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Co-stimulator contributions in $CD8^+$ T cell differentiation

by

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Abstract

The adaptive immune response against intracellular pathogens is largely mediated by $CD8^+$ T lymphocytes. The clonal expansion and expression of cytolytic and immune stimulatory proteins by $CD8^+$ T cells is responsible for their protective immune function. Prior to exhibiting effector activity, $CD8^+$ T cells exist in a naïve state and require three stimulatory signals for their optimal activation including the recognition of antigen, co-stimulator ligand engagement, and the presence of pro-inflammatory cytokines. When these activation requirements are met, $CD8^+$ T cells undergo a well described series of events including clonal expansion, cellular contraction, memory generation, and memory maintenance. The memory $CD8^+$ T cell population generated can survive for the life-time of the host, and provides more rapid and robust protection upon re-infection then their naïve precursors. While some factors that induce this sequence of events have been identified, the role of co-stimulation remains relatively undefined. This is due to the large number of co-stimulator receptors expressed by $CD8^+$ T cells that may be ligated individually and/or in combination, to instruct the development of specific effector and memory $CD8^+$ T cell phenotypes.

Using a bead-based ligand presentation system, I investigated the role of co-stimulation in directing naïve $CD8^+$ T cell activation, and the generation of T cell populations with distinct effector and memory fates. I identified ICAM-1 as the stimulatory molecule best able to induce naïve $CD8^+$ T cell proliferation and expression of the effector molecule granzyme B, while co-stimulation through

CD28 was required to induce IFN-γ. When provided in combination however, B7.1 and ICAM-1 co-stimulation generated $CD8^+$ T cells which were highly cytolytic and expressed high amounts of IFN-γ. These cells also exhibited enhanced survival once activated, with sustained expression of anti-apoptotic proteins and secretion of high amounts of IL-2, resulting in these cells exhibiting a terminal effector phenotype. While other co-stimulator combinations also enhanced $CD8^+$ T cell survival to some degree, their ability to sustain high expression of IL-2 was limited. This translated into less potent effector responses and preferential memory precursor development based on transcription factor expression.

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L ist of Abbreviations

2-ME	2-Mercaptoethanol
4-1BBL	4-1BB ligand
aAPC	artificial antigen presenting cell
Ab	Antibody
ACAD	activated cell autonomous cell death
ACT	adoptive cell transfer therapy
AICD	activation induced cell death
AIRE	autoimmune regulator
Akt	v-akt murine thymoma viral oncogene homolog-1, see PKB
AP-1	activator protein-1
APAF-1	apoptotic protease activating factor-1
APC	antigen presenting cell
BAK	BCL-2 antagonist/killer
BAX	BCL-2-associated protein X
BCL1	B cell lymphoma-1
Bcl-10	B cell lymphoma-10
Bcl-2	B cell lymphoma-2
Bcl-6	B cell lymphoma-6
Bcl-xL	B cell lymphoma-extra large
BCR	B cell receptor
BFA	Brefeldin A
Bid	BH3-interacting-domain death agonist
Bim	BCL-2-interacting mediator or cell death
Blimp-1	B-lymphocyte induced maturation protein-1
BSA	bovine serum albumin
BTLA	B- and T-lymphocyte attenuator
CARMA1	CARD-containing MAGUK protein-1
CCM	complete culture medium
CCR(7)	CC-chemokine receptor
CD	cluster of differentiation
CD30L	CD30-ligand
cDNA	complementary DNA
cFLIP	cFLICE-like inhibitory protein
ChIP	chromatin immunoprecipitation
CRD	cysteine-rich domains
CTL	cytotoxic T-lymphocyte
CTLA-4	cytotoxic T-lymphocyte antigen-4
DC	dendritic cell
DISC	death-inducing signalling complex
DNA	deoxyribonucleic acid
DP	double positive

ELISA	enzyme-linked immunosorbent assay
Eomes	Eomesodermin
ER	endoplasmic reticulum
Erk1/2	extracellular-signal-regulated kinase1/2
FADD	Fas-associated death domain
FasL	Fas-ligand
Fc	fragment, crystallisable (region)
FCS	fetal calf serum
Fig	figure
FOXO1	forkhead box protein O1
GITR	glucocorticoid-induced TNFR family related gene
GITRL	glucocorticoid-induced TNFR family related gene-ligand
GpD	glycoprotein-D
hi	high
Grb2	growth factor receptor-bound protein-2
HIV	human immunodeficiency virus
HLA	human leukocyte antigen
HPV	human papilloma virus
hrs	hours
HSV-1	Herpes-simplex virus-1
HVEM	Herpes-virus entry mediator
ICAM-1	intercellular adhesion molecule-1
ICOS-L	inducible T-cell co-stimulator-ligand
ICOS	inducible T-cell co-stimulator
Id3	inhibitor of DNA-binding-3
IFN	interferon
Ig	immunoglobulin
IL	interleukin
Int	intermediate
iono	ionomycin
IRF4	interferon regulatory factor-4
ITIM	immunoreceptor tyrosine-based inhibitory motif
JAK	janus kinase
JNK	jun-kinase
KD	kilo Dalton
KLRG1	killer cell lectin-like receptor subfamily G member-1
LTα	lymphotoxin-alpha
LCMV	lymphocytic choriomeningitis virus
LFA-1	lymphocyte function-associated antigen-1
lo	low
LIGHT	lymphotoxin-like, exhibits inducible expression, and competes with herpes simplex virus glycoprotein D for HVEM, a receptor expressed by T lymphocytes

LPS	lipopolysaccharide
mAb	monoclonal antibody
MAPK	mitogen-activated protein kinase
Mcl-1	myeloid cell leukemia sequence-1
MFI	mean fluorescence intensity
MHC	major histocompatibility complex
min	minute
mL	millilitre
mM	Milli molar
MPEC	memory precursor effector cells
mRNA	messager RNA
mTOR	mammalian target of rapamycin
mTORC1	mTOR complex-1
mTORC2	mTOR complex-2
NFAT	nuclear factor of activated T-cells
NF-kB	nuclear factor kappa-B
ng	Nano gram
NK	natural killer cell
NKT	natural killer T cell
nm	nanometres
nM	Nano molar
OT-1	OVA-transgenic-1
OVA	ovalbumin
OX-40L	OX-40 ligand
p38	mitogen activated protein kinase-38
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PD-1	programmed death receptor-1
PD-L1/2	programmed death receptor-1 ligand1/2
PI3K	phosphoinositide 3-kinase
PKB	protein kinase B, see Akt
PMA	Phorbol 12-myristate 13-acetate
PP2A	protein phosphatase 2 alpha isoform
PRR	pattern recognition receptor
PYK2	protein tyrosine kinase-2
qPCR	quantitative PCR
rADCC	reverse antibody dependant cellular cytotoxicity
rB7.1	recombinant B7.1
rCD70	recombinant CD70
rICAM-1	recombinant ICAM-1
RIG	retinoic acid-inducible gene
RNA	ribonucleic acid
RORγt	RAR-related orphan receptor gamma-terminus

RPL24	ribosomal protein L24
RPMI	Roswell Park Memorial Institute medium
Runx3	runt-related transcription factor-3
SHP-2	Src homology z domain containing protein tyrosine phosphatase 2
SLEC	short-lived effectors cells
SMAC	supramolecular cluster
SP	single positive
STAT	signal transducer and activator of transcription
TAP	transporters associated with antigen presentation
TBE	T-box DNA binding element
T-bet	T-box transcription factor-21
Tcf-1	T cell factor-1
Tcm	central memory T cells
TCR	T cell receptor
Tem	effector memory T cells
Tfh	T follicular helper cells
TGF	tumour growth factor
Th	T helper
TLR	toll-like receptor
TLT-2	Trem-like transcript-2
TNFR	tumour necrosis family receptor
TRAF	TNF receptor associated factor
TRAIL	tumour necrosis factor-related apoptosis-inducing ligand
Treg	regulatory T cell
TREM	triggering receptor expressed on myeloid cell
U	units
µCi	Micro Curie
µg	microgram
µL	Micro litre
VSV	vesicular stomatitis virus
Xbp-1	X-box binding protein-1

Chapter 1. General Introduction

1. Overview of innate and adaptive immunity

The immune system is charged with the task of detecting and eliminating a vast array of potential pathogens which continually attempt to invade and infect any given host. In mammals, the immune system is comprised of two distinct yet overlapping arms that cooperate to eliminate infection: the innate immune system, and the adaptive immune system [1]. The innate immune system is the first line of defense against invading pathogens. Armed with an array of pattern recognition receptors (PRRs) capable of broadly distinguishing "non-self" bacterial and viral components from "self", cells of the innate immune system detect the presence of infectious microbes and initiate the immune response against them [1, 2]. The innate immune response is rapid and acts mostly to contain, if not eliminate, infection until an adaptive immune response can be initiated. The cellular components of the innate immune system include monocytes, macrophages, dendritic cells (DCs), neutrophils, basophils, mast cells, and eosinophils. Natural killer (NK) cells are also classically considered part of the innate immune system, although they recently have been described as having both innate and adaptive properties [3, 4]. Other cells considered innate but with adaptive-like qualities are NKT and γδ T cells. Cells of the innate immune system are also responsible for the uptake, processing and presentation of "non-self" antigenic components to induce adaptive immune responses.

The adaptive immune system is comprised of two distinct cell lineages: antibody producing B lymphocytes (B cells), and T lymphocytes (T cells), which

are responsible for cell-mediated immunity. Cells of the adaptive immune system recognize pathogen-derived antigenic peptides with a high degree of specificity, but typically take between 5-6 days to become fully effective. The specificity of this recognition is mediated by the expression of antigen-specific surface receptors, the B-cell receptor (BCR), and the T-cell receptor (TCR). The BCR and TCR are generated by random gene recombination events during B and T cell development, respectively [5, 6]. Moreover, unlike cells of the innate immune system, B and T cells are capable of generating immunological memory that is maintained following the elimination of infection. Memory T and B cells mount more rapid and robust responses following re-infection with the originating pathogen than naïve cells, leading to enhanced control and elimination of infection upon secondary exposure [7, 8].

1.1 T cells and T cell subsets

T cells are involved in cell-mediated immunity and can be broadly classified into two major types, $CD4^+$ and $CD8^+$ T cells. In mice, both $CD4^+$ and $CD8^+$ T cells recognize antigen presented by the major histocompatibility complex (MHC) via their surface T cell receptors, but differ in their ability to recognize distinct types of MHC proteins. $CD8^+$ T cells recognize peptide antigen presented by MHC class I molecules, while $CD4^+$ T cells require peptide presentation in the context of MHC class II. $CD4^+$ and $CD8^+$ T cell also differ greatly in terms on their function [9]. $CD8^+$ T cells are cytotoxic effector cells which, after their activation, patrol the periphery and are capable of directly lysing infected target cells displaying foreign antigen [10]. Effector $CD8^+$ T cells can

also differ in their expression of specific cytolytic molecules and cytokines, indicating that some heterogeneity in the $CD8^+$ T cell effector population does exist [10, 11]. However, the mechanisms which might influence the generation of potential $CD8^+$ effector T cell subsets are not known.

$CD4^+$ T cells are often referred to as "helper" T cells, and exert their function through the release of cytokines that influence the magnitude and type of the immune response generated. $CD4^+$ T helper (Th) cells can be further classified into smaller subsets, each with unique cytokine producing capabilities, and the major subsets are: Th1, Th2, Th17 and regulatory T cell (Treg) subsets [12]. Each of these subsets, with the exception of Tregs, is believed to arise from a single multi-potent naïve $CD4^+$ T cell population. While defined $CD4^+$ T cell subsets are often associated with the production of specific cytokines and use of particular transcriptional regulators, it is important to note that these subsets are relatively plastic in nature [12-14]. Th1 and Th2 $CD4^+$ T cells represent two opposite poles of $CD4^+$ T cell differentiation. Th1 cells generally produce large amounts of IFN-γ that promotes cell-mediated immunity, while Th2 cells secrete the cytokines IL-4, IL-5 and IL-13, that support humoral immunity against parasites and other extracellular pathogens [12, 14]. Tregs function to suppress T cell responses in the periphery which helps to maintain peripheral-tolerance. Tregs develop both in the thymus (referred to as natural Tregs), and in the periphery in the presence of the immunosuppressive cytokines such as IL-10and TGF-β [15]. Finally, the Th17 subset of $CD4^+$ T cells arises from $CD4^+$ T cell precursors activated in the presence of IL-6 and TGF-β. Considered a pro-

inflammatory cell subset, Th17 $CD4^+$ T cells can recruit macrophages and neutrophils to the site of infection through the secretion of IL-17 and IL-21. However, Th17 cells are also linked to several autoimmune diseases including rheumatoid arthritis and lupus [16].

1.2 T cell Development

T cell precursors are generated in the bone marrow and migrate to the thymus for their development. Once in the thymus, immature thymocytes undergo a series of gene rearrangement events that give rise to the TCR. The conventional TCR is comprised of disulphide-linked α- and β-chains, which are derived from the random recombination of germ-line encoded, V (variable)-, D (diversity)-, and J (joining)- TCR gene elements. In conventional αβ T cells, double-negative (DN) thymocytes, which lack the expression of the CD4 and CD8 co-receptors, first rearrange V_β, D_β, and J_β gene segments to form a functional β-chain. The newly synthesized β-chain forms a complex with a pre-Tα chain and the CD3 signalling unit to form the pre-TCR. Ligation of the pre-TCR signals functional β-chain production, and leads to the rearrangement of V_α, and J_α gene segments to form the TCR α-chain. Once formed, the α-chain heterodimerizes with β-chain to form a mature αβ TCR complex [5, 17, 18]. At this stage, TCR-expressing thymocytes express both CD4 and CD8 co-receptors and are called double-positive thymocytes (DP). DP thymocytes then undergo the process of positive selection to ensure MHC restriction.

Due to the random nature of the TCR gene rearrangement events, ~10^{18} αβ TCR combinations can be generated, each with variable capacity to recognize

self-MHC complexes [5]. The process of positive selection is responsible for restricting T cell recognition of self-MHC by providing survival signals to T cells possessing TCRs which recognize self-MHC complexes with moderate affinity. The remaining thymocytes, which express either useless TCRs, or recognize MHC complexes with too high affinity, die by neglect or undergo clonal deletion, respectively. Thus, positive selection ensures that the resulting T cells can recognize self –MHC complexes, but not with overly high affinity as to induce self-reactivity [17]. During positive selection, TCR-mediated signalling also results in CD4 and CD8 co-receptor selection. This process is initiated in DP thymocytes by reducing their expression of CD8 to become $CD4^{+}CD8^{low}$. In the case of sustained TCR signalling, $CD4^{+}CD8^{low}$ cells are believed to develop into $CD4^{+}$ single positive (SP) mature thymocytes, while the $CD8^{+}$ co-receptor is selected if TCR signalling ceases [19]. Finally, SP mature thymocytes undergo the last stage of T cell development, negative selection. During negative selection, MHC-restricted SP thymocytes interact with thymic stromal cells bearing self-antigen MHC complexes. The expression of peripheral self-antigen in the thymus is driven by key transcription factors including AIRE which functions to decrease the number of surviving SP thymocytes expressing TCRs that recognizes self-peptides, thus promoting central tolerance [20, 21]. Self-reactive thymocytes are eliminated by apoptosis, while non-self-reactive thymocytes develop into mature $CD4^{+}$ and $CD8^{+}$ T lymphocytes.

1.3 CD8$^+$ T cell lifecycle

CD8$^+$ T cells are responsible for the cell-mediated control of intracellular pathogens. CD8$^+$ T cells undergo a well described series of events or phases following acute infection which comprise their entire lifecycle. These include: 1) Naive T cell *priming*, in which antigen specific CD8$^+$ T cells undergo massive clonal expansion, acquire cytolytic effector function, and home to peripheral sites to combat infection, 2) Cellular *contraction*, in which the majority of the expanded CD8$^+$ T cell population (90-95%) die by apoptotic mechanisms following antigen clearance, leaving behind a long-lived memory T cell population, and 3) Memory *maintenance*, in which the memory CD8$^+$ T cell population is sustained for the life-time of the host without the presence of antigen (Fig. 1-1) [8, 22, 23]. Memory CD8$^+$ T cells can be further subdivided into a heterogeneous mix of CD8$^+$ T effector memory cells (Tem), which reside in peripheral tissue and are highly cytolytic, and CD8$^+$ T central memory cells (Tcm), which preferentially home to lymph nodes and are highly proliferative [23, 24]. Tcm can be identified by their high expression of the homing receptors CC-chemokine receptor-7 (CCR7), CD44 and CD62L, while Tem are CD44^{hi} and CD62L^{lo} [24, 25]. While the sequence of events in the CD8$^+$ T cell lifecycle have been well characterized, the combination of antigenic, co-stimulatory, and cytokine signals required for the optimal generation of CD8$^+$ T cell effectors, has not been well defined. Furthermore it is currently unknown if these signalling events can be adapted to enhance either CD8$^+$ T cell effector generation or memory development.

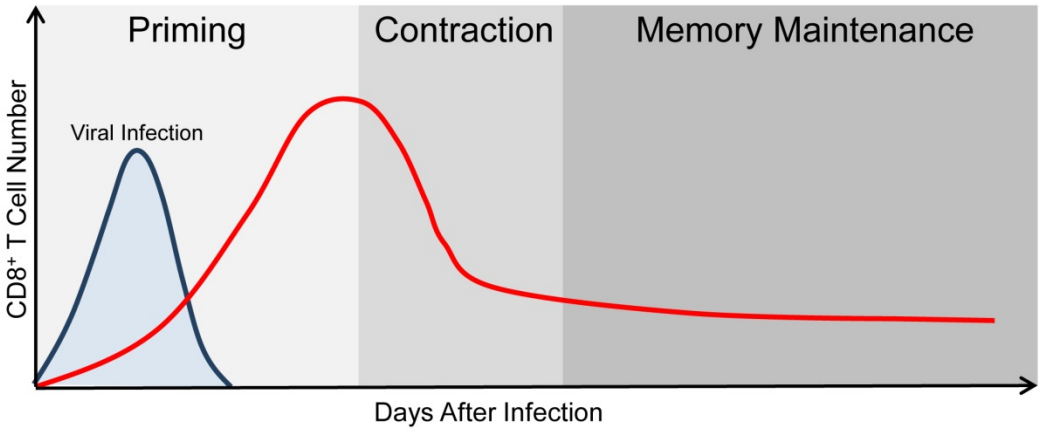


Figure 1-1. Overview of the CD8$^+$ T cell lifecycle following acute infection. Naïve CD8$^+$ T cells undergo a well described series of events following acute infection. In phase I, naïve CD8$^+$ T cells are primed by foreign peptide bearing APCs, which induces T cell clonal expansion, expression of effector molecules, and cytolytic function. Following infection resolution, CD8$^+$ T cells undergo cellular contraction (phase II), in which the clonally expanded T cell population is reduced in size by 90-95% via extrinsic and intrinsic apoptosis inducing mechanisms. During phase III, memory maintenance, cells that survive contraction differentiate into long-lived memory CD8$^+$ T cells, and are maintained by the cytokines IL-15 and IL-7. Memory CD8$^+$ T cells provide enhanced protection following re-infection, and can be activated with more rapid kinetics than their naïve precursors.

1.4 $CD8^+$ T cells mechanism of action

Once activated by antigen, naïve $CD8^+$ T cells undergo extensive clonal expansion, which increases the size of the antigen-specific T cell population from ~ 40 – 200 cells prior to activation, by ~10^4 – 10^5 fold after antigen recognition [8, 26, 27]. In addition, activated naïve $CD8^+$ T cells lose their expression of the lymph node homing molecules CD62L and CCR7 and increase their expression of CD44, allowing them to migrate to the site of infection in peripheral tissues [25]. During clonal expansion, $CD8^+$ T cells express effector molecules that are critical to their function. $CD8^+$ T cell cytotoxicity is mediated by their expression of the pore-forming protein perforin, serine proteinases called granzymes, and the death inducing ligands, Fas ligand (FasL) and Tumor necrosis factor (TNF) [10, 28]. The expression of these effector molecules requires only one cellular division and is maintained for approximately 6 divisions in *in vitro* culture [29]. In mice, activated cytotoxic T lymphocytes (CTL) store perforin and the serine proteinase granzyme B in cytolytic vesicles that orient toward the CTL:target cell interface when the TCR is engaged [30]. FasL, on the other hand, is stored in a distinct and yet unidentified vesicle that traffics to the CTL cell-surface to initiate death receptor signalling in cell targets, following binding to its receptor Fas [31]. Fas ligation induces target cell apoptosis through downstream caspase activation [28]. Perforin induces target cell damage by binding and forming pores in the target cell membrane in a Ca^{2+}-dependent manner [32]. Perforin also produces holes in the granzyme B-bearing endosomes in the target cell following endocytosis of CTL cytolytic molecules. This action expels granzyme B into the target cells

cytoplasm [33]. Granzyme B induces target cell apoptosis by directly cleaving caspase-8 and caspase-3, and by activating pro-apoptotic molecules such as Bid, which ultimately results in cytochrome-c release from the mitochondria [28].

As a final effector mechanism, $CD8^+$ T cells are potent producers of the pro-inflammatory cytokines TNF-α and IFN-γ [10, 23]. IFN-γ and TNFα release by these cells enhances cell-mediated responses by creating a pro-inflammatory environment that skews $CD4^+$ T cell development towards a Th1 phenotype, and augments the classical activation of macrophages [12, 34]. Additionally, IFN-γ release greatly enhances antigen processing by promoting the formation of the immunoproteasome, which is highly efficient at processing peptides for presentation on class I MHC [35]. Hence, the expression of $CD8^+$ T cell effector molecules is critical for mediating $CD8^+$ T cell activity in response to infection. However, it is not known if certain signals provided to naïve $CD8^+$ T cell during their activation can influence the production of specific effector molecules, or can be used to generate $CD8^+$ T cell populations with distinct effector phenotypes.

2. Overview of naïve $CD8^+$ T cell activation

Prior to their activation, naïve $CD8^+$ T cells circulate primarily between the blood and lymphoid tissues, including the spleen and lymph nodes. Homing to these sites is mediated by the expression of CD62L and CCR7, which allow naïve T cells to transverse the high endothelial venules of lymph nodes [25, 36]. Once they have trafficked to lymphoid tissue, naïve $CD8^+$ T cells await activation from peptide-bearing antigen presenting cells (APCs) that acquire antigen following infection. Optimal activation of naïve $CD8^+$ T cells by APCs is

believed to require three distinct signals: 1) Recognition of antigenic peptide in the context of class I MHC by the T cells TCR, 2) Ligation of surface co-stimulatory molecules, and 3) The presence of pro-inflammatory cytokines such as IL-12 and type-I IFN [37]. These signals are described in further detail below.

2.1 DC antigen presentation and priming of naïve $CD8^+$ T cells

The activation of naïve $CD8^+$ T cells begins with the recognition of antigenic peptide by their TCR. In most cases, foreign antigen is acquired, processed and presented to $CD8^+$ T cells by DCs, which are a specialized type of (APC). The DC population is quite heterogeneous, with specific DC subsets homing to distinct locations and possessing slightly different antigen presentation capacities [38, 39]. One of these subsets, termed CD8α DCs, are highly specialized in the processing of antigen for class I MHC, which is presented to $CD8^+$ T cells in lymphoid tissue [38, 40, 41]. In general, foreign antigen is recognized and captured by DCs through the expression of PRRs, namely toll-like receptors (TLRs) and C-type lectins [2, 42, 43]. Ligation of these PRRs initiates signalling pathways within DCs that leads to their activation. Activated DCs have enhanced antigen processing capacity, and secrete large amounts of pro-inflammatory cytokines such as IL-12 [44-46]. Activated DCs also express T cell co-stimulator ligands whose expression are often induced following ligation of DC surface CD40L molecules [38, 47]. In non-infected somatic cells, peptides for class I MHC are derived from newly synthesized endogenous proteins which are degraded by the proteasome. These peptides are then transported and loaded onto class I MHC molecules in the endoplasmic reticulum (ER) by way of the

transporters associated with antigen presentation (TAP) [48]. The mechanism of class I MHC peptide loading by uninfected CD8α DCs however, is still widely debated. Termed antigen cross-presentation, protein captured by DCs is believed to escape the phagosome and enter the cytoplasm, where it is processed and loaded onto MHC by conventional mechanisms. Alternatively, some evidence suggests that components of the ER, including peptide loading machinery, can also associate with phagosomes for antigen processing [48, 49]. Finally, antigenic peptide may also be transferred from class I MHC-bearing peripheral DCs to lymph node CD8α DCs as a means of antigen acquisition [40].

Experimentally, it has been determined that naïve $CD8^+$ T cells require only a brief encounter (2-8hrs) with antigen-bearing APCs both *in vitro* and *in vivo* to undergo clonal expansion and develop cytolytic function. Indeed, naïve $CD8^+$ T cells primed by DCs for only one hour exhibit a similar capacity for IFN-γ production and display comparable secondary responses as those primed for 48 hours [50, 51]. Optimal $CD8^+$ T cell activation, however, requires naïve $CD8^+$ T cell to interact with DCs for longer periods, as the absolute number of T cells at the peak of clonal expansion is markedly lower after only a brief encounter [51]. Multiphoton intravital microscopy, which can be used to visualize DC and T cell interactions in lymph nodes, has determined that the typical presentation of antigen to $CD8^+$ T cells *in vivo* takes ~44 hours and occurs via a three-stage process [52, 53]. In the first phase, $CD8^+$ T cells make brief transient contacts with DCs whereby they comb the DCs surface for the presence antigen. These primary interactions induce the expression of $CD8^+$ T cell activation markers

CD69 and CD44, and set a threshold for naïve $CD8^+$ T cell activation. Following this initial interaction, activated T cells form stable long-term contacts with DCs that last for approximately 5-12 hours (phase II). During this time, $CD8^+$ T cells become further committed to activation and express IL-2 and IFN-γ. The transition from phase I to phase II is dependent on both peptide quality and concentration, with higher doses of peptide decreasing transition time and results in more rapid activation [54]. Finally, during phase III, T cell:DC contacts again become transient, and the T cells proliferate. Of note, peptide quality is extremely important in setting the requirements for T cell activation as peptides recognized by the TCR with high affinity may override the requirement for T cell co-stimulation [55-57]. Furthermore, the presentation of low affinity antigen by DCs may reduce the magnitude of the $CD8^+$ T cell response, despite complete differentiation and memory generation [58]. Thus, the recognition of antigen, and the interactions between $CD8^+$ T cells and DCs are critical for $CD8^+$ T cell activation, and for shaping the T cell response to infection.

2.2 T cell Co-stimulation:

Co-stimulation is an absolute requirement for optimal $CD8^+$ T cell activation. First proposed by Lafferty and Cunningham in 1975 [59] as an extension of the two-signal requirement for T cell activation proposed by Bretscher and Cohn [60], T cell co-stimulation is defined by the engagement of T cell surface receptors, distinct from the TCR, which provide a "second" supporting signal for TCR mediated signalling. The current model of co-stimulation favours a signal strength model in which co-stimulator ligation

augments signals generated by the TCR, as well as triggering unique signalling cascades [56]. Co-stimulation is critical when antigen dose is low or peptide recognition by the TCR is sub-optimal. T cell co-stimulator molecules can be divided into two major co-stimulatory families, the CD28:B7 family and the Tumor necrosis family (TNFR/TNF) (Table 1-1, 1-2). Individual co-stimulatory molecules and their ligands often differ in the timing of their expression, impacting distinct periods of the T cell lifecycle [61, 62]. Of the T cell properties modulated by co-stimulation, proliferation, survival, effector molecule production and memory generation are often significantly affected. Thus, co-stimulatory molecules have become a key target of adjuvant and vaccination development. The contributions of individual co-stimulatory molecules, or their combinations, in generating optimal primary and secondary $CD8^+$ T cell responses following viral infection however, remain ill-defined.

2.2.1 CD28/B7 Superfamily

The CD28/B7 family of co-stimulator molecules consists of both activating and inhibitory members that can positively and negatively modulate T cell responses, respectively [61]. All members of the CD28/B7 co-stimulator family are immunoglobulin (Ig) proteins and consist of an extracellular IgV (variable) domain and a short intracellular tail that mediates signalling. Ligands for CD28/B7 co-stimulatory receptors are also Ig-family members, but have both IgV and IgC (conserved) extracellular domains [63]. Activating members of the

Table 1-1. CD28/B7 Superfamily

Co-stimulatory molecule	Ligand	Pattern of Expression	Known T cell Activity	Reference
CD28	B7.1/B7.2 (CD80/CD86)	Naïve T cell, increases with activation	IL-2, Proliferation, Effector molecules, Survival	[61, 64],[65],[66],
ICOS (CD278)	ICOS-L	Activated T cells	Cytokine production, $CD4^+$ T cell differentiation, Proliferation, Isotype switching	[67],[68],[69]
CTLA-4 (CD152)	B7.1/B7.2 (CD80/CD86)	Activated T cells	Inhibitory, Decreased IL-2, Inhibits TCR signalling	[61, 70],[71],
PD-1 (CD279)	PD-L1/PD-L2 (CD274/CD273)	Activated T cells	Inhibitory, T cell exhaustion, Inhibits proliferation, cytokine production	[61],[67],[72]
BTLA (CD272)	HVEM	Activated T cells	Inhibitory, Inhibits TCR signalling, decreased proliferation, cytokines	[67],[73],[74]

Table 1-2. TNF/TNFR Co-stimulator Superfamily

Co-stimulatory molecule	Ligand	Pattern of Expression	Known T cell Activity	References
CD27	CD70	Naïve and activated T cells	Survival, Effector function	[75],[76],[77]
4-1BB (CD137)	4-1BBL (CD137L)	Activated T cell	Survival, Proliferation, Effector function, Memory maintenance	[77, 78],[79], [80],
OX-40 (CD134)	OX-40L (CD252)	Activated T cells	Survival, Cytokine production, Proliferation, Effector function	[77, 81],[82],
HVEM (CD270)	LIGHT, GpD, LTα	Naïve T cells	Proliferation, Cytokines, Effector function	[73],[83],[84]
CD30	CD30L (CD153)	Activated T cells	Proliferation, Cytokine production	[62, 77],
GITR (CD357)	GITRL	Naïve T cell, increases with activation	Proliferation, Cytokine production,	[77, 85],

CD28/B7 family include CD28 which pairs with the B7.1 and B7.2 ligands, and ICOS, which ligates ICOS-L (B7-H2). Inhibitory members include CTLA-4 which also pairs with B7-1 and B7-2, PD-1 which ligates PD-L1 and PD-L2, and BTLA, which interacts with the TNFR family member HVEM. Ligation of these molecules on $CD8^+$ T cells functions to inhibit and downregulate T cell responses, thus helping to maintain peripheral tolerance. Additionally, B7-H3 and B7-H4 are two newly identified CD28/B7 family member ligands. B7-H3 has been demonstrated to bind the receptor Triggering receptor expressed on myeloid cells (TREM)-like Transcript 2 (TLT-2) on $CD8^+$ T cells, inducing T cell proliferation and cytokine production [86]. Conversely, the ligand for B7-H4 on T cells is currently unknown; however it is believed to have an inhibitory effect on T cell activation [87, 88]. As they pertain specifically to this thesis, CD28, CTLA-4 and PD-1 co-stimulatory molecules will be described in more detail. Due to time constraints, the role of ICOS, BTLA, B7-H3 and B7-H4 in $CD8^+$ T cell function was not investigated, but are well reviewed elsewhere [66].

2.2.1.1a CD28 and CTLA4-structure, signalling and function

CD28 and CTLA-4 are the best characterized members of the CD28/B7 superfamily and play key roles in T cell activation (CD28) and tolerance (CTLA-4) [70]. Ligation of CD28 has been demonstrated to enhance T cell activation by increasing T cell proliferation and IL-2 production, and can also prevent apoptosis [57]. CTLA-4 ligation on the other hand is often associated with the termination of T cell responses and the inhibition of T cell IL-2 production [61]. The CD28 and CTLA-4 genes are located in close proximity on chromosome 2 in mice and

share common structural elements, suggesting a gene duplication event [89, 90]. Both type I transmembrane proteins, CD28 and CTLA-4 have short cytoplasmic tails containing a tyrosine signalling motif. Similarly, both of these molecules contain a conserved cysteine residue in their stalk region for covalent homodimerization [63]. CD28 and CTLA-4 both recognize the ligands B7.1 (CD80) and B7.2 (CD86), which are exclusively expressed by activated APCs. CTLA-4, however, interacts with these receptors with an approximately 10-100 fold higher affinity than CD28 [91]. This difference in affinity is driven by differences in B7.1/2 ligand binding as CTLA-4 adopts a more open conformation than CD28 allowing for a bivalent interaction. CD28, on the other hand, can only interact with B7.1 and B7.2 in a monomeric fashion due to differences in protein orientation and steric hindrance [63, 90].

CD28 and CTLA-4 also differ in the timing of their expression. CD28 is expressed at low levels on naïve T cells and is rapidly upregulated following T cell activation [71]. Thus, CD28 plays a dominant role in the T cell activation process. CTLA-4 is expressed following TCR and CD28 ligation, thereby mediating inhibitory responses in previously activated T cells [71]. CD28 signalling is triggered upon the tyrosine phosphorylation of the YMNM motif found in its cytoplasmic tail; this leads to the recruitment of the kinase PI3K and the adaptor protein Grb2 [71, 92, 93]. The recruitment and activation of PI3K triggers the activation of PKB/Akt [71, 94, 95], which, in cooperation with TCR mediated signalling, enhances T cell proliferation, survival, and cellular metabolism [95]. Grb2 recruitment is necessary for CD28-induced IL-2

production [71, 92, 96]. Additionally, CD28 ligation has also been demonstrated to induce NF-κB activation through the CARMA1-Bcl-10 signalling pathway [71, 97, 98].

CTLA-4 ligation induces an inhibitory effect on activated T cells despite the lack of an immunoreceptor tyrosine-based inhibitory motif (ITIM) in its cytoplasmic tail [71]. This inhibitory function is crucial for the control and termination of T cell responses as evidenced by the severe immunopathology observed from CTLA-4 deficient mice. CTLA-4 has been hypothesized to mediate negative effects on T cell activation and TCR/CD28 signalling in a number of ways. Because of its high affinity binding to B7.1/B7.2, CTLA-4 may sequester binding of these shared ligands to prevent CD28 ligation [89]. Furthermore, CTLA-4 has been proposed to disrupt lipid raft TCR/CD28 signalling microclusters, reducing T cell signalling following TCR ligation [99, 100]. Finally, despite the lack of an inhibitory ITIM motif, CTLA-4 has been demonstrated to recruit the phosphatases SHP-2 and PP2A which promote inhibitory signalling [71, 101, 102]. Ultimately, the ligation of CTLA-4 leads to decreased IL-2 production and inhibition of T cell signalling which terminates T cell responses following activation [61].

2.2.1.1b CD28 co-stimulation in vivo

The requirement for CD28 co-stimulation in response to viral infection and memory generation is controversial. The use of the Lymphocytic choriomeningitis virus (LCMV) infection model, which induces very robust immune responses, has led to conflicting results [56, 57]. In other infection

models, including VSV (Vesicular stomatitis virus) and influenza, CD28 ligation increases the size of the effector T cell population, suggesting that CD28 co-stimulation is vital for the immune response against certain pathogens [103] [68] [56]. Thus, the requirement for CD28-mediated co-stimulation in response to primary infection likely depends on the infection model being used [55-57]. CD28 is also necessary for secondary responses following HSV-1 (Herpes simplex virus-1) and influenza infection as memory $CD8^+$ T cells exhibit less expansion and cytolytic capacity in B7-deficient animals [104]. Additionally, the absence of CD28 co-stimulatory signalling by memory $CD8^+$ T cell reduces the expression of the anti-apoptotic protein Bcl-xL and decreases cellular expansion [104-106]. Therefore, CD28 ligation is vital not only for initiating primary $CD8^+$ T cell responses following viral infection, but also in memory cell reactivation upon re-infection.

2.2.1.2 PD-1 structure, signalling, and function

PD-1 is a prominent inhibitory member of the CD28/B7 superfamily, and is structurally distinct from other CD28/B7 family members [63]. It is comprised of an external IgV variable domain and does not dimerize due to the lack of a cysteine residue in its stalk region. Furthermore, PD-1 contains an ITIM motif in its cytoplasmic tail which mediates negative signalling through the recruitment of the phosphatase SHP-2. SHP-2 dephosphorylates TCR-mediated signalling molecules leading to the inhibition of T cell responses [61] [63].

PD-1 is only detected on the surface of T cells following their activation [61] [67], indicating a role for PD-1 in the inhibition of T cell responses. The

ligands for PD-1, PD-L1 and PD-L2, are also inducible and exhibit distinct patterns of expression. PD-L2 is mostly found on activated APCs, B cells and T cells, while PD-L1 is preferentially expressed on cells of non-hematopoietic origin including endothelial cells [67]. Interestingly, the ligation of PD-1 has been demonstrated to directly inhibit anti-apoptotic Bcl-xL expression by T cells, thereby augmenting T cell contraction mechanisms [107]. Indeed, mice lacking PD-1 exhibit splenomegaly and increased occurrence of autoimmune disease, due to the requirement of PD-1 for the inhibition of T cell proliferation and cytokine production [67, 108, 109]. These findings indicate that PD-1 also plays a role in maintaining T cell tolerance [110]. *In vivo*, PD-1 ligation often negatively regulates $CD8^+$ T cell antiviral/antitumor activity as blockade of PD-1/PD-L1/2 binding enhances $CD8^+$ T responses to viral infection, vaccination, and tumours [111-113]. Sustained PD-1 expression is also associated with the development of T cell exhaustion during chronic infection [72]. Defined by $CD8^+$ T cell dysfunction and inability to maintain cytolytic activity [114], T cell exhaustion can be broken by inhibiting PD-1 ligand binding [72]. Thus PD-1 is a good immunotherapy target for the treatment of chronic infections such as HIV and cancer.

2.2.2. TNFR/TNF Co-stimulator Family

The TNF/TNFR family is composed of 19 TNF ligands and 29 TNF receptors, many of which play key roles in modulating immune function [63]. Ligands of the TNF family are all type II transmembrane proteins containing TNF homology domains critical for the non-covalent trimerization of these ligands.

TNF receptors are type I transmembrane proteins with cysteine-rich repeats in their extracellular domain responsible for disulphide bridge formation. TNF receptors likely exist as both monomers and oligomers, but trimerize when interacting with the appropriate ligand [63] [62]. Because these receptors lack intracellular signalling motifs, the trimerization of TNF receptors is required to promote the recruitment of signalling adaptor proteins [63]. Of the TNF/TNFR family members, several have co-stimulatory function for T cells and these receptors and their ligands include: 4-1BB (CD137):4-1BBL, OX-40 (CD134):OX-40L, CD27:CD70, HVEM:LIGHT, CD30:CD30L, and GITR:GITRL. In general, co-stimulatory members of the TNF/TNFR family signal through TRAF (TNFR-associated factor) signalling adaptor proteins [62, 115], and are important in increasing the overall number of T cell effectors following activation [77] [116]. This is achieved by promoting the proliferation and survival of activated T cells, and by increasing the expression of anti-apoptotic proteins and cell cycle factors [77] [62, 116]. Not only does TNFR co-stimulatory signalling augment the accumulation of T cell effectors during primary responses, but also the increases the survival of T cells following their activation, therefore contributing to an increase in the size of the memory T cell pool. Of the co-stimulatory TNF/TNFR family members, CD27, 4-1BB and HVEM will be discussed in detail below, as they relate specifically to the content of this thesis. OX-40, CD30, and GITR, which were not investigated due to time constraints, are well reviewed in Croft, Nature Reviews Immunology, 2003 and Watts, Annual Review of Immunology, 2005 [62, 77].

2.2.2.1 CD27 expression and function

The TNFR co-stimulator family member CD27 is expressed at low to intermediate levels on the surface of naïve $CD8^+$ T cells while its ligand, CD70, is induced on the surface of macrophages, DCs and B cells following their activation [77, 117, 118]. CD27-mediated signalling is initiated by the recruitment of the signalling adaptors TRAF2, 3, and 5 [77, 115, 119], and ultimately leads to the activation of the JNK pathway and NF-κB when provided in combination with TCR-mediated signals [117, 120]. Like CD28, the expression of CD27 on T cells prior to their activation indicates that CD27 likely plays a role in dictating early $CD8^+$ T cell responses. Indeed, the expression of CD27 peaks following 24 hours of T cell stimulation and has been reported to decrease on robust effector populations [77, 120, 121]. Ligation of CD27 supports $CD8^+$ T cell survival, accumulation, and cytotoxicity both *in vitro*, and *in vivo* following challenge with peptide antigen and viral infection. This is accomplished, in part by increasing the expression of Bcl-xL [75, 76, 122, 123]. Importantly, CD27 can also support $CD8^+$ T cell responses in animal's deficient in CD28 signalling, and in the absence of IL-2 [75, 124]. Moreover, mice deficient in CD27 exhibit reduced T cell accumulation in non-lymphoid tissues following influenza infection due to reduced IL-2 production, indicating a role for CD27 in the production of this cytokine [125]. With regard to T cell memory, CD27 is highly expressed by central memory T cells, suggesting the potential for CD27 to influence memory T cell reactivation [126]. In addition, mice previously infected with influenza or immunized with antigenic peptide and soluble CD70 display enhanced secondary

responses indicating that CD27 is important in memory T cell generation [76, 122]. CD27 co-stimulation is believed to influence memory T cell generation by enhancing the survival of T cell effectors, leading to a larger memory T cell pool [76, 77].

2.2.2.2 4-1BB expression and function

The TNFR co-stimulator family member 4-1BB has emerged as an important factor for dictating the generation and homeostasis of memory $CD8^+$ T cells. Expressed at high levels following 48 hours of T cell stimulation [77], 4-1BB co-stimulator signalling primarily influences T cell survival, and functions at later stages following naïve $CD8^+$ T cell activation. Indeed, mice lacking 4-1BB are unimpaired in their initial T cell responses, but display weakened function at later time points following infection [62, 127, 128]. Like other members of the TNF family, the ligand for 4-1BB, 4-1BBL, is expressed on APCs following their activation. Ligation of 4-1BB by 4-1BBL induces the recruitment of signalling adaptors TRAF 1, 2, and 3 [62, 129], which augments the activation of the JNK/p38 signalling pathway and NF-κB, in cooperation with the TCR [62, 129-131]. In addition, 4-1BB has been demonstrated to influence cell cycle progression and increase the expression of the anti-apoptotic protein Bcl-xL, while inhibiting the expression of the pro-apoptotic protein Bim [79, 132]. Thus, ligation of 4-1BB at later time points following T cell activation appears to increase the magnitude of the primary response by maintaining T cell numbers. Furthermore, 4-1BB co-stimulation also enhances $CD8^+$ T cell effector responses by increasing their IFN-γ production and cytotoxicity [80, 127]. As mentioned

above, 4-1BB has a unique role in $CD8^+$ T cell memory establishment and survival. 4-1BB deficient animals have reduced memory T cell survival following influenza infection [78], and decreased numbers of peptide-specific, IFN- γ (+) $CD8^+$ T following memory cell reactivation, in comparison to wild type animals [127]. Additionally, memory $CD8^+$ T cell cytotoxicity and secondary expansion are reduced following primary infection with Murine Gamma Herpes Virus-68 in 4-1BB-deficient mice [133]. Interestingly, the role of 4-1BB in memory cell survival appears to be associated with IL-15. IL-15 has been demonstrated to selectively enhance 4-1BB expression on $CD8^+$ T cells which in turn, provides a survival signal for memory $CD8^+$ T cells [78]. Thus, the ability of 4-1BB to sustain T cell numbers and survival at later times following primary infection, and during memory cell maintenance, makes 4-1BB co-stimulation crucial for the development of $CD8^+$ T cell effector and memory cell populations.

2.2.2.3 HVEM expression and function

The TNFR co-stimulator family member HVEM (Herpes virus entry mediator), is unique in that it provides an activating signal to T cells when bound to its TNF ligand LIGHT, and also function as a ligand for the CD28/B7 co-inhibitory family member BTLA [73]. HVEM also interacts with several other proteins including CD160, lymphotoxin α, and the herpes simplex virus glycoprotein D (GpD) [73]. The unique ligand-binding properties of HVEM are mediated by its external domain which is comprised of four cysteine-rich domains (CRD1-4) that interact preferentially with specific HVEM ligands [73, 134]. Like

other TNFR co-stimulator family members, the interaction between HVEM and its activating TNF ligand LIGHT, induces downstream signalling initiated by the recruitment of TRAF signalling adaptors, specifically TRAF1, 2, 3 and 5 [135]. This in turn ultimately induces the activation of NF-κB and JNK/AP-1 signalling [83, 135], influencing T cell proliferation, cytokine production and survival [62, 73]. Expressed at moderate to high levels on naïve $CD8^+$ T cells and lost with T cell activation, HVEM co-stimulatory signalling is believed to modulate the early activation of naïve $CD8^+$ T cells [62, 136]. Indeed, stimulation with the HVEM ligand LIGHT increases T cell proliferation and cytokine production [73, 83, 137], while mice lacking LIGHT exhibit decreased cytotoxicity and cytokine production in response to antigenic challenge [84]. Thus, further investigation into how HVEM co-stimulation influences $CD8^+$ T cell activation and effector phenotype differentiation is much warranted.

2.2.3. Integrins and T cell activation

In addition to co-stimulator molecule signalling, naïve $CD8^+$ T cell responses are greatly influenced by integrins and their downstream signalling. *In vivo*, integrins are responsible for the tethering and extravasation of T cells to and from blood vessels, and for establishing the T cell:APC contacts required for peptide recognition and efficient TCR signalling [138]. The integrin family member lymphocyte function-association antigen-1 (LFA-1) is particularly crucial for regulating these actions in T cells. Composed of α and β heterodimeric subunits, LFA-1 interacts with its binding partner ICAM-1 (intercellular adhesion molecule-1) to regulate T cell adhesion and signalling. LFA-1:ICAM-1

interactions are responsible for stabilizing naïve T cell and APC contacts, and for the formation of the immune synapse in which T cell signalling components are clustered in an organized fashion between the T cell:APC interface [139]. Called a supramolecular activation cluster (SMAC), the clustering of immune receptors in the SMAC is mediated by thousands of LFA-1 and ICAM-1 molecular interactions which form an outer adhesive ring [138]. The high affinity interaction between LFA-1 to ICAM-1 required for stable SMAC development is a result of TCR "inside-out" signalling, that results in a conformation change in LFA-1 that greatly increases the affinity of this interaction ($_{KD}$ 1mM – $_{KD}$ 100nM) [138, 139].

LFA-1 ligation can also provide T cell activating signals, however these have been difficult to uncouple from its adhesive effects which enhance TCR-mediated signalling. More specifically, LFA-1 mediated signalling has been demonstrated to influence PI3K and AP-1 activation, and the activity of the MAPK pathway by modifying the activity of Ras at the plasma membrane in cooperation with the TCR [138, 140-142]. In addition, LFA-1 signalling also increases IL-2 production by stabilizing IL-2 mRNA [138, 143, 144]. Notably, the LFA-1/ICAM-1 interaction is critical for memory $CD8^+$ T cell generation. ICAM-1-deficient mice exhibit a significantly reduced ability to form long-lasting contacts with DCs, both *in vitro* and *in vivo*, which greatly reduces the formation of $CD8^+$ T cell memory [145]. Thus, both the adhesive and potential signalling roles of LFA-1 appear to be vital for the induction of optimal effector and

memory T cell responses. However, the mechanism by which LFA-1 does so in cooperation with other $CD8^+$ T cell stimulator molecules is not known.

2.3 Pro-inflammatory cytokines

Evidence suggests that pro-inflammatory cytokines, secreted by DCs and other APCs following their activation, are required for the *optimal* activation of naïve $CD8^+$ T cells. Referred to as "signal 3", the addition of the pro-inflammatory cytokines IL-12, or type I IFNs (IFNα or IFNβ), to naïve $CD8^+$ T cells during their activation *in vitro*, enhances their proliferation and expression of cytolytic molecules such as granzyme B [37]. Moreover, naïve $CD8^+$ T cells stimulated *in vitro* without IL-12 have been found to be unable to lyse target cells in killing assays, despite being able to degranulate [146]. A similar phenomenon also occurs *in vivo* following the adoptive transfer of OT-I TCR-transgenic naïve $CD8^+$ T cells into mice which are later challenged with OVA peptide. Concurrent treatment of mice with IL-12 enhances $CD8^+$ T cell proliferation and cytolytic capacity to a similar degree as LPS, suggesting that IL-12 has a similar effect on developing CTL function *in vivo* as adjuvant [147]. Thus, pro-inflammatory cytokines appear to be important for improving the magnitude $CD8^+$ T cell response required for the control of infection.

Recent work suggests that pro-inflammatory cytokines also influence $CD8^+$ T cell responses on a transcriptional level, impacting their effector and memory development. Specifically, the presence of inflammatory stimuli, including IL-12, increases $CD8^+$ T cell expression of the effector-associated transcription factor T-bet, leading to preferential generation of short-lived effector

cells (SLECs), rather than memory precursor effector cells (MPECs) [148, 149]. The concurrent addition of IL-12 to antigen primed, CD28-co-stimulated $CD8^+$ T cells, has been demonstrated to regulate the expression of approximately 355 genes involved in T cell effector function and differentiation including T-bet, Eomes, CD25, granzyme B and IFN-γ [150]. While the expression of many of these genes is induced at early time points following TCR ligation and CD28 co-stimulation alone, their prolonged expression requires pro-inflammatory cytokine addition which can enhance chromatin accessibility at key gene loci by inhibiting the action of histone deacetylases [150]. However, full effector development can be induced by the addition of IL-2 alone to $CD8^+$ T cells during their activation, suggesting that pro-inflammatory cytokines may not be absolutely required for effector generation under some conditions [66]. Instead, pro-inflammatory cytokines may be more important in promoting the development of $CD8^+$ T cell effectors with distinct phenotypes. Thus the prerequisite for pro-inflammatory cytokines during T cell activation and differentiation may be stimuli specific and requires further investigation.

