, which detects antibodies for viral proteins. ELISA has a high sensitivity but low specificity for the diagnosis of HIV infection (22). Consequently, a positive ELISA test should be confirmed with western blot, which also detects antibodies against viral proteins (22). However, both of these tests are often negative in the first 1-2 months after HIV infection, the period which is needed for antibody production against viral particles. Consequently, the diagnosis of acute HIV infection is made with P24 antigen testing or HIV viral RNA (21, 22)

  - HIV RNA

    HIV RNA is usually measured using quantitative polymerase chain reaction (qPCR), nucleic acid amplification, or branched DNA assays. HIV RNA can be detectable 11-12 days after infection (21, 22).

  - HIV P24 Ag

    This test which is positive 14-15 days after infection, measures levels of HIV P24, which is part of *gag* structural protein (21, 22). However, the sensitivity of the test declines within six to eight weeks after infection, when the increase in HIV P24 antibodies results in decreased levels of HIV P24 Ag (21, 22).

- Monitoring
  - Plasma viral load

    Viral load is the preferred test for monitoring the progress of HIV infection (23, 24). Viral load testing should be performed at 6 and 12 months after the start of ART and every 12 months thereafter (24). According to WHO guidelines, viral load >1,000 copies/ml is considered a

treatment failure. If the viral load is >1,000 copies/ml, the patient's adherence to ART should be assessed first, and the test is repeated after 3-6 months. If the viral load remains more than 1,000 copies/ml, a switch to second-line therapy is indicated (24).

- CD4 cell count

  CD4 count should be measured in patients who enter or re-enter to care as a treatment baseline. However, if viral load monitoring is available, monitoring of CD4 cell count can be stopped in patients who have suppressed viral load and are stable on ART (24).

1.1.7 HIV Treatment

- ART

  There are around 30 different medications available for HIV treatment which belong to the following groups (25):

- non-nucleoside reverse transcriptase inhibitors (NNRTIs)
- nucleoside reverse transcriptase inhibitors (NRTIs)
- protease inhibitors (PIs)
- post-attachment inhibitors
- integrase strand transfer inhibitors (INSTIs)
- CCR5 antagonists
- fusion inhibitors

In 1985, the first clinical trials started with NRTIs (25), the first being zidovudine (ZDV) (26, 27). Despite its limited effects on survival, the use of ZDV was approved for patients with advanced HIV infection in 1987 (25). Shortly after, three other NRTIs were approved for use in HIV-infected individuals: didanosine (ddI), zalcitabine (ddC), and stavudine (d4T). The most beneficial effect of the NRTIs therapy was decreased transmission of HIV infection from pregnant women to their children (28). However, since

these drugs were associated with high toxicities, they were usually used alternatively (29). Although alternative use of NRTIs reduced some of the observed adverse effects, this approach was not effective, and patients still had a poor clinical outcome. The next approach was the combination NRTI therapy. While this approach was associated with an increase in CD4 T cell count and improved survival, the results were not durable, and tolerability remained poor (30). The development of NNRTIs and PIs resulted in the next advancement in HIV treatment. The approval of Indinavir, a protease inhibitor in 1996, heralded the highly active antiretroviral therapy (HAART) era (31). In the first International AIDS Society Conference held in Vancouver in 1996, the success of triple combination therapy for HIV infection was reported (25, 32). The conference supported the complete suppression of HIV replication to stop the occurrence of new mutations in the virus. In addition, it emphasized continuous monitoring of treatment by measuring HIV plasma viral load (32). Shortly after the recommendations of the first International AIDS Society Conference were published, effective use of PI-containing regimens resulted in a significant decrease in HIV-infection associated morbidity and mortality (33). In fact, HIV infection, which was the leading cause of death in the industrialized world (34), turned out to become a chronic and manageable condition. However, patients still had low quality of life for some time due to drug toxicities and the need to use multiple doses of a large number of pills. In recent years, the availability of new potent and safer drugs has made progress in ART. Moreover, single tablet, once-daily, fixed-dose combinations have become more available, which result in a higher success rate of treatment due to improved adherence (35–37). With the current advances in ART, except for the patients with low baseline CD4 count, there has been a dramatic improvement in the life expectancy of HIV patients (38, 39).

- Benefits of ART

ART has several goals in HIV treatment. It dramatically reduces HIV viral replication and titer. As a result, the risk of viral transmission drops significantly. Studies have shown that HIV-infected patients on ART with an undetectable viral load are less likely to transmit HIV to other people through sex, sharing needles, and during pregnancy and childbirth (40, 41). Moreover, ART decreases HIV-associated morbidity and mortality (42).

- When to start ART

One of the initial concerns with ART was determining when the initiation of treatment was most beneficial. According to the previous guidelines, ART was initiated in symptomatic HIV-infected individuals and asymptomatic individuals with CD4 counts below a certain threshold level. However, the threshold was different for various guideline and was changing over time (43). Recently, the results from the Strategic Timing of Antiretroviral Treatment (START) trial suggest that HIV-infected people should start treatment immediately after diagnosis (44). This suggestion was based on their observation that early treatment was associated with a 72% and 39% decreased risk of serious AIDS-related and non-AIDS related events, respectively (44). The Association of Medical Microbiology and Infectious Disease Canada (AMMI) supports this notion in their statement: "AMMI Canada lends its support to the recommendation for early initiation of ART in Canada based on the individual, as well as potential public health benefits" (45).

1.1.8 Immune response to HIV infection

1.1.8.1 Innate immune response

Innate immune cells are the first line of defence against the virus after it enters the body.

- Macrophages: Macrophages help clear infectious agents by ingestion and killing. They also present viral particles to T cells via antigen human leukocyte antigens (HLAs) complex. However, in the case of HIV infection, since macrophages are also infected by HIV, they lose their ability to ingest, kill, and present HIV antigens to T cells, which contributes to further immune dysfunction (46).
- NK cells: NK cells attack and lyse cells that have diminished expression of HLA-type I. Because HIV nef protein decreases HLA-I expression on the cell surface, they can be targeted by NK cells (46).

1.1.8.2 Adaptive immune response

- Humoral response: The antibody response to HIV is mainly directed towards viral structural proteins. The antibodies against the core (P24), envelope (gp120 and gp41), and matrix (P17) proteins are detectable in the plasma of HIV-infected individuals within few weeks of infection (47, 48). However, the elicited antibodies don't exhibit efficient neutralizing activity against HIV (49). Although neutralization is the most effective way of antibody-directed response against viral infections, non-neutralizing antibodies can also lead to viral clearance through phagocytosis, activation of the complement system and cytotoxic T cells (50). However, in the case of HIV infection, the role of non-neutralizing antibodies is not yet clear. In addition, some of these antibodies have shown to enhance HIV infection *in vitro*, which may oppose the protective role of these antibodies (50, 51).
- Cellular response
    - CD4 T cells

      CD4 T cells (T helper cells) have the primary role in the adaptive immune system. After recognizing the antigens presented on HLA class II on the surface of Antigen-presenting cells (APCs), they become activated and help to activate or regulate other immune cells

as well by the release of cytokines. CD4 T cells have an essential role in the activation of B cells, CD8 T cells and phagocytic cells, such as macrophages (18). Although CD4 T cells are the target cells in HIV infection, they maintain their functionality in acute phase of infection. However, as the infection progresses, CD4 T cells' response decreases, resulting in the progressive inability of the host to control HIV infection (52, 53)

- CD8 T cells

  CD8 T cells or cytotoxic T cells (CTL) are T lymphocytes that kill virally infected or cancerous cells or cells that are damaged by other causes. Binding of T cell receptor (TCR) on CD8 T cells to its specific antigen on the HLA class I molecule in the presence of co-stimulatory signals results in CD8 T cell activation. Upon activation, CD8 T cells use several mechanisms to kill target cells, mainly through the production of cytotoxic molecules such as perforin and granzymeB (GzmB) (54). Granzymes are serine proteases that are stored within cytotoxic granules in CD8 T cells and NK cells (55). There are five types of granzymes in human (A, B, H, K, and M), with the GzmB being the most focus of studies. This is due to the presence of GzmB in mice, humans, and rats, three species that are primarily used in research and its high expression in both NK cells and CD8 T cells. Granzymes are proteases that induce apoptosis in target cells through several mechanisms, including the activation of pro-apoptotic proteins, DNA fragmentation, and release of reactive oxygen species (ROS) (55, 56). Granzyme mediated apoptosis is carried out by a group of enzymes known as caspases that are found as zymogens that are activated upon the cleavage of their aspartic residues. GzmB activates caspases 3, 7, 8, and 10, with caspase 3 having the most critical role in CD8 T cells mediated cytotoxicity (57–59). Caspase 3 activation results in cleavage of other substrates such as Inhibitor of Caspase Activated DNase (ICAD), Poly (ADP-ribose)

Polymerase (PARP) and gelsolin, which result in DNA fragmentation, inhibition of DNA repair, and cytoskeletal changes, respectively (60–62). GzmB also induces target cell apoptosis through a caspase-independent mechanism by activating Bid, a pro-apoptotic protein. Bid activation results in target cell necrosis and death through mitochondrial outer membrane permeabilization (MOMP) (59, 63) with subsequent release of other pro-apoptotic factors such as second mitochondrial activator of caspases (SMAC) and cytochrome c (59, 64, 65).

Perforin is a glycoprotein that forms membrane-spanning pores through oligomerization in the presence of neutral PH condition and calcium (66). The inactivity of perforin under the acidic environment of granules prevents the degradation of the storage granules that contain the cytotoxic mediators (66, 67). The previous assumption was that perforin creates large pores in the plasma membrane that allow the passage of granzymes to the cytosol (67, 68). However, recently this model has been criticized mainly due to two reasons. First, the concentration of perforin used in these studies to create large pores to permit the passage of granzymes is not physiologically relevant and results in necrosis of the target cells through the loss of membrane integrity rather than granzyme-induced apoptosis (69, 70). Second, the pores that are created in the plasma membrane by physiologic concentrations of perforin are too small to allow the entry of granzymes to the cells (71, 72). It is now accepted that perforin mediated pores allow the passage of small molecules such as calcium to the target cells. The calcium influx triggers a repair process in the membrane which induces endocytosis of the damaged membrane compartments with the uptake of granzymes and perforin into a big endosome. This is followed by the formation of perforin-induced pores in large endosomes to allow the passage of granzymes into the

cytosol to perform its pro-apoptotic function (69). Thus, granzymes need to be co-endocytosed with perforin to mediate their function (73).

- T cell exhaustion

  CD8 T cells lose their effector function in the setting of persistent antigen stimulation, such as chronic infections and cancers (Fig. 1). This phenomenon is accompanied by enhanced expression of various co-inhibitory receptors, such as programmed cell death protein 1 (PD-1), T cell immunoreceptor with Ig and ITIM domains (TIGIT), and T-cell immunoglobulin and mucin-domain containing-3 (TIM-3) on the surface of CD8 T cells (74) (Fig. 2). Although transient elevations of these co-inhibitory receptors occur in the setting of acute infections, persistent elevation of multiple co-inhibitory receptors is the hallmark of T cell exhaustion (75). These co-inhibitory receptors interact with their ligands expressed on various immune and non-immune cells to deliver inhibitory signals to CD8 T cells (74, 75). The pathways that lead to CD8 T cell dysfunction are different for each co-inhibitory receptor.

Fig 1. Schematic presentation of CD8 T cells exhaustion. CD8 T cell exhaustion is accompanied by hierarchal loss of function of CD8 T cells and up-regulation of multiple co-inhibitory receptors (Adopted from Okoye et al. Frontiers in immunology, 2017).

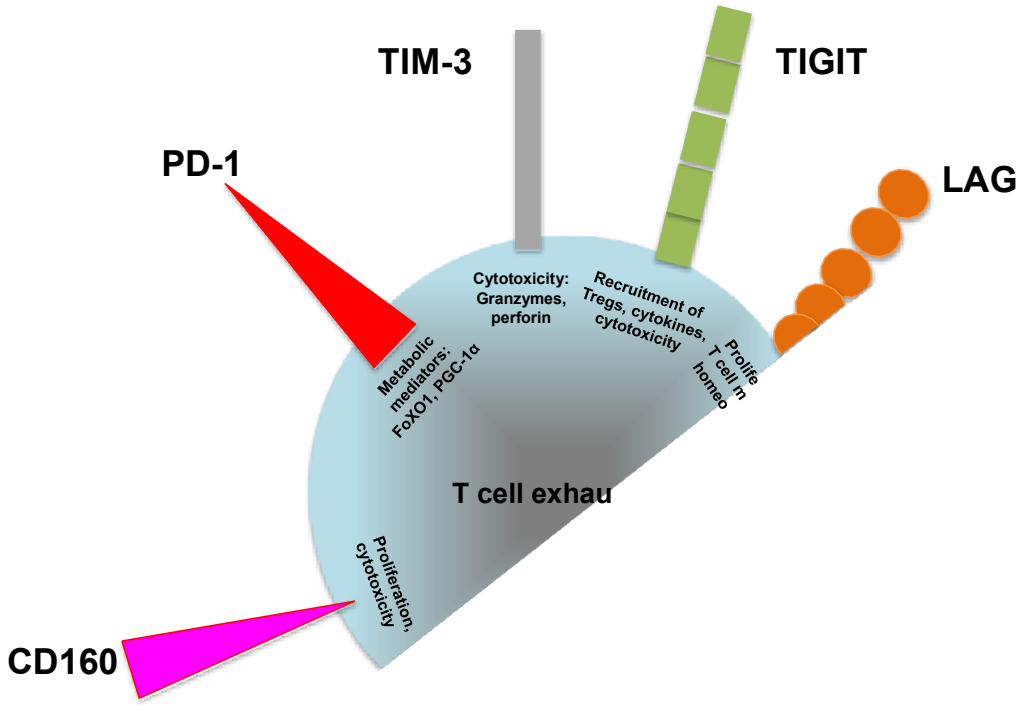

Fig 2. The diagram shows several co-inhibitory receptors that are associated with T cell exhaustion (Adopted from Okoye et al. Frontiers in immunology, 2017).

- PD-1: The expression and continuous upregulation of PD-1 on antigen-specific CD8 T cells is the main indicator of CD8 T cell exhaustion in various chronic conditions such as cancers and viral infections. It has been shown that HIV-specific CD8 T cells in HIV patients not receiving ART exhibit higher expression of PD-1 on their surface. In addition, the level of PD-1 correlates positively and negatively with the plasma viral load and CD4 T cell count, respectively (76). PD-1 interacts with its ligands PD-L1 and PD-L2 expressed on antigen

presenting or tumor cells and mediates T cell exhaustion through several mechanisms, including induction of genes with inhibitory functions such as BAFT (77) and inhibition of costimulatory molecule CD28 in CD8 T cells (78). In addition, the intracellular domain of PD-1 has an immunoreceptor tyrosine-based switch motif (ITSM), which recruits tyrosine-protein phosphatase SHP1 and/or SHP2. SHP1 and SHP2 further inhibit many activating signalling cascades in CD8 T cells (74).

The expression of PD-1 on exhausted CD8 T cells is regulated by various transcription factors to control immune response to antigen stimulation. One of the described mechanisms is that increased antigen stimulation impairs the activity of mechanistic target of rapamycin (mTOR) and AKT (protein kinase B) in exhausted CD8 T cells with a concomitant raise in the expression of FoxO1(79). FoxO1 promotes the expression and maintenance of PD-1 on exhausted CD8 T cells (80). Manipulation of this pathway and other pathways that regulate PD-1 expression can be used to promote CD8 T cells responses in chronic conditions.

- TIGIT: It has been shown that the expression of TIGIT on CD8 T cells is associated with their impaired ability to control viral replication (81). Additionally, the frequency of TIGIT+ CD8 T cells correlates directly with disease progression in HIV-infected individuals (81). Notably, the TIGIT+ CD8 T cells co-express PD-1 particularly in patients with poor control of HIV infection and the frequency of PD-1+TIGIT+ CD8 T cells showed positive and negative correlation with plasma viral load and CD4 T cell counts, respectively (81). Moreover, compared to PD-1+ or TIGIT+ CD8 T cells, TIGIT+PD-1+ $CD8^+$ T cells

exhibited impaired production of TNF-α, IFN-γ, IL-2 (81). While blockage of both TIGIT and PD-L1 *in vitro* improved the proliferative capacity of antigen-specific CD8 T cells in chronically infected patients, coblockade of PD-L1 and TIGIT *in vivo* only resulted in an increased in the production of IL-2 by antigen-specific CD8 T cells (81). These results demonstrate that co-inhibitory molecules use various pathways to exert their suppressive function on CD8 T cells.

- TIM-3: The role of TIM-3 on CD8 T cells from HIV-infected individuals has been the subject of different studies. One study showed that TIM-3 expression increases on CD8 T cells from HIV-infected individuals and the level of TIM-3+ CD8 T cells correlates positively with the viral load and inversely correlates with absolute CD4 T cells count. The authors further showed that there is a distinct population of TIM-3+ PD-1- CD8 T cells in chronically HIV-infected individuals. However, they didn't investigate whether TIM-3+ PD-1+ CD8 T cells exhibit a more exhausted phenotype compared to TIM+PD-1- CD8 T cells. Finally the authors showed that the blocking of TIM-3 interaction with Gal-9 using soluble TIM-3 could reverse the impaired functionality of HIV-specific CD8 T cells (82).

  TIM-3 interacts with its ligand Galectin-9 (Gal-9) on lymphocytes and other cells to deliver inhibitory signals to CD8 T cells (83). The cytoplasmic tail of TIM-3 contains five tyrosine residues, including Y256 and Y263, that can interact with tyrosine kinase FYN and HLA-B-associated transcript 3 (BAT3). In the absence of the TIM-3 ligation, Bat3 binds through Y256 and Y263 to the TIM-3 cytoplasmic tail and recruits Lck, thus preserving and maintaining T cell

signalling. On the contrary, Gal-9 binding to TIM-3 leads to Y256 and Y263 phosphorylation and release of Bat-3 from the TIM-3 cytoplasmic tail with subsequent FYN signalling and T cell inhibition (84, 85).

1.1.9 Long-term non-progressors (LTNPs)

LTNPs are a rare group of HIV-infected individuals capable of maintaining a low viral load and high CD4 count in the absence of ART. LTNPs constitute less than 1-5% of HIV-infected patients (86) and are defined as patients who have been infected with HIV for more than 11 years, with a consistent CD4 T cell count of over 500 cells/µl or CD4% over 28% and viral load <10,000 copies/ml in the absence of ART (87). Although most LTNPs remain symptom-free in the absence of ART for decades and do not progress to AIDS, some defined as delayed progressors (DP) may progress to AIDS at some point mainly due to a viral mutation or immune evasion (87, 88). There are several factors associated with the LTNP phenotype, both related to the virus and host. However, most studies support that host-related factors mainly contribute to viral control.

- Viral-associated factors

  The consideration of viral related factors as the cause of non-progression originates from one of the first studies in Sydney Blood Bank Cohort (SBBC), where a single LTNP donor was the source of infection for all infected subjects via blood transfusion (89). Full HIV genome sequencing of the infected individuals revealed a highly attenuated deletion in nef/long terminal repeat (LTR) (90). However, genome sequencing of viral isolates from other cohorts of LTNPs has shown that LTNPs are rarely infected with the nef-deleted, less virulent strains of HIV (91, 92). In fact, many LTNPs are infected with replication-competent strains of the HIV virion (93, 94). Other studies revealed that *Nef* proteins in viruses obtained from LTNPs are defective in enhancing viral replication and viral infectivity (95). Other reports have shown decreased function of *nef* and *vif* due to a premature stop codon in LTNPs (96). Moreover, *env* gene in LTNPs can have subtle

differences from chronic progressors, which results in the reduced viral capability to enter the host cells (97).

- Host-associated factors

  Both host genetic and immune components can contribute to the non-progression phenotype. For example, specific genetic polymorphisms have been reported in the CCR5 gene locus. These polymorphisms result in the expression of a truncated protein of CCR5 that cannot exit the endoplasmic reticulum with a subsequent lack of CCR5 expression on the surface of the cells (98–100). In addition, the presence of several HLA alleles in the host have been associated with protection against HIV infection, the most important being HLA-B*27 and HLA-B*-57 (101). These HLAs are called protective HLA alleles. The protective alleles present highly conserved HIV capsid protein antigens located on *gag* peptide (102). Fore example, HLA-B57 presents amino acids (aa) 240 to 249 (103), but HLA-B27 presents aa 263 to 272 of the gag peptide (104). For a viral escape to occur, several mutations are required in these epitopes, resulting in a low probability of the viral escape (102). Furthermore, HIV specific CD8 T cells in LTNPs exhibit high proliferative capacity and polyfunctionality, characterized by a higher production of inflammatory cytokines such as TNF-α, IFN-γ, and IL-2 and cytotoxic molecules (e.g. GzmB and perforin)(105, 106). Although the reason for the high polyfunctionality of CD8 T cells in LTNPs is not well-defined, the loss of polyfunctionality is associated with progression to AIDS in these patients. For instance, CD8 T cells restricted by HLA-B27/B57 alleles when encountering their cognate epitope do not express TIM-3, while CD8 T cells restricted by non-protective alleles (e.g. HLA-A03, A02, B08, etc.) do. As a result, CD8 T cells expressing TIM-3 will be prone to Treg-mediated suppression via TIM-3: Gal-9 (107). Thus, CD8 T cells restricted by protective HLA alleles maintain their functionality through the course of HIV infection by resisting Treg mediated suppression (87).

The role of CD4 T cells in non-progression has also been investigated in different cohorts. It has been shown that CD4 T cells from LTNPs are more resistant to HIV infection through the up-regulation of cyclin-dependent kinase inhibitor P21 (108). P21 upregulation results in less susceptibility of CD4 T cells in LTNPs to HIV infection due to less effective reverse transcription of viral RNA and impaired mRNA transcription of proviral DNA (108).

Unfortunately, some of LTNPs may progress to AIDS over time in the absence of ART (88). Several mechanisms have proposed for disease progression in this subset of LTNPs. As mentioned above, HLA-B27 recognizes a highly conserved epitope, but an uncommon point mutation from arginine to lysine/glycine at residue 264 results in disease progression by escaping from appropriate CD8 T cell response (104, 109). Furthermore, our group has shown that CD8 T cells restricted by protective alleles become more susceptible to Treg mediated suppression over time in DPs (87). The mechanisms of suppression is related to the upregulation of TIM-3 on CD8 T cells which enables Treg-mediated suppression of CD8 T cells via TIM-3: Gal-9 (87).

1.2 Galectin-9 (Gal-9)

1.2.1 Structure and function

Gal-9 is a member of the β-galactoside-binding lectin family of galectins, which is ubiquitously expressed in different tissues and cells, including various immune cells (B cells, T cells, and mast cells) (110). More specifically, Gal-9 is a 34-39kDa protein that has two carbohydrate recognition domains (CRDs) connected by a linker peptide (111). There are three isomers of Gal-9 (Gal-9 short, medium, and long) that differ in their length of linker peptides, which accounts for the observed range in the molecular weight of this protein (112). Gal-9 binds to various receptors such as protein disulfide isomerase (PDI), CD137, CD44, TIM-3, and IgE, (113–116), which results in its diverse roles such as cell adhesion, migration, apoptosis and chemoattraction of the immune cells (111, 115). Also, Gal-9 regulates immune

responses through the induction of Tregs and suppression of Th1 and Th17 cells (117). Gal-9 decreases Th1 and Th17 cells by binding to its receptor, TIM-3, which induces an influx of Calcium with subsequent caspase activation and death of the cells (115).

Gal-9 is synthesized in free ribosomes, and since it lacks the signalling sequence to be directed to the endoplasmic reticulum (ER), it is possibly secreted via a non-classical pathway (111). This ER/Gogi independent pathway of secretion is further verified by the inability of brefeldin A and monensin to block Gal-9 secretion (118).

1.2.2 Gal-9 and HIV infection

Gal-9 has multiple roles in HIV infection. The plasma Gal-9 is elevated in the acute stage of HIV infection, and its plasma level correlates with the viral load (119–121). We have reported that HIV patients with higher viral load (>10,000 copies/ml) had significantly higher levels of plasma Gal-9 compared to those with lower viral load (<10,000 copies/ml) (122). Considering the association of Gal-9 with high viral load, our group revealed that Gal-9 interaction with TIM-3 on the surface of activated CD4 T cells renders them less prone to HIV-1 infection and replication. This results from the down-regulation and up-regulation of HIV-1 coreceptors and the cyclin-dependent kinase inhibitor p21 on activated CD4 T cells, respectively (122). In contrast to activated CD4 T cells, Gal-9 interaction with PDI on the resting CD4 T cells enhances their permissibility to HIV infection (122). We concluded that the effect of Gal-9 on CD4 T cells depends on the stage of the disease and the resting state of CD4 T cells. Resting CD4 T cells express PDI that interacts with Gal-9 to make them more prone to the HIV infection. On the other hand, activated CD4 T cells upregulate TIM-3 which render them less permissive to HIV infection through interaction with Gal-9.

Another study has shown that the recombinant Gal-9 (rGal-9) reverses HIV latency in the J-Lat HIV latency model and in primary CD4 T cells from HIV-infected patients. They further revealed that rGal-9 interacts with N-linked oligosaccharides and O-linked hexasaccharide on the surface of CD4 T cells to

modulate the gene expression of key transcription initiation, chromatin remodelling factors and promoter proximal-pausing that regulate HIV latency (123). Moreover, rGal-9 induces the expression of the host antiviral deaminase APOBEC3G both *in vitro* and *ex vivo* resulting in reduced infectivity of the virus. Thus, while rGal-9 increases the reactivity of HIV infection, it decreases the probability of replenishment of the HIV reservoirs when the latency is reversed (123). The same group showed that Gal-9 modulates HIV transcription via T cell receptor-mediated activation of extracellular signal-regulated kinase 1/2 (ERK1/2) in an Lck-dependent manner (124). This signalling pathway also underlies the pro-inflammatory response and hyperimmune activation observed in HIV-infected patients (124). Understanding the mechanism(s) to force provirus out of its reservoir to be exposed to the immune system or ART is defined as the "shock and kill" strategy (125). This strategy has gained interest recent years as one of the biggest impediments to HIV cure (125). As such, using drugs such as rapamycin that uncouples Gal-9-mediated viral reactivation from unwanted pro-inflammatory effects of Gal-9, might allow utilization of rGal-9 for the reversal of viral latency in HIV-infected patients (125).

1.3 V domain-containing Ig suppressor of T-cell activation (VISTA)

1.3.1 Structure

VISTA, also known as DD1α (126), PD-1H (127), and Dies1 (128), is a member of the immune globulin (Ig) superfamily that has homology to B7 family ligands PD-L1 and PD-L2. Murine VISTA is a type I transmembrane protein with a single extracellular IgV domain linked to a stalk region, a transmembrane segment, and a cytoplasmic domain (129). However, some features of VISTA such as the presence of a long loop in the middle of the C" and D strands, multiple cysteine residues in the Ig-V domain and the stalk region, and the lack of a second Ig domain differentiates VISTA from other B7 family members (129). VISTA is a highly conserved molecule among the B7 members, with Human VISTA having 78% identity with murine VISTA. Human VISTA is a 279 aa protein comprising 162aa, 21aa and 96aa extracellular, transmembrane, and cytoplasmic domains, respectively (130). The cytoplasmic domain of

VISTA lacks immunoreceptor tyrosine-based signalling motifs. Instead, it contains multiple phosphokinase C phosphorylation and casein kinase two sites that could contribute to signal transduction.

1.3.2 Distribution

VISTA is expressed on antigen-presenting cells (APCs) and T cells and functions both as the ligand and receptor (130). As a ligand, VISTA expressed on APCs binds a yet unknown receptor on T cells to inhibit T cell activation (129, 131). As a receptor, VISTA expressed on T cells interacts with its ligands to transduce intracellular inhibitory signals (132). Recently, V-set and Ig domain containing 3 (VSIG3) has been identified as a ligand for VISTA (133), with an anti-VISTA antibody called VSTB blocking VSIG3-VISTA interaction (130).

In Human, VISTA gene transcripts are predominantly higher in hematopoietic cells and tissues that are highly infiltrated with lymphocytes (131). Among immune cells, VISTA is expressed on T cells, CD14+ monocytes, myeloid CD11c+ Dendritic cells (DCs) and neutrophils. However, there is no expression of VISTA on B cells or $CD56^{Hi}$ NK cells (134).

1.3.3 Function

- Role of VISTA in T cell regulation

  The inhibitory role of VISTA on T cells was first shown in acute graft-*versus*-host disease (GVHD) in which agonistic antibodies targeting VISTA inhibited GVHD induction (135). Another study showed that selective agonism of VISTA in donor alloreactive T cells is sufficient to prevent GVHD (136). Of relevance, other studies have found that blockade of VISTA significantly reduced corneal allograft and skin graft survival (137, 138).

  The physiologic role of VISTA on immune regulation has been widely studied in the *Vsir* knockout (KO) mice ($Vsir^{-/-}$ or VKO). VISTA KO mice are born healthy with normal hematopoietic

development but gradually develop spontaneous T cells activation with the accumulation of inflammatory cytokines such as TNF-α, IL-17A and IFN-γ (139). The observed T cell activation in VISTA KO mice suggests that VISTA deficiency has increased the TCR-mediated activation of T cells towards self-antigens. In agreement, breeding of VKO mice with 2D2 TCR transgenic mice (with a TCR that recognizes the self-antigen MOG, which predisposes them to develop experimental autoimmune encephalomyelitis (EAE)) showed that VISTA deficiency results in a significant increase in disease incidence and intensity. Interestingly, EAE development was accompanied by enhanced activation of encephalitogenic CD4 T cell in the peripheral blood and central nervous system (CNS) (139, 140). Similar results were obtained in the concanavalin A (ConA)-induced hepatitis model, in which VKO mice developed a more severe liver injury (132). Since such observations were mediated through pathologic activation of CD4 T cells, these results demonstrate the inhibitory role of VISTA. Such regulatory function of VISTA on T cells is performed through both cell-intrinsic and extrinsic mechanisms.

- The extrinsic roles of VISTA on T cells as a ligand

  The ligand function of VISTA has been investigated in studies with an immobilized ectodomain (ECD) of VISTA, which resulted in suppression of T cell proliferation and inflammatory cytokine production in a dose-dependent manner (129, 131). VISTA-associated suppression of T cells was accompanied by inhibition of phosphorylation of TCR proximal signalling molecules such as LAT and PLC-γ1 and downregulation of several activation markers (e.g. CD25, CD44, and CD69) in T cells (129, 131, 140). It was further showed that VISTA-ECD mediated T cell suppression results from direct inhibition of both naïve and memory T cells and promotion of conversion of FOXP3- CD4 T cells into FOXP3+ Tregs in the presence of TGF-β. These studies using VISTA expressed on APCs or tumour cells as the ligand confirmed the extrinsic roles of VISTA on T cells.

- The intrinsic roles of VISTA expressed on T cells

  The intrinsic role of VISTA has been primarily studied *in vitro* by stimulating wild type (WT) or VKO CD4 T cells with WT or VKO APCs (132). While VISTA deletion on both APCs and T cells results in robust proliferation of T cells, VISTA deficiency on either APCs or T cells results in less robust proliferation of T cells. Moreover, the VISTA-specific mAb MH5A inhibited allogenic T cells response and GVHD progression in the murine model of acute GVHD (135). Interestingly, while MH5A suppressed the proliferation and function of WT donor T cells, it had no effect on VKO donor T cells, suggesting that the expression of VISTA on donor T cells is required for the suppressive function of MH5A Also. MH5A treatment also resulted in the expansion of Tregs and promoted the polarization of naïve CD4 T cells to FOXP3+Tregs *in vivo*. This is in agreement with the role of VISTA in promoting Treg induction in tumour-bearing mice (141, 142).

  However, the downstream signalling pathways of VISTA has not been identified yet. As such, despite the evidence showing that VSIGs can interact with VISTA, whether and how their interactions lead to T cells suppression has not yet been determined (130).

- The role of VISTA in tumour progression

The role of VISTA in tumour progression has been well studied. For example, in gastric tumour tissues, the high expression of VISTA was reported on tumour-infiltrating lymphocytes (TILs) (143). Moreover, VISTA expression was correlated with tumour localization, Lauren classification, Epstein-Barr virus infection, PD-L1 expression, and KRAS-and PIK3CA-mutational status (143). Another study in human oral squamous cell carcinoma (OSCC) revealed that the level of the VISTA protein within tumour tissues was increased compared to the normal mucosa (144). In addition, a higher expression of VISTA protein together with the low expression of CD8α within the tumour tissues of OSCC was correlated with poor patients' survival (144).

Another study examined the expression of several co-inhibitory receptors following ipilimumab therapy in human prostate cancer and melanoma. Their results showed a higher percentage of VISTA+ and PD-L1+ TILs and a lower percentage of CD68+ macrophages following therapy, which suggest that both VISTA and PD-L1 contribute to the immunosuppressive tumor microenvironment (145).

A recent study investigated the expression and function of VISTA on immunosuppressive myeloid-derived suppressor cells (MDSCs) and other dendritic cells (DCs) in tumor environment (146). They showed that VISTA is expressed on tumor associated central dendritic cells (cDCs), inflammatory DCs (Inf-DCs), G-MDSCs, M-MDSCs, and CD103+ DCs. (146). VISTA blockade resulted in decrease frequency of both G- and M-MDSCs and expansion of inflammatory DCs within tumor tissue (146).In addition ,VISTA blockade augmented the secretion of inflammatory cytokines such as IL-12p140 from M-MDSCs, cDCS, Inf-DCs, and CD103+DCs (146). Moreover, VISTA blockade reversed the suppressive function of M-MDSCs, Inf-DCs, and cDCs (146). These results demonstrates that VISTA blockade promotes immunity against tumoral cells by reprogramming tolerogenic and DCs within tumor environment (146).

1.4 CD73

1.4.1 Structure and distribution

CD73, known as ecto-5'-nucleotidase (ecto-5'-NT), is a 70-kDa ectoenzyme that exists as a noncovalent dimer on the cell surface (147). Each subunit of the CD73 dimer consists of a N-terminal and C-terminal domain, which are linked by a single α helix that allows the large domain movements and subsequent shift between the closed and open conformations of CD73 (148). CD73 is anchored to the plasma membrane through a glycosyl-phosphatidylinositol (GPI)-anchor, which is sensitive to hydrolysis by endogenous phospholipases (149, 150). CD73 is expressed on various tissues and immune cells,

including neutrophils, monocytes, dendritic cells, myeloid-derived suppressor cells, B cells and T cells (151).

1.4.2 Function

The biological actions of cell membrane CD73 mainly result from the regulated enzymatic activity of CD73 on extracellular nucleotides. This ectoenzyme works in tandem with CD39 to generate adenosine from ATP. While CD39 mediates the conversion of ATP to ADP and then AMP, CD73 converts AMP to adenosine (151).

Extracellular ATP binds to purinergic P2 receptors P2X and P2Y, which are cation-selective receptor channels and G protein-coupled receptors, respectively. These receptors are expressed on different cells, including immune cells, where ATP binding induces pro-inflammatory responses (144). For example, ATP induces necrosis in T cells through P2X7 receptor activation (152). Adenosine signals through four adenosine receptors: A1, A2A, A2B, and A3, with A2A and A2B receptors mediating the suppressor effects of adenosine on neutrophils macrophages, T cells, and other immune cells (153). The A2A receptor (A2AR) is the dominant adenosine receptor on T cells which is induced upon T cell activation (154). Adenosine binding to A2AR inhibits the activation and proliferation of T cells with concomitant decreases in their cytotoxicity and cytokine production (155, 156). Moreover, it has been shown that A2AR stimulation results in increased and decreased production of TGF-B and IL-6 from splenocytes, respectively. This process promotes the formation of Tregs and inhibits the differentiation of naïve T cells to Th17 T cells (157). Another study confirmed this observation in the presence of A2AR agonist, which resulted in the expansion of Tregs with more potent immunosuppressive function (158). Collectively, CD39 and CD73 work together to dampen the immune response by converting highly inflammatory ATP to immunosuppressive adenosine.

However, CD73 was initially described as a lymphocyte differentiation antigen. CD73 functions as a co-stimulatory molecule on T cells, where CD73 cross-linking with monoclonal antibodies in the presence of

PMA or submitogenic doses of anti-CD3 antibody results in human T cell proliferation with subsequent CD25 expression and IL2 secretion (159, 160). This role of CD73 was further confirmed in another study showing that naïve CD8 T cells that express CD73 were less responsive to anti-CD3 activation, which can be overcome through simultaneous cross-linking of CD3 and CD73 by immobilized anti-CD3 and anti-CD73 antibodies (161). Thus, CD73 can reduce the activation threshold of naïve T cells with their first encounter with antigens (162). Also, CD73 is involved in CD8 T cells adhesion to endothelial cells and lymphocyte extravasation in a lymphocyte function-associated antigen-1 (LFA-1)-dependent mechanism (163). This suggests that CD73 may play a role in immune cells movements in the tissue.

1.5 Tregs

1.5.1 Definition and characterization

Immune homeostasis is maintained through several sophisticated regulatory mechanisms, with Tregs playing an indispensable role in preventing aberrant or exaggerated immune responses. Traditionally, Tregs were defined in both humans and mice as CD4 T cells expressing CD25, an IL-2α receptor. However, the fact that human T cells upregulate CD25 upon activation makes CD25 inefficient as the sole marker for human Treg characterization (79). Similarly, although the identification of forkhead box P3 (FOXP3) as a key transcription factor for Treg development and function has assisted in Treg identification in mice, the expression of FOXP3 on non-regulatory activated T cells in humans precludes it as a useful marker for human Tregs (164). Despite these limitations, most studies in human use CD25 and FOXP3 for Tregs characterization. However, the expression of FOXP3 inside the nucleus limits its use for Treg isolation. Further search for Treg-specific surface markers revealed that CD127 (IL-7R) was downregulated on Tregs, suggesting that CD127 can be used for human Treg isolation. In fact, there is a high (~90%) correlation between FOXP3 expression and CD4+ CD25+ CD127-/low T cells (165). In addition, recent studies have shown that compared with activated effector T cells, Tregs express a higher level of folate receptor 4 (FR4) (166). Similar to T-helper-cells, Tregs constitute a heterogeneous

population of cells and can be divided into two main subsets of effector Tregs (eTregs) and central Tregs (cTregs) (167, 168). cTregs and eTregs can be distinguished based on the expression of CD45RA, CD62L, and CCR7 (169, 170). While cTregs are mainly localized in secondary lymphoid tissues, eTregs are primarily found in non-lymphoid tissues and secondary lymphoid organs (168).

1.5.2 Mechanism of action

Tregs use several diverse mechanisms to exert their immunoregulatory properties. These mechanisms can be divided into multiple categories such as the production of inhibitory cytokines, metabolic disruption, induction of cytolysis, DCs suppression, cell to cell interaction, and the expression of T cell subset-specific transcription factors.

- Production of inhibitory cytokines

  Inhibitory cytokines such as TGF-β, IL-10, and IL-35 have essential roles in Treg-mediated immune suppression. These cytokines can suppress both T and B cells by their direct suppression and induction of Tregs and Regulatory B cells (Bregs) (171–174). Moreover, TGF-β and IL-10 also induce Tregs by inhibiting antigenic presentation and the resultant generation of tolerogenic dendritic cells (175–177).

- Metabolic disruption

  The high expression level of CD25 by Tregs empowers them with a competitive advantage for the consumption and subsequent deprivation of effecter cells of IL-2 that they need for survival (178). This mechanism seems more significant for CD8 rather than CD4 T cell suppression, which is due to the different sensitivity of CD4 and CD8 T cells to IL-2 (179). Another mechanism used by Tegs to suppress effector cells is through the expression of CD39 and CD73 and concomitant adenosine production (155). Adenosine inhibits effector T cells mainly through binding to the A2A receptor, which results in the blockade of TCR signalling due to an increase in intracellular cAMP (180). Moreover, activation of T cells in the presence of A2AR agonism leads to Tregs

expansion and the generation of Tregs with higher immunoregulatory activity (181). In addition, Tregs can suppress the function of effector T cells by direct transfer of potent inhibitory cAMP into effector T cells via membrane gap junctions (182).

- Induction of cytolysis

  The first notion that Tregs possess cytolytic function was first reported in a study showing decreased suppressive activity of granzyme-B-deficient mouse Tregs. This Treg dependent suppression of T cells was perforin independent and resulted in Treg-induced apoptosis in target T cells (183). More recent studies have shown that Tregs suppress the killing function of NK cells and CD8 T cells on tumour cells by killing these cells in perforin and granzyme-B-dependent manner (184). In addition to the production of granzyme-B and perforin, Treg-induced apoptosis of target T cells can occur through the TNF-related apoptosis-inducing ligand–death receptor 5 (TRAIL–DR5) pathway (185). Moreover, galectin-1, which induces T-cell apoptosis, is expressed by mice and humans Tregs and plays a role in Tregs' suppressor function (186).

- DCs suppression

  Antigen-specific suppression of Tregs is mainly due to Treg–DC interaction by recognising the antigen (Ag) presented on MHC-II on DCs through Treg TCR. This interaction results in the inability of DCs to present the Ag and induction of Ag-specific tolerogenic DCs. However, the mechanisms of DCs-induced suppression in Tregs are diverse. They include binding of CTLA-4 on Tregs to CD80/86 on DCs (187), trans-endocytosis, which involves removal of MHC-II-Ag from the surface of DCs with preservation of DCs' ability to present other antigens (188), and CTLA-4-mediated increase in indoleamine 2,3-dioxygenase (IDO) DCs, which decreases the tryptophan concentration necessary for effector T cells proliferation (189). Tregs may also inhibit DC maturation through the expression of lymphocyte-activation gene 3 (LAG3 or CD223). LAG3 is a CD4 homologue that binds to MHC class II molecules on the surface of immature DCs, resulting

in the activation of an immunoreceptor tyrosine-based activation motif (ITAM)-dependent inhibitory signalling pathway that suppresses DC maturation and function (190). Altogether, although the mechanism(s) that results in Treg-mediated suppression of target cells through DCs needs to be more elucidated, this mechanism(s) has a pivotal role in suppressing antigen-specific immune responses.

- Cell to cell interaction

  It has been shown that Tregs express PD-L1, which binds to PD-1 on T cells, resulting in SHP-1/2 recruitment and dephosphorylation of STAT molecule in T cells. This results in destabilization of the transcription moleculars of Th1 CD4 T cells and subsequent regulation of Th1 cells (191). In addition, Tregs express Gal-9 on their surface with induces killing of effector CD8 T cells through interaction with TIM-3 on the surface of CD8 T cells (192).

- Expression of T cell subset-specific transcription factors

  Tregs can suppress a particular CD4 T cell subset by the expression of the specific transcription factor of this subset. For example, it has been shown that a Treg subset that overexpressed T-bet in response to IFN-γ had a prominent role for the suppression of Th1-mediated immune responses (193). Similarly, expression of IRF-4 (a transcription factor expressed in Th2 and Th17 cells) in Tregs was required to suppress Th2-medicated immune responses. In contrast, the expression of certain CD4 T cell subset transcription factors in Tregs might result in their loss of FOXP3 expression and suppressive function. For example, STAT3 downregulates FOXP3 expression, and interestingly, STAT3 is upregulated by cytokines that decrease FOXP3 expression and Tregs' function such as IL-6, IL-23, or IL-27 (194–196). Accordingly, loss of expression of STAT3 in T cell transfer-induced colitis in mice increased Treg induction and decreased the severity of the disease (197).

However, the mechanisms of Tregs suppression through CD4 T cell subset transcription factors is not fully understood. It has been shown that transcription factors such as IRF-4, STAT3 and RORγt interact with FOXP3. In addition, these transcription factors regulate the expression of some genes, such as chemokine receptors that enable Tregs to migrate and expand at the site of active immune response (174). In agreement, the suppressive function of IRF-4 Tregs was unimpaired *in vitro*, and GATA-3 was selectively upregulated in Tregs at the site of active immune response (174).

1.5.3 HIV and Tregs

The role of Tregs in HIV infection has been the topic of debates and controversies. Following TCR activation, Tregs up-regulated the expression of the main HIV coreceptors CXCR4 and CCR5, rendering Tregs susceptible to HIV infection *in vitro* (198). However, whether Tregs are more prone to HIV infection compared to conventional CD4 T cells is still debated. First reports suggested that Tregs might be a more susceptible target of HIV compared to memory CD4 T cells (199). However, subsequent studies demonstrated lower expression of HIV-DNA in CD4 CD127low T cells compared to CD4 CD127high T cells (200). Finally, *in vivo* studies revealed no preferential HIV infection of circulating Tregs compared to effector T cells (201).

Similar to the susceptivity of Tregs to HIV, there are also conflicting data on the frequency of Tregs in HIV-infected individuals. In acute HIV infection, the frequency of Tregs among CD4 T cells has been reported to be higher (202) or lower (203) compared with healthy controls (HCs). Moreover, some reports have shown a higher frequency of Tregs in untreated HIV-infected individuals compared to HCs (204, 205) and a direct correlation between Treg frequency and CD4 T cell count (206–208). Other reports suggested a lower frequency of Tregs in chronic HIV-infected individuals (209, 210). Some longitudinal studies have evaluated the effect of ART on Treg frequency in HIV-infected individuals. Although the low number of patients was the limitation of these studies, most studies demonstrated

that ART decreases the frequency of Tregs or even normalizes it to levels similar to that of HCs (205, 211).

Interestingly, this reduction mainly was observed in patients who responded well to ART, with Treg frequency remaining higher in patients with inadequate response to treatment (212). In contrast to progressors, it has been shown that the level of Tregs in LTNPs is similar (213) or even lower than HCs (214). Low frequency of Tregs could contribute to high HIV-specific CD8 T cell responses described in most LTNPs, but also to a higher propensity for immune activation observed in these patients (214).

1.6 Hypothesis and aims

T cell exhaustion is characterized by decreased effector functions of T cells, such as cytokine production, proliferation and cytotoxicity, accompanied by the up-regulation of several co-inhibitory receptors (74). Upregulation of co-inhibitory receptors such as CTLA-4, PD-1, LAG-3, and TIM-3 on T cells in HIV infection with consequent T cell exhaustion is well-defined (215–217). For example, it has been shown that TIM-3 is upregulated on both CD4 and CD8 T cells in HIV-infected individuals, and its upregulation contributes to an exhaustion phenotype (82, 218). Furthermore, in another study, TIM-3 expression correlated positively and negatively with the viral load and CD4 T cell count, respectively (219). As mentioned previously, the plasma level of Gal-9 (the ligand of TIM-3) is significantly higher in HIV-infected patients compared to healthy controls (121, 122). Moreover, our group has shown that Gal-9 interacts with TIM-3 on activated CD4 T cells to render them less susceptible to HIV infection and replication (122). Another study by our group demonstrated that Tregs suppress CD8 T cells through the interaction of Gal-9 with TIM-3 on the surface of CD8 T cells. This study found that upon cognate epitope recognition, CD8 T cells restricted by protective HLA-B27 and HLA-B57 alleles upregulate lower TIM-3 compared to CD8 T cells restricted by non-protective alleles, resulting in their reduced susceptibility to Treg medicated suppression via TIM-3:Gal-9 interactions (192). These results explain why individuals carrying HLA-B27/B57 alleles have more functional CD8 T cells with subsequent natural immunity

against HIV infection. Based on a wide range of evidence, it is clear that Gal-9 has essential roles in HIV pathogenesis (123). In addition, the role of other immune checkpoints such as VISTA in HIV infection was unknown. More importantly, it is unclear how the expression of co-inhibitory receptors impacts persistent viral reservoir, which is a prerequisite to the development of HIV eradication strategies. Based on these observations, we hypothesized to identify undescribed mechanism(s) by which HIV infection impacts T cells phenotype and function. This hypothesis will be tested in the following aims:

***Aim 1*:** To determine the expression of co-inhibitory receptors on T cells from HIV-infected individuals on antiretroviral therapy (ART) versus Long-term Non-progressors (LTNPs).

Hypothesis 1: LTNPs express significantly lower levels of co-inhibitory receptors on their T cells compared to patients on ART, which results in higher CD8 T cell polyfunctionality.

Recent data from both HIV-infected individuals and mice models of chronic infections have shown that the function of virus-specific CD8 T cells appears to be regulated by multiple non-redundant inhibitory pathways that are triggered by distinct co-inhibitory receptors.

***Aim 2*:** To understand the mechanisms associated with lower CD73 expression on CD8 T cells in HIV-infected patients.

Hypothesis 2: Chronic inflammation in HIV-infected individuals downregulates the expression of CD73 on CD8 T cells, which in turn inhibits their access to reservoir niches in tissue.

One of the significant barriers to HIV eradication is establishing a pool of HIV reservoirs in immune cells susceptible to HIV infection. These cells can rarely be found in the peripheral blood in the presence of ART, but they are located in anatomical reservoirs, such as the spleen, lymph nodes, gut-associated lymphoid tissue (GALT) and the central nervous system (CNS) (220). It has been reported that CD73 on lymphocytes facilitates the migration of lymphocytes to the draining lymph nodes via afferent lymphatic vessels (221). Moreover, the expression of CD73 on CD4 T cells is required for the efficient entry of T cells into the CNS during the development of an EAE model (222). These results suggest that CD73 on

CD8 T cells may have a role in their migration to the tissues and subsequently accessing HIV reservoirs. In addition, CD73 works with CD39 to convert ATP to immunosuppressive adenosine. Consequently, lower CD73 observed on T cells in HIV patients can lead to the accumulation of ATP in the plasma of these patients. Increased ATP level contributes to chronic inflammation and occurrence of non-AIDS related morbidities such as cardiovascular diseases and cognitive impairment in HIV-infected patients (223, 224). Thus, finding the mechanisms that result in lower CD73 expression on T cells enables us to discover novel therapeutic approaches to prevent the downregulation of CD73 on T cells from HIV-infected individuals.

Aim 3: Characterization of Treg function and phenotype in HIV-infected individuals.

Hypothesis 3: HIV infection modulates the phenotypical characterization and function of Tregs.

In addition to CD4 expression, Tregs express the main HIV coreceptors CXCR4 and CCR5, making them susceptible to HIV infection (198). Since Tregs can inhibit effector CD8 T cells responses against the HIV antigens, understanding their role in different groups of HIV-infected patients will clarify the extrinsic inhibitory mechanisms that contribute to CD8 T cells dysfunction in HIV-infected individuals.

1.7 Scope of the thesis

This study was framed to test these hypotheses and address three objectives as outlines in three chapters, with two additional chapters for the introduction and literature review and a final chapter for the general discussion and conclusion.

In chapter 2, the expression of different co-inhibitory receptors on both CD4 and CD8 T cells was studied. We, for the very first time, observed elevated levels of Gal-9 and VISTA on T cells in HIV-infected individuals. Our further studies examined the functionality of CD8 T cells in regard to the expression of co-inhibitory receptors, in particular Gal-9 and VISTA. The results related to this chapter are already published in the Journal of Immunology (Shahbaz et al. 2020 May 1;204(9):2474-2491.). This chapter tested our first hypothesis.

Chapter 3 was focused on the role of CD73 on CD8 T cells in HIV infection. The expression of CD73 at the protein and gene level was investigated in CD8 T cells from different groups of HIV-infected individuals. This was followed by further characterization of CD73-expressing CD8 T cells. Finally, the role of ATP via upregulation of miR30 family as the mechanism underlying CD73 downregulation in CD8 T cells was proposed. The results related to this chapter are submitted as a research article to the Journal of Immunology (Shahbaz et al. 2021, under revision). This chapter tested the second hypothesis of this thesis.

Chapter 4 analyzed Tregs in HIV infected individuals either on ART or LTNPs compared to healthy controls. RNAseq was performed, followed by functional assays, which identified differential transcription profile and pathways for Tregs in different study groups. The results related to this chapter are already published in the Journal of Clinical and Translational Immunology (Shahbaz et al. 2021;10: e 1289 doi:1002/cti2.1289). This chapter tested our third hypothesis.

Taken together, the outcome of these studies, their interrelationship and significance in the setting of HIV infection and other chronic diseases are discussed in the last chapter. Also, the study limitations and the future direction are highlighted.

1.8 References

1. WHO | The Global Health Observatory. HIV/AIDS.
2. Canada, P. H. A. of. 2018. Estimates of HIV incidence, prevalence and Canada's progress on meeting the 90-90-90 HIV targets. *aem* .
3. Chen, Q., D. Zeng, Y. She, Y. Lyu, X. Gong, M. J. Feinstein, Y. Yang, and H. Jiang. 2019. Different transmission routes and the risk of advanced HIV disease: A systematic review and network meta-analysis of observational studies. *EClinicalMedicine* 16: 121–128.
4. Sharp, P. M., and B. H. Hahn. 2011. Origins of HIV and the AIDS Pandemic. *Cold Spring Harb Perspect Med* 1: a006841.
5. 2015. History of HIV and AIDS overview. *Avert* .
6. Hageman, J. R. 2018. A Historical Perspective of HIV Infection. *Pediatr Ann* 47: e226.
7. Simon, V., D. D. Ho, and Q. A. Karim. 2006. HIV/AIDS epidemiology, pathogenesis, prevention, and treatment. *Lancet* 368: 489–504.
8. Durand, S., and A. Cimarelli. 2011. The Inside Out of Lentiviral Vectors. *Viruses* 3: 132–159.
9. Blut), G. A. C. B. (Arbeitskreis, and S. 'Assessment of P. T. by Blood'. 2016. Human Immunodeficiency Virus (HIV). *TMH* 43: 203–222.

10. Mushahwar, I. K. 2006. Human Immunodeficiency Viruses: Molecular Virology, Pathogenesis, Diagnosis and Treatment. In *Perspectives in Medical Virology*. Congenital and Other Related Infectious Diseases of the Newborn vol. 13. I. K. Mushahwar, ed. Elsevier. 75–87.
11. Deeks, S. G., J. Overbaugh, A. Phillips, and S. Buchbinder. 2015. HIV infection. *Nature Reviews Disease Primers* 1: 1–22.
12. Tsunetsugu-Yokota, Y., and M. Muhsen. 2013. Development of human dendritic cells and their role in HIV infection: antiviral immunity versus HIV transmission. *Front Microbiol* 4: 178.
13. Vicenzi, E., P. Liò, and G. Poli. 2013. The puzzling role of CXCR4 in human immunodeficiency virus infection. *Theranostics* 3: 18–25.
14. Steinman, R. M., A. Granelli-Piperno, M. Pope, C. Trumpfheller, R. Ignatius, G. Arrode, P. Racz, and K. Tenner-Racz. 2003. The interaction of immunodeficiency viruses with dendritic cells. *Curr Top Microbiol Immunol* 276: 1–30.
15. Koppensteiner, H., R. Brack-Werner, and M. Schindler. 2012. Macrophages and their relevance in Human Immunodeficiency Virus Type I infection. *Retrovirology* 9: 82.
16. Piatak, M., M. S. Saag, L. C. Yang, S. J. Clark, J. C. Kappes, K. C. Luk, B. H. Hahn, G. M. Shaw, and J. D. Lifson. 1993. High levels of HIV-1 in plasma during all stages of infection determined by competitive PCR. *Science* 259: 1749–1754.
17. Streeck, H., R. Lu, N. Beckwith, M. Milazzo, M. Liu, J.-P. Routy, S. Little, H. Jessen, A. D. Kelleher, F. Hecht, R.-P. Sekaly, G. Alter, D. Heckerman, M. Carrington, E. S. Rosenberg, and M. Altfeld. 2014. Emergence of individual HIV-specific CD8 T cell responses during primary HIV-1 infection can determine long-term disease outcome. *J Virol* 88: 12793–12801.
18. Luckheeram, R. V., R. Zhou, A. D. Verma, and B. Xia. 2012. $CD4^+$T cells: differentiation and functions. *Clin Dev Immunol* 2012: 925135.
19. Sant, A. J., and A. McMichael. 2012. Revealing the role of CD4+ T cells in viral immunity. *J Exp Med* 209: 1391–1395.
20. Zhu, J., and W. E. Paul. 2008. CD4 T cells: fates, functions, and faults. *Blood* 112: 1557–1569.
21. Henn, A., C. Flateau, and S. Gallien. 2017. Primary HIV Infection: Clinical Presentation, Testing, and Treatment. *Curr Infect Dis Rep* 19: 37.
22. Chu, C., and P. A. Selwyn. 2010. Diagnosis and initial management of acute HIV infection. *Am Fam Physician* 81: 1239–1244.
23. Vajpayee, M., and T. Mohan. 2011. Current practices in laboratory monitoring of HIV infection. *Indian J Med Res* 134: 801–822.
24. WHO | What's new in treatment monitoring: viral load and CD4 testing. *WHO* .
25. Vella, S., B. Schwartländer, S. P. Sow, S. P. Eholie, and R. L. Murphy. 2012. The history of antiretroviral therapy and of its implementation in resource-limited areas of the world. *AIDS* 26: 1231–1241.
26. Furman, P. A., J. A. Fyfe, M. H. St Clair, K. Weinhold, J. L. Rideout, G. A. Freeman, S. N. Lehrman, D. P. Bolognesi, S. Broder, and H. Mitsuya. 1986. Phosphorylation of 3'-azido-3'-deoxythymidine and selective interaction of the 5'-triphosphate with human immunodeficiency virus reverse transcriptase. *Proc Natl Acad Sci U S A* 83: 8333–8337.
27. St Clair, M. H., C. A. Richards, T. Spector, K. J. Weinhold, W. H. Miller, A. J. Langlois, and P. A. Furman. 1987. 3'-Azido-3'-deoxythymidine triphosphate as an inhibitor and substrate of purified human immunodeficiency virus reverse transcriptase. *Antimicrob Agents Chemother* 31: 1972–1977.
28. Connor, E. M., R. S. Sperling, R. Gelber, P. Kiselev, G. Scott, M. J. O'Sullivan, R. VanDyke, M. Bey, W. Shearer, and R. L. Jacobson. 1994. Reduction of maternal-infant transmission of human immunodeficiency virus type 1 with zidovudine treatment. Pediatric AIDS Clinical Trials Group Protocol 076 Study Group. *N Engl J Med* 331: 1173–1180.

29. Skowron, G., S. A. Bozzette, L. Lim, C. B. Pettinelli, H. H. Schaumburg, J. Arezzo, M. A. Fischl, W. G. Powderly, D. J. Gocke, D. D. Richman, J. C. Pottage, D. Antoniskis, G. F. McKinley, N. E. Hyslop, G. Ray, G. Simon, N. Reed, M. L. LoFaro, R. B. Uttamchandani, L. D. Gelb, S. J. Sperber, R. L. Murphy, J. M. Leedom, M. H. Grieco, J. Zachary, M. S. Hirsch, S. A. Spector, J. Bigley, W. Soo, and T. C. Merigan. 1993. Alternating and intermittent regimens of zidovudine and dideoxycytidine in patients with AIDS or AIDS-related complex. *Ann Intern Med* 118: 321–330.
30. 1996. Delta: a randomised double-blind controlled trial comparing combinations of zidovudine plus didanosine or zalcitabine with zidovudine alone in HIV-infected individuals. Delta Coordinating Committee. *Lancet* 348: 283–291.
31. Hammer, S. M., K. E. Squires, M. D. Hughes, J. M. Grimes, L. M. Demeter, J. S. Currier, J. J. Eron, J. E. Feinberg, H. H. Balfour, L. R. Deyton, J. A. Chodakewitz, and M. A. Fischl. 1997. A controlled trial of two nucleoside analogues plus indinavir in persons with human immunodeficiency virus infection and CD4 cell counts of 200 per cubic millimeter or less. AIDS Clinical Trials Group 320 Study Team. *N Engl J Med* 337: 725–733.
32. Carpenter, C. C., M. A. Fischl, S. M. Hammer, M. S. Hirsch, D. M. Jacobsen, D. A. Katzenstein, J. S. Montaner, D. D. Richman, M. S. Sáag, R. T. Schooley, M. A. Thompson, S. Vella, P. G. Yeni, and P. A. Volberding. 1996. Antiretroviral therapy for HIV infection in 1996. Recommendations of an international panel. International AIDS Society-USA. *JAMA* 276: 146–154.
33. Palella, F. J., K. M. Delaney, A. C. Moorman, M. O. Loveless, J. Fuhrer, G. A. Satten, D. J. Aschman, and S. D. Holmberg. 1998. Declining morbidity and mortality among patients with advanced human immunodeficiency virus infection. HIV Outpatient Study Investigators. *N Engl J Med* 338: 853–860.
34. Selik, R. M., S. Y. Chu, and J. W. Buehler. 1993. HIV infection as leading cause of death among young adults in US cities and states. *JAMA* 269: 2991–2994.
35. Arribas, J. R., A. L. Pozniak, J. E. Gallant, E. Dejesus, B. Gazzard, R. E. Campo, S.-S. Chen, D. McColl, C. B. Holmes, J. Enejosa, J. J. Toole, and A. K. Cheng. 2008. Tenofovir disoproxil fumarate, emtricitabine, and efavirenz compared with zidovudine/lamivudine and efavirenz in treatment-naive patients: 144-week analysis. *J Acquir Immune Defic Syndr* 47: 74–78.
36. Molina, J.-M., J. Andrade-Villanueva, J. Echevarria, P. Chetchotisakd, J. Corral, N. David, G. Moyle, M. Mancini, L. Percival, R. Yang, A. Thiry, D. McGrath, and CASTLE Study Team. 2008. Once-daily atazanavir/ritonavir versus twice-daily lopinavir/ritonavir, each in combination with tenofovir and emtricitabine, for management of antiretroviral-naive HIV-1-infected patients: 48 week efficacy and safety results of the CASTLE study. *Lancet* 372: 646–655.
37. Sax, P. E., C. Tierney, A. C. Collier, M. A. Fischl, K. Mollan, L. Peeples, C. Godfrey, N. C. Jahed, L. Myers, D. Katzenstein, A. Farajallah, J. F. Rooney, B. Ha, W. C. Woodward, S. L. Koletar, V. A. Johnson, P. J. Geiseler, E. S. Daar, and AIDS Clinical Trials Group Study A5202 Team. 2009. Abacavir-lamivudine versus tenofovir-emtricitabine for initial HIV-1 therapy. *N Engl J Med* 361: 2230–2240.
38. Kitahata, M. M., S. J. Gange, A. G. Abraham, B. Merriman, M. S. Saag, A. C. Justice, R. S. Hogg, S. G. Deeks, J. J. Eron, J. T. Brooks, S. B. Rourke, M. J. Gill, R. J. Bosch, J. N. Martin, M. B. Klein, L. P. Jacobson, B. Rodriguez, T. R. Sterling, G. D. Kirk, S. Napravnik, A. R. Rachlis, L. M. Calzavara, M. A. Horberg, M. J. Silverberg, K. A. Gebo, J. J. Goedert, C. A. Benson, A. C. Collier, S. E. Van Rompaey, H. M. Crane, R. G. McKaig, B. Lau, A. M. Freeman, R. D. Moore, and NA-ACCORD Investigators. 2009. Effect of early versus deferred antiretroviral therapy for HIV on survival. *N Engl J Med* 360: 1815–1826.
39. Lima, V. D., R. Harrigan, D. R. Bangsberg, R. S. Hogg, R. Gross, B. Yip, and J. S. G. Montaner. 2009. The combined effect of modern highly active antiretroviral therapy regimens and adherence on mortality over time. *J Acquir Immune Defic Syndr* 50: 529–536.
40. Cohen, M. S., Y. Q. Chen, M. McCauley, T. Gamble, M. C. Hosseinipour, N. Kumarasamy, J. G. Hakim, J. Kumwenda, B. Grinsztejn, J. H. S. Pilotto, S. V. Godbole, S. Mehendale, S. Chariyalertsak, B. R. Santos, K. H. Mayer, I. F. Hoffman, S. H. Eshleman, E. Piwowar-Manning, L. Wang, J. Makhema, L. A. Mills, G. de

Bruyn, I. Sanne, J. Eron, J. Gallant, D. Havlir, S. Swindells, H. Ribaudo, V. Elharrar, D. Burns, T. E. Taha, K. Nielsen-Saines, D. Celentano, M. Essex, T. R. Fleming, and HPTN 052 Study Team. 2011. Prevention of HIV-1 infection with early antiretroviral therapy. *N Engl J Med* 365: 493–505.
41. Fowler, M. G., M. Qin, S. A. Fiscus, J. S. Currier, P. M. Flynn, T. Chipato, J. McIntyre, D. Gnanashanmugam, G. K. Siberry, A. S. Coletti, T. E. Taha, K. L. Klingman, F. E. Martinson, M. Owor, A. Violari, D. Moodley, G. B. Theron, R. Bhosale, R. Bobat, B. H. Chi, R. Strehlau, P. Mlay, A. J. Loftis, R. Browning, T. Fenton, L. Purdue, M. Basar, D. E. Shapiro, L. M. Mofenson, and IMPAACT 1077BF/1077FF PROMISE Study Team. 2016. Benefits and Risks of Antiretroviral Therapy for Perinatal HIV Prevention. *N Engl J Med* 375: 1726–1737.
42. Kasamba, I., K. Baisley, B. N. Mayanja, D. Maher, and H. Grosskurth. 2012. The impact of antiretroviral treatment on mortality trends of HIV-positive adults in rural Uganda: a longitudinal population-based study, 1999–2009. *Trop Med Int Health* 17: e66–e73.
43. Sellers, C. J., and D. A. Wohl. 2014. Antiretroviral Therapy: When to Start. *Infect Dis Clin North Am* 28: 403–420.
44. INSIGHT START Study Group, J. D. Lundgren, A. G. Babiker, F. Gordin, S. Emery, B. Grund, S. Sharma, A. Avihingsanon, D. A. Cooper, G. Fätkenheuer, J. M. Llibre, J.-M. Molina, P. Munderi, M. Schechter, R. Wood, K. L. Klingman, S. Collins, H. C. Lane, A. N. Phillips, and J. D. Neaton. 2015. Initiation of Antiretroviral Therapy in Early Asymptomatic HIV Infection. *N Engl J Med* 373: 795–807.
45. Becker, M., J. Cox, G. Evans, S. Haider, and S. Shafran. 2016. AMMI Canada position statement: The use of early antiretroviral therapy in HIV-infected persons. *Official Journal of the Association of Medical Microbiology and Infectious Disease Canada* 1: 1–11.
46. Mogensen, T. H., J. Melchjorsen, C. S. Larsen, and S. R. Paludan. 2010. Innate immune recognition and activation during HIV infection. *Retrovirology* 7: 54.
47. Binley, J. M., P. J. Klasse, Y. Cao, I. Jones, M. Markowitz, D. D. Ho, and J. P. Moore. 1997. Differential regulation of the antibody responses to Gag and Env proteins of human immunodeficiency virus type 1. *J Virol* 71: 2799–2809.
48. Richman, D. D., T. Wrin, S. J. Little, and C. J. Petropoulos. 2003. Rapid evolution of the neutralizing antibody response to HIV type 1 infection. *Proc Natl Acad Sci U S A* 100: 4144–4149.
49. Parren, P. W., J. P. Moore, D. R. Burton, and Q. J. Sattentau. 1999. The neutralizing antibody response to HIV-1: viral evasion and escape from humoral immunity. *AIDS* 13 Suppl A: S137-162.
50. Hangartner, L., R. M. Zellweger, M. Giobbi, J. Weber, B. Eschli, K. D. McCoy, N. Harris, M. Recher, R. M. Zinkernagel, and H. Hengartner. 2006. Nonneutralizing antibodies binding to the surface glycoprotein of lymphocytic choriomeningitis virus reduce early virus spread. *J Exp Med* 203: 2033–2042.
51. Huber, M., and A. Trkola. 2007. Humoral immunity to HIV-1: neutralization and beyond. *J Intern Med* 262: 5–25.
52. Rosenberg, E. S., M. Altfeld, S. H. Poon, M. N. Phillips, B. M. Wilkes, R. L. Eldridge, G. K. Robbins, R. T. D'Aquila, P. J. Goulder, and B. D. Walker. 2000. Immune control of HIV-1 after early treatment of acute infection. *Nature* 407: 523–526.
53. Walker, B., and A. McMichael. 2012. The T-cell response to HIV. *Cold Spring Harb Perspect Med* 2.
54. Zhang, N., and M. J. Bevan. 2011. CD8(+) T cells: foot soldiers of the immune system. *Immunity* 35: 161–168.
55. Krzewski, K., and J. E. Coligan. 2012. Human NK cell lytic granules and regulation of their exocytosis. *Front Immunol* 3: 335.
56. Aguiló, J. I., A. Anel, E. Catalán, A. Sebastián, R. Acín-Pérez, J. Naval, R. Wallich, M. M. Simon, and J. Pardo. 2010. Granzyme B of cytotoxic T cells induces extramitochondrial reactive oxygen species production via caspase-dependent NADPH oxidase activation. *Immunol Cell Biol* 88: 545–554.

57. Darmon, A. J., T. J. Ley, D. W. Nicholson, and R. C. Bleackley. 1996. Cleavage of CPP32 by granzyme B represents a critical role for granzyme B in the induction of target cell DNA fragmentation. *J Biol Chem* 271: 21709–21712.
58. Atkinson, E. A., M. Barry, A. J. Darmon, I. Shostak, P. C. Turner, R. W. Moyer, and R. C. Bleackley. 1998. Cytotoxic T lymphocyte-assisted suicide. Caspase 3 activation is primarily the result of the direct action of granzyme B. *J Biol Chem* 273: 21261–21266.
59. Lord, S. J., R. V. Rajotte, G. S. Korbutt, and R. C. Bleackley. 2003. Granzyme B: a natural born killer. *Immunol Rev* 193: 31–38.
60. Nicholson, D. W., A. Ali, N. A. Thornberry, J. P. Vaillancourt, C. K. Ding, M. Gallant, Y. Gareau, P. R. Griffin, M. Labelle, and Y. A. Lazebnik. 1995. Identification and inhibition of the ICE/CED-3 protease necessary for mammalian apoptosis. *Nature* 376: 37–43.
61. Kothakota, S., T. Azuma, C. Reinhard, A. Klippel, J. Tang, K. Chu, T. J. McGarry, M. W. Kirschner, K. Koths, D. J. Kwiatkowski, and L. T. Williams. 1997. Caspase-3-generated fragment of gelsolin: effector of morphological change in apoptosis. *Science* 278: 294–298.
62. Enari, M., H. Sakahira, H. Yokoyama, K. Okawa, A. Iwamatsu, and S. Nagata. 1998. A caspase-activated DNase that degrades DNA during apoptosis, and its inhibitor ICAD. *Nature* 391: 43–50.
63. Barry, M., J. A. Heibein, M. J. Pinkoski, S. F. Lee, R. W. Moyer, D. R. Green, and R. C. Bleackley. 2000. Granzyme B short-circuits the need for caspase 8 activity during granule-mediated cytotoxic T-lymphocyte killing by directly cleaving Bid. *Mol Cell Biol* 20: 3781–3794.
64. Li, P., D. Nijhawan, I. Budihardjo, S. M. Srinivasula, M. Ahmad, E. S. Alnemri, and X. Wang. 1997. Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. *Cell* 91: 479–489.
65. Bleackley, R. C., and J. A. Heibein. 2001. Enzymatic control of apoptosis. *Nat Prod Rep* 18: 431–440.
66. Voskoboinik, I., J. C. Whisstock, and J. A. Trapani. 2015. Perforin and granzymes: function, dysfunction and human pathology. *Nat Rev Immunol* 15: 388–400.
67. Tschopp, J., D. Masson, and K. K. Stanley. 1986. Structural/functional similarity between proteins involved in complement- and cytotoxic T-lymphocyte-mediated cytolysis. *Nature* 322: 831–834.
68. Podack, E. R., J. D. Young, and Z. A. Cohn. 1985. Isolation and biochemical and functional characterization of perforin 1 from cytolytic T-cell granules. *Proc Natl Acad Sci U S A* 82: 8629–8633.
69. Thiery, J., D. Keefe, S. Boulant, E. Boucrot, M. Walch, D. Martinvalet, I. S. Goping, R. C. Bleackley, T. Kirchhausen, and J. Lieberman. 2011. Perforin pores in the endosomal membrane trigger the release of endocytosed granzyme B into the cytosol of target cells. *Nature Immunology* 12: 770–777.
70. Gordy, C., and Y.-W. He. 2012. Endocytosis by target cells: an essential means for perforin- and granzyme-mediated killing. *Cellular & Molecular Immunology* 9: 5–6.
71. Metkar, S. S., B. Wang, M. Aguilar-Santelises, S. M. Raja, L. Uhlin-Hansen, E. Podack, J. A. Trapani, and C. J. Froelich. 2002. Cytotoxic cell granule-mediated apoptosis: perforin delivers granzyme B-serglycin complexes into target cells without plasma membrane pore formation. *Immunity* 16: 417–428.
72. Keefe, D., L. Shi, S. Feske, R. Massol, F. Navarro, T. Kirchhausen, and J. Lieberman. 2005. Perforin triggers a plasma membrane-repair response that facilitates CTL induction of apoptosis. *Immunity* 23: 249–262.
73. Shi, L., D. Keefe, E. Durand, H. Feng, D. Zhang, and J. Lieberman. 2005. Granzyme B binds to target cells mostly by charge and must be added at the same time as perforin to trigger apoptosis. *J Immunol* 174: 5456–5461.
74. Wherry, E. J., and M. Kurachi. 2015. Molecular and cellular insights into T cell exhaustion. *Nat Rev Immunol* 15: 486–499.
75. Okoye, I. S., M. Houghton, L. Tyrrell, K. Barakat, and S. Elahi. 2017. Coinhibitory Receptor Expression and Immune Checkpoint Blockade: Maintaining a Balance in CD8+ T Cell Responses to Chronic Viral Infections and Cancer. *Front Immunol* 8: 1215.

76. Day, C. L., D. E. Kaufmann, P. Kiepiela, J. A. Brown, E. S. Moodley, S. Reddy, E. W. Mackey, J. D. Miller, A. J. Leslie, C. DePierres, Z. Mncube, J. Duraiswamy, B. Zhu, Q. Eichbaum, M. Altfeld, E. J. Wherry, H. M. Coovadia, P. J. R. Goulder, P. Klenerman, R. Ahmed, G. J. Freeman, and B. D. Walker. 2006. PD-1 expression on HIV-specific T cells is associated with T-cell exhaustion and disease progression. *Nature* 443: 350–354.
77. Quigley, M., F. Pereyra, B. Nilsson, F. Porichis, C. Fonseca, Q. Eichbaum, B. Julg, J. L. Jesneck, K. Brosnahan, S. Imam, K. Russell, I. Toth, A. Piechocka-Trocha, D. Dolfi, J. Angelosanto, A. Crawford, H. Shin, D. S. Kwon, J. Zupkosky, L. Francisco, G. J. Freeman, E. J. Wherry, D. E. Kaufmann, B. D. Walker, B. Ebert, and W. N. Haining. 2010. Transcriptional analysis of HIV-specific CD8+ T cells shows that PD-1 inhibits T cell function by upregulating BATF. *Nat Med* 16: 1147–1151.
78. Hui, E., J. Cheung, J. Zhu, X. Su, M. J. Taylor, H. A. Wallweber, D. K. Sasmal, J. Huang, J. M. Kim, I. Mellman, and R. D. Vale. 2017. T cell costimulatory receptor CD28 is a primary target for PD-1-mediated inhibition. *Science* 355: 1428–1433.
79. Fontenot, J. D., J. P. Rasmussen, L. M. Williams, J. L. Dooley, A. G. Farr, and A. Y. Rudensky. 2005. Regulatory T cell lineage specification by the forkhead transcription factor foxp3. *Immunity* 22: 329–341.
80. Staron, M. M., S. M. Gray, H. D. Marshall, I. A. Parish, J. H. Chen, C. J. Perry, G. Cui, M. O. Li, and S. M. Kaech. 2014. The transcription factor FoxO1 sustains expression of the inhibitory receptor PD-1 and survival of antiviral CD8(+) T cells during chronic infection. *Immunity* 41: 802–814.
81. Chew, G. M., T. Fujita, G. M. Webb, B. J. Burwitz, H. L. Wu, J. S. Reed, K. B. Hammond, K. L. Clayton, N. Ishii, M. Abdel-Mohsen, T. Liegler, B. I. Mitchell, F. M. Hecht, M. Ostrowski, C. M. Shikuma, S. G. Hansen, M. Maurer, A. J. Korman, S. G. Deeks, J. B. Sacha, and L. C. Ndhlovu. 2016. TIGIT Marks Exhausted T Cells, Correlates with Disease Progression, and Serves as a Target for Immune Restoration in HIV and SIV Infection. *PLoS Pathog* 12: e1005349.
82. Jones, R. B., L. C. Ndhlovu, J. D. Barbour, P. M. Sheth, A. R. Jha, B. R. Long, J. C. Wong, M. Satkunarajah, M. Schweneker, J. M. Chapman, G. Gyenes, B. Vali, M. D. Hyrcza, F. Y. Yue, C. Kovacs, A. Sassi, M. Loutfy, R. Halpenny, D. Persad, G. Spotts, F. M. Hecht, T.-W. Chun, J. M. McCune, R. Kaul, J. M. Rini, D. F. Nixon, and M. A. Ostrowski. 2008. Tim-3 expression defines a novel population of dysfunctional T cells with highly elevated frequencies in progressive HIV-1 infection. *J Exp Med* 205: 2763–2779.
83. Zhu, C., A. C. Anderson, A. Schubart, H. Xiong, J. Imitola, S. J. Khoury, X. X. Zheng, T. B. Strom, and V. K. Kuchroo. 2005. The Tim-3 ligand galectin-9 negatively regulates T helper type 1 immunity. *Nat Immunol* 6: 1245–1252.
84. Rangachari, M., C. Zhu, K. Sakuishi, S. Xiao, J. Karman, A. Chen, M. Angin, A. Wakeham, E. A. Greenfield, R. A. Sobel, H. Okada, P. J. McKinnon, T. W. Mak, M. M. Addo, A. C. Anderson, and V. K. Kuchroo. 2012. Bat3 promotes T cell responses and autoimmunity by repressing Tim-3–mediated cell death and exhaustion. *Nat Med* 18: 1394–1400.
85. Zeidan, A. M., R. S. Komrokji, and A. M. Brunner. 2021. TIM-3 pathway dysregulation and targeting in cancer. *Expert Rev Anticancer Ther* 1–12.
86. Kumar, P. 2013. Long term non-progressor (LTNP) HIV infection. *Indian J Med Res* 138: 291–293.
87. Elahi, S., W. L. Dinges, N. Lejarcegui, K. J. Laing, A. C. Collier, D. M. Koelle, M. J. McElrath, and H. Horton. 2011. Protective HIV-specific CD8+ T cells evade Treg cell suppression. *Nat Med* 17: 989–995.
88. Poropatich, K., and D. J. Sullivan. 2011. Human immunodeficiency virus type 1 long-term non-progressors: the viral, genetic and immunological basis for disease non-progression. *J Gen Virol* 92: 247–268.
89. Learmont, J., B. Tindall, L. Evans, A. Cunningham, P. Cunningham, J. Wells, R. Penny, J. Kaldor, and D. A. Cooper. 1992. Long-term symptomless HIV-1 infection in recipients of blood products from a single donor. *Lancet* 340: 863–867.

90. Deacon, N. J., A. Tsykin, A. Solomon, K. Smith, M. Ludford-Menting, D. J. Hooker, D. A. McPhee, A. L. Greenway, A. Ellett, C. Chatfield, V. A. Lawson, S. Crowe, A. Maerz, S. Sonza, J. Learmont, J. S. Sullivan, A. Cunningham, D. Dwyer, D. Dowton, and J. Mills. 1995. Genomic structure of an attenuated quasi species of HIV-1 from a blood transfusion donor and recipients. *Science* 270: 988–991.
91. Miura, T., M. A. Brockman, C. J. Brumme, Z. L. Brumme, J. M. Carlson, F. Pereyra, A. Trocha, M. M. Addo, B. L. Block, A. C. Rothchild, B. M. Baker, T. Flynn, A. Schneidewind, B. Li, Y. E. Wang, D. Heckerman, T. M. Allen, and B. D. Walker. 2008. Genetic characterization of human immunodeficiency virus type 1 in elite controllers: lack of gross genetic defects or common amino acid changes. *J Virol* 82: 8422–8430.
92. Pushker, R., J.-M. Jacqué, and D. C. Shields. 2010. Meta-analysis to test the association of HIV-1 nef amino acid differences and deletions with disease progression. *J Virol* 84: 3644–3653.
93. Blankson, J. N., J. R. Bailey, S. Thayil, H.-C. Yang, K. Lassen, J. Lai, S. K. Gandhi, J. D. Siliciano, T. M. Williams, and R. F. Siliciano. 2007. Isolation and characterization of replication-competent human immunodeficiency virus type 1 from a subset of elite suppressors. *J Virol* 81: 2508–2518.
94. Lamine, A., A. Caumont-Sarcos, M.-L. Chaix, A. Saez-Cirion, C. Rouzioux, J.-F. Delfraissy, G. Pancino, and O. Lambotte. 2007. Replication-competent HIV strains infect HIV controllers despite undetectable viremia (ANRS EP36 study). *AIDS* 21: 1043–1045.
95. Carl, S., R. Daniels, A. J. Iafrate, P. Easterbrook, T. C. Greenough, J. Skowronski, and F. Kirchhoff. 2000. Partial “Repair” of Defective NEF Genes in a Long-Term Nonprogressor with Human Immunodeficiency Virus Type 1 Infection. *J Infect Dis* 181: 132–140.
96. Cruz, N. V. G., R. Amorim, F. E. Oliveira, F. A. C. Speranza, and L. J. Costa. 2013. Mutations in the nef and vif genes associated with progression to AIDS in elite controller and slow-progressor patients. *J Med Virol* 85: 563–574.
97. Lassen, K. G., M. A. Lobritz, J. R. Bailey, S. Johnston, S. Nguyen, B. Lee, T. Chou, R. F. Siliciano, M. Markowitz, and E. J. Arts. 2009. Elite suppressor-derived HIV-1 envelope glycoproteins exhibit reduced entry efficiency and kinetics. *PLoS Pathog* 5: e1000377.
98. Morawetz, R. A., G. P. Rizzardi, D. Glauser, O. Rutschmann, B. Hirschel, L. Perrin, M. Opravil, M. Flepp, J. von Overbeck, M. P. Glauser, S. Ghezzi, E. Vicenzi, G. Poli, A. Lazzarin, and G. Pantaleo. 1997. Genetic polymorphism of CCR5 gene and HIV disease: the heterozygous (CCR5/delta ccr5) genotype is neither essential nor sufficient for protection against disease progression. Swiss HIV Cohort. *Eur J Immunol* 27: 3223–3227.
99. Misrahi, M., J. P. Teglas, N. N’Go, M. Burgard, M. J. Mayaux, C. Rouzioux, J. F. Delfraissy, and S. Blanche. 1998. CCR5 chemokine receptor variant in HIV-1 mother-to-child transmission and disease progression in children. French Pediatric HIV Infection Study Group. *JAMA* 279: 277–280.
100. Romiti, M. L., C. Colognesi, C. Cancrini, A. Mas, M. Berrino, F. Salvatori, P. Orlandi, M. Jansson, E. Palomba, A. Plebani, J. M. Bertran, M. Hernandez, M. de Martino, A. Amoroso, P. A. Tovo, P. Rossi, T. Espanol, and G. Scarlatti. 2000. Prognostic value of a CCR5 defective allele in pediatric HIV-1 infection. *Mol Med* 6: 28–36.
101. Carrington, M., and S. J. O’Brien. 2003. The influence of HLA genotype on AIDS. *Annu Rev Med* 54: 535–551.
102. Neumann-Haefelin, C. 2013. HLA-B27-mediated protection in HIV and hepatitis C virus infection and pathogenesis in spondyloarthritis: two sides of the same coin? *Curr Opin Rheumatol* 25: 426–433.
103. Miura, T., M. A. Brockman, A. Schneidewind, M. Lobritz, F. Pereyra, A. Rathod, B. L. Block, Z. L. Brumme, C. J. Brumme, B. Baker, A. C. Rothchild, B. Li, A. Trocha, E. Cutrell, N. Frahm, C. Brander, I. Toth, E. J. Arts, T. M. Allen, and B. D. Walker. 2009. HLA-B57/B*5801 Human Immunodeficiency Virus Type 1 Elite Controllers Select for Rare Gag Variants Associated with Reduced Viral Replication Capacity and Strong Cytotoxic T-Lymphotye Recognition. *J Virol* 83: 2743–2755.
104. Kelleher, A. D., C. Long, E. C. Holmes, R. L. Allen, J. Wilson, C. Conlon, C. Workman, S. Shaunak, K. Olson, P. Goulder, C. Brander, G. Ogg, J. S. Sullivan, W. Dyer, I. Jones, A. J. McMichael, S. Rowland-Jones,

and R. E. Phillips. 2001. Clustered mutations in HIV-1 gag are consistently required for escape from HLA-B27-restricted cytotoxic T lymphocyte responses. *J Exp Med* 193: 375–386.
105. Hersperger, A. R., F. Pereyra, M. Nason, K. Demers, P. Sheth, L. Y. Shin, C. M. Kovacs, B. Rodriguez, S. F. Sieg, L. Teixeira-Johnson, D. Gudonis, P. A. Goepfert, M. M. Lederman, I. Frank, G. Makedonas, R. Kaul, B. D. Walker, and M. R. Betts. 2010. Perforin expression directly ex vivo by HIV-specific CD8 T-cells is a correlate of HIV elite control. *PLoS Pathog* 6: e1000917.
106. Migueles, S. A., A. C. Laborico, W. L. Shupert, M. S. Sabbaghian, R. Rabin, C. W. Hallahan, D. Van Baarle, S. Kostense, F. Miedema, M. McLaughlin, L. Ehler, J. Metcalf, S. Liu, and M. Connors. 2002. HIV-specific CD8+ T cell proliferation is coupled to perforin expression and is maintained in nonprogressors. *Nat Immunol* 3: 1061–1068.
107. Elahi, S., W. L. Dinges, N. Lejarcegui, K. J. Laing, A. C. Collier, D. M. Koelle, M. J. McElrath, and H. Horton. 2011. Protective HIV-specific CD8+ T cells evade Treg cell suppression. *Nat Med* 17: 989–995.
108. Chen, H., C. Li, J. Huang, T. Cung, K. Seiss, J. Beamon, M. F. Carrington, L. C. Porter, P. S. Burke, Y. Yang, B. J. Ryan, R. Liu, R. H. Weiss, F. Pereyra, W. D. Cress, A. L. Brass, E. S. Rosenberg, B. D. Walker, X. G. Yu, and M. Lichterfeld. 2011. CD4+ T cells from elite controllers resist HIV-1 infection by selective upregulation of p21. *J Clin Invest* 121: 1549–1560.
109. Goulder, P. J., R. E. Phillips, R. A. Colbert, S. McAdam, G. Ogg, M. A. Nowak, P. Giangrande, G. Luzzi, B. Morgan, A. Edwards, A. J. McMichael, and S. Rowland-Jones. 1997. Late escape from an immunodominant cytotoxic T-lymphocyte response associated with progression to AIDS. *Nat Med* 3: 212–217.
110. Oomizu, S., T. Arikawa, T. Niki, T. Kadowaki, M. Ueno, N. Nishi, A. Yamauchi, T. Hattori, T. Masaki, and M. Hirashima. 2012. Cell surface galectin-9 expressing Th cells regulate Th17 and Foxp3+ Treg development by galectin-9 secretion. *PLoS One* 7: e48574.
111. John, S., and R. Mishra. 2016. Galectin-9: From cell biology to complex disease dynamics. *J Biosci* 41: 507–534.
112. O'Brien, M. J., Q. Shu, W. A. Stinson, P.-S. Tsou, J. H. Ruth, T. Isozaki, P. L. Campbell, R. A. Ohara, A. E. Koch, D. A. Fox, and M. A. Amin. 2018. A unique role for galectin-9 in angiogenesis and inflammatory arthritis. *Arthritis Res Ther* 20: 31.
113. Bi, S., P. W. Hong, B. Lee, and L. G. Baum. 2011. Galectin-9 binding to cell surface protein disulfide isomerase regulates the redox environment to enhance T-cell migration and HIV entry. *Proc Natl Acad Sci U S A* 108: 10650–10655.
114. Wu, C., T. Thalhamer, R. F. Franca, S. Xiao, C. Wang, C. Hotta, C. Zhu, M. Hirashima, A. C. Anderson, and V. K. Kuchroo. 2014. Galectin-9-CD44 interaction enhances stability and function of adaptive regulatory T cells. *Immunity* 41: 270–282.
115. Merani, S., W. Chen, and S. Elahi. 2015. The bitter side of sweet: the role of Galectin-9 in immunopathogenesis of viral infections. *Rev Med Virol* 25: 175–186.
116. Madireddi, S., S.-Y. Eun, S.-W. Lee, I. Nemčovičová, A. K. Mehta, D. M. Zajonc, N. Nishi, T. Niki, M. Hirashima, and M. Croft. 2014. Galectin-9 controls the therapeutic activity of 4-1BB-targeting antibodies. *J Exp Med* 211: 1433–1448.
117. Niki, T., K. Fujita, H. Rosen, M. Hirashima, T. Masaki, T. Hattori, and K. Hoshino. 2018. Plasma Galectin-9 Concentrations in Normal and Diseased Condition. *Cell Physiol Biochem* 50: 1856–1868.
118. Nickel, W. 2003. The mystery of nonclassical protein secretion. A current view on cargo proteins and potential export routes. *Eur J Biochem* 270: 2109–2119.
119. Saitoh, H., Y. Ashino, H. Chagan-Yasutan, T. Niki, M. Hirashima, and T. Hattori. 2012. Rapid decrease of plasma galectin-9 levels in patients with acute HIV infection after therapy. *Tohoku J Exp Med* 228: 157–161.

