RECIPROCAL REGULATION OF $CD4^+$ AND $CD8^+$ T LYMPHOCYTE EFFECTOR AND MEMORY FATES BY INTERLEUKIN 12 AND TYPE I INTERFERON

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DEDICATION

To Lacey, Mom, Grandma, Grandpa, Linda and Paul

for your love, support and inspiration

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The work presented here is in no way a solitary effort. What I have accomplished to this point in my career, is attributed to all that have provided me the support and opportunities to aspire to greatness and realize my goals in life.

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Cytokine signaling networks play an important role in bridging the innate and adaptive immune responses. For example, the innate cytokines Interleukin-12 (IL-12) and type I interferon (IFN-α/β) are induced to high levels by intracellular bacterial and viral infections and have been shown to promote adaptive T lymphocyte responses to infection. While the role for IL-12 on the development of T lymphocyte effector responses has been well characterized, the exact role for IFN-α/β on these responses has been controversial. Therefore, the present study

set forth to characterize the distinct roles for IL-12 and IFN-α/β on the development of effector and memory responses in human $CD4^+$ and $CD8^+$ T cells. My work has found that IL-12 drives the development of effector $CD4^+$ and $CD8^+$ T cells. In contrast, IFN-α/β was incapable of promoting these responses and this was due to a difference in the kinetics of activation of two downstream transcription factors STAT4 and T-bet. Further examination of $CD8^+$ T cells revealed a distinct role for IFN-α/β in the development of a population of central memory T cells (T_{CM}). Alternatively, IL-12 drove the development of effector memory cells (T_{EM}). The variegated development of T_{CM} and T_{EM} was dictated by differential cytokine receptor expression and further, the strength of primary T cell receptor (TCR) activation determined the responsiveness to cytokine polarization. Finally, these studies uncovered a novel role for $CD8^+$ T cell licensing of CTL activity through the costimulatory CD27/CD70 pathway. Therefore, taken together, these findings support a novel model in which TCR activation and costimulation act to shape the ability for IL-12 and IFN-α/β to differentially program the development of distinct classes of effector and memory $CD8^+$ T lymphocytes. These studies have direct bearing on the design and development of effective therapeutics and vaccines and demonstrate a new understanding on the modulation of the adaptive immune response to intracellular infection.

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LIST OF ABBREVIATIONS

Ab – antibody

AICD –activation induced cell death

AP-1 – activation protein-1

APC – allophycocyanin

APC – antigen presenting cell

BSA – bovine serum albumin

CBA – cytometric bead array

CCR – chemokine receptor

cDC –conventional dendritic cell

cDMEM – complete Dubelcco's modified Eagle medium

cDNA – complementary DNA

CFSE – carboxyfluorescein diacetate succinimidyl ester

cIMDM – complete Iscove's modified Dubelcco's medium

CMV – cytomegalovirus

CTL – cytotoxic T lymphocyte

DAI – DNA-dependent activator of interferon regulator factors

DC – dendritic cell

DNA – deoxyribonucleic acid

ds – double-stranded

Effector-27 – population of $CD8^{+}CD45RA^{+}CD27^{-}$ T cells

eIF2α – eukaryotic initiation factor 2α

Eomes – eomesodermin

ELISA – enzyme-linked immunosorbent assay

FBS – fetal bovine serum

FITC – fluorescein isothiocyanate

GAS – γ-IFN activation sites

GFP – green fluorescent protein

h – human

HCV – hepatitis C virus

HRP–horseradish peroxidase

IFN – interferon

IFNAR – interferon-α/β receptor

Ig – immunoglobulin

IKK – inhibitor of κB kinase

IL – interleukin

iNOS – inducer of nitric oxide synthesis

IRAK – interleukin 1 receptor-associated kinase

IRF – interferon regulatory factor

ISG – interferon stimulated gene

ISGF3 – interferon stimulated gene factor 3

ISRE – interferon stimulated response element

LCMV – lymphocytic choriomeningitis virus

LN – lymph nodes

LPS – lippopolysaccharide

MAP kinase – mitogen-activated protein kinase

MΦ – macrophage

MHC – major histocompatibility complex

Mx – myxovirus resistance protein

MyD88 – myeloid differentiation factor 88

Naïve-27 – population of $CD8^+CD45RA^+CD27^+$ T cells

Naïve-RA – population of $CD8^+CD45RA^+$ T cells

NF-κB – nuclear factor κB

NK – natural killer cell

NKGSF – natural killer cell growth factor

NO – nitric oxide

NOD – nucleotide-binding oligomerization domain

OAS – 2'-5' oligoadenylate synthase

p35 – 35 kDa subunit of the IL-12 cytokine

p40 – 40 kDa subunit of the shared IL-12/23 cytokines

p70 – 70 kDa complete IL-12 cytokine

PAMP – Pathogen associated molecular pattern

PBMC – peripheral blood mononuclear cell

PBS – phosphate buffered saline

PBSE – pacific blue succinimydyl ester

PCR – polymerase chain reaction

pDC – plasmacytoid dendritic cell

PE – phycoerythrin

PGN – peptidylglycan

PI-3 kinase – phosphotidylinositol 3-kinase

PKR – protein kinase R

PhA – phoenix amphotropic

PHA – phytohemmaglutinin

PMA – phorbol 12-myristate 13-acetate

PMN – poly morpho neutrophil

PRR – pattern recognition receptor

PTP1D – protein tyrosine phosphotase

qRT-PCR – quantitative real-time polymerase chain reaction

rh – recombinant human

RIG-I – retinoic acid inducible gene I

RIPA – radioimmune precipitation assay

RNA – ribonucleic acid

RSV – respiratory syncytial virus

RV – retrovirus

SA – streptavidin

7AAD – 7-amino-actinomycin

SH2 – Src homology 2 domain

SOCS – suppressors of cytokine signaling

Signal 1 – TCR activation of naïve T cells

Signal 2 – Costimulation of naïve T cells

Signal3 – Innate cytokine activation of naïve T cells

ss – single-stranded

STAT – signal transducer and activator of transcription

T-bet – T-box expressed in T cells

TBK-1 – Tank-binding kinase 1

T_{CM} – central memory T cells

T_{CMR7} – central memory $CCR7^{hi/}CXCR3^{lo}$

T_{EM} – effector memory T cells

T_{EMXR3} –effector memory $CCR7^{lo}/CXCR3^{hi}$

TCR – T cell receptor

Tc1 – T cytotoxic type I

Tc2 – T cytotoxic type 2

Th1 – T helper type I

Th2 – T helper type 2

TIR – Toll/IL-1 Receptor

TLR – Toll-like receptor

TNF – tumor necrosis factor

TRAF6 – tumor necrosis factor receptor-associated factor 6

TRIF – Toll/IL-1 receptor domain-containing adaptor inducing interferon beta

U – units

VSV – vesicular stomatitis virus

VV– Vaccinia virus

CHAPTER ONE

INTRODUCTION

During infection, multiple classes of immune cells must recognize invading pathogenic microorganisms and coordinately act to eliminate them from the body. This proceeds through explicit recognition of the invading pathogen followed by an immune program tailored towards eradication of the specific infectious agent. To that regard, two arms of the immune response exist. Innate immunity, which detects infection and directs the development of specific immune responses and adaptive immunity, which responds to innate cues to form a productive immune response and memory to secondary infection. Cross-talk between the innate and adaptive immune systems is mediated through a complex network of soluble signaling molecules known as cytokines that act to directly shape the immune response such that it is specific for the invading pathogen.

T lymphocytes play a critical role in the adaptive immune response to infection and are directly responsive to cytokine cues set forth by innate immune cells. Therefore, depending upon the type of infection "sensed" by innate immune cells, different T cell developmental pathways are initiated to promote clearance of infection. Infection by intracellular organisms such as viruses promotes the development of type I, $CD4^+$ (Th1) and $CD8^+$ (Tc1) T lymphocyte

responses, marked by interferon-gamma (IFN-γ) and tumor necrosis factor-alpha (TNF-α) secretion and clearance of intracellular infection through cytolytic pathways (1-3). Multiple cytokines are secreted during intracellular infection and have the capacity to shape the adaptive immune response. These include the interleukins, interleukin-1 (IL-1), IL-6, IL-21, IL-12 family members, tumor-necrosis factor-alpha (TNF-α), and the type I (IFN-α/β) and type II (IFN-γ) interferons (4, 5). IL-12 and IFN-α/β, in particular, promote efficient induction of innate immunity as well as have been shown to participate in the development of adaptive Th1 and Tc1 responses to intracellular infection (5, 6).

The current understanding in the field suggests that IL-12 and IFN-α/β act as redundant signals to promote the development of both Th1 and Tc1 populations of cells. However, while a direct role for IL-12 on the development of effector T lymphocytes has been well characterized, the exact role of IFN-α/β on effector response or the independent roles of these cytokines on the development of secondary memory have not been well established. Therefore, this study set forth to identify the direct and independent roles of IL-12 and IFN-α/β on the development of $CD4^+$ and $CD8^+$ T cell effector and memory responses to intracellular infection. Unlike the previous understanding in the field, this study has identified reciprocal roles for IL-12 and IFN-α/β in promotion of type I responses and reveals a complex system in which innate cytokines uniquely and

independently promote effector and memory responses during infection. These results provide a new perspective into the roles of innate cytokines on adaptive T lymphocyte development and are the basis for a newfound understanding of these responses as they relate to immuno-modulatory therapies and vaccine development strategies.

CHAPTER TWO

LITERATURE REVIEW

Innate recognition of infection and induction of cytokine signaling networks

Cells of the innate immune system act as the first line of defense against invading microorganisms and play a crucial role in shaping the intensity and specificity by which a productive adaptive response ensues (4, 7). The innate response relies on a variety of cell types, namely neutrophils (PMN), macrophages (MΦ) and dendritic cells (DCs), which patrol the host periphery in search of pathogenic breach (8). In addition to their phagocytic functions and their ability to sense initial infection, DC subsets have been shown to act as the major antigen presenting cells (APC) for T cell priming. Therefore, among the early innate responders, DCs are considered to be the most efficient at promoting adaptive responses to infection (8, 9). This is due in part, to expression and signaling through a series of germline encoded surface and cytoplasmic pattern recognition receptors (PRRs). These include the Toll-like receptor (TLR) transmembrane members of the Toll/IL-1R family, the cytosolic nucleotide oligomerization domain (NOD) receptors, cytosolic RNA helicases, retinoic acid

inducible gene-I (RIG-I) and MDA5 and the recently discovered cytosolic DNA receptor, DNA-dependent activator of interferon regulator factors (DAI) (10-14).

The TLR family of PRRs have been extensively characterized and are thought to be major contributors in shaping adaptive immunity (15). To date, 11 TLR family members have been discovered in mouse and humans, each with the capacity to respond to distinct pathogen-associated molecular patterns (PAMPs) expressed on a diverse group of organisms including bacteria, fungi, protozoa and viral pathogens (4). These include the surface receptors, TLR1, 2, 4, 5, 6 which primarily detect PAMPs derived from extracellular infection and the endosomal receptors TLR3, 7, 8, 9 which detect nucleic acid PAMPs (4). Recognition of cognate ligands by TLRs have been shown to promote a process of maturation in DC subsets important in the shift from sentinel to APC (6, 16) . These include, increased expression of the Class I major-histocompatibility complex (MHC-I) and the Class II major-histocompatability complex (MHC-II) and increased expression of co-stimulatory molecules such as CD40, CD80, and CD86 which allow for efficient antigen presentation and T cell activation (8, 17). Further, PRR signals also modulate expression of chemokine receptors (CCR) such as CCR7, CCR1, CCR5 which allow for DC trafficking to draining lymph nodes (LN) where they can interact with naïve T cells and prime their development (8, 18, 19).

Importantly, signaling through TLRs and other PRRs also drives the expression of innate cytokines. For example in response to extracellular bacterial infection, TLR2 and TLR4 can recognize peptidylglycan (PGN) and lippopolysaccharide (LPS) respectively (20, 21). In addition, the cytosolic sensors NOD1 and NOD2 can recognize PGN moieties exposed within the cytosol (11, 22). These responses induce signaling pathways which direct the activation of nuclear factor κB (NF-κB) and mitogen-activated protein kinase 38 (p38 MAP kinase) leading to activation of Th1 responses via secretion of pro-inflammatory cytokines such as IL-12, IL-6 and TNF-α (9, 23-25). Alternatively, in response to intracellular bacterial or viral infection, sensing of nucleic acids by TLRs 3, 7, 8 and 9, RIG/MDA5 or DAI activate members of the interferon regulatory factor (IRF) family of proteins, especially IRF-3 and IRF-7 which play important roles in the induction of IFN-β and IFN-α respectively (4, 12-14). Therefore, depending upon the type of infection sensed, either IL-12 alone or both IL-12 and IFN-α/β may be induced. This has direct repercussions on the downstream T lymphocyte programs induced as IL-12 and IFN-α/β have been implicated in the development of Th1 and Tc1 effector responses (24, 26-31).

Pattern Recognition pathways in DC subsets and the control of Interleukin-12 and Type I interferon expression

In humans, multiple subsets of peripheral blood DCs have been described and shown to participate in secretion of IL-12 and IFN-α/β (6, 32). Of these, conventional dendritic cells (cDCs), and the natural interferon producing or plasmacytoid dendritic cells (pDC) have been well characterized in regards to PRR expression, cytokine secretion and the activation of downstream T cell responses (6) . Further, both human and murine subsets of cDCs and pDCs have the capacity to secrete IL-12 and IFN-α/β (6, 33, 34), although it is well understood that cDCs are the predominant producers of IL-12 whereas pDCs predominantly express IFN-α/β in response to infection (34-38)

The varied expression of IL-12 and IFN-α/β in these subsets is primarily due to variable expression of PRRs and signaling pathways between cDCs and pDCs (20). cDCs express high levels of the surface TLRs 1-6 as well as NOD1 and NOD2 which leads to a strong induction of IL-12 during bacterial infection. In addition, TLRs 2, 4, 6 can also recognize viral glycoproteins and therefore cDCs are primed to respond to both bacterial or viral infection by secreting IL-12 in response to their PRR ligation (6, 20, 35). Alternatively, pDCs express high levels of TLR7 and TLR9 and despite a capacity to drive IL-12 expression, these

PRR pathways predominantly drive the expression of IFN-α in response to viral infection (6, 39-42).

With the exception of TLR3 (the sensor for double stranded RNA (dsRNA)) all of the TLR family members either directly or indirectly interact with the adaptor protein, myeloid differentiation primary-response gene 88 (MyD88), through its conserved Toll/IL-1 Receptor (TIR) domain (4, 43). This leads to the MyD88 driven recruitment of members of the IL-1R-associated kinases (IRAKs)1, 4 and TNF-receptor associated factor-6 (TRAF6) which then initiate a signaling cascade which culminates in the activation of the Iκb kinase (IKK) family members IKKα and IKKβ (4). Similarly, NOD proteins activate IKKα and IKKβ (44). These kinases, relieve repression of NF-κB and allow for its translocation to the nucleus where it can interact with AP-1 to induce its target genes such as IL-12 (4, 24). Therefore, activation of NF-κB facilitates the main pathway by which cDCs promote the development of $CD4^+$ Th1 effector responses in an IL-12 dependent manner (Fig. 1A) (24).

Expression of IFN-β in cDCs occurs primarily through recognition of dsRNA by TLR3. This is mediated by a non-MyD88 pathway involving the TIR domain-containing adapter inducing IFN-β (TRIF). Activation of TRIF leads to activation of the non-canonical IKK related kinases tank binding kinase-1 (TBK-1) and IKKε and results in both NF-kB and IFN-β induction in MΦ and cDCs but

not pDCs (Fig. 1A) (45-49). A similar pathway which is also functional in most somatic cells is mediated by cytosolic recognition of dsRNA by RIG-I and MDA5 which through interactions with the adaptor mitochondrial associated viral sensor (MAVS/IPS-1) leads to the induction of IFN-β in a TBK-1 and IKKε dependent manner (Fig. 1A, C) (12, 14, 50-52).

Alternatively, pDCs, responding to single stranded RNA (ssRNA) by TLR7 or double-stranded unmethylated CpG DNA by TLR9, promote the activation of large quantities of IFN-α (6). This occurs through the phosphorylation and dimerization of IRF-7 and proceeds in a MyD88, IRAK and TRAF-6 dependent manner (53, 54). Activation of IRF-7 culminates in its translocation to the nucleus where it directly promotes the expression of IFN-α and other interferon stimulated genes (ISGs) (53, 54). Although TLR7 and TLR9 activation induce both the NF-kB and IRF signaling pathways, it is important to note that pDCs produce much higher levels of type I IFN than IL-12 in response to TLR ligation or infection (Fig. 1B) (35, 37, 55).

The variability of PRR signaling pathways on DC subsets may play an important role in their ability to prime type I lymphocyte responses. In addition to their ability to secrete IL-12, cDCs have been characterized as the most efficient APC subset (6). Further, this correlates well to their documented capacity to prime $CD4^+$ Th1 development in an IL-12 dependent manner (24). Initially, it was reported that pDCs could promote Th1 responses after infection

with viruses such as Influenza virus (6, 56). However, the current understanding suggests that pDCs are not efficient at antigen presentation when compared to cDCs arguing that cDCs priming may function as the primary pathway for type I response during T cell activation (57, 58). Interestingly, IFN-α/β secreted by pDCs can promote cDC maturation and $CD4^+$ T cell activation in the lymph node during viral infection and therefore pDCs may provide an important source of IFN-α/β during T cell priming (59).

Role of IFN-α/β in the immune response to infection

The interferons were identified and named on the basis of their ability to "interfere" with viral replication and therefore are regarded as one of the most important classes of cytokines produced during infection (60, 61). Multiple cell types have the capacity to secrete type I interferons (IFNs) and the initial phase is regulated through pattern recognition pathways. Infection of non-immune cells leads to the activation of the RIG-I/MDA5, and potentially the DAI pathways facilitating the production of large quantities of IFN-β (12–14). This IFN-β acts as the first line of defense during infection and can then feed back on cells in either an autocrine or paracrine manner facilitating the production of other IFN-α subtypes as well as a multitude of interferon response stimulated genes (ISGs) to promote an anti-viral state (62).

The type I IFNs are a diverse class of cytokines and in humans are composed of 13 IFN-α subtypes, IFN-κ, IFN-ε, IFN-ω and IFN-β (63). They signal through two distinct receptor chains termed the interferon alpha receptor 1 (IFNAR1) and IFNAR2 which are thought to be constitutively expressed on all somatic cells. Signaling through the IFNAR proceeds through the activation of the Janus Kinase family members TYK2 and JAK1 which associate with IFNAR1 and IFNAR2 respectively (64, 65). Following cytokine activation, JAK1 and TYK2 undergo auto-phosphorylation on specific tyrosine residues which allows for the docking of downstream signal transducer and activators of transcription (STAT) family of transcription factors via Src homology 2 domains (SH2) (65). In humans, this includes the members STAT1, STAT2, STAT3 and additionally, STAT4, which is predominantly active in T lymphocytes (30, 62). STAT1 and STAT2 can heterodimerize and interact with another transcription factor, p48/IRF-9 and together this forms a complex termed the interferon stimulatory gene factor 3 (ISGF3) (66). This facilitates the majority of responses by IFN-α/β and upon activation, this complex is translocated to the nucleus where it binds specific interferon responsive elements (ISRE) in the promoter regions of ISGs promoting the antiviral response (62). Alternatively, STAT4 can homodimerize in response to IFN-α/β in T lymphocytes and NK cells. This is similar to the induction by IL-12 signaling and has been proposed to be an alternative pathway for Th1 and Tc1 development (30, 67, 68).

In addition to the direct induction of activation of STATs and induction of anti-viral genes, signaling through the IFNAR leads to the activation of MAP Kinases (69), phosphotidylinositol 3-kinases (PI-3 Kinase) (70) and the Vav proto-oncogene (71). Further, in T lymphocyte cell lines, IFN-α/β signaling was also able to activate the protein tyrosine phosphatase (PTP1D), CD45, Lck and Zap70 pathways (62, 72). As these pathways play important roles in cell growth and proliferation, this suggests a role for IFN-α/β in these responses. However it is unclear as to the functional relevance of these signaling responses in primary T lymphocytes *in vitro* or *in vivo*.

The predominant role for type I IFN has been shown to be the promotion of the cell-intrinsic anti-viral state. This occurs through induction of multiple ISGs of which several can promote the antiviral state through a variety of strategies (73). During viral infection, inhibition of protein synthesis is an important host mechanism for preventing viral spread. In response to IFN-α/β or dsRNA, the cellular dsRNA sensor, RNA-activated protein kinase (PKR) becomes auto-phosphorylated. This leads to phosphorylation and inactivation of the eukaryotic protein synthesis initiation factor 2 (eIF2α), and leads to the inhibition of protein synthesis and apoptosis (74, 75). This is similar to the blockade of cellular eIF3 which is induced by the ISG, ISG56 and has been shown to be important in controlling hepatitis C virus (HCV) infection (76). Inhibition of viral replication and assembly by ISGs has also been documented.

IFN-α/β promotes the degradation of viral RNA via activation of the 2'5' oligoadenylate synthase pathway which promotes activation of RnaseL and inhibition of viral replication (77, 78). In addition, the induction of the MX protein is also linked to anti-viral responses and been shown to inhibit Influenza and vesicular stomatitis virus (VSV) infection via potential blockade of viral assembly and trafficking (73). Therefore, signaling via IFN-α/β is critical to promoting an antiviral state in response to infection.

In addition to cell intrinsic responses, IFN-α/β also contributes to both the innate and adaptive immune responses to viral infection. Signaling by IFN-α/β promotes the expression of MHC-I on non-immune cells as well as MHC-I and MHC-II on APCs, allowing for recognition by viral specific $CD8^+$ T cells or $CD4^+$ T cells respectively (17). Additionally, IFN-α/β can enhance factors important to antigen processing and presentation as well as surface expression of CD80 and CD86 allowing for enhanced T cell priming by cDCs and MΦ (8). IFN-α/β can also modulate cytokine expression in DCs and MΦs. For example, IFN-α/β signaling can promote induction of IL-15, a cytokine know to be important in effector responses as well as the maintenance of $CD8^+$ T cell memory (79). Alternatively, IFN-α/β can act to inhibit the type I polarizing cytokine IL-12 in DCs and has been proposed as a mechanism for regulating the immuno-pathology during intracellular infection (80-82). Further it plays a

positive role in activating the IFN-γ response in NK cells as well as both $CD4^+$ and $CD8^+$ T cells (30, 60, 83, 84), as well as a regulatory role via inhibition of both ISG expression and IFN-γ production via the induction of suppressor of cytokine signaling (SOCS) proteins (85). Therefore, IFN-α/β is not only critical in the cell-intrinsic response to intracellular infection, but also plays a major role in shaping the pro-inflammatory as well as regulatory pathways involved in innate and adaptive anti-viral immunity.

The necessity for IFN-α/β during intracellular infection is highlighted by the observation that mice deficient in the type I IFN receptor lack both the ability to resist infection by a diverse group of viruses including VSV, Lympho-choriomenengitis virus (LCMV), Vaccinia virus (VV) and Semliki forest virus as well as show deficiencies in certain adaptive immune responses (78). This is coupled to the identification of diverse viruses which encode viral inhibitors of IFN-α/β pathways. For example, HSV has been shown to block the IFN-α/β pathway at multiple stages, including disruption of the IRF pathways by the ICP0 protein or blockade of STAT1 activation by ICP27 (86-88). Blockade of the IRF response has also been observed by HCV-NS3/4A (89), Influenza virus-NS1 (90), VV-E3L (91) and the VP35 protein from Ebola viruses (92). In addition, VV encodes the soluble decoy receptor B18, which is homologous to the IFNAR and therefore acts to block IFN signaling (93). Taken together, type I IFNs represent an important signaling component critical to virus-host interaction and its

deregulation has the capacity to disrupt cell-intrinsic, innate and potentially adaptive responses to intracellular infection.

The role of Interleukin-12 on intracellular infection

In addition to IFN-α/β, Interleukin-12 (IL-12) also plays a critical role in the immune response to intracellular infection. IL-12 was first identified as a natural killer (NK) cell growth factor (NKGSF) that promoted innate IFN-γ expression and the induction of T lymphocyte proliferation (94, 95). It was subsequently termed IL-12 and shown to directly promote IFN-γ and type I responses from both $CD4^+$ and $CD8^+$ T lymphocytes (27, 28, 96, 97). In addition, signaling via IL-12 also promotes cytolytic activity in NK and $CD8^+$ T cells via induction of perforin and granzyme B and has the potential to promote B cell class switching through indirect activation of $CD4^+$ T cells (5, 98). Therefore, IL-12 represents an important innate cytokine in shaping both innate and adaptive immune responses to infection.

IL-12 is a heterodimeric member of the IL-12 family of cytokines which includes IL-23 and IL-27; all of which have been implicated in the development of type I responses (83, 99). It is composed of two subunits, a 40-kDa subunit (p40) which it shares with IL-23 and a unique 35-kDa subunit (p35) which together form the intact signaling molecule of 70-kDa termed IL-12p70 (94, 99).

Signaling in response to IL-12 proceeds through a heterodimeric receptor composed of the IL-12Rβ1 and the signaling IL-12Rβ2 chains. The IL-12R is expressed predominantly on NK cells and T cells. However, it can also be expressed on some B cell and DC subsets, though its functions in these cells are unclear (5). Importantly, NK cells appear to express higher levels of the IL-12R in their unprimed state allowing for rapid induction of IFN-γ and proliferation in response to stimulation by IL-12 (5). Alternatively in naïve $CD4^+$ and $CD8^+$ T cells, expression of the IL-12R is very low. Further, while IL-12Rβ1 is uniformly expressed, induction of the high affinity IL-12Rβ2 chain requires signaling provided by TCR activation, IFN-γ, IFN-α/β, IL-27 or IL-12 itself for the induction of type I development (83, 100).

Signaling via the biologically active IL-12p70 leads to activation of the Janus Kinase family members TYK2 and JAK2 which are associated with IL-12Rβ1 and IL-12Rβ2 respectively (6). These recruit downstream STAT molecules inducing their phosphoryation. Phosphorylation of STAT4 in response to IL-12 leads to its homodimerization and translocation to the nucleus where it can specifically target genes involved in IFN-γ expression and Th1/Tc1 development (30, 101, 102). Further, STAT4 appears to be crucial to Th1 development as $STAT4^{-/-}$ mice resemble $IL\text{-}12p40^{-/-}$ mice with decreased IFN-γ production and a block in Th1 development (103, 104). In addition, other

cytokines have been shown to contribute to Th1 responses. For example IL-18 can synergize with IL-12 to enhance IFN-γ expression on mature Th1 cells (105). This occurs in the absence of antigen stimulation and proceeds through IL-12 activation of STAT4 and the induction of NF-κB and AP-1 by IL-18 (105). Alternatively, the p40 chain of IL-12 can pair with another molecule p19 to form the IL-12 family member, IL-23 (106). Originally, signaling through IL-23 was thought to promote similar type I responses due to its ability to activate STAT4. However, more recent data demonstrates that IL-23 predominantly activates STAT3 and not STAT4 and is important in the development of the recently defined pro-inflammatory Th-17 cells in a STAT3 dependent manner (107). Further another IL-12 family member, IL-27 has been shown to play an important role in $CD4^+$ Th1 development via induction of the IL-12Rβ2, although it is unclear as to its functional response during infection (83).

As mentioned above, monocytes, macrophages and DC subsets can produce IL-12 in response to ligation of PRRs and further this has been shown to be important in the formation of protective immunity against a variety of agents including the protozoa *Leishmania* (108) and *Toxoplasma* (109) , bacteria such as *Listeria* (110, 111) and murine-cytomegalo (MCMV) and Influenza virus (112, 113) . Although induction of IL-12 is protective, further studies have revealed that PRR alone is insufficient to promote the induction of biologically active IL-12p70. Instead, additional signals such as T cell mediated CD40 activation on

DCs or signaling by IFN-γ may be important in the regulation of the p35 subunit and the secretion of fully active IL-12p70 (114, 115). Further, the p40 chain has also been shown to be secreted independently of linkage to p35 or p19 and although this does not signal via the IL-12R, it may prevent p70 signaling and act as a negative regulator of IL-12 induced inflammation (5, 6). Therefore, this suggests an important feedback loop in the induction of type I responses by IL-12 which can ensure that inflammation occurs as a necessity and is limited to situations in which only both the innate and adaptive arms of the immune response have been activated.

$CD4^+$ and $CD8^+$ T lymphocyte differentiation and the role of IFN-γ and the CTL response to intracellular infection

T lymphocytes have the capacity to diverge into distinct subsets dependent upon the type of infection sensed. This effect was initially described by Mosmann and Coffman who identified two distinct subsets of murine $CD4^+$ T-helper clones. Th1 cells which secreted IL-2, IFN-γ and TNF-β and were important to intracellular immunity and Th2 cells which secreted IL-4, IL-5, and IL-13 and were important to extracellular and helimith immunity (3). Subsequent studies identified analogous populations of $CD8^+$ T cells, termed Tc1 and Tc2 cells in both mouse and human (1, 2). These cells also were capable of

secreting IFN-γ and IL-4 respectively, however the functional relevance of the Tc2 population during infection is not well understood (2).

The development of Th2 and Tc2 cells has been shown to occur through signaling by IL-4 (2). Alternatively, the development of Th1 and Tc1 cells occurs through signaling via IL-12 and has been shown to promote immunity to intracellular pathogens including protozoans, bacteria and viruses (108-113, 116). Immunity to these pathogens is in part related to the induction of type I cytokines such as IFN-γ and TNF-α. However IFN-γ signaling has been shown to have the most pleotropic effects on type I effector responses. This is highlighted by examination of mice deficient in IFN-γ signaling pathways which have defects in their ability to respond to a broad range of pathogens including *Listeria*, *Mycobacterium*, *Leishmania* and Vaccinia virus (78, 117-120). Further, during infection by *Mycobacterium* and *Listeria,* IFN-γ expression from both $CD4^+$ and $CD8^+$ T cells has been shown to be critical to immunity (117, 121). Therefore T lymphocyte secreted IFN-γ acts as a major type I cytokine in the immune response to infection.

Signaling by IFN-γ leads to the activation of STAT1. Like other STAT family members, STAT1 forms homodimers in response to activation and translocates to the nucleus where it activates specific genes containing γ-IFN activation sites (GAS) within their promoters (116, 119). This can lead to direct

anti-viral responses such as enhancement of the PKR and 2'5' OAS pathways as well as induction of apoptotic pathways in infected cells in a manner similar to IFN-α/β (119). In addition to intrinsic responses, secretion of IFN-γ by T cells has a broad effect. For example, in response to IFN-γ, MHC-I and MHC-II levels as well as IL-12 production is enhanced in MΦs, leading to the enhancement of T cell priming to the type I fate (119, 122). Alternatively, recognition of both IFN-γ and TNF-α increased phagocytic capacity and the expression of nitric oxide (NO), hydrogen peroxide and other superoxides which increases MΦ clearance of internalized pathogens (119, 123, 124). In addition to phagocyte activation, IFN-γ secretion by $CD4^+$ T cells has been shown to promote murine B cell class switching to IgG_{2a}, an isotype important in bacterial and viral infection via its ability to fix complement and promote opsonization (119, 125). Further, IFN-γ produced by $CD4^+$ T cells can enhance promotion of murine cytolytic $CD8^+$ T cell responses, which are critical to clearance of intracellular bacterial and viral infection (119, 126). Therefore, lymphocyte secretion of IFN-γ plays an important and pleotropic role in the development of multiple pathways and cellular responses during intracellular infection.

Despite a clear role for IFN-γ in providing T cell mediated "help" to innate and adaptive responses during intracellular infection it has been suggested that IFN-γ secretion can play a direct role in anti-viral immunity. To this regard,

neutralizing experiments demonstrated a critical role for IFN-γ in the antiviral activity of $CD4^+$ but not $CD8^+$ T cells against MCMV (127). More recently, this has been extended to gammaherpesviruses68 infection and suggests an role for this pathway *in vivo* (128, 129). In line with these observations, recent data from our lab has found that supernatants from cytokine polarized $CD4^+$ T cells can directly inhibit viral replication as well as infectivity in response to VSV, HCV and RSV infection *in vitro.* This is dependent on both IFN-γ and TNF-α and suggests that $CD4^+$ T cells may exhibit direct effects on limiting viral infection and spread through cytokine secretion (130).

In addition to IFN-γ secretion, cytolytic activity is an important aspect of the T lymphocyte response to infection. This is predominantly active in the $CD8^+$ T cells which acquire the ability to lyse intracellular infected cells in an MHC-I dependent manner. In response to Tc1 priming, $CD8^+$ T cells acquire the expression of molecules such as perforin and the granzyme family of serine proteases which are crucial to the induction of the apoptotic pathway in a two step manner including membrane pore formation and the activation of cytosolic caspases (98, 131-135). Induction of apoptosis is also facilitated through engagement of the TNF-receptor family members including CD95/CD95L or FAS/FASL which plays a major role both in eradication of infected cells as well as in activation induced cell death (AICD) pathway of immune regulation (131, 135, 136). These pathways may also be functional in $CD4^+$ T cells, however the

role of this during *in vivo* infection is unclear (137, 138). Taken together however, the acquisition of type I effector responses by both $CD4^+$ and $CD8^+$ T cells is critical in multiple aspects of the immune response and to the clearance of intracellular infection and the prevention of progression of disease.

The role of IL-12 and IFN-α/β on T lymphocyte effector responses

Both $CD4^+$ and $CD8^+$ T cells are critical mediators of adaptive immune responses to intracellular pathogens. Activation through multiple signals including TCR engagement, costimulatory activation and cytokine programming are required for their efficient expansion and acquisition of effector functions such as cytokine secretion and lytic activity. These signals are delivered primarily by DCs in the draining lymph nodes. Recently a three signal model for the development of T lymphocyte effector responses has been proposed (Fig. 2) (27-29, 31). This model suggests that three distinct signals are required for the full induction of T cell responses. Activation of the TCR in an antigen-dependent manner facilitates "signal 1", this is followed by CD80/86-CD28 or IL-2 mediated co-stimulatory activation "signal 2", and signaling provided by innate inflammatory cytokines "signal 3" (139). While signals 1 and 2 prime naïve $CD4^+$ and $CD8^+$ T cells and initiate cell division, signal 3 cytokines program effector functions and ensure clonal survival (Fig. 2) (139, 140).

This model correlates nicely with the observed maturation of DC subsets responding to intracellular infection. PRR and interferon signaling can enhance the expression of MHC-I and MHC-II and processing pathways important for antigen presentation therefore allowing for signal 1 activation on $CD4^+$ or $CD8^+$ T cells respectively (17). Further, induction of CD80 and CD86 on DCs then allows for the activation of T cells via CD28 and activation through signal 2 (140, 141) . Depending upon the type of infection, DCs have the capacity to secrete a variety of cytokines which have the potential to act as signal 3 for $CD4^+$ and $CD8^+$ T cells (2-4, 28, 29). In particular, IL-12 and IFN-α/β, promote efficient induction of innate immunity as well as the development of adaptive type 1 responses to intracellular infection (5, 6). Thus, IL-12 and IFN-α/β which are secreted by activated DC may represent the predominant signal 3 during intracellular infection.

IL-12 signaling acts as the major determinant for $CD4^+$ Th1 and $CD8^+$ Tc1 development (5). In $CD4^+$ T cells, this occurs through the induction STAT4 which functions to promote Th1 development and the expression of IFN-γ in both mice and humans (102, 104, 142). Furthermore, as described above, disruption of this pathway in mice through targeted deletion (103, 104) or via disruptive mutations in humans (143) leads to a dramatic defect in Th1 responses. Initial observations in mice demonstrated that in addition to the IL-12/STAT4 pathway, the induction of a key transcription factor, T-box expressed in T cells (T-

bet/TBX21), was critical for the induction of IFN-γ in $CD4^+$ but not $CD8^+$ T cells (144-146). Furthermore, it was shown that this was due to the ability of T-bet to promote the induction of the IL-12Rβ2, allowing for IL-12 responsiveness and further Th1 polarization by IL-12 (147, 148). The expression of T-bet in mice is induced through TCR activation as well as by IFN-γ (144, 147). Additionally, this induction occurs upstream of IL-12 signaling and proceeds in a STAT1-dependent and STAT4-independent manner (147, 148). Therefore, in mice this suggests a two step model in which IFN-γ sensitizes naïve T cells for downstream IL-12 polarization through the induction of the T-bet and the IL-12Rβ2 chain **(**Fig. 3A**)**.

In contrast to mice, IFN-γ is a poor inducer of T-bet expression in human $CD4^+$ T cells, suggesting a differential pathway for Th1 development in humans might exist (83). Early reports suggested that this might occur through IFN-α/β, as its signaling was capable of promoting expression of both T-bet and IL-12Rβ2 (83, 149). In addition, IFN-α/β was shown to promote Th1 development from human peripheral blood mononuclear cells (hPBMCs) (150, 151) as well as synergize with IL-18 to promote IFN-γ from mature Th1 cells and NK cells (152, 153). Subsequently, IFN-α/β, was observed to promote STAT4 activation and IFN-γ production in human $CD4^+$ T cells, however this response was to a lesser degree than observed with IL-12 (30, 101, 154).

Adding complexity to role of IFN-α/β on Th1 development, parallel studies involving murine T cells demonstrated that IFN-α/β was insufficient to promote STAT4 phosphorylation (30, 154) or Th1 development (30, 155) in comparison to human $CD4^+$ T cells, indicating a potential species-specific role for IFN-α/β in regulating Th1 development. This was originally thought to be due to a role for human but not murine STAT2 in the recruitment of STAT4 to the human IFNAR (154, 156). However, further examination of $CD4^+$ T cells from STAT2 knock-in mice that expressed a humanized *Stat2* gene failed to exhibit either STAT4 phosphorylation or Th1 commitment in response to IFN-α/β activation (157). Therefore, these results suggested that although STAT2 was required for STAT4 activation in human cells, it was not sufficient to restore this pathway when expressed in the context of the murine IFNAR.

Based on this observation, further studies examined the ability of IFN-α/β to promote Th1 responses in mice. While some studies have called this species-specific pathway into question (68, 158), other studies have confirmed a defect in the ability of IFN-α/β to promote Th1 development in mice (155, 159). Further, recent studies in human $CD4^+$ T cells reveal that IFN-α/β induces attenuated phosphorylation of STAT4 in comparison to IL-12 and suggest that while IFN-α/β may augment Th1 responses, IL-12 may act as the predominant promoter of Th1 development in humans (100, 160). Therefore although multiple studies

have examined the role of these cytokines on Th1 development, it is still unclear as to the true contribution of IFN-α/β on $CD4^+$ Th1 development in either the human or the mouse system **(**Fig. 3**)**.

Although initially, IL-12 promotion of Tc1 development was thought to occur in a similar manner, studies by Biron and colleagues revealed that during infection with LCMV, IFN-α/β but not IL-12 was required for IFN-γ production from murine $CD8^+$ T cells (158, 161, 162). Further studies suggested that the induction of IFN-γ secretion and lytic activity in murine $CD8^+$ T cells was not only independent of IL-12 but STAT4, and T-bet as well (146, 163). Identification of another T-box family, Eomesodermin (Eomes), by Reiner and colleagues then suggested that differences between $CD4^+$ and $CD8^+$ T cells could be explained by a redundant role of Eomes in promoting IFN-γ and CTL activity in the absence of T-bet (164, 165). However, more recent studies revealed that during *in vivo* infection with either Influenza virus or *Toxoplasma gondii*, T-bet was in fact essential for the induction of IFN-γ expression and $CD8^+$ effector function (166). In line with this observation, studies by Mescher and colleagues then demonstrated that IL-12 signaling provided a necessary third signal that regulated $CD8^+$ effector T cell development and this was dependent on STAT4 (28, 29).

Although IL-12 was sufficient to promote effector cell development *in vitro*, IL-12R-deficient $CD8^+$ T cells were still capable of mounting both primary

effector and memory cell responses to *in vivo* infections with adenovirus[11] and *Listeria* (167), suggesting the possibility that further innate cytokines may promote $CD8^+$ effector cell development. To that regard, more recent studies have indicated that IFN-α/β can also act as signal 3 for the acquisition of cytokine secretion, clonal expansion and cytolytic activity in murine $CD8^+$ T cells (29). This correlates with the studies by Biron and colleagues (161, 162) and suggests that similar to the observations in human $CD4^+$ T cells, IL-12 and IFN-α/β can act as redundant signals to promote the development of effector responses in murine $CD8^+$ T cells. However sufficient examination of these pathways in humans is lacking and while these responses mirror the original human $CD4^+$ observations, they are quite distinct from the role of IFN-α/β on murine $CD4^+$ T cells. Therefore, as with $CD4^+$ Th1 development, the role of IFN-α/β on the development of $CD8^+$ Tc1 development is still unclear (Fig. 3B).

IL-12 and IFN-α/β in $CD4^+$ and $CD8^+$ T cell memory responses

Two main subsets of T lymphocytes arise from primary infection by intracellular pathogens. First, the effectors which are immediately responsible for clearance of the infectious organism and second, memory cells which "remember" the infection and are poised for a rapid response upon secondary challenge. Whereas effector cells are short lived and function only in the primary

responses, memory cells are long lived and traffic between lymph nodes and peripheral tissue in search of their cognate pathogen. Further, memory cells demonstrate properties which are distinct from naïve cells such as rapid proliferation and the requirements for signal 1 but not 2 and 3 for efficient activation (168).

In humans two populations of memory cells, the central memory (T_{CM}) and effector memory (T_{EM}) have been identified and well characterized in both the $CD4^+$ and $CD8^+$ compartment (169). Both T_{CM} and T_{EM} are low for the surface expression of CD45RA which is downregulated upon TCR activation. Therefore, memory cells represent a population of antigen experienced T cells (169). T_{CM} and T_{EM} are distinguished phenotypically on the basis of surface expression of the lymph node-homing chemokine receptor, CCR7 such that T_{CM} are $CD45RA^{lo}$ $CCR7^{hi}$ and T_{EM} are $CD45RA^{lo}$ $CCR7^{lo}$ (169). In addition to expression of surface receptors which promote their traffic to lymph nodes, T_{CM} cells lack immediate effector function but are capable of rapid proliferation and the pluripotent repopulation of both effectors and memory cells in response to secondary activation. Alternatively, T_{EM} traffic to the periphery, display immediate effector function, are more terminally differentiated and proliferate poorly to secondary activation (170, 171).

More rigorous examination of memory populations found in human peripheral blood has revealed that in addition to CD45 and CCR7, other markers

such as CD27 and CD28 can serve to further demarcate subpopulations of effector and memory cells (172, 173). The functional relevance of these populations and their development during infection are not yet clear, but their separation by surface markers involved in T cell costimulation suggests that their responsiveness and function during infection may be varied. In mice, populations of T_{CM} and T_{EM} have been identified using the markers CD62L and CD44. However, unlike in humans, T_{CM} displayed equivalent IFN-γ expression when compared to T_{EM} populations, although T_{EM} cells remained incapable of expressing IL-2 (171). Therefore, in both mice and man, during infection, the development of memory is diverged into at least two distinct groups with unique capacities to shape the secondary response to infection.

While there is clear evidence to support the development of multiple populations of memory cells with distinct phenotypic characteristics, the development of these subsets and the lineage relationships between these cells remains unclear in both the $CD4^+$ and $CD8^+$ compartment (174). Multiple models of lineage development have been proposed. Originally, Sallusto and Lanzavecchia proposed development of memory progressed in a linear stage from naive$\rightarrow T_{CM}\rightarrow T_{EM}$, with the T_{CM} compartment progressively giving rise to new T_{EM} cells (Fig. 4) (169, 175). This pathway appears to occur predominantly in $CD4^+$ T cells and its recapitulation in the $CD8^+$ compartment is not clear (176). However, two other models have emerged for both $CD4^+$ and $CD8^+$ T cell

memory development (177-180). These models hinge on either one of two principles. First that T_{CM} and T_{EM} are derived from a common progenitor or second that development of T_{CM} and T_{EM} progresses via differential development of distinct lineages of cells (171, 174, 180) (Fig. 4).

