Neutrophils promote T cell activation in HIV infection through the regulated release of CD44 bound, cell surface Galectin-9

by

Garett John Dunsmore

A thesis submitted in partial fulfillment of the requirements for the degree of

Master of Science

In Immunology

Department of Medical Microbiology and Immunology
University of Alberta

Abstract

Neutrophils are the most abundant leukocyte in the human immune system constituting 50-80% of all white blood cells in the peripheral blood. Neutrophils are potent source of protective immune responses through the production of reactive oxygen species (ROS), extracellular trap formation, cytokine release, and phagocytosis of pathogens. Previous work has investigated the ability of neutrophils to impact T cell function through activation (HLA-DR, CD40, CD80, CD86, and ROS) or inhibition (Arginase-1, PDL-1) in HIV infection. Despite these studies, the interaction between neutrophils and T cells in the context of HIV infection remains a largely unexplored field that may reveal crucial implications that benefit human health.

Our study identifies a novel mechanism by which activated neutrophils in HIV infection promote increased activation of T cells by shedding Galectin-9 (Gal-9). In addition to this we propose that exogenous Gal-9 binds to cell surface CD44 on T cells to promote LCK activation and subsequentially enhances T cell activation.

Neutrophils also bind Gal-9 to surface CD44, which does not appear to promote cell signalling. Instead, we identify a novel mechanism of Gal-9 shedding by stimulated neutrophils. Unstimulated neutrophils express high levels of surface Gal-9 that is bound to CD44, upon stimulation neutrophils depalmitoylate CD44 and induce its movement out of the lipid raft. This process causes the release of Gal-9 from the surface of neutrophils. Furthermore, this process is regulated by the activity of glycolysis and can be inhibited by IL-10.

Together our data reveal a novel mechanism of Gal-9 shedding from the surface of neutrophils. This could explain the elevated plasma Gal-9 levels in HIV-infected individuals as the underlying mechanism of the well-characterized chronic immune activation. This study provides a novel role for the Gal-9 in HIV pathogenesis.

Preface

Publications

2020

1) Shahbaz S., **Dunsmore G**., Koleva P., Xu L., Houston S., & Elahi S. Galectin-9 and VISTA expression define terminally exhausted T cells in HIV-1 infection. Journal of Immunology. doi : 10.4049/jimmunol.1901481

2019

1) Namdar N., Dunsmore G., Koleva P., Shahbaz S., Jovel J., Branton W., Power C., Houston S., & Elahi S. $CD71^{+}$ Erythroid Cells Exacerbate HIV-1 Susceptibility, Mediate trans-Infection, and Harbor Infective Viral Particles. mBio 2019. doi : 10.1128/mBio.02767-19.

2) Motamedi M., Shahbaz S., Fu L., Dunsmore G., Xu L., Harrington R., Houston S., & Elahi S. Galectin-9 Expression Defines a Subpopulation of NK Cells with Impaired Cytotoxic Effector Molecules but Enhanced IFN-γ Production, Dichotomous to TIGIT, in HIV-1 Infection. ImmunoHorizons 2019. doi: 10.4049/immunohorizons.1900087.

2018

1) **Dunsmore G**., Koleva P., Sutton R.T., Ambrosio L., Huang V., & Elahi S. Mode of delivery by an ulcerative colitis mother in a case of twins: immunological differences in cord blood and placenta. World Journal of Gastroenterology 2018. doi: 10.3748/wjg.v24.i42.4787.

2) Meng X., **Dunsmore G**., Koleva P., Elloumi Y., Wu R.Y., Sutton R.T., Ambrosio L., Hotte N., Nguyen V., Madsen K.L., Dieleman L.A., Chen H., Huang V., & Elahi S. The profile of human milk metabolome, cytokines and antibodies in inflammatory bowel diseases versus healthy mothers and potential impact on the newborn. Journal of Crohns Colitis 2018. doi: 10.1093/ecco-jcc/jjy186

3) **Dunsmore G**., Koleva P., Ghobakhloo N., Sutton R.T., Ambrosio L., Meng X., Hotte N., Nguyen V., Madsen K.L., Dieleman L.A., Huang V., & Elahi S. Lower Abundance and Impaired Function of CD71+ Erythroid cells in Inflammatory Bowel Disease Patients During Pregnancy. Journal of Crohn's and Colitis 2018. doi: 10.1093/ecco-jcc/jjy147.

4) Delyea C., Bozorgmehr N., Koleva P., **Dunsmore G**., Shahbaz S., Huang V., & Elahi S. CD71+ Erythroid Suppressor Cells Promote Fetomaternal Tolerance through Arginase-2 and PDL-1. Journal of Immunology 2018. doi: 10.4049/jimmunol.1800113.

2017

1) **Dunsmore G.,** Bozorgmehr N., Delyea C., Koleva P., Namdar A., & Elahi S. Erythroid suppressor cells compromise neonatal immune response against Bordetella

pertussis. Journal of Immunology – Featured Publication, 2017. doi: 10.4049/jimmunol.1700742.

Table of Contents

List of Figures

Introduction

Neutrophils are the most abundant leukocyte in the blood, constituting 60-80% of all white blood cells in circulation. The activation of neutrophils in circulation causes cellular polarization, which enhances neutrophils ability to engage in rolling adhesion to the endothelium of blood vessels and enhances tissue extravasation(1). Signalling involved in neutrophil polarization causes the movement of a variety of extracellular adhesion molecules that facilitate neutrophil rolling adhesion (selectins, integrins and CD44) (2, 3). Polarization of adhesion molecules to the leading or trailing end of a crawling neutrophil also promotes extravasation and movement of neutrophils through tissues(2-4). Reactive oxygen species (ROS) is involved in efficient chemotaxis and directionality of cytoskeleton remodelling in neutrophils and produced at the pseudopodia (front of the cell) by the NADPH-oxidase enzyme NOX2, which contributes to actin polymerization and directional movement of neutrophils (5).

Neutrophils primarily use glycolysis and pentose phosphate pathway for energy metabolism(6). One of the mechanisms of neutrophil protection that relies on glycolysis is ROS production by NADPH-oxidation upon stimulation. NADPH is produced by canonical glycolysis and pentose phosphate pathway in neutrophils and induces the production of ROS by the NOX2 enzyme(7). ROS can also mediate the activation of Calcium/calmodulin dependant protein kinase II (CaMKII), which contributes to cell migration in vascular smooth muscle and apoptosis (8-10). CaMKII is a serine/threonine-specific protein kinase that contributes to the phosphorylation, and transduction of a variety of signaling pathways. CaMKII can be activated by calcium/calmodulin or oxidation by hydrogen peroxide(8). Upon activation, CaMKII can contribute to neutrophil activation by a variety of mechanisms, for example, CaMKII can activate the inflammatory response proteins AP1, NF-kB, and HDAC4(11-13). CaMKII can facilitate CD44

phosphorylation and induces its interaction with the actin cytoskeleton, which is integral for the interaction of CD44 with its ligand hyaluronan(14).

CD44 is a glycoprotein that canonically has been shown to bind the polymer hyaluronic acid (hyaluronan) and promotes cell recruitment into tissues (15). CD44 is expressed on neutrophils and interacts with the extracellular polymer hyaluronan and E-cadherin that is expressed on the surface of endothelial cells and contributes to neutrophil crawling and extravasation(15, 16). In order for CD44 to perform its interaction with hyaluronan, it interacts with the cellular cytoskeleton through a cytoskeleton complex composed of talin-1, RAP-1, and ezrin. Upon binding directly to these proteins, CD44 will move to the trailing end of the cell where it will bind its ligands and promote cell movement(17). In agreement with these findings, previous studies have shown that during neutrophil polarization, CD44 leaves the lipid rafts and localizes to the uropod during cell polarization(18). The function of CD44 in neutrophil recruitment is not well understood; however, it is: involved in rolling adhesion, implicated in firm adhesion, and is required for neutrophils to enter liver sinusoids(19, 20). The regulation of CD44 movement to the trailing end of the cell during polarization is a regulated process facilitated by several proteins and CD44's post-translational modifications. CD44 has three characterized post-translational modifications: phosphorylation, palmitoylation, and glycosylation(21). Phosphorylation of CD44 at the Y395 residue, is integral for the movement of CD44 out of the lipid raft and the interaction with actin cytoskeleton in neutrophil polarization. The process of CD44 phosphorylation is regulated by CaMKII, calcium release and oxidation(8, 14). Y395 phosphorylation of CD44 has been speculated to cause a conformational change in the intracellular domain, which could regulate the depalmitoylation of CD44(17). The palmitoylation of CD44 anchors the protein to the lipid raft, similarly to other palmitoylated proteins. Once

CD44 is depalmitoylated it is able to leave lipid rafts and interact with the actin cytoskeleton of the cell. In neutrophils, this process facilitates the engagement of CD44 with its ligand hyaluronan.

The role of neutrophils in HIV infection has been contradicting. In general, neutrophil persistent in the site of inflammation can enhance chronic inflammation(22).

Mounting evidence supports a role for persistent inflammation during suppressive antiretroviral therapy (ART) as a contributing factor to non-AIDS-defining comorbidities (23-25).

For example, neutrophils appear to be more activated in HIV patients with low compared to those with a higher CD4 T cell count (26). Moreover, neutrophils from HIV-patients with low CD4 T cell count have increased chemotactic activity (26, 27). Neutrophils may support CD8+ T cell activation in the tissue and contribute to the inactivation of the HIV virion, but their persistence can cause collateral damage (28, 29). In contrast, neutrophils via mitochondrial ROS can suppress T cell functions (30).

T cell exhaustion is the hallmark of viral infections such as HIV and hepatitis C virus, as well as cancer(31). Exhausted T cells express multiple co-inhibitory receptors (e.g. PD-1, CTLA-4 and Tim-3), altered expression and use of key transcription factors and metabolic derangements that render patients unable to mount an effective immune response (32-35).

However, the expression of co-inhibitory receptors or their ligands on neutrophils in the context of HIV-infection has not been well studied. A recent study suggested that neutrophils by the expression of PD-L1 mediate immune suppression in PD-1 expressing T cells (36). However, this study was focused on low density weight neutrophils but not mature neutrophils. We have reported upregulation of Galectin-9 on NK and T cells in HIV-infected individuals(37, 38).

Gal-9 is a beta-galactoside binding protein in the galectin family of proteins that interacts with different receptors such as CD44, CD137, Protein disulfide isomerase (PDI), and TIM-3 resulting in different biological outcomes (39-41). Gal-9 can be released by a variety of immune and non-immune cells, causing a broad spectrum of functions(42).

The plasma Gal-9 increases in HIV infected patients (43) especially as the CD4 T cell count decreases (44). This indicates that plasma Gal-9 might be associated with disease progression in HIV-patients. Soluble Gal-9 can activate T cells, and is associated with reactivation and transcriptional enhancement of HIV by augmenting ERK signalling through LCK activation (45, 46). In addition to the exogenous Gal-9, surface bound Gal-9 has been identified on Tregs, T cells, NK cells, monocytes, basophils, and mast cells (37, 38, 47-49). Previous analysis has shown that neutrophils from healthy controls are capable of efficiently binding Gal-9; however, the receptor that facilitate this binding activity is unknown (50).

Recent studies have suggested that the interaction of CD44 and Gal-9 on T cells could increase T cell activation by enhancing ERK signaling (45, 46). In addition, CD44 can stabilize LCK interaction through direct zinc dependant binding, which is attributed to T cell spreading and movement(51).

In this study, we highlight the role of neutrophils in HIV infection. In particular, we show that neutrophils in HIV-infected individuals have differential gene and functional profiling depending on the CD4 T cell count. Moreover, we describe a novel mechanism and regulatory process that facilitates the release of surface bound Gal-9 from neutrophils which can activate T cells.

Thus, our study presents a novel link between T cell activation and neutrophils in HIV infection via Gal-9 shedding.

Materials and Methods

Study Population. Blood samples were taken from 50 healthy subjects (negative for HIV, HCV, and HBV) and 114 HIV patients (co-infected HIV patients with HCV or HBV were omitted from this study).

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 with protocol numbers Pro000046064 and Pro000070528.

Cell Isolation and culture. Fresh blood samples were collected in EDTA vacutainer blood tubes (BD). Peripheral blood mononuclear cells (PBMCs) were isolated by gradient separation using Ficoll-Paque Premium (GE). The red blood cell pellet containing neutrophils was removed and red blood cells were lysed using a red blood cell lysis buffer (0.155M NH_4Cl, 10mM $KHCO_3$, and 0.1mM EDTA). Freshly isolated polymorphonuclear cells were washed with 4°C PBS, and put in culture media (RPMI-1640 Sigma) supplemented with 10% fetal-bovine serum (Sigma), and a working concentration of pen/streptomycin (Sigma). Neutrophils were stimulated for one hour at 37°C (5% CO_2) in vitro by culturing $1x10^6$ PMNs in the presence or absence of lipopolysaccharide (LPS) at100 ng/mL in culture media supplemented with 10% fetal bovine serum, penicillin, and streptomycin.

Flow Cytometry. Antibodies used in this study were purchased mainly from BD Biosciences, ThermoFisher Scientific, and R&D: human anti-CD3 [SP34-2], anti-CD4 [RPA-T4], anti-CD8 [RPA-T8], anti-CD11b [ICRF44], anti-CD15 [W6D3], anti-CD16 [3G8], anti-CD25 [M-A251], anti-CD32 [FLI8.26], anti-CD38 [HB7], anti-CD44 [515], anti-CD49d [9F10], anti-CD69 [FN50], anti-CD101 [V7.1], anti-CD137 [4B4-1], anti-CD273 [MIH18], anti-CD274 [MIH1], anti-CD366 [7D3], anti-Gal-9 [9M1-3], anti-GLUT1 [FAAB1418F], anti-HLA-DR [G46-6], anti-phospho-LCK(Y505) [4], anti-VISTA [730804]. Cell viability was measured using an

Annexin V Staining Kit (BD Biosciences), and LIVE/DEAD Kit (Life Technologies). Reactive oxygen species production was measured using DCFDA / H2DCFDA – Cellular ROS Assay Kit (abcam). Data was acquired on an LSRFortessa X-20 (BD Biosciences) and analyzed using FlowJo Version 10.5.3.

ImageStream Analysis. Stained cells (1 x 10^6) were fixed in 4% paraformaldehyde and data was acquired on an Amnis ImageStream Mark II (EMD Millipore) according to our previous protocols (Dunsmore, 2017, 28779022). A minimum of 10,000 events were acquired for each sample. Data was analyzed using the IDEAS Analysis Software Package. Neutrophils were identified as having an area >100 and positively stained for CD15. T cells were identified by having an area >80 and stained positively for CD3. Co-localization was measured by calculating the Bright Detail Similarity of the colocalizing probes using the IDEAS wizard. Capping was measured using a delta centroid XY calculation.

RNA Sequencing. RNA libraries were assembled using TruSeq RNA Library Prep Kit v2 (Illumina) for sequencing by a NextSeq 500 Instrument (Illumina).

Differentially expressed genes were identified by subjecting raw counts to analysis using the EdgeR package (3.20.9). Comparisons identified 1651 genes differentially expressed that had a FDR <0.05 and a log fold change <-2 or >2. Heatmaps were generated using the pHeatmap package. Euclidean distance and Ward's Aggregation Criterion. Gene ontologies were generated by inputting a list of differentially expressed genes to the gene ontology consortium. Only significant ontologies associated with neutrophil function (FDR <0.05) were considered for analysis. The code used for EdgeR DEG RNA sequencing analysis and heatmapping can be found in supplemental text 1.

T cell stimulation studies. Freshly isolated T cells were stimulated with 0.25 ∝g/mL of anti-CD3 (BD Biosciences) and 0.1∝g/mL of anti-CD28 (BD Biosciences) antibodies. In some studies, recombinant human Gal-9 (Gal Pharma) 1∝g/mL (~31∝M) was added to T cells in the presence/absence of 100 ∝g/mL of an anti-CD44 blocking antibody (R&D). After 48 hours, T cell activation and phenotype were measured using flow cytometry. In other studies, T cells were stimulated for 48 hrs using anti-CD3/CD28 to upregulate TIM-3, then fresh autologous neutrophils obtained and added into the activated T cells in the presence/absence of 10 ∝g/mL of a neutralizing anti-TIM-3 antibody (R&D) for an additional 24 h, before analysis. In other experiments, freshly isolated and purified neutrophils were added at time of T cell stimulation at a 2:1 neutrophil to T cell ratio. T cells were treated with and without anti-TIM-3 antibody and in presence or absence of neutrophils.

Glycolysis inhibition. Neutrophil glycolysis was inhibited by the use of several chemical and biological inhibitors. LPS stimulated neutrophils were exposed to 250 ∝M of the chemical Glucose Transporter 1 (GLUT1) inhibitor Phloretin (Sigma). ROS production and Gal-9 shedding were measured using imagestream and flow cytometry respectively. In some studies, ROS production and Gal-9 shedding were quantified in the presence of recombinant IL-10 (R&D).

CD44 post-translational modification inhibitors. Neutrophils stimulated for one hour with 100 ng/mL of LPS were treated in the presence or absence of the depalmitoylation inhibitors Palmityl trifluoromethyl ketone (20 ∝M) (Cayman Chemical) and methyl arachidonyl fluorophosphonate (5 ∝M) (Cayman Chemical). To inhibit CaMKII activity, 20 ∝M of the CaMKII inhibitor CK59 (EMD milipore) was used. Cells were stained, fixed, and analyzed by flow cytometry and image stream.

Cytoskeletal inhibitors. Neutrophils were stimulated for one hour with 100 ng/mL of LPS and were treated with 100 ∝M of the RAP-1 inhibitor farnyslthiosialicyclic amide (Milipore Sigma). Ezrin was inhibited by treating neutrophils in the presence of LPS for one hour with an ezrin inhibitor NSC668394 (Calbiochem). Upon treatment with the inhibitors, neutrophils were imaged and analyzed by flow cytometry to measure the localization of CD44 and Gal-9.

Lipid raft depletion. Lipid rafts were depleted by exposing neutrophils to 30 mM methyl-beta-cyclodextran (Sigma) for 20 minutes at 4°C. Neutrophils were analyzed by flow cytometry and imaging to observe the impact of lipid raft depletion on Gal-9 expression and CD44 localization.

ELISA Assay. Plasma was collected from fresh blood samples and frozen at -80°C. Before carrying out the ELISA, the samples were thawed and the protocol was followed as described by the manufacturer. Gal-9 concentration in the plasma of HIV patients was measured using a Galectin-9 Duoset ELISA (R&D).

Statistical analysis. Statistical analysis was performed by utilizing prism version 8 using appropriate statistical tests depending on the data. Additionally, as previously mentioned all RNA sequencing statistical analysis was performed using the package edgeR. All statistically significant values were identified as having a p value or false-discovery rate of < 0.05.

