JLB

Brief Conclusive Report

$CD4^+CD25^+CD127^{dim}Foxp3^+$ T cells are cytotoxic for human neurons

Yohannes Haile, Dion Pasychniyk,* Diane Turner,* R. Chris Bleackley,† and Fabrizio Giuliani*,1*

*Division of Neurology/Department of Medicine and †Department of Biochemistry, University of Alberta, Edmonton, Alberta, Canada

RECEIVED DECEMBER 3, 2010; REVISED FEBRUARY 17, 2011; ACCEPTED FEBRUARY 20, 2011. DOI: 10.1189/jlb.1210654

ABSTRACT

MS lesions are characterized by destruction of myelin and significant neuronal and axonal loss. Preliminary studies with the use of T_{regs} in the mouse model of MS have been extremely encouraging. However, recent studies with human cells have shown the presence of different subpopulations of T cells within the $CD4^+CD25^+Foxp3^+$ T cell phenotype, some of which do not have regulatory functions. These findings suggest a potential difference between mouse and human in the regulatory phenotype. Here, we show that human activated $CD4^+CD25^+Foxp3^+$ T cells are neurotoxic in vitro. These cells expressed high levels of the cytotoxic molecule GrB and had no suppressive effect. On the contrary, they produced IFN-γ and low IL-17, suggesting a shift toward a T_H1 phenotype. Thus, our data confirm the presence of a nonregulatory cytotoxic subpopulation within the human $CD4^+CD25^+Foxp3^+$ T cells and suggest further studies on the human regulatory phenotype prior to any potential therapeutic application. *J. Leukoc. Biol.* 89: 927–934; 2011.

Introduction

Infection and autoimmunity can trigger the activation of the effector arm of the immune system. This activation is characterized by the differentiation of T_H1, T_H2, or T_H17 effector cells. Evidence exists that immune tolerance is maintained or promoted by another subpopulation of T cells, T_{regs} [1], a function that has encouraged the potential therapeutic use of T_{regs} in autoimmune diseases such as MS. Although the CNS is considered to be an immunologically privileged site, it is now known that this immune privilege is far from absolute [2]. Activated T cells transmigrate across the blood brain barrier, enter the CNS compartment, and accumulate as well as proliferate in response to antigen restimulation [3, 4]. Infiltration of these immune cells characterizes lesions of inflammatory diseases of the CNS such as MS. Within MS lesions, there is an almost complete destruction of myelin, which ensheathes neuronal axons. As a consequence, MS has been classically described as a demyelinating disease of the CNS. The recent rediscovery of axonal and neuronal injury in MS [5] has initiated a shift in the way we think about this disease [3]. Therefore, MS is now considered a demyelinating and a neurodegenerative disease. Little is known about the causes and mechanisms that lead to myelin and/or axonal injury in MS [6]. Pathological studies have shown a correlation among demyelination, axonal damage [7], and infiltrating $CD8^+$ T cells and macrophages [8]. Furthermore, in the animal model of MS, EAE, the destabilization of a microtubule network, colocalized with the infiltration of immune cells [9]. The presence of $CD4^+$ and $CD8^+$ T cells in MS and EAE lesions is proposed to mediate demyelination and axonal injury [10]. In an in vitro study, Giuliani et al. [11] reported that human T cells, activated by a soluble anti-CD3 antibody, induce neurotoxicity in allogeneic and syngeneic systems. It has been shown previously that CTLs expressing the serine protease GrB were found frequently in close contact with injured axons within actively demyelinating lesions [12]. Activated T cells release soluble factors such as GrB [13] and proinflammatory cytokines and are thought to mediate axonal and myelin injury [14]. Microarray-based analysis revealed that the expression of GrA and GrB was increased in cerebrospinal fluid of MS patients [15]. However, GrB is best characterized because of its strong proapoptotic activities [13].

In normal conditions, regulatory mechanisms are in place to prevent autoreactive T cells from causing an autoimmune disease. T_{regs} have been shown to be crucial for maintenance of self-antigens and tolerance even in post-transplantation [1]. $CD4^+CD25^+$ T cells are endowed with suppressor functions and characterized by high expression of Foxp3 [16]. In vivo, $CD4^+CD25^+Foxp3$ T_{regs} are described as neuroprotective after brain ischemia [17]. In the experimental allergic encephalomyelitis, it has been shown that T_{regs} may prevent the traffick-

Abbreviations: EAE=experimental autoimmune encephalomyelitis, Foxp3=forkhead box transcription factor, GrB=granzyme B, HFN=human fetal neuron, HPBMC=human PBMC, MAP-2=microtubule-associated protein-2, MS=multiple sclerosis, T_{reg}=regulatory T cell

1. Correspondence: Division of Neurology/Department of Medicine, 9-101 Clinical Sciences Building, University of Alberta, Edmonton, Alberta, T6G 2G3, Canada. E-mail: giuliani@ualberta.ca

ing of effector cells into the CNS [18]. On the other hand, under neurodegenerative conditions in the CNS of two mice strains (C57BL/6J and BALB/c/OLA), $CD4^+CD25^+$ T_{regs} displayed opposite, beneficial and destructive, effects [19]. Indeed, according to other reports, the induction and importance of T_{regs} and their regulatory pathways in human disease are not thoroughly understood. Miyara et al. [20] have shown recently that human $Foxp3^+$ T cells can be dissected in at least three subpopulations, and not all of them have a regulatory phenotype.

In this study, we investigate the potential role of $CD4^+CD25^+Foxp3^+$ T cells in inflammatory-mediated neurodegeneration. In particular, we examine the differential neurotoxicity and expression of GrB by the different T cell subsets $CD4^+$, $CD8^+$, and $CD4^+CD25^+CD127^{dim}Foxp3^+$ T cells. The results described here show that human $CD4^+CD25^+CD127^{dim}Foxp3^+$ T cells, when activated, produce high levels of GrB and IFN-γ and induce neuronal killing in vitro. This study shows for the first time that the $CD4^+CD25^+CD127^{dim}Foxp3^+$ T cell population includes effector T cells able to induce neurotoxicity. These findings warrant further studies about the human T cell phenotype currently attributed to T_{regs}.

MATERIALS AND METHODS

Isolation and culture of HFNs and T cells

Human brain tissue was obtained from legally and medically aborted 15 to 20 week-aged fetuses, according to the guidelines approved by the local institutional ethics committee. HFNs and HPBMCs were isolated and cultured as reported previously [11]. The maturity of HFNs has been described previously [21]. T cell subsets were negatively sorted into $CD4^+$ or $CD8^+$; then, $CD4^+CD25^+$ or $CD4^+CD25^+CD127^{dim}$ subpopulations were positively selected from the whole $CD4^+$ T cells using magnetic cell sorting (MACS separation columns, Miltenyi Biotec, Germany), according to the manufacturer's protocol.

