Nonapoptotic and Extracellular Activity of Granzyme B Mediates Resistance to Regulatory T Cell (Treg) Suppression by HLA-DR<sup>-</sup>CD25<sup>hi</sup>CD127<sup>lo</sup> Tregs in Multiple Sclerosis and in Response to IL-6

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In autoimmune patients, regulatory T cells (Tregs) are increasingly found to be unable to suppress patient-derived T cells, an outcome referred to as Treg resistance. In this study, we show that CD4 T cells from patients with multiple sclerosis resist suppression by patient-derived or healthy donor–derived ex vivo Tregs. Importantly, we report that granzyme B (GzmB) contributes to this Treg resistance via a novel, apoptosis-independent mechanism. We show that memory CD4<sup>+</sup>CD127<sup>lo</sup>FOXP3<sup>+</sup>Tregs in Multiple Sclerosis and HLA-DR<sup>-</sup> populations

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Abbreviations used in this article: GzmB, granzyme B; MS, multiple sclerosis; PD-L1, programmed death ligand 1; RA, rheumatoid arthritis; rh, recombinant human; Tcm, central memory T; Treg, regulatory T cell; Tresp, responder T.

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CD39, which have also been suggested to suppress by inducing target cell apoptosis via granzyme B (GzmB). In this case, although a third of murine Tregs express and suppress via GzmB (18), in humans, GzmB is highly expressed by nonregulatory CD4+ T cells (19) and was actually shown to inhibit Treg suppression by the highly pro-apoptotic CD127- HLA-DR+ natural Treg subset (DR+ Tregs) (19), as well as by human Tregs isolated from patients with HIV (20). In both of these studies, GzmB produced by the target cell population was shown to inhibit Treg suppression as it was delivered into the Tregs to induce their apoptosis, causing the loss in Treg viability and yield, and it reduced suppression.

GzmB is a serine protease, maintained in granules within cytolytic NK and CD8 T cells, that is known to initiate apoptosis via caspase activation upon its granule-mediated delivery into the cytosol of target cells (21). In contrast to this well-studied mechanism of intracellular GzmB-mediated apoptosis, it has recently become apparent that novel, nonapoptotic activities for extracellular GzmB also exist, and that a number of other cell types can produce GzmB. Extracellular GzmB has been shown to be functionally active and digest extracellular matrix proteins as well as variable cell surface proteins that express the specific amino acid sequences that make them sensitive to GzmB cleavage (22-24).

Additionally, extracellular GzmB has been proposed to play a role in altering cell migration. Furthermore, increases in extracellular GzmB have been associated with autoimmunity, as high levels are correlated in the presence of extracellular GzmB.

In this study, we examined the hypotheses that GzmB 1) regulates suppression by the most prevalent human Treg subset, the CD127- HLA-DR+ Treg, via a mechanism that does not involve Treg apoptosis; 2) is differentially used by distinct types of human Tregs; and 3) may be responsible for Treg resistance by CD4 T cells from patients with MS. We show that induced Tregs (CD127+CD25+), which are defective in MS (25), strongly express GzmB and suppress via GzmB-mediated apoptosis, whereas the more prevalent CD127+ natural Tregs, which are defective in patients with MS, do not express GzmB and are inhibited by its activity. Importantly, in contrast to its apoptotic effect on the DR+ CD127+ Tregs (19), we find that GzmB inhibits suppression by the DR- CD127+ Tregs without reducing their viability. In mixed donor Treg/Tresp cell cocultures with cells from healthy donors and patients with MS, we show that neutralizing GzmB enhances suppression of patient-derived, previously Treg-resistant, CD4 T cells. Importantly, we also demonstrate that the administration of extracellular human GzmB abrogates suppression, that the loss of suppression mediated by IL-6 involves its induction of GzmB, and that the frequency of Tregs expressing CD39 and programmed death ligand 1 (PD-L1) is significantly lower when they are stimulated in the presence of extracellular GzmB.

**Materials and Methods**

**Study subjects**

Peripheral venous blood was obtained from healthy individuals and subjects with relapsing-remitting MS (untreated), in compliance with the Institutional Review Board protocols at the Brigham and Women’s Hospital. For paired studies, healthy donors and MS patients were age- and sex-matched. See Table I for the patient demographic and clinical information.

