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Impaired Development of CD4$^+$ CD25$^+$ Regulatory T Cells in the Absence of STAT1: Increased Susceptibility to Autoimmune Disease

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Abstract

Type I and II interferons (IFNs) exert opposing effects on the progression of multiple sclerosis, even though both IFNs use the signal transducer and activator of transcription 1 (STAT1) as a signaling mediator. Here we report that STAT1-deficient mice expressing a transgenic T cell receptor against myelin basic protein spontaneously develop experimental autoimmune encephalomyelitis with dramatically increased frequency. The heightened susceptibility to this autoimmune disease appears to be triggered by a reduced number as well as a functional impairment of the CD4$^+$ CD25$^+$ regulatory T cells in STAT1-deficient animals. Adoptive transfer of wild-type regulatory T cells into STAT1-deficient hosts is sufficient to prevent the development of autoimmune disease. These results demonstrate an essential role of STAT1 in the maintenance of immunological self-tolerance.

Key words: STAT1 • EAE • autoimmune disease • regulatory T cells

Introduction

Multiple sclerosis (MS) is a T cell–mediated, demyelinating autoimmune disease of the central nervous system characterized by T cells reactive to the major components of the myelin sheaths, such as myelin basic protein (MBP), myelin oligodendrocyte glycoprotein, and proteolipid protein (1). In animals, MS-like symptoms can be induced by immunization with MBP or with peptides representing specific epitopes of proteins associated with the myelin sheats, leading to the development of experimental autoimmune encephalomyelitis (EAE; 2).

Similarly, MS-like disease symptoms emerge in mice that express a transgenic TCR against MBP (3) after immunization of such animals with MBP. In this case, the TCR α and β chains were derived from a CD4$^+$ encephalomyelitogenic T cell clone that recognizes MBP in an H-2 I-A^u–restricted context. Due to allelic exclusion, <5% of CD4$^+$ T cells in these animals express antigen receptors of different antigen specificity. In transgenic mice of H-2^u haplotype, 14% of the animals develop the disease within 12 mo, whereas no symptoms arise in an H-2^b background. One of the most striking discoveries resulted from the cross of MBP TCR transgenic mice with RAG-1–deficient animals. The RAG-1 protein is required for the successful rearrangement of antibody and TCR genes. Thus, in RAG-1–deficient mice, lymphocytes can only mature if they express functional antigen receptors as transgenes. RAG-1–deficient mice transgenic for the MBP-directed TCR succumb to EAE at a rate of 100% by 12 mo (4). These findings unequivocally demonstrate that nontransgenic lymphocytes, which are absent in the RAG-1–deficient animals, exert a protective effect against the development of the autoimmune disorder.

One cell population that can mediate a protective effect has been identified as a subset of CD4$^+$ T cells that coexpress CD25, but are distinct from activated T cells (5). Based on their function, this T cell subpopulation has been termed "suppressor" or "regulatory" T cells (6–8). Importantly, adoptive transfer of wild-type CD4$^+$ cells into RAG-1–deficient, MBP TCR transgenic mice was found to protect the animals from developing EAE (9). Once activated, CD4$^+$ CD25$^+$ cells act in an antigen-independent manner (10), and appear to represent a unique lineage of CD4$^+$ T cells, albeit of elusive origin.

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Abbreviations used in this paper: CFSE, carboxyfluorescein diacetate succinimidyl ester; CNS, central nervous system; EAE, experimental autoimmune encephalomyelitis; MBP, myelin basic protein; MS, multiple sclerosis; STAT1, signal transducer and activator of transcription 1; TDS, T cell–depleted spleen cells.

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Among several immunomodulatory therapies for MS treatment, IFN-β administration has become the treatment of choice. IFNs as well as other cytokines and growth factors mediate their antiviral and antiproliferative effects through the induction of immediate early response genes (11). The signal transducer and activator of transcription 1 (STAT1) transcription factor has been identified as an important part of the IFN-α/β and IFN-γ–induced signaling cascades (12).

Several years ago, two labs independently generated STAT1-deficient mice to elucidate the role of STAT1 in vivo (13, 14). As expected, the most prominent phenotype was a significantly increased sensitivity of these animals to viral infections due to their inability to respond to the antiviral effects of IFNs. The lack of IFN-mediated up-regulation of MHC expression also results in an inability to mount an effective immune response to bacterial infections. Interestingly, it appeared that lymphocyte development was not substantially altered in the absence of STAT1 (13, 14). As such, STAT1$^{-/-}$ mice display a normal CD4/CD8 profile in the thymus, and no obvious defects were found in the B cell compartment. A later report described that splenocytes derived from STAT1$^{-/-}$ animals displayed a reduced rate of apoptosis due to a lack of caspase 1 and 11 expression (15).

IFNs were first used in MS therapy because of their antiviral properties, as it has been observed that acute viral infection triggers the onset and clinical relapses of the disease. Initially, IFN-γ was administered, however, the IFN itself provoked significant exacerbation of the disease (16). This observation consequently led to the therapeutic application of IFN-α/β because type I IFNs were known to counteract several actions of IFN-γ.

The opposing effects of IFN-α/β and IFN-γ in the progression of MS raise the intriguing question as to what role STAT1 plays in the pathogenic process because both IFNs use STAT1 as the primary mediator of their biological actions. To address this issue we used EAE as a model for MS and examined the effects of STAT1 expression on the induction and progression of this autoimmune disease.

Materials and Methods

Animals. Wild-type and STAT1$^{-/-}$ 129/SvEv (H-2$^{b/b}$) mice were purchased from Taconic Inc. and mice expressing the transgenic TCR α and β chains (TCR^{+}) specific for the MBP (MBP1-9) were provided by S. Tonegawa (Massachusetts Institute of Technology, Cambridge, MA). These animals were crossed to generate STAT1$^{+/-}$ TCR^{+} H-2$^{u/u}$ or H-2$^{b/u}$ and STAT1$^{-/-}$ TCR^{+} H-2$^{u/u}$ or H-2$^{b/u}$ mice. All mice used in these experiments were maintained in a pathogen-free environment and were bred and cared for in accordance with University of California San Diego Animal Care Facility regulations.

EAE Scoring and Immunizations. Clinical symptoms of EAE were graded as follows (17): level 0, no symptoms; level 1, limp tail; level 2, partial paralysis of hind legs; level 3, complete paralysis of hind legs; level 4, paralysis of fore and hind legs; level 5, moribund. For some experiments, 6–10-wk-old STAT1$^{+/-}$ TCR αβ^{+} H-2$^{b/u}$ mice were immunized with 200 μg MBP per mouse emulsified in CFA.