The linear model of T cell memory is based on the assumption that memory cells are derived from the same progenitor and upon activation, a group of effector CTLs is generated, undergoes clonal expansion and upon contraction the remaining rested cells survive as the classical T_{CM} population (171, 174, 180, 181) (177, 180). Several studies are in accordance with the linear model of development (171, 181-183). For example, in response to either *Listeria* or LCMV infection, T_{EM} cells were observed to develop during primary infection and only after a period of rest, did these convert to a T_{CM} phenotype (171). Further, this has been recapitulated in other systems and recently this model has been extended to $CD4^+$ T cells as well (181, 182).

Alternatively, multiple studies suggest that effector and memory cells develop from distinct lineages which may arise as early as the first division after antigen encounter (178, 184-186). In accordance with this model Baron *et al.* demonstrated that TCRβ expression in Influenza specific, human $CD8^+$ T cells was distinct between the T_{CM} and T_{EM} compartment (184). Further, cells were found to develop in an asymmetrical fashion with T_{EM} phenotypes being separable even at the first division (186). This is consistent with previous data

that suggests that cytokine polarization influences the development of both effector and memory cells from the same T cell pool *in vitro* (185). More recent data has demonstrated that this can also occur *in vivo*, and in this study, infection with VV or *Listeria* led to the development of T_{CM} and T_{EM} concurrently even at early time points (178). Despite the wealth of data concerning lineage development of T_{CM} and T_{EM} phenotypes, it still remains unclear as to which of these models represents the predominant pathway for memory generation during infection.

In addition to the uncertainty of lineage development, the exact cytokine signals crucial in programming the T_{CM} and T_{EM} fates are not fully understood. While the common γ-chain (γc) cytokines IL-2, IL-7 and IL-15 have been shown to be critical to $CD8^+$ memory responses, they have mainly been attributed to homeostatic proliferation, cellular survival and maintenance of memory over time and not their differentiation into T_{CM} or T_{EM} phenotypes (187-189). Recently, IL-12 has emerged as a potential candidate for this role; however signaling through IL-12 has been shown to promote T_{EM} and not T_{CM} fate generation (190, 191). For example, infection of IL-12p35$^{-/-}$ with *Listeria* led to decreased $CD8^+$ effector responses but increased development of T_{CM} cells (190). Further, Joshi *et al*. then demonstrated that IL-12 could promote T-bet in a dose-dependent manner and this was directly related to the development of a T_{EM}-like fate suggesting a negative role for IL-12 in the generation of T_{CM} cells (192). These

studies were followed by the observation that deficiency in IL-12 signaling was directly related to decreased T-bet and increased Eomes during infection and this correlated with an increase in T_{CM} formation (191). This coupled to the subsequent identification of increased T-bet in sorted T_{EM} and increased Eomes in T_{CM} populations led to the hypothesis that Eomes was responsible for T_{CM} development; although this observation has yet to be demonstrated experimentally (193).

The role of IFN-α/β on memory formation is less clear. Initial studies indicated that it could promote memory indirectly by activating DCs to produce IL-15 (79). This was followed by the identification of a direct pathway in which IFN-α/β could prevent apoptosis of both $CD4^+$ and $CD8^+$ T cells and limit AICD (194). In agreement with this, examination of IFNAR-deficiency during infection with LCMV, demonstrated an inability for $CD4^+$ and $CD8^+$ T cells to survive and develop into memory cells in an adoptive transfer model (195, 196). Further, IFNAR-deficient $CD8^+$ T cells did not have any defects in their primary response to infections, suggesting a unique role for IFN-α/β in the development of memory (196, 197). Although more studies are necessary to gain a complete understanding of the role of innate cytokines on $CD8^+$ T cell memory formation, it is clear that IL-12 and IFN-α/β have the capacity to shape this developmental process. Further examination into the direct contributions of these cytokines is

key to our understanding of their role in the global T lymphocyte response to infection.

Concluding remarks

The generation of T lymphocyte effector and memory responses requires multiple signals, including antigen-specific TCR engagement, activation of costimulatory pathways and the programming of T lymphocytes via innate cytokine secretion. IL-12 and IFN-α/β are two cytokines which are positioned to promote these aspects of the T lymphocyte response and therefore, these cytokines represent major signals shaping immunity to intracellular infection. At the start of these studies, IL-12 and IFN-α/β were thought to be redundant in their ability to promote type I responses. While the role of IL-12 in these responses had been well characterized, several contradictory observations left the role for IFN-α/β unclear. Initial observations in humans suggested that IFN-α/β could prime Th1 development due to its ability to activate STAT4 (30, 101). However, IFN-α/β was unable to promote this response in mice altogether (30, 154, 155). While the findings that differences between human and mouse STAT2 might explain the species-specific differences (154), further observations revealed that this was not the case (157). Adding to this confusion, murine $CD8^+$ T cells were then found to be highly responsive to IFN-α/β (158, 161). Therefore as in $CD4^+$

T cells it was thought that IL-12 and IFN-α/β were redundant in priming Tc1 development (28, 29). In an attempt to rectify the controversial role of IFN-α/β in type I development, this study set out to examine the direct contributions of IL-12 and IFN-α/β on the development of both human $CD4^+$ and $CD8^+$ T lymphocyte effector and memory responses. Importantly, my work has revealed that unlike previously suggested, these cytokines are not simply redundant in their roles. Instead, IL-12 and IFN-α/β possess independent activities which promote distinct aspects of the lymphocyte response. While IL-12 remains key in promoting effector responses in $CD4^+$ and $CD8^+$ T cells, IFN-α/β is insufficient to do so and instead acts uniquely to shape the development of a distinct population of T_{CM} cells. Together, the results of this study highlight the importance of the regulation of IL-12 and IFN-α/β in terms of programming T lymphocyte responses. Furthermore, these findings have broad implications on how we should develop therapies and vaccine strategies which rely on IL-12, IFN-α/β and T lymphocyte mediated responses.

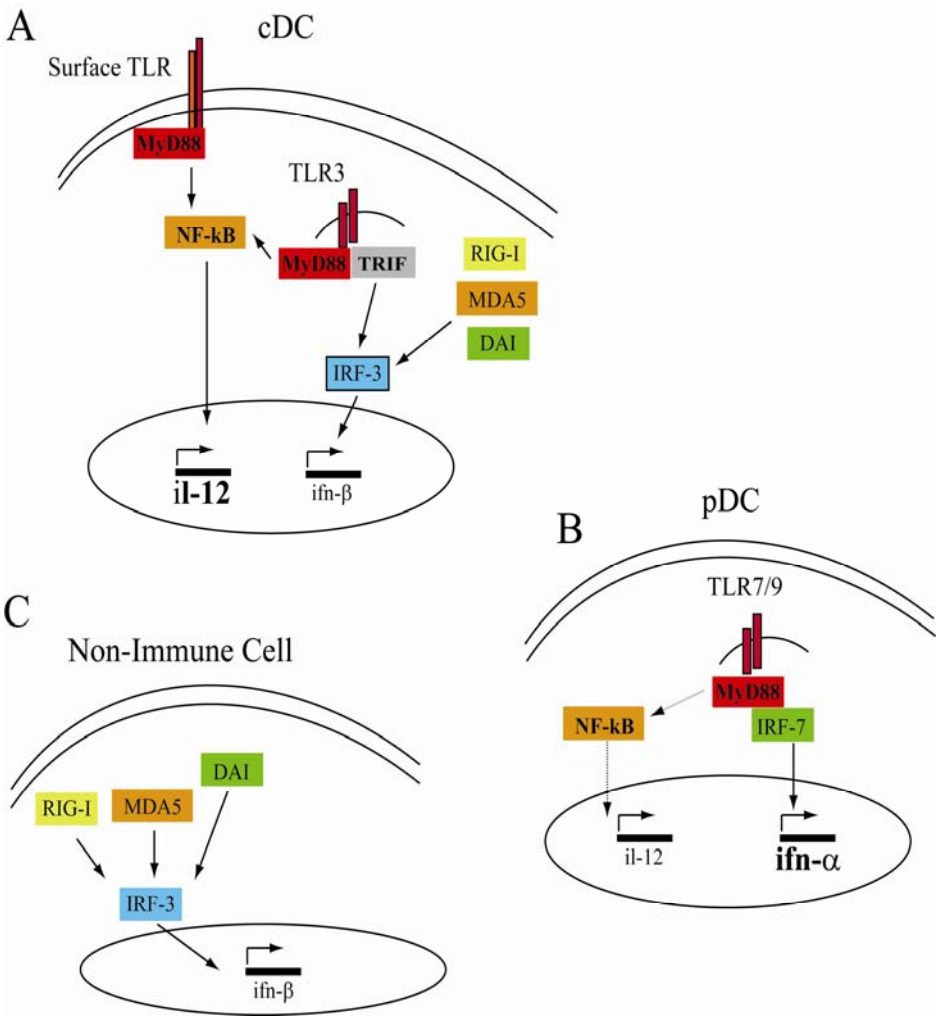

Figure 1. Pattern recognition pathways and IL-12 and IFN-α/β production. (**A**). cDCs utilize 3 pathways for innate cytokine induction. Signaling vial TLRs triggers NFκB expression and the induction of IL-12. Signaling via TLR3/TRIF as well as RIG-I/MDA5 and DAI trigger activation of IRF-3 and IFN-β expression. (**B**). pDCs utilize the TLR7/9 MyD88 pathway to induce IFN-α in an IRF-7 dependent manner. (**C**). Non-immune cells utilize the cytosolic receptors RIG-I/MDA5 and DAI to induce IFN-β in an IRF-3 dependent manner.

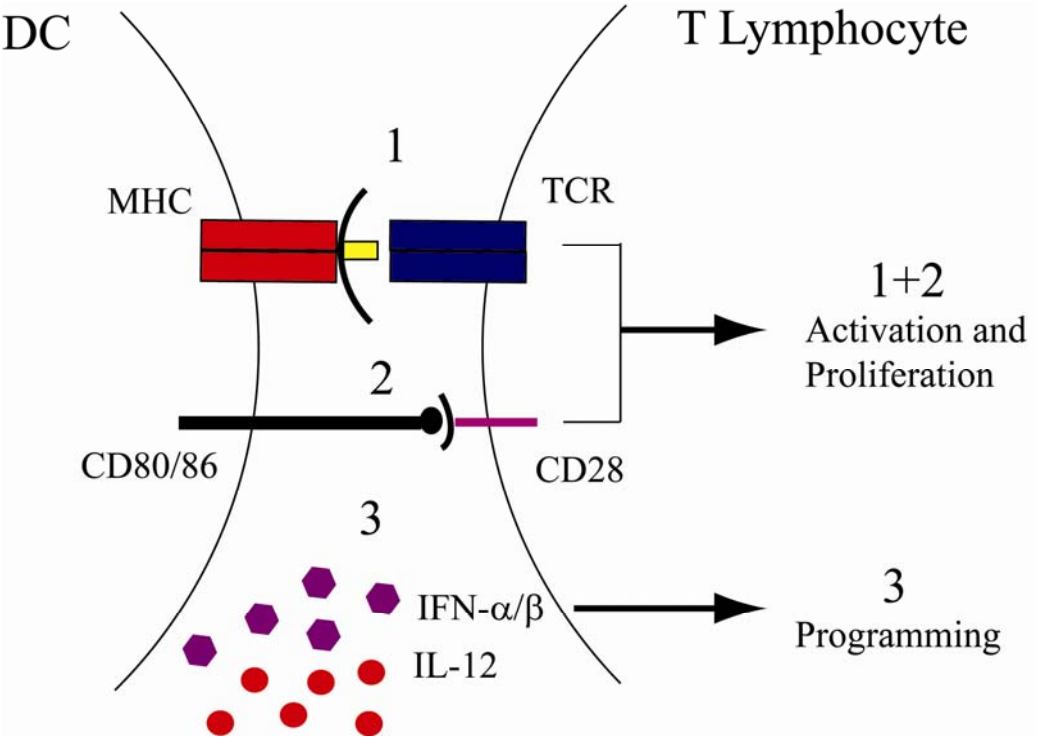

Figure 2. The " 3 signal model" for T lymphocyte activation. Three signals are required for optimal T cell activation. Signal 1 is delivered from DCs to T cells via MHC-I/MHC-II (red) presenting peptide to the TCR (blue). Signal 2 is delivered from DCs via CD80/86 ligation to CD28. Signal 3 is provided by innate cytokines such as IL-12 and IFN-α/β. While signals 1 and 2 promote activation and proliferation, signal 3 cytokines shape developmental programming.

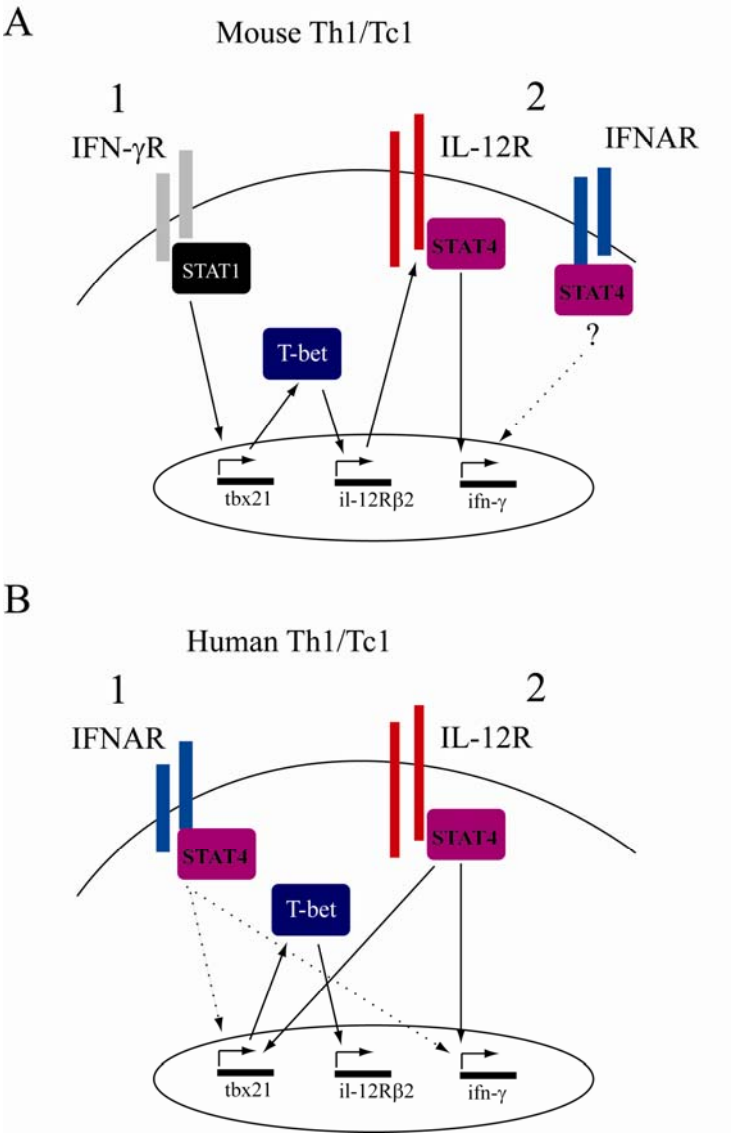

Figure 3. Type I development in mice and man. (A). Th1/Tc1 development is a two step process in mice. "1" IFN-γ/STAT1 promotes T-bet and IL-12Rβ2 induction and this primes "2" IL-12/STAT4 mediated IFN-γ production. The role of IFN-α/β induced STAT4 is unclear (dashed line). **(B)**. Both IFNAR and IL-12 act to primeIL-12 responsiveness "1". However, IL-12 promotes IFN-γ and Th1 via prolonged STAT4 (solid line) "2" while IFN-α/β is insufficient to induce Th1 development due to acute STAT4 and T-bet activation (dashed line).

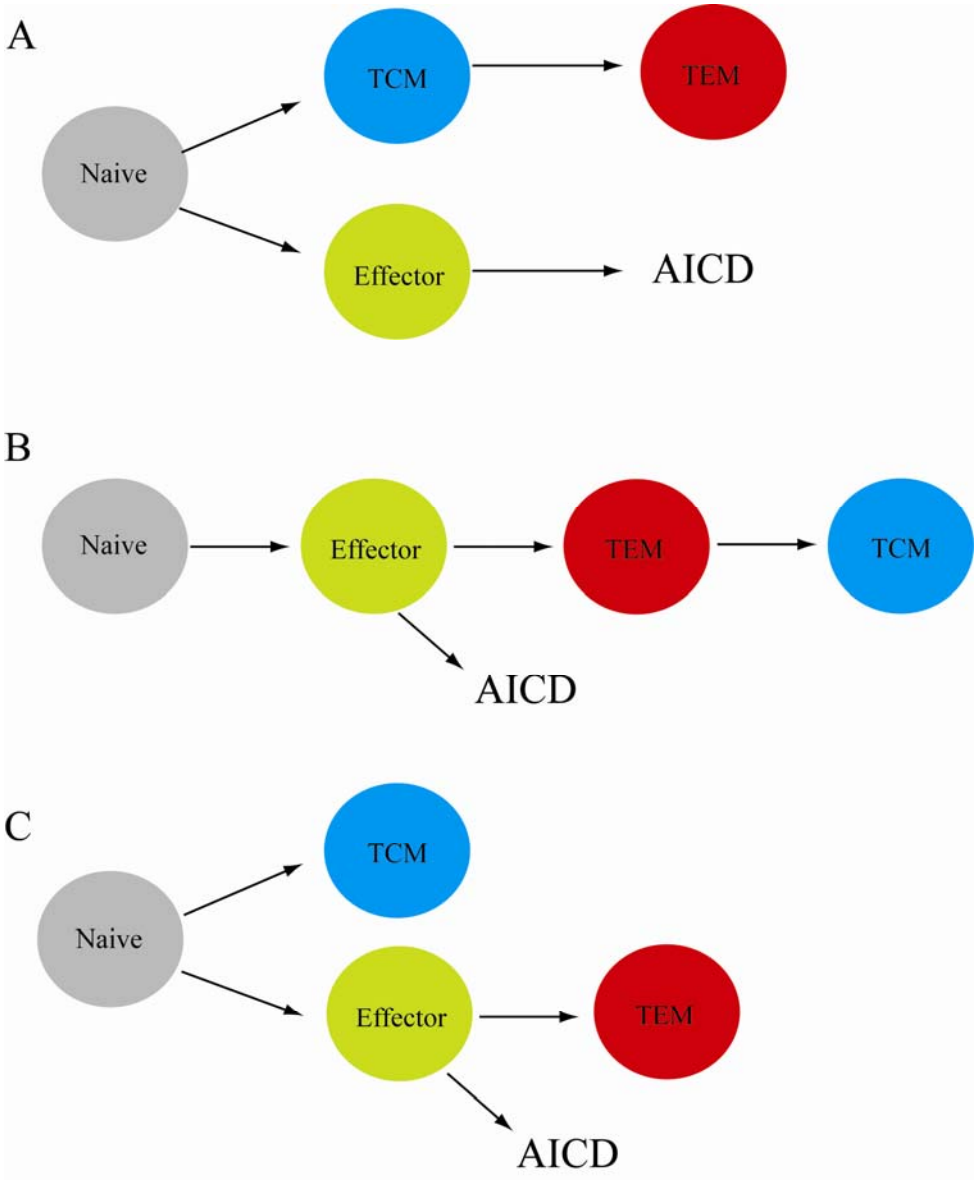

Figure 4. Models for T lymphocyte Lineage Development. (**A**). Naïve cells give rise to immediate effectors which undergo AICD or to T_{CM} cells which repopulate T_{EM} cells. (**B**) Memory cells develop in a linear manner from naïve to effector to T_{EM} to T_{CM}. (**C**) Two distinct lineages of cells arise during priming, effectors which give rise to T_{EM} or a distinct T_{CM} lineage.

CHAPTER THREE

MATERIALS AND METHODS

Human subjects

120 ml of peripheral blood was collected by venipuncture from healthy adult donors. Informed consent was obtained from each donor in accordance with guidelines established by the Internal Review Board (University of Texas Southwestern Medical Center, Dallas, TX).

Mice

DO11.10 TCR transgenic mice were housed in specific pathogen-free conditions in accordance with guidelines established by the Institutional Animal Care and Use Committee (UT Southwestern Medical Center). Spleen tissues from Ubp43-deficient and wild-type FVB mice were a generous gift from Dr. Dong-Er Zhang (Scripps Institute, La Jolla, CA).

Cell Lines

The human monocytic lymphoma, THP-1 cell line was purchased from the American Type Culture Collection (Manassas, VA). The Phoenix-amphotropic

and emphotropic cell lines used for retroviral packaging have been previously described (198) and were purchased from Orbigen, (San Diego, CA).

Cytokines, antibodies, and reagents for $CD4^+$ and $CD8^+$ T cell analysis

Cytokines: Recombinant human IL-12, IFN-α(A), IFN-γ and recombinant murine IL-12 were purchased from R&D Systems (Minneapolis, MN). Recombinant universal IFN-αA/D was purchased from PBL laboratories (Piscataway, NJ). Recombinant human IL-2 was a generous gift from M. Bennett (UT Southwestern Med. Center). Recombinant human IL-18 was purchased from Biosource (Camarillo, CA) and recombinant human IFN-β1a was generous gift from Dr. M. Racke (University of Ohio). The recombinant human cytokines IL-18 and IFN-B1a were solely used in human $CD4^+$ T cell studies.

Antibodies (cellular activation): Activating anti-human CD3 (clone, OKT3) and anti-human CD28 (clone, 37.51) were purchased from Bio Legend (San Diego, CA). Neutralizing anti-human IL-4 was purchased from R&D Systems (Minneapolis, MN). Neutralizing anti-human IFNAR2 was purchased from PBL laboratories (Piscataway, NJ) or Calbiochem (La Jolla, CA). Neutralizing anti-human IL-12 (clone, 20C2), anti-human IFN-γ (clone, 4SB3), and anti-mouse IL-12 (clone, TOSH), anti-mouse IFN-γ (clone, R46A2) and anti-mouse IL-4 (clone, 11B11) were generated in house.

Antibodies (flow-cytometric analysis): The Allophycocyanin (APC)-conjugated anti-human (h)CCR7 antibody was purchased from R&D Systems (Minneapolis, MN). The Fluorescein Isothiocyanate (FITC)-conjugated, anti-hIFN-γ , Pacific-Blue-conjugated, anti-hCD45RA, phycoerythrin (PE)- and tricolor (TC)-conjugated, anti-hCD4, anti-murine-CD4, and anti-murine-IFN-γ antibodies were purchased from Caltag Laboratories (Burlingame, CA). The APC-conjugated, anti-hTNF-α, PE-Cy5-conjugated, anti-hCD8, PE conjugated, anti-hPerforin, PE and APC-Cy7-conjugated, anti-hCD27, and AlexaFluor 700-conjugated, anti-hIL-2 antibodies were purchased from BioLegend (San Diego, CA). The FITC-conjugated, anti-hCD45RA, PE-conjugated, anti-hIL-12 receptor (IL-12Rβ2), PE-conjugated anti-hCXCR3, FITC-conjugated, anti-hPerforin, Alexafluor-700-conjugate, anti-hGranzymeB, PE-conjugated, anti-hCD70 and PE-Cy7-conjugated, anti-hIFN-γ antibodies were purchased from BD Pharmingen (San Diego, CA). The PE-conjugated, anti-hIL-15 receptor (IL-15Rα) and PE-Cy5-conjugated anti-hIL-7 receptor (IL-7Rα) and PE-Cy7 conjugated anti-hCD28 antibodies were purchased from eBioscience (San Diego, CA). The polyclonal rabbit α-hSTAT4, α-hSTAT1, and α-hT-bet antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The polyclonal rabbit-anti-human Eomesodermin antibody was purchased from Abcam (Cambridge, MA). Anti-phospho-STAT4 was purchased from Zymed (San Francisco, CA), and anti-

phospho-STAT1 was purchased from Upstate (Lake Placid, NY). The unconjugated, anti-human Lamin antibody was purchased from Cell Signaling Technology (Danvers, MA). The biotin conjugated, goat anti-rabbit Immunoglobulin antiserum FAB was purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA).

Reagents: The Horseradish peroxidase (HRP)-conjugated goat α-rabbit immunoglobulin antiserum was purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). Streptavidin (SA)-conjugated Qdot655 was purchased from Invitrogen (Carlsbad, CAN). SA-PerCP, Annexin V-FITC and 7-amino-actinomycin D (7AAD) were purchased from BD Pharmingen (San Diego, CA). Carboxyfluorescein diacetate succinimidyl ester (CFSE) was purchased from Sigma-Aldrich (St. Louis, MO) and Pacific-blue succinimidyl ester (PBSE) was purchased from Invitrogen (Carlsbad, CA). Phytohemagglutin (PHA) was purchased from Calbiochem (La Jolla, CA). Phorbol-12-myristate-13-acetate (PMA) was purchased from (A.G. Scientific, Inc. San Diego, CA) and ionomycin from (Sigma-Aldrich St. Louis, MO). Brefeldin A, was purchased from Epicentre, (Madison, WI).

Isolation and activation of hPBMCs and naïve $CD4^+$ T cells from peripheral blood

Human peripheral blood mononuclear cells (hPBMCs) were isolated by ficol density centrifugation using Lymphocyte Separation Media (Cellgro, Manassas, VA) and cultured in complete Iscove's Modified Dubelcco's Medium (HyClone, Logan, UT) supplemented with 1 mM sodium pyruvate (Hyclone), 2 mM L-glutamine (Hyclone), 10 U/ml penicillin (Hyclone), 10 μg/ml streptomycin (Hyclone), 50 μM β-mercaptoethanol (Bio-Rad Laboratories, Hercules, CA), non-essential amino acids (Hyclone), and 10% fetal bovine serum (FBS) (Valley Biomedical, Inc., Winchester, VA), (cIMDM). hPBMCs utilized for purification of naïve cells were stained with anti-human CD45RA-FITC and either anti-human CD4-PE or anti-human CD4-TC. Cells were sorted on a MoFlo cytometer (Dako Cytomation, Fort Collins, CO) to >93% purity. Both hPBMCs and naïve $CD4^+$ T cells were activated for 3 days at 1-5 x 10^6 cells/ml in cIMDM containing 50-100 U/ml rhIL-2 using either 1 μg/ml PHA (Calbiochem) for hPBMCs, or culture plates coated with 5 μg/ml anti-CD3 (clone, OKT3) and 5 μg/ml anti-CD28 (clone, 37.51) for purified T cells. Cytokines and neutralizing anti-cytokine antibodies were added at the following concentrations, unless otherwise indicated in the figure legends: rhIL-12, 10 ng/ml; rhIFN-αA, 1000 U/ml; rhIFN-γ, 10 ng/ml; anti-human IL-12 (clone, 20C2), 5 μg/ml; anti-human IFNAR2, 2-5 μg/ml;

anti-human IFN-γ (clone, 4SB3), 5 μg/ml; anti-human IL-4, 5 μg/ml. Cell were polarized under the following conditions: "Neutralized" (anti-IL-4, anti-IL-12, anti-IFNAR2, and anti-IFN-γ) or "IL-12 alone" (rhIL-12 (10 ng/ml), anti-IL-4, anti-IFNAR2, and anti-IFN-γ), or "IFN-α alone" (rhIFN-αA (1000 U/ml), anti-IL-4, anti-IL-12, and anti-IFN-γ), or "IL-12 + IFN-α" (rhIL-12, rhIFN-αA, anti-IL-4, and anti-IFN-γ). On day 3, cells were split into cIMDM containing 50U/ml rhIL-2 and rested to day 7. Restimulation was performed using either 0.8 μg/ml PMA (A.G. Scientific) + 1 μM ionomycin (Sigma-Aldrich) or 96 well plates coated with 5 μg/ml anti-CD3 (OKT3).

Isolation and activation of human $CD8^+$ T cells from peripheral blood

Human peripheral blood mononuclear cells (hPBMCs) were isolated by Ficol density centrifugation and cultured in cIMDM as described above for $CD4^+$ T cells. $CD8^+CD45RA^+$ cells were either sorted via flow cytometry or isolated by magnetic bead enrichment. For cell sorting, hPBMCs were stained with anti-human CD45RA-FITC and either anti-human CD8-PE or anti-human CD8-PE-Cy5 and sorted on a MoFLo cytometer (DakoCytomation) to greater than 90% purity. Alternatively for sorting of $CD27^+$ cells, hPBMCs were stained with anti-human CD45RA-FITC, anti-human CD8-PE and anti-human CD27-APC-Cy7

and cells were sorted as $CD8^+CD45RA^+CD27^+$ or $CD8^+CD45RA^+CD27^-$. For magnetic bead isolation, $CD8^+$ $CD45RA^+$ cells were negatively selected from hPBMCs using a human naïve $CD8^+$ enrichment set (BD Bioscience) following the manufacturer's instructions with purities greater than 90%. Purified $CD8^+CD45RA^+$ cells were cultured at $0.5\text{-}1\times10^6$ cells/ml on anti-CD3/anti-CD28-coated plates (1.5-5μg/ml as indicated in the figure legends) in cIMDM with IL-2 (50-200 U/ml) under polarizing cytokine conditions using cytokine and antibody concentrations as described for $CD4^+$ T cells activation. $CD8^+$ T cells were polarized in groups as follows: "Neutralized" (anti-IL-4, anti-IL-12, anti-IFNAR2, and anti-IFN-γ), IL-12 alone (rhIL-12 (5-10 ng/ml), anti-IL-4, anti-IFNAR2, and anti-IFN-γ), IFN-α alone (rhIFN-αA (1000 U/ml), anti-IL-4, anti-IL-12, and anti-IFN-γ), or IL-12 + IFN-α (rhIL-12, rhIFN-αA, anti-IL-4, and anti-IFN-γ). On day 3 of culture, cells were harvested for immediate analysis or split 1:10 in fresh media supplemented with 50-100 U/ml rh-IL-2 and cultured to day 7. At day 7, restimulation was performed as described above for $CD4^+$ T cells.

Isolation and activation of murine splenocytes

Whole spleens from WT FVB mice or $UBP43^{-/-}$ mice were harvested and shipped on ice by Dr. Dong-Er Zhang (Scripps Institute, La Jolla, CA). Leukocytes were isolated from spleen through gentle grinding, followed by lysis

of red blood cells. Cells were cultured in cIMDM on wells of 96 well plate pre-coated with anti-mouse CD3 (clone 2C11) at 2.5μg/ml in the presence of polarizing cytokines as follows: "Neutralized" (anti-mIL-4 (clone 11B11), anti-IL-12 (TOSH), and anti-IFN-γ (R46A2)) or "IL-12 alone" (rmIL-12 (10 ng/ml), anti-mIL-4, and anti-mIFN-γ), or "IFN-α alone" (rm/hIFN-α(A/D) (1000 U/ml), anti-mIL-4, anti-mIL-12, and anti-mIFN-γ), or "IL-12 + IFN-α" (rmIL-12, rm/hIFN-αA/D, anti-mIL-4, and anti-mIFN-γ). On day 3, cells were either harvested for RNA extraction or split into cIMDM containing 50 U/ml rhIL-2 and rested to day 7. Restimulation was performed using either 0.8 μg/ml PMA)(A.G. Scientific) + 1 μM ionomycin (Sigma-Aldrich) or plates coated with 5 μg/ml anti-CD3 (OKT3).

Flow cytometric analysis

Naïve human $CD4^+$ T cells were polarized for 7 days or naïve $CD8^+$ T cells were polarized for either 3, 5 or 7 days as described above. On day 3, 5 or 7, cells were washed and rested overnight at 2 x 10^6 cells/ml (for $CD4^+$ T cells) or at 1 x 10^6 cells/ml (for $CD8^+$ T cells) in cIMDM without IL-2. Cells were left unstimulated or were restimulated for 4 hours at 37°C, 5% CO_2 with 0.8 μg/ml PMA + 1 μM ionomycin in the presence of 1 μg/ml Brefeldin A. Intracellular

staining was performed as per the following protocol: Cells were harvested and washed in phosphate buffered saline (PBS) (Hyclone) followed by fixation for 20 minutes at room temperature in PBS containing 5% formalin (Mallinckrodt, Hazelwood, MO). Cells were then washed extensively and allowed to permeabilize for 10 minutes at 25^{o}C in PBS containing 0.5% bovine serum albumin (BSA) (Sigma-Aldrich) and 0.1% saponin (Sigma-Aldrich). Cells were labeled for 20 minutes at room temperature with fluorescently conjugated antibodies to surface or intracellular proteins as described in the figure legends, followed by washing in PBS supplemented with 0.5% BSA and 0.1% Saponin. Cells were resuspended at 1-2×10^{6} cells/ml in PBS supplement with 0.5% BSA and analyzed by flow cytometry on a FACScan, a FACSCalibur or LSRII cytometer (BD Bioscience). The data was assessed using FlowJo software (Treestar, Inc).

CD4^{+} Th1 effector function assays: Cells were stimulated with either PMA + ionomycin, 10 ng/ml rhIL-12, 1000 U/ml IFN-αA, 50 ng/ml IL-18, or a combination of IL-12/IL-18 or IFN-αA/IL-18. After stimulation, cells were harvested and intracellular cytokine staining for IFN-γ was performed as described above.

Analysis of Intracellular T-bet and Eomesodermin: Day 7 polarized cells were stained under intracellular staining conditions with the following modifications. Rested cells were split into two parallel pools and were fixed and

permeablized and stained in three layers. Permeabilized cells were first stained with either un-conjugated rabbit anti-human T-bet (Santa Cruz) or rabbit anti-human Eomes (Abcam), followed by a biotinylated goat anti-rabbit Immunoglobulin FAB secondary (Jackson) and either a SA-conjugate to PerCP (BD Pharmingen) or Q-dot655 (Invitrogen). Cells events were collected on an LSRII and analyzed using FloJo software as described above.

Analysis of cell survival: For analysis of apoptosis, unstimulated cells were stained in the absence of saponin, with 7-AAD and FITC-conjugated Annexin V (BD Pharmingen) in cIMDM containing 10% FBS. Cells were collected on an LSRII and analyzed as described above using FloJo software.

Intracellular phospho-STAT1 and STAT4 staining

.

Whole STAT1 and STAT4 and tyrosine phosphorylated STAT1 and STAT4 were detected by intracellular staining as follows: Freshly isolated PBMCs ($CD4^+$ T cell experiments) or Day 6 CFSE labeled, IL-12+IFN-α polarized $CD8^+$ T cells ($CD8^+$ T cell experiments) were activated with IL-12 or IFN-α for 30 min as indicated in the text , harvested and then incubated in 5% formalin/PBS followed by fixation in cold 100% methanol. Following fixation, cells were washed extensively in staining buffer (PBS, 0.5% BSA, and 1 mM $NaVO_4$) followed by permeabilization in staining buffer containing 0.1% saponin.

For $CD4^+$ T cell experiments, cells were stained with 0.5 μg of anti-STAT4 (SC-486, Santa Cruz) or with anti-P-tyr STAT4 (Zymed). For $CD8^+$ T cell experiments, cells were stained with 0.5 μg of either anti-STAT4 (SC-486, Santa Cruz) or anti-hSTAT1 (Santa Cruz Biotechnology) or with anti-P-tyr STAT4 (Zymed) or anti-phospho-STAT1 (Upstate). A goat-anti-mouse Ig-biotin (Jackson Immunoresearch) was used for secondary detection followed by staining with streptavidin-PE-Cy5 for $CD4^+$ T cell experiments or streptavidin-PerCP for $CD8^+$ T cell experiments. PBMCs were also stained with anti-hCD4-PE and anti-hCD45RA-FITC. $CD8^+$ T cells were assessed for division by CFSE dilution in addition to STAT staining. For $CD4^+$ T cell experiments, cells were collected on a FACScan (Becton Dickinson), and the data were processed through CellQuest software. For $CD8^+$ T cell experiments, cells were collected on an LSRII (BD Biosciences) and analyzed using FloJo Software.

Analysis of cytoplasmic and nuclear STAT4

Human PBMCs activated with IL-12 or IFN-α for various times were stained for CD4, CD45RA and P-tyr-STAT4 as described above and counterstained with 20 ng/ml propidium iodide. Alternatively, the biotinylated P-tyr-STAT4 was stained with streptavidin FITC and the nucleus counterstained with 5 μM DRAQ5™ (Axxora, San Diego, CA). These cells were also stained

with anti-hCD4-PE. Image files of 5000 to 10,000 events were collected for each sample using the ImageStream® imaging flow cytometer (Amnis Corp., Seattle, WA) and analyzed using IDEAS® software (Amnis). In-focus single cells were identified by gating on propidium iodide or DRAQ5 positive events with high nuclear aspect ratios (minor to major axis ratio, a measure of circularity) and high nuclear contrast (as measured by the Gradient Max feature). $CD4^+CD45RA^+$ lymphocytes (low SSC / low area cells) were then gated. Nuclear localization of P-tyr-STAT4 within these cells was measured using the Similarity score, which quantifies the correlation of pixel values of the nuclear and P-tyr-STAT4 images on a per-cell basis (199). If the transcription factor is nuclear, the two images will be similar and have large positive values. If it is cytoplasmic, the two images will be anti-similar and have large negative values. Events with positive values greater than 1 had visually apparent nuclear distributions of transcription factor, and were gated to quantify the percentage of cells within the $CD4^+CD45RA^+$ lymphocyte population with nuclear-localized P-Y-STAT

Analysis of Cell Division by CFSE and PBSE Dilution

For analysis of cell division, cells were resuspended at 1×10^7 cells/ml in PBS and treated with 1.25μM carboxyfluorescein diacetate succinimidyl ester (CFSE), or 12μg/ml Pacific-Blue succinimidyl ester (PBSE) for 10 min at room

temperature. Following treatment, cells were washed 1 times with cIMDM containing 20% FBS and two times with cIMDM containing 10% FBS. For primary activations, freshly purified $CD8^+$ $CD45RA^+$ T cells were suspended at 1×10^6 cells/ml in cIMDM and plated on anti-hCD3 and anti-hCD28 coated 96 well microtiter plates under polarizing conditions as described above. Cells were assessed for CFSE/PBSE dilution at either d3, d5 or d7 post activation. For secondary activation, day 7 polarized cells were resuspended at $3\text{-}5\times10^5$ cells/ml in cIMDM under either neutralizing or IL-12 +/- IFN-α polarizing conditions and examined for functional secondary responses at either day3 or day5 post activation. All data were collected on an LSRII flow cytometer (BD) and analyzed using FloJo software.

Live cell chemokine receptor and CFSE Sorting.

For day 7 chemokine receptor sorting experiments, $CD8^+$ $CD45RA^+$ sorted cells were polarized for 7 days under the "IL-12+IFN-α" condition either with primary activation (1.5-2.5μg/ml anti-human CD3 and anti-human CD28) or left as unstimulated (no TCR) controls. Day 7 cells were harvested and rested overnight in the absence of IL-2. Following rest, cells were labeled with either CFSE or PBSE as described above or stained with antibodies to human CCR7 and CXCR3. Stained cells were sorted using a FACS Aria (BD Biosciences) for

either $CCR7^{hi}$ (T_{CM}) or $CXCR3^{hi}$(T_{EM}) or $CFSE^{hi}$ (T_{CM}) or $CFSE^{lo}$ (T_{EM}) to <90% purity. Chemokine receptor sorted cells were either rested overnight and subjected to re-directed lysis as described above or were re-activated on wells of 96 well plates coated with 1.5μg/ml anti-human CD3 (OKT3) in either "neutralizing" or "IL-12+IFN-α" conditions for day 3 proliferation, phenotypic analysis and re-directed lysis assays. RNA was harvested from CFSE or PBSE sorted cells for use in an Illumina Bead array analysis.

Cytometric Bead Array and ELISA analyses of human cytokines

For human $CD4^+$ analysis, naïve human $CD4^+$ T cells were activated for 7 days as described above, and were then restimulated at 5 x 10^5 cells/ml for 24 hours on anti-CD3 (clone, OKT3)-coated plates (5μg/ml). Supernatants were analyzed for IL-4, IL-5, and IFN-γ cytokine concentrations by either cytometric bead arrays (BD Pharmingen) or ELISAs (eBioscience, San Diego, CA) according to the manufacturer's protocols.

For human $CD8^+$ analysis, naïve human $CD8^+$ T cells were activated for 7 days as described above, and were then restimulated at 1 x 10^6 cells/ml for 24 hours on anti-CD3 (clone, OKT3)-coated plates (2.5 μg/ml). Supernatants were harvested at 24hrs post activation and assessed for cytokine concentration using a Biolegend ELISA kit via the manufacturer's protocol.

Immunoprecipitation and immunoblotting

Human PBMCs were stimulated and expanded for two consecutive weeks with PHA under Th1 conditions (10 ng/ml rhIL-12). On day 14, cells were washed in cIMDM and rested at 1 x 10^7 cells/ml for 30 minutes at 37^{o}C, 5% CO_2. Cells were stimulated with either 10 ng/ml rhIL-12 or 1000 U/ml rhIFN-αA for 30min, 1hr, 3hr, 6hr or 24hrs at 37^{o}C, 5% CO_2. Lysis was performed for 1 hour at 4^{o}C in radioimmune precipitation assay (RIPA) buffer containing proteinase inhibitors (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% sodium dodecyl sulfate (SDS), 0.5% sodium deoxycholate, 1% Tween-20, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM sodium pervanadate ($NaVO_4$), 1 mM dithiothreitol (DTT), 10 μg/ml leupeptin). Samples were pre-cleared using rabbit pre-immune serum, and immunoprecipitation of human STAT4 was performed using 3 μg per sample rabbit anti-human STAT4 bound to Protein G Sepharose beads (Amersham Biosciences, Uppsala, Sweden). Immunoprecipitates were resolved by 7% SDS-PAGE and transferred to PVDF membranes (Bio-Rad Laboratories, Hercules, CA). Western blotting was performed using rabbit anti-phosphorylated STAT4 with an HRP-conjugated goat anti-rabbit secondary antibody. Detection was performed using ECL Detection Reagents followed by exposure to Hyperfilm ECL (Amersham Biosciences). The membranes were then stripped and reprobed

with anti-human STAT4 polyclonal antiserum followed by HRP-conjugated goat anti-rabbit immunoglobulin.

^{51}Cr Release by Re-directed lysis

Day 7, cytokine polarized $CD8^+$ T cells and target THP-1 monocytes were harvested, counted, and rested overnight at 1×10^6 cells/ml in the absence of IL-2. After overnight rest, THP-1 cells were cultured in the presence of 150μCi ^{51}Cr ($Na_2(Cr0_4)$) for 90 minutes. Following incubation, THP-1 cells were either left untreated or coated with anti-hCD3 (OKT3) at 1.5μg/ml. Coated and ^{51}Cr labeled THP-1 cells were incubated with polarized $CD8^+$ $CD45RA^+$ T cells at various effector:target (E:T) ratios for 4hrs at 37°C and CTL activity was assessed by β radiation emission in harvested supernatants quantified on a scintillation counter.

Retrovirus cDNA expression constructs

The full-length murine IFNAR2 cDNA was amplified from a cDNA clone (Clone ID: 4187603, Invitrogen) with primers: 5'- AAAAAGATCTAGCTGAGCAG GATGCGTTCACGG and 5'-AAAAAGATCTCTTTGGAGTCATCTCATGAT GTAGCC. The PCR product was digested with BglII and clone into the BamHI site of GFPRV.

