REGULATION OF HUMAN CYTOTOXIC T LYMPHOCYTE FUNCTIONS BY IL-12

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DEDICATION

In ever so loving memory of my friend, Nabil

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REGULATION OF HUMAN CYTOTOXIC T LYMPHOCYTE FUNCTIONS BY IL-12

by

FATEMA ZAHRA CHOWDHURY

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REGULATION OF HUMAN CYTOTOXIC T LYMPHOCYTE FUNCTIONS BY IL-12

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$CD8^+$ T cells or cytotoxic T lymphocytes (CTLs) play a major role in our defense against intracellular pathogens by secreting effector cytokines and directly killing infected cells. Upon sensing of pathogen, antigen presenting cells secrete innate cytokines such as IL-12 and IFN-α. CTL functions are specified by both antigen recognition and innate cytokines yielding a diverse population comprised of short-term effectors and long-lived memory cells. We have demonstrated that IL-12, in particular, programs effector CTL differentiation in human. Our objective was to elucidate the key pathways programmed by IL-12 that leads to effector function.

Using whole transcriptome analyses, we have identified a serine/threonine protein kinase MAP3K8 or Tpl2 to be selectively induced by IL-12. Furthermore, the functionally identified effector memory CTL population expressed higher levels of MAP3K8 mRNA *ex vivo* compared to the naïve/central memory CTL population. MAP3K8 or Tpl2 has been shown to play an important role in activating the MAP kinase pathways in innate immune cells such as macrophages. MAP kinase pathways are three-tiered kinase cascades that coordinate many different cellular responses by relaying the instructions from extracellular signals. Using specific small molecule inhibitors, we have demonstrated that MAP3K8/Tpl2 in human CTLs functions upstream of MEK/ERK MAP kinase pathway, leading to effector functions. An inhibitor for MAP3K8/Tpl2 blocked IFN-γ and TNF-α secretion as well as cytolysis of target cells in a dose-dependent manner. However, MAP3K8/Tpl2 deficient murine CTLs did not exhibit any functional deficiency either *in vitro* or *in vivo*. Furthermore, we have found that the Tpl2 inhibitor did not block IFN-γ and TNF-α secretion from murine effector CTLs. In summary, we found that IL-12, not IFN-α, programs effector function in human CTLs at the genetic level. Additionally, MAP3K8/Tpl2, a member of the MAP kinase pathway, is regulated by IL-12 during CTL priming in human and is a critical regulator of antigen receptor-mediated effector functions. Taken together, we have discovered a species-specific role for IL-12 regulated MAP3K8/Tpl2 in effector function of human CTLs, which plays a major role in adaptive immune responses to intracellular pathogens and tumors.

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LIST OF ABBREVIATIONS

Ab – antibody

AICD – activation induced cell death

AP-1 – activation protein-1

APC – allophycocyanin (for flow cytometry)

APC – antigen presenting cell

BHI – brain heart infusion

BSA – bovine serum albumin

CCR – chemokine receptor

ChIP – chromatim immunoprecipitation

CD – cluster of differentiation

cDC – conventional dendritic cell

cDMEM – complete Dulbecco's modified eagle medium

cDNA – complementary DNA

CFSE – carboxyfluorescein diacetate succinimidyl ester

CFU – colony forming unit

cIMDM – complete Iscove’s modified Dulbecco’s medium

COT – cancer Osaka thyroid

CTL – cytotoxic T lymphocyte

DC – dendritic cell

DNA – deoxyribonucleic acid

dpi – days post infection

ds – double-stranded

eIF2α – eukaryotic initiation factor 2α

Eomes – eomesodermin

ELISA – enzyme-linked immunosorbent assay

ERK – extracellular signal-regulated kinase

FACS – fluorescence-activated cell sorting

FBS – fetal bovine serum

FITC – fluorescein isothiocyanate

GAS –IFN-γ activation sites

h – human

HKLM – heat killed *Listeria monocytogenes*

HRP–horseradish peroxidase

IC – intracellular

IFN – interferon

IFNAR – interferon-α/β receptor

IFNLR – interferon-λ receptor

Ig – immunoglobulin

IKK – inhibitor of κB kinase

IL – interleukin

iNOS – inducible nitric oxide synthase

IR – infrared

IRF – interferon regulatory factor

ISG – interferon-stimulated gene

ISGF3 – interferon stimulated gene factor 3

ISRE – interferon stimulated response element

JAK – Janus kinase

KO – knock out

LM – *Listeria monocytogenes*

LM-OVA – ovalbumin expressing *Listeria monocytogenes*

LN – lymph nodes

LPS – lipopolysaccharide

MAP kinase – mitogen-activated protein kinase

MΦ – macrophage

MHC – major histocompatibility complex

Mx – myxovirus resistance protein

MyD88 – myeloid differentiation factor 88

NADPH – nicotinamide adenine dinucleotide phosphate

NF-κB – nuclear factor κB

NK – natural killer cell

NO – nitric oxide

NOD – nucleotide-binding oligomerization domain

OVA – ovalbumin

p35 – 35 kDa subunit of the IL-12 cytokine

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

p70 – 70 kDa complete IL-12 cytokine

PBMC – peripheral blood mononuclear cell

PBS – phosphate buffered saline

PBSE – pacific blue succinimydyl ester

PCR – polymerase chain reaction

PE – phycoerythrin

PMA – phorbol 12-myristate 13-acetate

qRT-PCR – quantitative real-time polymerase chain reaction

rh – recombinant human

rm – recombinant mouse

RIG-I – retinoic acid inducible gene I

RIPA – radioimmune precipitation assay

SA – streptavidin

7AAD – 7-amino-actinomycin

SEM – standard error of mean

SD – standard deviation

Signal 1 – TCR activation of T cells

Signal 2 – costimulation of T cells

Signal 3 – innate cytokine activation of T cells

STAT – signal transducer and activator of transcription

T-bet – T-box expressed in T cells, TBX21

T_{CM} – central memory T cells

T_{EM} – effector memory T cells

T_N – naïve T cells

TCR – T cell receptor

Tc1 – T cytotoxic type 1

Tc2 – T cytotoxic type 2

Th1 – T helper type 1

Th2 – T helper type 2

TLR – Toll-like receptor

TNF – tumor necrosis factor

Tpl2 – tumor progression locus 2

U – units

WT – wild type

CHAPTER ONE

INTRODUCTION AND LITERATURE REVIEW

Part of this chapter is adapted from a review article published in JAK-STAT, volume 2, issue 1 (Chowdhury and Farrar, 2013). Published by Landes Bioscience and published under the Creative Commons Attribution-NonCommercial 3.0 Unported License. Used with permission.

Overview

The immune system is comprised of varieties of cell types that vary in their development and function. The innate immune system is our first line of defense in the protection against pathogens and other environmental assaults. In case of pathogens, the cells of innate immunity can recognize the presence of pathogens via the pattern recognition receptors (PRR). This recognition leads to a state of inflammation that directs the body to wage a battle against the invading organisms. Expression and secretion of soluble chemical signals, known as chemokines and cytokines, can usher in and activate more innate immune cells. The cells of adaptive immune system can be alerted by the inflammatory cytokines and thus recruited to the site of infection for further assistance in eliminating the pathogen. The cytokines secreted by the innate immune system can also educate the adaptive immune system in executing the appropriate mechanism of action. Two such cytokines,

interleukin-12 (IL-12) and interferon-α (IFN-α), have been shown to be able to program T cell effector functions in mice and human. Although the adaptive immune system constitutes of both T and B lymphocyte, I will focus on T lymphocyte biology and more specifically $CD8^+$ T cells in this dissertation. The main goal of my project was to investigate the role of innate inflammatory cytokines in programming effector function of this particular subset of adaptive immune cell, $CD8^+$ T cell or cytotoxic T lymphocytes (CTL).

Innate recognition of pathogens leads to inflammation

Sentinel cells of the innate immune response such as dendritic cells (DCs) patrol the peripheral tissues for potential pathogenic invasion (Palucka et al., 1999). These cells can detect pathogens of viral, bacterial, and parasitic origins by recognition of conserved pathogen-associated molecular patterns (PAMPs) via PRRs (Janeway and Medzhitov et al., 2002). Mammalian PRRs include cell surface and endosomal transmembrane receptors known as Toll-like receptors (TLRs) as well as cytoplasmic sensors such as nucleotide-binding oligomerization domain (NOD) receptors and retinoic acid-inducible gene I (RIG-I) (Inohara et al., 2001; Iwasaki and Medzhitov, 2004; Takeuchi and Akira 2008). Each of these PRRs can bind to their cognate PAMP and trigger a cascade of intracellular signaling events leading to maturation and differentiation of the DCs (van Vliet et al., 2007). Upon sensing the presence of pathogens, APCs can initiate the ‘danger signal’ by

secretion of pro-inflammatory cytokines such as IL-6, IL-8, IL-1β, TNF-α, IL-12, and, in the case of viral infections, IFN-α/β. These pro-inflammatory cytokines can recruit and activate other members of the innate immune response such as neutrophils and macrophages that act early in the response to limit the infection (Harada et al., 1994; Kaplanski et al., 2003; Murphy et al., 2012). Additionally, IL-12 and IFN-α have been shown to influence the adaptive immune response by inducing type I response marked by IFN-γ production. Thus, innate immune system can initiate inflammatory response leading to recruitment and activation of adaptive immune response.

IL-12 and IFN-α/β are important anti-microbial cytokines

Monocytes, dendritic cells, and macrophages are the major producers of IL-12 upon PRR activation (Macatonia et al., 1995; Gautier et al., 2005). IL-12 is composed of p35 and p40 subunits that form the active p70 heterodimer prior to secretion (Kobayashi et al., 1989; Trinchieri et al., 2003). IL-12 binds to the heterodimeric receptor composed of two type I transmembrane glycoprotein chains, IL12Rβ1 and IL12Rβ2 (Perskey et al., 1996). Co-expression of both chains is required for the generation of high affinity IL-12-binding sites, and the IL-12Rβ2 subunit functions as the signal-transducing component of the high-affinity receptor complex. Binding of IL-12 to the IL-12R transduces downstream signaling events starting with the induction of tyrosine phosphorylation of JAK2 and TYK2 (Trinchieri et al., 2003). IL-12Rβ2 is thus phosphorylated and serves as a docking site for

STAT4. STAT4 binds to the receptor chain and is phosphorylated. STAT4 homodimers then translocate to the nucleus where they bind to STAT binding sites in the IFN-γ promoter and induces gene expression (Trinchieri et al., 2003).

The anti-microbial effects of IL-12 are propagated via its influences on NK and T cells to make IFN-γ. IL-12 plays a critical role in resistance to most bacteria, intracellular protozoa, and fungal infection by inducing Th1 differentiation (Trinchieri et al., 1998). For example, IL-12 is required for immunity to *Toxoplasma gondii* and *Leishmania major* (Yap et al., 2000; Park et al., 2000; Wilson et al., 2010). IL-12 was shown to directly inhibit IL-4 production in $CD4^+$ and promote IFN-γ in both $CD4^+$ and $CD8^+$ T lymphocytes (Curtsinger et al., 1999; Curtsinger et al., 2003; Manetti et al., 1993; Macatonia et al., 1995). In addition, IL-12 also promotes cytotoxic capabilities of both NK cells and CTLs via induction of perforin and granzyme B (Trinchieri et al., 2003; Curtsinger et al., 2005). Therefore, IL-12 is an important innate pro-inflammatory cytokine that plays a critical role in our immunity to infections.

Interferons were first identified in the 1950s for their ability to 'interfere' with viral replication (Isaacs and Lindenmann, 1957; Issacs et al., 1957). Type I interferon or IFN-α/β is one such anti-viral cytokine produced from virally infected cells. Upon recognition, viral infections trigger a cascade of intracellular events that lead to the production and secretion of these anti-viral cytokines (Colonna et al., 2004; O'Neil et al., 2010). In human, predominant producer of IFN-α/β are the plasmacytoid DCs or

pDCs (Barcher et al., 2005). IFN-α/β inhibits viral replication within the infected cells and creates a state of resistance in non-infected cells, effectively limiting viral spread.

In humans, type I interferons are encoded by ~16 α genes and individual genes encoding β, κ, ω and ε. Their gene products are highly structurally related and all bind to a single receptor (IFNAR) consisting of heterodimeric R1 and R2 subunits (Stark et al., 1998). IFNAR recruit and activate STATs 1, 2 and 3. While STATs 1 and 3 are promiscuously activated by a variety of other cytokines and growth factor receptors, STAT2 is selectively recruited to the IFNAR, as well as to the IFNLR in response to type III interferon known as IFN-λ. STAT2 recruitment and activation by both receptors involves tyrosine phosphorylation of STAT2 by JAK kinases and subsequent oligomerization with STAT1 and IRF-9 (Horvath et al., 1996; Bluyssen et al., 1997). The canonical interferon-stimulated gene factor-3 (ISGF3) complex of STAT2:STAT1:IRF-9 regulates a large fraction of the interferon pathway genes (Brierley et al., 2005).

Type I interferon regulates the expression of interferon stimulated genes (ISGs) (Wadell et al., 2010). Some examples include 2–5'-oligoadenylate synthase (OAS), which decorates viral RNAs with branched polyadenosine, and RNA endonucle-ase L, which promptly degrades RNAs containing these poly-adenosine modifications (Silverman et al., 2007). Most ISGs, however, have not been well characterized. Over 300 ISGs have been identified by microarray and genomic

analysis, which is curious given the magnitude of antiviral activity exhibited by select individual ISGs (de Veer et al., 2001; Sadler et al., 2008). For example, MxA potently inhibits the replication of a wide range of viruses and can do so in the absence of other ISGs or interferon signaling if ectopically expressed (Haller et al., 2011). Not all ISGs exhibit such robust effects individually; nonetheless, when combined with other ISGs, the host can mount an impressive arsenal of antiviral proteins that could conceptually block every step in the viral life cycle (Schoggins et al., 2011). In addition to its role in interferon mediated antiviral effect, IFN-α/β confers anti-proliferative and apoptotic activities on a variety of cell types via the STAT2 (Steen et al., 2012). This activity is perhaps one of the most fundamental actions of interferon to inhibit viral spread by blocking the replication of virally infected cells. Additionally, much attention has been focused on the use of IFN-α/β to inhibit cancer outgrowth and metastasis given its unique ability to inhibit proliferation of a variety of tumor cell types (Gamero et al., 2006; Maher et al., 2007). Thus, type I interferon is an important antiviral cytokine with diverse effects on cell proliferation and immunity.

Roles of IL-12 and IFN-α in providing 'signal 3' for CTL programming

$CD8^+$ T cells or cytotoxic T lymphocytes (CTLs) are essential for immunity against intracellular pathogens and even tumors. For example, protection against malaria and *Mycobacterium tuberculosis*, as well as viral infections such as Epstein-Barr virus and cytomegalovirus require efficient CTL response (van Leeuwen et al.,

2006; Schmidt et al., 2008; Einarsdottir et al., 2009; Reyes-Sandoval et al., 2011;). Naïve CTLs require three distinct signals for complete activation, expansion, and differentiation (Curtsinger et al., 2003; Mescher et al., 2006). Signal 1 comes from T cell receptor (TCR) engagement while co-stimulation through CD28 provides signal 2 (Figure 1.1). DCs can present antigen in the context of MHC I to engage TCR and provide co-stimulation via CD80/86 (on DCs) and CD28 (on CTLs) interaction within the immunological synapse. PRR pathway activation can up-regulate the MHC molecules as well as up-regulate the expression of CD80/86 on activated DCs, enabling more efficient signal 1 and 2. These two signals together propagate instructions for activation and proliferation of CTLs. A third signal comes from the innate inflammatory cytokines secreted by the mature DCs that program the CTLs to become functional (Curtsinger et al., 1999). In the absence of this third signal CTLs have been shown to become anergic and tolerant (Curtsinger et al., 2003; Curtsinger et al., 2005). All three signals combined, promotes a number of signaling cascades that ultimately determine cell fate through regulating cytokine production, cell survival, proliferation, and differentiation.

Ramos *et al.* have demonstrated that IL-12, but not IFN-α, programs effector function in human CTLs in vitro (Ramos et al., 2009). Additionally, IL-12 signaling was shown to provide ‘signal 3’ for differentiation and activation of human neonatal CTLs (McCarron et al., 2010). Although, a subsequent study reported that IFN-α has some ability to program effector function in human CTL, there was no comparison

with IL-12 and the magnitude of effector response was minimal (Hervas-Stubbs et al., 2010). In contrast, both IL-12 and IFN-α have been shown be provide the 'signal 3' to allow acquisition of effector function in Tc1 CTL development in mice. *In vitro* studies have demonstrated that IL-12 and IFN-α may play redundant roles in induction of cytokine secretion, cytolytic activity, and clonal expansion of murine naïve CTLs (Valenzuela et al., 2002; Schmidt et al., 2002; Chang et al., 2004; Curtsinger *et al*., 2005). Using microarray analysis of *in vitro* primed murine CTLs, Agarwal *et al.* have shown that IL-12 and IFN-α enforce a common genetic programming leading to effector functions (Agarwal et al., 2009). Furthermore, mice lacking IL-12 and IFN-α signals have impaired cytokine production and cytolytic activity in the context of lymphocytic choreomeningitis virus, vesicular stomatitis virus and *Listeria monocytogenes* infections (Kolumam et al., 2005; Thompson et al., 2006; Keppler et al., 2012). Thus, IL-12 and IFN-α are two critical innate inflammatory cytokine with influential instructions for adaptive immune response.

Effector cytokine secretion and cytolysis constitute CTL effector functions

IFN-γ secreting CTLs have been termed Tc1 due to 'type I response', defined by IFN-γ mediated immune response. IFN-γ and TNF-α are the hallmark effector cytokines secreted by the Tc1 CTLs. Atypical Tc17 (IL-17 producing CTLs), Tc9 (IL-9 and IL-13 producing CTLs) and Tc2 (IL-4 producing CTLs) subsets of CTLs have been reported in the literature (Sad et al., 1995; Murphy et al., 2000; Seder et al.,

2003; Peixoto et al., 2007; Intlekofer et al., 2008; Zhu et al., 2010; Visekruna et al., 2013). This may be due to differential use of transcription factors such as T-bet, Eomes, and GATA3. For example, virus specific murine $CD8^+$ T cells lacking both T-bet and Eomes differentiate into IL-17 producing CTLs (Intlekofer et al., 2008). Although the frequency of the anomalous GATA3 expressing Tc2 increases in patients with psoriasis vulgaris and systemic sclerosis, Tc1 remains the predominant CTL subset in human (Sad et al., 1995, Inaoki et al., 2003, Medsger et al., 2011). I will use the term CTL or $CD8^+$ T cells to refer to Tc1 subset throughout the dissertation.

Recognition of antigen presented in the context of MHC class I activates effector and T_{EM} CTLs and leads to secretion of type I effector cytokines, IFN-γ and TNF-α. IFN-γ and TNF-α up-regulate inducible nitric oxide synthase (iNOS) and nicotinamide adenine dinucleotide phosphate oxidase (NADPH oxidase) in monocytes, macrophages, and neutrophils at the site of infection (Berton et al., 1986; Perussia et al., 1987; Dalton et al., 1993). This leads to rapid release of nitric oxide, hydrogen peroxide, and other oxygen radicals into the phagosomes culminating to pathogen clearance by oxidative burst (Nathan et al., 1983; Ding et al., 1998; Cassatella et al., 1989). IFN-γ secreted from antigen-specific CTLs can elicit protective immunity against pathogens such as *Listeria monocytogenes,* while TNF-α secreted by CTLs can play a direct role in inducing apoptosis of the infected cells (Messingham et al., 2007). Additionally, IFN-γ and TNF-α can have potent

antiviral effect, which requires the type I interferon receptor expression (Davis et al., 2008). Thus, effector CTLs play direct and indirect roles in pathogen clearance through secretion of IFN-γ and TNF-α.

Cytolysis or killing of infected cells is the second major effector function of CTLs. CTLs kill infected cells using perforin and granzyme mediated lysis in a MHC class I dependent manner (Berke, 1995; Revell et al., 2005). CTLs acquire pore-forming molecules such as perforin and the granzyme family of serine proteases such as granzyme B, in response to Tc1 polarizing conditions. Once CTLs recognize the cognate antigen, perforin and granzymes are released in the direction of infected cells. Release of both perforin and granzymes are essential in cytotoxicity towards the infected cells (Kägi et al., 1994; Chowdhury et al., 2008). This is because of the need for coordinated two-step process in cytolysis that involves membrane pore formation and the activation of cytosolic caspases (Andrade et al., 1998). Effector CTLs also facilitate the induction of apoptosis through FAS/FASL (Rensing-Ehl et al., 1995). Once infection is resolved, this FAS/FASL mediated apoptosis is a major player in activation induced cell death (AICD) of T lymphocytes (Barry et al, 2003; Sallusto et al., 2004).

Taken together, effector cytokine secretion and cytolytic capabilities make CTL response very effective in eliminating intracellular infections and tumors. It is well established that CTLs can recognize tumor-associated antigen and respond by cytolysis of tumor cells (Ochsenbein et al., 2001; Schuler et al., 2003; Lotem et al.,

2008). Additionally, Farrar *et al*. have demonstrated that IFN-γ secreted by CTLs can maintain murine B cell lymphoma dormancy. IFN-γ can induce apoptosis and have anti-proliferative effects on tumor cells (Trubiani et al., 1994; Bromberg et al., 1996; Farrar et al., 1999). Thus, effector responses by CTLs have a critical role in multiple aspects of the adaptive immune response and in the clearance of intracellular infections as well as anti-tumor response.

Memory CTLs exert more rapid and robust response upon re-infection

Memory formation is the hallmark of adaptive immune response and the basis for immunization (Gaucher et al., 2008). Once primary infection subsides, majority of the effector CTLs undergo apoptosis by AICD and only less than about five percent survive (Badovinac et al., 2006; Kaech et al., 2012). These small numbers of surviving cells acquire the ability to 'remember' the pathogen and respond quickly upon secondary challenge by secreting IFN-γ and killing infected cells. While effector CTLs are short lived and die upon exertion of effector function, memory CTLs are long lived and have the ability to respond more rapidly and robustly upon reinfection with the same pathogen (Kaech et al., 2007). Works in murine model have suggested that long-lived memory CTL programming requires prolonged exposure to antigen but low levels of cellular activation (Bachmann et al., 2006; Jung et al., 2010; Beuneu et al., 2010). Pathways leading to memory CTL development are not fully understood and there may not be a linear mechanism for acquiring the memory

properties (Reiner et al., 2007; Bannard et al., 2009; Ahmed et al., 2009). However, memory CTLs undergo histone modifications, which facilitates rapid transcription of effector molecules through open chromatin structures (Fann et al., 2006; Araki et al., 2008; Pipkin et al., 2010; Zediak et al., 2010). Therefore, memory cells demonstrate properties that are distinct from naïve cells such as rapid proliferation and effector function (Kaech et al., 2002).

There are two main kinds of memory CTLs that have been identified based on their functional capabilities: effector memory (T_{EM}) and central memory (T_{CM}) (Sallusto et al., 2004). T_{EM} CTLs have the ability to directly respond to the infected cells upon recognition of antigen and do not require any co-stimulation or the presence of any innate inflammatory cytokines (London et al., 2000; Rufer et al., 2003). Since all nucleated cells in the body can express MHC class I, T_{EM} CTLs can rapidly respond to infected cells in the periphery. On the other hand, T_{CM} CTLs proliferate in response to DC mediated activation in the lymph nodes (Roberts et al., 2005). The daughter cells then acquire the effector molecules and home to the site of infection (Meng et al., 2006). A number of cell surface markers have been used to distinguish the different subtypes of naïve and memory CTLs. For example, the chemokine receptor CCR7 is expressed on naïve and T_{CM} allowing these cells to home to the lymph node in response to the chemokines CCL19 and CCL21 (Wherry et al., 2003; Tough 2003). On the other hand, effector cells express the chemokine receptor CXCR3 and home to the site of infection in response to IFN-γ induced

chemokines, MIG and IP10 (Weng et al., 1998; Hu et al., 2011). A decreased $CXCR3^+CD8^+$ population can contribute to T_{EM} CTL dysfunction in immunocompromised subjects, such as in the case of advanced HIV infection (Brainard et al., 2007). T_{EM} CTLs can rapidly secrete IFN-γ and TNF-α upon TCR stimulation alone. Also, T_{EM} CTLs have preformed perforin and granzyme, which licenses these cells to respond to any infected cell by cytotoxicity (Chowdhury et al., 2011). Thus, while T_{EM} CTLs respond quickly at the early stages of infection, T_{CM} CTLs clonally expand and dominate in later time points in eliminating infections.

MAP Kinase pathways in immune cell activation

One important aspect of signal transduction across different immune cells is the MAP kinase pathway. All mammalian cells utilize the mitogen activated protein kinases or MAP kinases to regulate a diverse set of cellular responses that range from embryogenesis to cell proliferation and even apoptosis (Raman et al., 2007). MAP kinases are evolutionarily conserved three-tiered kinase cascade that coordinate many different cellular responses by relaying the instructions from extracellular signals (Qi and Elion, 2005). The message from outside the cell is relayed to a serine-threonine protein kinase (MAP3 kinase or MAPKKK) that, in turn, phosphorylates and activates a dual-specificity protein kinase (MAP2 kinase or MAPKK), which phosphorylates and activates the final MAP kinase (MAPK). MAPK can activate a number of downstream target proteins such as transcription factors

and metabolic enzymes. In mammalian cells there are three main families of MAPK: extracellular signal-regulated kinases (ERK), c-jun N-terminal kinases (JNK), and p38.

ERK, JNK, and p38 MAP kinase pathways play important roles in activation of both innate and adaptive immune cells (Symons et al., 2005; Smith-Garvin et al., 2009). PRR activation and signaling in innate immune cells such as DC and Mϕ modulates cell migration, phagocytosis, anti-microbial defense, tissue repair, and even programming of adaptive immune response. These processes downstream of PRR engagement involve a coordinated activation of NF-κB transcription factors and differential use of each of the MAPK pathways (Gaestel et al., 2009; Banerjee and Gerondakis, 2007). Additionally, biosynthesis as well as response to pro-inflammatory cytokines, such as TNF-α and IL-1β, require MAP kinase activation (Vallabhapurapu and Karin, 2009).

Signal transduction in CTL activation: TCR-dependent and –independent pathways

TCR-dependent activation of CTLs is a complex process that leads to gene transcription via activation of MAP kinase pathways and transcription factors (Figure 1.2; Smith-Garvin et al., 2009). TCR is a membrane bound heterodimer composed of highly variable alpha and beta chains in majority of the CTLs. TCR heterodimer, two CD3 co-receptors, and two ζ chains together form the T cell receptor complex. In

CTLs, CD8 is the co-receptor that provides specific binding to MHC Class I. The signal transduction begins with TCR binding to antigen in MHC-restricted manner, which in experimental models can be provided by anti-CD3 antibody. Briefly, zeta-chain associated protein kinase (Zap-70) is recruited to the TCR/CD3 complex where it becomes activated, promoting recruitment and phosphorylation of downstream adapter or scaffold proteins. Downstream phosphorylation of phospholipase C γ1 (PLCγ1) results in the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP2) to produce the second messenger diacylglycerol (DAG) and inositol trisphosphate (IP3). In Ca^{2+} dependent manner, IP3 promotes activation of the phosphatase calcineurin, leading to translocation of the transcription factor NFAT (Qi et al., 2007). DAG activates PKCθ and the MAPK pathways, and promotes transcription factor NF-κB activation (Marsland and Kopf, 2008; Cheng et al., 2011). In TCR mediated activation pathway, Raf-1 is the MAP3 kinase that leads to ERK-1/2 MAP kinase activation (Smith-Garvin et al., 2009). Feedback regulation at several points within these pathways allows for different outcomes, depending on the environment. The incorporation of signals from other cell surface receptors (such as CD28) further regulates cellular response by providing signal 2 or co-stimulation (Sharpe et al., 2002; Sharpe et al., 2009; Park et al., 2009).

There is a TCR-independent pathway for activation of T cells by IL-12 and IL-18, culminating to IFN-γ production (Figure 1.3). This pathway for T cell activation has been found to be operational in the differentiated Th1 cells and memory CTLs

(Rincón et al., 1998; Raué et al., 2012). IFN-γ secretion is dependent upon synergistic response to IL-12 and IL-18. Signals from both IL-12 and IL-18 lead to synthesis of GADD45β, which in turn activates the p38 MAP kinase pathway (Yang et al., 2001). Additionally, both p38 mediated transcriptional activities and STAT4 are required for IFN-γ production during TCR-independent activation of Th1 cells (Zhang et al., 2000; Nakahira et al., 2002). It has been shown in murine models that in the absence of cognate antigens, this TCR-independent pathway can contribute to immune protection against such pathogens as vaccinia virus and *Listeria monocytogenes* (Gherardi et al., 2003; Berg et al., 2003).

Concluding remarks

APCs produce IL-12 and IFN-α in response to pathogen invasion. Along with their defined anti-microbial effects, these two cytokines have been shown to bridge the gap between innate and adaptive immunity by providing a 'signal 3' to T lymphocytes. However, the precise roles of IL-12 and IFN-α in programming CTL effector functions are still debatable. In mice, these two cytokines seem to play a redundant role in programming effector CTLs. Moreover, in mice, either or both of these cytokines seem to be important for generation of stable memory CTLs depending on the pathogen challenge. Analysis of human CTL function in the presence of defined cytokine milieu revealed that IL-12, but not IFN-α, is sufficient in programming effector function as measured by effector cytokine secretion and

cytolysis. There are several questions that still remain unanswered: Does effector function from memory CTLs depend on stable programming by IL-12 in human? And if so, what the molecular mechanism by which IL-12 programs effector response? The work presented in this dissertation will seek to answer these questions (Figure 1.4). First, I will compare the gene expression profile of IL-12 treated cells to the gene expression pattern of T_{EM} CTLs sorted from healthy human peripheral blood. Second, I will use an unbiased approach to determine if the T_{EM} CTLs bear any cytokine responsive gene signature. Finally, once a cytokine induced pathway is identified from the abovementioned screen; I will perform mechanistic studies to determine if blocking that pathway has any consequences on human CTL effector function.