**Cell isolation and coculture**

PBMCs were separated by Ficoll-Hypaque (GE Healthcare) gradient centrifugation. Total CD4+ T cells were isolated via a CD4+ T cell negative isolation kit II (Miltenyi Biotec) and FACS-sorted on a FACSAria (BD Biosciences) as reported previously (8) to typically >98% purity in postsort analysis. Cocultures contained CD127+CD25+ Treg cells (2.5 × 10^4/well) and were cultured alone or with (1.25 × 10^4/well) different CD25+ Treg subsets as reported previously (8). Unless specified, Tregs were cocultured with autologous Tresp cells. Cocultures were stimulated with either anti-CD3/anti-CD2 beads or plate-bound anti-CD3 and irradiated T-depleted APCs, and proliferation was measured by CFSE dilution on day 5 or by overnight pulse with 1 μCi [3H]thymidine. Some cultures were set up in the absence or presence of GzmB inhibitor (Z-AAD-CMK, 1 μg/ml; EMDC/Calbiochem), active recombinant human (rh)GzmB (0.1 μg/ml; Enzo Life Sciences), or rhIL-6 (10 ng/ml; R&D Systems). Viability was determined by staining with the fixable viability dye (Invitrogen), apoptosis was measured via annexin V, and cytokines secreted in culture supernatants were measured using cytometric bead arrays (BD Pharmingen). In the indicated experiments, CD4 central memory T (Tcm) cells were stimulated after isolation by FACS-sorting CCR7+CD29+ cells from the negatively isolated CD4 T cells.

**Cell culture reagents**

Cells were cultured in 96-well U-bottom plates (Corning) in RPMI 1640 medium (BioWhittaker) supplemented as described previously (4) with 5% human AB serum (Cellgro; Mediatech). Anti-CD3/CD2 beads were generated by coupling anti-CD3 (UCHT1 at 1, 2, or 3 μg/10^6 beads) and anti-CD2 (BMA 0111 [Dade Behring] at 1, 2 or 3 μg/10^6 beads) to tosyl-activated beads (Dynal Biotech) to generate the weak, intermediate, and strong stimulation beads, respectively. For other stimuli, plate-bound anti-human CD3 (UCHT1 at 0.1 μg/ml) and soluble anti-human CD28 (clone 28.2 at 0.5 μg/ml) from BD Biosciences were used. IL-2 was obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, National Institutes of Health: rhIL-2 was from Dr. Maurice Gately (Hoffmann-La Roche) and was used at 20 U/ml. Static V and IAK kinase inhibitor I were purchased from Calbiochem.

In some assays, active rhGzmB was added to the culture media at 0.1 μg/ml (similar to other reports using GzmB at 100-250 nM (29–31). We used this lower amount of rhGzmB because it exhibited the active equivalence of 25 ng/ml or 1.5 nM (GzmB activity assay; BioVision), which is within the range of GzmB that can be detected in human plasma (32). The range of GzmB secreted into the media by stimulated human CD4 T cells (at 0.08–0.25 μg/ml/10^6 cells) (19, 32). In some assays, the FACS-sorted cells were precultured in this amount of GzmB for 1 h at 37°C before being washed and combined in coculture or stained. To identify how extracellular GzmB affected human Tregs, 2–4 × 10^4 FACSSorted CD127+CD25+HLA-DR+ Tregs were stimulated with plate-bound anti-CD3 (UCHT1, 0.15 μg/ml) and soluble anti-CD28 (clone 28.2, 0.2 μg/ml) and rhIL-2 (20 U/ml) with or without exogenous rhGzmB (0.1 μg/ml) for 5 d before they were harvested and stained for surface expression of CTLA4, PD-L1, CD39, or LAG3, followed by intracellular staining for FOXP3.

**Flow cytometry**

Intracellular staining was done using the eBioscience FOXP3 staining kit with Abs for FOXP3 (2006, BioLegend) and GzmB (GB11, eBioscience). In some experiments, loss of cell viability was assessed via a viability dye (Invitrogen) before fixation. In other experiments, stimulated cells were labeled with annexin V (BD Biosciences). In some experiments, the cells were also surface stained for CTLA4 (clone BN13, BD Biosciences), PD-L1 (clone 29F.2A3, BioLegend), CD39 (clone A1, BioLegend), or LAG3 (clone 17B4, Enzo Life Sciences). Data were acquired on a FACSCalibur or LSR II flow cytometer (BD Biosciences) using CellQuest software and analyzed using FlowJo software (Tree Star).

