Factors controlling effector T cell responses during central nervous system autoimmunity

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Abstract

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IL-17-producing $CD4^+$ T (Th17) cells, along with IFN-gamma-expressing Th1 cells, represent two major pathogenic T cell subsets in experimental autoimmune encephalomyelitis (EAE), the animal model of multiple sclerosis (MS). The cytokines and transcription factors involved in the development and effector functions of Th1 and Th17 cells have been largely characterized. Among them, IL-23 is essential for the generation of stable and encephalitogenic Th17 cells and for the development of EAE. The IL-7/IL-7R signaling axis participates in cell survival, and perturbation of this pathway has been associated with enhanced susceptibility to MS. A link between IL-23-driven pathogenic T cells and IL-7/IL-7R signaling has previously been proposed but has not been formally addressed. Here, we showed that Th17 cells from mice with EAE express high levels of IL-7Ralpha compared to Th1 cells. Using mice that constitutively express IL-7Ralpha on T cells, we determined that sustained IL-7R expression in IL-23R deficient mice could not drive pathogenic T cells and the development of EAE. IL-7 inhibited the differentiation of Th17 cells but promoted IFN-gamma and GM-CSF secretion *in vitro*. *In vivo* IL-

7/anti-IL-7 mAb complexes selectively expanded and enhanced the proliferation of CXCR3-expressing Th1 cells but did not impact Th17 cells and EAE development in wild-type and IL-23R-deficient mice. Importantly, high IL-7 expression was detected in the CNS during EAE and could drive the plasticity of Th17 cells to IFN-gamma-producing T cells. Together, these data address the contribution of IL-23/IL-23R and IL-7/IL-7R signaling in Th17 and Th1 cell dynamics during CNS autoimmunity.

Moreover, we asked how the transcription factor STAT1 regulates the development of EAE. It is well established that the transcription factor STAT1 and its upstream cytokines, interferons and IL-27, serve a protective role in MS and EAE. Although STAT1 is critical in the stabilization of IFN-γ-producing CD4+ T helper (Th1) cells, which, together with IL-17-Th17 cells, drive the initiation of EAE, STAT1-deficient mice are highly susceptible to EAE. However, the mechanisms driving this effect remain elusive, particularly the role of STAT1 in CD4+ T cell responses. Here, we found that by using $STAT1^{fl/fl}$/CD4-Cre mice, STAT1-deficient Th17 cells could not induce EAE, despite the fact that these cells produced higher levels of IL-17. Moreover, STAT1 deficiency in T cells rendered mice protected from EAE development. We found that STAT1 did not impact the proliferation or survival of CD4+ T cells activated in vitro, but in vivo activated STAT1-deficient T cells failed to proliferate and expand. We uncovered a novel role for STAT1 in driving the upregulation of MHC class I molecules on CD4+ T cells upon activation, thus making them susceptible to NK cell targeting. After depletion of NK cells, we observed improved and restored survival and expansion of STAT1-deficient CD4+ T cells, fully capable of inducing EAE. Our findings provide a novel role of STAT1 in protecting CD4+ T cells from NK cell-mediated cytotoxicity, furthering our understanding of the effect of interferon in the pathogenesis of MS.

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DEDICATION

To my mamá, Danira Lima, for her unconditional love, patience, understanding, and support.

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CHAPTER 1

Introduction

The Immune System: Our War Hero

Our immune system protects us from a wide spectrum of pathogenic microbes such as viruses, bacteria, and parasites by engaging both the innate and adaptive arms. These systems developed in an evolutionary arms race with pathogens by providing us with a first line of defense against molecules that are not commonly found in our bodies, recognizing evolutionarily-conserved pathogen-associated molecular patterns (PAMPs) through Toll-like receptors (TLRs) (1). This is the main task of the innate immune system. Innate immune cells, such as natural killer (NK) cells, neutrophils, macrophages, and dendritic cells (DCs), are the first attackers against invaders and respond by phagocytizing and killing cells directly, producing a plethora of soluble factors that inhibit pathogen replication, and giving signals to activate and recruit cells of the adaptive immune system.

Natural Killer Cells: The Innate Lymphocyte

The innate immune system relies on NK cells to eliminate cells that are not derived from self-tissues (2). Although they have been historically classified as innate cells because of their ability to respond rapidly against their target cells, there is increasing evidence that NK cells share some attributes with lymphocytes of the adaptive immune system, B and T cells. NK cells contribute to the innate immune defense against microbial pathogens and tumors by sensing an exquisite arsenal of receptors that allows them to distinguish between normal cells and infected

cells or transformed cells (3). These cells express transcripts of IFN-γ and pre-formed cytotoxic granules, but require activation by IL-2, IL-12, IL-15, or IFN-β in order to lyse target cells (4). Once activated, NK cells recognize infected or transformed cells that express certain activating receptors and lack inhibitory receptors. Although the role of these cells in killing infected and transformed cells has long been known, NK cell-mediated lysis extends to autoreactive cells, as is becoming more evident (5). NK cell activity is tightly constrained by MHC class I molecule expression on the surface of immune cells. In fact, continuous engagement of inhibitory receptors with MHC class I molecules is required to dictate NK cell responsiveness (3). However, highly activated cells do not manage to escape NK cell lysis, which is thought to regulate clonal expansion of both CD4+ and CD8+ T cells in order to prevent excessive immunopathology or autoimmunity (6-8). In fact, the absence of one particular MHC class I molecule on T cells, Qa-1, makes them highly susceptible to NK cell killing (9). The mechanisms by which NK cells modulate immune responses require further work that will reveal insights about the selection processes that took place during the evolution of the adaptive immune system.

The Adaptive Immune System

To combat the plethora of ever-evolving pathogens, vertebrate animals developed a unique and exquisite mechanism: the adaptive immune system. One of the unique features of this system is that it has the capacity to establish memory against previously encountered pathogens and will therefore provide permanent protection against these pathogens. Because microbes are better at replicating their genome at higher rate, the adaptive system counters this feature by developing B and T cells, which are capable of recognizing virtually an infinite number of antigens. B cell and T cell receptors (TCRs) thus develop with unique specificity through the expression of the

recombination-activating genes (RAGs), resulting in the random generation of antigen receptors (10). Once a B or T cell becomes activated by its cognate antigen, it rapidly proliferates and becomes an effector cell. While B cells provide humoral immunity by secreting antibodies, which neutralize pathogens, T cells respond by producing cytokines that help activate and recruit more cells of the innate immune system and by killing affected cells directly. Activated cells rapidly expand clonally to become effector cells. After the immune response subsides, most of the effector cells die, but a subset of these cells goes on to become memory cells (11). Memory cells behave as sentinels that will respond rapidly in response to re-exposure with the same pathogen.

Effector CD4+ T cell Differentiation

T cells are comprised of CD4+ and CD8+ T cells. While CD8+ T cells normally function as cytotoxic T cells by killing their target cell upon recognition of the antigen, the primary purpose of CD4+ T cells is to serve as helper cells (12). The differentiation of the CD4+ T cell lineage into effector cells is crucial for successful adaptive immune responses aimed at distinct classes of pathogens. CD4+ T cells become helper T cells by receiving 3 signals (13): engagement of their TCR to a peptide-MHC complex (signal 1), binding to costimulatory molecules (signal 2), and ligation of cytokine receptors (signal 3). These 3 signals are coordinated by the innate immune system upon encounter of a pathogen. The functional specialization of this unique set of cells is coordinated by genetic programs that use different transcription factors to guide the expression of distinct soluble mediators and surface molecules that support interactions with other immune cells. Historically, and for illustrative simplistic reasons, naïve CD4+ T cells have been characterized as having different effector programs controlled by different transcription factors. IFN-γ-producing type I T (Th1) cells, which differentiate in the presence of IL-12, are controlled by the master

transcription factor T-bet and are important for the clearance of intracellular pathogens by activating macrophages. The Th1 program is initiated by engagement of the TCR on naïve CD4+ T cells, leading to low expression of T-bet and expression of IL-12Rβ2, which makes the cells responsive to IL-12 signaling mediated by STAT4 (14). Transactivation of IFN-γ expression by T-bet expressed by differentiating cells feeds back onto the cells through STAT1 and stabilizes the Th1 phenotype. On the other hand, type 2 (Th2) cells require IL-4 and the transcription factor GATA-3 and secrete IL-4, IL-5, and IL-13 in response to extracellular pathogens, such as parasites, and help B cells produce class-switched antibodies.

For decades, Th1 cells were believed to be the main inducers of tissue-specific autoimmune inflammation, whereas Th2 cells were viewed as having protective roles in autoimmunity, since IL-4 antagonizes the Th1 cell program. This paradigm of Th1 and Th2 cells, described in 1986 by Mosmann and Coffman (15), was expanded with the discovery of IL-17-producing Th17 cells in 2003 (16, 17). These cells require IL-23 and were initially found to be essential for the development of experimental models of the autoimmune diseases arthritis and multiple sclerosis (16, 18), since they can potently induce tissue inflammation by secretion of IL-17. The importance of Th17 cells in fungal infections has also been highlighted by multiple studies since the discovery of IL-23 (19). The robust Th17-inducing conditions of TGF-β and IL-6 and the identification of ROR-γt as the lineage defining transcription factor finalized support of Th17 cell as a distinct subset (20, 21). However, these cells have been shown to display one of the widest ranges of plasticity among all effector CD4+ T cell subsets. *In vitro*-generated Th17 cells show a STAT4- and T-bet-dependent conversion toward a Th1 profile (22), though IFN-γ-producing Th17 cells persist in the absence of T-bet and STAT4 (23). The conversion of Th17 cells into a Th1 profile is so drastic during EAE that some studies have shown that most of the Th1 cells found in the CNS

of EAE mice are “ex-Th17” cells, which are Th17 cells that have extinguished expression of IL-17 and strictly express IFN-γ (24).

Because having autoreactive CD4+ T cells capable of inducing so much tissue destruction was not a highly desirable feature during the course of the evolution of the adaptive immune system, regulatory T cells with a suppressive capacity also exist. The most widely studied regulatory T cell is the Foxp3+CD25+ CD4+ T cell, which arise naturally in the thymus or can be generated in the periphery in the presence of TGF-β and enhanced with IL-2 and retinoic acid (25). These cells are very important in maintaining self-tolerance (discussed below).

Cytokines produced by the innate immune system in the course of an immune response, in concert with cytokines produced by effector T cells, can also have proliferative or inhibitory roles on effector CD4+ T cell differentiation. IL-2 is critical for the proliferation of Th1 cells, while Foxp3+ regulatory T cells heavily depend on IL-2 for their suppressive function. However, IL-2, through STAT5, constrains Th17 cell polarization (26). IL-10, which can be secreted by regulatory T cells, is highly suppressive of inflammation. IFN-γ can inhibit Th2 and Th17 cells by upregulating T-bet. Thus, a balance of cytokine signaling through phosphorylation of various STATs orchestrates the differentiation programs of effector CD4+ T cells.

The JAK-STAT pathway dictates the effector program

Because CD4+ T cells differentiate in the presence of several cytokines, and these cytokines are absolutely required for their expansion and maintenance, the JAK-STAT signaling pathway plays an essential role in establishing the immune system (27). Upon engagement by a ligand, receptor-associated Janus kinases (JAKs) become activated and phosphorylate both each other and the intracellular tail of the receptors, forming docking sites for cytoplasmic transcription

factors called signal transducers and activators of transcription (STATs). This signaling pathway, downstream of cytokines, leads to JAK-mediated phosphorylation of STATs, which in turn translocate to the nucleus, bind DNA sites, and regulate gene expression. Cytokines have the tendency to activate a specific STAT, though interactive promiscuity between cytokines exists so that multiple STATs operate in parallel to varying degrees. Th17 cells depend heavily on STAT3 due to their need for IL-6 or IL-21 but are inhibited by STAT1, even though these cytokines activate both STATs (28). A model has emerged in which two antagonistic STATs will frequently be activated, but the ratio of the amount of phosphorylation determines the outcome of the differentiation program (29). IL-27, which inhibits Th17 differentiation through STAT1, can induce Th17 cells in the absence of STAT1 because STAT3 phosphorylation is preferred (30). Moreover, IL-12 signals through STAT4 to initiate the Th1 cell program, which then leads to IFN-γ expression that requires STAT1 to create a feedback loop in order to stabilize and expand Th1 cells (14). These STATs regulate gene expression in each subset by promoting a particular program while inhibiting another. For example, while TGF-β induces Foxp3+ expression, which guides CD4+ T cells toward a suppressive or regulatory subset (discussed below), IL-6, through STAT3 and upregulation of ROR-γt, counters the effect of TGF-β and initiates the Th17 cell program (20).

The complexity and essential nature of JAK-STAT signaling in cytokine biology is underscored by mutations in JAKs and STATs that affect the common γ-chain cytokines. In the context of lymphocyte development, the common γ-chain cytokines IL-2 and IL-7, which signal through STAT5, are crucial in T cell development in the thymus and proliferation in the periphery (31, 32). In severe combined immunodeficiency (SCID), mutations in JAK3 mimic mutations in the common γ-chain, leading to devastating primary immunodeficiency in which the combination of nonfunctional T cells and defective immunoglobulin production results in a syndrome of

recurrent infection, diarrhea, atopic dermatitis, and failure to thrive (33). Mutations in other STATs and JAKs can affect a variety of diseases, including cancer, infections, and autoimmunity, but the mechanisms that affect the different cell types in different contexts remain to be elucidated. Much work remains in dissecting the roles of individual STATs in the contribution to disease.

Establishing Self-Tolerance

Immunological tolerance is maintained at multiple levels by both the innate and adaptive immune systems. A hallmark of the adaptive immune system is the generation of diverse immune receptors for the anticipated encounter with rapidly evolving pathogens. However, this clever tactic for host defense brings considerable challenges. Because T cell receptors (TCRs) are selected by highly diverse endogenous ligands, i.e., self-peptide-MHC complexes, potentially pathogenic autoreactive T cells can be generated. Therefore, the adaptive immune system must proceed cautiously in generating T cells that fail to mount deleterious responses against self and food antigens, commensal microorganisms, and environmental antigens, which may or may not shape the TCR repertoire in the thymus. Moreover, when mounting effective immune responses against pathogens, there must be a mechanism at play that restrains inflammation in order to spare the host from excessive damage to its own tissues.

Self-tolerance is achieved through a combination of central and peripheral mechanisms guided by the adaptive immune system (34, 35). The first opportunity to eliminate self-reactive T cells occurs in the thymus, where central tolerance takes place to eliminate, by clonal deletion, T cells bearing TCRs with too high affinity or avidity for self-antigen-MHC complexes presented on antigen-presenting cells (APCs) through negative selection (36). Despite this, it is obvious that many autoreactive T cells escape into the periphery, where peripheral tolerance is required to prevent autoimmunity. If a T cell recognizes a self-peptide-MHC complex in the absence of an

inflammatory environment, chronic engagement of the TCR will induce cell death or state of unresponsiveness termed anergy. Peripheral tolerance is also reinforced by the requirement of simultaneous engagement of TCRs by a cognate peptide-MHC complex and the T cell costimulatory receptor CD28 by CD80 and CD86. CD80 and CD86 are induced on APCs upon the activation of innate immune system directly in response to microbial or viral products or through sensors of metabolic changes invoked by pathogens.

Mechanisms of tolerance, operating in a cell-intrinsic manner, and the two-signal requirement for the induction of a productive immune response appear insufficient to counter the threat of immune-mediated pathology. Thus, the discovery of Foxp3+ regulatory T cells, which can develop naturally in the thymus or can be induced in the periphery (37), are key players in maintaining peripheral tolerance. Ultimately, the activation of autoreactive cells stems from complex interactions between cells of both the innate and adaptive immune systems. When these mechanisms fail to eliminate self-reactive T cells, these cells may become activated, migrate to their target tissue where their cognate antigen is expressed, and initiate autoimmune disease.

Autoimmunity: When Our Hero Becomes a Traitor

Multiple sclerosis, psoriasis, type I diabetes, and rheumatoid arthritis are prime examples of autoimmune diseases that involve the activation of autoreactive Th1 and Th17 cells, which mediate destruction of the target organs. These diseases can be caused by a complex interplay between genetic and environmental factors, including infections. Polymorphisms in the MHC loci confer the highest genetic risk in many autoimmune diseases, pointing to a critical role for antigen-T cell interactions in disease pathogenesis.

Multiple Sclerosis

Multiple sclerosis (MS) is chronic, progressive, demyelinating autoimmune disorder of the central nervous system (CNS) that affects over 4 million people worldwide. The incidence of this autoimmune disease, and others, has been increasing over the last century, particularly in developed countries. The disease is characterized by inflammatory plaques that are visible through magnetic resonance imaging (MRI) scans (38). These lesions contain inflammatory cells, which have led scientists to believe that this disease is caused by the immune system. Further work in the animal of model of MS, experimental autoimmune encephalomyelitis (EAE), has reinforced these findings. EAE has been critical in understanding the mechanisms underlying the pathogenesis of MS and the cells and factors required for the disease (39). One of these is the widely accepted hypothesis that these diseases are driven by myelin-specific autoreactive CD4+ T cells, which then propagate a broader neurodegenerative process (40). Activated myelin-specific CD4+ T cells cross the blood brain barrier (BBB), access the CNS, and initiate the disease by expanding there and producing a wide variety of soluble factors, such as IFN-γ, IL-17, and GM-CSF. These cells target the myelin sheath that covers the axons of neurons, which leads to slowing down of signal conduction. Axonal damage and demyelination will cause paralysis and loss of motor function.

MS comes in a wide variety of forms, the most common being relapsing-remitting MS. Most patients advance to a more progressive form called secondary progressive MS, with continuing loss of neurological function, which is believed to be independent of inflammation. Thus, the goal of most clinicians is to decrease the number of relapses and increase the length of the recovery, which is accompanied by a return of some functions.

Current therapies for MS involve reducing inflammation using immunomodulatory drugs, such as type I interferon (IFN-β), glatiramer acetate, and natalizumab. However, these drugs are

only efficacious for so long and some of them are associated with bad side effects and infections (41). Although IFN-β is prescribed as the main line of treatment for MS, over half of the patients become unresponsive to the drug, and a small subset of patients develop worsened relapses. The mechanisms of action underlying the effectiveness of IFN-β, as well as the loss of its efficacy, have remained elusive, especially because this cytokine has pleiotropic effects, depending on the cell type.

EAE has served as a useful animal model for MS, since many of the pathologies observed in the CNS of mice with EAE bear strong similarity to those found in the CNS of MS patients (42). In both EAE and MS, the white matter of the CNS presents with demyelinating lesions associated with infiltrating T cells, macrophages, and B cells. Indeed, many therapeutics tested in multiple sclerosis patients are in fact based on concepts derived from the EAE model.

EAE can be induced in the C57BL/6 model by immunization with the myelin oligodendrocyte glycoprotein (MOG) emulsified in complete Freund's adjuvant (CFA). This results in a chronic progressive form of EAE in which afflicted mice develop ascending paralysis with lesions in the brain, spinal cord, and optic nerve. In addition to disease induction by active immunization, the disease can be induced by passive transfer of myelin-specific T cells, which can be generated from immunized mice or from 2D2 MOG_{35-55}-specific TCR transgenic mice (43). Myelin-specific CD4+ T cells induce EAE by producing the hallmark cytokines of Th1 and Th17 cells, IFN-γ and IL-17, respectively.

Th1 and Th17 cells can both induce EAE independently of each other, each with unique pathological features and tissue localization (44-46). Thus, both effector subsets contribute to EAE development. There is debate as to which subset plays a dominant role in EAE, particularly because MS patients can have different forms of the disease dominated by either Th1 or Th17 cells

and may experience different outcomes in response to IFN-β whereby non-responders have higher levels of IL-17F (47).

Tissue inflammation by autoimmune responses is induced when autoreactive CD4+ effector cells are activated and acquire appropriate pro-inflammatory phenotypes. Therefore, the factors that control CD4+ T cell effector differentiation and memory formation have been the subject of much investigation. Common γ-chain cytokines, such as IL-2 and IL-7, are critical in the development of an efficient T cell response at different time points. IL-7 is a very complex cytokine that is required for T cell development in the thymus and survival of memory cells in the periphery (31). Although its role in the encephalogenicity of CD4+ T cells has been studied *in vitro*, its role *in vivo* has not been characterized as well.

Factors that govern the differentiation, maintenance, and plasticity of Th17 cells have been implicated in the pathogenesis of EAE. IL-6, which can polarize Th17 cells, acts through STAT3 to activate ROR-γt and initiate Th17 cell differentiation. Deficiency of IL-6, IL-6 receptor, STAT3, or ROR-γt lead to protection from EAE development. IL-23, which was originally shown to maintain Th17 cells (48, 49), can induce the conversion into Th1 cells and is absolutely required for EAE development (22-24). IL-23 receptor (IL-23R) deficiency protects mice from EAE development, but the mechanisms underlying this phenotype are not entirely clear. Failure to upregulate the IL-7 receptor on IL-23R-deficient Th17 cells to drive EAE was cited as a mechanism by Cua et al. (48). However, these results could not be reconciled with the fact that Th17 cells fail to terminally differentiate in IL-23R-deficient mice.