Results

Neutrophils from HIV patients are transcriptionally pro-inflammatory

To determine the transcriptional profile of neutrophils in HIV patients, we conducted RNAseq analysis on total RNA extracted from enriched neutrophils of HIV-infected individuals on ART, with high (> 0.45) and low CD4 T cell count (< 0.45) in comparison with neutrophils obtained from healthy controls (HCs). When hierarchical clustering was conducted on Euclidian distances between samples, neutrophils from HIV patients clearly showed a different gene expression

profile than the HCs (Fig 1A). For the most part, healthy samples formed separated branches on a dendrogram, while HIV samples exhibited a more erratic distribution along such dendrogram depending on the CD4 T cell count (Fig 1A). These results were partially recapitulated in principal component analysis (PCA) on the Euclidian distances between samples (Fig 1B). In essence, neutrophils from HIV-infected individuals were clearly separated from HCs. However, three out of five samples from those with a higher CD4 T cell count were closer to HCs in PCA (Fig. 1B). To further evaluate the data, significantly differentiated expressed genes (FDR <0.05) with a log fold change difference < -2, or > 2, were heatmapped (Fig 1C) and comparisons of gene expression were shown by volcano plots (Supplemental Figure 1A-C). These analyses revealed five clusters of differentially expressed genes between the groups (HIV patients with high or low CD4 T cell count and HCs) (Fig 1C). To evaluate the biological processes associated with these differentially expressed clusters of genes, the transcripts within each cluster of the heatmap were subjected to gene ontology analysis. Some of the significantly related biological processes were graphically represented demonstrating the processes related to each cluster (Fig 1D and Supplemental Figure 2A). Additionally, the genes associated with these ontologies were heatmapped and annotated with their associated gene ontology (Fig 1E), which indicated upregulation of genes associated with NETosis, p38MAPK cascade, calcineurin-NFAT signaling cascade, FC-gamma receptor signaling, cellular response to hydrogen peroxide and the regulation of cell polarity in neutrophils from HIV-infected individuals versus HCs. Furthermore, genes associated with neutrophil activation were increased in HIV infected patients (Supplemental Figure 2B). These observations confirm a more activated transcriptional profile in neutrophils from HIV-infected individuals versus HCs.

Differential effects of neutrophils on T cell activation depending on the CD4 T cell count of HIV-infected individuals

To evaluate whether neutrophils from HIV patients impact T cell activation, we activated T cells with anti-CD3/CD28 antibodies for 48 hrs in the presence or absence of neutrophils (Supplemental Figure 3A). We observed a differential effect on the expression of T cell activation markers (CD69 and CD38) by neutrophils from HIV-patients with low versus high CD4 T cell counts (Fig. 2A and B). While neutrophils from HIV-infected individuals with high CD4 T cells count and healthy controls did not impact T cells activation, neutrophils from HIV-infected individuals with low CD4 T cell count significantly increased the expression of activation markers CD38 and CD69 on both $CD4^+$ and $CD8^+$ T cells in vitro (Fig 2A and B). Although based on the transcriptional profile neutrophils from HIV-infected patients were transcriptionally inflammatory, they exhibit differential effects on T cell activation.

Neutrophils express differential levels of Gal-9

To evaluate how neutrophils from HIV-infected individuals with low CD4 T cell count were enhancing T cell activation, we speculated that neutrophils via soluble factors such as cytokines or cell-cell interactions may influence T cell activation. To answer this question, we analyzed the transcriptional prolife of cytokines that may modulate T cell activation. Interestingly, no difference in the transcriptional expression of a variety of cytokines was noticed among the groups (Fig 3A). Next, we assessed the expression of co-inhibitory receptors, their ligands and co-stimulatory molecules at the transcriptional levels. Although at the transcriptional levels some co-inhibitory/co-stimulatory molecules were highly expressed and some had very low expression, we did not observe any significant difference in the expression of these molecules in neutrophils of HIV patients regardless of their CD4 T cell count with HCs (Fig 3B). To further

investigate the potential role of these molecules in T cell activation, their abundance at the protein levels were measured using flow cytometry. Using the gating strategy as depicted in supplemental figure 3B, we did not find any significant difference at the cell surface expression levels for a number of these highly-expressed molecules at the gene levels (Fig. 3 C-G). In contrast to the other evaluated molecules, we found a significant difference in the surface expression of Gal-9 on neutrophils from HCs versus HIV-patients. While > 80% of neutrophils from healthy controls expressed Gal-9, neutrophils from HIV-infected individuals exhibited significantly lower levels of Gal-9 (Fig. 3G). These observations suggested that differential Gal-9 expression on neutrophils may play a role in neutrophils mediated T cell activation.

Surface Gal-9 expression on Neutrophil decreases as HIV progresses

Upon observing the differential expression of Gal-9 on neutrophils from HCs versus HIV-infected individuals (Fig 3G and 3H), we aimed to further investigate the diversity of Gal-9 expression on neutrophils in HIV-infected individuals. We observed a significant decrease in the expression of surface Gal-9 on neutrophils in HIV patients as the disease progresses. As shown in Fig 4A and B, the decline in Gal-9 expression on neutrophils was more pronounced in HIV-infected individuals with low compared to high CD4 T cell count, and these groups had significantly lower levels of Gal-9 compared to HCs. This decline was significant at the percentages of Gal-9 expressing neutrophils and also the intensity of Gal-9 expression (Fig. 4B and C). These observations suggested that neutrophils from HIV-infected individuals may lose Gal-9 on their surface. To evaluate whether this Gal-9 was being shed or internalized, we measured the amount of soluble Gal-9 in the supernatant of unstimulated and cultured neutrophils (2×10^6) for 8 hrs. Interestingly, we found significantly higher levels of soluble Gal-9 in the culture supernatants of cultured neutrophils from HIV-infected individuals with low

CD4 T cell count compared to those with high CD4 T cell count and HCs (Fig 4D). Additionally, we found a positive correlation between CD4 T cell count and Gal-9 expressing neutrophils in HIV-infected individuals (Fig 4E). Moreover, an inverse correlation between the plasma Gal-9 concentrations and the percentages of Gal-9+ neutrophils was observed (Fig 4F). Similar pattern was observed for the plasma Gal-9 and CD4 T cell count in HIV-infected individuals (Fig 4G). In contrast to the expression of Gal-9, we found that the expression of CD32 was significantly higher in neutrophils of HIV-infected individuals, in particular, in those with low CD4 T cell count compared to HCs (Fig 4H). Further analysis confirmed a negative correlation between CD4 count with CD32 expression on neutrophils in HIV-infected individuals (Fig 4I). These observations suggest that activation status of neutrophils from HIV-infected individuals may result in Gal-9 shedding, which may play a role in HIV pathogenesis.

Soluble Gal-9 intensifies T cell activation

To evaluate the potential effects of secreted Gal-9 by neutrophils on T cells activation, total PBMCs from HCs were stimulated with anti-CD3/CD28 in the presence or absence of different concentrations of recombinant Gal-9 in vitro. We found that recombinant Gal-9 at higher concentrations enhanced T cells activation as measured by the expression levels of CD38 and HLA-DR on both CD4+ and CD8+ T cells (Fig 5A-H). These observations suggest that neutrophils from HIV-infected individuals shed higher concentrations of Gal-9, which explains their differential effects on T cells activation in vitro.

Gal-9 enhances T cell activation through CD44 binding and LCK signaling

Although the interaction of cytoplasmic membrane Gal-9 on antigen presenting cells or Tregs with TIM-3 on T cells renders them to an exhausted phenotype (31, 52, 53), soluble Gal-9 enhances T cells activation (54). As described above, we observed that soluble Gal-9 enhances T

cells activation in vitro. Thus, we decided to determine how soluble Gal-9 increases T cell activation. We began by measuring the colocalization of surface Gal-9 expression on T cells with different markers that have been identified to interact/bind to Gal-9. We found that co-culture of neutrophils from HIV-infected individuals with their T cells resulted in the colocalization of Gal-9 with CD44 on the surface of T cells (Fig 6A and B). However, this was not the case for other potential Gal-9 receptors such as CD137 (Fig. 6B). To mimic these observations, T cells were incubated with 1000 ng/mL of recombinant Gal-9 for one hour and then subjected to flow cytometry analysis. We found that the incubation of T cells with recombinant Gal-9 resulted in a significant increase in the percentages of Gal-9 expressing T cells (Fig 6C), suggesting the binding of recombinant Gal-9 to T cells. Subsequently, Gal-9 binding to T cells increased CD44 clustering on T cells (Fig 6D). These observations indicated that Gal-9 interacts with CD44 on T cells. To determine whether such interactions result in T cells activation, we examined T cell activation markers such as CD38 and HLA-DR following stimulation with anti-CD3/CD28 antibodies in the presence or absence of recombinant Gal-9 and anti-CD44 blocking antibody for 48 hours. Although these studies confirmed that the recombinant Gal-9 enhances T cells activation as shown by the upregulation of CD38 and HLA-DR, anti-CD44 blocking antibody significantly prevents such effects (Fig 6E-J). These observations suggest that recombinant Gal-9 via interactions by CD44 enhance T cells activation. Importantly, we found that this occurs through LCK signaling as recombinant Gal-9 (1000 pg/ml) decreases phospho-LCK (Y505) intensity in T cells (Fig 6K-M) in both CD4+ and CD8+ T cells, respectively. Interestingly, pre-treatment of T cells with LCK inhibitor, which suppresses LCK phosphorylation on Tyr-505 abrogated stimulatory effects of Gal-9 on T cells possibly through calcium mobilization (54).

These results indicate that exogenous Gal-9 interactions with CD44 contributes to T cell activation.

Neutrophils shed Gal-9 by CD44 depalmitoylation

Upon observing that Gal-9 shedding occurs in neutrophils of HIV-infected individuals, we decided to mechanistically explore how neutrophils shed Gal-9. We observed that Gal-9 colocalizes with CD44 on the surface of neutrophils even at greater extend compared to T cells (Fig 7A and B). Furthermore, we utilized imaging cytometry to analyze the organization of CD44 on neutrophils by the gating strategy detailed in Supplemental Figure 3C. We observed that when CD44 capping occurs (high radial delta centroid), Gal-9 diminishes from the surface of neutrophils (Fig 7C and D). Moreover, we found that stimulation of neutrophils by LPS induces (1hr) CD44 capping (Fig 7E and F). Subsequently, stimulation of neutrophils by LPS significantly reduced the surface expression of Gal-9 (Fig 7G-I). Based on these observations we found a reverse correlation between CD44 capping and % Gal-9 expressing neutrophils (Fig 7J). To further evaluate whether the movement of CD44 from the lipid raft contributes to the Gal-9 shedding, we exposed neutrophils to 30 mM of methyl-beta-cyclodextran (MBCD) for 20 minutes (depleting lipid rafts). Upon depletion of lipid rafts, we observed a decrease in the expression of Gal-9 (Fig 7K). Upon release from the lipid raft, CD44 has been shown to interact with the actin cytoskeleton through an interaction with the molecules RAP-1 and ezrin (55). We decided to determine if the inhibition of ezrin and RAP-1 can prevent Gal-9 shedding. However, our observations indicated that Ezrin and RAP-1 inhibitors did not inhibit Gal-9 shedding following stimulation of neutrophils by LPS (Fig 7L and 7M). It has been reported that to facilitate the movement of CD44 from the lipid rafts, depalmitoylation enzymes will depalmitoylate lipid raft associated CD44 (Donatello, 2012, 23031255). We found that the

processes of depalmitoylation contributes to the shedding CD44 capping and shedding of Gal-9 on neutrophils (Fig 7N and 7O). These results indicate that the movement of CD44 from the lipid raft, and more specifically the depalmitoylation of CD44 induces the shedding of Gal-9 from neutrophils.

Glycolysis and ROS production regulates CD44 polarization and Gal-9 shedding in neutrophils

Upon mechanistically identifying how Gal-9 is shed from neutrophils during activation, we aimed to investigate the regulatory process contributing to the Gal-9 shedding. By utilizing the small molecule inhibitor of GLUT1, phloretin, we observed the inhibition of Gal-9 shedding in LPS stimulated neutrophils (Fig 8A-C). Although neutrophil activation increases CD44 capping (Fig. 7E and 7F), this CD44 capping was impaired by phloretin (Fig 8D). In addition, we observed that stimulation of neutrophils with LPS resulted in a significant increase in the expression of ROS (Fig 8E and 8F). To determine whether phloretin can impact ROS production by neutrophils, we stimulated neutrophils with LPS in the presence or absence of 100 nM phloretin for one hour. We found that phloretin significantly inhibited ROS expression by neutrophils (Fig 8G and 8H). In order to better understand the role of ROS in Gal-9 shedding, we treated neutrophils with LPS in the presence or absence of Apocynin (Apoc) according to our previous report (56). As shown in Fig 8I and as predicated, LPS stimulation significantly reduced Gal-9 expression but the inhibition of ROS with apocynin treatment restored Gal-9 levels on neutrophils. Knowing that ROS oxidizes CaMKII (57), we evaluated whether Gal-9 shedding can be prevented by treating activated neutrophils with a CaMKII inhibitor. Remarkably, we found that although LPS stimulation sheds Gal-9 from the surface of neutrophils, treatment with a CamKII inhibitor prevented shedding of Gal-9 (Fig 8J) and

subsequently reduced CD44 capping (Fig 8K). Taken together these results demonstrate that glucose metabolism, ROS production, and CamKII activation regulate neutrophil shedding of Gal-9.

Neutrophil shedding of Gal-9 is regulated by IL-10 in HIV infection

As previously shown (Fig 1), the transcriptional profile of neutrophils from HIV-infected individuals exhibited an inflammatory profile. However, the expression of Gal-9 was different depending on the CD4 T cell count of the HIV-infected subjects. Upon identifying a regulatory mechanism for Gal-9 shedding in neutrophils, we wanted to evaluate whether these regulatory mechanisms could explain the differences in Gal-9 expression on neutrophils from HIV-infected patients with high CD4 T cell count versus those with low CD4 T cell count. We measured the surface expression of Gal-9 on neutrophils and found that HIV patients with high CD4 T cell count had lower percentages of GLUT-1 expressing neutrophils and also lower intensity of GLUT-1 (Fig 9A-C). In addition, we found an inverse correlation between percentages of GLUT-1+ neutrophils and CD4 T cell count in HIV-infected individuals (Fig 9D). To evaluate whether GLUT-1 expression is modulated by a plasma soluble factor, plasma specimens (10% plasma in culture media for one hour) from HIV-patients with low CD4 T cell count or high CD4 T cell count were added to unstimulated neutrophils of HCs. We observed that GLUT-1 expression was significantly reduced when neutrophils were treated with plasma from HIV-patients with high CD4 T cell count but not from those with low CD4 T cell count (Fig 9E). Interestingly, the plasma from HIV-infected individuals with low CD4 T cell count significantly increased GLUT-1 expression whereas the opposite effect was observed by the plasma from HIV-infected individuals with high CD4 T cell count (Fig 9F). However, plasma from healthy controls did not change GLUT-1 expression but treatment of neutrophils with LPS significantly

increased GLUT-1 expression (Fig 9F). To better understand the underlying mechanism of differential effects of plasma on GLUT-1 expression, the plasma of HIV-infected individuals was subjected to cytokine ELISAs. Among the detected cytokines in the plasma, we noticed HIV-patients with high CD4 T cell count had substantially elevated concentrations of IL-10 compared to the plasma from HIV-patients with low CD4 T cell count, while IL-10 in the plasma of HCs was undetectable (Fig 9G). In agreement with previous studies, the IL-10 responding gene DDIT4, was upregulated in neutrophils from HIV infected patients with high CD4 count (Supplemental figure 4A) (58). To better understand the role of IL-10 on GLUT-1 expression in neutrophils, we cultured neutrophils in the presence of IL-10 either unstimulated or stimulated with LPS. As predicated, LPS upregulated GLUT-1 expression on neutrophils, however, addition of IL-10 (for one hour) abrogated the LPS effects and significantly reduced GLUT-1 expression (Fig 9H). This agrees with previous reports that IL-10 decreases GLUT-1 translocation(58). Using the same approach utilized by Ip W.K., et al. we found that GLUT-1 translocation was inhibited by IL-10 (Fig 9I-J). Moreover, we observed that although LPS enhanced ROS production in neutrophils, IL-10 reversed this effect and significantly reduced ROS production by activated neutrophils (Fig 9K and 9L). Consequentially, Gal-9 shedding was inhibited by IL-10 treatment (Fig 9M) suggesting that Gal-9 shedding in HIV-patients with high CD4 T cell count was inhibited by IL-10.

Discussion

In this study, we identified a novel role for neutrophils in T cell activation during HIV infection. We found that neutrophils in HIV-infected individuals have an activated phenotype and shed Gal-9, which contributes to T cell function by a newly identified mechanism.

This is in agreement with previous studies that have shown phenotypically neutrophils are activated and have increased adhesion marker expression and ROS production in HIV-infected patients (26, 27, 59). Here, we show that neutrophils in HIV infection have a transcriptional profile that is associated with an activated phenotype in comparison to neutrophils from healthy controls. These observations provide informative evidence regarding specific genes and pathways that are contributing to the activation of neutrophils in HIV infection. Previous work has suggested that neutrophil activation in HIV infection is through IL-18, IL-17, and IL-8 signaling (59). Despite these important observations, previous studies have not distinguished which are contributing to neutrophil activation. Our data suggest that neutrophils have increased expression of specific genes associated with the p38 MAPK cascade, NFAT signaling, and FcgR signaling.

Our study also reveals a novel observation, that neutrophils from HIV patients with progressing infection (low CD4 T cell count) are more activated. Through analysis of transcriptional profiles of HIV infected patients, we found that neutrophils from HIV patients with low CD4 T cell count have increased expression of genes associated with a variety of signaling activities. These observations are in agreement with previous studies that have shown neutrophils from HIV patients with low CD4 T cell count are more activated than HIV patients with high CD4 T cell count (26). These results indicate a change in neutrophil biology in HIV patients as disease progresses, which is important to take into consideration when assessing HIV infected patients on ART. One possible explanation for differential neutrophil activation might be because of the increased bacterial product in the periphery via translocation from the gut as previously described (60). Our study did not specifically identify sources of neutrophil activation

during HIV infection; however, future studies should investigate the potential involvement of microbial translocation on neutrophil activation.

With the knowledge that neutrophils from HIV infected patients are more activated, we wanted to evaluate their impact on T cell activation. During HIV infection, the activation of T cells can be associated with HIV disease progression and therefore, increased T cell activation could be promoting HIV pathogenesis (61). Previous studies have attributed neutrophil function to the activation and/or suppression of T cells in HIV infection (36, 62). Our study suggests that neutrophils are capable of enhancing T cell activation if they are from HIV patients with low CD4 count only. In healthy individuals or HIV patients with high CD4 count we did not observe a change in T cell activation when co-cultured with neutrophils. These observations suggest that neutrophils are not created equally in all HIV-infected individuals and depending on their phenotype can influence T cell activation.

To identify which T cell activation modulating proteins neutrophils express to contribute to increasing T cell activation, we observed the expression of specific genes from the neutrophils of healthy controls, and HIV patients with high and low CD4 T cell count.

During viral infections, previous work has shown neutrophils express HLA-DR (MHC class II), CD80, and CD40, which contributes to antigen specific $CD4^+$ T cell responses(63). However, the expression of HLA-DR, CD80 and CD40 on neutrophils requires antigen exposure and a long-term neutrophil cell culture (6 days) which may not be physiologically relevant as neutrophils have a short half-life. In another study, the expression of HLA-DR has been shown to be expressed on the surface of neutrophils from patients treated with recombinant IFN-γ (44 hours) (64-66). Despite these reports, our studies did not indicate HLA-DR expression on the surface of neutrophils.

Our results also demonstrate that neutrophils in HIV infection do not express PD-L1 or PD-L2. Initially, these observations may appear as being inconsistent with a study by Bowes N. L. et al., but this study evaluated the suppression of T cells through PD-L1 on low density weight (LDW) neutrophils, while our study only focuses on normal density neutrophils. LDW neutrophils are immature neutrophils that do not have a fully segmented nucleus, that expand in HIV infection(67). These LDW neutrophils are considered immature and are functionally unique in comparison to mature neutrophils(68). Bowers N. L., et al. showed that PD-L1 is expressed on LDW neutrophils in HIV infection, which can suppress T cell function via PD-1:PDL-1. In contrast our study evaluated the activity of mature neutrophils, which do not express PD-L1 on their surface. This explains why we were unable to see the transcriptional expression and protein expression of PD-L1 on neutrophils.