A full-length human T-bet cDNA clone (Accession #BC039739) was purchased from ATCC (Manassas, VA). The open reading frame of this cDNA clone was amplified by PCR with primers: 5'-AAAACTCGAGCCCGGATGGGCATC GTGGAG and 5'-AAAACTCGAGCTGCTCAGTTGGGAAAATAGTTAT AAAACTGTCC. The PCR product was digested with XhoI and ligated into the XhoI site within the retrovirus vector GFPRV (200), and all constructs were confirmed by DNA sequencing. The full-length human Eomesodermin cDNA clone (Accession # BC037568) was purchased from ATCC (Manassas, VA). The open reading frame of this cDNA clone was amplified by PCR with primers containing exogenous XhoI sites (5'-CTCGAG-3'): 5'- AAAACTCGAG-AAAGCATGCAGTTAGGGGAGCAGCTC-3' and 5'-AAAACTCGAGTCTCT TAGGGAGTGTGTAAAAAGCATAATACC-3'. The ensuing product was digested with XhoI and ligated within the retrovirus vector GFPRV.

Retroviral transduction of primary naïve human $CD4^+$ and $CD8^+$ T cells

The GFPRV (200) and T-bet-GFPRV (100) retroviral expression constructs have been described. Retroviral supernatants were generated by calcium chloride transfection of the Phoenix amphotropic (Pha) packaging cell line (Orbigen, San Diego, CA) as previously described (198). PhA cells were cultured in Dubelcco's Modified Eagle Medium (Hyclone) supplemented with 1

mM sodium pyruvate, 2 mM L-glutamine, 10 U/ml penicillin, 10 μg/ml streptomycin, 0.015% sodium bicarbonate ($NaHCO_3$), non-essential amino acids, and 10% FBS (cDMEM). Prior to transfection, cells were washed and cultured for 15 min. in fresh cDMEM supplemented with 25 μM chloroquine. For transfection, a mastermix containing 25-30 μg plasmid DNA suspended in a solution of 0.244 M calcium chloride ($CaCl_2$) was mixed 1:1 with 2 X HBS (50 mM HEPES, pH 7.05, 10 mM KCl, 280 mM NaCl, 12 mM dextrose, 1.5 mM Na_2HPO_4) for 10-15 seconds and then added dropwise with shaking to PhA cells. Media was replaced every 8 hrs for 16 hours. After, 16hrs, cells were incubated at 32^{o}C, and retroviral supernatants were harvested at 24 hour intervals and stored at -80^{o}C.

Naïve $CD4^+CD45RA^+$ or $CD8^+CD45RA^+$ human T cells were purified by cell sorting as described above. Purified cells were activated with plate-bound anti-CD3/anti-CD28 in complete medium with 600 U/ml hIL-2 for 24 hours. The cells were subjected to two rounds of retroviral transduction by spinning the cells at 1000 x *g* for 90 min in the presence of retroviral supernatants containing 5 μg/ml polybrene. 24 hours after the first round of retroviral transduction, cytokines or anti-cytokine antibodies were added to the cultures as indicated in the text and figure legends. Cells were expanded on day 7 by restimulating the cells on anti-CD3 coated plates or harvested and sorted for GFP^+ cells for direct

assessment of phenotype. Expanded cells were rested to day 14 prior to intracellular cytokine analysis.

Retroviral transduction was performed in murine $CD4^+$ T cells as previously described (198). Briefly, infectious retrovirus supernatants were generated in the Phoenix-ectropic packaging cell line and transfected with retroviral plasmids using the Calcium chloride method described above. D011.10 splenic T cells were transduced with retrovirus supernatants supplemented with 2μg/ml protamine sulfate and 10ng/ml IL-12 to generate polarized Th1 cells. Transduced T cells were purified on day 7 after activation by flow cytometric sorting based on GFP expression. Purified cells were expanded by restimulation with OVA peptide and irradiated BALB/c splenocytes.

Assessment of gene expression by Quantitative real-time polymerase chain reaction (qPCR) analysis

Total RNA was harvested from day3 or day7 polarized $CD8^+$ $CD45RA^+$ cells using the RNeasy Mini kit (Qiagen) according to the manufacturer's instructions. Samples were subjected to dNaseI (Qiagen) digestion for 15 min at 25°C followed by reverse-transcription to cDNA at 42°C for 1hr. cDNA and was then subjected to qPCR with SYBR Green Master Mix (Stratagene) on an ABI7300 real-time thermocycler (Applied Bisosystems). Relative changes in

expression of mRNA were calculated by the $2^{-\Delta\Delta CT}$ method (201), and treatment groups were referenced to a neutralized control. hGAPDH was used as a reference gene for mRNA expression. The primer sets used to detect expression were purchased from Integrated DNA Technologies (Coralville, IA) and are a listed in table 1.

Statistical analysis

Statistical analysis was performed either by Two-tailed Students T-Test for $CD4^+$ T cell experiments or by one-way and two-way ANOVA for $CD8^+$ T cell experiments using GraphPad Prism software, version 5.00 for Windows (GraphPad Software, San Diego, CA). Values of $p < 0.05$ were taken as significant.

hIFN-γ	For	5’-TGATTACAAGGCTTTATCTCAGGG-3’
	Rev	5’-GGCAGTAACTGGATAGTATCACTTCAC-3’
hIL-12Rβ2	For	5’-ACCTTCCCACCCATGATGGC-3’
	Rev	5’-GAAAACAGAAAGGGAGATGTGCTG-3’
hT-bet	For	5’-CCTCTCCTACCCAACCAGTATCCT-3’
	Rev	5’-ATGCAGGCTTCATGCTGACTG-3’
hEomes	For	5’- GTCTCCTAATACTGGTCCCCACTGG-3’
	Rev	5’-CCACGCCATCCTCTGTAACTTC-3’
hSOCS-1	For	5’-AATGCAGTCTCCACAGCAGCAGA-3’
	Rev	5’-TAATCGGCGTGCGAACGGAATGT-3’
hGAPDH	For	5’-CTGCACCACCAACTGCTTAGCA-3’
	Rev	5’-TGATGTTCTGGAGAGCCCCG-3’
mT-bet	For	5’-AACCAGTATCCTGTTCCCAGC-3’
	Rev	5’-TGTCGCCACTGGAAGGATAG-3’
mIFN-γ	For	5’-TCAAGTGGCATAGATGTGGAAGAA-3’
	Rev	5’-TGGCTCTGCAGGATTTTCATG-3’
mHPRT	For	5’-TTGCAAGCTTGCTGGTGAAA-3’
	Rev	5’-GTGATTCAAATCCCTGAAGTACTAA-3’

Table 1. qRT-PCR Primer sets

CHAPTER FOUR

IFN-α IS NOT SUFFICIENT TO DRIVE TH1 DEVELOPMENT DUE TO LACK OF STABLE T-BET EXPRESSION

The following study has been published in the *Journal of Immunology*, volume 179, pages 3792-3803 (100) and is reproduced with the permission of the *Journal of Immunology*. This work represents an equal collaboration between Hilario J. Ramos and Ann M. Davis. Unless otherwise indicated in the figure legends, experiments were carried out by Hilario J. Ramos.

Introduction

Innate cytokines play important roles in bridging innate and adaptive immunity. They do so by acting coordinately with activation through the TCR as well as costimulatory pathways to program the development of lymphocyte responses that are both robust and specific to the invading pathogens (28, 139). During intracellular infection multiple cytokines are secreted by activated DCs (4, 5). Importantly, these include IL-12 and IFN-α/β which have been shown to act both in an innate manner as well as to provide instructive cues for adaptive T cell

responses (55). Although, these cytokines are thought to promote similar T lymphocyte responses during infection, several observations derived from mouse and human studies suggest that this might not be the case. Therefore in this study I wished to assess the direct roles of these cytokines on the development of human $CD4^+$ Th1 development.

A role for IL-12 in promoting Th1 development has been well established(5). IL-12 signaling promotes the phosphorylation of signal-transducer and activator of transcription-4 (STAT4) (102, 142) that is required for Th1 commitment (104). Further, IL-12-dependent STAT4 activation is conserved across species, and disruptive mutations in this signaling pathway significantly impair Th1 responses in both mice (103, 104) and humans (143). Likewise, early reports demonstrated a potential role for IFN-α/β in promoting Th1 development in human peripheral blood mononuclear cells (PBMCs) (150, 151). These initial studies were followed by findings that IFN-α, like IL-12, could promote STAT4 activation in human lymphocytes (30, 101, 154). These early reports concluded that IFN-α/β could directly regulate Th1 commitment in $CD4^+$ T cells through the activation of STAT4 (202).

However, unlike IL-12, the role of IFN-α/β in promoting Th1 development has been met with considerable controversy. Initial reports demonstrating IFN-α/β-dependent STAT4 activation and Th1 development were performed with human PBMCs or with human T and NK cell lines (101, 150,

151). In parallel studies with murine T cells, IFN-α/β was insufficient to promote Th1 development (30, 155) or to activate STAT4 when compared to human $CD4^+$ T cells (30, 154), indicating a potential species-specific role for IFN-α/β in regulating Th1 development. Further, human STAT2 was implicated in facilitating STAT4 recruitment to the human IFN-α/β receptor (IFNAR) complex in a species-specific manner (154, 156). These findings seemed to explain the presumed ability of IFN-α/β to promote STAT4 activation and Th1 development in human, but not murine $CD4^+$ T cells. However, $CD4^+$ T cells from STAT2 knock-in mice that expressed a humanized *Stat2* gene failed to exhibit either STAT4 phosphorylation or Th1 commitment in response to IFN-α/β activation (157). These results suggested that although human STAT2 was required for STAT4 activation in human cells, it was not sufficient to restore this pathway when expressed in the context of the murine IFNAR.

Recent studies have called this species-specific pathway into question (68, 158). Biron and colleagues demonstrated that IFN-α/β could promote STAT4 phosphorylation in murine T cells, although this effect was more pronounced in $CD8^+$ than in $CD4^+$ T cells (158). A further examination comparing various subtypes of IFN-α demonstrated that although mIFN-αA indeed could induce weak STAT4 phosphorylation, it was insufficient to drive IFN-γ production and Th1 development in murine $CD4^+$ T cells (203). In a related study, Hilkens and

colleagues demonstrated that even in human T cells, IFN-α/β-driven STAT4 activation was attenuated compared to the effects of IL-12 (160). This study suggested that IL-12 was more efficient than IFN-α/β at promoting Th1 development in human $CD4^+$ T cells. One explanation was that IL-12 stimulation was able to maintain STAT4 phosphorylation over a longer period of time compared to IFN-α/β. We have recently confirmed this observation in murine $CD4^+$ T cells (159), however a molecular explanation for this difference in signaling between IFN-α and IL-12 has not been examined. At the core of this issue is whether IL-12 and IFN-α/β share redundant roles in T helper commitment, and whether there truly is a species-specific pathway that operates in human but not murine T cells.

In this study, we have addressed this central issue by comparing the ability of IL-12 and IFN-α/β to direct the commitment of primary human $CD4^+$ T cells toward the Th1 fate. We found that IFN-α does not promote Th1 development from naïve human $CD4^+$ T cells, and we have linked this developmental defect to the inability of IFN-α to induce sustained T-bet expression. Further, we demonstrate that in primary naïve human $CD4^+$ T cells, T-bet expression is sufficient to drive Th1 commitment in a cytokine-independent manner. Thus, in contrast to previous reports, IFN-α/β does not promote Th1 development in the

absence of IL-12 due to the lack of sustained T-bet expression, and this effect is consistent across species.

Results

IFN-α does not promote Th1 development in the absence of IL-12

In order to assess the direct roles of IL-12 and IFN-α/β this study utilized an *in vitro* polarization model in which we could directly control the cytokine environment in which naïve $CD4^+$ T cells were developing. For these experiments, human $CD4^+$/$CD45RA^+$ cells were sorted to >95% purity from peripheral blood obtained from healthy adult donors. Sorted cells were then immediately activated on plate-bound anti-CD3/anti-CD28-coated plates in the presence of either purified cytokines or neutralizing anti-cytokine monoclonal antibodies. I began this study by assessing the ability of IL-12 and IFN-α/β to promote IFN-γ expression and Th1 development. In response to activation with PMA + ionomycin, day 7 polarized T cells expressed varying levels of IFN-γ (Fig. 5). As expected, IL-12 was capable of enhancing IFN-γ expression, and this effect was more pronounced when IL-4 was neutralized during primary stimulation (Fig. 5A). Further, IFN-α did not inhibit the ability of IL-12 to induce

IFN-γ expression either in the absence or presence of neutralizing anti-IL-4 antibody (Fig. 5A). Stimulation with IFN-γ alone did not promote Th1 development, as previously reported in human cells (Fig. 5A) (149, 155). However, in contrast to previous reports (30, 68, 158), IFN-α was also ineffective at promoting Th1 development in the absence of IL-12, and the percentage of cells capable of secreting IFN-γ was less in IFN-α-treated cells compared to neutralizing conditions (Fig. 5A, compare conditions 1 and 2). This response was highly reproducible among donors however we observed heterogeneity in the overall percentages of cells that were capable of secreting IFN-γ from one donor to the next (Fig. 5B). Comparison of 3 separate healthy human donors activated under neutralizing conditions (anti-IL-12 + anti-IFNAR2, "Control"), or with IL-12 + anti-IFNAR2 ("IL-12") or IFN-αA + anti-IL-12 ("IFN-αA") demonstrated that although the percentage of IFN-γ-secreting $CD4^+$ T cells that developed in the absence of IL-12 or IFN-α varied significantly from each of the 3 donors in all cases, IL-12 stimulation increased while IFN-α decreased the percentage of cells capable of secreting IFN-γ compared to cells activated under neutralizing conditions. Further, in studies done by Ann Davis, it was observed that cells primed with IFN-α did not induce IFN-γ mRNA in response to anti-CD3 stimulation (Fig. 6A), therefore indicating a general defect in Th1 commitment both at the protein as well as mRNA level.

It was possible that although IFN-α was incapable of promoting Th1 development it was instead promoting Th2 development. However, examination of this response by Ann Davis demonstrated that similar to Th1 development, IFN-α was not sufficient to promote Th2 development (Fig. 6B). In these experiments, purified naïve human $CD4^+$ T cells were activated under polarizing conditions as described above, and IFN-γ, IL-4 and IL-5 secretion was measured by cytometric bead arrays (Fig. 6B). As observed in figure 5, IL-12 stimulation enhanced IFN-γ secretion and did not promote IL-4 or IL-5 secretion compared to neutralizing conditions (Fig. 6B). As expected, stimulation of cells in the presence of IL-4 inhibited IFN-γ secretion while enhancing IL-4 and IL-5 secretion (Fig. 6B). Although IFN-α did not inhibit the ability of IL-12 to promote IFN-γ secretion in the absence of IL-12 or IL-4, IFN-α did not promote the secretion of either IFN-γ or IL-4 and IL-5 (Fig. 6B). Thus, in the absence of IL-12 or IL-4, IFN-α did not promote either Th1 or Th2 development.

Early reports demonstrating a role for IFN-α in Th1 development used mixed populations of peripheral or cord blood lymphocytes (150, 151). Thus, it was possible that IFN-α was acting indirectly on other populations of cells to promote Th1 development in $CD4^+$ T cells. Therefore, I addressed this issue by attempting to reproduce those early studies. For these experiments, non-fractionated human PBMCs were activated with plate-bound anti-CD3 + anti-

CD28 in the presence of cytokines or neutralizing anti-cytokine antibodies as indicated in Fig.7A. Cells were expanded in culture for 7 days followed by restimulation with PMA and ionomycin in order to assess IFN-γ expression by intracellular cytokine staining. As expected, IL-12 was sufficient to increase the percentage of $CD4^+$ T cells capable of secreting IFN-γ, (Fig. 7A), and the absence or presence of neutralizing anti-IL-4 antibody did not significantly enhance this effect. However, differentiation of human PBMCs in the presence of IFN-α alone was not sufficient to expand IFN-γ-secreting $CD4^+$ T cells compared to neutralizing conditions (Fig. 7A, compare conditions 1 and 2). Although IFN-α failed to enhance Th1 development, it did not inhibit the ability of IL-12 to expand a population of IFN-γ-secreting $CD4^+$ T cells (Fig. 7A).

The differences observed between our data and previous reports could have resulted from our strict control of endogenous IL-12 levels with the use of neutralizing anti-IL-12 antibodies with cells cultured in the presence of IFN-α. In some cases, previous studies did not neutralize IL-12 during the primary activation (150, 151). To address this, human PBMCs were activated in the presence of IL-12 or IFN-α in the absence or presence of neutralizing antibodies for 7 days (Fig. 7B). IFN-γ expression from $CD4^+$ T cells was assessed as described above. Even in the absence of neutralizing antibodies against IL-12, IFN-α alone was still unable to induce Th1 development in $CD4^+$ T cells (Fig 7B,

compare conditions 6 and 7). Therefore unlike previously reported, IFN-α is insufficient to promote Th1 development from either naïve $CD4^+$ T cells or bulk PBMC populations of cells. These observations then suggest that the activity of IFN-α/β in previous hPBMCs studies might be due to indirect action of IL-12 which was not blocked in these studies and in the complete absence of IL-12, IFN-α/β is not sufficient to promte these responses.

The possibility that subtypes of IFN-α/β other that IFN-αA may differentially regulate Th1 commitment was considered. To address this, naïve human $CD4^+$ T cells were activated in parallel cultures with IFN-αB2, IFN-αD, IFN-αA/D, and IFN-β. Consistent with results obtained with IFN-αA (Figs. 5 and 7), the other IFN-α subtypes (Ann davis, data not shown), or IFN-β (Fig. 8A) failed to promote Th1 development. In each case, these cultures were activated with 1000 U/ml of IFN-α/β. Previous reports demonstrating a role for IFN-α/β in murine Th1 development used IFN-α at concentrations ranging from 50,000 – 100,000 U/ml (68, 158). Thus, it was possible that extremely high concentrations of IFN-α/β were required to promote Th1 development. However, I titrated IFN-αA in primary stimulation cultures and found that IFN-αA at concentrations up to 100,000 U/ml was still incapable of driving Th1 development in human $CD4^+$ T cells (Fig. 8B). Studies of *in vitro*-activated murine T cells suggest that repeated stimulation under Th1 polarizing conditions tends to reinforce Th1 commitment

as measured by increased IFN-γ secretion and the inability of these cells to be redirected to the Th2 phenotype (204). However, examination of IFN-γ expression either at the mRNA or protein level in response to two weeks of polarization by IFN-α still revealed a deficiency in its ability to prime Th1 development (data not shown, Ann Davis and Hilario Ramos collaboration). Therefore IFN-α/β is truly insufficient to prime Th1 development in the absence of IL-12.

Cytokine-driven STAT4 phosphorylation and acute IFN-γ expression

IL-12 has been shown to drive Th1 development in $CD4^+$ T cells by efficiently promoting STAT4 tyrosine phosphorylation (102, 205). In addition, IL-12, in synergy with IL-18, induces sustained secretion of IFN-γ from Th1 cells that, in mice, has been shown to be dependent upon STAT4 (152, 153). Thus, IL-12-dependent STAT4 activation plays significant roles in both Th1 development and effector function. Although both IL-12 and IFN-α/β can promote STAT4 phosphorylation in human $CD4^+$ T cells (152, 153), several reports have suggested that either the magnitude or kinetics of IFN-α-induced STAT4 phosphorylation is decreased compared to IL-12 signaling (152, 160). Based on these observations, we wished to determine which aspects of Th1

development and effector function could be directly regulated by IFN-α through STAT4 activation.

First, the kinetics of IL-12- and IFN-α-dependent STAT4 tyrosine phosphorylation were assessed in human peripheral blood mononuclear cells (PBMC) by immunoblotting (Fig. 9A and B). IL-12 stimulation of Th1-polarized PBMCs promoted STAT4 phosphorylation that was sustained up to 24 hours post-stimulation (Fig. 9A, upper panel, lanes 2-7). In contrast, IFN-αA-dependent STAT4 phosphorylation was attenuated (Fig. 9A, upper panel, lanes 8-13), peaking at 0.5-2 hours and extinguished by 6 hours (Fig. 9B, lanes 8-13). In addition, IFN-α-dependent STAT1 phosphorylation was sustained up to 6 hours post-stimulation (Fig. 9A, lower panel, lanes 8-13), indicating that the rate at which STAT4 and STAT1 are dephosphorylated may be regulated through different mechanisms.

We wished to determine whether the kinetics of IFN-α-dependent STAT4 tyrosine phosphorylation differed between fully polarized Th1 cells and uncommitted naïve $CD4^+$ T cell progenitors. To address this, studies by Dr. David Farrar assessed the kinetics of STAT4 tyrosine phosphorylation in naïve $CD4^+$/$CD45RA^+$ T cells within freshly isolated human PBMCs (Fig. 10A through E). For these experiments, both total STAT4 protein and tyrosine phosphorylated (P-Y) STAT4 was measured by intracellular staining from cells activated with either IL-12 or IFN-α between 0-24 hours. The overall expression of STAT4

protein in $CD4^+/CD45RA^+$ cells was not altered by cytokine treatment (Fig. 10A) and remained constant throughout the 24 hour time course (data not shown). In naïve $CD4^+/CD45RA^+$ T cells, IL-12 did not increase the percentage of cells displaying elevated P-Y STAT4 compared to unstimulated cells (Fig. 10B), presumably due to the lack of IL-12Rβ2 expression (149). However, IFN-α stimulation promoted STAT4 phosphorylation in naïve $CD4^+$ T cells (Fig. 10B). Further, consistent with our observations in Th1 cells (Fig. 9A and B), we found that IFN-α-driven STAT4 phosphorylation was rapidly induced by 30 min and then extinguished by 6 hours post-stimulation in naïve $CD4^+$ T cells. Further, the kinetics of IFN-α-dependent STAT4 phosphorylation was similar in the $CD45RA^+$ and $CD45RA^-$ populations (Fig. 10D and E). We also observed that IL-12 promoted STAT4 phosphorylation in approximately 15-20% of the $CD45RA^-$ cells (Fig. 10D) indicating that these cells were responsive to IL-12. Further, IL-12-dependent STAT4 phosphorylation was maintained in this population through the entire 24 hour time course. Taken together, these data indicate that unlike IL-12, IFN-α does not promote sustained STAT4 tyrosine phosphorylation in naïve human $CD4^+$ T cells.

It was possible that the reason IFN-α failed to promote Th1 development was due to lack of sustained STAT4 phosphorylation. However, it was also possible that phosphorylated STAT4 was not efficiently translocated to the nucleus in response to IFN-α. This possibility was addressed by visualizing the

relative nuclear accumulation of phosphorylated STAT4 within naïve human $CD4^+$ T cells (Fig. 11A and B). In collaboration with Dr. Thaddeus George, we utilized a novel technology that combines multiparametric flow cytometry with single cell microscopy. For these experiments, freshly isolated human PBMCs were stimulated with either IL-12 or IFN-α followed by intracellular staining for CD4, CD45RA and P-Y STAT4 as described above. Cells were imaged with the use of the ImageStream® flow cytometer. Single cell images were processed through the live, $CD4^+$ and $CD45RA^+$ gates. Cells that exhibited an increase in phosphorylated STAT4 signal were divided into populations that displayed either P-Y STAT4 cytoplasmic accumulation (Fig. 11 left panel) or nuclear accumulation (Fig. 11A, right panel). As shown in Fig. 11B, approximately 50% of the $CD4^+$/$CD45RA^+$/P-Y $STAT4^+$ cells displayed accumulation of P-Y STAT4 in the nuclei in response to IFN-α. Although the level of P-Y STAT4 was significantly diminished by 6 hours (Fig. 10E), the residual amount of P-Y STAT4 within those cells was still retained within the nucleus (Fig. 11B). Thus, these data suggest that although IFN-α-induced STAT4 phosphorylation was attenuated compared to IL-12, STAT4 nuclear translocation remained intact in naïve human $CD4^+$ T cells.

I next wished to correlate the duration of IFN-α-dependent STAT4 phosphorylation with Th1 effector function. IFN-γ secretion by Th1 cells is regulated independently by both antigen activation and by innate cytokines (206-

208). In the case of innate cytokines, IL-12 synergizes with IL-18 to promote IFN-γ secretion from fully polarized Th1 cells in a STAT4-dependent manner. Although IFN-α + IL-18 stimulation was reported to induce IFN-γ secretion from human T cells (153), we suspected that IFN-α and IL-12 differentially regulated acute IFN-γ secretion due to the altered kinetics of STAT4 activation observed above. To test this possibility, human $CD4^+$ T cells were activated under Th1-inducing conditions (IL-12 + α-IL-4) in order to generate a population of cells capable of responding to IL-12 and secreting IFN-γ upon restimulation with innate cytokines. Human Th1 cells re-stimulated for 4 hours with either IL-12 + IL-18 or IFN-αA + IL-18 secreted IFN-γ to equivalent levels (Fig. 12A and *B*). Stimulation with each cytokine alone failed to promote IFN-γ secretion (Fig. 12B) as previously reported (152, 207). However, if cells were stimulated for 24 hours, the level of IFN-γ secreted into the culture supernatants was significantly lower in response to IFN-α + IL-18 as compared to IL-12 + IL-18 (Fig. 12C). These data correlate with the attenuated activation of STAT4 in response to IFN-α observed above.

Ectopic IFNAR2 expression enhances IFN-α-dependent STAT4 phosphorylation and acute IFN-γ secretion.

Several mechanisms could account for the differential activation of STAT4 by IL-12 versus IFN-α. First, IFN-α signaling may induce the expression of negative regulators of JAK/STAT signaling such as SOCS proteins or Ubp43. To that regard, I first examined the expression of SOCS-1 mRNA in 48hr polarized cells. Examination of SOCS mRNA showed no difference in the induction of SOCS-1 in cells activated with either IL-12 or IFN-α. This was representative of two separate human donors and therefore suggests that the inability for IFN-α/β to promote Th1 development is independent of SOCS-1 expression (Fig. 13). In addition to SOCS proteins, Ubp43 has recently been shown to specifically inhibit JAK kinase activation by the IFNAR (209), and Ubp43-deficient mice display enhanced anti-viral and anti-bacterial responses to IFN-α signaling (210, 211). Therefore we examined STAT4, activation in response to cytokine treatment in Ubp43-deficient mice and found no difference when compared to control (data not shown) Alternatively, I examined the effect of cytokines on expression of T-bet and IFN-γ by RT-PCR in Ubp43-deficient T cells. Here I detected no differences in the expression of these proteins compared to strain-matched wild type control T cells (Fig. 14). Atlhough I did observed that

UBP43 deficiency led to a general increase in IFN-γ under all cytokine treatments (Fig. 14). Therefore deficiency in Ubp43 does not explain the defect in IFN-α/β ability to promote Th1 development.

Previous studies have suggested that acute IFN-α/β signaling negatively regulates expression of the human IFNAR1 subunit, thereby extinguishing downstream signaling events (160). However, Berenson *et. a*l. found that the murine IFNAR1 subunit was stably expressed on murine $CD4^+$ T cells following IFN-α activation (159). This recent study concluded that like in human T cells, STAT4 tyrosine phosphorylation was attenuated in response to IFN-α compared to IL-12. Further, we recently demonstrated that the IFNAR2 subunit plays an important role in STAT4 activation (212). Here, we found that the STAT4 N-domain interacts with the cytoplasmic domain of the IFNAR2 subunit. Based on these collective observations, we wished to determine if ectopic overexpression of either the IFNAR1 or IFNAR2 subunit could restore IFN-α-dependent STAT4 activation in murine T cells. To test this, we expressed the full length murine IFNAR1 and IFNAR2 subunits in murine Th1 cells and tested both the kinetics of STAT4 phosphorylation and acute IFN-γ secretion in response to IFN-α. Retroviral expression of the IFNAR1 did not alter either the kinetics of STAT4 phosphorylation or the secretion of IFN-γ in response to IFN-α + IL-18 stimulation (data not shown). However, as shown in Fig. 15A, we found that

expression of the IFNAR2 subunit increased both the magnitude and duration of IFN-α-dependent STAT4 tyrosine phosphorylation. In contrast, IL-12-induced STAT4 activation was not altered by expression of the IFNAR2 subunit. The increased duration of STAT4 activation correlated well with enhanced induction of IFN-γ in response to IFN-α + IL-18 stimulation (Fig 15B). Here, purified transduced cells were activated for 24 hours with either IL-12 + IL-18 or with IFN-α + IL-18 in order to assess IFN-γ secretion. IL-12 + IL-18 activated cells displayed ~60% GFP^+ cells, and this effect was independent of the expression of the IFNAR2 subunit. However, we found that expression of the IFNAR2 subunit increased the IFN-γ^+ population by 10 fold in response to IFN-α + IL-18 activation (Fig. 15B and C).

Differential induction of T-bet expression by IL-12 and IFN-α

The differential kinetics of STAT4 phosphorylation observed in response to IL-12 and IFN-α could result in differences in commitment of naïve human $CD4^+$ T cells to the Th1 phenotype. One aspect of Th1 commitment is the acquisition of IL-12 responsiveness, mediated through induction of the IL-12Rβ2 subunit (147, 213). As both IL-12 and IFN-α have been reported to induce IL-12Rβ2 expression (149), we examined whether IL-12 and IFN-α differed in their

induction of IL-12Rβ2 in developing human $CD4^+$ T cells. For these experiments, naïve human $CD4^+/CD45RA^+$ cells were purified from human peripheral blood and activated with plate-bound anti-CD3 + anti-CD28 with the cytokines or neutralizing anti-cytokine antibodies indicated in Fig. 16. When analyzed at 48 hours post-stimulation, both IL-12 and IFN-α were able to promote expression of IL-12Rβ2 cell surface protein (Fig. 16A) and mRNA transcripts (Fig. 16B, upper panel) to similar levels. In addition, IL-12 + IFN-α acted synergistically to promote enhanced IL-12Rβ2 expression at 48 hours (Fig. 16A, and Fig. 16B, upper panel). However, IFN-α failed to maintain IL-12Rβ2 expression in cells activated for 7 days (Fig. 16*B*).

In mice, T-bet is known to be involved in the regulation of IL-12Rβ2 (144, 147) and also acts downstream of IL-12 signaling to promote Th1 development (145, 146, 148). In addition, previous studies have demonstrated that IFN-α induces expression of both IL-12β2 (149) and T-bet (83) in human $CD4^+$ T cells. Thus, it has been assumed that IL-12 and IFN-α play redundant roles in Th1 commitment in human T cells through the induction of T-bet. However, given that IFN-α failed to induce Th1 development (Figs. 5-7), we re-examined the ability of IL-12 and IFN-α to regulate T-bet expression during early phases of Th1 commitment and in fully polarized Th1 cells. Similar to the regulation of IL-12Rβ2, both IL-12 and IFN-α independently induced expression

of T-bet at 48 hours, and IL-12 + IFN-α co-stimulation further enhanced T-bet expression (Fig. 16B, lower panel). However, T-bet expression was not maintained in response to IFN-α alone in fully polarized cells analyzed on day 7 (Fig. 16B, lower panel). The low expression of T-bet mRNA in response to IFN-α correlated well with decreased T-bet protein accumulation in these cells (Fig. 16C).

Based on these observations, we considered the possibility that IFN-α failed to promote Th1 development due to the lack of stable T-bet expression within polarized Th cells. If this hypothesis is correct, then Th1 development should be restored by ectopic T-bet expression regardless of initial polarizing conditions. To test this hypothesis, I expressed T-bet by retroviral transduction of primary naïve human $CD4^+$ T cells (Fig. 17). For these experiments, purified $CD4^+/CD45RA^+$ T cells were activated with plate-bound anti-CD3 + anti-CD28 and transduced with retrovirus constructs expressing GFP only (GFPRV) or human T-bet and GFP (hT-bet-GFP). Upon retroviral transduction, polarizing cytokine conditions were imposed with the addition of cytokines or neutralizing anti-cytokine antibodies as indicated in Fig. 17. These cells were expanded on day 7 with plate-bound anti-CD3, and IFN-γ expression was assessed on day 14 by intracellular cytokine staining. We were able to achieve approximately 15-20% transduction efficiency as measured by GFP expression, and the non-GFP expressing cells served as an additional internal negative control. As shown in

Fig. 17A, cells transduced with the hT-bet-GFP construct were gated on GFP^- and GFP^+ populations. Within the GFP- population, IL-12 increased the frequency of IFN-γ^+ cells, whereas IFN-α did not promote IFN-γ expression, as expected. However, expression of T-bet within the GFP^+ population increased the percentage of cells capable of secreting IFN-γ, and this effect was independent of initial cytokine conditions (Fig. 17A), compare left and right panels). Expression of GFP alone from the GFPRV vector had little effect on IFN-γ expression (Fig. 17B). Further, T-bet was sufficient to promote IFN-γ expression even when cells were polarized under Th2-inducing conditions (Fig. 17B), bottom panels, and Fig. 17B, condition 8). Thus, these data place T-bet down-stream of STAT4 and suggest that T-bet is sufficient to mediate Th1 development in human $CD4^+$ T cells independent of innate cytokine priming. These data also suggest that IFN-α fails to mediate Th1 development due to lack of sustained T-bet expression.

Discussion

Type I interferon is one of the first lines of defense against many viral and some bacterial infections. In recent years, the importance of type I interferon during innate responses has been highlighted by the discovery of IFN-α-secreting plasmacytoid dendritic cells (pDCs) (214, 215) as well as Toll-like receptor

signaling pathways which promote IFN-α/β secretion from innate cells (7, 216). Thus, IFN-α/β represents an innate cytokine with the potential to shape adaptive immune responses. Further, *in vitro* virus infection of human pDC ($CD4^+/CD11c^-$ lin^-) promotes their differentiation into high IFN-α/β-producing cells (IPCs) (217), suggesting a role for these cells in regulating adaptive responses. However, in the absence of other innate signals, the ability of IFN-α/β to promote Th1 development is controversial. The role of IFN-α/β in promoting type I responses has been suggested by studies of LCMV infections in mice (158). Here, Biron and colleagues correlated the induction of IFN-γ from splenocytes with LCMV infection that was IFN-α/β- and STAT4-dependent, but IL-12-independent. Collectively, these studies have concluded that like IL-12, IFN-α/β can directly promote Th1 development through the activation of STAT4 in naïve $CD4^+$ T cells. However, more recent studies raise the possibility that the effects of IFN-α/β observed *in vivo* might be more pronounced in $CD8^+$ than in $CD4^+$ T cells (157, 160, 203).

In this study, we found that in the absence of IL-12, IFN-α was insufficient to promote Th1 development. The lack of activity, was correlated with attenuated STAT4 tyrosine phosphorylation and the lack of stable expression of the Th1-specific transcription factor T-bet. Whether STAT4 directly regulates the T-bet promoter in human $CD4^+$ T cells is unclear at this point. However, we

found that ectopic expression of T-bet within primary naïve human $CD4^+$ T cells circumvented the requirement for innate cytokines in Th1 commitment.

Although we found that IL-12 was sufficient to promote Th1 development, human $CD4^+$ T cells failed to commit to the Th1 fate in response to IFN-α/β. In some cases, we also observed that cells cultured with IL-12 + IFN-α expressed less IFN-γ that cells differentiated with IL-12 alone. However, this effect was not consistent from one donor to the next. Nonetheless, we consistently observed that IFN-α alone failed to promote Th1 development, and in some cases suppressed IFN-γ expression compared to cells developing under neutralizing conditions. Given these results, how can our observations be reconciled with previous reports suggesting that IFN-α/β regulates type I responses *in vivo*? Clearly, there are many examples of virus infections that promote IFN-α/β secretion and generate populations of T cells capable of secreting IFN-γ (158, 161, 218-220) . In such cases, adaptive antiviral responses rely heavily on the activation and expansion of $CD8^+$ cells that commit to IFN-γ expression independent of either IL-12 or STAT4 (163). Thus, it is possible that the type I responses observed *in vivo* do not originate from $CD4^+$ cells, but rely on other cell types such as $CD8^+$ and NK cells. Indeed, type I responses to certain viruses such as LCMV are diminished in mice deficient in STAT4 and IFN-α/β

receptor signaling (158). However, these defects were observed predominantly in the $CD8^+$ T cell compartment.

Alternatively, it is possible that IFN-α, in combination with other innate cytokines, could promote $CD4^+$ Th1 development *in vivo*. Our data indicate only that IFN-α is insufficient to promote Th1 development. IFN-α may be necessary *in vivo*, in the context of other innate signals, to drive Th1 development when IL-12 is limiting. For example, IL-18 and IFN-α were demonstrated to be required for productive IFN-γ responses from $CD8^+$ T cells during LCMV infections (221). These data suggest that IFN-α/β collaborates with IL-18 to promote IFN-γ secretion from $CD8^+$ T cells *in vivo*. However, the sufficiency of IFN-α/β to drive $CD4^+$ Th1 development *in vivo* has not been demonstrated. Taken together, our results suggest that IFN-α/β may act in synergy with IL-12 to positively regulate early stages of Th1 commitment, but in the absence of IL-12, IFN-α/β is insufficient to promote full commitment of human $CD4^+$ T cells to the Th1 lineage (Fig. 18).

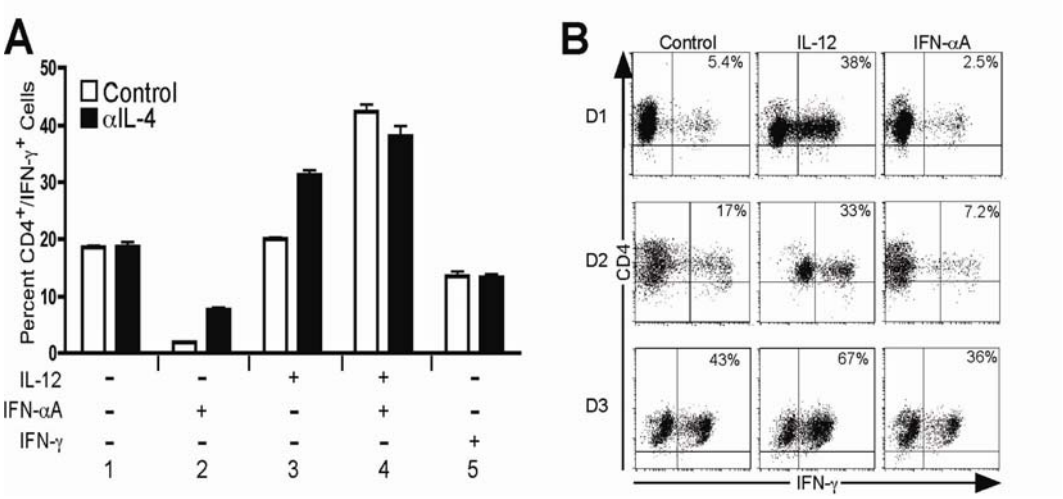

Figure 5. IL-12, but not IFN-α, promotes Th1 development in highly purified naïve human CD4$^+$ T cells. Purified human CD4$^+$CD45RA$^+$ T cells were activated with plate-bound anti-CD3 and anti-CD28 in the presence of IL-2, anti-IL-4, and the indicated cytokines and neutralizing antibodies, where "+" indicates addition of cytokine and "–" indicates addition of neutralizing anti-cytokine antibody. On day 3, cells were diluted into fresh medium containing IL-2 and rested to day 7. (**A**), Parallel cultures were stimulated in the absence (open bars) or presence (closed bars) of neutralizing anti-hIL-4 antibody. Cells were restimulated for 4 hours in the presence of PMA + ionomycin. Intracellular cytokine staining was performed with antibodies specific for hCD4 and hIFN-γ. Data were gated on live cell populations and expressed as a percentage of CD4$^+$/IFN-γ$^+$ cells. (**B**) Purified CD4$^+$/CD45RA$^+$ T cells from 3 separate donors (D1, D2, and D3) were stimulated under neutralizing conditions ("Control") or with IL-12 + anti-IFNAR2 ("IL-12") or anti-IL-12 + IFN-αA ("IFN-αA"). On day 7, cells were restimulated and stained for CD4 and intracellular IFN-γ as described above. Data were gated on live cell populations.

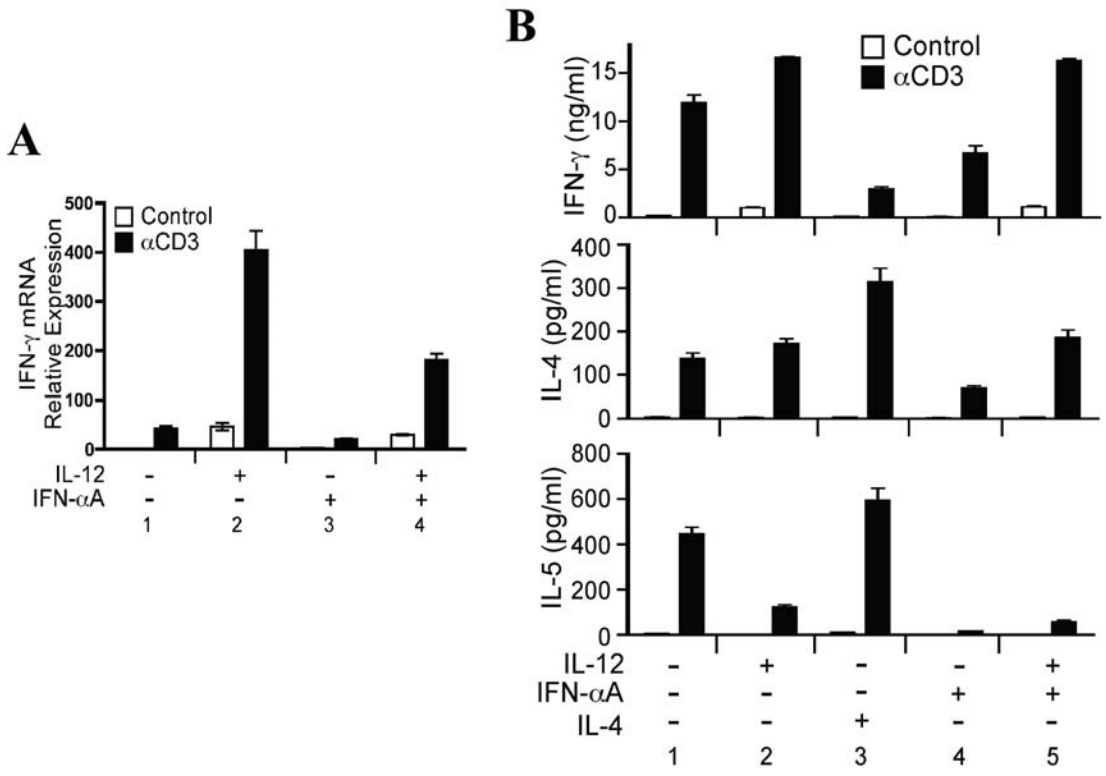

Figure 6. IFN-α is insufficient to promote Th1 or Th2 development. $CD4^+CD45RA^+$ Cells were activated as in figure 5. (**A**) Total RNA was isolated from cells that were resting (open bars) or restimulated (closed bars) with plate-bound anti-CD3 for 2 hours. Analysis of IFN-γ transcript levels was performed by qPCR, and transcript levels were normalized to GAPDH. Data were normalized relative to non-stimulated (Control) cells activated under neutralizing conditions. (**B**), Cells were rested (open bars) or restimulated for 24 hours with plate-bound anti-CD3 (closed bars). Cell culture supernatants were analyzed for the presence of IFN-γ (upper panel), IL-4 (middle panel), and IL-5 (lower panel) by cytometric bead array. This work was done by Ann Davis.