It is well established that the factors required for Th17 differentiation, IL-6, STAT3, ROR-γt, and IL-23, are required for induction of EAE (18, 21, 50, 51). However, IL-17-deficient mice remain resistant to EAE (52). Conversely, while mice deficient in IFN-γ, IFN-γ receptor, and

STAT1 mice are highly susceptible to EAE (53-55), T-bet and STAT4 deficiency protects mice from EAE development (55, 56). These paradoxes highlight the complex nature of the pathogenesis of EAE and multiple sclerosis, which stimulates further work to elucidate the impact of these factors and pathways to understand the mechanisms by which Th1 and Th17 cells become pathogenic and induce autoimmune disease.

Questions to Address

The factors controlling autoreactive effector CD4+ T cells are numerous and complex in nature. The IL-23/IL-23 receptor (IL-23R) signaling axis is one example. An interdependent and co-regulation between the IL-7/IL-7 receptor (IL-7R) and the IL-23/IL-23R pathways has been suggested but not formally tested. Furthermore, although the *in vitro* effects of IL-7 on T helper differentiation has been studied, the *in vivo* effects of IL-7 have not been determined, particularly since IL-7 or IL-7R blockade leads to a profound defect in survival and proliferation of T cells. A recently retracted study (Zhang), which attributed a Th17-promoting role to IL-7, caused much confusion in the field, since IL-7 signals through STAT5 and STAT5 constrains Th17 cell differentiation. Thus, the interplay between IL-23 and IL-7R signaling and the effects of IL-7 on the differentiation profiles of pathogenic effector CD4+ T cells necessitate more in-depth exploration, which is one of the aims of this thesis. Because the IL-7/IL-7R axis has been proposed to be targeted for treatment of MS, a better understanding of its impact on EAE development and cytokine production by CD4+ T cells is important.

STAT1 promotes Th1 cell differentiation while limiting Th17 cell development. Since Th17 cells are critical for the development of EAE, it has been proposed that STAT1-deficient mice might have exacerbated EAE due to enhanced Th17 response (57). STAT1 has also been

shown to inhibit lymphocyte proliferation (58) and negatively regulate apoptosis in other cell types, particularly in cancer (59). Other reports have suggested a protective effect of type I interferon on myeloid cells (60). Therefore, how modulation of STAT1 signaling in T cells affects EAE development has not been addressed. Here, we show that deletion of STAT1 only in T cells protects mice from EAE and that there is no defect in the proliferation and survival of these cells *in vitro*. T cells required STAT1 for their survival and expansion in lymphopenic settings and in response to their cognate antigen. STAT1 promoted the expression of MHC class I molecules on T cells after activation, which protected them from NK cell-mediated cytotoxicity. Elimination of NK cells restores the ability of STAT1-deficient CD4+ T cells to survive and become effective pathogenic effector cells.

CHAPTER 2

IL-7/IL-7 receptor signaling differentially affects effector CD4$^+$ T cell subsets involved in experimental autoimmune encephalomyelitis

Introduction

Multiple sclerosis (MS) is a demyelinating autoimmune disease of the central nervous system (CNS), leading to axonal damage and physical impairment. Experimental autoimmune encephalomyelitis (EAE), the mouse model of MS, has been useful in identifying the pathogenic mechanisms at play in MS and in determining that CD4$^+$ T helper (Th) cells are essential for the detrimental inflammation characteristic of MS and EAE (28). Historically, Th1 and Th17 cells have been known to drive the inflammatory processes within the CNS by producing IFN-γ and IL-17, respectively (61). Although Th1 or Th17 cells can induce EAE independently, the clinical signs, pathological features, and cells recruited may differ. Th1-polarized cells promote the expression of monocyte attracting chemokines and macrophage-rich infiltrates into the spinal cord, whereas IL-23 polarized Th17 cells activate neutrophil-attracting chemokines, promote neutrophil recruitment, especially in the brain (62), and drive the formation of ectopic lymphoid aggregates (45).

IL-23 is a dimeric cytokine composed of the p40 subunit common with IL-12 and the unique p19 subunit which is essential for the development of EAE, since both IL-23p19 KO and IL-23 receptor-deficient (IL-23R KO) mice are resistant to the development of EAE (48, 49, 63). IL-23 maintains and expands Th17 cells (64), induces the production of GM-CSF (65, 66), and promotes the plasticity of Th17 cells into a Th1 cell phenotype (22, 23). Indeed, while Th17 cells

differentiated *in vitro* have a clear and distinct phenotype under strong Th17-polarizing conditions, Th17 cells found in the CNS of mice with EAE modulate their cytokine expression and express IFN-γ (23, 24, 67). Few cytokines have been shown to modulate the plasticity of Th17 cells (22, 68) and the identity of the cytokine milieu, which modulates the balance between these effector populations *in vivo*, remains elusive.

Several cytokines are believed to be important in expanding $CD4^+$ T cells. IL-2 is an important survival factor for activated effector T cells, particularly Th1 cells, but limits Th17 cell differentiation and expansion (26). IL-7, besides being crucial for T cell development in the thymus, is important in maintaining the naïve and memory T cell populations in the periphery (69). IL-7/IL-7 receptor (IL-7R) signaling has gained considerable interest in MS because single-nucleotide polymorphisms (SNPs) in the IL-7Rα, which dimerizes with the common γ chain to form the IL-7R, have been associated with increased risk for developing MS (70, 71). The SNP, rs6897932, which is located within the alternatively spliced exon 6 of *Il7R*, increases the rate of IL7Rα mRNA splicing and results in increased levels of soluble IL7Ra, which competes with membrane-bound IL-7Rα to increase the bioavailability of IL-7 (72). Furthermore, IL-7Rα blockade leads to amelioration of EAE, which is associated with a reduction in $CD4^+$ and $CD8^+$ T cell numbers (73, 74).

Attempts to investigate the effects of IL-7-mediated signaling on pathogenic T cells have yielded conflicting results. It has been proposed that IL-23/IL-23R signaling is essential for the upregulation of IL-7Rα on differentiated Th17 cells and for their expansion (48). Another study further linked Th17 cells with IL-7 by suggesting that IL-7 selectively expands Th17 cells from mice with EAE and MS patients (75). However, the results of this latter study have been questioned, and another laboratory provided evidence that IL-7 does not affect Th17 cell

differentiation (73). Although the role of IL-7 has been well studied in development, homeostatic proliferation, and survival of T cells, how the IL-7/IL-7 signaling axis influences disease settings and pathogenic T cell responses, particularly *in vivo*, has not been fully addressed.

Here, we investigated the effects of IL-7-mediated signaling on the generation of pathogenic Th1 and Th17 cells. We found that IL-7Rα was enriched on Th17 cells in the periphery and CNS of mice with EAE at the peak and onset of the disease. Moreover, we provided evidence that IL-23R-deficient mice, which cannot generate stable Th17 cells, remain resistant to EAE when IL-7Rα was constitutively expressed and when a highly bioactive form of IL-7 complexed to IL-7 antibody (IL-7c) was administered. IL-17 production in IL-23R-deficient mice was not rescued in the presence of constitutively active IL-7Rα or treatment with IL-7c. We further demonstrated that IL-7 selectively promotes Th1 differentiation and the plasticity of Th17 cells. These results are relevant for the development of EAE and MS, since large amounts of IL-7 are produced in the CNS of mice and humans with autoimmunity.

Materials and Methods

Mice

C57BL/6 (B6) mice were purchased from the Jackson Laboratories and bred in the Benaroya Research Institute (BRI) animal facility. Foxp3-RFP/IL-17A-GFP/IFN-γ-Thy1.1triple reporter mice have been described previously (23). These mice were generated by breeding IL-17A-GFP (Biocytogen), IFN-γ knock-in Thy1.1(76), and Foxp3-RFP (77) reporter mice together. IL-23R knock-in (KI) and IL-23R homozygous KI (IL-23R$^{-/-}$) GFP reporter mice were previously described (49). IL-7RαTg and ROR-γt-GFP reporter mice were previously described (21, 78). All strains are on the C57BL/6 background. All animals were bred and maintained under specific pathogen-free conditions at the Benaroya Research Institute (Seattle, WA) and all experiments were performed in accordance with the guidelines of the Benaroya Research Institute Animal Care and Use Committee.

CD4$^+$ T cell preparation and T cell differentiation

For T cell differentiation, naïve CD4$^+$CD62L^{hi}CD44^{lo}CD25− T cells were isolated by FACS sorting (FACSAria, BD Biosciences) and cultured with irradiated (4000 rads) CD4-depleted spleen cells from wild-type (WT) mice and anti-CD3 (2.5 μg/ml, clone 145-2C11) for 5 days in complete RPMI. For Th17 differentiation, 2.5 ng/ml rhTGF-β (R&D Systems), 30 ng/ml rmIL-6 (Peprotech), 10 μg/ml anti-IFN-γ and 10 μg/ml anti-IL-4 (NIH/NCI BRB Preclinical Repository) were used. 10 ng/ml rmIL-12 was used for Th1 conditions. For the indicated conditions, 10 ng/ml rmIL-7 (eBioscience) was added to the cultures. For restimulation, T cells were collected and

activated with irradiated splenocytes, anti-CD3 and indicated cytokines (rmIL-7 was used at 10 ng/ml, eBioscience).

Antibodies and flow cytometry

For intracellular cytokine staining analysis, cells were incubated 5 hours in complete RPMI containing 50ng/ml phorbol myristate acetate (PMA), 1μg/ml ionomycin (Sigma-Aldrich) and Golgi Stop (BD Biosciences). Cells were then washed with cold PBS and blocked for 10 min with anti-CD16/32 purified antibody (clone 2.4G2, BioXCell). Viability of the cells was assessed by staining with fixable dye eFluor780 (eBioscience). Cells were then stained with anti-CD4 antibody and washed with PBS. Cells were then fixed for 20 min with fixation buffer (BD Biosciences), permeabilized with BD permeabilization/wash buffer (BD Biosciences) and stained with anti-IFN-γ, and anti-IL-17 specific antibodies in permeabilization buffer. Cells were acquired on LSRII (BD Biosciences), and data were analyzed with FlowJo software. The following antibodies were used in our experiments: Antibodies to CD4 (clone L3T4) conjugated to PerCP-Cy5.5 or Alexa700, IL-7Rα (CD127) (clone A7R34) conjugated to PerCP-Cy5.5, IFN-γ (clone XMG1.2) conjugated to PE-Cy7 or PE, were purchased from eBioscience and antibody to IL-17 (clone TC11-18H10.1) conjugated to APC was purchased from Biolegend.

EAE induction

EAE was induced by subcutaneous immunization of mice into the flanks with an emulsion of MOG_{35-55} peptide (100 μg) emulsified in complete Freund adjuvant supplemented with 4mg/ml of *M. tuberculosis* extract H37Ra (Difco). In addition, the animals received 200 ng of pertussis toxin (List Biological Laboratories) i.p. on days 0 and 2. Clinical signs of EAE were assessed

according to the following score: 0, no signs of disease; 1, loss of tail tonicity; 2, hind limb weakness; 3, hind limb paralysis; 4, hind and forelimb paralysis; 5, moribund.

Isolation of CNS mononuclear cells

Mice were sacrificed at the peak of disease and perfused with cold PBS. Brain and spinal cords were isolated and digested for 30 min at 37°C with Collagenase D at a concentration of 2.5mg/ml (Roche). Mononuclear cells were isolated over a 37% / 70% Percoll gradient (VWR), washed twice with complete medium and collected in medium for further analysis.

IL-7/M25 complex treatment

Recombinant mouse IL-7 was purchased from eBioscience (San Diego, CA). M25 anti-IL-7 antibody was purchased from Bio X Cell (West Lebanon, NH). IL-7/M25 complexes (IL-7c) were generated as previously described (79). Briefly, each mouse received complexes generated from a 30-minute incubation at 37°C of 1.5μg IL-7 with 15μg mAb M25. WT mice immunized with MOG_{35-55} in CFA received 3 injections of IL-7c every other day starting at day 1 after immunization. ROR-γt-GFP mice were sacrificed six days after the first injection.

Statistical analysis

Statistical analyses were conducted with GraphPad Prism software. P values were calculated with Student's paired *t*-test. P values of less than 0.05 were considered significant, *≤ 0.05, **≤0.01, ***≤0.001. Error bars denote ± SEM as indicated.

Results

IL-7 receptor is expressed at low levels on Foxp3+ regulatory T cells

Because high levels of IL-7 receptor have been proposed to render cells more responsive to IL-7-mediated signaling (69), we examined IL-7 receptor (IL-7Rα) expression on these subsets *in vivo* during the course of EAE. We took advantage of a triple reporter mouse (Foxp3-RFP/IL-17A-GFP/IFN-γ-Thy1.1) in which cells expressing Foxp3, IL-17, and IFN-γ can be detected based on RFP, GFP, and Thy1.1 expression, respectively, to identify the proportion of $Foxp3^+$ T and cytokine-producing effector Th17 and Th1 cells *ex vivo*. This strategy allowed the detection of cytokine-producing cells without permeabilization and activation with PMA/ionomycin, which can impact IL-7Rα expression. We analyzed at the peak of EAE IL-7Rα expression on $Foxp3^-$ and $Foxp3^+$ $CD4^+$ T cells, which contain effector and regulatory T cells respectively (**Figure 2.1A**). Consistent with published data, in the spleen and lymph nodes (LNs) of mice at the peak and onset of EAE, $CD4^+$ $Foxp3^-$ effector T cells expressed higher levels of IL-7Rα, while $Foxp3^+$ T cells showed diminished expression of the receptor (**Figure 2.1B-C** and not shown).

IL-7 receptor is highly expressed on Th17 cells

Th17 and Th1 cells can induce EAE with different pathological phenotypes and distinct clinical signs (45, 62). Thus, we examined the patterns of IL-7Rα expression on effector Th1 and Th17 cells after immunization with MOG_{35-55} and CFA to induce EAE development. Unexpectedly, Th17 cells expressed high amounts of IL-7Rα in both the spleen and LNs at the onset of the disease, while the expression of IL-7Rα on Th1 cells was significantly lower

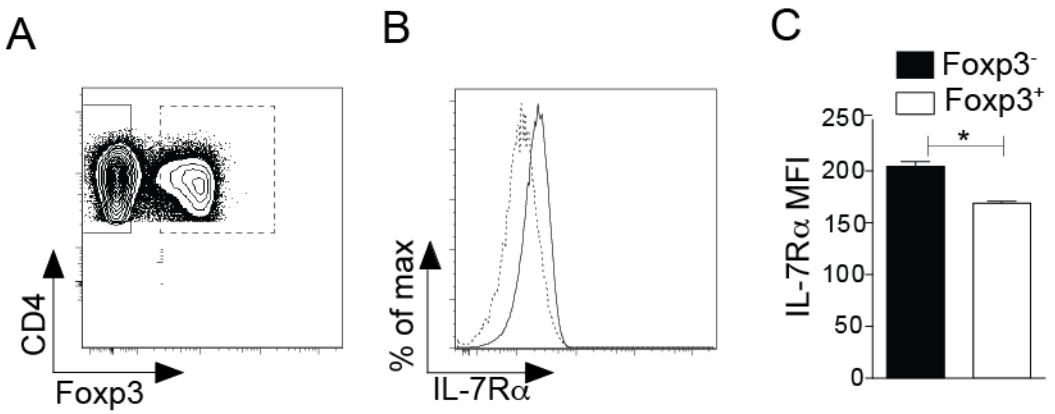

Figure 2.1. Foxp3+ regulatory CD4+ T cells express low levels of IL-7Ra relative to effector cells. (**A-I**) Foxp3-RFP/IL-17A-GFP/IFN-γ-Thy1.1 triple reporter mice were immunized for EAE with MOG_{35-55} emulsified in CFA. (**A-C**) Surface expression of IL-7Rα (**B**) was analyzed on CD4+ $Foxp3^-$ cells (solid line gate and histogram) and CD4+ $Foxp3^+$ cells (dashed line gate and histogram) from the spleen/lymph nodes of immunized mice (as gated in A). (**C**) Summary of IL-7Rα MFI on these populations. Statistics were performed with Student's t test ($^*p < 0.05$, $^{**}p < 0.01$). Data are representative of at least 2 independent experiments with at least 3 mice each experiment.

(Figure 2.2A-C). The enrichment of IL-7Rα on Th17 cells was also observed at the peak of the disease (**Figure 2.2D-F**). Therefore the differential expression of IL-7Rα on Th1 and Th17 cells remained constant at all measured stages of the disease, including at the peak of EAE.

IL-23R identifies IL-7Rα-expressing Th17 cells

IL-23R is required for the maintenance of Th17 cells and is expressed on effector T cells as they differentiate into Th17 cells (48). Thus, we analyzed the expression of IL-7Rα on IL-$23R^+$ cells from the draining LNs of MOG-immunized IL-23R-GFP reporter mice before disease onset. We found that IL-7Rα was more highly expressed on IL-$23R^+$ $CD4^+$ T cells than IL-$23R^-$ $CD4^+$ T cells (**Figure 2.3A-B**), consistent with our results that IL-7R expression is upregulated on Th17 cells at the onset and peak of EAE development. Thus, we observed that Th17 cells constantly express higher levels of IL-7Rα during all stages of the disease course. The elevated levels of IL-7Rα on Th17 cells compared to Th1 and $Foxp3^+$ T cells suggest that Th17 cells might be more sensitive and responsive to IL-7 and expand in response to this cytokine. Alternatively, the higher expression of IL-7Rα on Th17 cells could indicate that these cells did not respond to IL-7 and therefore did not downregulate IL-7Rα.

CD4+ T cells from IL-7RαTg/IL-23R$^{-/-}$ mice have highly functional IL-7/IL-7R signaling and preferentially expand in lymphopenic conditions

To address this question, we first tested the sensitivity to and requirement of IL-7/IL-7R signaling in Th17 cells by providing sustained IL-7R expression on T cells. T cells from IL-23R deficient (IL-23R $^{-/-}$) mice immunized with MOG_{35-55} have impaired Th17 cell expansion and

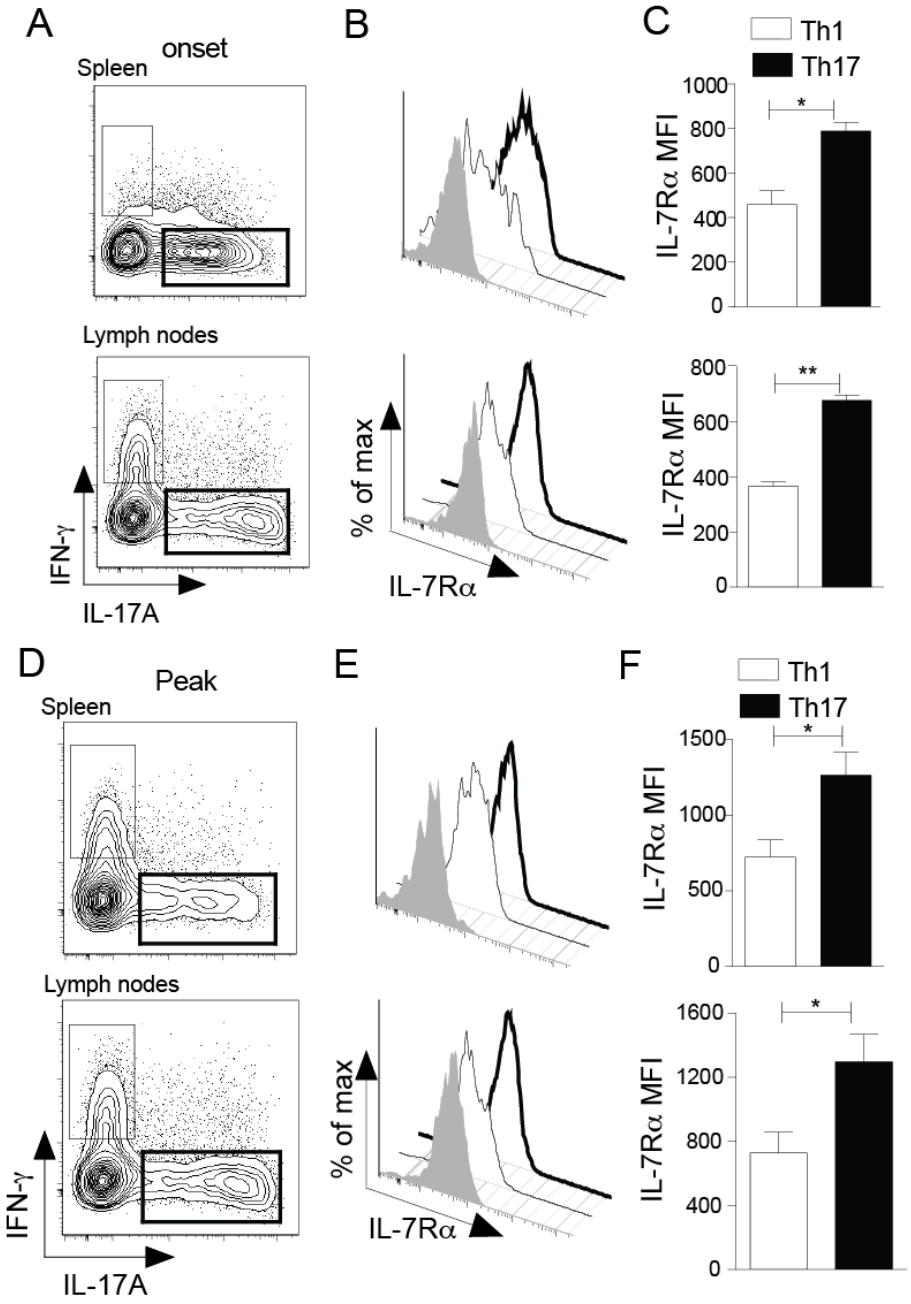

Figure 2.2. Th17 cells express high levels of IL-7Rα. (**A-I**) Foxp3-RFP/IL-17A-GFP/IFN-γ-Thy1.1 triple reporter mice were immunized for EAE. (**A-C**) Expression of GFP (IL-17A), indicative of Th17 cells (bold line), and Thy1.1 (IFN-γ), representing Th1 cells (thin line), on $CD4^+CD44^+$ T cells from the spleen and lymph nodes and IL-7Rα expression on these T cell populations were determined at the onset (day 9-12, **A-C**) and peak (day 14-18, **D-F**) of the disease. (**B** and **E**) Surface expression of IL-7Rα on $IL\text{-}17A^+$ Th17 cells (bold line histogram) and $IFN\text{-}\gamma^+$ Th1 cells (thin line histogram) is compared to isotype control (shaded grey histogram). (**C** and **F**) Summary of IL-7Rα expression quantified by mean fluorescent intensity (MFI) on gated subsets. Statistics were performed with Student's t test ($^*p < 0.05$, $^{**}p < 0.01$).