**Statistical analysis**

A standard two-tailed t test was typically used for statistical analysis; p values ≤ 0.05 were considered significant, although the one-way ANOVA analysis was used for data examining Treg activity under multiple conditions with autologous and allogeneic Tresp cells.

**Results**

Unlike CD127+ -induced Tregs, CD127lo Tregs do not express or suppress via GzmB

Because of the contradiction where we and others have shown that GzmB inhibits suppression by human DR+ Tregs, whereas GzmB is often proposed to be a component of the Treg suppressive repertoire, we examined the role of GzmB in Treg suppression in more
As shown in Fig. 1B, viable CD127+ Tresp cells and viable CD127+ ability dye followed by intracellular detection of GzmB and FOXP3. Their expression of GzmB, additional cultures of healthy donor–de-
tologous CD4 Tresp cells when isolated from patients with MS (8).

To determine whether these three Treg populations differed in their sensitivity to or utilization of GzmB during suppression, each Treg population was isolated from healthy donor PBMCs and tested for the ability to suppress with and without the GzmB inhibitor peptide (Fig. 1A). Although, both DR− and DR+ CD127lo populations both showed temporally distinct deficiencies in suppression of autologous CD4 Tresp cells when isolated from patients with MS (8).

To determine whether the three memory Treg subsets differed in their expression of GzmB during suppression, each Treg population was isolated from healthy donor PBMCs and tested for the ability to suppress with and without the GzmB inhibitor peptide (Fig. 1A). Although, both DR− and DR+ CD127lo populations both showed temporally distinct deficiencies in suppression of autologous CD4 Tresp cells when isolated from patients with MS (8). As shown in Fig. 1B, viable CD127+ Tresp cells and viable CD127+ FOXP3+ cells expressed high levels of intracellular GzmB whereas viable HLA-DR− or HLA-DR+ CD127loFOXP3+ Tregs did not. When testing for relative expression of GzmB transcripts, real-time PCR analysis of mRNA from 24 h–stimulated Tresp cell and Treg populations again demonstrated that DR+ Tregs were highly sensitive to GzmB-mediated apoptosis (19).

To determine whether the three memory Treg subsets differed in their expression of GzmB, additional cultures of healthy donor–derived cells were set up and analyzed by staining with a fixable viability dye followed by intracellular detection of GzmB and FOXP3. As shown in Fig. 1B, viable CD127+ Tresp cells and viable CD127+ FOXP3+ cells expressed high levels of intracellular GzmB whereas viable HLA-DR− or HLA-DR− CD127loFOXP3+ Tregs did not. When testing for relative expression of GzmB transcripts, real-time PCR analysis of mRNA from 24 h–stimulated Tresp cell and Treg populations again demonstrated that GzmB was strongly expressed by activated Tresp cells and CD127+ Tregs, but not by the DR− or DR− CD127lo Tregs (Fig. 1C). The data also indicate that the GzmB message is not highly expressed in freshly isolated CD4 Tresp cells, which correlates with previously published reports showing that only 1–4% of ex vivo CD4 T cells are expressing GzmB (33), but that it is strongly induced upon T cell activation (19, 34). Thus, these data indicate that only the CD127+CD25hi Tregs coexpress FOXP3 and GzmB and suppress via a GzmB–mediated mechanism.

We next examined whether GzmB similarly affected the viability of the different memory Tregs when they were activated in vitro with Tresp cells under conditions of increasing strength stimulation, which is known to decrease Treg suppression (13) and increase GzmB expression by Tresp cells (19). As we previously reported and therefore expected, reduced numbers of viable DR+ Tregs (i.e., lacking annexin V) were isolated from cocultures that had received strong stimulation, and the frequency of viable cells increased in cultures that received the GzmB inhibitor. In contrast, because the addition of the GzmB inhibitor did not significantly alter the yield of viable FOXP3+DR− CD127lo Tregs (Fig. 1D), the data suggest that within the CD127lo Treg population, the DR− Tregs lack the proapoptotic sensitivity to GzmB that is exhibited by the DR+ Tregs (19). Because inhibiting GzmB did not affect the viability, but did enhance the suppressive activity, of the DR− CD127lo Tregs, we focused on these cells to further study how GzmB affected suppression by these cells.

