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## Cutting Edge: Contact-Mediated Suppression by CD4<sup>+</sup>CD25<sup>+</sup> Regulatory Cells Involves a Granzyme B-Dependent, Perforin-Independent Mechanism

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## CUTTING EDGE

## Cutting Edge: Contact-Mediated Suppression by CD4<sup>+</sup>CD25<sup>+</sup> Regulatory Cells Involves a Granzyme B-Dependent, Perforin-Independent Mechanism<sup>1</sup>

David C. Gondek,<sup>2\*</sup> Li-Fan Lu,<sup>2\*</sup> Sergio A. Quezada,<sup>\*</sup> Shimon Sakaguchi,<sup>†</sup> and Randolph J. Noelle<sup>3\*</sup>

CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells (T<sub>reg</sub>) 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 T<sub>reg</sub>-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 T<sub>reg</sub> is shown by the reduced ability of T<sub>reg</sub> from GZ-B<sup>-/-</sup> mice to suppress as efficiently as T<sub>reg</sub> from WT mice. GZ-B-mediated suppression is perforin independent, because suppression by T<sub>reg</sub> from perforin<sup>-/-</sup> and WT is indistinguishable. Additionally, suppression mediated by T<sub>reg</sub> appears to be mediated, in part, by the induction of apoptosis in the CD4<sup>+</sup>CD25<sup>-</sup> effector cell. In summary, GZ-B is one of the key mechanisms through which CD4<sup>+</sup>CD25<sup>+</sup> T<sub>reg</sub> induce cell contact-mediated suppression. *The Journal of Immunology*, 2005, 174: 1783–1786.

Recent studies have underscored the importance of regulatory T cells (T<sub>reg</sub>)<sup>4</sup> in preventing the emergence of autoimmune disease, dampening the intensity of immune responses to pathogens and mediating peripheral transplantation tolerance. Multiple subsets of T<sub>reg</sub> have been implicated in these processes and include the thymically derived CD4<sup>+</sup>CD25<sup>+</sup> T<sub>reg</sub> (1), as well as an inducible regulatory T cell (Tr1) subset (2). A major focus of study has been to molecularly characterize the mechanisms that mediate T<sub>reg</sub> 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<sup>+</sup>CD25<sup>+</sup> T<sub>reg</sub> (4–6). In addition to suppression via soluble factors, the CD4<sup>+</sup>CD25<sup>+</sup> T<sub>reg</sub> have been shown to mediate suppression via a contact-dependent mechanism (7). The mo-

lecular basis for contact-dependent suppression by CD4<sup>+</sup>CD25<sup>+</sup> T<sub>reg</sub> is not known.

Glucocorticoid-induced TNF-like receptor (GITR or TNFSF18) is a member of the TNFR family that is constitutively expressed on T<sub>reg</sub> and inducibly expressed on CD4<sup>+</sup>CD25<sup>-</sup> effector T cells (T<sub>eff</sub>) (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 T<sub>reg</sub>, and anti-GITR-treated activated T<sub>reg</sub> 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 T<sub>reg</sub> 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<sup>+</sup> T cells are also able to synthesize GZ-B and perforin (12, 13). Furthermore, studies by Ley and coworkers as well as others (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<sup>+</sup>CD25<sup>+</sup> T<sub>reg</sub> 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<sup>+</sup>CD25<sup>+</sup> T<sub>reg</sub>.

## Materials and Methods

### Mice and materials

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

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<sup>4</sup> Abbreviations used in this paper: T<sub>reg</sub>, regulatory T cell; T<sub>eff</sub>, effector T cell; GITR, glucocorticoid-induced TNF-like receptor; GZ-B, granzyme B; GZ-A, granzyme A; PI, propidium iodide.

### 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<sup>+</sup>CD25<sup>-</sup> and CD4<sup>+</sup>CD25<sup>+</sup> 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<sup>+</sup>CD25<sup>-</sup> and CD4<sup>+</sup>CD25<sup>+</sup> T cells were 90–95%, respectively. Freshly isolated cells have been inoculated (10<sup>6</sup>/ml; complete RPMI 1640/10% FBS supplemented with 100 U of IL2) 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<sup>+</sup>CD25<sup>+</sup> 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<sup>-/-</sup>, or GZ-B<sup>-/-</sup> mice were magnetic bead sorted as stated above. Further purification of the T<sub>eff</sub> subset was accomplished with a CD4<sup>+</sup> T cell Isolation kit (Miltenyi Biotec). Effector cells were >95% pure at the end of this isolation. In a polyclonal T<sub>reg</sub> suppressor assay, CD4<sup>+</sup>CD25<sup>-</sup> T<sub>eff</sub> cells (5 × 10<sup>4</sup>) were cocultured with irradiated T-depleted splenocytes (1 × 10<sup>5</sup>), 5 μg/ml anti-CD3, and indicated numbers of CD4<sup>+</sup>CD25<sup>+</sup> cells for 3 days. In some experiments, 5 μg/ml anti-GITR was also added to the wells. Proliferation was assessed by incorporation of [<sup>3</sup>H]thymidine (1 μCi/well), which was added for the last 8 h of culture.

