TRANSCRIPTION FACTOR REQUIREMENTS FOR THE DEVELOPMENT AND ANTI-VIRAL FUNCTION OF IL-17-SECRETING CD8 T CELLS

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ABSTRACT

Norman Yeh

TRANSCRIPTION FACTOR REQUIREMENTS FOR THE DEVELOPMENT AND ANTI-VIRAL FUNCTION OF IL-17-SECRETING CD8 T CELLS

Inflammatory immune responses are regulated by T cell subsets that secrete specific panels of cytokines. While $CD8^+$ T cells that secrete IFN-γ and cytotoxic molecules (Tc1 cells) are known to mediate antiviral immunity, IL-17-secreting $CD8^+$ T (Tc17) cells have only recently been described and the development and function of these cells has not been clearly examined. Using in vitro T cell cultures and mice deficient in transcription factors regulating lineage development, we defined Tc17 development and function. Similar to IL-17 secretion from CD4 T cells, IL-17 secretion from Tc17 cells is dependent on the transcription factor Stat3 and inhibited by Stat1. Expression of transcription factors important for Tc1 function, T-bet and Eomesodermin (Eomes), is reduced in Tc17 cells and consistent with this, Tc17 cells are non-cytotoxic in vitro. However, Tc17 cells are unstable and switch to cytotoxic IFN-γ producing cells when exposed to a Tc1 inducing cytokine, IL-12. Overexpression of the lineage promoting transcription factors T-bet and Eomes is unable to induce a Tc1 phenotype in Tc17 cells and Stat3 is also unable to switch Tc1 cells into Tc17 cells, suggesting additional signals are involved in CD8 T cell lineage commitment. In vivo, Tc17 cells are induced by vaccinia virus, dependant on

Stat3, and are capable of mediating antiviral immunity. Tc17 cells acquire an IFN-γ-secreting phenotype after encounter with virus in vivo, however, viral clearance by Tc17 cells is independent of IFN-γ. Instead, viral clearance is correlated with a gain in T-bet expression and cytotoxic function in Tc17 cells which have encountered virus. The development of anti-viral activity independent of IFN-γ, suggests that Tc17 cells may mediate anti-viral immunity through novel mechanisms that depend on the ability of Tc17 cells to acquire other phenotypes.

Mark H. Kaplan, Ph.D.-Chair

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Ab	antibody
APC	antigen presenting cell
CD	cluster of differentiation
cDNA	complementary DNA
BSA	bovine serum albumin
CD	cluster of differentiation
cDNA	complementary DNA
CFSE	carboxyfluorescein succinimidyl ester
CHS	contact hypersensitivity
DEPC	diethyl pyrocarbonate
DMEM	Dulbecco's Modified Eagle's Medium
DNA	deoxyribonucleic acid
DTH	delayed type hypersensitivity
DTT	dithiothreitol
EAE	experimental autoimmune encephalomyelitis
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
FACS	Fluorescence Activated Cell Sorting
FBS	fetal bovine serum
GFP	green fluorescent protein
hCD4	human CD4
ICS	intracellular staining

IFN-γ	interferon gamma
IL	interleukin
IgG	immunoglobulin G
LCMV	lymphocytic choriomeningitis virus
MHC	major histocompatibility complex
mRNA	messenger RNA
NK	natural killer
PBS	phosphate buffered saline
PCR	polymerase chain reaction
pfu	plaque forming units
PMA	phorbol 12-myristate 13-acetate
ROR	retinoic acid receptor-related orphan receptor
RNA	ribonucleic acid
RT	room temperature
STAT	signal transducer and activator of transcription
Tc	cytotoxic CD8 T cell
TGF	transforming growth factor
Th	helper CD4 T cell
VV	Vaccinia virus
WT	wild type

INTRODUCTION

Innate and adaptive immune systems

Despite constant exposure to microorganisms in the environment, we rarely become sick due to protective epithelial barriers and our innate and adaptive immune systems. Pathogens that break through skin or mucosal surfaces are quickly detected by innate immune cells which are capable of directly killing the pathogen and also activating adaptive immune cells to amplify the immune response.

Innate immune cells constitute the first line of host immune defense and include phagocytes (macrophages, neutrophils, dendritic cells), granulocytes (neutrophils, eosinophils, mast cells, basophils), natural killer (NK) cells, and $\gamma\delta$ T cells. Phagocytes contain receptors for many common pathogen associated molecular patterns (PAMPs) allowing them to recognize general pathogen macromolecules. Macrophages are widely distributed in body tissues and are the first immune cell to encounter pathogens. Macrophages form reactive oxygen species (ROS) to potently destroy engulfed pathogens and are reinforced by the arrival of neutrophils from the blood. Neutrophils are the most numerous component of the innate immune response and function as both phagocytes and as granulocytes, which secrete antimicrobial granules from their cytoplasm. Another phagocytic cell located in tissues is the dendritic cell which is specialized to process and display antigens to the adaptive immune system. Dendritic cells, along with macrophages and B cells compose the professional antigen

presenting cells (APCs) which activate T cells to become armed effector cells (Janeway and Medzhitov, 2002; Murphy et al., 2008).

Other innate cells are associated with fighting more specific classes of pathogens. Eosinophils, mast cells and basophils are important for immunity against parasites and contribute to allergic responses. NK cells attack and destroy virus infected cells and tumor cells expressing low levels of surface major histocompatibility complex (MHC) class I. $\gamma\delta$ T cell function is still unclear; however they are especially localized to the gut mucosa compared to other lymphoid tissue suggesting they may be important players at this site (Murphy et al., 2008).

In addition to providing the first line of immune defense, activated innate immune cells amplify the immune response by priming helper CD4 T cells, cytotoxic CD8 T cells, and B cells of the adaptive immune system at the site of infection or in secondary lymphoid organs. Activation of T cells requires two signals from the same APC – one provided by a MHC molecule presenting a specific antigen to the T cell receptor, and the second signal provided by a costimulatory B7 molecule activating the T cell CD28 receptor. Activated helper T cells can then induce B cell proliferation and class switching and also help activated CD8 T cells proliferate and transition into memory cells. The cellular and humoral arms of the adaptive immune system provide both specific and long term protective

immunity to reinfection by the same pathogen (Janeway, 2001; Murphy et al., 2008).

The importance of the innate and adaptive immune system is illustrated by diseases where immune cells are deficient or functionally impaired. For example, people with chronic granulomatous disease have impaired production of ROS from phagocytes and cannot kill engulfed microorgansims. They often have recurrent bacterial and fungal infections. In addition, a deficiency in B and T cells characterizes one of the most common forms of severe combined immune deficiencies (SCID). People with SCID are highly susceptible to bacterial, viral, and fungal infections (Murphy et al., 2008).

T helper cell subsets

MHC class II molecules display antigens from pathogens multiplying in cytoplasmic vesicles and ingested from extracellular fluid to activate naïve CD4 T helper cells. Upon antigen stimulation, naïve CD4 T cells differentiate into at least three proinflammatory subsets – Th1, Th2, and Th17 depending on the strength of the MHC:TCR interaction, the costimulators used to drive the response, and the cytokines present in the environment. These subsets mobilize different arms of the immune system and are characterized by their pattern of cytokine secretion – Th1 cells secrete IFN-γ and lymphotoxin-α and are important for cell mediated immunity against intracellular pathogens, while Th2 cells produce IL-4, IL-5, and IL-13 to mediate humoral immune responses

against extracellular bacteria and parasites (Murphy and Reiner, 2002). Th17 cells secrete IL-17A, IL-17F, IL-21, and IL-22 to induce inflammation and promote cell mediated antibacterial and antifungal immunity (Weaver et al., 2007). T helper cell function is tightly regulated to provide optimal host defense and can lead to pathological immune responses if they are not properly regulated or if they are activated against self proteins. Chronic Th2 cells contribute to asthma and allergies while Th1 and Th17 cells can lead to autoimmune diseases (Ouyang et al., 2008).

Transcription factor requirements of T helper cell subsets

The local cytokine microenvironment induces specific transcription factors in an activating T cell to promote T helper development. Activation of signal transducer and activator of transcription (STAT) proteins induce lineage specific master regulators and together orchestrate the differentiation of CD4 T cells. In addition to promoting characteristic cytokine secretion, these factors regulate the expression of cytokine receptors on the surface of the T cell and inhibit other transcription factors to maintain and stabilize T cell lineages.

For Th1 development, two major pathways are essential – IL-12 (derived from activated APCs) → Stat4 → IFN-γ (Kaplan et al., 1996a; Magram et al., 1996; Thierfelder et al., 1996) and an autocrine IFN-γ pathway, IFN-γ → Stat1 → T-bet → IFN-γ (Durbin et al., 1996; Szabo et al., 2000; Afkarian et al., 2002). Stat4 and Stat1 proteins are critical for direct induction of IFN-γ as well as upregulation of T-

bet. The IL-12 specific receptor subunit, IL12Rβ2, is expressed at low levels in naïve CD4 T cells and is induced by T-bet to augment Th1 development (Afkarian et al., 2002). Th2 development is dependent on the IL-4 → Stat6 → GATA-3 → IL-4 (Kaplan et al., 1996a; Zheng and Flavell, 1997) pathway. The original source of IL-4 is unknown and GATA-3 is able to autoactivate its own expression in a Stat6-independent fashion to maintain Th2 commitment (Zheng and Flavell, 1997). The Th1 and Th2 lineages are stabilized by positive feedback loops with IFN-γ and IL-4 respectively and also by inhibition of the other lineage – GATA-3 inhibits Stat4 activation (Usui et al., 2003) and IL12Rβ2 upregulation, while T-bet inhibits GATA-3 expression and function. In addition, the IFN-γ signaling pathway potently inhibits Th17 development.

Development of Th17 cells occurs in three steps – induction, amplification, and maintenance (Ivanov et al., 2007). Induction requires both the anti inflammatory cytokine TGFβ and the acute phase cytokine IL-6 (Bettelli et al., 2006; Mangan et al., 2006; Veldhoen et al., 2006) and signals through a TGFβ + IL-6 → Stat3 → RORγt → IL-17 pathway (Ivanov et al., 2006; Mathur et al., 2007; Yang et al., 2007a). At the same time, TGFβ + IL-6 induces IL-21 which combines with TGFβ in a feedforward loop to amplify Th17 development and upregulate IL23R. IL-21 induction is STAT3 dependant and TGFβ + IL-21 also signal through Stat3 and RORγt (Wei et al., 2007). Finally, IL-23 signals through Stat4 in addition to Stat3 to maintain Th17 development (Mathur et al., 2007).

Stability of T helper cell subsets

T helper cell differentiation has been described as a terminal process leading to the development of subsets expressing mutually exclusive cytokine profiles (Ansel et al., 2003; Rowell and Wilson, 2009; Zhou et al., 2009). Early studies demonstrated that Th1 and Th2 cells are committed to their respective lineages after multiple rounds of stimulation (Murphy et al., 1996). However, observations of cells coexpressing IL-17 and IFN-γ in vivo have questioned T cell commitment (Mangan et al., 2006; Korn et al., 2007; Suryani and Sutton, 2007). In vitro, Th17 cells contain chromatin modifications at Th1 and Th2 transcription factors suggestive of a poised state ready for subsequent silencing or activation and transition into opposite lineages (Wei et al., 2009). Consistent with this, Th17 cells have the capacity to switch to Th1 and Th2 cells upon challenge with instructive cytokines of either lineage (Lexberg et al., 2008; Stritesky et al., 2008; Lee et al., 2009). These data have raised new questions about T cell stability and the relationship between T helper lineages during an ongoing immune response.

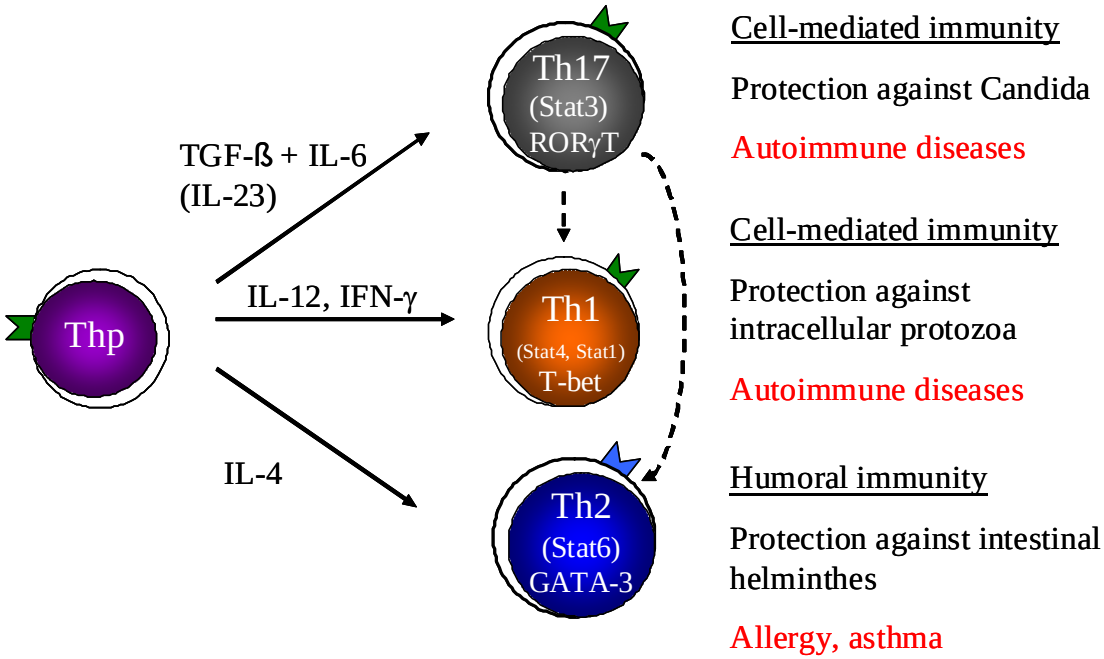


Figure 1. T helper cell development. Naive T helper precursors (Thp) differentiate into CD4 T cell subsets depending on cytokines in the environment and transcription factors activated. TGFβ + IL-6 activate Stat3 and Rorγt to promote Th17 development with IL-23 maintaining the Th17 lineage. IL-12 activates Stat4 and IFNγ activates Stat1 and T-bet to promote Th1 development. IL-4 activates Stat6 and GATA-3 to promote Th2 development.

Cytotoxic T Lymphocytes

Function and memory formation

CD8 T cells respond to an acute infection through three phases - (1) activation, (2) contraction, and (3) memory formation. During the activation phase, pathogen specific CD8 T cells recognize intracellular antigens presented by MHC class I molecules and expand several fold, sometimes accounting for up to 50% of the total CD8 T cells at the peak of the response (Butz and Bevan, 1998). Activating CD8 T cell populations upregulate multiple effector proteins – perforin and granzymes (both contained in granules) and surface expression of Fas Ligand to become effector cytotoxic T lymphocytes (CTLs). Perforin allows

granzymes to traffic into target cells and induce apoptosis through caspase cleavage and by releasing apoptotic factors from the mitochondria. Fas ligand can also induce caspase cleavage after binding to the Fas receptor. CTLs therefore induce apoptosis and also secrete IFN-γ to mediate antiviral immunity and tumor immunosurveillance (Russell and Ley, 2002).

In the subsequent contraction phase, 90-95% of these effector CD8 T cells undergo apoptosis. The remaining 5-10% of the CD8 T cells specific for the pathogen enter the memory pool and, following reexposure to the pathogen, undergo a rapid recall response to quickly expand and acquire effector function. In humans, memory CD8 T cells have been observed for up to 75 years after vaccination, presumably providing lifelong immunity in some instances (Hammarlund et al., 2003). The magnitude of the CD8 T cell response and the number of memory cells generated can be modulated by several factors including antigen availability, inflammatory stimuli, and CD4 T cell help (Williams and Bevan, 2007; Harty and Badovinac, 2008; Murphy et al., 2008).

Subsets

Activation in the presence of various proinflammatory cytokines, however, can alter the innate CTL programming of CD8 T cells and lead to at least three proinflammatory subsets characterized by their panel of secreted cytokines – Tc1, Tc2, and Tc17, analogous to CD4 T helper cell subsets (Carter and Dutton, 1996; Mosmann and Sad, 1996; Liu et al., 2007). IL-12 augments innate CD8 T

cell programming to increase expression of IFN-γ and cytotoxic molecules and induce Tc1 development (Croft et al., 1994). IL-4 diverts innate CD8 T cell programming to develop Tc2 cells expressing IL-4, IL-5, IL-13 and reduced amounts of IFN-γ (Seder et al., 1992; Kelso and Groves, 1997). The Tc17 subset develops in the presence of TGFβ and IL-6 and is characterized by secretion of IL-17A, IL-17F, IL-21, and IL-22 (Intlekofer et al., 2008; Ciric et al., 2009; Curtis et al., 2009; Hamada et al., 2009; Huber et al., 2009).

The role of Tc1 cells in delayed type hypsersensitivity (DTH) reactions and helping the immune system eradicate tumors and viruses is well established. In addition, putative roles for Tc2 and Tc17 cells in these traditional Tc1 functions have been suggested. Similar to Tc1 cells, Tc2 cells are antitumor in an in vivo tumor metastasis model (Dobrzanski et al., 1999), and recruit inflammatory effector cells to a DTH reaction. However, the mechanisms by which Tc1 and Tc2 cells operate may differ. In cell mediated cytotoxicity, both utilize the perforin pathway while Tc1, but not Tc2 cells, display significant lysis through Fas-mediated pathways (Carter and Dutton, 1995). In a metastatic melanoma model, Tc1 cells kill the tumor directly while Tc2 cells chemoattract host IFN-γ secreting cells to fight the tumor (Dobrzanski et al., 2001).

IL-17 secreting cells have a controversial role in tumor immunity which could depend on the level of IL-17 in the environment. Tumors secrete large quantities of TGF-β and tumor bearing mice have greater numbers of Th17 and Tc17 cells

(Kryczek et al., 2007b). A study has demonstrated that IL-17 secreting T cells promote tumor survival (Nam et al., 2008), however adoptive transfer of Tc17 cells clears tumor in a melanoma model (Hinrichs et al., 2009). Tc17 cells have also been shown to be antiviral in an influenza virus model. In this model, Tc17 cells are induced and clear virus in a perforin independent and IFN-γ dependant mechanism (Hamada et al., 2009).

Tc17 cells are also induced or correlated with several allergic or autoimmune diseases. IL-17 secreting CD8 T cells are critical for the elicitation of contact hypersensitivity CHS) (He et al., 2006). CD8 T cell clones from psoriatic lesions can express IL-17 (Teunissen et al., 1998). Active lesions in multiple sclerosis contain a high numbers of Tc17 and Th17 cells (Tzartos et al., 2008; Goverman, 2009). Autoreactive Tc17 and Th17 cells are also induced in experimental autoimmune uveitis (Peng et al., 2007). In addition, IL-6-driven expansion of Tc17 cells induces colitis (Tajima et al., 2008).

Collectively, these reports suggest that CD8 T cell subsets are able to clear tumors and intracellular pathogens most likely utilizing unique mechanisms. In addition, IL-17 secreting CD8 T cells may be involved in Th17 related host immunity and autoimmune diseases.

Transcription factor requirements of T cytotoxic cell subsets

Important TCR induced proteins for development of cytolytic effector cells include the related T-box factors T-bet and Eomes. Pre-existing Runx3 also plays a critical role in CTL development and a deficiency in any of these factors diminishes effector function (Taniuchi et al., 2002; Pearce et al., 2003; Sullivan et al., 2003). Based on overexpression studies, gene deficient cells, and expression kinetics, a two stage model has been proposed where T-bet is necessary for early development and Runx3 → Eomes signals are important for late CTL function (Cruz-Guilloty et al., 2009). This innate CTL programming is altered by cytokines in the microenvironment to develop T cytotoxic cell subsets utilizing similar signaling pathways which drive T helper cell subsets.

IL-12 → STAT4 → T-bet signals (Takemoto et al., 2006) augment CTL programming to induce Tc1 cells (Curtsinger et al., 1999; Curtsinger et al., 2003) and further upregulate IFN-γ (Li et al., 2006) and cytotoxic molecules (Curtsinger et al., 2005). Interestingly, IL-12 represses Eomes expression as it augments CTL programming (Takemoto et al., 2006). T-bet induction through IL-12 → STAT4 signals contrasts to CD4 T cells where T-bet is regulated through IFN-γ → STAT1 pathways and is possibly due to higher induction of IL12Rβ2 in CD8 T cells leading to increased sensitivity of CD8 T cells to IL-12 (Yang et al., 2007b). STAT1 does not appear to play a role in Tc1 development (Casey and Mescher, 2007) where the main function described of STAT1 is for CD8 T cell clonal expansion and memory formation (Gil et al., 2006; Quigley et al., 2008).

Tc2 cells develop via the same IL-4 → Stat6 → GATA3 (Kaplan et al., 1999; Omori et al., 2003; Yamaguchi et al., 2004) signaling pathway utilized to drive Th2 development and can secrete similar levels of IL-5 and IL-13 as Th2 cells. However, IL-4 production (Croft et al., 1994) and histone hyperacetylation at the IL-4 gene locus is much lower in Tc2 cells. This is proposed to be due to decreased GATA-3 expression and increased expression of an inhibitor of GATA-3 function, ROG (Repressor of GATA) compared to Th2 cells (Omori et al., 2003).

Cytokines and transcription factors determining Tc17 development also appear identical to those involved in Th17 development. The combination of TGFβ and either IL-6 or IL-21 induces the generation of Tc17 cells (Stumhofer et al., 2006; Liu et al., 2007) through a Stat3 → RORγt pathway. IL-23 can also induce IL-17 secretion from Tc17 cells and has been shown to induce the secretion of IL-17 in naïve CD8 T cells in contrast to its actions on naïve CD4 T cells (Vanden Eijnden et al., 2005). The IFN-γ → Stat1 pathway in addition to T-bet and Eomes strongly inhibit Tc17 development (Intlekofer et al., 2008; Ciric et al., 2009).

CD8 T cell subsets develop in a similar fashion as CD4 T cell subsets. However, the extent of polarization and the amount of cytokines secreted between respective CD8 and CD4 T cell subsets often differ due to preferential expression of certain transcription factors in naïve CD8 vs CD4 T cells (Runx3, Eomes) and

differential upregulation of lineage specific transcription factors (ROG) and cytokine receptors (ie. IL12Rβ2).

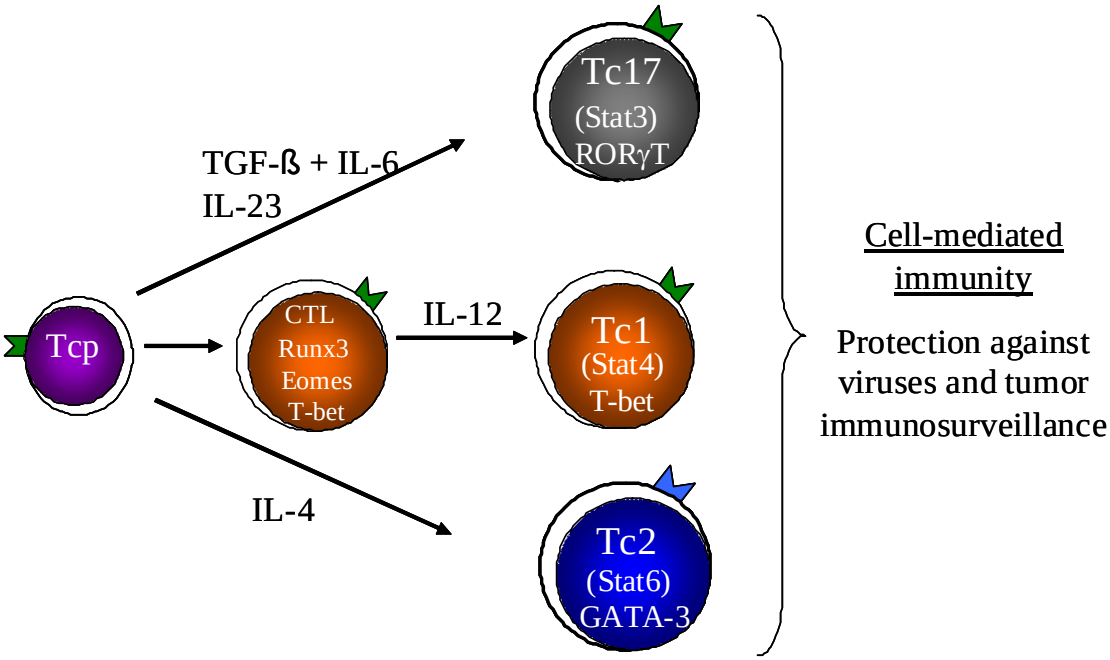


Figure 2. T cytotoxic cell development. Naive T cytotoxic precursors (Tcp) differentiate into CD8 T cell subsets depending on cytokines in the environment and transcription factors activated. TGFβ + IL-6 activate Stat3 and Rorγt to promote Tc17 development and IL-23 contributes to Tc17 formation. IL-12 activates Stat4 and T-bet to promote Tc1 development. IL-4 activates Stat6 and GATA-3 to promote Th2 development.

Antiviral Immunity

The life cycle of a virus can be described in four stages –

1. Viral attachment and entry into a host cell.
2. Migration into the appropriate cellular compartment.
3. Transcription and translation of the viral genome for viral replication.

4. Assembly and release of mature virions to reinfect additional cells.

Host immune responses interfere with each stage to promote antiviral immunity through cytolytic and non-cytolytic mechanisms.

Upon viral detection, the innate immune system produces high levels of Type I Interferons (IFN-α and IFN-β) to promote an antiviral state both directly, by inhibiting viral replication, and indirectly by activating the innate and adaptive immune systems. IFN-α and IFN-β can stimulate effector functions of NK cells, CTLs, and macrophages as well as induce MHC class I and MHC class II to increase immunogenicity of infected cells. NK cells are important for early viral control and kills either infected cells before MHC class I is upregulated or infected cells which normally express no or low levels of MHC class I. NK cells can also mediate noncytolytic control of viral infections by secreting cytokines such as IFN-γ and TNF-α.

Antigen presentation and clonal expansion of the adaptive immune response occurs over several days after which CD8 T cells, CD4 T cells, and B cells all play important roles in viral control. Similar to NK cells, CD8 T cells mediate viral clearance by killing infected cells and by producing IFN-γ and TNF-α. B cells produce neutralizing antibodies to prevent viral reinfection of additional cells or viral infection during a memory response. CD4 T cells produce IFN-γ and help memory formation of CD8 T cells and antibody isotype switching from B cells.

Immune response to vaccinia virus

Upon infection with vaccinia virus, NK cells (Bukowski et al., 1983; Stitz et al., 1986) and γδ T cells (Selin et al., 2001) contribute to vaccinia clearance. Vaccinia virus infection also induces an adaptive immune response which precedes viral clearance. This response peaks about a week after infection in mice and includes clonal expansion of CD4+ and CD8+ T cells and virus specific antibodies. CD4 T cells and B cells have a critical role in vaccinia clearance (Xu et al., 2004), while CD8 cells contribute to viral clearance during a memory response or in the absence of CD4 T cells (Xu et al., 2004). Inoculation with as few as 10 pfu of vaccinia virus leads to generalized vaccinia infection and death in athymic nude mice illustrating the importance of T cells in vaccinia clearance (Muller et al., 1994). Effects of antibody depletion or genetic deficiency of important antiviral factors are summarized in Table 1.

The role of IL-17 in vaccinia virus clearance is unclear. A recombinant vaccinia virus overexpressing IL-17 has been shown to have both increased (Patera et al., 2002) and decreased (Kohyama et al., 2007) viral load in an infected mouse. A role for IL-17 in vaccinia survival is supported by a report demonstrating IL-17 inhibition of NK cell function (Kawakami et al., 2009), while a role for IL-17 in vaccinia clearance is suggested in a study demonstrating increased vaccinia virus induced mortality in *Il17* deficient mice (Kohyama et al., 2007).

IL-17 has also been shown to play a role in other viral infection models. IL-17 was first cloned and described from a homologous gene in Herpesvirus Samirii (Yao et al., 1995). Deficiency of this gene does not affect H. samirii replication and is thought to be involved in apathogenic viral persistence in its host (Arens et al., 2008). IL-17 is induced after infection with several viruses including HIV (Maek et al., 2007), murine CMV (Arens et al., 2008), and influenza (Hamada et al., 2009). In a model of Theiler's murine encephalomyelitis virus infection, IL-17 enhances viral survival by upregulating antiapoptotic molecules (Hou et al., 2009). In contrast, IL-17 enhances viral killing in an influenza model (Hamada et al., 2009; McKinstry et al., 2009) suggesting that the function of IL-17 in viral immunity may be virus specific.

Table 1. Immune cell and antiviral effector requirements for immunity against vaccinia virus.

Cell Type	NK cells	γδ T cells	CD8 T cells	CD4 T cells	B cells
Requirement for primary VV clearance	+	+	-	+	+
Reference	(Bukowski et al., 1983; Stitz et al., 1986)	(Selin et al., 2001)	(Spriggs et al., 1992; Xu et al., 2004)	(Xu et al., 2004)	(Xu et al., 2004)

Effector Mechanism	IFN-α/βR	IFN-γR	IFN-γ	Perforin	Fas Ligand
Requirement for primary VV clearance	+	+	+	-	-
Reference	(Muller et al., 1994)	(Huang et al., 1993; Muller et al., 1994; Cantin et al., 1999)	(Cantin et al., 1999)	(Kagi et al., 1995)	(Kagi et al., 1995)

Research Goals

In the past decade, IL-17 has been shown to play a critical role in antibacterial and antifungal immunity and also to contribute to several autoimmune diseases such as multiple sclerosis and rheumatoid arthritis. IL-17 production and regulation has mostly been studied in CD4 T helper cells and its role from CD8 T cytotoxic cell has not been conclusively established. Therefore, the goal of this research is to determine IL-17 regulation from CD8 T cells and its role in CD8 T cell immunity.

Several reports have shown the existence of Tc17 cells; however, cytokines and transcription factors regulating their development have not been studied as extensively as factors regulating development in Th17 and IFN-γ secreting CD8 T cells. We wanted to examine the role of cytokines and important downstream Th17 and Tc1 transcription factors in Tc17 development utilizing in vitro T cell cultures and mice deficient in transcription factors regulating lineage development.

To determine the role of Tc17 cells in antiviral immunity, we utilized a vaccinia virus infection model and gene deficient mice with either defective or enhanced IL-17 production. In addition, adoptive transfer of in vitro generated IL-17-secreting CD8 T cells was utilized to determine the antiviral potential of Tc17 cells.

MATERIALS AND METHODS

Mice

The generation of *Stat4*$^{-/-}$ (Kaplan et al., 1996b), *Stat1*$^{-/-}$ (Meraz et al., 1996) (Taconic Farms, Germantown, NY), *Tbx21*$^{-/-}$ (Szabo et al., 2000), *Stat3*$^{fl/fl}$ mice with a CD4-Cre transgene (*Stat3*$^{CD4-/-}$) (Raz et al., 1999; Chiarle et al., 2005), *Ifng*$^{-/-}$ (Dalton et al., 1993) (Jackson Laboratories, Bar Harbor, ME) and *Il17*$^{-/-}$ mice (Nakae et al., 2002) were previously described. All mice were on a C57BL/6 background except for *Stat1*$^{-/-}$ mice which were on a 129S6 background and *Il17*$^{-/-}$ mice which were on a Balb/c background. C57BL/6 and Balb/c mice were purchased from Harlan Bioscience (Indianapolis, IN), 129S6 and OT-I / *Rag1*$^{-/-}$ mice were purchased from Taconic Farms, C3H/HeJ mice were purchased from Jackson Laboratories and BoyJ mice were purchased from the IU Simon Cancer Center In Vivo Therapeutics Core (Indianapolis, IN). Mice were kept in pathogen-free conditions and all studies were approved by the Indiana University School of Medicine Animal Care and Use Committee.

Viruses

VV (Western Reserve strain) and VV-SIINFEKL was generated in the human osteosarcoma TK-143B cell line, followed by sucrose purification and titer determination by the Poxvirus Core Facility at Indiana University School of Medicine as described (Li et al., 2005).

T cell and APC Isolation

Spleen and inguinal, axillary, brachial, and mesenteric lymph nodes were collected from mice and single cell suspensions were made in RPMI 1640 (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Atlanta Biologicals, Norcross, GA), 1mM glutamine, 100 Units/ml penicillin, 100 μg/ml streptomycin, 10 mM Hepes buffer, 0.5 x nonessential amino acids, 1mM sodium pyruvate (all from BioWhitaker, Walkersville, MD), and 50 μM β-mercaptoethanol (Sigma, St. Louis, MO). Cell suspensions were transferred into a 50 mL conical tube through a strainer (BD Biosciences, San Jose, CA) to remove debris. The cells were centrifuged at 1,500 rpm for 5 minutes at 4°C and the supernatant was discarded. Cell pellets were resuspended in 4-5 mL of red blood cell (RBC) lysis buffer (8.3 g/L NH_4Cl in 0.01 M Tris-HCl; pH: 7.5) and incubated for 1-2 minutes at room temperature. After RBC lysis, 5 mL of phosphate buffered saline (PBS) were added and samples were centrifuged at 1,500 rpm for 5 minutes at 4°C. Cell pellets were washed once in MACS Buffer (2mM EDTA and 0.5% bovine serum albumin (BSA) in PBS) and re-suspended in 900 μl of MACS buffer per spleen. Total CD4+ and CD8+ cells were isolated by positive selection according to manufacturer's protocol with magnetic beads (magnetic cell sorting [MACS] isolation system; Miltenyi Biotec, Auburn, CA). CD45.2+ cells were isolated by incubating with 25 μl anti-CD45.2 PE per spleen for 15 minutes, washing two times with MACS buffer, and then incubating with 25 μl anti-PE microbeads per spleen for an additional 15 minutes. In other experiments, APC's were obtained by collecting the flow through of cells from the column after positive selection.

APCs were resuspended in 5 mL of supplemented RPMI media in a 15 mL conical tube and irradiated with 3000 rads. Irradiated APC's were washed 3 times with PBS before use in cocultures with T cells.

In vitro T Cell Differentiation

For Tc cell differentiation, CD8+ cells ($0.25x10^6$ cells/ml) were activated with soluble anti-CD3 (4 μg/ml 145–2C11) and anti-CD28 (1 μg/ml; BD Pharmingen) in the presence of $CD8^+$ depleted irradiated splenocytes (1:5). OT-I CD8+ cells were activated with soluble SIINFEKL peptide (1μM; Bio-Synthesis Inc) and anti-CD28 (1 μg/ml). Tc17 primed cells were cultured with hTGFβ1 (2 ng/ml; R&D Systems), IL-6 (100 ng/ml; PeproTech), and anti-IFNγ (10 μg/ml R4/6A2 or XMG) and Tc1 primed cells were differentiated with IL-12 (5 ng/ml; R&D Systems). In some experiments, CD8+ cells were cultured with IL-21 (50ng/ml; eBioscience) or IL-23 (10ng/ml; R&D Systems). Cells were maintained in 5% CO_2 at 37°C. After 3 days of incubation, cells were expanded into 25 cm^2 flasks in the presence of 20 units/ml recombinant hIL-2. After 2 more days of culture, cells were washed, counted and analyzed by RT-PCR, flow cytometry, and ELISA. For Th cell differentiation, CD4+ cells ($1x10^6$ cells/ml) were activated and differentiated with cytokines and neutralizing antibodies at the same concentrations as described above. After 3 days of incubation, cells were expanded into larger wells in the presence of 20 units/ml recombinant hIL-2 and counted and analyzed after an additional 2 days of culture.

In some experiments, cells underwent 2 rounds of stimulation. For the second five days of culture, cells were replated ($0.25x10^6$ cells/ml) and stimulated with soluble anti-CD3 (1 μg/ml) and anti-CD28 (0.5 μg/ml) in the presence of $CD8^+$-depleted irradiated splenocytes (1:5). OT-I cells were activated with soluble SIINFEKL peptide (0.5 μM) and anti-CD28 (0.5 μg/ml). Cells were stimulated and maintained with the same cytokine and neutralizing antibody concentrations as used in the first five days.

Retroviral Vectors and Transductions

The retroviral vector MIEG-hCD4 was made as previously described (Chang et al., 2005) and modified for coexpression of Eomes by digesting the coding region of murine Eomes cDNA (Open Biosystems) with EcoRI and NotI and cloning into MIEG-hCD4. Additional vectors MIEG-GFP, Tbet-GFP, MSCV-$H2K^k$, STAT3C-$H2K^k$ have been previously described (Mathur et al., 2006; Mathur et al., 2007) and RORγt-GFP was received from Dr. Gilbert Kersh, Emory University.

Retroviral supernatants were generated by transfecting a Phoenix-Eco packaging cell line in chloroquine containing DMEM media with 15 μg of purified plasmid by calcium phosphate precipitation. Cells were cultured at 37°C and one day after transfection, chloroquine containing DMEM media was replaced with fresh DMEM media. After 1 more day, the supernatants containing retrovirus were collected, filtered through a 0.45 μm filter and stored at -80°C. Wild type CD8+ T cells were cultured under the indicated conditions for 24 hours. Cells were

transduced with 4 ml of retroviral supernatant containing 4 μg/ml of polybrene, and centrifuged at 2000 rpm at 20°C for 1 hr. For double transductions, 3 ml of each virus were mixed together for the spin infection. After the centrifugation, the retroviral supernatant was replaced with the original conditioned media. Transduced cell populations were either restimulated and gated on the selectable marker for analysis by ICS or sorted for positively transduced cells and analyzed by RT-PCR, flow cytometry, or ELISA assay.

RNA Isolation and cDNA Conversion

Cells ($0.5\text{-}1x10^6$) were lysed in Trizol (1 mL) and used immediately or stored at -80°C. Lysed cells were incubated for 5 minutes at room temperature before adding 200 μl of chloroform and shaking vigorously for 15 seconds. Homogenate was incubated at room temperature an additional 2 minutes and then spun at 12,000g for 15 minutes at 4°C. The aqueous phase was transferred to a new tube and RNA was precipitated by the addition of 0.5 ml isopropanol and incubation at -20^oC overnight. The next day, RNA was spun at 12,000g for 10 minutes and washed one time with cold 75% ethanol before air drying and resuspending in 20 μl diethylpolycarbonate (DEPC) treated ddH_20. Isolated RNA was reverse transcribed into cDNA using the Cloned AMV First-Strand cDNA Synthesis Kit (Invitrogen) and random hexamer primers in a 20 μl total volume. cDNA was then diluted with DEPC ddH_20 to a 100 μl total volume before using in real time RT PCR reactions. Nuclease-free filtered pipette tips were used for all RNA work.

Real-Time RT-PCR

cDNA (5 μl) was added to 1.25 μl primer (inventoried FAM-labeled TaqMan® Gene Expression Assays, Applied Biosystems), and 12.5 μl Taqman Universal PCR Master Mix (Applied Biosystems). DEPC H_20 was added to a final reaction volume of 25 μl. Samples were analyzed in duplicate, mixed in 96-well optical reaction plates, and capped with optical reaction caps (Applied Biosystems) Gene expression was measured on an Applied Biosystems ABI PRISM 7500 Realtime PCR system. Cycle number was normalized to β_2-microglobulin expression. Data were analyzed using the provided software and cycle threshold (Ct) was calculated using the Auto Ct function.

Cell Surface Staining

Total splenocytes or isolated T cells ($0.25\text{-}1x10^6$) were placed in 12x75 mm flow cytometry tubes. Following centrifugation at 1,500 rpm for 5 minutes at 4°C, cells were washed once in FACS/ELISA buffer (2% BSA, 0.01% NaN_3 in PBS) and re-suspended with formaldehyde at a final concentration of 2% and a total volume of 100 μl. Cells were incubated for 10 minutes at room temperature and washed twice with FACS/ELISA buffer. Buffer was removed and purified FcγIII/II receptor (1 μg per $1x10^6$ cells; BD Biosciences) was added to samples containing APC's, mixed by vortex and incubated at 4°C for 10 minutes. Cells were surface stained for CD4, CD8, CD44, CD122, CD62L, CD45.1, or CD45.2 using fluorochrome conjugated antibodies (eBioscience) for 20 minutes at 4°C. Cells were washed one time in 1 mL of FACS/ELISA buffer and permeabilized for intracellular

staining or analyzed by flow cytometry on FACS-Calibur machines (BD Biosciences). In each sample 8,000 gated events were collected and data were analyzed using WinMDI software.

Intracellular Cytokine Staining

CD4+ or CD8+ T cells (0.25-1x10^6) were stimulated with phorbol-myristate acetate (PMA) (50 ng/mL; Sigma) and ionomycin (500 ng/ml; Sigma) for 4 hours in the presence of GolgiPlug (BD Biosciences). In some experiments, OT-I cells were stimulated with SIINFEKL peptide (1 μM) or CD8 cells were stimulated with vaccinia virus B8R peptide (1 μg/ml) for 4-5 hours in the presence of GolgiPlug. Cells were fixed for 10 minutes at room temperature with formaldehyde at a final concentration of 2%, stained for surface markers as described above, and permeabilized by washing twice with FACS/ELISA buffer plus 0.1% saponin. Cells were stained for IL-17, IFN-γ, IL-2 (eBiosciences) and TNF-α (BD Biosciences) using fluorescently conjugated antibodies for 30 minutes at 4°C. Cells were washed one time in FACS/ELISA buffer plus 0.1% saponin. Samples were analyzed by flow cytometry using FACS-Caliber machines and data were analyzed using WinMDI software.

Granzyme B and Transcription Factor Staining

CD4+ or CD8+ T cells (0.25-0.5x10^6) were stimulated with PMA (50 ng/mL) and ionomycin (500 ng/ml; Sigma) for 4 hours in the presence of GolgiPlug for Granzyme B staining or left unstimulated for T-bet, Eomes, Rorγt, and phospho-

Stat4 staining. Cells were collected and fixed for 10 minutes at room temperature with formaldehyde at a final concentration of 2%. Cells were pelleted by centrifugation and resuspended in 1 mL of 100% methanol for 10 minutes at 4°C or overnight at -20°C. Cells were then washed three times with 2 mL FACS/ELISA buffer and stained for Granzyme B, T-bet, Eomes and Rorγt using fluorescently conjugated antibodies (eBiosciences) for 30 minutes at 4°C. Cells were washed in 1 mL FACS/ELISA buffer and the samples were analyzed by flow cytometry using FACS-Calibur machines. Data were analyzed using WinMDI software.

Detection of Cytokines Using ELISA

To generate cell free supernatants for analysis, T cells differentiated for 5 days were washed and stimulated ($1x10^6$ CD4+ T cells/ml or $0.25x10^6$ CD8+ T cells/ml) with plate bound anti-CD3 (4 μg/ml) for 24 hours or freshly isolated CD4+ or CD8+ T cells ($1x10^6$ CD4+ T cells/ml) were stimulated with plate bound anti-CD3 (4 μg/ml) for 48-72 hrs. For antigen specific stimulation, ex vivo isolated CD8+ cells were stimulated with 1μM SIINFEKL peptide for 48-72 hrs. To test for cytokine secretion, 2 μg/ml of anti-IFN-γ, anti-IL-17, anti-IL-21, or anti-IL-22 capture antibodies were dissolved in 0.1 M $NaHCO_3$ (pH 9) or 2 μg/ml of anti-IL-10 capture antibody was dissolved in 0.1 M Na_2HPO_4 (pH 7) and 50 μl/well was used to coat a 96 well Immunosorbent plate. Plates were incubated at 4°C overnight and washed two times in ELISA Wash buffer (0.1% Tween-20 in PBS) and blocked for at least two hours at room temperature in FACS/ELISA

buffer (100 μl/well). FACS/ELISA buffer was removed and supernatants and cytokine standards (R+D Systems) were added and incubated overnight at 4°C. Plates were washed four times with ELISA wash buffer and incubated at room temperature with 1 μg/mL of biotinylated detection antibodies dissolved in FACS/ELISA buffer for at least two hours. Plates were washed four times with ELISA wash buffer and incubated with. streptavidin alkaline phosphotase (1:2000 dilution; Sigma) in FACS/ELISA buffer for at least one hour. Cytokine levels were determined following the addition of Sigma 104 phosphatase substrate (5 mg/ml; Sigma) dissolved in ELISA substrate buffer (10% diethanoloamine, 0.05 mM $MgCl_2$, 0.02% NaN_3, pH 9.8) by measuring the absorbance at 415 nm (BIO-RAD microplate reader model 550).

^{51}Cr Release Cytotoxicity Assay

Cytotoxic activity of OT-I Tc17 and Tc1 effector cells were measured in standard ^{51}Cr release assays. EL4 and EG.7 (EL4 cells transfected with chicken ovalbumin) target cells were grown in RPMI supplemented with 10%FBS, 1mM glutamine, and 10 μg/ml gentamycin and split 1:5 the day before the assay.

On the day of the assay, EL4 and EG.7 target cells ($6x10^6$) were prepared by incubating with 200 μCi of Na_2CrO_4 (0.04 ml of 5 mCi/ml Chromium-51 radionuclide solution; Perkin Elmer) for 1 hr at 37°C. Target cells were incubated in a total volume of 1 mL in a 50 mL conical tube for 1 hr at 37°C and washed 3 times with supplemented RPMI media.

Effector cells were added to a round bottom 96-well microtiter plate in triplicate and serially diluted three fold to generate effector:target ratios ranging from 30:1 to 0.03:1. Next, target cells (100 μl, $2x10^4$ cells per well) were added to the microtiter plate. Reactions were conducted in a total volume of 200 μl per well. Plates were incubated for 6 hrs at 37°C, spun down, and 100 μl of supernatant was harvested from each well. Radioactivity was counted in a Perkin Elmer gamma counter. The % specific lysis was calculated as $(cpm_{sample} - cpm_{spontaneous})/(cpm_{maximum} - cpm_{spontaneous}) \times 100$, where cpm is counts per minute. Spontaneous release represents the radioactivity released by target cells in the presence of media alone, and maximum release represents the radioactivity released by target cells lysed with 5% Triton X-100. Spontaneous release was less than 20% of the maximum in chromium release assays.

CFSE Cytotoxicity Assay

In other experiments, cytotoxic activity of OT-I effector cells were measured by labeling the target cells with 2 different concentrations of CFSE. EL4 and EG.7 target cells were labeled with 1 and 0.01 μM CFSE respectively. Reactions were performed in a 96 well round bottom microtiter plate with 50 μl labeled EL4 cells, 50 μl labeled EG.7 cells, and 100 μl effector OT-I cells. After 4 hrs of incubation at 37°C, cells were collected into 15x75 mm flow cytometry tubes. 7-AAD (eBioscience) was added to identify dead cells and reactions were analyzed using flow cytometry. Percentage of antigen specific cytotoxicity was calculated by the formula: $\%cytotoxicity = 100 \times [1 - (E.G7/EL4)_{experimental}/(E.G7/EL4)_{control}]$.

Infections

For experiments analyzing the effects of genetic deficiencies in VV infection, 2-6 mice per group were infected intraperitoneally (i.p.) with a sublethal dose ($1x10^6$ or $5x10^6$ pfu/mouse) of VV (WR strain). Mice were sacrificed after 7 or 11 days of infection and ovaries and spleens were collected. For adoptive transfer experiments, 2-5 mice per group were infected either intranasally (i.n.) ($2x10^6$) or i.p. ($2x10^7$) with VV-SIINFEKL. Infection was performed 1 day before or after cell transfer. Changes in body weight were calculated as the percent body weight on the day of measurement minus the percent body weight on the day of infection. Mice were sacrificed 4-8 days after infection and spleens, ovaries, and lungs were collected.

Adoptive T Cell Transfer

Purified naïve CD8+ cells ($1x10^6$) or 5 day differentiated Tc17 or Tc1 cells ($1x10^6$) from OT-I mice were transferred via tail vein injection. Cells were transferred in 500 μl of PBS using 22G 1" needles and 1 mL syringes. In some experiments, purified cells were labeled with CFSE (Molecular Probes, Invitrogen, Carlsbad, CA) according to manufacturers protocol. Prior to transfer, all cells were washed twice in PBS.

VV Titer Assay

Mice were sacrificed 3-11 days after infection. Two ovaries or two lungs from each mouse were removed and stored in 1 ml of DMEM at -80°C. The day before

the assay, CV-1 cells were plated at ~1.7x10^5 cells/well in a 12 well plate. On the day of the assay, organs were thawed at 37°C and kept on ice. Organs were liquefied in the 1 ml of DMEM they were frozen in using a Tissue-tearor homogenizer. Blades of the homogenizer were sterilized with EtOH before beginning, and between each sample blades were rinsed with a 10% bleach solution, PBS 2 times, and then DMEM. 6 serial 1:10 dilutions of homogenized ovaries in DMEM were made and diluted ovaries were kept on ice. Plates with confluent CV-1 cells were prepared by washing one time with PBS and adding 60 μl of DMEM to each well. Ovary dilutions were measured in duplicate and 40 μl of each dilution was added to the plate to make a total volume of 100 μl. Plates were incubated at 37°C for 30 minutes with rocking every 5 minutes. 1 mL of DMEM supplemented with 10% FCS, penicillin/streptomycin, and 2 mM Glutamine was added to each well. Plates were incubated for an additional 72 hrs. After 72 hours, media was removed from the wells and 0.2% Crystal Violet (in 20% EtOH) was added to cover the bottom of the wells for 5 minutes. Wells were aspirated and allowed to dry before counting plaques.

Th17 Enrichment

Th17 cells were activated with plate-bound anti-CD3 (4 μg/ml). After 4 h, Th17 cells were labeled with 75 μl of previously crosslinked (Controlled Protein-Protein Crosslinking Kit; Pierce) anti-CD45 (clone 30-F11; BD Pharmingen) and anti-IL-17 (clone Tc11-18H10; BD Pharmingen) Abs (0.2 mg/ml) for 5 min on ice. Labeled cells were then diluted in prewarmed complete RPMI 1640 to a

concentration of 10^5 cells/ml and rotated for 1 h at 37°C. After capture, the Th17 cells were stained with 100 μl of biotin-labeled anti-IL-17 (0.2 mg/ml; clone Tc11-8H4.1; BD Pharmingen) for 15 min before washing and incubating 10 min with Streptavidin-PE (BD Pharmingen). IL-17 captured cells were sorted using a FACS Aria cell sorter (BD Biosciences).