### Extracellular, active GzmB can abrogate suppression by DR− CD127lo Tregs

Whereas GzmB–induced apoptosis involves the granule-mediated delivery of GzmB into the cytoplasm of target cells, free GzmB can also be found in extracellular fluids where it can exhibit non-apoptotic protease activity (22, 23). Because GzmB–mediated inhibition of DR− CD127lo Treg suppression was not associated with changes in Treg viability, we next asked whether non-apoptotic activities of extracellular GzmB may affect DR− Treg suppression. It is known that the simple addition of GzmB to cultured cells does not lead to cell death, as additional molecules are needed (such as perforin or streptolysin O) to enable GzmB uptake and cytosolic release (21, 35). To test whether extracellular, active GzmB affected Treg suppression, we cultured CFSE-labeled Tresp cells with or without DR− Tregs and different combinations of rhGzmB and the same GzmB inhibitor peptide. By comparing the number of cells that remained CFSE+ after 5 d, it was apparent that the addition of rhGzmB abrogated suppression by DR− Tregs, whereas the concomitant addition of the GzmB inhibitor allowed suppression to occur (Fig. 2A). Importantly, this demonstrated the specificity for the GzmB inhibitor and also indicated that extracellular GzmB inhibits Treg suppression. The results from repeated coculture assays with cells from additional donors are shown (Fig. 2B). Additionally, as the number of viable, FOXP3+ Tregs in the cocultures that had been given the exogenous GzmB was not lower than the number of Tregs in the other cocultures (Fig. 2C), it is apparent that the addition of extracellular GzmB did not inhibit DR− CD127lo Treg suppression by inducing Treg apoptosis. To examine whether GzmB negatively affected the viability of the DR− CD127lo Tregs when stimulated in the absence of Tresp cells, we also separately cultured the DR− Tregs and Tresp cells in the presence or absence of the same concentration of GzmB (Fig. 2D). Upon analyzing the number of cells that remained viable at 36 h, it was again apparent that the addition of 0.1 μg/ml extracellular GzmB did not adversely affect the viability of either cell type.

### Extracellular GzmB inactivates DR− CD127lo Treg suppression

Although the addition of GzmB abrogated suppression in cocultures containing both Treg and Tresp cells, it was unclear as to which population was being affected by the presence of the GzmB. Thus, to determine whether GzmB inactivated the suppressive activity of the Treg or decreased the sensitivity of the Tresp cell to Treg suppression, we pretreated each freshly isolated population with GzmB or mock (media alone) and then combined them in different combination cocultures (Fig. 3). As shown in Fig. 3A, the addition of GzmB to cocultures containing each mock-treated population again inhibited suppression. Furthermore, although the

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**Table I. Patient sample clinical and demographic information (all patients were untreated)**

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*Patient was black or African American; all other patients were white. RR, relapsing-remitting disease.
GzmB-pretreated Tresp cells still demonstrated sensitization to suppression by mock-treated Tregs, the GzmB-treated Tregs had lost the capacity to suppress the mock-treated Tresp cells (Fig. 3A).

To examine whether this effect involved GzmB internalization, freshly isolated Tresp cells and Tregs were pretreated with GzmB or media and examined for internalization of GzmB, which would be indicated by increased detection of intracellular GzmB. After preincubating freshly isolated Tresp or DR-CD127lo Tregs with or without GzmB, the cells were either stained to detect total GzmB by staining them after fixation and permeabilization, or for intracellular GzmB by blocking cell surface GzmB with the unlabeled mAb before permeabilization (Fig. 3B). To demonstrate the efficacy of this GzmB blockade procedure, PBMC-derived CD8 T cells were stained for intracellular GzmB with or without (top) preincubation with the unlabeled anti-GzmB mAb (Fig. 3C). The data indicate that in contrast to other reports showing that HeLa cells could internalize GzmB after being incubated in 100-fold more GzmB (31), the low level of GzmB used in our pretreatment did not result in GzmB internalization by the Tregs or the Tresp cells (Fig. 3B). The data do indicate that GzmB pretreatment resulted in GzmB being detected on the surface of ~0.3% of the treated Tresp cells and the Tregs. However, the different coculture outcomes when the pretreated versions of these two different cell types were assayed suggest that this low level of surface GzmB does not account for the subsequent inactivation of Treg suppression.