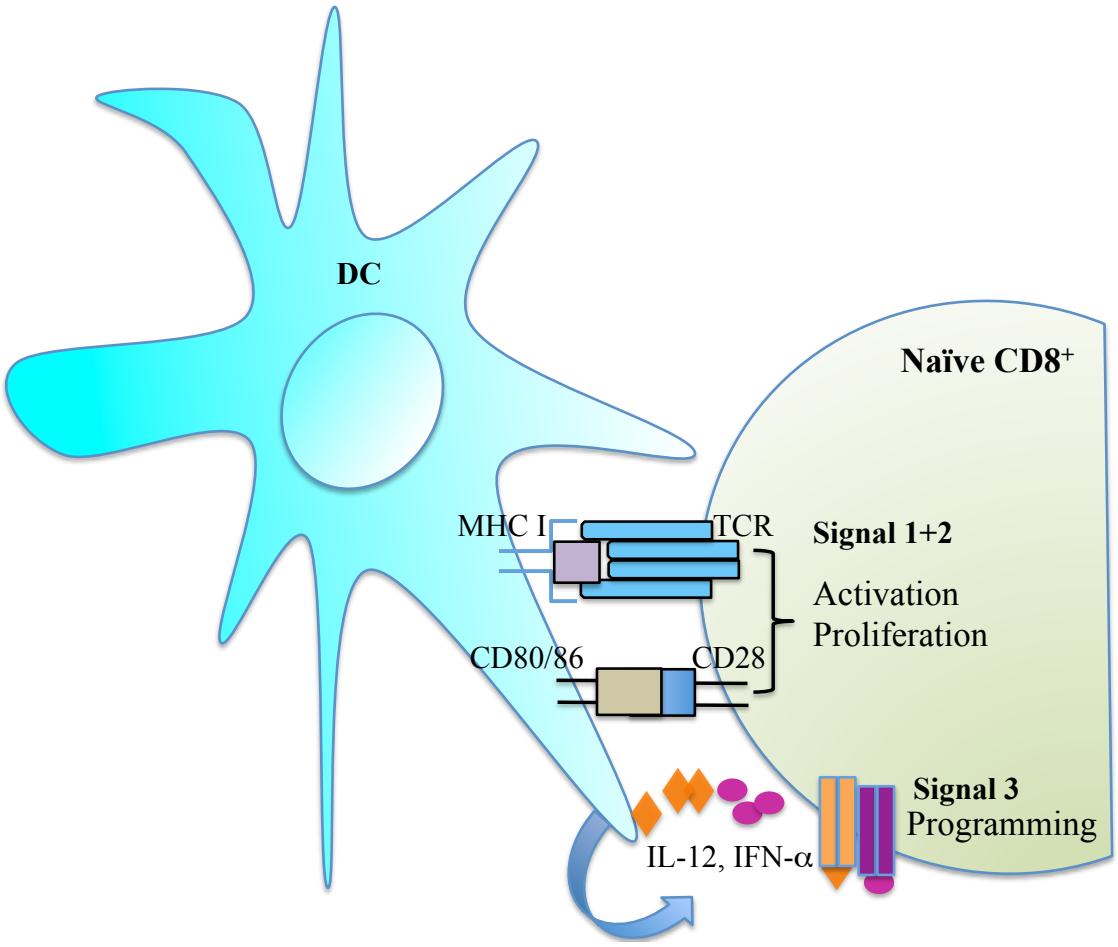

Figure 1.1: Activation of naïve CTLs require three signals. 'Signal 1' comes from TCR engagement, 'Signal 2' comes from co-stimulation, and 'Signal 3' is provided by IL-12 or IFN-α.

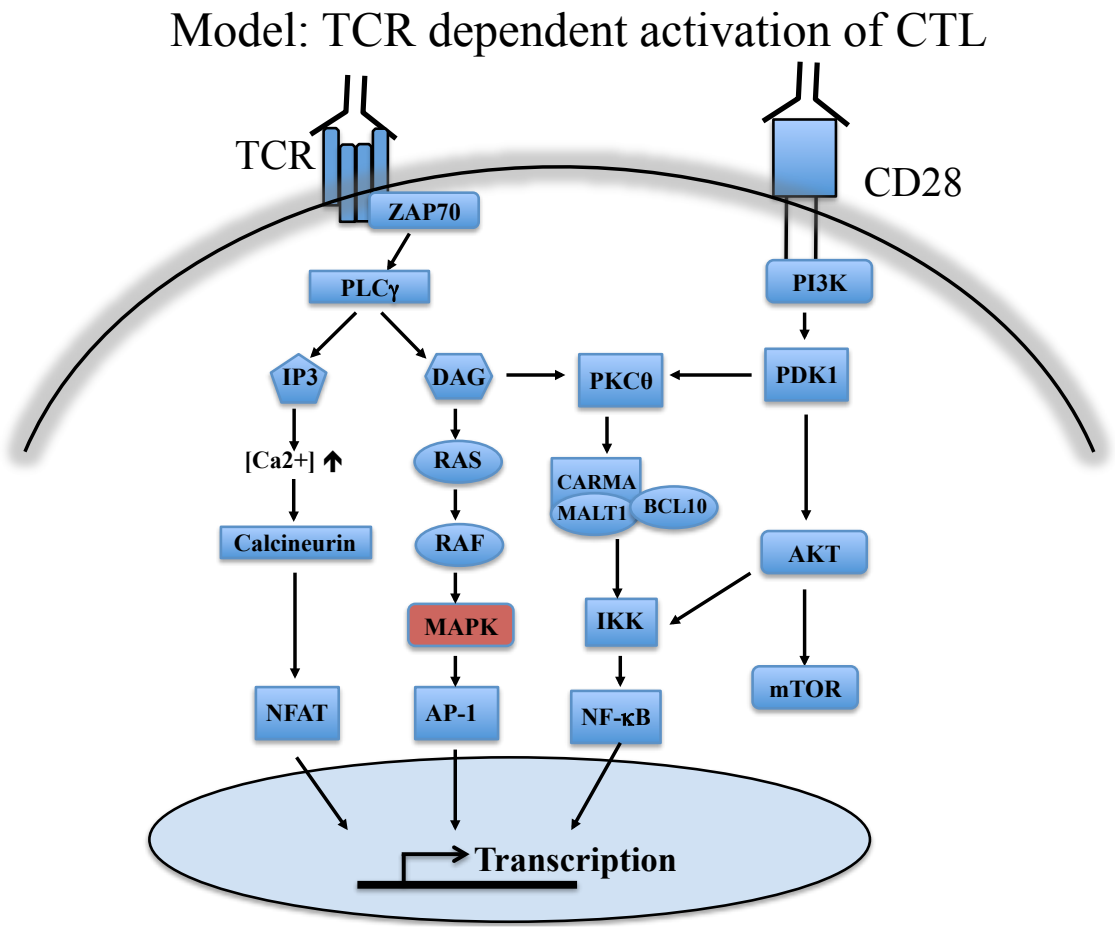

Figure 1.2: Abbreviated model of TCR mediated activation of CTLs. Adapted from www.cellsignal.com.

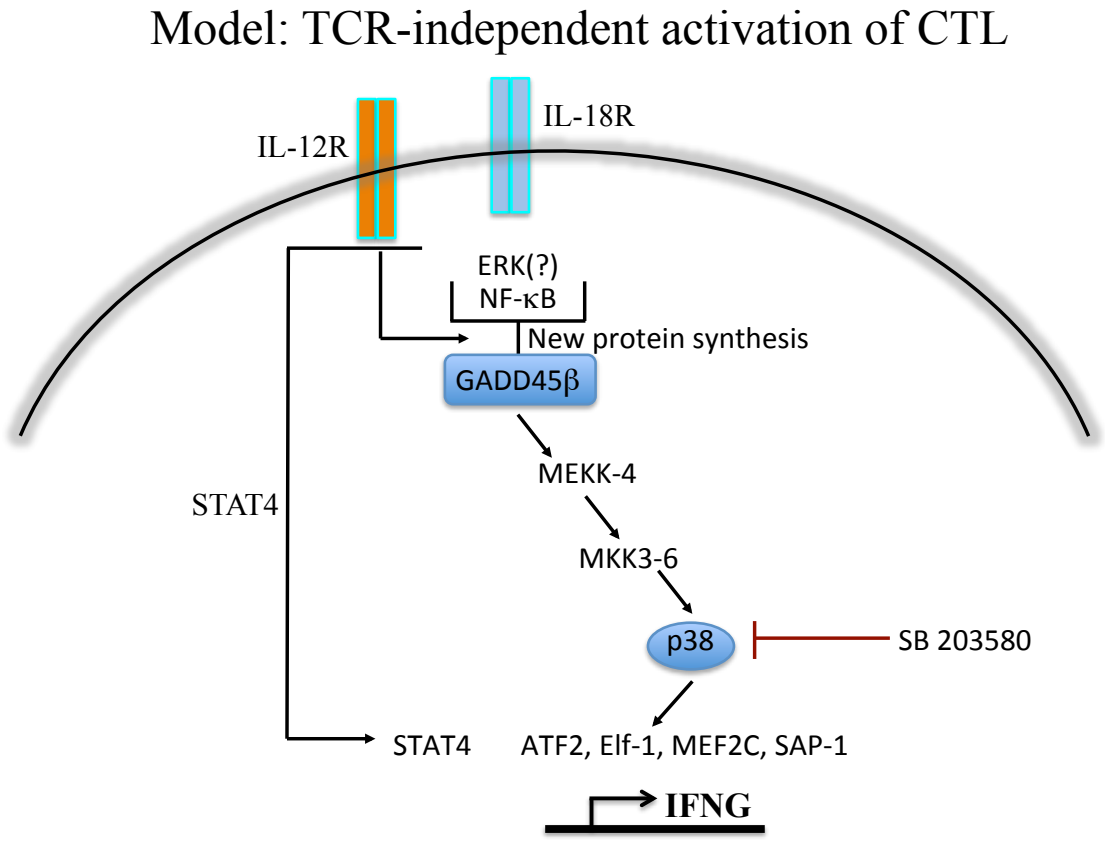

Figure 1.3: Abbreviated model for TCR-independent activation of CTL, leading to IFNG transcription. Adapted from Tsuji-Takayamaet al., 1997; Yang et al., 2001; Fortin et al., 2009.

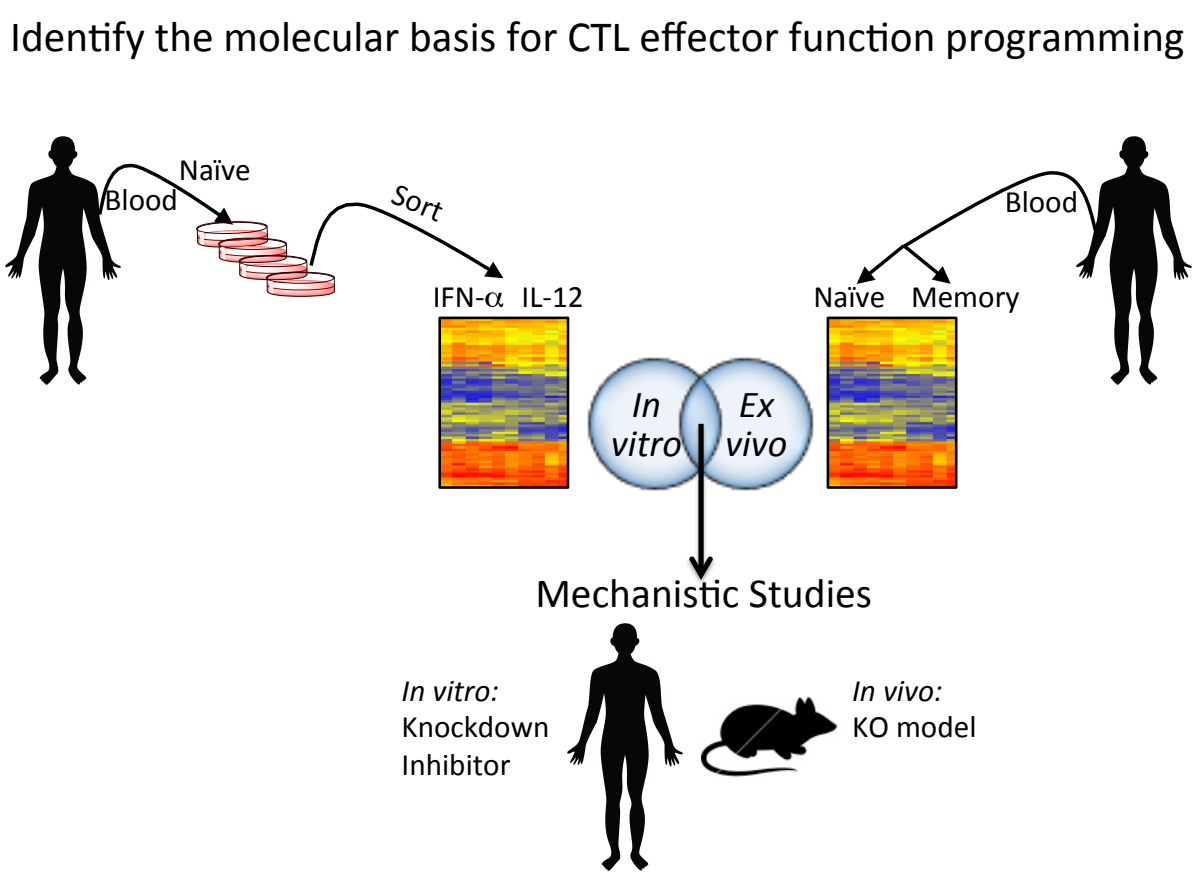

Figure 1.4: Model for proposed workflow to identify the molecular basis for CTL effector function programming by IL-12 and/or IFN-α.

CHAPTER TWO

MATERIALS AND METHODS

Human Subjects

Informed consent was obtained and peripheral blood was collected by venipuncture from healthy adult donors as approved by the Internal Review Board at the University of Texas Southwestern Medical Center. We also purchased buffy coats from Carter Blood Care for some experiments and the same blood preparation protocol as that for whole blood was used to isolate $CD8^+$ T cells from these buffy coats.

Isolation and culture of human $CD8^+CD45RA^+$ T cells

Human peripheral blood mononuclear cells (PBMCs) were isolated from either whole blood collected in lab or buffy coats purchased from Carter BloodCare by ficoll density centrifugation using Lymphocyte Separation Media (Cellgro, Manassas, VA). Briefly, blood was diluted 1:1 with sterile 1X PBS and PBMC was separated using differential centrifugation upon underlaying with Lymphocyte Separation Media. $CD8^+CD45RA^+$ cells were isolated by negative selection with BD IMag™ kit (Human Naïve $CD8^+$ T Cell Enrichment Set – DM) and purity was routinely greater than 90%. Purified $CD8^+CD45RA^+$ cells were either labeled with CFSE and cultured at $1e^6$ cells/mL in 96-well tissue culture treated plates coated

with 1.5 μg/mL of anti-CD3+anti-CD28 antibodies in complete IMDM supplemented with 10% FBS or cultured without any labeling. All cells received rhIL-2 (200 U/ml) and anti-human IFN-γ (5.0 μg/mL) while the variable cytokine polarization conditions included neutralized (anti-human IL-12 [5.0 μg/mL]), IL-12 (recombinant-human IL-12 [5.0 ng/mL]), IFN-α (anti-human IL-12 [5.0 μg/mL], recombinant-human IFN-α(A) [1000U/mL]), and IL-12+IFN-α (recombinant-human IL-12 [10.0 ng/mL], recombinant-human IFN-a(A) [1000U/mL]). Unless otherwise noted in the figure legends, cells were split 1:10 with 100U/mL rhIL-2 added. Also, 200U/mL rhIL-2 was added during secondary stimulation unless otherwise specified in the figure legends. All secondary stimulations for ELISA were done in the presence of plate-bound anti-CD3 stimulation only. Intracellular staining the stimulation was either PMA (0.8 µg/ml)+Ionomycin (1.0 µM)+Monensin (2.0 µM) or anti-CD3+Monensin.

Panning to enrich for human and murine T cells

150mmX25mm tissue culture treated dishes were coated with panning antibody diluted in Tris Coating Buffer (pH 9.5) and incubated either at 37^{o}C for 2h or overnight at room temperature. The following antibodies were used: AffinityPure Goat Anti-Human IgA + IgG + IgM (H+L) for panning of human T cells and AffinityPure Goat Anti-Mouse IgG + IgM (H+L) for panning of murine T cells. Both antibodies were purchased from Jackson ImmunoResearch at stock concentration of 2.4mg/mL and used 5µL per plate for coating. Before use, plates were washed once

with 1X PBS and cells suspended in cIMDM+10% FBS were transferred carefully into the plate. Plates were incubated at 37^{o}C for 45 min to 1 h and non-adherent cells were removed for further isolation processing.

CFSE labeling of human and murine CTL

CFSE (carboxyfluorescein diacetate succinimidyl ester) is a fluorescent dye that is cell permeable and covalently binds to all intracellular proteins. Upon each cell division the dye is diluted in half in the daughter cells and thus can be used to monitor cell division where each peak in the histogram for fluorescence corresponds to a cell division cycle. The protocols used here were improved upon consulting previously published protocols to assess cell division using CFSE (Lyons, 2000; Quah et al., 2007).

Isolated CTLs were washed in 1X PBS for 2-3 times and counted 1:10 using trypan blue. Human cells were then re-suspended at 10e^{6} cells/mL and directly labeled with 1.25μM of CFSE $[1mM]_{stock}$. cIMDM with 20% FBS was added to quench the excess dye. Cells were washed and plated at 1e^{6} cells/mL for desired stimulation condition. For murine CTLs, cells were re-suspended at 2e^{6} cells/mL and directly labeled with 1.25μM of CFSE while the rest of the protocol was unchanged.

Live cell sorting by FACS

Live cell sorting was performed by labeling cells with fluorescently labeled antibodies against cell surface markers or chemokine receptors followed by FACS sorting. FACSAria (BD) or MoFlo^{TM} (Beckman-Coulter) was used for cell sorting. Briefly, cells were pelleted and re-suspended in dreg containing desired quantity of antibody cocktail. Cells were then incubated on ice, in dark for 20 min. Stained cells were washed, counted using trypan blue, and re-suspended in cIMDM+10%FBS at 20X10^6 cells/mL for sorting on FACSAria and at 6-10X10^6 cells/mL for sorting on MoFlo^{TM}. Cell suspensions were always filtered using nylon mesh filters prior to sorting in order to avoid clogging the sorters with any cell clumps. Sorted cells were collected in either 15mL conical or 5mL polypropylene tubes containing ~3mL or ~1mL of cIMDM+10%FBS respectively. Sorted cells were pooled and pelleted in 15mL conical and counted prior to culturing or processing for RNA collection.

Cell lines

THP-1, a human monocytic lymphoma cell line was purchased from the American Type Culture Collection (Manassas, VA). Aliquots of 1X10^{6}cells/ml were stored at -80^{o}C in freezing media (90% FBS+10% DMSO). Cells were grown in cDMEM+10%FBS for at least 7 days prior to use.

Cytotoxicity Assays (re-directed lysis assay)

$CD8^+$ cells were sorted by FACS based on chemokine receptors and were subjected to a redirected lysis assay as previously described (Ramos et al., 2009). Briefly, anti-CD3-coated THP-1 target cells were labeled by culturing in the presence of 150 μCi $Na_2[^{51}Cr]O_4$ in complete growth media for 1.5h. Target cells were washed and incubated with CTLs at various effector:target (E:T) ratios for 4h at 37°C. Specific cytotoxicity was measured by scintillation counting of ^{51}Cr released in the media using a beta counter.

RNA isolation and microarray analysis

Undivided or $CFSE^{hi}$ and divided or $CFSE^{lo}$ cells were isolated from in vitro cultured cells by FACS sorting. Alternatively, cells were sorted based on CD8, CCR7, and CXCR3 expression by FACS sorting. RNA was isolated according to manufacturer's recommendations using Arcturus® PicoPure® RNA Isolation Kit. RNA was submitted to University of Texas Southwestern Medical Center Genomics and Microarray Core Facility where the integrity and quality of the RNA was tested using Bioanalyzer Chip. cDNA synthesis and hybridization onto Illumina SingleColor HumanWG-6_V3_0_R1 platform was according to the manufacturer's instructions. The microarray results were submitted to GEO and assigned the accession number GSE27337.

Raw image files from the scanned chip were first analyzed using GenomeStudio Data Analysis Software to obtain signal intensity values, and sample

probe profiles were generated without normalization. Data were then exported to GeneSpring GX 11 software (Agilent Technologies) for further statistical analysis and visualization. Using GeneSpring a gene-level expression file was obtained with quantile normalization of all samples. Raw data were normalized, and a 1-way ANOVA test with unequal variance was performed to identify genes that were differentially expressed among the donor samples and under any of the culture conditions. Genes with at least a fold change of >1.7-fold were identified based on comparison to neutralized condition for the *in vitro* polarized cells and by comparison to $CD8^{+}CXCR3^{lo}CCR7^{hi}$ cells for the *ex vivo* sorted cells. Gene ontology analysis on selected gene sets as well as pathway search was performed using the web-based tool: Database for Annotation, Visualization, and Integrated Discovery (DAVID) (Huang et al., Nat. Protoc. 2009; Huang et al., Nucleic Acids Res. 2009).

Mice

All mice were housed in the special pathogen-free facilities at UT Southwestern (UTSW) Medical Center except for when infections of mice were carried out. Mouse experiments and handling were done in accordance with guidelines established by the Institutional Animal Care and Use Committee at UTSW Medical Center. C57BL/6 WT mice were purchased from the UT Southwestern Mouse Core facility. IFNAR OTI mice were bred and maintained in house. $Tpl2^{-/-}$ mice were a gift from Dr. Philip Tsichlis of Tufts University and subsequently

maintained in house (~75% C57BL/6 background). All mice were genotyped using DNA isolated from tail clips.

Isolation of murine CD8$^+$ T cells

I used both spleen and lymph nodes as the source of murine CD8$^+$ T cells unless otherwise indicated. The organs were harvested using sterile surgical tools and submerged in cIMDM with 10% FBS. The organs were homogenized using sterile frosted glass slides. Red blood cells were lysed using RBC Lysing Buffer from Sigma (Cat# R7757) by re-suspending cells in 3mL of lysing buffer per mouse and incubating at room temperature for 2-3 minutes. Upon neutralization of the lysing buffer, cells were passed through cell strainer to remove any tissue clumps. Total CD8$^+$ T cells were subsequently isolated according to the manufacturer's suggestions using Dynal Mouse CD8 Negative Isolation Kit (Invitrogen, Cat# 114-17D). The purity of the isolated cells were routinely >85%. Isolated cells were either cultured immediately or kept on ice for no longer than an hour prior to culturing.

***In vitro* culture of murine CTLs**

Murine CTLs were stimulated in vitro with plate bound anti-CD3 (Clone 2C11, gift from Dr. Nicolai van Oars) and anti-CD28 (Clone 37.51, purchased from eBiosciences). For optimal primary stimulation, 0.5μg/mL of each stimulating antibody was used to coat tissue culture treated plates prior to stimulation. Cells

were cultured under neutralized (anti-mouse IL-4 + anti-mouse IFN-γ + anti-mouse IL-12 + rhIL-2 (cross-reactive with mouse) [200u/mL]), IL-12 (anti-mouse IL-4 + anti-mouse IFN-γ + rmIL-12 [10ng/mL] + rhIL-2 [200u/mL]), or IFN-α (anti-mouse IL-4 + anti-mouse IFN-γ + anti-mouse IL-12 + rhIFN-α (universal Type I, A/D) [1000u/mL] + rhIL-2 [200u/mL]) conditions. Cells were split 1:10 on day 2-3 post stimulation for long term cultures in cIMDM+10%FBS and 100U/mL rhIL-2. For culture conditions beyond 14 days, dead cells were removed using ficoll density centrifugation prior to re-stimulation. A list of all the cytokines and antibodies is provided below.

Cytokines and antibodies for tissue culture

Cytokines: Recombinant human IL-12, IFN-α(A), IFN-γ and recombinant murine IL-12 were purchased from R&D Systems (Minneapolis, MN). Recombinant universal IFN-αA/D was purchased from PBL laboratories (Piscataway, NJ). Recombinant human IL-2 was a generous gift from M. Bennett (UT Southwestern Medical Center). Recombinant human IL-18 was purchased from Biosource (Camarillo, CA).

Antibodies (cellular activation): Activating anti-human CD3 (clone, OKT3) and anti-human CD28 (clone, 37.51) were purchased from Biolegend (San Diego, CA). Neutralizing anti-human IL-4 was purchased from R&D Systems (Minneapolis, MN). Neutralizing anti-human IL-12 (clone, 20C2), anti-human IFN-γ (clone, 4SB3), and

anti-mouse IL-12 (clone, TOSH), anti-mouse IFN-γ (clone, R46A2) and anti-mouse IL-4 (clone, 11B11) were generated in house.

Antibodies for flow-cytometric analysis and FACS sorting

Antibodies used for flow-cytometric analysis and FACS sorting is summarized in Table 2.1 for anti-human antibodies and in Table 2.2 for anti-mouse antibodies.

***Listeria monocytogenes* infection of mice**

All infections and handling of infected animals and tissue samples were carried out with approval from Institutional Animal Care and Use Committee (IACUC) at UTSW Medical Center. We used ovalbumin expressing *Listeria monocytogenes* (LM-OVA) as the bacterial pathogen to infect mice intravenously (i.v.). Bacteria was cultured in BHI growth media containing ampicillin for selection and prepared in 1X PBS for injection. All injections were performed in accordance with IACUC guidelines in special biohazard suite of the animal care facility. We used 2,000 CFU/mouse for primary infection and 20,000 CFU/mouse for secondary infection. CFU counts for injected bacteria were confirmed by colony count from bacterial growth on BHI agar plates. Blood was collected retro-orbitally to test for expansion of $CD8^+$ T cells upon primary infection. Mice were sacrificed, followed by harvest of spleen and lymph nodes for isolation of splenocytes.

Analysis of cell viability using flow cytometry

Flow cytometry was used to analyze cell viability by staining the cells with both 7-AAD (Purchased from Biolegend) and Annexin V conjugated to PE (Purchased from BD Pharmingen™). 7-AAD is DNA binding vital dye, which is excluded by live intact cells and only dead or damaged cells are permeable to it. PE Annexin V staining recognizes cells with exposed membrane phospholipid phosphatidylserine in apoptotic cells. Staining with 7-AAD and PE Annexin V was carried out entirely in cIMDM+10% FBS. Flow cytometric analysis was performed using FACSCalibur (BD) and data was analyzed using the FloJo Software (Tree Star, Inc). Cells were considered viable if stained negative for both 7-AAD and Annexin V in the ungated population.

Quantitative Real-Time PCR (qRT-PCR) for assessment of gene expression

Total cellular RNA was collected using column purification with RNeasy® Mini Kit (Qiagen, Valencia, CA) according to manufacturer's instruction. Prior to elution an on column DNase I digestion treatment was carried out using RNase-free DNase from Qiagen and incubating at room temperature for 15 min. RNA was quantified using NanoDrop Spectrophotometer (Thermo Scientific) and either used right away or stored at -80^{o}C for later use. RNA was reverse transcribed into cDNA for 2 hours at 37^{o}C using the ABI High Capacity cDNA Reverse Transcription Kit from Applied Biosystems. cDNA was either used right away for qRT-PCR or stored at -20^{o}C for a

later use. Using the newly synthesized cDNA as template qRT-PCR was setup in accordance with manufacturer's instructions with either Brilliant® II SYBR Green QPCR Master Mix (Stratagne, Agilent Technologies) or Maxima SYBR Green qPCR Master Mix (Thermo Scientific). We used ABI7300 cycler (Applied Biosystems) for running qRT-PCR analysis and ROX as passive reference dye. Primers directed against PPIA were used as a reference for both human and murine gene expression. Relative expression of mRNA was calculated by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). All primers were synthesized by and purchased from either Integrated DNA Technologies (Coralville, IA) or Sigma Aldrich (St. Louis, MO). A list of the primer sequences for the genes reported in this dissertation is provided in Table 2.3.

Kinase inhibitor treatment

The use of three different kinase inhibitors is reported in this dissertation: Tpl2 Kinase Inhibitor (Santa Cruz Biotechnology, Cat# sc-204351), U-0126 or MEK1/2 inhibitor (Santa Cruz Biotechnilogy, Cat# sc-222395), and SB 203580 or p38 inhibitor (Calbiochem, Cat# 559389). All kinase inhibitors used here were commercially available and were purchased in lyophilized forms. Upon arrival they were stored at -20^{o}C. All kinase inhibitors were completely re-suspended in 100% DMSO according to the manufacturer's instructions for a stock concentration of 10mM. Aliquots were made and stored at -20^{o}C until use. Aliquots of inhibitors were

never freeze-thawed and always used at a dilution of 1:1000 for treatment of cells. Inhibitors were added to the cell suspensions prior to stimulation without any particular pre-incubation time with the inhibitors.

siRNA: Transfection and Nucleoporation

siRNA mediated knockdown of MAP3K8 in primary human $CD8^+$ T cells were attempted using both nucleoporation and transfection techniques. $CD8^+$ T cells were isolated as described above from either healthy adult donors or buffy coats purchased from Carter BloodCare. Isolated cells were used immediately for nucleoporation or transfection. I have also stimulated isolated $CD8^+$ T cells in the presence of IL-12 prior to knockdown attempts. I used ON-TARGETPlus™ SMARTpool siRNA mix specific for MAP3K8 (Dharmacon, Thermo Scientific) and Non-targeting Control Pool as negative control. siGLO RISC-free Control siRNA (Dharmacon, Thermo Scientific) was used as the fluorescent indicator for transfection efficiency. I used two different transfection reagents TransIT-siQUEST Transfection Reagent (gift from Mirus, Cat# MIR 2111) and TransIT-TKO Transfection Reagent (gift from Mirus, Cat# MIR 2152). These two transfection reagents are proprietary transfection media that form complexes with siRNA in order to transport them across the cell membrane. Isolated CTLs were plated at $5X10^6$ cells/mL in serum-free and antibiotic-free cIMDM for transfection procedure according to manufacturer's instruction. Serum was added to the culture after 6h and

transfection efficiency was measured 24-48h post transfection. For nucleoporation, cells were re-suspended in Human T cell Nucleofector Solution, siRNA was added, and transferred to a Nucleofection cuvette. I used U-014 or V-024 Nucleofector Program for Amaxa Nucleoporator. Cells were transferred immediately to tissue culture wells containing fresh media and efficiency was measured 24-48h post nucleoporation. Gene knockdown was assessed by qRT-PCR for MAP3K8 expression in siRNA treated cells and compared with control or mock treated cells.

