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A tale of two *STAT6* knock out mice in the induction of experimental autoimmune encephalomyelitis[★]

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Abstract

T helper 2 (Th2) cytokines are known to be important in protection against experimental autoimmune encephalomyelitis (EAE). To investigate the role of the signal transducer and activator of transcription factor 6 (STAT6) in EAE we used mice with two different targeted disruptions of the STAT6 gene. In this report, we show that mice with targeted deletion of the first coding exon of the SH2 domain of STAT6 induce Th2 cell differentiation and are resistant to EAE induction. By contrast, *STAT6*^{-/-} mice generated by deletion of amino acids 505 to 584 encoding the SH2 domain of STAT6 are defective in Th2 cell differentiation and develop very severe EAE. These results suggest that an altered STAT6 gene can be more efficient than wild type STAT6 in regulating the autoimmune response in EAE.

Keywords

T Cells; Th1/Th2 cells; EAE/MS; Cytokines; Transgenic/knockout mice

1. Introduction

Upon encountering antigen, naïve CD4⁺ T cells can differentiate into three distinct subsets, Th1, Th2 or Th17, that are distinguished by their cytokine expression profiles (Romagnani, 2006; Stummvoll et al., 2008). Th1 cells produce IFN- γ , IL-2 and TNF- α , while Th2 cells produce IL-4, IL-5 and IL-13 and Th17 cells produce IL-17 and IL-22 (Kurts, 2008). In addition to their cytokine profiles, Th1, Th2 and Th17 cells possess a signature array of transcription factors. T-bet and STAT1¹ are required for the development of IFN- γ -producing CD4 Th1 cells while STAT6 and GATA-3 are essential for the development of Th2 cells (Kurts, 2008).

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¹References used in this paper: STAT, Signal Transducer and Activator of Transcription; ROR γ , retinoic acid-related orphan receptor; ROR γ t, thymic-specific ROR γ ; EAE, Experimental Autoimmune Encephalomyelitis; MBP, Myelin Basic Protein, PLP, Proteolipid Protein; MOG, Myelin Oligodendrocyte Glycoprotein; STAT6 Δ 1EX, STAT6^{-/-} mouse made by Ihle et al.; STAT6 Δ SH2, STAT6^{-/-} mouse made by Grusby et. al.; PTX, Pertussis Toxin.

ROR γ t has been shown to be an essential transcription factor for development of the Th17 subset (Weaver et al., 2006). Recent studies show that T-bet, which plays an essential role in Th1 cells development, is also expressed in IL-17 producing cells and influences the development of Th17 cells (Gocke et al., 2007).

Both IL-4 and IL-13 signaling are regulated by STAT6, a Th2 signaling molecule known to have over 30 target genes (Hebenstreit et al., 2006). Following IL-4 receptor engagement, Janus Kinases (JAK1 and JAK3) phosphorylate the cytoplasmic tail of the receptor, recruiting STAT6, which is subsequently phosphorylated by the same JAKs. STAT6 then dimerizes and translocates to the nucleus, where it is able to upregulate IL-4 responsive gene transcription (Hebenstreit et al., 2006; Kurata et al., 1999). The STAT6 pathway contributes to the increasingly stable expression of Th2 cytokines, thereby regulating Th2 development. The mechanism by which STAT6 mediates development of Th2 responses is not completely understood, but the transcription factor has a central role in the progressive changes in chromatin structure of the IL4, IL-5 and IL-13 loci during Th2 differentiation (Agarwal and Rao, 1998; Fields et al., 2002) as well as the stable up-regulation of GATA3 (Ouyang et al., 2000; Ouyang et al., 1998).

Experimental autoimmune encephalomyelitis (EAE) is a chronic autoimmune disease of the central nervous system (CNS) induced in susceptible mouse strains. EAE bears many of the clinical and pathological features of MS and is considered a viable model for the disease. In mice, EAE can be actively induced in susceptible mouse strains following immunization with myelin components, such as myelin basic protein (MBP), proteolipid protein (PLP), or myelin oligodendrocyte glycoprotein (MOG), or by passive transfer of myelin antigen-specific T cells (Buenafe et al., 1997; Wekerle et al., 1994). These antigen-specific activated T cells, which are mostly CD4 positive, differentiate into effector Th1 or Th17 cells that migrate to the CNS where they mount an inflammatory response against myelin components (Ivanov et al., 2006; Komiyama et al., 2006; Kroenke and Segal, 2007; Langrish et al., 2005). As in MS, this inflammatory response results in demyelination and loss of neuronal function, leading to paralysis.

Both Th1 and Th17 cells and their production of the proinflammatory cytokines IFN γ and IL-17, respectively, have been implicated in the pathogenesis of EAE with regard to disease progression and exacerbation (Begolka et al., 1998; Ivanov et al., 2006; Komiyama et al., 2006; Langrish et al., 2005). Th2 cells and their associated anti-inflammatory cytokines are implicated in suppression, relapse prevention, remission and recovery from EAE (Cash et al., 1994; Issazadeh et al., 1995, 1998; Khoury et al., 1992; Ochoa-Reparaz et al., 2008; Racke et al., 1994; Rott et al., 1994). However, the precise effect of Th2 cytokines in EAE resistance is not completely understood, and contradictory results have been reported. In one study, it was reported that induction of a Th2 response (Falcone and Bloom, 1997) or transferring MBP specific Th2 clones (Cua et al., 1995) imparted resistance to EAE. Also, treatments with the Th2 cytokines IL-13 or IL-10 could prevent or suppress EAE (Cash et al., 1994; Rott et al., 1994). In apparent contradiction, IL4 $^{-/-}$ mice suffer similar disease to wild type mice (Bettelli et al., 1998; Liblau et al., 1997). However, other studies reported that transgenic over-expression of IL-4 in T cells could not protect from EAE (Bettelli et al., 1998). These apparently conflicting results may be due to redundancy, cascade; synergy and antagonism, or Th1/Th2 cross-talk, suggesting that Th2 cytokines play multiple roles in EAE immune response.

STAT6 knockout mice (STAT6 $^{-/-}$) have provided an opportunity to test the role of Th2 cytokine-signaling in the immune response. Studies using STAT6 $^{-/-}$ mice showed that these mice suffer more severe EAE compared to wild-type mice (Chitnis et al., 2001). This finding further supports the idea that Th2 cells are involved in EAE suppression and protection. In this report, we present findings of different EAE phenotypes in two different STAT6 $^{-/-}$ mice. We

show that *STAT6*^{-/-} mice, depending on the *STAT6* gene mutation, suffer either milder or more severe EAE compared to wild type mice.