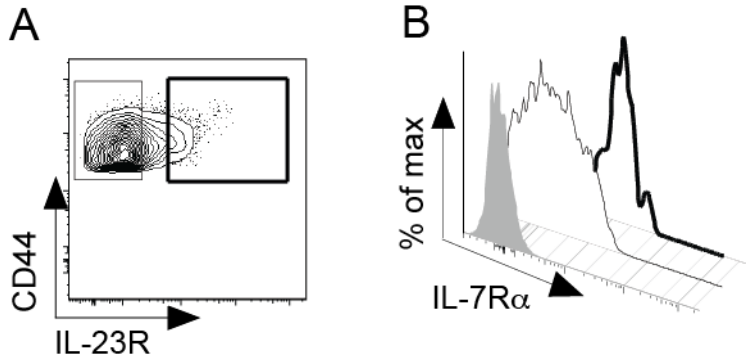

Figure 2.3. IL-23R identifies IL-7Rα-expressing Th17 cells (**A**) Identification of IL-23R$^+$ (bold line gate) and IL-23R$^-$ (thin line gate) effector CD4$^+$CD44$^+$ T cells in the draining lymph nodes of IL-23R-GFP mice 8-10 days after immunization with MOG_{35-55}/CFA. (**B**) Staining of IL-7Rα on IL-23R$^-$ (thin line histogram) and IL-23R$^+$ (bold line histogram) CD4$^+$ T cells as gated in (**A**). Statistics were performed with Student's t test ($^*p < 0.05$, $^{**}p < 0.01$). Data are representative of at least 2 independent experiments with at least 3 mice each experiment.

effector function, making them resistant to the development of EAE (48, 49). It was proposed that IL-23/IL-23R interactions drive IL-7Rα expression, which is essential for Th17 cell proliferation and expansion (48), further suggesting that forced expression of IL-7R on IL-23R$^{-/-}$ T cells could rescue the expansion of Th17 cells. To test this hypothesis, we took advantage of mice that constitutively express the IL-7Rα on CD4^{+} and CD8^{+} T cells (IL-7RαTg) (78). Since IL-7Rα is quickly downregulated upon TCR ligation, IL-7/IL-7R signaling, and other cytokine signals, we first tested the ability of IL-23R$^{-/-}$/IL-7RαTg CD4^{+} T cells to maintain high levels of IL-7Rα expression. Eight days after immunization with MOG$_{35-55}$/CFA, CD4^{+} T cells isolated from the draining LNs of IL-7RαTg and IL-23R$^{-/-}$/IL-7RαTg mice maintained high levels of IL-7Rα expression, whereas IL-23R$^{-/-}$ CD4^{+} T cells downregulated the receptor (**Figure 2.4A-B**). Furthermore, IL-7/IL-7R signaling was highly functional, since cells from IL-23R$^{-/-}$/IL-7RαTg mice were also capable of phosphorylating STAT5 at higher levels in response to IL-7 compared to cells from IL-23R$^{-/-}$ mice (**Figure 2.4C**). Thus, constitutive IL-7Rα expression and sustained STAT5 signaling in IL-23R$^{-/-}$/IL-7RαTg mice bypass the possible requirement of IL-23 for IL-7Rα expression and make the mice suitable to address whether IL-7/IL-7R signaling could compensate for the lack of IL-23 in the development of EAE.

To determine how constitutive expression of IL-7Rα affects the proliferation and expansion of CD4^{+} T cells, we sorted naïve (CD62L^{hi}CD44^{lo}) CD4^{+} T cells derived from IL-23R$^{-/-}$ and IL-23R$^{-/-}$/IL-7RαTg mice and allowed them to expand under lymphopenic conditions in TCRβδ-deficient mice. In this system, transferred cells have access to an abundant source of IL-7, which allows them to survive and expand very rapidly in an IL-7-dependent manner. Seven days after transfer, we recovered fewer IL-23R$^{-/-}$ cells, compared to IL-23R$^{-/-}$/IL-7RαTg cells

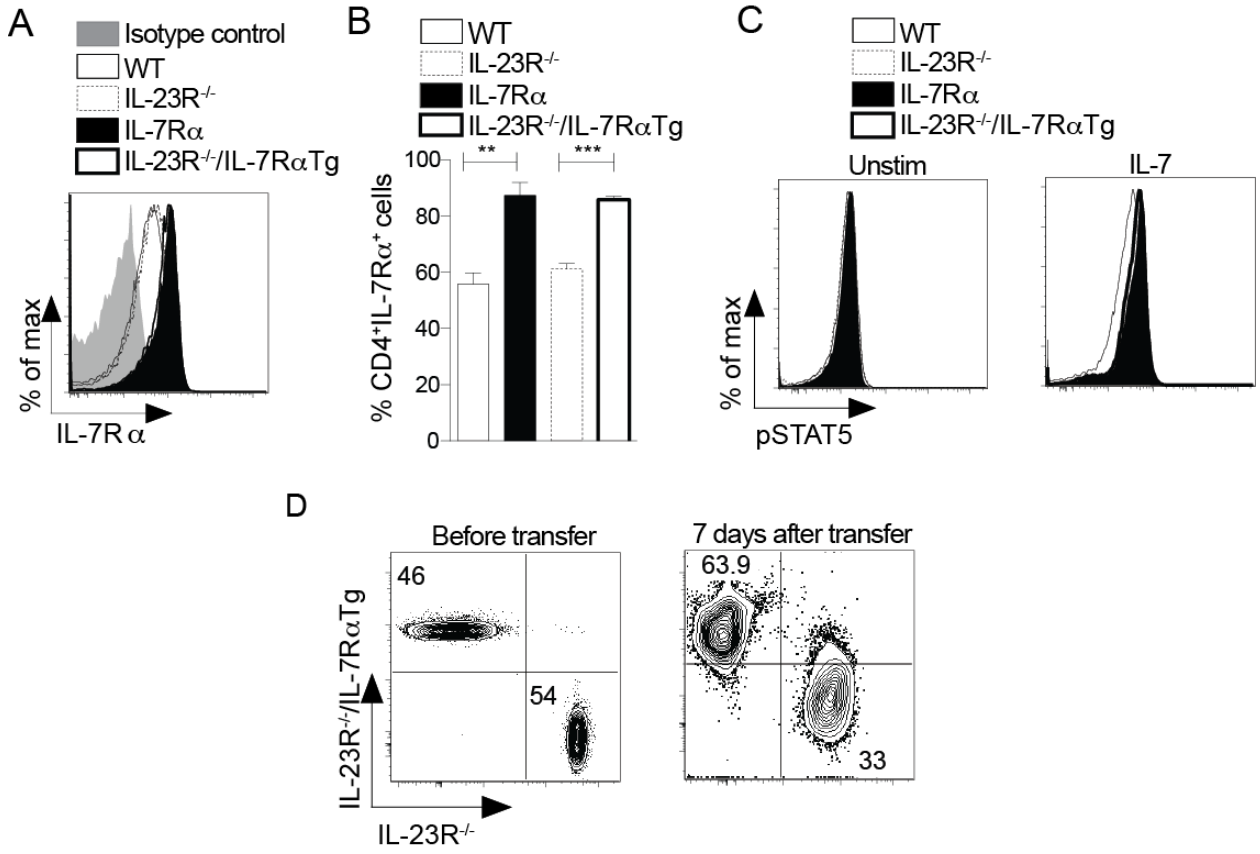

Figure 2.4. IL-7RαTg mice constitutively signal through IL-7 and survive better in lymphopenic conditions. (**A-C**) IL-7RαTg, IL-23R$^{-/-}$/IL-7RαTg, and IL-23R$^{-/-}$ mice were immunized with MOG_{35-55} in CFA. Eight to ten days after immunization, CD4^{+} T cells from the draining lymph nodes were analyzed for IL-7Rα expression (**A**) and quantified in (**B**). (**C**) STAT5 phosphorylation from CD4^{+} T cells isolated from the draining lymph nodes of MOG-immunized mice, in response to IL-7 or no stimulation. (**D**) Frequency of IL-23R$^{-/-}$ (CD45.1) and IL-23R$^{-/-}$ /IL-7RαTg (CD45.2) TCR-β^{+}CD4^{+} cells recovered in the spleen 7 days after transfer into TCR-βδ$^{-/-}$ mice, assessed by flow cytometry. Numbers indicate percentage of each cell population among CD4^{+} TCR-β^{+} T cells. Data are representative of at least 2 independent experiments with n≥2 mice in each experiment.

(**Figure 2.4D**), showing that increased expression and signaling through the IL-7R induces preferential expansion and survival of IL-23R-deficient T cells.

IL-7 signaling does not restore cytokine production in the absence of IL-23R deficiency

To test whether constitutive expression of IL-7Rα on IL-23R-deficient cells can affect cytokine production, we immunized WT, IL-7RαTg, IL-23R$^{-/-}$, and IL-23R$^{-/-}$/IL-7RαTg mice with MOG_{35-55}/CFA and determined cytokine production of CD4^{+} T cells by intracellular cytokine staining. IL-7RαTg mice had similar frequencies and percentages of IL-17^{+} and IFN-γ^{+} CD4^{+} T cells to WT mice (**Figure 2.5A-B**). Consistent with a role for IL-23 in the maintenance and expansion of Th17 cells, IL-23R-deficient mice had fewer IL-17-producing T cells and similar proportions of IFN-γ^{+} cells. Importantly, cytokine production by Th cells in IL-23R$^{-/-}$/IL-7RαTg mice phenocopied that of IL-23R deficiency (**Figure 2.5A-B**).

Sustained IL-7R signaling does not replace IL-23R signaling to restore EAE development

Although we did not see changes in cytokine production from CD4^{+} T cells isolated from IL-23R$^{-/-}$ and IL-23R$^{-/-}$/IL-7RαTg mice, we tested whether constant IL-7Rα expression on T cells can bypass the requirement for IL-23R in EAE development. To this end, we immunized WT, IL-23R$^{-/-}$, IL-23R$^{+/-}$/IL-7RαTg, and IL-7RαTg mice for EAE development with MOG_{35-55}/CFA. Whereas IL-7RαTg mice developed classical signs of EAE to the same extent as WT mice (**Figure 2.6A**), IL-23R$^{-/-}$ and IL-23R$^{-/-}$/IL-7RαTg mice did not develop any disease (**Figure 2.6A**). As a result, T cells failed to infiltrate the CNS of IL-23R$^{-/-}$ and IL-23R$^{-/-}$/IL-7RαTg mice (data not shown). Constitutive expression of IL-7Rα on T cells did not impact the balance of Th1, Th17, and IFN-γ- producing Th17 (Th1/17) cells in the CNS of mice that developed EAE

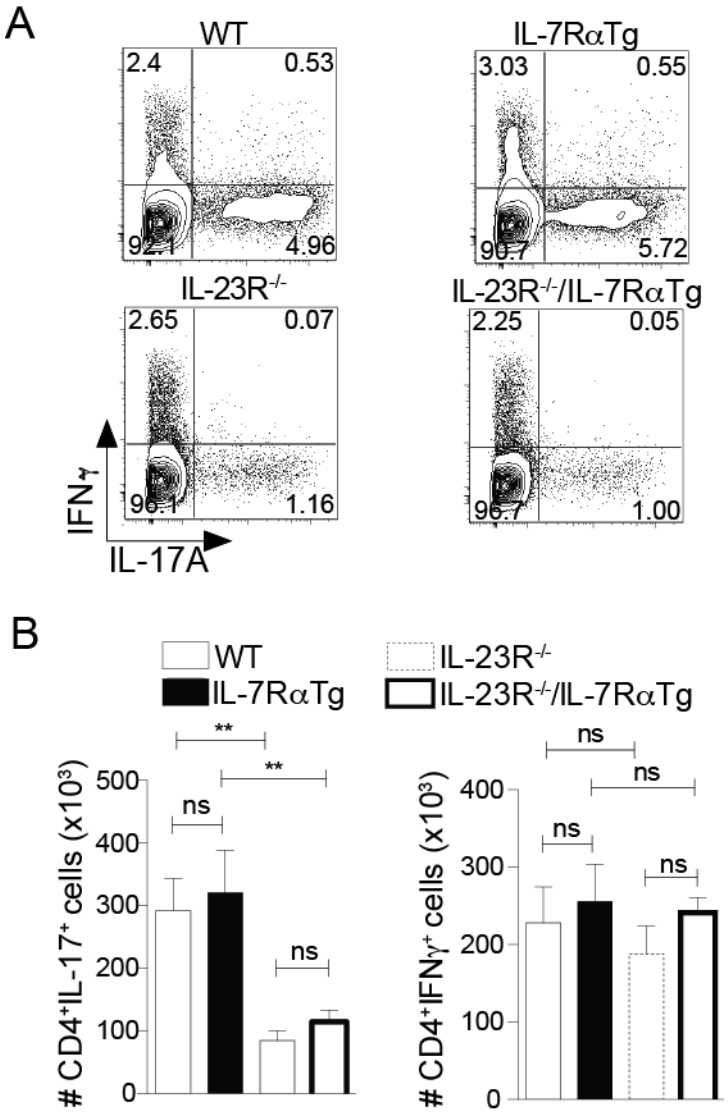

Figure 2.5. IL-7 signaling does not restore cytokine production in the absence of IL-23R deficiency. (**A-B**) Wild-type (WT), IL-7RαTg, IL-23R$^{-/-}$, and IL-23R$^{-/-}$/IL-7RαTg mice were immunized with MOG/CFA. (**A** and **B**) The percentage of IL-17^{+} and IFN-γ^{+} CD4^{+} T cells (representative dot plot, **A** and summary, **B**) from the lymph nodes of these animals 10 days after immunization was determined by intracellular cytokine staining. Data from 3 combined experiments are shown. Statistics were performed with Student's t test ($^{*}p < 0.05$, $^{**}p < 0.01$).

(**Figure 2.6B**). However, we found an increase in GM-CSF-producing $CD4^+$ T cells in the CNS of IL-7RTg mice afflicted with EAE (**Figure 2.6B**). Thus, providing constitutive expression of IL-7Rα on IL-$23R^{-/-}$ T cells does not rescue disease susceptibility or significantly impact the percentage of IL-17^+ $CD4^+$ T cells, showing that sustained IL-7R expression does not compensate for the absence of IL-23/IL-23R signaling in the generation of pathogenic Th17 cells.

IL-7 differentially affects the differentiation of $CD4^+$ T cells in vitro

While the IL-7/IL-7R axis cannot rescue the pathogenic T cell program driven by IL-23, it is possible that it works in concert with or sequentially after IL-23. To address this possibility, we investigated the effect of IL-7 on T helper cell differentiation of Th1 and Th17 cells. We activated WT naive $CD4^+$ T cells with anti-CD3 and irradiated antigen presenting cells (APCs) under neutral (Th0), Th1, or Th17 conditions in the presence or absence of IL-7 and further analyzed the frequencies of cytokine-producing T cells in each condition by intracellular cytokine staining. We found that IL-7 significantly induced IFN-γ under neutral and Th1 conditions (**Figure 2.7A-B**). In contrast, IL-7 did not promote but rather impaired Th17 cell differentiation *in vitro* (**Figure 2.7A-B**). Because GM-CSF is expressed by encephalitogenic Th1 and Th17 cells (65, 66), we analyzed its expression by *in vitro*-polarized cells in response to IL-7. We found that addition of IL-7 to the Th0, Th1, and Th17 cell cultures increased the frequency of GM-CSF^+ cells (**Figure 2.8A-B**). Together these data demonstrate the capacity of IL-7 to selectively expand Th1 cells, while inhibiting Th17 cells, and to drive GM-CSF production.

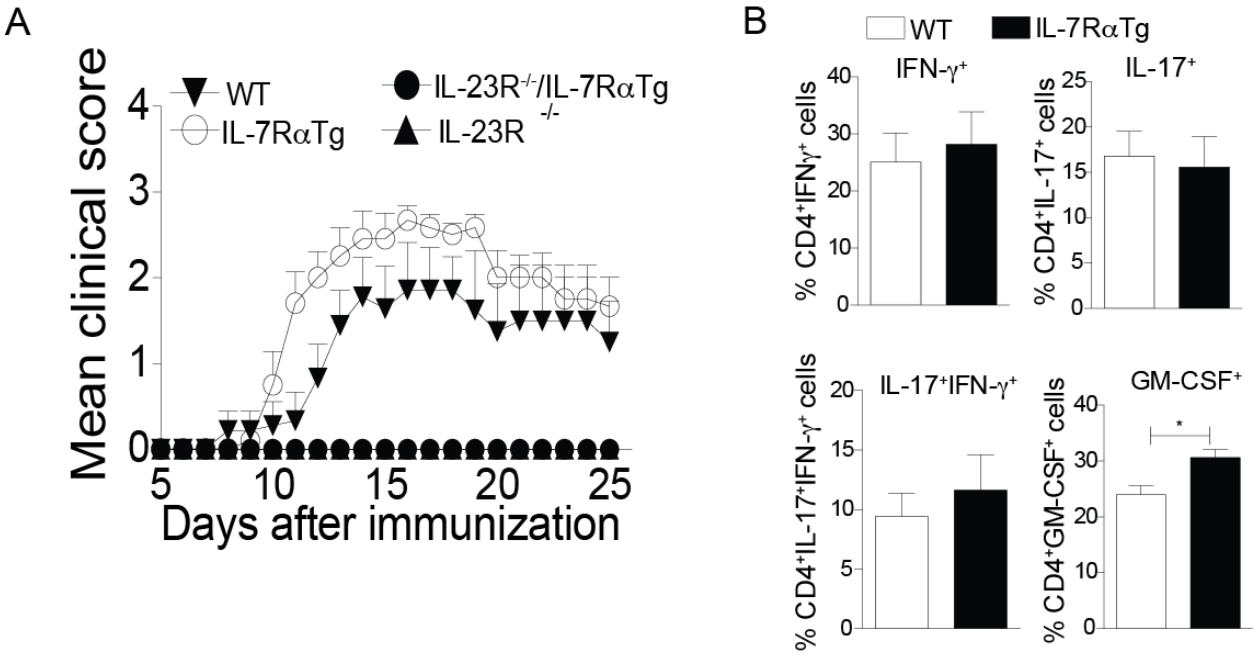

Figure 2.6. Sustained IL-7R signaling does not replace IL-23R signaling to restore EAE development. (**A**) Clinical course of EAE over time in these mice. Results are shown as mean clinical score ± SEM ($n \geq 5$ mice per group/experiment). Data from 3 combined experiments are shown. (**B**) Percentages of IL-17$^+$, IFN-γ$^+$, IL-17$^+$IFN-γ$^+$, and GM-CSF$^+$ CD4$^+$ T cells from the CNS of WT and IL-7RαTg mice at the peak of the disease are shown. Results from one representative experiment of two independent experiments are shown. Statistics were performed with Student's t test ($^*p < 0.05$).