Western blot analysis and infrared imaging

Cell suspension was pelleted and washed 2X with 1X PBS before lysis. Cells were then lysed at 5 x 10^7 cells/ml by incubating for 1 hour at 4^{o}C 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) plus proteinase and phosphatase inhibitors (1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM dithiothreitol (DTT), 10 μg/ml leupeptin, 1 mM benzamidine, 1 μM pepstatin, and 1 mM Na_3VO_4). Protein was quantified using Bradford reagent (Protein Assay, Bio-Rad) in 96 well microtitre plates and absorbance was read at 595nm using iMark^{TM} Microplate Absorbance Reader (Bio-Rad). However, due to the presence of SDS in the lysis buffer, it was difficult to obtain a consistent read. Thus, for protein quantitation Pierce BCA Protein Assay Kit is a better detergent compatible assay and has been used for the quantitation performed after 2012. Total protein lysate

was resolved by SDS-PAGE on a 12.5% polyacrylamide gel and transferred to polyvinylidene difluoride (PVDF) membranes (Bio-Rad, Hercules, CA) using semi-dry transfer apparatus (Bio-Rad). Specific protein was detected by Western blotting with rabbit polyclonal antibody against MAP3K8/Tpl2 (Santa Cruz Biotechnology, Cot (M-20), Cat# sc-720). Rabbit polyclonal antibody against Lamin A/C was used as loading control (Cell Signaling Technology, Cat#2032) and later switched to GAPDH as the loading control (Sigma, Cat#G8795). Goat anti-rabbit conjugated to IRDye® 800CW secondary antibody (Li-Cor Biosciences; Cat# 827-08365) and goat anti-mouse conjugated to IRDye® 680 secondary antibody (Li-Cor Biosciences; Cat#827-08366) were used to detect primary antibody. IR signal was quantified by scanning the membrane with Odyssey® Infrared Imaging System.

Measuring cytokine in supernatant by ELISA

Enzyme-linked immunosorbent assay or ELISA was used to measure cytokine secretion in the culture supernatants. We have used these following kits perform ELISA: Human TNF-α ELISA MAX™ Standard Set (Biolegend®), Human IFN-γ ELISA MAX™ Standard Set (Biolegend®), Human IL-2 ELISA MAX™ Standard Set (Biolegend®), Mouse TNF-α ELISA MAX™ Standard Set (Biolegend®). We bought the individual components separately for mouse IFN-γ ELISA: Purified Rat anti-Mouse IFN-g (purchased from BD Pharmingen™, Cat# 551216, Clone: R4-6A2) was used as the capture antibody, Biotin anti-Mouse IFN-γ (purchased from

Biolegend®, Cat# 505804, Clone: XMG1.2) was used as the detection antibody, and Streptavidin-HRP (Biolegend®, Cat# 405103) was used for enzymatic label for detection of biotinylated detection antibody. We used BD OptEIA™ TMB Substrate Reagent Set for colorimetric detection. ELISA for both mouse and human cytokines were performed according to manufacturer's instructions. Briefly, desired number of wells in NUNC Maxisorp 96-well ELISA plate were coated with capture antibody diluted in bicarbonate buffer and incubated overnight at $4^{o}C$. Plate was washed in wash buffer (1X PBS+0.05% Tween 20) and blocked with assay diluent (1X PBS+1% BSA) for approximately 1 hour. Analytes and standards were prepared by diluting in the assay diluent and transferred to the appropriate wells for incubation at room temperature for approximately 2 hours. Plate was washed and detection antibody diluted in assay diluent was added for about 1 hour incubation at room temperature. Plate was washed and SA-HRP diluted in assay diluent was added for 30 min incubation at room temperature. Plate was washed for the last time and substrate solution added for development of color. Reaction was stopped by adding H_2SO_4 in less than 30 min based on color development of the standard. Absorbance was measured using iMark™ Microplate Absorbance Reader (Bio-Rad) at 450nm.

Antibody	Fluorochrome	Clone	Vendor	Catalog No.
CD8α	Pacific Orange	3B5	Invitrogen	MHCD0830
CD8α	PE	HIT8a	Biolegend	300908
CD45RA	FITC	HI100	BD Pharmingen	555488
CD45RA	Pacific Blue	MEM-56	Invitrogen	MHCD45RA28
CCR7	PE	3D12	BD Pharmingen	552176
CCR7	APC	150503	R&D Systems	FAB197A
CXCR3	Alexa Fluor 488	1C6/CXCR3	BD Pharmingen	558047
CXCR3	PE	1C6/CXCR3	BD Pharmingen	550633
Perforin	PE	dG9	Biolegend	308105
Perforin	FITC	27-35	BD Pharmingen	556577
Granzyme B	Alexa Fluor 700	GB11	BD Pharmingen	557971
IFN-γ	PE-Cy7	4S.B3	BD Pharmingen	557844
IFN-γ	FITC	4S.B3	eBioscience	11-7319-81
TNF-α	APC	MAb11	Biolegend	502912
CD25	Pacific Blue	BC96	Biolegend	302620

Table 2.1: Anti-human antibodies used in flow cytometry and FACS sorting

Antibody	Fluorochrome	Clone	Vendor	Catalog No.
CD8α	Pacific Blue	53-6.7	Biolegend	100725
CD8α	APC	53-6.7	Biolegend	100712
CD8α	PE	53-6.7	Biolegend	100708
CD4	APC	GK1.5	eBioscience	17-0041-82
CD4	APC-Cy7	GK1.5	BD Pharmingen	552051
CD4	PE	RM4-5	Biolegend	100512
CD4	PerCP	RM4-5	BD Pharmingen	553052
CD62L	PE-Cy7	MEL-14	Biolegend	104418
CD62L	APC	MEL-14	Biolegend	104412
CD44	PE	IM7	BD Pharmingen	553134
CD44	FITC	IM7.8.1	Caltag Laboratories	RM5701
IFN-γ	FITC	XMG1.2	Biolegend	505806
IFN-γ	PerCP/Cy5.5	XMG1.2	Biolegend	505822
TNF-α	FITC	MP6-XT22	Biolegend	506304
CD45.2	Alexa Fluor 700	104	Biolegend	109822

Table 2.2: Anti-mouse antibodies used in flow cytometry and FACS sorting

Gene	Direction	Sequence
hIFNG	Forward	5’-TGATTACAAGGCTTTATCTCAGGG-3’
	Reverse	5’-GGCAGTAACTGGATAGTATCACTTCAC-3’
hEOMES	Forward	5’-GTCTCCTAATACTGGTTCCCACTGG-3’
	Reverse	5’-CCACGCCATCCTCTGTAACTTC-3’
hMAP3K8	Forward	5’-CAGTAATCAAAACGATGAGCGTTCTA-3’
	Reverse	5’-GAACATCGGAATCTATTTGGTAACGT-3’
hPPIA	Forward	5’-GCGTCTCCTTTGAGCTGTTTGC-3’
	Reverse	5’-ATGGACTTGCCACCAGTGCC-3’
hTBX21 (Tbet)	Forward	5’-CGT CCA ACA ATG TGA CCC AG -3’
	Reverse	5’-GCA GTC ACG GCA ATG AAC TG -3’
mIFNG	Forward	5’-ACAATCAGGCCATCAGCAACAAC-3’
	Reverse	5’-CAGCGACTCCTTTTCCGCTTC-3’
mMAP3K8	Forward	5’-TCAGTCCCCAGAATGGCCGCT-3’
	Reverse	5’-AGAACAGACCCTCCCTCGCCG-3’
mPPIA	Forward	5’-TTATTCCAGGATTCATGTGCCAGGG-3’
	Reverse	5’-TCATGCCTTCTTTCACCTTCCCAA-3’

Table 2.3: List of primers used in qRT-PCR analysis of mRNA expression

CHAPTER THREE

IL-12 SELECTIVELY PROGRAMS EFFECTOR PATHWAYS THAT ARE STABLY EXPRESSED IN HUMAN CD8$^+$ EFFECTOR MEMORY T CELLS *IN VIVO*

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Introduction

The pool of circulating CD8$^+$ cytotoxic T lymphocytes (CTLs) is remarkably heterogeneous, consisting of antigen inexperienced naïve cells as well as multiple populations of effector and memory subsets (Sallusto et al., 2004). Various models have been proposed to explain the genesis of memory subsets, and a significant body of evidence suggests that long-lived memory cells are derived from early effector cells that survive contraction as antigen loads wane (Wherry et al., 2003). If so, then the diverse spectrum of memory cells that fall into either effector memory (T_{EM}) or central memory (T_{CM}) categories would also be predicted to be derived from a common pool of primary effectors (Harrington et al., 2008). Regardless of their

origins, T_{EM} and T_{CM} differ substantially in their functional capabilities, and the extrinsic signals that regulate their development are complex and multifactorial. Among these factors, innate cytokines are key signals that regulate effector cell differentiation, with IL-12 and type I interferon (IFN-α/β) playing important roles in both effector and memory cell development (Mescher et al., 2006; Butler et al., 2010)

In human, $CD8^+$ subsets have been distinguished based on their differential expression of various markers including CD45RA, CD27, CD28, CCR7, CD62L, CD127, and CXCR3 (Topp et al., 2003; Paiardini et al., 2005; Kobayashi et al., 2006; Takata et al., 2006). Based solely on the expression of CCR7 and CD45RA, early studies defined four main subsets: naïve ($CD45RA^+/CCR7^+$), T_{CM} ($CD45RA^-/CCR7^+$), T_{EM} ($CD45RA^-/CCR7^-$), and effector memory-RA (T_{EMRA}, $CD45RA^+/CCR7^-$) (Sallusto et al., 1999). Aside from their differential expression of CD45RA and other cell surface markers, T_{EM} uniformly exhibit rapid effector functions in response to antigen activation. Further, T_{EM} do not generally require CD28-mediated co-stimulation or innate cytokines in order to secrete pro-inflammatory cytokines or to lyse target cells (London et al., 2000; Rufer et al., 2003). Thus, if T_{EM} cells are direct descendants of effector CTLs, then the maintenance of effector functions within T_{EM} would suggest early programming during the priming phase of infection.

In mice, both IL-12 and IFN-α/β exert somewhat overlapping and redundant effects by driving effector cell development (Valenzuela et al., 2002; Curtsinger et

al., 2003; Mescher et al., 2006; Xiao et al., 2009; Kohlmeier et al., 2010). Although IL-12 seems to be more potent at promoting IFN-γ secretion, both IL-12 and IFN-α/β are equally effective at regulating lytic activity in murine $CD8^+$ T cells. In contrast, IL-12 and IFN-α/β appear to play very distinct roles in priming human $CD8^+$ T cell responses. Recently, Ramos *et al.* demonstrated a preferential role for IL-12 over IFN-α/β in driving cytokine expression and lytic activity in human CTLs (Ramos et al., 2009). Conversely, IFN-α/β enhanced the development of a subpopulation of cells that displayed phenotypic and functional characteristics of T_{CM}. Programming of effectors by IL-12 was accompanied by progression of cells through cell division and was altered by the strength of TCR engagement. IL-12 programmed effector cell development, which was regulated by induction of the IL-12 receptor at each division as cells proliferated in response to TCR activation. In contrast, IFN-α/β tended to slow the progression of cell division in some cells, and this effect correlated with induction of the IFN-α/β receptor-2 (IFNAR2) subunit on cells that acquired a T_{CM} phenotype (Ramos et al., 2009). These observations suggest that the functional heterogeneity observed within the pool of memory $CD8^+$ T cells *in vivo* may result from the differential responsiveness of memory precursors to IL-12 and IFN-α/β during priming (Huber et al., 2011).

In this study, we wished to determine whether the initial signals delivered by IL-12 and IFN-α/β regulated either distinct or overlapping programs of gene expression, and whether these patterns were altered during proliferation. Further, we

sought to assess the differential gene expression patterns of memory subpopulations from peripheral blood. This approach allowed for the identification of a subset of genes that were both acutely regulated by IL-12 during priming and also stably expressed in T_{EM} *in vivo*. As a result, we have identified a defined program of IL-12-mediated gene expression with the potential to regulate effector cell development and maintenance of the T_{EM} phenotype.

Results

IL-12 selectively programs the development of CCR7^{lo}CXCR3^{hi} effector CTLs

Recently, Ramos *et al*. demonstrated that the innate cytokines IL-12 and IFN-α/β differentially regulated the development of effector and memory functions in human CD8$^+$ T cells (Ramos et al., 2009). By tracking cell division in response to TCR activation, the IL12Rβ2 subunit was increased by IL-12 activation at each cell division, thereby increasing IL-12 responsiveness. This observation suggests that effector functions regulated by IL-12 would be increased at each cell division. To address this, CD8$^+$CD45RA$^+$ T cells were purified from peripheral blood of healthy adult human donors, labeled with CFSE, and then activated with plate-bound anti-CD3/anti-CD28 for 3 days under various defined cytokine conditions. Based on prior titration experiments, the cytokine concentrations used here were chosen to maximize the relative frequency of both divided and undivided populations that differentially responded to IL-12 and IFN-α (Ramos et al., 2009). As expected, we

identified populations of cells that differentially expressed CCR7 and CXCR3 regardless of whether the cells were activated with either IL-12 or IFN-α, (Figure 3.1A). Further, cells that were retained in the undivided population were found to be predominantly CCR7^{hi} and CXCR3^{lo}. In contrast, cells that rapidly divided lost expression of CCR7 and were CXCR3^{hi}, which is consistent with previously reported effector CTL markers. Further, regardless of cytokine activation, the dividing cells displayed higher expression of CXCR3 than the undivided population, which remained predominantly CCR7^{hi}.

CTLs cultured *in vitro* were also examined for effector molecule expression. As the cells divided, daughter cells progressively expressed increased levels of IFN-γ and TNF-α that was significantly enhanced by IL-12, but not IFN-α (Figure 3.1B). Granzyme B was expressed uniformly in the most divided population of cells, and IL-12 enhanced granzyme B during earlier divisions (Figure 3.1B). Although CXCR3 expression increased in divided cells regardless of innate cytokine exposure, IL-12, but not IFN-α, significantly increased IFN-γ, TNF-α and granzyme B expression within cells as a function of proliferation. Collectively, these data indicate that enhanced sensitivity to IL-12, as previously reported, directly correlates with acquisition of effector functions and suggests an instructive program of effector cell differentiation by IL-12.

$CCR7^{lo}CXCR3^{hi}$ $CD8^+$ T cells in human peripheral blood are functional effectors

The differential expression of various markers, including CD45RA, CCR7, and CXCR3, on human $CD8^+$ cell populations reflects the functional behaviors of these cells *in vivo* (Kobayashi et al., 2006; Takata et al., 2006). For example, CXCR3 plays an important role in migration of the effector CTLs to sites of infection and thus has been associated as a marker for distinguishing the effector subsets (Taqueti et al., 2006; Brainard et al., 2007). The induction of the $CXCR3^{hi}/CCR7^{lo}$ cells in our *in vitro* assays were verified as a parallel functional subset within the $CD8^+$ T cell pool by staining peripheral blood mononuclear cells (PBMCs) for the surface markers CD8, CD45RA, CCR7, and CXCR3 (Figure 3.2A). Both $CD45RA^+$ and $CD45RA^-$ $CD8^+$ cells contained variegated populations of cells that express CCR7 and CXCR3 (Figure 3.2A, top panel). A proportion of the $CD8^+$ T cells with low expression of CCR7 co-expressed the lytic effector molecules perforin and granzyme B regardless of their expression of CD45RA (Figure 3.2A, lower panels, quadrant *III*). Using these chemokine receptors as cell surface markers, we purified the following $CD8^+$ T cell subsets directly from human peripheral blood by FACS sorting: $CCR7^{hi}CXCR3^{lo}$, $CCR7^{hi}CXCR3^{hi}$, and $CCR7^{lo}CXCR3^{hi}$. Because all of these populations exist within both the $CD45RA^+$ and $CD45RA^-$ population, CD45 was not included as a marker for sorting in this experiment. Immediately following purification, these subsets were then assessed for their ability to kill target cells in a redirected lysis assay.

Expression of perforin and granzyme B within the $CCR7^{lo}CXCR3^{hi}$ population (Figure 3.2A) correlated precisely with their ability to mediate killing activity in a redirected lysis assay (Figure 3.2B). These data demonstrate that lytic activity within the $CCR7^{lo}CXCR3^{hi}$ population is a stable property of these cells in homeostasis.

We further assessed the function of these purified cell populations in response to TCR activation by determining whether $CCR7^{lo}CXCR3^{hi}$ cells were capable of proliferation and pro-inflammatory cytokine expression. Cells were purified from peripheral blood as described above and assessed for proliferation and effector function as the cells divided in response to TCR activation. In the absence of exogenous IL-2, only the $CCR7^{lo}CXCR3^{hi}$ cells were capable of rapid proliferation and acquisition of IFN-γ expression (Figure 3.3A, lower panels). However, addition of IL-2 was sufficient to promote cell division within both the $CCR7^{hi}CXCR3^{lo}$ and $CCR7^{hi}CXCR3^{hi}$ populations (Figure 3.3A, right panel). Regardless of their proliferative capacity, IFN-γ was expressed predominantly by cells within the $CCR7^{lo}CXCR3^{hi}$ population (Figure 3.3B). In response to anti-CD3 stimulation, IFN-γ and TNF-α secretion correlated well with the selective cytokine expression pattern within the $CCR7^{lo}CXCR3^{hi}$ population (Figure 3.3C and D). Taken together, these data demonstrate that the circulating T_{EM} ($CCR7^{lo}CXCR3^{hi}$) population within human peripheral blood possesses immediate effector capabilities (Figures 3.2 and 3.3), and their functions and phenotype are paralleled by cells that differentiated in response to IL-12 *in vitro* (Figure 3.1) (Ramos et al., 2009).

IL-12 selectively regulates an effector CTL gene expression signature

CTL effector cell differentiation is dynamic, being regulated by multiple signals that are integrated as the cells divide in response to antigen stimulation. If stable populations of T_{EM} cells retain some of the original transcriptional programs that regulated their development, then comparing their gene expression signatures to cells acutely activated by IL-12 *in vitro* could reveal these pathways. As a first approach, we examined changes in gene expression profiles as cells differentiated in response to IL-12, IFN-α, or IL-12 + IFN-α. $CD8^+CD45RA^+$ T cells were isolated from peripheral blood of 5 healthy human subjects, labeled with CFSE, and stimulated *in vitro* under defined cytokine conditions for 3.5 days. $CFSE^{hi}$ (undivided) and $CFSE^{lo}$ (divided) were purified by cell sorting as described in Figure 3.1 followed by RNA isolation and microarray analysis.

We first performed an unsupervised 2-way hierarchical cluster analysis of genes that were significantly altered by 1.7-fold compared to the neutralized control, and the normalized intensity values of these genes are displayed in the heat maps (Figure 3.4). As shown in Figure 3.4A, cells that divided in the presence of IL-12 displayed a unique gene expression signature that was distinct from that of IFN-α, and this pattern was conserved among the 5 donors. Further, this signature of IL-12-regulated genes was observed in both the absence and presence of IFN-α. Although the differences in gene expression profiles were less pronounced within the

undivided population, we identified a cluster of genes that were distinctly regulated by IFN-α (Figure 3.4B, bracket). The differential regulation of IL-12-induced genes was further pronounced when comparing fold-change values of sets of genes regulated by IL-12 within the undivided versus divided populations (Figure 3.4C). For each gene that was regulated by IL-12, the magnitude of induction was greater in the divided population compared to the undivided cells. Thus, the differential induction of IL-12-regulated genes within the actively dividing population correlated precisely with increased sensitivity to IL-12 and acquisition of effector function observed above (Figure 3.1) and in previous studies (Ramos et al., 2009).

In mice, both IL-12 and IFN-α can promote effector CTL development, albeit IL-12 being more potent in this activity than IFN-α (Xiao et al., 2009). Mescher and colleagues have further demonstrated that these cytokine signaling pathways regulate an overlapping spectrum of effector response genes such as IFN-γ and granzyme B, suggesting some level of redundancy between these signals in mice (Agarwal et al., 2009). While IL-12 was more potent, we also found that both IL-12 and IFN-α induced IFN-γ secretion in murine $CD8^+$ OT-I cells, and the effect of IFN-α was abolished in IFNAR-deficient cells (Figure 3.8). In contrast, we found very little effect of IFN-α in regulating any aspect of human CTL effector function above the level displayed by cells activated under neutralizing conditions (Figure 3.1 and Ramos, et al., 2009), suggesting that IL-12 and IFN-α play non-redundant roles in regulating CTL effector functions. In order to distinguish genes that were either

regulated distinctly or commonly by IL-12 and IFN-α, we compared the gene lists by Venn analysis. The differences in activation by IL-12 and IFN-α were reflected by the unique spectrum of genes that were differentially regulated within the dividing population. Of the 222 genes that were assessed in Figure 3.4A, we found that only 2 genes (*Cxcl10* and *Fasl*) were commonly regulated by both IL-12 and IFN-α (Figure 3.5A, Venn segment VII). In contrast, 101 genes were regulated exclusively by IL-12 regardless of the presence of IFN-α (Figure 3.5A, Venn segment IV). These genes were further analyzed with the "DAVID" bioinformatics resource by assessing biological meaning of this gene list based on gene ontologies (Huang et al., Nat. Protoc. 2009; Huang et al., Nucleic Acids Res. 2009; Huang et al., Curr Protoc Bioinformatics. 2009). The major ontology terms that explained the gene pool included the following ontology definitions: immune response, cell surface receptor linked signal transduction, regulation of programmed cell death, defense response, inflammatory response, and response to virus (Figure 3.5B). These ontology terms included genes that were selectively induced by IL-12 in the dividing population such as IFNG, TNF, IL8, and LTA (lymphotoxin-α) (Figure 3.5C). Conversely, genes regulated exclusively by IFN-α were included only within the immune response, defense response, and response to virus categories, which included the canonical interferon sensitive genes IRF7, OASL, MX1, and ISG15. The robust induction of these genes within the IFN-α-treated population underscores their selective responsiveness to IFN-α in this assay. Thus, the patterns of gene expression and

the functional annotation of those genes highlight the unique pathways that are distinctly regulated by IL-12 and IFN-α in human $CD8^+$ T cells. Overall, we found that IL-12, but not IFN-α, selectively programmed effector functions within actively dividing $CD8^+$ T cells, and these functions corresponded to the known biological activities of the genes that were selectively regulated by IL-12.

Gene expression profiles of effector and naïve/central memory $CD8^+$ T cells in peripheral blood

As demonstrated above (Figures 3.2 and 3.3), circulating $CD8^+$ T cells within the $CCR7^{lo}/CXCR3^{hi}$ subpopulation were capable of robust effector response upon TCR stimulation. Based on our functional analysis, we predicted that these T_{EM} cells would stably express a subset of genes whose functions would be predictive of the effector phenotype of this population. We addressed this by comparing the gene expression profiles of $CCR7^{hi}CXCR3^{lo}$ and $CCR7^{lo}CXCR3^{hi}$ cells purified directly from peripheral blood of 4 healthy adults by cell sorting as described above. The $CCR7^{hi}CXCR3^{lo}$ potentially contained mixed populations of both naïve and central memory cells, yet are functionally similar given their relatively low levels of cytokine secretion shown above. We generated a list of significantly regulated genes with differential expression of >1.7 fold by performing a pair-wise t-test (p-value<0.01) between the 2 cell subsets from each of the 4 donor samples. A 2-way unsupervised hierarchical clustering of these genes revealed a clear segregation of gene

expression patterns that differed between the $CCR7^{hi}CXCR3^{lo}$ and $CCR7^{lo}CXCR3^{hi}$ populations, and the normalized expression values of these genes are depicted in the heat map in Figure 3.6A. Small differences in the absolute expression values for each gene were detected among the 4 donors, yet these differences did not significantly impact the hierarchy of the individual clusters of genes differentially expressed by the two subpopulations (Figure 3.6A).

To further assess the potential biological processes of these genes, functional annotation clustering was performed within DAVID, which grouped related ontologies and ranked their overall importance by an enrichment score (Figure 3.6B). A high enrichment score (≥ 1.3) is indicative of a more important role played by a specific biological process within the gene set of interest. Specific ontologies describing the gene sets that were more highly expressed in $CXCR3^{hi}$ cells than in the $CCR7^{hi}$ population were clustered in groups representing lymphocyte activation and differentiation, programmed cell death, unfolded protein response, and effector response. In contrast, gene sets that were elevated in $CCR7^{hi}$ cells compared to $CXCR3^{hi}$ cells clustered uniquely in groups describing regulation of cellular metabolic processes and RNA processing. As predicted by their effector phenotype and reflected by the ontology analysis, we found that many of the genes known to mediate effector functions were more highly expressed in the $CXCR3^{hi}$ population including IFN-γ, CCL5, and FasL (Figure 3.6C). In addition, granzymes A and K were elevated in the $CXCR3^{hi}$ cells, but, interestingly, perforin and granzyme B were

not significantly different between the two populations (Figure 3.6C). Effector cell surface receptors such as IL-18R1, KLRG1, and IL-2Rβ were also significantly enriched within the CXCR3^{hi} subset. Thus in summary, the genes that have significantly higher expression in T_{EM} CXCR3^{hi} cells were found to be part of functional clusters relating to immune response.

IL-12 drove the differentiation of acute effector cells that exhibited many of the functional attributes of circulating T_{EM} cells *in vivo*. However, it was not clear what components of this acute signaling pathway were stably expressed during the memory cell transition. Given their common functional activities, we predicted that we would find similarities in gene expression patterns between cells activated by IL-12 *in vitro* and the CXCR3^{hi} CD8^{+} T_{EM} cells sorted from peripheral blood. Conversely, the differences in gene expression between these two cell types may reveal subsets of genes transiently expressed during acute stimulation, which may lead to secondary gene expression pathways in T_{EM} cells persistent under homeostatic conditions *in vivo*. This analysis focused on the IL-12-induced genes that were selectively regulated in the dividing population described in Figure 3.4A. IL-12- and IL-12+IFN-α-induced genes from dividing cells were compared to the set of genes that were more highly expressed in CXCR3^{hi} cells purified directly from peripheral blood. Of the 308 genes elevated within the CXCR3^{hi} population *in vivo*, Venn analysis identified 8 of these genes that were regulated acutely by IL-12 *in vitro*, most notably IFN-γ and Eomes (Figure 3.7A, Venn center segment). However,

the majority of the IL-12-induced genes expressed in acutely activated dividing cells were not differentially expressed in the CXCR3^{hi} cells, indicating transience in the majority of the IL-12-regulated pathways or differences in activation status.

This Venn analysis enabled the identification of specific genes that represented 3 possible forms of differential regulation: 1) induced by IL-12 and elevated in CXCR3^{hi} cells, 2) induced by IL-12 but not differentially expressed in CXCR3^{hi} cells, and 3) no acute regulation by IL-12, but elevated in CXCR3^{hi} cells. As would be expected based on the differences in the activation state of the cells, only a small subset of genes were induced by IL-12 and remained elevated in T_{EM} compared to their T_{CM} counterparts (Figure 3.7A, center segment). By focusing on molecular regulators such as signaling intermediates and transcription factors, we found that DUSP5, MAP3K8, ERN1, CDKN2D, and EOMES, were acutely induced by IL-12 and selectively elevated within the CXCR3^{hi} population (Figure 3.7A Venn center segment and Figure 3.7B). Interestingly, the T-box family member TBX21 (T-bet), which is closely related to EOMES and involved in Th1 development, was neither regulated by IL-12 nor differentially expressed in T_{EM} vs T_{CM} cell *in vivo* (Figure 3.7B, bottom panel). In contrast, PIK3AP1 and CEBPD were acutely induced by IL-12, and although marginally elevated in CXCR3^{hi} cells, their normalized expression values were not significantly different from CCR7^{hi} cells (Figure 3.7C). Finally, the transcriptional regulators ZEB2, PRDM1 (Blimp-1), and BCL6 were not significantly regulated by either IL-12 or IFN-α *in vitro*, yet they were significantly

elevated in the CXCR3^{hi} cells, suggesting an alternate mode of regulation (Figure 3.7C). Thus, this combinatorial analysis revealed specific transcriptional pathways regulated acutely by IL-12 that give rise to stable patterns of gene expression and are unique to the effector phenotype of T_{EM}.

Discussion

Innate cytokines are critical signals that drive this differentiation process, with IL-12 and IFN-α/β playing central roles in the context of intracellular pathogen infections (Mescher et al., 2006; Huber et al., 2011). Our previous studies defined unique roles for IL-12 and IFN-α/β in regulating effector and memory functions in both human CD4$^+$ and CD8$^+$ T cells (Davis et al., 2008; Ramos et al., 2009). Although IL-12 was unique in its ability to regulate effector cell commitment, the downstream targets of IL-12 that mediate this activity have not been elucidated. In the current study, we used a hypothesis driven approach to dissect IL-12-driven gene expression pathways governing human CD8$^+$ T effector cell development. This analysis revealed both the acute transcriptional changes that occurred in response to IL-12 and the stable patterns of gene expression adopted by T_{EM} cells *in vivo*.

The *in vitro* culture system used here allowed for the strict control of innate cytokine stimulation during priming as well as distinguishing dividing versus non-dividing populations. As cells divided in response to TCR activation, IL-12 instructed a program of effector development in the daughter cells as assessed by increased

lytic activity and cytokine secretion. The differential expression of effector genes within this population directly paralleled their effector function and underscored the unique effects exerted by IL-12, but not IFN-α. Although many genes were induced by IL-12 in the undivided population, the magnitude of induction of those genes was greater as the cells divided, suggesting greater sensitivity to IL-12 (Ramos et al., 2009). The differences in gene expression signatures regulated by IL-12 versus IFN-α were striking. Regardless of whether the cells were dividing or not, less than 10% of the genes altered by IL-12 were commonly regulated by IFN-α. Importantly, the main effector cytokine genes IFNG, TNF, IL8, and LTA were selectively induced by IL-12, but not by IFN-α. This result is in contrast to observations in mice where both IFN-α and IL-12 share some common effects by promoting effector cell development (Curtsinger et al., 2005; Xiao et al., 2009). Thus, these data suggest that, unlike mice, IL-12 is unique in its ability to program effector cell development in human $CD8^+$ T cells, and further supports the conclusion that IL-12 and IFN-α are not redundant signals.

Acute effector cells die *en mass* following the resolution of an infection, yet small pools of antigen-experienced T_{EM} cells escape this attrition and retain their effector phenotype. These endogenous T_{EM} displayed lytic activity and secreted inflammatory cytokines and paralleled cells that were activated with IL-12 *in vitro*. Although the T_{EM} that we identified *ex vivo* potentially contained various additional populations of cells that differed in their effector function, we used these cells to

determine whether any subsets of genes that were acutely regulated by IL-12 *in vitro* were stably expressed in T_{EM} *in vivo*. First, compared to the $CCR7^{hi}$ subpopulation, the gene signatures in T_{EM} isolated *ex vivo* revealed a strong bias for specific ontology terms describing lymphocyte activation, immune response, and other biological processes consistent with their effector phenotype. Previous studies have examined the functional abilities and gene expression profiles of several subsets of $CD8^+$ T_{EM} cells, and our analysis is in agreement with those defined as effector memory cells (Kaech et al., 2002; Willinger et al., 2005; Haining et al., 2008; Gaucher et al., 2008). As expected based on the purification scheme, the T_{EM} population expressed higher levels of CXCR3 along with selective expression of KLRG1 and IL18R1. Further, even though these cells were not overtly activated, the steady-state levels of IFNG, TNF, and CCL5 were elevated, further indicating effector potential. Although we were able to detect higher intracellular protein levels of perforin and granzyme B in $CCR7^{lo}CXCR3^{hi}$ $CD8^+$ T cells, only granzyme K displayed statistically significant differential expression (Figure 3.6C). Granzyme K expressing $CD8^+$ T cells can exert cytotoxicity in the granzyme $AB^{-/-}$ mice (Jenkins et al., 2008). Additionally, our result is in agreement with previous studies that have shown that although resting memory $CD8^+$ T cells have open chromatin sites at Prf1 and GrzB, relatively low levels of active transcription occur at these genes without antigen stimulation (Zediak et al 2011). Overall, the T_{EM} isolated from peripheral

blood express gene signatures reflective of their unique effector function that we demonstrated to be acutely regulated *in vitro* by IL-12.

