DEVELOPMENT AND STABILITY OF IL-17-SECRETING T CELLS

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IL-17-producing T cells are critical to the development of pathogen and tumor immunity, but also contribute to the pathology of autoimmune diseases and allergic inflammation. $CD8^+$ (Tc17) and $CD4^+$ (Th17) IL-17-secreting T cells develop in response to a cytokine environment that activates Signal Transducer and Activator of Transcription (STAT) proteins, though the mechanisms underlying Tc17/Th17 development and stability are still unclear. In vivo, Tc17 cells clear vaccinia virus infection and acquire cytotoxic potential, that is independent of IL-17 production and the acquisition of IFN-γ-secreting potential, but partially dependent on Fas ligand, suggesting that Tc17-mediated vaccinia virus clearance is through cell killing independent of an acquired Tc1 phenotype. In contrast, memory Th cells and NKT cells display STAT4-dependent IL-23-induced IL-17 production that correlates with *Il23r* expression. IL-23 does not activate STAT4 nor do other STAT4-activating cytokines induce *Il23r* expression in these populations, suggesting a T cell-extrinsic role for STAT4 in mediating IL-23 responsiveness. Although IL-23 is important for the maintenance of IL-17-secreting T cells, it also promotes their instability, often resulting in a pathogenic Th1-like phenotype in vitro and in vivo. In vitro-derived Th17 cells are also flexible when cultured under polarizing conditions that promote Th2 or Th9 differentiation, adopting the respective effector programs, and decreasing IL-17

production. However, in models of allergic airway disease, Th17 cells do not secrete alternative cytokines nor adopt other effector programs, and remain stable IL-17-secretors. In contrast to Th1-biased pro-inflammatory environments that induce Th17 instability in vivo, during allergic inflammatory disease, Th17 cells are comparatively stable, and retain the potential to produce IL-17. Together these data document that the inflammatory environment has distinct effects on the stability of IL-17-secreting T cells in vivo.

Mark H. Kaplan, Ph.D.-Chair

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AAD	Allergic airway disease
AHR	Airway hyperresponsiveness
AHR	Aryl hydrocarbon receptor
Aiolos	Ikaros family zinc finger protein 3
Alum	Aluminum hydroxide
AP-1	Activator protein 1
APC	Antigen presenting cells
ASP	*Aspergillus fumigatus*
BAL	Bronchoalveolar lavage
BATF	Basic leucine zipper transcription factor ATF like
BCL	B cell lymphoma
BCR	B cell receptor
BMDC	Bone marrow derived dendritic cell
BrdU	Bromodeoxyuridine
BSA	Bovine serum albumin
CBF-β	Core binding factor, beta subunit
CBP	CREB-binding protein
CCL	C-C chemokine ligand
CCR	Chemokine (C-C motif) receptor
CD	Cluster of differentiation
CFA	Complete Freund’s adjuvant
CNS	Conserved non-coding sequence

CREB	cAMP response element-binding
CTL	Cytotoxic T lymphocyte
CTLA	Cytotoxic T lymphocyte antigen
CXCL	Chemokine (C-X-C motif) ligand
CXCR	C-X-C chemokine receptor
DAPA	DNA affinity precipitation assay
DC	Dendritic cell
DEPC	Diethylpyrocarbonate
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
EAE	Experimental autoimmune encephalomyelitis
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
Eomes	Eomesodermin
ERK	Extracellular signal-regulated kinase
Ets	E-twenty six
ETV5	ETS variant gene 5
FasL	Fas ligand
FBS	Fetal bovine serum
FACS	Fluorescence-activated cell sorting
Foxp3	Forkhead box protein 3
G-CSF	Granulocyte colony-stimulating factor
GalCer	Galactosylceramide

GATA3	GATA binding protein 3
Gfi-1	Growth factor independence 1
GFP	Green fluorescent protein
GM-CSF	Granulocyte macrophage colony-stimulating factor
HDM	House dust mite
Hlx	H2.0-like homeobox-1
IBD	Inflammatory bowel disease
ICOS	Inducible co-stimulator
ICS	Intracellular staining
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
ILL	Innate like lymphocyte
IN	intranasal
IP	intraperitoneal
IPEX	Immune dysregulation, polyendocrinopathy, X-linked
IRF	Interferon regulatory factor
Itκ	IL-2-inducible T cell kinase
iTreg	inducible T regulatory
IV	Intravenous
IκB	Inhibitor of κB
JAK	Janus family tyrosine kinase
JunB	Jun B proto-oncogene

LPS	Lipopolysaccharide
LT-α	Lymphotoxin alpha
Lti	Lymphoid tissue inducer
Maf	Musculoaponeurotic fibrosarcoma
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
MOG	Myelin oligodendrocyte glycoprotein
mRNA	messenger ribonucleic acid
MS	Multiple Sclerosis
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NFAT	Nuclear factor of activated T cells
NK	Natural killer
NKT	Natural killer T
nTreg	Natural T regulatory
OVA	Ovalbumin
PAMP	Pathogen-associated molecular pattern
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PI3	Phosphatidylinositol 3
PMA	Phorbol 12-myristake 13-acetate
Poly(I:C)	Polyinosinic-polycytidylic acid
PRR	Pattern recognition receptor

pSTAT	Phosphorylated STAT
PU.1	SFFV proviral integration 1
qRT-PCR	quantitative real-time polymerase chain reaction
RA	Rheumatoid arthritis
ROG	Repressor of GATA
ROR	Retinoid-acid-related orphan receptor
Runx	Runt-related transcription factor
SC	Subcutaneous
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
Sfpi1	Spleen focus forming virus proviral integration site-1
SH2	Src-homology domain
SLE	Systemic lupus erythematosus
SOCS	Suppressor of cytokine signaling
STAT	Signal transducer and activator of transcription
SWI/SNF	SWItch/Sucrose NonFermentable
T-bet	T-box expressed in T cells
Tbx21	T-box transcription factor 21
Tc	T cytotoxic
TCF	T cell factor 1
TCR	T cell receptor
TGF	Transforming growth factor
Th	T helper
TLR	Toll-like receptor

TLSP	Thymic stromal lymphopoietin
TNF	Tumor necrosis factor
Treg	T regulatory
TYK	Tyrosine Kinase
VV	Vaccinia virus
WT	Wild type
YFP	Yellow fluorescent protein
GalCer	Galactosylceramide

INTRODUCTION

Innate and adaptive immunity

Innate and adaptive immunity evolved to protect the body from infectious and foreign agents, which could otherwise be harmful to the host. Innate immunity is the first line of defense in protecting the host from most microorganisms through immediate effector responses. When an infectious organism breaches the first line of defense, an adaptive immune response will ensue. Antigen-specific effector cells that target the infectious organism are generated, followed by the generation of memory cells, which provide long-lasting immunity upon reinfection with the same pathogen. Thus, innate and adaptive immune responses work together to protect the host from pathogenic and foreign agents (1).

Microorganisms that cause disease enter the body at various sites and the first line of defense lies at epithelial surfaces of the body, such as the skin, gut, lung, eyes, and nose. Physical barriers, such as epithelial cells held together by tight junctions, cilia that continuously move and refresh mucus layers, and tears, are all important for the first line of defense. Epithelial cells at surface layers also produce protective chemical substances, such as enzymes and antibacterial peptides that prevent microbial growth. Additionally, epithelial surfaces are also associated with commensal microorganisms, which produce antimicrobial peptides or compete with infectious pathogens for nutrients (1, 2).

Once a microorganism crosses the epithelial barrier, various types of innate immune cells take part in the recognition and clearance of the pathogen. Phagocytic cells, such as macrophages, neutrophils and dendritic cells (DCs) express cell surface receptors, known as pattern recognition receptors (PRRs), which recognize pathogen-associated molecular patterns (PAMPs) that are present on many microorganisms. For example, the PRR, toll like receptor 4 (TLR4), recognizes the structural pattern of lipopolysaccharide (LPS), a major component of the outer membrane of Gram-negative bacteria. Macrophages are resident in most tissues and upon immediate recognition of a pathogen, they will engulf and kill the pathogen. Neutrophils, which do not normally reside in healthy tissues, can be recruited to sites of infection in large numbers and aid in the clearance of the pathogen through phagocytosis as well. Upon phagocytosis, macrophages and neutrophils produce toxic molecules that kill the engulfed pathogen, such as antimicrobial peptides and nitric oxide. DCs are also phagocytic, and they can further take up extracellular fluid and its contents through macropinocytosis. Upon maturation, DCs have an important role in presenting antigen to resting T cells. Basophils and eosinophils are innate immune cells that mediate defense against parasites through the release of cytokines, enzymes and toxic proteins. Mast cells are important for the clearance of parasitic worms and mediate inflammation through the release of agents, such as histamine. Natural killer cells (NK cells) recognize and kill abnormal cells, such as tumor cells, or cells infected with pathogenic agents, such as viruses.

With the exception of NK cells, which belong to the lymphocyte lineage, the aforementioned cells are derived from the myeloid lineage (1, 2).

The immune system must be able to recognize and mount an immune response against a wide variety of pathogens. With the exception of NK cells, lymphocytes display antigen specificity through the expression of highly variable antigen receptors on the cell surface that bind specific antigens. Each lymphocyte has a unique variant of a prototype antigen receptor, which is generated during development by the rearrangement of receptor gene segments. This allows for the generation of a pool of lymphocytes that express a diverse repertoire of receptors, ensuring that there will always be a population of lymphocytes circulating the body that can recognize a particular foreign antigen. Lymphocytes are comprised of B cells and T cells which recognize antigen through the B cell receptor (BCR) or T cell receptor (TCR), respectively. Most of these cells are part of the adaptive immune response, however there are some lymphocytes that are important for innate immunity and are referred to as innate-like lymphocytes (ILLs). These cells express antigen receptors that have limited diversity and can respond to antigens quickly, as they do not need to undergo clonal expansion before eliciting their effector response. γδ T cells have an antigen receptor composed of a γ chain and a δ chain, and unlike αβ T cells, which express an antigen receptor composed of an α chain and a β chain, they can recognize and target antigens directly. There are two types of γδ T cells, those found in lymphoid organs that have highly diversified T cell receptors and others known

as intraepithelial γδ T cells, which localize to a particular site in the host, such as the skin, have receptors with limited diversity, and recognize molecules other than pathogen-specific antigens. Natural killer T cells (NKT) are another subset of ILLs and they are present in the thymus and peripheral lymphoid organs. NKT cells express an invariant T cell receptor α chain and one of three β chains. These cells recognize glycolipid antigens presented by the non-classical major histocompatibility complex (MHC) molecule, CD1d, on the host cell surface. Upon stimulation, γδ T cells and NKT cells release cytokines, such as IFN-γ, IL-4 and IL-17 leading to increased inflammation. Together, myeloid cells, NK cells and ILLs work as a first line of defense in protecting the host against infectious and foreign agents (1, 2).

Adaptive immunity is characterized by antigen-specific responses driven by B cells and αβ T cells. Naïve lymphocytes circulate the blood and lymph and upon encounter with antigen and subsequent activation, they become effector lymphocytes. Naïve B cells become activated upon antigen binding to its BCR. The activated B cell proliferates and differentiates into a plasma cell, which produces large amounts of antibodies, also referred to as an immunoglobulin (Ig), that are specific for the antigen that activated the B cell. B cells produce five main classes of immunoglobulins, some of which can be further subdivided into subclasses, and all of which have different functions in adaptive immunity such as binding and neutralizing infectious agents. The antibody repertoire can be further diversified in activated B cells through somatic hypermutation, which

enhances the ability of the antibody to recognize and bind to foreign antigens and increases the effector abilities of the antibody (1, 2).

In contrast to the B cell receptor, the T cell receptor recognizes antigens, called peptides, which are presented on the surface of other types of host cells, referred to as antigen presenting cells (APCs). The three professional APCs are DCs, macrophages and B cells. APCs break down foreign proteins into peptides, which are loaded onto MHC molecules and expressed on the cell surface. Naïve αβ T cells become activated upon encounter with the TCR and a peptide:MHC complex on an APC. T cells express either a CD4 or CD8 co-receptor on the cell surface, which is important for antigen recognition and T cell activation. CD4 T cells recognize peptide presented on MHC class II molecules and CD8 T cells recognize peptide presented on MHC class I molecules. Upon antigen recognition and co-stimulation of the T cell, the T cell becomes activated and undergoes clonal expansion and differentiation into an effector T cell. T cell differentiation is influenced by signals in the local environment and depending on the type of infection, different T cells subsets, each with a specific effector function, will be generated to clear the infection. CD8 T cells can differentiate into cytotoxic T cells that specialize in the killing of abnormal cells, such as tumor cells or virally infected cells, through the release of pro-inflammatory cytokines or cytotoxic molecules. CD4 T cells, known as T helper cells, differentiate into effector subsets and are characterized by the cytokines they secrete, giving them specific function. For example, Th1 cells secrete IFN-γ, which is important in

activating macrophages and increasing their microbicidal activity. Regulatory T cells suppress the activity of other lymphocytes and help control the immune response. Over the course of an immune response, a subset of B cells and T cells that have been activated by antigen will differentiate into memory cells. Upon a second exposure with the same antigen, memory cells will quickly differentiate into effector cells and provide long-lasting immunity to the infectious agent. While the adaptive immune response is critical for host defense, it can also be triggered by non-infectious antigens or self-antigens, leading to the development of allergic and autoimmune diseases, respectively. Thus, innate and adaptive immune responses are crucial for host defense and normal health, however an inappropriate response can be detrimental to the host causing disease (1, 2).

JAK-STAT pathway

The Janus kinase (JAK)-signal transducer and activator of transcription (STAT) pathway has an essential role in mediating cytokine and growth factor-induced immune responses. The JAK family is made up of four members: JAK1, JAK2, JAK3 and tyrosine Kinase 2 (Tyk2), each of which constitutively binds to the cytoplasmic end of membrane spanning cell surface receptors. Upon cytokine binding to its receptor, receptor dimerization is triggered followed by activation of JAKs. Specific tyrosine residues on the intracellular domain of the receptors are phosphorylated by activated JAKs and serve as docking sites for STATs. STAT proteins are a family of cytosolic latent transcription factors, of which there are 7

members: STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B and STAT6. STAT molecules bind to phosphorylated receptors via their SRC homology 2 (SH2) domain and are phosphorylated by JAKs on a conserved tyrosine residue. Activated STAT monomers dimerize with one another through interactions with their phosphorylated-tyrosine residues and SH2 domains to form homodimers or heterodimers. STAT dimerization leads to the translocation of STAT proteins to the nucleus where they bind DNA and modulate expression of target genes. Cytokines and growth factors activate specific STAT family members and regulate a number of immune cells, including T cells, thereby playing an essential role in the development, differentiation, stability and function of T cells (3).

T helper cells

T helper cell differentiation is a critical component to adaptive immunity. To begin the differentiation process, a naïve $CD4^+$ T cell must be activated by a specific antigen. This occurs through an interaction between the TCR and peptide:MHC II complex on an APC, that in conjunction with the CD4 co-receptor, induces T cell activation. Ligation of co-stimulatory molecules on the T cell, such as CD28, with its receptor on the APC, CD80/CD86 (for CD28 co-stimulation), is required for proper T cell proliferation and survival. Upon T cell activation, CD4 T cells will differentiate into one of several T helper lineages depending on the cytokines present in the local environment. Cytokine signals induce a network of transcription factors that are important for the development of each T helper lineage. T helper subsets are often characterized by the cytokines they secrete,

which are essential for their effector functions in the immune response. Each T helper subset has a specialized role in immune homeostasis, host-defense or the development of inflammatory disease (1). A summary of T helper cell differentiation is illustrated in Figure 1.

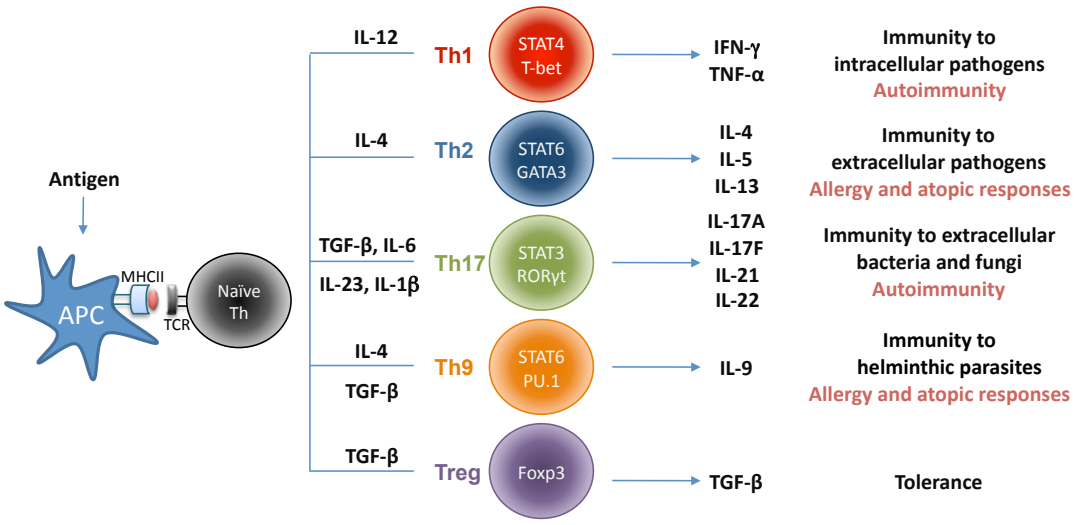


Figure 1. Differentiation and function of T helper cell lineages. Naïve $CD4^+$ T cells differentiate into several effector lineages based on the signals they receive upon interaction with APCs. T helper cells produce effector molecules and have specific functions in mediating inflammatory responses.

Th1 cells

T helper type 1 cells (Th1) mediate host defense against intracellular pathogens, however they can also contribute to the pathology of allergic and autoimmune diseases. Th1 cells primarily secrete IFN-γ, TNF-α, and LT-α and are important

for cell-mediated immunity, characterized by cellular cytolytic activity. In addition to their promotion of IgG2a, the products of Th1 cells are important in the differentiation and migration of macrophages, and the production of IFN-γ is essential in activating macrophages and increasing their microbicidal activity (4). Th1 cells are essential for the clearance of intracellular bacteria, such as *Mycobacterium avium, Salmonella typhimurium* and *Listeria monocytogenes*, of protozoal parasites such as, *Leishmania major,* of fungi such as, *Cryptococcus neoformans* and of viruses, such as influenza virus, herpes simplex virus and vaccinia virus. Th1 cells have further been implicated in tumor immunity. In contrast to their role in mediating pathogen and tumor immunity, Th1 cells can induce detrimental inflammatory responses and contribute to the development of inflammatory bowel disease (IBD), type 1 diabetes, rheumatoid arthritis (RA) and asthma (5).

IL-12 and IFN-γ signals promote the development of Th1 cells. IL-12 and IFN-γ activate STAT4 and STAT1, respectively, both of which are required for optimal Th1 development (6-10). The expression of T-bet (T-box expressed in T cells), which has been established as the master regulator of the Th1 lineage and is required for its development (11), is induced early in Th1 differentiation by TCR-IFN-γ-STAT1 signals and later by IL-12-STAT4 signals (12). STAT4 and T-bet cooperate to activate a number of Th1 genes, although each can activate a subset of Th1 genes in the absence of the other (13). STAT4 and T-bet induce the expression of runt-related transcription factor 3 (Runx3), which is also

important for Th1 development. The expression of IFN-γ is induced by STAT4 and T-bet in cooperation with other transcription factors, such as Runx3 and Hlx (H2.0-like homebox-1) (13-16). Thus, a number of signals are required for the development of the Th1 lineage, which is essential for host immunity, but also induces disease pathology.

Th2 cells

T helper type 2 cells (Th2) mediate host defense against extracellular pathogens, however they are also a major contributor to the pathology of atopic disease. Th2 cells secrete IL-4, IL-5, IL-9, IL-10, and IL-13 and induce IgG1 and IgE class-switching. They are an essential component of the humoral immune response elicited to neutralize extracellular pathogens and are indispensable for the clearance of extracellular parasites, such as helminthes and nematodes. Importantly, Th2 cells also mediate allergic disease. IL-4 induces IgE-class switching, and IgE surface bound allergen complexes activate innate immune cells, such as basophils and mast cells by cross-linking their high affinity Fc receptors for IgE, resulting in their degranulation. Upon activation, basophils and mast cells secrete a number of inflammatory mediators, including chemokines, cytokines, heparin, histamine, proteases and serotonin, which induce smooth muscle constriction, vascular permeability and the recruitment of additional inflammatory cells. In mucosal tissues, such as the lung and intestine, Th2 cells can induce eosinophilia and mast cell hyperplasia through the recruitment of eosinophils and mast cells, regulated by IL-5 and IL-9, respectively. The IL-4, IL-

9 and IL-13 produced acts directly on epithelial cells leading to goblet cell hyperplasia and mucus production; IL-4 and IL-13 can act directly on smooth muscle cells. In the lung, these Th2-mediated effects can induce airway hyperresponsiveness (AHR) (17, 18). Thus, while Th2-mediated responses are necessary for host immunity, an inappropriate Th2 response can be detrimental to the host.

IL-4 and IL-2 signals promote the development of Th2 cells. IL-4 and IL-2 activate STAT6 and STAT5, respectively, both of which are required for optimal Th2 development (19-23). IL-4 activated STAT6 induces the expression of GATA-binding protein 3 (GATA3), the master regulator of the Th2 lineage. GATA3 is required for the development of Th2 cells (24, 25). STAT6-independent pathways, such as the Notch-signaling pathway and Wnt signaling pathway, through β-catenin and T cell Factor 1 (TCF1), also promote GATA3 expression (26, 27). GATA3 induces expression of the hallmark Th2 effector cytokines, IL-4, IL-5 and IL-13 by binding to the promoter and enhancer regions of the *Il4-Il5-Il13* loci (28-31). Many other transcription factors also play a role in the development of Th2 cells. IL-4 activated STAT6 induces the expression of growth factor independent 1 (Gfi-1) and basic leucine zipper transcriptional factor ATF-like (BATF), which are important for optimal cell growth (32, 33). Interferon regulatory factor 4 (IRF4) is also essential for Th2 development and interacts with NFAT2c (nuclear factor of activated T cells) to induce expression of IL-4 (34-36). Another transcription factor, c-Maf (musculoaponeurotic fibrosarcoma), also induces the

expression of IL-4 in Th2 cells (37). JunB (jun B proto-oncogene) cooperates with c-Maf to induce IL-4 expression by binding to the *Il4* promoter (38). c-Maf further promotes Th2 development by upregulating the expression of CD25 (IL-2Rα) (39). In addition to STAT6, STAT5 and STAT3 are also important for Th2 development. STAT5 binds to regulatory elements in the *Il4* locus and cooperates with GATA3 to increase the accessibility and expression of the *Il4* gene (22, 23). STAT5 further induces the expression of IL-4Rα (40). During Th2 differentiation, STAT3 also becomes activated, and is required for STAT6 binding to target genes in Th2 cells (41). These findings illustrate that a complex transcriptional network controls the development of the Th2 lineage.

Th9 cells

T helper type 9 cells (Th9) are the most recently identified T helper subset, and are currently under intense investigation. Th9 cells primarily produce IL-9, but can also produce IL-10 and IL-21. They are important for the clearance of extracellular parasites, such as the nematodes *Trichuris muris* and *Trichinella spiralis*, but are most notably recognized for their role in the development of allergic disease. IL-9 affects a number of immune cells, including epithelial cells, hematopoietic progenitor cells, lymphocytes, mast cells, and smooth muscle cells. IL-9 stimulates the cell growth and expansion of mast cells, and further stimulates their production of proteases and cytokines involved in allergic responses. IL-9 also enhances IL-4-induced IgE class switching in B cells. IL-9 can promote the expression of mucus genes in human airway epithelial cells and

has been shown to promote goblet cell metaplasia in mice, an event dependent on IL-13. IL-9 also promotes the production of CCL11 and IL-8 from human smooth muscle cells. CCL11 and IL-8 are important for the recruitment of eosinophils and neutrophils, respectively, both of which contribute to allergic disease. Thus, although Th9 cells have a protective role in immunity against extracellular parasites, they also contribute to the pathology of allergic inflammation (42, 43).

TGF-β and IL-4 promote the development of the Th9 lineage (44, 45). TFG-β induces the expression of PU.1, an Ets-family transcription factor, through Smad activation in Th9 cells (46-48). PU.1 induces IL-9 expression, the signature cytokine of Th9 cells, by directly binding to the promoter of *Il9* (46, 47). In addition to TGF-β signals, Th9 development is dependent on the IL-4-STAT6 signaling pathway. IL-4-STAT6 signals induce IRF4, BATF, c-maf and GATA3. IRF4 regulates IL-9 production by binding to the *Il9* promoter and is required for Th9 development (49). BATF is also required for IL-9 production from Th9 cells and cooperates with IRF4 in binding to the *Il9* locus (50). c-Maf does not appear to directly regulate *Il9*, but may have a role in the regulation of IL-21 (47, 51). GATA3 is highly expressed in Th9 cells, albeit at lower levels than in Th2 cells, however GATA3 also does not appear to directly regulate the *Il9* gene and although it is required for Th9 development, its precise function is unclear (44, 45, 47). In addition to TGF-β and IL-4, other cytokines have also been illustrated to promote Th9 development. Although STAT6 binds *Il9* very poorly upon IL-4-

induced activation (47), STAT5 can directly regulate IL-9 production by binding to the *Il9* promoter upon activation by IL-2 or thymic stromal lymphopoietin (TSLP) stimulation (52, 53). IL-1 and IL-33 may also contribute to Th9 development through the production of IL-9, possibly through NF-κB activation (54, 55). Th9 cells express greater amounts of the IL-25 receptor chain, IL-17RB in comparison to other T helper subsets and IL-25 may have a role in the promotion of Th9 development (56). Lastly, the Notch-signaling pathway and TGF-β cooperate in the induction of IL-9 through the Notch intracellular domain (NICD), RBP-Jκ and Smad3 (48). These findings demonstrate that several signaling networks are required for the development of the Th9 lineage, contributing to their role in host immunity and the development of allergic disease.

Th17 cells

T helper type 17 (Th17) cells primarily secrete IL-17A, IL-17F, IL-21 and IL-22 and are important in host immunity to extracellular bacteria and fungi, but also promote autoimmune and allergic responses. IL-17A and IL-17F are essential for the recruitment, activation and migration of neutrophils and induce the expression of inflammatory mediators, such as IL-6, TNF-α, GM-CSF, G-CSF, nitric oxide, matrix metalloproteinases and chemokines from non-immune cells such as fibroblasts, epithelial cells, endothelial cells, and smooth muscle cells. IL-21 primarily has effects on T cells and B cells, and IL-22 is important for the immune barrier function of epithelial cells through the induction of antimicrobial agents and β-defensins. These effector cytokines are essential for Th17 cell-

mediated clearance of extracellular bacteria, such as *Klebsiella pneumonia* and fungi, such as *Candida albicans*. However, Th17 cells also contribute to disease pathology in autoimmune diseases such as RA, systemic lupus erythematosus (SLE), multiple sclerosis (MS), psoriasis, and IBD in addition to their role in allergic disease (5, 57).

TGF-β, IL-6, IL-1β and IL-23 promote Th17 cell development through the induction of a network of transcription factors, including the master regulator of Th17 cells, RORγt (retinoid-acid-related orphan receptor-γt), and others, such as IRF4, BATF, aryl hydrocarbon receptor (AHR), Runx1, and the nuclear IκB family member, IκBζ (58-66). IL-6, IL-21 and IL-23 activate STAT3, which binds to the transcription factor encoding genes *Rorc*, *Rora*, *Ahr*, *Irf4*, *Batf* and *maf*, promoting the development of the Th17 lineage (67). TGF-β induces the Smad2 pathway, inducing the expression of RORγt together with activated STAT3 (68). TGF-β-induced signals and IL-6-activated STAT3, induce expression of another related nuclear receptor, RORα, which cooperates with RORγt and STAT3 in binding to the *Il17* and *Il21* promoters (69). While early Th17 differentiation is thought to be primarily driven by TGF-β and IL-6-induced signals, naïve $CD4^+$ T cells express low levels of IL-21R and IL-21 produced early in Th17 differentiation can further induce expression of IL-21R, activating an autocrine IL-21 feedback loop important for Th17 development (70). IL-23, a heterodimer composed of the unique p19 subunit and the p40 subunit, which is shared with IL-12, binds to a receptor complex composed of the constitutively expressed IL-

12Rβ1 subunit, and the IL-23R subunit on T cells (71, 72). IL-23 is necessary for the maintenance of the Th17 cell lineage and while IL-23R is not expressed on naïve $CD4^+$ T cells, it is induced by IL-6, IL-21 and IL-23 in a STAT3-dependent manner (61, 62, 73-75). IL-23-induced IL-17 production from differentiated Th17 cells has also been shown to be dependent on STAT4 (76). IL-6 enhances the expression of IL-1R on differentiating Th17 cells, and together with IL-6 and IL-21, IL-1β regulates the expression of IRF4, possibly through activation of NF-κB (77, 78). BATF is essential for Th17 development, interacting with JunB and binding to the promoters of *Il17*, *Il21*, and *Il22* as well as two intergenic regions between the *Il17a* and *Il17f* genes (79). BATF and Jun complexes are essential for IRF4-mediated transcription in T cells and BATF, IRF4 and RORγt have been shown to cooperate in the induction of Th17 genes (80, 81). Furthermore, inducible co-stimulator (ICOS)-signals are important for c-maf and IL-21 expression in Th17 cells and AHR is necessary for IL-22 production (82, 83). Runx1 promotes Th17 development through induction of the *Rorc* gene, and can then cooperate with RORγt in the induction of the *Il17* gene (84). Etv5, an Ets-family transcription factor is induced by STAT3 and promotes Th17 development through regulation of the *Il17a* and *Il17f* genes (85). IκBζ also cooperates with RORγt and RORα during the development of the Th17 lineage (86). Socs3, a suppressor of cytokine signaling family member, is a cytokine-inducible negative regulator of STAT3, thereby limiting Th17 responses (87). Additionally, Twist1, a basic-helix-loop-helix protein, which is an IL-12-STAT4 induced negative regulator of Th1 cells that represses *Il12rb2,* subsequently reducing STAT4

activation, also affects Th17 differentiation. Twist1 is induced following stimulation with STAT3-activating cytokines in Th17 cells and negatively regulates the Th17 effector program by binding to and repressing *Il6ra*, subsequently reducing STAT3 activation (88, 89). Taken together, these findings demonstrate that the coordination of numerous signaling pathways is essential for the regulation and development of the Th17 lineage, promoting its function in host immunity and disease pathogenesis.

Regulatory T cells

Regulatory T cells (Treg), essential for immune homeostasis, regulate a magnitude of effector responses and the establishment of immunological tolerance. Two types of Treg cells have been identified: natural Treg cells (nTreg), which develop in the thymus, and inducible Treg cells (iTreg), which are extrathymically derived and develop in peripheral lymphoid organs. Treg cells are characterized by their secretion of IL-10, TGF-β and IL-2, and expression of the transcription factor, Forkhead box protein 3 (Foxp3), the master regulator of Treg cells, the inhibitory molecule, CTLA-4, and IL-2Rα (90-94). Treg cells mediate their suppressive functions through several mechanisms, including cell-cell interactions and their secretion of cytokines. Null mutations in the X-linked *Foxp3* gene results in the development of autoimmune diseases in humans and mice, demonstrating the critical role for Treg cells in the regulation of immune responses (95, 96). The induction of iTreg cells from naïve $CD4^+$ T cells occurs upon TCR stimulation in the presence of TGF-β and IL-2. TCR stimulation

induces NFAT activation, whereas TGF-β activates Smad3, and both NFAT and Smad3 cooperate in remodeling the *Foxp3* enhancer region and promote *Foxp3* expression (97). IL-2 activated STAT5 binds to the promoter and first intron of the *Foxp3* gene (98-100). STAT5 also regulates expression of IL-2Rα and the anti-apoptotic protein, BCL-2, promoting the survival and function of Treg cells (101). Runx1 also promotes Treg development through the regulation of Foxp3 and IL-2 (102-104). Other factors such as Nr4a2 and the Foxo (forkhead box O) family members, Foxo1 and Foxo3, promote Treg development through regulation of the *Foxp3* or *Ctla4* genes, respectively (105, 106). Thus, a number of signaling pathways are required for the development of the Treg lineage, promoting their role in the regulation of immune responses.

Cross-regulation of T helper cells

During T helper cell differentiation, the cytokine milieu and activation of cell surface receptors leads to the induction of a network of transcription factors, which are essential in promoting the expression of lineage-specific genes, driving the development of a specific T helper subset. Another critical component of T helper differentiation is cross-inhibition of other lineage fates during the development of a specific T helper subset. For example, the Th1-associated cytokine, IFN-γ and the Th2-associated cytokine, IL-4, can antagonize the development of Th2 and Th1 cells, respectively, and both cytokines inhibit the development of Th17 cells. Cytokine-mediated cross-regulation of T helper cells is dependent on downstream transcription factors, which affect gene transcription

through changes in transcription and epigenetic modifications. Thus, transcriptional networks affect T helper cell differentiation by promoting the expression of genes specific to one T helper lineage and repressing those important for the development of other T helper fates.

In addition to its role in Th9 development, TGF-β is essential for the differentiation of Th17 and Treg cells. In the absence of other pro-inflammatory cytokines, TGF-β induces the expression of Foxp3 and RORγt, however Foxp3 can directly interact with RORγt, repressing its function. High levels of TGF-β are optimal for Foxp3 expression and promote the repression of *Rorc*, thereby promoting Treg development. In the presence of pro-inflammatory cytokines, such as IL-6, IL-21 or IL-23, and low concentrations of TGF-β, RORγt expression is increased and the expression and function of Foxp3 is inhibited (107). The balance between Th17 and Treg cell development can further be affected by Runx1, which interacts with RORγt and Foxp3. RORγt and Runx1 cooperate to induce *Il17* expression in Th17 cells, whereas Foxp3 and Runx1 cooperate to inhibit Th17 development by repressing IL-17 production (84).

T-bet, a critical factor in Th1 differentiation, can repress the function of both GATA3 and RORγt. T-bet becomes phosphorylated by IL-2-inducible T cell kinase (Itκ), and phosphorylated T-bet interacts with GATA3 and prevents the binding of GATA3 with its target DNA, thereby inhibiting Th2 development (108). Furthermore, T-bet induces Runx3 in Th1 cells, and the two factors cooperate in

the production of IFN-γ by binding to the *Ifng* promoter, and in inhibition of IL-4 production by binding to the *Il4* silencer (14, 109). T-bet inhibits Th17 development by binding to the *Irf4* promoter, directly repressing IRF4 expression, in addition to interacting with Runx1 and inhibiting Runx1-mediated transactivation of *Rorc* (110, 111). GATA3 has been shown to affect Th1 development through the downregulation of STAT4 (112). Furthermore, GATA3 expression in Th2 cells has been shown to repress the expression of the Th1-associated genes, *Tbx21* and *Il12rb2*, and the Th17-associated gene, *Rorc* (113). RORγt inhibits IL-4 and IL-12 induced responses in Th17 cells through the repression of *Il4ra* and *Il12rb2* (81). Gfi-1, a factor important for Th2 differentiation, inhibits Th17 and Treg development and Tcf-1, which is important for STAT6-independent GATA3 expression in Th2 cells, represses Th1 and Th17 development through effects on IFN-γ production, or binding and repressing the *Il17* gene, respectively (27, 32, 114, 115).

STATs are also important for the positive and negative regulation of gene expression in T helper cell differentiation. STAT4 and STAT6 can bind to target genes and reciprocally regulate their expression. For example, in Th2 cells, STAT6 induces the expression of the Th2-associated gene, *Ccr8*, however in Th1 cells, STAT4 inhibits the expression of *Ccr8* (116). Furthermore, there is a considerable amount of overlap between STAT3 and STAT5 binding sites in the *Il17* gene. Thus, IL-2 activated STAT5 can directly compete with IL-6 activated STAT3 for binding to *the Il17 locus*, thereby inhibiting STAT3 binding and limiting

Il17 gene expression (117). STAT3 can indirectly repress IL-2 production in Th17 cells through its cooperation with AHR in the upregulation of Aiolos, a member of the Ikaros family of transcription factors, which directly represses the *Il2* gene (118). IL-12-activated STAT4, IL-4-activated STAT6 and IL-6-activated STAT3 all repress Foxp3 expression through epigenetic modifications at the *Foxp3* locus, thereby repressing Treg development (101).

Together, these findings demonstrate that complex transcription factor networks work together to promote the expression of T helper lineage-specific genes and repress those of other T helper cell fates, thereby driving the development of a specific T helper lineage.

T helper cell stability

T helper cells were originally thought to be terminally differentiated lineages with limited flexibility. However, more recent findings have revealed that extensive plasticity occurs between the different T helper lineages, contributing to T helper cell-mediated immunity. The flexible nature of T helper cells may enable them to promote a particular function at the beginning of an immune response, and then depending on the cytokine milieu, they could transition to a different lineage. This could allow T helper cells to exert a separate effector function necessary for immune homeostasis or host-defense. T helper cell plasticity has significant implications on our current understanding of T helper cell-mediated disease pathogenesis.

A number of studies have demonstrated the instability of Th17 cells. Th17 cells acquire an IFN-γ-secreting Th1-like phenotype in vitro, which is dependent on IL-12, IL-23, STAT4 and T-bet (119). In the mouse model of MS, experimental autoimmune encephalomyelitis (EAE), Th17 cells extinguish IL-17 production and upregulate expression of IFN-γ, which is associated with an increase in IL-23 in the environment, a pro-inflammatory cytokine that has previously been demonstrated to induce a pathogenic Th17 cell phenotype (120-122). In contrast, Th17 cells in an acute *Candida albicans* cutaneous infection do not upregulate alternative cytokines, though IL-17 production is diminished, which is associated with increased IL-10 in the skin (121). Thus, Th17 cells can acquire a Th1-like phenotype in vitro and in vivo.