RESULTS

Tc17 cells develop in vitro under similar conditions as Th17 cells

Naïve CD4 T cells develop into Th17 cells in the presence of TGFβ and IL-6. To determine if these cytokines also promote IL-17 production from CD8 T cells, we activated total CD8 cells in the presence of TGFβ, IL-6, and IFN-γ neutralizing antibodies for 5 days. Compared to CD4 cells, CD8 cells cultured in IL-17 promoting conditions routinely contained higher percentages of IL-17 and IFN-γ expressing cells (Fig 3A). The percentage of IL-17 expressing cells varied between experiments, but consistently peaked by Day 3 of culture, even without irradiated APCs, and maintained expression for at least 6 days (Fig 3B). Similar to Th17 cells, both TGFβ and IL-6 were required for IL-17 production because minimal IL-17 populations were produced in the presence of each cytokine alone (Fig 3C). As expected, IL-12 stimulated CD8 T cells to produce high levels of IFN-γ. In the presence of all three cytokines, a slightly reduced Tc17 population developed compared to TGFβ + IL-6 stimulated cultures. Meanwhile, the Tc1 population was greatly reduced compared to cultures stimulated with IL-12 alone indicating that Tc17 development is dominant to Tc1 development in this situation. IL-23 is a cytokine known to be critical for maintenance of Th17 cells but is unable to differentiate Th17 cells from naïve CD4 T cells. Similarly, IL-23 was unable to develop Tc17 cells from total CD8 T cells. In addition, IFN-γ inhibited Tc17 development because in the absence of IFN-γ neutralizing antibodies, IL-17 production was reduced approximately 2 fold (Fig 3C).

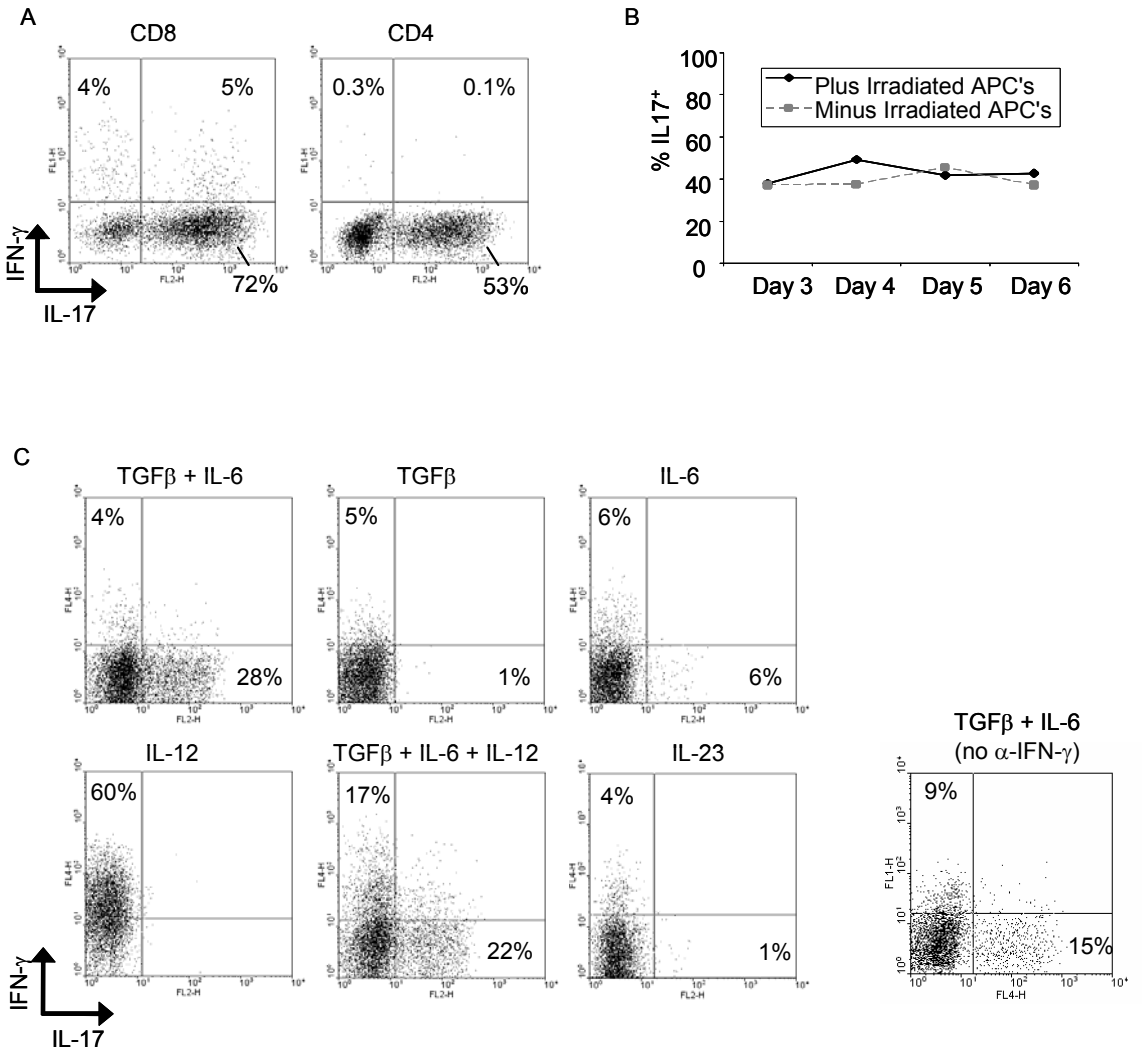


Figure 3. TGFβ + IL-6 induces Tc17 cells. (A) Total CD8+ or CD4+ T cells were stimulated for 5 days with irradiated APC's in IL-17 skewing conditions (TGFβ + IL-6 and neutralizing antibodies to IFN-γ and IL-4). IL-17+ and IFN-γ+ cells were detected by intracellular cytokine staining. (B) Total CD8+ T cells were cultured in Tc17 conditions (TGFβ + IL-6 and anti-IFN-γ) with or without irradiated APC's. IL-17+ cells were detected by ICS on various days. (C) Total CD8 T cells were stimulated for 5 days in the presence of IL-12 or anti-IFN-γ and the indicated cytokines. IL-17 and IFN-γ were detected by ICS. Data are representative of at least 2 experiments.

Previously, total CD8 T cells, which contain both memory and naïve CD8 T cells, were used to develop Tc17 cells. To test the development of Tc17 cells from a naïve population alone, CD8 cells were isolated from OT-I / *Rag1*$^{-/-}$ mice. These CD8 T cells have a transgenic TCR to $Ova_{257-264}$ with the amino acid sequence – SIINFEKL, and are naïve with a $CD44^{lo}CD122^{lo}$ surface phenotype (Fig 4A). Again, Tc17 development required the combination of TGFβ and IL-6 and was dominant to Tc1 development in OT-I cells stimulated with TGFβ+IL-6+IL-12 (Fig 4B). Unlike total CD8 cells, naïve CD8 T cells stimulated with IL-23 developed a small Tc17 population. This has also been seen with CD8+ T cells from human cord blood (Vanden Eijnden et al., 2005), but is somewhat unexpected because naïve CD4 cells cannot develop into Th17 cells with IL-23 stimulation. It is possible that naïve CD8 cells already express the IL-23 receptor or upregulate the receptor faster than CD4 cells do upon TCR stimulation. Th17 cell development also includes an autocrine feedforward step where IL-21 is produced and can replace IL-6 to induce Th17 cells in the presence of TGFβ (Wei et al., 2007). Likewise, TGFβ+IL-21 induce a significant population of Tc17 cells that was dependent on the presence of both cytokines (Fig 4B).

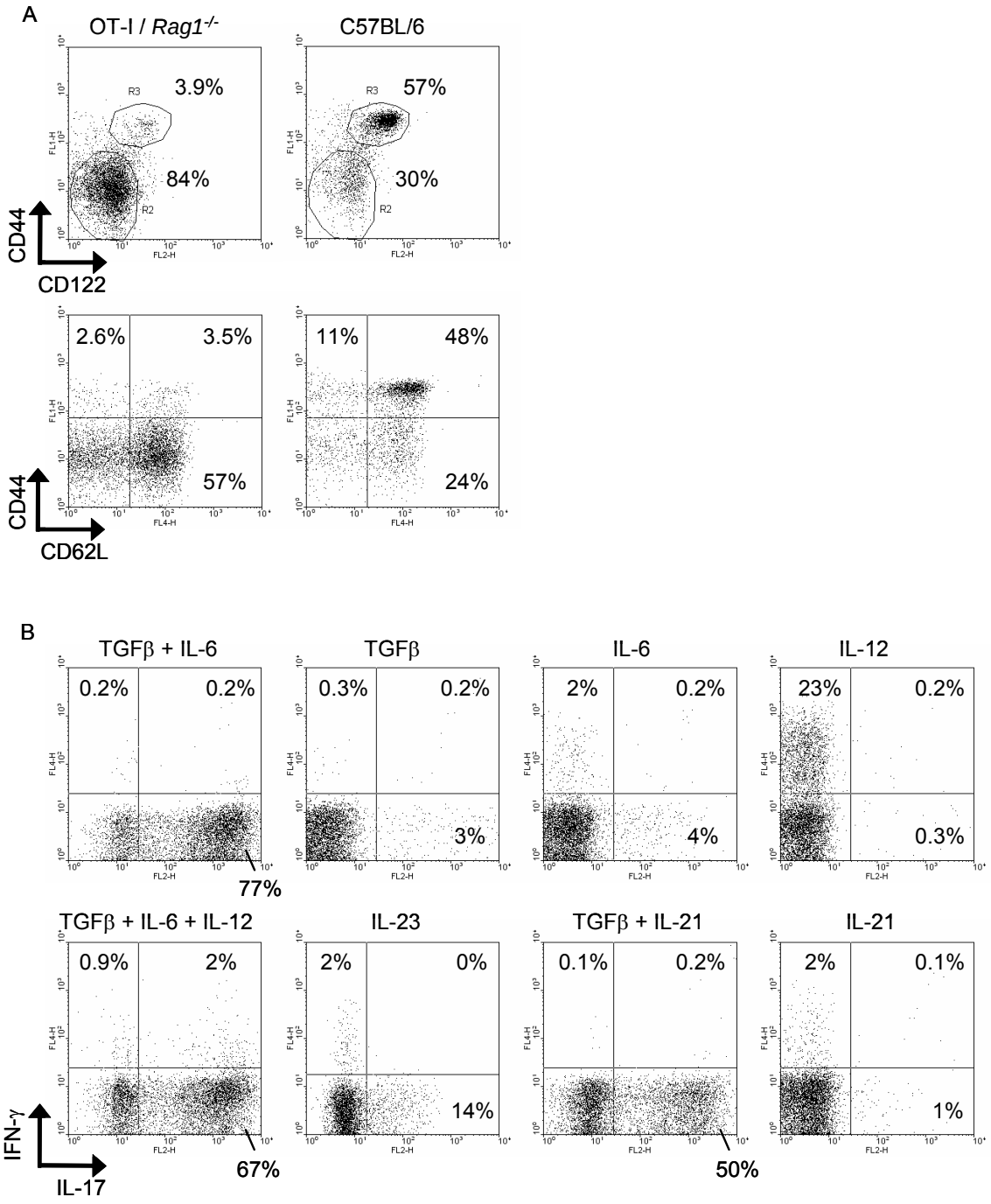


Figure 4. OT-I / *Rag1*$^{-/-}$ CD8 T cells have similar cytokine requirements for Tc17 developoment as total CD8 T cells. (A) Isolated splenic CD8+ T cells from OT-I / *Rag1*$^{-/-}$ and C57BL/6 mice were stained for surface expression of CD44, CD122, and CD62L. (B) OT-I / *Rag1*$^{-/-}$ CD8 T cells were stimulated for 5 days in the presence of IL-12 or α-IFN-γ and the indicated cytokines after which IL-17 and IFN-γ were detected using ICS. Data are representative of at least 2 experiments.

Th17 cells are characterized by their ability to secrete IL-17A (IL-17), but also produce IL-17F, IL-21, and IL-22 to mediate their functions. Th17 cells also upregulate the IL-23 receptor. Likewise, Tc17 cells express elevated levels of IL17F, IL-21, and IL-22 and upregulate IL23R when compared to Tc1 cells (Fig 5A, B).

Th17 cytokines are proinflammatory and promote immune responses. However, if TGFβ is present without IL-6 or other inflammatory cytokines during CD4 T cell priming, a regulatory T cell characterized by Foxp3 expression develops to suppress immune responses. When cultured with TGFβ, CD8 T cells also develop a Foxp3-expressing population which is downregulated in the presence of IL-6 or IL-21 (Fig 5C). These experiments illustrate that compared to Th17 cells, Tc17 cells are induced by the same cytokines and express many of the same characteristic cytokines and surface receptors.

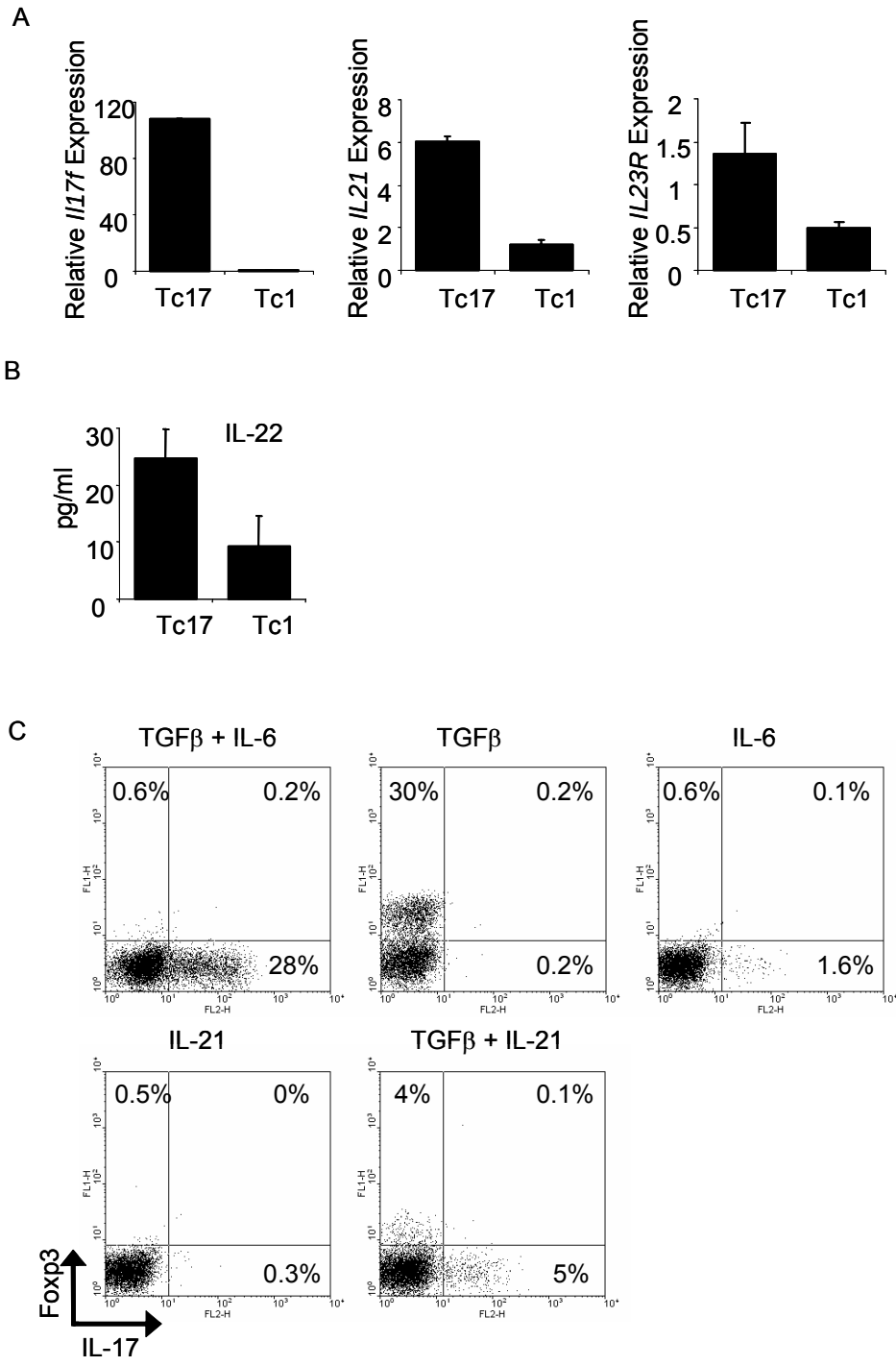

A
Relative Il17f Expression
120
80
40
0
Tc17
Tc1
Relative IL21 Expression
8
6
4
2
0
Tc17
Tc1
Relative IL23R Expression
2
1.5
1
0.5
0
Tc17
Tc1
B
IL-22
pg/ml
30
20
10
0
Tc17
Tc1
C
TGFβ + IL-6
0.6%
0.2%
28%
TGFβ
30%
0.2%
0.2%
IL-6
0.6%
0.1%
1.6%
IL-21
0.5%
0%
0.3%
TGFβ + IL-21
4%
0.1%
5%
FL1-H
FL2-H
Foxp3
IL-17

Figure 5. Tc17 cells express IL-17F, IL-21, and IL-22 but not Foxp3. (A) Total CD8+ T cells were stimulated in Tc17 or Tc1 conditions. After 5 days, cells were restimulated and RNA expression for the indicated genes was measured by real-time PCR. Cycle number is normalized to β2-microglobulin expression and results are represented as fold induction relative to Tc1 samples. (B) 5 day differentiated Tc17 or Tc1 cells were restimulated and cell free supernatants were used to measure IL-22 protein levels by ELISA assay. (C) Total CD8 T cells were stimulated for 5 days in the presence of anti-IFN-γ and the indicated cytokines. Cells were restimulated and IL-17 and Foxp3 were detected by ICS. Data are representative of 2 experiments.

Tc17 cells are noncytotoxic

Compared to other CD8 T cell subsets, Tc17 cells express low levels of IFN-γ. To determine if Tc17 cells also differ functionally from other CD8 T cells in their ability to mediate cell death, we performed standard chromium release assays. OT-I cells differentiated for 5 days were used as effector cells and EG.7 cells (an EL-4 cell line expressing ova) were used as the specific target. Tc17 populations were unable to lyse the target cells even at the highest effector to target ratio while Tc1 cells showed increased killing with the addition of increasing amounts of effector cells (Fig 6A). Tc17 cells also remained noncytotoxic with the addition of ConA to increase their stimulation (Fig 6B). Since Tc17 cells were noncytotoxic, we wanted to determine if they had a regulatory function and could inhibit Tc1 mediated lysis. Increasing amounts of Tc17 cells were added to a ratio of Tc1 cells which lysed ~60% of the target cells. Typically, regulatory T cells can suppress proliferation in vitro when they are in similar numbers as the target cells. However, Tc17 cells were only able to compete and lower Tc1 mediated lysis when the Tc17 cells were at a 10 fold excess to the Tc1 cells (Fig 6C). Therefore, in addition to being noncytotoxic, Tc17 cells do not actively inhibit Tc1 mediated killing.

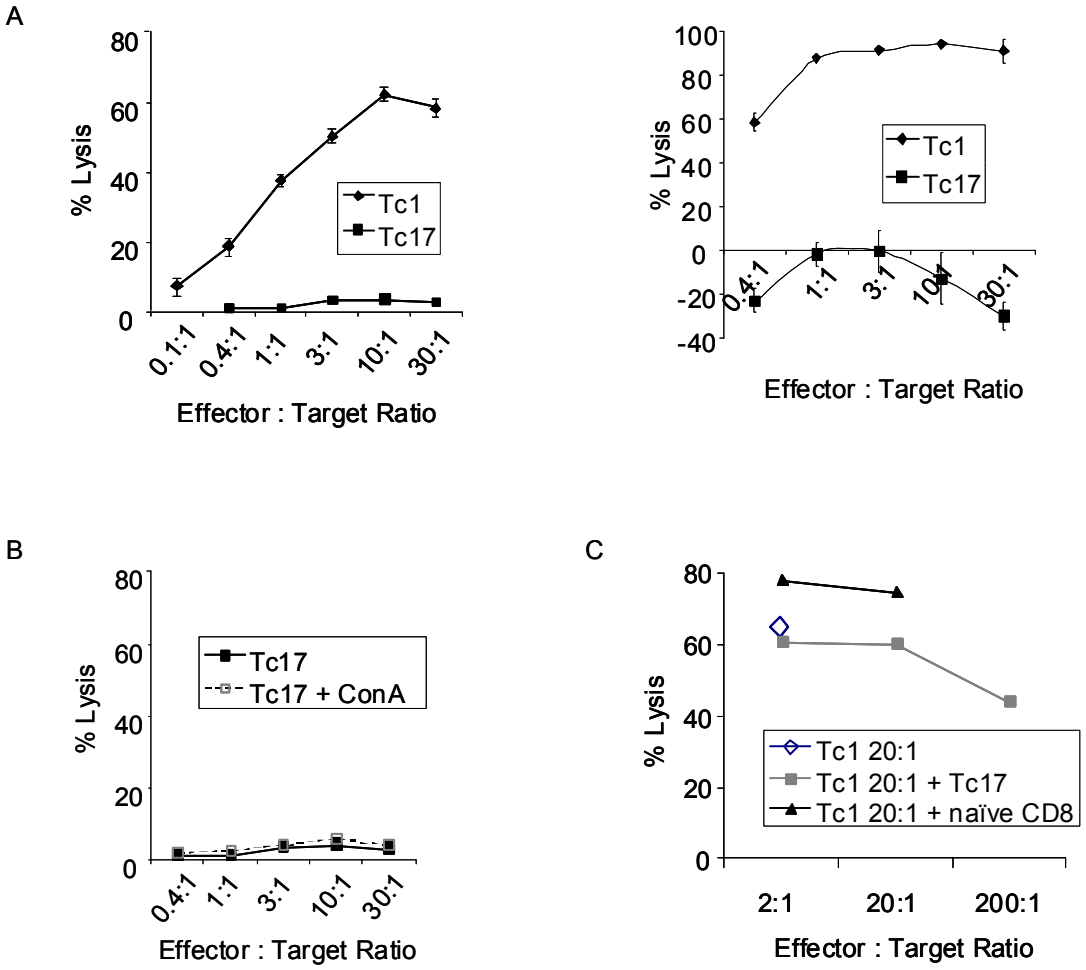
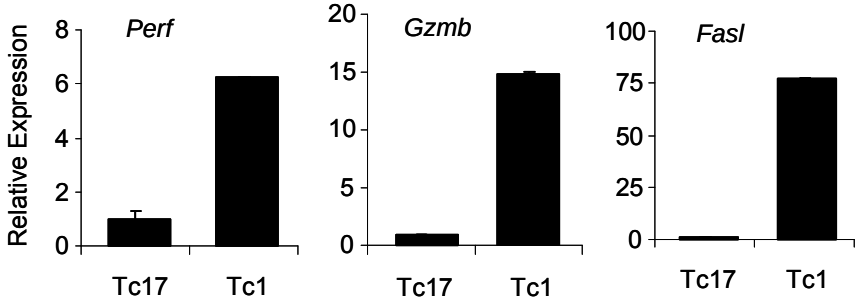


Figure 6. Tc17 cells are non-cytotoxic. (A) OT-I / *Rag1*$^{-/-}$ CD8+ T cells were stimulated in Tc17 or Tc1 conditions to generate effector cells. After 5 days, (left panel) effectors were added at increasing ratios to EG.7 target cells labeled with Cr^{51}. The killing assay was incubated for 6 hrs before measuring Cr^{51} released into the supernatant. (right panel) Nonspecific EL-4 and specific EG.7 target cells were labelled with two different concentrations of CFSE and mixed in a 1 to 1 ratio. Effector cells were added at increasing ratios to the target cells and incubated for 4 hrs. 7-AAD was added into the CTL reaction to identify dead cells before analysis by flow cytometry. Ag specific cytotoxicity was calculated by the formula: % lysis = 100 x [1 – $(E.G7/EL4)_{experimental}$ / $(E.G7/EL4)_{control}$]. (B) Cr^{51} release assays were performed with increasing ratios of Tc17 effectors incubated plus (□) or minus (■) 2.5 μg/ml ConA included in the assay. (C) Cr^{51} release assays were performed with Tc1 cells at a 20 to 1 effector to target cell ratio. Increasing numbers of Tc17 (■) or naïve (▲) CD8 T cells were included with the Tc1 cells before measuring Cr^{51} release. Data are representative of at least 2 experiments.

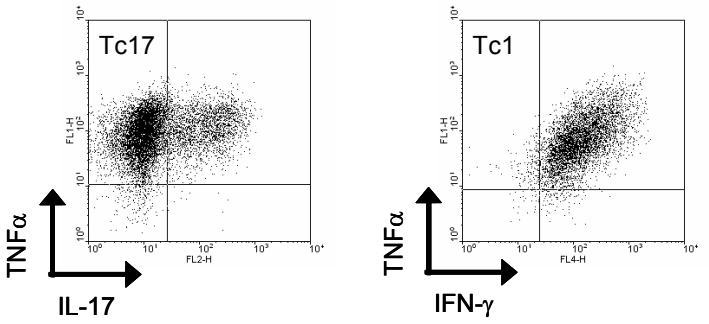
Cytotoxic CD8 T cells induce apoptosis in target cells by secreting effector molecules, such as perforin and granzyme B, and through Fas-FasL interactions. To determine if the noncytotoxic phenotype seen in Tc17 populations was associated with low expression of these effector molecules, we differentiated CD8 T cells in Tc17 and Tc1 conditions for 5 days. Compared to Tc1 cells, Tc17 cells expressed low levels of *Perf*, *Gzmb*, and *Fasl* consistent with their noncytotoxic function (Fig 7A). TNFα has also been reported to contribute to cytotoxicity in some instances (Qin et al., 2004; Zhang et al., 2008; Chavez-Galan et al., 2009) and is expressed at high levels in both Tc17 and Tc1 cells (Fig 7B). However, the previous chromium release assay illustrated that high TNFα expression from Tc17 cells was insufficient to induce cell mediated cytotoxicity.

To determine if low expression of cytotoxic molecules was specific to cells cultured in Tc17 inducing conditions or a result of stimulation from TGFβ alone, we measured granzyme B expression in cell populations cultured with various cytokines for 5 days. Low percentages of granzyme B expressing cells were selectively seen in IL-17 promoting conditions, while CD8 T cells cultured with individual cytokines alone had high percentages of granzyme B expressing cells (Fig 7C). This suggested that Tc17 inducing conditions downregulated granzyme B expression. We were unable to detect perforin and Fas Ligand by intracellular or cell surface staining, therefore, granzyme B was used as a representative cytotoxic molecule in future experiments.

A


Relative Expression
Perf
Gzmb
Fasl
Tc17
Tc1
Tc17
Tc1
Tc17
Tc1

B


Tc17
TNFα
IL-17
Tc1
TNFα
IFN-γ

C

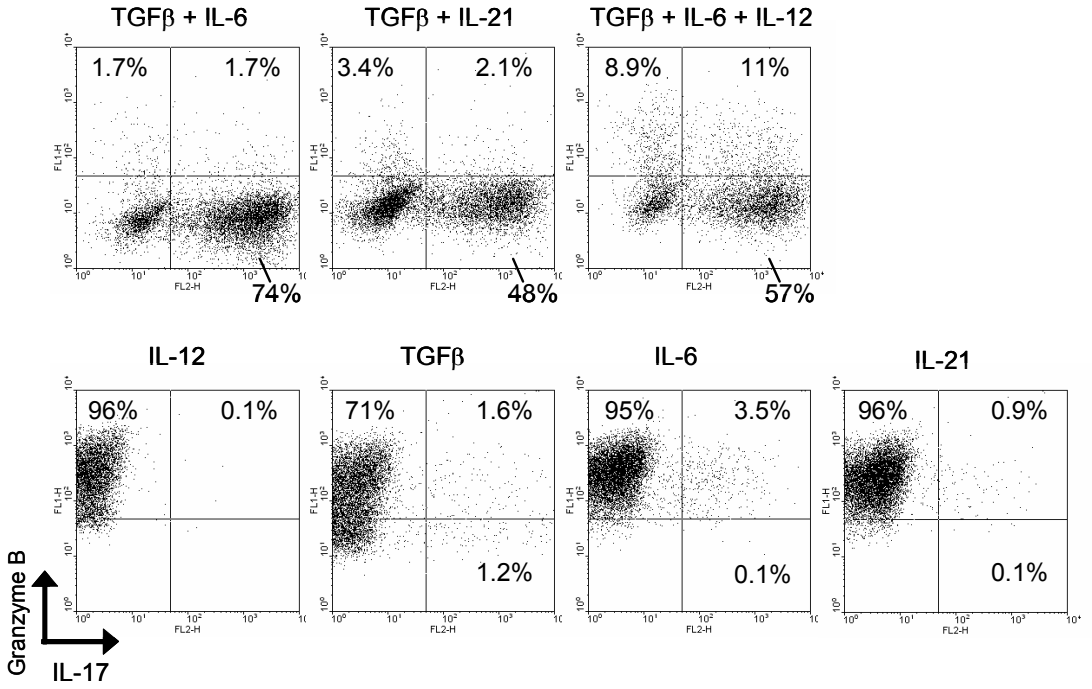

TGFβ + IL-6
TGFβ + IL-21
TGFβ + IL-6 + IL-12
1.7%
1.7%
74%
3.4%
2.1%
48%
8.9%
11%
57%
IL-12
TGFβ
IL-6
IL-21
96%
0.1%
71%
1.6%
1.2%
95%
3.5%
0.1%
96%
0.9%
0.1%
Granzyme B
IL-17

Figure 7. Tc17 cells have low expression of Perf, Gzmb, and Fasl. (A) Total CD8+ T cells were stimulated in Tc17 or Tc1 conditions. After 5 days, cells were restimulated and RNA expression for the indicated genes was measured using real-time PCR. Cycle number is normalized to β2-microglobulin expression and results are represented as fold induction relative to Tc17 samples. (B) 5 day differentiated Tc17 and Tc1 cells were restimulated and TNFα, IL-17, and IFN-γ were detected using ICS. (C) Total CD8 T cells were stimulated for 5 days in the presence of IL-12 or anti-IFN-γ and the indicated cytokines. Cells were restimulated and Granzyme B and IL-17 were detected using ICS. Data are representative of 2 experiments.

Expression of Th17 and Tc1 transcription factors in Tc17 cells

RORγt is both necessary and sufficient for Th17 development. Meanwhile T-bet and Eomes are each critical for optimal expression of IFN-γ and cytotoxic molecules in CD8 T cells. To begin to understand the molecular mechanisms regulating Tc17 cells, we analyzed transcription factor expression in cells activated for 5 days in the presence of Tc17 and Tc1 skewing cytokines alone or in various combinations. *Rorc* was selectively induced in cells cultured under Tc17 conditions and was undetectable or had low expression in the presence of individual cytokines (Fig 8). Induction of *Tbx21* was highest in Tc1 cells, while TGFβ+IL6- and TGFβ+IL21-induced Tc17 cells had selectively low *Tbx21* expression with ~2 fold less *Tbx21* expression than cells stimulated with TGFβ alone. IL-12 added to TGFβ+IL6 conditions was only able to partially recover *Tbx21* expression compared to IL-12 alone. Conditions inducing IL-17 expression also had selectively low levels of *Eomes* compared to incubation with single cytokines alone. *Runx3* is another protein shown to be critical for Tc1 development; however there was no significant difference in *Runx3* expression between Tc17 cells and Tc1 cells (Fig 8). Therefore, induction of IL-17 and repression of IFN-γ and granzyme B in Tc17 cells correlates with high *Rorc* expression and low *Tbx21* and *Eomes* mRNA levels.

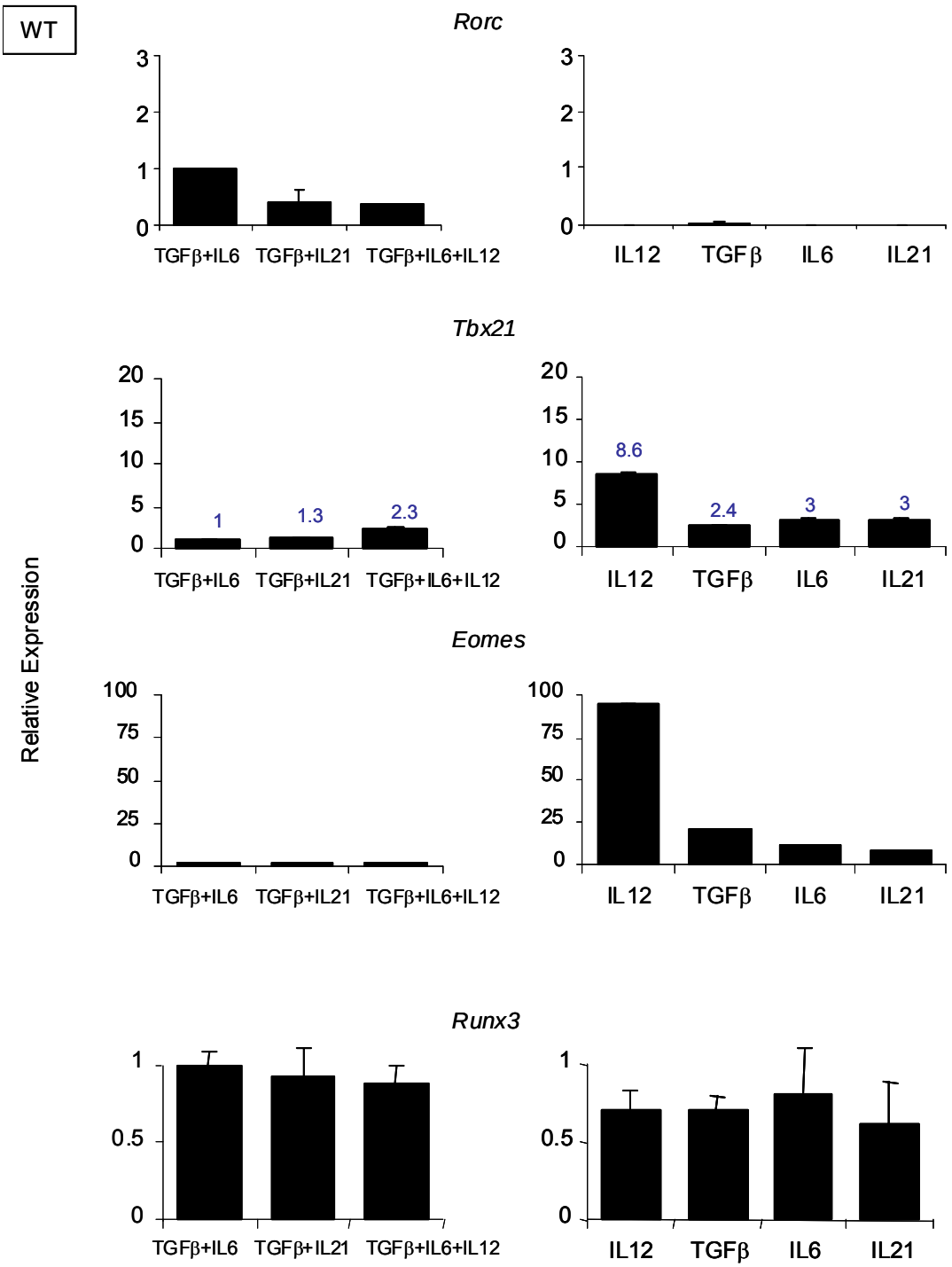


Figure 8. Tc17 cells express *Rorc* and inhibit *Tbx21* and *Eomes*. Total CD8+ T cells were stimulated in the presence of IL-12 or anti-IFN-γ and the indicated cytokines. After 5 days, RNA expression for the indicated genes was measured by real-time PCR. Cycle number is normalized to β_2-microglobulin expression. Results are represented as fold induction relative to TGFβ+IL-6 cultured samples and presented as mean ± SD of duplicate sample. Data are representative of 2 experiments.

CD8 T cells secrete IFN-γ upon stimulation through the TCR. Even though IFN-γ was neutralized in the previous figure, we also utilized IFN-γ deficient CD8 T cells in experiments side-by-side with those in Fig 8 to eliminate the possibility of autocrine IFN-γ effects on the regulation of these transcription factors. Similar to the results in the previous figure, conditions which induce IL-17 selectively upregulate *Rorc* and downregulate *Tbx21* and *Eomes* (Fig 9).

IFN-γ was previously shown to inhibit Tc17 development. Consistent with this, *Ifng*$^{-/-}$ Tc17 cells have higher expression of *Rorc* (top panel of Fig 9 vs top panel of Fig 8).

In CD4 T cells, T-bet can be induced through IFN-γ → Stat1 signals. Tc17 cells secrete lower levels of IFN-γ than cells stimulated with TGFβ or IL-6 alone. Low *Tbx21* expression in Tc17 cells is not an indirect result of lower IFN-γ expression because Tc17 cells lacking IFN-γ still express lower levels of *Tbx21* than *Ifng*$^{-/-}$ cells stimulated with TGFβ or IL-6 alone.

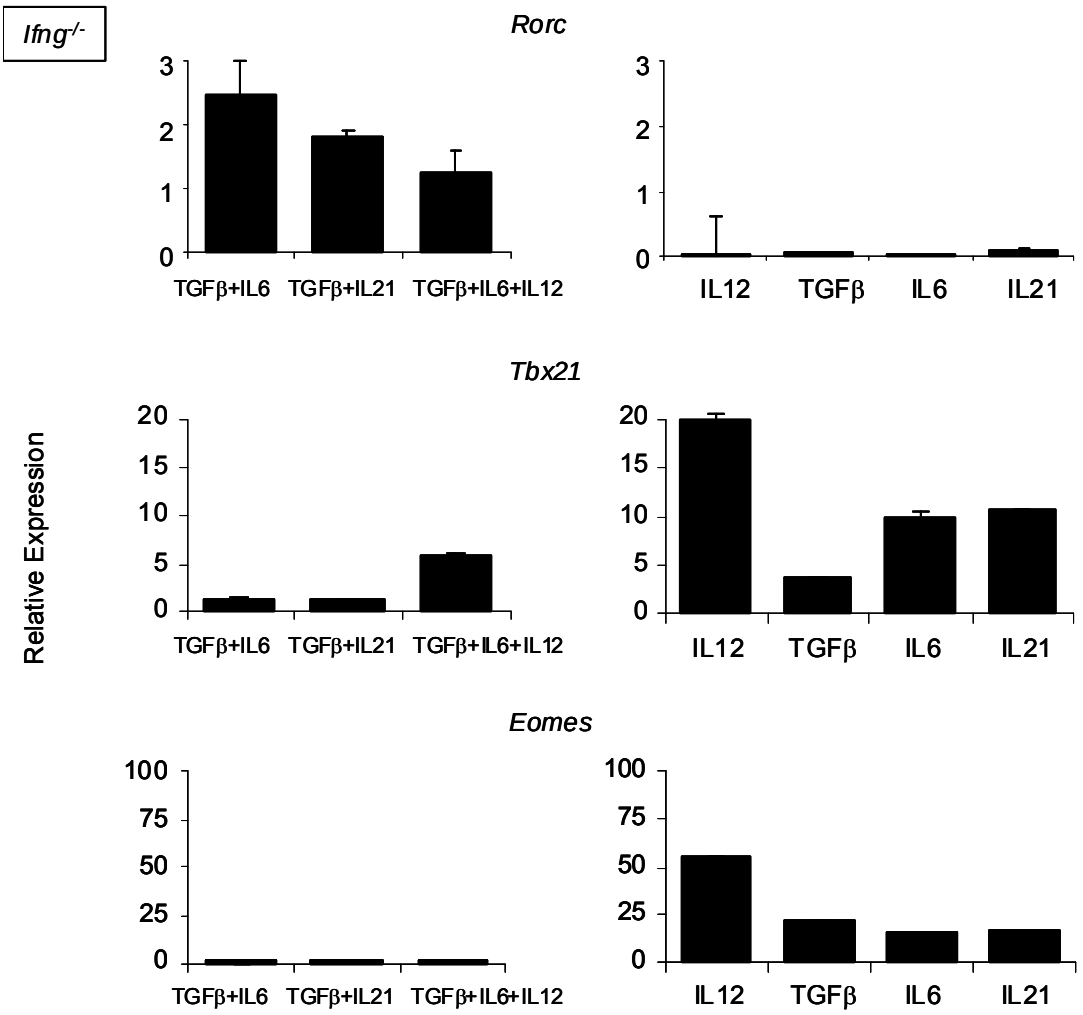
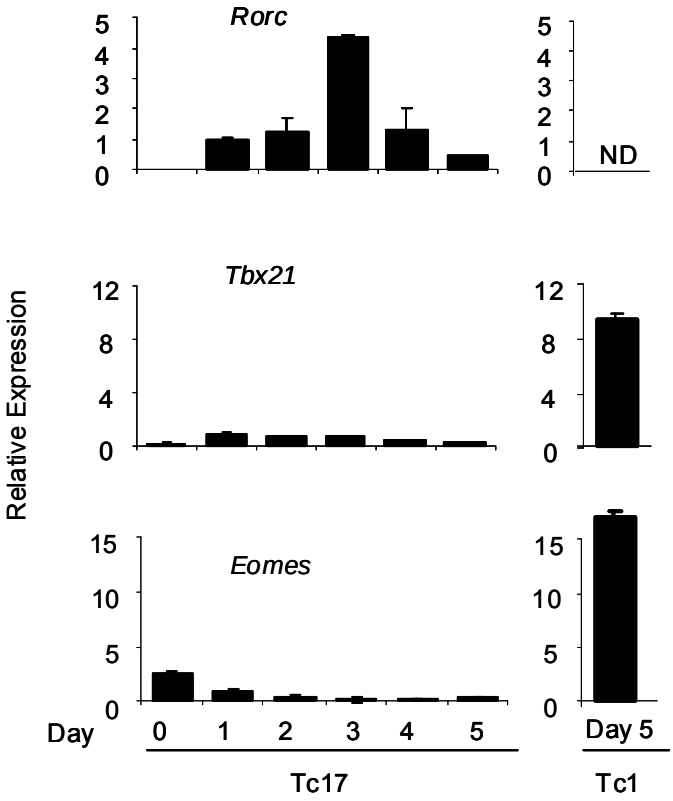


Figure 9. IFN-γ inhibits *Rorc* and is not necessary for *Tbx21* or *Eomes* expression. *Ifng*$^{-/-}$ CD8+ T cells were stimulated in the presence of IL-12 or anti-IFN-γ and the indicated cytokines. After 5 days, RNA expression for the indicated genes was measured by real-time PCR. Cycle number is normalized to β_2-microglobulin expression. Results are represented as fold induction relative to WT TGFβ+IL-6 cultured samples from Fig 8 and presented as mean ± SD of duplicate samples. Data are representative of 2 experiments.

To better understand transcriptional regulation of Tc17 cells, the temporal regulation of *Rorc*, *Tbx21*, and *Eomes* were examined in developing Tc17 cells. The expression of *Rorc* in Tc17 cells peaked at Day 3 of culture before decreasing (Fig 10A, B), although IL-17 expression was stable from Day 3 to Day 6 of development (Fig 3B). T-bet expression in Tc17 cells decreased during the 5 day culture to levels significantly below Tc1 cells after an approximately 4 fold induction of T-bet on Day 1 (Fig 10A). Previous reports have shown that Eomes is induced upon TCR stimulation and inhibited by IL-12. Consistent with this, the highest amount of Eomes protein was detected in neutralizing conditions, with Tc17 populations inhibiting Eomes even more than Tc1 populations (Fig 10B). Tc17 cells therefore transiently upregulate a Th17 master regulator and downregulate Tc1 transcription factors during their transition into IL-17 secreting T cells.

A


Rorc
Tbx21
Eomes
ND
Relative Expression
Day
0 1 2 3 4 5
Day 5
Tc17
Tc1

B

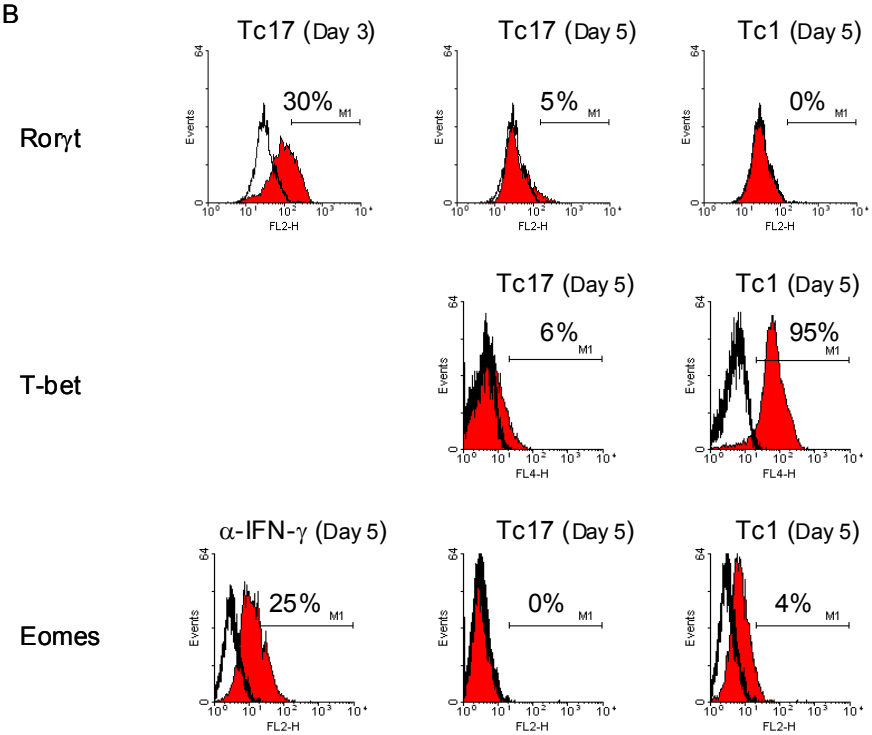

Rorγt
T-bet
Eomes
Tc17 (Day 3)
Tc17 (Day 5)
Tc1 (Day 5)
α-IFN-γ (Day 5)
30%
5%
0%
6%
95%
25%
4%
Events
FL2-H
FL4-H

Figure 10. Temporal regulation of *Tbx21*, *Eomes*, and *Rorc* in Tc17 cells. (A) CD8+ T cells were stimulated in Tc17 or Tc1 conditions and separated from the APCs in culture using histopaque on the indicated days. RNA was isolated and gene expression was analyzed by qPCR and normalized to β2-microglobulin expression. Data represent fold induction relative to Day 1 Tc17 cells and presented as mean ± SD of duplicate samples. (B) Protein expression in Tc17 and Tc1 populations was measured using ICS. Histograms are displayed as the indicated sample (red histogram) with the percent positive based on an overlaid control (dark black line). Tc1 cells (Day 5) were overlaid for a Rorγt negative control, *Tbx21*$^{-/-}$ Tc17 cells (Day 5) were overlaid for a T-bet negative control, and unstained cells (Day 5) were overlaid for an Eomes negative control. Data are representative of at least 2 experiments.

Role of Stat Proteins and T-bet in CD8 T cell subset development

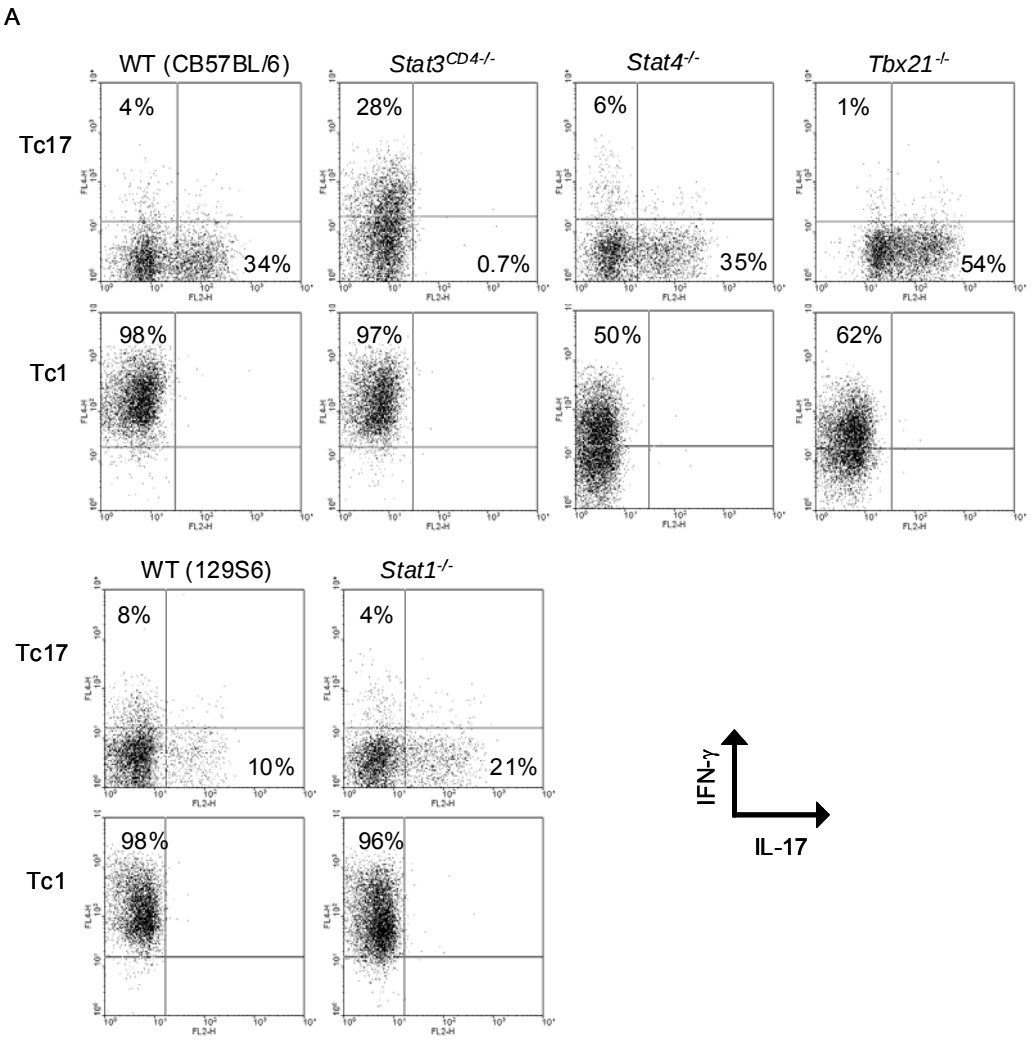
In CD4 T cells, Stat proteins are well established as upstream to master regulators of lineage development. IL-6 → Stat3 → RORγt signals are critical for Th17 development, while IFN-γ → Stat1 → T-bet and IL-12 → Stat4 signals are important for Th1 development. The Th1 factors Stat1 and T-bet also inhibit Th17 formation. Meanwhile, the role of Stat proteins in CD8 T cells is not as clear, although in most instances, it appears that IL-12 → Stat4 signals are necessary for optimal IFN-γ production while IFN-γ → Stat1 signals are dispensable.