### Cell surface, intracellular staining, and flow cytometry

Approximately 2 × 10<sup>5</sup> 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 1 × annexin staining buffer and treated with Annexin V<sup>FITC</sup> (BD Pharmingen) and propidium iodide (PI; Sigma-Aldrich). For GZ-B expression assay, following isolation for fresh T<sub>reg</sub> or 24–72 h for cultured T<sub>reg</sub> cells were stained with anti-CD4-FITC (clone RM 4-5) and anti-CD25-PE (clone PC-61). Samples were then fixed and permeabilized (Cytofix/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<sup>+</sup> cells were labeled with 5 μM CFSE and added to suppressor assay as described above.

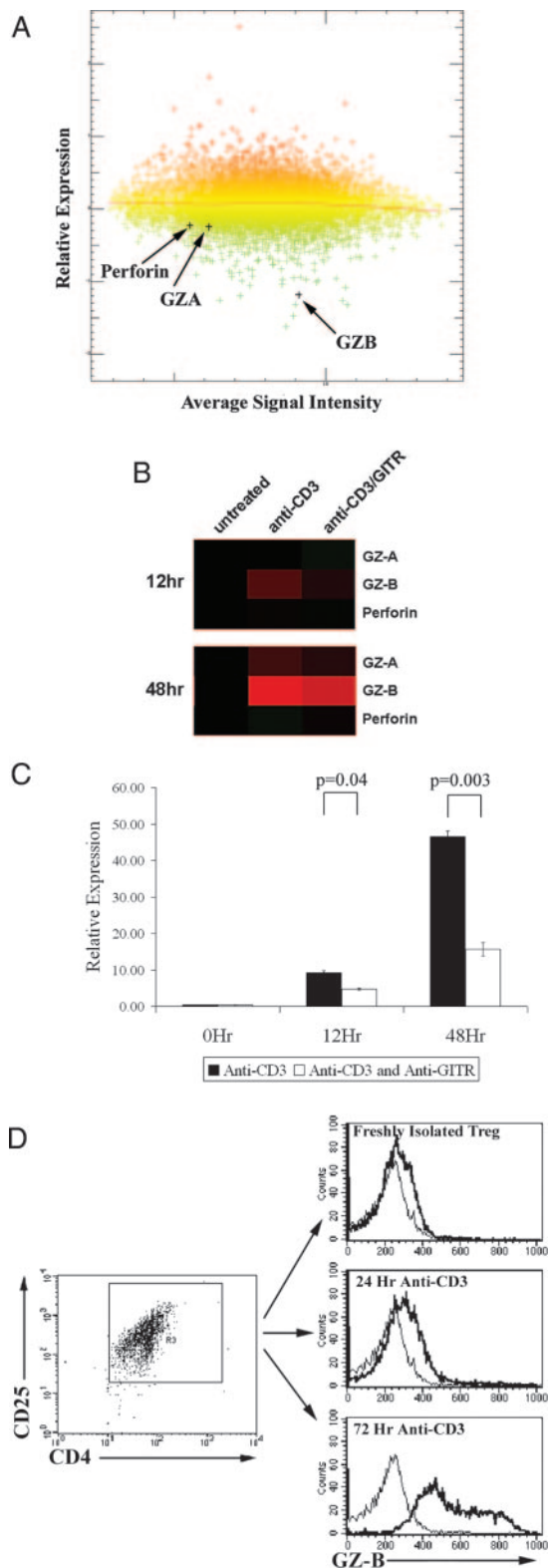
### Statistical analysis

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 T<sub>reg</sub>

CD4<sup>+</sup>CD25<sup>+</sup> T<sub>reg</sub> are suppressive to naive CD4<sup>+</sup> T<sub>eff</sub> 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 T<sub>reg</sub> treated or untreated with anti-GITR was used to identify candidate genes involved in suppression. We examined naive and activated T<sub>eff</sub> (purified CD4<sup>+</sup>CD25<sup>-</sup> T cells) and T<sub>reg</sub> (purified CD4<sup>+</sup>CD25<sup>+</sup> 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 T<sub>reg</sub> 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 T<sub>reg</sub> activation via anti-CD3 alone (9, 17). Studies presented herein show that 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. 1*C*). At both the 12- and 48-h time point, the levels GZ-B expression are 2-fold greater with anti-CD3 alone treatment vs combining with anti-GITR stimula-