The stability of Treg cells remains controversial, with evidence supporting both a stable and unstable phenotype. In a model of lethal oral infection with *Toxoplasma gondii*, Treg cells adopt a Th1-like phenotype, accompanied by enhanced T-bet and IFN-γ expression, and this Treg to Th1 transition leads to immunopathogenesis (123). In a transfer model of colitis, Treg cells maintain their suppressive function in conjunction with adopting a Th1 or Th17-like phenotype, secreting IFN-γ or IL-17, respectively, or a $Foxp3^{+}IFN\text{-}\gamma^{+}$ and/or $IL\text{-}17^{+}$ phenotype (124). Treg cells remain a stable phenotype in vivo under physiological conditions, and upon infection with a sublethal dose of *Listeria monocytogenes*, Treg cells maintain Foxp3 expression, but upregulate T-bet and IFN-γ. Furthermore, in a model of diabetes that promotes Th17 cell development,

Treg cells maintain expression of Foxp3 and do not upregulate alternative cytokines, such as IL-17 (125). Furthermore, it has been suggested that nTreg cells are a heterogenous population, with the majority being committed Treg cells and a minor population being uncommitted non-regulatory $Foxp3^+$ T cells. The latter population could then retain plasticity and transition to other T helper phenotypes under the appropriate cytokine milieu (126). Thus, the stability of Treg cells remains unclear.

Th2 cells have also been shown to adopt other T helper phenotypes. In a model of lymphocytic choriomeningitis virus infection, $GATA3^+$ Th2 cells adopt a $GATA3^+T\text{-}bet^+$ phenotype, secreting IL-4 and IFN-γ, which appear to be important in mediating viral persistence and fatal immunopathology. IL-12, type I and type II interferons, and T-bet, are important in reprogramming Th2 cells in this model of infection (127). Furthermore, $GATA3^+ROR\gamma t^+$ effector/memory T helper cells that produce IL-4 and IL-17 have been identified in asthmatic patients. Whether they originate from a Th2 or Th17 cell is unclear (128, 129).

T helper differentiation and plasticity are governed by epigenetic changes in chromatin structure, histone and DNA modifications and the expression of small noncoding RNAs. The N-terminal tails of histone proteins can be covalently modified by acetylation, methylation or phosphorylation, which either relax or condense the chromatin structure, permitting the activation or repression of gene transcription. Trimethylation of histone H3 lysine 4 (H3K4me3) is a permissive

mark associated with gene activation, while trimethylation of histone 3 lysine 27 (H3K27me3) is a repressive mark associated with the repression of gene expression. Some genomic regions display both H3K4me3 and H3K27me3 chromatin marks, and are termed 'bivalent,' which may poise genes ready for activation or repression during T helper cell differentiation (130).

Recent studies have examined genome-wide histone modifications associated with changes in gene expression in different T helper lineages, which have revealed new insights into the underlying mechanisms of T helper cell stability. Cytokine genes associated with each T helper cell lineage often display permissive H3K4me3 marks, while those associated with other lineages often display repressive H3K27me3 marks. For example, H3K4me3 marks are evident at the *Ifng* locus in Th1 cells while H3K27me3 marks are present at the *Il4* and *Il17* loci, while in Th2 cells H3K4me3 marks are present at the *Il4* locus and H3K27me3 modifications are present at the *Ifng* and *Il17* loci. H3K4me3 marks are evident at the *Il17a* locus and H3K27me3 modifications are found at the *Ifng* and *Il4* loci in Th17 cells. In contrast, genes encoding master regulator transcription factors display a more complex set of modifications. For example, the *Tbx21*, *Gata3* and *Rorc* loci all display bivalent H3K4me3 and H3K27me3 chromatin modifications in Treg cells, indicating that Treg cells could be an unstable phenotype. The *Tbx21* locus displays bivalent marks in Th2 and Th17 cells, and the *Gata3* locus displays bivalent marks in Th1 and Th17 cells, suggesting that T-bet could be expressed in Th2 and Th17 cells and Gata3 could

be expressed in Th1 and Th17 cells. The *Rorc* and *Foxp3* loci display H3K27me3 chromatin modifications in Th1 and Th2 cells, suggesting that RORγt and Foxp3 would not be expressed in Th1 and Th2 cells (131). Thus, epigenetic mechanisms likely underlie T helper cell stability.

To further explore the underlying mechanism of T helper cell plasticity a more recent study analyzed epigenetic modifications across the *Il17* and *Ifng* loci in Th17 cells that had been stimulated under Th1-polarizing conditions for a second round of culture in vitro. Upon stimulation under Th1-polarizing conditions, Th17 cells display a rapid increase in IFN-γ production and downregulation of IL-17 production. Th17 cell precursors display remodeling of the *Ifng* locus and undergo additional modifications when stimulated under Th1-polarizing conditions, permitting high expression of *Ifng*. Furthermore, repressive modifications are present at the *Rorc* and *Il17* loci in Th17 cells stimulated under Th1-polarizing conditions, leading to the repression of *Rorc* and *Il17a* in a STAT4 and T-bet-dependent manner (132). Thus, these findings confirm that Th17 cells can adopt a Th1-like phenotype in vitro and further demonstrate the instability of chromatin structure of key genes in the Th17 lineage. This demonstrates that T helper cell fates can be modulated through effects on the epigenetic status of cell lineage factors.

A common method used to identify or track the fate of T helper lineages is the use of cytokine reporter or lineage tracer mouse strains, respectively. Cytokine

reporter mice can be used to detect the level of activation of a particular cytokine promoter due to the presence of a reporter gene, such as green fluorescent protein (GFP), downstream of the selected promoter. For example, an IL-17 cytokine reporter mouse generated by the insertion of the gene encoding GFP into the *Il17* locus, results in GFP expression when *Il17* is induced during Th17 cell development. GFP remains expressed in Th17 cells for the continuation of *Il17* induction, however if a Th17 cell converts to a Th1 cell, thereby repressing *Il17*, GFP will be extinguished (Figure 2A). In contrast to cytokine reporter mice, lineage tracer mice track the fate of a specific cell lineage. For example, an IL-17 lineage tracer mouse can be generated by the insertion of the gene encoding Cre recombinase into the *Il17* locus and further mating these mice to a reporter mouse strain that conditionally expresses yellow fluorescent protein (YFP) upon Cre expression, resulting in permanent expression of YFP. Thus, when *Il17* is induced during Th17 cell development, Cre is expressed and conditionally induces permanent YFP expression in these cells, even when Th17 cells convert to a Th1 cell and *Il17* is repressed (Figure 2B). Studies using IL-17-reporter mice have contributed to our understanding of the development of Th17 cells through the identification of cells that actively express *Il17*, however as the reporter expression is extinguished upon loss of *Il17* expression, this model is limited in its ability to track the fate of IL-17-expressing cells (119, 133). The recent generation of IL-17 lineage tracer mouse models has been useful to trace the fate of IL-17-secreting cells in vitro and in vivo, furthering our understanding of Th17 cell stability and its effect on T cell-mediated immune responses (120, 121).

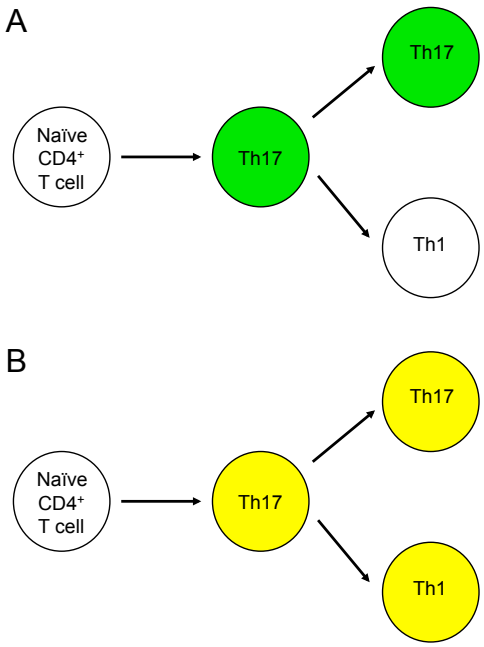


Figure 2. Distinct uses of cytokine reporter and lineage tracer mouse models. *A*, An IL-17 GFP reporter mouse enables GFP expression upon *Il17* induction during the development of Th17 cells. GFP expression is maintained in Th17 cells expressing *Il17*, however is lost upon repression of *Il17* in the conversion of a Th17 to a Th1 cell. *B*, An IL-17 Cre-YFP lineage tracer mouse enables Cre expression upon *Il17* induction, followed by permanent Cre-induced YFP expression during the development of Th17 cells. Cre-induced YFP expression is maintained upon repression of *Il17* in the conversion of a Th17 to a Th1 cell. GFP-expressing cell (green); YFP-expressing cell (yellow).

CD8 T cells

Like CD4 T helper cells, CD8 T cells are a crucial component of adaptive immunity. CD8 T cells can differentiate into a cytotoxic phenotype with cells referred to as CD8 cytotoxic T cells, or cytotoxic T lymphocytes (CTLs). CD8 T cells require similar signals as CD4 T cells to undergo differentiation, however

there are some differences. CD8 T cells encounter specific antigen through an interaction between the TCR and peptide:MHC I complex on the APC, in conjunction with the CD8 co-receptor. Furthermore, CD8 T cells require more co-stimulation than naïve CD4 T cells for effective differentiation. Effector CD8 T cells are most known for their role in defense against intracellular pathogens, such as viruses and their ability to kill infected cells or abnormal cells, such as tumor cells. While less is currently understood about CD8 T cell differentiation than T helper cell differentiation, CD8 T cells differentiate into different subsets that have specific functions in the immune response (1).

Tc1

Type 1 cytotoxic T cells (Tc1) are important for tumor and viral immunity. Tc1 cells are characterized by the production of pro-inflammatory cytokines, such as IFN-γ, TNF-α and LT-α, and their expression of cytolytic effector molecules, such as perforin, granzymes and Fas ligand. Upon release from cytotoxic granules, perforin acts in the delivery of granzymes into the cytosol of a target cell and granzymes further induce apoptosis in the target cell through caspase activation and the release of cytotoxic factors from the mitochondria. Fas ligand is expressed on the cell surface and can trigger caspase-mediated apoptosis in a target cell upon binding to the Fas receptor. IFN-γ directly inhibits viral replication and induces the expression of MHC-I in infected cells. Furthermore, IFN-γ, TNF-α and LT-α cooperate in macrophage activation and the killing of some target cells (1, 134).

Tc1 development is promoted by IL-12 and the transcription factors STAT4, T-bet, eomesodermin (Eomes) and Runx3. TCR signaling leads to rapid activation of T-bet, which is further augmented by IL-12-activated STAT4 and leads to the production of IFN-γ (135-137). T-bet upregulates expression of IL-12Rβ2 and initiates a positive feedback loop by increasing the sensitivity to IL-12 (136). While, Runx3 is required for Eomes expression, IL-12 signals repress the expression of Eomes (136, 138). It is thought that T-bet is important for early Tc1 development and expression of IFN-γ, whereas Eomes is expressed later in development and sustains IFN-γ production. Both Eomes and Runx3 bind to the *Prfn* locus, whereas T-bet is not necessary for perforin expression. Runx3 also binds to the promoter region of the genes encoding IFN-γ and granzyme B (138). Whether or not Runx3 cooperates with T-bet or Eomes in the induction of Tc1-associated genes remains unclear. However, a complex transcriptional network is involved in the development of Tc1 cells.

Tc2

Type 2 cytotoxic T cells (Tc2) are important in tumor immunity and primarily secrete IL-4, IL-5 and IL-13 (139, 140). In contrast to Tc1-mediated killing of tumor cells, Tc2 cells are important for the recruitment of other effector cells to the tumor site, which aid in the clearance of tumor cells (141, 142). Tc2 development is promoted by IL-4 and the transcription factors STAT6 and GATA3. Tc2 cells secrete similar levels of IL-5 and IL-13 as Th2 cells, however they secrete lower levels of IL-4, which is associated with decreased acetylation

of the *Il4* locus in Tc2 cells. This phenomenon is due to increased expression of ROG (repressor of GATA) in Tc2 cells, which inhibits GATA3 function in histone hyperacetylation and gene activation of the *Il4* locus (139, 143). Thus, Tc2 development requires some shared factors with Th2 cells, and is important in the effective clearance of tumors.

Tc17

Similar to Tc1 cells, type 17 cytotoxic T cells (Tc17) are important for viral and tumor immunity, but also have a role in the development of autoimmune disease (144-147). Tc17 cells primarily secrete IL-17A, IL-17F, IL-21 and IL-22. Tc17 development is promoted by IL-6 and TGF-β, and the transcription factors STAT3 and RORγt. IL-21 and IL-23 have also been shown to promote IL-17 production from CD8 T cells (145, 147-152). Tc17 development is strongly inhibited by T-bet and Eomes, as well as IFN-γ activated STAT1 (148, 150). Importantly, the ability of Tc17 cells to promote immunity in vivo is coupled with an acquired Tc1-like phenotype (153). Upon adoptive transfer, Tc17 cells mediate the regression of established tumors, accompanied by a transition to an IFN-γ-secreting Tc1-like phenotype (144). Tc17 cells mediate protection against lethal influenza through the recruitment of neutrophils and acquisition of an IFN-γ-secreting phenotype (145). In vitro, STAT4, T-bet and IFN-γ contribute to the ability of Tc17 cells to acquire a Tc1-like phenotype (154). Thus, Tc17 development requires some shared factors with Th17 cells, and the unstable phenotype of Tc17 cells is observed during viral and tumor immunity.

Together, CD8 T cells are important for viral and tumor immunity, but similar to T helper cells, they also have a role in the development of disease.

Innate IL-17-producing cells

IL-17 is produced early in an inflammatory response to stress, injury and pathogens, before the adaptive immune response has been established, leading to the discovery of IL-17-secreting innate immune cells. Innate IL-17-producing cells often reside at barrier surfaces such as the lungs, intestinal mucosa and skin, where they each play an important role in tissue surveillance. Innate IL-17-producing cells express an array of PRRs and a number of them constitutively express IL-1R, IL-23R and transcriptional regulators for IL-17 production. IL-17-producing innate immune cells can be activated in response to cytokines alone, or in combination with recognition of PAMPs, or antigen and MHC complexes, and can rapidly produce IL-17 during an inflammatory response. IL-17 induces epithelial cell secretion of factors, such as G-CSF and CCL20, which recruit neutrophils important for the rapid control of pathogens. A number of IL-17-secreting innate cells have been described in recent literature, including subsets of γδ T cells, lymphoid tissue inducer-like (LTi-like) cells, NKT cells, NK cells, and myeloid cells, such as paneth cells and neutrophils (155, 156).

IL-17-prodcuing γδ T cells are the most characterized subset of innate IL-17-producing cells. γδ T cells constitutively express CCR6, IL-23R, RORγt and AHR and display rapid IL-17 production when stimulated with IL-23 and IL-1β, in vitro and in vivo (157, 158). IL-17-producing γδ T cells appear to be more important

than αβ T cells in the resolution of a skin infection with *Staphylococcus aureus* (159, 160). In the lung, early IL-17 production by γδ T cells is important for immunity to bacteria, such as *Klebsiella pneumonia* and *Mycobacterium tuberculosis*, mediating its effects through the recruitment of other lymphocytes (161-166). In the liver, early IL-17 production by γδ T cells is required for the optimal recruitment of neutrophils, and is an important mediator in the resistance to infection with intracellular pathogens, such as *Listeria monocytogenes* and *Toxoplasma gondii* (167, 168). In addition to their role in host defense, IL-17 producing γδ T cells also contribute to disease pathology. In EAE, IL-23 and IL-1β induce early IL-17 production from γδ T cells, which amplifies Th17 responses and disease susceptibility (157). IL-17-producing γδ T cells also impair proper Treg cell responses in EAE, thereby enhancing autoimmunity (169). Furthermore, γδ T cells are an important source of early IL-17 production in response to IL-23 and IL-1β in collagen-induced arthritis, and IL-17 contributes to disease pathogenesis (170). Thus, IL-17 production by γδ T cells is critical for host defense and the development of disease.

Similar to γδ T cells, NKT cells also constitutively express IL-23R, RORγt and AHR and display rapid production of IL-17 upon stimulation with IL-23 and IL-1β, which is further enhanced upon TCR activation (155, 171). The highest levels of IL-17 production from NKT cells are produced from the $CD4^{-}NK1.1^{-}$ population (172, 173). IL-17-producing NKT cells are found in the peripheral blood of simian immunodeficiency virus infected rhesus macaques and are associated with viral

pathogenesis (174). In addition, IL-17 production by NKT cells is critical for mediating allergic responses in the airways, such as neutrophilia (172, 175-177). While, IL-17-producing NKT cells are associated with detrimental immune responses, it is not clear if they have a role in host defense.

LTi-like cells share phenotypic similarities with LTi- cells, which are critical in the development of secondary lymphoid organs and dependent on RORγt (155, 178). Splenic LTi-like cells constitutively express IL-23R, CCR6, RORγt and AHR and rapidly produce IL-17 in response to IL-23 stimulation in vitro or upon in vivo challenge with the yeast cell wall product, zymosan. Furthermore, STAT3 is important for optimal IL-23-induced IL-17 production from LTi-like cells (179). It remains unclear if IL-17 production from LTi-like cells contributes to the development of secondary lymphoid organs or host defense. Subsets of NK cells, paneth cells and neutrophils can also produce IL-17, which may contribute to immune responses through IL-17-mediated effects on neutrophil recruitment (155).

Thus, innate immune cells have an early role in initiating IL-17-dependent immune responses. Early IL-17 induces the production of antimicrobial peptides and factors important for neutrophil chemotaxis from epithelial barrier cells. Innate IL-17 production also amplifies T helper responses, and together, innate and adaptive IL-17 producing cells play a critical role in host defense and disease pathogenesis.

Research goals

Elucidating the factors involved in the development of Th17 cells has been an intense area of research, however less is known about the development of Tc17 cells and innate IL-17-producing T cells. Furthermore, IL-17-producing cells are critical to the development of pathogen and tumor immunity, but also contribute to the pathology of autoimmune diseases and allergic inflammation. The contribution of IL-17-producting T cells to some of these immune responses has been linked to an unstable phenotype. The overall goal of this research is to further define the development, function and stability of IL-17-secreting T cells. A novel mouse model designed to identify IL-17 producing cells and trace their fate once IL-17 has been extinguished will be generated and used to address the stability of Th17 cells. Together, these studies will further our understanding of the development and stability of IL-17-secreting cells in addition to their contribution to disease pathogenesis, having further implications in the treatment of IL-17-mediated diseases.

MATERIALS AND METHODS

Mice

For the generation of *Il17f* ^{Cost} mice, a vector spanning the *Il17f* locus was generated to replace the 3' end of exon 1 with an EGFP-Cre fusion protein and an *frt*-flanked neomycin resistance cassette (Vega Biolabs). The targeting vector was transfected into C57BL/6 ES cells. Neo-selected clones were screened for correct recombination and ES cells were injected into albino C57BL/6 blastocyts. The resulting founder mice were bred to establish germline transmission. To excise the *frt*-flanked neomycin-resistance cassette, *Il17f* ^{Cost} mice were crossed to FLPeR mice (have the *Saccharomyces cerevisiae FLP1* recombinase gene inserted into the *Gt(ROSA)26Sor* locus). *Il17f* ^{Cost}*Rs*^{YFP} mice were generated by crossing *Il17f* ^{Cost} mice to R26-stop-EYFP reporter mice (have a *loxP*-flanked stop sequence followed by the eYFP gene inserted into the *Gt(ROSA)26Sor* locus). *Il17f* ^{Cost}*Rs*^{YFP}-OT-II mice were generated by crossing *Il17f* ^{Cost}*Rs*^{YFP} mice to OT-II mice (express a transgenic T cell receptor specific for chicken ovalbumin 323-339). The generation of *Stat4*$^{-/-}$ (7), *Stat3*$^{CD4-/-}$ (180) and OT-I *Ifng*$^{-/-}$ (145) mice were previously described. C57BL/6 and BALB/c mice were purchased from Harlan Laboratories, OT-I *Rag1*$^{-/-}$ mice were purchased from Taconic Farms, and *Rorc*^{GFP}, C3H/HeJ, FLPeR mice, R26-stop-EFYP mice, OT-II mice, and *Il12a*$^{-/-}$ mice were purchased from Jackson Laboratory. BoyJ mice were obtained from the IU Simon Cancer Center In Vivo Therapeutics Core. For studies using *Stat4*$^{-/-}$ mice, mice were used on a C57BL/6 or BALB/c background for analysis of innate immune cells or memory Th cells, respectively. Mice were

kept in pathogen-free conditions and all studies were approved by the Indiana University School of Medicine Animal Care and Use Committee. The following primers and PCR cycling conditions were used for genotyping *Il17f*^{Cost}*Rs*^{YFP} mice: for *Cre*, F (forward) 5'-GTGAAACAGCATTGCTGTCACTT-3' and R (reverse) 5'-GCGGTCTGGCAGTAAAAACTATC-3', 96 bp; for *Il17f*, F 5'-GACATACCCAGGAAGACATACTTAGAAG-3' and R 5'-ACCATGATGGTCACTGGAGATAACCAG-3', 388 bp; for *Flp*, F 5'-GCTGCTGAACTAACCTATTTATGTTGG-3' and R 5'-CTTTAATGAGGCTTCCAGAATTGTTGC-3', 182 bp; for *YFP*, F 5'-TGGCCCACCCTCGTGACCACCTTCGG-3' and R 5'CATGGCGGACTTGAAGAAGTCGTGCTG-3', 96 bp; initial denaturation at 94^{o}C for 2 min, 29 cycles of 94^{o}C for 45 sec, 60^{o}C for 45 sec, 72^{o}C for 45 sec, and a final extension for 10 min at 72^{o}C. For original PCR screening of *Il17f*^{Cost} mice mated to FLPeR mice, *Cre* and *Il17f* were screened as noted before and *Flp* was screened according to the Jackson Laboratory protocol. For original PCR screening of *Il17f*^{Cost} mice mated to R26-stop-EYFP mice, *Cre* and *Il17f* were screened as noted before and *YFP* was screened according to the Jackson Laboratory protocol. The Jackson Laboratory protocols cannot distinguish between *Flp* and *YFP*, nor can they distinguish between *YFP* and *GFP*, therefore new primers were designed when *Il17f*^{Cost}FLPeR mice were mated with R26-stop-EYFP mice to generate *Il17f*^{Cost}*Rs*^{YFP} mice.

CD8 T cell differentiation

Total $CD8^+$ cells were isolated from spleens and lymph nodes using a MACS isolation system (Miltenyi Biotec). For Tc cell differentiation, $CD8^+$ cells were activated with soluble anti-CD3 (4 mg/ml, 145-2C11) and anti-CD28 (1 µg/ml) 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 hTGF-β1 (2 ng/ml), IL-6 (100 ng/ml), and anti-IFN-γ (10 µg/ml R4/6A2 or XMG) and Tc1 primed cells were differentiated with IL-12 (5 ng/ml). After 3 days of incubation, cells were expanded in the presence of recombinant hIL-2 (20 units/ml) and analyzed after an additional 2 days of culture. In some experiments, cells underwent 2 rounds of stimulation. For the second five-day culture, cells were re-plated 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) with the same cytokine and neutralizing antibody concentrations. OT-I cells were activated with soluble SIINFEKL peptide (0.5 µM) and anti-CD28 (0.5 µg/ml). Cytokines and antibodies were purchased from BD Biosciences (anti-CD28), Peprotech (IL-2, IL-6) or R&D Systems (IL-12, hTGF-β1).

CD4 T cell differentiation

Naïve $CD4^+CD62L^+$ T cells were purified from spleens via magnetic isolation (Miltenyi Bitoec) and activated with plate-bound anti-CD3 (2-4 µg/ml, 2C11) and soluble anti-CD28 (1-2 µg/ml). Cells were polarized to generate Th1 (5 ng/ml IL-

12; 50 units/ml IL-2; 10 μg/ml anti-IL-4, 11B11), Th2 (10 ng/ml IL-4; 10 μg/ml anti-IFN-γ, XMG), Th9 (20 ng/ml IL-4; 2 ng/ml hTGF-β1; 10 μg/ml anti-IFN-γ) and Th17 (100 ng/ml IL-6; 2 ng/ml hTGF-β1; 10 ng/ml IL-23; 10 ng/ml IL-1β; 10 μg/ml anti-IL-4; 10 μg/ml anti-IFN-γ) cells. Cells were expanded after 3 days with fresh media alone for Th1 and Th2 cells or in the presence of additional cytokines for Th9 (20 ng/ml IL-4; 2 ng/ml hTGF-β1) and Th17 (50 ng/ml IL-6; 5 ng/ml IL-23; 5 ng/ml IL-1β) cells. Cells were harvested after five days in culture for analysis. For long-term Th17 cultures, cells were cultured as noted above for five days. Cells were then harvested and re-activated under long-term Th17-polarizing conditions (1 μg/ml anti-CD3; 10 ng/ml IL-23; 10 ng/ml IL-1β; 10 μg/ml anti-IL-4; 40 μg/ml anti-IFN-γ). Cells were expanded after 3 days in the presence of additional cytokines (5 ng/ml each IL-23 and IL-1β). Cells were harvested on the fifth day of the second round of culture and live YFP^+ cells were sorted by flow cytometry. Sorted cells were re-activated with plate bound anti-CD3 (1 μg/ml) and cultured for a third round under Th1, Th2, Th9 or long-term Th17-polarizing conditions. Cells were expanded after 3 days as noted above for Th1, Th2, Th9 and long-term Th17 conditions. Cells were harvested on the fifth day of the third round of culture for further analysis. For experiments using cells from OT-II mice, purified naïve $CD4^+CD62L^+$ T cells were activated with soluble $OVA_{323-339}$ peptide (5 μg/ml; GenScript) and soluble anti-CD28 (1 μg/ml) in the presence of $CD4^+$ depleted splenocytes (1:5) that were first treated with Mitomycin C (Calbiochem) according to the manufacture's instructions. Cytokines and antibodies were purchased from Bio X Cell (anti-CD3, anti-IFN-γ and anti-IL-4), BD Biosciences

(anti-CD28), eBioscience (IL-1β), Miltenyi Biotec (hTGF-β1) Peprotech (IL-2, IL-4, IL-6) or R&D Systems (IL-12, IL-23).

Surface and intracellular staining

For Tc analyses, $CD8^+$ T cells were stimulated for 4 h with PMA (50 ng/mL; Sigma) and ionomycin (500 ng/ml; Sigma) in the presence of GolgiPlug (BD Biosciences), and OT-I cells were stimulated for 4 h with SIINFEKL peptide (1 μM) in the presence of GolgiPlug at 37°C before intracellular staining. Cells were fixed at RT for 10 min with 2% formaldehyde, permeabilized in FACS buffer (2% BSA, 0.01% NaN_3 in PBS) containing 0.1% saponin for cytokine staining, or 100% cold methanol for granzyme B and T-bet, and stained with fluorescently labeled antibodies for IL-17, IFN-γ, granzyme B or T-bet for 30 min at 4°C. For surface staining, cells were stained for CD4, CD8, CD45.1 or CD45.2 for 30 min at 4°C following treatment with Fc Block (BD Biosciences) for 10 min at 4°C for samples containing APCs. All antibodies were purchased from eBioscience. All samples were analyzed by flow cytometry using FACScalibur instruments and data were analyzed using WinMDI software.

For studies with *Stat4*$^{-/-}$ mice, splenocytes were surface stained for CD4, CD44, CD62L, CCR6, γδ TCR, TCR-β, PBS57-loaded CD1d-Tetramer and/or NK1.1 for 30 min at 4°C, following incubation with FC Block for 10 min at 4°C. Cells were incubated with 7-AAD viability die (eBioscience) for 10 min on ice and live memory Th cells ($CD4^+CD62L^-CD44^{hi}$), $CCR6^+$ memory Th cells

(CCR6^{+}CD4^{+}CD62L^{-}CD44^{hi}), γδ T cells (γδ TCR^{+}), LTi-like cells (CD4^{+}CD3^{-} CD11c^{-}B220^{-}), NK cells (CD3^{-}NK1.1^{+}), NKT cells (TCR-β^{+}CD1-d-Tetramer^{+}) and NK1.1^{-} NKT cells (TCR-β^{+}CD1-d-Tetramer^{+}NK1.1^{-}) were analyzed by flow cytometry with an LSR II (Becton Dickinson) or sorted using a Reflection sorter (iCyt). Flow cytometry results were analyzed with FlowJo. The PBS-57 (α-galactosylceramide analaog)-loaded CD1d-tetramer was obtained from the NIH Tetramer Core Facilities (Emory University) and all other antibodies were purchased from BD Biosciences or eBioscience.

For Th studies, cytokine analysis from in vitro differentiated Th cells was performed by first re-stimulating cells with PMA and ionomycin for 5 h at 37^{o}C with the addition of monensin during the last 3 h of stimulation. Cells were collected, stained with a fixable viability dye according to the manufacturer's instructions (eBioscience), fixed at RT with 2% final formaldehyde for 10 min, permeabilized with FACS buffer containing 0.1% saponin, and stained with fluorochrome-conjugated anti-mouse IFN-γ, IL-9, IL-13, IL-17A or IL-17F for 30 min at 4^{o}C. For transcription factor analysis, live YFP^{+} Th cells were sorted by flow cytometry. Unstimulated cells were fixed and permeabilized using a transcription factor staining buffer set (eBioscience) according to the manufacturer's instructions, and stained with fluorochrome-conjugated anti-mouse Gata3, RORγt or T-bet for 30 min at 4^{o}C. For ex-vivo analyses, splenocytes, total BAL (bronchoalveolar lavage) cells, total lung cells or mononuclear cells from the brain or lung were stimulated with PMA and

ionomycin as described above. Cells were collected, incubated with Fc Block for 10 min at 4^{o}C and stained with fluorochrome-conjugated anti-mouse CD4 for 30 min at 4^{o}C. Cells were then washed, stained with a fixable viability dye and fixed, permeabilized and stained with anti-mouse cytokine antibodies as described above. Antibodies were purchased from BD Biosciences, BioLegend or eBioscience. Cells were analyzed by flow cytometry using an LSR II and results were analyzed with FlowJo.

Phospho-STAT protein analysis

For the memory Th cell analysis, WT mice were administered OVA (20 µg; Sigma) and aluminum hydroxide (2 mg; Sigma) via intraperitoneal (i.p.) injection on days 0 and 7 of the protocol and mice were sacrificed 7 wks-post the last i.p. injection. Splenic CD4^{+} T cells were sorted via MACS bead selection (Miltenyi Biotec) and stimulated for 5 h with PMA and ionomycin at 37^{o}C. After 2 h of stimulation, GolgiPlug was added with or without the addition of IL-23 (50 ng/ml) or IL-12 (5 ng/ml) for the remaining 3 h of stimulation. IL-17A-secreting cells were labeled using the mouse IL-17 secretion assay cell enrichment and detection kit (Miltenyi Biotec) according to the manufacturer's instructions. Cells were fixed for 10 min with 2% formaldehyde at RT and permeabilized for 15 min at 4^{o}C with 100% methanol. Cells were then stained for CD4, CD44 and pSTAT3 or pSTAT4 for 30 min at RT. pSTAT expression was detected in the IL-17A^{+} or IL-17A^{-} CD4^{+}CD44^{hi} cell population. For the NKT cell analysis, NKT cells were sorted from the spleens of naïve WT mice and left unstimulated or stimulated with IL-23

(50 ng/ml) or IL-12 (5 ng/ml) for 3 h at 37^{o}C. Cells were fixed and permeabilized as mentioned above and stained for pSTAT4 for 30 min at RT. Cells were analyzed by flow cytometry with an LSR ll and results were analyzed with FlowJo. Antibodies were purchased from BD Biosciences or eBioscience.

Enzyme-linked immunosorbent assay (ELISA)

To generate cell free supernatants, cells were stimulated with plate-bound anti-CD3 (4 µg/ml), SIINFEKL peptide (1 µM), or with IL-23 (20 ng/ml) and IL-1β (25 ng/ml) for 24-48 h at 37^{o}C and cell-free supernatants were collected. In some experiments, cells were stimulated with OVA (250 µg/ml) for 72 h at 37^{o}C. To assess cytokine production, 96-well NUNC MaxiSorp plates were coated with capture antibodies (2 µg/ml; BD Biosciences) dissolved in 0.1 M $NaHC0_3$ buffer (pH 9) at 4^{o}C overnight. Wells were washed three times with ELISA wash buffer (0.1% Tween-20 in PBS) and blocked with ELISA buffer (2% BSA, 0.01% NaN_3 in PBS) for 2 h at RT. Diluted cytokine standards and cell-free supernatants were added to plates and incubated at 4^{o}C overnight. Wells were washed three times with ELISA wash buffer, biotinylated antibodies (1 µg/ml; BD Biosciences) dissolved in ELISA buffer were added and plates were incubated at RT for 2 h. Wells were washed three times with ELISA wash buffer and incubated with streptavidin alkaline phosphatase (1:2000; Sigma) dissolved in ELISA buffer at RT for 1 h. Wells were washed three times with ELISA wash buffer and phosphatase substrate (5 mg/ml; Sigma 104) dissolved in ELISA substrate buffer

(10% diethanolamine, 0.05 mM $MgCl_2$, 0.02% NaN_3; pH 9.8) was added. The absorbance was read at 405 nm (BioRad microplate reader model 680).

Gene expression analysis (quantitative RT-PCR)

Total RNA was isolated from unstimulated cells (for genes encoding transcription factors and surface proteins) or anti-CD3 (2 µg/ml) stimulated cells (for cytokine and cytotoxic genes) using Trizol reagent (Ambion Life Technologies) and reverse transcribed to make cDNA according to the manufacturer's instructions. (Invitrogen). Quantitative PCR reactions were set up by adding cDNA, primers (Applied Biosystems), TaqMan Fast Universal Master Mix (Applied Biosystems) and DEPC water to a final volume of 10 µl in MicroAmo Fast Optical 96-well plates (Applied Biosystems). Quantitative PCR was performed on duplicate samples using the 7500 Fast Real-Time PCR system. Samples were normalized to the expression of β_2-microglobulin mRNA and relative expression was calculated using the change-in-threshold ($-\Delta\Delta C_T$) method.

Cell proliferation analysis

For ex vivo proliferation assays, BAL and lung cells from OVA and alum-induced allergic mice were labeled with Violet CellTrace (Molecular Probes) according to the manufacturer's instructions. Cells were stimulated with 100 µg BSA or 100 µg OVA at 37^{o}C for 72 h. Monensin was added to cultures after 68 h of stimulation, to visualize cytokine expression by flow cytometry. Cells were fixed with 2% formaldehyde at RT for 10 min, premeabilized with FACS buffer containing 0.1%

saponin, incubated with FC block at 4^{o}C for 10 min, stained for CD4 and IL-17A for 30 min at 4^{o}C, and cell proliferation was measured by flow cytometry. For in vivo proliferation studies, mice were injected i.p. with 1 mg BrdU (BD Biosciences) and sacrificed 24 h later. Splenocytes were incubated with FC Block and cell surface antigens were stained for 30 min 4^{o}C as mentioned above. Cells were fixed, permeabilized and BrdU and total DNA were stained according to the manufacturer's instructions and cell cycle analysis was determined by flow cytometry. For some experiments, mice were injected i.p. with 1 mg BrdU with or without IL-12 (.5 µg) or IL-23 (.5 µg). After 12 h, mice received a second i.p. injection with IL-12 (.5 µg) or IL-23 (.5 µg) or PBS. Mice were sacrificed 12 h after the final injection and splenocytes were treated and analyzed as noted above. Antibodies were purchased from BD Biosciences or eBioscience. Cells were analyzed by flow cytometry using an LSR ll and results were analyzed with FlowJo.

Southern blot analysis

Genomic DNA was isolated from mouse tails and cleaved with Pvu ll (Invitrogen) at 37^{o}C overnight. DNA loading dye was added to each digestion and 40 µl of sample was added/ per well to a 1.1% agarose gel (made with a 25-well comb). The gel was run at 10 Volts/cm. To denature the DNA, the gel was gently rocked in denature buffer (1.5 M NaCl, 0.5 M NaOH) for 45 min, rinsed with dd$H_2$0, gently rocked in neutralization buffer (1 M Tris-HCl, 1.5 M NaCl, pH 7.4) for 45

min, rinsed with ddH_20 and put in 6 x SSC (made from 20 x SSC [175.3 g/L NaCl, 88.2 g/L NaCitrate]).

To transfer the gel to a membrane, 20 x SSC was poured into a transfer chamber and 2 pieces of 3MM paper were soaked in 20 x SSC and laid partially overlapped onto a plastic platform to ensure that SSC wicked up the paper. A tube was used to roll out any bubbles beneath the filter. The gel was placed over the filter up-side-down and a piece of Nytran membrane, same size as the gel, was soaked in 6 x SSC and placed on top of the gel. Another two pieces of SSC soaked 3MM were laid on top and a piece of saran wrap was used to cover the entire plastic platform, but was cut around 3 sides of the gel and pulled back, to leave the gel, membrane and 3MM papers 'open.' A stack of paper towels was placed on top and a plastic lid was placed on top of the paper towels with an approximately one pound weight on it. The transfer was carried out overnight. The gel and membrane were removed, wells were marked with a pencil, and successful transfer of DNA was checked with an ultraviolet light box. The membrane was incubated in 6 x SSC for 5 min, dried, and subjected to ultraviolet crosslink with Stratalink.

To begin the hybridization process, the membrane was transferred to a hybridization tube, with the side with the crosslinked DNA facing inward. Pre-hybridization was performed by adding ~ 20 ml of hybridization buffer (6 x SSC, 5 x Denhardt's reagent [50 x made with 5 g/L each Ficoll-400, polyvinylpyrrolidone

and BSA], 0.5% SDS, 50% formamide, 100 µg/ml denatured sheared salmon sperm DNA [incubated at 100^{o}C for 5 min and chilled on ice]) to the tube followed by incubation in the hybridization oven for 2 h at 42^{o}C.

