REGULATION OF THE EFFECTOR AND MEMORY DEVELOPMENT OF HUMAN CD4$^+$ T CELLS BY INTERLEUKIN 12 AND TYPE I INTERFERON

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DEDICATION

To my husband, Matt, for everything.

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by

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Innate cytokines induced at the onset of infection regulate the development of adaptive immune responses such as $CD4^+$ T helper cell development. For instance, the innate cytokines interleukin 12 (IL-12) and type I interferon (IFN-α/β) are produced in response to intracellular bacterial and viral infections. While the effects of IL-12 on $CD4^+$ T cell differentiation are relatively well-understood, the role of IFN-α/β, despite extensive study, has remained controversial. The present work seeks to clarify the effects of IFN-α/β on $CD4^+$ T cell development, effector functions, and memory generation. Previous reports had suggested that IFN-α, like IL-12, could promote Th1 development

in human $CD4^+$ T cells. However, my work demonstrates that IFN-α is insufficient to induce Th1 differentiation because of an inability to maintain stable STAT4 phosphorylation or T-bet expression. Furthermore, IL-12, but not IFN-α, induces the secretion of IFN-γ and TNF-α from human $CD4^+$ T cells. These two cytokines, in addition to promoting bacterial clearance, can directly participate in antiviral immunity via a signaling pathway which involves the type I IFN receptor. Finally, a combination of IL-12 and IFN-α influences memory $CD4^+$ T cell function by strongly inducing IL-2 secretion from a subset of cells in a T-bet-independent manner. These IL-2-producing cells demonstrate both phenotypic and functional characteristics of long-lived and pluripotent central memory. Taken together, these data provide a new understanding of the role of innate cytokines in shaping adaptive $CD4^+$ T cell responses. Given the numerous medical uses of IFN-α/β, these findings could have a broad impact on the design of vaccines and antiviral therapeutics.

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LIST OF ABBREVIATIONS

α – anti

Ab – antibody

ADAR – double-stranded RNA-specific adenosine deaminase

AP-1 – activation protein-1

APC – allophycocyanin

APC – antigen presenting cell

APOBEC3G – apolipoprotein B mRNA-editing enzyme, catalytic polypeptide-like 3G

BSA – bovine serum albumin

CBA – cytometric bead array

CCL – chemokine (C-C motif) ligand

CCR – chemokine (C-C motif) receptor

cDMEM – complete Dubelcco's modified Eagle medium

cDNA – complementary DNA

CFSE – carboxyfluorescein diacetate succinimidyl ester

cfu – colony forming unit

cIMDM – complete Iscove's modified Dubelcco's medium

CMV – cytomegalovirus

CTL – cytotoxic T lymphocyte

CXCL – chemokine (C-X-C motif) ligand

CXCR – chemokine (C-X-C motif) receptor

DC – dendritic cell

DNA – deoxyribonucleic acid

ds – double-stranded

eIF2α – eukaryotic initiation factor 2α

Eomes – eomesodermin

ELISA – enzyme-linked immunosorbent assay

FBS – fetal bovine serum

FITC – fluorescein isothiocyanate

GATA-3 – GATA binding protein 3

γc – common gamma chain

GFP – green fluorescent protein

γHV68 – gammaherpesvirus 68

h – human

HCV – hepatitis C virus

IFN – interferon

IFNAR – interferon-α/β receptor

Ig – immunoglobulin

IKKε – inhibitor of κB kinase ε

IL – interleukin

iNOS – inducer of nitric oxide synthesis

IRAK4 – interleukin 1 receptor-associated kinase 4

IRF – interferon regulatory factor

ISG – interferon stimulated gene

ISGF3 – interferon stimulated gene factor 3

LCMV – lymphocytic choriomeningitis virus

LT – lymphotoxin

MAMP – microbe-associated molecular pattern

MHC – major histocompatibility complex

m/hST2ki – chimeric murine/human STAT2 knock-in mouse

MS – multiple sclerosis

Mx – myxovirus resistance protein

MyD88 – myeloid differentiation factor 88

NF-κB – nuclear factor κB

NK – natural killer cell

NO – nitric oxide

NOD – nucleotide-binding oligomerization domain

OAS – 2'-5' oligoadenylate synthase

p – phosphorylated

PBMC – peripheral blood mononuclear cell

PBS – phosphate buffered saline

PCR – polymerase chain reaction

PDC – plasmacytoid dendritic cell

PE – phycoerythrin

pfu – plaque forming unit

PKR – protein kinase R

PMA – phorbol 12-myristate 13-acetate

p-MHC II – peptide-class II MHC complex

PRR – pattern recognition receptor

qPCR – quantitative real-time polymerase chain reaction

R – receptor

RA – rheumatoid arthritis

rh – recombinant human

RIG-I – retinoic acid inducible gene I

RIPA – radioimmune precipitation assay

RNA – ribonucleic acid

RSV – respiratory syncytial virus

RV - retrovirus

SLE – systemic lupus erythematosus

ss – single-stranded

STAT – signal transducer and activator of transcription

T-bet – T-box expressed in T cells

TBK-1 – Tank-binding kinase 1

TCM – T cell conditioned media

T_{CM} – central memory T cells

T_{EM} – effector memory T cells

TCR – T cell receptor

Th – T helper

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

CHAPTER I

INTRODUCTION

Overview

The responses of the adaptive immune system are regulated by the production of cytokines by innate cells at the onset of infection. During intracellular bacterial and viral infections, the differentiation of $CD4^+$ T helper cells has been proposed to be instructed by interleukin 12 and type I interferon produced by dendritic cells responding to the presence of pathogens. The functions of interleukin 12 in promoting $CD4^+$ T cell responses have been well-studied, but the roles of type I interferon in regulating the differentiation, effector functions, and memory generation of T helper cells remain incompletely understood.

Innate recognition of pathogens results in production of interleukin 12 and type I interferon

Initial contact between host and pathogen is mediated by phagocytic cells, particularly dendritic cells (DCs), which are situated throughout peripheral tissues at potential sites of pathogen entry. As professional antigen presenting cells (APCs), DCs play an extremely important role in the initiation of immune responses (1-4). These cells express a class of receptors known as pattern recognition receptors (PRRs), which

transmit "danger signals" at the onset of infection. These receptors include the Toll-like receptors (TLRs), transmembrane receptors which are members of the Toll/interleukin-1 (IL-1) superfamily, as well as intracellular sensors such as the nucleotide-binding oligomerization domain (NOD) proteins and retinoic acid-inducible gene I (RIG-I) (5-8). PRRs recognize a conserved set of pathogen-associated molecular patterns (PAMPs) found on viral, bacterial, fungal, and protozoan microorganisms (9, 10). PRR ligation by a cognate PAMP results in DC maturation and upregulation of class II major histocompatibility complex (MHC II) as well as costimulatory molecules such as CD80 and CD86 (5, 11, 12). Activated DCs upregulate expression of the chemokine receptor C-C chemokine receptor 7 (CCR7), which promotes migration to lymph nodes. Once there, mature DCs induce the maturation of naïve T cells into armed effectors (1, 2).

In addition to maturation, TLR ligation also induces DCs to secrete a variety of inflammatory cytokines (5, 12). These innate cytokines participate in the differentiation of naïve $CD4^+$ T cells by favoring the development of diverse functional subsets. In particular, interleukin 12 (IL-12) and type I interferons (IFNs), both of which are produced by DCs in response to bacterial and viral infections, have been proposed to play a role in the development of the T helper 1 (Th1) subset of $CD4^+$ T cells (10, 13-18).

Several subsets of DCs can produce both IL-12 and type I IFN in response to TLR signaling (Fig. 1). Human and murine plasmacytoid dendritic cells (PDCs) and murine $CD8\alpha^+$ DCs, express TLR7 and/or TLR9 in endosomal compartments (17, 19-22). These two TLRs recognize single-stranded RNA (ssRNA) and unmethylated CpG motifs in double-stranded DNA (dsDNA), respectively, which are components of many viral and bacterial pathogens (23, 24). Activation of TLR7 or TLR9 results in

recruitment of the Toll/IL-1 receptor (TIR) domain-containing adaptor molecule myeloid differentiation factor 88 (MyD88) (25-27). MyD88 associates with IL-1 receptor-associated kinase (IRAK), which phosphorylates and activates TNF receptor-associated factor 6 (TRAF6) (25, 28-30). TRAF6 then initiates a cascade of intracellular signaling events which culminates in the release of the transcription factors nuclear factor κB (NFκB) and activation protein-1 (AP-1) to the nucleus (25, 28, 31). Activated NFκB and AP-1 induce secretion of IL-12 as well as other proinflammatory innate cytokines (28, 32-34). MyD88 can also promote phosphorylation and activation of the transcription factor interferon regulatory factor 7 (IRF-7) in an IRAK- and TRAF6-dependent manner (35-38). Phosphorylated IRF-7 then dimerizes and translocates to the nucleus to induce expression of IFN-α as well as other interferon-stimulated genes (ISGs) (37, 39). Therefore, PDCs and $CD8\alpha^+$ DCs can secrete both IL-12 and IFN-α in response to viral and bacterial infections. It should be noted, however, that PDCs produce substantially more IFN-α than other DC subtypes, whereas $CD8\alpha^+$ DCs are the most significant source of IL-12 during intracellular infections (21, 40).

Another subset of DCs, the conventional DCs (cDCs), can also secrete both IL-12 and type I IFN. These cells express high levels of TLR3, an endosomal receptor recognizing double-stranded RNA (dsRNA) (41), and TLR4, a cell-surface receptor which is activated by bacterial lipopolysaccharide (LPS) and viral coat proteins (42, 43). TLR4 stimulation induces IL-12 secretion through MyD88 in the same manner as activation of TLR7 and TLR9. Additionally, TLR3 and TLR4 utilize a MyD88-independent signal transduction cascade involving the adaptor molecule TIR domain-

containing adaptor inducing IFN-β (TRIF) (44-47). In addition to NFκB, TRIF promotes activation of two kinases: Tank-binding kinase 1 (TBK-1) and inhibitor of κB kinase ε (IKKε) (48, 49). These two kinases phosphorylate and activate another interferon regulatory factor, IRF-3 (49, 50). Homodimerization and nuclear translocation of phosphorylated IRF-3 results in IFN-β secretion (51-53). Thus, like PDCs and CD8α^+ DCs, mDCs are also capable of producing both IL-12 and type I IFN in response to bacterial and viral infections.

IFN-α plays an important role in innate immunity

In addition to induction in professional APCs, type I IFNs can also be produced by nearly all somatic cells following intracellular recognition of viral infection through ubiquitously expressed PRRs such as MDA5 and RIG-I (54, 55). These molecules are cytosolic sensors which recognize dsRNA within infected cells. Binding of RIG-I or MDA5 to dsRNA initiates a cascade of signaling events which culminates in the activation of both NFκB and IRF-3, leading to the production of IFN-β (54-56). IFN-β signals the infected cell in an autocrine manner to induce expression of interferon stimulated genes (ISGs) as well as other type I IFN subtypes, initiating a viral resistance program. Furthermore, secretion of type I IFNs provides a paracrine signal to neighboring cells to promote resistance to viral spread (57).

In humans, the type I IFN family of cytokines is comprised of 13 functional IFN-α genes and one IFN-β gene, as well as the more recently discovered IFN-ε, IFN-κ, and

IFN-ω subtypes (58-60). All of these cytokines signal through a shared cell surface receptor, the IFN-α/β receptor (IFNAR). The IFNAR is a type 1 transmembrane receptor with two subunits, IFNAR1 and IFNAR2, and it is associated with a pair of Janus family kinases, Jak1 and Tyk2 (61-66). Binding of type I IFN to the IFNAR results in phosphorylation of the cytoplasmic tails of the receptor by these kinases, enabling docking of the SH2 domain-containing proteins signal transducers and activators of transcription (STATs) 1 and 2 (67-69). Phosphorylated STAT1 and STAT2, together with the p48/IRF-9 transcription factor, form the trimeric interferon-stimulated gene factor 3 (ISGF3) complex (70). ISGF3 translocates to the nucleus and regulates the expression of a substantial number of ISGs, which are involved in host resistance to intracellular infections.

While the roles of many ISGs in the innate response to viral infection are unknown, a number of ISGs have been ascribed one or more antiviral functions. The most well-studied of these is protein kinase R (PKR). PKR becomes activated by binding dsRNA in the cytosol of virally infected cells. Following activation, PKR phosphorylates and inactivates eukaryotic initiation factor 2α (eIF2α), an indispensable component of the protein translational machinery. This results in a general shutoff of *de novo* mRNA translation within infected cells (71-74). Since viruses are reliant on host cell machinery for their replication, a blockade of translation is a particularly effective mechanism of viral resistance.

In addition to PKR, several other ISGs have been shown to have important antiviral functions. For instance, the 2'-5' oligoadenylate synthase (OAS) proteins

polymerize ATP by way of unique 2'-5' linkages to create 2'-5' oligoadenylates. These small molecules activate RNase L, which cleaves mRNAs within virally infected cells, inhibiting protein synthesis (75-77). Several ISGs also have specific activities against a narrow range of viruses. The myxovirus resistance (Mx) proteins specifically inhibit influenza virus and vesicular stomatitis virus by suppressing viral translation (78). Furthermore, type I IFN-induced deaminases such as ADAR and APOBEC3G have been shown to promote lethal hypermutation of retroviral genomes during reverse translation and viral packaging (79-81). The ISG56 protein specifically inhibits the translation of the hepatitis C virus (HCV) polyprotein to mediate anti-HCV effects of IFN-α (82). Finally, in addition to inducing expression of ISGs, type I IFNs directly promote apoptosis in virally infected cells, thereby helping to prevent viral replication and spread (83, 84).

The importance of the type I IFN system in viral resistance is underscored by the variety of strategies employed by viral pathogens to subvert this signaling cascade. Poxviruses encode a soluble version of the IFNAR, which neutralizes free type I IFN and prevents signaling (85). Many viruses also interfere with induction of type I IFNs via the RIG-I pathway or with proximal type I IFN signal transduction (86-92). Finally, a range of viruses encode one or more proteins which inhibit the actions of PKR, OAS, Mx proteins, and other ISGs (93-97). Clearly, type I IFN signaling is an essential component of innate immunity to viral infections.

The innate cytokines IL-12 and IFN-α direct CD4$^+$ Th1 development

CD4$^+$, or helper, T cells are adaptive immune mediators which provide crucially important instructions to other cells of the immune system during infection. The differentiation of naïve CD4$^+$ T cell precursors into effector cells begins with engagement of the T cell receptor (TCR) with a cognate antigenic peptide presented by MHC II (p-MHC II) on the surface of an activated DC (98). In addition to p-MHC II, mature DCs also express high levels of CD80 and CD86, which provide naïve CD4$^+$ T cells with a necessary costimulatory signal via interactions with CD28 (5, 11, 12). TCR ligation and costimulation result in a cascade of intracellular signaling events which culminates in maturation and clonal expansion of naïve T helper cells, providing a large pool of effectors to combat infection (99, 100).

In addition to TCR ligation and costimulation, the differentiation of naïve CD4$^+$ T cell involves stimulation by innate cytokines secreted by DCs. Different patterns of innate cytokines favor the development of distinct T helper subsets which are specialized for various classes of infection. In particular, IL-12 regulates differentiation to the Th1 subset in both human and murine CD4$^+$ T cells (18, 101-104) (Fig. 2). The IL-12 receptor (IL-12R) is a type 1 transmembrane receptor composed of two subunits, IL-12Rβ1 and IL-12Rβ2 (105). IL-12Rβ2 is not expressed on naïve CD4$^+$ T cells and must therefore be upregulated in order to confer IL-12 responsiveness (15, 106). Interferon gamma (IFN-γ) secreted by natural killer (NK) cells induces phosphorylation and activation of STAT1 in developing T helper cells. Dimers of phosphorylated STAT1 translocate to the nucleus and induce expression of T-box expressed in T cells (T-

bet/Tbx21), the master regulator of Th1 development (106-108). T-bet upregulates surface expression of IL-12Rβ2, licensing Th1 differentiation (106, 108-110).

The IL-12R is associated with the Janus family kinases Jak2 and Tyk2 (111). Upon binding of IL-12 to its receptor, these kinases phosphorylate conserved tyrosines in the cytoplasmic tails of the receptor subunits, promoting docking and subsequent phosphorylation of the essential transcription factor STAT4 (112-115). Like STAT1, phosphorylated STAT4 forms homodimers which translocate to the nucleus and initiate transcription of a Th1-specific gene program (112, 115, 116). STAT4 phosphorylation is indispensable for Th1 development; mice and humans which carry mutations in STAT4 show defects in Th1 responses and are susceptible to a variety of intracellular infections (117-120).

STAT4 activation is directly required to mediate high-level secretion of IFN-γ by mature Th1 cells (117-119). IL-12 can also promote TCR-independent recall secretion of IFN-γ from fully polarized Th1 cells in a STAT4-dependent manner; this effect occurs in synergy with the innate cytokine IL-18, which is secreted by macrophages during inflammatory responses (121-124). Finally, IL-12 stimulation of developing $CD4^+$ T cells also leads to further upregulation of IL-12Rβ2 and T-bet, reinforcing Th1 commitment (15, 110, 114, 125, 126). Hence, IL-12 controls multiple aspects of Th1 development and function. T-bet, in addition to potentiating Th1 commitment by inducing IL-12Rβ2, also directly regulates IFN-γ expression; $T\text{-}bet^{-/-}$ animals, like $STAT4^{-/-}$ animals, show profound defects in the generation of Th1 responses (107-110,

127). Thus, a series of overlapping events, controlled by innate cytokines, regulates the development of the Th1 subset.

In addition to their critical role in viral resistance, type I IFNs, like IL-12, have been proposed to play a role in Th1 development in human, but not murine, $CD4^+$ T cells (Fig. 3). As mentioned previously, the IFNAR, like the IL-12R, is also a type 1 transmembrane receptor with two subunits, IFNAR1 and IFNAR2, and is associated with two Janus family kinases, Jak1 and Tyk2 (61-66, 111). Initial reports noted that IFN-α, like IL-12, could induce IFN-γ secretion from human peripheral blood mononuclear cells (hPBMCs), cord blood T cells, or unfractionated $CD4^+$ T cells (14, 15, 114). A combination of IFN-α and IL-18 has also been shown to promote antigen-independent IFN-γ production in a manner similar to IL-12 + IL-18 (128, 129). It was further noted that brief stimulation of human $CD4^+$ T cells with IFN-α resulted in STAT4 phosphorylation similar to that observed with IL-12 (15, 113, 114, 130). Finally, IFN-α has been shown to induce expression of both IL-12Rβ2 and T-bet in human T helper cells (15, 114, 125, 131). Hence, IFN-α appears to regulate multiple aspects of Th1 differentiation in human $CD4^+$ T cells.

In contrast, murine $CD4^+$ T cells failed to demonstrate IFN-γ secretion or STAT4 phosphorylation in response to IFN-α (130, 132). It was proposed that this species difference was the result of a minisatellite insertion in murine STAT2 which rendered this protein incapable of mediating preassociation of STAT4 with the IFNAR (130). However, IFN-α still failed to promote STAT4 activation in murine T helper cells from mice carrying a humanized version of STAT2 (m/hST2ki) (133, 134). Instead, it has

been shown that species differences in the ability of the N-terminal domain of STAT4 to preassociate with human versus murine IFNAR2 resulted in the observed differences in IFN-α signaling (135, 136).

More recent work has called these initial observations regarding the role of IFN-α in Th1 development into question. Several groups have suggested that IFN-α might, in fact, promote Th1 development in murine $CD4^+$ T cells. Recent reports have demonstrated a role for IFN-α in promoting IFN-γ secretion during infections with lymphocytic choriomeningitis virus (LCMV) or Gram-positive bacteria, although in some cases the responding cells were likely $CD8^+$ T cells (137-141). Furthermore, Biron and colleagues have demonstrated that in the absence of STAT1, IFN-α can promote STAT4 phosphorylation in murine $CD4^+$ T cells (140). In contrast, although work by Berenson et. al. confirmed that IFN-α could promote a minimal level of STAT4 phosphorylation in murine T helper cells, the observed STAT4 phosphorylation was insufficient to promote full Th1 commitment, as measured by IFN-γ secretion, even at concentrations of IFN-α up to 100,000 U/ml (142, 143). Furthermore, publications from several groups have demonstrated that even in human $CD4^+$ T cells, IFN-α induced an attenuated phosphorylation of STAT4 compared to that seen with IL-12, calling into question the ability of IFN-α to direct human Th1 commitment (144, 145). Therefore, despite extensive study, the role of type I IFN in both murine and human Th1 development remains controversial.

Effector Th1 cells direct inflammatory immune responses

As noted above, fully differentiated $CD4^+$ T cells are divided into functional subsets based upon patterns of cytokine secretion and participation in responses in various classes of infection. In particular, the Th1 subset has been proposed to be important for coordinating immune responses against intracellular bacterial and viral pathogens (146-151). These cells secrete an array of proinflammatory mediators, most importantly IFN-γ, tumor necrosis factor alpha (TNF-α), and lymphotoxin (LT/TNF-β) (152, 153). The direct roles of IFN-γ and TNF-α in promoting bacterial clearance are well-defined (Fig. 4A). Macrophages which have engulfed invading bacteria present bacterial antigens to mature Th1 cells as p-MHC II. Recognition of bacterial peptides induces Th1 effectors to secrete IFN-γ and TNF-α. IFN-γ and TNF-α upregulate inducible nitric oxide synthase (iNOS) and nicotinamide adenine dinucleotide phosphate oxidase (NADPH oxidase) in a STAT1-dependent manner in responding macrophages, leading to induction of the oxidative burst. This culminates in release of nitric oxide (NO), hydrogen peroxide (H_2O_2), and oxygen radicals into the phagosome, resulting in bacterial clearance (149, 154-160). These two cytokines have a similar effect on the antimicrobial activities of neutrophils (161-164). Thus, Th1 cells participate directly in bacterial killing by IFN-γ and TNF-α production.

In addition to their effects on phagocytosis and bacterial killing, Th1 cytokines also regulate other aspects of an antibacterial immune response. IFN-γ favors B cell production of opsonizing and complement-fixing antibody isotypes such as IgG_{2a},

enhancing phagocytosis and destruction of bacterial pathogens (165, 166). IFN-γ also upregulates the expression of MHC II and costimulatory molecules on phagocytes and B cells, enhancing antigen presentation to $CD4^+$ T cells (160, 167-170); additionally, this cytokine promotes elevated expression of FcγRI on phagocytic cells, which increases engulfment of opsonized bacteria (164, 169). Furthermore, TNF-α and proinflammatory chemokines produced by Th1 cells can promote extravasation and chemotaxis of leukocytes to sites of infection, enhancing the inflammatory response (171).

In contrast to bacterial infections, the role of Th1 cells in viral immunity is incompletely understood. Clearance of viral infections is generally thought to be less dependent upon the actions of $CD4^+$ T cells, relying instead upon the effector functions of NK cells and $CD8^+$ cytotoxic T lymphocytes (CTLs) and the activity of neutralizing antibodies (172-174). However, T helper cells can play a supporting role in numerous antiviral immune processes, again by secretion of effector cytokines (Fig. 4B). $CD4^+$ T cells and/or IFN-γ are known to activate NK cells and CTLs, enhancing elimination of virally infected cells (160, 175, 176). Furthermore, Th1 cells can also provide help to B cells to promote production of neutralizing antiviral antibodies (173, 177-180). *In vitro* studies with purified cytokines have shown that TNF-α can induce antiviral responses directly in infected cells, often in synergy with IFN-γ (181-185). A few reports have also suggested that a subset of Th1 cells can develop cytotoxic functions, including expression of CD95L/Fas ligand and the release of cytolytic granules (178, 186-191).

A small number of *in vivo* murine studies have also probed a direct role for $CD4^+$ T cells in antiviral immunity. A helper-independent role for $CD4^+$ T cells has been

proposed in the clearance of influenza virus and Sendai virus (SeV) in animals lacking B cells and/or $CD8^+$ T cells (192-196). Furthermore, $CD4^+$ effector T cells have also been shown to be involved in clearance of vesicular stomatitis virus (VSV), cytomegalovirus (CMV), and gammaherpesvirus 68 (γHV68), and in these cases, IFN-γ was show to be essential for antiviral activity (172, 197-199). However, in these studies, the source and target of the IFN-γ, as well as the precise functions of the antiviral $CD4^+$ T cells, were undetermined. Thus, a full exploration of the direct role of Th1 effector cells in antiviral immunity has yet to be conducted. A better understanding of these processes would provide a clearer picture of antiviral adaptive immunity.

Memory T cells arise during the primary response and provide protection from secondary infection

One of the hallmarks of the adaptive immune system is the generation of pathogen-specific memory responses. Following pathogen clearance, effector $CD4^+$ T cells undergo a pronounced contraction phase which involves apoptosis of the vast majority of the antigen-specific cells. However, a small fraction of cells is retained as a memory pool. These memory cells constitute an antigen-specific population which is qualitatively distinct from naïve precursors; they have a lower threshold of activation, are not dependent upon costimulation, and undergo homeostatic turnover at a higher rate (200, 201). Therefore, memory T cells provide optimal protection against reinfection of the host.

Memory T cells constitute a heterogeneous population which can be divided into phenotypic and functional subsets based upon the expression of cell surface markers. Initial reports described two subpopulations of memory $CD4^+$ T cells, termed central memory (T_{CM}) and effector memory (T_{EM}). T_{CM} cells are $CD45RA^-$, indicating that they have received activation through the TCR, but these cells also demonstrate high expression of the lymph node-homing chemokine receptor CCR7, previously considered a marker of naïve T cells and DCs (202). T_{CM} cells express low levels of the Th1 and Th2 effector cytokines IFN-γ, IL-4, and IL-5, but they secrete copious amounts of IL-2, a growth factor for activated T cells (202, 203). Furthermore, these cells traffic predominantly to secondary lymphoid organs, and they show substantial proliferative capacity and functional plasticity upon secondary activation (204-207). In contrast, T_{EM} cells are $CD45RA^-$ and $CCR7^{lo}$ (202). These cells secrete little IL-2 but substantial amounts of IFN-γ, IL-4, and IL-5 (202, 203). They are preferentially localized to peripheral tissues and display a more terminally differentiated phenotype, with little functional plasticity and a lower rate of proliferation (205, 207). More recent reports have expanded these initial observations based on expression of the cell surface markers CD27 and CD28 (208-210); however, the original distinction between T_{CM} and T_{EM} remains the most reliable and well-studied classification.

While the functional differences between T_{CM} and T_{EM} are relatively clear, there has been substantial controversy regarding the development and lineage relationships of these memory T cell subsets (Fig. 5). Some groups have proposed that T_{CM} and T_{EM} represent fully distinct lineages (211, 212). This hypothesis was supported by the finding that cohorts of human T_{CM} and T_{EM} clones showed only marginally overlapping usage of

TCR rearrangements (211). Furthermore, recent work by Reiner and colleagues has suggested that naïve $CD8^+$ T cells undergo asymmetric cell division upon initial activation by DCs, with one daughter cell inheriting determinants of effector phenotype and the other receiving a predominance of several molecules associated with memory functions (213).

Other work has suggested that T_{CM} cells might represent a memory cell reservoir which replenishes the T_{EM} pool. Upon secondary activation, T_{CM} cells can be induced to differentiate to a T_{EM} phenotype and secrete IFN-γ (202, 206). Furthermore, T cells which receive an intermediate or weak activation signal, such as those which arrive in the draining lymph nodes late in an immune response when DCs are already exhausted, undergo only a few rounds of division and display phenotypic characteristics of T_{CM}. In contrast, cells which receive a stronger TCR signal undergo more rounds of division and differentiate to an effector or T_{EM} fate (214-216). Based on these observations, it has been proposed that T_{CM} represent an incompletely differentiated pool which serves as a reservoir from which new T_{EM} cells are drawn upon secondary infection.

Finally, it has been suggested that memory T cells can arise via a linear differentiation pathway. In this model, naïve cells differentiate first to effectors and then to T_{EM}. Finally, T_{EM} undergo a dedifferentiation process to give rise to long-lived T_{CM}. Several groups have demonstrated that T_{EM} can convert to a T_{CM} phenotype upon restimulation or adoptive transfer to a naïve host (217, 218), although Marzo et. al. found that this phenomenon only occurred in cases in which high precursor frequency led to substantial competition for antigen (219). Furthermore, it has also been shown that adoptive transfer of a heterogeneous population of primary activated $CD4^+$ T cells or of

purified IFN-γ-producing Th1 cells can give rise to long-lived antigen-specific cells and a protective memory response in a naïve host (220-224). Clearly, the lineage relationships among memory T cell subsets, and the mechanisms by which they arise, remain unclear.

In addition to the efforts at understanding lineage relationships among memory T cell subsets, considerable interest has been devoted to the signals which control the generation of these cells. The common gamma chain (γc) cytokines IL-2, IL-7, and IL-15 have been proposed to play significant roles in memory cell survival and proliferation. Ku et. al. demonstrated that IL-15 enhanced the homeostatic proliferation of $CD8^+$ memory T cells, while IL-2, a growth factor which is required for effector cell development, inhibited memory cell proliferation (225). Later, it was reported that IL-7, a growth factor required by naïve T cells, enhanced the survival of memory $CD8^+$ T cells as well (226). Initial observations with $CD4^+$ memory T cells indicated that unlike $CD8^+$ T cells, these cells were not dependent upon IL-7 or IL-15 for their maintenance (226). However, further reports have demonstrated that $CD4^+$ memory T cells do, in fact, require IL-7 and IL-15 signaling for their survival and homeostatic proliferation (227-230). While these two cytokines are clearly important for memory T cell survival, neither IL-7 nor IL-15 directly enhances the generation or functions of either $CD4^+$ or $CD8^+$ memory T cells, nor do these cytokines regulate the balance between the T_{CM} and T_{EM} subsets (231, 232). Therefore, other signals present during the primary phase of the immune response are likely to be involved in the development of memory T cells.

In addition to the γc cytokines, innate cytokines have also been shown to play a role in the survival and expansion of memory T cells. Mescher and colleagues noted that in addition to TCR ligation and costimulation, naïve $CD8^+$ T cells require a stimulus from

innate cytokines, termed the "third signal", in order to achieve full activation and progress to memory formation. Furthermore, this "third signal" could be provided by either IL-12 or IFN-α (233). Additionally, several groups have demonstrated that IFN-α is required for survival and clonal expansion of responding $CD4^+$ and $CD8^+$ T cells during viral infections; in the absence of IFNAR signaling, T cells expanded poorly and failed to generate a protective memory pool (234-237). Hence, the innate cytokines which regulate effector $CD4^+$ T cell differentiation may also have a role to play in the formation of memory populations. A precise understanding of the roles of these cytokines in memory generation will be important in improving vaccine strategies and antiviral therapies.

Concluding Remarks

$CD4^+$ T helper cells play a central role in the adaptive immune response. In particular, the Th1 subset of $CD4^+$ T cells is important in the clearance of intracellular bacterial and viral pathogens through a variety of mechanisms. The ability of these cells to generate a long-lived memory pool is crucial in order to prevent secondary infection. Two innate cytokines, IL-12 and type I IFN, have been proposed to be involved in the effector and memory development of human T cells. While the role of IL-12 in Th1 differentiation is relatively well-studied, the effects of IFN-α on human $CD4^+$ T cell effector and memory generation remain incompletely understood. Intriguingly, IFN-α is used to treat a range of human diseases, including viral infections and autoimmune disorders, but, for reasons which remain unclear, these treatments are only effective in a

fraction of patients (238-243). Furthermore, agents which induce the expression of IL-12 and IFN-α by APCs are currently under consideration as a new generation of vaccine adjuvants (244-248). Hence, it is crucial to define the role of type I IFN in the development of T cell responses in order to gain a better understanding of the impact of this cytokine on human health. My work, therefore, seeks to answer three distinct questions (Fig. 6). First, what is the precise role of IFN-α in promoting the molecular signaling events leading to Th1 differentiation? Second, what is the contribution of IFN-α to the genesis of $CD4^+$ T cell effector functions? Finally, how does IFN-α participate in the process of $CD4^+$ T cell memory formation?

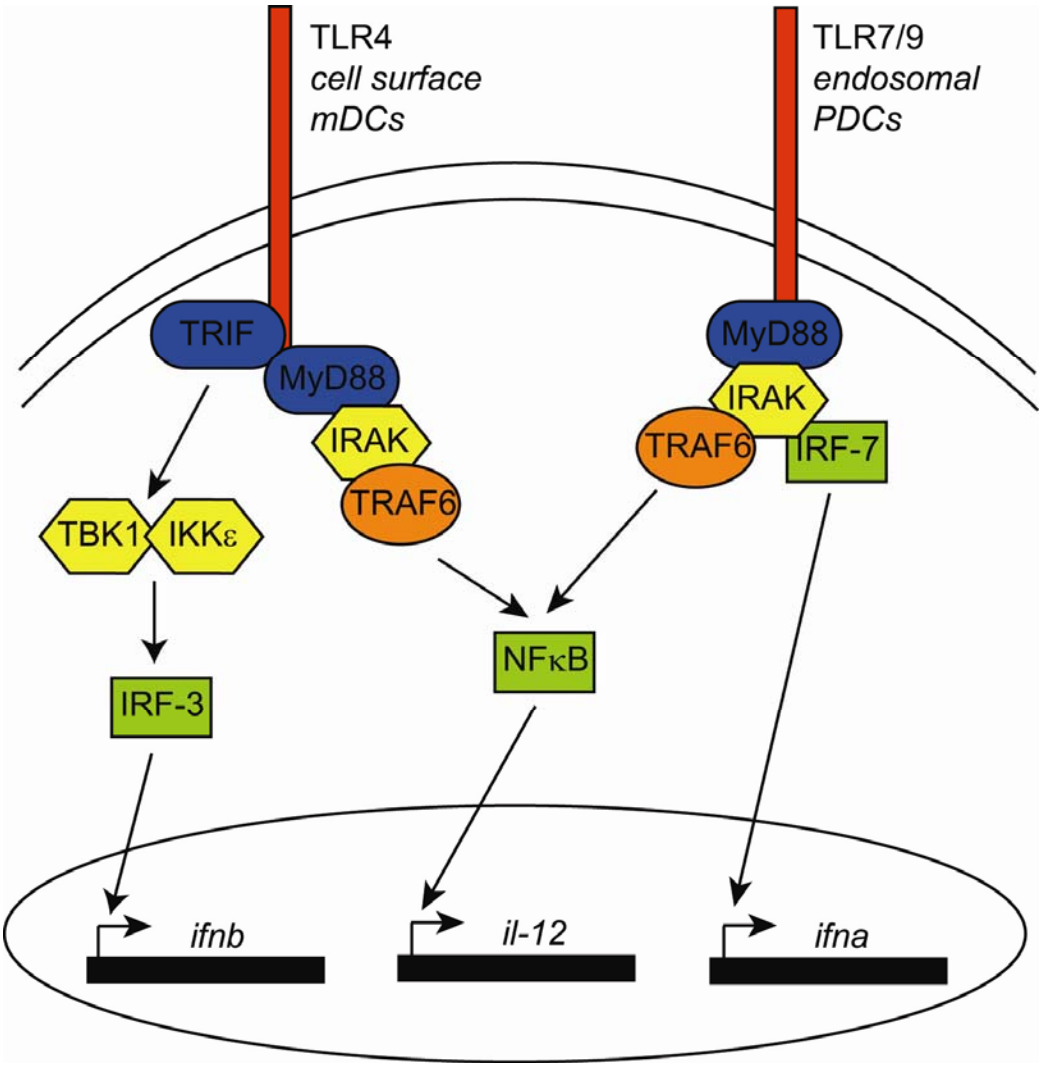

Figure 1. Induction of IL-12 and type I IFN by TLR signaling. Signals transduced through TLR7 and TLR9 expressed in the endosomal compartments of PDCs and CD8α^+ DCs induce expression of both IL-12 and IFN-α through MyD88-dependent pathways. In contrast, mDCs can secrete both IL-12 and IFN-β in response to TLR4 stimulation via MyD88-dependent and MyD88-independent pathways.

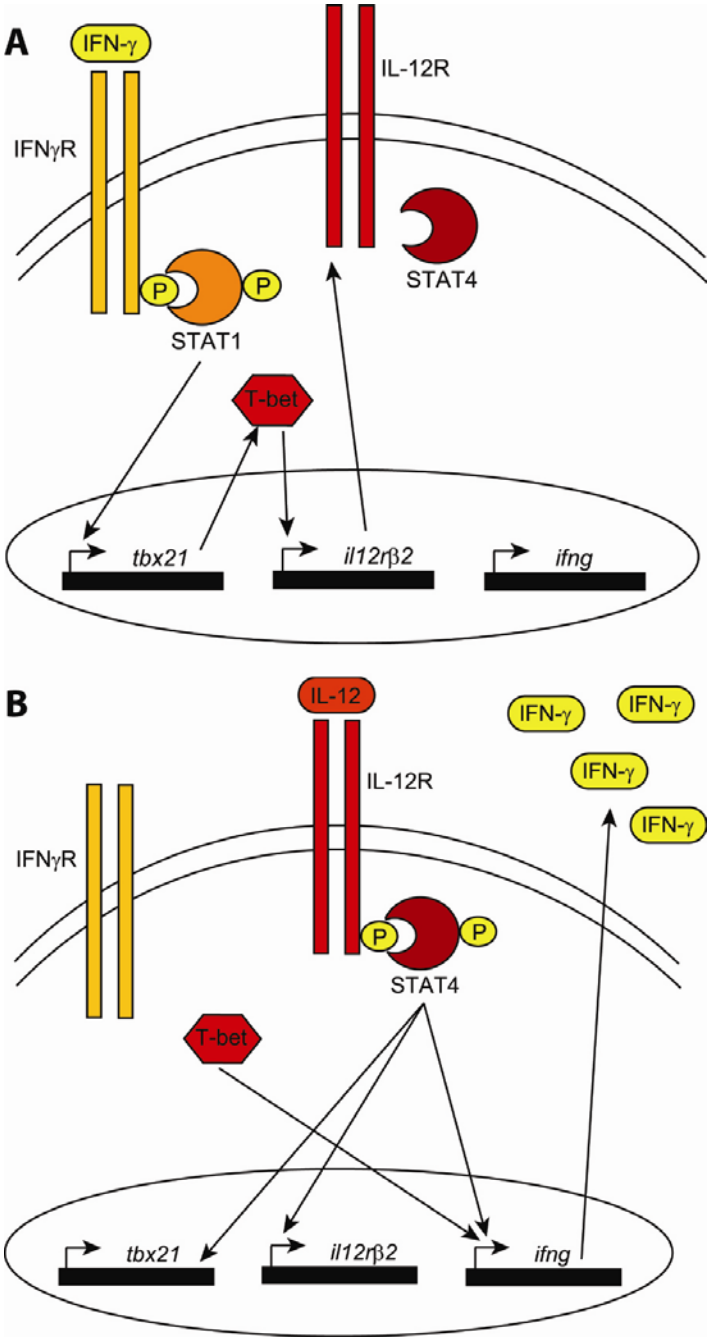

Figure 2. Overview of Th1 differentiation. (A) Naïve $CD4^+$ T cells must become IL-12 responsive through upregulation of the IL-12Rβ2 via IFN-γ-induced T-bet. (B) IL-12-activated STAT4 induces expression of both IL-12Rβ2 and T-bet. STAT4 and T-bet both promote IFN-γ secretion from fully polarized Th1 cells.

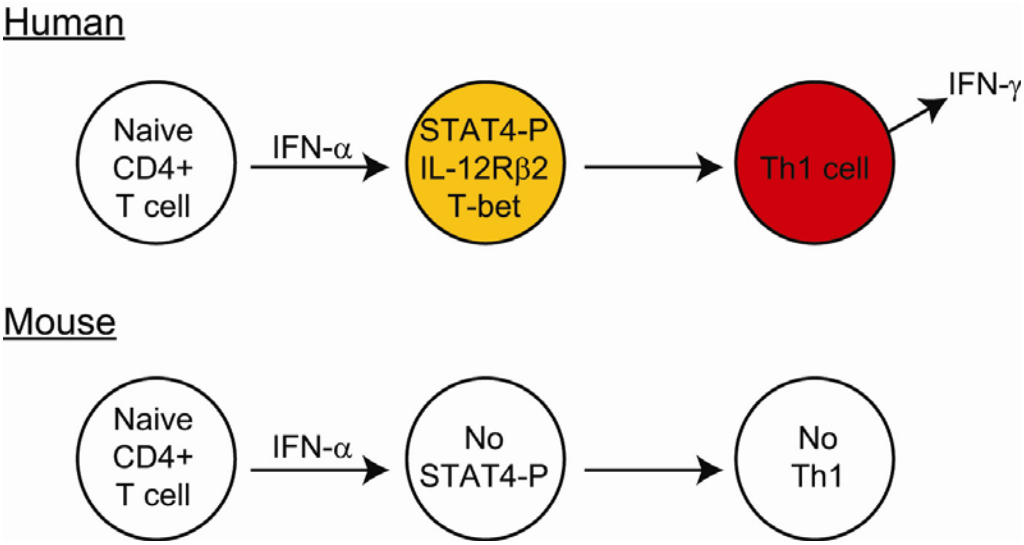

Figure 3. Proposed species-specific role for IFN-α in Th1 development. IFN-α has been shown to promote STAT4 phosphorylation and IL-12Rβ2 and T-bet expression in human $CD4^+$ T cells, leading to IFN-γ secretion. In contrast, IFN-α fails to induce STAT4 activation or IFN-γ secretion in murine T helper cells.