Flow Cytometry and Intracellular Cytokine Staining. FITC-labeled anti-CD4 mAb, PE-labeled anti-CD8 mAb, FITC-labeled anti-Vβ8 mAb, PE-labeled anti-CD69, biotin-conjugated anti-CD25 mAb, and biotin-conjugated anti–γ/δ TCR mAb were obtained from BD Biosciences. Biotin-conjugated anti-B220, anti-CD4 mAbs, and APC-conjugated streptavidin were purchased from eBioscience. Anti-CD3ε mAb (clone 2C11) and IL-2 supernatant were provided by S. Hedrick (University of California San Diego, San Diego, CA).

For immunostaining, single cell suspensions were prepared from thymus, spleen, and peripheral lymph node cells. After removing red blood cells, one million cells were resuspended in the staining buffer (PBS, 1% FCS, 0.02% NaN_3) and incubated with the antibodies for 30 min on ice. For intracellular cytokine staining, cells were stimulated with 50 ng/ml PMA and 500 ng/ml ionomycin for 4 h. 10 μg/ml brefeldin A was added during the last 2 h of the incubation. Stimulated cells were stained with FITC-labeled anti-CD4 Ab before fixing with 4% paraformaldehyde. Cells were resuspended in permeabilization buffer (PBS, 1% saponin, 1% FCS) and stained with PE-labeled anti–IFN-γ mAb (BD Biosciences) for 30 min on ice.

T Cell and APC Purification. CD4^{+} T cells from spleen and thymus were isolated by magnetic sorting using magnetic microbeads (Miltenyi Biotec). In brief, single cell suspensions were incubated with biotinylated anti-CD8, anti-B220, anti-MAC1, anti-Gr1, and anti–γ/δ TCR antibodies (BD Biosciences) for 30 min on ice, washed, and further incubated with anti-biotin beads (Miltenyi Biotec) for 15 min. Cells were then applied to a SuperMacs magnet column and CD4^{+} T cells that did not bind to the

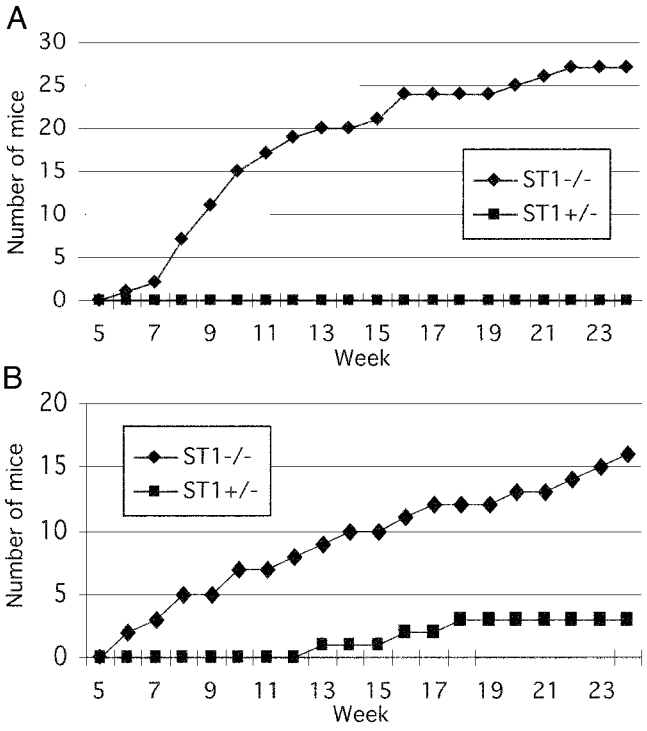

Figure 1. Spontaneous EAE development in wild-type and STAT1-deficient mice. (A) TCR^{+} STAT1$^{-/-}$ H-2$^{b/u}$ mice and their TCR^{+} STAT1$^{+/-}$ H-2$^{b/u}$ littermates (30 animals/group) were monitored for 6 mo and the spontaneous development of EAE (level 5) was recorded. (B) Same as in A, but TCR^{+} STAT1$^{-/-}$ H-2$^{u/u}$ mice and their TCR^{+} STAT1$^{+/-}$ H-2$^{u/u}$ littermates (20 animals/group) were used.

column were collected. This fraction was then stained with PE-labeled anti-CD25 (PC61) and FITC-labeled anti-CD4 and FACS® sorted to obtain $CD4^+$ $CD25^+$ and $CD4^+$ $CD25^-$ T cells. For depletion experiments, $CD4^+$ T cells were additionally labeled with biotinylated anti-CD25 antibody at the same time and $CD4^+$ $CD25^-$ cells were collected. T cell–depleted spleen cells (TDS) as APCs were obtained by removal of T cells with biotinylated anti-CD4, anti-CD8, and anti–γ/δ TCR antibodies as outlined above. APCs were treated with 50 μg/ml mitomycin C for 30 min.

Carboxyfluorescein Diacetate Succinimidyl Ester (CFSE) Assays. CFSE was obtained from Molecular Probes, Inc. Purified T cell populations of interest were washed and resuspended in PBS containing 5 μM CFSE. After incubation for 8 min, media containing serum was added and labeled cells were washed and subjected to proliferation assays. T cells (10^6 cells/well) were cultured with mitomycin-treated TDS (3×10^6 cells/well) as APCs in a 24-well flat-bottom plate in the absence or presence of 1.0 μg/ml Con A. After 3 d of incubation, stained cells were analyzed with a FACScan™ (Becton Dickinson). To analyze the suppressive properties of regulatory T cells, $CD4^+$ $CD25^-$ T cells were labeled with CFSE and cultured in 96-well plates (10^5 cells/well) with TDS as APCs (3×10^5 cells/well), 25 ng/ml anti-CD3 antibody, and the indicated numbers (1:0, 1:0.1, 1:0.2, 1:0.4, and 1:0.8) of unlabeled wild-type or STAT1-deficient $CD4^+$ $CD25^+$ cells. After 3 d of incubation, stained cells were analyzed with FACScan™ (Becton Dickinson).

Proliferation Assays by [^{3}H]thymidine Incorporation. Purified T cells (2×10^4 cells/well) were cultured with mitomycin-treated TDS (6×10^4 cells/well) as APCs in a 96-well round-bottom plate. For stimulation either 1.0 μg/ml Con A or 50 ng/ml anti-CD3 antibody in combination with 50 U/ml human rIL-2 were added. Stimulated cells were cultured in complete medium for 2 d, pulsed with 1 μCi [^{3}H]thymidine per well, and harvested 16 h later. All data shown represent an average of at least three independent experiments.

