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*J Immunol* 2005; 174:1783-1786; doi: 10.4049/jimmunol.174.4.1783
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Cutting Edge: Contact-Mediated Suppression by CD4+CD25+ Regulatory Cells Involves a Granzyme B-Dependent, Perforin-Independent Mechanism

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CD4+CD25+ regulatory T cells (Treg) are potent immunosuppressive cells that are pivotal in the regulation of peripheral tolerance. In this report, we identify granzyme B (GZ-B) as one of the key components of Treg-mediated suppression. Induction of regulatory activity is correlated with the up-regulation of GZ-B expression. Proof of a functional involvement of GZ-B in contact-mediated suppression by Treg is shown by the reduced ability of Treg from GZ-B−/− mice to suppress as efficiently as Treg from WT mice. GZ-B-mediated suppression is perforin independent, because suppression by Treg from perforin−/− and WT is indistinguishable. Additionally, suppression mediated by Treg appears to be mediated, in part, by the induction of apoptosis in the CD4+CD25+ effector cell. In summary, GZ-B is one of the key mechanisms through which CD4+CD25+ Treg induce cell contact-mediated suppression.


Recent studies have underscored the importance of regulatory T cells (Treg) in preventing the emergence of autoimmune disease, dampening the intensity of immune responses to pathogens and mediating peripheral transplantation tolerance. Multiple subsets of Treg have been implicated in these processes and include the thymically derived CD4+CD25+ Treg (1), as well as inducible regulatory T cells (Tr1) subset (2). A major focus of study has been to molecularly characterize the mechanisms that mediate Treg suppression of immunity. It has been shown that Tr1 suppress predominantly by a cytokine-dependent mechanism characterized by IL-10 and TGF-β secretion (3). Similarly, a TGF-β-dependent mechanism has also been implicated in suppression by CD4+CD25+ Treg (4–6). In addition to suppression via soluble factors, the CD4+CD25+ Treg have been shown to mediate suppression via a contact-dependent mechanism (7).

The molecular basis for contact-dependent suppression by CD4+CD25+ Treg is not known. Glucocorticoid-induced TNF-like receptor (GITR or TN-FSF18) is a member of the TNFR family that is constitutively expressed on Treg and inducibly expressed on CD4+CD25+ effector T cells (Teff) (8, 9). Triggering of GITR has been shown to extinguish their contact-dependent suppressive activity (8, 10). Based on this overt change in biological function, transcriptional profiling of resting, activated Treg, and anti-GITR-treated activated Treg has led to the identification of a number of candidate molecules that may be involved in contact-dependent suppression. One such molecule that was identified as up-regulated in activated Treg and whose expression was reduced via GITR-triggering is granzyme B (GZ-B).

GZ-B is a serine protease, secreted mainly by NK cells and CTLs (11), and is largely responsible for the induction of apoptosis in the target cell. However, recent reports have shown that human CD4+ T cells are also able to synthesize GZ-B and perforin (12, 13). Furthermore, studies by Ley and coworkers (14, 15) have shown that GZ-B is highly up-regulated in activated human T cells bearing a Tr1 phenotype. Moreover, Ley and coworkers (16) have shown CD4+CD25+ Treg in the human system mediate suppression with requirement for granzyme A (GZ-A). These results suggest a possible role for granzyme in mediating T cell suppression. The data presented in this study implicate that GZ-B plays a pivotal role in the suppressive capacity of murine CD4+CD25+ Treg.

Materials and Methods

Mice and materials

Congenic strains CD45.1 or CD45.2 C57BL/6 and perforin−/− mice, 8–10 wk old, were purchased from The Jackson Laboratory. C57BL/6 GZ-B−/− mice (15) were bred and maintained in our facility at Dartmouth Medical School.

Received for publication August 18, 2004. Accepted for publication December 7, 2004.

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1 This work was supported by National Institutes of Health Grants CA91436-01 and AI48667.

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4 Abbreviations used in this paper: Treg, regulatory T cell; Teff, effector T cell; GITR, glucocorticoid-induced TNF-like receptor; GZ-B, granzyme B; GZ-A, granzyme A; PI, propidium iodide.