In response to TCR activation, both the dividing and non-dividing cells responded to IL-12 as evidenced by the differential regulation of both induced and repressed genes. We focused on the IL-12-induced genes within the dividing population in order to identify specific subsets of genes expressed at higher levels in the $CXCR3^{hi}CCR7^{lo}$ subset isolated *ex vivo*. We identified genes with the intrinsic potential to regulate fate commitment. Within this set of genes, we identified 5 molecular regulators (signaling intermediates and transcription factors) that were both induced by IL-12 and more highly expressed in the T_{EM} population. Not surprisingly given its importance in regulating effector CTLs in mice, Eomes was included in this group, and this is the first demonstration that Eomes is an IL-12-induced gene in human $CD8^+$ T cells (Pearce et al., 2003; Intlekofer et al., 2005). In contrast, the related T-box family member T-bet was not differentially regulated by IL-12 or IFN-α, nor was it differentially expressed within the $CXCR3^{hi}$ population *in vivo*. This observation supports previous studies suggesting a reciprocal relationship between Eomes and T-bet in regulating effector functions in $CD8^+$ versus $CD4^+$ T cells (Pearce et al., 2003; Takemoto et al., 2006). However, in mice, IL-12 induces T-bet expression in $CD8^+$ T cells and the graded expression of T-bet drives terminal effector differentiation as cells respond to infection (Szabo et al., 2002; Joshi et al., 2007; Agarwal et al., 2009). By contrast, we found no differential

regulation of T-bet by IL-12, nor were there any significant difference in its expression in human $CD8^+$ T cells *in vivo*. Further, in human, both Eomes and T-bet are regulated by IL-12 in $CD8^+$ and $CD4^+$ T cells, respectively (Ramos et al., Davis et al., 2008; Ramos et al., 2009). As such, it is unclear how the IL-12 signaling pathway regulates only one of the two transcription factors in each cell type and whether species differences can account for this regulation.

In addition to Eomes, this analysis revealed 4 additional unique IL-12-induced intracellular regulators that were expressed in T_{EM} *in vivo*. First, we identified both a dual-specificity phosphatase (DUSP5) and a MAP kinase (MAP3K8), both of which have been implicated in directly regulating effector T cell activity (Kovanen et al., 2008; Watford et al., 2008; Mielke et al., 2009). Given their known enzymatic activities, it is possible that enhanced expression of DUSP5 and MAP3K8 may create a molecular on/off switch to transmit signals during recall responses to antigen. In addition, ERN1, an endoplasmic reticulum protein (ER) that acts as a ER stress signaling molecule, was also induced by IL-12. Stable expression of ERN1 within the T_{EM} might allow effectors to maintain viability during synthesis of large quantities of effector proteins, such as cytokines (Hetz et al., 2009). Finally, the cyclin-dependent kinase inhibitor family member CDKN2D was expressed in T_{EM}, and its activity may be involved in the differential ability of T_{EM} versus T_{CM} to divide in response to antigen stimulation in the absence of exogenous IL-2, as demonstrated in Figure 3.2A. Collectively, the induction of these 5 genes by IL-12 and their stable

expression within T_{EM} may represent a unique pathway that defines their phenotype and regulates their function.

Perhaps the most intriguing result of this analysis was the identification of three genes that were highly expressed within the T_{EM}, yet were not regulated by either IL-12 or IFN-α: ZEB2, PRDM1, and BCL6. All three of these factors have been demonstrated as transcriptional repressors. ZEB2 is often found to be mutated in Mowat-Wilson syndrome, and regulates epithelial-to-mesenchymal transition (EMT) during embryogenesis (Cecconi et al., 2008; Mongroo et al., 2010). Until now, expression of ZEB2 has not been described in T cells, although a recent study demonstrated a critical role for this factor during murine hematopoiesis (Goossens et al., 2011). In contrast, BCL6 and PRDM1 (Blimp-1) have been shown to exert distinct activities during memory cell development that seem to balance the ratios of T_{EM} to T_{CM} (Crotty et al., 2010). In murine $CD8^+$ T cells, Blimp-1 and Bcl6 are reciprocally expressed in T_{EM} and T_{CM}, and elevated Blimp-1 levels drive terminal effector cell differentiation associated with an exhausted phenotype during persistent viral infections (Rutishauser et al., 2009; Ichii et al., 2004; Shin et al., 2009). In human $CD8^+$ T cells, we found that both BCL6 and PRDM1 were elevated within the T_{EM} as compared to T_{CM}, which seems to contradict the notion that the two factors stand in opposing negative regulation of each other. However, given the strong and selective regulation of effector function by IL-12, it was surprising to find that neither BCL6 nor PRDM1 were regulated by IL-12. Perhaps other signals such at TCR

activation or IL-2 promote induction of these genes at early timepoints, while IL-12 retains expression of these factors at later time points that were not assessed in this study. Alternatively, ZEB2, BCL6, and PRDM1 could be downstream targets of other acute IL-12-induced genes such as EOMES, which could promote terminal differentiation of effectors during later points of activation or during secondary responses to antigen. Indeed, Wirth et. al. reported increased expression of both EOMES and PRDM1 in murine $CD8^+$ T cells undergoing repeated rounds of antigen stimulation, suggesting a contribution by TCR stimulation to the stable induction of these genes (Wirth et al., 2010).

In summary, IL-12 is a primary innate cytokine that directly regulates effector cell development in human $CD8^+$ T cells. While some gene signatures are shared between IL-12 and IFN-α, IL-12 uniquely regulates a subset of genes that are either direct effector molecules such as IFN-γ, or are regulators of their expression. Future studies will focus on the coordinated action of this subset of regulatory proteins and determine specific downstream targets during infection and vaccination.

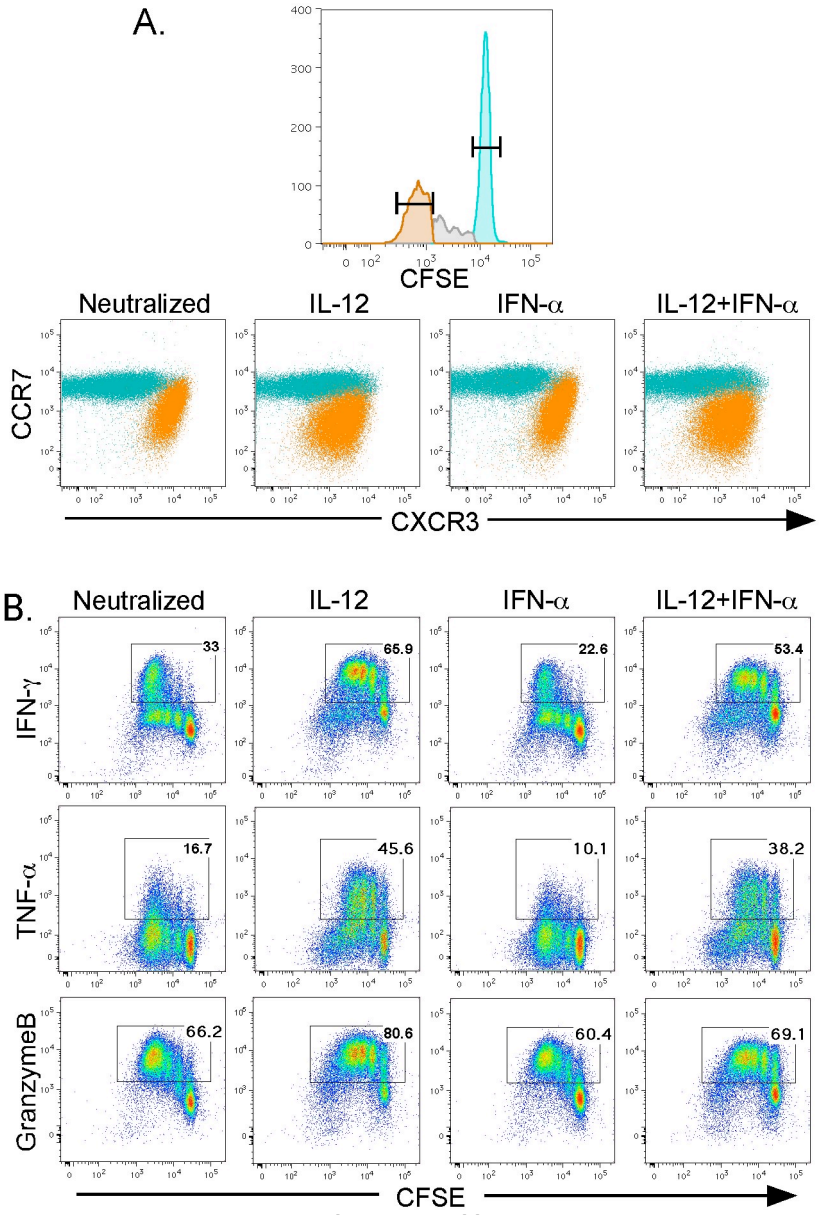


Figure 3.1. IL-12 programs CCR7^{lo}CXCR3^{hi} effector CD8^{+} T cells *in vitro*. *A,* CD8^{+}CD45RA^{+} T cells were labeled with CFSE and stimulated with plate-bound anti-CD3+anti-CD28 for 3 days in the presence of indicated cytokine conditions. On day 3 the cells were split 1:10 with 100 U/mL of rhIL-2. On d 4, cells were stained for CCR7 and CXCR3. *B,* Cytokine polarized CD8^{+} T cells (same as above) were re-stimulated with 80 ng/mL of PMA and 1μM of ionomycin in the presence of monensin for 4h. Cells were then fixed and stained for intracellular IFN-γ, TNF-α, and granzyme B.

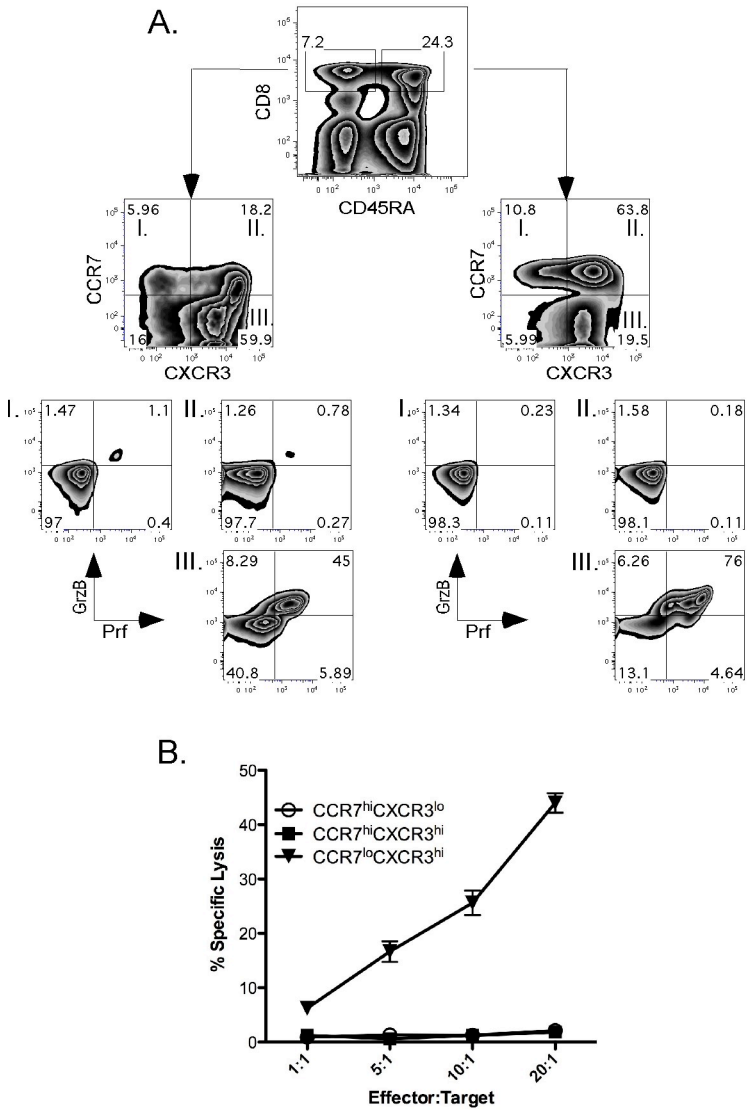


Figure 3.2. CCR7^{lo}CXCR3^{hi} CD8^{+} T cells display an effector phenotype. *A,* PBMCs from healthy human blood were stained for surface receptors CD8, CD45RA, CXCR3, CCR7, and intracellular perforin and granzyme B. Four individual donors were used in replicate experiments and representative plots from one experiment are shown. *B,* Healthy human PBMCs were stained with antibodies specific for CD8, CCR7, and CXCR3 and sorted into 3 separate CD8^{+} populations as indicated in the figure. Re-directed lysis assay was carried out at the indicated E:T ratios using anti-human anti-CD3-coated THP-1 target cells. The result was repeated with cells isolated from two additional healthy donor samples.

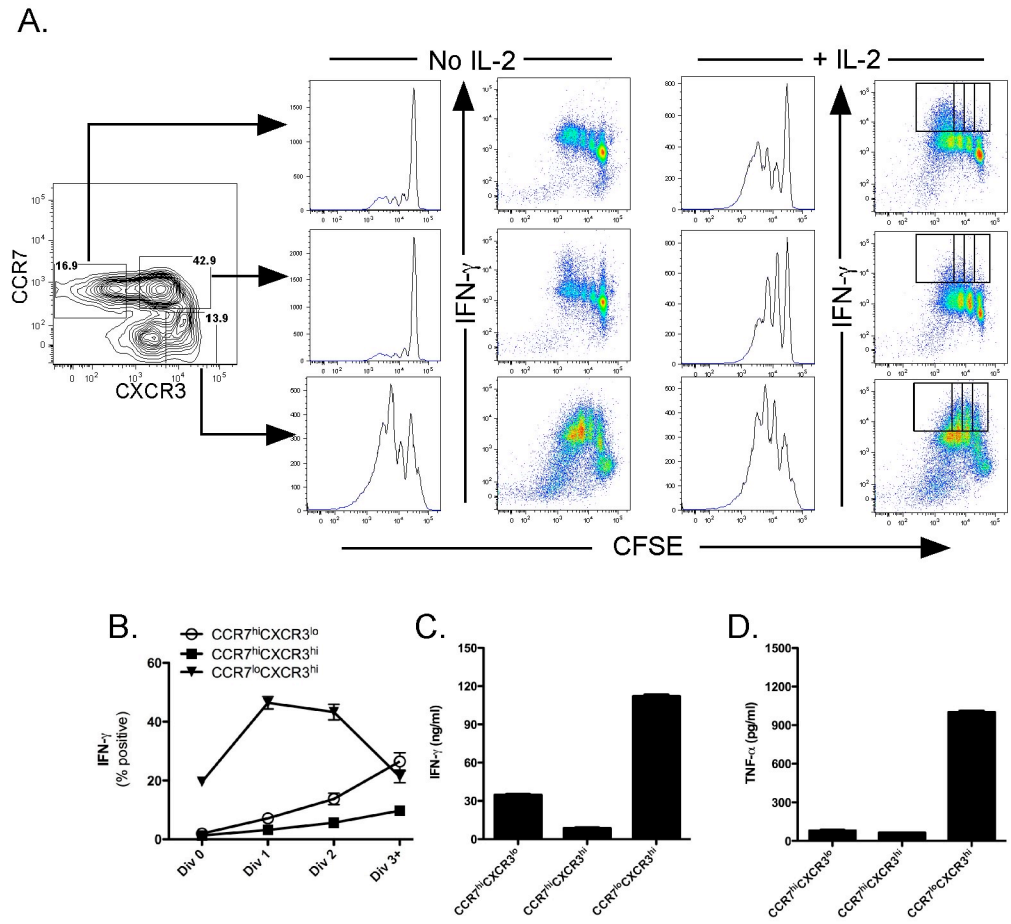


Figure 3.3. CCR7^{lo}CXCR3^{hi} effector memory CD8^{+} T cells secrete effector cytokines. CCR7^{hi}CXCR3^{lo}, CCR7^{hi}CXCR3^{hi}, and CCR7^{lo}CXCR3^{hi} CD8^{+} T cells were sorted from healthy human PBMC and labeled with CFSE. The cells were stimulated with plate-bound anti-CD3 for 3 days in the absence or presence of 200 U/ml of rhIL-2. The cells were restimulated with PMA and ionomycin in the presence of brefeldin A for 4h on d 3 post-stimulation. Proliferation was measured by CFSE dilution, and IFN-γ was measured by intracellular staining. *B,* Percent of cells positive for IFN-γ expression is plotted as mean ±SEM for the cells receiving exogenous IL-2. Effector cytokines in the supernatant was measured by ELISA for human IFN-γ (*C*) and TNF-α in (*D*).

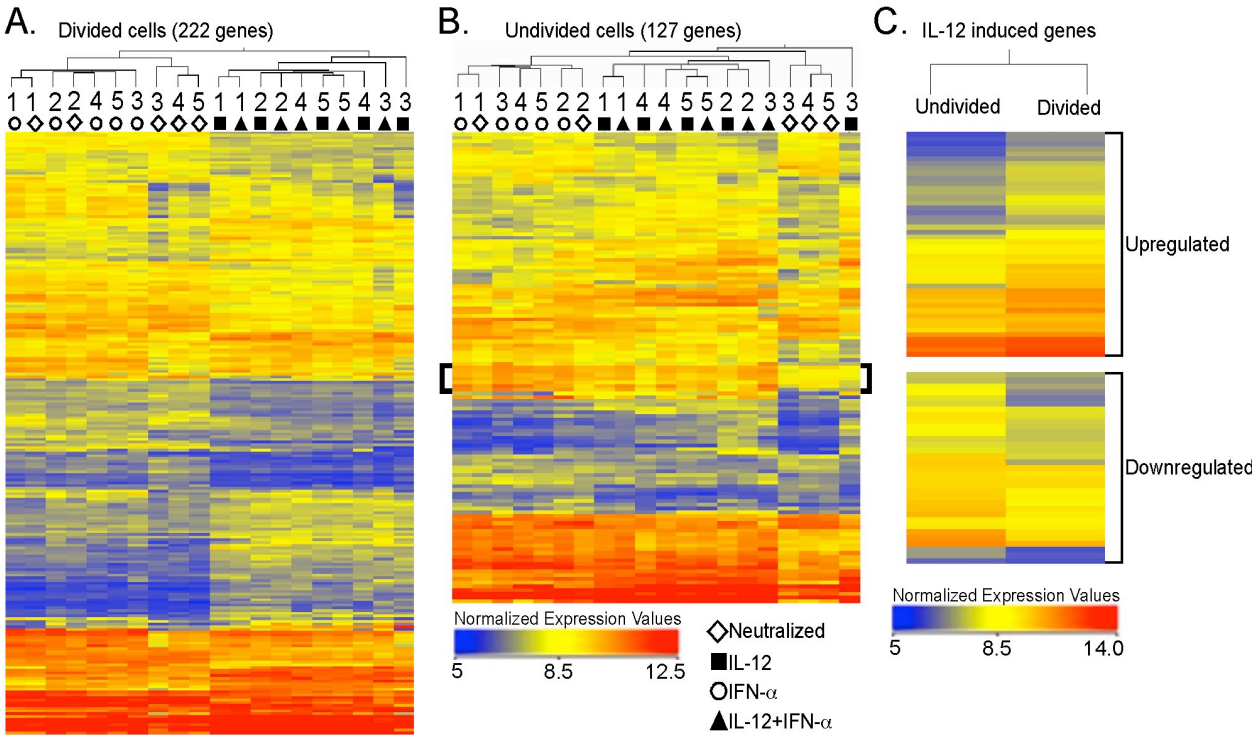


Figure 3.4. IL-12 and IFN-α regulate expression of unique gene sets in human CD8$^+$ T cells. *A & B,* Negatively isolated CD8$^+$CD45RA$^+$ T cells were polarized *in vitro* for 3 days under the defined conditions as indicated: Neutralized (◇), IL-12 (■), IFN-a (○), and IL-12+IFN-a (▲) and CFSE^{lo} (shown in orange in Figure 3.1A) and CFSE^{hi} (shown in teal in Figure 3.1A) cells were sorted for RNA extraction and microarray analysis. A statistically significant gene list (*p-value* ≤ 0.01) was obtained using normalized expression values of the 5 donors, and ANOVA Unequal Variance (Welch) was used as the statistical test. Cytokine stimulated samples were compared to the Neutralized condition to obtain a list of genes with at least 1.7 fold difference. Hierarchical clustering of both conditions and entities (genes) is represented in the heat-maps of normalized expression values, both within divided (*A*) and undivided (*B*) populations. Individual donors are indicated as numbers under the dendrogram. The bracket in 4*B* indicates a cluster of IFN-a stimulated genes. *C*, Genes regulated by IL-12 (compared to neutralized) were hierarchically clustered based on their expression pattern within the divided and undivided populations.

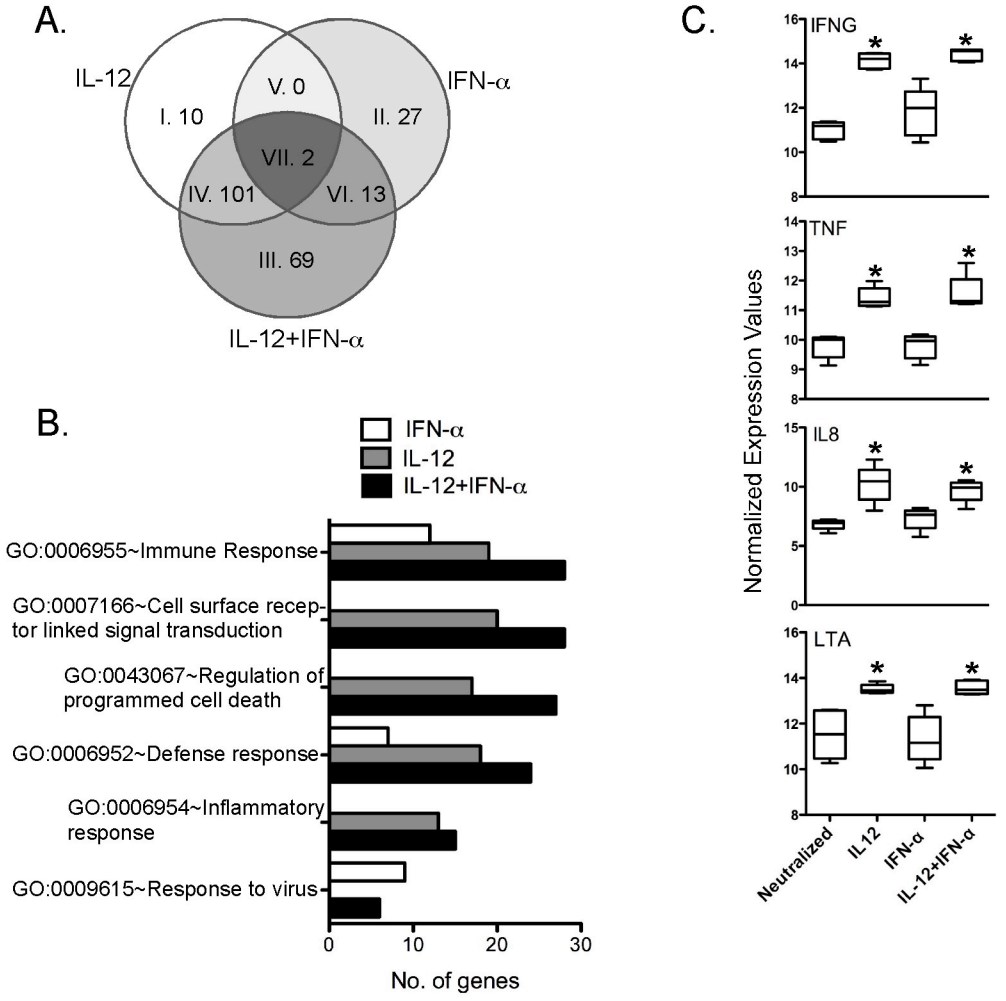


Figure 3.5. IL-12, but not IFN-α, regulates effector gene expression in human CD8$^+$ T cells. Differentially regulated genes by IL-12, IFN-α, and IL-12+IFN-α were analyzed by Venn diagram to identify any genes commonly regulated by the different cytokine conditions. *A,* Genes differentially regulated by IL-12 (≥ 1.7 fold) when compared to the neutralized counterpart were clustered based on normalized expression values (same scale as 3.4A) in undivided (CFSE^{hi} sorted) and divided (CFSE^{lo}) CTLs. *B,* Genes up regulated by at least 1.7 fold were used to determine the Gene Ontology (GO) of the biological processes. The number of genes within a specific GO term is used as an indication of the magnitude of their functional correlation. *C,* Normalized expression values of selected genes representing effector molecules are plotted as Box-Whisker bars (Whiskers: Min to Max).

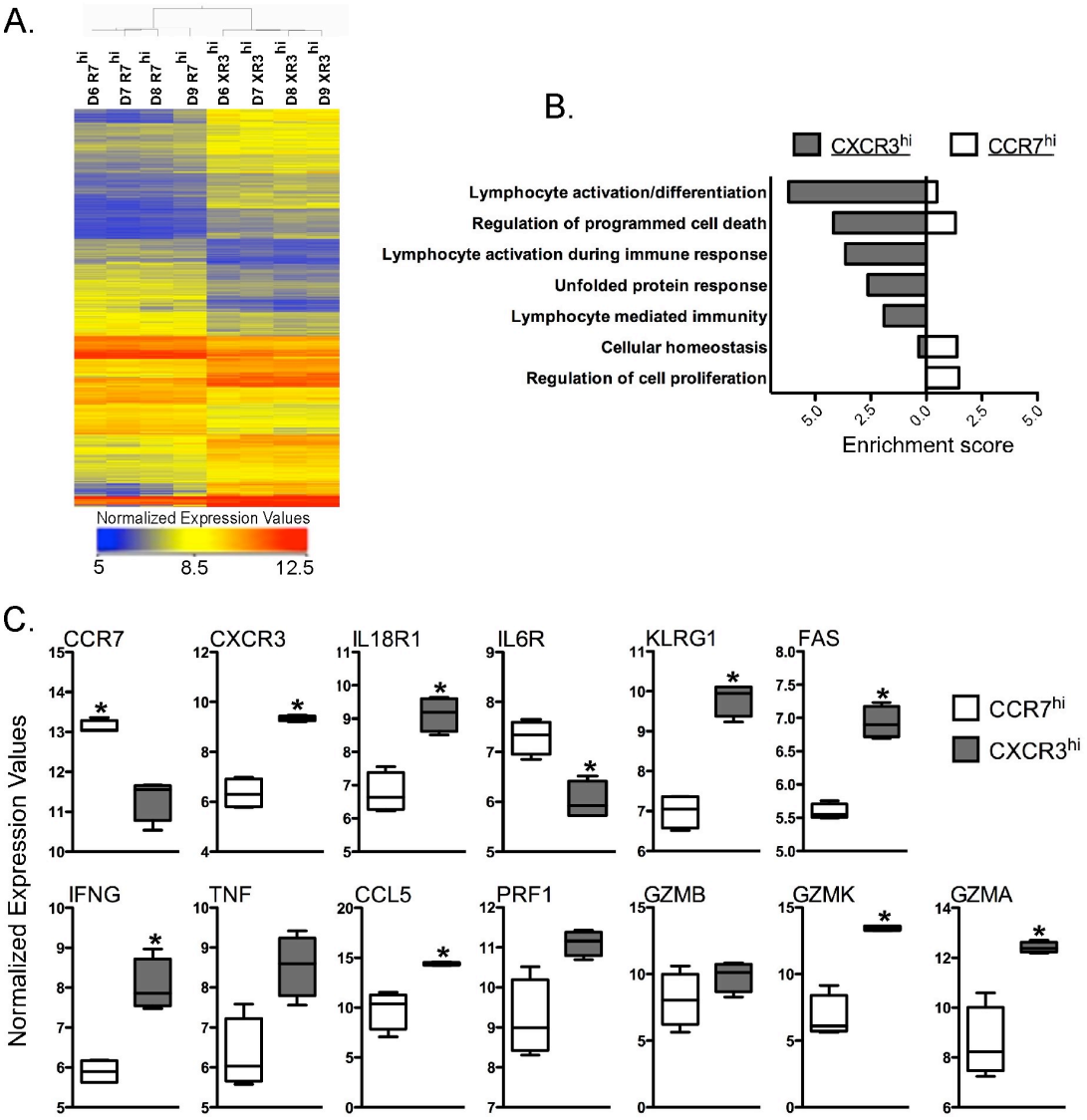


Figure 3.6. CCR7^{lo}CXCR3^{hi} CD8^{+} cells sorted from healthy human peripheral blood show effector/ effector memory gene expression pattern. *A,* The heat-map represents the normalized expression values of CD8^{+}CCR7^{hi}CXCR3^{lo} (R7^{hi}) and CD8^{+}CCR7^{lo}CXCR3^{hi} (XR3^{hi}) cells isolated from 4 individual healthy human donors (D6-D9) by FACS sorting. A statistically significant (*p-value* ≤ 0.01) gene list was obtained from the two subsets using a pair-wise t-test. A list of differentially regulated genes (≥1.7 fold) in 4 donors was generated, and hierarchical clustering of both cell types and entities was performed. *B.* Genes with higher expression values in CXCR3^{hi} or CCR7^{hi} cells (when compared to each other) were used to determine Functional Annotation Clustering of Biological Processes using DAVID. Enrichment score of the cluster is used as an indication for how strongly groups of differentially expressed genes are involved within each functional cluster. *C,* Normalized

expression values of selected genes are plotted as Box-Whisker bars (Whiskers: Min to Max).