Interestingly, neutrophil cytokine expression, and co-inhibitory/co-activation receptors expression did change between HIV infected or healthy control groups; however, we found that Gal-9 protein surface expression was reduced on neutrophils from HIV patients. This observation lead us to further investigate the mechanism underlying Gal-9 downregulation from the surface of neutrophils.

As neutrophils become activated in HIV-infected individuals, we speculated that Gal-9 gets cleaved or shed. The amount of plasma soluble Gal-9 in a variety of pathological conditions has been shown to increase(69). Interestingly, many of these pathological states are often associated with neutrophil activation as a contributing factor to immune protection or enhancement of disease symptoms. For example, in acute HIV infection, plasma concentrations of Gal-9 is increased similarly to TNF-a, IFN-a, and IL-10(43). Additionally, this increased in plasma Gal-9 level occurs during acute infection, suggesting that Gal-9 release is a part of innate

immune response. In agreement, our findings show that the activity of neutrophils from HIV patients is correlated with the level of exogenous Gal-9 in the plasma. Lower expression of Gal-9 on the surface of neutrophils had an inverse effect on the concentration of soluble Gal-9 in the plasma. These observations reveal a potential source of Gal-9 in a number of pathological conditions that have the involvement of neutrophils. Our results show that neutrophils from HIV infected patients with low CD4 cell count are capable of shedding substantial quantity of Gal-9, and the surface Gal-9 expression of neutrophils negatively correlates with its plasma levels. While it is difficult to claim that neutrophils are the only major source of soluble Gal-9 in the plasma because of the diverse expression of Gal-9 in the body, based on our data, neutrophils are a potent source of soluble Gal-9 in vitro. When considering neutrophil abundance in the blood, these results indicate that activated neutrophils might be a major source of soluble Gal-9 in the white blood cell compartment.

The soluble Gal-9 has been associated with T cell activation through ERK signaling, and LCK activation(46, 54). In agreement with these observations, we found that soluble Gal-9 is capable of enhancing the activation of T cells at >1000 pg/mL of, but not at lower concentrations. Previous in vivo findings, show that Gal-9 activates T cells in a dose dependent manner and promotes the formation of Th1 and central memory T cells(70). Although we did not investigate the polarization of T cells into specific T cell subsets, future studies should be performed to gain a better understanding of how the soluble Gal-9 may be impacting T cell differentiation in the context of HIV infection.

We also found that Gal-9 is capable of binding to CD44 on the cell surface, which causes the stabilization of dephosphorylated LCK and enhancement of T cell activation. This is in agreement with previous studies that have identified that soluble Gal-9 is capable of contributing

to T cell activation through the activation of LCK and ERK signaling(54). We for the first time have identified that exogenous Gal-9 facilitates this process by binding to CD44, and possibly stabilizing LCK activation.

We observed an increase in CD44 clustering on T cells in the presence of Gal-9. Previous studies have shown that CD44 clustering and crosslinking (by antibodies) can impact T cell signaling through the stabilization of LCK activation and PKC mediated movement(71, 72).

LCK is an integral T cell signaling molecule that promotes intracellular signaling during T cell activation. LCK can be phosphorylated at the tyrosine 394 residue (the activating phosphorylation site) and the tyrosine 505 residue (inactivation phosphorylation site). CD45 dephosphorylates CD44 at both the Y505 and Y395 residue, which can both positively or negatively impact T cell activation(73). Interestingly, CD45 is restricted from the lipid raft in inactive T cells and contributes to ERK activation(74). As a result of CD45 exclusion from lipid rafts, CD44 binds LCK and remains in the lipid rafts of T cells which contributes to the stabilization of dephosphorylated (primed) or activated LCK. This stabilization of LCK in lipid rafts can be enhanced by the crosslinking of CD44. Our results suggest that Gal-9 can contribute to the clustering of CD44 in T cells, which acts as a biological crosslinking factor of CD44 and contributes to the stabilization of dephosphorylated/active LCK. Our findings demonstrate a novel mechanism for Gal-9 in the activation of T cells through increased CD44 clustering and LCK activation.

To confirm that CD44 was involved in the activation of T cells in presence of Gal-9, we inhibited CD44 binding using a neutralizing antibody. Without CD44 we observed reduced CD4+ and CD8+ T cell activation in the presence of exogenous Gal-9. These results are in agreement with other studies that have shown that Gal-9 promotes T cell activation and that T

cell stimulation is reduced by inhibition of CD44(54, 75). However, we show that Gal-9 binds CD44 which reduces inactive LCK in T cells. Previous work has shown that Gal-9 can contribute to ERK signaling and consequentially increases transcription of latent HIV in vitro(45, 46). Taking these results into consideration, it is difficult to assume whether Gal-9 is detrimental to HIV infection, or beneficial. Our study shows that T cells treated with Gal-9 increase the co-expression of CD38 and HLA-DR (activation markers associated with effector T cells). Effector/effector memory T cells are more susceptible to HIV infection and therefore might be detrimentally impacted by Gal-9 presence (76, 77). These observations could contribute to HIV disease progression. On the other side, we have already shown that the interaction of exogenous Gal-9 with TIM-3 on CD4+ T cells reduces HIV-infection/replication(78). In contrast, LCK activity has been associated with differentially modulating CD8+ T cell polarization and could be protective in CD8+ T cell mediated immune responses(79, 80). $CD8^+$ effector memory T cells have more LCK in the active conformation, which suggests increased LCK signaling could produce a more robust and functional $CD8^+$ T cell response(79). Thus, the increased Gal-9 signaling in T cells from HIV patients can either increase or decrease viral replication depending on the interacting ligand target (e.g. PDI or TIM-3)(40, 78). However, enhanced $CD8^+$ T cell activation may be a double-edged sword. Activated CD8+ T cells by eliminating virally infected cells can play a protective role however prolonged immune activation can drive T cell exhaustion and chronic hyperimmune activation in HIV-infected individuals.

In light of our observations, we aimed to further investigate the unexplored mechanisms dictating neutrophil shedding of Gal-9 in response to immune activation. We found that neutrophils regulate the shedding of Gal-9 by cytoskeletal remodeling of surface CD44 expression through a regulated process.

During neutrophil activation, we observed the reorganization of surface CD44 in response to LPS stimulation in HIV infected individuals with low CD4 T cell count. The movement of CD44 in neutrophils is a regulated process that facilitates the binding of CD44 to hyaluronan, an integral step in the recruitment of neutrophils to a variety of tissues (15). Specifically, this process is essential for neutrophil extravasation into the sinusoids of the liver and are implicated to the movement into other tissues(2, 16, 19, 20). Our study identifies that neutrophils regulate this process by several inducible signaling molecules and enzymes. More specifically, neutrophil polarization is linked to the induction of ROS, calcium release, and the engagement of CD44 with the actin cytoskeleton.

Our study shows that neutrophils bind Gal-9 on their surface by interacting with CD44. The binding of Gal-9 to neutrophils has been implicated to reduce neutrophil recruitment to the lungs, and suggests a potential need for neutrophils to shed surface bound Gal-9 for optimal recruitment to the inflamed tissue (81). It should be noted that these studies identify the impact of Gal-9 on neutrophils in an allergic mouse model. In contrast, we showed that Gal-9 is not normally bound to murine neutrophils under homeostatic conditions and therefore, we cannot assume that these interactions will be reproducible in the human system (Supplemental figure 4B-D). Although we have no evidence to suggest that the increased Gal-9 binding on human neutrophils is capable of suppressing the biochemical movement of CD44 out of lipid rafts, we reveal that through an indirect manner, neutrophils regulate and promote the shedding of Gal-9 through the cytoskeletal reorientation of CD44 in human neutrophils upon activation.

When activated, neutrophils depalmitoylate CD44 and promote its interaction with ezrin to move CD44 to one end of the neutrophil(21). Depalmitoylation facilitates the movement of CD44 out of the lipid raft, where CD44 can interact with ezrin and the actin cytoskeleton(17).

We found that the depalmitoylation of CD44 during neutrophil activation facilitates the movement of CD44, and subsequently Gal-9 shedding. The process of shedding Gal-9 is regulated specifically by the depalmitoylation of CD44 and not interaction with the cytoskeleton (binding ezrin/RAP-1). Our observations suggest that depalmitoylation is a critical step in polarization of CD44 and Gal-9 shedding from the surface of neutrophils. We next evaluated the regulatory mechanisms that control CD44 depalmitoylation and Gal-9 shedding.

Neutrophil activation is primarily fueled by glycolysis metabolism and our study identified that Gal-9 shedding is dependent on the activity of glycolysis(82). These results indicate that glycolysis fuels a regulatory mechanism that is essential for Gal-9 shedding. Additionally, our results suggest that GLUT-1 is a prominent glucose transporter in neutrophils and contributes significantly to the function of neutrophils. These results are in agreement with other studies that have shown neutrophils primarily rely on glycolysis metabolism and GLUT-1 for glucose transport(82). To further evaluate the impact that glycolysis may have on neutrophil activity, we measured ROS production by neutrophils stimulated with LPS and phloretin and found that the inhibition of GLUT-1 impaired the production of ROS by neutrophils. Neutrophils use glycolysis to fuel the pentose-pyruvate phosphatase pathway (PPP) to generate NADPH. NADPH will then be used to fuel NOX2, a ROS producing enzyme that is essential in immune protection facilitated by neutrophils(7). This process appears to be essential for Gal-9 shedding as our observations indicated that ROS, and glycolysis are important mediators in the depalmitoylation of CD44. Interestingly, we found that if we inhibited ROS production or activity (by a ROS scavenger) Gal-9 release was inhibited; therefore, suggesting an association between Gal-9 shedding, neutrophil metabolism, and ROS production. From these observations we identified that neutrophil shedding of Gal-9, is regulated by CaMKII.

CaMKII is a calcium/ROS activated kinase that phosphorylates CD44 at the Ser325 residue, which causes a conformational change in CD44 allowing of depalmitoylation (2, 17, 57). As a result of CaMKII regulating CD44 depalmitoylation, we showed that CaMKII is responsible for the regulation of Gal-9 shedding. This agrees with a previous study by Lewis C. A., et al. showing that ser325 phosphorylation of CD44 is required for binding to hyaluronan(14), which indicates an integral role for CaMKII activation in CD44 mediated extravasation. We show that this process is also involved in inducing the depalmitoylation of CD44 and facilitating the shedding of Gal-9. CaMKII is an essential regulatory element of Gal-9 shedding because the depalmitoylation enzymes LYPLA1, LYPLA2, PPT1, ABHD12, ABHD17A, and ABHD17B are expressed by neutrophils and lack regulation (Supplemental figure 5A)(83). This biochemical observation suggests that the conformational changes in the induction of CD44 by CaMKII is the primary mechanism to promote CD44 depalmitoylation.

Interestingly, CaMKII can be activated by ROS, and therefore, helps to explain our observations that glycolysis, and ROS production promote neutrophil shedding of Gal-9 in response to activation. Our model suggests that during HIV infection, neutrophils are activated and produce ROS through NOX2 activity. The release of ROS from neutrophils in HIV infection becomes H_2O_2, which can pass back through the cell membrane of the neutrophils to activate CaMKII. This mechanism is reflected with increased ROS circulating in HIV patients (59, 84). These previous studies show that HIV infection causes increased levels of circulating ROS, which may contribute to neutrophil shedding of Gal-9 in HIV infection. Our results show an interesting regulatory mechanism for ROS production and glycolysis that are activated in HIV infection which can induce Gal-9 shedding from neutrophils. Although these observations provide important information regarding the processes that contribute to Gal-9 shedding by

neutrophils in HIV infection, do not explain why neutrophils from HIV patients with low CD4 T cells count have decreased expression of Gal-9 despite their similar activation transcriptional profile as previously discussed. These results suggest the possibility of other regulatory mechanisms in Gal-9 shedding by neutrophils in HIV infection.

In HIV infection, patients with low CD4 T cell count have lower surface Gal-9 expression in comparison with HIV patients with higher CD4 T cell count. Interestingly, based on our transcriptional analysis, this decrease in Gal-9 expression cannot be fully associated with the activation status of neutrophils as majority of pathways are similarly activated in both. These discrepancies lead us to investigate the potential role of ROS/glycolysis modifying proteins that are present in HIV infection.

Myeloid cell activation and metabolism has previously been shown to be modulated by IL-10 signaling(58). During macrophage activation, IL-10 signals through the IL-10 receptor causing the increase of DDIT4 expression and inhibition of secretory vesicle translocation, consequentially reducing the surface expression of GLUT-1(58). Interestingly, our RNA sequencing findings show an increase in the expression of DDIT4 in HIV patients with high CD4 T cell count, but not low CD4 T cells count (Supplemental figure 4A). Furthermore, genes associated with glycolysis were expressed more in HIV infected patients with low CD4 count in comparison to HIV patients with high CD4 count, or healthy control neutrophils (Supplemental Figure 5B). Additionally, we observed lesser surface expression of GLUT-1 on the surface of neutrophils from patients with high CD4 T cell count. These results suggest that neutrophils are similarly impacted by IL-10 in HIV infection. In the presence of plasma from HIV patients with high CD4 T cell count and recombinant human IL-10 reduction of GLUT1 on the surface of

neutrophils was observed. Thus, ROS and glycolysis in HIV infected patients contribute to Gal-9 shedding through the activation of CaMKII.

Together our findings identify a novel mechanism of Gal-9 shedding that can contribute to T cell activation. The appreciation of interactions between T cells and neutrophils has been only lightly investigated; however, our results suggest a sophisticated interaction between neutrophils and T cells through Gal-9 binding. By harnessing the shedding and activity of Gal-9 from neutrophils could be a novel therapeutic target to prevent further HIV replication or enhance CD8+ T cell responses.

Together these results contribute substantially to the role of neutrophils in T cell response and their activity in HIV infection. With the knowledge that neutrophils can impact T cell function through the regulated shedding of Gal-9 further investigation into how neutrophils may impact T cells in other chronic diseases, acute infections, and how modulation of neutrophil function could be used for therapeutics.

Cited Works

1. Lokuta MA, Nuzzi PA, Huttenlocher A. Analysis of neutrophil polarization and chemotaxis. Methods Mol Biol. 2007;412:211-29.
2. Khan AI, Kerfoot SM, Heit B, Liu L, Andonegui G, Ruffell B, et al. Role of CD44 and hyaluronan in neutrophil recruitment. J Immunol. 2004;173(12):7594-601.
3. McEver RP. Selectins: initiators of leucocyte adhesion and signalling at the vascular wall. Cardiovasc Res. 2015;107(3):331-9.
4. Morikis VA, Simon SI. Neutrophil Mechanosignaling Promotes Integrin Engagement With Endothelial Cells and Motility Within Inflamed Vessels. Front Immunol. 2018;9:2774.
5. Hattori H, Subramanian KK, Sakai J, Luo HR. Reactive oxygen species as signaling molecules in neutrophil chemotaxis. Commun Integr Biol. 2010;3(3):278-81.
6. Chacko BK, Kramer PA, Ravi S, Johnson MS, Hardy RW, Ballinger SW, et al. Methods for defining distinct bioenergetic profiles in platelets, lymphocytes, monocytes, and neutrophils, and the oxidative burst from human blood. Lab Invest. 2013;93(6):690-700.
7. Park DW, Zmijewski JW. Mitochondrial Dysfunction and Immune Cell Metabolism in Sepsis. Infect Chemother. 2017;49(1):10-21.
8. Erickson JR, Joiner ML, Guan X, Kutschke W, Yang J, Oddis CV, et al. A dynamic pathway for calcium-independent activation of CaMKII by methionine oxidation. Cell. 2008;133(3):462-74.
9. Maione AS, Cipolletta E, Sorriento D, Borriello F, Soprano M, Rusciano MR, et al. Cellular subtype expression and activation of CaMKII regulate the fate of atherosclerotic plaque. Atherosclerosis. 2017;256:53-61.
10. Gaines P, Lamoureux J, Marisetty A, Chi J, Berliner N. A cascade of Ca(2+)/calmodulin-dependent protein kinases regulates the differentiation and functional activation of murine neutrophils. Exp Hematol. 2008;36(7):832-44.
11. Backs J, Song K, Bezprozvannaya S, Chang S, Olson EN. CaM kinase II selectively signals to histone deacetylase 4 during cardiomyocyte hypertrophy. J Clin Invest. 2006;116(7):1853-64.
12. Kashiwase K, Higuchi Y, Hirotani S, Yamaguchi O, Hikoso S, Takeda T, et al. CaMKII activates ASK1 and NF-kappaB to induce cardiomyocyte hypertrophy. Biochem Biophys Res Commun. 2005;327(1):136-42.
13. Antoine M, Gaiddon C, Loeffler JP. Ca2+/calmodulin kinase types II and IV regulate c-fos transcription in the AtT20 corticotroph cell line. Mol Cell Endocrinol. 1996;120(1):1-8.
14. Lewis CA, Townsend PA, Isacke CM. Ca(2+)/calmodulin-dependent protein kinase mediates the phosphorylation of CD44 required for cell migration on hyaluronan. Biochem J. 2001;357(Pt 3):843-50.
15. Lesley J, Hascall VC, Tammi M, Hyman R. Hyaluronan binding by cell surface CD44. J Biol Chem. 2000;275(35):26967-75.
16. Katayama Y, Hidalgo A, Chang J, Peired A, Frenette PS. CD44 is a physiological E-selectin ligand on neutrophils. J Exp Med. 2005;201(8):1183-9.
17. Donatello S, Babina IS, Hazelwood LD, Hill AD, Nabi IR, Hopkins AM. Lipid raft association restricts CD44-ezrin interaction and promotion of breast cancer cell migration. Am J Pathol. 2012;181(6):2172-87.
18. Pierini LM, Eddy RJ, Fuortes M, Seveau S, Casulo C, Maxfield FR. Membrane lipid organization is critical for human neutrophil polarization. J Biol Chem. 2003;278(12):10831-41.