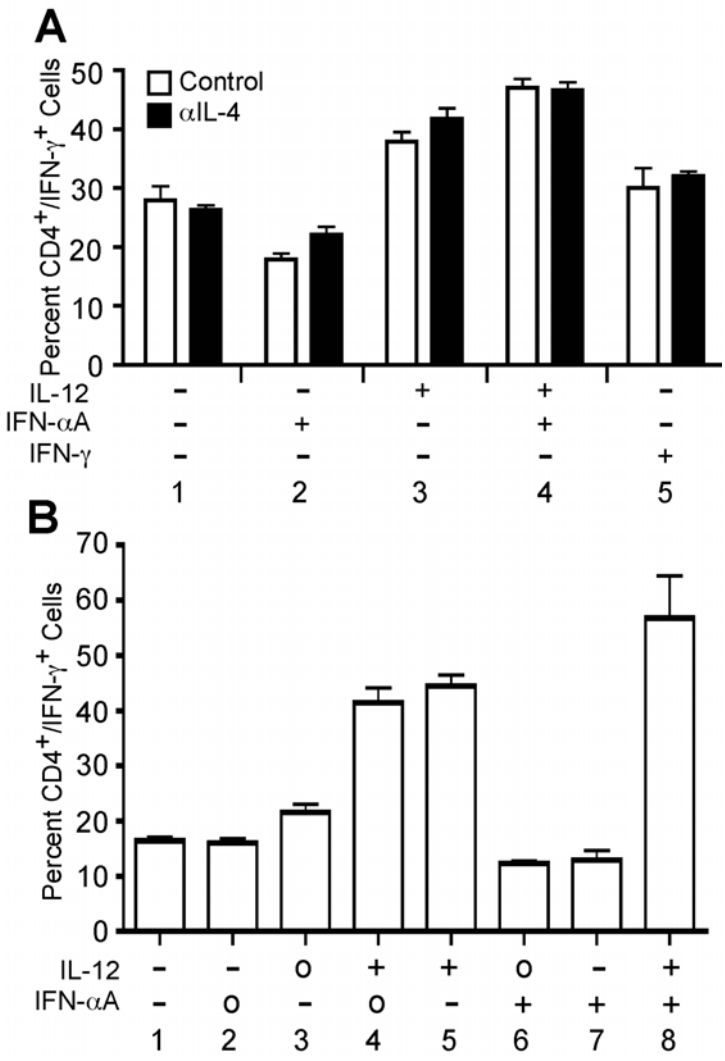

Figure 7. IL-12, but not IFN-α, promotes Th1 development in human PBMC cultures. (**A**), Human PBMCs were stimulated with plate-bound anti-CD3 + anti-CD28 in the presence of IL-2 and the indicated cytokines and neutralizing antibodies, where "+" indicates addition of cytokine, "–" indicates addition of neutralizing anti-cytokine antibody, and "**o**" indicates that the cytokine was not manipulated. Parallel cultures were stimulated in the absence (open bars) or presence (closed bars) of neutralizing anti-hIL-4 antibody. (**B**), Human PBMCs were activated as described above. On day 3, cells were diluted 1:8 into fresh medium containing IL-2 and rested to day 7. Cells were restimulated for 4 hours in the presence of PMA + ionomycin. Intracellular cytokine staining was performed with antibodies specific for hCD4 and hIFN-γ. Data were gated on live cell populations and expressed as a percentage of $CD4^+/IFN\text{-}\gamma^+$ cells.

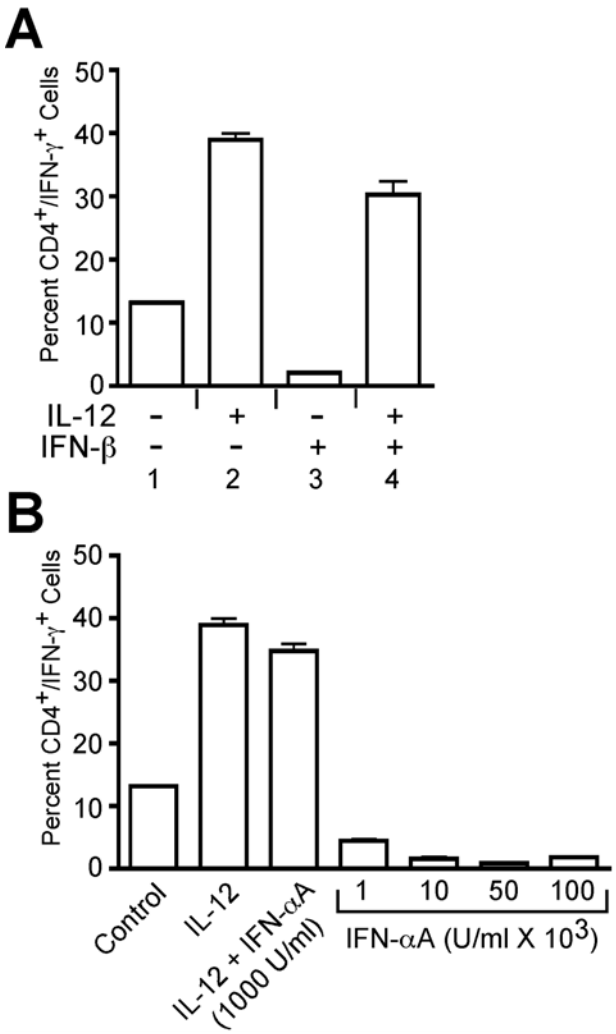

Figure 8. Type I interferon does not promote Th1 commitment. (**A**), Purified naïve $CD4^+$ T cells were stimulated with plate bound anti-CD3/anti-CD28 in the presence of IL-12 or IFN-β (1000 U/ml) as indicated in the figure. On day 7, cells were restimulated with PMA + ionomycin, and IFN-γ expression was measured by intracellular staining. (**B**), Purified naïve $CD4^+$ T cells were stimulated with IFN-α at concentrations indicated in the figure, and IFN-γ was measured by intracellular staining as describe above.

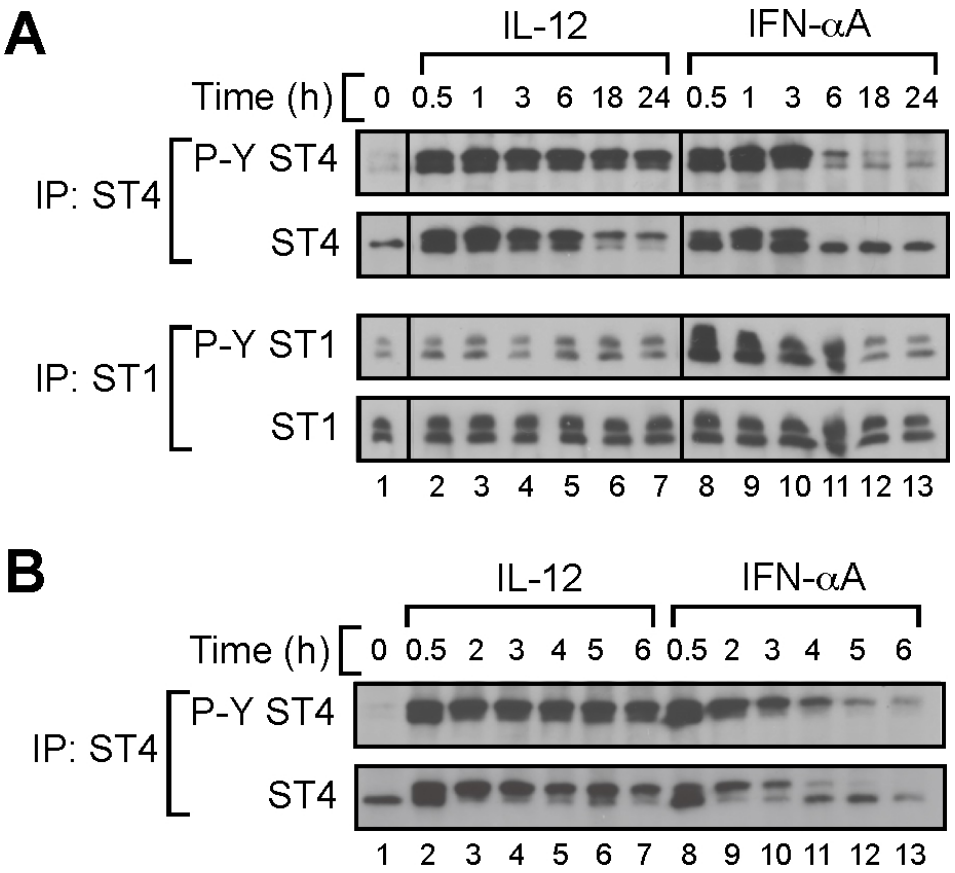

Figure 9. The kinetics of STAT4 phosphorylation are differentially induced by IL-12 and IFN-α. Human PBMCs were activated for two consecutive weeks in the presence of PHA, IL-2, and IL-12. On day 14, cells were rested in fresh media for 30', then restimulated for 0-24 hours (**A**) or 0-6 hours (**B**) in the presence of IL-12 or IFN-α. Cell were lysed at the indicated time-points and immunoprecipitated for STAT1 or STAT4. Western blotting was used to visualize phosphorylated STAT4 (A and B) or phosphorylated STAT1 (A). Total STAT4 and STAT1 were examined as loading controls. This work was performed in collaboration with Ann Davis.

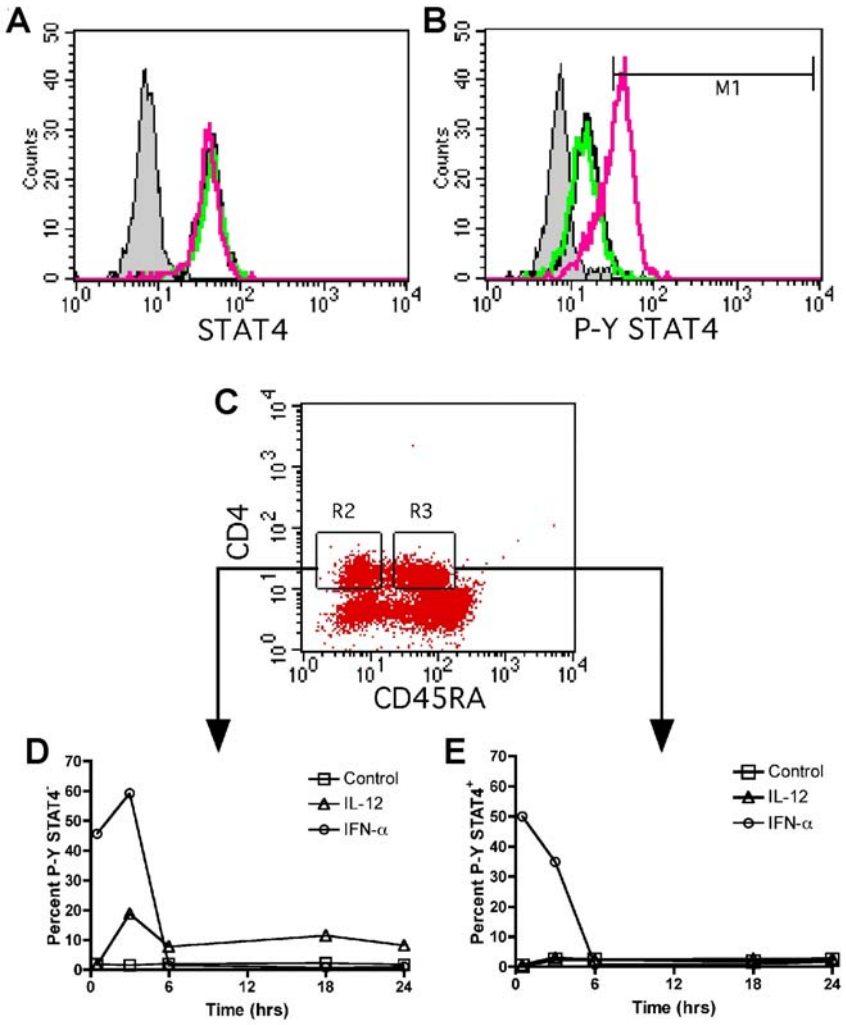

Figure 10. STAT4 phosphorylation is differentially regulated by IL-12 and IFN-α in freshly isolated human T cells. Freshly isolated PBMCs were activated with medium alone, IL-12, or IFN-α at time-points from 30'- 24 hours and stained for CD4, CD45RA, or total STAT4 and phosphorylated STAT4. (**A,B**) Representative histograms of total intracellular STAT4 (**A**) or phosphorylated STAT4 at 30' post activation (**B**). Data are gated on live, $CD4^+$, and $CD45RA^+$ cells. Black line, unstimulated; green line, IL-12 stimulated; red line, IFN-α stimulated; gray shaded, non-immune rabbit Ig control. (**C**), Gating scheme is shown for the analysis of $CD4^+$, $CD45RA^-$ (R2) and $CD45RA^+$ (R3) cells. (D,E) $CD45RA^-$ (**D**) and $CD45RA^+$ (**E**) gated cells are represented as a percentage of cells that display increased P-Y STAT4 staining over a 24 hour period. □, unstimulated; Δ, IL-12 stimulated ; ○, IFN-α stimulated. This work was performed by Dr. J. David Farrar.

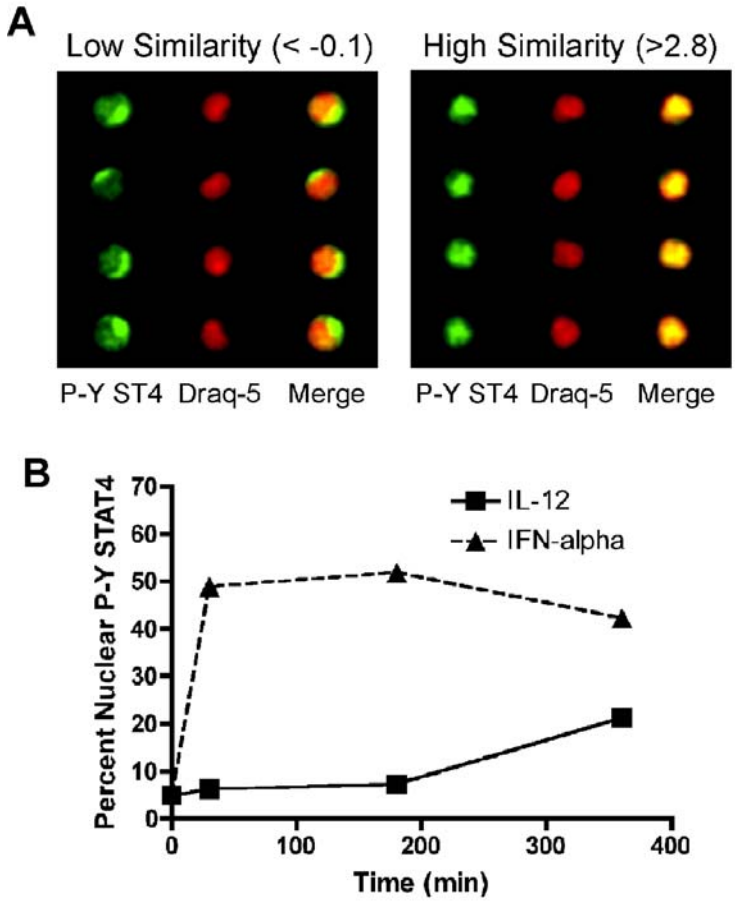

Figure 11. Nuclear localization of phosphorylated STAT4 in CD4+CD45RA+ T cells. hPBMCs were activated and stained with CD4, CD45RA and phosphor STAT4 as in Fig. 4. Cells were then analyzed on an ImageStream flow cytometer and single cell images were gated as live, $CD4^+$ $CD45RA^+$. Nuclear localization of cells staining positive for phospho-STAT4 were categorized as either low similarity (left panel) or high similarity (right panel) based on co-localization with the nuclear dye, Draq-5 (**A**). The percent of cells displaying nuclear localization of phosphorylated STAT4 in response to IL-12 or IFN-α was quantified (**B**). ■, IL-12; ▲, IFN-α. This work was performed by Dr. J. David Farrar and Dr. Thaddeus C. George.

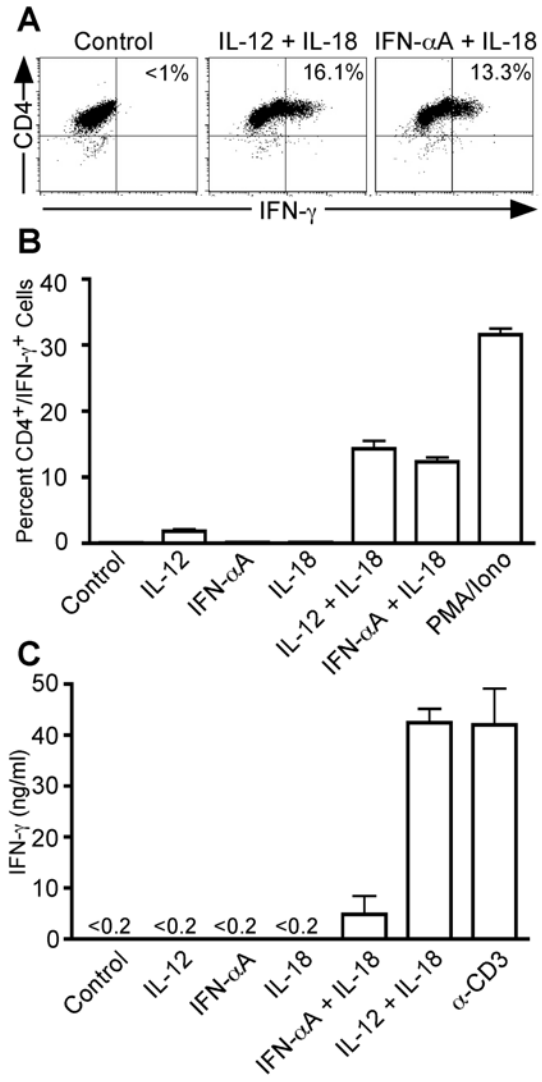

Figure 12. Cytokine-dependent IFN-γ secretion from fully polarized human Th1 cells. Purified human $CD4^+/CD45RA^+$ T cells were activated with plate-bound anti-CD3 and anti-CD28 in the presence of IL-2, anti-IL-4, anti-IFNAR2, and IL-12 for 3 days (Th1-inducing conditions). Cells were diluted into fresh medium and rested to day 7. (**A**,**B)**, Cells were restimulated for 4 hours in the presence of IL-12, IFN-αA, IL-18, or a combination of these cytokines. PMA + ionomycin was used as a positive control. Intracellular cytokine staining was performed with antibodies specific for hCD4 and hIFN-γ. Data were gated on live cell populations. (**A**), Representative dot plots showing unstimulated cells and cells stimulated in the presence of IL-12 + IL-18 or IFN-αA + IL-18. (**B**), Graphical representation of the proportion of $CD4^+$ IFN-γ^+ cells. *C*, Cells were restimulated for 24 hrs in the presence of the indicated cytokines or with plate-bound anti-CD3 antibody as described above. Cell culture supernatants were harvested and analyzed for the presence of IFN-γ protein by ELISA.

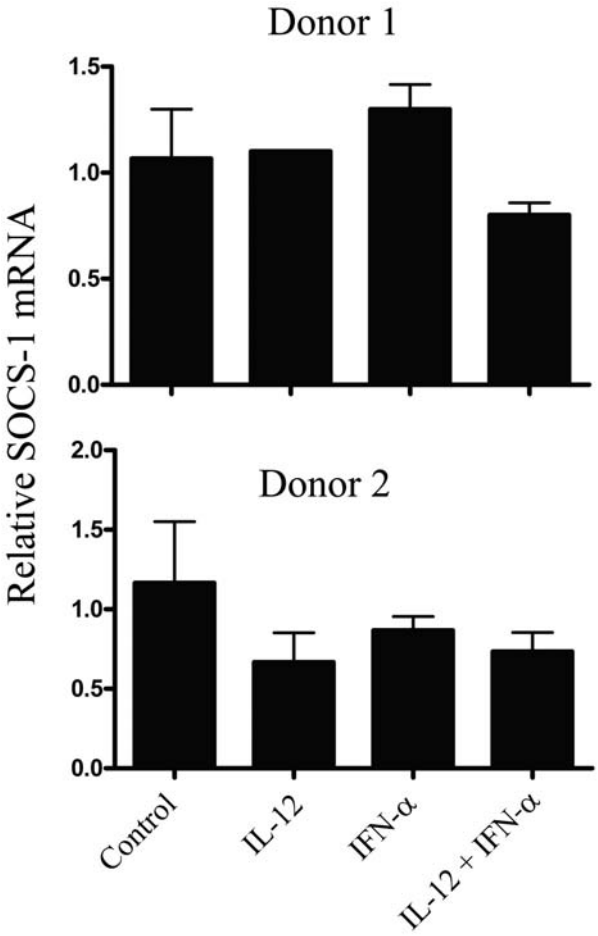

Figure 13. The inability of IFN-α to promote Th1 development is not correlated with SOCS-1 expression. $CD4^+$ $CD45RA^+$ cells were cultured to 48hrs in the presence of polarizing cytokines as indicated in the figure and allowed to rest overnight. Total RNA was harvested from 2 independent donors, and qRT-PCR was performed using primers directed against human SOCS-1. Primers directed against human GAPDH were used as a control for relative expression.

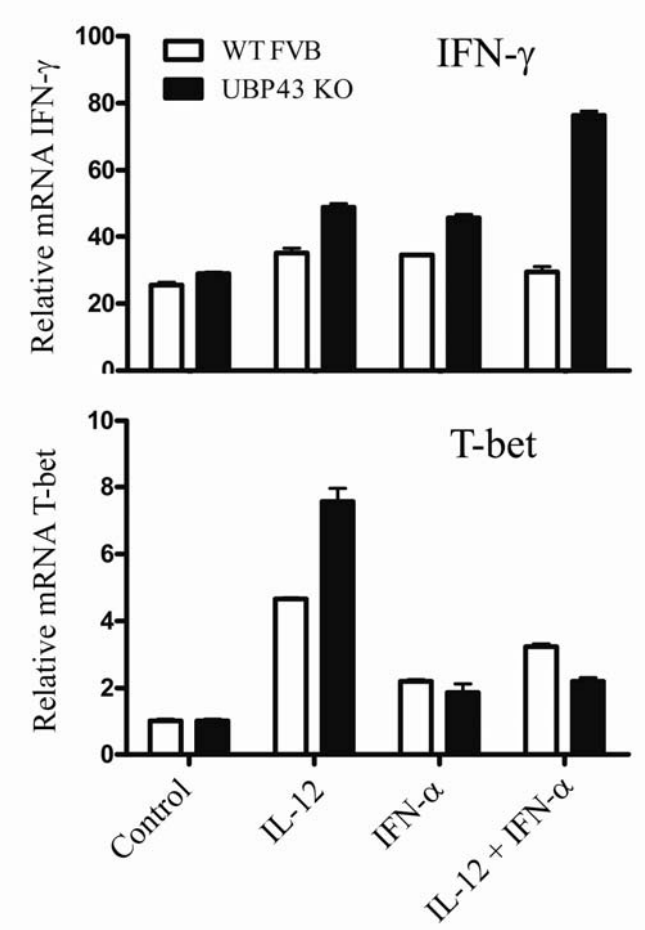

Figure 14. UBP43 deficiency does not restore IFN–α–dependent Th1 commitment in murine CD4+ T cells. Spleens from UPB43$^{-/-}$ mice and their control WT FVB counterparts were harvest and single cells suspensions were generated. Cells were polarized under the indicated cytokine conditions and allowed to activate for 7 days. On day 7, total RNA was harvested , and qRT-PCR was performed using primers directed against mouse IFN-γ and mouse T-bet. Primers directed against mouse HPRTwere used as a control for relative expression.

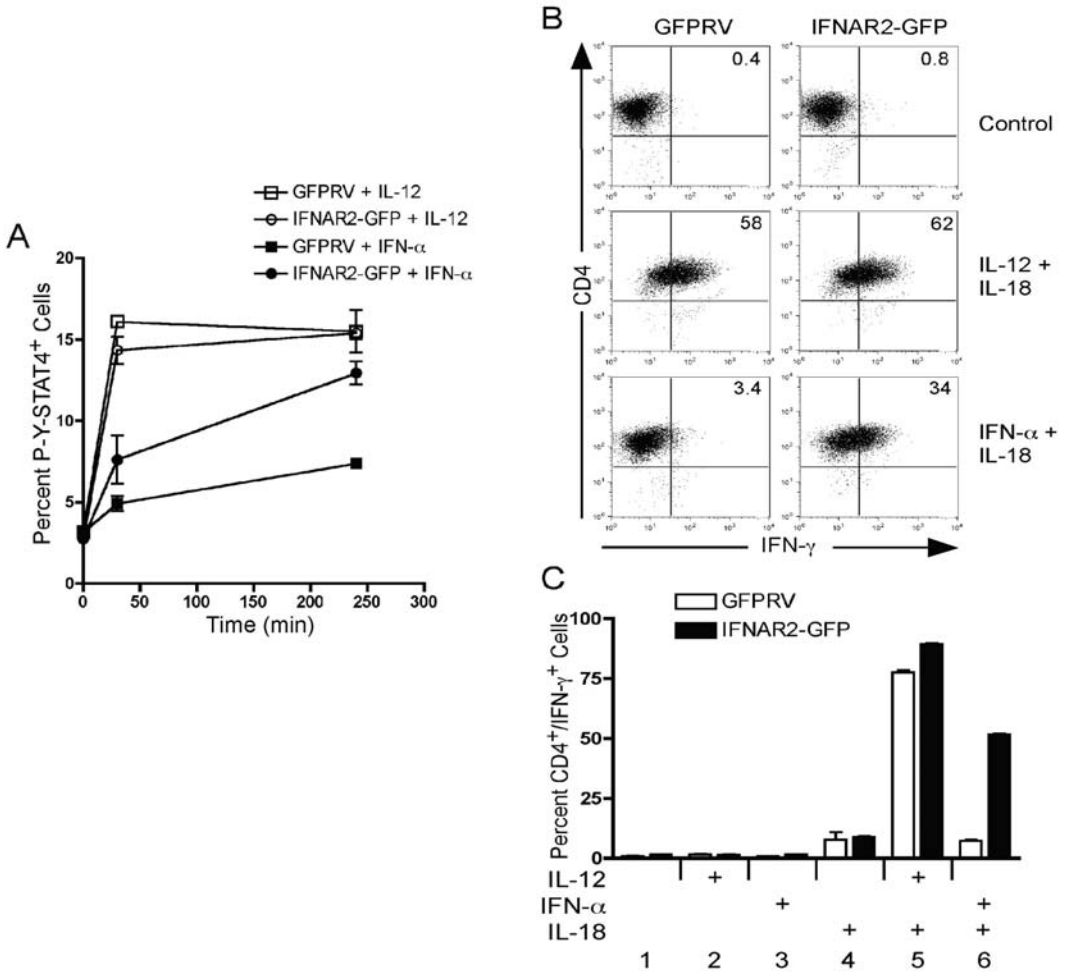

Figure 15. Ectopic IFNAR2 expression promotes sustained STAT4 phosphorylation and IFN-γ secretion in response to IFN-α. (**A**) Spleen and lymph node cells from DO11.10 mice were activated with OVA peptide under Th1-inducing conditions and transduced with retrovirus vectors expressing GFP alone (GFPRV) or the full-length mIFNAR2 subunit. Cells were sorted on day 7 based on GFP expression and restimulated with irradiated BALB/c splenocytes and OVA peptide. Following expansion for an additional 7 days, resting cells were activated with either IL-12 or IFN-α for the times indicated in the figure. Cells were then stained and analyzed for intracellular tyrosine-phosphorylated STAT4 as described in Fig. 5. (**B,C)** Day 14 transduced Th1 cells were activated with either IL-12 + IL-18, IFN-α + IL-18, or with the individual cytokines indicated in the figure for 24 hours. Brefelden A was added during the last 4 hours of stimulation. Cells were then stained for mCD4 and IFN-γ and analyzed by flow cytometry. Data were gated on live cells and GFP expression. This experiment was performed by Dr. David Farrar.

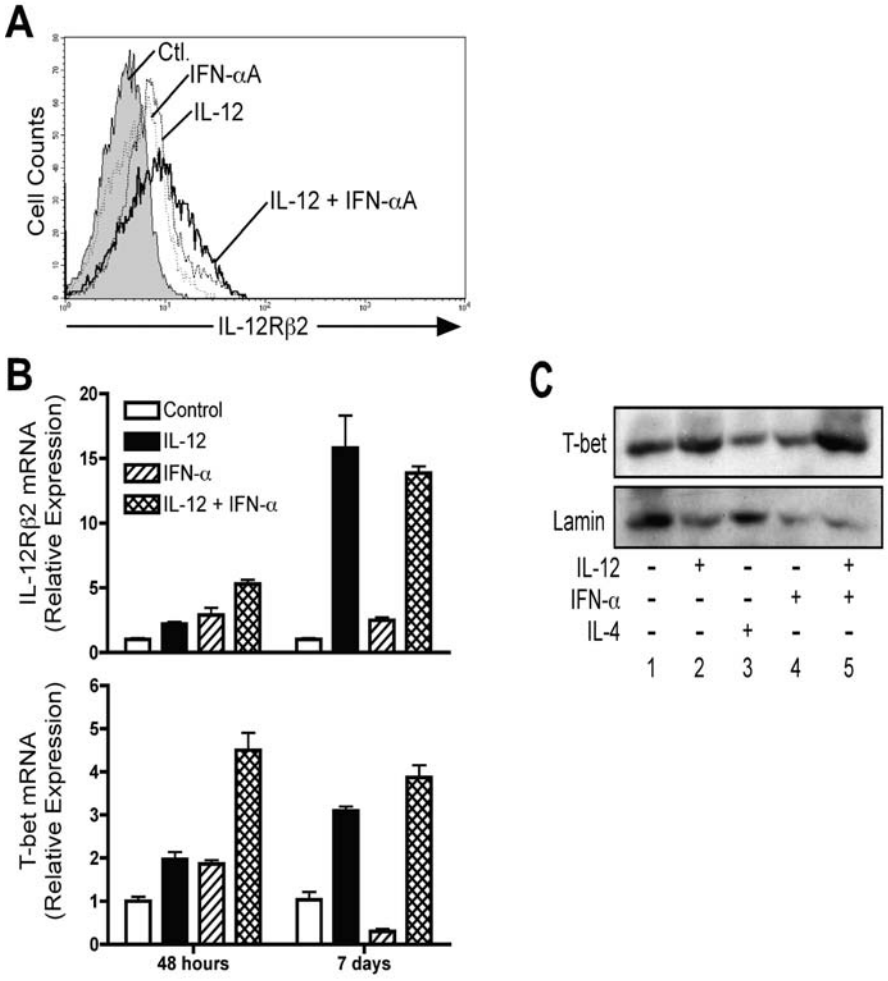

Figure 16. IFN-α does not promote stable T-bet expression in human CD4+ T cells. Purified human $CD4^+/CD45RA^+$ T cells were activated with plate-bound anti-CD3 + anti-CD28, IL-2, and anti-IL-4, and with either anti-IFNAR2 and anti-IL-12 ("Ctl"), anti-IFNAR2 and IL-12 ("IL-12"), IFN-αA and anti-IL-12 ("IFN-aA"), or with IFN-αA and IL-12 ("IL-12 + IFN-αA"). (**A**) After 72 hours, cells were stained for surface expression of IL-12Rβ2: filled histogram, neutralizing antibodies alone; dashed line, IL-12 + anti-IFNAR2; dotted line, IFN-αA + αIL-12; solid line, IL-12 + IFN-αA. (**B**), Total RNA was isolated from cells harvested 48 hours or 7 days after activation. Analysis of IL-12Rβ2 and T-bet transcript levels was performed by quantitative real-time PCR (qPCR) using the primers listed in Materials and Methods. Transcript levels for each condition were normalized to GAPDH, and the data were further normalized relative to cells activated under neutralizing conditions. (**C**), Whole-cell lysates were prepared from day 7 activated cells and assessed for expression of T-bet protein by Western blotting (upper panel). Blots were stripped and re-probed for Lamin protein expression (lower panel). Panels B and C were done by Ann Davis.

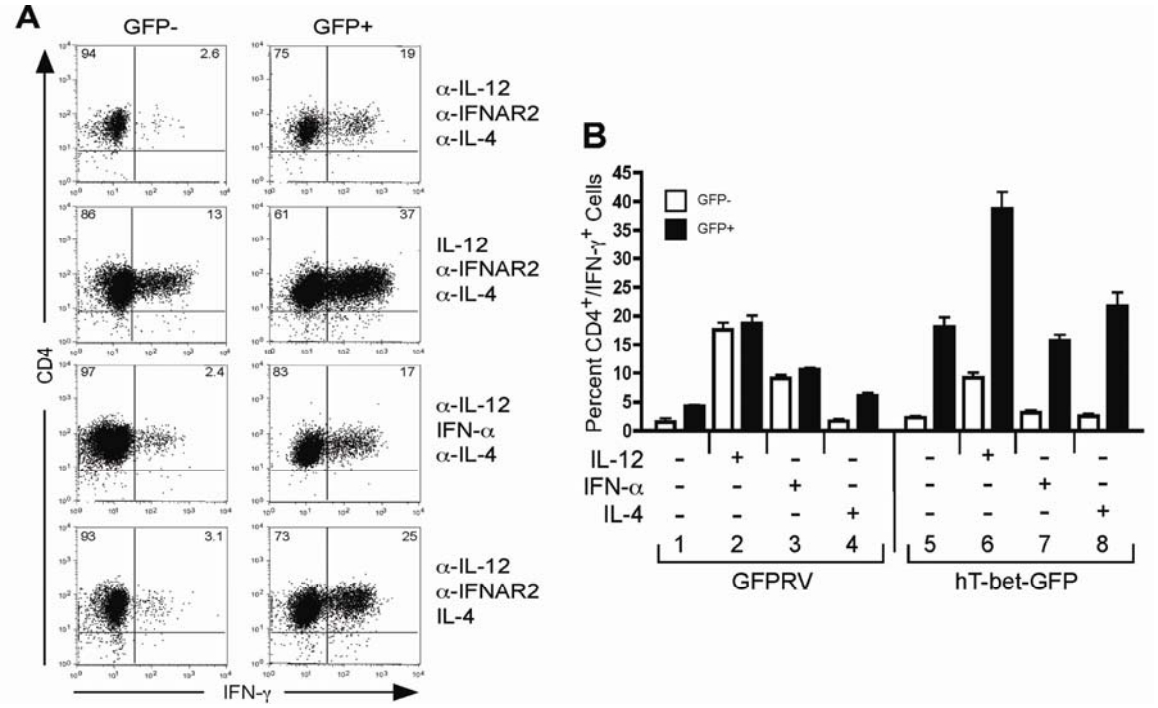

Figure 17. Ectopic T-bet expression promotes Th1 development independent of IL-12 or IFN-α in naïve human CD4+ T cells. Purified naïve human $CD4^+$ T cells were transduced with retrovirus constructs expressing GFP only (GFPRV) or with human T-bet (hT-bet-GFPRV). During retroviral transduction, separate groups of cells were simultaneously activated in the presence or absence of cytokines or anti-cytokine antibodies as indicated in the figure. Cells were expanded on day 7 by restimulation on anti-CD3-coated plates. On day 14, resting cells were restimulated with PMA + ionomycin and analyzed for IFN-γ expression by intracellular cytokine staining. (**A**) hT-bet-GFP-transduced cells were gated on live and either GFP negative (GFP-, left panels) or positive (GFP^+, right panels) populations. The percentages of $CD4^+$ and either IFN-γ^- or IFN-γ^+ populations are indicated within their respective quadrants. (**B**), Triplicate cultures were analyzed for IFN-γ expression by intracellular cytokine staining. The percentage of $CD4^+$/IFN-γ^+ cells transduced with either the control GFPRV or hT-bet-GFP vectors are compared between the GFP^- (open bars) and GFP^+ (closed bars) populations.

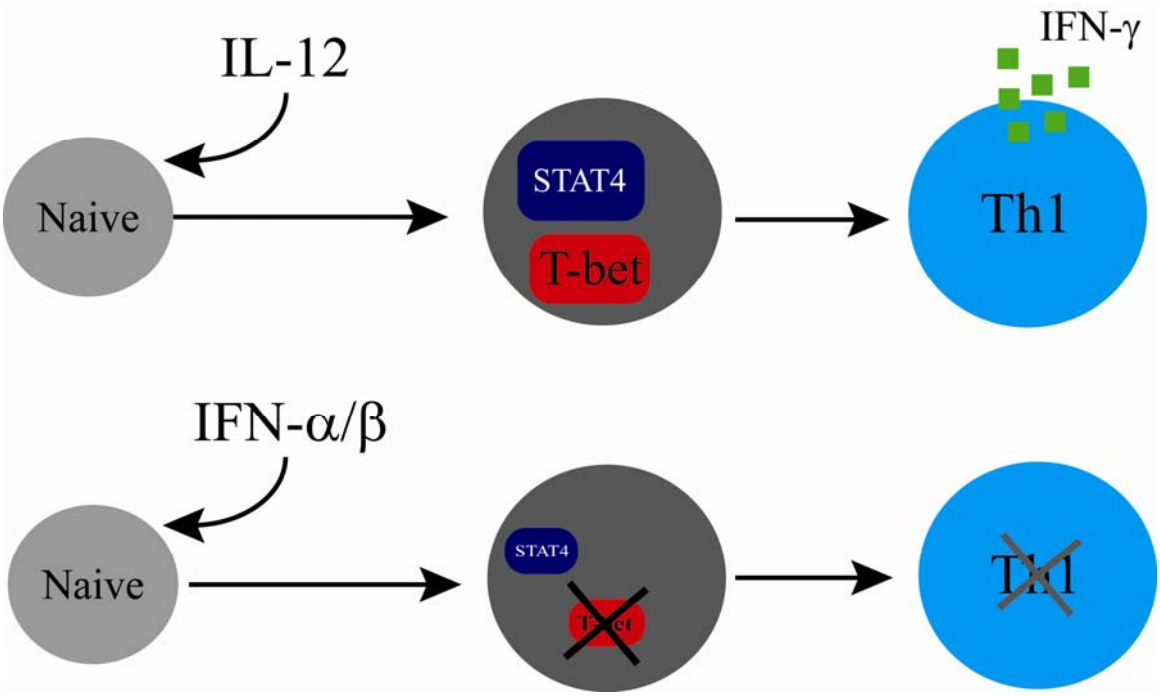

Figure 18. A model for the role of IFN-α/β on CD4+ Th1 development. In response to signaling by IL-12, naïve CD4+ T cells induce robust and prolonged phosphorylation of STAT4. This leads either directly or indirectly to the maintenance of T-bet and the development of Th1 cells which express IFN-γ. In contrast, signaling via IFN-α/β (bottom) leads to an acute STAT4 phosphorylation event. This is insufficient to maintain T-bet expression and therefore, these cells do not become Th1 cells.

CHAPTER FIVE

RECIPROCAL RESPONSIVENESS TO IL-12 AND IFN-α SPECIFIES HUMAN CD8$^+$ EFFECTOR VERSUS CENTRAL MEMORY T CELL FATES

The following study is currently accepted for publication in the Journal, *Blood*. Ramos, H.J., Davis, A.M., Cole, A.G., Schatzle, J.D., Forman, J., Farrar, J.D. *Reciprocal responsiveness to IL-12 and IFN-α specifies human CD8$^+$ effector versus central memory T cell fates*. *Blood*. Prepublised online March 18, 2009; DOI: 10.1182/Blood-2008-11-18-8458, and is reproduced with the permission of the *Journal of the American Society of Hemotology. Copyright 2009*. All experiments in this study were carried out by Hilario J. Ramos.

Introduction

My previous examination of human CD4$^+$ T cells revealed a distinct defect in the ability of IFN-α/β to promote Th1 development (100). This is in contrast to the current understanding in CD8$^+$ T cells, in which IL-12 and IFN-α/β are thought to act in a redundant manner to shape Tc1 development. While data suggest this might be the case in mice (29, 81, 139, 158), very little is known about the role of IFN-α/β on the development of human CD8$^+$ T cells responses.

Further, although most studies have analyzed the ability of cytokines to prime immediate effector responses, the role of both IL-12 and IFN-α/β on the generation of memory is still unclear. Therefore, I set out to address the direct roles of IL-12 and IFN-α/β in programming both the effector response as well as memory formation in human $CD8^+$ T cells.

$CD8^+$ T cells are critical mediators of adaptive inflammatory responses to intracellular pathogens. They require a series of signals for efficient expansion and acquisition of effector functions such as cytokine secretion and lytic activity. These signals are delivered by professional antigen presenting cells (APC) and include antigen recognition ("signal 1"), co-stimulatory activation ("signal 2"), and signaling provided by innate inflammatory cytokines ("signal 3") (139). While signals 1 and 2 prime naïve $CD8^+$ T cells and initiate cell division, signal 3 cytokines program effector functions and ensure clonal survival. A variety of cytokines have the potential to act as signal 3 in $CD4^+$ and $CD8^+$ T cells, including IL-1, IL-21, IL-12 and IFN-α/β (28, 29, 222, 223). In particular, IL-12 and IFN-α/β, promote efficient induction of innate immunity as well as the development of adaptive type 1 responses to intracellular infection (65, 161). Therefore, IL-12 and IFN-α/β have the potential to act as the predominant signal 3 during intracellular infection.

Unraveling the roles of IL-12 and IFN-α/β in regulating the effector function of $CD8^+$ T cells has been controversial. While IL-12 regulates Th1 development in $CD4^+$ T cells, early reports suggested that the induction of IFN-γ secretion and lytic activity in $CD8^+$ T cells was independent of IL-12, STAT4, and T-bet (146, 163, 224). However, these studies were soon followed by the observation that antigen-driven cytolytic function and IFN-γ secretion were severely impaired in T-bet-deficient $CD8^+$ T cells, suggesting a critical role for T-bet in effector function (166). Likewise, Mescher and colleagues found that IL-12 provided a necessary third signal that regulated $CD8^+$ effector T cell development (28, 223). Further, these effects were dependent on STAT4, indicating that IL-12 signaling provides a necessary third signal for the regulation of $CD8^+$ T cell development (29, 167, 225). Although IL-12 was sufficient to promote effector cell development *in vitro*, IL-12R-deficient $CD8^+$ T cells were still capable of mounting both primary effector and memory cell responses to *in vivo* infections with adenovirus (225) and *L. monocytogenes* (167), suggesting that additional innate cytokines may regulate $CD8^+$ effector cell development. More recent studies have indicated that IFN-α/β can act in a manner similar to IL-12 to provide signal 3 and promote the induction of cytokine secretion, cytolytic activity, and clonal expansion in murine $CD8^+$ T cells (28, 29). Collectively, these studies suggested that IL-12 and IFN-α can act as redundant signals to promote the development of effector responses in murine $CD8^+$ T cells.

In addition to enhancing effector cell development, IFN-α/β was implicated in the generation of memory $CD8^+$ T cells *in vivo*. In these studies, IFNAR-deficient, TCR-transgenic (P14) $CD8^+$ T cells failed to expand and generate memory populations in response to *in vivo* LCMV infection despite their ability to proliferate efficiently *in vitro* (196). Alternatively, IL-$12^{-/-}$ mice displayed defective primary effector responses, whereas development of central memory (T_{CM}) cells was markedly enhanced compared to wild-type, indicating that IL-12 signaling suppresses T_{CM} development (191-193). Similar effects were observed in T-$bet^{-/-}$ $CD8^+$ T cells. Here, Kaech and colleagues demonstrated that T-bet was highly induced by IL-12, but not IFN-γ, in wild-type $CD8^+$ T cells (192). Further, as T-bet expression increased in response to IL-12, the balance between effector and memory shifted away from T_{CM} to more effector phenotypes. This effect was reversed in the absence of T-bet where the majority of T-$bet^{-/-}$ $CD8^+$ T cells committed to the T_{CM} phenotype regardless of whether IL-12 was present during priming (191). This result was confirmed by Reiner and colleagues, who further demonstrated that the marked increase in $CD8^+$ effector cells that developed in the absence of $CD4^+$ T cell help was shifted towards T_{CM} in the absence of T-bet (193). Collectively, these data illustrate a potential role for IFN-α in the development of central memory responses and a separate role for IL-12 in effector and effector memory cell development.