Adoptive T Cell Transfer. $CD4^+$ T cells or $CD4^+$ $CD25^+$ T cells derived from $STAT1^{+/-}$ TCR^+ $H\text{-}2^{b/u}$ mice were purified as outlined above, and injected intravenously in 200 μl PBS into 37–41-d-old $STAT1^{-/-}$ TCR^+ $H\text{-}2^{b/u}$ littermates. Recipient mice were monitored every other day for EAE development and progression.

Results

STAT1-deficient Mice Are Highly Susceptible to Autoimmune Disease. Mice transgenic for an MBP-reactive T cell receptor and of the required $H\text{-}2^{u/u}$ haplotype (4) succumb to EAE after immunization with MBP. These transgenes were originally injected into C57BL/6 eggs, and the required $H\text{-}2^{u/u}$ haplotype was introduced into the transgenic background by crosses with PL/J mice (4). Several reports illustrated the need for a homozygous $H\text{-}2^{u/u}$ background, as mice of $H\text{-}2^{b/u}$ or $H\text{-}2^{b/b}$ genotype failed to develop EAE.

To address the role of STAT1 in autoimmune disease development, we crossed these transgenic mice onto $STAT1^{-/-}$ animals with the goal to obtain STAT1-defi-

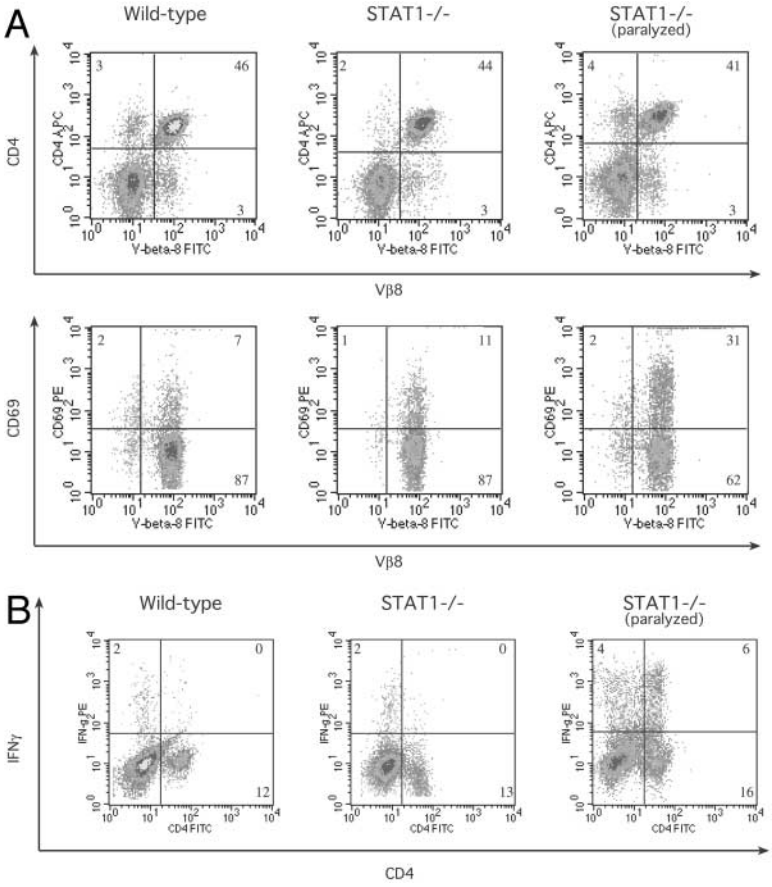

Figure 2. Flow cytometric analysis of wild-type, asymptomatic, or paralyzed STAT1-deficient mice. (A) Cells derived from lymph nodes of wild-type, asymptomatic, or paralyzed STAT1-deficient animals were stained for CD4, CD69, and Vβ8. Horizontal axis represents Vβ8 staining, vertical axis corresponds to CD4 (top) or CD69 (bottom) staining of $CD8^-$ cells. Cells displayed in lower panels are $CD4^+$. (B) Splenocytes derived from wild-type, asymptomatic, or paralyzed STAT1-deficient animals were stimulated with 50 ng/ml PMA/500 ng/ml ionomycin for 4 h (10 μg/ml brefeldin A was added during the last 2 h) and stained for CD4, CD8, and intracellular IFN-γ. CD4 staining (horizontal axis) and IFN-γ production (vertical axis) of $CD8^-$ cells is shown.

cient mice bearing the transgenic TCR on an H-$2^{u/u}$ background. Surprisingly, we observed already in the F2 generation that several STAT1-deficient animals carrying the transgenic TCR (TCR^+ $STAT1^{-/-}$ H-$2^{b/u}$) spontaneously developed progressive paralysis, even though these mice were of H-$2^{b/u}$ genotype.

By the age of 10 wk, 50% of these animals had developed level 5 paralysis, and by 24 wk 90% of the mice had succumbed to EAE (Fig. 1 A). In contrast, none of their TCR^+ $STAT1^{+/-}$ H-$2^{b/u}$ littermates displayed any symptoms. Strikingly, even repeated immunization of TCR^+ $ST^{+/-}$ H-$2^{b/u}$ mice with MBP was unable to provoke EAE development, confirming previously published results on the importance of the H-$2^{u/u}$ background (4).

Identical to mice of H-$2^{b/u}$ genotype, TCR^+ $STAT1^{-/-}$ H-$2^{u/u}$ mice succumb to spontaneous EAE at a drastically increased rate when compared with TCR^+ $STAT1^{+/-}$ H-$2^{u/u}$ animals, whereas TCR^+ $STAT1^{+/-}$ H-$2^{u/u}$ mice also spontaneously developed EAE at the previously published frequency (4) of ~15% (Fig. 1 B).

