Cutting Edge: Responder T Cells Regulate Human DR \(^{+}\) Effector Regulatory T Cell Activity via Granzyme B

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Pressing activity remains largely unknown, their activity is population.


The production of GzmB by strongly activated CD4 T cells represents a mechanism by which CD4 T cells re-emerges if GzmB is neutralized. We show that re-esterified DR Tregs that lack HLA class II expression (DR Tregs) play a central role in maintaining self-tolerant CD4 cells or by blocking GzmB activity, thus preventing DR Treg death. By silencing the expression of GzmB in responder CD4 cells, the expression of GzmB in the cocultured DR Tregs was also inhibited, indicating that the expression of GzmB in the Tresp and in strongly stimulated Tregs is linked. These data demonstrate a novel role for GzmB to down-regulate effector DR Treg function.

In this report, we investigated how the activity of the highly suppressive DR Treg population may be regulated. We demonstrate that activated CD4 responder T cells (Tresp) produced granzyme B (GzmB) to inactivate DR Tregs. The DR Tregs did not express GzmB or granzyme A (GzmA) during their suppression of weakly stimulated responder cells that expressed low levels of GzmB. Conversely, high GzmB expression by Tresp cells and the appearance of GzmB in the Tregs accompanied the loss in suppression that occurs with strong TCR stimulation. Importantly, the suppressive capacity of DR Tregs was enhanced by inhibiting the expression of GzmB in responder CD4 cells or by blocking GzmB activity, thus preventing DR Treg death. By silencing the expression of GzmB in responder CD4 cells, the expression of GzmB in the cocultured DR Tregs was also inhibited, indicating that the expression of GzmB in the Tresp and in strongly stimulated Tregs is linked. These data demonstrate a novel role for GzmB to down-regulate effector DR Treg function.

Materials and Methods

Cell isolation

Whole mononuclear cells were isolated from human blood drawn as approved by our institutional review board by Ficoll-Hypaque (GE Healthcare Bio-Sciences) gradient centrifugation. Total CD4 T cells were isolated via the CD4 cell negative isolation kit II from Miltenyi Biotec. Tregs were FACS sorted as reported previously (4), gating on HLA DR subsets of CD4 CD62L high CD25 high Tregs (referred to as DR Treg) that express higher ex vivo levels of FoxP3, induce a more vigorous and rapid T cell suppression, and exhibit a much higher capacity to expand than the CD25 high Tregs that lack HLA class II expression (DR Tregs) (4). These features indicate that the DR Tregs represent an effector Treg population.

Although the mechanism by which Tregs exert their suppressive activity remains largely unknown, their activity is highly regulated by cell contact and apoptosis (5). Dependence on cell contact and high expression of FoxP3 are the hallmark attributes used to distinguish Tregs from inhibitory FoxP3 low Tr1 or Th3 cells that suppress via the secretion of IL-10 or TGFβ (6). Recent reports suggest that Tregs can suppress by either inactivating or killing target CD4 T cells (7, 8). The Tregs themselves have also been shown to be highly sensitive to apoptosis that is mitigated by IL-2 (2–15).

More recently, the highly proapoptotic state of the Treg was shown to be modulated by changes in expression of anti-apoptotic Bcl-2 family genes that can be altered by PD1, CD30, or CD28 signals (9, 10).

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MHC class II expression identifies an effector subset of human CD4+CD25 high FoxP3 high natural regulatory T cells (DR Tregs) that induces more rapid suppression and exhibits higher FoxP3 expression than the remaining Treg population. Although Tregs are known to be highly sensitive to apoptosis, in this study we demonstrate that this sensitivity is primarily a feature of DR Tregs. Granzyme B (GzmB) is strongly expressed by nonresponder regulatory CD4 T cells, whereas effector DR Tregs express little GzmB. Strong TCR stimulation markedly increases the expression of GzmB in all dividing responder CD4 T cells and mitigates the suppression by DR Tregs. DR Treg suppressive activity reemerges if GzmB is neutralized. We show that responder cells actively kill effector Tregs by producing GzmB in response to strong TCR stimulation. Thus, the production of GzmB by strongly activated CD4 T cells represents a mechanism by which CD4 T cells resist Treg suppression. The Journal of Immunology, 2009, 183: 4843–4847.

Cutting Edge

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1 This work was supported by National Institutes of Health Grants NS24242, U01 DK6192601, R01 NS2424710, P01 AI39671, and P01 NS38037, by National Multiple Sclerosis Society Grants RG3828A1, RG2172C9, and RG3308A10, by the 2004 Federation of Clinical Immunology Societies Centers of Excellence Amgen Award, and by the 2007 Data Scholars Award.