We studied the regulatory functions of Th2 associated cytokines, transcription factors and CD4⁺/CD25⁺/Foxp3⁺ T regulatory (Treg) cells in EAE in the two *STAT6* knock outs compared to wild type. Our findings show that *STAT6*Δ1EX mice, which were generated by deleting the first coding exon of the SH2 domain of *STAT6* and carry a truncated form of *STAT6*, are protected from EAE. We show that cells from these mice also have the ability to differentiate into Th2 cells and produce Th2 cell cytokines, while cells from these mice have reduced IFNγ production but maintain the ability to develop Treg cells. *STAT6*ΔSH2 mice were generated by deletion of amino acids 505 to 584 encoding the SH2 domain of *STAT6*. These mice develop severe EAE, are unable to signal Th2 cell differentiation, and do not develop Treg cells and produce IFN-γ levels similar to wild type mice. The studies from these two *STAT6* models can lead to identifying aspects of *STAT6* signaling that can be targeted for therapeutic intervention in T cell-mediated autoimmune diseases.

2. Materials and methods

2.1. Mice

*STAT6*ΔSH2 mice made by Grusby's group (Kaplan et al., 1996), were generated by replacing amino acids 505–584 of *STAT6* encoding the SH2 domain with a neomycin resistance (*neo*^r) cassette. *STAT6*ΔSH2 mice on the C57BL/6 background were kindly provided by Dr. Samia Khoury. *STAT6*Δ1EX mice were generated by Ihle's group (Shimoda et al., 1996) by replacing the first coding exon of the SH2 domain of *STAT6* with a *neo*^r cassette (Shimoda et al., 1996). The MBP–TCR–Tg mice (Hardardottir et al., 1995) on a B10.PL background were crossed to *STAT6*Δ1EX on C57BL/6 background for nine generations to generate MBP *STAT6*Δ1EX on a B10.PL background. C57BL/6 mice were purchased from Jackson Laboratory (Bar Harbor, Maine, USA). The maintenance of the facility and use of animals is in full compliance with the Laboratory Animal Welfare Act and the Health Research Extensions Act. Female or male mice at 6–8 weeks of age were used for experiments. Experiments were conducted under a protocol approved by the Institutional Animal Care and Use Committee of Cornell University.

2.2. EAE induction with MOG

Myelin oligodendrocyte glycoprotein peptide (MOG_{35–55}, (MOG) 3 mg/ml in PBS) (M–E–V–G–W–Y–R–S–P–F–S–R–O–V–H–L–Y–R–N–G–K) (Invitrogen, Carlsbad, CA) was emulsified with an equal volume of complete Freund's adjuvant (CFA, Sigma), and 50 μl was injected subcutaneously in both flanks of each mouse. Pertussis toxin (PTX, 200 μl of 100 ng/ml) (EMD Biosciences, Gibbstown NJ), a G protein inhibitor commonly used to promote lymphocyte infiltration into the CNS during immunization with MOG_{35–55}–CFA to induce EAE, was injected intravenously (i.v.) at the time of immunization and again 48 h later. Mice were scored daily for clinical signs of disease and a numerical score was assigned based on the severity of disease symptoms. 0: no disease; 0.5 weak tail; 1: limp tail; 2: limp tail and partial hind limb paralysis; 3: total hind limb paralysis; 4: both hind limb and fore limb paralysis; 5: moribund (mice with a score of 4 were euthanized).

2.3. Immunization and cell culture

For *in vitro* experiments, mice were immunized with CFA–MOG_{35–55}, 50 μl in each flank. After 10 days, a single cell suspension was prepared from the draining lymph nodes or spleens. Red blood cells were lysed using ACK lysis buffer (0.15M NH₄Cl, 1 mM KHCO₃, 0.1 mM EDTA, PH 7.3) washed and counted. Cells were cultured in Bruff's media (Invitrogen,

Carlsbad, CA) containing 10% FBS in 96-well plates. Cells were incubated at 37 °C in humidified air containing 5% CO₂.

2.4. Naïve mouse CD4⁺ T cell separation and culture for polarization

A single cell suspension was prepared from draining lymph nodes or spleens from naïve mice, red blood cells were lysed using ACK lysis buffer, cells were washed, and then incubated (4 °C, 30 min) with cocktail of antibodies [CD8 (Tib-105), IA^{b,d,v,p,q,r} (212.A1), FcR(2.4-G2), B220 (TIB-164), NK1.1 (HB191); all hybridoma supernatants generated in our lab] for negative enrichment of CD4⁺ cells. Cells were then washed and incubated with beads containing BioMag goat-anti mouse IgG, goat-anti mouse IgM, and goat anti-rat IgG (Qiagen, Valencia, CA), that are specific for the antibody cocktail used. After magnetic enrichment, the unlabeled CD4⁺ T cells were used either as a total CD4⁺ population or further sorted into specific populations. The negative selected cells were stained with anti-CD4 (RM4-5), and the post sort CD4⁺ purify was routinely >99%. All commercial antibodies were purchased from BD Biosciences (San Jose, CA).

2.5. Generation of Th1, Th2 and Th17 polarized cells from naïve CD4⁺ T cells

Isolated mouse naïve CD4⁺ T cells were cultured in 2 ml Bruff's medium with plate-bound anti-mouse CD3 mAb (5 µg/ml, Clone 2C11, BD Biosciences, San Jose, CA) and anti-mouse CD28 mAb (1 µg/ml, Clone 37.51, BD Biosciences, San Jose, CA) in the presence of Th1 or Th2 cytokines. For differentiation into Th1 cells, mouse IL-12 (5 ng/ml, eBioscience, San Diego, CA), mouse IL-2 (25 U/ml, cell culture supernatant generated in our laboratory) and αIL-4 (10 µg/ml) (clone 11B11, generated in our laboratory) were added to the cell cultures. For Th2 cells, mouse IL-4 (10 ng/ml, cell culture supernatant generated in our laboratory), mouse IL-2 (25 U/ml), and αIFN-γ (1 µg/ml, Clone XMG1.2, generated in our laboratory) were added to the cultures. For Th17 cells, IL2 (25 U/ml), IL6 (20 ng/ml, eBioscience, San Diego, CA), TGFβ (1 ng/ml, R&D Systems, Minneapolis, MN), αIL4 (10 µg/ml), αIL12 (10 µg/ml, Clone JES6-1A12, BD Biosciences, San Jose, CA), and αIFNγ (10 µg/ml) were added to the cell cultures. At day 3 post-stimulation, cells were expanded for an additional 4 days in 20 ml of fresh Bruff's medium containing 25 U/ml mouse IL-2. At day 7, cells were extensively washed and restimulated with plate-bound anti-mouse CD3 and soluble anti-mouse CD28 plus mouse IL-2 (25 U/ml) for 48 h. Cell culture supernatant was collected for ELISA, and differentiated T cells were collected for real time PCR analysis. To determine the naïve CD4⁺ T cell phenotype, naïve CD4⁺ T cells were cultured in Bruff's medium with plate-bound anti-mouse CD3 (5 µg/ml) and anti-mouse CD28 (1 µg/ml) in the presence of IL-2 (25 U/ml) for 48 h, and supernatants were collected for ELISA and cells for real time PCR analysis.