RNA isolation and real-time RT-PCR

Cultured cells were lysed and homogenized in TRIzol (Invitrogen, Carlsbad, CA, USA). Total RNA was collected and purified using an RNeasy mini kit, according to the stated protocol (Qiagen, Valencia, CA, USA). cDNA was synthesized using oligo-d(T) and Superscript II RT (Invitrogen), according to the manufacturer's recommended instructions. Semiquantitative RT-PCR was carried out using Bio-Rad iQ SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA, USA) on the iQ5 program or i-Cycler (Bio-Rad Laboratories), according to the manufacturer's protocol. Primers for GrB (forward: GCG GTG GCT TCC TGA TAC AAG, reverse: CCC CCA AGG TGA CAT TTA TGG) and Foxp3 (forward: CAA GTT CCA CAA CAT GCG AC, reverse: ATT GAG TGT CCG CTG CTT CT) amplifications were used at an annealing temperature of 60°C.

Immunocytochemistry

HFNs and T cells (1:1) were cocultured for 24 h and then fixed with 4% PFA for ~20 min. The viability of neurons was assessed using mouse primary mAb against human MAP-2 as reported previously [11].

Flow cytometry

The intracellular protein levels of GrB, Foxp3, and IL-17 were assessed using flow cytometry. Activated or unactivated $CD4^+CD25^+$ or $CD4^+CD25^+CD127^{dim/-}$ ($CD4^+CD25^+Foxp3^+$) T cells were incubated with 1 μg/ml ionomycin and 10 ng/ml PMA (both Sigma-Aldrich, Oakville, ON, Canada) at 37°C and 5% CO_2 for 2 h. A mixture of transport inhibitors [3 μg/ml Brefeldin A (Sigma-Aldrich) and 2 μM Monensin solution (eBioscience, San Diego, CA, USA)] was added into each well, and the cell culture was incubated further for 2 h. Cells were harvested and went through several washing steps with PBS, followed by incubation in freshly prepared eBioscience Foxp3 fixation/permeabilization buffer for 30 min at 4°C in dark. After 2× washing in permeabilzation buffer, cells were incubated with intracellular staining for GrB, Foxp3, and IL-17 (all eBioscience) for 45 min–2 h at 4°C. Stained cells were washed 2× in permeabilization buffer, resuspended in PBS + 2% FBS, and analyzed on a FACSCanto flow cytometer using FACSDiva software for acquisition (BD Biosciences, San Diego, CA, USA). Multicolors were compensated using Compbeads (BD Biosciences). Cytometry data were analyzed using FlowJo 7.2.2 software (Tree Star, Ashland, OR, USA).

Suppression assay

To examine the suppressive function, 50,000 $CD4^+CD25^+CD127^{dim/-}$ T cells were cocultured for 24 h with 50,000 $CD4^+CD25^-CD127^{dim/-}$ T cells. AIM-V media (25–30 μl), containing 1 μCu ^{3}H-thymidine, were added into each well. After incubating for 18 h at 37°C, the cells were harvested, and the radioactivity was measured. Finally, the stimulation index of the wells was calculated, and then, the CPM of proliferating cells were analyzed.

Cytokine-ELISA

The phenotype of $CD4^+CD25^+Foxp3^+$ T cells was evaluated using human IFN-γ-ELISA (Invitrogen), according to the manufacturer's instruction.

Statistical analysis

Results were statistically analyzed as mean ± SD using GraphPad Prism 5. The groups were compared using one-factor ANOVA followed by Tukey post hoc test for normally distributed data. Two-tailed unpaired *t* test was applied to compare two groups with normally distributed data, and data were presented as mean ± SD. *P* values of <0.05 were considered significant. Asterisks correspond to $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$.

RESULTS AND DISCUSSION

Activated T cell subsets show different levels of neurotoxicity

We have reported previously that both subpopulations of $CD4^+$ and $CD8^+$ T cells display a neurotoxic effect in allogeneic and syngeneic systems [11]. In the present study, we have further evaluated and compared the differential neurotoxic effect and GrB expression of different subsets of human T cells such as $CD4^+$, $CD8^+$, and $CD4^+CD25^+$ T cells. A neuronal killing assay was performed using HFNs cocultured with T cells. T lymphocytes were plated on anti-CD3/anti-CD28-immobilized or control uncoated plates for 3 days before adding them to HFNs. After 72 h in culture, neurons in the control group were treated with only AIM-V medium, without exposure to T cells. Conversely, in the experimental groups, unactivated, activated HPBMCs, and activated $CD4^+$ or $CD8^+$ T cells were added to neurons in a 1:1 ratio and kept for 24 h. The neurotoxic effect of T cells was measured by immunocytochemistry using anti-MAP-2 antibody. MAP-2 was chosen as a marker of neuronal viability, as the disappearance of its immunoreactivity has been associated with neuronal damage and death [11, 22]. As indicated in **Fig. 1A**, a–c, the density of neurons, untreated or treated with unactivated T cells, remained unchanged. In contrast, the density of neurons in the cul-