IL-6 induces GzmB in CD4 T cells and abrogates DR-CD127lo Treg suppression via GzmB

Because IL-6 is known to inhibit suppression by Tregs (14, 36, 37), we next asked whether it might do so via a GzmB-related mechanism. We first examined whether IL-6 regulated the expression of GzmB in healthy donor-derived CD4 T cells. As shown in Fig. 4A, the presence of rhIL-6 strongly induced GzmB in both CD4 T cells and CD8 T cells. IL-6 is known to induce GzmB in CD8 T cells (38-40). Indicated by the two different bars, IL-6 significantly increased the percentage of cells expressing the highest levels of GzmB. In Fig. 4B, we examined the role of JAK/STAT activation in the IL-6-mediated induction of GzmB in both CD4 and CD8 T cells. IL-6 and specific inhibitors for STAT3 (StatticV) or the JAKs (JAK inhibitor I) were added to the cultures of PBMCs or Tresp cells from the outset, with expression of GzmB being measured after 4 d. STAT3 inhibition abrogated the IL-6-mediated increase in GzmB expression in both CD4 and CD8 T cells, whereas inhibiting all three JAKs resulted in a further decrease in GzmB expression only in the CD4 T cells. Additionally, although the T cells in the PBMC cultures showed stronger proliferation and greater levels of GzmB production, the ability of IL-6 to enhance GzmB in the less stimulatory cultures of purified CD4 T cells indicated that IL-6 can directly enhance GzmB expression in CD4 T cells without additional factors potentially provided by other cell types.

To determine whether this IL-6 induction of GzmB contributes to the reported ability of IL-6 to abrogate Treg suppression, cocultures of healthy donor-derived Tresp cells and DR-CD4 Tregs were stimulated in the presence or absence of rhIL-6 and with and without GzmB inhibitor (Fig. 5). By measuring CFSE dilution and expression of GzmB, it was apparent that the addition of IL-6 abrogated Treg suppression as expected, and it increased Tresp cell proliferation and expression of GzmB, whereas the simultaneous addition of IL-6 with blockade of GzmB resulted in lower Tresp cell proliferation (as compared with the addition of IL-6 only) and allowed Treg suppression to occur (Fig. 5B). These data indicate that GzmB is induced by IL-6, plays a role in IL-6-mediated loss of Treg suppression, and that it can also enhance Tresp cell proliferation in the presence of IL-6. Cells from three additional donors were tested in replicate assays to determine the reproducibility of the response (Fig. 5B). Again, because providing the GzmB inhibitor did not result in an increased yield of FOXP3+CFSE Tregs in the 5-d cocultures, it is apparent that IL-6 reduced Treg suppression via a GzmB-mediated mechanism that did not cause Treg apoptosis (Fig. 5C).

GzmB mediates DR-CD127lo Treg resistance by CD4 T cells in patients with MS

We previously showed that both the DR+ and DR- populations of CD127loCD25hi human Tregs were less efficient at suppressing the
activation of autologous responder CD4 T cells when isolated from patients with MS as compared with cocultures established with cells from healthy donors (8). In the present study, we set out to examine whether this was due to a defect in the suppressive capacity of patient-derived Tregs or resistance to suppression by patient-derived CD4 T cells. To do this, we cocultured Tresp cells (CD4+CD25-2/lo) with half as many FACS-sorted DR-CD127lo Tregs (HLA-DR2CD127loCD25hi) than isolated from arbitrarily paired peripheral blood samples from patients with MS (untreated) and from sex-matched healthy controls (Table I).