2.6. Determination of cytokines by ELISA

ELISA experiments were performed using OptEIA mouse IFN-γ, IL-4, IL-2 and IL13 kits (BD Biosciences, San Jose, CA) following the manufacturer's protocol. ELISA plates were read at 450 nm with a BioTEK ELx800 (Winooski, VT). IL17 ELISA data was generated using a DuoSet ELISA Development System mouse IL17 kit (R&D Systems, Minneapolis, MN).

2.7. Cytokine and transcription factor gene expression

RNA was isolated immediately after cell collection by homogenization in TRIzol reagent (Invitrogen, Carlsbad, CA). Reverse transcription (RT) was carried out with 2 µg of total RNA using Reverse-iT 1st strand Synthesis Kit (ABgene, Rochester, NY). The cDNA obtained was subjected to relative-quantitative Real Time PCR (QRT) using the ABSolute SYBR Green Rox Mix kit (ABgene, Rochester, NY), on an ABI Fast 7500 Real-Time machine (Applied Biosystems, Foster City, CA). The primers used are as follows: mouse T-bet: sense: 5'-GAATTGGAAGGTGCCCCACTAACTT-3', anti-sense: 5'-

GGGACACTCCTGTATTATTTCTT-3'; mouse GATA-3: sense: 5'-TCCAAGTGTGCGAAGAGTTC-3', anti-sense: 5'-CATTTTGCTTTCTGCCTTCA-3'; mouse IFN- γ sense: 5'-CTTTAACAGGCCAGACA-3', anti-sense: 5'-GCGAGTTATTTGTCATTCGG-3'; mouse IL-4: sense: 5'-GTCTGCGGCATATTCTG-3', anti-sense: 5'-GGCATTCTCATTGAGATTC-3'; GAPDH: sense: 5'-CCCCAATGTGTCCGTCGTG-3', anti-sense: 5'-GCCTTCACCACCTTCT-3'. The annealing temperatures used were as follows: mouse T-bet 60 °C; mouse GATA-3: 62 °C; mouse IFN- γ : 55.5 °C; mouse IL-4: 65 °C; mouse GAPDH: 58 °C.

2.8. Immunofluorescent staining of intracellular cytokines for flow cytometric analysis

Cells for intracellular cytokine staining were prepared by washing in cold FACS buffer (1% BSA in PBS), followed by staining (on ice for 30 min) for cell surface molecules (CD4-FITC or CD4 APC (BD Biosciences, San Jose, CA), CD25PE (Caltag, Invitrogen, Carlsbad, CA)). Cells were then washed and fixed in 3% paraformaldehyde (on ice for 15 min) in 1% BSA/PBS, followed by permeabilization in saponin buffer at room temperature for 10 min. Cells staining for Foxp3 (Foxp3-APC, eBioscience, San Diego, CA) were incubated at 4 °C overnight, and 2 h for IFN- γ , IL-4 and IL17 (IFN γ FITC and IL-4PE, BD Biosciences, San Jose, CA; IL17 APC, eBioscience, San Diego, CA) cytokines. Cells were then washed and analyzed using a BD FACScalibur (BD Biosciences, San Jose, CA).

2.9. Proliferation assay in vitro

For proliferation assays, CD4⁺ cells collected from mice on day 15 post-immunization were cultured along with irradiated antigen presenting cells (APC) at a 1:5 ratio in 0, 1, 10, and 100 μ g/ml of MOG antigen. CD4⁺ cells were also harvested from naïve mice and cultured with APC (1:5) in 10 μ g/ml MOG. To determine proliferation, polarized T helper and control cells were extensively washed, resuspended in Bruff's medium containing IL-2 (20 ng/ml) and restimulated with MOG peptide (10 μ g/ml) and APC (primed cells: APCs=1:10). After 48 h of culture, 1 μ Ci ³H-thymidine was added in 10 μ l of media to each well for another 18 h. Cells were harvested using a Tomtec Mach III (Hamden, CT) harvester and quantified using a Betaplate (Wallac, Perkin Elmer, Waltham, MA) scintillation counter.

2.10. Immunohistochemistry

Spinal cords and brains were collected from PBS-perfused mice on days 14–33 post-immunization. Spinal cord and brain tissues were embedded in OCT, snap frozen in liquid nitrogen, and kept at –80 °C until sectioning. Tissue sections (5 μ m) were cut using a cryostat (Microm, San Marcos, CA) and affixed to Superfrost/Plus microscope slides (Fisher, Pittsburgh, PA). Slides were fixed in acetone and stored at –80 °C. Prior to immunostaining, slides were thawed and fixed in 75% acetone/25% ethanol. Tissues sections were treated with 0.03% hydrogen peroxide in PBS to block endogenous peroxidase activity, washed in PBS, and blocked in 2 $\times$ casein blocking agent (Vector, Burlingame, CA) in normal goat serum (Zymed, San Francisco, CA). Primary antibodies [purified monoclonal anti-mouse CD4 (L3T4, BD Biosciences, San Jose, CA), anti-mouse CD45 (YW62.3, Biosource, Carlsbad, CA)] were incubated for 90 min at 37 °C at a 1:30 dilution in 1 $\times$ casein/PBS. Slides were then washed in PBS and incubated with biotinylated goat anti-rat antibody (Jackson ImmunoResearch), and then streptavidin–HRP (Zymed, Invitrogen, Carlsbad, CA). Tissues were developed with an AEC (Red) substrate kit (Zymed, Invitrogen, Carlsbad, CA), and counterstained with hematoxylin (Fisher, Pittsburgh, PA). The cover slips were mounted with Fluoromount-G (SouthernBiotech, Birmingham, AL), and photographed using light microscopy (Zeiss, Thornwood, NY).