To determine the regulation of Tc17 cells by Stat proteins and important CD4 lineage transcription factors, we utilized various Stat deficient and also T-bet deficient CD8 T cells and analyzed their ability to produce IL-17 in 5 day in vitro cultures activated in Tc17 skewing conditions.

In the absence of Stat3 almost no Tc17 cells developed and instead, there was a larger percentage of IFN-γ secreting cells. Meanwhile, Stat4 was not required and did not inhibit Tc17 development. In the absence of either T-bet or Stat1, an increased percentage of Tc17 cells was observed (Fig 11A). The requirement of Stat3 in Tc17 development and Tc17 inhibition by T-bet and Stat1 was also observed when protein levels were analyzed using ELISA assays (Fig 11B).

5 day differentiated Tc1 cells secrete high levels of IFN-γ and were used to better understand Stat protein regulation of IFN-γ production in our system. In the absence of T-bet, Tc1 development was impaired and the percentage of IFN-γ secreting cells was decreased even more in the absence of Stat4. Tc1 cells developed normally in the absence of Stat1 (Fig 11A, B).

These data suggest that Stat protein and T-bet regulation of Tc17 cells is similar to their regulation of Th17 cells. Meanwhile, in our system, optimal Tc1 development requires Stat4 and T-bet.


A
WT (CB57BL/6)
Stat3CD4-/-
Stat4-/-
Tbx21-/-
Tc17
Tc1
4%
34%
28%
0.7%
6%
35%
1%
54%
98%
97%
50%
62%
WT (129S6)
Stat1-/-
8%
10%
4%
21%
98%
96%
IFN-γ
IL-17

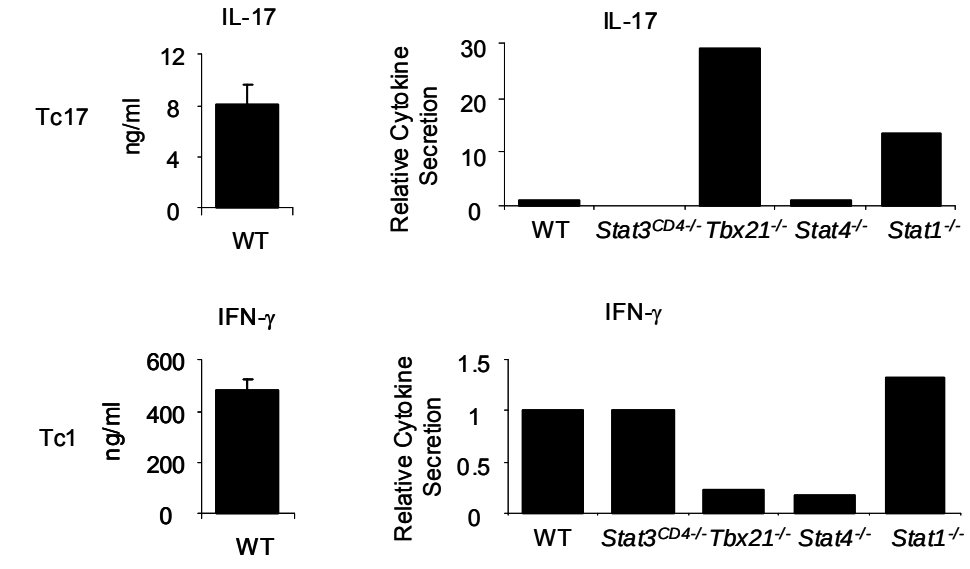

B
IL-17
Tc17
ng/ml
12
8
4
0
WT
IL-17
Relative Cytokine Secretion
30
20
10
0
WT
Stat3CD4-/-
Tbx21-/-
Stat4-/-
Stat1-/-
IFN-γ
Tc1
ng/ml
600
400
200
0
WT
IFN-γ
Relative Cytokine Secretion
1.5
1
0.5
0
WT
Stat3CD4-/-
Tbx21-/-
Stat4-/-
Stat1-/-

Figure 11. Stat protein regulation of Tc17 and Tc1 development. (A) Total CD8+ T cells from *Stat3*$^{CD4-/-}$, *Stat4*$^{-/-}$, *Tbx21*$^{-/-}$, *Stat1*$^{-/-}$ and strain matched wild type mice were stimulated in Tc17 or Tc1 conditions for 5 days. IL-17 and IFN-γ were detected by ICS. (B) Five day differentiated Tc17 or Tc1 cells were restimulated and cell free supernatants were used to measure IL-17 and IFN-γ protein levels by ELISA assay. Typical cytokine amounts are shown on the left side of the panel and ELISA fold induction relative to wild type samples are shown on the right. Data are representative of at least 2 experiments.

Role of Stat3 and RORγt in Tc17 development

To further analyze positive and negative regulation of the Tc17 program, we determined the effect of Stat protein deficiency on the production of additional Tc17 cytokines, surface receptors, and transcription factors.

In CD4 T cells, Stat3 is required not only for IL-17 expression, but also for additional proteins associated with the Th17 phenotype. To determine if Stat3 is required for multiple aspects of the Tc17 phenotype, expression of *Rorc*, *Il23r*, and *Il21* was determined by real-time PCR and found to be greatly reduced in *Stat3*$^{CD4-/-}$ Tc17 cells (Fig 12A). In addition, *Stat3*$^{CD4-/-}$ Tc17 cells expressed higher levels of the Tc1 transcription factors T-bet and Eomes (Fig 12B), consistent with the partial Tc1 phenotype seen in Fig 11A. *Perf*, *Gzmb*, and *Fasl* expression were also increased ~2, 8, and 7 fold respectively in Stat3 deficient Tc17 cells (Fig 12C). Therefore, in Tc17 cells, Stat3 is critical for multiple aspects of the IL-17 program and at the same time inhibits Tc1 transcription factors and cytotoxic gene expression.

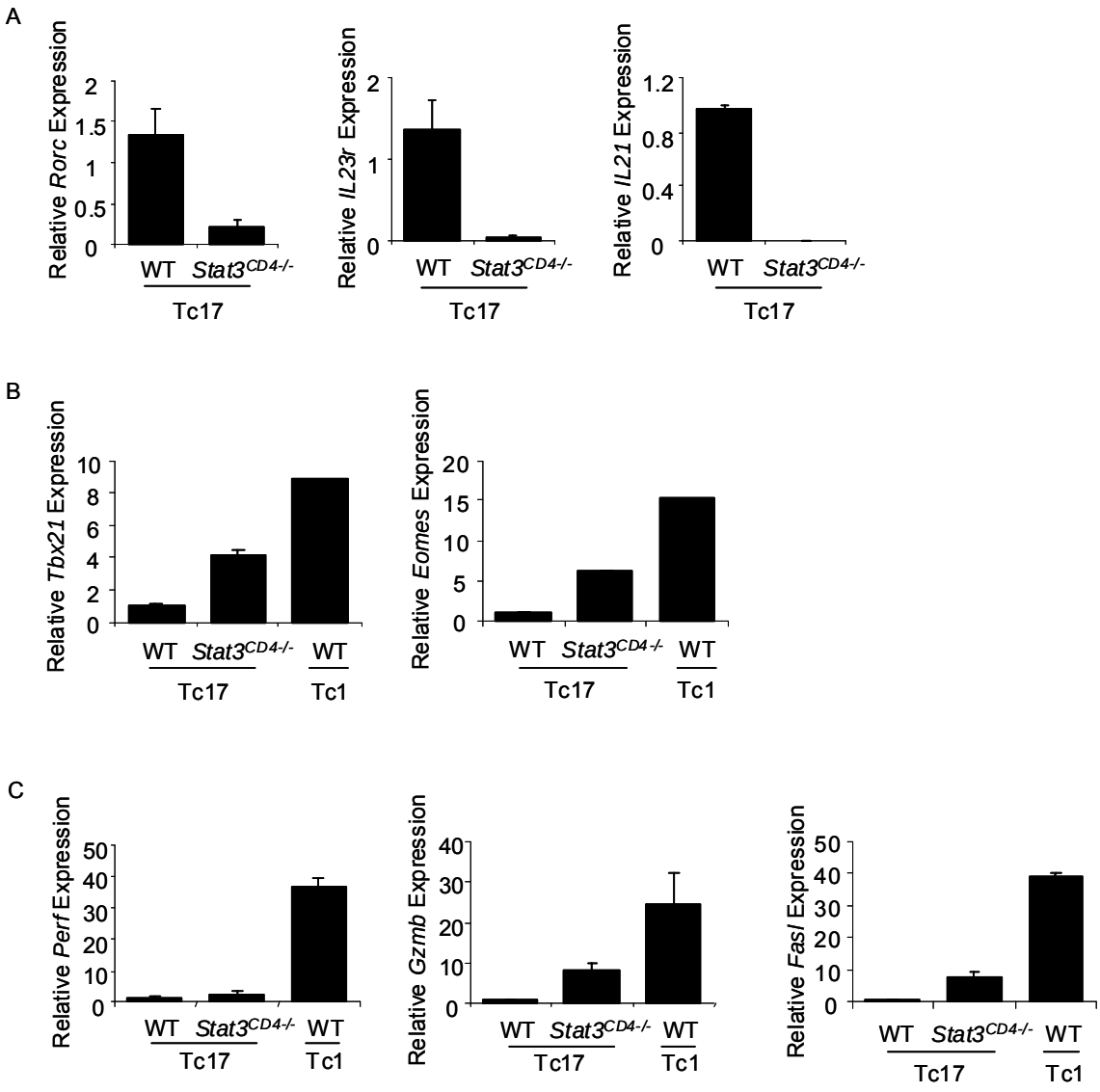


Figure 12. Stat3 is required for the Tc17 program and inhibits Tc1 genes. (A,B,C) WT or *Stat3*$^{CD4-/-}$ CD8+ T cells were stimulated in Tc17 or Tc1 conditions. After 5 days, RNA expression for the indicated genes was measured by real-time PCR. Expression of transcription factors were analyzed from unstimulated samples and expression of cytokines, cytokine receptors, and cytotoxic molecules were analyzed from stimulated samples. Cycle number is normalized to β_2-microglobulin expression and results are represented as fold induction relative to WT Tc17 samples. Data are representative of 2 experiments.

Since Stat3 is necessary for the development of IL-17-secreting CD8 T cells, we wanted to assess the ability of Stat3 to promote IL-17 development in a condition which does not normally promote Tc17 development. To test this, WT CD8 T cells differentiated in Tc1 conditions were transduced with a bicistronic retrovirus containing a H-2K^k selectable marker and either no insert or expressing a constitutively active Stat3 (Stat3C). After 5 days, H-2K^k positive cells were purified by FACS sorting and cytokine expression was analyzed using ELISA and ICS. Expression of Stat3C in Tc1 cells increased IL-17 production and inhibited IFN-γ secretion (Fig 13 A, B) suggesting that Stat3 can promote the development of IL-17 secretion even in Tc1 conditions.

The increase of granzyme B in Stat3 deficient Tc17 cells (Fig 12C) suggested the possibility that Stat3 could inhibit granzyme B. To determine if Stat3 overexpression could reduce granzyme B levels, differentiating Tc1 cells were transduced with a bicistronic retrovirus containing a H-2K^k selectable marker and either no insert or Stat3C. After 5 days, Stat3C transduced Tc1 cells maintained high levels of granzyme B (Fig 13C). Thus, Stat3 may inhibit granzyme B production in Tc17 cells, but does not appear to do so in Tc1 cells.

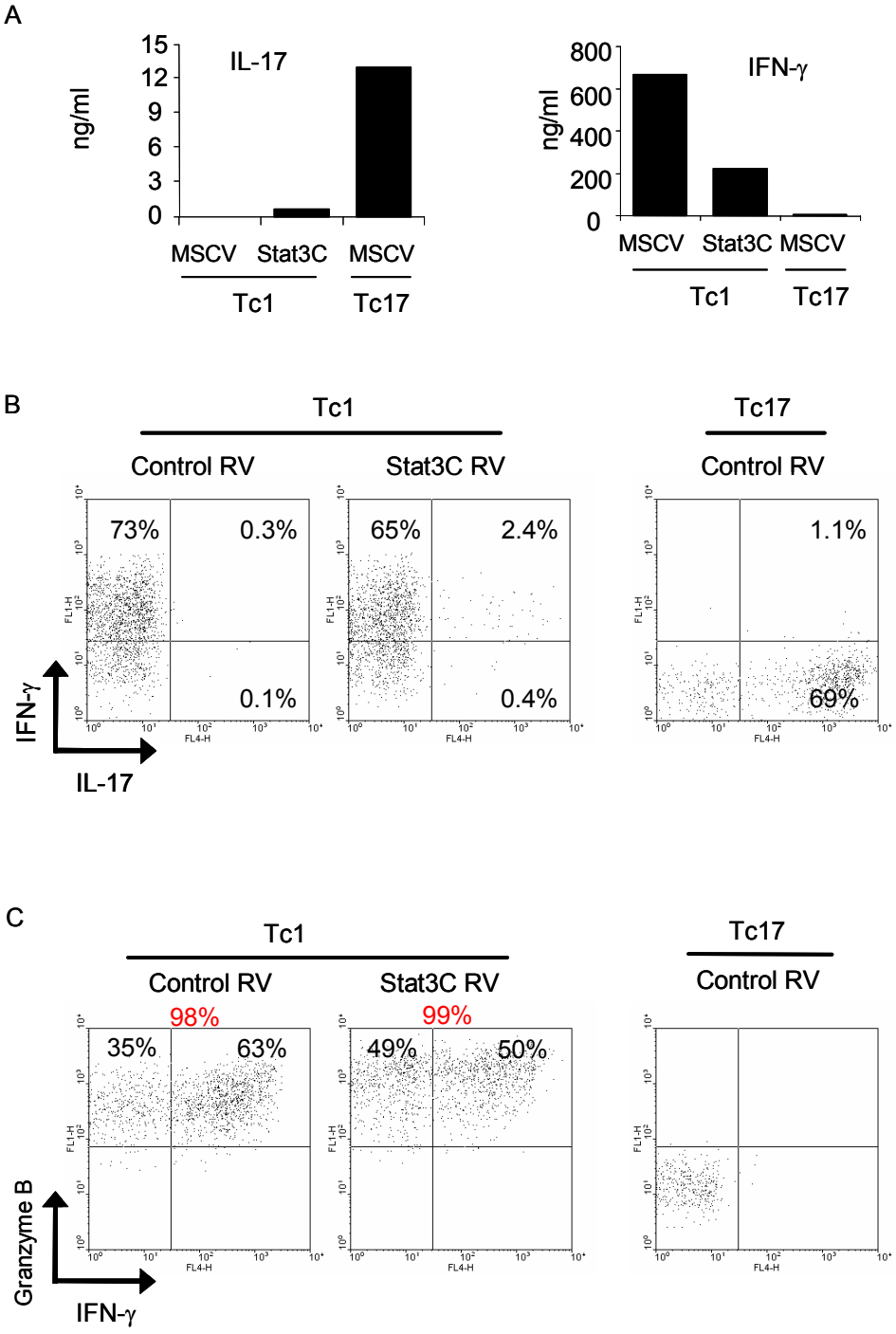

A
IL-17
ng/ml
15
12
9
6
3
0
MSCV
Stat3C
MSCV
Tc1
Tc17
IFN-γ
ng/ml
800
600
400
200
0
MSCV
Stat3C
MSCV
Tc1
Tc17
B
Tc1
Tc17
Control RV
Stat3C RV
Control RV
73%
0.3%
0.1%
65%
2.4%
0.4%
1.1%
69%
IFN-γ
IL-17
C
Tc1
Tc17
Control RV
Stat3C RV
Control RV
98%
99%
35%
63%
49%
50%
Granzyme B
IFN-γ

Figure 13. Stat3C increases IL-17 production and inhibits IFN-γ but not granzyme B. CD8+ T cells were stimulated in Tc1 or Tc17 conditions. After 1 day of culture, cells were transduced with an MSCV control or Stat3C-expressing retrovirus as indicated. Cells were analyzed after an additional 4 days of culture. (A) H-2K^k positive cells were purified by FACs sorting and restimulated with anti-CD3 for 24 hrs. Cell free supernatants were used to measure IL-17 and IFN-γ protein levels by ELISA. (B, C) IFN-γ, Granzyme B and IL-17 were detected using ICS. Plots are gated on live H-2K^k+ cells. Data are representative of 2 experiments.

Stat3 induces several proteins including RORγt. Therefore, we wanted to assess the ability of RORγt alone to promote IL-17 development. To test this, WT CD8 T cells differentiated in Tc1 conditions were transduced with a bicistronic retrovirus containing a GFP selectable marker and either no insert or an insert coding for RORγt. After 5 days, GFP positive cells were purified by FACS sorting and restimulated for ELISA analysis or analyzed using ICS and gated on the GFP^+ population. Expression of RORγt in Tc1 cells increased IL-17 production to almost half the amount seen in Tc17 cells using ELISA, although this increase was less dramatic when analyzed by ICS (Fig 14 A, B). Unexpectedly, RORγt also increased IFN-γ measured by ELISA although not by ICS (Fig 14 A, B) suggesting that RORγt can promote IL-17 and IFN-γ secretion.

While Stat3 overexpression did not inhibit granzyme B production in Tc1 cells, we also wanted to determine if RORγt overexpression alone could affect granzyme B production. Differentiating Tc1 cells were transduced with RORγt, and after 5 days, RORγt transduced cells were found to have approximately 20% less Granzyme B as control transduced cells (Fig 14C). Thus, even though Stat3C does not appear to inhibit granzyme B in Tc1 cells, RORγt overexpression shows slight granzyme B reduction.

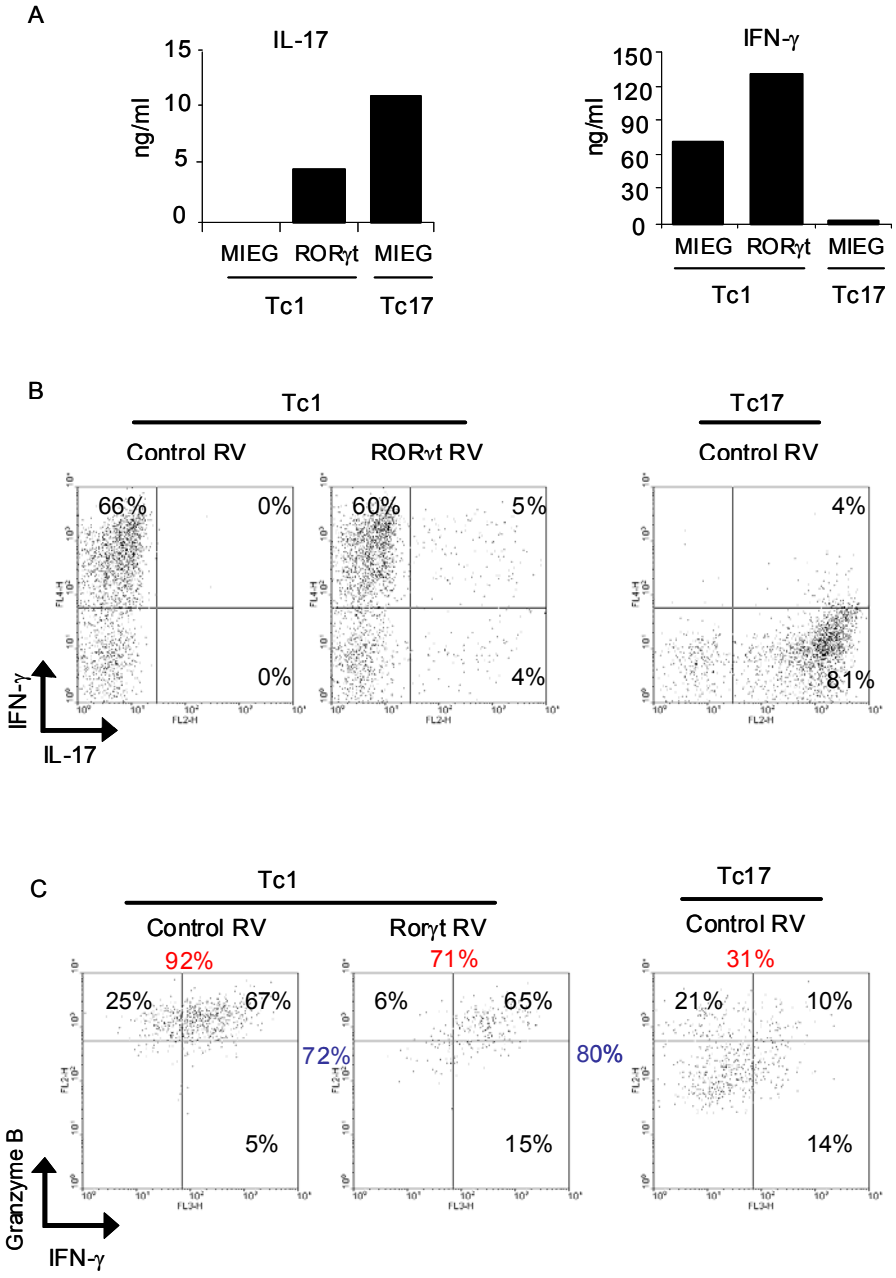


Figure 14. RORγt increases IL-17 and IFN-γ production and inhibits granzyme B. CD8+ T cells were stimulated in Tc1 or Tc17 conditions. After 1 day of culture, cells were transduced with MIEG control or RORγt-expressing retroviruses. Cells were analyzed after an additional 4 days of culture. (A) GFP positive cells were purified by FACs sorting and restimulated with anti-CD3 for 24 hrs. Cell free supernatants were used to measure IL-17 and IFN-γ protein levels by ELISA. (B, C) IFN-γ, Granzyme B and IL-17 were detected using ICS. Plots are gated on live GFP+ cells. Data are representative of 2 experiments.

To confirm the granzyme B reduction seen upon RORγt transduction, we also analyzed *Gzmb* RNA. Tc1 cells overexpressing RORγt contained an approximately 2 fold reduction in *Gzmb* RNA compared to control transduced cells, although inhibition of cytotoxic genes seemed to be *Gzmb* specific as *Perf* and *Fasl* were not reduced with RORγt overexpression (Fig 15A).

To determine if increased IFN-γ in RORγt transduced cells could be due to changes in Tc1 transcription factors, *Tbx21* and *Eomes* expression was also analyzed. *Tbx21* is increased 2-3 fold higher in transduced cells while there was no significant change in *Eomes* expression (Fig 15B). Therefore, the increased IFN-γ seen with RORγt overexpression correlates with increased *Tbx21* expression.

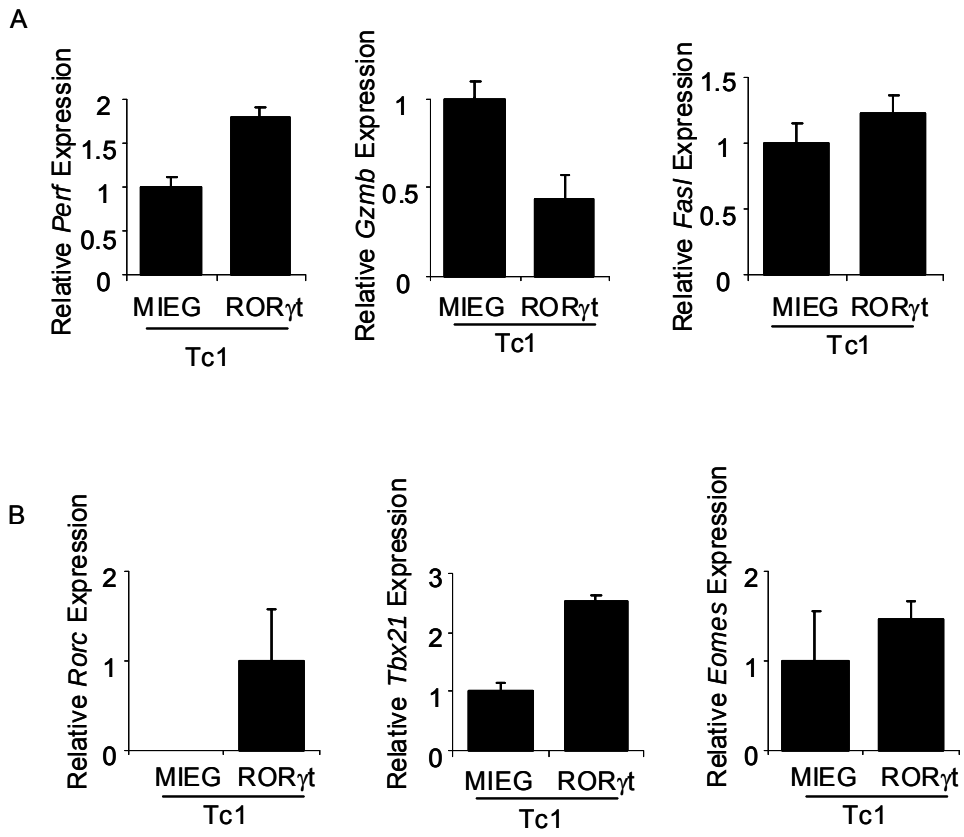


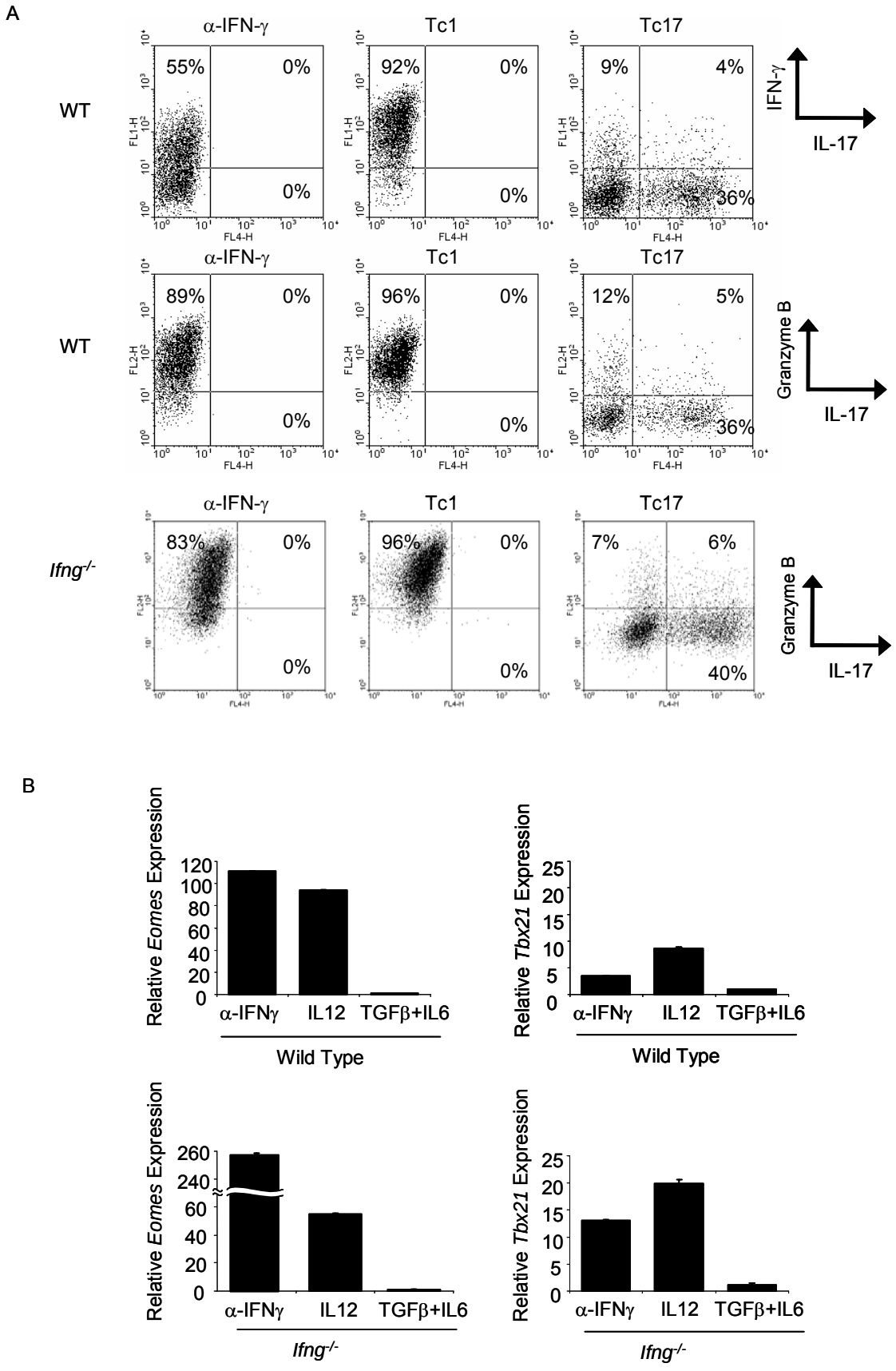
Figure 15. Effects of RORγt overexpression on cytotoxicity genes and Tc1 transcription factors. (A, B) Total WT CD8+ T cells were stimulated in Tc1 conditions. After 1 day of culture, cells were transduced with either a MIEG control or RORγt-expressing retrovirus. Cells were analyzed after an additional 4 days of culture. RNA expression for the indicated genes was measured by real-time PCR. Expression of cytotoxic molecules was analyzed from stimulated samples and expression of transcription factors were analyzed from unstimulated samples. Cycle number is normalized to β2-microglobulin expression and results for all samples except *Rorc* are represented as fold induction relative to MIEG transduced Tc1 samples. *Rorc* expression is represented as fold induction relative to RORγt transduced Tc1 cells. Data are representative of 2 experiments.

Previously, we found that Tc17 cells produced much less granzyme B than CD8 T cells stimulated in the presence of individual cytokines alone (Fig 7C). Since RORγt was able to slightly reduce granzyme B in Tc1 polarizing conditions, we wanted to assess the ability of RORγt to affect cytokine production in CD8 T cells in the absence of differentiating signals.

First, to determine the cytokine production of cells stimulated in the absence of exogenous cytokines, we activated CD8 cells for 5 days in the presence of IFN-γ neutralizing antibodies and compared to CD8 cells stimulated in Tc1 and Tc17 inducing conditions. After 5 days of culture in the presence of IFN-γ neutralizing antibodies, 55% of CD8 T cells were positive for IFN-γ. The percentage of IFN-γ producing cells was increased to 92% in Tc1 conditions and reduced to 13% in Tc17 conditions (Fig 16A, top panel). Almost all cells (89%) were positive for granzyme B upon activation alone and similar to IFN-γ expression, granzyme B expressing cells were decreased in Tc17 conditions (Fig 16A, middle panel). To ensure that IFN-γ was being neutralized and did not have an autocrine effect to induce granzyme B expression, *Ifng*$^{-/-}$ CD8 T cells were stimulated in the absence of exogenous cytokines and also found to develop a high percentage of granzyme B expressing cells(Fig 16A, bottom panel).

Next we analyzed expression of Tc1 transcription factors. Eomes expression was highest in neutralizing conditions during a 5 day culture and was inhibited with the addition of IL-12 as previously reported (Takemoto et al., 2006), while

Tc17 conditions further reduced Eomes. T-bet expression was induced in the presence of IL-12 and inhibited in IL-17 promoting conditions (Fig 16B). Previously, we observed that IFN-γ did not induce Eomes or T-bet in multiple conditions (Figs 8 and 9). Similarly, IFN-γ was not necessary for Eomes or T-bet expression in CD8 T cells stimulated in the absence of differentiating signals (Fig 16B). Therefore CD8 T cells stimulated through the TCR alone for 5 days induce Eomes and express T-bet to produce IFN-γ and high levels of granzyme B.


A
α-IFN-γ
Tc1
Tc17
WT
55%
0%
0%
92%
0%
0%
9%
4%
36%
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IL-17
WT
89%
0%
0%
96%
0%
0%
12%
5%
36%
Granzyme B
IL-17
Ifng-/-
83%
0%
0%
96%
0%
0%
7%
6%
40%
Granzyme B
IL-17
B
Relative Eomes Expression
120
100
80
60
40
20
0
α-IFNγ
IL12
TGFβ+IL6
Wild Type
Relative Tbx21 Expression
25
20
15
10
5
0
α-IFNγ
IL12
TGFβ+IL6
Wild Type
Relative Eomes Expression
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240
60
40
20
0
α-IFNγ
IL12
TGFβ+IL6
Ifng-/-
Relative Tbx21 Expression
25
20
15
10
5
0
α-IFNγ
IL12
TGFβ+IL6
Ifng-/-

Figure 16. TCR stimulation induces IFN-γ and granzyme B. (A) $CD8^+$ T cells were stimulated for 5 days with irradiated APC's and α-IFN-γ or in Tc1 or Tc17 skewing conditions. IL-17^+, IFN-γ^+, and granzyme B^+ cells were detected by intracellular cytokine staining. (B) RNA expression for the indicated genes was measured by real-time PCR from unstimulated samples. Cycle number is normalized to β_2-microglobulin expression and results are represented as fold induction relative to samples incubated with TGFβ+IL6. Data are representative of at least 2 experiments.

Next, we wanted to assess the ability of RORγt to inhibit granzyme B production in the absence of exogenous cytokines. CD8 T cells were differentiated in the presence of neutralizing IFN-γ antibodies and transduced with a bicistronic retrovirus containing a GFP selectable marker and either no insert or expressing RORγt. After 5 days, GFP positive cells were analyzed using ICS and gated on the GFP+ population. Expression of RORγt slightly decreased granzyme B expression (78% to 69%) (Fig 17A), similar to Fig 14C where RORγt was overexpressed in Tc1 cells. In Fig 7, we found that stimulation in the presence of TGFβ alone was able to slightly reduce granzyme B (96% down to 71%). To explore the possibility that TGFβ and RORγt synergize to reduce granzyme B in Tc17 cells, we transduced WT CD8 T cells stimulated in the presence of TGFβ with an RORγt expressing retrovirus. Again, RORγt slightly reduced granzyme B expression, but did not inhibit granzyme B to levels seen in Tc17 cells (Fig 17B). It is not known why control transduction of CD8 T cells differentiated in Tc17 conditions often contained lower percentages of IL-17 expressing cells than normally seen in untransduced Tc17 populations.

RORγt transduction into cells stimulated in neutralizing conditions or with TGFβ slightly increased the percentage of IFN-γ expressing cells consistent with the effects seen when RORγt was transduced into Tc1 cells (Fig 17B).

RORγt overexpression is therefore only partially responsible for the inhibition of granzyme B in Tc17 cells.

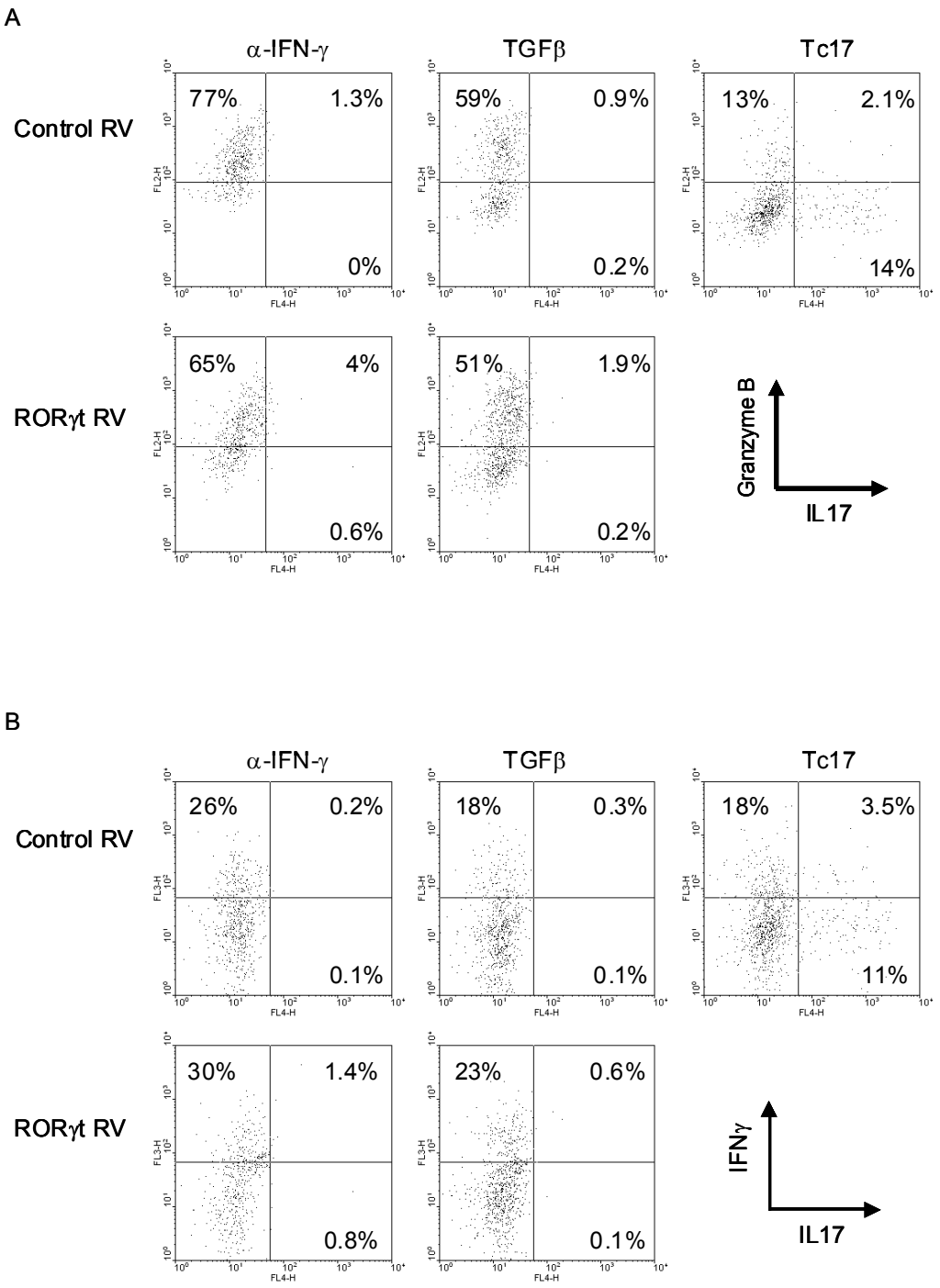


Figure 17. RORγt overexpression reduces granzyme B. (A, B) CD8+ T cells were stimulated in the presence of α-IFN-γ, TGFβ + α-IFN-γ, or TGFβ + IL-6 + α-IFN-γ (Tc17). After 1 day of culture, cells were transduced with MIEG control or RORγt-expressing retroviruses. Cells were analyzed after an additional 4 days of culture. IFN-γ, Granzyme B and IL-17 were detected using ICS. Plots are gated on live GFP+ cells. Data are representative of 2 experiments.

Role of Stat1 and T-bet in Tc17 development

To determine if Stat1 inhibits other Tc17 transcription factors and cytokines in addition to IL-17, we analyzed expression of *Rorc*, *Il17f*, *Il21*, and *Il22* in *Stat1*$^{-/-}$ Tc17 populations. Similar to IL-17, these Tc17 molecules were upregulated in *Stat1*$^{-/-}$ Tc17 cells suggesting that Stat1 inhibits several aspects of the Tc17 program (Fig 18A). Stat1 deficiency decreased *Tbx21* expression consistent with previous studies which have shown that Stat1, along with Stat4, are upstream regulators of T-bet in CD8 cells (Morishima et al., 2005; Yang et al., 2007b). Stat1 deficiency had little effect on Eomes expression in Tc17 populations (Fig 18B).

To determine if T-bet also inhibited the Tc17 program, expression of Tc17 factors were analyzed in *Tbx21*$^{-/-}$ Tc17 cells. Similar to *Stat1*$^{-/-}$ Tc17 cells, *Rorc*, *Il17f*, and *Il21* were all upregulated in *Tbx21*$^{-/-}$ Tc17 (Fig 18C). It was not determined if Stat1 mediated its inhibitory effects in Tc17 cells by inducing T-bet and it would have been interesting to see if ectopic T-bet expression in *Stat1*$^{-/-}$ Tc17 cells could reduce IL-17 and other Tc17 factors to wild type Tc17 levels.

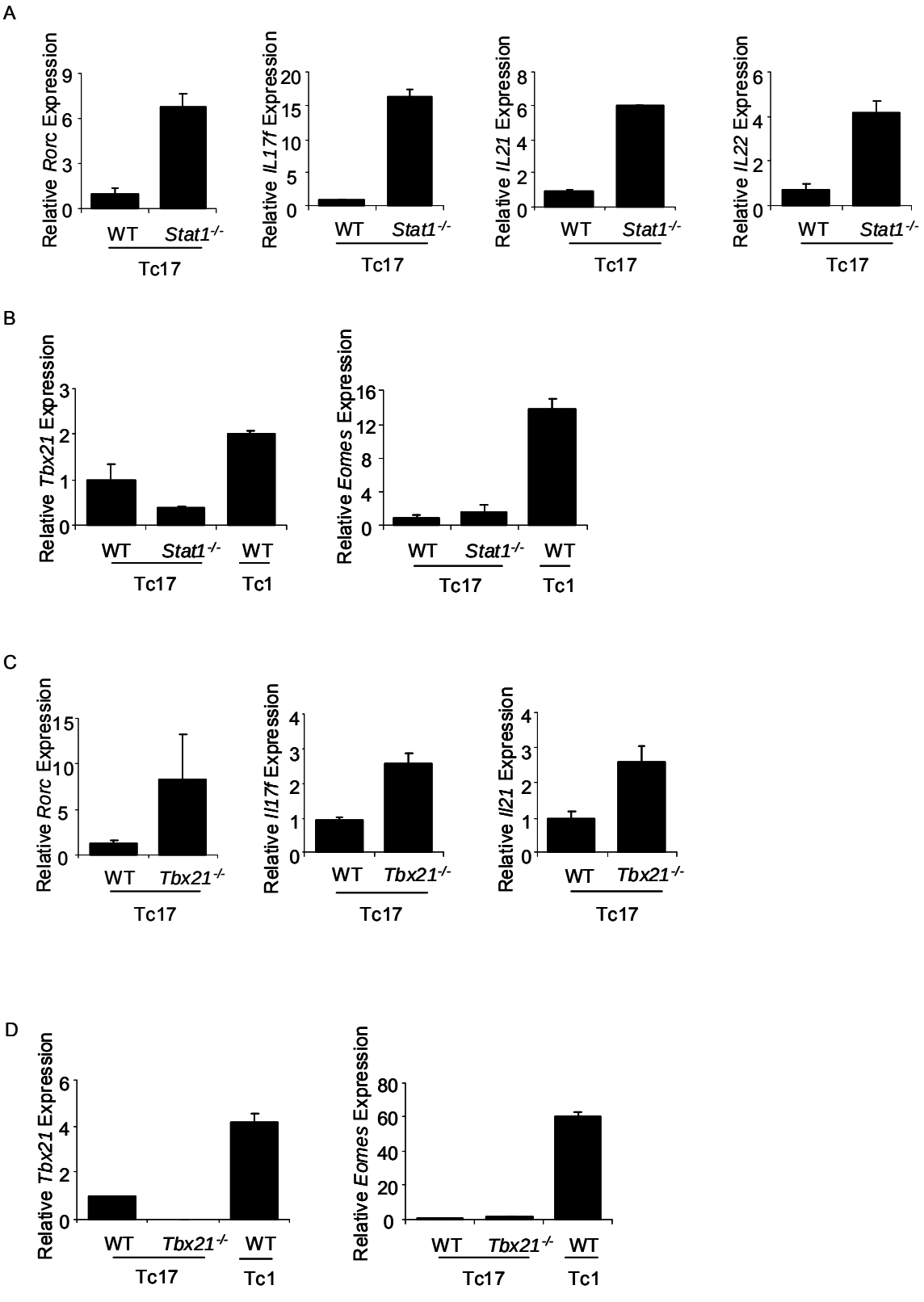

A
Relative Rorc Expression
9
6
3
0
WT
Stat1-/-
Tc17
Relative IL17f Expression
20
15
10
5
0
WT
Stat1-/-
Tc17
Relative IL21 Expression
8
6
4
2
0
WT
Stat1-/-
Tc17
Relative IL22 Expression
6
4
2
0
WT
Stat1-/-
Tc17
B
Relative Tbx21 Expression
3
2
1
0
WT
Stat1-/-
WT
Tc17
Tc1
Relative Eomes Expression
16
12
8
4
0
WT
Stat1-/-
WT
Tc17
Tc1
C
Relative Rorc Expression
15
10
5
0
WT
Tbx21-/-
Tc17
Relative Il17f Expression
4
3
2
1
0
WT
Tbx21-/-
Tc17
Relative Il21 Expression
4
3
2
1
0
WT
Tbx21-/-
Tc17
D
Relative Tbx21 Expression
6
4
2
0
WT
Tbx21-/-
WT
Tc17
Tc1
Relative Eomes Expression
80
60
40
20
0
WT
Tbx21-/-
WT
Tc17
Tc1

Figure 18. Both Stat1 and T-bet inhibit the Tc17 program. (A,B) WT or *Stat1*$^{-/-}$ CD8+ T cells were stimulated in Tc17 or Tc1 conditions. After 5 days, RNA expression for the indicated genes was measured by real-time PCR. Expression of transcription factors were analyzed from unstimulated samples and expression of cytokines were analyzed from stimulated samples. Cycle number is normalized to β_2-microglobulin expression and results are represented as fold induction relative to WT Tc17 samples. (C,D) WT or *Tbx21*$^{-/-}$ CD8+ T cells were stimulated and analyzed as in parts A and B. Data are representative of 2 experiments.

Role of Stat4 and T-bet in Tc1 development

In our system, we found that optimal Tc1 development was dependent on Stat4 and T-bet. To determine if Stat4 could be regulating Tc1 development through T-bet or another Tc1 transcription factor, Eomes, we analyzed expression of these factors in 5 day in vitro differentiated Tc1 cells deficient in Stat4. *Stat4*$^{-/-}$ Tc1 cells had similar *Tbx21* RNA expression as wild type Tc1 cells and slightly decreased T-bet protein (71% vs. 88%) (Fig 19A). Meanwhile, Eomes expression was increased in *Stat4*$^{-/-}$ Tc1 cells, although apparently not enough to overcome a decrease in IFN-γ production. To determine if Stat4 regulated cytotoxicity genes in addition to IFN-γ in Tc1 cells, RNA levels of *Perf*, *Gzmb*, and *Fasl* were also measured. *Stat4*$^{-/-}$ Tc1 cells had decreased levels of all three genes even though they were still expressed higher than in WT Tc17 cells (Fig 19B). Granzyme B protein was also analyzed by ICS and similar to the RNA data had decreased levels in *Stat4*$^{-/-}$ Tc1 cells (Fig 19C). Therefore, Stat4 is a positive regulator of IFN-γ and cytotoxicity genes in IL-12 induced Tc1 cells. Since Eomes is not decreased in *Stat4*$^{-/-}$ cells, Stat4 regulation of these genes is Eomes independent. However, decreased T-bet protein levels in *Stat4*$^{-/-}$ Tc1 cells suggest that Stat4 may partially regulate IFN-γ and cytotoxicity genes through T-bet stabilization.