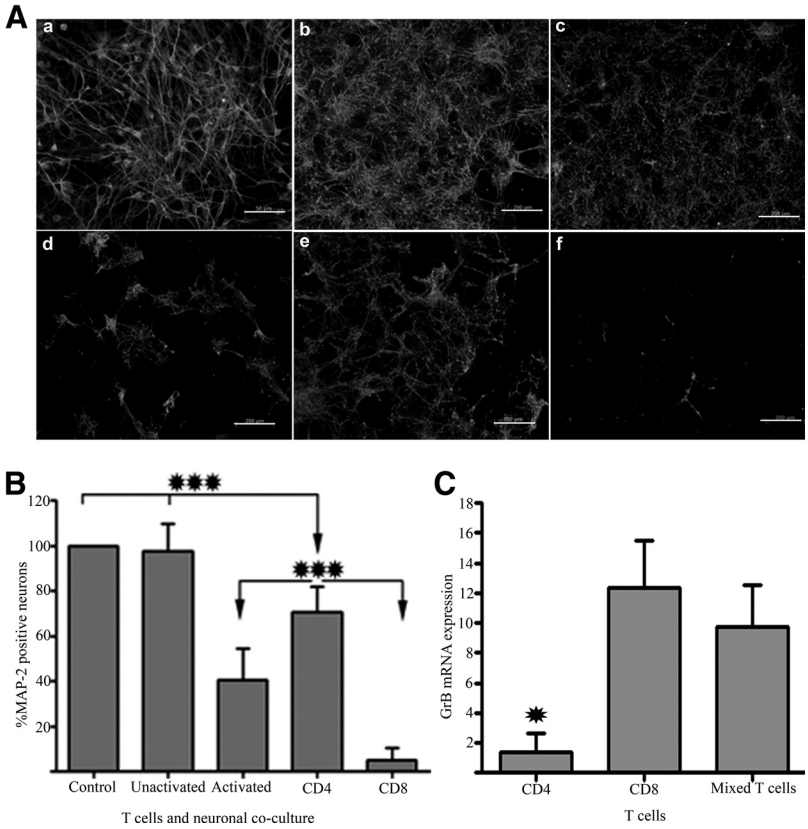

Figure 1. Neuronal killing assay by T cells activated after sorting. Whole human T lymphocytes or subsets sorted using magnetic beads into CD4$^+$ or CD8$^+$ T cells were activated using anti-CD3/anti-CD28 antibody for 3 days. HFNs were cocultured with (A, a) only AIM-V media as a control (×40), (A, b) lower magnification of A, a (×10), (A, c) unactivated T cells, (A, d) activated mixed population of T cells, (A, e) activated CD4$^+$ T cells, and (A, f) activated CD8$^+$ T cells. The culture was immunocytochemically stained with the neuronal marker anti-MAP-2. Data are representative of at least three individual experiments. (B) Manual quantification of MAP-2-positive neurons, demonstrating the differential neurotoxic effects of a mixed T cell population, CD4$^+$, or CD8$^+$ T cells. The most severe neurotoxicity was documented in the group treated with CD8$^+$ T cells ($P<0.001$). Data are representative of three individual experiments. (C) Total RNA was extracted from the mixed T lymphocytes, CD4$^+$, or CD8$^+$ T cells to measure GrB mRNA expression. RT-PCR revealed that CD4$^+$ T cells expressed significantly less GrB transcript than the rest of the groups ($P<0.05$). Data are representative of at least two individual experiments. Note that the expression of GrB in the unactivated group was negligible so that the scale and comparison were based on CD4$^+$ T cells [original scale bars: (A, a) 50 μm; (A, b–f) 200 μm].

ture treated with activated T cells was decreased significantly (37.4±14.5% vs. 100% in the control group). Despite significant neuronal loss, the group treated with CD4$^+$ T cells showed a significant amount of surviving neurons (69.6±11.9%). However, CD8$^+$ T cells induced severe neuronal injury, resulting in only a few surviving neurons (7.3±5.9%; $P<0.001$; Fig. 1A and B). In contrast with our previous findings [11], where CD4$^+$ and CD8$^+$ T cells induced comparable neuronal loss, these results show that the majority of the cytotoxic effect was induced by CD8$^+$ T cells. This discrepancy can be explained by the different activation system that we used in this study (immobilized anti-CD3 vs. soluble OKT3 in the previous work).

It is known that effector T cells express GrB upon activation in the presence of costimulatory molecules or cytokines or during pathogenic infections. It has been shown previously that GrB can mediate neuronal apoptosis [13]. In a rat model of cerebral ischemia, GrB mediated post-ischemic neuronal death [23]. Conversely, the absence of GrB has been associated with decreased neuronal injury [24]. We measured the expression of GrB in different subsets of T cells. Subpopulations of T cells (CD4$^+$ or CD8$^+$) were activated, and the total RNA was extracted. Although all of the cells expressed GrB after activation, RT-PCR revealed that CD4$^+$ T cells displayed very low levels of GrB (1.35±1.31) compared with CD8$^+$ and activated HPBMCs, which showed significantly higher levels of GrB (12.38±3.07 and 9.70±2.83, respectively; Fig. 1C; $P<0.001$). The expression of GrB in the unactivated T cells was negligible so that the scale and comparison were based on CD4$^+$ T cells. The levels of GrB expression correlated directly with the neurotoxic effect of CD4$^+$, CD8$^+$, and the activated whole T cell population (Fig. 1A and B). Neuronal killing by GrB containing conditioned supernatant from CD4$^+$ and CD8$^+$ T cells has been reported previously [13]. However, this supernatant-mediated neuronal killing was very mild (20%) compared with the T cell-mediated killing of >60% in the present study. Using a transwell system, where neurons and T cells were cocultured in two separate compartments, divided by a porous membrane allowing only the exchange of soluble factors, we have reported previously that no neurotoxicity occurred [11]. These findings underline the importance of cell–cell contact for effective neurotoxicity.

CD4$^+$CD25$^+$ T cells induce severe neurotoxicity

CD4$^+$CD25$^+$ T cells are reported frequently for their role in modulating a diverse array of immunological responses and maintenance of peripheral tolerances by acting on the effector cells. They are characterized by high expression of Foxp3 [16]. Thus, we hypothesized that these cells would suppress the severe neurotoxicity induced by CD8$^+$ T cells. To validate this hypothesis, CD4$^+$ and CD8$^+$ T cells were negatively selected through magnetic separation from HPBMCs and activated for 72 h using an anti-CD3 antibody. CD25$^+$ T cells were positively isolated from CD4$^+$ T cells after activation. A mixture of CD4$^+$CD25$^+$ and CD8$^+$ T cells (1:1 ratio) was added to HFN cultures. Cocultures of CD8$^+$ or CD4$^+$CD25$^+$ T cells with HFNs were used as controls. Immunocytochemical analysis using anti-MAP-2 antibody revealed that CD4$^+$CD25$^+$ T cells were not able to suppress the cytotoxic effect induced by

$CD8^+$ T cells; instead, they induced a neuronal killing comparable with $CD8^+$ T cells (data not shown). The failure of $CD4^+CD25^+$ T cells in preventing neuronal killing can be explained in two ways: first, when $CD4^+CD25^+$ and $CD8^+$ T cells were mixed at the same time of the coculture with neurons, there was not enough time to induce a suppressive response to prevent neuronal killing; second, CD25 is expressed by activated T cells [25], thus activation of $CD4^+CD25^+$ T cells could enhance proliferation of non-T_{regs} [26]. Therefore, to minimize these confounding effects, $CD25^+$ T cells were positively isolated from $CD4^+$ T cells before activation. $CD4^+CD25^+$ T cells were activated alone or in a mixture (1:1), with the negatively selected $CD8^+$ T cells for 72 h and cocultured with HFNs, as stated above. Following 24 h coculture, neurons were stained with MAP-2 for viability assessment. Surprisingly, almost 90% of neurons were killed in the control group treated with $CD4^+CD25^+$ T cells only. The count of MAP-2-positive viable neurons resulted in 10.5 ± 4.8% in $CD4^+CD25^+$ and 11.9 ± 7.2% in $CD8^+$ T cell-treated groups, respectively. Furthermore, the percent of surviving neurons in the coculture with the combination of $CD4^+CD25^+$ plus $CD8^+$ T cells was only 1.6 ± 1.3% (**Fig. 2A**; $P<0.001$). The results indicate that prior incubation of these two subsets of T cells is not able to inhibit the severe $CD8^+$-mediated neurotoxicity. In addition, our findings clearly show that $CD4^+CD25^+$ T cells are also neurotoxic after activation.