2.11. FACS staining of lymphocytes

Freshly isolated splenocytes from mice 10 days post immunization with CFA-MOG_{35–55} were stained for CD44, CD45RB (BD Biosciences, San Jose, CA), CD62L and CD4 (Caltag, Invitrogen, Carlsbad, CA). Cells were analyzed on a FACScalibur (BD Biosciences, San Jose, CA) and data was analyzed using FlowJo software (Tree Star, Ashland, OR).

2.12. Western blotting

Lymphocytes (30×10^6) were isolated from naïve mice of the three strains, and whole cell lysate was run on SDS-PAGE. Protein was transferred to PVDF (Millipore, Bedford, MA) membrane overnight using a wet transfer method. The blot was probed with α STAT6 (Clone M-20, Santa Cruz Biotechnology, Santa Cruz, CA), stripped and probed for β actin (Rockland Immunochemicals, Gilbertsville, PA).

3. Results

3.1. STAT6^{-/-} mice are resistant to EAE

STAT6 signaling is required for the promotion of a Th2 immune response, and the Th2 cytokines IL-4 and or IL-13 are protective against EAE (Cash et al., 1994; Ochoa-Reparaz et al., 2008). To determine whether Th2 cytokines (IL-4 and or IL-13) are important in protecting mice from developing EAE in an epicutaneous immunization model (Bynoe et al., 2003), we induced EAE in MBP-TCR-Tg mice whose T cell receptor (TCR) are transgenic (Tg) for a peptide of myelin basic protein [MBP, (Hardardottir et al., 1995)] that were bred to STAT6^{-/-} mice generated by Ihle's group (Shimoda et al., 1996) (MBP-TCR-Tg STAT6^{-/-}). Since IL-4 and IL-13 are both activators of STAT6 and are protective against EAE and previous studies showed that STAT6^{-/-} mice develop more severe EAE than wild type (Chitnis et al., 2001), we expected these mice would develop more severe EAE than wild type mice. Contrary to expectations, MBP-TCR-Tg STAT6^{-/-} mice were completely resistant to EAE (Fig. 1A). After confirming by PCR that these mice were indeed STAT6 deficient (data not shown), we induced EAE directly in STAT6^{-/-} mice on the C57BL/6 (MOG-induced EAE model) background to insure that the EAE inhibition we observed was not due to an aberration of the transgene or a difference in genetic background. We observed the same EAE phenotype, whereby C57BL/6-STAT6^{-/-} mice were resistant to EAE compared to their wild type littermates (Fig. 1B), indicating that the unexpected disease phenotype was indeed due to the disruption of STAT6.

3.2. Different models of STAT6^{-/-} mice

Three different STAT6 knock out models were generated by three different groups [(Akira's group) (Takeda et al., 1996), (Grusby's group) (Kaplan et al., 1996) and (Ihle's group) (Shimoda et al., 1996)]. Akira's group carried out targeted disruption of three exons containing the SH2 domain of STAT6 (Takeda et al., 1996), Grusby's group deleted amino acids 505–584 encoding the SH2 domain (Kaplan et al., 1996) (STAT6 Δ SH2) and Ihle's group deleted the first coding exon of the STAT6 gene (Shimoda et al., 1996) (STAT6 Δ 1EX). Our initial studies were performed in STAT6^{-/-} mice generated by Ihle's group (STAT6 Δ 1EX). We next obtained STAT6^{-/-} mice generated by Grusby's group (STAT6 Δ SH2) to compare EAE responses. Importantly, both of these strains were extensively backcrossed onto a C57BL/6 background and thus, are genetically identical. EAE induction was induced in both STAT6 Δ 1EX and STAT6 Δ SH2, as well as wild type C57BL/6 mice as controls. Our data show that STAT6 Δ 1EX mice are resistant to the development of clinical EAE (mean maximal grade: 1.32 ± 0.21), while STAT6 Δ SH2 mice experience a more severe form of disease (mean maximal grade 3.25 ± 0.25) as compared to wild-type mice (mean maximal grade: 2.31 ± 0.19) as expected (26) (Fig. 1C and Table 1).

While STAT6 Δ 1EX mice have a lower incidence of disease (<70% $p<0.05$) compared with wild-type and STAT6 Δ SH2 mice (>90%, $p<0.05$), STAT6 Δ 1EX mice experience a similar day of disease onset as the wild-type, but show a very mild clinical course (Table 1). In contrast, STAT6 Δ SH2 mice have an earlier onset of disease (onset day: 8.1 ± 0.28) compared to wild-type (onset day: 10.9 ± 0.55 , $p<0.05$) and STAT6 Δ 1EX mice (onset day: 11.7 ± 0.42 , $p<0.001$) (Table 1) and attain a higher disease grade and mortality rate compared to either mouse strain. These experiments suggest that targeted disruption of the STAT6 gene can lead to either increased clinical EAE severity in the case of the complete removal of the SH2 domain (STAT6 Δ SH2) or in decreased severity of EAE when only the first exon of the SH2 domain coding region is deleted (STAT6 Δ 1EX).

3.3. Histological analysis of CNS in EAE induced mice

Spinal cords and brains were collected on day 30 post-immunization. Tissues were cryopreserved and stained for the presence of infiltrating lymphocytes (Fig. 1D). Hematoxylin and Eosin staining illustrates a higher degree of infiltration and inflammation in the CNS of STAT6 Δ SH2 mice in spinal cord and cerebellum (a and d) when compared to STAT6 Δ 1EX (b and e) or wild type mice (c and f). This increased infiltration was characterized by multiple neurologic lesions with extensive invasion of the parenchyma. These histology data correlate with the clinical scores, providing further evidence that different targeted deletions of the SH2 domain of STAT6 result in altered EAE disease.

3.4. STAT6 protein is expressed in lymphocytes from STAT6 Δ 1EX but not in STAT6 Δ SH2 mice

Ihle's group reported the presence of a "small minor" STAT6 protein in the STAT6 Δ 1EX mice (Shimoda et al., 1996) while Grusby's group reported the absence of any STAT6 transcript in their model (Kaplan et al., 1996). We examined STAT6 protein levels in lymphocytes from STAT6 Δ 1EX, STAT6 Δ SH2 and wild-type mice (genotypes confirmed as knockouts by PCR, data not shown). The results show a low amount of a truncated STAT6 protein (about 105 kDa) in lymphocytes from STAT6 Δ 1EX, consistent with the published report (Shimoda et al., 1996) (Fig. 2A), when compared with wild-type mice (117 kDa). Furthermore, the amount of STAT6 protein in STAT6 Δ 1EX is dramatically lower (almost 20-fold) than in wild-type (Fig. 2B). No STAT6 protein was detected in lymphocytes from STAT6 Δ SH2 mice (Fig. 2A). Since the only apparent difference between STAT6 Δ 1EX and STAT6 Δ SH2 is the presence of a truncated STAT6 protein, we hypothesize that the small amount of STAT6 protein might be responsible for potentiating a partial or weak STAT6 signal in STAT6 Δ 1EX mice resulting in disease suppression. Alternatively, this mutated STAT6 protein may have enhanced affinity for its promoter or act in a constitutively active dominant negative fashion.