In cocultures containing autologous Tresp cells and DR-2 Tregs (Fig. 6A), the suppression in the patient samples was significantly lower \((p, 0.05)\) than the suppression in healthy donor cultures. However, when these cells were set up in mixed-donor cocultures where the patient-derived Tregs were cocultured with healthy donor Tresp cells (Fig. 6B), the patient-derived Tregs exhibited increased suppressive activity on the healthy donor Tresp cells. In the similar mixed donor assays of healthy donor–derived Tregs, the healthy donor–derived Tregs exhibited significantly less suppression with the patient-derived Tresp cells than with the healthy donor Tresp cells. These results indicate that the patient-derived Tresp cells are more difficult to suppress and thus resist Treg-mediated suppression.

Having previously found that GzmB can regulate Treg suppression by HLA-DR+ Tregs (19), replicates of all of these HLA-DR2 Treg cocultures had also been established in the presence of the same specific GzmB peptide inhibitor (Fig. 6C). By blocking the activity of GzmB, suppression was increased in all cocultures. However, within the completely autologous cocultures, the blockade of GzmB only significantly increased the suppression in the patient-derived cells, although the increase in healthy donor cultures did not.
reach significance. Thus, these data indicated that the GzmB produced in the cocultures of patient cells was a major factor leading to a significant decrease in suppression by HLA-DR\^2 Tregs. Although finding that GzmB limits suppression by patient-derived cells is a novel finding, it is also apparent that some suppression resistance is independent of GzmB.

To determine whether patient-derived Tresp cells produce greater amounts of GzmB than do healthy donor–derived Tresp cells, and whether inhibition of GzmB affected cytokine secretion, CD4 T cells from three healthy donors and three patients with MS were stimulated with anti-CD3 and autologous APCs in the presence and absence of rhIL-6 (10 ng/ml) and inhibitors for STAT3 (10 ng/ml) or JAKs. *p < 0.025 (B). The cells were isolated from healthy donors and are representative of independent repeats of cells from four different donors. *p < 0.025.

Because adding exogenous rhGzmB inhibited Treg suppression, whereas blocking GzmB activity enhanced suppression in cocultures between Tregs and the GzmB-producing CD4 Tresp cells, how GzmB mechanistically was affecting Treg suppressive capacity was the next clear question. To start to address this point, we asked whether Tregs that were stimulated in the presence or absence of exogenous GzmB exhibited reduced expression of the suppression-associated molecules CD39 and PD-L1.

**FIGURE 4.** IL-6 induces CD4 T cells to express GzmB. Intracellular expression of GzmB was determined in CD4 and CD8 T cells from PBMC cultures stimulated with and without rhIL-6 (10 ng/ml) for 4 d (A) and in CD4 and CD8 cells from PBMC cultures or in cultures of Tresp CD4 cells stimulated in the presence or absence of rhIL-6 (10 ng/ml) and inhibitors for STAT3 (10 ng/ml) or JAKs. *p < 0.025 (B). The cells were isolated from healthy donors and are representative of independent repeats of cells from four different donors. *p < 0.025.

**FIGURE 5.** GzmB contributes to the suppression-abrogating activity of IL-6. Using cells from healthy donors, DR\^2 Tregs were cocultured with twice as many Tresp cells and stimulated with anti-CD3/APCs alone or in the presence of IL-6 with or without GzmB inhibitor peptide (2 \(\mu\)g/ml). After 5 d, the cells were analyzed for CFSE dilution and intracellular GzmB expression (A). Cells from four additional donors were tested under the same assay conditions to determine reproducibility; significance was determined by repeated measures ANOVA (B). The yield of viable Tregs in the cocultures was determined after FOXP3 staining (C). Thus, in total, cells from five different donors were tested in independent repeat experiments.
absence of exogenous GzmB exhibited any alterations in cell surface expression of inhibitory immune checkpoint molecules that are known to be components of the Treg suppressive arsenal. In Fig. 7, we show that the presence of extracellular GzmB during the culture of DR$^{+}$CD127$^{lo}$ Tregs resulted in a significant reduction in the frequency of Tregs expressing CD39 or PD-L1. Similar to previous findings, the cultures given exogenous GzmB did not produce lower numbers of viable, FOXP3$^{+}$ Tregs (data not shown). Thus, although there are many layers of mechanism, these studies indicate that the reduction in expression of CD39 and PD-L1 are two possible ways in which extracellular GzmB could negatively influence Treg suppressive capacity.