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3 Abbreviations used in this paper: Treg, regulatory T cell; FasL, Fas ligand; GzmA, granzyme A; GzmB, granzyme B; siRNA, small interfering RNA; Tresp, responder T cell.

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expression (A), each well with 1
proliferation was monitored on day 4 by CFSE dilution (11) or by pulsing (24 h)
expression was completely nonviable and lacked all staining (data not shown). The frequencies given relate to the total Treg or total Tresp population. These data are representative
in contrast to the Tresp cells, of which
membrane integrity and intracellular FoxP3 expression (B)
(BD Biosciences) using CellQuest software and analyzed using FlowJo software
V (BD Pharmingen). Data were acquired on a FACSCalibur flow cytometer
was assessed by a 20-min incubation with a live/dead viability dye (Invitrogen)
(eBioGrB; eBioscience), or isotypes. In some experiments, loss of cell viability
expression was performed using the buffers from the eBioscience FoxP3 staining
rapid early suppressive activity of DR+
pathway in human T cells (12). We previously demonstrated
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Results
Strong TCR signals inhibit DR+ Treg suppression and enhance GzmB expression
In contrast to the murine system, CD2 is a major costimulatory pathway in human T cells (12). We previously demonstrated that CD2 and not CD28 costimulatory signals induced the rapid early suppressive activity of DR+ Tregs (4). Because
CD4+ CD25+ Tresp were plated at 2.5 × 10^5/well and the DR+ or DR− Tregs at 1.25 × 10^5/well, resulting in a 2:1 ratio. CFSE dilution (11) or by pulsing (24 h)
each well with 1 μCi of [3H]thymidine (PerkinElmer). Intracellular protein expression was performed using the buffers from the eBioscience FoxP3 staining
with mAbs for FoxP3 (206D BioLegend or PCH101; eBioscience), GzmB
expression of GzmB and Treg suppression.
no modulation of responder cell expression of GzmB. These
data suggested an inverse relationship between responder cell expression of GzmB and Treg suppression.

Figure 1
Strong anti-CD3/anti-CD2 (αCD3αCD2) stimulation increases GzmB in Tresp and decreases suppression and viability of DR+ Tregs. Both DR+ and DR− Tregs were cultured alone or with CFSE-labeled Tresp at low or high anti-CD3 with anti-CD2 costimulation and analyzed for high and low GzmB expression (A, top) and CFSE dilution (A, bottom). The initial sorted ex vivo Treg populations and the CFSE− Tregs from 4-day cocultures, were analyzed for membrane integrity and intracellular FoxP3 expression (B). In contrast to the Tresp cells, of which <10% were nonviable, the Tregs from Treg-only cultures were completely nonviable and lacked all staining (data not shown). The frequencies given relate to the total Treg or total Tresp population. These data are representative of three experiments with cells from different donors.

Cell culture reagents
Cells were cultured in RPMI 1640 medium supplemented as described previously (4) with 5% human AB serum (Cellgro Mediatech). The anti-CD3 (UCHT1 at 1 or 3 μg/10^6 beads) and anti-CD2 (BMA 0111 at 1 μg/10^6 beads) Abs were purchased from BD Pharmingen and Dade Behring, coupled to tosyl-activated beads (DynaBiotech), and used at 1 × 10^5 beads/well. GzmB inhibitor 1 (z-AAD-CMK), purchased from EMD/Calbiochem, was added at 1.0 μM.

In vitro micro coculture assay and flow cytometry analyses
Sorted cell populations were plated directly after ex vivo isolation; the CD4+ CD25+ Tresp were plated at 2.5 × 10^5/well and the DR+ or DR− Tregs at 1.25 × 10^5/well, resulting in a 2:1 ratio. CFSE dilution (11) or by pulsing (24 h)
each well with 1 μCi of [3H]thymidine (PerkinElmer). Intracellular protein expression was performed using the buffers from the eBioscience FoxP3 staining

GzmB quantification
One hundred microliters of supernatants collected from day 4 cell cultures were interrogated for GzmB content via the human soluble protein flex system from

Small interfering RNA (siRNA) transduction
CD4+ CD25− Tresp (1 × 10^6) were electrophorated with 2.3 nmol of each individual annealed and HPLC-purified siRNA oligonucleotides purchased from Ambion as suggested by the human T cell siRNA transduction kit (Ambaxa). The Ambion catalogue numbers for the siRNA oligonucleotides were s6387 (siGzmB) and 4635 (siControl) (Ambion).