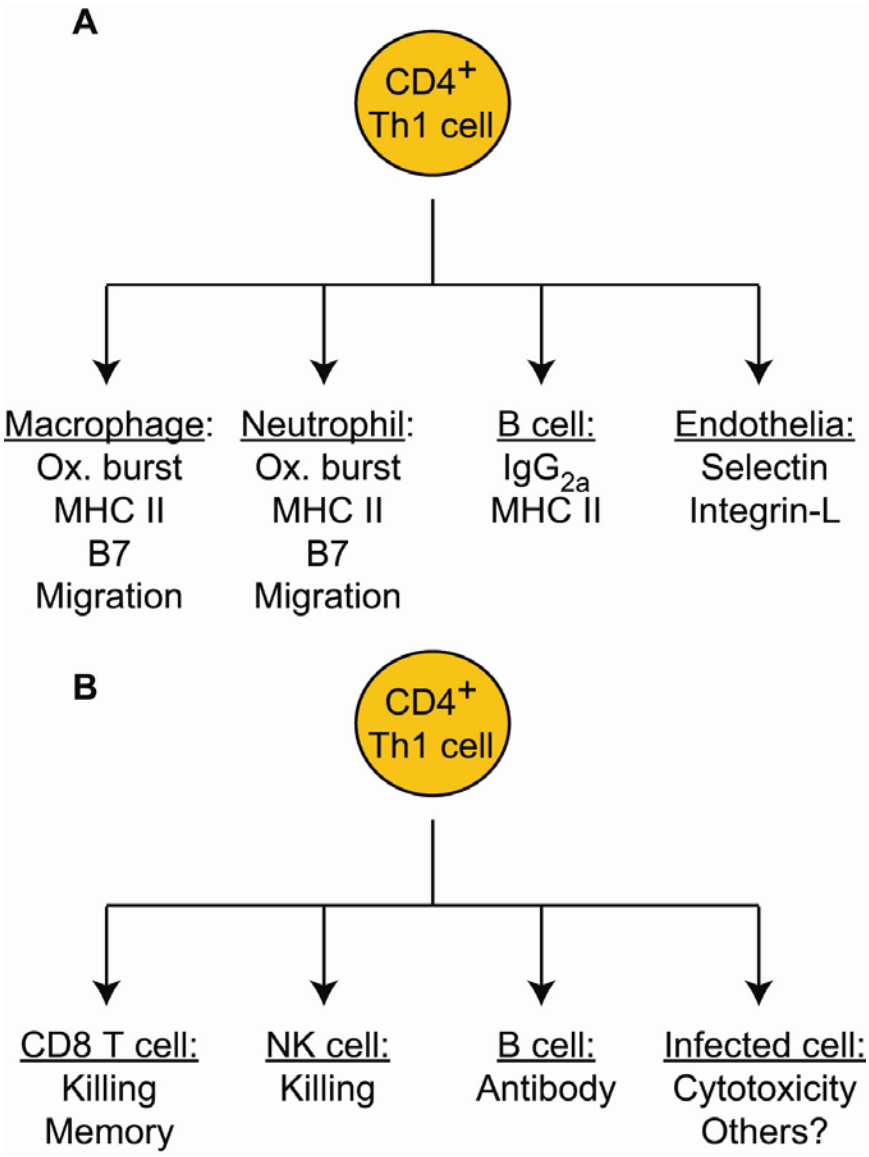

Figure 4. Th1 effector cells are important in resistance to intracellular infections. (A) Th1 cells participate directly in bacterial clearance by secretion of IFN-γ and TNF-α, which activate phagocytic macrophages and neutrophils. Additionally, IFN-γ and TNF-α promote B cell antibody production, antigen presentation, and leukocyte migration. (B) Th1 cells can participate in multiple facets of viral clearance. IFN-γ activates NK cell and CD^+ T cell cytolytic functions and B cell antibody production. Th1 cells can also be directly cytotoxic and may have other antiviral functions.

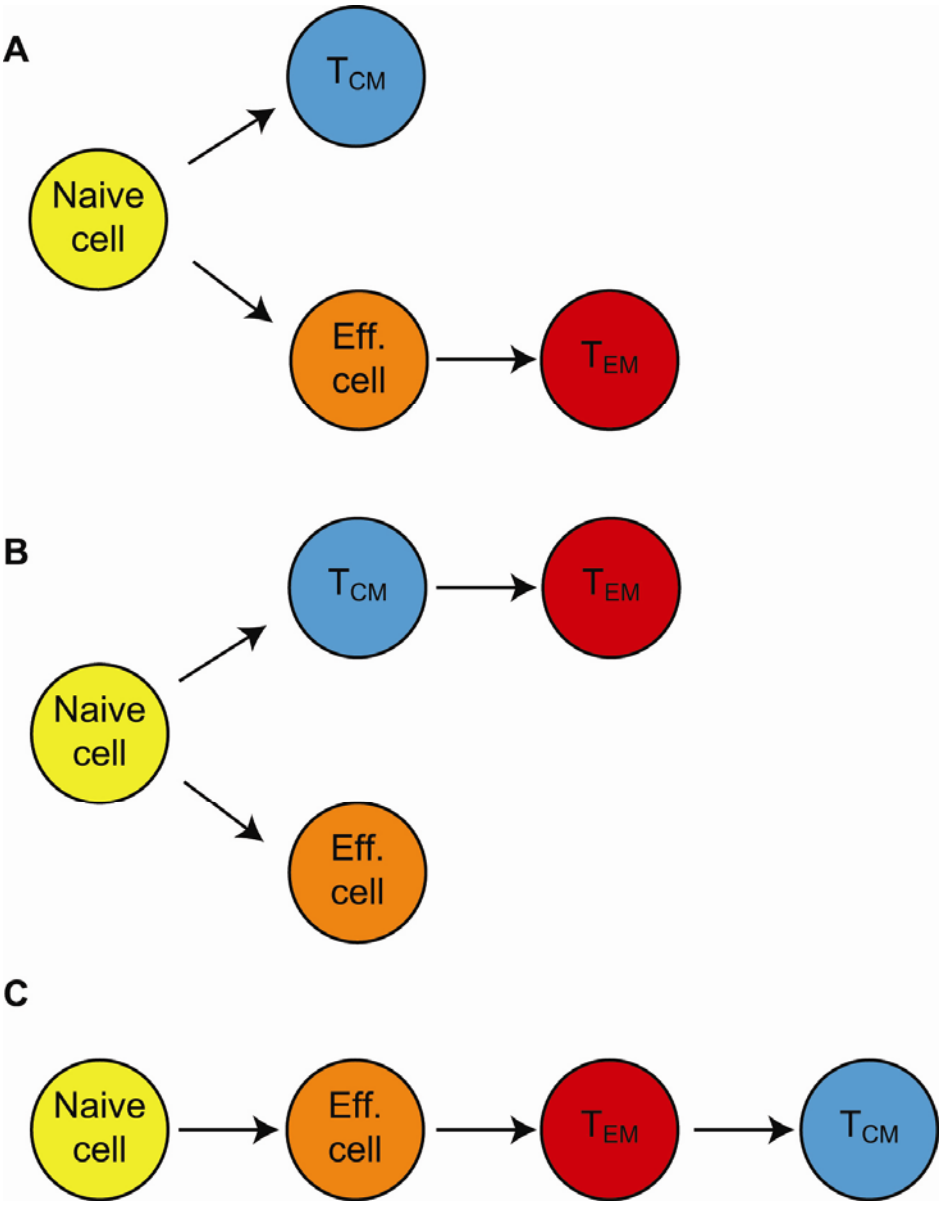

Figure 5. Models of memory cell generation. (A) Central and effector memory cells may arise from distinct lineages generated during an initial infection. (B) It is also possible that effector memory cells may differentiate from central memory cells upon secondary infection. (C) Finally, a linear differentiation model has been proposed for the development of effector and memory subsets.

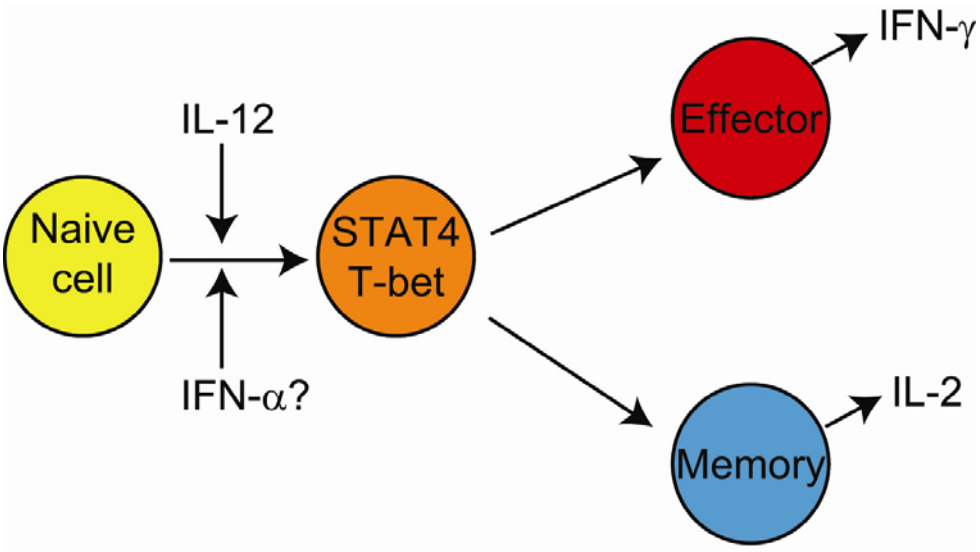

Figure 6. Possible roles for type I IFN in CD4$^+$ T cell development. The effects of IL-12 on Th1 differentiation are relatively well-understood. Like IL-12, IFN-α has been proposed to play a role in the initial signaling events which lead to Th1 commitment. IFN-α has also been suggested to regulate Th1 effector cytokine secretion. Finally, IFN-α may be playing a role in the genesis of memory CD4$^+$ T cells.

CHAPTER II

MATERIALS AND METHODS

Human subjects

100-120 ml of peripheral blood was obtained from healthy adult volunteers by venipuncture. Informed consent was obtained from each donor according to guidelines established by the Internal Review Board (University of Texas Southwestern Medical Center).

Cell lines

THP-1 cells, a human monocytic lymphoma line, CV-1 cells, a green monkey fibroblast line, and HeLa cells, a human cervical carcinoma line, were purchased from American Type Culture Collection (Manassas, VA). 2fTGH cells, a human fibroblast line, and 2fTGH-derived IFNAR2-deficient U5A cells were a generous gift of G. Stark (Cleveland Clinic) (67, 249, 250). A7 replicon cells, a human hepatoma line carrying a replicating hepatitis C virus genome, have been previously described (251).

Cytokines, antibodies, and reagents

Recombinant human IL-4 (rhIL-4), rhIL-12, rhIFN-γ, rhTNF-α, rhCCL19, rhCXCL10, and rhLT$\alpha_1\beta_2$, and the anti-human IL-4 (hIL-4), anti-hIFN-γ receptor (IFNγR1), anti-hLT, and allophycocyanin (APC)-conjugated anti-hCCR7 antibodies were purchased from R&D Systems (Minneapolis, MN). rhIL-18 was purchased from BioSource International (Carlsbad, CA). rhIFN-αA and rhIFN-ω, the anti-hIFN-α/β receptor (IFNAR2) and anti-hIFN-ω antibodies, and polyclonal antisera against hIFN-α and hIFN-β were purchased from PBL Laboratories (Piscataway, NJ). rhIFN-β1a was a generous gift of M. Racke (University of Ohio). rhIL-2 was a generous gift of M. Bennett (University of Texas Southwestern Medical Center). The anti-hCD3, anti-hCD28, anti-hTNF-α, APC-conjugated anti-hTNF-α, and AlexaFluor 700-conjugated anti-hIL-2 antibodies were purchased from BioLegend (San Diego, CA). The phycoerythrin (PE)-conjugated anti-hCD4, Pacific Blue-conjugated anti-hCD45RA, and fluorescein isothiocyanate (FITC)-conjugated anti-hIFN-γ antibodies were purchased from Caltag Laboratories (Burlingame, CA). The FITC-conjugated anti-hCD45RA, PE-conjugated anti-hIL-12 receptor (IL-12Rβ2), PE-conjugated anti-hCXCR3, and PE-Cy7-conjugated anti-hIFN-γ antibodies were purchased from BD Pharmingen (San Diego, CA). The PE-Cy5-conjugated anti-hIL-7 receptor (IL-7Rα) and PE-conjugated anti-hIL-15 receptor (IL-15Rα) antibodies were purchased from eBioscience (San Diego, CA). Rabbit polyclonal antisera against hT-bet, hSTAT1, and hSTAT4 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit polyclonal antiserum against

phosphorylated STAT4 (p-STAT4) was purchased from Zymed Laboratories (Carlsbad, CA). Rabbit polyclonal antiserum against p-STAT1 was purchased from Upstate Biotechnology (Billerica, MA). The anti-NS5A antibody was a generous gift of J. Ye (University of Texas Southwestern Medical Center). The anti-hISG56 antibody was a generous gift of G. Sen (Cleveland Clinic) (252). The anti-hGAPDH antibody was purchased from Abcam (Cambridge, MA). Rabbit polyclonal antiserum against hLamin A/C was purchased from Cell Signaling Technology, Inc. (Danvers, MA). HRP-conjugated goat anti-rabbit Ig antiserum and biotin-conjugated goat anti-rabbit Fab fragment were purchased from Jackson ImmunoResearch (West Grove, PA). The Qdot 655-conjugated streptavidin was purchased from Invitrogen (Carlsbad, CA).

Human $CD4^+$ T cell cultures

Peripheral blood mononuclear cells (PBMCs) and naïve human $CD4^+$ T cells were isolated from whole blood of healthy adult volunteers. Heparinized whole blood was subjected to density centrifugation using Lymphocyte Separation Media (Mediatech, Inc., Herndon, VA). PBMCs were isolated from buffy coats; cells were activated with 1 μg/ml phytohemagglutinin (PHA) (Calbiochem, San Diego, CA), and cytokines were added as described below.

To obtain naïve human $CD4^+$ T cells, PBMCs were stained with FITC-conjugated anti-hCD45RA and PE- or PE-Cy5-conjugated anti-hCD4 antibodies, and $CD45RA^+$ $CD4^+$ cells were sorted on a MoFlo cell sorter (Dako Cytomation, Fort Collins, CO) at > 90% purity. Alternately, untouched $CD45RA^+$ $CD4^+$ cells were

isolated using the Human Naive CD4 T Cell Enrichment Set (BD Pharmingen) according to the manufacturer's instructions.

Cells were activated at 2-2.5 x 10^6 cells/ml for three days in 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) on culture plates coated with 1-5 μg/ml anti-hCD3 + 1-5 μg/ml anti-hCD28 in the presence of 50 units (U)/ml rhIL-2. Cytokines and neutralizing antibodies were added at the following concentrations: anti-hIFN-γ (4S.B3), 5 μg/ml; anti-hIL-4, 2 μg/ml; anti-hIL-12 (20C2), 5 μg/ml; anti-hIFNAR2; 2 μg/ml; rhIL-12, 10 ng/ml; rhIL-4, 10 ng/ml; rhIFN-αA, 1000 U/ml. Cytokine activation conditions were as follows unless otherwise indicated: Neutralized – anti-hIFN-γ, anti-hIL-4, anti-hIL-12, and anti-hIFNAR2; IL-12 – anti-hIFN-γ, anti-hIL-4, rhIL-12, and anti-hIFNAR2; IL-4 – anti-hIFN-γ, rhIL-4, anti-hIL-12, and anti-hIFNAR2; IFN-α – anti-hIFN-γ, anti-hIL-4, anti-hIL-12, and rhIFN-αA; IL-12+IFN-α – anti-hIFN-γ, anti-hIL-4, rhIL-12, and rhIFN-αA. On day three, cells were split into fresh media containing IL-2 and were rested to day 7. For some experiments, cells were restimulated on day 7 for a further 7 days as described above.

Live cell sorting of IL-2- and IFN-γ-producing cells

Naïve human $CD4^+$ T cells were differentiated for 7 days in the presence of rhIL-2, rhIL-12, rhIFN-αA, anti-hIL-4, and anti-hIFN-γ as described above. On day 7, cells were washed and rested overnight in cIMDM without IL-2. Cells were stimulated for 2 hours at 37°C, 5% CO_2 with 0.8 μg/ml phorbol 12-myristate 13-acetate (PMA) (A.G. Scientific, Inc., San Diego, CA) + 1 μM ionomycin (Sigma-Aldrich, St. Louis, MO), and labeling was performed using MACS Cytokine Secretion Assay Kits for IL-2 and IFN-γ (Miltenyi Biotech, Auburn, CA) according to the manufacturer's instructions. Cells were isolated on a FACSAria cell sorter (Becton Dickinson) at > 90% purity.

Retroviral transduction of human T cells

The GFPRV (253) and T-bet-GFPRV (254) retroviral expression constructs have been described. Retroviral supernatants for transduction were generated by calcium chloride transfection of the Phoenix amphotropic (PhA) packaging cell line (Orbigen, San Diego, CA). PhA cells were maintained 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). Immediately prior to transfection, cells were given fresh media supplemented with 25 μM chloroquine. The transfection mixture was prepared as follows: 25-30 μg plasmid DNA was suspended in a solution of 0.244 M

calcium chloride ($CaCl_2$). The DNA/$CaCl_2$ mixture was combined 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) and mixed vigorously for 10-15 seconds. This mixture was added dropwise to PhA cells, and the cells were incubated at 37°C, 5% CO_2 for 8 hours. The transfection mixture was then exchanged for fresh cDMEM, and the cells were rested for 16 hours. Media was then exchanged for fresh cDMEM, cells were incubated at 32°C, and retroviral supernatants were harvested at 24 hour intervals and stored at -80°C. Efficiency of transfection was monitored by flow cytometry for GFP expression.

For retroviral transduction, naïve human $CD4^+$ T cells isolated as described above were activated at 2.5 x 10^6 cells/ml on culture plates coated with 2 μg/ml anti-hCD3 + 2 μg/ml anti-hCD28 in cIMDM in the presence of anti-hIL-12, anti-hIL-4, anti-hIFN-γ, and anti-hIFNAR2 and 600 U/ml rhIL-2. Cells were incubated at 37°C, 5% CO_2 for 16 hours and then transduced with retroviral supernatants in the presence of 5 μg/ml polybrene and neutralizing antibodies and rhIL-2 as described above for 90 minutes at 1000 x *g*. Transduction was repeated for three consecutive days. On the second and third days of transduction, cytokines and neutralizing antibodies were added at the concentrations listed above as described in the figure legends. On day 4, cells were split 1:10 into fresh cIMDM containing 50 U/ml rhIL-2 and rested to day 7 or day 14.

Vesicular stomatitis virus infections

Cells were washed and resuspended in cIMDM at 6 x 10^6 cells/ml (THP-1 cells) or 3 x 10^6 cells/ml (2fTGH and U5A cells). Cells were infected with recombinant

vesicular stomatitis virus carrying the GFP transgene (VSV-GFP) (generous gift of M. Whitt, University of Tennessee) (255) at 0.05 – 0.8 plaque-forming units (pfu)/cell for 2 minutes at room temperature. Cells were then transferred into wells of a 96-well plate containing cytokines or T cell conditioned media and incubated for 16 hours at 37°C, 5% CO_2. Following infection, cells were washed and fixed, and analysis for GFP expression was performed on a FACScan or FACSCalibur cytometer (Becton Dickinson, Franklin Lakes, NJ), and the data was processed using FlowJo software (TreeStar, Ashland, OR). For experiments in which αhIFNAR2 or αhIFNγR1 neutralizing antibodies were used, cells were incubated with 5 μg/ml anti-hIFNAR2 or 10 μg/ml anti-hIFNγR1 for 2 minutes at room temperature immediately prior to infection.

For quantitation of VSV-GFP infection by plaque assay, THP-1 cells were cultured for 24 hours at 37°C, 5% CO_2 in cIMDM in the absence or presence of 100 U/ml rhIFN-αA or T cell conditioned media (10% v/v). Cells were washed and resuspended at 6 x 10^6 cells/ml in cIMDM. Cells were infected with VSV-GFP at 0.7 pfu/cell for 15 minutes at room temperature. Cells were then washed in cIMDM, transferred to wells of a 96-well plate, and incubated for 24 hours at 37°C, 5% CO_2. Confluent CV-1 cells were infected with supernatants from infected THP-1 cells at dilutions from 10^1 - 10^7 for 45 minutes at 37°C, 5% CO_2. CV-1 cells were then washed and overlaid with cDMEM containing 0.6% agarose and cultured for 24-72 hours at 37°C, 5% CO_2. Cells were stained with crystal violet for quantitation of plaque formation.

A7 replicon cells and Western blotting

The generation and maintenance of the A7 replicon cell line has been previously described (251). A7 replicon cells were maintained in Dubelcco's modified Eagle medium (Mediatech, Inc.) supplemented with 10% FBS (cDMEM) and 200 μg/ml G418 (Gemini Bio-products, Sacramento, CA). 24 hours prior to treatment, cells were washed with PBS and given cDMEM without antibiotic. The following day, media was removed, and cells were cultured in cDMEM containing cytokines or 5% (v/v) T cell conditioned media as indicated in the figures. Concentrations of cytokines were as follows: rhIFN-αA, 100 U/ml; rhIFN-γ, 5 ng/ml; rhTNF-α, 2.5 ng/ml. 48 hours later, cells were harvested and lysed in radioimmune precipitation assay (RIPA) buffer (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), and proteins were separated by SDS-PAGE. Western blotting was performed using antibodies against HCV NS5A, hISG56, or hGAPDH. For experiments in which neutralizing antibodies were used, cells were incubated with 5 μg/ml anti-hIFNAR2, 10 μg/ml anti-hIFNγR1, or 5 μg/ml anti-hTNF-α for 1 hour immediately prior to treatment with cytokine or T cell conditioned media and supplemented with the same antibodies 24 hours after the initiation of treatment.

Respiratory syncytial virus infections

HeLa cells were washed and resuspended in cDMEM at 10 x 10^6 cells/ml. Cells were infected with recombinant respiratory syncytial virus carrying the GFP transgene (RSV-GFP) (generous gift of M. Peeples, Columbus Children's Research Institute) (256) at 2 – 2.5 pfu/cell for 2 minutes at room temperature. Cells were then transferred into wells of a 96-well plate containing cytokines or T cell conditioned media and incubated for 72 hours at 37°C, 5% CO_2. Following infection, cells were washed and fixed, and analysis for GFP expression was performed on a FACScan or FACSCalibur cytometer, and the data was processed using FlowJo software.

Listeria innocua *infections*

THP-1 cells were washed and resuspended in antibiotic-free cIMDM at 2 x 10^6 cells/ml. Cells were activated with 0.8 μg/ml PMA for 48 hours at 37°C, 5% CO_2 (257). Cells were washed, and cytokines or T cell conditioned media were then added for a further 48 hours at the concentrations indicated in the figures. Concentrations of cytokines were as follows: rhIFN-αA, 100 U/ml; rhIFN-γ, 10 ng/ml; rhTNF-α, 10 ng/ml. Cells were washed and infected with 3 colony-forming units (cfu)/cell *Listeria innocua* (generous gift of L. Hooper, University of Texas Southwestern Medical Center) for 45 minutes at 37°C, 5% CO_2. Gentamicin (Sigma-Aldrich, location) was added at 50-100 μg/ml, and cytokines or T cell conditioned media were added at the concentrations indicated in the figures. Cells were incubated for 16 hours at 37°C, 5% CO_2. Infected

cells were hypertonically lysed to release intracellular bacteria, and infection was assessed by plating on BHI agar plates and counting colonies following a 16 hour incubation at 37^{o}C.

Assessment of STAT1 and STAT4 phosphorylation by Western blotting

PBMCs isolated as described above were activated for two consecutive weeks in the presence of 1 μg/ml PHA, 50 U/ml rhIL-2, and 10 ng/ml rhIL-12. On day 14, cells were washed and rested for 30 minutes at 37^{o}C, 5% CO_2 at 1 x 10^7 cells/ml in cIMDM without IL-2. Cells were then stimulated with 10 ng/ml rhIL-12 or 1000 U/ml rhIFN-αA for 30 minutes – 24 hours at 37^{o}C, 5% CO_2. Lysis was performed at 5 x 10^7 cells/ml in RIPA buffer for 1 hour at 4^{o}C. Samples were subjected to immunoprecipitation using 3 μg/sample of polyclonal rabbit antisera against human STAT1 or STAT4 bound to Protein G sepharose beads (Amersham Biosciences, Piscataway, NJ). Immunoprecipitated proteins were resolved by SDS-PAGE on a 7% polyacrylamide gel and transferred to polyvinylidene difluoride membranes (Bio-Rad, Hercules, CA). Western blotting was performed using rabbit polyclonal antisera against phosphorylated STAT1 or phosphorylated STAT4 and HRP-conjugated polyclonal goat anti-rabbit Ig secondary antiserum. Detection was performed using ECL detection reagents (Amersham Biosciences) according to the manufacturer's instructions. Membranes were stripped and reprobed using polyclonal rabbit antisera against total human STAT1 or STAT4 as a loading control.

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

RNA was extracted from cultured cells using an RNeasy Mini Kit (QIAgen, Valencia, CA) according to the manufacturer's instructions. Following isolation, RNase-free DNase I digestion was performed for 15 minutes at 37°C. RNA was reverse transcribed for 1 hour at 42°C. The resultant cDNA was subjected to qPCR analysis on an ABI7300 cycler (Applied Biosystems, Foster City, CA) using primers directed against human interferon transcripts. Primers directed against human GAPDH were used as a reference. Relative transcript expression was calculated by the $2^{-\Delta\Delta Ct}$ method (258). All primers were synthesized by Integrated DNA Technologies (Coralville, IA). Primer sequences are found in Table 1. Primer sequences for hIFN-α1/13 and hIFN-β were taken from Remoli et. al. (259). Primer sequences for hIFN-γ, hIL-12Rβ2, hT-bet, and hGAPDH have been published previously (254).

Assessment of gene expression by Illumina BeadArray

RNA was extracted from cultured cells as described above and submitted to the University of Texas Southwestern Medical Center Microarray Core Facility for cDNA and cRNA synthesis, labeling, and hybridization to HumanWG-6 BeadChips (Illumina, Inc., San Diego, CA). These chips contain approximately 48,000 probes spanning the entire human genome. Initial data extraction was performed in BeadArray software, v. 3.0 (Illumina, Inc.), using cubic spline normalization and a custom error model provided

by the manufacturer. In order to determine relative gene expression, z-values were calculated using Excel software (Microsoft Corporation, Redmond, WA), as follows: $z = (x - \mu)/\sigma$, where x is the fluorescence intensity of a given sample for a given gene, μ is the mean fluorescence intensity for that gene across all samples, and σ is the standard deviation of the fluorescence intensity for that gene across all samples. Values of z for which the detection above background was significant at a level of $p = 0.01$ or better and the difference from the control sample (neutral unstimulated) was significant at a level of $p = 0.05$ or better were subjected to two-way hierarchical cluster analysis using Cluster software (M. Eisen, Stanford University, Stanford, CA), and cluster analyses were visualized using TreeView software (M. Eisen, Stanford University, Stanford, CA) (260).

Quantitation of cytokine secretion

Naïve human $CD4^+$ T cells were differentiated for one or two consecutive weeks as described above. On day 7 or day 14, cells were washed and replated in fresh cIMDM without IL-2 at 2 x 10^6 cells/ml in the absence or presence of 5 μg/ml plate-bound αhCD3. Cell-free supernatants were harvested after 24 hours of activation. Concentrations of human cytokines and chemokines were determined by enzyme-linked immunosorbet assay (ELISA) using ELISA MAX kits (BioLegend) or by cytometric bead array (CBA) analysis using CBA kits (BD Pharmingen) according to the manufacturer's instructions. Alternately, this T cell-conditioned media (TCM) was assayed for antiviral activity by *in vitro* infection as described above.

Flow cytometric analysis

Naïve human $CD4^+$ T cells were differentiated for 7 days as described above. On day 7, cells were washed and rested overnight at 2 x 10^6 cells/ml 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 (Epicentre, Madison, WI). Intracellular staining was performed as follows. Cells were washed in phosphate buffered saline (PBS) (Hyclone) and fixed for 20 minutes at room temperature in PBS containing 5% formalin (Mallinckrodt Baker, Inc., Phillipsburg, NJ). Cells were then washed and permeabilized for 10 minutes at room temperature 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 as noted in the figures. For analysis of apoptosis, unstimulated cells were washed and stained with 7-AAD and FITC-conjugated Annexin V (BD Pharmingen) in cIMDM. Cells were analyzed on a FACSCalibur or LSR II cytometer (Becton Dickinson), and the data were processed using FlowJo software.

For assessment of proliferation, freshly isolated naïve or cytokine-producing human $CD4^+$ T cells were washed and resuspended in PBS at 1 x 10^7 cells/ml. Cells were labeled with 1.25 μM carboxyfluorescein diacetate succinimidyl ester (CFSE) (Sigma-Aldrich) for 10 minutes at room temperature, and labeling was stopped by addition of cIMDM containing 20% FBS. Cells were washed extensively in cIMDM and differentiated for 3-7 days as described above. Dilution of CFSE was analyzed on an LSR II cytometer, and the data were processed using FlowJo software.

Cell migration assays

Naïve human $CD4^+$ T cells were differentiated for 7 days in the presence of rhIL-2, rhIL-12, rhIFN-αA, anti-hIL-4, and anti-hIFN-γ as described above. On day 7, cells were washed and resuspended in cIMDM without IL-2 at 1-5 x 10^6 cells/ml. Cells were added to the upper chambers of 24-well Transwell plates containing a polycarbonate membrane with a 5 μm pore (Corning, Lowell, MA), in which the lower chambers contained 10 ng/ml rhCCL19 or 10 ng/ml rhCXCL10 in cIMDM. cIMDM alone was used as a control. Plates were incubated for 2 hours at 37°C, 5% CO_2 and then on ice for 10 minutes to release adherent cells from the membrane. Cells which had migrated to the lower chamber 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, and intracellular staining was performed as described above.

Statistical analysis

Statistical analysis was performed by one-way and two-way ANOVA using GraphPad Prism software, version 5.00 for Windows (GraphPad Software, San Diego, CA). One-way ANOVA was used for univariate data sets, and two-way ANOVA was used with data sets containing two or more groupings. Values of $p < 0.05$ were taken as significant.

Table I. Primers used in qPCR analysis of gene expression.

Gene	Dir.	Sequence
hIFN-α1/13	F	5'-TGGCTGTGAAGAAATACTTCCG-3'
	R	5'-TGTTTTCATGTTGGACCAGATG-3'
hIFN-β	F	5'-GTGTCTCCTCCAAATTGCTCTC-3'
	R	5'-CCACAGGAGCTTCTGACACTG-3'
hIFN-ε	F	5'-CTTGTCAATTCAGCAGTGTCTACC-3'
	R	5'-CAACCATCCAGAGAAATATTTGC-3'
hIFN-κ	F	5'-AATCTGAGACATCTGAGTAGTATGAGC-3'
	R	5'-TTGCTGATCAAGTCCTATTTGG-3'
hIFN-ω	F	5'-GGAACACCTTGGTGCTTCTGC-3'
	R	5'-GCTGCAGCATCTCATGGAGG-3'
hIFN-γ	F	5'-TGATTACAAGGCTTTATCTCAGGG-3'
	R	5'-GGCAGTAACTGGATAGTATCACTTCAC-3'
hIL-12Rβ2	F	5'-ACCTTCCCACCCATGATGGC-3'
	R	5'-GAAAACAGAAAGGGAGATGTGCTG-3'
hT-bet	F	5'-CGTCCAACAATGTGACCCAG-3'
	R	5'-GCAGTCACGGCAATGAACTG-3'
hGAPDH	F	5'-ACATCGCTCAGACACCATGG-3'
	R	5'-CATGTAGTTGAGGTCAATGAAGGG-3'

CHAPTER III

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

The work presented in this chapter represents an equal and collaborative effort between Ann M. Davis and Hilario J. Ramos. It has been published in the *Journal of Immunology*, volume 179, pages 3792-3803 (254). This work is reproduced with the permission of the *Journal of Immunology*. Copyright 2008 The American Association of Immunologists, Inc. Experiments were performed by Ann M. Davis unless otherwise indicated in the text and figure legends.

Introduction

$CD4^+$, or helper, T cells are crucial mediators of adaptive immunity to infections. These cells secrete cytokines which coordinate the responses of other cells of the immune system against a variety of pathogens. $CD4^+$ T cells have conventionally been divided into subsets based upon their pattern of cytokine secretion and their involvement in immunity against distinct classes of pathogens. In particular, Th1 cells are central to immune responses against intracellular bacterial and viral pathogens and are classified by secretion of IFN-γ, TNF-α, and TNF-β/lymphotoxin (LT) (152, 153, 261).

The differentiation of human $CD4^+$ T cells into functional subsets occurs in response to innate cytokines produced at the onset of infection. IL-12 is the primary

cytokine which regulates commitment to the Th1 fate in both murine and human $CD4^+$ T cells (18, 101-103). Stimulation of naïve $CD4^+$ T cells with IL-12 results in phosphorylation of a key signaling mediator, signal transducer and activator of transcription 4 (STAT4) (112, 115). STAT4 activation is required for Th1 commitment and secretion of IFN-γ by $CD4^+$ T cells; mice and humans which lack the *stat4* gene are deficient in the generation of Th1 responses and fail to control a variety of intracellular infections (117-120, 122, 262, 263). IL-12 also upregulates expression of the β2 chain of its own receptor (IL-12Rβ2), allowing further commitment to the Th1 fate (15, 114, 125, 126). Furthermore, IL-12 induces expression of T-box expressed in T cells (T-bet/Tbx21), a transcription factor which acts as the "master regulator" of Th1 development (107, 110, 126, 264). Thus, IL-12 controls multiple aspects of Th1 differentiation.

In addition to IL-12, type I interferons (IFN-α/β) have also been suggested to participate in Th1 development. Early reports suggested a species difference in the response of $CD4^+$ T cells to IFN-α. In human T helper cells, IFN-α was reported to induce phosphorylation of STAT4 and elevated expression of T-bet and IL-12Rβ2 (15, 113, 114, 125, 126, 130, 131). Furthermore, activation of human PBMCs or $CD4^+$ T cells with IFN-α resulted in secretion of IFN-γ (14, 15, 114). In contrast, in murine $CD4^+$ T cells, IFN-α failed to promote appreciable STAT4 phosphorylation or IFN-γ secretion (114, 130, 132, 134). Initially, it was postulated that this apparent species difference was the result of a minisatellite insertion in the C-terminus of murine STAT2 which prevented STAT2-dependent STAT4 activation by IFN-α/β signaling (130, 134). However, further

work has shown that species differences in the N-terminal preassociation of STAT4 with the type I interferon receptor (IFNAR) account for the observed differences in signaling (133, 135, 136).

Recent reports have called these initial observations into question. First, IFN-γ secretion and STAT4 activation in response to IFN-α have been observed in mice *in vivo* during lymphocytic choriomeningitis virus (LCMV) and Gram positive bacterial infections, although in the case of LCMV, the responding cells were likely $CD8^+$ T cells (137-141). In *vitro* studies by Berenson et. al. found weak STAT4 activation in murine $CD4^+$ T cells in response to IFN-α, but this STAT4 phosphorylation did not result in IFN-γ secretion from murine T helper cells, even at IFN-α concentrations up to 100,000 U/ml (142, 143). Furthermore, the ability of IFN-α to promote Th1 differentiation in human T helper cells has been called into question by the observation that IFN-α induced attenuated STAT4 phosphorylation and IFN-γ production in purified human $CD4^+$ T cells compared to that induced by IL-12 (125, 144, 145). Therefore, the role of IFN-α in both murine and human Th1 differentiation remains unclear.

The present work seeks to precisely delineate the role of IFN-α in Th1 development in human $CD4^+$ T cells. Using *in vitro* activation of purified human naïve $CD4^+$ T cells and carefully regulated exposure to combinations of innate cytokines, I have shown that IFN-α alone is insufficient to engender commitment to the Th1 fate. IFN-α did induce STAT4 phosphorylation in developing human T helper cells; however, this activation was attenuated compared with that observed for IL-12 signaling. This shorter duration of STAT4 activation resulted in a transient rather than stable induction of

T-bet and IL-12Rβ2 expression. Because of these attenuated signaling events, IFN-α alone failed to promote IFN-γ secretion from human $CD4^+$ T cells.

Results

Type I IFN is insufficient to promote Th1 or Th2 development

Recent reports have demonstrated conflicting results regarding the role of IFN-α in human Th1 development. In order to delineate the individual roles of innate cytokines in human Th1 differentiation, purified naïve ($CD45RA^+$) human $CD4^+$ T cells were activated with plate-bound anti-CD3 + anti-CD28 in the presence of IL-2 and various cytokine polarization conditions. For these experiments, cells were given either innate cytokines or neutralizing anti-cytokine antibodies in order to carefully control cytokine exposure during the course of differentiation. In this way, it was possible to monitor the contributions of individual cytokines to T helper cell development. After 1-2 weeks in culture, cells were washed extensively in clean media without IL-2 and restimulated with either plate-bound anti-CD3 or PMA + ionomycin.

Naïve human $CD4^+$ T cells activated for 7 days in the presence of IL-12, IFN-α, or a combination of IL-12 and IFN-α were first analyzed for IFN-γ transcript expression by qPCR. Following anti-CD3 restimulation, cells activated in the presence of IL-12 induced very high levels of IFN-γ transcript in comparison to cells activated in the presence of neutralizing antibodies alone (Fig. 7A). In contrast to previous reports (14, 15, 114, 128, 129), cells activated in the presence of IFN-α alone did not show induction of IFN-γ mRNA in response to anti-CD3 and in fact demonstrated a modest decrease in comparison to neutralized controls (Fig. 7A). However, cells differentiated in the

presence of both innate cytokines were able to induce IFN-γ mRNA expression, indicating that IFN-α does not inhibit the ability of IL-12 to promote Th1 commitment. While a slight decrease in IFN-γ expression in cells activated in the presence of both cytokines was occasionally observed, this effect was likely donor specific.

It has been reported that *in vitro* activation of murine $CD4^+$ T cells in the presence of IL-12 for multiple consecutive weeks results in stronger Th1 commitment, as measured by IFN-γ secretion and resistance to Th2 polarization (265). Thus, it was possible that repeated exposure of $CD4^+$ T cells to IFN-α was required to achieve full Th1 differentiation. In order to examine this hypothesis, naïve cells were activated for one week in the absence or presence of IL-12 or IFN-α and then reactivated for a second week in the absence or presence of IFN-α. Even after two consecutive weeks of polarization with IFN-α, human $CD4^+$ T cells failed to demonstrate elevated IFN-γ mRNA expression in response to restimulation (Fig. 7B). In contrast, cells activated in the presence of IL-12 did express high levels of IFN-γ, and IFN-α neither enhanced nor inhibited this effect (Fig. 7B).

There are 11 subtypes of IFN-α as well as one IFN-β (58). The experiments detailed above were all performed using IFN-α subtype A (IFN-αA). It was possible that different subtypes of type I IFN could have different effects on human Th1 development. Therefore, human naïve $CD4^+$ T cells were activated in the presence of IL-12, IFN-αA, IFN-αB2, IFN-αD, or IFN-β. Cells were restimulated in the presence of PMA + ionomycin on day 7, and IFN-γ mRNA expression was assessed by qPCR. None of the

type I IFN subtypes examined was able to promote IFN-γ expression above the level of neutralized control cells (Fig. 7C).

Secretion of Th1 and Th2 cytokine protein by differentiated human $CD4^+$ T cells was examined by cytometric bead array (CBA). Cells polarized for two consecutive weeks in the presence of neutralizing antibodies produced a substantial level of IFN-γ, IL-4, and IL-5 (Fig. 2). This baseline level of cytokine secretion differed widely among different human donors (data not shown). However, activation of human naïve $CD4^+$ T cells in the presence of IL-12 resulted in substantially elevated secretion of IFN-γ and not IL-4 or IL-5. Conversely, IL-4 stimulation promoted IL-4 and IL-5 production but not IFN-γ secretion (Fig. 8). In agreement with the observed patterns of IFN-γ mRNA expression, IFN-α alone did not promote secretion of IFN-γ and even appeared to modestly repress IFN-γ production (Fig. 8). Furthermore, IFN-α did not promote Th2 development, as assessed by IL-4 and IL-5 secretion. However, as observed previously, IFN-α did not prevent IL-12 from inducing elevated IFN-γ secretion.