Increased IFN-γ Production and CD69 Up-regulation in STAT1-deficient Paralyzed Mice. T cells expressing the transgenic TCR can be identified by immunostaining with Vβ8-specific antibodies (4). To verify that the ab-

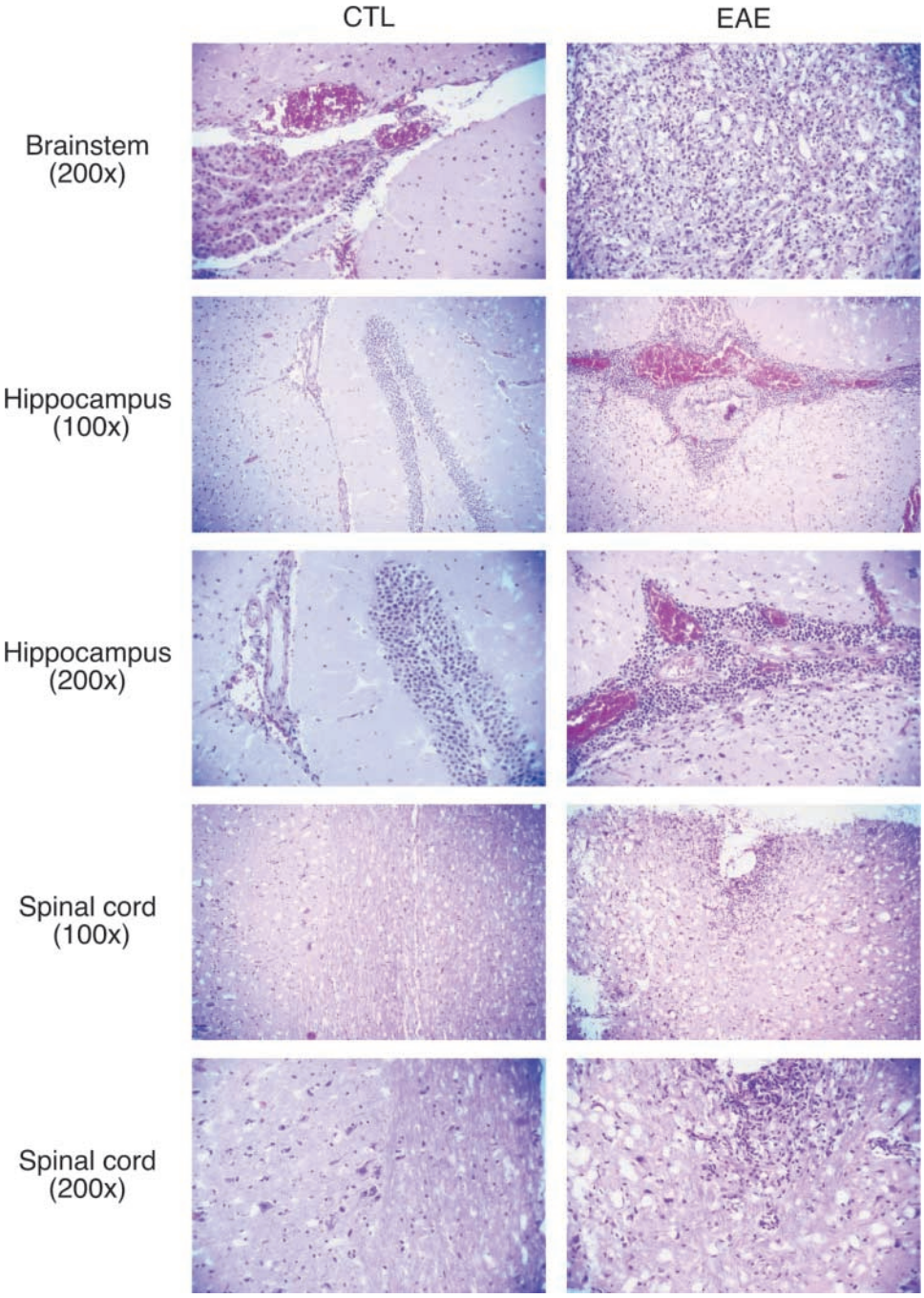

Figure 3. Immunohistological analysis of CNS tissue from asymptomatic and paralyzed STAT1-deficient mice. Brains and spines were removed from asymptomatic or paralyzed (level 5) STAT1-deficient mice and fixed in formaldehyde. Paraffin-embedded sections were stained with hematoxylin and eosin.

sence of STAT1 does not affect the expression of the transgenic TCR, peripheral lymph node T cells derived from either 10–12-wk-old asymptomatic or level 4 paralyzed TCR^+ $STAT1^{-/-}$ $H\text{-}2^{b/u}$ mice, or from their heterozygous littermates, were analyzed for Vβ8 expression. As shown in Fig. 2 A, the fraction of $CD4^+$ $V\beta8^+$ cells (>90%) is virtually identical among the three animal types. Notably, only $V\beta8^+$ cells displayed increased CD69 expression in the paralyzed $STAT1^{-/-}$ mice, whereas no such up-regulation is observed in $CD4^+$ $V\beta8^-$ cells. In accordance with the manifestation that MS/EAE is a Th1 cell–mediated autoimmune disorder, $CD4^+$ T cells from level 4 paralyzed TCR^+ $STAT1^{-/-}$ $H\text{-}2^{b/u}$ mice displayed a significant up-regulation in intracellular IFN-γ production (Fig. 2 B).

Increased Lymphocyte/Macrophage Infiltration in the Central Nervous System (CNS) of STAT1-deficient Paralyzed Mice. To ensure that the observed paralysis was indeed the consequence of an inflammatory response of the CNS, brains and spines were removed from asymptomatic or paralyzed STAT1-deficient TCR^+ $H\text{-}2^{b/u}$ mice and subjected to histopathological analysis after hematoxylin/eosin staining. As shown in Fig. 3, there is a multifocal to diffuse encephalitis composed of an admixture of neutrophils and mononuclear cells visible in the brain stem and hippocampus of the paralyzed animals. In addition, the spinal cord sections of EAE^+ mice display a focal cavitating necropurulent area at the distal aspect of the section. As such, the histological analysis confirms the presence of massive inflammatory processes and typical EAE lesions in the CNS of TCR^+ $STAT1^{-/-}$ $H\text{-}2^{b/u}$ mice that had progressed to level 5 paralysis.

Hyperproliferation of STAT1-deficient $CD4^+$ T Cells In Vitro. Total splenocytes isolated from STAT1-deficient animals have been shown to hyperproliferate in vitro (15). To explore specifically the mitogenic behavior of $CD4^+$ cells, these cells were purified from wild-type and $STAT1^{-/-}$ 129/SvEv mice as outlined in Materials and Methods, and subjected to in vitro proliferation assays in the presence of mitomycin-treated, wild-type APCs and 1 μg/ml Con A. Under these conditions STAT1-deficient cells displayed an approximately fourfold increased prolif-