3.5. No difference in proliferation of T cells from STAT6 Δ 1EX and STAT6 Δ SH2 mice

To determine whether the less severe disease observed in STAT6 Δ 1EX mice was due to a diminished capacity of T cells from these mice to respond to the autoantigen MOG, we performed *in vitro* proliferation analyses by culturing CD4⁺ T cells from naïve or MOG-immunized STAT6 Δ 1EX, STAT6 Δ SH2 or wild-type mice with irradiated antigen presenting cells (APCs) in the presence of different concentrations of exogenous MOG antigen (Fig. 3A). We detected no difference in proliferation of CD4⁺ T cells from STAT6 Δ EX, STAT6 Δ SH2 or wild type mice in response to MOG stimulation in culture (Fig. 3A). Similar to the *in vitro* data, we observed no difference in the *in vivo* proliferation of CFSE-labeled CD4⁺ T cells adoptively transferred into naïve TCR- $\alpha^{-/-}$ recipients (which are devoid of endogenous T cells) (data not shown). We conclude that the disease suppression/inhibition observed in STAT6 Δ 1EX mice is not due to an inability of CD4⁺ T cells in these mice to respond to antigen stimulation.

3.6. STAT6 Δ 1EX T cells proliferated normally under Th2-polarizing conditions

Naïve CD4 T cells isolated from STAT6 Δ SH2, STAT6 Δ 1EX or wild-type mice were subjected to Th1 and Th2 differentiation *in vitro*. We assessed proliferation of *in vitro* polarized-Th1 and Th2 cells from all three groups of mice (Fig. 3B) ($p < 0.05$). Polarized Th2 cells from STAT6 Δ 1EX mice proliferated significantly more than those from STAT6 Δ SH2 mice ($p = 0.007$) and almost reached the level of wild type proliferation (Fig. 3B). However, when polarized under Th1 conditions, STAT6 Δ EX CD4T cells proliferated significantly less than STAT6 Δ SH2 cells ($p = 0.002$) (Fig. 3B) and wild type cells. CD4 polarized Th1 and Th2 cells from wild type mice proliferated more than those from either STAT6 Δ 1EX or STAT6 Δ SH2 mice (Fig. 3B). These data suggest that the SH2 disruption in the STAT6 Δ 1EX mice does not impair CD4⁺ Th2 cell proliferative capacity, but might indirectly negatively influence CD4⁺ Th1 cell proliferation. Since disease progress is associated with a Th1 response and protection and recovery is associated with Th2 response, the proliferation data are consistent with the clinical finding that STAT6 Δ SH2 mice suffer more severe EAE, while STAT6 Δ 1EX mice appear resistant to EAE induction (Fig. 1).

3.7. CD4⁺ T cells from STAT6 Δ SH2 mice show impaired Th2 cytokine production compared to STAT6 Δ 1EX and wild type mice

To determine whether the proliferation profile of polarized cells is consistent with cytokine profiles in these two models, we analyzed the cytokines produced by these proliferating cells. We found that IFN- γ produced by *in vitro* Th1-differentiated cells from STAT6 Δ 1EX mice, although modest, was significantly less than from STAT6 Δ SH2 ($p = 0.009$) and wild-type mice ($p = 0.02$) (Fig. 4A), which correlates with our proliferation data (Fig. 3B). In fact, Th1 cells from STAT6 Δ SH2 mice produced similar levels of IFN- γ as wild type Th1 cells. In contrast, IL-4 produced by Th2-differentiated cells from STAT6 Δ 1EX mice was significantly higher than STAT6 Δ SH2 Th2 cells (IL-4, $p = 0.0001$) but lower than wild-type mice ($p = 0.027$) (Fig. 4B). IL-13 production by Th2-differentiated cells from STAT6 Δ SH2 mice was less than STAT6 Δ 1EX or wild-type mice ($p = 0.002$ and $p = 0.003$ respectively) (Fig. 4C). No difference in IL-2 production in undifferentiated cells was observed between the three groups (data not shown). Similar to the cytokine profile in culture supernatants, intracellular cytokine analysis shows that IL-4 production in STAT6 Δ 1EX mice was less than wild-type mice but greater than STAT6 Δ SH2 mice. Consistent with the ELISA data, intracellular IFN- γ staining showed little to no IFN- γ producing CD4 T cells from STAT6 Δ 1EX lymphocytes compared to cells from STAT6 Δ SH2 or wild-type mice (Fig. 4D). These data establish that primed STAT6 Δ SH2 and STAT6 Δ 1EX lymphocytes respond differently to antigen.

3.8. Differential expression of T-bet and GATA3 in T cells from STAT6 Δ 1EX and STAT6 Δ SH2 mice

To confirm the differentiation status of these cells, mRNA expression of IFN- γ plus the Th1-associated transcription factor T-bet, along with Th2 associated IL-4 and the transcription factor GATA3 were analyzed by quantitative real-time PCR (Fig. 5). Our data show increased levels of T-bet (Fig. 5A and B) and IFN- γ (Fig. 5C and D) cDNA in Th1-polarized cells from STAT6 Δ SH2 mice when compared to wild type mice, and almost no IL-4 (Fig. 5C and D) cDNA and very low levels of GATA3 (Fig. 5A and E) expression in STAT6 Δ SH2 Th2-polarized cells when compared to wild type cells. Conversely, Th1-polarized cells from STAT6 Δ 1EX mice express low levels of T-bet (Fig. 5A and B) and IFN- γ (Fig. 5C and D) and moderate GATA3 (Fig. 5A and E) and IL-4 (Fig. 5C and D) expression in the Th2-polarized cells when compared to wild type. These findings illustrate a lack of IL-4 expression and alteration in Th2 cell differentiation in STAT6 Δ SH2 mice, substantiating a vital role for the STAT6 pathway in mediating signals required for Th2 cell differentiation. We observed that T lymphocytes from STAT6 Δ SH2 mice are almost completely impaired in their ability to

differentiate into Th2 cells based upon these results (Figs. 4 and 5). These results suggest that signaling through the truncated STAT6 in STAT6 Δ EX mice shifts the cytokine profile to Th2 in these mice.