Considering that IFN-α/β has been implicated in effector and memory cell development, it is unclear how this signal regulates both events and whether any of these activities operate in human $CD8^+$ T cells. Further, it is not clear how IL-12 and IFN-α/β signals are integrated to balance effector and memory cell development as many intracellular pathogens elicit the secretion of both of these innate cytokines from professional APCs. To examine these responses in human $CD8^+$ T cells, we have utilized an *in vitro* polarization system to test the independent and combined roles of IL-12 and IFN-α in the development of both the effector and memory human $CD8^+$ T cell response. Probing the function of cytokines in the regulation of human $CD8^+$ T cell differentiation requires strict control of the initial *in vitro* priming conditions. Thus, we chose to utilize anti-CD3/anti-CD28 to prime cells, which allows for control of both innate cytokine stimulation and for delivery of varied TCR signal strengths.

In this study, we demonstrate a novel pathway for the variegated programming of human $CD8^+$ T cell effector and memory development by IL-12 and IFN-α. Here, we show that IL-12 and IFN-α are not redundant signals in the development of $CD8^+$ T cell responses and instead act in concert in the context of signals 1 and 2 to balance the development of effector and memory cell populations.

Results

IL-12, but not IFN-α, regulates effector CD8$^+$ T cell development

In my first series of experiments, I assessed the development of effector cytokine secretion and lytic activity in purified human CD8$^+$CD45RA$^+$ T cells in response to IL-12 and IFN-α. In order to strictly control specific cytokine signals, cells were activated with anti-CD3/anti-CD28 in the absence or presence of cytokines or anti-cytokine antibodies as described in the Methods section. As previously reported, IL-12 markedly induced the secretion of IFN-γ and TNF-αcompared to neutralizing conditions (Fig. 19, A and B). In contrast, treatment with IFN-α alone was insufficient to induce either expression or secretion of IFN-γ or TNF-α to levels above the neutralized control. Importantly, IFN-α did not inhibit the ability of IL-12 to induce cytokine expression and secretion (Fig. 19, A and B; compare IFN-α with IL-12 + IFN-α conditions), indicating that IL-12 drove effector cytokine expression independent IFN-α signaling. I next examined the ability of IL-12 and IFN-α to promote the expression of IL-2. Unlike IFN-γ and TNF-α, IL-2 expression was only marginally regulated by cytokine polarization as assessed by intracellular staining, with the majority of expression being induced by T cell receptor (TCR) activation alone (Fig. 20 A). As expected, the levels of IL-2 secreted by human CD8$^+$ T cells by ELISA was >10-

fold lower than levels secreted by $CD4^+$ T cells (data not shown). However, it was observed that $CD8^+$ T cells secreted elevated levels of IL-2 secretion in response to IL-12 + IFN-α compared to activation with either IL-12 or IFN-α alone (Fig. 20 B), suggesting a collaborative role for these cytokines in retaining IL-2 expression.

I next measured expression of the cytolytic effector molecules perforin and granzyme B. Both IL-12 and IFN-α were capable of inducing the expression of these molecules however, the magnitude and pattern by which these cytokines affected this induction was variable among the donors tested (Fig. 21). Therefore, to address the precise role of IL-12 and IFN-α on functional cytotoxicity, I assessed the ability of cytokine polarized cells to directly kill target cells in a re-directed lysis assay. Unlike expression of perforin and granzyme B, IL-12 but not IFN-α promoted strong lytic activity compared to cells activated under neutralizing conditions consistently in all donors (Figure 19 C). Although not enhanced by IFN-α alone, IL-12-mediated lytic activity was not inhibited by the presence of IFN-α during priming. Further, the lytic activity observed by these cells was completely inhibited by concanamycin A, demonstrating an exclusive role for perforin and granzyme as opposed to Fas/FasL-mediated killing in these assays (226, 227) (Fig. 22). These data demonstrate that IL-12, but not IFN-α, is

sufficient to program both effector cytokine secretion and perforin-mediated CTL activity in human $CD8^+$ T cells.

IFN-α drives the development of human $CD8^+$ T_{CM} cells

Considering recent studies in mice suggesting that IFN-α could modulate primary expansion and subsequent T_{CM} development (196, 228). I next assessed the ability of IL-12 and IFN-α to influence the expansion of human $CD8^+$ effector (T_{EM}) and central memory (T_{CM}) cells based on expression of a variety of molecular signatures. T_{EM} and T_{CM} are characterized by low expression of CD45RA and differential expression of CCR7 such that T_{EM} are $CCR7^{lo}$ and T_{CM} are $CCR7^{hi}$, whereas naïve cells are $CD45RA^{hi}/CCR7^{hi}$ [169, 229]. In response to activation, the majority of cells down-regulated CD45RA expression and adopted either T_{CM} or T_{EM} phenotypes even when activated under neutralizing conditions (Fig. 23 A, upper panels). However, IFN-α markedly enhanced the percentage of $CCR7^{hi}/CD45RA^{lo}$ T_{CM} cells (Fig. 23, A and B, upper panels). Further, the induction of T_{CM} development by IFN-α was not inhibited in the presence of IL-12, suggesting a dominant role for IFN-α in regulating T_{CM} differentiation. In contrast, the induction of $CCR7^{lo}/CD45RA^{lo}$ T_{EM} cells by IL-12, and the development of these cells was enhanced by IL-12 in both the absence and

presence of IFN-α. I next assessed two differentially expressed chemokine receptors: CXCR3, which allows for traffic to the periphery and is associated with effector phenotypes (230-232), and CCR7, which allows for efficient trafficking to lymph nodes and is associated with the T_{CM} phenotype (233, 234). Consistent with the induction of $CCR7^{hi}/CD45RA^{lo}$ T_{CM} cells, IFN-α promoted the development $CXCR3^{lo}/CCR7^{hi}$ populations either in the presence or absence of IL-12 (Fig. 23, A and B, lower panels). These cells are composed of a subset of the $CCR7^{hi}/CD45RA^{lo}$ cells, and in agreement with previous studies (230), we propose that the $CCR7^{hi}/CXCR3^{lo}$ (referred to hereafter as T_{CMR7}) subset represents a more precisely defined T_{CM} population. Thus, IFN-α positively regulates the development of T_{CM} cells, and this pathway occurs independently of IL-12.

I also assessed expression of the IL-7Rα (CD127), which is expressed by T_{EM} and to a greater extent on T_{CM}, but not on immediate effector cells. IL-12, but not IFN-α, enhanced CD127 expression on the population as a whole (Fig. 24, A and B). In addition, IL-12 enhanced CD127 expression on T_{EM} ($CCR7^{lo}$ cells) compared to cells developing in the presence of IFN-α alone (Fig. 24 C). Importantly, development of $CCR7^{hi}$ cells in response to IFN-α was accompanied by maintenance of CD127 expression, further supporting their characterization as T_{CM} cells. Together, these analyses reveal that IL-12 acts independently of IFN-α

to program the development of T_{EM}, while signaling via IFN-α promotes the development of T_{CM} phenotypes.

IFN-α-driven T_{CM} cells display functional memory activities

In order to determine whether the *in vitro* development of T_{CM} and T_{EM} paralleled their known functional roles, I examined effector molecule expression as a function of T_{CM} and T_{EM} cell surface phenotypes. Activation of naïve $CD8^+$ T cells led to the development of two distinct populations of cells; those which expressed either granzyme B alone, or both granzyme B and perforin (Fig. 25 A, upper panel, left and right gates). We assessed expression of CXCR3 and CCR7 within these two populations. The majority of cells expressing both perforin and granzyme B (Fig. 25 A, right panels, orange gate) uniformly expressed high levels of CXCR3 and low CCR7 regardless of cytokine treatment. In contrast, cells expressing granzyme B alone (Fig. 25 A, left panels, magenta gate), displayed a heterogeneous expression of CCR7 and CXCR3. Here, IFN-α enhanced a population of T_{CMR7} cells, demonstrating that the genesis of the T_{CM} cells in these cultures derives from within the granzyme B single positive population. This analysis was confirmed by examining expression of perforin and granzyme B in populations that differentially expressed CCR7 and CD127. Cells within the $CCR7^{lo}/CD127^{lo}$ gate displayed high frequencies of perforin and granzyme B

double positive cells (Fig. 25 B, lower right panel, orange gate). Alternatively, cells within the $CCR7^{hi}/CD127^{hi}$ gate expressed granzyme B with very low levels of perforin (Fig. 25 B, lower left panel, magenta gate), demonstrating a direct link between effector potential and decreased expression of the IL-7R. Finally, cells expressing CXCR3 in the absence of CCR7 overwhelmingly secreted high levels of IFN-γ in response to secondary activation (Fig. 25 C, population "D," orange gate). In contrast, only 50% of cells that co-expressed CXCR3 and CCR7 (Fig. 25 C, population "C," teal gate) and less that 1% of T_{CMR7} cells (Fig. 25 C, population "B," magenta gate) were capable of secreting IFN-γ upon reactivation. These data directly link T_{EM} phenotypes with secretion of effector molecules and demonstrate that IL-12 and IFN-α differentially regulate both the phenotype and function of T_{EM} and T_{CM}, respectively.

T-bet and Eomesodermin (Eomes) have been linked to the development of productive $CD8^+$ T cell effector responses (145, 165), and IL-12 signaling has been shown to play a major role in modulating T-bet and Eomes expression (191, 193). Therefore, I examined their expression in day 7 polarized $CD8^+$ T cells. I observed marked induction of T-bet by both IL-12 and IFN-α/β at the mRNA as well as protein level (Fig. 26, top panels). Further, cells responding to the IL-12 + IFN-α conditions displayed enhanced expression of T-bet (Fig. 26, top panels). In stark contrast to T-bet, regulation of Eomes was solely induced by IFN-α/β

both at the protein as well as mRNA levels (Fig 26 bottom panels). Therefore this suggested a potential role for IL-12 driven T-bet in T_{EM} generation and IFN-α/β driven Eomes in the development of T_{CM} cells. In order to address this further, I examined the expression of T-bet and Eomes within effector or memory sub-populations of cells. First, we examined expression of T-bet and Eomes within IL-2- and IFN-γ-secreting sub-populations. Cells secreting IFN-γ, regardless of their co-expression of IL-2, displayed the highest levels of T-bet, whereas the IL-2 single expressing cells displayed lower levels of T-bet (Fig. 27 A, center panel). The pattern of T-bet expression was distinct from that of Eomes, as Eomes was uniformly expressed in all cells regardless of their differential expression of IL-2 and IFN-γ (Fig. 27A, right panel). This analysis was extended by assessing T-bet and Eomes expression within cells that differentially expressed CXCR3 and CCR7 (Fig. 27 B) as well as perforin and granzyme B (Fig. 27 C). In each case, the presence of high levels of T-bet expression correlated with the development of T_{EMXR3}, while cells expressing lower T-bet levels acquired a T_{CMR7} phenotype. Therefore despite a strong induction of Eomes by IFN-α/β in the total population of $CD8^+$ T cells, surprisingly, I found no differential expression of Eomes within T_{EMXR3} versus T_{CMR7}, suggesting that selective expression of T-bet, but not Eomes, contribute to the development of T_{EM} and T_{CM} phenotypes.

In order to fully address the roles of T-bet and Eomes in effector and memory development, I assessed the role of overexpression of these molecules by

retroviral transduction. I had previously generated a retroviral construct encoding both T-bet and GFP (T-betRV) (100). In addition, I generated a similar construct expressing the human Eomes (Eomes-RV). Retroviral transduction with T-betRV or Eomes-RV promoted strong expression of effector markers including IFN-γ and perforin (data not shown). However this response was also observed in cells transduced with the control GFPRV alone. These observations suggest that unlike human $CD4^+$ T cells, human $CD8^+$ T cells may have an intrinsic ability to respond to infection by retrovirus. Therefore we were unable to utilize this system to fully assess the role of these transcription factors in the development of effector and memory responses.

T_{EM} cells exert immediate effector functions, are considered to be more terminally differentiated, and generally do not expand efficiently upon reactivation. In contrast, T_{CM} cells divide rapidly to secondary challenge giving rise to additional effector cells (170, 171). Therefore, we sought to determine whether IFN-α-driven T_{CM} cells were capable of rapid proliferation and generation of secondary effector cells. To address this, cells were polarized in the presence of both IL-12 + IFN-α for 7 days and sorted based on the following gates: T_{EMXR3}, $CCR7^{lo}/CXCR3^{hi}$, and T_{CMR7}, $CCR7^{hi}/CXCR3^{lo}$ (Fig. 28 A). With these purified populations, we first examined direct CTL activity by re-directed lysis assays. T_{EMXR3} cells displayed strong CTL activity, whereas the T_{CMR7} cells were incapable of immediate lytic activity (Fig. 28 B). These data are in

agreement with our previous observations that T_{EMXR3} cells expressed higher levels of perforin compared to T_{CMR7} cells (Fig. 25 A) and demonstrate functional differences in effector capabilities between T_{EMXR3} and T_{CMR7} $CD8^+$ T cells.

We next assessed the ability of sorted cells to proliferate and expand in response to secondary activation. As expected, activated T_{EMXR3} cells did not divide, whereas, T_{CMR7} cells displayed robust proliferation in response to anti-CD3 stimulation (Fig. 28 C). The lack of proliferation in the T_{EMXR3} cells correlated well with a decrease in the total live population of cells as assessed by forward/side scatter analysis and by 7-amino-actinomycin D staining, whereas the T_{CMR7} cells maintained a live lymphocyte profile (Fig. 29 A and B). We examined the ability of sorted cells to expand and give rise to new effector and memory sub-populations in response to secondary activation. Here, the T_{EMXR3} cells expressed perforin and granzyme B (Fig. 28 D, right panels), and displayed equivalent lytic activity regardless of whether they were restimulated with anti-CD3 following sorting (Fig. 28 E, right panel). In contrast, T_{CMR7} cells did not express either perforin or granzyme B (Fig. 28 D, left panels) and displayed poor lytic activity if they were not restimulated following sorting (Fig. 28 E, left panels). However, in response to reactivation, T_{CMR7} cells gave rise to perforin- and granzyme-expressing cells. The induction of perforin and granzyme in these cells correlated directly to the acquisition of lytic activity (Fig. 28, D and E, left

panels), demonstrating a high degree of plasticity in their ability to reconstitute effector cell populations.

It was possible that T_{CMR7} cells were derived from populations that remained naïve during the first week of the primary activation. Therefore, in order to characterize differences in primary and secondary activation of T_{CMR7} cells, we compared cells that received IL-12 + IFN-α alone ("Naïve $CCR7^{hi}$") with cells that were activated with IL-12 + IFN-α in the presence of anti-CD3/anti-CD28 during the primary stimulation ("Memory $CCR7^{hi}$") (Fig. 30). We measured the ability of these cells to divide in response to secondary activation with anti-CD3 alone, as memory cells do not require costimulation for proliferation. Naïve $CCR7^{hi}$ cells derived from day 7 non activated cultures displayed a resting lymphocyte profile, failed to divide, and did not express granzyme B in response to anti-CD3 (Fig. 30, left panels). In contrast, memory $CCR7^{hi}$ cells, which received primary activation, displayed a blasting morphology and expressed granzyme B as a function of division in response to secondary anti-CD3 stimulation (Fig. 30, right panels). Therefore, the memory $CCR7^{hi}$ cells are a unique population of memory cells and not simply naïve cells that failed to receive primary activation. Taken together, these data demonstrate that IL-12-driven T_{EM} cells behave in a more terminally differentiated manner, with poor survival and proliferation and an inability to give rise to heterogeneous populations upon secondary activation. In contrast, IFN-α-regulated T_{CM} cells

displayed strong survival and division to secondary activation and were endowed with the ability to give rise to functional effector populations.

Reciprocal regulation of the IL-12R and IFNAR in T_{EM} and T_{CM}

In $CD4^+$ T cells, Th1 commitment is regulated by IL-12 through the induction of the IL-12Rβ2 subunit (5). However, the IFN-α/β receptor (IFNAR) is thought to be constitutively expressed on all cells, enabling them to respond in an autocrine fashion to IFN-α that is secreted during viral infections (65). Thus, selective IFNAR expression has not been examined during T cell differentiation. It was possible that differential sensitivities to IL-12 and IFN-a may account for the concomitant development of T_{EMXR3} and T_{CMR7} in response to combined activation with IL-12 + IFN-α allowing for selective outgrowth or differential programming of these two subpopulations. To address this possibility we measured both IL-12Rβ2 and IFNAR2 expression in response to TCR and cytokine activation. As expected, IL-12 + IFN-α stimulation dramatically enhanced IL-12Rβ2 expression by day 3 of culture (Fig. 31 A, left panel). Surprisingly, we also found that the IFNAR2 was markedly induced by IL-12 + IFN-α compared to the neutralized control (Fig. 31 A, right panel). Further, analysis of co-expression with CCR7 demonstrated the development of a distinct

sub-population of cells in which the IL-12Rβ2 was inversely expressed with CCR7. In contrast, CCR7 expression was directly correlated with induction of IFNAR2 on a sub-population of cells (Fig. 31 B). These data demonstrate that IL-12 and IFN-α differentially regulate the expression of their surface receptors, implicating a potential role for this response in determining effector or memory development.

Recent studies have suggested that increased proliferation during the primary expansion leads to more terminally differentiated phenotypes of $CD8^+$ T cells that acquire a T_{EM} phenotype (235, 236). If this observation is related to IL-12 responsiveness, then the development of effector and memory cells may hinge on the differential acquisition of cytokine responsiveness over the course of division. To examine this, we monitored expression of IL-12Rβ2 and IFNAR2 as a function of division on day 3 of culture. First, we observed that IFN-α slowed the progression of cell division compared to activation of cells with either neutralizing conditions or with IL-12, and this effect was evident even in the presence of IL-12 (Fig. 32). Further, IL-12Rβ2 expression was enhanced in response to IL-12 and IFN-α alone at each progressive division and even more dramatically induced in the presence of both IL-12 + IFN-α, indicating a cooperative role for IL-12 and IFN-α in regulating IL-12 responsiveness as a function of cell division (Fig. 32 A,B). In contrast, expression of IFNAR2 was

progressively diminished at each cell division, and this effect was marginally influenced by IL-12 and IFN-α on day 3 of culture.

At each division, cells progressively gained expression of IL-12Rβ2 while losing expression of IFNAR2. Thus, cells that had progressed through fewer divisions had the potential to be more responsive to IFN-α and less responsive to IL-12. I compared the expression of multiple effector and memory markers in the context of cytokine receptors on day 5 of culture in response to IL-12 + IFN-α stimulation (Fig. 33). On day 5 of culture, we observed a greater induction of IL-12Rβ2 and IFNAR2 by IL-12 + IFN-α than on day 3 (Fig. 33). Here, the magnitude of modulation of IL-12Rβ2 and IFNAR2 was dramatically increased, clearly demonstrating the induction of IL-12Rβ2 as a function of division and the retention of IFNAR2 on less divided cells. In addition, IL-12 + IFN-α preserved a subpopulation of cells that remained undivided compared to neutralizing conditions (Fig. 34 A). Strikingly, cells that had undergone extensive division displayed a T_{EM} phenotype marked by high expression of CXCR3 and IL-12Rβ2 and low levels of CCR7 and IFNAR (Fig. 34 B) and correlated with expression and secretion of perforin, granzyme B, and IFN-γ (Fig. 35). Those cells that were retained in the undivided population in response to IL-12 + IFN-α displayed characteristic T_{CM} phenotypes including low CXCR3 and high CCR7 expression and high IFNAR (Fig. 34 B) with lower levels of perforin, granzyme B, and IFN-γ

(Fig. 35). Cytokine titration revealed that at lower concentrations of IFN-α (10-100U/ml) the effects of IL-12 signaling dominate, promoting the development of T_{EMXR3} cells over that of T_{CMR7} cells (Fig. 36). However, even in the context of IL-12, as the concentration of IFN-α is increased, the development of T_{CMR7} cells is enhanced. This result suggests that IL-12 and IFN-α work independently to induce T_{EM} and T_{CM} phenotypes. As the ratios of these cytokines are shifted, the development of T_{CM} and T_{EM} follow accordingly. Collectively, these data demonstrate reciprocal regulation of IL-12Rβ2 and IFNAR2 on cells that commit to T_{EM} and T_{CM} fates. T_{EM} cells are derived from rapidly dividing cells and are regulated by IL-12 through the progressive acquisition of IL-12 responsiveness at each cell division. In contrast, T_{CM} cells developing in response to IFN-α are retained at earlier divisions, have the greatest sensitivity to IFN-α, and express the lowest levels of the IL-12Rβ2.

The reciprocal regulation of the IL-12R and IFNAR suggested that T_{CM} and T_{EM} development was balanced by differential responsiveness to cytokines. Thus, we measured cytokine-driven STAT phosphorylation as a function of division. First, total STAT1 and STAT4 protein was not altered in response to IFN-α and IL-12, respectively (Figure 34C). Further, we observed strong induction of phospho-STAT1 in response to IFN-α signaling as well as phospho-STAT4 in response to IL-12 in the total population (Figure 34 C) demonstrating a

clear responsiveness to cytokine treatment. Interestingly, IFN-α-mediated STAT1 phosphorylation was observed at various levels in all cells, including cells that had progressed through >4 divisions (Figure 34 D). In contrast, IL-12-driven STAT4 phosphorylation was not observed in division 0. However, as cell division progressed, levels of phospho-STAT4 increased 5-6 fold over that of the control cells. Taken together, the ratio of IL-12 : IFN-α responsiveness increased progressively with each cell division, correlating directly with the expression of the respective receptor ratios.

In addition to cell division, the strength of signal delivered through TCR engagement has also been implicated in the regulation of memory cell development (235) Some studies have suggested that a strong and prolonged antigen signal promotes efficient generation of T_{EM} leading to the eventual development of T_{CM} (171). An alternative view posits that T_{EM} and T_{CM} develop in parallel and are balanced by TCR signal strength in which some clones receive a strong signal leading to rapid proliferation and T_{EM} development, while other clones receive a weaker or less sustained signal leading to T_{CM} (174). In the present study, the *in vitro* priming conditions were based on concentrations of anti-CD3 traditionally used to promote efficient proliferation and effector cell development (237). However, this particular culture condition may only provide a single view of how cells interpret IL-12 and IFN-α signals as they divide and differentiate. I wished to determine whether the strength of TCR engagement

altered the balance between T_{EM} and T_{CM} as cells develop in response to IL-12 + IFN-α. To address this, I examined the effect of increasing concentrations of anti-CD3/anti-CD28 under either neutralizing conditions or with IL-12 + IFN-α.

Analysis of cell division revealed that increasing TCR stimulation promoted more efficient and rapid cell division (Fig. 37 A). However, IL-12 + IFN-α treatment slowed cell division at each concentration of anti-CD3 compared to the neutralized control. Although each culture condition induced proliferation, twice as many cells were retained within divisions 0-3 when activated with the lowest concentration of anti-CD3/anti-CD28 in the presence of IL-12 + IFN-α compared to cells activated under neutralizing conditions (Fig. 37 A). As described above (Fig. 34, 35), cells that remained in the undivided population exhibit all of the cell surface phenotypes and functional characteristics of T_{CM}. Despite the pronounced acceleration of cell division driven by increased TCR signal strength, IL-12 + IFN-α signaling slowed the progression of cell division and enhanced T_{CMR7} cells even at the highest concentration of anti-CD3/CD28 (Fig. 37 B).

We next assessed expression of the IL-12Rβ2 and IFNAR2 at each concentration of anti-CD3/anti-CD28. Under neutralizing conditions, the IL-12Rβ2 was expressed at low levels and remained constant at each concentration of anti-CD3/anti-CD28 and at each cell division (Fig. 38 A, top panels). A

similar trend was observed with IFNAR2 expression, although a 2-3 fold increase in expression was observed at 2.5 and 5 μg/ml compared to 1 μg/ml of anti-CD3/anti-CD28 (Fig. 38 B, top panels). However, in the presence of IL-12 + IFN-α, we observed a striking regulation of both the IL-12Rβ2 and IFNAR2 as a function of anti-CD3 concentration. First, IL-12Rβ2 remained low on cells that did not progress into the first division regardless of anti-CD3/anti-CD28 concentration (Fig. 38 A, lower panels). As cells divided, IL-12Rβ2 was markedly induced up to division 3 in the presence of 2.5 and 5 μg/ml, but not 1 μg/ml of anti-CD3/anti-CD28. In stark contrast, IFNAR2 was most highly induced in response to IL-12 + IFN-α on cells that were retained in the undivided population, and this effect was amplified in response to increasing anti-CD3/anti-CD28 (Fig. 38 B, lower panels). IFNAR2 levels then declined precipitously at each cell division. Taken together, we found that at division 0, IL-12Rβ2 remained low while IFNAR2 expression increased dramatically in response to IL-12 + IFN-α stimulation and as a function of increased TCR signal strength. These T_{CM} cells bear the highest potential for IFN-α sensitivity and the lowest potential for IL-12 responsiveness. As cells divided in response to TCR stimulation, they rapidly induced IL-12Rβ2 while simultaneously down regulating the IFNAR2. Cells in later divisions lose sensitivity to IFN-α while gaining responsiveness to IL-12, and these cells are characterized phenotypically and functionally as T_{EM}.

These results demonstrate that the strength of the TCR signal dictates the level at which cells become responsive to IL-12 and IFN-α. Thus, as the TCR signal threshold is altered, the reciprocal regulation of the IL-12R and IFNAR allow for the simultaneous commitment of precursors to the T_{EM} and T_{CM} fates.

Discussion

In this study, we have systematically examined the independent and combined roles of IL-12 and IFN-α in the regulation of human $CD8^+$ T cell differentiation. For the first time, we report non-redundant roles for IL-12 and IFN-α/β in the development of human $CD8^+$ T responses. Together, our data support a model in which IL-12 and IFN-α/β act in concert with signals 1 and 2 to promote the variegated development of effector and memory populations of human $CD8^+$ T cells (Fig. 39).

Recently, IL-12 and IFN-α were proposed to act in a redundant fashion to promote effector cell development in murine $CD8^+$ T cells (28, 29, 223). However, our examination of IL-12- and IFN-α–driven cytokine secretion and lytic activity in human $CD8^+$ T cells revealed striking dissimilarities. Here, IL-12, but not IFN-α, drove effector cell development characterized by marked secretion of IFN-γ and TNF-α and enhanced lytic activity. Surprisingly, while IFN-α did not inhibit IL-12-regulated effector cell development, IFN-α signaling

was not sufficient to promote this response in the absence of IL-12. Thus, IL-12 remains unique in its ability to drive effector functions and suggests that IL-12 and IFN-α are not redundant signals in this regard.

While human $CD8^+$ T cells clearly do not adopt effector functions in response to IFN-α, we found a remarkable role for IFN-α in driving memory cell development. In line with observations that $IFNAR^{-/-}$ $CD8^+$ T cells develop effector phenotypes but lack functional memory (195, 196), we found that IFN-α markedly enhanced human $CD8^+$ T cells displaying a T_{CM} surface phenotype. Several models have been proposed to explain the development of effector and memory cells from the same pool of naïve precursors (174). For example, one model proposes that T_{CM} cells develop in a linear manner from a pool of rested effector cells (171, 181). Alternatively, multiple studies suggest that effector and memory cells develop from distinct lineages which may arise as early as the first division after Ag encounter (186). In the present study, we found that cells activated with both IL-12 + IFN-α simultaneously segregated to both the T_{CM} and T_{EM} fates, suggesting that signaling by both cytokines regulates their parallel development rather than sequential development (Fig. 39). The first evidence for this model comes from our observation that signals derived from IFN-α program the development of a population of T_{CM} cells. These cells express high levels of the lymphoid homing receptor CCR7, lack immediate effector function, and display the hallmark characteristics of T_{CM} cells upon a secondary activation.

Importantly, development of T_{CM} in response to IFN-α/β occurs concomitantly with the generation of T_{EM} cells that develop in response to IL-12 when both cytokines are present.

Recent studies have linked T-bet and Eomes expression to the regulation of effector and memory cells, respectively (165, 191). In this regard, we observed a steady increase in T-bet expression in cells displaying effector phenotypes. This was similar to observations by Joshi *et.a*l. in which the development of effector responses was found to coincide with high expression of T-bet (192). Thus, it is possible that the balance between T_{CM} and T_{EM} occurs via skewing of Eomes:T-bet ratios. This is similar to a model proposed by Reiner and colleagues, in which expression of T-bet and Eomes are thought to be divergently expressed in effector and memory cell populations, respectively (165, 191, 193). In these studies, T-bet expression was found to be highest during the effector phase of infection, whereas enhancement of Eomes was seen in long-lived memory populations. Therefore, in human $CD8^+$ T cells, development of effector responses is directly linked to higher levels of T-bet expression while conversely inhibiting T_{CM} development.

The data presented here support a model of co-linear commitment to T_{EM} and T_{CM} that is regulated independently by IL-12 and IFN-α, respectively (Fig. 39). First, we found that IL-12 promoted T_{EM} development, and this program was not altered by the presence of IFN-α. Likewise, IFN-α, either alone or in combination with IL-12, enhanced T_{CM} commitment. Importantly, IL-12 and

IFN-α drove these divergent pathways through the reciprocal regulation of the IL-12R and the IFNAR. In the absence of innate cytokines, we clearly observed the outgrowth of cells that phenotypically resembled T_{EM} by their selective expression of CXCR3 and low expression of CCR7. However, in the absence of IL-12, these cells were incapable of effector functions. Thus, IL-12 acted in an instructive manner to regulate increased cytokine expression and lytic activity. This correlated precisely with the induction of the IL-12Rβ2, as its expression was markedly increased at each cell division in response to innate cytokines. Further, we observed an even greater enhancement of IL-12Rβ2 expression in response to increasing concentrations of anti-CD3. In contrast, IFN-α-regulated T_{CM} cells were primarily derived from sub-populations that were retained either in the undivided population or had undergone only 1-2 divisions rather than T_{EM} cells that divided extensively. As the initial TCR signal strength was increased, far fewer cells were retained at earlier divisions giving rise to T_{EM} phenotypes at later divisions. Importantly, IFN-α enhanced the proportion of T_{CM} at every concentration of anti-CD3 tested. These T_{CM} cells expressed low levels of IL-12R and high levels of IFNAR, endowing them with the greatest sensitivity to IFN-α.

TCR signal strength has been implicated as a regulatory component for effector and memory formation (235). In some models, low TCR engagement, either through decreased Ag concentration or an acute TCR activation favors the

development of memory over effector cells. Whereas, cells receiving strong or prolonged activation develop primarily into effector cells (238). These observations have been extended to *in vivo* infection models, in which acute infection promotes stronger memory responses than that of chronic infection (180, 239). Indeed, our results support this model as cells that map to the T_{CM} phenotype were primarily derived from sub-populations of cells that were retained either in the undivided population or had undergone only 1-2 divisions compared to T_{EM} cells that divided extensively. As the initial TCR strength was increased, far fewer cells were retained at earlier divisions giving rise to T_{EM} phenotypes at later divisions. Importantly, IFN-α enhanced the proportion of T_{CM} by modulating the TCR signal strength and slowing the progression of cell division in some cells. The cyclin dependent kinase family members CDK2 and CDK6 have been implicated in the rapid division of memory cells to secondary activation (240). In addition, the CDK inhibitor, p27kip1 has been shown to be highly expressed in cells which do not actively divide (240, 241). Thus, differential regulation of these factors by IL-12 and IFN-α may explain the variegated behavior of cells as they commit to T_{EM} and T_{CM} phenotypes.

This study provides new and important insight into the development of effector and memory human $CD8^+$ T cells. Although these responses were derived from cells developing in response to signals 1 and 2 *in vitro*, this approach allowed us to methodically examine the direct and independent roles of

IL-12 and IFN-α/β in the generation of $CD8^+$ T cell effector and memory responses. Our data support a cooperative model in which TCR strength and innate cytokines act as rheostats to fine tune the balance between effector and memory cell development. If the demands are great and antigen levels are high, IL-12 dominates, and effector cells develop at the expense of memory cells. Alternatively, when Ag levels wane, cytokines then act to modulate the development of both effector and memory subpopulations. While this study suggests that T_{EM} and T_{CM} development can occur in parallel, it does not rule out the possibility that T_{CM} can be derived from rested T_{EM} cells as many recent studies have suggested (181, 182, 242). Nonetheless, the present study has broad implications to the field of $CD8^+$ T cell biology and suggests that optimal memory generation requires a precise balance of TCR signals and innate cytokines. This study marks the first discovery of independent roles for IL-12 and IFN-α/β in the development of human $CD8^+$ T cell responses and underscores the importance of these two cytokines in regulating effector and memory responses to infection.

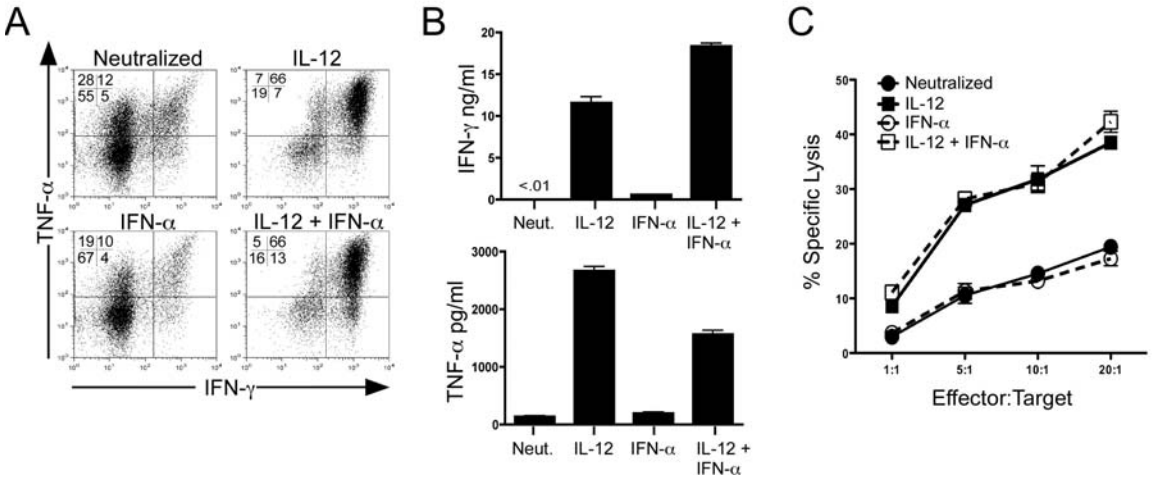

Figure 19. IL-12 but not IFN-α is sufficient to program human CD8$^+$ T cell effector functions. (**A**) Intracellular expression of human IFN-γ, and TNF-α from day 7 *in vitro* polarized human CD8$^+$ T cells. Rested cells were reactivated for 4hrs with PMA and ionomycin in the presence of brefeldin A, and IFN-γ and TNF-α were assessed by intracellular stain and flow cytometric analysis. Data are gated on live, CD8$^+$ cells. (**B**) Day 7 polarized cells were left un-stimulated or stimulated with anti-CD3 for 24hrs, and supernatants were harvested for ELISA. (**C**) Characterization of CTL activity by ^{51}Cr-release assay. Day 7 polarized CD8$^+$ T cells were incubated for 4hrs with ^{51}Cr-labeled THP-1 cells (target) at the E:T ratios shown. CTL activity was assessed by quantification of ^{51}Cr released into the supernatant by β emission. These experiments were performed with 5 different healthy donors with similar results.

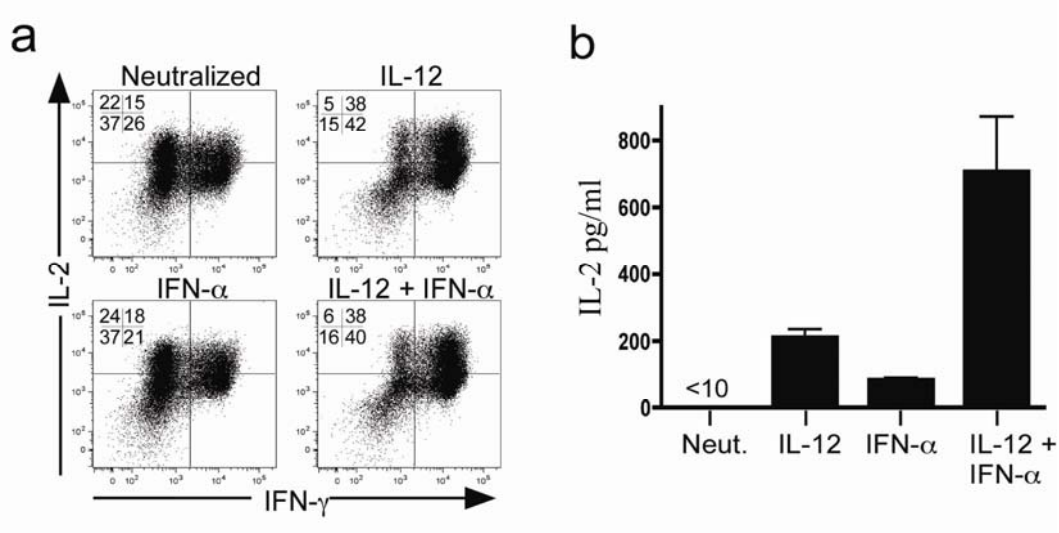

Figure 20. Regulation of cytokine secretion by IL-12 and IFN-α. (**A**) Intracellular expression of IFN-γ and IL-2 from day 7 *in vitro* polarized human $CD8^+$ T cells. $CD8^+$ $CD45RA^+$ sorted cells were polarized under neutralized, IL-12, IFN-α or IL-12+IFN-α conditions to day 7, harvested and rested overnight. Rested cells were reactivated for 4hrs with PMA and ionomycin in the presence of brefeldin A, and IFN-γ and IL-2 were assessed by intracellular staining and flow cytometric analysis. Data are gated on live, $CD8^+$ cells. (**B**) Day 7 polarized cells were stimulated with anti-CD3 for 24hrs, and supernatants were harvested for ELISA.

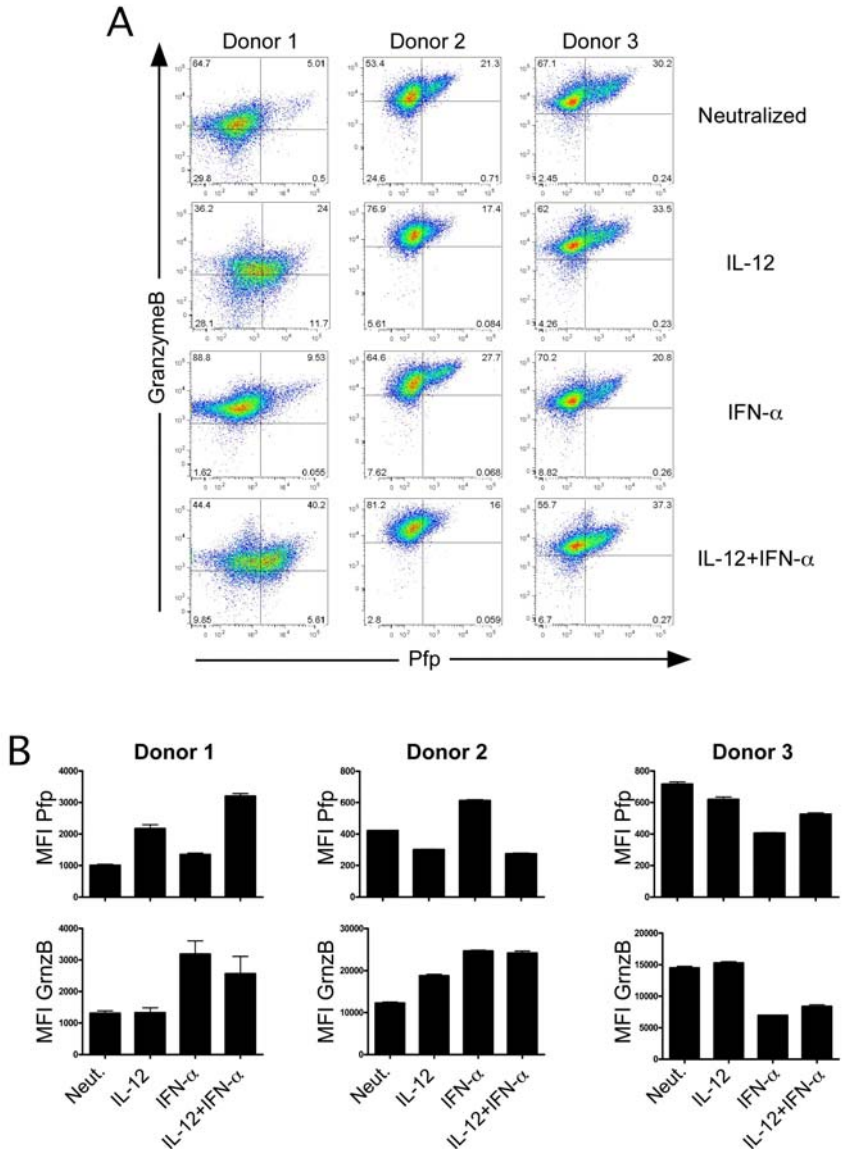

Figure 21. IL-12 and IFN-α/β directed Perforin and Granzyme B Expression. $CD8^+$ $CD45RA^+$ were purified from peripheral blood and cultured in the presence or absence of polarizing cytokines for 7 days. (**A**) Rested cells were harvested and stained for intracellular content of perforin and granzyme B in 3 separate donors and assessed for expression by flow cytometry. (**B**) Quantification of mean fluorescence intensities for perforin (top panels) or granzyme B (bottom panels) in three separate human donors.

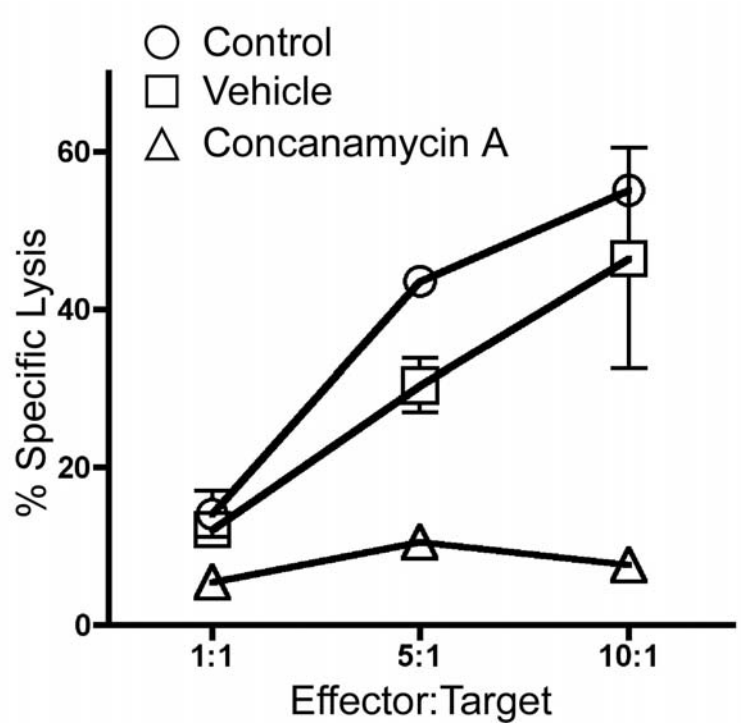

Figure 22. IL-12 driven CTL activity is perforin-dependent. Day 7, IL-12 polarized cells were used as effectors in a ^{51}Cr re-directed lysis assay. THP-1 target cells were prepared as described in figure 1 and effector cells were either left untreated (open circle) or treated with concanamycin A (1μM) for 90 minutes (open triangle). As a control for the vehicle, effectors were treated in 1μM DMSO (open square). Cells were incubated with target cells at the indicated effector:target ratios for 4hrs and CTL activity was assessed by quantification of release of ^{51}Cr into the supernatant by β emission counting.