**Discussion**

In this study, we examined a potential role for extracellular, non-Treg–derived GzmB in generating resistance to Treg-mediated suppression. In mixed donor cocultures, we found that ex vivo Tregs isolated from patients with MS were as suppressive as those isolated from healthy donors whereas the patient-derived CD4 responder T cells were resistant to Treg suppression. By specifically blocking GzmB activity, we showed that GzmB contributes significantly to this Treg resistance when cocultured with CD4 T cells from patients with MS. We find that CD4 T cells from patients with MS exhibit an intrinsically greater tendency to produce more GzmB than do cells from healthy donors upon TCR stimulation, and that GzmB expression in healthy donor–derived CD4 T cells is enhanced by IL-6. We also demonstrated that only the CD127$^{+}$ Tregs, which are functional in autoimmune patients, express and suppress via GzmB. In contrast, the DR$^{+}$CD127$^{lo}$ Tregs, previously shown to be less suppressive in patients with MS (8), are functionally inhibited by GzmB. Importantly, the simple addition of exogenous, active rhGzmB to cocultures containing CD127$^{lo}$ Tregs inhibited suppression without affecting Treg viability, suggesting that GzmB regulates CD127$^{lo}$ Treg suppressive activity via its nonapoptotic protease activities (23, 41). Upon showing that the suppression-abrogating cytokine IL-6 enhances GzmB expression in CD4 T cells and mediates at least part of its suppression-inactivating activity via GzmB, we also found that Tregs stimulated in the presence of extracellular GzmB exhibit reduced expression of the inhibitory molecules CD39 and PD-L1.

Treg resistance has been increasingly documented to be a feature of the effector T cells that are found in many autoimmune diseases. Enhanced STAT3, protein kinase C $\beta$, and Akt activation, as well as refractory TGF-$\beta$ signaling, have all been shown to play fundamental roles in mediating resistance to Treg suppression (42–45). Finding that Teff cells may resist Treg suppression suggests altered interpretations of the original reports that documented Treg functional deficiencies in the face of increased Treg frequencies in the joint in RA/juvenile idiopathic arthritis (46) and in the CNS of mice induced for experimental autoimmune encephalomyelitis as a model of human MS (47). By specifically blocking GzmB activity, we showed in the present study that GzmB also contributes significantly to Treg resistance by cells from patients with MS. Furthermore, as administering GzmB with or without its inhibitor to cocultures of healthy donor–derived cells did not alter DR$^{+}$ Treg viability but markedly affected Treg suppression, it appears that the ability of GzmB to regulate Treg-mediated suppression is a novel nonapoptotic activity of GzmB.

Although a number of cytokines can block Treg-mediated suppression, much research has focused on IL-6, as it is highly expressed in the joint, in serum, or in extracellular fluids in many autoimmune diseases (48, 49). IL-6 was first demonstrated to have

![Diagram](Image)
suppression-inactivating properties in 2003 (14), and it has also recently been shown to be involved in the resistance of CD4 responder cells from patients with MS to suppression by a population of in vitro–generated, adaptive Tregs (15). In that report, the patient Tresp cells exhibited increased IL-6–Re expression and elevated IL-6 signaling (15). Goodman et al. (36) demonstrated that STAT3 regulates the level of Treg suppression in cultures given increasing stimulation stimuli. Our data, showing that IL-6 induces GzmB expression in CD4 T cells and in CD8 T cells (38–40) and abrogates suppression in cultures of healthy donor–derived cells via GzmB, suggest a fundamental role of GzmB in regulating sensitivity to Treg suppression.

GzmB is known to have a number of nonapoptotic, extracellular proteolytic substrates. Because extracellular GzmB inhibits suppression without negatively impacting the number of viable Tregs, we hypothesized that, by cleaving GzmB-sensitive cell surface molecules such as PAR-1, FGFR, or TCRz (23, 24), extracellular GzmB may alter intra-Treg signaling and lead to a reduced expression of cell surface molecules involved in Treg suppressive activity. Thus, we compared whether GzmB altered the expression of a panel of suppression-associated molecules (50) and found that Tregs stimulated in the presence of GzmB showed no change in expression of CTLA4 or LAG3 but did show significantly reduced expression of CD39 and PD-L1. CD39 is an ectoenzyme that removes diphosphates from extracellular, proinflammatory ATP molecules in the rate-limiting step that leads to ultimate production of inhibitory adenosine molecules (51). In contrast, PD-L1 is not an enzyme, but rather is a ligand that binds to PD-1 and CD80 on target cells where it causes the transduction of inhibitory signals and results in reduced proliferation, although it can also control Treg motility and immune synapse formation (52, 53). Both molecules have been shown to be components of the Treg suppressive repertoire (50, 53).