120. Jost, S., U. Y. Moreno-Nieves, W. F. Garcia-Beltran, K. Rands, J. Reardon, I. Toth, A. Piechocka-Trocha, M. Altfeld, and M. M. Addo. 2013. Dysregulated Tim-3 expression on natural killer cells is associated with increased Galectin-9 levels in HIV-1 infection. *Retrovirology* 10: 74.
121. Tandon, R., G. M. Chew, M. M. Byron, P. Borrow, T. Niki, M. Hirashima, J. D. Barbour, P. J. Norris, M. C. Lanteri, J. N. Martin, S. G. Deeks, and L. C. Ndhlovu. 2014. Galectin-9 is rapidly released during acute HIV-1 infection and remains sustained at high levels despite viral suppression even in elite controllers. *AIDS Res Hum Retroviruses* 30: 654–664.
122. Elahi, S., T. Niki, M. Hirashima, and H. Horton. 2012. Galectin-9 binding to Tim-3 renders activated human CD4+ T cells less susceptible to HIV-1 infection. *Blood* 119: 4192–4204.
123. Abdel-Mohsen, M., L. Chavez, R. Tandon, G. M. Chew, X. Deng, A. Danesh, S. Keating, M. Lanteri, M. L. Samuels, R. Hoh, J. B. Sacha, P. J. Norris, T. Niki, C. M. Shikuma, M. Hirashima, S. G. Deeks, L. C. Ndhlovu, and S. K. Pillai. 2016. Human Galectin-9 Is a Potent Mediator of HIV Transcription and Reactivation. *PLoS Pathog* 12.
124. Colomb, F., L. B. Giron, T. A. Premeaux, B. I. Mitchell, T. Niki, E. Papasavvas, L. J. Montaner, L. C. Ndhlovu, and M. Abdel-Mohsen. 2019. Galectin-9 Mediates HIV Transcription by Inducing TCR-Dependent ERK Signaling. *Front. Immunol.* 10.
125. Vanhamel, J., A. Bruggemans, and Z. Debyser. Establishment of latent HIV-1 reservoirs: what do we really know? *J Virus Erad* 5: 3–9.
126. Yoon, K. W., S. Byun, E. Kwon, S.-Y. Hwang, K. Chu, M. Hiraki, S.-H. Jo, A. Weins, S. Hakroush, A. Cebulla, D. B. Sykes, A. Greka, P. Mundel, D. E. Fisher, A. Mandinova, and S. W. Lee. 2015. Control of signaling-mediated clearance of apoptotic cells by the tumor suppressor p53. *Science* 349.
127. Michalek, R. D., V. A. Gerriets, S. R. Jacobs, A. N. Macintyre, N. J. MacIver, E. F. Mason, S. A. Sullivan, A. G. Nichols, and J. C. Rathmell. 2011. Cutting Edge: Distinct Glycolytic and Lipid Oxidative Metabolic Programs Are Essential for Effector and Regulatory CD4+ T Cell Subsets. *The Journal of Immunology* 186: 3299–3303.
128. Aloia, L., S. Parisi, L. Fusco, L. Pastore, and T. Russo. 2010. Differentiation of Embryonic Stem Cells 1 (Dies1) Is a Component of Bone Morphogenetic Protein 4 (BMP4) Signaling Pathway Required for Proper Differentiation of Mouse Embryonic Stem Cells *. *Journal of Biological Chemistry* 285: 7776–7783.
129. Wang, L., R. Rubinstein, J. L. Lines, A. Wasiuk, C. Ahonen, Y. Guo, L.-F. Lu, D. Gondek, Y. Wang, R. A. Fava, A. Fiser, S. Almo, and R. J. Noelle. 2011. VISTA, a novel mouse Ig superfamily ligand that negatively regulates T cell responses. *J Exp Med* 208: 577–592.
130. Mehta, N., S. Maddineni, I. I. Mathews, R. Andres Parra Sperberg, P.-S. Huang, and J. R. Cochran. 2019. Structure and Functional Binding Epitope of V-domain Ig Suppressor of T Cell Activation. *Cell Rep* 28: 2509-2516.e5.
131. Lines, J. L., E. Pantazi, J. Mak, L. F. Sempere, L. Wang, S. O'Connell, S. Ceeraz, A. A. Suriawinata, S. Yan, M. S. Ernstoff, and R. Noelle. 2014. VISTA is an immune checkpoint molecule for human T cells. *Cancer Res* 74: 1924–1932.
132. Flies, D. B., X. Han, T. Higuchi, L. Zheng, J. Sun, J. J. Ye, and L. Chen. 2014. Coinhibitory receptor PD-1H preferentially suppresses $CD4^+$ T cell–mediated immunity. *J Clin Invest* 124: 1966–1975.
133. Wang, J., G. Wu, B. Manick, V. Hernandez, M. Renelt, C. Erickson, J. Guan, R. Singh, S. Rollins, A. Solorz, M. Bi, J. Li, D. Grabowski, J. Dirkx, C. Tracy, T. Stuart, C. Ellinghuysen, D. Desmond, C. Foster, and V. Kalabokis. 2019. VSIG-3 as a ligand of VISTA inhibits human T-cell function. *Immunology* 156: 74–85.
134. Bharaj, P., H. S. Chahar, O. K. Alozie, L. Rodarte, A. Bansal, P. A. Goepfert, A. Dwivedi, N. Manjunath, and P. Shankar. 2014. Characterization of programmed death-1 homologue-1 (PD-1H) expression and function in normal and HIV infected individuals. *PLoS One* 9: e109103.
135. Flies, D. B., S. Wang, H. Xu, and L. Chen. 2011. Cutting edge: A monoclonal antibody specific for the programmed death-1 homolog prevents graft-versus-host disease in mouse models. *J Immunol* 187: 1537–1541.

136. ElTanbouly, M. A., Y. Zhao, E. Nowak, J. Li, E. Schaafsma, I. L. Mercier, S. Ceeraz, J. L. Lines, C. Peng, C. Carriere, X. Huang, M. Day, B. Koehn, S. W. Lee, M. S. Morales, K. A. Hogquist, S. C. Jameson, D. Mueller, J. Rothstein, B. R. Blazar, C. Cheng, and R. J. Noelle. 2020. VISTA is a checkpoint regulator for naïve T cell quiescence and peripheral tolerance. *Science* 367.
137. Kunishige, T., H. Taniguchi, T. Ohno, M. Azuma, and J. Hori. 2019. VISTA Is Crucial for Corneal Allograft Survival and Maintenance of Immune Privilege. *Invest. Ophthalmol. Vis. Sci.* 60: 4958–4965.
138. ElTanbouly, M. A., E. Schaafsma, R. J. Noelle, and J. L. Lines. 2020. VISTA: Coming of age as a multi-lineage immune checkpoint. *Clin Exp Immunol* 200: 120–130.
139. Wang, L., I. Le Mercier, J. Putra, W. Chen, J. Liu, A. D. Schenk, E. C. Nowak, A. A. Suriawinata, J. Li, and R. J. Noelle. 2014. Disruption of the immune-checkpoint VISTA gene imparts a proinflammatory phenotype with predisposition to the development of autoimmunity. *Proc Natl Acad Sci U S A* 111: 14846–14851.
140. Liu, J., Y. Yuan, W. Chen, J. Putra, A. A. Suriawinata, A. D. Schenk, H. E. Miller, I. Guleria, R. J. Barth, Y. H. Huang, and L. Wang. 2015. Immune-checkpoint proteins VISTA and PD-1 nonredundantly regulate murine T-cell responses. *Proc Natl Acad Sci U S A* 112: 6682–6687.
141. Flies, D. B., T. Higuchi, and L. Chen. 2015. Mechanistic Assessment of PD-1H Coinhibitory Receptor-Induced T Cell Tolerance to Allogeneic Antigens. *J Immunol* 194: 5294–5304.
142. Le Mercier, I., W. Chen, J. L. Lines, M. Day, J. Li, P. Sergent, R. J. Noelle, and L. Wang. 2014. VISTA Regulates the Development of Protective Antitumor Immunity. *Cancer Res* 74: 1933–1944.
143. Böger, C., H.-M. Behrens, S. Krüger, and C. Röcken. 2017. The novel negative checkpoint regulator VISTA is expressed in gastric carcinoma and associated with PD-L1/PD-1: A future perspective for a combined gastric cancer therapy? *Oncoimmunology* 6: e1293215.
144. Wu, L., W.-W. Deng, C.-F. Huang, L.-L. Bu, G.-T. Yu, L. Mao, W.-F. Zhang, B. Liu, and Z.-J. Sun. 2017. Expression of VISTA correlated with immunosuppression and synergized with CD8 to predict survival in human oral squamous cell carcinoma. *Cancer Immunol Immunother* 66: 627–636.
145. Gao, J., J. F. Ward, C. A. Pettaway, L. Z. Shi, S. K. Subudhi, L. M. Vence, H. Zhao, J. Chen, H. Chen, E. Efstathiou, P. Troncoso, J. P. Allison, C. J. Logothetis, I. I. Wistuba, M. A. Sepulveda, J. Sun, J. Wargo, J. Blando, and P. Sharma. 2017. VISTA is an inhibitory immune checkpoint that is increased after ipilimumab therapy in patients with prostate cancer. *Nat Med* 23: 551–555.
146. Xu, W., J. Dong, Y. Zheng, J. Zhou, Y. Yuan, H. M. Ta, H. E. Miller, M. Olson, K. Rajasekaran, M. S. Ernstoff, D. Wang, S. Malarkannan, and L. Wang. 2019. Immune checkpoint protein VISTA regulates antitumor immunity by controlling myeloid cell–mediated inflammation and immunosuppression. *Cancer Immunol Res* 7: 1497–1510.
147. Martínez-Martínez, A., E. Muñoz-Delgado, F. J. Campoy, C. Flores-Flores, J. N. Rodríguez-López, C. Fini, and C. J. Vidal. 2000. The ecto-5′-nucleotidase subunits in dimers are not linked by disulfide bridges but by non-covalent bonds. *Biochimica et Biophysica Acta (BBA) - Protein Structure and Molecular Enzymology* 1478: 300–308.
148. Knapp, K., M. Zebisch, J. Pippel, A. El-Tayeb, C. E. Müller, and N. Sträter. 2012. Crystal Structure of the Human Ecto-5′-Nucleotidase (CD73): Insights into the Regulation of Purinergic Signaling. *Structure* 20: 2161–2173.
149. Klemens, M. R., W. R. Sherman, N. J. Holmberg, J. M. Ruedi, M. G. Low, and L. F. Thompson. 1990. Characterization of soluble vs membrane-bound human placental 5′-nucleotidase. *Biochemical and Biophysical Research Communications* 172: 1371–1377.
150. Vogel, M., H. Kowalewski, H. Zimmermann, N. M. Hooper, and A. J. Turner. 1992. Soluble low-Km 5′-nucleotidase from electric-ray (Torpedo marmorata) electric organ and bovine cerebral cortex is derived from the glycosyl-phosphatidylinositol-anchored ectoenzyme by phospholipase C cleavage. *Biochemical Journal* 284: 621–624.

151. Bono, M. R., D. Fernández, F. Flores-Santibáñez, M. Rosemblatt, and D. Sauma. 2015. CD73 and CD39 ectonucleotidases in T cell differentiation: Beyond immunosuppression. *FEBS Letters* 589: 3454–3460.
152. Aswad, F., H. Kawamura, and G. Dennert. 2005. High sensitivity of CD4+CD25+ regulatory T cells to extracellular metabolites nicotinamide adenine dinucleotide and ATP: a role for P2X7 receptors. *J Immunol* 175: 3075–3083.
153. Stagg, J., and M. J. Smyth. 2010. Extracellular adenosine triphosphate and adenosine in cancer. *Oncogene* 29: 5346–5358.
154. Antonioli, L., P. Pacher, E. S. Vizi, and G. Haskó. 2013. CD39 and CD73 in immunity and inflammation. *Trends Mol Med* 19: 355–367.
155. Deaglio, S., K. M. Dwyer, W. Gao, D. Friedman, A. Usheva, A. Erat, J.-F. Chen, K. Enjyoji, J. Linden, M. Oukka, V. K. Kuchroo, T. B. Strom, and S. C. Robson. 2007. Adenosine generation catalyzed by CD39 and CD73 expressed on regulatory T cells mediates immune suppression. *J Exp Med* 204: 1257–1265.
156. Ohta, A., A. Ohta, M. Madasu, R. Kini, M. Subramanian, N. Goel, and M. Sitkovsky. 2009. A2A adenosine receptor may allow expansion of T cells lacking effector functions in extracellular adenosine-rich microenvironments. *J Immunol* 183: 5487–5493.
157. Zarek, P. E., C.-T. Huang, E. R. Lutz, J. Kowalski, M. R. Horton, J. Linden, C. G. Drake, and J. D. Powell. 2008. A2A receptor signaling promotes peripheral tolerance by inducing T-cell anergy and the generation of adaptive regulatory T cells. *Blood* 111: 251–259.
158. Ohta, A., R. Kini, A. Ohta, M. Subramanian, M. Madasu, and M. Sitkovsky. 2012. The development and immunosuppressive functions of CD4(+) CD25(+) FoxP3(+) regulatory T cells are under influence of the adenosine-A2A adenosine receptor pathway. *Front Immunol* 3: 190.
159. Massaia, M., L. Perrin, A. Bianchi, J. Ruedi, C. Attisano, D. Altieri, G. T. Rijkers, and L. F. Thompson. 1990. Human T cell activation. Synergy between CD73 (ecto-5'-nucleotidase) and signals delivered through CD3 and CD2 molecules. *J Immunol* 145: 1664–1674.
160. Thompson, L. F., J. M. Ruedi, A. Glass, M. G. Low, and A. H. Lucas. 1989. Antibodies to 5'-nucleotidase (CD73), a glycosyl-phosphatidylinositol-anchored protein, cause human peripheral blood T cells to proliferate. *J Immunol* 143: 1815–1821.
161. Dianzani, U., V. Redoglia, M. Bragardo, C. Attisano, A. Bianchi, D. D. Franco, U. Ramenghi, H. Wolff, L. F. Thompson, and A. Pileri. 1993. Co-stimulatory signal delivered by CD73 molecule to human CD45RAhiCD45ROlo (naive) CD8+ T lymphocytes. *The Journal of Immunology* 151: 3961–3970.
162. Resta, R., Y. Yamashita, and L. F. Thompson. 1998. Ecto-enzyme and signaling functions of lymphocyte CD 7 3. *Immunological Reviews* 161: 95–109.
163. Airas, L., J. Niemelä, and S. Jalkanen. 2000. CD73 Engagement Promotes Lymphocyte Binding to Endothelial Cells Via a Lymphocyte Function-Associated Antigen-1-Dependent Mechanism. *The Journal of Immunology* 165: 5411–5417.
164. Allan, S. E., S. Q. Crome, N. K. Crellin, L. Passerini, T. S. Steiner, R. Bacchetta, M. G. Roncarolo, and M. K. Levings. 2007. Activation-induced FOXP3 in human T effector cells does not suppress proliferation or cytokine production. *Int Immunol* 19: 345–354.
165. Liu, W., A. L. Putnam, Z. Xu-yu, G. L. Szot, M. R. Lee, S. Zhu, P. A. Gottlieb, P. Kapranov, T. R. Gingeras, B. F. de St. Groth, C. Clayberger, D. M. Soper, S. F. Ziegler, and J. A. Bluestone. 2006. CD127 expression inversely correlates with FoxP3 and suppressive function of human CD4+ T reg cells. *J Exp Med* 203: 1701–1711.
166. Yamaguchi, T., K. Hirota, K. Nagahama, K. Ohkawa, T. Takahashi, T. Nomura, and S. Sakaguchi. 2007. Control of immune responses by antigen-specific regulatory T cells expressing the folate receptor. *Immunity* 27: 145–159.
167. Shevach, E. M. 2009. Mechanisms of foxp3+ T regulatory cell-mediated suppression. *Immunity* 30: 636–645.

168. Rothstein, D. M., and G. Camirand. 2015. New insights into the mechanisms of Treg function. *Curr Opin Organ Transplant* 20: 376–384.
169. Lee, J. H., S. G. Kang, and C. H. Kim. 2007. FoxP3+ T cells undergo conventional first switch to lymphoid tissue homing receptors in thymus but accelerated second switch to nonlymphoid tissue homing receptors in secondary lymphoid tissues. *J Immunol* 178: 301–311.
170. Mao, F.-Y., H. Kong, Y.-L. Zhao, L.-S. Peng, W. Chen, J.-Y. Zhang, P. Cheng, T.-T. Wang, Y.-P. Lv, Y.-S. Teng, X.-L. Fu, Y.-G. Liu, X.-L. Wu, C.-J. Hao, N. You, P. Luo, P.-W. Yu, Q.-M. Zou, G. Guo, and Y. Zhuang. 2017. Increased tumor-infiltrating CD45RA-CCR7- regulatory T-cell subset with immunosuppressive properties foster gastric cancer progress. *Cell Death Dis* 8: e3002.
171. Zhu, Z., Y. Zhang, J. Ye, X. Wang, X. Fu, Y. Yin, J. Wen, X. Wu, and Z. Xia. 2018. IL-35 promoted STAT3 phosphorylation and IL-10 production in B cells, but its production was reduced in patients with coronary artery diseases. *Hum Immunol* 79: 869–875.
172. Tsuchida, Y., S. Sumitomo, K. Ishigaki, A. Suzuki, Y. Kochi, H. Tsuchiya, M. Ota, T. Komai, M. Inoue, K. Morita, T. Okamura, K. Yamamoto, and K. Fujio. 2017. TGF-β3 Inhibits Antibody Production by Human B Cells. *PLoS One* 12: e0169646.
173. Dooley, A., M. Quintana, M. Cheung, L. L. Sun, and N. Gupta. 2018. The B cell IL-10 receptor suppresses antibody production. *The Journal of Immunology* 200: 164.9-164.9.
174. Schmidt, A., N. Oberle, and P. H. Krammer. 2012. Molecular mechanisms of treg-mediated T cell suppression. *Front Immunol* 3: 51.
175. Boks, M. A., J. R. Kager-Groenland, M. S. P. Haasjes, J. J. Zwaginga, S. M. van Ham, and A. ten Brinke. 2012. IL-10-generated tolerogenic dendritic cells are optimal for functional regulatory T cell induction--a comparative study of human clinical-applicable DC. *Clin Immunol* 142: 332–342.
176. Strobl, H., and W. Knapp. 1999. TGF-beta1 regulation of dendritic cells. *Microbes Infect* 1: 1283–1290.
177. Thepmalee, C., A. Panya, M. Junking, T. Chieochansin, and P.-T. Yenchitsomanus. 2018. Inhibition of IL-10 and TGF-β receptors on dendritic cells enhances activation of effector T-cells to kill cholangiocarcinoma cells. *Hum Vaccin Immunother* 14: 1423–1431.
178. de la Rosa, M., S. Rutz, H. Dorninger, and A. Scheffold. 2004. Interleukin-2 is essential for CD4+CD25+ regulatory T cell function. *Eur J Immunol* 34: 2480–2488.
179. Chinen, T., A. K. Kannan, A. G. Levine, X. Fan, U. Klein, Y. Zheng, G. Gasteiger, Y. Feng, J. D. Fontenot, and A. Y. Rudensky. 2016. An essential role for the IL-2 receptor in T reg cell function. *Nature Immunology* 17: 1322–1333.
180. Linnemann, C., F. A. Schildberg, A. Schurich, L. Diehl, S. I. Hegenbarth, E. Endl, S. Lacher, C. E. Müller, J. Frey, L. Simeoni, B. Schraven, D. Stabenow, and P. A. Knolle. 2009. Adenosine regulates CD8 T-cell priming by inhibition of membrane-proximal T-cell receptor signalling. *Immunology* 128: e728–e737.
181. Ohta, A., R. Kini, A. Ohta, M. Subramanian, M. Madasu, and M. Sitkovsky. 2012. The development and immunosuppressive functions of CD4(+) CD25(+) FoxP3(+) regulatory T cells are under influence of the adenosine-A2A adenosine receptor pathway. *Front Immunol* 3: 190.
182. Bopp, T., C. Becker, M. Klein, S. Klein-Hessling, A. Palmetshofer, E. Serfling, V. Heib, M. Becker, J. Kubach, S. Schmitt, S. Stoll, H. Schild, M. S. Staege, M. Stassen, H. Jonuleit, and E. Schmitt. 2007. Cyclic adenosine monophosphate is a key component of regulatory T cell-mediated suppression. *J Exp Med* 204: 1303–1310.
183. Gondek, D. C., L.-F. Lu, S. A. Quezada, S. Sakaguchi, and R. J. Noelle. 2005. Cutting edge: contact-mediated suppression by CD4+CD25+ regulatory cells involves a granzyme B-dependent, perforin-independent mechanism. *J Immunol* 174: 1783–1786.
184. Cao, X., S. F. Cai, T. A. Fehniger, J. Song, L. I. Collins, D. R. Piwnica-Worms, and T. J. Ley. 2007. Granzyme B and perforin are important for regulatory T cell-mediated suppression of tumor clearance. *Immunity* 27: 635–646.

185. Ren, X., F. Ye, Z. Jiang, Y. Chu, S. Xiong, and Y. Wang. 2007. Involvement of cellular death in TRAIL/DR5-dependent suppression induced by CD4(+)CD25(+) regulatory T cells. *Cell Death Differ* 14: 2076–2084.
186. Garín, M. I., C.-C. Chu, D. Golshayan, E. Cernuda-Morollón, R. Wait, and R. I. Lechler. 2007. Galectin-1: a key effector of regulation mediated by CD4+CD25+ T cells. *Blood* 109: 2058–2065.
187. Onishi, Y., Z. Fehervari, T. Yamaguchi, and S. Sakaguchi. 2008. Foxp3+ natural regulatory T cells preferentially form aggregates on dendritic cells in vitro and actively inhibit their maturation. *Proc Natl Acad Sci U S A* 105: 10113–10118.
188. Akkaya, B., M. Akkaya, O. Kamenyeva, J. Kabat, D. W. Dorward, A. H. Holstein, M. P. Maz, and E. M. Shevach. 2017. Unique interaction dynamics and peptide-MHC class II (pMHC II) transendocytosis lead to antigen-specific T regulatory cell (Treg)-mediated suppression. *The Journal of Immunology* 198: 80.8-80.8.
189. Fallarino, F., U. Grohmann, K. W. Hwang, C. Orabona, C. Vacca, R. Bianchi, M. L. Belladonna, M. C. Fioretti, M.-L. Alegre, and P. Puccetti. 2003. Modulation of tryptophan catabolism by regulatory T cells. *Nat Immunol* 4: 1206–1212.
190. Liang, B., C. Workman, J. Lee, C. Chew, B. M. Dale, L. Colonna, M. Flores, N. Li, E. Schweighoffer, S. Greenberg, V. Tybulewicz, D. Vignali, and R. Clynes. 2008. Regulatory T Cells Inhibit Dendritic Cells by Lymphocyte Activation Gene-3 Engagement of MHC Class II. *The Journal of Immunology* 180: 5916–5926.
191. Cai, J., D. Wang, G. Zhang, and X. Guo. 2019. The Role Of PD-1/PD-L1 Axis In Treg Development And Function: Implications For Cancer Immunotherapy. *Onco Targets Ther* 12: 8437–8445.
192. Elahi, S., W. L. Dinges, N. Lejarcegui, K. J. Laing, A. C. Collier, D. M. Koelle, M. J. McElrath, and H. Horton. 2011. Protective HIV-specific CD8+ T cells evade Treg cell suppression. *Nat Med* 17: 989–995.
193. Koch, M. A., G. Tucker-Heard, N. R. Perdue, J. R. Killebrew, K. B. Urdahl, and D. J. Campbell. 2009. The transcription factor T-bet controls regulatory T cell homeostasis and function during type 1 inflammation. *Nat Immunol* 10: 595–602.
194. Huber, M., V. Steinwald, A. Guralnik, A. Brüstle, P. Kleemann, C. Rosenplänter, T. Decker, and M. Lohoff. 2008. IL-27 inhibits the development of regulatory T cells via STAT3. *Int Immunol* 20: 223–234.
195. Ahern, P. P., C. Schiering, S. Buonocore, M. J. McGeachy, D. J. Cua, K. J. Maloy, and F. Powrie. 2010. Interleukin-23 drives intestinal inflammation through direct activity on T cells. *Immunity* 33: 279–288.
196. Yang, X. O., R. Nurieva, G. J. Martinez, H. S. Kang, Y. Chung, B. P. Pappu, B. Shah, S. H. Chang, K. S. Schluns, S. S. Watowich, X.-H. Feng, A. M. Jetten, and C. Dong. 2008. Molecular antagonism and plasticity of regulatory and inflammatory T cell programs. *Immunity* 29: 44–56.
197. Durant, L., W. T. Watford, H. L. Ramos, A. Laurence, G. Vahedi, L. Wei, H. Takahashi, H.-W. Sun, Y. Kanno, F. Powrie, and J. J. O'Shea. 2010. Diverse targets of the transcription factor STAT3 contribute to T cell pathogenicity and homeostasis. *Immunity* 32: 605–615.
198. Antons, A. K., R. Wang, K. Oswald-Richter, M. Tseng, C. W. Arendt, S. A. Kalams, and D. Unutmaz. 2008. Naive precursors of human regulatory T cells require FoxP3 for suppression and are susceptible to HIV infection. *Journal of Immunology (Baltimore, Md.: 1950)* 180: 764–773.
199. Oswald-Richter, K., S. M. Grill, N. Shariat, M. Leelawong, M. S. Sundrud, D. W. Haas, and D. Unutmaz. 2004. HIV infection of naturally occurring and genetically reprogrammed human regulatory T-cells. *PLoS biology* 2: E198.
200. Zaunders, J. J., S. Ip, M. L. Munier, D. E. Kaufmann, K. Suzuki, C. Brereton, S. C. Sasson, N. Seddiki, K. Koelsch, A. Landay, P. Grey, R. Finlayson, J. Kaldor, E. S. Rosenberg, B. D. Walker, B. Fazekas de St Groth, D. A. Cooper, and A. D. Kelleher. 2006. Infection of CD127+ (interleukin-7 receptor+) CD4+ cells and overexpression of CTLA-4 are linked to loss of antigen-specific CD4 T cells during primary human immunodeficiency virus type 1 infection. *J Virol* 80: 10162–10172.

201. Moreno-Fernandez, M. E., W. Zapata, J. T. Blackard, G. Franchini, and C. A. Chougnet. 2009. Human regulatory T cells are targets for human immunodeficiency Virus (HIV) infection, and their susceptibility differs depending on the HIV type 1 strain. *J Virol* 83: 12925–12933.
202. Angin, M., D. S. Kwon, H. Streeck, F. Wen, M. King, A. Rezai, K. Law, T. C. Hongo, A. Pyo, A. Piechocka-Trocha, I. Toth, F. Pereyra, M. Ghebremichael, S. J. Rodig, D. A. Milner, J. M. Richter, M. Altfeld, D. E. Kaufmann, B. D. Walker, and M. M. Addo. 2012. Preserved function of regulatory T cells in chronic HIV-1 infection despite decreased numbers in blood and tissue. *J Infect Dis* 205: 1495–1500.
203. Simonetta, F., C. Lecuroux, I. Girault, C. Goujard, M. Sinet, O. Lambotte, A. Venet, and C. Bourgeois. 2012. Early and long-lasting alteration of effector CD45RA(-)Foxp3(high) regulatory T-cell homeostasis during HIV infection. *J Infect Dis* 205: 1510–1519.
204. Epple, H.-J., C. Loddenkemper, D. Kunkel, H. Tröger, J. Maul, V. Moos, E. Berg, R. Ullrich, J.-D. Schulzke, H. Stein, R. Duchmann, M. Zeitz, and T. Schneider. 2006. Mucosal but not peripheral FOXP3+ regulatory T cells are highly increased in untreated HIV infection and normalize after suppressive HAART. *Blood* 108: 3072–3078.
205. Montes, M., C. Sanchez, D. E. Lewis, E. A. Graviss, C. Seas, E. Gotuzzo, and A. C. White. 2011. Normalization of FoxP3(+) regulatory T cells in response to effective antiretroviral therapy. *J Infect Dis* 203: 496–499.
206. Lim, A., D. Tan, P. Price, A. Kamarulzaman, H.-Y. Tan, I. James, and M. A. French. 2007. Proportions of circulating T cells with a regulatory cell phenotype increase with HIV-associated immune activation and remain high on antiretroviral therapy. *AIDS* 21: 1525–1534.
207. Hunt, P. W., A. L. Landay, E. Sinclair, J. A. Martinson, H. Hatano, B. Emu, P. J. Norris, M. P. Busch, J. N. Martin, C. Brooks, J. M. McCune, and S. G. Deeks. 2011. A low T regulatory cell response may contribute to both viral control and generalized immune activation in HIV controllers. *PLoS One* 6: e15924.
208. Nikolova, M., M. Carriere, M.-A. Jenabian, S. Limou, M. Younas, A. Kök, S. Huë, N. Seddiki, A. Hulin, O. Delaneau, H. Schuitemaker, J. T. Herbeck, J. I. Mullins, M. Muhtarova, A. Bensussan, J.-F. Zagury, J.-D. Lelievre, and Y. Lévy. 2011. CD39/adenosine pathway is involved in AIDS progression. *PLoS pathogens* 7: e1002110.
209. Andersson, J., A. Boasso, J. Nilsson, R. Zhang, N. J. Shire, S. Lindback, G. M. Shearer, and C. A. Chougnet. 2005. The prevalence of regulatory T cells in lymphoid tissue is correlated with viral load in HIV-infected patients. *J. Immunol.* 174: 3143–3147.
210. Apoil, P. A., B. Puissant, F. Roubinet, M. Abbal, P. Massip, and A. Blancher. 2005. FOXP3 mRNA levels are decreased in peripheral blood CD4+ lymphocytes from HIV-positive patients. *J. Acquir. Immune Defic. Syndr.* 39: 381–385.
211. Xing, S., J. Fu, Z. Zhang, Y. Gao, Y. Jiao, F. Kang, J. Zhang, C. Zhou, H. Wu, and F.-S. Wang. 2010. Increased turnover of FoxP3high regulatory T cells is associated with hyperactivation and disease progression of chronic HIV-1 infection. *J Acquir Immune Defic Syndr* 54: 455–462.
212. Piconi, S., D. Trabattoni, A. Gori, S. Parisotto, C. Magni, P. Meraviglia, A. Bandera, A. Capetti, G. Rizzardini, and M. Clerici. 2010. Immune activation, apoptosis, and Treg activity are associated with persistently reduced CD4+ T-cell counts during antiretroviral therapy. *AIDS* 24: 1991–2000.
213. Chase, A. J., H.-C. Yang, H. Zhang, J. N. Blankson, and R. F. Siliciano. 2008. Preservation of FoxP3+ regulatory T cells in the peripheral blood of human immunodeficiency virus type 1-infected elite suppressors correlates with low CD4+ T-cell activation. *J. Virol.* 82: 8307–8315.
214. Hunt, P. W., A. L. Landay, E. Sinclair, J. A. Martinson, H. Hatano, B. Emu, P. J. Norris, M. P. Busch, J. N. Martin, C. Brooks, J. M. McCune, and S. G. Deeks. 2011. A low T regulatory cell response may contribute to both viral control and generalized immune activation in HIV controllers. *PloS One* 6: e15924.

215. Fuertes Marraco, S. A., N. J. Neubert, G. Verdeil, and D. E. Speiser. 2015. Inhibitory Receptors Beyond T Cell Exhaustion. *Front Immunol* 6: 310.
216. Yasuma-Mitobe, K., and M. Matsuoka. 2018. The Roles of Coinhibitory Receptors in Pathogenesis of Human Retroviral Infections. *Front Immunol* 9: 2755.
217. Okoye, I., A. Namdar, L. Xu, N. Crux, and S. Elahi. 2017. Atorvastatin downregulates co-inhibitory receptor expression by targeting Ras-activated mTOR signalling. *Oncotarget* 8: 98215–98232.
218. Amancha, P. K., J. J. Hong, A. A. Ansari, and F. Villinger. 2015. Up-regulation of Tim-3 on T cells during acute simian immunodeficiency virus infection and on antigen specific responders. *AIDS* 29: 531–536.
219. Sakuishi, K., L. Apetoh, J. M. Sullivan, B. R. Blazar, V. K. Kuchroo, and A. C. Anderson. 2010. Targeting Tim-3 and PD-1 pathways to reverse T cell exhaustion and restore anti-tumor immunity. *J Exp Med* 207: 2187–2194.
220. Cao, S., and K. A. Woodrow. 2019. Nanotechnology approaches to eradicating HIV reservoirs. *European Journal of Pharmaceutics and Biopharmaceutics* 138: 48–63.
221. Ålgars, A., M. Karikoski, G. G. Yegutkin, P. Stoitzner, J. Niemelä, M. Salmi, and S. Jalkanen. 2011. Different role of CD73 in leukocyte trafficking via blood and lymph vessels. *Blood* 117: 4387–4393.
222. Mills, J. H., L. F. Thompson, C. Mueller, A. T. Waickman, S. Jalkanen, J. Niemela, L. Airas, and M. S. Bynoe. 2008. CD73 is required for efficient entry of lymphocytes into the central nervous system during experimental autoimmune encephalomyelitis. *PNAS* 105: 9325–9330.
223. Stachon, P., S. Geis, A. Peikert, A. Heidenreich, N. A. Michel, F. Ünal, N. Hoppe, B. Dufner, L. Schulte, T. Marchini, S. Cicko, K. Ayata, A. Zech, D. Wolf, I. Hilgendorf, F. Willecke, J. Reinöhl, C. von Zur Mühlen, C. Bode, M. Idzko, and A. Zirlik. 2016. Extracellular ATP Induces Vascular Inflammation and Atherosclerosis via Purinergic Receptor Y2 in Mice. *Arterioscler Thromb Vasc Biol* 36: 1577–1586.
224. Velasquez, S., L. Prevedel, S. Valdebenito, A. M. Gorska, M. Golovko, N. Khan, J. Geiger, and E. A. Eugenin. 2020. Circulating levels of ATP is a biomarker of HIV cognitive impairment. *EBioMedicine* 51: 102503.

Chapter 2: Galectin-9 and VISTA expression define terminally exhausted T cells in HIV-1 infection

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2.1 Introduction

Antigen-specific CD8 T cells play a crucial role in eliminating virus-infected and tumour cells. However, during chronic viral infections and cancer, which involve persistent signals from antigens or inflammation, the program of CD8 T cell differentiation is markedly altered. This scenario is often associated with deteriorating cytotoxic T lymphocyte (CTL) function, a state of dysfunction commonly called exhaustion (1). CD8 T cell exhaustion is the hallmark of chronic viral infections such as HIV and hepatitis C virus, as well as cancer. Exhausted CD8 T cells lose robust effector functions such as their ability to produce cytokines, capacity to proliferate, and eventually the ability to kill target cells. Instead, they manifest sustained upregulation and co-expression of multiple co-inhibitory receptors such as program cell death (PD)-1, cytotoxic T lymphocyte-associated protein 4 (CTLA-4) and T-cell immunoglobulin and mucin domain-3 (TIM-3). Subsequently, altered expression and use of key transcription factors and metabolic derangements in exhausted CD8 T cells render patients unable to mount an effective immune response against chronic infections and tumours (2-5).

V-domain Ig Suppressor of T cell Activation (VISTA) is a novel negative regulator of T cell functions (6). It shares homology to PDL-1, belongs to the B7 family, highly expressed on both CD4 and CD8 T cells in different tumor models and impairs anti-tumor immunity (7, 8). VISTA is associated with TGF-β production and its role in the promotion of naïve T cells to regulatory T cells (Tregs) has been documented (9). However, its overexpression on T cells in HIV-infected individuals has not been investigated, to our knowledge.

Although transient expression of these co-inhibitory receptors or immune checkpoints is crucial for controlling autoreactivity and immunopathology, their persistent overexpression renders CD8 T cells to an impaired phenotype (4, 10). However, exhausted CD8 T cells comprise heterogeneous cell populations, and the functional nature of exhausted T cells may vary according to the nature of eliciting antigens (1, 11).

Similarly, robust and functional CD4 T cells play an instrumental role in viral infections. For instance, loss of CD4 T cells is associated with HIV disease progression to AIDS (12, 13). In addition, CD4 T cells not only enhance the magnitude and quality of CD8 T cells responses but also improve their clonal expansion and differentiation into effector and memory CTLs (14, 15). However, CD4 T cells also become exhausted during chronic viral infections. Overexpression of co-inhibitory receptors is centrally involved in both CD4 and CD8 T cell impairment. Although exhausted CD4 T cells share some transcription modules with exhausted CD8 T cells, they utilize other gene expression programs that distinguish them functionally and phenotypically (16).

Even though the overexpression of co-inhibitory receptors on T cells in chronic viral infections has widely been investigated, the upregulation of their corresponding ligands on T cells has not been well studied.

Galectin-9 (Gal-9), the ligand for TIM-3, is a member of the β-galactoside-binding lectin family of galectins (17). It is ubiquitously expressed in different tissues and cells in humans and mice (18) and localized on the membrane, cytoplasm, and nucleus of different cells (19). The wide presence of Gal-9 on host cells indicate an essential but sophisticated role for this lectin, whose biological effects are exerted by multiple receptors with distinct and often opposing effects such as TIM-3 (20), a cell surface protein disulfide isomerase (PDI) (21), IgE (22), 4-1BB (CD137)(23) and CD44 (24). Therefore, having multiple interacting receptors make Gal-9 a highly immunomodulatory molecule depending on different circumstances (17). We and others have shown that Tregs express Gal-9 (25, 26), which via interaction with its receptor, TIM-3, suppresses antigen-specific CD8 T cells effector functions (27, 28). Further evidence suggests that exogenous Gal-9 prevents the differentiation and function of Th17 cells but promotes the induction of Tregs (20, 29, 30). Most recent evidence indicates that Gal-9 and CD44 interactions promote Foxp3 expression to enforce induced Treg (iTreg) differentiation and maintenance (31). In addition, the interaction of Gal-9 with the TIM-3 receptor on Th1 cells induces T cell apoptosis and regulates CD8 T cell

responses (20, 32).

From a different perspective, Gal-9 plays an important role in HIV pathogenesis. We and others have reported an elevation of Gal-9 levels in the plasma (33-35) and a direct correlation between Gal-9 levels and the plasma viral load in HIV-infected individuals (34).

Furthermore, in agreement with another study (36), we have reported that soluble Gal-9:PDI interactions on non-stimulated CD4 T cells enhance HIV-1 entry and infection (34). In contrast, the interaction of soluble Gal-9 with TIM-3 on stimulated cells decreases HIV infection and replication (34). More recent studies have provided additional evidence that soluble Gal-9 regulates HIV transcription and viral production by reactivating latent HIV in CD4 T cells (37) through TCR signalling transduction(38). Although the role of surface Gal-9 expression on Tregs and recombinant Gal-9 in HIV and other models have extensively been investigated, surface expression of Gal-9 on T cells in the context of chronic viral infections has never been reported.

Therefore, here for the very first time, in our knowledge, we demonstrate that Gal-9 is highly expressed on the surface of both CD4 and CD8 T cells in HIV-infected individuals compared to healthy controls. Overexpression of Gal-9 is associated with an impaired CD4 and CD8 T cells phenotype in HIV. Further characterization of Gal-9+ T cells revealed that these cells are mainly of the effector T cell phenotype with high expression of CD25 and CD122, the IL-2α and IL-2β receptors, respectively. Moreover, Gal-9+ T cells express genes such as T-bet, EOMES, and blimp-1, which are associated with an impaired T cell phenotype. Gal-9+ T cells display an impaired phenotype, and its co-expression with PD-1 and other co-inhibitory receptors provide a synergistic impaired effector function.

2.2 Material and methods

2.2.1 Study population

Our cohort consisted of a total of 132 human subjects, including 1) Twenty two healthy controls, which were HIV, HCV, and HBV seronegative 2) Twenty-five HIV-infected but ART-naïve individuals 3) Sixty-

three HIV-infected individuals on ART 4) Nine HIV and HCV co-infected individuals on ART 5) Thirteen Long-term non-progressors, defined based on our previous report (28), who had CD4 count > 400, plasma viral load < 10,000 copies/mL, ART-naive and have been infected with HIV > 11 years.

2.2.2 Ethics statement

This study was approved by the institutional research review boards at the University of Alberta, and written informed consent was obtained from all the participants in the study protocol numbers Pro000046064 and Pro000070528.

2.2.3 Cell isolation

The peripheral blood mononuclear cells (PBMCs) were isolated from the fresh blood of either HIV or healthy individuals using Ficoll-Paque gradients and cultured in RPMI 1640 (Sigma) supplemented with 10% fetal bovine serum (Sigma) and 1% penicillin/streptomycin (Sigma). In some studies, total T cells or CD8 T cells were isolated from fresh PBMCs according to the manufacturing instruction (STEMCELL Technologies) and our previous reports (28, 39), with a purity exceeding 95% (Supplementary Fig. 1A). In other studies, total T cells were labelled with FITC-conjugated anti-Gal-9 mAb (9M1-3), followed by anti-FITC microbeads (Miltenyi Biotec) and passed through MACS separation columns (Miltenyi Biotec) for isolating Gal-9+ and Gal-9- by positive and negative selections, respectively.

For proliferation assay, isolated T cells were labelled with CellTrace™ carboxyfluorescein succinimidyl ester (CFSE) and stimulated with anti-CD3/CD28 beads (Thermo Fisher Scientific) for 96 hours.

2.2.4 Flow Cytometry

Fluorophore antibodies specific to human cell antigens and cytokines were purchased mainly from BD, Thermo Fisher Scientific, Biolegend and R&D. The following antibodies were used in our study: anti-CD3 (SK7), anti-CD4 (RPA-T4), anti-CD8 (RPA-T8), anti-VISTA (FAB71261P), anti-PD-1 (MIH4), anti-2B4 (eBioDM244), anti-TIGIT (MBSA43), anti-CD160 (BY55), anti-TIM-3(7D3), anti-CD39 (TU66), anti-Gal-9 (9M1-3), anti-CD25 (M-A251), anti-CD127 (HIL-7R-M21), anti-CD107a (H4A3), pSTAT5 (pY694), anti-

Perforin (δG9), anti-Granzyme B (GB11), anti-CD4+5RO (UCHL1), CD4+5RA (HL100), anti-CD27 (0323), anti-GATA3 (L50-823), anti- RORγt (Q21-559), anti-T-bet (eBio4B10), anti-FOXP3 (150D/E4), anti-CD122 (Mik-β3), anti-HLA-DR (LN3), anti-CD154 (TRAP1), anti-CD62L (DREG-56), anti-CD38 (HIT2), anti-TCRαβ (T10B9.1A-31), anti-pERK1/2 (MILAN8R), anti-LCK (pY505), anti-IL12Rβ (69310), anti-IL-4Rα (25463), anti-Glut1 (202915), anti-IL-2 (MQ1-17H12), anti-TNF-α (MAB11), and IFN-γ (4S.B3). Purified NA/LE mouse anti-human CD3 (UCHT1), anti-human CD28 (CD28.2), Protein Transport Inhibitor (Containing Brefeldin A), and Protein Transport Inhibitor (Containing Monensin) were purchased from the BD Bioscience. Cell stimulation cocktail (PMA/Ionomycin) was purchased from the Biolegend. The apoptotic assay was performed using the PE Annexin V Apoptosis Detection Kit I (BD Bioscience) according to the manufacturing protocol. To assess cell viability, we used the LIVE/DEAD Kit (Life Technologies). Surface staining and intracytoplasmic cytokine staining (ICS) were performed as we described previously (28, 39, 40). We also performed additional assays in the presence or absence of lactose (Sigma-Aldrich; 30 mM), anti-Galectin-9 antibody (Millipore Sigma), and anti-TIM-3 antibody (R&D). CD107a staining was performed according to the previous report (41), Phospho-STAT5 and phospho-ERK 1/2 were performed according to the manufacturing instructions (BD Bioscience). After fixation with Paraformaldehyde (PFA 2%), stained cells were acquired on a Fortessa-X20 or LSR Fortessa-SORP (BD Bioscience) and analyzed using the FlowJo software (version 10). A representative gating strategy for Gal-9+ T cells is included (Supplementary Fig. 1B).

2.2.5 Image cytometry

PBMCs were surface stained and fixed with PFA for ImageStream analysis. We collected ≥ 3000 images for each condition using Amnis ImageStream Mark II (EMD Millipore). Analysis was performed by choosing a high aspect ratio, choosing only in-focus images, and calculating the maximum pixel intensity of the fluorochrome dye according to our previous report (39).

2.2.6 Gal-9 detection by ELISA

The Gal-9 concentration in the plasma of HIV-infected and culture supernatants were measured as previously described(34) using an ELISA kit (R&D).

2.2.7 Gene expression analysis

RNA isolation and qPCR were conducted according to our published data (9, 42). The resulting cDNA (5 ng/µl) was used as a template for TaqMan qPCR (Applied Biosystems) with the following gene expression probe assays EOMES (QT00026495), TNF-α (QT00029162), IFN-γ (QT00000525), T-bet (QT00042217), FOX01 (Hs01125659_m1) FBX038 (Hs00257443_m1). Each sample was run in duplicates on CFX96 Touch™ Real-Time PCR Detection System (BioRad). Beta-actin (Hs01060665_g1) was used as a reference gene, and the gene expression of the targeted genes was calculated by the $2^{-\Delta\Delta Ct}$ method.

2.2.8 Statistical analysis

P values displayed in cumulative flow cytometry plots or PCR were determined by non-parametric Mann-Whitney test. When more than two groups were compared, one-way ANOVA followed by Tukey's test was used to compare the results. Prism software was used for statistical analysis. Results are presented as mean ± SEM, with p-value < 0.05 being considered as statistically significant.

2.3 Results

2.3.1 The expression pattern of co-inhibitory receptors on T cells of HIV-infected individuals

In order to determine the expression status of different co-inhibitory receptors on T cells of HIV-infected individuals, we profiled the expression of Gal-9, PD-1, CD160, VISTA, 2B4, CD39, TIGIT and TIM-3 by flow cytometry using fresh peripheral blood mononuclear cells (PBMCs) of 110 HIV-infected individuals and 22 HIV-negative healthy controls (HCs). In agreement with previous reports (43, 44), we found higher percentage of 2B4, TIGIT, PD-1, CD160 and CD39 expressing CD4 and CD8 T cells in HIV-infected individuals compared to HCs (Fig. 1A, B). We also observed significantly higher percentage of V-domain Immunoglobulin Suppressor of T cell Activation (VISTA) (9) expressing CD4 and CD8 T cells in HIV-infected individuals versus HCs (Fig. 1A-C). Although there was higher percentage of TIM-3 expressing

CD4 T cells in HIV-infected individuals compared to HCs, this was not the case for CD8 T cells in our cohort (Fig. 1A, B). Strikingly, for the very first time, we observed significantly higher percentage of Gal-9 expressing CD4 and CD8 T cells in HIV-infected individuals compared to HCs (Fig. 1A, B, D). In addition, we investigated Gal-9 expressing T cells of different HIV subgroups in our cohort and compared them to HCs. As shown in Fig. 1E, F, the frequency of Gal-9+ CD4 T cells was significantly expanded in ART-naïve, LTNPs, ART-treated and HIV/HCV co-infected individuals compared to HCs. Furthermore, there was higher percentage of Gal-9 expressing CD4 T cells from ART-treated subjects compared to LTNPs (Fig. 1E). Similarly, there was higher percentage of Gal-9 expressing CD8 T cells from ART-treated/naive and HIV/HCV co-infected individuals compared to HCs (Fig. 1F). However, LTNPs did not show a higher expression of Gal-9 expressing CD8 T cells compared to HCs (Fig. 1F). Moreover, ART-treated subjects and HIV/HCV patients expressed significantly higher Gal-9 expressing CD8 T cells compared to ART-naïve and LTNPs (Fig. 1F).

We and others have previously shown that a subset of Tregs express Gal-9 and exert part of their immunosuppressive properties via Gal-9: TIM-3 interactions (26, 28). Thus, to determine whether Gal-9 expressing CD4 T cells belonged to the Treg subset, we assessed the frequency of Gal-9 expressing CD4 CD25+FOXP3+ T cells in PBMCs of HIV+ individuals. As depicted in Fig. 1G, we noticed that only a portion of CD4 CD25+FOXP3+ T cells expressed Gal-9. In addition, we assessed the expression of Gal-9 on CD25+CD127$^{lo/-}$CD4 T cells. Similarly, a small subset of CD25+CD127$^{lo/-}$CD4 T cells expressed Gal-9 on their surface (Fig. 1H). Furthermore, we observed higher MFI for Gal-9 on CD25$^{-/lo}$CD127^{hi}CD4 T cells versus CD25+CD127$^{lo/-}$CD4 T cells (Supplementary Fig. 1C, D).These observations confirmed that all Gal-9+CD4 T cells didn't belong to Tregs. Overall, our data demonstrate the significant expansion of Gal-9+ CD4 and Gal-9+CD8 T cells in HIV-infected individuals versus HCs.

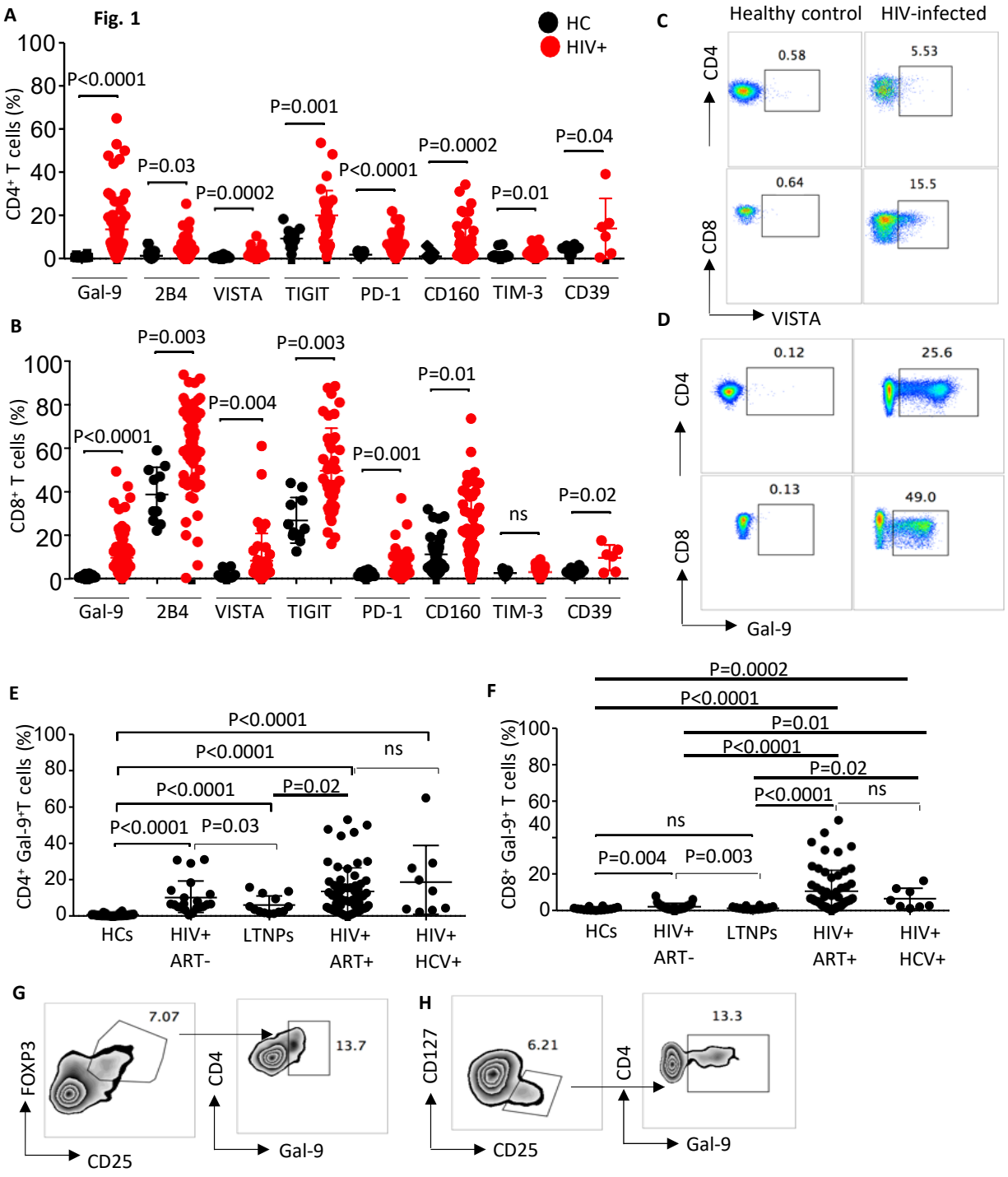


Fig. 1. Overexpression of Gal-9+ T cells in HIV-infected individuals. **A**) Cumulative data showing the percentage of CD4 and **B**) CD8 T cells expressing multiple co-inhibitory receptors in HCs versus HIV-infected individuals. **C**) Representative flow cytometry plots of surface expression of VISTA on CD4 and CD8 T cells. **D**) Representative flow cytometry plots of surface expression of Gal-9 on CD4 and CD8 T cells. **E**) Cumulative data showing the percentage of Gal-9 expressing CD4 and **F**) CD8 T cells in HCs

compared to HIV+ART naïve, LTNPS, HIV+ on ART and HIV/HCV co-infected individuals. **G**) Representative flow cytometry plot of surface Gal-9 expression on FOXP3+, and **H**) CD25+CD127- Tregs in an HIV-infected individual.

2.3.2 Co-expression of Gal-9 with other co-inhibitory receptors on T cells of HIV-infected individuals

Next, we decided to determine the simultaneous expression of Gal-9 with other co-inhibitory receptors on T cells. We found Gal-9 was co-expressed at different levels with different co-inhibitory receptors (e.g., 2B4, TIGIT, PD-1, CD160, TIM-3, VISTA, and CD39) on both CD4 and CD8 T cells in all groups of HIV-infected individuals (Fig. 2A). Nevertheless, we observed that the majority of PD-1 expressing CD4 or CD8 T cells co-expressed Gal-9 on their surface in all HIV-infected groups (Fig. 2A-C). Not only PD-1 was co-expressed with Gal-9 but also the MFI of Gal-9 was significantly higher on PD-1+ T cells compared to PD-1- CD4 and CD8 T cells (Fig. 2D-F). Although Gal-9 and PD-1 were co-expressed on T cells, their co-localization appeared to be very low as shown by the image stream (Fig. 2G, H and Supplementary Fig. 1E). However, further in depth analysis is required to confirm this concept.

2.3.3 Expression of Gal-9 on CD4 and CD8 T cells defines a dysfunctional phenotype

We then decided to characterize the functionally of Gal-9+ T cell *in vitro*. Initially, cytokine production (TNF-α, IFN-γ and IL-2) in Gal-9+ vs Gal-9- CD4 or CD8 T cells stimulated with anti-CD3/CD28 was assessed by intercellular cytokine staining (ICS). As shown in Fig. 3A-F, Gal-9+CD4 and Gal-9+CD8 T cells exhibited impaired functionality by producing significantly lesser TNF-α, IFN-γ and IL-2 compared to Gal-9- CD4 and CD8 T cells. As comparison, we also evaluated cytokine production in 2B4+ vs 2B4-, VISTA+ vs VISTA-, TIGIT+ vs TIGIT-, CD160+ vs CD160-, PD-1+ vs PD-1-, and TIM-3+ vs TIM-3- T cells. We found that PD-1+, TIM-3+, CD160+, and VISTA+ CD4 and CD8 T cells produced significantly lower levels of TNF-α and IFN-γ compared to PD-1-, TIM-3-, CD160-, and VISTA- CD4 and CD8 T cells, respectively (Fig. 3C, D and Supplementary Fig. 1F, G), which is consistent with the previous reports (44-46). In contrast, there was

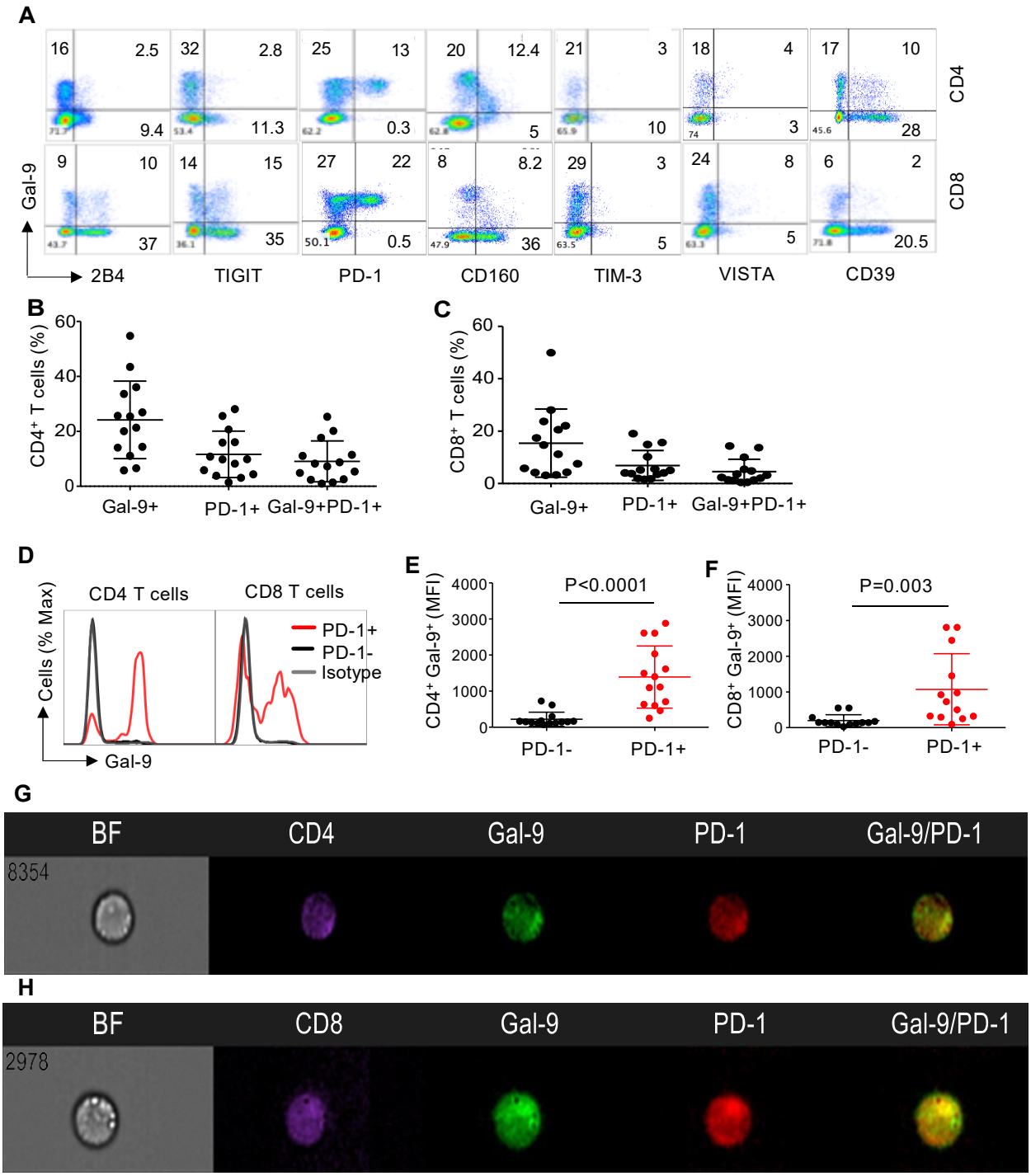


Fig. 2. Co-expression of Gal-9 with other co-inhibitory receptors. A) Representative flow cytometry plots showing surface co-expression of Gal-9 with different co-inhibitory receptors on both CD4 and CD8 T cells in HIV-infected individuals. **B**) Cumulative data showing surface co-expression levels of Gal-9 with PD-1 on CD4, and **C**) CD8 T cells from HIV-infected individuals. **D**) Representative histogram, and **E**, **F**) quantification of geometric mean fluorescence intensity (MFI) ± SEM of surface expression for Gal-9 on PD-1+ CD4 and PD-1+ CD8 T cells versus PD-1- T cells, respectively. **G**) Representative image stream plots showing co-expression of Gal-9 and PD-1 on either CD4 or **H**) CD8 T cells in an HIV-infected individual.

no significant difference in TNF-α and IFN-γ production by 2B4+ CD4 T cells vs 2B4- CD4 T cells and interestingly, 2B4+ CD8 T cells produced significantly more cytokines compared to 2B4- CD8 T cells (Supplementary Fig. 2a). Notably, the observed pattern of cytokine production was the same in all groups of HIV-infected patients (data not shown). The striking observation was that Gal-9 expression appeared to be the most dominant marker associated with CTL dysfunction, as shown in Supplementary Fig. 2A-C. In addition, we measured the proliferative capacity of Gal-9+ T cells by CFSE staining. We found that in response to TCR stimulation, Gal-9+ T cells exhibited impaired proliferative capacity compared to Gal-9- T cells (Fig. 3G, H). Although PD-1+ T cells proliferated less compared to PD-1- T cells (Fig. 3I), co-expression of Gal-9 and PD-1 exhibited a synergistic impaired effect on T cells proliferative capability (Fig. 3I). Taken together, our observations indicate that the expression of Gal-9 on T cells in HIV-infected individuals is associated with a functionally impaired phenotype.

2.3.4 Gal-9 expressing CD4 and CD8 T cells are intrinsically dysfunctional

We and others have already shown that Gal-9 interacts with different receptors expressed on immune cells (e.g., TIM-3, PDI, and CD44) (28, 31, 34, 36). Therefore, we decided to determine if impaired functionality of Gal-9+ T cells was mediated via cell-cell interactions (e.g., Gal-9: TIM-3) since total PBMCs were used in previous studies (Fig. 3). We decided to exclude T cell interaction with other cells by measuring cytokine production in isolated T cells instead of the whole PBMC. We observed that even in the absence of other immune cell components of PBMCs, both Gal-9+ CD4 and CD8 T cells exhibited impaired functionality and produced significantly lesser TNF-α and IFN-γ compared to Gal-9- CD4 and CD8 T cells (Fig. 4A-F). To reconfirm our observations, we blocked the interactions of Gal-9 with its potential receptors using an anti-Gal-9 blocking antibody. Interestingly, the Gal-9 blockade did not reverse the dysfunctional characteristic of Gal-9+ T cells when PBMCs were stimulated with anti-CD3/CD28 antibodies (Fig. 4G). This was also the case for anti-TIM-3 blocking antibody and lactose when

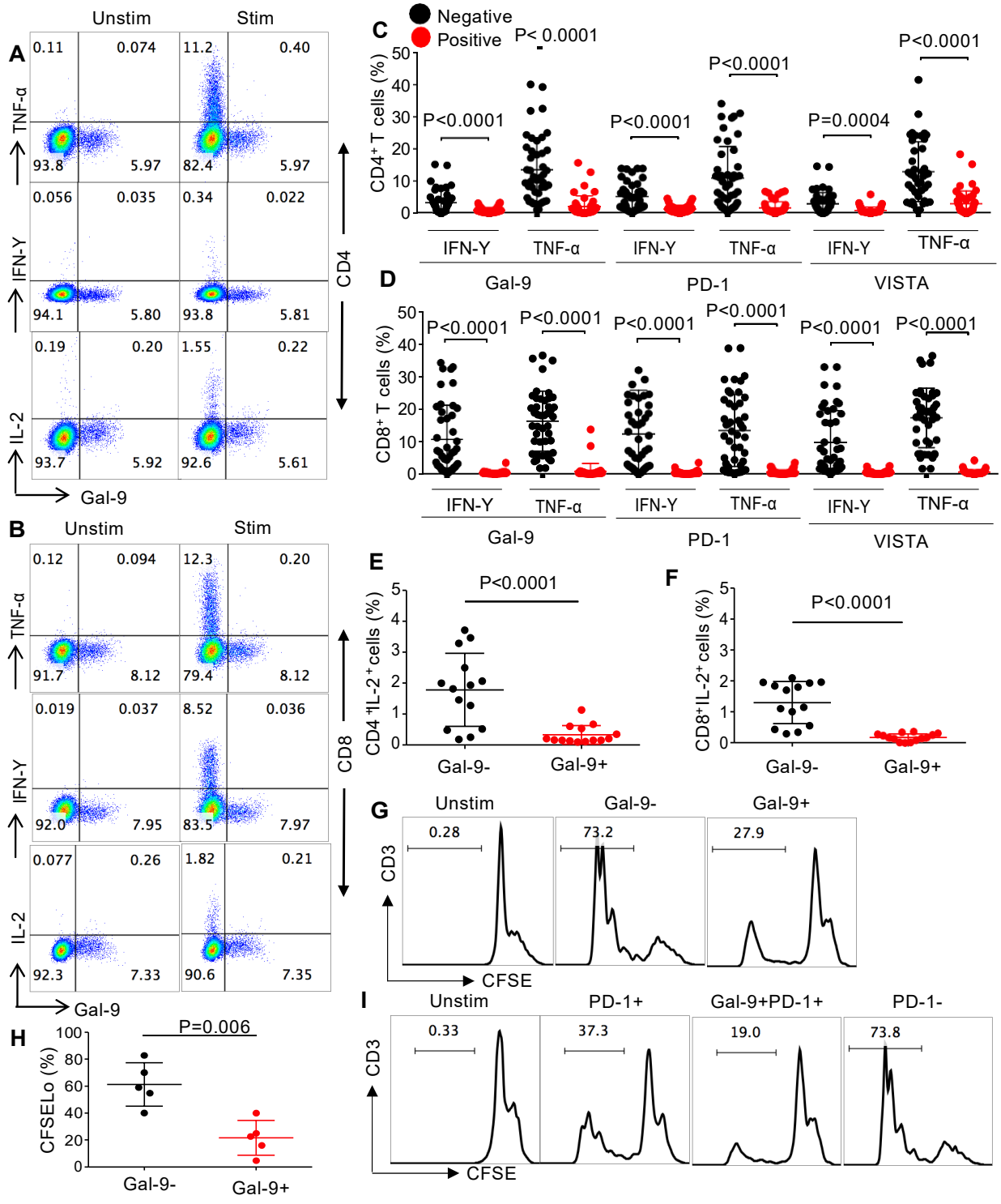


Fig. 3. Impaired effector functions of Gal-9+ T cells in PBMCs of HIV-infected individuals. A) Representative flow cytometry plots of TNF-α, IFN-γ and IL-2 production by Gal-9+ CD4 T cells, and **B**) Gal-9+ CD8 T cells versus Gal-9- T cells. **C**) Cumulative data of IFN-γ and TNF-α production levels in Gal-

9+ versus Gal-9- compared to PD-1+/PD-1- and VISTA+/VISTA- CD4 T cells, and **D**) CD8 T cells in HIV-infected individuals. **E**) Cumulative data of IL-2 production by Gal-9+ CD4, and **F**) Gal-9+ CD8 T cells versus Gal-9- T cells in HIV-infected individuals. **G**) Representative flow cytometry plots of proliferation (CFSE Lo) in Gal-9+ versus Gal-9- T cells. **H**) Cumulative data of T cells proliferation in Gal-9+ versus Gal-9- T cells. **I**) Representative flow cytometry plots of proliferation (CFSE Lo) in PD-1+ versus Gal-9+PD-1+ or PD-1- T cells.

added to stimulated PBMCs with anti-CD3/CD28 antibodies for 6 hr (Fig. 4H). Similarly, the addition of anti-TIM-3 antibody or lactose did not affect the cytokine production ability of Gal-9+ T cells when isolated T cells were stimulated with anti-CD3/CD28 antibodies (Fig. 4I). We even extended the time for cell culture studies (24 hours) in the presence of blocking antibodies against Gal-9/TIM-3 or lactose. Again, we found no reversal in the impaired functionality of Gal-9+ T cells. As shown in Fig. 2, co-expression of PD-1 and Gal-9 tempted us to restore Gal-9+ T cells functionality by using the anti-PD-1 antibody (Pembrolizumab). However, our attempts failed, and the anti-PD-1 antibody did not revive the functionality of Gal-9+ T cells *in vitro* (data not shown). Thus, our data indicate that Gal-9+ T cells are intrinsically dysfunctional, and co-expression of Gal-9 with PD-1 results in an irreversible dysfunctional phenotype.

2.3.5 The upregulation of Gal-9 is associated with impaired degranulation capacities of CD8 T cells in HIV-infected individuals

To determine the cytotoxic ability of Gal-9 expressing CD8 T cells, we compared the perforin and Granzyme B (GzmB) content of Gal-9+ and Gal-9- CD8 T cells. For the detection of perforin, we used δG9 clone, which detects the granule-associated form of perforin (47). We observed that the upregulation of Gal-9 on CD8 T cells was associated with a reduction in their granule-associated perforin compared to Gal-9- CD8 T cells (Fig. 5A, B). However, the frequency of GzmB expression in Gal-9 expressing CD8 T cells was not different from Gal-9- CD8 T cells (Fig. 5A-C). As the induction of apoptosis by cytotoxic T lymphocytes (CTLs) require the synergic action of both perforin and GzmB, we

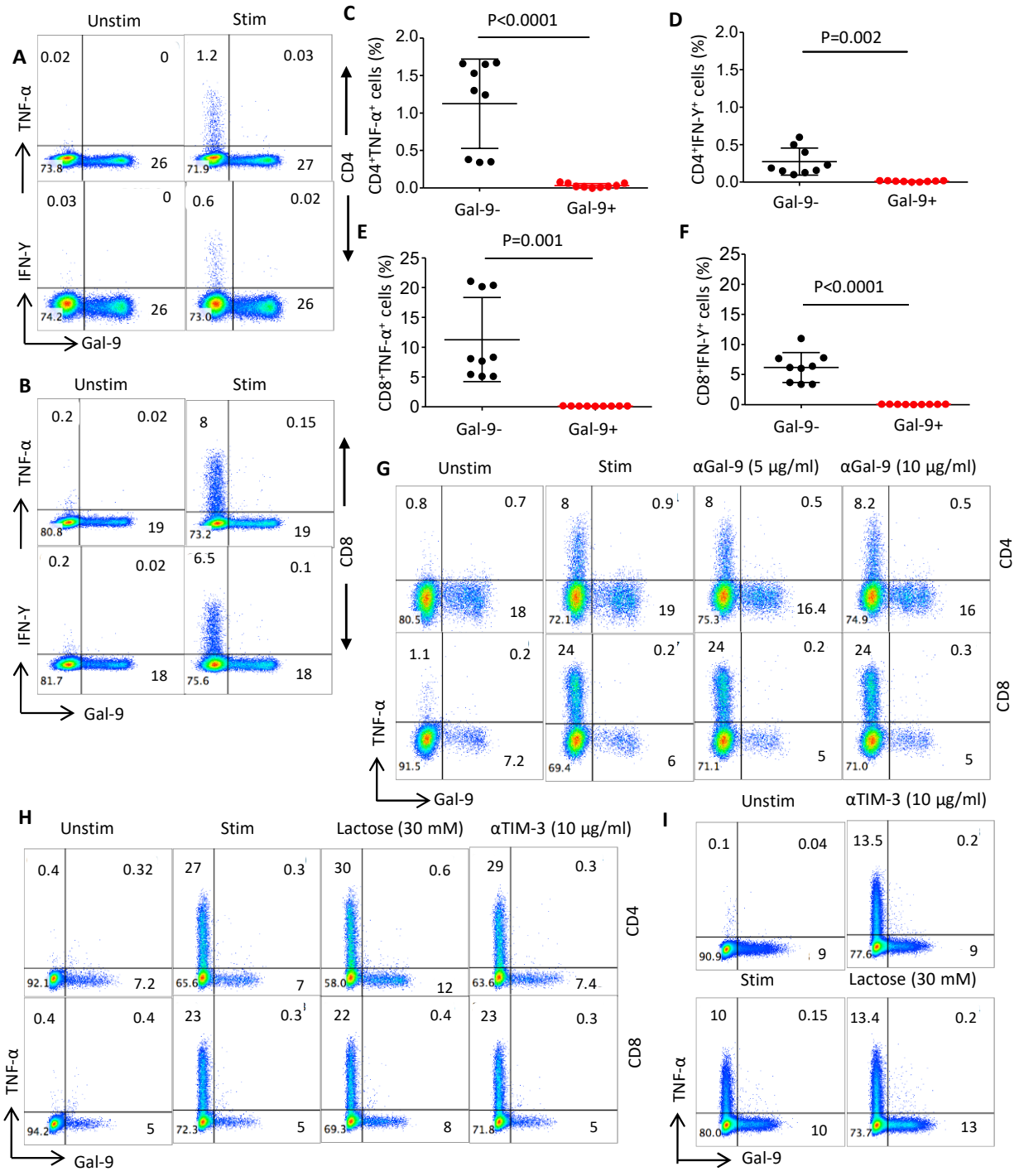


Fig. 4. Impaired effector functions of Gal-9+ T cells when isolated from PBMCs. A) Representative flow cytometry plots of TNF-α and IFN-γ by Gal-9+ CD4 T cells, and **B**) Gal-9+ CD8 T cells versus Gal-9- T cells in isolated T cells of an HIV-infected individual. **C**) Cumulative data of TNF-α and **D**) IFN-γ production in Gal-9+ versus Gal-9- CD4 T cells. **E**) Cumulative data of TNF-α and **F**) IFN-γ production in Gal-9+ versus Gal-9- CD8 T cells in isolated T cells of HIV-infected individuals. **G**) Representative flow cytometry plots

of TNF-α production by Gal-9+ CD4 and CD8 T cells in the presence or absence of anti-Gal-9 (aGal-9 5 and 10 μg/ml) blocking antibody. **H**) Representative flow cytometry plots of TNF-α production by Gal-9+ CD4 and CD8 T cells in the presence or absence of lactose (30 mM) or anti-TIM-3 blocking antibody (aTIM-3 10μg/ml) when total PBMCs were used. **I**) Representative flow cytometry plots of TNF-α production by Gal-9+ T cells in the presence or absence of aTIM-3 (10μg/ml) or lactose (30 mM) when isolated T cells were used.

examined co-expression of perforin and GzmB in Gal-9+ vs Gal-9- CD8 T cells. As shown in Fig. 5D, we observed Gal-9+ CD8 T cells had a significantly lower frequency of perforin+ GzmB+ cells compared to Gal-9- CD8 T cells. These observations suggest that Gal-9+ CD8 T cells may exhibit lower perforin content due to constant degranulation. In addition, this phenotype was consistently observed in different groups of HIV-infected patients (data not shown). This led us to investigate the degranulation ability of Gal-9+ vs Gal-9- CD8 T cells by evaluating the expression of the lysosomal-associated membrane protein 1 (LAMP1, or CD107a). PBMCs from HIV-infected patients were stimulated with anti-CD3/CD28 for 6 hours during the time that they were stained for CD107a. We found that both before and after TCR stimulation, Gal-9+ CD8 T cells expressed significantly higher CD107a compared to Gal-9- CD8 T cells (Fig. 5E, F). However, after TCR stimulation, in contrast to Gal-9- CD8 T cells, which significantly upregulated CD107a, the expression of CD107a in Gal-9+ CD8 T cells remained unchanged (Fig. 5E-H). These observations suggest that Gal-9+ CD8 T cells might have been activated *in vivo* and, due to constant degranulation of their contents, exhibit lesser bulk of perforin in their granules. This suggests that they have been activated in vivo and are defective in terms of re-stimulation. In agreement with this data, it has been reported that tumour-infiltrating PD-1+ CD8 T cells have impaired cytotoxic activity (48). Thus, we compared the perforin-GzmB content of the cytotoxic granules and the degranulation status of PD-1+ and PD-1- CD8 T cells. Similar to Gal-9+ CD8 T cells, PD-1+ CD8 T cells had a lower frequency of perforin+ GzmB+ cells compared to PD-1- CD8 T cells (Fig. 5I, J). In addition, PD-1+ CD8 T cells had significantly more CD107a before TCR stimulation (Fig. 5K). However, similar to Gal-9+ CD8 T cells, while

PD-1+ CD8 T cells were unable to further increase CD107a expression after stimulation with anti-CD3/CD28, expression of CD107a was increased significantly in PD-1- CD8 T cells (Fig. 5K-M).

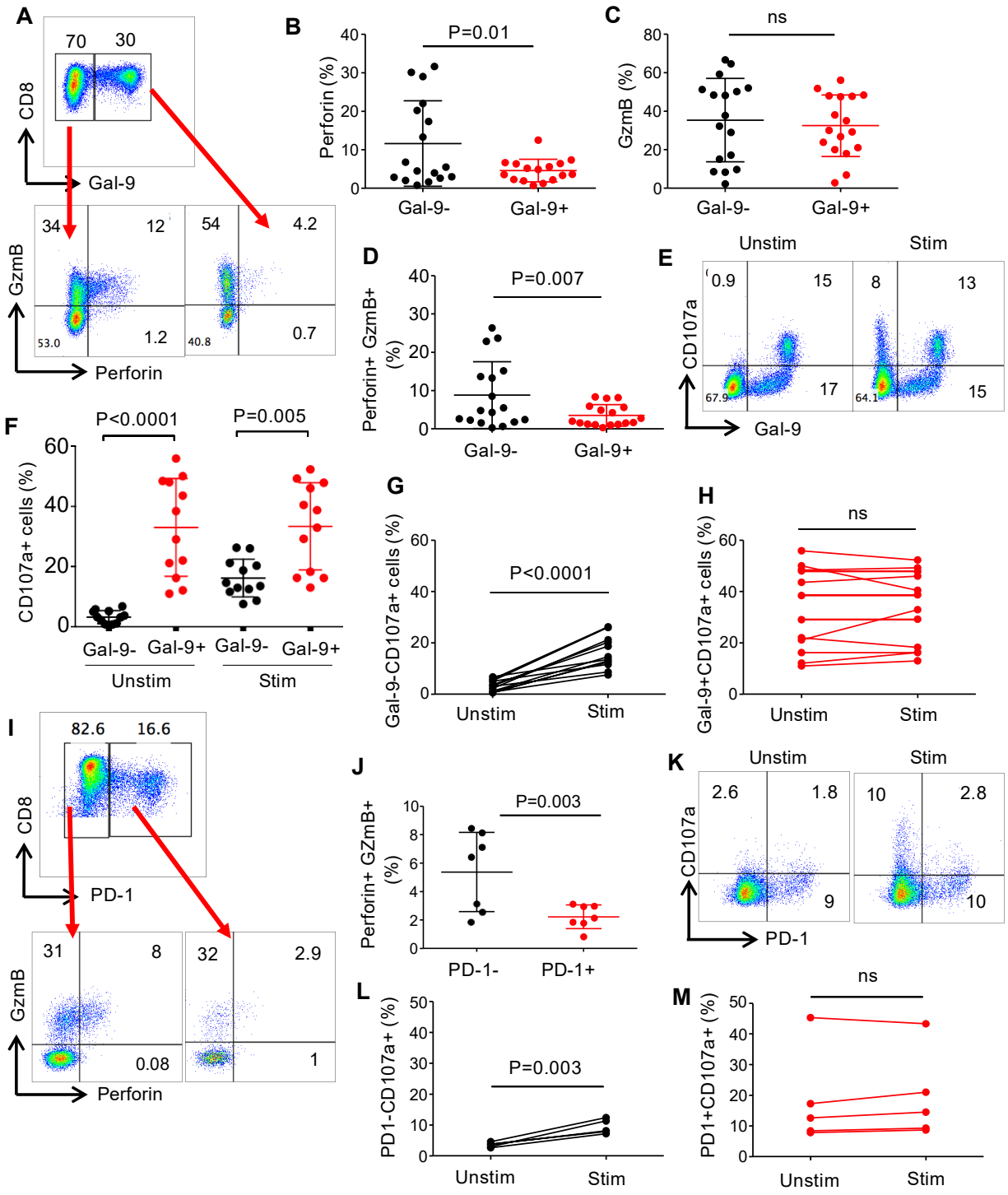


Fig. 5. Lower perforin and GzmB in Gal-9+ T cells. A) Representative flow cytometry plots showing expression of GzmB and perforin in Gal-9+ versus Gal-9- CD8 T cells. **B**) Cumulative data showing % perforin and **C**) % GzmB producing Gal-9- versus Gal-9+ CD8T cells. **D**) Cumulative data of % perforin+GzmB+ in Gal-9$^-$ versus Gal-9+ CD8 T cells. **E**) Representative flow cytometry plots of CD107a in Gal9-/Gal-9+ CD8 T cells pre and post-stimulation with anti-CD3/CD28. **F**) Cumulative data of CD107a in Gal9-/Gal-9+ CD8 T cells pre and post-stimulation with anti-CD3/CD28. **G**) Cumulative data of % CD107a in Gal-9$^-$, and **H**) Gal-9+ CD8 T cells with and without stimulation with anti-CD3/CD28. **I**) Representative flow cytometry plots showing expression of GzmB/perforin in PD-1+ versus PD-1- CD8 T cells. **J**) Cumulative data showing % GzmB in PD-1-/PD-1+CD8T cells. **K**) Representative flow cytometry plots of CD107a in PD-1-/PD-1+ CD8 T cells pre and post-stimulation with anti-CD3/CD28. **L**) Cumulative data of % CD107a in PD-1-, and **M**) PD-1+ CD8 T cells with and without stimulation with anti-CD3/CD28.

2.3.6 Gal-9 expressing T cells in HIV-infected individuals are effector T cells and mainly exhibit Th1 phenotype

Phenotypical characterization of Gal-9+ T cells was analyzed for the expression of FOXP3, T-bet, RORγt, and GATA-3, the lineage markers for Tregs, Th1, Th17, and Th2, respectively. Gal-9+ CD4 T cells appeared to be proportionally enriched with T-bet, followed by FOXP3, RORγt, and GATA-3 (Fig. 6A, B). Similarly, Gal-9+ CD8 T cells had higher expression of T-bet but appeared to express similar levels of RORγt and FOXP3, followed by lower GATA-3 expression (Fig. 6C, D).

In addition, we examined the expression of CD45RO, CD45RA, CD62L, and CD27 on Gal-9+ T cells to define their maturation and differentiation status. We observed that Gal-9+ CD4 T cells expressed high levels of CD45RA but very low levels of CD45RO and CD62L, suggesting an effector phenotype rather than naïve or memory phenotype (Fig. 6E, F). This was different for Gal-9+ CD8 T cells, which dominantly expressed CD45RA but moderate levels of CD45RO and CD62L (Fig. 6G, H). This indicated that Gal-9+ CD8 T cells were more of a heterogenous cell population. We also observed higher CD27+ T cells among Gal-9+ CD4 and CD8 T cells, which emphasises the heterogeneity of Gal-9+ T cells (Fig. 6F, H). However, we found that the intensity of CD27 was lower on Gal-9+ CD4 and CD8 T cells compared to Gal-9- T cells (Fig. 6I, J). Again, we found that different groups of HIV-infected individuals exhibit the same phenotype (data not shown). Our observations indicate that although Gal-9+ T cells represent an effector phenotype, they are a heterogeneous subpopulation of T cells in HIV-infected individuals.

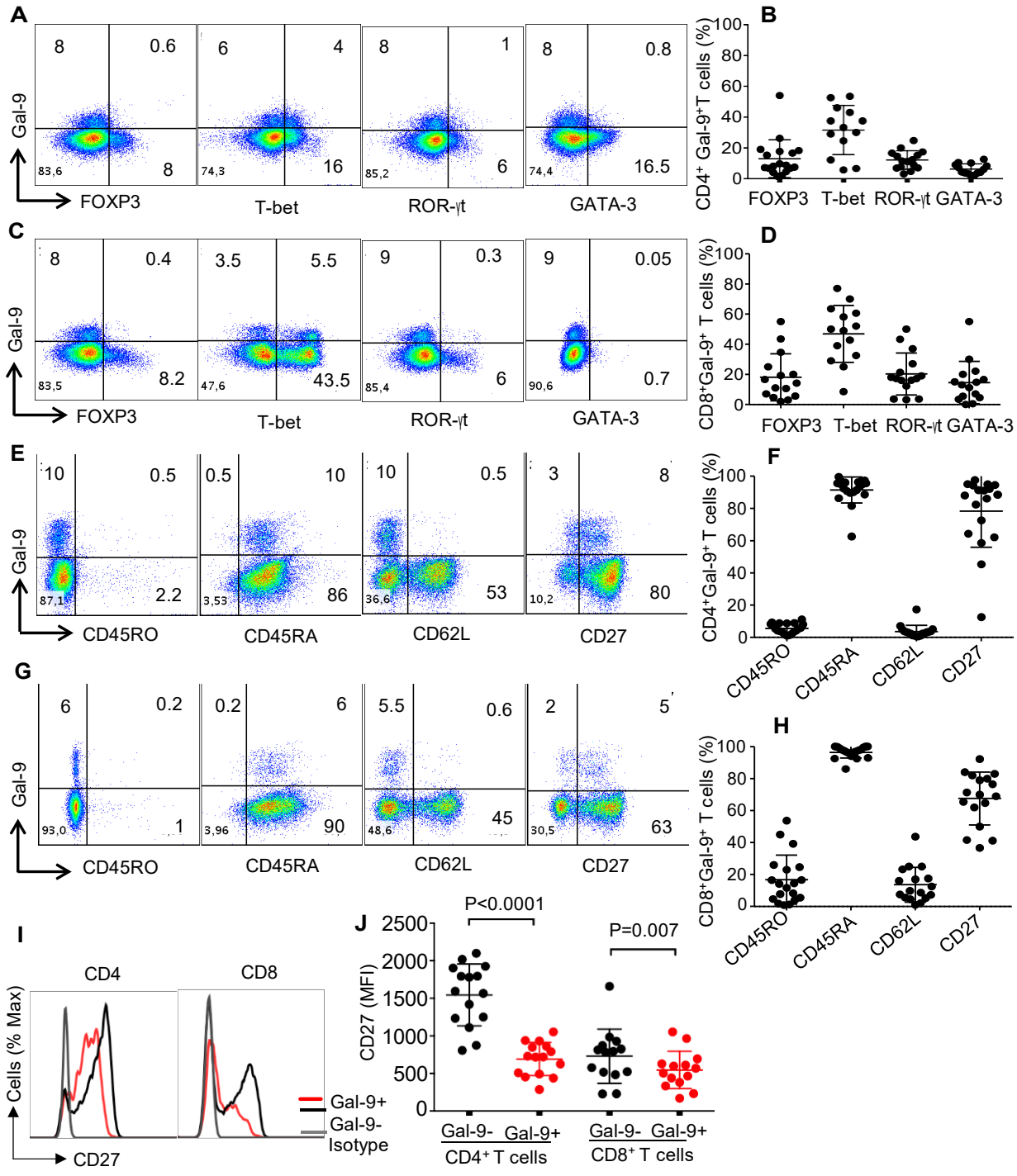


Fig. 6. Phenotypic characterization of Gal-9+ T cells in HIV-infected individuals. A) Representative flow cytometry plots of expression of FOXP3, T-bet, ROR-γt and GATA-3 in Gal-9⁺CD4 T cells. **B**) Cumulative data showing expression of FOXP3, T-bet, ROR-γt and GATA-3 in Gal-9⁺CD4 T cells. **C**) Representative flow cytometry plots of expression of FOXP3, T-bet, ROR-γt and GATA-3 in Gal-9⁺CD8 T cells. **D**)

Cumulative data showing expression of FOXP3, T-bet, ROR-γt and GATA-3 in Gal-9$^+$CD8 T cells. **E**) Representative flow cytometry plots of expression of CD45RO, CD45RA, CD62L and CD27 in Gal-9$^+$CD4 T cells. **F**) Cumulative data showing % expression of CD45RO, CD45RA, CD62L and CD27 in Gal-9$^+$CD4 T cells. **G**) Representative flow cytometry plots of expression of CD45RO, CD45RA, CD62L and CD27 in Gal-9$^+$CD8 T cells. **H**) Cumulative data showing % expression of CD45RO, CD45RA, CD62L and CD27 in Gal-9$^+$CD8 T cells. **I**) Representative flow cytometry plots of expression of CD27 in Gal-9$^+$CD4 and CD8 T cells. **J)** Quantification of MFI ± SEM of CD27 surface expression in Gal-9$^-$/Gal-9+ CD4 and CD8 T cells from HIV-infected individuals.