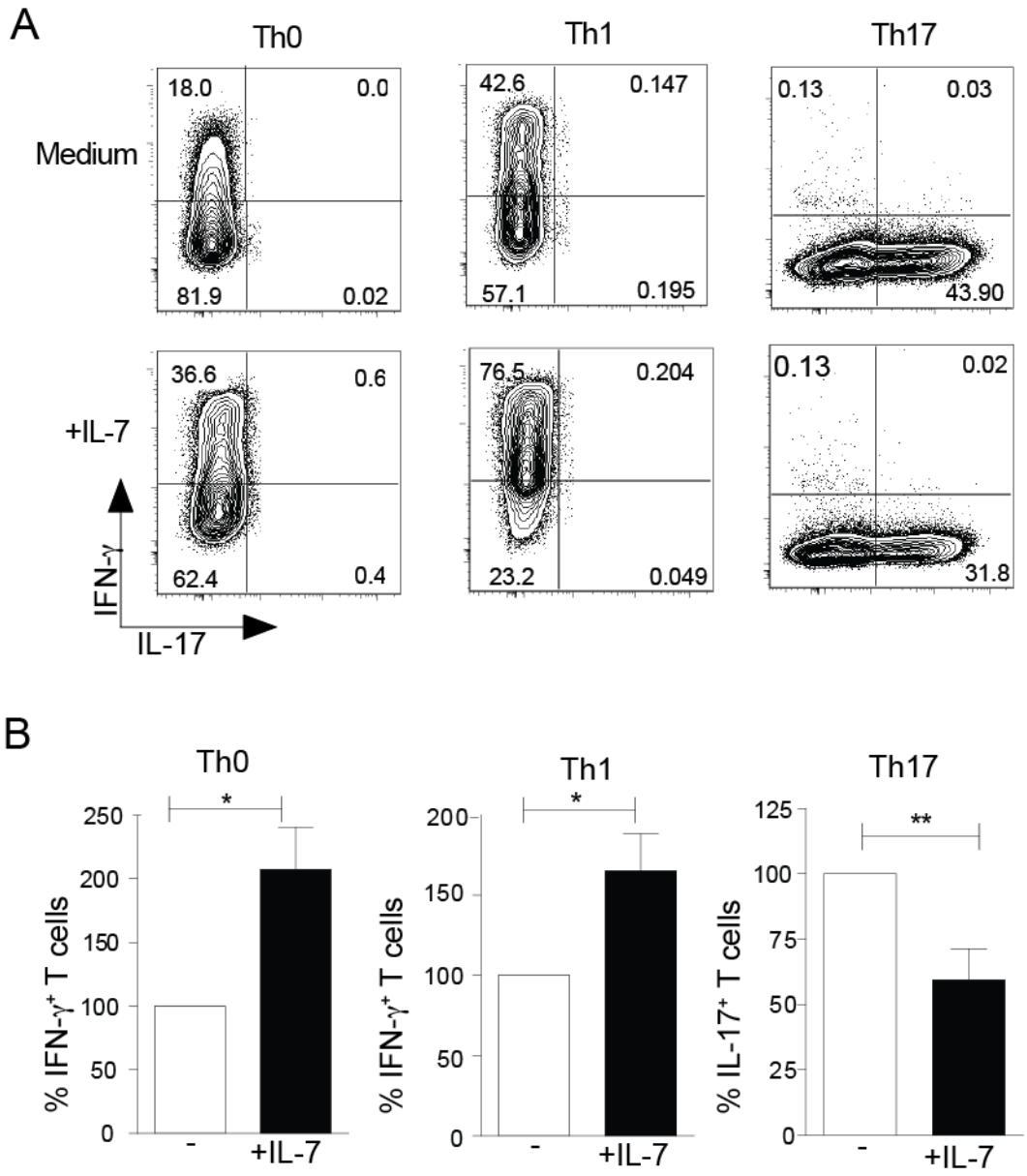

Figure 2.7. IL-7 enhances Th1 but inhibits Th17 differentiation *in vitro*. (**A-B**) Sorted naïve ($CD44^{lo}CD62L^{hi}CD25^{-}$) $CD4^{+}$ T cells were stimulated with antigen-presenting cells (APCs) and αCD3 (Th0), plus IL-12 (Th1), or IL-6 and TGF-β (Th17) in the presence or absence of IL-7. Five days after stimulation, cells were stimulated with PMA/ionomycin and intracellular cytokine staining was performed for IL-17 and IFN-γ (**A**) on live $CD4^{+}$ T cells. (**B**) Summary of the mean percentage of IFN-γ- and IL-17-expressing $CD4^{+}$ T cells is shown relative to the condition with medium alone. The percentages of cytokine-producing T cells are represented as mean ± SEM in each differentiation condition in the presence or absence of IL-7. Data are representative of at least 4 independent experiments. Statistics were performed with Student's t test ($^{*}p < 0.05$, $^{**}p < 0.01$, ns, not significant).

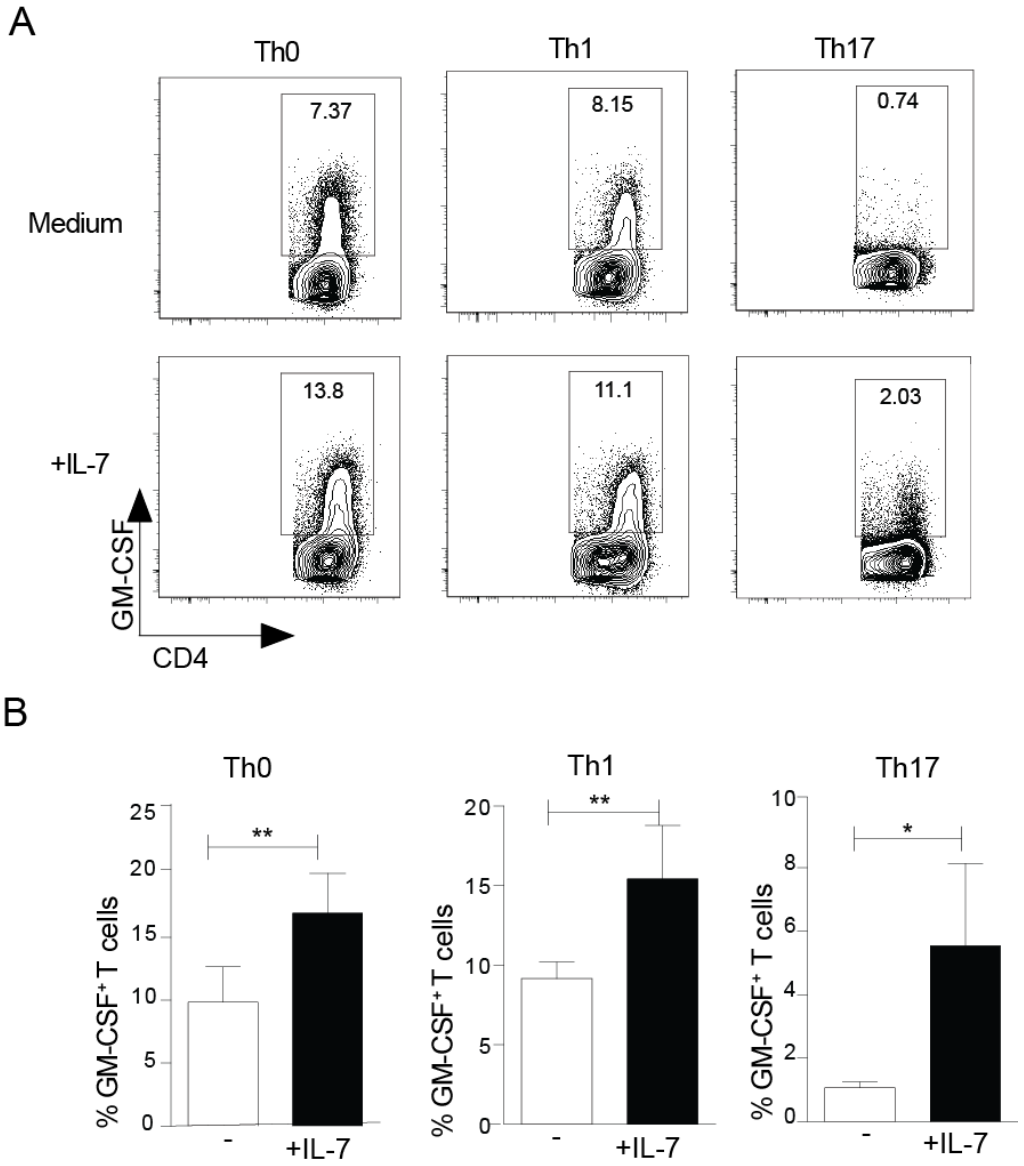

Figure 2.8. IL-7 enhances GM-CSF production *in vitro*. (**A, B**) Sorted naïve ($CD44^{lo}CD62L^{hi}CD25^{-}$) $CD4^{+}$ T cells were stimulated with antigen-presenting cells (APCs) and αCD3 (Th0), plus IL-12 (Th1), or IL-6 and TGF-β (Th17) in the presence or absence of IL-7. Five days after stimulation, cells were stimulated with PMA/ionomycin and intracellular cytokine staining was performed for GM-CSF (**A**) on live $CD4^{+}$ T cells. (**B**) Summary of the mean percentage of GM-CSF-expressing $CD4^{+}$ T cells is shown relative to the condition with medium alone. The percentages of cytokine-producing T cells are represented as mean ± SEM in each differentiation condition in the presence or absence of IL-7. Data are representative of at least 4 independent experiments. Statistics were performed with Student's t test ($^{*}p < 0.05$, $^{**}p < 0.01$, ns, not significant).

IL-7/M25 complexes expand CD4$^+$ T cells but do not impact the numbers of Foxp3$^+$ after immunization with myelin antigen

Although IL-7RαTg provided sustained expression of IL-7Rα on T cells, we considered the possibility that IL-7 availability *in vivo* might be scarce. Therefore, to test the importance of IL-7 on T cell expansion *in vivo*, and to ensure that a constant supply of IL-7 was available, we generated and administered IL-7 complexes (IL-7/M25 complexes: IL-7c) to WT mice. Treatment with IL-7c increases the potency of IL-7 50- to 100-fold, compared to IL-7 alone, and boosts lymphocyte numbers, particularly T cells, 2- to 3-fold (79). As a result, this strategy enabled us to test the effects of IL-7 on CD4$^+$ T cell expansion and survival *in vivo*. We treated ROR-γt-reporter mice (21) with IL-7c or PBS at days 0, 2, and 4, and harvested cells from the spleen at day 6. Total numbers of CD4$^+$ T cells were elevated 2 to 2.5 times in IL-7c treated mice compared to PBS treated mice (**Figure 2.9A**). We examined how IL-7 affects the pool of Foxp3$^+$ T cells upon treatment with IL-7c. IL-7c mildly decreased the frequency of Foxp3$^+$ CD4$^+$ T cells but due to the increase in total T cell numbers, the absolute number of these cells was significantly increased compared to PBS-treated groups (**Figure 2.9B**), an effect that may be reflective of the low levels of expression by Foxp3$^+$ cells. These results are in agreement with the reported effects of IL-7 on Foxp3$^+$ T cells (80).

Because Th17 cells have been shown to display a large degree of plasticity *in vivo* by expressing IFN-γ, we investigated the presence of Th17 cells via the expression of their hallmark transcription factor ROR-γt. We analyzed the frequency and absolute number of ROR-γt$^+$ Th17 and CXCR3$^+$ Th1 cells 6 days after the first injection of IL-7c. Flow cytometric analysis of T cells from the spleen of treated mice revealed a reduced frequency of ROR-γt-expressing CD4$^+$ T

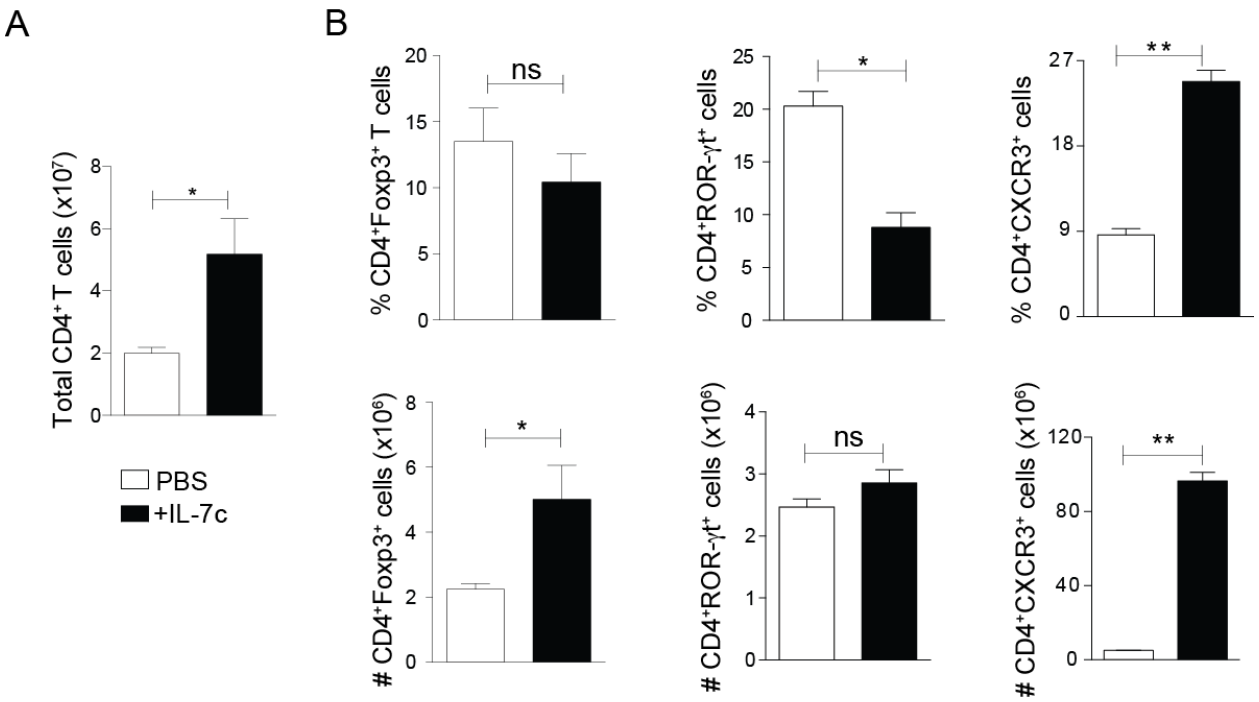

Figure 2.9. Expansion of CX3CR1$^+$ T cells by IL-7c *in vivo* after immunization with MOG$_{35-55}$/CFA. (**A, B**) ROR-γt-GFP reporter mice were treated with IL-7/M25 complexes (IL-7c) or PBS three times at 2-day intervals. (**A**) On day 6, cells from the spleen were quantified for total CD4$^+$ T cells. The frequencies and numbers of Foxp3$^+$, ROR-γt$^+$ Th17, and CXCR3$^+$ Th1 cells were determined by intranuclear and surface staining (GFP and CXCR3) respectively on CD4$^+$ T cells from the spleen. (**B**) Summary of frequency (top) and number (bottom) of Foxp3-, ROR-γt-, CXCR3-expressing CD4$^+$ T cells.

cells in IL-7c-treated mice, while no change in the absolute number of ROR-γt$^+$ T cells was observed between both groups (**Figure 2.9B**). However, we found that IL-7c boosted the frequency and number of CXCR3$^+$ Th1 cells compared to PBS-treated mice (**Figure 2.9B**). Thus, IL-7 enhanced IFN-γ production and the number of Th1 cells but did not expand Th17 or Treg cells *in vivo*, supporting our *in vitro* data.

IL-7/M25 complexes promote Th1 differentiation after immunization with MOG$_{35-55}$/CFA

Next, we assessed the effects of IL-7 complexes in the generation and expansion of Th1 and Th17 cells in wild type control and IL-23R-deficient mice immunized with MOG$_{35-55}$ and treated with IL-7c as described above. Total numbers of CD4$^+$ T cells from the LNs increased 2.5-fold in IL-7c treated WT mice compared to PBS treated mice (**Figure 2.10A**). In accordance with data from the ROR-γt-GFP reporter mice (**Figure 2.9B**), IL-7c treatment significantly enhanced the absolute number of Th1 cells in WT and IL-23R$^{-/-}$ mice compared to PBS treatment (**Figure 2.10B-C**). However, we did not observe a change in the number and frequency of Th17 cells generated after *in vivo* treatment with IL-7c upon immunization (**Figure 2.10B-C**). The generation of IFN-γ$^+$IL-17$^+$ T cells remained unchanged between PBS- and IL-7C-treated B6 and IL-23R$^{-/-}$ mice (**Figure 2.10B**). These results, together with the results observed in IL-7RαTg mice, demonstrate that increased IL-7/IL-7R signaling through administration of IL-7c does not rescue Th17 differentiation but acts to promote Th1 differentiation, reflective of what we see *in vitro*.

IL-7/M25 complexes promote the proliferation of Th1 and not Th17 cells

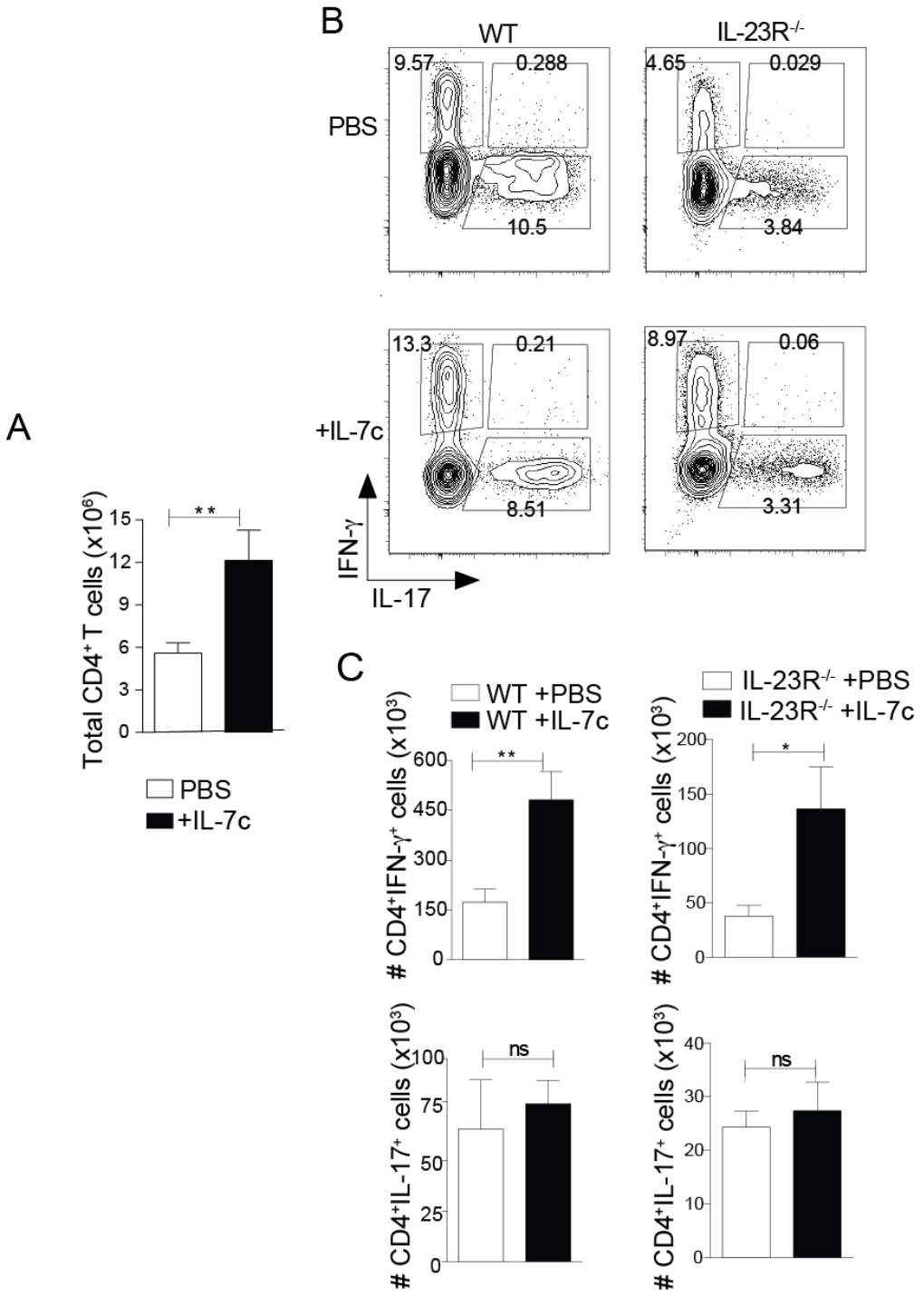

Figure 2.10. Expansion of IFN-γ$^+$ T and not IL-17$^+$ cells by IL-7c *in vivo* after immunization with MOG$_{35-55}$/CFA. (**A-C**) WT and IL-23R$^{-/-}$ mice were immunized with MOG$_{35-55}$/CFA and treated with (IL-7c) or PBS on days 1, 3, and 5 after immunization. (**A**) Eight days after immunization, CD4$^+$ T cells from the draining lymph nodes were counted. (**B-C**) CD4$^+$ T cells were stimulated with PMA/ionomycin for intracellular cytokine staining to assess the frequencies and numbers of Th1 and Th17 cells in each strain (**B**, representative dot plot and **C**, summary).

Together our data point to a role for IL-7 in promoting the differentiation of Th1 cells and increasing the numbers of Th1 cells. However, it is unclear whether IL-7 also played a role in the proliferation of Th1 cells. To determine whether this was the case, we analyzed Ki67 expression in Th1 and Th17 cells from PBS- or IL-7c-treated mice. Interestingly, Th17 cells proliferated more rapidly than Th1 cells in immunized mice that received PBS and their proliferation was not affected by IL-7c (**Figure 2.11**). The increased proliferation of Th17 cells, compared to Th1 cells that we observed, is in accordance with previous reports (24). In contrast, Th1 cells proliferated to a much larger extent in the presence of IL-7c (**Figure 2.11**). Collectively, these results demonstrate that IL-7c selectively promotes the proliferation of Th1 but not Th17 cells.

IL-7/M25 complexes do not affect the development of EAE

To test how increased levels of IL-7 by exogenous administration affects EAE development, we immunized WT and IL-23R-deficient mice with MOG_{35-55} to induce disease and treated them with IL-7c. We observed a slight increase, though not statistically significant, in the severity of the disease of IL-7c-treated compared to PBS-treated WT mice (**Figure 2.12**). This was associated with an increase number of IFN-γ^+ $CD4^+$ T cells generated in the spleen of WT mice treated with IL-7c (data not shown). On the other hand, IL-23R-deficient mice, whether or not treated with IL-7c, were resistant to the development of EAE (**Figure 2.12**). These results show that IL-7 complexes selectively expand Th1 cells but not Th17 cells in the periphery. As a result, IL-7c treatment, similarly to IL-7Rα overexpression, is incapable of restoring disease susceptibility in IL-23R-deficient mice.