3. $CD8^+$ T cell contraction and memory generation

$CD8^+$ T cell clonal expansion in response to antigen is followed by T cell contraction, otherwise known as programmed cell death. The process of contraction reduces the number of the expanded $CD8^+$ T cells by 90-95%. This maintains cellular homeostasis, and also selects for a subset of memory $CD8^+$ T cells responsible for providing long-lived protection against the infecting pathogen. The process of T cell contraction is driven by both extrinsic death

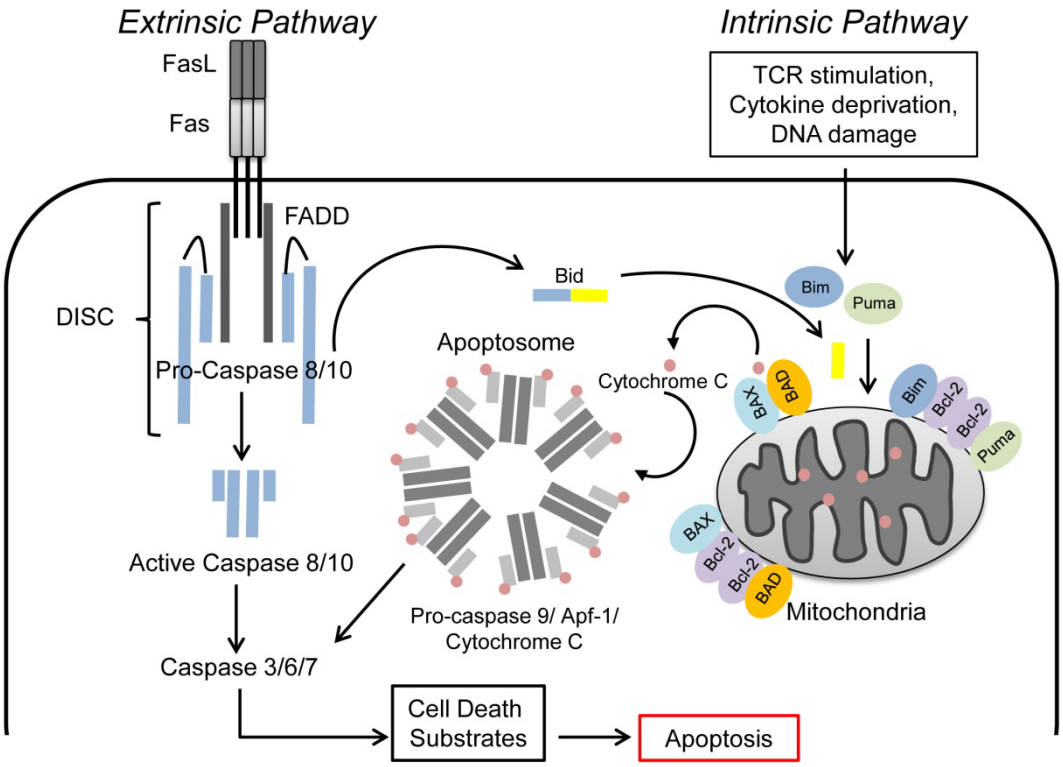


Figure 1-2. Extrinsic and intrinsic pathways of T cell apoptosis. T cell contraction is driven by two apoptosis inducing pathways: the extrinsic pathway, and the intrinsic pathway. The extrinsic pathway is initiated by death receptor engagement (ie. Fas:FasL), which leads to the formation of the DISC complex at the cell membrane. The DISC complex is comprised of the cytoplasmic tail of Fas, the adaptor protein FADD, and the pro-caspase 8 or 10. DISC complex formation leads to caspase 8/10 activation which in turn activates the executioner caspases 3/6/7, which cleave cell death substrates leading to apoptosis. Active caspase 8 can also cleave the pro-apoptotic protein Bid, augmenting the intrinsic pathway of apoptosis. The intrinsic pathway of cell death in T cells is triggered by excessive TCR stimulation and cytokine deprivation, leading to the enhanced expression of the pro-apoptotic proteins Bim and PUMA. Bim and PUMA interact with the anti-apoptotic proteins Bcl-2 and Bcl-xL (not depicted), preventing their inhibition of BAX and BAK mediated mitochondrial pore formation. Pore formation by BAX and BAK leads to cytochrome-c release from the mitochondria. Cytochrome-c interacts with pro-caspase 9, and APAF-1 to induce apoptosome formation. Apoptosome complex formation actives caspase 9, which then activates the executioner caspases 3/6/7. DISC, death-inducing signalling complex, FADD, Fas-associated death domain, Bid, BH3-interacting-domain death agonist, Bim, BCL-2-interacting mediator or cell death, PUMA, p53-upregulated modulator of apoptosis, Bcl-2, B cell lymphoma-2, Bcl-xL B cell lymphoma extra-long, BAX, BCL-2-assocaiated protein X, BAK, BCL-2 antagonist/killer, APAF-1, apoptotic-protease-activating factor 1.

receptor ligation (ie. Fas, TRAIL), as well as T cell intrinsic mechanisms involving pro- and anti- apoptotic proteins situated in the mitochondrial membrane (Fig. 1-2) [151, 152]. Details of these mechanisms can be found in the review "Life and Death of Peripheral T cells" by Krammer (2007) [151], and will be discussed further in Chapter 4. The population of $CD8^+$ T cells that survives contraction is often destined to become long-lived memory $CD8^+$ T cells which can reactivate with more rapid kinetics than naïve $CD8^+$ T cells, thereby providing enhanced protection against re-infection. Importantly, this transition to memory is critically dependant on the presence of $CD4^+$ T cells as $CD8^+$ T cell secondary responses are greatly diminished in mice which lack this cell subset [153] . As mentioned previously, the memory $CD8^+$ T cell population can be further broken down into Tem and Tcm subsets, which reside in peripheral tissues or secondary lymphoid tissue, respectively.

The memory $CD8^+$ T cell compartment continually changes, and grows in size with immunological experience [154]. In general, antigen-specific $CD8^+$ T cells detected 6-8 weeks after infection are typically considered memory T cells [8]. This population can be identified by expression of distinct surface markers and cytokine receptors, which are often required for memory T cell homeostasis and function [155]. Recently, memory $CD8^+$ T cell gene expression profiles have been extensively studied as a means to both identify this specialized T cell population, and to help understand their function. Interestingly, the gene expression profile of memory T cells only differs from naïve T cells by ~5%; however, memory T cell chromatin structure and gene accessibility are often

altered [156, 157]. Memory $CD8^+$ T cells contain many gene loci considered to be in a "poised state" in which the surrounding chromatin is in an open conformation. These "poised" gene loci are believed to be responsible for the rapid responses of memory T cells in comparison to those with a naïve phenotype [156, 157]. Thus, the epigenetic basis for memory T cells is a growing avenue of research which warrants further investigation.

3.1 Models of $CD8^+$ T cell memory generation

It was originally presumed that the transition of a naïve $CD8^+$ T cell to a memory T cell was a linear process in which the memory population was selected based on both the size of originating effector T cell population, and the survival of cells in response to growth factor withdrawal at the peak of their expansion [8, 158]. While these factors may indeed contribute to the process of memory T cell differentiation, the transition of naïve $CD8^+$ T cells to memory likely is not linear and instead may involve a dynamic interaction between activating signals, cytokines, and transcription factors. Thus, several models of $CD8^+$ T cell memory generation have been developed to describe not only the transition of naïve $CD8^+$ T cells to memory, but also the generation of Tem and Tcm $CD8^+$ T cell subsets.

The models of memory differentiation have been well reviewed by Kaech and Wherry [8], and are depicted in Figure 1-3. Two important $CD8^+$ T cell populations involved in the transition to memory are short-lived effectors cells (SLECs), which are highly proliferative and express large amounts of effector molecules, and $CD8^+$ T cells with increased memory potential called memory precursor effector cells (MPECs). MPECs are not memory T cells per se, but

represent a distinct population of activated $CD8^+$ T cells with increased potential for memory generation if they receive the proper signals. Individual models vary in the plasticity of the SLEC and MPEC subsets, and the signals required for the generation of each. In brief the models for memory $CD8^+$ T cell differentiation are (Fig. 1-3):

1) *Uniform potential* – Cells have equal memory potential following their activation. Extrinsic factors (ie. cytokines) select for MPECs that differentiate from a transitional Tem state to a stable Tcm population.
2) *Decreasing potential* – T cells begin with equal potential for memory generation, but this decreases with prolonged antigen exposure. Cells which receive limited stimulation differentiate into Tcm, and cells exposed to high amounts of antigen differentiate into SLECs, then Tem.
3) *Fixed lineage* – Activated $CD8^+$ T cells immediately mature into either SLECs or mature memory cells without transitioning through an effector phase. The resultant memory cells differentiate into Tcm.
4) *Fate commitment with progressive differentiation* – T cell fate is determined by signal strength. Cells that receive high amounts of stimulation become terminally differentiated SLECs whereas cells that receive lower amounts of stimulation progressively differentiate in to MPECs, followed by Tem, and finally Tcm. The MPEC population is plastic in nature and can develop into SLECs if activating stimuli are increased.

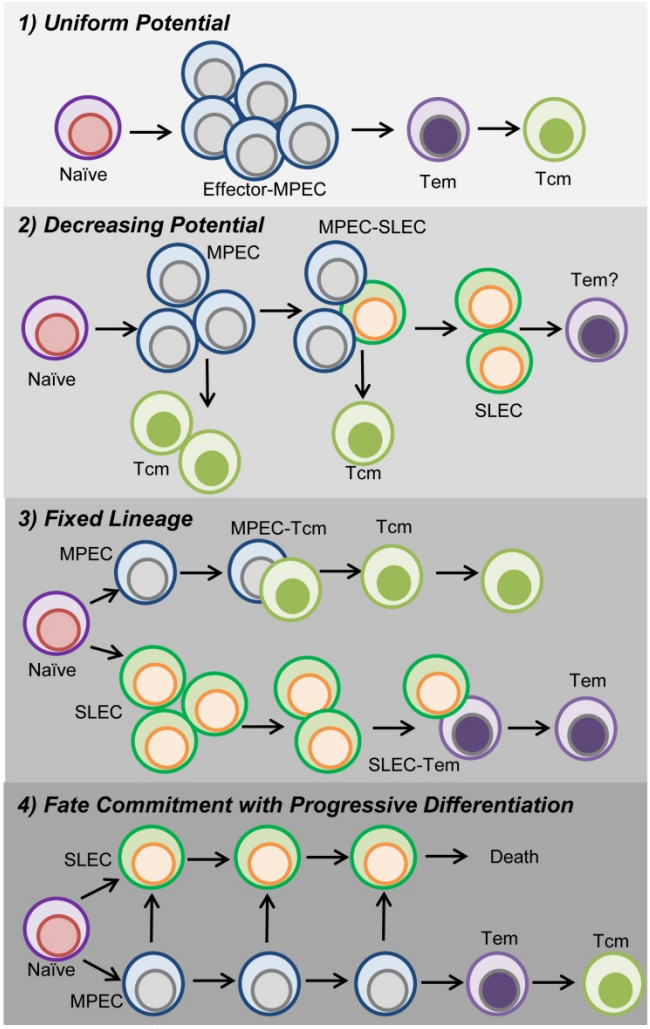


Figure 1-3. Models of CD8$^+$ T cell effector and memory differentiation following acute infection.

1. Uniform potential. All activated CD8$^+$ T cell effectors have MPEC potential. Extrinsic factors lead to T cell contraction and selects for memory CD8$^+$ T cells that differentiate into Tem, then Tcm.

2. Decreasing Potential. All activated CD8$^+$ T cell effectors have potential for MPEC development, but this decreases with continued antigen exposure. Cells which receive a short activating stimulus develop into MPECs then Tcm. Longer stimulation leads to SLEC development and end-stage Tem formation.

3. Fixed Lineage. Activated CD8$^+$ T cells immediately differentiate into either SLECs or MPECs. MPECs may by-pass the effector stage and develop into Tcm. SLEC development may lead to end-stage Tem formation.

4. Fate commitment with progressive differentiation. Differentiation into SLECs and MPECS is determined by signal strength. MPECs acquire some effector function and can differentiate into SLECs in the signal strength is maintained or increases. MPECs gradually differentiate into Tem followed by Tcm. SLEC differentiation is terminal.

Support for each of these models can be found in the literature. For example, asymmetric cell division of activated $CD8^+$ T cells, which results in mother and daughter $CD8^+$ T cells with distinct effector and memory potential [159], supports the model of fixed lineage commitment. Furthermore, increasing amounts of inflammation (ie. IL-12), decreases MPEC production and increases SLEC development [148, 149], favouring a model of fate commitment with progressive differentiation. The progressive differentiation of effector $CD8^+$ T cells to memory is also supported by genomic studies in which successive changes in gene expression gradually occur as memory T cell precursors become terminally differentiated [160]. Alternatively, these models may not be mutually exclusive as a combined model of fixed lineage commitment and fate commitment, whereby signal strength directly drives lineage choice, has been proposed [155]. Consequently, confirmation of a specific model of T cell memory differentiation will likely require the identification of precise signals that augment the transition of effector $CD8^+$ T cells to memory, which currently are not completely understood.

4. The influence of cytokines and transcription factors in $CD8^+$ T cell differentiation

The expression of certain cytokines and cytokine receptors, as well as specific transcriptional regulators, has been associated with the function and homeostasis of memory $CD8^+$ T cells. In particular, IL-2, IL-7, and IL-15 have been demonstrated to play a key role not only in the homeostasis of memory $CD8^+$ T cells, but also in the regulation of naïve and effector $CD8^+$ T cell

responses. Furthermore, specific transcription factors such as T-bet and Eomes, and the transcriptional regulators Blimp-1 and Bcl-6, have also been utilized for the identification of effector and memory $CD8^+$ T cells, based on their role in effector and memory $CD8^+$ T cell function. These effector and memory T cell specific factors are described in more detail below.

4.1 The common-γ_c family of cytokines and cytokine receptors

Cytokines play a key role in the $CD8^+$ T cell lifecycle dictating not only cell survival and proliferation, but also T cell differentiation. Therefore, the ability of T cell-activating stimuli to modulate either the production of cytokines or the expression of specific cytokine receptors is critical in dictating events in the $CD8^+$ T cell lifecycle. IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21 are all members of the common-γ_c family of cytokines and thus are named as each individual receptor contains a common γ_c signalling unit (CD132) [161]. Binding of these receptors initiates downstream through the JAK-STAT (Janus kinase- signal transducer and activator of transcription) pathway. JAK1/3 associate with the cytoplasmic tails of these cytokine receptors and propagate downstream signals through the activation of STAT proteins, influencing gene transcription and T cell function [161]. Of these family members, the cytokine IL-2, IL-7, and IL-15 have particular importance in the $CD8^+$ T cell lifecycle, controlling cell survival and fate.

4.1.1 IL-2 and the IL-2 receptor

IL-2 was the first identified member of the common γ_c cytokine family and has proven to be critical for many aspects of the $CD8^+$ T cell response. $CD4^+$

T cells are the major producers of IL-2, but IL-2 can also be produced, albeit to a lesser degree, by $CD8^+$ T cells, DCs, and NK cells [162, 163]. The IL-2 cytokine receptor is trimeric and comprised of β-chain (CD122) and γ-chain signalling subunits that bind IL-2 with low affinity, and a high-affinity α-chain (CD25) [164, 165]. CD25 is expressed at low levels on naïve $CD8^+$ T cells but is rapidly increased upon TCR engagement and the presence of IL-2 [163, 166]. The majority of IL-2 signalling is believed to occur through STAT5; however STAT1 and STAT3 have also been implicated [161, 167]. MAPK activation, as well as the activation of the PI3K/Akt pathway, also occurs downstream of the IL-2 receptor, implicating the metabolic regulator mTOR in IL-2 responses [161, 167]. While IL-2 and IL-2 receptor signalling are not absolute requirements for $CD8^+$ T cell proliferation [168], deficiencies in either IL-2 production or expression of the IL-2 receptor, leads to diminished $CD8^+$ T cell numbers and effector responses following naïve $CD8^+$ T cell activation [164, 168, 169]. Moreover, completely IL-2 deficient mice suffer from lethal autoimmune disease due to a lack of Treg development [170]. IL-2 has also been shown to be required during priming for optimal secondary $CD8^+$ T cell responses as CD25-deficient $CD8^+$ T cells exhibit poor proliferation and cytokine production following re-infection [171, 172]. Interestingly, the IL-2 required for optimal secondary T cell responses is produced by $CD8^+$ T cells themselves, as memory T cell reactivation is only diminished when $CD8^+$ T cells are IL-2-deficient [173]. IL-2 has also been found to enhance T cell effector responses and terminal effector differentiation in a dose-dependent manner [66, 174]. $CD8^+$ T cell effectors generated in the presence of high

amounts of IL-2 display enhanced expression of the effector molecules perforin and granzyme B, and exhibit better target cell lysis in *in vitro* killing assays [66]. Along with a robust effector phenotype, high amounts of IL-2 also induce the expression of the effector-associated transcriptional regulators Blimp-1 and Eomes [66, 175, 176]. Prolonged exposure to high amounts of IL-2 and sustained IL-2 signalling, however, can predispose activated $CD8^+$ T cells for apoptosis by increasing T cell expression of the death receptors TRAIL and Fas (CD95) [164, 174, 177]. Thus, IL-2 is critical not only in driving $CD8^+$ T cell differentiation, but also in generating a balance between vigorous effector responses, and the initiation of T cell contraction at the peak of antigen challenge.

4.1.2 IL-7 and the IL-7 receptor

IL-7 is produced by stromal cells found in both lymphoid and peripheral tissue [178, 179]. The receptor for IL-7 is composed of two subunits, the IL-7 receptor α-chain (CD127), and the common γ-chain. As opposed to other common-γ_c cytokine receptors, the IL-7 receptor, including the α-subunit CD127, is expressed at very high levels on naïve $CD8^+$ T cells [161, 166, 180]. This correlates with the importance of IL-7 in maintaining the survival of the naïve $CD8^+$ T cell subset. Indeed, IL-7-deficient mice exhibit decreased naive $CD8^+$ T cell numbers and homeostatic proliferation in correlation with the ability of IL-7 to induce T cell turnover [181, 182]. Like IL-2, signals from the IL-7 receptor activate the STAT proteins STAT5, STAT3 and STAT1 [161, 178]. In a similar fashion to IL-2, IL-7 receptor cytokine binding also activates the PI3K/Akt pathway, and ultimately increases the expression of the anti-apoptotic proteins

Bcl-2 and Mcl-1 [178, 180, 183, 184]. Following $CD8^+$ T cell activation, CD127 expression is lost from the cells surface and replaced with the expression of the IL-2 receptor α-chain CD25, indicating a minimal role for IL-7 in effector $CD8^+$ T cell differentiation [166, 185]. At later time points following activation, the IL-7 receptor α-chain is re-expressed on a small percentage (~10%) of $CD8^+$ T cells [185]. Further analysis of this population has revealed enhanced expression of several memory markers including Bcl-2 and CD62L, indicating a correlation between MPEC development and CD127 re-expression [148, 185]. Despite the re-expression of CD127 on memory destined $CD8^+$ T cells, IL-7 is not an absolute requirement for memory generation, but does enhance memory cell proliferation and survival [186]. Thus, IL-7 is important for both naïve and memory T cell turn-over and survival. Other cytokines however, are also critical for maintaining the memory T cell subset.

4.1.3 IL-15 and trans-presentation

Similar to IL-2, the IL-15 cytokine receptor is comprised of three subunits, the common γ-chain, a β-chain (CD122), and an IL-15-binding α-chain. Also reminiscent of the IL-2 receptor, the IL-15 receptor is associated with JAK1 and JAK3 kinases and propagates downstream signals through STAT5 [161]. Key differences in IL-15 and IL-2, however, include the expression of their respective α-chains, the source of IL-2 and IL-15 cytokines, and the $CD8^+$ T cell subsets in which these receptors are preferentially expressed. The IL-15 receptor is found at low to intermediate levels of naïve $CD8^+$ T cells, and is increased and maintained following T cell activation and memory generation [161, 166, 187]. While the

expression of the IL-15 receptor α-chain is elevated on memory $CD8^+$ T cells, its role in $CD8^+$ T cell memory generation is unclear as $CD8^+$ memory T cells can develop without IL-15 receptor α-chain expression [187]. IL-15 is produced by DCs, macrophages, and epithelial cells, which are also involved in the trans-presentation of IL-15 to T cells [188, 189]. These cells express the IL-15 receptor α-chain and can trans-present IL-15 to T cells in either a paracrine or autocrine fashion [188, 189]. In the paracrine model, soluble IL-15 produced by neighbouring cells associates with the IL-15 receptor α-chain on DCs and macrophages, which can then trans-present the IL-15 to IL-15 receptor bearing T cells. In the autocrine model, IL-15 producing cells generate pre-assembled IL-15 and IL-15 receptor α-chain complexes for trans-presentation.

The importance for IL-15 in the survival of memory T cells is evidence by the significantly reduced frequency of memory T cells in IL-15-deficient animals [190]. IL-15 enhances naïve $CD8^+$T cell homeostasis in cooperation with IL-7 [189, 191], and increases T cell survival at the peak of expansion by augmenting the expression of Bcl-2 downstream of STAT5 [184, 191]. Importantly, IL-15 is critical in maintaining T cell memory as it induces homeostatic proliferation of this T cell subset [166, 181, 192], particularly $CD8^+$ Tcm [193]. Together then, IL-15 and IL-7 cooperate to control the proliferation and survival of naïve and memory $CD8^+$ T cells.

4.2. T-bet and Eomes

The transcription factors T-bet (*Tbx21*) and Eomesodermin (*Eomes*) are considered master regulators of $CD8^+$ T cell effector and memory differentiation.

T-bet and Eomes are members of the T-box family of transcription factors, and are unique in that they are expressed exclusively in leukocytes [194], indicating a key role for T-bet and Eomes in dictating immune responses. T-bet was originally identified by a yeast one-hybrid screen attempting to identify novel proteins which interacted with the IL-2 promoter in T cells [195]. Since then, many reports have demonstrated T-bet and Eomes to be key factors dictating the lineage choices made by both $CD4^+$ and $CD8^+$ T cells.

4.2.1 T-bet and Eomes in T cell effector responses

Initial reports investigating the role of T-bet in T cell differentiation identified T-bet as crucial for the Th1 lineage commitment of $CD4^+$ T cells [195, 196]. $CD4^+$ T cells isolated from T-bet-deficient mice failed to produce significant amounts of IFN-γ following TCR and CD28 stimulation, even in the presence of the IFN-γ-promoting cytokine IL-12 [196]. The loss of T-bet expression by $CD4^+$ T cells also led to their preferential production of the Th2-related cytokines IL-4 and IL-5, indicating a problem with Th1 differentiation [196]. $CD8^+$ T cell effector differentiation is also dis-regulated in T-bet null mice as T-bet-deficient $CD8^+$ T cells demonstrate reduced IFN-γ production and cytolytic activity in ^{51}Cr release assays [197]. *In vivo*, lysis of antigen-bearing target cells following the adoptive transfer of T-bet-deficient $CD8^+$ T cells into naïve animals is also reduced [197]. Likewise, the T-bet paralogue Eomes was found to play an equally important role in $CD8^+$ T cell effector function. Eomes-deficient $CD8^+$ T cells exhibited diminished expression of IFN-γ and granzyme B following stimulation, which was further exacerbated by a combined deficiency in

T-bet [198]. Overall, these studies indicate that T-bet and Eomes cooperate to induce optimal $CD8^+$ T cell effector function and differentiation following $CD8^+$ T cell activation.

The specific roles that T-bet and Eomes play in $CD8^+$ T cell effector function and differentiation are becoming clearer. Recent evidence suggests that T-bet and Eomes are expressed with different kinetics following $CD8^+$ T cell activation and that their combined expression sustains effector responses and predisposing cells for terminal effector differentiation [199]. T-bet expression in $CD8^+$ T cells can be detected as early as 24 hours following TCR stimulation. After its induction, T-bet enhances the expression of IFN-γ and granzyme B by directly binding the IFN-γ and granzyme B promoters [199, 200]. Interestingly, the T-bet promoter contains STAT4 and STAT1 binding elements, which enhance T-bet expression during inflammatory conditions induced by IL-12 and IFN-γ treatment [148, 149, 201]. No such change in Eomes expression has been reported following inflammatory cytokine addition [149], defining a key difference in when T-bet and Eomes are preferentially induced. Eomes expression by T cells is often not detected until 48 hours after T-bet induction [199]. Once expressed, Eomes can directly bind the perforin and IFN-γ promoter elements [66, 199], increasing and sustaining the effector function of $CD8^+$ T cells in cooperation with T-bet. The transcription factor Runx3 is believed to be partially involved in regulating Eomes expression. Runx3-deficient $CD8^+$ T cells fail to express the Eomes protein and exhibit reduced effector responses following antigen stimulation [199]. Additionally, Eomes expression is also regulated by

the Wnt:Tfc-1 (Wnt:T cell factor-1) pathway [202], and through the metabolic regulator and serine/threonine kinase mTOR [203].

4.2.2 T-bet and Eomes in T cell memory generation

In addition to their role in regulating effector differentiation, T-bet and Eomes have also been demonstrated to be important in $CD8^+$ T cell memory generation. Mice which are homozygous *tbx21*$^{-/-}$ and heterozygous *eomes*$^{+/-}$ (homozygous *eomes* $^{-/-}$ is embryonically lethal), display a phenotype reflective of IL-15-deficient animals with no NK cell or memory T cell development [204]. This phenotype suggested that T-bet and Eomes may be involved in regulating IL-15 receptor expression on these cell types. Indeed, mice lacking both T-bet and Eomes were found to be deficient in their expression of the IL-15 receptor β-chain, CD122 [204]. In addition, reduced CD122 expression was found to be more dramatic following a loss in Eomes expression then T-bet, suggesting a preferential role for Eomes in inducing expression of this cytokine receptor and sustaining memory $CD8^+$ T cell survival [204]). Further experiments proved this to be the case as Eomes was found to directly bind the CD122 promoter [204]. This finding, in addition to the high preferential expression of Eomes in memory $CD8^+$ T cells, has led to the association of Eomes expression with memory development [205]. Thus, T-bet and Eomes expression by $CD8^+$ T cells appears to be critical to identifying effector and memory T cell progenitors, respectively, making these transcription factors central to vaccine and adoptive cell transfer therapy development.

4.3 Blimp-1 and Bcl-6

Blimp-1 and Bcl-6 are transcriptional repressors recently described in association with $CD8^+$ T cell effector and memory states. While both have well described roles in B cell maturation and memory development, their roles in $CD8^+$ T cell differentiation are just beginning to be elucidated. Blimp-1 is preferentially expressed by terminal effector and effector memory $CD8^+$ T cells, while Bcl-6 expression is increased in $CD8^+$ memory T cells, particularly the central memory T cell subset [206]. Both proteins contain a zinc-finger DNA-binding domain which can repress transcriptional activity, in addition to recruiting factors that modify histone complexes [207-209]. Interestingly, Blimp-1 and Bcl-6 are repressors of each other's transcription and can control each other's expression in addition to other genes associated with effector and memory T cell fates [206]. Thus, Blimp-1 and Bcl-6 represent attractive targets for monitoring $CD8^+$ T cell effector and memory differentiation.

4.3.1 Blimp-1 activity and lymphocyte differentiation

Blimp-1 (B-lymphocyte induced maturation protein-1, gene name *pdrm*) was originally identified to play a key role in B cell differentiation as it drives plasma cell maturation and Ig-secretion from the B cell lymphoma cell line BCL1 [210]. In B cells, Blimp-1 modifies the expression of several important genes including transcription factor Xbp-1, which is critical for plasma cell function [211], *c-myc* and Id3, which are involved in cell proliferation, as well as genes involved in apoptosis [207, 212, 213]. Blimp-1 expression is induced by several different mechanism including BCR engagement, TLR/RIG-I signalling, NF-κB,

AP-1, and IRF4 [207]. In addition, IL-2, IL-6, IL-10 and IL-21 have all been associated with increased Blimp-1 expression in B cells [207, 214]. In T cells however, Blimp-1 expression is strongly upregulated by IL-2 in combination with signals from the TCR [66, 207].

Blimp-1 has been demonstrated to play an important role in the differentiation of both $CD4^+$ and $CD8^+$ T cells. Blimp-1 is expressed at higher levels by Th2 differentiated $CD4^+$ T cells, compared with Th1 [207]. Indeed, Blimp-1-deficient animals often suffer from the inflammatory bowel disorder colitis, and exhibit enhanced secretion of IFN-γ and reduced production of IL-10 [215, 216]. In terms of the $CD8^+$ T cell subset, Blimp-1 expression is associated with terminal effector differentiation, but can also be detected at slightly elevated levels from effector memory $CD8^+$ T cells [217, 218]. The association between Blimp-1 expression and effector development stems from Blimp-1-deficient $CD8^+$ T cells expressing lower amounts of the effector molecules granzyme B and perforin than Blimp-1 positive cells [217, 218]. In response to influenza infection, Blimp-1-deficient animals have a reduced capacity to clear the virus and exhibit enhanced MPEC development, demonstrating a requirement for Blimp-1 in $CD8^+$ T cell effector generation [217, 218]. Blimp-1-deficient $CD8^+$ T cells also exhibit reduced expression of T-bet and enhanced expression of the memory-associated transcription factors Eomes and Bcl-6 [217, 218]. Importantly, in T cells, Blimp-1 appears to play a central role in IL-2 regulation [175, 219]. While IL-2 increases the expression of Blimp-1 in T cells, likely through STAT5, Blimp-1 negatively effects IL-2 expression by directly binding

and repressing the IL-2 promoter, thus completing an autoregulatory feedback loop [175]. This reduction in IL-2 expression by T cells both limits their proliferative capacity and likely initiates the process of T cell contraction. In addition to its enhanced expression following T cell activation, continued antigen exposure sustains Blimp-1 expression and is associated with T cell exhaustion and enhanced expression of the T cell co-inhibitory molecule PD-1 [220].

4.3.2 Bcl-6 activity and lymphocyte differentiation

The Blimp-1 antagonist Bcl-6 is a transcriptional repressor also identified in B-lymphocytes. Bcl-6 is required for B cell germinal centre and memory cell formation as mice lacking Bcl-6 lack germinal centre B cells and do not undergo affinity maturation [221, 222]. In T lymphocytes, Bcl-6 also directs cell differentiation. Bcl-6 is an absolute requirement for $CD4^+$ T cell helper follicular cell (T_{FH}) development [223], a special lineage of $CD4^+$ T cells dedicated to providing B cell help. $CD8^+$ T cells, on the other hand, require Bcl-6 for optimal memory generation [224]. Mice lacking Bcl-6 exhibit a diminished memory compartment and display a reduced capacity for proliferation upon stimulation [225]. Not only is Bcl-6 expression required for $CD8^+$ T cell memory generation, but Bcl-6 is capable of directly binding and inhibiting the granzyme B promoter, hampering full effector differentiation [226]. The specificity of Bcl-6 for certain promoter elements is determined by co-repressor binding, which interact along with Bcl-6 to repress the expression of individual genes [206]. Overall, the regulated expression of Blimp-1 and Bcl-6 appears to be critical in $CD8^+$ T cell effector and memory generation. Since the regulation of their expression in $CD8^+$

T cells is just now being uncovered, investigation into whether activating stimuli, including co-stimulation, can influence the expression of these transcription factors is much warranted.

5. Adoptive Cell Transfer Therapy and Artificial Antigen Presentation

Adoptive cell transfer therapy (ACT) is an immunotherapy in which patients are transfused with *ex vivo* expanded cytolytic $CD8^+$ T cells capable of recognizing and lysing cancerous or virally-infected cells. While still in a pre-clinical stage of development, ACT has been successful in the treatment metastatic melanoma [227, 228], and holds some promise for usage against other types of cancer and viral infections, including HIV. In brief, ACT involves the isolation of tumour-antigen specific $CD8^+$ T cells from the peripheral blood or tumor infiltrates of patients which are then expanded *in vitro* using peptide bearing human leukocyte antigen (HLA) complexes and co-stimulatory ligands, and re-introduced back into the individual [227, 229]. Ideally, the *in vitro* expansion of naïve antigen-specific $CD8^+$ T cells would utilize patient-derived DCs, but these are difficult to isolate and to maintain in culture, making their use in a clinical setting impractical. Because of this limitation, ACT development has turned to the use of peptide-HLA and co-stimulator ligand-coated beads as artificial APCs (aAPCs), which can be specifically manufactured for individual patients [230]. While bead aAPCs may lack the tight cell-to-cell adhesion reminiscent of *in vivo* antigen presentation, many groups have demonstrated the ability to effectively activate naïve murine and human $CD8^+$ T cells using stimulatory bead complexes *in vitro* [231, 232]. In addition, several co-

stimulatory molecules including CD28, CD27, and 4-1BB, and the adhesion molecule LFA-1, have demonstrated promise for use in ACT [233-237]. Thus, further research into how beads bearing different combinations of antigenic and co-stimulator ligands can be utilized to drive the generation of effector and memory $CD8^+$ T cell subsets would greatly benefit not only our understanding of basic immune function, but could also translate to advancing the field of ACT.

6. Project Overview:

Naive $CD8^+$ T cells integrate several different cues which dictate their activation, effector phenotype, and differentiation into memory T cell precursors. These include antigenic signal strength, co-stimulation, and cytokines. This thesis is focused on the role of co-stimulation in the T cell activation and differentiation process. Specifically, I am interested in determining if co-stimulator ligands, or their combination, can be used to direct activated $CD8^+$ T cell toward specific effector phenotypes with varying capacities for proliferation, cytokine production, cytotoxicity and survival. Additionally, I aim to determine if specific co-stimulatory conditions can be used to push naïve $CD8^+$ T cells toward their SLEC or MPEC development (Fig. 1-4). To address these questions, I have employed the use of a bead-based ligand presentation system in which co-stimulator ligands can be presented to naïve $CD8^+$ T cells both individually and in combination, in finely controlled fashion. The generated $CD8^+$ T cell population will be assessed for their effector and memory differentiation by examining their proliferative, and cytolytic phenotypes, as well as their expression of cytokine receptors and transcription factors associated with effector and memory $CD8^+$ T cell fate.

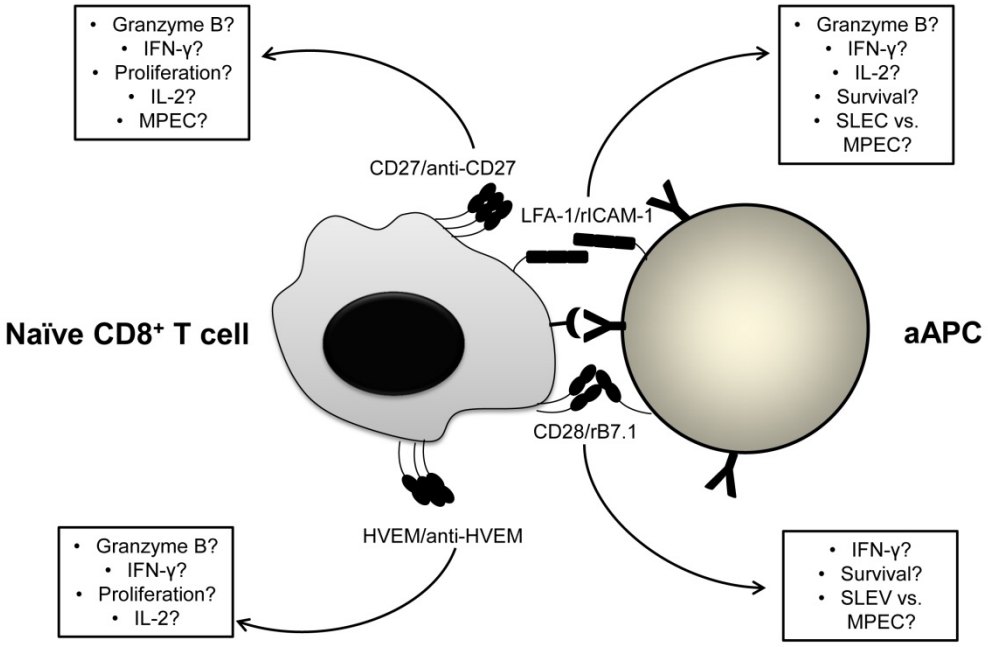


Figure 1-4. General questions to be addressed by this thesis.
Polystyrene beads will be used as an aAPC to determine the role of co-stimulatory molecule ligation in naive CD8+ T cell effector molecule, IL-2 production, cell survival, and differentiation after activation. T cell co-stimulators to be ligated include CD28, LFA-1, CD27, and HVEM. Ligation of these proteins will be through bead immobilized recombinant co-stimulatory ligands, or cross-linking antibodies, provided in combination with a suboptimal amount of CD3 cross-linking antibody. Co-stimulator ligands will be added in different quantities and combinations to examine their influence on CD8+ T cell activation and differentiation.

Hypothesis:

I hypothesize that co-stimulator molecules are non-redundant and that their ligation, either individually or in combination, can be used to generate $CD8^+$ T cell effector populations with distinct phenotypes and capacity for terminal effector and memory differentiation.

Research Goals:

1) Determine how various co-stimulator ligands and their combinations, can be used control the extent of naïve $CD8^+$ T cell activation and the generation of $CD8^+$ T cell populations with distinct effector phenotypes

2) Identify the co-stimulation induced factors which contribute to $CD8^+$ T cell survival following activation, and if their expression plays a role in modulating the effector phenotypes generated.

3) Determine if the co-stimulation generated $CD8^+$ T cell populations display a phenotype indicative of either terminal effector or memory differentiation through phenotypic marker analysis and effector vs. memory associated transcription factor expression.

Chapter 2. Materials and Methods

Mice and Reagents

Conventionally housed 6-8 week old female C57BL/6 mice purchased from Charles River were used for all experiments. Recombinant mouse B7-1Fc and ICAM-1Fc/chimeric and recombinant CD70 co-stimulatory proteins were purchased from R&D systems and reconstituted as per the manufacturer's instructions. Sterile functional grade (endotoxin $\leq$ 0.001ng/μg antibody, sodium azide free) anti-CD27 cross-linking antibody (clone LG.3A10) was purchased from BD Biosciences, while functional grade anti-CD28 (clone 37.51), anti-HVEM (clone LH1) and anti-CD3ε (clone 145-2C11) were purchased from eBioscience. The conjugated forms of the monoclonal antibodies and their isotypes used for flow cytometry analysis are detailed in Table 2-1. Goat anti-Armenian hamster IgG was purchased from Cedarlane. Streptavidin PeCy5.5 (Cat. #35-4317) was purchased from eBiosciences. Propidium iodide (Cat. # 00-6990), annexin V APC/FITC (Cat # 88-8007), and annexin V binding buffer were all purchased from eBioscience. Recombinant murine IL-2 was generated in our laboratory and titrated against a standard concentration of IL-2 (2000U/mL), using AB1 T cell clones. Phorbol 12-myristate 13-acetate (PMA) was purchased from Sigma-Aldrich and used at a concentration of 5ng/mL. Ionomycin calcium salt was also purchased from Sigma-Aldrich and used at a concentration of 500ng/mL.

Polystyrene bead preparation

Five or six micron polystyrene bead constructs were prepared by incubating 5 x 10^6 or 1 x10^7 sulphate-modified polystyrene beads (Invitrogen) with 0.156nM - 10nM of recombinant proteins or 0.0156µg - 2µg of cross-linking antibody at 4°C with rotation for 20 min in sterile PBS. Unbound sites were blocked with the addition of 1% BSA/PBS followed by an additional 20 min incubation at 4°C with rotation. Bead constructs were then washed and resuspended in 0.1% BSA/PBS. Densities of immobilized proteins or antibodies were analysed by flow cytometry using ligand or antibody Fc-specific Abs.

Isolation of ex vivo CD44^{lo} CD8$^+$T cells

Mice were anesthetized with halothane (Sigma-Aldrich) or isoflurane (Benson) and euthanized by cervical dislocation. Spleens and lymph nodes (axillary, brachial, inguinal and superficial cervical) were isolated, batched, and gently disrupted with a tissue homogenizer. CD44^{lo} CD8$^+$ T cells were isolated using EasySep CD8$^+$ T cell negative enrichment kits (StemCell Technologies) modified with the addition of 0.03µg/1 x 10^6 cells of anti-CD44 biotin (clone IM7, eBioscience) during the initial negative selection step (Appendix 4-1). Negatively enriched CD8$^+$ T cell preparations were typically between 95% - 98% CD8α$^+$ as determined by flow cytometric analysis.

Cell trace Violet Labeling

To label cells with cell trace violet, whose dilution was used to measure cell proliferation, CD8$^+$ T cells were washed with 0.1% BSA (Sigma-Aldrich) in PBS (Invitrogen) and resuspended at a density of 1 x10^7 cells/mL in 0.1% BSA/PBS.

One micromole per 1 x 10^6 $CD8^+$ T cells of cell trace violet (Invitrogen) was added to the cell suspensions and incubated for 20 min at 37°C. The dye was then quenched with the addition of complete cell culture medium (CCM) added at 5 x the volume of resuspended cells for 5 min at 37°C, followed by two washes using complete culture medium. Complete culture media consisted of RPMI 1640 supplemented with 10% FCS, 2mM $_L$-glutamine, 100U/mL penicillin, 100µg/mL streptomycin, and 0.055mM 2-ME (Invitrogen).

$CD44^{lo}CD8^+$ T cell stimulation with polystyrene beads

Two hundred and fifty thousand $CD44^{lo}$ $CD8^+$ T cells were cultured with 0.5 x 10^6 polystyrene beads in 96-well flat-bottom culture plates (Corning) at a final volume of 0.25mL in CCM. Cultures were incubated at 37°C with 5% CO_2 and harvested 48hrs after bead stimulation for the indicated flow cytometric analysis and Na_2 (^{51}Cr) O_4 (^{51}Cr) release assays. Activated $CD8^+$ T cells:bead cultures were transferred to 24-well flat-bottom culture plates (Corning) at a final volume of 1mL after 48 hrs of stimulation, for long-term 72-96 hr cultures.

Cell trace violet dilution and intracellular effector molecule analysis

Brefeldin A (Invitrogen) was added to cultures at a final concentration of 1µg/mL for 3 hrs before harvest. Cell trace violet-labeled $CD44^{lo}CD8^+$ T cells were harvested, fixed for 20 min with 4% formaldehyde (Fischer Scientific) at 4°C, and permeablized with eBioscience Permeabilization buffer (eBioscience). Cells were then stained with fluorochrome-conjugated anti-IFN-γ and anti-granzyme B mAbs. All staining was performed at 4°C for 15 min. Flow cytometric acquisition was performed using a Becton Dickinson FACSCanto II. 20,000

gated events were acquired for each sample. Flow cytometric analysis was conducted using FCS Express software (De Novo Software).

^{51}Cr release killing assays

Five to ten million Fc receptor-bearing P815 target cells were washed with 2% FCS/RPMI and resuspended in 80µL of FCS and labeled with 100uL (100µCi) of ^{51}Cr (Perkin Elmer) for 1hr at 37°C. P815 targets were then washed three times with 5% bovine calf serum (BCS)/RPMI and resuspended at a final concentration of 2 x 10^5cells/mL in CCM. 50µL of resuspended ^{51}Cr labeled P815 target cells were plated in 96-well V-bottom plates (Costar) and incubated with anti-CD3 antibody (clone 145.2C11) at a final concentration of 40µg/mL, diluted in RPMI, for 15min at room temperature. In parallel, control P815 targets were incubated in complete media instead of antibody. $CD44^{lo}CD8^{+}$ T cells stimulated with polystyrene bead constructs for 48hrs were harvested, washed in 2% FCS/RPMI, and resuspended at a final concentration of 2 x 10^6cells/mL in complete culture medium. One hundred microliters of effector cells were added to each well of P815 targets for an effector to target ratio of 20:1. Cells were pelleted by centrifugation and incubated for 4hrs at 37°C. Following incubation, cell suspensions were pelleted by centrifugation and 25µL of supernatant was collected for ^{51}Cr release analysis. Supernatants were mixed for 10 min by shaking with 100µL of OptiPhase "SuperMix" (Perkin Elmer) and analysed for the presence of ^{51}Cr using a Trilux 1450 Microbeta counter. Percent specific cell lysis was calculated as follows:

$$[experimental\ release - spontaneous\ release \Big/ total\ release - spontaneous\ release] \ X\ 100$$

Annexin V/Propidium Iodide staining

Activated $CD8^+$ T cell cultures were harvested at 24 or 48hrs following stimulation and washed with PBS and annexin V binding buffer (eBiosciences Cat. # BMS500BB). Cell pellets were resuspended in 100µL of annexin V binding buffer and 1.25µL of annexin V was added per sample, followed by a 15min incubation at room temperature. After the incubation period, cells were washed twice with annexin V binding buffer and resuspended at a final volume 200µL. One and a quarter microliters of propidium iodide was directly added to the resuspended cell and analyzed by flow cytometry within 2 hrs of its addition. Flow cytometric acquisition was performed using a Becton Dickinson FACSCanto II or LSR II. 10,000-20,000 gated events were acquired for each sample. Flow cytometric analysis was conducted using FCS Express software (De Novo Software).

IL-2 detection by ELISA and intracellular flow cytometry

For IL-2 ELISA, supernatants were collected 24 and 48hrs following $CD8^+$ T cell stimulation with polystyrene beads constructs. Harvested cells were pelleted in 96-well V-bottom plates (Costar) and approximately 100µL of supernatant was collected from each well. Supernatants were pooled when stimulations were performed in duplicate to ensure a large enough volume for multiple ELISA samples. IL-2 concentration was determined using a Ready-Set-Go IL-2 ELISA Kit purchased from eBioscience (Cat. # 88-7024-22). Absorbance was detected using a ν_{max} Kinetic microplate reader (Molecular Devices) at wavelength of 450nm - 570nm. The concentration of IL-2 was calculated using Softmax Pro

software. To detect intracellular IL-2 by flow cytometry, cells were treated for 4hrs with 3µg/mL of BFA (Invitrogen), harvested, washed with cold 2% FCS /PBS, and their surface stained with fluorochrome conjugated CD8 and CD4 antibodies for 15min at 4°C for gating purposes. Following surface staining, the cells were washed and fixed with 4% formaldehyde diluted in PBS at 4°C for 20min. Cells were then permeablized with eBioscience Permeabilization buffer, and stained using anti-IL-2 fluorochrome-conjugated monoclonal antibodies for 15min at 4°C. Flow cytometric acquisition was performed using a Becton Dickinson FACSCanto II or LSR II. 20,000 gated events were acquired for each sample. Flow cytometric analysis was conducted using FCS Express software (De Novo Software).

Transcription factor and anti-apoptotic protein detection

For intracellular detection of transcription factors (T-bet, Eomes, phosphorylated STAT5) and anti-apoptotic protein expression (Bcl-2, Bcl-xL), $CD8^+$ T cell:bead cultures were harvested at the indicated time points, washed with 2% FCS /PBS, and fixed with 4% formaldehyde diluted in PBS for 20 min at 4°C. Cells were then permeabilized using eBioscience Permeabilization buffer, and stained with anti-Bcl-2, anti-Bcl-xL, anti-T-bet, anti-Eomes or anti-STAT5 pY694 fluorochrome-conjugated monoclonal antibodies. Antibody clones and manufacturers can be found in Table 2-1. Staining was performed in the presence of cell permeabilization buffer for 15min at 4°C after which the cells were then washed and fixed with 4% formaldehyde diluted in PBS. Flow cytometric acquisition was performed using a Becton Dickinson FACSCanto II or LSR II.

20,000 gated events were acquired for each sample. Flow cytometric analysis was conducted using FCS Express software (De Novo Software)

Image Capture

Microscopy images of $CD8^+$ T cell:bead cultures at 24 and 48hrs were captured using an inverted microscope with an attached Retiga Q-image charge-coupled device camera, using OpenLab Software (Improvision, Walthma, MA). Images were manipulated in ImageJ (National Institutes of Health) and adjusted with Photoshop CS5.1 software.

RNA extraction

Prior to RNA extraction, approximately one million $CD8^+$ T cells, stimulated with various co-stimulatory combinations, were harvested, pelleted, and then frozen at -80°C until use. RNA was extracted from cell pellets using an RNeasy Mini Kit available from Qiagen (Cat. # 74104), as per the manufacturer's instructions. During this procedure, cells were lysed using commercially available QiaShredder columns (Qiagen, Cat. # 79654), and on the column DNA digestion was performed using an RNase-Free DNase Set kit (Qiagen, Cat. # 79254). Following extraction, RNA concentration was determined using a Nanodrop 2000 spectophotometer (Thermo Scientific) and Nanodrop 2000 software (Thermo Scientific).

Generation of cDNA template

cDNA for qPCR was generated by mixing 100ng of extracted RNA template from each stimulatory condition with RNase/DNase-free water, and the components of qScript cDNA Supermix (Quanta Biosciences), as per the manufactures

instructions. cDNA synthesis was performed using a Mastercycler Gradient PCR thermocycler (Eppendorf) following the reaction protocol specified by the manufacturer. The cDNA was stored at -20°C until use.

Quantitative PCR

Synthetic primers for the qPCR reaction were purchased from Sigma-Aldrich. Forward and reverse primers were mixed and diluted to a concentration of 1.6µM for storage. Forward and reverse primer sequences for Blimp-1, Bcl-6 and RPL24 can be found in Table 2-2. For each qPCR reaction, cDNA from each stimulation condition was diluted 1:4 in RNase/DNase- free water and mixed with 5µL of PerfeCta SYBR Green FastMix qPRC reagent (Quanta Biosciences) and 2.5µL of 1.6µM forward and reverse primers, for a final reaction volume of 10µL. qPCR samples were added to Twin.tec real-time PCR plates (Eppendorf), sealed, mixed by vortexing, and spun down before each qPCR reaction. Quantitative PCR was performed using an Eppendorf Realplex2 Mastercycler epigradients PCR machine using a PCR cycle suggested by Quanta Biosciences. Sample analysis was performed using Ep Realplex Software (Eppendorf). Fold increase in Blimp-1 and Bcl-6 transcripts was calculated relative to their expression from naïve $CD8^+$ T cells using RPL24 as an internal control gene for each stimulatory condition. Blimp-1 and Bcl-6 expression relative to RPL24 alone can be found in Appendix, Figure 7-3.