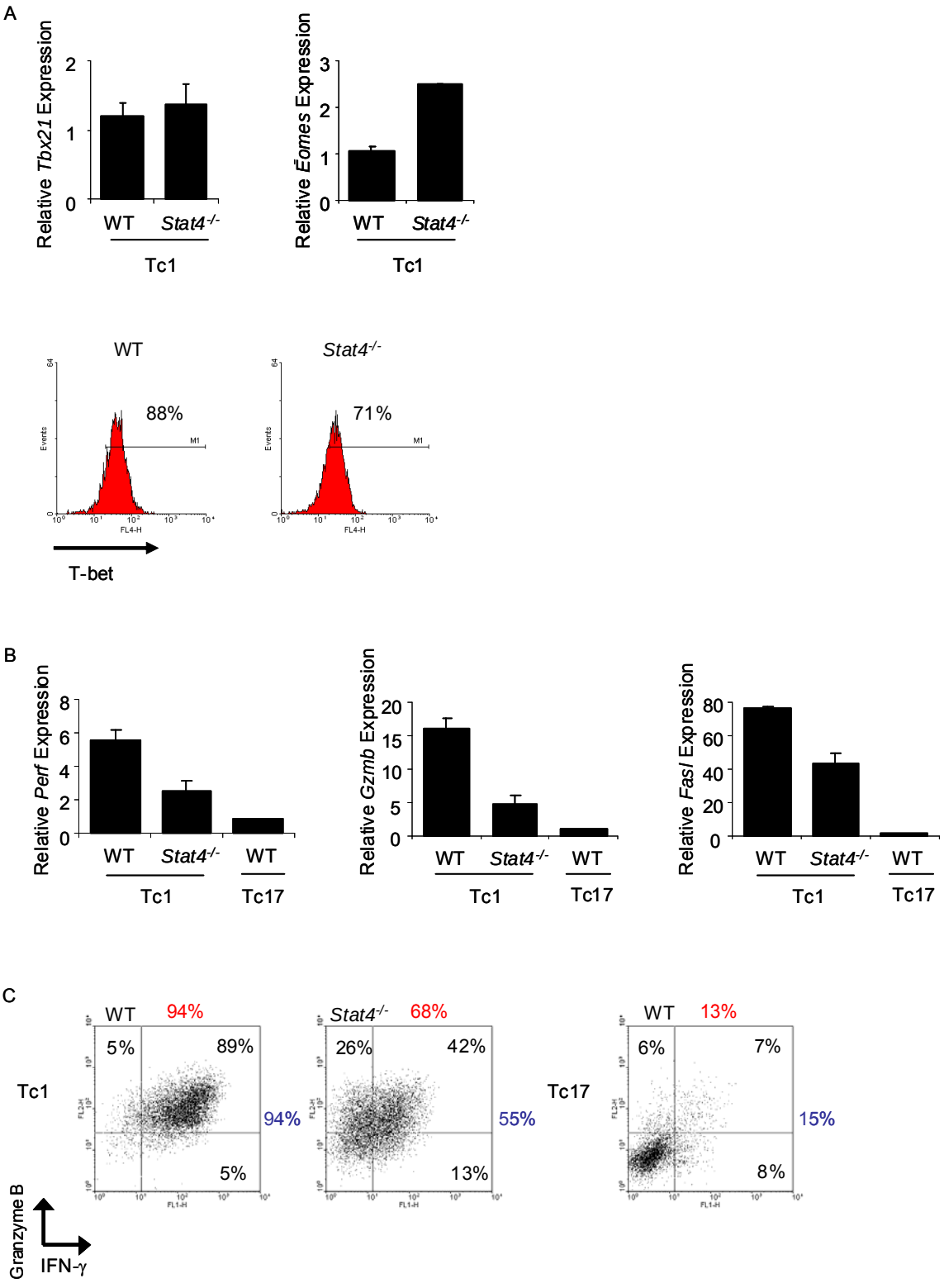

A
Relative Tbx21 Expression
2
1
0
WT
Stat4-/-
Tc1
Relative Eomes Expression
3
2
1
0
WT
Stat4-/-
Tc1
WT
88%
Stat4-/-
71%
T-bet
B
Relative Perf Expression
8
6
4
2
0
WT
Stat4-/-
WT
Tc1
Tc17
Relative Gzmb Expression
20
15
10
5
0
WT
Stat4-/-
WT
Tc1
Tc17
Relative Fasl Expression
80
60
40
20
0
WT
Stat4-/-
WT
Tc1
Tc17
C
Tc1
WT
94%
5%
89%
94%
5%
Stat4-/-
68%
26%
42%
55%
13%
Tc17
WT
13%
6%
7%
15%
8%
Granzyme B
IFN-γ

Figure 19. Stat4 is required for optimal expression of cytotoxic genes in Tc1 cells. (A,B,C) WT or *Stat4*$^{-/-}$ CD8^{+} T cells were stimulated for 5 days with irradiated APC's in Tc1 or Tc17 skewing conditions. RNA expression for the indicated genes was measured using real-time PCR with the cycle number normalized to β_2-microglobulin expression. Results are represented as fold induction relative to WT Tc1 samples for transcription factors and relative to WT Tc17 samples for cytotoxic genes. Intracellular staining was used to detect IFN-γ and granzyme B from stimulated cells and T-bet from unstimulated cells. Data are representative of at least 2 experiments.

To determine if T-bet could be regulating Tc1 development through Eomes, we analyzed Eomes expression in 5 day in vitro differentiated Tc1 cells deficient in T-bet. *Tbx21*$^{-/-}$ Tc1 cells had similar *Eomes* RNA expression as wild type Tc1 cells (Fig 20A) therefore T-bet did not seem to be mediating its effects through *Eomes* induction. We also wanted to see if T-bet regulated cytotoxicity genes in addition to IFN-γ in Tc1 cells. To do this, RNA levels of *Perf*, *Gzmb*, and *Fasl* were measured. *Tbx21*$^{-/-}$ Tc1 cells had decreased levels of *Perf* and similar levels of *Gzmb* and *Fasl* compared to WT Tc1 cells (Fig 20B). Granzyme B protein was also analyzed by ICS and granzyme B expressing cells decreased from 94% to 84% in Tc1 cells deficient in T-bet (Fig 20C). Therefore, T-bet is important for optimal IFN-γ, *Perf* and *Gzmb* expression in Tc1 cells, but not for *Fasl* expression. Since the defects seen in *Tbx21*$^{-/-}$ compared to *Stat4*$^{-/-}$ Tc1 cells were lesser in magnitude for some genes (*Ifng*, *Perf*, *Gzmb*) and absent in another gene analyzed (*Fasl*), Stat4 most likely has some functions which are independent of T-bet as well as possible functions dependent on T-bet.

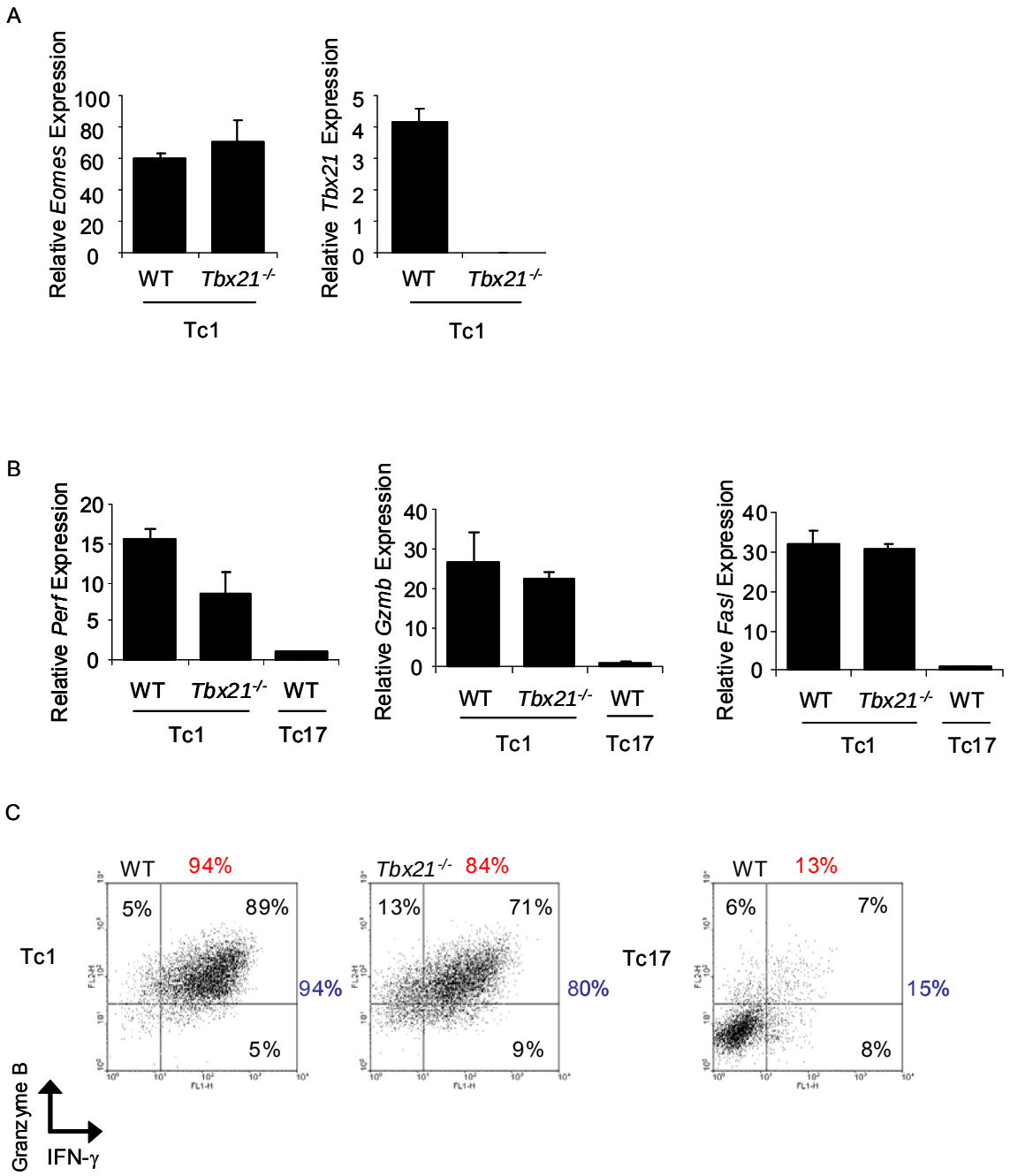


Figure 20. T-bet is required for optimal expression of Perforin and granzyme B in Tc1 cells. (A,B,C) WT or *Tbx21*$^{-/-}$ CD8^{+} T cells were stimulated for 5 days with irradiated APC's in Tc1 or Tc17 skewing conditions. RNA expression for the indicated genes was measured using real-time PCR with the cycle number normalized to β_2-microglobulin expression. Results are represented as fold induction relative to WT Tc17 samples. Intracellular staining was used to detect IFN-γ and granzyme B. Data are representative of at least 2 experiments.

Tc17 cells have low expression of T-bet, IFN-γ, and granzyme B. Since T-bet contributes to IFN-γ and granzyme B expression in Tc1 cells, we wanted to determine if IL-12 could induce T-bet in Tc17 cells and subsequently induce IFN-γ and granzyme B expression. WT CD8 T cells were stimulated for 5 days in Tc17 conditions and increasing doses of IL-12 after which T-bet and Eomes RNA levels were determined and IFN-γ, granzyme B, and IL-17 expression were measured using ICS. Our standard dose of IL-12 used in Tc1 conditions (5 ng/ml) increased *Tbx21* expression in Tc17 cells approximately 3 fold; however, increasing the IL-12 dose did not further increase *Tbx21* induction. IL-12 also did not increase *Eomes* expression in Tc17 cells (Fig 21A). All doses of IL-12 partially increased IFN-γ and granzyme B, which correlated with the *Tbx21* induction, and did not significantly decrease IL-17 (Fig 21B).

TGFβ is known to downregulate *Il12Rb2* in CD4 cells (Gorham et al., 1998) and one explanation for the lack of an IL-12 dose response in Tc17 cells is that IL-12 signaling is diminished in these cells. To explore this possibility, Stat4 phosphorylation was examined and found to be decreased in Tc17 cells restimulated with IL-12 (Fig 21C). Decreased IL-12 responsiveness, therefore, is likely one reason why increasing IL-12 doses cannot recover *Tbx21*, IFN-γ or granzyme B in Tc17 cells.

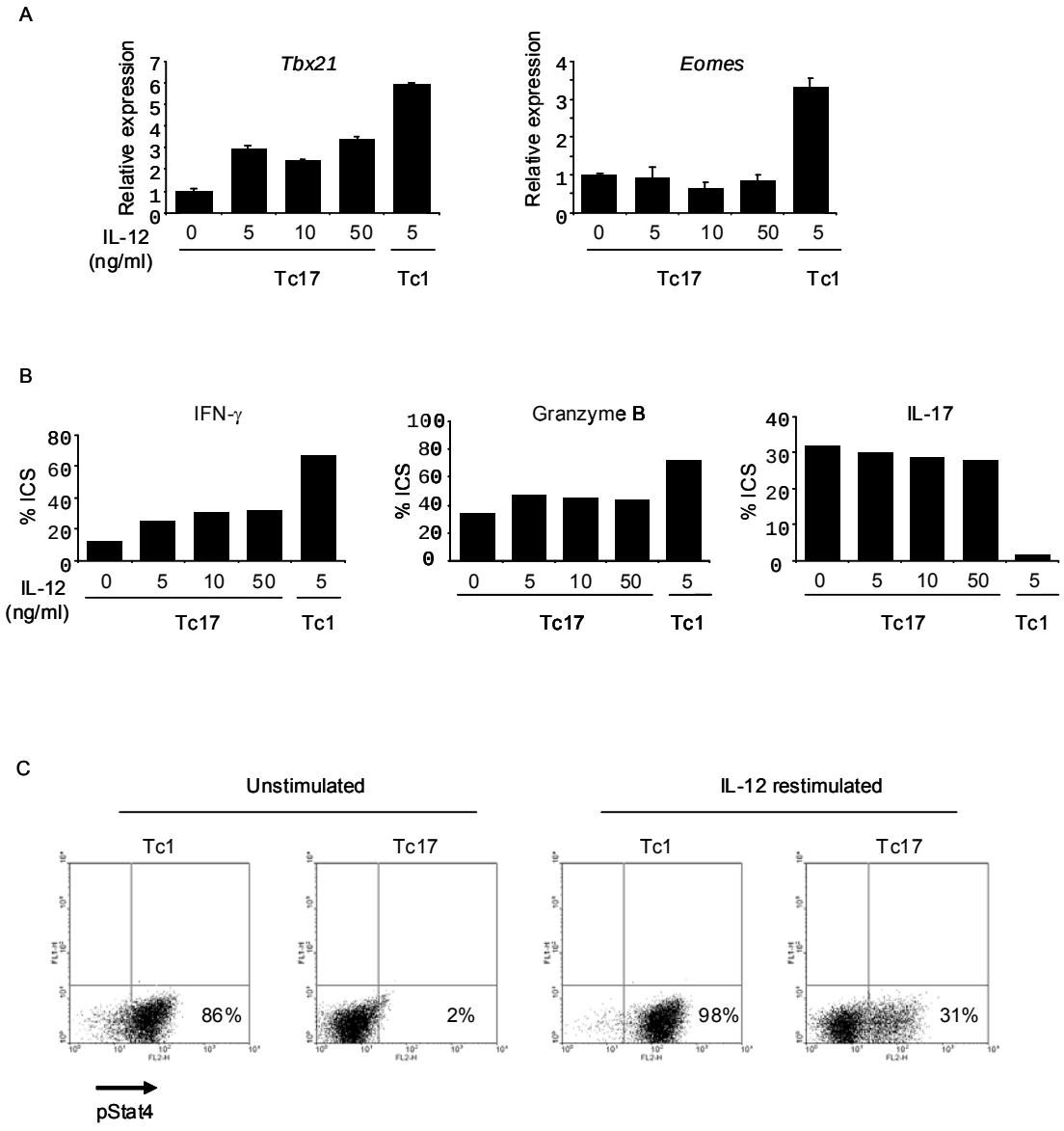


Figure 21. Tc17 cells have reduced IL-12 responsiveness. (A,B) CD8+ T cells were stimulated in Tc17 conditions with increasing doses of IL-12 or in Tc1 conditions for five days. RNA expression was measured using real-time PCR with the cycle number normalized to β2-microglobulin expression. Results are represented as fold induction relative to Tc17 cells plus 0 ng/ml IL-12. IFN-γ, Granzyme B and IL-17 were detected using ICS with the results from the dot plots summarized in a bar graph. (C) Five day differentiated Tc1 or Tc17 cells were left unstimulated or stimulated with 5 ng/ml IL-12 for 30 minutes before staining for phosphorylated Stat4. Data are representative of 2 experiments.

To circumvent the low IL-12 responsiveness and study the role of T-bet in Tc17 cells, T-bet overexpression experiments were performed. WT CD8 T cells differentiated in Tc17 conditions were transduced with a bicistronic retrovirus containing a GFP selectable marker and either no insert or expressing T-bet. After 5 days, GFP positive cells were analyzed using ICS. Expression of T-bet in developing Tc17 populations led to a minimal increase in the percentage of IFN-γ secreting cells and inhibited the percentage of IL-17 secreting cells (Fig 22A). To determine if IFN-γ could be upregulated in Tc17 cells by Eomes overexpression, WT CD8 T cells differentiated in Tc17 conditions were transduced with a bicistronic retrovirus containing a hCD4 selectable marker and either no insert or expressing Eomes. Eomes transduced cells also led to a minimal increase in the percentage of IFN-γ secreting cells and, unexpectedly, also increased the percentage of IL-17 secreting cells. A report has suggested that T-bet and Eomes may function in a synergistic manner (Intlekofer et al., 2008); therefore, we wanted to determine if overexpression of T-bet and Eomes together could upregulate IFN-γ in Tc17 cells. Transduction of both retroviruses did not significantly upregulate IFN-γ in Tc17 cells suggesting that in addition to downregulating IL-12 signaling, Tc17 cells are also refractory to Eomes and T-bet induced IFN-γ (Fig 22A). To determine if T-bet or Eomes could increase granzyme B in Tc17 cells, differentiating Tc17 cells were transduced with T-bet or Eomes or both factors. After 5 days, either factor alone recovered the percentage of granzyme B secreting cells compared to Tc1 populations with Eomes having a slightly greater effect than T-bet (Fig 22B).

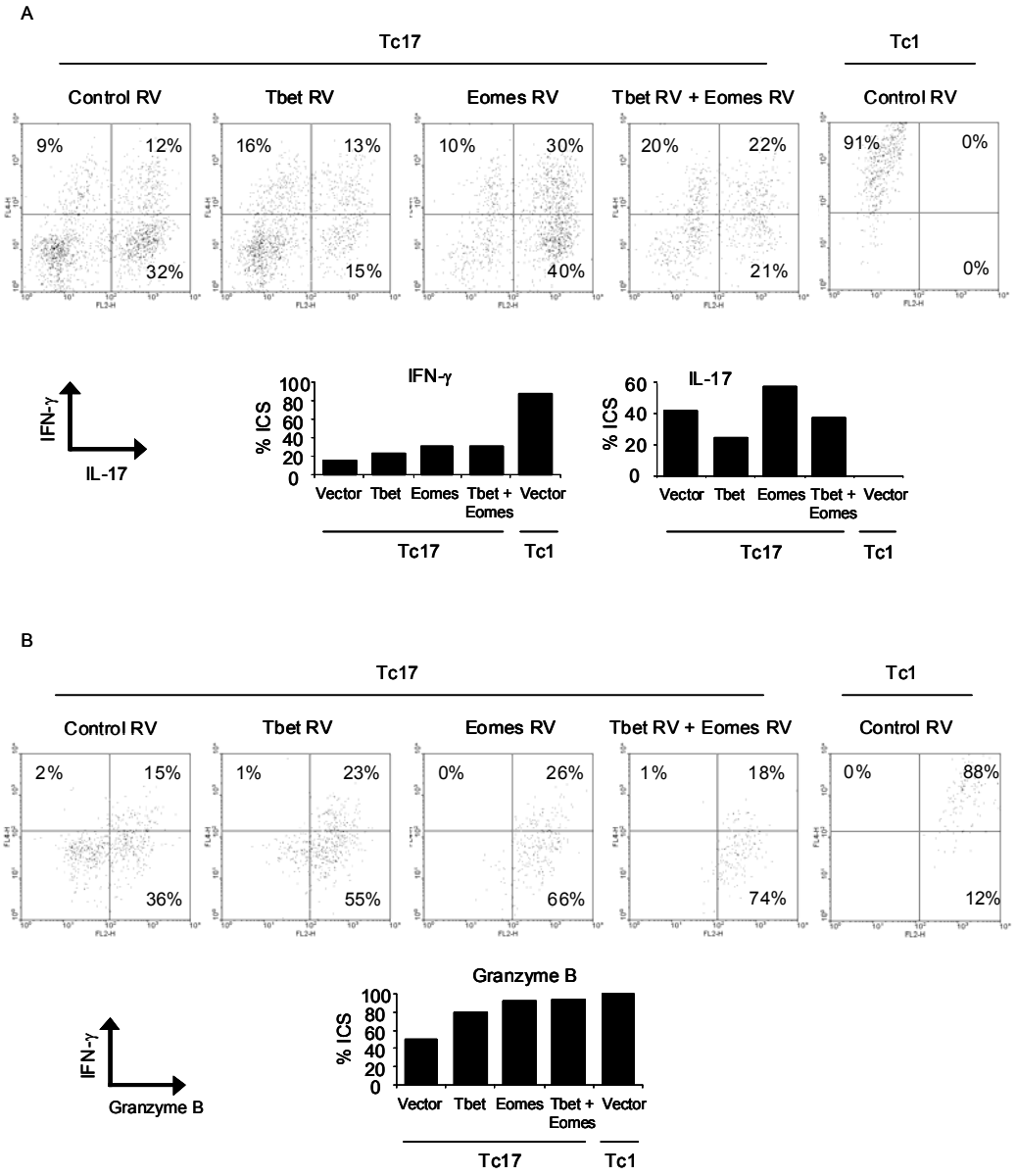
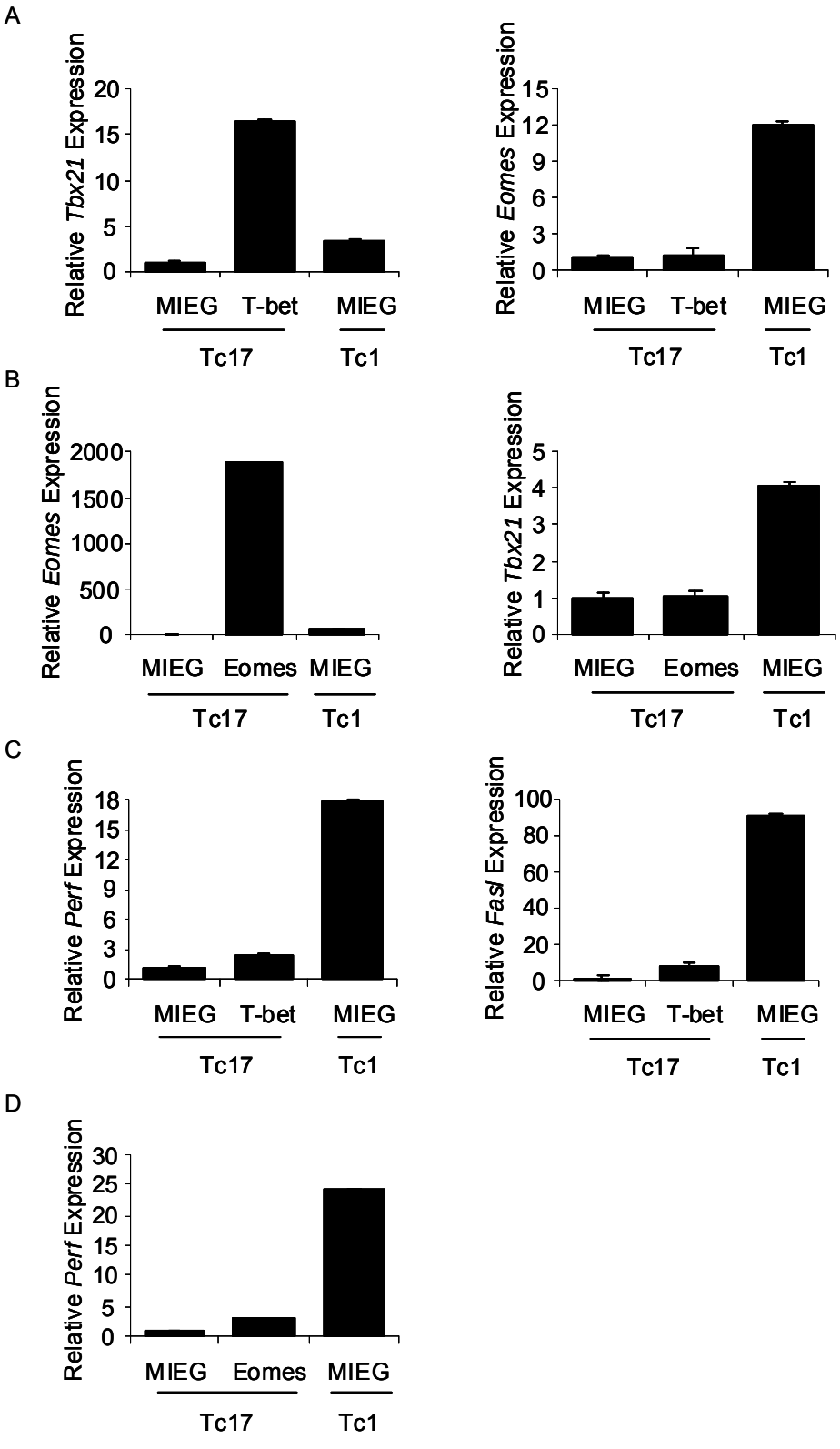


Figure 22. Effects of T-bet and Eomes overexpression on cytokine production from Tc17 cells. (A, B) CD8+ T cells were stimulated in Tc17 or Tc1 conditions. After 1 day of culture, cells were simultaneously transduced with two bicistronic retroviruses – one containing a GFP selectable marker and either no insert or expressing T-bet and the other containing a hCD4 selectable marker and either no insert or expressing Eomes. Vector transduced cells were double transduced with hCD4 and GFP controls, Tbet double transductions = Tbet+hCD4, Eomes double transduction = Eomes+GFP, and Tbet+Eomes equals cells which were transduced with both genes of interest. Cells were analyzed after an additional 4 days of culture. IFN-γ, Granzyme B and IL-17 were detected using ICS. Plots are gated on live $GFP^{+}hCD4^{+}$ cells and cytokine staining is summarized in bar graphs below the dot plots. Data are representative of 1 experiment.

T-bet overexpression was confirmed in Tc17 cells by RNA and consistent with *Tbx21*$^{-/-}$ Tc1 cells (Fig 20A), had no effect on Eomes expression (Fig 23A). Likewise, Eomes overexpression had no effect on T-bet RNA levels (Fig 23B). We also wanted to determine if overexpression of these transcription factors could upregulate other cytotoxic genes in addition to granzyme B. T-bet overexpression increased *Perf* RNA levels 2.3 fold and *Fasl* expression 8.4 fold. While Eomes expression increased *Perf* RNA levels 3.1 fold (Fig 23C, D).


A
Relative Tbx21 Expression
20
15
10
5
0
MIEG
T-bet
MIEG
Tc17
Tc1
Relative Eomes Expression
15
12
9
6
3
0
MIEG
T-bet
MIEG
Tc17
Tc1
B
Relative Eomes Expression
2000
1500
1000
500
0
MIEG
Eomes
MIEG
Tc17
Tc1
Relative Tbx21 Expression
5
4
3
2
1
0
MIEG
Eomes
MIEG
Tc17
Tc1
C
Relative Perf Expression
18
15
12
9
6
3
0
MIEG
T-bet
MIEG
Tc17
Tc1
Relative Fasl Expression
100
80
60
40
20
0
MIEG
T-bet
MIEG
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Tc1
D
Relative Perf Expression
30
25
20
15
10
5
0
MIEG
Eomes
MIEG
Tc17
Tc1

Figure 23. Effects of T-bet and Eomes overexpression on transcription factors and cytotoxicity genes from Tc17 cells. (A,C) CD8+ T cells were stimulated in Tc17 or Tc1 conditions. After 1 day of culture, cells were transduced with a bicistronic retrovirus containing a GFP selectable marker and either no insert or expressing T-bet. After an additional 4 days of culture, GFP+ cells were purified using Facs sorting and RNA expression for the indicated genes was measured by real-time PCR. Cycle number is normalized to β2-microglobulin expression and results are represented as fold induction relative to control transduced Tc17 samples. (B,D) CD8+ T cells were activated and transduced as above with a bicistronic retrovirus containing a hCD4 selectable marker and either no insert or expressing Eomes. Transduced cells were analyzed as in parts A and C. Data are representative of 1 experiment.

Role of Stat1 in Tc1 development

Previously we observed decreased *Tbx21* RNA in *Stat1*$^{-/-}$ Tc17 cells (Fig 18B). To determine if *Tbx21* expression was also decreased in Tc1 cells deficient in Stat1, we examined 5 day in vitro differentiated Tc1 cells. *Stat1*$^{-/-}$ Tc1 cells express normal levels of *Tbx21* and increased *Eomes* (Fig 24A). In addition, similar to the normal IFN-γ production seen in these cells, there was normal or increased expression of the cytotoxicity genes *Perf*, *Gzmb*, and *Fasl* (Fig 24B). Therefore, Stat1 is dispensable for normal IL-12 induced Tc1 programming.

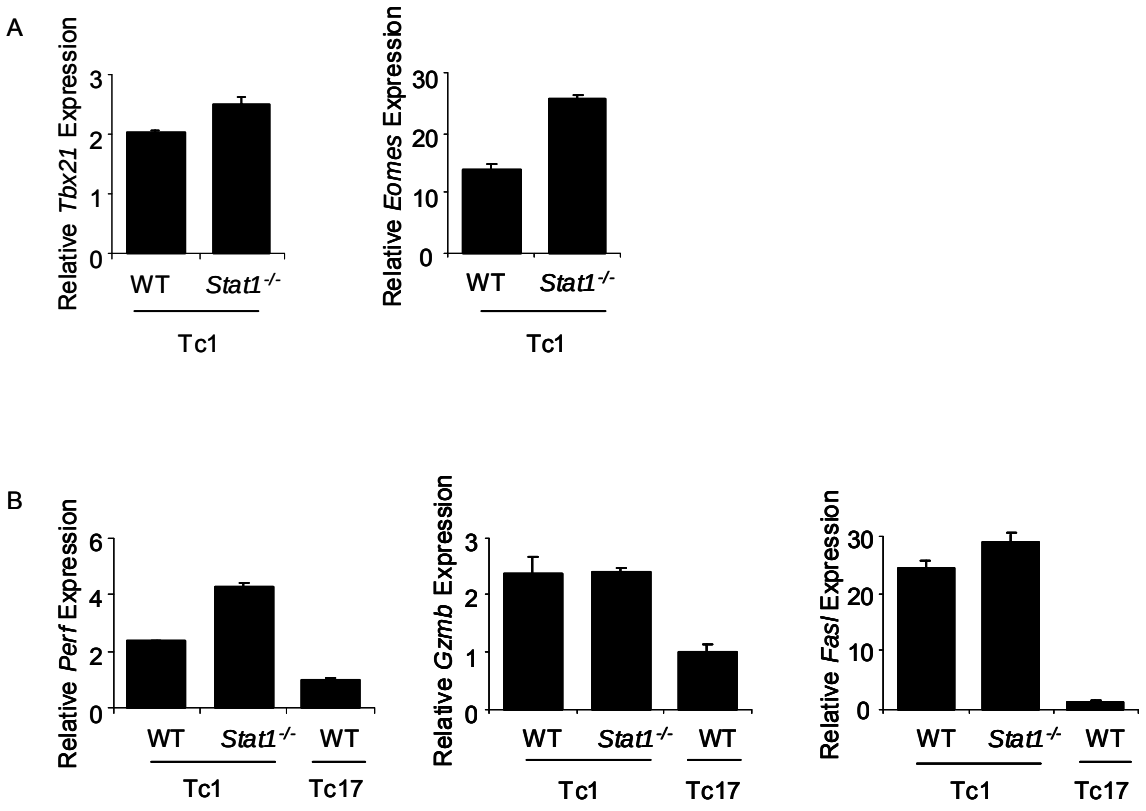


Figure 24. Stat1 is dispensable for Tc1 development. (A,B) WT (129S6) or *Stat1*$^{-/-}$ CD8+ T cells were stimulated in Tc1 or Tc17 skewing conditions. After 5 days, RNA expression for the indicated genes was measured by real-time PCR. Cycle number is normalized to β_2-microglobulin expression and results are represented as fold induction relative to WT Tc17 samples. Data are representative of 2 experiments.

Summary of the molecular regulation of Tc17 cells

We propose a model of Tc17 development as depicted in Fig 25. Upon TCR stimulation, induction of Eomes and T-bet promote a cytotoxic T lymphocyte phenotype expressing IFN-γ, granzyme B, and perforin. IL-12 induction of Stat4 augments CTL development to secrete even higher levels of IFN-γ. Alternatively, in the presence of TGFβ and IL-6, Stat3 and RORγt are induced, Eomes and T-bet are inhibited, and a Tc17 phenotype develops expressing IL-17A, IL-17F, IL-21, and IL-22 and upregulating the IL-23R. The Tc17 phenotype is under negative regulation by Stat1 and T-bet. The plasticity of naïve CD8 T cells reveals additional complexities of CD8 T cell differentiation and the importance of Stat proteins and lineage specific transcription factors in determining CD8 T cell fate.

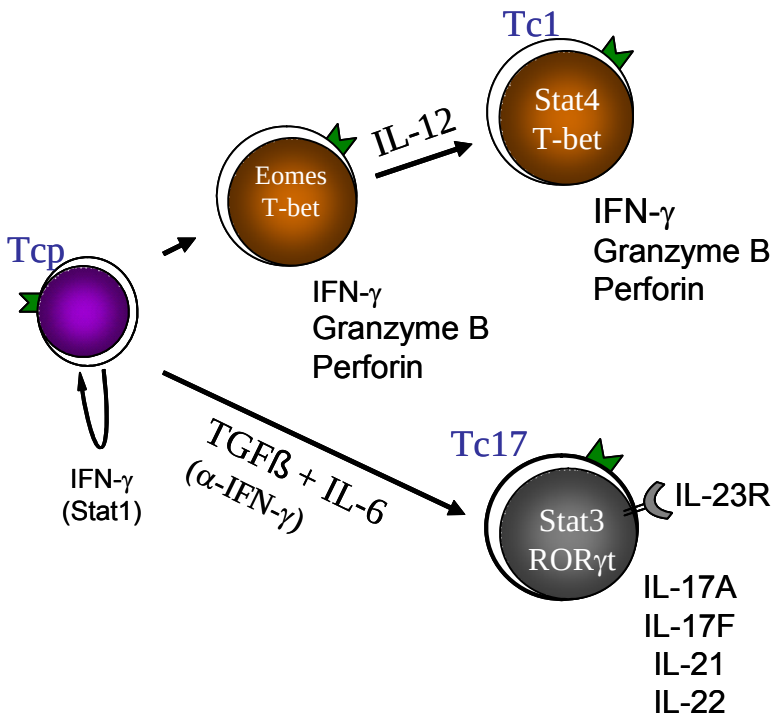


Figure 25. Development of CD8 T cell subsets. TCR stimulation induces the CTL transcription factors Eomes and T-bet. The fate of the developing CD8 T cell can be modified by cytokines in the environment and the transcription factors that are activated. IL-12 activates Stat4 and T-bet to induce Tc1 cells and TGFβ with IL-6 activates Stat3 and RORγt to induce Tc17 cells. These subsets are characterized by the cytokines they secrete – Tc1 cells secrete IFN-γ, granzyme B, and perforin while Tc17 cells secrete IL-17A, IL-17F, IL-21, and IL-22.

Tc17 Instability

CD8 T cells can produce high levels of IL-17 which is not completely suppressed by IL-12 stimulation or overexpression of T-bet and Eomes in a one week culture. However, CD4 cells producing both IL-17 and IFN-γ are often observed in in vivo disease models and several groups, including ours, have shown that Th17 cells are unstable upon restimulation in multiple week cultures.

Upregulation of IFN-γ and granzyme B in Tc17 cells

To determine if Tc17 cells are also unstable, we restimulated five day differentiated Tc17 for an additional five days in conditions containing various cytokines and neutralizing antibodies – a neutralizing condition (α-IFN-γ), a Tc17 skewing condition (TGFβ+IL-6+α-IFN-γ), a Tc1 condition (IL-12), or a combined Tc17/Tc1 condition (TGFβ+IL-6+IL-12+α-IFN-γ) and analyzed cytokine production. Tc17 cells restimulated in a neutralizing condition for a second week (Tc17→α-IFN-γ) downregulated the percentage of IL-17 secreting cells and increased the percentage of IFN-γ secreting cells (Fig 26A). This switch from a Tc17 population to a Tc1 population was greatly enhanced when the cells were restimulated for a second round in the presence of IL-12. Tc17 populations restimulated in Tc17 conditions further increased the percentage of IL-17 secreting cells while also slightly upregulating the percentage of IFN-γ secreting cells. In the presence of Tc17 and Tc1 instructive signals together, the

percentage of both IFN-γ and IL-17 secreting cells were upregulated compared to one week Tc17 populations (Fig 26A).

To determine the role of IFN-γ in Tc17 instability, *Ifng*$^{-/-}$ Tc17 populations were restimulated for a 2nd week with the same cytokine / antibody combinations described above. In the absence of IFN-γ, neutralizing conditions for the 2nd week only slightly lowered the percentage of IL-17 secreting cells (Fig 26B) suggesting that in the WT Tc17→α-IFN-γ condition, IL-17 is reduced via IFN-γ signals. *Ifng*$^{-/-}$ Tc17 populations contained more IL-17 secreting cells in each respective 2 week condition compared to WT Tc17 cells with a similar trend when placed in Tc17, combined Tc17/Tc1, or Tc1 conditions for a second week of culture consistent with the inhibitory role of IFN-γ in a one week culture (Fig 3C).

CD8 T cells cultured for one week are not always unstable, however, as seen by the inability of Tc1→Tc17 cells to produce IL-17 (Fig 26C). Therefore, Tc17 cells are uniquely unstable and in a second week of culture produce cytokines which are reflective of the instructive cytokines present in the environment – if TGFβ and IL-6 are present, Tc17 polarization is maintained; in the presence of IL-12, Tc17 populations switch to a Tc1 population; and if all three cytokines are present, both IFN-γ- and IL-17-secreting cells are developed.

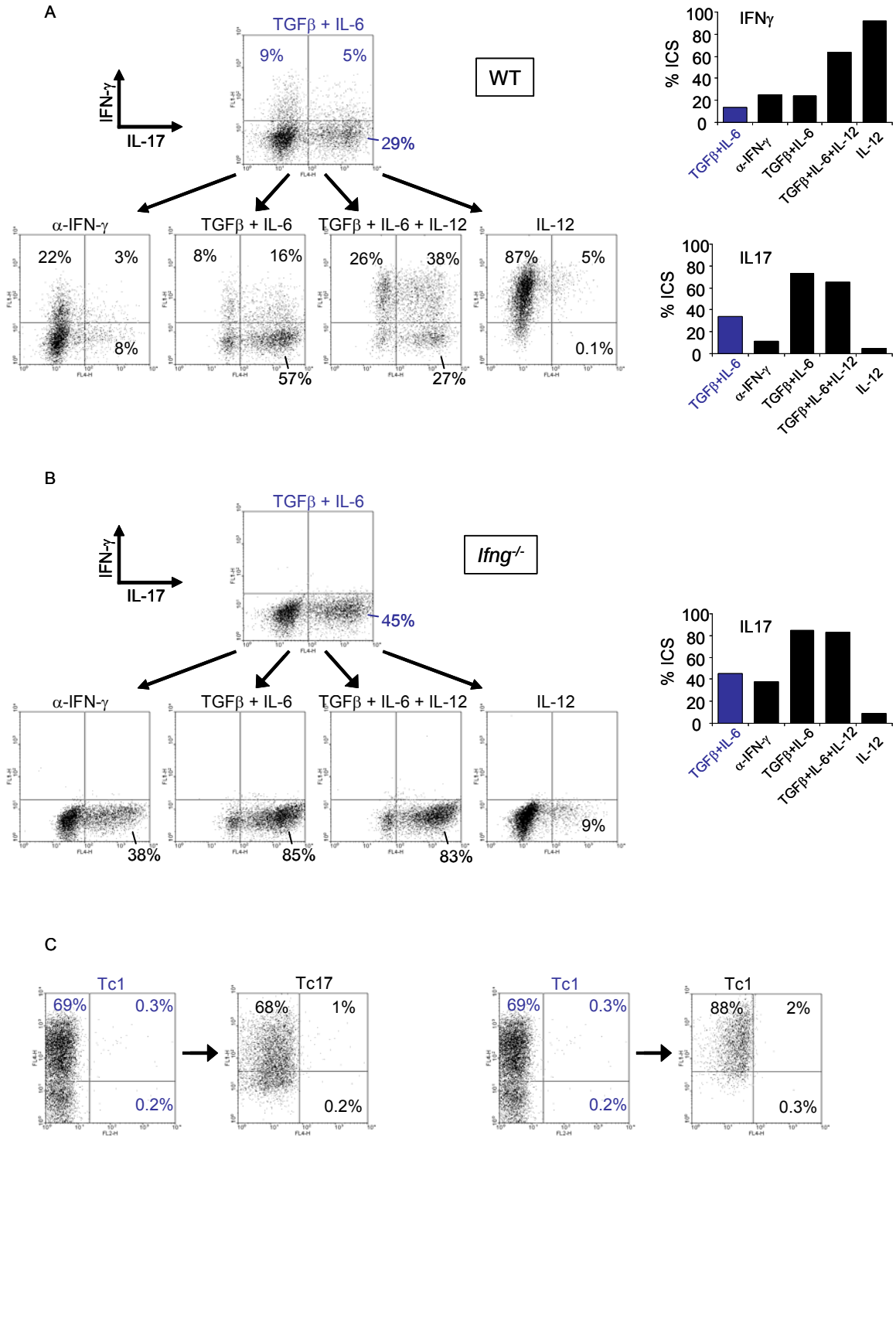

A
TGFβ + IL-6
9%
5%
29%
IFN-γ
IL-17
WT
α-IFN-γ
TGFβ + IL-6
TGFβ + IL-6 + IL-12
IL-12
22%
3%
8%
8%
16%
57%
26%
38%
27%
87%
5%
0.1%
IFNγ
% ICS
100
80
60
40
20
0
TGFβ+IL-6
α-IFN-γ
TGFβ+IL-6
TGFβ+IL-6+IL-12
IL-12
IL17
B
TGFβ + IL-6
45%
IFN-γ
IL-17
Ifng-/-
α-IFN-γ
TGFβ + IL-6
TGFβ + IL-6 + IL-12
IL-12
38%
85%
83%
9%
IL17
% ICS
C
Tc1
Tc17
69%
0.3%
0.2%
68%
1%
0.2%
Tc1
Tc1
69%
0.3%
0.2%
88%
2%
0.3%

Figure 26. Tc17 cells exhibit plasticity. (A) WT or (B) *Ifng*$^{-/-}$ CD8+ T cells were stimulated in Tc17 conditions. After five days, Tc17 cells were restimulated in the presence of α-IFN-γ and the indicated cytokines or IL-12 alone for an additional five days. IL-17+ and IFN-γ+ cells were detected using intracellular cytokine staining after the first round of stimulation (Day 5) and also at the end of the second round of stimulation (Day 10). Data are represented as dot plots or summarized in bar graphs with 5 day differentiated cells labeled in blue. (C) WT CD8+ T cells were stimulated in Tc1 (IL-12) conditions and after five days, restimulated in Tc17 (TGFβ + IL-6 + α-IFN-γ) or Tc1 conditions for an additional five days. IL-17+ and IFN-γ+ cells were detected after the first and second rounds of stimulation. Data are representative of at least 2 experiments.

We also wanted to determine if the expression of cytotoxic molecules was unstable in Tc17 cells by analyzing granzyme B expression in 2 week cultures. One week differentiated Tc17 cells were restimulated for an additional week in the presence of the same cytokine and antibody combinations described in the previous figure. Upon restimulation for a second week in neutralizing conditions alone, granzyme B is induced from 11% to 84% of the live cell population and this increase is enhanced in the presence of IL-12 (Fig 27A). The TCR mediated induction of granzyme B is mostly diminished when TGFβ and IL-6 are added to the IFN-γ neutralizing antibodies and is not recovered when additionally stimulated with IL-12. The trend in granzyme B instability remained the same when IFN-γ signals were completely removed using *Ifng*$^{-/-}$ Tc17 cells in a second week of culture (Fig 27B).

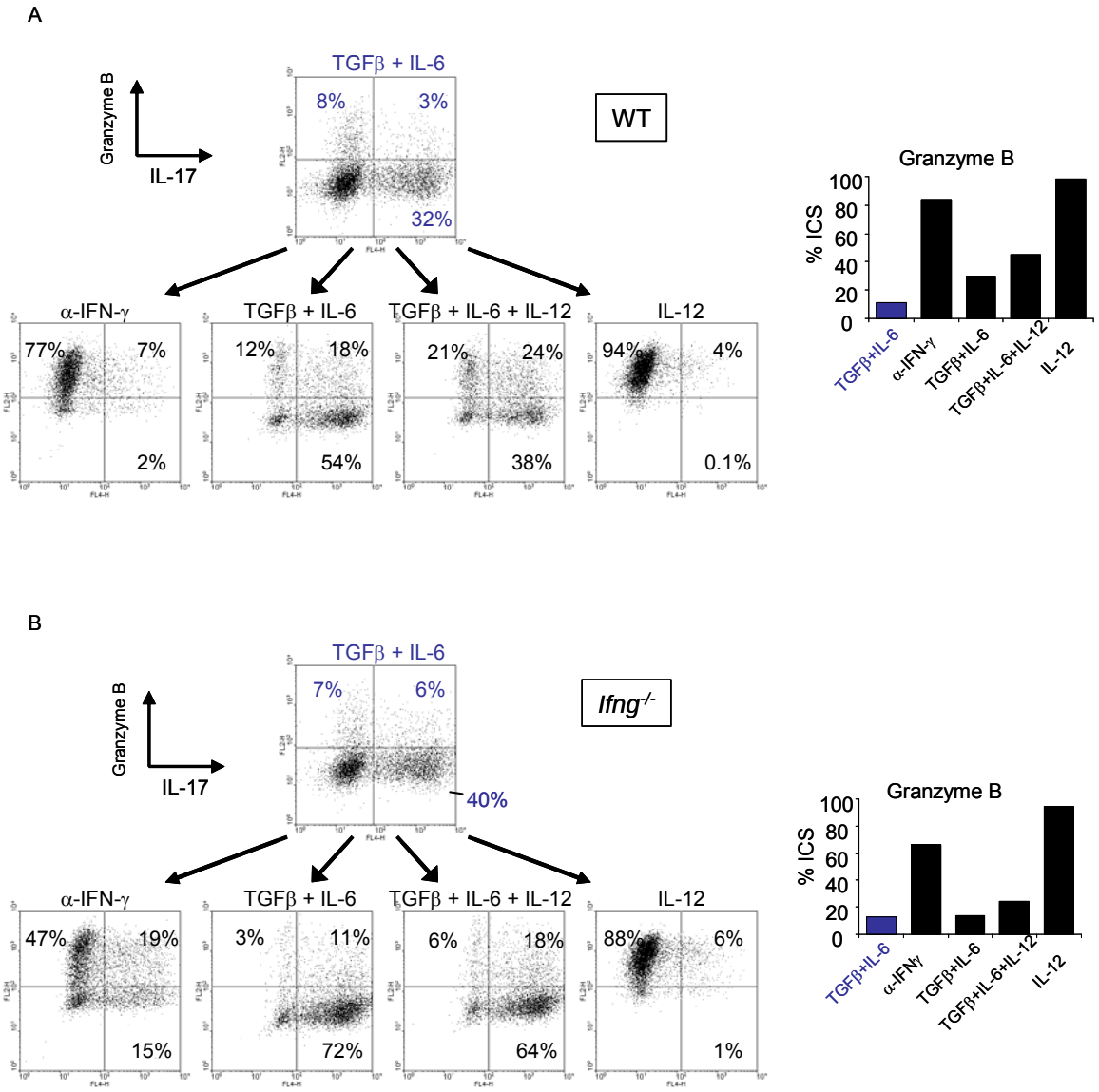


Figure 27. 2 week Tc17 cells upregulate granzyme B. (A) WT or (B) *Ifng*$^{-/-}$ CD8+ T cells were stimulated in Tc17 conditions. After five days, Tc17 cells were restimulated in the presence α-IFN-γ and the indicated cytokines or IL-12 alone for an additional five days. IL-17+ and granzyme B+ cells were detected using intracellular cytokine staining after the first round of stimulation (Day 5) and also at the end of the second round of stimulation (Day 10). Data are represented as dot plots or summarized in bar graphs with 5 day differentiated cells labeled in blue. Data are representative of at least 2 experiments.

Next, we analyzed the ability of switched Tc17 cells to mediate cytotoxicity. One week differentiated Tc17 cells from OT-I mice were restimulated for an additional week in the presence of neutralizing IFN-γ antibodies (Tc17→α-IFN-γ), in Tc17 conditions (Tc17→Tc17), or in Tc1 conditions (Tc17→Tc1) and analyzed in a standard chromium release assay. Tc17→α-IFN-γ cells gained cytotoxic ability compared to 5 day differentiated Tc17 cells and were able to lyse ~20% of target cells at the highest effector to target ratio (30:1) (Fig 28A). Tc17→Tc17 cells displayed similar cytotoxic ability and Tc17→Tc1 cells had increased cytotoxicity although not to the level of Tc1→Tc1 cells. The gain of cytotoxic function however did not correlate with granzyme B expression (Fig 28A vs. Fig 27A) – Tc17→α-IFN-γ and Tc17→Tc17 cells had similar cytotoxic potential while Tc17→α-IFN-γ cells had much higher expression of granzyme B than Tc17→Tc17 cells, suggesting that optimal cytototoxic function in these cells is mediated by many factors.

Cell mediated cytotoxicity was also tested in a CFSE based cytotoxicity assay. In this assay, Tc17→α-IFN-γ and Tc17→Tc17 cells displayed minimal to no killing compared to the chromium release assay, while the trends for the cells restimulated in Tc1 conditions were similar to the chromium release assay (Fig 28B). The slight difference in the results seen in these two assays could be due to differences in sensitivity.