2.3.7 Differential expression of activation markers on Gal-9+ and Gal-9- T cells

To determine the activation status of Gal-9+ T cells, we investigated the expression of both early (CD25, CD122, and CD69) and late (HLA-DR, CD38) T cell activation markers (49-51) on Gal-9+ and Gal-9- T cells. Our results showed that compared to Gal-9- CD4 and CD8 T-cells, Gal-9+ CD4 and CD8 T cells had significantly higher CD25 expression without any *in vitro* stimulation at the baseline (Fig. 7A, B). Since our findings showed that PD-1+ CD4 and CD8 T cells exhibited similar functional characteristics as Gal-9+ CD4 and CD8 T cells (Fig. 5), we assessed the expression of CD25 on PD-1+ and PD-1- T cells. Interestingly we observed a similar pattern of CD25 expression on PD-1+ T cells compared to PD-1- T cells (Fig. 7C, D). In addition, we examined the expression of CD122 on Gal-9+ vs Gal-9- T cells and found a significantly higher expression of CD122 on Gal-9+ compared to Gal-9- T cells (Fig. 7E, F). Since CD25 and CD122 are the α and β subunits of the IL-2 receptor complex, we concluded that Gal-9+ T cells express higher IL-2 receptors on their surface. Although we did not observe any significant difference in CD69 expression levels between Gal-9+ and Gal-9- T cells at the baseline, *in vitro* stimulation with anti-CD3/CD28 enhanced CD69 expression on Gal-9- T cells but not on Gal-9+ T cells (data not shown).
Furthermore, we compared the expression of CD38 and HLA-DR on Gal-9+ vs Gal-9- T cells. Our studies indicated that while Gal-9+ CD4 T cells expressed significantly higher levels of HLA-DR compared to Gal-9- CD4 T cells, there was no significant difference in HLA-DR expression between Gal-9+ and Gal-9- CD8 T cells (Fig. 7G, H). In contrast, Gal-9+ CD8 expressed significantly higher CD38 compared to Gal-9- CD8 T

cells but there was no significant difference in CD38 expression on Gal-9+ and Gal-9- CD4 T cells (Fig. 7I-K). Finally, we compared the expression of Ki67, a nuclear protein that is expressed by proliferating cells

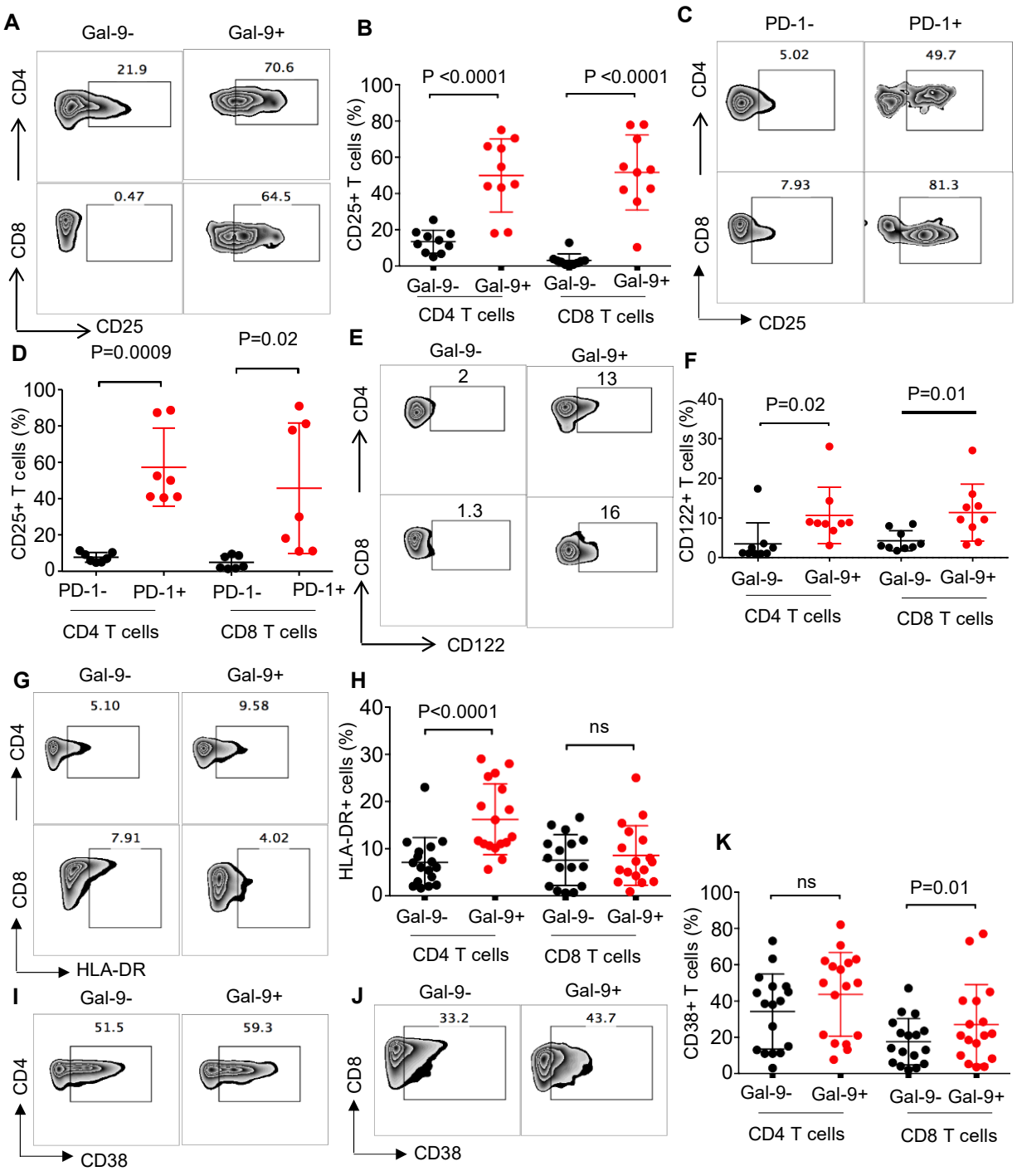


Fig. 7. Differential activation status of Gal-9+ T cells. A) Representative flow cytometry plots of CD25 expression on Gal-9-/Gal-9+ CD4 and CD8 T cells. **B**) Cumulative data of % CD25 expression on Gal-9-/Gal-9+ CD4 and CD8 T cells. **C**) Representative flow cytometry plots of CD25 expression on PD-1-/PD-1+

CD4 and CD8 T cells. **D**) Cumulative data of % CD25 expression on PD-1-/PD-1+ CD4 and CD8 T cells. **E**) Representative flow cytometry plots of CD122 expression on Gal-9-/Gal-9+ CD4 and CD8 T cells. **F**) Cumulative data of % CD122 expression on Gal-9-/Gal-9+ CD4 and CD8 T cells. **G**) Representative flow cytometry plots of HLA-DR expression on Gal-9-/Gal-9+ CD4 and CD8 T cells. **H**) Cumulative data of % HLA-DR expression on Gal-9-/Gal-9+ CD4 and CD8 T cells. **I**) Representative flow cytometry plots of CD38 expression on Gal-9-/Gal-9+ CD4+, and **J**) CD8 T cells. **K**) Cumulative data of % CD38 expression on Gal-9-/Gal-9+ CD4 and CD8 T cells.

(52). However, we did not observe any difference in Ki67 expression levels between Gal-9+ and Gal-9- T cells (data not shown).

2.3.8 Gal-9 expressing CD4 and CD8 T cells exhibit higher STAT-5 phosphorylation

Since we found higher expression of IL-2Rα and β subunits on Gal-9+ T cells, we decided to investigate the IL-2 downstream signalling pathways in Gal-9 expressing CD4 and CD8 T cells. IL-2 signalling occurs through two main pathways, STAT-5 and Raf-ERK MAP kinase cascade. When the STAT-5 pathway was analyzed, we found Gal-9+ CD4 T cells had a higher basal level of STAT-5 phosphorylation compared to Gal-9- CD4 T cells (Supplementary Fig. 2D, E). However, after *in vitro* stimulation with IL-2, while Gal-9- CD4 T cells increased phosphorylation of this molecule, it remained unchanged in Gal-9+ CD4 T cells (Supplementary Fig. 2F-I). This was also the case for Gal-9+ and Gal-9- CD8 T cells at the baseline (Supplementary Fig. 2J, K) and post IL-2 stimulation (Supplementary Fig. 2L-O). Hence, the phospho-STAT-5 remained unchanged following IL-2 stimulation in both Gal-9+ CD4 and CD8 T cells.

To determine the activity of the Raf-ERK MAP kinase cascade, we examined the phosphorylation of ERK. Contrary to STAT-5, we found that at the baseline, the ERK phosphorylation was not different between Gal-9+ and Gal-9- CD4 T cells (Supplementary Fig. 3A, B). However, in response to *in vitro* stimulation with PMA/Ionomycin, ERK phosphorylation increased significantly in Gal-9- CD4 T cells (Supplementary Fig. 3C-E). However, this was not the case for Gal-9+ CD4 T cells. Similar observations were noted for Gal-9+ and Gal-9- CD8 T cells (Supplementary Fig. 3F-J).

Because of the differential regulation of STAT-5 in Gal-9+ versus Gal-9- T cells, we decided to determine the expression of IL-4Rα and IL-12Rβ. Following Phosphorylation, STAT-5 does translocate into the

nucleus, where it induces the transcription of several genes. For instance, it upregulates the transcription of IL-4Rα and IL-12Rβ (53, 54). Therefore, we decided to determine the expression of these two cytokine receptors on Gal-9+ vs Gal-9- T cells. Interestingly, we found that the expression of IL-4Rα was significantly higher on both Gal-9+ CD4 and CD8 T cells compared to Gal-9- CD4 and CD8 T cells (Supplementary Fig. 3K-M). Similarly, Gal-9 expressing CD4 and CD8 T cells had significantly higher levels of IL-12Rβ compared to their negative counterparts (Supplementary Fig. 3N-P). In addition, it has been shown that IL-2 and IL-15 maintain CD154 (CD40L) expression on previously activated CD4 T cells through binding of STAT-5 to CD154 transcription promotor (55, 56). As such, we checked the expression of CD154 on Gal-9+ vs Gal-9- CD4 and CD8 T cells and observed a higher expression on Gal-9+ CD4 and CD8 T cells (Supplementary Fig. 3Q-S). Thus, our observations indicate that Gal-9+ T cells have a unique characteristic, distinguishing them from Gal-9- T cells.

2.3.9 Gal-9+ T cells are metabolically exhausted and express high EOMES and T-bet

Functional T cell exhaustion is accompanied by metabolic exhaustion characterized by increased glycolytic activity and high expression of glucose transporter-1 (Glut1) on their surface (57). Furthermore, activation of STAT-5 is associated with increased Glut1 on the human stem and progenitor cells (58). Therefore, we decided to examine the expression of Glut1 on T cells with respect to Gal-9 expression. Consistent with their exhausted phenotype, we observed that Gal-9+ CD4 and CD8 T cells had significantly higher expression of Glut1 on their surface (Fig. 8A-C).

T cell exhaustion is also accompanied by transcription programming with subsequent changes in the expression pattern of several genes. For example, T-bet and EOMES are two transcription factors, which work together to maintain the pool of exhausted CD8 T cells (59). We quantified the gene expression for T-bet and EOMES using qPCR in Gal-9+ and Gal-9- T cells isolated from the PBMCs of HIV-infected individuals. Interestingly, we found that Gal-9+ T cells had significantly higher expression of mRNA of both T-bet and EOMES (Fig. 8D, E). We also examined the expression of FOXO1 and FBXO38 genes,

which are associated with the maintenance and degradation of PD-1 on the T cell surface, respectively (60, 61). However, we did not observe any significant difference in the expression of these two genes between Gal-9+ and Gal-9- T cells (Fig. 8F, G).

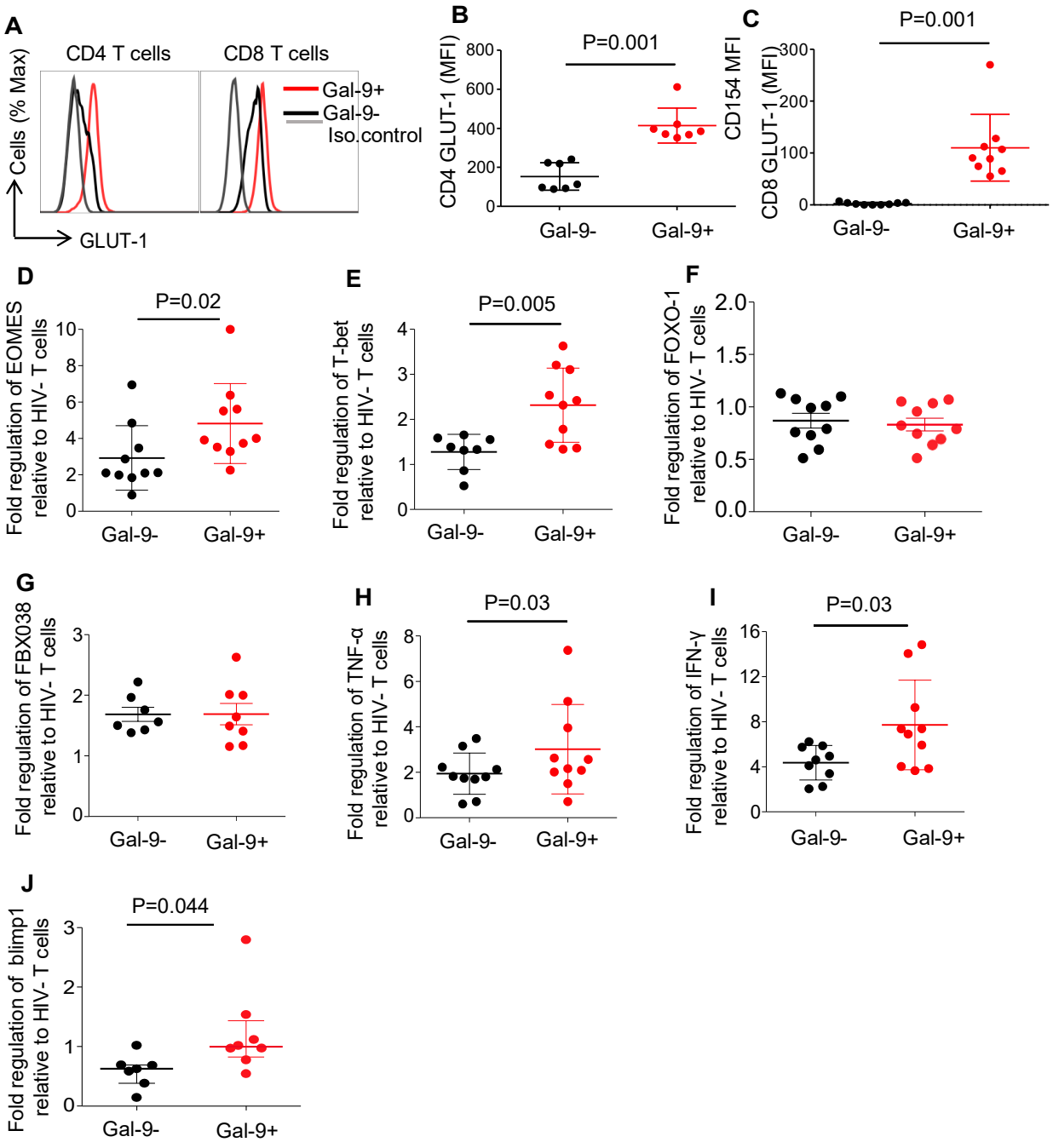


Fig. 8. Gal-9+ T cells are metabolically exhausted and express high EOMES and T-bet. A) Representative flow cytometry plots of GLUT-1 expression in Gal-9+/Gal-9- CD4 and CD8 T cells of HIV-infected individuals. **B**) Quantification of MFI ± SEM of GLUT-1 expression in Gal-9-/Gal-9+ CD4 and **C**) Gal-9-/Gal-

9+ CD8 T cells. **D**) Fold regulation of EOMES, **E**) T-bet, **F**) FOXO-1, **G**) FBX038, **H**) TNF-α, **I**) IFN-γ and **J**) blimp1 genes in Gal-9- versus Gal-9+ T cells relative to HIV-negative T cells quantified by qPCR.

Interestingly, we found significantly higher expression of mRNA for both TNF-α and IFN-γ in Gal-9+ versus Gal-9- T cells (Fig. 8H, I), which is in agreement with other studies showing continued higher mRNA expression for cytokines despite the severe defect in cytokine secretion ability of exhausted CD8 T cells (62). Finally, we found significant upregulation of blimp-1, which is associated with short-lived effector T cells of clonally exhausted T cells (63), in Gal-9+ versus Gal-9- T cells (Fig 8J).

2.3.10 T cell stimulation increases surface Gal-9 on T cells through activation of protein kinase C

What brings Gal-9 to the surface of T cells in HIV infected patients? It has been suggested that TIM-3 contributes to T cell exhaustion through enhanced TCR signalling (64). Furthermore, TCR signalling induces the expression of CD25 and prolongs STAT-5 activation (65, 66). Therefore, we examined CD3 expression on Gal-9+ T cells and compared it to Gal-9- T cells. Interestingly, we found significantly increased expression levels of CD3 on Gal-9+ compared to Gal-9- CD4 and CD8 T cells (Fig. 9A, B). Similarly, we found a significantly higher expression of CD3 on PD-1+ compared to PD-1- T cells (Fig. 9C, D). One of the downstream pathways of TCR signalling is the activation of protein kinase C (PKC) (67, 68). Since PMA also acts through PKC activation (69), we hypothesized that PMA stimulation might increase the expression of Gal-9 on the surface of T cells. To test this, PBMCs from HIV-infected individuals were stimulated with PMA (50 ng/ml) in the presence or absence of PKC Inhibitor (PKC Inh.). We observed that PMA stimulation increased Gal-9 expression on both CD4 and CD8 T cells (Fig. 9E-G), which was partially abrogated by PKC Inh (Fig. 9F, G).

Moreover, we decided to determine whether Gal-9 expressing T cells have Gal-9 secreting capability, as reported for a small subset of CD4 T cells (70). We isolated Gal-9+ and Gal-9- T cells from the PBMC of HIV-infected individuals and cultured them for 48 hrs with or without TCR stimulation. After this time, culture supernatants were subjected to Gal-9 quantification by ELISA. Although TCR stimulation did not significantly enhance Gal-9 secretion by Gal-9+ T cells, our results demonstrated that Gal-9+ T cells from

HIV-infected individuals were capable of secreting significantly higher levels of soluble Gal-9 compared to Gal-9- T cells *in vitro* (Fig. 9H).

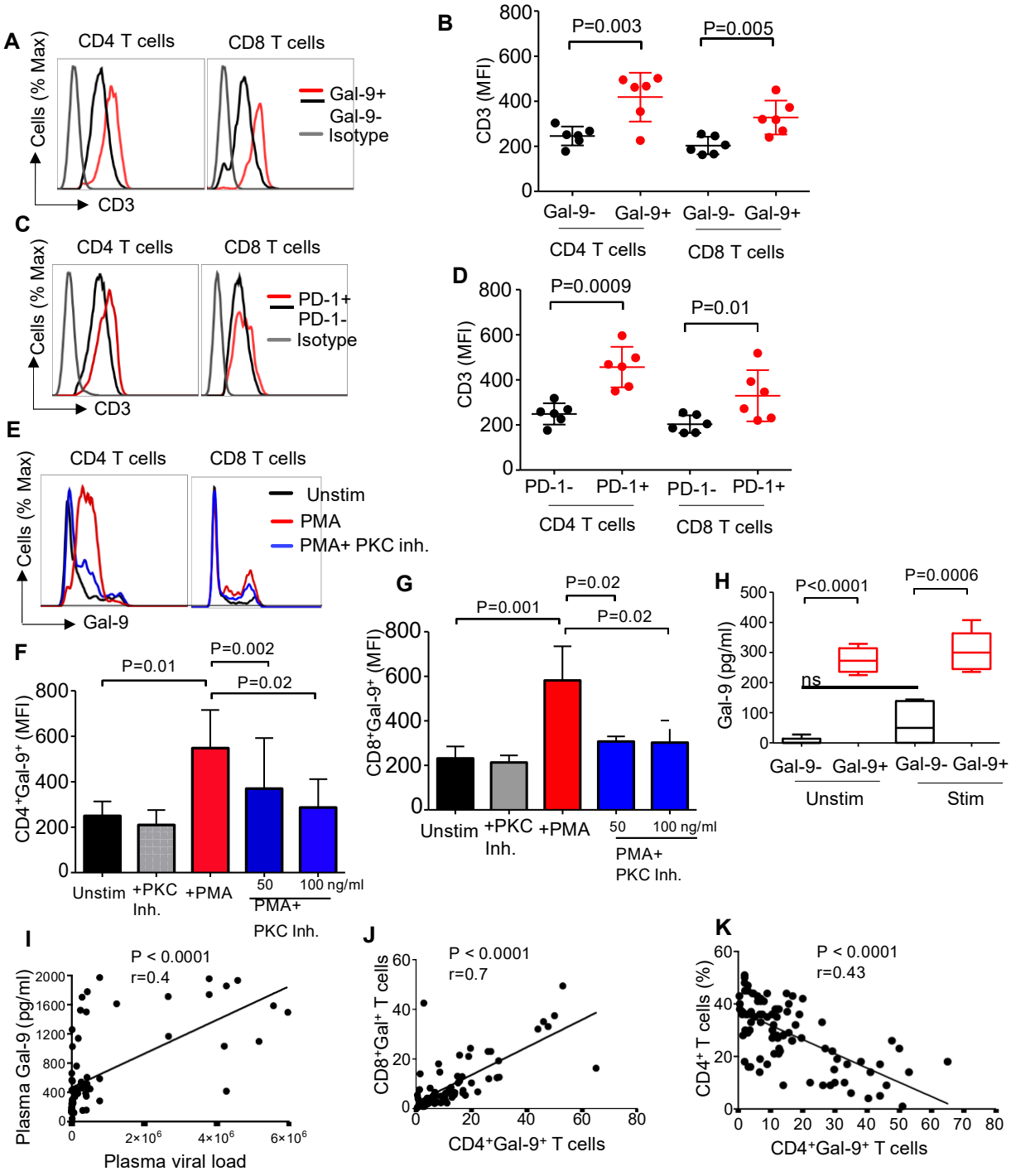


Fig. 9. Immune activation increases surface Gal-9 expression on T cells. A) Representative flow cytometry histogram of CD3 expression on Gal-9-/Gal-9+ CD4 and CD8 T cells of HIV-infected individuals. **B**) Quantification of MFI ± SEM of CD3 expression on Gal-9-/Gal-9+ CD4 and CD8 T cells. **C**) Representative flow cytometry histogram of CD3 expression on PD-1-/PD-1+ CD4 and CD8 T cells of HIV-infected individuals. **D**) Quantification of MFI ± SEM of CD3 expression on PD-1-/PD-1+ CD4 and CD8 T cells. **E**) Representative flow cytometry histogram of Gal-9 expression on CD4 and CD8 T cells of HIV-infected individuals following stimulation with PMA in the presence or absence of protein Kinase C inhibitor (PKC inh, abcam) (50 and 100 ng/ml). **F**) Quantification of MFI ± SEM of Gal-9 expression on CD4 T cells in the presence of PMA and/or PKC inhibitor and **G**) CD8 T cells. **H**) Quantification of soluble Gal-9 levels secreted by Gal-9- versus Gal-9+ T cells of HIV-infected individuals in the presence or absence of stimulation with anti-CD3/CD28 overnight as measured by ELISA. **I**) Cumulative data showing the correlation between the plasma Gal-9 levels with plasma viral load in HIV-infected individuals, either ART-interrupted or ART-naïve. **J**) Cumulative data showing the correlation of Gal-9 expression on CD4 with CD8 T cells in HIV-infected individuals on ART. **K**) Cumulative data of correlation between CD4 T cells count with Gal-9 expression on CD4 T cells in HIV-infected individuals on ART.

In agreement with our previous report (34), a positive correlation between the plasma viral load and plasma Gal-9 was observed (Fig. 9I). Although we did not find any correlation between the plasma Gal-9 levels in HIV-infected individuals with the frequency of Gal-9+ T cells (Supplementary Fig. 4A, B), a significant positive correlation between the frequency of Gal-9 expressing CD4 with Gal-9+ CD8 T cells was observed (Fig. 9J). On the contrary, a reverse correlation between the percent CD4 T cells in patients with the frequency of Gal-9+CD4 T cells was noted (Fig. 9K). This may explain higher apoptosis in Gal-9+ T cells (Supplementary Fig. 4C, D) as reported for PD-1+ T cells (71).

2.3.11 Gal-9 clustering CD44 influences T cells activation

A recent study has shown that recombinant Gal-9 (rGal-9) interacts with TCR, leading to the activation of TCR downstream signalling pathways (38). Since we found Gal-9+ T cells secrete rGal-9 and have higher expression of TCR, we asked whether Gal-9 binds to TCR on the surface of T cells. Also, because Gal-9 lacks a signal sequence, we decided to understand better how the surface expression of Gal-9 was associated with an impaired T cell phenotype. We observed that Gal-9 was colocalized with CD44 and CD3 on T cells (FIG. 10A and Supplementary Fig. 4E, F). More importantly, this interaction resulted in CD44 clustering (Fig. 10B-E). Under normal physiological conditions, CD44 may support T cell activation

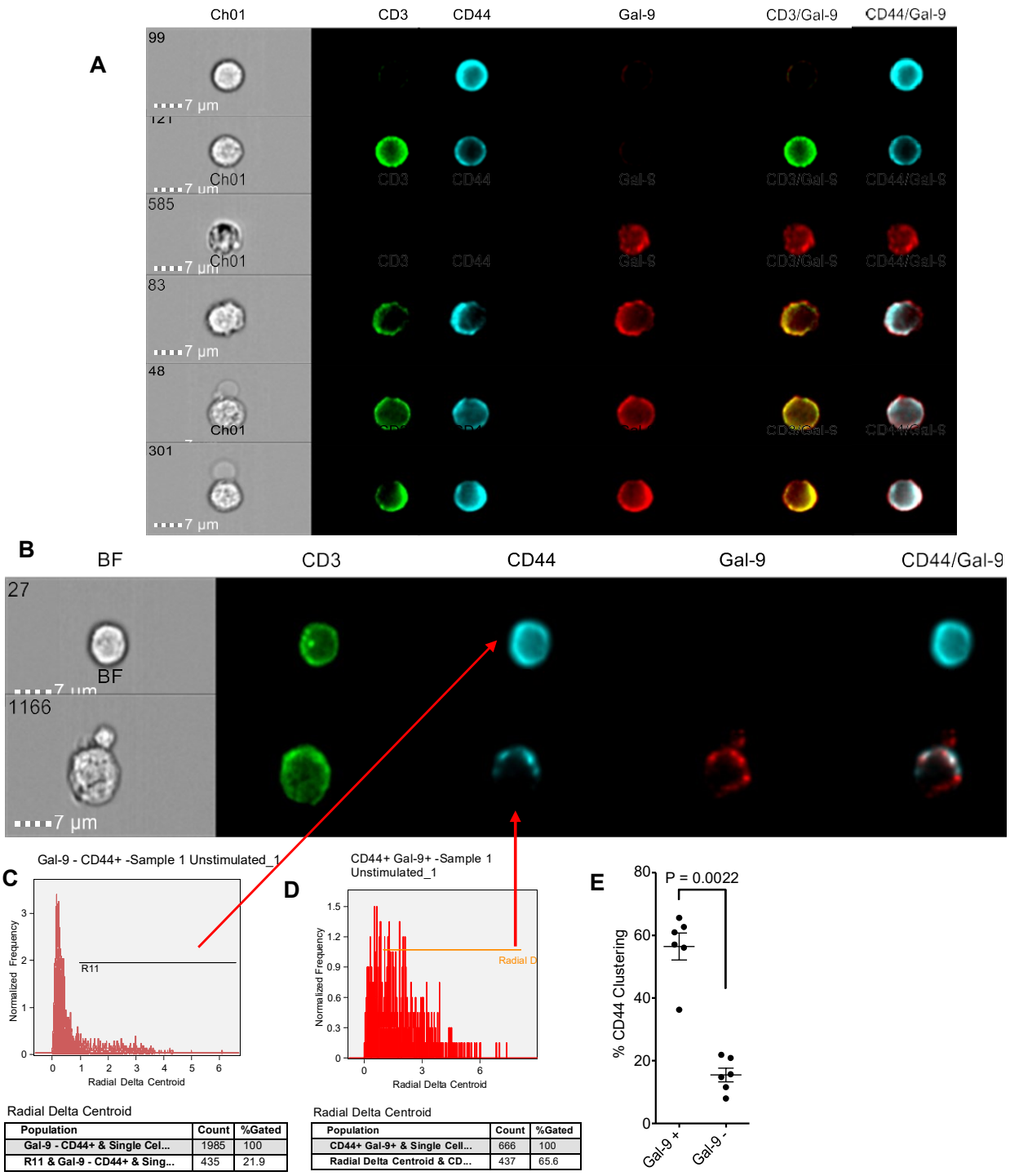


Fig. 10. Gal-9 clustering CD44 influences T cells activation. A) Image stream plots of Gal-9 colocalization with CD3 and CD44. **B)** Representative Image stream of Gal-9 clustering CD44 on T cells. **C**) Plot showing % clustering of CD44 on Gal-9- and **D**) Gal-9+ T cells. **E)** Cumulative data showing % CD44 clustering on Gal-9+ versus Gal9- T cells.

and apoptosis by clustering of the CD3/TCR complex and recruiting additional membrane receptors (72). Therefore, the interaction of Gal-9 with either CD44 or CD3 can directly or indirectly influence T cell function.

2.3.12 Upregulation of Gal-9 on T cells in other chronic conditions

To determine whether overexpression of Gal-9 is a feature of HIV infection or can be observed in other chronic viral infections, we measured the expression of Gal-9 on PBMCs from the hepatitis B virus-infected (HBV) and human papillomavirus-associated head and neck cancer patients. Our preliminary data confirm the upregulation of Gal-9 on T cells in these patients (Supplementary Fig. 4G, H).

2.4 Discussion

Consistent with previous studies, in this report, we show significantly higher expression of TIGIT, 2B4, CD39, PD-1, and CD160 expressing CD4 and CD8 T cells from HIV-infected individuals compared to HCs (44, 45, 73). The expression of TIM-3+ CD4 T cells from HIV-infected individuals was significantly higher than HCs in our cohort. However, contrary to the previous reports (46), we did not observe an increased percentage of TIM-3 expressing CD8 T cells in our HIV cohort compared to HCs. This discrepancy might be due to the differences in sample size and other characteristics of different HIV-cohorts. More importantly, our studies were conducted on freshly isolated PBMCs from HIV-infected and healthy individuals, which has not been the case for the previous studies, where frozen PBMCs have been used. We observed a significantly higher abundance of VISTA expressing CD4 and CD8 T cells in HIV-infected individuals compared to HCs. Although overexpression of VISTA on monocytes from HIV-infected individuals has already been reported (74), this is the first report, to our knowledge, to show overexpansion of VISTA+ CD4 and CD8 T cells and its association with T cell impairment in HIV-infected individuals.

Although we and others have reported surface Gal-9 expression in a small subpopulation of Th1 and Tregs (25, 26, 70), upregulation of Gal-9+ T cells in chronic viral infections has never been reported

before. Thus, here for the very first time, we report higher percentage of Gal-9 expressing CD4 and CD8 T cells from HIV-infected individuals compared to HCs. The presence of soluble Gal-9 in the plasma of HIV-infected individuals has been studied and can bear multiple effects. For instance, the serum level of Gal-9 increases rapidly during the acute phase of HIV infection and although it decreases after the onset of ART, it remains at elevated levels throughout the course of the disease (34, 75, 76). We previously showed that while the interaction of soluble Gal-9 with TIM-3 on the surface of activated CD4 T cells renders them more resistant to HIV infection, it enhances HIV infection in resting CD4 T cells when interacts with a different receptor, PDI (11). We also demonstrated that Gal-9 expressing Tregs via interaction with TIM-3 suppress the proliferative ability of HIV-specific CD8 T cells (25, 28). In addition, it has been reported that Gal-9 reverses HIV latency in the J-Lat HIV latency model and in primary CD4 T cells from HIV-infected patients. Moreover, rGal-9 induces the expression of the host antiviral deaminase APOBEC3G both *in vitro* and *ex vivo* resulting in reduced infectivity of the virus. Thus, while rGal-9 increases the reactivity of HIV infection, it reduces the replenishment of HIV reservoirs after viral reactivation (37). More recently, we reported upregulation of Gal-9 expressing NK cells in HIV-infected individuals (77). Gal-9 expressing NK cells exhibited impaired expression of cytotoxic effector molecules GzmB, perforin and granulysin but enhanced IFN-γ production (77).

Here we report that HIV infection results in the upregulation of both Gal-9 expressing CD4 and CD8 T cells. Importantly, HIV-infected individuals on ART exhibited significantly higher Gal-9+ CD4 and CD8 T cells compared to naïve and LTNPs. This might be associated with the general hyper-immune activation observed in HIV patients despite ART (78). Lower percentage of Gal-9+ CD8 T cells in LTNPs might be another potential mechanism associated with natural protective immunity and polyfunctional of CD8 T cells in these individuals (27, 28).

Although co-expression of Gal-9 with multiple co-inhibitory receptors such as CD160, 2B4, TIGIT, CD39 and VISTA was noted, strikingly often PD-1 was co-expressed with Gal-9 on T cells. The simultaneous co-

expression of different co-inhibitory receptors on CD8 T cells correlates with the level of CD8 T cell exhaustion (1, 44). In particular, co-expression of PD-1 and TIM-3 defines highly dysfunctional T cells (79). In agreement with this, we found that Gal-9+ PD-1+ T cells have a lower proliferative capacity and cytokine production ability compared to T cells expressing either Gal-9 or PD-1. In parallel with previous reports, we saw a dramatic reduction in the production of cytokines by T cells expressing PD-1, CD160, CD39, TIM-3 and VISTA (1, 7, 80). In contrast to other co-inhibitory molecules, the pattern of cytokine production was not different between 2B4+ and 2B4- CD4 T cells and interestingly, 2B4+ CD8 T cells exhibited higher cytokine production capabilities compared to 2B4- CD8 T cells. This agrees with previous studies showing either stimulatory or inhibitory signals by 2B4 depending on its surface density, interaction with its ligand, cell type, and the relative abundance of adaptor molecules (63, 81). Our data demonstrates that the co-expression of Gal-9 with other co-inhibitory molecules is associated with a more dysfunctional T cell phenotype.

Our demonstration that blockade of the Gal-9 pathway cannot enhance T cell responses *ex vivo* clearly demonstrates that Gal-9 expression on T cells is associated with an irreversible dysfunctional phenotype. It should be noted that Gal-9 expressing T cells remain dysfunctional even in the absence of other immune cell lineages (e.g., antigen-presenting cells (APCs)), which suggests an intrinsic defect rather than cell-cell interaction explains their impaired phenotype. This agrees with a previous study showing that the effects of Gal-9 on NK cells cannot be revived in the presence of an anti-TIM-3 blocking antibody (82). Despite the fact that often Gal-9+ was co-expressed with PD-1, blockade of the PD-1 pathway alone or in combination with the Gal-9 pathway could not reverse their exhausted phenotype. This suggests that the upregulation of Gal-9 on T cells is associated with a more terminally differentiated subset of exhausted T cells, which cannot be 'reinvigorated' by antibody blockade (63).

Our further studies to characterise Gal-9+ T cells revealed that these cells primarily represent effector T cells (T_{EFF}) phenotype (CD45RA+CD45RO-CD62L-CD27-) and exhibit higher expression of t-bet, which is

associated with terminal differentiation of T_{EFF} during the late phase of the immune response (83). Terminally differentiated T_{EFF} degranulate rapidly in response to stimulation and produce a high amount of TNF-*α* and IFN-*γ* (84). Similarly, Gal-9+ CD8 T cells express more CD107a *ex vivo* and TNF-*α* and IFN-*γ* genes. Terminally differentiated T_{EFF} are IL-2 dependent (83, 84) and accordingly, compared to Gal-9- T cells, Gal-9+ T cells express higher levels of CD25 and CD122, the α and β subunits of IL-2 receptors, respectively. Analysis of signalling downstream of the IL-2 pathway revealed that the STAT-5 pathway was differentially activated in Gal-9+ cells compared to Gal-9- cells, the pathway which is upregulated in terminally differentiated $T_{EFF\ (84)}$. Subsequently, Gal-9 expressing T cells could not produce more cytokines or degranulate following TCR stimulation, which was similar to PD-1+ T cells. Moreover, the impaired proliferative capability of Gal-9+ T cells was noted. Therefore, chronic antigenic stimulation in the course of HIV infection derives the formation of exhausted T cells from terminally differentiated T_{EFF} (85, 86). Similarly, IL2Rβ-mediated signals during chronic viral infections induce terminal exhaustion in CD8 T cells (86). These findings demonstrate that Gal-9+ T cells represent a subpopulation of terminally differentiated T_{EFF} cells that go through exhaustion, possibly by chronic antigenic stimulation. The chronic immune activation results in spontaneous IFN-γ secretion (87), which subsequently can enhance the expression of Gal-9 (88).

It is worth noting that higher expression of IL-2 receptors on Gal-9+ T cells imparts an activation signal, including upregulation of IL-4R and IL-12R. Consequently, it renders them susceptible to the input from IL-12 and IL-4, the cytokines which promote the formation of Th1 and Th2 cells, respectively (54, 89). Higher expression of CD154 (CD40L) and IL-12R on Gal-9+ CD4 T cells might explain the mechanism of T-bet upregulation as CD154 derives the development of Th1 cells from CD4 T cells both directly and indirectly through the production of IL-12 by APCs (90, 91). Although it has been described that CD154 defines antigen-specific CD4 T cells in chronic infection (92), its high expression on both CD4 and CD8 T cells in our study argues against the antigen specificity of CD154 expressing CD4 T cells.

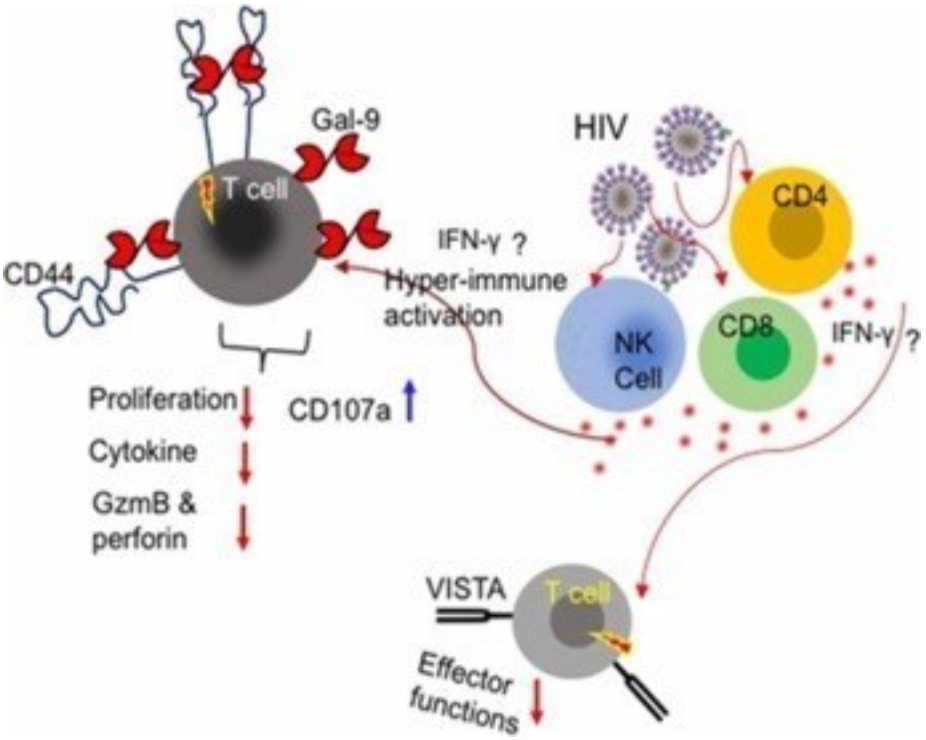
Glycolysis, accompanied by increased surface expression of Glut1 is considered to be the major metabolic pathway fueling effector functions following TCR activation (93). We found that Gal-9+ T cells, despite very limited effector functions, showed a marked increase in CD3 and Glut1 expression, which is consistent with the exhausted T cells phenotype (57, 94). Greater PKC activity in Gal-9+ T cells may explain the surface trafficking of Glut1 (95), which subsequently increases metabolic products secondary to glycolysis induced acidosis and apoptosis (96, 97). This has been reported for Glut1+ CD4 T in HIV-1 infection (57) and appears to be the case for Gal-9+ T cells.

The enhanced expression of Gal-9 following PMA stimulation via PKC could indicate persistent TCR stimulation as a potential factor. This suggests that Gal-9+ T cells may be highly activated *in vivo* and are defective in terms of re-stimulation *in vitro*. This was further emphasized by their higher degranulation capacity before *in vitro* re-stimulation. Persistent TCR signalling as a contributing factor for exhausted TIM-3+ T cells has already been reported (64). Similar to Gal-9+ T cells, higher STAT-5 phosphorylation in TIM-3+ CD8 T cells is reported (46). However, in contrary to TIM-3+ CD8 T cells, which have higher basal phosphorylation of Erk1/2 (46), Gal-9+ T cells have lower basal phosphorylation of Erk1/2. This suggests that both the co-inhibitory receptor (TIM-3) and its ligand (Gal-9) might utilize similar pathways to induce T cell exhaustion.

Gal-9 induces T cells activation or apoptosis using two different signalling pathways *in vitro* (98). The interactions of galectins with their surface ligands form a restrictive scaffold that inhibits TCR clustering required for TCR signalling (99). Our findings are based on the extracellular presence of Gal-9; however, the source of Gal-9 is unknown. We and others reported the abundance of Gal-9 in the plasma of HIV patients versus healthy controls (34, 76). Therefore, there is a possibility that the plasma Gal-9 binds to CD44 or CD3 on T cells, or it may be transported by unconventional pathways to the plasma membrane of T cells. Regardless of the source, our observations indicate that Gal-9 interacts with CD44 and/or CD3, which clusters CD44 on T cells. This CD44 clustering likely reorganizes the cytoskeleton and clusters

CD3/TCR complex that would result in lowering the threshold for signal transduction via CD3/TCR complex. As such, prolonged Gal-9 interaction with CD44 and CD3 influences membrane receptor reorganization accompanied by direct/indirect recruitment of signalling molecules. Therefore, Gal-9:CD44 possibly enhances T cell activation and/or apoptosis, which may provide further support for the increased T cells activation of Gal-9+ T cells and subsequently exhaustion in persistent antigen abundance scenarios. A schematic mechanism of Gal-9 function on CD8 T cells is shown in figure 11.

Fig. 11. The summary of the function of Gal-9 on T cells.



Taken together, our findings clearly demonstrate overexpression of Gal-9 and VISTA expressing T cells in HIV patients. More importantly, the co-expression of Gal-9 with other co-inhibitory is associated with a synergistic inhibitory signal. More importantly, as our preliminary data support, further studies are required to determine the role of Gal-9 in T cell exhaustion in other chronic viral infections. Such studies

will enable us to better understand the mechanism(s) associated with Gal-9 overexpression on T cells and how can be prevented. These approaches might allow for a personalized therapeutic intervention and a more comprehensive reversal of T cell exhaustion in HIV and other chronic viral infections.

2.5 Acknowledgement

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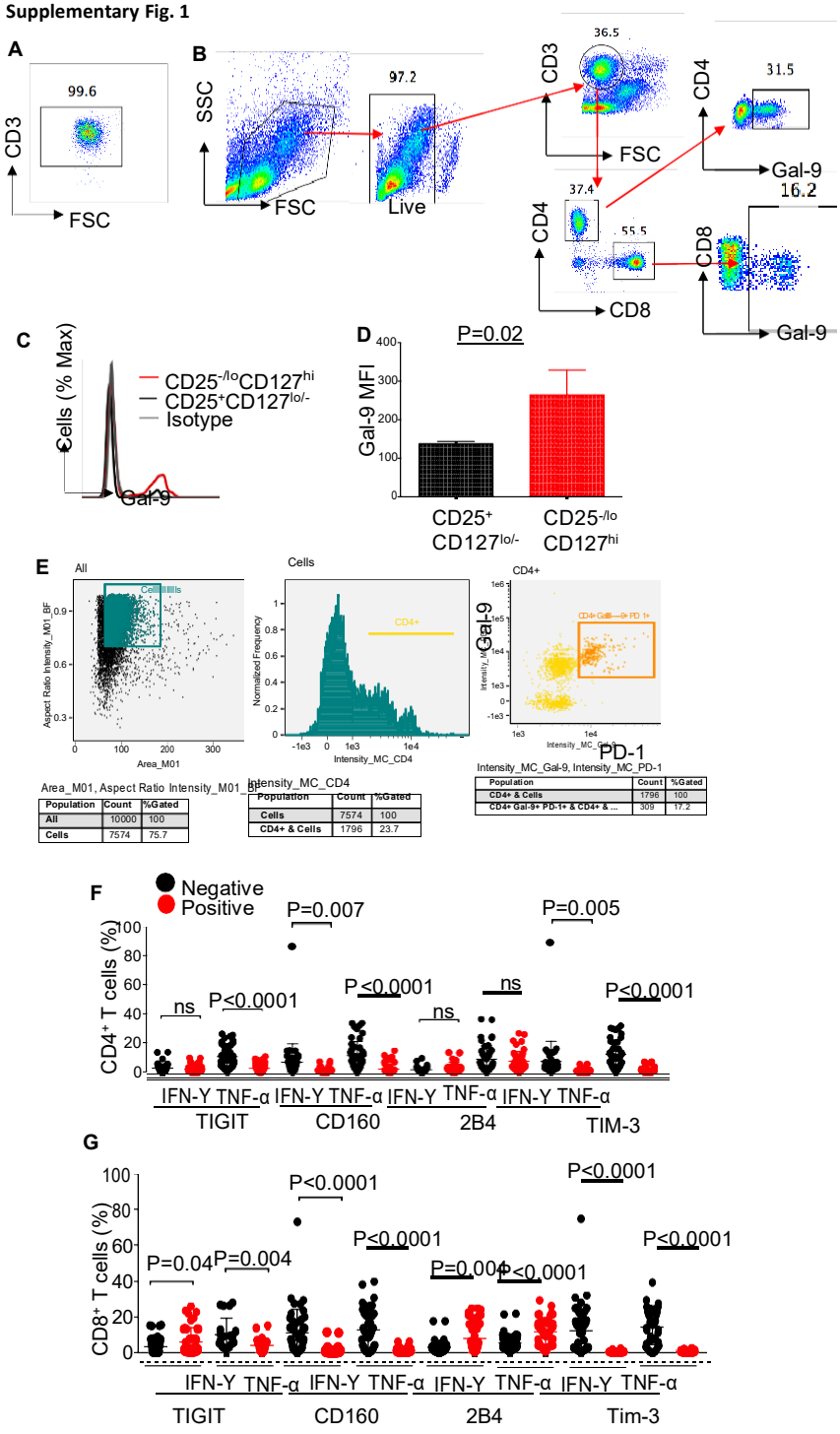
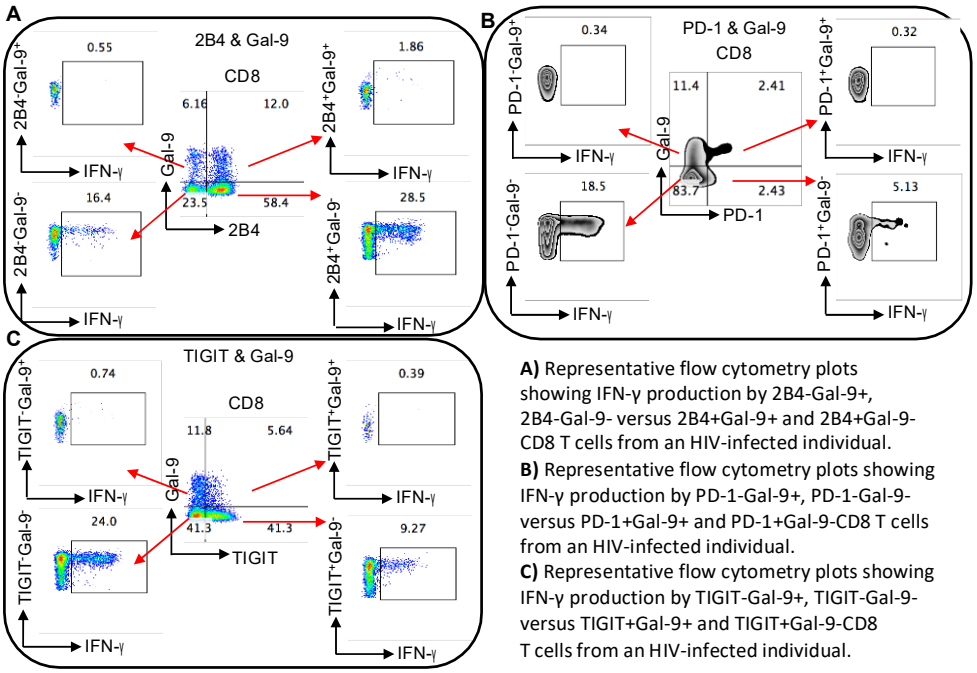
2.6 Supplementary figures

Supplementary Fig. 1

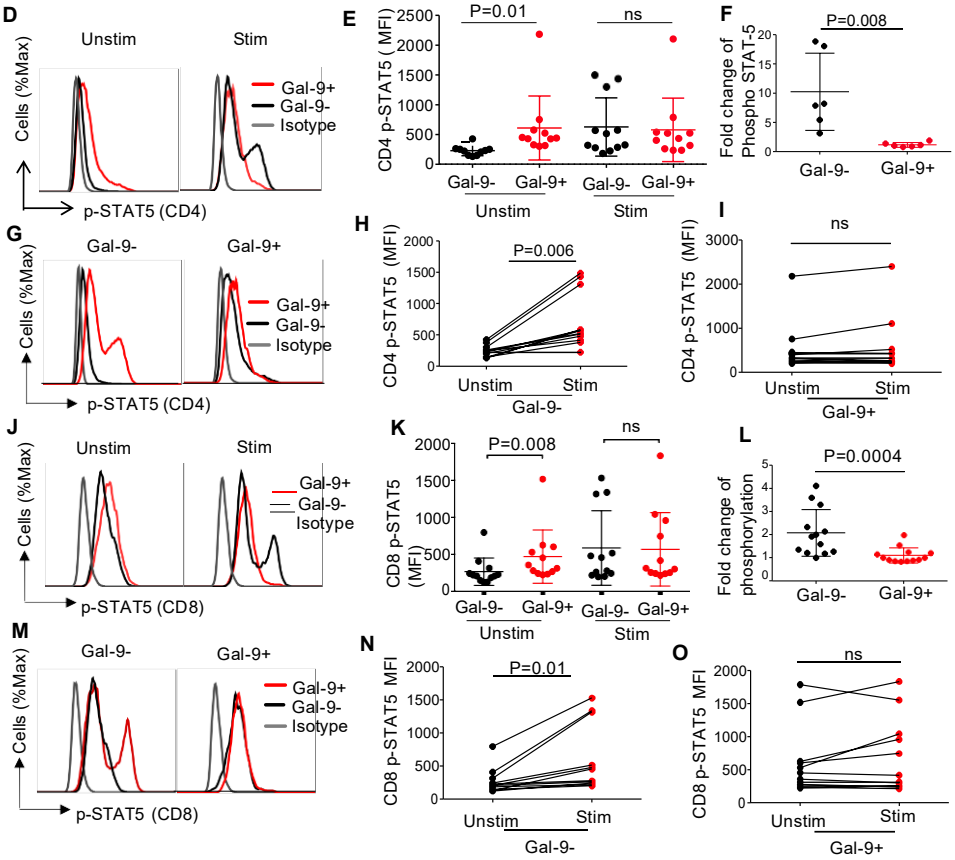


A) Flow cytometry plot showing the purity of isolated T cells. **B**) Representative plots showing gating strategy for defining Gal-9+ T cells. **C)** Representative flow cytometry histogram of Gal-9 expression on CD4+CD25-/^{lo}CD127^{hi} versus CD4+CD25+CD127$^{lo/-}$ of an HIV-infected individual. **D)** Quantification of MFI± SEM of Gal-9 expression on CD4+CD25-/^{lo}CD127^{hi} versus CD4+CD25+CD127$^{lo/-}$ of 10 HIV-infected individuals. **E**) Representative flow plots using image stream showing co-expression of Gal-9+PD-1+ on CD4 T cells. **F**) Cumulative data showing % TNF-α and IFN-γ production by CD4 T cells expressing TIGIT, CD160, TB4 and TIM-3 versus their negative counterparts. **G**) Cumulative data showing % TNF-α and IFN-γ production by CD8 T cells expressing TIGIT, CD160, TB4 and TIM-3 versus their negative counterparts.

Supplementary Fig. 2

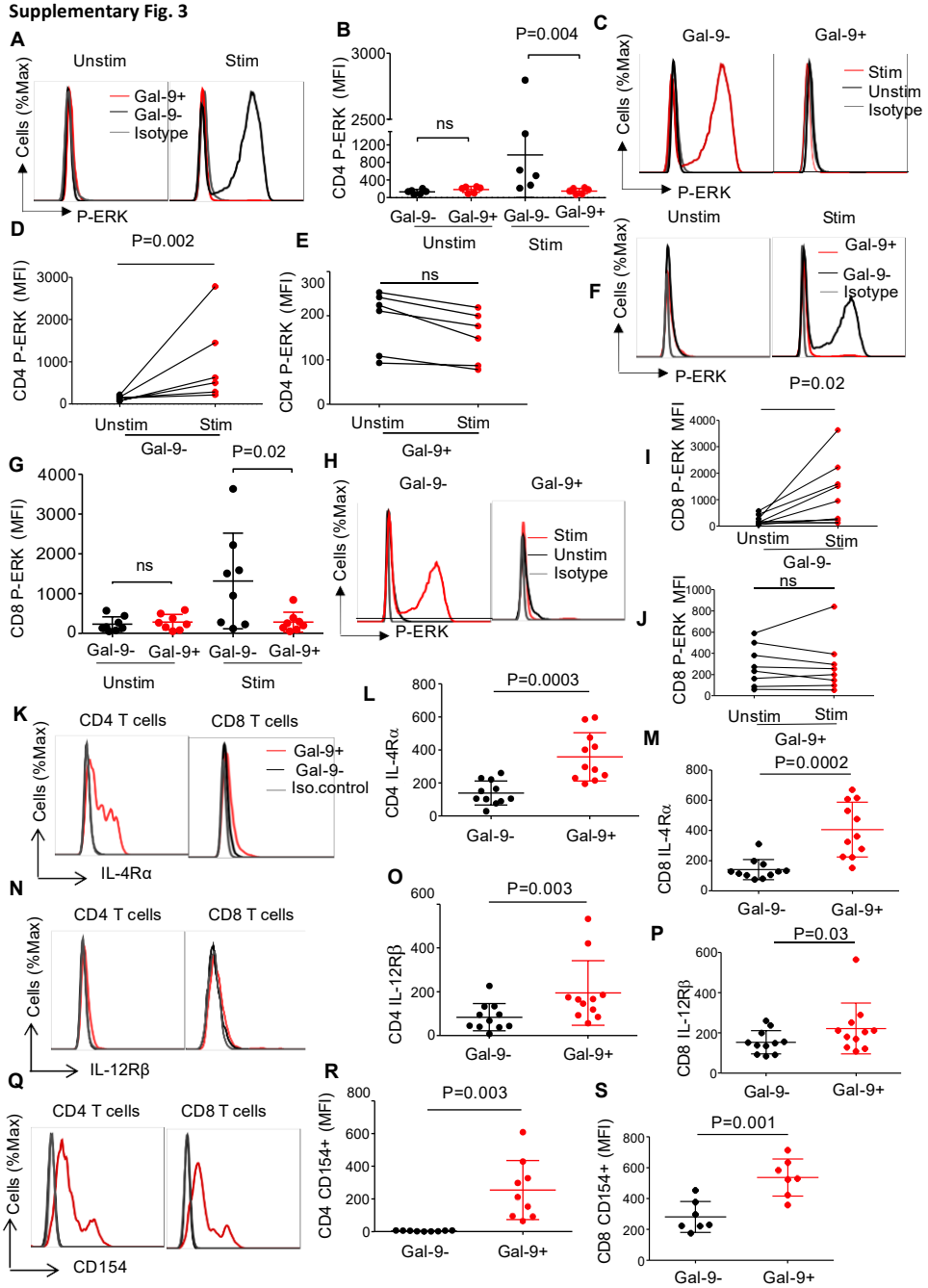


A) Representative flow cytometry plots showing IFN-γ production by 2B4-Gal-9+, 2B4-Gal-9- versus 2B4+Gal-9+ and 2B4+Gal-9- CD8 T cells from an HIV-infected individual.
B) Representative flow cytometry plots showing IFN-γ production by PD-1-Gal-9+, PD-1-Gal-9- versus PD-1+Gal-9+ and PD-1+Gal-9-CD8 T cells from an HIV-infected individual.
C) Representative flow cytometry plots showing IFN-γ production by TIGIT-Gal-9+, TIGIT-Gal-9- versus TIGIT+Gal-9+ and TIGIT+Gal-9-CD8 T cells from an HIV-infected individual.



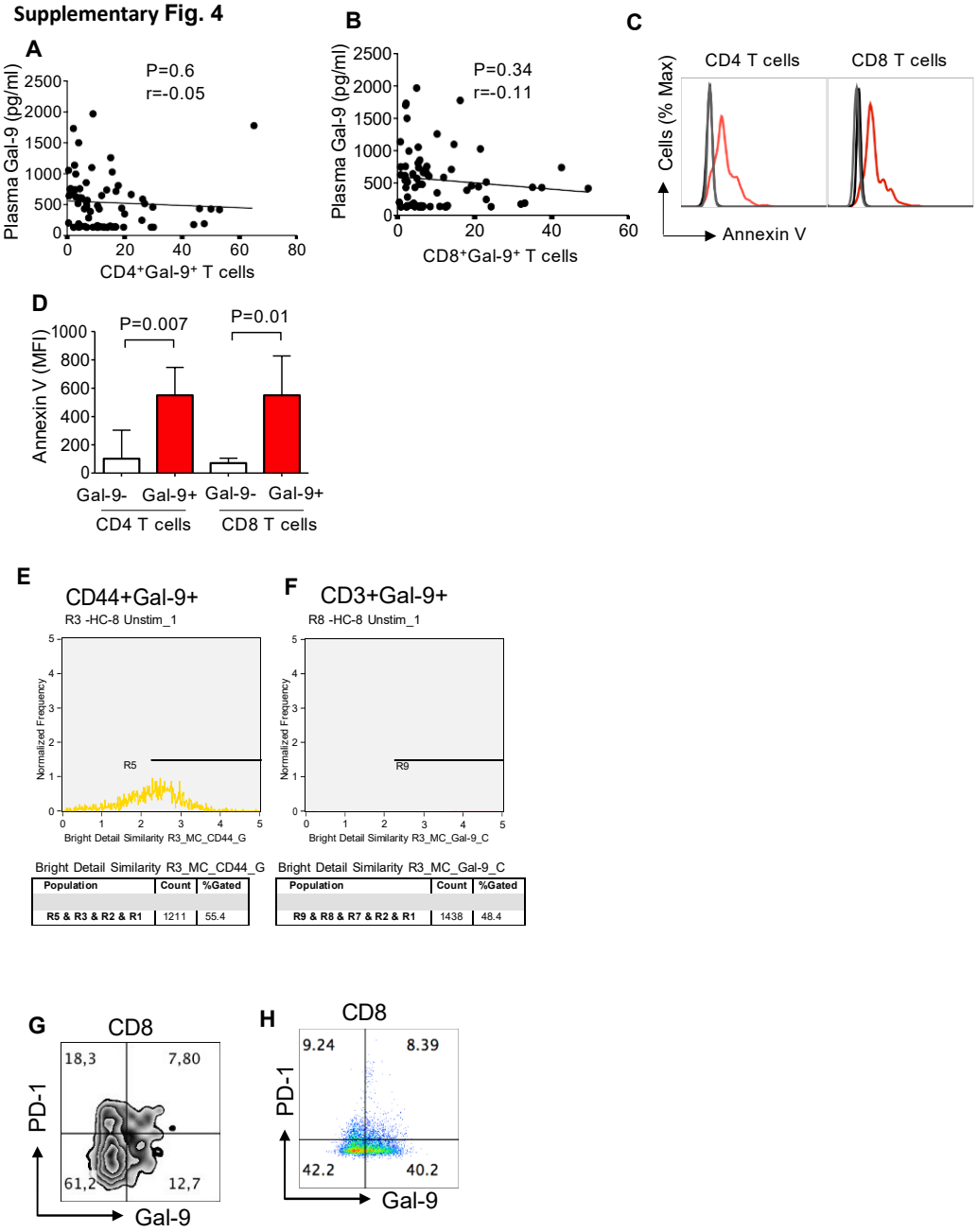
D) Representative flow cytometry plots of p-STAT5 in Gal-9+/Gal-9-CD4 T cells pre and post-IL-2 stimulation. **E**) MFI ± SEM of p-STAT5 expression in Gal-9-/Gal-9+ CD4 T cells pre and post-IL-2 stimulation (50 U/ml for 15 min). **F**) Fold change of p-STAT5 in Gal-9- versus Gal-9+ CD4 T cells. **G**) Representative flow cytometry plots of p-STAT5 in Gal-9+/Gal-9-CD4 T cells. **H**) MFI ± SEM of p-STAT5 expression in Gal-9-, and **I**) Gal-9+ CD4 T cells pre- and post-IL-2 stimulation. **J**) Representative flow cytometry plots of p-STAT5 in Gal-9+/Gal-9-CD8 T cells pre and post-IL-2 stimulation. **K**) MFI ± SEM of p-STAT5 expression in Gal-9-/Gal-9+ CD8 T cells pre and post-IL-2 stimulation (50 U/ml for 15 min). **L**) Fold change of p-STAT5 in Gal-9- versus Gal-9+ CD4 T cells. **M**) Representative flow cytometry plots of p-STAT5 in Gal-9+/Gal-9-CD8 T cells. **N**) MFI ± SEM of p-STAT5 expression in Gal-9-, and **O**) Gal-9+ CD8 T cells pre- and post-IL-2 stimulation.

Supplementary Fig. 3



A) Representative flow cytometry plots of p-ERK in Gal-9+/Gal-9- CD4 T cells pre and post-IL-2 stimulation. **B)** MFI ± SEM of p-ERK expression in Gal-9-/Gal-9+ CD4 T cells pre and post-PMA/ionomycin stimulation (81 nM & 1.3 ∝M). **C**) Representative flow cytometry plots of p-ERK in Gal-9+/Gal-9-CD4 T cells. **D**) MFI ± SEM of p-ERK expression in Gal-9-, and **E**) Gal-9+ CD4 T cells pre- and post-IL-2 stimulation. **F**) Representative flow cytometry plots of p-ERK in Gal-9+/Gal-9-CD8 T cells pre and post-IL-2 stimulation. **G**) MFI ± SEM of p-ERK expression in Gal-9-/Gal-9+ CD8 T cells pre and post-PMA/ionomycin stimulation ((81 nM & 1.3 ∝M). **H**) Representative flow cytometry plots of p-ERK in Gal-9+/Gal-9-CD8 T cells. **I**) MFI ± SEM of p-ERK expression in Gal-9-, and **J**) Gal-9+ CD8 T cells pre- and post-IL-2 stimulation. **K**) Representative flow cytometry histogram of IL-4 receptor-α (Rα) expression on Gal-9+/Gal-9- CD4 and CD8 T cells. **L**) MFI ± SEM of IL-4Rα expression on Gal-9-/Gal-9+ CD4, and **M**) CD8 T cells. **N**) Representative flow cytometry histogram of IL-12 receptor-β (Rβ) expression on Gal-9+/Gal-9- CD4 and CD8 T cells. **O**) MFI ± SEM of IL-12Rβ expression on Gal-9-/Gal-9+ CD4, and **P**) CD8 T cells. **Q**) Representative histogram plots of CD54 expression on Gal-9+/Gal-9- CD4 and CD8 T cells. **R**) MFI ± SEM of CD54 expression on Gal-9-/Gal-9+ CD4, and **S**)CD8 T cells.

Supplementary Fig. 4



A) Cumulative data showing correlation of plasma Gal-9 with the percentages of Gal-9+CD4 T cells, and **B**) Gal-9+CD8 T cells in HIV-infected individuals. **C**) Representative flow cytometry histogram of Annexin V expression on Gal-9+ versus Gal-9- CD4 and CD8 T cells in a HIV-patient. **D**) MFI ± SEM of Annexin V expression on Gal-9- and Gal-9+ CD4 and CD8 T cells. **E**) Data showing % colocalization of CD44 & Gal-9, and **F**) Colocalization of CD3 & Gal-9 on T cells. **G**) Representative plots showing PD-1/Gal-9 co-expression on CD8T cells from a HBV patient, and **H**) a patient with HPV associated head and neck cancer.

2.7 References

1. Okoye, I. S., M. Houghton, L. Tyrrell, K. Barakat, and S. Elahi. 2017. Coinhibitory Receptor Expression and Immune Checkpoint Blockade: Maintaining a Balance in CD8(+) T Cell Responses to Chronic Viral Infections and Cancer. *Frontiers in immunology* 8: 1215.
2. Doering, T. A., A. Crawford, J. M. Angelosanto, M. A. Paley, C. G. Ziegler, and E. J. Wherry. 2012. Network analysis reveals centrally connected genes and pathways involved in CD8+ T cell exhaustion versus memory. *Immunity* 37: 1130-1144.
3. Schietinger, A., and P. D. Greenberg. 2014. Tolerance and exhaustion: defining mechanisms of T cell dysfunction. *Trends Immunol* 35: 51-60.
4. Wherry, E. J. 2011. T cell exhaustion. *Nature immunology* 12: 492-499.
5. Wherry, E. J., and M. Kurachi. 2015. Molecular and cellular insights into T cell exhaustion. *Nature reviews. Immunology* 15: 486-499.
6. Lines, J. L., E. Pantazi, J. Mak, L. F. Sempere, L. Wang, S. O'Connell, S. Ceeraz, A. A. Suriawinata, S. Yan, M. S. Ernstoff, and R. Noelle. 2014. VISTA is an immune checkpoint molecule for human T cells. *Cancer Res* 74: 1924-1932.
7. Lines, J. L., L. F. Sempere, T. Broughton, L. Wang, and R. Noelle. 2014. VISTA is a novel broad-spectrum negative checkpoint regulator for cancer immunotherapy. *Cancer immunology research* 2: 510-517.
8. Mulati, K., J. Hamanishi, N. Matsumura, K. Chamoto, N. Mise, K. Abiko, T. Baba, K. Yamaguchi, N. Horikawa, R. Murakami, M. Taki, K. Budiman, X. Zeng, Y. Hosoe, M. Azuma, I. Konishi, and M. Mandai. 2019. VISTA expressed in tumour cells regulates T cell function. *Br J Cancer* 120: 115-127.
9. Shahbaz, S., N. Bozorgmehr, P. Koleva, A. Namdar, J. Jovel, R. A. Fava, and S. Elahi. 2018. CD71+VISTA+ erythroid cells promote the development and function of regulatory T cells through TGF-beta. *PLoS Biol* 16: e2006649.
10. Virgin, H. W., E. J. Wherry, and R. Ahmed. 2009. Redefining chronic viral infection. *Cell* 138: 30-50.
11. Hashimoto, M., A. O. Kamphorst, S. J. Im, H. T. Kissick, R. N. Pillai, S. S. Ramalingam, K. Araki, and R. Ahmed. 2018. CD8 T Cell Exhaustion in Chronic Infection and Cancer: Opportunities for Interventions. *Annu Rev Med* 69: 301-318.
12. Doitsh, G., and W. C. Greene. 2016. Dissecting How CD4 T Cells Are Lost During HIV Infection. *Cell host & microbe* 19: 280-291.
13. Elahi, S., R. H. Weiss, and S. Merani. 2016. Atorvastatin restricts HIV replication in CD4+ T cells by upregulation of p21. *Aids* 30: 171-183.
14. Janssen, E. M., E. E. Lemmens, T. Wolfe, U. Christen, M. G. von Herrath, and S. P. Schoenberger. 2003. CD4+ T cells are required for secondary expansion and memory in CD8+ T lymphocytes. *Nature* 421: 852-856.
15. Wang, J. C. E., and A. M. Livingstone. 2003. Cutting edge: CD4(+) T cell help can be essential for primary CD8(+) T cell responses in vivo. *Journal of immunology* 171: 6339-6343.
16. Crawford, A., J. M. Angelosanto, C. Kao, T. A. Doering, P. M. Odorizzi, B. E. Barnett, and E. J. Wherry. 2014. Molecular and transcription basis of CD4(+) T cell dysfunction during chronic infection. *Immunity* 40: 289-302.
17. Merani, S., W. Chen, and S. Elahi. 2015. The bitter side of sweet: the role of Galectin-9 in immunopathogenesis of viral infections. *Reviews in medical virology* 25: 175-186.
18. Li, Y., J. Feng, S. Geng, H. Wei, G. Chen, X. Li, L. Wang, R. Wang, H. Peng, G. Han, and B. Shen. 2011. The N- and C-terminal carbohydrate recognition domains of galectin-9 contribute differently to its multiple functions in innate immunity and adaptive immunity. *Mol Immunol* 48: 670-677.
19. Hirashima, M., Y. Kashio, N. Nishi, A. Yamauchi, T. A. Imaizumi, T. Kageshita, N. Saita, and T. Nakamura. 2004. Galectin-9 in physiological and pathological conditions. *Glycoconjugate journal* 19: 593-600.

20. Zhu, C., A. C. Anderson, A. Schubart, H. Xiong, J. Imitola, S. J. Khoury, X. X. Zheng, T. B. Strom, and V. K. Kuchroo. 2005. The TIM-3 ligand galectin-9 negatively regulates T helper type 1 immunity. *Nature immunology* 6: 1245-1252.
21. Bi, S., P. W. Hong, B. Lee, and L. G. Baum. 2011. Galectin-9 binding to cell surface protein disulfide isomerase regulates the redox environment to enhance T-cell migration and HIV entry. *Proc Natl Acad Sci U S A* 108: 10650-10655.
22. Niki, T., S. Tsutsui, S. Hirose, S. Aradono, Y. Sugimoto, K. Takeshita, N. Nishi, and M. Hirashima. 2009. Galectin-9 is a high affinity IgE-binding lectin with anti-allergic effect by blocking IgE-antigen complex formation. *The Journal of biological chemistry* 284: 32344-32352.
23. Madireddi, S., S. Y. Eun, S. W. Lee, I. Nemcovicova, A. K. Mehta, D. M. Zajonc, N. Nishi, T. Niki, M. Hirashima, and M. Croft. 2014. Galectin-9 controls the therapeutic activity of 4-1BB-targeting antibodies. *J Exp Med* 211: 1433-1448.
24. Tanikawa, R., T. Tanikawa, M. Hirashima, A. Yamauchi, and Y. Tanaka. 2010. Galectin-9 induces osteoblast differentiation through the CD44/Smad signaling pathway. *Biochem Biophys Res Commun* 394: 317-322.
25. Elahi, S., and H. Horton. 2012. Association of HLA-alleles with the immune regulation of chronic viral infections. *The international journal of biochemistry & cell biology* 44: 1361-1365.
26. Wang, F., L. Wan, C. Zhang, X. Zheng, J. Li, and Z. K. Chen. 2009. TIM-3-Galectin-9 pathway involves the suppression induced by CD4+CD25+ regulatory T cells. *Immunobiology* 214: 342-349.
27. Crux, N. B., and S. Elahi. 2017. Human Leukocyte Antigen (HLA) and Immune Regulation: How Do Classical and Non-Classical HLA Alleles Modulate Immune Response to Human Immunodeficiency Virus and Hepatitis C Virus Infections? *Frontiers in immunology* 8: 832.
28. Elahi, S., W. L. Dinges, N. Lejarcegui, K. J. Laing, A. C. Collier, D. M. Koelle, M. J. McElrath, and H. Horton. 2011. Protective HIV-specific CD8+ T cells evade Treg cell suppression. *Nature medicine* 17: 989-995.
29. Niwa, H., T. Satoh, Y. Matsushima, K. Hosoya, K. Saeki, T. Niki, M. Hirashima, and H. Yokozeki. 2009. Stable form of galectin-9, a TIM-3 ligand, inhibits contact hypersensitivity and psoriatic reactions: a potent therapeutic tool for Th1- and/or Th17-mediated skin inflammation. *Clin Immunol* 132: 184-194.
30. Seki, M., S. Oomizu, K. M. Sakata, A. Sakata, T. Arikawa, K. Watanabe, K. Ito, K. Takeshita, T. Niki, N. Saita, N. Nishi, A. Yamauchi, S. Katoh, A. Matsukawa, V. Kuchroo, and M. Hirashima. 2008. Galectin-9 suppresses the generation of Th17, promotes the induction of regulatory T cells, and regulates experimental autoimmune arthritis. *Clin Immunol* 127: 78-88.
31. Wu, C., T. Thalhamer, R. F. Franca, S. Xiao, C. Wang, C. Hotta, C. Zhu, M. Hirashima, A. C. Anderson, and V. K. Kuchroo. 2014. Galectin-9-CD44 Interaction Enhances Stability and Function of Adaptive Regulatory T Cells. *Immunity* 41: 270-282.
32. Sharma, S., A. Sundararajan, A. Suryawanshi, N. Kumar, T. Veiga-Parga, V. K. Kuchroo, P. G. Thomas, M. Y. Sangster, and B. T. Rouse. 2011. T cell immunoglobulin and mucin protein-3 (TIM-3)/Galectin-9 interaction regulates influenza A virus-specific humoral and CD8 T-cell responses. *Proceedings of the National Academy of Sciences of the United States of America* 108: 19001-19006.
33. Chagan-Yasutan, H., H. Saitoh, Y. Ashino, T. Arikawa, M. Hirashima, S. Li, M. Usuzawa, S. Oguma, O. T. EF, C. L. Obi, and T. Hattori. 2009. Persistent elevation of plasma osteopontin levels in HIV patients despite highly active antiretroviral therapy. *Tohoku J Exp Med* 218: 285-292.
34. Elahi, S., T. Niki, M. Hirashima, and H. Horton. 2012. Galectin-9 binding to TIM-3 renders activated human CD4+ T cells less susceptible to HIV-1 infection. *Blood* 119: 4192-4204.
35. Tandon, R., G. M. Chew, M. M. Byron, P. Borrow, T. Niki, M. Hirashima, J. D. Barbour, P. J. Norris, M. C. Lanteri, J. N. Martin, S. G. Deeks, and L. C. Ndhlovu. 2014. Galectin-9 Is Rapidly Released During Acute HIV-1 Infection and Remains Sustained at High Levels Despite Viral Suppression Even in Elite Controllers. *AIDS research and human retroviruses*.

36. Bi, S. G., P. W. Hong, B. Lee, and L. G. Baum. 2011. Galectin-9 binding to cell surface protein disulfide isomerase regulates the redox environment to enhance T-cell migration and HIV entry. *Proceedings of the National Academy of Sciences of the United States of America* 108: 10650-10655.
37. Abdel-Mohsen, M., L. Chavez, R. Tandon, G. M. Chew, X. T. Deng, A. Danesh, S. Keating, M. Lanteri, M. L. Samuels, R. Hoh, J. B. Sacha, P. J. Norris, T. Niki, C. M. Shikuma, M. Hirashima, S. G. Deeks, L. C. Ndhlovu, and S. K. Pillai. 2016. Human Galectin-9 Is a Potent Mediator of HIV Transcription and Reactivation. *Plos Pathogens* 12.
38. Colomb, F., L. B. Giron, T. A. Premeaux, B. I. Mitchell, T. Niki, E. Papasavvas, L. J. Montaner, L. C. Ndhlovu, and M. Abdel-Mohsen. 2019. Galectin-9 Mediates HIV Transcription by Inducing TCR-Dependent ERK Signaling. *Frontiers in immunology* 10: 267.
39. Dunsmore, G., N. Bozorgmehr, C. Delyea, P. Koleva, A. Namdar, and S. Elahi. 2017. Erythroid Suppressor Cells Compromise Neonatal Immune Response against Bordetella pertussis. *Journal of immunology* 199: 2081-2095.
40. Namdar, A., P. Koleva, S. Shahbaz, S. Strom, V. Gerdts, and S. Elahi. 2017. CD71+ erythroid suppressor cells impair adaptive immunity against Bordetella pertussis. *Sci Rep* 7: 7728.
41. Lorenzo-Herrero, S., C. Sordo-Bahamonde, S. Gonzalez, and A. Lopez-Soto. 2019. CD107a Degranulation Assay to Evaluate Immune Cell Antitumor Activity. *Methods Mol Biol* 1884: 119-130.
42. Dunsmore, G., P. Koleva, N. Ghobakhloo, R. T. Sutton, L. Ambrosio, X. Meng, N. Hotte, V. Nguyen, K. L. Madsen, L. A. Dieleman, V. Huang, and S. Elahi. 2018. Lower Abundance and Impaired Function of CD71+ Erythroid Cells in Inflammatory Bowel Disease Patients During Pregnancy. *J Crohns Colitis*.
43. Gupta, P. K., J. Godec, D. Wolski, E. Adland, K. Yates, K. E. Pauken, C. Cosgrove, C. Ledderose, W. G. Junger, S. C. Robson, E. J. Wherry, G. Alter, P. J. Goulder, P. Klenerman, A. H. Sharpe, G. M. Lauer, and W. N. Haining. 2015. CD39 Expression Identifies Terminally Exhausted CD8+ T Cells. *PLoS Pathog* 11: e1005177.
44. Yamamoto, T., D. A. Price, J. P. Casazza, G. Ferrari, M. Nason, P. K. Chattopadhyay, M. Roederer, E. Gostick, P. D. Katsikis, D. C. Douek, R. Haubrich, C. Petrovas, and R. A. Koup. 2011. Surface expression patterns of negative regulatory molecules identify determinants of virus-specific CD8+ T-cell exhaustion in HIV infection. *Blood* 117: 4805-4815.
45. Chew, G. M., T. Fujita, G. M. Webb, B. J. Burwitz, H. L. Wu, J. S. Reed, K. B. Hammond, K. L. Clayton, N. Ishii, M. Abdel-Mohsen, T. Liegler, B. I. Mitchell, F. M. Hecht, M. Ostrowski, C. M. Shikuma, S. G. Hansen, M. Maurer, A. J. Korman, S. G. Deeks, J. B. Sacha, and L. C. Ndhlovu. 2016. TIGIT Marks Exhausted T Cells, Correlates with Disease Progression, and Serves as a Target for Immune Restoration in HIV and SIV Infection. *Plos Pathogens* 12.
46. Jones, R. B., L. C. Ndhlovu, J. D. Barbour, P. M. Sheth, A. R. Jha, B. R. Long, J. C. Wong, M. Satkunarajah, M. Schweneker, J. M. Chapman, G. Gyenes, B. Vali, M. D. Hyrcza, F. Y. Yue, C. Kovacs, A. Sassi, M. Loutfy, R. Halpenny, D. Persad, G. Spotts, F. M. Hecht, T. W. Chun, J. M. McCune, R. Kaul, J. M. Rini, D. F. Nixon, and M. A. Ostrowski. 2008. TIM-3 expression defines a novel population of dysfunctional T cells with highly elevated frequencies in progressive HIV-1 infection. *The Journal of experimental medicine* 205: 2763-2779.
47. Hersperger, A. R., G. Makedonas, and M. R. Betts. 2008. Flow cytometric detection of perforin upregulation in human CD8 T cells. *Cytometry A* 73: 1050-1057.
48. Wu, X., H. Zhang, Q. Xing, J. Cui, J. Li, Y. Li, Y. Tan, and S. Wang. 2014. PD-1(+) CD8(+) T cells are exhausted in tumours and functional in draining lymph nodes of colorectal cancer patients. *Br J Cancer* 111: 1391-1399.
49. Bajnok, A., M. Ivanova, J. Rigo, Jr., and G. Toldi. 2017. The Distribution of Activation Markers and Selectins on Peripheral T Lymphocytes in Preeclampsia. *Mediators Inflamm* 2017: 8045161.
50. Buggert, M., J. Frederiksen, O. Lund, M. R. Betts, A. Biague, M. Nielsen, J. Tauriainen, H. Norrgren, P. Medstrand, A. C. Karlsson, M. Jansson, and S. C. group. 2016. CD4+ T cells with an activated and

exhausted phenotype distinguish immunodeficiency during aviremic HIV-2 infection. *Aids* 30: 2415-2426.
51. Motamedi, M., L. Xu, and S. Elahi. 2016. Correlation of transferrin receptor (CD71) with Ki67 expression on stimulated human and mouse T cells: The kinetics of expression of T cell activation markers. *J Immunol Methods* 437: 43-52.
52. Gerdes, J., H. Lemke, H. Baisch, H. H. Wacker, U. Schwab, and H. Stein. 1984. Cell cycle analysis of a cell proliferation-associated human nuclear antigen defined by the monoclonal antibody Ki-67. *Journal of immunology* 133: 1710-1715.
53. Liao, W., J. X. Lin, L. Wang, P. Li, and W. J. Leonard. 2011. Modulation of cytokine receptors by IL-2 broadly regulates differentiation into helper T cell lineages. *Nature immunology* 12: 551-U247.
54. Liao, W., D. E. Schones, J. Oh, Y. Cui, K. Cui, T. Y. Roh, K. Zhao, and W. J. Leonard. 2008. Priming for T helper type 2 differentiation by interleukin 2-mediated induction of interleukin 4 receptor alpha-chain expression. *Nature immunology* 9: 1288-1296.
55. Lowe, R. M., A. Genin, N. Orgun, and R. Q. Cron. 2014. IL-15 prolongs CD154 expression on human CD4 T cells via STAT5 binding to the CD154 transcription promoter. *Genes Immun* 15: 137-144.
56. Skov, S., M. Bonyhadi, N. Odum, and J. A. Ledbetter. 2000. IL-2 and IL-15 regulate CD154 expression on activated CD4 T cells. *Journal of immunology* 164: 3500-3505.
57. Palmer, C. S., M. Ostrowski, M. Gouillou, L. Tsai, D. Yu, J. L. Zhou, D. C. Henstridge, A. Maisa, A. C. Hearps, S. R. Lewin, A. Landay, A. Jaworowski, J. M. McCune, and S. M. Crowe. 2014. Increased glucose metabolic activity is associated with CD4 R T-cell activation and depletion during chronic HIV infection. *Aids* 28: 297-309.
58. Fatrai, S., A. T. J. Wierenga, S. M. G. J. Daenen, E. Vellenga, and J. J. Schuringa. 2011. Identification of HIF2 alpha as an important STAT5 target gene in human hematopoietic stem cells. *Blood* 117: 3320-3330.
59. Paley, M. A., D. C. Kroy, P. M. Odorizzi, J. B. Johnnidis, D. V. Dolfi, B. E. Barnett, E. K. Bikoff, E. J. Robertson, G. M. Lauer, S. L. Reiner, and E. J. Wherry. 2012. Progenitor and terminal subsets of CD8+ T cells cooperate to contain chronic viral infection. *Science* 338: 1220-1225.
60. Meng, X., X. Liu, X. Guo, S. Jiang, T. Chen, Z. Hu, H. Liu, Y. Bai, M. Xue, R. Hu, S. C. Sun, X. Liu, P. Zhou, X. Huang, L. Wei, W. Yang, and C. Xu. 2018. FBXO38 mediates PD-1 ubiquitination and regulates anti-tumour immunity of T cells. *Nature* 564: 130-135.
61. Staron, M. M., S. M. Gray, H. D. Marshall, I. A. Parish, J. H. Chen, C. J. Perry, G. Cui, M. O. Li, and S. M. Kaech. 2014. The transcription factor FoxO1 sustains expression of the inhibitory receptor PD-1 and survival of antiviral CD8(+) T cells during chronic infection. *Immunity* 41: 802-814.
62. Wherry, E. J., S. J. Ha, S. M. Kaech, W. N. Haining, S. Sarkar, V. Kalia, S. Subramaniam, J. N. Blattman, D. L. Barber, and R. Ahmed. 2007. Molecular signature of CD8+ T cell exhaustion during chronic viral infection. *Immunity* 27: 670-684.
63. Shin, H., S. D. Blackburn, A. M. Intlekofer, C. Kao, J. M. Angelosanto, S. L. Reiner, and E. J. Wherry. 2009. A role for the transcription repressor Blimp-1 in CD8(+) T cell exhaustion during chronic viral infection. *Immunity* 31: 309-320.
64. Ferris, R. L., B. Lu, and L. P. Kane. 2014. Too much of a good thing? TIM-3 and TCR signaling in T cell exhaustion. *Journal of immunology* 193: 1525-1530.
65. Nel, A. E. 2002. T-cell activation through the antigen receptor. Part 1: Signaling components. signaling pathways, and signal integration at the T-cell antigen receptor synapse (vol 109, pg 758, 2002). *J Allergy Clin Immun* 110: 25-25.
66. Smith, J. S., and N. J. Robinson. 2002. Age-specific prevalence of infection with herpes simplex virus types 2 and 1: a global review. *The Journal of infectious diseases* 186 Suppl 1: S3-28.
67. Hayashi, K., and A. Altman. 2007. Protein kinase C theta (PKCtheta): a key player in T cell life and death. *Pharmacol Res* 55: 537-544.

68. Phetsouphanh, C., and A. D. Kelleher. 2015. The role of PKC-theta in CD4+T cells and HIV infection: to the nucleus and back again. *Frontiers in immunology* 6.
69. Trushin, S. A., G. D. Bren, S. Asin, K. N. Pennington, C. V. Paya, and A. D. Badley. 2005. Human immunodeficiency virus reactivation by phorbol esters or T-cell receptor Ligation requires both PKC alpha and PKC theta. *Journal of virology* 79: 9821-9830.
70. Oomizu, S., T. Arikawa, T. Niki, T. Kadowaki, M. Ueno, N. Nishi, A. Yamauchi, T. Hattori, T. Masaki, and M. Hirashima. 2012. Cell surface galectin-9 expressing Th cells regulate Th17 and Foxp3+ Treg development by galectin-9 secretion. *PloS one* 7: e48574.
71. David, P., D. A. Megger, T. Kaiser, T. Werner, J. Liu, L. Chen, B. Sitek, U. Dittmer, and G. Zelinskyy. 2019. The PD-1/PD-L1 Pathway Affects the Expansion and Function of Cytotoxic CD8(+) T Cells During an Acute Retroviral Infection. *Frontiers in immunology* 10: 54.
72. Foger, N., R. Marhaba, and M. Zoller. 2000. CD44 supports T cell proliferation and apoptosis by apposition of protein kinases. *European journal of immunology* 30: 2888-2899.
73. Leal, D. B. R., C. A. Streher, C. D. Bertoncheli, L. F. D. Carli, C. A. M. Leal, J. E. P. da Silva, V. M. Morsch, and M. R. C. Schetinger. 2005. HIV infection is associated with increased NTPDase activity that correlates with CD39-positive lymphocytes. *Bba-Mol Cell Res* 1746: 129-134.
74. Bharaj, P., H. S. Chahar, O. K. Alozie, L. Rodarte, A. Bansal, P. A. Goepfert, A. Dwivedi, N. Manjunath, and P. Shankar. 2014. Characterization of programmed death-1 homologue-1 (PD-1H) expression and function in normal and HIV infected individuals. *PloS one* 9: e109103.
75. Saitoh, H., Y. Ashino, H. Chagan-Yasutan, T. Niki, M. Hirashima, and T. Hattori. 2012. Rapid decrease of plasma galectin-9 levels in patients with acute HIV infection after therapy. *Tohoku J Exp Med* 228: 157-161.
76. Tandon, R., G. M. Chew, M. M. Byron, P. Borrow, T. Niki, M. Hirashima, J. D. Barbour, P. J. Norris, M. C. Lanteri, J. N. Martin, S. G. Deeks, and L. C. Ndhlovu. 2014. Galectin-9 Is Rapidly Released During Acute HIV-1 Infection and Remains Sustained at High Levels Despite Viral Suppression Even in Elite Controllers. *Aids Res Hum Retrov* 30: 654-664.
77. Motamedi, M., S. Shahbaz, L. Fu, G. Dunsmore, L. Xu, R. Harrington, S. Houston, and S. Elahi. 2019. Galectin-9 Expression Defines a Subpopulation of NK Cells with Impaired Cytotoxic Effector Molecules but Enhanced IFN-gamma Production, Dichotomous to TIGIT, in HIV-1 Infection. *Immunohorizons* 3: 531-546.
78. Sigal, A., and D. Baltimore. 2012. As good as it gets? The problem of HIV persistence despite antiretroviral drugs. *Cell host & microbe* 12: 132-138.
79. Zhou, Q., M. E. Munger, R. G. Veenstra, B. J. Weigel, M. Hirashima, D. H. Munn, W. J. Murphy, M. Azuma, A. C. Anderson, V. K. Kuchroo, and B. R. Blazar. 2011. Coexpression of TIM-3 and PD-1 identifies a CD8+ T-cell exhaustion phenotype in mice with disseminated acute myelogenous leukemia. *Blood* 117: 4501-4510.
80. Okoye, I., A. Namdar, L. Xu, N. Crux, and S. Elahi. 2017. Atorvastatin downregulates co-inhibitory receptor expression by targeting Ras-activated mTOR signalling. *Oncotarget* 8: 98215-98232.
81. Chlewicki, L. K., C. A. Velikovsky, V. Balakrishnan, R. A. Mariuzza, and V. Kumar. 2008. Molecular basis of the dual functions of 2B4 (CD244). *Journal of immunology* 180: 8159-8167.
82. Nishio, A., T. Tatsumi, T. Nawa, T. Suda, T. Yoshioka, Y. Onishi, S. Aono, M. Shigekawa, H. Hikita, R. Sakamori, D. Okuzaki, T. Fukuhara, Y. Matsuura, N. Hiramatsu, and T. Takehara. 2017. CD14(+) monocyte-derived galectin-9 induces natural killer cell cytotoxicity in chronic hepatitis C. *Hepatology* 65: 18-31.
83. Kalia, V., S. Sarkar, S. Subramaniam, W. N. Haining, K. A. Smith, and R. Ahmed. 2010. Prolonged interleukin-2Ralpha expression on virus-specific CD8+ T cells favors terminal-effector differentiation in vivo. *Immunity* 32: 91-103.