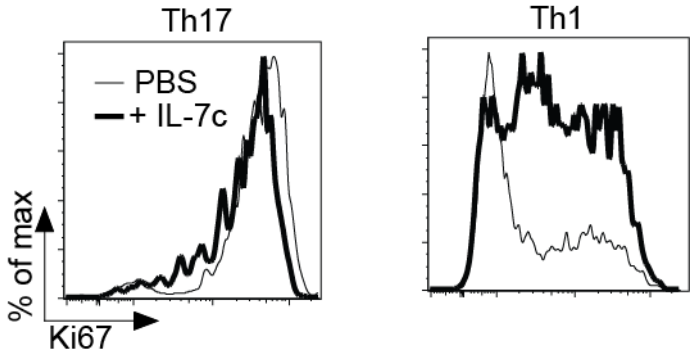

Figure 2.11. IL-7/M25 complexes promote the proliferation of Th1 and not Th17 cells. WT mice were immunized with MOG_{35-55}/CFA and treated with (IL-7c) or PBS on days 1, 3, and 5 after immunization. Proliferation of Th17 (left) and Th1 cells (right) from immunized PBS- and IL-7c-treated WT mice was compared by Ki67 staining in IL-17^+ and IFN-γ^+ $CD4^+$ T cells respectively. The graphs represent cumulative data of 2-5 mice per group, from at least 2 independent experiments (mean ±SEM). Statistics were performed with Student's t test ($^*p < 0.05$, $^{**}p < 0.01$, ns, not significant).

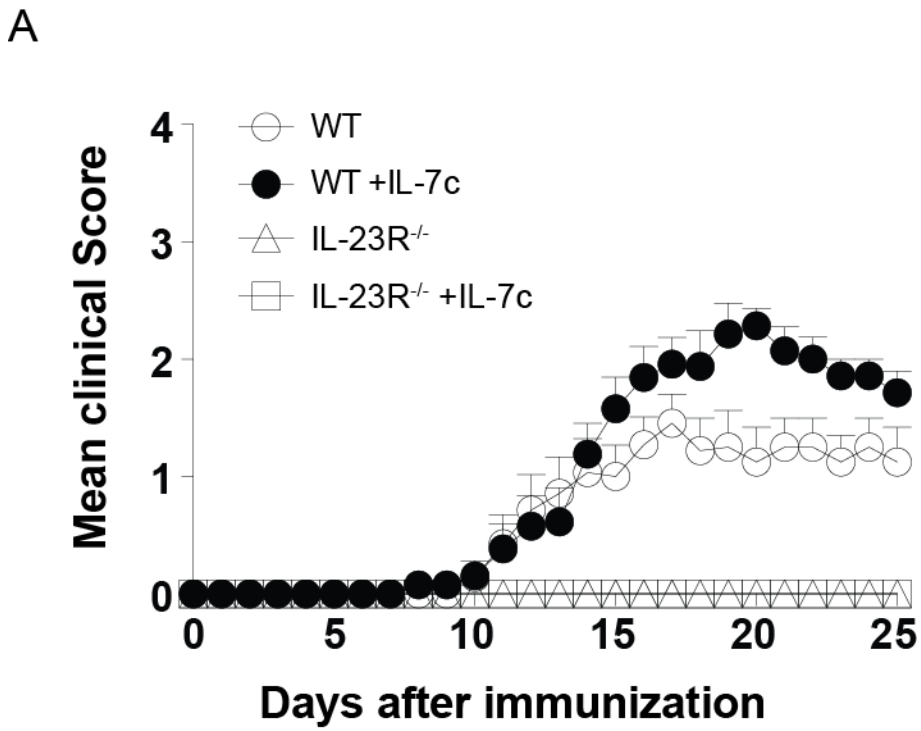

Figure 2.12. IL7/M25 complexes do not affect the development of EAE. Clinical scores of WT and IL-23R$^{-/-}$ mice immunized with MOG_{35-55}/CFA and treated with PBS or IL-7c at days 1, 3, and 5 after immunization. Results are shown as mean ± SEM over time ($n \geq 6$ mice per group). Data from 2 combined experiments are shown.

IL-7 promotes the conversion of Th17 cells to a Th1 phenotype

$CD4^+$ T cells infiltrating the target organ in various autoimmune diseases are phenotypically very different from $CD4^+$ T cells present in peripheral immune tissues because they become plastic and modulate their cytokine production. Therefore, we next examined whether CNS infiltrating $CD4^+$ T cells during EAE could have a specific IL-7Rα expression profile and IL-7 responsiveness compared to $CD4^+$ T cells from peripheral immune tissues. We analyzed the expression of IL-7 in the CNS of naive mice and mice with EAE. IL-7 mRNA was very highly expressed in the CNS of both naive mice and mice with EAE signs (**Figure 2.13A**), suggesting that it could play an important role in modulating the balance between IFN-γ^+ and IL-17^+ T cells.

To determine how the substantial presence of IL-7 in the CNS correlates with IL-7R positivity on Th1, Th17, and Th1/17 cells, we immunized the Foxp3-RFP/IL-17A-GFP/IFN-γ-Thy1.1 triple reporter mice for EAE and analyzed the cytokine expression by $CD4^+$ T cells in the CNS at the peak of the disease. Consistent with peripheral T cells (**Figure 2.13A-K**), a hierarchy of IL-7Rα expression was apparent, with IL-7Rα being more expressed on Th17 cells, followed by Th1/17 cells, and then Th1 cells (**Figure 2.13B-D**). Similar results were found when the CNS was separated into the brain and spinal cord or when the CNS was examined at the onset of the disease (data not shown). Because low IL-7Rα levels indicate a recent IL-7-mediated signaling event, this finding suggests that Th17 cells that maintain their phenotype (IL-17 production) do not respond to IL-7, while those that respond downregulate the IL-7Rα and express IFN-γ.

To address this hypothesis, we differentiated naïve T cells into Th17 cells and chronically stimulated Th17 cells in the presence of IL-7. Whereas restimulation alone did not impact Th17 cells, the presence of IL-7 induced IFN-γ production by Th17 cells (**Figure 2.13E**). These data not only confirm the inherent plastic nature of Th17 cells but they also suggest that restimulation of Th17 cells in the presence of IL-7 induces Th1/17 or ex-Th17 cells. Thus, high levels of IL-7 in

the CNS of mice, during EAE, induce the production of IFN-γ from Th17 cells and drive the development of Th1/17 and Th1 cells.

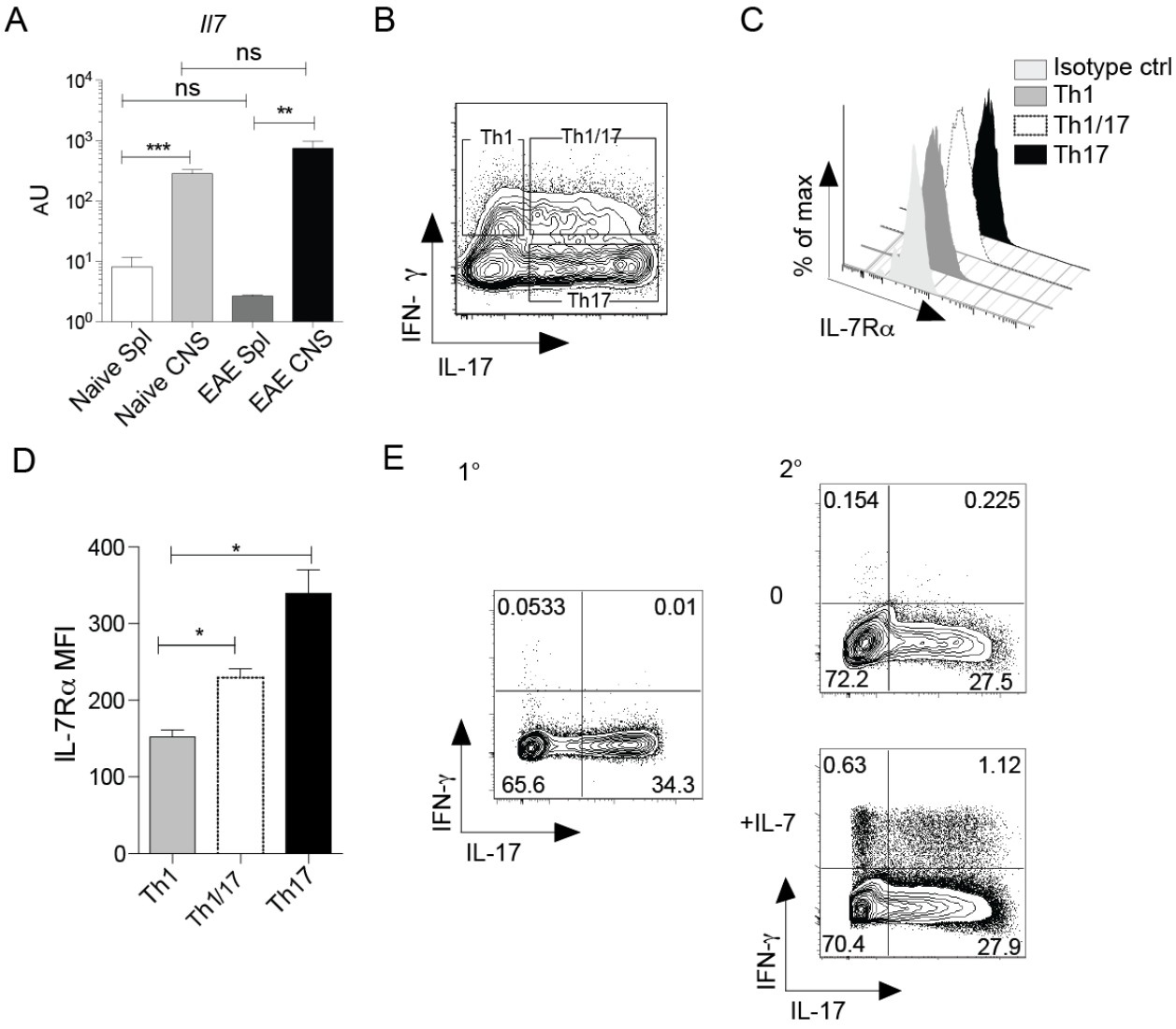

Figure 2.13. Role of IL-7-IL-7R signaling in Th17 plasticity. (**A**) IL-7 mRNA expression in the spleen (Spl) and CNS of naive and mice with EAE at the peak of the disease, was measured by real time PCR. (**B-D**) CNS-infiltrating Th1, Th1/17, and Th17 cells were detected by Thy1.1 and GFP surface expression in triple reporter (Foxp3-RFP/IFN-γ-Thy1.1/IL-17-GFP) mice with EAE. (C) IL-7Rα expression on the different effector $CD4^+$ T cells gated in (**B**). (**D**) Summary of IL-7Rα MFI on Th1, Th1/17, and Th17 cells. (E) Naïve $CD4^+$ T cells were differentiated into Th17 cells for the primary stimulation (1°). Seven days later the cells were restimulated with APCs and anti-CD3 in the absence or presence of IL-7 (2°). Five days after each stimulation, the cells were stained intracellularly for IFN-γ and IL-17 after PMA/ionomycin stimulation. Results are from at least 2 independent experiments of n≥2 mice/group.

Discussion

Th1 and Th17 cells play a central pathogenic role in both EAE and MS (28, 62). IL-23 is essential for the generation of pathogenic $CD4^+$ T cells and for the induction of EAE (18, 48, 49). On the other hand, IL-7 is required for lymphopoiesis and in many aspects of naïve and memory T cell survival and maintenance in the periphery (81). A connection between IL-7 and IL-23-induced pathogenic Th17 cells has emerged based on previous studies showing that IL-7 promoted Th17 expansion and that IL-23 induced the expression of IL-7Rα on Th17 cells (48, 75). In this report, we directly tested the hypothesis that IL-7/IL-7R signaling could promote Th17 expansion and pathogenic activity independently of IL-23. We found that constitutive expression of IL-7Rα on T cells does not compensate for the lack of IL-23R expression, that IL-7 does not impact Th differentiation in the absence of IL-23R, and that IL-23 does not modulate IL-7Rα expression. Moreover, we determined that IL-7 enhanced the differentiation and expansion of Th1 cells, and induced the plasticity of Th17 cells by converting them into IFN-γ-producing Th17 cells.

IL-7 receptor is rapidly downregulated on T cells after ligation and/or activation via the T cell receptor but is subsequently re-expressed, particularly after the cells have differentiated into an effector/memory phenotype (69). Because memory T cells require IL-7, upregulation of this receptor is necessary for their survival (82). In IL-23R-deficient mice, Th17 cells are generated early after immunization, but they fail to expand, rendering mice resistant to the development of EAE (18, 48, 49). Based on these observations and a study showing that IL-7 promotes Th17 cell expansion (75), it was therefore reasonable to speculate that IL-23R-deficient T cells failed to upregulate IL-7Rα receptor, making them less sensitive to IL-7 and less likely to survive (48). In this report, we determined that IL-7Rα expression is more highly expressed on Th17 cells and on $IL\text{-}23R^+$ T cells compared to Th1 cells. These results may explain why low expression of IL-7Rα

on T cells could be detected in IL-23R-deficient mice, which failed to generate stable Th17 cells (48). By providing IL-7Rα expression on IL-23R-deficient T cells, we investigated whether IL-7/IL-7R signaling could promote Th17 expansion and stability, thereby inducing EAE. Against our expectations, IL-7Rα expression on T cells from IL-23R$^{-/-}$ mice did not restore EAE susceptibility and did not promote the expansion of Th17 cells. We further determined that IL-7Rα expression was not modulated by the IL-23/IL-23R signaling axis (data not shown). To conclusively rule out the possibility that IL-23R-mediated IL-7/IL-7R signaling is essential for Th17 differentiation, we provided exogenous IL-7 and found that Th17 cell differentiation was not impacted after administration of IL-7, but IFN-γ expression was enhanced. Hence, we investigated the reasons behind these observations and our data help reconcile several published reports exploring the role of IL-7 during EAE development.

Several lines of evidence point to a disease-stimulating effect of IL-7: complete deficiency of IL-7Rα or selective deficiency of IL-7Rα on peripheral hematopoietic cells prevent or reduce clinical signs of EAE (74, 83). Furthermore, treatment of WT mice with an anti-IL-7Rα antibody limits EAE severity (73, 83). While IL-7 has been implicated in affecting both Th1 and Th17 cells, it remains unclear whether these two pathogenic T cell populations are equally affected by IL-7 *in vivo*. Indeed, the effects of IL-7 on T cell differentiation have been controversial. Liu et al. had reported that IL-7 did not affect Th1 cells and instead served a crucial role in the survival and expansion of Th17 cells, since blockade of the IL-7/IL-7R signaling pathway with an anti-IL-7Rα antibody altered the Th17 cell response (75). These results were questioned in light of another study, which reported that IL-7 promoted Th1 differentiation in human and murine naïve CD4^{+} T cells, while it had no effect on IL-17 production *in vitro* (73). The *in vitro* data obtained on Th differentiation contrasted with *in vivo* data presented in the same report and showed that treatment

of mice afflicted with EAE with an anti-IL-7Rα antibody limited both Th1 and Th17 responses (73). However, the anti-IL-7Rα blocking antibody has a broad depleting effect on the T cell compartment, making it difficult to investigate how IL-7 affects T helper cell differentiation (73). In our study, we took the approach of providing sustained IL-7/IL-7R signaling via the use of IL-7/M25 complexes. One major obstacle for addressing the effects of IL-7 *in vivo* is the presence of an IL-7 sink, whereby IL-7 is rapidly consumed after exogenous delivery, making IL-7 activity very brief. By complexing IL-7 to the monoclonal anti-IL-7 clone M25 antibody, the cytokine is more potent and stable (84). Our data show a preferential expansion and proliferation of Th1 cells over Th17 cells after IL-7/IL-7R complex injection in MOG_{35-55}-immunized mice. In addition, *in vitro* differentiation of Th1 cells, but not Th17 cells, is enhanced in the presence of IL-7 (**Figure 2.7**). Because Th1 cells are more prone to apoptosis than Th17 cells (85, 86), and because IL-7 can sustain Bcl-2 expression and promote cell survival (87), it is also possible that IL-7 prevents apoptosis in Th1 cells and therefore promote their preferential expansion (Figure 4F).

Furthermore, consistent with the idea that IL-7 modulates the differentiation of effector T cells, we found that in all Th-polarizing conditions tested IL-7 promotes GM-CSF production, a cytokine that has been implicated in the pathogenicity of T cells during EAE (65, 66). We also found an increase in GM-CSF production in the CNS of mice that constitutively expressed the IL-7R. These results were confirmed by an independent study published while this manuscript was in preparation (88). In addition, we determined that IL-7 induced IFN-γ production by Th17 cells (**Figure 2.13E**). Our lab (23) and others (24) have highlighted the pathogenicity of Th17 cells in the CNS of mice during EAE. We reported that IL-23 is a critical factor that leads to the generation of these cells (23). In this study, we show that while IL-7 does not promote IL-17 production or the expansion of Th17 cells, it likely facilitates the plasticity of Th17 cells and their production of IFN-γ (**Figure**

2.13E). Most Th1 cells in the CNS of mice afflicted with EAE have been recognized as ex-Th17 cells (Th17 cells that have extinguished IL-17 expression and express IFN-γ) (24). Our data suggest that although IL-23 plays an essential role in the emergence of IL-17$^+$ IFN-γ$^+$ (23), IL-7 could also participate in this process and sustain the plasticity of these cells, since IL-7 is highly expressed in the CNS during neuroinflammation (**Figure 2.13A**). This could also be relevant in MS since IL-7 expression is increased in brain lesions (89, 90). Together, our results suggest that IL-7, which is abundantly expressed in the CNS, could participate in EAE pathogenesis by converting Th17 cells into Th1 cells and expanding Th1 cells. These data build upon previous findings linking IL-7/IL-7R signaling to the promotion of Th1 cells and higher levels of serum IL-7 with a therapeutic benefit of IFN-β (47, 73). Thus, our results further provide evidence that IL-7Rα may serve as a target in treatment strategies for Th1-mediated multiple sclerosis.

Single-nucleotide polymorphisms (SNPs) in the IL-7/IL-7R pathway are associated with an increased risk of multiple sclerosis (MS). In particular, the skewed levels of IL-7 and IL-7Rα expression, resulting from specific SNPs, are believed to influence responsiveness of IL-7Rα$^+$ cells. Because the level of IL-7Rα expression has traditionally been proposed to correlate with the level of responsiveness of the cells, it was therefore of particular interest to analyze the expression of IL-7Rα on different subsets of T helper cells in lymphoid organs and CNS during the course of EAE. We detected high expression levels of IL-7Rα on Th17 cells. This enhanced expression of IL-7Rα on IL-17$^+$ ROR-γt$^+$ Th17 cells was not only observed in the periphery (**Figure 2.2** and **2.3**) but also on Th17 cells present in the CNS during EAE (**Figure 2.13B-D**), indicating that IL-7Rα, thus, serves as marker of Th17 cells. It was therefore surprising to observe a limited effect of IL-7 on Th17 cells, which are higher for IL-7Rα expression, and a promoting effect of IL-7 on Th1 cells, which express lower levels of the receptor. Our data support the novel notion that IL-7Rα is

enriched on Th17 cells because they are refractory to IL-7 signaling and therefore IL-7-mediated downregulation of the IL-7Rα. Indeed, because IL-7 complexes do not expand Th17 cells *in vivo* and Th17 cells maintain a high level of expression of the IL-7Rα, we propose that most Th17 cells do not readily respond to IL-7, or if they do, they lose IL-17 expression and acquire IFN-γ expression, whereas Th1 cells respond to IL-7/IL-7R signaling and downregulate IL-7Rα. In summary, we provide a model in which IL-7R is highly enriched on Th17 cells during EAE and is not involved in the protection of IL-23R-deficient mice to EAE development. As Th17 cells are converted to IFN-γ-producing cells, they downregulate the receptor. Thus, this study provides a novel mechanism by which Th17 cells can be converted to Th1 cells in response to chronic IL-7 stimulation in the CNS during EAE.

CHAPTER 3

STAT1-induction of missing self on CD4+ T cells prevents EAE development

Introduction

Recognition of self-markers is unequivocally one of the most important responsibilities of the immune system, because the immune system is tasked with preventing severe immunopathology and autoimmune diseases, while also being effective at eliminating and clearing cells that have been infected with pathogens. NK cells are a critical component of the innate immune arm by acting as a main line of defense in recognizing and killing infected cells, while leaving healthy cells intact.

A break in tolerance will sometimes lead to autoimmunity. The initiation and progression of autoimmune disease stem from complex interactions between cells of both the innate immune system and adaptive immune system. Multiple sclerosis is an autoimmune disease of the central nervous system (CNS) that inflicts axonal damage and demyelination through a complex interplay between the innate and adaptive arms of the mammalian immune system (61). Most of the knowledge gained about MS comes from its mouse model experimental autoimmune encephalomyelitis (EAE). CD4+ T cells have been shown to be crucial players in the development of the disease by recognizing myelin antigen and secreting pro-inflammatory cytokines such as IFN-γ for Th1 cells and IL-17 for Th17 cells.