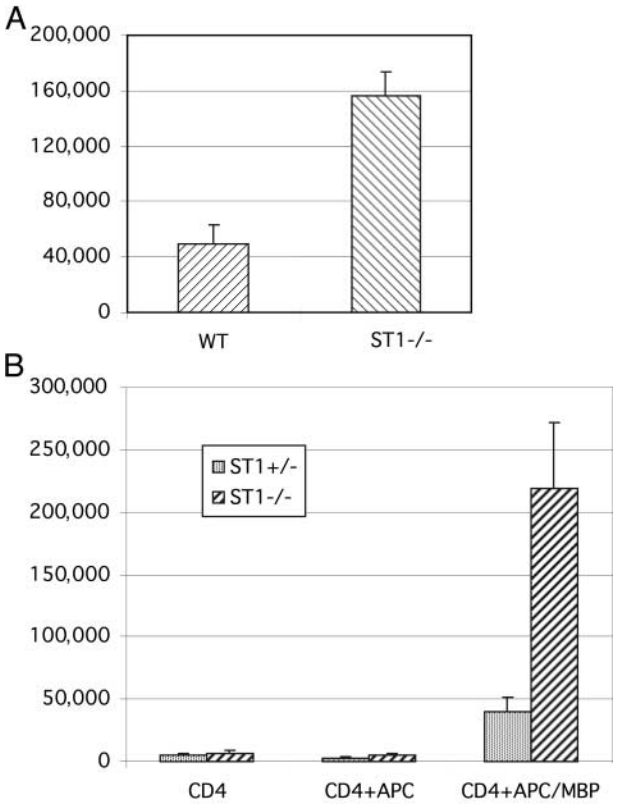

Figure 4. Proliferation of wild-type and STAT1-deficient $CD4^+$ cells. (A) $CD4^+$ cells were purified from the spleens of wild-type or STAT1-deficient mice as described in Materials and Methods, and proliferation was assessed by thymidine incorporation in response to stimulation with 1.0 μg/ml Con A in the presence of mitomycin C–treated APCs. (B) $CD4^+$ cells were purified from the spleens of TCR^+ $STAT1^{-/-}$ $H\text{-}2^{b/u}$ mice and their TCR^+ $STAT1^{+/-}$ $H\text{-}2^{b/u}$ littermates, and proliferation was measured by thymidine incorporation. Mitomycin C–treated APCs were generated from $STAT1^{+/-}$ $H\text{-}2^{b/u}$ animals and preincubated with 100 μg/ml MBP as indicated.

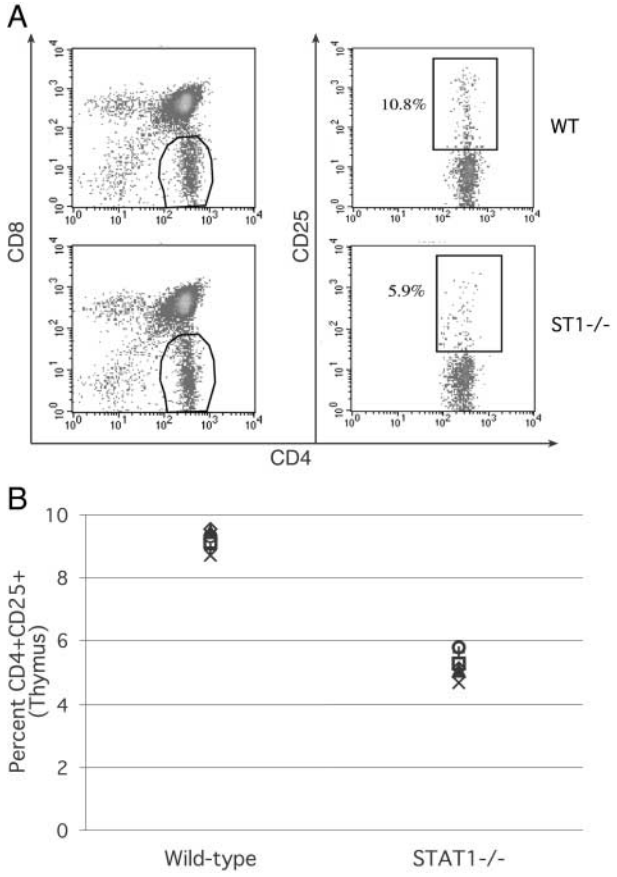

Figure 5. (A and B) Flow cytometric analysis of thymocytes in wild-type and STAT1-deficient mice. Cells derived from thymi of wild-type or STAT1-deficient animals were stained for CD4, CD8, and CD25. The ratio of $CD4^+$ $CD8^-$ $CD25^+$ cells as the percentage of total $CD4^+$ $CD8^-$ cells is shown.

eration rate when compared with their wild-type counterparts (Fig. 4 A).

To confirm this difference in cell growth in T cells expressing the transgenic TCR and in response to the dominant autoantigen of EAE, $CD4^+$ cells were also isolated from TCR^+ $STAT1^{-/-}$ $H\text{-}2^{b/u}$ mice and their TCR^+ $STAT1^{+/-}$ $H\text{-}2^{b/u}$ littermates. Mitomycin C–treated APCs were generated from $STAT1^{+/-}$ $H\text{-}2^{b/u}$ animals and preincubated with 100 μg/ml MBP as indicated. Similar to nonspecific stimulation by Con A, $CD4^+$ T cells derived from TCR^+ $STAT1^{-/-}$ $H\text{-}2^{b/u}$ mice responded with substantially increased proliferation in the presence of APCs presenting MBP antigen, whereas no mitogenesis was observed without APCs or with APCs in the absence of MBP (Fig. 4 B).