Work performed by Hilario Ramos also supported the above observations (254). Flow cytometric analysis of differentiated human naïve $CD4^+$ T cells revealed that IL-12, but not IFN-α, enhanced the population of IFN-γ-producing cells, and this effect was consistent in the absence or presence of a neutralizing antibody against IL-4 (Fig. 9). Furthermore, while baseline IFN-γ production in cells activated in the presence of neutralizing antibodies was widely varied among different human donors, IL-12, but not IFN-α, consistently induced IFN-γ secretion from human $CD4^+$ T cells (Fig. 9). Finally, up to 100,000 U/ml of IFN-αA failed to promote Th1 development, and IFN-β also did

not induce IFN-γ secretion (Fig. 9). Taken together, these results indicate that IL-12 is responsible for Th1 development in human $CD4^+$ T cells. In contrast, IFN-α alone is insufficient to induce human Th1 differentiation, but it does not impede the ability of IL-12 to promote commitment to the Th1 fate.

Differential kinetics of STAT4 activation in response to IL-12 vs. IFN-α

Several groups have reported that both IL-12 and IFN-α promote STAT4 phosphorylation in human $CD4^+$ T cells (15, 113, 114, 130). However, recent work from other groups has demonstrated distinct kinetics of STAT4 activation in response to these two cytokines (144, 145). Because qualitative differences in STAT4 activation could provide an explanation for the inability of IFN-α to promote Th1 development, I examined the phosphorylation of STAT4 in response to IL-12 and IFN-α in collaboration with Hilario Ramos (Fig. 10). Human PBMCs were differentiated for two consecutive weeks in the presence of IL-12. These cells were then washed and activated for 30 minutes – 24 hours in the presence of either IL-12 or IFN-α, and STAT4 phosphorylation was assessed by immunoprecipitation and Western blotting.

Stimulation of human Th1 PBMCs with IL-12 resulted in rapid and robust STAT4 phosphorylation, and this activation was maintained to 24 hours post-stimulation (Fig. 10A and B). In agreement with previous reports, activation of Th1 PBMCs with IFN-α also resulted in rapid STAT4 phosphorylation, and the initial level activation was comparable to that seen with IL-12 (Fig. 10A and B). However, IFN-α-induced STAT4

phosphorylation peaked at 3 hours post-activation and returned to baseline levels by 6 hours, demonstrating that IFN-α and IL-12 differ in the kinetics of STAT4 activation (Fig. 10A and B). In contrast to STAT4, STAT1 was strongly activated by IFN-α but not IL-12, and this activation was maintained to 6 hours post-stimulation (Fig. 10A). Therefore, the results observed for STAT4 did not represent a general attenuation of STAT activation by IFN-α but rather were specific for this signaling intermediate.

Phosphorylation of STAT4 by IL-12 and IFN-α was further assessed by flow cytometric staining and analysis of nuclear localization by J. David Farrar and Thaddeus T. George. Freshly isolated human PBMCs were activated for 30 minutes – 24 hours in the presence of IL-12 or IFN-α, and intracellular staining was performed to determine the level of STAT4 phosphorylation (Fig. 11A and B). In $CD4^+$ cells gated $CD45RA^+$ (naïve) or $CD45RA^-$ (antigen-experienced), IFN-α induced strong but brief STAT4 phosphorylation (Fig. 11C-E). In contrast, IL-12 promoted prolonged STAT4 activation to 24 hours in cells gated $CD45RA^-$ (Fig. 11C-E). These results are in direct agreement with the data presented above for STAT4 phosphorylation by Western blotting.

Nuclear localization of phosphorylated STAT4 was assessed using an ImageStream flow cytometer, which combines flow cytometric analysis with intracellular microscopy (Fig. 12A). In this case, activation of human PBMCs with either IFN-α or IL-12 resulted in nuclear localization of phosphorylated STAT4 (Fig. 12B). Thus, while IFN-α induced attenuated STAT4 activation, there was no defect in the ability of phosphorylated STAT4 to traffic to the nucleus following activation. Taken together, these data demonstrate that while both IL-12 and IFN-α promote STAT4 phosphorylation

in human $CD4^+$ T cells, they differ substantially in the quality of the signal which is delivered. IL-12 induces a very prolonged activation of STAT4. In contrast, STAT4 phosphorylation induced by IFN-α is attenuated, peaking at 3 hours and returning to baseline levels by 6 hours post-stimulation.

Type I IFN does not promote Th1 development because of lack of stable T-bet expression

Differences between IL-12 and IFN-α in the kinetics of STAT4 phosphorylation could have resulted in differences in downstream signaling events. I therefore examined the expression of IL-12Rβ2 and T-bet in naïve human $CD4^+$ T cells activated in the presence of IL-12, IFN-α, or a combination of IL-12 and IFN-α by quantitative real-time polymerase chain reaction (qPCR). In accordance with previous reports (15, 114, 125, 126, 131), both IL-12 and IFN-α induced expression of IL-12Rβ2 and T-bet 48 hours following primary activation (Fig. 13A). Furthermore, differentiation of human naïve $CD4^+$ T cells in the presence of both IL-12 and IFN-α enhanced the expression of IL-12Rβ2 and T-bet in an additive manner. Expression of these two genes in response to IL-12 was maintained at high levels even 7 days following the primary activation (Fig. 13A). In contrast, IFN-α-induced expression of IL-12Rβ2 and T-bet was entirely reversed by day 7. However, IFN-α did not inhibit IL-12-induced expression at day 7, in agreement with the results observed for IFN-γ expression.

Expression of T-bet protein in differentiated human $CD4^+$ T cells 7 days after primary activation was also examined by Western blotting. In agreement with the results

obtained for transcript expression, T-bet protein levels were elevated in response to IL-12 but not IFN-α at day 7 (Fig. 13B). IFN-α also did not prevent the IL-12-dependent induction of T-bet protein. Thus, while both IL-12 and IFN-α promote upregulation of IL-12Rβ2 and T-bet early during T cell activation, only IL-12 maintains expression of these two genes. IFN-α-dependent upregulation of IL-12Rβ2 and T-bet is not maintained to day 7 following primary activation.

Since the T-bet transcription factor is the master regulator of Th1 development, it was possible that the inability of IFN-α to maintain T-bet expression could have resulted in a failure to promote Th1 commitment. If this was the case, then ectopic T-bet expression should restore Th1 differentiation in naïve human $CD4^+$ T cells activated in the presence of IFN-α alone. To test this hypothesis, Hilario Ramos overexpressed T-bet in developing human naïve $CD4^+$ T cells using a bicistronic retrovirus (254). In these experiments, cells expressing GFP are positively transduced with the retroviral expression construct, whereas cells which do not express GFP remain untransduced and serve as an internal negative control. In cells transduced with an empty retrovirus (GFPRV), IL-12 but not IFN-α induced substantially elevated IFN-γ expression in both GFP^- and GFP^+ cells (Fig. 14A and B). Hence, retroviral transduction alone did not promote Th1 development in these cells. The same pattern of IFN-γ expression was seen in untransduced (GFP^-) cells from cultures transduced with GFPRV carrying a T-bet transgene (hT-bet-GFP). However, GFP^+ cells from cultures transduced with hT-bet-GFP showed greatly increased expression of IFN-γ compared to GFP^- controls, regardless of cytokines which were present during primary activation. Importantly, the level of

IFN-γ expression in T-bet-transduced cells differentiated in the presence of IFN-α was similar to that seen in untransduced cells differentiated in the presence of IL-12. Hence, T-bet overexpression was able to rescue the defect in Th1 development seen with IFN-α alone. These results indicate that the inability of IFN-α to promote commitment to the Th1 fate likely results from the failure of IFN-α to maintain stable expression of Th1 genes in developing human $CD4^+$ T cells.

Discussion

There has been considerable controversy surrounding the role of type I IFN in promoting Th1 development. While IFN-α was unable to promote STAT 4 activation or IFN-γ secretion in murine $CD4^+$ T cells, several groups had shown that IFN-α could positively regulate several aspects of Th1 development in human T helper cells. However, the data presented here indicate that IL-12, but not IFN-α, is responsible for Th1 differentiation in human $CD4^+$ T cells. In contrast to previous reports, IFN-α was insufficient to promote Th1 commitment *in vitro* as measured by IFN-γ secretion. This insufficiency was due to an inability of IFN-α to induce stable phosphorylation of STAT4 or long-term expression of T-bet. Hence, it now appears that IFN-α does not promote Th1 development in either murine or human T helper cells (Fig. 15).

The results presented here stand in direct contrast to a number of previously published reports showing that IFN-α could induce Th1 development in human $CD4^+$ T cells. The present data were generated using highly purified naïve $CD4^+$ T cells differentiated under defined *in vitro* culture conditions using a combination of innate cytokines and neutralizing anti-cytokine antibodies to carefully regulate cytokine exposure. In contrast, previous work was typically performed using peripheral blood or cord blood populations (14, 15, 129). Hence, the heterogeneity of the cell populations used could account for the observed differences; it is possible, for instance, that $CD8^+$ or NK cells, both of which can be activated by IFN-α, could have been responsible for IFN-γ secretion in these experiments (233, 266, 267). Furthermore, the exposure of

developing human $CD4^+$ T cells to cytokines has not previously been tightly controlled; hence, small amounts of IL-12 present during differentiation in the presence of IFN-α could have induced Th1 development. During the course of the above studies, Hilario Ramos also differentiated unfractionated human PBMCs in the presence of IL-12 or IFN-α, either in the presence or absence of a neutralizing antibody against IL-12 (254). However, neutralization of IL-12 in these experiments was not sufficient to engender Th1 development in response to IFN-α, even in PBMC cultures. It therefore remains unclear why the results presented here differ so strikingly from previously published reports.

This work also confirms and extends other, more recent findings regarding the role of IFN-α in human Th1 differentiation. Numerous studies have shown that IFN-α, like IL-12, can promote STAT4 activation in human $CD4^+$ T cells (15, 113, 114, 130). The findings described here clearly confirm this observation. However, Athie-Morales et. al. and others have observed a difference in the kinetics of STAT4 phosphorylation in human $CD4^+$ T cells in response to IL-12 vs. IFN-α (144, 145). Indeed, I also found that IFN-α stimulation of human Th1 cells resulted in an attenuated STAT4 activation as compared to that seen with IL-12; while IL-12 promoted STAT4 phosphorylation beyond 24 hours post-stimulation, IFN-α-induced activation of STAT4 peaked at 3 hours and returned to baseline by 6 hours.

Other groups have also reported that both IL-12 and IFN-α could induce expression of IL-12Rβ2 and T-bet in human $CD4^+$ T cells (15, 114, 125, 126, 131). I demonstrated similar levels of IL-12Rβ2 and T-bet induction by these two cytokines in developing human T helper cells 48 hours following initial activation. It is also of

interest to note that, in contrast to murine $CD4^+$ T cells, IFN-γ did not affect expression of IL-12Rβ2 or T-bet, and this corresponded to a lack of participation of IFN-γ in human Th1 development (254). However, previous studies had not examined the long-term effects of innate cytokines on T-bet and IL-12Rβ2 expression. I found that, in contrast to IL-12, IFN-α-induced expression of these two genes was transient, such that expression of both had dropped below the levels seen in control cells by 7 days of differentiation. Furthermore, overexpression of T-bet in human $CD4^+$ T cells developing in response to IFN-α was sufficient to restore Th1 development, indicating that prolonged expression of T-bet is crucial for full Th1 commitment (254). These data, together with the findings for STAT4, provide evidence that the quality of the cytokine signals received by a developing $CD4^+$ T cell is critically important; brief expression of the necessary transcription factors is not sufficient to generate fully polarize effector cells.

Fully polarized Th1 cells can secrete IFN-γ in an antigen-independent manner upon stimulation with a combination of IL-12 and IL-18, and this effect is dependent upon STAT4 (122). Matikainen and colleagues have reported that a combination of IFN-α and IL-18 can also induce TCR-independent secretion of IFN-γ from mature Th1 cells (128, 129). Given that IL-12 and IFN-α differ in the kinetics of STAT4 activation, it was possible that there could be qualitative differences in IL-12 + IL-18 vs IFN-α + IL-18 restimulation of human $CD4^+$ T cells. During the course of these studies, Hilario Ramos showed by flow cytometry that IFN-α + IL-18 stimulation does indeed promote IFN-γ secretion from polarized Th1 cells (254). However, in agreement with the differential kinetics of STAT4 activation between IL-12 and IFN-α, IFN-γ production in response to

IFN-α + IL-18 was significantly less than that seen in response to IL-12 + IL-18 over a longer period of activation. These data indicate that differences in STAT4 phosphorylation translate into functional differences between IL-12 and IFN-α in promoting secondary IFN-γ secretion. Furthermore, while type I IFN produced at the site of infection may participate in bystander $CD4^+$ T cell responses, IFN-α stimulation is still inferior to IL-12 stimulation in promoting TCR-independent IFN-γ secretion.

Based upon previous studies which found that human, but not murine, $CD4^+$ T cells could differentiate to the Th1 fate in response to IFN-α, it has been proposed that wild-type mice might not provide an accurate model for Th1 responses to viral infections (134). The above findings, in combination with published reports from other groups, indicate that IFN-α does not, in fact, direct Th1 development in either murine or human $CD4^+$ T cells (142-145). However, there are still molecular signaling differences between murine and human $CD4^+$ T cells in response to IFN-α. For instance, activation of murine $CD4^+$ T cells from mice carrying a wild-type or a humanized version of the *stat2* gene in the presence of type I IFN results in substantially different patterns of gene expression (M. Persky and J. D. Farrar, personal communication). Furthermore, there is a clear biochemical difference in the preassociation of STAT4 with the human and murine IFNAR chains, which could affect transduction of downstream signals (136). These signaling differences may have unexplored consequences for the immune response to pathogens in mice and humans.

This work clearly demonstrates that IFN-α is not involved, either positively or negatively, in regulating IFN-γ secretion from human $CD4^+$ T cells. However, IFN-α

unquestionably does activate both STAT1 and STAT4, two transcription factors which are essential for Th1 development. IFN-α also promotes transient upregulation of IL-12Rβ2 and the Th1 master regulator T-bet, and fully polarized human Th1 cells can respond to a combination of IFN-α and IL-18 by producing IFN-γ. Hence, IFN-α, while unable to independently induce full Th1 commitment, may play an important accessory role in IL-12-regulated Th1 differentiation during viral infections. IFN-α also does not promote Th2 development, as measured by IL-4 and IL-5 secretion. Aman et. al. reported that IFN-α induced elevated IL-10 secretion by human $CD4^+$ T cells (268), but I have not been able to confirm this finding *in vitro* using purified human $CD4^+$ T cells and the defined cytokine conditions described above (Fig. 37). It is, however, possible that IFN-α may be regulating the expression by T helper cells of other cytokines which have not yet been examined. Furthermore, type I IFN induces the expression of a substantial number of genes in human T cells, and the roles of many of these genes in T helper cell development have yet to be fully explored (269). A clearer understanding of the role of type I IFN in the generation of human $CD4^+$ T cell responses will be important for the design of a new generation of vaccines and antiviral therapies.

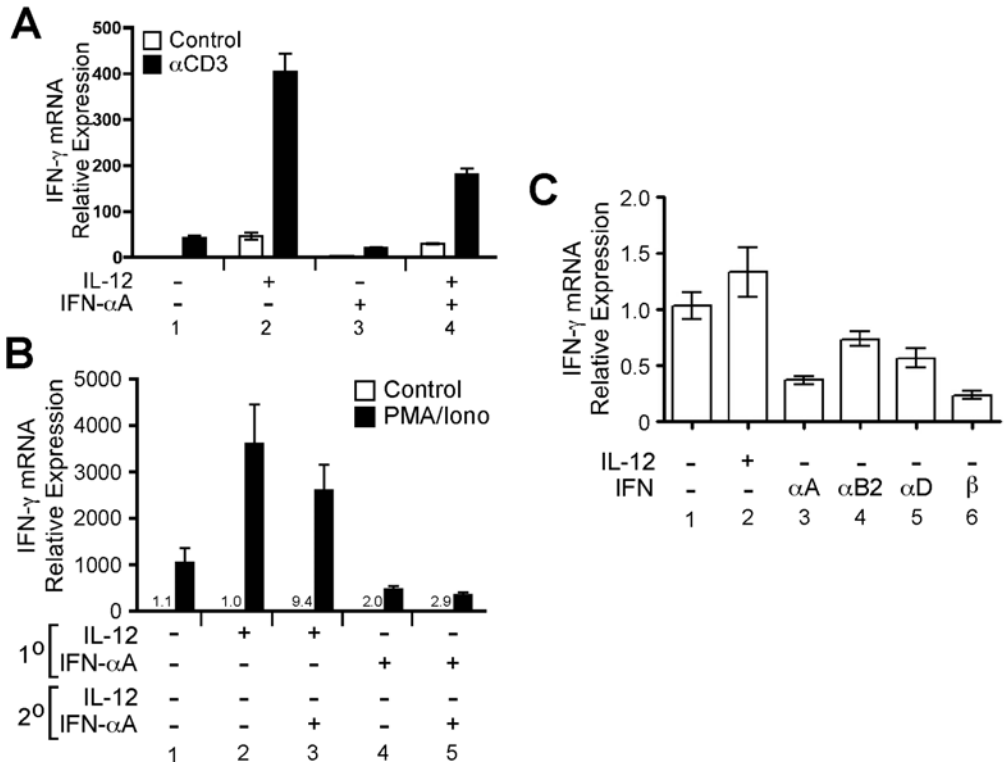

Figure 7. IFN-α is insufficient to promote Th1 development in human CD4$^+$ T cells. (A) Human naïve CD4$^+$ T cells were activated for 7 days with plate-bound anti-CD3 + anti-CD28 in the presence of IL-2 and cytokines and neutralizing anti-cytokine antibodies as indicated in the figures. A "+" symbol indicates that a cytokine was added; a "–" symbol indicates that the cytokine was neutralized. On day 7, cells were washed and reactivated for 2 hours in the absence (open bars) or presence (filled bars) of plate-bound anti-CD3. Total RNA was harvested, and qPCR was performed using primers directed against human IFN-γ. Primers directed against human GAPDH were used as a control. (B) Naïve human CD4$^+$ T cells were differentiated for 7 days as described in (A) (1^o). On day 7, cells were washed and reactivated for a second week with plate-bound anti-CD3 + anti-CD28 in the presence of IL-2 and the indicated cytokines and neutralizing antibodies (2^o). On day 14, cells were restimulated for 2 hours in the absence (open bars) or presence (filled bars) of PMA + ionomycin. Total RNA was harvested, and qPCR analysis was performed as in (A). (C) Naïve human CD4$^+$ T cells were differentiated for 7 days as described in (A) in the presence of the indicated type I IFN subtypes. On day 7, cells were washed and restimulated for 2 hours in the presence of PMA + ionomycin. Total RNA was harvested, and qPCR analysis was performed as described in (A).

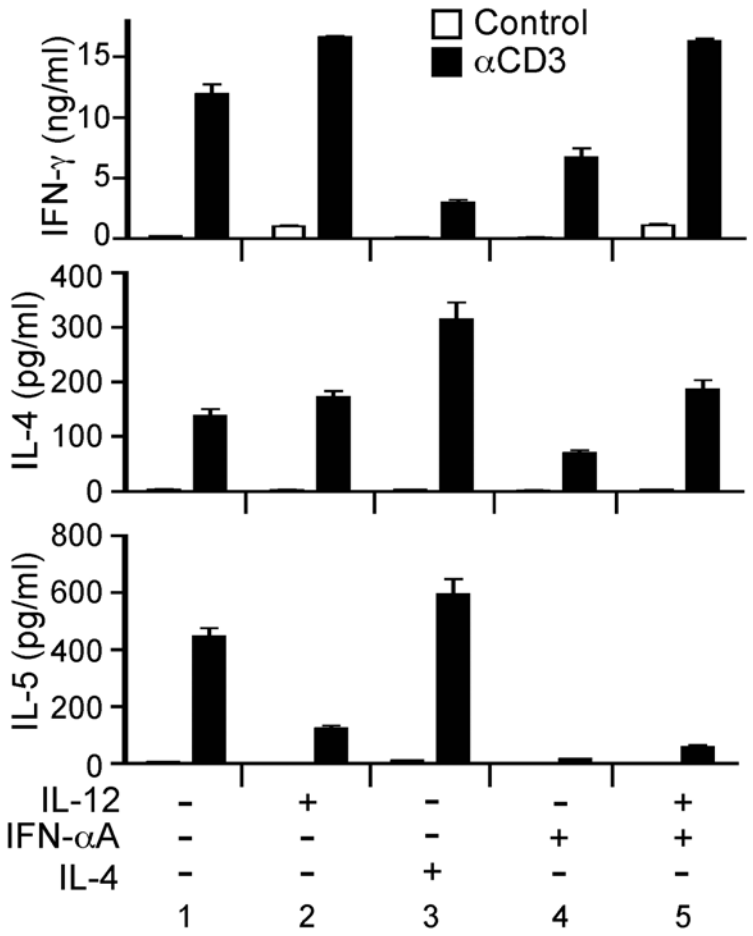

Figure 8. IFN-α does not induce Th1 or Th2 development in human CD4$^+$ T cells. Human naïve CD4$^+$ T cells were activated for 2 consecutive weeks with plate-bound anti-CD3 + anti-CD28 in the presence of IL-2 and the indicated cytokines and/or neutralizing antibodies. On day 14, cells were washed and restimulated for 24 hours in the absence (open bars) or presence (filled bars) of plate-bound anti-CD3. Concentrations of IFN-γ, IL-4, and IL-5 in supernatants were measured by cytometric bead array (CBA).

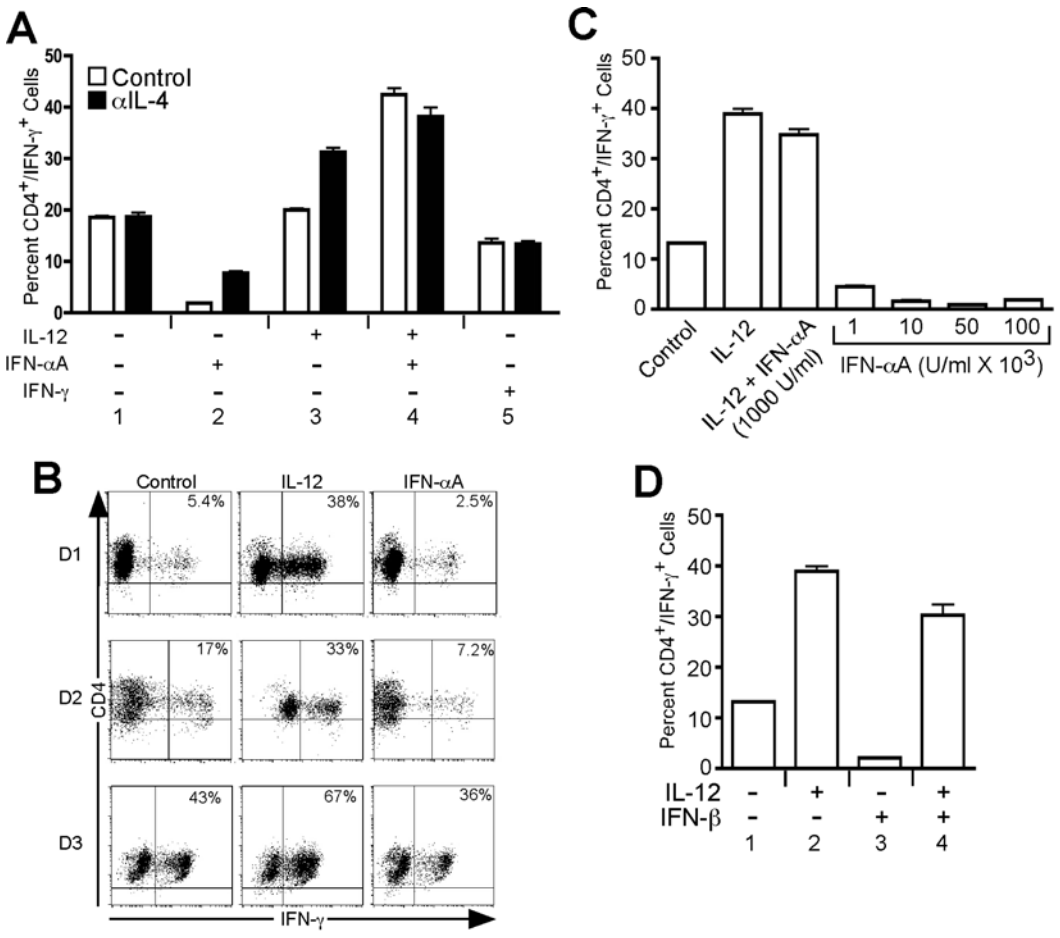

Figure 9. IFN-α is insufficient to promote Th1 development in human CD4$^+$ T cells. Human naïve CD4$^+$ T cells were activated for 7 days with plate-bound anti-CD3 + anti-CD28 in the presence of IL-2 and cytokines and neutralizing anti-cytokine antibodies as indicated in the figures. A "+" symbol indicates that a cytokine was added; a "–" symbol indicates that the cytokine was neutralized. On day 7, cells were rested overnight in the absence of IL-2 and then restimulated for 4 hours with PMA + ionomycin in the presence of Brefeldin A. Cells were gated on live lymphocytes, and data are expressed as the percent of CD4$^+$ cells expressing IFN-γ. (A) Cells were activated in the absence (open bars) or presence (filled bars) of a neutralizing anti-hIL-4 antibody. (B) Cells from three separate human donors were activated as described. (C) Cells were activated in the presence of IL-12, IL-12 + IFN-α, or increasing concentrations of IFN-α as indicated. (D) Cells were activated in the presence of IL-12, IFN-β, or IL-12 + IFN-β as indicated.

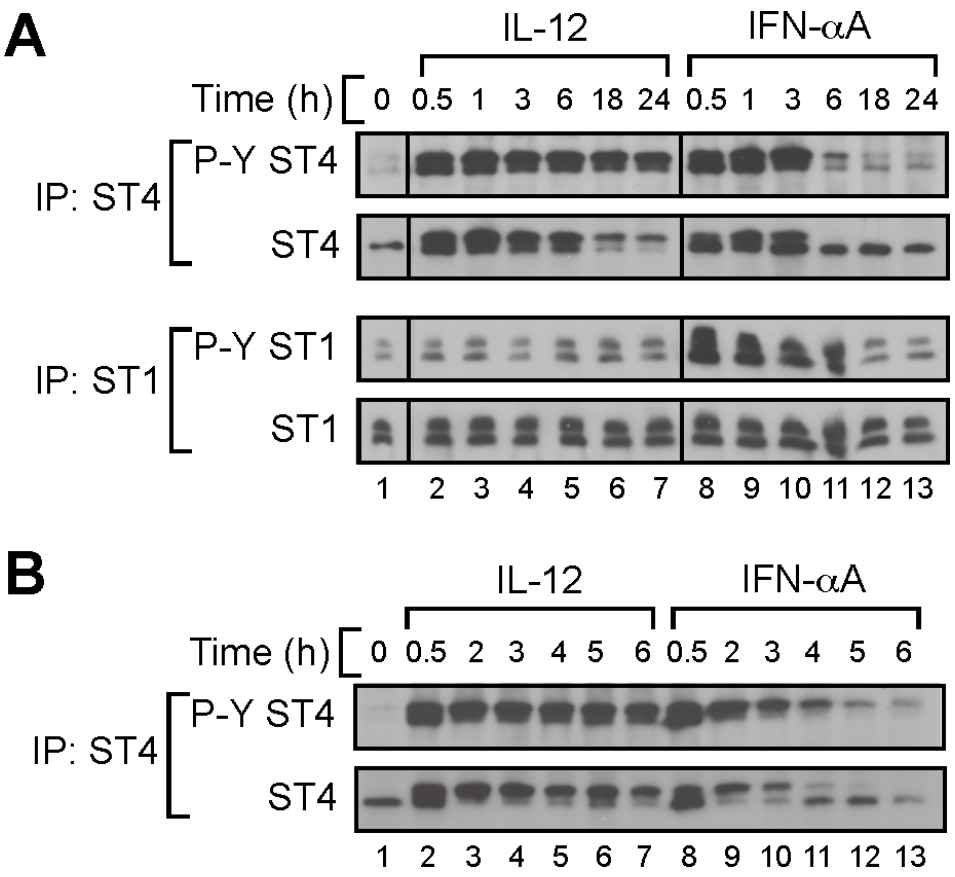

Figure 10. Distinct kinetics of STAT4 phosphorylation in response to IL-12 vs. IFN-α. Human PBMCs were activated for two consecutive weeks in the presence of PHA, IL-2, and IL-12. On day 14, cells were washed and restimulated for 0-24 hours (A) or 0-6 hours (B) in the presence of IL-12 or IFN-α. Cell lysates were subjected to immunoprecipitation, and Western blotting was performed for 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 Hilario J. Ramos.

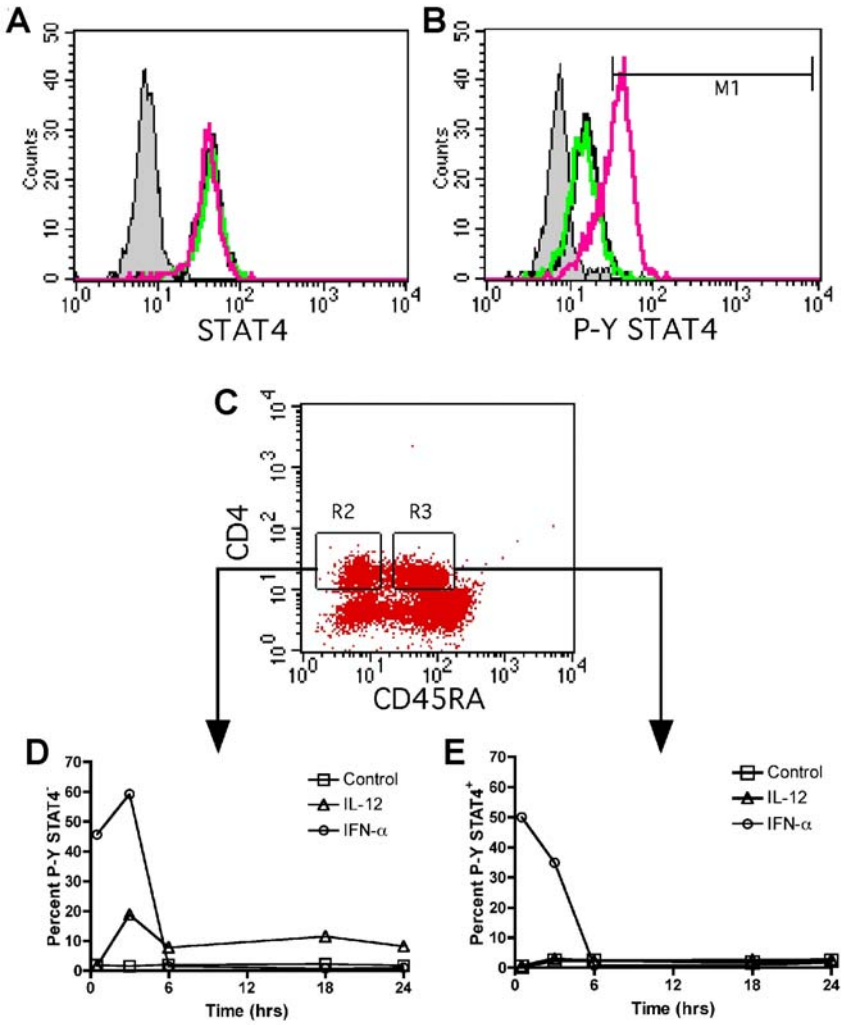

Figure 11. Differential STAT4 phosphorylation mediated by IL-12 and IFN-α in freshly isolated human T cells. Freshly isolated human PBMCs were activated for 30 minutes – 24 hours in the presence of IL-12 or IFN-α, and intracellular flow cytometry was performed for CD4 and CD45RA as well as phosphorylated or total STAT4. (A – B) Representative histograms showing total STAT4 (A) and phosphorylated STAT4 (B) staining. Filled histogram, nonimmune rabbit Ig control; black line, unstimulated; green line, IL-12; pink line, IFN-α. (C – E) Cells were gated on expression of CD4 and CD45RA (C), and the percent of cells demonstrating phosphorylated STAT4 in response to IL-12 or IFN-α was quantified for cells which were $CD4^+$ $CD45RA^-$ (D) or $CD4^+$ $CD45RA^+$ (E). □, unstimulated; Δ, IL-12; ○, IFN-α. This work was performed by Dr. J. David Farrar.

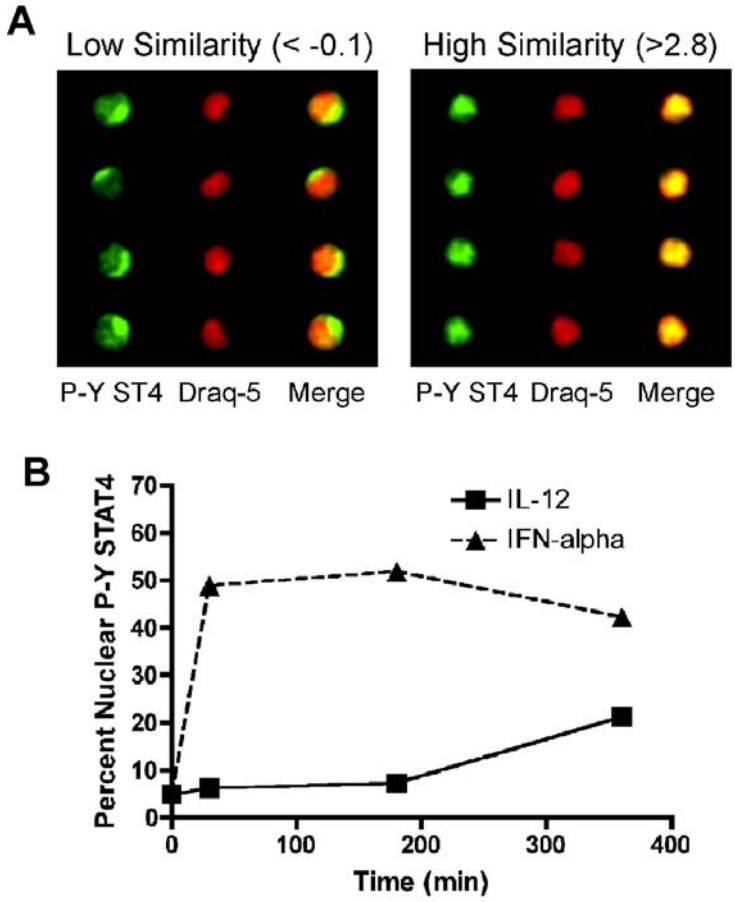

Figure 12. Nuclear localization of phosphorylated STAT4 in human naïve CD4$^+$ T cells. Cells were stained as in Fig. 4 and gated CD4$^+$ CD45RA$^+$. Cells were then analyzed on an ImageStream flow cytometer. Nuclear localization of phosphorylated STAT4 was classified as low similarity or high similarity based on staining with a nuclear dye (F). The percent of cells displaying nuclear localization of phosphorylated STAT4 in response to IL-12 or IFN-α was quantified (G). ■, IL-12; ▲, IFN-α. This work was performed by Dr. J. David Farrar and Dr. Thaddeus C. George.

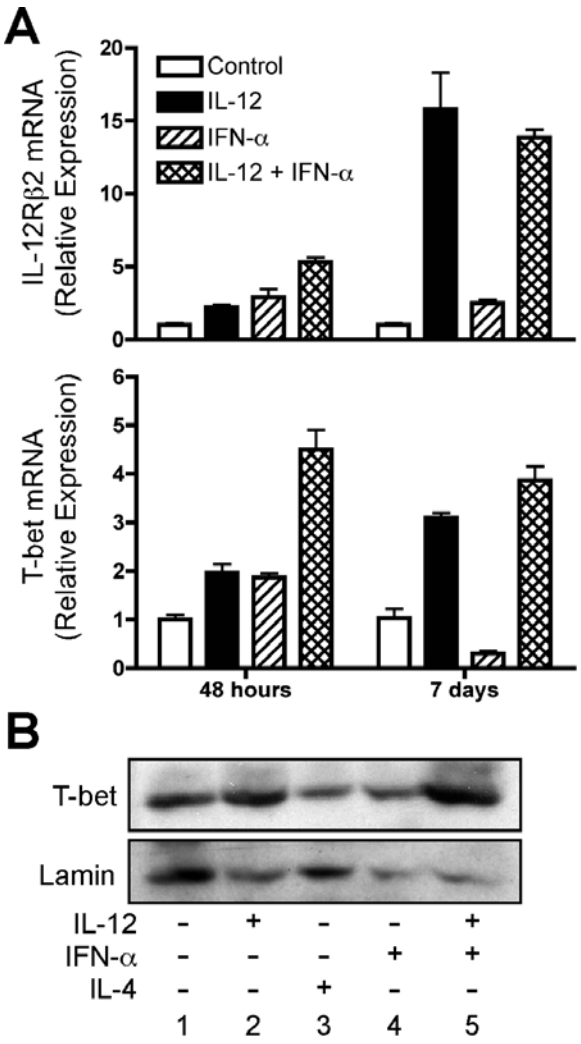

Figure 13. IFN-α is insufficient to maintain expression of IL-12Rβ2 or T-bet. (A) Naïve human $CD4^+$ T cells were activated with plate-bound anti-CD3 + anti-CD28 in the presence of IL-2 and cytokines and neutralizing antibodies as indicated. 48 hours or 7 days following primary activation, total RNA was harvested, and qPCR was performed using primers directed against human IL-12Rβ2 or human T-bet. Primers directed against human GAPDH were used as a control. (B) Naïve human $CD4^+$ T cells were activated with plate-bound anti-CD3 + anti-CD28 in the presence of IL-2 and cytokines and neutralizing antibodies as indicated. On day 3, cells were split into clean media containing IL-2 and rested to day 7. On day 7, cell lysates were prepared, and Western blotting was performed using antibodies against human T-bet or human Lamin A/C.

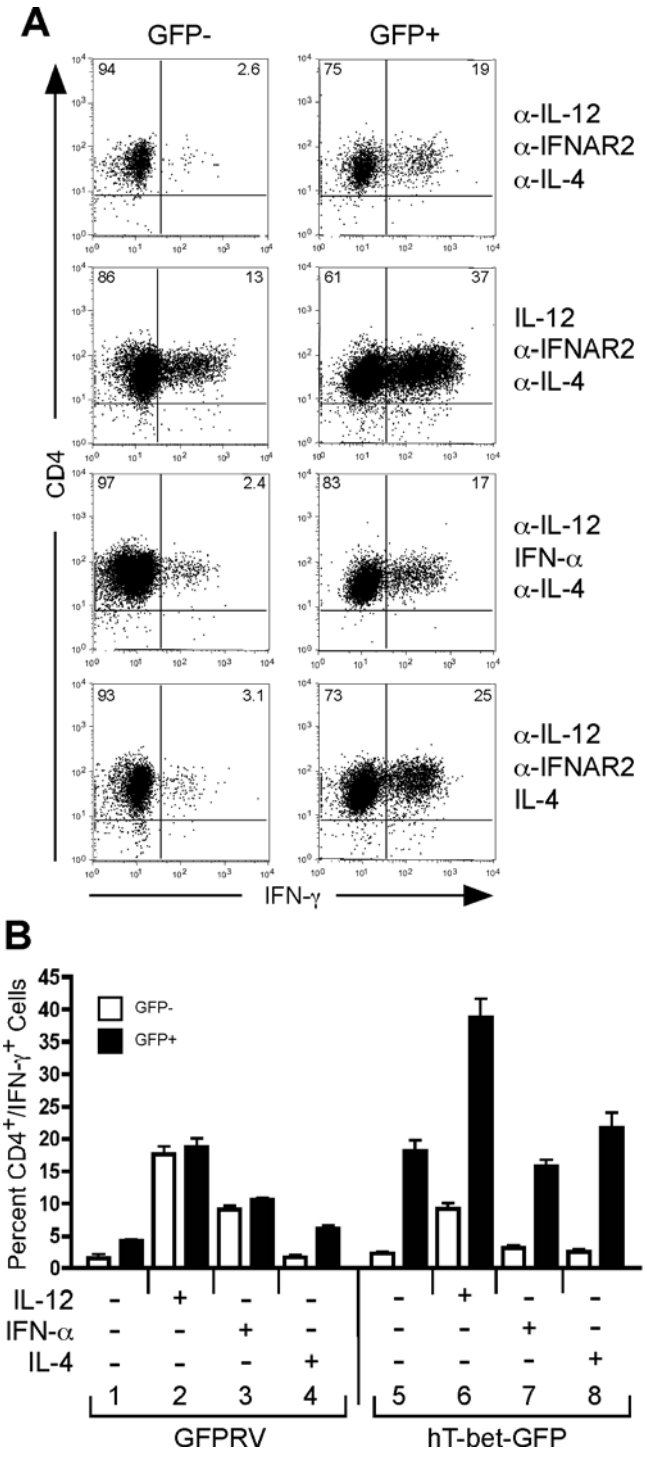

Figure 14. Overexpression of T-bet in developing human CD4$^+$ T cells rescues Th1 development. Human naïve CD4$^+$ T cells were activated with plate-bound anti-CD3 + anti-CD28 in the presence of IL-2 and neutralizing antibodies and transduced with an empty retrovirus (GFPRV) or a retrovirus containing human T-bet (hT-bet-GFP). Cells were then cultured in the presence of cytokines or neutralizing antibodies as indicated. On day 7, cells were expanded with plate-bound anti-CD3 for a further 7 days. On day 14, cells were washed and restimulated with PMA + ionomycin, and intracellular flow cytometry was performed using antibodies against IFN-γ and CD4. Cells were gated on expression of GFP. (A) Representative dot plots showing hIFN-γ secretion from GFPRV and hT-bet-GFP transduced cells. (B) Quantification of IFN-γ secretion from cells gated GFP$^-$ (open bars) or GFP$^+$ (filled bars). This work was performed by Hilario J. Ramos.

