Tob1 plays a critical role in the activation of encephalitogenic T cells in CNS autoimmunity

Ulf Schulze-Topphoff,1 Simona Casazza,1 Michel Varrin-Doyer,1 Kara Pekarek,1 Raymond A. Sobel,2 Stephen L. Hauser,1 Jorge R. Oksenberg,1 Scott S. Zamvil,1 and Sergio E. Baranzini1

1Department of Neurology, University of California, San Francisco, San Francisco, CA 94143
2Department of Pathology, Stanford University School of Medicine, Stanford, CA 94305

Reliable biomarkers corresponding to disease progression or therapeutic responsiveness in multiple sclerosis (MS) have not been yet identified. We previously reported that low expression of the antiproliferative gene TOB1 in CD4+ T cells of individuals presenting with an initial central nervous system (CNS) demyelinating event (a clinically isolated syndrome), correlated with high risk for progression to MS. We report that experimental autoimmune encephalomyelitis (EAE) in Tob1−/− mice was associated with augmented CNS inflammation, increased infiltrating CD4+ and CD8+ T cell counts, and increased myelin-reactive Th1 and Th17 cells, with reduced numbers of regulatory T cells. Reconstitution of Rag1−/− mice with Tob1−/− CD4+ T cells recapitulated the aggressive EAE phenotype observed in Tob1−/− mice. Furthermore, severe spontaneous EAE was observed when Tob1−/− mice were crossed to myelin oligodendrocyte glycoprotein–specific T cell receptor transgenic (2D2) mice. Collectively, our results reveal a critical role for Tob1 in adaptive T cell immune responses that drive development of EAE, thus providing support for the development of Tob1 as a biomarker for demyelinating disease activity.

© 2013 Schulze-Topphoff et al. This article is distributed under the terms of an Attribution–Noncommercial–Share Alike–No Mirror Sites license for the first six months after the publication date (see http://www.rupress.org/terms). After six months it is available under a Creative Commons License (Attribution–Noncommercial–Share Alike 3.0 Unported license, as described at http://creativecommons.org/licenses/by-nc-sa/3.0/).

The initial event in multiple sclerosis (MS) is commonly an acute neurological attack caused by inflammation in one or more sites in the central nervous system (CNS), a presentation referred to as a clinically isolated syndrome (CIS). Approximately 80% of CIS patients develop clinically definitive MS (CDMS) within 3 yr (half within 2 yr), and only 10% do not advance to MS after 15 yr (Brex et al., 2002; Hauser and Goodin, 2012). We previously identified a gene expression signature in peripheral blood CD4+ T cells of individuals at CIS diagnosis that highly correlates with a rapid evolution to CDMS (Corvol et al., 2008). This signature includes the up-regulation of genes that promote T cell activation, proliferation, and survival, as well as down-regulation of genes that promote apoptosis and cell quiescence. One of the most differentially expressed genes in that signature was TOB1 (transducer of ERBB2-1), showing a sevenfold down-regulation compared with expression in CIS subjects who progressed at a slower pace. Remarkably, 92% of patients with this signature converted into CDMS within 9 mo of CIS diagnosis, whereas only 20% of patients without this gene expression profile converted in the same period of time. TOB1 is a member of the Tob/Btg1 family of antiproliferative (APRO) proteins that regulate cell growth. Tob1 has been shown to modulate the activity of several transcription factors and other molecules involved in cellular differentiation and quiescence (Yoshida et al., 1997), including SMADs, ERKs, and CTNNB, underscoring its potential functional diversity within cell differentiation and proliferation pathways (Yoshida et al., 2003a; Xiong et al., 2006; Tzachanis et al., 2007; Kennedy et al., 2009; Winkler, 2010). Tob1 was found to be highly expressed in anergic

CORRESPONDENCE
Sergio E. Baranzini: sebaran@cgl.ucsf.edu

Abbreviations used: CDMS, clinically definite MS; CIS, clinically isolated syndrome; CNS, central nervous system; EAE, experimental autoimmune encephalomyelitis; MOG, myelin oligodendrocyte glycoprotein; MS, multiple sclerosis; ptx, pertussis toxin.
or quiescent CD4+ lymphocytes and its inhibition augmented CD3-mediated responses, whereas Tob1 overexpression in primary T cells led to cell cycle arrest (Tzachanis et al., 2001). Thus, TOB1 deficiency (or down-regulation), as observed in CIS patients at risk of conversion to CDMS, may contribute to differentiation and proliferation of proinflammatory T cells that, in turn, promote CNS autoimmunity.