84. Reiser, J., and A. Banerjee. 2016. Effector, Memory, and Dysfunctional CD8(+) T Cell Fates in the Antitumor Immune Response. *J Immunol Res* 2016: 8941260.
85. Klebanoff, C. A., L. Gattinoni, and N. P. Restifo. 2006. CD8+ T-cell memory in tumor immunology and immunotherapy. *Immunol Rev* 211: 214-224.
86. Yi, J. S., M. A. Cox, and A. J. Zajac. 2010. T-cell exhaustion: characteristics, causes and conversion. *Immunology* 129: 474-481.
87. Sparks, R., D. M. Koelle, and S. Dhanireddy. 2015. Elevated Spontaneous Interferon-Gamma Secretion in HIV-Infected Persons. *J Allergy Clin Immun* 135: Ab100-Ab100.
88. Imaizumi, T., M. Kumagai, N. Sasaki, H. Kurotaki, F. Mori, M. Seki, N. Nishi, K. Fujimoto, K. Tanji, T. Shibata, W. Tamo, T. Matsumiya, H. Yoshida, X. F. Cui, S. Takanashi, K. Hanada, K. Okumura, S. Yagihashi, K. Wakabayashi, T. Nakamura, M. Hirashima, and K. Satoh. 2002. Interferon-gamma stimulates the expression of galectin-9 in cultured human endothelial cells. *J Leukocyte Biol* 72: 486-491.
89. Beltra, J. C., S. Bourbonnais, N. Bedard, T. Charpentier, M. Boulange, E. Michaud, I. Boufaied, J. Bruneau, N. H. Shoukry, A. Lamarre, and H. Decaluwe. 2016. IL2Rbeta-dependent signals drive terminal exhaustion and suppress memory development during chronic viral infection. *Proceedings of the National Academy of Sciences of the United States of America* 113: E5444-5453.
90. Howland, K. C., L. J. Ausubel, C. A. London, and A. K. Abbas. 2000. The roles of CD28 and CD40 ligand in T cell activation and tolerance. *Journal of immunology* 164: 4465-4470.
91. Shu, U., M. Kiniwa, C. Y. Wu, C. Maliszewski, N. Vezzio, J. Hakimi, M. Gately, and G. Delespesse. 1995. Activated T cells induce interleukin-12 production by monocytes via CD40-CD40 ligand interaction. *European journal of immunology* 25: 1125-1128.
92. Moller, J. F., B. Moller, B. Wiedenmann, T. Berg, and E. Schott. 2011. CD154, a marker of antigen-specific stimulation of CD4 T cells, is associated with response to treatment in patients with chronic HCV infection. *J Viral Hepatitis* 18: E341-E349.
93. MacIver, N. J., R. D. Michalek, and J. C. Rathmell. 2013. Metabolic regulation of T lymphocytes. *Annual review of immunology* 31: 259-283.
94. Schurich, A., L. J. Pallett, D. Jajbhay, J. Wijngaarden, I. Otano, U. S. Gill, N. Hansi, P. T. Kennedy, E. Nastouli, R. Gilson, C. Frezza, S. M. Henson, and M. K. Maini. 2016. Distinct Metabolic Requirements of Exhausted and Functional Virus-Specific CD8 T Cells in the Same Host. *Cell Rep* 16: 1243-1252.
95. Siska, P. J., and J. C. Rathmell. 2015. PKCs Sweeten Cell Metabolism by Phosphorylation of Glut1. *Mol Cell* 58: 711-712.
96. Gatenby, R. A., E. T. Gawlinski, A. F. Gmitro, B. Kaylor, and R. J. Gillies. 2006. Acid-mediated tumor invasion: a multidisciplinary study. *Cancer Res* 66: 5216-5223.
97. Gatenby, R. A., and R. J. Gillies. 2004. Why do cancers have high aerobic glycolysis? *Nat Rev Cancer* 4: 891-899.
98. Lhuillier, C., C. Barjon, T. Niki, A. Gelin, F. Praz, O. Morales, S. Souquere, M. Hirashima, M. Wei, O. Dellis, and P. Busson. 2015. Impact of Exogenous Galectin-9 on Human T Cells: CONTRIBUTION OF THE T CELL RECEPTOR COMPLEX TO ANTIGEN-INDEPENDENT ACTIVATION BUT NOT TO APOPTOSIS INDUCTION. *The Journal of biological chemistry* 290: 16797-16811.
99. Hsu, D. K., H. Y. Chen, and F. T. Liu. 2009. Galectin-3 regulates T-cell functions. *Immunol Rev* 230: 114-127.

Chapter 3: Elevated ATP via enhanced miRNA-30b, 30c, and 30e downregulates the expression of CD73 in $CD8^+$ T cells of HIV-infected individuals

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3.1 Introduction

With the success of antiretroviral therapy (ART), the prognosis of HIV infection has substantially improved (1). As such, HIV infection is no longer considered a fatal disease but instead a chronic and manageable infection (2). Human HIV infection is suppressed but not eliminated by ART; thus, the viral antigen persistence is a significant cause of substantial immune activation and chronic inflammation, both of which are associated with the 'inflammaging' (3). This chronic immune activation is linked to microbial translocation and increased levels of coagulation factors, which contribute to non-HIV-related comorbidities observed in HIV-infected individuals (4). As such, identifying the mechanism(s) underlying immune activation and how it can be prevented has been the focus of utmost interest in recent years.

CD73 (NT5E), an ectoenzyme, is expressed on the surface of various immune cells, including monocytes, dendritic cells, neutrophils, B and T cells (5). CD73 works mainly with CD39, another cell surface ectoenzyme to convert highly inflammatory ATP to immunosuppressive adenosine in a tightly regulated process (6). While the conversion of ATP to ADP and subsequently AMP is catalyzed by CD39, CD73 catalyzes the conversion of AMP into adenosine (6). In addition to its enzymatic activity, CD73 functions as a co-stimulatory molecule on T cells, reducing the threshold for naïve T cells' activation following antigen encounter (7). Also, CD73 acts as an adhesion molecule on T cells, mediating lymphocyte binding to endothelial cells through engagement with the Lymphocyte Function-Associated Antigen-1 (LFA-1) (8). HIV-infected individuals show significantly lower frequency of CD73 expressing CD4 and CD8 T cells compared with healthy individuals (9). Interestingly, the level of ATP, the upstream molecule if the CD39/CD73 pathway is upregulated in the plasma of HIV-infected individuals (10). CD73 gene is located on the long arm of chromosome 6, and its expression is controlled by several transcription factors (11). The promotor region of CD73 has binding sites for transcription factors SP1, AP-2, cAMP-responsive elements, as well as SMAD proteins (12, 13). Besides, the hypoxia-inducible factor (HIF-1) can elevate CD73 expression by direct binding to the CD73 promotor, which accounts for the functional role of CD73

in hypoxia adaptation (14, 15). Moreover, different cytokines may impact CD73 gene expression directly or through the regulation of other transcription factors. For example, while IL-6 enhances CD73 expression through stat3 activation, TGF-β-mediated increase in CD73 expression results from the suppression of transcription factor Gfi-1, which represses CD73 expression (16). Finally, FOXP3 the transcription factor of regulatory T cells (Tregs) has been shown to enhance CD73 expression in murine Tregs (17).

In addition to the above-mentioned transcription factors, several microRNAs (miRNAs) are suggested to regulate CD73 expression by direct targeting of CD73 miRNA or indirect targeting of CD73 transcription factors (11). Among these miRNAs, miR-30a, miR-30b, miR-187, and miR-193b are reported to control CD73 expression by directly regulating the CD73 gene (18–21). On the other hand, miR-30a-e and miR-200c target SMAD2, a transcription activator of CD73, resulting in downregulation of CD73 expression (22). Similarly, miR-16 and miR-142-5p can downregulate CD73 expression by SMAD-3 inhibition, another transcription regulator of the CD73 gene (23).

In this study, we aimed to evaluate the surface expression of CD73 in both CD4 and CD8 T cells in HIV-infected individuals compared to healthy controls (HCs). We also measured CD73 at the intracellular and gene levels in CD8 T cells of HIV-infected patients compared to HCs. Notably, we evaluated the functionality of CD73 expressing CD8 T cells in HIV infected and HCs. We further proposed that the elevated ATP in the plasma of HIV patients downregulates CD73 expression in CD8 T cells through enhanced expression of miRNA-30c-e.

3.2 Material and methods

3.2.1 Study population

124 human subjects were recruited for our studies (Supplementary Table 1), including 1) 26 HIV-infected but antiretroviral therapy (ART)-naive individuals; 2) 63 HIV-infected individuals on ART (ART); and 3) 13 Long-term non-progressors (LTNPs), who had a viral plasma load <10,000 copies/ml and CD4 count >400

as defined in our previous reports (24, 25). We also recruited 22 HCs who were HIV, hepatitis B and C viruses seronegative.

3.2.2 Ethics statement

This study was approved by the Research Ethics Board at the University of Alberta (protocol # Pro000046064 and Pro000070528). A written informed consent form was obtained from all study participants. Similarly, animal studies were approved by the Research Ethics Board at the University of Alberta (protocol # AUP00001021).

3.2.3 Animal studies

BALB/c mice were used for studying the frequency of CD73+ CD8 T cells in different mice tissues. The research ethics boards at the University of Alberta approved these studies (AUP00001021).

3.2.4 Cell isolation and processing

The peripheral blood mononuclear cells (PBMCs) were isolated from the blood of either HIV-infected or HCs using Ficoll-Paque gradients. Cell cultures were performed in RPMI 1640 (Sigma-Aldrich) supplemented with 10% FBS (Sigma-Aldrich) and 1% penicillin/streptomycin (Sigma-Aldrich). Our studies were mainly conducted on freshly isolated cells, but occasionally, PBMCs were cryopreserved for further use. In some studies, total T cells or CD8 T cells were isolated from PBMCs according to the manufacturing instruction (STEMCELL Technologies), with a purity exceeding 95% (Supplementary Fig. 1A). In other studies, CD8 T cells were labelled with the biotin-conjugated anti–CD73 mAb (AD2), followed by the anti-biotin microbeads (Miltenyi Biotec), and passed through MACS separation columns (Miltenyi Biotec) for isolating $CD73^+$ and $CD73^-$ CD8 T cells by positive and negative selections, respectively (Supplementary Fig. 1B).

For the effector T cell isolation, isolated total T cells were labelled with the FITC-conjugated anti-CCR7 and passed through MACS separation columns (Miltenyi Biotec) to isolate the negative fraction (Supplementary Fig. 1C). For the proliferation assay, isolated effector T cells were labelled with 1.25 μM

CFSE (Thermo Fisher Scientific) as described elsewhere (24) before stimulation with anti-CD3/CD28 microbeads and then analyzed 3-4 days later.

In some experiments, isolated CD8 T cells were cultured with the anti-CD3 (3μg/mL) and anti-CD28 (1μg/mL) antibodies in the presence or absence of recombinant IL-2 (NIH HIV-reagents), IL-15 (BioLegend), IL-16 (R&D), TNF-α (R&D), IFN-α (Abcam), IL-10 (R&D), TGF-β (BioLegend), IFN-γ (STEMCELL Technologies) and IL-15 (BioLegend). In other experiments, CD8 T cells were cultured with 100 μM ATP (Thermo Fisher Scientific).

3.2.5 Flow cytometry

Fluorophore antibodies with specificity to human cell antigens and cytokines were purchased mainly from the BD Biosciences, Thermo Fisher Scientific, and BioLegend. The following Abs were used in our study: anti-CD3 (SK7), anti-CD4 (RPA-T4), anti-CD8 (RPA-T8), anti-CD39 (TU66), anti-CD107a (H4A3), anti-Perforin (dG9), anti–Granzyme B (GzmB; GB11), anti-CD45RA (HL100), anti-CD62L (DREG-56), anti-CD73 (AD2), anti-CCR7 (2-L1-A), anti-FasL (NOK-1), anti-IL-2 (MQ1-17H12), anti-TNF-α (MAB11), anti-IFN-γ (4S.B3), and anti-Integrin β7 (FIB504). Purified anti-human CD3 (UCHT1), anti-human CD28 (CD28.2), Protein Transport Inhibitor (containing brefeldin A), and Protein Transport Inhibitor (containing monensin) were purchased from the BD Biosciences. Cell stimulation mixture (PMA/ionomycin) was purchased from BioLegend. Surface and intracytoplasmic cytokine staining (ICS) was performed according to our previous reports (25, 26). For the ICS, depending on the experiment, PMBCs were cultured and stimulated with the HIV-derived Gag peptide pool for 24 hours, or PMA (2 μL/ml), anti-CD3 (3 μg/mL) and anti-CD28 (5 μg/mL) antibodies for 5 hr in the presence of Brefeldin A (1 μl/ml). CD107a staining was performed as described elsewhere (27). Finally, stained cells were fixed in paraformaldehyde (2%) and were acquired on an LSRFortessa-SORP or Fortessa- X20 (BD Biosciences) and analyzed using the FlowJo software (version 10).

3.2.6 Gene expression analysis

The RNA was isolated from approximately 1×10^6 CD8 T cells using the Direct-zol RNA MicroPrep kit (Zymo Research). The isolated RNA (100 ng) was used for cDNA synthesis. cDNA samples for miRNA and mRNA expression were synthesised using the miScript II RT kit (Qiagen), as described previously (27). cDNA samples for mRNA expression using RT2 primers were synthesised using the RT2 Reverse Transcription kit (Qiagen). For quantitative real-time PCR (RT-PCR), samples were run in duplicate using Quantitect or and RT^2 Primer Assays (mRNAs) and miScript Primer Assays (miRNAs) (Qiagen) using the CFX96 Touch Real-Time PCR Detection System (BioRad). Expression of the following mRNAs and miRNAs were analysed: *NT5E, TNFA, IFNG, IL2, FoxP3, PRF, GZMB,* miR-30a, -30b, -30c, -30d, and -30e. Beta-2-microglobulin and RNU6 were used as a reference gene for mRNAs and miRNAs, respectively. Data analysis was carried out using the 2^{-ddCT} method.

3.2.7 Nucleofection assay

CD8 T cells were stimulated with the anti-CD3 (3 μg/mL), anti-CD28 (2 μg/mL) and IL-2 (25 IU/mL) for 48 hours. The cell pellet (2×10^6 cells) was resuspended in 100 μl of P3 primary cell nucleofector solution (Lonza). The cells were then immediately loaded into the supplied nucleocuvette vessel (Lonza), and 300 μMolar of miRNA inhibitors was added to each vessel and tapped gently to mix while avoiding bubbles. The cuvette was inserted into the Lonza 4D-Nucleofector, and nucleofected with the human T cells stimulated condition. The cuvette was removed, and 400μl of prewarmed X-VIVO 15 medium (Lonza) was immediately added into each cuvette vessel. The medium/cell was pipetted into the appropriate cell culture plate, and the cells were cultured for 48 hours. The inhibition of miRNAs was further confirmed by RT-PCR.

3.2.8 ELISA

The concentration of CD73 in the plasma of HIV-infected and healthy controls was measured using an ELISA kit (R&D Systems).

3.2.9 ATP assay

The ATP concentration was measured in the plasma using the ATPlite luminescence assay system (PerkinElmer, MA). The samples (100 µL) were combined with 100 µL of ATPlite reagent, and the luminescence was measured using a BioTek Synergy H1 Multi-Mode Reader. The concentration of ATP was calculated by comparing the luminescence of the samples to a standard curve generated using ATP standards according to the manufacture's instruction.

3.2.10 Statistical analysis

The *P* values shown in cumulative flow cytometry graphs or PCR were determined by non-parametric Mann-Whitney *U*-test. Prism software was used for statistical analysis, and results are expressed as mean ± SEM with p-value < 0.05 considered statistically significant.

3.3 Results

3.3.1 Reduction of CD73 expressing T cells in HIV-infected individuals

Since CD39 and CD73 are two associated ectoenzymes, we first determined their frequency in PBMCs of HIV-infected individuals and HCs. In agreement with previous reports (25, 28), we found that the percentage of CD39 expressing CD4 T cells was significantly elevated in HIV-infected individuals compared to HCs (Fig. 1A and 1B, and the gating strategy Supplementary Fig. 1D). However, the percentages of CD39 expressing CD8 T cells were not significantly different in HIV-infected individuals compared to HCs (Fig. 1C and 1D). In contrast, we found percentages of CD73 expressing CD4 and CD8 T cells were significantly decreased in HIV-infected individuals compared to HCs (Fig. 1A-D), which is in agreement with a previous report (9). Then, we decided to investigate the frequency of CD73 expressing T cells in different HIV infected subgroups compared to HCs. In agreement with the previous report (9), we found that the frequency of CD73+ CD4 T cells was significantly decreased in individuals on ART, LTNPs, and ART-naïve individuals compared to HCs (Fig. 1E and 1F). Similarly, the frequency of CD73 expressing CD8 T cells was significantly lower in individuals on ART, LTNPs, and ART-naive individuals compared to HCs (Fig. 1E and 1G). However, there was no significant difference in the frequency of CD73 expressing CD4 and CD8 T cells

between different groups of HIV-infected individuals. It is worth mentioning that the reduction in CD73+ cells was beyond T cells, and it appeared to be a general phenomenon in HIV infection (Supplementary Fig. 1E). Thus, our results show higher percentages of CD39+ but lower CD73+ T cells in HIV-infected individuals compared to HCs.

3.3.2 The lower expression of CD73 at the intracellular protein and gene levels in CD8 T cells of HIV-infected individuals

The focus of our study was CD8 T cells as the main anti-virus effector cells in HIV infection (29–31). Thus, we did not purse CD4 T cells in regard to CD73 expression. To assess whether the lower frequency of CD73+ CD8 T cells results from the surface shedding of CD73 or a lower synthesis of CD73 in CD8 T cells in HIV-infected individuals, we first measured the intracellular CD73 protein expression in CD8 T cells using intracytoplasmic cytokine staining (ICS). We observed that the expression of intracellular CD73 protein was significantly lower in CD8 T cells of ART-naive, LTNPs, and individuals on ART compared to HCs (Fig. 2A and 2B). Notably, the intracellular expression of CD73 was significantly lower in CD8 T cells from patients on ART compared to LTNPs (Fig. 2B). Also, we examined the expression of the CD73 gene in CD8 T cells from different subpopulation of HIV-infected individuals compared to HCs. Interestingly we found a lower expression of the CD73 gene in CD8 T cells in HIV-infected individuals on ART and LTNPs compared to HCs (Fig. 2C). Although the expression of the CD73 gene was lower in CD8 T cells of the ART-naïve group, it did not reach a significant level. Thus, our results showed a lower expression of CD73 in CD8 T cells at the gene and protein levels in HIV-infected individuals. Moreover, we compared the plasma level of CD73, where we found lower plasma levels of CD73 in HIV-infected individuals compared to HCs (Supplementary Fig. 1E). These observations further confirmed that the reduced CD73 expression was not due to its shedding from T cells in HIV-infected individuals.

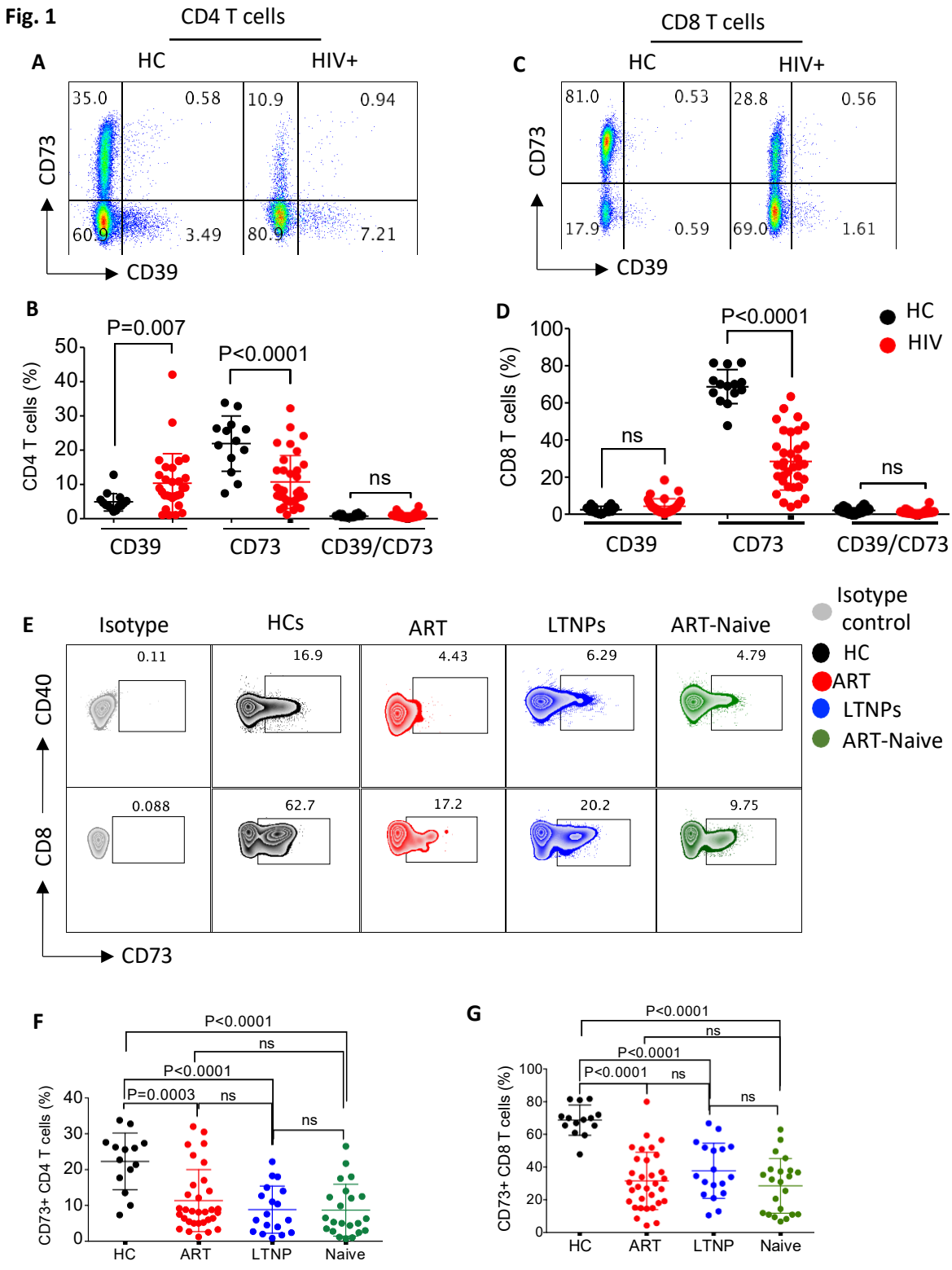

Fig. 1. Lower frequency of CD73+ T cells in the peripheral blood of HIV-infected individuals. (**A**) Representative flow cytometry plots, and (**B**) Cumulative data showing percentages of CD39+, CD73+ and CD39+/CD73+ CD4 T cells in healthy controls (HCs) versus HIV-infected individuals. (**C**) Representative flow

cytometry plots, and (**D**) Cumulative data showing percentages of CD39+, CD73+ and CD39+/CD73+ CD8 T cells in HCs versus HIV-infected individuals. (**E**) Representative flow cytometry plots, (**F**) Cumulative data showing percentages of CD73+ CD4 T cells, and CD73+ CD8 T cells in HCs compared to HIV-infected individuals either on ART, ART-naive or LTNPs. Each point represents one human subject, either HC or HIV-infected individual. Data are obtained from multiple independent experiments.

3.3.3 The frequency of CD73+ CD8 T cells is reduced in HIV-infected individuals regardless of their differentiation status

Antigen stimulation results in the generation of effector and memory CD8 T cells that are functionally and phenotypically different from naïve T cells (32–34). Therefore, we questioned whether the decreased frequency of CD73+ CD8 T cells is associated with a specific CD8 T cell subset in HIV-infected individuals. We first divided CD8 T cells to naïve ($CD45RA^{+}CD62L^{+}$), central memory (CM) ($CD45RA^{-}CD62L^{+}$), effector memory (EM) ($CD45RA^{-}$ $CD62L^{-}$), and effector cells ($CD45RA^{+}$ $CD62L^{-}$) (Fig. 2D). Next, we compared the percentages of CD73+ cells in each subset of CD8 T cells in HCs and HIV-infected individuals. We found that the frequency of CD73 expressing cells was significantly lower among different CD8 T cell subsets in HIV-infected individuals compared to HCs (Fig. 2E and 2F). These results show that the lower frequency of CD73 expressing cells is not restricted to any specific subset of CD8 T cells but rather a general phenomenon in HIV-infected individuals.

3.3.4 CD73 expressing CD8 T cells exhibit a dysfunctional phenotype

Since we were limited with the number of cells from ART-naïve and LTNPs, our functional studies were performed on HIV-infected individuals on ART. To characterize the functionally of CD73+ CD8 T cell *in vitro*, PBMCs from HIV-infected individuals on ART were stimulated with anti-CD3/CD28 antibodies, and cytokine production (TNF-α, IFN-γ, and IL-2) was assessed by intercellular cytokine staining in CD73+ versus CD73-CD8 T cells. In contrast to what has been shown (9, 35), we observed that CD73+ CD8 T cells exhibited impaired functionality by expressing significantly lesser TNF-α, IFN-γ, and IL-2 compared to their negative counterparts (Fig. 3A and 3B). The same phenotype was observed in $CD73^{+}$CD8 T cells in HCs

upon stimulation of their PBMCs with anti-CD3/CD28 antibodies (Supplementary Fig. 1G). Since other groups have used phorbol-12-myristate-13-acetate (PMA) for their studies (9), we stimulated PBMCs with PMA instead of anti-CD3/CD28 antibodies and once again, we found significantly lower cytokine

Fig. 2

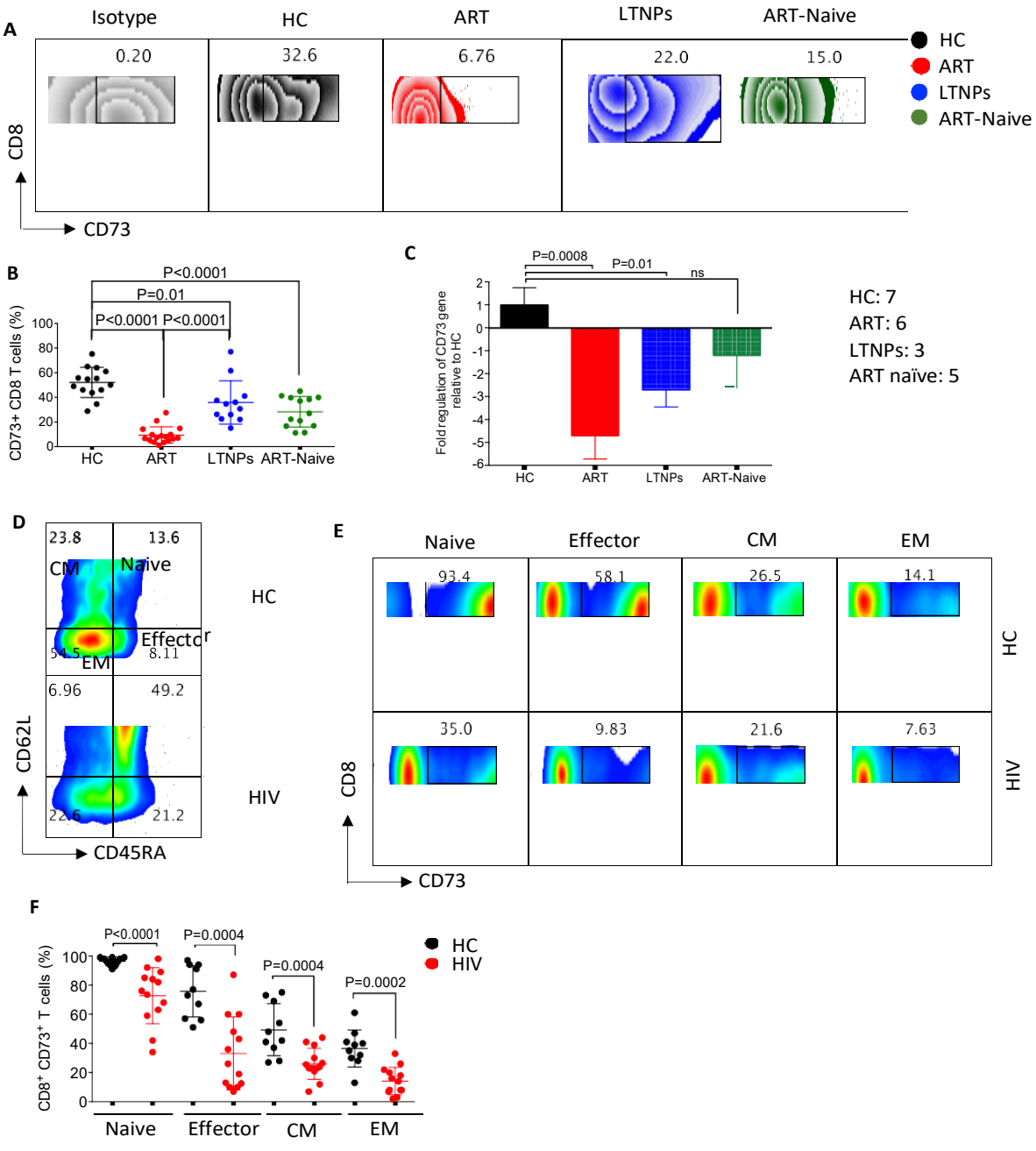

Fig. 2. Global reduction in the frequency of CD73+ CD8 T cells in HIV-infected individuals. (A) Representative flow cytometry plots and (**B**) Cumulative data showing the intra-cellular expression level of CD73 in CD8 T cells in HCs compared to HIV-infected individuals either on ART, ART-naive or LTNP. (**C**) Fold regulation of CD73 gene in CD8 T cells of HIV-infected individuals (on ART, ART-naïve and LTNPs) relative to HCs quantified by qPCR. (**D**) Representative flow cytometry plot showing different subpopulations of CD8 T cells in an HC versus an HIV-infected individual on ART. (**E**) Representative flow cytometry plots and (**F**) Cumulative data showing expression of CD73 on different subsets of CD8 T cells in HCs compared with HIV-infected individuals. Each point represents one human subject, either HC or HIV-infected individual. Data are obtained from multiple independent experiments.

expression in CD73+ versus CD73- CD8 T cells in HIV-infected individuals (Supplementary Fig. 1H). We also observed lower cytokine production in CD73+ versus CD73- CD8 T cells after stimulation of PBMCs with PMA in HCs (data not shown). To investigate whether the decreased ability to produce cytokine is consistent among different CD8 T cell subpopulations, we examined the production of TNF-α and IFN-γ by various CD8 T cell subsets. As we expected, effector and EM CD8 T cells had the highest cytokine expression compared to naïve and CM subsets. However, those expressing CD73 had significantly lower levels of TNF-α and IFN-γ compared to their negative counterparts among effector and EM subsets (Fig. 3C and 3D, and Supplementary Fig.1I). Furthermore, we found that CD73+ CD8 T cells had significantly lower mRNA expression for TNF-α, IFN-γ, and IL-2 compared to their negative counterparts (Fig. 3E-G). Moreover, we assessed the frequency of antigen-specific T cells among CD73+/CD73- CD8 T cells in HIV-infected individuals following stimulating of their PBMCs with the HIV gag peptide pool for cytokine response as we have reported elsewhere using the CD137 (24, 36) (Fig. 3H). We found that antigen-recognizing CD8 T cells (CD137+) were mainly CD73- (Fig. 3H) and CD137+ CD73+ CD8 T cells expressed significantly lower TNF-α and IFN-γ compared to CD137+CD73- CD8 T cells (Fig. 3H and 3I). It is reported that CD73 acts as a receptor for different ligands such as tenascin C, fibronectin, laminin, and extracellular matrix proteins (ECM) (37–39). Because some of these ligands (e.g. fibronectin) are also expressed on immune cells (40), we decided to determine if impaired functionality of CD73+ CD8 T cells was mediated via cell-cell interactions. To test this, we excluded the interaction of CD8 T cells with other cells by measuring cytokine expression in isolated CD8 T cells in HIV-infected individuals instead of using the whole

PBMCs. However, we observed that even in the absence of other immune cell components of PBMCs, CD73+ CD8 T cells exhibited impaired functionality and expressed significantly lower TNF-α, IFN-γ, and IL-2 compared to CD73- CD8 T cells (Supplementary Fig. 2A-2C). Also, we measured the activation markers CD38 and HLA-DR in CD73+CD8 T cells and found that these cells had significantly lower HLA-DR expression compared to their negative counterparts while CD38 expression remained unchanged on CD73+/CD73- CD8 T cells in HIV-infected Individuals (Supplementary Fig. 2D). Therefore, our results show that CD73+CD8 T cells have lower cytokine production capability compared to their negative counterparts.

3.3.5 The expression of CD73 is associated with impaired degranulation capacity of CD8 T cells

To determine the impact of CD73 expression on the cytotoxic ability of CD8 T cells, we compared perforin and GzmB content of CD73+ versus CD73-CD8 T cells in HIV-infected individuals. We observed that the presence of CD73 in CD8 T cells was associated with a significant loss of expression of both perforin and GzmB expression (Fig. 4A and 5B). Our further results showed that CD73+CD8 T cells from HCs also showed impaired expression of perforin and GzmB (data not shown). We also found significantly lower perforin and GzmB at the gene levels in CD73+CD8 T cells compared to their CD73- counterparts (Fig. 4C and 4D). As the induction of apoptosis in target cells by CD8 T cells requires the synergistic action of both perforin and GzmB, our observations suggest that CD73+CD8 T cells have profound cytotoxic dysfunction. We also investigated the degranulation capacity of CD73+ versus CD73-CD8 T cells by measuring the expression of the lysosomal-associated membrane protein 1 (LAMP1; or CD107a). PBMCs from HIV-infected individuals were stimulated with anti-CD3/CD28 antibodies for 6h during the time they were stained for CD107a. We found that the expression of CD107a was not significantly different between CD73+ and CD73-CD8 T cells in the absence of TCR stimulation (Fig. 4E and 4F). However, upon TCR stimulation, CD73+CD8 T cells exhibited impaired degranulation capacity compared to CD73-CD8 T cells (Fig. 4E and 4F). Since CD8 T cell-mediated cytotoxicity can also occur via the interaction of FasL: Fas on target cells (41, 42), we examined

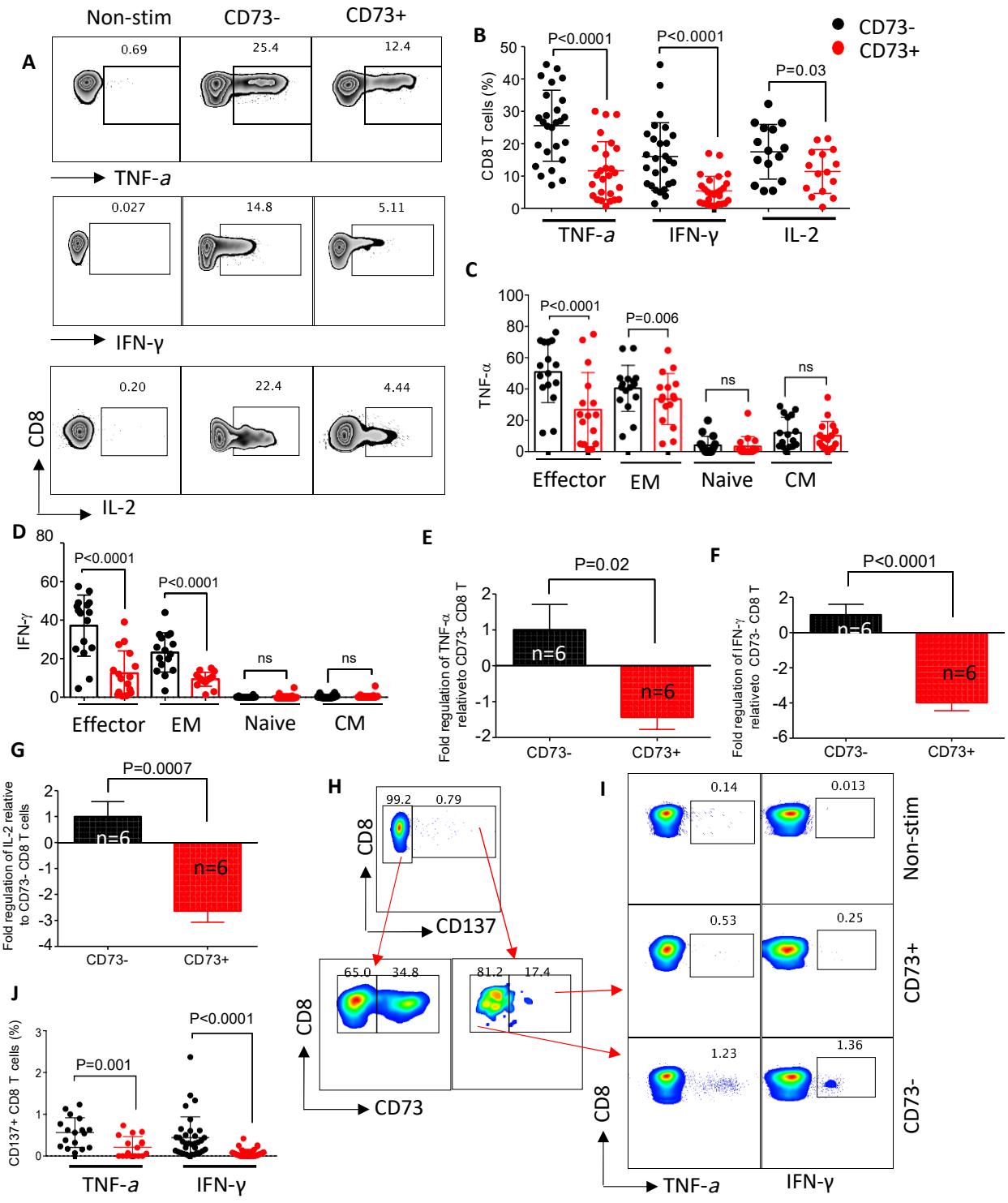

Fig. 3. Impaired effector functions of CD73+ CD8 T cells compared to CD73- CD8 T cells in PBMCs of HIV-infected individuals. (**A**) Representative flow cytometry plots, and (**B**) Cumulative data of TNF-α, IFN-γ, and IL-2 expression in CD73+ versus CD73- CD8 T cells upon stimulation of PBMCs from HIV-infected individuals with anti-CD3/CD28. (**C**) Cumulative data of TNF-α, and (**D**) IFN-γ expression in CD73+ versus

CD73- in different subsets of CD8 T cells of HIV-infected individuals. (**E-G**) Fold regulation of TNF-α, IFN-γ, and IL-2 genes in CD73+CD8 T cells relative to CD73- CD8 T cells of HIV-infected individuals quantified by qPCR. (**H**) Gating strategy and the representative plots showing CD73+ cells among CD137+ CD8 T cells compared to CD137-CD8 T cells, and (**I**) Cumulative data of TNF-α and IFN-γ expression in CD73+ versus CD73- CD137+ CD8 T cells upon stimulation of PBMCs from HIV-infected individuals with the Gag peptide pool. Each point represents one HIV-infected individual. Data are obtained from multiple independent experiments.

the expression of FASL on CD73+ CD8 T cells which showed lower expression of FASL compared to CD73- CD8 T cells (Fig. 4G and 4H). However, we did not find any difference in the proliferative capacity of CD73+ versus CD73-CD8 T cells (Fig. 4I and 4J). These results show that CD73 expressing CD8 T cells exhibit an impaired cytotoxic phenotype compared to their negative siblings within the same individual.

3.3.6 CD73+CD8 T cells exhibit a greater migratory capability

It is reported that CD73 is involved in CD8 T cells adhesion to endothelial cells and lymphocyte extravasation in an LFA-1-dependent mechanism (8). Moreover, the role of CD73 in CD4 T cells for the efficient entry into the central nervous system during the development of experimental autoimmune encephalomyelitis has been documented (43). Also, CD73 participates in the migration of lymphocytes via the afferent lymphatic vessels and intestinal lymphoid tissues (44, 45). To delineate the potential role of CD73 in T cell trafficking, we compared the expression of CCR7 and *a*4β7 integrin on CD73+ and CD73- CD8 T cells. We found that CD73+CD8 T cells expressed significantly higher levels of both CCR7 and *a*4β7 integrin compared to their negative counterparts (Fig. 5A- 5D). These observations suggest a differential capability of trafficking for CD73+CD8 T cells since these molecules are associated with T cell trafficking to the secondary lymph nodes and the gut (46, 47). We further confirmed this concept by measuring the expression of CD73 in CD8 T cells in the gut tissue versus the peripheral blood. As shown in Fig. 5E and 5F, we found a significant abundance of CD73+CD8 T cells in the gut tissue compared to the peripheral blood in mice. These results may support the concept of a higher capacity of CD73+ T cells to migrate to lymph nodes and gut-associated lymphoid tissues (GALT), the sites that harbour HIV reservoirs (48). As proof of

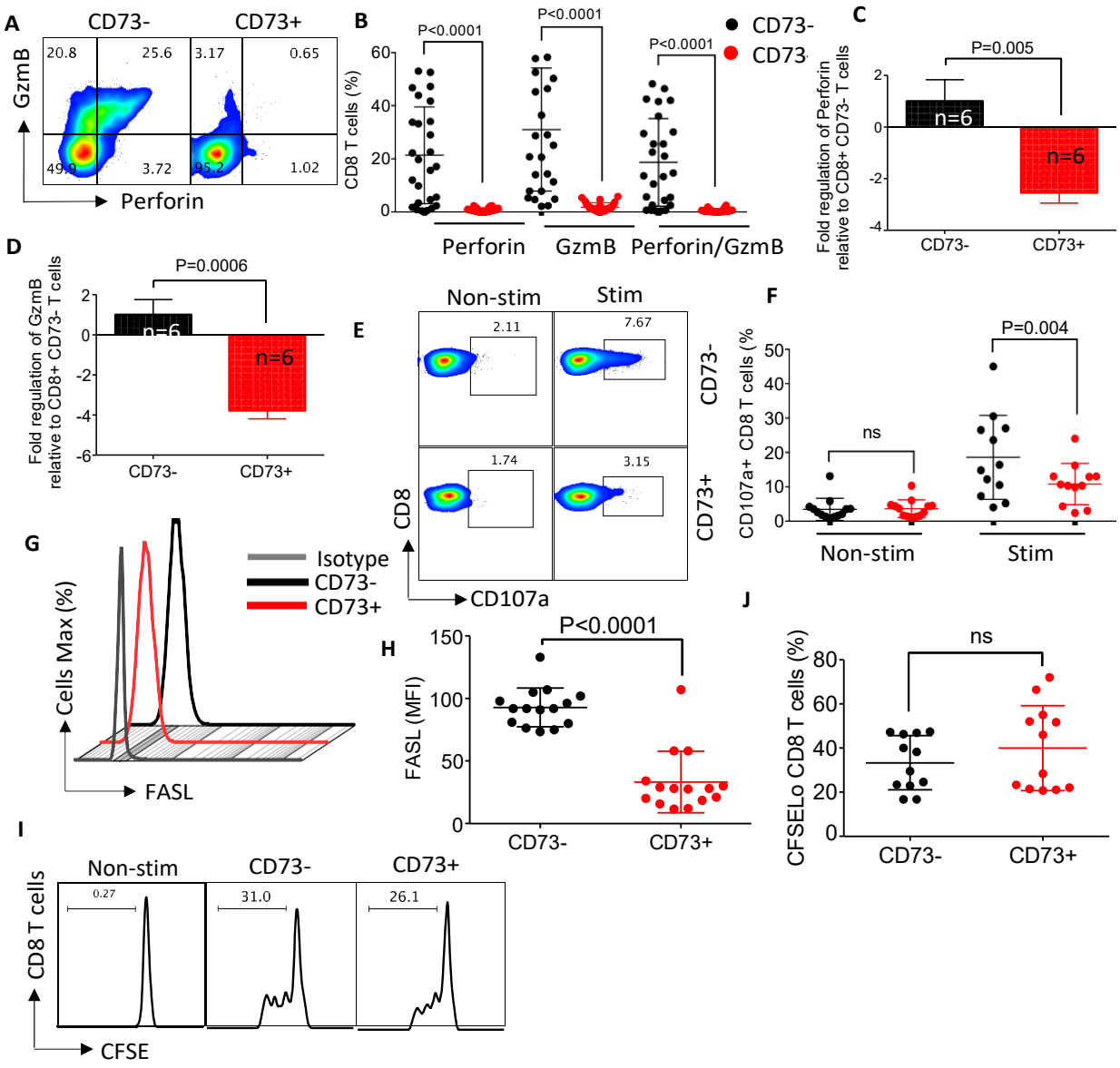

Fig. 4. Impaired expression of cytolytic molecules in CD73+CD8 T cells. (**A**) Representative flow cytometry plots, and (**B**) cumulative data showing the expression of perforin, GzmB and perforin/GzmB in CD73+ versus CD73- CD8 T cells. (**C**) Fold regulation of perforin gene, and (**D**) GzmB gene in CD73+ relative to CD73-CD8 T cells in HIV-infected individuals quantified by qPCR. (**E**) Representative flow cytometry plots of CD107a, and (**F**) cumulative data of CD107a in CD73- versus CD73+ CD8 T cells pre- and post-stimulation with anti-CD3/CD28 antibodies. (**G**) Representative histogram plots, and (**H**) cumulative data of FASL expression as measured by the mean fluorescence intensity (MFI) in CD73- versus CD73+ CD8 T cells in HIV-infected individuals. (**I**) Representative flow cytometry plots, and (**J**) cumulative data of CD73+ versus CD73- CD8+ T cells proliferation measured by CFSE after 3 days of stimulation with anti-CD3/CD28 antibodies. Each point represents one HIV-infected individual. Data are obtained from multiple independent experiments.

concept, we investigated the frequency of CD73 expressing CD8+ T cells in the cerebrospinal fluids (CSF) of relapsing-remitting multiple sclerosis (MS) patients at the time of disease remission versus relapse. Interestingly, we found a significantly higher abundance of CD73+CD8 T cells in the CSF of MS patients at time of relapse (Supplementary Fig. 2E and F). These observations suggest that CD73+ CD8 T cells might exhibit a greater trafficking capacity.

3.3.7 A lower expression of CD73 corresponds with FOXP3 in $CD8^+$ T cells in HIV-infected individuals

We then decided to investigate the underlying mechanism(s) associated with a lower frequency of CD73 expressing CD8 T cells in HIV-infected individuals. It is reported that chronic immune activation due to persistent HIV antigens results in increased plasma levels of several cytokines, such as IL-2, IL-8, IL-10, IL-15, IL-16, IFN-α, IFN-γ, TNF-α, and TGF-β (27, 49–55). Thus, we investigated the effect of these cytokines on the expression of the CD73 gene in isolated CD8 T cells from HCs following stimulation with anti-CD3/CD28 antibodies *in vitro*. Interestingly, we observed that T cell activation, in general, downregulates CD73 at the mRNA level (Supplementary Fig. 3A-3G). While the addition of IL-2, IL-15, IL-16, TNF-α, and IFN-α had no synergistic effect on the downregulation of CD73 mRNA, the addition of IL-10 and TGF-β upregulated the expression of CD73 in $CD8^+$ T cells (Supplementary Fig. 3A-3G). In contrast, we observed a synergistic effect for IFN-γ and IL-8 cytokines and T cell stimulation, resulting in a significant downregulation of CD73 mRNA in $CD8^+$ T cells (Fig. 5G and 5H).

Next, we measured the expression level of several transcription factors and miRNAs that are reported to regulate CD73 expression in CD8 T cells. For example, it is well recognized that FOXP3 upregulates the expression of CD73 in Tregs through permissive H3 modification (17). Thus, we quantified the expression of the FOXP3 gene in CD8 T cells of HIV-infected individuals compared to HCs. We found significantly lower FOXP3 expression at the gene and protein levels in CD8 T cells of HIV-infected individuals versus HCs (Fig. 5I-5K). These observations were further supported by the positive correlation between the expression of

FOXP3 and CD73 at the gene and protein levels in CD8 T cells of both HIV-infected individuals and HCs (Fig. 5L and 5M).

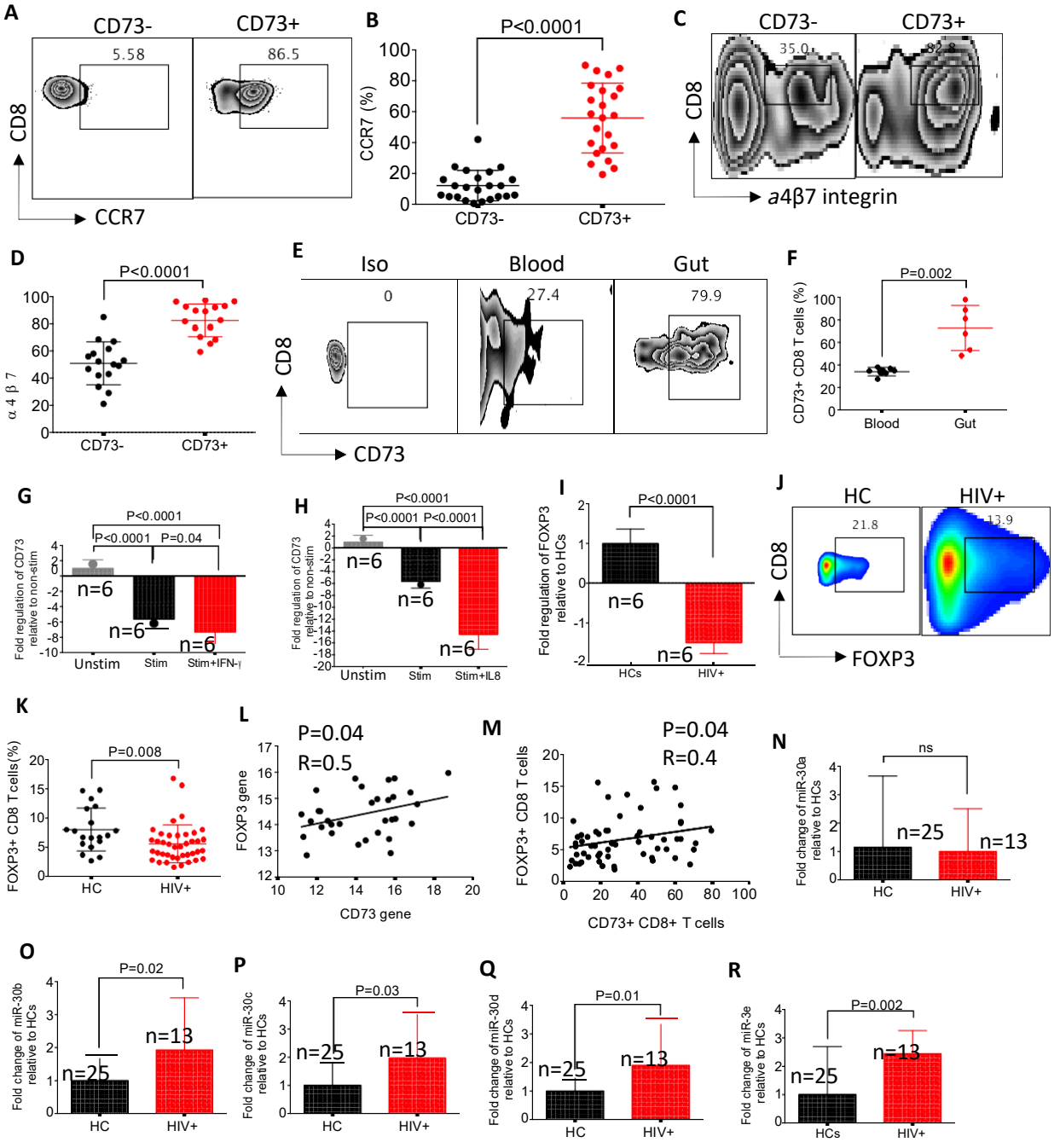

Fig. 5. Upregulation of miRNA30b-30e in CD8 T cells of HIV-infected individuals. (**A**) Representative flow cytometry plots and (**B**) cumulative data of CCR7 expression on CD73- versus CD73+ CD8 T cells. (**C**) Representative flow cytometry plots and (**D**) cumulative data of *a*4β7 integrin expression on CD73- versus CD73+ CD8 T cells. (**E**) Representative flow cytometry plots and (**F**) cumulative data of the percentage of

CD73 expressing CD8 T cells in the peripheral blood and the gut tissue of mice. (**G**) Fold regulation of CD73 gene in isolated CD8 T cells either unstimulated (unstim) or stimulated (stim) with anti-CD3/CD28 antibodies in the presence or absence of IFN-γ (100 ng/mL), and (**H**) IL-8 (100 ng/mL). (**I**) Fold regulation of FOXP3 gene in CD8 T cells isolated from PMBCs of HIV-infected individuals relative to HCs quantified by qPCR. (**J**) Representative flow cytometry plots, and (**K**) cumulative data of FOXP3 expression in CD8 T cells from HCs vs HIV-infected individuals. (**L**) Cumulative data of the correlation between the CD73 gene and the FOXP3 gene in CD8 T cells of HIV-infected individuals and HCs. (**M**) Cumulative data of the correlation between the cell surface CD73 and FOXP3 expression in CD8 T cells of HIV-infected individuals and HCs. (**N**) Fold change of miRNA-30a, (**O**) miRNA-30b, (**P**) miRNA-30c, (**Q**) miRNA-30d, and (**R**) miRNA30-e in CD8 T cells of HIV-infected individuals relative to HCs quantified by qPCR.

3.3.8 The high plasma ATP downregulates CD73 in CD8 T cells through upregulation of miR-30b, 30c, and 30e

Since the role of miRNAs in CD73 expression in different cell lines has been suggested (11), we aimed to determine whether miRNAs regulate the expression of CD73 in CD8 T cells. It is shown that the miR-30 family downregulates CD73 expression by direct binding to its promotor (56) or by the downregulation of SMAD2 expression (22). Thus, we analyzed the expression of miR-30 family members in CD8 T cells isolated from both HIV-infected individuals and HCs. We found that the expression of miR-30a was not significantly different in HCs vs HIV-infected individuals (Fig. 5N). However, we observed a significantly higher expression of miR-30b-e in CD8 T cells isolated from HIV-infected individuals compared to HCs (Fig. 5O-5R). Also, we quantified the expression of a wide range of other miRNAs (e.g. miRNA193, miRNA187, miRNA422, miRNA146, etc.) in CD8 T cells of HIV-infected versus healthy individuals but we did not find any meaningful difference.

To investigate whether overexpression of miR-30b-30e results in the downregulation of CD73 in CD8 T cells of HIV-infected individuals, we inhibited these miRNAs using miRNA inhibitors and quantified the expression of CD73 at the gene level. First, we confirmed that miRNA inhibitors significantly downregulated the expression of our target miRNAs (Supplementary Fig. 3H-K). We found that inhibition of miR-30b,30c, and 30e resulted in the upregulation of the CD73 gene in CD8 T cells (Fig. 6A-6C). However, this was not observed when the miR-30d was inhibited (Fig. 6D). These observations suggest

that the upregulation of miR-30b, 30c, and 30e may contribute to the decreased expression of CD73 in CD8 T cells in HIV-infected individuals.

To provide an insight into the potential mechanism(s) linked to the upregulation of miR30b-e in CD8 T cells of HIV-infected individuals, we decided to quantify ATP in the plasma of patients. In general, CD73 acts in tandem with CD39 on the cell surface to convert ATP to AMP and then adenosine (5). Thus, we speculated whether decreased CD73 results in the accumulation of upstream ATP in the plasma of HIV-infected individuals. Interestingly, and in agreement with the previous report (10), we observed significantly higher levels of ATP in the plasma of HIV-infected individuals compared to HCs (Fig. 6E). We further decided to determine the effect of increased ATP on CD73 expression in CD8 T cells. Therefore, we treated PMBCs of HIV-infected individuals with ATP (100 μM) for 72 hours and then examined for the expression of CD73 in CD8 T cells. Interestingly, we found that ATP treatment significantly decreased the expression of CD73 in CD8 T cells (Fig. 6F and 6G). Similarly, treatment of PBMCs from HCs with ATP downregulated the expression of CD73 in CD8 T cells (data not shown). To determine if the effect of ATP on CD73 is mediated through the elevation of miR-30s, we measured the levels of miR-30b-e in CD8 T cells treated with ATP. We found that ATP significantly upregulated the expression levels of miR-30b-e in CD8 T cells (Fig. 6H-6K). Therefore, our results suggest that increased ATP levels in the plasma of HIV-infected individuals might act through miRNAs to induce the downregulation of CD73 in CD8 T cells.

3.4 Discussion

In this report, we show a significantly lower frequency of CD73 expressing CD4 and CD8 T cells in HIV-infected individuals, which is in agreement with another report (9). Our results show that CD73 expressing T cells consistently decreased among both CD4 and CD8 T cells of all HIV-infected subgroups (e.g. LTNPs, ART-naïve, and those on ART). It has been reported that CD73 is downregulated upon CD8 T cell activation (5). However, our results demonstrate a reduction in CD73 expressing cells in different subsets of CD8 T

Fig. 6

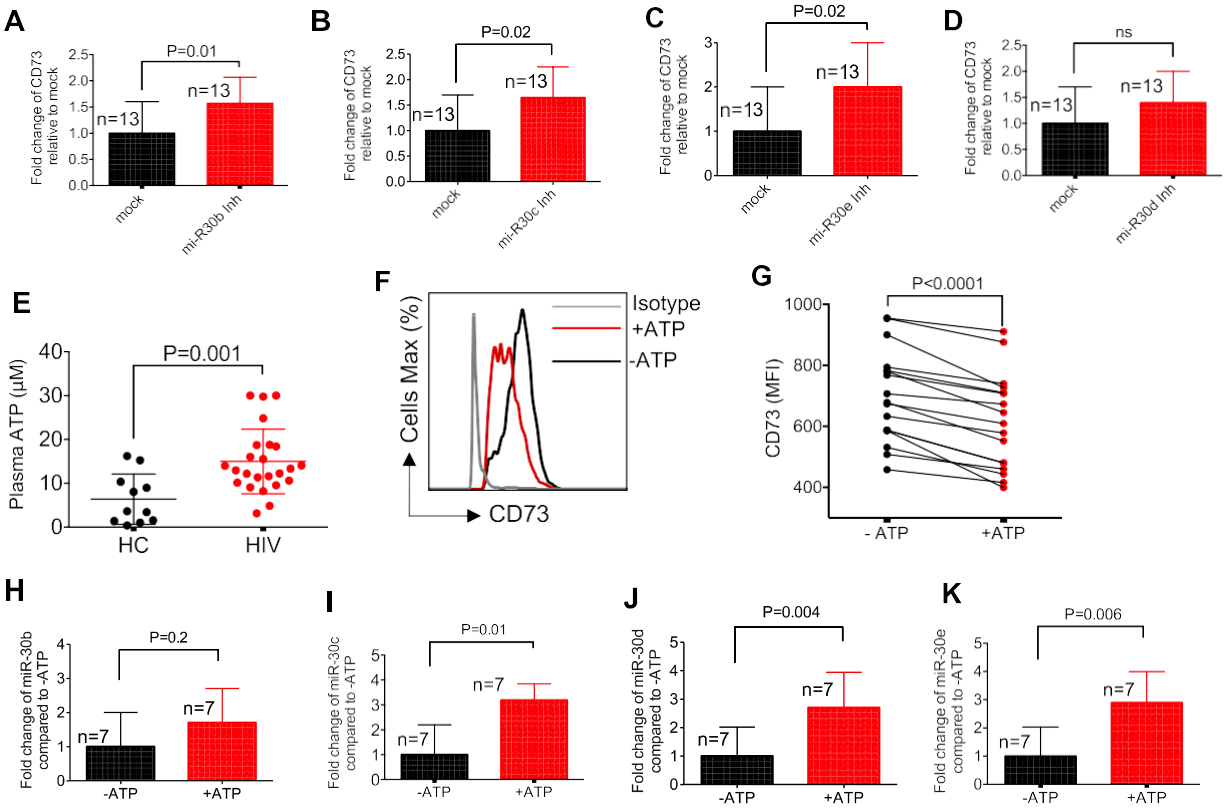

Fig. 6. Higher levels of the plasma ATP may upregulate the expression of miRNA30b-30e in HIV-infected individuals. Fold regulation of the CD73 gene in isolated CD8 T cells after treatment with (**A**) the miR-30b, (**B**) miR-30c, (**C**) miR-30d, and (**D**) miR-30e inhibitors. (**E**) Quantification of ATP levels in the plasma of HIV infected individuals versus HCs using ATP light kit. (**F**) Representative histogram plots of CD73 expression in CD8 T cells with/without treatment with ATP (100 μmol) for 72 hr. (**G**) Cumulative data showing MFI of CD73 in CD8 T cells after their treatment with ATP. (**H**) Fold change of miR-30b, (**I**) miR-30c, (**J**) miR-30d, and (**K**) miR-30e in isolated CD8 T cells after treatment with ATP (100 μmol) for 2 hr.

cells, suggesting that the decreased CD73 is a general feature of CD8 T cell regardless of their phenotype (e.g. memory, effector, or naïve) in HIV infection.

CD73 is a heterodimer anchored to the plasma membrane through a GPI-anchor, which is sensitive to hydrolysis by endogenous phospholipases (57, 58). Intestinally, both membrane-bound and soluble forms of CD73 have a similar affinity for AMP and exhibit similar AMPase activity (57) that can be inhibited by adenosine 5'-(α,β-methylene)-diphosphate (APCP), a specific CD73 inhibitor (59). This led us to speculate

whether decreased CD73 expressing CD8 T cells results from the shedding of CD73 from the cell surface. However, our results refuted this concept as we observed lower soluble CD73 in the plasma samples and reduced expression of CD73 at the gene level in CD8 T cells of HIV-infected individuals, which supports a lower CD73 synthesis.

Our further studies to characterize the functionality of CD73+CD8 T cells revealed that these cells exhibit impaired effector functions including lower inflammatory cytokines production, cytolytic molecules expression and degranulation capacity compared to their negative counterparts following *in vitro* global (e.g. anti-CD3/CD28 and/or PMA) or HIV Gag peptide pool stimulation. It is worth mentioning that CD73+CD8 T cells in HCs had similar effector functions compared to CD73-CD8 T cells. Our further studies showed that the expression of CD73 was not restricted to a subset of T cells but expressed by different CD8 T cell subsets with the highest expression among naïve followed by effector T cells. This led us to examine the functionality of CD73+ effector and EM CD8 T cells, which once again confirmed their impaired effector functions. Thus, CD73+CD8 T cells exhibit an impaired phenotype regardless of their differentiation status. We and others have shown that terminally impaired CD8 T cells express a higher amount of mRNA for cytokines despite the severe defect in their cytokine production capacity in HIV-infected individuals (25, 60). Nevertheless, we noted even lower expression of cytokines at the mRNA level in CD73+ CD8 T cells, which suggests that CD73+ CD8 T cells have impaired pro-inflammatory cytokine production capability at the gene level. This may suggest that CD73+ T cells suppress intrinsic signaling pathways through adenosine production as the depletion of CD73 results in a pro-inflammatory phenotype in the endothelial cells (61). However, our results are in disagreement with a previous report claiming that CD73+ EM CD8 T cells produce more IL-2 and TNF-α following stimulation with PMA and HIV peptides, respectively (9). Another group has shown that CM, EM, and effector CD73+CD8 T cells produce significantly higher IL-2 upon TCR stimulation compared to their negative counterpart in patients with acute myeloid leukemia (AML) (35). They also showed that antigen-specific CD73+CD8 T cells produce

higher levels of TNF-α, IFN-γ, and IL-2 in response to WT-1, a tumour-associated antigen (35). This discrepancy might be related to different and questionable protocols used for T cell stimulation *in vitro*. Interestingly, in one of the studies, PBMCs were stimulated overnight with the PMA in the presence of a Golgi blocker. Since the recommended time for the treatment of cells with the Golgi blocker is 4-6hr, it's unclear how 1) this group prevented the toxic effects of the Golgi blocker in such a long incubation time? 2) The recommended concentration of Golgi blocker is 1 μg/ml but this group has used (10 g/ml) (9). The other group has expanded CD8 T cells from AML patients in the presence of exogenous IL-2 for 6 days before conducting functional analysis (35). In contrast, we performed our functional studies on freshly isolated PBMCs from HIV-infected individuals and HCs without any additional *in vitro* manipulation. Therefore, we believe our results reflect the true nature of CD73+CD8 T cells. Besides, we examined the expression of cytokines at the mRNA level and other effector functions such as cytolytic molecules expression and degranulation capacity in T cells. These observations altogether confirmed an impaired effector phenotype in CD73+CD8 T cells.

The impact of CD73 downregulation in the context of HIV infection is not well understood. There is a possibility that the lower CD73 expression on T cells impairs the conversion process of AMP to the anti-inflammatory modulator, adenosine. As such, lower adenosine may facilitate the observed chronic inflammation in HIV-infected patients (4). In turn, chronic immune activation is accompanied by increased levels of multiple cytokines and ATP in the plasma of HIV-infected individuals (10, 62). Interestingly, we showed that the addition of IFN-γ and IL-8 downregulate CD73 expression in CD8 T cells. Similarly, we observed exogenous ATP downregulates CD73 expression in CD8 T cells when examined *in vitro*. These observations suggest that higher plasma IFN-γ and IL-8 might be linked to the downregulation of CD73 expression in HIV-infected individuals; both of these cytokines are elevated in the plasma of HIV-infected individuals (63, 64). Notably, we found that increased ATP facilitates the overexpression of miR-30b,30c, and 30e in $CD8^+$ T cells. Therefore, our results propose that the increased ATP level in the plasma of HIV-

infected individuals via enhanced miR-30b, 30c, and 30e downregulates the expression of CD73 in CD8 T cells.

The major unanswered question is that what are the biological properties of CD73+CD8 T cells when they have minimal effector functions? First of all, as we have shown, CD73 is highly expressed on naïve CD8 T cells. Our results suggest that CD73 downregulation following CD8 T cell activation may be essential to prevent autocrine adenosine signalling during T cell activation, which is necessary for the transition of naïve to effector cells (5). However, our observations show that CD73 is not restricted to naïve but also effector and memory $CD8^+$ T cells. Thus, we believe CD73 is required for CD8 T cells migration into the tissue. In CD73 knockout mice, migration of lymphocytes to the draining lymph nodes via afferent lymphatic vessels is impaired (45). Also, CD73 promotes the binding of lymphocytes to endothelial cells through an LFA1-dependent mechanism (8). Moreover, the expression of CD73 on CD4 T cells is required for the efficient entry into the central nervous system during the development of experimental autoimmune encephalomyelitis (EAE) (43). Consistent with this finding, it has been shown that individuals with HIV infection have a reduced risk of developing MS and/or experience lower relapse rates (65). This is further supported by our observation of a significant increase in the presence of CD73+CD8 T cells in the CSF of MS patients. Moreover, we found a high co-expression of CD73 with CCR7 and *a*4β7integrin in CD8 T cells, markers associated with CD8 T cells ability to migrate to secondary lymphoid tissues, such as lymph nodes and the gut (66, 67). It is worth noting that one of the significant barriers to HIV eradication is the presence of HIV reservoirs in different tissues, such as the spleen, lymph nodes (LNs), gut-associated lymphoid tissue (GALT), and central nervous system (CNS) (48). We speculate that the lower frequency of CD73+CD8 T cells in HIV-infected individuals might account for the deficient access of CD8 T cells to target tissues that harbour viral reservoirs. However, further studies are required to examine this possibility.

On the other hand, CD73 works with CD39 to convert ATP to immunosuppressive adenosine. Consequently, lower CD73 expression on T cells and other immune cells (9, 68) in the context of HIV may

lead to the accumulation of ATP in the plasma. As such, this scenario establishes a vicious cycle through which ATP decreases the expression of CD73, further contributing to the accumulation of ATP in the plasma of HIV-infected individuals with its adverse consequences.

We are aware of multiple study limitations such as lack of access to biopsies from HCs and HIV-infected individuals to determine if there are differences in the frequency of tissue residents CD73+ CD8 T cells. Also, CSF collection from HCs is not common, and such samples are highly informative to compare the frequency of CD73+ CD8 T cells in the CFS of HCs compared to MS patients. Moreover, we were limited with the number of MS patients in studies; therefore, performing similar studies on a larger cohort is required.

Taken together, our findings demonstrate a lower expression of CD73 on T cells in HIV patients. However, further studies are required to determine the role of CD73 on T cells in other chronic conditions such as viral infections and cancer. Such studies will enable us to better understand the mechanism (s) associated with the lower expression of CD73 on T cells and how it can be prevented. Such studies enable us to develop therapeutic interventions that reverse CD73 downregulation on CD8 T cells in HIV infection and beyond.

3.5 Acknowledgement

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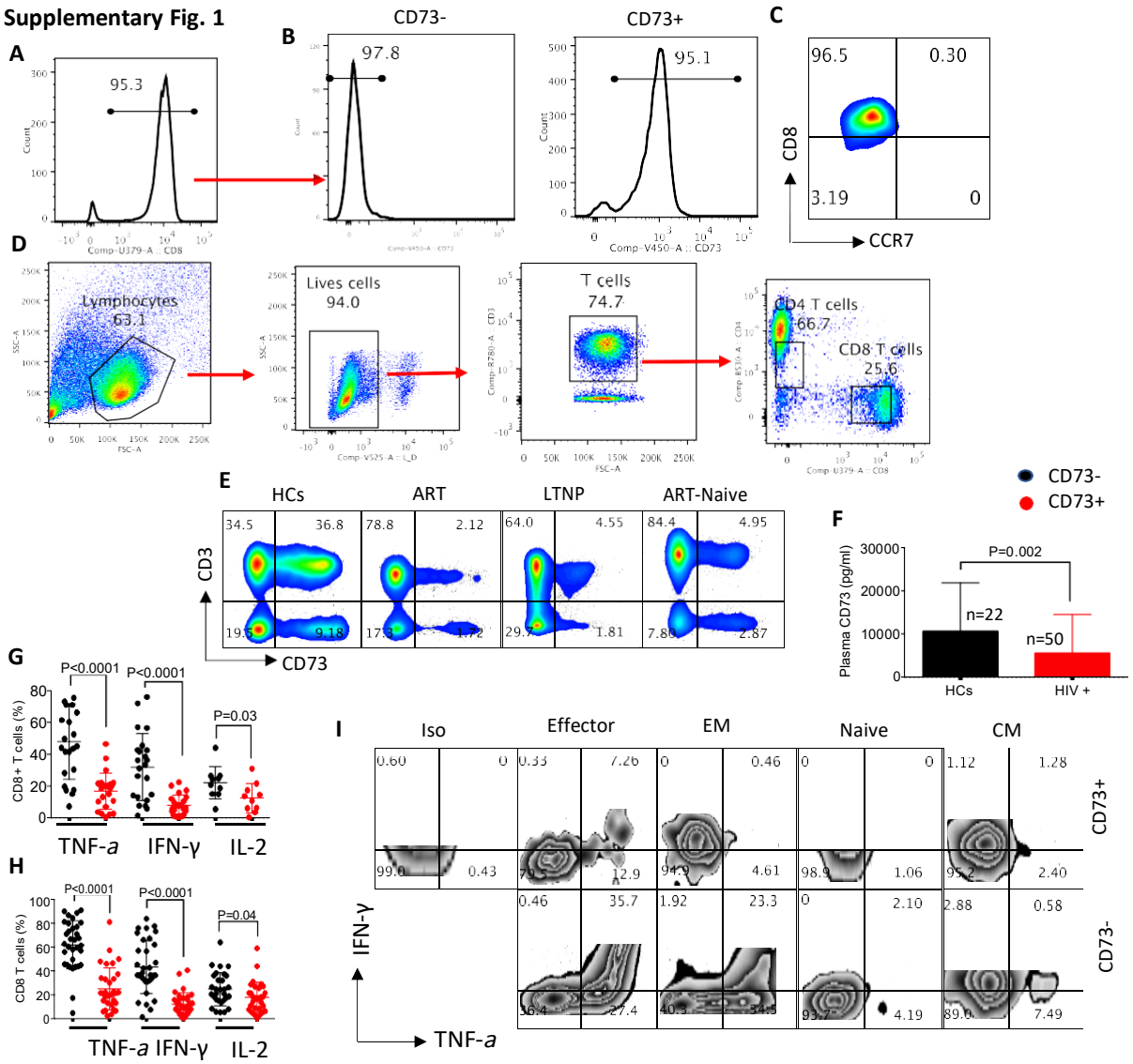
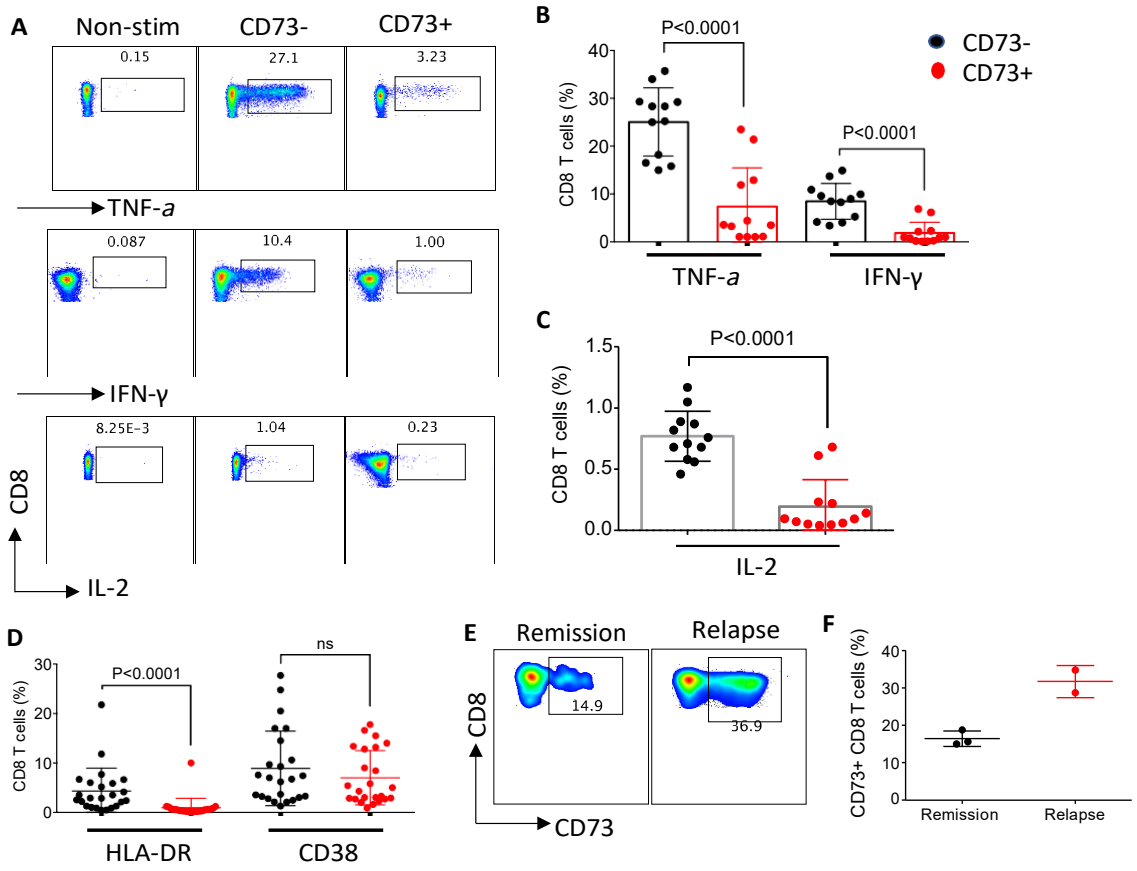
3.6 Supplementary Figures

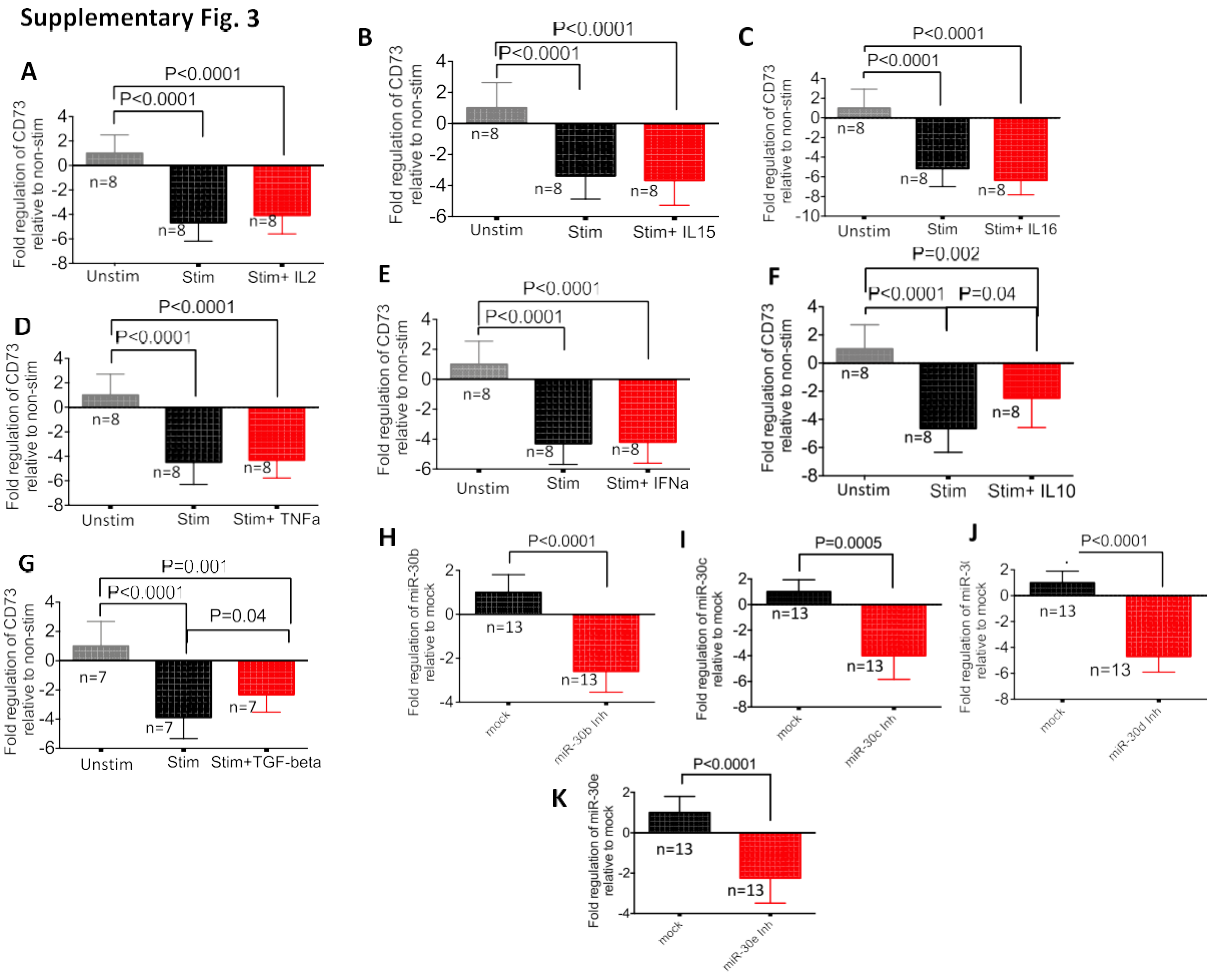


A) The representative histogram plots showing the purity of total CD8, **B**) CD73-, CD73+, and **C**) effector CD8 T cells. **D**) The gating strategy for CD4 and CD8 T cells. **E**) Representative plots of CD73 expression in CD3+/CD3- cells. **F**) Cumulative results of the plasma CD73 levels in HCs and HIV-infected individuals. **G**) Cumulative data for TNF-*a*, IFN-γ, and IL-2 expression in CD73- versus CD73+ CD8 T cells from HCs following stimulation with anti-CD3/CD28. **H**) Cumulative data of TNF-α, IFN-γ, and IL-2 production by CD73+ versus CD73- CD8 T cells upon stimulation of PBMCs from HIV-infected individuals with PMA for 6 hr as measured by ICS. **I**) Representative plots of TNF-*a* and IFN-γ expression in different subsets of CD73+ or CD73- CD8 T cells. Each dot represents results from a human subject. Data are obtained from multiple independent experiments.

Supplementary Fig. 2



A) Representative flow cytometry plots, and **B**, **C**) cumulative data of cytokine expression in CD73- versus CD73+ isolated CD8 T cells upon stimulation with anti-CD3/CD28 antibodies for 6 hr as measured by ICS. **D**) Cumulative data of HLA-DR and CD38 expression on CD73-/CD73+CD8 T cells of HIV-infected individuals. **E**) Representative flow cytometry plots of CD73+CD8 T cells in CSF of a MS patient at the remission and replace times. **F**) Cumulative data of percentages of CD73+CD8 T cells in CSF of three MS patients while on remission and two MS patients upon flare-up. Each dot represents a human subject either HIV-infected individual or MS patient. Data are obtained from multiple independent experiments.



A) Cumulative data showing fold regulation of CD73 gene in CD8 T cells in either unstimulated (Unstim), stimulated (Stim) or stimulated p lus IL-2 (50 IU/ml). **B**) Cumulative data of fold regulation of CD73 gene in CD8 T cells in either unstimulated, stimulated or stimulated plus IL-15 (100 ng/ml). **C**) Cumulative data of fold regulation of CD73 gene in CD8 T cells in either unstimulated, stimulated or stimulated plus IL-16 (1 μg/ml). **D**) Cumulative data of fold regulation of CD73 gene in CD8 T cells in either unstimulated, stimulated or stimulated plus TNF-α (50 ng/ml). **E**) Cumulative data showing fold regulation of CD73 gene in CD8 T cells in either unstimulated, stimulated or stimulated plus IFN-α (100 ng/ml). **F**) Cumulative data showing fold regulation of CD73 gene in CD8 T cells in either unstimulated, stimulated or stimulated plus IL-10 (100 ng/ml). **G**) Cumulative data of fold regulation of CD73 gene in CD8 T cells in either unstimulated, stimulated or stimulated plus TGF-beta (20 ng/ml). (**H-K**) Fold regulation of miRNA30b-30e in isolated CD8 T cells after their treatment with their corresponding miRNA-inhibitors quantified by qPCR.

3.7 Supplementary Tables

Supplementary Table 3.7.1 Participants clinical data

PTID	Sex	Plasma viral load (copies/mL^{-1})	CD4 count (mL^{-1})	On ART
LTNP-1	M	792	570	No
LTNP-2	M	<50	522	No
LTNP-3	M	<50	473	No
LTNP-4	F	800	421	No
LTNP-5	M	<50	796	No
LTNP-6	M	<50	832	No
LTNP-7	M	125	783	No
LTNP-8	M	<50	759	No
LTNP-9	F	<50	773	No
LTNP-10	M	65	426	No
LTNP-11	F	<50	643	No
LTNP-12	F	1073	574	No
LTNP-13	M	140	555	No
ART-1	M	<30	720	yes
ART-2	M	<30	570	yes
ART-3	M	<30	1080	yes
ART-4	M	<30	450	yes
ART-5	M	1399	800	yes
ART-6	M	<30	720	yes
ART-7	M	<30	830	yes
ART-8	M	<30	460	yes
ART-9	M	<30	470	yes
ART-10	F	<30	410	yes
ART-11	M	<30	350	yes
ART-12	M	<30	850	yes
ART-13	M	<30	740	yes
ART-14	M	<30	680	yes
ART-15	M	<30	470	yes
ART-16	M	<30	550	yes
ART-17	M	208	410	yes
ART-18	M	<30	420	yes
ART-19	M	<30	580	yes
ART-20	F	<30	590	yes
ART-21	M	<30	680	yes
ART-22	M	<30	570	yes
ART-23	M	<30	420	yes
ART-24	M	<30	210	yes
ART-25	M	<30	500	yes
ART-26	M	<30	640	yes
ART-27	M	<30	260	yes

ART-28	F	<30	700	yes
ART-29	F	<30	720	yes
ART-30	M	<30	910	yes
ART-31	F	168	290	yes
ART-32	F	<30	180	yes
ART-33	M	<30	680	yes
ART-34	M	<30	510	yes
ART-35	M	<30	1210	yes
ART-36	F	<30	550	yes
ART-37	F	60	910	yes
ART-38	F	<30	190	yes
ART-39	M	<30	340	yes
ART-40	F	160	60	yes
ART-41	M	<30	390	yes
ART-42	M	<30	250	yes
ART-43	M	<30	520	yes
ART-44	M	<30	610	yes
ART-45	M	<30	360	yes
ART-46	M	<30	290	yes
ART-47	M	<30	250	yes
ART-48	M	<30	260	yes
AET-49	M	<30	342	yes
ART-50	F	<30	457	yes
ART-51	M	<30	632	yes
ART-52	M	540	578	yes
ART-53	M	990	550	yes
ART-54	M	<30	602	yes
ART-55	M	<30	575	yes
ART-56	F	800	451	yes
ART-57	M	<30	790	yes
ART-58	M	<30	850	yes
ART-59	M	125	783	yes
ART-60	M	<30	765	yes
ART-61	M	<30	562	yes
ART-62	F	<30	690	yes
ART-63	F	<30	720	yes
ART-naïve-1	F	88800	307	No
ART-naïve-2	F	20000	333	No
ART-naïve-3	M	40370	463	No
ART-naïve-4	F	368000	305	No
ART-naïve-5	M	15200	447	No
ART-naïve-6	M	172200	479	No
ART-naïve-7	M	32300	798	No
ART-naïve-8	M	73300	156	No
ART-naïve-9	M	17200	382	No
ART-naïve-10	F	18800	390	No

ART-naïve-11	M	37400	335	No
ART-naïve-12	M	13700	414	No
ART-naïve-13	M	25300	259	No
ART-naïve-14	F	383000	551	No
ART-naïve-15	M	153000	329	No
ART-naïve-16	M	385000	239	No
ART-naïve-17	M	93300	396	No
ART-naïve-18	M	153000	321	No
ART-naïve-19	M	121000	408	No
ART-naïve-20	F	19500	637	No
ART-naïve-21	M	40100	748	No
ART-naïve-22	M	476000	556	No
ART-naïve-23	M	225000	621	No
ART-naïve-24	M	71500	547	No
ART-naïve-25	M	67200	448	No
ART-naïve-26	F	24100	787	No

Male (M) and female (F)

3.8 References

1. Shahbaz, S., M. Manicardi, G. Guaraldi, and P. Raggi. 2015. Cardiovascular disease in human immunodeficiency virus infected patients: A true or perceived risk? *World Journal of Cardiology* 7: 633–644.

2. Deeks, S. G., S. R. Lewin, and D. V. Havlir. 2013. The End of AIDS: HIV Infection as a Chronic Disease. *Lancet* 382: 1525–1533.

3. Elahi, S., R. H. Weiss, and S. Merani. 2016. Atorvastatin restricts HIV replication in CD4+ T cells by upregulation of p21. *AIDS* 30: 171–183.

4. Paiardini, M., and M. Müller-Trutwin. 2013. HIV-associated chronic immune activation. *Immunol Rev* 254: 78–101.

5. Bono, M. R., D. Fernández, F. Flores-Santibáñez, M. Rosemblatt, and D. Sauma. 2015. CD73 and CD39 ectonucleotidases in T cell differentiation: Beyond immunosuppression. *FEBS Letters* 589: 3454–3460.

6. Beavis, Paul. A., J. Stagg, P. K. Darcy, and M. J. Smyth. 2012. CD73: a potent suppressor of antitumor immune responses. *Trends in Immunology* 33: 231–237.

7. Resta, R., Y. Yamashita, and L. F. Thompson. 1998. Ecto-enzyme and signaling functions of lymphocyte CD 7 3. *Immunological Reviews* 161: 95–109.

8. Airas, L., J. Niemelä, and S. Jalkanen. 2000. CD73 Engagement Promotes Lymphocyte Binding to Endothelial Cells Via a Lymphocyte Function-Associated Antigen-1-Dependent Mechanism. *The Journal of Immunology* 165: 5411–5417.

9. Tóth, I., A. Q. Le, P. Hartjen, A. Thomssen, V. Matzat, C. Lehmann, C. Scheurich, C. Beisel, P. Busch, O. Degen, A. W. Lohse, T. Eiermann, G. Fätkenheuer, D. Meyer-Olson, M. Bockhorn, J. Hauber, J. van Lunzen, and J. Schulze Zur Wiesch. 2013. Decreased frequency of CD73+CD8+ T cells of HIV-infected patients correlates with immune activation and T cell exhaustion. *Journal of Leukocyte Biology* 94: 551–561.

10. Velasquez, S., L. Prevedel, S. Valdebenito, A. M. Gorska, M. Golovko, N. Khan, J. Geiger, and E. A. Eugenin. 2020. Circulating levels of ATP is a biomarker of HIV cognitive impairment. *EBioMedicine* 51: 102503.

11. Kordaß, T., W. Osen, and S. B. Eichmüller. 2018. Controlling the Immune Suppressor: Transcription Factors and MicroRNAs Regulating CD73/NT5E. *Front Immunol* 9.