For making a DNA probe, the DNA was purified from PCR product using GeneClean Kit (Qbiogene). For incorporation labeling (Random Primed DNA labeling kit Ambion), DNA (25 ng) was mixed with decamers and water, boiled at 100^{o}C for 5 min, quickly frozen in liquid nitrogen, thawed on ice for 5 min and the mixture was briefly collected by centrifugation. dCTP-containing buffer, α-^{32}P dCTP and klenow enzyme were added to the reaction and incubated at 37^{o}C for 30 min. The reaction was terminated with the addition of EDTA. The radiolabeled probe was purified through ProbeQuant G-50 Micro Columns (Amersham Biosciences). The probe was denatured by adding 10 mM EDTA to each probe and incubating it at 100^{o}C for 5 min. The denatured probe was immediately added to the tube containing the pre-hybridized membrane and the tube was rotated in the hybridization oven overnight at 42 ^{o}C. The following morning, the hybridization buffer was removed and the membrane was rinsed with 2 x SSC/0.1% SDS. The membrane was consecutively washed with 2 x SSC/0.1% SDS for 20 min at 60^{o}C and 0.2 x SSC/0.1% SDS for 20 min at 60^{o}C. The membrane was removed from the tube, blotted on paper towels, placed on 3MM paper, covered in plastic wrap, placed in a cassette and a film was put on top of the membrane in the dark and placed at -80^{o}C for 1-3 days before development.

To remove the DNA probe, 0.1 x SSC/0.5% SDS was boiled and the membrane was soaked in the buffer (off-heat) for 15 min and rinsed in 6 x SSC.

Microscopy analysis

Th1 or Th17 cells were stimulated with PMA and ionomycin for 3 h. Cells were washed and loaded onto a 35 mm glass-bottom microwell dish (MatTek Corporation), the dish was mounted on the microscope stage and EGFP and YFP expression was analyzed. Cells were imaged on a laser scanning Zeiss LSM-510 confocal microscope (Carl Zeiss, Jena, Germany). Images were analyzed using Zeiss LSM software (Carl Zeiss).

Viruses

Vaccinia virus (VV) (Western Reserve strain) and VV-SIINFEKL (originally provided by J. Yewdell and J. Bennick, LVD, NIAID/NIH) were propagated in the human osteosarcoma TK-143B cell line, followed by sucrose gradient purification and titer determination by the VV Core Facility at the Indiana University School of Medicine as described (181).

Vaccinia virus infections

For standard infections, 2-6 mice per group were infected i.p. with a sublethal dose ($1x10^6$ - $5x10^6$ pfu/mouse) of VV-WR. Mice were sacrificed after 5-21 days of infection and ovaries and spleens were collected. For adoptive transfer experiments, 2-7 mice per group were infected either intranasally (i.n.) ($2x10^6$

pfu/mouse) or i.p ($2x10^7$ pfu/mouse) with VV-SIINFEKL or VV-WR. Infection was performed 1 day before or after cell transfer. Mice were sacrificed 4-8 days after infection and spleens and ovaries were collected. VV was titered using plaque assays as previously described (181).

Adoptive Tc cell transfer and isolation

BMDCs were cultured as described in Lutz et al (182) and stimulated with LPS (1 µg/ml; Sigma) in the presence or absence of SIINFEKL peptide (1 µM) for 24 h. Prepared BMDCs ($1x10^6$) or 5 day-differentiated OT-I Tc17 or Tc1 cells ($1x10^6$) were transferred via intravenous (i.v.) injection in the tail in 0.5 ml PBS. In some experiments, $CD45.2^+$ cells were isolated from infected mice using anti-CD45.2 antibodies and magnetic cell selection (Miltenyi Biotec).

Cytolytic activity analysis

Cytotoxic activity of OT-I Tc17 and Tc1 effector cells was measured in a standard ^{51}Cr release assay (183). In brief, $6x10^6$ EL4 and EG.7 (EL4 cells transfected with chicken ovalbumin) target cells were incubated with 200 µCi of $Na_2{}^{51}CrO_4$ (Perkin Elmer) in 1 ml for 1 h 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 cell ratios ranging from 60:1 to 0.1:1. Target cells were then added to the microtiter plate at a concentration of $2x10^4$ cells/well. Reactions were conducted in a total volume of 200 µl per well. Plates were incubated for 6 h at 37°C, spun down, and 100 µl of

cell free supernatant was harvested from each well. Radioactivity was counted in a Perkin Elmer gamma counter. The percentage of specific lysis was calculated as ($cpm_{sample} - cpm_{spontaneous}$)/($cpm_{maximum} - cpm_{spontaneous}$) x 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 (Sigma). In some experiments, as noted in figures, control or anti-FasL (10 μg/ml; BioLegend) was added to cytotoxicity assays.

Induction of allergic airway disease (AAD)

Induction of allergic airway disease using OVA and alum has been described previously (46). In brief, 8-10 week old mice were sensitized by i.p. injection of OVA (20 μg; Sigma) adsorbed with alum (2 mg; Sigma) on days 0 and 7. Mice were challenged with OVA (100 μg) i.n. from days 14-19 and were sacrificed 24 h after the last challenge. For some experiments, mice were sensitized on days 0 and 7 as mentioned above and in vitro-derived antigen specific-YFP^+ Th17 cells ($1x10^5$) were transferred by i.v. injection to the tail on day 20. Mice were challenged with OVA (100 μg) i.n. from days 21-26 and were sacrificed 24 h after the last challenge. In some experiments, mice were sensitized on days 0 and 7 as mentioned above, challenged with OVA (100 μg) or OVA (100 μg) and LPS (1 μg; Sigma) i.n. from days 14-16, challenged with OVA (100 μg) i.n. from days 23-25 and were sacrificed 24 h after the last challenge. For experiments with *Stat4*$^{-/-}$ mice, mice were sensitized on days 0 and 7 as mentioned above and were

challenged with OVA from days 14-18 and were sacrificed 48 h after the last challenge. To induce allergic airway disease using house dust mite (HDM), 8-10 week old mice were administered i.n. doses of HDM (50 µg; whole body *Dermatophagoides pteronyssinus*; Greer) for 3 consecutive days each week for 5 weeks. Mice were sacrificed 24 h after the last dose was administered. To induce allergic airway disease using *Aspergillus fumigatus* (ASP), 8-10 week old mice were administered i.n. doses of ASP (100 µg; cellular antigen; Greer) every other day for 21 days (11 challenges total). Mice were sacrificed 24 h after the last dose was administered. For all models of AAD, mice were sacrificed by i.p. injections of pentobarbital (5 mg/mouse). The trachea was cannulated and the lungs were lavaged 3 times with 1 ml PBS. The cells recovered in the BAL fluid were counted with a hemacytometer. The lungs were isolated and single cell-suspensions were prepared using a gentleMACS Dissociator (Miltenyi Biotec) according to the manufacturer's instructions.

Induction of α-GalCer-induced inflammation

Mice were injected i.v. with PBS, 1 µg α-galactosylceramide (α-GalCer; NIH Tetramer Core Facility, Emory University) or 1 µg α-GalCer and 1 µg IL-23 and sacrificed 6 h later. Spleen and/or blood (via cardiac puncture) were collected and serum was isolated from the blood. In other experiments, mice received i.n. doses of PBS, 1 µg α-GalCer or 1 µg α-GalCer and 1 µg IL-23 and were sacrificed 24 h later. BAL and lung were collected and lung mononuclear cells were isolated from part of the lung using a gentleMACS Dissociator (Miltenyi

Biotec) according to the manufacturer's instructions and a Ficoll–Hypaque gradient was performed. In some experiments, mice were administered i.n. doses of PBS or 2 μg α-GalCer and 125 μg Poly(I:C) (InvivoGen) and were sacrificed 48 h later. BAL cells and lung were isolated and NKT cells were sorted by flow cytometry from the BAL.

Induction of experimental autoimmune encephalomyelitis (EAE)

Induction of EAE disease has been previously described (184). In brief, 8-10 week old female mice were immunized subcutaneously (s.c.) on days 0 and 7 with myelin oligodendrocyte glycoprotein (MOGp35-55) antigen peptide (100 μg; Genemed Synthesis) emulsified in complete Freund's adjuvant (150 μl; Sigma). Mice were injected i.p. with pertussis toxin (100 ng; Sigma) on days 0 and 2. Mice were sacrificed 19 days after induction of disease and spleen and brain were harvested. Mononuclear cells were isolated from the brain using a 30/70% Percoll gradient.

Statistical analysis

The Student's *t* test or one-way ANOVA was used for statistical comparison. *P* values of 0.05 or less were considered as significant.

RESULTS

Part I- Tc17 cells are capable of mediating immunity to vaccinia virus by acquisition of a cytotoxic phenotype

STAT3 and RORγt are essential for optimal IL-17 production from Tc17 cells

While it is known that STAT3 and RORγt are required for the development of the Th17 effector program, including IL-17 production, less is known about their role in the development of Tc17 cells. To analyze the differentiation of $CD8^+$ T cells in the absence of STAT3 or RORγt, we isolated $CD8^+$ T cells from the spleen of wild type, *Stat3*$^{CD4-/-}$ and *Rorc*$^{GFP/GFP}$ mice, stimulated them under Tc1 or Tc17-polarizing conditions for five days and analyzed cytokine production in re-stimulated cells. STAT3-deficient Tc17 cells display abrogated IL-17 production in comparison to wild type cells (Figure 3A). Furthermore, IL-17 production is also largely diminished in RORγt-deficient Tc17 cells (Figure 3A). RORγt-deficiency did not have an effect on IFN-γ or Granzyme B production from Tc1 cells, whereas RORγt-deficient Tc17 cells displayed a slight enhancement of IFN-γ and Granzyme B production in comparison to wild type Tc17 cells (Figure 3A and B). STAT3-deficient Tc1 and Tc17 cells displayed an increase in IFN-γ production, however Granzyme B production was only enhanced in STAT3-deficient Tc17 cells (Figure 3A and B). Together, these data demonstrate a requirement for STAT3 and RORγt for optimal IL-17 production from Tc17 cells, and indicate that STAT3 represses IFN-γ and Granzyme B production for in vitro

derived Tc cells, whereas RORγt may have a smaller role in the repression of these cytokines in the Tc17 cell subset.

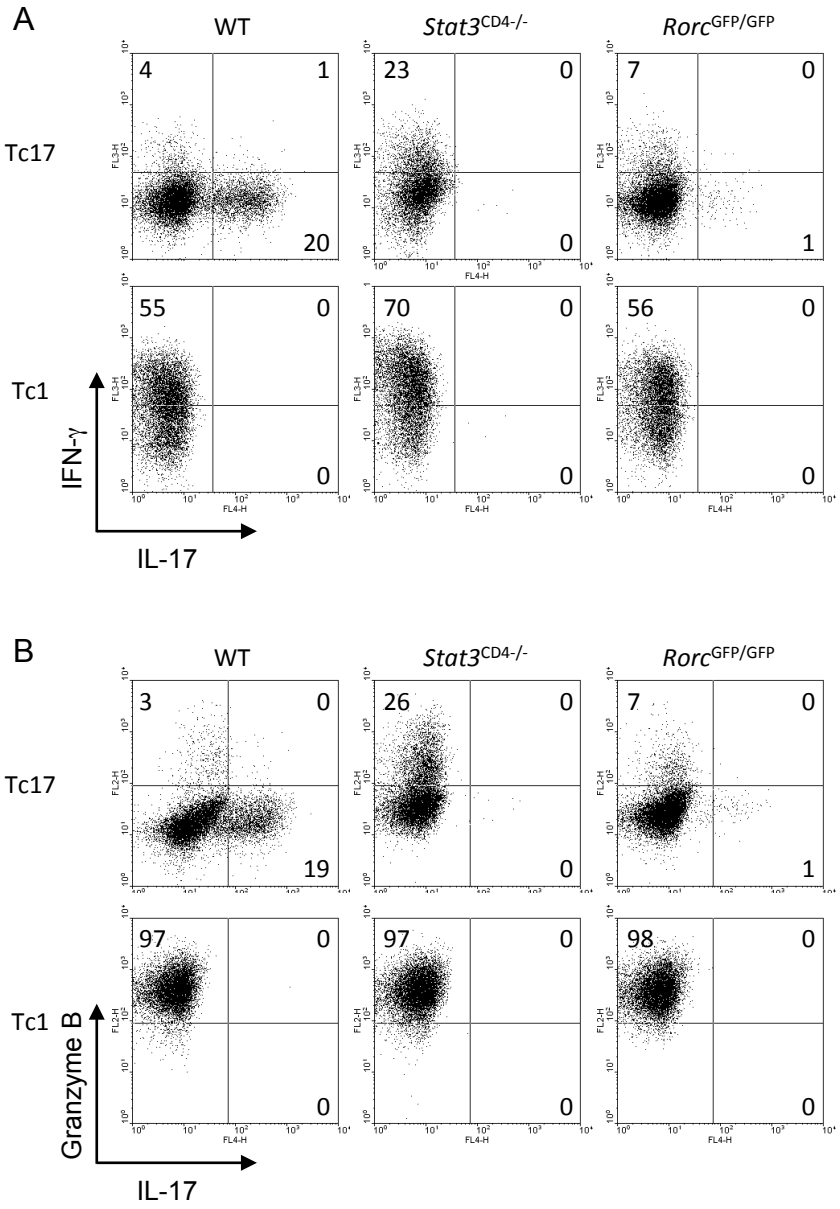


Figure 3. STAT3 and RORγt are essential for IL-17 production from Tc17 cells. *A-B*, $CD8^+$ T cells from WT, *Stat3*$^{CD4-/-}$ and *Rorc*$^{GFP/GFP}$ mice were primed under Tc1 or Tc17 conditions for five days. Cells were re-stimulated with PMA and ionomycin and IL-17-, IFN-γ- (*A*), and Granzyme B-positive (*B*) cells were

detected using intracellular cytokine staining. Representative dot plots are shown and data are representative of 2 mice.

Tc17 cells switch to a Tc1 phenotype in vitro

Previous reports have shown that the IL-17-secreting T cell phenotype is unstable, particularly in vivo. To explore this phenomenon further, we cultured in vitro-derived Tc17 cells for an additional round of differentiation under various culture conditions. Tc17 cultures maintained for a second round under Tc17 skewing conditions (TGFβ + IL-6) developed a higher percentage of IL-17-secreting cells but still maintained a small population of IFN-γ-secreting cells (Figure 4). Tc17 cells cultured for a second round in the absence of cytokines (condition labeled as "α-IFN-γ") demonstrated decreased IL-17 production and increased IFN-γ production, and culture of the cells in Tc1 conditions (IL-12) enhanced this phenotype (Figure 4). When IL-12 was added to cells under Tc17 conditions for the second round, the cells that developed were more heterogeneous, with a significant population positive for both IFN-γ and IL-17 (Figure 4).

We also examined Granzyme B expression in these cultures. Only a small percentage of Tc17 cells after one week of culture demonstrated expression of Granzyme B (Figure 4). The percentage of Granzyme B^+ cells increased after a second round of culture in Tc17 conditions (Figure 4). Granzyme B^+ cells increased if Tc17 cells were cultured in the absence of cytokines, an effect that was enhanced by culture with IL-12 (Figure 4). Moreover, IL-12 induced

Granzyme B expression even when cells were maintained in Tc17 culture conditions (Figure 4). Thus, continued exposure to Tc17 skewing cytokines is required to maintain the Tc17 phenotype, while IL-12 contributes to the conversion of Tc17 cells to a Tc1 phenotype.

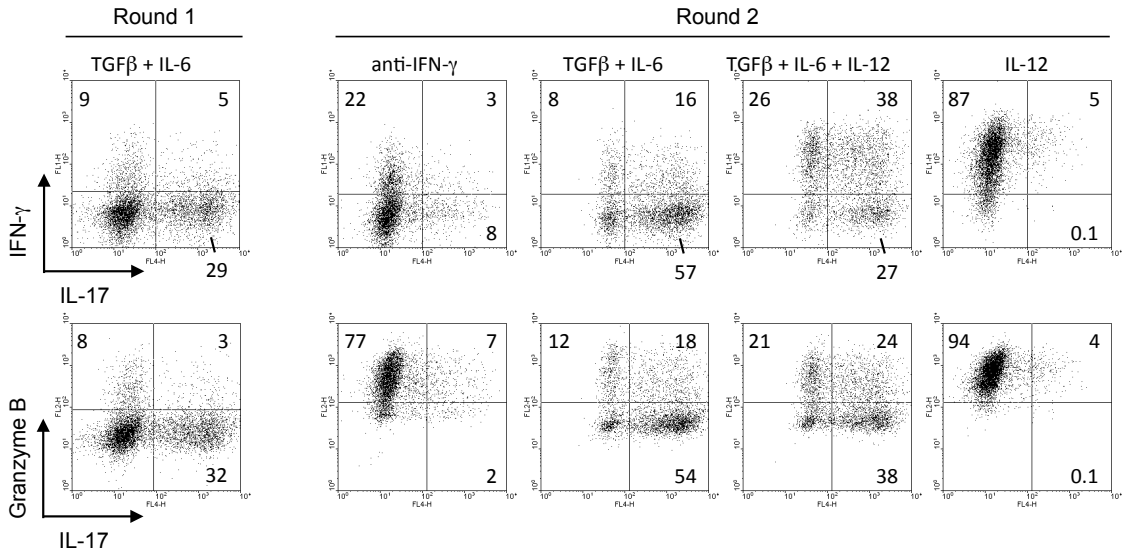


Figure 4. Tc17 cells are unstable and have increased Granzyme B production in a second round of culture. WT $CD8^+$ T cells were primed under Tc17 conditions. After five days, Tc17 cells were re-stimulated in the presence of α-IFN-γ alone, or with the addition of the indicated cytokines, or IL-12 alone for an additional five days. Cells were re-stimulated with PMA and ionomycin and IL-17-, IFN-γ-, and Granzyme B-positive cells were detected using intracellular cytokine staining after the first round or second round of stimulation as indicated. Representative dot plots are shown and data are representative of 2-3 mice. Experiments were performed in collaboration with Norman Yeh.

Expression of cytotoxicity genes in Tc17 cells

Since Tc17 cultures switched to Tc1 conditions were able to acquire IFN-γ and Granzyme B expression, we then tested further whether other genes in these cultures that are associated with cytotoxicity demonstrated changes in expression. Similar to results of intracellular cytokine staining, *Il17a* was expressed in Tc17 cells but not in Tc1 cultures, and expression was diminished in Tc17 cells cultured for a second week in Tc1 conditions (Figure 5). *Ifng* and *Gzmb* also showed similar patterns to intracellular staining; higher expression in Tc1 cells and acquired expression in Tc17 cells switched to Tc1 conditions, though still lower than in Tc1 cells. Expression of *Tnf* was not different between Tc1 and Tc17 cells. Somewhat surprisingly, Tc17 cells switched to culture under Tc1 conditions did not demonstrate increased expression of *Prf1* or *Tnfsf10* (encoding TRAIL), but rather maintained low expression, compared to Tc1 cells, regardless of culture condition (Figure 5). In contrast to the pattern of expression of other genes, *Fasl*, which was expressed at considerably lower levels in cells cultured for one round in Tc17 conditions than in Tc1 cells, demonstrated expression comparable to Tc1 cells in Tc17 cultures that were switched to either Tc1 or Tc17 culture conditions for the second round (Figure 5).

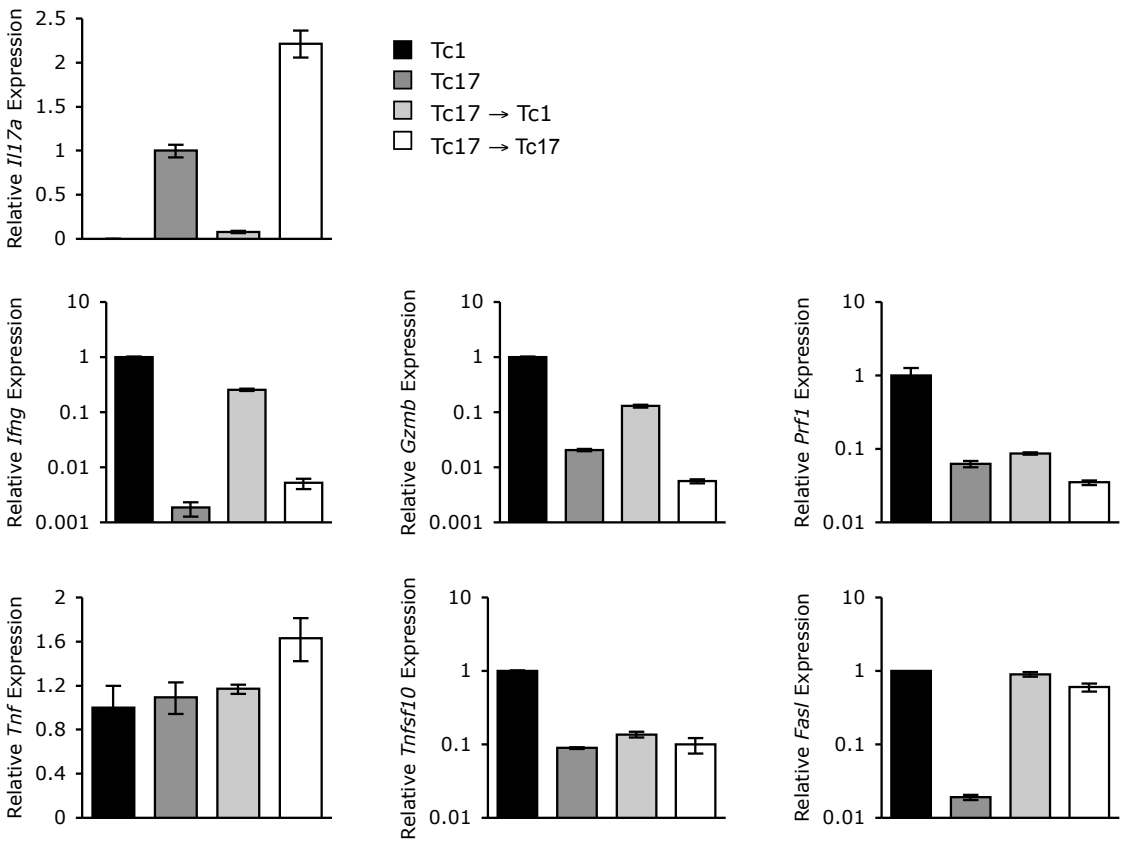


Figure 5. Expression of cytotoxicity-associated genes in Tc17 cultures. OT-I $CD8^+$ T cells were cultured under Tc1 or Tc17 conditions. After five days, Tc17 cells were re-stimulated under Tc1 or Tc17 conditions for an additional five days. RNA was isolated from differentiated cells after 4 h of re-stimulation with SIINFEKL peptide. Expression for the indicated genes was measured using quantitative PCR with samples being normalized to the expression of β_2-microglobulin mRNA and are relative to levels in Tc1 cells, with the exception of *Il17a*. A logarithmic scale was used to display the relative expression of all genes with the exception of *Il17a* and *Tnf*.

Tc17 cells in vivo

While considerable attention has been paid to the function of in vitro-derived Tc17 cells, little work has been performed on in vivo-derived IL-17-secreting $CD8^+$ T cells. To test the ability of $CD8^+$ T cells from different strains of mice to

produce IL-17, we isolated $CD8^+$ T cells from naïve Balb/c, C57BL/6 and C3H mice, stimulated them with anti-CD3 and assessed the production of IL-17 and IFN-γ using ELISA. While amounts of IL-17 were significantly lower than IFN-γ, IL-17 production was detectable in cultures from all three strains, and highest in C57BL/6 (Figure 6A). To determine if this population expanded in response to viral infection, we innoculated mice with VV and assessed IL-17 and IFN-γ production in $CD4^+$ and $CD8^+$ T cells at time points after infection (Figure 6B). While both $CD4^+$ and $CD8^+$ T cells showed transient increases in IFN-γ-producing cells, with levels decreasing following viral clearance (Figure 6B and C), only $CD8^+$ T cells showed an increase in the number of IL-17-secreting cells (Figure 6B). While the number of IL-17^+ cells was much lower than the number of IFN-γ-producing cells, the fold-induction of cells, compared to uninfected mice, was similar to that observed for IFN-γ-producing cells. We performed a similar experiment in mice that were infected twice with VV to examine the memory response, and while there was no significant increase in IL-17-secreting $CD4^+$ T cells, there was a significant increase in IL-17-secreting $CD8^+$ T cells, compared to uninfected mice (Figure 6D).

To determine if the increased numbers of IL-17-secreting $CD8^+$ T cells elicited in vivo were specific for viral antigen, we adoptively transferred naïve OT-I T cells to recipient mice that were subsequently infected with VV expressing the ovalbumin SIINFEKL peptide. Five days after either intranasal or intraperitoneal infection, splenocytes were stimulated with peptide and analyzed using intracellular

cytokine staining and ELISA. A significant increase in epitope-specific IL-17-secreting cells was observed in mice following both routes of infection (Figure 6E and F).

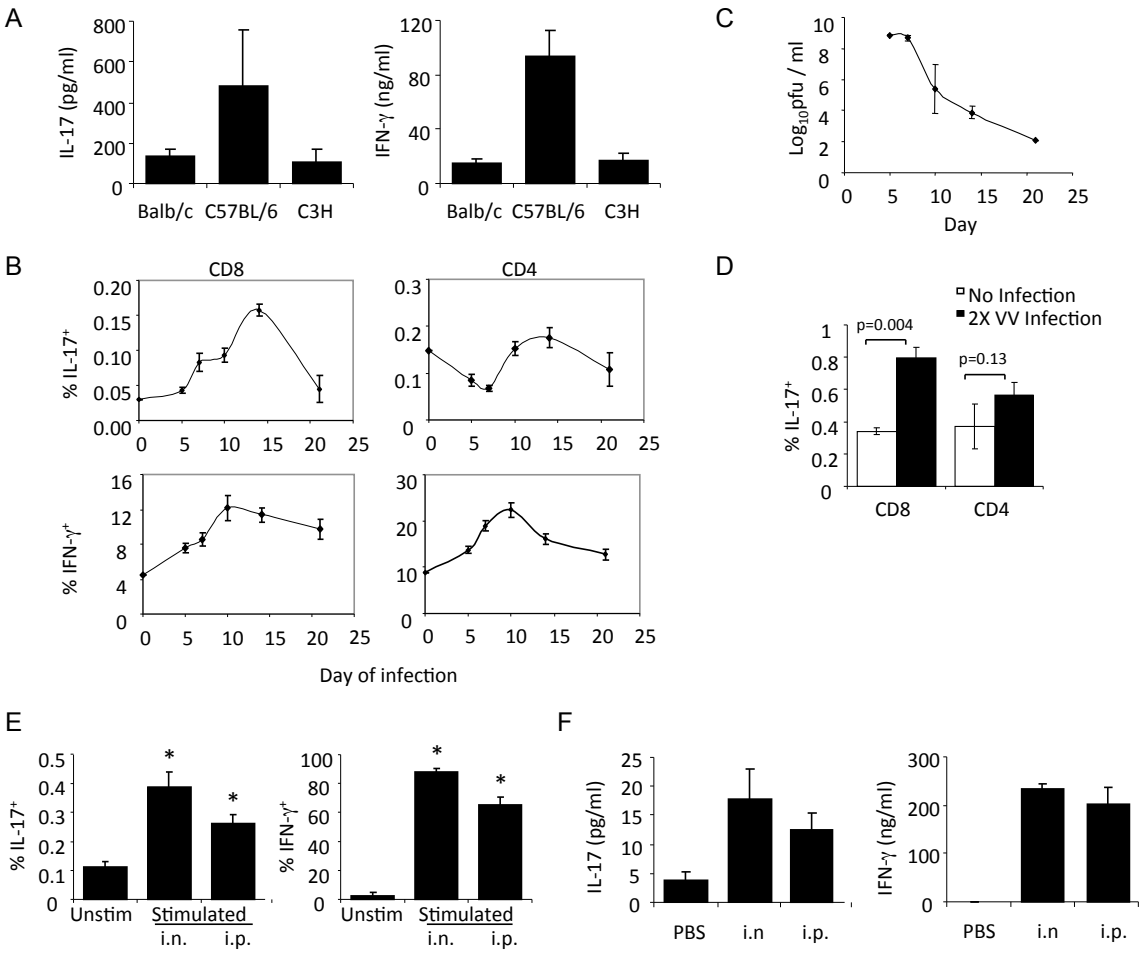


Figure 6. Tc17 cells are induced in response to infection with vaccinia virus. *A*, CD8$^+$ T cells were isolated from the spleens of Balb/c, C57BL/6, or C3H/HeJ mice and stimulated for 48 h before cell free supernatants were used to measure IL-17 and IFN-γ protein levels using ELISA. Results are presented as the mean ± SEM of CD8$^+$ T cells from 3 mice. *B-C*, C57BL/6 mice were infected i.p. with 5x10^6 pfu VV per mouse. Spleens and ovaries were respectively isolated for cytokine analysis following re-stimulation (*B)* or viral titers (*C*) at 5, 7, 10, 14, and 21 days post-infection. The data are presented as an average of values from

4 mice per time point ± SEM. *D*, C57BL/6 mice were infected on days 0 and 21 with $1x10^6$ pfu VV, i.p. per mouse at each time point. Five days after the second infection, $CD8^+$ and $CD4^+$ cells were isolated from the spleens and re-stimulated before analyzing IL-17 and IFN-γ production using ICS. The data shown are an average of values from 2-4 mice ± SEM. *E-F*, BoyJ homozygous ($CD45.1^+$) or heterozygous ($CD45.1^+CD45.2^+$) mice were injected i.v. with $1x10^6$ OT-I/*Rag1*$^{-/-}$ $CD8^+$ ($CD45.2^+$) 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. (*E*) Five days after infection, splenocytes were stimulated with SIINFEKL peptide or left unstimulated. $CD45.1^-$ $CD45.2^+$ IL-17^+ and IFN-γ^+ cells were detected using ICS. The data are the average ± SEM of 5 mice. (*F*) Splenocytes were stimulated with SIINFEKL peptide for 48 h and IL-17 and IFN-γ protein levels in cell free supernatants were measured using ELISA. Statistics in *D* and *E* were performed using a Student's *t* test. *$p<0.05$ compared to unstimulated condition. Experiments were performed in collaboration with Norman Yeh.

Anti-viral activity of Tc17 cells in vivo

We next tested the ability of Tc17 cells to mediate viral clearance. OT-I cells were differentiated in vitro to Tc1 or Tc17 phenotypes, and cultured cells were adoptively transferred to mice one day after i.p. infection with VV-SIINFEKL. At day 7 post-infection, viral titers in the ovaries were determined. Transferred Tc1 or Tc17 cells were equally capable of enhancing the clearance of virus (Figure 7A). While transferred Tc17 cells showed greater expansion than Tc1 cells, they lost IL-17-secreting potential and demonstrated increased production of IFN-γ; however the levels were still lower than Tc1 cells (Figure 7B and C). Analysis of post-transfer cells following intranasal VV inoculation showed a switch to more IFN-γ and less IL-17 production, though the polarization was less dramatic compared to intraperitoneal infection (Figure 7C and D).

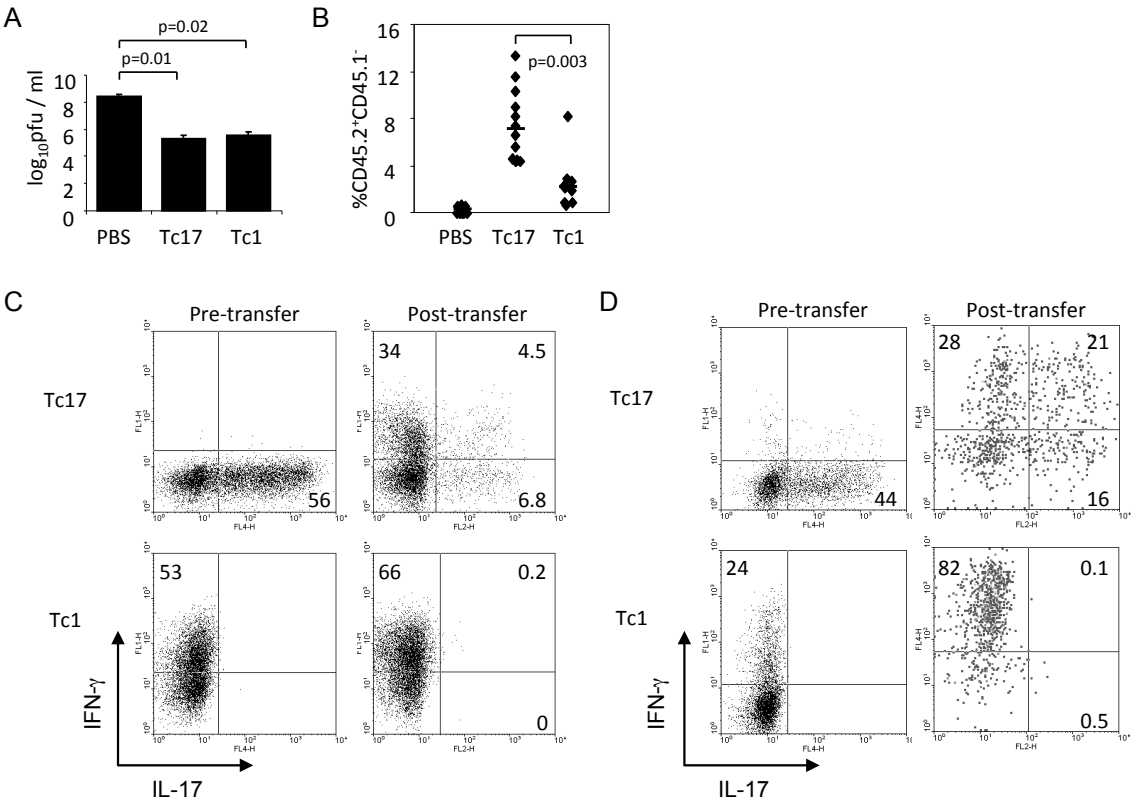
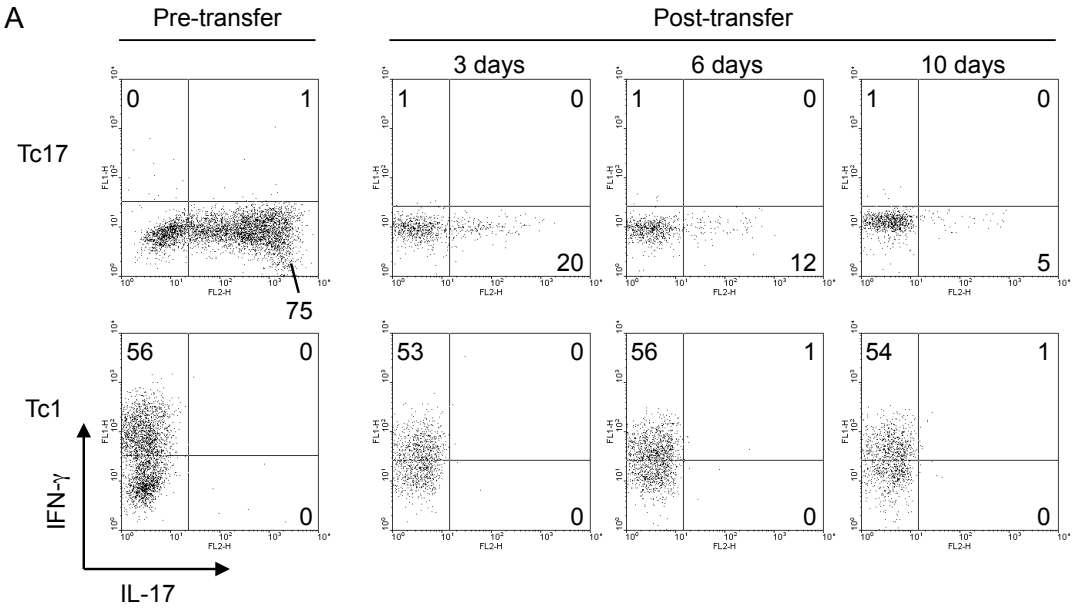


Figure 7. Adoptively transferred Tc17 cells promote VV-SIINFEKL clearance and convert to an IFN-γ-secreting phenotype. *A-C*, BoyJ (CD45.1$^+$) mice were infected with 2x10^7 pfu VV-SIINFEKL, i.p., and injected i.v. with differentiated OT-I Tc17 or Tc1 cells (1x10^6, CD45.2$^+$) or PBS after 24 h. Six days later ovaries were harvested for viral titer determination (*A*), and splenocytes were surface stained with antibodies to CD45.1 and CD45.2 to identify transferred cells (*B*). (*C*) IL-17$^+$ and IFN-γ$^+$ cells were identified in differentiated OT-I Tc17 or Tc1 cells immediately before adoptive transfer (left panels) and in splenocytes six days after adoptive transfer (right panels). Transferred cells in the right panels are gated on CD45.1$^-$CD45.2$^+$ cells. Data are the average of 4-5 mice ± SEM (*A*) or are representative experiments (*B, C*). *D*, Differentiated OT-I Tc17 or Tc1 cells (1x10^6) or PBS were injected i.v. into BoyJ mice. One day later, mice were infected with 2x10^6 pfu VV-SIINFEKL intranasally. IL-17$^+$ and IFN-γ$^+$ cells were determined in Tc17 or Tc1 cells immediately before adoptive transfer (left panels) and five days after adoptive transfer in isolated splenocytes (right panels). Transferred cells in the right panels are gated on the CD45.1$^-$CD45.2$^+$ cells. The data are representative of 6-7 mice. Statistics in *A* and *B* were performed using a Student's *t* test. Experiments were performed in collaboration with Norman Yeh.