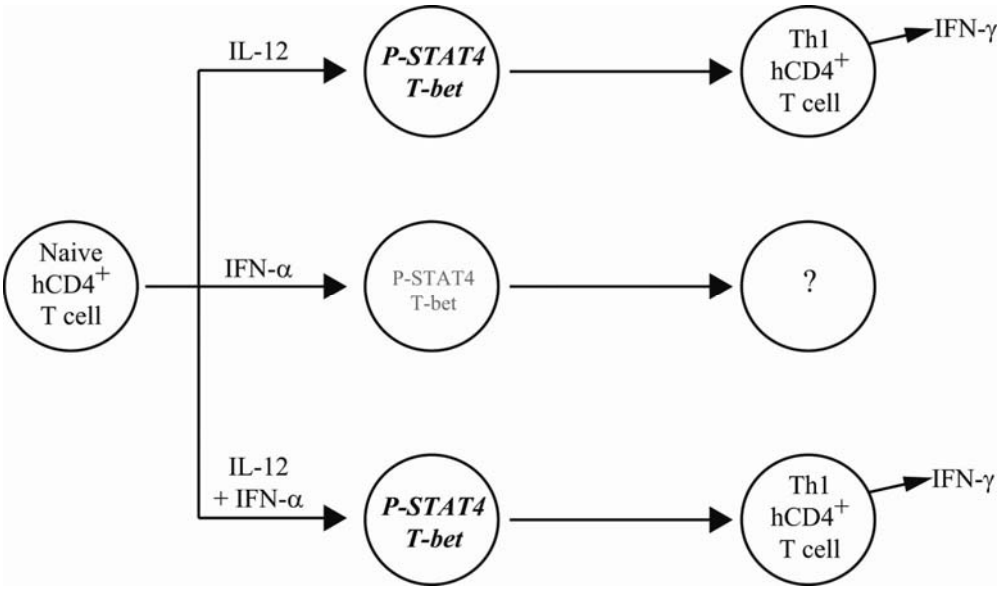

Figure 15. Model for the distinct roles of IL-12 and IFN-α in human Th1 development. Activation of human naïve $CD4^+$ T cells in the presence of IL-12 leads to prolonged phosphorylation of STAT4, resulting in extended expression of T-bet. This culminates in productive Th1 development, as indicated by IFN-γ secretion upon secondary activation. In contrast, activation of human naïve $CD4^+$ T cells in the presence of IFN-α alone results in attenuated STAT4 phosphorylation and transient rather than stable T-bet induction. Because of the lack of long-term T-bet expression, IFN-α fails to promote commitment to the Th1 fate. However, IFN-α also does not inhibit the ability of IL-12 to induce Th1 differentiation.

CHAPTER IV

BLOCKADE OF VIRUS INFECTION BY HUMAN CD4$^+$ T CELLS VIA A CYTOKINE RELAY NETWORK

The work presented in this chapter has been published in the *Journal of Immunology*, volume 180, pages 6923-6932 (270). This work is reproduced with the permission of the *Journal of Immunology*. Experiments were performed by Ann M. Davis unless otherwise noted in the text and figure legends.

Introduction

Adaptive immune responses play a critical role in the clearance of infectious diseases and in providing long-term resistance against re-infection. CD4$^+$ and CD8$^+$ T cells orchestrate inflammatory processes through both cytolytic and cytokine-mediated effector mechanisms. In response to bacterial infections, CD4$^+$ T helper type 1 (Th1) cells promote the recruitment and activation of phagocytic cells, such as macrophages and neutrophils, into sites of infection through the secretion of the chemokines CXCL8/IL-8 and CCL3/MIP-1α and the cytokines interferon gamma (IFN-γ) and tumor necrosis factor alpha (TNF-α). IFN-γ and TNF-α act in concert to promote the production of reactive oxygen and nitrogen radicals from phagocytic cells, which effectively sterilizes the site of infection (149, 154, 158). Thus, CD4$^+$ T cells participate directly in antibacterial immunity through the secretion of proinflammatory cytokines. In

contrast, viral infections are considered to rely predominantly on $CD8^+$ T cell responses (271). $CD4^+$ T cells clearly play a supporting role during viral infections through cytokine secretion and by providing critical help for B cell antibody production (176-178, 272-276). Additionally, there are some reports detailing a population of $CD4^+$ cytotoxic T cells which can directly lyse infected targets by cell-cell contact (178, 186-191). However, the ability of $CD4^+$ T cells to directly inhibit viral replication and spread has not been thoroughly examined.

Viral infections initiate a cascade of innate and adaptive immune responses that are collectively regulated by cytokines. Type I interferon (IFN-α/β) is one of the first cytokines secreted by virally infected cells and from professional antigen presenting cells through the activation of various pattern recognition receptors such as Toll-like receptors and RIG-I (277). IFN-α/β exerts potent antiviral activities directly on infected cells by inducing the expression of interferon sensitive genes (ISGs), thereby inhibiting virus replication and spread (57). In addition to these innate activities, IFN-α/β also enhances effector functions of natural killer (NK) cells and $CD8^+$ cytolytic T lymphocytes (CTL) (233, 266, 267, 278). However, the role of IFN-α/β in regulating $CD4^+$ effector functions has been controversial. Early reports suggested that IFN-α/β could promote Th1 development through activation of STAT4 in an IL-12-independent manner (14, 114, 129, 131, 132, 134, 136, 140). However, recent studies have demonstrated that IFN-α/β completely lacks the ability to drive Th1 development in human $CD4^+$ T cells because unlike IL-12, IFN-α/β does not induce stable expression of the Th1-specific transcription factor T-bet (142, 143, 145, 254). However, in these studies, IFN-α/β did not inhibit the

ability of IL-12 to promote Th1 development as assessed only by IFN-γ secretion. As both IL-12 and IFN-α/β are secreted to high levels by dendritic cells in response to viral infections (279), it is possible that IFN-α/β synergizes with IL-12 to regulate other potential $CD4^+$ effector cytokines that may play important roles in inhibiting viral infections.

In addition to IFN-α/β, several other proinflammatory cytokines have been shown to exert antiviral activity. For example, IFN-γ shares various antiviral activities with IFN-α/β, such as upregulation of class I MHC, inhibition of viral replication, and the induction of an overlapping set of ISGs (57). In addition, TNF-α and TNF-β (lymphotoxin) have also been shown to inhibit viral replication directly as well as indirectly through the induction of IFN-β within infected cells (181-185, 280, 281). $CD4^+$ Th1 cells represent a significant source of IFN-γ and TNF-α, and it is possible that $CD4^+$ T cells play a much more central role in the course of viral infections than has previously been attributed to this subset. Indeed, studies in mice have demonstrated a $CD4^+$-dependent component to clearance of Sendai virus, influenza A virus, and γ-herpesvirus (192, 194-196, 198). In cases of γ-herpesvirus infections, $CD4^+$ T cells were shown to inhibit reactivation from latency, and neutralization of IFN-γ could inhibit this activity. However, administration of IFN-γ was not sufficient to maintain latency, particularly within infected B cells (198, 199). Based on these observations, it is likely that $CD4^+$ T cells play a significant role in the inhibition of viral replication through the action of a complex mixture of cytokines, the nature of which has not been investigated.

I therefore sought to answer two distinct questions. First, how do innate cytokines present during viral infections shape effector $CD4^+$ T cell responses? Second, can cytokines secreted by effector $CD4^+$ T cells directly impact viral infections? I found that IL-12 is primarily responsible for the generation of antiviral $CD4^+$ T cell effector cytokine responses. IL-12 drives the secretion of IFN-γ and TNF-α, which induce potent antiviral responses against a number of viruses. Further, this antiviral effect on VSV infection requires IFN-α/β receptor (IFNAR) expression on the target cell, indicating the presence of a novel cytokine relay network.

Results

Human CD4$^+$ T cells secrete an antiviral activity

Th1 cells play a direct role in clearance of bacterial infections by secretion of IFN-γ. Since CD4$^+$ T cells are known to secrete a variety of soluble mediators, I hypothesized that these cells may also play a role in viral clearance by direct cytokine signaling to infected cells. To test this hypothesis, an *in vitro* infection model was established whereby THP-1 cells, a human monocyte line, were infected with vesicular stomatitis virus carrying a transgene for green fluorescent protein (VSV-GFP). The percentage of infected cells was monitored by flow cytometry (Fig. 16A and B), whereas the relative secretion of live virus was quantified by plaque assay (Fig. 17C). In this model, VSV-GFP infection was blocked by treatment of infected cells with type I IFN (Fig. 1A and B), and this effect was reversed by blocking the human type I IFN receptor (IFNAR) by a neutralizing antibody against the IFNAR2 subunit (Fig. 16A). IFN-α significantly reduced the percentage of infected cells, which correlated well with a significant decrease in secretion of live virus (Fig. 16B and Fig. 17C).

The effect of CD4$^+$ T cell-derived effector cytokines on VSV-GFP infection of THP-1 cells was then examined. In order to isolate the individual contributions of innate cues to the generation of antiviral effector responses, naïve (CD45RA$^+$) human CD4$^+$ T cells were differentiated with plate-bound anti-CD3 and anti-CD28 in the presence of cytokines or anti-cytokine neutralizing antibodies for 7-14 days. The use of neutralizing antibodies allowed careful regulation of the exposure of the developing T helper cells to

innate cytokine signals. These cells were then washed extensively in clean media and restimulated for 24 hours with plate-bound anti-CD3, and the conditioned media from these cells was harvested and used to treat VSV-GFP-infected THP-1 cells. Treatment of THP-1 cells with T cell conditioned media at the time of infection inhibited VSV-GFP infection as measured by GFP expression, and this effect was dose-dependent (Fig. 17A). Furthermore, conditioned media from resting $CD4^+$ T cells did not inhibit VSV-GFP infection, indicating that the secretion of antiviral activity required secondary T cell activation (Fig. 17B, $p < 0.05$, anti-CD3-restimulated versus unstimulated, all conditions). Pre-treatment of THP-1 cells with T cell conditioned media for 24 hours prior to infection significantly inhibited VSV-GFP virus production from these cells as measured by plaque assay (Fig. 17C). Furthermore, T cell conditioned media generated from T cells differentiated in the presence of IL-12 or a combination of IL-12 and IFN-α was consistently more effective at reducing VSV-GFP infection in the THP-1 cells than T cell conditioned media generated from T cells differentiated in the presence of neutralizing antibodies or IFN-α alone (Fig. 17A, $p < 0.05$ versus neutralized). Conversely, T cell conditioned media generated from T cells differentiated in the presence of IL-4 had no effect on VSV-GFP infection (Fig. 17C). Occasionally, a slight difference was observed in antiviral activity generated from $CD4^+$ T cells differentiated in the presence of IL-12 alone versus IL-12 with IFN-α (Fig. 17A); however, this difference was not present in most experiments and was likely a result of donor variation.

In order to determine whether the activity secreted by human $CD4^+$ T cells represented a general antiviral mechanism, the ability of T cell conditioned media to inhibit infection with two other viruses was examined: respiratory syncytial virus (RSV)

and hepatitis C virus (HCV). HeLa cells were infected for 72 hours with RSV carrying a GFP transgene (RSV-GFP) by Gagan Bajwa in the laboratory of Dr. Michelle A. Gill. Treatment of HeLa cells with T cell conditioned media at the time of infection significantly reduced RSV-GFP infection (Fig. 18A). In agreement with previous results, conditioned media generated from T cells activated in the presence of IL-12, alone or in combination with IFN-α, contained greater antiviral activity against RSV-GFP than conditioned media from T cells activated under either neutralizing or IFN-α conditions ($p < 0.05$ versus neutralized).

Inhibition of hepatitis C virus (HCV) infection by T cell conditioned media was examined by Kristan A. Hagan in the laboratory of Dr. Michael Gale, Jr. using the A7 HCV replicon cell line. These cells carry a full-length, replicating HCV genome and express HCV proteins (251). Addition of 5% (v/v) T cell conditioned media to these cells reduced HCV NS5A protein synthesis, and this antiviral activity also required restimulation of the T cells by anti-CD3 crosslinking (Fig. 18B). At these concentrations, obvious differences were not observed between conditioned media from different T cell conditions, which suggested that NS5A expression was particularly sensitive to very low levels of antiviral factors secreted by human $CD4^+$ T cells. Taken together, these results demonstrate for the first time that effector cytokines secreted by human $CD4^+$ T cells can directly inhibit viral infection in target cells.

IFN-γ and TNF-α secreted by CD4$^+$ T cells demonstrate antiviral activity

The greatest antiviral activity against VSV and RSV was observed in conditioned media generated from T cells differentiated in the presence of IL-12, suggesting that the secreted factor was a Th1 cytokine. In accordance with previously published work (254), secretion of IFN-γ and TNF-α from human T helper cells depended on IL-12, and IFN-α/β neither enhanced nor inhibited this effect (Fig. 19). Priming with IL-12 significantly enhanced the percentage of IFN-γ secreting cells (Fig. 19A), and the IFN-γ secreting cells were found to also secrete TNF-α (Fig. 19A and B). Furthermore, approximately 90% of cells were found to secrete TNF-α regardless of whether the cells were activated under neutralizing conditions or with IL-12 (Fig. 19A and B). However, cells differentiated in the presence of IL-12 secreted significantly higher concentrations of IFN-γ (16-18 fold) and TNF-α (3-5 fold) protein as compared to cells polarized under neutralizing conditions (Fig. 19C and D).

IFN-γ and TNF-α are proinflammatory cytokines that markedly inhibit intracellular bacterial infections. These cytokines act in concert to promote the oxidative burst within phagocytic cells (149, 154, 158). In order to confirm that the T cell conditioned media contained functionally relevant levels of these proinflammatory cytokines, cell-free supernatants were tested for their ability to control *Listeria innocua* infection within the THP-1 monocyte cell line by Loderick Matthews. THP-1 cells were differentiated to a macrophage state with PMA and cultured in the presence or absence of recombinant cytokines (Fig. 20A) or T cell conditioned media (Fig. 20B) for 48 hours.

The cells were subsequently infected with *L. innocua*, again in the presence or absence of recombinant cytokines or T cell conditioned media. As expected, bacterial replication was markedly inhibited by combined treatment with recombinant IFN-γ and TNF-α (Fig. 20A). Recombinant IFN-α also inhibited *L. innocua* infection (Fig. 20A), as has been previously reported (282). Additionally, treatment with T cell conditioned media significantly inhibited *L. innocua* infection, and conditioned media from cells differentiated in the presence of IL-12 displayed the greatest antibacterial activity in this assay (Fig. 20B). Thus, T cell conditioned media contain relevant levels of IFN-γ and TNF-α sufficient to inhibit intracellular bacterial replication. These data further indicate that the THP-1 monocyte cell line is sensitive to both IFN-γ and TNF-α signaling.

As noted previously, T cell conditioned media inhibited HCV infection in A7 replicon cells (Fig. 18B). HCV suppresses antiviral signaling by type I IFN in infected host cells by a variety of mechanisms, including inhibition IFN-α/β synthesis through disruption of the RIG-I pathway (86, 91, 92). However, several reports have indicated that HCV is susceptible to the antiviral effects of IFN-γ (283-285). Therefore, the role of IFN-γ and TNF-α secretion by human $CD4^+$ T cells in inhibition of HCV infection was examined by Kristan A. Hagan in the laboratory of Dr. Michael Gale, Jr. (Fig. 21). While recombinant TNF-α alone displayed no effect on HCV NS5A expression, recombinant IFN-γ alone inhibited HCV NS5A (Fig. 21A, compare lanes 2 and 3). Furthermore, addition of TNF-α marginally enhanced the antiviral effect of IFN-γ (Fig. 21A, compare lanes 3 and 5).

As was previously observed, addition of T cell conditioned media to A7 HCV replicon cells reduced HCV NS5A protein synthesis (Fig. 21A). Neutralization of the R1 chain of the IFN-γ receptor (IFNγR1) on target cells, combined with neutralization of TNF-α in T cell conditioned media, reversed the previously observed antiviral activity of T cell conditioned media in this assay system (Fig. 21A, compare lanes 8 and 11), demonstrating an antiviral role for T cell-secreted IFN-γ and TNF-α in HCV infection. As expected, addition of neutralizing anti-hIFNAR2 antibody failed to reverse the antiviral effect of T cell conditioned media (Fig. 21B, compare lanes 11 and 12).

To elucidate a possible molecular mechanism for the antiviral effects of T cell conditioned media in the HCV replicon system, the expression of ISG56, an interferon-stimulated gene known to inhibit HCV replication (82), was examined. Treatment of A7 replicon cells with recombinant IFN-α induced ISG56 expression, as expected (Fig. 21A, lane 2). Unexpectedly, recombinant IFN-γ also induced ISG56 expression in these cells, and addition of recombinant TNF-α enhanced this effect (Fig. 21A, lanes 3 and 5). Furthermore, ISG56 was induced by T cell conditioned media from T cells restimulated with αCD3 (Fig. 21A and B), and this effect was reversed by blockade of IFN-γ and TNF-α signaling (Fig. 21A, lanes 8 and 11) but not by neutralization of IFNAR2 (Fig. 21B, lane 12).

IFN-γ and TNF-α signal through a cytokine relay network involving the type I IFN receptor

As IFN-γ and TNF-α were found to potently inhibit HCV gene expression, it was of interest to determine whether these two proinflammatory cytokines were also responsible for the antiviral activity of T cell conditioned media in VSV-GFP infection. Recombinant TNF-α alone showed little antiviral activity at concentrations up to 50 ng/ml, while recombinant IFN-γ alone had a modest and dose-dependent effect on VSV infection (Fig. 22A). However, the combination of IFN-γ and TNF-α displayed a very potent and synrgistic antiviral activity, comparable to the activity of 100 U/ml rhIFN-αA in this assay (Fig. 22A). Th1 cells also secrete lymphotoxin (LT), a member of the TNF superfamily (152), and some recent reports have demonstrated that LT secreted by NK cells has noncytopathic antiviral properties (280, 281). However, recombinant LT failed to demonstrate antiviral activity, either alone or in combination with IFN-γ (Fig. 23A and B). Furthermore, neutralization of LT in TCM resulted in no reversal of the observed antiviral activity (Fig. 23C). Thus, VSV infection is specifically sensitive to the combined effects of IFN-γ and TNF-α.

As demonstrated above, T cell conditioned media markedly inhibited VSV-GFP infection, and this activity was partially inhibited by blocking either the IFNγR1 or TNF-α (Fig. 22B). Further, neutralization of both cytokines resulted in much greater reversal (Fig. 22B, condition 7, $p < 0.05$ versus T cell conditioned media alone). As a control, target cells were preincubated with neutralizing anti-IFNAR2 before VSV-GFP infection

in the presence of T cell conditioned media. Surprisingly, neutralization of IFNAR2 reversed the antiviral effect of T cell conditioned media as effectively as blockade of IFN-γ and TNF-α (Fig. 22B, condition 4, $p < 0.05$ versus T cell conditioned media alone).

No previous reports have demonstrated secretion of type I interferon by $CD4^+$ T cells. Thus, it was surprising to find that neutralization of the type I interferon receptor on target cells prevented the antiviral activity of T cell conditioned media against VSV-GFP. Therefore, the role of type I interferon signaling in the observed antiviral activity secreted by human $CD4^+$ T cells was further pursued. The antiviral effect of recombinant IFN-γ and TNF-α was reversed by neutralization of IFNAR2, indicating that this effect is dependent upon type I IFN signaling (Fig. 24A, $p < 0.05$, no antibody versus anti-IFNAR2). As noted previously, pre-treatment of THP-1 cells with neutralizing anti-IFNAR2 also abolished the antiviral effect of T cell conditioned media, indicating that the secreted activity requires this receptor (Fig. 24B, $p < 0.05$, no antibody versus anti-IFNAR2, all conditions). These data suggest the existence of a previously undescribed cytokine relay network whereby IFN-γ and TNF-α synergize to induce type I IFN signaling, which promotes viral clearance.

Either the T helper cells or the THP-1 target cells could have been a source of type I IFN. In either case, neutralization of soluble type I IFN would reverse the antiviral activity. Therefore, neutralizing anti-cytokine antibodies were employed to examine the identity of the type I IFN involved in the observed antiviral activity. As noted previously, pre-treatment of THP-1 target cells with anti-IFNAR2 reversed the antiviral activity of T cell conditioned media (Fig. 25A, condition 4, $p < 0.05$ versus T cell

conditioned media alone). However, addition of neutralizing anti-IFN-α, anti-IFN-β, or anti-IFN-ω antibodies to VSV-GFP infections failed to reverse the antiviral activity of T cell conditioned media, demonstrating that neither $CD4^+$ T cells nor infected THP-1 cells secrete type I IFNs (Fig. 25A, conditions 5-8). Addition of each antibody was sufficient to block 10-100 U/ml of its corresponding type I IFN activity in this assay (Fig. 25B), demonstrating that these antibodies possess the capacity to neutralize each specific type I interferon.

Human $CD4^+$ T cells were also assayed for secretion of IFN-α and IFN-β by ELISA. No detectable IFN-α or IFN-β protein was found in T cell conditioned media (Fig. 26A and B). Additionally, quantification of IFN-β secretion from untreated and T cell conditioned media-treated uninfected and VSV-GFP-infected THP-1 cells was performed, but no detectable secretion of IFN-β from these cells was observed (Fig. 26C and data not shown). Finally, both human $CD4^+$ T cells and THP-1 cells were examined for induction of mRNA transcripts for IFN-α1/13, IFN-β, IFN-ω, IFN-ε, and IFN-κ by quantitative real-time polymerase chain reaction (qPCR), but no transcripts were detected (Fig. 26D and data not shown). Taken together, these data demonstrated no detectable type I IFN production from either $CD4^+$ T cells or THP-1 target cells.

Given the lack of detectable type I IFN production in this assay, it was possible that the anti-IFNAR2 antibody was inhibiting the previously observed antiviral activity through pathways not involving the human IFNAR. Therefore, efforts were made to further verify the role of type I IFN signaling in the observed antiviral activity. A genetically modified human fibroblast cell line, U5A, was used. In this cell line, the gene

for the human IFNAR2 subunit has been ablated (67, 249, 250). VSV-GFP infection in these cells was compared to the parent cell line, 2fTGH, which expresses an intact IFNAR. In agreement with the results in THP-1 cells, treatment of VSV-GFP-infected wild-type 2fTGH cells with a combination of recombinant IFN-γ and TNF-α at the time of infection significantly reduced viral infection. This antiviral activity was reversed in the IFNAR2-deficient U5A cells (Fig. 27A, $p < 0.05$ versus 2fTGH), confirming that the antiviral activity of IFN-γ and TNF-α is dependent upon type I IFN signaling. Furthermore, infection of wild-type 2fTGH cells with VSV-GFP could be inhibited by treatment at the time of infection with recombinant human IFN-αA or T cell conditioned media. However, the antiviral effects of both IFN-αA and T cell conditioned media were severely attenuated in the IFNAR2-deficient U5A cells (Fig. 27A and B, $p < 0.05$ versus 2fTGH). These results confirm the involvement of type I IFN signaling in VSV-GFP inhibition by effector cytokines secreted by human $CD4^+$ T cells.

Discussion

The present study has demonstrated that secretion of IFN-γ and TNF-α represents a direct, cytokine-mediated antiviral activity of human $CD4^+$ T cells. Elevated secretion of these cytokines was directed by IL-12; there was no significant contribution, positive or negative, by IFN-α/β. A combination of IFN-γ and TNF-α produced by Th1 cells promotes antiviral responses by two distinct mechanisms (Fig. 28). First, IFN-γ and TNF-α can transmit an antiviral signal via a type I IFN-independent pathway, as in the case of HCV infection. In this case, the antiviral activity could be mediated by direct effects of IFN-γ and TNF-α or through the induction of another, non-IFN-α/β cytokine. Alternatively, the activity can be mediated through a cytokine relay network, as in the case of VSV infection, in which type I IFN signaling is required for the antiviral effect.

In agreement with these results, several other groups have shown that $CD4^+$ T cells have the capacity to promote viral clearance in vivo in a "helper-independent" fashion. For instance, clearance of Sendai virus, gammaherpesvirus (γHV68), or influenza A virus can proceed in a $CD4^+$ T cell-dependent fashion in the absence of B cells and $CD8^+$ T cells (192, 194-196, 198). Additionally, memory T helper cells generated against VSV in CTL-nonresponsive mice provide protection in an antibody-independent manner (172). In many cases, a deficiency in IFN-γ *in vivo* abolished the antiviral capacity of $CD4^+$ T cells (172, 195, 286), and adoptive transfer of an antigen-specific Th1 clone conferred protection from γHV68 infection (199). However, the target of IFN-γ was undetermined in these studies. Therefore, it was possible that viral

clearance could have been mediated by a population of innate cells, such as NK cells, which were activated in the presence of IFN-γ. Here, I definitively demonstrate for the first time that cytokines secreted by human $CD4^+$ T cells directly impact viral clearance from infected targets.

Furthermore, $CD4^+$ T cell-mediated control of cytomegalovirus (CMV) in salivary glands requires IFN-γ, but, paradoxically, treatment of virally infected mice with recombinant IFN-γ failed to clear the virus (197). The studies described above have demonstrated that both IFN-γ and TNF-α are required to achieve robust viral inhibition by Th1 cell-secreted factors. Therefore, *in vivo* treatment of CMV-infected animals with a combination of recombinant IFN-γ and TNF-α could promote viral clearance when neither cytokine alone possessed this activity.

Several groups have reported that TNF-α can induce secretion of IFN-β from target cells and that this IFN-β can synergize with IFN-γ for viral inhibition (181, 182, 184, 185, 287, 288). However, this effect relied upon pre-treatment of target cells with cytokines for 16-24 hours before *in vitro* infection. In contrast, the current work delineates an antiviral activity of IFN-γ and TNF-α which does not require pre-treatment of target cells. Thus, secretion of these cytokines by $CD4^+$ T cells at peripheral sites could have beneficial effects even after cells were already infected.

The antiviral activity of T cell-secreted IFN-γ and TNF-α was independent of type I IFN signaling in the case of HCV infection. Surprisingly, this activity was completely dependent upon the presence of a functional IFNAR in the case of VSV infection. It is currently unclear whether this phenomenon is specific to VSV or

represents a more general antiviral mechanism. However, it was noted during the course of these studies that Sendai virus, which blocks type I IFN signaling in infected cells, was also completely resistant to the antiviral effects of T cell conditioned media (Kristan A. Hagan and Michael Gale, Jr., unpublished observations).

While the observed antiviral effect of IFN-γ and TNF-α was dependent upon signaling through the IFNAR in the case of VSV, there was no induction of any known type I IFN genes in target cells. This further excludes induction of IFN-β by TNF-α as a mechanism for the observed antiviral effect. Many possible explanations exist for this unique antiviral effect of IFN-γ and TNF-α during VSV infection. For instance, IFN-γ and TNF-α may be inducing expression of a novel type I IFN gene in virally infected target cells. Several new type I IFN genes have been described in recent years (58-60); a more extensive search may reveal other, distantly related family members located within or even outside the IFN locus.

Alternatively, IFN-γ and TNF-α may synergize to directly activate IFNAR signaling via a mechanism such as receptor sharing in order to induce type I IFN-like effects in specialized situations. There are many known cases in which two or more unrelated receptors are activated by the same ligand. For instance, glial cell-derived neurotrophic factor (GDNF) signals through both the receptor tyrosine kinase RET and the Ig-domain-containing receptor NCAM (289). Alternately, a single receptor subunit can be shared among multiple distinct receptors, as in the case of the common gamma chain (γc) which is used for cytokine signaling (290). Consistent with the present *in vitro* studies, it is interesting to note that Müller et. al. demonstrated that the antiviral effects of

IFN-γ against VSV were impaired in murine cells lacking IFNAR expression (291). However, other IFN-γ signaling pathways were unaffected in cells from IFNAR$^{-/-}$ mice, and IFNγR$^{-/-}$ mice showed no defect in VSV clearance.

Many viruses encode intracellular or extracellular mechanisms to antagonize antiviral cytokine secretion and signaling by infected host cells. For instance, poxviruses encode soluble, secreted forms of the IFNAR, IFNγR, and TNFR which can neutralize host cytokines (85, 292, 293). A variety of viruses, including HCV, influenza A virus, and Sendai virus, also inhibit intracellular induction of type I IFN by blockade of the RIG-I pathway (87, 89-91). In such cases, exogenously delivered cytokines from CD4^{+} T cells could provide alternative pathways to overcome these blocks and promote pathogen clearance in a noncytopathic manner.

IFN-α is widely used to treat HCV infections, but many patients fail to respond to this therapy. HCV and other flaviviruses, such as West Nile Virus, inhibit IFNAR signal transduction in target cells through inactivation of downstream signaling intermediates (86, 88, 92). In accordance with previous reports, I have demonstrated that IFN-γ possessed substantial antiviral activity against HCV (283-285). However, Frese et. al. found no role for TNF-α, either alone or in combination with IFN-γ, in inhibition of HCV replication (284). In contrast, I observed cooperation between IFN-γ and TNF-α in suppressing HCV NS5A protein expression. Furthermore, these data show that IFN-γ and TNF-α inhibit HCV infection by a type I IFN-independent mechanism. Therefore, Th1 responses generated during infections with this virus could represent an important alternative mechanism for pathogen clearance when type I IFN is ineffective.

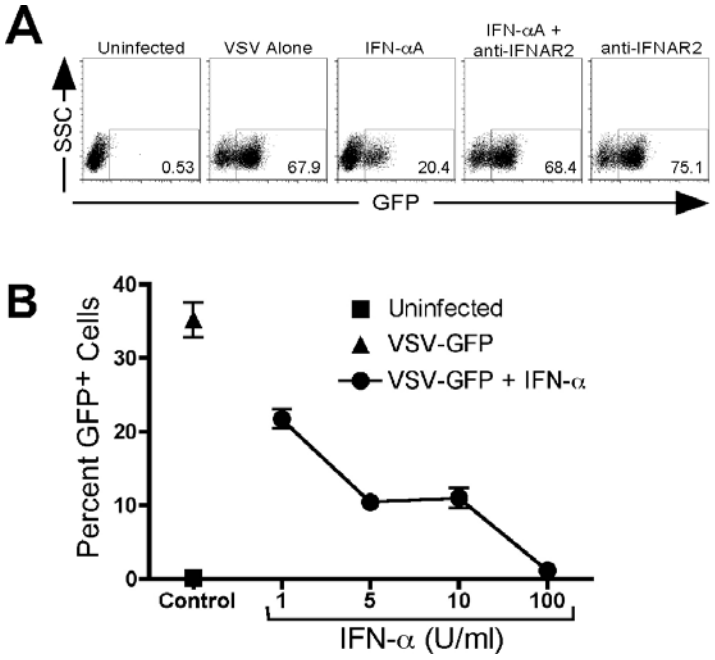

Figure 16. Optimization of VSV-GFP infection of THP-1 monocytes. (A) THP-1 cells were infected for 16 hours with VSV-GFP. GFP expression was analyzed by flow cytometry. Representative FACS plots showing THP-1 cells left uninfected or infected with VSV-GFP in the presence or absence of 100 U/ml rhIFN-αA and/or a neutralizing anti-hIFNAR2. (B) THP-1 cells were left uninfected (square) or were infected for 16 hours with VSV-GFP in the absence (triangle) or presence (circles) of increasing concentrations of rhIFN-αA. GFP expression was analyzed by flow cytometry. This work was performed by Loderick A. Matthews.

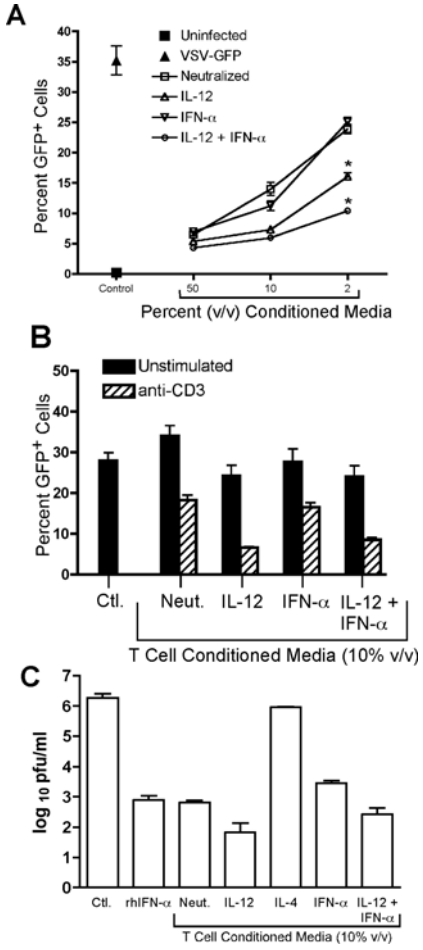

Figure 17. Soluble factors secreted by human CD4$^+$ T cells inhibit VSV infection. (A) THP-1 cells were left uninfected (■) or were infected for 16 hours with VSV-GFP. Cells were treated at the time of infection with either media alone (▲) or with increasing doses of TCM from T cells polarized with the following conditions: Neutralized, □; IL-12, Δ; IFN-αA, ∇; or IL-12 + IFN-αA, ○. GFP expression was analyzed by flow cytometry. *, $p < 0.05$ versus neutralized. This work was performed by Loderick A. Matthews. (B) THP-1 cells were infected for 16 hours with VSV-GFP in the absence or presence of TCM from T cells which were left unstimulated (black bars) or were restimulated on day 7 for 24 hours with plate-bound anti-CD3 (hatched bars). GFP expression was analyzed by flow cytometry. (C) THP-1 cells were cultured for 24 hours in the absence or presence of 100 U/ml rhIFN-αA or TCM derived from cells polarized as indicated in the figure. Cells were then washed and then infected for an additional 24 hours with VSV-GFP, and viral replication was quantified by plaque assay.

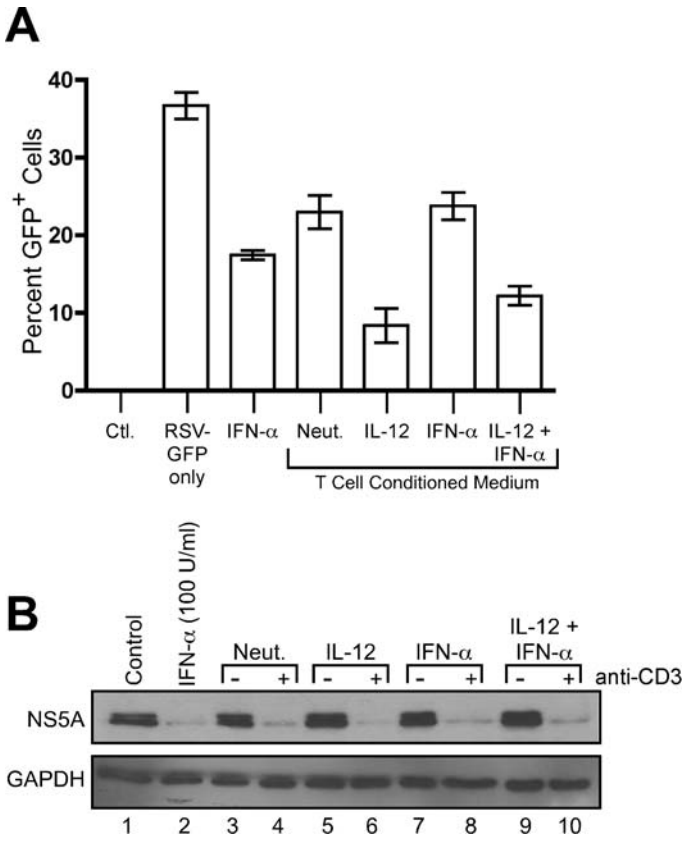

Figure 18. Inhibition of RSV and HCV replication factors secreted by human CD4$^+$ T cells. (A) HeLa cells were infected for 72 hours with RSV-GFP in the absence or presence of 100 U/ml rhIFN-αA or 10% (v/v) TCM as indicated. GFP expression was analyzed by flow cytometry. This work was performed by Gagan Bajwa in the laboratory of Dr. Michelle A. Gill. (B) A7 replicon cells were incubated for 24 hours in the absence (lane 1) or presence of 100 U/ml rhIFN-αA (lane 2) or 5% (v/v) TCM (lanes 3-10) as indicated. Cell lysates were prepared, and Western blotting was performed using antibodies against HCV NS5A and human GAPDH. This work was performed by Kristan A. Hagan in the laboratory of Dr. Michael Gale, Jr.

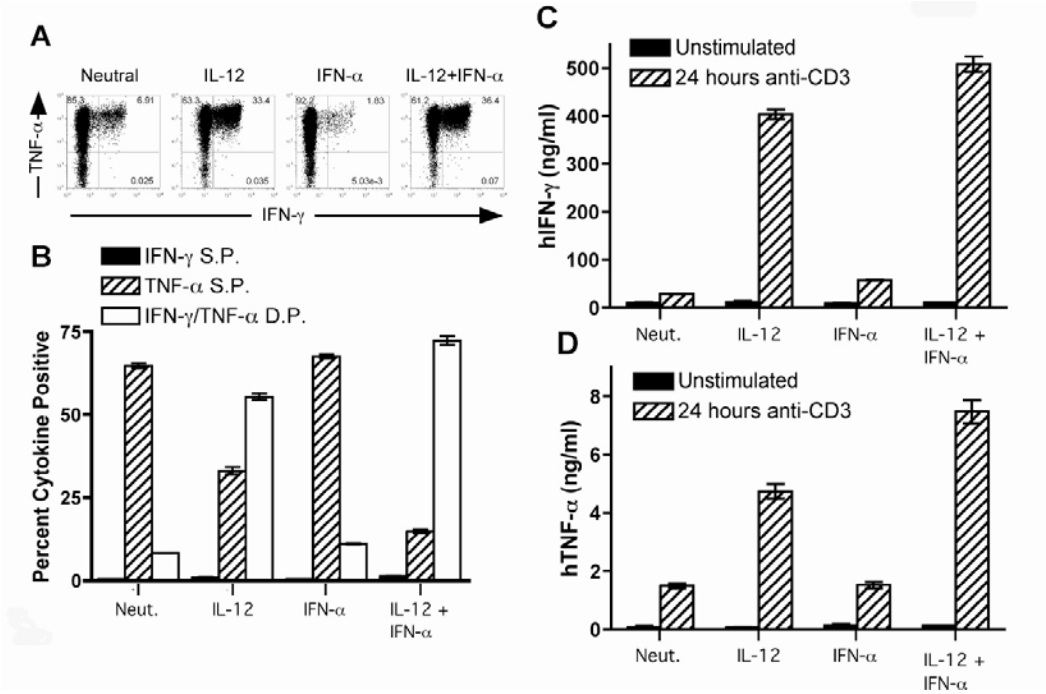

Figure 19. IL-12 promotes IFN-γ and TNF-α secretion by human CD4$^+$ T cells. (A – D) Naïve (CD45RA$^+$ CD4$^+$) T cells were sorted from peripheral blood of healthy donors and activated for 7 (A, C – D) or 14 (B) days with plate-bound αCD3 and αCD28 in the presence of cytokines and neutralizing antibodies as indicated. (A – B) Cells were restimulated for 4 hours with PMA and ionomycin, and flow cytometry was performed using antibodies against human IFN-γ and human TNF-α. Black bars, percent IFN-γ single positive cells; hatched bars, percent TNF-α single positive cells; open bars, percent IFN-γ/TNF-α double positive cells. (C and D) Cells were washed and left unstimulated (open bars) or restimulated for 24 hours with plate-bound anti-CD3 (black bars). Supernatants were harvested, and ELISAs were performed for hIFN-γ (C) or hTNF-α (D).