12. Hansen, K. R., R. Resta, C. F. Webb, and L. F. Thompson. 1995. Isolation and characterization of the promoter of the human 5′-nucleotidase (CD73)-encoding gene. *Gene* 167: 307–312.
13. Fausther, M., N. Sheung, Y. Saiman, M. B. Bansal, and J. A. Dranoff. 2012. Activated hepatic stellate cells upregulate transcription of ecto-5′-nucleotidase/CD73 via specific SP1 and SMAD promoter elements. *American Journal of Physiology-Gastrointestinal and Liver Physiology* 303: G904–G914.
14. Synnestvedt, K., G. T. Furuta, K. M. Comerford, N. Louis, J. Karhausen, H. K. Eltzschig, K. R. Hansen, L. F. Thompson, and S. P. Colgan. 2002. Ecto-5′-nucleotidase (CD73) regulation by hypoxia-inducible factor-1 mediates permeability changes in intestinal epithelia. *J Clin Invest* 110: 993–1002.
15. Colgan, S. P., H. K. Eltzschig, T. Eckle, and L. F. Thompson. 2006. Physiological roles for ecto-5′-nucleotidase (CD73). *Purinergic Signalling* 2: 351.
16. Chalmin, F., G. Mignot, M. Bruchard, A. Chevriaux, F. Végran, A. Hichami, S. Ladoire, V. Derangère, J. Vincent, D. Masson, S. C. Robson, G. Eberl, J. R. Pallandre, C. Borg, B. Ryffel, L. Apetoh, C. Rébé, and F. Ghiringhelli. 2012. Stat3 and Gfi-1 Transcription Factors Control Th17 Cell Immunosuppressive Activity via the Regulation of Ectonucleotidase Expression. *Immunity* 36: 362–373.
17. Zheng, Y., S. Z. Josefowicz, A. Kas, T.-T. Chu, M. A. Gavin, and A. Y. Rudensky. 2007. Genome-wide analysis of Foxp3 target genes in developing and mature regulatory T cells. *Nature* 445: 936–940.
18. Zhu, J., Y. Zeng, W. Li, H. Qin, Z. Lei, D. Shen, D. Gu, J. Huang, and Z. Liu. 2017. CD73/NT5E is a target of miR-30a-5p and plays an important role in the pathogenesis of non-small cell lung cancer. *Molecular Cancer* 16: 34.
19. Wang, N., X. Xiang, K. Chen, P. Liu, and A. Zhu. 2018. Targeting of NT5E by miR-30b and miR-340 attenuates proliferation, invasion and migration of gallbladder carcinoma. *Biochimie* 146: 56–67.
20. Zhang, F., Y. Luo, Z. Shao, L. Xu, X. Liu, Y. Niu, J. Shi, X. Sun, Y. Liu, Y. Ding, and L. Zhao. 2016. MicroRNA-187, a downstream effector of TGFβ pathway, suppresses Smad-mediated epithelial–mesenchymal transition in colorectal cancer. *Cancer Letters* 373: 203–213.
21. Ikeda, Y., E. Tanji, N. Makino, S. Kawata, and T. Furukawa. 2012. MicroRNAs Associated with Mitogen-Activated Protein Kinase in Human Pancreatic Cancer. *Mol Cancer Res* 10: 259–269.
22. Braun, J., C. Hoang-Vu, H. Dralle, and S. Hüttelmaier. 2010. Downregulation of microRNAs directs the EMT and invasive potential of anaplastic thyroid carcinomas. *Oncogene* 29: 4237–4244.
23. Chanda, S., S. Nandi, and M. Chawla-Sarkar. 2016. Rotavirus-induced miR-142-5p elicits proviral milieu by targeting non-canonical transforming growth factor beta signalling and apoptosis in cells. *Cellular Microbiology* 18: 733–747.
24. Elahi, S., W. L. Dinges, N. Lejarcegui, K. J. Laing, A. C. Collier, D. M. Koelle, M. J. McElrath, and H. Horton. 2011. Protective HIV-specific CD8+ T cells evade Treg cell suppression. *Nat Med* 17: 989–995.
25. Shahbaz, S., G. Dunsmore, P. Koleva, L. Xu, S. Houston, and S. Elahi. 2020. Galectin-9 and VISTA Expression Define Terminally Exhausted T Cells in HIV-1 Infection. *J Immunol* 204: 2474–2491.
26. Dunsmore, G., N. Bozorgmehr, C. Delyea, P. Koleva, A. Namdar, and S. Elahi. 2017. Erythroid Suppressor Cells Compromise Neonatal Immune Response against Bordetella pertussis. *J. Immunol.* 199: 2081–2095.
27. Lorenzo-Herrero, S., C. Sordo-Bahamonde, S. Gonzalez, and A. López-Soto. 2019. CD107a Degranulation Assay to Evaluate Immune Cell Antitumor Activity. *Methods Mol Biol* 1884: 119–130.
28. Leal, D. B. R., C. A. Streher, C. de M. Bertoncheli, L. F. D. Carli, C. A. M. Leal, J. E. P. da Silva, V. M. Morsch, and M. R. C. Schetinger. 2005. HIV infection is associated with increased NTPDase activity that correlates with CD39-positive lymphocytes. *Biochim Biophys Acta* 1746: 129–134.
29. Migueles, S. A., and M. Connors. 2001. Frequency and function of HIV-specific CD8(+) T cells. *Immunol Lett* 79: 141–150.
30. Collins, D. R., G. D. Gaiha, and B. D. Walker. 2020. CD8 + T cells in HIV control, cure and prevention. *Nature Reviews Immunology* 20: 471–482.

31. Perdomo-Celis, F., N. A. Taborda, and M. T. Rugeles. 2019. CD8+ T-Cell Response to HIV Infection in the Era of Antiretroviral Therapy. *Front Immunol* 10.
32. Gonzalez, S. M., N. A. Taborda, and M. T. Rugeles. 2017. Role of Different Subpopulations of CD8+ T Cells during HIV Exposure and Infection. *Front Immunol* 8.
33. Lau, L. L., B. D. Jamieson, T. Somasundaram, and R. Ahmed. 1994. Cytotoxic T-cell memory without antigen. *Nature* 369: 648–652.
34. Persistence of Memory CD8 T Cells in MHC Class I-Deficient Mice | Science. .
35. Kong, Y., B. Jia, C. Zhao, D. F. Claxton, A. Sharma, C. Annageldiyev, J. S. Fotos, H. Zeng, R. F. Paulson, K. S. Prabhu, and H. Zheng. 2019. Downregulation of CD73 associates with T cell exhaustion in AML patients. *J Hematol Oncol* 12: 40.
36. Wehler, T. C., M. Karg, E. Distler, A. Konur, M. Nonn, R. G. Meyer, C. Huber, U. F. Hartwig, and W. Herr. 2008. Rapid identification and sorting of viable virus-reactive CD4+ and CD8+ T cells based on antigen-triggered CD137 expression. *Journal of Immunological Methods* 339: 23–37.
37. Olmo, N., J. Turnay, G. Risse, R. Deutzmann, K. von der Mark, and M. A. Lizarbe. 1992. Modulation of 5'-nucleotidase activity in plasma membranes and intact cells by the extracellular matrix proteins laminin and fibronectin. *Biochem J* 282: 181–188.
38. Sadej, R., K. Inai, Z. Rajfur, A. Ostapkowicz, J. Kohler, A. C. Skladanowski, B. S. Mitchell, and J. Spychala. 2008. Tenascin C interacts with ecto-5'-nucleotidase (eN) and regulates adenosine generation in cancer cells. *Biochim Biophys Acta* 1782: 35–40.
39. Adzic, M., and N. Nedeljkovic. 2018. Unveiling the Role of Ecto-5'-Nucleotidase/CD73 in Astrocyte Migration by Using Pharmacological Tools. *Front Pharmacol* 9.
40. Wagner, C., A. Bürger, M. Radsak, S. Blum, F. Hug, and G. M. Hänsch. 2000. Fibronectin synthesis by activated T lymphocytes: up-regulation of a surface-associated isoform with signalling function. *Immunology* 99: 532–539.
41. Kägi, D., F. Vignaux, B. Ledermann, K. Bürki, V. Depraetere, S. Nagata, H. Hengartner, and P. Golstein. 1994. Fas and perforin pathways as major mechanisms of T cell-mediated cytotoxicity. *Science* 265: 528–530.
42. Rouvier, E., M. F. Luciani, and P. Golstein. 1993. Fas involvement in Ca(2+)-independent T cell-mediated cytotoxicity. *J Exp Med* 177: 195–200.
43. Mills, J. H., L. F. Thompson, C. Mueller, A. T. Waickman, S. Jalkanen, J. Niemela, L. Airas, and M. S. Bynoe. 2008. CD73 is required for efficient entry of lymphocytes into the central nervous system during experimental autoimmune encephalomyelitis. *PNAS* 105: 9325–9330.
44. Rodriguez, M. W., A. C. Paquet, Y. H. Yang, and D. J. Erle. 2004. Differential gene expression by integrin β7+ and β7- memory T helper cells. *BMC Immunol* 5: 13.
45. Ålgars, A., M. Karikoski, G. G. Yegutkin, P. Stoitzner, J. Niemelä, M. Salmi, and S. Jalkanen. 2011. Different role of CD73 in leukocyte trafficking via blood and lymph vessels. *Blood* 117: 4387–4393.
46. SALMI, M., and S. JALKANEN. 1999. Molecules controlling lymphocyte migration to the gut. *Gut* 45: 148–153.
47. Höpken, U. E., J. Droese, J.-P. Li, J. Joergensen, D. Breitfeld, H.-G. Zerwes, and M. Lipp. 2004. The chemokine receptor CCR7 controls lymph node-dependent cytotoxic T cell priming in alloimmune responses. *Eur J Immunol* 34: 461–470.
48. Cao, S., and K. A. Woodrow. 2019. Nanotechnology approaches to eradicating HIV reservoirs. *European Journal of Pharmaceutics and Biopharmaceutics* 138: 48–63.
49. Kahle, E. M., M. Bolton, J. P. Hughes, D. Donnell, C. Celum, J. R. Lingappa, A. Ronald, C. R. Cohen, G. de Bruyn, Y. Fong, E. Katabira, M. J. McElrath, J. M. Baeten, C. Celum, A. Wald, J. Lingappa, A. Magaret, J. P. Hughes, L. Corey, J. M. Baeten, M. J. McElrath, D. Coetzee, K. Fife, E. Were, M. Essex, J. Makhema, E. Katabira, A. Ronald, E. Bukusi, C. Cohen, S. Allen, W. Kanweka, S. Kapiga, R. Manongi, C. Farquhar, G. John-Stewart, J. Kiarie, S. Allen, M. Inambao, O. Farm, S. Delany-Moretlwe, H. Rees, G. de Bruyn, G. Gray,

J. McIntyre, and N. R. Mugo. 2015. Plasma Cytokine Levels and Risk of HIV Type 1 (HIV-1) Transmission and Acquisition: A Nested Case-Control Study Among HIV-1–Serodiscordant Couples. *J Infect Dis* 211: 1451–1460.
50. Orsilles, M. A., E. Pieri, P. Cooke, and C. Caula. 2006. IL-2 and IL-10 serum levels in HIV-1-infected patients with or without active antiretroviral therapy. *APMIS* 114: 55–60.
51. Salazar-Gonzalez, J. F., O. Martinez-Maza, N. Aziz, J. A. Kolberg, T. Yeghiazarian, L. P. Shen, and J. L. Fahey. 1997. Relationship of plasma HIV-RNA levels and levels of TNF-alpha and immune activation products in HIV infection. *Clin Immunol Immunopathol* 84: 36–45.
52. Wiercińska-Drapalo, A., R. Flisiak, J. Jaroszewicz, and D. Prokopowicz. 2004. Increased plasma transforming growth factor-beta1 is associated with disease progression in HIV-1-infected patients. *Viral Immunol* 17: 109–113.
53. Amiel, C., E. Darcissac, M.-J. Truong, J. Dewulf, M. Loyens, Y. Mouton, A. Capron, and G. M. Bahr. 1999. Interleukin-16 (IL-16) Inhibits Human Immunodeficiency Virus Replication in Cells from Infected Subjects, and Serum IL-16 Levels Drop with Disease Progression. *The Journal of Infectious Diseases* 179: 83–91.
54. Osunkalu, V. O., A. Adewumi, C. Onwuzuluigbo, and O. F. Olowoselu. 2015. Interleukin-8 A Marker of Disease Progression and Therapeutic Response in HIV Infection. *Nigerian Hospital Practice* 15: 21–26.
55. Hardy, G. A. D., S. Sieg, B. Rodriguez, D. Anthony, R. Asaad, W. Jiang, J. Mudd, T. Schacker, N. T. Funderburg, H. A. Pilch-Cooper, R. Debernardo, R. L. Rabin, M. M. Lederman, and C. V. Harding. 2013. Interferon-α is the primary plasma type-I IFN in HIV-1 infection and correlates with immune activation and disease markers. *PLoS One* 8: e56527.
56. Xie, M., H. Qin, Q. Luo, Q. Huang, X. He, Z. Yang, P. Lan, and L. Lian. 2017. MicroRNA-30a regulates cell proliferation and tumor growth of colorectal cancer by targeting CD73. *BMC Cancer* 17.
57. Klemens, M. R., W. R. Sherman, N. J. Holmberg, J. M. Ruedi, M. G. Low, and L. F. Thompson. 1990. Characterization of soluble vs membrane-bound human placental 5′-nucleotidase. *Biochemical and Biophysical Research Communications* 172: 1371–1377.
58. Vogel, M., H. Kowalewski, H. Zimmermann, N. M. Hooper, and A. J. Turner. 1992. Soluble low-Km 5′-nucleotidase from electric-ray (Torpedo marmorata) electric organ and bovine cerebral cortex is derived from the glycosyl-phosphatidylinositol-anchored ectoenzyme by phospholipase C cleavage. *Biochemical Journal* 284: 621–624.
59. Morello, S., M. Capone, C. Sorrentino, D. Giannarelli, G. Madonna, D. Mallardo, A. M. Grimaldi, A. Pinto, and P. A. Ascierto. 2017. Soluble CD73 as biomarker in patients with metastatic melanoma patients treated with nivolumab. *Journal of Translational Medicine* 15: 244.
60. Wherry, E. J., S.-J. Ha, S. M. Kaech, W. N. Haining, S. Sarkar, V. Kalia, S. Subramaniam, J. N. Blattman, D. L. Barber, and R. Ahmed. 2007. Molecular signature of CD8+ T cell exhaustion during chronic viral infection. *Immunity* 27: 670–684.
61. Grünewald, J. K., and A. J. Ridley. 2010. CD73 represses pro-inflammatory responses in human endothelial cells. *Journal of Inflammation* 7: 10.
62. Kedzierska, K., and S. M. Crowe. 2001. Cytokines and HIV-1: interactions and clinical implications. *Antivir Chem Chemother* 12: 133–150.
63. Ellwanger, J. H., J. M. Valverde-Villegas, V. de L. Kaminski, R. M. de Medeiros, S. E. de M. Almeida, B. R. Santos, M. G. de Melo, F. S. Hackenhaar, and J. A. B. Chies. 2020. Increased IL-8 levels in HIV-infected individuals who initiated ART with CD4+ T cell counts <350 cells/mm3 – A potential hallmark of chronic inflammation. *Microbes and Infection* 22: 474–480.
64. Roff, S. R., E. N. Noon-Song, and J. K. Yamamoto. 2014. The Significance of Interferon-γ in HIV-1 Pathogenesis, Therapy, and Prophylaxis. *Front Immunol* 4.
65. Stefanou, M.-I., M. Krumbholz, U. Ziemann, and M. C. Kowarik. 2019. Human immunodeficiency virus and multiple sclerosis: a review of the literature. *Neurological Research and Practice* 1: 24.

66. Petrovic, A., O. Alpdogan, L. M. Willis, J. M. Eng, A. S. Greenberg, B. J. Kappel, C. Liu, G. J. Murphy, G. Heller, and M. R. M. van den Brink. 2004. LPAM (alpha 4 beta 7 integrin) is an important homing integrin on alloreactive T cells in the development of intestinal graft-versus-host disease. *Blood* 103: 1542–1547.
67. Hunter, M. C., A. Teijeira, and C. Halin. 2016. T Cell Trafficking through Lymphatic Vessels. *Front. Immunol.* 7.
68. Kim, E.-S., C. Ackermann, I. Tóth, P. Dierks, J. M. Eberhard, R. Wroblewski, F. Scherg, M. Geyer, R. E. Schmidt, C. Beisel, M. Bockhorn, F. Haag, J. van Lunzen, and J. S. zur Wiesch. 2017. Down-regulation of CD73 on B cells of patients with viremic HIV correlates with B cell activation and disease progression. *Journal of Leukocyte Biology* 101: 1263–1271.

Chapter 4: Differential transcription and functional properties of regulatory T cells in HIV-infected individuals on antiretroviral therapy and Long-term non-progressors

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4.1 Introduction

Human immunodeficiency virus 1 (HIV-1) infection is characterized by the progressive depletion of CD4 T cells and persistent immune activation resulting in the gradual deterioration of the immune system (1, 2). Persistent hyper-immune activation is linked to endothelial inflammation and subsequently increased rates of non-AIDS-associated comorbidities such as cardiovascular diseases (3). Furthermore, activated CD4 T cells become an attractive target of HIV-1 infection by upregulating CCR5 and CXCR4 expression, two main HIV-1 co-receptors (4). Regulatory T cells (Tregs) are one of the main cornerstones responsible for controlling inappropriate HIV-1 associated hyper-immune activation (5). Tregs are a subset of CD4 T cells that are characterized by the high surface expression of IL-2Rα (CD25) and the transcription factor FOXP3 (6, 7). Tregs constitute a heterogeneous population and can be divided into two main subsets of effector Tregs (eTregs) and central Tregs (cTregs) (8, 9). cTregs and eTregs can be distinguished based on the expression of CD45RA, CD62L, and CCR7 (10, 11). While cTregs are mainly localized in secondary lymphoid tissues, eTregs are primarily found in non-lymphoid tissues as well as secondary lymphoid organs (9). Tregs exert their regulatory functions through several mechanisms, including secretion of inhibitory cytokines or soluble factors (e.g. TGF-β, IL-35, and IL-10), the direct killing of target cells (through cytolytic molecules such as granzyme B (GzmB)), inhibition of dendritic cells maturation and function through engagement by co-inhibitory receptors (e.g. CTLA4:CD80/CD86), metabolic deprivation (e.g. CD25-mediated IL-2-deprivation-induced apoptosis) and cell: cell interactions (e.g. PD-1/PDL-1 and Gal-9:Tim-3) (12). Although Tregs may have potential benefits in HIV-1 infection by suppressing an inadvertent immune response, this could be counteracted by their detrimental effects on viral control through the suppression of HIV-1-specific immune response.

Long-term non-progressors (LTNPs) are a rare group of HIV-1 infected individuals with low viral load (< 10,000 copies mL^{-1}), high CD4 T cell count and antiretroviral therapy (ART) naive (13). Several mechanisms have been proposed to explain the robust viral replication control in LTNPs, such as the possession of

certain HLA alleles, primarily HLA-B*27 and HLA-B*57 alleles (14). Previously, we showed that CD8 T cells restricted by HLA-B27 and -B57 evade Tregs-mediated suppression and continue to proliferate and kill virally-infected cells through secretion of lytic molecules such as GzmB (13). In contrast, CD8 T cells restricted by non-HLA-B*27/B*57 alleles upon recognition of their cognate epitopes upregulate Tim-3 and, therefore, become suppressed by Tregs via Tim-3: Galectin-9 (Gal-9) interaction (13). More recently, we reported that CD8 T cells restricted by HLA-B*35Px and HLA-B*53 alleles associated with rapid disease progression to AIDS also evade Tregs mediated suppression by not upregulating Tim-3 upon cognate epitope recognition (15). Nevertheless, the phenotype and global function of Tregs in LTNPs versus patients on ART have not been well characterized. Several studies have shown that LTNPs exhibit similar or decreased frequency of Tregs compared to healthy controls (HCs); however, the function of Tregs was not fully examined in these studies (16–18). The role of Tregs in HIV-1-infection has been the topic of debate and controversies (19). This becomes more complicated when there are conflicting data on the frequency of Tregs in HIV-infected individuals. For example, some studies have reported higher and others lower Tregs percentages in HIV-1 infected patients (20–22).

The present study was designed to conduct a comprehensive RNAseq analysis to better understand differences in Tregs at the transcriptome levels in LTNPs, HIV-infected individuals receiving ART (ART), and HCs. Furthermore, based on the RNAseq data, we performed additional studies to better characterize the functionality of Tregs in different study groups. Our results show that Tregs from ART patients upregulate pathways downstream of TCR and IL-2 stimulation, resulting in a more suppressive (activated) phenotype. In contrast, Tregs in LTNPs shows the upregulation of pathways associated with less suppressive effector functions. We further confirmed our observations by showing that Tregs from patients on ART were more potent in suppressing the proliferation of effector T cells compared to Tregs from other groups. Thus, our studies provide a novel insight into the functionality of Tregs in different groups of HIV-infected individuals.

4.2 Material and methods

4.2.1 Study population

We recruited 112 human subjects for our study, consisting of (1) 38 HCs, which were HIV-1, hepatitis C virus (HCV), and hepatitis B virus (HBV) seronegative; (2) 50 HIV-infected individuals on ART; (3) 24 LTNPs, who were ART-naïve, had been infected with HIV-1 > 11 years with CD4 T cell count > 400 (cell mL^{-1} of blood) and plasma viral load < 10,000 copies mL^{-1} (13, 23). Some LTNPs were recruited through the University of Washington/Fred Hutch Centre for AIDS Research (CFAR). The remaining patients were recruited from the cohort of the Northern Alberta HIV program.

4.2.2 Cell isolation

We isolated the peripheral blood mononuclear cells (PBMCs) from the fresh blood of either healthy subjects or HIV-infected patients using Ficoll-Paque gradients. PBMCs were cultured in RPMI 1640 (Sigma-Aldrich, Toronto) supplemented with 10% FBS (Sigma-Aldrich) and 1% penicillin/streptomycin (Sigma-Aldrich). In some studies, Tregs were isolated from fresh PBMCs according to the manufacturing instruction (STEMCELL Technologies, Vancouver), with a purity exceeding 95% (Supplementary figure 1a).

4.2.3 Flow cytometry analysis

Fluorophore antibodies with specificity to antigens and cytokines of human cells were purchased mainly from BD Biosciences (San Jose), Thermo Fisher Scientific (Waltham), and BioLegend (San Diego). The following antibodies were used specifically in our study: anti- CD3 (SK7), anti-CD4 (RPA-T4), anti-CD8 (RPA-T8), anti-TIGIT (MBSA43), anti–TIM-3 (7D3), anti-CD39 (TU66), anti–Gal-9 (9M1-3), anti-CD25 (M-A251), anti-CD127 (HIL-7R-M21), anti-pSTAT5 (pY694), anti-CD45RA (HL100), anti-FOXP3 (150D/E4), anti-FOXP3 (PCH101), anti–HLA-DR (LN3), anti-CD62L (DREG-56), anti-pZap70 (J34-602), anti-Ki67 (20Raj1), anti-pERK1/2 (MILAN8R), anti-CD73 (AD2), anti-CTLA-4 (BNI3), anti-CCR7 (2-L1-A), anti-ICOS (C398.A4), anti-pS235/pS236 (N7-548) and anti-HLA-F (3D11).

For stimulation, mouse anti-human CD3 (UCHT1) was purchased from BD Biosciences. The apoptotic assay was performed using the PE Annexin V Apoptosis Detection Kit (BD Biosciences) according to the manufacturing protocol. Surface and intranuclear staining were performed as we previously described elsewhere (13, 24). To assess cell viability, the LIVE/DEAD Kit (Thermo Fisher Scientific) was used. Stained cells were fixed with paraformaldehyde (2%) and were acquired on a Fortessa- X20 or LSRFortessa-SORP (BD Biosciences). Data were analyzed using FlowJo software (version 10). For Tregs staining, PBMCs were subjected to surface staining for the viability dye (Therm Fisher Scientific) and anti-CD3, CD4, CD25 and CD127 antibodies followed by washing. Then cells were permeabilized using the permeabilization buffer (Thermo Fisher Scientific) per the manufacture's instruction before staining for the FOXP3, as we have reported elsewhere (13). For comparing surface versus intracellular staining for CD73, we first stained the cells with the anti-CD73 antibody for surface staining followed by fixation/permeabilization and intracellular staining using the same clone but a different fluorophore.

4.2.4 Phospho-flow assays

Phospho-STAT5, phospho-ERK, phospho-Zap70, and pS235/pS236 1/2 staining were performed according to the manufacturing instructions (BD Biosciences). In brief, PBMCs were cultured in RPMI media containing (0.5 % FBS) in a 96 well plate and were stimulated for 30 min with IL-2 (100 IU) for STAT5, with PMA (2 $\mu L\ mL^{-1}$) for ZAP70 and pERK, and with IL-6 (100 $ng\ mL^{-1}$) for STAT3. Then cells were stained for surface antigens followed by fixation with 2% PFA. Cells were incubated for 30 min at room temperature in the dark, and then the cell pellet was resuspended in 100 µL of ice-cold 90-100% methanol. Next, the plate was incubated at 2-8$^{\circ}C$ for 30 min and washed with 2%FBS in PBS. The cell pellet was resuspended in 50 µL of 2%FBS in PBS followed by the addition of an appropriate concentration of conjugated phospho-antibodies for 45 min incubation at room temperature. Finally, the cell pellet was washed and resuspended in 2%FBS in PBS for analysis by the flow cytometer.

4.2.5 Proliferation assay

For functional assays, T cells were isolated (STEMCELL Technologies) and labelled with CFSE dye (Thermo Fisher Scientific) and cultured in 96 well plates in the absence or presence of Tregs at a 1:1 ratio. Cell stimulation was performed using anti-CD3 (1µg mL^{-1}) and mitomycin-treated whole PBMCs, as we have reported elsewhere (25). The % suppression of proliferation calculated by (% CFSEloCD3 w/out Tregs - % CFSEloCD3 with Tregs)/CFSEloCD3 w/out Tregs x100 as reported elsewhere (13).

4.2.6 Library construction and sequencing

Total RNA was extracted with TRIzol reagent (Invitrogen, Waltham) as per the manufacturer's instructions. RNAseq libraries were made from 100 ng of total RNA using the TruSeq RNA Library Prep Kit v2 (Illumina). Polyadenylated mRNAs were pulled down with oligo dTs conjugated to paramagnetic beads by sequential EtOH washes that removed non-polyadenylated transcripts. Recovered mRNAs were chemically fragmented and used for first- and second-strand cDNA synthesis. cDNAs were blunted and A-tailed; T-A ligation was used to add, and finally, Illumina adapters containing multiplexing barcodes were incorporated by 12 cycles of PCR. Sequencing was done on a HiSeq 2500 instrument with a paired-end 150 cycles protocol. Demultiplexing was carried out in-instrument. Data generated are publicly available from the SRA portal of NCBI under accession number PRJNA671810.

4.2.7 Bioinformatic analysis

Fragments were aligned to the human cDNA database (GRCh38) using Kallisto (26), with 100 permutations during pseudo-alignments and bias correction. Differential expression (DE) analysis of count data was conducted using negative binomial generalized linear models with the DESeq2 R package (27). Gene abundance differences with a corrected *P*-value (P_{adj}) < 0.05 and a $\log_2$ -1 < fold change > +1 were considered differentially expressed. Plots were generated with R scripts.

4.2.8 Statistical analysis

The *P*-values shown in the graphs were determined by the non-parametric Mann Whitney *U*-test. Statistical analysis was performed using the Prism software, and non-parametrical measures are

expressed as median with interquartile range (MIR) and a *P*-value < 0.05 was considered to be statistically significant.

4.2.9 Ethics statement

The institutional review boards at the University of Alberta approved our study with the ethics # Pro00070528 and Pro000064046. All study participants gave written informed consent to participate in the study.

4.3 Results

4.3.1 Differential gene expression profile of Tregs

For this study, we recruited 38 HCs, 50 HIV-infected individuals who have been on ART for a minimum of 2 years, and 24 LTNPs defined as ART-naïve, infected with HIV-1 >11 years, $CD4^+$ T cell count > 400 (cell mL^{-1} of blood), and plasma viral load < 10,000 copies mL^{-1} (Supplementary Table 1). To determine possible differences between the transcription profile of Tregs in ART, LTNPs, and HCs, we conducted RNA sequencing (RNAseq) on the total RNA extracted from the enriched Tregs of 5 individuals per group (Supplementary Table 2). Isolated Tregs had a purity of > 95% using (13) CD25 and FOXP3 staining (Supplementary Fig. 1A).

A transcript was considered differentially expressed (DE) if it had an FDR < 0.05 and a minimum of $\log_2$ fold-change (L2FC) smaller than -1 or greater than +1. The greatest differences in transcriptions were observed when HCs and ART subjects were compared (Fig. 1A, B, Supplementary Fig. 1B). Principal component analysis (PCA) based on Euclidean distances separated samples in HCs and ART groups in a two-dimensional plot, although one ART subject deployed a transcription profile similar to that of HCs (Supplementary Fig. 1C). More specifically, compared with HCs, 2956 and 2278 transcripts were found up and downregulated in ART Tregs, respectively (Fig. 1A). Among the most upregulated transcripts in the ART group were those belonging to genes in the human leukocyte antigen (HLA) complex, class I (22 transcripts) and class II (six transcripts), some of which expressed with a Log2FC higher than 20. Thus, we

concluded that the most upregulated transcripts in ART patients were transcripts belonging to the HLA class I and II complexes. Nonetheless, nine of class I and two of class II genes were downregulated in the ART group. DDX39B was the second most up-regulated HLA-associated transcript in the ART group as a component of the TREX complex and has been reported to stimulate viral RNA synthesis (28). TAP2 was the third up-regulated transcript in ART Tregs compared to HC Tregs. TAP2 is involved in HLA-1 protein assembly and expression, which results in the generation of $CD8^+$ T cell-specific immune response against viral particles (29).

Among the most downregulated transcripts were those from the splicing factor 1 (SF1) gene, which is a proto-oncogene involved in HIV-1 replication (30). Heterogeneous nuclear ribonucleoprotein H1 (HNRNPH1) was the next downregulated transcript in ART Tregs compared to HCs Tregs, which belongs to HIV-1 dependency factors (HDF) (31). Although HDFs are essential for HIV-1 replication, silencing of their expression is not lethal to the host cell (31, 32). Moreover, ten transcripts of the double bromodomain-containing protein (BRD2) were drastically downregulated in ART Tregs. BRD2 suppresses HIV-1 transcription in latent cells, contributing to HIV-1 dormant state in its reservoirs (33). The identities (IDs) of genes that contain the 10 most down or upregulated transcripts in this comparison are shown in Supplementary figure 1b. When Tregs from HCs were compared to LTNP subjects, the observed transcription changes were less dramatic than in the previous group (Fig. 1B, C, Supplementary Fig. 1D). In this comparison, we identified 419 and 262 transcripts that were up and downregulated, respectively. Although HCs and LTNPs were well-separated by the first two components of a PCA (Supplementary Fig. 1E), it was evident that LTNPs constituted two subgroups with three subjects deploying a transcription profile more similar to subjects in the HCs. The IDs of the genes that contain the 10 most down or upregulated transcripts in this comparison are shown in Supplementary figure 1D.

Among the three most upregulated transcripts were HLA-F gene and genes associated with HLAs such as DDX39B, and to a lesser extent, heat shock protein family A (Hsp70) member 1B (HSPA1B). Hsp70 binds

to HIV-1 Gag polyprotein chains during transport to the plasma membrane and maintains their assembly-competent conformation (34, 35). Besides, after the entry of virions to the target cells, virion-associated Hsp70 precipitates in the early phases of infection (36). Thus, Hsp70 proteins contribute to HIV-1 antigen presentation and promote viral replication inside the host cell.

SF1 was also the most downregulated gene in the LTNPs, as in the case of the ART group. The RNA binding protein SMN1 and protein phosphatase 1 regulatory inhibitory subunit 11 (PPP1R11) were also amongst the most downregulated genes. PPP1R11 is a negative regulator of cytokine expression upon T cell activation and might contribute to Treg polarization (37). Finally, the comparison between LTNP and ART subjects revealed the upregulation and downregulation of 92 and 63 transcripts in ART compared with LTNPs, respectively (Fig. 1B, D, Supplementary Fig. 1F). PCA analysis showed that although samples in the ART and LTNP groups were different, the latter one showed two subgroups (Supplementary Fig. 1G). A series of class I and II HLA genes were found dramatically upregulated in the ART group compared to LTNPs; however, a smaller number of class I HLA genes were found to be downregulated. The survival of motor neuron 1 (SMN2) and exocyst complex component 3 (EXOC3) were the next up-regulated transcripts in ART compared to LTNPs. EXOC3 involves in the targeted transfer of Nef between cells of the immune system, leading to the viral particle spreading between immune cells (38).

As mentioned before, a series of class I HLA genes were drastically downregulated in ART compared with LTNP Tregs. BRD2 and asparagine-linked glycosylation 13 (ALG13) were the next down-regulated genes in ART Tregs. ALG13 heterodimerizes with ALG14 to form UDP-GlcNAc glycosyltransferase (39). GlcNAcylation inhibits the activity of the HIV-1 promotor in infected host cells, resulting in decreased viral transcription (40). These analyses revealed substantial differences at the transcriptome levels between Tregs in HCs with patients on ART compared to the other group.

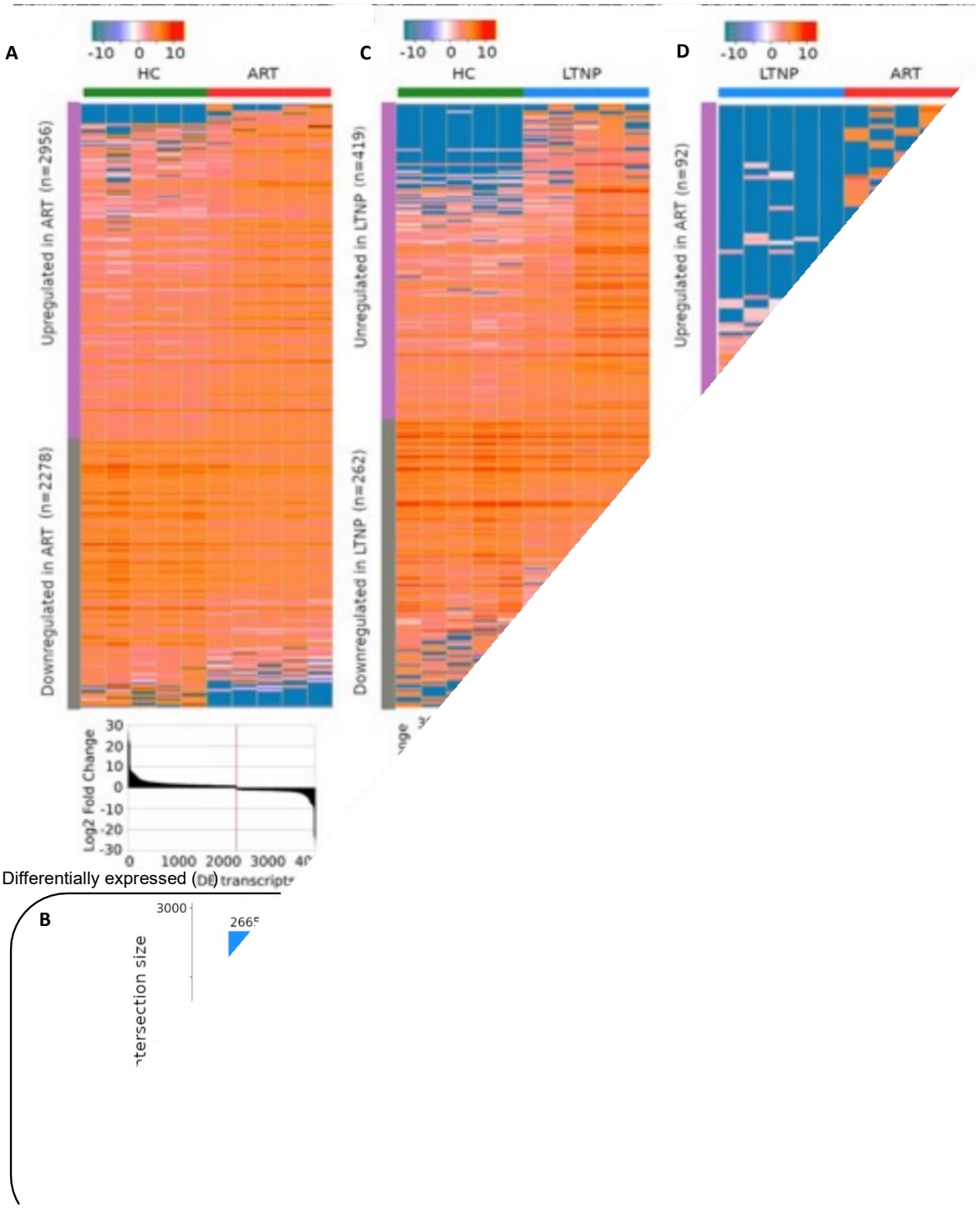

Fig. 1. Heat maps describing normalized abundance of differentially expressed transcripts. A) Differentially expressed transcripts in HCs vs ARTs. **B**) UpSet plots depicting transcripts DE in one or more comparisons. Upregulated (left panel) and downregulated (right panel) transcripts are shown separately. Side bars represent number of transcripts DE in each comparison (red and green bars correspond to upregulated or downregulated DE transcripts, respectively). Dodger-blue vertical bars represent the

intersection size, i.e., the number of transcripts that were found DE in one or more comparisons. Blue dots represent comparisons in which transcripts were found DE. For instance, the left-most vertical blue bar in each panel indicates that 2665 and 2138 transcripts, respectively, were found up or downregulated only in the comparison of HC versus ART. Correspondingly, the right-most blue bar in each panel indicates that 19 and 26 transcripts, respectively, were found up or downregulated in the comparisons of HCs versus ARTs, and LTNPs versus ARTs. Blue dots connected with a line indicate transcripts DE in more than one comparison. **C**) Differentially expressed transcripts in HCs vs LTNPs. **D**) Differentially expressed transcripts in LTNPs vs ARTs. Regularized logarithmic transformation was applied to the raw counts prior to heat map plotting. Only transcripts with an FDR < 0.05 and an absolute $\log_2$ fold-change value > 1 are included. The number of upregulated or downregulated transcripts in each comparison is indicated with purple or dark-grey vertical bars on the right of each heat map and included in parentheses. Below each heat map, the magnitude of the $\log_2$ fold-change is depicted in the graph bar. As indicated, RNAseq was obtained from 5 human subjects in each group.

4.3.2 Higher TCR and IL-2 signalling in Tregs of ART compared to HCs

We subsequently used the weighted IPA analysis to computationally analyze the upstream regulators that govern the differential gene expression pattern in HCs vs the ART group. Our analysis revealed a higher expression of CD3, Fibronectin 1 (FN1), and IL2 upstream regulators in ART Tregs compared to Tregs in HCs (Fig. 2A). Since IL2 signalling occurs through the phosphorylation of STAT5, we examined the phosphorylation of STAT5 in ART compared to HCs. Our observation revealed a higher expression of IL2RA (CD25) transcript (Fig. 2B) and the mean fluorescent intensity (MFI) of both CD25 and phospho-STAT5 in ART compared to HCs (Fig. 2C-F). Although there was no significant difference in the expression of CD25 in ARTs versus LTNPs, the MFI for phospho-STAT5 was significantly lower in Tregs of LTNPs compared to their counterparts in the ART group (Fig. 2F). The FN1 works as a costimulatory molecule in T-cell receptor (TCR)-mediated T cell activation (41) and TCR signalling is required for Treg effector differentiation and function, which is mediated through the phosphorylation of ZAP70. In agreement, we found higher phosphorylation of ZAP70 in Tregs of ART compared to HCs (Fig. 2G, H). Similarly, the MFI of ZAP70 was significantly higher in Tregs of LTNPs compared to HCs, but it was lower in Tregs of LTNPs when compared to Tregs from patients on ART (Fig. 2G, H). Thus, upstream analyses revealed higher TCR and IL2 activity and up-regulation of their downstream signalling pathways in Tregs of ART compared to HCs and LTNPs.

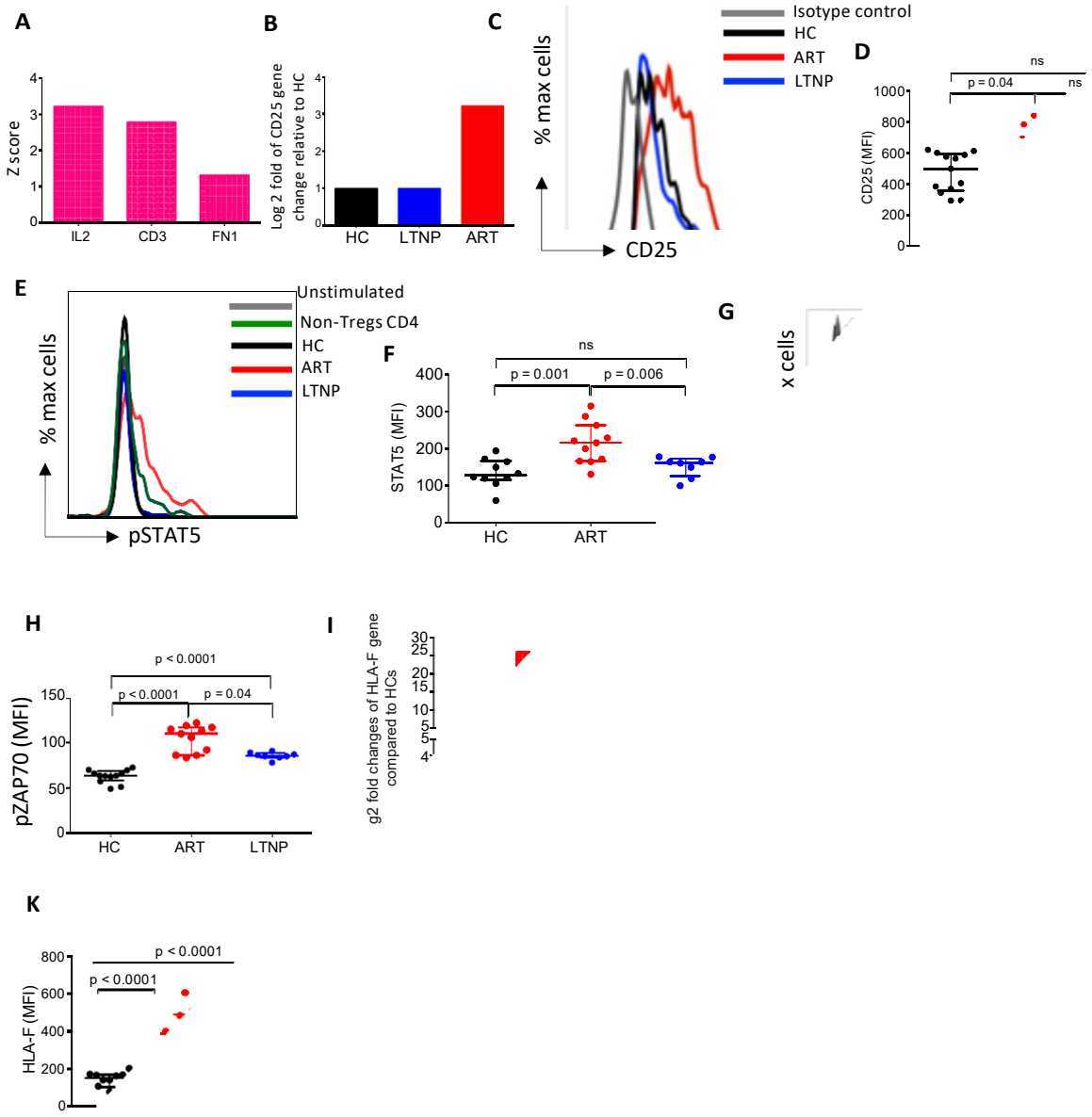

Fig 2. Higher TCR and IL-2 signalling in Tregs of ARTs than in those of HCs. A) Upstream regulators predicted to account for transcription differences between HCs versus ART patients. **B**) Log_2 fold change in the CD25 gene in Tregs from ART compared to HCs. **C**) Representative histogram of CD25 expression, and **D**) the mean fluorescence intensity (MFI) ± Median with interquartile range (MIR) of CD25 expression in Tregs of different study groups (n = 13 HC, n = 17 ART, n = 9 LTNP). **E**) Representative histogram, and **F**) the MFI ± MIR of pSTAT5 expression in Tregs of different study groups (n = 10 HC, n = 11 ART, n = 8 LTNP). **G**) Representative histogram, and **H**) the MFI ± MIR of pZAP70 expression in Tregs of different study groups (n = 12 HC, n = 11 ART, n = 8 LTNP). **I**) Log_2 fold change of HLA-F gene in Tregs of ARTs and LTNPs compared to HCs. **J**) Representative histogram, and **K**) the MFI ± MIR of HLA-F expression in Tregs of HIV-infected individuals versus HCs (n = 10 HC, n = 10 ART, n = 8 LTNP). **L**)

Representative histogram, and **M**) the MFI ± MIR of HLA-F expression in unstimulated (n = 14 Unstim.) and stimulated $CD4^+$ T cells from the ART group (n = 14 Stim.) $CD4^+$ T cells with anti-CD3/CD28 for 5 h. Each dot represents a human subject either HC or HIV-infected. Data were obtained from a single RNAseq analysis or from 3 to 5 independent experiments.

4.3.3 High frequency of HLA-F in Tregs from HIV-infected individuals compared to HC

Our RNAseq analysis revealed that Tregs from HIV-infected patients, either ART or LTNPs express significantly higher mRNA levels for HLA-F compared to HCs (Fig. 2I). HLA-F is one of the non-classical HLA class Ib members with a tolerogenic role (42). The same pattern was observed at the protein level, and Tregs from HIV-infected individuals, regardless of their status (ART or LTNPs) expressed significantly higher surface HLA-F than Tregs from HCs (Fig. 2J, K). As reported elsewhere (43), we found that activation of CD4 T cells was associated with the upregulation of HLA-F (Fig. 2L, M). These observations suggest that HLA-F may play an important role in Tregs effector functions in HIV patients, which merits further investigations.

4.3.4 Tregs in the ART group exhibit a greater effector phenotype than Tregs in HCs

We first quantified Tregs frequency in HIV infected individuals and HCs. However, we did not observe any significant difference in their percentages between groups (Supplementary Fig. 2A-C). We then used IPA analysis to computationally translate the observed gene expression patterns into directional changes of canonical pathways in Tregs of HCs vs ART. As shown in Figure 3A, the mTOR pathway was the most upregulated pathway in Tregs from ART compared to Tregs from HCs. mTOR has a key role in many cellular processes in Tregs, including cell proliferation, metabolism, and suppressive functions (44–47). Since the expression of mTOR has been reported to be higher in eTregs than in cTregs (45, 47), we examined the expression of CD45RA and CCR7 in Tregs to compare their maturation and differentiation status in ART and HCs. Our results showed a significantly higher percentage of CD45RA+CCR7- Tregs in ART and LTNP than in HCs, compatible with a greater effector phenotype (Fig. 3B, C). Moreover, we found a higher expression of other genes that are associated with eTregs in Tregs from ART than those from HCs (Fig. 3D

and Supplementary Table 3). Among these genes, we identified the interferon regulatory factor 4 (IRF4), which is a downstream molecule of the TCR signalling pathway. It controls the expression of several genes that are related to activation, proliferation, differentiation, and immunosuppression by Tregs. Then, we further analyzed the expression of other genes that are under the control of IRF4 and found that many of IRF4 target genes were highly upregulated in Tregs from ART compared to HCs (Fig. 3E and Supplementary Table 4). It has been shown that IRF4+ Tregs express higher levels of genes related to the NF-kB members such as RELA and RELB and lower expression of transcripts associated with quiescent Tregs such as CCR7, LEF1, and TCF7 (48, 49). In agreement, we found higher expression of RELA and RELB but lower expression of CCR7, LEF1, and TCF7 in Tregs from ART compared to HCs (Fig. 3F and Supplementary Table 5).

To confirm our gene expression results at the protein level, we examined the expression of several markers that have been reported to be highly upregulated on the surface of eTregs, such as HLA-DR, Tim-3, and inducible costimulatory molecule (ICOS) (48, 50, 51). Interestingly, we found that Tregs from ART patients expressed significantly higher levels of HLA-DR, Tim-3, and ICOS than Tregs from HCs (Fig. 3G-K, and Supplementary Fig. 2D). Moreover, Tregs from ART expressed significantly higher CTLA-4 protein than Tregs from HCs, confirming the gene expression analysis (Fig. 3L and Supplementary Fig. 2E). In agreement with our gene expression analysis, Tregs from ART had a significantly lower surface expression of CCR7 than HCs (Supplementary Fig. 2F, G). Since mTOR contributes to a high proliferation rate in Tregs, we measured the proliferation of Tregs in ART and HCs using Ki67. Interestingly, we found higher Ki67 expression in Tregs from ART than in their counterparts in HCs (Fig. 3M, N). Also, we compared the activation status of Tregs versus non-Tregs-CD4 T cells in each group by comparing the expression of Ki67 and HLA-DR. These analyses revealed that Tregs compared to non-Tregs-CD4 T cells express significantly higher Ki67 and HLA-DR in HCs and patients on ART while this was the case only for HLA-DR in LTNPs (Supplementary Fig 3A, B). However, we did not find any significant difference in the expression of Ki67 and HLA-DR in non-Tregs-CD4 T cells between groups (Supplementary Fig. 3A, B). Finally, we compared

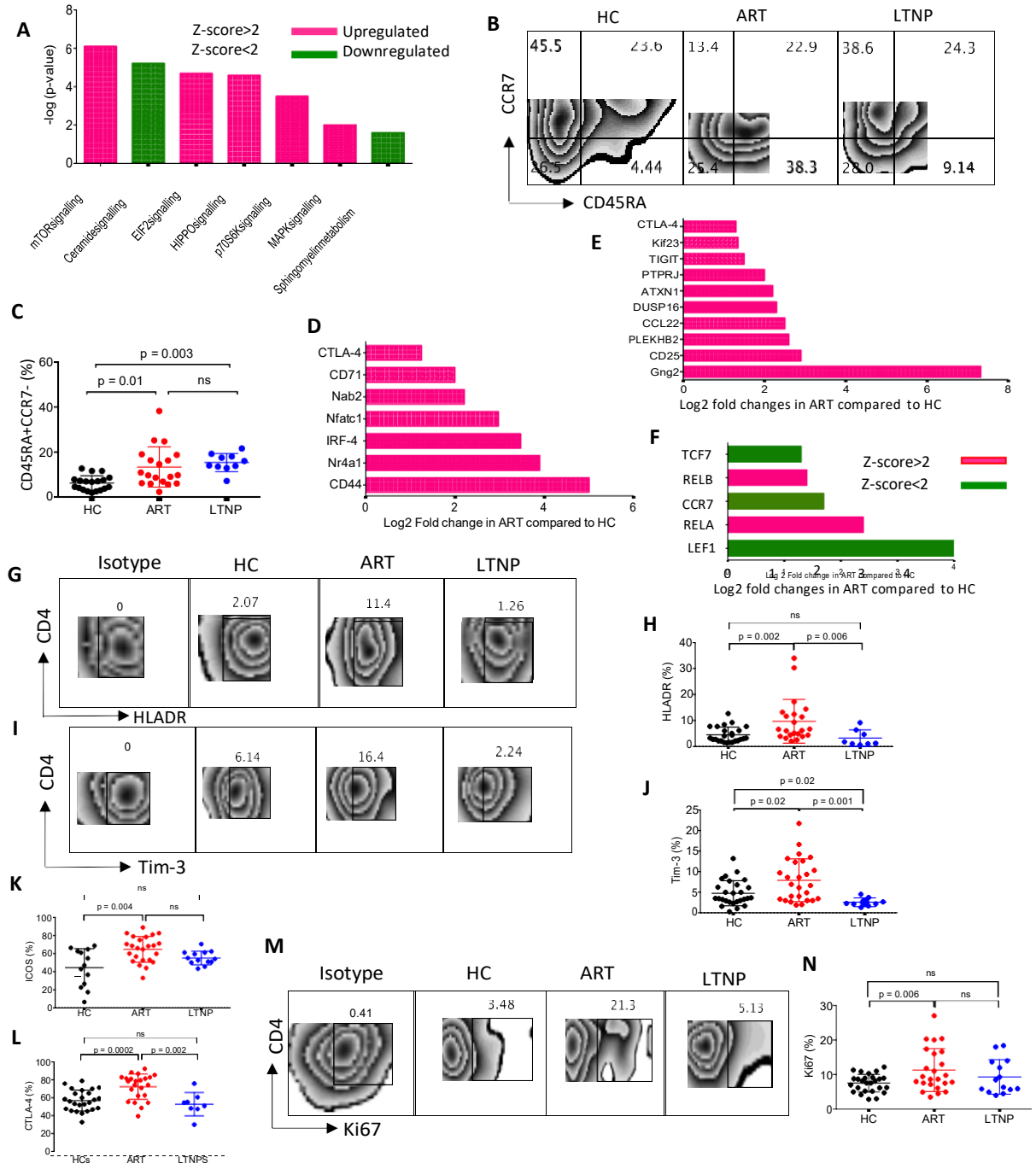

Fig. 3. Higher effector phenotype in Tregs from the ART group than in those of HCs A) Canonical pathways enriched in differentially regulated genes in Tregs from HCs vs ARTs using IPA analysis. **B**) Representative flow cytometry plots and **C**) cumulative data of the expression of CD45RA and CCR7 in Tregs of different study groups (n = 19 HC, n = 19 ART, n = 10 LTNP). **D**) Log_2 fold change of genes associated with eTreg phenotype of ARTs compared to HCs. **E**) Log_2 fold change of the genes directly controlled by the IRF4 binding to the genome in Tregs from ARTs compared to HCs. **F**) Log_2 fold change of NF-kB family member genes in Tregs of ARTs compared to HCs. **G**) Representative flow cytometry plots, and **H**) cumulative data of HLA-DR expression in Tregs of different study groups (n = 20 HC, n = 21 ART, n = 8 LTNP). **I**) Representative flow cytometry plots, and **J**) cumulative data of Tim-3 expression in Tregs of

different study groups (n = 26 HC, n = 26 ART, n = 8 LTNP). **K**) Cumulative data of the expression of ICOS in Tregs of different study groups (n = 13 HC, n = 24 ART, n = 13 LTNP). **L**) Cumulative data of CTLA-4 expression in Tregs of different study groups (n = 26 HC, n = 23 ART, n = 8 LTNP). **M**) Representative flow cytometry plots, and **N**) cumulative data of Ki67 expression in Tregs of different study groups (n = 25 HC, n = 24 ART, n = 14 LTNP). Data were obtained from a single RNAseq analysis, from 3 to 6 independent experiments.

Tregs frequency using different FOXP3 clones such as the clone 150D/E4 versus the clone 235A/E7; however, we did not find any significant difference in $FOXP3^+$ Tregs frequency using these two different antibodies (Supplementary Fig 3C, D). Therefore, our results show that Tregs from ART demonstrate a greater effector phenotype than Tregs from HCs.

4.3.5 Low ceramide expression contributes to the activation of mTOR and protein synthesis in Tregs from ART

Next, we examined the role of other pathways that show differential expression in Tregs of ART vs HCs. The ceramide pathway was the most downregulated in Tregs of ART compared to HCs (Fig. 3A). Ceramide is an endogenous activator of protein phosphatase 2A (PP2A), the core enzyme of the ceramide pathway (52). Ceramide is generated inside the cell through enzymatic cleavage of sphingomyelins in the cell membrane by the acid or neutral sphingomyelinases (53). The levels of both acid and neutral sphingomyelinases were lower in Tregs from the ART group than those from HCs (Fig. 4A), which is reflected in the downregulation of the sphingomyelin metabolism pathway observed in the ART group (Fig. 3A). PP2A inhibits mTORC1 and EIF2 pathways (54), which may contribute to the upregulation of both EIF2A and mTOR pathways observed in Tregs of ART patients. The second highly upregulated pathway after the mTOR pathway in Tregs of ART patients was the EIF2 pathway (Fig. 3A). The EIF2 pathway contributes to protein synthesis following the TCR stimulation (55, 56). As such, among 21 ribosomal proteins whose expression was assessed in our data set, 18 had higher expression in Tregs from ART than those in HCs (Fig. 4B and Supplementary Table 6). Moreover, the expression of several enzymes associated with protein synthesis, including ribosomal protein S6 kinases B1 (p70S6 kinase), was higher in Tregs from

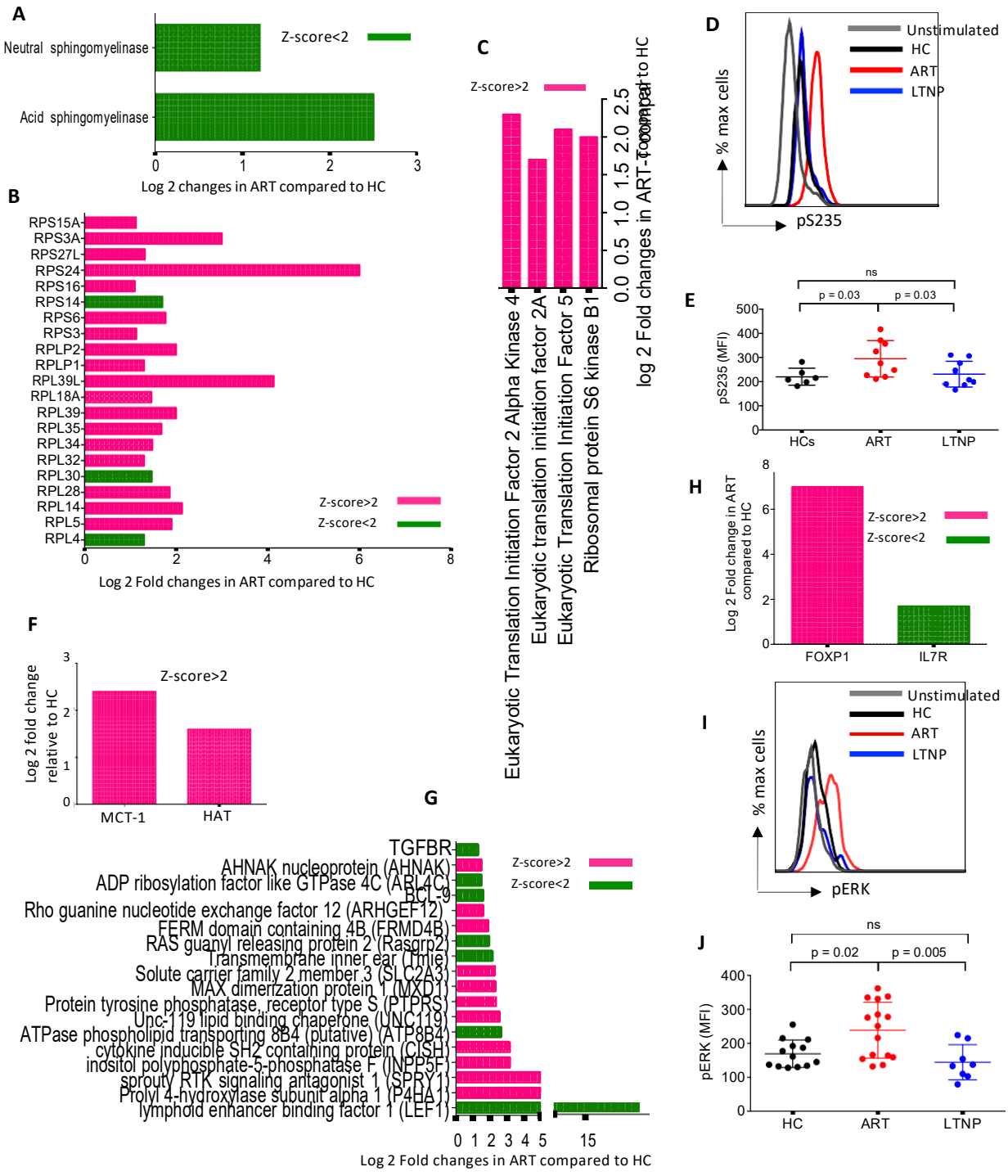

Fig. 4. Low ceramide expression contributes to the activation of mTOR and protein synthesis in Tregs from ARTs patients with a more suppressive phenotype. A) Log_2 fold change of the acid and neutral sphingomyelinase genes in Tregs of ARTs compared to HCs. **B**) Log_2 fold change of the ribosomal protein genes in Tregs of ARTs compared to HCs. **C**) Log_2 fold change of enzymes associated with protein synthesis in Tregs of ARTs compared to HCs. **D**) Representative histogram, and **E**) the MFI ± MIR of p-S235-236 expression in Tregs of different study groups (n = 6 HC, n = 9 ART, n = 9 LTNP). **F**) Log_2 fold change of MST-

1 and HAT genes in Tregs of ARTs compared to HCs. **G)** Log_2 fold change of the genes controlled by pSTAT5 binding to the genome in Tregs of ARTs compared to HCs. **H**) Log_2 fold change of *FOXP1* and IL7R genes in Tregs of ARTs compared to HCs. **I**) Representative histogram, and **J**) the MFI ± MIR of p-ERK expression in Tregs of different study groups (n = 13 HC, n = 15 ART, n = 8 LTNP). Each dot represents a human subject, either HC or HIV-infected. Data were obtained from a single RNAseq analysis and from three independent experiments.

ART than in Tregs of HCs (Fig. 4C and Supplementary Table 7). The P70S6 kinase as a target of mTOR increases the activity of ribosomal protein S6 through its phosphorylation (57). Thus, we assessed the activity of P70S6 kinase by examining the phosphorylation of ribosomal protein S6 at S235 and S236 serine residues. Our results showed significantly higher levels of p-S235-236 in Tregs from ART than in those from HCs and LTNPs (Fig. 4D, E). These data suggest enhancement of protein synthesis in Tregs of ART patients, which is required for synthesizing necessary materials for Tregs activation and proliferation.

4.3.6 Tregs from the ART group exhibit a more suppressive phenotype

The Hippo pathway was the third highly upregulated pathway in Tregs of ART compared to Tregs of HCs. The hippo pathway mainly controls the size of animal organs with well-recognized roles in the differentiation and function of Tregs (58, 59). MST-1, the key enzyme of the hippo pathway, enhances *FOXP3* acetylation through the activity of Histone acetyltransferase p300 (HAT), resulting in higher stability of *FOXP3* (60). We found a higher expression of both MST-1 and HAT transcripts in Tregs from ART than in Tregs of HCs (Fig. 4F). Moreover, the upstream IL-2 signalling facilitates STAT5 phosphorylation and activation through MST-1. As we showed in Figure 2A-F, IL2 signalling and pSTAT5 levels were upregulated in Tregs of ART compared to HCs. TCR signalling and STAT5 pathways control distinct sets of genes that contribute to Tregs suppressive activity (61, 62). Therefore, we investigated the expression of genes that were STAT5 activation-dependent in our RNAseq datasets. Our analysis showed the upregulation of genes associated with STAT5 activation in Tregs of ART, whereas genes indicative of STAT5 inhibition were downregulated in ART Tregs (Fig. 4G and Supplementary Table 8). Although CD25 enhances the stability of the *FOXP3* gene through the activation of pSTAT5, there seems to be a positive

feedback loop between CD25 and FOXP3, in which FOXP3 binds to several binding sites in CD25 locus, leading to increased expression of CD25 (63). The *FOXP1* gene was another highly up-regulated gene in ART Tregs from ART patients, which enhances *FOXP3*-mediated expression of several Treg lineage genes including CD25 (63). *FOXP1* also represses the IL7ra locus, resulting in decreased IL-7 dependent proliferation in Tregs (64). Our analysis of the expression level of *FOXP1* and IL7ra genes in our dataset revealed higher and lower levels of *FOXP1* and IL7ra genes in ART Tregs than those in HCs, respectively (Fig. 4H). Collectively, the comparison of Tregs from HCs with ART revealed upregulation of the pathways that are associated with more suppressive phenotype in ART Tregs. Also, we observed the upregulation of MAPK signaling and elevated levels of pERK in Tregs of ARTs compared to HCs (Fig. 4I, J), which are associated with a more suppressive phenotype in ART Tregs (65).

4.3.7 Higher glycolysis but lower fatty acid oxidation (FAO) in Tregs of LTNPs than in those of HCs

To better understand the upstream regulators of the differential gene expression pattern in Tregs of HCs vs LTNPs, we compared the gene expression profile in these two groups. Our results showed upregulation of the MYC, IL15, TCR, and Hypoxia-inducible factor-1A (HIF1A) upstream regulators and downregulation of IL10 regulator in Tregs of LTNPs vs HCs (Fig. 5A). We further verified increased TCR signalling in LTNPs Tregs by showing higher pZAP70 expression than that of HCs (Fig. 2G-H). IL15 and IL10 mitigate and increase the suppressive function of Tregs, respectively (66–68). Moreover, HIF-1α is involved in the intracellular glycolysis pathway (69), and C-Myc is a general transcription factor that regulates the expression of several genes involved in cell metabolism and glycolysis (69). We then analyzed the canonical pathways that were differentially expressed in Tregs of HCs vs LTNPs. Our analysis revealed that the EIF2 signalling was the most upregulated pathway in Tregs of LTNPs, followed by glycolysis and gluconeogenesis (Fig. 5B). In contrast, FAO and PPAR signalling were downregulated in Tregs of LTNPs compared to HCs. As shown in Figure 5C, we found a significantly higher expression of transcripts of several enzymes that are involved in glycolysis in Tregs of LTNPs. However, we identified lower expression

of multiple genes belonging to the aldehyde dehydrogenase family in Tregs of LTNPs than in Tregs of HCs, possibly resulting in lesser oxidation of long-chain aldehydes into fatty acids (Fig. 5D). FAO involves the generation of acetyl-CoA through sequential removal of two-carbon units from the acyl chain as it enters the tricarboxylic acid (TCA) cycle in mitochondria to regulate mitochondrial OXPHOS (70). Interestingly, glycolysis and OXPHOS have reciprocal effects on Tregs-mediated suppression. In contrast to glycolysis which reduces *FOXP3* and hence Treg stability, oxidative phosphorylation (OXPHOS) increases the suppressive function of Tregs (70, 71). Thus, the observed changes in the glycolysis and FAO suggest a lower suppressive function for Tregs in LTNPs than those in HCs. We also observed a higher activation of the gluconeogenesis pathway in Tregs in LTNPs than those in HCs (Fig. 5B). Therefore, the increased glucose production through gluconeogenesis may fuel the necessary glucose required for glycolysis in Tregs.

4.3.8 High PPAR signalling results in a lower STAT3 in Tregs of LTNPs

Our results showed the downregulation of PPAR signalling in Tregs of LTNPs vs HCs (Fig. 5B). The PPAR signalling results in the activation of FAO and the suppression of glycolysis (72). Moreover, the loss of PPARα in Tregs is associated with a decline in their suppressive activity (73–75). Since PPARγ leads to the inactivation of STAT3 (76), we analyzed the expression of STAT3 transcript in our dataset. We observed a higher expression of the STAT3 gene in Tregs of LTNPs than those in HCs (Fig. 5E). We also noted a higher expression of IL6 signal transducer (IL6 ST) in Tregs of LTNPs (Fig. 5E), which is responsible for STAT3 phosphorylation and activation (77). Accordingly, we found higher phosphorylation of STAT3 in Tregs of LTNPs than those of HCs (Fig. 5F-G). STAT3 binds to STAT5 binding sites on *FOXP3* loci, resulting in Treg destabilization (78, 79). The loss of function in Tregs is accompanied by higher production of inflammatory cytokines such as IL-2 and IFN-γ (80, 81). In agreement, we found a significantly higher expression of the IL2 gene and a lower expression of the TGF-beta gene in Tregs of LTNPs than those of

HCs (Fig. 5E). Also, we investigated the expression of other genes that are associated with Tregs stability and functionality. Interestingly, we observed a lower expression of TNF receptor superfamily member 25

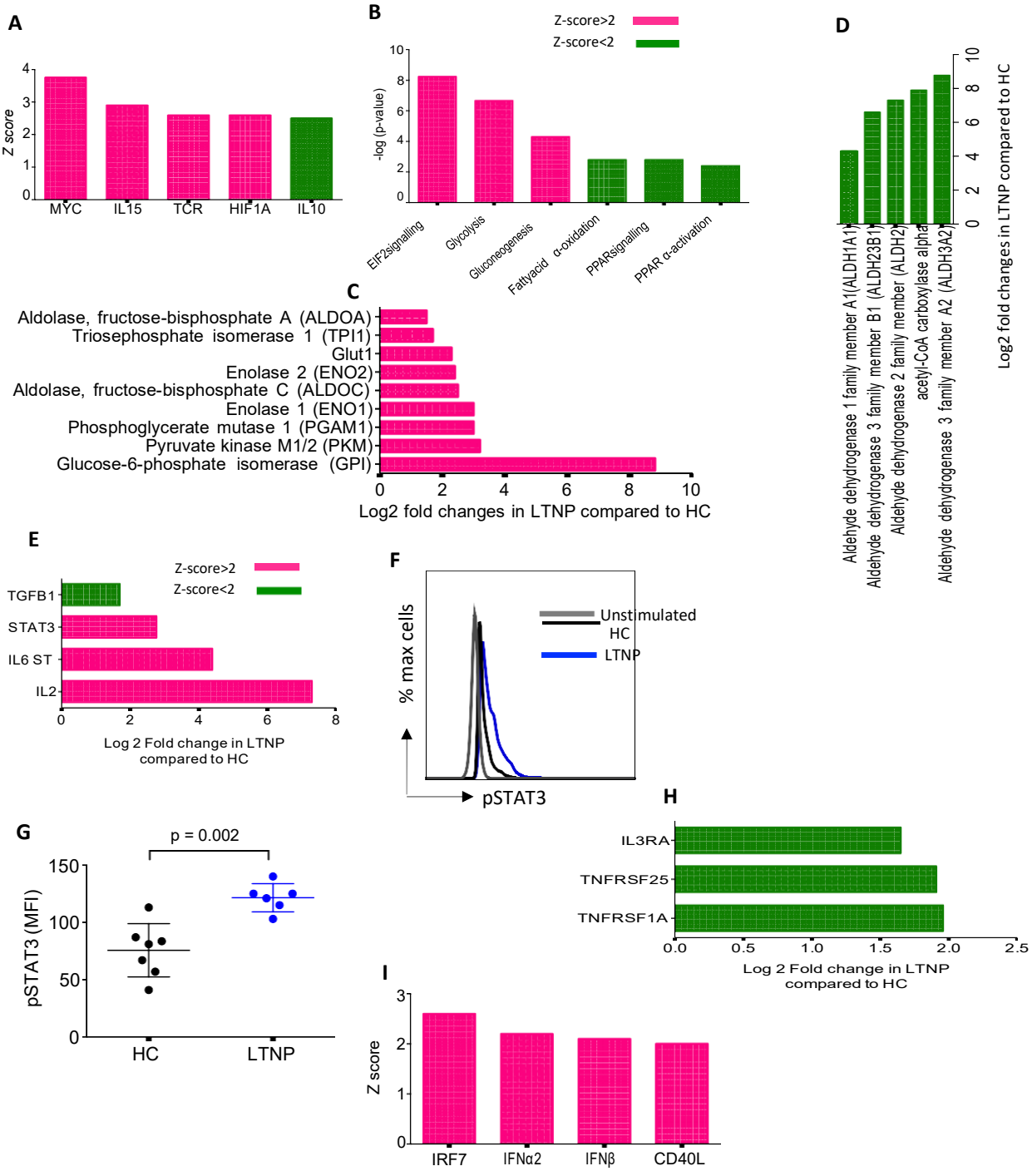

Fig. 5. High glycolysis and low FAO in Tregs of LTNPs compared to HCs. A) Upstream regulators predicted to account for transcription differences between Tregs in HCs versus LTNPs. **B**) Canonical pathways enriched in differentially regulated genes between Tregs of HCs and LTNPs, as determined using IPA. **C**) Log_2 fold change of transcripts of several enzymes that are involved in glycolysis in Tregs of LTNPs compared to HCs. **D**) Log_2 fold change of transcripts of several aldehyde dehydrogenase family members in Tregs of LTNPs compared to HCs. **E**) Log_2 fold change of TGFB1, IL2, STAT3, and IL6 ST genes in Tregs from LTNPs compared to HCs. **F**) Representative histogram, and **G**) cumulative data of the MFI ± MIR of p-STAT3 in Tregs of LTNPs (n = 6) vs HCs (n = 7). **H**) Log_2 fold change of IL3RA, TNFRSF25, and TNFRSF1A in Tregs of LTNPs compared to HCs. **I**) Upstream regulators predicted to account for transcription differences between Tregs in the ART group versus LTNPs. Data were obtained from a single RNAseq analysis. Each dot represents a human subject, either HC or HIV-infected and pSTAT3 studies are representative of two independent experiments.

(TNFRSF25), TNF receptor superfamily member 1A (TNFRSF1A) and IL3 receptor IL3RA genes that are associated with the stability of Tregs (Fig. 5H) (82–84). Finally, we found a higher activity for IFNα and β, IFN regulatory factor 7 (IRF7), and CD40L upstream regulators in ART Tregs than in Tregs from LTNPs (Fig. 5I). IFNα/βsignalling promotes Treg development and function, which occurs through key transcription regulators such as IRF7and IRF9 (85). Similarly, CD40L ligation results in Treg activation (86). Taken together, these results revealed an increase in glycolysis but a decrease in the suppressive potential of Tregs in LTNPs compared to their counterparts in HCs. Moreover, our data support the enhanced activity of Tregs in the ART group compared to LTNPs.

4.3.9 Tregs from ART exhibit more suppressive properties compared to their counterparts in HCs and LTNPs

To further evaluate the functionality of Tregs in our different study cohorts, we measured effector T cell proliferation in the presence/absence of Tregs (at 1:1 ratio). Interestingly, we observed higher suppression of the responder T cells to Tregs from ART than that to HCs and LTNPs (Fig. 6A-B). However, we did not find any significant difference between the proliferative capacity of effector T cells in the presence of Tregs from HCs or LTNPs (Fig. 6A-B). We and others have previously shown that Tregs express Gal-1, Gal-9, and PD-L1 and exert some of their regulatory functions through the interaction of these ligands with their corresponding receptors on effector T cells (13, 87, 88). Thus, we decided to investigate the expression of the above ligands in Tregs of our different study cohorts. We observed higher expression

of Gal-1, Gal-9, and PD-L1 transcripts in Tregs from ARTs than those in HCs (Fig. 6c). We also found a higher gene expression of Gal-8 in Tregs of ART subjects (Fig. 6c), which is associated with enhanced IL-2 signalling

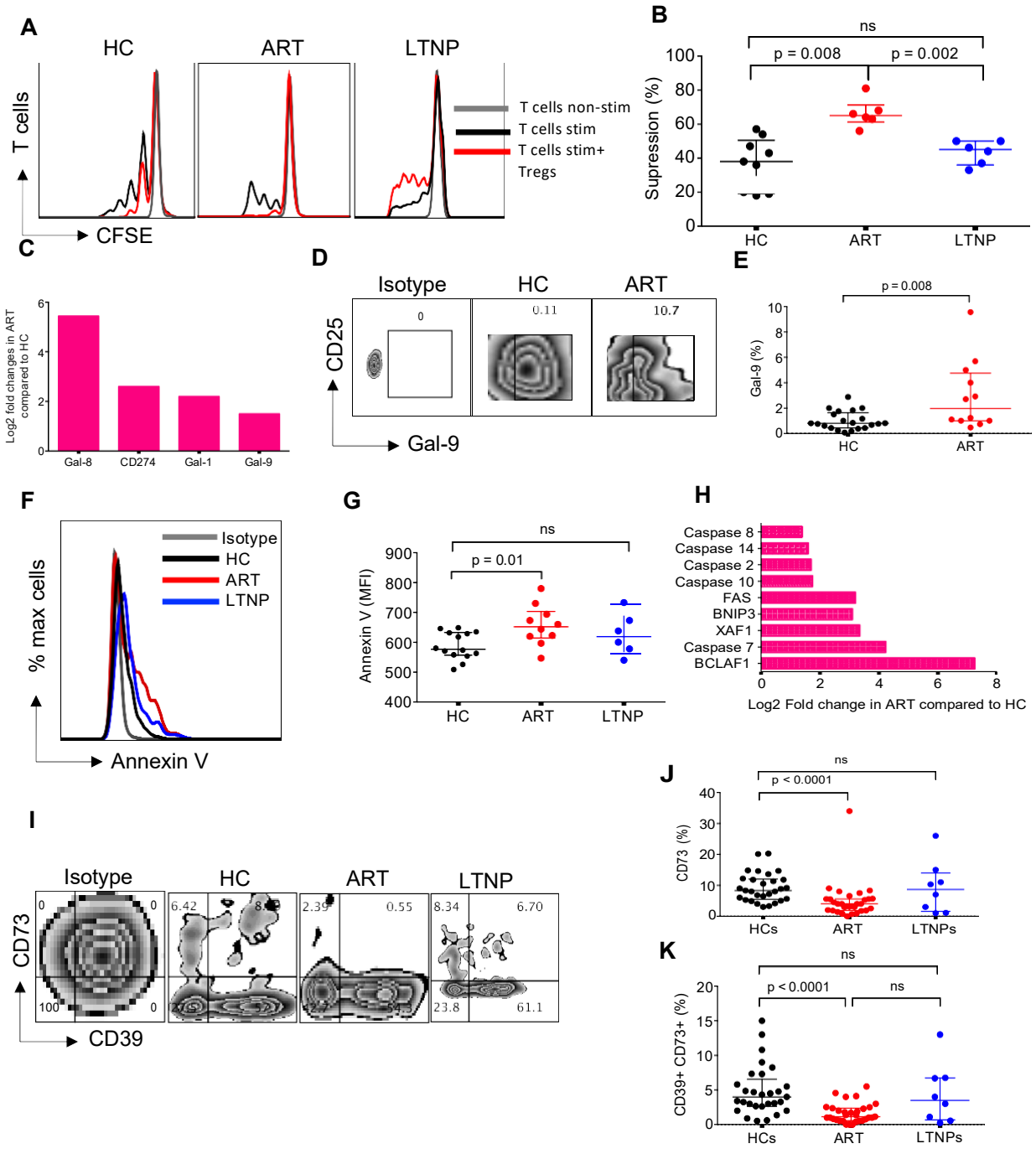

Fig. 6. Greater suppressive properties of Tregs in ARTs than in their counterparts in HCs and LTNPs. A) Representative plots, and **B**) cumulative data of % suppression of proliferation by Tregs from different groups (n = 9 HC, n = 6 ART, n = 6 LTNP). Isolated T cells from different groups were labelled with CFSE dye

and stimulated with anti-CD3 and mitomycine-treated whole PBMCs in the presence and absence of Tregs **C**) Log_2 fold change of Gal-1, Gal-8, Gal-9 and CD274 in Tregs of ART compared to HCs. **D**) Representative flow cytometry plots, and **E**) cumulative data of Gal-9 expression in Tregs of ART patient's (n = 12) vs HCs (n = 21). **F**) Representative histogram, and **G**) cumulative data of the MFI ± MRI of Annexin V expression in Tregs of different study groups (n = 14 HC, n = 10 ART, n = 6 LTNP). **H**) Log_2 fold change of several genes associated with apoptosis in Tregs of ART compared to HCs. **I**) Representative flow cytometry plots of CD39 and CD73 expression in Tregs of different study groups. **J**) Cumulative data of % of $CD73^+$ Tregs in different study cohorts (n = 29 HC, n = 31 ART, n = 8 LTNP). **K**) Cumulative data showing % of $CD39^+CD73^+$ Tregs in different study cohorts (n = 29 HC, n = 31 ART, n = 8 LTNP). Each dot represents a human subject, either HC or HIV-infected. Data were obtained from a single RNAseq analysis and from 2 to 6 independent experiments.

in Tregs (89). However, we did not observe any difference in the gene expression of the above molecules between Tregs of LTNPs and HCs. We further found that Tregs in ARTs expressed significantly higher levels of Gal-9 on their surface than those in HCs (Fig. 6D-E). It has been shown that apoptotic Tregs have more suppressive functions than non-apoptotic Tregs (90). As such, we assessed the apoptosis of Tregs in our different study groups. Interestingly, we found that Tregs from ARTs had significantly higher expression of annexin V than Tregs from HCs and LTNPs (Fig. 6F-G). This was accompanied by the upregulation of several genes related to apoptosis in Tregs from ARTs, such as BCL-2 interacting protein 3 (BNIP3), XIAP, BCL2 associated transcription factor 1 (BCLAF1), FAS, caspase 2,7,8,10, and caspase 14 (Fig. 6H) (82, 90–92). Apoptotic Tregs release ATP that is converted to adenosine through the tandem function of CD39 and CD73, two ectoenzymes that are expressed on the surface of Tregs (90). Contrary to previous reports (51), we didn't find any significant difference in the expression of CD39 on Tregs of different study groups (Fig. 6I and Supplementary fig. 4A). However, we observed decreased frequency of $CD73^+$ Tregs and hence $CD39^+CD73^+$ Tregs in ART compared with HCs and LTNPs (Fig. 6I–k) (93). It has been shown that in contrast to murine Tregs, CD73 is mainly expressed intracellularly in human Tregs (94). However, we did not observe any significant difference in the percentage of intracellular $CD73^+$ cells in our different study cohorts (Supplementary fig. 4B-C). These data suggest that a higher apoptosis rate in ART Tregs may provide more ATP for the adenosine production in Tregs from the ART group.

4.4 Discussion

It has been reported that infection of Tregs with HIV-1 impairs their suppressive potential through DNA methylation of the *FOXP3* gene (21, 95), but ART reverses this impaired function (96). In contrast, some studies have reported that Tregs functions are not altered in the setting of HIV infection (97–99). However, the potential differences in Tregs of different HIV subgroups at the transcription level or functionality have not been fully elucidated.

In this report, we show that Tregs from the ART group demonstrate a more suppressive phenotype than Tregs from LTNPs and HCs. Our IPA analysis revealed the upregulation of IL2 and TCR signalling in Tregs of ARTs compared to HCs. Also, we observed the upregulation of mTOR, hippo, and MAPK signalling pathways that are associated with Tregs activation in patients on ART (44–47, 57, 59). The mTOR pathway has a crucial role in the activation and differentiation of cTregs to eTregs, and is crucial for the peripheral T cell tolerance and homeostasis (100). The TCR signalling induces IRF4 and contributes to the acquisition of an effector phenotype in Tregs (101, 102). The mTOR controls the expression of IRF4 at the post-transcription level (100) and induces the expression of ICOS, which derives eTreg differentiation (103). Therefore, a higher expression of IRF4 and its controlled genes in Tregs of ARTs than those in HCs may support their suppressive effector phenotype. A higher mTOR activity contributes to the higher expression of the large neutral amino acid–transporter CD98 (LAT1) gene and the transferrin receptor (CD71) in Tregs of ARTs, which are required for Tregs activation (44).

We also observed the hippo pathway upregulation in Tregs of patients on ART. The hippo pathway controls the size of animal organs through tight regulation of cell proliferation and apoptosis (58, 104). The core enzyme of the hippo pathway, MST1, has several key roles in Tregs stability. For example, it mediates IL2 induced STAT5 phosphorylation, resulting in the expression of the genes that are associated with the inhibitory activity of Tregs (59). Also, MST1 increases FOXP3 acetylation (60). While FOXP3 is expressed transiently in effector T cells, stable expression of FOXP3 is required for the development and establishment of a Treg phenotype (105, 106). Moreover, the interaction of MST1 with the DOCK-8-LRCHs

complex controls T cell migration (59). Interestingly, both DOCK8 and LRCH were upregulated in Tregs of ARTs compared to HCs (Supplementary Fig. 4D). This suggests a higher migratory ability for Tregs in the ART group, which may enhance their access to the effector site.

In contrast, we observed a lower ceramide signalling in Tregs of the ART group. Protein phosphatase 2A (PP2A), the core enzyme of the ceramide pathway, is a highly conserved and ubiquitously expressed serine-threonine phosphatase, which regulates many cellular functions (107, 108). This enzyme has 3 different subunits, with the Ac subunit being the catalytic and Aa subunit as the scaffold subunit. The abundance of catalytic PP2Ac is similar in different cells. However, its activity is regulated through the phosphorylation of the carboxy-terminal end of PP2Ac at the Tyr307 residue (109). The SET oncoprotein inhibits PP2A activity through its phosphorylation (107, 108). Besides, ceramide interacts with the SET oncoprotein and inhibits its function (Supplementary Fig. 4E), thus, preventing its inhibitory effect on PP2A (52). It has been shown that the inhibition of acid sphingomyelinase increases the suppressive activity of Tregs through the increased CTLA-4 turnover (110). Therefore, decreased ceramide levels and subsequent decline in PP2A activity in Tregs of ART may enhance their suppressive activity through the upregulation of the mTOR pathway (54). However, increased ceramide level in CD4 T cells and monocyte reduce their infectivity to HIV-1 (111). As such, decreased ceramide levels in Tregs of ARTs may enhance their susceptibility to HIV-1 infection and make them a favourite target for the virus.

Our further analysis revealed an increase in MYC, IL5, TCR, HIF1A but a decrease in IL10 signalling in LTNPs Tregs versus HCs. The upregulation of genes involved in IL15 and the downregulation of genes involved in IL10 signalling suggest the decreased suppressive function of Tregs in LTNPs (68, 112). MYC and HIF1A can increase the glycolysis in Tregs (69), which was demonstrated by the upregulation of the glycolysis pathway in Tregs of LTNPs. HIF-1α also reduces the suppression role of Tregs through the degradation and destabilization of the *FOXP3* gene (113, 114).

Compared to other subsets of T cells such as Th1, Th2, and Th17 cells, Tregs rely less on glycolysis but mainly on mitochondrial metabolism and oxidative phosphorylation (OXPHOS) as their source of energy (115) because glycolysis inhibits Treg suppressive function (70, 71, 116, 117). As such, FOXP3 and CTLA4 suppress glycolysis and enhance OXPHOS, resulting in reprogramming of Tregs metabolism (118). Interestingly, the expression of glucose transporter Glut-1, the rate-limiting step in glycolysis (119), was higher in Tregs of LTNPs than in those of HCs. It has been shown that the overexpression of Glut-1 decreases the expression of FOXP3 in Tregs (71). In contrast to glycolysis, OXPHOS results in the production of reactive oxygen species (ROS) in T cells (120), preventing nuclear transport of BTB domain and CNC homolog 2 (Batch2) through Bach2 deSUMOylation (121). DeSUMOylation of BACH2 prevents its nuclear export, which leads to a stable FOXP3 expression and stability of Tregs (121). Another important observation was the upregulation of gluconeogenesis in Tregs of LTNPs. Although gluconeogenesis mainly occurs in the liver and kidney (122), it has been reported to occur in memory T cells (123). The similarity of the metabolic pathway in memory T cells and Tregs (115) may account for the observed gluconeogenesis pathway in Tregs. Overall, the metabolic pattern of Tregs in LTNPs and the activation of the IL6/STAT3 pathway suggest impaired suppressive properties of Tregs in this group. This was further demonstrated when we noted a significant deficiency in Tregs-mediated suppression of effector T cell proliferation by Tregs of LTNPs versus those in the ART group. We further found that Tregs in ARTs were more apoptotic and showed higher expression of surface co-inhibitory receptors (e.g., CTLA-4 and ICOS), which might contribute to their enhanced suppressive functions. Apoptotic Tregs exhibit more suppressive function through the release of ATP and its conversion to adenosine by the enzymatic activity of CD39 and CD73 ectoenzymes (90). Of note, we observed a significant elevation of HLA-F mRNA and HLA-F protein in Tregs from HIV-infected individuals (both ART and LTNP) compared to HCs. Upregulation of HLA-F in Tregs of HIV-infected individuals might be related to their hyper-activation status. HLA-F is primarily located in the endoplasmic reticulum, and its expression is tightly regulated (124). This suggests

that HLA-F surface expression on Tregs of HIV-infected patients might serve as a marker of cell stress, including endoplasmic reticulum stress. Additionally, HIV-1 infection of CD4 T cells can increase the transcription of HLA-F mRNA but decrease the binding of its ligand (KIR3DS1) as a potential mechanism of avoiding recognition by NK cells (125). Thus, it's possible to speculate that the ability of KIR3DS1 to bind to HLA-F expressed on Tregs in HIV-infected patients provides a mechanistic link between Tregs and NK cells. This hypothesis merits further investigation and might also have relevance in other infectious diseases, autoimmune disorders, and cancers. Taken together, we have provided a novel insight into the differential properties of Tregs in HIV-infected individuals. In agreement with these observations, we have previously reported that $CD8^+$ T cells restricted by HLA-B*27 and-B*57 upon recognition of their cognate epitopes do not upregulate TIM-3 and therefore evade Tregs-mediated suppression (13). In contrast, $CD8^+$ T cells restricted by non-protective HLA-alleles upon encounter with their cognate epitopes upregulate TIM-3 and therefore become suppressed by Tregs (13). Thus, T cell evasion of Tregs-mediated immune regulation (13) and lower suppressive properties of Tregs in LTNPs may explain the higher propensity of autoimmune diseases in individuals possessing HLA-B*27, HLA-B*57 (42). In contrast, it is possible to speculate that the robust immune response against HIV infection in LTNPs may in part be associated with impaired Tregs functions. More recently, we demonstrated that CD8 T cells restricted by HLA-B*35Px were not suppressed by Tregs in HIV-infected progressors (15). Although due to the cell limitation, we were unable to conduct RNAseq analysis on Tregs from HIV-infected progressors, we have shown that Tregs in these individuals exhibit impaired suppressive properties. This hypothesis can be further supported by the higher susceptibility of individuals restricted by HLA-B*35Px to autoimmune diseases such as subacute thyroiditis (126).

We are aware of several study limitations. For example, we performed limited functional studies on Tregs *in vitro*. Isolating a large number of Tregs was not practical for functional assays, mainly because of the cell limitation. Similarly, the discrepancy in the sample size in some experiments was related to the same

issue. There were no selection criteria for the samples in each set of experiments, and samples were randomly selected; however, we tried to use the same subset of samples for experiments that were related to each other (e.g., phospho assays). The period of HIV infection may influence the functionality of Tregs, and we were unable to obtain accurate information for our patients on ART, but they have been on ART beyond 2 years. The sex of HIV-infected individuals is also an important factor to be taken into consideration, but our patients were a combination of men and women. The activation status of T cells in HCs versus HIV patients may influence the results. As such higher activated T cells in one group compared to the other may influence the transcription levels. However, our observations do not support this concept since non-Tregs $CD4^+$ T cells exhibited similar activation status as measured by Ki67 and HLA-DR in our cohort. In this study, we were unable to examine the effects of ART on Tregs transcription profile/functionality. Nevertheless, future studies comparing Tregs transcription profiles and effector functions pre- and post-treatment are warranted.