Foxp3 has been identified as a specific and most up-to-date marker of T_{regs} [27] and is required for their development [28]. To characterize further whether $CD4^+CD25^+$ T cells acquire a regulatory phenotype, a RT-PCR experiment was performed using a Foxp3 primer. In this case, two sets of cells, $CD25^+$ and $CD25^-$ depleted, were selected from $CD4^+$ T cells and activated for 72 h. Following RNA extraction, RT-PCR results demonstrated that Foxp3 expression was 7.45-fold increased in activated $CD4^+CD25^+$ compared with CD25-depleted $CD4^+$ T cells (Fig. 2B; $P<0.001$). This was in line with earlier findings, where $CD4^+CD25^+$ T cells expressed high levels of Foxp3 compared with $CD4^+CD25^-$ [29]. It has been shown that T_{regs} use GrB to kill target effector cells [30]. In our study, we assessed GrB expression by $CD25^+$ T cell subsets. Interestingly, RT-PCR revealed that activated $CD4^+CD25^+$ T cells expressed ~66-fold more GrB than activated $CD4^+CD25^-$ T cells and above 96-fold change more than the unactivated T cells (Fig. 2C; $P<0.001$). GrB expression in these cells was extremely high, and this may account for the extreme neurotoxic effect observed in our experiments.

$CD4^+CD25^+$ T cells express GrB and Foxp3 but low IL-17

As described above, RT-PCR revealed that $CD4^+CD25^+$ T cells express Foxp3 and GrB at a transcript level (Fig. 2B and C, respectively). To confirm whether these high expressions translated into high-protein production, cells underwent intracellular staining for Foxp3 and GrB. Flow cytometric analysis indicated that only 9% of unactivated $CD4^+CD25^+$ T cells expressed GrB, whereas 4.51% were positive for GrB and Foxp3 (**Fig. 3A**, middle panel). However, after activation, ~76% of $CD4^+CD25^+$ T cells expressed GrB. In addition, 38.84% of activated $CD4^+CD25^+$ T cells expressed GrB and Foxp3, and ~47% were positive for Foxp3. On the other hand, >50% of activated $CD4^+CD25^+$ T cells were negative for Foxp3 expression (Fig. 3A, right panel). As the neurotoxic effect could have been related to the Foxp3-negative fraction, we further purified $Foxp3^+$ cells. The absence of a surface marker for Foxp3

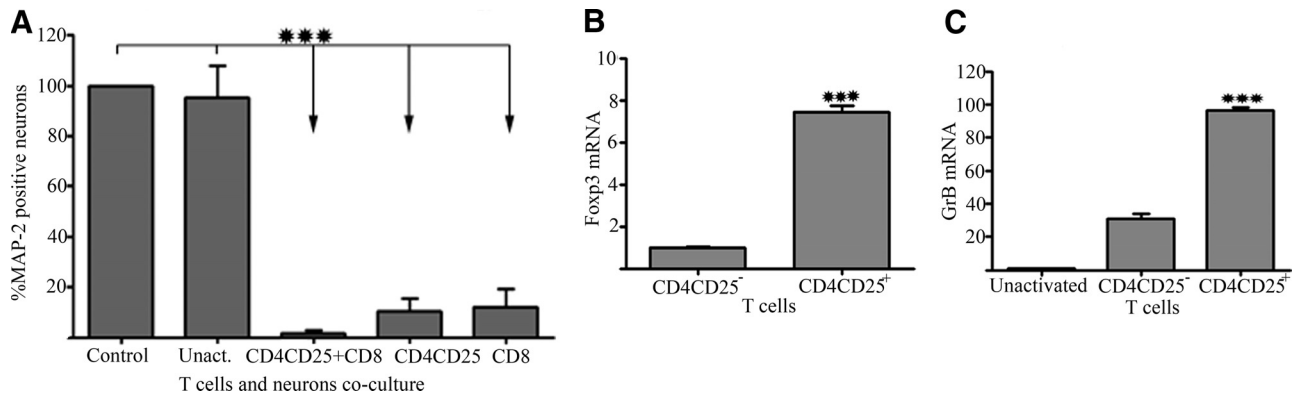

Figure 2. Neuronal killing assay by postselection-activated $CD4^+CD25^+$ T cells. Human T lymphocytes were positively sorted into $CD4^+CD25^+$ subsets of T cells using magnetic-conjugated antibody and activated for 3 days by anti-CD3/anti-CD28 antibody prior to coculture with neurons. (A) HFNs were treated with the coculture media as a control (left-most bar) or cocultured with unactivated (Unact.), activated in a mixture of $CD4^+CD25^+$ and $CD8^+$ T cells (1:1), postselection-activated $CD4^+CD25^+$ T cells, or $CD8^+$ T cells (only 50%). After 24 h in culture, cells were immunocytochemically stained using MAP-2 and manually quantified. As demonstrated in the graph, $CD4^+CD25^+$ T cells failed to reverse the $CD8^+$ T cell-induced neurotoxicity ($P<0.001$). Data are representative of three individual experiments. (B) Expression of the transcription factor Foxp3 by $CD4^+CD25^+$ T cells. Total RNA was extracted from each group (activated $CD4^+CD25^+$ or activated $CD4^+CD25^-$) to measure the Foxp3 mRNA expression. RT-PCR revealed that $CD4^+CD25^+$ T cells expressed above sevenfold more Foxp3 in comparison with $CD4^+CD25^-$ T cells ($P<0.001$). Data are representative of three individual experiments. (C) GrB expression was measured, and activated $CD4^+CD25^+$ T cells expressed more than 96- and 60-fold increases compared with unactivated whole T cell populations and activated $CD4^+CD25^-$ T cells, respectively ($P<0.001$). Data are representative of at least two individual experiments.