A lot of studies have focused on the role of transcription factors and signaling pathways, which impact Th1 and Th17 cells. T-bet, STAT1, and STAT4 have been shown to control Th1 differentiation, while ROR-γt and STAT3 are key determinants of Th17 differentiation and maintenance. While deficiency in ROR-γt, STAT3, or STAT4 leads to resistance to EAE

development, STAT1-deficient mice develop exacerbated disease (55). The paradoxical effects of STAT1 in EAE have been phenocopied in mice deficient for type I interferon (91) and IFN-γ (53), which signal mainly through STAT1. One explanation is that STAT1 inhibits IL-17 production by CD4+ T cells, making $STAT1^{-/-}$ mice more adept at making Th17 cells (92). However, further work needs to be done to prove that the increase in IL-17 expression from CD4+ T cells and augmentation of proliferation in STAT1-deficient mice cause exacerbated CNS autoimmunity.

Multiple studies have suggested that NK cells can influence the magnitude of an autoantigen-specific response by direct cytotoxic effects against self-tissues and/or immunoregulatory activity (93). NK cells evolved to rapidly respond to a variety of insults with cytolytic activity and cytokine secretion (94). These cells are equipped with the lytic machinery to target different cells of the body, including activated T cells, which limits clonal expansion of T cells activated by foreign or self-antigens *in vivo*. NK cells have been shown in many infection models to regulate the number of effector T cells (6, 7, 95). For these reasons, NK cells have been shown to play a key role not just in controlling T cell responses but also in regulating autoimmune diseases (96). Multiple cytokines have been shown to be crucial for the development, survival, and activation of NK cells, including type I IFN, IFN-γ, IL-2, and IL-15. IL-2 potently induces perforin and granzyme, which are critical components for lysis mediated by NK cells (97), can be secreted by activated CD4+ cells to boost NK cell cytotoxicity. Thus, NK cells can limit T cell responses in a variety of contexts.

In the present study, we defined a novel role for STAT1 in CD4+ T cells. In stark contrast to the protective effect of global STAT1 signaling in EAE, STAT1-deficient Th17 cells were incapable of inducing EAE despite increased IL-17 production. Moreover, mice with a

selective deficiency of STAT1 in T cells ($STAT1^{fl/fl}$/CD4-Cre) were protected from EAE development. CD4+ T cell proliferation and survival were profoundly altered *in vivo* but not *in vitro* in the absence of STAT1. After co-transfer into lymphopenic settings and in response to their cognate antigen, STAT1-deficient CD4+ T cells failed to survive and expand. We found that NK cells were responsible for this phenotype as CD4+ T cells failed to upregulate MHC class I molecules when they lacked STAT1. Elimination of NK cells via antibody depletion or genetic mutation using IL-15-deficient mice restored the ability for STAT1-deficient CD4+ T cells to survive and proliferate *in vivo*. Furthermore, transfer of STAT1-deficient CD4+ T cells into Rag-deficient hosts led to restoration of EAE development only in the absence of NK cells. These results implicate STAT1 as essential for recognition of CD4+ T cells as self in order to maintain NK cell tolerance and illustrate the importance of this signaling pathway in the control of CD4+ effector T cell responses.

Materials and Methods

Mice

C57BL/6 (B6), B6/SJL (CD45.1), and RAG1$^{-/-}$ mice were purchased from the Jackson Laboratories and bred in the Benaroya Research Institute (BRI) animal facility. STAT1$^{fl/fl}$ mice, generously provided by Dr. Daniel Campbell, were crossed to CD4-Cre mice. STAT1$^{fl/fl}$/FIC were kindly provided by Dr. Daniel Campbell. 2D2 TCR transgenic mice have been previously described (43). Rag2$^{-/-}$/IL-15$^{-/-}$ mice were kindly provided by Dr. Mohamed Oukka. All strains are on the C57BL/6 background. For depletion of NK cells, antibody to NK1.1 (PK136) was injected at 200μg/mouse one day prior to transfer of CD4+ T cells. All animals were bred and maintained under specific pathogen-free conditions at the Benaroya Research Institute (Seattle, WA) and all experiments were performed in accordance with the guidelines of the Benaroya Research Institute Animal Care and Use Committee.

CD4$^+$ T cell preparation, T cell differentiation, and proliferation assay

For T cell differentiation, naïve CD4$^+$CD62L^{hi}CD44^{lo}CD25− T cells were isolated by FACS sorting (FACSAria, BD Biosciences) and cultured with irradiated (4000 rads) CD4-depleted spleen cells from wild-type (WT) mice and anti-CD3 (2.5 μg/ml, clone 145-2C11) for 5 days in complete RPMI. For Th17 differentiation, 2.5 ng/ml rhTGF-β (R&D Systems), 30 ng/ml rmIL-6 (Peprotech), 10 μg/ml anti-IFN-γ and 10 μg/ml anti-IL-4 (NIH/NCI BRB Preclinical Repository) were used. For transfer experiments, IL-23 was added and α-IFN-γ was excluded from the cultures. 10 ng/ml rmIL-12 was used for Th1 conditions.

Antibodies, flow cytometry, and cell sorting

For surface staining, cells were incubated at 4°C for 30 minutes in staining buffer (PBS, 2% FCS) with the following directly conjugated antibodies for murine proteins (from Biolegend unless otherwise specified): CD4 (clone L3T4) conjugated to PerCP-Cy5.5, Alexa700, or BV650, CD44 (IM7) conjugated to PE-Cy7 or APC-Cy7, CD25 (PC61) conjugated to PE, CD62L (MEL-14) conjugated to Pacific Blue, CD45.2 and CD45.1 conjugated to Pacific Blue and PE-Cy7, H-2K^b in PE, and Qa2 in APC. For intracellular cytokine staining analysis, cells were incubated 5 hours in complete RPMI containing 50ng/ml phorbol myristate acetate (PMA), 1μg/ml ionomycin (Sigma-Aldrich) and Golgi Stop (BD Biosciences). Cells were then washed with cold PBS and blocked for 10 min with anti-CD16/32 purified antibody (clone 2.4G2, BioXCell). Viability of the cells was assessed by staining with fixable dye eFluor780 (eBioscience). Cells were then stained with surface antibodies and washed with PBS. Cells were then fixed for 20 min with fixation buffer (BD Biosciences), permeabilized with BD permeabilization/wash buffer (BD Biosciences) and stained with anti-IFN-γ, and anti-IL-17 specific antibodies in permeabilization buffer. Cells were acquired on LSRII (BD Biosciences), and data were analyzed with FlowJo software. The following antibodies were used in our experiments: IL-17 (clone TC11-18H10.1) conjugated to APC from Biolegend and IFN-γ (clone XMG1.2) conjugated to PE-Cy7 or PE from eBioscience.

Phospho-S6 staining

Cells were stimulated for 2 hours at 37°C, 5% CO_2, in complete RPMI with plate-bound αCD3 and αCD28 (2.5μg). Cells were harvested and fixed for 20 minutes in BD fix/perm buffer at RT (BD Biosciences), washed with BD Perm Wash buffer, and fixed in 90% ice cold methanol

for 30 minutes. Cells were washed with BD Perm Wash and stained with antibodies against CD4 and phospho-S6 (Ser235/236) from Cell Signaling for 45 minutes at RT.

Proliferation assays

For *in vitro* proliferation assays, naïve CD4+ T cells were labeled with either 5μM CFSE (eBioscience) or 5μM Cell Trace Violet (Molecular Probes) for 10 minutes at 37°C in PBS and washed with complete RPMI and stimulated with 2.5μg plate-bound αCD3 and αCD28 for 3 days.

For *in vitro* recall responses of MOG-specific cells, draining lymph nodes (dLN) if immunized mice were collected 8-10 days after immunization with MOG_{35-55}/CFA. Cells were cultured at 5×10^6 cells/mL in the presence of different concentrations of MOG for 72 h. During the last 16 h, cells were pulsed with 1 μCi [^{3}H]thymidine. [^{3}H]thymidine incorporation was measured using a β-counter.

Adoptive transfer of CD4$^+$ T cells

For co-transfer experiments into a lymphopenic environment, $0.5x10^6$ sorted naïve CD4+ T cells from congenically marked control (Ctrl) and STAT1$^{fl/fl}$/CD4-Cre were transferred into Rag2$^{-/-}$ mice for 7 days. For antigen-dependent proliferation of co-transferred cells, $0.5x10^6$ sorted naïve CD4+ T cells from congenically marked control (2D2) and STAT1$^{fl/fl}$/CD4-Cre/2D2 were transferred into Rag2$^{-/-}$ mice 1 day prior to immunization with 50 μg/mouse of MOG_{35-55} emulsified in complete Freund's adjuvant (CFA).

EAE induction

EAE was induced by subcutaneous immunization of mice into the flanks with an emulsion of MOG_{35-55} peptide (100 μg) emulsified in complete Freund adjuvant supplemented with 4mg/ml of *M. tuberculosis* extract H37Ra (Difco). In addition, the animals received 200 ng of pertussis toxin (List Biological Laboratories) i.p. on days 0 and 2. Clinical signs of EAE were assessed according to the following score: 0, no signs of disease; 1, loss of tail tonicity; 2, righting reflex; 3, partial hind limb paralysis; 4, complete hind paralysis; 5, complete hind and forelimb paralysis; 6, moribund.

Passive EAE induction

50,000 naïve CD4+ T cells from 2D2 and $STAT1^{fl/fl}$/CD4-Cre/2D2 mice were transferred into $Rag2^{-/-}$ mice and immunized s.c. with 25μg MOG_{35-55} in CFA and 200 ng pertussis toxin was injected i.p the same and 2 days after. For transfer of Th17 cells, sorted naïve CD4+ T cells from 2D2 and $STAT1^{fl/fl}$/CD4-Cre/2D2 mice were differentiated *in* vitro for 5 days in the absence of α-IFN-γ with IL-23, as described above, and $5x10^6$ cells from each mouse were transferred into $Rag2^{-/-}$ mice with pertussis toxin injected the same day and 2 days later. Clinical signs of EAE were assessed according to the following score: 0, no signs of disease; 1, loss of tail tonicity; 2, righting reflex; 3, partial hind limb paralysis; 4, complete hind paralysis; 5, complete hind and forelimb paralysis; 6, moribund.

Isolation of CNS mononuclear cells

Mice were sacrificed at the peak of disease and perfused with cold PBS. Brain and spinal cords were isolated and digested for 30 min at 37°C with Collagenase D at a concentration of

2.5mg/ml (Roche). Mononuclear cells were isolated over a 37% / 70% Percoll gradient (VWR), washed twice with complete medium and collected in medium for further analysis.

Statistical analysis

Statistical analyses were conducted with GraphPad Prism software. P values were calculated with Student's paired *t*-test. P values of less than 0.05 were considered significant, *≤ 0.05, **≤0.01, ***≤0.001. Error bars denote ± SEM as indicated.

Results

STAT1-deficient Th17 cells do not induce EAE

Given that STAT1 deficiency leads to exacerbated EAE (55) and that CD4+ T cells are the main drivers of EAE development, we initially were interested in defining the contribution of STAT1 to the pathogenicity of effector CD4+ T cell differentiation during EAE. We differentiated Th1 and Th17 cells from naïve $CD62L^{hi}CD44^{lo}$ CD4+ T cells derived from 2D2 mice, which have TCR transgenic CD4+ T cells specific for MOG_{35-55}, and $STAT1^{fl/fl}$/CD4-Cre/2D2 mice, which lack STAT1 signaling in T cells. Although IFN-γ production by Th1 cells was reduced when the cells lacked STAT1, IL-17 expression in both Th1- and Th17-skewed conditions was elevated (**Figure 3.1A**). These results are consistent with previous studies illustrating the importance of STAT1 in aiding the differentiation and stability of the Th1 cell phenotype, while repressing the Th17 cell program (14, 92). Hence, it is possible that STAT1 deficiency dramatically increases the susceptibility to EAE development due to enhanced Th17 cell differentiation. We thus reasoned that STAT1 deficiency in CD4+ T cells impaired Th1 differentiation, affecting EAE development, but because Th17 cells have been shown to be potent inducers of EAE development (28, 45), Th17 cells alone could transfer disease. Because antigen-specific Th17 cells can induce EAE independently of Th1 cells, we transferred Th17-diffentiated 2D2 and $STAT1^{fl/fl}$/CD4-Cre/2D2 cells into Rag1-deficient ($RAG1^{-/-}$) hosts. Surprisingly, even though STAT1-deficient Th17 cells differentiated efficiently and better than WT Th17 cells (**Figure 3.1A),** mice transferred with STAT1-deficient Th17 cells did not transfer disease as efficiently as control Th17 cells (**Figure 3.1B**). These data point to a role for STAT1 in guiding T cells toward an encephalitogenic phenotype.

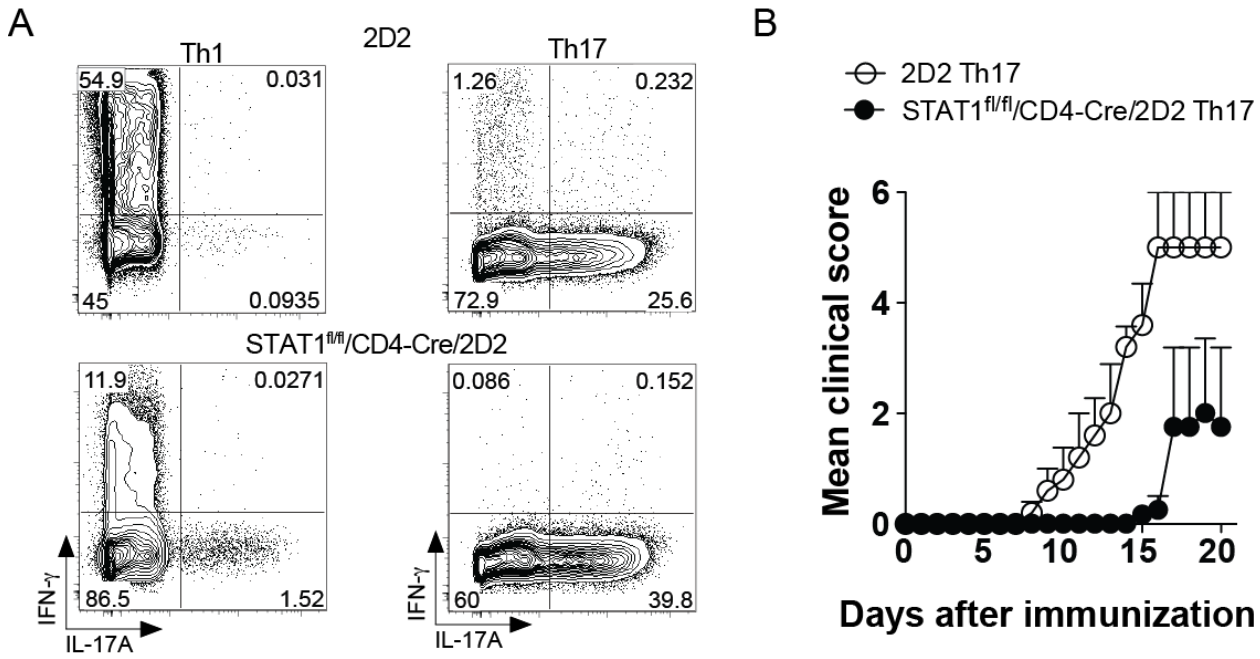

Figure 3.1. STAT1-deficient Th17 cells do not induce EAE. (**A**) Naïve CD4+ T cells from 2D2 and STAT1$^{fl/fl}$/CD4-Cre mice were differentiated with MOG_{35-55} and antigen-presenting cells (APCs) into Th1 and Th17 cells. Cells were stimulated with PMA and ionomycin for intracellular cytokine staining of IFN-γ and IL-17, gated on live CD4+ cells.). Data are representative of at least 2 experiments. (**B**) Th17 cells from 2D2 and STAT1$^{fl/fl}$/CD4-Cre/2D2 mice stimulated *in vitro* with anti-CD3 and APCs in the presence of IL-23 for 4 days were transferred into RAG1$^{-/-}$ hosts. Mice were given pertussis toxin at day 0 and 2 after transfer. Clinical scores of mice are shown. Results are shown as mean ± SEM over time ($n \geq 6$ mice per group). Data are representative of 2 experiments with $n \geq 6$.

STAT1 deficiency in CD4+ T cells protects mice from EAE development

The fact that STAT1-deficient Th17 cells could not transfer disease suggested that the effect of STAT1 extended beyond Th17 cells. Therefore, we tested whether $STAT1^{fl/fl}$/CD4-Cre mice were also protected from EAE development. We immunized $STAT1^{fl/WT}$/CD4-Cre mice, referred to as control mice from now on, and $STAT1^{fl/fl}$/CD4-Cre mice with MOG_{35-55} emulsified in complete Freund's adjuvant (CFA) and monitored the mice for EAE development. In stark contrast to STAT1-deficient ($STAT1^{-/-}$) mice, $STAT1^{fl/fl}$/CD4-Cre mice were protected from disease EAE development (**Figure 3.2A**). These mice developed delayed signs, which were also less severe, compared to control mice (**Figure 3.2A**). Because T cell infiltration into the CNS propagates EAE, we enumerated CD4+ T cells in the brain and spinal cord of the mice that were equally sick late in the disease. Correlating with disease severity, we found that the numbers of CD4+ T cells were significantly reduced (**Figure 3.2B**). Accordingly, we also observed an impairment of cytokine-producing T cells in the CNS of $STAT1^{fl/fl}$/CD4-Cre mice (not shown).

To exclude the possibility that other non-T cell populations were affected by STAT1 deletion, we used an adoptive transfer system to induce EAE. We thus co-transferred naïve CD4+ T cells from 2D2 and $STAT1^{fl/fl}$/CD4-Cre/2D2 mice at a 1:1 ratio into RAG1-deficient hosts in order to quantify the infiltration of STAT1-deficient T cells into the CNS when the mice developed EAE. This approach allowed us to confirm that the protection from EAE development in $STAT1^{fl/fl}$/CD4-Cre mice did not extend beyond T cells. One day after transfer, we immunized the transferred $Rag^{-/-}$ hosts with MOG_{35-55} to induce EAE. At the peak of the disease (around day 12), we found that very few STAT1-deficient T cells migrated into the CNS, especially compared to their WT counterparts (**Figure 3.2C**). These results showcase the absence of pathogenic STAT1-deficient CD4+ T cells in the CNS, which could be a consequence of a modulation of T cell

trafficking, generation, and expansion of pathogenic T cells in the periphery when the cells lack STAT1. Alternatively, deficiency of STAT1 could impact Foxp3+ regulatory T cell generation, which we tested directly.

STAT1 deficiency in Foxp3+ regulatory T cells does not impact EAE development

Because STAT1 has been shown to affect the generation of Foxp3+ regulatory T (Treg) cells (98), we examined how Foxp3-expressing T cells were affected by the absence of STAT1 during EAE. $STAT1^{fl/fl}$/CD4-Cre mice with EAE had similar frequencies of Foxp3+ cells (not shown), with lower numbers of these cells compared to control mice. Moreover, to exclude the possibility that STAT1 was affecting the suppressive capabilities of Treg cells independently of their frequencies or numbers, we immunized $STAT1^{fl/fl}$/Foxp3-IRES-Cre ($STAT1^{fl/fl}$/FIC) for EAE development. C57BL/6 control mice had a similar disease incidence and severity as $STAT1^{fl/fl}$/FIC mice (**Figure 3.3**), showing that specific elimination of STAT1 in the Foxp3+ Treg cells does not confer protection.