3.9. IL-17 is not impaired in CD4 T cells from STAT6 Δ EX mice

Recent studies have implicated Th17 cells and their IL-17 production in EAE pathogenesis (Ivanov et al., 2006; Komiyama et al., 2006; Languish et al., 2005; Park et al., 2005). To determine whether the protection against EAE observed in STAT6 Δ EX mice was due to suppressed IL-17, we analyzed tissue culture supernatants from *in vitro* differentiated MOG-stimulated Th1, Th2 and Th17 CD4 T cells from STAT6 Δ SH2, STAT6 Δ EX and wild type mice (Fig. 6). Th1 cells from STAT6 Δ SH2 and STAT6 Δ EX mice produced similar levels of IL-17, compared to wild type mice that produced two-fold more IL-17 (Fig. 6A). Interestingly, IL-17 production was increased in STAT6 Δ EX Th2 cells compared to STAT6 Δ SH2 and wild type Th2 cells. Under Th17 polarization, CD4 T cells from wild type mice produced two-fold more IL-17 than CD4T cells from STAT6 Δ SH2 mice that produced just above background levels of IL-17 under both polarized and non-polarized conditions (Fig. 6). Interestingly, CD4 T cells from STAT6 Δ EX mice produced two to four fold more IL-17 than wild type cells under both polarizing and non-polarizing conditions respectively (Fig. 6B). These results show that the less severe EAE observed in STAT6 Δ EX mice is not due to a defect in IL-17 production by CD4⁺ T cells in STAT6 Δ EX mice.

3.10. CD4⁺CD25⁺ Foxp3⁺ Regulatory T cell generation is impaired in STAT6 Δ SH2 mice

It has been recently reported that the STAT6 pathway is involved in the development of CD4⁺CD25⁺Foxp3⁺ Regulatory T cells (Treg) cells (Sanchez-Guajardo et al., 2007). Foxp3 is a key marker of CD4⁺CD25⁺ Treg cell lineage (Maloy and Powrie, 2001; Sakaguchi, 2000; Shevach, 2000). CD4 T cells from naive STAT6 Δ SH2, STAT6 Δ EX and wild type mice were stimulated *in vitro* with 10 μ g/ml MOG antigen followed by intracellular Foxp3 staining. We found a complete absence of Treg cells in STAT6 Δ SH2 mice, while STAT6 Δ EX mice had a reduced population (about half) of Treg cells compared to wild-type mice (Fig. 7). The results in STAT6 Δ SH2 mice are consistent with the previous reports showing that a lack of STAT6 signaling prohibits Treg cell development (Sanchez-Guajardo et al., 2007). The presence of Treg cells in STAT6 Δ EX mice, albeit in reduced numbers compared to wild type, suggests that the truncated STAT6 signal is sufficient to generate low numbers of Treg cells.

4. Discussion

STAT6 Δ SH2 mice were generated by replacing amino acids 505–584 of the SH2 domain of the STAT6 gene with a neomycin resistance cassette (neo^r) (Kaplan et al., 1996), while STAT6 Δ EX mice were generated by replacing the first coding exon of the SH2 domain of the STAT6 gene with a neo^r cassette (Shimoda et al., 1996). Here we report the effect of the different targeted disruptions of STAT6 on the development of EAE. We observe that STAT6 Δ EX mice have mild disease and are protected from EAE, while STAT6 Δ SH2 mice suffer more severe disease than either STAT6 Δ EX or wild-type controls. The molecular basis for the different EAE phenotype appears to be due to a difference in the approaches used to disable the STAT6 gene. In STAT6 Δ EX mice, replacing the first coding exon of the SH2 domain of STAT6 resulted in a truncated STAT6 protein, while removal of the SH2 domain in STAT6 Δ SH2 resulted in complete ablation of the STAT6 gene and complete absence of any STAT6 protein.

To determine the basis of these differences, we studied cytokine profiles, proliferation, differentiation and Treg cell development of CD4⁺ T cells in the two different STAT6 knockout mice and compared them to wild type mice. CD4⁺ T cells in the STAT6 Δ EX mice show a

significantly diminished capacity to differentiate into Th1 cells while Th2 cell differentiation is unimpaired. Our proliferation data show that STAT6 Δ 1EX derived cells proliferate as well as wild-type cells. Therefore there is no defect in the ability of CD4 Th1 or Th2 differentiated cells to proliferate in response to antigen stimulation. Second, STAT6 Δ 1EX mice express the appropriate cytokine profile that is consistent with protection from disease. That is, CD4⁺ T cells from immunized STAT6 Δ 1EX mice stimulated with MOG–CFA produce significantly lower levels of IFN- γ , while IL-4 and IL-13 levels are similar to wild type. A STAT6 Δ SH2 Th2 response was almost non-existent, while the Th1 response appears normal. Furthermore, we found low T-bet and IFN- γ siRNA expression in Th1-polarized cells and appropriate GATA3 and IL-4 mRNA expression in Th2-polarized cells from STAT6 Δ 1EX mice. The precise mechanisms underlying spontaneous down-modulation of the inflammatory process in EAE, as well as prevention of spontaneous relapses, is not fully understood. Evidence exists for a role for IL-4 and the subsequent STAT6-driven Th2 response being part of a natural suppressor mechanism of EAE (Cua et al., 1995; Falcone and Bloom, 1997). Supporting this hypothesis are reports correlating genetic resistance to EAE with preferential development of T cells of Th2 phenotype (Cua et al., 1995; Mustafa et al., 1994), thus suggesting that IL-4 plays an essential role in down-regulation of antigen specific autoreactive cells in local tissue. The caveat to this is that other studies have demonstrated a lack of this protective role of IL4 in EAE progression (Buenafe et al., 1997; Liblau et al., 1997). The STAT6 protein and the transcription factor GATA3 may bind to chemokine promoters (Kusam et al., 2003), and activated STAT6 signaling in turn may activate the expression of Th2-driven chemokines which may control the tissue migration of effector cells (Luster, 1998; Sallusto et al., 2000; Zhang et al., 2000). Antigen-specific wild-type Th2 cells, when adoptively transferred into STAT6^{-/-} mice, failed to induce asthma, consistent with the presence of functional STAT6 binding sites in the promoters of Th2 driven chemokines (Mathew et al., 2001). Therefore, the STAT6 protein is a key factor in the Th2 cytokine signaling pathway that leads to differentiation and recruitment of STATG-driven differentiated Th2 cells that protect from EAE.