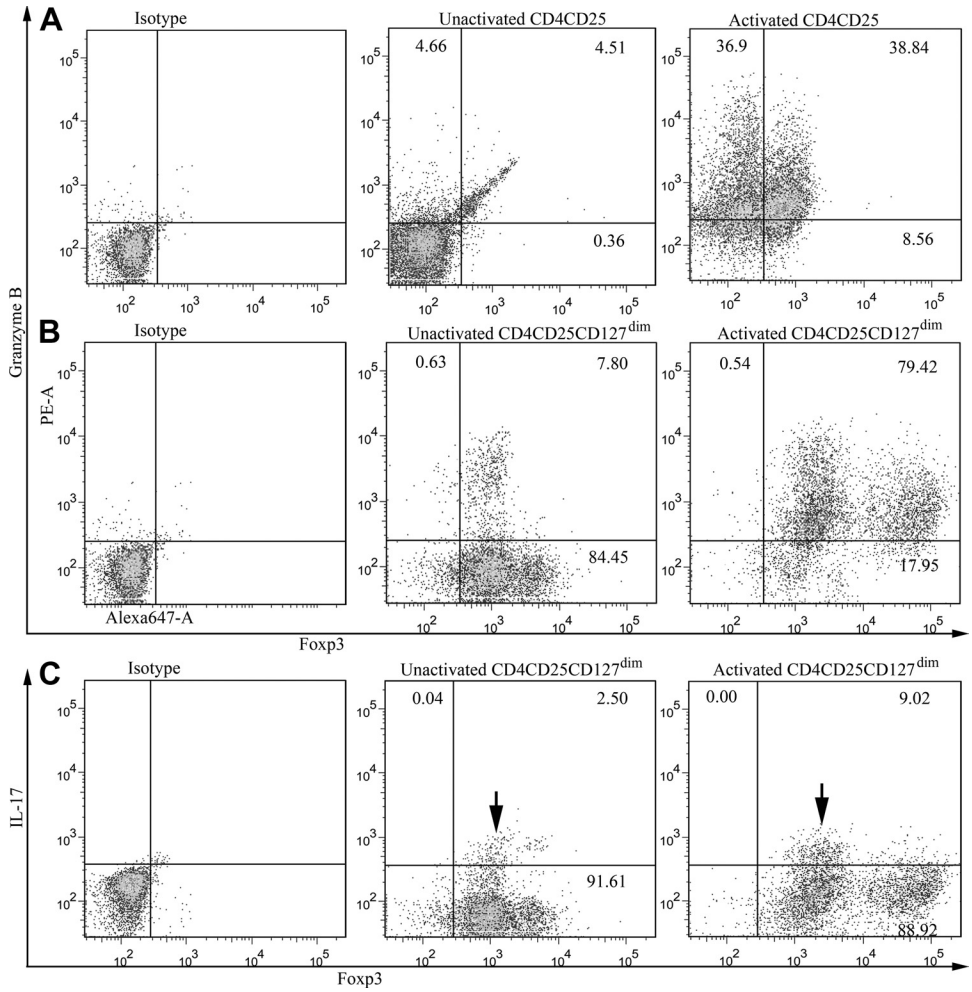

Figure 3. Showing the expressions of intracellular GrB, Foxp3, and IL-17. (A) Flow cytometry data showing CD4$^+$CD25$^+$ T cells expressing GrB and Foxp3. Only 4.51% of unactivated CD4$^+$CD25$^+$ T cells were positive for GrB and Foxp3 (middle panel), whereas the expression of GrB and Foxp3 was increased significantly in the activated CD4$^+$CD25$^+$ T cells, but still more than half of the cells were negative for Foxp3 (right panel, Activated CD4CD25). (B) Foxp3$^+$ T cells were enriched further by depletion of CD127^{high} T cells so that close to 97% of the cells were Foxp3-positive (right panel). However, the Foxp3 expression is bimodal. In activated CD4$^+$CD25$^+$CD127^{dim} T cells, 79.42% of the cells were positive for GrB and Foxp3. (C) Micrograph showing expression of IL-17 and Foxp3 by CD4$^+$CD25$^+$CD127^{dim} T cells. Among activated CD4$^+$CD25$^+$CD127^{dim} T cells, ~98% expressed Foxp3, whereas only 9.02% were positive for the intracellular IL-17 staining (right panel). In unactivated and activated CD4$^+$CD25$^+$CD127^{dim} T cells, the majority of IL-17-expressing cells was Foxp3^{low} (arrows). Data are representative of three individual experiments.

limits the isolation of pure Foxp3$^+$ T cells. It has been shown previously that CD127 (surface marker) is down-regulated in human T_{regs} and inversely correlated to Foxp3 expression [31]. Consequently, the CD127^{high} cells were eliminated from the CD4$^+$CD25$^+$ T cell population using magnetic separation. Flow cytometry showed that 92% unactivated and 98% activated CD4$^+$CD25$^+$CD127^{dim} T cells were Foxp3$^+$, and 79.42% were positive for GrB and Foxp3 (Fig. 3B). By selecting CD4$^+$CD25$^+$CD127^{dim}, the purity of Foxp3$^+$ T cells reached 98% (Fig. 3B and C, right panels). However, the distribution of Foxp3-expressing cells was bimodal. This dual expression of Foxp3 has been described recently by Miyara et al. [20], who showed that three subpopulations of cells were present within the Foxp3$^+$CD127^{dim} population. Nevertheless, ~80% of both subpopulations (Foxp3 low or high) of activated CD4$^+$CD25$^+$CD127^{dim} T cells expressed a high level of GrB (Fig. 3B, right panel).

Plasticity is a characteristic of CD4$^+$ T cell subsets [32], and there is increasing evidence that certain subsets of T cells, defined by their function and transcription factors, are not stable. It has been shown previously that T cells with a T_{reg} phenotype can shift to a T_H1, T_H2 [32], and T_H17 phenotype [33]. Furthermore, we have reported previously that T_H17 cells express high levels of GrB and induce neuronal killing [34]. In addition, T_H17 and T_{regs} share a common lineage so that they may demonstrate plasticity toward each other in the presence of IL-6 or IL-10, respectively. Therefore, an intracellular staining for IL-17, a cytokine expressed by T_H17 T cells, was performed on unactivated or activated CD4$^+$CD25$^+$CD127^{dim} T cells. Out of 98% activated CD4$^+$CD25$^+$Foxp3$^+$ T cells, 9.02% expressed IL-17. Interestingly, IL-17-expressing cells were mainly from the Foxp3^{low} subpopulations (Fig. 3C, arrows). CD4$^+$CD25$^+$CD127$^{dim/-}$ T cells are enriched for Foxp3, so we will refer to them as CD4$^+$CD25$^+$Foxp3$^+$.

Purified CD4$^+$CD25$^+$Foxp3$^+$ T cells are neurotoxic

To exclude the possibility that the neurotoxic effect induced by CD4$^+$CD25$^+$ T cells could be caused by the Foxp3-negative fraction of the cells shown in Fig. 3A, right panel, a neuronal killing assay was performed using purified CD4$^+$CD25$^+$Foxp3$^+$ T cells displayed in Fig. 3B and C. HFNs were treated with AIM-V serum-free media (untreated control), cocultured with unactivated or activated CD4$^+$CD25$^+$Foxp3$^+$ T cells. After 24 h in culture, immunocytochemical staining for MAP-2 revealed that neurons treated only with media or unactivated T cells remained healthy and dense (**Fig. 4A**, a and b, respectively). In contrast, the group treated with activated CD4$^+$CD25$^+$Foxp3$^+$ T cells showed