Results
Strong TCR signals inhibit DR+ Treg suppression and enhance GzmB expression
In contrast to the murine system, CD2 is a major costimulatory pathway in human T cells (12). We previously demonstrated that CD2 and not CD28 costimulatory signals induced the rapid early suppressive activity of DR+ Tregs (4). Because

Figure 2
Strong stimulation induces the secretion of active GzmB. Supernatants from 4-day weak or strong anti-CD3/anti-CD2 (αCD3αCD2) stimulations of Tresp cells, DR+ or DR− Tregs, and 1:0.5 cocultures were examined for total GzmB content (A) and GzmB activity (B). The shaded area denotes GzmB levels in unstimulated cultures. CFSE− Tresp or anti-CD3/anti-CD2-stimulated DR+ Treg cocultures exhibited markedly less suppression and no modulation of responder cell expression of GzmB. These data suggested an inverse relationship between responder cell expression of GzmB and Treg suppression.
Strong stimulation led to Treg apoptosis, the DR of stimulation. To determine whether the GzmB induced by strong stimulation had no effect on the level or activity of the GzmB secreted with edly reduced GzmB secretion, whereas the addition of Tregs to Tresp with either type of Treg under weak stimulation mark-induced by weak stimulation that was inactive. Coculturing with stronger stimulation in contrast to the low level of GzmB that Tresp secreted a greater amount of enzymatically active GzmB.Tregs were stained for viability and FoxP3 after 4 days of co-culture with strong stimulation (Fig. 1A). The data indicated that although the DR Tregs expressed the highest levels of FoxP3 ex vivo, they responded to strong stimulation with a striking loss of viability and concomitant loss in FoxP3 expression, whereas only a small fraction of DR Tregs lost viability and FoxP3 expression.

Because strong stimuli drastically increased GzmB expression in Tresps, we next measured whether stimulation strength affected the secretion and activity of GzmB. As shown in Fig. 2, Tresps secreted a greater amount of enzymatically active GzmB with stronger stimulation in contrast to the low level of GzmB induced by weak stimulation that was inactive. Coculturing Tresp with either type of Treg under weak stimulation markedly reduced GzmB secretion, whereas the addition of Tregs had no effect on the level or activity of the GzmB secreted with strong stimulation.

DR Tregs are highly sensitive to apoptosis

Because the entire Treg population has been suggested to be highly sensitive to apoptosis, we next examined whether the DR and DR Tregs exhibited differential sensitivity to apoptosis and expressed different ratios of BclxL/Bax. Measuring the expression of Bcl family proteins in weakly stimulated and actively suppressing Tregs, the DR Tregs exhibited a significantly lower level of BclxL vs Bax and a higher expression of annexin V (>50%) than the DR Tregs (Fig. 2C). In additional experiments, we found that treatment with recombinant human GzmB augmented DR Treg apoptosis, whereas it had no effect on Tresp or DR Treg viability (supplemental Fig. 1). These data suggest that the DR Tregs are much more sensitive to apoptosis than the DR Tregs, which only exhibit 20% annexin V expression.

GzmB inhibits DR Treg-induced suppression by inducing their apoptosis

To determine whether GzmB directly inhibited suppression in DR Treg cocultures, we established DR Treg cocultures with different strength stimuli where GzmB was or was not blocked from the outset with an irreversible cell-permeable peptide inhibitor that specifically binds to the active site of GzmB (GzmB inhibitor I, z-AAD-CMK). Examining whether inhibiting GzmB in the context of strong TCR stimulation reduced the level of DR Treg apoptosis, we measured annexin V expression in replicate cultures established under low-dose (1 μg/ml) or high-dose (3 μg) anti-CD3/anti-CD2 stimulation.

The stronger TCR stimulation again induced the DR Tregs to become extremely apoptotic (Fig. 3B, bottom), as >70% expressed annexin V in response to high-dose anti-CD3/anti-CD2, while 45% expressed annexin V in the lower stimulation cocultures (Fig. 3A). When GzmB activity was blocked, the frequency of apoptotic DR Tregs was reduced to 48% in these strong stimulation cocultures and the level of suppression in DR Treg cocultures was markedly enhanced, as the frequency of CFSEhigh responder cells more than doubled from 27 to 58% (Fig. 3B). In contrast, the addition of the inhibitor had no effect on the complete suppression induced in the low-strength stimulation cocultures or on the poor suppression induced by anti-CD3/anti-CD28 stimulation (supplemental Fig. 2). Thus, inactivating GzmB increased suppression and DR Treg viability in strong anti-CD3/anti-CD2 stimulated cocultures.