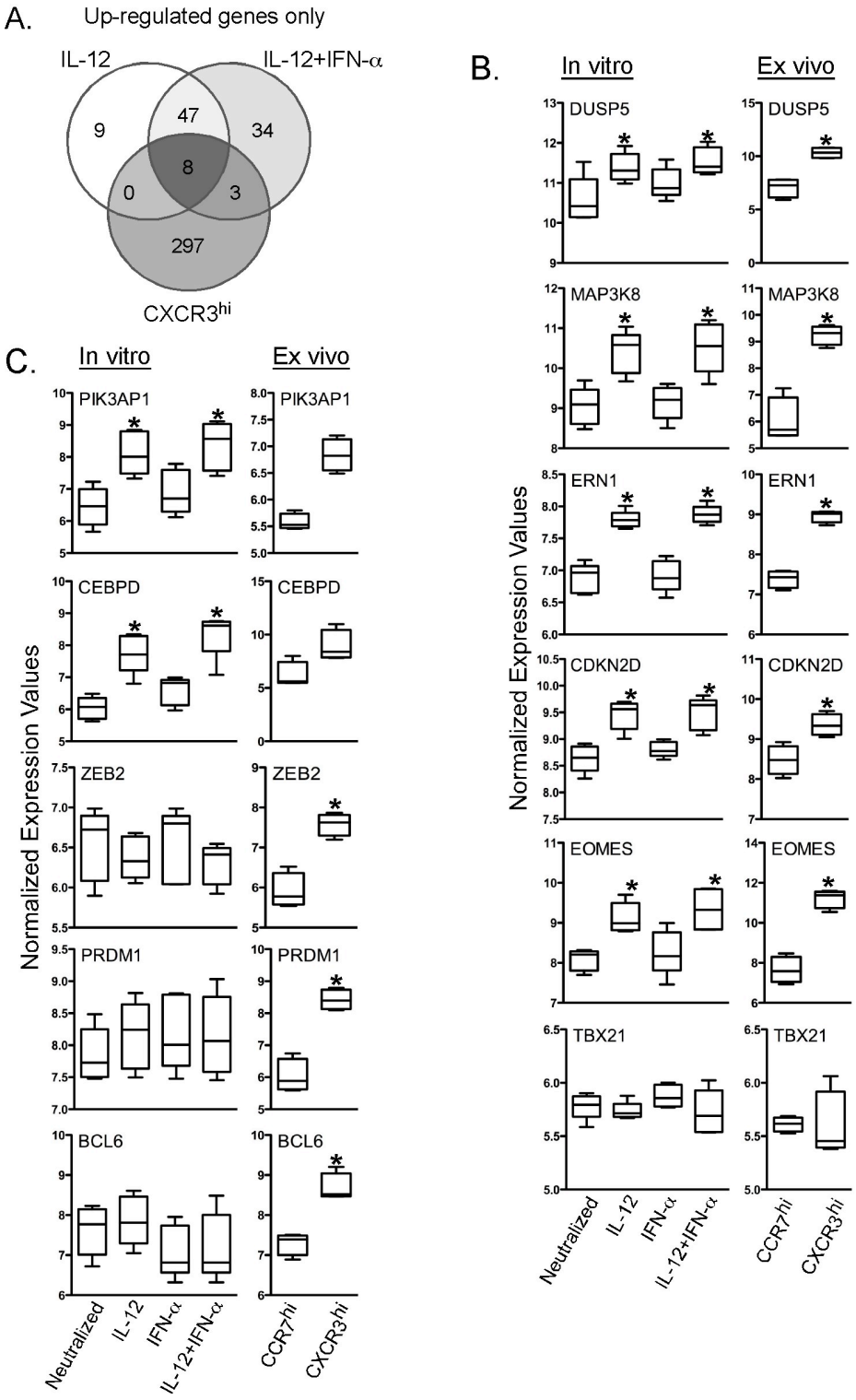


Figure 3.7. Effector memory CD8$^+$ T cells isolated *ex vivo* and IL-12 programmed effector CD8$^+$ T cells generated *in vitro* share a set of commonly regulated genes. Differentially expressed genes from the *ex vivo* samples were

reassessed to identify genes expressed >1.7 fold higher in all donors within the $CXCR3^{hi}$ compared to the $CCR7^{hi}$ cells. These genes were then assessed for commonality with the induced genes identified in cells activated with IL-12 and IL-12 + IFN-α culture conditions. *A*, Genes up-regulated by either IL-12 or IL-12+IFN-α and also increased in $CD8^{+}CCR7^{lo}CXCR3^{hi}$ cells are depicted in the Venn diagram. *B & C,* Normalized expression values of selected genes are plotted as Box-Whisker bars (Whiskers: Min to Max).

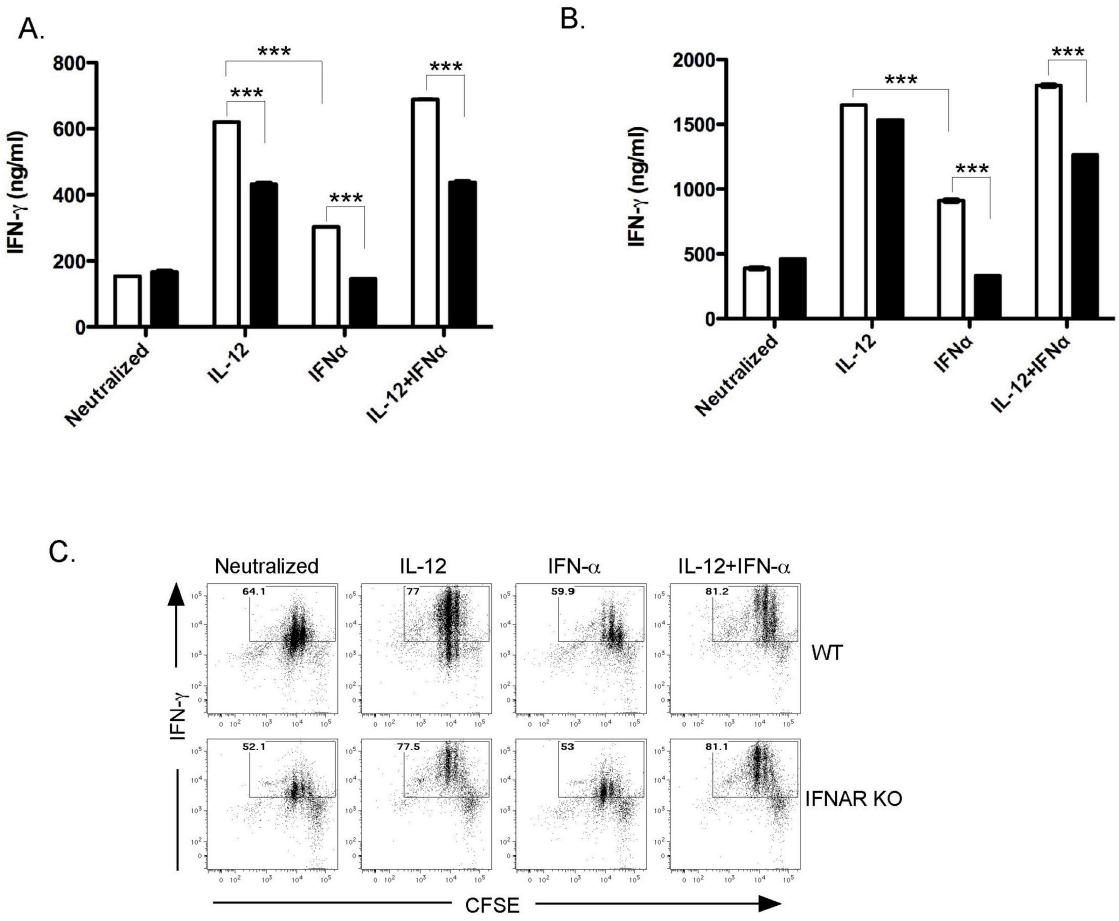


Figure 3.8. IL-12 and IFN-α regulate IFN-γ secretion from murine CD8$^+$ T cells. (A and B) CD8$^+$ T cells were isolated from spleen and lymph nodes of WT OTI (open bars) and IFNAR KO OTI (solid bars) (C57BL/6 background). CD8$^+$ T cells were stimulated for 3 days with plate-bound anti-CD3+anti-CD28 under defined cytokine conditions: neutralized (anti-IL12), IL-12 (10 ng/mL IL-12), IFN-α (anti–IL-12, 1000 U/mL IFN-α), or IL-12+IFN-α. All conditions also received anti–IL-4, anti–IFN-γ, and 200 U/mL rIL-2. Cells were rested overnight and restimulated for 36h with plate-bound anti-CD3 (A) or PMA and Ionomycin (B). IFN-γ in the supernatant was measured by ELISA. *** p≤0.01 by ANOVA. C. CD8$^+$ T cells were labeled with CFSE and cultured for 2 days with irradiated splenocytes from B6-Ly5.1 mice in the presence of 10nM SIINFEKL peptide under defined cytokine conditions: neutralized, IL-12, IFN-α, or IL-12+IFN-α. Cells were restimulated with PMA and Ionomycin in the presence of monensin for 4h. CFSE dilution and intracellular IFN-γ were measured flow cytometry, and data were gated on Ly5.2$^+$ cells.

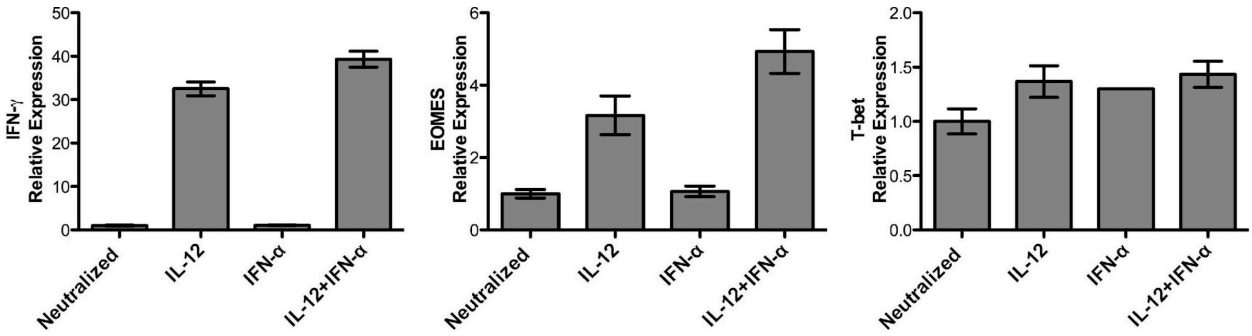


Figure 3.9: qRT-PCR of selected genes in the *in vitro* cytokine polarized CD8$^+$ T cells. CD8$^+$CD45RA$^+$ cells were isolated from healthy human peripheral blood and stimulated under different defined cytokine conditions as indicated. Based on CFSE dilution the divided populations were sorted on BD FACS ARIA. Total RNA was isolated using Arcturus® PicoPure® RNA isolation kit according to manufacturer's recommendations followed by reverse transcription using ABI High Capacity Reverse Transcription kit. Relative mRNA was measured by quantitative RT-PCR analysis with SYBR Green Master Mix (Stratagene, La Jolla, CA) on an ABI7300 real-time thermocycler (Applied Biosystems, Foster City, CA). Human PPIA was used as the reference gene. Relative changes in mRNA expression were calculated and all treatment groups were in referenced to the neutralized control.

CHAPTER FOUR

MAP3K8/TPL2 IS REGULATED BY IL-12 AND REQUIRED FOR EFFECTOR FUNCTION OF HUMAN CYTOTOXIC T LYMPHOCYTES

The work presented in chapter four is unpublished data. Fatema Chowdhury carried out all the experiments unless otherwise indicated.

Nota bene: I have used the terms MAP3K8 and Tpl2 interchangeably in the text to refer to the same entity. I have used MAP3K8 to designate mRNA and Tpl2 for the protein when appropriate.

Introduction

$CD8^+$ T cell or cytotoxic T lymphocyte (CTL) function is essential for immunity to intracellular infections and tumors. Functions of activated effector CTLs include cytotoxicity towards infected cells as well as secretion of effector cytokines, IFN-γ and TNF-α. Both antigen recognition and innate cytokine signaling specify these functions. IL-12 and IFN-α/β are potent "signal 3" inflammatory cytokines that are involved in activation and programming of naïve $CD8^+$ T cells (T_N) in mice during intracellular infections (Valenzuela et al., 2002; Schmidt et al., 2002; Chang et al., 2004; Curtsinger et al., 2005; Agarwal et al., 2009). Once infection is resolved, cell death occurs in majority of the antigen-specific CTLs, and a small subset of memory

CTLs persist with the ability to respond more rapidly and robustly upon reinfection with the same pathogen. There are two types of memory $CD8^+$ T cells: central memory $CD8^+$ T cells (T_{CM}) and effector memory $CD8^+$ T cells (T_{EM}). While T_{CM} require cell division to give rise to effector CTLs, the T_{EM} cells are capable of immediate effector function upon antigen presentation without the need for additional signals (Sallusto et al., 2004). We have previously described that IL-12, but not IFN-α, programs effector function in human $CD8^+$ T cells (Ramos et al., 2009; Chowdhury et al., 2011). We have determined that IL-12 regulates a distinct set of genes involved in effector function. Moreover, some of the IL-12 regulated genes were also stably expressed within the T_{EM} CTLs *ex vivo,* when compared to the T_{N+CM} CTLs (Chowdhury et al., 2011). Within this gene signature we identified MAP3K8 to be an IL-12 induced gene. The goal of this study is to test the role of MAP3K8 in effector function of human $CD8^+$ T cells.

The mitogen activated protein kinase (MAPK) pathways are evolutionarily conserved kinase cascades that control many different cellular responses such as cell survival, cellular metabolism, and proliferation. The process begins with activation of a serine-threonine protein kinase or MAPKKK in response to an extracellular signal. MAPKKK phosphorylates and activates a MAPKK. The activated MAPKK finally activates a MAPK such as ERK, JNK, or p38. This three-kinase cascade can be activated in response to many different stimuli and in turn have the ability to phosphorylate other downstream kinases and transcription factors, which

then guides cellular activation and gene expression (Raman et al., 2007). Both TCR-dependent and –independent activation of CTLs require the function of MAP kinase pathways (Yang et al., 2003; Smith-Garvin et al., 2009). MAP3K8 or Tpl2 (also known as or Cot or c-Cot) is a serine-threonine protein kinase and belongs to the MAPKKK family (Gantke et al., 2012). LPS, TNF-α, and IL-1β signaling can activate Tpl2 in the innate immune cells such as DC and Mϕ. Macrophages from Tpl2 KO mice exhibit a defect in ERK phosphorylation as well as decreased TNF-α secretion in response to LPS stimulation (Dumitru et al., 2000; Eliopoulos et al., 2003). While Tpl2 can directly phosphorylate MEK1/2, leading to ERK phosphorylation, it is largely dispensable for p38 and JNK activation (Gantke et al., 2012). Tpl2 dependent activation of T helper 1 cells is required for optimal IFN-γ production and subsequent clearance of *Toxoplasma gondii* in mice (Watford et al., 2008). ERK activation has been shown to be important for $CD8^+$ T cell development in the thymus (Fischer et al., 2005; Graham et al., 2006). T lymphocyte metabolism as well as $CD8^+$ T cell proliferation and survival have been linked to ERK activation (D'Souza et al., 2008; Carr et al., 2010). However, the role of Tpl2 in triggering ERK pathway in the activation of CTL and effector function is still unknown.

In this study we have demonstrated that Tpl2 is induced by IL-12 in human CTLs. When the requirement for Tpl2 was tested in a murine model we found that unlike the innate immune cells, Tpl2 is dispensable for CTL effector function both *in vivo* and *in vitro*. We have also examined the requirement for Tpl2 activation in

human CTL effector function by using a commercially available small molecule inhibitor. We have shown that blocking Tpl2 function significantly decreased human CTL effector function. IL-12 programmed effector cytokine secretion was also greatly diminished in the presence of MEK1/2 inhibitor, but not p38 inhibitor. Thus, TCR mediated activation of CTLs is likely dependent upon Tpl2-MEK-ERK cascade in human CTLs.

Results

Higher MAP3K8 mRNA transcript is maintained in human T_{EM} CTLs

We have previously demonstrated that $CD8^+CCR7^{lo}CXCR3^{hi}$ T cells isolated from healthy human peripheral blood include T_{EM} CTLs with immediate effector function in the absence of any inflammatory cytokines or 'signal 3' (Chowdhury et al., 2011). I tested to see if CCR7 could be used as the sole marker to functionally demarcate T_{EM} and T_{N+CM} CTL populations. We sorted $CD8^+CCR7^{hi}$ and $CD8^+CCR7^{lo}$ CTLs from healthy human PBMCs (Figure 4.1A,B). $CD8^+CCR7^{lo}$ T cells secreted a significant amount of IFN-γ and TNF-α upon plate-bound anti-CD3 stimulation alone when compared to the $CD8^+CCR7^{hi}$ T cells (Figure 4.1A). $CD8^+CCR7^{lo}$ T cells also displayed immediate cytotoxicity towards the target cells while $CD8^+CCR7^{hi}$ CTLs lacked any significant killing of the target cells (Figure 4.1B). Based on our microarray analysis, we predicted that MAP3K8 expression would be higher in the T_{EM} cells when compared to the naïve cells (Figure 3.7B). As

quantitated by RT-PCR, MAP3K8 mRNA expression is indeed higher in the $CD8^+CCR7^{lo}$ population when compared with the $CD8^+CCR7^{hi}$ cells (Figure 4.1C). Although, the $CD8^+CCR7^{lo}$ sorted cells from healthy human PBMCs is a heterogeneous population, the immediate effector function indicates that it is likely comprised of T_{EM} CTLs when compared to $CD8^+CCR7^{hi}$ CTLs. A higher expression of MAP3K8 mRNA is thus maintained in the T_{EM} CTLs under homeostatic conditions in human.

Tpl2 activation is necessary for human T_{EM} CTL effector function

I utilized a commercially available small molecule inhibitor of Tpl2 activation (Tpl2 inhibitor) to test if human T_{EM} CTL effector function requires the activation of Tpl2. This inhibitor is cell permeable, reversible, and blocks Tpl2 activation in an ATP-dependent manner (Garvin et al., 2005; Wu et al., 2009). Tpl2 has a proline instead of the conserved glycine in the glycine-rich ATP binding loop, making it a selective binding site for the naphthyridine derivative Tpl2 inhibitor, which in turn, blocks phosphorylation and activation of Tpl2 (Luciano et al., 2004; Garvin et al., 2005). Both IFN-γ (Figure 4.2A, top) and TNF-α (Figure 4.2A, bottom) secretion were significantly reduced in a dose-dependent manner when $CD8^+CCR7^{lo}$ sorted CTLs were stimulated with plate-bound anti-CD3 and in the presence of increasing concentration of the Tpl2 inhibitor (Figure 4.2A). DMSO was used as the vehicle control treatment in all experiments, which did not have any significant effect on the

cytokine secretion compared to media alone. When I tested the cytotoxicity *in vitro*, there was marked reduction in the T_{EM} CTLs' ability to kill target cells in the presence of the Tpl2 inhibitor in a dose dependent manner (Figure 4.2B).

Tpl2 may activate the MEK-ERK pathway in TCR mediated activation of human T_{EM} CTLs

In order to delineate the MAP kinase pathways involved in human T_{EM} CTL function, I used commercially available small molecule inhibitors, U0126 and SB 203580. U0126 blocks activation of MEK1/2, which phosphorylates ERK, and SB 203580 directly blocks p38 activation. I observed a dose-dependent reduction in IFN-γ and TNF-α secretion from T_{EM} CTLs when stimulated with plate-bound anti-CD3 in the presence of U0126, but not SB 203580, (Figure 4.2A). Together, these data suggested that TCR mediated activation of human T_{EM} CTLs is Tpl2-MEK-ERK dependent but p38 independent.

There is a TCR-independent mechanism of T cell activation. IL-12 and IL-18 together, but not alone, can activate memory T cells that leads to the secretion of effector cytokines and in mice, it has been shown to be p38 dependent. $CD8^{+}CCR7^{lo}$ or T_{EM} CTLs were sorted from healthy human peripheral blood and activated in the presence of rhIL-12 and rhIL-18. Significant amount of IFN-γ was measured from the supernatant only when the cells were treated with IL-12 and IL-18 together (Figure 4.3). I was unable to detect any significant amount of TNF-α secretion under the

same conditions. While there was a dose-dependent reduction in IFN-γ secretion in the presence of the Tpl2 inhibitor, secretion was completely blocked in the presence of even the lowest amount (0.5uM) of SB 203580 used. This suggests that unlike TCR-mediated activation, the cytokine-mediated activation of CTL is p38 dependent in human. Although Tpl2 inhibitor also blocked cytokine-mediated activation of CTLs, I cannot be sure whether Tpl2 acts upstream of ERK or p38 because U0126 was not used in these experiments. However, TCR-independent activation of T cells require NF-κB mediated synthesis of GADD45β prior to p38 activation (Yang et al., 2001; Figure 1.3). Additionally, IL-18 has been shown to activate ERK in human neutrophils and murine Th1 clones (Tsuji-Takayama et al., 1997; Fortin et al., 2009). Thus, it is possible that Tpl2 inhibitor is blocking cytokine stimulated IFN-γ production from human CTLs by blocking ERK activation upstream of p38.

MAP3K8/Tpl2 expression is regulated by IL-12 in human CTLs *in vitro*

Our microarray analysis indicated that MAP3K8 mRNA is significantly up-regulated by IL-12 in the divided effector CTLs *in vitro*. To confirm the microarray analysis, I examined both total mRNA and total protein in polarized $CD8^+$ T cells, 3.5 days post-stimulation (Figure 4.4A). IL-12, but not IFN-α, was able to significantly up regulate the expression of MAP3K8 mRNA in CTLs *in vitro* when compared to the neutralized condition (Figure 4.4B). This correlates with our observation from the microarray analysis (Figure 3.7). The same trend was observed when I blotted for

Tpl2 in total cell lysate where IL-12 alone significantly up-regulated Tpl2 protein (Figure 4.4C). After many attempts, I was unsuccessful at staining for intracellular Tpl2 protein since the antibody is raised against the c-term of the protein, which is likely unexposed in the cytoplasm due to complex formation with p105 and Abin2. However, RT-PCR and Western blot data suggests that IL-12 up-regulates MAP3K8/Tpl2 *in vitro* in human $CD8^+$ T cells.

Tpl2 is necessary for IL-12 programmed effector function of human CTLs

I have confirmed that IL-12, but not IFN-α, programs effector function in human $CD8^+$ T cells *in vitro* as measured by IFN-γ and TNF-α secretion, as described previously (Figure 4.5B, Ramos et al., 2009). Next I wanted to test whether Tpl2 was necessary for effector cytokine secretion from IL-12 programmed CTLs *in vitro* upon secondary stimulation with anti-CD3 alone (Figure 4.5A). Tpl2 inhibitor treatment significantly reduced both IFN-γ (Figure 4.5C, top) and TNF-α (Figure 4.5C, bottom) secretion from IL-12 polarized CTLs in a dose-dependent manner. Moreover, significant reduction in both of the effector cytokine secretion was observed when activation of MEK1/2 was blocked using U0126 (Figure 4.5C). However, the p38 inhibitor (SB 203580) did not have any significant affect on IFN-γ or TNF-α secretion after TCR-mediated activation of CTLs (Figure 4.5C). Together, the data suggests that TCR-mediated activation of IL-12 programmed effector CTLs

in vitro leads to IFN-γ and TNF-α secretion and this process is mediated by Tpl2-MEK-ERK pathway.

The Tpl2 inhibitor does not alter the expression of activation marker, CD25, or the viability of CTLs *in vitro*

Since blocking Tpl2 activation significantly reduces both effector cytokine secretion and cytotoxicity of human CTLs, I wanted to test whether this is a global block in CTL activation. I chose to quantify CD25 expression, which is a cell surface marker for T cell activation. Human $CD8^+$ T cells were polarized under the same conditions as above (Figure 4.5A). After day 7 post primary stimulation, the rested cells were either left unstimulated or re-stimulated with plate-bound anti-CD3 only. Upon secondary stimulation, ~80% of the live cells expressed CD25, regardless of the primary cytokine conditions (Figure 4.6A). The percentage of cells expressing CD25 also did not change with the treatment of any of the inhibitors for 24h during the secondary stimulation (Figure 4.6B).

Next, I tested whether there was increased cell death due to the presence of the various kinase inhibitors. Cell viability was tested by staining the T_{EM} CTLs for the cell death markers annexin V and 7AAD 24h post stimulation. CTL viability was the same across the treatment conditions regardless of the dose of Tpl2 inhibitor (Figure 4.7A). Presence of SB 203580 (p38 inhibitor) also did not affect cell viability under the same conditions (data not shown). Although by forward and side scatter

the cells treated with Tpl2 inhibitor appeared to be smaller in size, there was no significant difference in the percentage of annexinV^-7AAD^- or live cells when ungated total population was analyzed (Figure 4.7B). Thus, treatment of the CTLs by Tpl2 inhibitor does not affect the expression of the activation marker CD25 or cell viability but rather blocks IL-12 programmed effector cytokine secretion.

MAP3K8 knockdown approaches in primary human $CD8^+$ T cells were unsuccessful

Since small molecule inhibitors may have off-target effects, we wanted to test if knock down of MAP3K8 in primary human $CD8^+$ T cells might reduce effector function. There is only a single report on successful knockdown in primary human $CD8^+$ T cells and it was accomplished using Amaxa nucleoporation method to knock down GATA3 in $CD8^+$ T cells isolated from systemic sclerosis patients (Medsger et al., 2011). My attempts at siRNA mediated knockdown of MAP3K8 mRNA using both nucleoporation as well as transfection methods were unsuccessful in both human sorted T_{EM} CTLs *ex vivo* and IL-12 treated effector CTLs *in vitro*. I used siGLO as a fluorescent indicator for visual indication of siRNA delivery. Punctate staining was visible by confocal microscopy and increased in intensity with higher concentration of siGLO used (Figure 4.8A). However, the siRNA seemed to be localized to the endosomes rather than disseminating in the cytoplasm as per visual inspection up to 72h post nucleoporation/transfection. Up to 20% of $siGLO^+$ cells were also detected

by flow cytometry (Figure 4.8B). Nevertheless, there was no significant down regulation in the relative expression of MAP3K8 mRNA by specific siRNA when compared to the control treated cells (Figure 4.8C). Thus, knockdown of MAP3K8 in primary human $CD8^+$ T cells was not successful, perhaps due to inadequate release of the siRNA in the cytoplasm.

MAP3K8/Tpl2 is dispensable for effector cytokine expression and secretion in murine CTLs

Given that blocking Tpl2 activation reduces effector function of human CTLs, I wanted to examine if CTL effector function is affected by the absence of Tpl2 in mice. First, I wanted to determine if murine MAP3K8 mRNA is regulated by IL-12 *in vitro* in the same manner as human CTLs. I used murine $CD8^+$ T cells from WT C57BL/6 mice and measured mRNA expression from *in vitro* stimulated cells (Figure 4.9A). However, MAP3K8 expression was not significantly regulated by IL-12 in murine $CD8^+$ T cells *in vitro* (Figure 4.9B-C). Next, I used *Listeria monocytogenes* as a model for an intracellular bacterial pathogen and identified T_{EM}, T_{CM}, and T_N based on the cell surface expression pattern of CD44 and CD62L (Figure 4.10). CD44 and CD62L are the two surface markers used commonly in the analysis of murine $CD8^+$ T cells memory subsets (Wherry et al., 2003; Banerjee et al., 2010). Using quantitative RT-PCR, I measured relative expression of MAP3K8 and IFNG mRNA levels within these sorted $CD8^+$ T cell memory subpopulations. Both $T_{Effector}$ and T_{CM}

expressed significantly higher level of MAP3K8 when compared to T_N and the expression pattern correlated with IFNG expression (Figure 4.11).

In order to test if the lack of Tpl2 expression leads to an altered effector function, we wanted to utilize the knockout mouse model (a gift from Dr. P. Tsichlis). There was no gross difference between the $Tpl2^{+/+}$, $Tpl2^{+/-}$, and $Tpl2^{-/-}$ mice in terms of CD4 and CD8 expression patterns in thymus, blood, LN, and spleen (Figure 4.12). This is in agreement with already published description of the knockout stating that $Tpl2^{-/-}$ mice developed normally with normal ratios of immune cells and without any obvious phenotype (Dumitru et al., 2000; Gugasyan et al., 2011). In accordance with the already published observations, IL-12 treated murine $CD8^+$ T cells were able to secrete significantly higher amounts of IFN-γ *in vitro* upon primary stimulation when compared with either neutralized or IFN-α treated cells (data not shown). However, CTLs from $Tpl2^{+/+}$, $Tpl2^{+/-}$, and $Tpl2^{-/-}$ mice were equally competent in their ability to secrete both IFN-γ and TNF-α (Figure 4.13 B, C). Moreover, no difference in IFN-γ and TNF-α secretion was observed upon secondary stimulation of IL-12 polarized murine CTLs *in vitro* (Figure 4.14 B, C). I also cultured murine CTLs *in vitro* for up to 14 days and did not observe any difference between the different strains in their ability to secrete effector cytokines (data not shown).

Since we detected higher MAP3K8 mRNA level in $T_{Effector}$ and T_{CM} CTLs in response to LM *in vivo*, I wanted to test if $Tpl2^{-/-}$ $CD8^+$ T cells are at a disadvantage in effector function when generated in response to the same pathogen *in vivo*. I

isolated $CD8^+$ T cells from both $Tpl2^{+/+}$ and $Tpl2^{-/-}$ mice and adoptively transferred them into CD45.1 recipient mice (Figure 4.15). The mice were then infected with OVA expressing *Listeria monocytogenes* (LM-OVA) intravenously. I harvested the spleens 7 days post infection and re-stimulated the splenocytes in the presence of SIINFEKL, HKLM, anti-CD3, or left unstimulated. Using CD45.2 as a marker for the donor cells, I was able to detect antigen specific IFN-γ and TNF-α expression intracellularly only when the mice were infected with LM-OVA (Figure 4.16). Three recipient mice per donor for a total of three donor of each strain were used for statistical strength in this experiment. However, there was no discernable difference in either IFN-γ or TNF-α expression between WT and $Tpl2^{-/-}$ $CD8^+$ T cells under any of the re-stimulation conditions (Figure 4.17 A, B).

Given that $Tpl2^{-/-}$ CTLs were not defective in effector cytokine secretion but human CTL function is sensitive to the Tpl2 inhibitor, we wanted to determine if the Tpl2 inhibitor also blocks effector cytokine secretion from murine CTLs *in vitro*. Human and mouse Tpl2 are 93% identical at the amino acid level (Luciano et al., 2004). Additionally, the Tpl2 inhibitor has been shown to block TNF-α secretion from murine bone marrow derived macrophages and RAW264.7 cell line, in response to LPS stimulation (Hirata et al., 2010; Lawrenz et al., 2011). Thus, the commercially available Tpl2 inhibitor has the ability to block Tpl2 activation in both mouse and human. I cultured murine $CD8^+$ T cells *in vitro* in the presence of IL-12 and then re-stimulated the harvested cells with plate-bound anti-CD3 alone in the presence of

the same kinase inhibitors previously used to block activation of Tpl2, MEK1/2, or p38. To our surprise, only U0126 was able to significantly block TNF-α secretion from both $Tpl2^{+/+}$ and $Tpl2^{-/-}$ CTLs (Figure 4.18 A, B). Taken together these data suggest that MAP3K8 function may be dispensable for effector cytokine secretion from murine CTLs.