Table 2-1. Antibody clone and isotype list

Antibody	Clone	Isotype	Source
CD3ε	145-2C11	Armenian Hamster IgG	eBioscience
CD28	37.51	Golden Syrian Hamster IgG	eBioscience
CD27 Functional Grade	LG.3A10	Armenian Hamster IgG1	BD Biosciences
CD27 staining	LG.7F9	Armenian Hamster IgG	eBioscience
HVEM	LH1	Armenian Hamster IgG	eBioscience
CD8α	53.6.7	Rat IgG2a	eBioscience
CD44	IM7	Rat IgG2b	eBioscience
CD62L	MEL-14	Rat IgG2a	eBioscience
CD25	PC61.5	Rat IgG1	eBioscience
CD127	A7R34	Rat IgG2a	eBioscience
ICAM-1/CD54	YN1/1.7.4	Rat IgG2b	eBioscience
LFA	M17/4	Rat IgG2a	eBioscience
B7.1	16-10A1	Armenian Hamster IgG2	BD Biosciences
CD70	FR70	Rat IgG2b	eBioscience
IFN-γ	XMG1.2	Rat IgG1	eBioscience
Granzyme B	16G6	Rat IgG2b	eBioscience
FasL	MFL3	Armenian Hamster IgG	eBioscience
PD-1	J43	Armenian Hamster IgG	eBioscience
CTLA-4	UC10-4B9	Armenian Hamster IgG	eBioscience
Bcl-2	3F11	Armenian Hamster IgG1	BD Biosciences
Bcl-xL	Cat # sc-8392	Mouse IgG1	Santa Cruz
CD4	RM4-4 or GK1.5	Rat IgG2b	eBioscience
IL-2	JES6-5H4	Rat IgG2b	eBioscience
CD122	Tm-b1	Rat IgG2b	eBioscience
IL-15Rα	DNT15Ra	Rat IgG1	eBioscience
4-1BB/CD137	17B5	Golden Syrian Hamster IgG	eBioscience
KLRG1	2F1	Golden Syrian Hamster IgG	eBioscience
STAT5 pY694	47	Mouse IgG1	BD Biosciences
T-bet	eBio4B10	Mouse IgG1	eBioscience
Eomes	Dan11mag	Rat IgG2a	eBioscience

Table 2-2. qPCR Primer List

Primer	Sequence
Blimp-1 Forward Primer	5’-GCGTCAGTACTGCTCAGCCCG-3’
Blimp-1 Reverse Primer	5’-CTTGGGGGCAGCCAAGGTCGTA-3’
Bcl-6 Forward Primer	5’-GCCTGCAGCGGCCTGTTCTAC-3’
Bcl-6 Reverse Primer	5’-TTGCCTTCCCTCAGGTTGAGCC-3’
RPL24 Forward Primer	5’-GGACCGACGGGAAGGTTTTCCA-3’
RPL24 Reverse Primer	5’-TTGACTGCACGGCGGGTTCT-3’

Chapter 3. Differential and Complementary Contributions of Co-stimulator Ligation to Naïve $CD8^+$ T cell Activation

Introduction

$CD8^+$ T cell responses play a critical role in controlling infections involving intracellular pathogens. Activation and expansion of $CD8^+$ T cell populations in response to infection is a complex process involving the interplay between antigen recognition and co-stimulator receptors present on naïve $CD8^+$ T cells interacting with stimulating ligands on APCs, with potential influences from the local cytokine environment. Specifically, to be optimally activated, naïve $CD8^+$ T cells receive three distinct activating signals and these are: 1) Recognition of foreign antigen by the $CD8^+$ T cells via its TCR, 2) Ligation of co-stimulators which fine-tune T cell activation and inhibit the development of T cell anergy and, 3) Pro-inflammatory cytokines which can enhance the production of $CD8^+$ effector molecules such as IFN-γ and granzyme B [37, 147, 238]. T cell receptor mediated signalling itself can greatly influence the fate of $CD8^+$ T cells, with the strength and duration of TCR signalling correlating with increased T cell fitness, enhanced effector function, and memory cell generation [26, 239, 240]. Ligation of co-stimulators on naive T cells during TCR stimulation can amplify and diversify TCR-mediated signals, thereby lowering the threshold for naïve T cell activation and further promote effector T cell responses [239, 241].

T cell co-stimulators and ligands typically fall into one of two distinct families; the CD28/B7 family, or the TNFR family. The CD28/B7 family contains both activating and inhibitory members with the most characterized

member being the activating co-stimulatory molecule CD28. The ligands for CD28, B7.1 and B7.2, are both expressed on activated APCs and DCs [61]. Signalling through CD28 ultimately results in enhanced TCR signalling, increased activation of NF-κB, production of the cytokine IL-2, and upregulation of anti-apoptotic factors such as Bcl-xL, promoting T cell survival [61, 65, 242]. Co-inhibitory members of the CD28/B7 family include CTLA-4 and PD-1, that pair with ligands B7.1 or B7.2 and PD-L1 or PD-L2, respectively [67]. Inhibitory members of the CD28/B7 family potentiate negative signalling, leading to overall inhibition and eventual termination of T cell responses [61]. The TNFR family of co-stimulators on the other hand is comprised nearly entirely of activating receptors. These receptors and their ligands include CD27:CD70, 4-1BB:4-1BBL, OX-40: OX-40L and HVEM which potentiates positive signalling when stimulated by its activating ligand LIGHT [77]. Of these receptors, only CD27 and HVEM are constitutively expressed on the surface of naïve $CD8^+$ T cells, while 4-1BB and OX-40 expression peaks 48hrs following T cell activation [77]. All TNFR family members activate pro-inflammatory signalling pathways leading to the activation of NF-κB [116]. Signalling through TNFR family members also promotes T cell survival, as anti-apoptotic factors such as Bcl-xL are upregulated following receptor ligation, and can synergize with TCR mediated signals to increase cell-cycle-progression [75, 79, 116, 131]. Co-stimulation through both the CD28/B7 and TNFR family members is predicted to enhance $CD8^+$ T cell proliferation and survival as well as pro-inflammatory cytokine production. However, the existence of multiple co-stimulator-ligand pairs suggests that these

receptors are not completely redundant and ligation of individual receptors, or combinations thereof, could potentially lead to different T cell fates and functions, perhaps even promoting $CD8^+$ T cell subsets better suited for effector or memory T cell roles.

Prior to activation, naïve $CD8^+$ T cells express only a small number of activating co-stimulator molecules capable of influencing their immediate activation and these are CD28, CD27, HVEM and the adhesion protein stimulator, LFA-1. *In vivo* mouse models addressing the role of these receptors during the activation of naïve $CD8^+$ T cells have revealed that partial redundancies in these co-stimulators do exist. Mice lacking expression of CD28 have been demonstrated to maintain a reduced yet functional effector T cell population following viral infection; however this was dependent on the presence of CD27 which was found to promote the survival of generated $CD8^+$ T cell effector populations [75]. Moreover, CD27 induces the proliferation of $CD8^+$ T cells in the absence of IL-2 [124], suggesting that CD27 may be capable of expanding effector T cell populations in the absence of CD28-mediated IL-2 production. Stimulation of HVEM with its activating ligand LIGHT increases naïve $CD8^+$ activation by promoting T cell proliferation and the production of IFN-γ [83, 137]. Indeed, mice which lack the expression of LIGHT have deficiencies in $CD8^+$ T cell cytotoxicity and cytokine production following injection and re-stimulation with the HPV-16 peptide E7 [84]. While not considered a conventional co-stimulatory molecule, ligation of the adhesion molecule LFA-1 by ICAM-1 in combination with TCR stimulation, induces T cell proliferation and

IL-2 production from $CD8^+$ T cells [234]. In addition, several studies have indicated a requirement for LFA-1:ICAM-1 interactions for the generation of functional memory $CD8^+$ T cells [145, 243]. LFA-1:ICAM-1 ligation is associated with the formation of stable contacts between T cells and DCs, and has been demonstrated to both augment and propagate unique signalling cascades when ligated in combination with a TCR stimulus [138].

While some have approached the role of individual co-stimulators in the activation of naïve $CD8^+$ T cells and acquisition of their effector phenotype, defining how co-stimulators function in combination to fine-tune the activation and generation of specific effector $CD8^+$ T cell populations has been difficult to establish. *In vivo* models often address the function of these molecules by generating functional knock-outs of a particular receptor or ligand, but fail to take into account additional remaining co-stimulators which may partially compensate for the loss of the receptor of interest. In addition, co-stimulation of naïve $CD8^+$ T cells is a complex process likely involving a number of co-stimulator and co-inhibitor ligands. The ability to precisely control which co-stimulator ligands are present and in what quantity during $CD8^+$ T cell activation is key to addressing if co-stimulation through specific receptors can result in distinct $CD8^+$ T cell effector populations. In this study, cell-sized beads were used as a presentation platform to investigate the role of individual co-stimulator ligands, and combinations thereof, in the activation and effector cell development of naïve $CD8^+$ T cells. Recombinant co-stimulator ligands and/or cross-linking antibodies against co-stimulators were displayed in a highly controlled manner either

individually or in combination with suboptimal amounts of TCR stimulation, to determine the contributions of selected co-stimulators in the activation of naïve $CD8^+$ T cells.

In this chapter I demonstrate that, of the co-stimulators present on the surface of naïve $CD8^+$ T cells during activation, LFA-1 ligation with recombinant ICAM-1 is the most efficient at stimulating $CD8^+$ proliferation and cytolytic abilities, but fails to induce expression of the pro-inflammatory cytokine IFN-γ. IFN-γ production could only be detected following co-stimulation with recombinant B7.1. However when ICAM-1 and B7.1 were provided in combination, the proliferation and cytolytic capacity of the generated effector $CD8^+$ T cell populations was increased even further than with B7.1 or ICAM-1 co-stimulation alone, and greatly enhanced the production of IFN-γ. This generation of potent effector $CD8^+$ T cells was not observed following naïve $CD8^+$ T cell stimulation through any other combination of co-stimulator molecules including CD27 or HVEM. This suggests that while some $CD8^+$ T cell proliferation and effector molecule production can be induced following stimulation with co-stimulator ligands individually, optimal generation of functional $CD8^+$ T cell effectors likely requires the engagement of multiple co-stimulator ligands.

Results

Presentation of co-stimulator ligands to CD44^{lo} naïve CD8^{+} T cells

For optimal activation of naive CD8^{+} T cells in response to antigen, CD8^{+} T cells need not only stimulation of their TCR with relevant peptide antigen presented in the context of MHC class I, but also a second activating signal provided by ligation of surface co-stimulator receptor(s). Ligation of these receptors may lead to optimal activation of naive CD8^{+} T cells, but the precise role and quantitative contributions that each receptor/ligand pair or combinations thereof provide toward activation, have been difficult to define. To investigate the role of co-stimulators and co-stimulator combinations in the activation of naïve T cells, TCR-specific antibodies were combined with single or multiple recombinant co-stimulator ligands or cross-linking antibodies on polystyrene beads for presentation to CD44^{lo}CD8^{+} T cells. Under these conditions, co-stimulator ligands can be presented to CD8^{+} T cells in a highly controlled and quantifiable manner to study the influence of various co-stimulatory conditions on the activation of *ex vivo* CD44^{lo} naive CD8^{+} T cells.

To ensure proper display of both recombinant co-stimulator ligands and cross-linking antibodies on beads for presentation to CD8^{+} T cells, bead constructs were prepared by adding increasing amounts of recombinant B7.1, CD70, or ICAM-1 proteins (0.156nM – 10nM) or CD3ε cross-linking antibody (0.0156µg – 1µg) (Fig. 3-1A). Flow cytometric analysis of the bead constructs confirmed that each of the co-stimulator ligands, and the cross-linking antibody, could be immobilized onto the surface of the beads in a controlled manner with

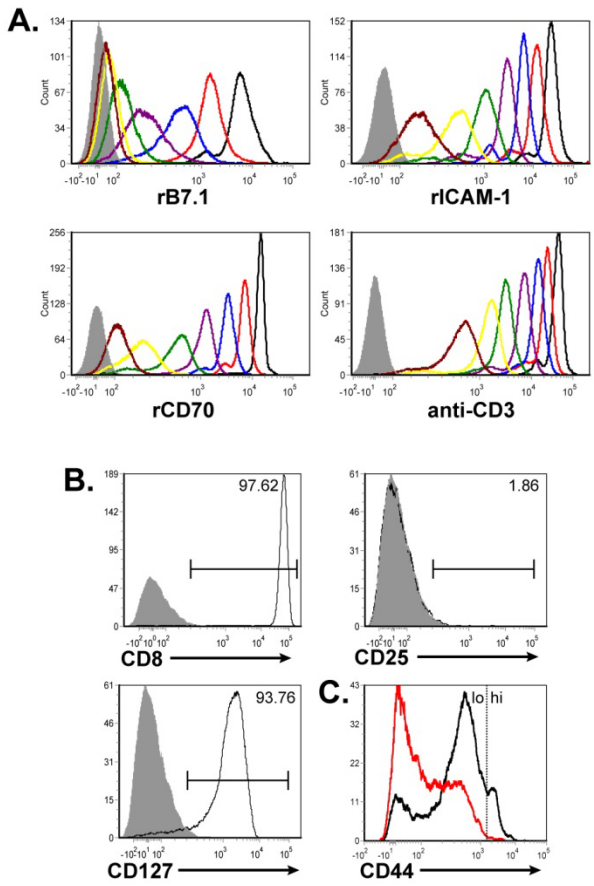
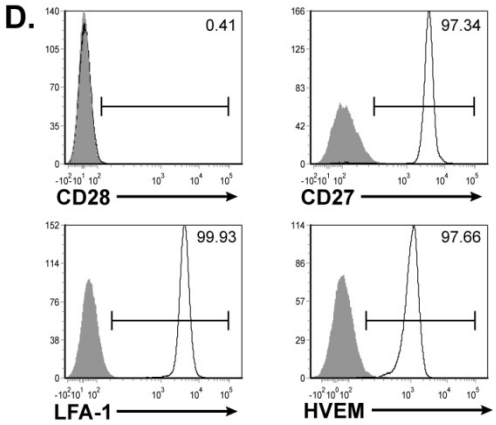


Figure 3-1. Co-stimulator ligand display on polystyrene beads and surface marker expression on *ex vivo* CD44^{lo} CD8^{+} T cells. (*A*) Recombinant co-stimulator ligands B7.1, CD70 and ICAM-1 (0.156nM – 10nM) as well as anti-CD3 cross-linking antibody (0.0156µg – 1µg) were immobilized on the surface of polystyrene beads and their adherence determined by monoclonal antibody binding and flow cytometry. Anti-CD3 binding to the beads was assessed using FITC-coupled goat anti-Armenian hamster antibody. Shaded histograms represent antibody stained BSA-coated negative control beads, while solid lines represent antibody stained bead constructs. In each panel, the maroon line indicates the lowest amount of stimulatory protein, while the black line indicates the highest. Other colours in order indicate increasing intermediate amounts of protein. (*B*) Freshly isolated naïve CD8^{+} T cells were stained with fluorescently conjugated mAbs against CD8α, CD25 and CD127 to determine cell purity and naïve phenotypic staining. Shaded histograms represent isolated CD8^{+} T cells stained with isotype matched controls. (*C*) Flow cytometry detection of CD44 expression from *ex vivo* splenocytes (black) stained using fluorochrome conjugated CD44 antibodies and from freshly isolated CD44^{lo} CD8^{+} T cells (red) stained using PeCy5.5 coupled streptavidin. (*D*) *Ex vivo* CD8^{+} T cells were stained with monoclonal antibodies to detect LFA-1, CD28, HVEM and CD27 respectively, and analyzed by flow cytometry. Shaded histograms represent cells stained with isotype control antibodies.


D.
0.41
CD28
97.34
CD27
99.93
LFA-1
97.66
HVEM

two fold increases in co-stimulator ligand addition leading to a proportional increase in bead surface staining (Fig. 3-1A). In addition, freshly isolated $CD44^{lo}$ $CD8^+$ T cells were stained for their $CD8^+$ T cell purity and naïve-like expression of the surface markers CD44, CD25 and CD127 (Fig 3-1B,C). The isolated $CD8^+$ T cells typically expressed high and uniform amounts of CD8α, low amounts of CD25 and CD44, and high levels of the IL-7 receptor α-chain CD127 (Fig. 3-1B, C). The expression of the co-stimulatory molecules LFA-1, CD28, CD27 and HVEM was also assessed on freshly isolated $CD8^+$ T cells by flow cytometry, and were found to have a typical naïve pattern of expression (Fig. 3-1D). Each of the co-stimulators could be detected to some degree on the surface of the *ex vivo* $CD44^{lo}$ $CD8^+$ T cells, with uniform high expression of LFA-1 and CD27, moderate expression of HVEM, and very low to undetectable expression of CD28 being detected (Fig. 3-1D). Thus, each of these receptors expressed by naive $CD8^+$ T cells may be ligated by co-stimulator ligands present on the prepared bead constructs, and thus the roles of selected co-stimulator ligands in the activation of $CD44^{lo}$ naive $CD8^+$ T cells could be assessed.

Increasing B7.1 co-stimulation increases naïve $CD44^{lo}CD8^+$ T cell activation

A previous report examining the role of B7.1 co-stimulation in the activation of naive $CD8^+$ T cells using a similar bead-based antigen presentation system indicated that increasing the amount of CD28/B7.1 co-stimulation increased the extent of $CD8^+$ T cell proliferation in a B7.1 dependent manner [232]. To demonstrate that the generated bead preparations could enhance $CD8^+$ T cell granzyme B and IFN-γ effector molecule production, as well as

proliferation in a similar manner, bead constructs were prepared by immobilizing increasing amounts of recombinant B7.1 in combination with a suboptimal amount (0.125µg) of cross-linking CD3ε antibody. The suboptimal amount of CD3ε antibody was experimentally determined to induce very low amounts of $CD44^{lo}$ $CD8^{+}$ T cell proliferation (~1%), granzyme B production (~7%) and IFN-γ production (~0.50%) (Fig. 3-2A,B). Thus, any increases in these parameters are due to co-stimulator ligation. Bead constructs were incubated for 48 hours with cell trace violet labeled *ex vivo* $CD44^{lo}$ $CD8^{+}$ isolated from naive C57BL/6 mice as described in *Materials and Methods*. The 48 hour time point was selected to assess the activation of the stimulated $CD8^{+}$ T cells as it is the approximate time frame required for the initiation of $CD8^{+}$ T cell responses following acute infection, and also provides sufficient time for the $CD8^{+}$ T cells to undergo several rounds of division and express effector molecules. In addition, there are an adequate number of suboptimally stimulated cells which can be assessed as controls. Following stimulation, the cells were treated with BFA, fixed, permeabilized and stained with fluorochrome-conjugated granzyme B and IFN-γ monoclonal antibodies, and also assessed for proliferation by flow cytometry. In the absence of any stimulation, $CD44^{lo}$ naïve $CD8^{+}$ T cells did not undergo proliferation as determined by cell trace violet dilution, and did not produce significant amounts of granzyme B or IFN-γ (Fig. 3-2A,B). In contrast, when recombinant B7.1 protein was presented in combination with a suboptimal amount of CD3 cross-linking antibody, the extent of $CD8^{+}$ T cell activation increased in proportion to the amount of B7.1 co-stimulator ligand provided

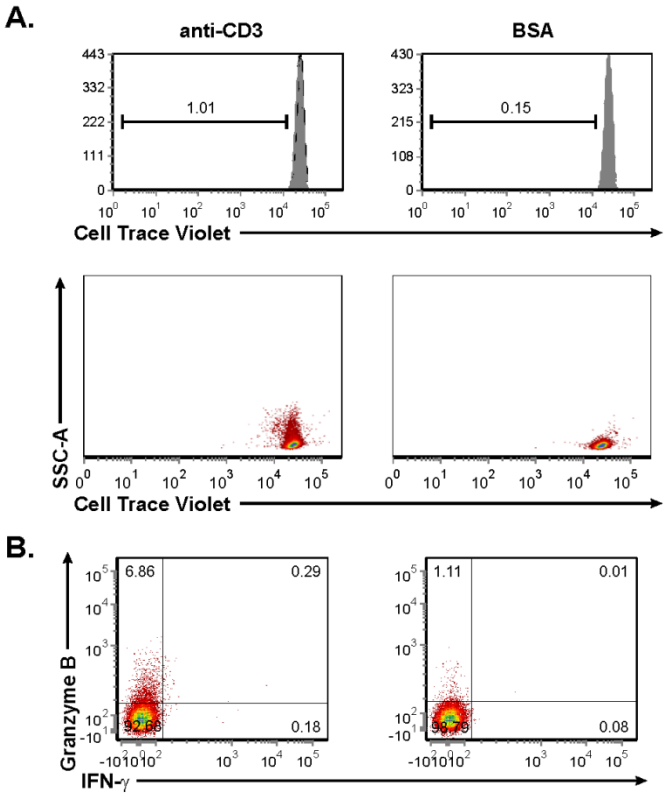
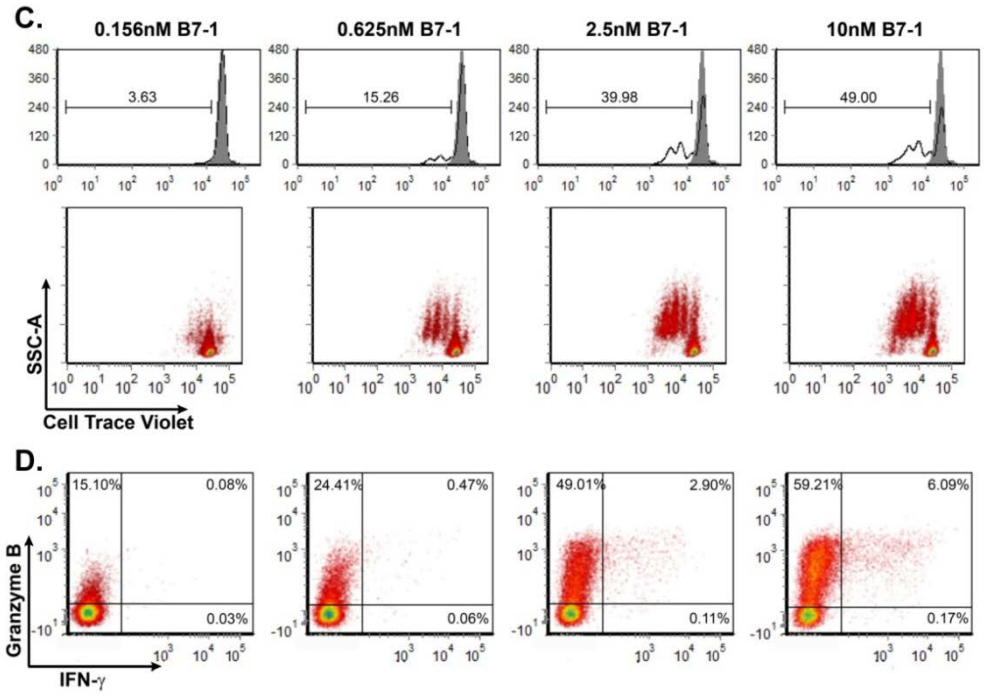


Figure 3-2. Enhanced CD44^{lo} CD8^{+} T cell proliferation, granzyme B and IFN-γ production following increased recombinant B7.1 co-stimulation relative to controls. *Ex vivo* CD44^{lo} CD8^{+} T cells were incubated for 48 hours with the indicated amounts of recombinant B7.1 protein applied to polystyrene beads in combination with a suboptimal amount of anti-CD3 antibody (0.125µg/1 x10^{7} beads) , anti-CD3 alone, or with BSA coated beads. (*A*) The extent of CD8^{+} T cell proliferation following stimulation anti-CD3 and BSA control beads was determined by flow cytometry using cell trace violet dye dilution, and is depicted in histograms (upper panels) or dot plots (lower panels). (*B*) The percentage of granzyme B positive and IFN-γ positive CD8^{+} cells following stimulation with control anti-CD3 and BSA coated beads. (C) CD8^{+} T cell proliferation in response to increased recombinant B7.1 immobilization to beads in combination with sub-optimal amounts of anti-CD3 determined by cell trace violet dilution. (D) The percentage of granzyme B positive and IFN-γ positive CD8^{+} cells following stimulation with increasing amounts in recombinant B7.1 protein. Cells were fixed and permeabilized as described in *Materials and Methods* and stained with monoclonal antibodies against mouse granzyme B and IFN-γ. Shaded histograms represent undivided BSA-bead incubated controls. Results are representative of three independent experiments preformed in duplicate.


C.
0.156nM B7-1
0.625nM B7-1
2.5nM B7-1
10nM B7-1
3.63
15.26
39.98
49.00
SSC-A
Cell Trace Violet
D.
15.10%
0.08%
0.03%
24.41%
0.47%
0.06%
49.01%
2.90%
0.11%
59.21%
6.09%
0.17%
Granzyme B
IFN-γ

during stimulation (Fig. 3-2C, D). Increasing B7.1 co-stimulation led to increases in proliferation of $CD44^{lo}$ naïve $CD8^+$ T cell as determined by cell trace violet dilution, as well as increases in the percentage of cells which underwent multiple divisions (Fig. 3-2C). Elevated production of intracellular granzyme B and IFN-γ was also observed in a B7.1 dependent manner (Fig. 3-2D). Intracellular granzyme B could be detected at all B7.1 densities tested and was increased substantially following high amounts of B7.1 co-stimulation (Fig. 3-2D). By contrast, intracellular IFN-γ could only be detected following co-stimulation with high amounts of B7.1. These results confirm that this approach induces activation of $CD44^{lo}$ naïve $CD8^+$ T cells, and that enhanced $CD8^+$ T cell proliferation, granzyme B and IFN-γ production correlate with increased B7.1 co-stimulation.

Differential activation of $CD44^{lo}$ naïve $CD8^+$ T cells following stimulation with increasing amounts of recombinant co-stimulator ligands and cross-linking antibodies

I next sought to determine how co-stimulation through LFA-1, CD27 and ultimately HVEM compared to $CD8^+$ T cell activation observed by increasing stimulation through CD28. To explore the efficiency of CD28, LFA-1 and CD27 in the activation of $CD44^{lo}$ naïve $CD8^+$ T cells, bead constructs were initially prepared displaying increasing amounts of recombinant B7.1, ICAM-1 or CD70 proteins, in combination with suboptimal amounts of CD3ε cross-linking antibody. Bead constructs were incubated with cell trace violet labeled $CD44^{lo}$ naïve $CD8^+$ T cells for 48 hours and assessed for the extent of their activation by cell trace violet dilution and intracellular staining for granzyme B and IFN-γ using

flow cytometry. Of the three recombinant molecules tested, each induced some activation of $CD44^{lo}$ naïve $CD8^+$ T cells but to different extents, which was dependent on the type of response examined. ICAM-1 co-stimulation was the most effective at inducing $CD8^+$ T cell proliferation and granzyme B production, involving up to 80-100% of the cells, compared to stimulation with either B7.1 or CD70 (Fig. 3-3A,B). For proliferation, co-stimulation by B7.1 plateaued at a level half that of ICAM-1 (Fig. 3-3A). While co-stimulation through B7.1 activated $CD44^{lo}$ naïve $CD8^+$ T cells with somewhat less efficiency than ICAM-1, based on proliferation and granzyme B production data, it was superior to ICAM-1 at inducing IFN-γ production (Fig. 3-3C). However, intracellular IFN-γ could only be detected in a relatively small percentage of cells following even high B7.1 co-stimulation. This may indicate that optimal production of this cytokine is limited to a small subset of cells, or perhaps may not be easily detected by stimulation through a single co-stimulator when provided in combination with a suboptimal TCR stimulus. The stimulated $CD8^+$ T cells were also assessed for their surface expression of the death inducing ligand FasL. Of the three recombinant co-stimulator molecules tested, ICAM-1 induced the highest expression of FasL at the 48 hour time point (Fig. 3-3D). However, the FasL expression induced by ICAM-1 co-stimulation was detected in a low percentage of cells, and was quite variable, preventing any conclusions being made as to whether this increase in FasL expression was significant. Finally, whether for proliferation, granzyme B or IFN-γ production, recombinant CD70 was found to be very inefficient at co-stimulating $CD44^{lo}$ naïve $CD8^+$ T cells. Of the three

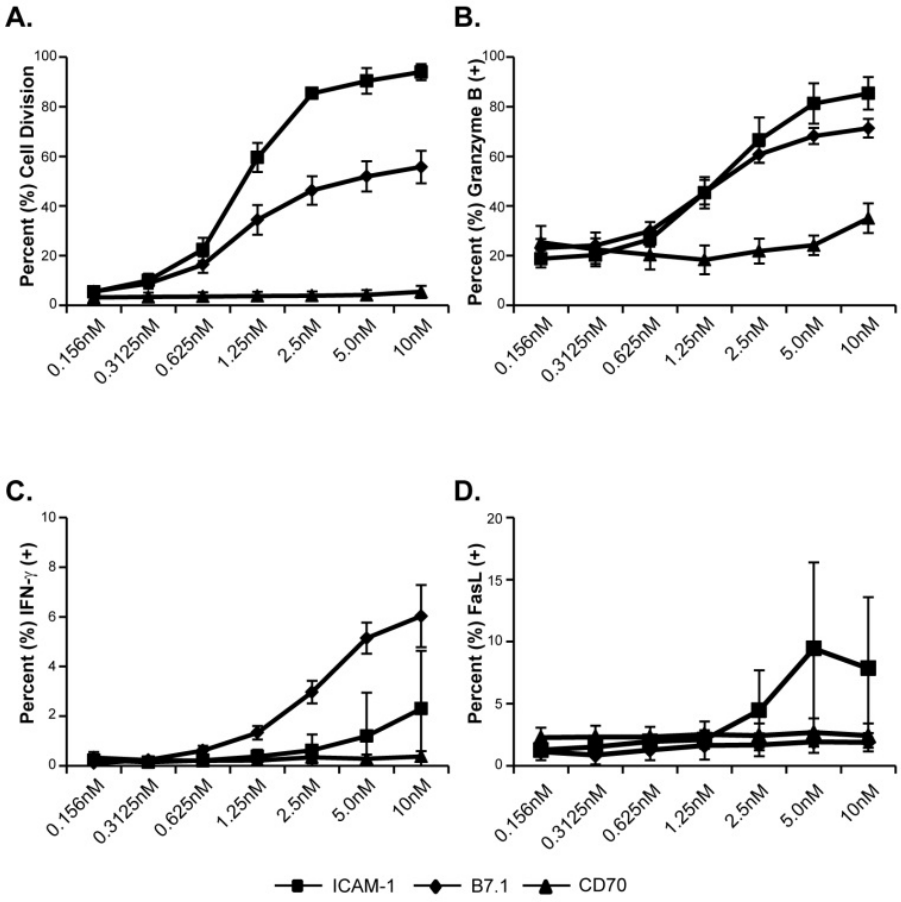


Figure 3-3. Differential activation and effector molecule production by CD44^{lo} CD8^{+} T cells stimulated with increasing amounts of recombinant co-stimulator ligands. CD44^{lo} CD8^{+} T cells were stimulated for 48 hours with increasing amounts (0.156nM-10nM) of recombinant B7.1, ICAM-1 and CD70 co-stimulator ligands immobilized on polystyrene beads, in combination with a suboptimal amount of anti-CD3 antibody (0.125µg/1 x 10^7 beads). Cells were assessed 48 hours later for (*A*) proliferation by cell trace violet dilution and for granzyme B (*B*) and IFN-γ (*C*) production by intracellular staining, detected by flow cytometry. Surface FasL expression (*D*) was also detected by flow cytometry using fluorochrome-conjugated FasL mAbs. Percent cell division was determined using undivided cells as a control, while granzyme B, IFN-γ, and FasL expression was determined relative to isotype stained control cells. Results represent the mean of three independent experiments performed in duplicate. Error bars represent standard deviation of experimental means.

different responses examined, only a slight increase in intracellular granzyme B could be observed following CD70 co-stimulation at high density (Fig. 3-3A-C). This indicates that recombinant CD70 is either incapable of inducing $CD44^{lo}$ naïve $CD8^{+}$ T cell stimulation when in combination with TCR ligation, or the recombinant protein is not effectively recognized.

To compare results between recombinant proteins and cross-linking antibodies, and to broaden the study to include HVEM co-stimulation, a similar set of co-stimulation experiments were performed utilizing co-stimulator-specific cross-linking antibodies. An antibody against HVEM was chosen for the ligation of this receptor as no recombinant LIGHT (an HVEM activating ligand) was commercially available. For these experiments, increasing amounts of monoclonal antibodies against CD28, CD27 and HVEM were displayed on the surface of beads in combination with suboptimal amounts of CD3ε cross-linking antibody. Prepared bead constructs were incubated with freshly isolated cell trace violet-labeled $CD44^{lo}$ naïve $CD8^{+}$ T cells for 48 hours whereupon the extent of $CD8^{+}$ T cell activation was assessed by cell trace violet dilution, intracellular detection of granzyme B and IFN-γ, as well as FasL surface expression. Similar to the results found with recombinant B7.1 co-stimulator ligand, CD28 cross-linking antibody was able to induce a significant percentage of $CD8^{+}$ T cells to proliferate and produce granzyme B, but induced IFN-γ production in only 2% of the cells (Fig. 3-4A-C*)*. Induction of $CD44^{lo}$ $CD8^{+}$ T cell proliferation and granzyme B production by a suboptimal TCR stimulation was found to be approximately twice

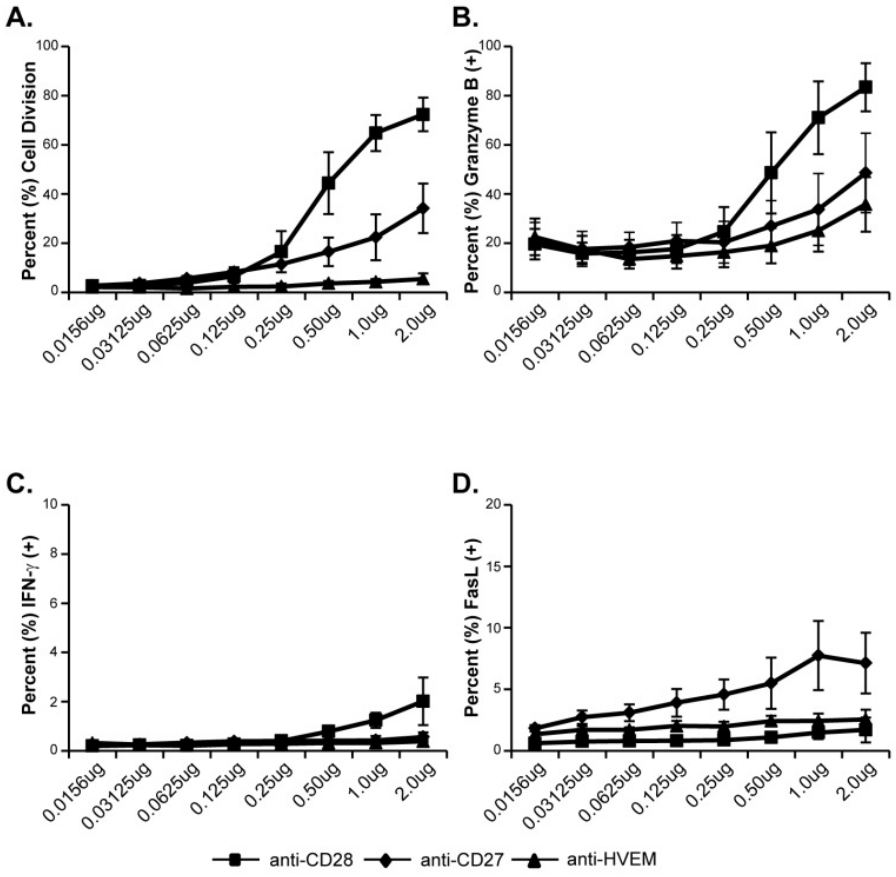


Figure 3-4. Differential activation and effector molecule production by CD44^{lo} CD8^{+} T cells stimulated with increasing amounts of co-stimulator ligand cross-linking antibodies. CD44^{lo} CD8^{+} T cells were stimulated for 48 hours with increasing amounts (0.0156µg – 2µg) of CD28, CD27 or HVEM specific cross-linking antibodies immobilized on polystyrene beads in combination with a suboptimal amount of anti-CD3 antibody. CD8^{+} T cell activation was determined by proliferation (*A*) using cell trace violet dilution, and by granzyme B (*B*) and IFN-γ (*C*) production by intracellular staining, detected by flow cytometry. Surface FasL expression (*D*) was also detected by flow cytometry using fluorochrome-conjugated FasL mAbs. Percent cell division was determined relative to undivided T cell controls, while granzyme B, IFN-γ, and FasL expression were determined relative to isotype stained control cells. Results represent the mean of three independent experiments performed in duplicate. Error bars represent standard deviation of experimental means.

as effective with CD28 cross-linking than with CD27 cross-linking, throughout the antibody density titrations (Fig. 3-4A,B). There was also a difference in $CD8^+$ T cell activation following the use of CD27 cross-linking antibody in replacement of CD70. CD27 cross-linking antibody was much more efficient than recombinant CD70 at inducing $CD44^{lo}$ naïve $CD8^+$ T cell proliferation and granzyme B production when provided in combination with a suboptimal amount of anti-CD3ε (Fig. 3-4A-C). This result indicates that while a co-stimulatory signal can be generated through CD27, the recombinant CD70 protein used in the previous experiments may be ineffective at doing so. Cross-linking antibodies to HVEM however did not trigger proliferation and IFN-γ production at any density, and only induced a slight increase in the percentage of $CD8^+$ T cells expressing intracellular granzyme B (Fig. 3-4A-C). These results suggest that, while HVEM ligation has been reported to enhance naïve $CD44^{lo}$ $CD8^+$ T cell activation, the use of cross-linking antibody to ligate HVEM does not appear to be ideal for inducing a strong co-stimulatory signal through this receptor. Finally, similar to the FasL expression detected following recombinant co-stimulatory molecule ligation, cross-linking antibodies to CD28, CD27 and HVEM also failed to induce a significant increase in FasL expression. However a slight increase in the percentage of cells positive for FasL (~7%) was observed when CD27 cross-linking antibodies were provided at high density (Fig. 3-4D).

Finally, to expand our analysis, $CD8^+$ T cells were assessed at the 48 hour time point following co-stimulation with recombinant B7.1 and ICAM-1 proteins, or CD28 and CD27 cross-linking antibodies to determine the percentage of cells

which had undergone two or more divisions, through comparison of the generated cellular division profiles. $CD8^+$ T cells which received a low amount (0.125nM or 0.625nM) of B7.1 or ICAM-1 co-stimulation in combination with suboptimal amounts of anti-CD3 antibody, underwent a similar number of divisions with a small percentage of cells induced to undergo up to three cellular divisions (Fig. 3-5A). However, when high amounts of the individual co-stimulatory ligands were provided (2.5nM and 10nM), ICAM-1 co-stimulation tended to induce a higher percentage of cells to undergo two and three divisions, in comparison to B7.1 (Fig. 3-5A). Approximately 35% of cells stimulated with 10nM of ICAM-1 underwent three divisions while only 13% of $CD8^+$ T cells stimulated with 10nM of B7.1 were induced to undergo three rounds of division (Fig. 3-5A). Thus, ICAM-1 stimulates greater entry into cell division (Fig. 3-3A), and a higher percentage of cells to undergo multiple divisions. In addition, when CD28 and CD27 cross-linking antibodies were compared for their ability to induce multiple rounds of cell division following ligation on naïve $CD44^{lo}$ $CD8^+$ T cells, CD28 cross-linking was able to induce more rounds of proliferation than CD27 cross-linking antibody when both were at higher densities (Fig. 3-5B). Finally, anti-CD28 was slightly better at inducing multiple rounds of T cells proliferation than B7.1 when provided at higher amounts (Fig. 3-5A,B). Together these findings, in addition to experiments assessing overall $CD8^+$ T cell proliferation and effector molecule production, indicate that stimulation with recombinant ICAM-1 results in more effective $CD44^{lo}$ naïve $CD8^+$ T cell activation than stimulation with any of the cross-linking antibodies or recombinant CD70. The same can be said for

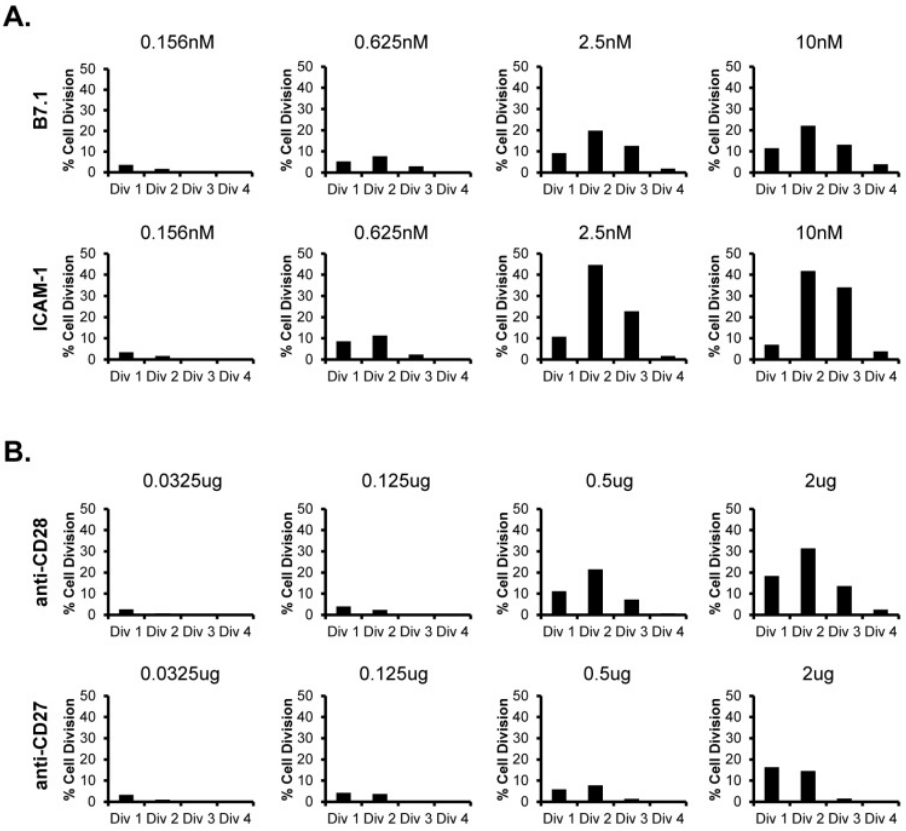


Figure 3-5. Naïve CD44^{lo} CD8^{+} T cells are stimulated to undergo multiple rounds of proliferation following co-stimulator molecule ligation. The percentage of CD8^{+} T cells which underwent one, two, three, or four cellular divisions was determined following 48hrs of naïve CD8^{+} T cell stimulation with a suboptimal amount of CD3 cross-linking antibody in combination with 0.125nM, 0.625nM, 2.5nM and 10nM of B7.1 or ICAM-1 (*A*) or 0.0325µg, 0.125µg, 0.5µg, and 2µg of CD28 or CD27 cross-linking antibody (*B*). The percentage of cells in each division was determined using cell trace violet dilution dye and calculated using FCS Express analysis software using undivided BSA stimulated cells as a negative control. Percentages represent the mean of three experiments performed in duplicate.

recombinant B7.1 with the exception that anti-CD28 does modestly improve the number of rounds of division relative to B7.1, and can also be used to induce significant $CD8^+$ T cell proliferation and granzyme B production when combined with a suboptimal TCR stimulus.

Combinations of recombinant co-stimulator ligands and cross-linking antibodies can be used to optimally activate naïve $CD44^{lo}$ $CD8^+$ T cells

While significant $CD44^{lo}$ naive $CD8^+$ T cell activation could be achieved following stimulation with bead constructs bearing individual co-stimulator ligands, I was also interested in how combinations of such ligands would affect the activation of naive $CD8^+$ T cells, for a number of reasons. First, it may be possible that some combinations of co-stimulator ligands are more effective at inducing T cell activation or effector molecule production than others, and could enhance activation over that which occurs when these co-stimulator ligands are provided individually. Secondly, the specific functional T cell responses induced may differ depending on the particular combination of co-stimulator ligands presented. Thirdly, it is likely that it is combinations of co-stimulator ligands expressed by dendritic cells that naive T cells will encounter *in vivo*.

To begin the investigation, bead constructs were prepared with combinations of increasing amounts of recombinant co-stimulator ligands B7.1, ICAM-1 and CD70. In these experiments, lower to moderate density (0.156nM – 1.25nM) of B7.1 and ICAM-1 were displayed on the surface of the bead, as high densities of these proteins individually were previously found to induce a significant amount of $CD8^+$ T cell activation (Fig. 3-3). Recombinant CD70,

however, was provided at a higher density (1.25nM -10nM), as it appeared to only affect $CD8^+$ T cell activation marginally when presented in high amounts in previous experiments (Fig. 3-3). Combinations of B7.1/ICAM-1, B7.1/CD70, and ICAM-1/CD70 recombinant protein were immobilized onto beads and incubated with freshly isolated $CD44^{lo}$ naïve $CD8^+$ T cells for 48 hours following which the cells were assessed for activation by cell proliferation, intracellular granzyme B and IFN-γ production. Of the co-stimulatory pairs tested, the combination of recombinant B7.1 and ICAM-1 resulted in the most extensive activation, with near maximal amounts of proliferation and granzyme B production (Fig. 3-6A). Of note, this combination of co-stimulator ligands also resulted in much higher IFN-γ detection than when either of these proteins was provided individually, indicating that this combination may be particularly effective at inducing production of this cytokine by $CD44^{lo}$ $CD8^+$ T cells. When B7.1 was paired with recombinant CD70 however, only slight increases were seen in $CD8^+$ T cell proliferation, granzyme B and IFN-γ production with most of the T cell activation occurring as a result of B7.1 increasing stimulation (Fig. 3-6B). In comparison, when ICAM-1 was paired with CD70, large amounts of $CD8^+$ T cell proliferation and granzyme B production could be detected but no IFN-γ, despite the appearance of efficient T cell activation (Fig. 3-6C). Similar to the combination of B7.1 and CD70 however, the increase in proliferation and granzyme B production following co-stimulation with ICAM-1 and CD70 appeared to be mostly due to increasing ICAM-1 density. This indicates that while recombinant CD70 may be able to induce a slight increase in naïve $CD44^{lo}$ $CD8^+$ T cell

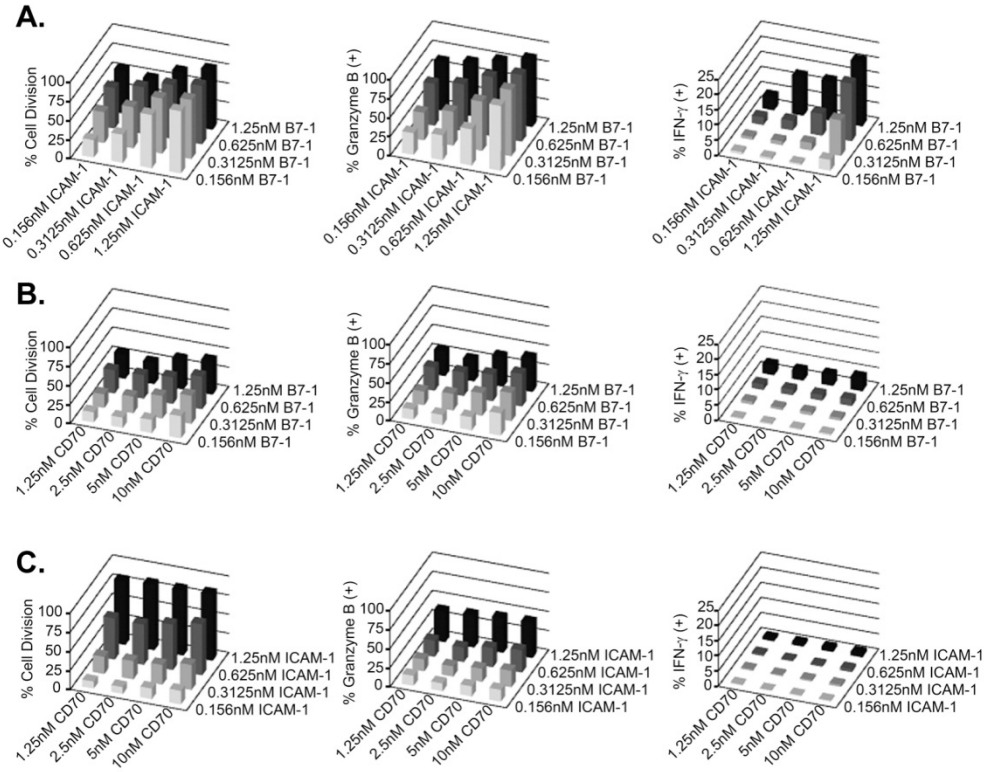


Figure 3-6. Cross-titration of recombinant co-stimulator ligands directs the generation of effector CD8$^+$ T cells with distinct phenotypes. *Ex vivo* CD44^{lo} CD8$^+$ T cells were labeled with cell trace violet and incubated for 48 hours with polystyrene beads presenting the indicated combinations of co-stimulator ligands, in addition to sub-optimal amounts of anti-CD3 antibody. Recombinant proteins B7.1 and ICAM-1 (0.156nM-1.25nM) (*A*), B7.1 (0.156nM-1.25nM) and CD70 (1.25nM-10nM) (*B*), and ICAM-1 (0.156nM-1.25nM) and CD70 (1.25nM-10nM) (*C*), were presented in combination as indicated to naïve CD44^{lo} CD8$^+$ T cell and their activation was assessed 48hrs later. T cell proliferation and the production of granzyme B and/or IFN-γ were used to determine T cell activation. Percent cell division was determined using cell trace violet dilution and calculated relative to undivided T cell controls. Granzyme B and IFN-γ were detected using granzyme B and IFN-γ mABs and flow cytometry as described in the *Material and Methods*. The percentage of cells positive for granzyme B and IFN-γ was determined relative to isotype stained controls. Data is representative of two experiments performed in duplicate.

activation when co-presented with another recombinant co-stimulatory protein, its contribution is minimal.