The discovery of Th17 cells and their production of IL-17 have recently been shown to play an important role in EAE pathogenicity (Ivanov et al., 2006; Komiyama et al., 2006; Park et al., 2005; Zhang et al., 2000). However, emerging studies demonstrate that the role of Th17 and Th1 cells in EAE is more complicated than originally reported. T-bet, which is essential for the differentiation of Th1 cells and IFN- γ production, is also important in the regulation and generation of Th17 cells and IL-17 production (Gocke et al., 2007). Silencing of T-bet with siRNA can ameliorate EAE in mice by inhibiting both Th1 and Th17 cells (Gocke et al., 2007). Further, a recent report showed that the ratio between Th17 and Th1 cells determines the level of pathogenicity in EAE (Stromnes et al., 2008). That is, T cell inflammation and infiltration in the CNS is exacerbated when Th17 cells predominate over Th1 cells (Stromnes et al., 2008). Interestingly, we observed that CD4⁺ T cells from STAT6 Δ 1EX mice produced much higher levels of IL-17 under Th2 polarizing, Th17 polarizing and non-polarizing conditions compared to wild type cells. However, CD4 T cells from STAT6 Δ SH2 mice appear defective in IL-17 production under these same conditions, yet get very severe disease. Therefore, the difference in the cytokine profile which can account for the decrease in disease severity between wild type and STAT6 Δ 1EX mice, as well as between STAT6 Δ SH2 and STAT6 Δ 1EX mice, is the reduced IFN- γ production by STAT6 Δ 1EX CD4 T cells. These data suggest that despite numerous reports demonstrating a predominant role for IL-17 and/or IFN- γ in EAE pathogenesis, that it is the relative expression of Th2:Th1 that ultimately regulates EAE pathogenesis.

The second interesting observation in our study that could further explain the difference in the disease phenotypes between STAT6 Δ 1EX and STAT6 Δ SH2 mice is that STAT6 Δ SH2 mice have an impaired generation of Treg cells compared to STAT6 Δ 1EX or wild-type mice. Regulatory CD4⁺CD25⁺ T lymphocytes play a major role in T cell homeostasis and in the

maintenance of tolerance by actively suppressing the activation and expansion of self-reactive T cells (Maloy and Powrie, 2001; Sakaguchi, 2000; Shevach, 2000). Indeed, removal of CD4⁺CD25⁺ T cells, which constitute 5–10% of CD4⁺ T cells in rodents and humans, leads to spontaneous development of various autoimmune diseases in otherwise normal mice (Sakaguchi et al., 1995). It has recently been shown that STAT6 is involved in the development of CD4⁺CD25⁺Foxp3⁺ regulatory T cells (Sanchez-Guajardo et al., 2007). The lack of Treg cells in STAT6ΔSH2 mice also contributes to the more severe EAE in these mice due to ineffective or lack of suppression of effector CD4⁺ T cells that can infiltrate the CNS and cause inflammation. This would result in induction of a more severe disease phenotype, as we have observed.

While the presence of Treg cells in STAT6Δ1EX mice could also contribute to the protection in EAE, it does not explain why these mice develop significantly milder EAE than wild type mice, as STAT6Δ1EX mice have half the number of Treg cells compared to wild type. Therefore, it must be the combination of the decrease in IFN-γ, the ability to generate Th2 cytokines and Tregs which contributes to protection in EAE. Further, it must be the decreased IFN-γ produced by STAT6Δ1EX mice that is the limiting factor that results in protection.

In summary, since STAT6 signaling is involved in the regulation of Th2 cells, their cytokines and their associated transcription factors, as well as Treg cells development, our data suggest that the STAT6 gene is essential for regulating the immune response during the induction of EAE. The differential disruption of the STAT6 gene, as seen in both STAT6Δ1EX and STAT6ΔSH2 mice, either ameliorates clinical disease and reduces inflammatory infiltrates in the CNS, or causes increased clinical and pathological disease, respectively. The fact that wild type mice develop more severe disease than STAT6Δ1EX mice suggest that the “modulated” STAT6 in *Ih1e* mice is more efficient than wild type STAT6 in protection against EAE. Therefore, the modulation of cytokine-signaling pathways and the balance between Th1 and Th2 cytokines through the target genes may be more important than complete elimination of a particular individual cytokine with regard to modulating autoimmune responses *in vivo*. The ability to specifically affect the cytokine shifting, Th1/Th2 balance, or Th1/Th2 cross-communication and Treg cell regulatory function through STAT6 target genes should be an essential tool in therapeutic regulation of various autoimmune disorders, such as multiple sclerosis.

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Fig. 1. *STAT6* Δ 1EX mice (on a BL/6 background) were crossed with MBP-Tg (on a B10.PL background) mice to form MBP-Tg *STAT6*^{-/-} mice. Mice were immunized sub-cutaneously (sc) with MOG₃₅₋₅₅-CFA along with IV pertussis (at immunization and 48 h later) and graded for disease daily. When compared with control MBP-Tg mice (filled squares), MBP-Tg *STAT6*^{-/-} (open squares) mice showed no clinical symptoms of EAE (A). This phenotype was tested on the original *STAT6* Δ 1EX mice (open diamonds) and compared with wild type BL/6 mice (filled diamonds) to determine if this unexpected result was due to the B10.PL background cross. We observed the same phenotype whereby *STAT6* Δ EX mice are resistant to EAE (B). EAE was induced in *STAT6* Δ 1EX, *STAT6* Δ SH2 and wild type mice. A representative experiment (of 4) shows disease progression in wild-type (filled triangles, STDV<0.33), *STAT6* Δ 1EX (open diamonds, STDV<0.34), and *STAT6* Δ SH2 (open circles, STDV<0.47) mice (C). The mean daily disease grade for each group ($n=5$ mice per group) is shown. Data is representative of one of four different experiments. For immunohistochemical analysis of inflammation and infiltration in mice with EAE, the CNS was removed from immunized mice after 30 days (D). Tissue was cryopreserved, sectioned and stained for CD4 (brown). Photomicrographs (x40) of representative spinal cord and brain sections on day 30 from post-immunized mice are shown, a and d are representative sections of spinal cord and cerebellum respectively in *STAT6* Δ SH2 mice; b and e show representative sections of spinal

cord and cerebellum respectively from STAT6 Δ 1EX mice; c and f depict representative sections of spinal cord and cerebellum respectively from wild-type mice.

Fig. 2.