Discussion

Extensive work has been done to examine the role of Tpl2 in innate immune cells such as dendritic cells and macrophages. Both knockout mice and the use of the inhibitor have demonstrated an essential role for Tpl2 in TNF-α production from murine DC and Mϕ (Dumitru et al., 2000; Symons et al., 2006; Gantke et al., 2011). Although it has been shown that Tpl2 deficiency renders Th1 cells inadequate at IFN-γ secretion, there is no work on what role Tpl2 plays in $CD8^+$ T cell function (Watford et al., 2008). When the TCR2C transgenic mice were crossed with $Tpl2^{-/-}$ mice, the transgenic $CD8^+$ T cells transformed into chronically stimulated T cells and developed into lymphomas which led the investigators to conclude that Tpl2 may be playing an anti-proliferative role in murine CTLs (Tsatsanis et al., 2008). The consequences of TCR2C transgenic $Tpl2^{-/-}$ CTLs in terms of effector function was not tested and it remains inconclusive if Tpl2 play any role in effector function from $CD8^+$ T cells. We have sought to answer that very question using both human and murine $CD8^+$ T cells in this study.

The main functions of CTLs, effector cytokine secretion and cytotoxicity, are both reduced under the treatment of Tpl2 kinase inhibitor *in vitro* (Figure 4.2, 4.5C). Additionally, using MEK1/2 as a surrogate for ERK1/2 MAP kinase activation we have demonstrated that effector cytokine secretion may be dependent in Tpl2-MEK-ERK pathway activation. We know that differentiated effector CTLs as well as T_{EM} CTLs can exert their effector function in response to antigen presentation alone. IL-12 induced Tpl2 stored in the effector and T_{EM} CTLs may be at the center of this signal 2+3 independent triggering of effector function in human T_{EM} CTLs while naïve CTL response requires additional co-stimulation and inflammatory cytokine signals. As such, it is possible that IL-12 programmed readily available Tpl2-MEK-ERK activation pathway in the effector and T_{EM} CTLs make these cells licensed to respond to any infected cells in the periphery.

I used a cell surface marker to test if the Tpl2 inhibitor blocked all aspects of CTL activation. IL2Rα or CD25 is up-regulated in response to TCR signaling and indicates activation of T cells (Cantrell et al., 1983; Obar et al., 2010). Human CTLs polarized *in vitro* expressed CD25 only in response to anti-CD3 stimulation (Figure 4.6A). Moreover, this expression was not down modulated by any of the kinase inhibitor treatment for 24h (Figure 4.6B). Thus, indicating that not all aspects of TCR-mediated CTL activation is dependent upon Tpl2-MEK-ERK pathway.

In the absence of cognate antigen, human memory $CD8^+$ T cells are capable of IFN-γ secretion in the presence of IL-12 and IL-18 (Smeltz 2007). It has been

shown that this TCR-independent pathway for IFN-γ production from murine $CD8^+$ T cells can confer immune protection from *Listeria monocytogenes* and vaccinia virus infection (Berg et al., 2002; Gherardi et al., 2003; Berg et al., 2003; Berg et al., 2005;). This synergistic role for IL-12/18 in activating CTLs also exist in $CD4^+$ T cells and it has been shown to be p38 MAP kinase pathway dependent in murine Th1 cells (Zhang et al., 2000; Yang et al, 2001; Berenson et al., 2006). In line with this observation we have found that human T_{EM} CTLs can be activated to produce IFN-γ, but not TNF-α, by IL-12 and IL-18 together (Figure 4.3). Moreover, unlike TCR-mediated activation of the same CTLs this process of TCR-independent pathway was very sensitive to the presence of p38 kinase inhibitor (SB 203580). IL-12 and IL-18 mediated IFN-γ secretion was also blocked by the presence of Tpl2 inhibitor in a dose dependent manner. We cannot rule out the possibility that Tpl2-MEK-ERK may be involved in IL-18 mediated expression of GADD45β, which in turn activates the p38 MAPK. We cannot be sure at this point if Tpl2 is exerting its kinase activity upstream of p38 or ERK without performing this experiment in the presence of either MEK1/3 or ERK inhibitor.

We have observed that knocking out Tpl2 in mice did not have any consequences for CTL effector function both *in vitro* as well as in vivo. However, the lack of Tpl2 in the innate immune cells has been shown to block TNF-α secretion among other phenotypes (Gantke et al., 2011; Gantke et al., 2012). Although we have not examined this any further, we can speculate as to why this might be the

case. First, there are at least 16 other MAP3 kinases that have been identified to have the dual-specificity kinase activity upstream of the major MAP kinase pathways (Raman et al., 2003). It is possible that any of these MAP3 kinases may be playing a compensatory role in activation of murine CTLs. Second, in contrast to human, we have not consistently observed an up-regulation of Tpl2 in murine CTLs in response to IL-12. Although IL-12 up-regulates Tpl2 in $CD4^+$ T cells and was found to be important for IFN-γ secretion from Th1 cells, we did not observe it in murine CTLs (Watford et al., 2008). Finally, ERK1/2 phosphorylation in CTLs from $Tpl2^{-/-}$ mice might still be intact which would indicate that there is an alternative pathway to ERK activation in murine CTLs when TCR stimulation is provided. ERK activity is essential for proper thymic development of T cells (Fischer et al., 2005; Graham et al., 2006).). But when we tested for the CD4 and CD8 profile of the thymocytes comparing $Tpl2^{-/-}$ and WT littermates, there was no difference in their expression pattern (Figure 4.12). Furthermore, blocking ERK activation in murine CTLs through MEK1/2 inhibition blocks TNF-α, but not IFN-γ, secretion while pharmacological inhibition of Tpl2 has no effect (Figure 4.18). Thus, we can conclude that there is likely a Tpl2 independent mechanism of ERK activation leading to effector cytokine secretion in murine CTLs.

Inflammation develops when the innate immune system recognizes a danger signal, and in response, secretes inflammatory cytokines as well as chemokines to recruit more cellular players of the immune response. The state of inflammation is

usually resolved upon pathogen clearance and tissue repair. However, chronic inflammation can have debilitating effects in certain autoimmune disorders such as rheumatoid arthritis, inflammatory bowel disease, etc. Blockade of TNF-α and IL1β secretion is the goal of treatments for such diseases since they are the major pathogenic factors in the disease manifestation. There has been remarkable interest in developing small molecule inhibitors for protein kinases since the discovery of their ability to block not only their kinase activity but also the production of pro-inflammatory cytokines such as IL-1β and TNF-α (Lindstrom et al., 2009). Tpl2 has been identified as a 'druggable' target by many because of its role in activating innate immune cells to produce TNF-α in mice and human (Hall et al., 2007; Wu et al., 2009; Hirata et al., 2010). For example, therapeutic use of the Tpl2 inhibitor has been suggested to impede prolonged and uncontrolled inflammation in diseases such as rheumatoid arthritis and inflammatory bowel disease based on some preliminary studies done in mice (Hu et al., 2006; Cohen 2009; Gaestel et al., 2009; Lawrenz et al., 2011). Here we have shown that human CTL effector function is drastically reduced when the cells are stimulated in the presence of Tpl2 inhibitor. As such, we have to be careful in moving forward with any plans to test the therapeutic efficacy of such kinase inhibitors in human since a global block in immune function may result in further health complications due to increased susceptibility to infections.

In summary, this study has shown that human CTL effector function is regulated by the IL-12 induced serine-threonine protein kinase Tpl2, likely upstream of MEK/ERK pathway. This may be the potential signaling intermediate that abolishes a need for co-stimulation in activating effector and T_{EM} CTLs. Blocking this pathway has dire consequences on effector function of human CTLs *in vitro*. The effects of blocking the different MAP kinases can have different consequences in a cell type dependent manner. Thus any therapeutic use of kinase inhibitors must be considered carefully by weighing out the cost-benefit of the outcome.

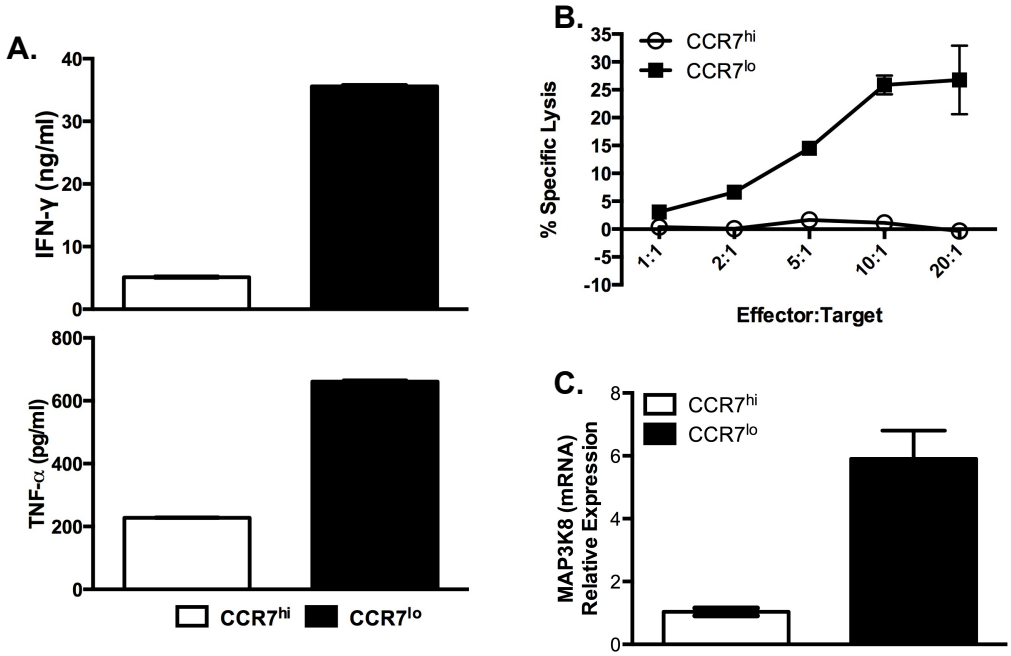


Figure 4.1. Higher relative mRNA expression of MAP3K8 is maintained in human T_{EM} CTLs. $CD8^+CCR7^{hi}$ and $CD8^+CCR7^{lo}$ cells were FACS sorted from healthy human PBMC. *A.* CTLs were stimulated with plate-bound anti-CD3 for 24h and cytokines (IFN-γ and TNF-α) were measured in the supernatants by ELISA. Mean±SEM is plotted for technical replicates. Data shown here is representative of multiple experiments. *B.* Sorted CTLs were used in redirected lysis assay at the indicated Effector:Target ratios using anti-hCD3 coated THP-1 target cells. Killing of target cells is plotted as percent specific lysis, mean±SD of technical replicates is plotted and representative of multiple experiments. *C.* Total RNA from the sorted CTLs were collected *ex vivo* without any stimulation. MAP3K8 expression in $CD8^+CCR7^{lo}$ CTLs is expressed relative to $CD8^+CCR7^{hi}$ CTLs. Mean±SD is plotted for technical replicates. This is also a validation of the microarray data presented in chapter five.

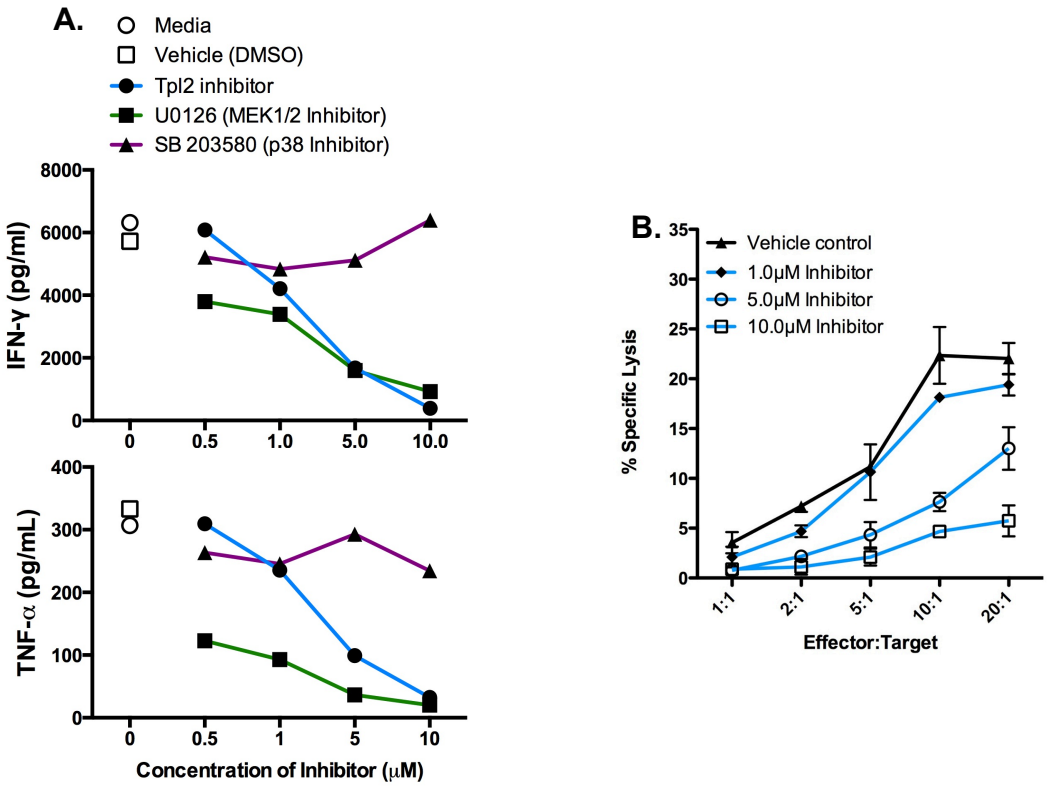


Figure 4.2. TCR mediated activation of human T_{EM} CTLs is dependent on Tpl2-MEK/ERK activation but not on p38. $CD8^{+}CCR7^{lo}$ CTLs were FACS sorted from healthy human PBMC. *A.* CTLs were stimulated with plate-bound anti-CD3 for 24h±indicated kinase inhibitors. Cytokines (IFN-γ and TNF-α) in the supernatants were measured by ELISA. Mean±SEM is plotted for technical replicates and this is representative of multiple experiments. *B.* The sorted CTLs were used in redirected lysis assay at the indicated Effector:Target ratios using anti-hCD3 coated THP-1 target cells±Tpl2 inhibitor at the indicated concentrations. Killing of target cells is plotted as percent specific lysis, mean±SD. Data shown is representative of two similar experiments.

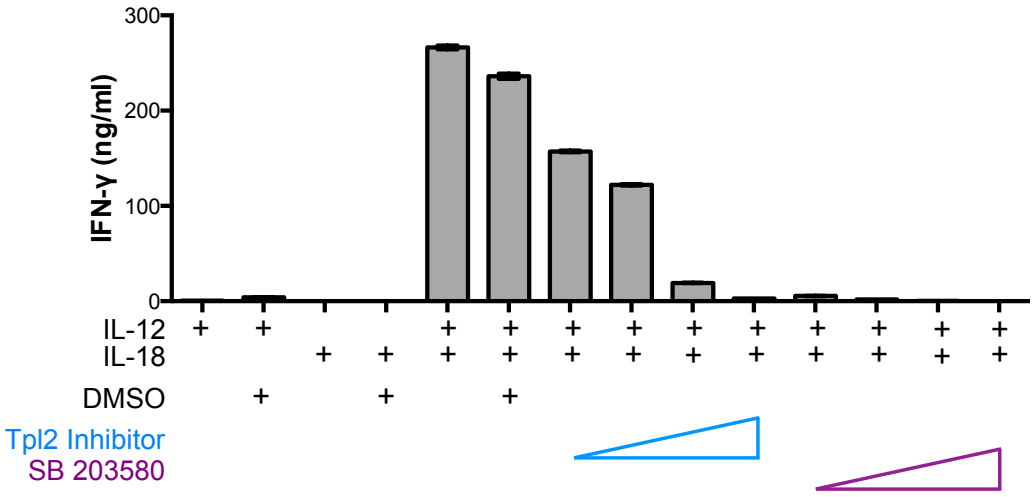


Figure 4.3. Cytokine mediated (TCR-independent) activation of human T_{EM} CTLs is dependent on both Tpl2 and p38 activation. $CD8^{+}CCR7^{lo}$ CTLs were FACS sorted from healthy human PBMC. CTLs were stimulated with a combination of IL-12 (10n g/mL) and IL-18 (80 ng/mL)±indicated kinase inhibitors at concentrations of 0.5, 1.0, 5.0, and 10.0μM indicated as a gradient in the figure. IFN-γ in the supernatant was measured by ELISA 36h post stimulation. Mean±SEM is plotted. The data is representative of two similar experiments.

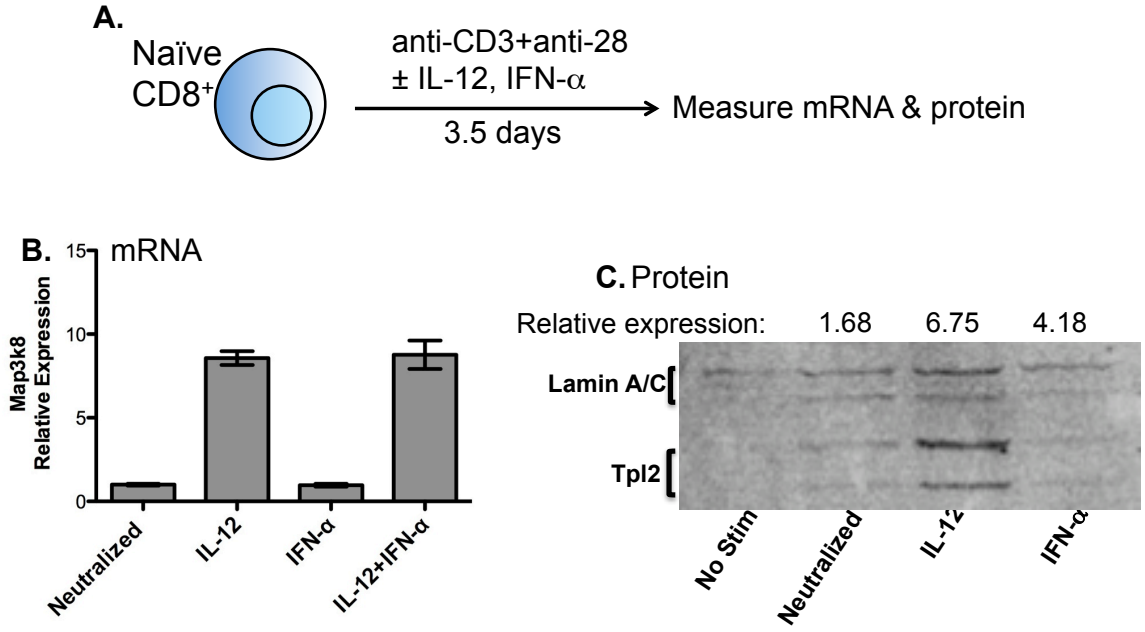


Figure 4.4. MAP3K8/Tpl2 expression is up regulated by IL-12 in human CTLs. *A.* Graphical representation of the experimental design. Naïve $CD8^+$ ($CD8^+CD45RA^+$) T cells were isolated by negative selection or FACS from healthy human PBMC and stimulated with plate-bound anti-CD3+anti-CD28 under neutralized, IL-12, IFN-α, or IL-12+IFN-α cytokine conditions. Total RNA or protein was collected 3.5 days post stimulation. *B.* MAP3K8 mRNA was measured by qRT-PCR and plotted (mean±SD) as relative expression compared to the neutralized condition. *C.* Total protein in the cell lysate was separated by SDS-PAGE and blotted for Tpl2 protein using rabbit polyclonal antibody against Tpl2. LaminA/C was used as a loading control. Relative expression of Tpl2 is represented on the top of the blot and it was calculated first as a ratio to loading control and then relative to the no stimulation (No Stim) lysate.

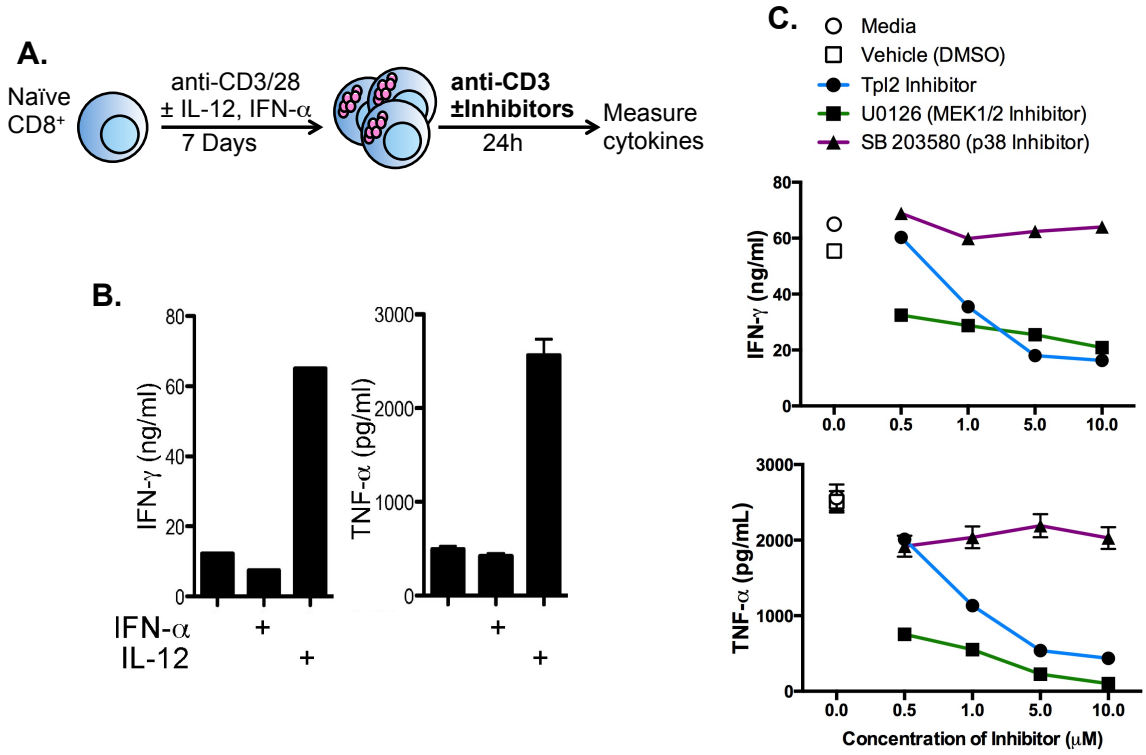


Figure 4.5. Tpl2 activation is necessary for IL-12 programmed effector function of human CTLs and is dependent on MEK-ERK pathway but not p38. *A.* Graphical depiction of the experimental design. Naïve $CD8^+$ ($CD8^+CD45RA^+$) T cells were isolated by negative selection or FACS from healthy human PBMC and stimulated with plate-bound anti-CD3+anti-CD28 under neutralized, IL-12, or IFN-α cytokine conditions. Cells were split 1:10 with 100U/mL IL-2 and cultured until day 7 when cells were counted and re-stimulated with plate-bound anti-CD3±indicated inhibitors. Data shown here are representative of several independent experiments. *B.* IFN-γ and TNF-α in the supernatant were measured by ELISA 24h post 2^o stimulation for the samples with indicated 1^o polarization conditions. Mean±SEM is plotted. *C.* IFN-γ and TNF-α in the supernatant were measured by ELISA 24h post 2^o stimulation for IL-12 polarized cells re-stimulated with plate-bound anti-CD3±indicated inhibitors. Mean±SEM is plotted.

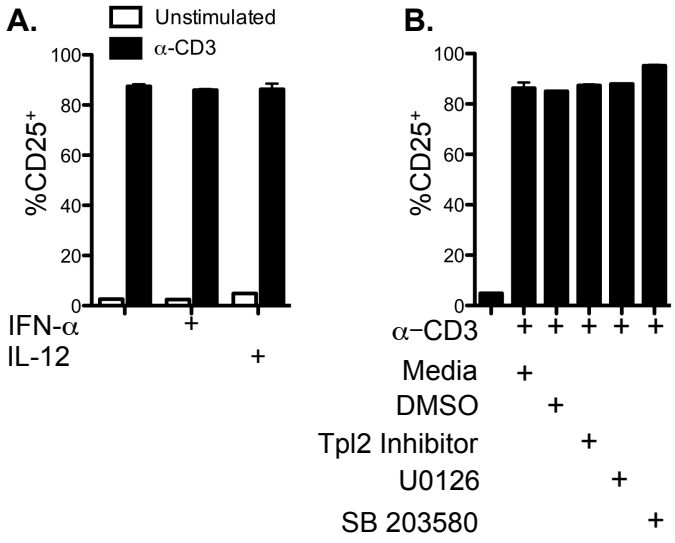


Figure 4.6. Treatment of CTLs with kinase inhibitors during 2^{o} stimulation does not affect expression of activation marker CD25. Naïve CD8^{+} (CD8^{+}CD45RA^{+}) T cells were isolated by negative selection or FACS from healthy human PBMC and stimulated with plate-bound anti-CD3+anti-CD28 under neutralized, IL-12, or IFN-α cytokine conditions. Cells were split 1:10 with 100U/mL IL-2 and cultured until day 7 when cells were counted and re-stimulated with plate-bound anti-CD3±indicated inhibitors or left unstimulated. CD25 expression was measured by staining for surface markers and analysis with flow cytometry 24h post 2^{o} stimulation. Data shown here are representative of several similar experiments *A.* Percent of CD25^{+} cells within live gate were determined and mean±SD plotted for samples treated under indicated 1^{o} polarizing conditions that were re-stimulated with anti-CD3 or left unstimulated. *B.* IL-12 polarized cells were re-stimulated under the indicated conditions and percent of CD25^{+} cells within live gate were determined and mean±SD plotted.

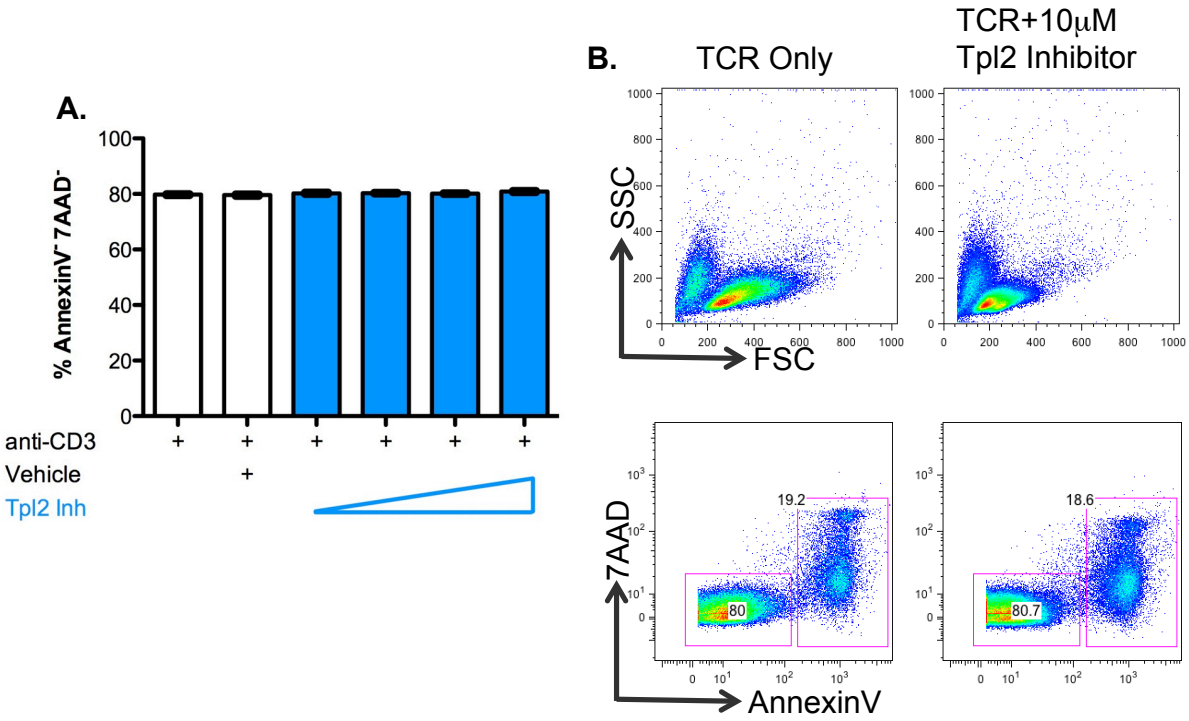


Figure 4.7. Treatment of human T_{EM} CTLs with kinase inhibitors for 24h does not affect the cell viability. $CD8^{+}CCR7^{lo}$ T cells were isolated from healthy human PBMC by FACS and stimulated with plate-bound anti-CD3 in the presence of increasing concentration (0.5μM, 1.0μM, 5.0μM, and 10.0 μM) of Tpl2 inhibitor as indicated by the gradient in the figure. Cell viability was measured by staining for the cell death markers AnnexinV and 7AAD 24h post stimulation. *A.* Percent of AnnexinV-7AAD- cells were measured within ungated populations and plotted as mean±SD. *B.* Forward and side scatter plots are shown on top for TCR stimulation only (anti-CD3) and TCR stimulation under the highest concentration of Tpl2 inhibitor treatment (10μM). Bottom panel shows AnnexinV vs 7AAD dot plot for previously ungated cells under the same conditions as the top panel.