As a second approach to evaluate the role of CD27 in the activation $CD44^{lo}$ naïve $CD8^{+}$ T cells, cross-linking antibody against CD27 was used in place of recombinant CD70 protein. An increasing amount (0.125µg – 1µg) of cross-linking CD27 antibody was presented in combination with an increasing amount of CD28 cross-linking antibody (0.0156µg – 0.125µg) and a fixed suboptimal amount of CD3ε antibody, and used to stimulate $CD44^{lo}$ $CD8^{+}$ T cells. A lower density of CD28 cross-linking antibody was selected for these experiments as higher amounts of this antibody led to near maximal activation of the T cells when in combination with anti-CD27, in initial experiments. As described above, cell trace violet labeled cells were incubated with bead constructs for 48 hours after which the cells were assessed for cell trace dilution, granzyme B, and IFN-γ production. Contrary to what was observed using recombinant CD70, when increasing amounts of CD27 cross-linking antibody were added in combination with CD28 cross-linking antibody, CD27 ligation led to enhanced $CD8^{+}$ T cell proliferation (Fig. 3-7A). However, despite enhancement of T cell proliferation, the combination of CD27 and CD28 cross-linking antibodies failed to result in a large increase in granzyme B or IFN-γ production (Fig. 3-7A). In a similar set of experiments, increasing amounts of cross-linking antibodies against CD28 or CD27 were also presented in combination with increasing amounts of HVEM cross-linking antibody. When anti-HVEM was paired with either anti-CD28 or anti-CD27 however, only slight

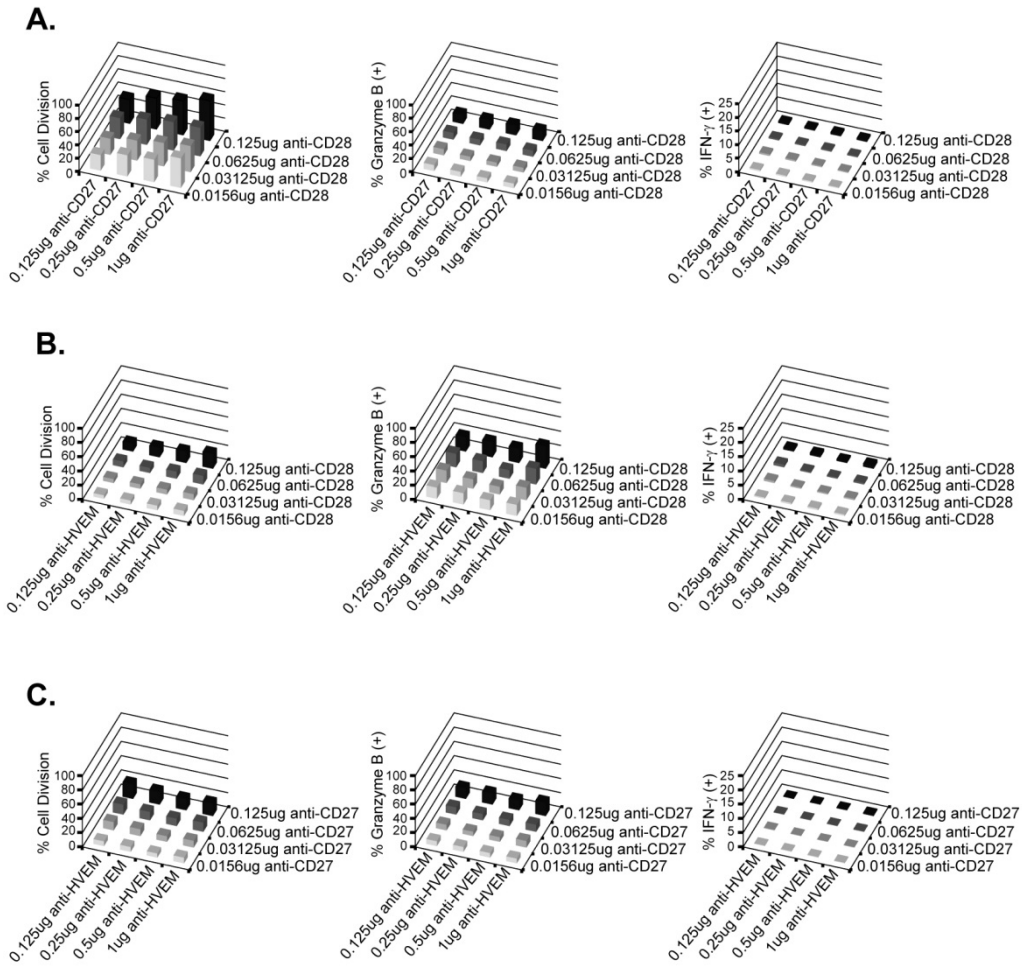


Figure 3-7. Cross-linking antibodies specific for the co-stimulatory molecules CD27 and HVEM, in combination with anti-CD28, induce minor increases in CD44^{lo} CD8^{+} T cell activation. *Ex vivo* CD44^{lo} CD8^{+} T cells were labeled with cell trace violet and incubated for 48 hours with polystyrene beads presenting the indicated combinations of cross-linking antibodies specific to CD28, CD27, and HVEM in addition to sub-optimal amounts of anti-CD3 antibody. Cross-linking antibodies to CD27 (1µg - 0.125µg) and to CD28 (0.125µg - 0.0156µg) (*A*), CD28 (0.125µg - 0.0156µg) and HVEM (1µg – 0.125µg) (*B*), or CD27 (1µg – 0.125µg) and HVEM (1µg – 0.125µg) (*C*) were presented in combination as indicated to naïve CD44^{lo} CD8^{+} T cells and their activation was assessed 48hrs later. T cell proliferation and the production of granzyme B and/or IFN-γ were used to determine T cell activation. Percent cell division was determined using cell trace violet dilution and calculated relative to undivided T cell controls. Granzyme B and IFN-γ were detected using granzyme B and IFN-γ mABs and flow cytometry as described in the *Material and Methods.* The percentage of cells positive for granzyme B and IFN-γ was determined relative to isotype stained controls. Data is representative of two experiments performed in duplicate.

increases in $CD8^+$ T cell activation could be detected with both combinations. Proliferation and granzyme B production from the activated $CD8^+$ T cells was only increased by ~15% when comparing high and low densities of the CD28/HVEM or CD27/ HVEM cross-linking antibody combinations (Fig. 3-7 B,C). Furthermore, following $CD8^+$ T cell co-stimulation with both of these cross-linking antibody combinations, no intracellular IFN-γ could be detected (Fig. 3-7B, C). This then further indicates that the HVEM cross-linking antibodies used in these experiments may not be ideal for potentiating HVEM activating co-stimulator signals.

As CD27 cross-linking antibody alone appeared to be more effective than CD70 at enhancing CD8 T cell proliferation and to some extent, granzyme B production (Fig. 3-3, 3-4), a final set of experiments was performed in which increasing amounts of CD27 cross-linking antibody was added to beads in combination with increasing amounts of recombinant B7.1 (0.156nM- 1.25nM) (Fig. 3-8A), or increasing amounts of recombinant ICAM-1 (0.156nM – 1.25nM) (Fig. 3-8B). Again, bead constructs were incubated for 48 hours with cell trace violet labeled *ex vivo* $CD44^{lo}$ naïve $CD8^+$ T cells and assessed for the extent of activation through cell trace dilution and effector molecule production. The combination of B7.1 and CD27 cross-linking antibody led to increased cell proliferation and granzyme B production (Fig. 3-8A) in comparison to when cross-linking antibodies against both CD27 and CD28 were used in combination (Fig. 3-7A). However the majority of these increases were due to increased CD28 signalling and intracellular IFN-γ could only be detected in cells when high

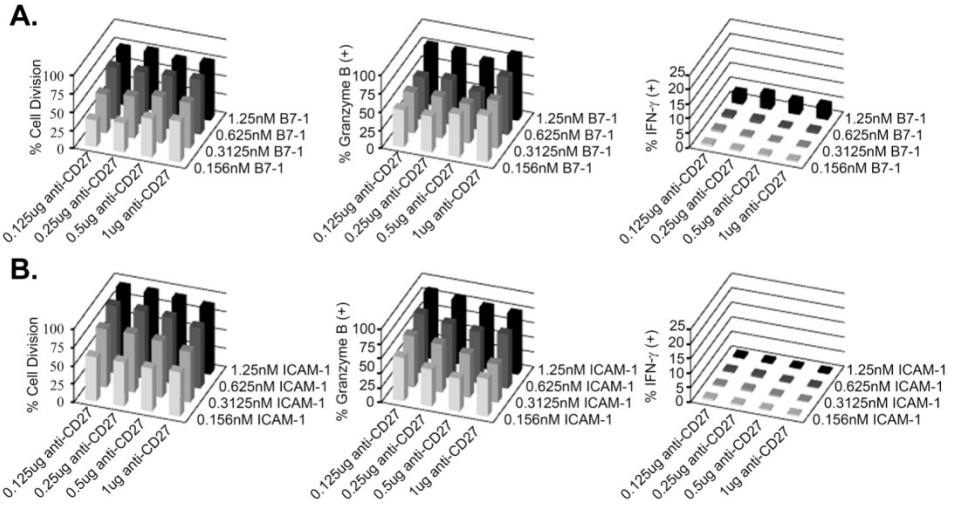


Figure 3-8. CD27 cross-linking antibody provided in combination with recombinant B7.1 and ICAM-1 co-stimulatory ligands augments CD8$^+$ T cell effector differentiation. *Ex vivo* CD44^{lo} CD8$^+$ T cells were labeled with cell trace violet and incubated for 48 hours with polystyrene beads presenting the indicated combinations of co-stimulator ligands or antibodies specific to co-stimulators, in addition to sub-optimal amounts of anti-CD3 antibody. Cross-linking antibodies to CD27 (1µg - 0.125µg) and recombinant B7.1 (0.156nM-1.25nM) (*A*), or recombinant ICAM-1 (0.156nM-1.25nM) (*B*), were presented in combination as indicated to naïve CD44^{lo} CD8$^+$ T cell and their activation was assessed 48hrs later. T cell proliferation, the production of granzyme B, and/or IFN-γ were used to determine T cell activation. Percent cell division was determined using cell trace violet dilution and calculated relative to undivided T cell controls. Granzyme B and IFN-γ were detected using granzyme B and IFN-γ mABs and flow cytometry as described in the *Material and Methods.* The percentage of cells positive for granzyme B and IFN-γ was determined relative to isotype stained controls. Data is representative of two experiments performed in duplicate.

amounts of B7.1 were used, indicating that the combination of B7.1 and ICAM-1 was still superior for the induction of this cytokine (Fig. 3-6A). Similarly, $CD8^+$ T cells stimulated with a combination of ICAM-1 and CD27 cross-linking antibody in comparison, lead to high percentages of T cells proliferating and producing granzyme B, but no intracellular IFN-γ could be detected at any density tested (Fig. 3-8B). Again, CD27 ligation did not drastically increase any of these parameters, and even tended to have an overall inhibitory effect on $CD8^+$ T cells proliferation and granzyme B production when co-presented with ICAM-1 (Fig. 3-8B). Specifically, when cells were provided with 0.625nM of ICAM-1 co-stimulation, the addition of increasing amounts of cross-linking CD27 antibody decreased cell proliferation by almost 10 % (91.45% - 82.69%) while the same addition of CD27 cross-linking antibody to 0.3125nM of ICAM-1 led to nearly a 20% decrease in granzyme B production (69.48% - 51.85%) (Fig. 3-8 B). A closer examination of the cellular proliferation profiles revealed that not only did increasing CD27 cross-linking antibody lower the total percentage of cells which underwent proliferation when combined with ICAM-1, fewer cells tended to reach the third round of division as the amount of CD27 co-stimulation increased (Fig. 3-9). Of note, no such inhibitory effect on proliferation was observed following increased CD27 co-stimulation when in combination with B7.1 (Fig. 3-8A, Fig. 3-9), suggesting that this co-stimulator combination may better cooperate in promoting effector $CD8^+$ T cell numbers, phenotype, and function. Taken together, these data suggest that the recombinant B7.1 and ICAM-1 protein combination is more effective at promoting $CD44^{lo}$ naïve $CD8^+$ T cells to display

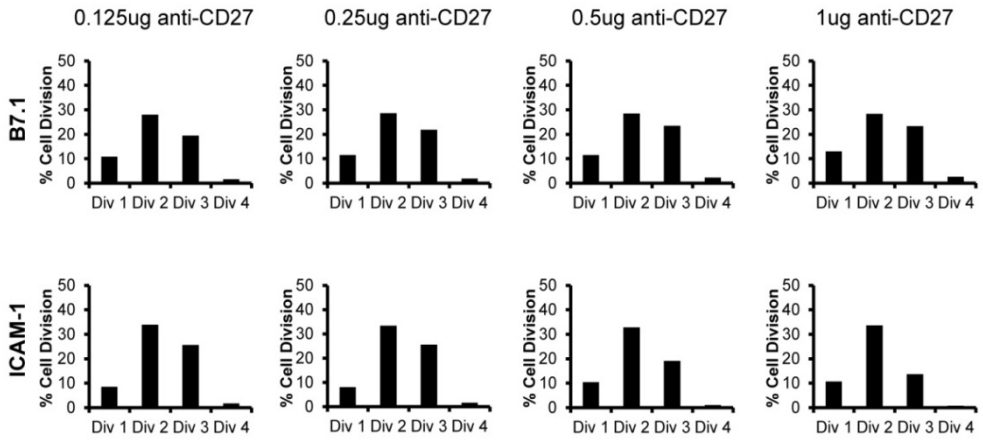


Figure 3-9. Increasing CD27 co-stimulation decreases the percentage of cells which undergo higher rounds of cell division when in combination with ICAM, but not B7.1. $CD44^{lo}$ $CD8^{+}$ T cells stimulated with either 0.625nM of B7.1 (top) or 0.625nM of ICAM-1 (bottom) in combination with increasing amounts of CD27 cross-linking antibody (0.125µg – 1µg) were assessed for the percentage of cells in each cellular division following stimulation. The percentage of cells in each division was determined by cell trace violet dye and calculated using FCS express analysis software with undivided BSA stimulated cells as a negative control. Data are representative of two independent experiments performed in duplicate.

a full spectrum of measured responses: proliferation, production of granzyme B and IFN-γ, than any of the other cross-linking antibody or recombinant protein combinations tested. Furthermore, the degree of cell proliferation, as well as the type and extent of effector molecule expression, can differ depending on the particular combination of co-stimulator ligands/antibodies presented to $CD44^{lo}$ naïve $CD8^{+}$ T cells.

B7.1 and ICAM-1 stimulated naïve $CD44^{lo}CD8^{+}$ T cells display cytolytic activity

Previous data indicated that naïve $CD44^{lo}$ $CD8^{+}$ T cells stimulated with high amounts of B7.1 and ICAM-1 recombinant proteins in combination with suboptimal CD3ε cross-linking antibody resulted in efficient T cell proliferation and granzyme B production (Fig. 3-3A,B). To test the functional capacity of the stimulated cells to lyse target cells, I employed rADCC assays in which $CD8^{+}$ T cells stimulated for 48 hours with bead constructs were incubated with ^{51}Cr-labeled P815 target cells bearing Fc-receptor bound with CD3ε cross-linking antibody to stimulate T cell-mediated cytolysis. In this way, I could determine whether the activated $CD8^{+}$ T cells had cytotoxic capacity, without requiring knowledge of antigen specificity following anti-CD3 stimulation in conjunction with co-stimulatory signals. Bead constructs were prepared by immobilization of high amounts (10nM) of recombinant B7.1 or ICAM-1 co-stimulator ligand or high amounts (1µg) of CD27 and HVEM cross-linking antibodies to beads in combination with suboptimal amounts of CD3ε cross-linking antibody. CD27 specific cross-linking antibody was chosen for these experiments as it was more efficient at activating $CD44^{lo}CD8^{+}$ T cells than the recombinant CD70 protein in

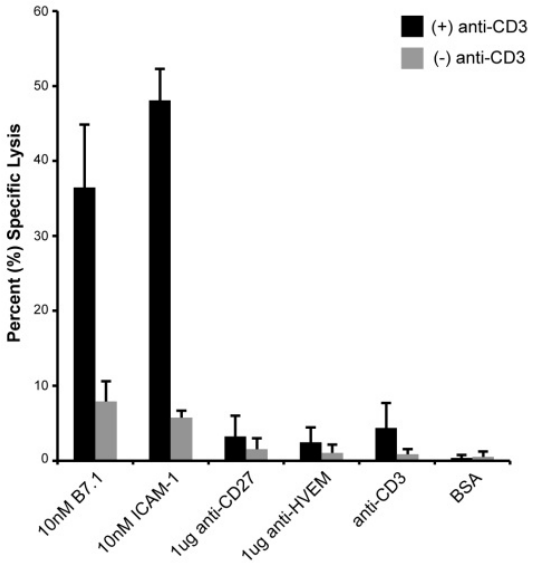


Figure 3-10. B7.1 or ICAM-1 co-stimulated CD44^{lo} CD8^{+} T cells lyse target cells in ^{51}Cr release assays. CD44^{lo} CD8^{+} T cells were stimulated for 48hrs with polystyrene beads coated with recombinant B7.1, ICAM-1 (10nM), anti-CD27, or anti-HVEM (1µg) cross-linking antibodies in combination with a suboptimal amount of anti-CD3. Beads coated with suboptimal anti-CD3 and BSA coated beads were used as controls for T cell stimulation. Stimulated CD8^{+} T cells were incubated for 4 hours in the presence of ^{51}Cr labeled P815 target cells and 40µg/mL of anti-CD3 cross-linking antibody (black bars), as described in *Materials and Methods*. P815 target cells and stimulated T cells incubated in the absence of anti-CD3 cross-linking antibody (grey bars) served as negative controls for cell lysis in the cytotoxicity assays. Following incubation with targets, supernatants were collected and analysed for ^{51}Cr release. The data and standard deviation are calculated from three independent experiments performed in triplicate.

previous assays (Fig. 3-3, 3-4). To serve as controls for $CD8^+$ T cell stimulation, beads were also prepared with suboptimal amounts of CD3ε cross-linking antibody alone, or were coated with BSA as an unstimulated control. Forty eight hours after incubation with the various bead constructs, the resulting T cells were tested in the rADCC assay. A significant level of target cell lysis was observed following incubation of naïve $CD44^{lo}$ $CD8^+$ T cells co-stimulated with recombinant B7.1 or ICAM-1 (Fig. 3-10). In correlation with slightly better stimulation of $CD8^+$ T cell proliferation and granzyme B production following recombinant ICAM-1 co-stimulation (Fig. 3-3B), recombinant ICAM-1 stimulated $CD44^{lo}$ $CD8^+$ T cells displayed better cytolytic activity on a per cell basis than recombinant B7.1 stimulated $CD8^+$ T cells in ^{51}Cr-release assays (~37% lysis by B7.1 stimulated cells compared to ~48% lysis by ICAM-1 stimulated cells) (Fig. 3-10). Little target cell lysis was observed following incubation of P815 targets with anti-CD27 or anti-HVEM co-stimulated $CD8^+$ T cells. Minimal lysis was also observed following target cell incubation with control $CD8^+$ T cells stimulated with suboptimal amounts of CD3ε cross-linking antibody, or unstimulated BSA controls (Fig. 3-10). These data demonstrate that not only does co-stimulation of naïve $CD44^{lo}$ $CD8^+$ T cells with recombinant B7.1 or ICAM-1 result in $CD8^+$ T cell proliferation, granzyme B and IFN-γ production, but the $CD8^+$ effector T cells generated display a functional capacity to lyse target cells.

B7.1 and ICAM-1 cooperate to enhance suboptimal CD44^{lo} naïve CD8^{+} T cell cytolytic capacity when provided in combination during CD8^{+} T cell activation

The data collected indicates that not only can high densities of specific single co-stimulatory ligands induce naïve CD44^{lo} CD8^{+} T cell activation, but when combined, intermediate densities of co-stimulator ligands can cooperate to lead to optimal activation of CD8^{+} T cells (Fig. 3-6 – 3-8). In particular, the combination of recombinant B7.1 and ICAM-1 co-stimulator ligands provided with suboptimal TCR stimulation, induced near maximal amounts of CD8^{+} T cell proliferation and granzyme B production, and also greatly enhanced detection of intracellular IFN-γ (Fig. 3-6A). To determine if combinations of co-stimulator ligands could also be used to enhance CD8^{+} T cell cytolytic capacity, bead constructs were prepared displaying intermediate amounts (1.25nM) of B7.1 and ICAM-1 recombinant co-stimulator ligands or high amounts (10nM) of recombinant CD70 protein either individually or in combination. All prepared bead constructs also contained suboptimal amounts of CD3ε cross-linking antibody which, on its own, also served as a control for T cell stimulation, in addition to BSA-coated negative control beads. When used at these intermediate densities as opposed to high densities used in Fig. 3-10, B7.1 stimulated CD44^{lo} CD8^{+} T cells displayed only a slight reduction in their cytolytic capacity towards ^{51}Cr labeled P815 targets (Fig. 3-10, Fig. 3-11 A,B). By comparison, ICAM-1 stimulated CD8^{+} T cells had a substantial decrease in cytolytic capacity, decreasing the cell lysis from ~48% at 10nM to ~13% when provided at an intermediate density of 1.25nM (Fig. 3-10, Fig. 3-11 A,B). These results were

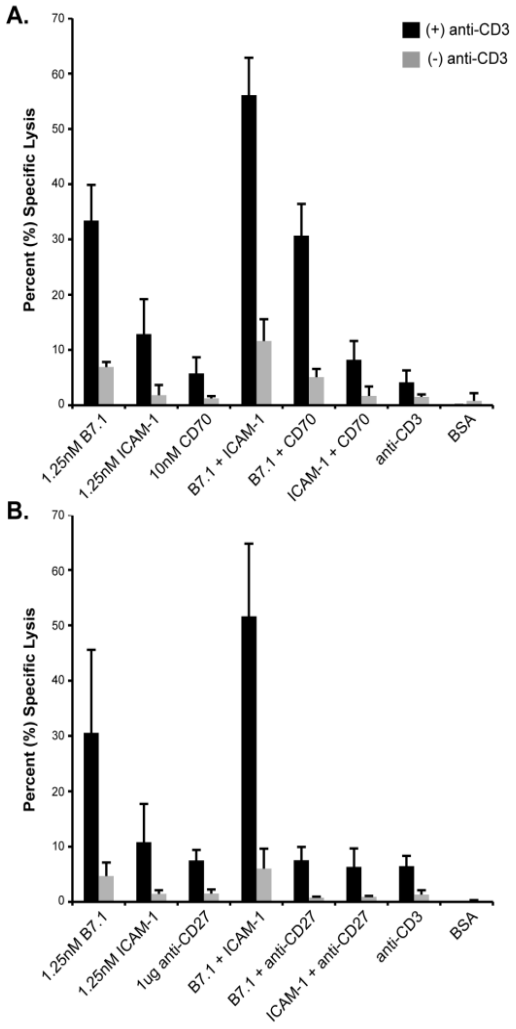


Figure 3-11. The combination of B7.1 and ICAM-1 co-stimulation is effective at generating CD8$^+$ T cells with cytotoxic capacity. CD44^{lo} CD8$^+$ T cells were stimulated for 48hrs with beads displaying suboptimal amounts (1.25nM) of recombinant B7.1, ICAM-1, or CD70 (10nM) co-stimulator ligands individually or in pair-wise combinations (*A*). (*B*) Co-stimulator ligands were used as in (*A*), but anti-CD27 cross-linking antibody (1µg) was substituted for CD70. All cultures included suboptimal stimulation with anti-CD3. Anti-CD3 cross-linking alone and BSA coated beads were used as controls for cell stimulation. Following 48 hours of stimulation, T cells were harvested and incubated for 4hrs with ^{51}Cr-labeled P815 target cells in the presence of 40µg/mL of anti-CD3 cross-linking antibody (black bars). Stimulated T cells incubated with P815 target cells in the absence of anti-CD3 cross-linking antibody (grey bars) served as a negative controls for cell lysis. Following incubation with targets, supernatants were collected and analysed for ^{51}Cr release. Data and standard deviations are calculated from three independent experiments performed in triplicate.

found to correlate with the flow cytometry data (Fig. 3-3B), in which at a density of 1.25nM, B7.1 co-stimulation of naïve $CD44^{lo}$ $CD8^{+}$ T cells showed only a minor reduction in proliferation and granzyme B production, whereas the decrease in $CD8^{+}$ T cell activation following 1.25nM of ICAM-1 co-stimulation was more dramatic. When provided in combination however, intermediate amounts of recombinant B7.1 and ICAM-1 co-stimulator ligand greatly enhanced the cytolytic ability of the stimulated $CD44^{lo}$ naïve $CD8^{+}$ T cells to kill P815 target cells in rADCC assays, increasing the percentage of specific lysis to an average of ~56% (Fig. 3-11A,B). Notably, the lysis which occurred when intermediate amounts of recombinant B7.1 and ICAM-1 were co-immobilized on beads met or exceeded the lysis observed when either recombinant co-stimulator ligand was provided individually at intermediate levels or at high density (Fig. 3-10, Fig. 3-11A,B). In particular, the inclusion of ICAM-1 with B7.1 improved the level of lysis observed relative to co-stimulation with a high density of B7.1 alone.

Co-stimulation with high amounts of recombinant CD70 (10nM) alone or in combination with either intermediate densities of B7.1 or ICAM-1, failed to increase $CD8^{+}$ T cell lysis of ^{51}Cr- labeled P815 targets above that observed with B7.1 or ICAM-1 co-stimulation alone (Fig. 3-11A). As an alternative, recombinant CD70 was replaced with high amounts (1µg) of CD27 cross-linking antibody (Fig. 3-11B), as co-stimulation through CD27 was found to be more effective using the cross-linking antibody than the recombinant CD70 protein in previous experiments (Fig. 3-3, 3-4). However, using CD27 cross-linking antibody either alone or in combination with intermediate amounts of

recombinant B7.1 or ICAM-1also failed to increase the cytolytic capacity of stimulated $CD44^{lo}$ naïve $CD8^{+}$ T cells in comparison to B7.1 and ICAM-1 co-stimulation alone (Fig. 3-11B). Not only did the combination of intermediate amounts of recombinant B7.1 with anti-CD27 fail to increase the lysis of P815 target cells, it inhibited the cytolytic capacity normally induced by B7.1 (Fig. 3-11B). Following stimulation, the percentage of specific lysis decreased from ~27% when B7.1 co-stimulation was provided alone, to ~7% when in combination with the CD27 cross-linking antibody (Fig. 3-11B). Of note, there was a significant amount of error calculated following lytic assays involving 1.25nM B7.1 co-stimulated $CD8^{+}$ T cell effectors (Fig. 3-11B), thus making the inhibitory effect of CD27 cross-linking antibodies on B7.1 induced lytic responses appear more dramatic than what may be occurring. In conclusion, these results demonstrate that not only does the combination of recombinant co-stimulator ligands B7.1 and ICAM-1 result in strong $CD44^{lo}$ $CD8^{+}$ T cell proliferation and effector molecule production following stimulation, but effector $CD8^{+}$ T cells generated in this manner also display superior cytolytic capacity against target cells in functional assays.

Discussion

Co-stimulation of naïve $CD8^+$ T cells is critical for the development of effector responses following antigen exposure [61, 62]. Not only is co-stimulation thought to fine tune effector responses, it may direct $CD8^+$ T cell populations to terminal effector or long-lived memory states. In this study, both the qualitative and density-dependent quantitative roles of major co-stimulator ligands in co-activating naïve $CD8^+$ T cells were explored with cell-sized beads onto which varying amounts of single and multiple co-stimulator ligands were displayed. Both recombinant co-stimulator ligands (B7.1, ICAM-1 and CD70) and receptor cross-linking antibodies (anti-CD28, anti-CD27, and anti-HVEM) in substitution for co-stimulator ligands, were used to quantitatively assess how co-stimulation through CD28, CD27, HVEM and LFA-1 compare in driving $CD8^+$ T cell proliferation, effector molecule production, and cytolytic capacity. Not only was I able to define differences in individual co-stimulator molecules in their ability to induce effector responses at specific ligand densities, but I was also able to determine which combinations of co-stimulator ligands could direct effector cells toward a specific phenotype.

In vivo, $CD8^+$ T cell proliferation in response to antigen is vital for the expansion of small numbers of responding T cells displaying the proper TCR, and augmentation of proliferative responses through co-stimulator ligation may be critical when antigen is limiting. In this study, proliferation was measured following naïve $CD8^+$ T cell TCR stimulation using low amounts of a high affinity CD3ε cross-linking antibody in addition to individual, as well as

combinations, of co-stimulator ligands. With respect to cellular expansion, the results indicate that co-stimulation using recombinant ICAM-1 protein is superior at inducing naïve $CD8^+$ T cell proliferation in comparison to CD28, CD27 and HVEM co-stimulation at all densities tested. Nearly 60% of the stimulated cells underwent at least one round of proliferation at an intermediate density (1.25nM) of ICAM-1, which increased to approximately 96% following stimulation with 10nM of ICAM-1. Not only did ICAM-1 induce a significant percentage of cells to divide, it also increased the percentage of cells which underwent multiple divisions. The increased $CD8^+$ T cell proliferation observed following ICAM-1 co-stimulation is likely a result of LFA-1 synergy with TCR-mediated signalling, as these signalling pathways have been reported to cooperate to promote T cell adhesion and effector generation through the common downstream signalling intermediate Protein Tyrosine Kinase 2 (PYK2) [244]. In addition, LFA-1 signalling in combination with signals from the TCR has been shown to enhance Erk1/2 MAPK signalling through increased activation and mobilization of Ras at the plasma membrane [141, 245]. Previous reports have also indicated that ICAM-1/LFA-1 signalling increases the production of the proliferation supporting cytokine IL-2 [144, 234], sustaining $CD8^+$ T cell division to a similar extent as CD28 co-stimulation [234]. However, it has also been reported that ICAM-1 co-stimulation may increase cellular apoptosis over prolonged periods [234]. Since our study is focused on the generation of short-term $CD8^+$ T cell effectors, and the assays were modified to account for differences in cell viability, the effector function observed in these experiments was likely not affected by apoptosis.

In addition to assessing cellular proliferation following stimulation with a single co-stimulator ligand, combinations of co-stimulator ligands were also tested to determine if certain combinations could enhance the extent of naïve $CD8^+$ T cell division in comparison to co-stimulator ligands provided individually. When intermediate amounts (1.25nM) of ICAM-1 and B7.1 were used in combination to stimulate naïve $CD8^+$ T cells, this combination lead to a near maximal percentage of cellular division. Not only was proliferation increased, but the combination of B7.1 and ICAM-1 appeared to synergize as the percentage of cells which underwent division was higher than the additive percentage of cells stimulated to divide when B7.1 and ICAM-1 were provided individually at these densities. However, other combinations of co-stimulatory ligands were not found to be as beneficial for enhancing cell division, as exemplified by the combination of CD27 cross-linking antibody and ICAM-1. While cross-linking CD27 alone did induce some $CD8^+$ T cell proliferation as previously reported [75, 122, 124], when paired with ICAM-1, increasing amounts of CD27 cross-linking led to a decrease in the overall percentage of cells stimulated to undergo at least one division, as well as a decrease in the number of cells which underwent three or more divisions. Recent work by Van Gisbergen et al. [246] investigating the role of CD27 co-stimulation during the activation of $CD8^+$ T cells with high affinity MHC-presented peptides or altered peptide ligands recognized by the TCR, suggests that CD27 co-stimulation may only enhance the survival of $CD8^+$ T cells generated with low antigen dose or low affinity antigen, and can increase cell death when provided in response to a high

dose of high affinity antigen. In this way, CD27 co-stimulation may be maintaining a wider antigen specific memory pool. Correspondingly, it is possible that the high affinity CD3ε cross-linking utilized in these experiments, despite low receptor engagement due to low anti-CD3 density, created a condition in which higher CD27 ligation lead to an overall inhibition of $CD8^+$ T cell responses. This property may be particularly relevant when paired with ICAM-1 as TCR signalling could be enhanced due to increased T cell-bead adhesion under these circumstances.

While the expansion of rare cell populations is important during acute infection, the generation of potent effector cells with the ability to lyse infected targets and secrete the appropriate cytokines is also critical for infection resolution [247, 248]. In addition, the generation of effector $CD8^+$ lymphocytes with specific phenotypes for either target cell killing, cytokine secretion, or both, may be essential for the clearance of certain infectious agents. Using this bead presentation system, I was able to directly assess the role that certain co-stimulator ligands or combinations of co-stimulator ligands, play in the differentiation of effector subsets. For the generation of effector $CD8^+$ T cells with cytolytic ability, the results indicate that both recombinant B7.1 and ICAM-1 co-stimulation can induce granzyme B production in activated $CD8^+$ T cells when provided at an intermediate density of 1.25nM. However, when the density of these co-stimulator ligands is increased, ICAM-1 is able to induce slightly more granzyme B expressing cells than B7.1. This finding is further supported by reverse ADCC experiments in which $CD8^+$ T cell effectors generated with high

amounts of ICAM-1 co-stimulation (10nM) lead to approximately 15% more target cell lysis than cells stimulated with 10nM of B7.1, suggesting that ICAM-1 co-stimulation is more effective at generating effectors with cytolytic capacity when provided at high density, than B7.1 on a per cell basis. When paired together however at an intermediate density of 1.25nM, equivalent to an approximately 10-fold reduction from the 10nM density used when B7.1 and ICAM-1 were tested individually at high density, the combination of B7.1 and ICAM-1 lead to the highest frequency of granzyme B expressing cells, and target cell lysis observed in reverse ADCC. This finding firmly supports that combinations of co-stimulator ligands, even when provided at lower densities, are effective at induction of $CD8^+$ T cell effector responses, and can greatly enhance T cell activation in comparison to single co-stimulator ligands provided individually at high density.

Ligation of CD27 and HVEM was found to induce little effector molecule production with only small increases in intracellular granzyme B detected with increasing amounts of cross-linking antibodies against these receptors. Additionally, target cell lysis could not be detected in reverse ADCC when T cells were activated with either CD27 or HVEM in combination with the TCR. This suggests that while some granzyme B could be detected in $CD8^+$ T cells following CD27 and HVEM cross-linking, the amount of granzyme B or other lytic components (e.g. perforin), produced from these stimulations may not be sufficient to support cytolytic activity. Of interest, when CD27 cross-linking was paired with B7.1, CD27 co-stimulation slightly enhanced granzyme B

production in the activated $CD8^+$ T cell effectors, but not when CD27 cross-linking was paired with high amounts of ICAM-1. CD27 co-stimulation however did not enhance target cell lysis in reverse ADCC assays with either B7.1 or ICAM-1, and appeared to even reduce the cytolytic capacity of the generated effectors, particularly when paired with B7.1, in comparison to when rB7.1 or rICAM-1 were provided alone. This suggests that, similar to effects of CD27 co-stimulation on T cell proliferation, high amounts of CD27 cross-linking may have an overall inhibitory effect on the generation of effector $CD8^+$ T cells with the ability to lyse targets efficiently, perhaps directing effectors away from this lytic phenotype.

In this study, I compared the ability of various co-stimulators and combinations of co-stimulators to induce the production of the cytokine IFN-γ, which is pivotal for the clearance of certain infectious microbes, enhancing antigen processing, and for promoting Th1 immune responses [12, 35, 249]. Direct assessment of co-stimulation for the production of IFN-γ showed that only B7.1 co-stimulation leads to the presence of intracellular IFN-γ at the 48 hour time point. Co-stimulation by ICAM-1 has previously been documented to induce secretion of IFN-γ from both human and murine $CD8^+$ T cells stimulated *in vitro* [144, 245]. The cultures in these studies used $CD8^+$ T cell populations that may have been comprised of both naïve and memory phenotype $CD8^+$ T cells, with the latter population perhaps particularly able to produce more IFN-γ than the $CD44^{lo}$ naïve $CD8^+$ T cells used in our assays. These differences in responding $CD8^+$ T cell populations may explain why I do not detect ICAM-1 co-stimulation of IFN-γ

production. In addition, the combined co-stimulator signals from LFA-1 and CD27 also failed to induce production of IFN-γ, suggesting that alternative co-stimulatory signals cannot compensate for CD28 co-stimulation in inducing IFN-γ. Despite this finding, the combination of intermediate amounts (1.25nM) of B7.1 and ICAM-1 resulted in the largest frequency of IFN-γ expressing cells detected in these experiments with over 20% of the stimulated $CD8^+$ T cells producing IFN-γ. This suggests that while B7.1 co-stimulation can induce some IFN-γ production when provided alone, the combination of co-stimulation through B7.1 and ICAM-1 may be ideal for generating effector $CD8^+$ T cells capable of producing IFN-γ. While the specific reason for differences in IFN-γ production following ligation of individual co-stimulator ligands and their combinations is not known, it is likely due to the convergence of individual co-stimulatory signalling pathways on transcription factors responsible for inducing IFN-γ expression. The IFN-γ promoter is under the control of several transcription factors which are activated following T cell activation including NFAT, AP-1, and NF-κB [250], several of which are target transcription factors of CD28, LFA-1, and TNFR family member signalling pathways [116, 242, 245, 250]. In addition to these, the transcription factors T-bet and Eomes are of particular importance in IFN-γ gene regulation in $CD8^+$ T cells [198, 204, 250]. Taking this into account, CD28 signalling may increase the number of activated transcription factors or sustain the activation of these transcription factors to a greater degree than signalling through LFA-1, or TNFR family member receptors, leading to increased IFN-γ production.

During *in vivo* immune responses to microbial pathogens, the effector $CD8^+$ T cell responses generated do not occur independently of other cell types as assessed in this study, but in the presence of other immune cells which provide $CD8^+$ T cells support in increasing or sustaining these effector responses. Inflammation and inflammatory responses generated at the site of infection have been shown to play a role not only in inducing the expression of co-stimulator ligands on dendritic cells and other APCs, but the inflammatory cytokines released by these cells can also influence the quality of effector $CD8^+$ T cells generated [251]. In particular, the pro-inflammatory cytokine IL-12 has been well documented for its ability to provide a "third signal" to naïve $CD8^+$ T cells, which promotes a stronger effector phenotype in responding $CD8^+$ T cells after activation resulting in increased IFN-γ and granzyme B production [146, 150], and higher expression of T-bet, which correlates with CTL generation [148, 150]. In our experiments, naïve $CD8^+$ T cells were activated in an environment presumably absent of inflammatory cytokines and were still capable of developing into potent effectors with the ability to both produce cytolytic molecules such as granzyme B and IFN-γ, and effectively lyse targets cells as measured by rADCC. These results then suggest that IL-12 and other pro-inflammatory cytokines such as IFN-α/β, are not absolutely necessary for the generation of cytotoxic T lymphocytes in short term responses, but likely augment and/or sustain such responses. These results also suggest that certain forms of co-stimulation, particularly the combination of B7.1 and ICAM-1, may be sufficient to drive the generation of $CD8^+$ T cells with an effector phenotype in the absence

of inflammation, as these cells expressed high amounts of effector molecules and were cytolytic without the addition of inflammatory cytokines. I was also able to identify some co-stimulator combinations such as ICAM-1 and anti-CD27/CD70 or B7.1 and anti-CD27/CD70, which may benefit from the presence of IL-12. The effectors generated under these conditions had a weaker effector phenotype and failed to efficiently lyse targets, suggesting that IL-12 may increase or sustain the production of granzyme B and IFN-γ in these circumstances, allowing for enhanced effector cell development.

HVEM co-stimulation has previously been reported to enhance the activation of naïve $CD8^+$ T cells when stimulated using the appropriate activating ligands [73]. In my hands however, ligation of HVEM through antibody cross-linking failed to produce significant naïve $CD8^+$ T cell activation when provided at a range of densities, or in combination with other co-stimulator ligands. Co-stimulation via HVEM is more complex than stimulation through the other co-stimulatory molecules tested in that it has an external domain comprised of four cysteine-rich domains that can be ligated by several proteins [134, 252]. It is possible that the antibody selected for these experiments did not bind the appropriate domain, or did not bind with high enough affinity to initiate positive signalling in the naïve $CD8^+$ T cells in these experiments. This, in combination with the rapid loss of HVEM from the surface of activated $CD8^+$ T cells [136], may make HVEM a poor candidate for the generation of effector $CD8^+$ T cells using this bead-based system. In addition to HVEM, CD27 co-stimulation was less effective than might be anticipated in generating effector $CD8^+$ T cells.

While CD27 has been described to partially rescue effector responses in the absence of B7.1 co-stimulation *in vivo* [75] and to enhance $CD8^+$ T cell activation and clonal expansion [76, 124], I found that ligation of CD27 using recombinant CD70 or cross-linking antibody failed to generate T cell populations with a strong effector phenotype when provided as the sole co-stimulator. The recombinant CD70 protein used in these assays might not have been effective at inducing CD27 signalling as CD70 ligates CD27 as a trimer *in vivo* [63], which may not have been replicated using the recombinant form of CD70 utilized in our assays. While CD27 ligation using cross-linking antibody did induce more $CD8^+$ T cell proliferation and granzyme B production when provided individually and in combination with B7.1 than CD70, increased amounts of this antibody tended to have an overall inhibitory effect on the generation of effector T cell populations with particularly strong effects on cell proliferation and lytic responses. As mentioned above, this effect may be due to the high affinity TCR ligation induced by the cross-linking antibody used in these experiments indicating that CD27 ligation may be better at promoting survival of effectors generated using lower affinity TCR stimulation or altered peptide ligands.

Finally, I aimed to qualitatively and quantitatively compare individual co-stimulator ligands and their combinations for their ability to generate what might be considered an "ideal" effector phenotype in the $CD8^+$ T cell populations, defined by the potential to undergo significant cellular expansion, produce effector molecules, and display cytolytic capacity. While the ideal effector phenotype will vary depending on the pathogen or target for which the $CD8^+$ T

cell populations may be generated, and the application for which they will be used, certain co-stimulator ligands and combinations thereof tended to promote a stronger effector phenotype than others. In terms of effector $CD8^+$ T cells generated for immediate use in applications requiring rapid cellular expansion and strong cytolytic responses, co-stimulation through ICAM-1 appears to be ideal as the stimulated cells undergo the most extensive proliferation with rapid acquisition of the cytolytic molecule granzyme B, and display the best ability to lyse target cells in *in vitro* killing assays. However, $CD8^+$ T cell effectors co-stimulated solely by ICAM-1 fail to produce or sustain the cytokine IFN-γ which may be critical in certain infections including those requiring the classical activation of macrophages for pathogen clearance [34, 249]. Co-stimulation using B7.1 appears to compensate for this deficiency as effectors derived using B7.1 co-stimulation alone produce low amounts of IFN-γ in addition to inducing cell proliferation and the production of functional amounts of granzyme B, albeit at lower quantities than ICAM-1. Assessment of all the stimulation conditions tested, however, revealed that the combination of B7.1 and ICAM-1 appears most ideal for generating strong phenotypic effector $CD8^+$ T cells. Even at intermediate levels this combination induced the generation of a high number of cytolytic $CD8^+$ T cells and stimulated the production of high amounts of IFN-γ from a significant portion of these cells, resulting in a cell population that appears to be adept both in lytic function as well as immune stimulation. In addition, the combination of B7.1 and ICAM-1 co-stimulation may be particularly relevant *in vivo* as DC:naïve $CD8^+$ T cell interactions may frequently involve stimulation

through CD28 and LFA-1, both of which have been shown to promote initial T cell activation, and may be required for optimal memory $CD8^+$ T cell generation [104, 106].

In conclusion, using bead-based T cell stimulation, I have quantitatively compared the major co-stimulator ligands and their combinations, for their ability to generate $CD8^+$ T cell effector populations from naïve precursors, and how co-stimulation can influence the phenotype of the effectors generated. Future applications using bead-based presentation include adoptive transfer of $CD8^+$ T cell effector populations generated *ex vivo* into either naïve or infected hosts, to examine the ability of the T cell populations to protect naïve animals against future infections, and to provide protection against ongoing infections. In addition, this work could be expanded to examine how the initial co-stimulation of naïve $CD8^+$ T cells can influence the generation of long-lived memory populations. Finally, the use of bead-based co-stimulation may be extended to ACT, as are other approaches to enhance co-stimulation [230, 235, 253], and may be a useful tool in future treatments of viral infection and cancer.

Chapter 4. Co-stimulation induced factors differentially regulate $CD8^+$ T cell survival and function following activation

Introduction

T cell contraction is a critical phase in the $CD8^+$ T cell lifecycle by which apoptotic cell death mechanisms reduce the size of expanded effector populations by 90-95%. The decrease in $CD8^+$ T cell numbers not only protects the host from excessive inflammation and autoimmunity following infection resolution, but also selects for cells destined to become long-lived memory $CD8^+$ T cells. Originally, it was hypothesized that the withdrawal of survival factors at the end of the expansion phase was the major contributor to this marked decrease in T cell numbers. However, it is now understood that extrinsic death receptor ligation and intrinsic mitochondrial induced cell death processes, in addition to cytokine deprivation, co-operate in regulating the T cell contraction process.

T cell contraction is believed to occur by two major mechanisms: 1) Activation induced cell death (AICD) and, 2) Activated cell autonomous death (ACAD) [151]. AICD is primarily observed following re-stimulation of activated T cells in the absence of co-stimulation. Re-stimulation under these conditions increases the expression of several death inducing surface receptors on T cells including Fas, the TNF receptor, and TRAIL [151, 152, 254]. Ligation of these receptors results in the induction of the caspase-dependant extrinsic cell death pathway. The extrinsic cell death pathway is initiated by the formation of the death inducing signalling complex (DISC) at the cell membrane, followed by the hallmark activation of initiator and executioner caspases, which induce T cell

apoptosis [151, 255]. Death receptor engagement is a very attractive mechanism of $CD8^+$ T cell contraction as the ligands for these receptors, including FasL and TNFα, are readily found in the T cell environment, and on activated $CD8^+$ T cell themselves [152, 256]. However, the role of death receptor induced T cell contraction *in vivo* has been controversial. While mice displaying specific mutations in either Fas or FasL develop autoimmunity and exhibit lymphoproliferative disorders [257, 258], studies involving the combined deletion of both Fas and TNF death receptors failed to result in a significant difference in T cell contraction following antigen encounter [259, 260]. This suggests that while death receptor engagement may be important in maintaining T cell homeostasis, other cell death mechanisms may also play a critical role in T cell contraction following the expansion phase.

ACAD, the second major T cell contraction mechanism [151], is associated with the deprivation of growth factors following T cell expansion, leading to cell death via intrinsic cell death mechanisms. The intrinsic pathway of cell death involves pro- and anti-apoptotic proteins situated in the mitochondria whose relative expression controls mitochondrial pore formation, cytochrome-c release, apoptosome complex formation, and T cell apoptosis [151, 152]. The major pro-apoptotic proteins associated with ACAD are the BH3-only protein Bim and its family member PUMA [151, 261, 262]. Evidence for Bim as a key player in ACAD stems from studies involving Bim knock-out mice, which exhibit elevated T cell numbers following the contraction phase [261]. Bim and PUMA induce apoptosis through their preferential interaction with the anti-apoptotic Bcl-

2 family members Bcl-2 and Bcl-xL [263]. Bcl-2 and Bcl-xL normally bind and inactivate the pro-apoptotic executioner proteins BAX and BAK found in the mitochondrial membrane. This interaction inhibits BAX and BAK oligomerization, mitochondrial pore formation, and apoptosis when T cells are healthy [151, 263]. In the presence of apoptosis inducing stimuli such as cytokine deprivation or DNA damage, the preferential interaction of the anti-apoptotic proteins Bcl-2 and Bcl-xL with Bim and PUMA, rather than BAX and BAK, results in increased BAX and BAK interactions and mitochondrial pore formation [263]. Therefore, it is believed that the balance between pro and anti-apoptotic proteins is what governs T cell survival following cytokine withdrawal *in vivo*. However, the relative expression of pro- and anti-apoptotic proteins can be modulated by T cell activating stimuli. Bcl-2 and Bcl-xL expression is increased following TCR engagement and NF-KB activation, and can also be influenced by co-stimulator molecule signalling [151, 264-266].

Co-stimulation is a crucial factor in the life and death decisions made by T cells following their activation. Co-stimulation through CD28 has been shown to decrease AICD by decreasing the expression of FasL [265], and by increasing the expression of cFLIP proteins (FLICE-like inhibitory protein). cFLIP proteins inhibit initiator caspase 8 activation preventing caspase cleavage and apoptosis induction downstream of CD95 (Fas) [267, 268]. In addition, CD28 signalling amplifies pro-survival signals mediated by the TCR and augments the activation of NF-κB [71], leading to increased expression of the anti-apoptotic protein Bcl-xL [266]. NF-κB activation also increases IL-2 secretion, which can provide

protection against intrinsic cell death mechanisms. IL-2 maintains mitochondrial Bcl-2 levels, while repressing the activation of pro-apoptotic Bim, and can also inhibit the expression of TRAIL following T cell activation [269-271]. The co-stimulators CD27 and 4-1BB have also been directly linked to $CD8^+$ T cell survival, and can increase T cell expression of the anti-apoptotic protein Bcl-xL [79, 123]. Moreover, 4-1BB ligation has also been demonstrated to inhibit Bim expression [132], further protecting activated T cells from ACAD. Finally, the adhesion molecule LFA-1 has also been reported to increase the expression of Bcl-xL, potentially enhancing memory $CD8^+$ T cell establishment [272].

These reported associations between co-stimulation and T cell survival prompted me to investigate if co-stimulation influences the survival of the $CD8^+$ T cell populations generated using our bead-based stimulatory system. Not only were certain co-stimulator ligands and their combinations found to predispose stimulated $CD8^+$ T cells for improved cell survival following activation, but co-stimulation was also found to influence anti-apoptotic protein expression and IL-2 secretion by $CD8^+$ T cells. Furthermore, enhanced IL-2 secretion induced following co-stimulation appeared to be linked to both the survival of $CD8^+$ T cells following activation, as well as granzyme B expression, demonstrating an important role for IL-2 in potent effector differentiation.