STAT1 is not required for T cell proliferation and expansion in vitro

To address whether STAT1 modulates the generation and expansion of pathogenic CD4+ T cells, we first evaluated whether these cells proliferated more efficiently in response to polyclonal and MOG_{35-55}-specific stimulation. We hypothesized that STAT1 deficiency selectively in CD4+ T cells was crucial for their pathogenicity and differentiation into effector and memory cells. STAT1 has been shown to improve the survival and proliferation of

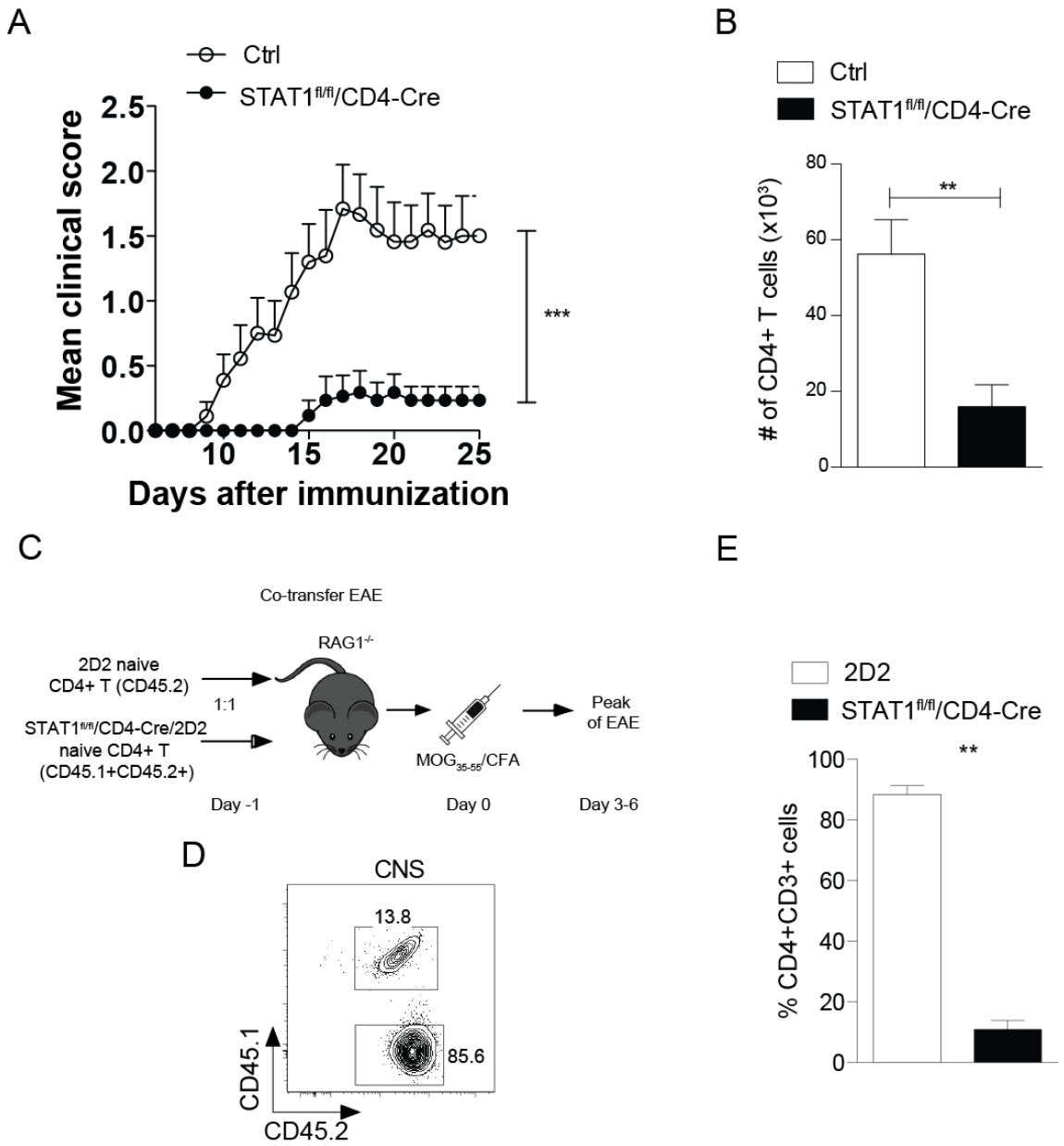

Figure 3.2. STAT1 deficiency in CD4+ T cells protects mice from EAE development. (**A**) Clinical scores of WT and $STAT1^{fl/fl}$/CD4-Cre immunized with MOG_{35-55}/CFA. Results are shown as mean ± SEM over time (n ≥ 6 mice per group). Data from 3 combined experiments are shown. (**B**) Total number of CD4+ T cells in the brain and spinal cord (CNS) from EAE mice from (**A**) at day 30 were harvested. (**C**) Diagram of experimental set-up in **d**: $5x10^5$ congenically marked naïve CD4+ T cells from 2D2 (CD45.2) and $STAT1^{fl/fl}$/CD4-Cre/2D2 (CD45.1+CD45.2+) were co-transferred into $RAG1^{-/-}$ mice at a 1:1 ratio 1 day before immunization with MOG_{35-55}/CFA. The CNS of mice with EAE was harvested at the peak. (**D**) Frequency of wild-type (CD45.2+) and STAT1-deficient (CD45.1+CD45.2+) donor CD4+ T cells in the CNS at the peak of EAE in $RAG1^{-/-}$, assessed by flow cytometry. Numbers adjacent to outlined areas indicate percent cells in each. Data are representative of 2 experiments with n≥5. Data are representative of 2 experiments with n≥5. ($^{**}p < 0.01$, $^{***}p < 0.001$).

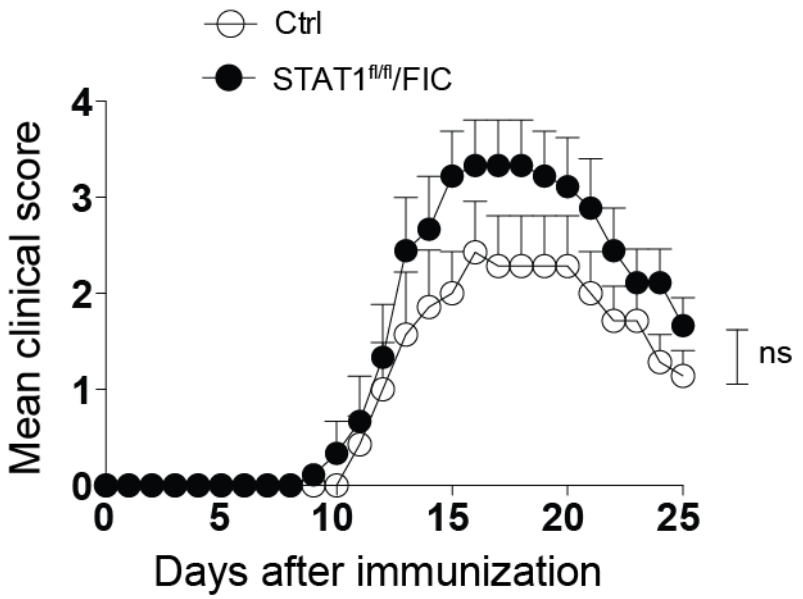

Figure 3.3. STAT1 deficiency in Foxp3+ regulatory T cells does not impact EAE development. Clinical scores of WT and STAT1$^{fl/fl}$/Foxp3-IRES-Cre (FIC) immunized with MOG_{35-55}/CFA. Results are shown as mean ± SEM over time (n ≥ 6 mice per group). Data are representative of 2 experiments with n≥5. (ns, not significant).

lymphocytes (58), which led us to explore how STAT1 affects the activation, survival, and proliferation of naïve CD4+ T cells *in vitro*. We found that when FACS-sorted naïve cells were stimulated with anti-CD3/CD28, no difference in their phosphorylation of S6, survival, or proliferation was detected (**Figure 3.4A**). MOG_{35-55}-specific T cell proliferation using naïve cells from 2D2 and $STAT1^{fl/fl}$/CD4-Cre/2D2 mice yielded similar results in terms of phosphorylation of S6 and proliferation of the cells (**Figure 3.4A-B**)

These data indicate that the effect of STAT1 in the enhanced survival and proliferation of CD4+ T cells is not cell intrinsic and must be due to other cell types that interact with CD4+ T cells *in vivo*. The fact that effector/memory cells were increased in $STAT1^{-/-}$ mice and that CD4+ T cells were phenotypically different *ex vivo* in intact mice (not shown) supported our hypothesis.

CD4+ T cell proliferation and survival are defective in the absence of STAT1

Next, we examined CD4+ T cell response to MOG_{35-55} *in vivo*. Although the frequencies of cytokine-producing Th1 and Th17 cells were equivalent in both control and $STAT1^{fl/fl}$/CD4-Cre mice 8 days after immunization, the numbers of these cells were reduced in the lymph nodes of these mice (**Figure 3.5A**). These results are in sharp contrast to what we have seen *in vitro* and the reported effect of STAT1 on Th1 and Th17 differentiation, proliferation, and survival. However, our data indicate that STAT1-deficient T cells failed to expand and proliferate efficiently in vivo, suggesting that STAT1 is required for the generation of efficient pathogenic responses in the periphery of mice. When we assessed the ability for T cells to proliferate in a recall response to MOG_{35-55}, we found that STAT1 deficiency in CD4+ T cells caused a crash in

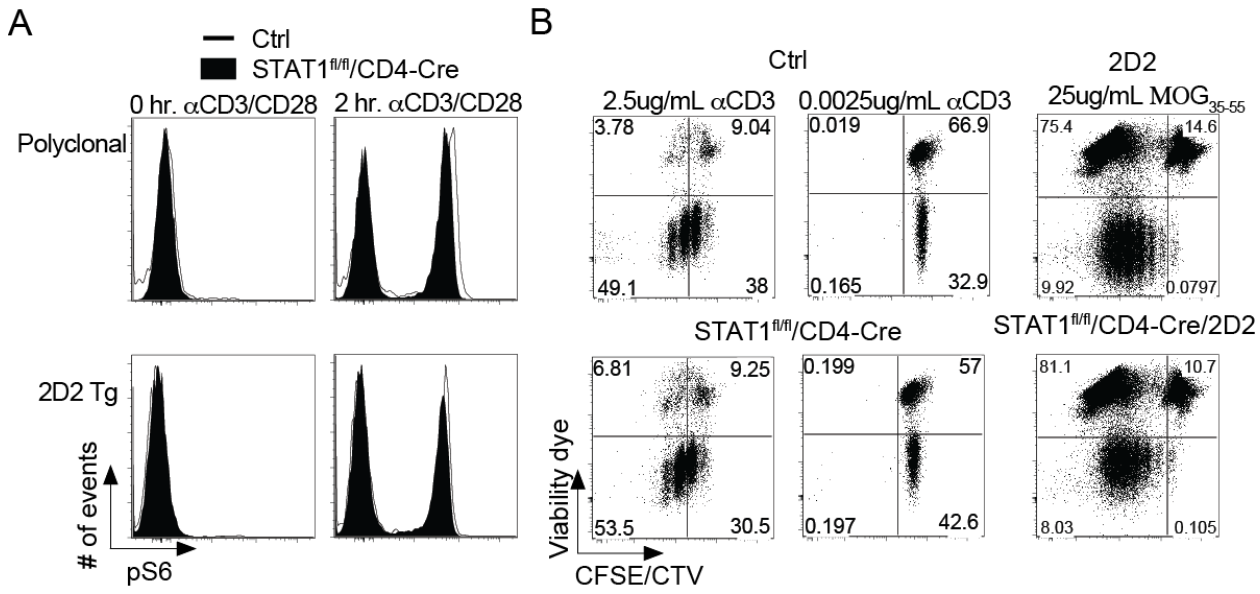

Figure 3.4. STAT1 is not required for T cell proliferation and expansion *in vitro*. (**A**) Naïve CD4+ T cells from wild-type Ctrl and STAT1$^{fl/fl}$/CD4-Cre (polyclonal, above) and 2D2 and STAT1$^{fl/fl}$/CD4-Cre/2D2 (2D2 Tg, below) were stimulated with plate-bound anti-CD3/CD28 (above) or MOG_{35-55} and APCs (below) for 2 hrs and phosphorylated S6 was assessed by flow cytometry. (**B)** CFSE- and CellTrace Violet (CTV)-labeled naïve CD4+ T cells from Ctrl and STAT1$^{fl/fl}$/CD4-Cre (left) and 2D2 and STAT1$^{fl/fl}$/CD4-Cre/2D2 (right) were stimulated with plate-bound the indicated concentrations of anti-CD3/CD28 (left) or MOG_{35-55} and APCs (right) for 3 days. Viability and proliferation were assessed by staining with a viability dye and following the dilution of CFSE and CTV, gated on CD4+ T cells. Data are representative of 3 experiments.

proliferation as assessed by thymidine incorporation (**Figure 3.5B**). Moreover, after in vitro MOG-restimulation, STAT1-deficient CD4+ T cells displayed a significant reduction in IFN-γ and IL-17 production (not shown). These results were corroborated by the observation that CD4+ T cells did not survive as well when STAT1 signaling is defective (**Figure 3.5C**).

STAT1-deficient CD4+ T cells fail to expand in vivo

Thus far, we have determined that STAT1 deficiency blocks T cell proliferation and survival when the cells were primed *in vivo* in response to immunization with MOG_{35-55}; when T cells were activated *in vitro*, we failed to see an effect of STAT1 deficiency. We hypothesized that the hyperproliferative phenotype of lymphocytes observed in the $STAT1^{-/-}$ mice, which is not observed in $STAT1^{fl/fl}$/CD4-Cre mice, is caused by cell-extrinsic factors that are not controlled by CD4+ T cells. To test further how these cells behave in a model of antigen-driven expansion, we co-transferred congenically labeled naïve CD4+ T cells from 2D2 and $STAT1^{fl/fl}$/CD4-Cre/2D2 mice into $RAG1^{-/-}$ hosts as outlined (**Figure 3.6A**). After co-transfer of the cells, we found a robust elimination of CD4+ T cells deficient for STAT1, compared to wild-type 2D2 cells, even as early as day 3 after transfer (**Figure 3.6B**).

Defective expansion of STAT1-deficient CD4+ T cells was also observed in a lymphoreplete model of antigen-dependent expansion in WT settings (**Figure 3.6C**), even as early as 2 days after transfer, when naïve CD4+ T cells from 2D2 and $STAT1^{fl/fl}$/CD4-Cre/2D2 mice were co-transferred into MOG-immunized WT recipients (**Figure 3.6D**), although the defect was not as profound as that observed in $RAG1^{-/-}$ mice.

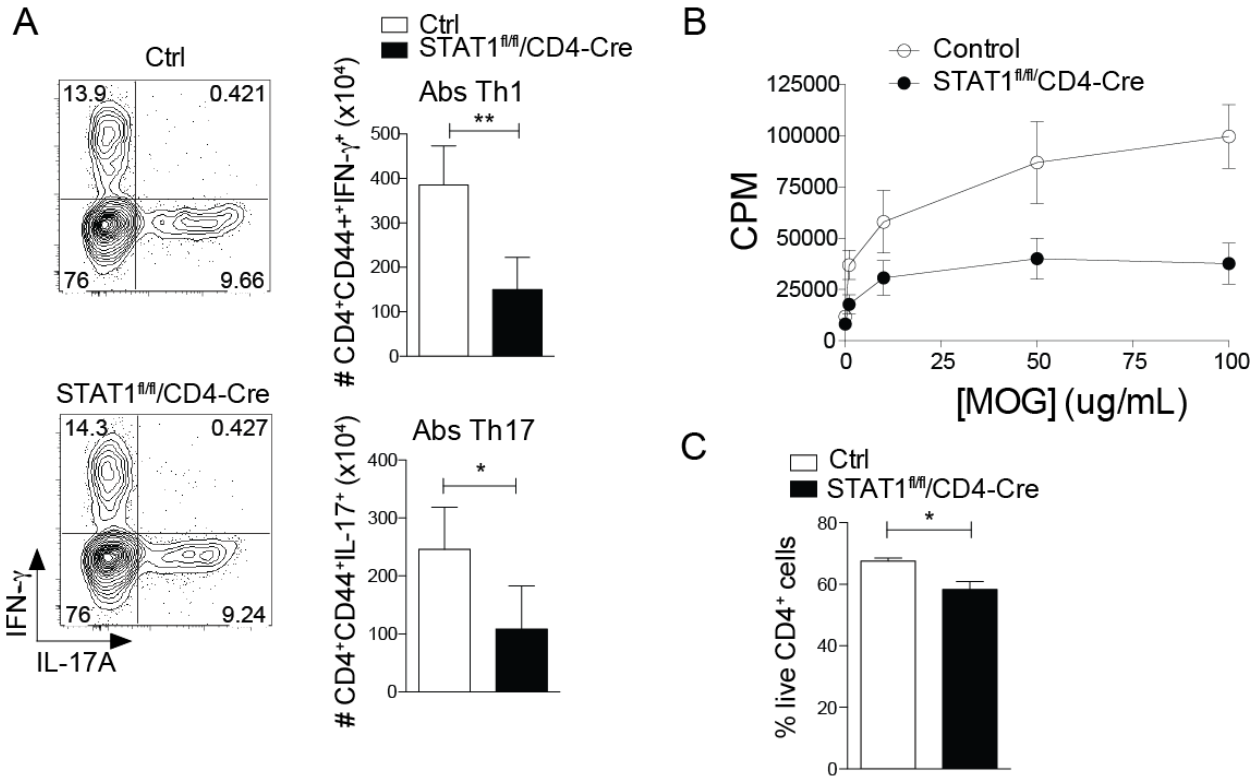

Figure 3.5. CD4+ T cell proliferation and survival are defective in the absence of STAT1. (**A-C**) Control (Ctrl) and STAT1$^{fl/fl}$/CD4-Cre mice were immunized with MOG_{35-55}/CFA. Eight to ten days after immunization, $CD4^+$ T cells from the draining lymph nodes were analyzed for cytokine production of IFN-γ and IL-17, measured by intracellular cytokine staining after PMA/ionomycin stimulation (**A**) Frequencies (left) and numbers (right) of cytokine-producing cells are shown. (**B**) Proliferative response of draining lymph node cells from immunized mice was assessed by [^{3}H]thymidine incorporation after restimulation with varying concentrations of MOG_{35-55}. (**C**) Draining lymph node cells were restimulated with MOG_{35-55} for 72 hours before labeling with a viability dye, gating on CD4+ T cells. Data are representative of at least 2 independent experiments with n≥3 mice in each experiment. ($^*p < 0.05$, $^{**}p < 0.01$)

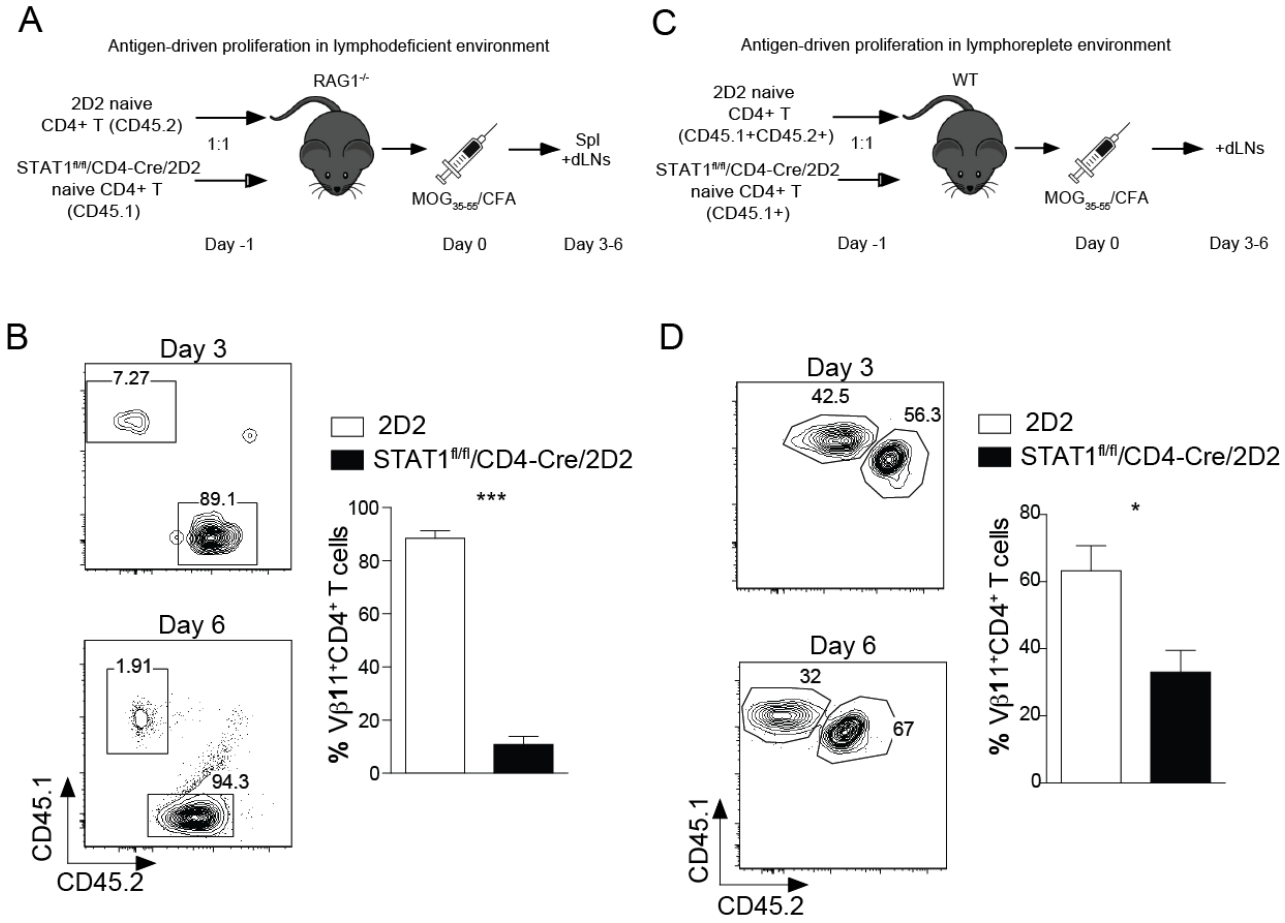

Figure 3.6. STAT1-deficient CD4+ T cells fail to expand *in vivo*. (**A**) Diagram of experimental set-up in **B**: $5x10^5$ congenically marked naïve CD4+ T cells from 2D2 (CD45.2+) and $STAT1^{fl/fl}$/CD4-Cre/2D2 (CD45.1+) were co-transferred into $RAG1^{-/-}$ mice at a 1:1 ratio 1 day before immunization with MOG_{35-55}/CFA. Three and six days after transfer, the draining lymph nodes were harvested. (**B**) Frequencies (left) of wild-type (CD45.2+) and STAT1-deficient (CD45.1+) donor CD4+ T cells, assessed by flow cytometry. (**C**) Diagram of experimental set-up in **D**: $5x10^5$ congenically marked naïve CD4+ T cells from 2D2 (CD45.1+CD45.2+) and $STAT1^{fl/fl}$/CD4-Cre/2D2 (CD45.2+) were co-transferred into wild-type (WT) mice at a 1:1 ratio 1 day before immunization with MOG_{35-55}/CFA. Three and six days after transfer, the draining lymph nodes were harvested. (**D**) Frequencies (left) of 2D2 (CD45.1+CD45.2+) and STAT1-deficient (CD45.1+) donor CD4+ T cells Numbers adjacent to outlined areas indicate percent cells in each. Data are representative of 3 experiments with n≥4 mice. Data are representative of at least 2 experiments with n≥4. ($^{*}p < 0.05$, $^{***}p < 0.001$).