Taken together, we demonstrated differential Tregs phenotype and function in different HIV subgroups versus HCs. In particular, by conducting RNAseq analysis, we provided a novel insight into the mechanism underlying differential Tregs properties in HIV-infected individuals.

4.5 Acknowledgement

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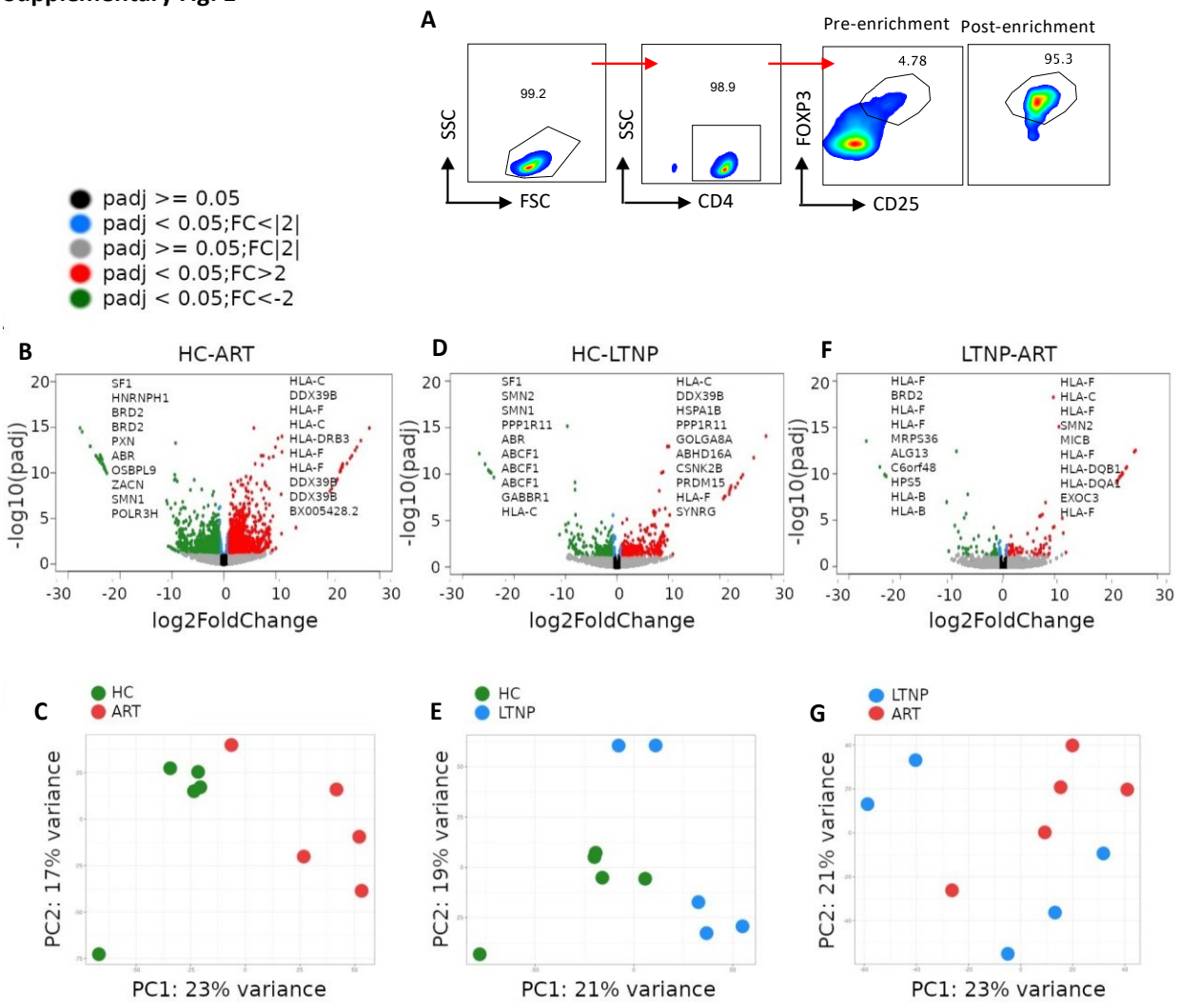
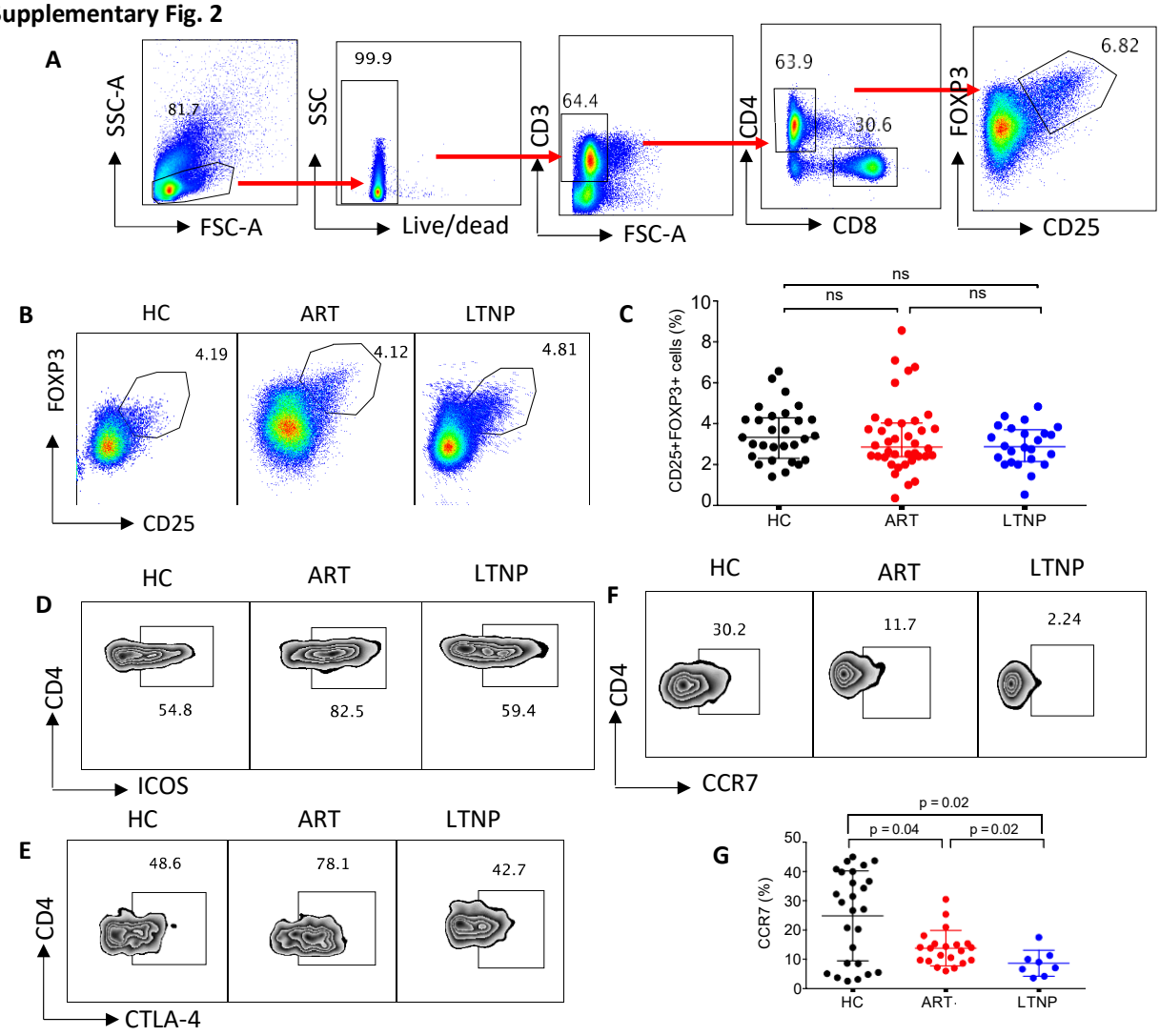
4.6 Supplementary Figures

Supplementary Fig. 1



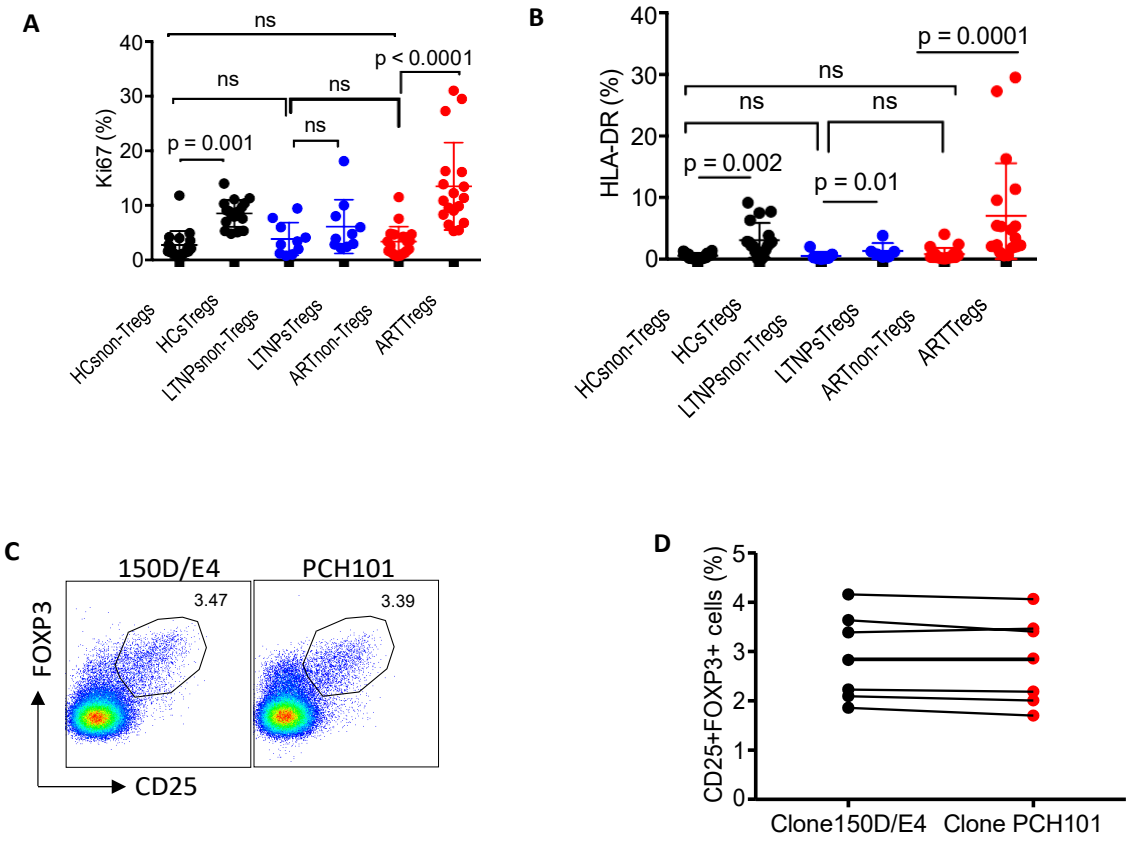
A) Representative flow cytometry plots showing the purity of Tregs pre-and post-enrichment. **B**), **D**), **F**) MA plots depicting abundance, fold-change and significance of transcripts in Tregs of different indicated groups. Black dots represent transcripts that were not differentially expressed (DE) between different groups. Grey dots represent DE transcripts with a -2 < fold-change < 2. Red dots represent DE transcripts with a fold-change > 2. Green dots represent DE transcripts with a fold-change < -2. **C**), **E**), **G**) Principal component analysis (PCA) on the Euclidian distances between Tregs of different study cohorts. HC (healthy control), ART (Anti-retroviral treated patients) and LTNP (long-term non-progressors). From one RNAseq analysis (n=5/group).

Supplementary Fig. 2



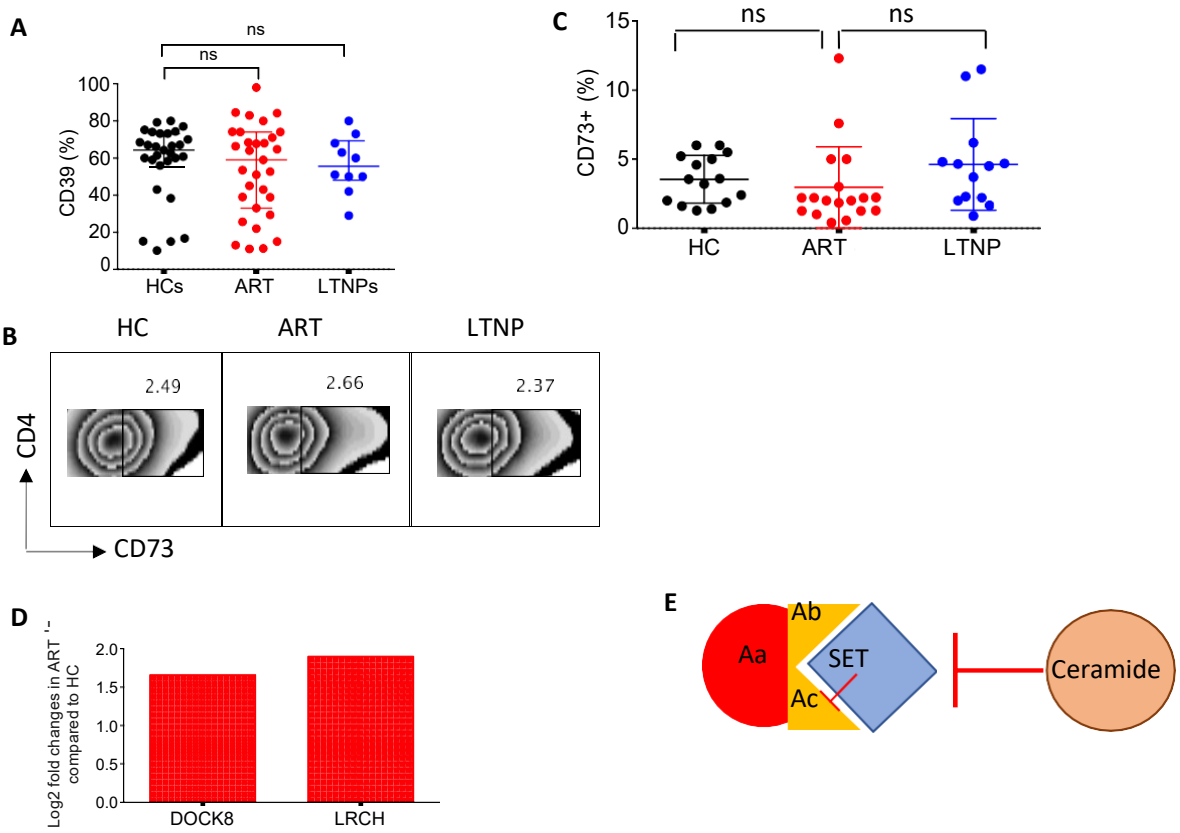
A) Representative plots of gating strategy for Tregs identification. **B**) Representative flow cytometry plots, and **C**) cumulative data of Tregs frequency in different study groups, (n=38 HC, n=50 ART. n=24 LTNP). **D**) Representative flow cytometry plots of ICOS expression in Tregs of different study groups. **E**) Representative flow cytometry plots of CTLA-4 expression in Tregs of different study groups. **F**) Representative flow cytometry plots, and **G**)cumulative data of CCR7 expression in Tregs of different study groups (n=26 HC, n=21 ART, n=8 LTNP). Data are obtained from 3 or 4 independent experiments.

Supplementary Fig. 3



A) Cumulative data showing percentage of Ki67, and **B**) HLA-DR expressing Tregs versus non-Tregs-CD4 T cells in HC (n=18), ART (n=18) and LTNP (n=10). **C**) Representative flow cytometry plots, and **D**) cumulative data of percentages of Tregs in the same HIV-infected individuals comparing two different clones for the anti-FOXP3 antibody (n=7). Data are obtained from 2 or 3 independent experiments.

Supplementary Fig. 4



A) Cumulative data showing percentage of CD39+ Tregs in different study cohorts (n=28 HC, n=31 ART, n=10 LTNP). **B)** Representative flow cytometry plots, and **C**) cumulative data of intracellular CD73 expression in Tregs of different study groups (n=15 HC, n=18 ART, n=13 LTNP). **D**) Log2 fold change of DOCK8 and LRCH genes in Tregs of the ART group compared to HCs. **E**) Simplified schematic of the effects of ceramide on the function of PP2A holoenzyme subunits via SET oncoprotein. Each dot represents a human subject either HC or HIV-infected. Data are obtained from 3 to 5 independent experiments. RNAseq data are from 5 samples/group.

4.7 Supplementary Tables

Supplementary Table 4.7.1 Participants clinical data

PTID	Sex	Plasma viral load (copies/mL^{-1})	CD4 count (mL^{-1})	On ART	
LTNP-1	M	792	570	no	
LTNP-2	M	<50	522	no	
LTNP-3	M	<50	473	no	
LTNP-4	F	800	421	no	
LTNP-5	M	<50	796	no	
LTNP-6	M	<50	832	no	
LTNP-7	M	125	783	no	
LTNP-8	M	<50	759	no	
LTNP-9	F	<50	773	no	
LTNP-10	M	65	426	no	
LTNP-11	F	<50	643	no	
LTNP-12	F	1073	574	no	
LTNP-13	M	140	555	no	
LTNP-14	M	<50	632	no	
LTNP-15	M	540	578	no	
LTNP-16	M	990	550	no	
LTNP-17	M	<50	602	no	
LTNP-18	M	<50	575	no	
LTNP-19	F	800	451	no	
LTNP-20	M	<50	790	no	
LTNP-21	M	<50	850	no	
LTNP-22	M	125	783	no	
LTNP-23	M	<50	765	no	
LTNP-24	M	4500	562	no	
ART-1	M	<30	720	yes	
ART-2	M	<30	570	yes	
ART-3	M	<30	1080	yes	
ART-4	M	<30	450	yes	
ART-5	M	1399	800	yes	
ART-6	M	<30	720	yes	
ART-7	M	<30	830	yes	
ART-8	M	<30	460	yes	
ART-9	M	<30	470	yes	
ART-10	F	<30	410	yes	
ART-11	M	<30	350	yes	

ART-12	M	<30	850	yes	
ART-13	M	<30	740	yes	
ART-14	M	<30	680	yes	
ART-15	M	<30	470	yes	
ART-16	M	<30	550	yes	
ART-17	M	208	410	yes	
ART-18	M	<30	420	yes	
ART-19	M	<30	580	yes	
ART-20	F	<30	590	yes	
ART-21	M	<30	680	yes	
ART-22	M	<30	570	yes	
ART-23	M	<30	420	yes	
ART-24	M	<30	210	yes	
ART-25	M	<30	500	yes	
ART-26	M	<30	640	yes	
ART-27	M	<30	260	yes	
ART-28	F	<30	700	yes	
ART-29	F	<30	720	yes	
ART-30	M	<30	910	yes	
ART-31	F	168	290	yes	
ART-32	F	<30	180	yes	
ART-33	M	<30	680	yes	
ART-34	M	<30	510	yes	
ART-35	M	<30	1210	yes	
ART-36	F	<30	550	yes	
ART-37	F	60	910	yes	
ART-38	F	<30	190	yes	
ART-39	M	<30	340	yes	
ART-40	F	160	60	yes	
ART-41	M	<30	390	yes	
ART-42	M	<30	250	yes	
ART-43	M	<30	520	yes	
ART-44	M	<30	610	yes	
ART-45	M	<30	360	yes	
ART-46	M	<30	290	yes	
ART-47	M	<30	250	yes	
ART-48	M	<30	260	yes	
AET-49	M	<30	342	yes	
ART-50	F	<30	457	yes	

Long-term non-progressor (LTNP)
On antiretroviral therapy (ART)
Male (M) and female (F)
The mean age for HCs was 37.5 (range 22-62), 21 males and 11 females.

Supplementary Table 4.7.2 Sex and age of participants that RNA-seq was done on their Tregs

PTID	Sex	Age
HC-1	M	43
HC-2	M	33
HC-3	M	33
HC-4	M	47
HC-5	M	55
LTNP-1	F	53
LTNP-2	M	41
LTNP-3	M	34
LTNP-4	M	49
LTNP-5	M	34
ART-1	M	42
ART-2	M	35
ART-3	M	36
ART-4	M	46
ART-5	M	54

Tregs from these subjects were used for the RNAseq analysis.
Healthy control (HC)
Long-term non-progressor (LTNP)
On antiretroviral therapy (ART)
Male (M) and female (F)

Supplementary Table 4.7.3 The log2 fold changes and p values of the genes presented in Fig 3D

Gene name	Log2 Fold change in ART compared to HC	P value
Cytotoxic T-lymphocyte associated protein 4 (CTLA-4)	1.25	0.001
Transferrin receptor (CD71)	2	0.0006
NGFI-A binding protein 2 (Nab2)	2.21	0.003
Nuclear factor of activated T cells 1 (Nfatc1)	2.97	0.0005
Interferon regulatory factor 4 (IRF-4)	3.47	9.05501E-07
Nuclear receptor subfamily 4 group A member 1 (Nr4a1)	3.9	0.002

CD44	5	0.0005

Supplementary Table 4.7.4 The log2 fold changes and p values of the genes presented in Fig 3E

Gene name	Log2 Fold change in ART compared to HC	P value
Cytotoxic T-lymphocyte associated protein 4 (CTLA-4)	1.25	0.001
Kinesin family member 23 (Kif23)	1.35	0.002
T cell immunoreceptor with Ig and ITIM domains (TIGIT)	1.47	4.85129E-05
Protein tyrosine phosphatase, receptor type J (PTPRJ)	2	5.97522E-06
Ataxin 1 (ATXN1)	2.2	3.91563E-05
Dual specificity phosphatase 16 (DUSP16)	2.28	0.000856893
C-C motif chemokine ligand 22 (CCL22)	2.48	5.83422E-05
Pleckstrin homology domain containing B2 (PLEKHB2)	2.58	0.000479517
CD25	2.93	0.000462961
G protein subunit gamma 2 (Gng2)	7.32	3.92287E-05

Supplementary Table 4.7.5 The log2 fold changes and p values of the genes presented in Fig 3F

Gene name	Log2 Fold change in ART compared to HC	P value
Transcription factor 7 (TCF7)	-1.3	0.000142302
RELB proto-oncogene, NF-kB subunit (RELB)	1.35	0.000842881
C-C motif chemokine receptor 7 (CCR7)	-1.7	6.9175E-05
RELA proto-oncogene, NF-kB subunit (RELA)	-2.5	0.000495637
Lymphoid enhancer binding factor 1 (LEF1)	-23.43	1.62451E-15

Supplementary Table 4.7.6 The log2 fold changes and p values of the genes presented in Fig 4B

Gene name	Log2 Fold change in ART compared to HC	P value
Ribosomal protein S3A (RPS3A)	3	3.57916E-06
Ribosomal protein S27 **(**RPS27L)	1.32	9.78056E-05
Ribosomal protein S15a (RPS15A)	1.12	0.000136744
Ribosomal protein S24 (RPS24)	5.94	0.00439609
Ribosomal protein S16 (RPS16)	1.1	2.2405E-05
Ribosomal protein S14 (RPS14)	-1.75	0.002522177
Ribosomal protein S6 (RPS6)	1.77	3.76778E-07
Ribosomal protein S3 (RPS3)	1.13	0.003304774
Ribosomal protein lateral stalk subunit P2 (RPLP2)	2	0.000125663
Ribosomal protein lateral stalk subunit P1 (RPLP1)	1.35	4.04459E-10
Ribosomal protein L39 like (RPL39L)	4.13	0.002418652
Ribosomal protein L18a (RPL18A)	1.46	9.05286E-05
Ribosomal protein L39 (RPL39)	2	0.000111302
Ribosomal protein L35 (RPL35)	1.68	0.000147745
Ribosomal protein L34 (RPL34)	1.48	0.000495182
Ribosomal protein L32 (RPL32)	1.27	8.2931E-05
Ribosomal protein L30 (RPL30)	-1.47	0.000734192
Ribosomal protein L28 (RPL28)	1.86	1.82958E-05

Ribosomal protein L14 (RPL14)	2.13	1.43181E-11
Ribosomal protein L5 (RPL5)	1.9	6.34523E-08
Ribosomal protein L4 (RPL4)	-1.3	0.002013026

Supplementary Table 4.7.7 The log2 fold changes and p values of the genes presented in Fig 4C

Gene name	Log2 Fold change in ART compared to HC	P value
Eukaryotic translation initiation factor 2A	1.7	0.002615925
Eukaryotic translation initiation factor 2 alpha kinase 2	2.73	0.003415744
Eukaryotic translation initiation factor 5	2.17	0.00313122
Ribosomal protein S6 kinase B1	2.22	5.60961E-05

Supplementary Table 4.7.8 The log2 fold changes and p values of the genes presented in Fig 4G

Gene name	Log2 Fold change in ART compared to HC	P value
Transforming growth factor beta receptor 2 (TGFBR)	-1.3	0.00032547
AHNAK nucleoprotein **(**AHNAK)	1.48	0.001762867
ADP ribosylation factor like GTPase 4C (ARL4C)	-1.5	0.000832051
BCL-9	-1.67	0.000281316
Rho guanine nucleotide exchange factor 12 (ARHGEF12)	1.6	2.2405E-05
FERM domain containing 4B (FRMD4B)	1.88	0.000216146
RAS guanyl releasing protein 2 **(**RASGRP2)	-2	0.000599225
Transmembrane inner ear (TMIE)	-2.25	0.003353353

Solute carrier family 2 member 3 **(**SLC2A3)	2.32	0.000711445
MAX dimerization protein 1 (MXD1)	2.35	0.00319058
Protein tyrosine phosphatase, receptor type S (PTPRS)	2.38	0.002341638
Unc-119 lipid binding chaperone (UNC119)	2.6	5.5049E-05
ATPase phospholipid transporting 8B4 (putative) (ATP8B4**)**	-2.66	0.000572542
Cytokine inducible SH2 containing protein (CISH)	3.2	1.72531E-06
Inositol polyphosphate-5-phosphatase F (INPP5F)	3.21	7.85463E-06
Sprouty RTK signaling antagonist 1 **(**SPRY1)	4.9	1.62247E-05
Prolyl 4-hydroxylase subunit alpha 1 (P4HA1)	5.9	0.000854751
lymphoid enhancer binding factor 1 (LEF1)	-23.4	1.62451E-15

4.8 References

1. Elahi, S., R. H. Weiss, and S. Merani. 2016. Atorvastatin restricts HIV replication in CD4+ T cells by upregulation of p21. *AIDS* 30: 171–183.
2. Massanella, M., E. Negredo, N. Pérez-Álvarez, J. Puig, R. Ruiz-Hernández, M. Bofill, B. Clotet, and J. Blanco. 2010. CD4 T-cell hyperactivation and susceptibility to cell death determine poor CD4 T-cell recovery during suppressive HAART. *AIDS* 24: 959–968.
3. López, M., J. San Román, V. Estrada, E. Vispo, F. Blanco, and V. Soriano. 2012. Endothelial dysfunction in HIV infection--the role of circulating endothelial cells, microparticles, endothelial progenitor cells and macrophages. *AIDS reviews* 14: 223–230.
4. Elahi, S., T. Niki, M. Hirashima, and H. Horton. 2012. Galectin-9 binding to TIM-3 renders activated human CD4+ T cells less susceptible to HIV-1 infection. *Blood* 119: 4192–4204.
5. Aandahl, E. M., J. Michaëlsson, W. J. Moretto, F. M. Hecht, and D. F. Nixon. 2004. Human CD4+ CD25+ Regulatory T Cells Control T-Cell Responses to Human Immunodeficiency Virus and Cytomegalovirus Antigens. *J Virol* 78: 2454–2459.

6. Sakaguchi, S. 2004. Naturally arising CD4+ regulatory t cells for immunologic self-tolerance and negative control of immune responses. *Annual Review of Immunology* 22: 531–562.
7. Hori, S., T. Nomura, and S. Sakaguchi. 2003. Control of regulatory T cell development by the transcription factor Foxp3. *Science (New York, N.Y.)* 299: 1057–1061.
8. Mechanisms of foxp3+ T regulatory cell-mediated suppression - PubMed. .
9. Rothstein, D. M., and G. Camirand. 2015. New insights into the mechanisms of Treg function. *Curr Opin Organ Transplant* 20: 376–384.
10. Lee, J. H., S. G. Kang, and C. H. Kim. 2007. FoxP3+ T cells undergo conventional first switch to lymphoid tissue homing receptors in thymus but accelerated second switch to nonlymphoid tissue homing receptors in secondary lymphoid tissues. *J Immunol* 178: 301–311.
11. Increased tumor-infiltrating CD45RA − CCR7 − regulatory T-cell subset with immunosuppressive properties foster gastric cancer progress | Cell Death & Disease. .
12. Elahi, S., and H. Horton. 2012. Association of HLA-alleles with the immune regulation of chronic viral infections. *Int J Biochem Cell Biol* 44: 1361–1365.
13. Elahi, S., W. L. Dinges, N. Lejarcegui, K. J. Laing, A. C. Collier, D. M. Koelle, M. J. McElrath, and H. Horton. 2011. Protective HIV-specific CD8+ T cells evade Treg cell suppression. *Nat Med* 17: 989–995.
14. The influence of HLA genotype on AIDS - PubMed. .
15. Elahi, S., S. Shahbaz, and S. Houston. 2020. Selective Upregulation of CTLA-4 on CD8+ T Cells Restricted by HLA-B*35Px Renders them to an Exhausted Phenotype in HIV-1 infection. *PLOS Pathogens* 16: e1008696.
16. Jiao, Y., J. Fu, S. Xing, B. Fu, Z. Zhang, M. Shi, X. Wang, J. Zhang, L. Jin, F. Kang, H. Wu, and F.-S. Wang. 2009. The decrease of regulatory T cells correlates with excessive activation and apoptosis of CD8+ T cells in HIV-1-infected typical progressors, but not in long-term non-progressors. *Immunology* 128: e366-375.
17. Hunt, P. W., A. L. Landay, E. Sinclair, J. A. Martinson, H. Hatano, B. Emu, P. J. Norris, M. P. Busch, J. N. Martin, C. Brooks, J. M. McCune, and S. G. Deeks. 2011. A low T regulatory cell response may contribute to both viral control and generalized immune activation in HIV controllers. *PloS One* 6: e15924.
18. Shaw, J. M., P. W. Hunt, J. W. Critchfield, D. H. McConnell, J. C. Garcia, R. B. Pollard, M. Somsouk, S. G. Deeks, and B. L. Shacklett. 2011. Increased frequency of regulatory T cells accompanies increased immune activation in rectal mucosae of HIV-positive noncontrollers. *Journal of Virology* 85: 11422–11434.
19. Chevalier, M. F., and L. Weiss. 2013. The split personality of regulatory T cells in HIV infection. *Blood* 121: 29–37.
20. Oswald-Richter, K., S. M. Grill, N. Shariat, M. Leelawong, M. S. Sundrud, D. W. Haas, and D. Unutmaz. 2004. HIV infection of naturally occurring and genetically reprogrammed human regulatory T-cells. *PLoS biology* 2: E198.
21. Antons, A. K., R. Wang, K. Oswald-Richter, M. Tseng, C. W. Arendt, S. A. Kalams, and D. Unutmaz. 2008. Naive precursors of human regulatory T cells require FoxP3 for suppression and are susceptible to HIV infection. *Journal of Immunology (Baltimore, Md.: 1950)* 180: 764–773.
22. Zaunders, J. J., S. Ip, M. L. Munier, D. E. Kaufmann, K. Suzuki, C. Brereton, S. C. Sasson, N. Seddiki, K. Koelsch, A. Landay, P. Grey, R. Finlayson, J. Kaldor, E. S. Rosenberg, B. D. Walker, B. Fazekas de St Groth, D. A. Cooper, and A. D. Kelleher. 2006. Infection of CD127+ (interleukin-7 receptor+) CD4+ cells and overexpression of CTLA-4 are linked to loss of antigen-specific CD4 T cells during primary human immunodeficiency virus type 1 infection. *Journal of Virology* 80: 10162–10172.
23. Shahbaz, S., G. Dunsmore, P. Koleva, L. Xu, S. Houston, and S. Elahi. 2020. Galectin-9 and VISTA Expression Define Terminally Exhausted T Cells in HIV-1 Infection. *J. Immunol.* 204: 2474–2491.

24. Dunsmore, G., N. Bozorgmehr, C. Delyea, P. Koleva, A. Namdar, and S. Elahi. 2017. Erythroid Suppressor Cells Compromise Neonatal Immune Response against Bordetella pertussis. *J. Immunol.* 199: 2081–2095.
25. Shahbaz, S., N. Bozorgmehr, P. Koleva, A. Namdar, J. Jovel, R. A. Fava, and S. Elahi. 2018. CD71+VISTA+ erythroid cells promote the development and function of regulatory T cells through TGF-β. *PLoS Biol* 16: e2006649.
26. Bray, N. L., H. Pimentel, P. Melsted, and L. Pachter. 2016. Near-optimal probabilistic RNA-seq quantification. *Nature Biotechnology* 34: 525–527.
27. Love, M. I., W. Huber, and S. Anders. 2014. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biology* 15: 550.
28. Kawaguchi, A., and K. Nagata. 2007. De novo replication of the influenza virus RNA genome is regulated by DNA replicative helicase, MCM. *The EMBO Journal* 26: 4566–4575.
29. Abitew, A. M., R. C. Sobti, V. L. Sharma, and A. Wanchu. 2020. Analysis of transporter associated with antigen presentation (TAP) genes polymorphisms with HIV-1 infection. *Mol Cell Biochem* 464: 65–71.
30. Tange, T. Ø., and J. Kjems. 2001. SF2/ASF binds to a splicing enhancer in the third HIV-1 tat exon and stimulates U2AF binding independently of the RS domain11Edited by J. Karn. *Journal of Molecular Biology* 312: 649–662.
31. Sertznig, H., F. Hillebrand, S. Erkelenz, H. Schaal, and M. Widera. 2018. Behind the scenes of HIV-1 replication: Alternative splicing as the dependency factor on the quiet. *Virology* 516: 176–188.
32. Brass, A. L., D. M. Dykxhoorn, Y. Benita, N. Yan, A. Engelman, R. J. Xavier, J. Lieberman, and S. J. Elledge. 2008. Identification of Host Proteins Required for HIV Infection Through a Functional Genomic Screen. *Science* 319: 921–926.
33. Boehm, D., V. Calvanese, R. D. Dar, S. Xing, S. Schroeder, L. Martins, K. Aull, P.-C. Li, V. Planelles, J. E. Bradner, M.-M. Zhou, R. F. Siliciano, L. Weinberger, E. Verdin, and M. Ott. 2013. BET bromodomain-targeting compounds reactivate HIV from latency via a Tat-independent mechanism. *Cell Cycle* 12: 452–462.
34. Hartl, F. U., and J. Martin. 1995. Molecular chaperones in cellular protein folding. *Curr Opin Struct Biol* 5: 92–102.
35. Gurer, C., A. Cimarelli, and J. Luban. 2002. Specific Incorporation of Heat Shock Protein 70 Family Members into Primate Lentiviral Virions. *J Virol* 76: 4666–4670.
36. Chappell, T. G., W. J. Welch, D. M. Schlossman, K. B. Palter, M. J. Schlesinger, and J. E. Rothman. 1986. Uncoating ATPase is a member of the 70 kilodalton family of stress proteins. *Cell* 45: 3–13.
37. Joshi, R. N., S. J. Fernandes, M. Shang, N. A. Kiani, D. Gomez-Cabrero, J. Tegnér, and A. Schmidt. 2019. Phosphatase inhibitor PPP1R11 modulates resistance of human T cells toward Treg-mediated suppression of cytokine expression. *J Leukoc Biol* 106: 413–430.
38. Mukerji, J., K. C. Olivieri, V. Misra, K. A. Agopian, and D. Gabuzda. 2012. Proteomic analysis of HIV-1 Nef cellular binding partners reveals a role for exocyst complex proteins in mediating enhancement of intercellular nanotube formation. *Retrovirology* 9: 33.
39. Membrane Topology of the Alg14 Endoplasmic Reticulum UDP-GlcNAc Transferase Subunit. .
40. Jochmann, R., M. Thurau, S. Jung, C. Hofmann, E. Naschberger, E. Kremmer, T. Harrer, M. Miller, N. Schaft, and M. Stürzl. 2009. O-linked N-acetylglucosaminylation of Sp1 inhibits the human immunodeficiency virus type 1 promoter. *J Virol* 83: 3704–3718.
41. Bearz, A., G. Tell, S. Formisano, S. Merluzzi, A. Colombatti, and C. Pucillo. 1999. Adhesion to fibronectin promotes the activation of the p125FAK/Zap-70 complex in human T cells. *Immunology* 98: 564–568.
42. Crux, N. B., and S. Elahi. 2017. Human Leukocyte Antigen (HLA) and Immune Regulation: How Do Classical and Non-Classical HLA Alleles Modulate Immune Response to Human Immunodeficiency Virus and Hepatitis C Virus Infections? *Front Immunol* 8.

43. Lee, N., A. Ishitani, and D. E. Geraghty. 2010. HLA-F is a surface marker on activated lymphocytes1. *Eur J Immunol* 40: 2308–2318.
44. Zeng, H., K. Yang, C. Cloer, G. Neale, P. Vogel, and H. Chi. 2013. mTORC1 couples immune signals and metabolic programming to establish Treg cell function. *Nature* 499: 485–490.
45. Sun, I.-H., M.-H. Oh, L. Zhao, Chirag, H. Patel, M. L. Arwood, W. Xu, A. J. Tam, R. L. Blosser, J. Wen, and J. D. Powell. 2018. mTORC1 signaling regulates the generation and function of central and effector FoxP3+ regulatory T cells. *J Immunol* 201: 481–492.
46. Chapman, N. M., and H. Chi. 2014. mTOR signaling, Tregs and immune modulation. *Immunotherapy* 6: 1295–1311.
47. Waickman, A. T., and J. D. Powell. 2012. mTOR, metabolism, and the regulation of T-cell differentiation and function. *Immunol Rev* 249: 43–58.
48. Alvisi, G., J. Brummelman, S. Puccio, E. M. Mazza, E. P. Tomada, A. Losurdo, V. Zanon, C. Peano, F. S. Colombo, A. Scarpa, M. Alloisio, A. Vasanthakumar, R. Roychoudhuri, M. Kallikourdis, M. Pagani, E. Lopci, P. Novellis, J. Blume, A. Kallies, G. Veronesi, and E. Lugli. 2020. IRF4 instructs effector Treg differentiation and immune suppression in human cancer. *J. Clin. Invest.* 130: 3137–3150.
49. Vasanthakumar, A., Y. Liao, P. Teh, M. F. Pascutti, A. E. Oja, A. L. Garnham, R. Gloury, J. C. Tempany, T. Sidwell, E. Cuadrado, P. Tuijnenburg, T. W. Kuijpers, N. Lalaoui, L. A. Mielke, V. L. Bryant, P. D. Hodgkin, J. Silke, G. K. Smyth, M. A. Nolte, W. Shi, and A. Kallies. 2017. The TNF Receptor Superfamily-NF-κB Axis Is Critical to Maintain Effector Regulatory T Cells in Lymphoid and Non-lymphoid Tissues. *Cell Reports* 20: 2906–2920.
50. Baecher-Allan, C., E. Wolf, and D. A. Hafler. 2006. MHC Class II Expression Identifies Functionally Distinct Human Regulatory T Cells. *The Journal of Immunology* 176: 4622–4631.
51. Schulze Zur Wiesch, J., A. Thomssen, P. Hartjen, I. Tóth, C. Lehmann, D. Meyer-Olson, K. Colberg, S. Frerk, D. Babikir, S. Schmiedel, O. Degen, S. Mauss, J. Rockstroh, S. Staszewski, P. Khaykin, A. Strasak, A. W. Lohse, G. Fätkenheuer, J. Hauber, and J. van Lunzen. 2011. Comprehensive analysis of frequency and phenotype of T regulatory cells in HIV infection: CD39 expression of FoxP3+ T regulatory cells correlates with progressive disease. *J. Virol.* 85: 1287–1297.
52. Mukhopadhyay, A., S. A. Saddoughi, P. Song, I. Sultan, S. Ponnusamy, C. E. Senkal, C. F. Snook, H. K. Arnold, R. C. Sears, Y. A. Hannun, and B. Ogretmen. 2009. Direct interaction between the inhibitor 2 and ceramide via sphingolipid-protein binding is involved in the regulation of protein phosphatase 2A activity and signaling. *FASEB J* 23: 751–763.
53. Smith, E. L., and E. H. Schuchman. 2008. The unexpected role of acid sphingomyelinase in cell death and the pathophysiology of common diseases. *FASEB J* 22: 3419–3431.
54. Apostolidis, S. A., N. Rodríguez-Rodríguez, A. Suárez-Fueyo, N. Dioufa, E. Ozcan, J. C. Crispín, M. G. Tsokos, and G. C. Tsokos. 2016. Protein phosphatase 2A is requisite for the function of regulatory T cells. *Nat Immunol* 17: 556–564.
55. Kleijn, M., and C. G. Proud. 2002. The regulation of protein synthesis and translation factors by CD3 and CD28 in human primary T lymphocytes. *BMC Biochem* 3: 11.
56. Asmal, M., J. Colgan, F. Naef, B. Yu, Y. Lee, M. Magnasco, and J. Luban. 2003. Production of Ribosome Components in Effector CD4+ T Cells Is Accelerated by TCR Stimulation and Coordinated by ERK-MAPK. *Immunity* 19: 535–548.
57. Li, W., D. Tan, Z. Zhang, J. J. Liang, and R. E. Brown. 2008. Activation of Akt-mTOR-p70S6K pathway in angiogenesis in hepatocellular carcinoma. *Oncol. Rep.* 20: 713–719.
58. Zhao, B., L. Li, and K.-L. Guan. 2010. Hippo signaling at a glance. *J Cell Sci* 123: 4001–4006.
59. Hippo kinases Mst1 and Mst2 sense and amplify IL-2R–STAT5 signaling in regulatory T cells to establish stable regulatory activity. .

60. Li, J., X. Du, H. Shi, K. Deng, H. Chi, and W. Tao. 2015. Mammalian Sterile 20-like Kinase 1 (Mst1) Enhances the Stability of Forkhead Box P3 (Foxp3) and the Function of Regulatory T Cells by Modulating Foxp3 Acetylation. *J Biol Chem* 290: 30762–30770.
61. Levine, A. G., A. Arvey, W. Jin, and A. Y. Rudensky. 2014. Continuous requirement for the TCR in regulatory T cell function. *Nat. Immunol.* 15: 1070–1078.
62. Chinen, T., A. K. Kannan, A. G. Levine, X. Fan, U. Klein, Y. Zheng, G. Gasteiger, Y. Feng, J. D. Fontenot, and A. Y. Rudensky. 2016. An essential role for the IL-2 receptor in T reg cell function. *Nature Immunology* 17: 1322–1333.
63. Transcription factor Foxp1 regulates Foxp3 chromatin binding and coordinates regulatory T cell function | Nature Immunology. .
64. Foxp1 is an essential transcription regulator for the generation of quiescent naive T cells during thymocyte development | Blood | American Society of Hematology. .
65. Adler, H. S., and K. Steinbrink. 2008. MAP kinase p38 and its relation to T cell anergy and suppressor function of regulatory T cells. *Cell Cycle* 7: 169–170.
66. Perna, S. K., B. De Angelis, D. Pagliara, L. Zhan, C. M. Rooney, H. E. Heslop, M. K. Brenner, G. Dotti, and B. Savoldo. 2009. IL15 Enhances Proliferation and Effector Function of Antigen-Specific Cytotoxic T Lymphocytes (CTLs) and Mitigates the Suppressive Action of Regulatory T Cells (Tregs). *Blood* 114: 4088–4088.
67. Chaudhry, A., R. M. Samstein, P. Treuting, Y. Liang, M. C. Pils, J.-M. Heinrich, R. S. Jack, F. T. Wunderlich, J. C. Brüning, W. Müller, and A. Y. Rudensky. 2011. Interleukin-10 signaling in regulatory T cells is required for suppression of Th17 cell-mediated inflammation. *Immunity* 34: 566–578.
68. Interleukin-10 deficiency impairs regulatory T cell-derived neuropilin-1 functions and promotes Th1 and Th17 immunity | Scientific Reports. .
69. Grzes, K. M., C. S. Field, and E. J. Pearce. 2017. Treg Cells Survive and Thrive in Inhospitable Environments. *Cell Metabolism* 25: 1213–1215.
70. Shi, H., and H. Chi. 2019. Metabolic Control of Treg Cell Stability, Plasticity, and Tissue-Specific Heterogeneity. *Front. Immunol.* 10.
71. Gerriets, V. A., R. J. Kishton, M. O. Johnson, S. Cohen, P. J. Siska, A. G. Nichols, M. O. Warmoes, A. A. de Cubas, N. J. MacIver, J. W. Locasale, L. A. Turka, A. D. Wells, and J. C. Rathmell. 2016. Foxp3 and Toll-like receptor signaling balance T reg cell anabolic metabolism for suppression. *Nature Immunology* 17: 1459–1466.
72. Le Menn, G., and J. G. Neels. 2018. Regulation of Immune Cell Function by PPARs and the Connection with Metabolic and Neurodegenerative Diseases. *Int J Mol Sci* 19.
73. Guri, A. J., S. K. Mohapatra, W. T. Horne, R. Hontecillas, and J. Bassaganya-Riera. 2010. The role of T cell PPAR gamma in mice with experimental inflammatory bowel disease. *BMC Gastroenterol* 10: 60.
74. Hontecillas, R., and J. Bassaganya-Riera. 2007. Peroxisome proliferator-activated receptor gamma is required for regulatory CD4+ T cell-mediated protection against colitis. *J. Immunol.* 178: 2940–2949.
75. Wohlfert, E. A., F. C. Nichols, E. Nevius, and R. B. Clark. 2007. Peroxisome proliferator-activated receptor gamma (PPARgamma) and immunoregulation: enhancement of regulatory T cells through PPARgamma-dependent and -independent mechanisms. *J. Immunol.* 178: 4129–4135.
76. Wang, L. H., X. Y. Yang, X. Zhang, J. Huang, J. Hou, J. Li, H. Xiong, K. Mihalic, H. Zhu, W. Xiao, and W. L. Farrar. 2004. Transcription Inactivation of STAT3 by PPARγ Suppresses IL-6-Responsive Multiple Myeloma Cells. *Immunity* 20: 205–218.
77. Zhong, Z., Z. Wen, and J. E. Darnell. 1994. Stat3: a STAT family member activated by tyrosine phosphorylation in response to epidermal growth factor and interleukin-6. *Science* 264: 95–98.
78. Fujino, M., and X.-K. Li. 2013. Role of STAT3 in regulatory T lymphocyte plasticity during acute graft-vs.-host-disease. *JAK-STAT* 2: e24529.

79. Laurence, A., S. Amarnath, J. Mariotti, Y. C. Kim, J. Foley, M. Eckhaus, J. J. O'Shea, and D. H. Fowler. 2012. STAT3 Transcription Factor Promotes Instability of nTreg Cells and Limits Generation of iTreg Cells During Acute Murine Graft Versus Host Disease. *Immunity* 37: 209–222.
80. Zhou, X., S. Bailey-Bucktrout, L. T. Jeker, and J. A. Bluestone. 2009. Plasticity of CD4+ FoxP3+ T cells. *Curr Opin Immunol* 21: 281–285.
81. Full article: Functional plasticity in human FOXP3+ regulatory T cells. .
82. Lin, B., D. Xu, and D. W. Leaman. 2016. XIAP-associated factor 1 (XAF1) regulates TNF receptor 1 complex stability. *FEBS Lett* 590: 4381–4392.
83. Srivastava, R. K., G. B. Tomar, A. P. Barhanpurkar, N. Gupta, S. T. Pote, G. C. Mishra, and M. R. Wani. 2011. IL-3 Attenuates Collagen-Induced Arthritis by Modulating the Development of Foxp3+ Regulatory T Cells. *The Journal of Immunology* 186: 2262–2272.
84. Hamano, R., J. Huang, T. Yoshimura, J. J. Oppenheim, and X. Chen. 2011. TNF by inducing co-stimulatory TNF receptor superfamily members TNFR2, 4-1BB and OX40 augments the number and function of mouse CD4+FoxP3+ regulatory T cells. *Eur J Immunol* 41: 2010–2020.
85. Metidji, A., S. A. Rieder, D. D. Glass, I. Cremer, G. A. Punkosdy, and E. M. Shevach. 2015. IFN-α/β receptor signaling promotes regulatory T cell development and function under stress conditions. *Journal of Immunology (Baltimore, Md.: 1950)* 194: 4265–4276.
86. Vogel, I. T., S. W. V. Gool, and J. L. Ceuppens. 2014. CD28/CTLA-4/B7 and CD40/CD40L costimulation and activation of regulatory T cells. *World Journal of Immunology* 4: 63–77.
87. Feng, C., L.-J. Cao, H.-F. Song, P. Xu, H. Chen, J.-C. Xu, X.-Y. Zhu, X.-G. Zhang, and X.-F. Wang. 2015. Expression of PD-L1 on CD4+CD25+Foxp3+ Regulatory T Cells of Patients with Chronic HBV Infection and Its Correlation with Clinical Parameters. *Viral Immunology* 28: 418–424.
88. Valli, E., H. A. Sterle, S. M. Huergo, I. Mascafroni, A. J. Klecha, M. L. B. Arcos, G. A. Rabinovich, and G. A. Cremaschi. 2013. Regulatory T cells mediate immunosupression induced by experimental hypothyroidism. (P1215). *The Journal of Immunology* 190: 188.10-188.10.
89. Sampson, J. F., A. Suryawanshi, W.-S. Chen, G. A. Rabinovich, and N. Panjwani. 2016. Galectin-8 promotes regulatory T cell differentiation by modulating IL-2 and TGFβ signaling. *Immunol Cell Biol* 94: 213–219.
90. Maj, T., W. Wang, J. Crespo, H. Zhang, W. Wang, S. Wei, L. Zhao, L. Vatan, I. Shao, W. Szeliga, C. Lyssiotis, J. R. Liu, I. Kryczek, and W. Zou. 2017. Oxidative stress controls regulatory T cell apoptosis and suppressor activity and PD-L1-blockade resistance in tumor. *Nature Immunology* 18: 1332–1341.
91. Zhang, J., and P. A. Ney. 2009. Role of BNIP3 and NIX in cell death, autophagy, and mitophagy. *Cell Death & Differentiation* 16: 939–946.
92. McPherson, J. P., H. Sarras, B. Lemmers, L. Tamblyn, E. Migon, E. Matysiak-Zablocki, A. Hakem, S. A. Azami, R. Cardoso, J. Fish, O. Sanchez, M. Post, and R. Hakem. 2009. Essential role for Bclaf1 in lung development and immune system function. *Cell Death & Differentiation* 16: 331–339.
93. Tóth, I., A. Q. Le, P. Hartjen, A. Thomssen, V. Matzat, C. Lehmann, C. Scheurich, C. Beisel, P. Busch, O. Degen, A. W. Lohse, T. Eiermann, G. Fätkenheuer, D. Meyer-Olson, M. Bockhorn, J. Hauber, J. van Lunzen, and J. Schulze Zur Wiesch. 2013. Decreased frequency of CD73+CD8+ T cells of HIV-infected patients correlates with immune activation and T cell exhaustion. *Journal of Leukocyte Biology* 94: 551–561.
94. Zhao, H., X. Liao, and Y. Kang. 2017. Tregs: Where We Are and What Comes Next? *Front. Immunol.* 8.
95. Angin, M., S. Sharma, M. King, T. T. Murooka, M. Ghebremichael, T. R. Mempel, B. D. Walker, M. K. Bhasin, and M. M. Addo. 2014. HIV-1 Infection Impairs Regulatory T-Cell Suppressive Capacity on a Per-Cell Basis. *J Infect Dis* 210: 899–903.
96. Pion, M., D. Jaramillo-Ruiz, A. Martínez, M. A. Muñoz-Fernández, and R. Correa-Rocha. 2013. HIV infection of human regulatory T cells downregulates Foxp3 expression by increasing DNMT3b levels and DNA methylation in the FOXP3 gene. *AIDS* 27: 2019–2029.

97. Eggena, M. P., B. Barugahare, N. Jones, M. Okello, S. Mutalya, C. Kityo, P. Mugyenyi, and H. Cao. 2005. Depletion of regulatory T cells in HIV infection is associated with immune activation. *J Immunol* 174: 4407–4414.
98. Kinter, A. L., M. Hennessey, A. Bell, S. Kern, Y. Lin, M. Daucher, M. Planta, M. McGlaughlin, R. Jackson, S. F. Ziegler, and A. S. Fauci. 2004. CD25+CD4+ Regulatory T Cells from the Peripheral Blood of Asymptomatic HIV-infected Individuals Regulate CD4+ and CD8+ HIV-specific T Cell Immune Responses In Vitro and Are Associated with Favorable Clinical Markers of Disease Status. *J Exp Med* 200: 331–343.
99. Kinter, A. L., R. Horak, M. Sion, L. Riggin, J. McNally, Y. Lin, R. Jackson, A. O'shea, G. Roby, C. Kovacs, M. Connors, S. A. Migueles, and A. S. Fauci. 2007. CD25+ regulatory T cells isolated from HIV-infected individuals suppress the cytolytic and nonlytic antiviral activity of HIV-specific CD8+ T cells in vitro. *AIDS Res Hum Retroviruses* 23: 438–450.
100. mTOR coordinates transcription programs and mitochondrial metabolism of activated T reg subsets to protect tissue homeostasis | Nature Communications. .
101. T cell receptor signalling in the control of regulatory T cell differentiation and function | Nature Reviews Immunology. .
102. Ono, M. 2020. Control of regulatory T-cell differentiation and function by T-cell receptor signalling and Foxp3 transcription factor complexes. *Immunology* 160: 24–37.
103. Zheng, Y., A. Chaudhry, A. Kas, P. deRoos, J. M. Kim, T.-T. Chu, L. Corcoran, P. Treuting, U. Klein, and A. Y. Rudensky. 2009. Regulatory T-cell suppressor program co-opts transcription factor IRF4 to control T(H)2 responses. *Nature* 458: 351–356.
104. Meng, Z., T. Moroishi, and K.-L. Guan. 2016. Mechanisms of Hippo pathway regulation. *Genes Dev* 30: 1–17.
105. van Loosdregt, J., Y. Vercoulen, T. Guichelaar, Y. Y. J. Gent, J. M. Beekman, O. van Beekum, A. B. Brenkman, D.-J. Hijnen, T. Mutis, E. Kalkhoven, B. J. Prakken, and P. J. Coffer. 2010. Regulation of Treg functionality by acetylation-mediated Foxp3 protein stabilization. *Blood* 115: 965–974.
106. Li, B., A. Samanta, X. Song, K. T. Iacono, K. Bembas, R. Tao, S. Basu, J. L. Riley, W. W. Hancock, Y. Shen, S. J. Saouaf, and M. I. Greene. 2007. FOXP3 interactions with histone acetyltransferase and class II histone deacetylases are required for repression. *Proceedings of the National Academy of Sciences of the United States of America* 104: 4571.
107. Li, M., A. Makkinje, and Z. Damuni. 1996. The myeloid leukemia-associated protein SET is a potent inhibitor of protein phosphatase 2A. *J. Biol. Chem.* 271: 11059–11062.
108. Trotta, R., D. Ciarlariello, J. D. Col, J. Allard, P. Neviani, R. Santhanam, H. Mao, B. Becknell, J. Yu, A. K. Ferketich, B. Thomas, A. Modi, B. W. Blaser, D. Perrotti, and M. A. Caligiuri. 2007. The PP2A inhibitor SET regulates natural killer cell IFN-γ production. *J Exp Med* 204: 2397–2405.
109. Wlodarchak, N., and Y. Xing. 2016. PP2A as a master regulator of the cell cycle. *Crit Rev Biochem Mol Biol* 51: 162–184.
110. Hollmann, C., S. Werner, E. Avota, D. Reuter, L. Japtok, B. Kleuser, E. Gulbins, K. A. Becker, J. Schneider-Schaulies, and N. Beyersdorf. 2016. Inhibition of Acid Sphingomyelinase Allows for Selective Targeting of CD4+ Conventional versus Foxp3+ Regulatory T Cells. *The Journal of Immunology* 197: 3130–3141.
111. Finnegan, C. M., S. S. Rawat, A. Puri, J. M. Wang, F. W. Ruscetti, and R. Blumenthal. 2004. Ceramide, a target for antiretroviral therapy. *Proc Natl Acad Sci U S A* 101: 15452–15457.
112. 178. IL-15 Reduces Suppressive Effects of Regulatory T Cells (Tregs) on Antigen-Specific Cytotoxic T Lymphocytes (CTLs): Molecular Therapy.
113. Dang, E. V., J. Barbi, H.-Y. Yang, D. Jinasena, H. Yu, Y. Zheng, Z. Bordman, J. Fu, Y. Kim, H.-R. Yen, W. Luo, K. Zeller, L. Shimoda, S. L. Topalian, G. L. Semenza, C. V. Dang, D. M. Pardoll, and F. Pan. 2011. Control of TH17/Treg Balance by Hypoxia-inducible Factor 1. *Cell* 146: 772–784.

114. Tao, J.-H., J. Barbi, and F. Pan. 2015. Hypoxia-inducible factors in T lymphocyte differentiation and function. A Review in the Theme: Cellular Responses to Hypoxia. *Am J Physiol Cell Physiol* 309: C580–C589.
115. Kempkes, R. W. M., I. Joosten, H. J. P. M. Koenen, and X. He. 2019. Metabolic Pathways Involved in Regulatory T Cell Functionality. *Front. Immunol.* 10.
116. Wei, J., L. Long, K. Yang, C. Guy, S. Shrestha, Z. Chen, C. Wu, P. Vogel, G. Neale, D. R. Green, and H. Chi. 2016. Autophagy enforces functional integrity of regulatory T cells by coupling environmental cues and metabolic homeostasis. *Nature Immunology* 17: 277–285.
117. Miska, J., C. Lee-Chang, A. Rashidi, M. E. Muroski, A. L. Chang, A. Lopez-Rosas, P. Zhang, W. K. Panek, A. Cordero, Y. Han, A. U. Ahmed, N. S. Chandel, and M. S. Lesniak. 2019. HIF-1α Is a Metabolic Switch between Glycolytic-Driven Migration and Oxidative Phosphorylation-Driven Immunosuppression of Tregs in Glioblastoma. *Cell Reports* 27: 226-237.e4.
118. Angelin, A., L. Gil-de-Gómez, S. Dahiya, J. Jiao, L. Guo, M. H. Levine, Z. Wang, W. J. Quinn, P. K. Kopinski, L. Wang, T. Akimova, Y. Liu, T. R. Bhatti, R. Han, B. L. Laskin, J. A. Baur, I. A. Blair, D. C. Wallace, W. W. Hancock, and U. H. Beier. 2017. Foxp3 Reprograms T Cell Metabolism to Function in Low-Glucose, High-Lactate Environments. *Cell Metabolism* 25: 1282-1293.e7.
119. Deng, D., C. Xu, P. Sun, J. Wu, C. Yan, M. Hu, and N. Yan. 2014. Crystal structure of the human glucose transporter GLUT1. *Nature* 510: 121–125.
120. Beier, U. H., A. Angelin, T. Akimova, L. Wang, Y. Liu, H. Xiao, M. A. Koike, S. A. Hancock, T. R. Bhatti, R. Han, J. Jiao, S. C. Veasey, C. A. Sims, J. A. Baur, D. C. Wallace, and W. W. Hancock. 2015. Essential role of mitochondrial energy metabolism in Foxp3+ T-regulatory cell function and allograft survival. *The FASEB Journal* 29: 2315–2326.
121. Yu, X., Y. Lao, X.-L. Teng, S. Li, Y. Zhou, F. Wang, X. Guo, S. Deng, Y. Chang, X. Wu, Z. Liu, L. Chen, L.-M. Lu, J. Cheng, B. Li, B. Su, J. Jiang, H.-B. Li, C. Huang, J. Yi, and Q. Zou. 2018. SENP3 maintains the stability and function of regulatory T cells via BACH2 deSUMOylation. *Nature Communications* 9: 3157.
122. Hanson, R. W., and O. E. Owen. 2013. Gluconeogenesis. In *Encyclopedia of Biological Chemistry (Second Edition)* W. J. Lennarz, and M. D. Lane, eds. Academic Press, Waltham. 381–386.
123. A Pck1-directed glycogen metabolic program regulates formation and maintenance of memory CD8 + T cells - PubMed. .
124. Selective Export of HLA-F by Its Cytoplasmic Tail | The Journal of Immunology. .
125. Garcia-Beltran, W. F., A. Hölzemer, G. Martrus, A. W. Chung, Y. Pacheco, C. R. Simoneau, M. Rucevic, P. A. Lamothe-Molina, T. Pertel, T.-E. Kim, H. Dugan, G. Alter, J. Dechanet-Merville, S. Jost, M. Carrington, and M. Altfeld. 2016. Open conformers of HLA-F are high-affinity ligands of the activating NK-cell receptor KIR3DS1. *Nature Immunology* 17: 1067–1074.
126. Kramer, A. B., C. Roozendaal, and R. P. F. Dullaart. 2004. Familial occurrence of subacute thyroiditis associated with human leukocyte antigen-B35. *Thyroid* 14: 544–547.

Chapter 5: General discussion and conclusion

5.1 Discussion

HIV-specific CTLs are crucial for limiting viral replication during primary HIV infection (1, 2). The T cell receptor (TCR) on CTLs recognize and binds to HIV-derived epitopes expressed on HLA class I molecules on infected cells. Following this direct cell-to-cell interaction, an immunological synapse is formed between infected cells and CTLs that results in a cascade of effector functions targeting the virally-infected cell (3). These effector functions include the production of cytotoxic molecules such as granzymes and perforin, Fas-ligand, and the secretion of various antiviral chemokines and cytokines, which collectively help to kill the infected cells, potentially before the synthesis and emergence of progeny virus (4). The importance of the CTL in controlling HIV infection is evident from the inverse correlation between the magnitude of CTL response and the viral load during acute HIV infection and the strong ability of HIV-specific CTLs isolated from HIV-infected individuals to kill virally infected cells *in vitro* (5). Moreover, depletion of CTLs during acute Simian immunodeficiency virus (SIV) infection increases viremia in a macaque model (6). In addition, the emergence of the mutations in viral epitopes targeted by CTLs as a mechanism of viral escape highlights the importance of CTLs in controlling HIV infection (7).

Despite the crucial role of CTLs in the acute phase of HIV infection, CTLs eventually fail to control viral replication and disease progression in HIV-infected individuals (8, 9). This is attributed to several factors including the acquisition of the CTL-resistant escape mutations in the viral epitopes (10), the disruption of HLA-class I antigen presentation pathway by the virus (11), the establishment of non-replicating provirus inside HIV target cells in latent virus reservoirs (12), and CTL dysfunction (13). CTLs lose their effector function in the setting of persistent antigen stimulation such as HIV infection. This phenomenon refered to as T cell exhaustion, is accompanied by reduced proliferation, decreased polyfunctionality, and enhanced apoptosis of antigen specific CD8 T cells (14). Exhausted CD8 T cells upregulate several co-inhibitory molecules, such as PD-1, CTLA-4, TIGIT, CD160, and TIM-3 (15–18). Notably, the co-expression

of several co-inhibitory receptors on CD8 T cells is associated with a more severe exhausted phenotype (19).

The role of Gal-9 as a ligand for TIM-3 has been described in several studies (20, 21). It is reported that the interaction of Gal-9 with TIM-3 on the surfaces of Th1 cells results in the induction of apoptosis and Th1 cell exhaustion (22). Similarly, Gal-9 induces apoptosis of Th17 cells and inhibits Th17 differentiation and function via interaction with TIM-3 on their surface (23). Additionally, stimulation of naïve CD4 T cells with TGF-β1 and IL-6 in the presence of Gal-9 results in the downregulation and upregulation of the IL-17 mRNA and FOXP3 mRNA, respectively (23). Moreover, recent pieces of evidence demonstrated that the interaction of Gal-9 with CD44 to promotes FOXP3 expression and Tregs induction through a Smad3 dependent pathway (24). Our group and other groups have shown that TIM-3+ CD8 T cell are expanded in chronic HIV-infection and the interaction of Gal-9 with TIM-3 on CD8 T cells results in CD8 T cell exhaustion (18, 25). However, the surface expression of Gal-9 on T cells in the setting of HIV infection had never been investigated prior to our study.

In our studies, for the very first time, we found increased frequency of Gal-9 expressing CD4 and CD8 T cells in HIV-infected individuals compared to HCs. Similarly, we observed upregulation of both VISTA expressing CD4 and CD8 T cells form HIV-infected individuals. We also demonstrated that the expression of these molecules on CD8 T cells was associated with a dysfunctional phenotype. Our further investigations revealed that Gal-9 was highly co-expressed with other co-inhibitory receptors, in particular, PD-1. We found this co-expression of Gal-9 with other co-inhibitory receptors was associated with a terminally exhausted T cell phenotype. More notably, Gal-9 expressing T cells exhibited an irreversible dysfunctional phenotype compared to other T cells expressing other co-inhibitory receptors (e.g. PD-1). Moreover, we demonstrated that isolated Gal-9 expressing CD8 T cells maintained their dysfunctional phenotype in the absence of other immune cells, suggesting an intrinsic functional defect. Of note, we found expressing CD8 T cells were not abundant in LTNPs, which provides a novel feature of

CTLs in this rare population. This is in agreement with a report of lower frequency of PD-1 and TIGIT expressing CD8 T cells restricted by HLA-B*57:01 within the first year of infection. However, upregulation of PD-1 and TIGIT was later associated with CD8 T cells dysfunction and disease progression in the HLA-B*57:01-restricted patients (26). Another group assessed the frequency of HIV-specific CD160+2B4+ CD8 T cells in elite controllers, a group of HIV-infected individuals with similar immunologic and clinical characteristic to LTNPs. They reported similar frequency of CD160+2B4+ HIV-specific CD8 T cells in elite controllers and chronic progressors (27). However, elite controllers harbored a subset of CD160+2B4+ HIV-specific CD8 T cells that possessed cytolytic properties, a subset that was not present in chronic progressors (27). In summary, our results demonstrated the abundance of Gal-9 and VISTA-expressing CD8 T cells in HIV-infected individuals. In particular, our data demonstrated Gal-9 and VISTA as novel immune checkpoints associated with intrinsic dysfunctional CTL phenotype in HIV infection.

We then focused on the next barrier to viral eradication in HIV-infection. Although ART suppresses viremia in HIV-infected individuals, the replication competent virus persists in some cells in certain anatomical locations in the body called “reservoirs” (28, 29). Since CD4 T cells are the main infected cells in HIV infection, they are the dominant reservoirs of HIV in virally suppressed patients receiving ART (30), with central memory and follicular helper T cells being the most important reservoirs of latent HIV in CD4 T cell subsets (31). In addition, myeloid cells such as macrophages and dendritic cells, particularly follicular dendritic cells in B cell follicles can be considered as HIV reservoirs. Although different anatomical locations can harbor HIV-infected cells, lymphoid tissues such as LNs, spleen, thymus, and GALT constitute the main locations of the virally infected cells, with viral DNA detectable in LNs after many years of receiving ART (32). Antigen sequestration in reservoirs limits the access of CD8 T cells to viral antigens during ART and is considered as the major barrier for viral eradication (33). For example, most CD8 T cells are not able to enter the B cell follicles due to lack of chemokine receptors (34), which

might account for 30-fold higher accumulation of latent virus in follicular CD4 Th cells compared to peripheral CD4 T cells (35). Similar to LNs, CD8 T cells fail to access to other immune-privileged sites including the CNS and testis (36, 37). In addition, tissue resident memory T cells that are harboring the HIV viral particles don't expose themselves to CD8 T cells by periodic regress (38). Thus, finding the strategies to improve access of CD8 T cells to HIV reservoirs can have great importance to achieve durable HIV remission.

We and others found that the frequency of CD73 expressing CD8 T cells was lower in HIV-infected individuals compared to HCs, as reported elsewhere (39). It is suggested that CD73 is involved in cell trafficking (40, 41), thus we propose it may facilitate the access of CD8 T cells to tissues such as LNs. Interestingly, we found a high co-expression of CD73 with CCR7 and *a*4β7integrin in CD8 T cells, markers associated with CD8 T cells ability to migrate to LNs and the gut, respectively. In agreement, we found a higher frequency of CD73+ CD8 T cells in the gut tissues compared with peripheral blood in mice. These results suggest that CD73 might have a role in homing of CD8 T cells to the GALT. This may account for the lower relapse rate and better prognosis of Inflammatory Bowel Disease (IBD) in HIV-infected individuals (42, 43). Although it has been suggested that CD4 T cell depletion in HIV-infection results in IBD remission, other studies have rejected this concept of a low CD4 count as the cause of IBD remission (44–46). Moreover, it is reported that individuals with HIV infection have a reduced risk of developing Multiple Sclerosis (MS) and/or experience lower relapse rates (47). This might result from deficient entry of T cells to the CNS as the expression of CD73 on CD4 T cells was required for their efficient entry into the CNS during the development of EAE in mice (40).

In addition to its role in homing of CD8 T cells to different tissues, CD73 also works with CD39 to convert ATP to AMP and then adenosine. Therefore, lower CD73 expressing immune cells in HIV-infected patients might contribute to the high plasma ATP observed in these patients (48). Higher ATP results in the release of HIV virions from infected macrophages, resulting in purging HIV reservoirs in HIV-infected

individuals (49). However, high ATP might account for the higher cardiovascular disease risk observed in HIV patients, even in those receiving ART (50, 51). Also it is shown that the plasma ATP level correlates with cognitive decline in HIV-infected patients, suggesting a role for ATP in the brain cell injury through increased inflammation (48). Our studies confirmed the elevation of ATP in the plasma of HIV-infected individuals. We found that the elevation of ATP was associated with the downregulation of CD73 mRNA expression in CD8 T cells. Our further investigations provided a novel concept in which a higher ATP level was linked to the upregulation of miR-30 family. In turn, the inhibition of miR-30b, 30c, and 30e prevented CD73 mRNA downregulation in CD8 T cells. Therefore, these observations suggest a positive feedback loop in which ATP decreases CD73 expression, whereas low CD73 results in ATP accumulation in the plasma. Therefore, treatment strategies to inhibit these miRNAs in HIV-infected individuals may provide a brake in this feedback loop to prevent CD73 downregulation in CD8 T cells. Such strategies may have therapeutic implications to improve the access of CD8 T cells to different tissues where HIV reservoirs reside.

Although co-inhibitory/co-stimulatory molecules play a crucial role in regulating effector CTL functions in the context of HIV, Tregs are significant subset of immune cells with a broad range of immunoregulatory properties. Despite their important role in chronic viral infections and cancer, their role in HIV has been the focus of debate and controversy. Therefore, we aimed to investigate their role in more depth. First, we used RNAseq to compare the transcriptional profile of Tregs from LTNPs, HIV-infected individuals receiving ART, and healthy controls. Our results showed upregulation of the pathways associated with higher immunosuppressive/activated phenotypes in Tregs of patients receiving ART. In contrast, we observed downregulation of the pathways associated with Tregs activation in Tregs from LTNPs. We furfure confirmed our results by showing higher suppressive function of Tregs in patients receiving ART compared to healthy controls and LTNPs. A previous report by our groups demonstrated that Tregs suppress CD8 T cells through interaction of Gal-9 on their surface with TIM-3 on the surface of CD8 T

cells. They also showed that upon cognate epitope recognition, CD8 T cells restricted by protective HLA-B27 and HLA-B57 alleles upregulate less TIM-3 compared to CD8 T cells restricted by non-protective alleles, resulting in their less susceptibility to Treg mediated suppression (52). However, since this study compared the function of different allele specific CD8 T cells in the same individual, the role of Tregs between different groups of patients was not addressed. Our results may explain why individuals carrying HLA-B27/B57 alleles have more functional CD8 T cells with subsequent natural immunity against HIV infection. Thus, our recent data demonstrate that beside intrinsic defects in CD8 T cells function observed in HIV patients, their function could be further compromised extrinsically via Tregs. Moreover, the decreased function of Tregs in LTNPs may account for preservation of CD8 T cells function in this rare group of HIV-infected patients.

5.2 Limitations and recommendations for the future direction

5.2.1 The first study: Galectin-9 and VISTA expression define terminally exhausted T cells in HIV-1 infection

We showed that Gal-9-expressing T cells exhibit decreased functionality compared to their negative counterpart. We also extrapolated that Gal-9-expressing CD8 T cells were less cytotoxic due to the fact that they expressed lower levels of GzmB, perforin and CD107a. Unfortunately, we were unable to perform cytotoxicity assay to compare the ability of Gal-9+ vs. Gal-9- CD8 T cells to kill target cells. We only examined the cytotoxic function of CD8 T cells thorough the expression of cytotolytic molecules and degranulation. Although we showed that Gal-9 interaction with CD44 might account for dysfunctional phenotypes observed in Gal-9 expressing T cells, the pathways associated with their phenotypes were not fully investigated. In this study, the expression of Gal-9 on antigen-specific CD8 T cells was not addressed, therefore, such studies are required to determine the frequency of Gal-9+ antigen-specific CTLs and their functional properties. Notably, we found that Gal-9 was highly co-

expressed with PD-1 on T cells from HIV-infected individuals. This is a very important observations, which suggests the interaction of PD-1 with Gal-9.

However, these observations led us to investigate the expression of Gal-9 on T cells in virus-associated solid tumors. Interestingly, we found that Gal-9 was also highly expressed on T cells of these patients and Gal-9-expressing T cells exhibited the same dysfunctional phenotype (Okoye et al. Journal for ImmunoTherapy of Cancer 2020) (53). Therapeutic monoclonal antibodies (mABs) targeting immune checkpoints recently have emerged as a game-changer in the cancer field. However, their potential protective role in HIV merits further investigations. We anticipate that manipulation of Gal-9 may reverse T cell exhaustion with therapeutic benefits in HIV and cancer patients. These examples indicate the significant impact of our discovery, which has implications beyond the HIV field.

5.2.2 The second study: Elevated ATP via enhanced miRNA-30b, 30c, and 30e downregulates the expression of CD73 in $CD8^+$ T cells of HIV-infected individuals

Our studies were based on RNAseq analysis, however, due to the logistic issues (e.g. the low frequency of Tregs) we were unable to have more than 5 sample/group. Therefore, our RNAseq data are concluded from a small sample size. For the same reason, we were unable to perform extensive functional assays using Tregs. In our studies, because of the low cell numbers from patients the functionality of Tregs towards HIV antigen specific CD8 T cells was not evaluated.

Future longitudinal studies examining the effect of ART on Tregs in HIV patients is warranted. Since Tregs are the major suppressor cells, manipulation of pathways associated with their higher activation will have therapeutic benefits in HIV-infected individuals in whom CD8 T cells function has been already compromised through CD8 T cells exhaustion. These therapeutic interventions can pave the way for manipulating such pathways in other chronic conditions.

5.2.3 The third study: Differential transcription and functional properties of regulatory T cells in HIV-infected individuals on antiretroviral therapy and Long-term non-progressors

One factor that should be considered in the interpretation of our results is the sample preparation. Due to the COVID-19 pandemic, our access to the HIV clinic for access to patients was terminated. Thus, for some experiments we had to thaw cryopreserved samples instead of freshly isolated cells, which might impacted the functional properties of the examined cells (54). Although previous reports have shown that CD73 has a role for the homing of CD8 T cells to different tissues, additional experiments using CD73 KO mice to unravel the role of CD73 in CD8 cell migration is required. We had access to a small sample size of CSF from MS patients. To further clarify the role of CD73 in MS pathogenesis, a larger sample size with access to the peripheral blood and CSF from patients will be highly valuable. Having access to CSF from healthy individuals is not feasible to obtain but will be highly informative for comparison.

Although we compared the level of ATP in the plasma of HIV-infected individuals to healthy controls, we did not have the right tools to measure the levels of ATP metabolites such as ADP and AMP in HIV-infected individuals. Finally, more studies to understand the mechanisms associated with CD73 down-regulation on CD8 T cells in HIV infection and possibly other chronic conditions and how it can be prevented are warranted. Such studies will enable us to develop therapeutic interventions to prevent CD73 downregulation on CD8 T cells in HIV infection and beyond.

5.3 Conclusions

In conclusion, within limitations of these cross-sectional study, the present studies highlighted 3 different mechanisms that might account for the persistence of HIV virus in the body. We showed that in ART patients, increased levels of Gal-9 on the surface of CD8 T cells results in their intrinsic inability to kill virally infected cells. Moreover, CD8 T cell function was further compromised externally by increased suppressive activity of Tregs observed in this group of patients. In contrast, Gal-9 was not up-regulated on CD8 T cells from LTNPs, resulting in maintenance of their function. In addition, CD8 T cells from LTNPs were less suppressed by Tregs. However, there was a universal downregulation of CD73 expressing CD8

T cells in different groups of HIV patients. Since CD73 has a role in trafficking of CD8 T cells to HIV reservoirs, lowered percentage of CD73 CD8 T cells might explain the persistent of HIV virion in LTNPs despite effective immune response against actively replicating virus. However, further studies are required to gain a deeper insight at the molecular and gene levels. In addition, long-term studies in larger cohorts will elucidate the effects of ART on the observed results. A summary of our findings is illustrated in Fig. 5.1.

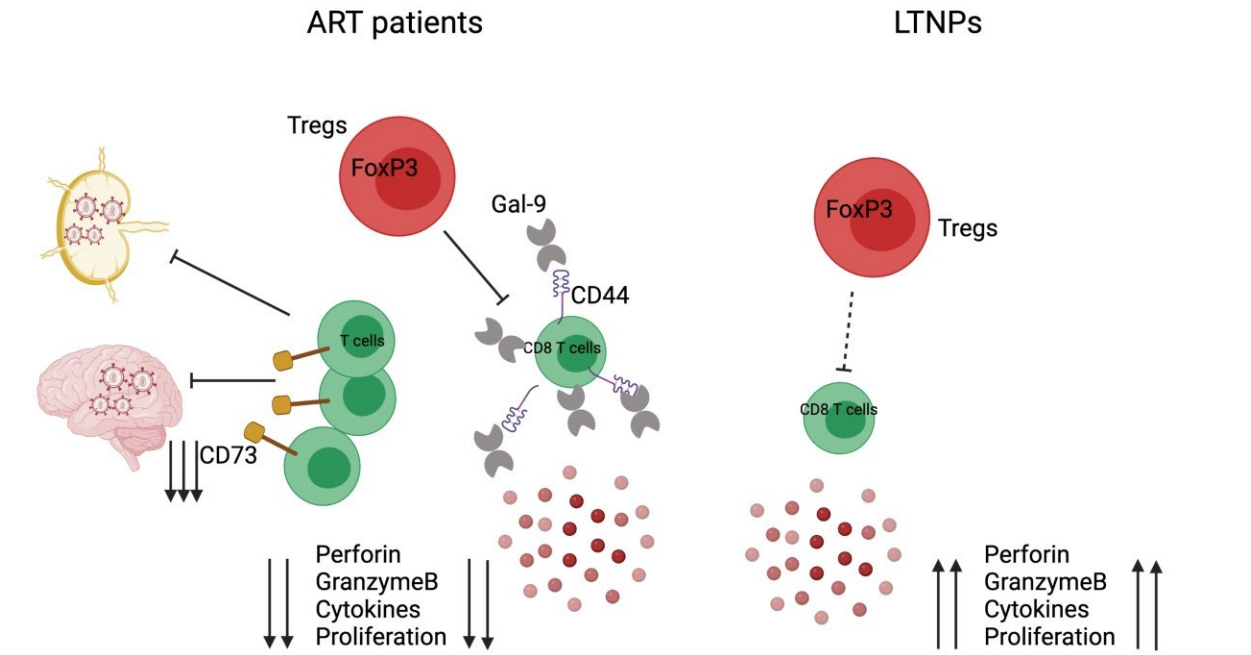


Fig 5.1. The summary figures showing the different mechanisms leading to the inability of the immune system to eradicate HIV-virus.

5.4 References

1. Balasubramaniam, M., J. Pandhare, and C. Dash. 2019. Immune Control of HIV. *J Life Sci (Westlake Village)* 1: 4–37.
2. Borrow, P., H. Lewicki, X. Wei, M. S. Horwitz, N. Peffer, H. Meyers, J. A. Nelson, J. E. Gairin, B. H. Hahn, M. B. Oldstone, and G. M. Shaw. 1997. Antiviral pressure exerted by HIV-1-specific cytotoxic T lymphocytes (CTLs) during primary infection demonstrated by rapid selection of CTL escape virus. *Nat Med* 3: 205–211.
3. Dustin, M. L., and E. O. Long. 2010. Cytotoxic immunological synapses. *Immunol Rev* 235: 24–34.
4. Halle, S., O. Halle, and R. Förster. 2017. Mechanisms and Dynamics of T Cell-Mediated Cytotoxicity In Vivo. *Trends Immunol* 38: 432–443.
5. Yang, O. O., S. A. Kalams, M. Rosenzweig, A. Trocha, N. Jones, M. Koziel, B. D. Walker, and R. P. Johnson. 1996. Efficient lysis of human immunodeficiency virus type 1-infected cells by cytotoxic T lymphocytes. *J Virol* 70: 5799–5806.
6. Schmitz, J. E., M. J. Kuroda, S. Santra, V. G. Sasseville, M. A. Simon, M. A. Lifton, P. Racz, K. Tenner-Racz, M. Dalesandro, B. J. Scallon, J. Ghrayeb, M. A. Forman, D. C. Montefiori, E. P. Rieber, N. L. Letvin, and K. A. Reimann. 1999. Control of viremia in simian immunodeficiency virus infection by CD8+ lymphocytes. *Science* 283: 857–860.
7. Allen, T. M., M. Altfeld, S. C. Geer, E. T. Kalife, C. Moore, K. M. O'sullivan, I. Desouza, M. E. Feeney, R. L. Eldridge, E. L. Maier, D. E. Kaufmann, M. P. Lahaie, L. Reyor, G. Tanzi, M. N. Johnston, C. Brander, R. Draenert, J. K. Rockstroh, H. Jessen, E. S. Rosenberg, S. A. Mallal, and B. D. Walker. 2005. Selective escape from CD8+ T-cell responses represents a major driving force of human immunodeficiency virus type 1 (HIV-1) sequence diversity and reveals constraints on HIV-1 evolution. *J Virol* 79: 13239–13249.
8. Zanussi, S., C. Simonelli, M. D'Andrea, C. Caffau, M. Clerici, U. Tirelli, and P. DePaoli. 1996. CD8+ lymphocyte phenotype and cytokine production in long-term non-progressor and in progressor patients with HIV-1 infection. *Clin Exp Immunol* 105: 220–224.
9. Migueles, S. A., and M. Connors. 2015. Success and failure of the cellular immune response against HIV-1. *Nat Immunol* 16: 563–570.
10. Moore, C. B., M. John, I. R. James, F. T. Christiansen, C. S. Witt, and S. A. Mallal. 2002. Evidence of HIV-1 adaptation to HLA-restricted immune responses at a population level. *Science* 296: 1439–1443.
11. Carlson, J. M., A. Q. Le, A. Shahid, and Z. L. Brumme. 2015. HIV-1 adaptation to HLA: a window into virus-host immune interactions. *Trends Microbiol* 23: 212–224.
12. Jones, R. B., and B. D. Walker. HIV-specific CD8+ T cells and HIV eradication. *J Clin Invest* 126: 455–463.
13. Wang, C., M. Singer, and A. C. Anderson. 2017. Molecular dissection of CD8+ T cell dysfunction. *Trends Immunol* 38: 567–576.
14. Fenwick, C., V. Joo, P. Jacquier, A. Noto, R. Banga, M. Perreau, and G. Pantaleo. 2019. T-cell exhaustion in HIV infection. *Immunol Rev* 292: 149–163.
15. Day, C. L., D. E. Kaufmann, P. Kiepiela, J. A. Brown, E. S. Moodley, S. Reddy, E. W. Mackey, J. D. Miller, A. J. Leslie, C. DePierres, Z. Mncube, J. Duraiswamy, B. Zhu, Q. Eichbaum, M. Altfeld, E. J. Wherry, H. M. Coovadia, P. J. R. Goulder, P. Klenerman, R. Ahmed, G. J. Freeman, and B. D. Walker. 2006. PD-1 expression on HIV-specific T cells is associated with T-cell exhaustion and disease progression. *Nature* 443: 350–354.
16. Peretz, Y., Z. He, Y. Shi, B. Yassine-Diab, J.-P. Goulet, R. Bordi, A. Filali-Mouhim, J.-B. Loubert, M. El-Far, F. P. Dupuy, M. R. Boulassel, C. Tremblay, J.-P. Routy, N. Bernard, R. Balderas, E. K. Haddad, and R.-P. Sékaly. 2012. CD160 and PD-1 co-expression on HIV-specific CD8 T cells defines a subset with advanced dysfunction. *PLoS Pathog* 8: e1002840.

17. Amancha, P. K., J. J. Hong, A. A. Ansari, and F. Villinger. 2015. Up-regulation of Tim-3 on T cells during acute simian immunodeficiency virus infection and on antigen specific responders. *AIDS* 29: 531–536.
18. Jones, R. B., L. C. Ndhlovu, J. D. Barbour, P. M. Sheth, A. R. Jha, B. R. Long, J. C. Wong, M. Satkunarajah, M. Schweneker, J. M. Chapman, G. Gyenes, B. Vali, M. D. Hyrcza, F. Y. Yue, C. Kovacs, A. Sassi, M. Loutfy, R. Halpenny, D. Persad, G. Spotts, F. M. Hecht, T.-W. Chun, J. M. McCune, R. Kaul, J. M. Rini, D. F. Nixon, and M. A. Ostrowski. 2008. Tim-3 expression defines a novel population of dysfunctional T cells with highly elevated frequencies in progressive HIV-1 infection. *J Exp Med* 205: 2763–2779.
19. Yamamoto, T., D. A. Price, J. P. Casazza, G. Ferrari, M. Nason, P. K. Chattopadhyay, M. Roederer, E. Gostick, P. D. Katsikis, D. C. Douek, R. Haubrich, C. Petrovas, and R. A. Koup. 2011. Surface expression patterns of negative regulatory molecules identify determinants of virus-specific CD8+ T-cell exhaustion in HIV infection. *Blood* 117: 4805–4815.
20. Merani, S., W. Chen, and S. Elahi. 2015. The bitter side of sweet: the role of Galectin-9 in immunopathogenesis of viral infections. *Rev Med Virol* 25: 175–186.
21. Chou, F.-C., S.-J. Shieh, and H.-K. Sytwu. 2009. Attenuation of Th1 response through galectin-9 and T-cell Ig mucin 3 interaction inhibits autoimmune diabetes in NOD mice. *European Journal of Immunology* 39: 2403–2411.
22. Zhu, C., A. C. Anderson, A. Schubart, H. Xiong, J. Imitola, S. J. Khoury, X. X. Zheng, T. B. Strom, and V. K. Kuchroo. 2005. The Tim-3 ligand galectin-9 negatively regulates T helper type 1 immunity. *Nat Immunol* 6: 1245–1252.
23. Seki, M., S. Oomizu, K.-M. Sakata, A. Sakata, T. Arikawa, K. Watanabe, K. Ito, K. Takeshita, T. Niki, N. Saita, N. Nishi, A. Yamauchi, S. Katoh, A. Matsukawa, V. Kuchroo, and M. Hirashima. 2008. Galectin-9 suppresses the generation of Th17, promotes the induction of regulatory T cells, and regulates experimental autoimmune arthritis. *Clin Immunol* 127: 78–88.
24. Wu, C., T. Thalhamer, R. F. Franca, S. Xiao, C. Wang, C. Hotta, C. Zhu, M. Hirashima, A. C. Anderson, and V. K. Kuchroo. 2014. Galectin-9-CD44 interaction enhances stability and function of adaptive regulatory T cells. *Immunity* 41: 270–282.
25. Elahi, S., W. L. Dinges, N. Lejarcegui, K. J. Laing, A. C. Collier, D. M. Koelle, M. J. McElrath, and H. Horton. 2011. Protective HIV-specific CD8+ T cells evade Treg cell suppression. *Nat Med* 17: 989–995.
26. Scharf, L., J. Tauriainen, M. Buggert, W. Hartogensis, D. J. Nolan, S. G. Deeks, M. Salemi, F. M. Hecht, and A. C. Karlsson. 2020. Delayed Expression of PD-1 and TIGIT on HIV-Specific CD8 T Cells in Untreated HLA-B*57:01 Individuals Followed from Early Infection. *Journal of Virology* 94.
27. Pombo, C., E. J. Wherry, E. Gostick, D. A. Price, and M. R. Betts. 2015. Elevated Expression of CD160 and 2B4 Defines a Cytolytic HIV-Specific CD8+ T-Cell Population in Elite Controllers. *J Infect Dis* 212: 1376–1386.
28. Eisele, E., and R. F. Siliciano. 2012. Redefining the viral reservoirs that prevent HIV-1 eradication. *Immunity* 37: 377–388.
29. Blankson, J. N., D. Persaud, and R. F. Siliciano. 2002. The challenge of viral reservoirs in HIV-1 infection. *Annu Rev Med* 53: 557–593.
30. Barton, K., A. Winckelmann, and S. Palmer. 2016. HIV-1 Reservoirs During Suppressive Therapy. *Trends Microbiol* 24: 345–355.
31. Kulpa, D. A., and N. Chomont. HIV persistence in the setting of antiretroviral therapy: when, where and how does HIV hide? *J Virus Erad* 1: 59–68.
32. Wong, J. K., and S. A. Yukl. 2016. Tissue reservoirs of HIV. *Curr Opin HIV AIDS* 11: 362–370.
33. Folkvord, J. M., C. Armon, and E. Connick. 2005. Lymphoid follicles are sites of heightened human immunodeficiency virus type 1 (HIV-1) replication and reduced antiretroviral effector mechanisms. *AIDS Res Hum Retroviruses* 21: 363–370.