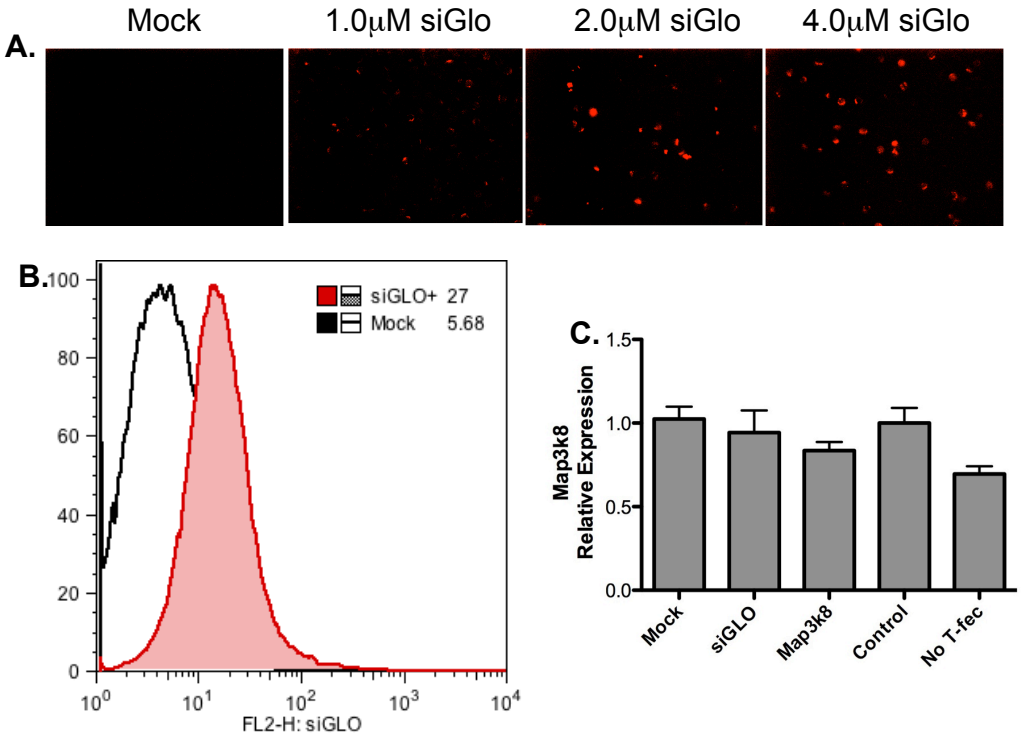


Figure 4.8. Knockdown of MAP3K8 in primary human CTLs was unsuccessful. *A.* Total $CD8^+$ T cells were isolated from Carter Care buffy coat and increasing concentrations of siRNA was nucleoporated using Amaxa Nucleoporator by following manufacturer's protocol. Confocal microscopy images are shown for siGLO fluorescent indicator. *B.* Total $CD8^+$ T cells were isolated from healthy human PBMC using negative selection and stimulated with plate-bound anti-CD3+anti-CD28+IL-12 for 24h. Mirus TransIT-TKO transfection reagent was used to transfect siGLO and transfection efficiency was measured by flow cytometry. *C.* Relative expression of MAP3K8 is measured by qRT-PCR and mean±SD plotted compared to mock transfected condition.

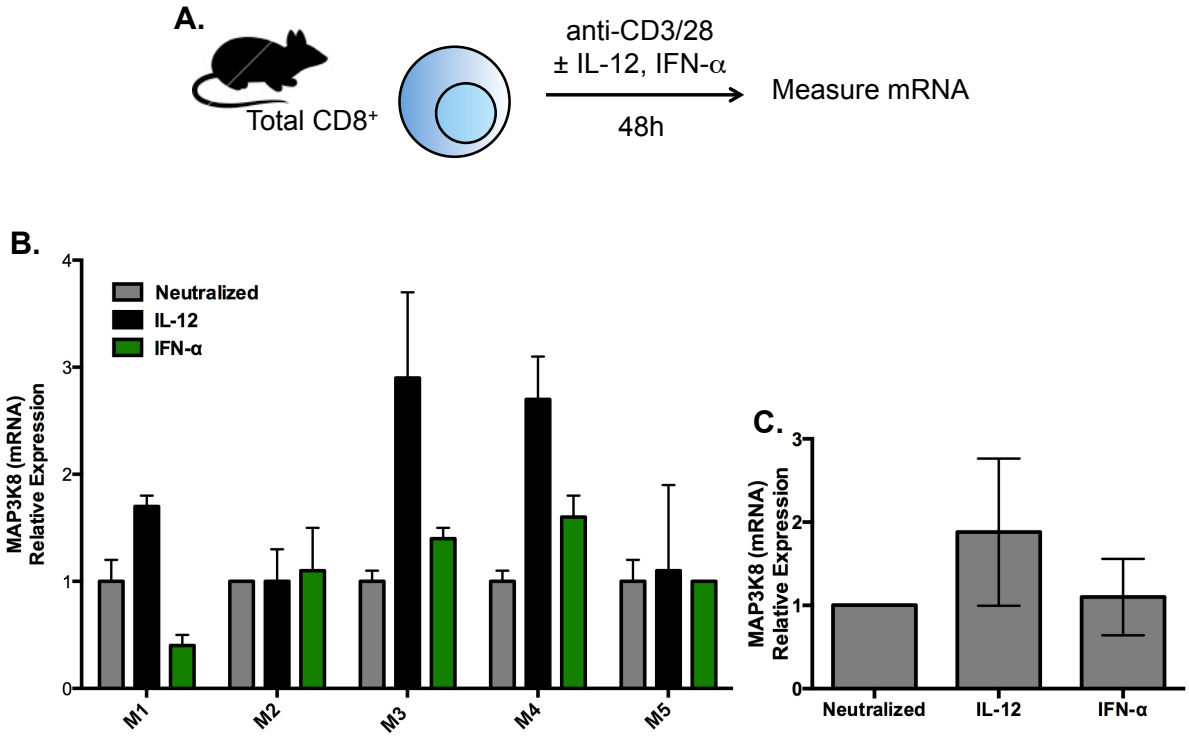


Figure 4.9. Neither IL-12 or IFN-α significantly regulate MAP3K8 mRNA in murine CD8⁺ T cells *in vitro*. *A,* Graphical representation of the experimental design. Total CD8⁺ T cells from C57BL/6 WT murine splenocytes were isolated using negative isolation kit and stimulated *in vitro* with plate-bound anti-CD3+anti-CD28 under defined cytokine conditions (Neutralized, IL-12, and IFN-α). Total RNA was collected 48h post stimulation. *B.* MAP3K8 mRNA was measured by qRT-PCR. M1-M5 indicates five different mice that were setup separately in the same experiment. Relative expression of MAP3K8 is determined for each mouse separately compared its own neutralized condition and mean±SD were plotted. *C.* Relative expression of MAP3K8 mRNA is plotted by using averages for all five mice under the same conditions.

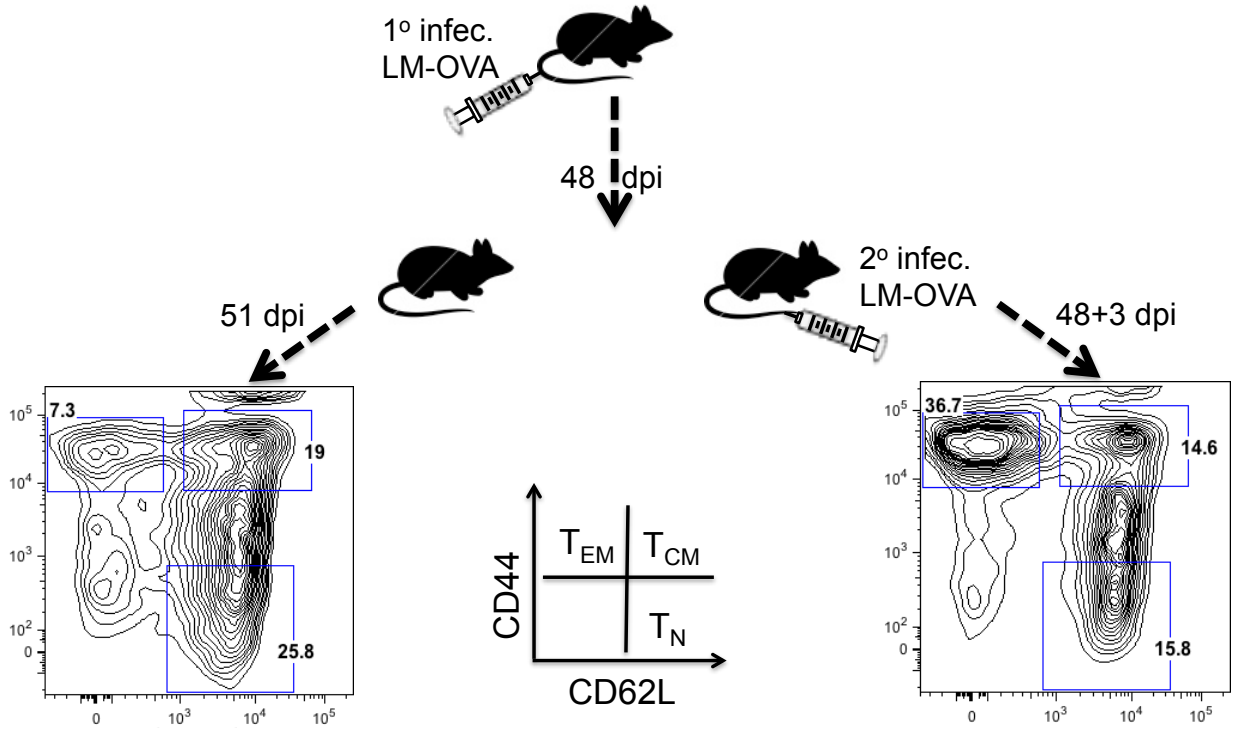
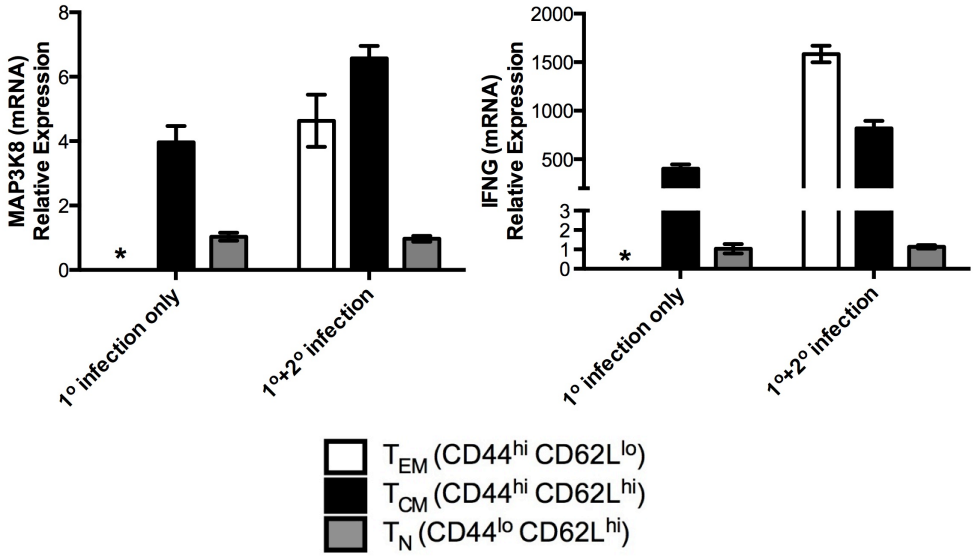


Figure 4.10. CTL memory subpopulations can be isolated after *Listeria monocytogenes* infection of WT C57BL/6 mice. WT C57BL/6 mice were infected intravenously with 2000 CFU of LM-OVA. One cohort was re-infected with 20,000 CFU of LM-OVA 48dpi. Spleen and LN from all mice were collected 51dpi (48+3 dpi for 2^o challenge) and surface staining was performed for CD8, CD44, and CD62L. T_{EM}, T_{CM}, and T_N CTLs were defined as indicated for FACS and total RNA was isolated immediately. All infections were performed with the generous help of Sean Murray. Since no adoptive transfer was performed there was not enough endogenous K^b-tetramer$^+$ cells for enough RNA and the cells were pooled from the mice under the same treatment group.



* No T_{EM} sample from mice with 1° infection only

Figure 4.11. MAP3K8 expression is higher in murine memory CTLs and correlates with relative expression of IFNG. MAP3K8 (Left Panel) and IFNG (Right Panel) mRNA were measured by qRT-PCR within the T_{EM}, T_{CM}, and T_N sorted CTLs and relative expression was determined by comparing to T_N sample from 1° infection only.

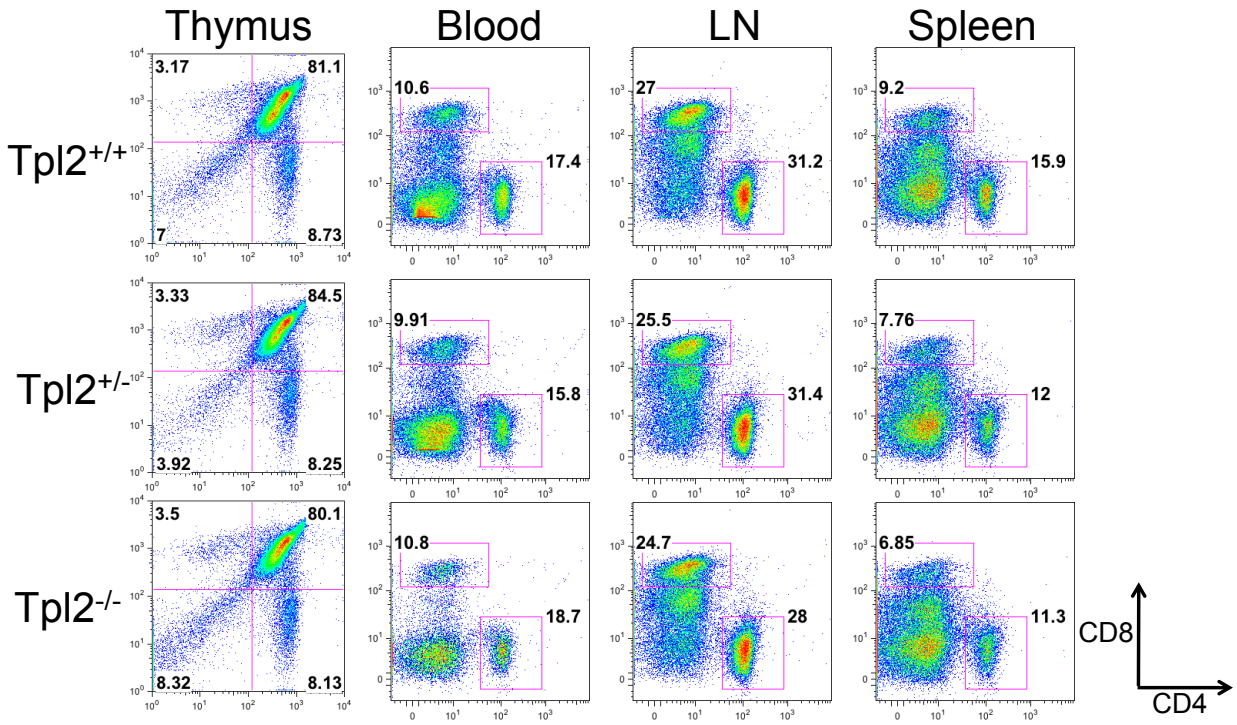


Figure 4.12. Tpl2$^{-/-}$ mice have CD4 and CD8 profile comparable to Tpl2$^{+/+}$ in different organs. Thymus, peripheral blood (retro-orbitally collected), lymph nodes, and spleen were harvested from 4 months old Tpl2$^{+/+}$, Tpl2$^{+/-}$, and Tpl2$^{-/-}$ mice. Cells were stained for surface expression of CD4 and CD8 *ex vivo* and analyzed by flow cytometry.

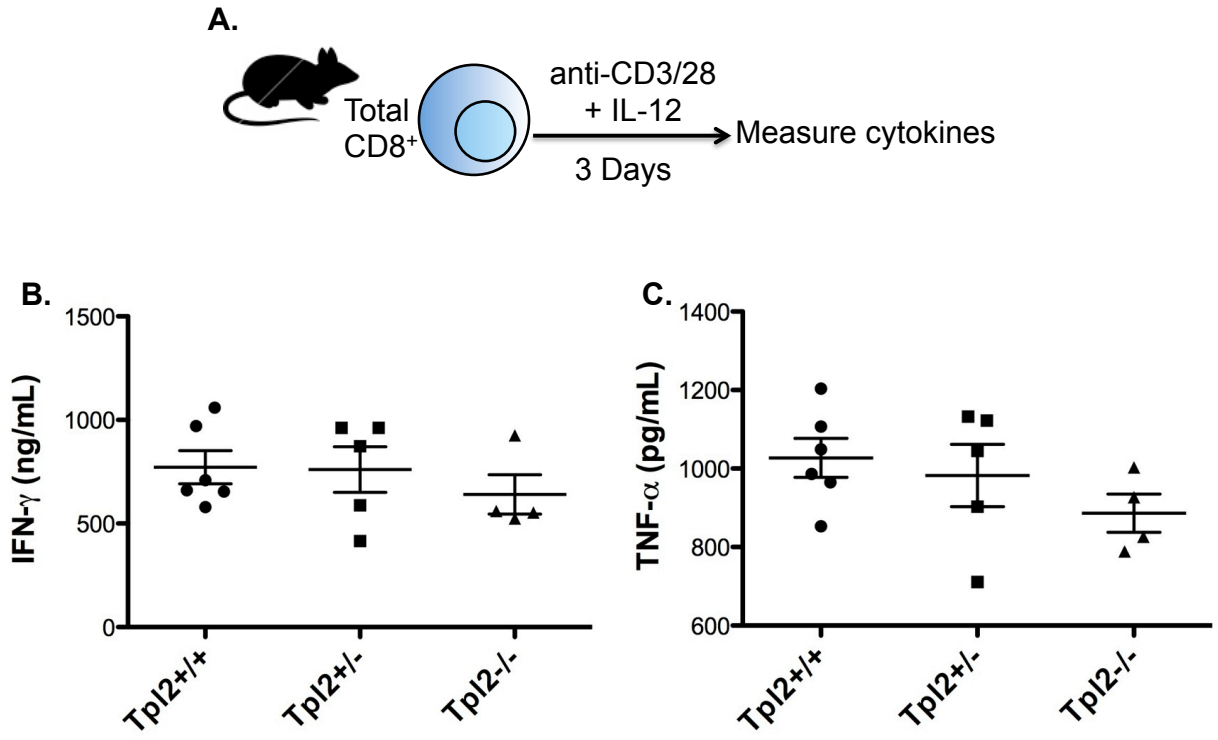


Figure 4.13. Tpl2$^{-/-}$ CTLs are competent at secreting effector cytokines after polarization with IL-12. *A.* Graphical depiction of experimental design. Total CD8^{+} T cells were isolated from spleens and LNs of Tpl2$^{+/+}$ (n=6), Tpl2$^{+/-}$ (n=5), and Tpl2$^{-/-}$ (n=4) mice using negative isolation kit. Cells were stimulated *in vitro* with plate-bound anti-CD3 and anti-CD28 in the presence of IL-12 for 3 days. IFN-γ (*B.*) and TNF-α (*C.*) in the supernatant were measured by ELISA. Each individual data point represents a separate mouse.

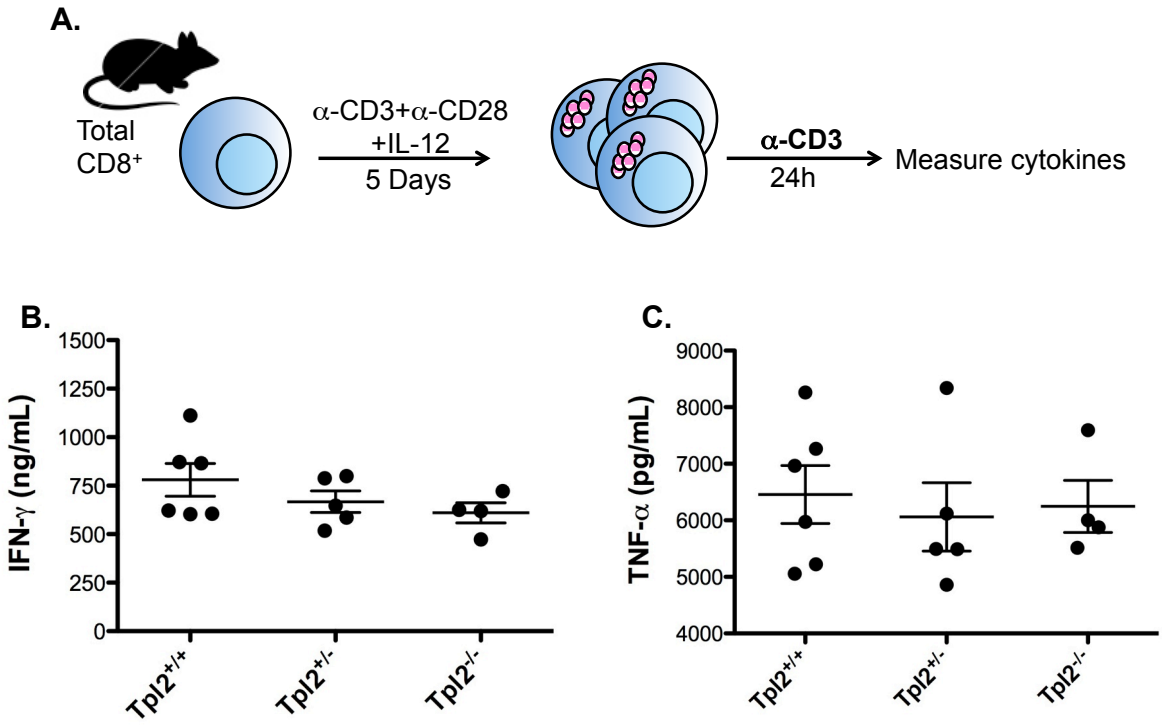


Figure 4.14. Tpl2$^{-/-}$ CTLs are competent at secreting effector cytokine upon 2^{o} stimulation *in vitro*. *A.* Graphical representation of experimental design. Negatively isolated total CD8+ T cells from Tpl2$^{+/+}$ (n=6), Tpl2$^{+/-}$ (n=5), and Tpl2$^{-/-}$ (n=4) mice were cultured *in vitro* for 5 days and then re-stimulated with plate-bound anti-CD3 only for 24h. IFN-γ (*B.*) and TNF-α (*C.*) in the supernatant were measured by ELISA. Individual dots represent a separate mouse.

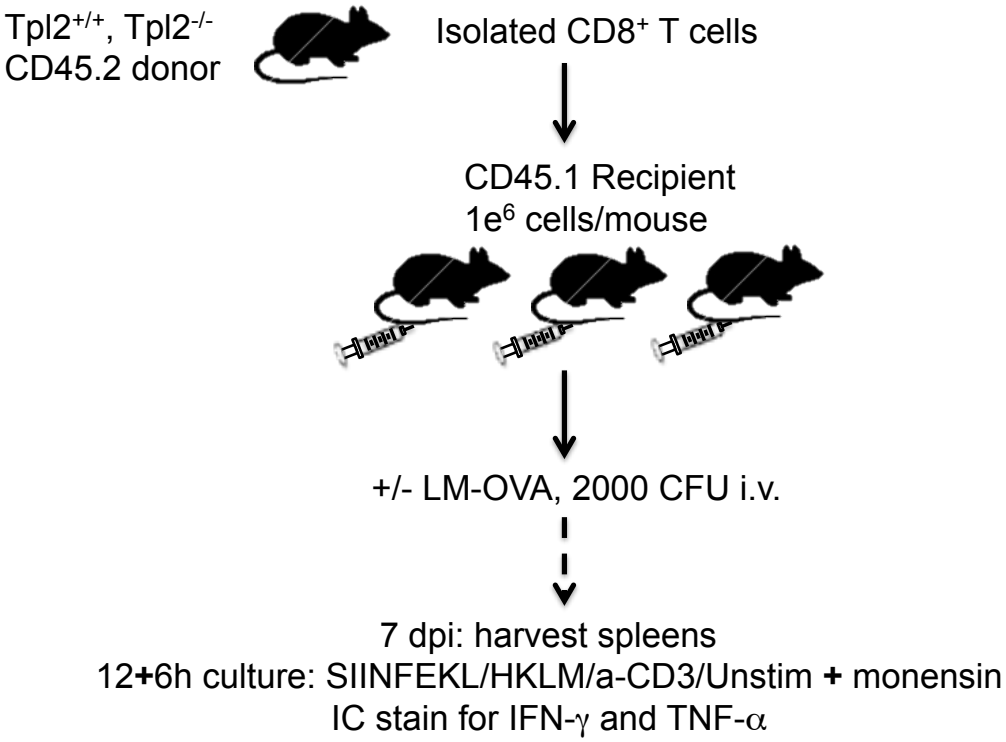


Figure 4.15. Experimental design for *Listeria monocytogenes* infection of adoptively transferred mouse CD8$^+$ T cells. Total CD8$^+$ T cells were isolated from Tpl2$^{+/+}$ (n=4) and Tpl2$^{-/-}$ (n=4) mice, which carried the CD45.2 congenic marker. Isolated cells from each mouse were separately injected into three CD45.1 recipient mice at 1e^6 cells per mouse intravenously. 24h post cell transfer mice were either infected with 2000 CFU of LM-OVA or left uninfected. All injections were carried out by Sean Murray. Spleens were harvested 7 dpi and splenocytes were cultured with SIINFEKL (10nM), HKLM (5μg/mL), soluble anti-CD3 (0.5μg/mL), or left unstimulated for 18h. Monensin was added to the cultures for the last 6h. Intracellular expression of IFN-γ and TNF-α were measured by flow cytometry along with surface markers.

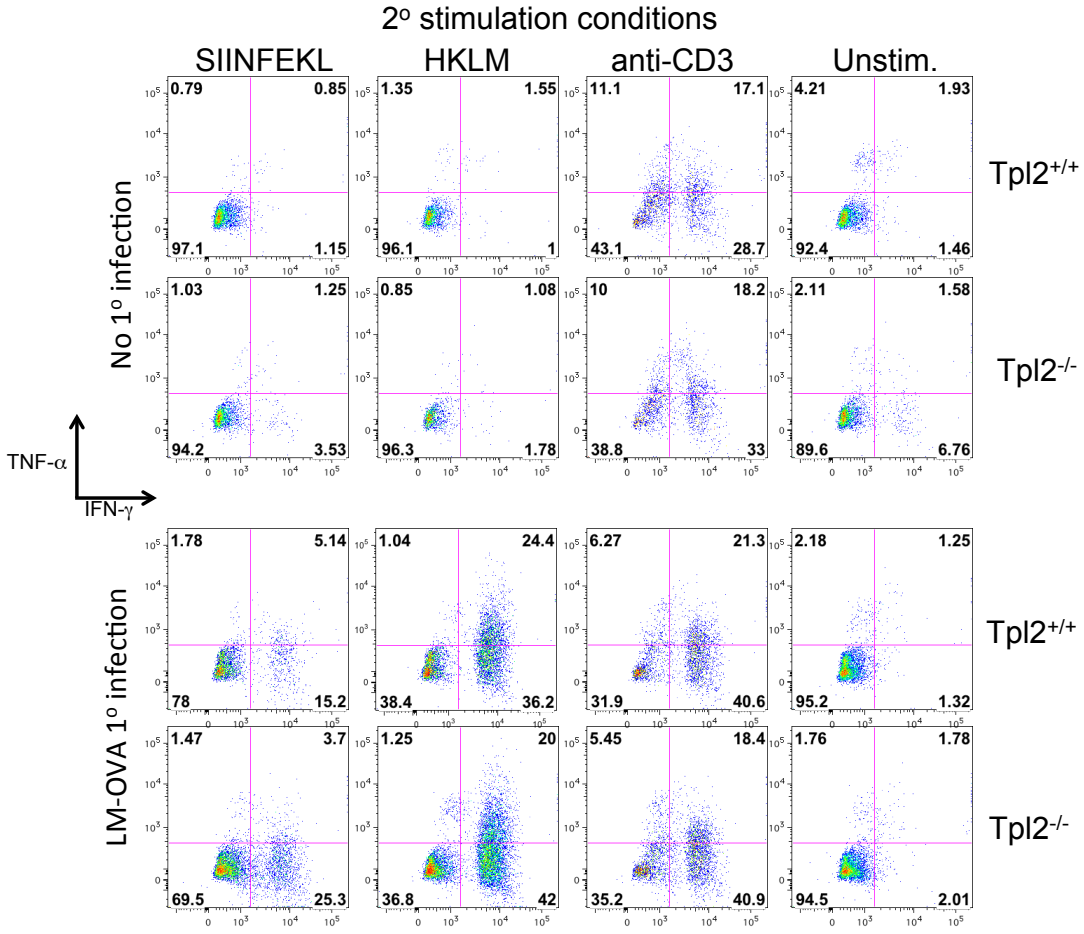


Figure 4.16. Antigen-specific effector cytokine expression can be detected in adoptively transferred non-transgenic CTLs. Intracellular IFN-γ (x-axis) and TNF-α (y-axis) expression after 2º stimulation is represented in dot plots for both Tpl2$^{+/+}$ and Tpl2$^{-/-}$ mice (indicated on the right side of the figure). Transferred cells were identified based on positive staining for CD45.2 surface marker. The infection conditions are indicated on the left side of the plots.

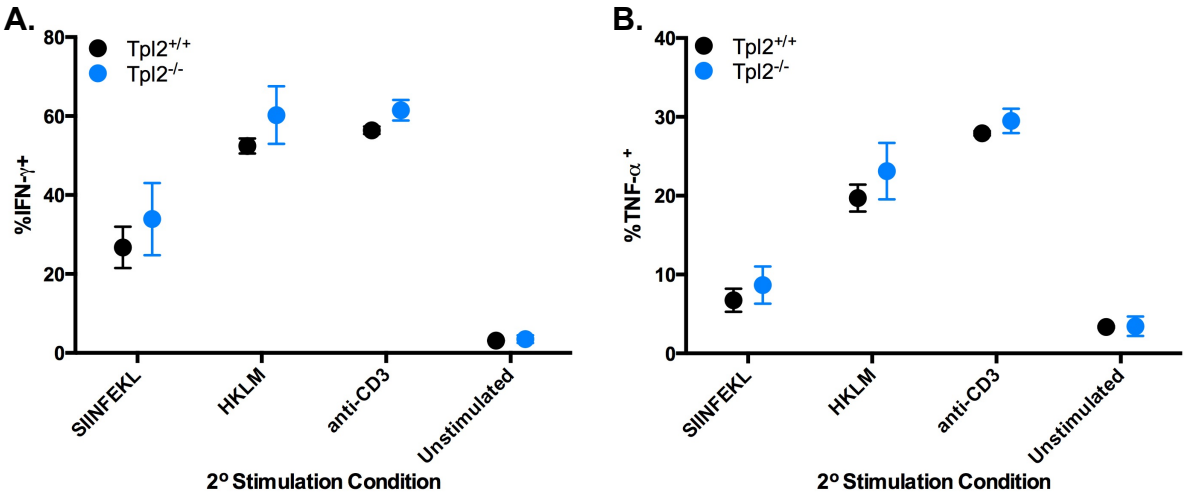


Figure 4.17. LM-OVA immunized Tpl2$^{-/-}$ CTLs are not defective in effector cytokine expression upon 2^{o} stimulation. Total IFN-γ (*A.*) and TNF-α (*B.*) expressing cells were quantified as a percentage of the identified total transferred CD8^{+}CD45.2^{+} T cells. Mean±SEM is plotted representing triplicate donor mice with Tpl2$^{+/+}$ in black and Tpl2$^{-/-}$ in blue dots.