FIGURE 3. Strong stimulation abrogates suppression and induces GzmB-dependent apoptosis of DR Tregs. CFSE-labeled Tresp cells were stimulated with weak (A) or strong (B) anti-CD3/anti-CD2 (αCD3αCD2) alone or together with DR or DR Tregs (at 1:0.5 ratio), in the presence or absence of a GzmB peptide inhibitor (z-AAD-CMK; 1 μg/ml) and monitored for proliferation (CFSE dilution; top) and apoptosis of CFSE Tregs (annexin V; bottom) on day 4. The frequencies refer to the percentages of Tregs or of Tresp cells, not the percentages of all cultured cells. The Tregs stimulated alone were completely dead and are not shown. Measures of coculture suppression by [3H]thymidine incorporation are shown for a representative experiment (C) and from four repeat experiments (D).

4 The online version of this article contains supplemental material.
The ability of a GzmB blockade to augment DR⁺ Treg suppression was also observed when proliferation was measured via [³H]thymidine-incorporation (Fig. 3C). In four separate experiments using cells from different donors, blocking the activity of GzmB typically resulted in a 30% increase in DR⁺ Treg suppression over that of control cocultures (Fig. 3D), whereas there was no effect on the DR⁻ Treg suppressive capacity. These data indicated that GzmB plays an active role in inhibiting the suppressive capacity of DR⁺ Tregs in response to strong TCR signals, while it has little effect on DR⁻ Treg suppression.

**CD4 Tregs produce GzmB to regulate DR⁻ Treg effector activity**

To determine whether the source of the “suppression-inactivating” GzmB was the CD4 Tresp or the Treg, we selectively silenced GzmB in Tresps by electroporating them with siRNA oligonucleotides specific for GzmB or a negative control siRNA, labeling them with CFSE, and using them as targets of suppression in Treg coculture assays. Responder T cells transduced with GzmB oligonucleotides expressed significantly less GzmB and underwent enhanced suppression after anti-CD3/anti-CD2 stimulation as compared with siControl responder cells (Fig. 4A, *top*). Thus, the frequency of nonproliferating, CFSEhigh responder cells increased from 59 to 97% for siGzmB-treated cells cocultured with DR⁺ Tregs, whereas the siControl responder cells or the DR⁻ Treg cocultures showed no change in suppression (Fig. 4A, *bottom*). Analyzing the CFSE⁻ Tregs in these cocultures, the DR⁺ Tregs were found to only express GzmB and an exacerbated loss of viability and FoxP3 expression if they had been stimulated in the presence of GzmB-expressing siControl Tresps (Fig. 4B).

**Discussion**

Activation-induced cell death and activated T cell autonomous death are two death pathways thought to maintain T lymphocyte homeostasis, contract the immune response, and delete inappropriate self-reactive cells (13). T cells can undergo apoptosis by the extrinsic pathway via signals from death receptors such as Fas/Fas ligand (FasL) or by the intrinsic pathway involving altered ratios of pro-apoptotic and anti-apoptotic Bcl-2 family members. In this study we show that strong TCR signals can also induce the death of effector Tregs via a third mechanism: by increasing their sensitivity to GzmB-mediated death.

A number of reports on Treg sensitivity to apoptosis under different experimental conditions have come to contrasting conclusions on the role of Fas or TCR signals in CD25high Treg apoptosis. Fritzsching et al. reported that long-term expanded human total CD25high Tregs were resistant to activation-induced cell death by TCR restimulation with anti-CD3/anti-CD28 but sensitive to apoptosis induced by CD95 cross-linking (14). More recently, Strauss et al. showed that CD25high Tregs were sensitive to death when cocultured with CD4 responder cells in the presence of high (150 U/ml) doses of IL-2, but resistant to apoptosis at extremely high (1000 U/ml) levels of IL-2 (15). Our data demonstrating that DR⁺ Tregs are highly sensitive to apoptosis mediated by GzmB produced by CD4 T cells suggest a mechanism for this observation, whereby GzmB-mediated induction of Treg apoptosis is a mechanism used by highly activated T cells to counteract highly suppressive Tregs.

In contrast to reports that granzymes are effector molecules produced by a subset of mouse Tregs or by CD46-induced human Tregs, our data indicate that GzmB actually inhibits the effector function of GzmB⁻ Tregs. Our data indicate that CD4 T cells are not susceptible to GzmB, supporting prior reports of strong CD4 T cell expression of the GzmB inhibitor PI-9 (16). Whether the DR⁻ and DR⁺ Tregs differentially express PI-9 in response to different strength stimulation is under investigation. Moreover, we find that GzmB is only expressed by DR⁺ Tregs when they are stimulated under conditions that will not lead to suppression, such as strong TCR stimulation in the presence of GzmB-sufficient responder CD4 T cells. Furthermore, as compared with the DR⁻ Tregs, the DR⁺ Tregs express less of the antiapoptotic Bcl proteins that can inhibit mitochondrial release of cytochrome c (13).