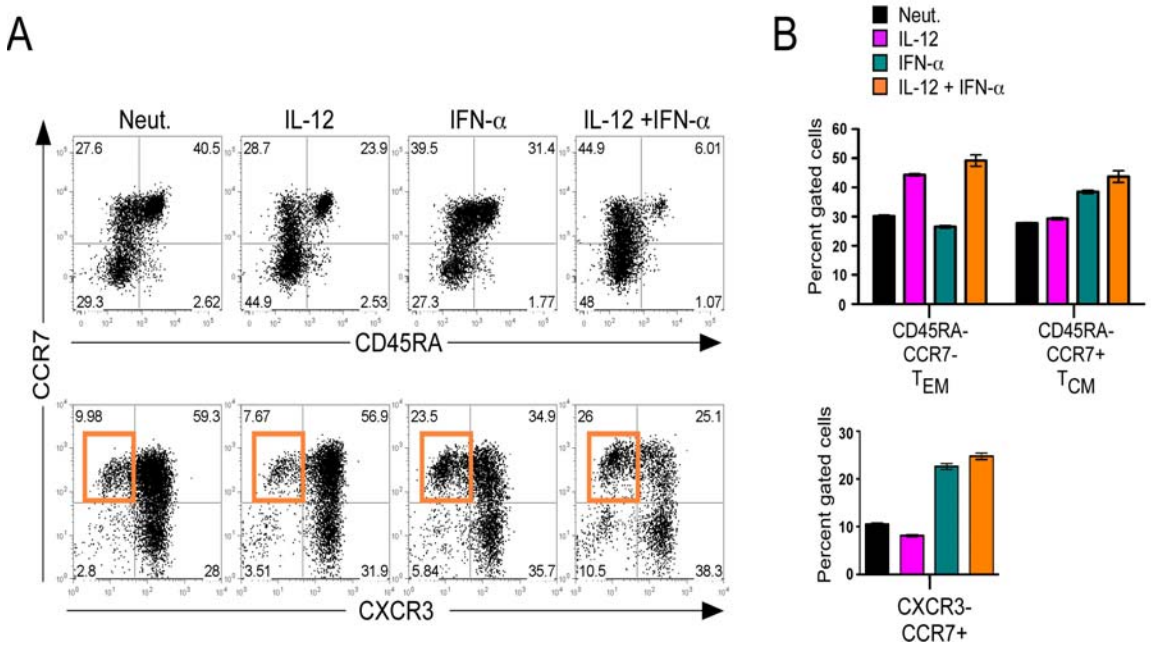

Figure 23. Regulation of human CD8$^+$ T_{CM} development by IFN-α. Day 7 cytokine polarized cells were stained with a panel of anti-human monoclonal antibodies, including CCR7, CD45RA, and CXCR3, to assess memory and effector phenotypes. (**A**) Analysis of surface markers CCR7 and CD45RA (top panel) and CCR7 and CXCR3 (bottom panel). The induction of CCR7^{hi}/CXCR3^{lo} cells by IFN-α is indicated by the orange gate. (**B**) Quantification of human effector and memory profile (top) and chemokine receptor profile (bottom) regulated by IL-12 and IFN-α. Black, (neutralized), magenta (IL-12), teal (IFN-α), orange (IL-12+IFN-α). These experiments were performed with 7 different healthy donors with similar results.

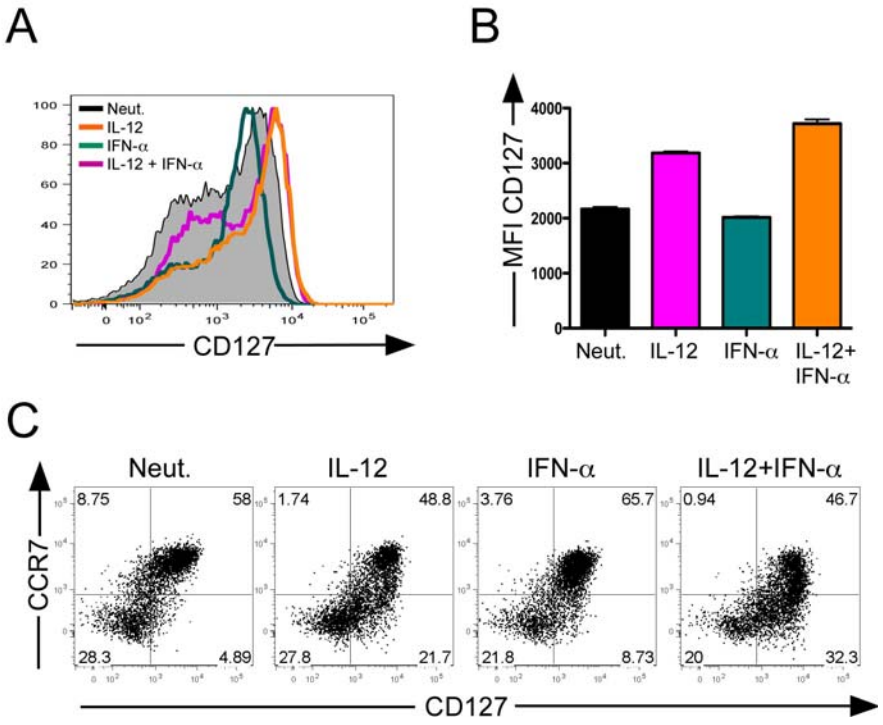

Figure 24. Differential regulation of IL-7Rα expression by IL-12 and IFN-α. $CD8^+$ $CD45RA^+$ sorted cells were polarized under neutralized, IL-12, IFN-α or IL-12+IFN-α conditions to day 7, harvested and rested overnight. (**A**) Cells were stained with anti-human monoclonal antibodies to CD127 and surface expression was assessed in the total population of live $CD8^+$ T cells. (**B**) Mean fluorescence intensity of cytokine polarized cells as expressed by histogram overlays for CD127. (**C**) Bi-variant analysis of CCR7 and CD127 expression in response to IL-12 and IFN-α.

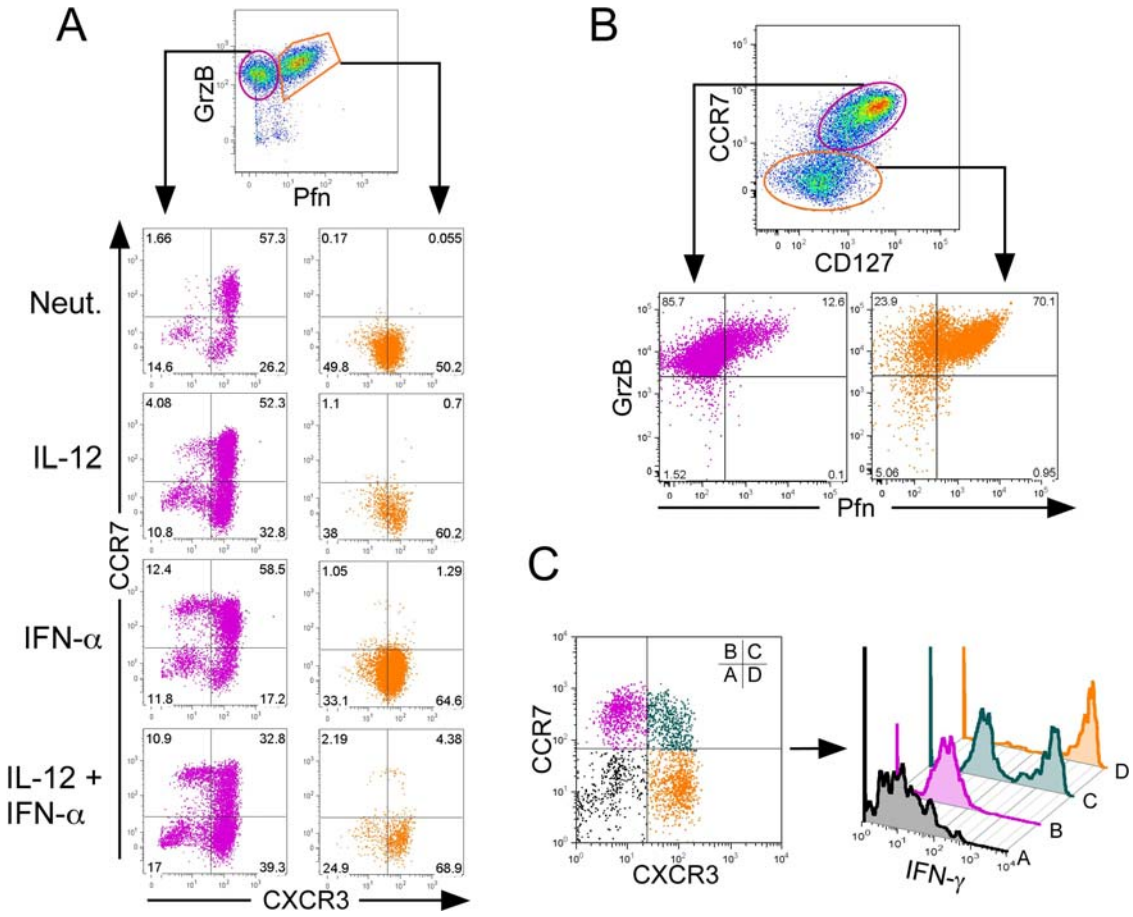

Figure 25. Human CD8$^+$ T_{EM} and T_{CM} cells display distinct effector properties. Day 7 cytokine polarized cells were assessed for surface marker and cytokine expression. (**A**) Cells were gated on live CD8$^+$ cells, and perforin and granzyme B levels were assessed. Cells were gated on either granzyme B single positive cells (magenta) or perforin-granzyme B double positive cells (orange) and examined for CCR7 and CXCR3 expression (lower panels). (**B**) Live CD8$^+$ cells were gated through either CCR7^{hi}, CD127^{hi} cells (magenta) or CCR7 and CD127^{lo} cells (orange) and assessed for perforin and granzyme B expression (lower panels). (**C**) PMA and Ionomycin activated cells were gated on either CCR7^{lo}/CXCR3^{lo} (A, black), CCR7^{hi}/CXCR3^{lo} (B, magenta), CCR7^{hi}/CXCR3^{hi}, (C, teal) or CCR7^{lo}/CXCR3^{hi} (D, orange) and examined for IFN-γ expression by intracellular staining (right panel).

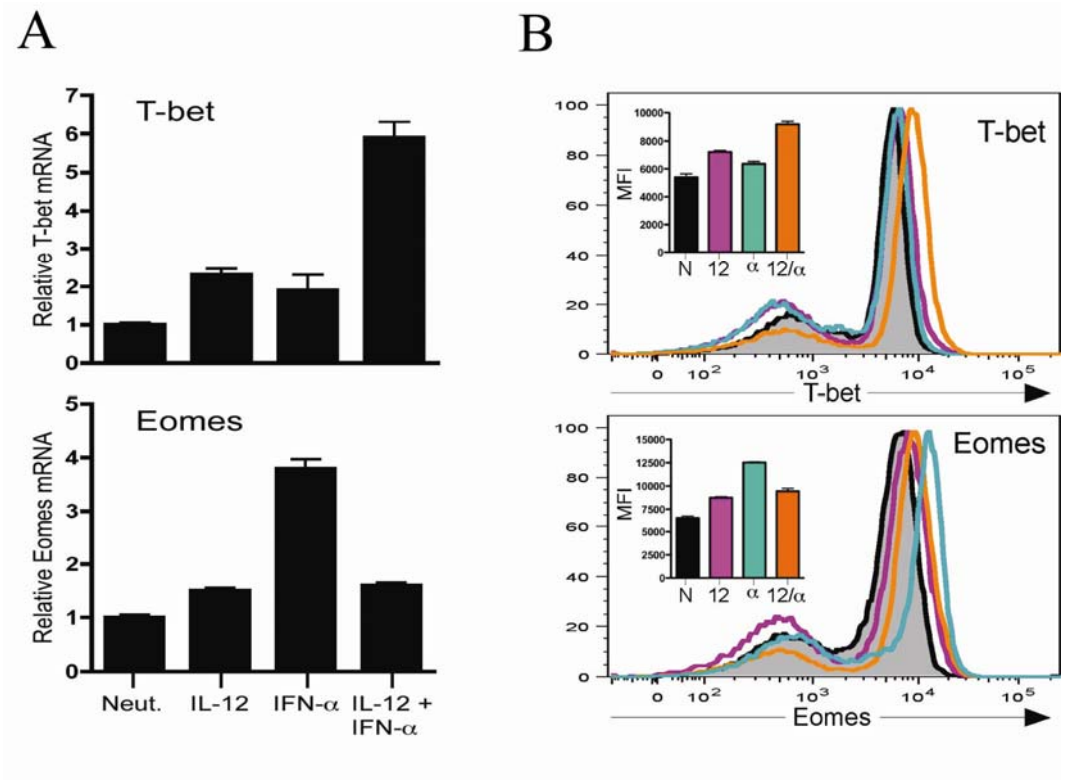

Figure 26. Differential regulation of T-bet and Eomes expression by IL-12 and IFN-α. $CD8^+$ $CD45RA^+$ sorted cells were polarized under neutralized, IL-12, IFN-α or IL-12+IFN-α conditions to day 7, harvested and rested overnight. (**A**) Cells were harvested for RNA and expression of T-bet (top) and Eomes (bottom) was assessed by qRT-PCR in relation to the GAPDH control. (**B**) Day 7 cells were stained intracellularly for T-bet (top) or Eomes (bottom) and assessed for expression by flow cytometry. Mean fluorescence intensity of cytokine polarized cells as expressed by histogram overlays for T-bet and Eomes are inset.

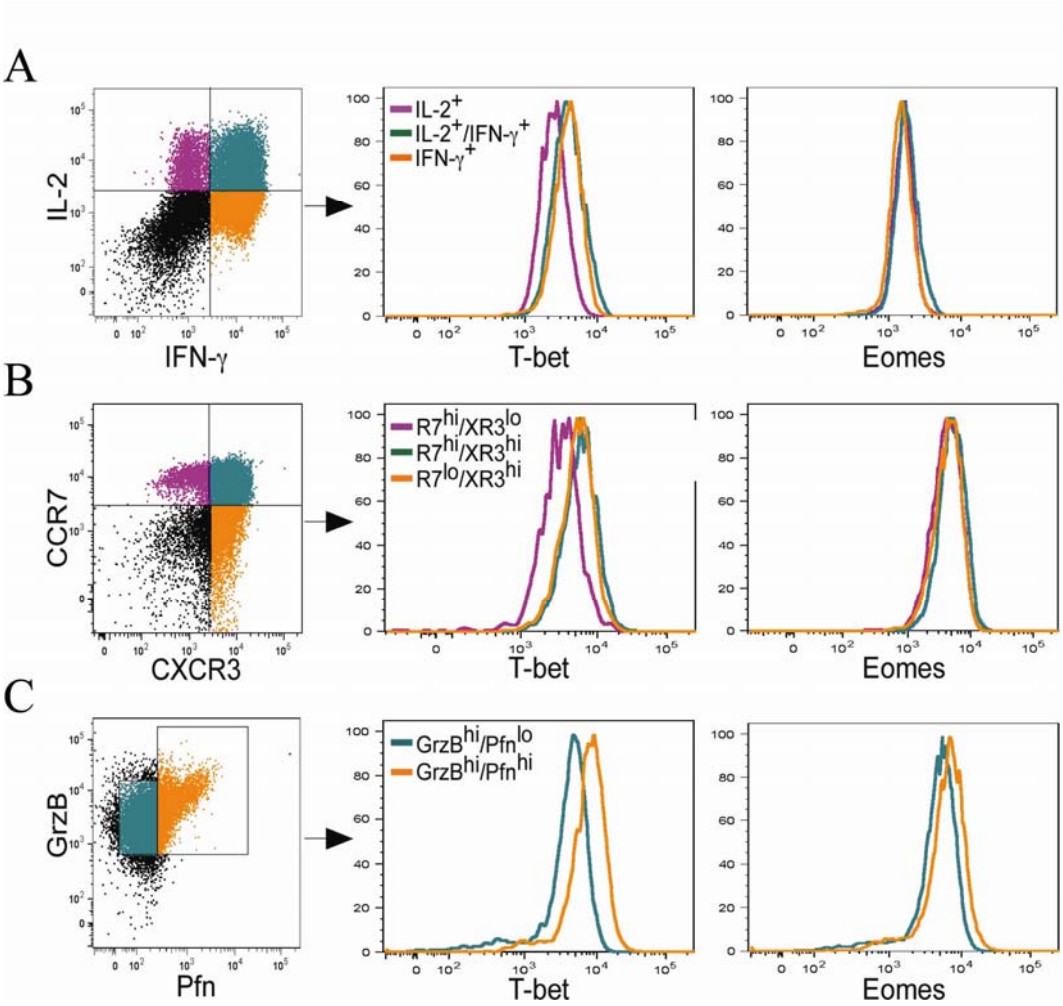

Figure 27. T-bet expression correlates to the T_{EM} phenotype in Human $CD8^+$ T cells. Examination of T-bet expression as a function of cytokine, chemokine receptor and lytic effector molecule expression. (**A**) Day 7 IL-12+IFN-α polarized Cells were activated for 4hrs with PMA and Ionomycin in the presence of brefeldin A, and live $CD8^+$ gated cells were examined for IL-2 and IFN-γ expression by bi-variant dot plot analysis. Cells were gated as shown and assessed for T-bet expression as a function of cytokine expression. (B and C) Resting $CD8^+$ cells were gated based on expression of CCR7 and CXCR3 (**B**) and perforin and granzyme (C) and expression of T-bet was assessed by intracellular flow cytometry.

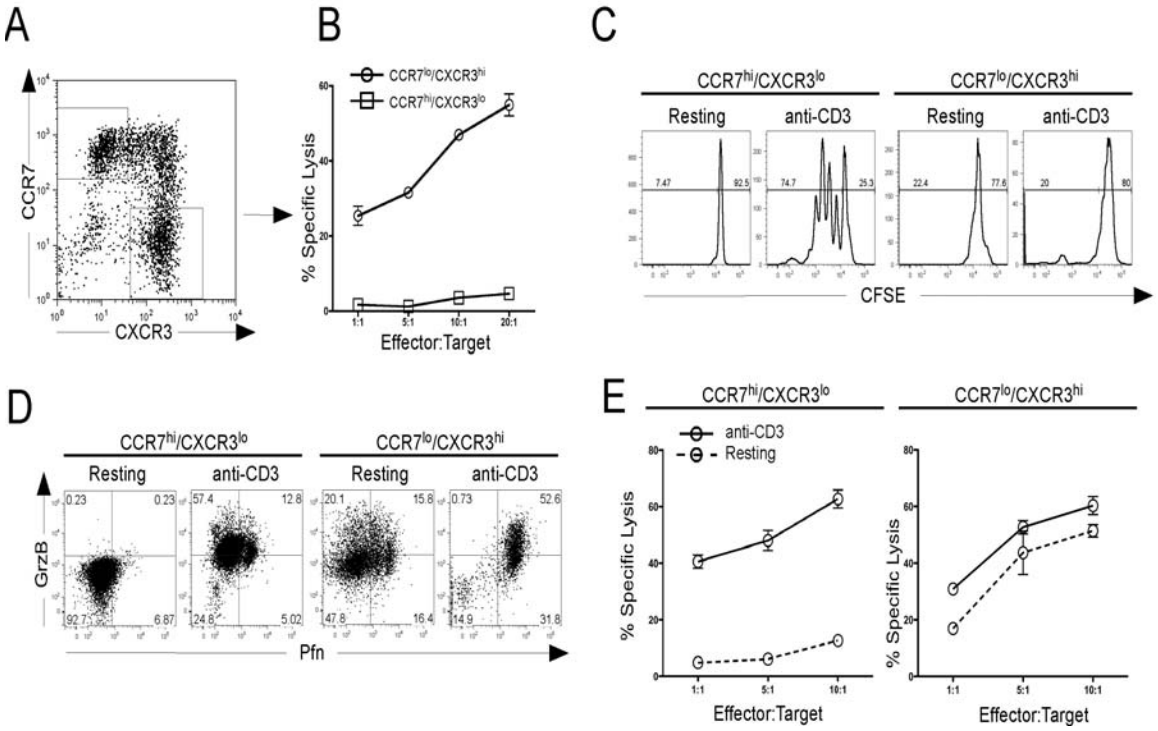

Figure 28. CCR7 and CXCR3 expression demarcates distinct sub-populations of human CD8$^+$ T cells with functional effector and memory properties. (**A**) Sorted CD8$^+$ CD45RA$^+$ cells were activated with IL-12 + IFN-α to day 7. Cells were then sorted into separate CCR7^{hi}/ CXCR3^{lo} or CXCR3^{hi}/CCR7^{lo} populations. (**B**) Sorted cells were rested overnight in the absence of IL-2 and subjected to a ^{51}Cr re-directed lysis assay with THP-1 target cells at the indicated E:T ratios. (**C**) Sorted cells were labeled with CFSE and left untreated (resting) or activated with 1.5μg/ml plate bound anti-human CD3 for 3 days (anti-CD3). On day 3, cells were assessed for proliferation by CFSE dilution. (**D**) Sorted cells were activated as described in panel **c** and examined at day 3 for expression of perforin and granzyme B by bi-variant dot plot analysis. (**E**) Sorted cells were either left untreated (resting) or activated with 1.5μg/ml anti-CD3 for 3 days (anti-CD3). On day 3, CCR7^{hi}/ CXCR3^{lo} cells (left panel) and CXCR3^{hi}/CCR7^{lo} cells (right panel) were subjected to a re-directed lysis assay as described above. Each of these experiments was performed twice with two separate healthy donors with similar results.

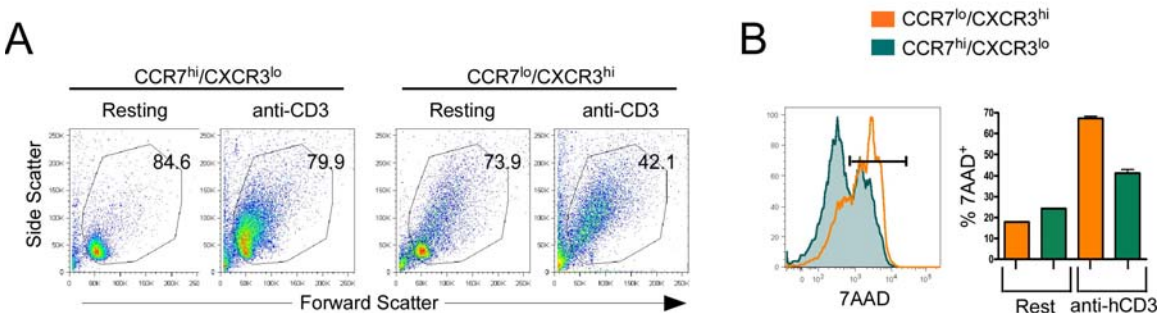

Figure 29. CCR7hi/CXCR3lo sorted cells maintain viability following restimulation. $CD8^+$ $CD45RA^+$ cells were polarized to day 7 with IL-12 + IFN-α and sorted into either $CCR7^{hi}/CXCR3^{lo}$ or $CXCR3^{hi}/CCR7^{lo}$ populations. Sorted cells were either left untreated (resting) or activated on 1.5μg/ml anti-CD3-coated plates for 3 days. At day 3, $CCR7^{hi}$ sorted (left panel) or $CXCR3^{hi}$ sorted (right panel) cell survival was assessed by forward and side scatter bi-variant dot plot profiles (**A**), and by 7-amino-actinomycin D staining (**B**).

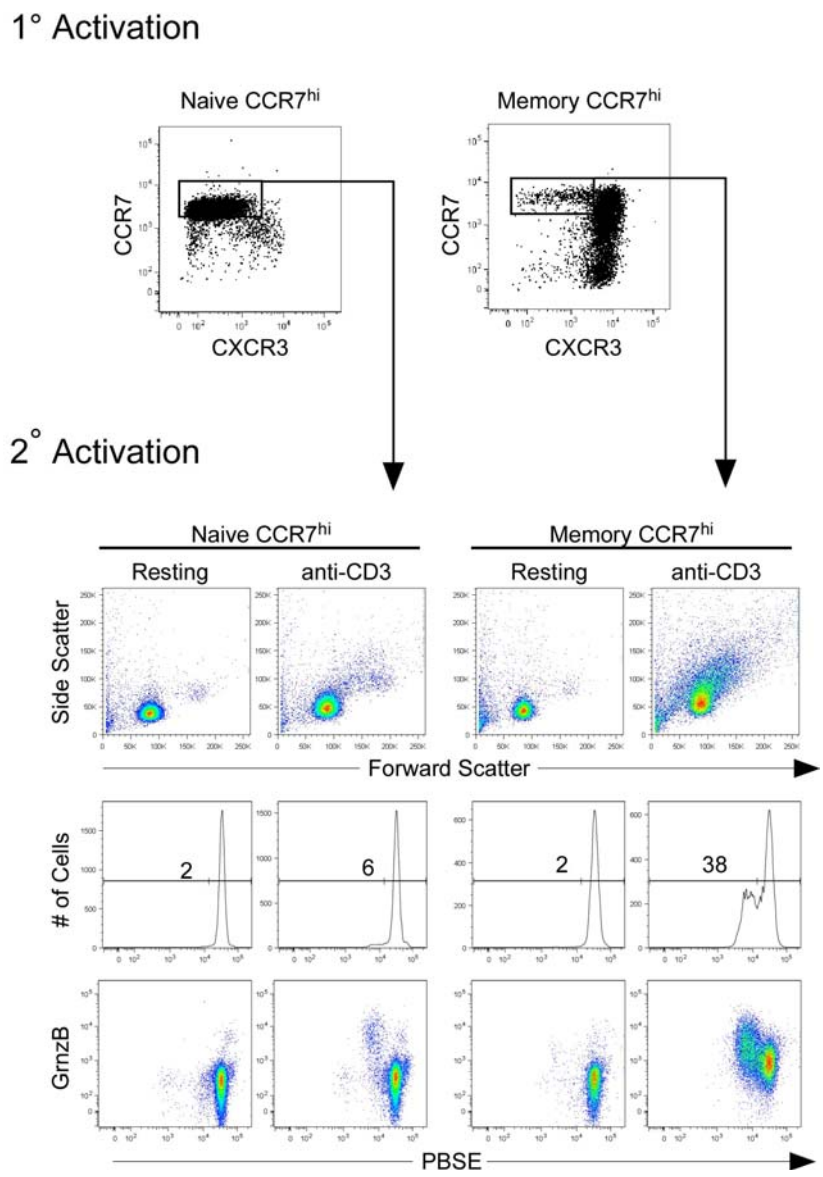

Figure 30. CCR7hi/CXCR3lo T_{CM} cells display functional memory responses and are distinct from naïve cells. $CD8^+$ $CD45RA^+$ cells were cultured to day 7 with IL-12 + IFN-α either in the absence (Naïve) or presence of anti-CD3/anti-CD28 (Memory). $CCR7^{hi}/CXCR3^{lo}$ cells were then sorted from these cultures (1^o Activation, top panels), labeled with PBSE, and then either left untreated (resting), or restimulated with 1.5μg/ml anti-hCD3 for 24hrs. Cells were assessed for forward and side scatter profile (2^o Activation, top panel), proliferation by PBSE dilution (2^o Activation, middle panel) or granzyme B as a function of division (2^o Activation, bottom panel).

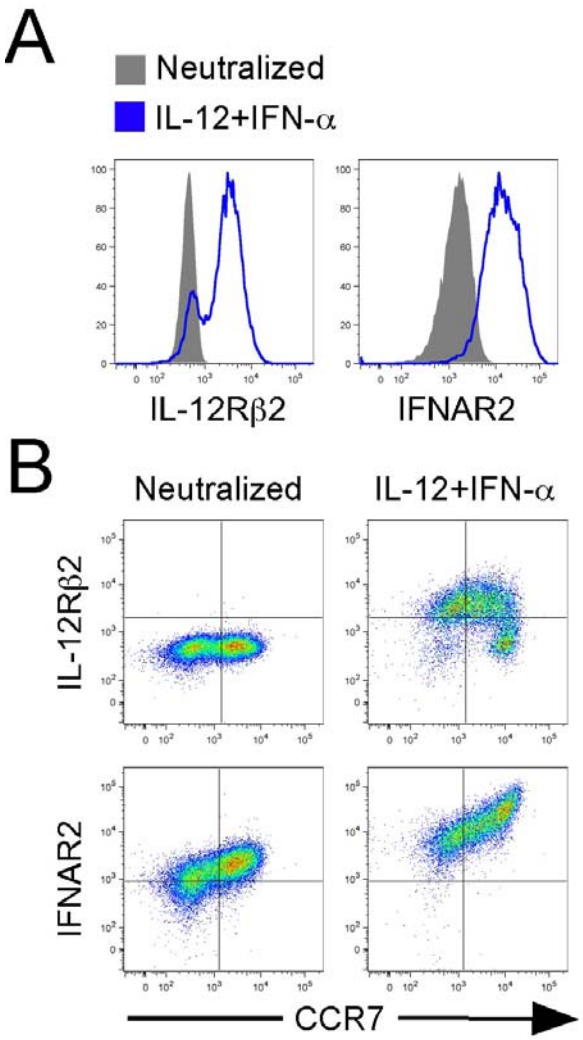

Figure 31. Reciprocal responsiveness to IL-12 and IFN-α/β correlates to development of T_{EM} and T_{CM} cells .
(**A**) $CD8^+$ $CD45RA^+$ cells were polarized to day 3, under neutralized or IL-12+IFN-α conditions. Activated cells were then assessed for surface expression of the IL-12Rβ2 or IFNAR2 or (**B**) bi-variant expression of CCR7,IL-12Rβ2 and IFNAR2 by flow cytometry.

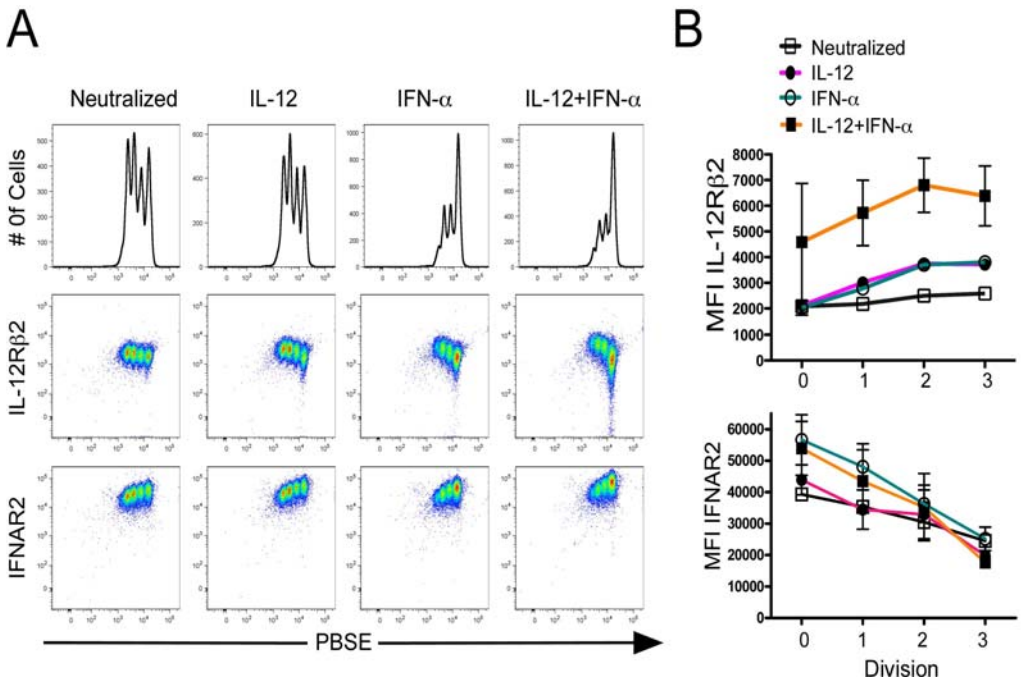

Figure 32. Reciprocal regulation of the IL-12Rβ2 and IFNAR2 on developing T_{EM} and T_{CM} cells. $CD8^+$ $CD45RA^+$ sorted cells were labeled with CFSE and cultured in the presence or absence of cytokines for 3 days. (**A**) Examination of expression of IL-12Rβ2 and IFNAR2 by division on day 3 of activation. Top: division of total live $CD8^+$ population; middle: IL-12Rβ2 expression; bottom: IFNAR2 expression. (**B**) Quantification of mean fluorescence intensities as a function of division for IL-12Rβ2 (top panel) or IFNAR2 (bottom panel). Black, open square (neutralized), magenta, closed circle (IL-12), teal, open circle (IFN-α), orange, closed square (IL-12+IFN-α).

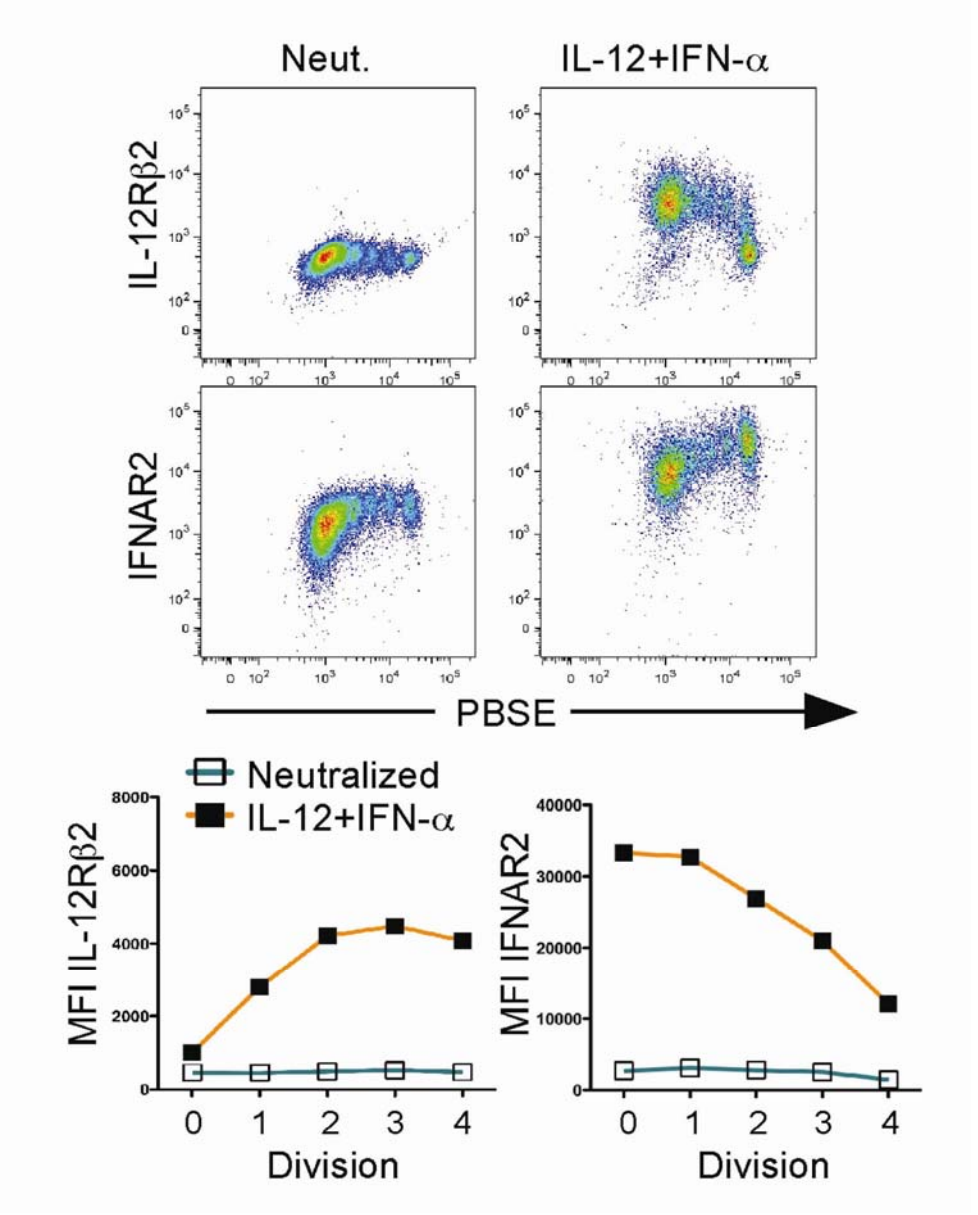

Figure 33. Expression of the IL-12R and IFNAR correlates to development of T_{EM} and T_{CM} cells . $CD8^+$ $CD45RA^+$ were labeled with PBSE and cells were polarized under neutralized or IL-12+IFN-α conditions. Polarized cells were cultured to day 5 and assessed by flow cytometry. On day 5, cells assessed for IL-12Rβ2 and IFNAR2 expression as a function of division (top), and relative mean fluorescence intensity was quantified (bottom).

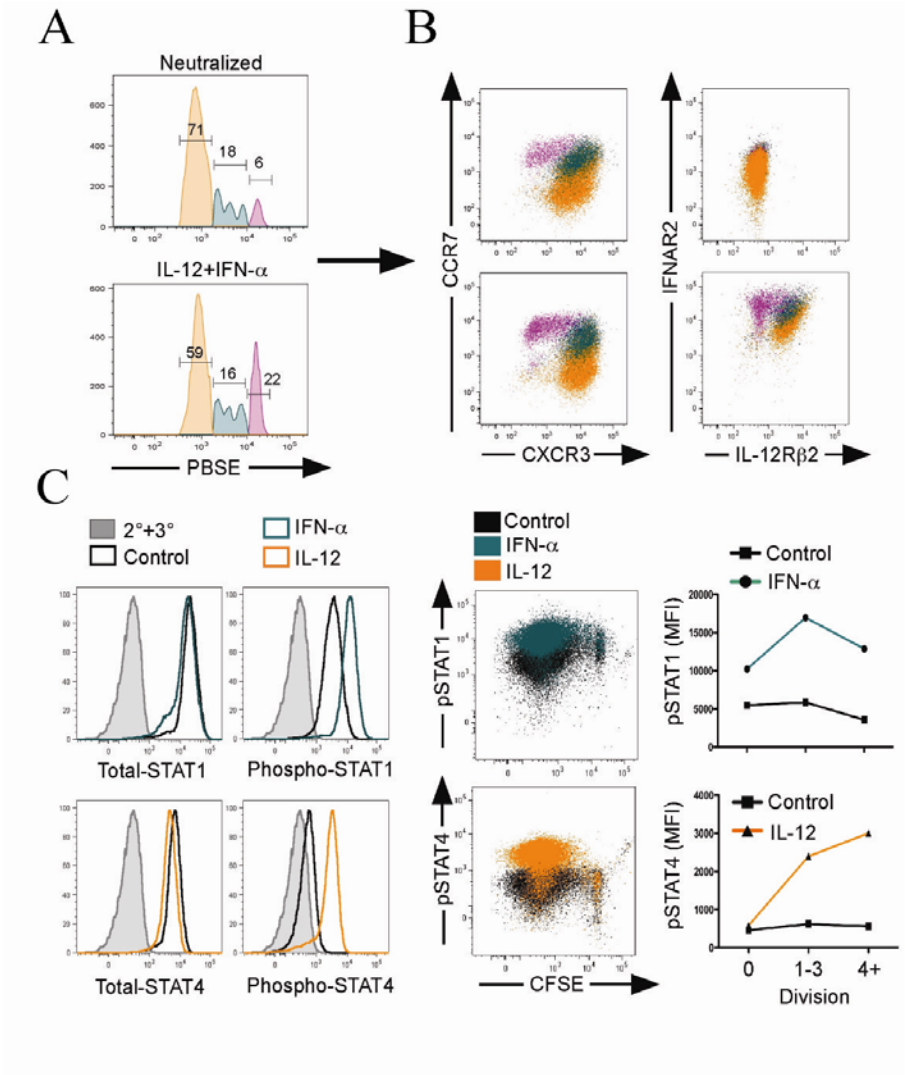

Figure 34. Reciprocal responsiveness to IL-12 and IFN-α/β in correlates to development of T_{EM} and T_{CM} cells . $CD8^+$ $CD45RA^+$ cells were culture to Day5 in the presence of PBSE under neutralizing or IL-12 + IFN-α conditions. (**A**) Day5, PBSE labeled cells were gated on division 0 (magenta), division 1-3 (teal), or division 4+ (orange), and chemokine receptor (middle panels) and cytokine receptor (right panels) expression was measured. (**B-C**) Day 5 cells were polarized with IL-12+IFN-α, reactivated with cytokines for 30 min. and assessed for intracellular STAT or phospho-STAT protein expression. (**C**) Total STAT, STAT4 (left panels) or phospho-STAT1, phospho-STAT4 (right panels) expression in live $CD8^+$ gated cells. 2°+3° antibody alone (grey), unstimulated (Black), IFN-α- (teal) or IL-12-treated (orange). (F) Dot plot overlays of phospho-STAT1 (Top panel, right) or phospho-STAT4 (bottom panel, left) expression as a function of CFSE dilution. Unstimulated (black), IFN-α- (teal) or IL-12-treated (orange). Quantification of mean fluorescence intensity as a function of CFSE dilution (right panels); phopho-STAT1 (top), phospho-STAT4 (bottom).

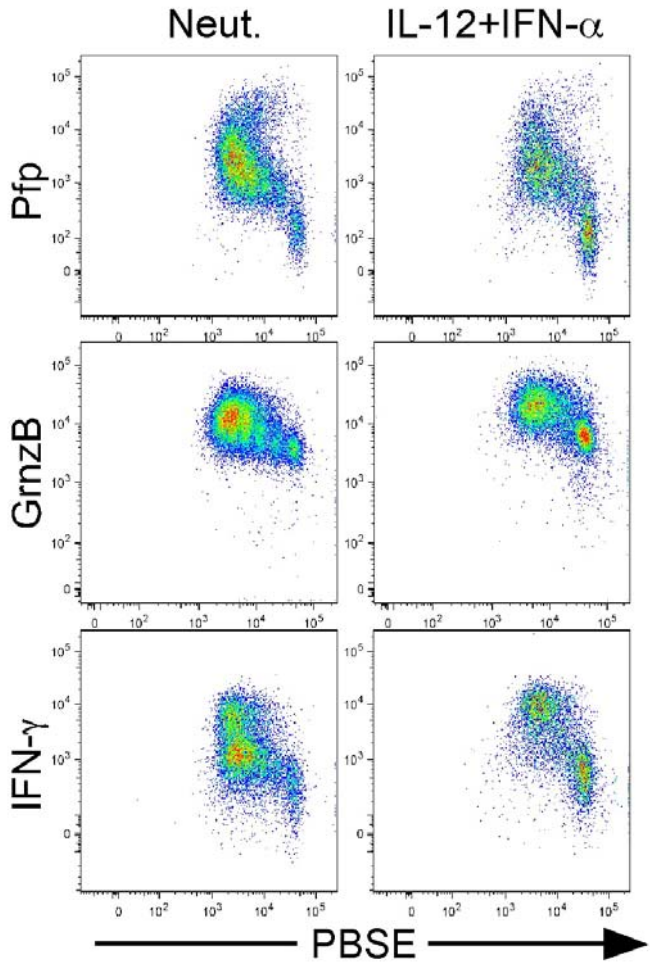

Figure 35. Development of effector cytokine expression is directly related to cellular proliferation. $CD8^+$ $CD45RA^+$ sorted cells were labeled with PBSE and polarized under neutralized or IL-12+IFN-α conditions to day 3. Cells were stained intracellularly for cytokines and assessed for expression of perforin (top), granzyme B (middle) or IFN-γ (bottom) as a function of division by flow cytometry.