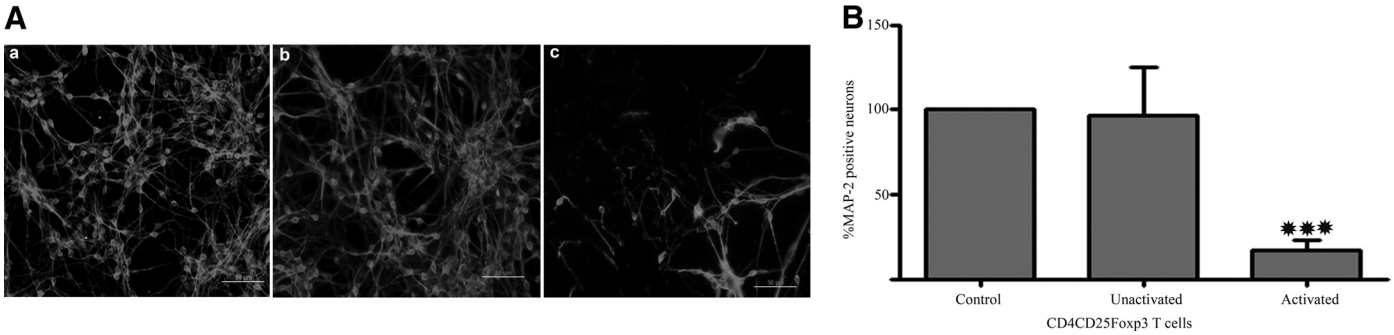

Figure 4. Micrographs showing that CD4$^+$CD25$^+$ Foxp3$^+$ T cells induced neuronal killing. After 3 days activation, CD4$^+$CD25$^+$Foxp3$^+$ T cells were cocultured with HFNs for 24 h, followed by immunocytochemical staining with MAP-2. HFNs were treated with: (A, a) AIM-V media (control), (A, b) unactivated, or (A, c) activated CD4$^+$CD25$^+$Foxp3$^+$ T cells. (B) MAP-2-stained neurons were enumerated, and the viability of neurons in A, c (treated with activated CD4$^+$CD25$^+$Foxp3$^+$ T cells) was significantly affected ($P<0.001$). Data are representative of three individual experiments (original scale bars: 50 μm).

severe neuronal loss (Fig. 4A, c). Quantification of the above qualitative observation confirmed that only 16.6 ± 6.5% MAP-2-positive, viable neurons were present in the group treated with activated CD4$^+$CD25$^+$Foxp3$^+$ T cells (Fig. 4B; $P<0.001$). The loss of >80% of neurons clearly showed that these cells were significantly neurotoxic, although they expressed a T_{reg} profile.

As indicated in Fig. 3B, the high level of GrB expression was reproduced in the CD4$^+$CD25$^+$Foxp3$^+$ T cell population. The GrB released by activated T cells has been shown to induce cytotoxicity on human neurons [35]. GrB and perforin are the most crucial effectors of T_{reg}-mediated cytolysis. In a GrB-deficient mouse model, the function of T_{regs} was reduced compared with NK and CD8$^+$ T cells [36]. Up-regulation of GrB expression in murine CD4$^+$CD25$^+$ T_{regs} correlated with induction of regulatory activity [37]. This reflects the importance of GrB for T_{reg} cytolysis activity. Given that GrB induces neurotoxicity [13], and T_{regs} use GrB to suppress their target cells [36] using the granular pathway [38] of cytotoxicity, we can speculate that in this study, GrB plays a role in the CD4$^+$CD25$^+$Foxp3$^+$ T cell-mediated killing of human cortical neurons. This possibility is supported by the finding that the CD4$^+$CD25$^+$Foxp3$^+$ T cells showed above 96-fold increases of GrB expression. In addition, ~80% of the cells were positive for GrB and Foxp3.

Activated CD4$^+$CD25$^+$Foxp3$^+$ T cells lack suppressive functions and express IFN-γ

T_{regs} maintain tolerance by suppressing the immune reaction of effector cells. The suppressive action of T_{regs} occurs by different mechanisms, such as inhibitory cytokines (e.g., IL-10, IL-35, and TGF-β), cytolysis, metabolic disruption, and modulation of DC maturation or function [39]. To address whether CD4$^+$CD25$^+$Foxp3$^+$ T cells acquire a suppressive function, CD4$^+$CD25$^+$CD127^{dim} were cocultured with CD4$^+$CD25$^-$CD127^{dim} in a 1:1 ratio. Control groups were CD4$^+$CD25$^+$CD127^{dim} or CD4$^+$CD25$^-$CD127^{dim} alone. After 18 h incubation, quantification of ^{3}H-thymidine-incorporating cells showed that CD4$^+$CD25$^+$CD127^{dim} cells did not increase in number, and the effector cells, CD4$^+$CD25$^-$CD127^{dim}, underwent significantly increased proliferation. More importantly, the proliferation of the effector cells was not inhibited by CD4$^+$CD25$^+$CD127^{dim} T cells (**Fig. 5A**; $P<0.001$), which demonstrates that acti-

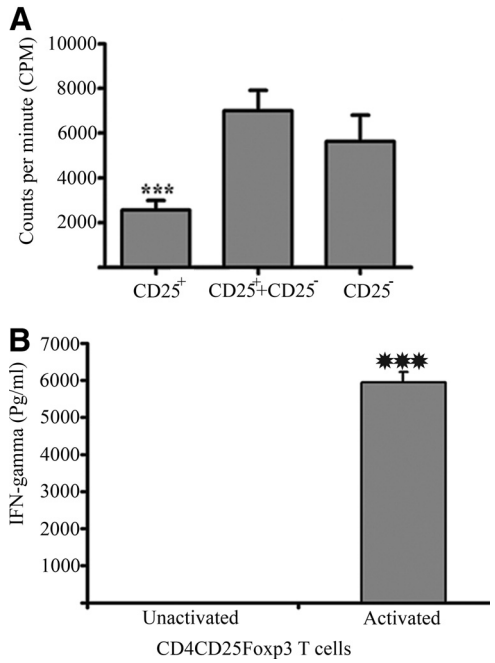

Figure 5. Showing CD4$^+$CD25$^+$ Foxp3$^+$ T cell suppression assay and expression of IFN-γ. (A) To evaluate the suppressive function, if any, of CD4$^+$CD25$^+$Foxp3$^+$ T cells, 50,000 CD4$^+$CD25$^+$CD127^{dim} T cells were cocultured with 50,000 CD4$^+$CD25$^-$CD127^{dim} effector T cells (denoted as CD25$^+$+CD25$^-$ in the middle bar). Controls are CD4$^+$CD25$^+$CD127^{dim} or CD4$^+$CD25$^-$CD127^{dim}. Proliferation of ^{3}H-thymidine-incorporated cells was quantified. Data are representative of two individual experiments. (B) IFN-γ-ELISA showing that activated CD4$^+$CD25$^+$Foxp3$^+$ T cells released significantly higher levels of IFN-γ ($P<0.001$). Data are representative of two individual experiments.

vated $CD4^+CD25^+Foxp3^+$ T cells had no suppressive function.