Results

Co-stimulation of CD44^{lo} CD8^{+} T cells results in differences in cell survival following activation

Antigenic stimulation of naïve CD8^{+} T cells leads to the development of a greatly expanded antigen-specific CD8^{+} T cell effector population. Homeostasis, following the clearance of antigen, requires the expanded T cell population to undergo cellular contraction in which the majority of the cells (~90-95%) die by apoptotic mechanisms [273]. The commencement of T cell contraction, and the survival of memory fated CD8^{+} T cells, is believed to be regulated by several mechanisms including co-stimulation, cytokine availability, extrinsic death receptor ligation, and the balance between anti-apoptotic and pro-apoptotic proteins [151]. Because co-stimulation can both directly and indirectly affect the survival and contraction of CD8^{+} T cells following activation, I assessed the role of co-stimulation in the survival of CD8^{+} T cell effectors generated using co-stimulator ligand bearing beads.

To begin my evaluation, I determined the percentage of CD8^{+} T cells which were recovered following 48 hours of co-stimulation with 10nM B7.1 or ICAM-1, 1ug anti-CD27 or anti-HVEM, suboptimal amounts of anti-CD3 alone, and BSA coated beads as a control. Cell recovery was calculated relative to the number of cells initially plated with percentages under 100% indicating cell loss, and percentages over 100% indicating CD8^{+} T cell accumulation. Of the tested co-stimulator ligands, only co-stimulation with 10nM B7.1 led to an accumulation of T cell effectors at the 48 hour time point with ~160% cell recovery (Fig. 4-1A).

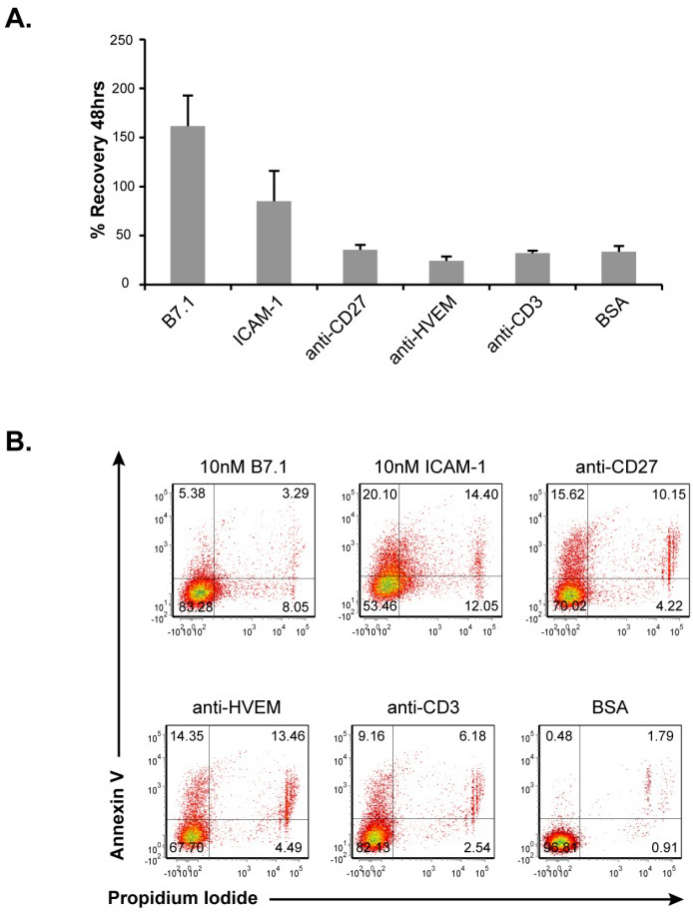


Figure 4-1. Activated CD8$^+$ T cell survival is influenced by co-stimulatory molecule ligation. Naïve CD44^{lo} CD8$^+$ T cells were stimulated for 48 hours with 10nM of B7.1 or ICAM-1, 1µg of anti-CD27 or anti-HVEM, suboptimal amounts of anti-CD3 or BSA coated beads, and assessed for their percent cell recovery (*A*) and Annexin V/propidium iodide staining (*B*). All co-stimulatory bead constructs included suboptimal amounts of anti-CD3. Percent cell recovery was calculated by dividing the total live CD8$^+$ T cell number following stimulation by the total number of CD8$^+$ T cells originally plated for each co-stimulatory condition. (*B*) Annexin V and Propidium iodide staining of the activated CD8$^+$ T cell was performed as described in the *Materials and Methods* using stimulated but unstained control samples for gate alignment. Data in (*A*) represents the mean of three independent experiments performed in duplicate. Error bars represent standard deviation of experimental means. Data in (*B*) is representative of three independent experiments.

Surprisingly, cells co-stimulated with 10nM ICAM-1 failed to accumulate (~85% cell recovery) (Fig. 4-1A), despite being the most proliferative in previous assays (Fig. 3-3A). To investigate potential mechanisms for this loss of cell recovery, the remaining $CD8^+$ T cells were stained with annexin V and propidium iodide to determine their relative amount of apoptosis (Fig. 4-1B). Annexin V detects cell membrane inner leaflet phospholipids translocated to the outer leaflet, which occurs during apoptosis, while propidium iodide binds DNA exposed during cell membrane breakdown. The presence of annexin V^+ populations could be detected 48 hours following all forms of stimulation tested, but was greatly reduced following 10nM B7.1 co-stimulation, and was nearly undetectable from BSA unstimulated cells (Fig. 4-1B). This data then suggests that B7.1 co-stimulation may result in better $CD8^+$ T cell accumulation after activation by reducing $CD8^+$ T cell death by apoptosis.

To further investigate the role of co-stimulation in $CD8^+$ T cell survival following activation, I tested whether combinations of co-stimulator ligands could be used to enhance the recovery of the generated $CD8^+$ T cell effectors following 48 hours of stimulation. Suboptimal amounts (1.25nM) of recombinant B7.1 and ICAM-1, and 1µg of anti-CD27, were used either individually or in combination to test the ability of multiple co-stimulator ligands to enhance the recovery of the stimulated cells. Co-stimulation with 1.25nM B7.1 and 1.25nM ICAM-1 individually resulted in approximately 70-90% $CD8^+$ T cell recovery, which was increased to ~140% when 1.25nM B7.1 and 1.25nM ICAM-1 were provided in combination (Fig. 4-2). Surprisingly, the combination of 1.25nM B7.1 + 1µg

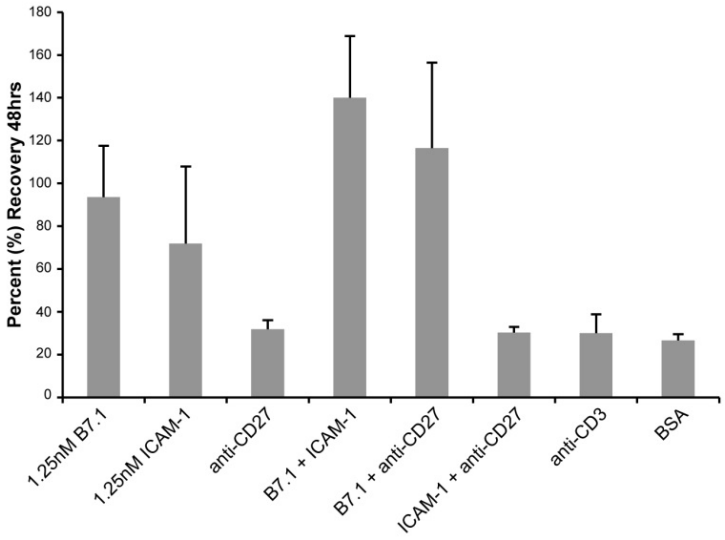


Figure 4-2. Combinations of co-stimulator ligands can be used to enhance activated CD8$^+$ T cell recovery following 48hrs of stimulation. Naïve CD44^{lo} CD8$^+$ T cells were stimulated with beads displaying suboptimal amounts (1.25nM) of recombinant B7-1 or ICAM-1 co-stimulator ligands, or 1µg of anti-CD27, either individually or in pair-wise combinations, and assessed for their percent cell recovery at 48hrs. All co-stimulatory bead constructs included suboptimal amounts of anti-CD3. Anti-CD3 cross-linking alone and BSA coated beads were used as controls for cell stimulation. Percent cell recovery was calculated by dividing the total number of live CD8$^+$ T cell remaining after stimulation by the total number of CD8$^+$ T cells originally plated for each co-stimulatory condition. Results represent the mean of three independent experiments performed in duplicate. Error bars represent standard deviation of experimental means.

anti-CD27 also led to an accumulation of activated $CD8^+$ T cells (~120% recovery) (Fig. 4-2), despite previous experiments indicating that this co-stimulator ligand combination leads to poorly cytolytic effectors (Fig. 3-11B). Lastly, the combination of 1.25nM ICAM-1 + 1µg anti-CD27 co-stimulation led to very poor cell recovery (~30%) following 48 hours of $CD8^+$ T cell stimulation (Fig. 4-2), suggesting that the combination of ICAM-1 + anti-CD27 not only has an inhibitory effect on T cell proliferation (Fig. 3-9), but also reduces the survival of the $CD8^+$ T cell effectors generated.

Multiple factors differentially induced by co-stimulation are responsible for differences in $CD8^+$ T cell survival following activation with stimulatory beads

The survival and accumulation of activated $CD8^+$ T cell populations following co-stimulation is likely due to both the expression of pro-survival factors produced by the T cells themselves, and the *in vitro* culture conditions in which the cells are grown. *In vivo*, the contraction of $CD8^+$ T cell populations is partially governed by a balance of co-stimulatory and co-inhibitory molecule ligation and signalling. Co-inhibitory molecule ligation is thought to be required for optimal pathogen clearance during infection, and for preventing excessive inflammation [274]. Ligation of co-inhibitory molecules have been shown to affect $CD8^+$ T cell survival directly by disrupting the balance between pro- and anti-apoptotic proteins [107], and indirectly by repressing the secretion of IL-2, which supports continued T cell proliferation and survival [275]. To determine if co-inhibitory molecule expression is preferentially induced following certain forms of co-stimulation, the generated $CD8^+$ T cell populations were assessed by

flow cytometry for the expression of two important co-inhibitory molecules, PD-1 and CTLA-4, following 48 hours of stimulation. PD-1 was found to be expressed on high percentage of activated $CD8^+$ T cell populations following most forms of co-stimulation (Fig. 4-3A). In particular, the highest expression of PD-1 was detected following co-stimulation with 10nM ICAM-1 as 92% of the cells were found to express this co-inhibitory molecule. This was reduced to only 86% following nearly a 10-fold decrease in ICAM-1 co-stimulation to 1.25nM (Fig. 4-3A). While still quite high, the percentage of PD-1 positive T cells was somewhat lower following co-stimulation through either CD28 or CD27 individually (Fig. 4-3A). CTLA-4 expression by comparison was much lower than PD-1 following all forms of $CD8^+$ T cell co-stimulation tested (Fig. 4-3B). CTLA-4 was expressed to the highest level on cells co-stimulated with 10nM B7.1, which were approximately 12% positive (Fig. 4-2B). Because blocking experiments of co-inhibitory molecules were not performed, and the expression of PD-1 and CTLA-4 does not directly correlate with differences in the $CD8^+$ T cell survival following 48 hours of stimulation, the role these co-inhibitory molecules are playing in these experiments remains unclear.

The intrinsic mechanism for cellular apoptosis is governed by a balance between pro- and anti-apoptotic proteins found in the mitochondrial membrane. Following $CD8^+$ T cell activation, there is a decrease in the expression of anti-apoptotic proteins resulting in reduced cell survival and more efficient induction of $CD8^+$ T cell contraction mechanisms [255, 261]. To determine if the relative expression of anti-apoptotic proteins induced by various forms of co-stimulation

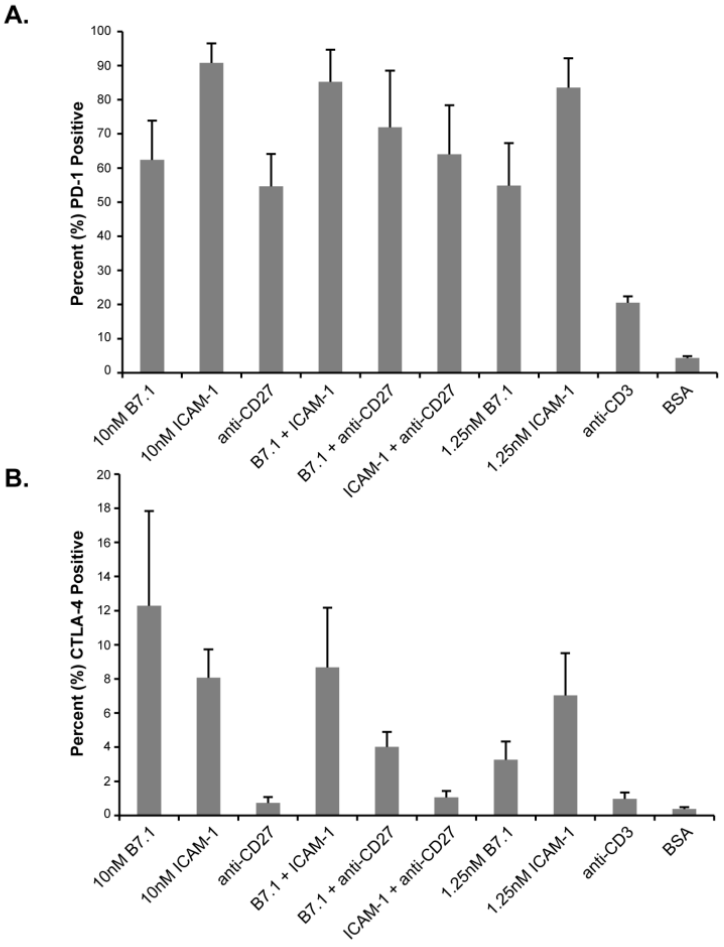


Figure 4-3. Co-inhibitory molecule expression is induced on the surface of activated CD8$^+$ T cells following co-stimulation. Co-inhibitory molecule expression was assessed following CD44^{lo} CD8$^+$ T cell stimulation with high (10nM) or intermediate (1.25nM) amounts of B7.1 or ICAM-1 or high amounts (1ug) of anti-CD27 either individually or in combination. Co-stimulator ligand combinations utilized intermediate amounts (1.25nM) of recombinant proteins, and all bead constructs included suboptimal amounts of anti-CD3, with the exception of BSA unstimulated controls. Following 48hrs of stimulation, CD8$^+$ T cells were stained for their expression of the co-inhibitory molecules PD-1 (*A*) and CTLA-4 (*B*) using fluorochrome conjugated anti-PD-1 and anti-CTLA-4 mAbs and flow cytometry. The percentage of cells positive for PD-1 and CTLA-4 was calculated relative to isotype stained controls. Data in (*A*) and (*B*) is an average of four experiments and error bars represent standard deviation of experimental means.

was related to the survival of the $CD8^+$ T cell populations, cells were monitored for 48 – 96 hours for their expression of the anti-apoptotic protein Bcl-2 by flow cytometry. Co-stimulatory conditions which resulted in the highest $CD8^+$ T cell survival at 48hrs (10nM B7.1, 10nM ICAM-1, B7.1 + ICAM-1, B7.1 + anti-CD27), tended to display the highest expression of Bcl-2 at the 48 and 72 hour time points (Fig. 4-4A). The highest percentage of T cells positive for Bcl-2 however, occurred following B7.1 + ICAM-1 co-stimulation as approximately 33% of the cells were Bcl-2 positive at 72 hours (Fig. 4-4A). In addition, the relative expression of Bcl-2 from B7.1 + ICAM-1 co-stimulated $CD8^+$ T cells was also higher than 10nM B7.1 or ICAM-1, and B7.1 + anti-CD27 co-stimulated cells at the 72 hour time point (Fig. 4-4B). However, the percentage of cells positive for Bcl-2 was greatly reduced following all forms of co-stimulation at 96 hours, especially when cells were stimulated with a combination of B7.1 + anti-CD27 (Fig. 4-4A). The relative expression of a second anti-apoptotic protein Bcl-xL, was also determined by flow cytometry between $CD8^+$ T cells co-stimulated with either 10nM B7.1, 10nM ICAM-1, B7.1 + ICAM-1 or B7.1 + anti-CD27. While Bcl-xL was found to be expressed to similar levels in cells co-stimulated with 10nM B7.1, ICAM-1 or B7.1 + ICAM-1 at 72 hours, $CD8^+$ T cells co-stimulated with B7.1 + anti-CD27 tended to have lower expression of this anti-apoptotic protein (Fig. 4-4C). This indicates that the B7.1 + anti-CD27 co-stimulatory combination may predispose activated $CD8^+$ T cells to a more rapid or earlier contraction in comparison to the other co-stimulatory conditions tested. Overall, the assessment of anti-apoptotic Bcl-2 and Bcl-xL expression indicates

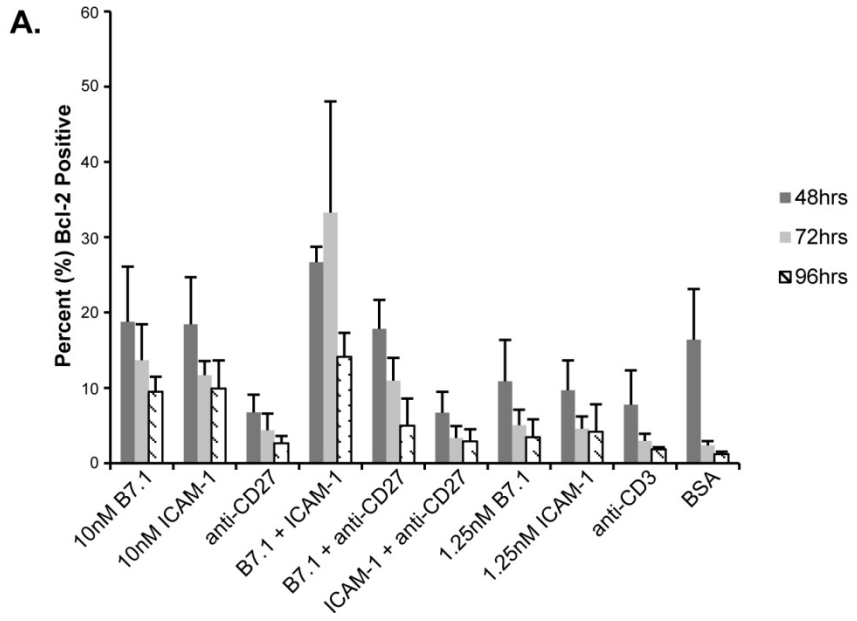
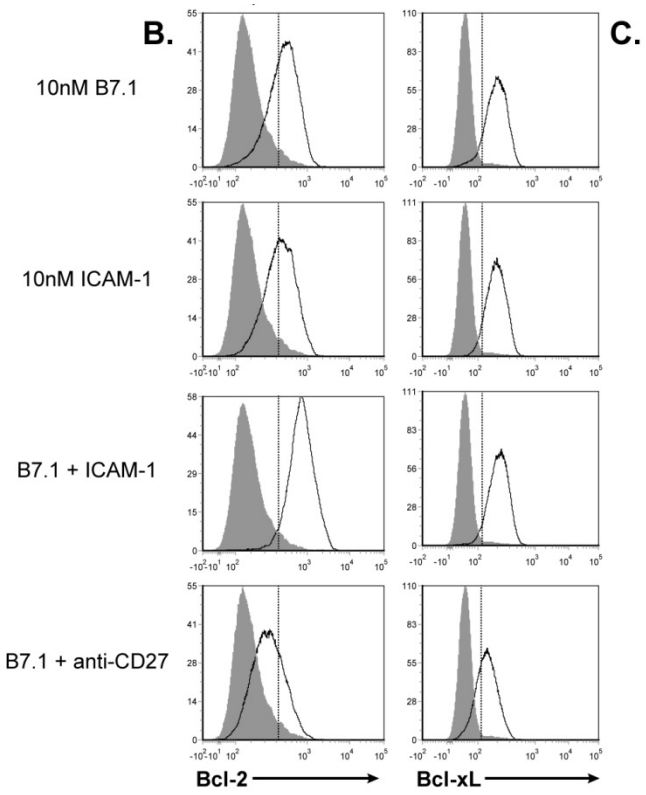


Figure 4-4. $CD8^+$ T cell contraction is reflective of co-stimulation induced Bcl-2 and Bcl-xL expression. Anti-apoptotic Bcl-2 and Bcl-xL protein expression was determined following naïve $CD44^{lo}$ $CD8^+$ T cells stimulation with high (10nM) or intermediate (1.25nM) amounts of B7.1 or ICAM-1 or high amounts (1µg) of anti-CD27 either individually or in combination at the indicated time points. Co-stimulator ligand combinations utilized intermediate amounts (1.25nM) of recombinant proteins, and all bead constructs included suboptimal amounts of anti-CD3, with the exception of BSA unstimulated controls. (*A*) Bcl-2 expression from $CD8^+$ T cells activated under the indicated co-stimulatory conditions at 48, 72, and 96hrs. Bcl-2 expression was determined by intracellular staining and flow cytometry, as described in the *Materials and Methods*. The percentage of cells positive for Bcl-2 was calculated relative to isotype stained controls. (*B, C*) Relative expression of the anti-apoptotic proteins Bcl-2 (*B*) and Bcl-xL (*C*) following $CD44^{lo}CD8^+$ T cell stimulation with the indicated co-stimulatory bead constructs for 72 hours. $CD8^+$ T cells were stained with fluorochrome conjugated Bcl-2 and Bcl-xL monoclonal antibodies and assessed for Bcl-2 and Bcl-xL expression by flow cytometry as described in the *Materials and Methods*. Shaded histograms represent unstimulated stained controls. Data in (*A*) represents the mean Bcl-2 expression following three independent experiments and error bars represent standard deviation of experimental means. Histograms depicting the relative expression of Bcl-2 (*B*) and Bcl-xL(*C*) are representative of three independent experiments.


B.
C.
10nM B7.1
10nM ICAM-1
B7.1 + ICAM-1
B7.1 + anti-CD27
Bcl-2
Bcl-xL

that the relative expression of these molecules following co-stimulation may be partially responsible for differences in $CD8^+$ T cell survival and contraction observed following activation.

The absence of appropriate survival signals during $CD8^+$ T cell expansion can increase cellular apoptosis through activated cell-autonomous death (ACAD) [151]. IL-2 is a necessary growth factor secreted by both $CD8^+$ and $CD4^+$ T cells following activation promoting both cell survival and proliferation during the expansion phase, as well as effector molecule production following antigen encounter [66, 163]. Because IL-2 is a vital growth factor for $CD8^+$ T cell expansion, and plays such a crucial role in dictating the full maturation of $CD8^+$ T cell effector responses, the production of IL-2 by the $CD8^+$ T cells was determined following 24 and 48 hours of co-stimulation. IL-2 ELISAs were performed on supernatants collected from $CD8^+$ T cells co-stimulated with either 10nM B7.1, 10nM ICAM-1, 1µg anti-CD27 or 1µg anti-HVEM. Significant IL-2 secretion could only be detected from $CD8^+$ T cells co-stimulated with 10nM B7.1 and 10nM ICAM-1 at both 24 and 48 hours (Fig. 4-5A,B). Greatly elevated levels of IL-2 were detected from PMA/ionomycin treated positive controls at both time points. Notably, minimal IL-2 secretion could be detected from $CD8^+$ T cells co-stimulated with CD27 and HVEM cross-linking antibodies (Fig. 4-5A,B). While IL-2 could be detected from both B7.1 and ICAM-1 co-stimulatory conditions, 10nM B7.1 co-stimulation induce approximately 3x more IL-2 secretion than $CD8^+$ T cells co-stimulated with 10nM ICAM-1 at both time points (Fig. 4-5A, B). The presence of IL-2 positive $CD8^+$ T cells could also be detected

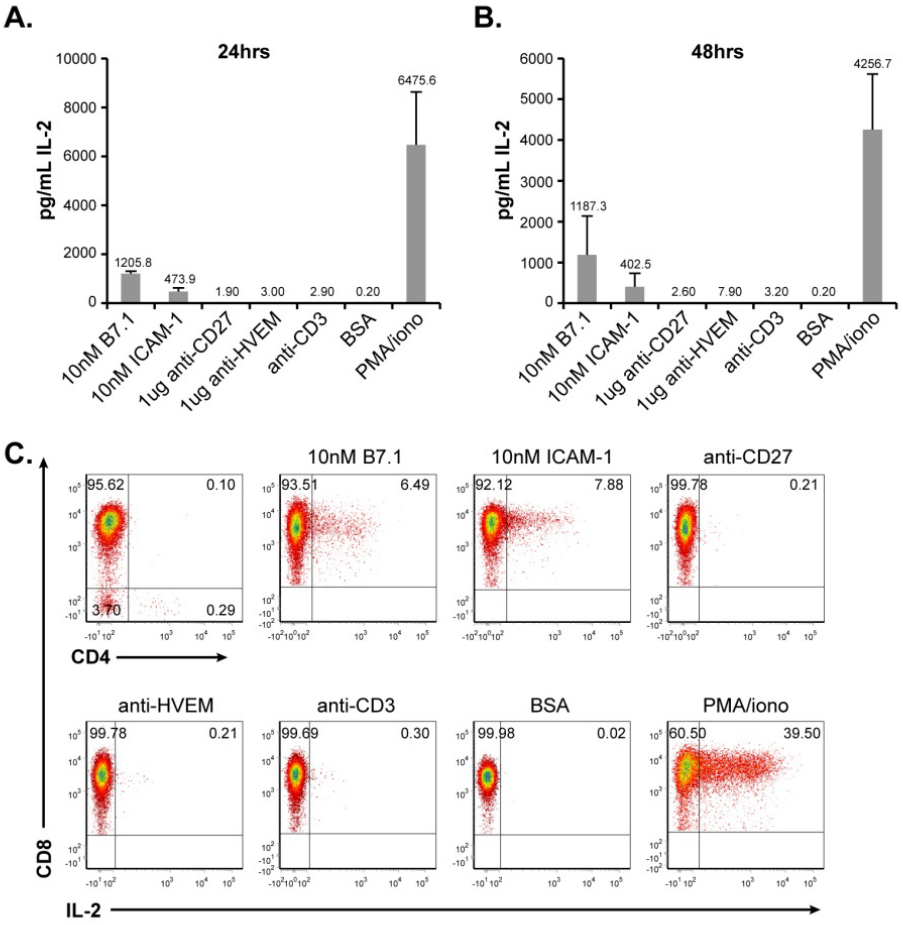


Figure 4-5. Detectable IL-2 production is induced following B7.1 and ICAM-1 co-stimulation of CD44^{lo} CD8^{+} T cells. Supernatants were collected from naïve CD44^{lo} CD8^{+} T cells stimulated under the indicated co-stimulatory conditions and the amount of secreted IL-2 determined by ELISA. All co-stimulatory constructs contained suboptimal amounts of anti-CD3. CD8^{+} T cells cultured with beads bearing suboptimal amounts of anti-CD3 cross-linking antibody and BSA, or PMA/ionomycin treated CD8^{+} T cells, served as controls. Numbers indicate the average concentration of IL-2 detected from each co-stimulatory condition at 24 (*A*) and 48(*B*) hours following three independent stimulations. Error bars depict standard deviations in the mean IL-2 concentration. (*C*) Intracellular IL-2 detected by flow cytometry following 24 hours of naïve CD8^{+} T cell stimulation with the co-stimulatory molecules listed, in combination with suboptimal amounts of anti-CD3. Cells were stained with CD8 and CD4 fluorescently-labeled monoclonal antibodies for gating purposes, and intracellular IL-2 was detected using fluorochrome conjugated monoclonal antibodies as described in the *Materials and Methods*. Data in (*C*) is representative of three independent experiments.

by intracellular staining and flow cytometry at the 24 hour time point (Fig. 4-5C). Intracellular IL-2 could not be detected from $CD8^+$ T cells following 48 hours of stimulation by flow cytometry and therefore has not been included. Flow cytometry however, was found to be less accurate for determining the expression level of IL-2 than ELISA as 10nM B7.1 and 10nM ICAM-1 co-stimulation led to a similar percentage of IL-2 positive cells (~6 - 8%) at 24 hours (Fig. 4-5C), despite 10nM B7.1 co-stimulated cells secreting 3x more IL-2 into culture supernatants. Nevertheless, the results from this experiment support the conclusion that co-stimulation induced IL-2 secretion may be related to the differences observed in $CD8^+$ T cell recovery following 48 hours of stimulation.

Increasing co-stimulation can enhance IL-2 production by $CD44^{lo}$ $CD8^+$ T cells

Since IL-2 secretion plays a major role in dictating $CD8^+$ T cell survival and effector function following activation, I sought to determine if co-stimulator ligands could be provided in combination to increase the production of IL-2 from $CD44^{lo}$ $CD8^+$ T cells. To address this, increasing amounts of recombinant ICAM-1 (0.156nM - 10nM) or CD27 cross-linking antibodies (0.0156µg – 1µg) were added to beads bearing a consistent intermediate amount (1.25nM) of recombinant B7.1. The generated bead constructs were incubated with $CD44^{lo}$ $CD8^+$ T cells and assessed 24 hours later for their production of IL-2 by ELISA and flow cytometry. Assessment of culture supernatants revealed that increasing amounts of ICAM-1 co-stimulation, in combination with B7.1, induced a very significant increase in IL-2 production with the concentration of IL-2 in the culture supernatants reaching the Nano gram range (~20ng/mL) when ICAM-1 density

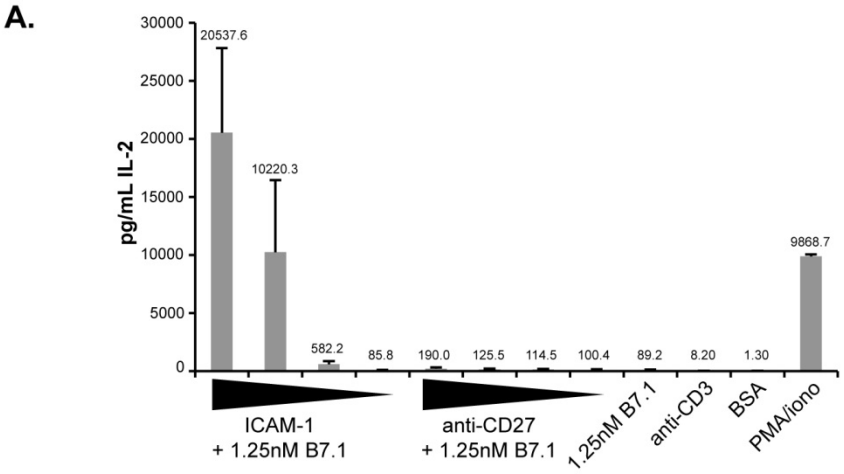
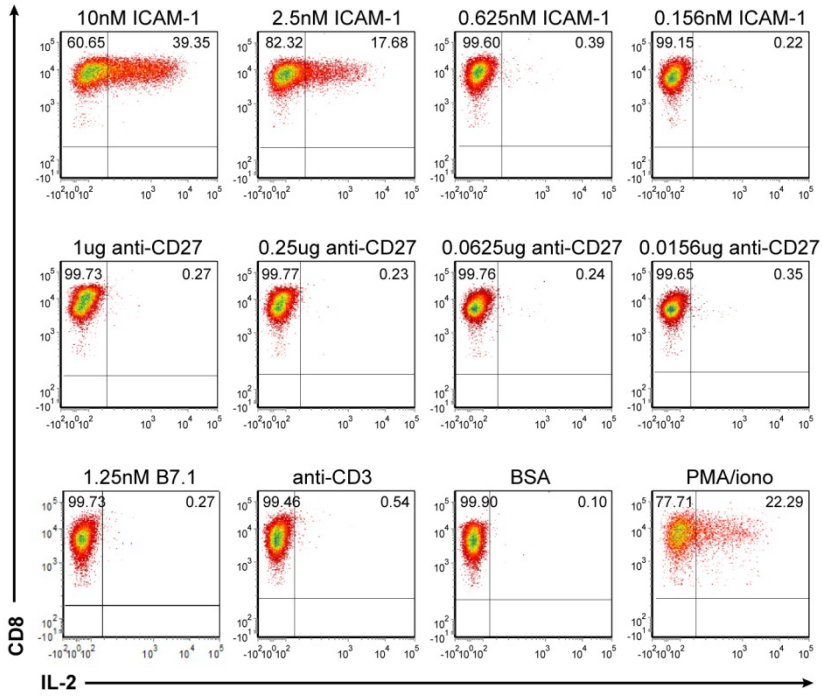


Figure 4-6. The co-stimulator ligand combination of ICAM-1 and B7.1 preferentially induces IL-2 production from naïve CD44^{lo} CD8^{+} T cells. (*A,B*) Naïve CD44^{lo} CD8^{+} T cells incubated for 24hrs with beads bearing decreasing amounts of ICAM-1 (10nM, 2.5nM, 0.625nM, and 0.156nM) or CD27 cross-linking antibodies (1µg, 0.25µg, 0.0625µg, 0.0156µg) in combination with an intermediate (1.25nM) of B7.1 and suboptimal amounts of anti-CD3, were assessed 24 hours later for production of IL-2. As controls, IL-2 production was determined following CD8^{+} T cell stimulation with an intermediate amount (1.25nM) of rB7.1 co-stimulation, suboptimal amounts of anti-CD3 and BSA coated beads, or following PMA/ionomycin treatment. (*A*) IL-2 ELISA of culture supernatants collected following 24hrs stimulation under the indicated co-stimulatory conditions. Numbers represent the average concentration of IL-2 following three experiments. (*B)* IL-2 intracellular staining of activated CD8^{+} T cells following 24hrs of stimulation under the indicated co-stimulatory conditions. CD8^{+} T cells were stained with fluorescently-labeled CD8 monoclonal antibodies for gating, and intracellular IL-2 was detected with fluorochrome conjugated mAbs as described in the *Materials and Methods*. Data in (*B*) is representative of three independent experiments.

B.


10nM ICAM-1
60.65
39.35
2.5nM ICAM-1
82.32
17.68
0.625nM ICAM-1
99.60
0.39
0.156nM ICAM-1
99.15
0.22
1ug anti-CD27
99.73
0.27
0.25ug anti-CD27
99.77
0.23
0.0625ug anti-CD27
99.76
0.24
0.0156ug anti-CD27
99.65
0.35
1.25nM B7.1
99.73
0.27
anti-CD3
99.46
0.54
BSA
99.90
0.10
PMA/iono
77.71
22.29
CD8
IL-2

was increased (Fig. 4-6A). Of note, the amount of IL-2 secreted by this co-stimulator combination was even greater than the IL-2 secreted from PMA/ionomycin treated positive control $CD8^+$ T cell controls. The high amount of IL-2 produced under these co-stimulatory conditions was also reflected in the higher percentage of T cells positive for intracellular IL-2 (~40%) detected by flow cytometry (Fig. 4-6B). In comparison to ICAM-1, increasing amounts of CD27 cross-linking antibody led to only a minor increase (~100-200pg/mL) in IL-2 secretion, detectable only by ELISA (Fig. 4-6A,B). These results indicate that co-stimulator combinations can be used to increase IL-2 production from $CD44^{lo}$ $CD8^+$ T cells, and, of the combinations tested, B7.1 and ICAM-1 is most effective at doing so.

Cytokine deprivation induced ACAD can be partially reversed by IL-2 addition

$CD8^+$ T cells which receive TCR stimulation in the absence of appropriate co-stimulatory signals may be predisposed to undergo activation induced cell death or AICD [151]. AICD can be detected in cell cultures by the presence of cell populations exhibiting high CD25 and annexin V staining. AICD often involves the engagement of surface T cell death receptors such as Fas and TRAIL, but can also occur via non-death receptor mediated mechanisms such as lysosomal cathepsin release [276, 277]. From previous assays, $CD44^{lo}$ $CD8^+$ T cells co-stimulated with 10nM ICAM-1 were found to be apoptotic despite their ability to secrete IL-2. This led me to investigate whether AICD was occurring in the co-stimulated $CD8^+$ T cell cultures. Staining the $CD8^+$ T cell populations at

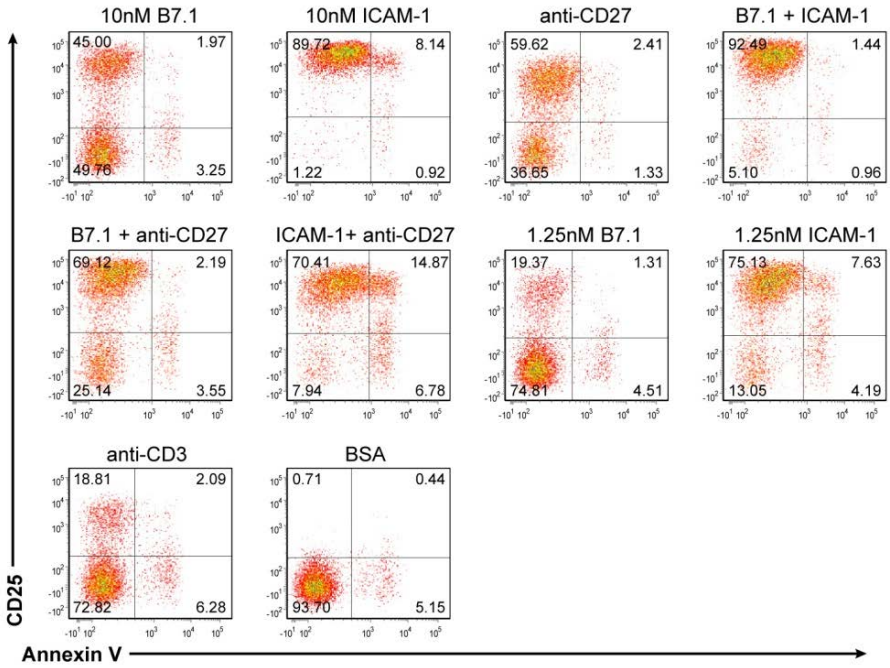


Figure 4-7. ICAM-1 co-stimulation results in an increased amount of AICD. Naïve $CD44^{lo}$ $CD8^{+}$ T cells were stimulated for 24hrs with high (10nM) or intermediate (1.25nM) amounts of rB7.1 or rICAM-1 or high amounts (1ug) of anti-CD27 either individually or in combination, and tested for the induction of AICD. Co-stimulatory combinations utilized intermediate amounts (1.25nM) of recombinant proteins, and all bead constructs included suboptimal amounts of anti-CD3, with the exception of BSA unstimulated controls. AICD was detected using annexin V and CD25 staining of $CD8^{+}$ T cells following 24 hours of stimulation under the indicated co-stimulatory conditions, and analyzed by flow cytometry as described in the *Materials and Methods*. Data is representative of three independent experiments.

24 hours following various forms of co-stimulation with annexin V and CD25 antibodies revealed that distinct populations of cells undergoing AICD were present in some cell cultures (Fig. 4-7). In particular, significant $CD25^{hi}$ annexin V^{hi} $CD8^{+}$ T cell populations could be detected following co-stimulation with 10nM ICAM-1 (8%), ICAM-1 + anti-CD27 (~15%) and 1.25nM ICAM-1 (~8%) (Fig. 4-7). This suggests that the poor cell recovery and increased apoptosis observed from $CD8^{+}$ T cells when co-stimulated under these conditions may be a result of AICD.

While $CD8^{+}$ T cells co-stimulated with ICAM-1 + anti-CD27 and 1.25nM ICAM-1 had $CD25^{hi}$ annexin V^{hi} cell populations, it is difficult to discern whether these populations arose from AICD mechanisms involving strong TCR stimulation with poor co-stimulatory support, or through the lack of available growth factors leading to the induction of ACAD. To test if the lack of growth factors was contributing to generation of the $CD25^{hi}$ annexin V^{hi} populations following co-stimulation, 40units/mL of recombinant murine IL-2 was added to the cell cultures at the time of plating, and the $CD8^{+}$ T cells were assessed 24 and 48 hours later for changes in the percentage of this apoptotic population. IL-2 addition however, can have both beneficial and detrimental effects on T cell survival as excessive IL-2 signalling can sensitize cells to AICD [174, 278, 279]. In general, the result of IL-2 addition to most T cell cultures was highly variable. However, the addition of exogenous IL-2 was found to be beneficial for $CD8^{+}$ T cells co-stimulated with a combination of 1.25nM ICAM-1 and anti-CD27, as these cells demonstrated nearly 35% reduction in the $CD25^{hi}$ annexin V^{hi}

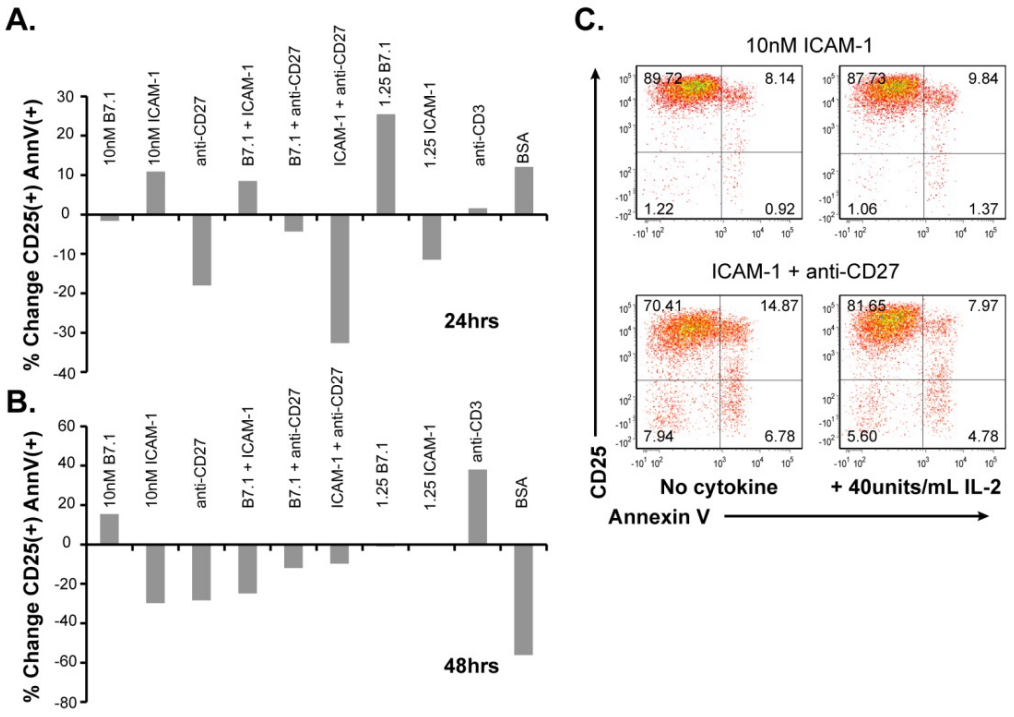


Figure 4-8. ACAD is variably reduced by the addition of IL-2.
Naïve CD44^{lo} CD8^{+} T cells were cultured with and without 40units/mL of recombinant murine IL-2, and stimulated with high (10nM) or intermediate (1.25nM) amounts of B7.1 or ICAM-1 or high amounts (1ug) of anti-CD27 either individually or in combination, for 24 (*A*) and 48 (*B*) hours. Co-stimulatory combinations utilized intermediate amounts (1.25nM) of recombinant proteins, and all bead constructs included suboptimal amounts of anti-CD3, with the exception of BSA unstimulated controls. The recovered CD8^{+} T cells were stained with fluorochrome conjugated annnexin V and CD25 antibodies to test for ACAD reduction. Change in the percentage of CD8^{+} T cells which were annexin V and CD25 positive after IL-2 addition was determined by flow cytometry, and calculated relative to untreated CD8^{+} T cell co-stimulatory controls. (*C*) Representative annexin V and CD25 staining of 10nM ICAM-1 and 1.25nM ICAM + 1ug-anti-CD27 co-stimulated CD8^{+} T cells at 24hrs with and without the addition of 40units/mL of IL-2, as analyzed by flow cytometry. Results depicted in (*A*) and (*B*) are an average of three independent experiments. Data in (*C*) is representative of three independent experiments.

population with IL-2 addition (Fig. 4-8A,C). A similar but less substantial reduction in this apoptotic population was also detected following IL-2 addition to cells co-stimulated with either 1ug-anti-CD27 (18% decrease) or 1.25nM ICAM-1 (11% decrease) at 24hrs (Fig. 4-8A). The reduction in the percentage of $CD8^+$ T cells which were $CD25^{hi}$ and annexin V^{hi} following IL-2 addition however was less substantial following 48 hours of stimulation (Fig. 4-8B). Nonetheless, this reduction in the $CD25^{hi}$ annexin V^{hi} populations generated with anti-CD27, ICAM-1 + anti-CD27 and 1.25nM ICAM-1 co-stimulation following the addition of IL-2 suggests that these cells were likely dying due to insufficient growth factors. 10nM ICAM-1 co-stimulated cells however, exhibited no such decrease in the percentage which were $CD25^{hi}$ annexin V^{hi} following IL-2 addition (Fig. 4-8A,C). In fact, exogenous IL-2 seemed to modestly exasperate this phenotype with the percentage of $CD25^{hi}$ annexin V^{hi} cells increasing from 8.14% to 9.84% when the $CD8^+$ T cells were assessed following 24 hours of stimulation (Fig. 4-8A,C). This indicates that 10nM ICAM-1 stimulated $CD8^+$ T cells may have been undergoing AICD rather than ACAD since the addition of IL-2 did not rescue T cells undergoing apoptosis following this form of co-stimulation at the 24 hour time point.

IL-2 addition induces changes in $CD8^+$ T cell activation state and granzyme B expression following 48 hours of co-stimulation

The presence of IL-2 can affect not only the survival of activated $CD8^+$ T cells, but can also have considerable effects on T cell effector function. IL-2 addition to $CD8^+$ T cell cultures has been shown to increase their expression of

CD25, and the effector molecules granzyme B and perforin [66, 174]. Because I had previously found that co-stimulatory molecule ligation leads to differential induction of IL-2 depending on the type of co-stimulation, I investigated whether the addition of exogenous IL-2 to co-stimulated cell cultures can enhance their expression of CD25, as well as granzyme B. Forty units/mL of recombinant murine IL-2 was added to bead:$CD8^+$ T cell cultures at the time of plating, and the cells were assessed 48 hours later for changes in their expression of CD25 and granzyme B, with and without the addition of IL-2. IL-2 addition greatly increased the expression of CD25 from most cell cultures with as much as a 10-fold increase being observed following IL-2 addition to B7.1 + anti-CD27 and ICAM-1 + anti-CD27 co-stimulated $CD8^+$ T cells (Fig. 4-9A). The exceptions were $CD8^+$ T cells co-stimulated with 10nM B7.1, 10nM ICAM-1 or a combination of 1.25nM B7.1 + ICAM-1 (Fig. 4-9A). As IL-2 itself has been previously demonstrated to increase the expression of the IL-2 receptor α-chain CD25 [174], the lack of increased CD25 expression following IL-2 addition from these co-stimulatory conditions suggests that amount of autocrine IL-2 produced by these $CD8^+$ T cells may already be saturating. A similar trend occurred when monitoring the cells for increased granzyme B expression following the addition of IL-2. While IL-2 addition did somewhat increase the expression of granzyme B following all co-stimulatory conditions tested (Fig. 4-9B), it had a greater effect on cells which likely produce little IL-2 themselves. This includes the co-stimulatory combination of B7.1 + anti-CD27 and ICAM-1 + anti-CD27 whose granzyme B expression increased approximately 2-fold following IL-2 addition

(Fig. 4-9B). Of note, the co-stimulatory conditions most affected by IL-2 addition were also the least cytolytic in previously described killing assays (Fig. 3-11B), suggesting that a suboptimal amount of granzyme B expression, due to poor IL-2 secretion, may have prevented their full cytolytic potential. Overall, the results from this experiment indicate that IL-2 addition can greatly enhance $CD8^+$ T cell CD25 and granzyme B expression when co-stimulatory molecule engagement fails to induce high expression of these molecules.

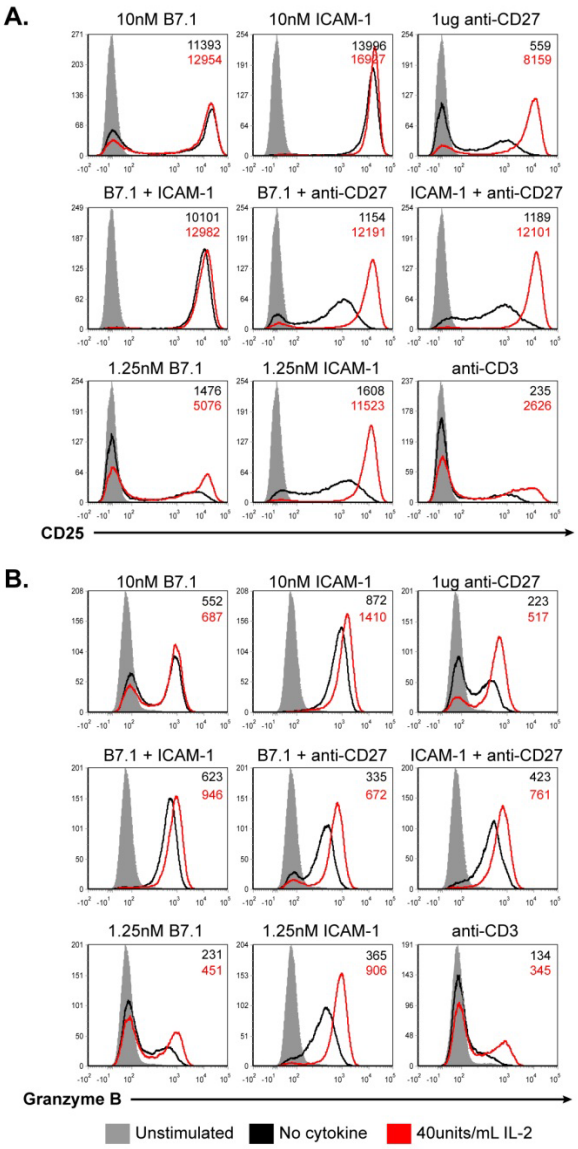


Figure 4-9. IL-2 addition enhances co-stimulation induced CD8$^+$ T cell CD25 and granzyme B expression. Naïve CD44^{lo} CD8$^+$ T cells, activated under the indicated co-stimulatory conditions, were cultured with (red) and without (black) the addition of 40units/mL of recombinant murine IL-2, and assessed 48 hours later for their expression of CD25 (*A*) and granzyme B (*B*) by flow cytometry. CD25 surface staining was performed using fluorochrome conjugated CD25 mAbs while intracellular staining for granzyme B was performed using granzyme B mAbs as described in the *Materials and Methods*. Shaded histograms in both (*A*) and (*B*) represent unstimulated stained CD8$^+$ T cell controls while the numbers denote mean fluorescence intensity. Data is representative of three independent experiments.