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Cell isolation, gene array, and real time

Single-cell suspensions were prepared from 8- to 10-wk-old mice and applied to CD4 enrichment. CD4+ CD25+ and CD4+ CD25- T cells were further purified by magnetic separation with MACS (Miltenyi Biotec) according to the manufacturer’s instructions. Enriched cell populations and purified cells were phenotypically analyzed by FACS. The purities of CD4+ CD25- and CD4+ CD25+ T cells were 90–95%, respectively. Freshly isolated cells have been inoculated (10^6/ml; complete RPMI 1640/10% FBS supplemented with 100 U of IL-2) into a 24-well plate precoated with 10 μg/ml anti-CD3 (clone 2C11) with or without 10 μg/ml anti-GITR (clone DTA-1; Ref. 8) cultured at 37°C for 0, 12, and 48 h. Purified RNA were then analyzed by using Affymetrix mouse genome A430 oligonucleotide arrays or by real-time PCR analysis.

Cell culture and T cell suppression assay

GZ-B expression was assessed in freshly isolated CD4+ CD25+ T cells or in cells cultured in vitro 24–72 h in the presence of plate-bound CD3 (1 μg/ml) with 100 U/ml IL-2.

Spleens and lymph nodes from wild-type, perforin -/-, or GZ-B -/- mice were magnetic bead sorted as stated above. Further purification of the Teff subset was accomplished with a CD4+ T cell isolation kit (Miltenyi Biotec). Effector cells were >95% pure at the end of this isolation. In a polyclonal Teff suppressor assay, CD4+ CD25- T eff cells (5×10^6) were cocultured with irradiated T-depleted splenocytes (1×10^7), 5 μg/ml anti-CD3, and indicated numbers of CD4+ CD25+ T cells for 3 days. In some experiments, 5 μg/ml anti-GITR was also added to the wells. Proliferation was assessed by incorporation of [3H]thymidine (1 μCi/well), which was added for the last 8 h of culture.

Cell surface, intracellular staining, and flow cytometry

Approximately 2×10^5 cells from each of triplicate wells were collected and pooled. Cells were labeled with anti-CD45.1-allophycocyanin (clone A20; eBioscience). Samples were then resuspended in 1X annexin staining buffer and treated with Annexin V/PI (BD Pharmingen) and propidium iodide (PI; Sigma-Aldrich). For GZ-B expression assay, following isolation for fresh Treg or in vitro cultured for 24 or 72 h for cultured Treg, cells were stained with anti-CD4-FITC (clone RM 4-5) and anti-CD25-PE (clone PC-61). Samples were then fixed and permeabilized (Cytotox/Cytoperm; BD Pharmingen) and stained with anti-human GZ-B-allophycocyanin (clone GB12; Caltag) diluted 1/200 in staining buffer. Throughout all steps, normal rat serum (5% v/v; Invitrogen Life Technologies) was used to block nonspecific binding. Samples were analyzed on FACScan (BD Biosciences). Anti-human GZ-B cross-reactivity with mouse GZ-B has been previously reported (15). For CFSE experiments, CD45.1+ cells were labeled with 5 μM CFSE and added to suppressor assay as described above.

Statistical analysis

Analysis of proliferation assays and real-time expression between the various treatment groups were analyzed by two-tailed, paired Student’s t test. Values of p < 0.05 were considered significant.

Results and Discussion

Anti-GITR causes down-regulation of GZ-B in in vitro-cultured Treg

CD4+ CD25+ Treg are suppressive to naive CD4+ T eff in vitro following polyclonal and Ag-specific activation. Furthermore, the in vitro suppressive capacity has been shown to be contact dependent and ablated following treatment with anti-GITR (8). Global gene analysis of activated Treg treated or untreated with anti-GITR was used to identify candidate genes involved in suppression. We examined naive and activated T eff (purified CD4+ CD25- T cells) and Treg (purified CD4+ CD25+ T cells) in the presence of anti-CD3 with or without anti-GITR for 12 or 48 h. Of the ~22,700 genes examined, 259 were up-regulated >1.5-fold and 99 were down-regulated >1.5-fold in Treg following treatment with anti-GITR and anti-CD3 relative to treatment with anti-CD3 alone. GZ-B, as has been shown previously, is up-regulated with Treg activation via anti-CD3 alone (9, 17). Studies presented herein show that GZ-B is down-regulated 2-fold with anti-CD3 in combination with anti-GITR (Fig. 1, A and B). The microarray data was confirmed by RT-PCR (Fig. 1C). At both the 12- and 48-hour time point, the levels GZ-B expression are 2-fold greater with anti-CD3 alone treatment vs combining with anti-GITR stimula-