19. McDonald B, Kubes P. Interactions between CD44 and Hyaluronan in Leukocyte Trafficking. Front Immunol. 2015;6:68.
20. McDonald B, McAvoy EF, Lam F, Gill V, de la Motte C, Savani RC, et al. Interaction of CD44 and hyaluronan is the dominant mechanism for neutrophil sequestration in inflamed liver sinusoids. J Exp Med. 2008;205(4):915-27.
21. Thorne RF, Legg JW, Isacke CM. The role of the CD44 transmembrane and cytoplasmic domains in co-ordinating adhesive and signalling events. J Cell Sci. 2004;117(Pt 3):373-80.
22. Soehnlein O, Steffens S, Hidalgo A, Weber C. Neutrophils as protagonists and targets in chronic inflammation. Nat Rev Immunol. 2017;17(4):248-61.
23. Ross AC, Rizk N, O'Riordan MA, Dogra V, El-Bejjani D, Storer N, et al. Relationship between inflammatory markers, endothelial activation markers, and carotid intima-media thickness in HIV-infected patients receiving antiretroviral therapy. Clin Infect Dis. 2009;49(7):1119-27.
24. Baker JV, Duprez D. Biomarkers and HIV-associated cardiovascular disease. Curr Opin HIV AIDS. 2010;5(6):511-6.
25. Leng SX, Margolick JB. Understanding frailty, aging, and inflammation in HIV infection. Curr HIV/AIDS Rep. 2015;12(1):25-32.
26. Dobmeyer TS, Raffel B, Dobmeyer JM, Findhammer S, Klein SA, Kabelitz D, et al. Decreased function of monocytes and granulocytes during HIV-1 infection correlates with CD4 cell counts. Eur J Med Res. 1995;1(1):9-15.
27. Elbim C, Prevot MH, Bouscarat F, Franzini E, Chollet-Martin S, Hakim J, et al. Polymorphonuclear neutrophils from human immunodeficiency virus-infected patients show enhanced activation, diminished fMLP-induced L-selectin shedding, and an impaired oxidative burst after cytokine priming. Blood. 1994;84(8):2759-66.
28. Tate MD, Brooks AG, Reading PC, Mintern JD. Neutrophils sustain effective CD8(+) T-cell responses in the respiratory tract following influenza infection. Immunol Cell Biol. 2012;90(2):197-205.
29. Sivanandham R, Brocca-Cofano E, Krampe N, Falwell E, Venkatraman SMK, Ribeiro RM, et al. Neutrophil extracellular trap production contributes to pathogenesis in SIV-infected nonhuman primates. J Clin Invest. 2018;128(11):5178-83.
30. Rice CM, Davies LC, Subleski JJ, Maio N, Gonzalez-Cotto M, Andrews C, et al. Tumour-elicited neutrophils engage mitochondrial metabolism to circumvent nutrient limitations and maintain immune suppression. Nat Commun. 2018;9(1):5099.
31. Okoye IS, Houghton M, Tyrrell L, Barakat K, Elahi S. Coinhibitory Receptor Expression and Immune Checkpoint Blockade: Maintaining a Balance in CD8(+) T Cell Responses to Chronic Viral Infections and Cancer. Frontiers in immunology. 2017;8:1215.
32. Wherry EJ, Kurachi M. Molecular and cellular insights into T cell exhaustion. Nature reviews Immunology. 2015;15(8):486-99.
33. Wherry EJ. T cell exhaustion. Nature immunology. 2011;12(6):492-9.
34. Schietinger A, Greenberg PD. Tolerance and exhaustion: defining mechanisms of T cell dysfunction. Trends Immunol. 2014;35(2):51-60.
35. Doering TA, Crawford A, Angelosanto JM, Paley MA, Ziegler CG, Wherry EJ. Network analysis reveals centrally connected genes and pathways involved in CD8+ T cell exhaustion versus memory. Immunity. 2012;37(6):1130-44.

36. Bowers NL, Helton ES, Huijbregts RP, Goepfert PA, Heath SL, Hel Z. Immune suppression by neutrophils in HIV-1 infection: role of PD-L1/PD-1 pathway. PLoS Pathog. 2014;10(3):e1003993.
37. Motamedi M, Shahbaz S, Fu L, Dunsmore G, Xu L, Harrington R, et al. 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. 2019;3(11):531-46.
38. Shahbaz S, Dunsmore G, Koleva P, Xu L, Houston S, Elahi S. Galectin-9 and VISTA Expression Define Terminally Exhausted T Cells in HIV-1 Infection. J Immunol. 2020;204(9):2474-91.
39. Zhu C, Anderson AC, Schubart A, Xiong H, Imitola J, Khoury SJ, et al. The Tim-3 ligand galectin-9 negatively regulates T helper type 1 immunity. Nature immunology. 2005;6(12):1245-52.
40. Bi S, Hong PW, Lee B, Baum LG. 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. 2011;108(26):10650-5.
41. Madireddi S, Eun SY, Lee SW, Nemcovicova I, Mehta AK, Zajonc DM, et al. Galectin-9 controls the therapeutic activity of 4-1BB-targeting antibodies. J Exp Med. 2014;211(7):1433-48.
42. Merani S, Chen W, Elahi S. The bitter side of sweet: the role of Galectin-9 in immunopathogenesis of viral infections. Rev Med Virol. 2015;25(3):175-86.
43. Tandon R, Chew GM, Byron MM, Borrow P, Niki T, Hirashima M, et al. 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. 2014;30(7):654-64.
44. Jost S, Moreno-Nieves UY, Garcia-Beltran WF, Rands K, Reardon J, Toth I, et al. Dysregulated Tim-3 expression on natural killer cells is associated with increased Galectin-9 levels in HIV-1 infection. Retrovirology. 2013;10:74.
45. Abdel-Mohsen M, Chavez L, Tandon R, Chew GM, Deng X, Danesh A, et al. Human Galectin-9 Is a Potent Mediator of HIV Transcription and Reactivation. PLoS Pathog. 2016;12(6):e1005677.
46. Colomb F, Giron LB, Premeaux TA, Mitchell BI, Niki T, Papasavvas E, et al. Galectin-9 Mediates HIV Transcription by Inducing TCR-Dependent ERK Signaling. Frontiers in immunology. 2019;10:267.
47. Harwood NM, Golden-Mason L, Cheng L, Rosen HR, Mengshol JA. HCV-infected cells and differentiation increase monocyte immunoregulatory galectin-9 production. J Leukoc Biol. 2016;99(3):495-503.
48. Oomizu S, Arikawa T, Niki T, Kadowaki T, Ueno M, Nishi N, et al. Cell surface galectin-9 expressing Th cells regulate Th17 and Foxp3+ Treg development by galectin-9 secretion. PloS one. 2012;7(11):e48574.
49. Shan M, Carrillo J, Yeste A, Gutzeit C, Segura-Garzon D, Walland AC, et al. Secreted IgD Amplifies Humoral T Helper 2 Cell Responses by Binding Basophils via Galectin-9 and CD44. Immunity. 2018;49(4):709-24 e8.

50. Wiersma VR, Clarke A, Pouwels SD, Perry E, Abdullah TM, Kelly C, et al. Galectin-9 Is a Possible Promoter of Immunopathology in Rheumatoid Arthritis by Activation of Peptidyl Arginine Deiminase 4 (PAD-4) in Granulocytes. Int J Mol Sci. 2019;20(16).
51. Lefebvre DC, Lai JC, Maeshima N, Ford JL, Wong AS, Cross JL, et al. CD44 interacts directly with Lck in a zinc-dependent manner. Mol Immunol. 2010;47(10):1882-9.
52. Elahi S, Dinges WL, Lejarcegui N, Laing KJ, Collier AC, Koelle DM, et al. Protective HIV-specific CD8+ T cells evade Treg cell suppression. Nature medicine. 2011;17(8):989-95.
53. Elahi S, Horton H. Association of HLA-alleles with the immune regulation of chronic viral infections. The international journal of biochemistry & cell biology. 2012;44(8):1361-5.
54. Lhuillier C, Barjon C, Niki T, Gelin A, Praz F, Morales O, et al. 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. 2015;290(27):16797-811.
55. Ross SH, Post A, Raaijmakers JH, Verlaan I, Gloerich M, Bos JL. Ezrin is required for efficient Rap1-induced cell spreading. J Cell Sci. 2011;124(Pt 11):1808-18.
56. Namdar A, Dunsmore G, Shahbaz S, Koleva P, Xu L, Jovel J, et al. CD71(+) Erythroid Cells Exacerbate HIV-1 Susceptibility, Mediate trans-Infection, and Harbor Infective Viral Particles. MBio. 2019;10(6).
57. Zhu LJ, Klutho PJ, Scott JA, Xie L, Luczak ED, Dibbern ME, et al. Oxidative activation of the Ca(2+)/calmodulin-dependent protein kinase II (CaMKII) regulates vascular smooth muscle migration and apoptosis. Vascul Pharmacol. 2014;60(2):75-83.
58. Ip WKE, Hoshi N, Shouval DS, Snapper S, Medzhitov R. Anti-inflammatory effect of IL-10 mediated by metabolic reprogramming of macrophages. Science. 2017;356(6337):513-9.
59. Campillo-Gimenez L, Casulli S, Dudoit Y, Seang S, Carcelain G, Lambert-Niclot S, et al. Neutrophils in antiretroviral therapy-controlled HIV demonstrate hyperactivation associated with a specific IL-17/IL-22 environment. J Allergy Clin Immunol. 2014;134(5):1142-52 e5.
60. Brenchley JM, Price DA, Schacker TW, Asher TE, Silvestri G, Rao S, et al. Microbial translocation is a cause of systemic immune activation in chronic HIV infection. Nat Med. 2006;12(12):1365-71.
61. Hunt PW, Martin JN, Sinclair E, Bredt B, Hagos E, Lampiris H, et al. T cell activation is associated with lower CD4+ T cell gains in human immunodeficiency virus-infected patients with sustained viral suppression during antiretroviral therapy. J Infect Dis. 2003;187(10):1534-43.
62. Yaseen MM, Abuharfeil NM, Yaseen MM, Shabsoug BM. The role of polymorphonuclear neutrophils during HIV-1 infection. Arch Virol. 2018;163(1):1-21.
63. Vono M, Lin A, Norrby-Teglund A, Koup RA, Liang F, Lore K. Neutrophils acquire the capacity for antigen presentation to memory CD4(+) T cells in vitro and ex vivo. Blood. 2017;129(14):1991-2001.
64. Reinisch W, Lichtenberger C, Steger G, Tillinger W, Scheiner O, Gangl A, et al. Donor dependent, interferon-gamma induced HLA-DR expression on human neutrophils in vivo. Clin Exp Immunol. 2003;133(3):476-84.
65. Smith WB, Guida L, Sun Q, Korpelainen EI, van den Heuvel C, Gillis D, et al. Neutrophils activated by granulocyte-macrophage colony-stimulating factor express receptors for interleukin-3 which mediate class II expression. Blood. 1995;86(10):3938-44.

66. Gosselin EJ, Wardwell K, Rigby WF, Guyre PM. Induction of MHC class II on human polymorphonuclear neutrophils by granulocyte/macrophage colony-stimulating factor, IFN-gamma, and IL-3. J Immunol. 1993;151(3):1482-90.
67. Hsu BE, Tabaries S, Johnson RM, Andrzejewski S, Senecal J, Lehuede C, et al. Immature Low-Density Neutrophils Exhibit Metabolic Flexibility that Facilitates Breast Cancer Liver Metastasis. Cell Rep. 2019;27(13):3902-15 e6.
68. Garley M, Jablonska E. Heterogeneity Among Neutrophils. Arch Immunol Ther Exp (Warsz). 2018;66(1):21-30.
69. Niki T, Fujita K, Rosen H, Hirashima M, Masaki T, Hattori T, et al. Plasma Galectin-9 Concentrations in Normal and Diseased Condition. Cell Physiol Biochem. 2018;50(5):1856-68.
70. Gooden MJ, Wiersma VR, Samplonius DF, Gerssen J, van Ginkel RJ, Nijman HW, et al. Galectin-9 activates and expands human T-helper 1 cells. PLoS One. 2013;8(5):e65616.
71. Fanning A, Volkov Y, Freeley M, Kelleher D, Long A. CD44 cross-linking induces protein kinase C-regulated migration of human T lymphocytes. Int Immunol. 2005;17(4):449-58.
72. Wong NK, Lai JC, Birkenhead D, Shaw AS, Johnson P. CD45 down-regulates Lck-mediated CD44 signaling and modulates actin rearrangement in T cells. J Immunol. 2008;181(10):7033-43.
73. Furlan G, Minowa T, Hanagata N, Kataoka-Hamai C, Kaizuka Y. Phosphatase CD45 both positively and negatively regulates T cell receptor phosphorylation in reconstituted membrane protein clusters. J Biol Chem. 2014;289(41):28514-25.
74. Zhang M, Moran M, Round J, Low TA, Patel VP, Tomassian T, et al. CD45 signals outside of lipid rafts to promote ERK activation, synaptic raft clustering, and IL-2 production. J Immunol. 2005;174(3):1479-90.
75. Baaten BJ, Li CR, Deiro MF, Lin MM, Linton PJ, Bradley LM. CD44 regulates survival and memory development in Th1 cells. Immunity. 2010;32(1):104-15.
76. Groot F, van Capel TM, Schuitemaker J, Berkhout B, de Jong EC. Differential susceptibility of naive, central memory and effector memory T cells to dendritic cell-mediated HIV-1 transmission. Retrovirology. 2006;3:52.
77. Brenchley JM, Hill BJ, Ambrozak DR, Price DA, Guenaga FJ, Casazza JP, et al. T-cell subsets that harbor human immunodeficiency virus (HIV) in vivo: implications for HIV pathogenesis. J Virol. 2004;78(3):1160-8.
78. Elahi S, Niki T, Hirashima M, Horton H. Galectin-9 binding to Tim-3 renders activated human CD4+ T cells less susceptible to HIV-1 infection. Blood. 2012;119(18):4192-204.
79. Moogk D, Zhong S, Yu Z, Liadi I, Rittase W, Fang V, et al. Constitutive Lck Activity Drives Sensitivity Differences between CD8+ Memory T Cell Subsets. J Immunol. 2016;197(2):644-54.
80. Tewari K, Walent J, Svaren J, Zamoyska R, Suresh M. Differential requirement for Lck during primary and memory CD8+ T cell responses. Proc Natl Acad Sci U S A. 2006;103(44):16388-93.
81. Horio Y, Ichiyasu H, Kojima K, Saita N, Migiyama Y, Iriki T, et al. Protective effect of Galectin-9 in murine model of lung emphysema: Involvement of neutrophil migration and MMP-9 production. PLoS One. 2017;12(7):e0180742.
82. Maratou E, Dimitriadis G, Kollias A, Boutati E, Lambadiari V, Mitrou P, et al. Glucose transporter expression on the plasma membrane of resting and activated white blood cells. Eur J Clin Invest. 2007;37(4):282-90.

83. Won SJ, Cheung See Kit M, Martin BR. Protein depalmitoylases. Crit Rev Biochem Mol Biol. 2018;53(1):83-98.
84. Hunter MV, Willoughby PM, Bruce AEE, Fernandez-Gonzalez R. Oxidative Stress Orchestrates Cell Polarity to Promote Embryonic Wound Healing. Dev Cell. 2018;47(3):377-87 e4.

Figure Legends

Figure 1

Neutrophils from HIV infected individuals have a transcriptionally activated phenotype. (**A**) Correlation heatmap representing the similarities between differentially expressed genes in neutrophils from healthy controls, HIV patients with high CD4 T cell count and low CD4 T cell count. (**B**) PCA plot representing the differences between HIV infected patients with high and low CD4 T cell count or healthy controls. (**C**) A clustered heatmap showing the differences between individual genes between HIV patients with low and high CD4 T cell count and healthy controls (Differentially expressed genes have an FDR <0.05, and a log FC > 2 or < -2). (**D**) Graphs representing gene ontologies significantly associated with the heatmap clusters (FDR < 0.05). (**E**) Significantly increased genes expressed in neutrophils from HIV patients contributing to gene ontologies.

Figure 2

Neutrophils from HIV infected patients with low CD4 T cell count contribute to T cell activation. (**A**) Representative plots of T cell activation in the presence or absence of neutrophils from healthy controls, HIV+ patients with high CD4 T cell count and low CD4 T cell count. (**B**) Cumulative data showing the percent expression of CD69 on $CD4^+$ T cells (**C**) and $CD8^+$ T cells in presence or absence of neutrophils. (**D**) Cumulative data showing the percent expression of CD38 on $CD4^+$ T cells, and (**E**) and $CD8^+$ T cells in presence or absence of neutrophils.

Figure 3

Neutrophils from HIV infected patients have differential expression of Gal-9, but not other T cell modulating molecules. (**A**) Heatmaps showing the expression of T cell modulating cytokine transcripts in healthy, HIV+ high or low CD4 T cell count patients. (**B**) Heatmaps indicating the magnitude co-inhibitory and co-activating receptors expression shown in

neutrophils. Neutrophil surface expression of (**C**) VISTA, (**D**) HLA-DR, (**E**) CD40, (**F**) TIM-3, and (**G**) Gal-9 and their corresponding cumulative data.

Figure 4

Neutrophils shed Gal-9 in HIV infection. (**A**) Representative plots showing surface expression of Gal-9 and CD32 on neutrophils. (**B**) Cumulative data comparing surface expression of Gal-9 in neutrophils from healthy controls (HC) versus HIV-patients with high or low CD4 T cell count. (**C**) MFI of surface Gal-9 on neutrophils from HIV patients with high CD4 or low CD4 T cell count and healthy controls. (**D**) ELISA results demonstrating the concentration of soluble Gal-9 from unstimulated neutrophils obtained from healthy controls or HIV-patients cultured for 8 hours. (**E**) Correlation between CD4 T cell count and Gal-9+ neutrophils. (**F**) Correlation between Gal-9+ neutrophils and plasma Gal-9 in HIV-patients. (**G**) Negative correlation between plasma Gal-9 and CD4 T cell count in HIV-patients. (**H**) % CD32 expression on neutrophils from HC, HIV patients with high and low CD4 T cell count. (**I**) Negative correlation between CD32 expression and CD4 T cell count in HIV-patients.

Figure 5

High concentrations of Gal-9 contribute to T cell activation. (**A**) Representative flow cytometry plots of $CD4^+$ T cells cultured in the absence or presence of exogenous Gal-9 in vitro. (**B**) Representative plot of $CD8^+$ T cells cultured in the absence or presence of exogenous Gal-9 in vitro. (**C**) Cumulative data showing CD38 expression on $CD4^+$ T cells, and (**D**) $CD8^+$ T cells. (**E**) Cumulative data showing HLA-DR expression on $CD4^+$, and (**F**) $CD8^+$ T cells. (**G**) Cumulative data showing co-expression of HLA-DR and CD38 on $CD4^+$, and (**H**) CD8+ T cells.

Figure 6

Gal-9 binds CD44 on T cells and enhances T cell activation. (**A**) Images showing the expression of CD44 and Gal-9 on the surface of T cells. (**B**) Cumulative data showing percent colocalization of Gal-9 with CD44 and CD137. (**C**) Percent of Gal-9 positive T cells in the presence or absence of recombinant Gal-9 following in vitro incubation. (**D**) Percent CD44 clustering in the presence and absence of Gal-9. (**E**) Representative plots showing HLA-DR and CD38 co-expression on $CD4^+$ and (**F**) $CD8^+$ T cells. (**G**) Cumulative data showing CD38 expression on $CD4^+$ T cell or (**H**) $CD8^+$ T cells expression following stimulation with anti-CD3/CD28 antibodies in the presence of exogenous Gal-9 and/or Gal-9 and anti-CD44 antibody. (**I**) Cumulative data showing HLA-DR expression on $CD4^+$ T cell or (**J**) $CD8^+$ T cells expression following stimulation with anti-CD3/CD28 antibodies in the presence of exogenous Gal-9 and/or Gal-9 and anti-CD44 antibody. (**K**) Cumulative data showing CD38/HLA-DR co-expression on $CD4^+$ T cell or (**L**) $CD8^+$ T cells expression following stimulation with anti-CD3/CD28 antibodies in the presence of exogenous Gal-9 and/or Gal-9 and anti-CD44 antibody. (**M**) Representative plots showing phospho-LCK (Y505) in CD4+ T cells in the absence and presence of Gal-9. (**N**) Cumulative data showing phosphor-LCK in $CD4^+$ and (**O**) $CD8^+$ T cells following treatment with Gal-9 in vitro.