Discussion

Co-stimulation of naïve $CD8^+$ T cells can direct effector development, and also plays a significant role in enhancing $CD8^+$ T cell survival following activation. Improved cell survival benefits $CD8^+$ T cell effector function, and increases the number of viable T cells available to form long-lived memory populations following T cell contraction. In this chapter, I investigated whether individual co-stimulator ligands or their combinations can be used to enhance $CD8^+$ T cell survival following activation. Not only did I discover a divergence in co-stimulation driven $CD8^+$ T cell survival and effector function, I also found differences in co-stimulation induced anti-apoptotic protein expression and IL-2 secretion that may play a significant role in both T cell survival and effector cell development.

Activation of naive $CD8^+$ T cells with the described co-stimulator ligands and combinations led to detectable differences in cell survival following activation. When individual co-stimulators were compared for their ability to enhance $CD8^+$ T cell recovery following activation, only co-stimulation with 10nM B7.1 led to a significant accumulation of activated $CD8^+$ T cells with minimal indication of apoptosis. Co-stimulation through CD28 has been reported to enhance T cell survival in a number of ways, including the inhibition FasL expression [265], as well as augmenting expression of the anti-apoptotic protein Bcl-xL, in cooperation with TCR mediated signalling [65]. On the other hand, co-stimulation with 10nM ICAM-1 led to poor cell recovery following T cell activation despite these cells being the most proliferative in previous experiments

(Fig. 3-4A). This exemplifies a divergence in co-stimulation induced $CD8^+$ T cell effector function and cell survival and suggests that the co-stimulatory conditions which induce the most potent effectors are not necessarily ideal for sustaining the survival of these T cells. While LFA-1 ligation by ICAM-1 has been demonstrated to enhance memory $CD8^+$ T cell generation by increasing the expression of Bcl-xL [272], it may be necessary to provide ICAM-1 in the presence of a second *bona fide* co-stimulatory signal to observe this effect. At early time points, the co-stimulatory combination of B7.1 and anti-CD27 exhibited a reciprocal phenotype in which these combined co-stimulatory signals enhanced $CD8^+$ T cell survival following activation, but did not lead to pronounced stimulation of T cell effector function in previous assays (Fig. 3-11B). This result was not unexpected as CD27 co-stimulation has been demonstrated to enhance early T cell survival by increasing anti-apoptotic protein expression [75, 123]. Moreover, this may exemplify an alternative function for CD27 and CD28 co-stimulation in enhancing $CD8^+$ T cell survival at the expense of effector function.

The two major factors found to correlate with $CD8^+$ T cell survival in these experiments were: 1) co-stimulation induced IL-2 secretion and, 2) expression of the anti-apoptotic protein Bcl-2. While co-inhibitory molecule expression was also investigated as a third potential mechanism affecting T cell survival, no direct correlation could be found between the expression of PD-1 and CTLA-4, and the cell survival differences observed in these experiments. PD-1 ligation has been demonstrated to inhibit the expression of Bcl-xL, leaving T cells

more vulnerable to intrinsic mechanisms of cell death [107]. While CTLA-4 ligation does not directly influence anti-apoptotic protein expression, the inhibition of IL-2 production induced by CTLA-4 has been demonstrated to be a major factor in the commencement of T cell contraction [89, 280]. While no clear association could be made between the expression of PD-1 and CTLA-4 on T cell survival, a question still remains as to the availability of their respective ligands. The expression of PD-L1 on the activated T cells themselves could not be confirmed as the antibody used for its detection appeared to interact with the T cells in a non-specific manner. This leaves the purpose of the elevated PD-1 expression on nearly all co-stimulation generated $CD8^+$ T cell populations unresolved. PD-1 expression is associated with T cell exhaustion [72], but has also been linked to increased T cell motility by inhibiting stable T cell:DC contacts [281]. In these experiments, T cell exhaustion may have occurred due to sustained TCR and co-stimulatory signalling generated by continued exposure of the activated $CD8^+$ T cells to the stimulatory beads. In particular, ICAM-1 and anti-CD3 coated beads initiate the most rapid and sustained T cell tethering (Appendix Figure 7-2), suggesting that the high PD-1 expression from ICAM-1 co-stimulated cells may be due overwhelming T cell stimulation leading to exhaustion. While the ligand for CTLA-4, B7.1, was obviously present in some instances, CTLA-4 expression was quite infrequent in comparison to PD-1, despite the presence of its stimulatory triggers, TCR and CD28 ligation [61, 71]. The low percentage of cells expressing CTLA-4 at the 48 hour time point suggests

that a longer period of stimulation may be required to detect a marked increase in the expression of this receptor.

Both anti-apoptotic protein expression and IL-2 secretion appeared to be directly associated with the observed differences in $CD8^+$ T cell survival following activation. Bcl-2 expression correlated well with the percentage of cell recovery observed in these experiments. Strong co-stimulatory conditions including 10nM B7.1, 10nM ICAM-1, B7.1 + ICAM-1 and B7.1 + anti-CD27, which had previously been demonstrated to enhance T cell survival and accumulation, all sustained high expression of Bcl-2 at the 48 hour time. In addition, poor co-stimulatory conditions, including anti-CD27 alone and ICAM-1 + anti-CD27, exhibited low levels of Bcl-2 suggesting their poor cell survival may be linked to anti-apoptotic protein expression. Importantly, Bcl-2 expression by T cells is also directly related to the presence of IL-2 and other T cell growth factors [184, 269, 282]. $CD8^+$ T cells lacking sufficient IL-2 are more prone to cell death due the loss of Bcl-2 expression than other extrinsic mechanisms [270]. IL-2 and IL-2 receptor signalling have also been demonstrated to increase the expression of another important anti-apoptotic protein Mcl-1, further increasing apoptotic thresholds [283]. This suggests that while CD28, CD27 and ICAM-1 co-stimulatory signalling can increase the expression of the anti-apoptotic proteins, their ability to induce autocrine IL-2 secretion from the $CD8^+$ T cells may trump any other protective cell death mechanism in these cell culture conditions.

The induction of IL-2 secretion by $CD8^+$ T cells following co-stimulation has been widely examined. CD28 ligation represents a classic example of co-

stimulation induced IL-2 production, with IL-2 secretion being reported to increase relative to the amount CD28 co-stimulation by both $CD4^+$ and $CD8^+$ T cells [64, 162]. IL-2 production has also been demonstrated to increase following concurrent TCR and LFA-1 engagement, and to some degree following CD27 ligation [125, 234]. IL-2 production is not only critical in enhancing $CD8^+$ T cell survival following activation, but it also plays a key role in dictating T cell activation state and effector molecule production. Exogenous IL-2 addition has been demonstrated to increase the expression of the IL-2 high-affinity receptor CD25 and to enhance the expression of both granzyme B and perforin [66, 174]. Thus, differential IL-2 secretion following co-stimulation appears to be critical in shaping several aspects of the $CD8^+$ T cell response. The head-to-head comparison between individual co-stimulator ligands for their ability to induce IL-2 secretion from $CD8^+$ T cells revealed that B7.1 was superior to ICAM-1, CD27 and HVEM in the ability to induce secretion of this cytokine. The combination of B7.1 and ICAM-1 co-stimulation, however, induced by far the largest amount of IL-2 secretion suggesting that this co-stimulator combination is not only ideal for inducing IL-2 production, but may direct activated $CD8^+$ T cells toward unique effector fates due to this elevated cytokine expression. Contrary to this, the lack of IL-2 produced following CD27 ligation was somewhat unexpected as CD27 has been demonstrated to induce some IL-2 secretion following ligation [125, 284]. The amount of IL-2 produced following combined B7.1 + anti-CD27 co-stimulation did slightly increase in a CD27 dependent manner, suggesting that CD27 co-stimulation can enhance IL-2 production, but

not to the same degree as ICAM-1. The ability of CD27 co-stimulation to induce IL-2 production in these experiments may have been limited by the activating conditions. For example, it is possible that if TCR stimulation is increased, or the amount of co-immobilized B7.1 is adjusted, the ability of CD27 to induce significant IL-2 production from $CD8^+$ T cells may be better detected.

The addition of exogenous IL-2 to the co-stimulated $CD8^+$ T cell cultures revealed a preferential ACAD mechanism for cell death following many co-stimulatory conditions. This is evidenced by the reduction in $CD25^{hi}$ annexin V^{hi} $CD8^+$ T cells observed following the addition of IL-2 to cells co-stimulated with 1.25nM ICAM-1 + anti-CD27, and 1µg anti-CD27 alone. Because ACAD is associated with growth factor withdrawal, the poor IL-2 production induced following anti-CD27 co-stimulation, even in combination with B7.1, further supports suboptimal IL-2 production as a potential cell death mechanism in these experiments. In addition, the reduced expression of Bcl-2 exhibited by these cells also correlates with the activation of intrinsic cell death mechanisms associated with ACAD. 10nM ICAM-1 co-stimulated $CD8^+$ T cells however, did not display a phenotype indicative of ACAD, at least at early time points, as these cells did secrete IL-2 and exhibited relatively high Bcl-2 expression. IL-2 addition also did not reduce the percentage of cells which were $CD25^{hi}$ annexin V^{hi} after 24 hours, indicating AICD may play a larger role in the death of 10nM ICAM-1 co-stimulated cells. AICD generally occurs following TCR stimulation in the absence of proper co-stimulation [151]. Because LFA-1 is not considered a traditional co-stimulatory molecule due to the difficulty in identifying unique

signalling cascades generated by this receptor, under these conditions, the ICAM-1:LFA-1 interaction may simply be augmenting TCR mediated signalling and not uniquely supporting the activation process, resulting in AICD.

IL-2 secretion and anti-apoptotic protein expression encompass only one mechanism of $CD8^+$ T cell contraction and that is the intrinsic ACAD pathway. AICD death receptor engagement, which is associated with T cell suicide and fratricide, is also a common mechanism of T cell death *in vitro* [151, 152]. T cell suicide and fratricide involves the ligation of surface death receptors Fas, TRAIL, and the TNF receptor, which are induced on the surface of $CD8^+$ T cells following activation, particularly in the absence of $CD4^+$ T cell help [152, 256]. While not thoroughly investigated, extrinsic cell death mechanisms may also contribute to the differences in $CD8^+$ T cell survival observed following co-stimulation. As mentioned above, co-stimulation of naive $CD8^+$ T cells with 10nM ICAM-1 does not result in cell death due to growth factor withdrawal, suggesting that death receptor engagement may be the underlying cause of the poor cell recovery observed by these $CD8^+$ T cells. While not confirmed, ICAM-1 co-stimulated $CD8^+$ T cells tended to have slightly elevated expression of FasL in previous experiments (Fig. 3-3D), indicating that these cells may have an increased risk of fratricide. To determine if Fas:FasL death receptor engagement is the cause of 10nM ICAM-1 co-stimulated T cell death, cells need to be better assessed for the expression of these molecules. Quantitative PCR (qPCR) will likely be required for the accurate detection of Fas and FasL, as the expression level of these receptors tended to be too low and variable to be confirmed by flow cytometry.

Co-stimulatory molecule engagement however, has been demonstrated to decrease AICD *in vitro* suggesting that combinations of co-stimulator ligands may be better able to prevent AICD. This is evidenced by the lack of AICD detected following the co-stimulation of naïve $CD8^+$ T cells with a combination of 1.25nM B7.1 + 1.25nM ICAM-1 at 24 hours (Fig. 4-7A,B). Co-stimulation via CD28 has been implicated in inhibiting AICD by limiting the expression of FasL [265], as well as inducing the expression of the cFLIP isoform $cFLIP_R$ [268]. While not investigated, the CD28 co-stimulation provided in these experiments may protect activated $CD8^+$ T cells from AICD via these mechanisms. Furthermore, co-stimulation through CD27 has also been demonstrated to reduce Fas expression on T cells, thereby reducing the chance of fratricide [285]. Because ACAD often masks any AICD phenotype however [270], the poor IL-2 production induced by CD27 ligation may make it difficult to discern if CD27 co-stimulation inhibits Fas mediated AICD in these experiments.

Finally, while exogenous IL-2 differentially affected $CD8^+$ T cell survival depending on the co-stimulatory condition, it did induce dramatic changes to $CD8^+$ T cell activation and granzyme B production following its addition to several co-stimulatory cultures. As demonstrated in the literature [174], IL-2 considerably increased the expression of CD25 from most $CD8^+$ T cell cultures indicating enhanced activation. Exceptions to this phenomenon included co-stimulation with 10nM B7.1, 10nM ICAM-1, and B7.1 + ICAM-1, likely due to saturating amounts of IL-2 already being present in the culture supernatants. A similar trend also occurred when assessing the $CD8^+$ T cells for an increase in

granzyme B expression following IL-2 addition. In particular, the co-stimulator ligand combinations of B7.1 + anti-CD27 and ICAM-1+ anti-CD27, which were poorly cytolytic in previous killing assays (Fig. 3-11B), both exhibited nearly a 2-fold increase in granzyme B expression with IL-2 treatment. This suggests that some co-stimulatory conditions may be sufficient to induce enough autocrine IL-2 to generate potent $CD8^+$ T cell effectors, while others may not. Thus, co-stimulator ligand combinations which fail to produce sufficient IL-2 to support full effector differentiation and survival, may greatly benefit from concurrent activation of IL-2 secreting $CD4^+$ T cell or the addition of exogenous IL-2 to achieve full activation following both *in vivo* and *in vitro* stimulation.

In conclusion, the results from these experiments suggest that co-stimulation can greatly affect the survival of $CD44^{lo}$ $CD8^+$ T cells following their activation. In particular, the co-stimulator ligand combination of 1.25nM B7.1 + 1.25nM ICAM-1 appears best for preserving T cell viability following T cell activation by their combined capacity to induce high relative expression of the anti-apoptotic proteins Bcl-2 and Bcl-xL, as well as their ability to induce large amounts of IL-2 secretion. IL-2 production from the activated $CD8^+$ T cells not only correlated with enhanced T cell survival and the inhibition of ACAD cell death mechanism, but also improved effector development by increasing the expression of CD25 and granzyme B by the $CD8^+$ T cells. This indicates that the ability of $CD8^+$ T cells to produce IL-2 is likely critical for their full effector differentiation, at least *in vitro,* and suggests that IL-2 addition or congruent $CD4^+$ T cell activation may be required for optimal $CD8^+$ T cell effector differentiation.

Chapter 5. Co-stimulation induced IL-2 secretion drives $CD8^+$ T cell terminal effector differentiation

Introduction

Acute viral infection results in an expanded population of antigen specific effector $CD8^+$ T cells which are ideally suited for the control and elimination of the originating pathogen. Following the expansion phase, the majority of these $CD8^+$ T cells effectors will die by apoptotic mechanisms during what is termed the cellular contraction [273]. The small numbers of cells which survive may go on to become long-lived memory $CD8^+$ T cells, which can be further subdivided into distinct effector memory (Tem) and central memory (Tcm) T cell subsets. While the magnitude of the $CD8^+$ T cells expansion phase may be directly related to the number of memory $CD8^+$ T cells which survive contraction [158, 286], the exact mechanisms which drive the differentiation of $CD8^+$ T cell terminal effector and memory subsets are still widely debated.

It is understood that the early interactions of naïve $CD8^+$ T cells with APCs during priming are critical in shaping the development of effector and memory T cell populations. Only a brief encounter between a single naïve $CD8^+$ T cell and an antigen presenting cell is sufficient to develop both $CD8^+$ T cell effector and memory populations [50, 287, 288]. This suggests that $CD8^+$ T cell differentiation to effector and memory subsets is not dictated by distinct pre-existing naïve clonotypes. In the fate commitment model with progressive differentiation, the generation of $CD8^+$ T cell effector and memory subsets is dictated by "signal strength", which encompasses TCR stimulation, co-

stimulatory molecule ligation, and cytokine availability. In this model, $CD8^+$ T cells which encounter strong antigenic signals preferentially differentiate into short-lived effector cells (SLECs), which display a robust effector phenotype with high expression of cytolytic molecules and IFN-γ. In contrast, $CD8^+$ T cells activated in the presence of a weak antigenic stimulus tend to be fated as memory precursor effector cells (MPECs) that exhibit less efficient effector function, but possess greater memory potential. This model also suggests that the MPEC population is relatively plastic in nature and can develop into SLECs if antigen stimulation is sustained or increased by environmental factors such as inflammation. Overall, this model proposes that strong antigenic stimulation and inflammation preferentially induces terminal effector development, while weaker antigenic stimulation in the presence of low amounts of inflammatory stimuli may preferentially result in memory $CD8^+$ T cell differentiation [8].

Terminal effector and memory precursor $CD8^+$ T cells can be distinguished based on their expression of several surface markers, including cytokine receptors and transcription factors, associated with effector and memory subsets. The common γ-chain family of cytokine receptors, and the cytokines IL-2, IL-7, and IL-15, have all been directly linked to memory generation. Specifically, the cytokines IL-7 and IL-15 have been demonstrated to be necessary for the survival of memory $CD8^+$ T cells [166, 181, 189, 192]. Furthermore, the distinct expression pattern of the IL-7 receptor α-chain (CD127) plays a key role in identifying memory progenitors. Following acute infection, CD127 expression is rapidly lost from the surface of $CD8^+$ T cells, however a

small proportion of activated $CD8^+$ T cells (~5-20%) re-express slightly elevated levels of the CD127. These $CD127^{hi}$ $CD8^+$ T cells are believed to be precursors for memory cell development based on their simultaneous expression of other memory associated markers including CD27, CD122, CD62L and Bcl-2 [185]. In contrast to their effector counter-parts, these $CD127^{hi}$ memory precursors also exhibit reduced expression of the effector associated surface marker KLRG1 [148, 185].

The cytokine IL-2 has also been linked to $CD8^+$ T cell effector and memory development. While the requirement for this cytokine during T cell activation is controversial [289, 290], IL-2 has been demonstrated to augment the expansion of $CD8^+$ T cells during the effector phase, and to increase the expression of several effector associated molecules including granzyme B and perforin [66, 174]. The presence of IL-2 nonetheless, is required for optimal memory $CD8^+$ T cell generation and function. Mice lacking the IL-2 receptor α-chain, CD25, exhibit poor $CD8^+$ T cell mediated protection following antigen re-encounter, suggesting that IL-2 signalling is necessary for robust memory $CD8^+$ T cell recall responses [171]. It is important to note however that while the expression of the IL-2, IL-7, and IL-15 cytokine receptors have been associated with memory differentiation, their expression does not necessarily indicate that $CD8^+$ T cells displaying these markers will be destined for memory development [291]. Thus, concurrent expression of effector and memory T cell associated transcription factors is also useful for distinguishing these T cell subsets.

Transcription factor expression has been linked to the generation of $CD8^+$ T cell terminal effector and memory precursor lineages. The T-box transcription factor T-bet, and its closely related family member Eomes, were the first described transcription factors associated with T cell fate. T-bet is associated with $CD8^+$ T cell effector differentiation as T-bet deficient cells displayed less cytolytic activity and IFN-γ secretion following antigen encounter [196, 197]. T-bet and Eomes have also been found to collaborate in inducing the expression of several effector molecules including granzyme B, perforin, and IFN-γ by $CD8^+$ T cells [198, 199, 204]. Importantly, Eomes has been established to play a crucial role in $CD8^+$ T cell memory generation by inducing the expression of the IL-2/15 receptor β-chain CD122, required for IL-15 receptor signalling [204]. More recently, the transcription factors Blimp-1 and Bcl-6 have also been linked to $CD8^+$ T cell effector and memory development. Blimp-1 expression has been demonstrated to favour terminal effector differentiation, while Bcl-6 expression is associated with memory generation [206, 217]. Blimp-1 and Bcl-6 are transcriptional repressors originally identified to dictate plasma and germinal centre cell formation in B lymphocytes [206]. Following antigen stimulation, naïve $CD8^+$ T cells are believed to differentiate into primed early effectors with moderate cytolytic ability, and are induced to express the terminal effector associated transcription factor Blimp-1 in the presence of continued antigen exposure. Alternatively, Bcl-6 is preferentially expressed by $CD8^+$ T cell effectors in the absence of further activating stimuli [206]. This model for Blimp-1 and Bcl-6 induction also appears to be closely linked to the progressive

differentiation model of T cell fate determination as each rely on signal strength and duration during priming to direct $CD8^+$ T cells toward effector and memory phenotypes.

The signal strength driving effector and memory generation of $CD8^+$ T cells includes the process of co-stimulation. While the co-stimulation of naïve T cells can enhance signals propagated by the TCR [56], this process also generates distinct signals. Either or both of these contributions of co-stimulation may favour effector or memory differentiation. CD28 co-stimulation has been demonstrated to aid in memory formation following viral infection *in vivo* [103, 104]. In addition, the interaction between the TNF family member CD27 and its ligand CD70 has been established to enhance memory T cell survival and maintenance after antigenic challenge [75, 76]. $CD8^+$ T cell stimulation through the adhesion molecule LFA-1 is also required for memory generation, as LFA-1 deficient mice fail to generate a functional $CD8^+$ T cell memory pool [145]. However, it is not currently known if co-stimulation of naive $CD8^+$ T cells can be used to preferentially generate terminal effectors or memory progenitors. If co-stimulator molecule ligation can indeed skew $CD8^+$ T cells toward these distinct T cell subsets, the mechanism by which co-stimulation does so requires investigation.

In this chapter, I examined whether co-stimulation can be used to direct naïve $CD8^+$ T cells toward effector and/or memory fates. Potential effector and memory $CD8^+$ T cell differentiation was determined through the detection of the SLEC and MPEC associated markers CD127, CD25, the IL-15 receptor α-chain,

4-1BB, and CD27, and through the relative expression of the transcription factors T-bet, Eomes, Blimp-1 and Bcl-6. Using polystyrene beads as a co-stimulator ligand bearing platform, I was able to determine which co-stimulator ligands and combinations promote skewing of $CD8^+$ T cells toward terminal effector and memory generation, based on their cytokine receptor, surface marker, and transcription factor expression. This approach identified the co-stimulator combination of B7.1 + ICAM-1 as preferentially generating $CD8^+$ T cell terminal effectors with elevated expression of Blimp-1 and T-bet. Not only did I establish the co-stimulatory conditions which favoured $CD8^+$ T cell effector and memory precursor development, I also identified co-stimulation-induced secretion of IL-2 as a potential mechanism by which $CD8^+$ T cell fate is established.

Results

B7.1 + ICAM-1 co-stimulated CD8$^+$ T cells exhibit the highest and most sustained expression of the IL-7 and IL-15 cytokine receptors

Co-stimulatory molecule ligation of naïve CD8$^+$ has the potential to direct not only the generation of CD8$^+$ T cells with distinct effector phenotypes, but may play a critical role in directing CD8$^+$ T cells toward terminal effector or memory precursor fates. Previous research has associated the generation of effector and memory CD8$^+$ T cells with the expression of various differentiation specific molecules including cytokine receptors, co-stimulatory molecules, and transcription factors. Not only is the presence or absence of these select phenotypic markers associated with T cell fate, but their relative expression is also critically important in the formation of distinct CD8$^+$ T cell subsets.

Cytokines and their receptors have proven to be especially important in driving CD8$^+$ T cell effector and memory generation. The common γ-chain family of cytokine receptors, which includes receptors for the cytokines IL-2, IL-7 and IL-15, has been found to be associated with specific CD8$^+$ T cell subsets due to the timing of their expression, and the cell survival signals propagated by these receptors [166, 189]. Because of their critical roles in CD8$^+$ T cell terminal effector and memory generation, the expression of these cytokine receptors was investigated to determine which certain co-stimulator ligands, or their combinations, are ideally suited to induce their expression.

IL-7 receptor α-chain (CD127) expression is regained preferentially on CD8$^+$ T cells destined for a memory fate [185]. CD127 expression was assessed

on the surface of $CD8^+$ T cells after 48, 72 and 96 hour of stimulation to determine if certain co-stimulatory conditions could direct $CD8^+$ T cells to display a $CD127^{hi}$ memory-associated phenotype. CD127 could be detected following most co-stimulatory conditions tested with variable kinetics (Fig. 5-1A). Co-stimulation of naïve $CD8^+$ T cells with 10nM of ICAM-1 resulted in the highest percentage of cells expressing CD127 as nearly 75% of the cells were CD127 positive after only 48 hours of stimulation (Fig. 5-1A,B). This high percentage of CD127 positive cells was eventually lost however, as only 40% of the $CD8^+$ T cells remained $CD127^{hi}$ after 96 hours of stimulation with 10nM ICAM-1 (Fig. 5-1A,B). Co-stimulation with 10nM B7.1 and B7.1 + anti-CD27 showed a similar trend in which CD127 was expressed to some degree with distinct $CD127^{hi}$ and $CD127^{lo}$ populations detected. This bi-phasic expression of CD127 indicates that the cells had been activated (Fig. 5-1A,B). A reduction in CD127 expression and thus, $CD8^+$ T cell activation, could also be detected from cells co-stimulated with 1µg anti-CD27, ICAM-1 + anti-CD27, 1.25nM B7.1, and 1.25nM ICAM-1, as each of these cell populations has a reduced percentage of cells positive for CD127 in comparison to anti-CD3 and BSA stimulated controls (Fig. 5-1A). CD127 however, was found to be re-expressed and sustained to the highest degree on cells co-stimulated with a combination of B7.1 + ICAM-1, which were approximately 85 – 95% CD127 positive at the 72 and 96 hour time points (Fig. 5-1A,B). The expression of CD127 from B7.1 + ICAM-1 co-stimulated cells was nearly as high as expression of the IL-7 receptor α-chain from naïve unstimulated $CD8^+$ T cells, which were ~ 90-95% CD127 positive for nearly

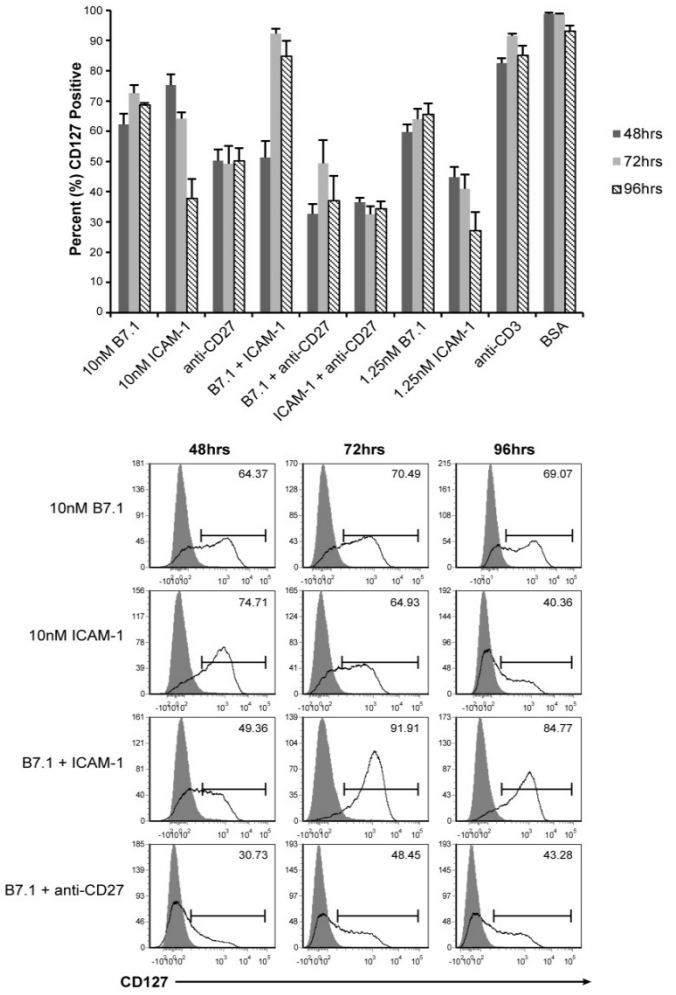


Figure 5-1. CD127 expression is re-expressed and sustained on CD8$^+$ T cells co-stimulated with a combination of B7.1 and ICAM-1. CD127 expression was assessed on CD44^{lo} CD8$^+$ T cells following 48, 72 and 96 hours of co-stimulation with high (10nM) or intermediate (1.25nM) amounts of B7.1 or ICAM-1 or high amounts (1µg) of anti-CD27 either individually or in combination (*A*). Co-stimulatory combinations utilized intermediate amounts (1.25nM) of recombinant proteins, and all bead constructs included suboptimal amounts of anti-CD3, with the exception of BSA unstimulated controls. Data in (*B*) depicts representative CD127 expression from CD8$^+$ T cells stimulated with 10nM B7.1, 10nM ICAM-1, 1.25nM B7.1 + ICAM-1 and 1.25nM B7.1 + 1µg anti-CD27 at 48, 72, and 96hrs. CD127 expression in both (*A*) and (*B*) was determined using fluorescently-conjugated CD127 antibodies analyzed by flow cytometry, and the percentage of cells positive for CD127 (*A*) was calculated relative to isotype stained controls. Results in (*A*) are an average of three independent experiments and error bars represent standard deviation in the experimental means. Flow cytometry histograms in (*B*) are representative of three experiments and shaded histograms (*B*) represent isotype control staining.

the entire experiment (Fig. 5-1A). From this data then, it appears that B7.1 + ICAM-1 co-stimulated cells may be best suited for IL-7 receptor signalling based on the extent of their CD127 re-expression.

CD122 is the common β-chain and signalling unit for both the IL-2 and the IL-15 cytokine receptors. Following cytokine binding, the IL-2 and IL-15 cytokine receptors are endocytosed, and their respective α-chains recycled back to the surface, while the β and γ-chains are targeted to late endosomal compartments for degradation [163, 292, 293]. Because CD122 is critically important in both IL-2 and IL-15 receptor signalling, its expression on $CD8^+$ T cells was monitored following various forms of co-stimulation. CD122 expression differed between co-stimulatory conditions in both the timing of its expression, and the percentage of cells positive for this receptor. CD122 expression was detected as early as 48 hours following several co-stimulatory conditions, including 1.25nM ICAM-1, and the combination of B7.1 + anti-CD27, which were approximately 55% and 50% positive for CD122 respectively (Fig. 5-2A). However, this expression was eventually lost suggesting that most of these cells may not be fit to support IL-15 receptor signalling during the contraction phase. A small percentage of cells positive for CD122 were also detected following co-stimulation with anti-CD27, anti-CD27 + ICAM-1, and 1.25nM B7.1. In each case however, CD122 expression was not maintained, with the exception of cells co-stimulated with anti-CD27, which were ~20% CD122 positive following 96 hours of stimulation. Surprisingly, CD122 expression was absent from cells co-stimulated with 10nM B7.1 and B7.1 + ICAM-1 at 48 hours. Significant CD122 expression

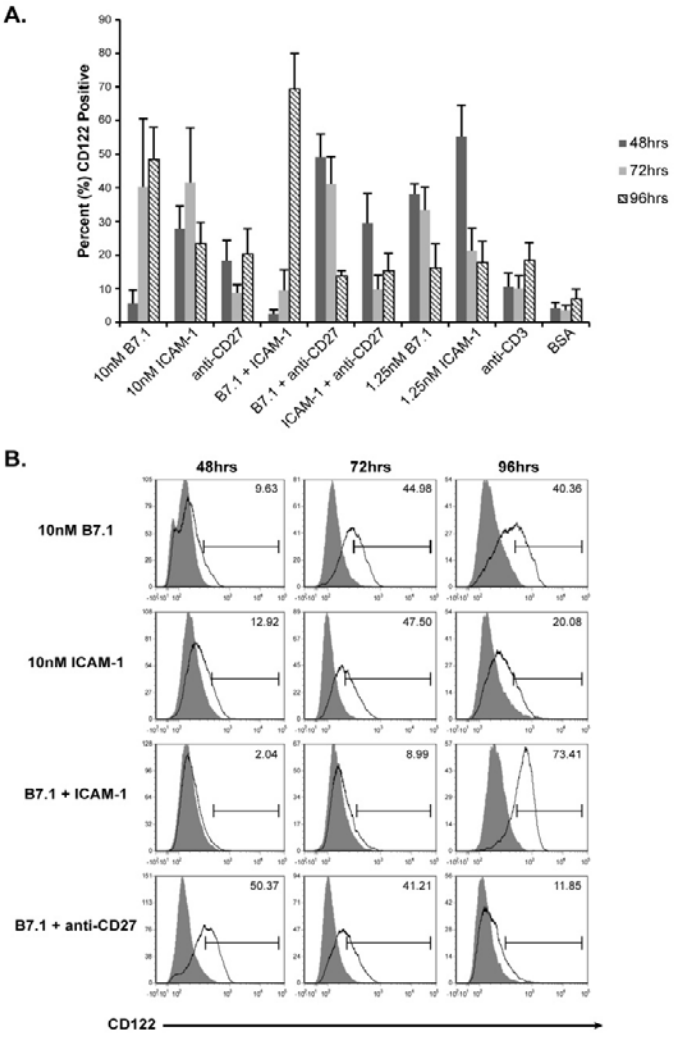


Figure 5-2. CD122 expression is induced with different kinetics following various forms of naïve CD44^{lo} CD8^{+} T cell co-stimulation. CD122 expression was assessed on CD44^{lo} CD8^{+} T cells following 48, 72 and 96 hours of co-stimulation with high (10nM) or intermediate (1.25nM) amounts of B7.1 or ICAM-1 or high amounts (1µg) of anti-CD27 either individually or in combination (*A*). Co-stimulatory combinations utilized intermediate amounts (1.25nM) of recombinant proteins, and all bead constructs included suboptimal amounts of anti-CD3, with the exception of BSA unstimulated controls. Data in (*B*) depicts representative CD122 expression from CD8^{+} T cells stimulated with 10nM B7.1, 10nM ICAM-1, 1.25nM B7.1 + ICAM-1 and 1.25nM B7.1 + 1µg anti-CD27 at 48, 72, and 96hrs. CD122 expression in both (*A*) and (*B*) was determined using fluorescently-conjugated CD122 antibodies and analyzed by flow cytometry, while the percentage of cells positive for CD122 (*A*) was calculated relative to isotype stained controls. Results in (*A*) are an average of three independent experiments and error bars represent standard deviation in the experimental means. Flow cytometry histograms in (*B*) are representative of three experiments and shaded histograms (*B*) represent isotype control staining.

was not detected until the 72 hour time point by CD8 $^{+}$ T cells co-stimulated with 10nM B7.1, and 96 hours following B7.1 + ICAM-1 co-stimulation (Fig. 5-2A,B). The high percentage of CD122 positive cells (~70% positive) detected following B7.1 + ICAM-1 co-stimulation at the 96 hour point suggests that these cells may be ideally suited to survive the contraction phase due to their potential for IL-15 receptor signalling.

Finally, the cytokine IL-15 itself is required for the generation and survival of memory CD8^{+} T cells *in vivo* [166, 192]. This indicates that the expression of the IL-15 receptor α-chain by CD8^{+} T cells may predispose these cells for enhanced memory cell survival. The role of the IL-15 receptor α-chain on CD8^{+} T cells however, is complicated, as IL-15 is believed to be trans-presented to T cells by distinct IL-15 receptor α-chain bearing cells [189]. Despite this, IL-15 receptor α-chain expression has been shown to sensitize CD8^{+} T cells to low concentrations of IL-15 cytokine, and may be important for maintaining cell viability when IL-15 levels are low [191]. Naïve CD44^{lo} CD8^{+} T cells were stimulated with the indicated co-stimulatory ligands and their combinations for 48 - 96 hours, and assessed for their expression of the IL-15 receptor α-chain by flow cytometry. In general, IL-15Rα expression was found to be low and variable in comparison to the other cytokine receptor subunits investigated (Fig. 5-3). Initially, IL-15Rα expression was induced on CD8^{+} T cells co-stimulated with 10nM ICAM-1 and 1.25nM ICAM-1. These cells were approximately 20% - 23% positive for the IL-15Rα-chain at the 48 hour time point, but this was reduced to 5% positive following 72 hours of stimulation (Fig.

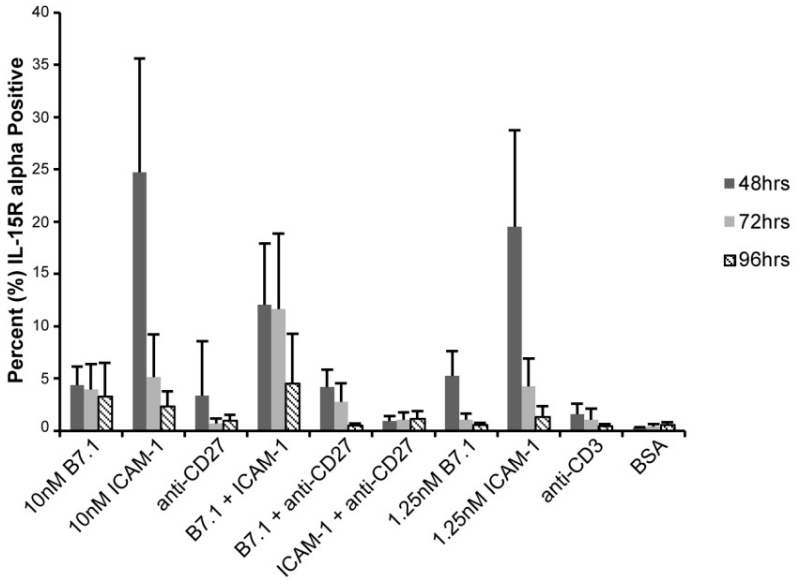


Figure 5-3. IL-15R alpha is preferentially expressed on $CD8^+$ T cells under co-stimulation conditions including ICAM-1. IL-15Rα expression was assessed on $CD44^{lo}$ $CD8^+$ T cells following 48, 72 and 96 hours of co-stimulation with high (10nM) or intermediate (1.25nM) amounts of B7.1 or ICAM-1 or high amounts (1µg) of anti-CD27 either individually or in combination. Co-stimulatory combinations utilized intermediate amounts (1.25nM) of recombinant proteins, and all bead constructs included suboptimal amounts of anti-CD3, with the exception of BSA unstimulated controls. IL-15Rα expression was determined using fluorescently-conjugated IL-15Rα monoclonal antibodies and analyzed by flow cytometry, while the percentage of cells positive for the IL-15 receptor alpha-chain was calculated relative to isotype stained controls. Results are an average of three independent experiments and error bars represent standard deviation in the experimental averages.

5-3). Little IL-15 receptor α-chain expression could be detected following $CD8^+$ T cell co-stimulation with the other conditions tested, with the exception of cells co-stimulated with a B7.1 + ICAM-1. Similar to CD127 and CD122 expression, co-stimulation with a combination of B7.1 + ICAM-1 appeared most effective for the induction and sustained expression of the IL-15 receptor α-chain, as moderate expression was detected up to the 72 hour point (Fig. 5-3). This again indicates that B7.1 + ICAM-1 co-stimulated $CD8^+$ T cells may be the best candidates for memory progenitor development and IL-15 receptor signalling. However, the expression of IL-15 receptor α-chain was significantly lower than the CD127 and CD122 expression detected in previous experiments, suggesting that IL-15 receptor α-chain may not be the best indicator for MPEC generation**.**

Sustained 4-1BB and CD27 expression from B7.1 + ICAM-1 co-stimulated $CD8^+$ T cells indicates a pro-longed effector phase.

Ligation of the TNF family members 4-1BB and CD27 during effector differentiation is believed to enhance the survival of $CD8^+$ T cells during the expansion phase, thereby increasing the number of $CD8^+$ T cells available for memory generation [56, 116]. This enhancement in cell survival stems from the ability of 4-1BB and CD27 to increase the expression of the anti-apoptotic protein Bcl-xL [79, 123]. As determined by flow cytometry, 48 hours of stimulation induced high 4-1BB expression following most forms of co-stimulation, with the exception of cells co-stimulated with either anti-CD27, or anti-CD27 + ICAM-1 in combination (Fig. 5-4A). High expression of 4-1BB however, was only maintained for 96 hours on $CD8^+$ T cells co-stimulated with a combination of

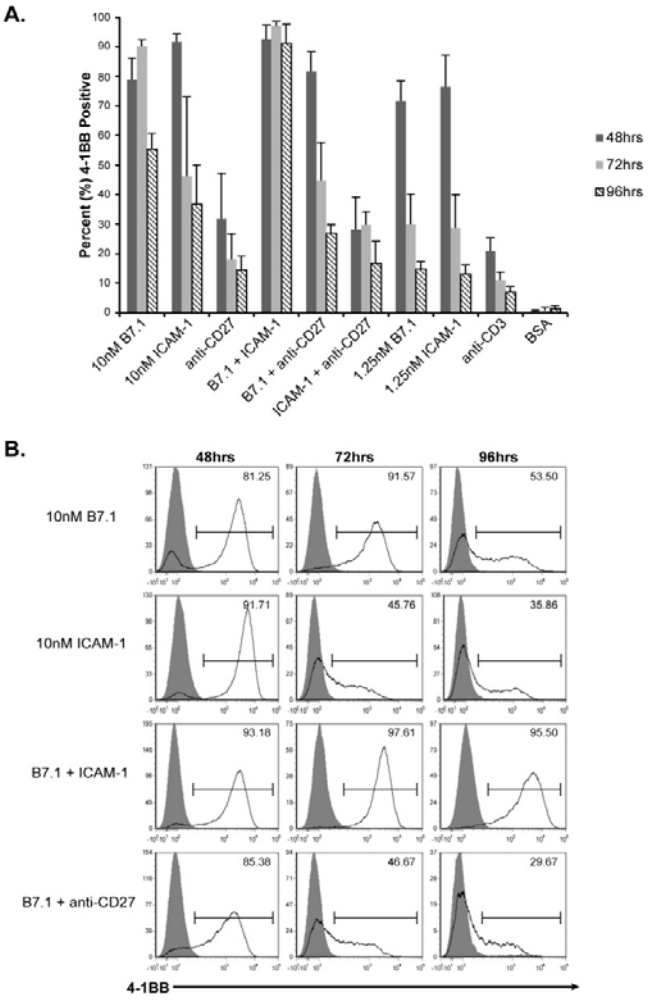


Figure 5-4. 4-1BB expression is preferentially induced and sustained on CD8$^+$ T cells co-stimulated with a combination of B7.1 and ICAM-1. 4-1BB expression was assessed on CD44^{lo} CD8$^+$ T cells following 48, 72 and 96 hours of co-stimulation with high (10nM) or intermediate (1.25nM) amounts of B7.1 or ICAM-1 or high amounts (1µg) of anti-CD27 either individually or in combination (*A*). Co-stimulatory combinations utilized intermediate amounts (1.25nM) of recombinant proteins, and all bead constructs included suboptimal amounts of anti-CD3, with the exception of BSA unstimulated controls. Data in (*B*) depicts representative 4-1BB expression from CD8$^+$ T cells stimulated with 10nM B7.1, 10nM ICAM-1, 1.25nM B7.1 + ICAM-1 and 1.25nM B7.1 + 1µg anti-CD27 at 48, 72, and 96hrs. 4-1BB expression in both (*A*) and (*B*) was determined using fluorescently-conjugated 4-1BB antibodies and analyzed by flow cytometry. The percentage of cells positive for 4-1BB (*A*) was calculated relative to isotype stained controls. Results in (*A*) are an average of three independent experiments and error bars represent standard deviation in the experimental means. Flow cytometry histograms in (*B*) are representative of three experiments and shaded histograms (*B*) represent isotype control staining.

B7.1 + ICAM-1 as these cells were up to 95% positive for 4-1BB at the 96 hour time point (Fig. 5-4A,B). This suggests that B7.1 + ICAM-1 co-stimulated $CD8^+$ T cells may be the best suited to receive 4-1BB propagated pro-survival signals.

Because CD27 signalling can also enhance anti-apoptotic protein expression [123], its expression was monitored following $CD44^{lo}$ $CD8^+$ T cell activation with various co-stimulatory ligands. Analysis of the CD27 expression profiles from the co-stimulation generated $CD8^+$ T cell populations showed that CD27 is expressed by a high percentage of cells co-stimulated with both high (10nM) and intermediate (1.25nM) amounts of recombinant B7.1 and ICAM-1, as well as B7.1 + ICAM-1 in combination (Fig. 5-5A). However, the analysis of CD27 expression is more complex than 4-1BB because of the expression of CD27 on naïve $CD8^+$ T cells. In these experiments, it appears that CD27 expression transitions from a low level found on unstimulated and poorly activated $CD8^+$ T cells, to high levels found on sufficiently stimulated cells (Fig. 5-5B). Once at this high level, CD27 expression tended to remain high, with the exception of cells co-stimulated with 1.25nM ICAM-1, which were ~30% CD27 positive following 96 hours of stimulation (Fig. 5-5A). Surprisingly, little CD27 expression could be detected from $CD8^+$ T cells activated in the presence of CD27 cross-linking antibodies, particularly following co-stimulation with anti-CD27 alone, and anti-CD27 + 1.25nM B7.1 (Fig. 5-5A,C). This was despite using two different antibody clones for stimulation and staining. Nonetheless, the combined high expression of both 4-1BB and CD27 from B7.1 + ICAM-1 co-stimulated

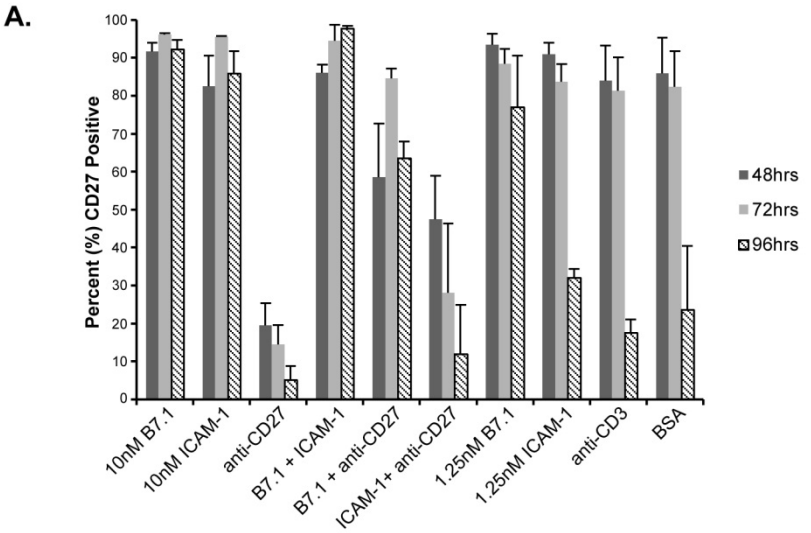
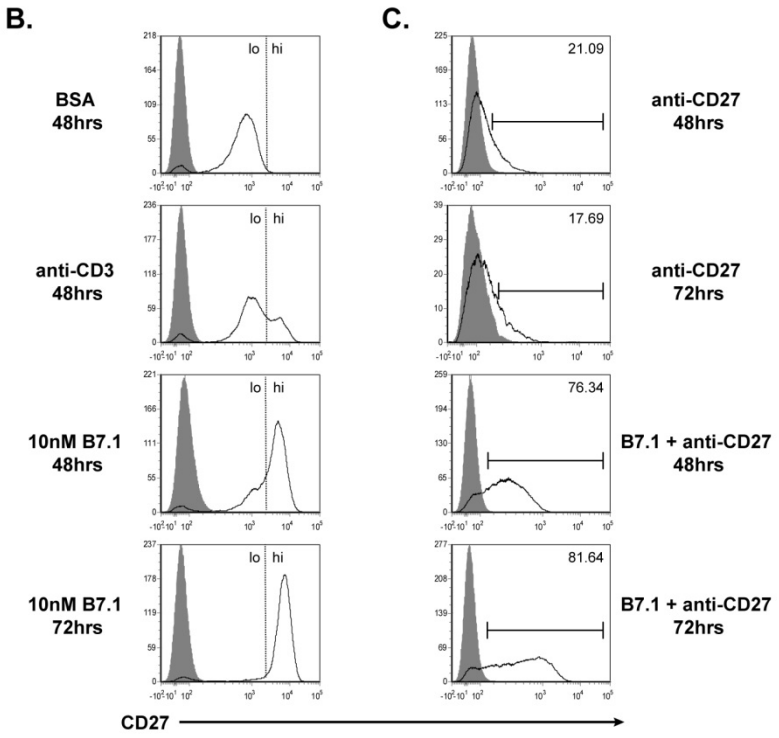


Figure 5-5. Strong co-stimulatory conditions induce and sustained high expression of CD27 on the surface of activated CD8$^+$ T cells. CD27 expression was assessed on CD44^{lo} CD8$^+$ T cells following 48, 72 and 96 hours of co-stimulation with high (10nM) or intermediate (1.25nM) amounts of rB7.1 or rICAM-1 or high amounts (1µg) of anti-CD27 either individually or in combination (*A*). Co-stimulatory combinations utilized intermediate amounts (1.25nM) of recombinant proteins, and all bead constructs included suboptimal amounts of anti-CD3, with the exception of BSA unstimulated controls. The percentage of cells positive for CD27 (*A*) was calculated relative to isotype stained controls. (B) Typical CD27 staining on BSA stimulated, anti-CD3 suboptimally stimulated, and 10nM B7.1 co-stimulated CD8$^+$ T cells at 48 and 72hrs following stimulation. CD27 shifts from low to high expression following CD44^{lo} CD8$^+$ T cell activation. (C) The percentage of CD27 expression at 48 and 72hrs on CD8$^+$ T cells co-stimulated with either 1µg anti-CD27 (top) or 1.25nM B7.1 + 1µg anti-CD27 (bottom). Histograms show reduced CD27 detection from both stimulations at both time points. CD27 expression in all panels was determined using fluorescently-conjugated CD27 antibodies and analyzed by flow cytometry. Results in (*A*) are an average of three independent experiments and error bars represent standard deviation in the experimental means. Flow cytometric data in (B) and (C) are representative of three independent experiments and shaded histograms represent isotype control staining.