Acquisition of a Tc1 phenotype in vivo requires virus-encoded antigen

Since we observed that Tc17 cells transferred to VV-infected mice altered their phenotype towards a potential for increased IFN-γ production and decreased IL-17 production, we wanted to determine if virus-encoded antigen was required for the altered phenotype. Transfer of Tc17 cells into naïve mice resulted in a time-dependent decrease in IL-17 production with no concomitant increase in IFN-γ production, while transferred Tc1 cells maintained the IFN-γ-secreting phenotype (Figure 8A and B). When OT-I Tc17 cells were transferred with BMDC or SIINFEKL-pulsed BMDC, there was a similar loss of IL-17-secreting potential, (significantly more loss of IL-17 in the mice receiving SIINFEKL-pulsed BMDC versus naïve mice) but little induction of IFN-γ (Figure 8B and data not shown), despite increased cell expansion in mice that received SIINFEKL-pulsed BMDCs (1.1 ± 0.06% $CD45.2^+$ cells in spleen versus only 0.31 ± 0.15% for BMDCs alone). We then tested whether VV infection alone, without relevant antigen, would alter the phenotype of the cells. Tc1 cells showed increased IFN-γ-secreting potential following infection (Figure 8C and D). However, transferred Tc17 cells still displayed a marked reduction in the percentage of IL-17-positive cells after infection, with no increase in IFN-γ production (Figure 8C and D). These results suggest that the combination of virus-encoded antigen and infection are required to promote conversion of cells to the Tc1 phenotype in vivo.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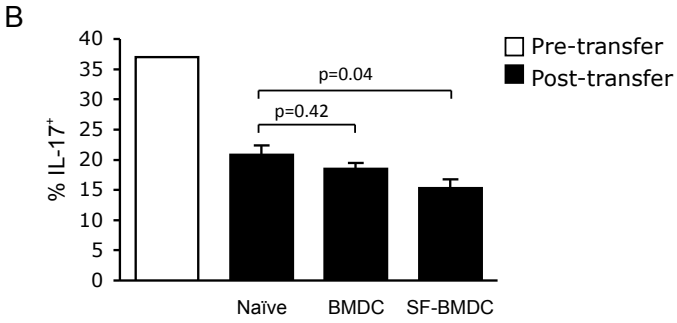
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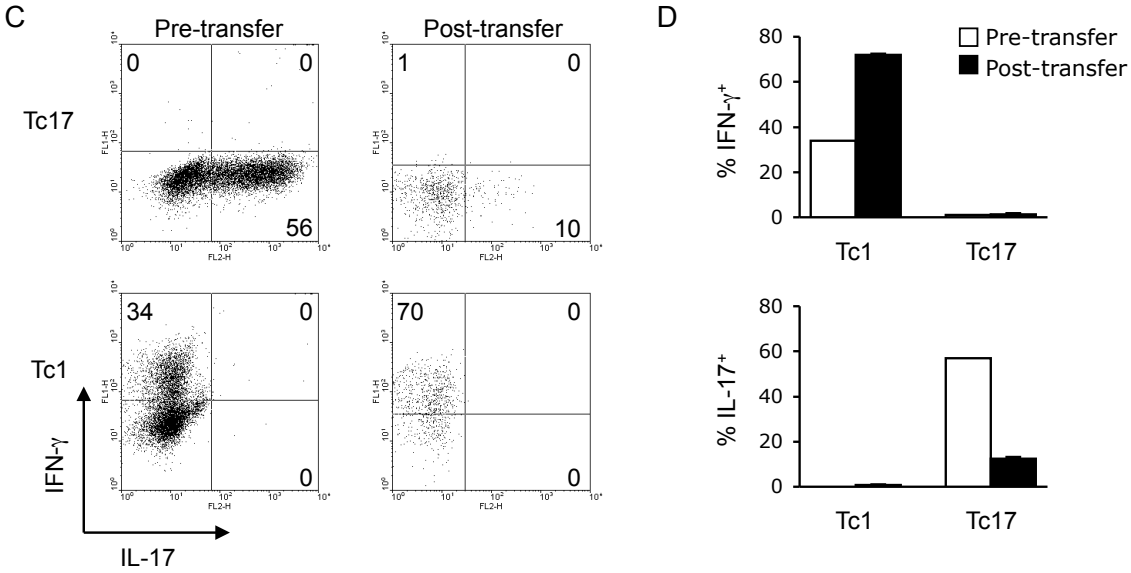
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D
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Pre-transfer
Post-transfer
Tc1
Tc17

Figure 8. VV-encoded antigen is required for Tc17 cells to convert to an IFN-γ-secreting phenotype in vivo. *A*, Naïve BoyJ (CD45.1$^+$) mice were injected i.v. with five day differentiated OT-I Tc17 or Tc1 cells (1x10^6, CD45.2$^+$) or PBS. After an additional 3, 6, or 10 days, spleens were isolated and total splenocytes were stimulated with SIINFEKL (SF) peptide. IL-17$^+$ and IFN-γ$^+$ cells were identified in differentiated Tc17 or Tc1 cells immediately before adoptive transfer (left panels) and 3, 6, and 10 days after adoptive transfer from splenocytes (right panels). Transferred cells in the right panels are gated on the CD45.1$^-$CD45.2$^+$ cells. The data are representative of two experiments. *B*, BoyJ mice were injected i.v. with BMDCs untreated or pulsed with SIINFEKL peptide (1x10^6, CD45.1$^+$) or PBS-treated and injected i.v. with differentiated OT-I Tc17 cells (1x10^6, CD45.2$^+$) 48 h later. After an additional 5 days, spleens were isolated and total splenocytes were stimulated with SIINFEKL peptide. IL-17$^+$ cells were identified in differentiated Tc17 cells immediately before adoptive transfer and 5 days after adoptive transfer from total splenocytes. Transferred cells are gated on CD45.1$^-$CD45.2$^+$ cells. The data are the average of 3-4 mice ± SEM and are representative of 2 or more experiments. *C-D*, BoyJ mice were infected i.p. with 2x10^7 pfu VV-WR and injected i.v. with differentiated OT-I Tc17 or Tc1 cells (1x10^6) or PBS one day later. After an additional six days, spleens were isolated and total splenocytes were stimulated with SIINFEKL peptide. (*C*) IL-17$^+$ and IFN-γ$^+$ cells were analyzed in differentiated OT-I Tc17 or Tc1 cells immediately before adoptive transfer (left panels) and six days after adoptive transfer in spleen (right panels). The transferred cells were gated on CD45.1$^-$ CD45.2$^+$ cells. Data are representative of 5 mice (*C*), or are the average ± SEM of 5 mice (*D*) and are representative of 2 or more experiments. Statistics in *B* were performed using a Student's *t* test.

Acquisition of cytotoxic function by Tc17 cells in vivo

To determine if IFN-γ was required for Tc17-mediated viral clearance, we repeated the experiment in Figure 7*A-C* using both wild type and *Ifng*$^{-/-}$ OT-I cells. Somewhat surprisingly, WT and *Ifng*$^{-/-}$ Tc17 cells were equally capable of clearing VV infection (Figure 9A). While *Ifng*$^{-/-}$ Tc17 cells demonstrated increased potential for production of IL-17, neither wild type nor *Ifng*$^{-/-}$ Tc17 cells showed acquisition of T-bet or Granzyme B expression (Figure 9B). Thus, conversion to

an IFN-γ-secreting phenotype is not a crucial component of Tc17-mediated viral clearance.

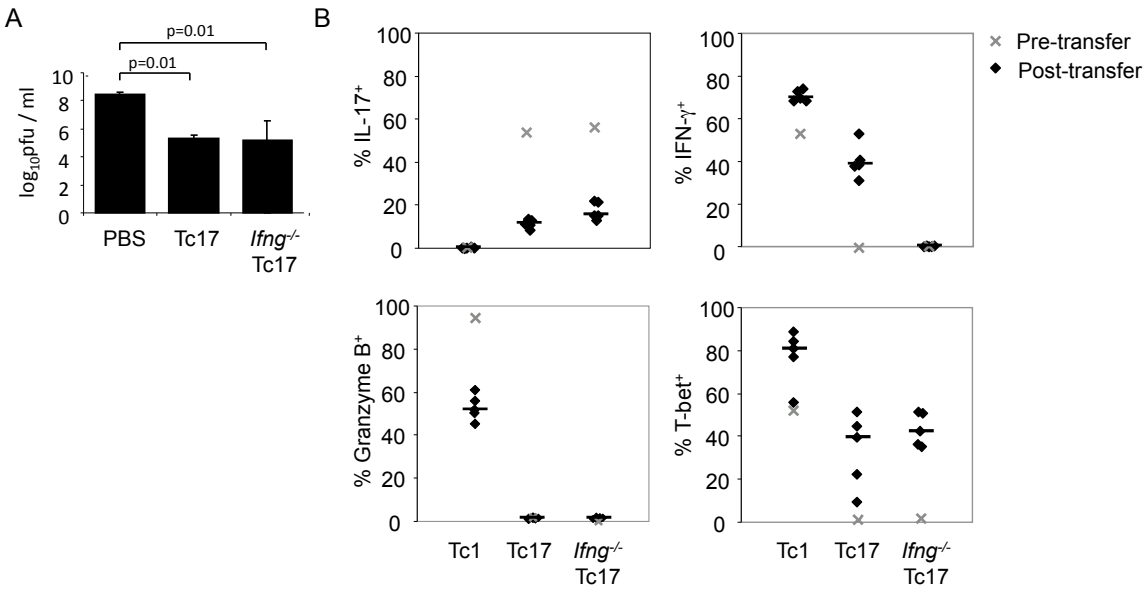

Figure 9. IFN-γ is not required for Tc17-mediated viral clearance. *A-B*, BoyJ (CD45.1$^+$) mice were infected with 2x10^7 pfu VV-SIINFEKL i.p. and injected i.v. with differentiated OT-I Tc17, *Ifng*$^{-/-}$ Tc17 and Tc1 cells (1x10^6, CD45.2$^+$) or PBS 24 h later. After six days, ovaries were harvested for viral titer determination (*A*), and IL-17$^+$, IFN-γ$^+$, Granzyme B$^+$, and T-bet$^+$ CD45.1$^-$CD45.2$^+$ splenic cells were analyzed using ICS (*B*). Median values are displayed as horizontal lines and symbols represent individual mice. Statistics in *A* was performed using a Student's *t* test. Experiments were performed in collaboration with Norman Yeh.

The ability of transferred Tc17 cells to mediate viral clearance, regardless of IFN-γ production, suggested that Tc17 cells might acquire a cytotoxic phenotype in vivo. As others have shown, and we have reproduced, Tc17 cells, after 1 week of culture, have limited cytotoxic activity in a standard ^{51}Cr-release assay (Figure 10A). However, after two rounds of culture in Tc17 conditions that maintain the

IL-17-secreting phenotype, Tc17 cells acquire cytotoxic activity, though this activity is still comparatively less efficient than killing by cells cultured under Tc1 conditions for two rounds (data not shown), or by cells switched from Tc17 to Tc1 conditions for the second round of culture (Figure 10A).

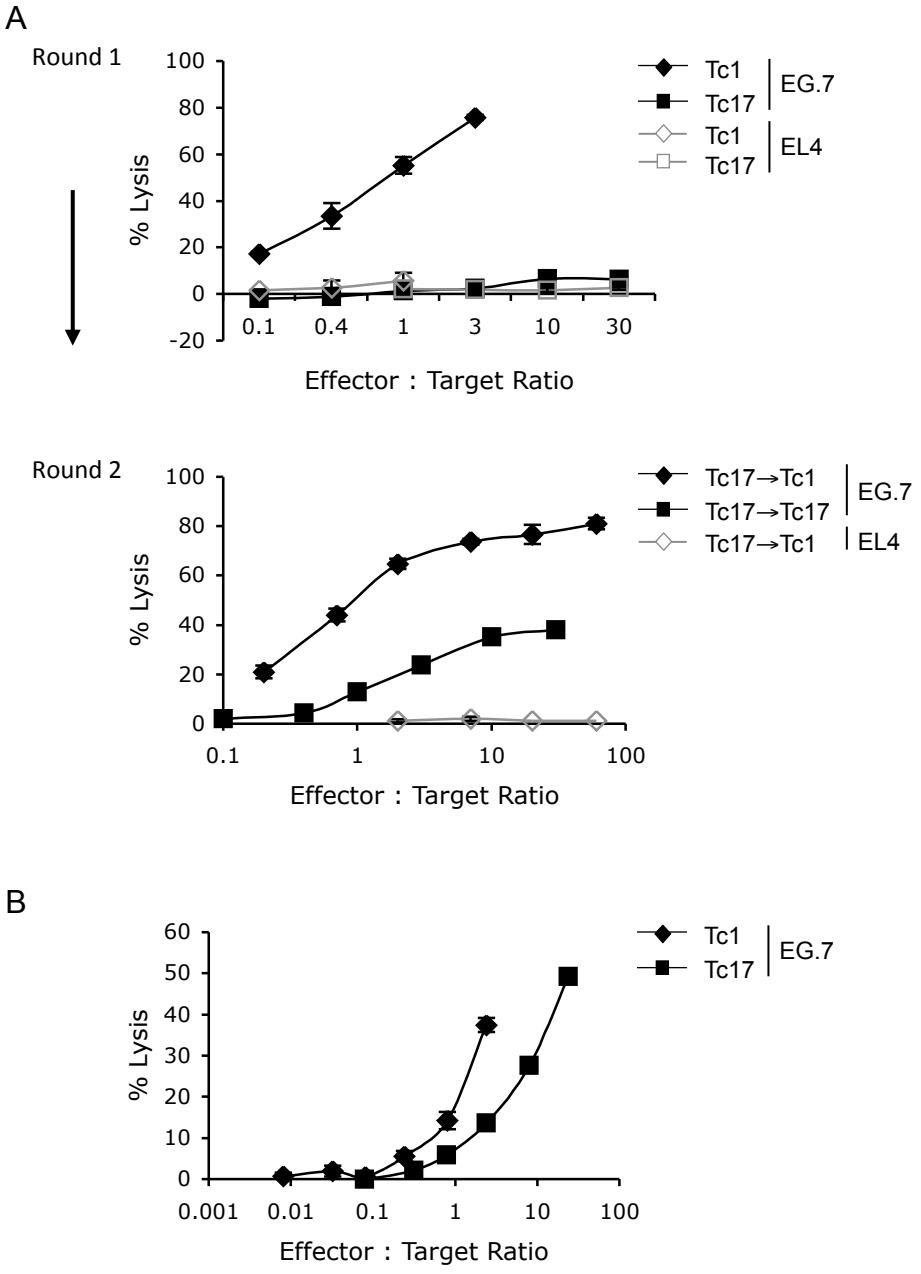

Figure 10. Adoptively transferred Tc17 cells acquire cytotoxic potential in vivo. *A*, After culture for one or two rounds (as indicated) under Tc17, Tc1 or Tc17 switched to Tc1 conditions, OT-I $CD8^+$ T cells were tested for cytotoxic potential using a ^{51}Cr-release assay. Effector cells were co-cultured with ^{51}Cr-labeled, OVA-expressing EG.7 target cells for 6 h at the indicated effector to target ratios. *B*, BoyJ ($CD45.1^+$) mice were infected with $2x10^7$ pfu VV-SIINFEKL i.p. and injected i.v. with differentiated OT-I Tc17 and Tc1 cells ($1x10^6$, $CD45.2^+$) 24 h later. Six days later, injected cells were isolated using anti-CD45.2 antibodies and magnetic selection. A ^{51}Cr release assay was performed as in (*A*). E:T ratios were normalized to the percentages of $CD45.1^-CD45.2^+$ cells in the purified populations. Cytotoxicity against EL4 targets was less than 1.5%.

We then wanted to determine if the acquisition of cytotoxic activity that potentially contributed to viral clearance also occurred in vivo. Transferred Tc1 and Tc17 OT-I cells were purified after VV-SIINFEKL infection and function in a cytotoxicity assay was tested. Purified Tc17 cells following infection demonstrated in vitro cytotoxic activity following adoptive transfer to virus-infected mice, although they were still less efficient than similarly purified Tc1 cells (Figure 10B).

Since *Fasl* expression was increased in long-term Tc17 cultures (Figure 5), and these cells acquired cytotoxic activity (Figure 10A), we tested whether there was increased *Fasl* expression in cytotoxic Tc17 cells isolated post-transfer. We observed that Tc17 cells isolated post-transfer demonstrate *Fasl* expression comparable to in vitro differentiated Tc1 cells (Figure 11A). To determine if FasL is responsible for the cytotoxic activity of Tc17 cells following infection we performed a cytotoxicity assay as in Figure 10B with the addition of control antibodies or anti-FasL. Incubation with anti-FasL, but not the control antibodies, resulted in a modest though significant decrease in Tc1-mediated cytotoxicity

(Figure 11B). Moreover, anti-FasL resulted in a 50% decrease in the cytotoxic activity of post-transfer Tc17 cells. Thus, Tc17 cells can acquire cytotoxic potential in vivo that is at least partially dependent upon FasL.

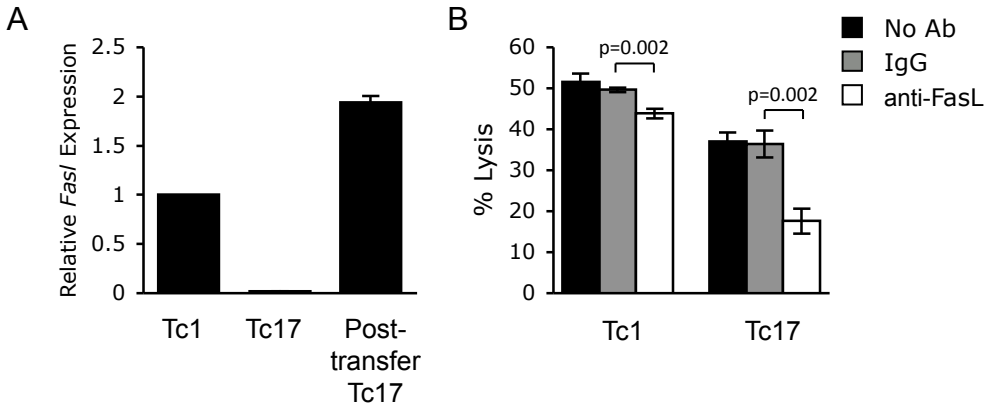

Figure 11. Cytotoxic potential of adoptively transferred Tc17 cells in vivo is partially dependent on Fas ligand. *A-B*, BoyJ (CD45.1$^+$) mice were infected with 2x10^7 pfu VV-SIINFEKL i.p. and injected i.v. with differentiated OT-I Tc17 and Tc1 cells (1x10^6, CD45.2$^+$) 24 h later. Six days later, injected cells were isolated using anti-CD45.2 antibodies and magnetic selection. (*A*) Expression of *Fasl* mRNA was determined in post-transfer Tc17 cells and compared to in vitro derived Tc1 and Tc17 cells using quantitative PCR. (*B*) Isolated cells were tested for cytotoxic potential using a ^{51}Cr-release assay with the addition of control antibodies or anti-FasL. Effector cells were co-cultured with ^{51}Cr-labeled, OVA-expressing EG.7 target cells for 6 h. E:T ratios for post-transfer Tc1 were 2.5:1 and for post-transfer Tc17 were 20:1. Cytotoxicity against EL4 targets was less than 1.5%. Statistics in *B* were performed using a Student's *t* test

Part II- STAT4 is required for IL-23 responsiveness in Th17 memory cells and NKT cells

STAT4 is required for IL-23-stimulated IL-17 production from memory Th cells and NKT cells

We previously demonstrated that STAT4-deficient $CD4^+$ T cells cultured in vitro with IL-23 display reduced IL-17 production, compared to wild type cells, in response to re-stimulation with IL-23 and IL-1β, cytokines known to synergize in the induction of IL-17 (76). To define the STAT4-dependent IL-17-secreting T cell population, we assessed the development and function of sorted populations from wild type and *Stat4*$^{-/-}$ spleen. We first determined if STAT4 was important for the development of IL-17-secreting lymphocytes by assessing cell numbers in spleen. γδ T cell and NKT cell numbers were similar between the two groups, however STAT4-deficient mice displayed significantly increased splenic memory $CD4^+$ T cell numbers with a trend toward reduced splenic memory $CCR6^+CD4^+$ T cell numbers in comparison to wild type mice (Figure 12A and B). We found the frequency of proliferative and apoptotic in vivo splenic memory $CD4^+$ T cells and memory $CCR6^+CD4^+$ T cells between wild type and STAT4-deficient mice to be similar. Furthermore, administration of the cytokines, IL-12 or IL-23, to wild type mice did not affect the proliferation of either population of cells (Figure 13A and B, data not shown).

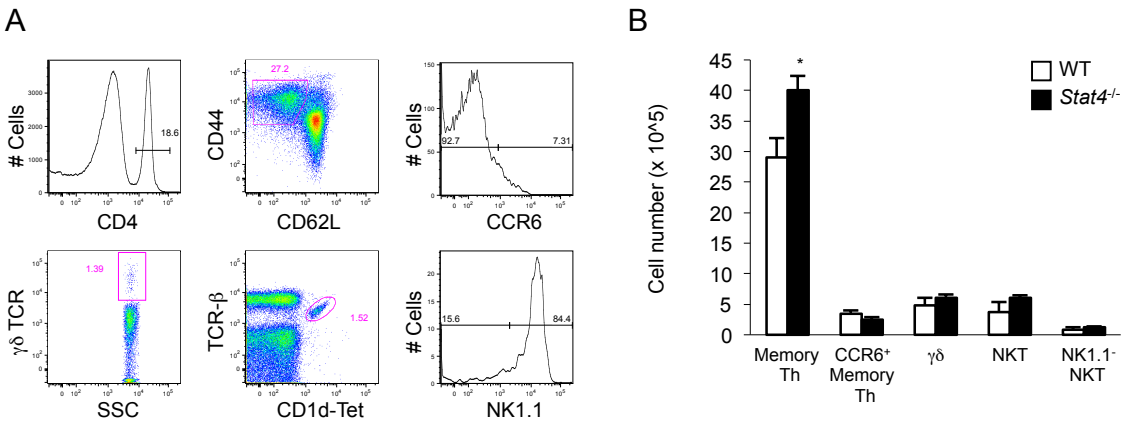

Figure 12. Development of IL-17-secreting T cells in the absence of STAT4. *A*, Representative flow cytometry dot plots demonstrating the gating strategy used to identify memory Th cell, γδ T cell, and NKT cell populations displayed in *B*. Splenocytes from WT and *Stat4*$^{-/-}$ mice were stained with antibodies and cell populations were identified as follows: memory Th cells (CD4^{+}CD62L^{-}CD44^{hi}), CCR6^{+} memory Th cells (CCR6^{+}CD4^{+}CD62L^{-}CD44^{hi}), γδ T cells (γδ TCR^{+}), NKT cells (TCR-β^{+}CD1d-Tetramer^{+}) and NK1.1^{-} NKT cells (TCR-β^{+}CD1d-Tetramer^{+}NK1.1^{-}). *B*, The cell number of the indicated lymphocyte populations was determined in the spleens of WT and *Stat4*$^{-/-}$ mice. The data are the mean ± SEM of 3-4 mice. Statistical analysis was performed using the Student's *t* test. *$p < 0.05$, compared with WT samples.

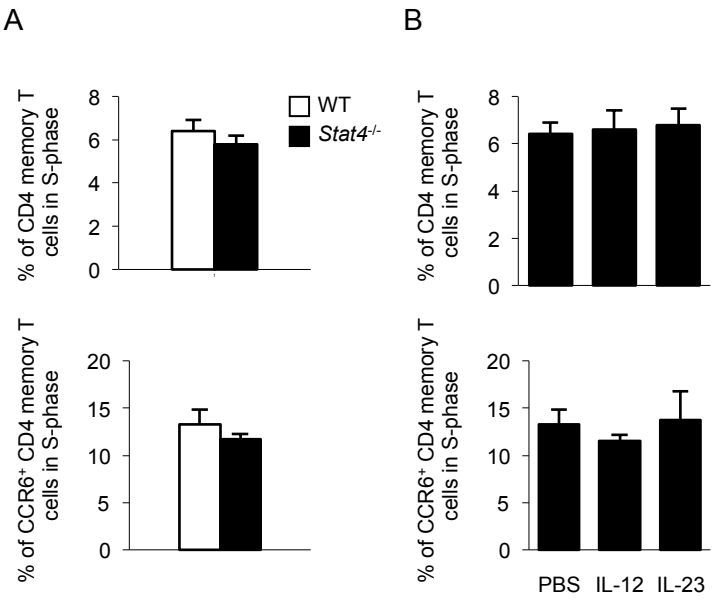

Figure 13. In vivo proliferation of memory Th cells in the absence of STAT4. *A*, WT and *Stat4*$^{-/-}$ mice were injected i.p. with 1 mg BrdU and sacrificed 24 h later. Cell proliferation was determined in splenic total memory Th cells or CCR6^{+} memory Th cells by flow cytometry. *B*, WT mice were injected i.p. with 1 mg BrdU with or without IL-12 (.5 µg) or IL-23 (.5 µg). After 12 h, mice received a second i.p. injection with IL-12 (.5 µg), IL-23 (.5 µg) or PBS. Mice were sacrificed 12 h after the final injection. Cell proliferation was determined in splenic total memory Th cells or CCR6^{+} memory Th cells by flow cytometry. The data are the mean ± SD of 3 mice (*A*) or 2-3 mice (*B*).

To define the requirement for STAT4 in IL-17 production from memory Th cells, total memory CD4^{+} T cells were sorted from the spleens of wild type and STAT4-deficient mice and stimulated ex vivo for 48 hours in the presence of anti-CD3 or IL-23 and IL-1β before IL-17 production was analyzed. STAT4-deficient memory Th cells displayed significantly less anti-CD3 and cytokine-induced IL-17 production in comparison to control cells (Figure 14A). As we observed a trend toward lower memory CCR6^{+} Th cell numbers in the spleens of STAT4-deficient

mice than in wild type mice ($2.5x10^5 \pm 0.3$ memory $CCR6^+$ Th cells in the spleen of STAT4-deficient mice versus $3.5x10^5 \pm 0.4$ from WT mice), we hypothesized that the reduced IL-17 production from total STAT4-deficient memory Th cells might simply be due to reduced memory $CCR6^+$ Th cells within the total Th memory cell pool. However, when sorted $CCR6^+$ memory Th cells were stimulated with IL-23 and IL-1β, we still observed reduced IL-17 production in the absence of STAT4, similar to what was seen with total memory Th cells (Figure 14B). This result suggests that the lower levels of cytokine-induced IL-17 production from STAT4-deficient memory Th cells is likely due to a combination of decreased numbers of $CCR6^+$ cells within the stimulated population and an intrinsic defect in the ability of STAT4-deficient $CD4^+$ $CCR6^+$ T cells to respond to IL-23. Taken together, these data imply that STAT4 contributes to IL-23-stimulated IL-17 production from memory Th cells.

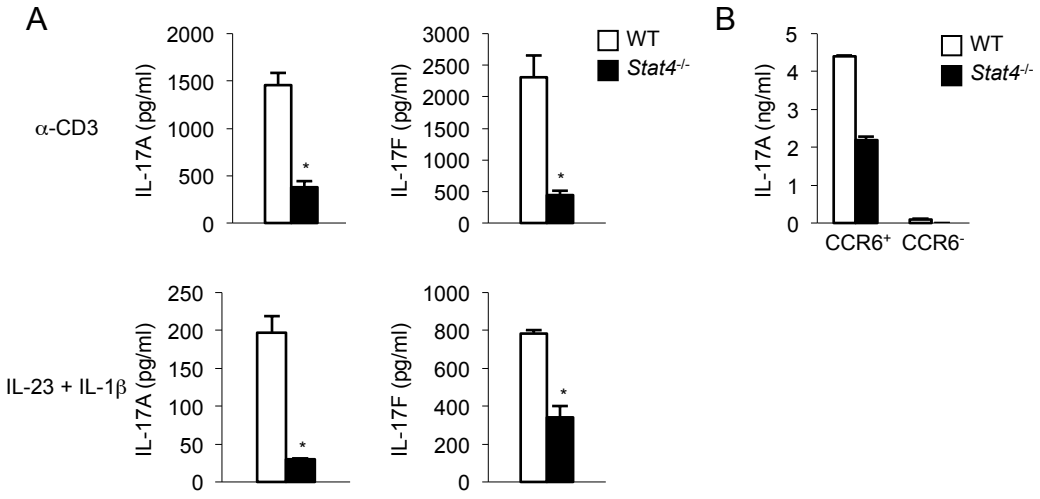

Figure 14. Reduced IL-17 production from memory Th cells in the absence of STAT4. *A*, Total memory Th cells were sorted by flow cytometry from the spleens of WT and *Stat4*$^{-/-}$ mice, stimulated with anti-CD3 or IL-23 and IL-1β for 48 h, cell-free supernatants were collected, and cytokine levels were determined via ELISA. *B,* Memory Th cells were sorted by flow cytometry from the spleens of WT and *Stat4*$^{-/-}$ mice based on CCR6 expression, simulated with IL-23 and IL-1β for 48 h and IL-17A production was determined by ELISA as in *A*. The data are the mean ± SEM of 3-4 mice (*A)* or are representative experiments (*B*). Statistical analysis was performed using the Student's *t* test. **p* < 0.05, compared with WT samples.

To determine if STAT4 is required for IL-23-dependent IL-17 production from innate IL-17-producing lymphocytes, we sorted γδ T cells, LTi-like cells ($CD4^+$ $CD3^-CD11c^-B220^-$), NK cells ($CD3^-NK1.1^+$) and NKT cells ($TCR\beta^+$CD1d-tetramer$^+$) from the spleens of wild type and STAT4-deficient mice and stimulated them ex vivo. Similar levels of IL-17 were observed between wild type and STAT4-deficient γδ T cells, LTi-like cells and NK cells in response to stimulation with IL-23 and IL-1β (Figure 15A). As $NK1.1^-$ NKT cells have been shown to be the dominant IL-17-producing NKT cell population (172, 173), we further sorted NKT cells based on NK1.1 expression. Upon stimulation with IL-23 and IL-1β, STAT4-deficient NKT cells secreted lower levels of IL-17 in comparison to control cells (Figure 15B). Thus, STAT4 is required for optimal IL-23-induced IL-17 production from NKT cells.

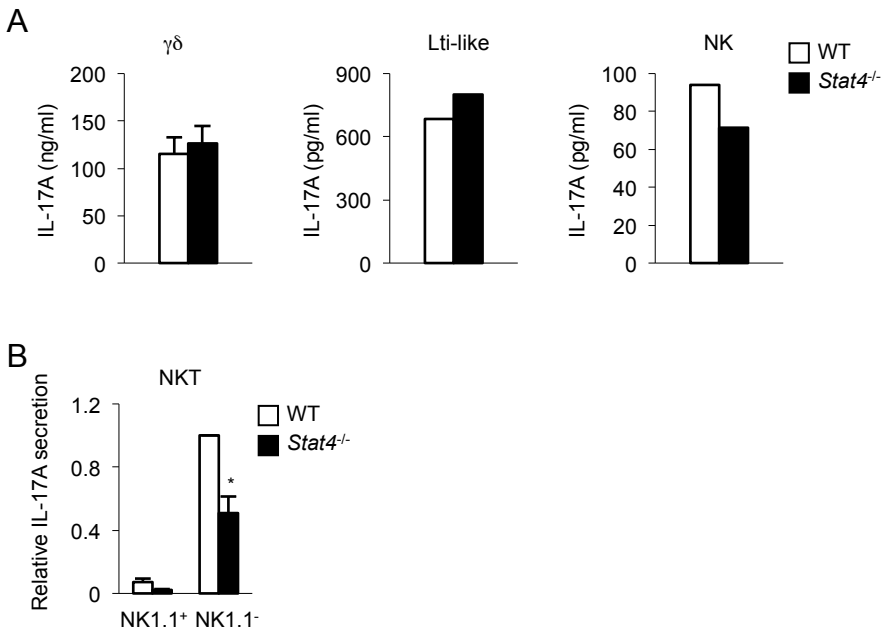

Figure 15. Reduced IL-23-induced IL-17 production from NKT cells in the absence of STAT4. *A*, γδ T cells (γδ TCR$^+$), LTi-like cells (CD4$^+$CD3$^-$CD11c$^-$ B220$^-$) and NK cells (CD3$^-$NK1.1$^+$) were sorted from the spleens of WT and *Stat4*$^{-/-}$ mice and stimulated with IL-23 and IL-1β for 48 h, cell-free supernatants were collected, and cytokine levels were determined via ELISA. *B*, NKT cells were sorted based on NK1.1 expression from the spleens of WT and *Stat4*$^{-/-}$ mice and stimulated and analyzed as in *A*. The data are the mean ± SEM of 3-4 mice (*A,* left graph*),* mean of cells pooled from 3-4 mice (*A*, middle and right graph) or mean ± SEM of 3 experiments with IL-17A secretion being normalized to WT NK1.1$^-$ levels. Statistical analysis was performed using the Student's *t* test. *$p <$ 0.05, compared with WT samples.

IL-23 does not activate STAT4 in memory Th cells or NKT cells

To begin to define the mechanism for STAT4 function in IL-17-producing cells, we tested whether IL-23 activated STAT4 in these cell populations. IL-23 binds to a heterodimeric receptor composed of the IL-12Rβ1 and IL-23R subunits. We next determined if IL-23 could induce STAT4 activation in wild type cell

populations that demonstrated STAT4-dependent IL-17 production. We have routinely observed that less than five percent of total memory Th cells from the spleens of naïve wild type mice produce IL-17 (data not shown). To increase the memory Th cell pool, we sensitized wild type mice with OVA and the adjuvant, alum. Seven weeks after the second OVA and alum sensitization, mice were sacrificed and $CD4^+$ T cells were isolated from the spleens of these mice, stimulated with cytokines and pSTAT activation was analyzed in memory Th cells. As expected, IL-23-induced STAT3 activation in IL-$17A^+$ memory Th cells (Figure 16A). However, while we observed IL-12-induced STAT4 activation in the IL-$17A^-$ memory Th population, IL-23 did not activate STAT4 in IL-$17A^+$ memory Th cells (Figure 16A). Furthermore, NKT cells sorted from naïve wild type mice displayed IL-12-induced STAT4 activation, whereas IL-23 did not induce STAT4 activation in this cell population (Figure 16B). Taken together, these data suggest that although we observe IL-23-stimulated and STAT4-dependent IL-17 production from memory Th cells and NKT cells, IL-23 does not induce STAT4 activation in these cell populations.

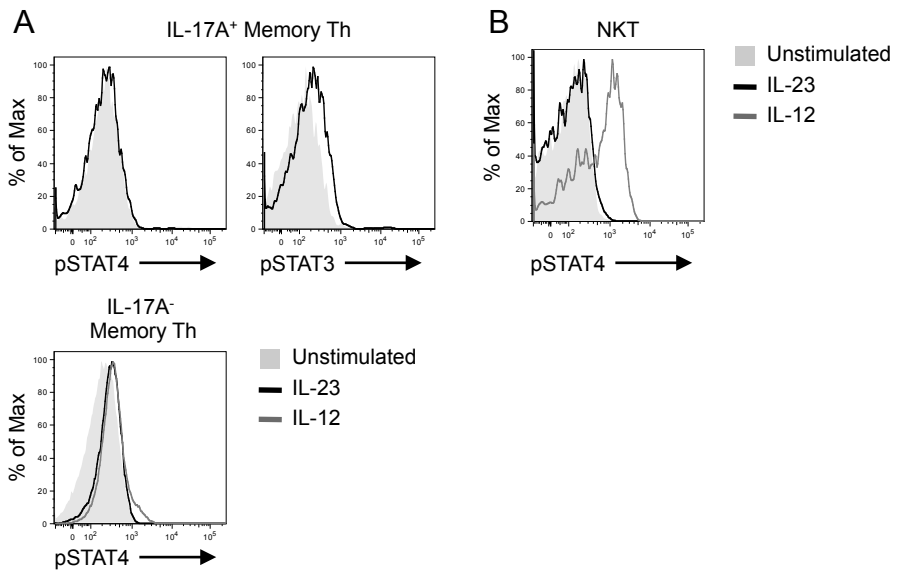

Figure 16. IL-23-induced STAT4 activation in memory Th cells and NKT cells. *A*, WT mice were sensitized i.p. with OVA and alum on days 0 and 7 and were sacrificed 7 weeks after the last sensitization. $CD4^+$ T cells were sorted via MACS bead selection from the spleens of allergen-exposed mice and were stimulated with PMA and ionomycin for 5 h. GolgiPlug was added after 2 h of stimulation with or without the addition of IL-23 (50 ng/ml) or IL-12 (5 ng/ml). IL-17A-secreting cells were labeled and cells were fixed and permeabilized. Cells were stained for CD4, CD44 and pSTAT3 or pSTAT4. pSTAT expression was detected in the $IL\text{-}17A^+$ or $IL\text{-}17A^-$ memory Th cell population ($CD4^+CD44^+$). *B*, Total NKT cells ($TCR\text{-}\beta^+CD1d\text{-}Tetramer^+$) were sorted by flow cytometry from the spleens of naïve WT mice, left unstimulated or stimulated with IL-23 (50 ng/ml) or IL-12 (5 ng/ml) for 3 h, and stained for pSTAT4. The data are representative of 3-5 mice (*A*) or are representative experiments (*B*).

STAT4 is necessary for optimal IL-23R expression in memory Th cells and NKT cells

Since STAT4 is not detectably activated by IL-23 in memory Th cells or NKT cells, it is possible that STAT4-deficiency results in diminished cellular responses

to IL-23. To examine this possibility, we analyzed the expression of IL-23R and IL-12Rβ1 in sorted memory Th cells an NKT cells isolated from the spleens of wild type and STAT4-deficient mice. Whereas memory Th cells from wild type and STAT4-deficient mice displayed similar levels of *Il12rb1* expression, significantly reduced *Il23r* expression was observed in memory Th cells from STAT4-deficient mice (Figure 17A). Memory Th cells from STAT4-deficient mice also expressed lower levels of *Rorc* and *Ccr6* in comparison to control memory Th cells, the latter coinciding with a trend toward lower numbers of $CCR6^+$ memory Th cells in mice deficient in STAT4 (Figure 17A and 12B). As STAT4-deficient memory Th cells displayed reduced expression of Th17-associated genes, we next wanted to determine if these cells had adopted the phenotype of other Th subsets in the absence of STAT4. We observed similar expression of the Th2-associated genes, *Gata3* and *Ccr8*, along with the Th1-associated genes, *Tbx21* and *Cxcr3*, in wild type and STAT4-deficient memory Th cells, suggesting that in the absence of STAT4, memory Th cells do not adopt a Th1 or Th2 phenotype in naïve mice (Figure 17A). We then performed a similar analysis with NKT cells. Whereas wild type and STAT4-deficient NKT cells expressed similar levels of *Rorc*, *Tbx21* and *Il12rb1*, STAT4-deficient NKT cells displayed significantly reduced levels of *Il23r* in comparison to control cells (Figure 17B). Together, these data illustrate that in the absence of STAT4, memory Th cells and NKT cells have reduced expression of IL-23R, rendering them less responsive to IL-23-dependent stimulation.