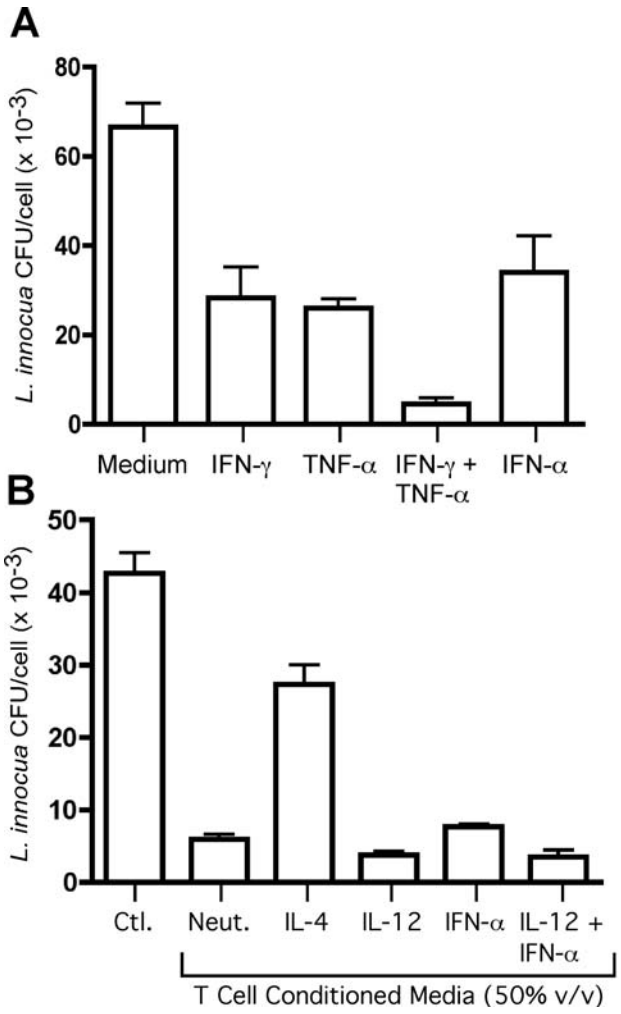

Figure 20. **Inhibition of *Listeria* replication by human CD4$^+$ T cells secreting IFN-γ and TNF-α.** THP-1 cells were infected with *Listeria innocua* in the absence or presence of recombinant cytokines (A) or 50% (v/v) T cell conditioned media (B) for 16 hours. Cell monolayers were harvested, and *L. innocua* was quantified by colony-forming assay from cell lysates. This work was performed by Loderick A. Matthews.

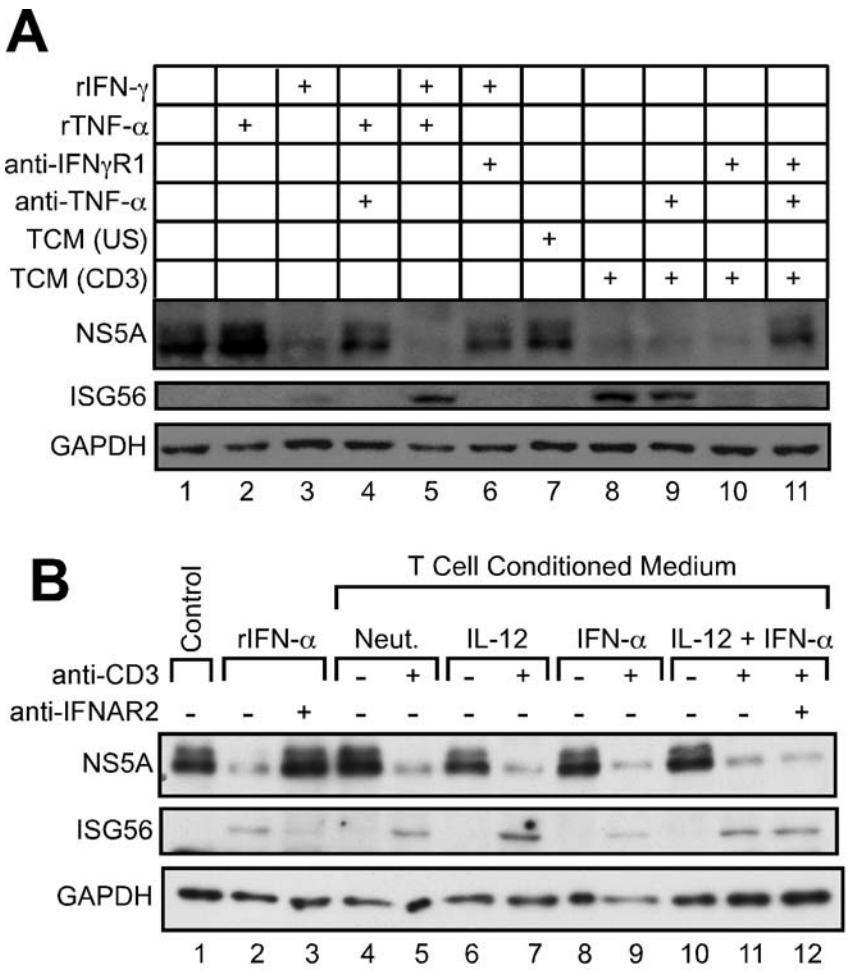

Figure 21. IFN-γ and TNF-α secreted by human CD4$^+$ T cells exert antiviral activity against HCV infection. (A) A7 replicon cells were incubated for 24 hours in the absence (lane 1) or presence (lanes 2-11) of cytokines, neutralizing anti-cytokine antibodies, and 5% (v/v) T cell conditioned media from IL-12 + IFN-α activated T cells as indicated. Cell lysates were prepared, and Western blotting was performed using antibodies against HCV NS5A, human ISG56, and human GAPDH. This work was performed by Kristan A. Hagan in the laboratory of Michael Gale, Jr. (B) A7 replicon cells were incubated for 24 hours in the absence (lane 1) or presence of 100 U/ml rhIFN-αA (lanes 2-3) or 5% (v/v) T cell conditioned media (lanes 4-12), in the absence or presence of anti-hIFNAR2, as indicated. Cell lysates were prepared, and Western blotting was performed using antibodies against HCV NS5A, human ISG56, and human GAPDH. This work was performed by Kristan A. Hagan in the laboratory of Dr. Michael Gale, Jr.

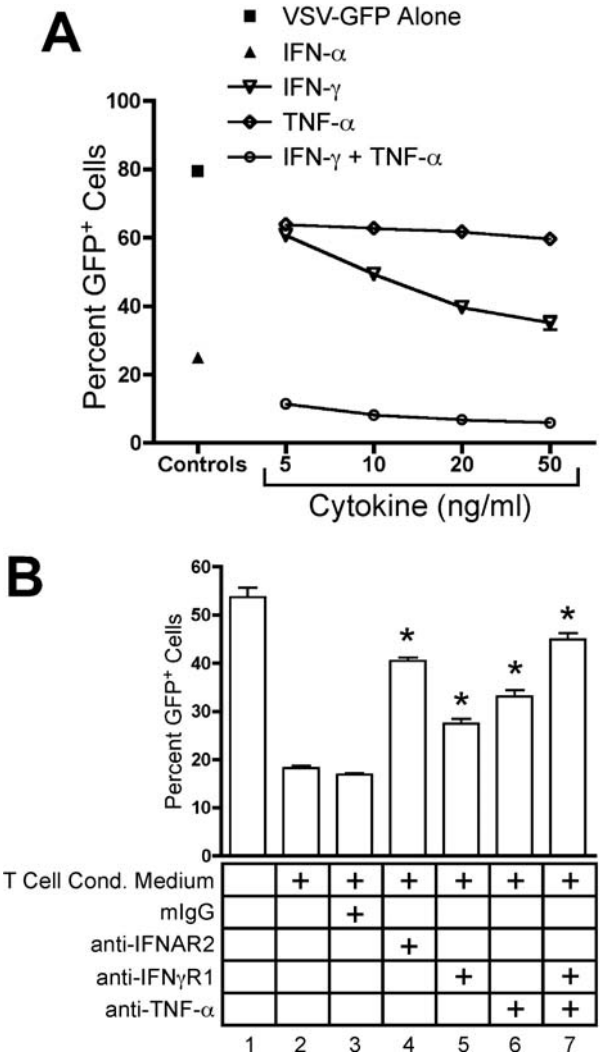

Figure 22. IFN-γ and TNF-α secreted by CD4$^+$ T cells inhibit VSV infection. THP-1 cells were infected for 16 hours with VSV-GFP. GFP expression was analyzed by flow cytometry. (A) THP-1 cells were infected in the absence (■) or presence of 100 U/ml rhIFN-αA (▲) or increasing concentrations of rhIFN-γ (▽), rhTNF-α (◇), or rhIFN-γ + rhTNF-α (○) as indicated. (B) THP-1 cells were infected in the absence (1) or presence (2-7) of 10% (v/v) T cell conditioned media from IL-12 + IFN-α activated T cells in the absence (2) or presence of mouse IgG_1 isotype control antibody (3), anti-hIFNAR2 (4), anti-hIFNγR1 (5), anti-hTNF-α (6), or a combination of anti-hIFNγR1 and anti-hTNF-α (7).

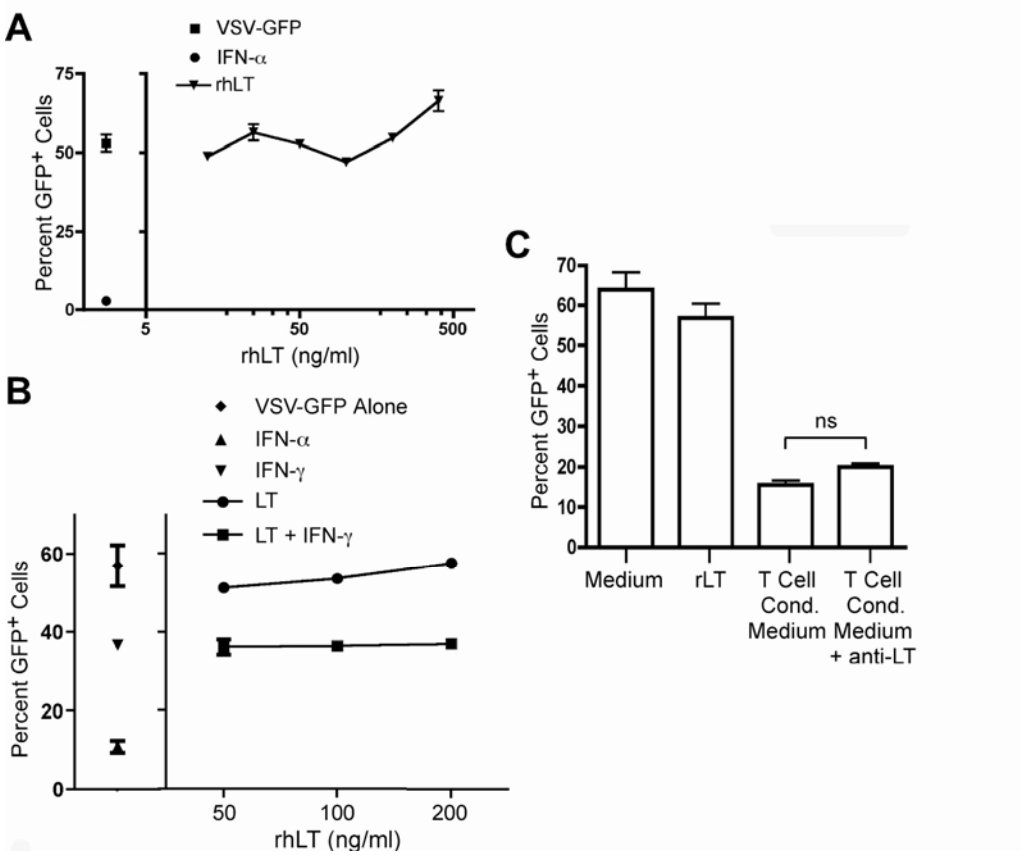

Figure 23. Lymphotoxin does not participate in the antiviral activity of human CD4$^+$ T cells. THP-1 cells were infected for 16 hours with VSV-GFP. GFP expression was analyzed by flow cytometry. (A) THP-1 cells were infected in the absence (■) or presence of 100 U/ml rhIFN-αA (●) or increasing concentrations of rhLT$\alpha_1\beta_2$ (▼) as indicated. (B) THP-1 cells were infected in the absence (♦) or presence of 100 U/ml rhIFN-αA (▲), 2.5 ng/ml rhIFN-γ (▼), or increasing concentrations of rhLT$\alpha_1\beta_2$ in the absence (●) or presence (■) of 2.5 ng/ml rhIFN-γ, as indicated. (C) THP-1 cells were infected in the absence or presence of 10 ng/ml rhLT$\alpha_1\beta_2$ or T cell conditioned media from IL-12 + IFN-α activated T cells in the absence or presence of neutralizing anti-hLT.

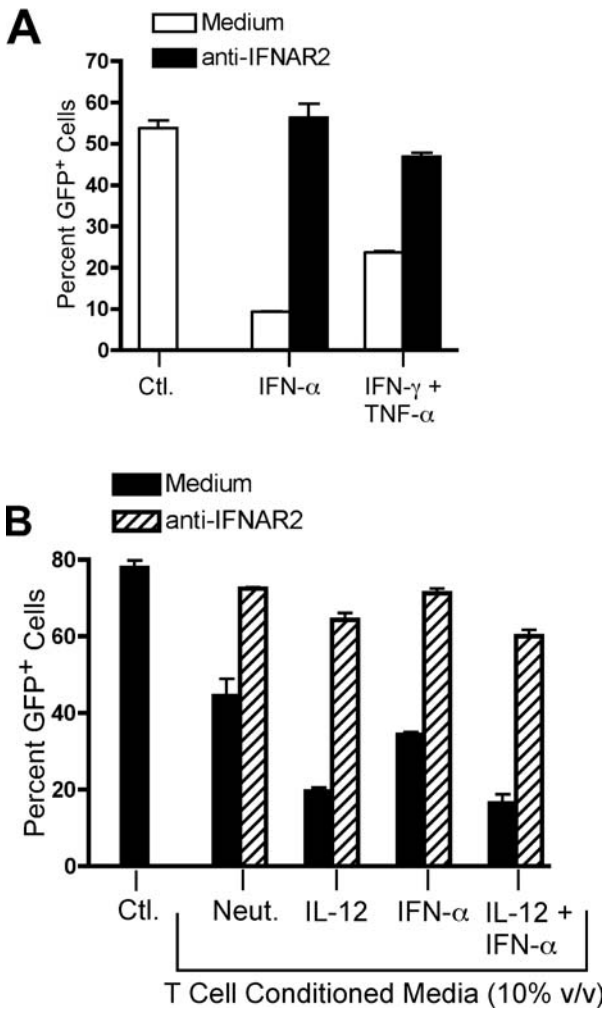

Figure 24. IFN-γ- and TNF-α-mediated antiviral activity requires availability of the IFNAR. THP-1 cells were infected for 16 hours with VSV-GFP. GFP expression was analyzed by flow cytometry. (A) THP-1 cells were infected in the absence or presence of rhIFN-αA or a combination of rhIFN-γ and rhTNF-α in the absence (open bars) or presence (black bars) of anti-hIFNAR2. *, $p < 0.05$, two-way ANOVA. (B) THP-1 cells were infected in the absence or presence of 10% (v/v) T cell conditioned media as indicated in the absence (black bars) or presence (hatched bars) of anti-hIFNAR2.

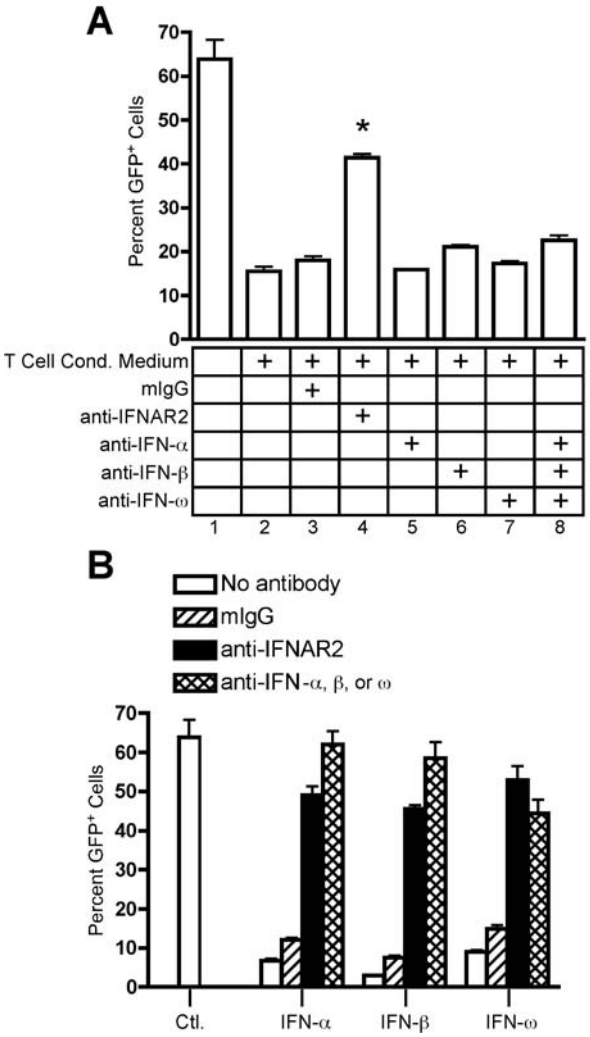

Figure 25. Neutralization of type I IFN fails to inhibit the antiviral activity of human CD4$^+$ T cells. THP-1 cells were infected for 16 hours with VSV-GFP. GFP expression was analyzed by flow cytometry. (A) THP-1 cells were infected in the absence (1) or presence (2-8) of 10% (v/v) TCM from IL-12 + IFN-α activated T cells in the absence (2) or presence of mouse IgG_1 isotype control antibody (3), αhIFNAR2 (4), anti-hIFN-α (5), anti-hIFN-β (6), anti-hIFN-ω (7), or a combination of anti-hIFN-α, anti-hIFN-β, and anti-hIFN-ω (8). (B) THP-1 cells were infected in the absence or presence of 100 U/ml rhIFN-αA or 100 U/ml rhIFN-β or 10 U/ml rhIFN-ω in the absence (open bars) or presence of mouse IgG_1 isotype control antibody (hatched bars), anti-hIFNAR2 antibody (black bars), or anti-hIFN-α antibody or anti-hIFN-β antibody or anti-hIFN-ω antibody (double hatched bars) as indicated.

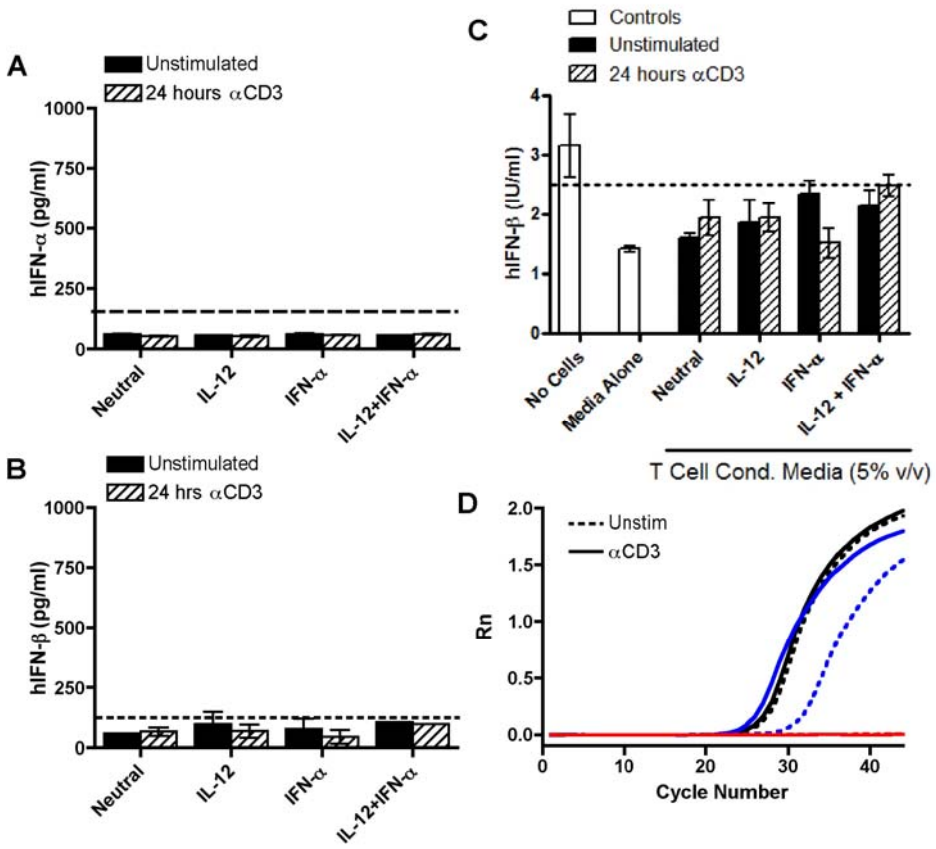

Figure 26. Human CD4$^+$ T cells and VSV-GFP-infected THP-1 cells do not secrete type I IFN. (A – B) Human naïve CD4$^+$ T cells were activated for 7 days with plate-bound αCD3 + αCD28 in the presence of cytokines and neutralizing antibodies as indicated. On day 7, cells were washed and left unstimulated (black bars) or restimulated with plate-bound anti-CD3 for 24 hours (hatched bars), and supernatants were subjected to ELISA for IFN-α (A) or IFN-β (B). Dashed line indicates the lower limit of detection of the assay. (C) THP-1 cells were infected for 16 hours with VSV-GFP in the absence (open bars) or presence of TCM from human CD4$^+$ T cells left unstimulated (black bars) or restimulated for 24 hours with anti-CD3 (hatched bars). Following infection, supernatants from infected THP-1 cells were subjected to ELISA for IFN-β. Media in which no cells were cultured was used as a negative control (No cells). Dashed line indicates the lower limit of detection of the assay. (D) CD4$^+$ T cells differentiated as above were left unstimulated (dashed lines) or were restimulated with plate-bound anti-CD3 for 2 hours (solid lines). Total RNA was harvested and reverse transcribed, and quantitative real-time PCR was performed using primers directed against IFN-γ (blue), IFN-ε (red), or GAPDH (black).

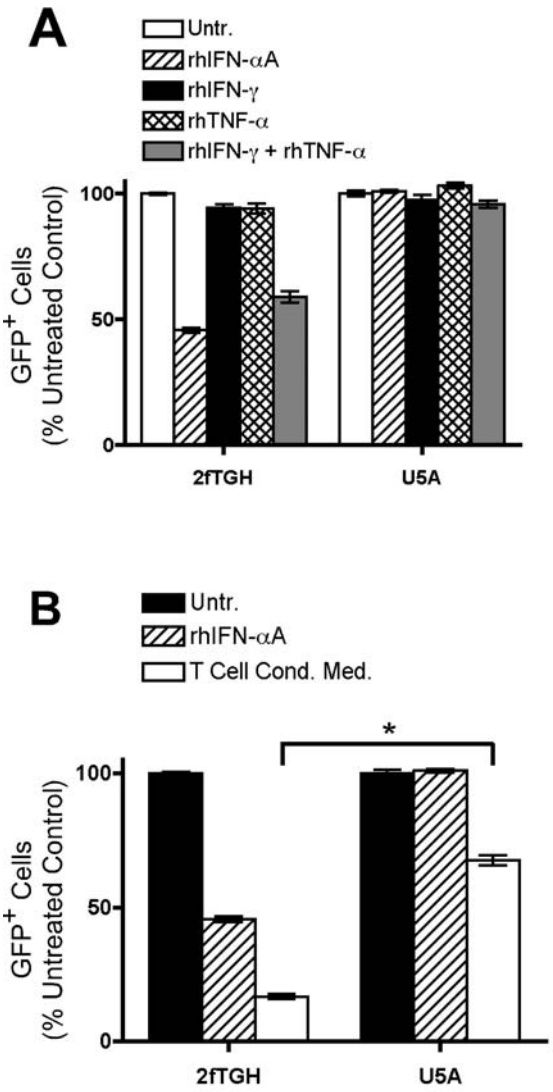

Figure 27. IFN-γ and TNF-α signal through a cytokine relay network involving the type I IFN receptor. Wild-type 2fTGH cells and hIFNAR2-deficient U5A cells were infected for 16 hours with VSV-GFP. GFP expression was analyzed by flow cytometry. (A) 2fTGH and U5A cells were infected in the absence (open bars) or presence of 100 U/ml rhIFN-αA (hatched bars), 2.5 ng/ml rhIFN-γ (black bars), 2.5 ng/ml rhTNF-α (double hatched bars), or a combination of 2.5 ng/ml rhIFN-γ and 2.5 ng/ml rhTNF-α (gray bars). (B) 2fTGH and U5A cells were infected in the absence (black bars) or presence of 100 U/ml rhIFN-αA (hatched bars) or 10% (v/v) TCM generated from IL-12 + IFN-α activated $hCD4^+$ T cells (white bars). *, $p < 0.05$, one-way ANOVA.

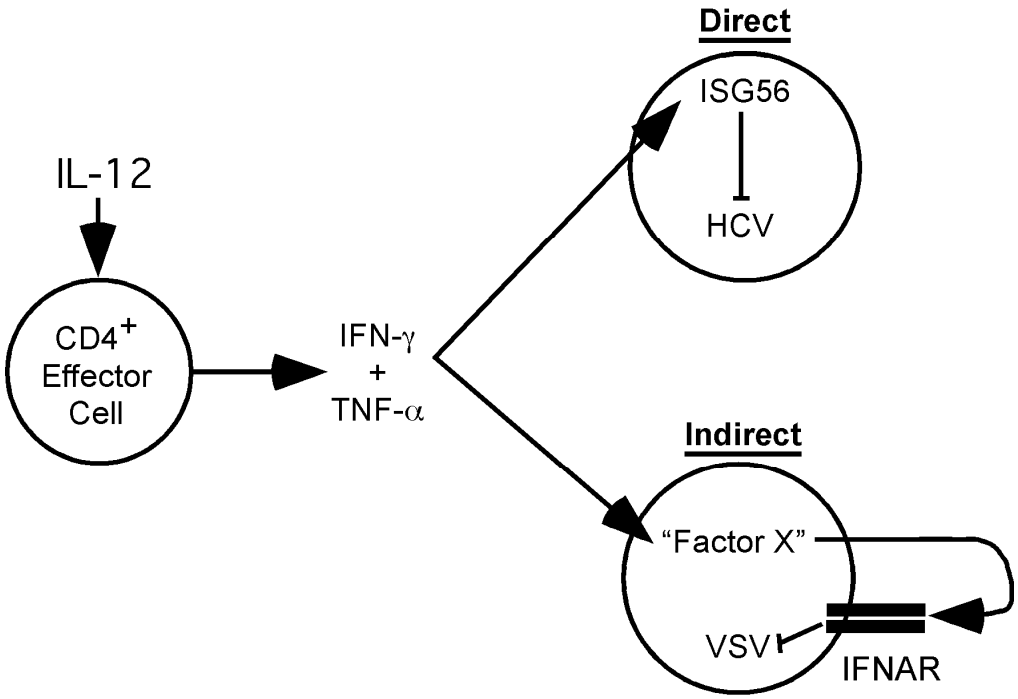

Figure 28. Proposed model for the antiviral activity of human CD4$^+$ T cells. IL-12 promotes the generation of a population of human CD4$^+$ T cells which secrete IFN-γ and TNF-α. These two cytokines show a synergistic antiviral activity which can have either a direct or an indirect effect on infected target cells. In the case of HCV infection, IFN-γ and TNF-α directly inhibit expression of viral proteins independently of type I IFN signaling. This activity likely involves the induction of ISG56, a host protein with known inhibitory effects on HCV. In contrast, in the case of VSV infection, IFN-γ and TNF-α indirectly inhibit infection through a mechanism which requires type I IFN signaling. This could be via induction of an unknown factor, such as a novel type I IFN gene, in target cells or through a receptor sharing mechanism.

CHAPTER V

INDUCTION OF GENE EXPRESSION IN NAÏVE HUMAN CD4$^+$ T CELLS BY IL-12, IL-4, AND TYPE I IFN

Introduction

The differentiation of distinct cell lineages within an organism is the result of a coordinated program of transcriptional events within the cell. For instance, activation of naïve CD4$^+$ T cells by TCR ligation and CD28 costimulation results in the coordinate up- and downregulation of a large panel of mRNA transcripts (294-296). These gene expression changes culminate in maturation and clonal expansion of naïve T helper cells into functional effectors (99, 100, 297). Furthermore, stimulation of developing CD4$^+$ T cells with either IL-12 or IL-4 promotes polarization to the Th1 or Th2 phenotypes, respectively (261). The generation of these functional subsets is associated with further divergent patterns of gene expression, including the induction of the lineage-specific transcription factors T-bet and GATA-3 and cytokines IFN-γ and IL-4 (107, 298). Finally, further developmental changes result in the generation of both effector cells, which secrete cytokines and directly participate in the primary response to infection, and memory cells, which provide long-term protection against secondary infection (200, 201). While the delineation of small numbers of subset-associated genes has been important for an understanding of the functions of these cells during infection, it is likely

that a number of other genes which play a role during $CD4^+$ T cell phenotype development have yet to be uncovered.

Gene microarray technology was first developed in the 1990's and has revolutionized the study of biological development. Previously, investigators were limited to the simultaneous assessment of only a handful of genes in developing cell populations using techniques such as Northern blotting and polymerase chain reaction (PCR). With the advent of microarray analysis, it is now possible to examine genome-wide transcriptional changes in a comprehensive and unbiased manner. Furthermore, newer technologies have provided increasingly reliable information, facilitating the discovery of novel pathways involved in cell subset development. Initial microarray technology used as probes complementary DNAs (cDNAs) or synthesized oligonucleotides spotted onto glass slides or nylon filters (299). This technology allowed analysis of broad patterns of transcriptional changes by comparing the binding of two fluorescently labeled samples. However, these spotted arrays were limited by high inter-array and intra-array variability as well as technical problems such as flawed spots (300). Newer technologies involving *in situ* generation of oligonucleotides probes on either nylon filters (301) or latex beads (302) have largely overcome these limitations, making it possible to generate highly reliable, reproducible analyses of gene expression.

A small number of studies have examined global gene expression changes in human and murine peripheral blood subsets in response to cytokines and developmental signals. It has been established that T cell receptor (TCR) ligation alone induces substantial transcriptional changes in human $CD4^+$ T cells (294). Additionally, the gene expression patterns of Th1 and Th2 cells differentiated *in vitro* or *in vivo* have been

examined, and these studies have confirmed the expression of genes known to be involved in T helper subset development as well as identifying a number of novel genes with as-yet unknown roles in Th1 and Th2 differentiation and effector functions (264, 294, 303-306). The effects of type I IFNs on the gene expression patterns of human $CD4^+$ T cells have also been examined by microarray analysis, and unique gene expression patterns have been identified (269, 307, 308). However, these studies have been limited by the use of heterogeneous $CD4^+$ populations from peripheral blood or cord blood, which are likely to contain multiple subpopulations with their own distinct tendencies for gene expression and responses to cytokine signals. To date, there has not been a genome-wide analysis of transcriptional changes in developing naïve human $CD4^+$ T cells in response to defined cytokine signals. I therefore used Illumina BeadArray microarray technology to examine the gene expression patterns of naïve human $CD4^+$ T cells which were differentiated in the presence of the innate cytokines IL-12, IL-4, IFN-α, and IFN-β.

Results

Substantial similarity in gene expression patterns induced by innate cytokines

While a number of microarray studies have examined the effects of innate cytokines on gene expression in $CD4^+$ T cells, none have investigated the effects of these cytokines in isolation on purified naïve cells (264, 294, 303-306). Therefore, human naive ($CD45RA^+$) $CD4^+$ T cells were isolated from a healthy subject and activated with plate-bound anti-CD3 and anti-CD28 in the presence of IL-2 and cytokines and/or neutralizing anti-cytokine antibodies for 2 consecutive weeks. The use of neutralizing antibodies facilitated assessment of the individual effects of IL-12, IL-4, IFN-α, IFN-β, and combinations of IL-12 with either IFN-α or IFN-β. On day 14, cells were washed and either left unstimulated or restimulated for 2 hours with plate-bound anti-CD3. RNA was prepared and used for Illumina BeadArray analysis. The resulting data was subjected to cubic spline normalization, and heat maps were generated by two-way hierarchical clustering analysis of genes for which expression was significantly different from the unstimulated neutral sample at a significance level of $p = 0.05$ or better. In these heat maps, a red color indicates upregulation of a gene, whereas a green color indicates downregulation.

The overall expression pattern of genes was surprisingly similar across a majority of the samples (Fig. 29). Furthermore, the cluster analysis revealed that the samples clustered into two major groups, with one group containing the unstimulated samples and the other group containing the samples which were restimulated with plate-bound anti-

CD3. The most predominant gene clusters were those in which expression was modulated by TCR restimulation (data not shown). Furthermore, a number of genes were differentially regulated by IL-12 or type I IFN, either alone or in combination (Fig. 30). However, many of these genes have no known function, making it difficult to assess the functional relevance of this gene expression pattern.

Expression of subset-specific genes by human CD4$^+$ T cells

A small number of publications have reported groups of genes whose expression is differentially regulated by IL-12 and IL-4. In order to assess the validity of the present data, expression of the genes reported by Lund et. al. was examined in this dataset (Fig. 31) (264). While there was not a strong pattern of upregulation of reportedly Th1-associated genes in the samples which had been differentiated in the presence of IL-12, each of these samples did show induction of one or more previously established markers of the Th1 phenotype, including IFN-γ, IL-8, and granzyme A (Fig. 31A) (153, 306). However, expression of T-bet, the master regulator of Th1 commitment (107), was not detected above background in any sample (data not shown). In contrast, differentiation in the presence of IL-4 upregulated expression of GATA-3 and SOCS1, as has been reported previously (298, 309), while the expression of many other reported Th2 genes was less strongly associated with IL-4 stimulation (Fig. 31B).

In order to further verify the effects of IL-12 and IL-4, genes for which the gene ontology description contained the words “cytokine” or “chemokine” were examined (Fig. 32). In this case, as expected, the samples were clustered based upon secondary

stimulation. Elevated expression for secreted cytokines and chemokines was predominantly seen within the cluster of samples which were restimulated with plate-bound anti-CD3, whereas these samples showed suppression of a number of cytokine and chemokine receptors. Specifically, cells differentiated in the presence of IL-4 upregulated IL-5 and IL-13 expression upon restimulation, as expected (153). Also, both the unstimulated and anti-CD3-restimulated IL-4-activated samples displayed elevated expression of CCR4, a chemokine receptor which has been associated with the Th2 phenotype (310-313). In accordance with previously published reports, cells activated in the presence of IL-12 showed elevated expression of transcripts for IFN-γ, IL-8, and CXCR3 (311-313). Intriguingly, cells differentiated in the presence of both IL-12 and IFN-α/β, but not IL-12 alone, exhibited induction of IL-2, IL-3, and IL-10. While IL-10 has been shown to be induced by IFN-α, it has generally been associated with Th2 or regulatory T cell responses rather than with the Th1 phenotype (268, 314). IL-2 and IL-3 are growth factors for T cells and hematopoietic stem cells, respectively (315, 316); the significance of the expression of these three cytokine genes by $CD4^+$ T cells in response to IL-12 + type I IFN is not immediately clear.

A few recent reports have also detailed differential expression of a number of genes between memory $CD4^+$ T cells with a central memory ($CD45RA^-$ $CCR7^{hi}$) or effector memory ($CD45RA^-$ $CCR7^{lo}$) phenotype (317, 318). These two subsets of memory $CD4^+$ T cells have been postulated to perform different functions in the maintenance of long-term protection against secondary infection, but the signals which regulate their development are currently unknown (202). In order to assess a potential role for innate cytokines in promoting central or effector memory phenotypes, expression

of reportedly central memory or effector memory-associated genes was examined (Fig. 33) (317). Unfortunately, no substantial patterns of gene expression were noted among the various cytokine activation conditions examined.

Finally, the expression of ISGs in response to type I IFN activation was assessed by examining genes for which the gene definition contained "interferon" (Fig. 34). In this case, the majority of the samples which were differentiated in the presence of IFN-α or IFN-β clustered together, and these samples showed a predominant induction of a number of known ISGs, including G1P2/ISG-15, G1P3/IFI-6-16, Mx1, IRF-7, IFI44, and several members of a family of interferon-induced proteins with tetratricopeptide repeats (Fig. 6). While the functions of many of these proteins are currently unknown, they have been reported to be induced by type I IFNs in a variety of cell types and therefore provide reliable markers for successful IFN-α/β stimulation of these cells (269, 307, 308).

Transcription factor expression regulated by IL-12 and type I IFN

Transcription factor expression plays a crucial role in the generation of T helper cell subsets (107, 298). In order to more closely examine the roles of innate cytokines in directing $CD4^+$ T cell differentiation, the expression patterns of transcription factors were examined by two-way hierarchical cluster analysis. For this analysis, only the unstimulated samples were used because secondary TCR activation can alter the patterns of transcription factor expression and thereby mask the effects of cytokines. As noted previously, the gene expression patterns were largely similar among all cytokine activation conditions (Fig. 35). The samples formed two clusters: the IL-12+IFN-α and

IL-12+IFN-β conditions were more closely related to the neutralized control, whereas individual stimulation with IL-12, IL-4, IFN-α, or IFN-β yielded similar results.

A small number of genes was observed to be induced by IL-12 and/or type I IFN but not by IL-4 (Fig. 36). Interestingly, IL-12 induced the expression of CREBBP/CBP and Jun/AP-1, two essential transcription factors involved in a number of immune signaling events, including TCR signal transduction and cytokine secretion (110, 297, 319, 320). Conversely, IFN-α/β upregulated the Ets1, ATF4, and Foxo1a transcription factors, all of which are known to be involved in apoptosis (321-323). This result is not surprising considering the known role of type I IFNs in promoting cell death in response to viral infections (83, 84). Finally, in addition to inducing CREBBP/CBP, Foxo1a, and Jun/AP-1, a combination of IL-12 and type I IFN also induced the expression of RORα, a transcription factor which has been shown to be involved in differentiation of the Th17 phenotype (324). No other markers of Th17 cells were upregulated in response to this combination of cytokines; hence, the significance of RORα induction is unclear.

Discussion

Previous microarray studies had not examined the individual effects of innate cytokines on the differentiation of naïve human $CD4^+$ T cells. I have found that the gene expression patterns induced by IL-12, IL-4, IFN-α/β, or a combination of IL-12 and IFN-α/β were largely similar. However, minor differences in gene expression were observed which are in agreement with published reports detailing the transcriptional effects of these cytokines on $CD4^+$ T cells.

This study is unfortunately limited by the fact that the analysis was only conducted on a single human donor. Humans are a highly genetically diverse population, and substantial number of subjects must often be analyzed for gene expression before a consistent pattern can be elucidated. For instance, previously reported microarray studies using primary human $CD4^+$ T cells have made use of samples from as many as 13 different individuals (308). Because of this, the results reported here will need to be validated by further microarray studies or by quantitative real-time PCR or Northern blot analysis of individual transcripts from cells taken from multiple human donors.

In spite of the limitations of this study, several patterns of gene expression were observed which validate the results obtained. First, cells activated in the presence of IL-12 showed elevated expression of transcripts for IFN-γ, IL-8, granzyme A, and CXCR3, all of which have previously been reported to be associated with the Th1 phenotype. Conversely, cells stimulated with IL-4 upregulated expression of GATA-3, SOCS1, IL-5, IL-13, and CCR4, which are known markers of Th2 cells. Finally, cells which were treated with type I IFN showed a concerted induction of a number of known ISGs. Taken

together, these data indicate that the naïve human $CD4^+$ T cells used for this experiment responded appropriately to innate cytokine signals. While the pattern of gene expression in response to a combination of IL-12 and type I IFN is somewhat puzzling, the joint effects of these two cytokines have not previously been addressed. Therefore, it is possible that IL-12 and type I IFN modulate one another's effects of T helper cell differentiation and may even synergize to induce the expression of genes not upregulated by either cytokine alone.

It was surprising that so few genes were differentially regulated between IL-12, IL-4, and type I IFNs. IL-12 and IL-4, for example, are known to promote the generation of the highly distinct Th1 and Th2 phenotypes, which have divergent functions *in vivo* (261). Furthermore, while IL-12 induces Th1 commitment in naïve $CD4^+$ T cells, type I IFNs fail to do so despite similar induction of STAT4 phosphorylation and T-bet expression early in the process of differentiation (254). Hence, it would be expected that differences between these cytokines in the induction of other transcriptional regulators or signaling molecules might play a role in the established differences in the regulation of T helper fate decisions. While a small number of transcriptional regulators were found to be differentially expressed in cells activated in the presence of IL-12, IL-4, and/or type I IFNs, none of these factors provides a good clue as to the signaling differences which might account for $CD4^+$ T cell subset development.