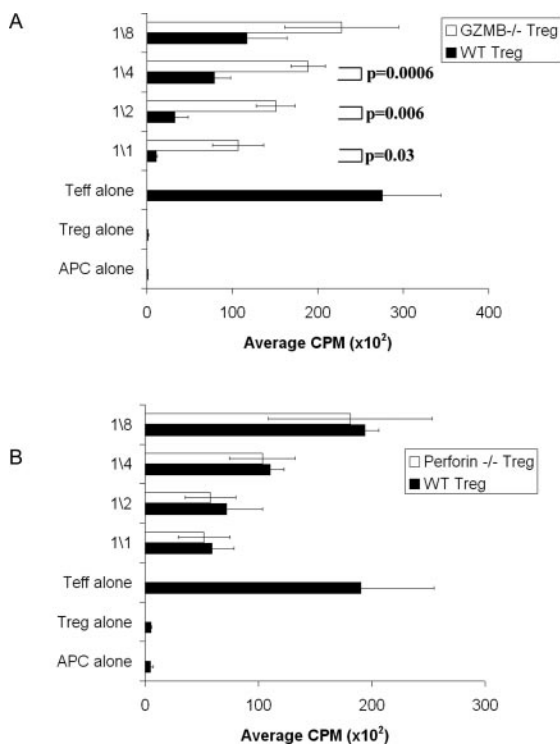


**FIGURE 1.** Activation-induced up-regulation of GZ-B in T<sub>reg</sub> and its regulation by GITR. *A*, Cells were purified via MACS columns to >90% purity and cultured in vitro with anti-CD3 and IL-2 with or without anti-GITR for 12 h. RNA was prepared and hybridized to the Affymetrix A430 array. Relative expression indicates the mean log<sub>2</sub> ratio of changes in T<sub>reg</sub> 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 T<sub>reg</sub> treated as described above. Figure is representative of two independent experiments. *D*, GZ-B expression in freshly isolated T<sub>reg</sub> 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,  $T_{reg}$  GZ-B mRNA expression is 20-fold greater than  $T_{eff}$  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,  $T_{reg}$  rapidly up-regulate GZ-B; however, GZ-A and perforin remain low in abundance relative to GZ-B expression.

#### *GZ-B<sup>-/-</sup> T<sub>reg</sub> have reduced suppressive capacity in vitro*

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



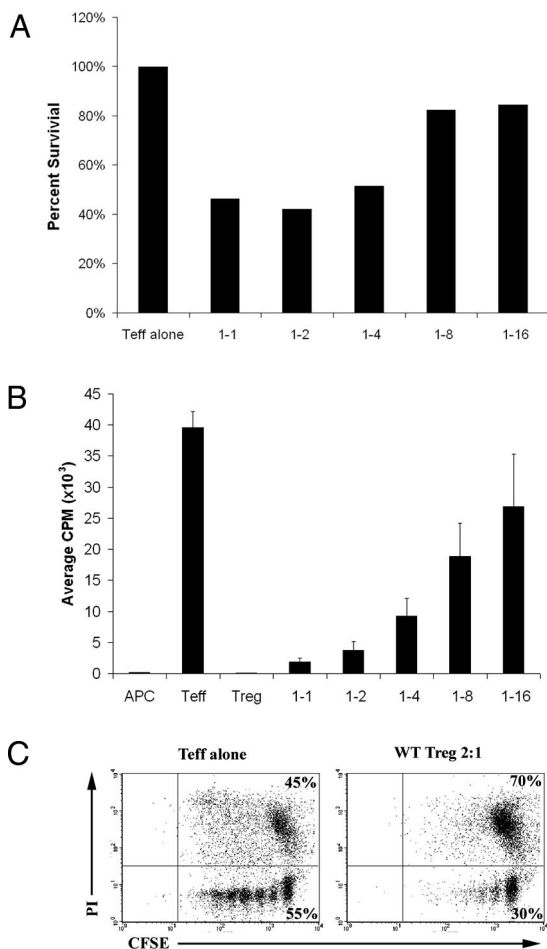
**FIGURE 2.** GZ-B mediates  $T_{reg}$  suppression via a perforin-independent mechanism. *A*,  $T_{reg}$  isolated from wild-type or GZ-B<sup>-/-</sup> mice were cocultured with CD4<sup>+</sup>CD25<sup>-</sup>  $T_{eff}$  and irradiated T-depleted APC with anti-CD3 for 72 h. Wells were pulsed with 1  $\mu$ Ci/well <sup>3</sup>H for the last 8 h of culture, and analyzed as described in *Materials and Methods*. *B*,  $T_{reg}$  isolated from wild-type or perforin<sup>-/-</sup> mice were cultured as above.