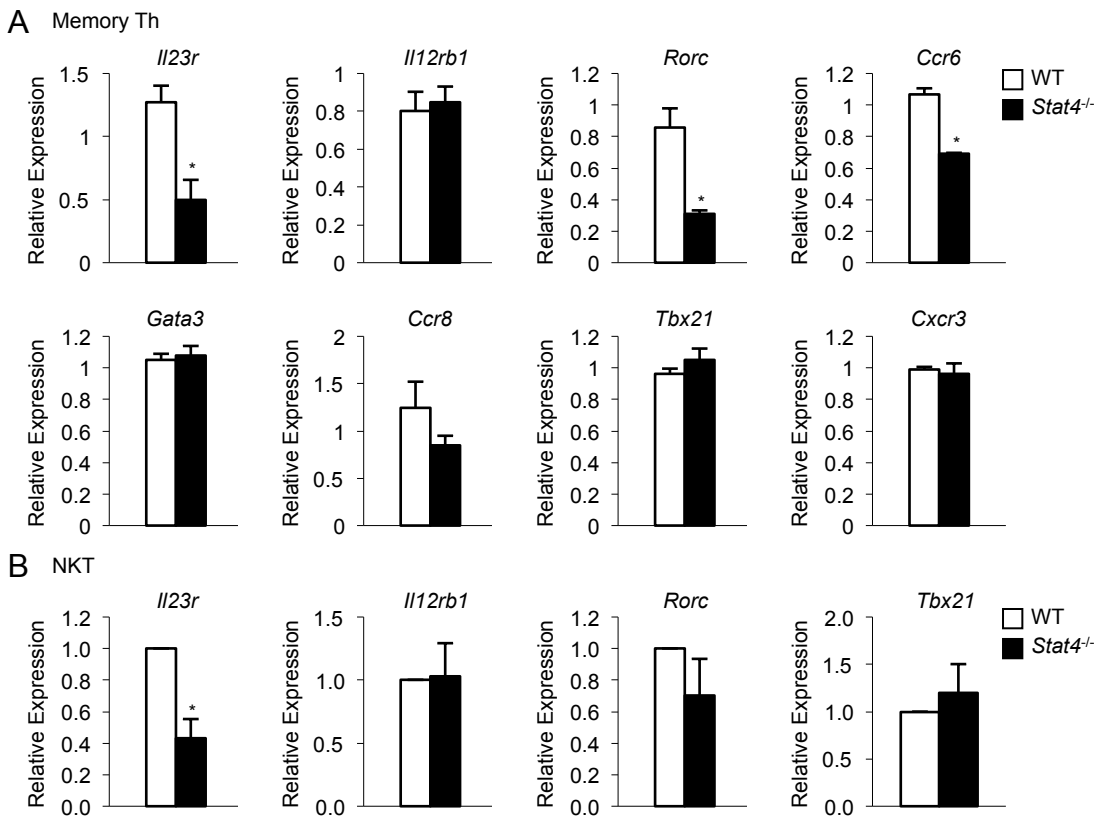

Figure 17. Reduced expression of Th17-associated genes in memory Th cells and NKT cells in the absence of STAT4. *A-B,* Memory Th cells ($CD4^+CD62L^-CD44^{hi}$) (*A*) or NKT cells (TCR-β^+CD1d-Tetramer$^+$) (*B*) were sorted from the spleens of WT and *Stat4*$^{-/-}$ mice. RNA was isolated from unstimulated cells. Expression of the indicated genes was measured using quantitative PCR; samples were normalized to the expression of β_2-microglobulin mRNA and are relative to levels in WT cells. The data are the mean ± SEM of 3 mice (*A*), or the mean ± SEM of 3 independent experiments (*B*). Statistical analysis was performed using the Student's *t* test. *$p < 0.05$, compared with WT samples.

As we did not observe IL-23-induced STAT4 activation in wild type memory Th cells or NKT cells, our data suggests that the reduced IL-23R expression and IL-23-stimulated IL-17 production observed in memory Th cells and NKT cells from STAT4-deficient mice is not due to a loss of direct IL-23-STAT4 signaling in these cells. We hypothesized that IL-12-STAT4 signaling could be necessary for

optimal IL-23R expression and IL-23-induced IL-17 production in T cells. To test this, we performed studies with mice deficient in the p35 subunit of IL-12 (IL-12α), which is not shared with IL-23. We sorted NKT cells from the spleen of wild type and IL-12α-deficient mice and stimulated them ex vivo. NKT cells from IL-12α-deficient mice displayed reduced IL-17A production when stimulated with IL-23 and IL-1β in comparison to control cells (Figure 18A). Furthermore, NKT cells from the spleen of wild type and IL-12α-deficient mice expressed similar levels of *Il12rb1*, whereas those from IL-12α-deficient mice displayed reduced levels of *Il23r* and *Rorc* in comparison to control cells (Figure 18B). Thus, similar to what we observed in NKT cells from STAT4-deficient mice, NKT cells from IL-12α-deficient mice display reduced IL-23R expression and IL-23-induced IL-17 production.

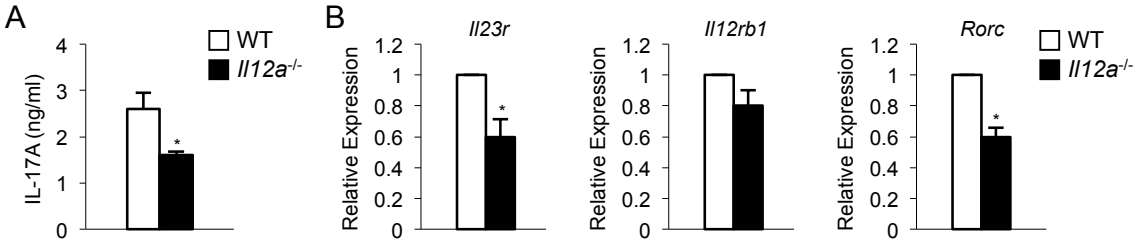

Figure 18. Gene expression and IL-23-induced IL-17 production from NKT cells in the absence of IL-12α (p35). *A*-*B*, Total NKT cells were sorted from the spleens of WT and *Il12a*$^{-/-}$ mice. (*A*) Cells were stimulated with IL-23 and IL-1β for 48 h, cell-free supernatants were collected, and cytokine levels were determined via ELISA. (*B*) RNA was isolated from unstimulated cells. Expression of the indicated genes was measured using quantitative PCR; samples were normalized to the expression of β_2-microglobulin mRNA and are relative to levels in WT cells. The data are the mean ± SEM of 3 independent experiments (*A*-*B*). Statistical analysis was performed using the Student's *t* test. *$p < 0.05$, compared with WT samples.

As the phenotype of NKT cells from STAT4-deficient mice and IL-12α-deficient mice was remarkably similar, we hypothesized that IL-12-STAT4 signaling could lead to the induction of IL-23R expression in T cells. To test this, we sorted NKT cells from the spleen of wild type mice and left them unstimulated or stimulated them with IL-23 or IL-12 for 12 hours. Whereas IL-23 stimulation resulted in the induction of *Il23r* expression, IL-12 did not induce *Il23r* expression in NKT cells, but did induce *Il18r1* (Figure 19). Taken together, these data suggest that the reduced IL-23R expression and IL-23-stimulated IL-17 production observed in memory Th cells and NKT cells from STAT4-deficient mice might not be directly due to a loss of STAT4 in these cells. However, there may be an indirect effect of STAT4-deficiency in other cell populations, which could prevent optimal IL-23R expression in memory Th cells and NKT cells from these mice, rendering them less responsive to IL-23 signals.

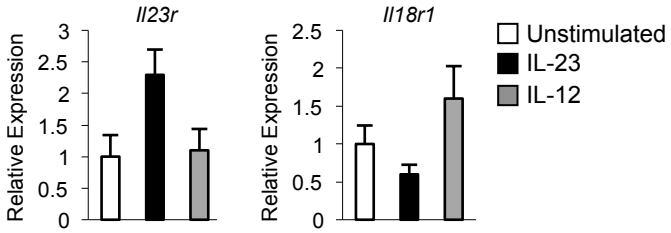

Figure 19. IL-12 does not induce IL-23R in NKT cells. NKT cells were sorted by flow cytometry from the spleens of WT mice and left unstimulated or stimulated with IL-23 (10 ng/ml) or IL-12 (5 ng/ml) for 18 h. RNA was isolated from cells and expression of the indicated genes was measured using quantitative PCR; samples were normalized to the expression of β_2-microglobulin mRNA and are relative to levels in unstimulated cells.

In vivo effects of STAT4-deficiency on memory Th and NKT cell IL-17 production

Our data suggest that reduced IL-23R expression and IL-23-stimulated IL-17 production observed in memory Th and NKT cells from STAT4-deficient mice might be due to an indirect effect of STAT4-deficiency in other cell populations. To further explore this in vivo, we used several mouse models of inflammation, that have previously been shown to induce IL-17 production from $CD4^+$ T cells or NKT cells, and analyzed the effects of STAT4-deficiency on the development of inflammation and subsequent IL-17 production in mice.

While Th2 and Th9 cells are induced in the development of allergic airway disease (AAD), Th17 cells are also induced in allergic disease. To test if Ag-induced IL-17 production is altered in allergic mice in the absence of STAT4, wild type and *Stat4*$^{-/-}$ mice were sensitized with OVA and alum followed by intranasal challenges with OVA. In comparison to control wild type mice challenged with PBS, wild type and STAT4-deficient mice challenged with OVA displayed an increase in the total bronchoalveolar lavage (BAL) cell number, with slightly elevated numbers observed in STAT4-deficient mice compared with wild-type mice (Figure 20A). Upon stimulation with OVA, splenoctyes and BAL cells from allergic wild type and STAT4-deficient mice displayed an increase in Ag-induced IL-17 production in comparison to wild type PBS control mice, and there was a trend toward lower IL-17 production from STAT4-deficient mice (Figure 20B). Although a trend toward lower Ag-induced IL-17 production was observed in allergic STAT4-deficient mice compared with wild type allergic mice, the data

suggest that STAT4 is not required for OVA induced IL-17 production from OVA and alum sensitized and challenged mice.

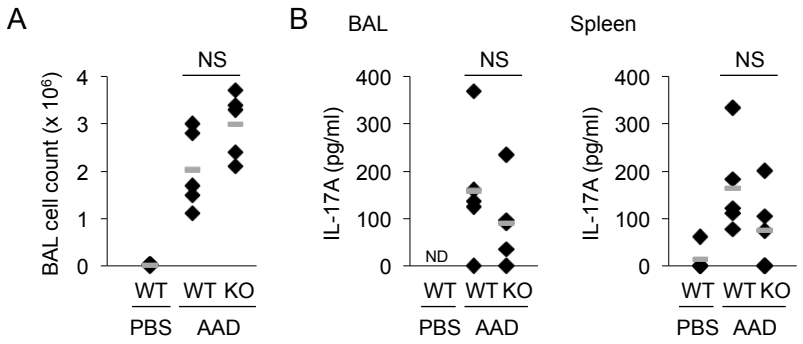

Figure 20. Ag-induced IL-17 production from allergic mice in the absence of STAT4. *A-B*, WT and *Stat4*$^{-/-}$ mice were sensitized i.p. with OVA and alum on days 0 and 7, challenged i.n. with PBS or OVA on days 14-18 and sacrificed 48 h after the final challenge. Cells were isolated from the BAL and spleen for further analysis. (*A*) Total cell number was calculated in the BAL of control (PBS) or allergic (AAD) mice. (*B*) BAL and spleen cells were stimulated with OVA for 72 h, cell-free supernatants were collected, and cytokine levels were determined via ELISA. Diamonds represent a single mouse and horizontal bars represent the mean of 3-5 mice. Statistical analysis was performed using the Student's *t* test. ND, Not Detected, NS, Not Significant.

It has previously been shown that mice treated with α-GalCer via intravenous injection display rapid production of IL-17 by NKT cells (171). To determine if STAT4 is required for optimal NKT cell IL-17 production in response to stimulation with α-GalCer, control and STAT4-deficient mice were administered α-GalCer intravenously and sacrificed 6 hours later to analyze cytokine production in the spleen and serum. In comparison to splenocytes from PBS-

treated wild type mice, splenocytes from α-GalCer-treated wild type mice displayed increased *Il17a, Il4 and Il9* expression, with expression levels being similar to that observed in α-GalCer treated STAT4-deficient mice (Figure 21A). *Il17f* expression levels were lower in splenocytes from both groups of mice treated with α-GalCer in comparison to PBS-treated groups (Figure 21A). *Ifng* expression was also increased in splenocytes from α-GalCer-treated wild type mice in comparison to PBS-treated mice and as expected, was diminished in STAT4-deficient α-GalCer treated mice (Figure 21A). IL-17A levels in the serum were similar between PBS and α-GalCer-treated mice and were not dependent on STAT4 (Figure 21B). Together, these data suggest that α-GalCer-stimulated NKT cell IL-17 production is not dependent on STAT4 in vivo.

As our in vitro data suggests that STAT4 is important for IL-23-stimulated IL-17 production from NKT cells, and we did not observe any difference in α-GalCer-induced NKT cell IL-17 production, we next assessed α-GalCer-induced IL-17 production from NKT cells in the context of exogenous IL-23. Control and STAT4-deficient mice were injected intravenously with PBS or α-GalCer and IL-23 and sacrificed 6 hours later. *Il17a* and *Il17f* expression levels were increased to similar levels in the spleen of wild type mice and STAT4-deficient mice treated with α-GalCer and IL-23 in comparison to PBS-treated mice (Figure 21C), suggesting that α-GalCer and IL-23-induced IL-17 production from NKT cells is not dependent on STAT4 in vivo (Figure 21C).

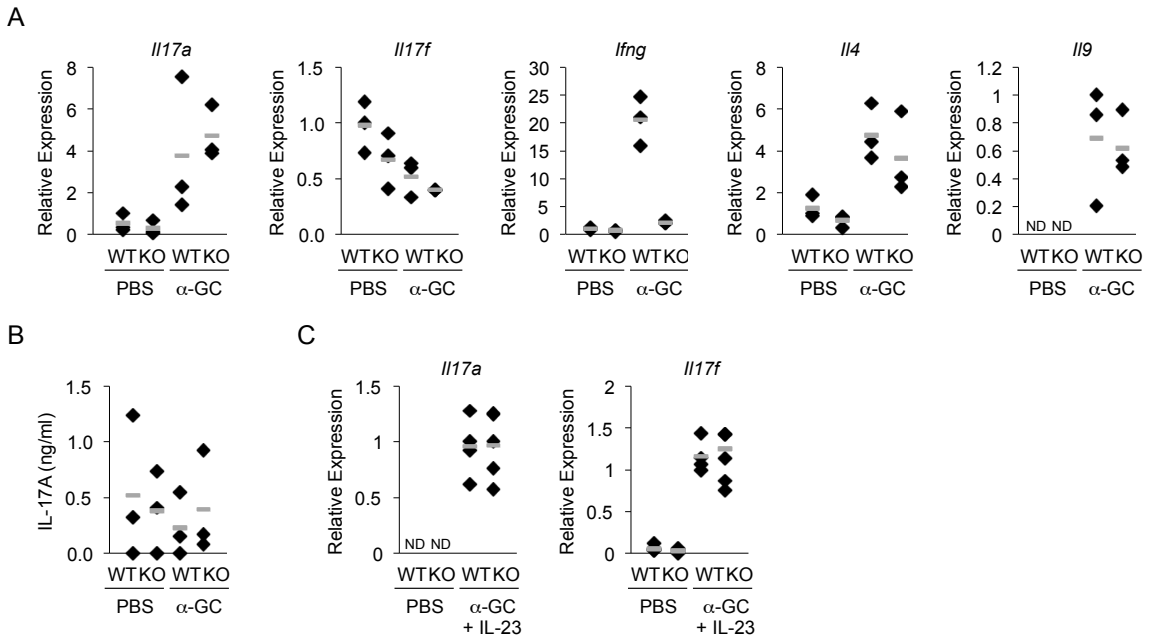

Figure 21. Analysis of α-GalCer-induced NKT cell cytokine expression in the absence of STAT4. *A-B*, WT and *Stat4*$^{-/-}$ mice were injected i.v. with PBS or 1 μg α-GalCer (α-GC), sacrificed 6 hr later, spleens and blood were collected and serum was isolated from the blood. (*A*) RNA was isolated from unstimulated spleen cells. Expression of the indicated genes was measured using quantitative PCR; samples were normalized to the expression of β_2-microglobulin mRNA and are relative to levels from WT PBS-treated mice or WT OVA-treated mice (*Il9*). (*B*) IL-17A levels were determined in the serum by ELISA. *C*, WT and *Stat4*$^{-/-}$ mice were injected i.v. with PBS or 1 μg α-GalCer and 1 μg IL-23, sacrificed 6 h later, and spleens were collected. RNA isolation and quantitative PCR was performed as in *A*; samples are relative to levels from WT α-GC + IL-23-treated mice. Diamonds represent a single mouse and horizontal bars represent the mean of 3-4 mice. ND, Not Detected.

Mice administered α-GalCer intranasally have been shown to display an NKT-cell dependent increase in inflammation and IL-17A production in the lung (172, 176). To further see if this phenomenon is dependent on STAT4, PBS, α-GalCer, or α-GalCer and IL-23 were administered intranasally to wild type and STAT4-

deficient mice, and mice were sacrificed 24 hours later. In comparison to control wild type mice treated with PBS, wild type mice treated with α-GalCer displayed an increase in the total BAL cell number, which was slightly higher than what was observed in STAT4-deficient mice treated under the same conditions (Figure 22A). Wild type and STAT4-deficient mice treated with α-GalCer and IL-23 displayed an increase in total BAL cell number in comparison to control wild type PBS-treated mice, however the cell number was somewhat lower than what was observed in those treated with α-GalCer alone (Figure 22A). We next analyzed IL-17A production in the BAL fluid of each group of mice and found that in comparison to control PBS-treated wild type mice, wild type mice treated with α-GalCer or α-GalCer and IL-23 displayed a modest increase in IL-17 production in the BAL fluid with levels being similar to those from STAT4-deficient mice treated with α-GalCer or α-GalCer and IL-23 (Figure 22B). We further analyzed cytokine gene expression in total lung or mononuclear cells isolated from the lung from the different groups of mice. In comparison to control PBS-treated wild type mice, *Ifng* expression was increased in the lung of wild type mice treated with α-GalCer and to a lesser extent in those treated with α-GalCer and IL-23 and this effect was largely dependent on STAT4 (Figure 22C). *Il17a* expression levels were highest in the lung of mice treated with α-GalCer and IL-23 in comparison to those treated with PBS or α-GalCer alone, however expression was independent of STAT4 (Figure 22C). No difference in *Il17a* expression was observed in lung mononuclear cells from wild type and STAT4-deficient mice treated under the three different conditions (Figure 22D). Together, these data suggest that in vivo

α-GalCer and α-GalCer and IL-23-stimulated IL-17 production from NKT cells in the lung is independent of STAT4.

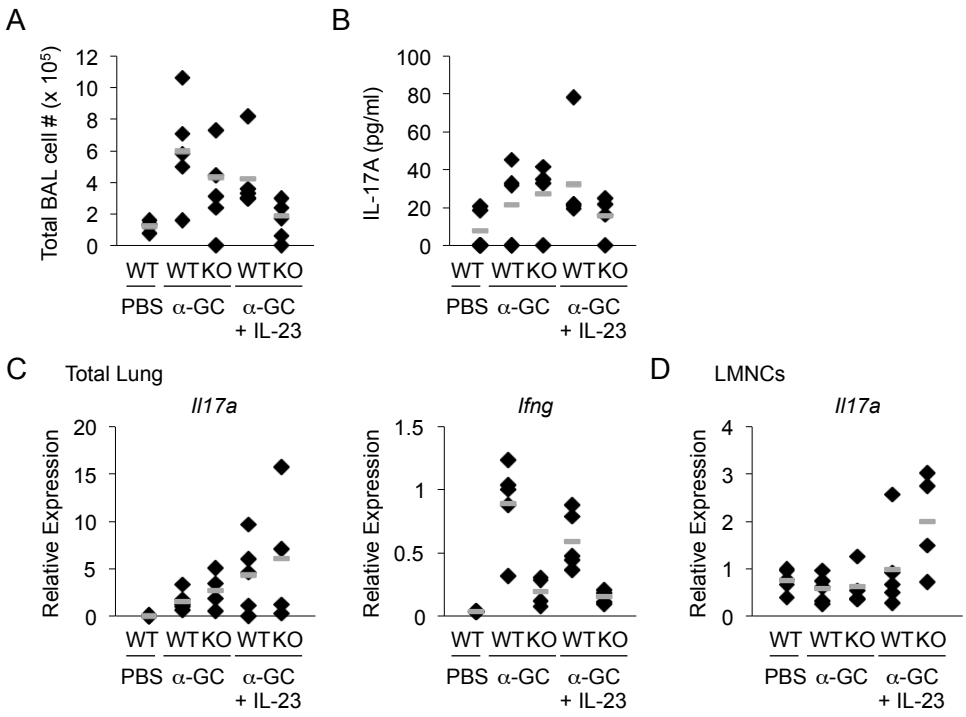

Figure 22. α-GalCer-induced NKT cell IL-17 production in the lung in the absence of STAT4. *A-D*, WT and *Stat4*$^{-/-}$ mice received PBS, 1 μg α-GalCer (α-GC) or 1 μg α-GalCer and 1 μg IL-23 (α-GC + IL-23) via i.n. administration and were sacrificed 24 h later. (*A*) Total cell number was calculated in the BAL. (*B*) IL-17A levels were determined in the BAL fluid by ELISA. (*C*) RNA was isolated from the lung. Expression of the indicated genes was measured using quantitative PCR; samples were normalized to the expression of β_2-microglobulin mRNA and are relative to levels from WT α-GC-treated mice. (*D*) RNA isolation and quantitative PCR was performed on lung mononuclear cells (LMNCs) as in *C*; samples are relative to levels from WT PBS-treated mice. Diamonds represent a single mouse and horizontal bars represent the mean of 4-5 mice.

Another study demonstrated that mice that had received intranasal does of α-GalCer in the presence of Poly(I:C), showed increased IL-17A expression in the lung in comparison to mice that received α-GalCer alone. The authors further showed these effects to be dependent on IL-23 (177). As our data indicate a role for STAT4 in IL-23-stimulated IL-17 production from NKT cells, we wanted to determine if STAT4 was important for optimal NKT cell IL-17 production in response to stimulation with α-GalCer and Poly(I:C). Wild type and STAT4-deficient mice were administered PBS or α-GalCer and Poly(I:C) intranasally and sacrificed 48 hours later. In comparison to their control PBS-treated counterparts, wild type and STAT4-deficient mice treated with α-GalCer and Poly(I:C) displayed a similar increase in the number of cells in the BAL (Figure 23A). Furthermore, *Il17a* expression levels in the lung were similarly increased in wild type and STAT4-deficient mice treated with α-GalCer and Poly(I:C) in comparison to their control PBS-treated counterparts (Figure 23B). To test if there were differences in *Il17a* expression levels in NKT cells, we sorted NKT cells from the BAL and analyzed *Il17a* expression from α-GalCer and Poly(I:C) wild type and STAT4-deficient mice and found similar expression levels between the two groups (Figure 23C). Together, these data illustrate that α-GalCer and Poly(I:C)-stimulated NKT cell IL-17 production occurs independently of STAT4-mediated signals.

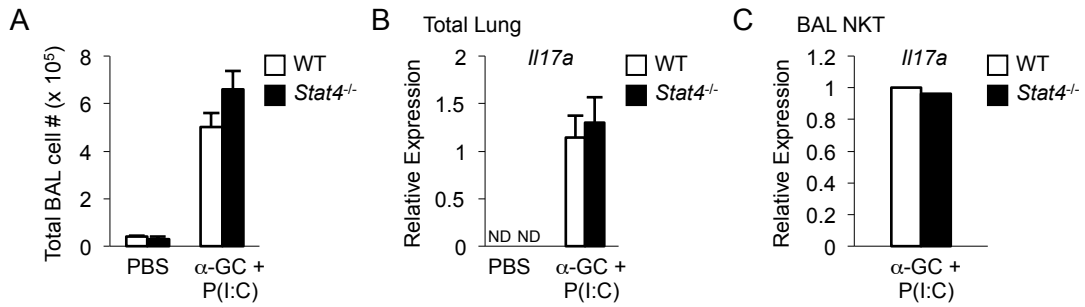

Figure 23. NKT cell IL-17 production in the lung in response to α-GalCer and Poly(I:C) stimulation in the absence of STAT4. *A-C*, WT and *Stat4*$^{-/-}$ mice received PBS or 2 μg α-GalCer and 125 μg Poly(I:C) (α-GC + P(I:C)) via i.n. administration and were sacrificed 48 h later. (*A*) Total cell number was calculated in the BAL. (*B*) RNA was isolated from the lung. Expression of the indicated genes was measured using quantitative PCR; samples were normalized to the expression of β_2-microglobulin mRNA and are relative to levels from WT α-GC + P(I:C)-treated mice. (*C*) NKT cells were sorted from the BAL of α-GC + P(I:C)-treated WT and *Stat4*$^{-/-}$ mice, RNA was isolated and quantitative PCR was performed as in *B*; samples are relative to levels from WT α-GC + P(I:C)-treated mice. The data are the mean ± SEM of 3-5 mice (*A, B*) or the value from cells pooled from 3-5 mice (*C*). ND, Not Detected.

Part III-Th17 cells demonstrate stable cytokine production during allergic inflammation

Generation and characterization of Il17f ^{Cost} mice

In recent years, a number of IL-17 reporter and lineage tracer mouse strains have been generated, serving as a tool to identify cells that currently produce IL-17 (reporter mouse) or which have produced IL-17 (lineage tracer mouse). Our goal was to generate a mouse strain that combined both approaches, to simultaneously identify cells that currently produce IL-17F or that previously produced IL-17F, but have since turned off the *Il17f* gene. *Il17f* is located in close proximity to *Il17a* on mouse chromosome 1 (Figure 24A). An EGFP-Cre recombinant fusion protein and an *frt*-flanked neomycin resistance cassette were inserted into exon 1 of *Il17f*, termed Cost (Cre on seventeen transcript) (Figure 24B). *Il17f* ^{Cost} mice express a fusion of EGFP and Cre recombinase when the *Il17f* locus is turned on.

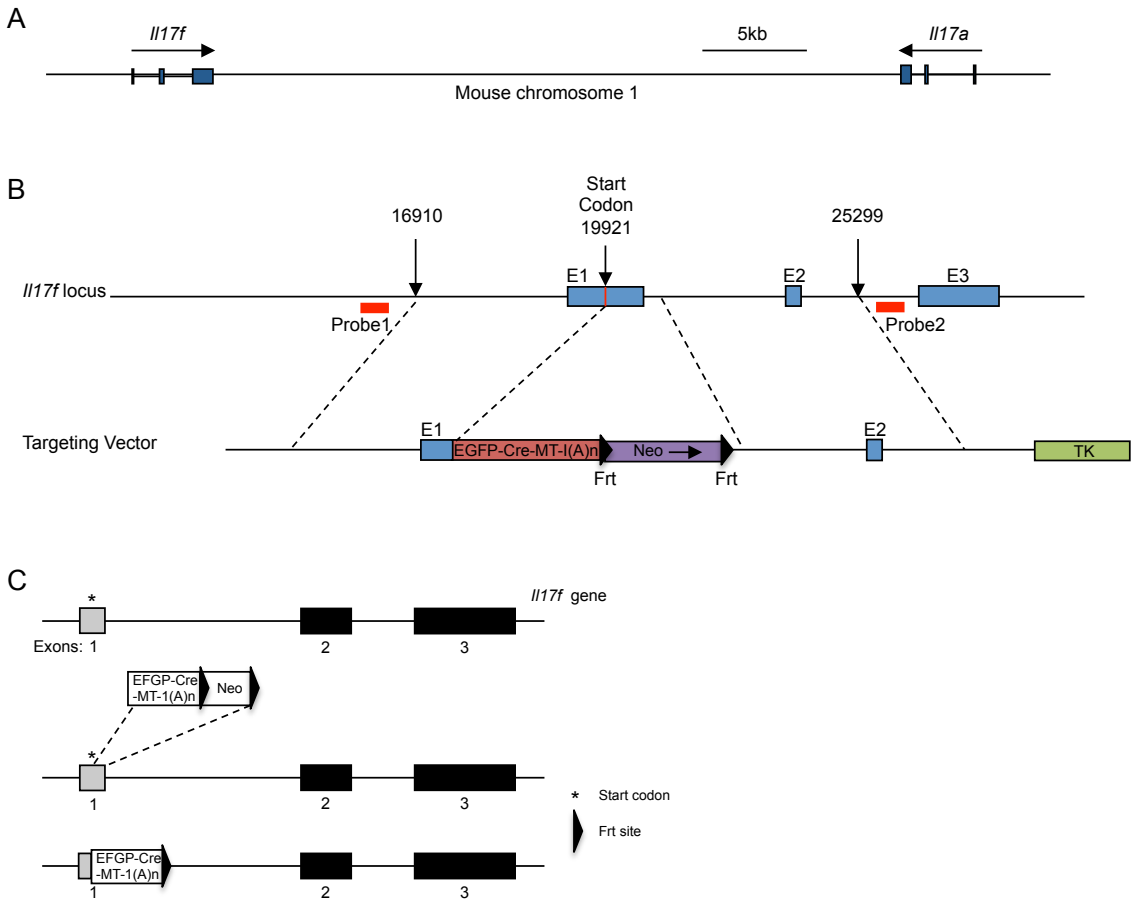

Figure 24. **Generation of *Il17f* ^{Cost} mice.** *A*, Schematic of the *Il17a and Il17f* loci on mouse chromosome 1. *B-C*, For the generation of *Il17f* ^{Cost} mice, a vector spanning the *Il17f* locus was generated to replace the 3' end of exon 1 with an EGFP-Cre fusion protein and an *frt*-flanked neomycin resistance cassette. (*B*) Detailed schematic of the targeted *Il17f* locus. DNA probes used for Southern blot analysis are show in red. (*C*) Simplified schematic of *Il17f* gene and targeted *Il17f* locus after FLP-mediated excision of the *frt*-flanked neomycin resistance cassette.

Southern blot and PCR analysis of genomic DNA from littermate control and *Il17f* ^{Cost} mice was performed to identify mice with the *Cost* allele (Figure 25A and B). To excise the *frt*-flanked neomycin-resistance cassette, *Il17f* ^{Cost} mice were crossed to FLPeR mice, which have a variant of the *Saccharomyces cerevisiae* *FLP1* recombinase (Figure 25C).

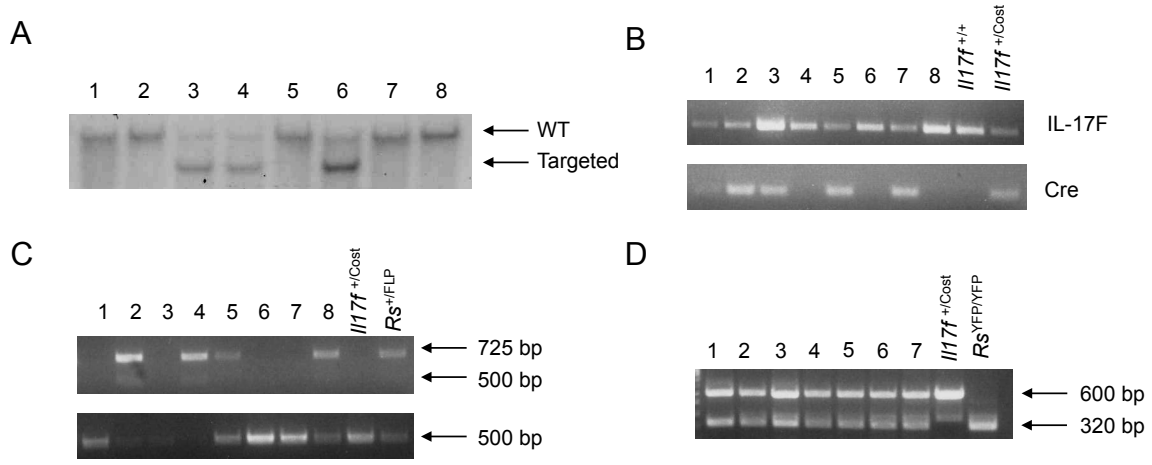

Figure 25. Southern blot and PCR screening of *Il17f*^{Cost} mice. *A*, Southern blot of genomic DNA isolated from the tails of *Il17f* $^{+/+}$ and *Il17f* $^{+/Cost}$ mice. The blot was probed with the 5' probe shown in Figure 24B and WT and targeted alleles are indicated. *B*, PCR on tail DNA from *Il17f* $^{+/+}$ and *Il17f* $^{+/Cost}$ mice using primers designed to amplify *Cre* or *Il17f*. *C*, PCR on tail DNA from *Il17f* $^{+/Cost}$ mice crossed to *Rs*$^{+/FLP}$ mice, using primers designed by the distributor with the following expected results: *Rs*$^{+/+}$ = 500 bp, *Rs*$^{+/FLP}$ = 500 and 725 bp, *Rs*$^{FLP/FLP}$ = 725 bp (not shown). *D*, PCR on tail DNA from *Il17f* $^{+/Cost}$ mice crossed to *Rs*$^{YFP/YFP}$ mice using primers designed by the distributor with the following expected results: *Rs*$^{+/+}$ = 600 bp, *Rs*$^{+/YFP}$ = 320 and 600 bp, *Rs*$^{YFP/YFP}$ = 320 bp.

To detect Cre activity, *Il17f* ^{Cost} mice were then crossed with a reporter mouse strain that permanently expresses YFP following expression of Cre (referred to as *Rs*^{YFP} here) (Figure 25D and 26A). Ideally, cells from *Il17f* ^{Cost}*Rs*^{YFP} mice would express EGFP and Cre when *Il17f* is induced, and retain Cre-induced YFP expression if *Il17f* expression were discontinued, allowing for the fate of former IL-17F-secreting cells to be monitored (Figure 26B).

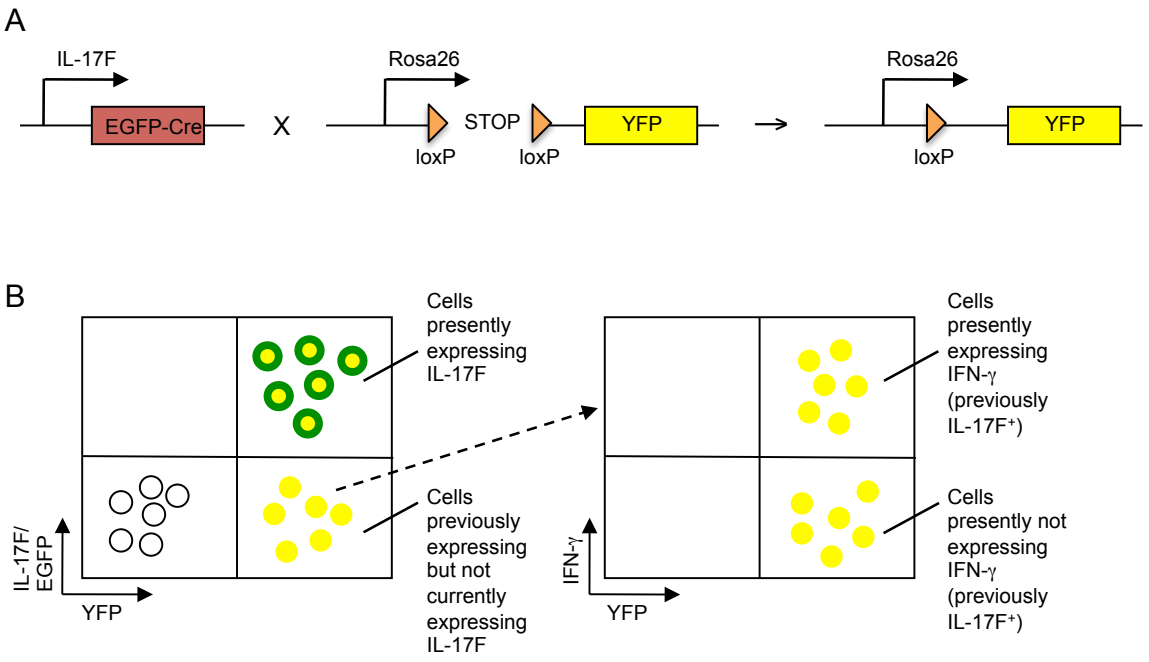

Figure 26. Predicted flow cytometry analysis using *Il17f*^{Cost}*Rs*^{YFP} mice. *A*, Schematic demonstrating YFP expression when *Il17f*^{Cost} mice are crossed with a reporter mouse strain that expresses YFP upon expression of Cre (*Rs*^{YFP}). *Il17f*^{Cost}*Rs*^{YFP} mice should express Cre when *Il17f* is turned on. In Cre-expressing cells, the *loxP*-flanked STOP codon upstream of YFP will be excised and YFP will be continuously expressed from the ubiquitous *Rosa26* promoter. *B*, Schematic of predicted flow cytometry analysis using cells from *Il17f*^{Cost}*Rs*^{YFP} mice. Cells from *Il17f*^{Cost}*Rs*^{YFP} mice should express EGFP and Cre when *Il17f* is turned on, and retain Cre-induced YFP expression if *Il17f* expression were discontinued, allowing for the fate of IL-17F-secreting cells to be monitored.