FIGURE 1. Activation-induced up-regulation of GZ-B in Treg and its regulation by GITR. A, Cells were purified via MACS columns to >90% purity and cultured in vitro with anti-CD3 and IL-2 with or without anti-GITR for 12 h. RNA was prepared and hybridized to the Affymetrix A430 array. Relative expression indicates the mean log2 ratio of changes in Treg expression between anti-CD3-alone treatment and anti-CD3 with anti-GITR. B, Gene chip signal intensity comparison of GZ-A, GZ-B, and perforin following treatments for 12 or 48 h. C, Real-time RT-PCR analysis of GZ-B expression in Treg treated as described above. Figure is representative of two independent experiments. D, GZ-B expression in freshly isolated Treg or in vitro cultured for 24 or 72 h with plate-bound anti-CD3 and 100 U of IL-2.


tion. Moreover, protein expression of GZ-B recapitulates the results found via RT-PCR by increasing the abundance of GZ-B from 24 to 72 h (Fig. 1D). Additionally, after 12 h in culture, Treg GZ-B mRNA expression is 20-fold greater than Teff with CD3 stimulation alone (data not shown). We also examined expression levels of GZ-A and perforin at all time points. For both molecules, we see similar regulation to that of GZ-B with anti-GITR treatment; however, expression is at a much lower intensity at all time points (Fig. 1, A and B). These data were also confirmed by RT-PCR (data not shown). These data indicate that, immediately following activation, Treg rapidly up-regulate GZ-B; however, GZ-A and perforin remain low in abundance relative to GZ-B expression.

GZ-B−/− Treg have reduced suppressive capacity in vitro

Coculture of Treg with wild-type Teff leads to suppression of proliferation in a dose-dependent manner. To functionally evaluate the role of GZ-B in the contact-mediated suppression by Treg, the suppressive activity of Treg from WT and GZ-B−/− mice was compared (Fig. 2A). Data presented show that Treg from WT mice at a 1:1 ratio suppress the proliferation of Teff >90%, whereas Treg from GZ-B−/− mice suppress Teff proliferation <50%. The reduced suppressive activity of Treg from GZ-B−/− mice is observed across a spectrum of Treg:Teff ratios, suggesting a functional role of GZ-B in contact-mediated suppression. A comparison of FoxP3 levels of GZ-B−/− Treg revealed no significant difference from those of WT Treg (data not shown). Because loss of GZ-B does not completely extinguish Treg suppression, additional contact-dependent mechanisms must be important in this system.

Typically, GZ-B requires cytosolic entry via perforin or a perforin-like molecule to induce cell death (18, 19). To determine whether suppression is mediated by the canonical GZ-B-perforin pathway, the suppressor activity of Treg from perforin−/− vs WT mice was determined (Fig. 2B). The suppressive activity of Treg from perforin−/− and WT mice was indistinguishable, suggesting that GZ-B-mediated suppression is via a novel, perforin-independent mechanism. Moreover, studies of perforin−/− mice on the BALB/c background showed similar results (data not shown). Several reports indicate that, at high concentrations of GZ-B, the necessity of perforin to release GZ-B from the endosomal compartment can be circumvented (20, 21). Additionally, Choy et al. (22) have shown that GZ-B is able to induce cell death in a perforin-independent manner mediated by a combination of intracellular and extracellular events.

A recent report by Grossman et al. (16) indicates that human CD4+CD25+ Treg mediate their suppressive effects via death induced by a GZ-A perforin-dependent mechanism. The differences between the use of GZ-A in humans and GZ-B in mice could be due to species differences, or subtle differences in the subsets and/or activation of T cells that were used. With regard to perforin dependency, the study by Ley (16) implicates perforin because of the fact that a calcium chelator relieves suppression. Although this is a reasonable assertion, they also show that CD18 is required, and it is known that this molecule requires calcium to form the tight synapse required for granzyme-mediated toxicity (23–25). In our studies using perforin knockout mice, suppression was indistinguishable from WT mice.