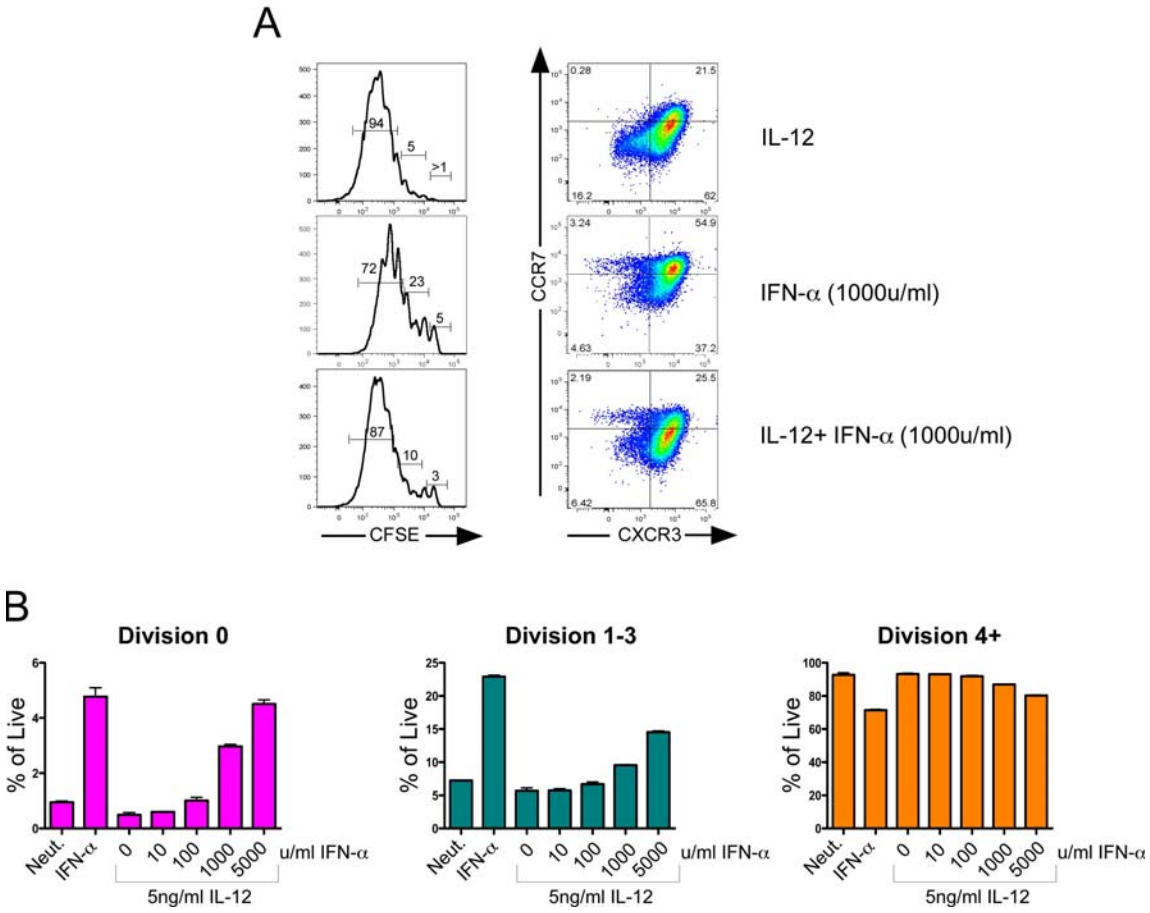

Figure 36. Development of T_{EM} and T_{CM} populations of human $CD8^+$ T cells is directly affected by the concentration of IL-12 and IFN-α. $CD8^+$ $CD45RA^+$ cells were purified, labeled with CFSE, and cultured to day 7 under polarizing cytokine conditions. Cells were either left untreated (Neut.), treated in the presence of IFN-α alone (1000u/ml) or treated with IL-12 and a titration of IFN-α at 0u/ml, 10u/ml, 100u/ml, 1000u/ml or 5000u/ml. (**A**) Rested cells were harvested and assessed for division by CFSE dilution (left panel) or development of $CCR7^{hi}$ T_{CM} and $CXCR3^{hi}$ T_{EM} (right panel) either in the presence of IL-12 alone (top panel) IFN-α alone (middle panel) or IL-12+IFN-α (bottom panel). (**B**) Quantification of the percent of live cells falling within either the division 0 (left panel, magenta), divisions 1-3 (middle panel, teal) or divisions 4+ (right panel, orange) as a function of primary cytokine polarizing conditions.

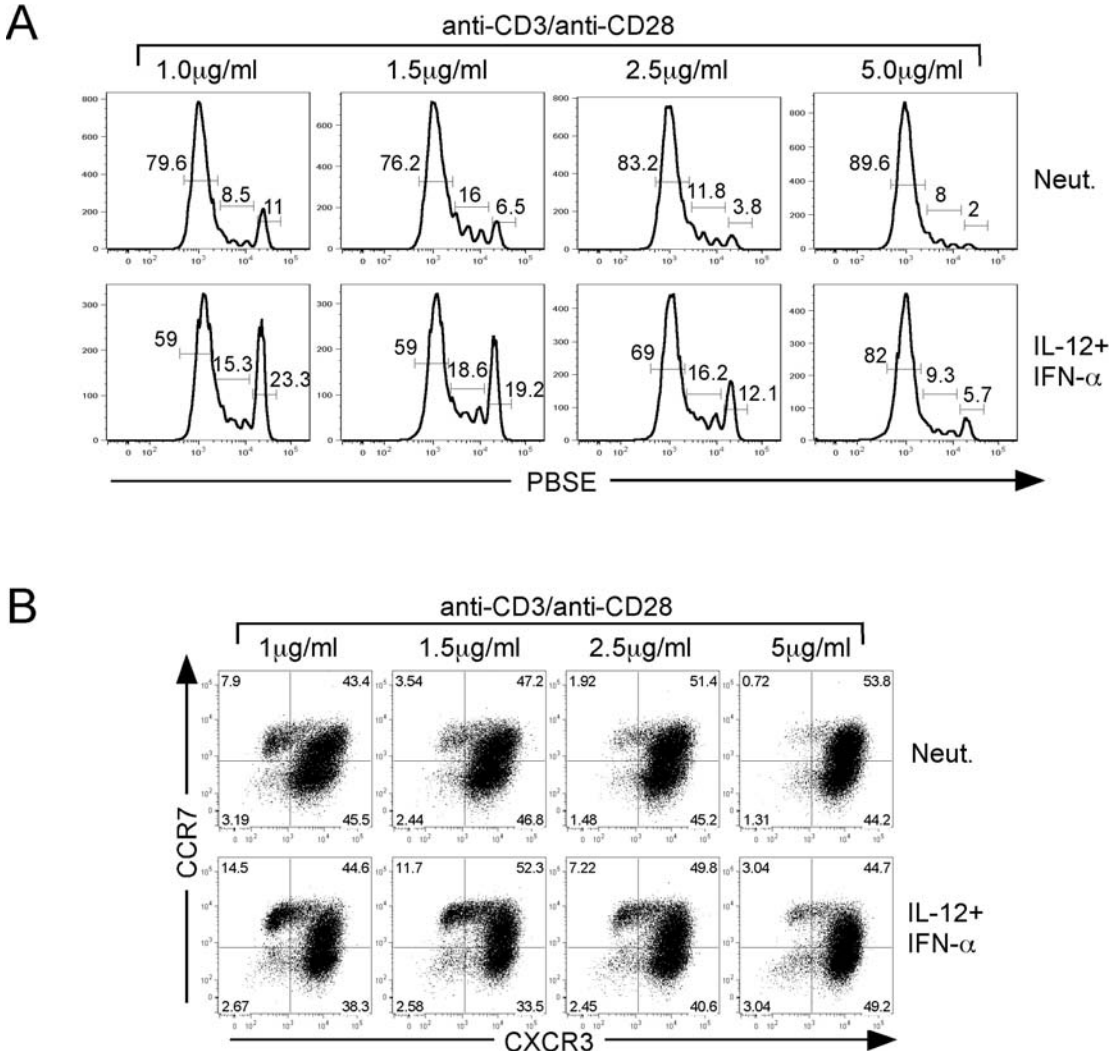

Figure 37. TCR signal strength regulates cytokine-dependent T_{CM} development. $CD8^+$ $CD45RA^+$ sorted cells were labeled with PBSE and polarized under either neutralizing or IL-12 + IFN-α conditions with 1μg/ml, 1.5μg/ml, 2.5μg/ml or 5μg/ml α-hCD3 and α-hCD28 for 5 days. (**A**) Assessment of division by PBSE dilution as a function of primary activation strength. The percentages of cells that are contained within each gate are indicated above the gate. (**B**) Cells were analyzed for expression of CXCR3 and CCR7 by bi-variant dot plot analysis.

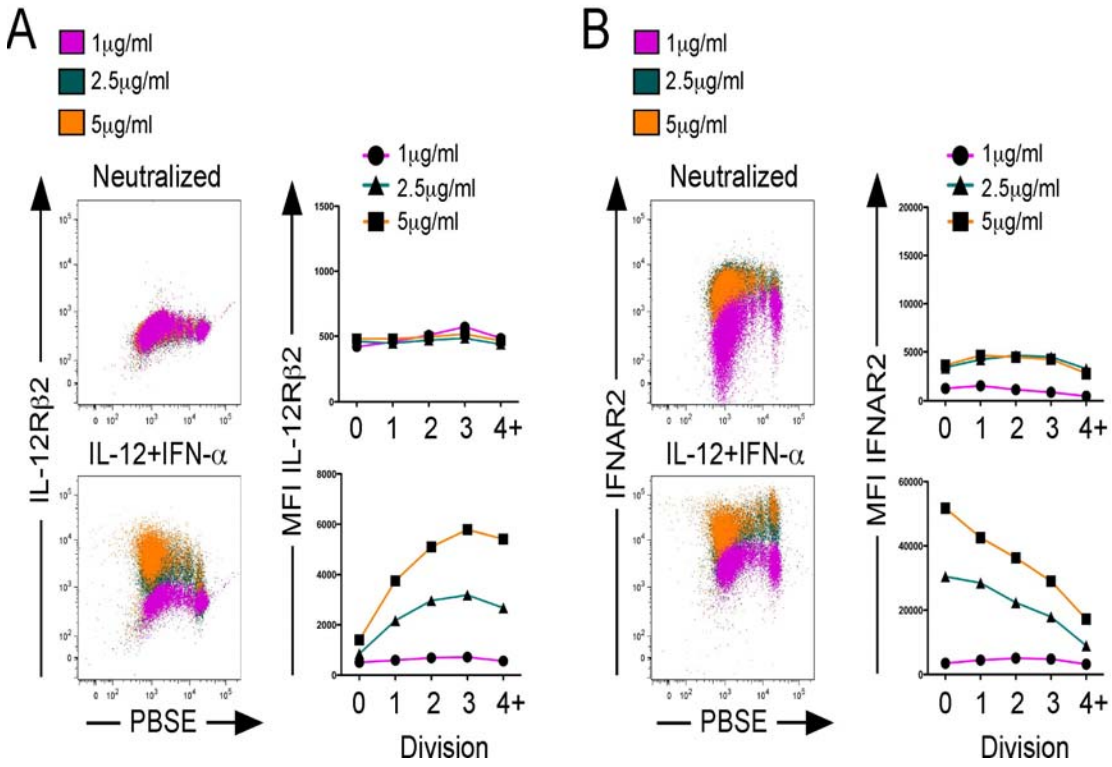

Figure 38. The development of human CD8$^+$ T_{EM} and T_{CM} cells is regulated by both cytokine signaling and strength of primary activation.
CD8$^+$ CD45RA$^+$ sorted cells were labeled with PBSE and polarized under neutralizing or IL-12 + IFN-α conditions with 1μg/ml, 2.5μg/ml or 5μg/ml anti-hCD3 and anti-hCD28 for 5 days. Cells were assessed for expression of IL-12Rβ2 (**A**) or IFNAR2 (**B**) as a function of division. Quantification of mean fluorescence intensity is displayed as a function of division for IL-12Rβ2 (A, right panels) or IFNAR2 (B, right panels) in neutralized (top panels) or IL-12+IFN-α (bottom panels) cells.

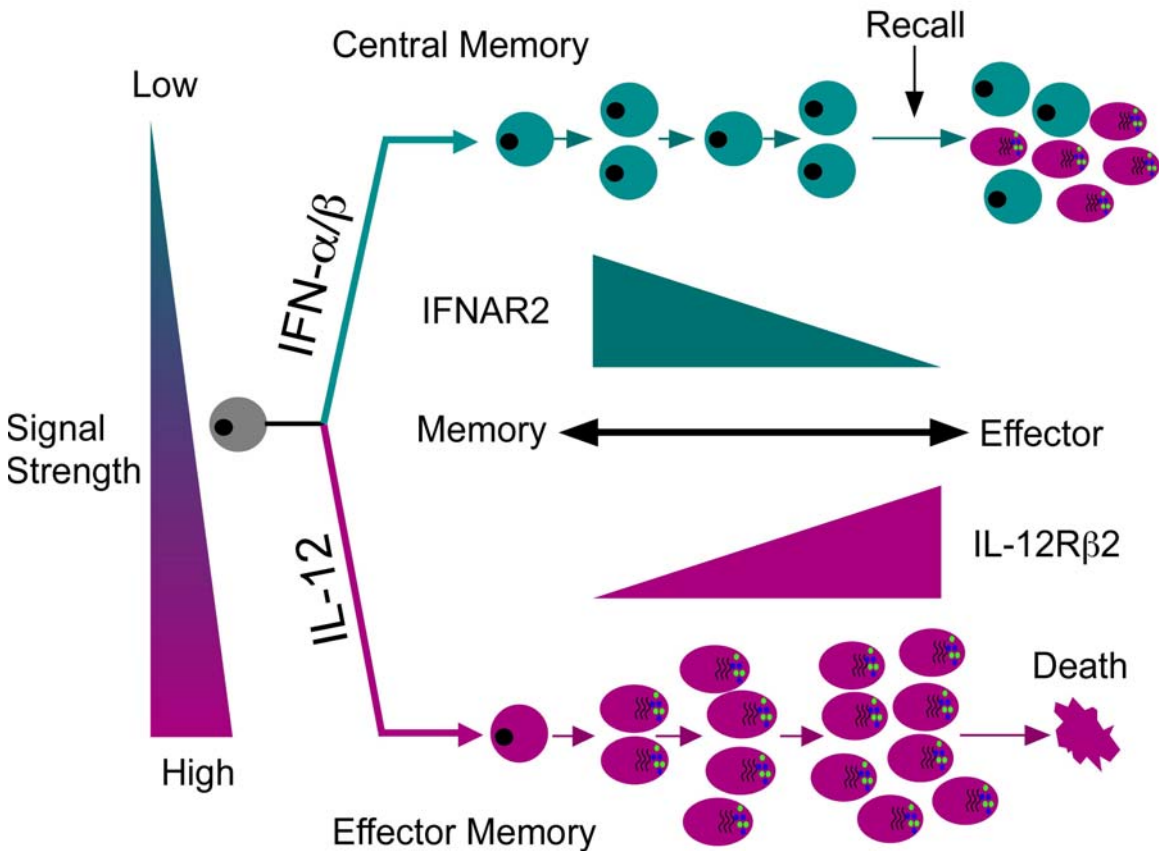

Figure 39. IL-12 and IFN-α/β direct the unique development of effector or central memory populations of human CD8$^+$ T cells.
Naïve CD8$^+$ CD45RA$^+$ T cells receiving signals 1 and 2 require further cytokine signals to develop into effector and memory populations. IL-12, programs the development of rapidly dividing cells, which acquire full effector properties. Alternatively, cells responding to IFN-α/β do not divide rapidly to primary activation but rather acquire phenotypic and functional attributes of T_{CM}. Programming of T_{EM} and T_{CM} occurs through the differential expression of the IL-12R and the IFNAR and directly correlates to differential downstream responsiveness to cytokine signaling. The pathways to T_{CM} and T_{EM} are further modulated by the strength of initial TCR activation. Under conditions of strong TCR activation, cells progress toward the T_{EM} fate, whereas weaker signal strength dampens this progression and favors a balance between T_{CM} and T_{EM}. Taken together, these results suggest a model in which development of T_{CM} and T_{EM} phenotypes occurs through signals obtained through by the TCR and cytokine signaling pathways and suggests that development of T_{CM} and T_{EM} during infection may be the direct result of the level of inflammatory signals received by developing T cells at the time of priming.

CHAPTER SIX

ROLE OF COSTIMULATORY SIGNALING VIA THE CD27/CD70 INTERFACE ON HUMAN CD8$^+$ T CELL EFFECTOR RESPONSES

The following chapter includes unpublished data which arose from the observations generated in chapter Five. All data was generated by Hilario Ramos.

Introduction

Three signals have been proposed to regulate the generation of effector and memory populations of T lymphocytes**.** These include TCR activation via MHC-peptide presentation, activation of co-stimulatory pathways, and innate cytokine signaling (28, 139). As discussed in depth in the previous chapter, innate cytokine cues (signal 3) play an important role in programming CD8$^+$ T cell fate. However in addition, I found that the strength of the primary signal (signal 1+2) plays a major role in priming the CD8$^+$ T cells ability to respond to cytokine cues. In these studies, higher strength of signal favored the development of effector cells whereas lower signals favored the generation of memory (243). While these responses are of great interest, this study did not separate signals 1 from signal 2, and therefore, I did not examine the direct effect of co-stimulatory signaling on effector or memory responses.

The classical pathway for T lymphocyte co-stimulation involves the activation of CD28 by CD80 or CD86 expressed on professional APCs (140). This leads to induction of IL-2 as well as multiple anti-apoptotic genes such as bcl-2 and bcl-xl which promote proliferation and cellular survival (244, 245). While this has been shown to be critical to the priming of naïve lymphocytes, several other surface receptors have been ascribed similar co-stimulatory roles (176, 245).

CD27 is a member of the TNF-α family of receptors (TNFR) which include the co-stimulatory molecules OX-40 and 4-IBB (246). It is expressed on B and T lymphocytes and in particular is highly expressed on all naïve $CD8^+$ T cells (176, 245). Unlike CD28, $CD8^+$ T cells express CD27 in the absence of activation and its expression is lost as they progress through differentiated effector stages (176, 247). This suggests a critical role for CD27 during the priming stage of naïve T lymphocyte development. In support of this, activation through its ligand CD70 has been shown promote proliferation in TCR activated $CD8^+$ T cells *in vitro* (248). Furthermore, signaling through CD27 has been shown to promote a variety of effector functions including enhanced CTL activity (249-251) and the prevention of apoptosis (246, 252). Adding to the importance of this pathway in T cell co-stimulation, examination of $CD27^{-/-}$ mice reveals a defect in primary expansion and T cell trafficking to the site of infection (Influenza virus infection) (253) as well as in secondary activation to LCMV

infection (176, 254). Therefore, CD27 signaling functions in co-stimulation for both the effector and memory T lymphocyte response to infection.

CD70 is the ligand for CD27 and can be expressed on activated DCs, B cells and T cells (255). However, DCs are widely thought to be the predominant source of CD70 co-stimulation for T lymphocytes. Further, CD70 expression is thought to play an important role in CD40 dependent priming of $CD8^+$ T cells both *in vitro* and *in vivo* (247, 256-258). In addition to DC mediated activation of CD27, more recent studies have suggested a role for lymphocytes in the activation of this pathway. For example, culture of $CD8^+$ T cells with purified B cells was shown to increase their CTL activity in a CD70 dependent manner (259). In addition, recent evidence shows that signaling through CD27 can be mediated by CD70 expressed on $CD8^+$ T cells *in vitro*. However this required high concentrations of exogenous IL-2 and therefore it is unclear as to the significance of this response *in vivo* (260). Importantly, recent studies have shown that CD27 expression can identify populations of memory $CD8^+$ with distinct effector cytolytic activity, suggesting potential roles for CD27 in not only priming but memory responses as well (172, 173).

Based on these observations, I wished to examine the capacity for naïve $CD8^+CD45RA^+CD27^+$ (referred to hereafter as "Naïve-27") to proliferate and develop effector functions in response cytokine polarization. In these studies, I found that Naïve-27 sorted cells were capable of inducing robust effector cytokine

secretion in response to primary activation. However, these cells were defective in cytolytic activity and especially the acquisition of perforin. In addition, this defect was linked to CD70 expression by a population of $CD8^+CD45RA^+CD27^-$ (referred to hereafter as "Effector-27" cells). Further, this suggested a possible role for Effector-27 cells in promoting CTL activity in the Naïve-27 population. Together, these observations suggest a novel pathway for the development of effector responses through $CD8^+$-$CD8^+$ T cell interactions.

Results

CD27 expression demarcates distinct subsets of human $CD8^+$ T lymphocytes

In my original examination of cytokine programming of human $CD8^+$ effector and memory responses, the starting pool of T cells was sorted only on the markers CD8 and CD45RA. However, recent studies have demonstrated that in addition to CD45RA, CD27 co-expression demarcates a truly naïve populations (172, 173). Therefore, I wished to assess the contribution of CD27 on the development of $CD8^+$ T cell responses. I examined the co-expression of CD27 within the $CD8^+CD45RA^+$ (referred to hereafter as "Naïve-RA") compartment of T cells freshly isolated from hPBMCs. Flow cytometric analysis of hPBMCs identified a large population of $CD8^+$ T cells which were $CD45RA^+$ (Fig. 40, top

left). Gating of this population revealed that the majority of these cells co-stained for CD27 (Naïve-27) (Fig. 40, top right). However, a minor population of cells which expressed high CD45RA but low CD27 (Effector-27) were also present (Fig 40, top right). This population occurred in multiple donors and ranged from roughly 10-20% of the total Naïve RA^+ cells (data not shown).

Further examination of these cells revealed that the Naïve-27 cells lacked expression of the cytolytic molecules perforin and granzyme B. (Fig. 40, bottom right). This was in contrast to the Effector-27 cells which almost exclusively co-expressed perforin/granzyme B. This suggested that as previously reported (172, 173), CD27 can demarcate distinct population of $CD8^+$ T cells; those which exhibit a truly naïve phenotype (Naïve-27) and those which are capable of immediate effector function directly from peripheral blood (Effector-27).

Naïve-27 cells do not develop CTL activity when separated from the Naïve-RA population

Naïve populations of cells have previously been described to lack effector molecule expression in the absence of activation. Therefore, the differences in the expression of perforin and granzyme B between peripheral blood populations of Naïve-27 and Effector-27 cells suggested that these cells might respond differentially to primary activation *in vitro*. To examine this, Naïve-27 cells were

isolated from hPBMCs by cell sorting and assessed for their development in response to cytokine polarization. Examination of Naïve-27 sorted cells revealed that the development of IFN-γ producing T cells at day 7 post activation was entirely regulated by IL-12 (Fig. 41). In addition, IFN-α/β was insufficient to prime this response although it did not inhibit the ability of IL-12 to do so (Fig. 41). These observations were strikingly similar to my previous observation in Naïve-RA T cell experiments and suggested that Naïve-27 cells behave similarly in response to cytokine polarization either in the presence or absence of Effector-27 Cells.

In order to further characterize the effector response, I examined the intracellular expression of perforin and granzyme B in Naïve-27 sorted cells. Surprisingly, cytokine polarization induced poor expression of perforin in these cells (Fig. 42A). However, levels of granzyme B were intact. Furthermore, Naïve-27 sorted cells were inefficient at promoting lysis in re-directed lysis assays (Fig. 42B). This was in contrast to my observations in Naïve-RA cells in which robust killing in response to IL-12 signaling was observed (243). Therefore, despite intact cytokine expression, the acquisition of perforin-dependent CTL activity was defective in Naïve-27 sorted cells and suggested a potential role for Effector-27 cells in these responses.

A variety of mechanisms could explain the potential role for Effector-27 cells in promoting CTL activity in Naïve-27 cells. First, it was possible that

Effector-27 cells were capable of outgrowing within the Naïve-RA compartment and that this could result in the acquisition of strong effector development. Alternatively, it was possible that Naïve-27 cells required stimulatory cues to drive CTL activity and this occurred in the presence of Effector-27 cells but not in their absence. In order to test these hypotheses, Naïve-27 and Effector-27 cells were sorted from the same pool of hPBMCs. These cells were then cultured independently of each other in the presence of polarizing cytokines and primary activation. Activated cells were then assessed for the acquisition of effector functions and proliferation. As expected Naïve-27 cells as well as Effector-27 cells acquired robust expression of IFN-γ (data not shown). However, examination of perforin and granzyme B expression revealed distinct differences between Naïve-27 and Effector -27 cells (Fig. 43). In accordance with my initial observation, Naïve-27 cells displayed poor expression of perforin in response to cytokine polarization (Fig. 43A). However, Effector-27 cells expressed high levels of both perforin and granzyme B even in the absence of innate cytokines (Fig. 43B). Interestingly Effector-27 cells polarized in the presence of IL-12 displayed less perforin than other conditions (Fig. 43B), suggesting a defect in IL-12 responsiveness by these cells. However, further examination of these cells revealed that IL-12 driven Effector-27 cells failed to survive the primary activation (data not shown) and thus the lack of perforin was likely due to the decreased viability and not responsiveness to IL-12. Additional comparison

between Effector-27 cells and Naïve-27 revealed a general defect in expansion of Effector-27 cells when compared to Naïve-27 cells. Therefore, these observations then suggest that it was unlikely that Effector-27 cells were outgrowing in culture. In order to directly assess this hypothesis, I next examined the capacity of Naïve-27 and Effector-27 cells to proliferate in response to primary *in vitro* activation.

In these experiments, Naïve-27 and Effector-27 sorted cells were independently labeled with CFSE and assessed for proliferation by CFSE dilution at day 3 post activation. Similar to our observations in Naïve-RA cells, Naïve-27 sorted cells were capable of proliferating in response to primary activation. Further, these cells responded differentially to cytokines with IL-12 increasing the number of cellular divisions in comparison to IFN-α/β (Fig. 44, top panel). In contrast Effector-27 sorted cells displayed a profound defect in their ability to proliferate in response to activation (Fig. 44, bottom panel). Therefore despite their increased effector responses *in vitro*, the outgrowth of Effector-27 cells in Naïve-RA cultures is unlikely to be the reason for the observed differences observed in Naïve-27 sorted cells.

It was possible that Effector-27 cells required signals from Naïve-27 cells to proliferate in culture. To address this, I next performed mixing experiments to assess the requirement of Naïve-27 cells to restore Effector-CD27 cells ability to proliferate in response to primary activation. For these experiments, Naïve-27 and Effector-27 cells were independently sorted as described above and activated

in two separate groups. Group one consisted of CFSE-labeled Naïve-27 cells mixed with unlabeled Effector CD27 cells. This group was used to assess division of Naïve-27 cells. Alternatively, the second group consisted of CFSE-labeled Effector-27 cells mixed with unlabeled Naïve-27 cells. This group was used to assess proliferation of Effector-27 cells. In both groups, cells were mixed back together in the ratios they were found in total hPBMCs and were assessed for the ability to proliferate at day 3 post primary activation (Fig. 45). As previously observed, the Naïve-27 cells expanded robustly to primary activation in the presence of Effector-27 cells (Fig. 45, top panel). Further, this was enhanced when compared to Naïve-27 cells cultured alone (Compare Fig. 44, top panel with Fig. 45, top panel). In contrast, co-culture of Effector-27 cells with Naïve-27 cells did not restore the ability of Effector-27 cells to proliferate (Fig. 45, bottom panels). Therefore based on this data, it is unlikely that Effector-27 cells outgrow in the Naïve-RA compartment. Instead, these observations suggested that Effector-27 cells were required for some signal which could "educate" Naïve-27 cells to develop CTL activity. However, it is important to note that this data is preliminary and further replication is necessary to fully demonstrate this significance of these findings.

Effector-27 cells educate Naïve-27 cells to acquire effector responses through the CD70- CD27 pathway

It was possible that Effector-27 cells were capable of providing "education" either in the form of a soluble mediator or a direct cellular interaction to promote the development of CTL activity in Naïve-27 cells. Several possible mechanisms could potentially mediate this response. However, since our observed phenotypes were directly linked to CD27 expression, I first focused on examining activation of the CD27 pathway as a mechanism for Effector-27 education of Naïve-27 cells. Ligation of CD27 by CD70 has been shown to promote the efficient development of both proliferation and effector CTL activity (248, 249). Therefore I addressed whether these two populations of $CD8^+$ T cells could interact through CD27 and CD70 and whether this could serve as a potential mechanism for the education of Naïve-27 T cell effector development. To begin these studies, Naïve-RA cells (which contain both the Naïve-27 and Effector-27 cells) were sorted and cultured in the presence of cytokines for 3 days. Cells were then examined for surface expression of both CD27 as well as CD70 (Here, cells were not directly sorted from each other. Therefore populations expressing high levels of CD27 are referred to as $CD27^{hi}$ and $CD27^{lo}$ populations). The majority of cells at day 3 expressed high levels of CD27 ($CD27^{hi}$) (84-90%), (Fig. 46). However a minor population was observed that expressed low levels of CD27 ($CD27^{lo}$) (Fig. 46). Interestingly, the $CD27^{lo}$ population maintained high expression of CD70, whereas the $CD27^{hi}$ cells were low for CD70. These data provide evidence that $CD27^{lo}$ cells could potentially

activate $CD27^{hi}$ cells via CD70. While this does not directly implicate this pathway in the education of $CD27^{hi}$ cells, it suggests that $CD27^{lo}$ cells could interact with $CD27^{hi}$ cells via the CD70/CD27 pathway. However at this time, I have not further examined or determined the direct requirement for CD27 and CD70 in promoting the observed effector responses. Clearly further analysis of this interaction is required to fully understand this process. For example neutralizing experiments in which CD70 activation is blocked might demonstrate a necessity for activation of CD27 and the development of CTL activity. Alternatively, exogenous activation of CD27 on Naïve-27 cells in the absence of Effector-27 could confirm this. However until these questions are addressed experimentally the mechanism by which Effector-27 cells educate the acquisition of full effector potential in Naïve-27 T cells remains outstanding.

Despite an incomplete understanding of the role of $CD8^+$ mediated CD27 activation, further experimental evidence suggested that this may play an important role in the regulation of $CD8^+$ effector cell development. In day 3 cultures, CD70 expression was uniquely expressed on $CD27^{lo}$ cells and innate cytokines played little role in its expression (Fig. 46). This suggested that the population of $CD27^{lo}$ cells was already programmed to express this molecule. I further examined the expression of this marker over time. Naïve-RA cells were sorted and cultured in the presence of cytokines. On day 7, one group of cells was harvested for analysis and additionally a second group was re-activated in the

presence of cytokines and culture to day 14 for analysis. Similar to day 3, day 7 cells expressed rather invariable amount of CD70 as a function of cytokine treatment (Fig. 47). In contrast, examination of CD27 expression revealed minor differences in expression. Here IFN-α/β was able to maintain higher expression of CD27 than cells polarized in the presence of IL-12.

Further, examination of day 14 cells revealed a more striking phenotype. Here IL-12 enhanced a population of CD70 even in the presence of IFN-α/β. This was coupled to the loss of CD27 expression in IL-12 polarized cells, suggesting a potential role for these cytokines in programming the expression of CD70 and CD27. Interestingly as CD27 expression has been linked to populations of memory cells (172, 173), the retention of CD27 on day 14 cells by IFN-α/β further illustrates an importance of this cytokine in memory development. While further replication of these experiments is necessary to fully define a trend in the regulation of the co-stimulatory markers, together our data suggest a potential role for CD8 mediated costimulation via the CD27/CD70 pathway. Further, the regulation of CD70 by IL-12 and CD27 by IFN-α/β may provide important clues as to the role of these markers in the segregation of effector and memory $CD8^+$ T cell responses to infection.

Discussion

My initial observation in this study, demonstrated that two populations of $CD8^+CD45RA^+$ exist within human peripheral blood; those cells which express high levels of CD27, (Naïve-27 cells) and those which do not express CD27, (Effector-27cells). These populations have been previously ascribed either naïve or effector-like properties respectively (172, 173). In previous studies I examined the role of IL-12 and IFN-α/β on the development of $CD8^+CD45RA^+$ effector and memory responses. Therefore, I wished to examine whether Naïve-27 and Effector-27 cells behaved similarly during the primary activation. Although I observed a general defect in cytolytic function, both proliferation and effector cytokine production was similar in Naïve-27 sorted cells when compared to the Naïve-RA sorted cells. This data suggested that the environment and perhaps cellular components present in the Naïve-RA compartment were necessary for cytolytic activity. By isolating Naïve-27 and Effector-27 cells from the same Naïve-RA pool of cells, I was able to identify distinct cytolytic properties between these two subsets. Further as the Effector-27 but not Naïve-27 cells demonstrated enhanced cytolytic capacity this suggested that they may be the cellular source for this response in the Naïve-RA sorted cells. I found that Effector-27 cells either in the absence or presence of Naïve-27 cells were unable to proliferate *in vitro*. This is in line with observations that $CD8^+CD45RA^+CD27^-$

cells are effector cells with a terminally differentiated phenotype (172, 173). Further this suggested that the mechanism for generation of CTL responses was not due to direct outgrowth of Effector-27 cells but might instead be explained by interactions between Effector-27 and Naïve-27 cells *in vitro*.

Although this study did not fully address the mechanism by which Effector-27 cells might function to "educate" Naïve-27 effector development, I was able to make some key observations to suggest a potential mechanism. Staining experiments for surface CD70 expression revealed that CD70 was solely expressed on the population of $CD27^{lo}$ cells. Further, I found that CD70 expression could be regulated by IL-12 at later time-points in development. As both IL-12 signaling (5) and CD70 activation (249, 251) are known to directly participate in induction of human $CD8^+$ CTL responses, it is possible that $CD8^+$ T cells may interact with each other via CD27 and CD70 to enhance effector function. Further, this pathway for "licensing" CTL activity in Naïve-27 cells may occur through CD70 expression on an effector subset of $CD8^+$ $CD27^{lo}$ cells. While these studies were unable to fully explore the directness of this interaction *in vitro*, the observation does pose the question as to the nature of CD8-CD8 T cell interactions *in vivo*.

One of the limiting factors in examining this observation *in vitro*, is the significance of CD8-CD8 interactions *in vivo*. While my work demonstrates clear defects in the acquisition of effector functions in Naïve-27 cells developing in the

absence of Effector-27 cells *in vitro*, it is unclear at what stages or anatomical locations these cellular interactions might occur at to promote this response *in vivo*. It is possible that our observations simply are an artifact of removing cells from the natural environment and not be fully capable of restoring accurate activation conditions *in vitro*. To this regard, circulating naïve T lymphocytes are part of a pool of hPBMCs circulating through blood and lymph. Upon activation in the lymph node, naïve $CD8^+$ T cells are exposed to DCs as well as B cells, $CD4^+$ T cells and other $CD8^+$ T cells populations. Therefore activation of CD27 by CD70 expressed on all these cells types may potentially play a role in effector CTL activation. In line with this hypothesis, recent studies have demonstrated that murine B cells and $CD8^+$ T cells can directly activate CD27 on naïve T cells in a CD70 dependent manner (259, 260). The data presented here clearly suggests a similar role for human $CD8^+$ T cells, and suggests that CD8-CD8 interactions may be more important in the generation of effector functions than previously thought.

Previous studies which have assessed the role of CD70/CD27 activation *in vivo* have shown an importance in the priming phase (256-258). This is likely to occur in a DC dependent manner in the draining lymph nodes. One potential interaction point for lymphocyte-lymphocyte or in particular CD8-CD8 co-stimulation may be in subsequent phases of development such as during clonal expansion. Clonal expansion leads to a massive increase of T lymphocytes in the

draining lymph nodes as well as in the periphery. This influx of cells within a confined environment could then facilitate increased T cell-T cell interactions and activation. Thus CD70 mediated co-activation may be important at the stage of clonal expansion. Since this pathway has been implicated in the induction of pro-survival molecules such as Bcl-2, it makes sense that this pathway be functional beyond the initial T cell-DC priming event to ensure survival long enough for T cells to participate in the effector response (255, 261). The identification of CD70 and CD27 expression on memory populations (172, 173) suggests that other locations such as the site of primary infection might be areas in which $CD8^+$-$CD8^+$ co-stimulation can occur as well and therefore the possibility for multiple stages of T cell response may be involved in this process. Clearly, it is uncertain at this point as to the significance of lymphocyte mediated co-stimulation of naïve $CD8^+$ T cells. Although my findings suggest that in human $CD8^+$ T cells, the interactions between effector ($CD27^-$) and naïve ($CD27^+$) may be crucial in priming a full effector response, further examination of these pathways *in vivo* as well as characterization of the necessity for this interaction in a $CD8^+$ dependent manner will be important to the global understanding of the role of CD27 in priming the $CD8^+$ T cell response to infection..

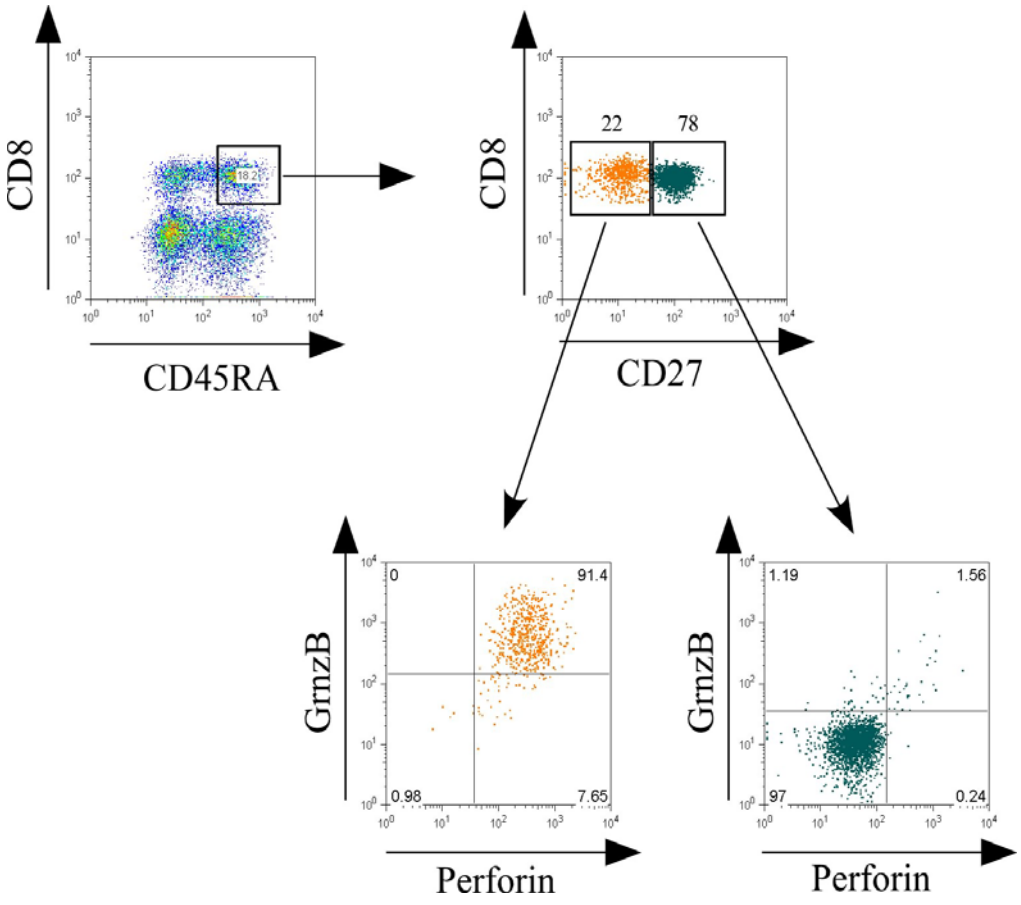

Figure 40. CD27 demarcates distinct populations of human CD8+CD45RA+ T cells. hPBMCs were isolated from whole peripheral blood by ficol density centrifugation. Freshly isolated cells were then stained for surface expression of CD8, CD45RA, CD27 and intracellular expression of perforin and granzyme B. Cells were gated on live and displayed as $CD8^+$ $CD45RA^+$ by bi-variant dot plot (top left). $CD8^+CD45RA^+$ cells were gated and expression of CD27 was assessed (top right). $CD27^+$ and $CD27^-$ cells were then gated and expression of perforin and granzyme B was assessed in these populations (bottom right).

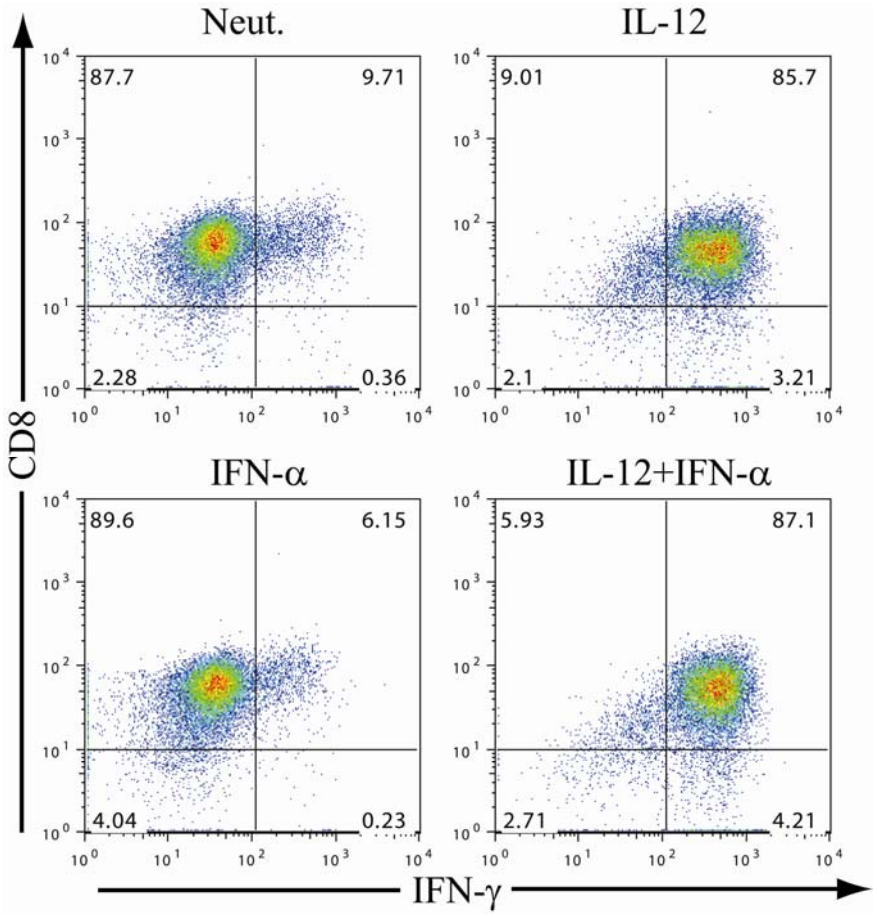

Figure 41. IL-12 regulates effector cytokine expression in Naïve-27 sorted cells
Cells were sorted as $CD8^{+}CD45RA^{+}CD27^{+}$ and polarized to day 7 in the presence of the indicated cytokine conditions. On day 7, cells were rested overnight followed by 4 hr activation with PMA/Ionomycin in the presence of Brefeldin A. Cells were stained for intracellular IFN-γ and surface CD8 and assessed for expression by bi-variant dot plot analysis.