Figure 7

Gal-9 is shed by neutrophil mediated depalmitoylation of CD44 upon activation. (**A**) Percent colocalization of CD44 and Gal-9 on neutrophils quantified using an amnis imagestream. (**B**) Cumulative percent colocalization of Gal-9 and CD44 on the surface of neutrophils. (**C**) Representative plot of neutrophils with a high and low delta centroid XY. (**D**) Representative images of capped and dispersed CD44 on neutrophils. (**E**) Representative plot of LPS treated neutrophil radial delta centroid. (**F**) Cumulative data of CD44 capping (delta centroid >1) in

unstimulated and LPS treated neutrophils. (**G**) Representative plot showing changing Gal-9 and CD32 expression on neutrophils untreated or stimulated with LPS. (**H**) Cumulative results showing the percent expression of surface Gal-9 on unstimulated and LPS stimulated neutrophils (**I**) Cumulative results showing the MFI of surface Gal-9 on unstimulated and LPS stimulated neutrophils. (**J**) Cumulative data showing correlation between % Gal-9 expression on neutrophils and CD44 capping. (**K**) Surface expression of Gal-9 on neutrophils untreated or treated with methyl-b-cyclodextran. (**L**) Surface expression of Gal-9 on neutrophils untreated or treated with LPS in the presence or absence of a RAP-1 inhibitor, (**M**) ezrin inhibitor, and a (**N**) depalmitoylation inhibitor

Figure 8

Gal-9 shedding by activated neutrophils is regulated by ROS and CaMKII. (**A**) Visual model showing the mechanistic action of Phloretin on the GLUT-1 transporter. (**B**) Representative plots showing the effect of phloretin on Gal-9 shedding. (**C**) Cumulative data showing surface Gal-9 expression in untreated or LPS treated neutrophil in the presence or absence of phloretin. (**D**) Cumulative data showing percent CD44 capping in the presence or absence of phloretin in untreated or LPS treated neutrophils. (**E**) Representative plots of ROS expression in neutrophils in the presence or absence of LPS. (**F**) Cumulative data representing the MFI of ROS in untreated and LPS treated neutrophils. (**G**) Representative plots showing the expression of ROS in the presence and absence of LPS and treated or untreated with phloretin. (**H**) Cumulative data showing the change in ROS expression in the presence or absence of LPS and phloretin. (**I**) Cumulative data showing percent Gal-9 expression in the absence or presence of LPS and apocynin. (**J**)Cumulative data showing percent Gal-9 expression and (**K**) CD44 capping in the absence or presence of LPS and a CaMKII inhibitor

Figure 9

Neutrophils from HIV patients with high CD4 T cell count have increased IL-10 activity, and decreased glycolysis. (**A**) Representative plots and (**B**) cumulative data representing GLUT-1 and Gal-9 expression on neutrophils of healthy controls versus HIV patients with low or high CD4 T cell count. (**C**) Cumulative data showing the MFI of GLUT-1 on neutrophils from healthy controls versus HIV patients with low or high CD4 T cell count. (**D**) Correlation of GLUT-1 expression and CD4 T cell count on neutrophils of HIV-infected patients. (**E**) Representative plot of GLUT-1 expression in neutrophils treated or untreated with plasma from HIV patients with low and high CD4 T cell count. (**F**) Cumulative data showing GLUT-1 expression in the presence of plasma from HIV patients with low or high CD4 T cell count and Healthy controls. (**G**) ELISA results showing IL-10 concentrations in the plasma of HIV-patients with low and high CD4 T cell count. (**H**) GLUT-1 expression in the presence or absence of IL-10 treatment and/or LPS. (**I**) Representative images of GLUT-1 and CD11b. (**J**) Cumulative data showing colocalization of CD11b and GLUT-1 in the presence or absence of IL-10 and/or LPS. (**K**) Representative plots of ROS expression in neutrophils in the presence or absence of IL-10 and LPS. (**L**) Cumulative data of ROS expression on neutrophils in the presence of IL-10 and LPS. (**K**) Cumulative data of Gal-9 expression on neutrophils in the presence or absence of IL-10 and/or LPS.

Supplemental Figure Legends

Supplemental Figure 1. Neutrophil transcriptional differences

a) Volcano plot showing the difference between DEGs upregulated in healthy control patient neutrophils or HIV patients with low CD4 count. 1b) Volcano plot showing the difference

between DEGs upregulated in healthy control patient neutrophils or HIV patients with high CD4 count. 1c) Volcano plot showing the difference between DEGs upregulated in HIV patients with low CD4 count or patient neutrophils or HIV patients with high CD4 count. 1d) Histograms representing gene ontologies associated cluster 2 and 4 in figure 1. 1e) Heatmap representing gene expression associated with the gene ontology "Neutrophil Activation".

Supplemental Figure 2 Experimental scheme for data represented in Figure 2.

Supplemental Figure 3 Gating strategy for human neutrophils.

Supplemental Figure 4: Image stream gating strategy for neutrophil colocalization of gal-9 and CD44.

Supplemental Figure 5: Murine neutrophil expression of gal-9

3a) Gating strategy for murine neutrophil identification and expression of gal-9. 3b) cumulative results of murine neutrophil expression of gal-9. 3c) Image of murine neutrophil expression of gal-9 and CD44

Supplemental Figure 6: Relative expression of DDIT4 in neutrophils from healthy controls and HIV patients with low CD4 count and high CD4 count.

Supplemental Figure 7: Neutrophil gene expression associated with the gene ontologies 7A) Neutrophil Extravasation, 7B) Protein depalmitoylation, and 7C), glycolytic process.

Supplemental Figure 8: Visual representation of the mechanism that neutrophils use to shed gal-9.

Appendix

Supplemental Text 1

```
# Package Load
library(pheatmap)
library(edgeR)
library(cluster)
library(statmod)

# Set Directory
setwd("~/Documents/RNA Sequencing/Neutrophil Phenotyping/")
# Read Tab Deliminanted file of RAW COUNTS - THIS DOES NOT MEAN
TPM/CPM/RPKM/htsqct/fastqc
Rawdata <- read.delim("HC CD4 Low and CD4 High.txt", row.names = "target_id")

# Group Samples based on Subject
length(Rawdata)

# DGEList Conversion
y <- DGEList(counts=Rawdata[,])

# Filtering and Normalizing
y$samples
```

Appendix

```
# Normalize the data by "Relative Log Expression" (RLE = Log2)
y <- calcNormFactors(y,method=c("RLE"))

#How many surviving samples
head(y$samples)

# Data Exploration

# Multidimensional Scaling
keep <- rowSums(cpm(y)>2) >= 5
y <- y[keep, , keep.lib.sizes=FALSE]

plotMDS(y, top = 5000, col=c(rep("black",5), rep("red",5), rep("blue",5)), gene.selection =
"common", pch = 16, cex = 2)
#Conditioning - Expreimental treatments to samples are taken into account for differential
equation analysis
condition <- factor(c("HC", "HC", "HC", "HC", "HC",
                      "CD4 High", "CD4 High", "CD4 High", "CD4 High", "CD4 High",
                      "CD4 Low", "CD4 Low", "CD4 Low", "CD4 Low", "CD4 Low"))

# Estimate Dispersion

# Organize the data by subject (individual/patient/mouse/etc.)
```

```
subject <- factor(c(1,2,3,4,5,6,7,8,9,10,11,12,13,14,15))
# Ensure the appropriate subject and conditions match the sample
data.frame(group=colnames(y),subject,condition)
# Make the expreimental design that will be utilized by the dispersion analysis
design <- model.matrix(~condition)
# Subject data to GLM Dispersion - Utilizes the
# Cox-Reid profile adjusted likelihood method (CR) to perform generalized linear model with
# design previously mentioned

# Subject data to GLM Trended Dispersion
countTable <- estimateGLMTrendedDisp(y, design)
names (countTable)
# Subject data to GLM Tagwise Dispersion
summary(countTable$trended.dispersion)
countTable <- estimateGLMTagwiseDisp(countTable, design)
names (countTable)
summary( countTable$tagwise.dispersion )
# Subject Data to GLM Common Dispersion - Verbose
countTable <- estimateGLMCommonDisp(countTable, design, verbose=TRUE)
names (countTable)
summary( countTable$common.dispersion )
# Plot the Biological Coefficient of Variation
plotBCV(countTable)
```

Appendix

```
# Plot Mean Variability to demonstrate the variation
meanVarPlot <- plotMeanVar( countTable ,show.raw.vars=TRUE , show.tagwise.vars=TRUE ,
                    show.binned.common.disp.vars=FALSE , show.ave.raw.vars=FALSE ,
                    NBline = TRUE , nbins = 100 ,
                    #these are arguments about what is plotted
                    pch = 16 , xlab ="Mean Expression (Log10 Scale)" ,
                    ylab = "Variance (Log10 Scale)" , main = "Mean-Variance Plot" )


# Differential Expression


# Fit the GLM data to the analysis
fit <- glmFit(countTable, design, robust=TRUE)
# Perform likelyhood ratio test on the GLM fit data -
# This test reads out Log Fold Change, Log CPM, Likelyhood Ratio, P value, and False
Discovery Rate
LRT <- glmLRT(fit, coef =2:3)
topTags(LRT,n=10)
# Export Stats findings to a table (n = number of genes being tested (Rows in Rawdata))
Take <- topTags(LRT,n=200000, sort.by = "none")
write.table(Take, 'Stats Neutrophils High v Low CD4 Coef2:3.txt', sep="\t")
top <- rownames(topTags(LRT))
cpm(y)[top,]
summary(decideTests(LRT))
```

```
# Plot Mean-Difference
plotMD(LRT)
# Add a line at logFC -2 and +2 as these are the cutoffs for heatmapping later on.
abline(h=c(-2,2), v=(107), col="green")
# Perform FDR analysis using the Beyer-Hardwick Method
FDR <- p.adjust(LRT$table$PValue, method="BH")
# FDR cutoff to demonstrate significant data
FDRajust <- sum(FDR < 0.05)
# How many genes are significantly different
summary(decideTests(FDR))

# Session info - keep a log of this with publishable formal analysis
sessionInfo()
#Heatmapping
# Package Load
library(pheatmap)
library(edgeR)
library(cluster)
library(statmod)
library(factoextra)
library(FactoMineR)
library(ggpubr)
```

Appendix

```
# Set Directory

setwd("~/Documents/RNA Sequencing/Neutrophil Phenotyping/")

# Read Tab Deliminanted file of RAW COUNTS - THIS DOES NOT MEAN

TPM/CPM/RPKM/htsqct/fastqc

Rawdata <- read.delim("DupGone.txt", row.names = "target_id")

PCARaw <- prcomp(Rawdata)

fviz_pca_ind(Rawdata)


# DGEList Conversion

y <- DGEList(counts=Rawdata[,])


# Filtering and Normalizing

y$samples

keep <- rowSums(cpm(y)>2) >= 5

y <- y[keep, , keep.lib.sizes=TRUE]

keep <- rowSums(cpm(y)>2) <= 10

y <- y[keep, , keep.lib.sizes=FALSE]


# Heat Mapping DGE Data

logcpm <- cpm(y, prior.count = 0, log=FALSE)


cal_z_score <- function(x){

  (x - mean(x)) / sd(x)
```

```
}


data_subset_norm <- t(apply(logcpm, 1, cal_z_score))
res <- pheatmap(data_subset_norm, show_rownames = F, clustering_method = "ward.D2",
clustering_distance_rows = "euclidean", clustering_distance_cols = "euclidean")




scaled <- scale(logcpm)
res <- pheatmap(logcpm, show_rownames = F, cutree_cols = 1, clustering_method = "ward.D2",
clustering_distance_rows = "euclidean", clustering_distance_cols = "euclidean")
logcpmClust <- cbind(data_subset_norm,
                     cluster = cutree(res$tree_row, k = 5))
pheatmap(logcpmClust, cutree_cols = 4, show_rownames = F,
         clustering_method = "ward.D2", clustering_distance_rows = "euclidean",
clustering_distance_cols = "euclidean")
write.table(logcpmClust, '2.txt', sep="\t")
```

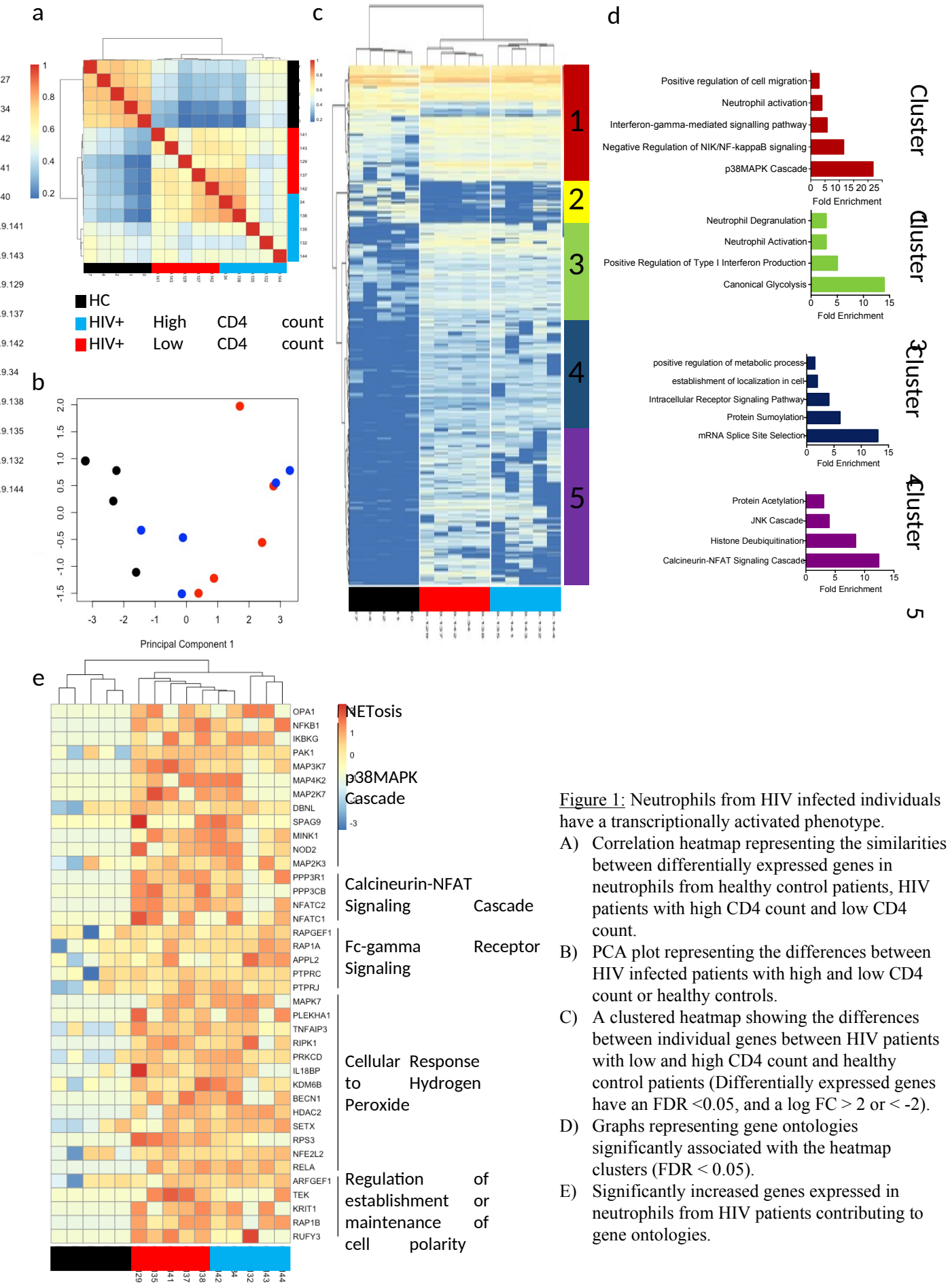


Figure 1: Neutrophils from HIV infected individuals have a transcriptionally activated phenotype.

A) Correlation heatmap representing the similarities between differentially expressed genes in neutrophils from healthy control patients, HIV patients with high CD4 count and low CD4 count.

B) PCA plot representing the differences between HIV infected patients with high and low CD4 count or healthy controls.

C) A clustered heatmap showing the differences between individual genes between HIV patients with low and high CD4 count and healthy control patients (Differentially expressed genes have an FDR <0.05, and a log FC > 2 or < -2).

D) Graphs representing gene ontologies significantly associated with the heatmap clusters (FDR < 0.05).

E) Significantly increased genes expressed in neutrophils from HIV patients contributing to gene ontologies.

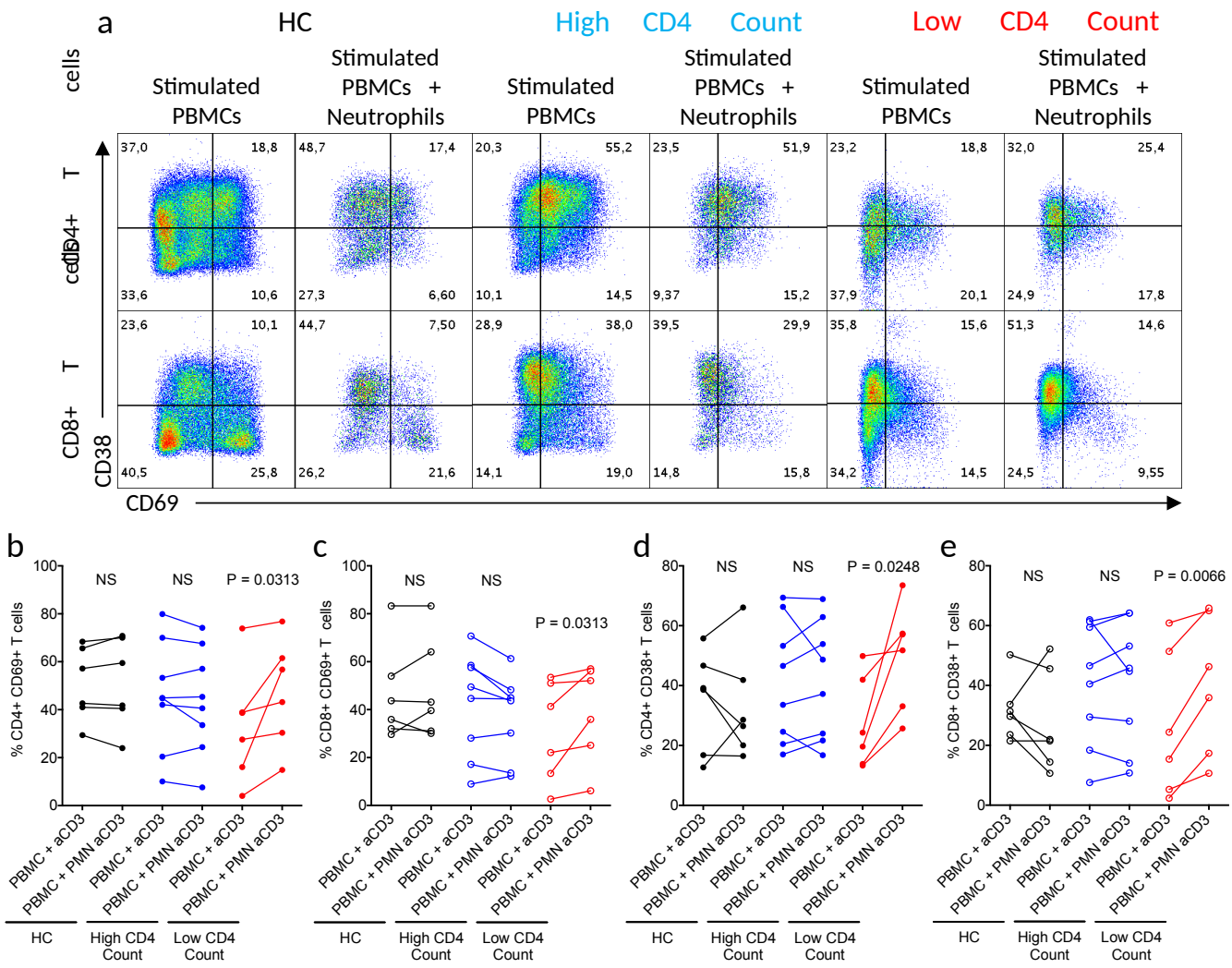


Figure 2: Neutrophils from HIV infected patients with low CD4 count contribute to T cell activation.

a) Representative plots of T cell activation in presence and absence of neutrophils from healthy controls, HIV+ patients with high CD4 count and low CD4 count.
b) Percent expression of CD69 on CD4+ T cells in presence or absence of neutrophils.
c) Percent expression of CD69 on CD8+ T cells in presence or absence of neutrophils.
d) Percent expression of CD38 on CD4+ T cells in presence or absence of neutrophils.
e) Percent expression of CD38 on CD8+ T cells in presence or absence of neutrophils.