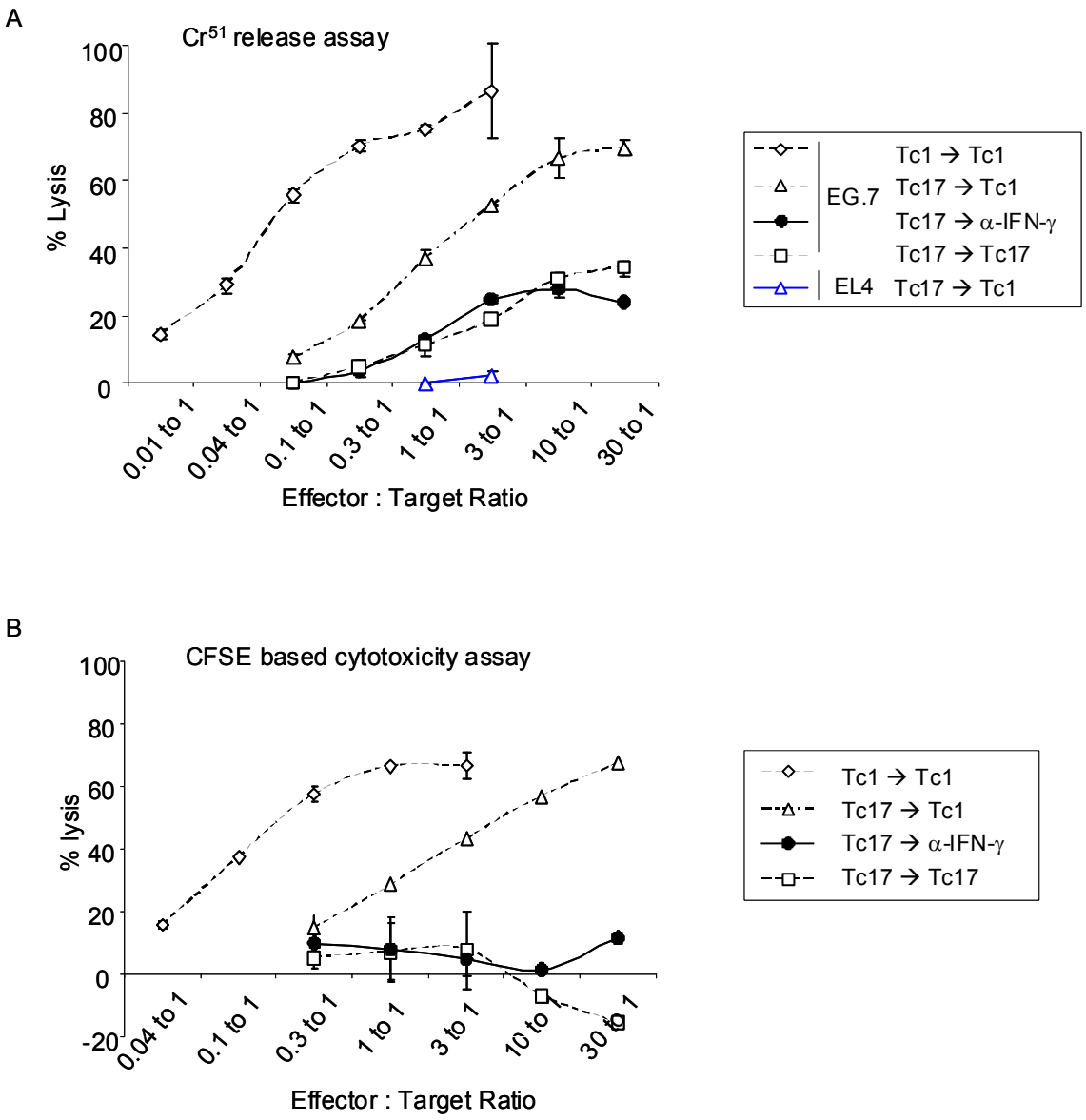


Figure 28. IL-12 induces Tc17 cells to become cytotoxic. OT-I CD8+ T cells were stimulated in Tc17 or Tc1 conditions. After 5 days, cells were restimulated in the presence of α-IFN-γ, in Tc17 conditions or in Tc1 conditions for an additional five days. (A) Effector cells were added at increasing ratios to ova expressing EG.7 target cells labeled with Cr^{51} and incubated for 6 hrs before measuring Cr^{51} released into the supernatant. (B) Nonspecific EL-4 and specific EG.7 target cells were labeled with two different concentrations of CFSE and mixed in a 1:1 ratio. Effector cells were added at increasing ratios to the target cells and incubated for 4 hrs. 7-AAD was added into the CTL reaction to identify dead cells before analysis by flow cytometry. Ag specific cytotoxicity was calculated by the formula: % lysis = 100 x [1 – $(E.G7/EL4)_{experimental}$ / $(E.G7/EL4)_{control}$]. Data are representative of at least 2 experiments.

In a one week Tc17 culture, high IL-17 expression and low IFN-γ and granzyme B expression correlate to an induction of *Rorc* expression and low levels of *Tbx21* and *Eomes* expression. To determine if these transcription factors also correlate to cytokine changes seen in switched Tc17 cells, we compared their expression levels in one week Tc17 cells to Tc17 cells restimulated in various cytokines and antibodies for a second week. WT Tc17→α-IFN-γ cultures downregulated *Rorc* expression and upregulated *Tbx21* and *Eomes* expression (Fig 29). This inhibition of *Rorc* was mostly absent in IFN-γ deficient Tc17 cells indicating that it was primarily due to TCR induced IFN-γ. *Tbx21* and *Eomes* expression were also increased in *Ifng*$^{-/-}$Tc17→α-IFN-γ cultures compared to WT Tc17 cells suggesting that IFN-γ also inhibits *Tbx21* and *Eomes* induction in this condition. When WT Tc17 cells were stimulated in the presence of TGFβ and IL-6, *Rorc* was further induced and *Eomes* and *Tbx21* expression remained low. The addition of IL-12 to Tc17 conditions in the second week inhibited *Rorc* induction in WT Tc17 cells, but did not induce *Eomes* or *Tbx21* expression. Similar to Tc17→α-IFN-γ cultures, *Rorc* inhibition in Tc17→TGFβ+IL-6+IL-12 conditions was mostly abrogated in the absence of IFN-γ, suggesting that inhibition is again mediated through IFN-γ. WT Tc17→Tc1 cells also inhibited *Rorc* and did not induce *Tbx21* or *Eomes* expression (Fig 29).

These trends in *Rorc*, *Tbx21*, and *Eomes* regulation correlate most closely with changes in IL-17 and granzyme B, and not with IFN-γ production suggesting that *Rorc* maintains IL-17 production in a two week culture and *Tbx21* and *Eomes*

upregulate granzyme B in the second week. However, IFN-γ induced from 2 week Tc17 cells by IL-12 may be mediated through T-bet and Eomes independent pathways.

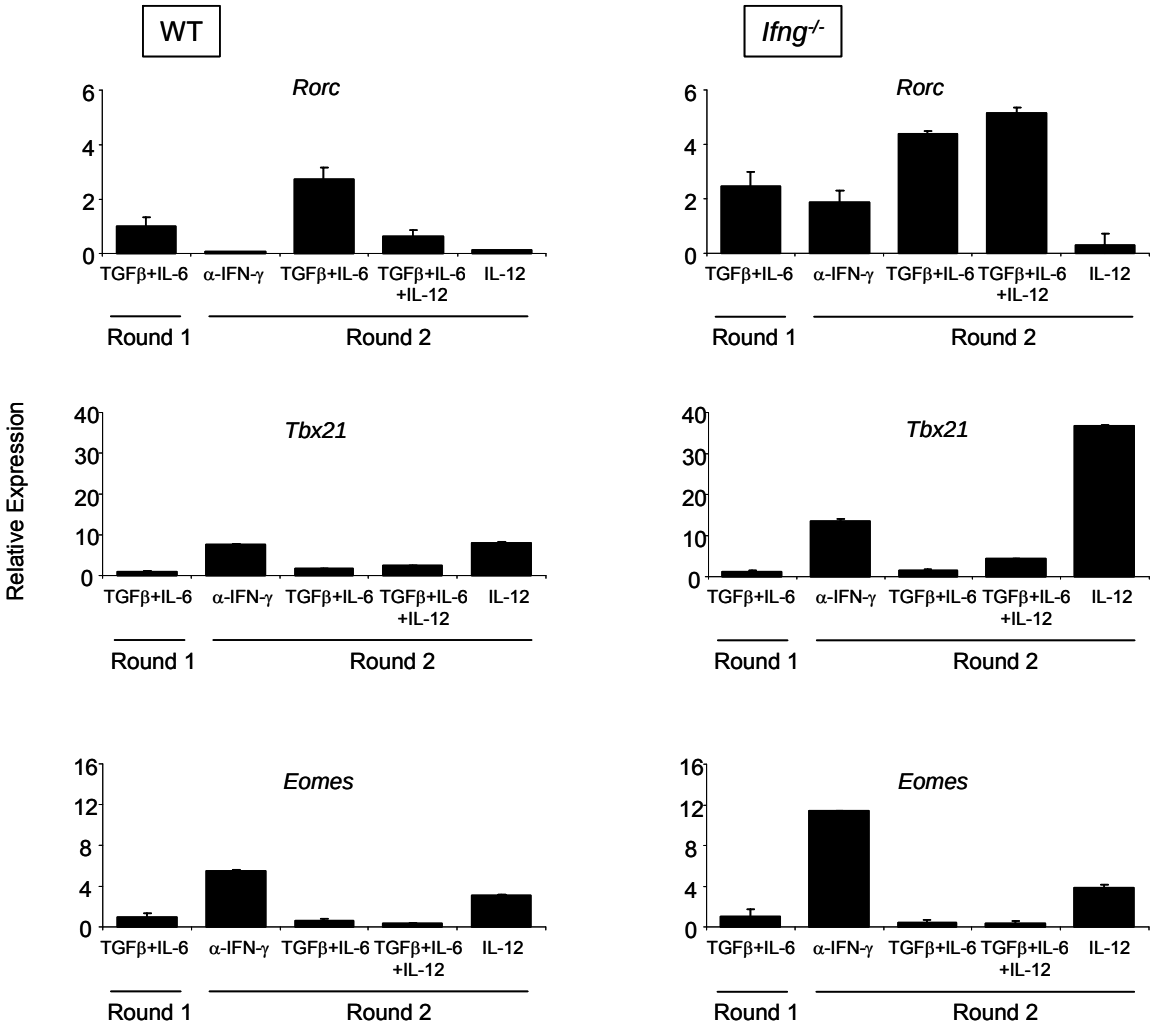


Figure 29. Five day differentiated Tc17 cells downregulate *Rorc* and upregulate *Tbx21* and *Eomes* when challenged with IL-12. WT (left panels) or *Ifng*$^{-/-}$ (right panels) CD8^{+} T cells were stimulated in Tc17 conditions. After five days, Tc17 cells were restimulated in the presence α-IFN-γ and the indicated cytokines or IL-12 alone for an additional five days. RNA expression for the indicated genes was measured by real-time PCR after one round of stimulation (Day 5) and after two rounds of stimulation (Day 10). Cycle number is normalized to β_2-microglobulin expression and results are represented as fold induction relative to WT Day 5 Tc17 samples. Data are representative of 2 experiments.

Role of Stat4 and T-bet in Tc17 instability

Stat4 and T-bet were both previously seen to be important for optimal IFN-γ production from Tc1 cells. To further study their role in IFN-γ producing cells and begin to understand their importance for IFN-γ upregulation in Tc17 cells, we first analyzed CD8 T cells deficient in both factors together or either factor alone in neutralizing conditions or Tc1 conditions for one week. When stimulated in neutralizing conditions, roughly half of the WT CD8 T cells produced IFN-γ and this percentage was reduced 2-3 fold in the absence of T-bet (Fig 30A). IFN-γ secreting cells were also slightly reduced in the absence of Stat4. The absence of both factors did not further decrease the percentage of IFN-γ-secreting cells compared to a deficiency in T-bet alone.

Tc1 conditions increased the percentage of IFN-γ-secreting cells compared to neutralizing conditions dependent on Stat4 because this increase was absent in Stat4 deficient cells. IL-12 induced IFN-γ had a larger decrease with a deficiency of both Stat4 and T-bet together compared to a deficiency in either transcription factor alone (Fig 30A). Together, these results suggest that IFN-γ is induced through TCR signals which are partially T-bet dependant and is further enhanced upon IL-12 activation of Stat4.

We also wanted to determine the role of T-bet or Stat4 for granzyme B induction. WT CD8 T cells stimulated in neutralizing conditions upregulated high levels of

granzyme B which was reduced in the absence of Stat4 (89% vs 70%) but not T-bet (Fig 30B). Tc1 cells were also dependent on Stat4 for maximal granzyme B production. Unlike in IFN-γ regulation, double knockout CD8 T cells did not have a greater decrease in granzyme B than a single knockout alone for either culture condition.

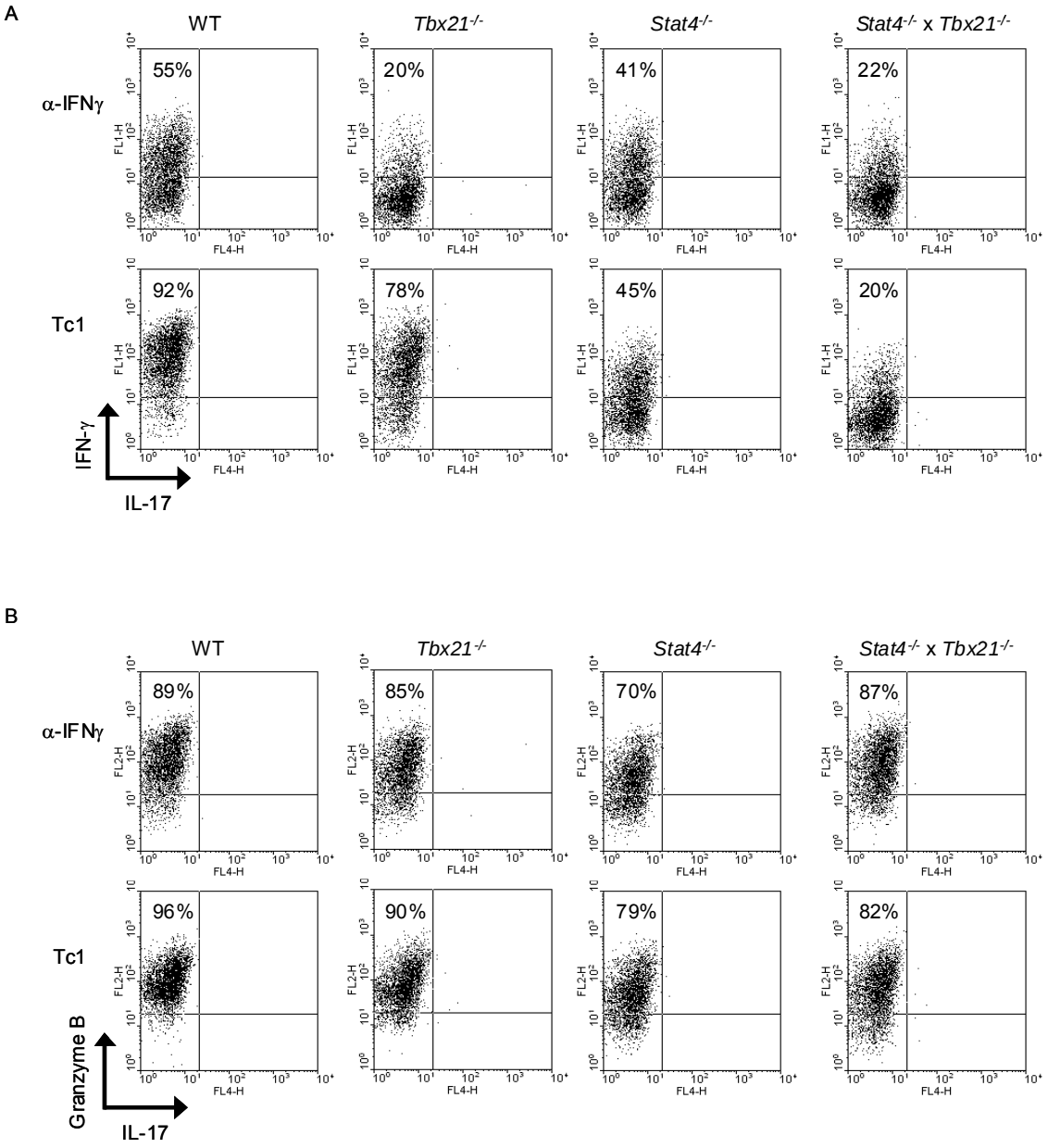


Figure 30. Optimal Tc1 development requires non-redundant Stat4 and T-bet signals. (A,B) WT, *Tbx21*$^{-/-}$, *Stat4*$^{-/-}$, or *Tbx21*$^{-/-}$ *x Stat4*$^{-/-}$ CD8^{+} T cells were stimulated for 5 days with α-IFN-γ alone or with IL-12 (Tc1). IL-17^{+}, IFN-γ^{+}, and granzyme B^{+} cells were detected by intracellular cytokine staining. Data are representative of 2 experiments.

As we analyzed the role of Stat4 and T-bet in IFN-γ production, we also wanted to determine T-bet and Eomes expression levels in the cells from the previous figure. Consistent with a role for T-bet in upregulating IFN-γ during neutralizing conditions, T-bet was induced to high levels in this condition and further induced in the presence of IL-12 (Fig 31A). T-bet expression was not dependent on Stat4 in neutralizing conditions, but was partially Stat4 dependent in Tc1 conditions. Eomes expression meanwhile had the highest expression level in neutralizing conditions and was inhibited by IL-12, consistent with previous reports. In neutralizing conditions, Eomes showed partial dependence on both T-bet and Stat4 (Fig 31B).

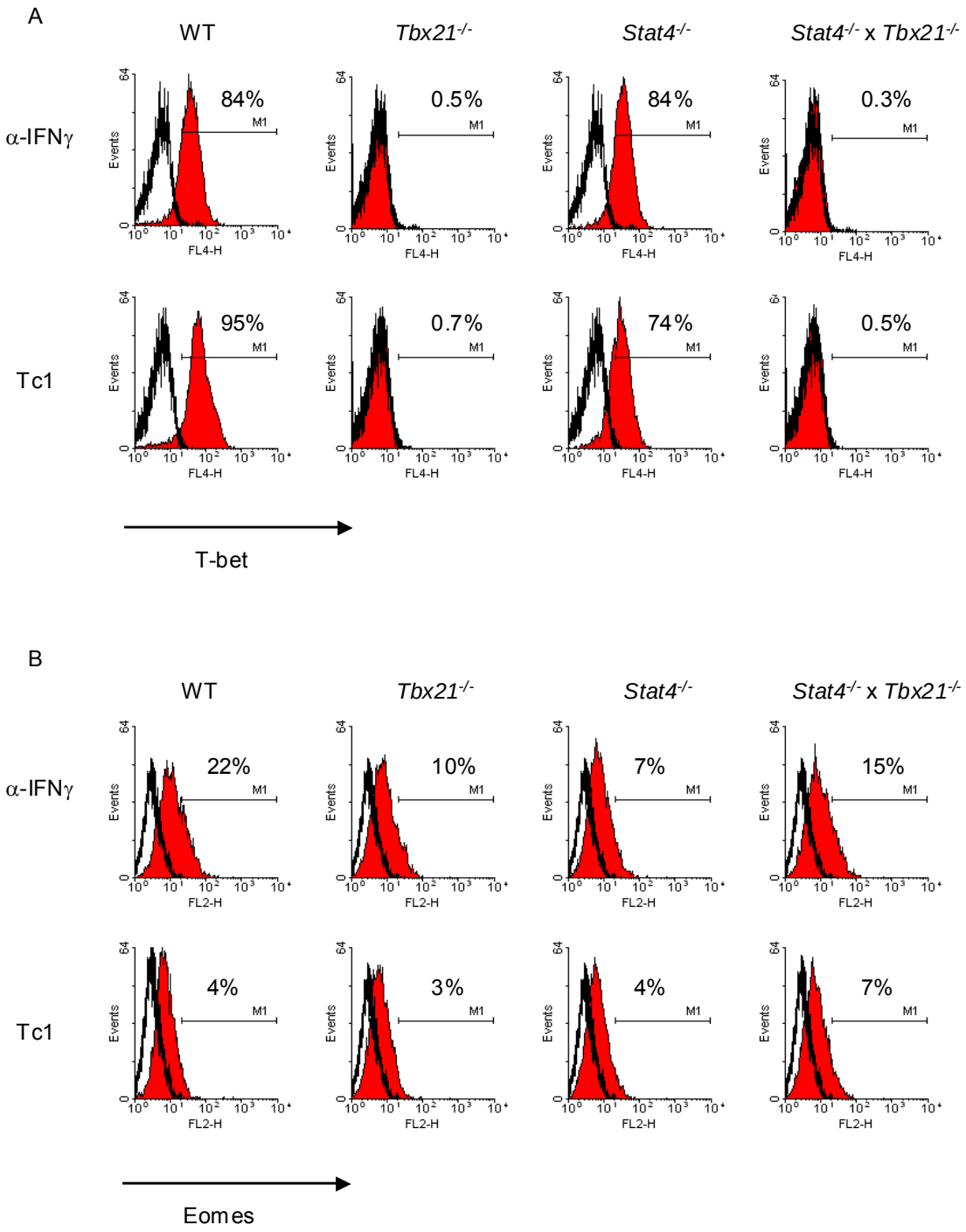
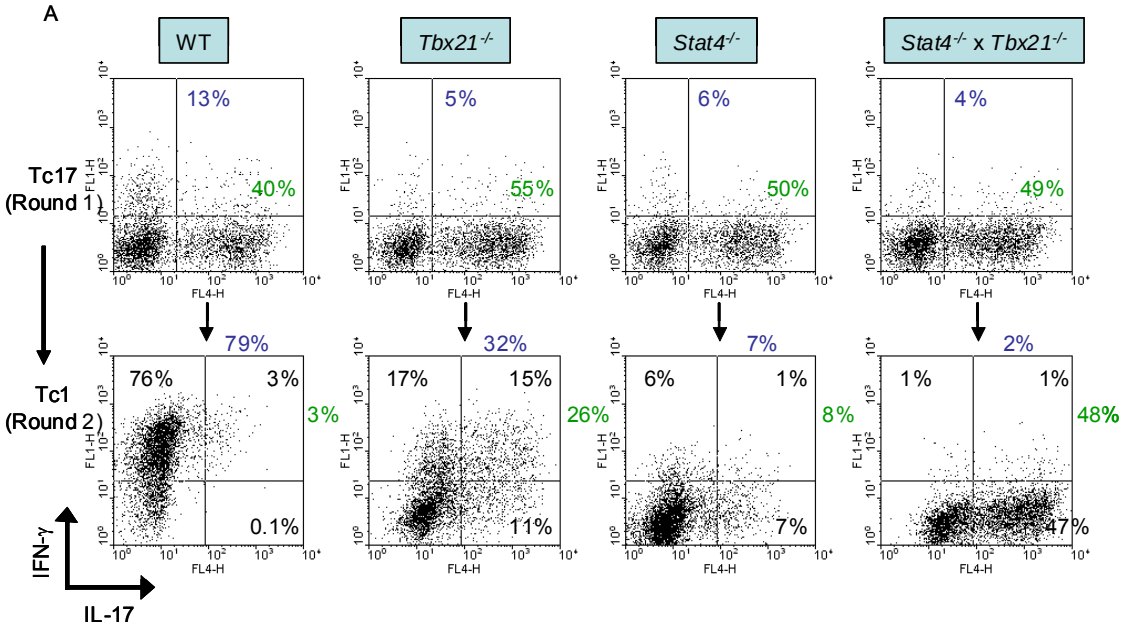


Figure 31. Role of T-bet and/or Stat4 in the expression of CTL transcription factors. WT, *Tbx21*$^{-/-}$, *Stat4*$^{-/-}$, or *Tbx21*$^{-/-}$ *x Stat4*$^{-/-}$ CD8^{+} T cells were stimulated for 5 days in the presence of α-IFN-γ alone or with IL-12 (Tc1). (A) T-bet and (B) Eomes were detected by intracellular cytokine staining. Data are representative of 2 experiments.

Previously, we observed that both of these transcription factors were important for optimal IFN-γ production from Tc1 cells, however in two week Tc17 cultures, IL-12 induced IFN-γ without an induction in *Tbx21* RNA. Therefore, we wanted to determine the requirements of Stat4 and T-bet in IFN-γ induction from Tc17→Tc1 cultures using cells deficient in these transcription factors. WT, *Tbx21*$^{-/-}$, *Stat4*$^{-/-}$, and *Tbx21 x Stat4* double knockout CD8 T cells were differentiated into Tc17 cells. After 5 days, Tc17 cells were restimulated in a neutralizing condition (α-IFN-γ) or in a Tc1 inducing condition (IL-12) and cultured for an additional 5 days before analysis. Previously, Tc17 cells stimulated in neutralizing conditions did not upregulate IFN-γ to a significant degree (Fig 26A), and the absence of T-bet and/or Stat4 did not affect the basal percentage of cells producing IFN-γ in Tc17→α-IFN-γ cultures (data not shown). However, when Tc17 cells were restimulated in the presence of IL-12, the increase of IFN-γ-secreting cells was impaired in T-bet deficient Tc17 cells (79% vs. 32%) and was further impaired in Stat4 deficient Tc17 cells (79% vs. 7%) (Fig 32A). Double knockout Tc17 populations developed almost no cells secreting IFN-γ when switched in Tc1 conditions. The importance of these factors was reversed in regards to IL-17 inhibition – IL-17 was mostly inhibited in the absence of Stat4, while T-bet deficiency had less IL-17 inhibition (3% vs. 26%), and in the absence of both transcription factors, IL-17 was no longer inhibited when switched in Tc1 conditions (Fig 32A). These data suggest that in the presence of IL-12, Tc17 cells induce IFN-γ and turn off IL-17 production through both Stat4 and T-bet signals.

T-bet levels were also analyzed in these cells and were found to have low expression in both five day differentiated Tc17 cells and Tc17→α-IFN-γ conditions. T-bet was greatly induced in Tc17→Tc1 conditions and this induction was decreased in the absence of Stat4 (Fig 32B).


A
WT
Tbx21-/-
Stat4-/-
Stat4-/- x Tbx21-/-
Tc17 (Round 1)
Tc1 (Round 2)
13%
40%
5%
55%
6%
50%
4%
49%
79%
3%
76%
3%
0.1%
32%
17%
15%
11%
7%
6%
1%
7%
2%
1%
1%
47%
IFN-γ
IL-17
FL1-H
FL4-H

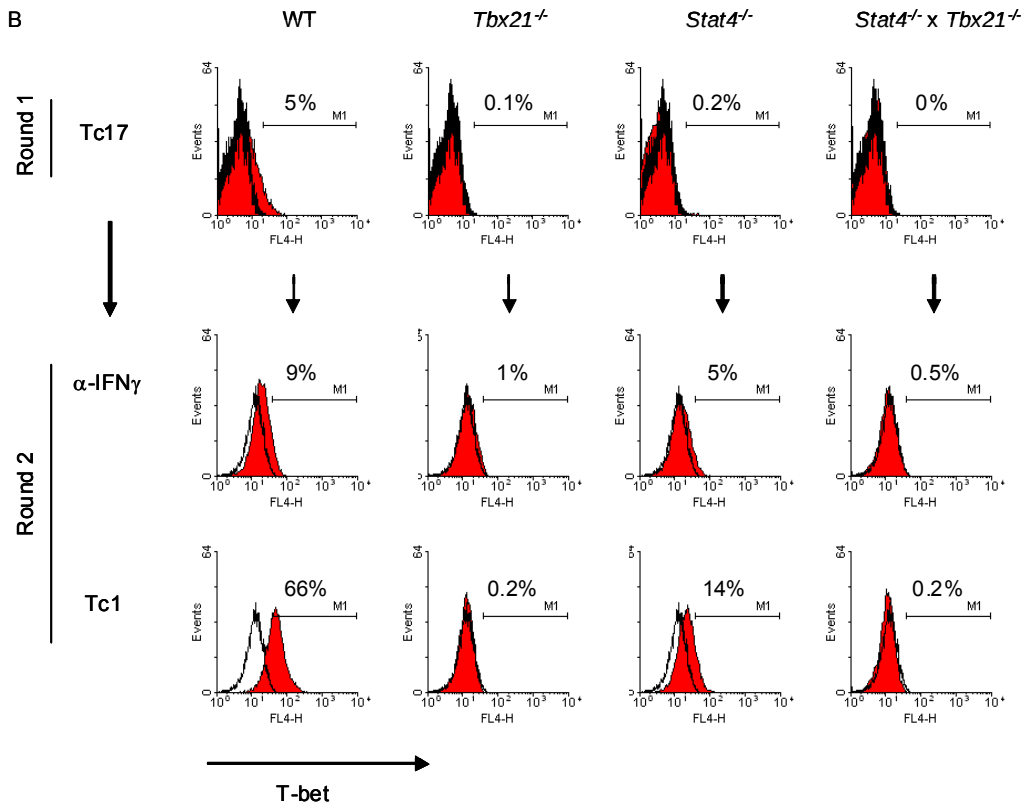

B
WT
Tbx21-/-
Stat4-/-
Stat4-/- x Tbx21-/-
Round 1
Tc17
5%
0.1%
0.2%
0%
Round 2
α-IFNγ
9%
1%
5%
0.5%
Tc1
66%
0.2%
14%
0.2%
M1
Events
FL4-H
T-bet

Figure 32. Tc17 plasticity is dependent on both Stat4 and T-bet. (A,B) WT, *Tbx21*$^{-/-}$, *Stat4*$^{-/-}$, or *Tbx21*$^{-/-}$ *x Stat4*$^{-/-}$ CD8^{+} T cells were stimulated in Tc17 conditions. After five days, Tc17 cells were restimulated in the presence of α-IFN-γ or IL-12 alone for an additional five days. IL-17, IFN-γ, and T-bet were detected using intracellular cytokine staining after the first round of stimulation (Day 5) and also at the end of the second round of stimulation (Day 10). Numbers in blue indicate total percentage of IFN-γ detected and numbers in green indicate total percentage of IL-17 detected. Data are representative of 2 experiments.

In Fig 30B, T-bet played a minimal role in optimal granzyme B expression in Tc1 cells, but Stat4 was required. However T-bet correlated with granzyme B induction in two week Tc17 cultures (Figs 27 and 29). Therefore, we wanted to determine the requirements of Stat4 and T-bet in granzyme B induction from Tc17→Tc1 cultures using cells deficient in these transcription factors. Gene deficient CD8 T cells were differentiated into Tc17 cells and after 5 days restimulated with neutralizing IFN-γ antibodies or with IL-12 and cultured for an additional 5 days before analysis. WT Tc17 cells restimulated for a second week in neutralizing conditions upregulated granzyme B and this upregulation was not dependant on T-bet or Stat4 (Fig 33). Granzyme B was further induced in WT Tc17 cells when they were incubated with IL-12 for a second week of culture. IL-12 induced granzyme B was dependent on both T-bet and Stat4 as Tc1 cells induced granzyme B to similar percentages as α-IFN-γ conditions in the absence of these factors. Therefore, TCR induction of granzyme B is increased with IL-12→Stat4 and IL-12→T-bet signals.

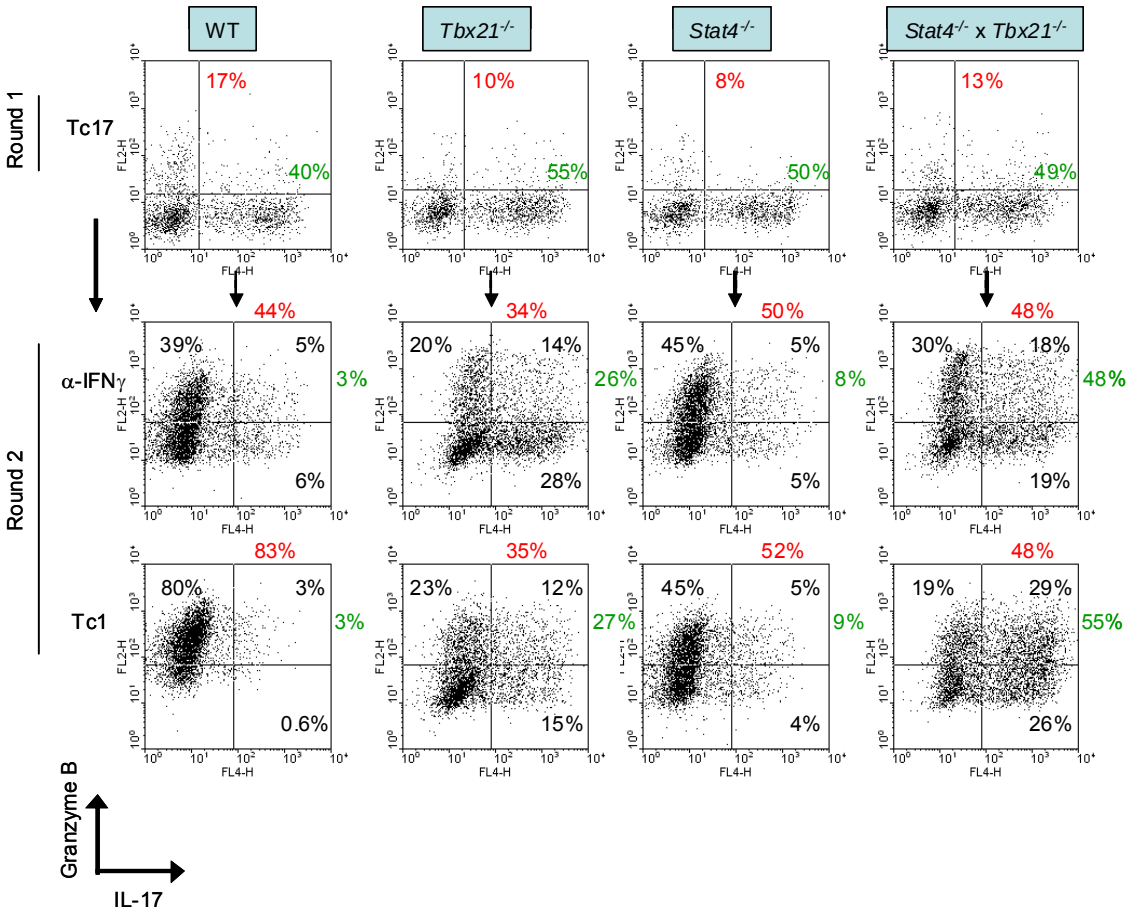


Figure 33. Optimal granzyme B induction in switched Tc17 cells is dependent on both Stat4 and T-bet. (A,B) WT, *Tbx21*$^{-/-}$, *Stat4*$^{-/-}$, or *Tbx21*$^{-/-}$ *x Stat4*$^{-/-}$ CD8^{+} T cells were stimulated in Tc17 conditions. After five days, Tc17 cells were restimulated in the presence α-IFNγ or IL-12 for an additional five days. Granzyme B and IL-17 were detected using intracellular cytokine staining after the first round of stimulation (Day 5) and at the end of the second round of stimulation (Day 10). Numbers in red indicate total percentage of granzyme B detected and numbers in green indicate total percentage of IL-17 detected. Data are representative of 2 experiments.

Tc17 cells exist in WT mice

Tc17 cells are easily induced in vitro upon stimulation in the presence of TGFβ and IL-6. To determine if IL-17-secreting CD8 T cells exist in vivo, CD8 T cells were isolated from the spleens and lymph nodes of Balb/c, C57Bl/6 and C3H mice and stimulated with anti-CD3. We hypothesized that Balb/c CD8 T cells would produce higher amounts of IL-17 than C57Bl/6 CD8 T cells because Balb/c mice are less prone to produce IFN-γ. We were also interested in C3H mice because IL-17 has been correlated to tumor development and C3H mice are prone to develop mammary tumors. IL-17 was detectable from splenic CD8 T cells of all mouse strains tested and surprisingly, C57Bl/6 CD8 T cells exhibited the highest IL-17 expression (Fig 34A). C57Bl/6 splenic CD8 T cells also produced the highest amounts of IFN-γ. In general, CD8 T cells isolated from the lymph nodes produced lower amounts of both IL-17 and IFN-γ compared to splenic CD8 T cells possibly due to increased naïve and decreased memory CD8 T cells in the lymph node (Fig 34B). With respect to lymph node CD8 T cells, the most IL-17 was produced by C57Bl/6 CD8 T cells and the most IFN-γ produced by C3H CD8 T cells. These data demonstrate that Tc17 cells exist in vivo and C57Bl/6 mice were used in future experiments due to their higher levels of IL-17-secreting CD8 T cells.

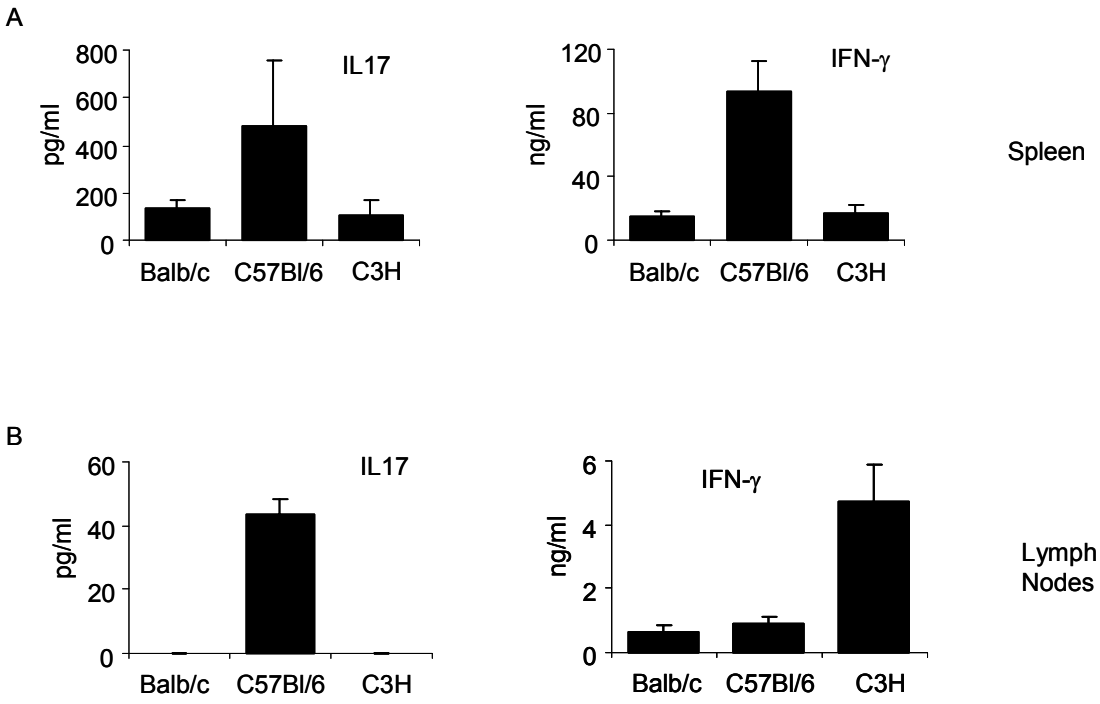


Figure 34. IL-17-secreting CD8 T cells exist in vivo. CD8+ T cells were isolated from the (A) spleen or (B) lymph nodes of Balb/c, C57Bl/6, or C3H wild type mice. CD8+ T cells were stimulated for 48 hrs and cell free supernatants were used to measure IL-17 and IFN-γ protein levels by ELISA assay. Results are presented as the mean ± SEM of CD8+ T cells from 3 individual mice. Data are representative of 2 experiments.

Vaccinia virus infection induces Tc17 cells

Since Tc17 cells exist in vivo, we next wanted to determine the ability of a viral infection to induce Tc17 cells. Vaccinia virus activates CD8 T cells upon infection and also interferes with IFN-γ signaling to evade the host immune system. To determine if vaccinia virus infection induces Tc17 development, mice were infected with vaccinia virus and CD4 and CD8 T cell populations were isolated at various time points to determine cytokine production. Tc17 cells increased approximately 3 fold after 14 days of a vaccinia virus infection before returning to baseline levels (Fig 35A). IL-17-secreting CD4 T cells meanwhile never significantly rose above baseline levels and were inhibited for the first week of infection. IFN-γ plays an important role in the control of vaccinia virus and was induced in both CD8 and CD4 T cells with a peak at Day 10 post-infection. The increase in Tc17 development and IFN-γ production correlated with a decrease in vaccinia virus detected in the ovaries of infected mice (Fig 35B).

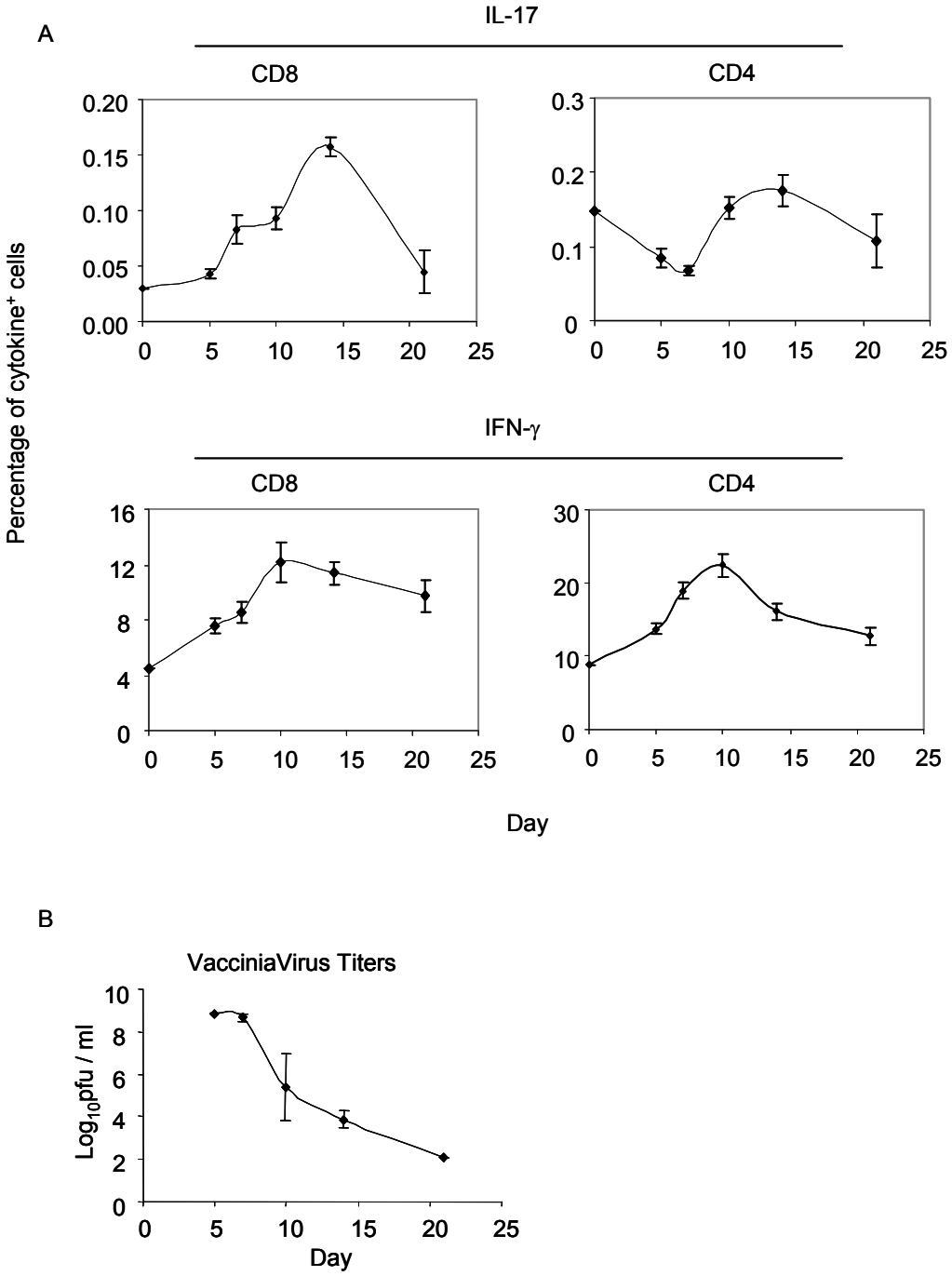


Figure 35. Vaccinia virus induces Tc17 development and IFN-γ production from T cells. C57Bl/6 mice were infected i.p. with $5x10^6$ pfu vaccinia virus per mouse (Day 0). 5, 7, 10, 14, and 21 days postinfection mice were sacrificed and ovaries and spleens isolated. (A) CD8 and CD4 cells were isolated from the spleens and restimulated. IL-17 and IFN-γ was detected using ICS. (B) Viral titers were measured from the ovaries of infected mice. Data corresponds to four mice per time point ± SEM.

Previous reports have shown that CD8 T cells are dispensable for primary vaccinia virus infection, but are important in the memory response to vaccinia re-infection (Xu et al., 2004). To determine if Tc17 cells develop in a memory response, we infected mice with vaccinia virus and re-infected the mice 3 weeks after primary infection. After another 5 days, cytokine production from T cells was analyzed. Mice infected twice with vaccinia virus induced IL-17 and granzyme B from CD8 T cells and IFN-γ from CD4 T cells (Fig 36). Smaller or insignificant changes were seen in IL-17 and granzyme B from CD4 T cells and IFN-γ from CD8 T cells. Therefore, Tc17 cells are induced in a memory response to vaccinia virus infection in addition to the primary response to vaccinia observed in the previous figure.

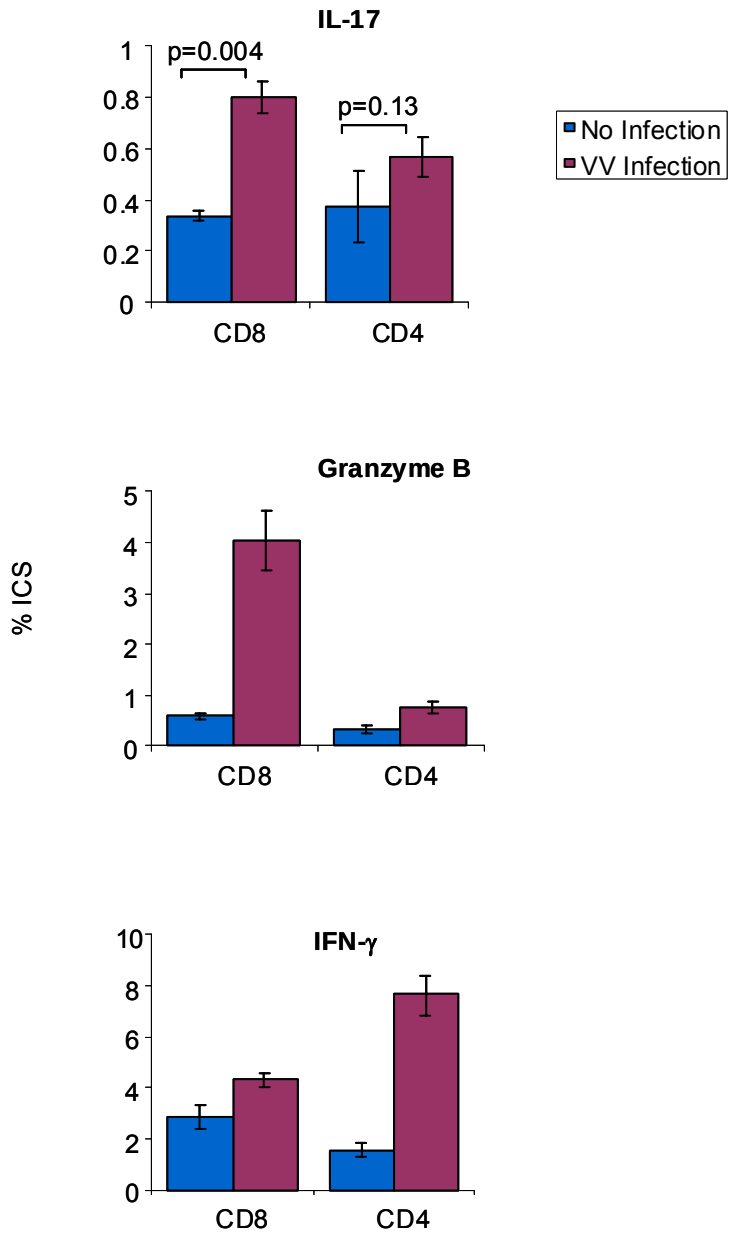


Figure 36. Tc17 cells are induced in a recall response. C57Bl/6 mice were infected once on Day 0 and infected again 3 wks after the primary infection with $1x10^6$ pfu vaccinia virus per mouse i.p each time. Five days after the second infection, mice were sacrificed. CD8 and CD4 cells were isolated from the spleens and restimulated before analyzing IL-17 and IFN-γ production using ICS. Data corresponds to 2-4 mice ± SEM.

Induction of Vaccinia virus specific Tc17 cells

B8R is a vaccinia virus protein which contains immunodominant epitopes and functions as a soluble IFN-γ receptor (Seet et al., 2003). To determine if vaccinia virus can induce virus specific Tc17 cells, we analyzed cytokine response upon restimulation with a B8R peptide. Mice were infected for one week before isolating CD8 T cells and stimulating with the B8R peptide. B8R stimulated CD8 T cells did not produce IL-17 or IFN-γ, however B8R stimulated CD8 T cells produced similar amounts of granzyme B as polyclonally stimulated CD8 T cells (Fig 37). B8R is only one of the immunodominant epitopes of vaccinia virus and it is possible that other vaccinia virus epitopes are capable of inducing the Tc17 cells seen after infection.

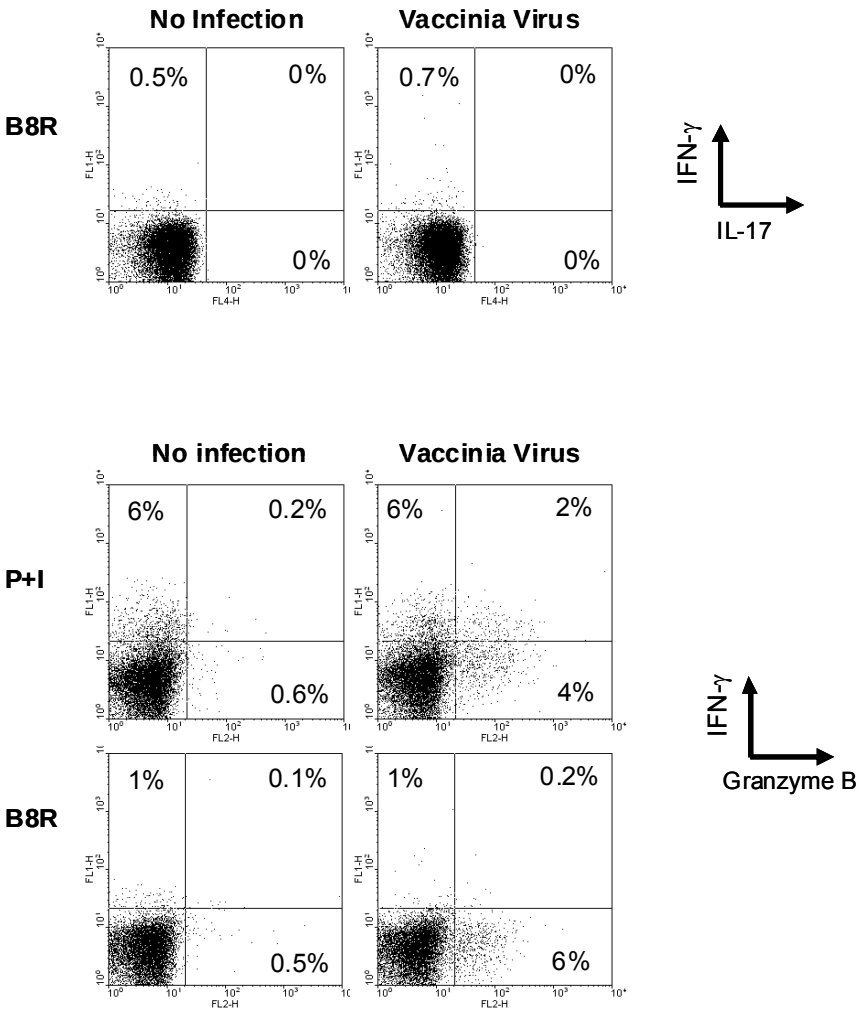


Figure 37. Vaccinia virus B8R peptide does not induce Tc17 cells. C57Bl/6 mice were infected i.p. with $1x10^6$ pfu vaccinia virus per mouse and sacrificed after 7 days of infection. CD8+ T cells were isolated from the spleen and restimulated with B8R peptide or PMA + Ionomycin (P+I). IL-17, IFN-γ, and granzyme B were detected using ICS. Data are representative of 2 experiments.