Fully polarized $CD4^+$ T cells are a heterogeneous population, both in vitro and in vivo. For instance, when naïve human $CD4^+$ T cells are activated in the presence of IL-12, only 10-70% of these cells develop the capacity to produce IFN-γ upon restimulation after one week in culture (254). Thus, it is possible that cytokine stimulation of naïve T

helper cells only promotes transcriptional changes in a subpopulation of cells. Since these analyses were conducted on bulk *in vitro* cultures, subtle changes in gene expression in only a percentage of cells would not have been highly evident. Therefore, methods such as multicolor flow cytometry and live cell sorting might provide a more accurate depiction of gene expression changes induced by innate cytokines in developing human $CD4^+$ T cells. These methods will allow analysis of phenotypic and functional changes in subpopulations of T helper cells in response to various cytokine signals.

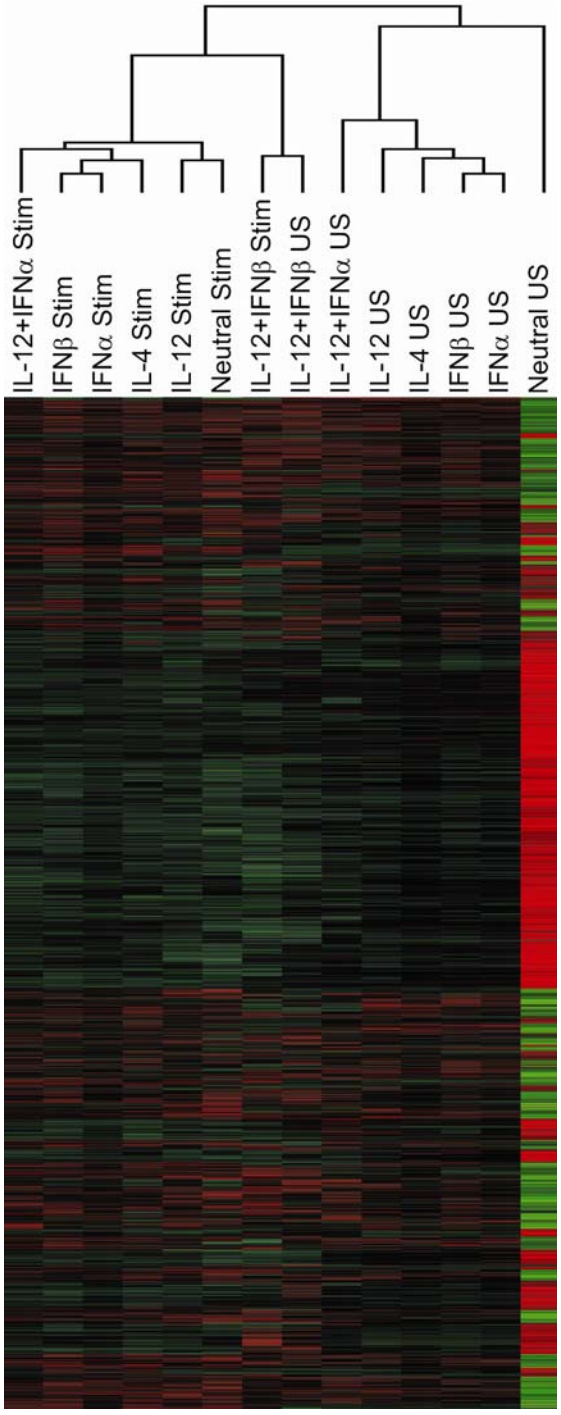

Figure 29. Genes significantly different from Neutral Unstimulated. US, unstimulated; Stim, 2 hours anti-CD3.

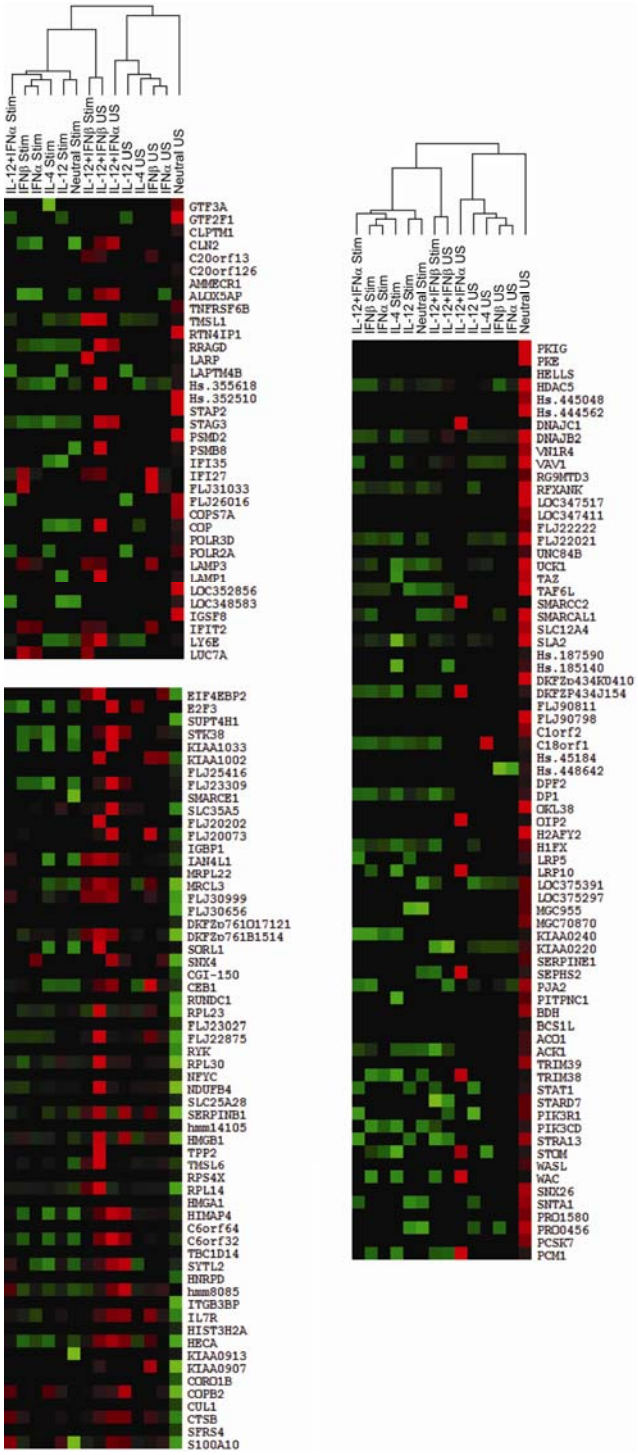

Figure 30. Clusters of genes differentially regulated by IL-12 and IFN-α.

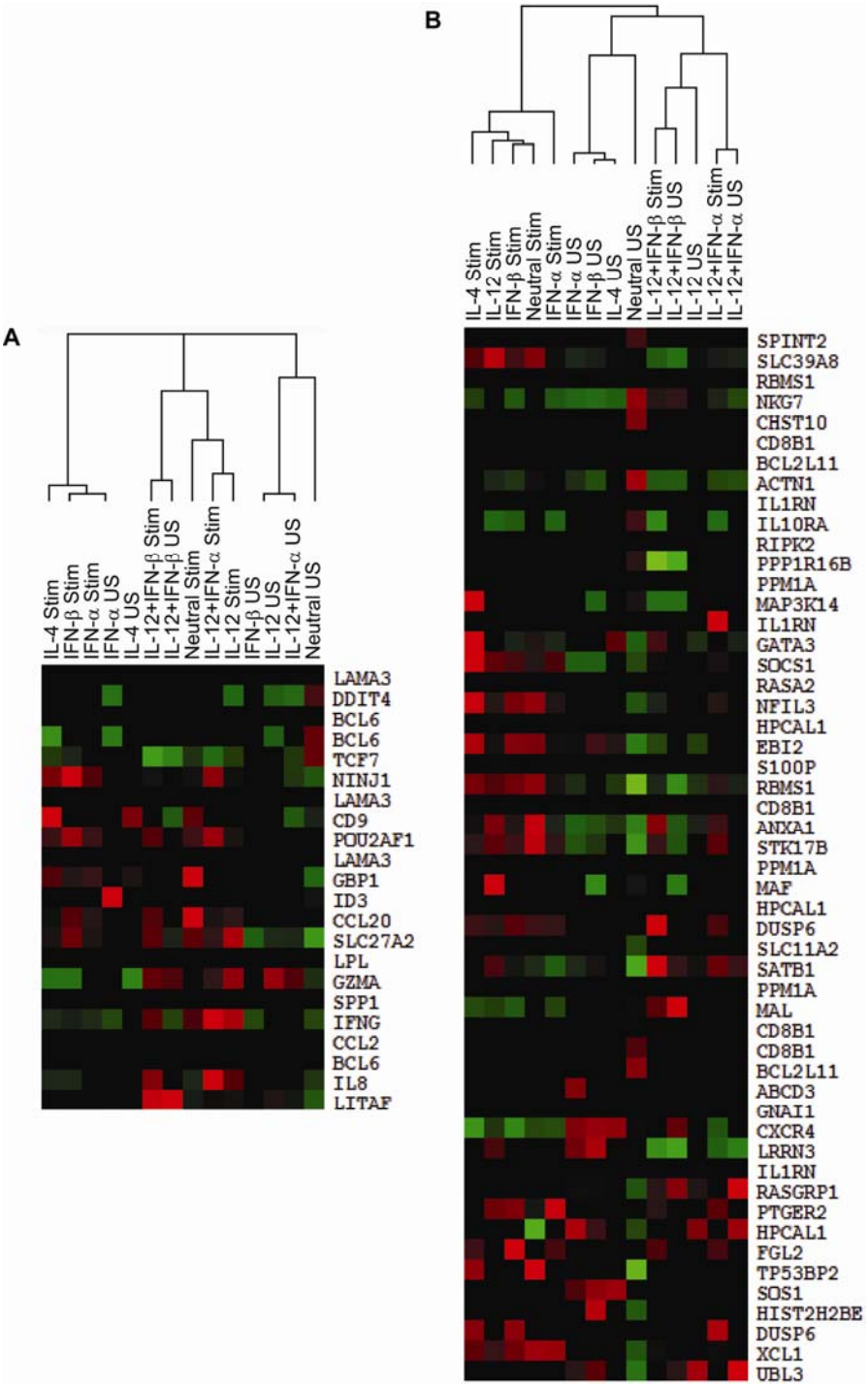

Figure 31. Expression patterns of genes which have been reported to be associated with the Th1 (A) or Th2 (B) phenotype in human $CD4^+$ T cells.

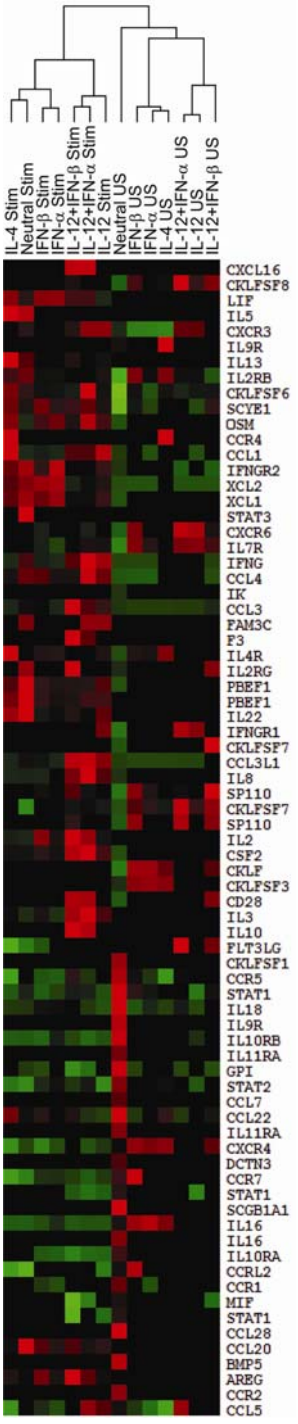

Figure 32. Expression pattern of genes designated as cytokine or chemokine by gene ontology.

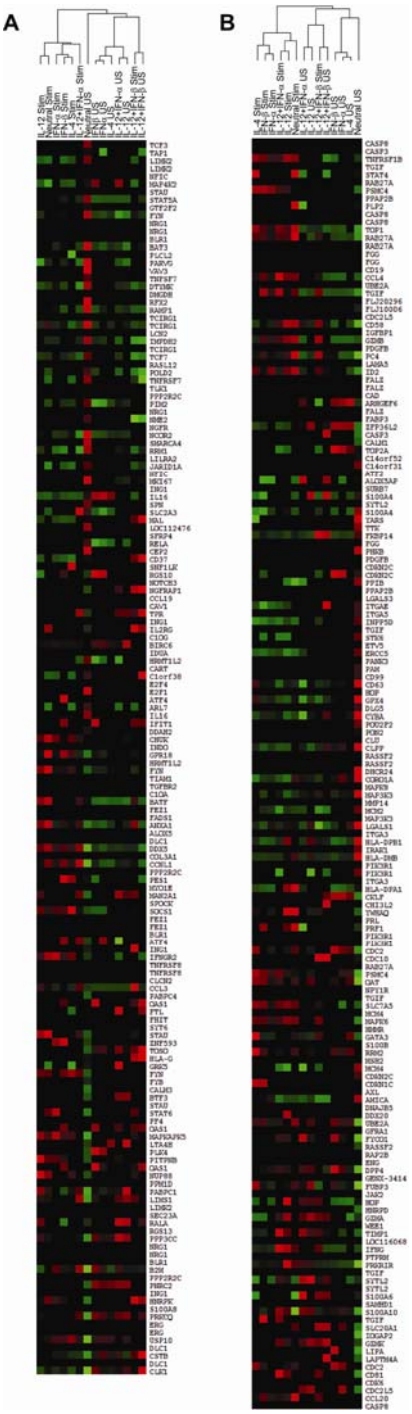

Figure 33. Expression patterns of genes which have been reported to be associated with the T_{CM} (A) or T_{EM} (B) phenotype in human $CD4^+$ T cells.

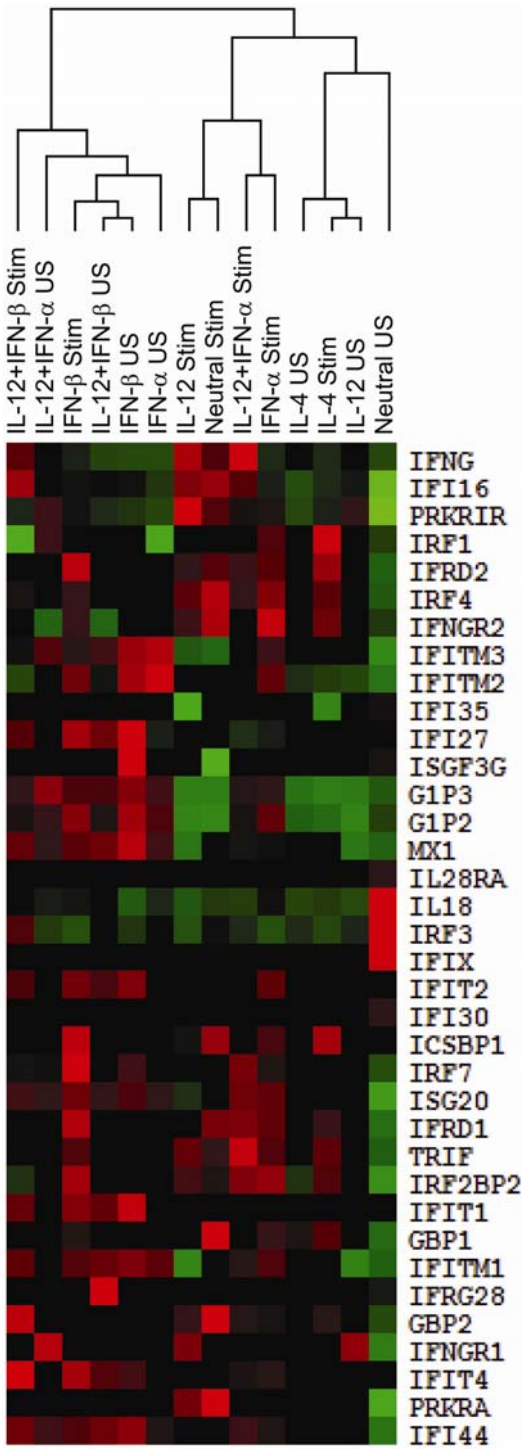

Figure 34. Expression pattern of genes with "interferon" in the gene definition.

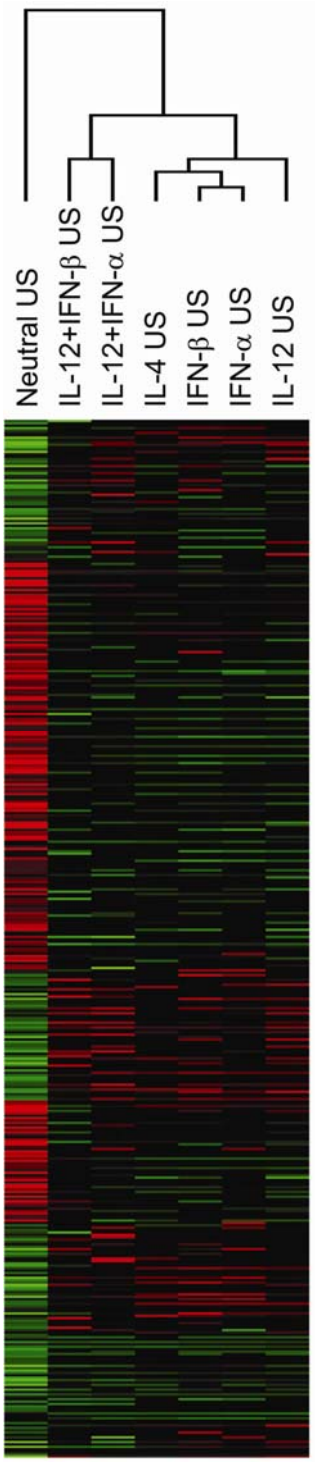

Figure 35. Transcription factors significantly different from Neutral Unstimulated.

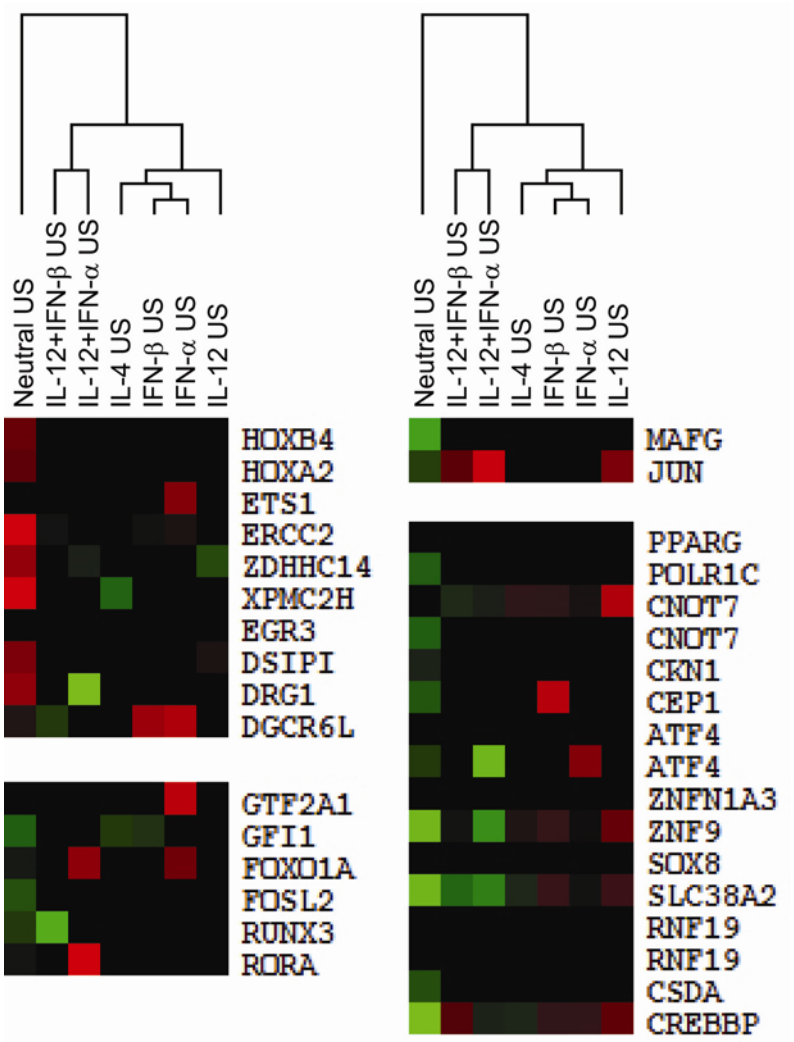

Figure 36. Clusters of transcription factors differentially regulated by IL-12 and IFN-α.

CHAPTER VI

A T-BET-INDEPENDENT ROLE FOR IFN-α/β IN REGULATING IL-2 SECRETION IN HUMAN CD4$^+$ CENTRAL MEMORY T CELLS

The work presented in this chapter is under consideration for publication at the *Journal of Immunology*. All experiments were performed by Ann M. Davis.

Introduction

The innate cytokines IL-12 and type I IFN (IFN-α/β) are key signals that set in motion a series of cellular responses aimed at eradicating a diversity of viral and bacterial infections. IL-12 promotes type I responses by inducing IFN-γ secretion from CD4$^+$ and CD8$^+$ T cells and NK cells via induction of STAT4 and T-bet (18, 110, 112, 115). In parallel, IFN-α/β can directly inhibit viral replication through the induction of a spectrum of IFN-α/β-induced genes (277). The role of IFN-α/β in regulating innate responses is well established; however, the ability of IFN-α/β to promote adaptive T cell responses remains controversial. Initially, IFN-α was proposed to mediate Th1 responses in human CD4$^+$ T cells through the activation of STAT4 and T-bet (114, 130, 131). However, more recent studies have demonstrated that, while IFN-α could promote STAT4 phosphorylation, this signaling pathway was not sustained and did not lead to stable T-bet expression, Th1 commitment, or effector function in human CD4$^+$ T cells (254, 270).

Thus, IL-12 is unique in its ability to drive Th1 effector cell development, while the effects of IFN-α on Th responses remain unclear.

Unlike IL-12, IFN-α does not maintain high T-bet expression or affect the development of effector functions in human $CD4^+$ T cells (254, 270). However, IFN-α has been demonstrated to play a role in the clonal expansion and long-term survival of memory $CD4^+$ T cells (234, 236). In recent years, considerable effort has been directed toward understanding the nature and genesis of $CD4^+$ T cell memory. Initially, memory $CD4^+$ T cells were divided into two subsets based upon expression of the lymph node-homing chemokine receptor CCR7 (202). Effector memory cells (T_{EM}), defined as $CD45RA^-$ $CCR7^{lo}$, secrete effector cytokines IFN-γ, IL-4, and IL-5, but they proliferate poorly upon secondary antigen stimulation (202, 203). These cells can be found predominantly in the spleen and peripheral organs and are relatively short-lived (205, 207). In contrast, central memory cells (T_{CM}), which are $CD45RA^-$ $CCR7^{hi}$, traffic to lymph nodes by virtue of their expression of CCR7 (202, 207). These cells secrete high levels of IL-2 but relatively low levels of effector cytokines compared to T_{EM} (202, 203). Further, T_{CM} tend to be longer-lived, display robust proliferation, and are able to generate secondary T_{EM} cells in response to recall antigen activation (202, 205). Although these classifications are useful in pursuing hypotheses, it has become clear that memory cells display significant heterogeneity with regard to cytokine production, trafficking, and their ability to expand *in vivo*.

Multiple signals contribute to the genesis and maintenance of $CD4^+$ T cell memory subsets. IL-7 and IL-15 play an essential role in the survival and long-term maintenance of $CD4^+$ memory T cells (229, 230). However, these signals alone are not

sufficient to directly promote or enhance the generation of these populations (231, 232). Determination of other factors which may play a direct role in memory cell generation will be important in the development of a new generation of vaccines as well as for a more complete understanding of disease responses.

In this study, I examined human $CD4^+$ T cells differentiated in the presence of IL-12, IFN-α, or a combination of IL-12 and IFN-α in order to more clearly delineate the roles of these two cytokines in promoting effector and memory functions. While IL-12 was found to control the development of effector functions in Th1 cells, activation in the presence of a combination of IL-12 and IFN-α enhanced the outgrowth of a subpopulation of Th1 cells with phenotypic and functional characteristics of central memory cells. Furthermore, the memory characteristics generated *in vitro* were independent of the effects of the T-bet transcription factor. These findings demonstrate for the first time the important role of the innate cytokine IFN-α in shaping human memory T cell responses to viral pathogens.

Results

Type I IFN enhances the generation of CD4$^+$ T cell memory phenotypes

Previous work has indicated that type I IFN does not influence the development of Th1 effectors as assessed by secretion of IFN-γ and TNF-α (254, 270). However, few studies have examined the ability of IFN-α to regulate expression of other cytokines or chemokines in CD4$^+$ T cells (325-327). Furthermore, there has been no comprehensive analysis of the effects of IFN-α, in isolation or in combination with other cytokines, on the differentiation of purified naïve human CD4$^+$ T cells. I therefore wished to examine more closely the effects of type I IFN on Th cytokine secretion in the absence or presence of IL-12. For these experiments, CD4$^+$ CD45RA$^+$ cells were purified from 5 healthy adult donors and activated with plate-bound anti-CD3 and anti-CD28 in the presence of IL-2 and cytokines and neutralizing antibodies for 7-14 days. After 1-2 weeks, cells were washed extensively in clean media and restimulated with either plate-bound anti-CD3 for cytometric bead array analysis or with PMA and ionomycin for flow cytometry.

Human CD4$^+$ T cells differentiated in the absence of innate cytokines were able to produce an array of cytokines and chemokines (Fig. 37). Additionally, T cells from different donors secreted a relatively wide range of concentrations of each cytokine when activated under neutralizing conditions. For example, IL-5 secretion ranged from 0.4 – 1.4 ng/ml (Fig. 37). In spite of this inherent heterogeneity, reliable trends were observed in the responses of cells from all 5 donors to innate cytokines. In accordance with previously published work (254, 270), IL-12 promoted elevated secretion of the Th1

cytokines IFN-γ and TNF-α, as well as the proinflammatory chemokine IL-8 (Fig. 37). As expected, the Th2-associated cytokines IL-4, IL-5, and IL-13 were positively regulated by IL-4, but not by IL-12 (Fig. 37). Compared to cells activated under neutralizing conditions, IFN-α alone did not induce secretion of any cytokine or chemokine assessed, including the Th1 cytokines IFN-γ and TNF-α and the Th2 cytokines IL-4, IL-5, and IL-10 (Fig. 37). However, IFN-α did not inhibit the ability of IL-12 to promote elevated secretion of proinflammatory cytokines or chemokines when these two cytokines were used in combination (Fig. 37). Therefore, IL-12, but not IFN-α, regulates the expression of Th1 effector cytokines and chemokines in human $CD4^+$ T cells.

In addition to effector cytokines and chemokines, the secretion of IL-2 by differentiated human $CD4^+$ T cells was also measured (Fig. 38). Activation of cells with either IL-12 or IFN-α alone resulted in a 2-20 fold induction of IL-2 secretion among the various donors compared to the neutralized condition. Surprisingly, and in contrast to the patterns of effector cytokine expression, IL-12 and IFN-α synergistically induced high levels of IL-2 secretion, ranging from 7-120 fold induction compared to the neutralized control. This induction has previously been observed at the transcript level by microarray analysis (Fig. 32). The synergy observed with IL-12 and IFN-α was consistent among all 5 donors (Fig. 38A, $p < 0.05$ vs. neutral). Furthermore, elevated IL-2 secretion was maintained for two consecutive weeks of activation, indicating that IL-12 and IFN-α potentiate a sustained induction of IL-2 (Fig. 38C). Flow cytometric analysis demonstrated that IL-12 and IFN-α enhanced the generation of both $IL\text{-}2^+$ $IFN\text{-}\gamma^-$ and IL-

2^+ IFN-γ^+ populations (Fig. 38B). Therefore, IFN-α synergizes with IL-12 to uniquely promote elevated IL-2 secretion from human $CD4^+$ T cells.

Because activation of human $CD4^+$ T cells in the presence of IL-12 and IFN-α resulted in high secretion of IL-2, the hallmark cytokine produced by T_{CM} cells, it was of interest to determine whether IFN-α could influence other aspects of the T_{CM} phenotype. Therefore, flow cytometric analysis was performed with human $CD4^+$ T cells differentiated for 7 days in the presence of IL-12, IFN-α, or a combination of IL-12 and IFN-α. As expected, the generation of populations of cells with both T_{CM} ($CD45RA^-$ $CCR7^{hi}$) and T_{EM} ($CD45RA^-$ $CCR7^{lo}$) phenotypes was observed even in the absence of innate cytokines (Fig. 39). However, there were also modest but consistent trends for IL-12 and IFN-α to shift the balance between these phenotypes. IFN-α modestly enhanced expression of CCR7, resulting in an increase in the percentage of cells with a T_{CM} phenotype (Fig. 39). Differentiation of cells in the presence of IFN-α also resulted in a moderately increased percentage of cells with a $CCR7^{hi}$ $CXCR3^{lo}$ phenotype (Fig. 39). In contrast, IL-12 modestly enhanced both the proportion of cells with a T_{EM} phenotype and the percentage which were $CCR7^{lo}$ $CXCR3^{hi}$ (Fig. 39), in accordance with previous work demonstrating that CXCR3 is a marker of Th1 effectors (311-313). IL-12 also enhanced the proportion of IL-$7R\alpha^{lo}$ cells and upregulated the IL-15R on both IL-$7R\alpha^{lo}$ and IL-$7R\alpha^{hi}$ cells, consistent with the role of IL-12 in regulating T_{EM} (Fig. 39) (227, 328). IFN-α alone modestly enhanced a proportion of IL-$7R\alpha^{hi}$ cells without influencing IL-15R expression (Fig. 39). In addition, IFN-α preserved the population of IL-$7R\alpha^{hi}$ cells in the presence of IL-12, suggesting that IFN-α may enhance a subpopulation of T_{CM} cells. The

expression of a number of other chemokine receptors in response to IL-12 and IFN-α was also analyzed, including CXCR4 and CCR5, which have been suggested to be involved in memory responses and Th effector cell trafficking (329). However, no other receptors were regulated by IFN-α and IL-12 in this manner (Fig. 40). Taken together, these results indicate that IFN-α modestly enhances the generation of cells with T_{CM} characteristics in developing human Th1 cells, while IL-12 regulates effector and T_{EM} phenotypes.

The overall percentages of naïve, T_{CM}, or T_{EM} were not as dramatically regulated as the robust induction of IL-2 secreting cells in response to IL-12 + IFN-α activation. Thus, it was not clear whether the IL-2-secreting cells that developed in response to IL-12 + IFN-α were T_{CM}. To address this issue, I focused my analysis on defining the nature of the IL-2-secreting cells that were markedly regulated in response to IL-12 and IFN-α. For these experiments, naïve human $CD4^+$ T cells were polarized with IL-12 + IFN-α to day 7 followed by multiparametric analysis for both cell surface markers and expression of IL-2 and IFN-γ. Cells were first gated as naïve ($CD45RA^+$ $CCR7^{hi}$), T_{CM} ($CD45RA^-$ $CCR7^{hi}$), or T_{EM} ($CD45RA^-$ $CCR7^{lo}$), and then analyzed for expression of IL-2, IFN-γ, IL-15R, and IL-7Rα (Fig. 5). As expected, each of the markers was expressed in the patterns which have been described previously (202, 203, 227, 328); naïve and T_{CM} cells were largely IL-7Rα^{hi} and produced primarily IL-2, whereas T_{EM} cells showed reduced expression of IL-7Rα, a higher proportion of cells expressing IL-15R, and secretion of substantially more IFN-γ and less IL-2 (Fig. 41). In parallel analyses, it was found that cells secreting only IL-2 showed uniformly high expression of CCR7 and IL-7Rα and

were of the naïve and T_{CM} phenotypes (Fig. 42). In contrast, cells which expressed only IFN-γ were CXCR3^{hi}, and a much higher proportion of these cells displayed T_{EM} and IL-7Rα^{lo} phenotypes (Fig. 42). Cells capable of secreting both IL-2 and IFN-γ demonstrated an intermediate phenotype, such that the majority of these cells were uniformly high for expression of IL-7Rα, but most were CXCR3^{hi} as well as CCR7^{hi}. Like the cells which produced only IFN-γ, cells capable of secreting both cytokines showed a higher proportion with a T_{EM} phenotype than cells which produced only IL-2 (Fig. 42). Thus, the IL-2-producing populations enhanced by IL-12 and IFN-α predominantly display T_{CM} characteristics.

The IFN-α-enhanced population demonstrates functional properties of memory

It was of interest to more closely examine the genesis of the central and effector memory phenotypes as these cells divided in culture in response to a combination of IL-12 and IFN-α. Purified naïve human CD4$^+$ T cells were labeled with CFSE prior to activation for 7 days in the presence of IL-12 and IFN-α. Cells were then restimulated and analyzed by flow cytometry. We found expression of CCR7, IL-7Rα, and IL-2, three markers of central memory, on cells at all divisions (Fig. 43A and B). In contrast, expression of CXCR3, IL-15R, and IFN-γ, three markers of Th1 effector and effector memory phenotypes, was only apparent in cells at the latest divisions (Fig. 43A and B). Based on this data, it would appear that the generation of human effector CD4$^+$ T cells is associated with maximal proliferation, as has been reported for murine T cells during

influenza infection (330). In contrast, central memory characteristics can be observed in cells at any point during proliferation and differentiation.

I next wished to examine the functional characteristics of the IL-2-secreting cells that developed in response to IL-12 and IFN-α. The migration potential of IL-2- and IFN-γ-producing $CD4^+$ T cells was first examined in order to correlate chemokine receptor expression with functional capacity. Naïve human $CD4^+$ T cells were differentiated for 7 days in the presence of IL-12 and IFN-α. Cells were then washed extensively and rested overnight. The following day, a transwell migration assay was performed using either CCL19, a CCR7 ligand, or CXCL10, a CXCR3 ligand. Cells that migrated across the transwell membrane were restimulated and analyzed for IFN-γ and IL-2 expression by flow cytometry (Fig. 44A). Cells with the capacity to produce IL-2 but not IFN-γ showed enhanced migration in response to CCL19 but not CXCL10 (Fig. 44B, $p < 0.05$ vs. media alone). In contrast, cells which secreted IFN-γ but not IL-2 showed significantly enhanced migration in response to CXCL10 but not CCL19 (Fig. 44B, $p < 0.05$ vs. media alone). Cells which were able to express both cytokines simultaneously showed equivalently enhanced migration in response to both chemokines (Fig. 44B, $p < 0.05$ vs. media alone), as would be expected based upon their co-expression of CCR7 and CXCR3. Therefore, the expression patterns of CCR7 and CXCR3 on subsets of IL-2- and IFN-γ-secreting human $CD4^+$ T cells correspond to functional differences in migration capacity. Further, these data demonstrate that the IL-2-secreting population that develops in response to IL-12 and IFN-α displays selective migration to CCR7 ligands, as expected of the T_{CM} subset.

The IL-2- and IFN-γ-secreting populations were next isolated in order to more closely examine the functions of these cells as individual subsets. Live cell sorting was performed to obtain IL-2- and IFN-γ-producing populations of cells after 7 days of activation of naïve human $CD4^+$ T cells in the presence of IL-12 and IFN-α (Fig. 45A and B). These cells were then restimulated with plate-bound anti-CD3 and anti-CD28 in the presence of IL-2, and functional properties were assessed.

It has been proposed that cells with T_{CM} phenotypes are able to give rise to new waves of effector cells upon secondary activation, whereas effector or T_{EM} $CD4^+$ T cells are more terminally differentiated (202, 212). However, work by other groups has shown that cells with effector or T_{EM} characteristics can give rise to T_{CM} cells (218, 220, 222). Hence, the lineage relationships between these populations remain unclear. Therefore, the functional plasticity of IL-2- and IFN-γ-producing populations was examined. Sorted cells were restimulated for a further 7 days in the presence of IL-12 and IFN-α. Cells which could produce only IL-2 or both IL-2 and IFN-γ were able to give rise to multiple populations of cytokine-expressing cells after a second round of activation in the presence of innate cytokines, indicating a degree of functional plasticity (Fig. 46A). However, cells which secreted only IFN-γ were unable to give rise to cells which only secreted IL-2, suggesting a more terminally differentiated phenotype.

T_{CM} cells have also been shown to be more proliferative and more resistant to apoptosis than their T_{EM} counterparts (202, 205). In order to probe the survival of IL-2- and IFN-γ-producing populations of cells, we reactivated sorted cells for 3 days with plate-bound anti-CD3 and anti-CD28 in the presence of IL-2 and then performed flow

cytometric analysis for Annexin V and 7-AAD labeling. Cells secreting only IL-2 showed the lowest rates of apoptosis, while cells expressing IFN-γ demonstrated a greatly enhanced tendency to undergo apoptosis (Fig. 46B). Furthermore, the percentage of cells which labeled with Annexin V and 7-AAD corresponded with the total percentage of live cells in the IL-2- and IFN-γ-expressing populations, as assessed by forward and side scatter (Fig. 46C).

Enhancement of memory phenotypes by IFN-α is independent of T-bet and Eomes

T-bet, a member of the T-box family of transcription factors, has been proposed to be the master regulator of Th1 effector development (107). T-bet regulates IFN-γ secretion from murine $CD4^+$ T cells, and mice deficient in T-bet show elevated IL-2 secretion and the generation of a large population of cells with T_{CM} characteristics (107, 331). Furthermore, graded expression of T-bet and eomesodermin (Eomes), another T-box family member, has also been proposed to play a role in the generation of $CD8^+$ T_{EM} vs. T_{CM} cells (332-334). In human $CD4^+$ T cells, T-bet has been shown to be responsive to both IL-12 and IFN-α, but unlike IL-12, IFN-α signaling does not maintain long-term T-bet expression (254). In contrast, Eomes has only recently been shown to be expressed in $CD4^+$ T cells, and the signals which might control its expression have not been identified (335). Thus, it was possible that the IL-2-secreting population enhanced by IFN-α might have differential expression of T-bet and Eomes as a result of local exposure to IL-12 vs. IFN-α during differentiation. Therefore, T-bet and Eomes

expression was examined by intracellular flow cytometry in human $CD4^+$ T cells activated for 7 days in the presence of a combination of IL-12 and IFN-α. Surprisingly, we found that cells with a $CD45RA^-CCR7^{lo}$ T_{EM} phenotype showed slightly lower expression of both T-bet and Eomes when compared with cells with $CD45RA^+CCR7^{hi}$ naïve or $CD45RA^-CCR7^{hi}$ T_{CM} phenotypes (Fig. 47). Furthermore, both IL-2- and IFN-γ-expressing cells demonstrated similar Eomes and T-bet content, whereas only a fraction of the cells that did not produce either cytokine were $T\text{-bet}^{lo}$ or $Eomes^{lo}$.

In order to more directly examine the functional role of T-bet in the generation of T_{EM} and T_{CM} phenotypes, T-bet was expressed in developing human $CD4^+$ T cells using a retroviral vector. Unexpectedly, ectopic expression of T-bet did not influence the expression of CCR7 or the proportions of T_{CM} and T_{EM} cells generated after 7 days of *in vitro* culture in the presence of IL-12 and IFN-α (Fig. 48). In contrast with previously published reports (107, 331), T-bet expression also failed to inhibit expression of IL-2. However, in accordance with published data from several labs (254, 336, 337), T-bet expression greatly enhanced the expression of CXCR3 and IFN-γ in $CD4^+$ T cells.

Expression of the IL-12R and IFNAR does not correlate with memory phenotype

While the type I IFN receptor (IFNAR) is constitutively expressed on nearly all somatic cells, the β2 chain of the IL-12 receptor (IL-12Rβ2) must be induced on developing $CD4^+$ T cells in order to confer IL-12 responsiveness and license IFN-γ secretion (15, 106). Both IL-12 and IFN-α can induce IL-12Rβ2 expression by human

$CD4^+$ T cells; however, induction of IL-12Rβ2 in response to IFN-α is transient, whereas IL-12-mediated expression is prolonged (254). It is therefore possible that local concentrations of IL-12 and IFN-α could give rise to populations of cells with differential relative expression of IL-12Rβ2 and IFNAR, that is, with differential cytokine responsiveness. In turn, differences in the ability of cells within a population to respond to IL-12 vs. IFN-α could result in the simultaneous development of multiple populations of cytokine-producing cells. Thus, if some cells receive a stronger IL-12 signal during differentiation by virtue of higher IL-12R expression, those cells might go on to become the IFN-γ-producing population. In contrast, cells which express IFNAR but relatively low levels of IL-12R might favor IL-2 secretion.