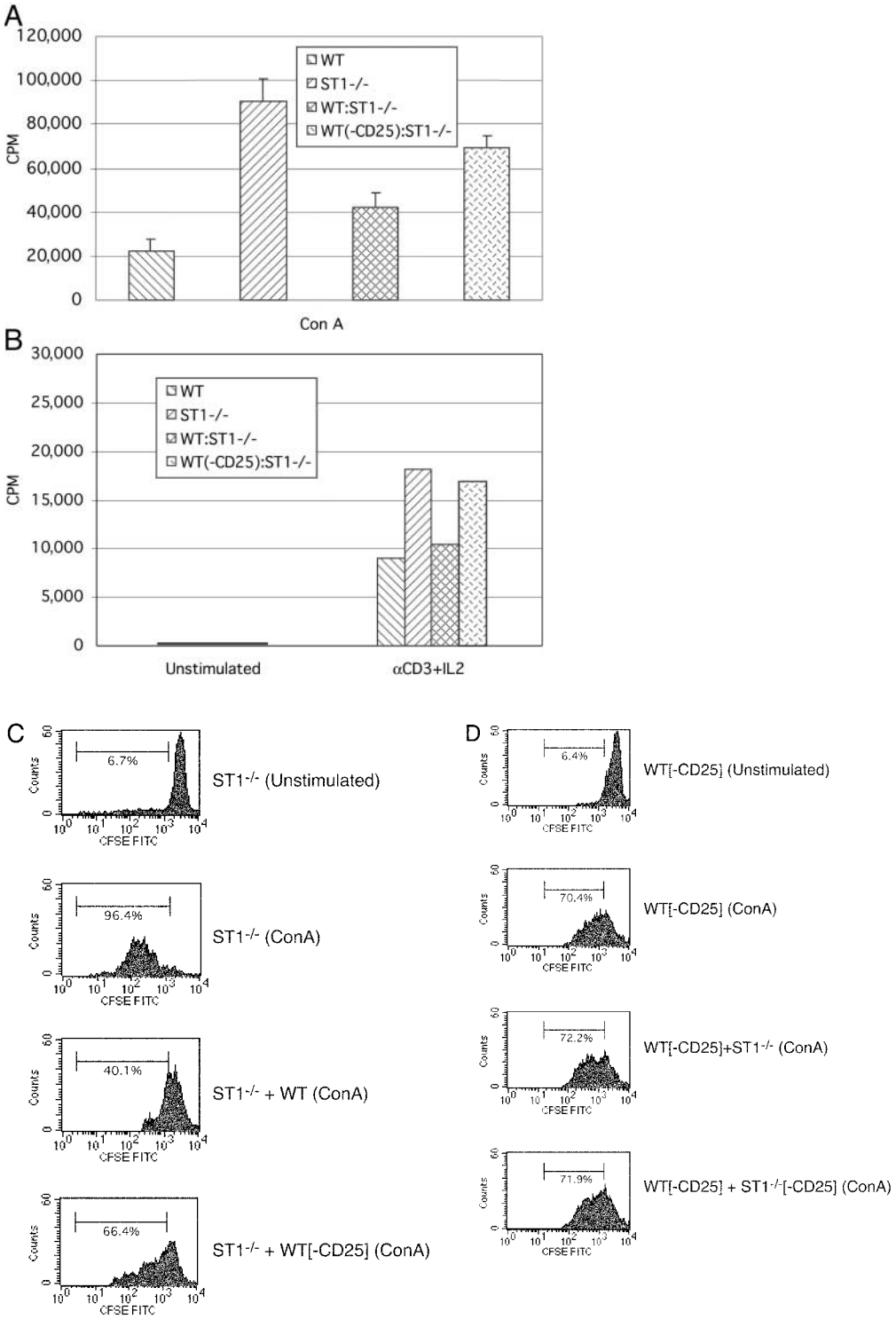

Figure 6. Effect of wild-type $CD4^+$ $CD25^+$ cells on the proliferation of $STAT1^{-/-}$ $CD4^+$ cells. (A) $CD4^+$ cells were purified from the spleens of wild-type or STAT1-deficient mice as described in Materials and Methods. Wild-type or STAT1-deficient $CD4^+$ cells were either incubated separately, mixed in a 1:1 ratio, or wild-type cells were CD25 depleted before mixing 1:1 with STAT1-deficient cells as indicated. Proliferation was then assessed by thymidine incorporation in response to stimulation with 1.0 μg/ml Con A in the presence of mitomycin C–treated APCs. (B) Same as in A, but cells were stimulated with 50 ng/ml anti-CD3 antibody in combination with 50 U/ml human rIL-2. (C) $CD4^+$ cells were purified from the spleens of wild-type or STAT1-deficient mice as described in Materials and Methods. STAT1-deficient cells were labeled with CFSE and their proliferation in response to 1.0 μg/ml Con A in the presence of either unlabeled wild-type $CD4^+$ cells or unlabeled CD25-depleted wild-type $CD4^+$ cells (1:1 ratio) was determined by flow cytometry. (D) $CD4^+$ cells were purified from the spleens of wild-type mice, depleted of $CD25^+$ cells, and labeled with CFSE. Proliferation in response to 1.0 μg/ml Con A in the presence of either unlabeled $STAT1^{-/-}$ $CD4^+$ cells or unlabeled CD25-depleted $STAT1^{-/-}$ $CD4^+$ cells (1:1 ratio) was determined by flow cytometry.

Reduced Number of CD4+ CD25+ Regulatory T Cells in the Absence of STAT1. The dramatically increased frequency of spontaneous EAE development in TCR^{MBP}-transgenic STAT1-deficient animals is reminiscent of the phenotype observed in mice expressing the TCR^{MBP} in the absence of RAG-1 (4). In this model, a deficiency of $CD4^+$ $CD25^+$ regulatory T cells was found to be ultimately responsible for the lack of immunological self-tolerance (9). Therefore, we decided to analyze thymic $CD4^+$ cells for the presence of the $CD25^+$ subpopulation in wild-type and $STAT1^{-/-}$ 129/SvEv mice. In wild-type animals, 9–10% of the $CD4^+$ cells were $CD25^+$, conforming to previously published findings. In contrast, only ~5% of $CD4^+$ cells accounted for $CD25^+$ regulatory T cells in STAT1-deficient animals (Fig. 5). Similar, albeit smaller differences were observed in the peripheral lymphoid organs (not depicted). We further analyzed the $CD4^+$ $CD25^+$ T cells from wild-type and $STAT1^{-/-}$ mice for the expression of additional cell surface markers such as CTLA-4, CD45RB, and CD62L, which are typical for regulatory T cells, but did not observe any significant differences between these animals (not depicted).

Wild-type CD4+ Cells Suppress the Hyperproliferation of STAT1-deficient T Cells. The finding of a reduced $CD4^+$ $CD25^+$ T subpopulation in $STAT1^{-/-}$ mice raised the possibility that the hyperproliferation of STAT1-deficient $CD4^+$ cells is due to a loss of the intrinsic suppressor effect of regulatory T cells. Therefore, we tested whether wild-type $CD4^+$ cells had the ability to restrain the proliferation of their STAT1-deficient counterparts. $CD4^+$ cells were purified from wild-type and $STAT1^{-/-}$ 129/SvEv mice and subjected to thymidine incorporation assays. As found previously, $STAT1^{-/-}$ cells displayed an approximately fourfold increase in the Con A–stimulated mitogenic response when compared with wild-type cells. However, mixing of wild-type and STAT1-deficient $CD4^+$ cells in a 50:50 ratio resulted in a proliferation rate significantly lower than the expected average of the two individual populations, suggesting that wild-type $CD4^+$ cells exert a suppressive effect on their $STAT1^{-/-}$ equivalents (Fig. 6 A). To further investigate whether the wild-type $CD4^+$ cells mediate their suppressive function truly through the activities of $CD4^+$ $CD25^+$ regulatory T cells, we performed analogous mixing experiments, this time with wild-type $CD4^+$ cells depleted of their $CD25^+$ subpopulation. Indeed, removal of $CD25^+$ cells from the wild-type $CD4^+$ cells resulted in significant reduction in their ability to exert antiproliferative effects upon STAT1-deficient cells (Fig. 6 A). This finding is not restricted to Con A stimulation, as a similar pattern of trans-suppression emerged when anti-CD3 antibodies were used for TCR stimulation in the presence of exogenous IL-2 (Fig. 6 B). The trans-suppressive effect required the coculture of the $CD4^+$ cells from both animals, as the transfer of conditioned media from wild-type cells into the cultures of their STAT1-deficient counterparts was unable to reproduce the antiproliferative effect (not depicted).