RESULTS AND DISCUSSION
We investigated the role of Tob1 in EAE, an animal model which reproduces many of the clinical, immunological, and histopathological aspects of MS (Zamvil and Steinman, 2003), including multifocal infiltration of autoreactive T lymphocytes across the blood–brain barrier, leading to CNS inflammation, demyelination (Raine et al., 1999; Lucchinetti et al., 2001). Tob1 deficiency (or down-regulation), as observed in CIS patients at risk of conversion to CDMS, may contribute to differentiation and proliferation of proinflammatory T cells that, in turn, promote CNS autoimmunity.
whereas the number and proportion of CD4+CD25+Foxp3+
in vivo (unpublished data).

quency of dendritic cells (CD11c+), monocytes (CD11b+), or
CD8+ compared with CD4+ T cells. No difference in the fre-
may be related to a less central role of CD28 costimulation in
anti-CD28–stimulated CD8+ T cells and speculated that this
served less marked down-regulation of T ob1 in anti-CD3/

nized TOB1

in T ob1

with MOG35-55 (Fig. 1 D) or nonselectively after T cell recep-
tor stimulation was detected in response to antigen challenge was detected
in response to antigen in vivo, we studied proliferative responses after re-
stimulated with T ob1

stimulated with T ob1

or indirectly affect de novo T cell immune responses to myelin
antigen in vivo, we studied proliferative responses after re-
plishing the T cell compartment of Rag1−/− mice with CD4+
cells from both T ob1−/− (CD45.2) and WT mice (C57BL/6-CD45.1) in a 1:1 ratio. In response to immunization
with MOG35-55, the frequency of T ob1−/− T cells (CD4+CD45.2+) in
the spleen was higher than the one of WT T cells (CD4+CD45.1+; Fig. 3 B). Together with our previous results,
these data strongly support a role for Tob1 during adaptive
T cell responses after MOG35-55 challenge in vivo. Furthermore,
we reconstituted the T cell compartment of Rag1−/− mice with CD4+
cells from Tob1−/− mice and immunized animals with MOG35-55. As a control, CD4+ T cells from WT mice
were used for reconstitution. After immunization, Rag1−/− reconstituted with Tob1−/− T cells showed an earlier
disease onset and a more severe disease than Rag1−/− replen-
ished with WT T cells, similar to that observed when EAE
was compared in Tob1−/− mice and WT controls (Fig. 3 C). We
also evaluated the proportion of Foxp3+ T cells in naive and
MOG35-55–immunized Rag1−/− mice reconstituted with
either Tob1−/− or WT CD4+ T cells, and detected significantly
fewer T reg cells in the spleen of mice replenished with
Tob1−/− T cells (Fig. 3, D and E). Interestingly, there was a
more considerable reduction in the frequency of T reg cells
in a lymphopenic environment (Fig. 3 D) in comparison
with the slight decrease observed in naive Tob1−/− mice.
Concomitantly, a higher proportion of activated (CD44+) IFN-γ– and IL-17–producing cells was observed in Rag1\(^{-/-}\) mice receiving Tob1\(^{-/-}\) T cells (Fig. 3 F). This suggests that the lower T reg cell population and increased Th1 and Th17 responses are intrinsic properties of T cells as a result of the loss of Tob1 expression. This is also supported by in vitro observations showing increased Th1 and Th17 differentiation (Fig. 3 G) and a reduced capability of naive Tob1\(^{-/-}\) T cells to differentiate into T reg cells (Fig. 3 H). These results confirm that Tob1 deficiency restricted to T cells promotes encephalitogenic...
Figure 3. Tob1 deficiency in T cells modulates T cell responses. (A) Whole spleen cells from immunized Tob1−/− and C57BL/6 mice were depleted of CD3+ cells and used as APC in co-culture with CD4+ T cells from Tob1−/− or WT T cells. [H]-thymidine incorporation in response to MOG35-55 is shown. (B) Rag1−/− mice were reconstituted with identical numbers of CD4+ cells from Tob1−/− (CD45.2) and transgenic WT (C57BL/6-CD45.1) mice. CD4+ T cells were isolated 12 d after immunization with MOG35-55 and quantified by FACS. Horizontal bars indicate mean. (C) Disease course after immunization with MOG35-55 of Rag1−/− mice reconstituted with CD4+ T cells from Tob1−/− or WT mice. Mean EAE disease score ± SEM is shown. (D and E) Proportion of splenic Foxp3-expressing cells (CD25+Foxp3+) by CD4+ cells in Rag1−/− mice either naive (D) or 8 d after immunization (E). (F) Proportion of splenic IFN-γ, IL-17–secreting cells gated on CD4+CD44+ cells 8 d after immunization. (G) Polarization of T cells into a Th1 lineage was induced by IL-12 and polarization into a Th17 lineage was induced by IL-23, IL-6, and TGF-β. (H) Polarization of T cells into T reg cells was achieved with TGF-β. Intracellular cytokine staining for IFN-γ and IL-17 after 3 d in culture, and histograms for Foxp3 expression (gated on CD4+CD25+ cells) after 5 d in culture, are shown. Representative FACS staining profiles are shown including quantification (C, n = 4; D, n = 6; E, n = 4). For all experiments, data shown are representative of at least two independent experiments. *, P < 0.05, Mann-Whitney U test. Error bars indicate SEM.
Figure 4.  Tob1 deficiency in myelin-specific T cells favors development of spontaneous EAE. (A) Naive (CD4+CD44−CD62L+) T cells were isolated from MOG35-55 TCR-transgenic mice (2D2) and 2D2-Tob1−/− mice and cultured in the presence of purified APC and antigen (MOG35-55). Polarization of T cells into a Th1 lineage was induced by IL-12, and polarization into a Th17 lineage was induced by IL-23, IL-6, and TGF-β. Intracellular cytokine staining for IFN-γ and IL-17 after 3 d in culture is shown. (B) Incidence of EAE after administration of ptx in 2D2 (n = 5) and 2D2-Tob1−/− (n = 5) mice. Mean EAE disease score ± SEM is shown. (C) Splenocytes (n = 7) and peripheral blood (n = 14) of age-matched (60–80 d old) symptom-free 2D2 and 2D2-Tob1−/− mice were analyzed for the proportion of Foxp3-expressing (CD25+Foxp3+) CD4+ cells. (D) Similar to C but the proportion of activated (CD44+CD69+) IFN-γ− and IL-17-secreting splenocytes is shown. Error bars indicate SEM. (E and F) The incidence (E) and disease severity (F) of spontaneous EAE in 2D2 (n = 25) and 2D2-Tob1−/− (n = 32) is increased compared with the 2D2 line. (G) Histology of representative spinal cord samples (Luxol fast blue–hematoxylin and eosin). Meningeal and parenchymal lymphoid infiltrates in a 93-d-old 2D2-Tob1−/− mouse (top). A representative spinal cord section from a 165-d-old 2D2 mouse is shown for comparison (bottom). Bar: (left) 200 µm; (right) 50 μm. (H) Lesion quantification of meningeal and parenchymal inflammatory foci. For all experiments, data shown are representative of two independent experiments. Error bars indicate SD. For all experiments, *, P < 0.05; **, P < 0.01; ***, P < 0.001, Mann-Whitney U test.
T cell responses and is sufficient to modulate adaptive T cell immune responses responsible for disease induction.