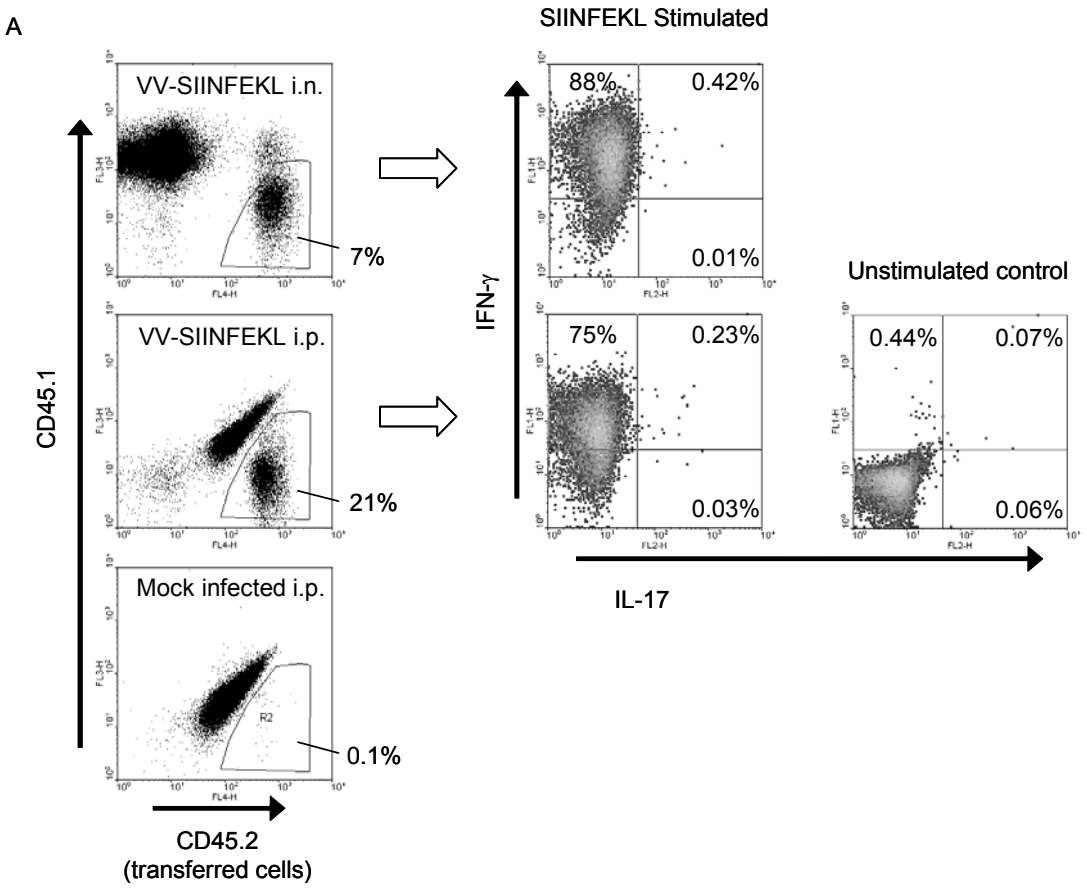
To further study antigen specific Tc17 cells in response to vaccinia virus, we utilized a vaccinia virus overexpressing an ova peptide with the sequence SIINFEKL (VV-SIINFEKL). CD45.1 congenic mice were injected with naïve OT-I cells (CD45.2), infected with VV-SIINFEKL either i.p. or i.n. one day later, and sacrificed five days after infection. After infection, the OT-I cells proliferated several fold (Fig 38A, 7% and 21% vs. 0.1%). Infection induced the OT-I cells into a predominantly Tc1 phenotype but also induced a small population of cells producing both IL-17 and IFN-γ (Fig 38A). The induction in SIINFEKL specific IL-17 and IFN-γ-secreting CD8 T cells was significant after both i.n. and i.p. infection (Fig 38B).

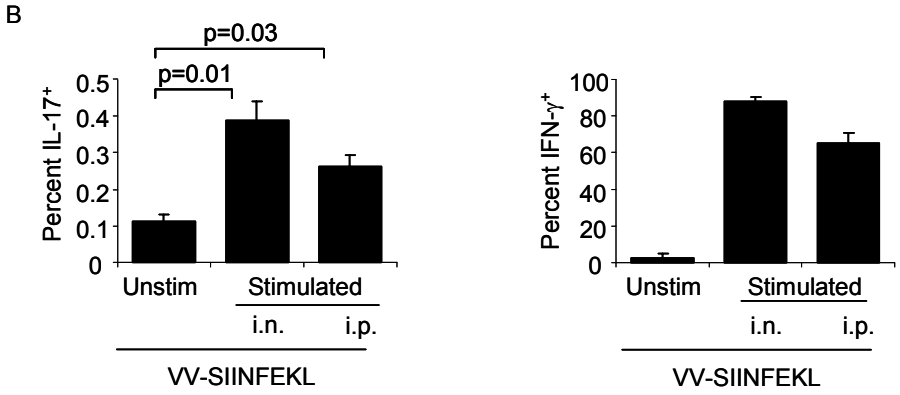
SIINFEKL specific IL-17 and IFN-γ producing cells was confirmed using an ELISA assay. Total splenocytes obtained in Fig 37A were incubated with SIINFEKL peptide for 48 hrs and cell free supernatants were analyzed for IL-17 and IFN-γ cytokines. Antigen specific secretion of IL-17 and IFN-γ were significantly higher in the splenocytes from infected mice compared to control mice given PBS (Fig 38C).

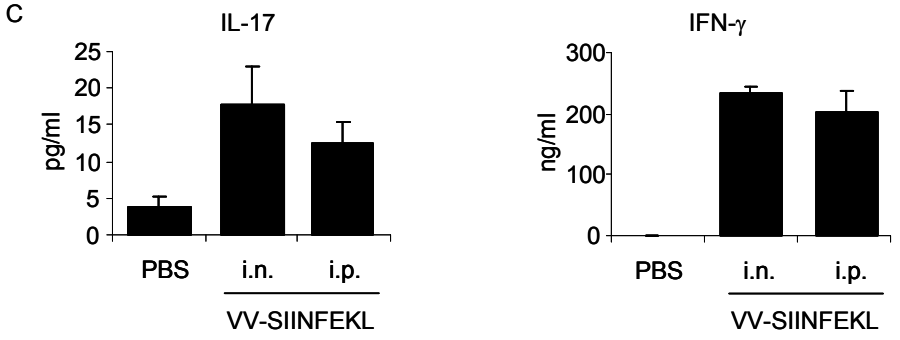
Vaccinia virus infection induces a heterogeneous CD8 T cell response. We wanted to analyze induction of additional proinflammatory cytokines and cytotoxic molecules and found antigen specific induction of both TNFα and granzyme B after VV-SIINFEKL infection. Similar to IL-17 and IFN-γ, infection via

the i.n. route induced higher percentages of both proteins compared to i.p. infection (Fig 38D).

To determine if OT-I cells found in the draining lymph nodes of i.n. infected mice produce higher percentage of cytokines as OT-I cells found in the spleen, we also analyzed the mediastinal lymph node. Mediastinal lymph nodes, compared to the spleen, contained a similar percentage of OT-I cells after i.n. infection and a lower percentage of OT-I cells after i.p. infection (Fig 38E vs. 38A). In addition, after an i.n. infection with VV-SIINFEKL, OT-I cells specifically trafficked to the mediastinal lymph nodes since they were not found in the distant inguinal lymph node (data not shown). OT-I cells in the mediastinal lymph node produced lower amounts of IFN-γ than in the spleen and IL-17 was not detected at this location.


A
SIINFEKL Stimulated
VV-SIINFEKL i.n.
7%
VV-SIINFEKL i.p.
21%
Mock infected i.p.
0.1%
CD45.1
CD45.2
(transferred cells)
IFN-γ
IL-17
88%
0.42%
0.01%
75%
0.23%
0.03%
Unstimulated control
0.44%
0.07%
0.06%


B
p=0.03
p=0.01
Percent IL-17+
Unstim
Stimulated
i.n.
i.p.
VV-SIINFEKL
Percent IFN-γ+


C
IL-17
pg/ml
PBS
i.n.
i.p.
VV-SIINFEKL
IFN-γ
ng/ml

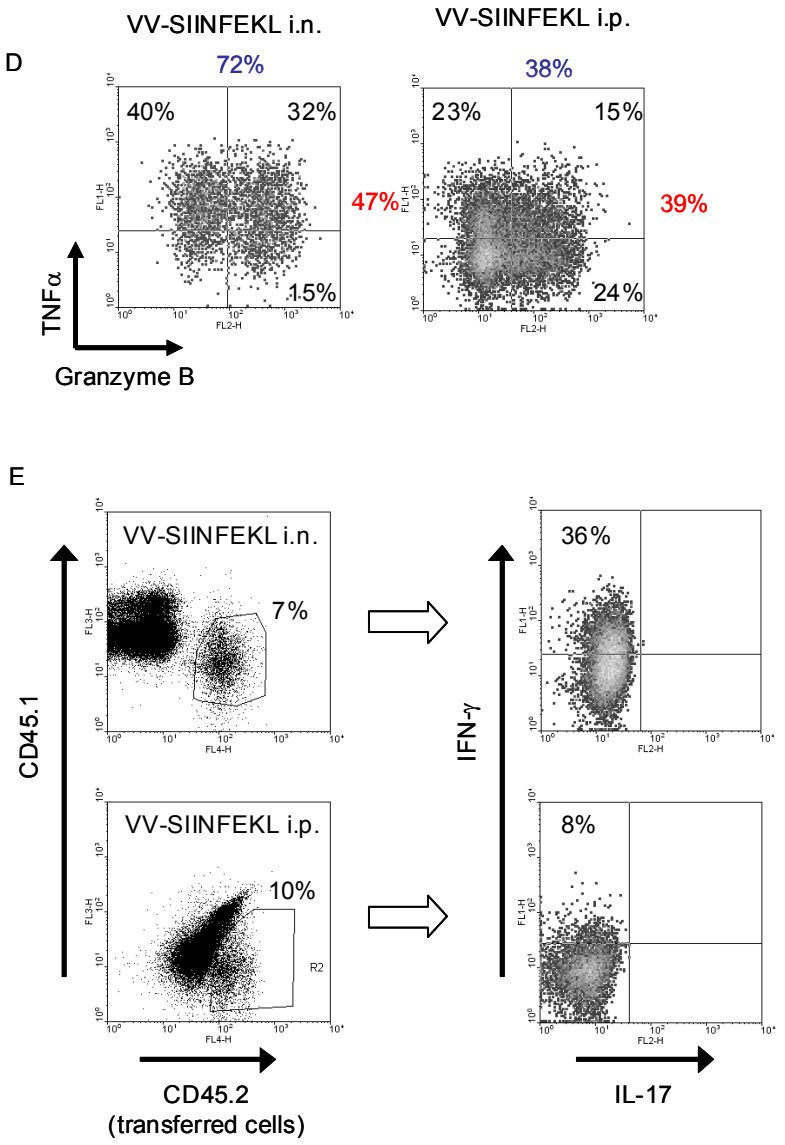


Figure 38. VV-SIINFEKL infection induces antigen specific Tc17 cells. BoyJ homozygous (CD45.1$^+$) or heterozygous (CD45.1$^+$CD45.2$^+$) mice were injected i.v. with 1x10^6 OT-I / *Rag1*$^{-/-}$ CD8 T cells. One day later, mice were infected with either 2x10^6 pfu VV-SIINFEKL i.n. or 2x10^7 pfu VV-SIINFEKL i.p. Five days after infection, (A) splenocytes were isolated and stimulated with SIINFEKL peptide or left unstimulated for a staining control. IL-17$^+$ and IFN-γ$^+$ cells were detected using ICS and panels on the right are gated on CD45.1$^-$CD45.2$^+$ cells. (B) Data from part (A) are summarized as the average ± SEM from 5 mice. (C) Splenocytes were stimulated with SIINFEKL peptide for 48 hrs and cell free supernatants were used to measure IL-17 and IFN-γ protein levels by ELISA assay. (D) TNFα$^+$ and Granzyme B$^+$ cells were detected using ICS and plots are gated on CD45.1$^-$CD45.2$^+$ cells. (E) Mediastinal lymph nodes were isolated and stimulated to detect IFN-γ and IL-17 using ICS. Plots on the right are gated on CD45.1$^-$CD45.2$^+$ cells. Data corresponds to 5 mice.

Along with viral titers in target organs, weight loss is also often used to determine the degree of vaccinia virus infection (Verardi et al., 2001; Xu et al., 2004; Jacobs et al., 2006). To determine if weight loss could be utilized in our system as a non-terminal measure of viral infection, we tracked weight changes in the mice from the previous experiments. After transfer of naïve OT-I cells and infection with VV-SIINFEKL (Fig 38), mice began to lose weight immediately when infected intraperitoneally, and began to lose weight after 3 days when infected intranasally (Fig 39A). These weight changes correlated with high viral titers in all infected mice (data not shown). Mice given no infection gained weight from the start of the experiment. When examining longer time points after infection with $5x10^6$ pfu per mouse i.p. (Fig 35), infected mice initially lost weight, but this trend was reversed around 5 days post-infection when infected mice began to gain weight again (Fig 39B). This data suggested that viral titers could possibly be correlated to initial weight loss. To further examine this correlation, we compared viral titers when mice were actively clearing virus (Day 10 and 14, Fig 35B), with their weight loss on Day 5 (Fig 39B). The viral titers on Day 10 had an increasing trend with increasing weight loss on Day 5 (Fig 39C) as expected; however, viral titers on Day 14 had a decreasing trend compared to increasing weight loss on Day 5. Weight loss also did not correlate well with experiments done with VV-SIINFEKL; therefore, viral titers in the target organs were exclusively utilized in future experiments to determine the degree of vaccinia virus infection.

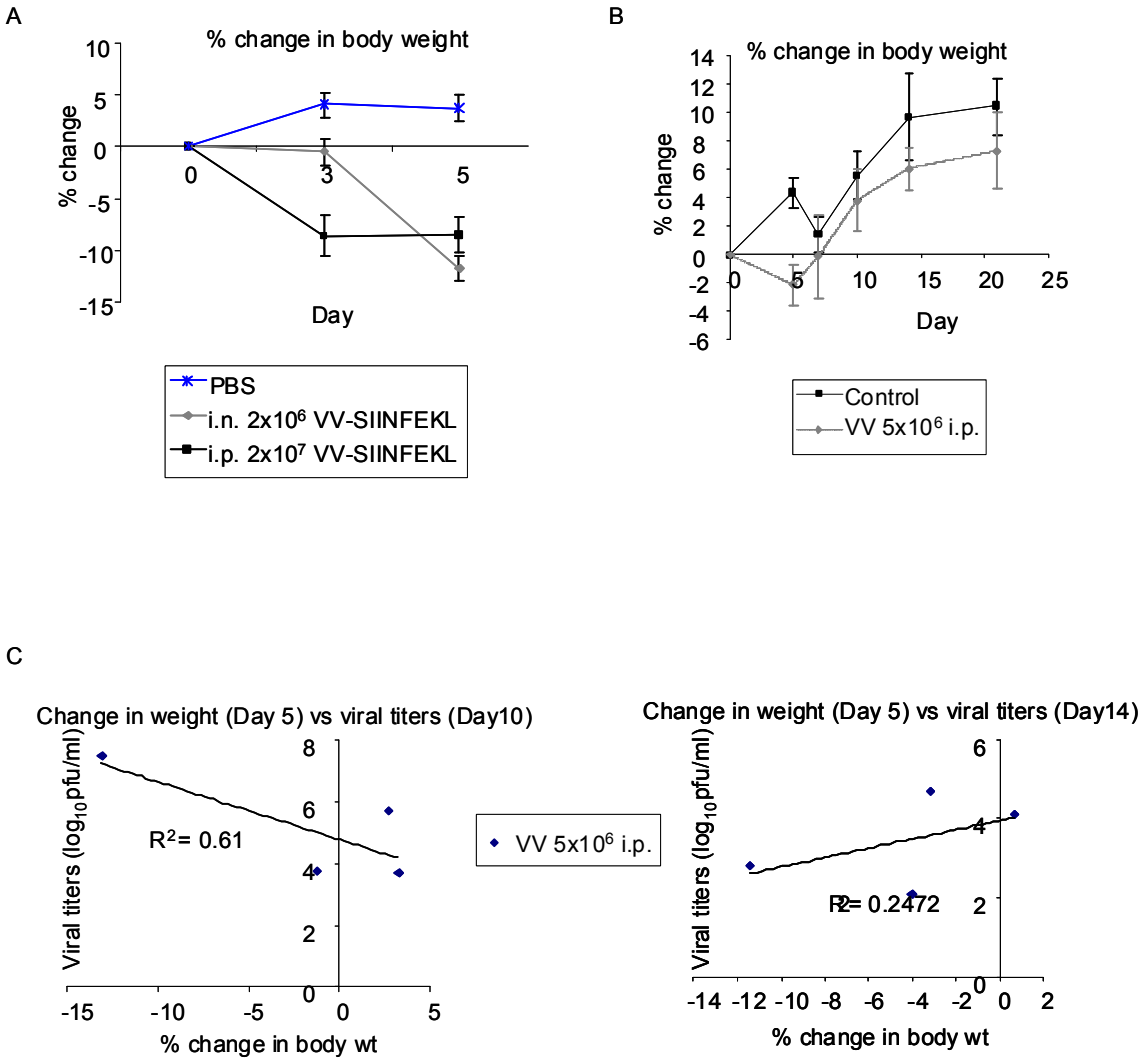


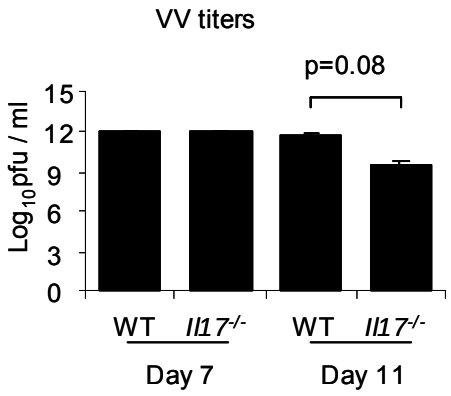
Figure 39. Weight loss induced by vaccinia infection does not correlate with viral titers. (A) Mice were injected with OT-I / *Rag1*$^{-/-}$ CD8 T cells i.v. and infected with VV-SIINFEKL one day later as described in Fig 38. Mice were weighed on Days 0, 3, and 5 and weight change was determined compared to weight of the same mouse on Day 0. Data represent the average ± SEM from 5 mice. (B) Mice were infected with VV i.p. as described in Fig 35 and weighed on Days 0, 5, 7, 10, 14, and 21. Weight change was determined compared to weight of the same mouse on Day 0. Data represent the average ± SEM from 4 mice. (C) Data represent the change in body weight on Day 5 post-infection versus viral titers on Day 10 or Day 14. R^2 values were determined from the best fit line.

Role of IL17 in vaccinia virus clearance

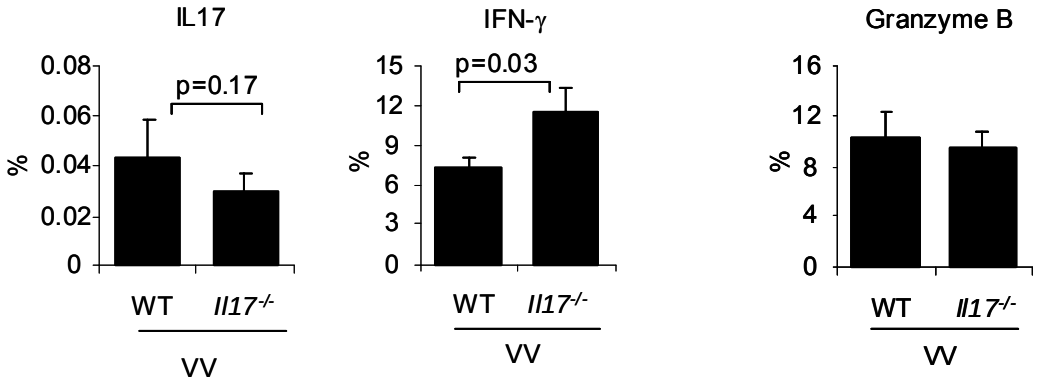
Previously, we demonstrated that antigen specific Tc17 cells are induced during a vaccinia virus infection. To begin to understand the role of IL-17 in the immune response against vaccinia virus, we infected various gene deficient mice with defective or enhanced IL-17 production and analyzed viral clearance.

First, we analyzed the ability of *Il17a*$^{-/-}$ (also referred to as *Il17*$^{-/-}$) mice to clear vaccinia virus. *Il17*$^{-/-}$ had similar viral titers 7 days after infection and lower viral titers 11 days after infection (Fig 40A) suggesting that IL-17 inhibits the host response to eliminate virus. CD8 T cells in these mice had significantly low levels of IL-17 (using ELISA analysis) and increased IFN-γ (Fig 40B). A significant decrease in IL-17 production from CD8 T cells was not detected using ICS possibly due to the lower sensitivity of this assay. Granzyme B was not affected by the absence of IL-17 and IL-17F was increased in the CD8 T cells (Fig 40B). IL-21 was not detected from the CD8 T cells analyzed (data not shown). CD4 T cells in *Il17*$^{-/-}$ mice infected with vaccinia virus also have minimal IL-17 as expected (Fig 40C). IFN-γ, granzyme B, and IL17F are not significantly affected and IL-21 is increased in the CD4 T cells. In addition to the deficiency in IL-17A, the altered regulation of several cytokines in *Il17*$^{-/-}$ mice after vaccinia virus infection could contribute to their increased anti-vaccinia response.

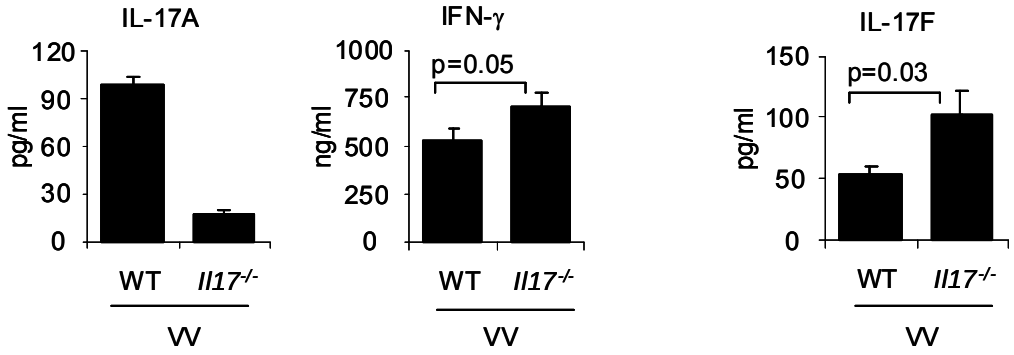
A


VV titers
p=0.08
Log10pfu / ml
15
12
9
6
3
0
WT
Il17-/-
WT
Il17-/-
Day 7
Day 11

B


IL17
p=0.17
%
0.08
0.06
0.04
0.02
0
WT
Il17-/-
VV
IFN-γ
p=0.03
%
15
12
9
6
3
0
WT
Il17-/-
VV
Granzyme B
%
16
12
8
4
0
WT
Il17-/-
VV

CD8


IL-17A
pg/ml
120
90
60
30
0
WT
Il17-/-
VV
IFN-γ
p=0.05
ng/ml
1000
750
500
250
0
WT
Il17-/-
VV
IL-17F
p=0.03
pg/ml
150
100
50
0
WT
Il17-/-
VV

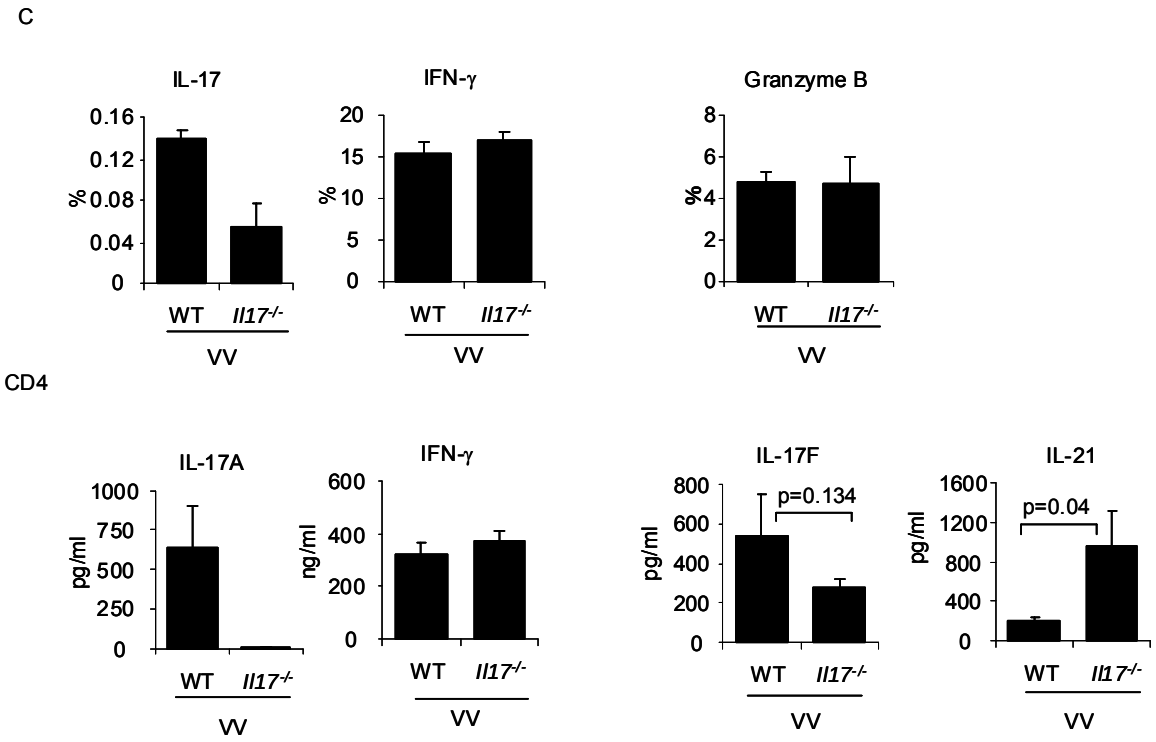
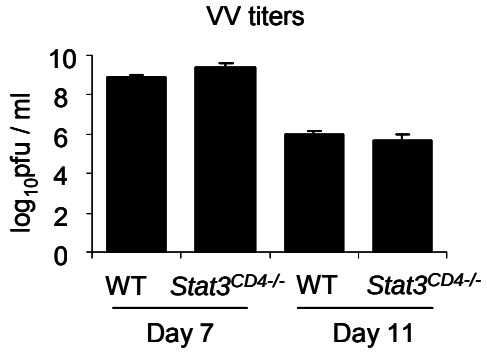


Figure 40. *Il17*$^{-/-}$ mice have increased clearance of vaccinia virus. (A) Balb/c or *Il17*$^{-/-}$ mice were infected with 5x10^6 pfu of vaccinia virus per mouse and sacrificed either 7 or 11 days after infection. Ovaries were harvested at the indicated time points for viral titers using a plaque forming assay. (B,C) Mice were infected with 1x10^6 pfu of vaccinia virus per mouse and sacrificed 7 days after infection. CD8+ (B) or CD4+ (C) cells were isolated from the spleen. Cells were either restimulated for 4 hrs with PMA + Ionomycin to analyze IL-17, IFN-γ, and granzyme B using ICS (top panels) or restimulated for 24 hrs with α-CD3 to generate cell free supernatants to analyze IL-17A, IL-17F, IL-21, and IFN-γ protein levels using an ELISA assay (bottom panels). Data corresponds to 4-5 mice ± SEM.

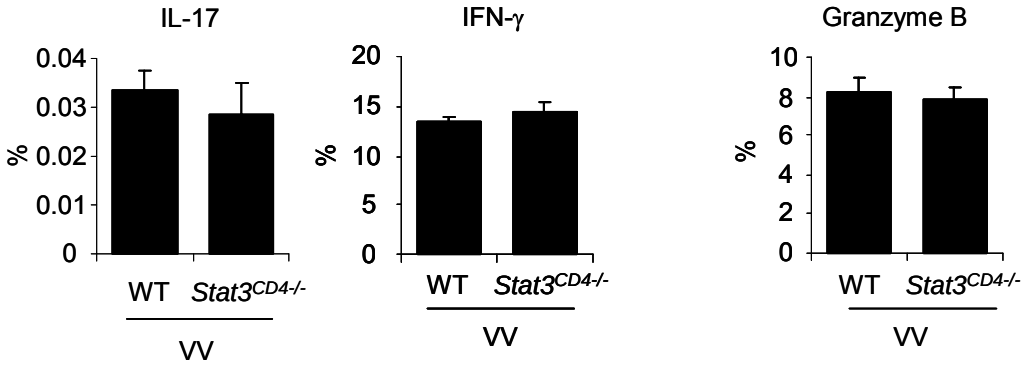
Role of Stat3 in vaccinia virus clearance

Since Stat3 is critical for IL-17 secretion from CD4 (Mathur et al., 2007; Yang et al., 2007a) and CD8 (Fig 11) T cells, we also analyzed the ability of *Stat3*$^{CD4-/-}$ mice to clear vaccinia virus. *Stat3*$^{CD4-/--}$ had a non-significant trend towards increased viral titers 7 days after infection and similar viral titers 11 days after infection (Fig 41A). CD8 T cells in these mice had low levels of IL-17A and IL-17F and no significant change in IFN-γ or granzyme B (Fig 41B). CD4 T cells in *Stat3*$^{CD4-/-}$ mice infected with vaccinia virus produce low levels of the Th17 cytokines IL-17A, IL-17F, and IL21 as expected (Fig 41C). Production of granzyme B in CD4 T cells of infected mice was not significantly changed; however, IFN-γ was increased. Altogether, the decreases in Tc17 and Th17 related cytokines and the increase in IFN-γ from CD4 T cells did not significantly affect the ability of Stat3 deficient mice to clear vaccinia virus.

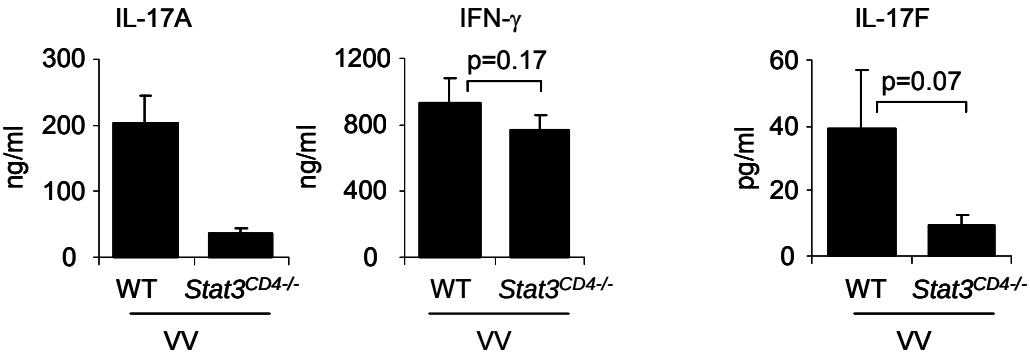
A


VV titers
log10pfu / ml
10
8
6
4
2
0
WT
Stat3CD4-/-
WT
Stat3CD4-/-
Day 7
Day 11

B


IL-17
IFN-γ
Granzyme B
%
0.04
0.03
0.02
0.01
0
20
15
10
5
0
10
8
6
4
2
0
WT
Stat3CD4-/-
VV

CD8


IL-17A
IFN-γ
IL-17F
ng/ml
pg/ml
300
200
100
0
1200
800
400
0
60
40
20
0
p=0.17
p=0.07
WT
Stat3CD4-/-
VV

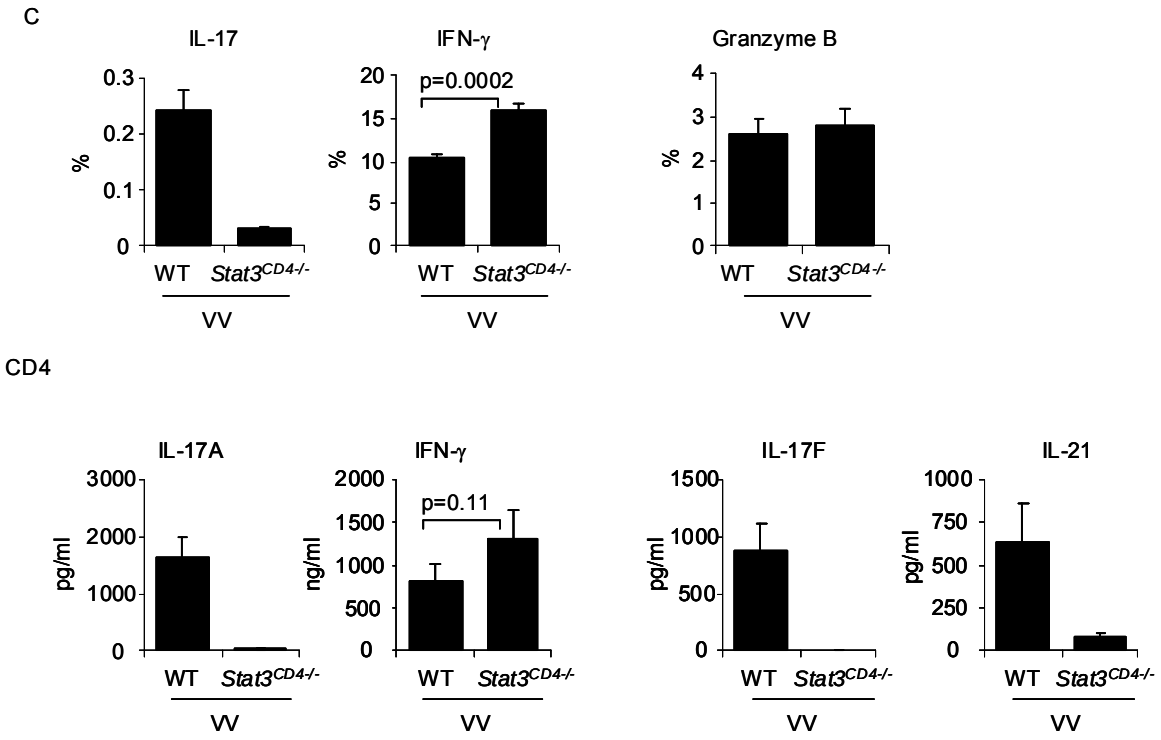


Figure 41. *Stat3*$^{CD4-/-}$ mice have normal clearance of vaccinia virus. (A) C57/Bl6 or *Stat3*$^{CD4-/-}$ mice were infected with 5x10^6 pfu of vaccinia virus per mouse and sacrificed either 7 or 11 days after infection. Ovaries were harvested at the indicated time points for viral titers using a plaque forming assay. (B,C) Mice were infected with 1x10^6 pfu of vaccinia virus per mouse and sacrificed 7 days after infection. CD8+ (B) or CD4+ (C) cells were isolated from the spleen. Cells were either restimulated for 4 hrs with PMA + Ionomycin to analyze IL-17, IFN-γ, and granzyme B using ICS (top panels) or restimulated for 24 hrs with α-CD3 to generate cell free supernatants to analyze IL-17A, IL-17F, IL-21, and IFN-γ protein levels using an ELISA assay (bottom panels). Data corresponds to 4-5 mice ± SEM.

Role of T-bet in vaccinia virus clearance

T-bet is a negative regulator of IL-17 production from CD4 and CD8 T cells (Harrington et al., 2005; Park et al., 2005 and Fig 11). We infected *Tbx21*$^{-/-}$ mice to determine if mice with increased IL-17 have altered clearance of vaccinia virus. *Tbx21*$^{-/-}$ mice had increased viral titers both 7 and 11 days after infection (Fig 42A). CD8 T cells in these mice had increased production of IL-17, decreased IFN-γ, and no change in granzyme B (Fig 42B). A previous report (Matsui et al., 2005) observed a decreased ability of *Tbx21*$^{-/-}$ mice to control vaccinia virus infection which was partially attributed to an increased production of IL-4 in addition to diminished function of VV specific CTLs. Consistent with this, we also observed an increased production of IL-4 from CD8 T cells in *Tbx21*$^{-/-}$ mice after vaccinia virus infection (Fig 42B). CD4 T cells in *Tbx21*$^{-/-}$ mice infected with vaccinia virus produce higher levels of IL-17, decreased IFN-γ, and no change in granzyme B (Fig 42C). Therefore, in addition to the changes in IFN-γ and IL-4 previously reported, IL17 induction may also contribute to the defective host response against vaccinia virus in *Tbx21*$^{-/-}$ mice.

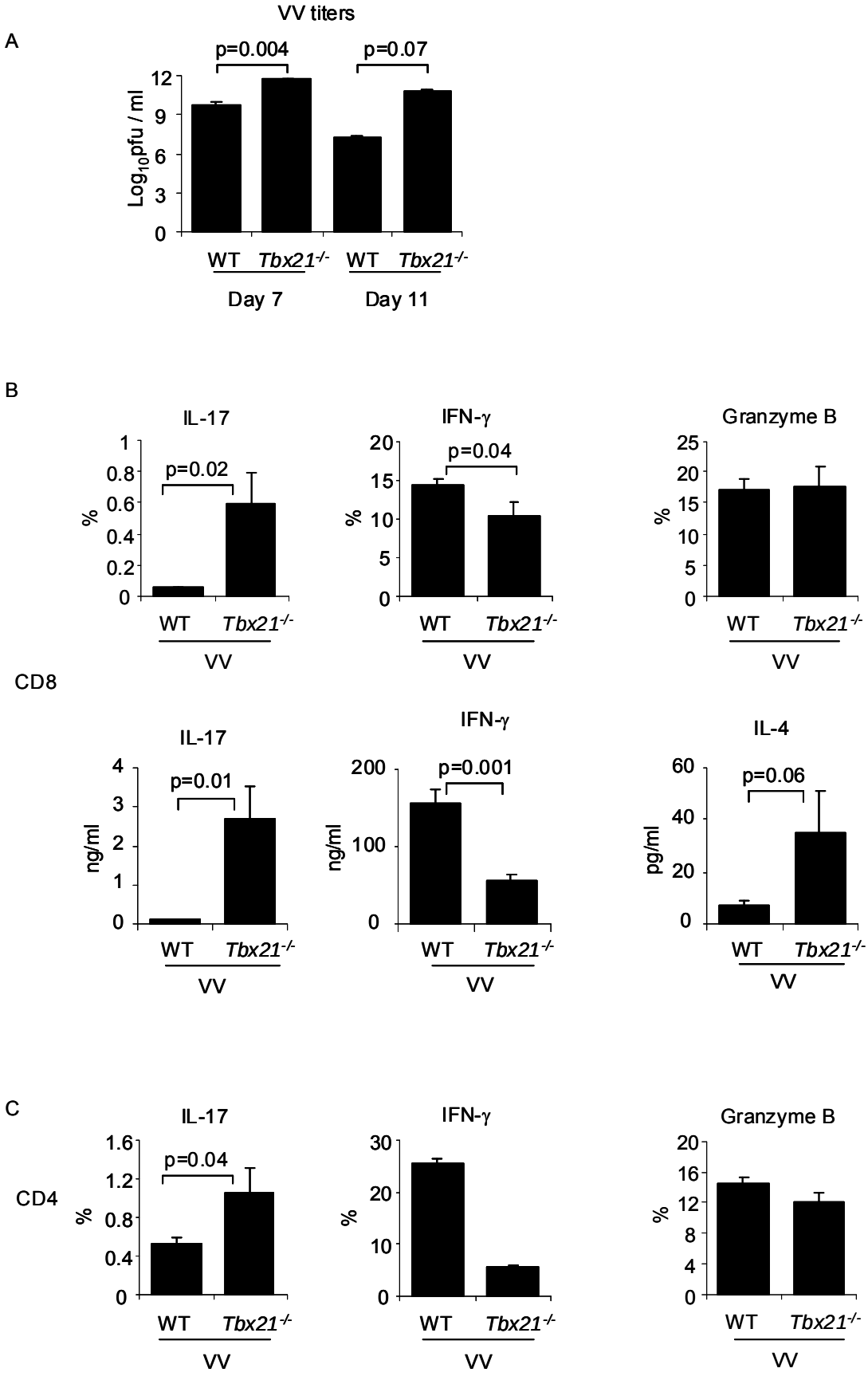

A
VV titers
p=0.004
p=0.07
Log10pfu / ml
12
9
6
3
0
WT
Tbx21-/-
WT
Tbx21-/-
Day 7
Day 11
B
IL-17
p=0.02
%
1
0.8
0.6
0.4
0.2
0
WT
Tbx21-/-
VV
IFN-γ
p=0.04
%
20
15
10
5
0
WT
Tbx21-/-
VV
Granzyme B
%
25
20
15
10
5
0
WT
Tbx21-/-
VV
CD8
IL-17
p=0.01
ng/ml
4
3
2
1
0
WT
Tbx21-/-
VV
IFN-γ
p=0.001
ng/ml
200
100
0
WT
Tbx21-/-
VV
IL-4
p=0.06
pg/ml
60
40
20
0
WT
Tbx21-/-
VV
C
CD4
IL-17
p=0.04
%
1.6
1.2
0.8
0.4
0
WT
Tbx21-/-
VV
IFN-γ
%
30
20
10
0
WT
Tbx21-/-
VV
Granzyme B
%
20
16
12
8
4
0
WT
Tbx21-/-
VV

Figure 42. *Tbx21*$^{-/-}$ mice have decreased clearance of vaccinia virus. C57Bl/6 or *Tbx21*$^{-/-}$ mice were infected with 5x10^6 pfu of vaccinia virus per mouse and sacrificed either 7 or 11 days after infection. (A) Ovaries were harvested at the indicated time points for viral titers using a plaque forming assay. (B) CD8+ cells were isolated from the spleen. Cells were either restimulated for 4 hrs with PMA + Ionomycin to analyze IL-17, IFN-γ, and granzyme B using ICS (top panels) or restimulated for 24 hrs with α-CD3 to generate cell free supernatants for analysis of IL-17, IFN-γ, and IL-4 protein levels using an ELISA assay (bottom panels). (C) CD4+ cells were isolated from the spleen and restimulated for 4 hrs with PMA + Ionomycin. IL-17$^+$, IFN-γ^+, and granzyme B$^+$ cells were detected using ICS. Data corresponds to 4-5 mice ± SEM.

Antiviral activity of adoptively transferred Tc17 cells

To more directly determine the function of Tc17 cells in vivo and their potential role in anti-vaccinia immunity, we performed adoptive transfer experiments with in vitro differentiated Tc17 cells and VV-SIINFEKL infected hosts. CD45.2 OT-I CD8 T cells were differentiated for 5 days in Tc17 conditions before i.v. transfer into CD45.1 hosts. Mice were infected i.n. with VV-SIINFEKL one day after T cell transfer and analyzed 4 days after infection. Infected mice which received Tc17 cells had a trend towards lower vaccinia virus titers in the lung, similar to infected mice which received Tc1 cells (Fig 43A). After a systemic infection, vaccinia virus preferentially localizes to the ovaries (Huang et al., 1993; Muller et al., 1994), however was not detected in the ovaries of any mice after i.n. infection (data not shown). In the infected mice, there was a larger migration to the draining lymph nodes of transferred Tc17 cells compared to transferred Tc1 cells and more Tc1 cells were detected in the spleens (Fig 43B). However, in both locations, OT-I Tc1 cells had a greater dilution of CFSE and proliferated to a greater extent than OT-I Tc17 cells. To determine if Tc17 cells secrete characteristic Tc17 cytokines after viral challenge in vivo or have an unstable phenotype as previously seen in vitro (Fig 26), we analyzed cytokine production from transferred cells detected in the spleen. After 4 days of infection with VV-SIINFEKL, IL-17 production from Tc17 cells was maintained while a portion of IL-17-secreting CD8 T cells also began to produce IFN-γ. Tc1 cells secreted a higher percentage of IFN-γ after adoptive transfer. These data suggest that Tc17 cells are potentially anti-viral in vivo and are unstable after viral challenge.

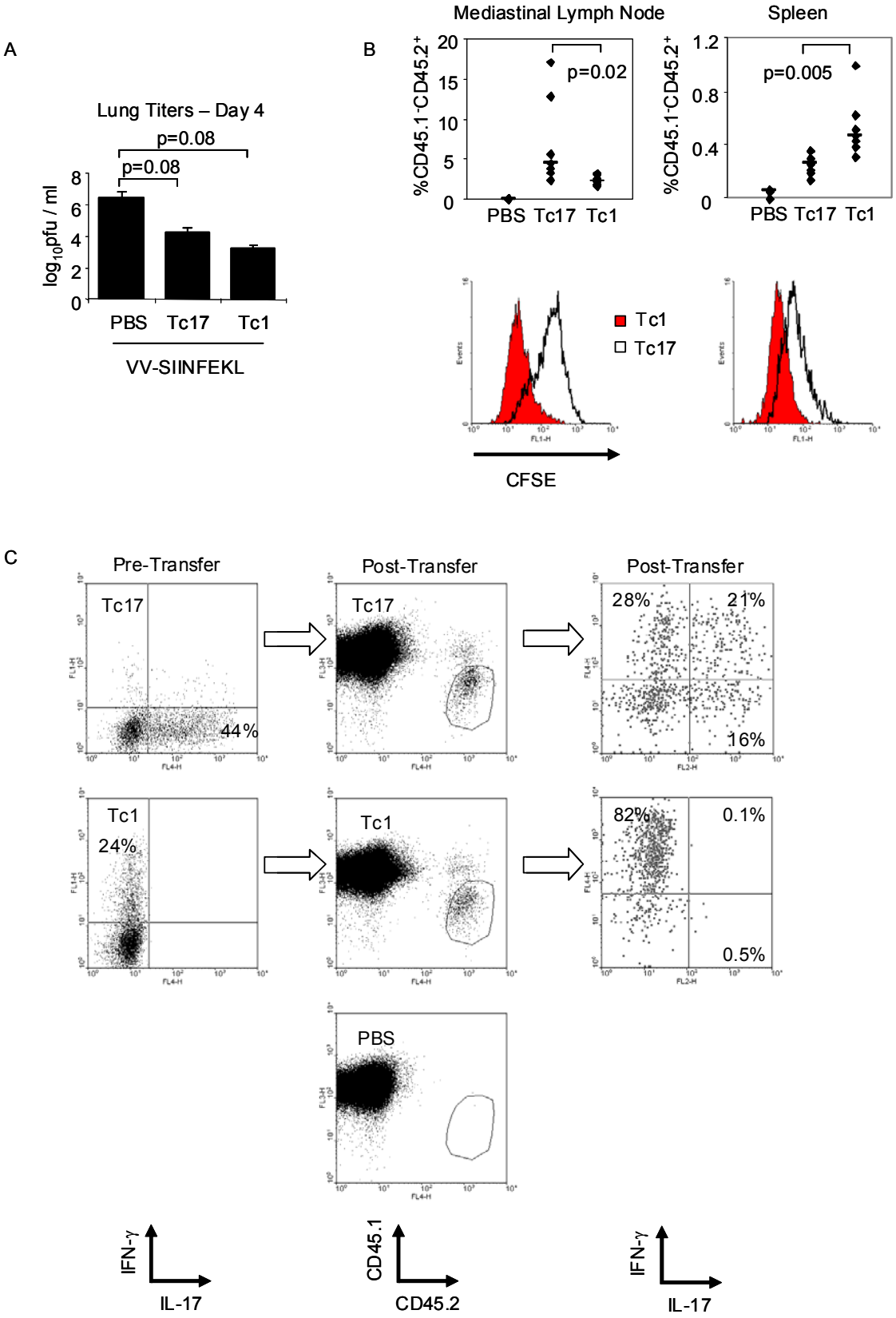

A
Lung Titers – Day 4
p=0.08
p=0.08
log10pfu / ml
8
6
4
2
0
PBS
Tc17
Tc1
VV-SIINFEKL
B
Mediastinal Lymph Node
Spleen
%CD45.1-CD45.2+
20
15
10
5
0
p=0.02
PBS Tc17 Tc1
1.2
0.8
0.4
0
p=0.005
PBS Tc17 Tc1
Tc1
Tc17
CFSE
C
Pre-Transfer
Post-Transfer
Post-Transfer
Tc17
44%
Tc17
28%
21%
16%
Tc1
24%
Tc1
82%
0.1%
0.5%
PBS
IFN-γ
IL-17
CD45.1
CD45.2
IFN-γ
IL-17

Figure 43. Adoptively transferred Tc17 cells reduce an i.n. VV-SIINFEKL infection and are unstable. Five day differentiated Tc17 or Tc1 cells ($1x10^6$, CD45.2) or PBS were injected i.v. into BoyJ ($CD45.1^+$) mice. Cells were also labeled with CFSE immediately prior to adoptive transfer. One day later, mice were infected with $2x10^6$ pfu VV-SIINFEKL i.n. Four days after infection, (A) lungs were harvested to determine viral titers using a plaque forming assay. (B) Single cell suspensions from mediastinal lymph nodes (left panels) and spleens (right panels) were surface stained with CD45.1 and CD45.2 to identify transferred cells. Bottom panels are gated on $CD45.1^-CD45.2^+$ cells and display CFSE dilution of transferred Tc17 and Tc1 cells. (C) $IL\text{-}17^+$ and $IFN\text{-}\gamma^+$ cells were identified in 5 day differentiated Tc17 or Tc1 cells immediately before adoptive transfer (left panels) and five days after adoptive transfer (or four days after infection, right panels). Transferred cells in the right panels are gated on the $CD45.1^-CD45.2^+$ cells illustrated in the middle panels. Data corresponds to 4-7 mice ± SEM or are typical representatives of the experimental group.