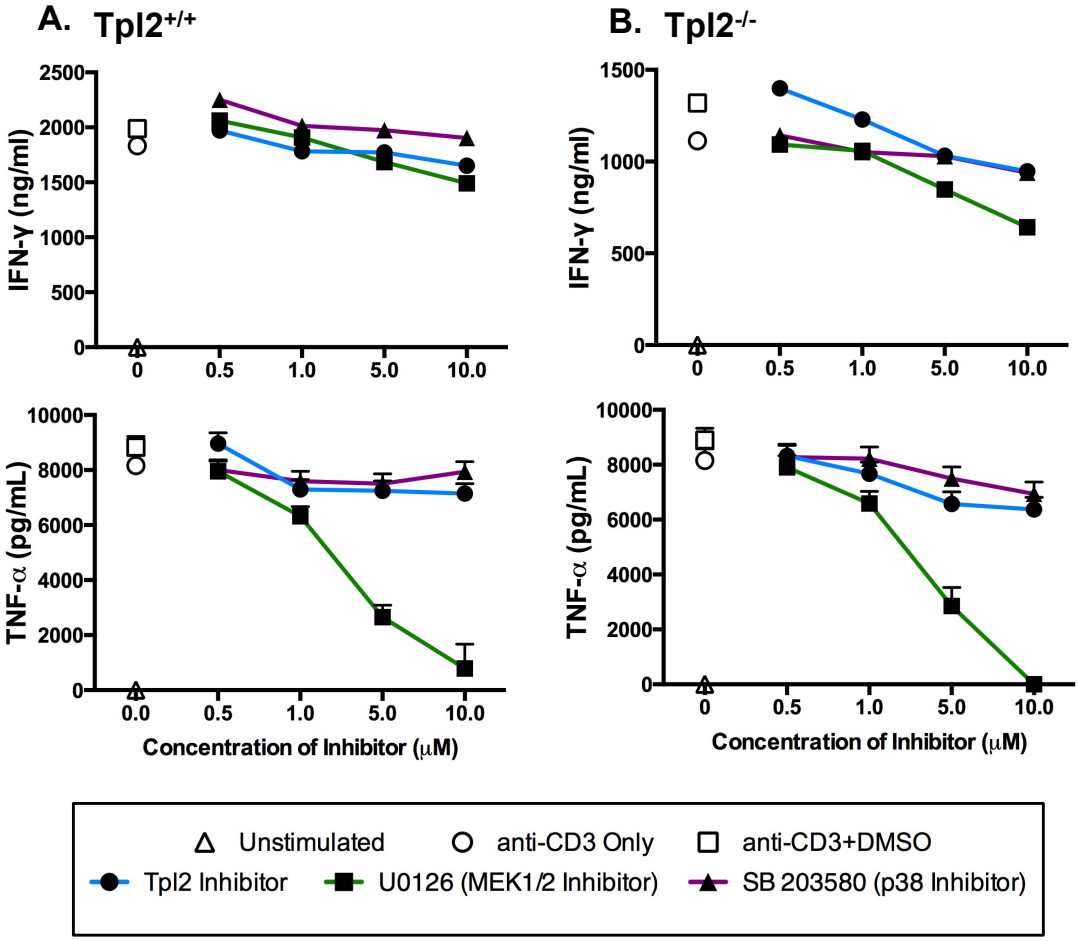


Figure 4.18. Tpl2 inhibitor does not block effector cytokine secretion from murine CD8$^+$ T cells. Isolated CD8$^+$ T cells from Tpl2$^{+/+}$ and Tpl2$^{-/-}$ mice were cultured *in vitro* under the same experimental setup as Figure 6.14. CTLs were counted and re-stimulated on day 5 with plate-bound anti-CD3±Tpl2 inhibitor, U0126, and SB 203580 at the indicated concentrations. Secreted IFN-γ (Top Panels) and TNF-α (Bottom Panels) in the supernatant were measured by ELISA for both Tpl2$^{+/+}$ (*A.*) and Tpl2$^{-/-}$ (*B.*). Mean±SEM were plotted for technical replicates.

CHAPTER FIVE

DISCUSSION

Role of IL-12 in human CTL programming

While the innate immune system acts as the first line of defense against pathogenic invasion, the adaptive immune response provides the heavy artillery for complete clearance of pathogens and tumors. Studies have shown that IL-12 and IFN-α play important roles in bridging the gap between innate and adaptive immune systems by programming functional specificity of T cells. However, their roles in programming CTL effector function have been rather contradictory in studies done between mouse and human. IL-12 and IFN-α seem to play a redundant role in programming murine effector CTLs (Curtsinger et al., 2003; Curtsinger et al., 2005; Agarwal et al., 2009). On the other hand, IL-12, but not IFN-α, programs the effector functions in human CTLs *in vitro* (Ramos et al., 2009; Chowdhury et al., 2011).

We have used primary human $CD8^+$ T cells to test the roles of IL-12 and IFN-α in programming CTL function. After *in vitro* priming we tested the CTLs' ability to perform cytolysis and effector cytokine secretion. Only IL-12 polarized CTLs excelled in both lysis of target cells as well as secretion of effector cytokines when compared to either neutralized or IFN-α treated cells. This is in contrast to the findings in murine CTLs. We further analyzed the roles of IL-12 and IFN-α in programming CTLs at the level of genetic programming. We have shown that at the genetic level

IL-12, but not IFN-α, programs effector function in human CTLs (Figure 5.1). Furthermore, we have discovered a distinct gene signature in isolated human T_{EM} CTLs *ex vivo* akin to that of IL-12 programmed genes *in vitro*. On the other hand, IFN-α up-regulates the signature IFN-regulated genes in human CTLs independent of IL-12 and no effector molecules were found to be among the IFN-regulates genes in human CTLs. This is also unlike the murine model, which shows a significant set of commonly regulated genes by both IL-12 and IFN-α and thus implying a redundant role for these two innate inflammatory cytokines in programming CTL function in mice. Agarwal *et al* finds that when IL-12 or IFN-α are provided as the signal 3 to murine $CD8^+$ T cells, a set of ~355 commonly regulated genes can be identified (Agarwal et al., 2009). These lists of commonly regulated genes include IFNG, GZB, CD25 etc., all involved in effector function of CTLs. In contrast, we find that only two genes are commonly regulated by IL-12 and IFN-α, CXCL10 and FASL. Although these two genes are involved in inflammation, neither is required for programming and deploy of effector molecules. Thus, IL-12, but not IFN-α, programs effector function in human $CD8^+$ T cells.

Regulation of Tpl2 expression and function

In our search for the regulation of effector function of CTL by IL-12 we have discovered that along with effector molecules (IFNG, TNF, IL-8, etc.) IL-12 also up-regulates a MAP kinase pathway intermediate MAP3K8 or Tpl2. MAP kinase

pathways play a crucial role in activation and function of CTLs. We decided to pursue the potential for a differentially regulated MAP kinase pathway as a signaling intermediate in activation of effector CTLs. We have found that human T_{EM} CTLs have a higher expression of MAP3K8 *ex vivo* when compared to T_{N+CM} CTLs. We have demonstrated that IL-12 can induce the expression of MAP3K8/Tpl2 mRNA and protein in human $CD8^+$ T cells.

The regulation and function of Tpl2 has been mainly studied in cell lines and innate cells from mice. The function of Tpl2 is known to be regulated by NF-κB1 (p105) in two ways. First, Tpl2 stability is dependent upon its complex formation with p105 and Abin2 in the cytoplasm (Lang et al., 2004; Papoutsopoulou et al., 2006; Verstrepen et al., 2009; Handoyo et al., 2009). Although all cellular Tpl2 is coupled with p105, only less than 5% p105 is in complex with Tpl2 in murine macrophages. Additionally, Tpl2 expression is drastically reduced in the cytoplasm in $nfkb1^{-/-}$ cells. Secondly, p105 interaction blocks the kinase domain of Tpl2 and in turn blocks its ability to phosphorylate MEK (Waterfield et al., 2003; Beinke et al., 2003). Thus, Tpl2 is sequestered in the cytoplasm by complex formation with p105 and Abin2.

Phosphorylation of Tpl2 at Thr-290 and Ser-400 are essential for activation of Tpl2-MEK-ERK pathway in response to TLR4 signaling, but the way in which these events take place is not clear (Luciano et al., 2004; Cho et al, PNAS 2005; Cho et al., J. Biol. Chem. 2005; Robinson et al., 2007). While we know that c-terminal truncation greatly increases Tpl2 kinase activity in T cell line such as Jurkat cells, we

have no knowledge of what activates or phosphorylates Tpl2 in the first place (Vougioukalaki et al., 2011). Thus, there is a lack of understanding of what signaling molecules lead to TCR mediated activation of Tpl2, which merits further studies.

Role of Tpl2 in health and disease: Clinical significance

Tpl2 or cot was first discovered in the early 1990s as a proto-oncogene. DNA isolated from a specific human thyroid carcinoma cell line showed the ability to transform the hamster embryonic cell line (SHOK) *in vitro*, which was the first description of the human Cot (cancer Osaka thyroid) oncogene (Miyoshi et al., 1991). The rat homolog of Cot, Tpl2 (tumor progression locus 2) was then identified as a target for provirus integration in Moloney murine leukemia virus-induced lymphomas and was found to transform NIH 3T3 fibroblasts *in vitro* (Patriotis et al., 1993). Both of these transforming cDNA contained an altered c-terminal domain. Subsequently, it was discovered that Tpl2/Cot overexpression could induce IL-2 secretion from Jurkat and EL-4 T cell lines through increased activation of NFAT and NF-κB and moreover, a c-terminal truncation enhanced the kinase activity of Tpl2/Cot (Ceci et al., 1997; Ballester et al., 1997; Tsatsanis et al., PNAS 1998; Tsatsanis et al., Oncogene 1998). The c-terminus of the wild-type Tpl2/Cot protein kinase contains a proposed degron, which is likely removed or altered in the mutant/overexpressed Tpl2, thus conferring sustained kinase activity to the protein (Gantke et al., 2011; Gantke et al., 2012).

Most studies on the role of Tpl2 in human health and disease only provide a cursory view. I have summarized the published reports on the findings regarding the involvement of Tpl2 in human diseases in Table 5.1. As evident from the list, Tpl2 has been found to be overexpressed in various cancers. However, these studies are largely correlative and in some cases contradictory. For example, only one report suggests that mutational activation may be involved in one specific case of lung cancer. In contrast to that report, Gkirtzimanaki *et al* recently revealed that reduced expression of Tpl2 in human lung cancer patients actually correlate with poor outcome and increased expression of Tpl2 might have tumor suppressive effects. In addition, by inspecting the transcriptome profiles registered in the Oncomine Database, Vougioukalaki *et al* have reported that MAP3K8 expression may be up- or down-modulated in certain cancers compared to the normal tissue (Vougioukalaki et al., 2011). Thus, an inadequate picture is present from these superficial views of the role of wild-type Tpl2 in tumor progression.

It is well established that CTLs can recognize tumor-associated antigen and respond by secreting IFN-γ and cytotoxicity (Farrar et al., 1999; Ochsenbein et al., 2001; Schuler et al., 2003; Lotem et al., 2008). Unfortunately, anti-tumor activity of CTLs can be hampered due to a multitude of reasons that includes loss of antigen presentation, increased apoptosis in immune cells, and suppressed immune function via myeloid suppressor cells, all orchestrated by the tumor growth (Whiteside 2008). Studies on tumor infiltrating T cells have suggested that CTLs isolated from the

tumor microenvironment can be functionally impaired (Zippelius et al., 2004; Radoja et al., 2001). However, anergic effector and effector memory CTLs isolated from tumors can be reactivated to produce effector cytokine and exert cytotoxicity against tumor cells upon IL-12 treatment in mice, both *in vitro* and *in vivo* (Curtsinger et al., 2007; Zhao et al., 2010; Harden et al., 2011). In mice, subsequent effector functions from IL-12 treated CTLs enhanced tumor clearance. In human, IL-12 treatment has already proven to be effective in restoring tumor resident T cell functions *in vitro* as well as *in vivo* (Broderick et al., J Immunol. 2005; Broderick et al., Clin. Immunol. 2005; Broderick et al., 2006; Kilinc et al., 2006; Cao et al., 2009). IL-12 treated CTLs had increased responsiveness to TCR activation (Broderick and Bankert, 2006). Clinical studies on metastatic HER-2 positive tumors have shown that IL-12 can be safely used as a supplemental therapy *in vivo* in human (Bekali-Saab et al., 2009). Patients who received IL-12 had increased ERK activation in PBMCs and increased levels of IFN-γ with clinical benefits. Together, these studies have led to the proposal that IL-12 may be used in cancer treatment to enhance CTL effector function (Egilmez et al., 2010; Lisiero et al., 2011). More work is warranted to decipher the mechanism of anti-tumor activity of CTLs upon IL-12 treatment, and if IL-12 induced Tpl2 plays a role in acquisition of the effector functions.

In contrast, Johannessen *et al* have found that in some melanoma patients, Tpl2 is likely involved in activating MEK-ERK pathway independent of RAF kinase in certain metastatic melanoma with high expression of Tpl2. RAF is a MAPKKK and

overactive in some melanoma tumors. Tpl2 may be reactivating the MAP kinase pathway when RAF inhibition is used as a treatment. Thus, PLX4032, which selectively blocks mutant RAF kinase activity in melanoma cells, has been rendered inactive and this led to the search for Tpl2 and MEK kinase inhibitors as treatment options for the resistant melanomas (Johannessen et al., 2010; Solit et al., 2010; Haas 2010). Our *in vitro* studies in human CTL suggest that IL-12 induced Tpl2 plays an essential role in TCR mediated activation of effector functions in these cells via Tpl2-MEK-ERK pathway. Using Tpl2 or MEK inhibitor may inhibit tumor growth, but it will also likely dampen total CTL responses. Therefore, using either the Tpl2 or MEK1/2 inhibitor may prove to be counter productive if antitumor effector CTLs require the kinase action of Tpl2 via MEK-ERK pathway.

Other disease processes caused by chronic inflammation such as obesity-induced metabolic disease, colitis, pancreatitis, etc. have been studied further in the $Tpl2^{-/-}$ mouse model. I have summarized the findings of these studies in Table 5.2. There are some conflicting results. For example, in experimental colitis model and inflammation associated with obesity Tpl2 was found to both suppress and increase susceptibility to disease. In some cases Tpl2 has been suggested as a 'druggable' target. Some researchers have proposed the use of Tpl2 inhibitor in therapy for rheumatoid arthritis, Crohn's disease, etc. based on murine studies (Hu et al., 2006; Cohen 2009; Gaestel et al., 2009; Lawrenz et al., 2011). Our *in vitro* data using human CTLs would suggest that treatment with Tpl2 inhibitor may also significantly

reduce effector function of CTLs and in turn increase danger of uninhibited infectious diseases.

Functional differences of Tpl2: Innate vs adaptive immunity

Studies in the mouse model has shown that Tpl2 mediated ERK1/2 activation regulates TNF-α secretion from bone marrow derived macrophages in response to LPS stimulation (Dimitru et al., 2000). ERK1/2 phosphorylates the protease TACE that eventually cleaves pre-TNF-α to the secreted form of TNF-α and thus Tpl2-deficient macrophages are unable to secrete TNF-α (Rousseau et al., 2007). In our studies we have examined the role of Tpl2 in CTL function. In human, CTL function is dependent on Tpl2 activation because in the presence of Tpl2 inhibitor TCR-dependent cytokine secretion and cytotoxicity are both reduced. In addition to TNF-α secretion, we have demonstrated that IFN-γ secretion from human CTLs is also dependent on Tpl2 function. However, Tpl2 function seems to be dispensable for murine CTL function.

An overview of the few reports of *in vivo* studies done using $Tpl2^{-/-}$ mice confirms that, at least in mice, Tpl2 function is necessary for defense against pathogens (Table 5.3). While $Tpl2^{-/-}$ mice are resistant to LPS mediated septic shock (Dumitru et al., 2000; Mielke et al., 2009), Tpl2 deficiency proved to be a disadvantage in fighting infectious diseases, mainly due to inadequate innate immune response (Table 5.3). The involvement of Tpl2 in adaptive immune

response is not very well studied except for in the case of *T. gondii* infection where lack of IFN-γ production from Tpl2$^{-/-}$ Th1 cells confers greater susceptibility to infection in Rag2-deficient background (Watford et al., 2008). We have discovered that Tpl2 function is necessary for adequate activation of human effector and T_{EM} CTLs *in vitro* but it is near impossible to study its requirement during the combat against infectious diseases. However, studies can be done to examine regulation of Tpl2 expression in human CD8^{+} T cells upon vaccination. This contrast in the role of Tpl2 between innate and adaptive immunity in mice complicates the interpretation of any *in vivo* data from Tpl2$^{-/-}$ mice. In addition, the differences we have discovered between mice and human in the need for Tpl2 in CTL function further convoluted the complete picture.

Role of Tpl2 in effector memory CTL activation

Effector memory CTL have the ability to functionally respond to infected cells that present antigen in the context of MHC I molecules. T_{EM} CTLs can secrete effector cytokines and kill the infected cells without the need for any co-stimulation and inflammatory cytokines. Our *in vitro* studies have supported this, and we have shown that a distinct programming of human CTLs by IL-12 is necessary for the uncoupling of signaling requirement for activation of T_{EM} CTLs. Effector/effector memory CTLs can bypass the need for co-stimulation and this may be through Tpl2-MEK-ERK activated pathway (Figure 5.2).

I have discussed previously that there is a distinct TCR-independent pathway for memory T cell activation via IL-12 and IL-18 signaling. This antigen non-specific T cell activation pathway can be crucial in viral and bacterial pathogen clearance (Berg et al., 2002; Gherardi et al., 2003; Berg et al., 2003; Berg et al., 2005) This T cell activation pathway has been shown to be mediated by p38 MAP kinase activity in murine CD4+ T cells (Zhang et al., 2000; Yang et al., 2001; Berenson et al., 2006). We have shown that this synergistic role of IL-12 and IL-18 is also operational in human T_{EM} CTL activation. While p38 activity is required for human T_{EM} CTLs to secrete IFN-γ, we have also found that blocking Tpl2 activation also reduced the amount of IFN-γ secreted from the same cells. It is possible that Tpl2 may be involved in kinase activity upstream of p38 in TCR-independent activation of human T_{EM} CTL. Additionally, studies in a mouse T cell clone (2D6) have shown that IL-12 activated STAT4 and IL-18 activated AP-1 together contributes to IFN-γ promoter activation leading to induction and secretion of the cytokine (Nakahira et al., 2002). The convergence of these two pathways in human T_{EM} CTL may require the activation of multiple MAP kinase pathways involving Tpl2, which remain to be tested.

Additional Future directions

Although we have been unable to knockdown the MAP3K8 mRNA in primary human CTL, we have not ventured to overexpress it in human CTLs. Retroviral

transduction methods have been used in our laboratory to express transcription factors such as GATA3 in primary $CD4^+$ T cells, and this technique may be successful in human primary CTLs as well (Huber et al., 2010). We can try to overexpress Tpl2 in naïve $CD8^+$ T cells and test for their functional ability to secrete cytokines and kill target cells. If overexpression of Tpl2 alone allows naïve human CTLs to perform at the level of IL-12 polarized CTLs, we can further conclude that indeed IL-12 programs effector function in CTLs via up-regulation of the MAP kinase signaling intermediate Tpl2.

A distinct role for IL-12 in programming human CTLs effector function is evident from the work presented in this dissertation. Ramos et al have shown that in response to IL-12 the cell surface expression of IL12Rβ2 increases with cell division. Moreover, an increase in phospho-STAT4 is also observed in response to IL-12 and linked to cell cycle progression in the same experimental setup as that used in my analysis (Ramos et al., 2009). We have discovered that the expression of Tpl2 in human $CD8^+$ T cells is IL-12 regulated through measurement of Tpl2 mRNA and protein levels after IL-12 polarization. But we have not demonstrated that MAP3K8 induction is a STAT4 dependent process. There are putative STAT4 binding sites on human MAP3K8 promoter region (data not shown). In order to conclude that Tpl2/MAP3K8 is a *bona fide* IL-12 regulated gene in human CTLs, I would like to perform STAT4 ChIP experiments to test direct binding of IL-12 activated STAT4 to MAP3K8 promoter.

Concluding remarks

We set out to answer the question of how cytotoxic T lymphocyte function is regulated by innate inflammatory cytokines. In the course of this study we have distinguished the role of IL-12 as programming innate cytokine for CTL effector function and discovered a possible IL-12 mediated pathway of programming effector function in CTL. Subsequently we have discovered a species-specific role for IL-12 induced serine threonine protein kinase MAP3K8 or Tpl2 in regulating effector function of human, but not murine CTL. Blocking Tpl2 function upstream of MEK-ERK activation markedly diminished effector cytokine secretion and cytolytic capabilities of human effector and T_{EM} CTLs. In addition, our works in mouse models indicate that Tpl2 is involved in divergent pathways with varied outcomes, further complicating the analysis. Although working with primary human CTL is technically challenging, we were able to provide direction to a biologically significant process important for our defense against pathogens and tumor.

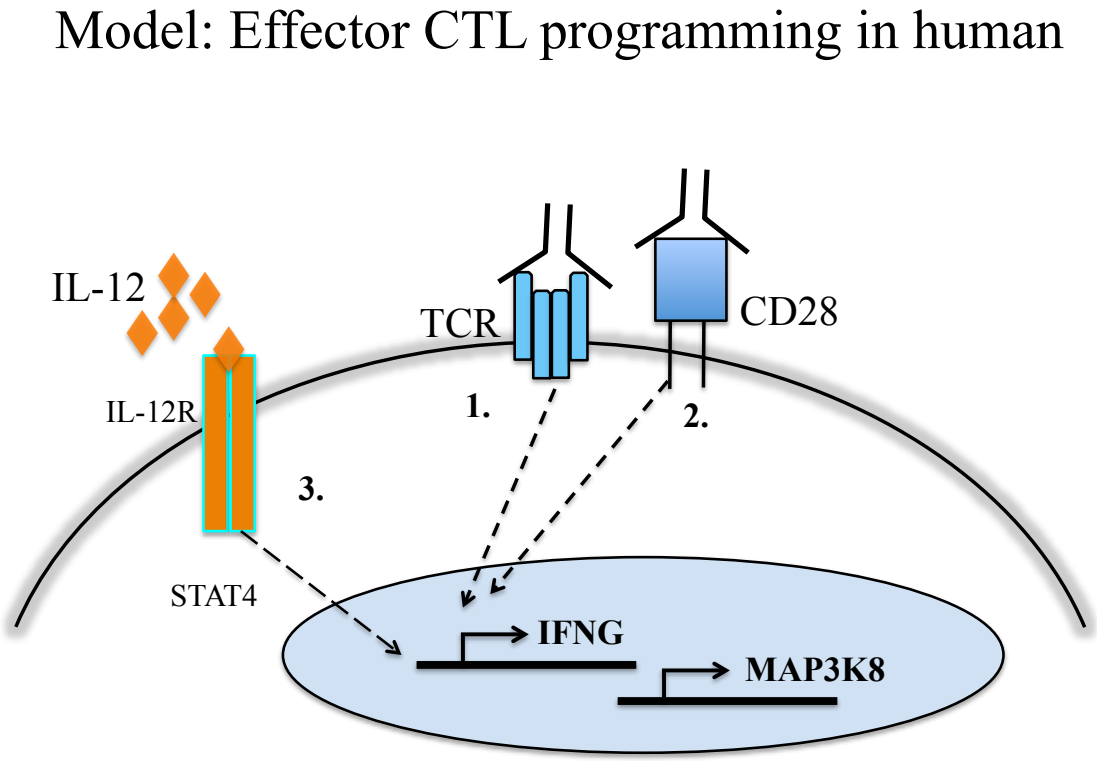


Figure 5.1. IL-12 provides "signal 3" in programming effector pathways in human CTLs. *In vitro* analysis using primary human $CD8^+$ T cells revealed that effector pathways (represented by regulation of MAP3K8 and IFNG) are programmed in the presence of 1. TCR stimulation from anti-CD3, 2. Co-stimulation from anti-CD28, and 3. rhIL-12.

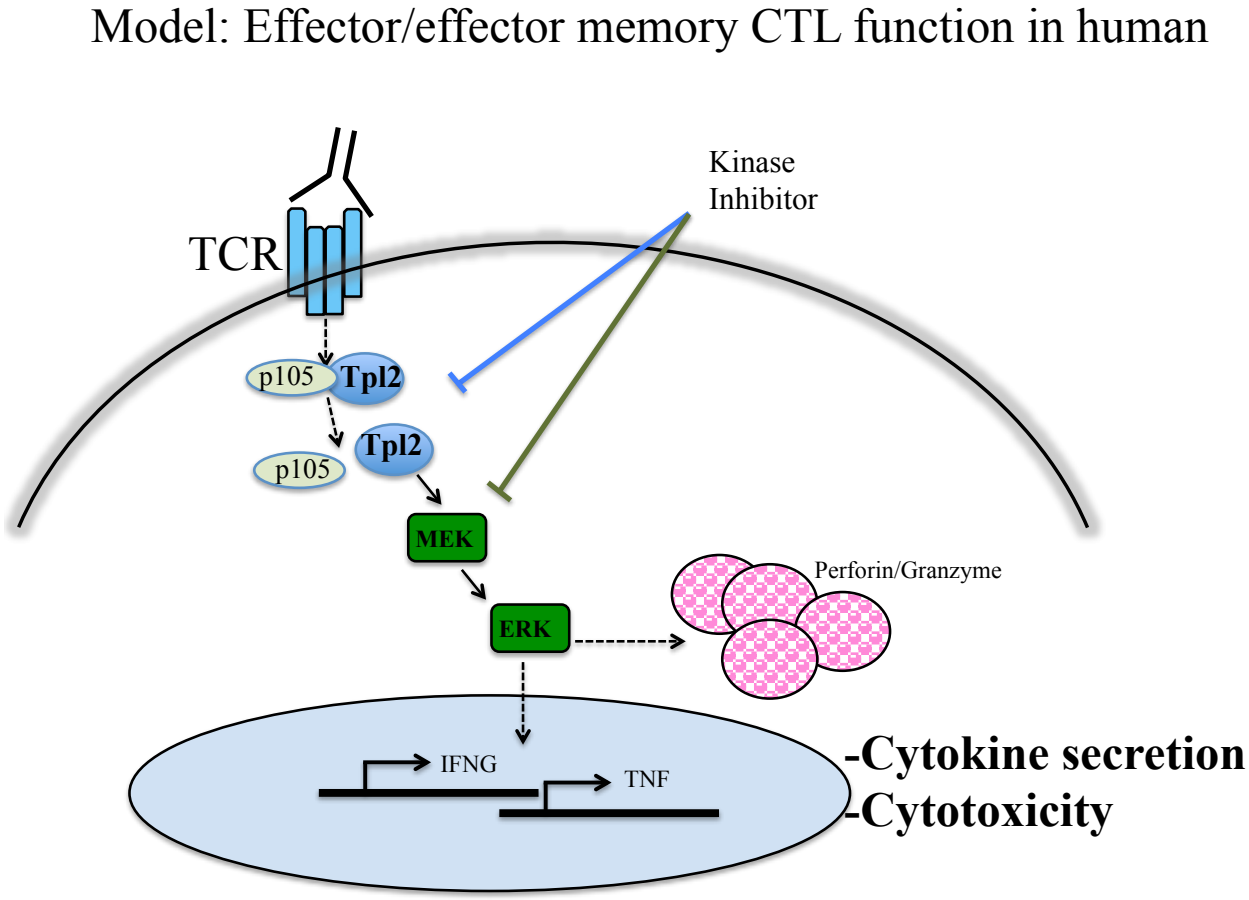


Figure 5.2. : Proposed model for activation of human effector/effector memory CTL. Activation of human effector/effector memory CTL is mediated by Tpl2-MEK-ERK pathway. This proposed model indicates that TEM CTLs can respond to TCR stimulation alone via up-regulated Tpl2 and pre-formed perforin/granzyme. Effector cytokine secretion and cytotoxicity from activated cells can be blocked by the treatment of specific kinase inhibitors.

Disease	MAP3K8/Tpl2 Expression	Phenotype/Outcome	References
Colorectal Cancer	Up-regulated mRNA	Associated with poor prognosis	Tunca et al., 2013
Myeloma	Constitutively active	Myeloma growth and survival	Hebron et al., 2012
Prostate cancer	Up-regulated protein	Increased cell growth	Jeong et al., 2011
Breast Cancer	Overexpression of mRNA	May serve as a marker	Sourvinos et al., 1999; Krcova et al., 2008
Malignant melanoma	Normal expression but RAF-independent MAP activation	Resistance to selective RAF inhibition	Johannessen et al., 2010
T-cell neoplasias	Overexpression of mRNA	Correlated with disease pathogenesis	Christoforidou et al., 2004
Lung Cancer	Mutational activation	Transforming gene in adenocarcinoma	Clark et al., 2004
	Reduced expression	Associated with poor survival	Gkirtzimanaki et al., 2013

Table 5.1: MAP3K8/Tpl2 in disease manifestation in human.

Disease	Outcome	Phenotype of Tpl2 KO	References
Acute pancreatitis	$Tpl2^{-/-}$ protected	Decreased pancreatic and lung inflammation	Acker et al., 2007
Ischaemia/ Reperfusion injury	$Tpl2^{-/-}$ protected	Decreased renal cell apoptosis	Yaomura et al., 2008
Inflammatory hypernociception	$Tpl2^{-/-}$ protected	Acute inflammatory response	Soria-Castro et al., 2010
Obesity	$Tpl2^{-/-}$ and inhibitor treatment protects	Reduced inflammation, improved insulin resistance	Jager et al., 2010; Perfield et al., 2011;
	No difference in outcome	No difference in obesity induced metabolic disease	Lancaster et al., 2012
Experimental Colitis	$Tpl2^{-/-}$ and inhibitor treatment protects	DSS mediated colitis due to overinflammation	Lawrenz et al., 2011;
	$Tpl2^{+/+}$ protected against tumors	Increased colitis associated tumorigenesis; increased polyp in APC^{min} mice	Koliaraki et al., 2012; Serebrennikova et al., 2012
Lung cancer	$Tpl2^{+/+}$ protected	Reduced function associated with tumor growth	Gkirtzimanaki et al., 2013
Skin carcinogenesis	$Tpl2^{+/+}$ protected	Enhanced tumorigenesis and inflammation	DeCicco-Skinner et al., 2010; DeCicco-Skinner et al., 2013

Table 5.2: MAP3K8/Tpl2 in disease manifestation in murine model.

Pathogen	Phenotype of Tpl2$^{-/-}$ mice	Reference
Toxoplasma gondii	↓IFN-γ and Th1 ↑parasitic load in direct infection ↑Increased susceptibility to *T. gondii* in RAG2 KO background	Watford et al., 2008
Leishmania major	↑ IL-12 production in response to CpG ↑ Th1 response but no difference in pathogen clearance	Susanta et al., 2010
Listeria monocytogenes	↑ susceptibility to bacteria ↓ TNF-α secretion in Mϕ ↓ IL-1β from both DC and Mϕ	Mielke et al., 2009
Group B streptococcal Disease	↓ Type I interferon and TNF-α production ↑ susceptibility to infection	Xiao et al., 2009

Table 5.3: Phenotype of Tpl2$^{-/-}$ mice in response to pathogens.

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