Activation and expansion of autoreactive myelin-specific Th1 and Th17 cells are known to take place during the initial phase of MS and EAE. To further evaluate whether Tob1 deficiency in myelin-specific T cells affects de novo development of Th1 and Th17 responses, we crossed MOG[35-55] T cell receptor transgenic mice (2D2) with Tob1−/− mice. Naive CD4+ T cells isolated from both 2D2 and double transgenic 2D2*Tob1−/− mice were labeled with CFSE and stimulated with anti-CD3/CD28. CFSE dilution revealed greater proliferation of 2D2*Tob1−/− T cells compared with those from 2D2 mice (unpublished data). More importantly, the number of Th1 and Th17 cells observed after in vitro polarization of naive CD4+ T cells from 2D2*Tob1−/− mice was increased compared with 2D2 controls (Fig. 4 A). Considering the importance of Th1 and Th17 cells in the development of MS and EAE, and given that a previous study showed that EAE in 2D2 mice can be triggered by non–antigen-specific stimulation as pertussis toxin (ptx; Bettelli et al., 2003), we wondered whether 2D2*Tob1−/− mice were more prone to develop EAE under these conditions. Strikingly, after ptx administration alone, 2D2*Tob1−/− mice showed an earlier disease onset compared with 2D2 mice (Fig. 4 B). In addition, disease incidence and severity were increased in 2D2*Tob1−/− mice in comparison to 2D2 controls.