The data we present here indicate that the highly suppressive DR⁺ effector Tregs are regulated by apoptosis and are significantly more prone to undergo cell death than the DR⁻ Tregs.
Similar to the DR− Tregs, naive Tregs from adult peripheral blood (CD45RA+CD25−) are also relatively resistant to apoptosis (17) and do not express HLA class II (data not shown). We show that strongly activated responder cells can regulate effector Tregs by producing GzmB in a mechanism that is distinct from the Fas/FasL signaling reported by others (15, 18). Importantly, these data also indicate that the higher frequency, proliferation-competent, DR+ Tregs remain viable in the presence of strong stimuli and that all CD4 Trespects have the capacity to express GzmB. Furthermore, although human Tregs have been suggested to function by killing targets via a GzmA-mediated mechanism (7), we found no evidence that human DR− or DR+ Tregs expressed GzmA in highly suppressive cocultures (data not shown). Rather, our data suggest that the GzmB produced by Tresps limits the suppressive capacity of human DR+ effector Tregs. Together with the previous findings that activated/cycling Tregs are sensitive to FasL-induced apoptosis (17), our finding that in vivo activated, nonproliferating effector Tregs expressed GzmA in highly suppressive cocultures (data not shown). Rather, our data suggest that the GzmB produced by Tresps limits the suppressive capacity of human DR+ effector Tregs. Together with the previous findings that activated/cycling Tregs are sensitive to FasL-induced apoptosis (17), our finding that in vivo activated, nonproliferating effector DR+ Tregs are sensitive to GzmB-induced apoptosis suggests the importance of apoptosis in Treg homeostasis, and indicates that different apoptotic pathways may be involved in regulating functionally distinct Treg populations.

In conclusion, these data indicate a previously unrecognized role for GzmB in regulating the interaction between responder CD4 T cells and effector DR+ Tregs, whereas it has little effect on the less suppressive DR− Tregs. Shedding light on the observations made a number of years ago linking strong stimulation with poor Treg suppression, these new findings suggest that strongly stimulated CD4 T cells use GzmB to resist suppression by natural Tregs.

Disclosures
The authors have no financial conflict of interest.

References


Supplemental Figure 1. DR+Treg are sensitive to rhGzmB mediated death. FACS sorted responder T cells, DR+Treg and DR-Treg were cultured for 12 hours without stimulation, in the presence of exogenous IL-2 (50U/ml) to prevent Treg death by cytokine deprivation, and in the absence (top) or presence (bottom) of rhGzmB (5μg/ml, R&D Sytems). After harvest, staining for loss of membrane integrity via the live/dead viability dye (Invitrogen), demonstrated that the DR+Treg were highly prone to die as one-fourth lost viability in the control cultures as compared to only 6% of the DR-Tregs even though IL-2 was present. Moreover, the addition of rhGzmB selectively augmented the death of the DR+Treg as it increased the frequency of non-viable DR+Tregs by over 50% while the rhGzmB had little effect on the similarly treated DR-Treg and Tresp populations. These data are representative of two repeat experiments using cells from different donors.

Supplemental Figure 2. The blockade of GzmB activity had no effect on suppression or death in response to αCD3αCD28 stimulation. CFSE labeled Tresp cells were cultured alone or in the presence of DR+ or DR-Tregs, and in the presence or absence of GzmB inhibitor peptide, and monitored for proliferation/suppression by CFSE dilution and death by Annexin V binding at day 4 (a). The cultures were stimulated with beads bound with αCD3 (UCHT1 at 1μg per 10^7 beads) and αCD28 (28.2 at 1μg per 10^7 beads). There was no detectable suppression in response to this stimulation regardless of whether GzmB inhibitor was present. This assay was performed with the same cells shown in Figure 3 of the manuscript where suppression re-emerged in co-cultures given strong αCD3αCD2 stimulation if GzmB activity was neutralized. The CD4 T cells were shown to express the highest levels of GzmB in response to the αCD3αCD28 stimulation as compared to the weak and strong αCD3αCD2 bead stimuli used in the accompanying manuscript (b).
Responder T cells only

No rhGzmB

rhGzmB (5μg/mL)

Co-culture

Cell #

Loss of Viability

A

1

24

6

3

37

8

24

1