To further characterize the profile of this T cell subpopulation, supernatants were collected from unactivated and activated $CD4^+CD25^+Foxp3^+$ T cells to evaluate the secretion of IFN-γ, a marker for cytotoxic T_H1 cells. Interestingly, IFN-γ ELISA showed that activated $CD4^+CD25^+Foxp3^+$ T cells released high amounts (6000 pg/ml) of IFN-γ into the culture media (Fig. 5B; $P<0.001$). In line with these findings, Zhou et al. [40] have shown that $Foxp3^+$ T_{regs} lose their Foxp3 expression and take on an effector-memory T_H1 phenotype producing high IFN-γ and in some instances, have the potential to cause rapid pancreatic cell destruction and immune-mediated diabetes [40, 41]. In our study, we found that upon activation, human $CD4^+CD25^+CD127^{dim}$ T cells did not lose the expression of $Foxp3^+$ and produced high levels of IFN-γ and low IL-17. To explain these findings, two hypotheses are possible. First, there is a shift to a T_H1 effector phenotype without the loss of Foxp3 or with transient Foxp3 expression. This hypothesis is in contrast with what has been reported previously in the mouse [40], which may highlight another difference between the mouse and human immune system. In addition, as suggested by Osorio et al. [42], Foxp3-expressing T_{regs} may play an unpredicted proinflammatory role on some occasions. They are involved in the rejection of certain organ transplants [1], which may depend on the microenvironment in which the cells were activated [16] or be affected by the local inflammatory cytokines in the target tissue [43].

Second, Miyara et al. [20] have reported within the population of $CD4^+CD25^+CD127^{dim}$ T cells a fraction of $Foxp3^+$ non-T_{regs} producing IL-17, IFN-γ, and IL-2. Thus, there is the possibility that after activation, we have expanded this nonregulatory effector population.

In conclusion, this study demonstrates that upon activation, human $CD4^+CD25^+Foxp3^+$ T cells become neurotoxic and present a shift toward a T_H1 phenotype. Our study provides important insight about the potential limitations of human T_{regs} for therapeutic applications and the requirement of serious caution in the use of these cells for disease-intervention strategies until we reach a better definition of the different subpopulations within the human $CD4^+CD25^+Foxp3^+$ phenotype.

AUTHORSHIP

Y.H. participated in the conception and design of the project, conducted most of the experiments, and wrote the manuscript. D.P. prepared and cultured human cortical neurons. D.T. conducted the suppression assay. R.C.B. participated in the conception and design of the project and revised the manuscript. F.G. supervised the entire study, participated in the conception and design of the project, and wrote the manuscript.

ACKNOWLEDGMENTS

The study was supported by the Multiple Sclerosis Society of Canada (F.G.), University of Alberta Hospital Foundation (F.G.), and the Canadian Institutes of Health Research (R.C.B.). We thank Dr. Chris Power and Kristofor Ellestad for their technical help.

REFERENCES

1. Brown, K., Wong, W. (2008) Diagnostic value of regulatory T cells: a new facet of a much studied cell population. *Transplantation* **86,** 1485–1491.
2. Galea, I., Bechmann, I., Perry, V. H. (2007) What is immune privilege (not)? *Trends Immunol.* **28,** 12–18.
3. Hauser, S. L., Oksenberg, J. R. (2006) The neurobiology of multiple sclerosis: genes, inflammation, and neurodegeneration. *Neuron* **52,** 61–76.
4. Correale, J., Villa, A. (2007) The blood-brain-barrier in multiple sclerosis: functional roles and therapeutic targeting. *Autoimmunity* **40,** 148–160.
5. Trapp, B. D., Peterson, J., Ransohoff, R. M., Rudick, R., Mörk, S., Bö, L. (1998) Axonal transection in the lesions of multiple sclerosis. *N. Engl. J. Med.* **338,** 278–285.
6. McFarland, H. F., Martin, R. (2007) Multiple sclerosis: a complicated picture of autoimmunity. *Nat. Immunol.* **8,** 913–919.
7. Lassmann, H., Bruck, W., Lucchinetti, C. F. (2007) The immunopathology of multiple sclerosis: an overview. *Brain Pathol.* **17,** 210–218.
8. Bitsch, A., Schuchardt, J., Bunkowski, S., Kuhlmann, T., Brück, W. (2000) Acute axonal injury in multiple sclerosis. Correlation with demyelination and inflammation. *Brain* **123,** 1174–1183.
9. Shriver, L. P., Dittel, B. N. (2006) T-cell-mediated disruption of the neuronal microtubule network: correlation with early reversible axonal dysfunction in acute experimental autoimmune encephalomyelitis. *Am. J. Pathol.* **169,** 999–1011.
10. Compston, A., Coles, A. (2002) Multiple sclerosis. *Lancet* **359,** 1221–1231.
11. Giuliani, F., Goodyer, C. G., Antel, J. P., Yong, V. W. (2003) Vulnerability of human neurons to T cell-mediated cytotoxicity. *J. Immunol.* **171,** 368–379.
12. Neumann, H., Medana, I. M., Bauer, J., Lassmann, H. (2002) Cytotoxic T lymphocytes in autoimmune and degenerative CNS diseases. *Trends Neurosci.* **25,** 313–319.
13. Wang, T., Allie, R., Conant, K., Haughey, N., Turchan-Chelowo, H., Hahn, K., Rosen, A., Steiner, J., Keswani, S., Jones, M., Calabresi, P. A., Nath, A. (2006) Granzyme B mediates neurotoxicity through a G-protein-coupled receptor. *FASEB J.* **20,** 1209–1211.
14. Giuliani, F., Yong, V. W. (2003) Immune-mediated neurodegeneration and neuroprotection in MS. *Int. MS J.* **10,** 122–130.
15. Malmeström, C., Lycke, J., Haghighi, S., Andersen, O., Carlsson, L., Wadenvik, H., Olsson, B. (2008) Relapses in multiple sclerosis are associated with increased CD8+ T-cell mediated cytotoxicity in CSF. *J. Neuroimmunol.* **196,** 159–165.
16. Von Boehmer, H. (2005) Mechanisms of suppression by suppressor T cells. *Nat. Immunol.* **6,** 338–344.
17. Liesz, A., Suri-Payer, E., Veltkamp, C., Doerr, H., Sommer, C., Rivest, S., Giese, T., Veltkamp, R. (2009) Regulatory T cells are key cerebroprotective immunomodulators in acute experimental stroke. *Nat. Med.* **15,** 192–199.
18. Kohm, A. P., Carpentier, P. A., Anger, H. A., Miller, S. D. (2002) Cutting edge: CD4+CD25+ regulatory T cells suppress antigen-specific autoreactive immune responses and central nervous system inflammation during active experimental autoimmune encephalomyelitis. *J. Immunol.* **169,** 4712–4716.
19. Kipnis, J., Avidan, H., Caspi, R. R., Schwartz, M. (2004) Dual effect of CD4+CD25+ regulatory T cells in neurodegeneration: a dialogue with microglia. *Proc. Natl. Acad. Sci. USA* **101** (Suppl. 2), 14663–14669.
20. Miyara, M., Yoshioka, Y., Kitoh, A., Shima, T., Wing, K., Niwa, A., Parizot, C., Taflin, C., Heike, T., Valeyre, D., et al. (2009) Functional delineation and differentiation dynamics of human CD4+ T cells expressing the FoxP3 transcription factor. *Immunity* **30,** 899–911.
21. Kerkovich, D. M., Sapp, D., Weidenheim, K., Brosnan, C. F., Pfeiffer, S. E., Yeh, H. H., Busciglio, J. (1999) Fetal human cortical neurons grown in culture: morphological differentiation, biochemical correlates and development of electrical activity. *Int. J. Dev. Neurosci.* **17,** 347–356.
22. Rosenstein, J. M. (1995) Diminished expression of microtubule-associated protein (MAP-2) and β-tubulin as a putative marker for ischemic injury in neocortical transplants. *Cell Transplant.* **4,** 83–91.
23. Chaitanya, G. V., Kolli, M., Babu, P. P. (2009) Granzyme-B mediated cell death in the spinal cord-injured rat model. *Neuropathology* **29,** 270–279.
24. Kroner, A., Ip, C. W., Thalhammer, J., Nave, K. A., Martini, R. (2010) Ectopic T-cell specificity and absence of perforin and granzyme B alleviate neural damage in oligodendrocyte mutant mice. *Am. J. Pathol.* **176,** 549–555.
25. Buckner, J. H. (2010) Mechanisms of impaired regulation by CD4(+)CD25(+)FOXP3(+) regulatory T cells in human autoimmune diseases. *Nat. Rev. Immunol.* **10,** 849–859.
26. Walker, M. R., Kasprowicz, D. J., Gersuk, V. H., Bernard, A., Van Landeghen, B., Buckner, J. H., Ziegler, S. F. (2003) Induction of FoxP3 and acquisition of T regulatory activity by stimulated human CD4+CD25– T cells. *J. Clin. Invest.* **112,** 1437–1443.