Therefore, the expression of IL-12Rβ2 and IFNAR was examined on naïve human $CD4^+$ T cells at day 3 and day 7 of differentiation in response to IL-12, IFN-α, or a combination of IL-12 and IFN-α. Cells were labeled with CFSE prior to activation in order to track the expression of these receptors as a function of cell division, and cells which received no anti-CD3/anti-CD28 stimulus or cytokines were used as a negative control. Surprisingly, expression of IFNAR was strongly upregulated at day 3 in response to TCR signals (Fig. 49A). However, this TCR-mediated induction was reversed by day 7 (Fig. 49B). Furthermore, no differences were observed between cytokine treatment groups in expression of either IL-12Rβ2 or IFNAR at either day 3 or day 7 (Fig. 49A and B), in contrast with previously reported findings for IL-12Rβ2 expression (254). The expression of these receptors also did not change appreciably over the course of multiple rounds of division (Fig. 49A and B), in contrast to previous

observations for established cell surface markers of the T_{CM} and T_{EM} phenotypes (Fig. 43).

Many of the phenotypic differences described above were observed only in IL-2- and IFN-γ-producing subpopulations and not in the total pool of cells. Therefore, multicolor gating analysis was performed to examine expression of IFNAR and IL-12Rβ2 in subsets of human $CD4^+$ T cells differentiated in response to IL-12 and IFN-α. At day 3, no differences were observed between IL-2- and IFN-γ-secreting cells in expression of either IFNAR or IL-12Rβ2 (Fig. 50A). At day 7, only minor differences were noted between these cytokine-producing populations in expression of IFNAR, and no differences were found in expression of IL-12Rβ2 (Fig. 50B). Therefore, the expression level of these two receptors does not regulate the development of the cytokine-producing populations in response to IL-12 and IFN-α.

Discussion

The work presented here demonstrates that differentiation of naïve human $CD4^+$ T cells in the presence of both IL-12 and IFN-α results in enhanced generation of a population of cells which secrete IL-2 upon reactivation (Fig. 51). These IL-2-secreting cells display functional characteristics of T_{CM} cells. IFN-γ-producing cells also arise in parallel with IL-2 producers, and these cells possess a T_{EM} phenotype. While T-bet regulates IFN-γ secretion and CXCR3 expression on developing Th1 cells, neither T-bet nor innate cytokine receptor expression impacts the generation of memory phenotypes.

Swain and colleagues have demonstrated that *in vitro* activation of naïve murine $CD4^+$ T cells generates a population which gives rise to cells with memory characteristics following a rest period as short as 4 days (220, 318). Using a similar set of assays, I now provide evidence of this same phenomenon in human $CD4^+$ T cells. According to these data, human cells differentiated *in vitro* represent a heterogeneous mixture, some of which possess the phenotypic determinants of long-lived memory cells. Furthermore, to date, no cytokine has been shown to directly enhance the generation or function of T helper cell memory. For the first time, the present work demonstrates such a role for IFN-α in the context of human Th1 development.

In early studies, IL-2 was considered to be a Th1-associated cytokine based upon its selective co-expression with IFN-γ, but not IL-4 (153, 338). However, as Th1 effector cells are passaged *in vitro* in the presence of IL-12, they rapidly lose the ability to secrete high levels of IL-2 compared with their naïve precursor counterparts (265, 331). This

may be due to IL-12-mediated induction of T-bet in effector cells, which is thought to negatively regulate IL-2 expression (107, 331). Furthermore, T_{EM} cells that migrate to peripheral sites selectively express IFN-γ, but not appreciable levels of IL-2 (207, 330). Rather, IL-2 is predominantly expressed by naïve and T_{CM} subsets as compared to effector or T_{EM} cells (202). The work described above shows that a combination of IL-12 and IFN-α strongly enhances secretion of IL-2 from human $CD4^+$ T cells. Furthermore, I have observed enhanced development both of cells that co-expressed IL-2 and IFN-γ as well as cells that expressed only IL-2 in response to IL-12 + IFN-α stimulation. IL-2 secretion is an inherent property of naïve $CD4^+$ T cells, and retention of IL-2 expression is an important characteristic of the T_{CM} compartment. However, the signals that retain IL-2 expression in memory cells have not previously been identified. Here, for the first time, a synergistic role for IL-12 and IFN-α is demonstrated in inducing high levels of IL-2 secretion in a population of activated human $CD4^+$ T cells.

In agreement with a number of published reports, the data presented here indicate that the ability to produce IL-2 correlates with the ability of a $CD4^+$ T cell to survive and regenerate new waves of effector cells (202, 207, 330). While the IL-2- and IFN-γ-producing cells described here show tendencies for the expression of certain cell surface markers, all of the cytokine-secreting populations which were examined retained a degree of heterogeneity in terms of CCR7, CXCR3, and IL-7Rα expression (Fig. 2B). However, these populations showed strong characteristics for memory vs. effector formation when analyzed in functional assays. This segregation of IL-2 and IFN-γ production to the T_{CM} and T_{EM} compartments, respectively, was proposed at the initial description of these

subsets (202). Wu et. al. have also demonstrated for murine T helper cells that IFN-γ^- but not IFN-γ^+ cells give rise to memory (212). In contrast, more recent work has shown that IFN-γ-producing cells can also generate a population of T helper cells with memory characteristics (222). However, the studies presented here suggest that cells that secrete only IFN-γ, but not IL-2, would have a severely impaired ability to reconstitute additional effector cells upon rechallenge. In agreement with these findings, Harrington et. al. demonstrated the generation of T_{EM} from primary IFN-γ-producing effector cells that also secrete IL-2 (222).

These data also demonstrate a distinct difference between the generation of memory phenotypes in $CD4^+$ vs. $CD8^+$ T cells. Recent studies have suggested that the levels of T-bet and Eomes expression contribute to the balance between central and effector memory development in $CD8^+$ T cells (332-334). In contrast, the present work suggests that T-bet expression in Th1 cells controls the generation of effector functions such as IFN-γ production and inflammatory chemokine responsiveness without impacting central memory phenotypes such as CCR7 expression and IL-2 secretion. Furthermore, the enhancement of T_{CM} phenotypes and IL-2 secretion in response to a combination of IL-12 and IFN-α also cannot be explained by differences in IL-12 vs. IFN-α responsiveness in IL-2- and IFN-γ-secreting populations; these populations did not differ in expression of the IL-12 and type I IFN receptors. Thus, the mechanism of memory phenotype enhancement by IFN-α remains unclear.

The findings presented here highlight the importance of IL-12 and IFN-α in modulating $CD4^+$ T cell effector and memory responses, respectively. Recently, there

has been substantial interest in the use of Toll-like receptor (TLR) ligands, particularly TLR9 agonists, as vaccine adjuvants and antiviral drugs (339). TLR9 stimulation of human DCs is known to induce secretion of substantial amounts of both IL-12 and IFN-α (13). My data suggest that co-production of these two cytokines will help to optimize the formation of memory cells which will provide long-term protection against viral pathogens. Thus, a better understanding of the roles of IL-12 and type I IFN in $CD4^+$ T cell development and memory formation will allow more efficient design of novel vaccines and antiviral therapies.

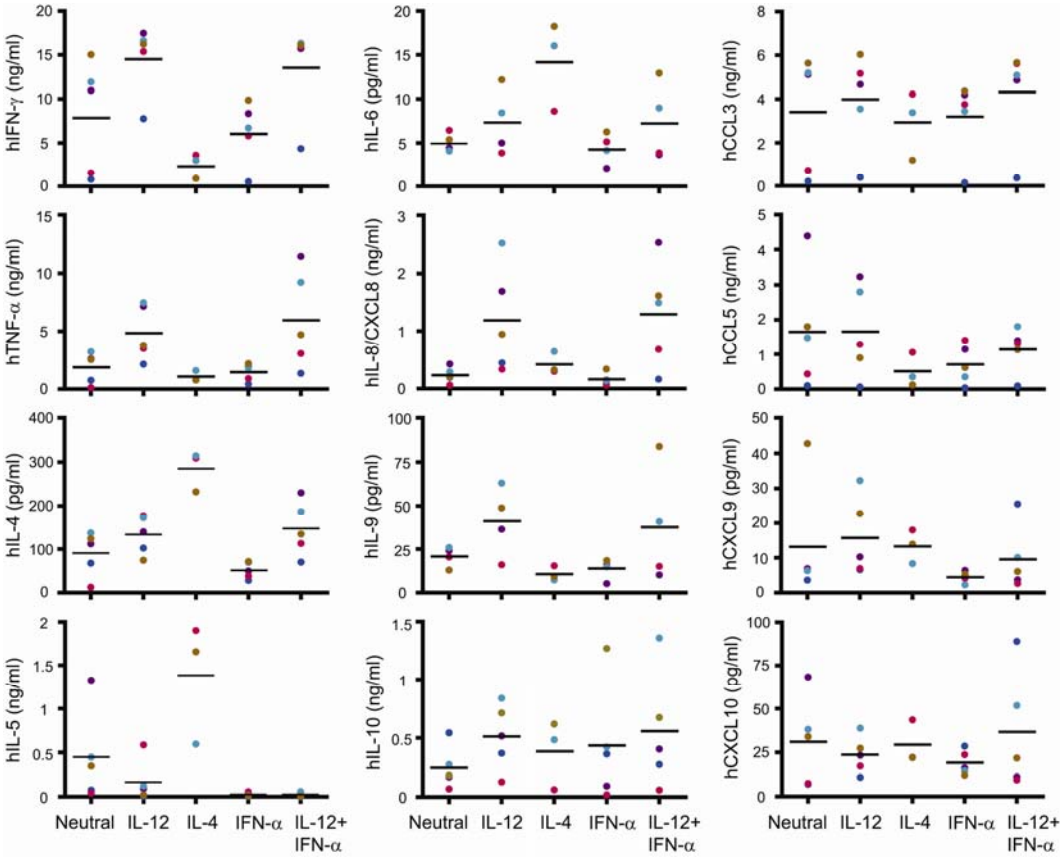

Figure 37. Type I IFN does not promote elevated secretion of effector cytokines or chemokines from human CD4$^+$ T cells. Naïve human CD4$^+$ T cells were activated for one or two consecutive weeks with plate-bound anti-CD3 and anti-CD28 in the presence of rhIL-2 and cytokines and neutralizing antibodies as indicated in the figure. On day 14, cells were washed and restimulated with plate-bound anti-CD3 for 24 hours. Supernatants were harvested, and production of the indicated cytokines and chemokines was assayed using Cytokine Bead Array kits. Each color is representative of a separate human subject.

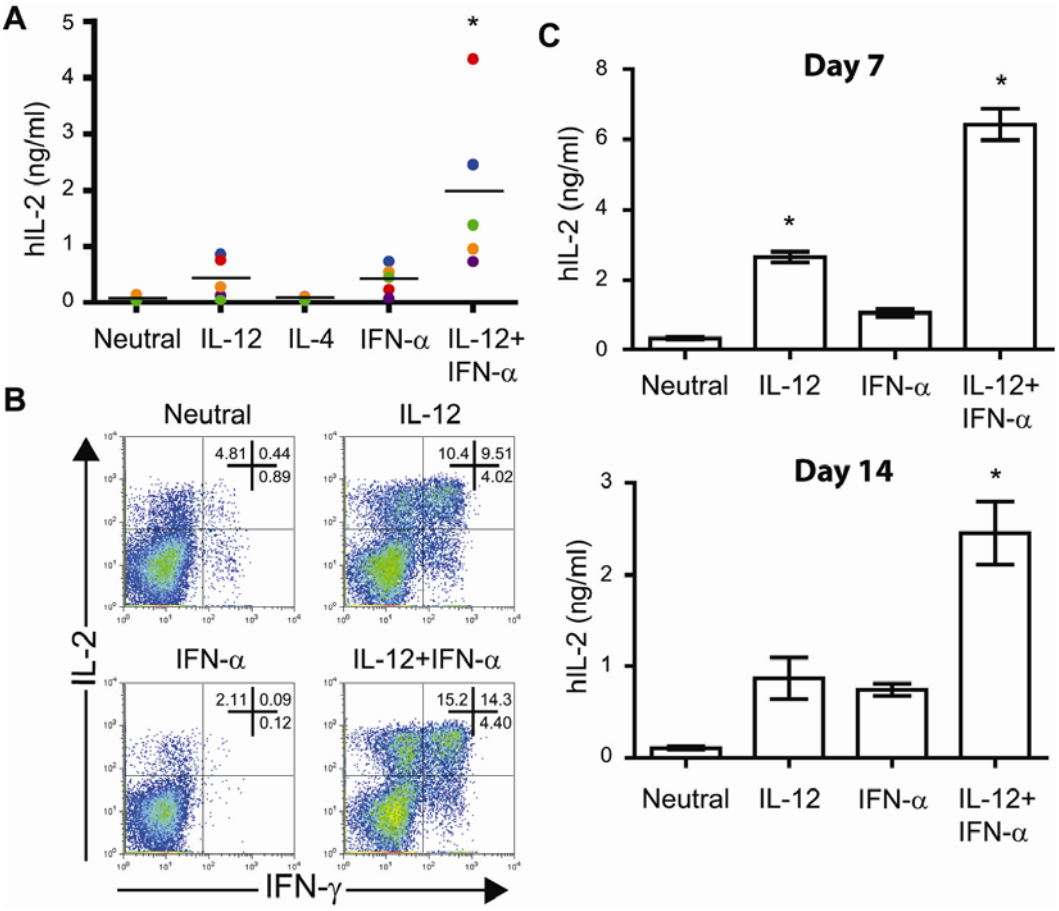

Figure 38. Type I IFN and IL-12 enhance the generation of human CD4$^+$ T cells secreting IL-2. Naïve human CD4$^+$ T cells were activated for one or two consecutive weeks with plate-bound anti-CD3 and anti-CD28 in the presence of rhIL-2 and cytokines and neutralizing antibodies as indicated in the figure. (A) On day 14, cells were washed and restimulated with plate-bound anti-CD3 for 24 hours. Supernatants were harvested, and IL-2 production was assayed using a Cytokine Bead Array kit. Each color is representative of a separate human subject. *, $p < 0.05$ compared to neutral. (B) On day 7, cells were washed and rested overnight in cIMDM in the absence of IL-2. Cells were restimulated for 4 hours with PMA + ionomycin, and intracellular staining was performed for IL-2 and IFN-γ. (C) On day 7 and day 14, cells were washed and restimulated in the presence or absence of plate-bound anti-CD3 for 24 hours. Supernatants were harvested, and IL-2 production was assayed using a Cytokine Bead Array kit. *, $p < 0.05$ compared to neutral.

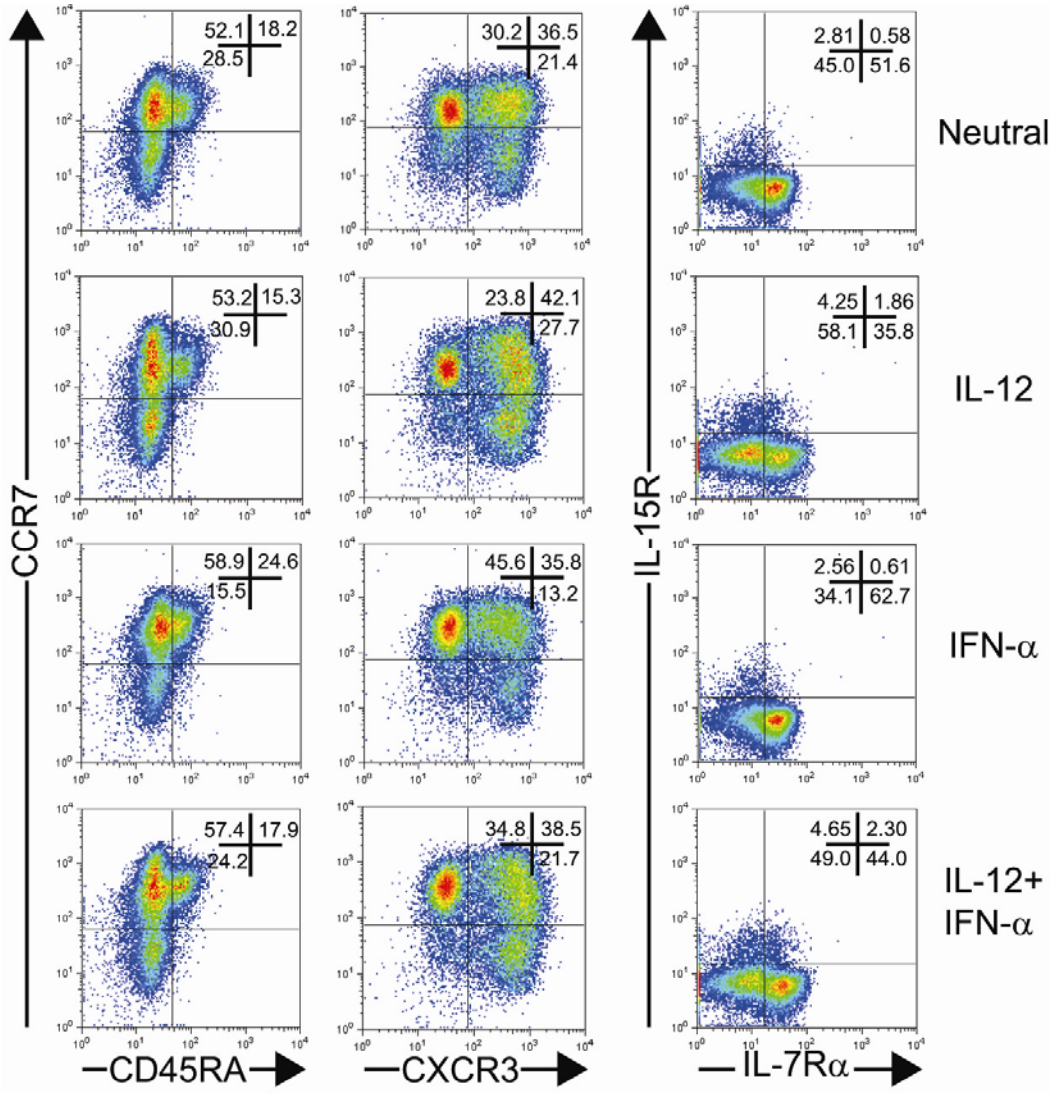

Figure 39. ***In vitro*** **differentiated human CD4$^+$ T cells display characteristics of central memory.** Naïve human CD4$^+$ T cells were activated for 7 days with plate-bound anti-CD3 and anti-CD28 in the presence of rhIL-2 and the indicated cytokines and/or neutralizing antibodies as described in Figure 1. On day 7, cells were analyzed by flow cytometry for the indicated markers.

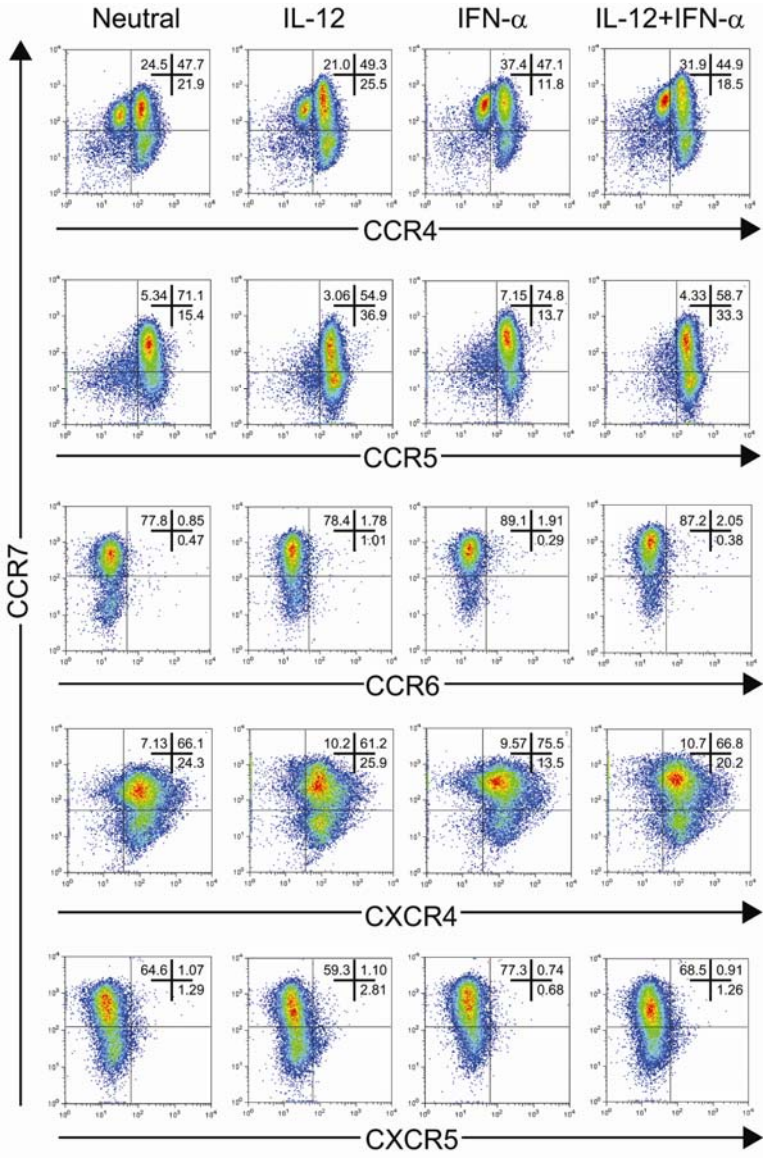

Figure 40. Analysis of chemokine receptor expression from *in vitro* activated human CD4$^+$ T cells. Naïve human CD4$^+$ T cells were activated for 7 days with plate-bound anti-CD3 and anti-CD28 in the presence of rhIL-2 and cytokines and neutralizing antibodies as indicated in the figure. On day 7, cells were washed and rested overnight in cIMDM in the absence of IL-2. Cells were then stained and analyzed for expression of the indicated chemokine receptors by flow cytometry. Data are gated on live cells.

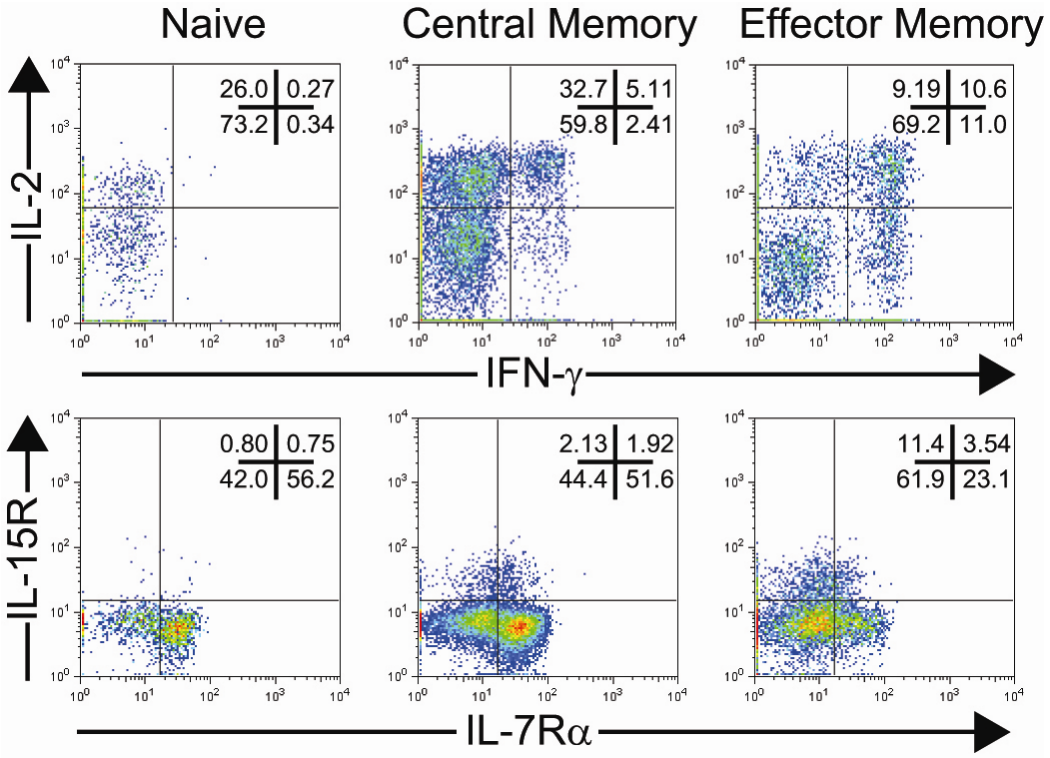

Figure 41. Central memory and effector memory phenotypes generated *in vitro* correspond to phenotypes observed *in vivo*. Naïve human $CD4^+$ T cells were activated for 7 days with plate-bound anti-CD3 and anti-CD28 in the presence of rhIL-2, rhIL-12, rhIFN-αA, anti-hIFN-γ, and anti-hIL-4. On day 7, cells were washed and rested overnight in cIMDM in the absence of IL-2. Cells were restimulated for 4 hours in the presence or absence of PMA + ionomycin, and intracellular staining was performed as indicated. Cells were gated as follows: Naïve, $CD45RA^+$ $CCR7^{hi}$; Central Memory, $CD45RA^-$ $CCR7^{hi}$; Effector Memory, $CD45RA^-$ $CCR7^{lo}$.

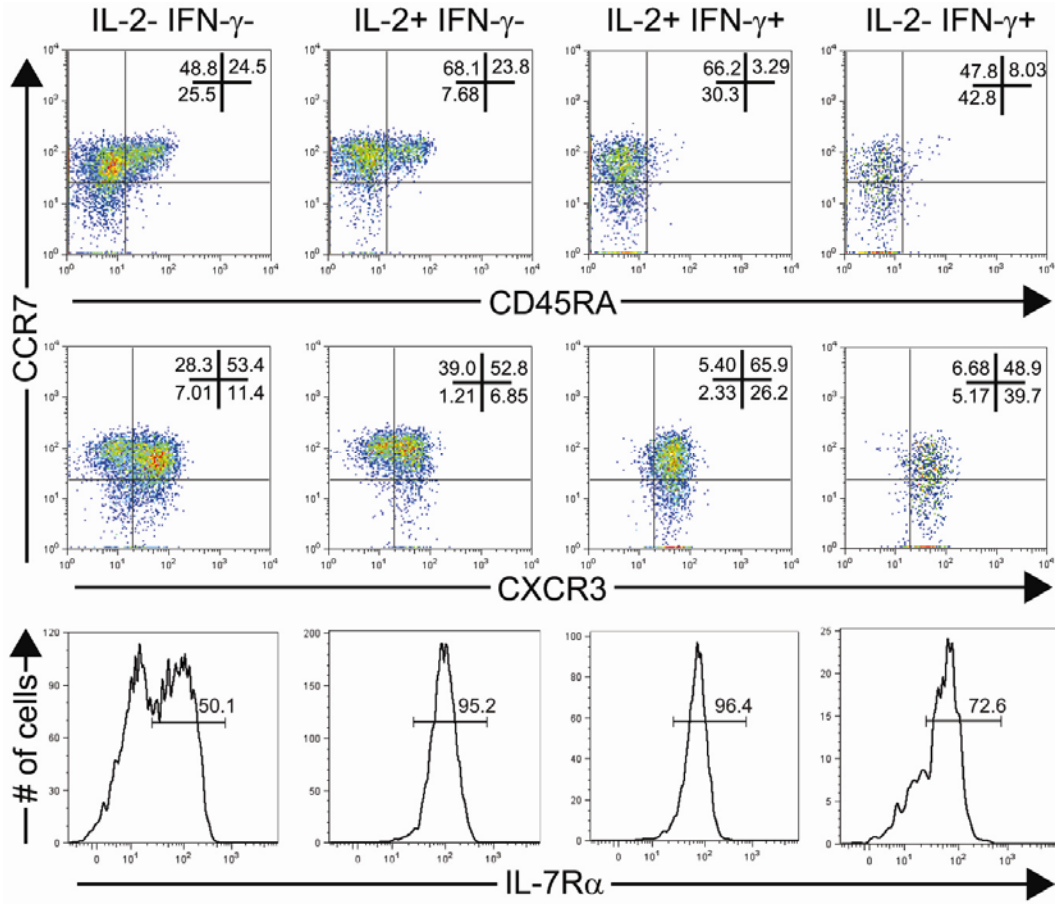

Figure 42. IL-2- and IFN-γ-secreting cells display surface phenotypes of central memory and effector memory cells. Naïve human $CD4^+$ T cells were activated for 7 days with plate-bound anti-CD3 and anti-CD28 in the presence of rhIL-2, rhIL-12, rhIFN-αA, anti-hIFN-γ, and anti-hIL-4. On day 7, cells were washed and rested overnight in cIMDM in the absence of IL-2. Cells were restimulated for 4 hours in the presence or absence of PMA + ionomycin, and intracellular staining was performed as indicated. Cells were gated on IL-2 and IFN-γ expression as indicated.

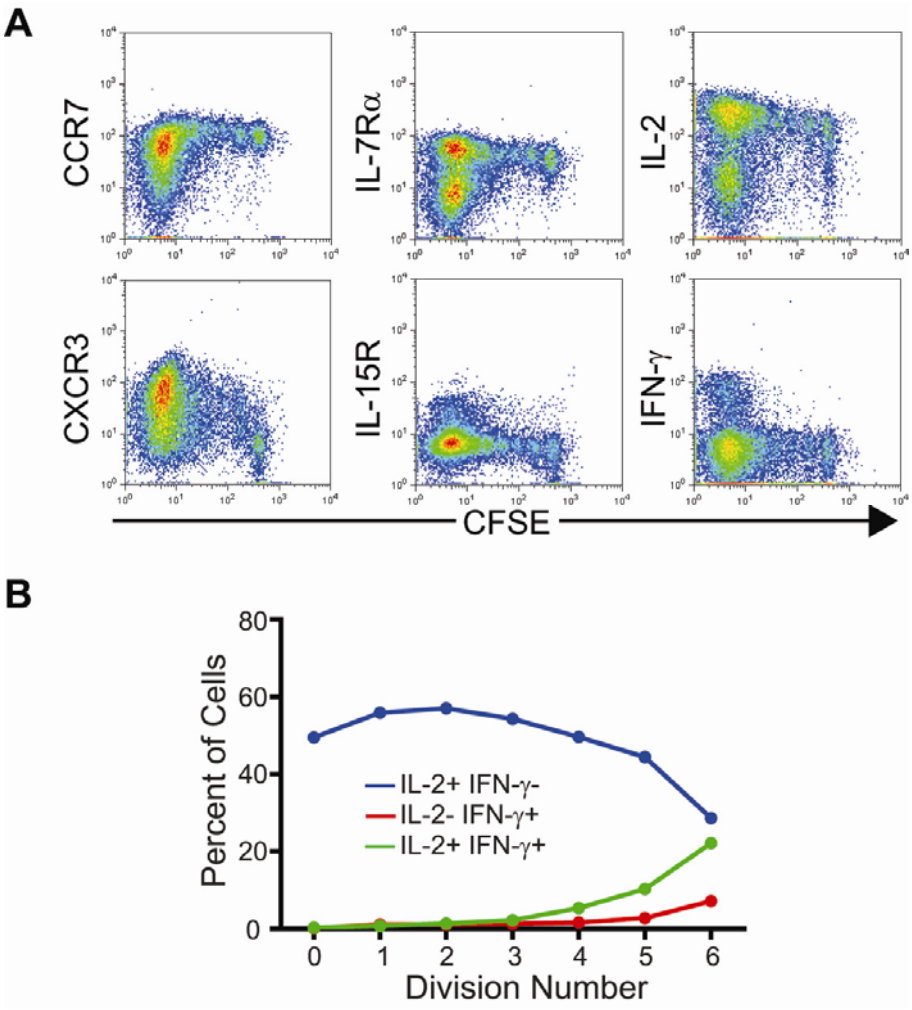

Figure 43. Human CD4$^+$ T cell effector phenotypes are associated with maximal proliferation. Naïve human CD4$^+$ T cells were labeled with CFSE and activated for 7 days with plate-bound anti-CD3 and anti-CD28 in the presence of rhIL-2, rhIL-12, rhIFN-αA, anti-hIFN-γ, and anti-hIL-4. On day 7, cells were washed and rested overnight in cIMDM in the absence of IL-2. Cells were restimulated for 4 hours in the presence or absence of PMA + ionomycin, and intracellular staining was performed as indicated. (A) Data were gated on live cells and analyzed for CFSE dilution (x axis) and CCR7, IL-7Rα, IL-2, CXCR3, IL-15R, and IFN-γ expression (y axes, as indicated). (B) Graphical representation of cytokine secretion as a function of division number. Data were gated on live cells and IL-2 and IFN-γ as follows: blue line, IL-2$^+$ IFN-γ$^-$ cells; green line, IL-2$^+$ IFN-γ$^+$ cells; red line, IL-2$^-$ IFN-γ$^+$ cells.

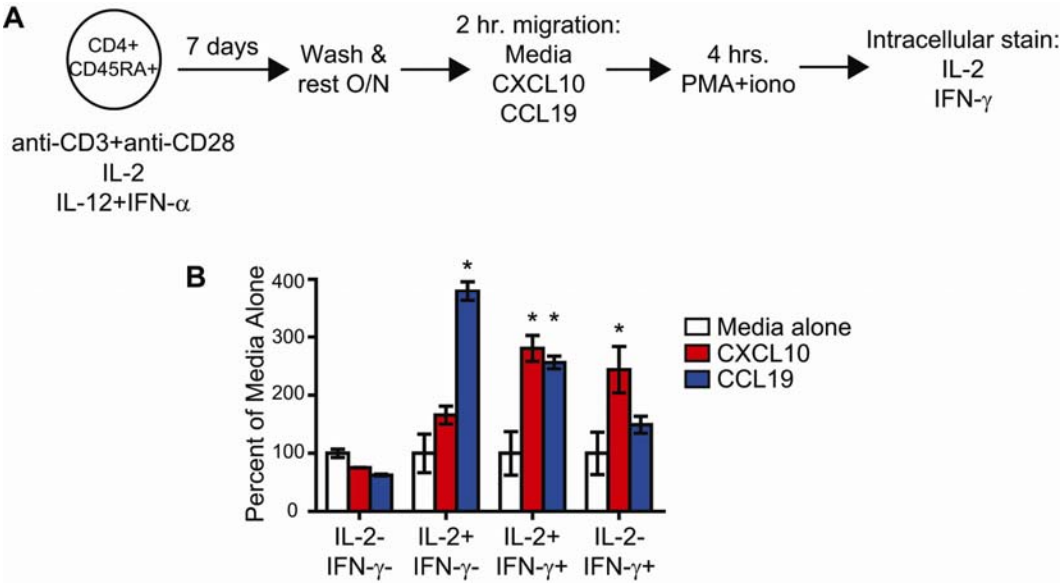

Figure 44. IL-2- and IFN-γ-secreting cells display differential migratory capacity to chemokine receptor ligands. Naïve human $CD4^+$ T cells were activated for 7 days with plate-bound anti-CD3 and anti-CD28 in the presence of rhIL-2, rhIL-12, rhIFN-αA, anti-hIFN-γ, and anti-hIL-4. On day 7, cells were washed and rested overnight in cIMDM in the absence of IL-2. A migration assay was performed for 2 hours in the presence of CXCL10 or CCL19. Cells were then washed and restimulated for 4 hours with PMA + ionomycin, and intracellular staining was performed for IL-2 and IFN-γ. (A) Flow chart of experimental design for chemotaxis experiments. (B) Graphical representation of the percentage of cytokine-producing cells which migrated in response to media alone (open bars), CXCL10 (red bars), or CCL19 (blue bars). *, $p < 0.05$ vs media alone.

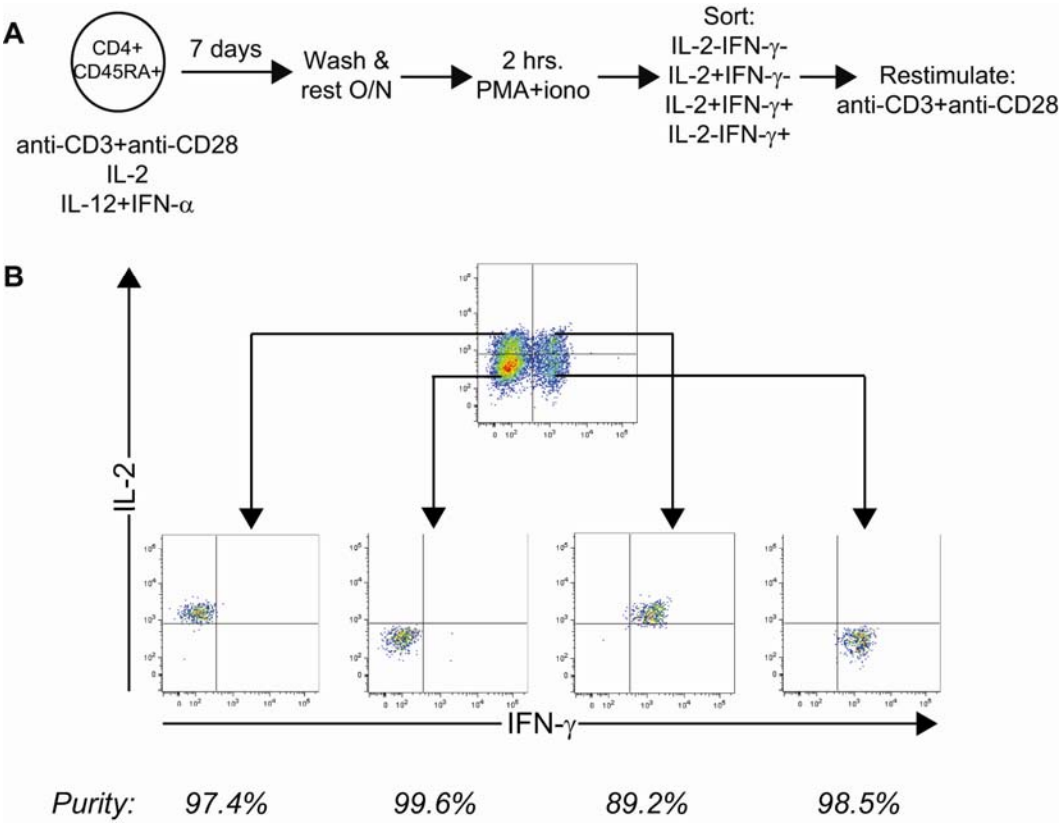

Figure 45. Isolation of IL-2- and IFN-γ-producing populations by live cell sorting. Naïve human $CD4^+$ T cells were activated for 7 days with plate-bound anti-CD3 and anti-CD28 in the presence of rhIL-2, rhIL-12, rhIFN-αA, anti-hIFN-γ, and anti-hIL-4. On day 7, cells were washed and rested overnight in cIMDM in the absence of IL-2. Cells were restimulated for 2 hours with PMA + ionomycin, and IL-2- and IFN-γ-producing cells were sorted using MACS Cytokine Secretion Assay kits. (A) Flow chart of experimental design for sorting experiments. (B) Typical post-sort analysis.

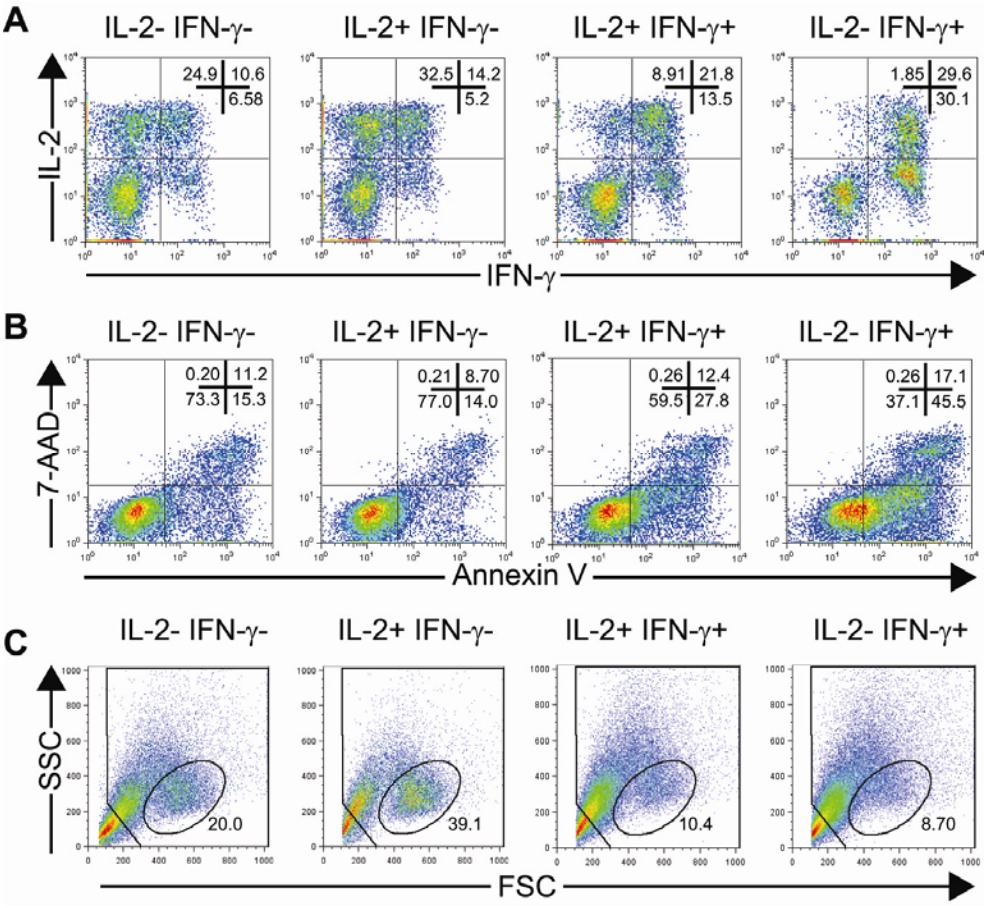

Figure 46. IL-2- and IFN-γ-secreting cells demonstrate functional characteristics of central memory and effector memory cells. Naïve human $CD4^+$ T cells were activated for 7 days with plate-bound anti-CD3 and anti-CD28 in the presence of rhIL-2, rhIL-12, rhIFN-αA, anti-hIFN-γ, and anti-hIL-4. On day 7, cells were washed and rested overnight in cIMDM in the absence of IL-2. Cells were restimulated for 2 hours with PMA + ionomycin, and IL-2- and IFN-γ-producing cells were sorted using MACS Cytokine Secretion Assay kits. (A) Cells were restimulated for 7 days with plate-bound anti-CD3 and anti-CD28 in the presence of rhIL-2, rhIL-12, rhIFN-αA, anti-hIFN-γ, and anti-hIL-4. On day 7, cells were washed and rested overnight in cIMDM in the absence of IL-2. Cells were restimulated for 4 hours with PMA + ionomycin, and intracellular staining was performed for IL-2 and IFN-γ. (B – C) Cells were restimulated for 3 days with plate-bound anti-CD3 + anti-CD28 in the presence of rhIL-2. On day 3, cells analyzed by flow cytometry for Annexin V and 7-AAD labeling (B) or the percentage of live cells by forward and side scatter (C).