Missing self on STAT1-deficient CD4+ T cells

The ability to upregulate and recognize MHC class I molecules allows cells to distinguish self and healthy from infected cells. NK cells are equipped with the cytotoxic machinery to target and destroy cells that are infected or foreign cells, which makes them instrumental in rejection of organ transplantation, mainly because the target cells fail to express normal self-markers such as MHC-I molecules. NK cells have been shown to inhibit T cell priming during viral infection by targeting recently activated T cells (6, 95). As CD4+ T cells become activated and begin to divide, they upregulate MHC-I molecules. We found that CD4+ T cells from immunized $STAT1^{fl/fl}$/CD4-Cre mice express low levels of classical ($H\text{-}2K^{b}$) and non-classical (Qa2) MHC-I molecules (**Figure 3.7A**). We found that as naïve CD4+ T cells are activated *in vitro*, they express higher levels of MHC class I molecules, but this effect is blunted when the cells lack STAT1 (**Figure 3.7B**). These results demonstrate that STAT1 is required for upregulation of self-markers and suggest that NK cells may be involved in the elimination of autoreactive T cells during EAE.

NK cells selectively target STAT1-deficient CD4+ T cells to suppress EAE

Lymphopenic RAG1-deficient mice were recently shown to have hyperactive NK cells (99), while STAT1$^{-/-}$ mice have decreased NK cell numbers and function (100, 101). We reasoned that the decreased survival of cells that lacked STAT1 was potentially due to specific targeting and lysis by NK cells. Genetic deletion of the IL-15 leads to a defect in NK cell development, since IL-15 is critical for their development. After co-transfer of naïve cells from 2D2 and STAT1$^{fl/fl}$/CD4-Cre/2D2 mice into doubly deficient mice (RAG1$^{-/-}$IL-15$^{-/-}$), we found that there was a full recovery of STAT1-deficient CD4+ T cells and these cells outcompeted their wild-type counterparts (**Figure 3.8A**). These results show the important role that STAT1 plays in controlling NK cell-mediated cytotoxicity.

If our hypothesis that activated STAT1-deficient CD4+ T cells do not induce EAE because they are eliminated by NK cells, then in the absence of NK cells, CD4+ T cells that lack STAT1 will be capable of transferring EAE. Our genetic model of NK cell depletion by deleting IL-15 allowed us to circumvent the issue of NK cells returning shortly after antibody-mediated depletion of these cells and the upregulation of NK1.1 by activated cells, particularly by antigen-specific CD4+ and CD8+ T cells (102). After transfer of naïve 2D2 CD4+ T cells into RAG1-deficient mice, these mice developed severe EAE, especially compared to EAE induced by STAT1-deficient 2D2 CD4+ T cells (**Figure 3.8B**). However, upon transfer of STAT1-deficient CD4+ T cells into RAG1$^{-/-}$/IL-15$^{-/-}$ mice, EAE was fully restored (**Figure 3.8B**), demonstrating that NK cells suppress T cells when they lack STAT1 and cannot express MHC-I molecules.

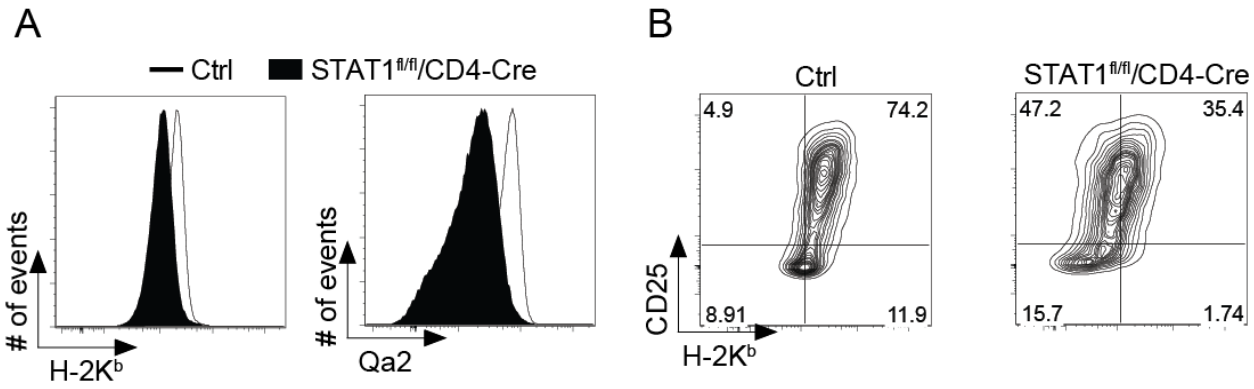

Figure 3.7. Missing self on STAT1-deficient CD4+ T cells. (**A**) Control (Ctrl) and $STAT1^{fl/fl}$/CD4-Cre mice were immunized with MOG_{35-55}/CFA. Eight to ten days after immunization, $CD4^+$ T cells from the draining lymph nodes were analyzed for expression of H-$2K^b$ (left) and Qa2 (right). Data are representative of 2 experiments with n≥3 mice. (**B**) Expression of H-$2K^b$ and CD25 on CD4+ T cells from control (Ctrl) and $STAT1^{fl/fl}$/CD4-Cre mice after 3 days of activation of naïve CD4+ T cells with plate-bound anti-CD3/CD28 for 3 days. Data are representative of 2 experiments.

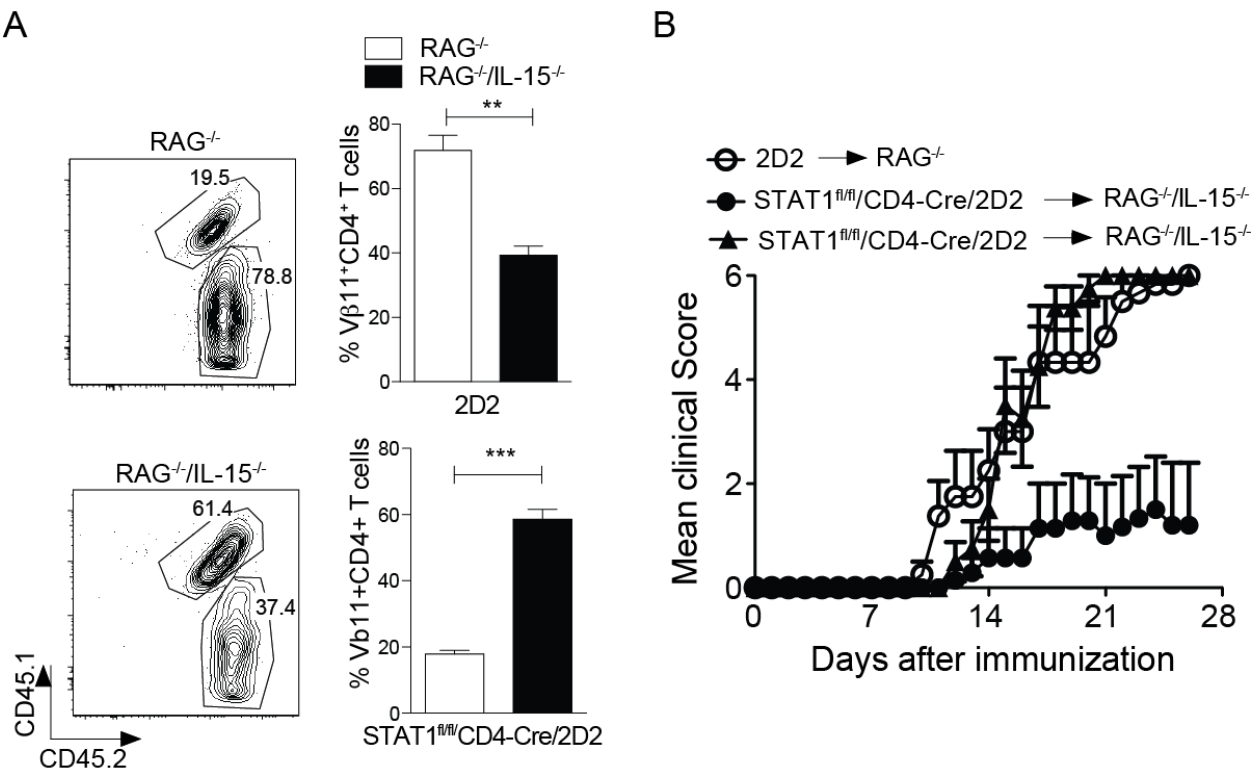

Figure 3.8. NK cells selectively target STAT1-deficient CD4+ T cells for elimination and suppress EAE. (A) $5x10^5$ congenically marked naïve CD4+ T cells from 2D2 (CD45.2+) and $STAT1^{fl/fl}$/CD4-Cre/2D2 (CD45.1+CD45.2+) were co-transferred into $RAG1^{-/-}$ and $RAG1^{-/-}$/IL-$15^{-/-}$ mice at a 1:1 ratio. Six days after transfer, the draining lymph nodes were harvested. Frequencies (left) of wild-type (CD45.2+) and STAT1-deficient (CD45.1+CD45.2+) donor CD4+ T cells, assessed by flow cytometry. (**B**) $5x10^4$ naïve CD4+ T cells from 2D2 and $STAT1^{fl/fl}$/CD4-Cre/2D2 were transferred into $RAG1^{-/-}$ and $RAG1^{-/-}$/IL-$15^{-/-}$ mice one day before immunization with MOG_{35-55}/CFA and treatment with pertussis toxin at time of immunization and 2 days later. Clinical scores of mice are shown. Results are shown as mean ± SEM over time. Data are representative of 2 experiments with n≥4 mice. ($^{**}p < 0.01$, $^{***}p < 0.001$).

Discussion

Although the phenotype of global STAT1 deficiency in EAE was reported over 10 years ago (55), the precise mechanisms underlying this phenomenon have not been uncovered. Some studies have proposed a protective role of type I IFN on myeloid cells (60). It has also been suggested that IL-17 dysregulation in these mice is responsible for the exacerbated disease (57). Consistent with previous reports, we determined that STAT1 deficiency limited IFN-γ production by Th1 cells and enhanced Th17 differentiation *in vitro* (57, 92). Our observation that STAT1-null Th17 cells are unable to transfer disease suggests that other mechanisms must be at play. To understand how STAT1 was affecting Th17 and other effector CD4+ T cells, we used $STAT1^{fl/fl/}$/CD4-Cre mice to understand the behavior of effector cells during EAE. Although studies have hinted at pro-apoptotic and anti-proliferative roles of STAT1 in T cells (58), we found that *in vitro*-activated STAT1-deficient T cells did not show enhanced proliferation or survival compared to controls. Instead, these cells were found at reduced numbers after activation with MOG_{35-55}/CFA and failed to proliferate in a recall response proliferation assay. Cytokines produced *in vivo*, such as IL-10 or TGF-β (103), could lead to the suppression of T cells, but we found no evidence of this (data not shown). Increased suppression by Foxp3+ regulatory T cells has been proposed previously in the context of graft-versus-host disease (98), but we ruled out this hypothesis by the observation that $STAT1^{fl/fl}$/Foxp3-IRES-Cre mice develop EAE similar to control mice. Although T cell activation, proliferation, and survival were intact in STAT1-sufficient and -deficient T cells primed *in vitro*, these cells failed to expand when co-transferred into RAG1-deficient recipients and after activation with their cognate antigen *in vivo*, revealing an *in vivo*-specific effect of STAT1 abrogation.

Although NK cells have historically been shown to be important in innate immunity to viral infections and tumor immunosurveillance, a role for NK cells in regulating autoimmunity, particularly multiple sclerosis, has emerged (104). Depletion of NK cells during EAE leads to exacerbated disease (96, 105). In humans, transient periods of decreased NK cell activity correlate with a symptomatic relapse and the functions of NK cells are affected during administration of drugs to treat MS. Our studies revealed a novel role of STAT1 in preventing NK cell-mediated killing of autoreactive CD4+ T cells.

Control of effector T cell responses by NK cells has been observed in the context of infection (6, 95) and suggested in autoimmunity (93), but direct evidence for NK cells affecting autoreactive CD4+ cells has not been observed to our knowledge. Our study demonstrated that T cells lacking the ability to signal through STAT1 were highly susceptible to elimination by NK cells during the course of EAE development. Therefore, the data presented here revealed that under certain circumstances, NK cells can constrain autoreactive T cell responses and prevent the development of autoimmunity. Two recent reports also found that type I interferon signaling was important for CD8+ T cells to prevent NK cell killing during the course of LCMV infection (106, 107). Our results nicely complement these previous studies and demonstrate that NK cells can regulate a wide spectrum of immune responses by targeting effector T cells.

A variety of activating and inhibitory signals orchestrate the function of NK cells. Recognition of MHC class I molecules on the surface of healthy cells from self-tissues is typically sufficient to prevent targeting by NK cells. However, activating receptors can also induce NK killing activity. NK cells also need to be activated by cytokine signals that take place during the course of an inflammation, such as high levels of type I interferon, IL-2, IL-12, IL-15, or IL-18. Thus, a balance between these signals is established to dictate the outcome of NK cell function.

STAT1-deficient CD4+ T cells in our study expressed low levels of MHC class I. Yet, these cells persisted in STAT1$^{fl/fl}$/CD4-Cre mice possibly because T cells need to be activated in order to express high levels of MHC class I, and possibly activating receptors, in order to be susceptible to NK cell targeting (7). Our data support the notion that STAT1-deficient CD4+ T cells need to be activated before they are killed by NK cells, which is consistent with a handful of studies (7, 95, 108, 109). The presence of activating ligands controlled by STAT1 signaling need to be investigated in future experiments.

The finding that MHC class I molecules are upregulated on wild-type CD4+ T cells, particularly following activation *in vitro*, implies that signaling through STAT1 somehow allows for the upregulation of MHC class I molecules, implicating the possibility that a STAT1 activating cytokine, most likely IFN-γ secreted by activated T cells, is required to generate effector cells and establish clonal selection. Further work identifying the cytokine upstream of STAT1 will reveal the mechanism underlying the upregulation of MHC class I molecules that protects CD4+ T cells from NK cell-mediated lysis.

In light of these results, an obvious question emerges: if STAT1-deficient CD4+ T cells are eliminated by NK cells, especially when activated, why are STAT1-null mice hyper-susceptible to EAE? STAT1-deficient mice have been shown to have reduced NK cell numbers and NK cell activity (100, 101), implicating the possibility that STAT1-deficient mice develop exacerbated EAE because their NK cells cannot control effector CD4+ T cells, despite their low expression of MHC class I molecules. All studies examining the behavior of lymphocytes in STAT1$^{-/-}$ mice neglected to take into consideration the impact of NK cell dysfunction in these mice. STAT1 has cell type-specific effects that have been highlighted by an infection model in which different cell types were deficient in STAT1 using the Cre-lox system with STAT1$^{fl/fl}$ mice

(110). Further work needs to be performed to test the hypothesis that STAT1-deficient mice develop exacerbated EAE because of the reduced numbers of NK cells and their reduced cytolytic activity, especially in targeting effector T cells.

In conclusion, we showed that STAT1 abrogation in CD4+ T cells abolished their pathogenic functions by failing to upregulate MHC class I molecules, making them susceptible to NK cell killing. These cells did not have a cell-intrinsic defect in their proliferation and survival, and this effect was not unique to any particular effector subset, since Th17 cells failed to induce EAE and expand after restimulation with myelin antigen. One important implication of the transfer studies presented in this study is that we must be careful in interpreting transfer experiments of CD4+ T cells. NK cells target CD4+ T cells that lack IFN/STAT1 signaling, impacting our interpretations of experiments with passive induction of EAE. Moreover, these results may have implications in multiple sclerosis, because the main line of treatment, type I interferon, acts through STAT1 and is capable of activating NK cells potently (111). Because NK cells can kill even cells that express MHC class I, as highlighted by previous studies using infection models (6, 95), it is possible that T cells need further upregulation of MHC class I in order to be protected from NK cell killing after activation. Alternatively, NK cells recognize a balance of activating and inhibitory receptors that may be modulated by type I interferon-mediated STAT1 signaling. Whether type I interferon or STAT1 signaling impacts the expression of NK cell activating and inhibitory receptors on autoreactive CD4+ T cells from MS patients and whether this makes them more susceptible to NK cell cytotoxicity remains subject of further investigation. If NK cells are defective or even refractory to type I interferon treatment, we predict that more autoreactive cells will persist. As evidenced by the transient valleys of NK cell function that manifest during relapses (112), this mechanism of type I interferon activation of NK cells should be tested in future studies.

In the case of MS patients treated with IFN-β, it is possible that the efficacy of treatment is dependent on its ability to turn on NK cells to kill highly activated responses driven by autoreactive T cells that express low levels of MHC class I molecules. Although lots of studies have shown defective NK cell function and numbers in MS patients with active lesions (112-115), further work is needed to elucidate whether IFN-β can activate NK cells and lyse autoreactive effector T cells.

CHAPTER 4

Concluding Remarks

Collectively, the results presented here illustrate the tight control required for our immune system to function properly without causing overt inflammation and autoimmunity. Several factors are required to maintain this balance and perturbation of certain cytokine signaling pathways, as highlighted by the results shown here, can lead to differential outcomes in the immune response, particularly autoimmunity. CD4+ T helper subsets are required to mount effective immune responses against microbial pathogens by recognizing virtually any antigen through highly variable T cell receptors generated by the RAG recombinase during their development, but the generation of autoreactive clones is inevitable. Dissecting the pathways and factors that determine lineage differentiation of autoreactive clones is imperative to understanding how autoimmune diseases, such as multiple sclerosis, arise in light of a very dynamic mechanism of self-tolerance establishment. In this work, we asked whether and how the IL-7/IL-7R and STAT1 signaling pathways contributed to the development of pathogenic CD4+ T cells to address two central questions in the field of multiple sclerosis and EAE: 1) What is the role of IL-7 in T helper differentiation, particularly in the absence of IL-23R signaling? 2) Since Th1 cells, which require STAT1 for efficient differentiation, can drive EAE development, why are STAT1-deficient mice so susceptible to EAE?

The discovery in 2003 that Th17 cells heavily influenced autoimmune diseases and that these cells required the IL-23R served as the impetus for the revision of the paradigm of CD4+ T helper subset differentiation. The past decade has been spent deciphering the molecular mechanisms and factors that dictate lineage commitment and function. Treatments targeting the

IL-23R signaling pathway were developed as a result of the wealth of information that was gained from the new paradigm of T cell differentiation. While psoriasis, and to a certain extent Crohn's disease, has shown promise when the IL-17/IL-23 signaling axis is blocked (116, 117), the same is not true of multiple sclerosis (118). Although multiple studies have focused on understanding the effect of IL-23 on Th17 cells, only one identified a possible mechanism (48). IL-23R-deficient mice were shown to lack Th17 cells by day 10, which was caused by a failure to upregulate IL-7R on CD4+ T cells. Our results presented here showed that Th17 cells expressed high levels of the receptor. In contrast, Th1 cells expressed very low levels of the IL-7R, possibly because they have recently received IL-7 signals or were activated recently. Thus, we reasoned that because of the lack of Th17 cells, IL-7R expression on CD4+ T cells will be significantly lower in the IL-23R-deficienct mice. In support of this hypothesis, provision of constitutive expression of the IL-7R did not rescue cytokine production or EAE development when they mice lacked IL-23R.

In our studies, we found that IL-7 promoted IFN-γ production but did not impact the expression of IL-17 by CD4+ T cells both *in vitro* and *in vivo*. These results established the effect of IL-7 on T cell differentiation and EAE. The initial study by Zhang et al. that identified a role for IL-7 on T cells and multiple sclerosis (75) was retracted and an independent study found contradicting results from the retracted study (73). Thus, it was necessary to define the role of IL-7 in T cell differentiation and pathogenic T cell generation, especially *in vivo*. Importantly, we identified a novel role in enhancing IFN-γ production not only from Th1 cells but also from Th17 cells. Thus, Th17 cells can be converted to Th1 cells with IL-12, IL-23, and IL-7. The high levels of IL-7 expression in the CNS of mice is a conducive environment to induce the plasticity of Th17 cells, which may explain the pattern of IL-7R expression on effector subsets, with Th17 cells expressing the highest level, IFN-γ-producing Th17 cells expressing intermediate levels, and Th1

cells expressing the lowest amount of the receptor. This hierarchy is representative of the amount of IL-7R downregulation as the cells become exposed to IL-7, which we presented in this thesis.

In dissecting how the transcription factor STAT1 impacts the generation of pathogenic CD4+ T cells during the course of EAE, we uncovered a novel role for this signaling pathway in establishing a relationship in which the innate immune system regulates clonal selection that is independent of T cell commitment and is instead dependent on activation of the cells. STAT1-deficient CD4+ T cells were activated and proliferated to the same extent as wild-type cells when the cells were stimulated *in vitro*. Instead, these cells were eliminated when they were primed *in vivo*, which we went on to show was due to NK cell-mediated cytotoxicity. These results argue that the mechanisms underlying the higher susceptibility to EAE in STAT1-deficient mice is caused by the inability of NK cells to control effector CD4+ T cell responses, since these mice have defective NK cell development and function (100, 101). Thus, our work demonstrates the cell type-specific roles of STAT1 signaling in the development of EAE, which informs scientists and clinicians about the effects of this signaling pathway during organ-specific autoimmunity. Although numerous studies have shown defective NK cell function and numbers in MS patients with active lesions (112-115), it will be interesting to learn how this signaling pathway impacts NK cell function in controlling autoreactive effector T cell responses.

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