Wild-type, but Not STAT1-deficient CD4+ CD25+ Cells Suppress T Cell Proliferation. Next, we wanted to ensure that the reduced rate of [^{3}H]thymidine incorporation in the mixed T cell populations was indeed due to a suppressive effect of the wild-type $CD4^+$ $CD25^+$ regulatory T cells on the proliferation of STAT1-deficient cells. To specifically measure the mitogenic response of only one of the T cell populations in the mixture, we used CFSE staining to analyze cell proliferation. $CD4^+$ cells were derived from wild-type and $STAT1^{-/-}$ mice, however, only STAT1-deficient cells were labeled with CFSE such that only proliferation of these cells, but not of added wild-type cells, would be monitored. Stimulation with 1 μg/ml Con A resulted in a massive proliferative response of $STAT1^{-/-}$ $CD4^+$ cells (Fig. 6 C, panels 1 and 2), which was considerably reduced when unlabeled, wild-type $CD4^+$ T cells were present during the incubation (Fig. 6 C, panel 3). Removal of $CD25^+$ cells from the wild-type cells before their addition to labeled, STAT1-deficient cells resulted in a significant, albeit not complete, abrogation of the antiproliferative effect (Fig. 6 C, panel 4).

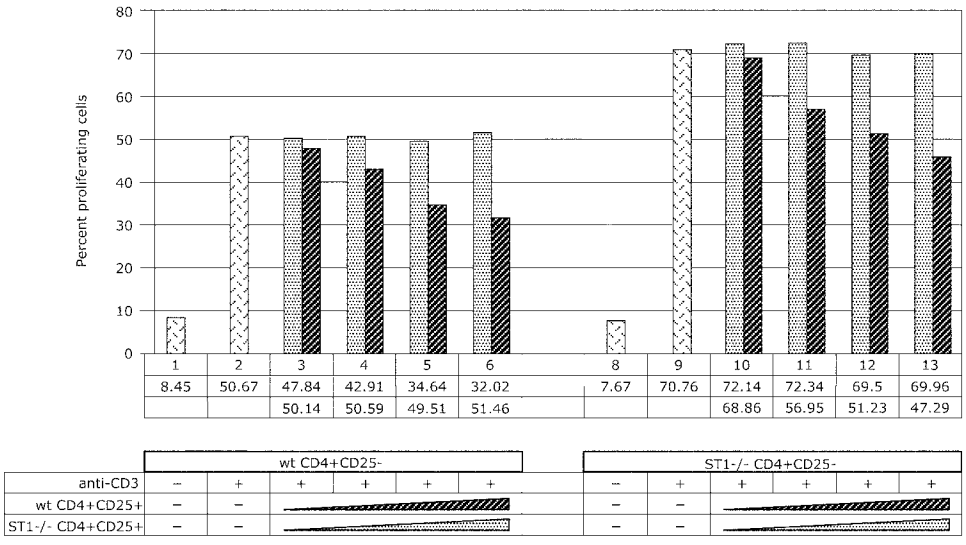

Figure 7. STAT1-deficient $CD4^+$ $CD25^+$ cells fail to suppress T cell proliferation. $CD4^+$ $CD25^+$ and $CD4^+$ $CD25^-$ cells were purified from spleens as described in Materials and Methods. Wild-type or STAT1-deficient $CD4^+$ $CD25^-$ cells were labeled with CFSE and their proliferation in response to 1.0 μg/ml Con A in the absence or presence of either unlabeled wild-type or STAT1-deficient $CD4^+$ $CD25^+$ cells was determined by flow cytometry (ratios of $CD4^+$ $CD25^-/CD4^+$ $CD25^+$ cells were 1:0.1, 1:0.2, 1:0.4, and 1:0.8 in lanes 3–6 and 10–13, respectively). A representative of three independent experiments is shown.

$CD4^+$ $CD25^+$ T cells can still be found in $STAT1^{-/-}$ mice, even though their population is ~50% reduced compared with wild-type animals. Because it seemed unlikely that the lower ratio of $CD4^+$ $CD25^+$ cells alone would account for the substantial differences in cell proliferation, we decided to analyze the suppressive ability of the remaining STAT1-deficient $CD4^+$ $CD25^+$ cells. Resembling the approach outlined above, wild-type $CD4^+$ cells were labeled with CFSE after they had been depleted of their own $CD25^+$ subpopulation. Subsequently, proliferation of these cells was monitored in the absence or presence of unlabeled STAT1-deficient $CD4^+$ cells. Interestingly, the addition of $STAT1^{-/-}$ $CD4^+$ cells was unable to trigger any change in the proliferation rate of their wild-type counterparts (Fig. 6 D, panels 2 and 3). Accordingly, the removal of $CD4^+$ $CD25^+$ cells from the STAT1-deficient population was also without any effect on the mitogenic response of the wild-type $CD4^+$ cells (Fig. 6 D, panel 4).

To further corroborate these results, we also added increasing numbers of purified wild-type or STAT1-deficient $CD4^+$ $CD25^+$ cells to either wild-type or STAT1-deficient $CD4^+$ $CD25^-$ cells that had been labeled with CFSE to analyze their proliferation. As shown in Fig. 7, wild-type $CD4^+$ $CD25^+$ cells were able to suppress the proliferation of their wild-type or STAT1-deficient $CD25^+$ counterparts in a dose-dependent manner (striped bars). In contrast, $STAT1^{-/-}$ $CD4^+$ $CD25^+$ cells failed to exert any growth inhibitory effect on either wild-type or $STAT1^{-/-}$ $CD4^+$ $CD25^-$ cells (dotted bars). These results not only demonstrate that $STAT1^{-/-}$ $CD4^+$ $CD25^+$ cells fail to act as functional regulatory T cells, but also illustrate that $STAT1^{-/-}$ $CD4^+$ $CD25^-$ cells retain their ability to respond to the inhibitory effects of wild-type $CD4^+$ $CD25^+$ cells.