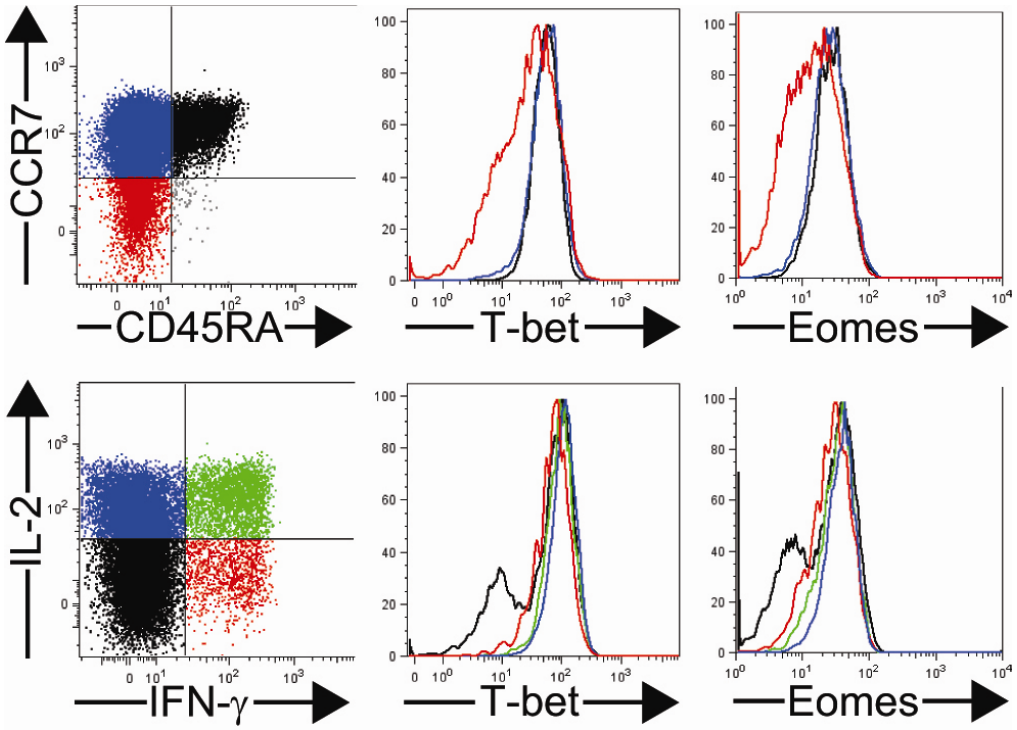

Figure 47. Expression of T-bet and Eomes does not correlate with memory phenotype or cytokine production. Naïve human $CD4^+$ T cells were activated for 7 days with plate-bound anti-CD3 and anti-CD28 in the presence of rhIL-2, rhIL-12, rhIFN-αA, anti-hIFN-γ, and anti-hIL-4. On day 7, cells were washed and rested overnight in cIMDM in the absence of IL-2. Cells were restimulated for 4 hours in the presence or absence of PMA + ionomycin, and intracellular staining was performed as indicated.

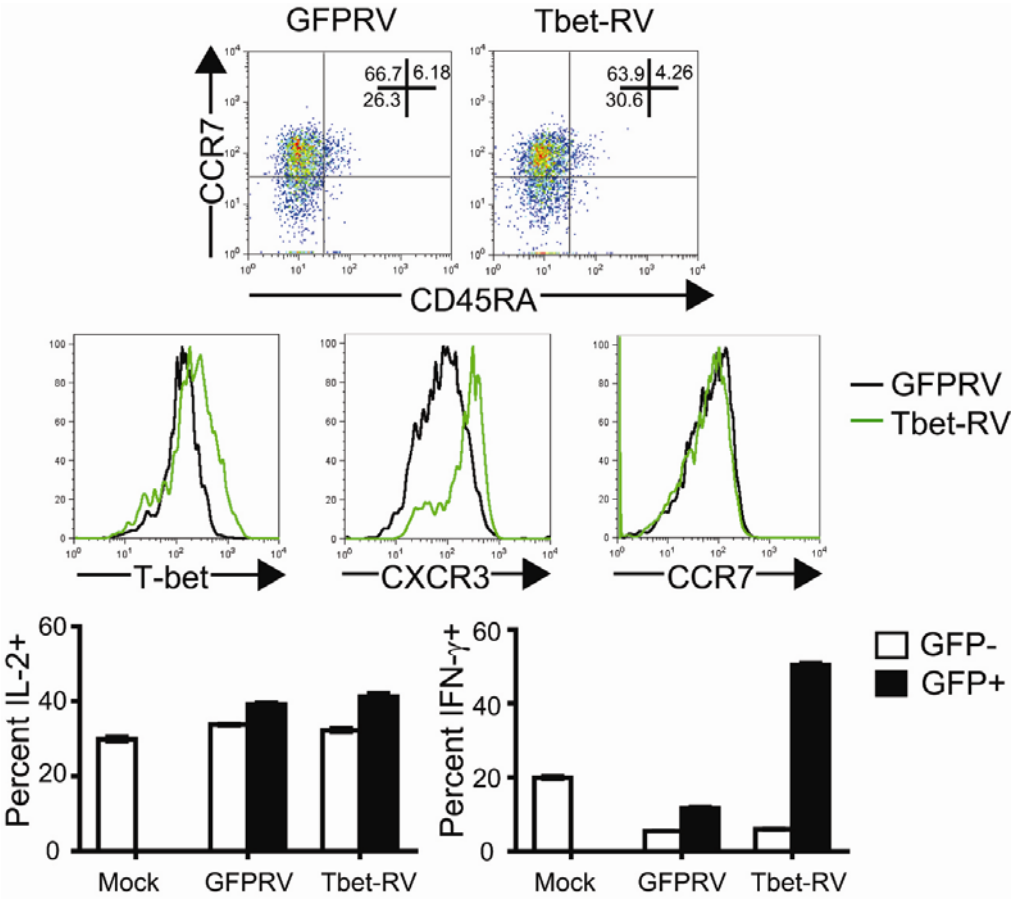

Figure 48. T-bet expression regulates effector but not memory phenotypes in human CD4$^+$ T cells. Naïve human CD4$^+$ T cells were activated overnight with plate-bound anti-CD3 and anti-CD28 in the presence of rhIL-2, anti-hIFN-γ, anti-hIL-4, anti-hIL-12, and anti-hIFNAR2. Transduction with GFPRV or T-bet-GFPRV was performed on three consecutive days, with rhIL-12 and rhIFN-αA added on days 2 and 3. On day 7, cells were washed and rested overnight in cIMDM in the absence of IL-2. Cells were restimulated for 4 hours in the presence or absence of PMA + ionomycin, and intracellular staining was performed as indicated in the figure. Dot plots and histograms were gated on GFP$^+$ cells. Histograms: Black, GFPRV; Green; Tbet-GFPRV. Bar graphs: Open bars, GFP$^-$ cells; filled bars, GFP$^+$ cells.

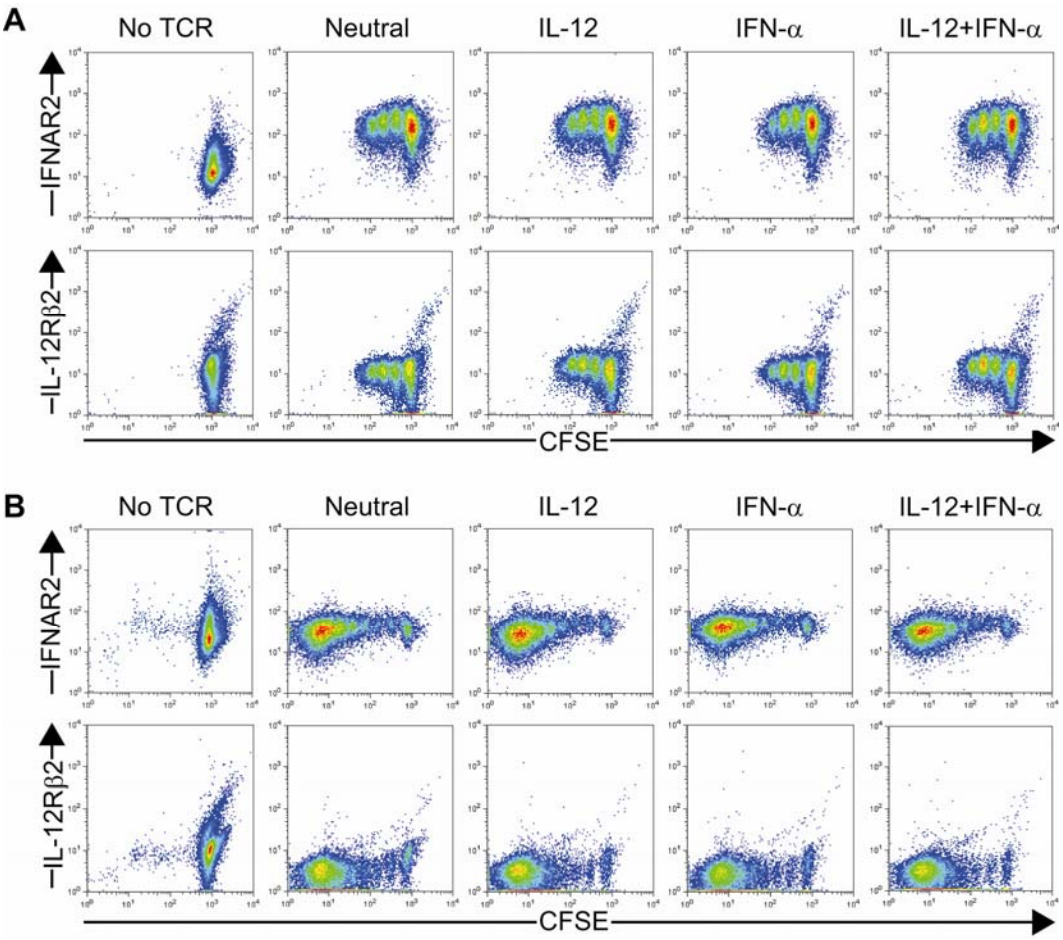

Figure 49. Expression of IL-12Rβ2 and IFNAR2 is regulated by TCR stimulation but not by innate cytokines. Naïve human $CD4^+$ T cells were labeled with CFSE and activated for 3 days (A) or 7 days (B) with plate-bound anti-CD3 and anti-CD28 in the presence of IL-2 and cytokines and neutralizing antibodies as indicated in the figure. "No TCR" indicates that the cells received neutralizing antibodies and IL-2 but not anti-CD3 + anti-CD28 stimulation. On day 3 or day 7, cells were washed and restimulated for 4 hours with PMA + ionomycin, and intracellular flow cytometry was performed as indicated.

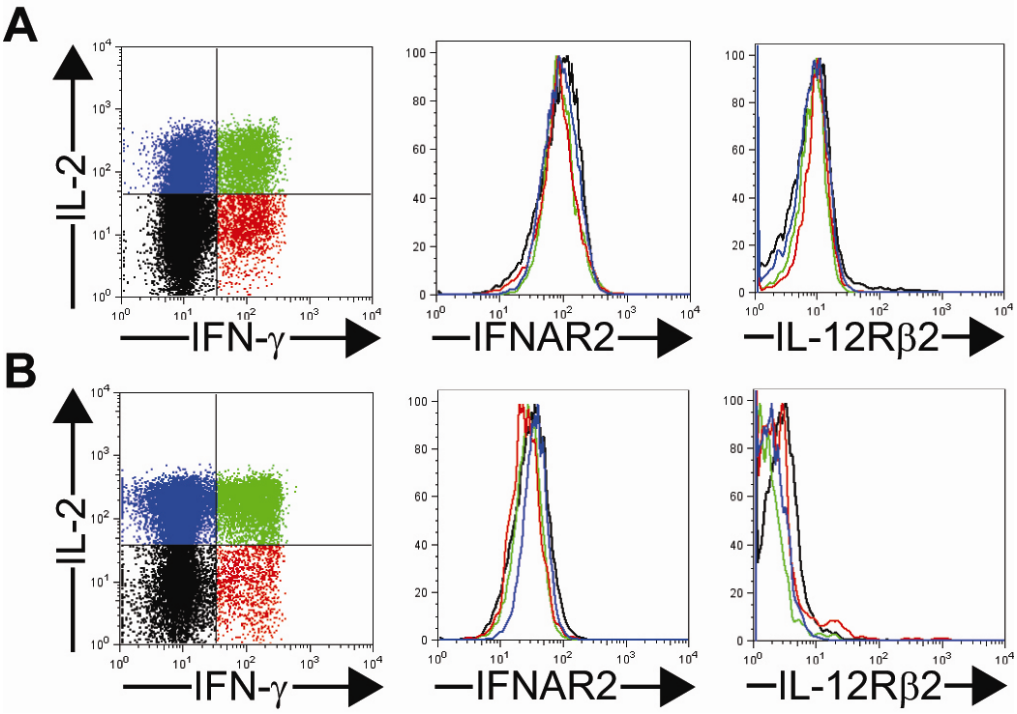

Figure 50. IL-2- and IFN-γ-producing populations do not differ substantially in expression of IL-12Rβ2 or IFNAR. Naïve human $CD4^+$ T cells were labeled with CFSE and activated for 3 days (A) or 7 days (B) with plate-bound anti-CD3 and anti-CD28 in the presence of IL-2 and cytokines and neutralizing antibodies as indicated in the figure. On day 3 or day 7, cells were washed and restimulated for 4 hours with PMA + ionomycin, and intracellular flow cytometry was performed as indicated.

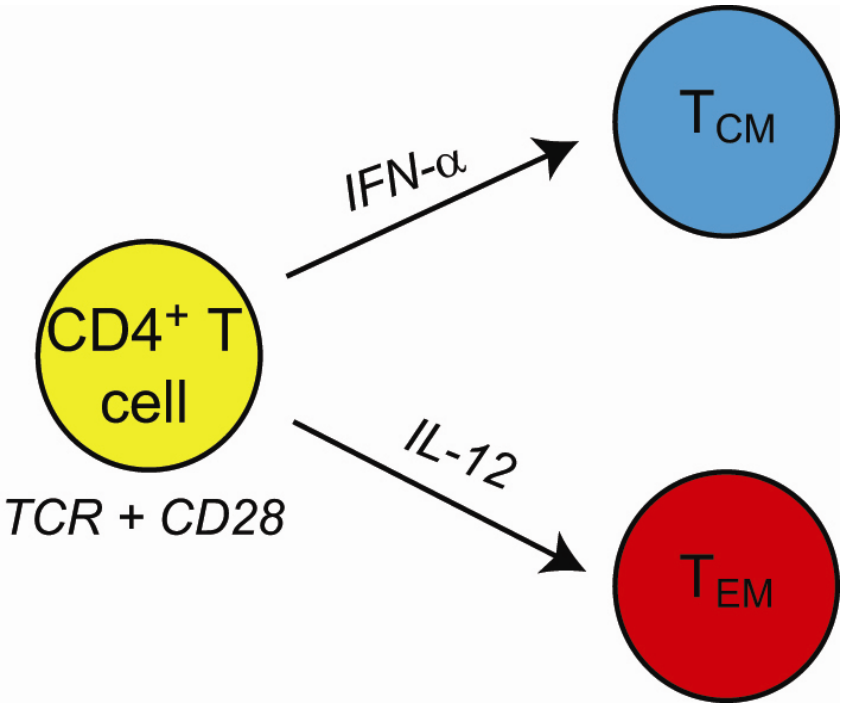

Figure 51. Type I IFN enhances the generation of IL-2-producing cells with a T_{CM} phenotype. $CD4^+$ T cells with a cell surface phenotype of T_{CM} or T_{EM} can develop even in the absence of innate cytokines. However, these cells are unable to secrete substantial amounts of IFN-γ or IL-2. In the presence of IL-12, memory generation is biased to T_{EM}, while a combined stimulus of IL-12 + IFN-α results in the generation of a larger population of IL-2-producing cells with T_{CM} phenotypes.

CHAPTER VII

DISCUSSION

Overview

Type I IFN is an innate cytokine produced to high levels by a variety of cells at the onset of intracellular infections. This cytokine has long been known to play a crucial role in innate host resistance to viruses. However, despite being exceptionally well-characterized and much-studied, the role of type I IFN in adaptive responses has remained difficult to decipher. The data which I have presented here have clarified several key points regarding the activities of IFN-α/β in regulating T helper cell development and function. First, in spite of signaling similarities with IL-12, IFN-α is insufficient to direct commitment to the Th1 phenotype because of qualitative differences in the activation of key intermediates. Second, IL-12, but not IFN-α, regulates the development of antiviral effector functions in human $CD4^+$ T cells; these effector functions are mediated by the synergistic actions of the Th1 cytokines IFN-γ and TNF-α and can proceed through a unique mechanism involving the type I IFN receptor. Finally, while IL-12 favors effector phenotypes, a combination of IL-12 and IFN-α enhances the generation of an IL-2-producing population of $CD4^+$ Th1 cells

with central memory characteristics. Given the predominance of type I IFN during antiviral responses, as well as the substantial interest in the medical use of IFN-α/β and agents which induce these cytokines, these data are likely to have an important impact on the design of novel vaccines and therapies.

Clarifying the role of IFN-α in Th1 commitment

Both IL-12 and IFN-α signal through type I transmembrane receptors by way of a pair of Janus kinases, and one member of the receptor-proximal signaling complex is shared between these two receptors (61-66, 105, 111). Given this, it is not surprising that these two cytokines are able to activate an overlapping set of downstream STAT transcription factors, including STAT4. However, the present work clearly demonstrates a substantial qualitative difference in STAT4 activation between IL-12 and IFN-α, and this distinction is borne out in differences in the induction of the T-bet transcription factor and the ability to promote Th1 commitment (254). At present, a biochemical explanation for this difference in signaling is unclear. It has been suggested that receptor-Janus kinase pairings could be specific for the functions which they regulate (340); if this is the case, the use of Jak1 rather than Jak2 by the IFNAR could affect STAT4 activation. Swapping the Jak binding domains of the IL-12R and IFNAR might lend some insight into this possibility (340). That is, if replacement

of Jak1 by Jak2 on the IFNAR increases the duration of STAT4 activation, this would indicate a substantial role for individual Janus kinase molecules in the receptor-specific activation of STATs.

Furthermore, the N-terminal domain of STAT4 is known to be latently preassociated with the IFNAR and IL-12R in unstimulated cells, and this preassociation is required for optimal signaling (135, 136, 341). It is possible that there are differences in the strength of preassociation of STAT4 with the IL-12R and IFNAR which could affect the membrane-proximal availability of this molecule and thus influence the kinetics of activation. A determination of the K_d of the association between each receptor and STAT4, while likely to be difficult as this interaction is relatively weak, could indicate whether there are differences between the IL-12R and IFNAR in this regard. Additionally, while STAT4 can directly interact with the IL-12R, STAT2 is required to mediate receptor docking and activation of STAT4 by type I IFNs (116, 130). Thus, it is also possible that STAT4 phosphorylation at the IFNAR is less efficient that activation by the IL-12R because of the greater separation from the IFNAR mediated by STAT2.

The suppressor of cytokine signaling (SOCS) molecules have also been suggested to play a role in the downmodulation of STAT activation in response to both IL-12 and IFN-α. Specifically, SOCS1 regulates both STAT1 and STAT4 phosphorylation in murine cells (342-344). However, Hilario Ramos found no differential expression of SOCS transcripts in human $CD4^+$ T cells in response to

IL-12 vs. IFN-α (254). Furthermore, the microarray data presented here showed the same conclusion; IL-4, but not IL-12 or IFN-α, mediated substantial upregulation of SOCS1. Therefore, it is unlikely that differential regulation of SOCS1 could play a role in the signaling differences between IL-12 and IFN-α in Th1 differentiation.

Given that antigen presentation and cytokine production by DCs is central to the generation of primary $CD4^+$ T cell responses, it was natural to deduce that IFN-α, which is produced by several DC subsets to high levels during viral infection, would be involved in the polarization of adaptive responses, particularly Th1 responses (17, 19-21). The apparent species-specific role of IFN-α in inducing Th1 differentiation was puzzling and gave rise to the hypothesis that mice provided an inaccurate model for human antiviral immunity (134). The work presented here clearly overturns the misconception that IFN-α directs Th1 commitment in human $CD4^+$ T cells (254). Furthermore, while type I IFN signaling has been demonstrated to be important during certain types of bacterial infections in mice (138, 139, 345), Berenson et. al. have definitively shown that IFN-α is biochemically insufficient to promote Th1 development in murine $CD4^+$ T cells (142, 143). Collectively, these findings demonstrate that, while IFN-α may participate in an ancillary role, IL-12 is the only innate cytokine known to direct complete Th1 differentiation in both human and murine T helper cells.

However, these data do not rule out the possibility of other species differences between mice and humans in adaptive antiviral responses. For instance, both human STAT2 and human IFNAR2 are divergent from their murine counterparts (130, 133, 134, 136). While these distinctions do not appear to mediate differences in the ability of human and murine IFN-α to regulate Th1 development, it is likely that there are other species-specific signaling differences which have not yet been investigated. In fact, microarray analysis of mice carrying a humanized knock-in version of the stat2 gene (m/hSt2ki) has shown that the human and murine C-termini of STAT2 mediate distinct patterns of gene expression following IFN-α stimulation (M. Persky and J. D. Farrar, personal communication). Further investigation of these and other species differences could lend important insights into the evolution of antiviral signaling pathways as well as improving our ability to model these responses in a laboratory setting.

Future exploration of the molecular signaling differences between IL-12 and type I IFN in T helper development could focus on the induction of downstream mediators along a time course of differentiation. The microarray experiment presented here has provided a snapshot of gene expression in fully polarized human $CD4^+$ T cells which were activated in the presence of various innate cytokines. However, a more thorough examination of transcript induction by IL-12 and IFN-α in cells from multiple human donors along several time points of differentiation could generate a more complete picture of the distinct

signaling cascades regulated by these two cytokines. This, in turn, might allow further insights into the differences and similarities between these two cytokines in the molecular regulation of $CD4^+$ Th1 development.

A new understanding of the role of $CD4^+$ T cells in antiviral immunity

The synergy of IFN-γ and TNF-α in antibacterial immunity is well known; secretion of these two cytokines by Th1 cells regulates a variety of inflammatory immune responses, including the oxidative burst response in phagocytic cells as well as upregulation of MHC II and $F_c\gamma R$ expression (149, 154, 156, 160, 164, 168, 169). Furthermore, IFN-γ is known to have a number of antiviral properties, such as activation of NK cells and upregulation of MHC I expression (160, 346). In addition to these important functions, my findings now demonstrate a direct role for the secretion of Th1 cytokines in viral clearance (270). However, the relative importance of this mechanism of antiviral immunity may be difficult to decipher. Studies *in vivo* in mice have also found a helper-independent role for $CD4^+$ T cells in viral clearance, but in these cases, the absence of $CD8^+$ T cells and B cells was often required in order to reveal this effect (192, 193, 195, 196, 198). Clearly, CTLs and neutralizing antibodies play a central role in mediating antiviral immune responses. However, in cases in which a viral pathogen is able to subvert or evade these responses, cytokine secretion by Th1 cells could provide

another line of defense. For instance, a number of viruses are able to downmodulate expression of MHC I, thereby avoiding detection by CTLs (347, 348). In this case, IFN-γ and TNF-α secretion by Th1 cells could inhibit viral infection as well as reversing the suppression of MHC I so that CTL responses could take effect.

Furthermore, the mechanism of the antiviral activity of IFN-γ and TNF-α against VSV is intriguing because it involves signaling of two cytokines through an unrelated receptor, the IFNAR. The most appealing explanation for this phenomenon was that these two cytokines could be inducing expression of type I IFN from infected cells; indeed, TNF-α is known to promote secretion of IFN-β during viral infections (181-185, 349). However, I was unable to detect the secretion of any known type I IFN from either differentiated Th1 cells or infected cells. In spite of this, it is possible that IFN-γ and TNF-α may be inducing the expression of a novel type I IFN. A number of novel type I IFN genes have been discovered in recent years, including IFN-κ, which is located distal to the other members of this gene family on human chromosome 9 (58-60). While a careful examination of the IFN locus itself did not reveal any promising candidates for unknown genes (J. D. Farrar, personal communication), there may be other, distantly related genes located elsewhere in the human genome. If this is the case, biochemical fractionation of VSV-infected, T cell conditioned media-treated target cells might yield secreted proteins of interest. Functional assays would

quickly identify the protein fraction of interest once IFN-γ and TNF-α were depleted. Alternately, microarray analysis of gene expression in TCM-treated target cells could provide candidate genes for novel secreted factors, although if the gene of interest has not yet been characterized, it could prove difficult to identify by this method.

A second, equally interesting possibility is that IFN-γ and TNF-α, or their receptors, are directly interacting with the IFNAR. There is precedent for receptor sharing in a number of biological systems, including cytokine signaling through γc (289, 290). Furthermore, signaling through the IFNAR is known to cross-regulate several other cytokine receptors, including the gp130 chain of the IL-6 receptor as well as the IFNγR (350-353). In fact, the IFNAR1 and IFNγR2 have been shown to physically associate in caveolar membrane domains of multiple cell types, and absence of type I IFN signaling results in reduced STAT1 phosphorylation by IFNγR (353). Additionally, TNF-α can cross-regulate several components of the IFN-γ and IFN-α signaling pathways, including Jak/STAT signaling and IRF-7 induction and activation (354-357). Direct interactions between individual chains of the IFNAR, IFNγR, and TNFR could be examined using a fluorescence resonance energy transfer (FRET) approach as well as by standard biochemical assays such as co-immunoprecipitation and yeast two-hybrid screening.

A unique role for IFN-α in the enhancement of CD4$^+$ T cell memory

While the γc cytokines IL-7 and IL-15 are central to CD4$^+$ memory T cell survival and homeostatic proliferation, they are unable to directly regulate the generation of the T_{CM} or T_{EM} phenotypes (231, 232). The work presented here demonstrates for the first time that an innate cytokine, IFN-α, can directly enhance the production of IL-2-secreting T_{CM} cells. IFN-α has previously been shown to be necessary for the clonal expansion and survival of activated CD4$^+$ as well as CD8$^+$ T cells during the primary response, such that mice lacking the IFNAR were unable to generate significant numbers of memory cells (234-237). My work now demonstrates that in addition to participating in the survival of activated CD4$^+$ T cells, IFN-α, in conjunction with IL-12, can also influence their function by increasing IL-2 production in a subset of developing Th1 cells.

This work also sets a precedent for the concept that innate cytokine signals can regulate the generation of adaptive memory responses. IFN-α is only induced during certain classes of immune responses, but IL-2-secreting memory CD4$^+$ T cells are generated under a variety of conditions (202, 277). Thus, it is likely that other cytokines are also involved in the enhancement of memory responses. In particular, type 2 responses are characterized by substantial production of IL-4 but

little IL-12 or type I IFN (261). The work presented here indicates that IL-4 does not induce elevated IL-2 secretion by human $CD4^+$ T cells. However, it would be of interest to determine whether activation of human $CD4^+$ T cells in the presence of IL-4 could influence any other phenotypic characteristics of memory cells.

Furthermore, both $CD4^+$ T cells and IL-2 have been shown to play a role in promoting the efficient generation of memory $CD8^+$ T cell responses. While the role of $CD4^+$ T cell help in $CD8^+$ T cell development remains controversial, several groups have provided evidence that $CD4^+$ T cells are important for optimal CTL responses against certain types of infections (175, 176, 273-276, 358, 359). Furthermore, Bevan and colleagues demonstrated an indispensable role for IL-2 in the generation of secondary, but not primary, $CD8^+$ T cell responses (360). This group also suggested that $CD4^+$ T cells were a likely candidate for the source of the essential IL-2 signal. The work presented here now adds a role for IFN-α in inducing optimal IL-2 production by T helper cells during viral infections. Along these lines, it is of interest to note that RSV has been shown to induce much lower levels of IFN-α production than other RNA viruses, and this virus also promotes only low levels of $CD8^+$ T cell memory (361-364). Furthermore, vaccination with formalin-inactivated RSV gives rise to a pathological Th2-mediated inflammation upon infection with live RSV, a process known as vaccine-enhanced disease (362, 365). Vaccine-enhanced disease is associated with lung eosinophilia, substantial production of IL-4, IL-5,

and IL-13 in the lung, and the absence of RSV-specific $CD8^+$ T cell memory. Exogenously provided IL-2 can promote a productive $CD8^+$ T cell response which suppresses vaccine-enhanced disease, suggesting that type 1 $CD4^+$ T cell responses which include substantial IL-2 secretion could participate in the generation of protective anti-RSV memory responses (366).

Unfortunately, no molecular mechanism could be elucidated for the enhancement of $CD4^+$ T cell central memory phenotypes by IFN-α. It was surprising to find that T-bet and Eomes were not involved given the critical importance of these two transcription factors for $CD8^+$ T cell memory generation (332-334). However, $CD4^+$ and $CD8^+$ T cells have been observed to differ in other aspects of memory formation (224, 367). Furthermore, T-bet has previously been reported to suppress IL-2 production and T_{CM} generation in murine T helper cells (107, 331). In contrast, I have shown that in $CD4^+$ T cells, T-bet clearly controls effector phenotypes, but the factor or factors regulating IL-2 production and memory generation remain to be elucidated. A detailed examination of the IL-2 promoter might yield clues regarding transcriptional regulators which could play a role in this process. Additionally, microarray analysis of $CD4^+$ T cells with T_{CM} and T_{EM} phenotypes could provide information regarding differential expression of transcription factors between these two subsets. Any transcriptional changes of interest could then be examined for potential regulation by type I IFN. Indeed, Riou et. al. have examined transcriptional differences between human

$CD4^+$ T_{CM} and T_{EM} cells; this group found substantial differences among genes involved in cell cycle progression and cell survival and apoptosis (317). One caveat to this approach is that signaling differences which rely upon posttranslational modifications such as phosphorylation cannot be detected by microarray; for instance, Riou et. al. also found an important difference in phosphorylation of the proapoptotic transcription factor Foxo3a between T_{CM} and T_{EM} cells, even though the absolute level of Foxo3a expression did not differ between these subsets (317).

No substantial differences in IL-12R or IFNAR expression were noted among IL-2- and IFN-γ-producing cells, indicating that differential responsiveness to innate cytokines is not playing a role in the generation of T_{CM} vs. T_{EM} cells. It is possible that one or more ISGs induced by IFN-α in the presence of IL-12 could be regulating memory phenotypes, and particularly IL-2 production, in a subset of developing Th1 cells. By microarray analysis, a number of ISGs were found to be upregulated in human $CD4^+$ T cells differentiated in the presence of a combination of IL-12 and IFN-α. Furthermore, the function of many known ISGs has yet to be elucidated, suggesting that one or more of these genes could definitely be involved in regulating memory formation.

The microarray experiment presented here was conducted on whole cultures of cells, whereas the T_{EM} and T_{CM} phenotypes which I have described arise simultaneously as subpopulations within the same culture. Therefore, it is

likely that critically important gene expression changes within these individual subsets were masked by the heterogeneity of the *in vitro* culture. In order to probe the gene expression profiles induced by IL-12 and IFN-α within developing T_{CM} and T_{EM} subpopulations, it will therefore be necessary to conduct microarray analyses on sorted IL-2- and IFN-γ-producing cells.

TCR signal strength has also been proposed to play a role in both the generation of Th1 and Th2 effector phenotypes as well as in the development of central and effector memory subsets. Specifically, weaker signals from exhausted DCs at the decline of an infection are thought to favor the development of non-terminally differentiated central memory populations (214-216, 368). Furthermore, Reiner and colleagues have proposed a model of asymmetric cell division for the generation of $CD8^+$ T cell memory, in which the daughter cell distal to the T cell – DC interaction receives a weaker signal and develops T_{CM} characteristics (213). In agreement with these findings, I have demonstrated that even in the absence of all innate cytokine signaling, $CD4^+$ T cells with both T_{CM} and T_{EM} phenotypes arise *in vitro*. It is possible that IFN-α may further modulate TCR signal strength and thereby favor a T_{CM} outcome in a subset of cells. In this vein, chemokine and cytokine receptors have been shown to interact with the TCR signaling complex and thereby modify the outcome of TCR stimulation. For instance, CXCR3 and CXCR4 physically associate with the TCR complex and utilize downstream TCR signaling components such as ZAP70, and stimulation of

$CD4^+$ T cells with the cognate chemokine ligands CXCL10 or CXCL12 enhances TCR-mediated events such as IL-2 production (369-372). On the other hand, TNF-α can modulate TCR signaling by suppressing expression of CD3ζ, resulting in inhibition of IL-2 secretion (373). Along these lines, it is of interest to note that a larger proportion of cells are retained as naïve ($CD45RA^+$ $CCR7^{hi}$) in cultures differentiated in the presence of IFN-α. However, IL-12- and IFN-α-stimulated cells do not differ in CFSE dilution, indicating that IFN-α does not retard $CD4^+$ T cell proliferation. Thus, any interaction of IFNAR signaling with TCR stimulation is likely to be complex. Initial analyses of this possibility might focus on the ability of the IFNAR to physically associate with components of the TCR complex by co-immunoprecipitation or fluorescence microscopy. Further investigations could probe whether the IFNAR shares downstream signaling components with the TCR complex, which could provide an explanation for the observed enhancement of IL-2 production.

It should also be noted that the data generated here support a model of simultaneous, rather than linear, development of T_{CM} and T_{EM} populations. Based upon these results, it does not appear that T_{CM} cells must pass through an effector phase before acquiring memory capabilities as cells with T_{CM} phenotypes could be observed at all points of division, whereas cells bearing effector markers were only evident at maximal division. This same observation has also been made *in vivo* in a murine model of influenza infection (330). Furthermore, Wu et. al. have

reported that IFN-γ^-, but not IFN-γ^+, antigen-specific $CD4^+$ T cells could provide protection from infection when transferred into naïve hosts (212). I have also shown that human $CD4^+$ T cells which could secrete only IFN-γ were unable to give rise to IL-2-producing T_{CM}-type cells, indicating that full effector differentiation is likely to be a terminal phenotype. In contrast, and in agreement with other reports, IL-2-secreting cells were able to survive reactivation and could generate a new population of effectors, as defined by IFN-γ production (202, 204, 328).

The results presented here provide a foundation for examination of the effects of innate cytokines on memory responses to viral infection *in vivo* in a murine model. Adoptive transfer of small numbers of congenically marked, antigen-specific wild-type or $IFNAR^{-/-}$ $CD4^+$ T cells would allow investigation of the role of type I IFN signaling in the generation of memory responses in an otherwise normal animal. In contrast, previous work examining the importance of IFN-α in promoting $CD4^+$ T cell memory made use of animals which were deficient for expression of IFNAR on all cells, such that the individual role of type I IFN signaling on T helper cells might have been difficult to elucidate (234-237). Furthermore, co-transfer of antigen-specific $CD4^+$ and $CD8^+$ cells would permit the analysis of the importance of type I IFN signals in the generation of productive $CD4^+$ T cell help for $CD8^+$ memory generation *in vivo*.

Relevance of the present work to human health and disease

It should be noted that all of this work was performed using primary human $CD4^+$ T cells. Given the potential for species differences between murine and human cells in the biochemical responses to innate signals, I found it important to use the most physiologically and medically relevant system available. While all of these experiments were performed *in vitro* using polyclonal activation, the focus of my studies was not on the effects of TCR activation itself but rather on the specific roles of innate cytokines in T helper cell development. Hence, this work came as close as is possible to modeling the developmental processes of human $CD4^+$ T cells in response to biological signals present during viral infections. Therefore, these findings are likely to provide important insights with direct relevant to human health.

IFN-α is currently used as a therapy for chronic HCV infection, either alone or in combination with the antiviral drug ribavirin, but only a fraction of patients respond successfully to treatment (239, 241). Obviously this cytokine is crucial for innate host resistance to viral infection, and innate mechanisms are likely to be one primary mode of action of IFN-α when used in medical settings. Furthermore, IFN-α activates $CD8^+$ T cells and NK cells, two cell types which are responsible for lysis of virally infected cells (233, 266, 267). It was thought that

IFN-α also promoted Th1 responses, which could have played a supporting role in adaptive immunity to chronic viral infections (14, 15, 114). However, based on my work, this does not appear to be the case. It may be that, in addition to mediating innate host responses, IFN-α partially alleviates the functional exhaustion of $CD4^+$ and $CD8^+$ T cells that is characteristic of chronic viral infections. T cell exhaustion is characterized by elevated expression of the pro-apoptotic cell surface marker PD-1 and progressive loss of cytokine production and, in the case of $CD8^+$ T cells, loss of cytotoxic function (374-378). However, $CD4^+$ T cells can alleviate $CD8^+$ T cell exhaustion, and this may occur partly through IL-2 secretion (359, 376, 379). Thus, IFN-α treatment could enhance IL-2 secretion by $CD4^+$ T cells, aiding in the restoration of anti-HCV CTL responses.

A gene expression "signature" of ISGs has been noted in peripheral blood of patients suffering from inflammatory autoimmune disorders such as rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE), and IFN-α has now been implicated in the pathogenesis of these diseases (380, 381). In contrast, IFN-β is used to treat the autoimmune disorder multiple sclerosis (MS), but the mechanism of action has not been clearly elucidated (382). Since type I IFN was thought to promote Th1 responses, which are believed to play a pathogenic role in autoinflammatory processes, the involvement of IFN-α in SLE and RA seemed logical, whereas the therapeutic benefits of IFN-β treatment were

somewhat paradoxical. However, my data shows that type I IFN does not promote Th1 development or effector responses in human $CD4^+$ T cells. Hence, IFN-α and IFN-β likely do not, in fact, induce pathogenic inflammatory T cells. Type I IFN has also been reported to induce IL-10 production from human T helper cells; thus, IFN-β might have acted to promote an anti-inflammatory environment (268). However, I did not observe any consistent trend for IFN-α to induce elevated IL-10 secretion, calling this theory into question as well. Thus, while some potential roles of type I IFN in autoimmune disorders have been ruled out, the involvement of these cytokines, and their mechanism of action, remain unclear.

The TLR ligand CpG DNA, a TLR9 agonist, is currently under investigation as a vaccine adjuvant. Vaccination of mice using CpG as an adjuvant results in improved recall responses (244-248). Importantly, TLR9 stimulation is known to result in the secretion of both IL-12 and type I IFN (13). As I have shown, this combination of cytokines favors efficient generation of both effector and memory $CD4^+$ T cell responses as well as promoting elevated levels of IL-2 production to support $CD8^+$ T cell development. Hence, the induction of IFN-α may be an important facet to consider in rational vaccine design. This observation could warrant further investigation by administration of IFN-α in combination with traditional adjuvants upon experimental vaccination of mice or by blockade of type I IFN signaling during vaccination. Based on my findings, it

could be predicted that administering IFN-α during priming might provide a more favorable memory pool, whereas lack of type I IFN signaling would have the opposite effect.

Furthermore, based upon the linear model of memory development, it has been proposed that strong stimulation to generate a large pool of highly active effector cells is the best way to promote memory generation (218, 220). In contrast, my data suggest that long-lived memory cells cannot develop from terminally differentiated effectors, so the generation of a large population of these cells likely does not represent an effective vaccine strategy. Instead, a vaccine which delivers a range of signal strengths might provide the optimal mixture of effector and memory responses to combat both primary and secondary infections.

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