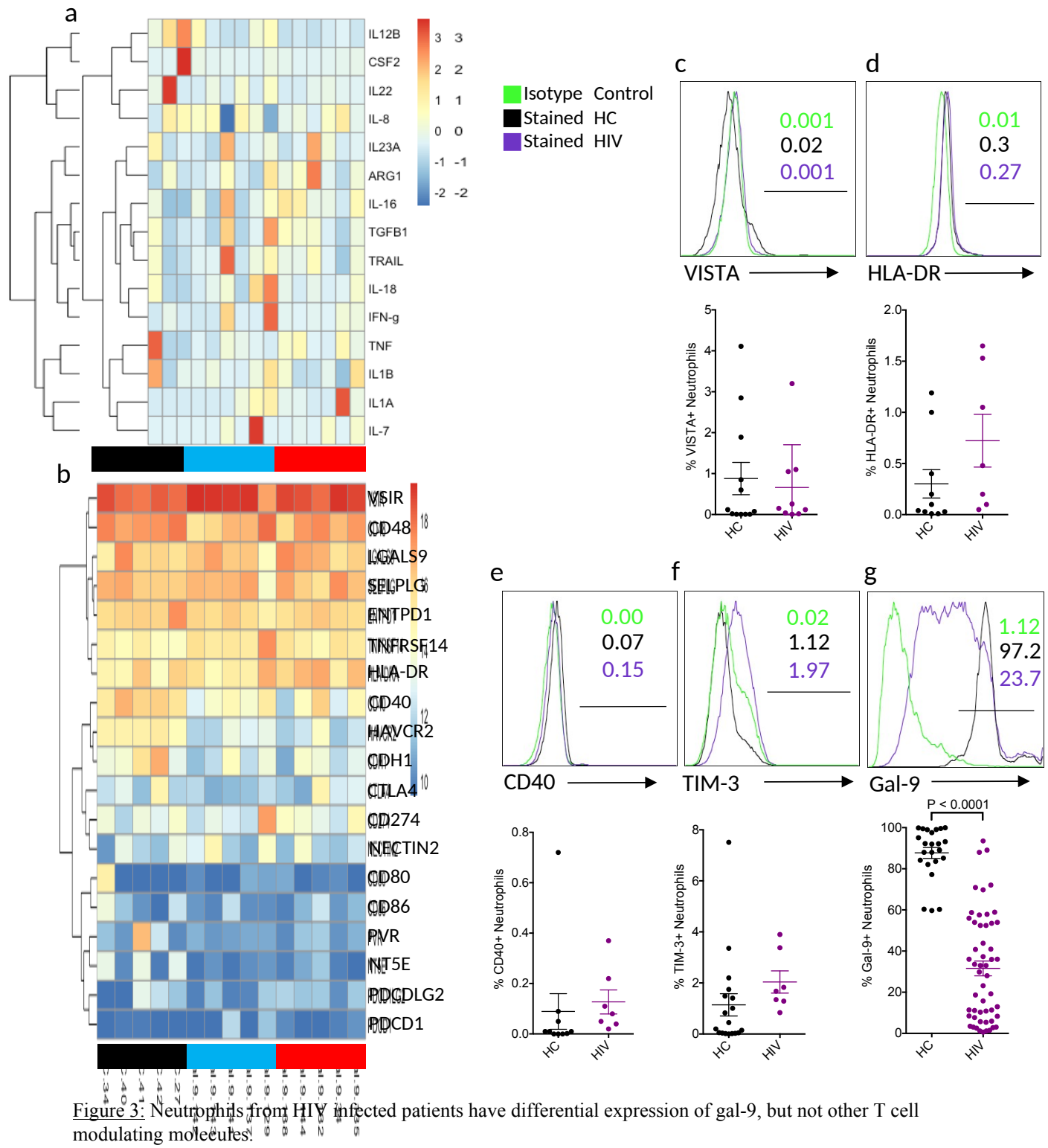


Figure 3: Neutrophils from HIV infected patients have differential expression of gal-9, but not other T cell modulating molecules.

a) Expression of T cell modulating cytokine transcripts in healthy, HIV+ high CD4 count patients and low CD4 count patients.
b) Magnitude of expression of co-inhibitory and co-activating receptors shown to modulate T cell function.
c) Neutrophil surface expression of VISTA
d) Neutrophil surface expression of HLA-DR
e) Neutrophil surface expression of CD40
f) Neutrophil surface expression of TIM-3
g) Neutrophil surface expression of gal-9

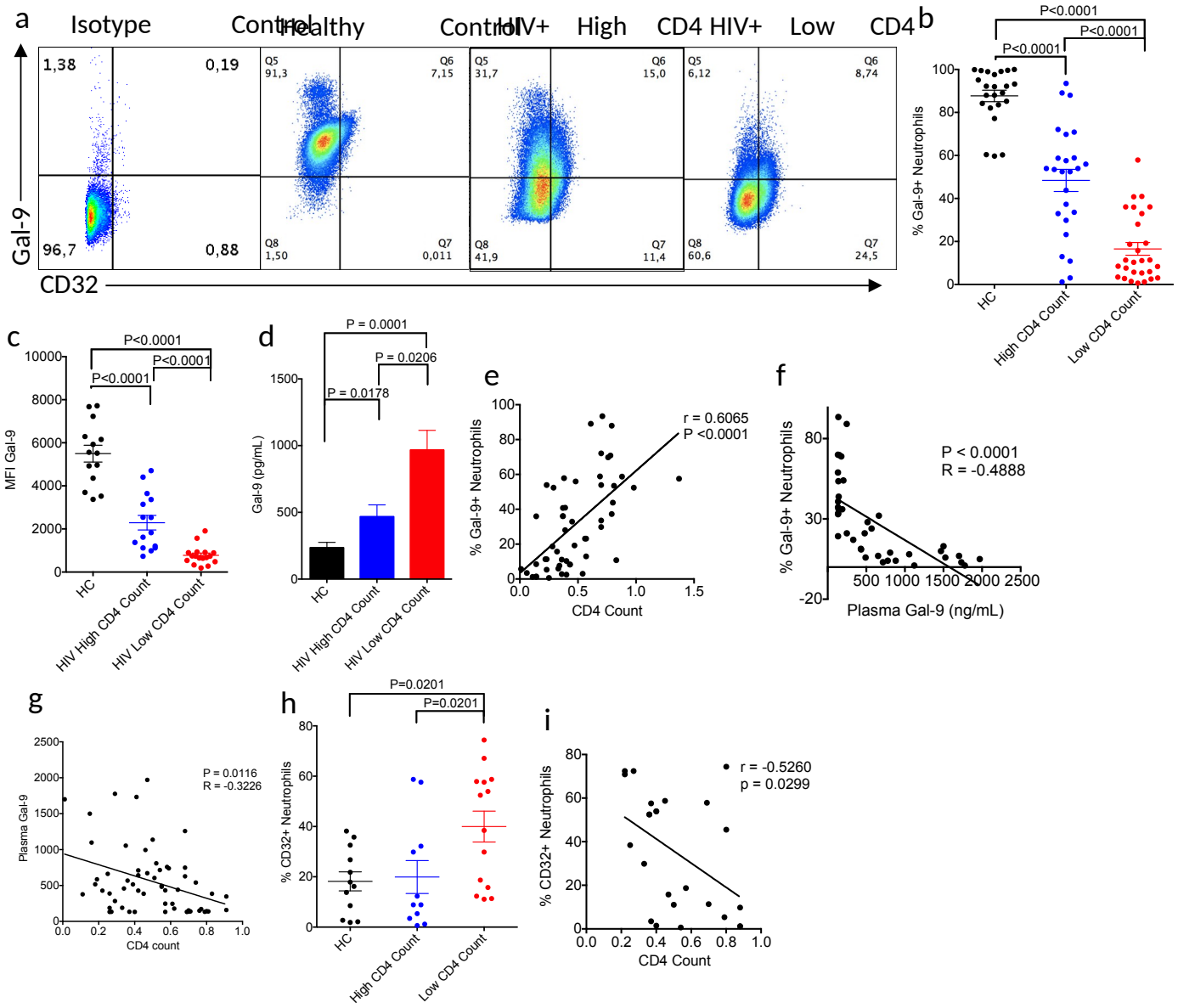


Figure 4: Neutrophils shed gal-9 in HIV infection.

a) Representative plots showing surface expression of Gal-9 and CD32 on neutrophils
b) Graphical representation of Gal-9 expression on neutrophils from healthy controls, HIV infected patients with low and high CD4 count
c) MFI of surface gal-9 on neutrophils from HIV patients with high CD4 count, low CD4 count and healthy controls
d) Concentration of soluble gal-9 from unstimulated neutrophils cultured for 8 hours
e) Correlation between CD4 count and Gal-9+ neutrophils
f) Correlation between gal-9+ neutrophils and plasma gal-9
g) Negative correlation between plasma gal-9 and CD4 count
h) % CD32 expression on neutrophils from HC, HIV patients with high and low CD4 count
i) Negative correlation between CD32 expression and CD4 count.

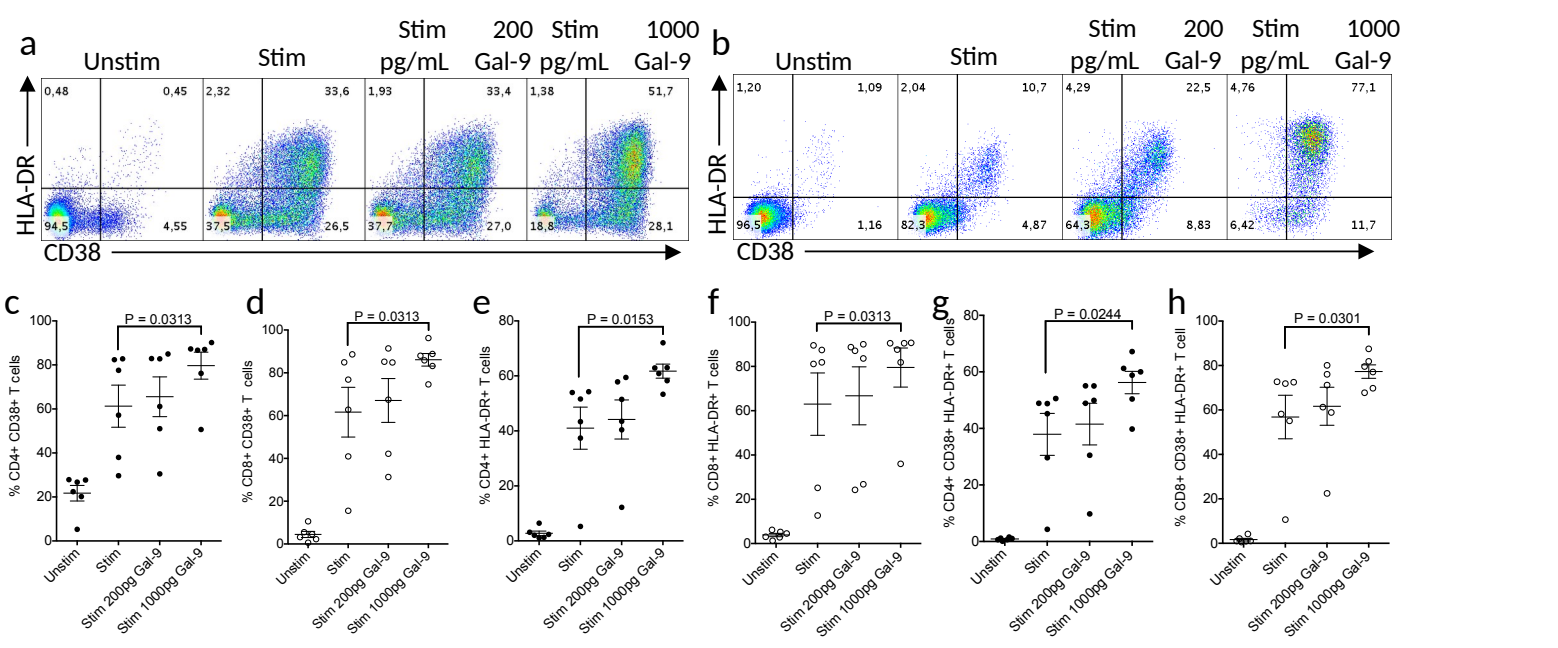


Figure 5: High concentrations of gal-9 contribute to T cell activation

a) Representative plot of CD4+ T cells cultured in absence or presence of increasing gal-9 concentrations
b) Representative plot of CD8+ T cells cultured in absence or presence of increasing gal-9 concentrations
c) Graphical representation of CD4+ T cell expression of CD38
d) Graphical representation of CD8+ T cell expression of CD38
e) Graphical representation of CD4+ T cell expression of HLA-DR
f) Graphical representation of CD8+ T cell expression of HLA-DR
g) Graphical representation of CD4+ T cell expression of CD38 and HLA-DR expression
h) Graphical representation of CD8+ T cell expression of CD38 and HLA-DR expression

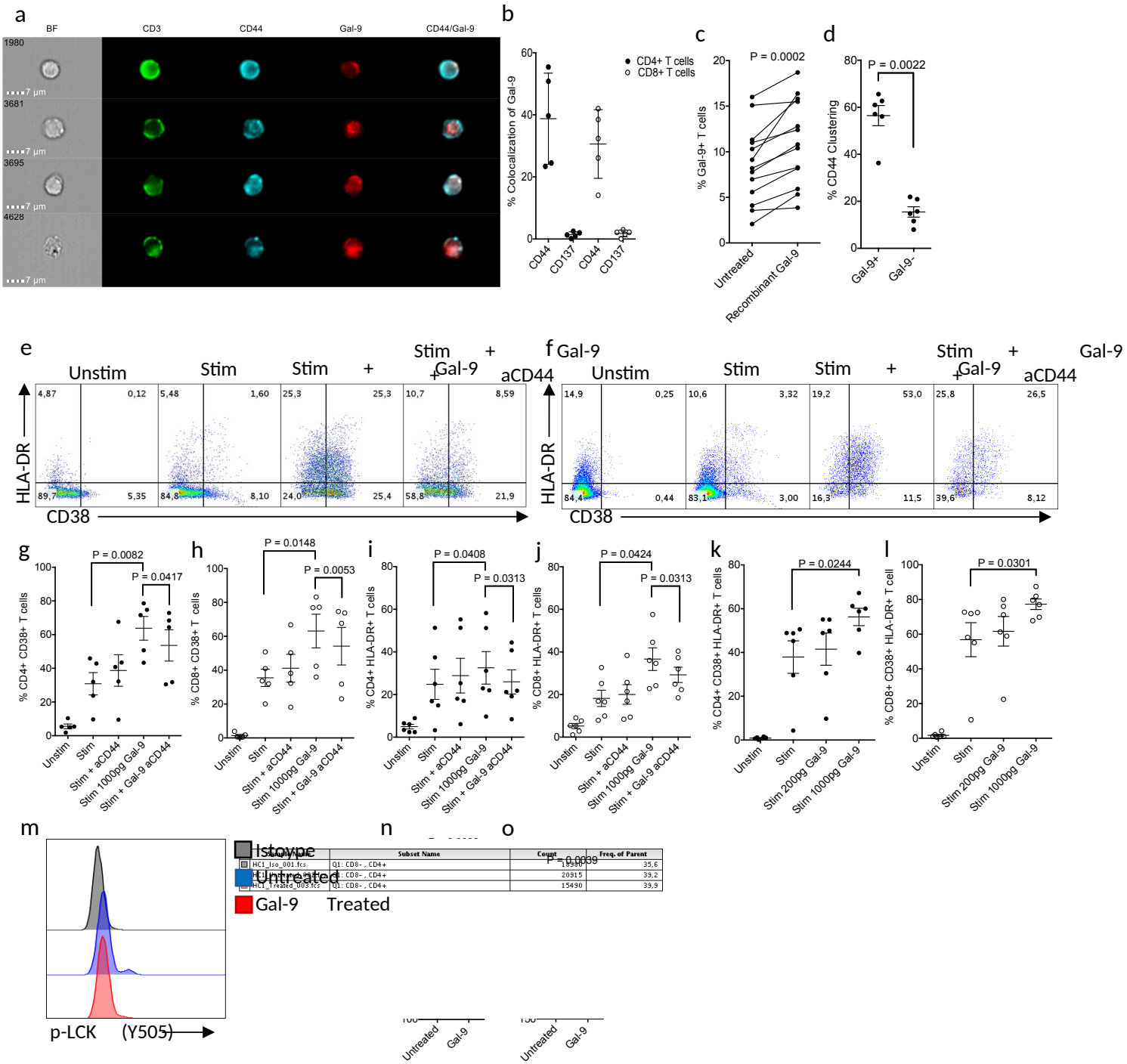


Figure 6: Galectin-9 binds CD44 on T cells and enhances T cell activation

a) Images demonstrating the organization of CD44 and gal-9 on the surface of T cells
b) Percent colocalization of gal-9 with the markers CD44 and CD137
c) Percent of gal-9 positive T cells in presence or absence of recombinant gal-9
d) Percent CD44 clustering in presence and absence of gal-9
e) Representative plots showing HLA-DR and CD38 expression on CD4+ T cells
f) Representative plots showing HLA-DR and CD38 expression on CD8+ T cells
g) Graphical representation of CD4+ T cell expression of CD38 in presence of recombinant gal-9
h) Graphical representation of CD8+ T cell expression of CD38 in presence of recombinant gal-9
i) Graphical representation of CD4+ T cell expression of HLA-DR in presence of recombinant gal-9
j) Graphical representation of CD8+ T cell expression of HLA-DR in presence of recombinant gal-9
k) Graphical representation of CD4+ T cell expression of CD38 and HLA-DR expression in presence of recombinant gal-9
l) Graphical representation of CD8+ T cell expression of CD38 and HLA-DR expression in presence of recombinant gal-9