B.
C.
BSA
48hrs
anti-CD3
48hrs
10nM B7.1
48hrs
10nM B7.1
72hrs
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17.69
76.34
81.64
anti-CD27
48hrs
anti-CD27
72hrs
B7.1 + anti-CD27
48hrs
B7.1 + anti-CD27
72hrs
CD27

cells indicates that these $CD8^+$ T cells may be best suited to receive pro-survival signals from ligation of these receptors.

Blimp-1 versus Bcl-6 transcription factor analysis indicates B7.1 + ICAM-1 co-stimulation directs $CD8^+$ T cells toward terminal effector differentiation

As a second approach to determine the preferential differentiation of co-stimulated $CD8^+$ T cells toward effector and memory $CD8^+$ T cell fates, quantitative PCR (qPCR) was used to monitor the $CD8^+$ T cells for their expression of the differentiation associated transcription factors Blimp-1 and Bcl-6. The relative expression of Blimp-1 and Bcl-6 has been associated with terminal effector and memory generation, respectively [206]. Since Blimp-1 expression is not induced to detectable levels following initial $CD8^+$ T cell stimulation [66, 215], cells were assessed for Blimp-1 and Bcl-6 expression after 96 hours of stimulation with the indicated co-stimulatory ligand bearing bead constructs. Detection of Blimp-1 and Bcl-6 transcripts was also attempted following five and six days of $CD8^+$ T cell stimulation, but poor cell survival, RNA quality and yield, hampered accurate assessment of transcript expression levels. Surprisingly, after 96 hours of stimulation, the combination of B7.1 + ICAM-1 co-stimulation resulted in a $CD8^+$ T cell population with a preferential SLEC phenotype, exhibiting an approximate 20-fold increase in Blimp-1 expression relative to naïve controls (Fig. 5-6A). Additionally, little expression of the memory associated transcript Bcl-6 could be detected (Fig. 5-6B). In contrast, $CD8^+$ T cells co-stimulated with either a combination of B7.1 + anti-CD27 or 10nM ICAM-1, had a transcription factor expression profile associated with

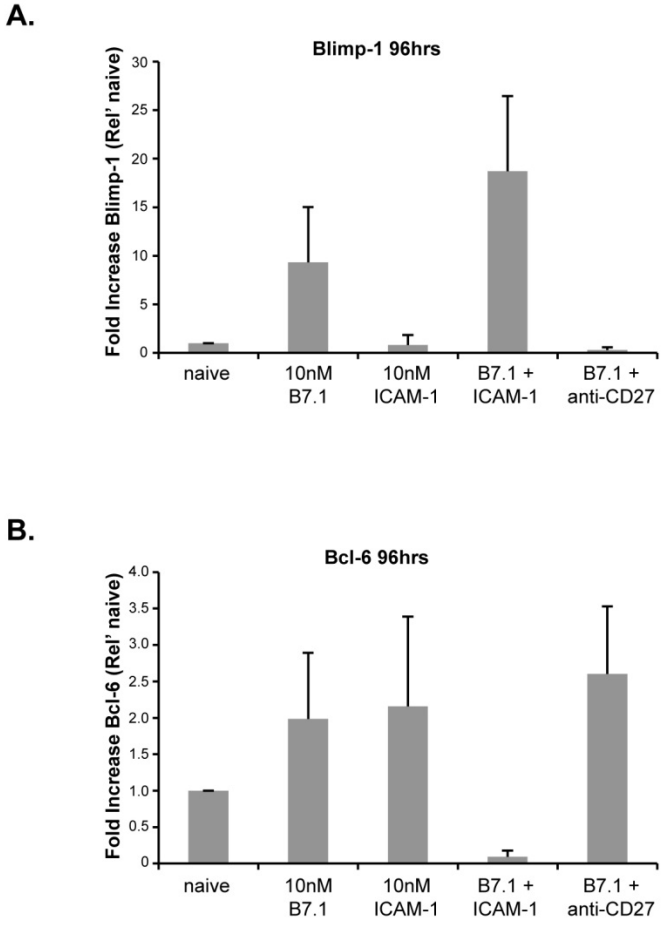

Figure 5-6. $CD44^{lo}$ $CD8^{+}$ T cells co-stimulated with a combination of B7.1 and ICAM-1 preferentially express the terminal effector associated transcription factor Blimp-1. Naive $CD44^{lo}$ $CD8^{+}$ T cells were stimulated for 96 hours with either 10nM B7.1, 10nM ICAM-1, 1.25nM B7.1/ICAM-1 or 1.25nM B7.1/1µg anti-CD27, in combination with suboptimal amounts of anti-CD3, and assessed for their relative expression of Blimp-1 (*A*) and Bcl-6 (*B*) by quantitative PCR (qPCR). The fold increase in both Blimp-1 and Bcl-6 expression was calculated relative to naïve $CD44^{lo}$ $CD8^{+}$ T cells using RPL24 gene expression as an internal control. Results are a mean of three independent experiments. Error bars represent standard deviation in the experimental means.

memory precursor generation as Bcl-6 expression was found to be slightly elevated (~2.5 fold increase in expression relative to naïve controls), while Blimp-1 transcript levels were relatively low (Fig. 5-6A,B). This approximate 2 – 2.5 fold increase in Bcl-6 expression relative to naïve controls is similar to the increase in Bcl-6 transcript levels reported for both $CD127^{hi}$ $KLRG1^{lo}$ MPEC-phenotype $CD8^+$ T cells, and memory $CD8^+$ T cells analyzed 150 days post-infection [217]. Finally, 10nM B7.1 co-stimulated $CD8^+$ T cells had an unexpected phenotype with detectable expression of both Blimp-1 and Bcl-6 (Fig. 5-6A,B). This is particularly interesting as Blimp-1 and Bcl-6 are transcriptional repressors of each other, suggesting 10nM B7.1 co-stimulation leads to a $CD8^+$ T cells with a mixed effector and/or memory phenotype.

Terminal effector differentiation is driven by co-stimulation induced IL-2 secretion and signalling

Current research indicates a direct relationship between IL-2 and Blimp-1 expression in $CD8^+$ T cells [66, 175]. IL-2 and Blimp-1 take part in an autoregulatory feedback loop in which IL-2 induces Blimp-1 expression, after which Blimp-1 binds the IL-2 promoter, repressing its production [175]. Since Blimp-1 was found to be differentially expressed under the co-stimulatory conditions tested, I sought to determine whether IL-2 secretion was enhanced following certain forms of co-stimulation, as a potential mechanism for Blimp-1 induction. As an initial experiment to determine if there was a correlation between co-stimulation induced IL-2 secretion and Blimp-1, supernatants were collected following 24 and 48 hours of $CD8^+$ T cell stimulation with the indicated

bead constructs, and tested for the presence of IL-2 by ELISA. As expected based on previous data (Fig. 4-6), the co-stimulator ligand combination of B7.1 + ICAM-1 induced the highest and most sustained secretion of IL-2, with cytokine concentrations approaching 15 -17ng/mL in the culture supernatants (Fig. 5-7A,B). The production of IL-2 following B7.1 + ICAM-1 co-stimulation was even greater than the IL-2 secretion induced following T cell stimulation with PMA and ionomycin (Fig. 5-7A,B). Importantly, this elevated IL-2 secretion correlates with the induction of Blimp-1 expression detected from B7.1 + ICAM-1 co-stimulated cells in the previous qPCR experiments (Fig. 5-6). IL-2 secretion could also be detected from $CD8^+$ T cell cultures co-stimulated with 10nM B7.1, 10nM ICAM-1 and 1.25nM B7.1 + anti-CD27 at both 24 and 48 hours; however the amount of secreted IL-2 was many fold lower, particularly from 10nM ICAM-1 and B7.1 + anti-CD27 co-stimulated cultures (Fig. 5-7). This again parallels the relative expression of Blimp-1 and Bcl-6 transcripts from the $CD8^+$ T cells co-stimulated with either B7.1 + anti-CD27 or 10nM ICAM-1, as these cells exhibited an increase in Bcl-6 expression with no induction of Blimp-1 (Fig. 5-6). From this data then, it appears that co-stimulation induced IL-2 secretion does indeed correlate with the relative expression levels of Blimp-1 and Bcl-6 determined by qPCR.

To further investigate the relationship between IL-2 and Blimp-1 expression, co-stimulation generated $CD8^+$ T cell populations were assessed for their expression of the high-affinity IL-2 receptor α-chain, CD25. High CD25 expression is associated with both the presence of IL-2, and the preferential

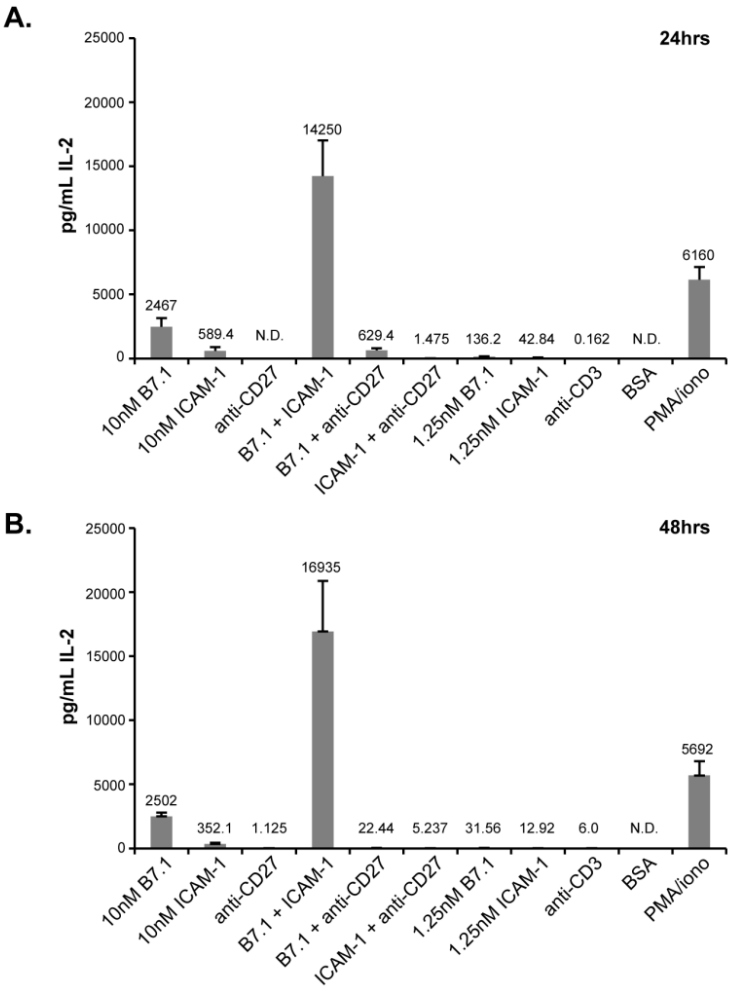

Figure 5-7. Co-stimulation induced IL-2 secretion may favour terminal effector differentiation of B7.1 + ICAM-1 co-stimulated CD8$^+$ T cells. To compare co-stimulated CD8$^+$ T cells for their IL-2 secretion, supernatants were collected from CD44^{lo} CD8$^+$ T cells co-stimulated with high (10nM) or intermediate (1.25nM) amounts of rB7.1 or rICAM-1 or high amounts (1µg) of anti-CD27 either individually or in combination at 24 (*A*) or 48 (*B*) hours following stimulation. Co-stimulatory combinations utilized intermediate amounts (1.25nM) of recombinant proteins, and all bead constructs included suboptimal amounts of anti-CD3, with the exception of BSA unstimulated controls. PMA/ionomycin treated naïve CD8$^+$ T cells were used as a positive control of IL-2 secretion. Collected supernatants were assessed for their IL-2 concentration at 24 and 48hrs by IL-2 ELISA. Numbers indicate the average concentration of IL-2 detected from each co-stimulatory condition following three independent stimulations. Error bars depict standard deviations in the mean IL-2 concentration.

generation of $CD8^+$ T cell terminal effectors [174]. CD25 expression was monitored 48, 72 and 96 hours after co-stimulation by flow cytometry. Following 48 hours of stimulation, all the co-stimulatory conditions tested displayed high expression of CD25, confirming the cells had been activated (Fig. 5-8). High expression of CD25 however, was only sustained on $CD8^+$ T cells co-stimulated with a combination of B7.1 + ICAM-1, which were nearly 100% CD25 positive at the 96 hour time point (Fig. 5-8B). This high expression of CD25 parallels the IL-2 secretion (Fig. 5-7), and Blimp-1 expression (Fig. 5-6 A), exhibited by these T cells in previous experiments. While the percentage of cells expressing CD25 following 10nM ICAM-1 and B7.1 + anti-CD27 co-stimulation appeared high at the 72 and 96 hour time points (~60-90% positive) (Fig. 5-8A), further analysis of the CD25 expression profiles revealed the presence of distinct $CD25^{hi}$ and $CD25^{lo}$ populations (Fig. 5-8B). Other groups have found that $CD25^{lo}$ $CD8^+$ T cells from similar bi-phasic populations tended to be fated toward memory generation [174]. Again this correlates with the elevated Bcl-6 expression (Fig. 5-6B), and lower IL-2 secretion (Fig. 5-7), exhibited from 10nM ICAM-1 and B7.1 + anti-CD27 co-stimulated cells in previous assays. Finally, 10nM B7.1 co-stimulated $CD8^+$ T cells displayed an intermediate CD25 expression pattern with a high percentage of CD25 positive cells (~85- 98% CD25 positive), which was maintained for the majority of the experiment. However, a slight progressive decline in CD25 expression level could be detected (Fig. 5-8A,B). In total, these data further establish a relationship between co-stimulation induced IL-2 production, IL-2 receptor signalling potential, and $CD8^+$ T cell differentiation.

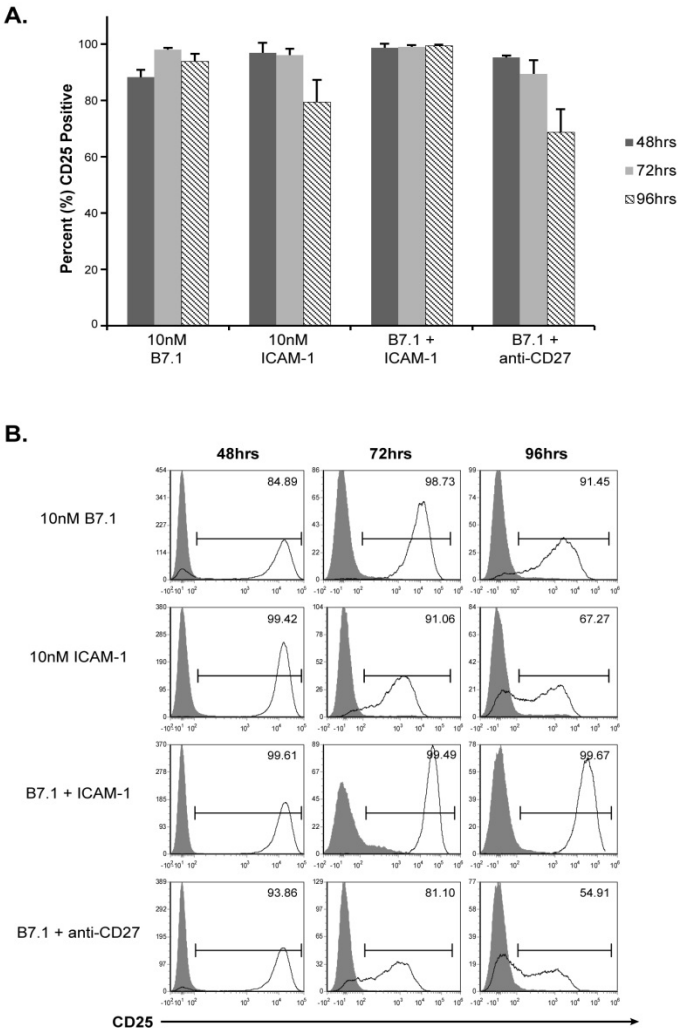

Figure 5-8. Co-stimulation of naïve CD44^{lo} CD8^{+} T cells results is differential expression of the IL-2 receptor alpha-chain. CD44^{lo} CD8^{+} T cells were stimulated with 10nM B7.1, 10nM ICAM-1, 1.25nM B7.1 + ICAM-1, or 1.25nM B7.1 + 1µg anti-CD27, in combination with suboptimal amounts of anti-CD3, and assessed 48, 72, and 96 hours later for their expression of CD25. CD25 expression was determined by cells surface staining using fluorochrome-conjugated CD25 mAbs, and analyzed by flow cytometry. The percentage of cells positive for CD25 at 48, 72, and 96hrs hours is depicted in panel (*A*), while data in (*B*) are representative CD25 expression profiles from CD8^{+} T cells following stimulation with the indicated co-stimulatory combinations at each time point. The percentage of cells positive for CD25 (*A*) was calculated relative to isotype stained controls and is an average of three independent experiments. Error bars in (*A*) represent standard deviation in the experimental means. Flow cytometry histograms in (*B*) are representative of three independent experiments and shaded histograms (*B*) represent isotype control staining.

To confirm an IL-2 centred mechanism for the differentiation of $CD8^+$ T cells following co-stimulation, I investigated STAT5 phosphorylation at various time points following T cell co-stimulation. STAT5 is a prominent signalling molecule and transcription factor phosphorylated downstream of the IL-2 receptor after cytokine binding [170]. As a mechanism for IL-2 induced expression of Blimp-1, it has been proposed that phosphorylated STAT5 may directly induce the production of Blimp-1 transcripts [175, 207]. Following their activation, a similar percentage of cells were found to be positive for STAT5 phosphorylation at the 24 and 48 hour time points with all the co-stimulatory combinations tested, as the percentage of STAT5 phosphorylation ranged between 30 - 60% (Fig. 5-9A). By 72 hours however, 10nM ICAM-1 and B7.1 + anti-CD27 co-stimulated $CD8^+$ T cells exhibited a 2-fold decrease in STAT5 phosphorylation relative to B7.1 + ICAM-1 co-stimulated cells, which were on average ~45% STAT5-P positive (Fig. 5-9A,B). Moreover, B7.1 + ICAM-1 co-stimulated cells sustained a high level of STAT5 phosphorylation following 72 hours of stimulation, relative to the other co-stimulatory conditions tested (Fig. 5-9B). Thus, the STAT5 phosphorylation exhibited by B7.1 + ICAM-1 co-stimulated $CD8^+$ T cells correlates with the co-stimulation induced IL-2 secretion, CD25 expression, and Blimp-1 induction displayed by these cells in previous assays. Again, $CD8^+$ T cells co-stimulated with 10nM B7.1 displayed an intermediate phenotype, with a slight decrease in STAT5 phosphorylation being detected at 72 hours relative to the other co-stimulatory conditions tested (Fig. 5-9A,B). In sum, this provides further evidence for an IL-2 centered mechanism of terminal effector and memory

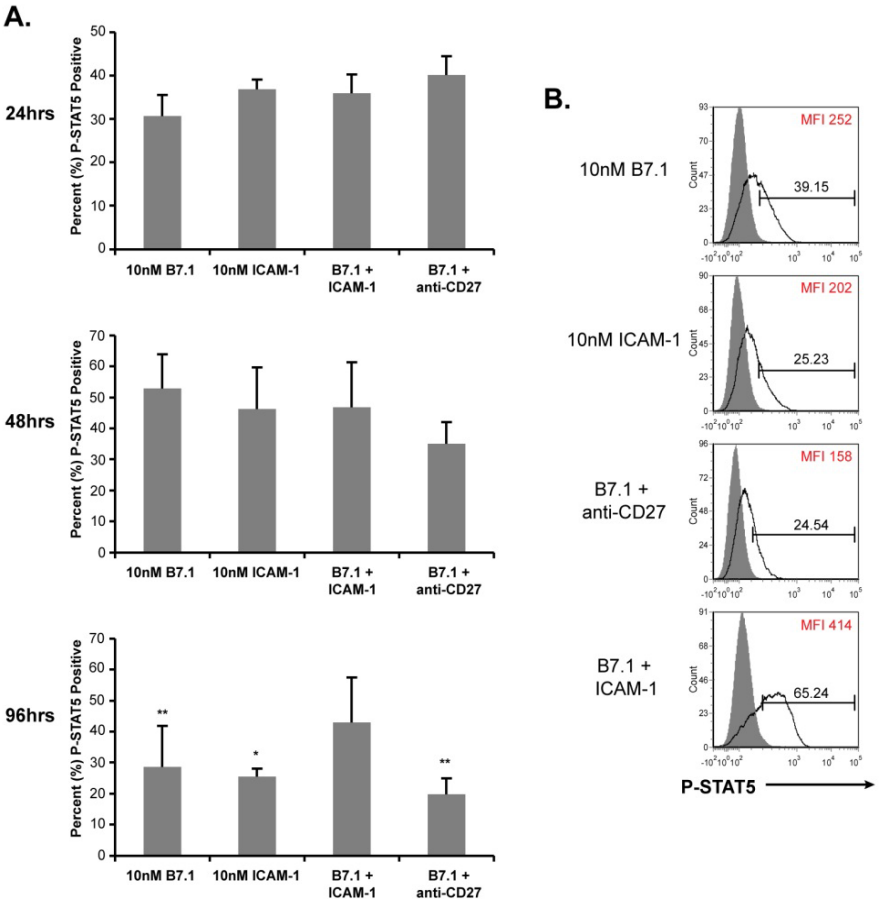

Figure 5-9. B7.1 + ICAM-1 co-stimulation preferentially sustains STAT-5 phosphorylation. To test for differences in co-stimulation associated STAT-5 phosphorylation, $CD44^{lo}$ $CD8^{+}$ T cells were stimulated with either 10nM B7.1, 10nM ICAM-1, 1.25nM B7.1 + ICAM-1, or 1.25nM B7.1 + 1µg anti-CD27, in combination with suboptimal amounts of anti-CD3, and assessed 24, 48, and 72 hours later for the presence of P-STAT5. STAT-5 phosphorylation was assessed at each time point by intracellular staining using fluorochrome conjugated P-STAT5 monoclonal antibodies and flow cytomtery as described in the *Materials and Methods*. The percentage of cells positive for phosphorylated STAT-5 at 24, 48, and 72 hours is depicted in panel (*A*), while data in (*B*) is representative staining of phosphorylated STAT5 following each co-stimulatory condition at the 72 hour time point. Data in (A) is an average of three independent experiments and error bars in (*A*) represent the standard deviation in the experimental means. Flow cytometry histograms in (*B*) are representative of three independent experiments and shaded histograms (*B*) represent isotype control staining. The representative mean fluorescence intensity (MFI) of phosphorylated STAT5 for each sample is depicted in red. MFI for cells stimulated with anti-CD3 alone was 154. (**) P ≤ than 0.01, (*) P ≤ than 0.05 compared to B7.1 + ICAM stimulations.

generation, and is consistent with a possible role for IL-2 receptor signalling in the establishment of differentiated $CD8^+$ T cell populations, following various forms of co-stimulation.

Terminal effector $CD8^+$ T cells have high and sustained expression of the transcription factor T-bet.

To verify the $CD8^+$ T cell differentiation phenotypes induced by co-stimulatory molecule ligation, I assessed the activated T cell populations for their relative expression of the effector and memory associated transcription factors T-bet and Eomes. T-bet was found to be expressed by a similar percentage of $CD8^+$ T cells following 48 hours stimulation with all the co-stimulator molecules and combinations tested (Fig. 5-10A). This expression was rapidly lost however from cells co-stimulated with a combination of B7.1 + anti-CD27, in which the percentage of cells positive for T-bet was reduced from approximately 85% at 48 hours, to 40% following 72 hours of co-stimulation (Fig. 5-10A). A slight reduction in T-bet expression could also be observed from 10nM ICAM-1 co-stimulated $CD8^+$ T cells at the 72hr time point (Fig. 5-10A). Contrary to this, T-bet expression was maintained for 72 hours by approximately 90% of the cells co-stimulated with 10nM B7.1, and 96 hours following B7.1 + ICAM-1 co-stimulation (Fig. 5-10A). Importantly, the high and sustained expression of T-bet displayed by B7.1 + ICAM-1 co-stimulated $CD8^+$ T cells correlates with the high IL-2 secretion and signalling, as well as the Blimp-1 expression, exhibited by

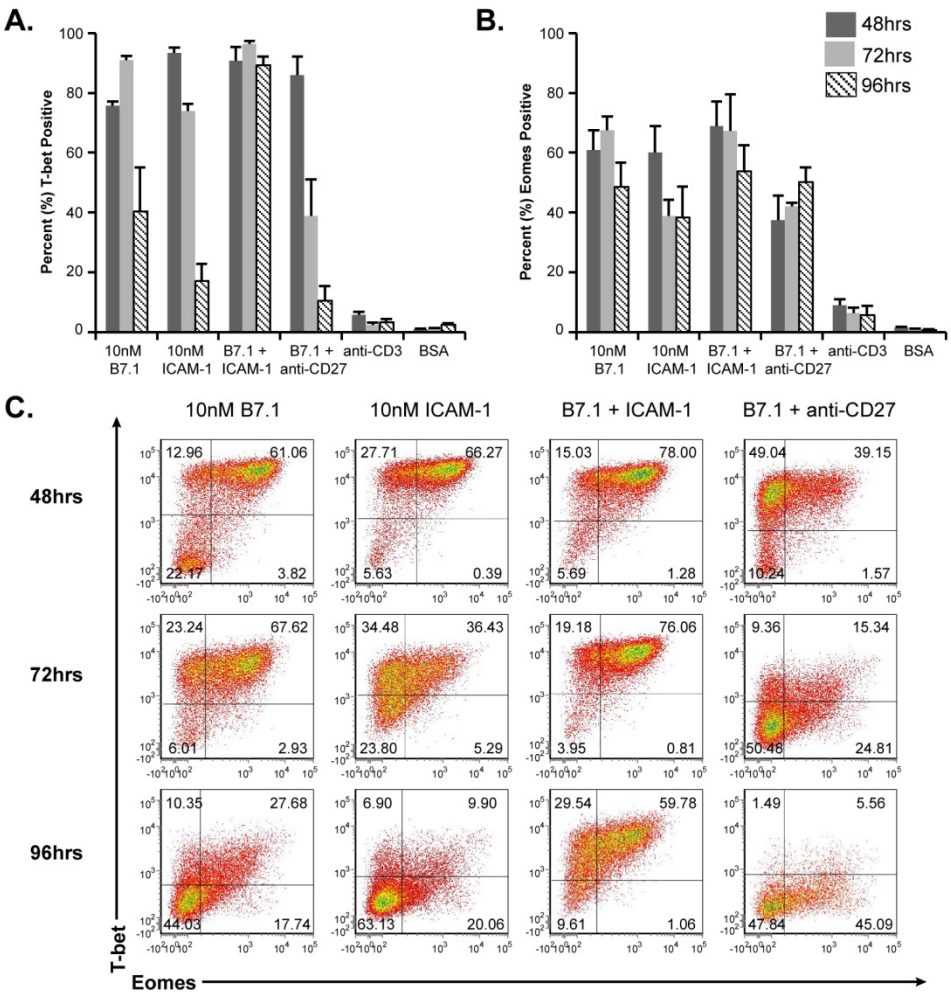

Figure 5-10. B7.1 + ICAM-1 co-stimulation induces high and sustained expression of the CD8$^+$ T cell effector associated transcription factor T-bet. CD44^{lo} CD8$^+$ T cells were stimulated with either 10nM B7.1, 10nM ICAM-1, 1.25nM B7.1 + ICAM-1, or 1.25nM B7.1 + 1ug anti-CD27, in combination with suboptimal amounts of anti-CD3, and assessed 48, 72, and 96 hours later for their expression of the transcription factors T-bet and Eomes by flow cytometry. CD8$^+$ T cells stimulated with a suboptimal amount of anti-CD3 and BSA coated beads served as negative controls for stimulation. The percentage of cells positive for T-bet (*A*) and Eomes (*B*) relative to isotype matched control staining was determined at the 48, 72, and 96 hour time point while representative staining for T-bet and Eomes from CD8$^+$ T cells stimulated under the indicated co-stimulatory conditions can be found in (*C*). T-bet and Eomes were detected using fluorochrome-conjugated mAbs and intracellular staining as described in the *Materials and Methods*. Data in (*A*) and (*B*) is an average of three independent experiments and error bars represent the standard deviation in these averages. Dot plots in (*C*) are representative of three independent experiments.

these cells in previous assays (Fig. 5-6 – 5-9). Eomes expression is required for full $CD8^+$ T cell effector function [198], but it is also associated with memory due to its role in inducing CD122 expression for IL-15 receptor signalling [204]. Eomes was found to be present in a similar percentage of $CD8^+$ T cells following all the co-stimulatory conditions tested at the 48 hour time point, with the exception of cells co-stimulated with B7.1 + anti-CD27 (Fig. 5-10B). These cells had approximate 2-fold reduction in Eomes expression at 48 hours, which may be reflective of the poor effector function demonstrated by these cells in previous experiments (Fig. 3-11B). However, the expression of Eomes by B7.1 + anti-CD27 co-stimulated cells was sustained, and even slightly increased, following 96 hours of stimulation (Fig. 5-10 B, C), correlating with the elevated expression of Bcl-6 exhibited by these cells in previous assays (Fig. 5-6). 10nM ICAM-1 co-stimulated $CD8^+$ T cells also demonstrated a similar but less pronounced T-bet versus Eomes expression pattern, in comparison to B7.1 + anti-CD27 co-stimulated $CD8^+$ T cells (Fig. 5-10 B, C). Of note, the majority of B7.1 + ICAM-1 co-stimulated cells remained both T-bet and Eomes positive for the entire experiment. Thus, the relative T-bet and Eomes transcription factor expression exhibited by these co-stimulation generated $CD8^+$ T cell populations, supports the terminal effector verses memory phenotypes predicted by Blimp-1 and Bcl-6 transcript levels in previous experiments. T-bet expression was preferentially sustained by $CD8^+$ T cells following B7.1 + ICAM-1 co-stimulation, in accordance with their elevated expression of terminal effector associated transcription factor Blimp-1, while anti-CD27 + B7.1 and 10nM ICAM-1 co-

stimulated cells, displayed reduced expression of T-bet and sustained expression of Eomes. In addition, the T-bet and Eomes expression patterns exhibited by the co-stimulated $CD8^+$ T cells also correlates to the effector phenotypes displayed by these $CD8^+$ T cell in previous assays, confirming an association between transcription factor expression and effector/memory development.

Discussion

Co-stimulation has the potential to direct naïve $CD8^+$ T cells toward terminal effector and memory precursor development. While the model for the transition of naïve $CD8^+$ T cells towards effector and memory states has not been firmly established, it likely involves a dynamic interaction between cytokine availability and co-stimulatory molecule ligation, resulting in differential transcription factor expression. In this chapter, I investigated if co-stimulation could be used to direct naïve $CD8^+$ T cells toward terminal effector and memory precursor development, based on the expression of effector and memory associated surface markers and transcription factors. Using stimulatory beads as a platform for co-stimulation and activation of naïve $CD8^+$ T cells, I was able to demonstrate a divergence in T cell effector and memory differentiation based on transcription factor expression. Furthermore, I uncovered co-stimulation induced IL-2 production as a potential mechanism by which $CD8^+$ T cell fate is determined.

Expression of the common-γ chain family of cytokine receptors was utilized as an initial approach for assessing the generation of $CD8^+$ T cell effector and memory subsets following various forms of co-stimulation. Based on the expression of CD127, CD122, and the IL-15 receptor α-chain, co-stimulation with a combination of B7.1 and ICAM-1 appears best suited for directing naïve $CD8^+$ T cells toward early memory development. When co-stimulated with this combination, $CD8^+$ T cells displayed the highest and most sustained re-expression of CD127, as well as sustained expression of the IL-15 receptor α-chain, both of

which are associated with optimal $CD8^+$ memory T cell establishment and survival [192, 273]. In addition, B7.1 + ICAM-1 co-stimulated cells also exhibited late and elevated expression of the IL-2/IL-15 receptor β-chain CD122, reflecting the potential for these cells to sustain IL-15 receptor signalling. Despite this phenotypic marker expression however, some doubt remains as to whether this memory precursor phenotype is genuine.

While re-expression of CD127 after $CD8^+$ T cell activation is believed to be associated with memory differentiation [185], other groups have found that this is not always the case. Following peptide immunization of naïve mice, Lacombe and others [291] demonstrated that high expression of CD127 was preferentially found on $CD8^+$ T cells at the peak of the effector response and did not correlate with later memory generation. In addition, CD127 expression and *bona fide* MPEC development is often linked to reduced expression of the surface marker KLRG1. Combined $KLRG1^{lo}$ $CD127^{hi}$ phenotypic marker expression by $CD8^+$ T cells is generally what designates memory precursor generation [148]. KLRG1 expression could not be detected in these experiments, likely due to its dependence on inflammatory cytokines for expression [294]. While high and sustained CD127 expression following B7.1 + ICAM-1 co-stimulation suggests the potential for memory related IL-7 cytokine receptor signalling, the specific $CD127^{hi}$ $KLRG1^{lo}$ expression profile associated with true MPEC generation could not be firmly established. Furthermore, while CD122 was found to be expressed from a high percentage of B7.1 + ICAM-1 co-stimulated cells at the 96 hour time point, this delayed expression of CD122 may be related to IL-2 signalling.

Because CD122 is endocytosed and degraded following cytokine binding [170], its low initial detection may be due to excessive IL-2 production induced by B7.1 + ICAM-1 co-stimulation, rather than IL-15 receptor expression. Overall, while B7.1 + ICAM-1 co-stimulated $CD8^+$ T cells appear to display a memory-like phenotype based on the expression of specific cytokine receptor components, this expression pattern may better reflect a pro-longed expansion phase rather than genuine memory generation.

Several other co-stimulators and co-stimulator combinations also induced expression of CD127, CD122 and the IL-15Rα-chain to some degree. Strong co-stimulatory conditions including 10nM B7.1, 10nM ICAM-1, and B7.1 + anti-CD27, did induce some re-expression of CD127, with distinct CD127 high and low populations being detected. This suggests that a portion of these co-stimulated $CD8^+$ T cells may be destined for memory generation. While detected very early following B7.1 + anti-CD27 co-stimulation, CD122 expression was also observed on these, 10nM ICAM-1, and 10nM B7.1 co-stimulated cells, indicating a potential for IL-15 receptor signalling. In addition, the IL-15Rα-chain could be detected from B7.1 + ICAM-1 and other ICAM-1 co-stimulated $CD8^+$ T cell populations, albeit at lower than anticipated levels. This is in accordance with previous work suggesting that LFA-1 signalling is required for the expression of this receptor, as LFA-1 knock-out mice fail to express the IL-15Rα-chain, resulting in an underdeveloped memory $CD8^+$ T cell compartment [272]. Together, these data suggest that the $CD8^+$ T cell populations generated following co-stimulation with 10nM B7.1, 10nM ICAM-1, and B7.1 + anti-CD27,

may also exhibit some memory potential. As with B7.1 + ICAM-1 co-stimulation, however, the expression of CD127, CD122, and the IL-15 receptor α-chain does not necessarily indicate genuine memory generation. Thus, IL-7 and IL-15 cytokine addition to long-term $CD8^+$ T cell cultures is required to demonstrate true memory differentiation using these phenotypic markers.

Engagement of specific co-stimulatory molecules during naïve $CD8^+$ T cell activation and expansion has been demonstrated to enhance T cell effector function, as well as memory generation. In the model of cooperative and sequential TNF family member ligation proposed by Croft in 2009 [116], the consecutive ligation of TNF co-stimulatory molecules, including CD27 and 4-1BB, enhances the activation of naïve $CD8^+$ T cells and increases their survival through the expansion phase. This ultimately results in an expanded number of T cells available for memory generation. 4-1BB and CD27 were both found to be expressed following many of the forms of co-stimulation tested including 10nM B7.1, 10nM ICAM-1 and B7.1 + ICAM-1. 4-1BB expression in particular could be detected at high levels following nearly all co-stimulatory conditions at the 48 hour time point, with the exception of $CD8^+$ T cells co-stimulated with either anti-CD27 or ICAM-1 + anti-CD27. While the reason for this reduced expression is not known, it may be related to the suboptimal activation induced by the ligation of CD27 and LFA-1 receptors. 4-1BB expression however, was only sustained for 96 hours following B7.1 + ICAM-1 co-stimulation. The availability of 4-1BB for ligation for this extended period of time not only suggests that these cells may be primed for memory generation, but may also indicate that these $CD8^+$ T cells

experience a prolonged expansion phase. To decisively conclude if the expression of 4-1BB in these experiments supports a prolonged effector phase, memory generation, or both, long-term cultures assessing the $CD8^+$ T cell survival following 4-1BB ligation are required. The ability of B7.1 + ICAM-1 co-stimulated cells to receive further anti-apoptotic signals during the effector phase is also demonstrated by their sustained high expression of CD27, which could be detected as early as 48 hours after stimulation. A similar shift in CD27 low to high expression was also observed following several other forms of co-stimulation including 10nM B7.1 and 10nM ICAM-1, in a manner that appeared to correlate with the extent of the $CD8^+$ T cell activation. Exceptions to this distinct CD27 staining pattern included co-stimulatory conditions involving CD27 cross-linking. Naïve $CD8^+$ T cells stimulated with the CD27 cross-linking antibody clone LG.3A10, displayed low and broad CD27 expression following staining of this receptor with a different antibody clone, LG.7F9. While not investigated further, this suggests that either these clones are in competition for the same CD27 epitope, or CD27 ligation with the LG.3A10 cross-linking antibody somehow inhibits the further expression of the CD27 receptor. In addition, CD27 may have been internalized.

The effector and memory associated transcription factors Blimp-1 and Bcl-6 were utilized to further define the potential for co-stimulation to generate $CD8^+$ T cell populations with distinct effector and memory fates. B7.1 + ICAM-1 co-stimulated cells displayed greatly elevated expression of Blimp-1 with relatively little Bcl-6 being detected, indicating a terminal effector phenotype.

This is contrary to the cytokine receptor expression displayed by these cells in earlier experiments. The elevated Blimp-1 transcription factor expression favours the theory that B7.1 + ICAM-1 co-stimulation skews $CD8^+$ T cell responses toward a pro-longed and more robust effector phase indicative of SLEC development, rather than memory differentiation. The high expression of Blimp-1 also parallels the effector molecule expression, cytolytic potential, and anti-apoptotic protein expression displayed by these cells in previous experiments. Contrary to this, co-stimulation with either B7.1 + anti-CD27 or 10nM ICAM-1 resulted in $CD8^+$ T cell effector populations with the phenotype of an early memory precursor, with slightly elevated expression of Bcl-6 and no detection of Blimp-1. Based on their cytokine receptor and 4-1BB expression, these co-stimulatory conditions were not the most likely candidates for MPEC generation. However the temporal expression of CD127 and CD122 memory associated markers did not exclude the possibility. Lastly, co-stimulation of naïve $CD44^{lo}$ $CD8^+$ T cells with 10nM B7.1 generated a T cell population with a mixed effector and memory phenotype displaying high amounts of Blimp-1, and slightly elevated levels Bcl-6. This transcription factor expression profile was unexpected as Blimp-1 and Bcl-6 are recognized as repressors of each other's expression [206]. Several scenarios however could have resulted in this unique expression pattern. First, it is possible that the effectors generated following 10nM B7.1 co-stimulation were unequally stimulated resulting in two distinct cell populations with high and low Blimp-1 expression levels. Secondly, this transcription factor profile may represent progressive differentiation as these cells could be

transitioning from a Bcl-6 expressing MPEC population, to SLECs expressing Blimp-1. Thirdly, this mixed phenotype may demonstrate asymmetric cell division in which mother and daughter cells have unequal distribution of their cytolytic contents, resulting in cell populations with distinct effector and memory potentials [159]. A similar uneven distribution of T-bet has also been demonstrated to occur [295].

Based on the Blimp-1 and Bcl-6 expression patterns presented here, it appears that co-stimulation can indeed skew $CD8^+$ T cells toward either terminal effector or memory precursor development. In addition, these results fit well with the fate commitment and progressive differentiation model of memory T cell differentiation [8], as strong co-stimulatory conditions, including the combination of B7.1 + ICAM-1, tend to direct cells toward SLEC development, while weaker co-stimulatory conditions including B7.1 + anti-CD27, tend to favour MPEC differentiation. These findings however need to be approached with caution. Due to the poor survival and RNA yield from co-stimulated $CD8^+$ T cell populations past the 96 hour time point, it cannot be verified that the Blimp-1 and Bcl-6 expression patterns observed are stable and do not change beyond 96 hours of stimulation. In particular, it is very likely that the B7.1 + ICAM-1 co-stimulated cells are being assessed during their effector phase and could shift from expressing high amounts of effector associated Blimp-1, to memory associated Bcl-6. Thus, irrefutable terminal effector generation or memory development cannot be determined unless the co-stimulation generated $CD8^+$ T cell populations are assessed at later time points for their differentiation stability. This can be

approached by either adoptively transferring co-stimulation generated $CD8^+$ T cells into naïve mice and assessing the cells two months later for the establishment of genuine memory populations, or by maintaining the co-stimulated $CD8^+$ T cell populations in long-term culture using the memory supporting cytokines IL-15 and IL-7. These long-term cultures could then be assessed for their preferential expression of Bcl-6 transcripts relative to naïve and effector $CD8^+$ T cell controls at later time points to detected memory cell development.

In this chapter, I proposed a model in which co-stimulation induced IL-2 secretion directs primed early $CD8^+$ T cell effectors towards terminal effector or memory precursor phenotypes (Fig. 5-11). The role IL-2 plays in $CD8^+$ T cell differentiation has been controversial as memory fated Blimp-1 deficient $CD8^+$ T cells have been found to produce higher amounts of IL-2 [215]. On the other hand, IL-2 addition has also been demonstrated to increase the expression of several effector associated molecules including granzyme B and perforin, thus promoting effector differentiation [66, 176]. Importantly, IL-2 signalling has been established to induce terminal effector associated Blimp-1 expression, likely through the transcription factor STAT5 [66, 175]. Based on a model in which high IL-2 concentration skews $CD8^+$ T cell responses toward effector development, I assessed the co-stimulation generated T cell populations for their production of IL-2 and their IL-2 receptor associated signalling potential. In support of their terminal effector differentiation and preferential expression of

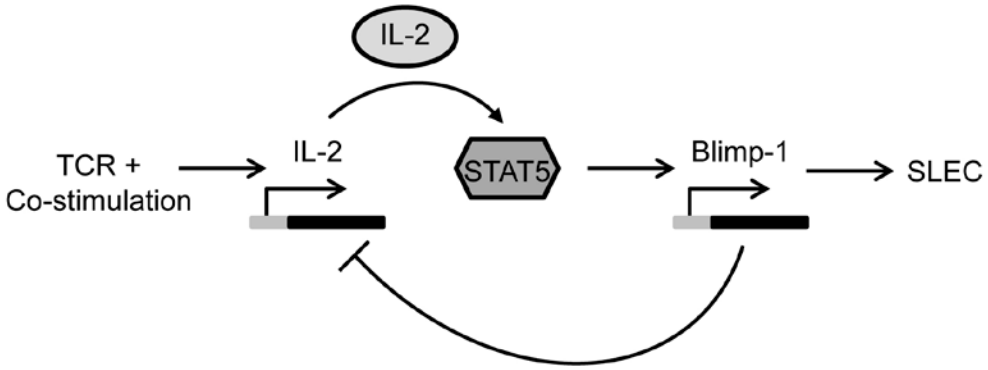

Figure 5-11. Model of co-stimulation induced IL-2 secretion directing CD8$^+$ T cell differentiation. TCR ligation and co-stimulatory combinations induce varying amounts of IL-2 transcription and secretion by CD8$^+$ T cells. Secreted IL-2 interacts with the IL-2 receptor on T cells in an autocrine fashion, inducing downstream STAT5 phosphorylation. High amounts of IL-2 secretion and IL-2 receptor signalling, lead to the induction of Blimp-1 expression, likely driven by STAT5. Blimp-1 expression by activated CD8$^+$ T cells leads to SLEC development and terminal effector differentiation.

Blimp-1, B7.1 + ICAM-1 co-stimulated $CD8^+$ T cells secreted a comparatively large amount of IL-2, and displayed sustained high expression of CD25 and phosphorylation of STAT5. Contrary to this, but still in support of an IL-2 based model for effector versus memory differentiation, $CD8^+$ T cells co-stimulated with either B7.1 + anti-CD27 or 10nM ICAM-1, secreted lower amounts of IL-2 and demonstrated a biphasic CD25 expression pattern associated with memory generation [174]. In addition, these cells also exhibited slightly reduced STAT5 phosphorylation relative to B7.1 + ICAM-1 co-stimulated $CD8^+$ T cells. For better comparison of STAT5 phosphorylation between co-stimulator combinations however, western blotting may be required. Nonetheless, these results suggest that co-stimulation induced IL-2 secretion from the $CD8^+$ T cell cultures themselves may be directly linked to the divergence of $CD8^+$ T cell terminal effector and early memory progenitors. In such a model, co-stimulation induced IL-2 secretion increases CD25 expression and IL-2 receptor signalling through STAT5, leading to the preferred induction of Blimp-1 (Fig. 5-11). Validation of this IL-2 based model however requires further experimentation involving the addition of exogenous IL-2 to co-stimulated cells cultures to detect a shift from Bcl-6 expressing MPECs to Blimp-1 expressing SLECs, or the addition of IL-2 neutralizing antibodies to observe preferential Bcl-6 expression.

Lastly, I confirmed the terminal effector and potential memory phenotypes exhibited by these co-stimulated $CD8^+$ T cells by monitoring their relative expression of the effector and memory associated transcription factors T-bet and Eomes. In accordance with the relatively high expression of Blimp-1 from B7.1 +

ICAM-1 co-stimulated $CD8^+$ T cells, these cells also displayed high and sustained expression of the effector associated transcription factor T-bet, as well as Eomes. This correlates with the high IFN-γ expression and cytolytic capacity demonstrated from these cells in previous experiments, both of which are controlled by T-bet to some degree [198, 199]. Thus, the sustained high expression of T-bet from B7.1 + ICAM-1 co-stimulated $CD8^+$ T cell provides further evidence of their terminal effector differentiation. Contrary to this, T-bet expression was rapidly lost from B7.1 + anti-CD27 co-stimulated $CD8^+$ T cells, and was in turn replaced with sustained and relatively solitary expression of Eomes. This preferential expression of Eomes at later time points correlates with the memory precursor phenotype predicted by the slightly elevated Bcl-6 expression from B7.1 + anti-CD27 co-stimulated cells. Interestingly, an association can also be drawn between the initial low expression of T-bet and Eomes exhibited by B7.1 + anti-CD27 co-stimulated $CD8^+$ T cells, and their poor lytic potential established in previous experiments, since Eomes has been demonstrated to directly regulate the expression of the cytolytic molecule perforin [66]. However, T-bet expression by $CD8^+$ T cells has been found to be somewhat deregulated in the absence of $CD4^+$ T cell help. $CD8^+$ T cells stimulated in the absence of $CD4^+$ T cells *in vivo* display an unusual phenotype in which activated $CD8^+$ T cells express exceptionally high amounts of T-bet, resulting in the generation of a greatly expanded effector-memory $CD8^+$ T cell pool at the expense of central memory generation [296]. Whether the absence of $CD4^+$ T cells affected the outcome of experiments presented here is currently unknown as

cells were not assessed for effector versus central memory development. However, the dynamic regulation of T-bet expression demonstrated by the $CD8^+$ T cells suggests that terminal effector development was not disrupted. Together, the relative T-bet and Eomes expression patterns displayed by these co-stimulated $CD8^+$ T cells further supports a model for co-stimulation induced terminal effector and memory differentiation. Not only does the expression of these transcription factors correlate with the generation of terminal effector and potential memory T cell populations, they also parallel the effector function displayed by the co-stimulation generated effector $CD8^+$ T cells in previous assays.

Whether a model of co-stimulation induced IL-2 secretion directing $CD8^+$ T cell fate can be applied *in vivo* remains an unanswered question. The presence of inflammatory cytokines induced by infection, and IL-2 secretion from activated bystander $CD4^+$ T cells, likely would skew responses toward effector generation in an *in vivo* environment. Inflammatory cytokines secreted by activated APCs have been demonstrated to enhance effector differentiation as well as function by increasing the expression of effector molecules such as granzyme B and perforin [146, 150]. In particular, IL-12 has been shown to directly increase T-bet expression and effector development in a dose dependent manner both *in vitro* and *in vivo* [148, 149]. Furthermore, congruent activation of IL-2 secreting $CD4^+$ T cells has the potential to skew $CD8^+$ T cells toward terminal effector differentiation by providing $CD8^+$ T cells with a sustained dose of IL-2 required for their continued effector function [37]. Nonetheless, a model supporting IL-2

induction of either SLEC or MPEC development may in some cases be favoured. For example, late arriving $CD8^+$ T cells activated in a stimulatory environment with reduced amounts of pro-inflammatory cytokines and IL-2 secretion may be preferentially directed toward memory precursor development rather than differentiate into terminal effectors. Furthermore, $CD8^+$ T cells that receive a sub-optimal activating stimulus, such as altered peptide ligand recognition, may still develop into memory precursors, resulting in a memory $CD8^+$ T cell pool with broader antigen specificity. Overall, the results from this chapter provide evidence that co-stimulation of naïve $CD8^+$ T cells can influence effector and memory development, via an IL-2 secretion mechanism. However, further investigation is required to confirm the stability of the effector and memory phenotypes generated under these *in vitro* co-stimulatory conditions, and to determine the role of *in vivo* stimuli in skewing these phenotypes when pursuing this method of $CD8^+$ T cell stimulation for adoptive cell transfer therapy.

Chapter 6. General Discussion

The manipulation of signals with potential to direct effector and memory $CD8^+$ T cell programming is critical for elucidating how T cell function and fate are determined. The process of T cell programming is complex with $CD8^+$ T cells integrating cues from both antigenic peptide bearing APCs, and from the local environment. Stimuli directly associated with the T cell differentiation process include the quality and quantity of antigen, ligation of T cell co-stimulatory and co-inhibitory molecules, and the presence of pro-inflammatory cytokines such as IL-12 and IFN-α/β [37]. Of these, the role of T cell co-stimulation remains relatively undefined as $CD8^+$ T cells express a large number of co-stimulatory and co-inhibitory receptors, each with potentially unique roles in governing T cell responses. Therefore, determining the co-stimulatory requirements involved in the programming of effector and memory $CD8^+$ T cells would greatly benefit our understanding of basic immune function.