Expression of EGFP and YFP was first tested from in vitro derived Th1 and Th17 cells from control and *Il17f*$^{+/Cost}$*Rs*$^{+/YFP}$ mice and we found that both EGFP and YFP could be detected in Th17 cells by immunofluorescence microscopy, but not in Th1 cells as expected (Figure 27). We then tested the expression of EGFP and YFP from in vitro derived Th1 and Th17 cells from different control and *Il17f*$^{Cost/Cost}$*Rs*$^{+/YFP}$ founder mice and we found that YFP, but not EGFP was detected

by flow cytometry (Figure 28). Thus, in subsequent experiments, *Il17f* ^{Cost}*Rs*^{YFP} mice were used as a lineage tracer mouse strain using flow cytometry analyses to define YFP^{+} cells as those that had activated the *Il17f* locus.

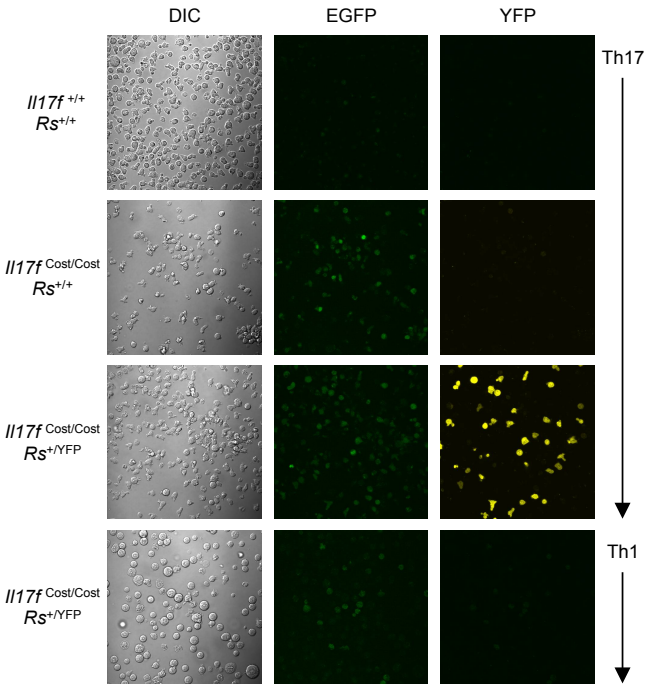

Figure 27. Detection of EGFP and YFP in Th17 cells from *Il17f* ^{Cost} mice by immunofluorescence microscopy. Naïve CD4^{+} T cells were isolated from the spleen of *Il17f* $^{+/+}$*Rs*$^{+/+}$, *Il17f* $^{Cost/Cost}$*Rs*$^{+/+}$ or *Il17f* $^{Cost/Cost}$*Rs*$^{+/YFP}$ mice and stimulated under Th1 or Th17-polarizing conditions for 5 days. Cells were re-stimulated with PMA and ionomycin for 3 h and EGFP and YFP expression were analyzed by immunofluorescence microscopy in unfixed cells. DIC, Differential interference contrast. Experiments were performed in collaboration with Heather Bruns.

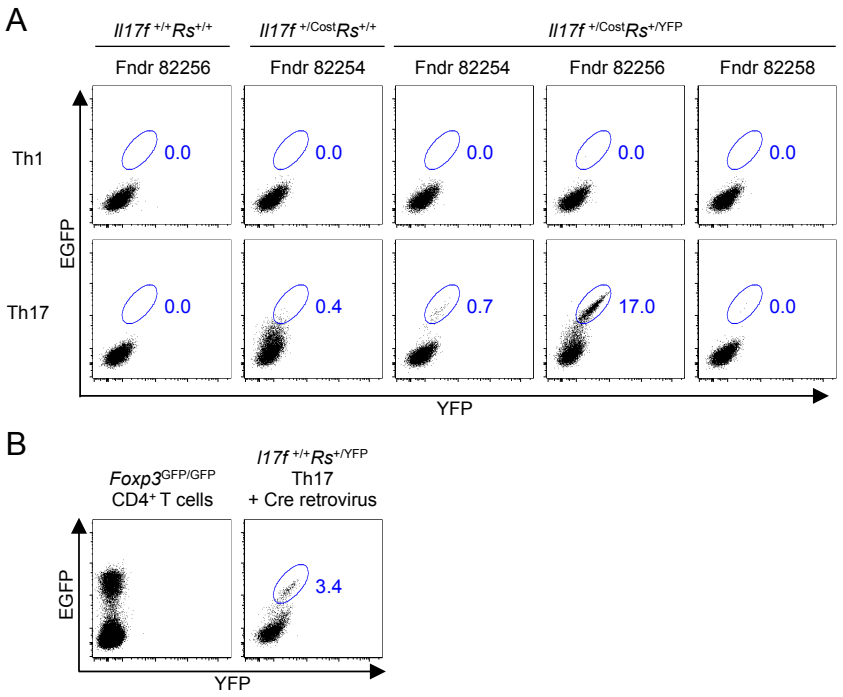

Figure 28. Detection of EGFP and YFP in Th17 cells from *Il17f* ^{Cost} founder mice by flow cytometry. *A*, Naïve CD4^{+} T cells were isolated from the spleen of *Il17f* $^{+/+}$*Rs*$^{+/+}$, *Il17f* $^{+/Cost}$*Rs*$^{+/+}$ or *Il17f* $^{+/Cost}$*Rs*$^{+/YFP}$ mice from different *Il17f* ^{Cost} founder (Fndr) mice and stimulated under Th1 or Th17-polarizing conditions for 5 days. Cells were re-stimulated with PMA and ionomycin and EGFP and YFP expression was analyzed in unfixed cells by flow cytometry. *B*, EGFP and YFP expression was analyzed in unfixed CD4^{+} T cells isolated from *Foxp3*$^{GFP/GFP}$ mice (left panel, positive EGFP control) or in Th17 cells differentiated from *Il17f* $^{+/+}$*Rs*$^{YFP/YFP}$ mice, which were transduced with a Cre-expressing retrovirus during differentiation (right panel, positive YFP control). Representative dot plots are shown.

To validate the *Il17f* ^{Cost}*Rs*^{YFP} mouse strain, naïve CD4^{+} T cells were isolated from the spleen of *Il17f* $^{+/+}$*Rs*$^{YFP/YFP}$, *Il17f* $^{+/Cost}$*Rs*$^{YFP/YFP}$ and *Il17f* $^{Cost/Cost}$*Rs*$^{YFP/YFP}$ mice and stimulated under Th17-polarizing conditions for five days. As expected, Th17 cells from *Il17f* $^{+/+}$*Rs*$^{YFP/YFP}$ did not express YFP, but did express high percentages of IL-17F and IL-17A-positive cells (Figure 29). Th17 cells from *Il17f* $^{+/Cost}$*Rs*$^{YFP/YFP}$ mice demonstrated expression of YFP and displayed an expected

reduction in IL-17F, as the cells have only one functioning *Il17f* allele (Figure 29). Th17 cells derived from *Il17f* $^{Cost/Cost}$*Rs*$^{YFP/YFP}$ mice displayed a further increase in YFP expression, but did not produce IL-17F, as they are *Il17f* null (Figure 29). Although only the *Il17f* locus was targeted in the generation of *Il17f* ^{Cost} mice, it is important to note that Th17 cells derived from *Il17f* $^{+/Cost}$*Rs*$^{YFP/YFP}$ and *Il17f* $^{Cost/Cost}$*Rs*$^{YFP/YFP}$ mice produced less IL-17A than control mice, suggesting that there is an effect of the *Cost* allele on the expression of the adjacent *Il17* allele (Figure 29). Importantly, in comparison to total Th17 cells derived from *Il17f* $^{+/Cost}$*Rs*$^{YFP/YFP}$ and *Il17f* $^{Cost/Cost}$*Rs*$^{YFP/YFP}$ mice, the YFP$^+$ population of cells had a higher expression level of IL-17A and IL-17F, demonstrating an enrichment of IL-17-producing cells in the YFP$^+$ population (Figure 29).

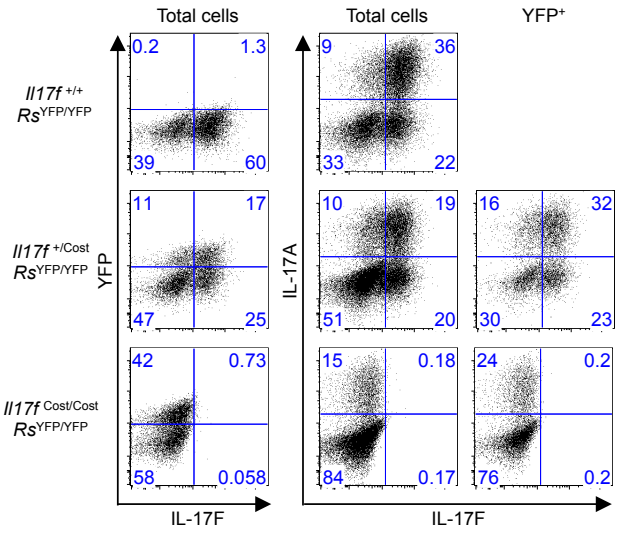

Figure 29. Verification of cytokine and YFP reporter expression in Th17 cells from *Il17f* ^{Cost} mice. Naïve CD4$^+$ T cells were isolated from the spleen of *Il17f* $^{+/+}$*Rs*$^{YFP/YFP}$, *Il17f* $^{+/Cost}$*Rs*$^{YFP/YFP}$ or *Il17f* $^{Cost/Cost}$*Rs*$^{YFP/YFP}$ mice and stimulated

under Th17-polarizing conditions for 5 days. Cells were re-stimulated with PMA and ionomycin and YFP and cytokine expression was analyzed by flow cytometry. Cells are gated on the live population (left and middle panels) and live YFP^+ population (right panel). Representative dot plots are shown.

To confirm that in vitro differentiated Th17 cells and not other Th subsets expressed YFP, we isolated naïve $CD4^+$ T cells from the spleen of *Il17f* $^{+/Cost}$*Rs*$^{YFP/YFP}$ mice and stimulated them under Th1, Th2, Th9 or Th17-polarizing conditions. YFP was expressed in Th17 cells, but not in Th1, Th2 or Th9 cells (Figure 30).

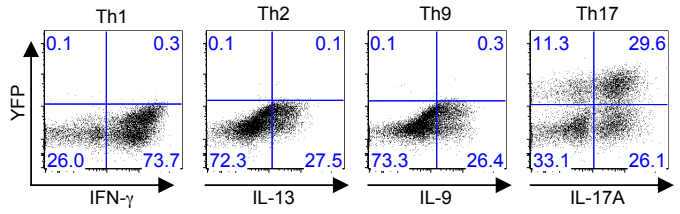

Figure 30. YFP reporter expression in Th subsets from *Il17f* ^{Cost} mice. Naïve $CD4^+$ T cells were isolated from the spleen of *Il17f* $^{+/Cost}$*Rs*$^{YFP/YFP}$ mice and stimulated under Th1, Th2, Th9 or Th17-polarizing conditions for 5 days. Cells were re-stimulated with PMA and ionomycin and YFP and cytokine expression was analyzed by flow cytometry in the live population of cells. Representative dot plots are shown.

To further validate the use of *Il17f* ^{Cost}*Rs*^{YFP} mice for in vivo studies intended to track the fate of IL-17F-expressing cells, we induced EAE in *Il17f* $^{+/Cost}$*Rs*$^{YFP/YFP}$ mice and analyzed cytokine secretion in $CD4^+$ cells from the brain. Similar to what has been previously reported (121), we found that $CD4^+$ cells from the brain

of mice at the peak of paralysis co-expressed IL-17A and IFN-γ (Figure 31). The cytokine-positive population was further enhanced in the $CD4^+YFP^+$ population demonstrating that in EAE, IFN-γ-secreting $CD4^+$ cells co-express IL-17F or arise from an IL-17F-positive precursor (Figure 31). Together, these data demonstrate that *Il17f* ^{Cost}*Rs*^{YFP} mice can be used for in vitro and in vivo studies to further explore the stability of IL-17F-secreting cells.

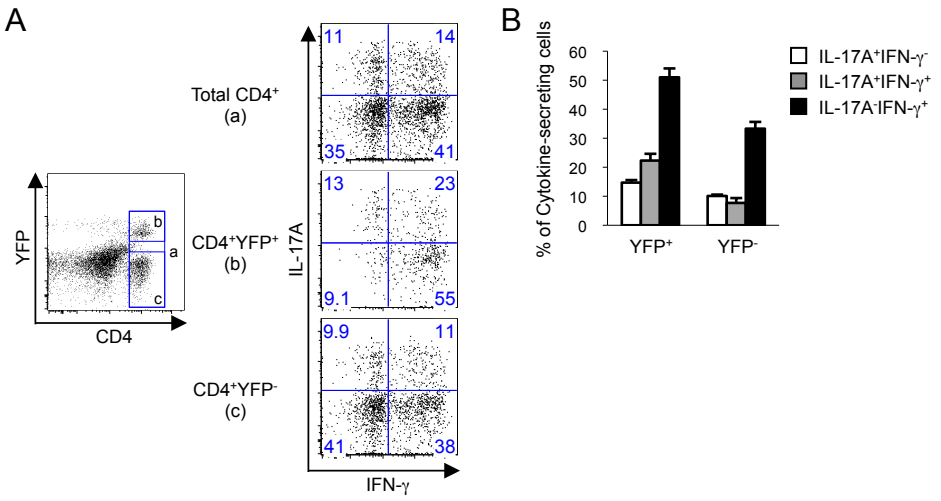

Figure 31. Verification of YFP reporter expression from *Il17f* ^{Cost} mice in vivo. *A-B Il17f* $^{+/Cost}$*Rs*$^{YFP/YFP}$ mice were immunized s.c. on days 0 and 7 with myelin oligodendrocyte glycoprotein (MOGp35-55) antigen peptide emulsified in complete Freund's adjuvant. Mice were injected i.p. with pertussis toxin on days 0 and 2. Mice were sacrificed 19 days after induction of disease and spleen and brain were harvested. Splenocytes (data not shown) and mononuclear cells from the brain were re-stimulated with PMA and ionomycin and cytokines were analyzed by flow cytometry (*A*) in live total $CD4^+$ (gate a), $CD4^+YFP^+$ (gate b) and $CD4^+YFP^-$ (gate c) lymphocytes. Representative dot plots are shown. (*B*) Graphical representation of the data displayed in *A*. Cells are gated on the live $CD4^+YFP^+$ or $CD4^+YFP^-$ population. The data are the mean ± SEM of 3 mice.

Kinetics of YFP reporter expression and cytokine production in Th17 cells

To determine the kinetics of YFP reporter expression, we isolated naïve $CD4^+$ T cells from the spleen of control and *Il17f* $^{Cost/Cost}$*Rs* $^{+/YFP}$ mice and stimulated them under Th17-polarizing conditions for five days, analyzing YFP expression each day. Cells expressed YFP after one day of culture under Th17-polarizing conditions (Figure 32A). While YFP expression increased nearly four-fold between 1 and 2 days of stimulation under Th17-polarizing conditions, YFP expression continuously increased, at modest levels, over days 3-5 of culture with ~44% of cells expressing YFP after 5 days of stimulation under Th17-skewing conditions (Figure 32A). We further analyzed the kinetics of YFP reporter and cytokine expression in Th17 cells from *Il17f* $^{Cost/Cost}$*Rs* $^{YFP/YFP}$ after five days of culture and re-stimulation with PMA and ionomycin over a 45-hour time course. The percentage of YFP^+ cells steadily increased over 25 hours of stimulation and leveled off between 25 and 45 hours of stimulation (Figure 32B). YFP^+ Th17 cells expressed low levels of IL-22, and IL-22 was expressed at the highest level after 5 and 10 hours of re-stimulation (Figure 32C). IL-17A and IL-21-secreting YFP^+ Th17 cells drastically increased after 5 hours of stimulation, with a higher percentage of the cells secreting IL-17A (Figure 32C). While IL-21 production steadily decreased in YFP^+ Th17 cells after 5 hours of stimulation, IL-17A levels were modestly increased from 5 to 10 hours of re-stimulation, and then declined over the remainder of the 25-hour time course (Figure 32C). To determine if there were differences in IL-17A and IL-21 expression in YFP^+ and YFP^- Th17 cells after re-stimulation, we compared cytokine expression in these

two populations over time. Over the course of 25 hours of stimulation, the highest percentage of YFP^+ cells were IL-$17A^+$IL-21^-, with lower levels of IL-$17A^+$IL-21^+ and IL-$17A^-$IL-21^+ being expressed in the YFP^+ population (Figure 32D). Interestingly, a similar percentage of YFP^- cells were L-$17A^+$IL-21^- and IL-$17A^-$IL-21^+, while the lowest percentage of YFP^- cells were IL-$17A^+$IL-21^+ (Figure 32D). Furthermore, while IL-$17A^+$IL-21^- and IL-$17A^+$IL-21^+ cells were more highly expressed in the YFP^+ population than in the YFP^- population, the percentage of IL-$17A^-$IL-21^+ cells in the YFP^+ and YFP^- population was similar (Figure 32D), suggesting that YFP reporter expression coincides with the expression of some (IL-17A), but not all (IL-21) Th17-associated cytokines.

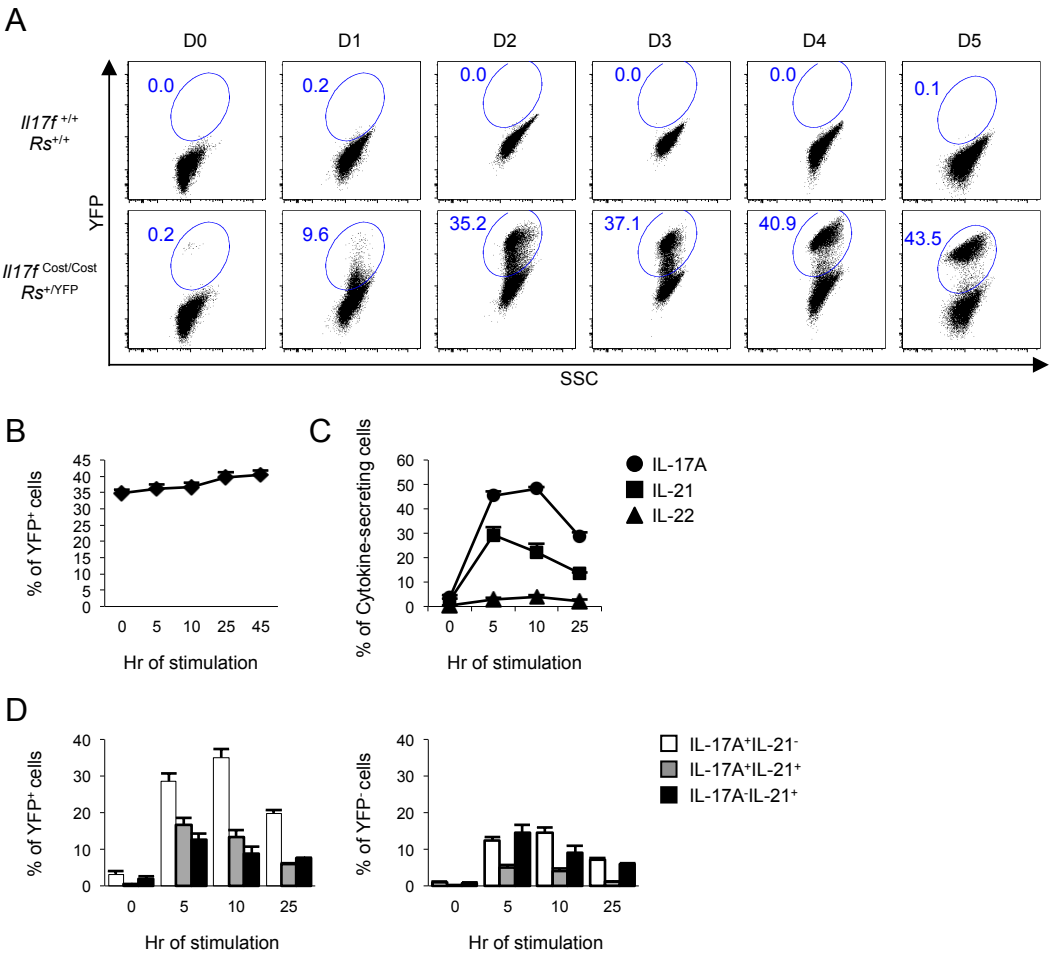

Figure 32. Kinetics of YFP reporter expression and cytokine production in Th17 cells. A, Naïve CD4$^+$ T cells were isolated from the spleen of *Il17f* $^{+/+}$*Rs*$^{+/+}$ and *Il17f* $^{Cost/Cost}$*Rs*$^{+/YFP}$ mice and stimulated under Th17-polarizing conditions for 5 days. YFP expression was analyzed by flow cytometry in unfixed naïve cells (Day 0) or after 1-5 days in culture. Representative dot plots are shown. *B-D*, Naïve CD4$^+$ T cells were isolated from the spleen of *Il17f* $^{Cost/Cost}$*Rs*$^{YFP/YFP}$ mice and stimulated under Th17-polarizing conditions for 5 days. Cells were left unstimulated (0 hr) or restimulated with PMA and ionomycin for 5, 10, 25 or 45 h, with the addition of monensin 3 h prior to each time point and cells were analyzed by flow cytometry. (*B*) Percentage of YFP$^+$ cells was determined at each time point. (*C*) Percentage of cytokine-secreting cells was determined in the YFP$^+$ population of cells at each time point. (*D*) Percentage of IL-17A and IL-21-secreting cells was determined in the YFP$^-$ and YFP$^+$ population. The data are the mean ± SEM of 4 mice (*B-D*).

Altered Th17 cytokine expression upon stimulation under Th1, Th2 or Th9-polarizing conditions

It has been established that Th17 cells can produce robust amounts of IFN-γ when stimulated in vitro under Th1-skewing conditions, however less is known about their cytokine profile when stimulated under Th2 or Th9-polarizing conditions. To examine this, we isolated naïve $CD4^+$ T cells from the spleen of *Il17f* $^{+/Cost}$*Rs*$^{YFP/YFP}$ mice and stimulated them under Th17-polarizing conditions for two rounds of culture to establish well-differentiated Th17 cells. YFP^+ cells were sorted and further stimulated under Th17-polarizing conditions or were stimulated under Th1, Th2 or Th9-polarizing conditions for a third round of culture. After two rounds of culture under Th17-polarizing conditions, YFP^+ cells produced IL-17A and IL-17F with little to no IFN-γ, IL-13 or IL-9 production (Figure 33A). YFP^+ cells stimulated for a third round of culture under Th17-polarizing conditions displayed an increase in IL-$17A^+$ cells, and this population was slightly reduced when YFP^+ Th17 cells were cultured under Th1, Th2 or Th9-polarizing conditions (Figure 33B and C). YFP^+ cells maintained under Th17-skewing conditions for a third round of culture also displayed an increase in IL-$17A^+$IL-$17F^+$ cells (Figure 33B and C). This population was reduced when YFP^+ Th17 cells were stimulated under Th1 or Th2-polarizing conditions and to a lesser extent when stimulated under Th9-polarizing conditions (Figure 33B and C). Thus, IL-17 expression is maintained under Th17-polarizing conditions, but overall is reduced when Th17 cells are stimulated under Th1, Th2 or Th9-polarizing conditions.

We further analyzed the co-expression of IL-17A and IFN-γ, IL-13 or IL-9 in YFP^+ Th17 cells stimulated under Th1, Th2 or Th9-polarizing conditions. YFP^+ Th17 cells stimulated under Th1-polarizing conditions displayed reduced IL-$17A^+$IFN-γ^- cells with increased IL-$17A^+$IFN-γ^+ and IL-$17A^-$IFN-γ^+ cells in comparison to cells maintained under Th17 conditions (Figure 33B and D). An increase in IL-$17A^+$IL-13^+ and IL-$17A^-$IL-13^+ cells was observed when YFP^+ Th17 cells were cultured under Th2-skewing conditions, and to a lesser extent upon stimulation under Th9-polarizing conditions, whereas the IL-$17A^+$IL-13^- population was decreased in comparison to cells maintained under Th17-skewing conditions (Figure 33B and E). Furthermore, YFP^+ Th17 cells cultured under Th9-polarizing conditions had an increase in IL-$17A^+$IL-9^+ cells along with a smaller increase in IL-$17A^-$IL-9^+ cells compared with cells maintained under Th17-skewing conditions (Figure 33B and F). YFP^+ Th17 cells cultured under Th2-polarizing conditions also displayed an increase in IL-$17A^-$IL-9^+ cells with a more moderate increase in IL-$17A^+$IL-9^+ cells, however both populations were produced at lower levels than cells cultured under Th9-polarizing conditions (Figure 33B and F). Enhanced IL-9 secretion from Th17 cells stimulated under Th2-skewing conditions could be an effect of prior TGF-β-induced signals during Th17 differentiation in combination with additional IL-4 stimulation under Th2-polarizing conditions. In addition, IL-$17A^+$IL-9^- cells were reduced when YFP^+ Th17 cells were cultured under Th9 conditions, and a further reduction was observed under Th2-skewing conditions, in comparison to cells maintained under Th17-polarizing conditions (Figure 33B and

F). Thus, YFP$^+$ Th17 cells stimulated under Th1, Th2 or Th9-polarizing conditions display an increase in IFN-γ, IL-13 and IL-9-producing cells, respectively, suggestive of a transition to the respective effector program.

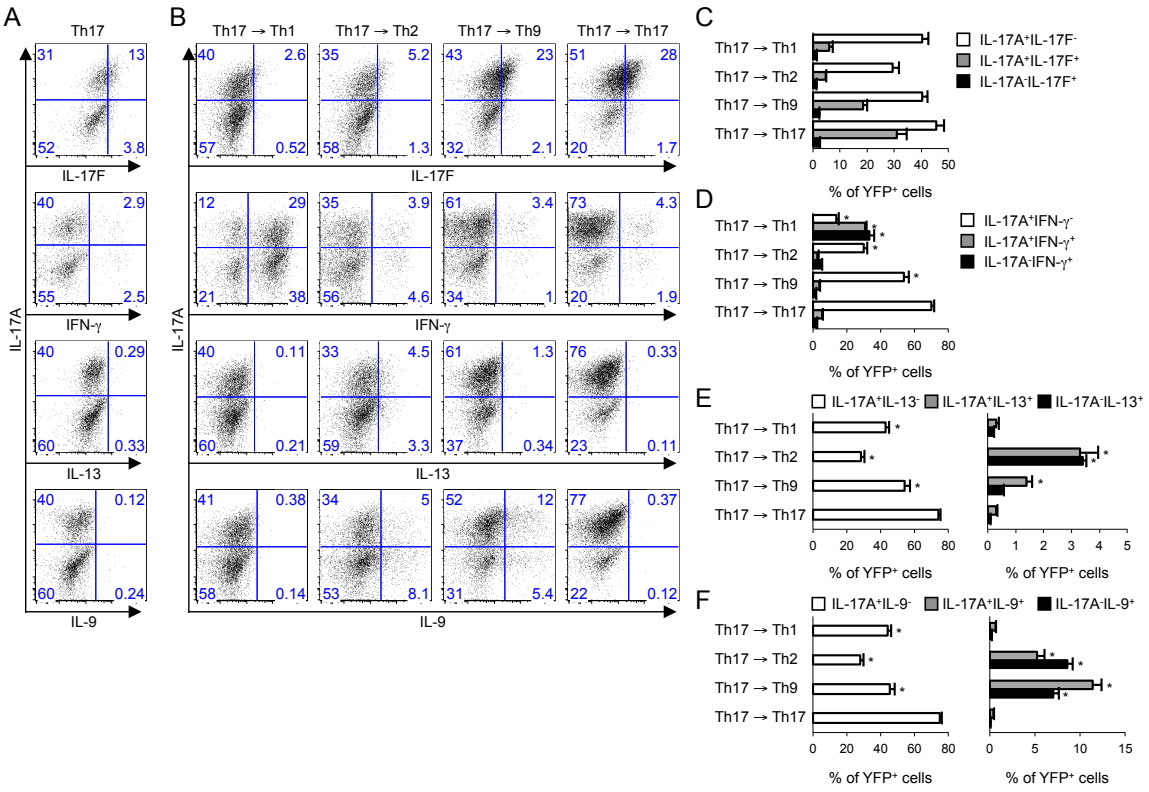

Figure 33. Th17 cells display an altered cytokine profile when cultured under Th1, Th2 or Th9-polarizing conditions. *A-F,* Naïve CD4$^+$ T cells were isolated from the spleen of *Il17f* $^{+/Cost}$*Rs*$^{YFP/YFP}$ mice and stimulated under Th17-polarizing conditions for 5 days (Round 1). Cells were re-stimulated and cultured under long-term Th17 polarizing conditions for another five days (Round 2). Live YFP$^+$ cells were sorted by flow cytometry and cultured under Th1, Th2, Th9 or long-term Th17-polarizing conditions for five days (Round 3). (*A*) After 2 rounds of stimulation under Th17-polarizing conditions, cells were re-stimulated with PMA and ionomycin and cytokines were analyzed by flow cytometry. Cells are gated on the live YFP$^+$ population. Representative dot plots are shown. (*B*) After culture under Th1, Th2, Th9 or Th17-polarizing conditions (Round 3), cells were re-stimulated, stained and analyzed as in *A*. Cells are gated on the live YFP$^+$

population. Representative dot plots are shown. (*C-F*), Graphical representation of the data displayed in *B*. The data are the mean ± SEM of 5 mice. Statistical analysis in *D-F* was performed using the one-way ANOVA. *$p < 0.05$, compared with Th17 → Th17 samples.

Similar to results of intracellular cytokine staining, *Il17a* and *Il17f* were expressed in YFP$^+$ Th17 cells cultured for two rounds under Th17-polarizing conditions, and their expression was further enhanced after a third round of culture under Th17-polarizing conditions (Figure 34). *Il17a* and *Il17f* levels were similar between YFP$^+$ Th17 cells cultured for two rounds under Th17-polarizing conditions as to those that were stimulated for a third round under Th9-polarizing conditions, however they were reduced in YFP$^+$ Th17 cells that were stimulated for a third round under Th1 and Th2-polarizing conditions (Figure 34). *Ifng* levels were increased in YFP$^+$ Th17 cells stimulated for a third round under Th1-polarizing conditions (Figure 34) and *Il9* expression was enhanced in YFP$^+$ Th17 cells stimulated for a third round under Th9-polarizing conditions, with a modest increase in those stimulated for a third round under Th2-skewing conditions (Figure 34). In comparison to YFP$^+$ Th17 cells cultured for two rounds under Th17-polarizing conditions, YFP$^+$ Th17 cells stimulated for a third round under Th2-skewing conditions displayed enhanced levels of *Il4*, *Il5* and *Il13*, whereas a more modest increase in *Il5* and *Il13* was observed in YFP$^+$ Th17 cells stimulated for a third round under Th9-polarizing conditions (Figure 34). Taken together, these data demonstrate that the expression of Th17-associated cytokines are differentially expressed when YFP$^+$ Th17 cells are stimulated under Th1, Th2, Th9 or Th17-polarizing conditions, while the expression of Th-associated

cytokines are increased when YFP$^+$ Th17 cells are stimulated under the respective Th polarizing-conditions.

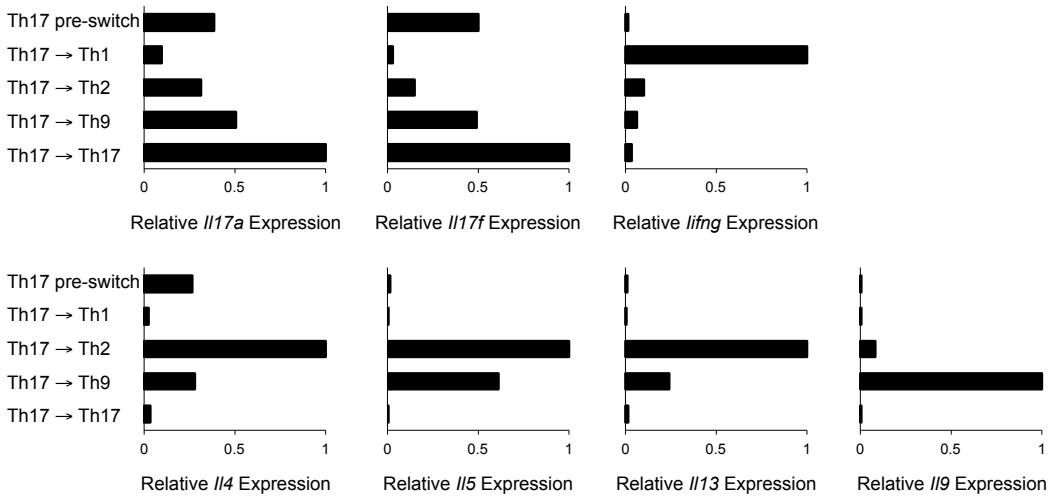

Figure 34. Th-associated cytokine gene expression in Th17 cells cultured under different Th cell-polarizing conditions. Naïve CD4$^+$ T cells were isolated from the spleen of *Il17f* $^{+/Cost}$*Rs*$^{YFP/YFP}$ mice and stimulated under Th17-polarizing conditions for 5 days (Round 1). Cells were re-stimulated and cultured under long-term Th17-polarizing conditions for another five days (Round 2). Live YFP$^+$ cells were sorted by flow cytometry and cultured under Th1, Th2, Th9 or long-term Th17-polarizing conditions for three days (Round 3). RNA was isolated from sorted live YFP$^+$ cells on the third day of round 2 (Th17 pre-switch) or after three days of the third round of culture. Before RNA isolation, cells were re-stimulated for 5 h with anti-CD3. Expression of the indicated genes was measured using quantitative PCR; samples were normalized to the expression of β_2-microglobulin mRNA and are relative to Th17 → Th17 cells (*Il17a, Il17f*), Th17 → Th1 cells (*Ifng*), Th17 → Th2 cells (*Il4, Il5, Il13*) or Th17 → Th9 cells (*Il9)*. The data are the value from one mouse and are representative of 2 experiments.

Stimulation of Th17 cells under different Th-polarizing conditions induces the expression of the respective Th-associated transcription factors

The changes in cytokine production observed in YFP$^+$ Th17 cells maintained under Th17-polarizing conditions or stimulated under Th1, Th2 or Th9-polarizing conditions led us to determine if there were also alterations in transcription factor expression among these cell populations. To examine this, naïve CD4$^+$ T cells from the spleen of *Il17f* $^{+/Cost}$*Rs*$^{YFP/YFP}$ mice were cultured as described for Figure 33 and YFP$^+$ cells were sorted after 3 days of the third round of stimulation under Th17-skewing conditions or Th1, Th2 or Th9-polarizing conditions. Although we saw a reduction in IL-17A$^+$ cells when YFP$^+$ Th17 cells were cultured under Th1, Th2 or Th9-skewing conditions in comparison to cells maintained under Th17-polarizing conditions (Figure 33B and C), there was not a significant difference in RORγt protein expression when YFP$^+$ Th17 cells were stimulated under other Th-skewing conditions (Figure 35A and B). However, in comparison to YFP$^+$ Th17 cells maintained under Th17-polarizing conditions, *Rorc* levels were reduced in cells stimulated under Th1, Th2 or Th9-polarizing conditions, with a significant reduction observed under Th1-polarizing conditions (Figure 35C).

YFP$^+$ Th17 cells stimulated under Th1-polarizing conditions expressed high levels of T-bet (Figure 35A-C), coinciding with the observed increase in IFN-γ production from these cells (Figure 33B and D). YFP$^+$ Th17 cells stimulated under Th2 or Th9-polarizing conditions displayed enhanced Gata3 levels in comparison to cells maintained under Th17-polarizing conditions (Figure 35A-C). Similarly, Th17 cells stimulated under Th9-polarizing conditions demonstrated increased levels of *Irf4* and *Maf*, both of which encode transcription factors

associated with the development of Th9 cells (Figure 35A-C). While BATF has been shown to play an important role in the development of a number of Th subsets, *Batf* levels were significantly lower in YFP$^+$ Th17 cells stimulated under Th1-polarizing conditions, but no difference was observed between the levels seen in cells stimulated under Th2, Th9 or Th17-polarizing conditions (Figure 35C). These data demonstrate that YFP$^+$ Th17 cells stimulated under Th1, Th2 or Th9-polarizing conditions induce the expression of transcription factors associated with the development of each Th subset.

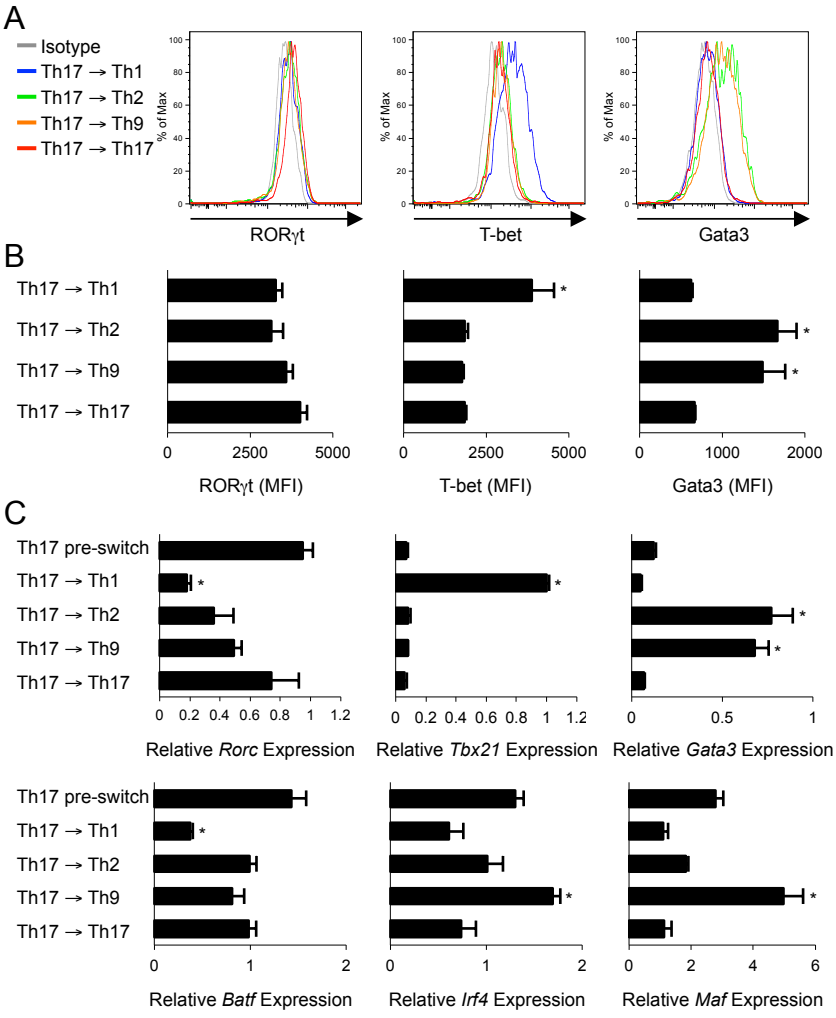

Figure 35. Expression of transcription factors in Th17 cells cultured under different Th cell-polarizing conditions. *A-C*, Naïve $CD4^+$ T cells were isolated from the spleen of *Il17f* $^{+/Cost}$*Rs*$^{YFP/YFP}$ mice and stimulated under Th17-polarizing conditions for 5 days (Round 1). Cells were re-stimulated and cultured under long-term Th17-polarizing conditions for another five days (Round 2). Live YFP^+ cells were sorted by flow cytometry and cultured under Th1, Th2, Th9 or long-term Th17-polarizing conditions for three days (Round 3) and live YFP^+ cells were sorted for further analysis. (*A*) Expression of the indicated transcription factors was assessed in unstimulated cells by flow cytometry. Representative histograms are shown. (*B*) MFI of the data shown in *A*. The data are the mean ± SEM of 3 mice. (*C*) RNA was isolated from sorted live YFP^+ cells on the third day of round 2 (Th17 pre-switch) or sorted live YFP^+ cells after three days of the third round of culture. Expression of the indicated genes was measured using quantitative PCR; samples were normalized to the expression of β_2-microglobulin mRNA and are relative to Th17 → Th1 cells (*Tbx21*), Th17 → Th2 cells (*Gata3*) or Th17 → Th17 cells (*Rorc*, *Batf*, *Irf4*, *Maf*). The data are the mean ± SEM of 3 mice. Statistical analysis in *B-C* was performed using the one-way ANOVA. *p < 0.05, compared with Th17 → Th17 samples.