In most EAE models, disease is induced by immunization with autoantigen supplemented with adjuvant and administration of B. ptx. Given the strong effect seen on T cell proliferation, increased Th1 and Th17 responses, and a decreased T reg cell population, we hypothesized that Tob1 deficiency would promote spontaneous EAE in 2D2*Tob1−/− mice. Compared with 2D2, age-matched 2D2*Tob1−/− mice were characterized by a proinflammatory phenotype with a reduced proportion of T reg cells in the spleen and blood (Fig. 4 C), and a higher proportion of activated (CD44+) IFN-γ- and IL-17-producing cells (Fig. 4 D). Although 2D2 mice can develop spontaneous EAE with a low incidence and a mild clinical disease score (Bettelli et al., 2003), these mice did not develop spontaneous disease in our facility. In contrast, a higher rate of spontaneous disease was observed in 2D2*Tob1−/− mice (44%) than 2D2 control mice (0%; Fig. 4, E and F; Table 2). Histological examination of spinal cords revealed inflammatory foci and demyelination in 2D2*Tob1−/− mice, but not in 2D2 controls (Fig. 4, G and H).

Altogether, our results allow us to speculate that interplay between the activation and expansion of autoreactive T cells and a low expression of TOB1 could underlie MS risk. Potentially, either or both of these contributors could be environmentally or genetically determined, or result from a combined influence of the two. In this regard, the latest genome–wide association study in MS revealed common DNA variants in >50 loci associated with disease susceptibility, many of which are involved in T cell activation pathways (International Multiple Sclerosis Genetics Consortium and Wellcome Trust Case Control Consortium 2 et al., 2011).

A recent study showed that dominant active β-catenin (DA-Cat) transgenic mice experience delayed myelination and remyelination upon injury, thus implicating the Wnt pathway in this process for the first time (Fancy et al., 2009). Tob1 has also been shown to interact and antagonize β-catenin in zebrafish embryos, thus controlling dorsal development (Xiong et al., 2006). We speculate that Tob1−/− mice might have derepressed β-catenin expression, thus mimicking the DA-Cat phenotype. If true, Tob1 would then be affecting the two connected, albeit distinct processes (i.e., inflammation and demyelination) underlying EAE and MS. Experiments to evaluate this hypothesis are underway.

In summary, these results demonstrate that Tob1 deficiency in T cells is capable of driving aberrant T cell immune responses favoring the development of autoimmune demyelinating disease. These data provide a biological rationale for the clinical observation that Tob1 expression influences the development of MS, support the development of Tob1-based expression profiling as a biomarker of disease activity in MS, and imply that increasing Tob1 expression on CD4+ cells represents a potential therapeutic strategy for MS and perhaps other autoimmune conditions.

### Table 2. Spontaneous EAE

<table>
<thead>
<tr>
<th></th>
<th>EAE Incidence</th>
<th>Mortality</th>
<th>Maximum clinical score</th>
<th>Day of onset</th>
</tr>
</thead>
<tbody>
<tr>
<td>2D2*Tob1−/−</td>
<td>14/32 (44%)</td>
<td>2/32 (6%)</td>
<td>3.3 ± 0.9</td>
<td>114 ± 33</td>
</tr>
<tr>
<td>2D2</td>
<td>0/25 (0%)</td>
<td>na</td>
<td>na</td>
<td>na</td>
</tr>
</tbody>
</table>

Data are presented as means ± SD.

### MATERIALS AND METHODS

#### Mice.

Mice. Tob1−/− mice on a C57BL/6 background were obtained from the RIKEN Bioresource Center. These mice are derived from a line produced and maintained by T.Yamamoto (University of Tokyo, Tokyo, Japan; Yoshida et al., 2003b). Genotyping was performed as previously described (Ho et al., 2010). C57BL/6 and Rag1−/− female mice, 5–8 wk of age, were purchased from The Jackson Laboratory. MOG[35-55]-specific TCR transgenic mice were provided by V.K. Kuchroo (Harvard, Boston, MA). Tob1−/− and WT (Tob1+/+) C57BL/6 mice were obtained from a heterozygous breeding of Tob1−/− mice and further kept as separate colonies. All animal procedures were performed in compliance with experimental guidelines approved by the University of California, San Francisco committee on animal research (CAR).

#### Peptides.

Mouse MOG[35-55] (MEVGWYRSPFRVHLYRNGK) was synthesized by AnaSpec.