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  $T_{reg}$  from perforin<sup>-/-</sup> vs WT mice was determined (Fig. 2B). The suppressive activity of  $T_{reg}$  from perforin<sup>-/-</sup> and WT mice was indistinguishable, suggesting that GZ-B-mediated suppression is via a novel, perforin-independent mechanism. Moreover, studies of perforin<sup>-/-</sup> 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<sup>+</sup>CD25<sup>+</sup>  $T_{reg}$  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 T<sub>eff</sub> apoptosis is a component of contact-mediated suppression*

Recent reports have re-examined apoptosis by  $T_{reg}$  of  $T_{eff}$  as a mechanism for suppression (15, 26). The molecule(s) that mediate the induction of  $T_{eff}$  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  $T_{reg}$  suppression, the ability of  $T_{reg}$  to induce  $T_{eff}$  apoptosis and cell death was re-examined. The induction of  $T_{eff}$  apoptosis by  $T_{reg}$  was determined following the in vitro coculture of activated  $T_{eff}$  and  $T_{reg}$ . Briefly, CD45.1<sup>+</sup> (Ly5.2<sup>+</sup>)  $T_{eff}$  were cocultured with increasing numbers of CD45.2<sup>+</sup> (Ly5.1<sup>+</sup>)  $T_{reg}$ , in the presence of anti-CD3. After 72 h of culture, apoptosis of the CD4<sup>+</sup>  $T_{eff}$  was determined by multiparameter flow cytometry. The data show that there is a dose-dependent increase in cell death of the  $T_{eff}$  cells when cocultured with  $T_{reg}$ , such that ~50% more  $T_{eff}$  are dead at a 1:1 ratio than at a 1:16 ratio of  $T_{reg}$  to  $T_{eff}$  (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  $T_{reg}$  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  $T_{reg}$ , we examined suppression and death with CFSE-labeled  $T_{eff}$  counterstained with PI. In Fig. 3C, we demonstrate that the  $T_{eff}$  have a greater percentage of PI<sup>+</sup> cells when cocultured with  $T_{reg}$ . Interestingly, in addition to the induction of cell death, the proliferation of PI<sup>-</sup>  $T_{eff}$  was also inhibited, which indicates multiple mechanisms are involved in  $T_{reg}$ -mediated suppression.



**FIGURE 3.** T<sub>reg</sub> suppression is mediated by enhanced death in T<sub>eff</sub>. *A*, Ly5.2<sup>+</sup> T<sub>eff</sub> and Ly5.1<sup>+</sup> T<sub>reg</sub> and APC were cocultured for 72 h in a standard suppressor assay. Wells were harvested and stained with anti-Ly5.2 to identify the CD4<sup>+</sup>CD25<sup>-</sup> T<sub>eff</sub>. Cells were then stained with PI and Annexin V<sup>FITC</sup>. *B*, Proliferation of cells in wells run simultaneously with stained wells. Cells were pulsed with 1μCi/well <sup>3</sup>H for the last 8 h of culture. *C*, CFSE-labeled cells were cultured for 72 h in standard suppressor assay, and then counterstained with PI.

The data presented above suggest that GZ-B is pivotal for complete suppression by T<sub>reg</sub>. The fact that the expression of GZ-B is up-regulated by T<sub>reg</sub> activation, and impaired by anti-GITR triggering is consistent with biological activity manifested by T<sub>reg</sub> under these conditions. Functionally, the role of GZ-B in T<sub>reg</sub> activities was strongly suggested by the reduced ability of T<sub>reg</sub> from GZ-B<sup>-/-</sup> mice to suppress T<sub>eff</sub> proliferation. Surprisingly, it appears that the GZ-B-dependent suppression of T<sub>eff</sub> activities is not dependent on perforin, because the T<sub>reg</sub> from perforin<sup>-/-</sup> mice suppressed T<sub>eff</sub> 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<sub>reg</sub> action was demonstrated by the fact that CD4<sup>+</sup>CD25<sup>+</sup> T<sub>reg</sub> in a dose-dependent manner, can induce apoptosis in T<sub>eff</sub>. The current studies raise many questions as to how GZ-B mediates T<sub>eff</sub> death, and the role of GZ-B in the in vivo function of T<sub>reg</sub>.

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