Adoptive Transfer of Wild-type $CD4^+$ $CD25^+$ Cells Prevents EAE Development in $STAT1^{-/-}$ TCR^+ Mice. To determine whether the observed deficiencies in $CD4^+$ $CD25^+$ regulatory T cell function are indeed responsible for the increased susceptibility of STAT1-deficient mice toward the development of EAE, we transferred either total $CD4^+$ cells or $CD4^+$ $CD25^+$ cells derived from TCR^+ $STAT1^{+/-}$ $H\text{-}2^{b/u}$ into their TCR^+ $STAT1^{-/-}$ $H\text{-}2^{b/u}$ littermates. As summarized in Table I, 80% of TCR^+ $STAT1^{+/-}$ $H\text{-}2^{b/u}$ mice developed level 5 paralysis at the age of 17 wk. In contrast, mice that had received wild-type $CD4^+$ cells succumbed to the disease at a substantially reduced (32%) rate. This protective effect was further increased when purified $CD4^+$ $CD25^+$ cells were transferred into the TCR^+ $STAT1^{+/-}$ $H\text{-}2^{b/u}$ mice, illustrating the physiological importance of this cell population in the protection against EAE.

Table I. *Protection of $STAT1^{-/-}$ TCR against EAE after Adoptive Transfer of $CD4^+$ T Cells*

Transferred WT cells	Lethality after 17 wk (%)
No transfer	24/30 (80)
$CD4^+$ (10^6 cells)	2/6 (32)
$CD4^+$ $CD25^+$ (5×10^4 cells)	1/6 (16)

Discussion

The immunomodulatory functions of IFNs have long provoked interest in the role of these cytokines in the development or treatment of numerous autoimmune disorders, particularly of MS (18). The opposing effects that types I and II IFNs elicit in the progression of the disease prompted intense investigation into the underlying mechanism (16). It is generally accepted that MS, as well as the widely used murine model EAE, represent Th1-mediated autoimmune diseases in which $CD4^+$ cells specific for MBP trigger demyelination of the CNS with devastating consequences (1). The immune system in STAT1-deficient mice is skewed toward Th2-type responses (19). Therefore, contrary to our observations, a reduced sensitivity toward Th1-mediated autoimmune diseases would be expected. Intriguingly, 35% of the $CD4^+$ cells in paralyzed TCR^+ $STAT1^{-/-}$ $H\text{-}2^{b/u}$ mice stained positive for intracellular IFN-γ, demonstrating that these animals were able to mount a strong Th1 response despite their inability to respond to IFN-γ.

Previous studies have shown that STAT1-deficient cells lack the expression of several caspase family members (15, 20), thus raising the possibility of an increased susceptibility toward autoimmune disease due to a defect in the negative selection of autoreactive T cells in nontransgenic mice. However, virtually identical numbers of TCR^{MBP}-transgenic T cells were found in the peripheral lymphoid organs of TCR^+ wild-type and STAT1-deficient animals, making the possibility of reduced negative selection as the reason for the increased EAE rate in $STAT1^{-/-}$ mice rather unlikely.

In mice carrying the transgene for the MBP-directed TCR, ~95% of the $CD4^+$ cells actually express the TCR^{MBP}, whereas the remaining cells harbor TCRs of undetermined specificity. This minor population of phenotypically nontransgenic $CD4^+$ T cells is absolutely crucial for maintaining immunological self-tolerance, as evidenced by crosses of the TCR^{MBP}-transgenic mice onto $Rag1^{-/-}$ animals (4). There, 100% of the animals succumb spontaneously to EAE due to the complete absence of regulatory T cells. Further support for the existence of regulatory T cells stems from the observation that reconstitution of immune-deficient mice with $CD4^+$ cells depleted of $CD4^+$ $CD25^+$ cells induces autoimmune disease, whereas complementation with $CD4^+$ $CD25^+$ cells prevents autoimmune syndromes elicited by transfer of $CD4^+$ $CD25^-$ cells (9, 21). Although the existence of such a "suppressor" T cell population was originally somewhat controversial, the presence of protective regulatory $CD4^+$ cells is now well accepted (7, 22–26).

Crosses of TCR^{MBP} mice onto STAT1-null mice results in essentially the same pathological phenotype as the respective cross to RAG-1–deficient animals and is accompanied by a significant reduction in the number of $CD4^+$

$CD25^+$ cells. Further evidence for a disturbed balance of $CD4^+$ $CD25^+$ cells in the absence of STAT1 comes from the finding that wild-type $CD4^+$ $CD25^+$ cells are able to suppress the proliferation of $STAT1^{-/-}$ T cells, whereas conversely, STAT1-deficient $CD4^+$ $CD25^+$ cells fail to exert any effect on $CD4^+$ $CD25^-$ cells derived from either wild-type or $STAT1^{-/-}$ mice. In accordance with previously published observations that the function of regulatory T cells requires cell to cell contact, conditioned media from wild-type T cell cultures was unable to inhibit the excessive proliferative response of STAT1-deficient $CD4^+$ cells. The final proof that diminished $CD4^+$ $CD25^+$ cell function accounts for the increased incidence of EAE in the absence of STAT1 comes from the fact that adoptive transfer of this cell population from wild-type mice into their $STAT1^{-/-}$ littermates protected the host animals against the development of the disease.

Many key aspects of the origin of regulatory T cells remain unsolved. B7 and CD28 are required elements in the generation of $CD4^+$ $CD25^+$ cells (27) as are CD62L (28) and IL-2 (29). IL-12 is also a crucial factor in the development of EAE, as $IL\text{-}12^{-/-}$ mice are completely resistant to the disease (30). The efficacy of type I IFNs in the treatment of MS is likely due to the suppressive effects of IFN-α/β on the IL-12 production by APCs (16). However, initial theories that IL-12 promotes EAE development by inducing IFN-γ are contradicted by the finding that IFN-γ– or IFN-γR–deficient mice still develop EAE, surprisingly with even higher incidence (31, 32). The latter finding prompted the hypothesis that IFN-γ plays an important role in the development and activation of regulatory T cells (33). The presence and functional integrity of regulatory T cells in IFN-γ– or IFN-γR–deficient mice remains to be determined. Intriguingly, a recent study reported that STAT1 and Jak2 expression are significantly increased after TCR stimulation in $CD4^+$ $CD25^+$ regulatory cells, but is not affected in $CD4^+$ $CD25^-$ T cells (34). The results presented in this work support the notion that STAT1 signaling might be required for the development and function of functional regulatory T cells.

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