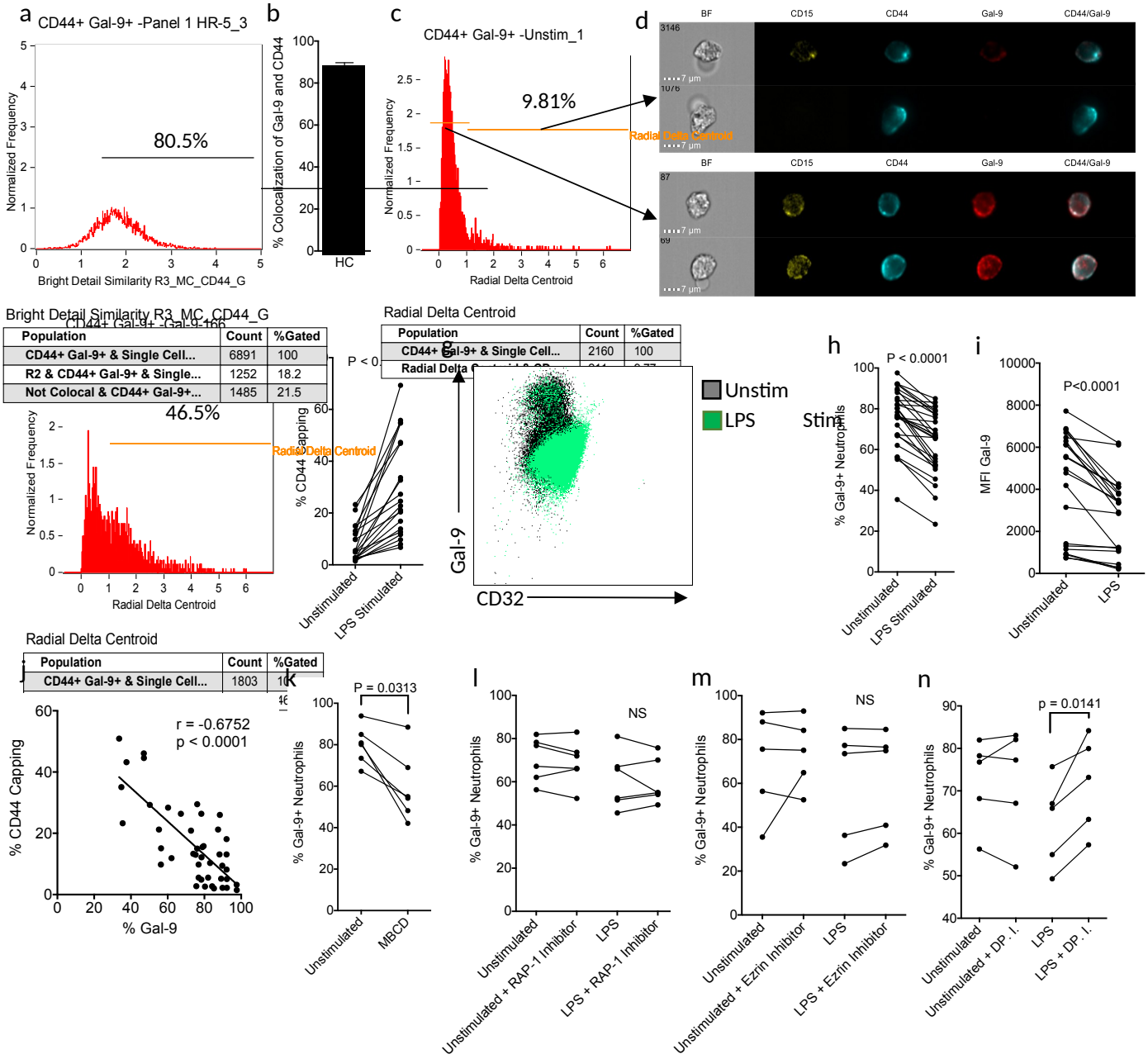


Figure 7: Gal-9 is shed by neutrophil mediated depalmitoylation of CD44 upon activation

a) Percent colocalization of CD44 and gal-9 on neutrophils quantified using an amnis imagestream
b) Cumulative percent colocalization of gal-9 aand CD44 on the surface of neutrophils
c) Representative plot of neutrophils with a high and low delta centroid XY
d) Representative of capped and dispersed neutrophil CD44.
e) Representative plot of LPS treated neutrophil radial delta centroid
f) Graphical representation of CD44 capping (delta centroid >1) in unstimulated and LPS treated neutrophils
g) Representative plot showing changing gal-9 and CD32 expression on neutrophils untreated and stimulated with LPS
h) Cumulative results showing the percent expression of surface gal-9 on unstimulated and LPS stimulated neutrophils
i) Cumulative results showing the MFI of surface gal-9 on unstimulated and LPS stimulated neutrophils
j) Correlation comparing % gal-9 expression on neutrophils and CD44 capping
k) Surface expression of gal-9 on neutrophils untreated or treated with methyl-b-cyclodextran
l) Surface expression of gal-9 on neutrophils untreated or treated with LPS in presence or absence of a RAP-1 inhibitor
m) Surface expression of gal-9 on neutrophils untreated or treated with LPS in presence or absence of a ezrin inhibitor
n) Surface expression of gal-9 on neutrophils untreated or treated with LPS in presence or absence of a depalmitoylation inhibitor

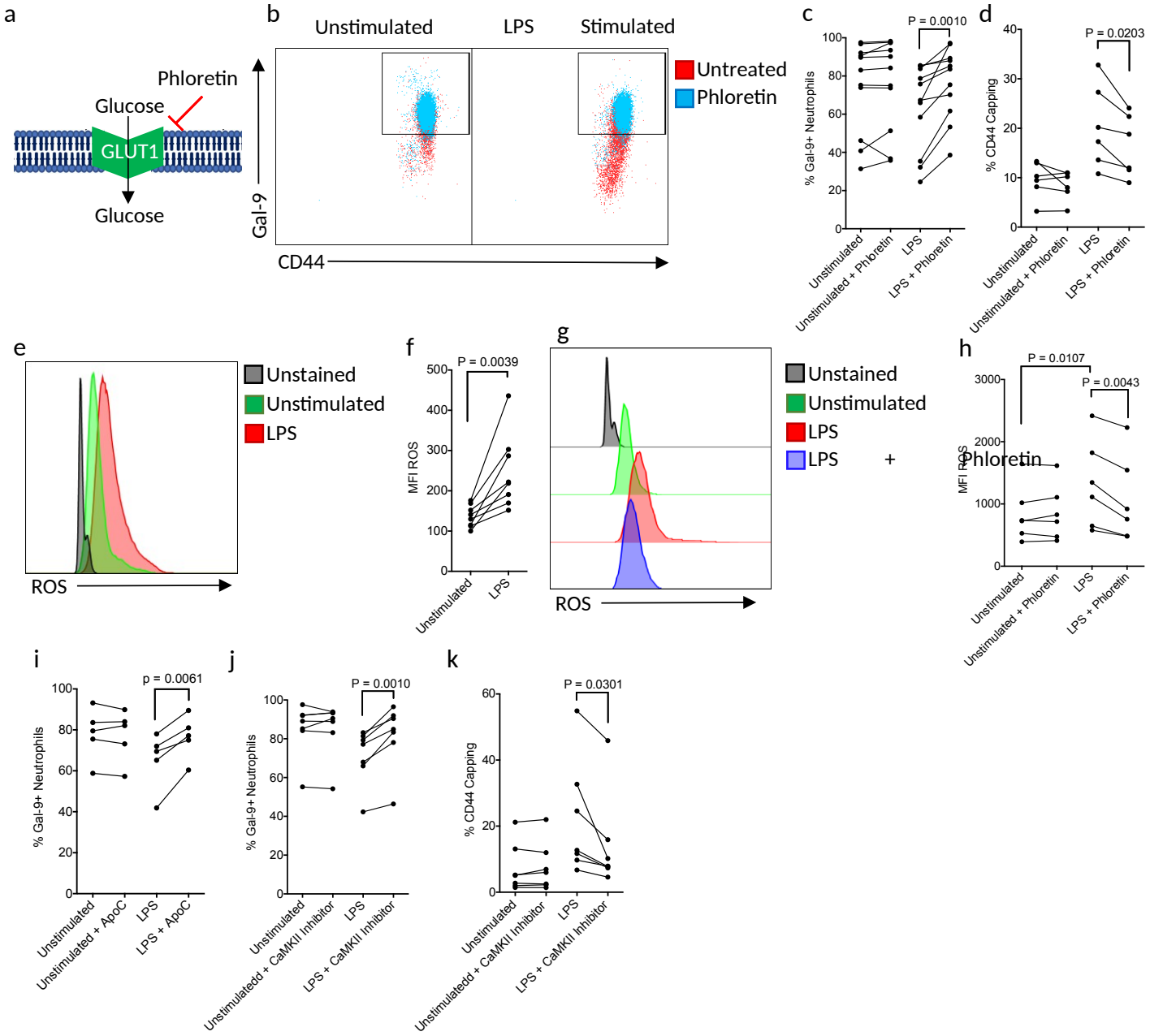


Figure 8: Gal-9 shedding by activated neutrophils is regulaated by ROS, and CaMKII

a) Visual showing the mechanistic action of Phloretin on the GLUT1 transporter
b) Representative plots showing the effect of phloretin on gal-9 shedding
c) Graphical representation of neutrophil surface gal-9 untreated or treated with LPS in presence or absence of phloretin
d) Percent CD44 capping in presence or absence of phloretin, untreated or treated with LPS
e) Representative plots of ROS production in neutrophils in presence or absence of LPS
f) Graphical representation of MFI of ROS production in untreated and LPS treated neutrophils
g) Representative plots showing the production of ROS in presence and absence of LPS and treated or untreated with phloretin
h) Cumulative results showing the change in ROS production in presence or absence of LPS and phloretin
i) Percent gal-9 expression in absence or presence of LPS and apocyanin
j) Percent gal-9 expression in absence or presence of LPS and a CaMKII inhibitor
k) Percent CD44 capping in absence or presence of LPS and a CaMKII inhibitor

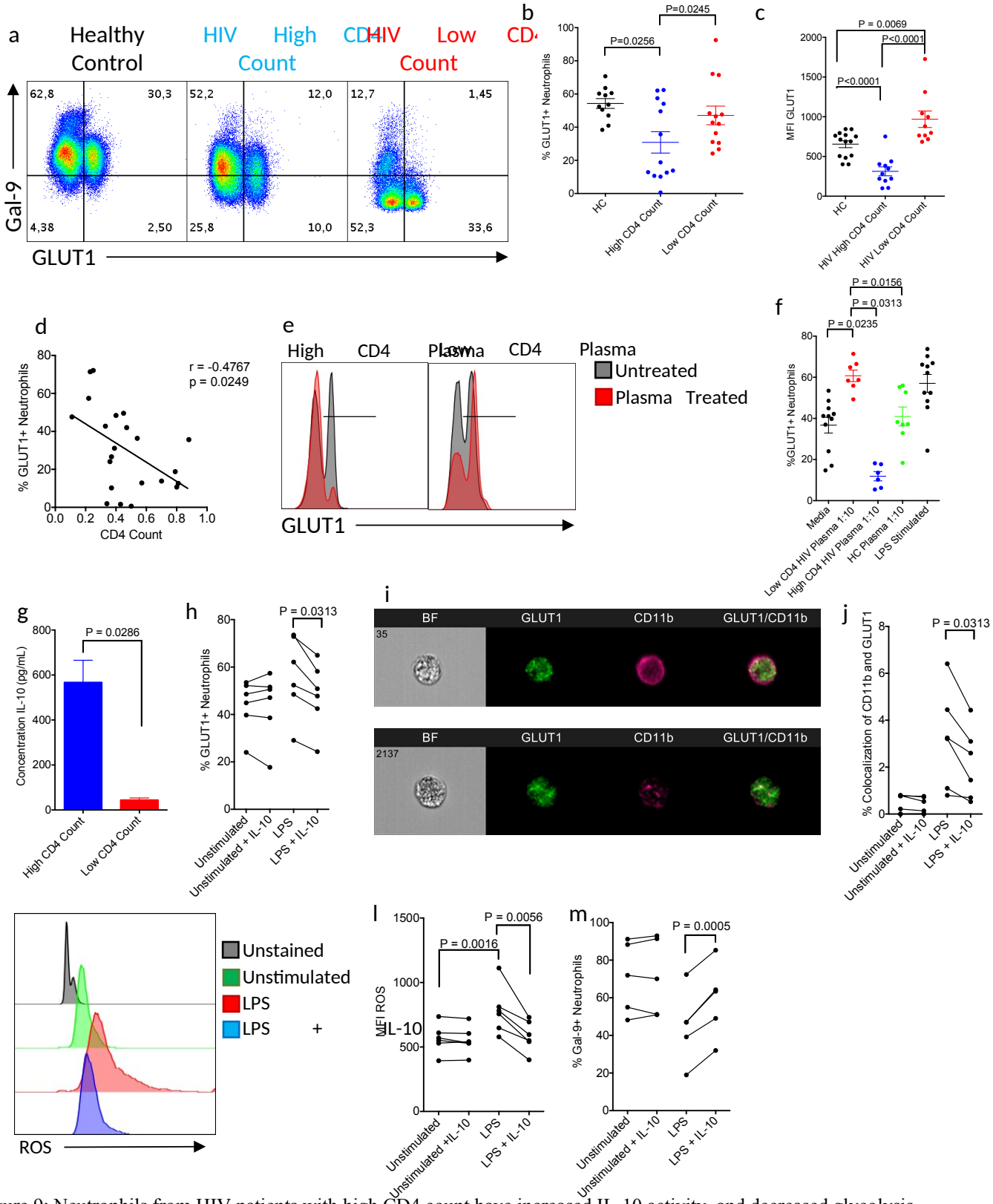
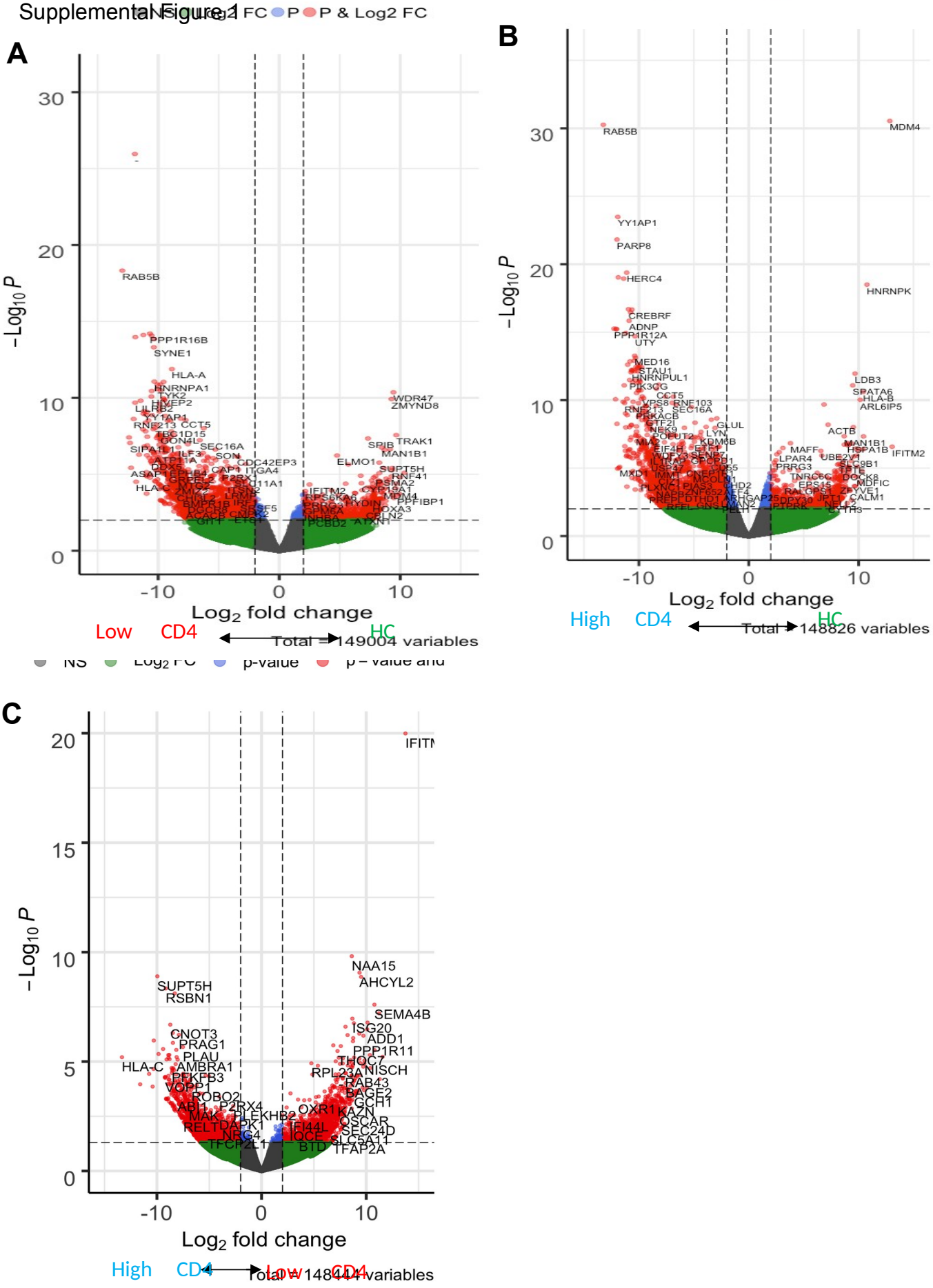


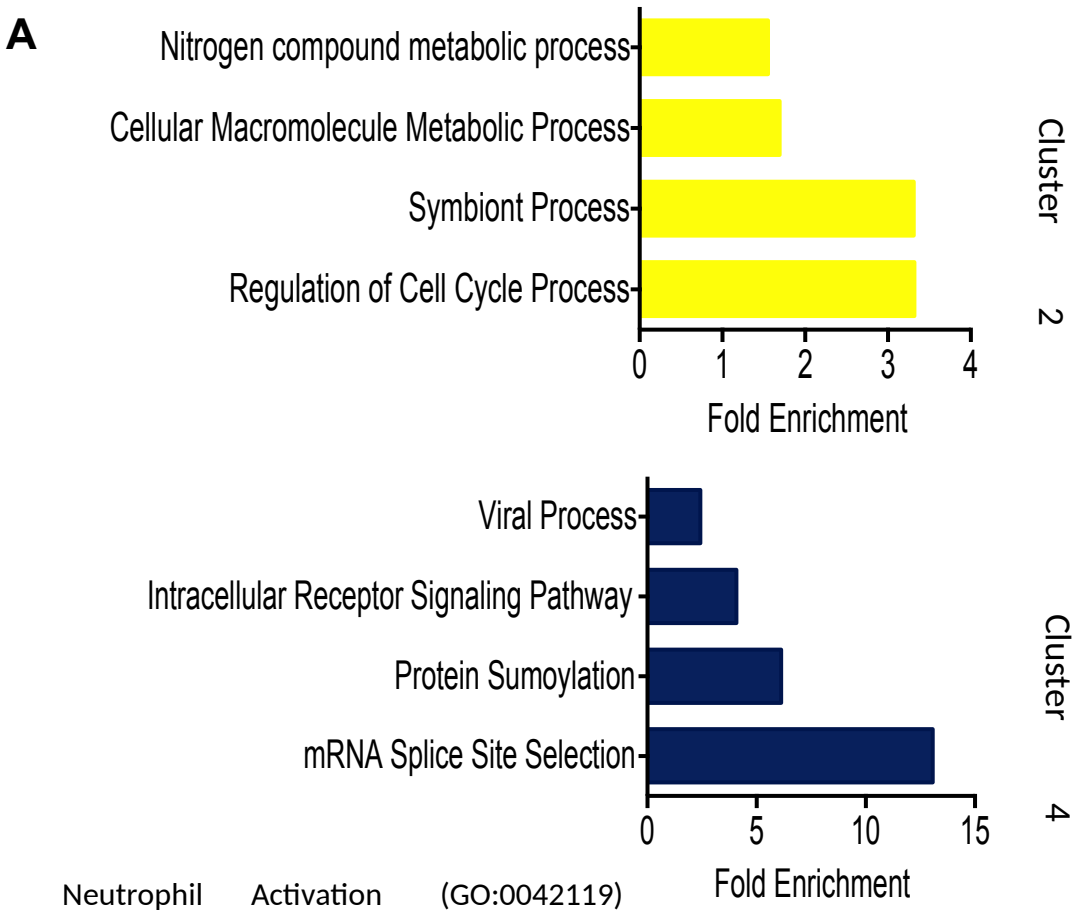
Figure 9: Neutrophils from HIV patients with high CD4 count have increased IL-10 activity, and decreased glycolysis