Interestingly, the novel finding that extracellular GzmB leads to reduced expression of CD39 on the Treg may provide the mechanism for observations that were published years ago by other groups studying the activity and phenotype of Tregs isolated from patients with MS. Interestingly, these two independent studies had both demonstrated that although the actual number of Tregs was not reduced in patients with MS, patient-derived Tregs expressed markedly less CD39 and less suppressive activity than did Tregs isolated from healthy donors (54, 55). No mechanistic rationale was provided to explain why the patient-derived Tregs should express lower levels of CD39. However, because we have shown (in the present study) that patient-derived CD4 Tcm cells produce significantly more GzmB than do healthy donor Tcm cells, that extracellular GzmB inhibits Treg suppressive ability, and that extracellular GzmB activity significantly reduces CD39 expression on Tregs, these data support the hypothesis that perhaps unchecked production of GzmB in the patient may cause the lower frequency of CD39-expressing Tregs.

In contrast to a number of previous studies, including our own work, that had indicated that aberrant Treg function was responsible for the loss of suppression in patient samples and that there was no association with IL-6 (6, 56, 57), the new data in the present study indicate that Treg resistance may account for a significant portion of the reduced suppression by patient cells. Although in this study we still do not associate loss of suppression in samples from patients with MS with the production of IL-6, we do demonstrate that IL-6 can inhibit suppression in cultures of healthy donor cells via a GzmB-mediated mechanism and, most importantly, that a major cause of decreased Treg suppression in samples from patients with MS is that the patient’s Tresp cells resist suppression via a mechanism that appears to involve the activity of GzmB.

Although it is important to note that Treg resistance does not preclude that patient-derived Tregs may also be functionally deficient, it is hard to understand how two such different results can arise from the same mixed donor suppression assay. We think that the most likely explanation for the marked change in outcome is the nature of the Tregs that were tested in these various studies. In the earlier studies, the Tregs were isolated via the single CD25hi feature, which from the present studies put forth, we now know would contain the functionally distinct, induced FOXP3+ cells (CD127+), as well as both the natural, CD127–DR+ Treg, and DR–Treg (8). Testing a combined mixture of these Tregs could easily lead to a suppressive outcome because the DR–Tregs are the most suppressive human Treg population (4), but they are also extremely sensitive to GzmB-mediated apoptosis (19), and, although the DR+ Tregs are not sensitive to GzmB-mediated apoptosis, they too are unable to suppress in the presence of GzmB (the present study). All the while, the patient-derived CD25hi inducible Tregs (CD127+) within the total Treg population are the cells that would be producing perhaps elevated levels of GzmB similar to what we have observed in the analysis of activated, patient-derived Tcm cells. Thus, as the CD127+ inducible Treg–derived GzmB would inhibit suppression by both the CD127hi DR– and DR+ Tregs, the assay of patient-derived total CD25hi Treg populations would likely give the previous results where patient-derived Tregs show decreased suppressive capacity.

Our current scenario of how GzmB may regulate Treg activity is based on time and signal strength. Because CD4 T cells produce more GzmB with stronger stimulation, early and greater production of GzmB would inactivate Tregs and favor immune activation in an immune response. In the healthy donor, as the levels of extracellular GzmB decrease over time, Treg suppression would be the favored outcome. However, in the autoimmune patient, the higher levels of GzmB that may be maintained due to ongoing activation would not allow suppression to emerge. Overall, our data suggest that the inactivation of specifically the extracellular GzmB could have potential therapeutic efficacy in the treatment of autoimmunity.
and transplantation and may restore the function of the patient’s own Tregs by blocking the GzmB-mediated loss of molecules important for Treg suppressive function. Blocking only the extracellular activity of this enzyme would also be therapeutically attractive because it would theoretically not affect the cytolytic activities of CD8 and NK cells, and thus leave the immune system in patients highly functional.

Disclosures

The authors have no financial conflicts of interest.

References