One function of IL-17 is to attract innate immune cells, especially neutrophils, to the site of an infection. To determine if adoptively transferred Tc17 cells attracted an inflammatory infiltrate into the lungs of VV-SIINFEKL infected mice, histological lung sections were analyzed. Tc17 cells or PBS was transferred into mice which were infected one day later with VV-SIINFEKL as in Fig 43. Four days after infection, mice were sacrificed. Lung sections from infected mice which had received PBS showed a perivascular inflammatory infiltrate in some areas consisting of both mononuclear and polymorphonuclear cells including eosinophils (Fig 44A). Infected mice which had received Tc17 cells appeared to have increased inflammation with a similar composition, however histological slides were not scored. To quantitate lung inflammation, we counted cell numbers in the BAL fluid and analyzed the percentage of BAL neutrophils (Fig 44B). Infected mice which received Tc1 cells had a significantly increased number of BAL cells and infected mice which received Tc17 cells had an increased trend in neutrophils in their BAL fluid, although this trend was not statistically significant. Smallpox vaccinations in military personnel have been associated with rare cases of myopericarditis (Maurer et al., 2003); however inflammatory infiltrates were not detected in the hearts of any of the mice examined.

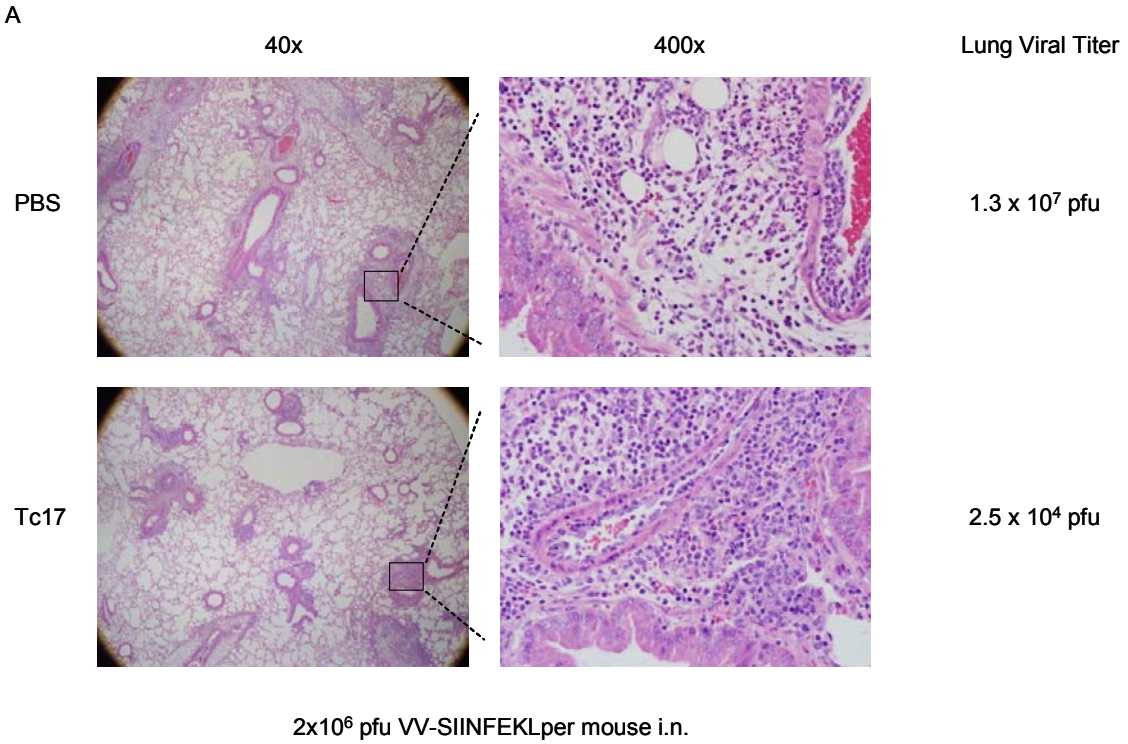


Figure 44. VV-SIINFEKL infection induces lung inflammation. Mice were injected with Tc17 or Tc1 cells or PBS i.v. and infected with VV-SIINFEKL one day later as described in Fig 43. Four days after infection (A) lung sections were fixed and stained with H&E. Two different magnifications are shown of lung sections from infected mice given PBS or Tc17 cells and the corresponding viral titers are displayed on the right side of the slides. (B) Cell numbers in the BAL fluid were counted (left panel) and surface stained (right panel). Neutrophils were determined as $B220^{-}CD3^{-}FSC^{lo}CCR3^{-}$ cells. Data corresponds to 4-5 mice ± SEM or representative slides.

In the previous figures (Figs 43 and 44), we utilized an intranasal infection model to localize vaccinia virus infection and to mimic natural vaccinia virus infection. However, our results with viral titers and inflammation were often inconsistent. Therefore, we switched to an intraperitoneal infection route for VV-SIINFEKL to determine the function of adoptively transferred Tc17 cells. Mice were infected i.p. with VV-SIINFEKL and one day after infection, 5 day differentiated Tc17 or Tc1 cells were transferred into the infected mice. Mice were sacrificed 6 days after T cell transfer (which was also 7 days after infection). Infected mice which received Tc17 cells had significantly lower vaccinia virus titers in their ovaries compared to infected mice which received PBS (Fig 45A). Adoptively transferred Tc17 cells reduced viral load to similar levels as transferred Tc1 cells and more Tc17 cells were detected in the spleens of infected cells compared to Tc1 cells (Fig 45B). Similar to Tc17 cells transferred into i.n. infected mice, Tc17 cells transferred into i.p. infected mice were unstable and upregulated IFN-γ after encounter with VV-SIINFEKL (Fig 45C). An increase in neutrophils was not detected in the blood or spleens of mice which received Tc17 cells (Fig 45D). Therefore, Tc17 cells are protective against vaccinia virus infection through mechanisms possibly involving IFN-γ.

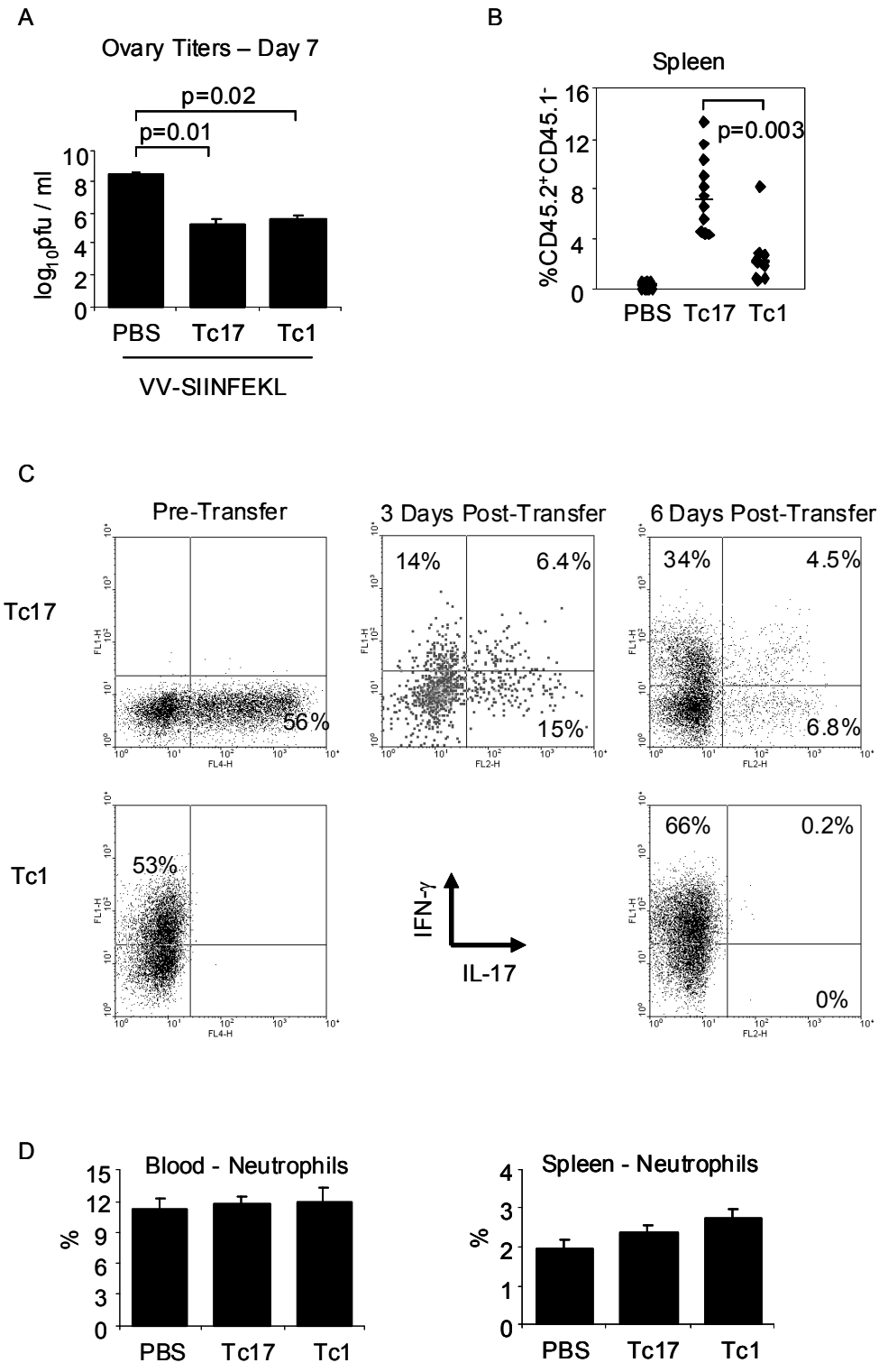

A
Ovary Titers – Day 7
p=0.02
p=0.01
log10pfu / ml
PBS
Tc17
Tc1
VV-SIINFEKL
B
Spleen
%CD45.2+CD45.1-
p=0.003
PBS Tc17 Tc1
C
Pre-Transfer
3 Days Post-Transfer
6 Days Post-Transfer
Tc17
Tc1
56%
14%
6.4%
15%
34%
4.5%
6.8%
53%
66%
0.2%
0%
IFN-γ
IL-17
D
Blood - Neutrophils
%
PBS
Tc17
Tc1
Spleen - Neutrophils
%
PBS
Tc17
Tc1

Figure 45. Adoptively transferred Tc17 cells are antiviral against an i.p. VV-SIINFEKL infection. BoyJ (CD45.1) mice were infected with $2x10^7$ pfu VV-SIINFEKL i.p. and injected i.v. with five day differentiated Tc17 or Tc1 cells ($1x10^6$, CD45.2) or PBS one day later. After an additional six days, (A) ovaries were harvested for viral titers. (B) Splenocytes were surface stained with CD45.1 and CD45.2 to identify transferred cells. (C) IL-17^+ and IFN-γ^+ cells were identified in 5 day differentiated Tc17 or Tc1 cells immediately before adoptive transfer (left panels), three and six days after adoptive transfer (middle and right panels). Transferred cells in the middle and right panels are gated on CD45.1^- CD45.2^+ cells. (D) After one week of infection, neutrophils were analyzed in the blood and spleen of infected mice. Neutrophils were gated on CD11b^{hi}Gr-1^{hi} cells. Data corresponds to 4-5 mice ± SEM or are typical representatives of the experimental group.

Role of IFN-γ in Tc17 mediated viral clearance

IFN-γ plays a critical role in the clearance of vaccinia virus and mice deficient in IFN-γ signaling are highly susceptible to vaccinia infection (Huang et al., 1993; Muller et al., 1994). To determine if IFN-γ induction in Tc17 cells was responsible for Tc17 mediated viral clearance, we repeated the previous experiment with *Ifng*$^{-/-}$ Tc17 cells. Mice were infected i.p. with VV-SIINFEKL and one day after infection, 5 day differentiated OT-I Tc17 or OT-I *Ifng*$^{-/-}$ Tc17 cells were transferred into the infected mice. Mice were sacrificed 6 days after T cell transfer or 7 days after infection. The decrease in IL-17 and induction of IFN-γ in transferred Tc17 cells was confirmed using ICS (Fig 46A). *Ifng*$^{-/-}$ Tc17 did not upregulate IFN-γ as expected and transferred Tc17 cells did not upregulate granzyme B. WT and *Ifng*$^{-/-}$ Tc17 induced T-bet to similar levels. Tc17 cells deficient in IFN-γ were able to clear vaccinia virus as efficiently as wild type Tc17 cells (Fig 46B) indicating that Tc17 cells are able to clear vaccinia virus via an IFN-γ independent mechanism.

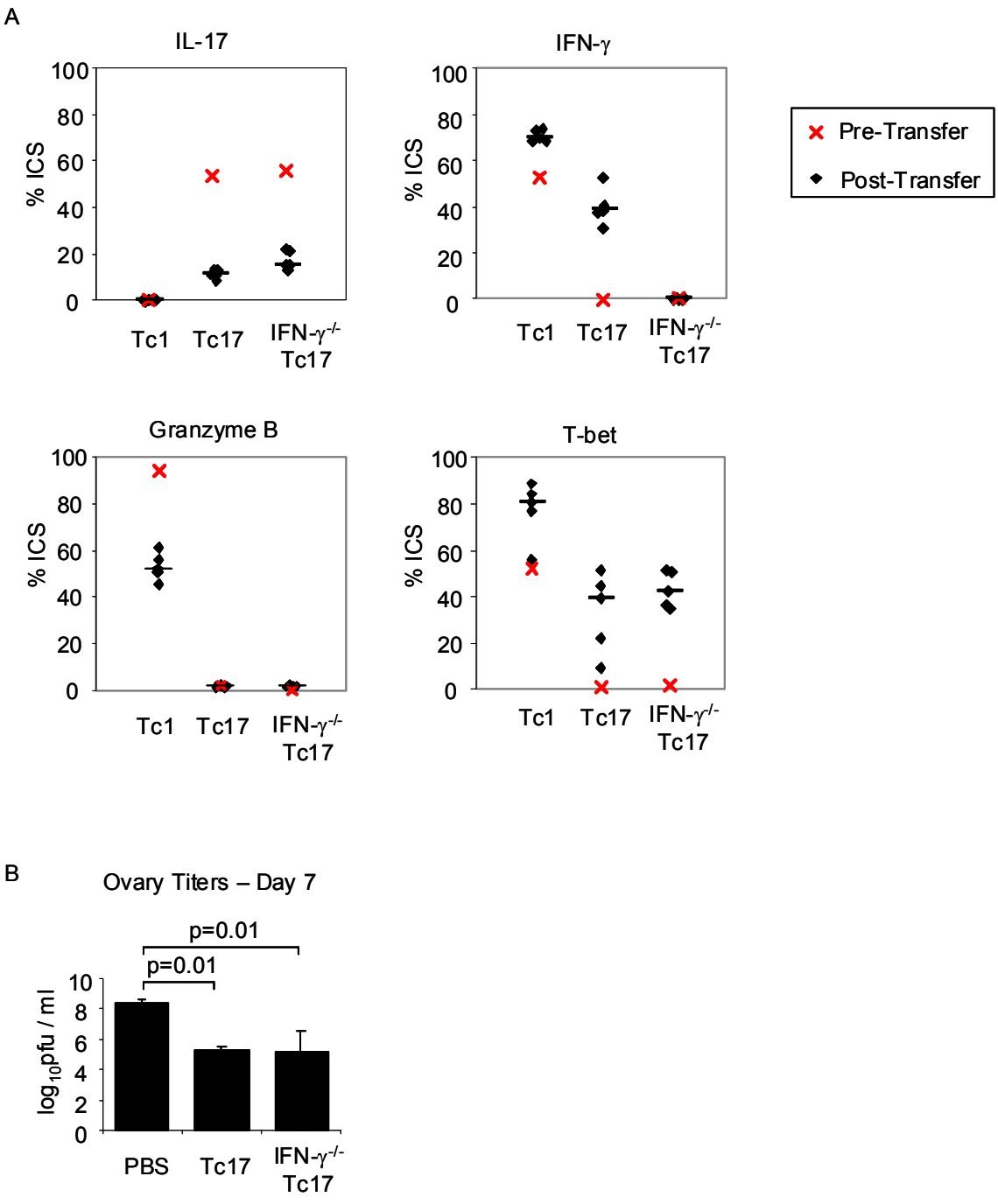
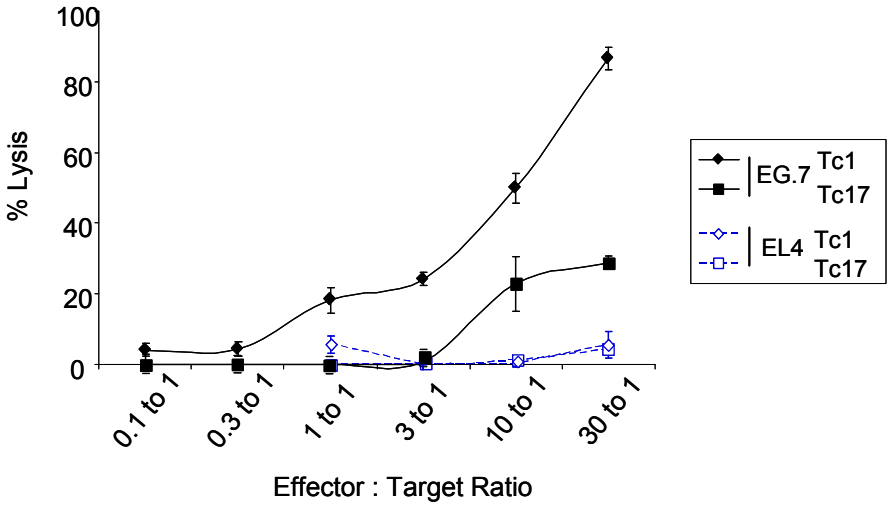


Figure 46. Adoptively transferred Tc17 cells are antiviral via an IFN-γ independent mechanism. BoyJ mice were infected with $2x10^7$ pfu VV-SIINFEKL i.p. and injected i.v. with five day differentiated Tc17 or Tc1 cells ($1x10^6$) or PBS one day later. After an additional six days, (A) IL-17$^+$, IFN-γ$^+$, granzyme B$^+$, and T-bet$^+$ cells from the CD45.1$^-$CD45.2$^+$ gate were determined using ICS. Median values are displayed as horizontal lines and diamonds represent individual mice (B) Ovaries were harvested for viral titers. Data corresponds to 4-5 mice ± SEM.

Cytotoxic potential of Tc17 cells after VV-SIINFEKL challenge

After restimulation in the presence of IL-12 in vitro, Tc17 cells upregulate IFN-γ and gain cytotoxic function. To determine if Tc17 cells can become cytotoxic during a VV-SIINFEKL infection, we reisolated adoptively transferred cells from infected hosts to test in a killing assay. Mice were infected i.p. with VV-SIINFEKL and one day after infection, 5 day differentiated Tc17 or Tc1 cells were transferred into the infected mice. After another 6 days, adoptively transferred cells were isolated based on their expression of CD45.2 and tested in a chromium release assay. After encounter with VV-SIINFEKL in vivo, Tc1 cells remain cytotoxic and Tc17 cells also gained cytotoxic ability (Fig 47A). As seen previously, the Tc17 cells expressed low levels of IL-17 and did not upregulate granzyme B (Fig 47B). Upregulation of IFN-γ was not observed in these Tc17 cells and we believe this was due to a technical issue of restimulating isolated cells with peptide alone instead of peptide with APCs. Thus, Tc17 cells become cytotoxic during clearance of vaccinia virus from infected mice.

A


100
80
60
40
20
0
% Lysis
0.1 to 1
0.3 to 1
1 to 1
3 to 1
10 to 1
30 to 1
Effector : Target Ratio
EG.7 Tc1
Tc17
EL4 Tc1
Tc17

B

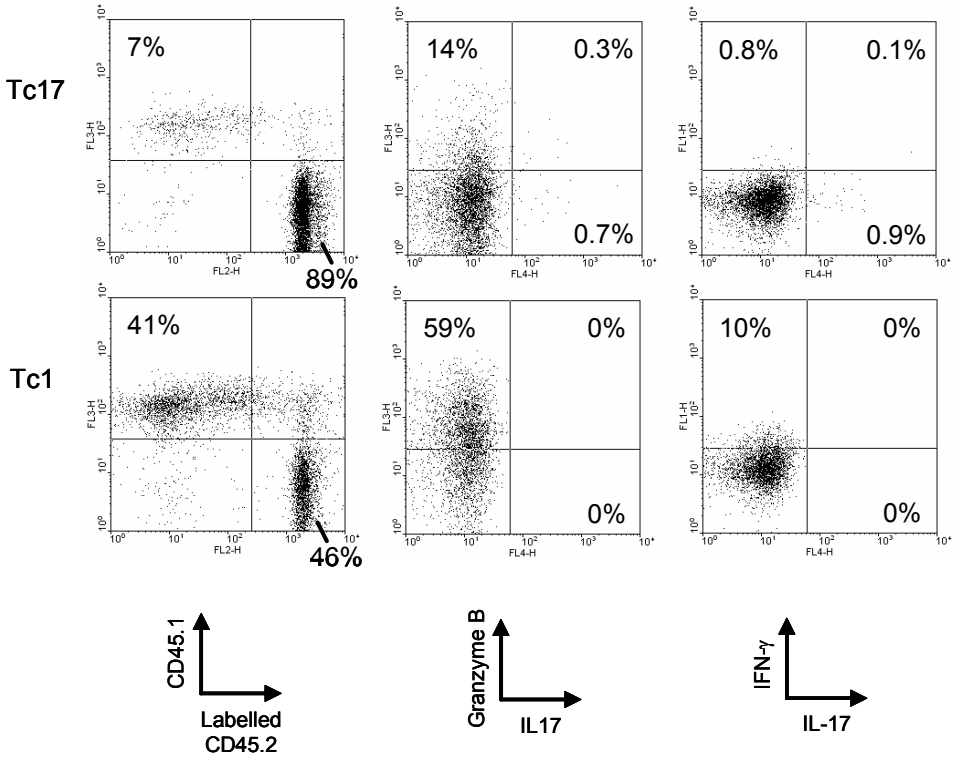

Tc17
Tc1
7%
89%
14%
0.3%
0.7%
0.8%
0.1%
0.9%
41%
46%
59%
0%
0%
10%
0%
0%
FL2-H
FL3-H
FL4-H
FL1-H
CD45.1
Labelled CD45.2
Granzyme B
IL17
IFN-γ
IL-17

Figure 47. Tc17 cells become cytotoxic after encountering vaccinia virus in vivo. Mice were infected with VV-SIINFEKL and injected with Tc17 or Tc1 cells as described in Fig 45. Injected cells were isolated after an additional six days using PE conjugated anti-CD45.2 antibodies and anti-PE microbeads. (A) Effector cells were added at increasing ratios to ova expressing EG.7 target cells or nonspecific EL4 target cells labeled with Cr^{51} and incubated for 6 hrs before measuring Cr^{51} released into the supernatant. (B) CD45.2 enriched cells were stained with CD45.1 to determine purity. IL-17^+, IFN-γ^+, and granzyme B^+ cells were determined using ICS. Data corresponds to 4-5 mice ± SEM or are typical representatives of the experimental group.

Summary of the antiviral potential of Tc17 cells

We propose a model of CD8 T cell mediated clearance of vaccinia virus as depicted in Fig 48. Upon recognition of an infected cell, a vaccinia virus specific CD8 T cells undergo clonal expansion to divide several fold. In the presence of IL-12, Tc1 cells are formed which likely mediate cell killing through IFN-γ dependant mechanisms. If TGFβ and IL-6 are present in the environment, a transient Tc17 cell develops which acquires a Tc1 phenotype upon reencounter with virus. Tc17 cells are clearly able to mediate viral clearing, however the role of its cytotoxic potential and unique phenotype are still unknown.

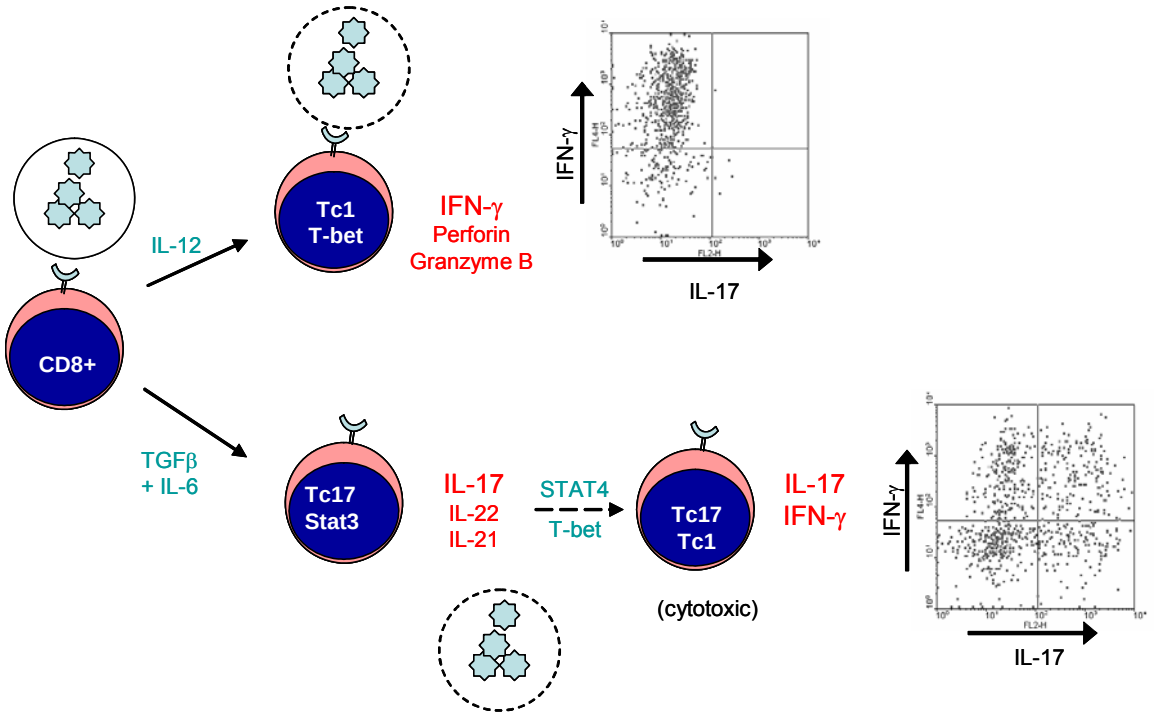


Figure 48. CD8 T cell clearance of vaccinia virus. Vaccinia virus specific CD8 T cells develop into a heterogeneous population after stimulation by vaccinia virus epitopes. Some develop into IFN-g expressing Tc1 cells while others secrete IL-17. IL-17 secreting CD8 T cells subsequently acquire cytotoxic potential and secrete IFN-γ as they mediate viral clearance.

IL-23 maintains IL-17 secretion without affecting Th17 cell proliferation or expansion

Although the requirement for IL-23 in the function of Th17 cells in vivo is established, the precise role of this cytokine in affecting the Th17 phenotype is unclear. Among other functions, IL-23 was proposed to act as a Th17 cell proliferation or survival factor. To directly test these functions, we developed a cytokine capture assay for IL-17-secreting cells to compare IL-23 functions in enriched IL-17-high and low secretor populations. Naive CD4 T cells were cultured with TGF-β1+IL-6+IL-1β for 5 days before stimulation with anti-CD3 before selection of IL-17-high and low cells by cell sorting (Fig 49A). Following sorting there was a 10–12-fold enrichment for IL-17 secreting cells in the IL-17-high population (Fig 49A). The separation of cells into distinct populations was confirmed by demonstrating segregated expression of IL-17, IL-22, *Il23r*, and *Rorc*, while IFN-γ production was indistinguishable between the two populations (Fig 49 B, C). Intracellular staining for IFN-γ in these populations demonstrated less than 0.5% in any population (data not shown).

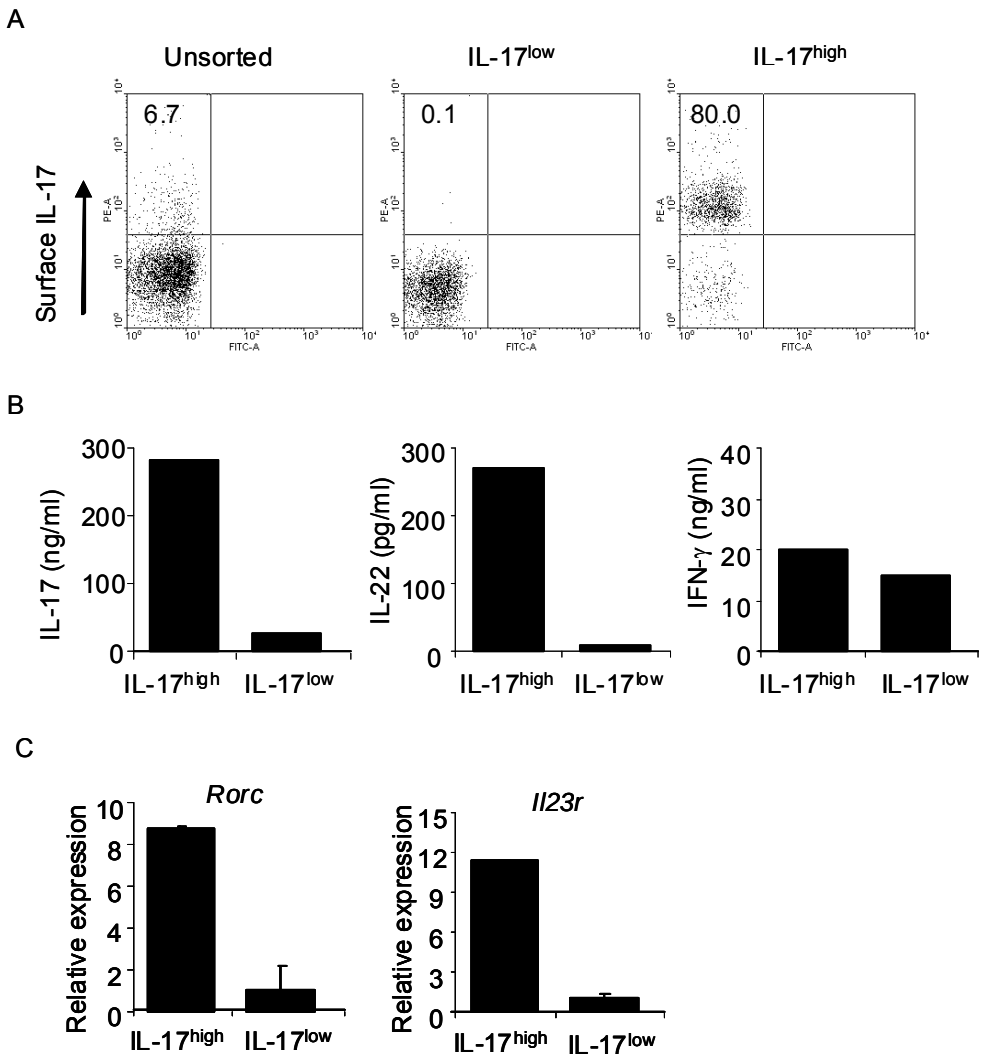

A
Unsorted
IL-17low
IL-17high
6.7
0.1
80.0
Surface IL-17
PE-A
FITC-A
B
IL-17 (ng/ml)
IL-22 (pg/ml)
IFN-γ (ng/ml)
IL-17high
IL-17low
C
Rorc
Il23r
Relative expression

Figure 49. Cytokine selection of IL-17+ Th17 cells. (A), Naive $CD4^+$ T cells were activated, cultured in TGF-β plus IL-6 plus IL-1β and blocking Abs (anti-IFN-γ and anti-IL-4) for 5 days and activated before surface staining for IL-17 using cytokine capture. Cells were then sorted into IL-17-high and low populations. (B), Supernatants from IL-17-high and -low cells stimulated with anti-CD3 were tested for cytokines using ELISA. (C), RNA was isolated from cells treated in (B*)* and gene expression was assessed using real-time PCR. Data correspond to 2 experiments.

One of the proposed functions of IL-23 is promoting the proliferation or expansion of Th17 cells. To test this directly, naive CD4 T cells were cultured with TGF-β+IL-6+IL-1β for 5 days, separated based on IL-17 production and labeled with CFSE (Fig 50A). CFSE-labeled IL-17-high and low populations were cultured in blocking Abs (anti-IFN-γ and anti-IL-4) alone or blocking Abs plus IL-23 for 24 or 48 h to assess proliferation. Although IL-17-high cells had an intrinsically higher rate of proliferation than IL-17-low cells, there was not a significant difference in proliferation between cultures incubated with or without IL-23 (Fig 50 B, C), suggesting that IL-23 does not promote a robust proliferative response.

IL-23 was also proposed to affect Th17 survival. However, in examining the overall cell growth in IL-17-high and low cells, we observed the increased proliferative capacity of IL-17-high cells that resulted in a 2–3-fold increase in cell number compared with IL-17-low cells, but only minor effects of IL-23 (Fig 50D). Similarly, IL-17-high and low cells cultured in the presence or absence of IL-23 had similar percentages of Annexin V^+ cells after 2 or 4 days of culture with little effect of IL-23 culture (Fig 50E).

In contrast, we did note that in IL-17-high populations cultured with IL-23, a higher percentage of cells, with a higher intensity of IL-17 staining, was maintained compared with cells cultured in the absence of IL-23 (Fig 50B). The effects of IL-23 on maintaining the IL-17-secreting phenotype were even more dramatic when cells were activated with anti-CD3. After 2 days of activation, IL-

17-high cells cultured in IL-23 still had >60% IL-17^{+} cells, while cultures incubated in the absence of IL-23 has <30% IL-17^{+} cells (Fig 50F). This effect was also observed in the IL-17-low cultures where IL-17+ cells comprised <10% of the population when cultured in the absence of IL-23 (Fig 50F). These data suggest that IL-23 maintains the IL-17-secreting phenotype without detectable effects on Th17 cell proliferation or expansion.

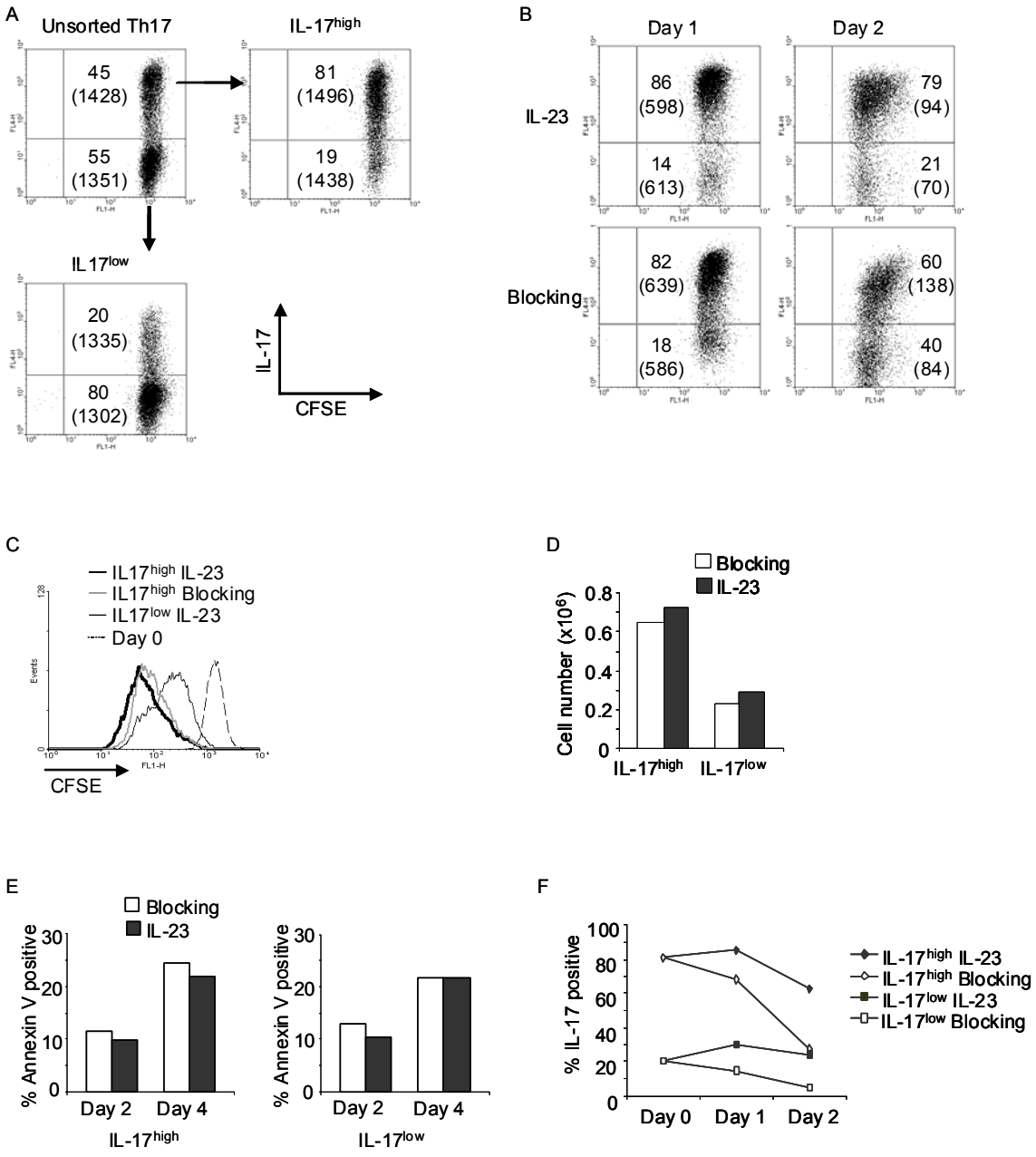


Figure 50. IL-23 maintains the IL-17-secreting phenotype without affecting cell expansion or survival. (A), Naive $CD4^+$ T cells were activated, cultured in TGF-β plus IL-6 plus IL-1β and blocking Abs (anti-IFN-γ and anti-IL-4) for 5 days before sorting into IL-17-high and low populations. Cells were then labeled with CFSE and for intracellular IL-17 following stimulation with PMA plus ionomycin. Numbers indicate percent of cells in each quadrant and bracketed numbers indicate CFSE mean fluorescence intensity. (B), IL-17-high cells were cultured with IL-23 and blocking Abs or blocking Abs alone for the indicated times before cells were stimulated and stained for intracellular IL-17. Numbers indicate percent of cells in each quadrant and bracketed numbers indicate CFSE mean

fluorescence intensity. (C), IL-17-high or -low CFSE-stained cells prepared as in (B) were cultured for 2 days with blocking Abs in the presence or absence of IL-23 as indicated. CFSE staining is shown from freshly stained cells (day 0) for comparison. (D), IL-17-high and -low cells cultured as in (B) were counted after 48 h. (E), IL-17-high and -low cells were cultured as in (B) and were analyzed for Annexin V staining after 2 or 4 days of culture in the presence or absence of IL-23. (F), IL-17-high and-low cells were stimulated with anti-CD3 and cultured with blocking Abs with or without IL-23. Cells were stimulated with PMA plus ionomyocin for 4 h and stained for intracellular IL-17. Data correspond to 2 experiments.

IL-23 maintains the Th17 phenotype in long-term cultures

To test the ability of IL-23 to maintain the Th17 phenotype over long term culture, we cultured naive CD4 T cells for 1 wk with TGF-β plus IL-6 plus IL-1β, the latter which we found amplifies IL-23 responsiveness in vitro (Veldhoen et al., 2006 and data not shown), and then for subsequent rounds of stimulation with either blocking Abs alone or IL-23 with blocking Abs. Despite a high level of IL-17 in the initial cultures, IL-23 was only partially effective in attenuating the decrease of IL-17 production from T cells following subsequent rounds of stimulation, compared with blocking Abs alone (Fig 51A). IL-23 was effective in limiting IFN-γ production from these cultures suggesting that the decrease in IL-17 production observed over multiple rounds of stimulation is not due to coincident increases in IFN-γ production or increases in the percentage of cells that are IFN-γ^+.

Given the similarity in autoimmune disease phenotype between IL-1RI- and IL-23p19-deficient mice, and that IL-1 enhances IL-23 responsiveness (Cua et al., 2003; Cho et al., 2006; Sutton et al., 2006), we next tested the ability of IL-1β to cooperate with IL-23 in long term cultures. Naive CD4 T cells were cultured as in Fig 3A for the first week and then cultured for subsequent rounds of stimulation with IL-23, IL-1β, or a combination of IL-23 and IL-1β. Although IL-1β was no more effective than IL-23 in attenuating the loss of IL-17 secretion over multiple rounds of stimulation, the combination of IL-23 and IL-1β was able to maintain a high level of IL-17 secretion over three rounds of stimulation (Fig 51B). There were not dramatic differences in the growth or survival among these cultures

over several rounds of culture (data not shown), suggesting that the effects of these cytokines are not on survival or expansion, but rather on maintaining the phenotype of the cells. The combination of IL-23 and IL-1β was similarly capable of maintaining higher levels of IL-21 and IL-22 secretion than IL-23 alone, although there were decreases in these cytokines over rounds of stimulation (Fig 51B).

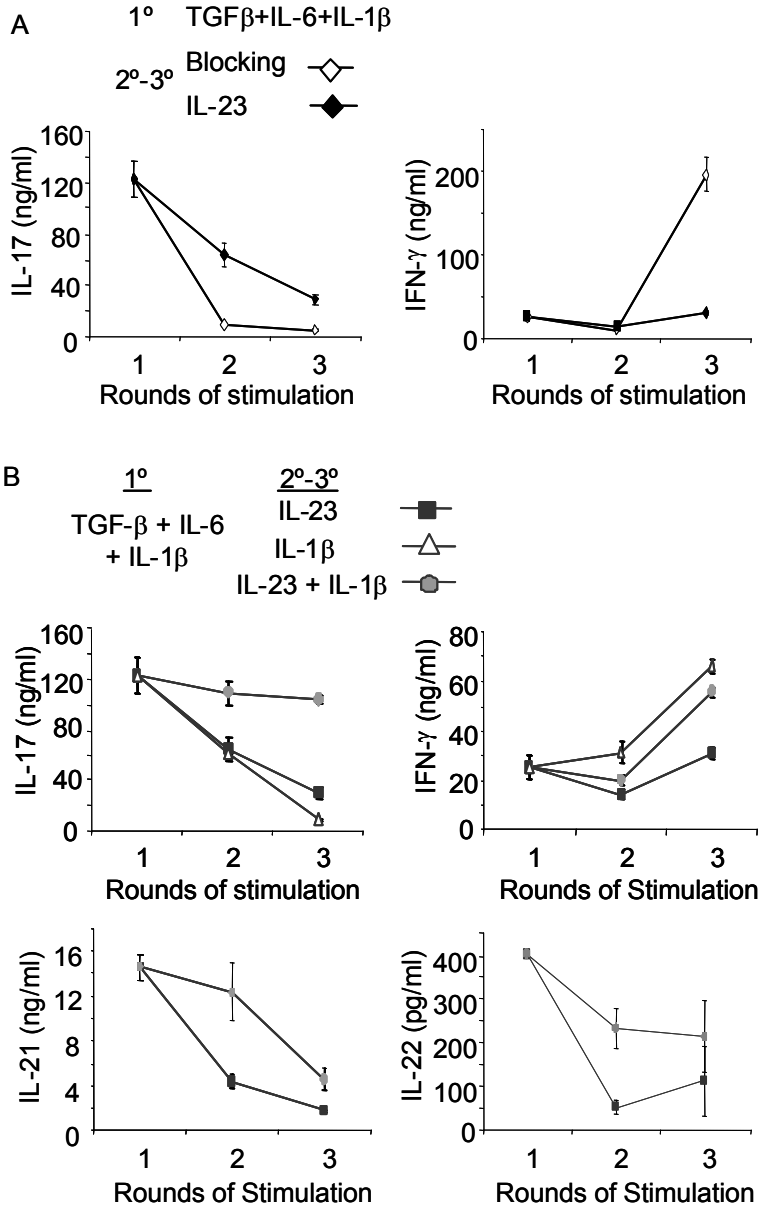


Figure 51. IL-1β increases IL-23 stimulated maintenance of the Th17 phenotype. (A), Naive T cells were activated, primed with TGF-β plus IL-6 plus IL-1β for the first round and cultured for two additional rounds of stimulation in the presence of blocking Abs with or without IL-23. After each round of culture cells were stimulated and cell-free supernatants were tested for cytokine production using ELISA. (B), Naive T cells were activated and primed as in (A) and cultured for two additional rounds of stimulation with IL-23, IL-1β, or IL-23 plus IL-1β as indicated. Cytokine production was measured using ELISA. Data correspond to 2 experiments.

To define the mechanism for the ability of IL-1β to augment IL-23 function we first analyzed IL-23R expression in cells cultured with IL-23, IL-1β, or both cytokines for the second and third rounds of stimulation. Although culture with IL-23 maintained or enhanced *Il23r* mRNA expression, there was no increased expression in cells cultured with IL-1β alone or with both cytokines (Fig 52A). We then examined IL-23 signaling using flow cytometry to assess levels of phospho-Stat3 following acute stimulation of cultures incubated for three rounds in IL-23, IL-1β, or both cytokines. IL-23 stimulated Stat3 phosphorylation in cells from each of the conditions with insignificant differences in the phospho-Stat3 levels among the conditions, suggesting that the effect of IL-1β was not altering IL-23 signaling (Fig 52B). To test whether IL-1β had altered the *Il17* gene to make it more responsive to IL-23, we took advantage of an assay we previously described for the acute stimulation of IL-17 production by a combination of IL-23 and IL-18 (Mathur et al., 2007). Naive CD4 T cells primed with TGF-β plus IL-6 plus IL-1β for the first round of stimulation and cultured in IL-23 or IL-23 plus IL-1β for two rounds of stimulation were restimulated with IL-23 plus IL-18. Cells that were cultured with IL-23 plus IL-1β generated higher amounts of IL-17 than cells cultured in IL-23 alone in response to IL-23 plus IL-18 (Fig 52C). Because analysis of *Il23r* expression in these cultures (Fig 52A) showed only minor differences in expression, with slightly lower levels in the IL-23 plus IL-1β cultured cells, it suggests that culture in IL-23 plus IL-1β is directly affecting the responsiveness of the *Il17* locus, although we did not find differences in the level of total histone acetylation between cells that were cultured with or without IL-1β

(data not shown). IL-1β may enhance IL-23 function by activating cooperative transcription factors, or through indirect mechanisms including the ability to limit the inhibitory effects of IL-2 on Th17 development (Kryczek et al., 2007a).