**Induction of Teff apoptosis is a component of contact-mediated suppression**

Recent reports have re-examined apoptosis by Treg as a mechanism for suppression (15, 26). The molecule(s) that mediate the induction of Teff apoptosis have not been resolved, and it is unlikely that FasL plays a central role (27). Based on the finding that GZ-B plays a functionally significant role in Treg suppression, the ability of Treg to induce Teff apoptosis and cell death was re-examined. The induction of Teff apoptosis by Treg was determined following the in vitro coculture of activated Teff and Treg. Briefly, CD45.1+ (Ly5.2+) Teff were cocultured with increasing numbers of CD45.2+ (Ly5.1+) Treg in the presence of anti-CD3. After 72 h of culture, apoptosis of the CD4+ Teff was determined by multiparameter flow cytometry. The data show that there is a dose-dependent increase in cell death of the Teff cells when cocultured with Treg such that ~50% more Teff are dead at a 1:1 ratio than at a 1:16 ratio of Treg to Teff (Fig. 3A). Moreover, addition of anti-GITR relieves the suppression and apoptosis as evidenced by enhanced proliferation and cell survival (data not shown). In parallel experiments, we examined thymidine incorporation in a standard suppressor assay with Treg from wild-type mice treated with anti-CD3 to determine levels of suppressive activity concurrent with PI/annexin staining (Fig. 3B). To distinguish between the antiproliferative and antiapoptotic effect of Treg, we examined suppression and death with CFSE-labeled Teff counterstained with PI. In Fig. 3C, we demonstrate that the Teff have a greater percentage of PI− cells when cocultured with Treg. Interestingly, in addition to the induction of cell death, the proliferation of PI− Teff was also inhibited, which indicates multiple mechanisms are involved in Treg-mediated suppression.

![FIGURE 2.](http://www.jimmunol.org/) GZ-B mediates Treg suppression via a perforin-independent mechanism. A, Treg isolated from wild-type or GZ-B−/− mice were cocultured with CD4+CD25+ Teff and irradiated T-depleted APC with anti-CD3 for 72 h. Wells were pulsed with 1 μCi/well [3H]thymidine for the last 8 h of culture, and analyzed as described in Materials and Methods. B, Treg isolated from wild-type or perforin−/− mice were cultured as above.
The data presented above suggest that GZ-B is pivotal for T-reg suppression is mediated by enhanced death in T-eff. Wells were harvested and stained with anti-Ly5.2 to identify the CD4+CD25+ T-reg Cells were then stained with PI and Annexin V-FITC. Proliferation of cells in wells run simultaneously with stained wells. Cells were pulsed with 1uCi/well 3Hf or the last 8h of culture. CFSE-labeled cells were cultured for 72 h in standard suppressor assay, and then counterstained with PI.

The presented data above suggest that GZ-B is pivotal for complete suppression by T-reg. The fact that the expression of GZ-B is up-regulated by T-reg activation, and impaired by anti-GITR triggering is consistent with biological activity manifested by T-reg under these conditions. Functionally, the role of GZ-B in T-reg activities was strongly suggested by the reduced ability of T-reg from GZ-B−/− mice to suppress T-eff proliferation. Surprisingly, it appears that the GZ-B-dependent suppression of T-eff activities is not dependent on perforin, because the T-reg from perforin−/− mice suppressed T-eff proliferation equivalent to that observed from wild-type mice. Hence, the precise mechanism of GZ-B-mediated suppression is enigmatic. In this context, GZ-B-mediated apoptosis in the absence of perforin has been reported in other systems. Finally, death as a consequence of T-reg action was demonstrated by the fact that CD4+CD25+ T-reg in a dose-dependent manner, can induce apoptosis in T-eff. The current studies raise many questions as to how GZ-B mediates T-reg death, and the role of GZ-B in the in vivo function of T-reg.

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