Th17 cell stability in acute allergic airway disease

Several reports have demonstrated that Th17 cells can adopt the IFN-γ-secreting phenotype of Th1 cells in vivo, and we have confirmed that using our reporter mouse (Figure 31). However it is less clear if Th17 cells can adopt a Th2 or Th9 effector program in an in vivo environment that promotes their development. To explore this possibility, we first examined the stability of Th17 cells in an acute model of AAD. *Il17f* $^{+/Cost}$*Rs*$^{YFP/YFP}$ mice were sensitized with OVA and alum followed by intranasal challenges with OVA. In comparison to control mice challenged with PBS, mice challenged with OVA displayed an increase in the total BAL cell number as well as the number of $CD4^+YFP^+$ cells isolated from the BAL (Figure 36A). While most of the $CD4^+$ T cells from the BAL were single

producers of IL-17A, IFN-γ or IL-13, there was a very small population of cells that were IL-17A^{+}IFN-γ^{+} or IL-17A^{+}IL-13^{+} (Figure 36B). Interestingly, CD4^{+}YFP^{+} cells from the BAL and lung produced IL-17A, but did not express the other Th cell-associated cytokines analyzed (Figure 36B and C, data not shown), suggesting a stable Th17 phenotype in acute AAD.

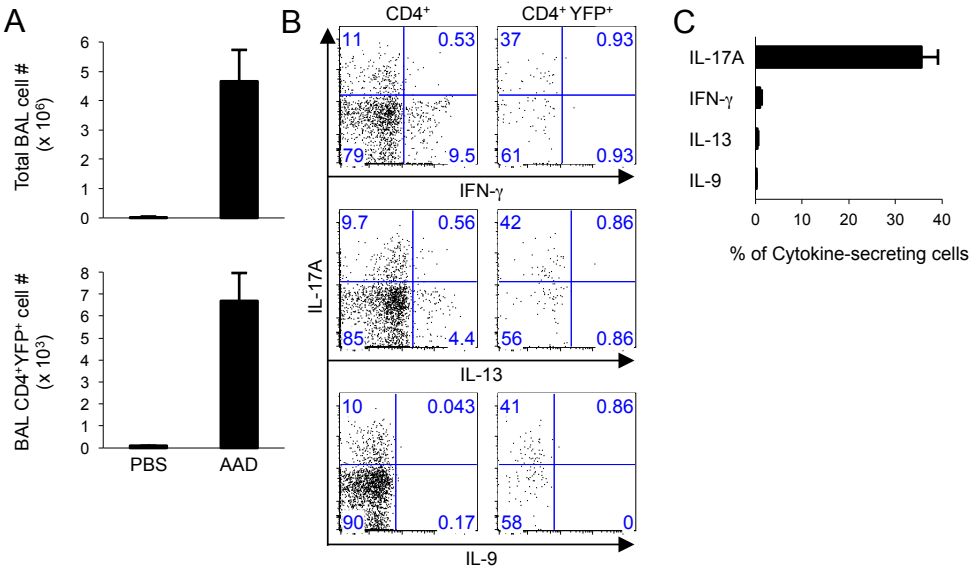

Figure 36. Stability of Th17 cells in an acute model of AAD. *A-C*, *Il17f* $^{+/Cost}$*Rs*$^{YFP/YFP}$ mice were sensitized i.p. with OVA and alum on days 0 and 7, challenged i.n. with PBS or OVA on days 14-19 and sacrificed 24 h after the final challenge. Cells were isolated from the BAL and lung (data not shown) for further analysis. (*A*) Total cell number was calculated in the BAL of control (PBS) or allergic (AAD) mice (top panel). CD4^{+}YFP^{+} cell number was determined from the BAL of control or allergic mice using flow cytometry (bottom panel). Data are the mean ± SEM of 3-5 mice. (*B*) BAL cells were stimulated with PMA and ionomycin and cytokines were analyzed by flow cytometry in live CD4^{+} (left panel) or CD4^{+}YFP^{+} lymphocytes (right panel). Representative dot plots are shown. (*C*) Graphical representation of the CD4^{+}YFP^{+} data displayed in *B*. The data are the mean ± SEM of 5 mice.

Although Th17 cells have been shown to adopt an IFN-γ-secreting Th1 phenotype in vivo, they did not do so during the development of acute AAD. As Ova and alum induced-AAD is largely associated with the development of Th2 and Th9 cells, we hypothesized that if we added a Th1-inducing agent to OVA and alum sensitized mice, this environment could promote Th17 cells to adopt an IFN-γ secreting phenotype upon challenge with OVA. To test this, *Il17f* $^{+/Cost}$*Rs*$^{YFP/YFP}$ mice were sensitized with OVA and alum followed by intranasal challenges with OVA or OVA and LPS (Figure 37A). In comparison to mice challenged with OVA alone, mice challenged with OVA and LPS displayed only a modest increase in the percentage of total BAL $CD4^+$ cells that produced IFN-γ. Mice challenged with OVA and LPS also displayed a small increase in $CD4^+$IL-$17A^+$ cells in comparison to OVA-challenged mice, and most of the $CD4^+$ cells were single producers of IFN-γ or IL-17A (Figure 37B). Furthermore, a small percentage of $CD4^+YFP^+$ cells from the BAL of mice challenged with OVA and LPS co-produced IL-17A and IFN-γ, however the percentage of $CD4^+YFP^+$ IFN-γ-producing cells was not different from the BAL of mice challenged with OVA and LPS or OVA alone. The majority of $CD4^+YFP^+$ cells were single producers of IL-17A, similar to what was observed from mice challenged with OVA alone (Figure 37B and C). These data indicate a stable Th17 phenotype during the development of acute AAD in combination with an LPS-induced inflammatory environment (Figure 37B and C), and suggest that this environment may promote the development and stability of Th17 cells.

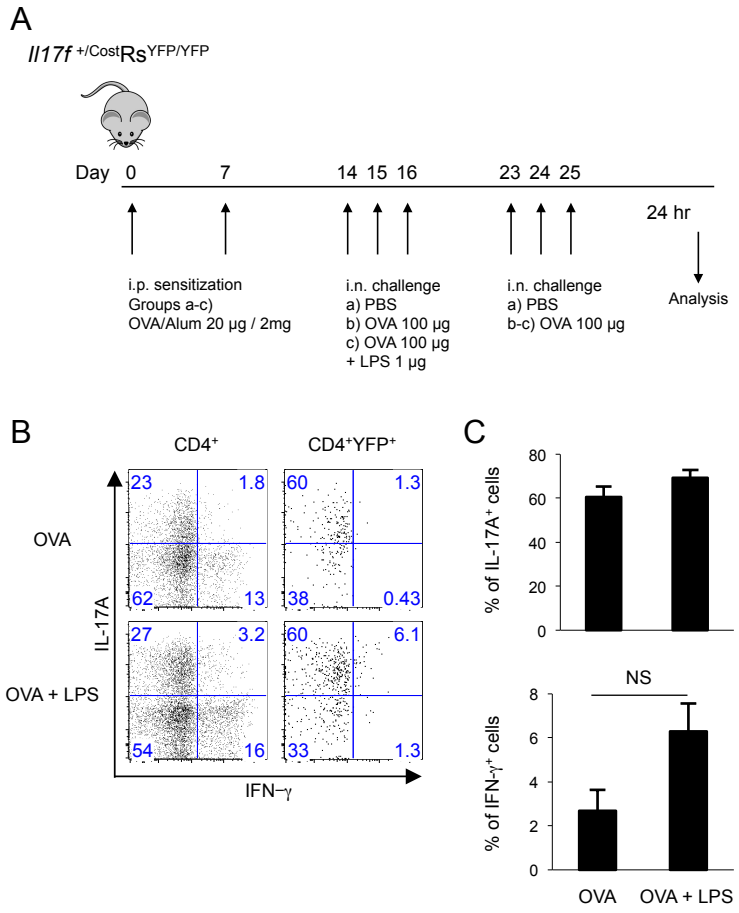

Figure 37. Stability of Th17 cells in OVA and alum-induced AAD in the context of LPS. *A-C*, *Il17f* $^{+/Cost}$*Rs*$^{YFP/YFP}$ mice were sensitized i.p. with OVA and alum on days 0 and 7 (groups a-c), challenged i.n. with PBS (group a), OVA (group b), or OVA and LPS (group c) on days 14-16, challenged i.n. with PBS (group a) or OVA (groups b-c) on days 23-25 and sacrificed 24 h after the final challenge. Cells were isolated from the BAL and lung (data not shown) for further analysis. (*A*) Schematic illustrating experimental procedure. (*B*) BAL cells were stimulated with PMA and ionomycin and cytokines were analyzed by flow cytometry in live CD4$^+$ (left panel) or CD4$^+$YFP$^+$ lymphocytes (right panel). Representative dot plots are shown from group b (OVA) or group c (OVA + LPS). (*C*) Graphical representation of the CD4$^+$YFP$^+$ data displayed in *B*. The data are the mean ± SEM of 4-5 mice. Statistical analysis was performed using the Student's *t* test. NS, Not Significant.

Th17 cell stability in chronic allergic airway disease

Because there are differences in the allergic environment induced by different models of AAD, we also explored the stability of Th17 cells in chronic models of AAD using house dust mite (HDM) or *Aspergillus fumigatus* (ASP) challenge. To test the stability of Th17 cells in HDM-induced AAD, *Il17f* $^{+/Cost}$*Rs*$^{YFP/YFP}$ mice received 3 consecutive intranasal challenges with HDM each week for five weeks. Allergic mice displayed an increase in the total number of cells that infiltrated the BAL along with an increase in $CD4^+YFP^+$ cells in the BAL in comparison to non-allergic control mice (Figure 38A). Similar to what was observed in the acute model of AAD, $CD4^+YFP^+$ cells from the BAL and lung expressed IL-17A, but did not express IFN-γ, IL-13 or IL-9 (Figure 38B and C).

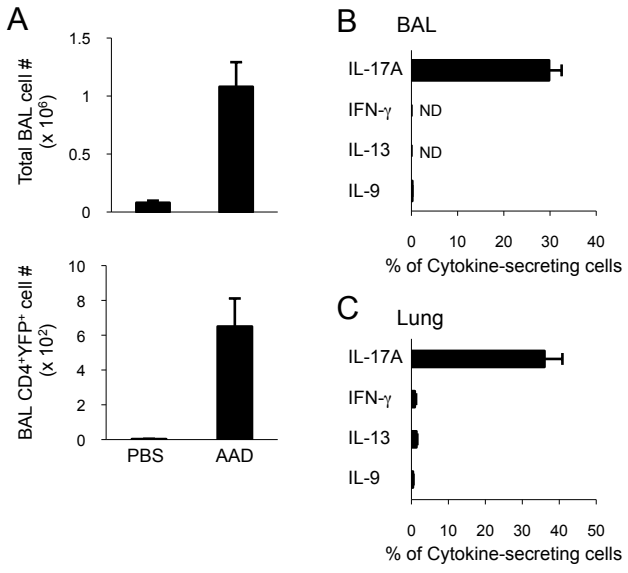

Figure 38. Stability of Th17 cells in the HDM-induced chronic model of AAD. *A-C*, *Il17f* $^{+/Cost}$*Rs*$^{YFP/YFP}$ mice received i.n. doses of HDM for 3 consecutive days each week for 5 weeks and were sacrificed 24 h after the final challenge. Cells

were isolated from the BAL and lung for further analysis. (*A*) The total cell number was calculated in the BAL of control (PBS) or allergic (AAD) mice (top panel). The $CD4^+YFP^+$ cell number was determined from the BAL of control or allergic mice using flow cytometry (bottom panel). Data are the mean ± SEM of 3-5 mice. (*B*-*C*) BAL cells (*B*) and lung cells (*C*) were stimulated with PMA and ionomycin and cytokines were analyzed by flow cytometry in live $CD4^+YFP^+$ lymphocytes. The data are the mean ± SEM of 3 mice. ND, Not Detected.

To further test the stability of Th17 cells in ASP-induced AAD, *Il17f* $^{+/Cost}$*Rs*$^{YFP/YFP}$ mice received intranasal challenges with ASP every other day for 21 days. Allergic mice displayed an increase in the total number of cells that infiltrated the BAL, however the percentage $CD4^+YFP^+$ cells in the BAL of ASP-induced allergic mice was much lower than that observed in response to OVA and alum or HDM-induced AAD, which prevented us from collecting enough events to accurately analyze cytokine production from these cells via flow cytometry (Figure 39A and B). Taken together, these data demonstrate that YFP^+ Th17 cells remain IL-17-producers and do not express cytokines associated with other Th subsets during the development of acute and chronic AAD.

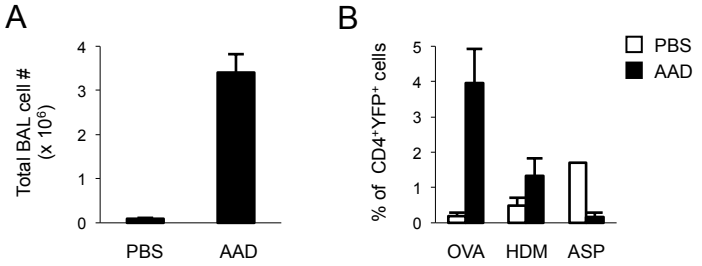

Figure 39. Percentage of Th17 cells in the airways of allergic mice. *A*-*B*, *Il17f* $^{+/Cost}$*Rs*$^{YFP/YFP}$ mice received i.n. doses of ASP every other day for 21 days and were sacrificed 24 h after the final challenge. Cells were isolated from the BAL

and lung (data not shown) for further analysis. (*A*) The total cell number was calculated in the BAL of control (PBS) or allergic (AAD) mice. (*B*) The percentage of $CD4^{+}YFP^{+}$ live lymphocytes was determined from the BAL of ASP-induced allergic mice using flow cytometry, and compared with that from OVA and alum-induced AAD (OVA) from Figure 36 and HDM-induced AAD (HDM) from Figure 38. Data are the mean ± SEM of 3-5 mice.

Differential cytokine receptor expression from in vitro and in vivo derived Th17 cells

Our data show that Th17 cells derived in vitro are capable of adopting a Th2 or Th9 effector program, however in the models of AAD tested, Th17 cells remain IL-17 secretors and do not adopt alternative T helper phenotypes. It is possible that Th cells must be actively proliferating to respond to signals in the environment, leading to a potential change in their effector program. As Th17 cells do not adopt alternative T helper phenotypes in the models of AAD tested, we wanted to confirm the Ag-specificity of YFP^{+} Th17 cells in the lung of OVA and alum-induced allergic mice. *Il17f* $^{+/Cost}$*Rs*$^{YFP/YFP}$ mice were sensitized with OVA and alum followed by intranasal challenges with OVA. Isolated cells from the lung and BAL were incubated with a dye used to trace cell proliferation, and stimulated ex vivo. We observed an increase in the percentage of BAL YFP^{+} and YFP^{-} $CD4^{+}$ proliferating cells in response to OVA stimulation in comparison to stimulation with BSA (Figure 40A and B). Furthermore, $CD4^{+}YFP^{+}$ cells displayed a significant increase in IL-17A production in response to OVA stimulation (Figure 40C and D). Together, these data suggest that YFP^{+} Th17 cells from OVA and alum-induced allergic mice display Ag-specificity and their inability to

adopt alternative Th phenotypes is not likely to due to a defect in Ag-induced responses.

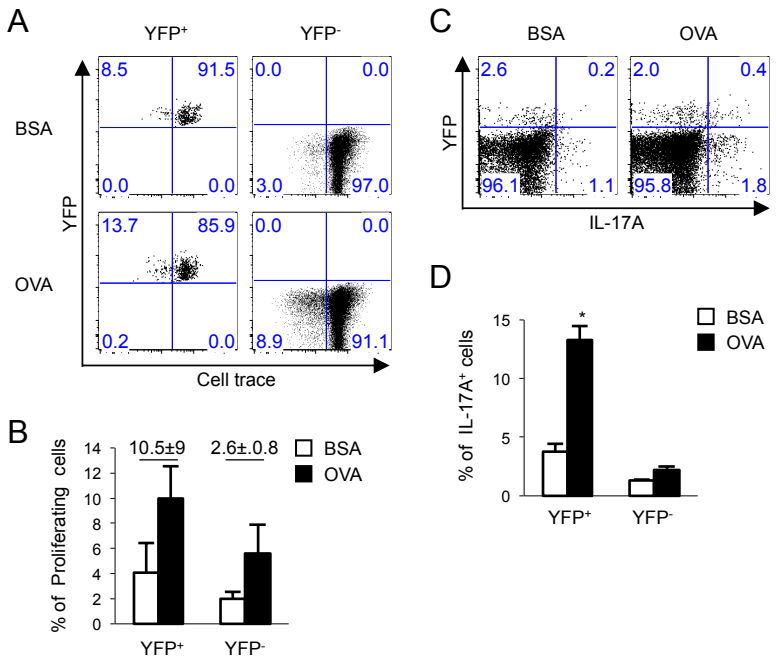

Figure 40. Ag-specificity of Th17 cells in OVA and alum-induced AAD. *A-D*, *Il17f* $^{+/Cost}$*Rs*$^{YFP/YFP}$ mice were sensitized i.p. with OVA and alum on days 0 and 7, challenged i.n. with OVA on days 14-19 and sacrificed 24 h after the final challenge. Cells were isolated from the BAL (data not shown) and lung for further analysis. Cells were incubated with Cell trace and re-stimulated with BSA or OVA for 24 h, with the addition of monensin during the final 3 h of re-stimulation. Proliferation and cytokine production were analyzed in CD4$^+$ cells. (*A*) Ag-specific proliferation was analyzed in YFP$^-$ and YFP$^+$ CD4$^+$ lymphocytes. Representative dot plots are shown. (*B*) Graphical representation of the data displayed in *A*. The data are the mean ± SEM of 3 mice, with the fold increase of OVA/BSA noted above the bars. (*C*) Ag-induced IL-17A expression was analyzed in CD4$^+$ lymphocytes. Representative dot plots are shown. (*D*) Percent of Ag-induced IL-17A$^+$ cells within the CD4$^+$YFP$^+$ or CD4$^+$YFP$^-$ population was determined. Data are the mean ± SEM of 3 mice. Statistical analysis was performed using the Student's *t* test. *$p < 0.05$, compared with BSA samples.

It is also possible that in vitro and in vivo derived Th17 cells display differences in cytokine receptor expression, affecting their responsiveness to cytokines in the environment. To explore this further, we first analyzed cytokine receptor expression on sorted YFP$^+$ Th17 cells derived from *Il17f* $^{+/Cost}$*Rs*$^{YFP/YFP}$ mice prior to stimulation under different Th-polarizing conditions (Th17 pre-switch). As expected, after two rounds of culture under Th17-polarizing conditions, YFP$^+$ Th17 pre-switch cells displayed substantial levels of *Il23r* expression (Figure 41A). In addition, YFP$^+$ Th17 pre-switch cells also displayed higher levels of *Il12rb2* and a trend toward increased *Il4ra* expression compared to littermate control naïve CD4$^+$ T cells (Figure 41A). We next assessed cytokine receptor expression on CD4$^+$ T cells in the BAL and lung of *Il17f* $^{+/Cost}$*Rs*$^{YFP/YFP}$ mice that had developed OVA and alum-induced AAD. CD4$^+$YFP$^-$ T cells from the BAL and lung of allergic mice displayed enhanced expression of IL-4Rα in comparison to control mice, which coincides with increased Th2 and Th9 cell development in allergic mice (Figure 41B, 36B and C, data not shown). However, CD4$^+$YFP$^+$ T cells from the BAL and lung of allergic mice did not express IL-4Rα (Figure 41B). IL-12Rβ2 was not expressed on CD4$^+$ T cells in the BAL and lung of control or allergic mice, and we did not observe a difference in TGF-βRII expression on YFP$^+$ or YFP$^-$ CD4$^+$ cells in control or allergic mice (Figure 41B). Together, these data suggest that in vitro derived Th17 cells express moderate levels of the cytokine receptors necessary to respond to cytokines essential to the development of other T helper effector programs whereas Th17 cells developed during AAD do not. This could be a possible explanation for the differences in

stability observed for in vitro derived Th17 cells and those developed during AAD.

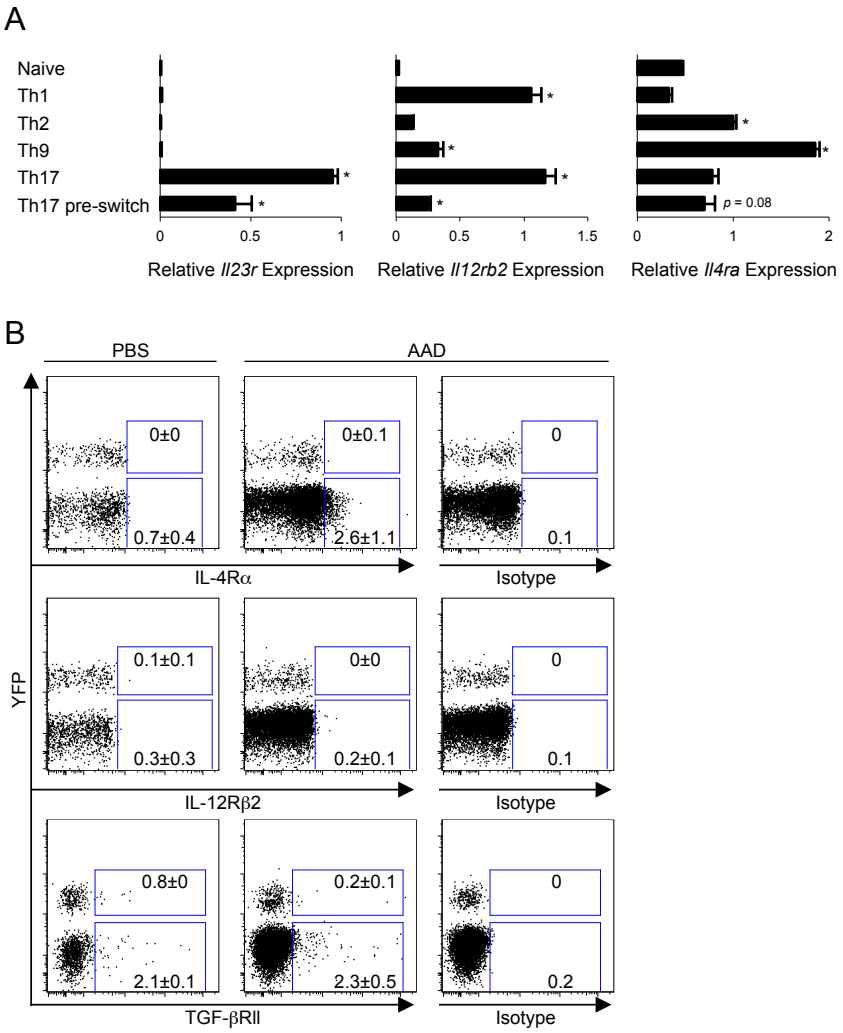

Figure 41. Cytokine receptor expression from Th17 cells derived in vitro or in vivo from OVA and alum-induced AAD mice. *A*, For Th17 pre-switch cells, naïve CD4$^+$ T cells were isolated from the spleen of *Il17f* $^{+/Cost}$*Rs*$^{YFP/YFP}$ mice and stimulated under Th17-polarizing conditions for 5 days (Round 1). Cells were re-stimulated and cultured under long-term Th17-polarizing conditions for another five days (Round 2) and live YFP$^+$ cells were sorted by flow cytometry. For other Th samples, naïve CD4$^+$ T cells were isolated from the spleen of *Il17f* $^{+/+}$*Rs*$^{YFP/YFP}$ littermate control mice and stimulated under Th1, Th2, Th9 or Th17-polarizing

conditions for five days. RNA was isolated from unstimulated naïve $CD4^+$ T cells, Th1, Th2, Th9, Th17 or Th17 pre-switch cells. Expression of the indicated genes was measured using quantitative PCR; samples were normalized to the expression of β_2-microglobulin mRNA and are relative to Th17 cells (*Il23r*), Th1 cells (*Il12rb2*) or Th2 cells (*Il4ra*). The data are the mean ± SEM of 3-5 mice. *B*, OVA and alum-induced AAD was developed in *Il17f* $^{+/Cost}$*Rs*$^{YFP/YFP}$ mice as in Figure 36. Cells were isolated from the BAL and lung (data not shown) and IL-4Rα, IL-12Rβ2 and TGF-βRII expression was analyzed in YFP^- and YFP^+ $CD4^+$ lymphocytes from control (PBS) or allergic (AAD) mice by flow cytometry. The cells are gated on live $CD4^+$ lymphocytes. Representative dot plots are shown with the mean ± SD of 2-4 mice displayed. Statistical analysis in *A* was performed using the one-way ANOVA. *$p < 0.05$, compared with Naive samples.

In vitro derived Ag-specific Th17 cells remain stable IL-17-secretors in an in vivo allergic environment

As our data demonstrate that Th17 cells derived in vitro express the cytokine receptors necessary to respond to cytokines required for the development of other T helper subsets, whereas those that developed during AAD do not, we wanted to determine if in vitro derived Th17 cells could adopt other T helper effector programs upon further stimulation with the addition of BAL fluid from allergic mice added to the cultures. We stimulated naïve $CD4^+$ T cells from *Il17f* $^{+/Cost}$*Rs*$^{YFP/YFP}$ mice under Th17-polarizing conditions for two rounds of culture. YFP^+ Th17 cells were sorted and stimulated for a third round of culture under Th17-polarizing conditions or in the presence of BAL fluid from control mice, allergic mice or allergic mice with the addition of anti-IL-4, to block any effects IL-4 present in the allergic BAL fluid may have on differentiation. In comparison to pre-switch Th17 cells, YFP^+ Th17 cells stimulated in the presence of all three BAL fluid conditions displayed similar levels of IL-17A production, with levels

expectedly being increased in cells stimulated for a third round of culture under Th17-polarizing conditions (Figure 42). While we did not observe a difference in the amount of IFN-γ produced between Th17 cells cultured under the three different BAL fluid conditions, YFP^+ Th17 cells stimulated for a third round of culture in the presence of all BAL fluid conditions displayed enhanced IFN-γ production in comparison to YFP^+ Th17 cells stimulated for two or three rounds of culture under Th17-skewing conditions (Figure 42), which could be attributed to the absence of anti-IFN-γ blocking antibodies in the in vitro cultures that contain BAL fluid. While a small increase in the percentage of $IL\text{-}13^+$ and $IL\text{-}9^+$ cells was observed when YFP^+ Th17 cells were simulated for a third round of culture in the presence of BAL fluid from allergic mice, YFP^+ Th17 cells stimulated for a third round of culture in the presence of BAL fluid from control mice displayed a similar increase in $IL\text{-}13^+$ and $IL\text{-}9^+$ cells. Of note, the increase in $IL\text{-}13^+$ and $IL\text{-}9^+$ cells observed for YFP^+ Th17 cells stimulated for a third round of culture in the presence of allergic BAL fluid was diminished in the presence of anti-IL-4, suggesting these effects may be due to IL-4-induced signals. Together, these data suggest that although in vitro derived YFP^+ Th17 cells express the cytokine receptors necessary to respond to cytokines required for the development of other T helper subsets, YFP^+ Th17 cells stimulated in vitro in the presence of BAL fluid from allergic mice remain stable IL-17-secretors and do not adopt alternative T helper effector programs.

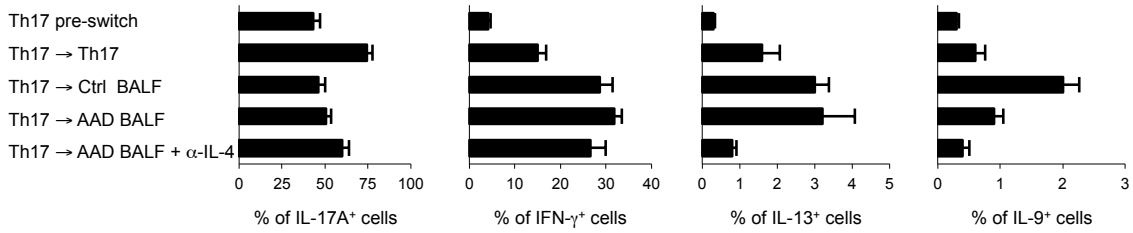

Figure 42. Stability of in vitro-derived Th17 cells upon culture with BAL fluid from allergic mice. Naïve CD4$^+$ T cells were isolated from the spleen of *Il17f* $^{+/Cost}$*Rs*$^{YFP/YFP}$ mice and stimulated under Th17-polarizing conditions for 5 days (Round 1). Cells were re-stimulated and cultured under long-term Th17 polarizing conditions for another five days (Round 2). Live YFP$^+$ cells were sorted by flow cytometry and stimulated under long-term Th17-polarizing conditions or in the presence of BAL fluid (BALF) from non-allergic mice (Ctrl BALF), OVA and alum-induced allergic mice (AAD BALF) or OVA and alum-induced allergic mice with the addition of anti-IL-4 (AAD BALF + α-IL-4) for five days (Round 3). Cells were re-stimulated with PMA and ionomycin and cytokines were analyzed by flow cytometry in the live YFP$^+$ population. The data are the mean ± SEM of 4-5 mice.

To further understand if the differences observed in the stability of Th17 cells derived in vitro or during the development of AAD is due to differences in cytokine receptor expression or can be attributed to the cytokine environment induced during AAD, we determined if in vitro derived Ag-specific Th17 cells could adopt other T helper effector programs upon transfer to an allergic environment. Ag-specific naïve CD4$^+$ T cells from *Il17f* $^{+/Cost}$*Rs*$^{YFP/YFP}$-OT-II mice were stimulated under Th17-polarizing conditions for two rounds of culture. YFP$^+$ Th17 cells were sorted, transferred intravenously to OVA and alum sensitized wild type mice and subsequently challenged with OVA. While endogenous CD4$^+$YFP$^-$ cells from the BAL and lung produce IL-17A, IFN-γ or IL-13,

transferred CD4^{+}YFP^{+} cells maintain IL-17A secretion, but do not produce IFN-γ, IL-13 or IL-9 (Figure 43B-D, data not shown). These data demonstrate that while in vitro-derived Th17 cells express IL-4Rα, respond to IL-4-induced signals and upregulate the expression of alternative cytokines in vitro (Figure 33B, E-F and 41A), when transferred to a Th2 and Th9-biased pro-inflammatory environment, they are refractory to these signals and maintain an IL-17-secreting phenotype without adopting alternative T helper effector programs.

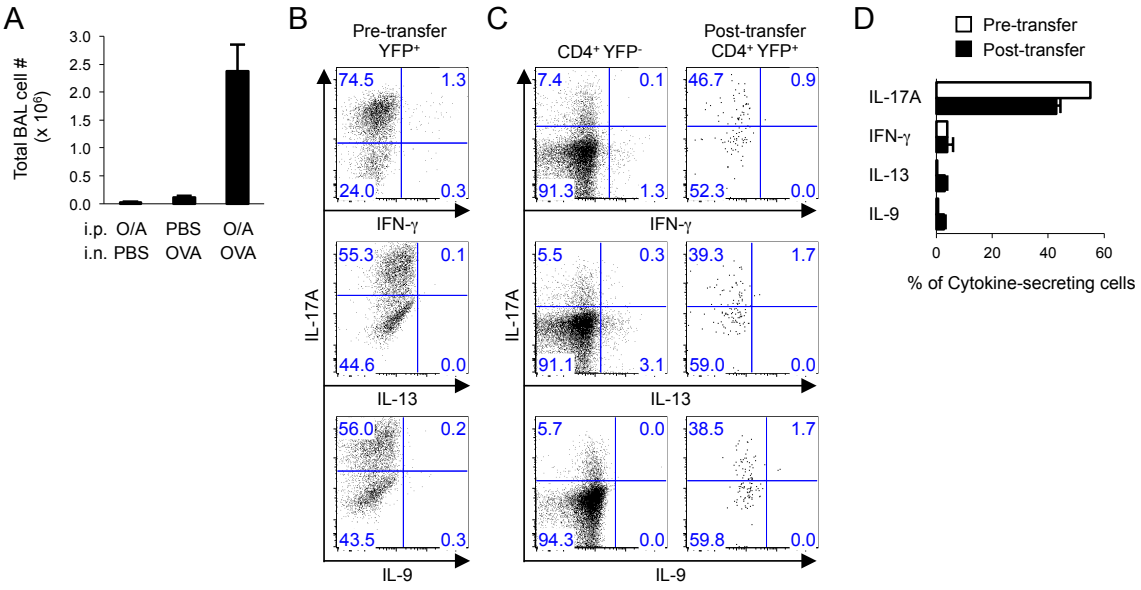

Figure 43. Stability of Ag-specific in vitro-derived Th17 cells upon adoptive transfer to allergic mice. *A-D,* WT mice were injected i.p. with PBS or sensitized i.p. with OVA and alum (O/A) on days 0 and 7. All mice were injected i.v. with sorted YFP$^+$ Th17 cells (1x10^5) differentiated from *Il17f* $^{+/Cost}$*Rs*$^{+/YFP}$ –OT-II mice (2 rounds under Th17-polarizing conditions) on day 20. Mice were challenged i.n. with PBS or OVA on days 21-26 and sacrificed 24 h after the final challenge. Cells were isolated from the BAL and lung for further analysis. (*A*) Total cell number was calculated in the BAL of each group of mice. Data are the mean ± SEM of 7-8 mice. (*B*) Sorted YFP$^+$ Th17 cells differentiated from *Il17f* $^{+/Cost}$*Rs*$^{+/YFP}$ –OT-II mice (2 rounds under Th17-polarizing conditions) were

stimulated with PMA and ionomycin and cytokines were analyzed by flow cytometry (Pre-transfer YFP^+). (*C*) BAL (data not shown) and lung cells were stimulated with PMA and ionomycin and cytokines were analyzed by flow cytometry in live YFP^- (endogenous) or YFP^+ (Post-transfer) $CD4^+$ lymphocytes. Representative dot plots are shown. (*D*) Graphical representation of the Pre-transfer YFP^+ and Post-transfer $CD4^+YFP^+$ data displayed in *B*-*C*. The data are from Th17 cells (Pre-transfer) or the mean ± SEM of 4 mice (Post-transfer).

DISCUSSION

IL-17-producing T cells are critical to the development of pathogen and tumor immunity, but also contribute to the pathology of autoimmune diseases and allergic inflammation. IL-17-secreting T cells develop in response to a cytokine environment that activates STAT molecules and other regulatory factors, though the mechanisms underlying the development, stability and function of IL-17-secreting T cells are still unclear.

CD8 T cells can acquire cytokine-secreting phenotypes paralleling cytokine production from Th cells. Tc17 cells have been shown to promote inflammation and mediate immunity to influenza. However, most reports have observed a lack of cytotoxic activity by Tc17 cells. We further explored the anti-viral activity of Tc17 cells using a vaccinia virus infection model. Tc17 cells expanded during VV infection, and TCR transgenic Tc17 cells were capable of clearing recombinant VV infection. In vivo, adoptively transferred Tc17 cells lost the IL-17-secreting phenotype even in the absence of stimulation, but did not acquire IFN-γ-secreting potential unless stimulated with a virus-encoded antigen. However, examination of cells following infection demonstrated that these cells acquired cytotoxic potential in vivo even in the absence of IFN-γ. Cytotoxic potential correlated with *Fasl* expression, and the cytotoxic activity of post-infection Tc17 cells was partially blocked by addition of anti-Fas ligand. Thus, Tc17 cells mediate VV clearance through expression of specific molecules associated with cytotoxicity, but independent of an acquired Tc1 phenotype.

STAT4 is a critical mediator of inflammatory immunity and is required for all known IL-12 biological responses, including the induction of IFN-γ and development of Th1 cells. The role of STAT4 in immunity is not restricted to IL-12 signaling as type I IFNs and IL-23 have also been shown to activate STAT4. We previously demonstrated that IL-23-induced IL-17 production was diminished in STAT4-deficient T cells, however in those experiments, performed early in our understanding of IL-17-producing T cells, the STAT4 effect was observed using IL-23 stimulation of total $CD4^+$ T cells. Thus it was not clear which cell populations were responding, and whether STAT4 was required in all cell types. We found that STAT4-deficiency results in reduced IL-23-mediated IL-17 production from memory Th cells and NKT cells. IL-23 stimulation does not activate STAT4 in these populations, however STAT4-deficiency results in reduced *Il23r* expression from memory Th cells and NKT cells, rendering them less responsive to IL-23-induced signals. Our data present evidence suggesting a T cell-extrinsic role for STAT4 in mediating IL-23 responsiveness.