STAT6 protein expression in lymphocytes from STAT6 Δ SH2, STAT6 Δ 1EX and wild type mice. Lymphocytes were isolated from STAT6 Δ 1EX, STAT6 Δ SH2 and wild type mice, and Western analysis was performed for STAT6 expression. A smaller than wild type or truncated STAT6 (about 105-kDa) protein is shown in STAT6 Δ 1EX mice (A). As a loading control, the blot was stripped and reprobed for β -actin (42 kDa), and STAT6 expression was normalized to β -actin expression (B).

Fig. 3.

In vivo proliferative response of naïve CD4⁺ T cells isolated from naïve STAT6 Δ 1EX (white bars) STAT6 Δ SH2 (gray bars) and wild-type (black bars) mice. *In vitro* proliferative response to MOG₃₅₋₅₅ of lymphocytes isolated from immunized wild-type, STAT6 Δ SH2 and STAT6 Δ 1EX mice. Spleen and lymph node cells were obtained on day 15 post-immunization from the three groups. Cells were cultured in the presence irradiated APCs with MOG₃₅₋₅₅ at 0, 1, 10, and 100 μ g/ml, and proliferation was measured by tritiated thymidine incorporation. Proliferation was not significantly different between STAT6 Δ 1EX mice (white bars), STAT6 Δ SH2 mice (gray bars) and wild-type mice (black bar) (*p* value not significant) (A). The proliferative response of naïve CD4⁺ cells under Th1/Th2 polarizing conditions was

measured. Th1 polarized CD4⁺ cells from STAT6ΔSH2 mice (gray bar) proliferated at a higher rate than STAT6Δ1EX (white bar) ($p < 0.01$), but less than wild-type mice (black bar) ($p < 0.05$). Under Th2 polarizing conditions, STAT6ΔSH2 cells (gray bar) proliferated at a significantly lower rate than STAT6Δ1EX cells (white bar) ($p < 0.01$) and wild-type cells (black bar) ($p < 0.001$) (B).

Fig. 4.

Cytokine analysis of *in vitro* differentiated T lymphocytes from STAT6 Δ EX, STAT6 Δ SH2 and wild-type mice. Th1-polarized cells from STAT6 Δ 1EX (white bar) produced less IFN- γ less than STAT6 Δ SH2 (gray bar) ($p < 0.01$) or wild-type cells (black bar) ($p < 0.01$) (A). IL-4 production by Th2-polarized cells from STAT6 Δ 1EX (white bar) was significantly higher than STAT6 Δ SH2 (gray bar) ($p < 0.001$) but lower than wild-type cells (black bar) ($p < 0.01$) (B). IL-13 produced by Th2-polarized cells from STAT6 Δ SH2 (gray bar) was significantly lower than STAT6 Δ 1EX (white bar) and wild-type cells (black bar) ($p < 0.001$) (C). Intracellular cytokine staining of CD4 $^{+}$ T cells from spleen and lymph nodes from STAT6 Δ SH2, STAT6 Δ 1EX and wild type mice correlate with these results. STAT6 Δ SH2 CD4 $^{+}$ lymphocytes produce less IL-4 (0.09%) compared to STAT6 Δ 1EX (0.44%) and wild-type (0.56%), while STAT6 Δ 1EX lymphocytes produce less IFN- γ (0.07%) compared with STAT6 Δ SH2 (0.33%) and wild-type (0.34%) (D).

Fig. 5.

T-bet, GATA-3 and cytokine gene expression in polarized Th1/Th2 cells. Naïve CD4⁺ T cells were collected from STAT6 Δ SH2, STAT6 Δ EX, and wild-type mice, and were stimulated *in vitro* under Th1 or Th2 polarizing conditions. Cells were harvested for RNA preparation and subsequent rtPCR, and supernatants were recovered for cytokine analysis. Gene expression was measured by semi-quantitative reverse-transcriptase-PCR and by relative real-time-PCR for T-bet and GATA-3 (A, B and E), or IFN- γ and L-4 (C and D) in polarized splenocytes and compared with undifferentiated splenocytes (control). Relative real-time-PCR data was normalized to GAPDH expression and presented as mean $\pm$ standard deviation of duplicate samples for one mouse.

Fig. 6.

IL-17 production by STAT6 Δ EX, STAT6 Δ SH2 and wild-type mice. CD4 T cells were isolated from spleen and lymph nodes of STAT6 Δ 1EX (white bar), STAT6 Δ SH2 (gray bar) and wild-type (black bar) mice and differentiated *in vitro* into Th1, Th2 (A) and Th17 cells (B). Tissue culture supernatants were analyzed by ELISA for IL-17 production. Both STAT6 Δ SH2 (gray bar) and STAT6 Δ 1EX (white bar) CD4+ cells produced less IL17 than wild type (black bar) under Th1 polarizing conditions (A). Under Th2 polarizing conditions, STAT6 Δ 1EX (white bar) CD4+ cells produced much higher amounts of IL17 than either STAT6 Δ SH2 (gray bar) or wild type (black bar) (A). Both STAT6 Δ SH2 (gray bar) and wild type (black bar) CD4+ cells produced less IL17 than STAT6 Δ 1EX (white bar) under no polarization and Th17 polarizing conditions (B). STAT6 Δ 1EX (white bar) CD4+ cells produced high levels of IL17 whether or not the cells were subjected to Th17 polarizing conditions (B).

Fig. 7.

Foxp3 expression in immunized wild-type, STAT6 Δ SH2 and STAT6 Δ 1EX mice.

Lymphocytes were isolated on day 10 post-immunization and were stimulated overnight with MOG₃₅₋₅₅. Cells were stained for CD4, CD25 and Foxp3, and analyzed using flow cytometry. Dot plots are gated on CD4⁺ total live cells. The percentage of CD4⁺CD25⁺Foxp3⁺ Regulatory Tcells observed in STAT6 Δ 1EX lymphocytes (2.79%) is higher than in STAT6 Δ SH2 lymphocytes (0.30%, $p < 0.05$), but is still lower than the population found in wild type mice (6.60%, $p < 0.05$).

Table 1Disease incidence, mean maximal grade, and day of onset in STAT6 Δ 1EX, STAT6 Δ SH2, and wild-type mice

Mouse type	Disease incidence	Mean maximal grade	Day of onset	Mortality rate
STAT6 Δ SH2	18/19	3.24 $\pm$ 0.25	8.1 $\pm$ 0.28	37.4%
STAT6 Δ 1EX	13/19	1.33 $\pm$ 0.21	11.7 $\pm$ 0.42	15.8%
WT	18/20	2.314 $\pm$ 0.19	10.9 $\pm$ 0.55	16.7%

This table represents the sum of 4 separate experiments.