27. Hori, S., Nomura, T., Sakaguchi, S. (2003) Control of regulatory T cell development by the transcription factor Foxp3. *Science* **299,** 1057–1061.
28. Fontenot, J. D., Gavin, M. A., Rudensky, A. Y. (2003) Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. *Nat. Immunol.* **4,** 330–336.
29. Grossman, W. J., Verbsky, J. W., Barchet, W., Colonna, M., Atkinson, J. P., Ley, T. J. (2004) Human T regulatory cells can use the perforin pathway to cause autologous target cell death. *Immunity* **21,** 589–601.
30. Vignali, D. (2008) How many mechanisms do regulatory T cells need? *Eur. J. Immunol.* **38,** 908–911.
31. Liu, W., Putnam, A. L., Xu-Yu, Z., Szot, G. L., Lee, M. R., Zhu, S., Gottlieb, P. A., Kapranov, P., Gingeras, T. R., Fazekas de St Groth, B., Clayberger, C., Soper, D. M., Ziegler, S. F., Bluestone, J. A. (2006) CD127 expression inversely correlates with FoxP3 and suppressive function of human CD4+ T reg cells. *J. Exp. Med.* **203,** 1701–1711.
32. Zhu, J., Paul, W. E. (2010) CD4+ T cell plasticity—Th2 cells join the crowd. *Immunity* **32,** 11–13.
33. Koenen, H. J., Smeets, R. L., Vink, P. M., van Rijssen, E., Boots, A. M., Joosten, I. (2008) Human CD25highFoxp3pos regulatory T cells differentiate into IL-17-producing cells. *Blood* **112,** 2340–2352.
34. Kebir, H., Kreymborg, K., Ifergan, I., Dodelet-Devillers, A., Cayrol, R., Bernard, M., Giuliani, F., Arbour, N., Becher, B., Prat, A. (2007) Human TH17 lymphocytes promote blood-brain barrier disruption and central nervous system inflammation. *Nat. Med.* **13,** 1173–1175.
35. Wang, T., Lee, M. H., Johnson, T., Allie, R., Hu, L., Calabresi, P. A., Nath, A. (2010) Activated T-cells inhibit neurogenesis by releasing granzyme B: rescue by kv1.3 blockers. *J. Neurosci.* **30,** 5020–5027.
36. Cao, X., Cai, S. F., Fehniger, T. A., Song, J., Collins, L. I., Piwnica-Worms, D. R., Ley, T. J. (2007) Granzyme B and perforin are important for regulatory T cell-mediated suppression of tumor clearance. *Immunity* **27,** 635–646.
37. Gondek, D. C., Lu, L. F., Quezada, S. A., Sakaguchi, S., Noelle, R. J. (2005) Cutting edge: contact-mediated suppression by CD4+CD25+ regulatory cells involves a granzyme B-dependent, perforin-independent mechanism. *J. Immunol.* **174,** 1783–1786.
38. Henkart, P. A. (1985) Mechanism of lymphocyte-mediated cytotoxicity. *Annu. Rev. Immunol.* **3,** 31–58.
39. Vignali, D. A., Collison, L. W., Workman, C. J. (2008) How regulatory T cells work. *Nat. Rev. Immunol.* **8,** 523–532.
40. Zhou, X., Bailey-Bucktrout, S. L., Jeker, L. T., Penaranda, C., Martínez-Llordella, M., Ashby, M., Nakayama, M., Rosenthal, W., Bluestone, J. A. (2009) Instability of the transcription factor Foxp3 leads to the generation of pathogenic memory T cells in vivo. *Nat. Immunol.* **10,** 1000–1007.
41. Zhou, X., Bailey-Bucktrout, S., Jeker, L. T., Bluestone, J. A. (2009) Plasticity of CD4(+) FoxP3(+) T cells. *Curr. Opin. Immunol.* **21,** 281–285.
42. Osorio, F., LeibundGut-Landmann, S., Lochner, M., Lahl, K., Sparwasser, T., Eberl, G., Reis e Sousa, C. (2008) DC activated via dectin-1 convert Treg into IL-17 producers. *Eur. J. Immunol.* **38,** 3274–3281.
43. Korn, T., Reddy, J., Gao, W., Bettelli, E,. Awasthi, A., Petersen, T. R., Bäckström, B. T., Sobel, R. A., Wucherpfennig, K. W., Strom, T. B., Oukka, M., Kuchroo, V. K. (2007) Myelin-specific regulatory T cells accumulate in the CNS but fail to control autoimmune inflammation. *Nat. Med.* **13,** 423–431.

KEY WORDS:
granzyme B · axonal injury · cytotoxicity · T_{regs} · neurodegeneration