#### EAE Induction.

7–10-wk-old female C57BL/6 and Tob1−/− mice were injected subcutaneously with 75 µg MOG[35-55] in complete Freund’s adjuvant (DIFCO Laboratories). After immunization and 2 d later, mice received 300 ng (C57BL/6) ptx i.p. Active EAE in C57BL/6 Rag1−/− mice: 8 × 10^6
MACS-purified splenic CD4+ cells isolated from C57BL/6 or Tob1-/- mice were injected i.v. into naive C57BL/6 Rag-F-/- mice. After cell transfer C57BL/6 Rag-F-/- mice were immunized as stated above. Suboptimal immunization: 2D2 and 2D2 ‘Tob1-/-, Tob1-/-, and WT mice (purity >96%; Miltenyi Biotec) were stimulated with 20 µg/ml MOG35-55 in the presence of WT-APC (T cell/APC ratio of 1:5). Th differentiation was induced using 3 ng/ml TGF-β, 20 ng/ml IL-23, and 20 ng/ml IL-6 for Th17 lineage or 10 ng/ml IL-12 for Th1 lineage (R&D Systems). 3 d after culture, cytokine production was analyzed using a FACS Canto flow cytometer (BD). T reg cell differentiation was conducted as previously described (Faminti et al., 2007).

Generation of Th1, Th17, and T reg cells. Naïve T cells (CD4+CD62L-CD44+) were obtained by magnetic cell sorting from TCR- knockout mice. CD4+ T cells were purified from Tob1-/- and C57BL/6 mice 14 d after immunization with MOG35-55 were depleted for CD3+ cells (Miltenyi Biotec) and used as APCs for CD4+ T cells. T cells were purified from Tob1-/- and C57BL/6 mice immunized with MOG35-55. Cells were cultured in 96-well microtiter plates at a concentration of 0.25 × 10^6 cells/ml and 20 µg/ml MOG35-55. Culture medium consisted of RPMI 1640 supplemented with 2 mM l-glutamine, 1 mM sodium pyruvate, 100 U/ml penicillin, 0.1 ng/ml streptomycin, 5 × 10^-3 M 2-mercaptoethanol, and 10% (vol/vol) fetal bovine serum. Cells were incubated for 48 h and pulsed for 18 h with 1 µCi/ml [3H]-thymidine before harvesting.

Isolation of CNS infiltrating mononuclear cells. Isolation of CNS infiltrating cells was performed as previously described (Schulze-Topphoff et al., 2009). Briefly, mice were perfused using PBS. CNS tissue was manually cut into small pieces and incubated for 20 min in Hank’s buffered saline solution containing collagenase. Homogenate was resuspended in 30% Percoll (Sigma-Aldrich) and underlayer with 70% Percoll and centrifuged for 30 min. Cells were harvested from the resulting interface.

Histology. Brains and spinal cords of mice were fixed in 10% neutral formalin, embedded in paraffin, and sectioned at 5 µm. Sections were stained with hematoxylin and eosin (H&E). Meningeal and parenchymal inflammatory lesions and areas of demyelination were quantified as previously described (Kuchroo et al., 1995; Stutte et al., 2006).

Flow cytometry. Single-cell suspensions were incubated with anti-CD16/CD32 (1:100) to prevent nonspecific antibody binding and stained with anti-CD4, -CD11c, -CD11b, -B220, and -CD3. Intracellular cytokine staining by CD4+ cells was analyzed by monitoring the expression of IFN-γ, IL-17, and GM-CSF (all 1:100; eBioscience). Foxp3 staining was performed according to the manufacturer’s protocol (eBioscience). For intracellular cytokine staining, T cells were stimulated with 50 ng/ml PMA plus 500 ng/ml ionomycin in the presence of 1 µl/ml GolgiStop (BD).

Statistical analysis. Data are presented as mean ± SEM or SD. We examined significance between groups using the Mann-Whitney U test. A value of P ≤ 0.05 was considered significant.

We thank Dr. S.J. Karp for providing material and protocols for the genotyping of Tob1-/- mice. This work was supported by the National Institutes of Health (NIH) R01 grants NS26799 (J.R. Oksenberg), NS049477 (J.R. Oksenberg), AI073733 (S.S. Zamvil), AI059709 (S.S. Zamvil), and NS063008 (S.S. Zamvil), and the National Multiple Sclerosis Society (NMSS) grants RG36822 and RG3913 (S.S. Zamvil). We also thank the generous contributions of the Robert Tillman Family Fund (S.E. Baranzini), Guthy Jackson Charitable Foundation (S.S. Zamvil), and the Māsion Foundation (S.S. Zamvil). S.E. Baranzini is a Harry Weaver Neuroscience scholar from the NMSS. U. Schulze-Topphoff is a fellow of the NMSS and the Deutsche Forschungsgemeinschaft (DFG; CHU2 587/1-1). M. Varrin-Doyer is a fellow of the NMSS. Authors declare no competing financial interests.

Submitted: 18 July 2012
Accepted: 5 June 2013

REFERENCES