34. Connick, E., T. Mattila, J. M. Folkvord, R. Schlichtemeier, A. L. Meditz, M. G. Ray, M. D. McCarter, S. Mawhinney, A. Hage, C. White, and P. J. Skinner. 2007. CTL fail to accumulate at sites of HIV-1 replication in lymphoid tissue. *J Immunol* 178: 6975–6983.
35. Fukazawa, Y., R. Lum, A. A. Okoye, H. Park, K. Matsuda, J. Y. Bae, S. I. Hagen, R. Shoemaker, C. Deleage, C. Lucero, D. Morcock, T. Swanson, A. W. Legasse, M. K. Axthelm, J. Hesselgesser, R. Geleziunas, V. M. Hirsch, P. T. Edlefsen, M. Piatak, J. D. Estes, J. D. Lifson, and L. J. Picker. 2015. B cell follicle sanctuary permits persistent productive simian immunodeficiency virus infection in elite controllers. *Nat Med* 21: 132–139.
36. Gray, L. R., M. Roche, J. K. Flynn, S. L. Wesselingh, P. R. Gorry, and M. J. Churchill. 2014. Is the central nervous system a reservoir of HIV-1? *Curr Opin HIV AIDS* 9: 552–558.
37. Galvin, S. R., and M. S. Cohen. 2006. Genital tract reservoirs. *Curr Opin HIV AIDS* 1: 162–166.
38. Park, C. O., and T. S. Kupper. 2015. The emerging role of resident memory T cells in protective immunity and inflammatory disease. *Nat Med* 21: 688–697.
39. Tóth, I., A. Q. Le, P. Hartjen, A. Thomssen, V. Matzat, C. Lehmann, C. Scheurich, C. Beisel, P. Busch, O. Degen, A. W. Lohse, T. Eiermann, G. Fätkenheuer, D. Meyer-Olson, M. Bockhorn, J. Hauber, J. van Lunzen, and J. Schulze Zur Wiesch. 2013. Decreased frequency of CD73+CD8+ T cells of HIV-infected patients correlates with immune activation and T cell exhaustion. *Journal of Leukocyte Biology* 94: 551–561.
40. Mills, J. H., L. F. Thompson, C. Mueller, A. T. Waickman, S. Jalkanen, J. Niemela, L. Airas, and M. S. Bynoe. 2008. CD73 is required for efficient entry of lymphocytes into the central nervous system during experimental autoimmune encephalomyelitis. *PNAS* 105: 9325–9330.
41. Ålgars, A., M. Karikoski, G. G. Yegutkin, P. Stoitzner, J. Niemelä, M. Salmi, and S. Jalkanen. 2011. Different role of CD73 in leukocyte trafficking via blood and lymph vessels. *Blood* 117: 4387–4393.
42. Aharoni Golan, M., R. Weisshof, Y. Wang, and D. T. Rubin. 2019. New Diagnosis of Acquired Immunodeficiency Syndrome in a Patient With Crohn's Disease. *ACG Case Rep J* 6: e00056.
43. Viazis, N., J. Vlachogiannakos, O. Georgiou, M. Rodias, D. Georgiadis, V. Papastamopoulos, I. G. Baraboutis, D. G. Karamanolis, and A. Skoutelis. 2010. Course of inflammatory bowel disease in patients infected with human immunodeficiency virus. *Inflamm Bowel Dis* 16: 507–511.
44. Bongiovanni, M., R. Ranieri, S. Ferrero, F. Casanova, and A. d'Arminio Monforte. 2006. Crohn's disease onset in an HIV/hepatitis C virus co-infected woman taking pegylated interferon alpha-2b plus ribavirin. *AIDS* 20: 1989–1990.
45. Bernstein, C. N., and W. J. Snape. 1991. Active idiopathic ulcerative colitis in a patient with ongoing HIV-related immunodepression. *Am J Gastroenterol* 86: 907–909.
46. Liebowitz, D., and D. McShane. 1986. Nonspecific chronic inflammatory bowel disease and AIDS. *J Clin Gastroenterol* 8: 66–68.
47. Stefanou, M.-I., M. Krumbholz, U. Ziemann, and M. C. Kowarik. 2019. Human immunodeficiency virus and multiple sclerosis: a review of the literature. *Neurological Research and Practice* 1: 24.
48. Velasquez, S., L. Prevedel, S. Valdebenito, A. M. Gorska, M. Golovko, N. Khan, J. Geiger, and E. A. Eugenin. 2020. Circulating levels of ATP is a biomarker of HIV cognitive impairment. *EBioMedicine* 51.
49. Graziano, F., M. Desdouits, L. Garzetti, P. Podini, M. Alfano, A. Rubartelli, R. Furlan, P. Benaroch, and G. Poli. 2015. Extracellular ATP induces the rapid release of HIV-1 from virus containing compartments of human macrophages. *PNAS* 112: E3265–E3273.
50. Stachon, P., S. Geis, A. Peikert, A. Heidenreich, N. A. Michel, F. Ünal, N. Hoppe, B. Dufner, L. Schulte, T. Marchini, S. Cicko, K. Ayata, A. Zech, D. Wolf, I. Hilgendorf, F. Willecke, J. Reinöhl, C. von Zur Mühlen, C. Bode, M. Idzko, and A. Zirlik. 2016. Extracellular ATP Induces Vascular Inflammation and Atherosclerosis via Purinergic Receptor Y2 in Mice. *Arterioscler Thromb Vasc Biol* 36: 1577–1586.

51. Shahbaz, S., M. Manicardi, G. Guaraldi, and P. Raggi. 2015. Cardiovascular disease in human immunodeficiency virus infected patients: A true or perceived risk? *World Journal of Cardiology* 7: 633–644.
52. Elahi, S., W. L. Dinges, N. Lejarcegui, K. J. Laing, A. C. Collier, D. M. Koelle, M. J. McElrath, and H. Horton. 2011. Protective HIV-specific CD8+ T cells evade Treg cell suppression. *Nat Med* 17: 989–995.
53. Okoye, I., L. Xu, M. Motamedi, P. Parashar, J. W. Walker, and S. Elahi. 2020. Galectin-9 expression defines exhausted T cells and impaired cytotoxic NK cells in patients with virus-associated solid tumors. *J Immunother Cancer* 8: e001849.
54. Oomizu, S., T. Arikawa, T. Niki, T. Kadowaki, M. Ueno, N. Nishi, A. Yamauchi, T. Hattori, T. Masaki, and M. Hirashima. 2012. Cell surface galectin-9 expressing Th cells regulate Th17 and Foxp3+ Treg development by galectin-9 secretion. *PLoS One* 7: e48574.

Bibliography

Chapter 1: Introduction

1. WHO | The Global Health Observatory. HIV/AIDS.
2. Canada, P. H. A. of. 2018. Estimates of HIV incidence, prevalence and Canada's progress on meeting the 90-90-90 HIV targets. *aem* .
3. Chen, Q., D. Zeng, Y. She, Y. Lyu, X. Gong, M. J. Feinstein, Y. Yang, and H. Jiang. 2019. Different transmission routes and the risk of advanced HIV disease: A systematic review and network meta-analysis of observational studies. *EClinicalMedicine* 16: 121–128.
4. Sharp, P. M., and B. H. Hahn. 2011. Origins of HIV and the AIDS Pandemic. *Cold Spring Harb Perspect Med* 1: a006841.
5. 2015. History of HIV and AIDS overview. *Avert* .
6. Hageman, J. R. 2018. A Historical Perspective of HIV Infection. *Pediatr Ann* 47: e226.
7. Simon, V., D. D. Ho, and Q. A. Karim. 2006. HIV/AIDS epidemiology, pathogenesis, prevention, and treatment. *Lancet* 368: 489–504.
8. Durand, S., and A. Cimarelli. 2011. The Inside Out of Lentiviral Vectors. *Viruses* 3: 132–159.
9. Blut), G. A. C. B. (Arbeitskreis, and S. 'Assessment of P. T. by Blood'. 2016. Human Immunodeficiency Virus (HIV). *TMH* 43: 203–222.
10. Mushahwar, I. K. 2006. Human Immunodeficiency Viruses: Molecular Virology, Pathogenesis, Diagnosis and Treatment. In *Perspectives in Medical Virology*. Congenital and Other Related Infectious Diseases of the Newborn vol. 13. I. K. Mushahwar, ed. Elsevier. 75–87.
11. Deeks, S. G., J. Overbaugh, A. Phillips, and S. Buchbinder. 2015. HIV infection. *Nature Reviews Disease Primers* 1: 1–22.
12. Tsunetsugu-Yokota, Y., and M. Muhsen. 2013. Development of human dendritic cells and their role in HIV infection: antiviral immunity versus HIV transmission. *Front Microbiol* 4: 178.
13. Vicenzi, E., P. Liò, and G. Poli. 2013. The puzzling role of CXCR4 in human immunodeficiency virus infection. *Theranostics* 3: 18–25.
14. Steinman, R. M., A. Granelli-Piperno, M. Pope, C. Trumpfheller, R. Ignatius, G. Arrode, P. Racz, and K. Tenner-Racz. 2003. The interaction of immunodeficiency viruses with dendritic cells. *Curr Top Microbiol Immunol* 276: 1–30.
15. Koppensteiner, H., R. Brack-Werner, and M. Schindler. 2012. Macrophages and their relevance in Human Immunodeficiency Virus Type I infection. *Retrovirology* 9: 82.
16. Piatak, M., M. S. Saag, L. C. Yang, S. J. Clark, J. C. Kappes, K. C. Luk, B. H. Hahn, G. M. Shaw, and J. D. Lifson. 1993. High levels of HIV-1 in plasma during all stages of infection determined by competitive PCR. *Science* 259: 1749–1754.
17. Streeck, H., R. Lu, N. Beckwith, M. Milazzo, M. Liu, J.-P. Routy, S. Little, H. Jessen, A. D. Kelleher, F. Hecht, R.-P. Sekaly, G. Alter, D. Heckerman, M. Carrington, E. S. Rosenberg, and M. Altfeld. 2014. Emergence of individual HIV-specific CD8 T cell responses during primary HIV-1 infection can determine long-term disease outcome. *J Virol* 88: 12793–12801.
18. Luckheeram, R. V., R. Zhou, A. D. Verma, and B. Xia. 2012. $CD4^+$T cells: differentiation and functions. *Clin Dev Immunol* 2012: 925135.
19. Sant, A. J., and A. McMichael. 2012. Revealing the role of CD4+ T cells in viral immunity. *J Exp Med* 209: 1391–1395.

20. Zhu, J., and W. E. Paul. 2008. CD4 T cells: fates, functions, and faults. *Blood* 112: 1557–1569.
21. Henn, A., C. Flateau, and S. Gallien. 2017. Primary HIV Infection: Clinical Presentation, Testing, and Treatment. *Curr Infect Dis Rep* 19: 37.
22. Chu, C., and P. A. Selwyn. 2010. Diagnosis and initial management of acute HIV infection. *Am Fam Physician* 81: 1239–1244.
23. Vajpayee, M., and T. Mohan. 2011. Current practices in laboratory monitoring of HIV infection. *Indian J Med Res* 134: 801–822.
24. WHO | What's new in treatment monitoring: viral load and CD4 testing. *WHO* .
25. Vella, S., B. Schwartländer, S. P. Sow, S. P. Eholie, and R. L. Murphy. 2012. The history of antiretroviral therapy and of its implementation in resource-limited areas of the world. *AIDS* 26: 1231–1241.
26. Furman, P. A., J. A. Fyfe, M. H. St Clair, K. Weinhold, J. L. Rideout, G. A. Freeman, S. N. Lehrman, D. P. Bolognesi, S. Broder, and H. Mitsuya. 1986. Phosphorylation of 3'-azido-3'-deoxythymidine and selective interaction of the 5'-triphosphate with human immunodeficiency virus reverse transcriptase. *Proc Natl Acad Sci U S A* 83: 8333–8337.
27. St Clair, M. H., C. A. Richards, T. Spector, K. J. Weinhold, W. H. Miller, A. J. Langlois, and P. A. Furman. 1987. 3'-Azido-3'-deoxythymidine triphosphate as an inhibitor and substrate of purified human immunodeficiency virus reverse transcriptase. *Antimicrob Agents Chemother* 31: 1972–1977.
28. Connor, E. M., R. S. Sperling, R. Gelber, P. Kiselev, G. Scott, M. J. O'Sullivan, R. VanDyke, M. Bey, W. Shearer, and R. L. Jacobson. 1994. Reduction of maternal-infant transmission of human immunodeficiency virus type 1 with zidovudine treatment. Pediatric AIDS Clinical Trials Group Protocol 076 Study Group. *N Engl J Med* 331: 1173–1180.
29. Skowron, G., S. A. Bozzette, L. Lim, C. B. Pettinelli, H. H. Schaumburg, J. Arezzo, M. A. Fischl, W. G. Powderly, D. J. Gocke, D. D. Richman, J. C. Pottage, D. Antoniskis, G. F. McKinley, N. E. Hyslop, G. Ray, G. Simon, N. Reed, M. L. LoFaro, R. B. Uttamchandani, L. D. Gelb, S. J. Sperber, R. L. Murphy, J. M. Leedom, M. H. Grieco, J. Zachary, M. S. Hirsch, S. A. Spector, J. Bigley, W. Soo, and T. C. Merigan. 1993. Alternating and intermittent regimens of zidovudine and dideoxycytidine in patients with AIDS or AIDS-related complex. *Ann Intern Med* 118: 321–330.
30. 1996. Delta: a randomised double-blind controlled trial comparing combinations of zidovudine plus didanosine or zalcitabine with zidovudine alone in HIV-infected individuals. Delta Coordinating Committee. *Lancet* 348: 283–291.
31. Hammer, S. M., K. E. Squires, M. D. Hughes, J. M. Grimes, L. M. Demeter, J. S. Currier, J. J. Eron, J. E. Feinberg, H. H. Balfour, L. R. Deyton, J. A. Chodakewitz, and M. A. Fischl. 1997. A controlled trial of two nucleoside analogues plus indinavir in persons with human immunodeficiency virus infection and CD4 cell counts of 200 per cubic millimeter or less. AIDS Clinical Trials Group 320 Study Team. *N Engl J Med* 337: 725–733.
32. Carpenter, C. C., M. A. Fischl, S. M. Hammer, M. S. Hirsch, D. M. Jacobsen, D. A. Katzenstein, J. S. Montaner, D. D. Richman, M. S. Sáag, R. T. Schooley, M. A. Thompson, S. Vella, P. G. Yeni, and P. A. Volberding. 1996. Antiretroviral therapy for HIV infection in 1996. Recommendations of an international panel. International AIDS Society-USA. *JAMA* 276: 146–154.
33. Palella, F. J., K. M. Delaney, A. C. Moorman, M. O. Loveless, J. Fuhrer, G. A. Satten, D. J. Aschman, and S. D. Holmberg. 1998. Declining morbidity and mortality among patients with advanced human immunodeficiency virus infection. HIV Outpatient Study Investigators. *N Engl J Med* 338: 853–860.
34. Selik, R. M., S. Y. Chu, and J. W. Buehler. 1993. HIV infection as leading cause of death among young adults in US cities and states. *JAMA* 269: 2991–2994.
35. Arribas, J. R., A. L. Pozniak, J. E. Gallant, E. Dejesus, B. Gazzard, R. E. Campo, S.-S. Chen, D. McColl, C. B. Holmes, J. Enejosa, J. J. Toole, and A. K. Cheng. 2008. Tenofovir disoproxil fumarate, emtricitabine,

and efavirenz compared with zidovudine/lamivudine and efavirenz in treatment-naive patients: 144-week analysis. *J Acquir Immune Defic Syndr* 47: 74–78.
36. Molina, J.-M., J. Andrade-Villanueva, J. Echevarria, P. Chetchotisakd, J. Corral, N. David, G. Moyle, M. Mancini, L. Percival, R. Yang, A. Thiry, D. McGrath, and CASTLE Study Team. 2008. Once-daily atazanavir/ritonavir versus twice-daily lopinavir/ritonavir, each in combination with tenofovir and emtricitabine, for management of antiretroviral-naive HIV-1-infected patients: 48 week efficacy and safety results of the CASTLE study. *Lancet* 372: 646–655.
37. Sax, P. E., C. Tierney, A. C. Collier, M. A. Fischl, K. Mollan, L. Peeples, C. Godfrey, N. C. Jahed, L. Myers, D. Katzenstein, A. Farajallah, J. F. Rooney, B. Ha, W. C. Woodward, S. L. Koletar, V. A. Johnson, P. J. Geiseler, E. S. Daar, and AIDS Clinical Trials Group Study A5202 Team. 2009. Abacavir-lamivudine versus tenofovir-emtricitabine for initial HIV-1 therapy. *N Engl J Med* 361: 2230–2240.
38. Kitahata, M. M., S. J. Gange, A. G. Abraham, B. Merriman, M. S. Saag, A. C. Justice, R. S. Hogg, S. G. Deeks, J. J. Eron, J. T. Brooks, S. B. Rourke, M. J. Gill, R. J. Bosch, J. N. Martin, M. B. Klein, L. P. Jacobson, B. Rodriguez, T. R. Sterling, G. D. Kirk, S. Napravnik, A. R. Rachlis, L. M. Calzavara, M. A. Horberg, M. J. Silverberg, K. A. Gebo, J. J. Goedert, C. A. Benson, A. C. Collier, S. E. Van Rompaey, H. M. Crane, R. G. McKaig, B. Lau, A. M. Freeman, R. D. Moore, and NA-ACCORD Investigators. 2009. Effect of early versus deferred antiretroviral therapy for HIV on survival. *N Engl J Med* 360: 1815–1826.
39. Lima, V. D., R. Harrigan, D. R. Bangsberg, R. S. Hogg, R. Gross, B. Yip, and J. S. G. Montaner. 2009. The combined effect of modern highly active antiretroviral therapy regimens and adherence on mortality over time. *J Acquir Immune Defic Syndr* 50: 529–536.
40. Cohen, M. S., Y. Q. Chen, M. McCauley, T. Gamble, M. C. Hosseinipour, N. Kumarasamy, J. G. Hakim, J. Kumwenda, B. Grinsztejn, J. H. S. Pilotto, S. V. Godbole, S. Mehendale, S. Chariyalertsak, B. R. Santos, K. H. Mayer, I. F. Hoffman, S. H. Eshleman, E. Piwowar-Manning, L. Wang, J. Makhema, L. A. Mills, G. de Bruyn, I. Sanne, J. Eron, J. Gallant, D. Havlir, S. Swindells, H. Ribaudo, V. Elharrar, D. Burns, T. E. Taha, K. Nielsen-Saines, D. Celentano, M. Essex, T. R. Fleming, and HPTN 052 Study Team. 2011. Prevention of HIV-1 infection with early antiretroviral therapy. *N Engl J Med* 365: 493–505.
41. Fowler, M. G., M. Qin, S. A. Fiscus, J. S. Currier, P. M. Flynn, T. Chipato, J. McIntyre, D. Gnanashanmugam, G. K. Siberry, A. S. Coletti, T. E. Taha, K. L. Klingman, F. E. Martinson, M. Owor, A. Violari, D. Moodley, G. B. Theron, R. Bhosale, R. Bobat, B. H. Chi, R. Strehlau, P. Mlay, A. J. Loftis, R. Browning, T. Fenton, L. Purdue, M. Basar, D. E. Shapiro, L. M. Mofenson, and IMPAACT 1077BF/1077FF PROMISE Study Team. 2016. Benefits and Risks of Antiretroviral Therapy for Perinatal HIV Prevention. *N Engl J Med* 375: 1726–1737.
42. Kasamba, I., K. Baisley, B. N. Mayanja, D. Maher, and H. Grosskurth. 2012. The impact of antiretroviral treatment on mortality trends of HIV-positive adults in rural Uganda: a longitudinal population-based study, 1999–2009. *Trop Med Int Health* 17: e66–e73.
43. Sellers, C. J., and D. A. Wohl. 2014. Antiretroviral Therapy: When to Start. *Infect Dis Clin North Am* 28: 403–420.
44. INSIGHT START Study Group, J. D. Lundgren, A. G. Babiker, F. Gordin, S. Emery, B. Grund, S. Sharma, A. Avihingsanon, D. A. Cooper, G. Fätkenheuer, J. M. Llibre, J.-M. Molina, P. Munderi, M. Schechter, R. Wood, K. L. Klingman, S. Collins, H. C. Lane, A. N. Phillips, and J. D. Neaton. 2015. Initiation of Antiretroviral Therapy in Early Asymptomatic HIV Infection. *N Engl J Med* 373: 795–807.
45. Becker, M., J. Cox, G. Evans, S. Haider, and S. Shafran. 2016. AMMI Canada position statement: The use of early antiretroviral therapy in HIV-infected persons. *Official Journal of the Association of Medical Microbiology and Infectious Disease Canada* 1: 1–11.
46. Mogensen, T. H., J. Melchjorsen, C. S. Larsen, and S. R. Paludan. 2010. Innate immune recognition and activation during HIV infection. *Retrovirology* 7: 54.

47. Binley, J. M., P. J. Klasse, Y. Cao, I. Jones, M. Markowitz, D. D. Ho, and J. P. Moore. 1997. Differential regulation of the antibody responses to Gag and Env proteins of human immunodeficiency virus type 1. *J Virol* 71: 2799–2809.
48. Richman, D. D., T. Wrin, S. J. Little, and C. J. Petropoulos. 2003. Rapid evolution of the neutralizing antibody response to HIV type 1 infection. *Proc Natl Acad Sci U S A* 100: 4144–4149.
49. Parren, P. W., J. P. Moore, D. R. Burton, and Q. J. Sattentau. 1999. The neutralizing antibody response to HIV-1: viral evasion and escape from humoral immunity. *AIDS* 13 Suppl A: S137-162.
50. Hangartner, L., R. M. Zellweger, M. Giobbi, J. Weber, B. Eschli, K. D. McCoy, N. Harris, M. Recher, R. M. Zinkernagel, and H. Hengartner. 2006. Nonneutralizing antibodies binding to the surface glycoprotein of lymphocytic choriomeningitis virus reduce early virus spread. *J Exp Med* 203: 2033–2042.
51. Huber, M., and A. Trkola. 2007. Humoral immunity to HIV-1: neutralization and beyond. *J Intern Med* 262: 5–25.
52. Rosenberg, E. S., M. Altfeld, S. H. Poon, M. N. Phillips, B. M. Wilkes, R. L. Eldridge, G. K. Robbins, R. T. D'Aquila, P. J. Goulder, and B. D. Walker. 2000. Immune control of HIV-1 after early treatment of acute infection. *Nature* 407: 523–526.
53. Walker, B., and A. McMichael. 2012. The T-cell response to HIV. *Cold Spring Harb Perspect Med* 2.
54. Zhang, N., and M. J. Bevan. 2011. CD8(+) T cells: foot soldiers of the immune system. *Immunity* 35: 161–168.
55. Krzewski, K., and J. E. Coligan. 2012. Human NK cell lytic granules and regulation of their exocytosis. *Front Immunol* 3: 335.
56. Aguiló, J. I., A. Anel, E. Catalán, A. Sebastián, R. Acín-Pérez, J. Naval, R. Wallich, M. M. Simon, and J. Pardo. 2010. Granzyme B of cytotoxic T cells induces extramitochondrial reactive oxygen species production via caspase-dependent NADPH oxidase activation. *Immunol Cell Biol* 88: 545–554.
57. Darmon, A. J., T. J. Ley, D. W. Nicholson, and R. C. Bleackley. 1996. Cleavage of CPP32 by granzyme B represents a critical role for granzyme B in the induction of target cell DNA fragmentation. *J Biol Chem* 271: 21709–21712.
58. Atkinson, E. A., M. Barry, A. J. Darmon, I. Shostak, P. C. Turner, R. W. Moyer, and R. C. Bleackley. 1998. Cytotoxic T lymphocyte-assisted suicide. Caspase 3 activation is primarily the result of the direct action of granzyme B. *J Biol Chem* 273: 21261–21266.
59. Lord, S. J., R. V. Rajotte, G. S. Korbutt, and R. C. Bleackley. 2003. Granzyme B: a natural born killer. *Immunol Rev* 193: 31–38.
60. Nicholson, D. W., A. Ali, N. A. Thornberry, J. P. Vaillancourt, C. K. Ding, M. Gallant, Y. Gareau, P. R. Griffin, M. Labelle, and Y. A. Lazebnik. 1995. Identification and inhibition of the ICE/CED-3 protease necessary for mammalian apoptosis. *Nature* 376: 37–43.
61. Kothakota, S., T. Azuma, C. Reinhard, A. Klippel, J. Tang, K. Chu, T. J. McGarry, M. W. Kirschner, K. Koths, D. J. Kwiatkowski, and L. T. Williams. 1997. Caspase-3-generated fragment of gelsolin: effector of morphological change in apoptosis. *Science* 278: 294–298.
62. Enari, M., H. Sakahira, H. Yokoyama, K. Okawa, A. Iwamatsu, and S. Nagata. 1998. A caspase-activated DNase that degrades DNA during apoptosis, and its inhibitor ICAD. *Nature* 391: 43–50.
63. Barry, M., J. A. Heibein, M. J. Pinkoski, S. F. Lee, R. W. Moyer, D. R. Green, and R. C. Bleackley. 2000. Granzyme B short-circuits the need for caspase 8 activity during granule-mediated cytotoxic T-lymphocyte killing by directly cleaving Bid. *Mol Cell Biol* 20: 3781–3794.
64. Li, P., D. Nijhawan, I. Budihardjo, S. M. Srinivasula, M. Ahmad, E. S. Alnemri, and X. Wang. 1997. Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. *Cell* 91: 479–489.
65. Bleackley, R. C., and J. A. Heibein. 2001. Enzymatic control of apoptosis. *Nat Prod Rep* 18: 431–440.
66. Voskoboinik, I., J. C. Whisstock, and J. A. Trapani. 2015. Perforin and granzymes: function, dysfunction and human pathology. *Nat Rev Immunol* 15: 388–400.

67. Tschopp, J., D. Masson, and K. K. Stanley. 1986. Structural/functional similarity between proteins involved in complement- and cytotoxic T-lymphocyte-mediated cytolysis. *Nature* 322: 831–834.
68. Podack, E. R., J. D. Young, and Z. A. Cohn. 1985. Isolation and biochemical and functional characterization of perforin 1 from cytolytic T-cell granules. *Proc Natl Acad Sci U S A* 82: 8629–8633.
69. Thiery, J., D. Keefe, S. Boulant, E. Boucrot, M. Walch, D. Martinvalet, I. S. Goping, R. C. Bleackley, T. Kirchhausen, and J. Lieberman. 2011. Perforin pores in the endosomal membrane trigger the release of endocytosed granzyme B into the cytosol of target cells. *Nature Immunology* 12: 770–777.
70. Gordy, C., and Y.-W. He. 2012. Endocytosis by target cells: an essential means for perforin- and granzyme-mediated killing. *Cellular & Molecular Immunology* 9: 5–6.
71. Metkar, S. S., B. Wang, M. Aguilar-Santelises, S. M. Raja, L. Uhlin-Hansen, E. Podack, J. A. Trapani, and C. J. Froelich. 2002. Cytotoxic cell granule-mediated apoptosis: perforin delivers granzyme B-serglycin complexes into target cells without plasma membrane pore formation. *Immunity* 16: 417–428.
72. Keefe, D., L. Shi, S. Feske, R. Massol, F. Navarro, T. Kirchhausen, and J. Lieberman. 2005. Perforin triggers a plasma membrane-repair response that facilitates CTL induction of apoptosis. *Immunity* 23: 249–262.
73. Shi, L., D. Keefe, E. Durand, H. Feng, D. Zhang, and J. Lieberman. 2005. Granzyme B binds to target cells mostly by charge and must be added at the same time as perforin to trigger apoptosis. *J Immunol* 174: 5456–5461.
74. Wherry, E. J., and M. Kurachi. 2015. Molecular and cellular insights into T cell exhaustion. *Nat Rev Immunol* 15: 486–499.
75. Okoye, I. S., M. Houghton, L. Tyrrell, K. Barakat, and S. Elahi. 2017. Coinhibitory Receptor Expression and Immune Checkpoint Blockade: Maintaining a Balance in CD8+ T Cell Responses to Chronic Viral Infections and Cancer. *Front Immunol* 8: 1215.
76. Day, C. L., D. E. Kaufmann, P. Kiepiela, J. A. Brown, E. S. Moodley, S. Reddy, E. W. Mackey, J. D. Miller, A. J. Leslie, C. DePierres, Z. Mncube, J. Duraiswamy, B. Zhu, Q. Eichbaum, M. Altfeld, E. J. Wherry, H. M. Coovadia, P. J. R. Goulder, P. Klenerman, R. Ahmed, G. J. Freeman, and B. D. Walker. 2006. PD-1 expression on HIV-specific T cells is associated with T-cell exhaustion and disease progression. *Nature* 443: 350–354.
77. Quigley, M., F. Pereyra, B. Nilsson, F. Porichis, C. Fonseca, Q. Eichbaum, B. Julg, J. L. Jesneck, K. Brosnahan, S. Imam, K. Russell, I. Toth, A. Piechocka-Trocha, D. Dolfi, J. Angelosanto, A. Crawford, H. Shin, D. S. Kwon, J. Zupkosky, L. Francisco, G. J. Freeman, E. J. Wherry, D. E. Kaufmann, B. D. Walker, B. Ebert, and W. N. Haining. 2010. Transcriptional analysis of HIV-specific CD8+ T cells shows that PD-1 inhibits T cell function by upregulating BATF. *Nat Med* 16: 1147–1151.
78. Hui, E., J. Cheung, J. Zhu, X. Su, M. J. Taylor, H. A. Wallweber, D. K. Sasmal, J. Huang, J. M. Kim, I. Mellman, and R. D. Vale. 2017. T cell costimulatory receptor CD28 is a primary target for PD-1-mediated inhibition. *Science* 355: 1428–1433.
79. Fontenot, J. D., J. P. Rasmussen, L. M. Williams, J. L. Dooley, A. G. Farr, and A. Y. Rudensky. 2005. Regulatory T cell lineage specification by the forkhead transcription factor foxp3. *Immunity* 22: 329–341.
80. Staron, M. M., S. M. Gray, H. D. Marshall, I. A. Parish, J. H. Chen, C. J. Perry, G. Cui, M. O. Li, and S. M. Kaech. 2014. The transcription factor FoxO1 sustains expression of the inhibitory receptor PD-1 and survival of antiviral CD8(+) T cells during chronic infection. *Immunity* 41: 802–814.
81. Chew, G. M., T. Fujita, G. M. Webb, B. J. Burwitz, H. L. Wu, J. S. Reed, K. B. Hammond, K. L. Clayton, N. Ishii, M. Abdel-Mohsen, T. Liegler, B. I. Mitchell, F. M. Hecht, M. Ostrowski, C. M. Shikuma, S. G. Hansen, M. Maurer, A. J. Korman, S. G. Deeks, J. B. Sacha, and L. C. Ndhlovu. 2016. TIGIT Marks Exhausted T Cells, Correlates with Disease Progression, and Serves as a Target for Immune Restoration in HIV and SIV Infection. *PLoS Pathog* 12: e1005349.
82. Jones, R. B., L. C. Ndhlovu, J. D. Barbour, P. M. Sheth, A. R. Jha, B. R. Long, J. C. Wong, M. Satkunarajah, M. Schweneker, J. M. Chapman, G. Gyenes, B. Vali, M. D. Hyrcza, F. Y. Yue, C. Kovacs, A.

Sassi, M. Loutfy, R. Halpenny, D. Persad, G. Spotts, F. M. Hecht, T.-W. Chun, J. M. McCune, R. Kaul, J. M. Rini, D. F. Nixon, and M. A. Ostrowski. 2008. Tim-3 expression defines a novel population of dysfunctional T cells with highly elevated frequencies in progressive HIV-1 infection. *J Exp Med* 205: 2763–2779.
83. Zhu, C., A. C. Anderson, A. Schubart, H. Xiong, J. Imitola, S. J. Khoury, X. X. Zheng, T. B. Strom, and V. K. Kuchroo. 2005. The Tim-3 ligand galectin-9 negatively regulates T helper type 1 immunity. *Nat Immunol* 6: 1245–1252.
84. Rangachari, M., C. Zhu, K. Sakuishi, S. Xiao, J. Karman, A. Chen, M. Angin, A. Wakeham, E. A. Greenfield, R. A. Sobel, H. Okada, P. J. McKinnon, T. W. Mak, M. M. Addo, A. C. Anderson, and V. K. Kuchroo. 2012. Bat3 promotes T cell responses and autoimmunity by repressing Tim-3–mediated cell death and exhaustion. *Nat Med* 18: 1394–1400.
85. Zeidan, A. M., R. S. Komrokji, and A. M. Brunner. 2021. TIM-3 pathway dysregulation and targeting in cancer. *Expert Rev Anticancer Ther* 1–12.
86. Kumar, P. 2013. Long term non-progressor (LTNP) HIV infection. *Indian J Med Res* 138: 291–293.
87. Elahi, S., W. L. Dinges, N. Lejarcegui, K. J. Laing, A. C. Collier, D. M. Koelle, M. J. McElrath, and H. Horton. 2011. Protective HIV-specific CD8+ T cells evade Treg cell suppression. *Nat Med* 17: 989–995.
88. Poropatich, K., and D. J. Sullivan. 2011. Human immunodeficiency virus type 1 long-term non-progressors: the viral, genetic and immunological basis for disease non-progression. *J Gen Virol* 92: 247–268.
89. Learmont, J., B. Tindall, L. Evans, A. Cunningham, P. Cunningham, J. Wells, R. Penny, J. Kaldor, and D. A. Cooper. 1992. Long-term symptomless HIV-1 infection in recipients of blood products from a single donor. *Lancet* 340: 863–867.
90. Deacon, N. J., A. Tsykin, A. Solomon, K. Smith, M. Ludford-Menting, D. J. Hooker, D. A. McPhee, A. L. Greenway, A. Ellett, C. Chatfield, V. A. Lawson, S. Crowe, A. Maerz, S. Sonza, J. Learmont, J. S. Sullivan, A. Cunningham, D. Dwyer, D. Dowton, and J. Mills. 1995. Genomic structure of an attenuated quasi species of HIV-1 from a blood transfusion donor and recipients. *Science* 270: 988–991.
91. Miura, T., M. A. Brockman, C. J. Brumme, Z. L. Brumme, J. M. Carlson, F. Pereyra, A. Trocha, M. M. Addo, B. L. Block, A. C. Rothchild, B. M. Baker, T. Flynn, A. Schneidewind, B. Li, Y. E. Wang, D. Heckerman, T. M. Allen, and B. D. Walker. 2008. Genetic characterization of human immunodeficiency virus type 1 in elite controllers: lack of gross genetic defects or common amino acid changes. *J Virol* 82: 8422–8430.
92. Pushker, R., J.-M. Jacqué, and D. C. Shields. 2010. Meta-analysis to test the association of HIV-1 nef amino acid differences and deletions with disease progression. *J Virol* 84: 3644–3653.
93. Blankson, J. N., J. R. Bailey, S. Thayil, H.-C. Yang, K. Lassen, J. Lai, S. K. Gandhi, J. D. Siliciano, T. M. Williams, and R. F. Siliciano. 2007. Isolation and characterization of replication-competent human immunodeficiency virus type 1 from a subset of elite suppressors. *J Virol* 81: 2508–2518.
94. Lamine, A., A. Caumont-Sarcos, M.-L. Chaix, A. Saez-Cirion, C. Rouzioux, J.-F. Delfraissy, G. Pancino, and O. Lambotte. 2007. Replication-competent HIV strains infect HIV controllers despite undetectable viremia (ANRS EP36 study). *AIDS* 21: 1043–1045.
95. Carl, S., R. Daniels, A. J. Iafrate, P. Easterbrook, T. C. Greenough, J. Skowronski, and F. Kirchhoff. 2000. Partial “Repair” of Defective NEF Genes in a Long-Term Nonprogressor with Human Immunodeficiency Virus Type 1 Infection. *J Infect Dis* 181: 132–140.
96. Cruz, N. V. G., R. Amorim, F. E. Oliveira, F. A. C. Speranza, and L. J. Costa. 2013. Mutations in the nef and vif genes associated with progression to AIDS in elite controller and slow-progressor patients. *J Med Virol* 85: 563–574.
97. Lassen, K. G., M. A. Lobritz, J. R. Bailey, S. Johnston, S. Nguyen, B. Lee, T. Chou, R. F. Siliciano, M. Markowitz, and E. J. Arts. 2009. Elite suppressor-derived HIV-1 envelope glycoproteins exhibit reduced entry efficiency and kinetics. *PLoS Pathog* 5: e1000377.

98. Morawetz, R. A., G. P. Rizzardi, D. Glauser, O. Rutschmann, B. Hirschel, L. Perrin, M. Opravil, M. Flepp, J. von Overbeck, M. P. Glauser, S. Ghezzi, E. Vicenzi, G. Poli, A. Lazzarin, and G. Pantaleo. 1997. Genetic polymorphism of CCR5 gene and HIV disease: the heterozygous (CCR5/delta ccr5) genotype is neither essential nor sufficient for protection against disease progression. Swiss HIV Cohort. *Eur J Immunol* 27: 3223–3227.
99. Misrahi, M., J. P. Teglas, N. N'Go, M. Burgard, M. J. Mayaux, C. Rouzioux, J. F. Delfraissy, and S. Blanche. 1998. CCR5 chemokine receptor variant in HIV-1 mother-to-child transmission and disease progression in children. French Pediatric HIV Infection Study Group. *JAMA* 279: 277–280.
100. Romiti, M. L., C. Colognesi, C. Cancrini, A. Mas, M. Berrino, F. Salvatori, P. Orlandi, M. Jansson, E. Palomba, A. Plebani, J. M. Bertran, M. Hernandez, M. de Martino, A. Amoroso, P. A. Tovo, P. Rossi, T. Espanol, and G. Scarlatti. 2000. Prognostic value of a CCR5 defective allele in pediatric HIV-1 infection. *Mol Med* 6: 28–36.
101. Carrington, M., and S. J. O'Brien. 2003. The influence of HLA genotype on AIDS. *Annu Rev Med* 54: 535–551.
102. Neumann-Haefelin, C. 2013. HLA-B27-mediated protection in HIV and hepatitis C virus infection and pathogenesis in spondyloarthritis: two sides of the same coin? *Curr Opin Rheumatol* 25: 426–433.
103. Miura, T., M. A. Brockman, A. Schneidewind, M. Lobritz, F. Pereyra, A. Rathod, B. L. Block, Z. L. Brumme, C. J. Brumme, B. Baker, A. C. Rothchild, B. Li, A. Trocha, E. Cutrell, N. Frahm, C. Brander, I. Toth, E. J. Arts, T. M. Allen, and B. D. Walker. 2009. HLA-B57/B*5801 Human Immunodeficiency Virus Type 1 Elite Controllers Select for Rare Gag Variants Associated with Reduced Viral Replication Capacity and Strong Cytotoxic T-Lymphotye Recognition. *J Virol* 83: 2743–2755.
104. Kelleher, A. D., C. Long, E. C. Holmes, R. L. Allen, J. Wilson, C. Conlon, C. Workman, S. Shaunak, K. Olson, P. Goulder, C. Brander, G. Ogg, J. S. Sullivan, W. Dyer, I. Jones, A. J. McMichael, S. Rowland-Jones, and R. E. Phillips. 2001. Clustered mutations in HIV-1 gag are consistently required for escape from HLA-B27-restricted cytotoxic T lymphocyte responses. *J Exp Med* 193: 375–386.
105. Hersperger, A. R., F. Pereyra, M. Nason, K. Demers, P. Sheth, L. Y. Shin, C. M. Kovacs, B. Rodriguez, S. F. Sieg, L. Teixeira-Johnson, D. Gudonis, P. A. Goepfert, M. M. Lederman, I. Frank, G. Makedonas, R. Kaul, B. D. Walker, and M. R. Betts. 2010. Perforin expression directly ex vivo by HIV-specific CD8 T-cells is a correlate of HIV elite control. *PLoS Pathog* 6: e1000917.
106. Migueles, S. A., A. C. Laborico, W. L. Shupert, M. S. Sabbaghian, R. Rabin, C. W. Hallahan, D. Van Baarle, S. Kostense, F. Miedema, M. McLaughlin, L. Ehler, J. Metcalf, S. Liu, and M. Connors. 2002. HIV-specific CD8+ T cell proliferation is coupled to perforin expression and is maintained in nonprogressors. *Nat Immunol* 3: 1061–1068.
107. Elahi, S., W. L. Dinges, N. Lejarcegui, K. J. Laing, A. C. Collier, D. M. Koelle, M. J. McElrath, and H. Horton. 2011. Protective HIV-specific CD8+ T cells evade Treg cell suppression. *Nat Med* 17: 989–995.
108. Chen, H., C. Li, J. Huang, T. Cung, K. Seiss, J. Beamon, M. F. Carrington, L. C. Porter, P. S. Burke, Y. Yang, B. J. Ryan, R. Liu, R. H. Weiss, F. Pereyra, W. D. Cress, A. L. Brass, E. S. Rosenberg, B. D. Walker, X. G. Yu, and M. Lichterfeld. 2011. CD4+ T cells from elite controllers resist HIV-1 infection by selective upregulation of p21. *J Clin Invest* 121: 1549–1560.
109. Goulder, P. J., R. E. Phillips, R. A. Colbert, S. McAdam, G. Ogg, M. A. Nowak, P. Giangrande, G. Luzzi, B. Morgan, A. Edwards, A. J. McMichael, and S. Rowland-Jones. 1997. Late escape from an immunodominant cytotoxic T-lymphocyte response associated with progression to AIDS. *Nat Med* 3: 212–217.
110. Oomizu, S., T. Arikawa, T. Niki, T. Kadowaki, M. Ueno, N. Nishi, A. Yamauchi, T. Hattori, T. Masaki, and M. Hirashima. 2012. Cell surface galectin-9 expressing Th cells regulate Th17 and Foxp3+ Treg development by galectin-9 secretion. *PLoS One* 7: e48574.
111. John, S., and R. Mishra. 2016. Galectin-9: From cell biology to complex disease dynamics. *J Biosci* 41: 507–534.

112. O'Brien, M. J., Q. Shu, W. A. Stinson, P.-S. Tsou, J. H. Ruth, T. Isozaki, P. L. Campbell, R. A. Ohara, A. E. Koch, D. A. Fox, and M. A. Amin. 2018. A unique role for galectin-9 in angiogenesis and inflammatory arthritis. *Arthritis Res Ther* 20: 31.
113. Bi, S., P. W. Hong, B. Lee, and L. G. Baum. 2011. Galectin-9 binding to cell surface protein disulfide isomerase regulates the redox environment to enhance T-cell migration and HIV entry. *Proc Natl Acad Sci U S A* 108: 10650–10655.
114. Wu, C., T. Thalhamer, R. F. Franca, S. Xiao, C. Wang, C. Hotta, C. Zhu, M. Hirashima, A. C. Anderson, and V. K. Kuchroo. 2014. Galectin-9-CD44 interaction enhances stability and function of adaptive regulatory T cells. *Immunity* 41: 270–282.
115. Merani, S., W. Chen, and S. Elahi. 2015. The bitter side of sweet: the role of Galectin-9 in immunopathogenesis of viral infections. *Rev Med Virol* 25: 175–186.
116. Madireddi, S., S.-Y. Eun, S.-W. Lee, I. Nemčovičová, A. K. Mehta, D. M. Zajonc, N. Nishi, T. Niki, M. Hirashima, and M. Croft. 2014. Galectin-9 controls the therapeutic activity of 4-1BB-targeting antibodies. *J Exp Med* 211: 1433–1448.
117. Niki, T., K. Fujita, H. Rosen, M. Hirashima, T. Masaki, T. Hattori, and K. Hoshino. 2018. Plasma Galectin-9 Concentrations in Normal and Diseased Condition. *Cell Physiol Biochem* 50: 1856–1868.
118. Nickel, W. 2003. The mystery of nonclassical protein secretion. A current view on cargo proteins and potential export routes. *Eur J Biochem* 270: 2109–2119.
119. Saitoh, H., Y. Ashino, H. Chagan-Yasutan, T. Niki, M. Hirashima, and T. Hattori. 2012. Rapid decrease of plasma galectin-9 levels in patients with acute HIV infection after therapy. *Tohoku J Exp Med* 228: 157–161.
120. Jost, S., U. Y. Moreno-Nieves, W. F. Garcia-Beltran, K. Rands, J. Reardon, I. Toth, A. Piechocka-Trocha, M. Altfeld, and M. M. Addo. 2013. Dysregulated Tim-3 expression on natural killer cells is associated with increased Galectin-9 levels in HIV-1 infection. *Retrovirology* 10: 74.
121. Tandon, R., G. M. Chew, M. M. Byron, P. Borrow, T. Niki, M. Hirashima, J. D. Barbour, P. J. Norris, M. C. Lanteri, J. N. Martin, S. G. Deeks, and L. C. Ndhlovu. 2014. Galectin-9 is rapidly released during acute HIV-1 infection and remains sustained at high levels despite viral suppression even in elite controllers. *AIDS Res Hum Retroviruses* 30: 654–664.
122. Elahi, S., T. Niki, M. Hirashima, and H. Horton. 2012. Galectin-9 binding to Tim-3 renders activated human CD4+ T cells less susceptible to HIV-1 infection. *Blood* 119: 4192–4204.
123. Abdel-Mohsen, M., L. Chavez, R. Tandon, G. M. Chew, X. Deng, A. Danesh, S. Keating, M. Lanteri, M. L. Samuels, R. Hoh, J. B. Sacha, P. J. Norris, T. Niki, C. M. Shikuma, M. Hirashima, S. G. Deeks, L. C. Ndhlovu, and S. K. Pillai. 2016. Human Galectin-9 Is a Potent Mediator of HIV Transcription and Reactivation. *PLoS Pathog* 12.
124. Colomb, F., L. B. Giron, T. A. Premeaux, B. I. Mitchell, T. Niki, E. Papasavvas, L. J. Montaner, L. C. Ndhlovu, and M. Abdel-Mohsen. 2019. Galectin-9 Mediates HIV Transcription by Inducing TCR-Dependent ERK Signaling. *Front. Immunol.* 10.
125. Vanhamel, J., A. Bruggemans, and Z. Debyser. Establishment of latent HIV-1 reservoirs: what do we really know? *J Virus Erad* 5: 3–9.
126. Yoon, K. W., S. Byun, E. Kwon, S.-Y. Hwang, K. Chu, M. Hiraki, S.-H. Jo, A. Weins, S. Hakroush, A. Cebulla, D. B. Sykes, A. Greka, P. Mundel, D. E. Fisher, A. Mandinova, and S. W. Lee. 2015. Control of signaling-mediated clearance of apoptotic cells by the tumor suppressor p53. *Science* 349.
127. Michalek, R. D., V. A. Gerriets, S. R. Jacobs, A. N. Macintyre, N. J. MacIver, E. F. Mason, S. A. Sullivan, A. G. Nichols, and J. C. Rathmell. 2011. Cutting Edge: Distinct Glycolytic and Lipid Oxidative Metabolic Programs Are Essential for Effector and Regulatory CD4+ T Cell Subsets. *The Journal of Immunology* 186: 3299–3303.

128. Aloia, L., S. Parisi, L. Fusco, L. Pastore, and T. Russo. 2010. Differentiation of Embryonic Stem Cells 1 (Dies1) Is a Component of Bone Morphogenetic Protein 4 (BMP4) Signaling Pathway Required for Proper Differentiation of Mouse Embryonic Stem Cells *. *Journal of Biological Chemistry* 285: 7776–7783.
129. Wang, L., R. Rubinstein, J. L. Lines, A. Wasiuk, C. Ahonen, Y. Guo, L.-F. Lu, D. Gondek, Y. Wang, R. A. Fava, A. Fiser, S. Almo, and R. J. Noelle. 2011. VISTA, a novel mouse Ig superfamily ligand that negatively regulates T cell responses. *J Exp Med* 208: 577–592.
130. Mehta, N., S. Maddineni, I. I. Mathews, R. Andres Parra Sperberg, P.-S. Huang, and J. R. Cochran. 2019. Structure and Functional Binding Epitope of V-domain Ig Suppressor of T Cell Activation. *Cell Rep* 28: 2509-2516.e5.
131. Lines, J. L., E. Pantazi, J. Mak, L. F. Sempere, L. Wang, S. O'Connell, S. Ceeraz, A. A. Suriawinata, S. Yan, M. S. Ernstoff, and R. Noelle. 2014. VISTA is an immune checkpoint molecule for human T cells. *Cancer Res* 74: 1924–1932.
132. Flies, D. B., X. Han, T. Higuchi, L. Zheng, J. Sun, J. J. Ye, and L. Chen. 2014. Coinhibitory receptor PD-1H preferentially suppresses $CD4^+$ T cell–mediated immunity. *J Clin Invest* 124: 1966–1975.
133. Wang, J., G. Wu, B. Manick, V. Hernandez, M. Renelt, C. Erickson, J. Guan, R. Singh, S. Rollins, A. Solorz, M. Bi, J. Li, D. Grabowski, J. Dirkx, C. Tracy, T. Stuart, C. Ellinghuysen, D. Desmond, C. Foster, and V. Kalabokis. 2019. VSIG-3 as a ligand of VISTA inhibits human T-cell function. *Immunology* 156: 74–85.
134. Bharaj, P., H. S. Chahar, O. K. Alozie, L. Rodarte, A. Bansal, P. A. Goepfert, A. Dwivedi, N. Manjunath, and P. Shankar. 2014. Characterization of programmed death-1 homologue-1 (PD-1H) expression and function in normal and HIV infected individuals. *PLoS One* 9: e109103.
135. Flies, D. B., S. Wang, H. Xu, and L. Chen. 2011. Cutting edge: A monoclonal antibody specific for the programmed death-1 homolog prevents graft-versus-host disease in mouse models. *J Immunol* 187: 1537–1541.
136. ElTanbouly, M. A., Y. Zhao, E. Nowak, J. Li, E. Schaafsma, I. L. Mercier, S. Ceeraz, J. L. Lines, C. Peng, C. Carriere, X. Huang, M. Day, B. Koehn, S. W. Lee, M. S. Morales, K. A. Hogquist, S. C. Jameson, D. Mueller, J. Rothstein, B. R. Blazar, C. Cheng, and R. J. Noelle. 2020. VISTA is a checkpoint regulator for naïve T cell quiescence and peripheral tolerance. *Science* 367.
137. Kunishige, T., H. Taniguchi, T. Ohno, M. Azuma, and J. Hori. 2019. VISTA Is Crucial for Corneal Allograft Survival and Maintenance of Immune Privilege. *Invest. Ophthalmol. Vis. Sci.* 60: 4958–4965.
138. ElTanbouly, M. A., E. Schaafsma, R. J. Noelle, and J. L. Lines. 2020. VISTA: Coming of age as a multi-lineage immune checkpoint. *Clin Exp Immunol* 200: 120–130.
139. Wang, L., I. Le Mercier, J. Putra, W. Chen, J. Liu, A. D. Schenk, E. C. Nowak, A. A. Suriawinata, J. Li, and R. J. Noelle. 2014. Disruption of the immune-checkpoint VISTA gene imparts a proinflammatory phenotype with predisposition to the development of autoimmunity. *Proc Natl Acad Sci U S A* 111: 14846–14851.
140. Liu, J., Y. Yuan, W. Chen, J. Putra, A. A. Suriawinata, A. D. Schenk, H. E. Miller, I. Guleria, R. J. Barth, Y. H. Huang, and L. Wang. 2015. Immune-checkpoint proteins VISTA and PD-1 nonredundantly regulate murine T-cell responses. *Proc Natl Acad Sci U S A* 112: 6682–6687.
141. Flies, D. B., T. Higuchi, and L. Chen. 2015. Mechanistic Assessment of PD-1H Coinhibitory Receptor-Induced T Cell Tolerance to Allogeneic Antigens. *J Immunol* 194: 5294–5304.
142. Le Mercier, I., W. Chen, J. L. Lines, M. Day, J. Li, P. Sergent, R. J. Noelle, and L. Wang. 2014. VISTA Regulates the Development of Protective Antitumor Immunity. *Cancer Res* 74: 1933–1944.
143. Böger, C., H.-M. Behrens, S. Krüger, and C. Röcken. 2017. The novel negative checkpoint regulator VISTA is expressed in gastric carcinoma and associated with PD-L1/PD-1: A future perspective for a combined gastric cancer therapy? *Oncoimmunology* 6: e1293215.
144. Wu, L., W.-W. Deng, C.-F. Huang, L.-L. Bu, G.-T. Yu, L. Mao, W.-F. Zhang, B. Liu, and Z.-J. Sun. 2017. Expression of VISTA correlated with immunosuppression and synergized with CD8 to predict survival in human oral squamous cell carcinoma. *Cancer Immunol Immunother* 66: 627–636.

145. Gao, J., J. F. Ward, C. A. Pettaway, L. Z. Shi, S. K. Subudhi, L. M. Vence, H. Zhao, J. Chen, H. Chen, E. Efstathiou, P. Troncoso, J. P. Allison, C. J. Logothetis, I. I. Wistuba, M. A. Sepulveda, J. Sun, J. Wargo, J. Blando, and P. Sharma. 2017. VISTA is an inhibitory immune checkpoint that is increased after ipilimumab therapy in patients with prostate cancer. *Nat Med* 23: 551–555.
146. Xu, W., J. Dong, Y. Zheng, J. Zhou, Y. Yuan, H. M. Ta, H. E. Miller, M. Olson, K. Rajasekaran, M. S. Ernstoff, D. Wang, S. Malarkannan, and L. Wang. 2019. Immune checkpoint protein VISTA regulates antitumor immunity by controlling myeloid cell–mediated inflammation and immunosuppression. *Cancer Immunol Res* 7: 1497–1510.
147. Martínez-Martínez, A., E. Muñoz-Delgado, F. J. Campoy, C. Flores-Flores, J. N. Rodríguez-López, C. Fini, and C. J. Vidal. 2000. The ecto-5'-nucleotidase subunits in dimers are not linked by disulfide bridges but by non-covalent bonds. *Biochimica et Biophysica Acta (BBA) - Protein Structure and Molecular Enzymology* 1478: 300–308.
148. Knapp, K., M. Zebisch, J. Pippel, A. El-Tayeb, C. E. Müller, and N. Sträter. 2012. Crystal Structure of the Human Ecto-5'-Nucleotidase (CD73): Insights into the Regulation of Purinergic Signaling. *Structure* 20: 2161–2173.
149. Klemens, M. R., W. R. Sherman, N. J. Holmberg, J. M. Ruedi, M. G. Low, and L. F. Thompson. 1990. Characterization of soluble vs membrane-bound human placental 5'-nucleotidase. *Biochemical and Biophysical Research Communications* 172: 1371–1377.
150. Vogel, M., H. Kowalewski, H. Zimmermann, N. M. Hooper, and A. J. Turner. 1992. Soluble low-Km 5'-nucleotidase from electric-ray (Torpedo marmorata) electric organ and bovine cerebral cortex is derived from the glycosyl-phosphatidylinositol-anchored ectoenzyme by phospholipase C cleavage. *Biochemical Journal* 284: 621–624.
151. Bono, M. R., D. Fernández, F. Flores-Santibáñez, M. Rosemblatt, and D. Sauma. 2015. CD73 and CD39 ectonucleotidases in T cell differentiation: Beyond immunosuppression. *FEBS Letters* 589: 3454–3460.
152. Aswad, F., H. Kawamura, and G. Dennert. 2005. High sensitivity of CD4+CD25+ regulatory T cells to extracellular metabolites nicotinamide adenine dinucleotide and ATP: a role for P2X7 receptors. *J Immunol* 175: 3075–3083.
153. Stagg, J., and M. J. Smyth. 2010. Extracellular adenosine triphosphate and adenosine in cancer. *Oncogene* 29: 5346–5358.
154. Antonioli, L., P. Pacher, E. S. Vizi, and G. Haskó. 2013. CD39 and CD73 in immunity and inflammation. *Trends Mol Med* 19: 355–367.
155. Deaglio, S., K. M. Dwyer, W. Gao, D. Friedman, A. Usheva, A. Erat, J.-F. Chen, K. Enjyoji, J. Linden, M. Oukka, V. K. Kuchroo, T. B. Strom, and S. C. Robson. 2007. Adenosine generation catalyzed by CD39 and CD73 expressed on regulatory T cells mediates immune suppression. *J Exp Med* 204: 1257–1265.
156. Ohta, A., A. Ohta, M. Madasu, R. Kini, M. Subramanian, N. Goel, and M. Sitkovsky. 2009. A2A adenosine receptor may allow expansion of T cells lacking effector functions in extracellular adenosine-rich microenvironments. *J Immunol* 183: 5487–5493.
157. Zarek, P. E., C.-T. Huang, E. R. Lutz, J. Kowalski, M. R. Horton, J. Linden, C. G. Drake, and J. D. Powell. 2008. A2A receptor signaling promotes peripheral tolerance by inducing T-cell anergy and the generation of adaptive regulatory T cells. *Blood* 111: 251–259.
158. Ohta, A., R. Kini, A. Ohta, M. Subramanian, M. Madasu, and M. Sitkovsky. 2012. The development and immunosuppressive functions of CD4(+) CD25(+) FoxP3(+) regulatory T cells are under influence of the adenosine-A2A adenosine receptor pathway. *Front Immunol* 3: 190.
159. Massaia, M., L. Perrin, A. Bianchi, J. Ruedi, C. Attisano, D. Altieri, G. T. Rijkers, and L. F. Thompson. 1990. Human T cell activation. Synergy between CD73 (ecto-5'-nucleotidase) and signals delivered through CD3 and CD2 molecules. *J Immunol* 145: 1664–1674.

160. Thompson, L. F., J. M. Ruedi, A. Glass, M. G. Low, and A. H. Lucas. 1989. Antibodies to 5'-nucleotidase (CD73), a glycosyl-phosphatidylinositol-anchored protein, cause human peripheral blood T cells to proliferate. *J Immunol* 143: 1815–1821.
161. Dianzani, U., V. Redoglia, M. Bragardo, C. Attisano, A. Bianchi, D. D. Franco, U. Ramenghi, H. Wolff, L. F. Thompson, and A. Pileri. 1993. Co-stimulatory signal delivered by CD73 molecule to human CD45RAhiCD45ROlo (naive) CD8+ T lymphocytes. *The Journal of Immunology* 151: 3961–3970.
162. Resta, R., Y. Yamashita, and L. F. Thompson. 1998. Ecto-enzyme and signaling functions of lymphocyte CD 7 3. *Immunological Reviews* 161: 95–109.
163. Airas, L., J. Niemelä, and S. Jalkanen. 2000. CD73 Engagement Promotes Lymphocyte Binding to Endothelial Cells Via a Lymphocyte Function-Associated Antigen-1-Dependent Mechanism. *The Journal of Immunology* 165: 5411–5417.
164. Allan, S. E., S. Q. Crome, N. K. Crellin, L. Passerini, T. S. Steiner, R. Bacchetta, M. G. Roncarolo, and M. K. Levings. 2007. Activation-induced FOXP3 in human T effector cells does not suppress proliferation or cytokine production. *Int Immunol* 19: 345–354.
165. Liu, W., A. L. Putnam, Z. Xu-yu, G. L. Szot, M. R. Lee, S. Zhu, P. A. Gottlieb, P. Kapranov, T. R. Gingeras, B. F. de St. Groth, C. Clayberger, D. M. Soper, S. F. Ziegler, and J. A. Bluestone. 2006. CD127 expression inversely correlates with FoxP3 and suppressive function of human CD4+ T reg cells. *J Exp Med* 203: 1701–1711.
166. Yamaguchi, T., K. Hirota, K. Nagahama, K. Ohkawa, T. Takahashi, T. Nomura, and S. Sakaguchi. 2007. Control of immune responses by antigen-specific regulatory T cells expressing the folate receptor. *Immunity* 27: 145–159.
167. Shevach, E. M. 2009. Mechanisms of foxp3+ T regulatory cell-mediated suppression. *Immunity* 30: 636–645.
168. Rothstein, D. M., and G. Camirand. 2015. New insights into the mechanisms of Treg function. *Curr Opin Organ Transplant* 20: 376–384.
169. Lee, J. H., S. G. Kang, and C. H. Kim. 2007. FoxP3+ T cells undergo conventional first switch to lymphoid tissue homing receptors in thymus but accelerated second switch to nonlymphoid tissue homing receptors in secondary lymphoid tissues. *J Immunol* 178: 301–311.
170. Mao, F.-Y., H. Kong, Y.-L. Zhao, L.-S. Peng, W. Chen, J.-Y. Zhang, P. Cheng, T.-T. Wang, Y.-P. Lv, Y.-S. Teng, X.-L. Fu, Y.-G. Liu, X.-L. Wu, C.-J. Hao, N. You, P. Luo, P.-W. Yu, Q.-M. Zou, G. Guo, and Y. Zhuang. 2017. Increased tumor-infiltrating CD45RA-CCR7- regulatory T-cell subset with immunosuppressive properties foster gastric cancer progress. *Cell Death Dis* 8: e3002.
171. Zhu, Z., Y. Zhang, J. Ye, X. Wang, X. Fu, Y. Yin, J. Wen, X. Wu, and Z. Xia. 2018. IL-35 promoted STAT3 phosphorylation and IL-10 production in B cells, but its production was reduced in patients with coronary artery diseases. *Hum Immunol* 79: 869–875.
172. Tsuchida, Y., S. Sumitomo, K. Ishigaki, A. Suzuki, Y. Kochi, H. Tsuchiya, M. Ota, T. Komai, M. Inoue, K. Morita, T. Okamura, K. Yamamoto, and K. Fujio. 2017. TGF-β3 Inhibits Antibody Production by Human B Cells. *PLoS One* 12: e0169646.
173. Dooley, A., M. Quintana, M. Cheung, L. L. Sun, and N. Gupta. 2018. The B cell IL-10 receptor suppresses antibody production. *The Journal of Immunology* 200: 164.9-164.9.
174. Schmidt, A., N. Oberle, and P. H. Krammer. 2012. Molecular mechanisms of treg-mediated T cell suppression. *Front Immunol* 3: 51.
175. Boks, M. A., J. R. Kager-Groenland, M. S. P. Haasjes, J. J. Zwaginga, S. M. van Ham, and A. ten Brinke. 2012. IL-10-generated tolerogenic dendritic cells are optimal for functional regulatory T cell induction--a comparative study of human clinical-applicable DC. *Clin Immunol* 142: 332–342.
176. Strobl, H., and W. Knapp. 1999. TGF-beta1 regulation of dendritic cells. *Microbes Infect* 1: 1283–1290.

177. Thepmalee, C., A. Panya, M. Junking, T. Chieochansin, and P.-T. Yenchitsomanus. 2018. Inhibition of IL-10 and TGF-β receptors on dendritic cells enhances activation of effector T-cells to kill cholangiocarcinoma cells. *Hum Vaccin Immunother* 14: 1423–1431.
178. de la Rosa, M., S. Rutz, H. Dorninger, and A. Scheffold. 2004. Interleukin-2 is essential for CD4+CD25+ regulatory T cell function. *Eur J Immunol* 34: 2480–2488.
179. Chinen, T., A. K. Kannan, A. G. Levine, X. Fan, U. Klein, Y. Zheng, G. Gasteiger, Y. Feng, J. D. Fontenot, and A. Y. Rudensky. 2016. An essential role for the IL-2 receptor in T reg cell function. *Nature Immunology* 17: 1322–1333.
180. Linnemann, C., F. A. Schildberg, A. Schurich, L. Diehl, S. I. Hegenbarth, E. Endl, S. Lacher, C. E. Müller, J. Frey, L. Simeoni, B. Schraven, D. Stabenow, and P. A. Knolle. 2009. Adenosine regulates CD8 T-cell priming by inhibition of membrane-proximal T-cell receptor signalling. *Immunology* 128: e728–e737.
181. Ohta, A., R. Kini, A. Ohta, M. Subramanian, M. Madasu, and M. Sitkovsky. 2012. The development and immunosuppressive functions of CD4(+) CD25(+) FoxP3(+) regulatory T cells are under influence of the adenosine-A2A adenosine receptor pathway. *Front Immunol* 3: 190.
182. Bopp, T., C. Becker, M. Klein, S. Klein-Hessling, A. Palmetshofer, E. Serfling, V. Heib, M. Becker, J. Kubach, S. Schmitt, S. Stoll, H. Schild, M. S. Staege, M. Stassen, H. Jonuleit, and E. Schmitt. 2007. Cyclic adenosine monophosphate is a key component of regulatory T cell-mediated suppression. *J Exp Med* 204: 1303–1310.
183. Gondek, D. C., L.-F. Lu, S. A. Quezada, S. Sakaguchi, and R. J. Noelle. 2005. Cutting edge: contact-mediated suppression by CD4+CD25+ regulatory cells involves a granzyme B-dependent, perforin-independent mechanism. *J Immunol* 174: 1783–1786.
184. Cao, X., S. F. Cai, T. A. Fehniger, J. Song, L. I. Collins, D. R. Piwnica-Worms, and T. J. Ley. 2007. Granzyme B and perforin are important for regulatory T cell-mediated suppression of tumor clearance. *Immunity* 27: 635–646.
185. Ren, X., F. Ye, Z. Jiang, Y. Chu, S. Xiong, and Y. Wang. 2007. Involvement of cellular death in TRAIL/DR5-dependent suppression induced by CD4(+)CD25(+) regulatory T cells. *Cell Death Differ* 14: 2076–2084.
186. Garín, M. I., C.-C. Chu, D. Golshayan, E. Cernuda-Morollón, R. Wait, and R. I. Lechler. 2007. Galectin-1: a key effector of regulation mediated by CD4+CD25+ T cells. *Blood* 109: 2058–2065.
187. Onishi, Y., Z. Fehervari, T. Yamaguchi, and S. Sakaguchi. 2008. Foxp3+ natural regulatory T cells preferentially form aggregates on dendritic cells in vitro and actively inhibit their maturation. *Proc Natl Acad Sci U S A* 105: 10113–10118.
188. Akkaya, B., M. Akkaya, O. Kamenyeva, J. Kabat, D. W. Dorward, A. H. Holstein, M. P. Maz, and E. M. Shevach. 2017. Unique interaction dynamics and peptide-MHC class II (pMHC II) transendocytosis lead to antigen-specific T regulatory cell (Treg)-mediated suppression. *The Journal of Immunology* 198: 80.8-80.8.
189. Fallarino, F., U. Grohmann, K. W. Hwang, C. Orabona, C. Vacca, R. Bianchi, M. L. Belladonna, M. C. Fioretti, M.-L. Alegre, and P. Puccetti. 2003. Modulation of tryptophan catabolism by regulatory T cells. *Nat Immunol* 4: 1206–1212.
190. Liang, B., C. Workman, J. Lee, C. Chew, B. M. Dale, L. Colonna, M. Flores, N. Li, E. Schweighoffer, S. Greenberg, V. Tybulewicz, D. Vignali, and R. Clynes. 2008. Regulatory T Cells Inhibit Dendritic Cells by Lymphocyte Activation Gene-3 Engagement of MHC Class II. *The Journal of Immunology* 180: 5916–5926.
191. Cai, J., D. Wang, G. Zhang, and X. Guo. 2019. The Role Of PD-1/PD-L1 Axis In Treg Development And Function: Implications For Cancer Immunotherapy. *Onco Targets Ther* 12: 8437–8445.
192. Elahi, S., W. L. Dinges, N. Lejarcegui, K. J. Laing, A. C. Collier, D. M. Koelle, M. J. McElrath, and H. Horton. 2011. Protective HIV-specific CD8+ T cells evade Treg cell suppression. *Nat Med* 17: 989–995.

193. Koch, M. A., G. Tucker-Heard, N. R. Perdue, J. R. Killebrew, K. B. Urdahl, and D. J. Campbell. 2009. The transcription factor T-bet controls regulatory T cell homeostasis and function during type 1 inflammation. *Nat Immunol* 10: 595–602.
194. Huber, M., V. Steinwald, A. Guralnik, A. Brüstle, P. Kleemann, C. Rosenplänter, T. Decker, and M. Lohoff. 2008. IL-27 inhibits the development of regulatory T cells via STAT3. *Int Immunol* 20: 223–234.
195. Ahern, P. P., C. Schiering, S. Buonocore, M. J. McGeachy, D. J. Cua, K. J. Maloy, and F. Powrie. 2010. Interleukin-23 drives intestinal inflammation through direct activity on T cells. *Immunity* 33: 279–288.
196. Yang, X. O., R. Nurieva, G. J. Martinez, H. S. Kang, Y. Chung, B. P. Pappu, B. Shah, S. H. Chang, K. S. Schluns, S. S. Watowich, X.-H. Feng, A. M. Jetten, and C. Dong. 2008. Molecular antagonism and plasticity of regulatory and inflammatory T cell programs. *Immunity* 29: 44–56.
197. Durant, L., W. T. Watford, H. L. Ramos, A. Laurence, G. Vahedi, L. Wei, H. Takahashi, H.-W. Sun, Y. Kanno, F. Powrie, and J. J. O'Shea. 2010. Diverse targets of the transcription factor STAT3 contribute to T cell pathogenicity and homeostasis. *Immunity* 32: 605–615.
198. Antons, A. K., R. Wang, K. Oswald-Richter, M. Tseng, C. W. Arendt, S. A. Kalams, and D. Unutmaz. 2008. Naive precursors of human regulatory T cells require FoxP3 for suppression and are susceptible to HIV infection. *Journal of Immunology (Baltimore, Md.: 1950)* 180: 764–773.
199. Oswald-Richter, K., S. M. Grill, N. Shariat, M. Leelawong, M. S. Sundrud, D. W. Haas, and D. Unutmaz. 2004. HIV infection of naturally occurring and genetically reprogrammed human regulatory T-cells. *PLoS biology* 2: E198.
200. Zaunders, J. J., S. Ip, M. L. Munier, D. E. Kaufmann, K. Suzuki, C. Brereton, S. C. Sasson, N. Seddiki, K. Koelsch, A. Landay, P. Grey, R. Finlayson, J. Kaldor, E. S. Rosenberg, B. D. Walker, B. Fazekas de St Groth, D. A. Cooper, and A. D. Kelleher. 2006. Infection of CD127+ (interleukin-7 receptor+) CD4+ cells and overexpression of CTLA-4 are linked to loss of antigen-specific CD4 T cells during primary human immunodeficiency virus type 1 infection. *J Virol* 80: 10162–10172.
201. Moreno-Fernandez, M. E., W. Zapata, J. T. Blackard, G. Franchini, and C. A. Chougnet. 2009. Human regulatory T cells are targets for human immunodeficiency Virus (HIV) infection, and their susceptibility differs depending on the HIV type 1 strain. *J Virol* 83: 12925–12933.
202. Angin, M., D. S. Kwon, H. Streeck, F. Wen, M. King, A. Rezai, K. Law, T. C. Hongo, A. Pyo, A. Piechocka-Trocha, I. Toth, F. Pereyra, M. Ghebremichael, S. J. Rodig, D. A. Milner, J. M. Richter, M. Altfeld, D. E. Kaufmann, B. D. Walker, and M. M. Addo. 2012. Preserved function of regulatory T cells in chronic HIV-1 infection despite decreased numbers in blood and tissue. *J Infect Dis* 205: 1495–1500.
203. Simonetta, F., C. Lecuroux, I. Girault, C. Goujard, M. Sinet, O. Lambotte, A. Venet, and C. Bourgeois. 2012. Early and long-lasting alteration of effector CD45RA(-)Foxp3(high) regulatory T-cell homeostasis during HIV infection. *J Infect Dis* 205: 1510–1519.
204. Epple, H.-J., C. Loddenkemper, D. Kunkel, H. Tröger, J. Maul, V. Moos, E. Berg, R. Ullrich, J.-D. Schulzke, H. Stein, R. Duchmann, M. Zeitz, and T. Schneider. 2006. Mucosal but not peripheral FOXP3+ regulatory T cells are highly increased in untreated HIV infection and normalize after suppressive HAART. *Blood* 108: 3072–3078.
205. Montes, M., C. Sanchez, D. E. Lewis, E. A. Graviss, C. Seas, E. Gotuzzo, and A. C. White. 2011. Normalization of FoxP3(+) regulatory T cells in response to effective antiretroviral therapy. *J Infect Dis* 203: 496–499.
206. Lim, A., D. Tan, P. Price, A. Kamarulzaman, H.-Y. Tan, I. James, and M. A. French. 2007. Proportions of circulating T cells with a regulatory cell phenotype increase with HIV-associated immune activation and remain high on antiretroviral therapy. *AIDS* 21: 1525–1534.
207. Hunt, P. W., A. L. Landay, E. Sinclair, J. A. Martinson, H. Hatano, B. Emu, P. J. Norris, M. P. Busch, J. N. Martin, C. Brooks, J. M. McCune, and S. G. Deeks. 2011. A low T regulatory cell response may contribute to both viral control and generalized immune activation in HIV controllers. *PLoS One* 6: e15924.

208. Nikolova, M., M. Carriere, M.-A. Jenabian, S. Limou, M. Younas, A. Kök, S. Huë, N. Seddiki, A. Hulin, O. Delaneau, H. Schuitemaker, J. T. Herbeck, J. I. Mullins, M. Muhtarova, A. Bensussan, J.-F. Zagury, J.-D. Lelievre, and Y. Lévy. 2011. CD39/adenosine pathway is involved in AIDS progression. *PLoS pathogens* 7: e1002110.
209. Andersson, J., A. Boasso, J. Nilsson, R. Zhang, N. J. Shire, S. Lindback, G. M. Shearer, and C. A. Chougnet. 2005. The prevalence of regulatory T cells in lymphoid tissue is correlated with viral load in HIV-infected patients. *J. Immunol.* 174: 3143–3147.
210. Apoil, P. A., B. Puissant, F. Roubinet, M. Abbal, P. Massip, and A. Blancher. 2005. FOXP3 mRNA levels are decreased in peripheral blood CD4+ lymphocytes from HIV-positive patients. *J. Acquir. Immune Defic. Syndr.* 39: 381–385.
211. Xing, S., J. Fu, Z. Zhang, Y. Gao, Y. Jiao, F. Kang, J. Zhang, C. Zhou, H. Wu, and F.-S. Wang. 2010. Increased turnover of FoxP3high regulatory T cells is associated with hyperactivation and disease progression of chronic HIV-1 infection. *J Acquir Immune Defic Syndr* 54: 455–462.
212. Piconi, S., D. Trabattoni, A. Gori, S. Parisotto, C. Magni, P. Meraviglia, A. Bandera, A. Capetti, G. Rizzardini, and M. Clerici. 2010. Immune activation, apoptosis, and Treg activity are associated with persistently reduced CD4+ T-cell counts during antiretroviral therapy. *AIDS* 24: 1991–2000.
213. Chase, A. J., H.-C. Yang, H. Zhang, J. N. Blankson, and R. F. Siliciano. 2008. Preservation of FoxP3+ regulatory T cells in the peripheral blood of human immunodeficiency virus type 1-infected elite suppressors correlates with low CD4+ T-cell activation. *J. Virol.* 82: 8307–8315.
214. Hunt, P. W., A. L. Landay, E. Sinclair, J. A. Martinson, H. Hatano, B. Emu, P. J. Norris, M. P. Busch, J. N. Martin, C. Brooks, J. M. McCune, and S. G. Deeks. 2011. A low T regulatory cell response may contribute to both viral control and generalized immune activation in HIV controllers. *PloS One* 6: e15924.
215. Fuertes Marraco, S. A., N. J. Neubert, G. Verdeil, and D. E. Speiser. 2015. Inhibitory Receptors Beyond T Cell Exhaustion. *Front Immunol* 6: 310.
216. Yasuma-Mitobe, K., and M. Matsuoka. 2018. The Roles of Coinhibitory Receptors in Pathogenesis of Human Retroviral Infections. *Front Immunol* 9: 2755.
217. Okoye, I., A. Namdar, L. Xu, N. Crux, and S. Elahi. 2017. Atorvastatin downregulates co-inhibitory receptor expression by targeting Ras-activated mTOR signalling. *Oncotarget* 8: 98215–98232.
218. Amancha, P. K., J. J. Hong, A. A. Ansari, and F. Villinger. 2015. Up-regulation of Tim-3 on T cells during acute simian immunodeficiency virus infection and on antigen specific responders. *AIDS* 29: 531–536.
219. Sakuishi, K., L. Apetoh, J. M. Sullivan, B. R. Blazar, V. K. Kuchroo, and A. C. Anderson. 2010. Targeting Tim-3 and PD-1 pathways to reverse T cell exhaustion and restore anti-tumor immunity. *J Exp Med* 207: 2187–2194.
220. Cao, S., and K. A. Woodrow. 2019. Nanotechnology approaches to eradicating HIV reservoirs. *European Journal of Pharmaceutics and Biopharmaceutics* 138: 48–63.
221. Ålgars, A., M. Karikoski, G. G. Yegutkin, P. Stoitzner, J. Niemelä, M. Salmi, and S. Jalkanen. 2011. Different role of CD73 in leukocyte trafficking via blood and lymph vessels. *Blood* 117: 4387–4393.
222. Mills, J. H., L. F. Thompson, C. Mueller, A. T. Waickman, S. Jalkanen, J. Niemela, L. Airas, and M. S. Bynoe. 2008. CD73 is required for efficient entry of lymphocytes into the central nervous system during experimental autoimmune encephalomyelitis. *PNAS* 105: 9325–9330.
223. Stachon, P., S. Geis, A. Peikert, A. Heidenreich, N. A. Michel, F. Ünal, N. Hoppe, B. Dufner, L. Schulte, T. Marchini, S. Cicko, K. Ayata, A. Zech, D. Wolf, I. Hilgendorf, F. Willecke, J. Reinöhl, C. von Zur Mühlen, C. Bode, M. Idzko, and A. Zirlik. 2016. Extracellular ATP Induces Vascular Inflammation and Atherosclerosis via Purinergic Receptor Y2 in Mice. *Arterioscler Thromb Vasc Biol* 36: 1577–1586.

224. Velasquez, S., L. Prevedel, S. Valdebenito, A. M. Gorska, M. Golovko, N. Khan, J. Geiger, and E. A. Eugenin. 2020. Circulating levels of ATP is a biomarker of HIV cognitive impairment. *EBioMedicine* 51: 102503.

Chapter 2: Galectin-9 and VISTA expression define terminally exhausted T cells in HIV-1 infection

1. Okoye, I. S., M. Houghton, L. Tyrrell, K. Barakat, and S. Elahi. 2017. Coinhibitory Receptor Expression and Immune Checkpoint Blockade: Maintaining a Balance in CD8(+) T Cell Responses to Chronic Viral Infections and Cancer. *Frontiers in immunology* 8: 1215.
2. Doering, T. A., A. Crawford, J. M. Angelosanto, M. A. Paley, C. G. Ziegler, and E. J. Wherry. 2012. Network analysis reveals centrally connected genes and pathways involved in CD8+ T cell exhaustion versus memory. *Immunity* 37: 1130-1144.
3. Schietinger, A., and P. D. Greenberg. 2014. Tolerance and exhaustion: defining mechanisms of T cell dysfunction. *Trends Immunol* 35: 51-60.
4. Wherry, E. J. 2011. T cell exhaustion. *Nature immunology* 12: 492-499.
5. Wherry, E. J., and M. Kurachi. 2015. Molecular and cellular insights into T cell exhaustion. *Nature reviews. Immunology* 15: 486-499.
6. Lines, J. L., E. Pantazi, J. Mak, L. F. Sempere, L. Wang, S. O'Connell, S. Ceeraz, A. A. Suriawinata, S. Yan, M. S. Ernstoff, and R. Noelle. 2014. VISTA is an immune checkpoint molecule for human T cells. *Cancer Res* 74: 1924-1932.
7. Lines, J. L., L. F. Sempere, T. Broughton, L. Wang, and R. Noelle. 2014. VISTA is a novel broad-spectrum negative checkpoint regulator for cancer immunotherapy. *Cancer immunology research* 2: 510-517.
8. Mulati, K., J. Hamanishi, N. Matsumura, K. Chamoto, N. Mise, K. Abiko, T. Baba, K. Yamaguchi, N. Horikawa, R. Murakami, M. Taki, K. Budiman, X. Zeng, Y. Hosoe, M. Azuma, I. Konishi, and M. Mandai. 2019. VISTA expressed in tumour cells regulates T cell function. *Br J Cancer* 120: 115-127.
9. Shahbaz, S., N. Bozorgmehr, P. Koleva, A. Namdar, J. Jovel, R. A. Fava, and S. Elahi. 2018. CD71+VISTA+ erythroid cells promote the development and function of regulatory T cells through TGF-beta. *PLoS Biol* 16: e2006649.
10. Virgin, H. W., E. J. Wherry, and R. Ahmed. 2009. Redefining chronic viral infection. *Cell* 138: 30-50.
11. Hashimoto, M., A. O. Kamphorst, S. J. Im, H. T. Kissick, R. N. Pillai, S. S. Ramalingam, K. Araki, and R. Ahmed. 2018. CD8 T Cell Exhaustion in Chronic Infection and Cancer: Opportunities for Interventions. *Annu Rev Med* 69: 301-318.
12. Doitsh, G., and W. C. Greene. 2016. Dissecting How CD4 T Cells Are Lost During HIV Infection. *Cell host & microbe* 19: 280-291.
13. Elahi, S., R. H. Weiss, and S. Merani. 2016. Atorvastatin restricts HIV replication in CD4+ T cells by upregulation of p21. *Aids* 30: 171-183.
14. Janssen, E. M., E. E. Lemmens, T. Wolfe, U. Christen, M. G. von Herrath, and S. P. Schoenberger. 2003. CD4+ T cells are required for secondary expansion and memory in CD8+ T lymphocytes. *Nature* 421: 852-856.
15. Wang, J. C. E., and A. M. Livingstone. 2003. Cutting edge: CD4(+) T cell help can be essential for primary CD8(+) T cell responses in vivo. *Journal of immunology* 171: 6339-6343.
16. Crawford, A., J. M. Angelosanto, C. Kao, T. A. Doering, P. M. Odorizzi, B. E. Barnett, and E. J. Wherry. 2014. Molecular and transcription basis of CD4(+) T cell dysfunction during chronic infection. *Immunity* 40: 289-302.

17. Merani, S., W. Chen, and S. Elahi. 2015. The bitter side of sweet: the role of Galectin-9 in immunopathogenesis of viral infections. *Reviews in medical virology* 25: 175-186.
18. Li, Y., J. Feng, S. Geng, H. Wei, G. Chen, X. Li, L. Wang, R. Wang, H. Peng, G. Han, and B. Shen. 2011. The N- and C-terminal carbohydrate recognition domains of galectin-9 contribute differently to its multiple functions in innate immunity and adaptive immunity. *Mol Immunol* 48: 670-677.
19. Hirashima, M., Y. Kashio, N. Nishi, A. Yamauchi, T. A. Imaizumi, T. Kageshita, N. Saita, and T. Nakamura. 2004. Galectin-9 in physiological and pathological conditions. *Glycoconjugate journal* 19: 593-600.
20. Zhu, C., A. C. Anderson, A. Schubart, H. Xiong, J. Imitola, S. J. Khoury, X. X. Zheng, T. B. Strom, and V. K. Kuchroo. 2005. The TIM-3 ligand galectin-9 negatively regulates T helper type 1 immunity. *Nature immunology* 6: 1245-1252.
21. Bi, S., P. W. Hong, B. Lee, and L. G. Baum. 2011. Galectin-9 binding to cell surface protein disulfide isomerase regulates the redox environment to enhance T-cell migration and HIV entry. *Proc Natl Acad Sci U S A* 108: 10650-10655.
22. Niki, T., S. Tsutsui, S. Hirose, S. Aradono, Y. Sugimoto, K. Takeshita, N. Nishi, and M. Hirashima. 2009. Galectin-9 is a high affinity IgE-binding lectin with anti-allergic effect by blocking IgE-antigen complex formation. *The Journal of biological chemistry* 284: 32344-32352.
23. Madireddi, S., S. Y. Eun, S. W. Lee, I. Nemcovicova, A. K. Mehta, D. M. Zajonc, N. Nishi, T. Niki, M. Hirashima, and M. Croft. 2014. Galectin-9 controls the therapeutic activity of 4-1BB-targeting antibodies. *J Exp Med* 211: 1433-1448.
24. Tanikawa, R., T. Tanikawa, M. Hirashima, A. Yamauchi, and Y. Tanaka. 2010. Galectin-9 induces osteoblast differentiation through the CD44/Smad signaling pathway. *Biochem Biophys Res Commun* 394: 317-322.
25. Elahi, S., and H. Horton. 2012. Association of HLA-alleles with the immune regulation of chronic viral infections. *The international journal of biochemistry & cell biology* 44: 1361-1365.
26. Wang, F., L. Wan, C. Zhang, X. Zheng, J. Li, and Z. K. Chen. 2009. TIM-3-Galectin-9 pathway involves the suppression induced by CD4+CD25+ regulatory T cells. *Immunobiology* 214: 342-349.
27. Crux, N. B., and S. Elahi. 2017. Human Leukocyte Antigen (HLA) and Immune Regulation: How Do Classical and Non-Classical HLA Alleles Modulate Immune Response to Human Immunodeficiency Virus and Hepatitis C Virus Infections? *Frontiers in immunology* 8: 832.
28. Elahi, S., W. L. Dinges, N. Lejarcegui, K. J. Laing, A. C. Collier, D. M. Koelle, M. J. McElrath, and H. Horton. 2011. Protective HIV-specific CD8+ T cells evade Treg cell suppression. *Nature medicine* 17: 989-995.
29. Niwa, H., T. Satoh, Y. Matsushima, K. Hosoya, K. Saeki, T. Niki, M. Hirashima, and H. Yokozeki. 2009. Stable form of galectin-9, a TIM-3 ligand, inhibits contact hypersensitivity and psoriatic reactions: a potent therapeutic tool for Th1- and/or Th17-mediated skin inflammation. *Clin Immunol* 132: 184-194.
30. Seki, M., S. Oomizu, K. M. Sakata, A. Sakata, T. Arikawa, K. Watanabe, K. Ito, K. Takeshita, T. Niki, N. Saita, N. Nishi, A. Yamauchi, S. Katoh, A. Matsukawa, V. Kuchroo, and M. Hirashima. 2008. Galectin-9 suppresses the generation of Th17, promotes the induction of regulatory T cells, and regulates experimental autoimmune arthritis. *Clin Immunol* 127: 78-88.
31. Wu, C., T. Thalhamer, R. F. Franca, S. Xiao, C. Wang, C. Hotta, C. Zhu, M. Hirashima, A. C. Anderson, and V. K. Kuchroo. 2014. Galectin-9-CD44 Interaction Enhances Stability and Function of Adaptive Regulatory T Cells. *Immunity* 41: 270-282.
32. Sharma, S., A. Sundararajan, A. Suryawanshi, N. Kumar, T. Veiga-Parga, V. K. Kuchroo, P. G. Thomas, M. Y. Sangster, and B. T. Rouse. 2011. T cell immunoglobulin and mucin protein-3 (TIM-3)/Galectin-9 interaction regulates influenza A virus-specific humoral and CD8 T-cell responses. *Proceedings of the National Academy of Sciences of the United States of America* 108: 19001-19006.