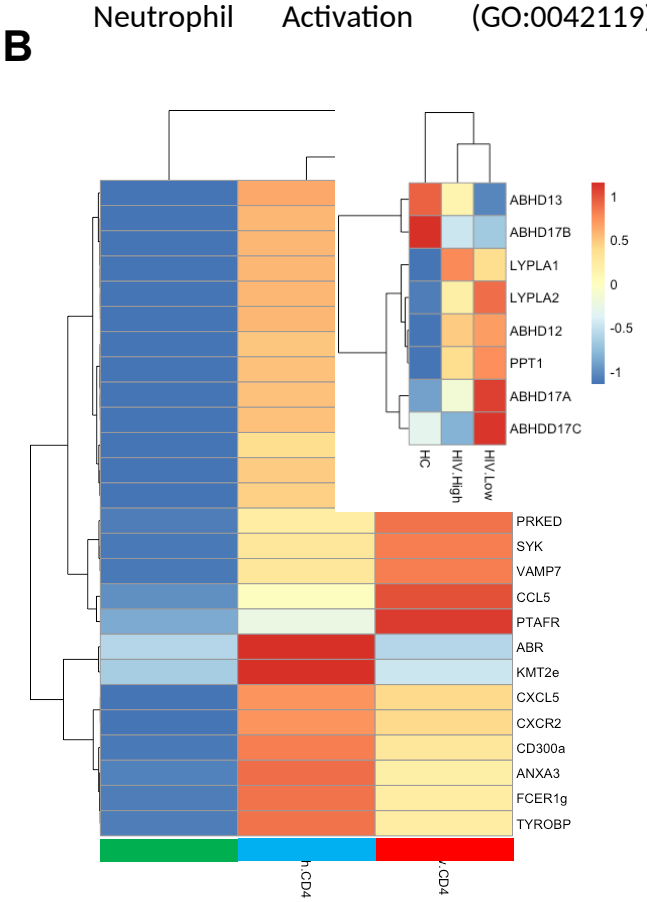
a) Representative plots of neutrophil GLUT1 and gal-9 expression from healthy controls, or HIV patients with low or high CD4 count
b) Graphical representation of the percent expression of GLUT1 on neutrophils from healthy controls, or HIV patients with low or high CD4 count
c) Graphical representation of the MFI of GLUT1 on neutrophils from healthy controls, or HIV patients with low or high CD4 count
d) Correlation of GLUT1 expression and CD4 count on neutrophils from HIV infected patients
e) Representative plot of GLUT1 expression in neutrophils treated or untreated with plasma from HIV patients with low CD4 count and high CD4 count

Supplemental Figure 1



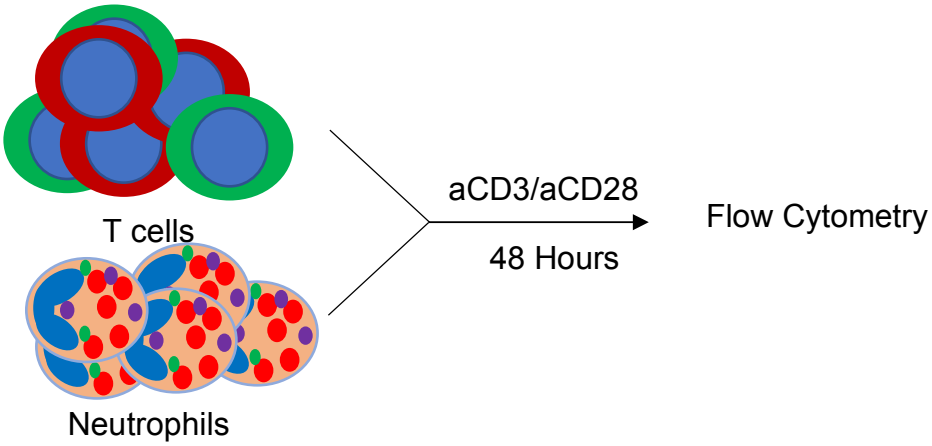
Supplemental Figure 2





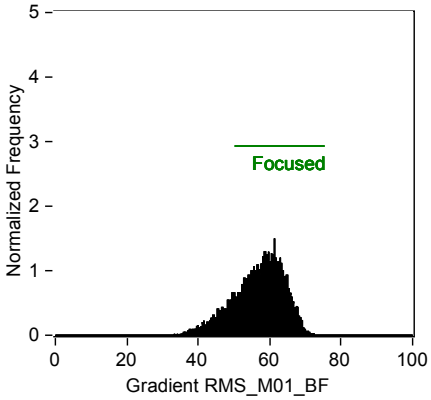
Supplemental Figure 3

A



B

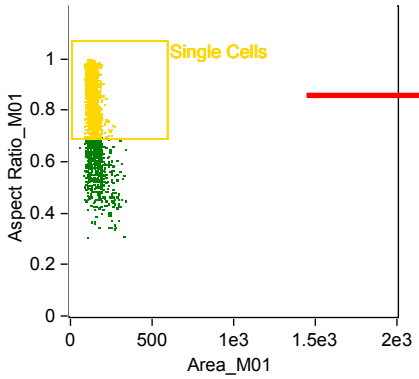
All-Panel 1 HR-5_3



Gradient RMS_M01_BF

Population	Count	%Gated
All	10000	100
Focused	8226	82.3

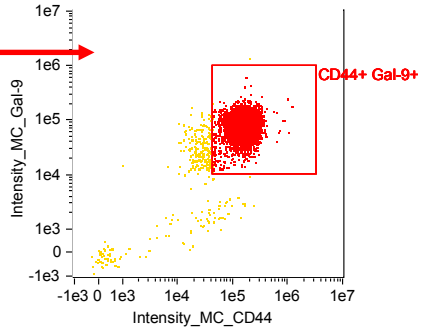
Focused-Panel 1 HR-5_3



Area_M01, Aspect Ratio_M01

Population	Count	%Gated
Focused	8226	100
Single Cells & Focused	7231	87.9

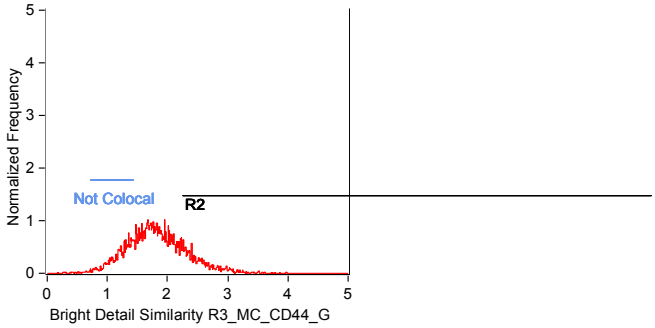
Single Cells -Panel 1 HR-5_3



Intensity_MC_CD44, Intensity_MC_Gal-9

Population	Count	%Gated
Single Cells & Focused	7231	100
CD44+ Gal-9+ & Single Cells...	6891	95.3

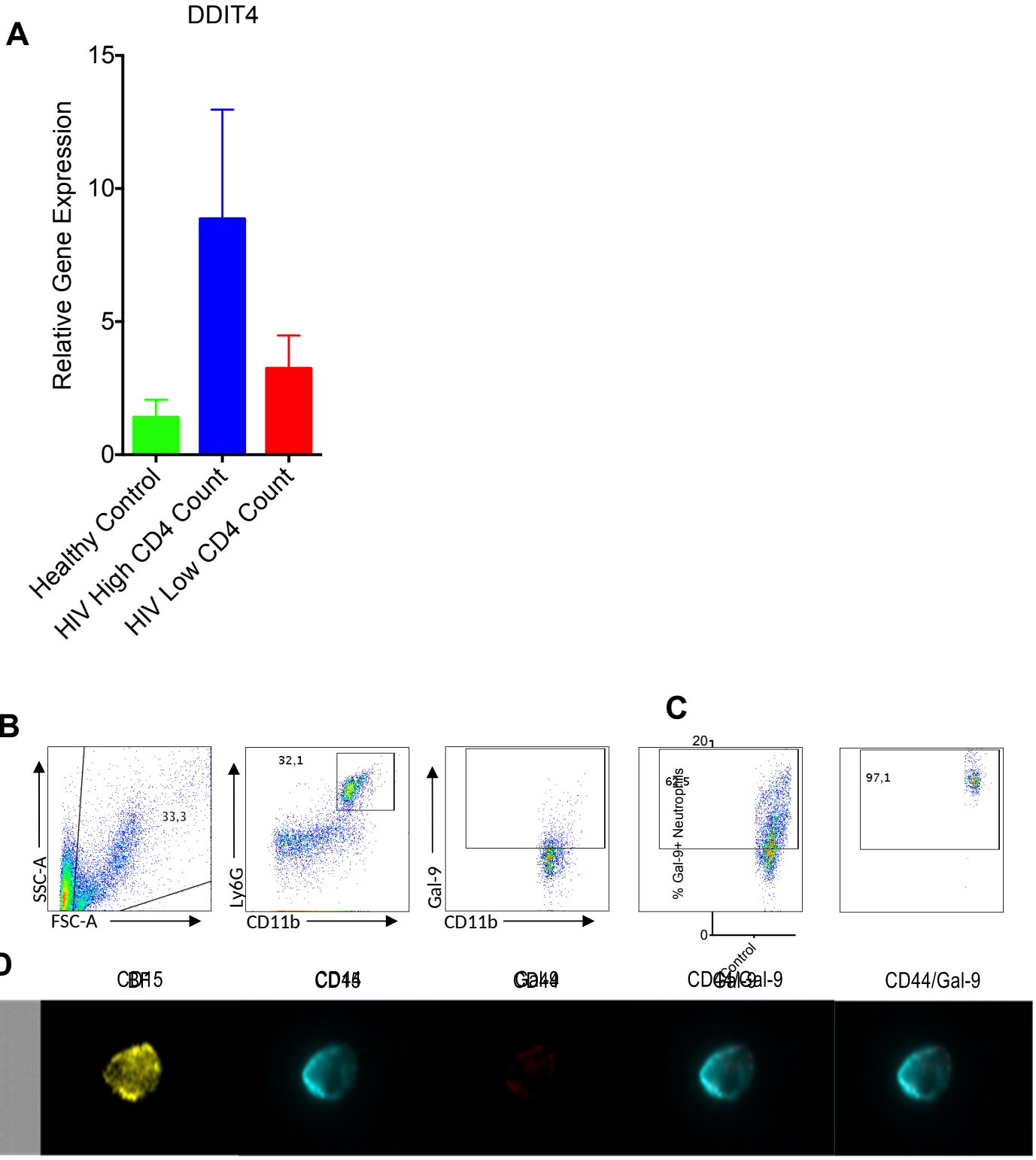
CD44+ Gal-9+ -Panel 1 HR-5_3



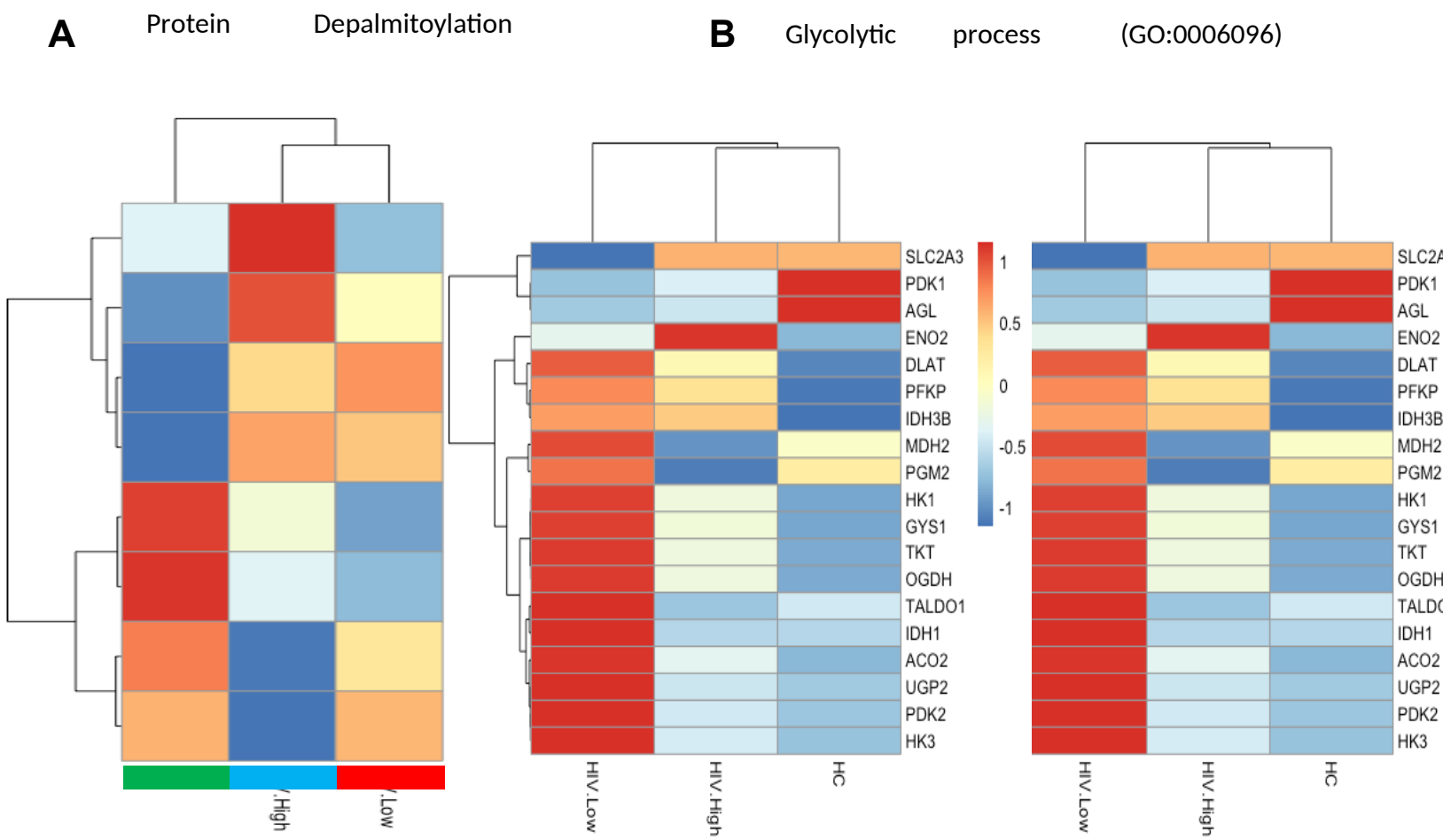
Bright Detail Similarity R3_MC_CD44_G

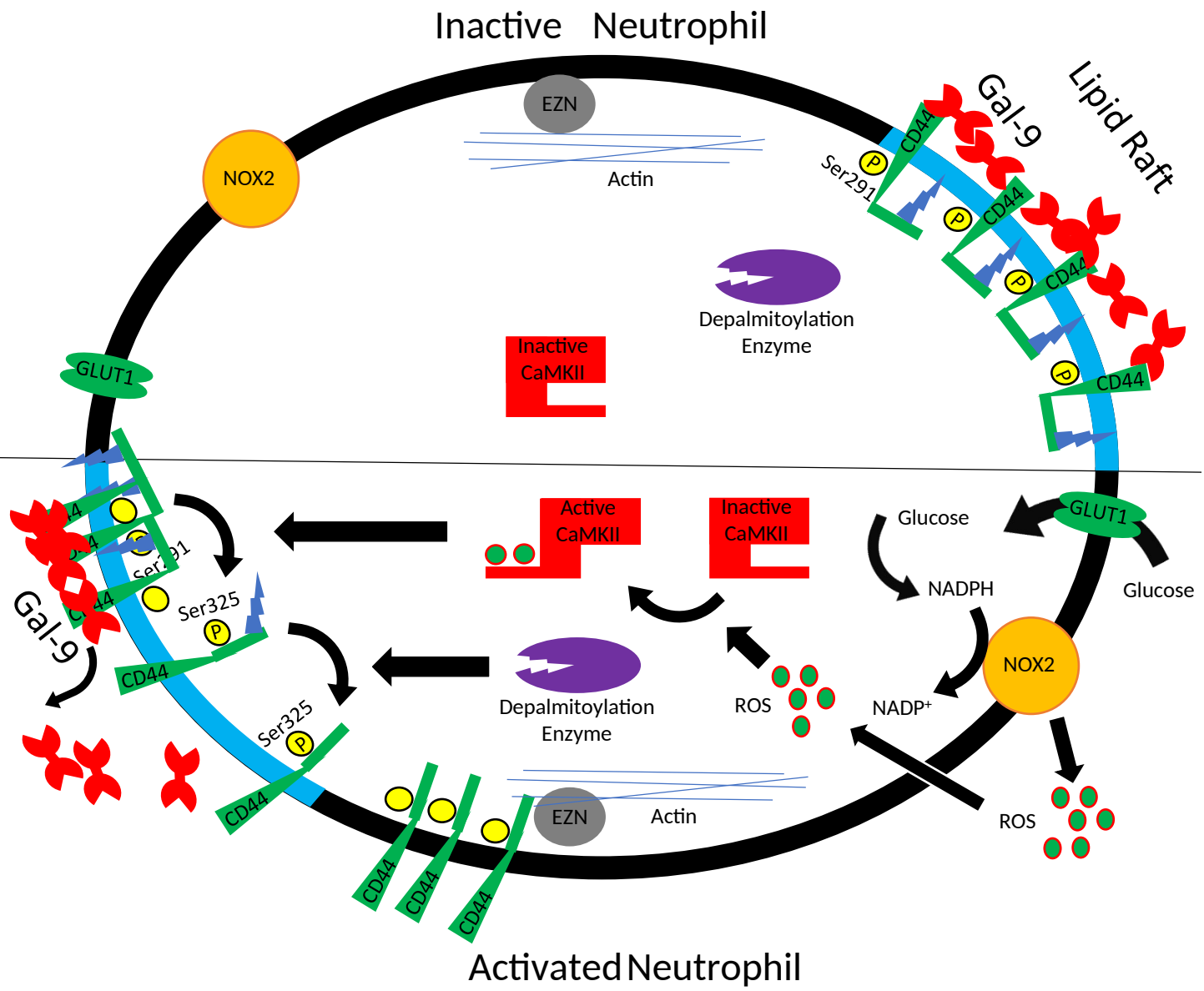
Population	Count	%Gated
CD44+ Gal-9+ & Single Cell...	6891	100
R2 & CD44+ Gal-9+ & Single...	1252	18.2
Not Colocal & CD44+ Gal-9+...	1485	21.5

Supplemental Figure 4



Supplemental Figure 5




Inactive Neutrophil
EZN
NOX2
Actin
Lipid Raft
Gal-9
CD44
Ser291
P
Depalmitoylation Enzyme
Inactive CaMKII
GLUT1
Active CaMKII
Glucose
NADPH
Ser325
ROS
NADP+
Activated Neutrophil