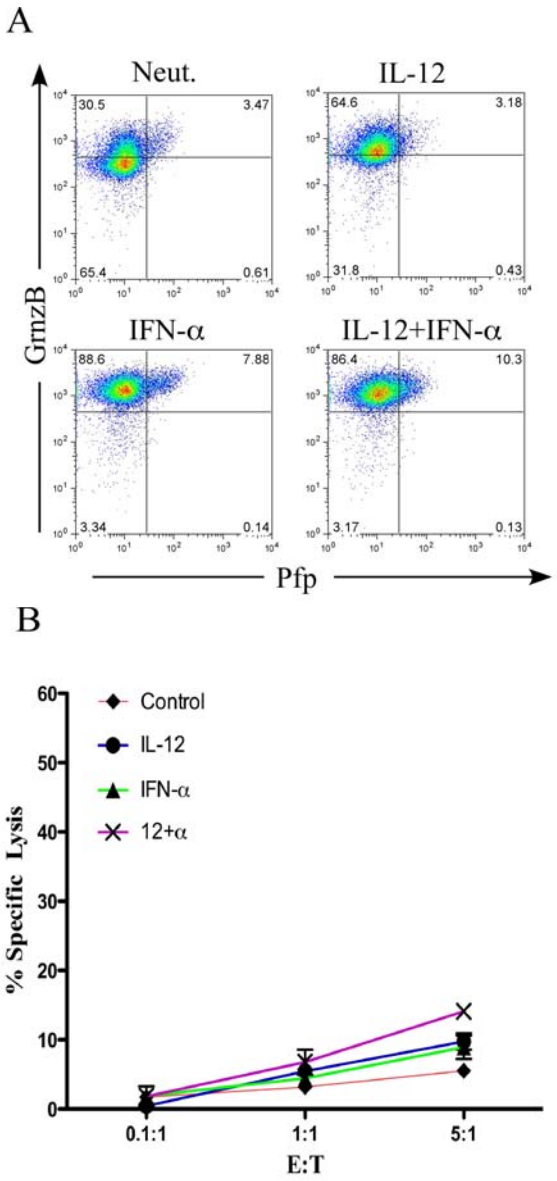

Figure 42. Naïve -27 sorted cells are defective in the ability to obtain cytolytic activity

Cells were sorted as described above and cultured for 7 days in the presence of cytokines as indicated in the figure. (**A**) Day 7 cells were rested overnight and then stained for intracellular perforin and granzyme B or (**B**) cells were used as effectors in a re-directed lysis assay with THP- monocytes. (Control-orange, IL-12-blue, IFN-α-green, IL-12+IFN-α-purple).

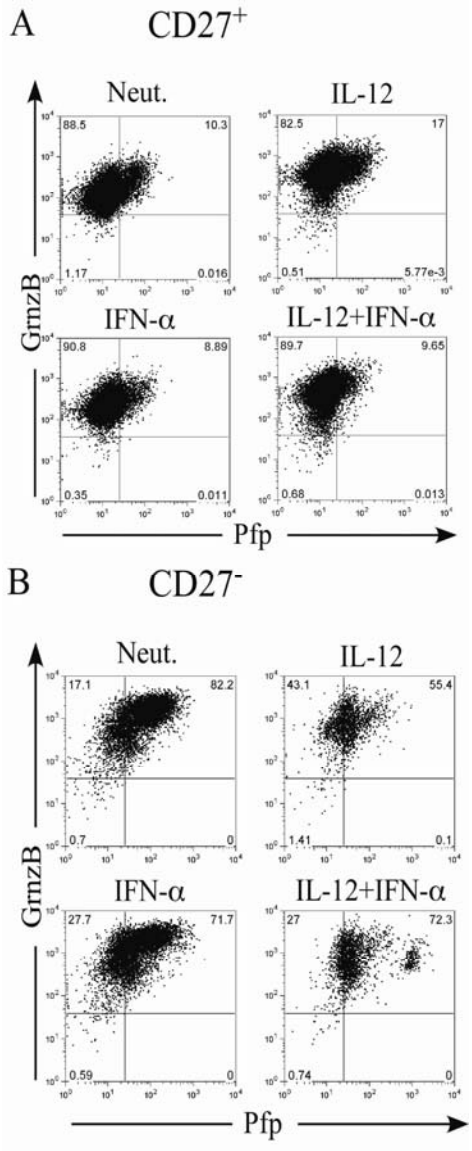

Figure 43. CD27+ and CD27- cell s display distinct effector potential.
Cells were sorted as either $CD8^{+}CD45RA^{+}CD27^{+}$ or $CD8^{+}CD45RA^{+}CD27^{-}$ and polarized to day 7 in the presence of the indicated cytokine. (**A**) Day 7 $CD27^{+}$ or (B) $CD27^{-}$ cells were rested overnight and the stained for intracellular perforin and granzyme B and assessed by bi-variant dot plot analysis.

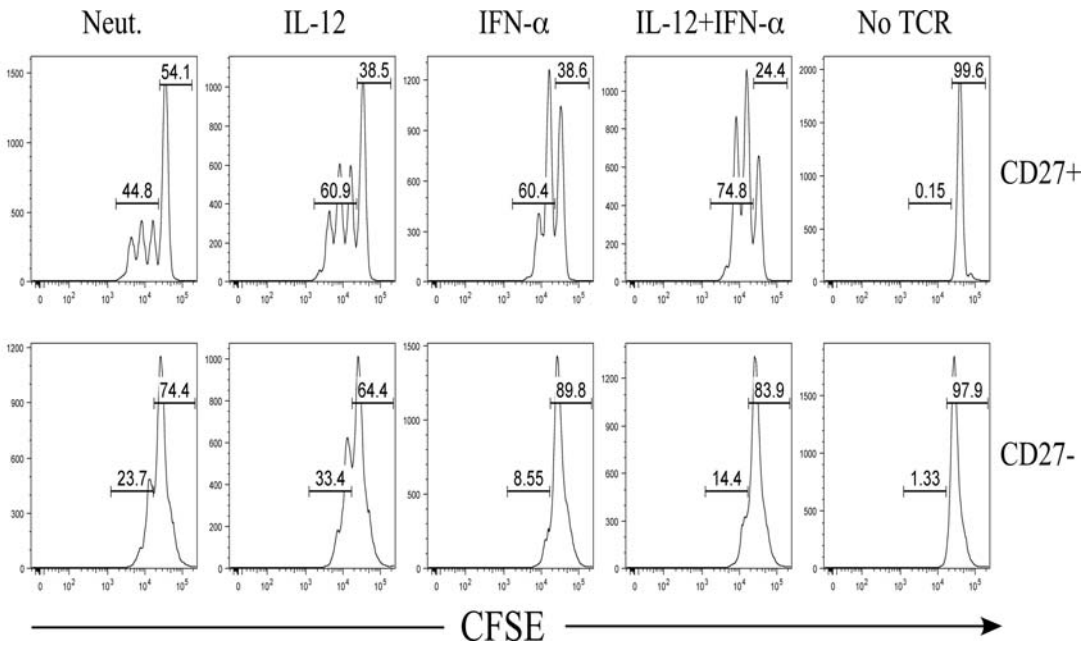

Figure 44. Effector CD27$^-$ sorted cells are unable to proliferate in response to Primary activation

Naïve-CD27$^+$ or Effector-CD27$^-$ cells were purified and labeled with CFSE. Labeled cells were activated under cytokine polarizing conditions as indicated in the figure and assessed for division by CFSE dilution at day 3 post activation. Cells were cultured independently as Naïve-CD27$^+$ alone (top panel) or as Effector-CD27$^-$ alone (bottom panel).

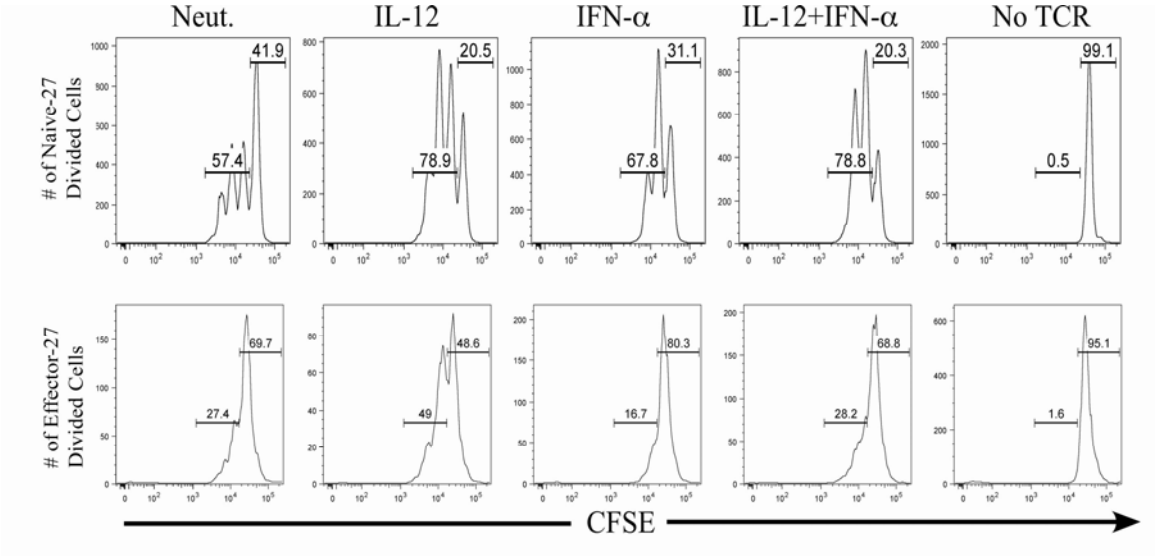

Figure 45. Co-culture of CD27^{+} and CD27^{-} cells does not restore the ability of CD27^{-} cells to proliferate.
Naïve-27^{+} Effector-27^{-} were purified and either labeled or not with CFSE. Labeled cells were activated under cytokine polarizing conditions as indicated in the figure and assessed for division by CFSE dilution at day 3 post activation. CFSE labeled Naïve-27 cells were cultured with unlabeled Effector-27 cells (top panels) or CFSE labeled Effector-27 cells were cultured with unlabeled Naïve-27 cells (bottom panels). Proliferation of Naïve-27 cells (top) or Effector-27 cells (bottom) was assessed by CFSE dilution.

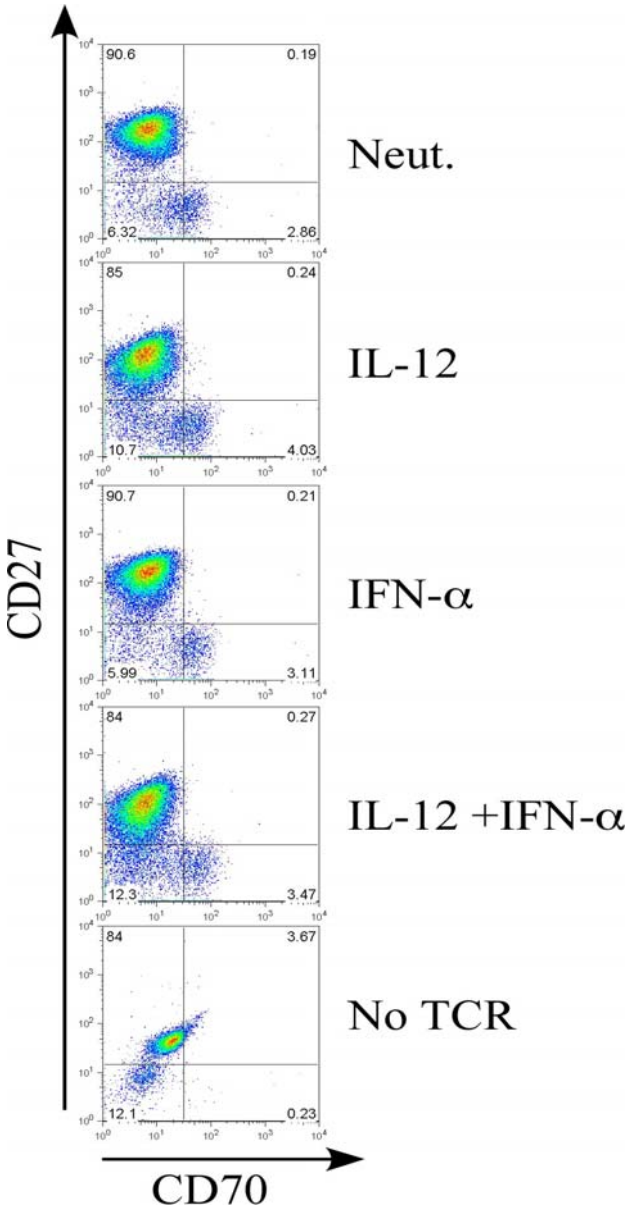

Figure 46. CD70 is expressed on Effector-CD27 cells. Cells were sorted as $CD8^{+}CD45RA^{+}$ and cultured to day 3 in the presence of polarizing cytokines. On day 3, cells were stained for surface CD70 and CD27 and assessed for expression by bi-variant dot plot analysis by flow cytometry.

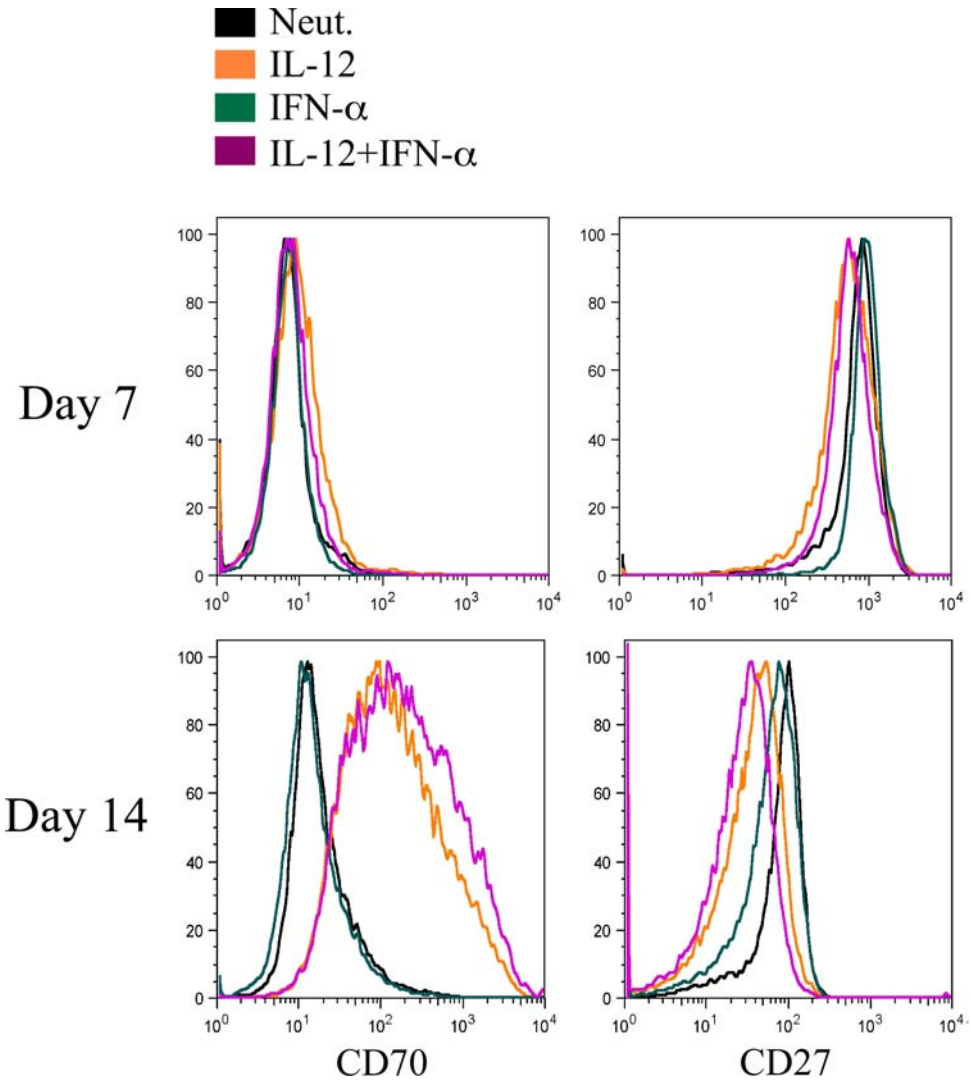

Figure 47. IL-12 and IFN-α/β differential regulate the expression of CD27 and CD70 on human CD8$^+$ T cells.
Cells were sorted as CD8$^+$CD45RA$^+$ and cultured to either day 7 (top panels) or day 14 (bottom panels) and stained for surface expression of CD27 and CD70. Cells were assessed for expression on day 7 (top panels) or day 14 (bottom panels) by histogram overlay. Black-Neutr., Orange-IL-12, Green-IFN-α, Magenta-IL-12+IFN-α

CHAPTER SEVEN

DISCUSSION

Overview

TCR activation (signal 1), costimulation (signal 2) and innate cytokine signaling (signal 3) act together to promote the development of effector and memory T lymphocyte responses to infection. Importantly, signal 3 cytokines act to directly bridge the innate and adaptive responses. This occurs through programming of T lymphocytes subsets with effector arsenals specifically geared toward the clearance of the infecting organisms. Both IL-12 and IFN-α/β are induced during viral infection and are thought to act as redundant signals for the programming of type I T lymphocyte responses. However, while IL-12 has been shown to critically regulate these activities the true necessity for IFN-α/β in directly programming T lymphocyte functions has remained controversial.

Therefore, these studies set out to separate the effects of IL-12 and IFN-α/β on T lymphocyte development. Here, several key observations have been made which further our understanding of the role of signal 3 cytokines in human T lymphocyte development. First, these studies have demonstrated that IL-12 and IFN-α/β are in fact not redundant in their abilities to promote type I effector

responses, and this is due to distinct differences in activation of downstream intermediates of Th1 development. Secondly, these cytokines display distinct roles in the development of $CD8^+$ T cell memory such that IL-12 promotes T_{EM} while IFN-α/β promotes T_{CM} fates. Finally, my work has uncovered unique interactions between signals 1, 2 and 3 which govern the overall interpretation of the inflammatory environment. These interactions help shape the responsiveness to cytokine signaling, and therefore suggest a model in which signals 1, 2, and 3 cooperate to ensure proper development of effector and memory responses to the specific pathogenic challenge. Together these observations shed important light on our understanding of the role of inflammation on the development of effector and memory responses. Further this work has direct implications on the development of therapeutics and vaccine strategies in which IL-12 and IFN-α/β signaling are important.

The development of type I responses by IFN-α/β revisited

Both IL-12 and IFN-α/β signal through the JAK/STAT pathway to activate their downstream targets. Importantly, signaling through both IL-12 and IFN-α/β can lead to activation of STAT4. As STAT4 is critical to Th1 development in human $CD4^+$ T cells, IL-12 and IFN-α/β are thought to act

redundantly to prime Th1 commitment in a STAT4 dependent manner in humans (5, 30, 62, 104). However my work now clarifies the role of these cytokines in STAT4 activation and defines a decreased ability for IFN-α/β to promote STAT4 when compared to IL-12. This manifests in differences in the maintenance of expression of T-bet, and ultimately an inability of IFN-α/β to prime Th1 commitment. Therefore, my work overturns previous assumptions and clearly demonstrates that IL-12 and IFN-α/β are not redundant in their capacities to drive Th1 development in human $CD4^+$ T cells.

The mechanism by which IL-12 and IFN-α/β differentially activate STAT4 is not entirely clear. While in my studies, I was able to identify a role for the IFNAR in mediating these differences, it is possible that other factors may further contribute to this response. I examined the expression of SOCS-1 and UBP43 as they are IFN-α/β regulated and have been shown to directly block JAK/STAT signaling.(209, 262-265). Surprisingly, I found no role for these proteins in the observed defect in STAT4 activation or Th1 commitment (100). As I examined these responses at distinct time-points after activation, it is possible that a more dynamic regulation at earlier time-points may play a role in this response. If this is true then further studies examining a kinetic analysis of SOCS-1 or UBP43 might shed light on whether these pathways may contribute to our observed defects in IFN-α/β mediated STAT4 activation.

In addition to regulation at the level of STAT phosphorylation, it is possible that differential recruitment or preferential association of STAT molecules between the IL-12R and IFNAR drive the variegated STAT4 activation. For example while IL-12 can activate STAT1 and STAT3, it preferentially activates STAT4 (5, 104, 116). In contrast, STAT4 activation by IFN-α/β appears to be secondary to that of STAT1 and STAT2 (62, 65). Therefore it is possible that the IFNAR preferentially associates with STAT1 and STAT2 at the expense of STAT4. If that is the case, then under conditions in which STAT1 or STAT2 expression is low, the activation of STAT4 should dominate. Studies by Biron and colleagues support this potential mechanism. Examination of murine T and NK cell responses to IFN-α/β activation demonstrated that as levels of STAT1 increase, the ability for IFN-α/β to phosphorylate STAT4 and induce IFN-γ decreases (162, 266). In addition, recent work by Berenson *et al*. show that in the absence of STAT1, STAT4 phosphorylation is increased in response to IFN-α/β in murine $CD4^+$ T cells (159). While this was not sufficient to promote Th1 development in mice, this at least suggests that the defect in IFN-α/β mediated STAT4 phosphorylation could in part be explained by the preferential activation of STAT1 by IFN-α/β.

Regulation of cytokine receptors may also play a role in this response. For example, it is well established that the IFNAR can be internalized and degraded in

response to IFN-α/β signaling (160, 267, 268). Alternatively, IL-12 signaling reinforces the expression of the IL-12Rβ2 chain (149, 269). Therefore this differential regulation of cytokine receptors may explain a decrease in IFN-α/β mediated STAT4 activation in comparison to IL-12. In line with this, we observed that overexpression of the hIFNAR2 in murine cells led to the enhancement of IFN-γ expression in response to IFN-α/β and IL-18 signaling. Further this corresponded to increased kinetics of STAT4 phosphorylation (100). This suggests that retention of IFNAR, or at least overexpression can restore IFN-α/β mediated STAT4 phosphorylation. Whether this is sufficient to promote Th1 development is unclear at this time. However, polarization studies in naïve cells transduced with the IFNAR subunits could provide important clues to the relevance of the enhanced STAT4 activation. Alternatively, it would be interesting to observe the status of STAT4 phosphorylation under the IL-12 + IFN-α/β condition. In this case, if STAT4 activation was still decreased it might suggested an active repression (such as SOCS or UBP43 activity) as opposed to differences in binding or receptor signaling between IL-12 and IFN-α/β.

Our findings here support a new model for Th1 development in humans in which IL-12 acts as the main modulator of this response. It is possible that IFN-α/β may act to augment these responses, however in the absence of IL-12 it is unable to do so. The role of STAT4 in the activation of T-bet is controversial and

thus it is not understood whether T-bet is a direct target of STAT4 activation. However, the finding here that defects in STAT4 activation are correlated to an inability to maintain T-bet expression suggested a linear model of Th1 commitment in which STAT4 is upstream of T-bet and IFN-γ. Further, our ability to restore IFN-α/β mediated Th1 commitment via overexpression of T-bet supports this hypothesis (100). While it has been suggested that STAT4 can promote IFN-γ expression via direct interactions with the IFN-γ promoter (270-272), it unclear whether STAT-4 can directly drive the expression of T-bet. Two recent studies have identified a potential STAT binding site in the enhancer region of murine T-bet which can allow for both STAT4 and STAT1 activation of its transcription (273, 274). Therefore this suggests that the decrease STAT4 activation by IFN-α/β may directly affect Th1 commitment through a lack of direct STAT4 mediated T-bet induction. Examination of the effect of forced expression of STAT4 on T-bet expression in human cells by retroviral transduction will aid in our understanding of this pathway in humans.

My observations in human $CD4^+$ T cells led me to question whether human $CD8^+$ T cells would respond in a similar manner to IFN-α/β polarization. While it is clear that IFN-α/β can not promote Th1 development in murine $CD4^+$ T cells (30, 154, 155, 159) murine $CD8^+$ T cells appear to be hyper-responsive to IFN-α/β *in vivo* (158, 161) . More recent studies by Curtsinger and colleagues

identify both IL-12 and IFN-α/β as redundant signal 3 cytokines for Tc1 effector development (28, 29). Surprisingly and in contrast to the observation in murine $CD8^+$ T cells, my observations clearly demonstrate that IFN-α/β is not sufficient to promote Tc1 development in human $CD8^+$ T cells. While it is unclear why we observe these species specific differences, it is possible that the signaling pathways between murine $CD8^+$ and human $CD8^+$ T cells differ. This was originally thought to be the case in $CD4^+$ T cells as both the human STAT2 and IFNAR2 displayed distinct insertions in their C-termini when compared to mice (156, 212, 275). While further studies demonstrated that expression of human STAT2 did not confer the ability of IFN-α/β to prime Th1 commitment (157), it is possible that these pathways or other as of yet unidentified species-specific differences may explain the observed Tc1 priming by IFN-α/β in mice but not humans.

A new role for type I IFN in the development of $CD8^+$ T lymphocyte memory

The development of memory against infectious organisms is crucial to our ability to survive re-infection by these pathogens. Although cytokines play a major role in multiple aspects of immunity, to date no cytokine has been described to directly promote the development of memory. For the first time, my work identifies a cytokine, IFN-α/β, with the ability to directly promote T_{CM}

development. While the common γ-chain cytokines IL-2, IL-7, and IL-15 have all been shown to be important in the survival and maintenance of memory, these cytokines do not directly promote T_{CM} and T_{EM} commitment (187-189). Further, other cytokines such as IL-12 have been implicated in the generation of memory but this is likely due to their role in the establishment of effector responses and not a direct role in programming T_{CM} (190, 191) A role for IFN-α/β in the development of memory has also been suggested by the finding that $IFNAR^{-/-}$ mice display decreased survival and reduced numbers of memory cells during infection (194-196). My data add to these observations and now demonstrate for the first time that $CD8^+$ T cells can directly respond to IFN-α/β by acquiring surface phenotypes and functional characteristics ascribed to T_{CM} cells.

In addition to the ability of IFN-α/β to drive T_{CM} in $CD8^+$ T cells, Ann Davis recently demonstrated a role for IFN-α/β in the development of an IL-2 producing $CD4^+$ T_{CM} population (276). This was also found in my study of $CD8^+$ T cells and therefore suggests a global role for IFN-α/β in the development of T cell memory in humans. IL-2 signaling has been linked to maintenance of $CD8^+$ T cell memory(187) and further, recent studies by Bevan and Williams have demonstrated an important role for $CD4^+$ T cells in the development of CD8 memory via an IL-2 dependent pathway (188). Whether or not $CD4^+$ T cells are required for the generation of $CD8^+$ T cell memory is still a point of contention.

However, these combined studies suggest that during intracellular infection in which IFN-α/β is highly produced, the induction of $CD8^+$ T_{CM} fate may occur directly via signaling by IFN-α/β and indirectly via IFN-α/β mediated IL-2 production. Whether these responses occur *in vivo* is yet to be determined, however adoptive transfer of IFNAR deficient $CD8^+$ T cells into infected hosts either in the absence or presence of IFNAR deficient $CD4^+$ T cells might shed light on the impact of direct and indirect memory development by IFN-α/β.

Beyond our observation that IFN-α/β drives T_{CM} development, I also found a direct role for IL-12 in the acquisition of a T_{EM} fate. This is in line with previous observations that abrogation of IL-12 signaling leads to decreased effector and T_{EM} development and therefore further reinforces the notion that IL-12 is a key regulator of type I T lymphocyte responses (190, 191, 277). Although IL-12 is thought to be the predominant Tc1 cytokine during intracellular infection, it is important to note that additional cytokines have been shown to promote Tc1 effector function in the context of other infection models. For example, IL-21 and IL-27 have recently been described to promote effector Tc1 responses against infiltrating tumors (222, 278, 279). In addition, IL-27 and IL-23 may also participate in promoting effector activity towards *Toxoplasma gondii* and HCV infection respectively (166, 280). Hence, depending upon the type of infection, cytokines other than IL-12 may promote Tc1 effector responses as well. It is unclear whether additional cytokines can direct the development of T_{CM} fates

either *in vitro* or *in vivo*. However, as IFN-α/β is predominantly produced during intracellular infections, it would not be surprising that other cytokines might promote similar memory responses depending upon the type of infection and inflammatory environment. Identification of the mechanisms by which IFN-α/β drives T_{CM} development and IL-12 drives T_{EM} development will certainly be useful in identifying other cytokine signaling pathways which are important to this response.

To address the mechanism behind the variegated development of T_{EM} and T_{CM} by cytokines, I examined the ability of IL-12 and IFN-α/β to regulate two key $CD8^+$ T cell transcription factors, T-bet and Eomes. Clearly T-bet is required for induction of IFN-γ and effector responses (145, 146, 224). Further, examination of T-$bet^{-/-}$ mice showed clear defects in immunity towards HSV, *T. gondii* and LCMV (166, 224, 281). Therefore it is likely that this pathway is crucial in development of T_{EM} against a variety of pathogens. My work confirms these observations in human cells. Here, I found a direct link between IL-12 signaling and T_{EM} development. Further, this is likely to progress through T-bet as T_{EM} cells express higher levels of T-bet than T_{CM} although the requirement for IL-12 for this response is still not clear (243).

The role for Eomes in the development of effector and memory is less clear. Originally it was thought to act redundantly to T-bet in driving $CD8^+$ effector functions (164, 165). Alternatively, more recent studies by Reiner and

colleagues suggest that Eomes is associated with and may be involved in the generation of T_{CM} cells (165, 191, 193). Surprisingly although a strong correlation between IFN-α/β signaling and the induction of Eomes was found, a direct correlation for Eomes in the T_{CM} population was not. This is in contrast to previous studies in mice, which have linked the T_{CM} fate with high expression of Eomes. However, this has only been speculated and the relevance of this pathway in mice is still not clear. Regardless, my studies suggest that at least in human $CD8^+$ T cells, IFN-α/β mediated T_{CM} development may not proceed in an Eomes-dependent manner.

It is possible that the development of T_{EM} and T_{CM} cells may not occur in a strict linear manner. Instead, the ratio of T-bet to Eomes within a cell may dictate effector vs. memory development. Thus T_{CM} cells could express a higher ratio of Eomes to T-bet. If that is the case then overexpression of Eomes in T-bet competent cells should drive T_{CM} development. I experienced a number of technical difficulties in attempts to express genes in primary human $CD8^+$ T cells by retroviral transduction. This procedure requires prolonged centrifugation of cells in the presence of retrovirus. It is possible that this component of the procedure may over-stimulate $CD8^+$ T cells as compared to $CD4^+$ T cells, leading to their cell death. Perhaps a shorter centrifugation cycle or fewer rounds of transduction could remedy this effect. Once these technical issues are resolved it

will be important to dissect the contribution of T-bet and Eomes to $CD8^+$ T_{EM} and T_{CM} development.

Recently other transcriptional regulators have been described to participate in $CD8^+$ T cell development. For example RUNX3 and Rel have been shown to be important in the acquisition of CTL activity and proliferative capacity in $CD8^+$ T cells (282, 283). Further, Bcl-2 has been shown to enhance cellular survival of T cells by blocking the induction of apoptosis during activation (284, 285) while Blimp-1 inhibits Bcl-2 and IL-2 production and is predominantly expressed in T_{EM} $CD8^+$ T cells (286, 287). Therefore it is possible that these factors may contribute to the variegated development of T_{EM} and T_{CM} in response to cytokines. If that is the case, then differential regulation of these factors by IL-12 and IFN-α/β may explain the divergent roles of these cytokines on T_{EM} and T_{CM} fates.

Comparison of T_{EM} and T_{CM} driven by IL-12 and IFN-α/β by microarray analysis might shed light on the factors involved in this variegation. Here it might be possible to identify target mRNAs which are induced by IFN-α/β in T_{CM} but not T_{EM} populations and that are independent of IL-12 regulation. Alternatively, factors involved in T_{EM} generation might be found in a reciprocal approach. Indeed, these approaches have been used previously to identify genetic differences in $CD8^+$ effector and memory cells (288) as well as in comparison of $CD4^+$ T_{CM} and T_{EM} cells (289). Here I propose the addition of cytokine

polarization to these comparisons as a means to likely narrow down target genes capable of mediating the variegated development of T_{EM} an T_{CM} in response to IL-12 and IFN-α/β.

Models of memory and the roles of signals 1, 2 and 3

Multiple models of T cell memory development have been proposed. For example some propose a linear development of memory cells from rested effectors (171, 181, 182) while others favor simultaneous development of memory cells from a starting pool of naïve cells (178, 184-186). My data clearly support a model for memory development in which $CD8^+$ T_{EM} and T_{CM} populations develop simultaneously from the same pool of naïve cells. Further this data excludes a linear model of development as T_{CM} and T_{EM} populations display distinct functional characteristic and can be distinguished from each other as early as the first cellular division. This is similar to observations by Reiner and Chang in which T_{CM} and T_{EM} development could occur through a process of asymmetric cell division leading to phenotypic differences in cells as early as the first division (186). Therefore, my studies demonstrate for the first time that innate cytokines can directly shape the simultaneous development of $CD8^+$ T_{CM} and T_{EM}.

IL-12 and IFN-α/β promote the variegated development of T_{EM} and T_{CM} through differential expression and responsiveness via their respective receptors. Moreover this was directly linked to the capacity of cells to proliferate *in vitro*. As IL-12 is known to enhance cellular proliferation (5), it was not surprising that cells responsive to IL-12 rapidly proliferated and attained effector phenotypes. In contrast, I found that cells expressing high levels IFNAR2 were highly responsive to IFN-α/β and displayed attenuated proliferation when compared to other cytokine driven populations. This is of interest, as the role of IFN-α/β on T cell proliferation has been paradoxical. For example, initial studies observed that IFN-α/β could slow the proliferation of both $CD4^+$ and $CD8^+$ cells *in vitro*(290) and in $CD8^+$ T cells during viral infection *in vivo* (291, 292). Alternatively, more recent *in vitro* studies by Marrack and colleagues (194) as well as *in vivo* studies by Murali-Krishna's group (195, 196) showed that IFN-α/β was required for the clonal expansion and survival of T cells. My data now clearly demonstrate that IFN-α/β acts directly to slow the progression of cell division in human $CD8^+$ T cells and this effect is directly correlated to the development of T_{CM} fate.

It is unclear how IFN-α/β inhibits division. However factors involved in cell cycle progression are likely involved. The cyclin dependent kinase family members CDK2 and CDK6 have been implicated in the rapid division of memory cells (240). Alternatively, the CDK inhibitor, $p27^{KIP-1}$ has been shown to be

highly expressed in cells which do not actively divide and further, a role for the cytokine/STAT pathway has been shown to regulate this response in T cells (240, 241, 293). In addition, IFN-α/β has been shown to modulate the expression of the p27 family member $p21^{WAF-1}$. Perhaps then it is possible that regulation of p21 or p27 or modulation of other factors involved in this pathway by IFN-α/β may play a role in slowing the progression of cellular division. If that is the case, then the use of pharmological inhibitors of either CDKs or molecules like p21 and p27 might reveal their importance of these factors in the variegated development of T_{EM} and T_{CM}. In addition, examination of the expression of these factors as a function of cytokine treatment will also be beneficial in tying their function to the specific abilities of IL-12 and IFN-α/β to drive T_{EM} and T_{CM} respectively.

The variegated development of T_{EM} and T_{CM} from the same starting pool of cells suggested a role for pathways other than just signal 3 cytokines in this process. The strength of TCR activation has been shown to directly influence the development of T_{EM} and T_{CM} populations of cells (235, 238, 294). I observed that the ability of cells to differentially respond to cytokine driven T_{CM} and T_{EM} development was directly linked to the strength of signal 1 during priming. My work now highlights an important role for cytokines in the previous observations and suggests that under conditions in which antigen is low, the IFN-α/β signal is interpreted and memory development occurs efficiently. Alternatively when antigen levels are high the importance is shifted to immediate clearance of

pathogen, therefore fewer cells are responsive to IFN-α/β and effector development occurs at the expense of memory. Moreover, these findings have direct correlations to what is observed during functional viral infections. For example during infection with acute viruses such as Influenza or Measles virus, antigen levels are relatively low and strong memory populations develop efficiently (294, 295). In contrast, examination of chronic infections with agents such as EBV, CMV, HIV, LCMV and HCV demonstrate poor memory formation, which is likely due to increased exposure of T lymphocytes to antigen (180, 239, 295, 296). Therefore in regards to development of vaccines and therapies against viral disease, it is likely that vaccines which induce a tempered inflammatory environment will be better suited a promoting efficient CD8 memory responses against secondary infection.

In addition to my observations regarding classical signal 1 and signal 2 on the activation of $CD8^+$ T cells, I observed a unique role for activation of CD27 in the generation of CTL activity. While the global significance of this observation is unclear, it is possible that this pathway increases the development of effector responses during infection. This is supported by our observation that the ligand for CD27, CD70 is positively regulated by IL-12. Therefore in addition to a role for IL-12 in programming T_{EM} and effector responses, it may also function in the indirect generation of CTL activity via the induction of CD70 on T lymphocytes. Both $CD4^+$ T cells and B cells have been ascribed roles in the activation of CD27

by CD70 (259, 297). Therefore it would be interesting to see whether IL-12 regulates the expression of CD70 on these populations as well. If so this might suggest that activation of the CD27 pathway by CD70 serves as a major mechanism by which effector CTL activity occurs. Further, adoptive transfer systems of $CD8^+$ T cells from a $CD27^{-/-}$ background may shed light as to the importance of this pathway *in vivo*. This could then be correlated to phenotypes observed in $IFNAR^{-/-}$ or $IL\text{-}12R^{-/-}$ systems to determine the effect of cytokines on this pathway.

Taken in the context of human health and disease, the work presented here has direct implications on the therapeutic modulation of T lymphocytes during disease. For instance, IFN-α/β has been used to treat multiple disease states including autoimmune disorders and a variety of viral infections including HCV and HIV (60, 65, 298). However, the effect of IFN-α/β on these diseases has been highly variable. For example, although IFN-α/β can be a beneficial treatment against HCV infection, only 15-20% of HCV infected patients respond productively to treatment with IFN-α/β (298). As HCV is a chronic infection and given the results presented here, it is possible that in the responsive patients, IFN-α/β is capable of rescuing the T cell pool from a predominantly terminal differentiated fates and instead shift T cells towards the T_{CM} phenotype and the

clearance of virus. Further understanding of the mechanism of IFN-α/β mediate T_{CM} development will be beneficial in modulating its therapeutic efficacy.

In addition, a role for IFN-α/β in the pathology of Lupus has been described. Here, microarray studies detected a strong interferon "signature" in whole blood PBMCs (299, 300). This suggested a potential role for IFN-α/β and ISGs in mediated disease. Alternatively, IFN-α/β has been used to treat patients with multiple sclerosis and the effects appear to be positive and to limit the auto-inflammatory environment (65, 301). While these studies are paradoxical, my work suggests potential mechanisms for interferon activity in these disease states. For example during Lupus, IFN-α/β may be enhancing the development of populations of T_{CM} which are recognizing self antigens and are therefore pathogenic in nature. Alternatively in MS patients, it is possible that the anti-proliferative effects of IFN-α/β act to decrease immediate effector function and limit disease. As these disease states are very complex there is no doubt that a variety of mechanisms and signaling pathways are involved in disease. However a clearer understanding of the how IFN-α/β mediates the block in effector function and the promotion of T_{CM} will surely be beneficial in enhancing its therapeutic role against these auto-immune disorders.

Future directions and concluding remarks

Given the complexity of antiviral responses, teasing apart the contributions of specific innate cytokines involved in the induction of type I T lymphocyte programs has been a challenging endeavor. I chose to examine the direct roles of IL-12 and IFN-α/β on type I development as these are two cytokines positioned to directly participate in programming of both $CD4^+$ and $CD8^+$ T lymphocytes. For example, during viral infection IL-12 and IFN-α/β can be produced in large quantities by cDCs, pDCs and MΦs (6, 24, 97, 302-304). Moreover, the secretion of both of these cytokines has been shown to occur in the draining lymph nodes by different subsets of DCs and is important to the T lymphocyte response to a variety of viruses (6, 24, 304-306). This suggests that at the time of T cell priming, IL-12 and IFN-α/β may play important roles in programming naïve T cells to their specific anti-viral fates. To address this directly, we established an *in vitro* cytokine polarization model system. While this system does not entirely mimic the inflammatory conditions found *in vivo*, it serves as the best model to directly tease apart the individual contributions of these cytokines on priming human T lymphocyte responses.

Two main questions have arisen from the observations of these studies and will be very interesting avenues of research going forward. First, what factor(s) are responsible for the variegated development of effector and central memory

responses by IL-12 and IFN-α/β and second the functional significance of these cytokines during complex *in vivo* infection. To address the question of what factors drive the variegated development of T_{EM} and T_{CM} by IL-12 and IFN-α/β, I have compared highly purified populations of T_{CM} and T_{EM} populations of cells arising from cytokine treatment by microarray analysis. In collaboration with Fatema Chowdhury in the lab, we have generated several functional comparisons of genes differentially regulated by cytokine in either the T_{CM} or T_{EM} populations. From these studies, it is possible that we will identify target genes which are regulated by either IFN-α/β alone or IL-12 alone and correlate to T_{CM} or T_{EM} populations respectively. Once targets have been identified and characterized, two approaches may be useful in demonstrating a role in the variegated development of T_{CM} and T_{EM}. First, we can use targeted siRNA approaches to knockdown the expression of target mRNAs and assess their role in effector and memory generation. Second, we can revise our techniques for retroviral transduction and examine the effects of overexpression of these target genes on the development of T_{EM} and T_{CM}.

In addition, it will be important to correlate our observations to *in vivo* responses. Here we can use IFNAR or IL-12R deficient mice to tease apart these pathways *in vivo* against a variety of intracellular infections. Recent work by Mescher's group has suggested that both IL-12R and IFNAR deficient mice have defects in the development of memory (307). My data takes this observation one

step further and now directly defines the development of human T_{EM} and T_{CM} occurs as a function of IL-12 and IFN-α/β respectively. Therefore, adoptive transfer models of IL-12 and IFNAR deficient cells during infection may allow us to directly link the development of T_{CM} and T_{EM} to cytokines *in vivo*. Alternatively, culture of human T lymphocytes with either PRR-primed or infected pDCs or cDCs may highlight the human "*in vivo"* relevance of IL-12 and IFN-α/β mediated effector and memory development.

The findings presented in these studies have direct implications on development of vaccine strategies to infection. The classic linear model of memory development suggests that the size of the initial clonal burst is directly related to the size of the ensuing memory population (171, 308). In contrast, my work clearly demonstrates a sequential development of T_{CM} and T_{EM} in human $CD8^+$ T lymphocytes and suggest that as the inflammatory signals are increased, the size of the terminally differentiated effector pool grows at the expense of T_{CM} development. Therefore it is likely that in order to elicit optimal human $CD8^+$ T cell memory to immunization, a vaccine must promote more tempered antigen activation.

Recent vaccine strategies have utilized TLR agonists such as CpG DNA and Poly:IC to enhance the effectiveness of immunization against HSV and Influenza virus (309-311). These PAMPs trigger TLR9 and TLR7 respectively and have been shown to activate pDC production of IFN-α/β (24, 39, 40, 57,

312). My work has clearly defined a role for IFN-α/β in the development of T_{CM}. Therefore it is likely that utilizing adjuvants which induce high levels of IFN-α/β during T cell priming enhance the development of T_{CM} generation and the induction of strong protective immunity. The results presented in these findings offer a new understanding of the interactions between signal 1, 2 and 3 during the activation of human $CD8^+$ T lymphocyte memory. Moreover, they demonstrate a necessity for crosstalk between signals 1, 2 which allows for signal 3 cytokines to promote the outgrowth of both T_{CM} and T_{EM} populations of cells during infection. Therefore, unlike the standard model for memory generation, my work clearly demonstrate that vaccine strategies which generate low level antigen activation in the presence of adjuvants which elicit high level IFN-α/β are likely key to the induction of protective $CD8^+$ T cell immunity.

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