33. Chagan-Yasutan, H., H. Saitoh, Y. Ashino, T. Arikawa, M. Hirashima, S. Li, M. Usuzawa, S. Oguma, O. T. EF, C. L. Obi, and T. Hattori. 2009. Persistent elevation of plasma osteopontin levels in HIV patients despite highly active antiretroviral therapy. *Tohoku J Exp Med* 218: 285-292.
34. Elahi, S., T. Niki, M. Hirashima, and H. Horton. 2012. Galectin-9 binding to TIM-3 renders activated human CD4+ T cells less susceptible to HIV-1 infection. *Blood* 119: 4192-4204.
35. Tandon, R., G. M. Chew, M. M. Byron, P. Borrow, T. Niki, M. Hirashima, J. D. Barbour, P. J. Norris, M. C. Lanteri, J. N. Martin, S. G. Deeks, and L. C. Ndhlovu. 2014. Galectin-9 Is Rapidly Released During Acute HIV-1 Infection and Remains Sustained at High Levels Despite Viral Suppression Even in Elite Controllers. *AIDS research and human retroviruses*.
36. Bi, S. G., P. W. Hong, B. Lee, and L. G. Baum. 2011. Galectin-9 binding to cell surface protein disulfide isomerase regulates the redox environment to enhance T-cell migration and HIV entry. *Proceedings of the National Academy of Sciences of the United States of America* 108: 10650-10655.
37. Abdel-Mohsen, M., L. Chavez, R. Tandon, G. M. Chew, X. T. Deng, A. Danesh, S. Keating, M. Lanteri, M. L. Samuels, R. Hoh, J. B. Sacha, P. J. Norris, T. Niki, C. M. Shikuma, M. Hirashima, S. G. Deeks, L. C. Ndhlovu, and S. K. Pillai. 2016. Human Galectin-9 Is a Potent Mediator of HIV Transcription and Reactivation. *Plos Pathogens* 12.
38. Colomb, F., L. B. Giron, T. A. Premeaux, B. I. Mitchell, T. Niki, E. Papasavvas, L. J. Montaner, L. C. Ndhlovu, and M. Abdel-Mohsen. 2019. Galectin-9 Mediates HIV Transcription by Inducing TCR-Dependent ERK Signaling. *Frontiers in immunology* 10: 267.
39. Dunsmore, G., N. Bozorgmehr, C. Delyea, P. Koleva, A. Namdar, and S. Elahi. 2017. Erythroid Suppressor Cells Compromise Neonatal Immune Response against Bordetella pertussis. *Journal of immunology* 199: 2081-2095.
40. Namdar, A., P. Koleva, S. Shahbaz, S. Strom, V. Gerdts, and S. Elahi. 2017. CD71+ erythroid suppressor cells impair adaptive immunity against Bordetella pertussis. *Sci Rep* 7: 7728.
41. Lorenzo-Herrero, S., C. Sordo-Bahamonde, S. Gonzalez, and A. Lopez-Soto. 2019. CD107a Degranulation Assay to Evaluate Immune Cell Antitumor Activity. *Methods Mol Biol* 1884: 119-130.
42. Dunsmore, G., P. Koleva, N. Ghobakhloo, R. T. Sutton, L. Ambrosio, X. Meng, N. Hotte, V. Nguyen, K. L. Madsen, L. A. Dieleman, V. Huang, and S. Elahi. 2018. Lower Abundance and Impaired Function of CD71+ Erythroid Cells in Inflammatory Bowel Disease Patients During Pregnancy. *J Crohns Colitis*.
43. Gupta, P. K., J. Godec, D. Wolski, E. Adland, K. Yates, K. E. Pauken, C. Cosgrove, C. Ledderose, W. G. Junger, S. C. Robson, E. J. Wherry, G. Alter, P. J. Goulder, P. Klenerman, A. H. Sharpe, G. M. Lauer, and W. N. Haining. 2015. CD39 Expression Identifies Terminally Exhausted CD8+ T Cells. *PLoS Pathog* 11: e1005177.
44. Yamamoto, T., D. A. Price, J. P. Casazza, G. Ferrari, M. Nason, P. K. Chattopadhyay, M. Roederer, E. Gostick, P. D. Katsikis, D. C. Douek, R. Haubrich, C. Petrovas, and R. A. Koup. 2011. Surface expression patterns of negative regulatory molecules identify determinants of virus-specific CD8+ T-cell exhaustion in HIV infection. *Blood* 117: 4805-4815.
45. Chew, G. M., T. Fujita, G. M. Webb, B. J. Burwitz, H. L. Wu, J. S. Reed, K. B. Hammond, K. L. Clayton, N. Ishii, M. Abdel-Mohsen, T. Liegler, B. I. Mitchell, F. M. Hecht, M. Ostrowski, C. M. Shikuma, S. G. Hansen, M. Maurer, A. J. Korman, S. G. Deeks, J. B. Sacha, and L. C. Ndhlovu. 2016. TIGIT Marks Exhausted T Cells, Correlates with Disease Progression, and Serves as a Target for Immune Restoration in HIV and SIV Infection. *Plos Pathogens* 12.
46. Jones, R. B., L. C. Ndhlovu, J. D. Barbour, P. M. Sheth, A. R. Jha, B. R. Long, J. C. Wong, M. Satkunarajah, M. Schweneker, J. M. Chapman, G. Gyenes, B. Vali, M. D. Hyrcza, F. Y. Yue, C. Kovacs, A. Sassi, M. Loutfy, R. Halpenny, D. Persad, G. Spotts, F. M. Hecht, T. W. Chun, J. M. McCune, R. Kaul, J. M. Rini, D. F. Nixon, and M. A. Ostrowski. 2008. TIM-3 expression defines a novel population of dysfunctional T cells with highly elevated frequencies in progressive HIV-1 infection. *The Journal of experimental medicine* 205: 2763-2779.

47. Hersperger, A. R., G. Makedonas, and M. R. Betts. 2008. Flow cytometric detection of perforin upregulation in human CD8 T cells. *Cytometry A* 73: 1050-1057.
48. Wu, X., H. Zhang, Q. Xing, J. Cui, J. Li, Y. Li, Y. Tan, and S. Wang. 2014. PD-1(+) CD8(+) T cells are exhausted in tumours and functional in draining lymph nodes of colorectal cancer patients. *Br J Cancer* 111: 1391-1399.
49. Bajnok, A., M. Ivanova, J. Rigo, Jr., and G. Toldi. 2017. The Distribution of Activation Markers and Selectins on Peripheral T Lymphocytes in Preeclampsia. *Mediators Inflamm* 2017: 8045161.
50. Buggert, M., J. Frederiksen, O. Lund, M. R. Betts, A. Biague, M. Nielsen, J. Tauriainen, H. Norrgren, P. Medstrand, A. C. Karlsson, M. Jansson, and S. C. group. 2016. CD4+ T cells with an activated and exhausted phenotype distinguish immunodeficiency during aviremic HIV-2 infection. *Aids* 30: 2415-2426.
51. Motamedi, M., L. Xu, and S. Elahi. 2016. Correlation of transferrin receptor (CD71) with Ki67 expression on stimulated human and mouse T cells: The kinetics of expression of T cell activation markers. *J Immunol Methods* 437: 43-52.
52. Gerdes, J., H. Lemke, H. Baisch, H. H. Wacker, U. Schwab, and H. Stein. 1984. Cell cycle analysis of a cell proliferation-associated human nuclear antigen defined by the monoclonal antibody Ki-67. *Journal of immunology* 133: 1710-1715.
53. Liao, W., J. X. Lin, L. Wang, P. Li, and W. J. Leonard. 2011. Modulation of cytokine receptors by IL-2 broadly regulates differentiation into helper T cell lineages. *Nature immunology* 12: 551-U247.
54. Liao, W., D. E. Schones, J. Oh, Y. Cui, K. Cui, T. Y. Roh, K. Zhao, and W. J. Leonard. 2008. Priming for T helper type 2 differentiation by interleukin 2-mediated induction of interleukin 4 receptor alpha-chain expression. *Nature immunology* 9: 1288-1296.
55. Lowe, R. M., A. Genin, N. Orgun, and R. Q. Cron. 2014. IL-15 prolongs CD154 expression on human CD4 T cells via STAT5 binding to the CD154 transcription promoter. *Genes Immun* 15: 137-144.
56. Skov, S., M. Bonyhadi, N. Odum, and J. A. Ledbetter. 2000. IL-2 and IL-15 regulate CD154 expression on activated CD4 T cells. *Journal of immunology* 164: 3500-3505.
57. Palmer, C. S., M. Ostrowski, M. Gouillou, L. Tsai, D. Yu, J. L. Zhou, D. C. Henstridge, A. Maisa, A. C. Hearps, S. R. Lewin, A. Landay, A. Jaworowski, J. M. McCune, and S. M. Crowe. 2014. Increased glucose metabolic activity is associated with CD4 R T-cell activation and depletion during chronic HIV infection. *Aids* 28: 297-309.
58. Fatrai, S., A. T. J. Wierenga, S. M. G. J. Daenen, E. Vellenga, and J. J. Schuringa. 2011. Identification of HIF2 alpha as an important STAT5 target gene in human hematopoietic stem cells. *Blood* 117: 3320-3330.
59. Paley, M. A., D. C. Kroy, P. M. Odorizzi, J. B. Johnnidis, D. V. Dolfi, B. E. Barnett, E. K. Bikoff, E. J. Robertson, G. M. Lauer, S. L. Reiner, and E. J. Wherry. 2012. Progenitor and terminal subsets of CD8+ T cells cooperate to contain chronic viral infection. *Science* 338: 1220-1225.
60. Meng, X., X. Liu, X. Guo, S. Jiang, T. Chen, Z. Hu, H. Liu, Y. Bai, M. Xue, R. Hu, S. C. Sun, X. Liu, P. Zhou, X. Huang, L. Wei, W. Yang, and C. Xu. 2018. FBXO38 mediates PD-1 ubiquitination and regulates anti-tumour immunity of T cells. *Nature* 564: 130-135.
61. Staron, M. M., S. M. Gray, H. D. Marshall, I. A. Parish, J. H. Chen, C. J. Perry, G. Cui, M. O. Li, and S. M. Kaech. 2014. The transcription factor FoxO1 sustains expression of the inhibitory receptor PD-1 and survival of antiviral CD8(+) T cells during chronic infection. *Immunity* 41: 802-814.
62. Wherry, E. J., S. J. Ha, S. M. Kaech, W. N. Haining, S. Sarkar, V. Kalia, S. Subramaniam, J. N. Blattman, D. L. Barber, and R. Ahmed. 2007. Molecular signature of CD8+ T cell exhaustion during chronic viral infection. *Immunity* 27: 670-684.
63. Shin, H., S. D. Blackburn, A. M. Intlekofer, C. Kao, J. M. Angelosanto, S. L. Reiner, and E. J. Wherry. 2009. A role for the transcription repressor Blimp-1 in CD8(+) T cell exhaustion during chronic viral infection. *Immunity* 31: 309-320.

64. Ferris, R. L., B. Lu, and L. P. Kane. 2014. Too much of a good thing? TIM-3 and TCR signaling in T cell exhaustion. *Journal of immunology* 193: 1525-1530.
65. Nel, A. E. 2002. T-cell activation through the antigen receptor. Part 1: Signaling components. signaling pathways, and signal integration at the T-cell antigen receptor synapse (vol 109, pg 758, 2002). *J Allergy Clin Immun* 110: 25-25.
66. Smith, J. S., and N. J. Robinson. 2002. Age-specific prevalence of infection with herpes simplex virus types 2 and 1: a global review. *The Journal of infectious diseases* 186 Suppl 1: S3-28.
67. Hayashi, K., and A. Altman. 2007. Protein kinase C theta (PKCtheta): a key player in T cell life and death. *Pharmacol Res* 55: 537-544.
68. Phetsouphanh, C., and A. D. Kelleher. 2015. The role of PKC-theta in CD4+T cells and HIV infection: to the nucleus and back again. *Frontiers in immunology* 6.
69. Trushin, S. A., G. D. Bren, S. Asin, K. N. Pennington, C. V. Paya, and A. D. Badley. 2005. Human immunodeficiency virus reactivation by phorbol esters or T-cell receptor Ligation requires both PKC alpha and PKC theta. *Journal of virology* 79: 9821-9830.
70. Oomizu, S., T. Arikawa, T. Niki, T. Kadowaki, M. Ueno, N. Nishi, A. Yamauchi, T. Hattori, T. Masaki, and M. Hirashima. 2012. Cell surface galectin-9 expressing Th cells regulate Th17 and Foxp3+ Treg development by galectin-9 secretion. *PloS one* 7: e48574.
71. David, P., D. A. Megger, T. Kaiser, T. Werner, J. Liu, L. Chen, B. Sitek, U. Dittmer, and G. Zelinskyy. 2019. The PD-1/PD-L1 Pathway Affects the Expansion and Function of Cytotoxic CD8(+) T Cells During an Acute Retroviral Infection. *Frontiers in immunology* 10: 54.
72. Foger, N., R. Marhaba, and M. Zoller. 2000. CD44 supports T cell proliferation and apoptosis by apposition of protein kinases. *European journal of immunology* 30: 2888-2899.
73. Leal, D. B. R., C. A. Streher, C. D. Bertoncheli, L. F. D. Carli, C. A. M. Leal, J. E. P. da Silva, V. M. Morsch, and M. R. C. Schetinger. 2005. HIV infection is associated with increased NTPDase activity that correlates with CD39-positive lymphocytes. *Bba-Mol Cell Res* 1746: 129-134.
74. Bharaj, P., H. S. Chahar, O. K. Alozie, L. Rodarte, A. Bansal, P. A. Goepfert, A. Dwivedi, N. Manjunath, and P. Shankar. 2014. Characterization of programmed death-1 homologue-1 (PD-1H) expression and function in normal and HIV infected individuals. *PloS one* 9: e109103.
75. Saitoh, H., Y. Ashino, H. Chagan-Yasutan, T. Niki, M. Hirashima, and T. Hattori. 2012. Rapid decrease of plasma galectin-9 levels in patients with acute HIV infection after therapy. *Tohoku J Exp Med* 228: 157-161.
76. Tandon, R., G. M. Chew, M. M. Byron, P. Borrow, T. Niki, M. Hirashima, J. D. Barbour, P. J. Norris, M. C. Lanteri, J. N. Martin, S. G. Deeks, and L. C. Ndhlovu. 2014. Galectin-9 Is Rapidly Released During Acute HIV-1 Infection and Remains Sustained at High Levels Despite Viral Suppression Even in Elite Controllers. *Aids Res Hum Retrov* 30: 654-664.
77. Motamedi, M., S. Shahbaz, L. Fu, G. Dunsmore, L. Xu, R. Harrington, S. Houston, and S. Elahi. 2019. Galectin-9 Expression Defines a Subpopulation of NK Cells with Impaired Cytotoxic Effector Molecules but Enhanced IFN-gamma Production, Dichotomous to TIGIT, in HIV-1 Infection. *Immunohorizons* 3: 531-546.
78. Sigal, A., and D. Baltimore. 2012. As good as it gets? The problem of HIV persistence despite antiretroviral drugs. *Cell host & microbe* 12: 132-138.
79. Zhou, Q., M. E. Munger, R. G. Veenstra, B. J. Weigel, M. Hirashima, D. H. Munn, W. J. Murphy, M. Azuma, A. C. Anderson, V. K. Kuchroo, and B. R. Blazar. 2011. Coexpression of TIM-3 and PD-1 identifies a CD8+ T-cell exhaustion phenotype in mice with disseminated acute myelogenous leukemia. *Blood* 117: 4501-4510.
80. Okoye, I., A. Namdar, L. Xu, N. Crux, and S. Elahi. 2017. Atorvastatin downregulates co-inhibitory receptor expression by targeting Ras-activated mTOR signalling. *Oncotarget* 8: 98215-98232.

81. Chlewicki, L. K., C. A. Velikovsky, V. Balakrishnan, R. A. Mariuzza, and V. Kumar. 2008. Molecular basis of the dual functions of 2B4 (CD244). *Journal of immunology* 180: 8159-8167.
82. Nishio, A., T. Tatsumi, T. Nawa, T. Suda, T. Yoshioka, Y. Onishi, S. Aono, M. Shigekawa, H. Hikita, R. Sakamori, D. Okuzaki, T. Fukuhara, Y. Matsuura, N. Hiramatsu, and T. Takehara. 2017. CD14(+) monocyte-derived galectin-9 induces natural killer cell cytotoxicity in chronic hepatitis C. *Hepatology* 65: 18-31.
83. Kalia, V., S. Sarkar, S. Subramaniam, W. N. Haining, K. A. Smith, and R. Ahmed. 2010. Prolonged interleukin-2Ralpha expression on virus-specific CD8+ T cells favors terminal-effector differentiation in vivo. *Immunity* 32: 91-103.
84. Reiser, J., and A. Banerjee. 2016. Effector, Memory, and Dysfunctional CD8(+) T Cell Fates in the Antitumor Immune Response. *J Immunol Res* 2016: 8941260.
85. Klebanoff, C. A., L. Gattinoni, and N. P. Restifo. 2006. CD8+ T-cell memory in tumor immunology and immunotherapy. *Immunol Rev* 211: 214-224.
86. Yi, J. S., M. A. Cox, and A. J. Zajac. 2010. T-cell exhaustion: characteristics, causes and conversion. *Immunology* 129: 474-481.
87. Sparks, R., D. M. Koelle, and S. Dhanireddy. 2015. Elevated Spontaneous Interferon-Gamma Secretion in HIV-Infected Persons. *J Allergy Clin Immun* 135: Ab100-Ab100.
88. Imaizumi, T., M. Kumagai, N. Sasaki, H. Kurotaki, F. Mori, M. Seki, N. Nishi, K. Fujimoto, K. Tanji, T. Shibata, W. Tamo, T. Matsumiya, H. Yoshida, X. F. Cui, S. Takanashi, K. Hanada, K. Okumura, S. Yagihashi, K. Wakabayashi, T. Nakamura, M. Hirashima, and K. Satoh. 2002. Interferon-gamma stimulates the expression of galectin-9 in cultured human endothelial cells. *J Leukocyte Biol* 72: 486-491.
89. Beltra, J. C., S. Bourbonnais, N. Bedard, T. Charpentier, M. Boulange, E. Michaud, I. Boufaied, J. Bruneau, N. H. Shoukry, A. Lamarre, and H. Decaluwe. 2016. IL2Rbeta-dependent signals drive terminal exhaustion and suppress memory development during chronic viral infection. *Proceedings of the National Academy of Sciences of the United States of America* 113: E5444-5453.
90. Howland, K. C., L. J. Ausubel, C. A. London, and A. K. Abbas. 2000. The roles of CD28 and CD40 ligand in T cell activation and tolerance. *Journal of immunology* 164: 4465-4470.
91. Shu, U., M. Kiniwa, C. Y. Wu, C. Maliszewski, N. Vezzio, J. Hakimi, M. Gately, and G. Delespesse. 1995. Activated T cells induce interleukin-12 production by monocytes via CD40-CD40 ligand interaction. *European journal of immunology* 25: 1125-1128.
92. Moller, J. F., B. Moller, B. Wiedenmann, T. Berg, and E. Schott. 2011. CD154, a marker of antigen-specific stimulation of CD4 T cells, is associated with response to treatment in patients with chronic HCV infection. *J Viral Hepatitis* 18: E341-E349.
93. MacIver, N. J., R. D. Michalek, and J. C. Rathmell. 2013. Metabolic regulation of T lymphocytes. *Annual review of immunology* 31: 259-283.
94. Schurich, A., L. J. Pallett, D. Jajbhay, J. Wijngaarden, I. Otano, U. S. Gill, N. Hansi, P. T. Kennedy, E. Nastouli, R. Gilson, C. Frezza, S. M. Henson, and M. K. Maini. 2016. Distinct Metabolic Requirements of Exhausted and Functional Virus-Specific CD8 T Cells in the Same Host. *Cell Rep* 16: 1243-1252.
95. Siska, P. J., and J. C. Rathmell. 2015. PKCs Sweeten Cell Metabolism by Phosphorylation of Glut1. *Mol Cell* 58: 711-712.
96. Gatenby, R. A., E. T. Gawlinski, A. F. Gmitro, B. Kaylor, and R. J. Gillies. 2006. Acid-mediated tumor invasion: a multidisciplinary study. *Cancer Res* 66: 5216-5223.
97. Gatenby, R. A., and R. J. Gillies. 2004. Why do cancers have high aerobic glycolysis? *Nat Rev Cancer* 4: 891-899.
98. Lhuillier, C., C. Barjon, T. Niki, A. Gelin, F. Praz, O. Morales, S. Souquere, M. Hirashima, M. Wei, O. Dellis, and P. Busson. 2015. Impact of Exogenous Galectin-9 on Human T Cells: CONTRIBUTION OF THE T CELL RECEPTOR COMPLEX TO ANTIGEN-INDEPENDENT ACTIVATION BUT NOT TO APOPTOSIS INDUCTION. *The Journal of biological chemistry* 290: 16797-16811.

99. Hsu, D. K., H. Y. Chen, and F. T. Liu. 2009. Galectin-3 regulates T-cell functions. *Immunol Rev* 230: 114-127.

Chapter 3: Elevated ATP via enhanced miRNA-30b, 30c, and 30e downregulates the expression of CD73 in $CD8^+$ T cells of HIV-infected individuals

1. Shahbaz, S., M. Manicardi, G. Guaraldi, and P. Raggi. 2015. Cardiovascular disease in human immunodeficiency virus infected patients: A true or perceived risk? *World Journal of Cardiology* 7: 633–644.
2. Deeks, S. G., S. R. Lewin, and D. V. Havlir. 2013. The End of AIDS: HIV Infection as a Chronic Disease. *Lancet* 382: 1525–1533.
3. Elahi, S., R. H. Weiss, and S. Merani. 2016. Atorvastatin restricts HIV replication in CD4+ T cells by upregulation of p21. *AIDS* 30: 171–183.
4. Paiardini, M., and M. Müller-Trutwin. 2013. HIV-associated chronic immune activation. *Immunol Rev* 254: 78–101.
5. Bono, M. R., D. Fernández, F. Flores-Santibáñez, M. Rosemblatt, and D. Sauma. 2015. CD73 and CD39 ectonucleotidases in T cell differentiation: Beyond immunosuppression. *FEBS Letters* 589: 3454–3460.
6. Beavis, Paul. A., J. Stagg, P. K. Darcy, and M. J. Smyth. 2012. CD73: a potent suppressor of antitumor immune responses. *Trends in Immunology* 33: 231–237.
7. Resta, R., Y. Yamashita, and L. F. Thompson. 1998. Ecto-enzyme and signaling functions of lymphocyte CD 7 3. *Immunological Reviews* 161: 95–109.
8. Airas, L., J. Niemelä, and S. Jalkanen. 2000. CD73 Engagement Promotes Lymphocyte Binding to Endothelial Cells Via a Lymphocyte Function-Associated Antigen-1-Dependent Mechanism. *The Journal of Immunology* 165: 5411–5417.
9. Tóth, I., A. Q. Le, P. Hartjen, A. Thomssen, V. Matzat, C. Lehmann, C. Scheurich, C. Beisel, P. Busch, O. Degen, A. W. Lohse, T. Eiermann, G. Fätkenheuer, D. Meyer-Olson, M. Bockhorn, J. Hauber, J. van Lunzen, and J. Schulze Zur Wiesch. 2013. Decreased frequency of CD73+CD8+ T cells of HIV-infected patients correlates with immune activation and T cell exhaustion. *Journal of Leukocyte Biology* 94: 551–561.
10. Velasquez, S., L. Prevedel, S. Valdebenito, A. M. Gorska, M. Golovko, N. Khan, J. Geiger, and E. A. Eugenin. 2020. Circulating levels of ATP is a biomarker of HIV cognitive impairment. *EBioMedicine* 51: 102503.
11. Kordaß, T., W. Osen, and S. B. Eichmüller. 2018. Controlling the Immune Suppressor: Transcription Factors and MicroRNAs Regulating CD73/NT5E. *Front Immunol* 9.
12. Hansen, K. R., R. Resta, C. F. Webb, and L. F. Thompson. 1995. Isolation and characterization of the promoter of the human 5'-nucleotidase (CD73)-encoding gene. *Gene* 167: 307–312.
13. Fausther, M., N. Sheung, Y. Saiman, M. B. Bansal, and J. A. Dranoff. 2012. Activated hepatic stellate cells upregulate transcription of ecto-5'-nucleotidase/CD73 via specific SP1 and SMAD promoter elements. *American Journal of Physiology-Gastrointestinal and Liver Physiology* 303: G904–G914.
14. Synnestvedt, K., G. T. Furuta, K. M. Comerford, N. Louis, J. Karhausen, H. K. Eltzschig, K. R. Hansen, L. F. Thompson, and S. P. Colgan. 2002. Ecto-5'-nucleotidase (CD73) regulation by hypoxia-inducible factor-1 mediates permeability changes in intestinal epithelia. *J Clin Invest* 110: 993–1002.
15. Colgan, S. P., H. K. Eltzschig, T. Eckle, and L. F. Thompson. 2006. Physiological roles for ecto-5'-nucleotidase (CD73). *Purinergic Signalling* 2: 351.
16. Chalmin, F., G. Mignot, M. Bruchard, A. Chevriaux, F. Végran, A. Hichami, S. Ladoire, V. Derangère, J. Vincent, D. Masson, S. C. Robson, G. Eberl, J. R. Pallandre, C. Borg, B. Ryffel, L. Apetoh, C. Rébé, and F.

Ghiringhelli. 2012. Stat3 and Gfi-1 Transcription Factors Control Th17 Cell Immunosuppressive Activity via the Regulation of Ectonucleotidase Expression. *Immunity* 36: 362–373.
17. Zheng, Y., S. Z. Josefowicz, A. Kas, T.-T. Chu, M. A. Gavin, and A. Y. Rudensky. 2007. Genome-wide analysis of Foxp3 target genes in developing and mature regulatory T cells. *Nature* 445: 936–940.
18. Zhu, J., Y. Zeng, W. Li, H. Qin, Z. Lei, D. Shen, D. Gu, J. Huang, and Z. Liu. 2017. CD73/NT5E is a target of miR-30a-5p and plays an important role in the pathogenesis of non-small cell lung cancer. *Molecular Cancer* 16: 34.
19. Wang, N., X. Xiang, K. Chen, P. Liu, and A. Zhu. 2018. Targeting of NT5E by miR-30b and miR-340 attenuates proliferation, invasion and migration of gallbladder carcinoma. *Biochimie* 146: 56–67.
20. Zhang, F., Y. Luo, Z. Shao, L. Xu, X. Liu, Y. Niu, J. Shi, X. Sun, Y. Liu, Y. Ding, and L. Zhao. 2016. MicroRNA-187, a downstream effector of TGFβ pathway, suppresses Smad-mediated epithelial–mesenchymal transition in colorectal cancer. *Cancer Letters* 373: 203–213.
21. Ikeda, Y., E. Tanji, N. Makino, S. Kawata, and T. Furukawa. 2012. MicroRNAs Associated with Mitogen-Activated Protein Kinase in Human Pancreatic Cancer. *Mol Cancer Res* 10: 259–269.
22. Braun, J., C. Hoang-Vu, H. Dralle, and S. Hüttelmaier. 2010. Downregulation of microRNAs directs the EMT and invasive potential of anaplastic thyroid carcinomas. *Oncogene* 29: 4237–4244.
23. Chanda, S., S. Nandi, and M. Chawla-Sarkar. 2016. Rotavirus-induced miR-142-5p elicits proviral milieu by targeting non-canonical transforming growth factor beta signalling and apoptosis in cells. *Cellular Microbiology* 18: 733–747.
24. Elahi, S., W. L. Dinges, N. Lejarcegui, K. J. Laing, A. C. Collier, D. M. Koelle, M. J. McElrath, and H. Horton. 2011. Protective HIV-specific CD8+ T cells evade Treg cell suppression. *Nat Med* 17: 989–995.
25. Shahbaz, S., G. Dunsmore, P. Koleva, L. Xu, S. Houston, and S. Elahi. 2020. Galectin-9 and VISTA Expression Define Terminally Exhausted T Cells in HIV-1 Infection. *J Immunol* 204: 2474–2491.
26. Dunsmore, G., N. Bozorgmehr, C. Delyea, P. Koleva, A. Namdar, and S. Elahi. 2017. Erythroid Suppressor Cells Compromise Neonatal Immune Response against Bordetella pertussis. *J. Immunol.* 199: 2081–2095.
27. Lorenzo-Herrero, S., C. Sordo-Bahamonde, S. Gonzalez, and A. López-Soto. 2019. CD107a Degranulation Assay to Evaluate Immune Cell Antitumor Activity. *Methods Mol Biol* 1884: 119–130.
28. Leal, D. B. R., C. A. Streher, C. de M. Bertoncheli, L. F. D. Carli, C. A. M. Leal, J. E. P. da Silva, V. M. Morsch, and M. R. C. Schetinger. 2005. HIV infection is associated with increased NTPDase activity that correlates with CD39-positive lymphocytes. *Biochim Biophys Acta* 1746: 129–134.
29. Migueles, S. A., and M. Connors. 2001. Frequency and function of HIV-specific CD8(+) T cells. *Immunol Lett* 79: 141–150.
30. Collins, D. R., G. D. Gaiha, and B. D. Walker. 2020. CD8 + T cells in HIV control, cure and prevention. *Nature Reviews Immunology* 20: 471–482.
31. Perdomo-Celis, F., N. A. Taborda, and M. T. Rugeles. 2019. CD8+ T-Cell Response to HIV Infection in the Era of Antiretroviral Therapy. *Front Immunol* 10.
32. Gonzalez, S. M., N. A. Taborda, and M. T. Rugeles. 2017. Role of Different Subpopulations of CD8+ T Cells during HIV Exposure and Infection. *Front Immunol* 8.
33. Lau, L. L., B. D. Jamieson, T. Somasundaram, and R. Ahmed. 1994. Cytotoxic T-cell memory without antigen. *Nature* 369: 648–652.
34. Persistence of Memory CD8 T Cells in MHC Class I-Deficient Mice | Science. .
35. Kong, Y., B. Jia, C. Zhao, D. F. Claxton, A. Sharma, C. Annageldiyev, J. S. Fotos, H. Zeng, R. F. Paulson, K. S. Prabhu, and H. Zheng. 2019. Downregulation of CD73 associates with T cell exhaustion in AML patients. *J Hematol Oncol* 12: 40.
36. Wehler, T. C., M. Karg, E. Distler, A. Konur, M. Nonn, R. G. Meyer, C. Huber, U. F. Hartwig, and W. Herr. 2008. Rapid identification and sorting of viable virus-reactive CD4+ and CD8+ T cells based on antigen-triggered CD137 expression. *Journal of Immunological Methods* 339: 23–37.

37. Olmo, N., J. Turnay, G. Risse, R. Deutzmann, K. von der Mark, and M. A. Lizarbe. 1992. Modulation of 5'-nucleotidase activity in plasma membranes and intact cells by the extracellular matrix proteins laminin and fibronectin. *Biochem J* 282: 181–188.
38. Sadej, R., K. Inai, Z. Rajfur, A. Ostapkowicz, J. Kohler, A. C. Skladanowski, B. S. Mitchell, and J. Spychala. 2008. Tenascin C interacts with ecto-5'-nucleotidase (eN) and regulates adenosine generation in cancer cells. *Biochim Biophys Acta* 1782: 35–40.
39. Adzic, M., and N. Nedeljkovic. 2018. Unveiling the Role of Ecto-5'-Nucleotidase/CD73 in Astrocyte Migration by Using Pharmacological Tools. *Front Pharmacol* 9.
40. Wagner, C., A. Bürger, M. Radsak, S. Blum, F. Hug, and G. M. Hänsch. 2000. Fibronectin synthesis by activated T lymphocytes: up-regulation of a surface-associated isoform with signalling function. *Immunology* 99: 532–539.
41. Kägi, D., F. Vignaux, B. Ledermann, K. Bürki, V. Depraetere, S. Nagata, H. Hengartner, and P. Golstein. 1994. Fas and perforin pathways as major mechanisms of T cell-mediated cytotoxicity. *Science* 265: 528–530.
42. Rouvier, E., M. F. Luciani, and P. Golstein. 1993. Fas involvement in Ca(2+)-independent T cell-mediated cytotoxicity. *J Exp Med* 177: 195–200.
43. Mills, J. H., L. F. Thompson, C. Mueller, A. T. Waickman, S. Jalkanen, J. Niemela, L. Airas, and M. S. Bynoe. 2008. CD73 is required for efficient entry of lymphocytes into the central nervous system during experimental autoimmune encephalomyelitis. *PNAS* 105: 9325–9330.
44. Rodriguez, M. W., A. C. Paquet, Y. H. Yang, and D. J. Erle. 2004. Differential gene expression by integrin β7+ and β7- memory T helper cells. *BMC Immunol* 5: 13.
45. Ålgars, A., M. Karikoski, G. G. Yegutkin, P. Stoitzner, J. Niemelä, M. Salmi, and S. Jalkanen. 2011. Different role of CD73 in leukocyte trafficking via blood and lymph vessels. *Blood* 117: 4387–4393.
46. SALMI, M., and S. JALKANEN. 1999. Molecules controlling lymphocyte migration to the gut. *Gut* 45: 148–153.
47. Höpken, U. E., J. Droese, J.-P. Li, J. Joergensen, D. Breitfeld, H.-G. Zerwes, and M. Lipp. 2004. The chemokine receptor CCR7 controls lymph node-dependent cytotoxic T cell priming in alloimmune responses. *Eur J Immunol* 34: 461–470.
48. Cao, S., and K. A. Woodrow. 2019. Nanotechnology approaches to eradicating HIV reservoirs. *European Journal of Pharmaceutics and Biopharmaceutics* 138: 48–63.
49. Kahle, E. M., M. Bolton, J. P. Hughes, D. Donnell, C. Celum, J. R. Lingappa, A. Ronald, C. R. Cohen, G. de Bruyn, Y. Fong, E. Katabira, M. J. McElrath, J. M. Baeten, C. Celum, A. Wald, J. Lingappa, A. Magaret, J. P. Hughes, L. Corey, J. M. Baeten, M. J. McElrath, D. Coetzee, K. Fife, E. Were, M. Essex, J. Makhema, E. Katabira, A. Ronald, E. Bukusi, C. Cohen, S. Allen, W. Kanweka, S. Kapiga, R. Manongi, C. Farquhar, G. John-Stewart, J. Kiarie, S. Allen, M. Inambao, O. Farm, S. Delany-Moretlwe, H. Rees, G. de Bruyn, G. Gray, J. McIntyre, and N. R. Mugo. 2015. Plasma Cytokine Levels and Risk of HIV Type 1 (HIV-1) Transmission and Acquisition: A Nested Case-Control Study Among HIV-1–Serodiscordant Couples. *J Infect Dis* 211: 1451–1460.
50. Orsilles, M. A., E. Pieri, P. Cooke, and C. Caula. 2006. IL-2 and IL-10 serum levels in HIV-1-infected patients with or without active antiretroviral therapy. *APMIS* 114: 55–60.
51. Salazar-Gonzalez, J. F., O. Martinez-Maza, N. Aziz, J. A. Kolberg, T. Yeghiazarian, L. P. Shen, and J. L. Fahey. 1997. Relationship of plasma HIV-RNA levels and levels of TNF-alpha and immune activation products in HIV infection. *Clin Immunol Immunopathol* 84: 36–45.
52. Wiercińska-Drapalo, A., R. Flisiak, J. Jaroszewicz, and D. Prokopowicz. 2004. Increased plasma transforming growth factor-beta1 is associated with disease progression in HIV-1-infected patients. *Viral Immunol* 17: 109–113.
53. Amiel, C., E. Darcissac, M.-J. Truong, J. Dewulf, M. Loyens, Y. Mouton, A. Capron, and G. M. Bahr. 1999. Interleukin-16 (IL-16) Inhibits Human Immunodeficiency Virus Replication in Cells from Infected

Subjects, and Serum IL-16 Levels Drop with Disease Progression. *The Journal of Infectious Diseases* 179: 83–91.
54. Osunkalu, V. O., A. Adewumi, C. Onwuzuluigbo, and O. F. Olowoselu. 2015. Interleukin-8 A Marker of Disease Progression and Therapeutic Response in HIV Infection. *Nigerian Hospital Practice* 15: 21–26.
55. Hardy, G. A. D., S. Sieg, B. Rodriguez, D. Anthony, R. Asaad, W. Jiang, J. Mudd, T. Schacker, N. T. Funderburg, H. A. Pilch-Cooper, R. Debernardo, R. L. Rabin, M. M. Lederman, and C. V. Harding. 2013. Interferon-α is the primary plasma type-I IFN in HIV-1 infection and correlates with immune activation and disease markers. *PLoS One* 8: e56527.
56. Xie, M., H. Qin, Q. Luo, Q. Huang, X. He, Z. Yang, P. Lan, and L. Lian. 2017. MicroRNA-30a regulates cell proliferation and tumor growth of colorectal cancer by targeting CD73. *BMC Cancer* 17.
57. Klemens, M. R., W. R. Sherman, N. J. Holmberg, J. M. Ruedi, M. G. Low, and L. F. Thompson. 1990. Characterization of soluble vs membrane-bound human placental 5'-nucleotidase. *Biochemical and Biophysical Research Communications* 172: 1371–1377.
58. Vogel, M., H. Kowalewski, H. Zimmermann, N. M. Hooper, and A. J. Turner. 1992. Soluble low-Km 5'-nucleotidase from electric-ray (Torpedo marmorata) electric organ and bovine cerebral cortex is derived from the glycosyl-phosphatidylinositol-anchored ectoenzyme by phospholipase C cleavage. *Biochemical Journal* 284: 621–624.
59. Morello, S., M. Capone, C. Sorrentino, D. Giannarelli, G. Madonna, D. Mallardo, A. M. Grimaldi, A. Pinto, and P. A. Ascierto. 2017. Soluble CD73 as biomarker in patients with metastatic melanoma patients treated with nivolumab. *Journal of Translational Medicine* 15: 244.
60. Wherry, E. J., S.-J. Ha, S. M. Kaech, W. N. Haining, S. Sarkar, V. Kalia, S. Subramaniam, J. N. Blattman, D. L. Barber, and R. Ahmed. 2007. Molecular signature of CD8+ T cell exhaustion during chronic viral infection. *Immunity* 27: 670–684.
61. Grünewald, J. K., and A. J. Ridley. 2010. CD73 represses pro-inflammatory responses in human endothelial cells. *Journal of Inflammation* 7: 10.
62. Kedzierska, K., and S. M. Crowe. 2001. Cytokines and HIV-1: interactions and clinical implications. *Antivir Chem Chemother* 12: 133–150.
63. Ellwanger, J. H., J. M. Valverde-Villegas, V. de L. Kaminski, R. M. de Medeiros, S. E. de M. Almeida, B. R. Santos, M. G. de Melo, F. S. Hackenhaar, and J. A. B. Chies. 2020. Increased IL-8 levels in HIV-infected individuals who initiated ART with CD4+ T cell counts <350 cells/mm3 – A potential hallmark of chronic inflammation. *Microbes and Infection* 22: 474–480.
64. Roff, S. R., E. N. Noon-Song, and J. K. Yamamoto. 2014. The Significance of Interferon-γ in HIV-1 Pathogenesis, Therapy, and Prophylaxis. *Front Immunol* 4.
65. Stefanou, M.-I., M. Krumbholz, U. Ziemann, and M. C. Kowarik. 2019. Human immunodeficiency virus and multiple sclerosis: a review of the literature. *Neurological Research and Practice* 1: 24.
66. Petrovic, A., O. Alpdogan, L. M. Willis, J. M. Eng, A. S. Greenberg, B. J. Kappel, C. Liu, G. J. Murphy, G. Heller, and M. R. M. van den Brink. 2004. LPAM (alpha 4 beta 7 integrin) is an important homing integrin on alloreactive T cells in the development of intestinal graft-versus-host disease. *Blood* 103: 1542–1547.
67. Hunter, M. C., A. Teijeira, and C. Halin. 2016. T Cell Trafficking through Lymphatic Vessels. *Front. Immunol.* 7.
68. Kim, E.-S., C. Ackermann, I. Tóth, P. Dierks, J. M. Eberhard, R. Wroblewski, F. Scherg, M. Geyer, R. E. Schmidt, C. Beisel, M. Bockhorn, F. Haag, J. van Lunzen, and J. S. zur Wiesch. 2017. Down-regulation of CD73 on B cells of patients with viremic HIV correlates with B cell activation and disease progression. *Journal of Leukocyte Biology* 101: 1263–1271.

Chapter 4: Differential transcription and functional properties of regulatory T cells in HIV-infected individuals on antiretroviral therapy and Long-term non-progressors

1. Elahi, S., R. H. Weiss, and S. Merani. 2016. Atorvastatin restricts HIV replication in CD4+ T cells by upregulation of p21. *AIDS* 30: 171–183.
2. Massanella, M., E. Negredo, N. Pérez-Álvarez, J. Puig, R. Ruiz-Hernández, M. Bofill, B. Clotet, and J. Blanco. 2010. CD4 T-cell hyperactivation and susceptibility to cell death determine poor CD4 T-cell recovery during suppressive HAART. *AIDS* 24: 959–968.
3. López, M., J. San Román, V. Estrada, E. Vispo, F. Blanco, and V. Soriano. 2012. Endothelial dysfunction in HIV infection--the role of circulating endothelial cells, microparticles, endothelial progenitor cells and macrophages. *AIDS reviews* 14: 223–230.
4. Elahi, S., T. Niki, M. Hirashima, and H. Horton. 2012. Galectin-9 binding to TIM-3 renders activated human CD4+ T cells less susceptible to HIV-1 infection. *Blood* 119: 4192–4204.
5. Aandahl, E. M., J. Michaëlsson, W. J. Moretto, F. M. Hecht, and D. F. Nixon. 2004. Human CD4+ CD25+ Regulatory T Cells Control T-Cell Responses to Human Immunodeficiency Virus and Cytomegalovirus Antigens. *J Virol* 78: 2454–2459.
6. Sakaguchi, S. 2004. Naturally arising CD4+ regulatory t cells for immunologic self-tolerance and negative control of immune responses. *Annual Review of Immunology* 22: 531–562.
7. Hori, S., T. Nomura, and S. Sakaguchi. 2003. Control of regulatory T cell development by the transcription factor Foxp3. *Science (New York, N.Y.)* 299: 1057–1061.
8. Mechanisms of foxp3+ T regulatory cell-mediated suppression - PubMed. .
9. Rothstein, D. M., and G. Camirand. 2015. New insights into the mechanisms of Treg function. *Curr Opin Organ Transplant* 20: 376–384.
10. Lee, J. H., S. G. Kang, and C. H. Kim. 2007. FoxP3+ T cells undergo conventional first switch to lymphoid tissue homing receptors in thymus but accelerated second switch to nonlymphoid tissue homing receptors in secondary lymphoid tissues. *J Immunol* 178: 301–311.
11. Increased tumor-infiltrating CD45RA − CCR7 − regulatory T-cell subset with immunosuppressive properties foster gastric cancer progress | Cell Death & Disease. .
12. Elahi, S., and H. Horton. 2012. Association of HLA-alleles with the immune regulation of chronic viral infections. *Int J Biochem Cell Biol* 44: 1361–1365.
13. Elahi, S., W. L. Dinges, N. Lejarcegui, K. J. Laing, A. C. Collier, D. M. Koelle, M. J. McElrath, and H. Horton. 2011. Protective HIV-specific CD8+ T cells evade Treg cell suppression. *Nat Med* 17: 989–995.
14. The influence of HLA genotype on AIDS - PubMed. .
15. Elahi, S., S. Shahbaz, and S. Houston. 2020. Selective Upregulation of CTLA-4 on CD8+ T Cells Restricted by HLA-B*35Px Renders them to an Exhausted Phenotype in HIV-1 infection. *PLOS Pathogens* 16: e1008696.
16. Jiao, Y., J. Fu, S. Xing, B. Fu, Z. Zhang, M. Shi, X. Wang, J. Zhang, L. Jin, F. Kang, H. Wu, and F.-S. Wang. 2009. The decrease of regulatory T cells correlates with excessive activation and apoptosis of CD8+ T cells in HIV-1-infected typical progressors, but not in long-term non-progressors. *Immunology* 128: e366-375.
17. Hunt, P. W., A. L. Landay, E. Sinclair, J. A. Martinson, H. Hatano, B. Emu, P. J. Norris, M. P. Busch, J. N. Martin, C. Brooks, J. M. McCune, and S. G. Deeks. 2011. A low T regulatory cell response may contribute to both viral control and generalized immune activation in HIV controllers. *PloS One* 6: e15924.
18. Shaw, J. M., P. W. Hunt, J. W. Critchfield, D. H. McConnell, J. C. Garcia, R. B. Pollard, M. Somsouk, S. G. Deeks, and B. L. Shacklett. 2011. Increased frequency of regulatory T cells accompanies increased

immune activation in rectal mucosae of HIV-positive noncontrollers. *Journal of Virology* 85: 11422–11434.
19. Chevalier, M. F., and L. Weiss. 2013. The split personality of regulatory T cells in HIV infection. *Blood* 121: 29–37.
20. Oswald-Richter, K., S. M. Grill, N. Shariat, M. Leelawong, M. S. Sundrud, D. W. Haas, and D. Unutmaz. 2004. HIV infection of naturally occurring and genetically reprogrammed human regulatory T-cells. *PLoS biology* 2: E198.
21. Antons, A. K., R. Wang, K. Oswald-Richter, M. Tseng, C. W. Arendt, S. A. Kalams, and D. Unutmaz. 2008. Naive precursors of human regulatory T cells require FoxP3 for suppression and are susceptible to HIV infection. *Journal of Immunology (Baltimore, Md.: 1950)* 180: 764–773.
22. Zaunders, J. J., S. Ip, M. L. Munier, D. E. Kaufmann, K. Suzuki, C. Brereton, S. C. Sasson, N. Seddiki, K. Koelsch, A. Landay, P. Grey, R. Finlayson, J. Kaldor, E. S. Rosenberg, B. D. Walker, B. Fazekas de St Groth, D. A. Cooper, and A. D. Kelleher. 2006. Infection of CD127+ (interleukin-7 receptor+) CD4+ cells and overexpression of CTLA-4 are linked to loss of antigen-specific CD4 T cells during primary human immunodeficiency virus type 1 infection. *Journal of Virology* 80: 10162–10172.
23. Shahbaz, S., G. Dunsmore, P. Koleva, L. Xu, S. Houston, and S. Elahi. 2020. Galectin-9 and VISTA Expression Define Terminally Exhausted T Cells in HIV-1 Infection. *J. Immunol.* 204: 2474–2491.
24. Dunsmore, G., N. Bozorgmehr, C. Delyea, P. Koleva, A. Namdar, and S. Elahi. 2017. Erythroid Suppressor Cells Compromise Neonatal Immune Response against Bordetella pertussis. *J. Immunol.* 199: 2081–2095.
25. Shahbaz, S., N. Bozorgmehr, P. Koleva, A. Namdar, J. Jovel, R. A. Fava, and S. Elahi. 2018. CD71+VISTA+ erythroid cells promote the development and function of regulatory T cells through TGF-β. *PLoS Biol* 16: e2006649.
26. Bray, N. L., H. Pimentel, P. Melsted, and L. Pachter. 2016. Near-optimal probabilistic RNA-seq quantification. *Nature Biotechnology* 34: 525–527.
27. Love, M. I., W. Huber, and S. Anders. 2014. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biology* 15: 550.
28. Kawaguchi, A., and K. Nagata. 2007. De novo replication of the influenza virus RNA genome is regulated by DNA replicative helicase, MCM. *The EMBO Journal* 26: 4566–4575.
29. Abitew, A. M., R. C. Sobti, V. L. Sharma, and A. Wanchu. 2020. Analysis of transporter associated with antigen presentation (TAP) genes polymorphisms with HIV-1 infection. *Mol Cell Biochem* 464: 65–71.
30. Tange, T. Ø., and J. Kjems. 2001. SF2/ASF binds to a splicing enhancer in the third HIV-1 tat exon and stimulates U2AF binding independently of the RS domain11Edited by J. Karn. *Journal of Molecular Biology* 312: 649–662.
31. Sertznig, H., F. Hillebrand, S. Erkelenz, H. Schaal, and M. Widera. 2018. Behind the scenes of HIV-1 replication: Alternative splicing as the dependency factor on the quiet. *Virology* 516: 176–188.
32. Brass, A. L., D. M. Dykxhoorn, Y. Benita, N. Yan, A. Engelman, R. J. Xavier, J. Lieberman, and S. J. Elledge. 2008. Identification of Host Proteins Required for HIV Infection Through a Functional Genomic Screen. *Science* 319: 921–926.
33. Boehm, D., V. Calvanese, R. D. Dar, S. Xing, S. Schroeder, L. Martins, K. Aull, P.-C. Li, V. Planelles, J. E. Bradner, M.-M. Zhou, R. F. Siliciano, L. Weinberger, E. Verdin, and M. Ott. 2013. BET bromodomain-targeting compounds reactivate HIV from latency via a Tat-independent mechanism. *Cell Cycle* 12: 452–462.
34. Hartl, F. U., and J. Martin. 1995. Molecular chaperones in cellular protein folding. *Curr Opin Struct Biol* 5: 92–102.
35. Gurer, C., A. Cimarelli, and J. Luban. 2002. Specific Incorporation of Heat Shock Protein 70 Family Members into Primate Lentiviral Virions. *J Virol* 76: 4666–4670.

36. Chappell, T. G., W. J. Welch, D. M. Schlossman, K. B. Palter, M. J. Schlesinger, and J. E. Rothman. 1986. Uncoating ATPase is a member of the 70 kilodalton family of stress proteins. *Cell* 45: 3–13.
37. Joshi, R. N., S. J. Fernandes, M. Shang, N. A. Kiani, D. Gomez-Cabrero, J. Tegnér, and A. Schmidt. 2019. Phosphatase inhibitor PPP1R11 modulates resistance of human T cells toward Treg-mediated suppression of cytokine expression. *J Leukoc Biol* 106: 413–430.
38. Mukerji, J., K. C. Olivieri, V. Misra, K. A. Agopian, and D. Gabuzda. 2012. Proteomic analysis of HIV-1 Nef cellular binding partners reveals a role for exocyst complex proteins in mediating enhancement of intercellular nanotube formation. *Retrovirology* 9: 33.
39. Membrane Topology of the Alg14 Endoplasmic Reticulum UDP-GlcNAc Transferase Subunit. .
40. Jochmann, R., M. Thurau, S. Jung, C. Hofmann, E. Naschberger, E. Kremmer, T. Harrer, M. Miller, N. Schaft, and M. Stürzl. 2009. O-linked N-acetylglucosaminylation of Sp1 inhibits the human immunodeficiency virus type 1 promoter. *J Virol* 83: 3704–3718.
41. Bearz, A., G. Tell, S. Formisano, S. Merluzzi, A. Colombatti, and C. Pucillo. 1999. Adhesion to fibronectin promotes the activation of the p125FAK/Zap-70 complex in human T cells. *Immunology* 98: 564–568.
42. Crux, N. B., and S. Elahi. 2017. Human Leukocyte Antigen (HLA) and Immune Regulation: How Do Classical and Non-Classical HLA Alleles Modulate Immune Response to Human Immunodeficiency Virus and Hepatitis C Virus Infections? *Front Immunol* 8.
43. Lee, N., A. Ishitani, and D. E. Geraghty. 2010. HLA-F is a surface marker on activated lymphocytes1. *Eur J Immunol* 40: 2308–2318.
44. Zeng, H., K. Yang, C. Cloer, G. Neale, P. Vogel, and H. Chi. 2013. mTORC1 couples immune signals and metabolic programming to establish Treg cell function. *Nature* 499: 485–490.
45. Sun, I.-H., M.-H. Oh, L. Zhao, Chirag, H. Patel, M. L. Arwood, W. Xu, A. J. Tam, R. L. Blosser, J. Wen, and J. D. Powell. 2018. mTORC1 signaling regulates the generation and function of central and effector FoxP3+ regulatory T cells. *J Immunol* 201: 481–492.
46. Chapman, N. M., and H. Chi. 2014. mTOR signaling, Tregs and immune modulation. *Immunotherapy* 6: 1295–1311.
47. Waickman, A. T., and J. D. Powell. 2012. mTOR, metabolism, and the regulation of T-cell differentiation and function. *Immunol Rev* 249: 43–58.
48. Alvisi, G., J. Brummelman, S. Puccio, E. M. Mazza, E. P. Tomada, A. Losurdo, V. Zanon, C. Peano, F. S. Colombo, A. Scarpa, M. Alloisio, A. Vasanthakumar, R. Roychoudhuri, M. Kallikourdis, M. Pagani, E. Lopci, P. Novellis, J. Blume, A. Kallies, G. Veronesi, and E. Lugli. 2020. IRF4 instructs effector Treg differentiation and immune suppression in human cancer. *J. Clin. Invest.* 130: 3137–3150.
49. Vasanthakumar, A., Y. Liao, P. Teh, M. F. Pascutti, A. E. Oja, A. L. Garnham, R. Gloury, J. C. Tempany, T. Sidwell, E. Cuadrado, P. Tuijnenburg, T. W. Kuijpers, N. Lalaoui, L. A. Mielke, V. L. Bryant, P. D. Hodgkin, J. Silke, G. K. Smyth, M. A. Nolte, W. Shi, and A. Kallies. 2017. The TNF Receptor Superfamily-NF-κB Axis Is Critical to Maintain Effector Regulatory T Cells in Lymphoid and Non-lymphoid Tissues. *Cell Reports* 20: 2906–2920.
50. Baecher-Allan, C., E. Wolf, and D. A. Hafler. 2006. MHC Class II Expression Identifies Functionally Distinct Human Regulatory T Cells. *The Journal of Immunology* 176: 4622–4631.
51. Schulze Zur Wiesch, J., A. Thomssen, P. Hartjen, I. Tóth, C. Lehmann, D. Meyer-Olson, K. Colberg, S. Frerk, D. Babikir, S. Schmiedel, O. Degen, S. Mauss, J. Rockstroh, S. Staszewski, P. Khaykin, A. Strasak, A. W. Lohse, G. Fätkenheuer, J. Hauber, and J. van Lunzen. 2011. Comprehensive analysis of frequency and phenotype of T regulatory cells in HIV infection: CD39 expression of FoxP3+ T regulatory cells correlates with progressive disease. *J. Virol.* 85: 1287–1297.
52. Mukhopadhyay, A., S. A. Saddoughi, P. Song, I. Sultan, S. Ponnusamy, C. E. Senkal, C. F. Snook, H. K. Arnold, R. C. Sears, Y. A. Hannun, and B. Ogretmen. 2009. Direct interaction between the inhibitor 2 and

ceramide via sphingolipid-protein binding is involved in the regulation of protein phosphatase 2A activity and signaling. *FASEB J* 23: 751–763.
53. Smith, E. L., and E. H. Schuchman. 2008. The unexpected role of acid sphingomyelinase in cell death and the pathophysiology of common diseases. *FASEB J* 22: 3419–3431.
54. Apostolidis, S. A., N. Rodríguez-Rodríguez, A. Suárez-Fueyo, N. Dioufa, E. Ozcan, J. C. Crispín, M. G. Tsokos, and G. C. Tsokos. 2016. Protein phosphatase 2A is requisite for the function of regulatory T cells. *Nat Immunol* 17: 556–564.
55. Kleijn, M., and C. G. Proud. 2002. The regulation of protein synthesis and translation factors by CD3 and CD28 in human primary T lymphocytes. *BMC Biochem* 3: 11.
56. Asmal, M., J. Colgan, F. Naef, B. Yu, Y. Lee, M. Magnasco, and J. Luban. 2003. Production of Ribosome Components in Effector CD4+ T Cells Is Accelerated by TCR Stimulation and Coordinated by ERK-MAPK. *Immunity* 19: 535–548.
57. Li, W., D. Tan, Z. Zhang, J. J. Liang, and R. E. Brown. 2008. Activation of Akt-mTOR-p70S6K pathway in angiogenesis in hepatocellular carcinoma. *Oncol. Rep.* 20: 713–719.
58. Zhao, B., L. Li, and K.-L. Guan. 2010. Hippo signaling at a glance. *J Cell Sci* 123: 4001–4006.
59. Hippo kinases Mst1 and Mst2 sense and amplify IL-2R–STAT5 signaling in regulatory T cells to establish stable regulatory activity. .
60. Li, J., X. Du, H. Shi, K. Deng, H. Chi, and W. Tao. 2015. Mammalian Sterile 20-like Kinase 1 (Mst1) Enhances the Stability of Forkhead Box P3 (Foxp3) and the Function of Regulatory T Cells by Modulating Foxp3 Acetylation. *J Biol Chem* 290: 30762–30770.
61. Levine, A. G., A. Arvey, W. Jin, and A. Y. Rudensky. 2014. Continuous requirement for the TCR in regulatory T cell function. *Nat. Immunol.* 15: 1070–1078.
62. Chinen, T., A. K. Kannan, A. G. Levine, X. Fan, U. Klein, Y. Zheng, G. Gasteiger, Y. Feng, J. D. Fontenot, and A. Y. Rudensky. 2016. An essential role for the IL-2 receptor in T reg cell function. *Nature Immunology* 17: 1322–1333.
63. Transcription factor Foxp1 regulates Foxp3 chromatin binding and coordinates regulatory T cell function | Nature Immunology. .
64. Foxp1 is an essential transcription regulator for the generation of quiescent naive T cells during thymocyte development | Blood | American Society of Hematology. .
65. Adler, H. S., and K. Steinbrink. 2008. MAP kinase p38 and its relation to T cell anergy and suppressor function of regulatory T cells. *Cell Cycle* 7: 169–170.
66. Perna, S. K., B. De Angelis, D. Pagliara, L. Zhan, C. M. Rooney, H. E. Heslop, M. K. Brenner, G. Dotti, and B. Savoldo. 2009. IL15 Enhances Proliferation and Effector Function of Antigen-Specific Cytotoxic T Lymphocytes (CTLs) and Mitigates the Suppressive Action of Regulatory T Cells (Tregs). *Blood* 114: 4088–4088.
67. Chaudhry, A., R. M. Samstein, P. Treuting, Y. Liang, M. C. Pils, J.-M. Heinrich, R. S. Jack, F. T. Wunderlich, J. C. Brüning, W. Müller, and A. Y. Rudensky. 2011. Interleukin-10 signaling in regulatory T cells is required for suppression of Th17 cell-mediated inflammation. *Immunity* 34: 566–578.
68. Interleukin-10 deficiency impairs regulatory T cell-derived neuropilin-1 functions and promotes Th1 and Th17 immunity | Scientific Reports. .
69. Grzes, K. M., C. S. Field, and E. J. Pearce. 2017. Treg Cells Survive and Thrive in Inhospitable Environments. *Cell Metabolism* 25: 1213–1215.
70. Shi, H., and H. Chi. 2019. Metabolic Control of Treg Cell Stability, Plasticity, and Tissue-Specific Heterogeneity. *Front. Immunol.* 10.
71. Gerriets, V. A., R. J. Kishton, M. O. Johnson, S. Cohen, P. J. Siska, A. G. Nichols, M. O. Warmoes, A. A. de Cubas, N. J. MacIver, J. W. Locasale, L. A. Turka, A. D. Wells, and J. C. Rathmell. 2016. Foxp3 and Toll-like receptor signaling balance T reg cell anabolic metabolism for suppression. *Nature Immunology* 17: 1459–1466.

72. Le Menn, G., and J. G. Neels. 2018. Regulation of Immune Cell Function by PPARs and the Connection with Metabolic and Neurodegenerative Diseases. *Int J Mol Sci* 19.
73. Guri, A. J., S. K. Mohapatra, W. T. Horne, R. Hontecillas, and J. Bassaganya-Riera. 2010. The role of T cell PPAR gamma in mice with experimental inflammatory bowel disease. *BMC Gastroenterol* 10: 60.
74. Hontecillas, R., and J. Bassaganya-Riera. 2007. Peroxisome proliferator-activated receptor gamma is required for regulatory CD4+ T cell-mediated protection against colitis. *J. Immunol.* 178: 2940–2949.
75. Wohlfert, E. A., F. C. Nichols, E. Nevius, and R. B. Clark. 2007. Peroxisome proliferator-activated receptor gamma (PPARgamma) and immunoregulation: enhancement of regulatory T cells through PPARgamma-dependent and -independent mechanisms. *J. Immunol.* 178: 4129–4135.
76. Wang, L. H., X. Y. Yang, X. Zhang, J. Huang, J. Hou, J. Li, H. Xiong, K. Mihalic, H. Zhu, W. Xiao, and W. L. Farrar. 2004. Transcription Inactivation of STAT3 by PPARγ Suppresses IL-6-Responsive Multiple Myeloma Cells. *Immunity* 20: 205–218.
77. Zhong, Z., Z. Wen, and J. E. Darnell. 1994. Stat3: a STAT family member activated by tyrosine phosphorylation in response to epidermal growth factor and interleukin-6. *Science* 264: 95–98.
78. Fujino, M., and X.-K. Li. 2013. Role of STAT3 in regulatory T lymphocyte plasticity during acute graft-vs.-host-disease. *JAK-STAT* 2: e24529.
79. Laurence, A., S. Amarnath, J. Mariotti, Y. C. Kim, J. Foley, M. Eckhaus, J. J. O'Shea, and D. H. Fowler. 2012. STAT3 Transcription Factor Promotes Instability of nTreg Cells and Limits Generation of iTreg Cells During Acute Murine Graft Versus Host Disease. *Immunity* 37: 209–222.
80. Zhou, X., S. Bailey-Bucktrout, L. T. Jeker, and J. A. Bluestone. 2009. Plasticity of CD4+ FoxP3+ T cells. *Curr Opin Immunol* 21: 281–285.
81. Full article: Functional plasticity in human FOXP3+ regulatory T cells. .
82. Lin, B., D. Xu, and D. W. Leaman. 2016. XIAP-associated factor 1 (XAF1) regulates TNF receptor 1 complex stability. *FEBS Lett* 590: 4381–4392.
83. Srivastava, R. K., G. B. Tomar, A. P. Barhanpurkar, N. Gupta, S. T. Pote, G. C. Mishra, and M. R. Wani. 2011. IL-3 Attenuates Collagen-Induced Arthritis by Modulating the Development of Foxp3+ Regulatory T Cells. *The Journal of Immunology* 186: 2262–2272.
84. Hamano, R., J. Huang, T. Yoshimura, J. J. Oppenheim, and X. Chen. 2011. TNF by inducing co-stimulatory TNF receptor superfamily members TNFR2, 4-1BB and OX40 augments the number and function of mouse CD4+FoxP3+ regulatory T cells. *Eur J Immunol* 41: 2010–2020.
85. Metidji, A., S. A. Rieder, D. D. Glass, I. Cremer, G. A. Punkosdy, and E. M. Shevach. 2015. IFN-α/β receptor signaling promotes regulatory T cell development and function under stress conditions. *Journal of Immunology (Baltimore, Md.: 1950)* 194: 4265–4276.
86. Vogel, I. T., S. W. V. Gool, and J. L. Ceuppens. 2014. CD28/CTLA-4/B7 and CD40/CD40L costimulation and activation of regulatory T cells. *World Journal of Immunology* 4: 63–77.
87. Feng, C., L.-J. Cao, H.-F. Song, P. Xu, H. Chen, J.-C. Xu, X.-Y. Zhu, X.-G. Zhang, and X.-F. Wang. 2015. Expression of PD-L1 on CD4+CD25+Foxp3+ Regulatory T Cells of Patients with Chronic HBV Infection and Its Correlation with Clinical Parameters. *Viral Immunology* 28: 418–424.
88. Valli, E., H. A. Sterle, S. M. Huergo, I. Mascafroni, A. J. Klecha, M. L. B. Arcos, G. A. Rabinovich, and G. A. Cremaschi. 2013. Regulatory T cells mediate immunosupression induced by experimental hypothyroidism. (P1215). *The Journal of Immunology* 190: 188.10-188.10.
89. Sampson, J. F., A. Suryawanshi, W.-S. Chen, G. A. Rabinovich, and N. Panjwani. 2016. Galectin-8 promotes regulatory T cell differentiation by modulating IL-2 and TGFβ signaling. *Immunol Cell Biol* 94: 213–219.
90. Maj, T., W. Wang, J. Crespo, H. Zhang, W. Wang, S. Wei, L. Zhao, L. Vatan, I. Shao, W. Szeliga, C. Lyssiotis, J. R. Liu, I. Kryczek, and W. Zou. 2017. Oxidative stress controls regulatory T cell apoptosis and suppressor activity and PD-L1-blockade resistance in tumor. *Nature Immunology* 18: 1332–1341.

91. Zhang, J., and P. A. Ney. 2009. Role of BNIP3 and NIX in cell death, autophagy, and mitophagy. *Cell Death & Differentiation* 16: 939–946.
92. McPherson, J. P., H. Sarras, B. Lemmers, L. Tamblyn, E. Migon, E. Matysiak-Zablocki, A. Hakem, S. A. Azami, R. Cardoso, J. Fish, O. Sanchez, M. Post, and R. Hakem. 2009. Essential role for Bclaf1 in lung development and immune system function. *Cell Death & Differentiation* 16: 331–339.
93. Tóth, I., A. Q. Le, P. Hartjen, A. Thomssen, V. Matzat, C. Lehmann, C. Scheurich, C. Beisel, P. Busch, O. Degen, A. W. Lohse, T. Eiermann, G. Fätkenheuer, D. Meyer-Olson, M. Bockhorn, J. Hauber, J. van Lunzen, and J. Schulze Zur Wiesch. 2013. Decreased frequency of CD73+CD8+ T cells of HIV-infected patients correlates with immune activation and T cell exhaustion. *Journal of Leukocyte Biology* 94: 551–561.
94. Zhao, H., X. Liao, and Y. Kang. 2017. Tregs: Where We Are and What Comes Next? *Front. Immunol.* 8.
95. Angin, M., S. Sharma, M. King, T. T. Murooka, M. Ghebremichael, T. R. Mempel, B. D. Walker, M. K. Bhasin, and M. M. Addo. 2014. HIV-1 Infection Impairs Regulatory T-Cell Suppressive Capacity on a Per-Cell Basis. *J Infect Dis* 210: 899–903.
96. Pion, M., D. Jaramillo-Ruiz, A. Martínez, M. A. Muñoz-Fernández, and R. Correa-Rocha. 2013. HIV infection of human regulatory T cells downregulates Foxp3 expression by increasing DNMT3b levels and DNA methylation in the FOXP3 gene. *AIDS* 27: 2019–2029.
97. Eggena, M. P., B. Barugahare, N. Jones, M. Okello, S. Mutalya, C. Kityo, P. Mugyenyi, and H. Cao. 2005. Depletion of regulatory T cells in HIV infection is associated with immune activation. *J Immunol* 174: 4407–4414.
98. Kinter, A. L., M. Hennessey, A. Bell, S. Kern, Y. Lin, M. Daucher, M. Planta, M. McGlaughlin, R. Jackson, S. F. Ziegler, and A. S. Fauci. 2004. CD25+CD4+ Regulatory T Cells from the Peripheral Blood of Asymptomatic HIV-infected Individuals Regulate CD4+ and CD8+ HIV-specific T Cell Immune Responses In Vitro and Are Associated with Favorable Clinical Markers of Disease Status. *J Exp Med* 200: 331–343.
99. Kinter, A. L., R. Horak, M. Sion, L. Riggin, J. McNally, Y. Lin, R. Jackson, A. O'shea, G. Roby, C. Kovacs, M. Connors, S. A. Migueles, and A. S. Fauci. 2007. CD25+ regulatory T cells isolated from HIV-infected individuals suppress the cytolytic and nonlytic antiviral activity of HIV-specific CD8+ T cells in vitro. *AIDS Res Hum Retroviruses* 23: 438–450.
100. mTOR coordinates transcription programs and mitochondrial metabolism of activated T reg subsets to protect tissue homeostasis | Nature Communications. .
101. T cell receptor signalling in the control of regulatory T cell differentiation and function | Nature Reviews Immunology. .
102. Ono, M. 2020. Control of regulatory T-cell differentiation and function by T-cell receptor signalling and Foxp3 transcription factor complexes. *Immunology* 160: 24–37.
103. Zheng, Y., A. Chaudhry, A. Kas, P. deRoos, J. M. Kim, T.-T. Chu, L. Corcoran, P. Treuting, U. Klein, and A. Y. Rudensky. 2009. Regulatory T-cell suppressor program co-opts transcription factor IRF4 to control T(H)2 responses. *Nature* 458: 351–356.
104. Meng, Z., T. Moroishi, and K.-L. Guan. 2016. Mechanisms of Hippo pathway regulation. *Genes Dev* 30: 1–17.
105. van Loosdregt, J., Y. Vercoulen, T. Guichelaar, Y. Y. J. Gent, J. M. Beekman, O. van Beekum, A. B. Brenkman, D.-J. Hijnen, T. Mutis, E. Kalkhoven, B. J. Prakken, and P. J. Coffer. 2010. Regulation of Treg functionality by acetylation-mediated Foxp3 protein stabilization. *Blood* 115: 965–974.
106. Li, B., A. Samanta, X. Song, K. T. Iacono, K. Bembas, R. Tao, S. Basu, J. L. Riley, W. W. Hancock, Y. Shen, S. J. Saouaf, and M. I. Greene. 2007. FOXP3 interactions with histone acetyltransferase and class II histone deacetylases are required for repression. *Proceedings of the National Academy of Sciences of the United States of America* 104: 4571.
107. Li, M., A. Makkinje, and Z. Damuni. 1996. The myeloid leukemia-associated protein SET is a potent inhibitor of protein phosphatase 2A. *J. Biol. Chem.* 271: 11059–11062.

108. Trotta, R., D. Ciarlariello, J. D. Col, J. Allard, P. Neviani, R. Santhanam, H. Mao, B. Becknell, J. Yu, A. K. Ferketich, B. Thomas, A. Modi, B. W. Blaser, D. Perrotti, and M. A. Caligiuri. 2007. The PP2A inhibitor SET regulates natural killer cell IFN-γ production. *J Exp Med* 204: 2397–2405.
109. Wlodarchak, N., and Y. Xing. 2016. PP2A as a master regulator of the cell cycle. *Crit Rev Biochem Mol Biol* 51: 162–184.
110. Hollmann, C., S. Werner, E. Avota, D. Reuter, L. Japtok, B. Kleuser, E. Gulbins, K. A. Becker, J. Schneider-Schaulies, and N. Beyersdorf. 2016. Inhibition of Acid Sphingomyelinase Allows for Selective Targeting of CD4+ Conventional versus Foxp3+ Regulatory T Cells. *The Journal of Immunology* 197: 3130–3141.
111. Finnegan, C. M., S. S. Rawat, A. Puri, J. M. Wang, F. W. Ruscetti, and R. Blumenthal. 2004. Ceramide, a target for antiretroviral therapy. *Proc Natl Acad Sci U S A* 101: 15452–15457.
112. 178. IL-15 Reduces Suppressive Effects of Regulatory T Cells (Tregs) on Antigen-Specific Cytotoxic T Lymphocytes (CTLs): Molecular Therapy.
113. Dang, E. V., J. Barbi, H.-Y. Yang, D. Jinasena, H. Yu, Y. Zheng, Z. Bordman, J. Fu, Y. Kim, H.-R. Yen, W. Luo, K. Zeller, L. Shimoda, S. L. Topalian, G. L. Semenza, C. V. Dang, D. M. Pardoll, and F. Pan. 2011. Control of TH17/Treg Balance by Hypoxia-inducible Factor 1. *Cell* 146: 772–784.
114. Tao, J.-H., J. Barbi, and F. Pan. 2015. Hypoxia-inducible factors in T lymphocyte differentiation and function. A Review in the Theme: Cellular Responses to Hypoxia. *Am J Physiol Cell Physiol* 309: C580–C589.
115. Kempkes, R. W. M., I. Joosten, H. J. P. M. Koenen, and X. He. 2019. Metabolic Pathways Involved in Regulatory T Cell Functionality. *Front. Immunol.* 10.
116. Wei, J., L. Long, K. Yang, C. Guy, S. Shrestha, Z. Chen, C. Wu, P. Vogel, G. Neale, D. R. Green, and H. Chi. 2016. Autophagy enforces functional integrity of regulatory T cells by coupling environmental cues and metabolic homeostasis. *Nature Immunology* 17: 277–285.
117. Miska, J., C. Lee-Chang, A. Rashidi, M. E. Muroski, A. L. Chang, A. Lopez-Rosas, P. Zhang, W. K. Panek, A. Cordero, Y. Han, A. U. Ahmed, N. S. Chandel, and M. S. Lesniak. 2019. HIF-1α Is a Metabolic Switch between Glycolytic-Driven Migration and Oxidative Phosphorylation-Driven Immunosuppression of Tregs in Glioblastoma. *Cell Reports* 27: 226-237.e4.
118. Angelin, A., L. Gil-de-Gómez, S. Dahiya, J. Jiao, L. Guo, M. H. Levine, Z. Wang, W. J. Quinn, P. K. Kopinski, L. Wang, T. Akimova, Y. Liu, T. R. Bhatti, R. Han, B. L. Laskin, J. A. Baur, I. A. Blair, D. C. Wallace, W. W. Hancock, and U. H. Beier. 2017. Foxp3 Reprograms T Cell Metabolism to Function in Low-Glucose, High-Lactate Environments. *Cell Metabolism* 25: 1282-1293.e7.
119. Deng, D., C. Xu, P. Sun, J. Wu, C. Yan, M. Hu, and N. Yan. 2014. Crystal structure of the human glucose transporter GLUT1. *Nature* 510: 121–125.
120. Beier, U. H., A. Angelin, T. Akimova, L. Wang, Y. Liu, H. Xiao, M. A. Koike, S. A. Hancock, T. R. Bhatti, R. Han, J. Jiao, S. C. Veasey, C. A. Sims, J. A. Baur, D. C. Wallace, and W. W. Hancock. 2015. Essential role of mitochondrial energy metabolism in Foxp3+ T-regulatory cell function and allograft survival. *The FASEB Journal* 29: 2315–2326.
121. Yu, X., Y. Lao, X.-L. Teng, S. Li, Y. Zhou, F. Wang, X. Guo, S. Deng, Y. Chang, X. Wu, Z. Liu, L. Chen, L.-M. Lu, J. Cheng, B. Li, B. Su, J. Jiang, H.-B. Li, C. Huang, J. Yi, and Q. Zou. 2018. SENP3 maintains the stability and function of regulatory T cells via BACH2 deSUMOylation. *Nature Communications* 9: 3157.
122. Hanson, R. W., and O. E. Owen. 2013. Gluconeogenesis. In *Encyclopedia of Biological Chemistry (Second Edition)* W. J. Lennarz, and M. D. Lane, eds. Academic Press, Waltham. 381–386.
123. A Pck1-directed glycogen metabolic program regulates formation and maintenance of memory CD8 + T cells - PubMed. .
124. Selective Export of HLA-F by Its Cytoplasmic Tail | The Journal of Immunology. .
125. Garcia-Beltran, W. F., A. Hölzemer, G. Martrus, A. W. Chung, Y. Pacheco, C. R. Simoneau, M. Rucevic, P. A. Lamothe-Molina, T. Pertel, T.-E. Kim, H. Dugan, G. Alter, J. Dechanet-Merville, S. Jost, M.

Carrington, and M. Altfeld. 2016. Open conformers of HLA-F are high-affinity ligands of the activating NK-cell receptor KIR3DS1. *Nature Immunology* 17: 1067–1074.
126. Kramer, A. B., C. Roozendaal, and R. P. F. Dullaart. 2004. Familial occurrence of subacute thyroiditis associated with human leukocyte antigen-B35. *Thyroid* 14: 544–547.

Chapter 5: General discussion and conclusion

1. Balasubramaniam, M., J. Pandhare, and C. Dash. 2019. Immune Control of HIV. *J Life Sci (Westlake Village)* 1: 4–37.
2. Borrow, P., H. Lewicki, X. Wei, M. S. Horwitz, N. Peffer, H. Meyers, J. A. Nelson, J. E. Gairin, B. H. Hahn, M. B. Oldstone, and G. M. Shaw. 1997. Antiviral pressure exerted by HIV-1-specific cytotoxic T lymphocytes (CTLs) during primary infection demonstrated by rapid selection of CTL escape virus. *Nat Med* 3: 205–211.
3. Dustin, M. L., and E. O. Long. 2010. Cytotoxic immunological synapses. *Immunol Rev* 235: 24–34.
4. Halle, S., O. Halle, and R. Förster. 2017. Mechanisms and Dynamics of T Cell-Mediated Cytotoxicity In Vivo. *Trends Immunol* 38: 432–443.
5. Yang, O. O., S. A. Kalams, M. Rosenzweig, A. Trocha, N. Jones, M. Koziel, B. D. Walker, and R. P. Johnson. 1996. Efficient lysis of human immunodeficiency virus type 1-infected cells by cytotoxic T lymphocytes. *J Virol* 70: 5799–5806.
6. Schmitz, J. E., M. J. Kuroda, S. Santra, V. G. Sasseville, M. A. Simon, M. A. Lifton, P. Racz, K. Tenner-Racz, M. Dalesandro, B. J. Scallon, J. Ghrayeb, M. A. Forman, D. C. Montefiori, E. P. Rieber, N. L. Letvin, and K. A. Reimann. 1999. Control of viremia in simian immunodeficiency virus infection by CD8+ lymphocytes. *Science* 283: 857–860.
7. Allen, T. M., M. Altfeld, S. C. Geer, E. T. Kalife, C. Moore, K. M. O'sullivan, I. Desouza, M. E. Feeney, R. L. Eldridge, E. L. Maier, D. E. Kaufmann, M. P. Lahaie, L. Reyor, G. Tanzi, M. N. Johnston, C. Brander, R. Draenert, J. K. Rockstroh, H. Jessen, E. S. Rosenberg, S. A. Mallal, and B. D. Walker. 2005. Selective escape from CD8+ T-cell responses represents a major driving force of human immunodeficiency virus type 1 (HIV-1) sequence diversity and reveals constraints on HIV-1 evolution. *J Virol* 79: 13239–13249.
8. Zanussi, S., C. Simonelli, M. D'Andrea, C. Caffau, M. Clerici, U. Tirelli, and P. DePaoli. 1996. CD8+ lymphocyte phenotype and cytokine production in long-term non-progressor and in progressor patients with HIV-1 infection. *Clin Exp Immunol* 105: 220–224.
9. Migueles, S. A., and M. Connors. 2015. Success and failure of the cellular immune response against HIV-1. *Nat Immunol* 16: 563–570.
10. Moore, C. B., M. John, I. R. James, F. T. Christiansen, C. S. Witt, and S. A. Mallal. 2002. Evidence of HIV-1 adaptation to HLA-restricted immune responses at a population level. *Science* 296: 1439–1443.
11. Carlson, J. M., A. Q. Le, A. Shahid, and Z. L. Brumme. 2015. HIV-1 adaptation to HLA: a window into virus-host immune interactions. *Trends Microbiol* 23: 212–224.
12. Jones, R. B., and B. D. Walker. HIV-specific CD8+ T cells and HIV eradication. *J Clin Invest* 126: 455–463.
13. Wang, C., M. Singer, and A. C. Anderson. 2017. Molecular dissection of CD8+ T cell dysfunction. *Trends Immunol* 38: 567–576.
14. Fenwick, C., V. Joo, P. Jacquier, A. Noto, R. Banga, M. Perreau, and G. Pantaleo. 2019. T-cell exhaustion in HIV infection. *Immunol Rev* 292: 149–163.
15. Day, C. L., D. E. Kaufmann, P. Kiepiela, J. A. Brown, E. S. Moodley, S. Reddy, E. W. Mackey, J. D. Miller, A. J. Leslie, C. DePierres, Z. Mncube, J. Duraiswamy, B. Zhu, Q. Eichbaum, M. Altfeld, E. J. Wherry, H. M. Coovadia, P. J. R. Goulder, P. Klenerman, R. Ahmed, G. J. Freeman, and B. D. Walker. 2006. PD-1

expression on HIV-specific T cells is associated with T-cell exhaustion and disease progression. *Nature* 443: 350–354.
16. Peretz, Y., Z. He, Y. Shi, B. Yassine-Diab, J.-P. Goulet, R. Bordi, A. Filali-Mouhim, J.-B. Loubert, M. El-Far, F. P. Dupuy, M. R. Boulassel, C. Tremblay, J.-P. Routy, N. Bernard, R. Balderas, E. K. Haddad, and R.-P. Sékaly. 2012. CD160 and PD-1 co-expression on HIV-specific CD8 T cells defines a subset with advanced dysfunction. *PLoS Pathog* 8: e1002840.
17. Amancha, P. K., J. J. Hong, A. A. Ansari, and F. Villinger. 2015. Up-regulation of Tim-3 on T cells during acute simian immunodeficiency virus infection and on antigen specific responders. *AIDS* 29: 531–536.
18. Jones, R. B., L. C. Ndhlovu, J. D. Barbour, P. M. Sheth, A. R. Jha, B. R. Long, J. C. Wong, M. Satkunarajah, M. Schweneker, J. M. Chapman, G. Gyenes, B. Vali, M. D. Hyrcza, F. Y. Yue, C. Kovacs, A. Sassi, M. Loutfy, R. Halpenny, D. Persad, G. Spotts, F. M. Hecht, T.-W. Chun, J. M. McCune, R. Kaul, J. M. Rini, D. F. Nixon, and M. A. Ostrowski. 2008. Tim-3 expression defines a novel population of dysfunctional T cells with highly elevated frequencies in progressive HIV-1 infection. *J Exp Med* 205: 2763–2779.
19. Yamamoto, T., D. A. Price, J. P. Casazza, G. Ferrari, M. Nason, P. K. Chattopadhyay, M. Roederer, E. Gostick, P. D. Katsikis, D. C. Douek, R. Haubrich, C. Petrovas, and R. A. Koup. 2011. Surface expression patterns of negative regulatory molecules identify determinants of virus-specific CD8+ T-cell exhaustion in HIV infection. *Blood* 117: 4805–4815.
20. Merani, S., W. Chen, and S. Elahi. 2015. The bitter side of sweet: the role of Galectin-9 in immunopathogenesis of viral infections. *Rev Med Virol* 25: 175–186.
21. Chou, F.-C., S.-J. Shieh, and H.-K. Sytwu. 2009. Attenuation of Th1 response through galectin-9 and T-cell Ig mucin 3 interaction inhibits autoimmune diabetes in NOD mice. *European Journal of Immunology* 39: 2403–2411.
22. Zhu, C., A. C. Anderson, A. Schubart, H. Xiong, J. Imitola, S. J. Khoury, X. X. Zheng, T. B. Strom, and V. K. Kuchroo. 2005. The Tim-3 ligand galectin-9 negatively regulates T helper type 1 immunity. *Nat Immunol* 6: 1245–1252.
23. Seki, M., S. Oomizu, K.-M. Sakata, A. Sakata, T. Arikawa, K. Watanabe, K. Ito, K. Takeshita, T. Niki, N. Saita, N. Nishi, A. Yamauchi, S. Katoh, A. Matsukawa, V. Kuchroo, and M. Hirashima. 2008. Galectin-9 suppresses the generation of Th17, promotes the induction of regulatory T cells, and regulates experimental autoimmune arthritis. *Clin Immunol* 127: 78–88.
24. Wu, C., T. Thalhamer, R. F. Franca, S. Xiao, C. Wang, C. Hotta, C. Zhu, M. Hirashima, A. C. Anderson, and V. K. Kuchroo. 2014. Galectin-9-CD44 interaction enhances stability and function of adaptive regulatory T cells. *Immunity* 41: 270–282.
25. Elahi, S., W. L. Dinges, N. Lejarcegui, K. J. Laing, A. C. Collier, D. M. Koelle, M. J. McElrath, and H. Horton. 2011. Protective HIV-specific CD8+ T cells evade Treg cell suppression. *Nat Med* 17: 989–995.
26. Scharf, L., J. Tauriainen, M. Buggert, W. Hartogensis, D. J. Nolan, S. G. Deeks, M. Salemi, F. M. Hecht, and A. C. Karlsson. 2020. Delayed Expression of PD-1 and TIGIT on HIV-Specific CD8 T Cells in Untreated HLA-B*57:01 Individuals Followed from Early Infection. *Journal of Virology* 94.
27. Pombo, C., E. J. Wherry, E. Gostick, D. A. Price, and M. R. Betts. 2015. Elevated Expression of CD160 and 2B4 Defines a Cytolytic HIV-Specific CD8+ T-Cell Population in Elite Controllers. *J Infect Dis* 212: 1376–1386.
28. Eisele, E., and R. F. Siliciano. 2012. Redefining the viral reservoirs that prevent HIV-1 eradication. *Immunity* 37: 377–388.
29. Blankson, J. N., D. Persaud, and R. F. Siliciano. 2002. The challenge of viral reservoirs in HIV-1 infection. *Annu Rev Med* 53: 557–593.
30. Barton, K., A. Winckelmann, and S. Palmer. 2016. HIV-1 Reservoirs During Suppressive Therapy. *Trends Microbiol* 24: 345–355.

31. Kulpa, D. A., and N. Chomont. HIV persistence in the setting of antiretroviral therapy: when, where and how does HIV hide? *J Virus Erad* 1: 59–68.
32. Wong, J. K., and S. A. Yukl. 2016. Tissue reservoirs of HIV. *Curr Opin HIV AIDS* 11: 362–370.
33. Folkvord, J. M., C. Armon, and E. Connick. 2005. Lymphoid follicles are sites of heightened human immunodeficiency virus type 1 (HIV-1) replication and reduced antiretroviral effector mechanisms. *AIDS Res Hum Retroviruses* 21: 363–370.
34. Connick, E., T. Mattila, J. M. Folkvord, R. Schlichtemeier, A. L. Meditz, M. G. Ray, M. D. McCarter, S. Mawhinney, A. Hage, C. White, and P. J. Skinner. 2007. CTL fail to accumulate at sites of HIV-1 replication in lymphoid tissue. *J Immunol* 178: 6975–6983.
35. Fukazawa, Y., R. Lum, A. A. Okoye, H. Park, K. Matsuda, J. Y. Bae, S. I. Hagen, R. Shoemaker, C. Deleage, C. Lucero, D. Morcock, T. Swanson, A. W. Legasse, M. K. Axthelm, J. Hesselgesser, R. Geleziunas, V. M. Hirsch, P. T. Edlefsen, M. Piatak, J. D. Estes, J. D. Lifson, and L. J. Picker. 2015. B cell follicle sanctuary permits persistent productive simian immunodeficiency virus infection in elite controllers. *Nat Med* 21: 132–139.
36. Gray, L. R., M. Roche, J. K. Flynn, S. L. Wesselingh, P. R. Gorry, and M. J. Churchill. 2014. Is the central nervous system a reservoir of HIV-1? *Curr Opin HIV AIDS* 9: 552–558.
37. Galvin, S. R., and M. S. Cohen. 2006. Genital tract reservoirs. *Curr Opin HIV AIDS* 1: 162–166.
38. Park, C. O., and T. S. Kupper. 2015. The emerging role of resident memory T cells in protective immunity and inflammatory disease. *Nat Med* 21: 688–697.
39. Tóth, I., A. Q. Le, P. Hartjen, A. Thomssen, V. Matzat, C. Lehmann, C. Scheurich, C. Beisel, P. Busch, O. Degen, A. W. Lohse, T. Eiermann, G. Fätkenheuer, D. Meyer-Olson, M. Bockhorn, J. Hauber, J. van Lunzen, and J. Schulze Zur Wiesch. 2013. Decreased frequency of CD73+CD8+ T cells of HIV-infected patients correlates with immune activation and T cell exhaustion. *Journal of Leukocyte Biology* 94: 551–561.
40. Mills, J. H., L. F. Thompson, C. Mueller, A. T. Waickman, S. Jalkanen, J. Niemela, L. Airas, and M. S. Bynoe. 2008. CD73 is required for efficient entry of lymphocytes into the central nervous system during experimental autoimmune encephalomyelitis. *PNAS* 105: 9325–9330.
41. Ålgars, A., M. Karikoski, G. G. Yegutkin, P. Stoitzner, J. Niemelä, M. Salmi, and S. Jalkanen. 2011. Different role of CD73 in leukocyte trafficking via blood and lymph vessels. *Blood* 117: 4387–4393.
42. Aharoni Golan, M., R. Weisshof, Y. Wang, and D. T. Rubin. 2019. New Diagnosis of Acquired Immunodeficiency Syndrome in a Patient With Crohn's Disease. *ACG Case Rep J* 6: e00056.
43. Viazis, N., J. Vlachogiannakos, O. Georgiou, M. Rodias, D. Georgiadis, V. Papastamopoulos, I. G. Baraboutis, D. G. Karamanolis, and A. Skoutelis. 2010. Course of inflammatory bowel disease in patients infected with human immunodeficiency virus. *Inflamm Bowel Dis* 16: 507–511.
44. Bongiovanni, M., R. Ranieri, S. Ferrero, F. Casanova, and A. d'Arminio Monforte. 2006. Crohn's disease onset in an HIV/hepatitis C virus co-infected woman taking pegylated interferon alpha-2b plus ribavirin. *AIDS* 20: 1989–1990.
45. Bernstein, C. N., and W. J. Snape. 1991. Active idiopathic ulcerative colitis in a patient with ongoing HIV-related immunodepression. *Am J Gastroenterol* 86: 907–909.
46. Liebowitz, D., and D. McShane. 1986. Nonspecific chronic inflammatory bowel disease and AIDS. *J Clin Gastroenterol* 8: 66–68.
47. Stefanou, M.-I., M. Krumbholz, U. Ziemann, and M. C. Kowarik. 2019. Human immunodeficiency virus and multiple sclerosis: a review of the literature. *Neurological Research and Practice* 1: 24.
48. Velasquez, S., L. Prevedel, S. Valdebenito, A. M. Gorska, M. Golovko, N. Khan, J. Geiger, and E. A. Eugenin. 2020. Circulating levels of ATP is a biomarker of HIV cognitive impairment. *EBioMedicine* 51.
49. Graziano, F., M. Desdouits, L. Garzetti, P. Podini, M. Alfano, A. Rubartelli, R. Furlan, P. Benaroch, and G. Poli. 2015. Extracellular ATP induces the rapid release of HIV-1 from virus containing compartments of human macrophages. *PNAS* 112: E3265–E3273.

50. Stachon, P., S. Geis, A. Peikert, A. Heidenreich, N. A. Michel, F. Ünal, N. Hoppe, B. Dufner, L. Schulte, T. Marchini, S. Cicko, K. Ayata, A. Zech, D. Wolf, I. Hilgendorf, F. Willecke, J. Reinöhl, C. von Zur Mühlen, C. Bode, M. Idzko, and A. Zirlik. 2016. Extracellular ATP Induces Vascular Inflammation and Atherosclerosis via Purinergic Receptor Y2 in Mice. *Arterioscler Thromb Vasc Biol* 36: 1577–1586.
51. Shahbaz, S., M. Manicardi, G. Guaraldi, and P. Raggi. 2015. Cardiovascular disease in human immunodeficiency virus infected patients: A true or perceived risk? *World Journal of Cardiology* 7: 633–644.
52. Elahi, S., W. L. Dinges, N. Lejarcegui, K. J. Laing, A. C. Collier, D. M. Koelle, M. J. McElrath, and H. Horton. 2011. Protective HIV-specific CD8+ T cells evade Treg cell suppression. *Nat Med* 17: 989–995.
53. Okoye, I., L. Xu, M. Motamedi, P. Parashar, J. W. Walker, and S. Elahi. 2020. Galectin-9 expression defines exhausted T cells and impaired cytotoxic NK cells in patients with virus-associated solid tumors. *J Immunother Cancer* 8: e001849.
54. Oomizu, S., T. Arikawa, T. Niki, T. Kadowaki, M. Ueno, N. Nishi, A. Yamauchi, T. Hattori, T. Masaki, and M. Hirashima. 2012. Cell surface galectin-9 expressing Th cells regulate Th17 and Foxp3+ Treg development by galectin-9 secretion. *PLoS One* 7: e48574.