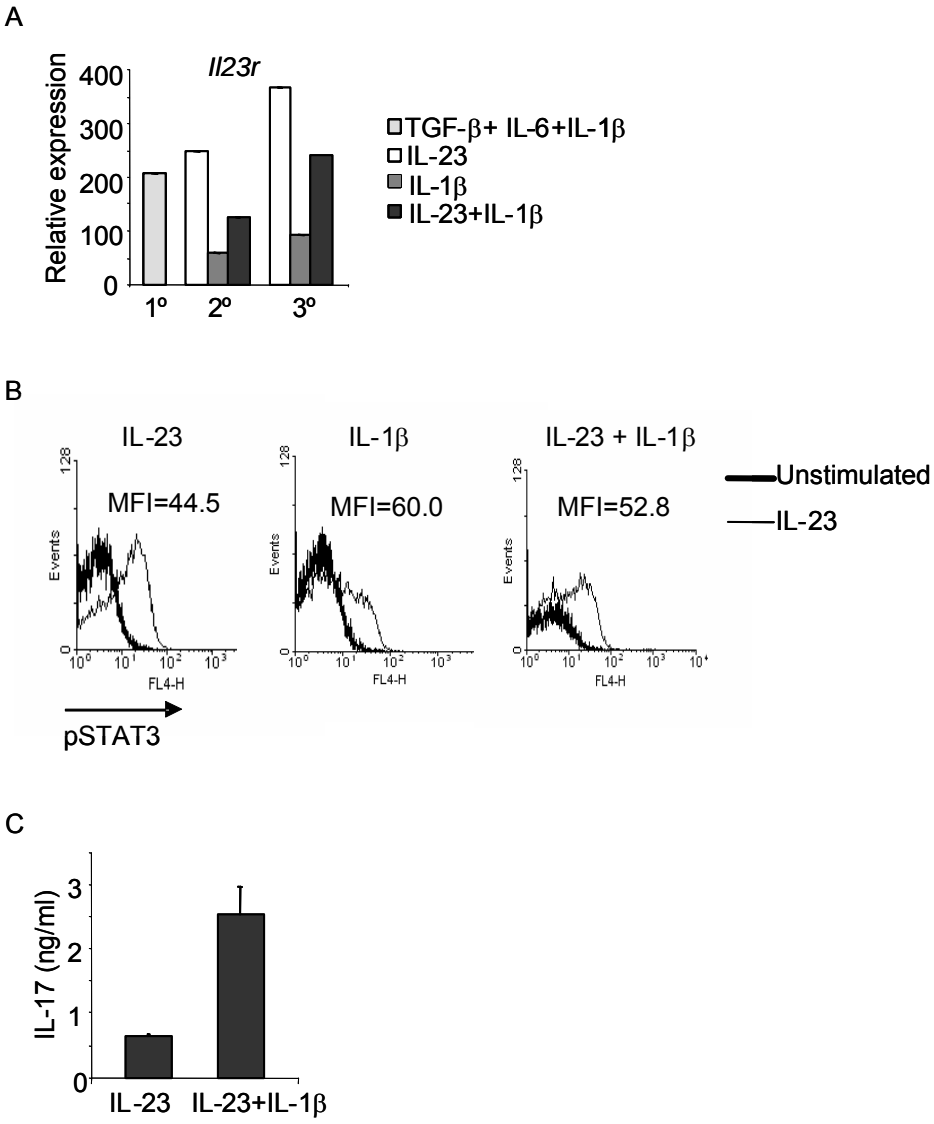


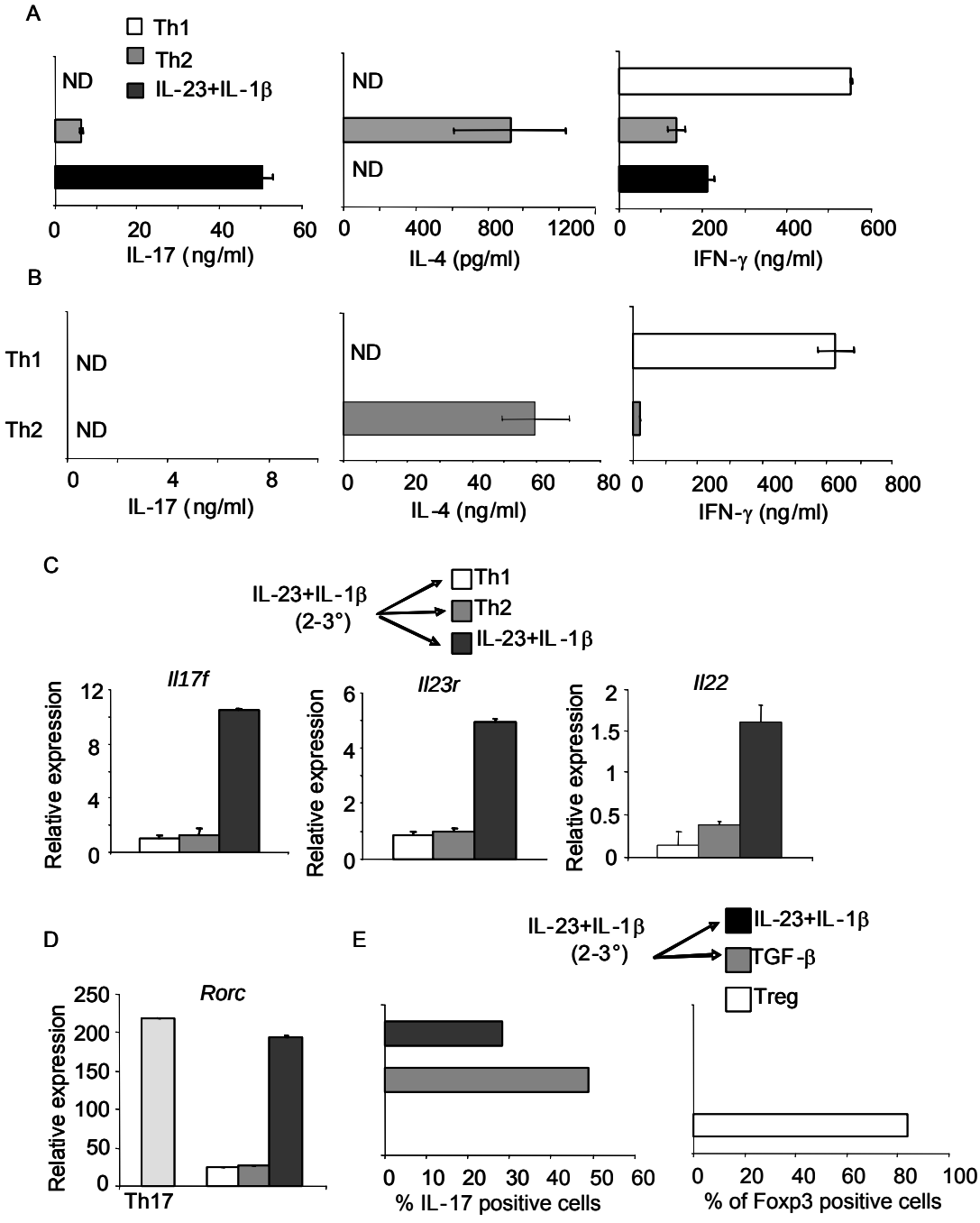
Figure 52. IL-1β increases responsiveness of the IL-17 locus. (A), Naive T cells were activated, primed with TGF-β plus IL-6 plus IL-1β for the first round and cultured for two additional rounds of stimulation with IL-23, IL-1β, or IL-23 plus IL-1β as indicated. At the end of the first, second, and third round of stimulation RNA was isolated from cells stimulated with anti-CD3 for 4 h. Quantitative PCR of *Il23r* expression is shown as relative to Th1 cultures after the third round of stimulation. (B), Cells cultured and stimulated as in (A) for three rounds were stimulated with IL-23 for 30 min before intracellular staining for phospho-STAT3. (C), Naive cells primed and cultured as in (A) were stimulated with IL-18 and IL-23 for 24 h. Cell-free supernatants were measured for IL-17 using ELISA. Data correspond to 2 experiments.

IL-23 does not mediate commitment to the Th17 lineage

Because IL-23 was capable of maintaining the IL-17-secreting phenotype, it allowed us to determine whether IL-23 mediated commitment to the Th17 lineage, with commitment being defined as the ability of cells to maintain the IL-17-secreting phenotype in the presence of cytokines promoting the development of other subsets. Th1 and Th2 cells are committed to their respective lineages after three rounds of stimulation (Murphy et al., 1996). Naive CD4 T cells were primed with TGF-β plus IL-6 plus IL-1β for the first round, cultured for two rounds in IL-23 plus IL-1β, and then either maintained in IL-23 plus IL-1β or switched to IL-12 or IL-4 containing medium for the fourth round of stimulation before stimulation with anti-CD3 to assess cytokine production. Although cells cultured for the fourth round in IL-23 plus IL-1β maintained the ability to produce IL-17, cells switched to Th1 or Th2 promoting conditions showed diminished IL-17 production and the induction of IFN-γ and IL-4, respectively (Fig 53 A, B). Similar results were generated using cultures derived from C57BL/6 or BALB/c mice (data not shown). The levels of IL-4 induced following switching into Th2 conditions were lower than seen from cells cultured for 4 wk under Th2 conditions, though the levels of IFN-γ secreted by Th17 cultures switched to Th1 conditions were comparable to long term Th1 cultures (Fig 53 A, B). Expression of other Th17 genes including *Il17f*, *Il23r*, *Il22*, and *Rorc* were also diminished in cultures switched to Th1 or Th2 conditions (Fig 53 C, D). In contrast, naive CD4 T cells primed with TGF-β plus IL-6 plus IL-1β for the first round, cultured for two rounds in IL-23 plus IL-1β, and subsequently switched to culture conditions that

promote Treg development were unable to develop into Foxp3-expressing cells (Fig 53E). Culture of cells with TGFβ plus IL-2 increased the percentage of IL-17$^+$ cells. Thus, Th17 cells cultured with IL-23 can adopt some, but not all CD4 T cell lineages.

Although T cells cultured for three rounds under these Th17 conditions produce large amounts of IL-17, there is still some production of IFN-γ, and it remained possible that a contaminating population of cells was expanding and overtaking the Th17 cells upon switching cultures to Th1 or Th2 conditions. To eliminate this possibility, we used the cytokine capture protocol to isolate IL-17-high cells from Th17 cultures after three rounds of stimulation (Fig 53F) and then maintained the cultures with IL-23 plus IL-1β or switched to conditions promoting Th1 or Th2 development. As observed with unseparated Th17 cultures, IL-17-high cells maintained their phenotype with continued culture in IL-23 plus IL-1β, but showed decreased IL-17 and IL-22 production, and increased IFN-γ and IL-4 production in Th1 and Th2 conditions, respectively (Fig 53 G, H). Thus, IL-17-high cells are not stable secretors of IL-17 and upon exposure to conditions promoting the development of other Th subsets, they acquire new cytokine secreting characteristics.


A
Th1
Th2
IL-23+IL-1β
ND
ND
ND
0 20 40 60
IL-17 (ng/ml)
0 400 800 1200
IL-4 (pg/ml)
0 200 400 600
IFN-γ (ng/ml)
B
Th1
Th2
ND
ND
ND
0 4 8
IL-17 (ng/ml)
0 20 40 60 80
IL-4 (ng/ml)
0 200 400 600 800
IFN-γ (ng/ml)
C
IL-23+IL-1β
(2-3°)
Th1
Th2
IL-23+IL-1β
Il17f
Il23r
Il22
Relative expression
0 4 8 12
0 2 4 6
0 0.5 1 1.5 2
D
Rorc
Relative expression
0 50 100 150 200 250
Th17
E
IL-23+IL-1β
(2-3°)
IL-23+IL-1β
TGF-β
Treg
0 10 20 30 40 50 60
% IL-17 positive cells
0 20 40 60 80 100
% of Foxp3 positive cells

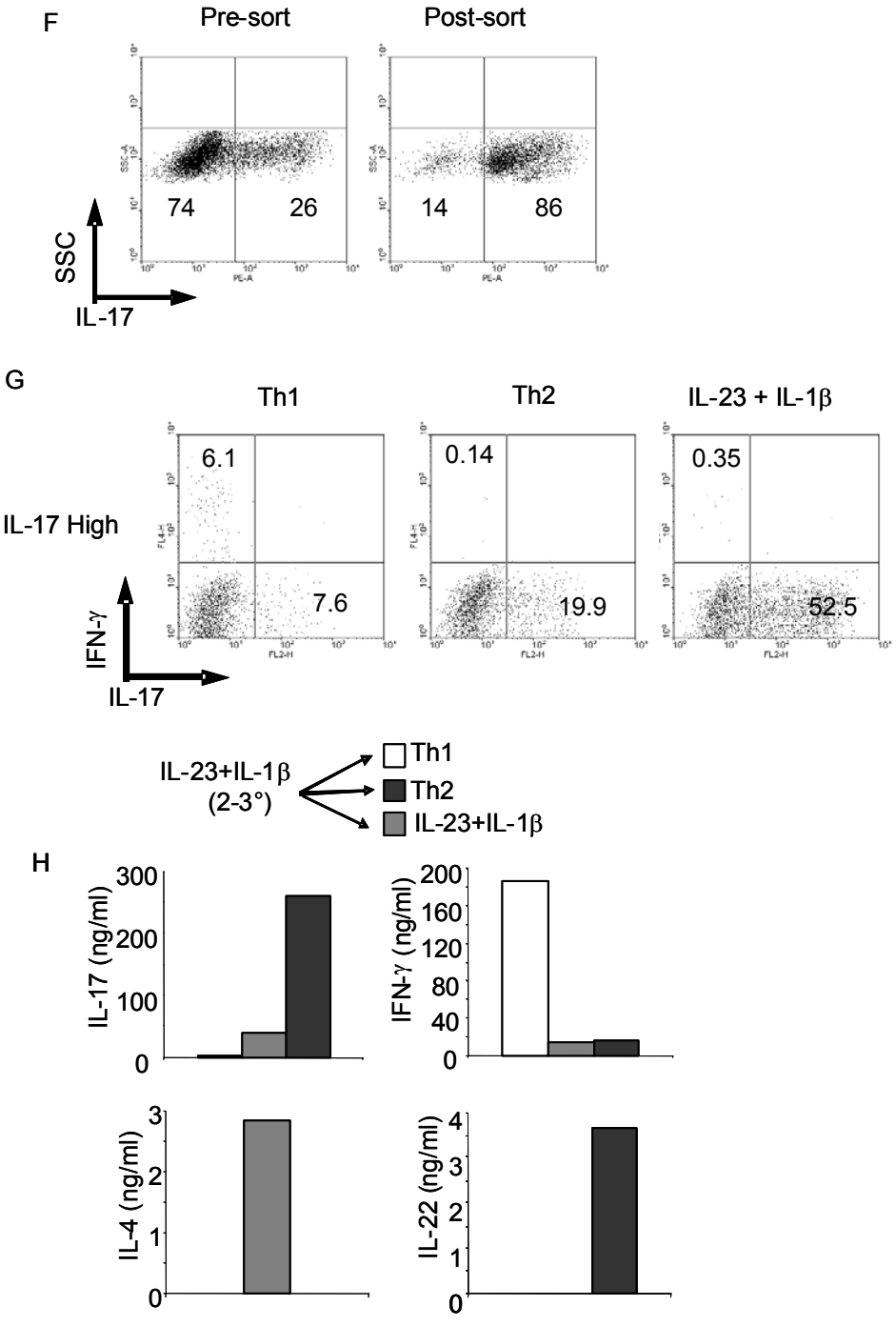

F
Pre-sort
Post-sort
74
26
14
86
SSC
IL-17
G
Th1
Th2
IL-23 + IL-1β
IL-17 High
6.1
7.6
0.14
19.9
0.35
52.5
IFN-γ
IL-17
IL-23+IL-1β
(2-3°)
Th1
Th2
IL-23+IL-1β
H
IL-17 (ng/ml)
300
200
100
0
IFN-γ (ng/ml)
200
160
120
80
40
0
IL-4 (ng/ml)
3
2
1
0
IL-22 (ng/ml)
4
3
2
1
0

Figure 53. IL-23 does not program commitment to the Th17 lineage. (A), Naive T cells were activated, primed with TGF-β plus IL-6 plus IL-1β for the first round followed by two rounds of stimulation in IL-23 plus IL-1β, were cultured for an additional round of stimulation in Th1 or Th2 conditions, or IL-23 plus IL-1β. Supernatants from anti-CD3 stimulated cells were tested for cytokine production using ELISA. (B), Naive $CD4^+$ T cells were activated and cultured under Th1 or Th2 priming conditions for four rounds of stimulation. At the end of the fourth round of stimulation, supernatants from anti-CD3 stimulated cells were tested for cytokine production using ELISA. (C), Cells stimulated and cultured as in *A* were stimulated with anti-CD3 for 4 h and RNA was isolated for quantitative PCR. (D), Cells were stimulated and cultured for three rounds as in (A). After 3 days of culture in Th1, Th2, or IL-23 plus IL-1β conditions, RNA was isolated from control or switched cultures for quantitative PCR. (E), Naive T cells were activated, primed with TGF-β plus IL-6 plus IL-1β for the first round followed by two rounds of stimulation in IL-23 plus IL-1β, were cultured for an additional round of stimulation in IL-23 plus IL-1β or TGF-β plus IL-2. The percentages of cells positive for Foxp3 or IL-17 intracellular staining are indicated with cells cultured for 1 wk in TGFβ plus IL-2 shown as a control for Foxp3 expression. (F), Naive $CD4^+$ T cells were primed and cultured as in *A*, and after the third round of culture, cells were enriched for IL-17-secreting cells by cytokine selection. Surface staining for IL-17 is shown pre- and post sort. (G), IL-17-high cells from (F) were cultured in Th1, Th2, or IL-23 plus IL-1β for an additional round of stimulation. Cells were stimulated for 4 h and stained for intracellular IL-17 and IFN-γ. (H), Supernatants from anti-CD3 stimulated cells cultured as in (G) were tested for cytokine production using ELISA. Data correspond to 2 experiments.

We then defined if the switch from Th17 to Th1 or Th2 was characterized by induction of the standard pathways and lineage determining factors. IL-4 signaling is qualitatively altered in Th1 cells through mechanisms that are still not clear but do not involve altered Stat6 activation (Huang and Paul, 1998), and IL-4 was able to activate Stat6 in cells cultured under Th1, Th2, or Th17 conditions (Fig 54A). During Th2 development, IL-12 signaling is extinguished, contributing to commitment in the Th2 lineage (Szabo et al., 1997). However, while *Il12rb2* expression was greatly decreased in cells cultured for three rounds in Th2 conditions, cells cultured in IL-23 plus IL-1β demonstrated expression of *Il12rb2* similar to Th1 cells (Fig 54B). To determine whether IL-12 signaling was functional, we examined Stat4 expression and IL-12-induced phosphorylation of Stat4 in Th1, Th2, and Th17 cultures after three rounds of stimulation. Although Stat4 expression is reduced and IL-12-induced Stat4 phosphorylation is eliminated in Th2 cultures, normal expression of Stat4 was retained in Th17 cultures. IL-12-induced Stat4 activation was only modestly diminished in IL-23 plus IL-1β and IL-23 cultured cells compared with Th1 cultures (Fig 54C). Despite the reduction in IL-12-induced Stat4, IL-12 and IL-4 were still able to promote an increase in *Tbx21* and *Gata3* expression in Th17 cultures switched to Th1 or Th2 conditions, respectively (Fig 54D). Thus, Th17 cells, even after long-term culture, are competent to assume a Th1 or Th2 phenotype.

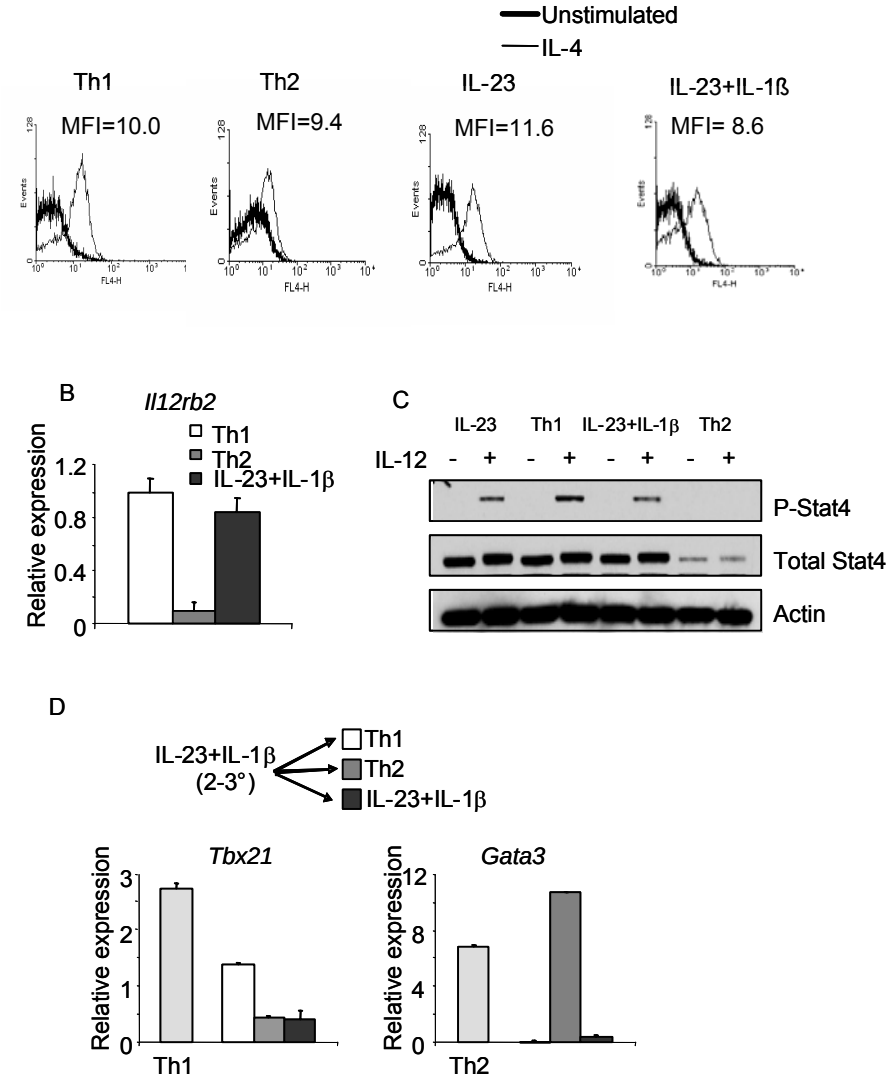


Figure 54. Signals promoting Th1 or Th2 development are intact in Th17 cultures. (A), Cells stimulated and cultured as in Fig 51, (A) and (B) were incubated with IL-4 for 30 min and stained for phospho-Stat6. (B), RNA from cells stimulated as in (A) for three rounds of stimulation was examined for relative levels of *Il12rb2* expression. Levels are relative to three-round Th1 cultures. (C), Cells stimulated and cultured as in (A) for three rounds of stimulation were then stimulated with IL-12 for 1 h. Phospho-Stat4, total Stat4, and actin were detected by immunoblot. (D), Cells were stimulated and cultured for three rounds as in (A). After 3 days of culture in Th1, Th2, or IL-23 plus IL-1β, RNA was isolated from control or switched cultures for quantitative PCR to test for expression of the indicated genes. Expression is relative to the level of expression of each gene in IL-23 plus IL-1β cultured cells before the fourth round of culture. Data correspond to 2 experiments.

DISCUSSION

Development of IL-17 secreting CD4 and CD8 T cells

Within the past 5 years Th17 cells have been discovered to be important mediators of inflammation including autoimmune diseases such as EAE (experimental autoimmune encephalomyelitis), a mouse model of multiple sclerosis. Cytokines and transcription factors regulating Th17 development are now fairly well established, making the factors that regulate IL-17 secretion from CD4 T cells an ideal framework for the study of IL-17 secretion from other cell types. IL-17 secreting CD8 T cells have been observed in several in vivo models; however the specific conditions and molecular mechanisms necessary for their development in vitro have not been fully elucidated. Similar to Th17 development, our data demonstrate that Tc17 development requires either IL-6 or IL-21 in combination with TGFβ, and STAT3. RORγt is also correlated with and can induce IL-17 secretion from CD8 T cells. Meanwhile, Tc17 development is inhibited by IFN-γ, STAT1, and T-bet. These observations regarding Tc17 regulation are consistent with several recent reports (Ciric et al., 2009; Hamada et al., 2009; Huber et al., 2009; Yen et al., 2009) which have also demonstrated a positive role in Tc17 development for IL-1 and TNFα. Other studies have shown inhibition of Tc17 development by IL-2, IL-27, and Type I interferons (Stumhofer et al., 2006; Curtis et al., 2009; Hamada et al., 2009). Altogether, similar regulation suggests that IL-17 inducing environments can induce both Th17 and Tc17 cells depending on the antigen presentation pathway. Indeed, both Th17 cells and Tc17 cells have been detected in infectious models of *Klebsiella*

pneumonia (Happel et al., 2003) and LCMV (Intlekofer et al., 2008) in addition to autoimmune models of contact hypersensitivity (He et al., 2006) and EAE (Huber et al., 2009). Similar regulation also suggests that mechanisms to inhibit IL-17 mediated pathologies would inhibit IL-17 secretion from both CD4 and CD8 T cells. This has been seen with T-bet and IL-27 mediated IL-17 inhibition in infectious models of *Trypanosoma cruzi* and *Toxoplasma gondii* respectively (Stumhofer et al., 2006; Guo et al., 2009). The emergence of IL-17 secreting CD8 T cells in vivo during inflammation implicate Tc17 cells as important mediators of IL-17 related pathologies and expose possible roles of IL-17 in CD8 T cell mediated functions.

Transcriptional regulation of IL-17 and IFN-γ secreting CD8 T cells

CD8 T cells mediate their functions through cytokine secretion and acquire the potential to secrete various cytokines after activating lineage specific transcription factors and proteins in the Stat signaling family. Utilizing gene deficient mice and siRNA, we and others (Huber et al., 2009; Yen et al., 2009) have demonstrated the critical role of Stat3 in Tc17 development. In addition, Stat3 is necessary for optimal expression of RORγt expression which can induce IL-17 production even in non-optimal conditions (Fig 14 and Huber et al., 2009).

IL-17 secreting cells have also been shown to be induced by Stat4 downstream of IL-23 (Mathur et al., 2007). IL-23 activates both Stat3 and Stat4 (Parham et al., 2002) and it is possible that Tc17 cells in vivo are induced through both TGFβ

/ IL-6 and IL-23 mediated pathways. Indeed, we found IL-23 promoted a small population of IL-17 secreting cells from a naïve CD8 population (Fig 4C). Even though Stat4 is not required for TGFβ / IL-6 to induce Tc17 cells (Fig 11A), it is still unknown whether Stat4 is important for IL-23 mediated Tc17 development.

The development of IFN-γ secreting CTLs is mediated through TCR induction of Eomes and T-bet. IL-12 activation of Stat4 further augments the potential for IFN-γ secretion and promotes memory development of Tc1 cells. In the absence of Stat4, IFN-γ production from Tc1 cells is greatly decreased despite similar expression of T-bet and higher expression of Eomes (Fig 19) suggesting that Stat4 acts in a non-redundant fashion with T-bet and Eomes to promote IFN-γ production. In contrast to IFN-γ expression from CD4 T cells, Stat1 is not necessary for optimal Tc1 development (Casey and Mescher, 2007 and Fig 11). Instead, the main role described for Stat1 in CD8 T cells has been in inhibiting CD8 T cell clonal expansion and promoting their contraction (Quigley et al., 2008). Our experiments also show that Stat1 functions to inhibit the Tc17 phenotype.

Stat proteins and master regulators not only promote the acquisition of cytokine secreting potentials, but also crossregulate T cell subsets to control various stages of the immune response and the immune response to different classes of pathogens. CD8 T cells which primarily produce IFN-γ after stimulation instead produce IL-17 in the absence of the critical CTL transcription factors Eomes and

T-bet (Intlekofer et al., 2008). This is associated with expression of the IL-17 transcription factor RORγt and a lack of cytotoxic activity from these cells. Conversely, CD8 T cells which are differentiated to produce IL-17 instead convert to a CTL phenotype and upregulate IFN-γ, Eomes, and T-bet in the absence of the critical Tc17 transcription factor Stat3. In addition, CTL associated proteins are not induced in conditions which induce IL-17 (Figs 7C, 8).

However, our overexpression studies suggest that factors in addition to Eomes, T-bet and Stat3 are involved in the crossregulation of IFN-γ and IL-17 secreting CD8 T cells. Overexpression of Stat3 is only able to reduce IFN-γ, but not granzyme B and overexpression of RORγt only slightly reduces granzyme B levels (Fig 13). Overexpression of Eomes and T-bet in Tc17 cells is also unable to completely suppress Tc17 programming and induce CTL cells (Fig 22).

Additional candidates to suppress Tc17 programming and induce CTL cells could include Stat1 and Stat4. Experiments with cells lacking Stat1 displayed increased IL-17 expression (Fig 11). In addition, IL-6 activates both Stat1 and Stat3 (Gerhartz et al., 1996) and despite Stat3 activation, only induces Tc17 cells in the presence of TGFβ. One possibility is that Stat1 is inhibiting activation of Stat3 and TGFβ relieves this inhibition since TGFβ has been shown to inhibit Stat1 function in epithelial cells (Reardon and McKay, 2007) and macrophages (Takaki et al., 2006). Stat4 may also be critical for the Tc17 / Tc1 crossregulation and in vitro Tc1 cultures require IL-12 induced Stat4 for optimal IFN-γ production.

One possibility is that Stat4 may be required for overexpressed T-bet or Eomes to induce optimal expression of IFN-γ in Tc17 cells as it is in CD4 cells where T-bet requires Stat4 to induce optimal IFN-γ production (Thieu et al., 2008).

Our overexpression studies were performed after 24 hrs of T cell activation. It is also possible that early gene expression is critical for the acquisition of cytokine secreting potential and genes expressed at a later time point have less effect in determining CD8 T cell fate. For example, a limited timeframe for CTL inhibition is seen when TGFβ and IL-6 are added to IL-12 induced cultures at different time points. When added at Day 0, TGFβ and IL-6 suppress T-bet, Eomes, IFN-γ, and granzyme B (Fig 21) and suppression of IFN-γ is absent when TGFβ and IL-6 are added to Tc1 cultures restimulated after 5 days (Fig 26C). Huber et al. (2009) also demonstrate that RORγt overexpression can more significantly upregulate IL-17 production under conditions of presumably early reduced Eomes expression, specifically cells cultured with IL-6 or IL-21 alone or with TGFβ (Fig 8). In these instances, it is possible that T-bet, Eomes, and Stat1 induced early during T cell activation are inhibiting the actions of the overexpressed Tc17 transcription factors.

Therefore even though Stat3/RORγt and Eomes/T-bet are critical transcription factors mediating the decision between IL-17 and IFN-γ production from a CD8 T cell, additional factors are likely necessary for optimal subset development.

Cytotoxicity of Tc17 cells

In addition to secreting cytokines, CD8 T cells also mediate their functions through direct cell killing. Tc1 cells secrete IFN-γ to activate macrophages and directly kill recognized targets via granzyme B / Perforin and Fas / FasL mediated apoptosis. Studies have shown conflicting results on the cytotoxic ability of Tc17 cells. We and others have demonstrated that Tc17 cells are noncytotoxic (Intlekofer et al., 2008; Hamada et al., 2009; Huber et al., 2009), although one study showed cytotoxic activity of Tc17 cells (Ciric et al., 2009). Two differences between our study and that of Ciric et al. include different types of cytotoxicity assays – Cr^{51} release vs CFSE based, and also different length of culture for Tc17 development – 5 days vs 3 days. To determine if these differences could account for the development of cytotoxic Tc17 cells, we analyzed the cytotoxic ability of our Tc17 cells in the assay described by Ciric et al. We found our Tc17 cells to be noncytotoxic in the CFSE based assay consistent with our Cr^{51} release results. We also found Tc17 cells to be noncytotoxic after 3 days in culture and were unable to determine the reason for the discrepancy in cytotoxic phenotypes.

Stability of IL-17 secreting T cells

Utilizing cytokine enrichment assays, we demonstrated that even after several rounds of stimulation, Th17 cells were unstable upon challenge with Th1 or Th2 instructive cytokines. Tc17 cells were also found to upregulate IFN-γ and cytotoxic function and downregulate IL-17 expression when challenged with IL-

12. This instability explains or correlates with several in vivo observations such as the presence of IFN-γ x IL-17 double producing cells in many disease models. Since Th1 cells do not convert to Th17 cells, cells producing both cytokines most likely first developed as Th17 cells. In addition, we find that the induction of IFN-γ in Tc17 cells is dependent on both STAT4 and T-bet. It is interesting that EAE, an autoimmune disease dependent on Th17 cells, is abrogated in the deficiency of STAT4 or T-bet, which are not required for the development of Th17 cells. It is possible that the instability of Th17 cells also requires Stat4 and T-bet and the Th17→Th1 switch is important for the propagation of EAE. The instability of IL-17 secreting cells raises the question of whether memory IL-17 secreting cells exist. However, it could also lead to several functional advantages. First, IL-17 secreting cells may facilitate proinflammatory cell access to immune privileged sites. IL-17 has been shown to disrupt blood brain barrier tight junctions leading to inflammation of the brain (Kebir et al., 2007). After immune access, proinflammatory cells can secrete different cytokines to effectively clear pathogens. Cells secreting both IL-17 and IFN-γ can also promote a dual function of localization and immune mediation. After pathogen invasion, neutrophils are found at the infection site followed by macrophages. Unstable T cells secreting IL-17 and then IFN-γ may mediate the temporal chemoattraction of innate immune cells to more effectively eradicate pathogens. In addition, dysregulated IL-17 responses have been shown to contribute towards autoimmune responses. The reduction of IL-17 secreting potential and

acquisition of IFN-γ secretion could be another mechanism of crossregulation to prevent IL-17 mediated pathology.

Effect of IL-17 in vaccinia virus infections

Clearance of vaccinia virus within the first 2 weeks of infection has mainly been attributed to immune cells producing Type I and II Interferons, NK cells, and B cell antibody production. A sublethal vaccinia virus infection of mice with isolated deficiencies of any of these mechanisms or immune cells does not cause death suggesting that mediators for viral clearance are able to compensate for one another (Muller et al., 1994; Huang et al., 1993; Cantin et al., 1999; Bukowski et al., 1983; Stitz et al., 1986; Xu et al., 2004). It is also possible that additional cells or cytokines, such as IL-17, have a role in vaccinia virus clearance which is masked by more dominant mechanisms. Previous reports analyzing the roles of cytokines in vaccinia virus infection have predominantly utilized recombinant vaccinia viruses expressing the cytokines of interest. These studies have made significant contributions to our understanding of the immune response to vaccinia virus, but have significant limitations. One limitation is that the level of cytokine expression may not correlate with physiological levels induced upon infection and cytokine expression cannot be separated from the infectious dose of virus. This can lead to nonphysiological or inconsistent cytokine expression and different immune responses. For example, in a model of tumor immunity, low IL-17 production is beneficial to the tumor by mechanisms including neovascularization. In contrast, high levels of IL-17 recruit innate immune cells

to the tumor site to promote tumor clearance. Therefore we chose alternative methods instead of recombinant cytokine expressing vaccinia viruses. Upon characterizing Tc17 cells in vitro, we determined the potential role of Tc17 cells in vaccinia virus in vivo utilizing gene deficient mice and adoptive transfer of Tc17 cells.

Altered IL-17 levels and vaccinia virus clearance

By analyzing vaccinia virus clearance from mice with altered IL-17 production, we did not find a clear correlation between IL-17 production and viral clearance, suggesting that a complex immune response to vaccinia which could be masking the role of IL-17. *Il17*$^{-/-}$ mice had a trend towards increased viral clearance upon infection with a sublethal dose of vaccinia virus. In contrast, Kohyama et al. (2007) reported decreased viral clearance with a lethal dose of vaccinia virus. The contradictory results could be due to the different doses used since a lethal viral dose could lead to nonphysiological immune responses caused by the increased release of cytokines into the bloodstream. In our experiments, we also observed increased IFN-γ production from CD8 T cells of infected *Il17*$^{-/-}$ mice which is likely contributing to the increased viral clearance. A recent report has demonstrated that IL-17 inhibits NK cell activity in a mouse model of eczema vaccinatum (Kawakami et al., 2009) suggesting that increased NK cell activity could also be contributing to increased viral clearance in our *Il17*$^{-/-}$ mice.

Clearance of vaccinia virus in mice deficient in IL-17 was also examined using *Stat3*$^{CD4-/-}$ mice. These mice have a conditional deletion of Stat3 in all T cells leading to defective T cell IL-17 production. *Stat3*$^{CD4-/-}$ had an increased trend in vaccinia titers during early time points and infected mice also had an increased trend in weight loss (data not shown) suggesting that IL-17 may contribute to vaccinia clearance. Interestingly, the trend in increased viral titers was seen despite increased IFN-γ production from CD4 cells in the Stat3 deficient mice. IL-17F and IL-21 production was also decreased in these mice. Stat3 is involved in several signaling pathways in T cells – IL-27 and IL-6 induction of IL-10 (Stumhofer et al., 2007), upregulation of CD25 (Akaishi et al., 1998) and SOCS3 (Qin et al., 2009), and *Stat3*$^{CD4-/-}$ mice have been observed to have increased inducible Foxp3^{+} regulatory T cells (Sehra, unpublished). Therefore, multiple factors could contribute to the increased vaccinia virus titers seen in these mice.

We also found decreased vaccinia virus clearance in mice with elevated expression of IL-17 due to a deficiency in T-bet. This is consistent with a report by Matsui et al. demonstrating that an absence of T-bet impairs VV-specific CTL function and IFN-γ production, and cytolytic function of NK cells. T-bet deficiency also leads to a partial shift in CD4 T cells to a Th2 like phenotype which had been attributed as a mechanism for viral survival (Matsui et al., 2005). Our data indicate that increased IL-17 may also contribute to viral survival in *Tbx21*$^{-/-}$ mice.

Tc17 cells

Adoptive cell transfer allowed us to directly analyze the antiviral potential of IL-17 secreting CD8 T cells. We found that transferred Tc17 cells were antiviral towards vaccinia virus, upregulated T-bet and IFN-γ, and acquired cytotoxic potential without upregulation of granzyme B. Antiviral function of Tc17 cells was independent of IFN-γ (Fig 46) and Stat4 (data not shown). We observed reduction of viral titers within 3 days after adoptive cell transfer making viral clearance by antibodies an unlikely mechanism during this short time period and the role of NK cells was not examined. IL-17 has been proposed to mediate immunity through neutrophil attraction, however, we did not observe neutrophilia upon transfer of Tc17 cells. In addition to acquiring cytotoxic potential, several other mechanisms could contribute to viral clearance by transferred Tc17 cells. Hamada et al. have suggested that Fas-FasL interactions play a role in Tc17 mediated clearance of influenza virus (personal communication). Indeed, we found that Tc17 cells upregulate FasL upon a second round of culture with IL-12 (data not shown). Tc17 cells also express TNFα which is important for viral clearance as suggested by the presence of a soluble TNF receptor in some strains of vaccinia virus (Reading et al., 2002). In addition, antimicrobial peptides have been shown to contribute to vaccinia virus killing (Howell et al., 2004). IL-22 upregulates antimicrobial peptides (Kolls et al., 2008) and IL-22 production from Tc17 cells could contribute to vaccinia clearance.

Several studies have observed upregulation of IFN-γ in IL-17 secreting T cells adoptively transferred into various disease models. Bending et al. (2009) and Martin-Orozco et al. (2009) both demonstrate that diabetes progression is dependent on IFN-γ and not IL-17 after transfer of Th17 cells. Muranski et al. (2008) demonstrate a dependence on IFN-γ production from Th17 cells in eradication of an established melanoma. Muranski et al. also show a survival advantage of transferred Th17 cells compared to Th1 and Th0 cells. With respect to Tc17 cells, Hamada et al. (2009) demonstrate that Tc17 cells mediate survival in an influenza virus infection in an IFN-γ dependant mechanism. Hinrichs et al. (2009) show conversion of Tc17 cells into Tc1 cells, but attribute increased Tc17 mediated melanoma clearance to increased persistence of Tc17 cells compared to Tc1 cells. Ciric et al. (2009) do not examine IFN-γ production and demonstrate that Tc17 cells mediate diabetes in an IL-17 dependant mechanism. In our model, Tc17 cells mediated vaccinia clearance in an IFN-γ independent mechanism and we demonstrate a novel cytotoxic potential of Tc17 cells after viral encounter in vivo. In these disease models, it is also possible that both IL-17 and IFN-γ contribute, and effects of one or both cytokines are masked by more dominant immune functions. In contact hypersensitivity responses, IL-17 and IFN-γ secreting CD8 T cells are both able to mediate the delayed type cellular response by recruiting distinct cellular infiltrates and having distinct potentials in inducing reactive oxygen species. Altogether, these studies suggest that the enhanced survival and unique ability of IL-17 secreting T cells to secrete

both IFN-γ and IL-17 allow them to be potent inflammatory cells contributing to the immune response in several disease states.

Tc2 cells and vaccinia virus

Even though Tc17 cells gain cytotoxicity and mediate viral clearance in vivo, they are noncytotoxic in vitro similar to other CD8 T cell subsets such as Tc2 cells. Both cell types are activated by viral antigens, however the role of Tc2 cells in vaccinia virus clearance is unknown. In a model of viral infection utilizing influenza virus, Tc2 cells were found to be less cytotoxic than Tc1 cells due to decreased chemoattraction (Cerwenka et al., 1999). In contrast, larger numbers of Tc17 cells were found in the draining lymphoid organs during our experiments. Additional studies exploring the mechanism of action of CD8 subsets in vivo could determine if Tc2 cells are also able to mediate vaccinia virus clearance.

Routes of viral inoculation and the immune response

The natural route of infection for vaccinia virus is the respiratory tract (Fenner et al., 1989), although several models have been developed to study the use of vaccinia virus as a vaccine vector and to better understand the immune response to vaccinia virus. In addition to the intranasal route of infection; intradermal, intraperitoneal, and intracranial routes of infection have been utilized (Turner, 1967; Buller et al., 1985; Williamson et al., 1990; Lee et al., 1992; Tscharke and Smith, 1999). However, these routes do not illicit identical immune responses since critical VV virulence factors often depend on the route of infection

(Tscharke et al., 2002). Our studies infected mice via i.n. and i.p. routes and found that adoptively transferred Tc17 cells mediated antiviral immunity through both routes while highlighting important observations. First, i.n. infection led to increased immunogenicity and larger amounts of cytokines being produced. This could be due to the virus having a higher potency in a more localized environment or to the innate cells in these environments having different priming capabilities. This is in contrast to a previous study which showed that CTL cells were impaired in the lung compared to the spleen after i.n. infection (DiNapoli et al., 2008). However, $\gamma\delta$ T cells, CD8α dendritic cells and alveolar macrophages have also been shown to be important in VV clearance and it is possible that these innate cells have differential effects on CTL vs Tc17 development at these sites (Selin et al., 2001; Belz et al., 2004; Rivera et al., 2007). A second observation noted was that during an i.n. infection, more Tc17 cells were recruited or proliferated in the draining lymph nodes compared to Tc1 cells while similar numbers were found in the spleen. It is possible that Tc17 cells have an intrinsic proliferation advantage or decrease in apoptosis since more Tc17 cells were also found in the spleen after an i.p. infection. Another possiblility is that Tc17 cells express higher levels of chemokine receptors to migrate to sites of infection. Even though adoptively transferred Tc17 cells were antiviral regardless of route, the route of infection may be critical in future experiments studying endogenous Tc17 cells.

IL-21 and Tc17 cells

Recent studies (Elsaesser et al., 2009; Frohlich et al., 2009; Yi et al., 2009) have demonstrated that IL-21 is an important cytokine necessary to sustain long term function of CD8 T cells. IL-21 prevents CD8 T cell exhaustion and is proposed to be one of the critical elements that allow CD4 T cells to help CD8 T cells combat chronic viral infection. Production of IL-21 by Tc17 cells raises the possibility that Tc17 cells may be uniquely suited to combat chronic viral infections – either through autocrine IL-21 signaling or paracrine IL-21 signaling towards IFN-γ secreting CD8 T cells. It is unclear how IL-21 prevents exhaustion, however, autocrine IL-21 signals may also lead to greater clonal expansion and memory generation of Tc17 cells. In addition, Tc17 cells could utilize their unstable phenotype to prevent exhaustion in IFN-γ secreting CD8 T cells which once secreted IL-17. Exhaustion of CD8 T cells was first described with LCMV (Zajac et al., 1998), and has also been seen in animal models of adenovirus (Krebs et al., 2005) and human chronic infections such as HIV (Shankar et al., 2000), HCV (Gruener et al., 2001), and HBV (Reignat et al., 2002). The role of Tc17 cells in these models is currently unknown. Interestingly, Stat3 is critical for both IL-21 signaling and Tc17 development suggesting that Tc17 cells may have an intrinsic ability to prevent exhaustion.

OVERALL CONCLUSION

$CD8^+$ T cells that secrete IFN-γ and cytotoxic molecules have been well characterized and function to mediate antiviral immunity, however, IL-17-secreting $CD8^+$ T cells are only beginning to be described. Using in vitro T cell cultures and mice deficient in transcription factors regulating lineage development, our studies defined Tc17 development and function. Similar to Th17 cells, Tc17 development is dependent on the transcription factor Stat3 and inhibited by Stat1. Compared to Tc1 cells, expression of T-bet and Eomes is reduced in Tc17 cells and consistent with this, Tc17 cells are non-cytotoxic in vitro. However, Tc17 cells exhibit plasticity and switch to cytotoxic IFN-γ producing cells when exposed to a Tc1 inducing cytokine, IL-12. Overexpression of the lineage promoting transcription factors T-bet and Eomes is unable to induce a Tc1 phenotype in Tc17 cells and Stat3 is also unable to switch Tc1 cells into Tc17 cells, suggesting additional signals are involved in CD8 T cell lineage commitment. Experiments utilizing a vaccinia virus infection model demonstrate that Tc17 cells mediate antiviral immunity in vivo and acquire a novel phenotype after viral encounter with the potential to secrete IFN-γ and mediate direct cell cytotoxicity. However, IFN-γ secretion is not necessary for vaccinia clearance by Tc17 cells and the role of cell mediated cytotoxicity is still unclear. These data suggest that Tc17 cells may mediate anti-viral immunity through novel mechanisms that depend on the ability of Tc17 cells to acquire other phenotypes.

FUTURE DIRECTIONS

Are endogenous Tc17 cells necessary for vaccinia virus clearance?

Even though CD8 cells are dispensable during primary vaccina virus clearance, they are critical for anti-vaccinia defense in the absence of CD4 T cells. However, it is unknown whether these CD8 T cells require IL-17 to mediate their function. Our experiments utilizing gene knockout mice with altered levels of IL-17 did not show a clear relationship between IL-17 and viral clearance or discriminate between IL-17 secretion by CD4 or CD8 T cells. To determine the role of endogenous IL-17 secreting CD8 T cells in vaccinia virus infection, first, a correlation could be determined utilizing infected mice deficient in CD4 T cells (either through antibody depletion or genetic deficiency such as $CIITA^{-/-}$ or $A\beta^{-/-}$ mice) and characterizing the cytokine response from the CD8 T cells. Next, mice lacking both CD4 and CD8 T cells, such as $Rag^{-/-}$ mice, could be infected and various cytokine deficient CD8 T cells adoptively transferred. Another strategy to determine if IL-17 producing cells are necessary for anti-vaccinia defense would utilize infected $Stat3^{CD4-/-}$ mice and adding back WT CD4 and CD8 T cell populations to determine which IL-17 secreting population promotes optimal viral clearance.

Are Tc17 cells intrinsically unstable and how do they mediate viral protection?

Tc17 cells convert to IFN-γ secreting CD8 cells when stimulated in the presence of IL-12 in vitro. In addition, Tc17 cells transferred into a virally infected mouse

acquire a cytotoxic phenotype as they mediate viral clearance. There are at least 4 possibilities for the requirements necessary to convert Tc17 cells to a cytotoxic phenotype – 1) Tc17 cells alter their phenotype upon homeostatic proliferation, 2) viral antigen is required for conversion, 3) an inflammatory milieu is required for conversion, 4) or both antigen and inflammatory cytokines are required. Determining the requirements for the transition of the Tc17 phenotype would provide insight into the mechanism of viral clearance by adoptively transferred Tc17 cells. If Tc17 cells require any combination of viral antigens or inflammatory cytokines to become cytotoxic, it is likely that this cytotoxic phenotype is necessary for viral clearance. Further in vitro cytotoxicity assays or in vivo infection experiments utilizing antibody neutralization or genetically deficient cells could determine which cytotoxic mediators – for example granzyme B, perforin, FasL, TNFα, or a novel cytotoxic molecule – are responsible for Tc17 mediated cytotoxicity. On the other hand, if Tc17 cells are intrinsically unstable, it would suggest that the cytotoxic phenotype is a byproduct of the Tc17 life cycle and raise the possibility that molecules involved in the Tc17 phenotype – such as IL-21 and IL-22 are responsible for Tc17 mediated viral clearance. These experiments can be performed utilizing transferred Tc17 cells and bone marrow derived dendritic cells either infected with vaccinia virus or uninfected and analyzing subsequent Tc17 phenotypes. Since *Stat4*$^{-/-}$ x *Tbx21*$^{-/-}$ double knockout CD8 cells maintain a Tc17 phenotype in vitro, antigen specific double knockout cells could also be utilized to answer a similar question of whether conversion of the Tc17 phenotype is necessary for viral clearance.

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Curriculum Vitae

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Education

2005-2011	Indiana University, Indianapolis, IN	Ph.D. Immunology
1996-2000	Purdue University, West Lafayette, IN	B.S. Biochemistry

Work Experience

2001-2003	Peace Corps, Ghana, West Africa	Teacher
2000-2001	National Institute of Health, Bethesda, MD	Internship

Awards and Honors

2003-Present	Medical Scientist Training Program Fellowship, Indiana University
2008-2009	Immunology and Infectious Disease Training Grant (T32 AI 060519)
2008	University Travel Fellowship
2007-2008	Cancer Biology Training Program Grant (Devault Endowment)
2000-2001	Cancer Research Training Award (CRTA), NIH

Publications

Yeh N*, Glosson N*, Wang N, McKinley C, Hamada H, Li Q, Dutton RW, Shrikant P, Zhou B, Brutkiewicz RR, Blum JS, Kaplan MH. Tc17 cells are capable of mediating immunity to vaccinia virus by acquisition of a cytotoxic phenotype. J Immunol. Accepted.

Good SR, Thieu VT, Mathur AN, Yu Q, Stritesky GL, **Yeh N**, O'Malley JT, Perumal NB, Kaplan MH. Temporal induction pattern of STAT4 target genes defines potential for Th1 lineage-specific programming. J Immunol. 2009 Sep 15;183(6):3839-47.

Thieu VT, Yu Q, Chang HC, **Yeh N**, Nguyen ET, Sehra S, Kaplan MH. Signal transducer and activator of transcription 4 is required for the transcription factor T-bet to promote T helper 1 cell-fate determination. Immunity. 2008 Nov; 29(5): 679-90.

Stritesky G*, **Yeh N*** and Kaplan MH. IL-23 promotes maintenance but not commitment to the Th17 lineage. J. Immunol. 2008 Nov 1; 181(9):5948-55.

Mann B, Huang J, Li P, Chang HC, Slee R, O'Sullivan A, Mathur A, **Yeh N**, Klemsz M, Brutkiewicz R, Blum J, Kaplan M. Vaccinia virus blocks STAT1-

dependent and -independent gene expression induced by type I and type II interferons. J Interferon Cytokine Res. 2008 Jun; 28(6):367-80.

Li W, Sofi MH, **Yeh N**, Sehra S, McCarthy BP, Patel DR, Brutkiewicz R, Kaplan M, Chang CH. Thymic selection pathway regulates the effector function of CD4 T cells. J Exp Med. 2007 Sep 3; 204(9):2145-57.

Hansen AM, Qui Y, **Yeh N**, Blattner FR, Durfee T, Jin DJ. SspA is required for acid resistance in stationary phase by downregulation of H-NS in Escherichia coli. Mol Microbiol. 2005 May;56(3):719-34.

*These authors contributed equally to the work.

Abstracts Presented

N Yeh, N Wang, R Brutkiewicz, J Blum, MH Kaplan. Development and anti-viral activity of IL-17-secreting CD8 T cells. Autumn Immunology Conference. Nov 2008.

N Yeh, DE Levy and MH Kaplan. Regulation of the differentiation of IL-17-secreting CD8 T cells. American Association of Immunologists Conference. Apr 2008.

N Yeh, DE Levy and MH Kaplan. Regulation of the differentiation of IL-17-secreting CD8 T cells. Autumn Immunology Conference. Nov 2007.