Th17 cells are critical for the clearance of extracellular bacteria and fungi, but also contribute to the pathology of autoimmune diseases and allergic inflammation. Following exposure to an appropriate cytokine environment, Th17 cells can acquire a Th1-like phenotype in vitro and in vivo, but less is known about their ability to adopt Th2 and Th9 effector programs. To explore this in more detail, we generated an IL-17F lineage tracer mouse strain that allows

tracking of cells that formerly expressed IL-17F and can be used to test stability of the Th17 phenotype. In vitro-derived Th17 cells cultured under polarizing conditions that promote Th1, Th2 or Th9 differentiation, adopted the respective effector programs, expressing lineage associated transcription factors, and secreting IFN-γ, IL-13 or IL-9, while diminishing IL-17 production. To explore the stability of Th17 cells in an in vivo environment that is associated with the development of Th2 and Th9 cells, we explored Th17 cell stability in both acute and chronic models of allergic airway disease. In the models of AAD tested, Th17 cells from the lungs of diseased mice did not secrete alternative cytokines nor adopt Th1, Th2 or Th9 effector programs, but remained stable IL-17-secretors. While in vitro derived Th17 cells expressed IL-4Rα, those induced during AAD did not, possibly rendering them unresponsive to IL-4-induced signals. Furthermore, in vitro-derived OVA-specific Th17 cells transferred in vivo to OVA and alum-sensitized mice remained IL-17-secretors and did not produce alternative cytokines upon subsequent OVA challenge, suggesting that the allergic airway environment either promotes the stability of Th17 cells or prevents their instability. Thus, although Th1-biased pro-inflammatory environments have been shown to induce alternative cytokine expression from Th17 cells in vivo, our data suggest that during allergic inflammatory disease, Th17 cells are comparatively stable, and retain the potential to produce IL-17.

Part I- Stability of Tc17 cells

Cytokines are critical effectors of T cell function, and CD8 T cells predominantly produce IFN-γ upon stimulation. However, CD8 T cells can acquire various cytokine-secreting potentials, including a phenotype resembling the Th17 phenotype, termed Tc17. Acquisition of this phenotype is directed by similar cytokines and transcription factors as the Th17 subset. However, the function of these cells is still not well defined. In several studies, Tc17 cells have been shown to mediate anti-viral immunity and inflammation. Whether Tc17-mediated immunity is through promoting inflammation or acquiring a cytotoxic phenotype is not clear.

Development and stability of Tc17 cells in vitro

One of the features of in vitro-derived Tc17 cells following in vivo injection is that these cells lose the IL-17-secreting phenotype. As shown in Th17 cells (185, 186), the development of Tc17 cells requires STAT3 and RORγt (Figure 3) (187-190). STAT3 and RORγt are required for IL-17 production from Tc17 cells and STAT3 appears to have a more suppressive effect than RORγt on IFN-γ and Granzyme B production in developing Tc17 cells (Figure 3). Using in vitro experiments, we determined that Tc17 cells could switch to a Tc1 phenotype, accompanied by the induction of IFN-γ and Granzyme B and repression of IL-17 (Figure 4). We further found that IFN-γ, STAT4 and T-bet contribute to the ability of Tc17 cells to switch phenotypes (154), yet in vivo, viral clearance and the acquisition of cytotoxic activity were independent of both IFN-γ and STAT4, and were not associated with an increase in Granzyme B expression (Figure 9 and

data not shown). Thus the signals that generate cytotoxic Tc17 cells remain undefined.

Anti-viral activity of Tc17 cells

While Tc17 cells can clearly develop in vitro, their existence in vivo is not well characterized. We demonstrated IL-17 production from unimmunized CD8 T cells, and an expansion of IL-17-secreting CD8 T cells following infection with VV. This is similar to observations following an influenza virus infection (191). We further show that there is development of IL-17-secreting CD8 T cells specific for a virus-derived peptide following VV infection. However, while this enhancement of IL-17-secreting CD8 T cells is specific, it is unlikely to be the sole mechanism responsible for VV clearance and anti-viral immunity. We further observed slightly enhanced or normal clearing of VV from *Il17*$^{-/-}$ and *Stat3*$^{CD4-/-}$ mice, respectively (data not shown). This suggests that IL-17 or Th17/Tc17 cells are not critical for viral clearance. However, IL-17 may regulate anti-viral immunity through additional mechanisms. A recombinant VV encoding IL-17 had increased virulence in a mouse model, suggesting IL-17 impaired anti-VV immunity (192). Moreover, IL-17 has been shown to limit Th1 development (193), and it has been suggested that VV uses induction of IL-17 as a mechanism of immune evasion, particularly in skin with atopic dermatitis (194, 195). In contrast, a recombinant VV expressing IL-23 enhanced anti-viral immunity through a mechanism that was at least partially IL-17-dependent (196). Thus, IL-17 may be involved in

regulating the effectiveness of the anti-viral response in a context-dependent manner.

Cytotoxic function of Tc17 cells

Perhaps the most critical question is how Tc17 cells mediate anti-VV immunity. Our data demonstrate that *Fasl* is unique among cytotoxicity-associated genes in that expression is lower in Tc17 cells compared to Tc1 cells after 1 week of culture, but expression increases after additional culture in vitro or post-transfer in vivo (Figure 5 and 11A). We further showed that blocking FasL significantly decreased post-transfer Tc17 cytotoxicity (Figure 11B), suggesting that Tc17 cytotoxicity is at least partially FasL-dependent. Whether other mediators are involved is still unclear. Hamada et al showed that Tc17-mediated influenza immunity was dependent on IFN-γ but not perforin (191). However, we showed that IFN-γ was not required for Tc17-mediated immunity to VV (Figure 9), consistent with a prior report that IFN-γ is not required for immunity to VV (197). Similarly, we saw no dependence on STAT4 (data not shown), suggesting that many features of the IL-12-induced genetic program are not required for Tc17-mediated immunity to VV. In agreement with this, we did not see any increase in expression of either perforin or Granzyme B when Tc17 cells acquired a cytotoxic phenotype (Figure 5 and 9). As TNF-α expression is not vastly different between Tc1 and Tc17 cells (Figure 5), it seems unlikely to be a candidate mechanism for Tc17-mediated killing in vitro. However, it could be involved in viral clearance. We observed some increased pulmonary inflammation in mice infected with VV

intranasally upon receiving transferred Tc17 cells, compared to mice receiving Tc1 cells, a result similar to observations in the influenza model (191). However, this was not uniform among all Tc17 recipients, and there was no correlation between inflammation and viral clearance (data not shown). It is likely that there are multiple overlapping mechanisms of Tc17-mediated viral clearance and the specific contribution of each mechanism may depend on the pathogen. Importantly, it is clear that Tc17 cells do function after transfer in vivo, and in some experiments function better than matched Tc1 cells (190, 198). Thus, after extended culture in vitro, or following exposure to antigen-encoded virus in vivo, Tc17 cells can acquire a cytotoxic phenotype that is at least partially dependent upon FasL.

While IL-17-secreting CD4 T cells are established as an important component of inflammatory immunity, the role of Tc17 cells is less clear. Several studies have shown that Tc17 cells expand during infection or inflammation, and that they can function in vivo. Importantly, the current study reveals that Tc17 cells, while initially non-cytotoxic, acquire a cytotoxic phenotype and can mediate clearance of VV in vivo. The requirement for FasL and the mechanisms of cytotoxic phenotype acquisition will be important to determine in the future.

Part II- Role of STAT4 in IL-23-induced IL-17 production

STAT4 is a critical mediator of inflammatory immunity and is required for all known IL-12 biological responses, including the induction of IFN-γ and development of Th1 cells (7, 8, 199, 200). However, the role of STAT4 in immunity is not restricted to IL-12 signaling. Type I IFNs activate STAT4 and IL-23 has also been suggested to activate STAT4 in human T cells (71, 201, 202). We previously demonstrated that IL-23-induced IL-17 production in mouse T cells was abrogated in the absence of STAT4, however the mechanisms underlying STAT4-mediated IL-23 responses in T cells are still unclear (76).

STAT4-dependent cellular responses in vitro

We previously demonstrated that IL-23-induced IL-17 production was diminished in STAT4-deficient T cells (76). In those experiments, performed early in our understanding of IL-17-producing T cells, the STAT4 effect was observed using IL-23 stimulation of total $CD4^+$ T cells. Thus it was not clear which cell populations were responding, and whether STAT4 was required in all cell types. While naïve $CD4^+$ T cells do not express IL-23R, it is induced during Th17 cell differentiation (72, 203). Conversely, IL-23R is constitutively expressed on IL-17 producing innate immune cells, enabling rapid IL-17 production from these populations in response to IL-23 (155). The regulation of IL-23-induced IL-17 secretion from IL-17-producing cells has not been fully established. We further explored the requirement for STAT4 in IL-23-induced IL-17 production from memory Th cells and innate immune cells that had previously been shown to

produce IL-17. We showed that IL-23-induced IL-17 production from γδ T cells, LTi-like cells and NK cells was independent of STAT4 (Figure 15A). A role for STAT3 in IL-17 production from LTi-like cells has been demonstrated (179), suggesting that STAT3 might be more important than STAT4 in IL-23-induced IL-17 production from this group of innate immune cells. We further demonstrated that memory Th cells and NKT cells displayed diminished IL-23-induced IL-17 production in the absence of STAT4 (Figure 14 and 15B), suggesting a requirement for STAT4 for optimal cytokine-induced IL-17 production from these populations.

Oppmann et al first demonstrated that IL-23 can activate STAT4 in human T cells, however to a lesser extent than STAT3 (71). We tested whether IL-23 could induce STAT4 activation in wild type T cell populations that demonstrated STAT4-dependent IL-17 production and found that IL-23 did not activate STAT4 in memory Th cells or NKT cells (Figure 16). We further explored if STAT4-deficiency resulted in diminished cellular responses to IL-23 and found *Il23r* expression to be reduced on both memory Th cells and NKT cells from STAT4-deficient mice (Figure 17). Since STAT4 was not detectably activated by IL-23 in these cell types, we hypothesized that IL-12 or type 1 IFN-STAT4 signals could be required for optimal IL-23R expression and IL-23-depenent IL-17 production in T cells. To further analyze the role of IL-12 in IL-23-dependent IL-17 responses, we performed experiments with mice deficient in the p35 subunit of IL-12 (IL-12α), which is not shared with IL-23. We demonstrated that NKT cells from IL-

12α-deficient mice displayed reduced expression of *Il23r* and abrogated IL-23-induced IL-17 production (Figure 18), similar to that from STAT4-deficient mice. However, when we tested if IL-12 could induce *Il23r* in wild type NKT cells, we found that it had no direct effect on *Il23r* expression (Figure 19). As type I IFNs also activate STAT4 in T cells, we tested if IFN-α could induce *Il23r* on wild type NKT cells and found that IFN-α stimulation did not induce *Il23r* expression on NKT cells (data not shown). Thus, as IL-23 does not activate STAT4 in memory Th cells or NKT cells, nor do other known STAT4-activating cytokines induce *Il23r* expression in these populations, our in vitro data suggest a T cell-extrinsic role for STAT4 in mediating IL-23 responsiveness.

STAT4 and IL-23-mediated inflammation

STAT4-deficient mice are resistant to the development of T cell-mediated autoimmune diseases, including arthritis, colitis, diabetes and EAE (204-208). While resistance was originally attributed to alterations in IL-12-driven Th1-development, inflammatory mediators from other Th subsets have proven to be essential for the pathology of some autoimmune responses. The generation of IL-23 p19-deficient mice identified IL-23, rather than IL-12, as the crucial factor in driving EAE and further showed a role for IL-23 and Th17 cells in the development of collagen-induced arthritis (209, 210). Not only is IL-23 important for the maintenance of Th17 cells, it is also critical for the development of pathogenic Th17 cells, which produce GM-CSF in addition to IL-17, IL-21 and IL-

22 and have been associated with the pathogenesis of several autoimmune and inflammatory diseases (61, 62, 73-75, 122, 203, 211, 212).

Our data suggest that reduced IL-23R expression and IL-23-stimulated IL-17 production observed in memory Th and NKT cells from STAT4-deficient mice might be due to an indirect effect of STAT4-deficiency in other cell populations. To address this possibility in vivo, we analyzed IL-17 production in mouse models of inflammation associated with the induction of IL-17 from $CD4^+$ T cells and NKT cells. IL-17 production from Th17 cells and NKT cells has been implicated in allergic disease, and reports have also demonstrated that STAT4 and IL-23-deficiency in mice results in impaired airway responses, including reduced IL-17 production in the lung (172, 213-215). We further analyzed the effects of STAT4-deficiency on the development of OVA and alum-induced allergic responses, including IL-17 production, and while we did not observe significant differences in inflammation and OVA-induced IL-17 production between wild type and STAT4-deficient mice with AAD, there was a trend toward reduced allergen induced-IL-17 production from the BAL cells and splenocytes of STAT4-deficient mice (Figure 20). As our in vitro data demonstrated a role for STAT4 in IL-23-mediated responses in memory Th cells and NKT cells, it maybe beneficial to adjust the protocol and challenge the mice at a later time point to analyze memory responses. Wingender et al demonstrated a role for IL-17 producing NKT cells and $CD4^+$ T cells in eliciting allergic responses in AAD using house dust extracts in place of alum as an adjuvant, however the requirement for

different STAT molecules in IL-17 production was not analyzed, but could be valuable to determine in the future (176).

A number of reports have demonstrated that administration of α-GalCer to mice elicits rapid production of IL-17 from NKT cells (171-173, 176). However the regulation of NKT cell IL-17 production in vivo remains unclear. We demonstrated that intravenous administration of α-GalCer induced IL-17 production in mice, however the levels were similar between wild type and STAT4-deficient mice, whereas IFN-γ levels were decreased in the absence of STAT4 (Figure 21A and B). As our studies demonstrated a role for STAT4 in IL-23-induced-IL-17 production in vitro, we also analyzed NKT cell IL-17 production in vivo in response to a combination of α-GalCer and IL-23. We showed that STAT4-deficiency did not have an effect on IL-17 production in response to intravenous administration of α-GalCer and IL-23 (Figure 21C) and further demonstrated that STAT4-deficiency did not affect IL-17 production in the lung in response to intranasal administration of α-GalCer and IL-23 (Figure 22B-D). IL-17A has been associated with virus-associated and steroid-resistant asthma with NKT cells being implicated in allergic disease (176, 216, 217). Vultaggio et al demonstrated that upon intratracheal administration of α-GalCer and the TLR3 ligand, Poly(I:C), airway responses and IL-17A levels were elevated in comparison to mice treated with α-GalCer alone, and this was attributed to effects on IL-23 and IL-1β (177). We tested the effects of STAT4-deficieny in a similar model using intranasal administration of α-GalCer and Poly(I:C) and

observed similar levels of IL-17 production in the total lung and from NKT cells isolated from the BAL, from wild type and STAT4-deficient treated mice (Figure 23B and C). As STAT4-deficiency did not affect IL-17 production from NKT cells in any of the inflammatory models tested, it is possible that STAT3 is able to compensate for the loss of STAT4, inducing normal IL-17 production from NKT cells in vivo, that STAT3 is more important than STAT4 in mediating IL-17 production from NKT cells in vivo or that cytokine-induced IL-17 production from NKT cells is not critical in vivo.

STAT4 and the IL-23-Th17 pathway have been implicated in the development of human autoimmune and inflammatory diseases, such as IBD, psoriasis, RA, SLE, allergy and asthma (57). Genome-wide association studies have implicated *STAT4* in diseases such as asthma, IBD, RA and SLE (218-220) and *IL23R* in IBD, psoriasis and RA (221-224). While STAT4 is involved in signaling by IL-12, IL-23 and type I IFNs, and IL-23R is upstream of STAT3 and STAT4, it can be difficult to determine the particular signaling pathway involved in disease pathogenesis. Importantly, these findings reveal that the IL-23 signaling pathway may be relevant to human disease.

Our in vitro data presented evidence suggesting a T cell-extrinsic role for STAT4 in mediating IL-23 responsiveness to memory Th and NKT cells in mice. It will be important to determine mechanisms underlying STAT4-dependent IL-23

responses in the future, to better understand IL-23-mediated inflammatory responses and disease pathogenesis.

Part III-Stability of Th17 cells

T helper cell biology is a critical component of adaptive immunity, with each T helper subset having a specialized role in immune homeostasis, host-defense or the development of inflammatory disease. T helper cell development has been studied extensively for more than 20 years, with the question of lineage stability and plasticity currently under intense investigation. Upon identification of the Th17 differentiation pathway, it became clear that there were overlapping components of the Th17 effector program with that of other T helper lineages, however the stability of Th17 cells is still not well defined. In several studies, Th17 cells have been shown to adopt an iTreg or Th1 effector program. Whether or not Th17 cells can adopt a Th2 or Th9 effector program is still not clear.

Validation of Il17f ^{Cost} mice

Several studies have shown that the fate of Th17 cells can change upon in vitro culture or adoptive transfer, however it was originally thought that their development in vivo was relatively stable (225-228). A number of reporter and lineage tracer mice have been generated to respectively, identify or trace, IL-17-secreting cells in vivo (119-121, 132, 229, 230). Our goal was to generate a mouse strain that combined both approaches, to simultaneously identify cells that currently produce IL-17F or that previously produced IL-17F, but have since turned off the *Il17f* gene. Ideally cells from *Il17f* ^{Cost}*Rs*^{YFP} mice should express EGFP and Cre when *Il17f* is turned on, and retain Cre-induced YFP expression if *Il17f* expression were discontinued, allowing for the fate of IL-17F-secreting cells

to be monitored. Expression of EGFP and YFP was tested from in vitro derived Th17 cells from *Il17f*^{Cost}*Rs*^{YFP} mice and we found that while both EGFP and YFP could be detected in Th17 cells by immunofluorescence microscopy (Figure 27), YFP, indicating active Cre expression, but not EGFP, was detected by flow cytometry (Figure 28), possibly due to insufficient expression of the fusion protein for cytometric detection. Thus, in subsequent experiments, *Il17f*^{Cost}*Rs*^{YFP} mice were used as a lineage tracer mouse strain using flow cytometry analyses to define YFP^{+} cells as those that had activated the *Il17f* locus.

We confirmed that in vitro differentiated Th17 cells and not other Th subsets expressed YFP (Figure 30) and that there was an enrichment of IL-17-producing cells in the YFP^{+} population (Figure 29). Furthermore, we provided evidence that our lineage tracer mouse can be used to monitor the fate of IL-17-secreting cells in an in vivo model of EAE, as others have previously shown with different IL-17-reporter mice (Figure 31) (120, 121). Although only the *Il17f* locus was targeted in the generation of *Il17f*^{Cost} mice, we found that Th17 cells derived from these mice produced less IL-17A than control Th17 cells, suggesting that there is an effect of the *Cost* allele on the expression of the adjacent *Il17* allele (Figure 29). Although the epigenetic processes and regulatory mechanisms that control Th17 cell differentiation have yet to be fully established, *Il17a* and *Il17f* appear to share similar regulatory elements and may be coordinately regulated (231-233). While no known regulatory elements were deleted in the generation of *Il17f*^{Cost} mice, it remains possible that the dynamics of the chromatin have been altered by the

Cost allele, affecting the regulation of *Il17a*. The apparent effect the *Cost* allele has on IL-17A expression from Th17 cells will be important to consider when using *Il17f* ^{Cost} mice to analyze inflammatory responses in vivo.

Stability of Th17 cells in vitro

Similar to other findings, we demonstrated that YFP$^+$ Th17 cells transition to a Th1-like effector program in vitro, accompanied by the upregulation of T-bet and IFN-γ and downregulation of *Rorc* and IL-17 (Figure 33-35) (119, 226). IL-12 induced STAT4 and T-bet have been shown to repress the *Rorc* locus and the *Il17a-Il17f* locus in developed Th17 cells in vitro, while permitting the induction of *Ifng* (119, 132), in addition to earlier findings that demonstrated a role for T-bet and STAT4 in the plasticity of Th17 cells in vitro (213, 234). Expression of *Il12rb2* on YFP$^+$ Th17 cells likely renders them responsive to IL-12 induced signals, supporting their transition to a Th1-like phenotype in vitro (Figure 33-35 and 41).

Similar to IL-12, IL-4 also negatively regulates the development of the Th17 lineage in vitro (59, 235). Prior to the development of IL-17-reporter mice, the stability of Th17 cells was analyzed by the isolation of a pure IL-17-secreting population of cells. Using this method of isolation, our laboratory and others, previously showed that IL-17$^+$ Th17 cells cultured under Th2-polarizing conditions displayed downregulation of IL-17 and *Rorc* and enhanced IL-4 and *Gata3* levels. As a small percentage of contaminating IL-17$^-$ cells existed in the sorted population, it remained possible that these Th2-like cells generated from

an IL-17$^-$ precursor (75, 226). With the use of an IL-17-reporter mouse, Nurieva et al showed that Th17 cells cultured for 4 days in vitro, adopted a Th2-like phenotype after a further 3 days of stimulation under Th2-polarizing conditions (133). Bivalent epigenetic marks are present at the *Tbx21* and *Gata3* loci from in vitro derived Th17 cells, suggesting that following exposure to the appropriate cytokine environment, Gata3 could be induced in Th17 cells, similar to T-bet (131). In our studies, we stimulated Th17 cells for two rounds under Th17-polarizing conditions to explore the stability of a more developed Th17 cell. We showed that YFP$^+$ Th17 cells adopted the Th2 effector program in vitro, with the upregulation of Gata3 and IL-13 and downregulation of IL-17 (Figure 33-35). Similarly, YFP$^+$ Th17 cells adopted the Th9 effector program in vitro, accompanied by the induction of *Il4ra*, *Irf4*, *Maf* and Gata3 and suppression of *Il23r* and IL-17 (Figure 33-35 and 41). In vitro derived YFP$^+$ Th17 cells expressed low levels of *Il4ra*, enabling them to respond to IL-4 signals (Figure 41A). *Il4ra* expression was increased in cells skewed with IL-4 toward the Th2 or Th9 lineage (Figure 41A), which was accompanied by increased GATA3 in cells skewed toward a Th2 or Th9 phenotype, and *Irf4* and *Maf* in cells skewed toward the Th9 lineage (Figure 35 and 37). IL-4 and STAT6 promote the expression of c-Maf, GATA3 and IRF4 during the development of Th2 and Th9 cells (236). Furthermore, GATA3 is required for the development of Th2 cells and turns on the *Il4-Il5-Il13* locus (24, 25, 237, 238). While GATA3 is also required for Th9 development, it does not appear to have a direct effect on the *Il9* gene. In contrast, while PU.1 negatively regulates the Th2 program, it is critical for the

development of Th9 cells. TGF-β induced PU.1 and IL-4-STAT6 induced IRF4, promote Th9 development by binding to the *Il9* promoter (46, 47, 49, 239, 240). *Irf4* levels were increased in YFP$^+$ Th17 cells stimulated under Th9-polarizing conditions, accompanied by increased IL-9 production (Figure 33-35), however we were unable to detect PU.1 (data not shown). We have routinely observed that PU.1 is expressed early during Th9 development (unpublished observations), therefore PU.1 expression could be downregulated in Th9 transitioned YFP$^+$ cells at the time the analysis was performed. These data establish that IL-4-induced signals play a role in the transition of YFP$^+$ Th17 cells to a Th2 or Th9-like phenotype in vitro, and while we have identified possible candidate transcription factors involved in the regulation of this flexibility, their requirement in this process will be determined in the future.

A closer analysis of cytokine production in YFP$^+$ Th17 cells stimulated under different Th-polarizing conditions in vitro revealed that IL-17$^+$ cells were significantly decreased in Th17 cells upon stimulation under Th1, Th2 or Th9-skewing conditions (Figure 33B and D-F). Furthermore, IL-17$^+$IFN-γ$^+$ and IFN-γ$^+$ cells were increased to similar levels in Th17 cells stimulated under Th1-polarizing conditions (Figure 33B and D), while IL-17$^+$IL-13$^+$ and IL-13$^+$ (Figure 33B and E) or IL-17$^+$IL-9$^+$ and IL-9$^+$ (Figure 33B and F) cells were increased to similar levels in Th2 or Th9 cells, respectively. It is possible that under Th1-polarizing conditions, RORγt and T-bet are differentially expressed in the IL-17$^+$, IL-17$^+$IFN-γ$^+$ or IFN-γ$^+$ population of YFP$^+$ cells, attributing to the cytokine-profile

of each population. Similarly, when YFP^+ Th17 cells are stimulated under Th2 or Th9-polarizing conditions, differential expression of RORγt, GATA3 and other transcription factors important for the development of each subset may occur in each cytokine-positive population of cells. It will be important to determine the co-expression of transcription factors and cytokines in these different populations to further understand the underlying mechanisms of Th17 cell plasticity.

Stability of Th17 cells in allergic airway disease

Similar to other reports, we demonstrated that Th17 cells can adopt the Th1 effector program in vivo, using a model of EAE (Figure 31), but their potential to adopt the Th2 or Th9 effector programs in vivo is not well defined. Allergic disease is often thought of as a Th2-mediated disease, however the development of AAD is orchestrated by a large number of immune cells, which include Th1, Th2, Th9 and Th17 cells (241). Two recent reports have identified a population of T effector/memory cells in humans that co-express GATA3 and RORγt, and coproduce Th2 and Th17 cytokines. This cell population was increased in asthmatic patients compared with non-asthmatics and further identified in the lung of allergic mice, however the origin of the Th2/Th17 effector/memory population is still unclear (128, 129). To further understand the stability of Th17 cells in allergic inflammation, we induced AAD in mice using the more acute, adjuvant-dependent OVA and alum model (Figure 36) and the chronic, adjuvant-independent house dust mite model (Figure 38). In both models of disease, we found that $CD4^+YFP^+$ cells from the lung of allergic mice

secreted IL-17, but did not produce IFN-γ, IL-13 or IL-9 (Figure 36B-C and 38B-C). We did detect a small population of $CD4^+$ cells in the lung of allergic mice that coproduced IL-17A and IL-13, but as they did not express YFP, it is possible that they arose from a Th2 rather than a Th17 origin, or may not have expressed IL-17F (Figure 36B).

IL-4 is required for Th2 and Th9 development, and we showed that Th17 cells express low levels of *Il4ra* (Figure 41A) and that IL-4-induced signals promote the development of Th2- and Th9-like cells from Th17 precursors in vitro (Figure 33-35). To determine the potential responsiveness of $CD4^+YFP^+$ cells in AAD, we further characterized cytokine receptor expression on $CD4^+$ cells from the lung of control and allergic mice and showed that while IL-4Rα expression is induced on $CD4^+YFP^-$ T cells during the development of AAD, it is not expressed on $CD4^+YFP^+$ cells from allergic mice, perhaps rendering them unresponsive to IL-4 signals (Figure 41B). To determine if differences in IL-4Rα expression on Th17 cells derived in vitro, or in vivo during allergic disease, accounted for the observed differences in stability, we transferred in vitro-derived OVA-specific YFP^+ Th17 cells to OVA and alum-sensitized mice and challenged them with OVA. Although in vitro derived YFP^+ Th17 cells express *Il4ra* (Figure 41A), upon transfer, they remained IL-17 secretors and did not secrete alternative cytokines in an allergic environment, similar to Th17 cells developed in the lung during AAD (Figure 43C and D). Hirota et al found that during the development of a skin infection with *Candida albicans*, Th17 cells repressed IL-17 expression and did

not secrete alternative cytokines upon clearance of the infection, which was accompanied by low IL-23 expression and increased IL-10 expression in the skin, suggesting a switch from a pro-inflammatory to an anti-inflammatory environment (121). Our studies did not address the kinetics of YFP and IL-17 expression over a period of time in allergic mice, however the level of IL-17 produced by in vitro derived YFP^+ Th17 cells remained the same before and after transfer to allergic mice, suggesting that the allergic environment either maintains the stability of Th17 cells or prevents their instability (Figure 43D). It will be important to determine the inflammatory mediators critical to the stability of Th17 cells in allergic inflammation in future studies.

Th17 cells play an important role in the development of allergic inflammation in mice by promoting neutrophillic airway inflammation and further enhancing Th2-mediated airway eosinophilia (242, 243). Th17 cells also promote steroid-resistant airway responses in mice, linking them to severe AAD (217). While IL-17 appears to be essential during allergic sensitization to establish airway inflammation, it has also been shown to repress airway responses in mice that have been sensitized to allergen, suggesting a dual regulatory role for IL-17 in AAD (244-246). Although there is an established role for Th17 cells in the development of allergic airway responses in mice, the role of IL-17 in human allergic disease is less clear. While IL-17 levels are increased in the lung of asthmatic patients, the cellular source of IL-17 and its association with disease severity remain controversial (247-250). The association of asthma and SNPs in

IL1R1 and *RORA* and clinical effects of trials targeting IL-17A and IL-17R in asthma, are suggestive of a role for the Th17 cell pathway in human allergic disease (251-254). Collectively, these findings demonstrate an association with Th17 cells and the development of allergic responses in the mouse and human lung, suggesting that Th17 cell stability may be essential for the development of allergic disease.

Using *Il17f* ^{Cost} mice, we demonstrated that Th17 cells can adopt the effector programs of Th1, Th2 or Th9 cells in vitro. However, in contrast to Th1-biased pro-inflammatory environments that induce Th17 instability in vivo, during allergic inflammatory disease, Th17 cells are comparatively stable, and retain the potential to produce IL-17. Thus, our data demonstrate that the inflammatory environment has distinct effects on the stability of IL-17-secreting T cells in vivo. Future studies will define inflammatory mediators involved in the regulation of Th17 cell stability in the allergic airway environment to further establish the underlying mechanisms of Th17 stability in allergic disease.

Function of Th17 stability in the immune response

It is possible that the inflammatory environment dictates the stability and plasticity of Th17 cells. Th17 cells develop early in an immune response and induce the recruitment of neutrophils, which can phagocytose microorgansims. Th1 cells develop later in the response and can activate neutrophil and macrophage antimicrobicidal activity, leading to the death and clearance of microorgansims.

As the environment becomes more pro-Th1, this can have a negative effect on the Th17 phenotype, and result in the downregulation of IL-17 and upregulation of a Th1-like phenotype. This may increase the ability of Th17-transitioned cells to target intracellular pathogens or pathogens that are resistant to neutrophilic immunity, in addition to dampening neutrophillic inflammation, which could otherwise lead to tissue damage. In contrast, it is possible that Th17 immunity is maintained and does not interfere with Th2 and Th9-mediated immune responses. Th2 and Th9-mediated immunity is directed toward parasites, which are often located at mucosal sites, and the maintenance of Th17 cells at these same sites is important for Th17-mediated host defense. These two scenarios demonstrate differential requirements for the plasticity of Th17 cells, where Th17-mediated immunity is ultimately dominated by Th1-mediated effects during the resistance and clearance of certain types of inflammation. In contrast, Th17-mediated effects may co-exist with type 2 inflammation, allowing for a broader immune response to other potential pathogens.

FUTURE DIRECTIONS

The data presented in this thesis contribute to the current understanding of the development, stability and function of IL-17-secreting T cells. We demonstrated that in vivo, Tc17 cells clear VV infection and acquire cytotoxic potential, that is independent of IL-17 production and the acquisition of IFN-γ-secreting potential, but partially dependent on Fas ligand, suggesting that Tc17-mediated VV clearance is through cell killing independent of an acquired Tc1 phenotype. In contrast, memory Th cells and NKT cells display STAT4-dependent IL-23-induced IL-17 production that correlates with *Il23r* expression. IL-23 does not activate STAT4 nor do other STAT4-activating cytokines induce *Il23r* expression in these populations, suggesting a T cell-extrinsic role for STAT4 in mediating IL-23 responsiveness. Furthermore, in vitro-derived Th17 cells are flexible when cultured under polarizing conditions that promote Th1, Th2 or Th9 differentiation, adopting the respective effector programs, and decreasing IL-17 production. However, in models of AAD, Th17 cells do not secrete alternative cytokines nor adopt other effector programs, and remain stable IL-17-secretors. In contrast to Th1-biased pro-inflammatory environments that induce Th17 instability in vivo, during allergic inflammatory disease, Th17 cells are comparatively stable, and retain the potential to produce IL-17. Thus, these data document that the inflammatory environment has distinct effects on the stability of IL-17-secreting T cells in vivo.

Kinetics of Th17 cells

To determine if there are differences in cytokine expression in YFP^+ and YFP^- in vitro derived Th17 cells after re-stimulation, we compared IL-17 and IL-21 expression in these two populations and found that YFP reporter expression coincides with the expression of some (IL-17A), but not all (IL-21) Th17-associated cytokines. It will be important to further analyze differences in transcription factor expression in YFP^+ and YFP^- Th17 cells and determine if YFP reporter expression coincides with the expression of Th17 cell-associated transcription factors such as RORγt, IRF4, BATF, c-Maf and Etv5. Furthermore, it is unclear if YFP^- cells generated during Th17 cell differentiation can become YFP^+ upon further stimulation under Th17-polarizing conditions, something we would like to clarify in the future. Together, these studies will increase our understanding of cytokine and transcription factor expression patterns during Th17 cell differentiation.

Mechanisms in Th17 conversion to alternative T helper fates

We demonstrated that the conversion of Th17 cells to a Th1, Th2 or Th9-like phenotype in vitro was accompanied by the adoption of the respective T helper lineage effector programs and loss of IL-17 production. Although we saw an increase in the expression of transcription factors critical for the development of each T helper lineage upon stimulation of Th17 cells under the respective T helper cell culture conditions, we did not define a requirement for these factors in this conversion. To further our understanding of Th17 stability, it will be important to ectopically express key transcription factors important for the development of a

specific lineage, either alone or in combination with one another, in Th17 cells to determine their ability to shut off the Th17 program and drive the differentiation of a specific lineage. For example, T-bet may be sufficient to shut off the Th17 program and drive IFN-γ expression, however Runx3 may be necessary as well. We could further analyze epigenetic modifications across transcription factor and cytokine loci, which are associated with the development of each T helper lineage before and after conversion of Th17 cells to other T helper cell lineages, such as Th2 and Th9 cells. These studies will elucidate the contribution of factors involved in the stability of Th17 cells and further our understanding of the underlying mechanism of Th17 cell stability.

Defining factors that impact Th17 cell stability

It will be important to define the factors that are involved in maintaining Th17 stability during the development of AAD. Previous reports demonstrated that the in vivo conversion of Th17 cells to an IFN-γ-secreting Th1-like phenotype during the development of EAE is dependent on IL-23 (121). This could be in part due to low levels of TGF-β in the EAE model as it has been shown that Th17 cells stimulated in vitro for a second round in the presence of IL-23 and TGF-β maintain a Th17 phenotype, however upon stimulation with IL-12 or IL-23 in the absence of TGF-β, Th17 cells are converted to an IFN-γ-secreting phenotype (119). It is possible that the levels of TGF-β in the allergic environment are suitable to maintain Th17 cell stability, perhaps in addition to IL-6, IL-1β, or IL-23 levels. It will be important to analyze the production of these cytokines in the BAL

fluid and lung of allergic mice to determine their possible contribution to Th17 cell stability in AAD.

Th17 cell stability in skin and gut inflammation

Before the discovery of IL-23 and Th17 cells, psoriasis, a chronic inflammatory disease of the skin caused by a dysregulated immune response, was thought to be a Th1/Tc1-mediated disease due to increased IFN-γ levels in psoriatic lesions. Recent findings have demonstrated the importance of Th17 cells in the development of disease (253). However, as IFN-γ-producing T cells are present in psoriatic lesions, it is possible that these cells arose from a Th17 origin. Atopic dermatitis (AD) is a skin disease associated with an allergic Th2-mediated response. Although Th17 cells do not seem to be a major contributor to disease pathology, AD patients have increased Th17 cells in their blood and IL-17-producing cells are present in skin lesions (253, 255). It would be beneficial to determine the stability of Th17 cells during the development of psoriasis and AD to further understand if Th17 cell plasticity is a contributing factor to disease pathology. Furthermore, Tc17 cells may also be important for the development of psoriasis, and IL-17-producing γδ T cells, which are present in the skin, may also have a role in the development of psoriasis or AD. Our data demonstrate that like Th17 cells, Tc17 cells are unstable in some in vivo environments, contributing to their function in the immune response, however less is known about the stability of innate IL-17-producing T cells. In addition to that of Th17 cells, it will be important to determine the stability of Tc17 cells and innate IL-17-producing T

cells, such as γδ T cells, and their contribution to the immunopathology of diseases, such as psoriasis and AD.

In addition to their role in allergic airway and skin inflammation, Th17 cells have also been implicated in food allergy. Food induced-intestinal allergic inflammation is initiated by allergic responses such as Th2 differentiation, IgE synthesis and mast cell recruitment and eosinophilia, leading to gastrointestinal inflammation and dysfunction. In addition to Th2-associated cytokines, increased levels of IL-17A are detected in the small intestine and mesenteric lymph nodes in mouse models of food allergy, in conjunction with increased Th17 cells in the mesenteric lymph nodes, implicating Th17 cells in disease pathogenesis (256, 257). It will be important to further define the role of Th17 cells in mediating food-induced allergic responses and determine if their stability is a contributing factor to disease pathogenesis.

Together, these studies will further our understanding of the development and stability of IL-17-secreting cells in addition to their contribution to disease pathogenesis. Furthermore, these studies have important implications in the treatment of IL-17-mediated diseases.

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Education

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Awards

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Abstracts

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Peer reviewed publications

2007	Glosson NL and Hudson AW. 2007. Human herpes virus-6A and 6B encode viral immunoevasins that downregulate class I MHC. *Virology.* 365(1):125-35.
2010	May NA, Glosson NL, Hudson AW. 2010. Human herpes 7 u21 downregulates classical and nonclassical class I major

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