The $CD8^+$ T cell response to acute infection is intriguing in that it appears to have both a pre-programmed and adaptive component that cooperate to direct T cell effector and memory development. Once the threshold for antigen recognition by the TCR has been exceeded, $CD8^+$ T cells are believed to undergo a defined process of clonal expansion and contraction, regardless of antigen dose [26, 297]. However, this programmed response can be manipulated by co-stimulatory and pro-inflammatory stimuli. In general, pro-inflammatory cytokines direct activated $CD8^+$ T cells toward effector development, increasing the production of cytolytic molecules such as granzyme B and perforin [146,

251]. This is achieved by increasing the accessibility of effector molecule gene loci to transcription factors, which prolongs and accentuates $CD8^+$ T cell effector function [150, 251]. T cell co-stimulation, on the other hand, is believed to be critical during several stages of the $CD8^+$ T cell lifecycle. During the priming stage, co-stimulation can augment TCR mediated signals, decreasing the threshold for naïve $CD8^+$ T cell activation, particularly when antigen quantity and quality is low [56]. Following this initial activation, co-stimulatory molecule signalling can enhance cell proliferation, effector molecule and cytokine production, as well as cell survival [61, 77]. This leads to a greater accumulation of effector $CD8^+$ T cells, ultimately resulting in a larger memory T cell pool. Additionally, ligation of co-stimulatory molecules during memory $CD8^+$ T cell reactivation is believed to enhance the expansion and function of this T cell subset [106]. Thus, co-stimulatory molecule ligation influences all aspects of the T cell lifecycle including activation, expansion, contraction, and memory generation. Therefore, further examination as to how co-stimulatory molecules cooperate to coerce this series of events is much warranted.

For this thesis, I addressed the question of how co-stimulation directs $CD8^+$ T cell activity through the use of a bead-based presentation system in which co-stimulator ligands were immobilized onto beads and presented to naïve $CD8^+$ T cells. Cell-sized beads are ideally suited for this purpose as the combination and quantity of co-stimulator ligands presented to naïve $CD8^+$ T cells can be precisely controlled. In addition, the *in vitro* culture system described here, allows for the direct comparison between co-stimulator ligands without external

influence from APCs, which themselves may express additional ligands, and secrete T cell influencing cytokines in a manner that cannot be controlled. Using this system, I was able to demonstrate that individual and combinations of co-stimulator ligands do differ in their ability to induce the activation and effector function of $CD8^+$ T cells. In addition, I determined which co-stimulatory conditions were best for enhancing $CD8^+$ T cell survival after their activation, as well as which were ideal for inducing the production of IL-2. Finally, I showed for the first time, how co-stimulator combinations compare in their ability to direct $CD8^+$ T cell terminal effector development and memory precursor generation. This analysis also revealed a potential mechanism in which co-stimulation induced IL-2 production influences $CD8^+$ T cell effector differentiation *in vitro*. Thus, the findings described in this thesis provide strong evidence for co-stimulation directed CD8+ T cell effector and memory development, which may greatly benefit vaccine and adjuvant design, as well as adoptive cell transfer therapy.

1. $CD8^+$ T cell co-stimulation and effector development:

Initial experiments presented in this thesis explored the role of co-stimulator ligands, and their combinations, in dictating $CD8^+$ T cell effector function. Using this bead-based method of co-stimulator ligand presentation, I was able to qualitatively and quantitatively assess the role of co-stimulation on the $CD8^+$ T cell activation process by directly measuring the cellular division, granzyme B and IFN-γ production, and cytolytic capacity of the generated $CD8^+$ T cell populations. Using this approach, I determined that ICAM-1 was best for

inducing naïve $CD8^+$ T cell proliferation and granzyme B production; however co-stimulation with B7.1 was required for the sustained production of IFN-γ. Furthermore, the co-stimulator combination of B7.1 and ICAM-1 was found to be ideal for the generation $CD8^+$ T effectors that were highly proliferative, and expressed large amounts of IFN-γ and granzyme B, which translated to efficient target cell lysis. The effector development observed in these experiments also appeared to be related to the ability of individual and combinations of co-stimulator ligand to augment $CD8^+$ T cell production IL-2, and to enhance the expression of effector associated transcription factors.

Co-stimulation induced transcription factor expression and $CD8^+$ T cell effector development

Results from chapter 3 demonstrated that co-stimulation does result in differential $CD8^+$ effector development in accordance with the co-stimulatory conditions provided. While most co-stimulator combinations enhanced T cell proliferation to some degree, they had a variable effect on IFN-γ and granzyme B expression, and $CD8^+$ T cell cytolysis. The IFN-γ, granzyme B, and perforin promoters are under the control of several transcription factors including NFAT, NF-κB and AP-1, as well as the leukocyte specific transcription factors T-bet and Eomes [26, 199, 298]. Because no inflammatory cytokines were added, enhanced expression of these effector molecules likely resulted from the optimal activation of these transcription factors downstream of the TCR, and the co-stimulatory molecules ligated. Thus, the expression of effector molecules may be somewhat related to the ability of individual co-stimulatory molecules to enhance TCR

mediated signals, or to propagate unique signals that lead to the expression/activation of these general transcription factors. T-bet and Eomes expression however, was examined more closely. This evaluation is highly significant as the ability of co-stimulators and their combinations to augment the expression of these crucial transcription factors, to my knowledge, has never been examined. Of the co-stimulatory conditions tested (10nM B7.1, 10nM ICAM-1, B7.1 + ICAM-1, anti-CD27 + B7.1), each induced distinct expression patterns of T-bet and Eomes that correlated to $CD8^+$ T cell effector function. For example, cells stimulated with a combination of B7.1 + ICAM-1 were positive for both T-bet and Eomes, and this was maintained up to 96 hours following their initial activation. The expression of T-bet and Eomes provides a partial explanation as to why the $CD8^+$ T cell effectors generated by this co-stimulator combination were highly cytolytic and produced large amounts of IFN-γ, as T-bet and Eomes enhance the expression of granzyme B and IFN-γ by binding the promoter region of these genes [199]. Contrary to this, cells stimulated with B7.1 + anti-CD27 were poorly cytolytic and expressed lower amounts of granzyme B and IFN-γ. In addition, the percentage of cells positive for T-bet and Eomes was lower and not maintained passed 48 hours of stimulation. In particular, the low expression of Eomes by B7.1 + anti-CD27 co-stimulated cells at 48 hours may be directly related to their poor cytolytic function, as Eomes is required for the optimal expression of perforin [66, 199]. While not addressed in these experiments, the low percentage of cells positive for Eomes after stimulation with B7.1 + anti-CD27, suggests that a lack of perforin production may be responsible for the poor

cytolytic activity of these cells, despite their moderate expression of granzyme B. For this to be confirmed however, perforin expression, particularly following stimulation with B7.1 + anti-CD27, needs to be examined more closely.

IL-2 has also been directly linked to the expression of several effector molecules including granzyme B and perforin, and can dramatically enhance the expression of both of these proteins [66, 174]. Thus, differences in co-stimulation induced IL-2 production may be partially responsible for modulating the amount of granzyme B and IFN-γ produced by the $CD8^+$ effector T cells generated in these experiments. In addition, high amounts of IL-2 have been demonstrated to augment the expression of Eomes, whose binding to the IFN-γ and perforin promoters is subsequently increased [66]. Naïve $CD8^+$ T cells co-stimulated with a combination of B7.1 + ICAM-1 secreted large amounts of IL-2 which correlated to their high expression of granzyme B and cytolytic activity in killing assays. B7.1 + anti-CD27 and ICAM-1 + anti-CD27 co-stimulated $CD8^+$ T cells on the other hand, failed to secrete large amounts of IL-2, and were poorly cytolytic. Since a substantial increase in granzyme B expression was detected from these $CD8^+$ T cell effectors following IL-2 addition, an inability to produce and sustain IL-2 secretion may be responsible for their poor effector development. Furthermore, since Eomes has been demonstrated to cooperate with STAT5 to enhance perforin expression [66], the lack of IL-2 secretion induced by this co-stimulator combination may greatly decrease their cytolytic potential. Hence, exogenous IL-2 addition, or concurrent activation of $CD4^+$ T cells, may be

required to generate potent $CD8^+$ effector T cells under these, and other, suboptimal co-stimulatory conditions.

Co-stimulation and IL-2 production

IL-2 production has many levels of regulation including epigenetic control, as well as transcriptional and post-transcriptional regulation [162]. In naive $CD8^+$ T cells, the IL-2 promoter is highly methylated, which inhibits the accessibility of the IL-2 gene loci to transcription factors [299]. Thus, IL-2 gene expression first requires promoter remodelling. This most often occurs through TCR and CD28-mediated signalling, which cooperate to increase histone acetylation, and reduce methylation, allowing for better promoter access [26, 299]. On a transcriptional level, IL-2 is a major downstream target of TCR signalling and its expression is controlled by several transcription factors including NF-κB, NFAT, and AP-1 [162, 299]. Each of these transcription factors are induced to some degree following TCR ligation, resulting in some IL-2 production. However, optimal IL-2 production requires co-stimulatory molecule signalling. Signalling through CD28 for example, has been demonstrated to increase NF-κB and AP-1 activity potentially enhancing IL-2 transcription in combination with TCR-mediated signals, in addition to enhancing promoter accessibility [71, 167, 299]. As a final method of IL-2 transcriptional control, IL-2 message can be also degraded post-transcriptionally. JNK activity however can increase the half-life of IL-2 message by targeting a stability element in the IL-2 transcript [162, 300]. Interestingly, ICAM-1 signalling has also been

demonstrated to enhance IL-2 message stability, revealing a potentially important role for ICAM-1 in regulating production of this cytokine [138, 143].

The elevated IL-2 production observed in these experiments, such as following B7.1 and ICAM-1 co-stimulation, may be a result of overcoming all levels of IL-2 transcriptional controls. CD28 co-stimulation alone was found to induce moderate expression of IL-2 (Fig. 4-5), perhaps by cooperating with the TCR to increase the accessibility of the IL-2 promoter, and augmenting the expression of key transcription factors including NF-κB and AP-1. The addition of ICAM-1/LFA-1 mediated signalling to CD28 co-stimulatory signals however, greatly enhanced the production of IL-2, perhaps by stabilizing the IL-2 transcript. Notably, ICAM-1/LFA-1 interactions also appeared to improve T cell:bead adhesion (Appendix figure 7-2), conceivably increasing and sustaining TCR-mediated signalling, resulting in further expression of IL-2 transcripts. Thus, this co-stimulatory combination may be ideal for the production of this cytokine by overcoming IL-2 transcriptional controls, and by pro-longing the activity of the signalling pathways required for its production.

The role of CD27 in IL-2 production is less defined. While reports have found a correlation between CD27 co-stimulation and IL-2 production [125, 284], these have not conclusively implicated CD27 signalling in the production of IL-2 from $CD8^+$ T cells. While CD27 ligation does influence the expression/activation of transcription factors responsible for IL-2 production such as NF-κB [116, 119], its ability to induce IL-2 production from the $CD8^+$ T cells in the experiments described here, was only minor. The lack of IL-2 produced from CD27 co-

stimulated $CD8^+$ T cells was particularly evident following T cell co-stimulation with a combination of CD27 and ICAM-1. The failure to induce significant IL-2 production following anti-CD27 + ICAM-1 co-stimulation may, in part, be responsible for the inhibitory effect of increasing CD27 co-stimulation on ICAM-1 induced T cell proliferation, observed in Figure 3-9. In this case, the lack of IL-2 secretion induced by the combination of anti-CD27 + ICAM-1, may have failed to support the high amount of T cell proliferation induced by this co-stimulator combination (Fig. 3-8 B). CD27 may also play a larger role in enhancing the secretion of IL-2 when ligated in combination with other co-stimulator molecules, such as CD28, which can enhance promoter access [125, 299]. While the experiments presented here demonstrated only a moderate increase in IL-2 secretion when an increasing amount of CD27 cross-linking antibody was added to B7.1 coated beads, this does not imply that CD27 is incapable of inducing secretion of this cytokine. Instead, CD27 may require a stronger TCR stimulus or B7.1 co-stimulator ligand density level to observe its ability to augment IL-2 production.

2. Co-stimulator molecule signalling and $CD8^+$ T cell survival

Enhanced $CD8^+$ T cell survival following activation has many benefits to the overall $CD8^+$ T cell response to infection. First, a healthy effector T cell is likely more efficient in its cytolytic and cytokine producing function, making it better able to control acute infection. Secondly, a larger number of viable $CD8^+$ T cells at the end of the expansion phase is believed to increase the number of $CD8^+$ T cell which survive contraction, leading to a larger, and perhaps more diverse,

memory T cell pool [22, 158]. T cell co-stimulatory molecules, particularly members of the TNFR co-stimulator family, have well documented roles in enhancing T cell survival by increasing the expression of anti-apoptotic molecules, such as Bcl-xL [77, 116]. When comparing individual and combinations of co-stimulators for their ability to augment $CD8^+$ T cell survival, I observed a divergence in co-stimulation induced effector function and survival that appeared to be related to both the expression of anti-apoptotic proteins, and the production of IL-2. In particular, while co-stimulation with 10nM ICAM-1 leads to highly proliferative and cytolytic effectors, these cells failed to accumulate after activation. Co-stimulation of naïve $CD8^+$ T cells with B7.1 + anti-CD27 on the other hand, led to poorly cytolytic effectors, but these survived better after activation. Thus, these experiments demonstrate that $CD8^+$ T cell effector function does not necessarily equate to enhanced T cell survival, and vice versa.

Co-stimulation and anti-apoptotic protein expression

Anti-apoptotic protein expression by $CD8^+$ T cells plays a large role in determining their fate following activation. Bcl-2 expression, for example, has a well-defined period of reduced expression following T cell activation that facilitates T cell contraction, followed by an increase in expression as $CD8^+$ T cells transition to memory [255]. The experiments described here revealed a strong correlation between co-stimulation induced Bcl-2 expression and T cell accumulation after activation. Yet, because Bcl-2 protein levels only decreased with time, it seems unlikely that the Bcl-2 expression observed indicates memory

generation, but rather a pro-longed effector phase. When monitoring the generated $CD8^+$ T cell effector populations for their expression of anti-apoptotic proteins however, an interesting observation was made in regards to TNFR co-simulator function and expression. Naive $CD8^+$ T cells stimulated with a combination of anti-CD27 + B7.1, were found to exhibit enhanced Bcl-2 expression following 48 hours of activation, in comparison to co-stimulation with 1.25nM B7.1 alone. However, the expression of Bcl-2 and Bcl-xL was not maintained to a similar degree as with other co-stimulatory combinations after 72 hours of stimulation (Fig. 4-4). This result may highlight the previously described temporal restrictions of CD27 co-stimulation and its ability to enhance CD8 + T cell survival early after activation [57, 76, 116]. Furthermore, 4-1BB, which also has described anti-apoptotic function [79, 132], was highly expressed on the surface of $CD8^+$ T cells following 48 and 72 hours of stimulation, with most co-stimulatory conditions tested (Fig. 5-4). Together then, these findings appear to support a cooperative and sequential model of co-stimulator molecule engagement in which consecutive ligation of different TNFR co-stimulatory molecules may be required for optimal effector and memory $CD8^+$ T cell survival and accumulation [116]. However, since the function of 4-1BB was not tested in these experiments, more work is required to confirm its ability to enhance T cell survival in a similar manner as CD27, and to determine its role in $CD8^+$ T cell responses when paired with other co-stimulators such as B7.1 and ICAM-1.

IL-2 production and CD8$^+$ T cell survival

As demonstrated in figure 4-5, different co-stimulatory conditions varied in their ability to induce IL-2 production from the stimulated CD8$^+$ T cells. IL-2 is a critical growth factor for CD8$^+$ T cells, and has been demonstrated to enhance T cell survival and accumulation following their activation [164, 168]. While IL-2 is believed to augment T cell survival in a number of ways, there has been a direct relationship established between IL-2 and the expression of Bcl-2 [269, 270, 282]. In the experiments described here, differences in IL-2 production appeared to be related to cell accumulation and Bcl-2 expression, but also correlated to ADAC induction under some co-stimulatory conditions. Co-stimulatory conditions which induced detectable increases in IL-2 production, particularly the combination of B7.1 and ICAM-1, exhibited higher Bcl-2 expression, especially at the 48 hour time point. Furthermore, co-stimulatory conditions which failed to induce IL-2 secretion, such as ICAM-1 + anti-CD27, had a larger population of cells which were CD25 and annexin V positive, indicating the occurrence of activation induced death. Because the apoptosis exhibited by this population was somewhat reversed following IL-2 addition, this suggests that these effectors were likely suffering from growth factor deprivation resulting in ACAD. Importantly, this finding has direct implications for ACT development, and suggests that certain CD8$^+$ T cell effector populations generated *in vitro* may require the addition of exogenous IL-2, or concurrent activation of CD4$^+$ T cells, not only for full effector development, but also for maintaining T cell viability. Therefore, future experiments using the bead-based stimulatory

system described here may greatly benefit from the addition of small amounts of IL-2 to protect cells from cytokine deprivation induced cell death.

3. Co-stimulation and SLEC vs. MPEC differentiation

Uncovering the cues which direct $CD8^+$ T cell toward either terminal effector or memory development has been a long-time goal of immunology. A better understanding of this process would be greatly beneficial for vaccine development, as well as ACT, both of which aim to provide individuals with long-lived protective $CD8^+$ T cell populations. Several models have been developed to aid in our understanding of $CD8^+$ T cell effector and memory differentiation, with more recent models highlighting the strength of the activating stimulus and pro-inflammatory cues in directing this process [8]. Because co-stimulation plays such a major role in augmenting and directing naïve T cell activation, the role of co-stimulation in directing effector vs. memory development warranted further investigation. Using the bead-based stimulatory system described here, I was able to demonstrate, for the first time, a potential role for co-stimulation in directing CD8+ T cell effector and memory fate. Specifically, the data collected suggests that the co-stimulatory combination of B7.1 and ICAM-1 results in $CD8^+$ T cells that exhibit an phenotype indicative of SLEC development, with high expression of the transcription factors Blimp-1 and T-bet. Contrary to this, naïve CD8 T cells stimulated with 10nM ICAM-1 or B7.1 + anti-CD27, exhibited a phenotype associated with MPEC development. Both of these T cell populations displayed slightly elevated expression of the memory associated transcription factor Bcl-6, and little detectable Blimp-1. High amounts of B7.1 co-stimulation, on the other

hand, resulted in an intermediate SLEC/MPEC phenotype. Interestingly, the data collected also provides some evidence for an IL-2 based mechanism for $CD8^+$ T cell differentiation, with co-stimulatory combinations inducing high amounts of IL-2 exhibiting more pronounced SLEC differentiation.

$CD8^+$ T cell co-stimulation and effector/memory associated transcription factor expression

In chapter 5, I compared specific co-stimulatory conditions for their ability to promote the generation of SLEC and MPEC $CD8^+$ T cell populations, by assessing the generated cells for their expression of the transcription factors Blimp-1, Bcl-6, T-bet and Eomes. Remarkably, the co-stimulatory conditions tested varied quite widely in the ability to induce SLEC and MPEC development. The co-stimulatory combination of B7.1 + ICAM-1 resulted in a $CD8^+$ T cell population that exhibited a transcription factor expression profile indicative of preferential SLEC development with high expression of Blimp-1, but little detectable Bcl-6. Furthermore, naïve $CD8^+$ T cells activated with this co-stimulator combination, also displayed elevated and sustained expression of T-bet and Eomes, whose combined expression is associated with a strong effector phenotype. The high expression of these effector associated transcription factors also correlated well with the potent effector function demonstrated by B7.1 + ICAM-1 co-stimulated cells in previous assays. If we assume T cell activating signal strength including TCR ligation, co-stimulation, and pro-inflammatory stimuli, directs the development of SLECs and MPECs as proposed by the fate determination model with progressive differentiation [8], the co-stimulatory

combination of B7.1 + ICAM-1 may skew $CD8^+$ T cell toward a SLEC phenotype for a number of reasons. Besides the enhanced production of IL-2, this combination likely results in strong and sustained TCR and CD28 signalling that favours SLEC development. This strong signal strength is also reflected in the T cell:bead interactions captured in Appendix Figure 7-2, where naïve $CD8^+$ T cell are rapidly tethered to beads coated in recombinant B7.1 and ICAM-1 proteins, resulting in stable, long-lived interactions.

Contrary to this, naïve $CD8^+$ T cells co-stimulated with a combination of B7.1 + anti-CD27 or 10nM ICAM-1 demonstrated an overall MPEC phenotype with slightly elevated expression of Bcl-6, and progressively increasing expression of Eomes. Furthermore, T-bet expression by these cells was not maintained, translating into the poor effector phenotype demonstrated by these cells in Chapter 3. It is important to note however, that these cells did not completely bypass effector development. The current model for MPEC generation does require $CD8^+$ T cells to go through an effector phase; however the end-point of this effector period is thought to be dictated by surrounding stimuli [206]. If the presence of antigen is sustained, Blimp-1 expression is thought to be induced. Alternatively, if antigen encounter is brief or the signal strength not as pronounced, preferential MPEC development and elevated expression of Bcl-6 results [206]. This is likely the case following $CD8^+$ T cell co-stimulation with a combination of B7.1 + anti-CD27. Since this combination lacks the presence of ICAM-1 for optimal cell:bead adherence, and an elevated density of B7.1, the

activating signal strength may not be sufficient to induce Blimp-1 expression, or sustain the expression of T-bet.

IL-2 and T cell differentiation

In chapter 5, I outlined how IL-2 may be directing SLEC development of $CD8^+$ T cells. I predict this to be due, in part, by IL-2 receptor signalling and STAT5 activation, inducing Blimp-1 expression. A clear correlation between IL-2 and Blimp-1 expression has been demonstrated in the literature, with IL-2 inducing Blimp-1 expression *in vitro* [66, 219]. In addition, IL-2 expression has been demonstrated to augment TCR-mediated Eomes expression [66]; however evidence for IL-2 induced T-bet expression is lacking. As discussed above, naïve $CD8^+$ T cell co-stimulation appears to greatly affect the ability of these cells to produce IL-2, resulting in a divergence in phenotype. Naive $CD8^+$ T cells stimulated with a combination of B7.1 + ICAM secreted large amounts of IL-2 that resulted in prolonged IL-2 receptor signalling and enhanced Blimp-1 expression, which may have repressed Bcl-6 transcript expression. In this scenario, the large burst of IL-2 induced by this co-stimulator combination seemed to preferentially induce SLEC development. Interestingly, a correlation can also be drawn between the intermediate and low production of IL-2 following 10nM B7.1 and 10nM ICAM-1 or B7.1 + anti-CD27, respectively, and their associated effector/memory fates. Thus, the differentiation of the T cells in these experiments appears to be highly dependent on the $CD8^+$ T cells ability to secrete IL-2, which enhances effector function, survival, and transcription factor expression. However, if T cell differentiation in these experiments is based solely

on IL-2 receptor signalling, is not known. Other factors, including TCR/co-stimulation signal strength, likely influence $CD8^+$ T cell differentiation to a large degree. Thus, if and how IL-2 and TCR/co-stimulatory signals are integrated by $CD8^+$ T cells to direct their effector and memory fates, requires further investigation.

In the experiments described in Chapter 5, STAT5 was used an indicator of IL-2 signalling, and was also suspected to be a means by which Blimp-1 expression was induced. While STAT5 is believed to be the signalling molecule/transcription factor involved in Blimp-1 induction [175, 207], this has not been unquestionably proven. While increasing amounts of IL-2 can indeed enhance Blimp-1 expression [66, 219], confirmation of STAT5 directly interacting with the Blimp-1 promoter, to my knowledge, has not been demonstrated. The assumption that STAT5 induces Blimp-1 expression in T cells is based on the strong induction of Blimp-1 expression following IL-2 addition, and STAT5 being a prominent signalling component and transcription factor downstream of the IL-2 receptor [170]. However, because other proteins, including STAT3, are also activated downstream of the IL-2 receptor, and the regulation of Blimp-1 expression in B cells appears to have many additional influences [161, 207], confirming STAT5 as the transcription factor directly inducing Blimp-1 expression, is still required. This could be done by examining the Blimp-1 promoter in T cells for STAT5 binding elements and chromatin immunoprecipitation (ChIP) assays. This would provide direct proof for IL-2 induced STAT5 phosphorylation in inducing the expression of Blimp-1, and thus,

directly influencing T cell differentiation through this mechanism. However, evidence for IL-2 promoting SLEC development still requires further experimentation involving the blocking of the IL-2/IL-2 receptor interaction to observe a decrease in Blimp-1 expression and SLEC development, or adding exogenous IL-2 to increase Blimp-1 expression.

4. Overall Model:

The experiments presented in this thesis describe for the first time, a head-to-head comparison between individual co-stimulator molecules and co-stimulator combinations, for their ability to direct $CD8^+$ T cell effector development, survival, and differentiation. The data generated also suggests that $CD8^+$ T cell SLEC and MPEC development may be somewhat regulated through a co-stimulation induced IL-2 secretion mechanism. As an overall model of how co-stimulation can influence effector development and $CD8^+$ T cell differentiation, I propose a variation of the fate commitment with progressive differentiation model (Fig. 6-1). As predicted by this model, I believe that naïve $CD8^+$ T cell differentiation is based on activating signal strength, which is influenced by TCR signal quality, pro-inflammatory cytokines, and importantly, co-stimulation. Naïve $CD8^+$ T cells which receive a strong co-stimulatory activating signal (ie. B7.1 + ICAM-1), tend to be directed toward terminal effector and SLEC development with elevated expression of effector molecules such as granzyme B and IFN-γ, and enhanced expression of effector associated transcription factors T-bet, Eomes, and Blimp-1. Importantly, this differentiation toward SLEC

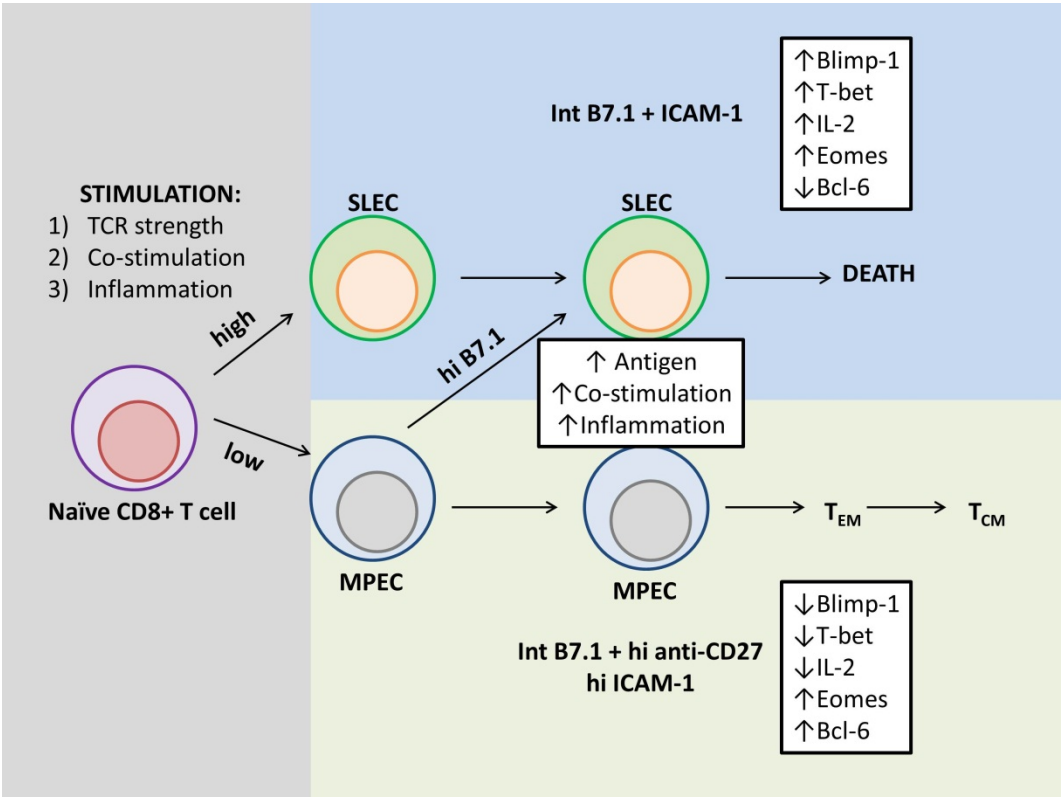

Figure 6-1. Overview of the role of co-stimulation in the fate commitment model of CD8$^+$ T cell differentiation. CD8$^+$ T cell differentiation is determined by activating signal strength, which includes T cell co-stimulation. Strong activating and co-stimulatory conditions lead to high amounts of IL-2 secretion, Blimp-1 expression, and terminal SLEC differentiation. Naïve CD8$^+$ T cells which receive a weak activating signal and suboptimal co-stimulation preferentially differentiate into MPECs with low expression of IL-2, T-bet, and Blimp-1, and elevated expression of Bcl-6. Naïve CD8$^+$ T cells which receive an activating signal and co-stimulation of intermediate strength produce a moderate of IL-2, and display a mixed SLEC/MPEC phenotype. Continued antigen exposure or an increase in activating stimuli can lead to the conversion of CD8$^+$ MPECs to terminal SLECs.

development is influenced by the co-stimulation induced production of IL-2. Conversely, naïve $CD8^+$ T cells which receive a weak co-stimulatory activating signal (ie. B7.1 + anti-CD27/10nM ICAM-1), first differentiate into intermediate effectors expressing some granzyme B, but eventually develop a phenotype associated with MPEC development. This preferential MPEC development is a result of suboptimal co-stimulator molecule ligation and unsustained IL-2 production. These factors also prevent the induction of Blimp-1 and the sustained expression of T-bet, leading to elevated levels of Bcl-6. Finally, naïve $CD8^+$ T cells which receive an intermediate activating co-stimulatory stimulus, such as 10nM B7.1, develop an intermediate SLEC/MPEC phenotype with moderate production of effector molecules and IL-2, and express transcription factors encompassing both SLEC and MPEC development. Importantly, these cells would seem ideally suited to convert to SLECs if activating stimuli are maintained or increased. Thus, the combination of co-stimulator molecule signalling strength, and IL-2 production, is involved in directing $CD8^+$ T cell effector and memory fate.

In terms of the effector and memory decisions made by $CD8^+$ T cells, I believe my results fit best with a fate commitment with progressive differentiation model of memory generation, in that the strength of the activating stimulus (which includes co-stimulation), leads to effector and memory fate decisions by $CD8^+$ T cells. However, I do not feel that these results absolutely exclude all of the other pathways for memory generation described in Figure 1-3. The findings presented here could also represent a model of fixed lineage commitment as cells stimulated

with 10nM B7.1 did display a mixed effector and memory phenotype. Furthermore, these experiments were not carried out for a long enough time period to exclude the uniform potential model. In fact, I believe that during the course of natural infection, the $CD8^+$ T cell population *as a whole* does go through stages of preferential effector then memory generation, but perhaps not a per cell basis as the uniform potential model suggests. Finally, the results of this research are least consistent with the decreasing potential model of memory generation as SLEC phenotype cells appeared to be generated without displaying a previous memory-like phenotype. Still, more research is required to absolutely exclude this possibility.

5. Future Directions:

$CD8^+$ T cell differentiation and adoptive transfer

The results described here indicate that naïve $CD8^+$ T cell co-stimulation can be used to generate T cell populations with SLEC and MPEC phenotypes *in vitro*. The next stage of this research then demands that these generated $CD8^+$ T cell effector and memory precursor populations be tested for their ability to maintain their function *in vivo*. These experiments will not only test the basic immunological findings presented here, but may also reflect how these $CD8^+$ T cell populations would respond if used for ACT. Previous work has indicated that the differentiation phenotype demonstrated by $CD8^+$ T cells *in vitro,* is not always maintained after the cells are adoptively transferred into naïve animals. In a study conducted by Pipkin et al. [66], *in vitro* generated $CD8^+$ T cell populations exhibiting a SLEC phenotype, were still capable of responding to secondary

antigenic challenge when adoptively transferred in naïve mice. While the number of adoptively transferred $CD8^+$ T cells with a SLEC phenotype did decrease with time, the small number of cells that survived reactivated with the same kinetics and potency as adoptively transferred T cells with a MPEC phenotype. This demonstrates that, while a T cell population may exhibit an overall SLEC phenotype, there likely are a certain proportion of those cells which do develop into memory precursors. Whether this population of cells results from SLEC differentiation into MPECs, or are simply too difficult to detect amongst cells with a strong SLEC phenotype, is unknown. Alternatively, an *in vivo* environment may induce or preferentially select for memory precursor populations. Thus, future experiments with these co-stimulation generated $CD8^+$ T cell populations require an adoptive transfer component to determine the stability of their phenotype. In addition, a detailed assessment of memory-associated $CD8^+$ T cell epigenetic changes, which may occur during these *in vitro* stimulations, might also shed light onto potential SLEC and MPEC development. While still a new and growing field of study, changes in chromatin structure and gene expression may be key to identifying the generation of a *bone fide* memory $CD8^+$ T cell population.

TCR signal strength and pro-inflammatory stimuli

Two major influences in naïve $CD8^+$ T cell activation not addressed in these experiments is the role of TCR signal strength and pro-inflammatory stimuli in directing T cell activation and memory development. TCR signal strength is critical in directing naïve $CD8^+$ T cell activation [26, 54, 239], and could be easily

manipulated using this bead-based stimulatory system. Not only could TCR ligation and signal strength be increased or decreased in future experiments, but the use of antigenic peptides and TCR transgenic $CD8^+$ T cells would also greatly broaden the scope of this study. Specifically, the co-stimulatory requirements for naive $CD8^+$ T cell activation in response to suboptimal or altered peptide ligands could be easily determined using this bead-based stimulatory system, and would be very relevant in terms of *in vivo* infection. Furthermore, pro-inflammatory cytokines such as IL-12 and IFN-α/β could be added to the previously described $CD8^+$ T cell:bead cultures in different combinations and amounts, to test their effect on naïve $CD8^+$ T cell activation. As noted earlier, the addition of pro-inflammatory cytokines may be important in increasing the activation and effector molecule production by naïve $CD8^+$ T cells when co-stimulatory conditions are suboptimal, such as following co-stimulation with ICAM-1 + anti-CD27. Again, this would also be highly relevant to *in vivo* infections as inflammation at the site of infection does occur to some degree. In terms of T cell differentiation, the fate commitment model with progressive differentiation predicts that increasing either of these parameters (TCR signal strength and inflammation), should increase $CD8^+$ T cell SLEC development [8]. Hence, infections which produce large amounts of foreign antigen and inflammation should lead to a more robust and effective effector $CD8^+$ T cell population, but perhaps increased injury [251]. While the role of co-stimulation in influencing T cell effector differentiation in the presence of additional activating stimuli is unknown, it can be predicted to

both pro-long and enhance the $CD8^+$ T effector response, and should be investigated further.

mTOR and T cell Co-stimulation

Recent work has identified the serine/threonine kinase mTOR (mammalian target of rapamycin), as a key protein regulating $CD8^+$ T cell differentiation [203, 301]. mTOR is centrally located downstream of several signalling pathways, and is required for the integration of environmental cues including nutrient availability and the presence of growth factors, with T cell stimulatory signals, to instruct effector and memory T cell development [302] (Fig. 6-2). The importance of mTOR in T cell differentiation was demonstrated by treating cells with the mTOR inhibitor rapamycin, which led to a change in T cell metabolism and an increase in T cell memory generation following LCMV infection [303, 304]. The mTOR protein itself forms two distinct complexes, mTORC1 and mTORC2, which are activated by signalling components downstream of the TCR including PI3K and Akt [302, 305]. In particular, Akt phosphorylation is required for the activation of mTORC1. Activated mTORC1 influences T cell differentiation by augmenting the expression of transcription factors such as T-bet and RORγt [302, 305]. The mechanisms regulating mTORC2 activity however, are not well understood. Once activated, mTORC2 has been demonstrated to phosphorylate Akt, leading to Akt activating yet another target protein, FOXO1 [301]. FOXO1 is specifically associated with $CD8^+$ T cell differentiation because it binds and activates the Eomes proximal promoter, favouring Eomes transcription and memory differentiation [301].

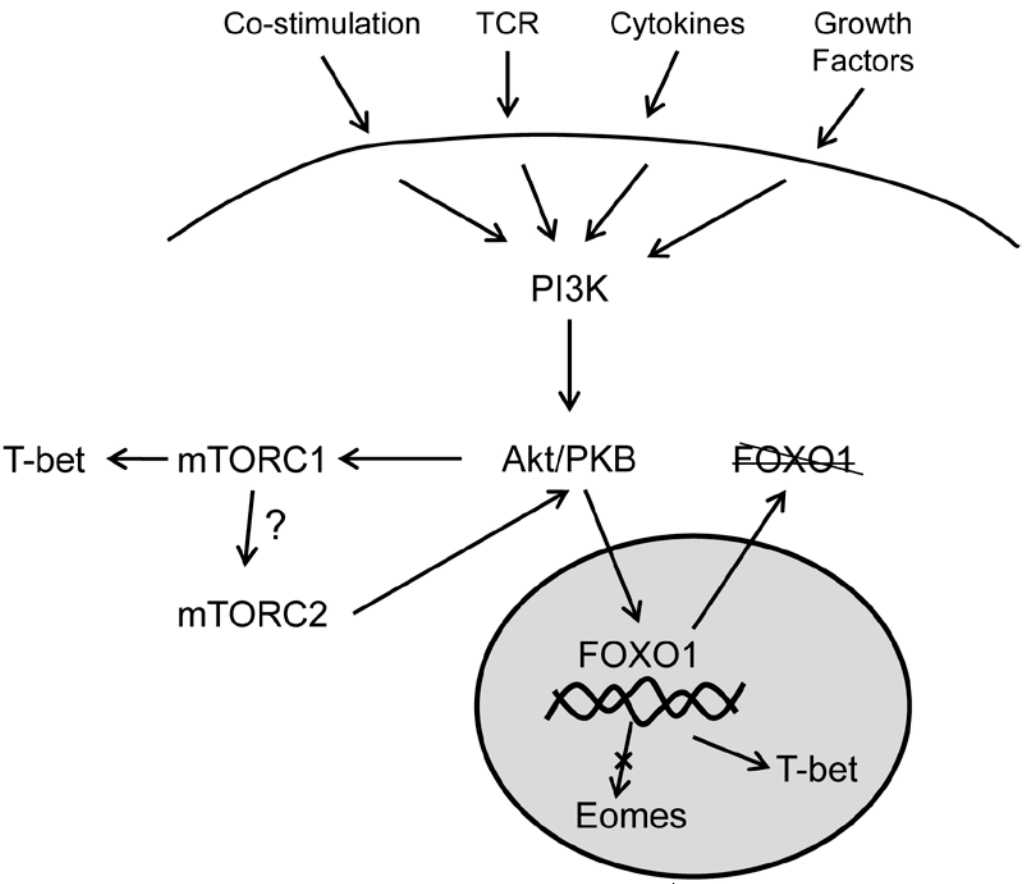

Figure 6-2. Simplified model of mTOR driven CD8$^+$ T cell differentiation. Multiple signals including TCR ligation, co-stimulation, cytokine presence, and nutrient availability converge on the PI3K/Akt/mTOR pathway, and direct CD8$^+$ T cell differentiation. In general, these stimuli lead to the downstream activation of PI3K, which converts PIP_3 to PIP_2 (not depicted). This leads to the eventual activation and phosphorylation of Akt. Akt in turn activates the mTOR complex, mTORC1, which augments the expression of T-bet. Alternatively, mTORC1 may activate mTORC2, which phosphorylates Akt at a second residue. This secondary phosphorylation of Akt leads to Akt phosphorylating FOXO1. Unmodified FOXO1 is normally located in the nucleus where it augments the expression of Eomes, favouring memory generation. Phosphorylation of FOXO1 causes it to be excluded from the nucleus and degraded, thus preventing Eomes expression, and relieving its indirect repression of T-bet. T-bet can then be transcribed, leading to preferential effector differentiation.

The phosphorylation of FOXO1 by Akt results in its nuclear exclusion and transport to the cytoplasm where it is then degraded [301]. The degradation of the FOXO1 leads to enhanced T-bet expression and effector differentiation due to the loss of indirect FOXO1 repression of the T-bet promoter [301]. Through this mechanism then, sustained mTOR activity leads to enhanced T-bet expression and $CD8^+$ T cell effector generation through downstream FOXO1 phosphorylation and degradation.

As mentioned above, the activity of mTOR can be modulated by immune stimulatory events. Antigen recognition by the TCR, co-stimulatory molecule ligation, and cytokine binding, including IL-2 and IL-12, all enhance effector differentiation by increasing and sustaining the activity of PI3K, AKT, and mTOR [305-308]. Specifically, co-stimulation through CD28 directly enhances the activity of the PI3K/Akt pathway, and ultimately mTOR [307], while ligation of OX-40 activates mTOR by forming a signalling complex that augments PI3K/Akt activation [309]. Thus, mTOR is critical for integrating signals from the TCR, co-stimulatory molecules, and cytokines, and likely is responsible for directing $CD8^+$ T cell effector and memory differentiation to some degree. Thus, future work investigating the role of T cell activating stimuli in directing $CD8^+$ T cell effector and memory development should target mTOR as key factor involved in this process. Important questions to address include which co-stimulatory combinations are best for activating mTOR, and whether mTOR activity is required to integrate IL-2 and co-stimulatory signals, which appear to direct $CD8^+$ T cell differentiation.

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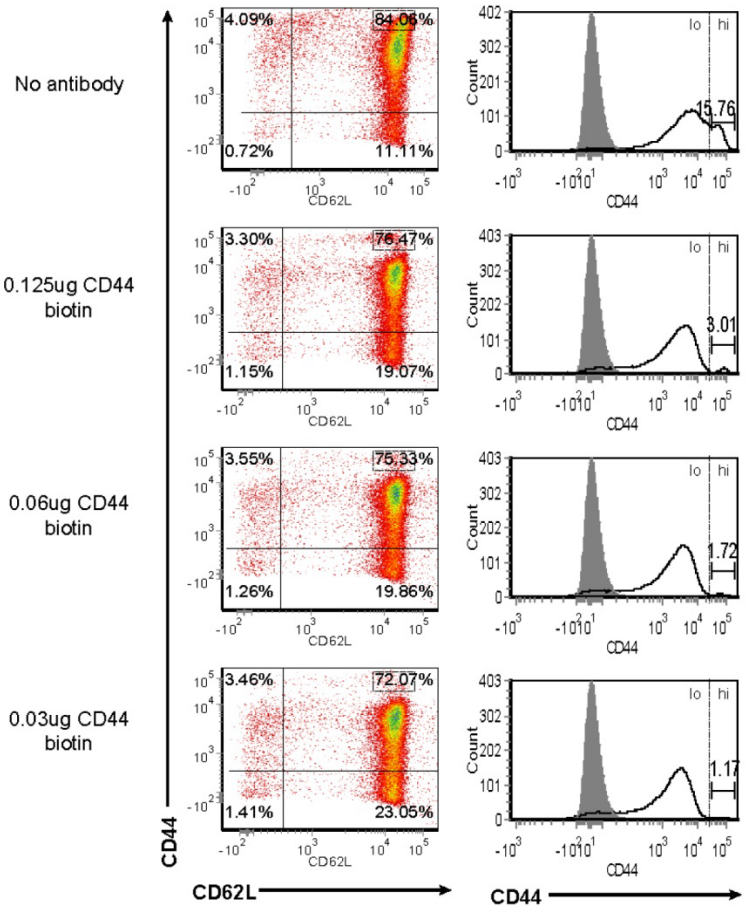
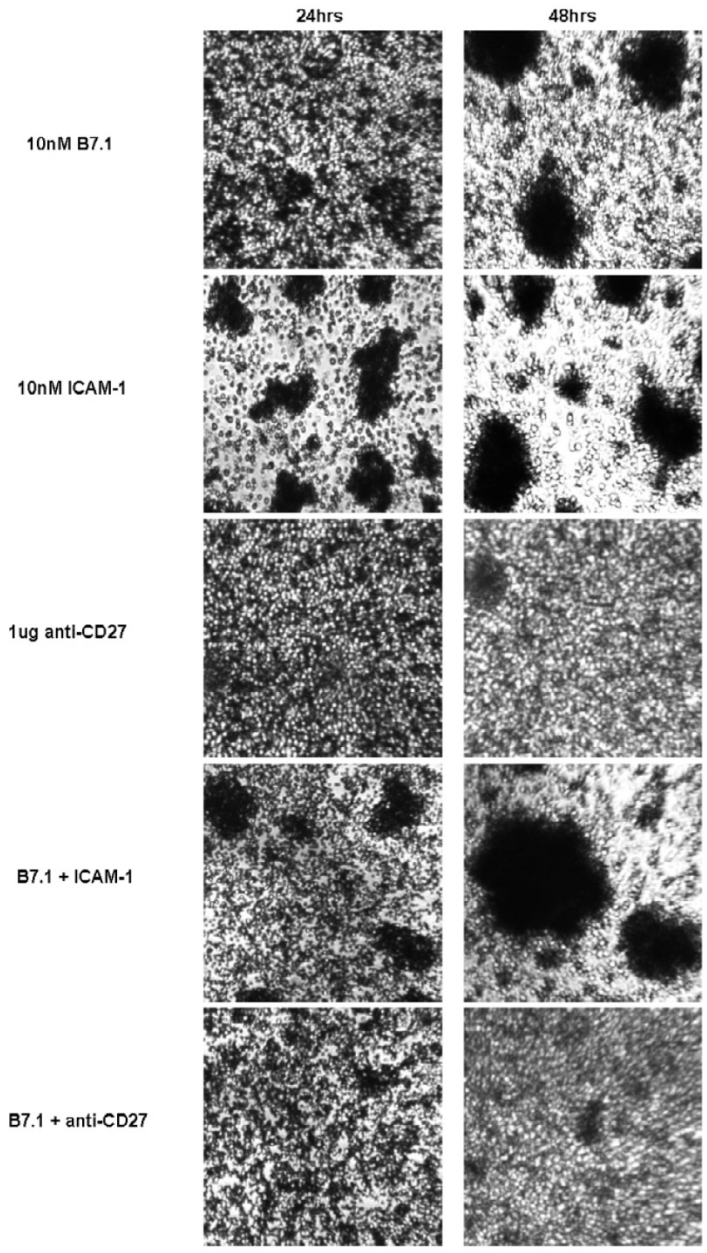
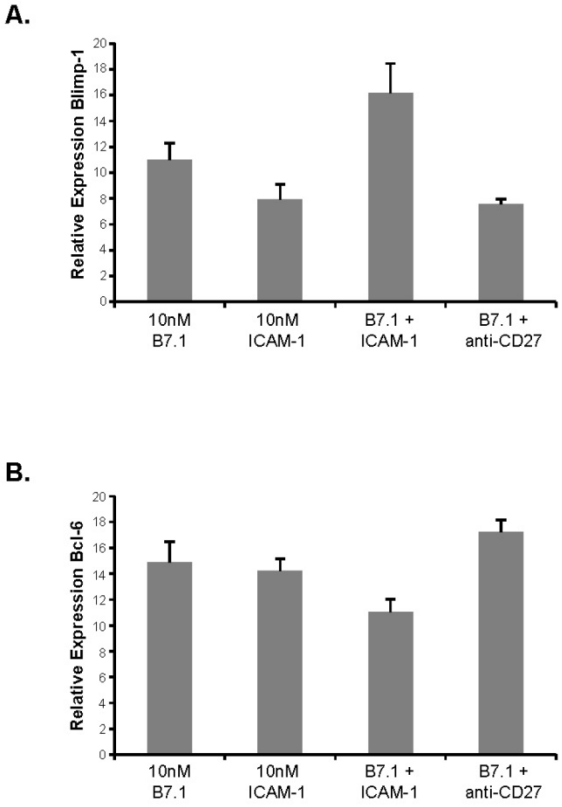
Chapter 7. Appendix



Appendix Figure 7-1. CD44 titration for purification of naïve CD8$^+$ T cells. OT-1 splenocytes were counted and mixed with 0.125µg, 0.06µg, or 0.03µg/1x10^8 cells of anti-CD44 biotin for purification. Following anti-CD44 addition, the splenocytes were incubated with the components of the CD8$^+$ T cell negative selection EasySep kit from Stemcell, as per the manufacturer's instructions. Purified cells were then stained for their expression of CD44 and CD62L to assess the cells purity, using fluorescently-conjugated mAbs. Representative data shows that 0.03µg/1x10^8 cells of anti-CD44 biotin successfully removes the CD44^{hi} effector/memory population from the CD8$^+$ T cell population.



Appendix Figure 7-2. Purified CD44^{lo} CD8^{+} T cells interact with co-stimulator ligand bearing beads with unique binding characteristics.
Purified CD44^{lo} CD8^{+} T cells were incubated with stimulatory beads bearing the indicated co-stimulators and co-stimulator combinations, and their interactions captured following 48 and 72 hours of stimulation. Each of the indicated bead constructs also contained suboptimal amounts of anti-CD3 (1.25ug). Images were captured by an inverted microscope attached to a Retiga Q-image charge-coupled device camera as described in the *Materials and Methods*. Images are representative of two independent experiments.



Appendix Figure 7-3. Blimp-1 and Bcl-6 expression relative to RPL24. Naive $CD44^{lo}$ $CD8^{+}$ T cells were stimulated for 96 hours with either 10nM B7.1, 10nM ICAM-1, 1.25nM B7.1/ICAM-1 or 1.25nM B7.1/1μg anti-CD27, in combination with suboptimal amounts of anti-CD3, and assessed for their relative expression of Blimp-1 (*A*) and Bcl-6 (*B*) by quantitative PCR (qPCR). The fold increase in both Blimp-1 and Bcl-6 expression was calculated relative to RPL24 internal control gene expression only. Results are a mean of three independent experiments. Error bars represent standard deviation in the experimental means.