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Cutting Edge: Regulatory T Cells Do Not Mediate Suppression via Programmed Cell Death Pathways

Andrea L. Szmyczak-Workman, Greg M. Delgoffe, Douglas R. Green, and Dario A. A. Vignali

Regulatory T cells (Tregs) play a critical role in the immune system to regulate peripheral tolerance and prevent autoimmunity. However, the relative importance of different mechanisms of Treg function remains obscure. In this article, we reveal a limited role for programmed cell death pathways in mediating Treg suppression of conventional T cells. We show that Tregs are able to suppress the proliferation of conventional T cells that are resistant to apoptosis (Bim\(^{-/-}\), Bim\(^{-/-}\), Puma\(^{-/-}\), Bcl-2 transgenic) or receptor-interacting serine-threonine kinase-dependent necrosis (also referred to as regulated necrosis or necroptosis) (Ripk3\(^{-/-}\)) in several in vitro and in vivo assays. These data suggest that programmed cell death pathways, such as apoptosis and receptor-interacting serine-threonine kinase-dependent necrosis, are not required for Treg-mediated suppression. The Journal of Immunology, 2011, 187: 4416–4420.

Regulatory T cells (Tregs) are potent mediators of immune regulation and play a key role in maintaining peripheral tolerance. A number of Treg populations have been identified, primarily based on their origin of development (thymus versus periphery) and the factors that induce their development (Foxp3, TGF-β, IL-2, retinoic acid, IL-10, IL-35) (1–3). Although a broad array of suppressive mechanisms has been proposed to mediate Treg function, the relative contribution and importance of these mechanisms remain controversial. It was proposed that Tregs suppress conventional T cells (Tconvs) by causing IL-2 deprivation-mediated apoptosis (4). High IL-2R (CD25) expression on Tregs may lead to increased IL-2 “consumption,” effectively depleting the local surroundings and, thereby, starving Tconvs of this important growth factor that is required for their survival. However, the relative contribution of this mechanism is controversial, because more recent studies showed that IL-2 depletion alone is not required for the suppression of human T cells (5, 6). Furthermore, the general contribution of cell death pathways in mediating Treg function remains unclear.

Two forms of programmed cell death have been described: apoptosis and receptor-interacting serine-threonine kinase (RIPK)-dependent necrosis. Apoptosis in response to a variety of stimuli is regulated by members of the B cell lymphoma 2 (Bcl-2) family (7). Cells from mice overexpressing Bcl-2, an antiapoptotic molecule that inhibits the mitochondrial death pathway, are resistant to apoptosis induced by growth factor and cytokine deprivation, radiation exposure, and treatment with glucocorticoids, phorbol esters, ionomycin, and sodium azide (8, 9). The proapoptotic molecule Bim (encoded by the Bcl2l11 gene), in its active state, binds to Bcl-2 in response to stress signals, such as growth factor deprivation, thereby priming the mitochondrial pathway of apoptosis (10). Bim\(^{-/-}\) T cells are resistant to apoptosis induced by cytokine or growth factor withdrawal, particularly IL-2 (11). The BH-3 only gene Puma (encoded by the Bbc3 gene) is a transcriptional target of the tumor suppressor p53 (12, 13). Lymphocytes from Puma\(^{-/-}\) mice are highly resistant to DNA-damaging drugs and gamma radiation. These cells also have decreased sensitivity to p53-independent cell death stimuli, such as growth factor deprivation and treatment with dexamethasone and phorbol esters (13). Analysis of Bim\(^{-/-}\), Puma\(^{-/-}\) mice showed that these two proteins cooperate in mediating apoptosis of T cells during development, following activation (14, 15) and upon cytokine withdrawal (16, 17). RIPK-dependent necrosis (also referred to as regulated necrosis or necroptosis) (18), is a recently described novel form of programmed cell death that requires the receptor-interacting serine-threonine kinases Ripk1 and Ripk3 (16, 19, 20). Although the mitochondrial pathway of apoptosis is a major mechanism of mammalian cell death, there are other relevant apoptotic and nonapoptotic cell-death pathways. These include the death receptor, inflammasome, and caspase-2 pathways of apoptosis, as well as active necrosis mediated by the mitochondrial permeability transition and by receptor-interacting protein kinases (21).

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Abbreviations used in this article: 7AAD, 7-aminoactinomycin D; Bcl-2, B cell lymphoma 2; RIPK, receptor-interacting serine-threonine kinase; Tconv, conventional T cell; Tg, transgenic; Treg, regulatory T cell; WT, wild-type.

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In this study, we assessed whether the two forms of programmed cell death, apoptosis and RIPK-dependent necrosis, contribute to the mechanisms used by Tregs to mediate suppression. This is particularly relevant given previous suggestions that Tregs mediate suppression via cytokine deprivation-mediated apoptosis (specifically IL-2), which is blocked by loss of Bim expression (4). Thus, we asked whether Tregs are capable of suppressing Tconvs that are resistant to apoptosis (Bim−/−, Bim−/−Puma−/−, Bcl-2 Tg) and RIPK-dependent necrosis (Ripk3−/−).

Materials and Methods

Mice

C57BL/6 (wild-type [WT]) mice were obtained from The Jackson Laboratory. Bim−/− mice were provided by Andrea Strasser (The Walter and Eliza Hall Institute of Medical Research, Parkville, Australia) (11). Puma−/− mice were provided by Gerard Zambetti (St. Jude Children’s Research Hospital) (13). Bcl-2 Tg mice were provided by John Reed (Salk Institute, La Jolla, CA) (8). Ripk3−/− mice were provided by Vishva Dixit (Genentech, South San Francisco, CA) (22). These, as well as Rag1−/−CD3ε−/− and Rag1−/− mice, were bred and maintained at St. Jude Children’s Research Hospital. All animal experiments were performed in American Association for the Accreditation of Laboratory Animal Care-accredited, specific pathogen-, Helicobacter-, and murine norovirus-free facilities following national, state, and institutional guidelines. Animal protocols were approved by the St. Jude Animal Care and Use Committee.

Cell purification and flow cytometry

Spleens and lymph nodes from mice were processed and stained with fluorochrome-conjugated Abs and purified by FACS (MoFlo; DakoCyto-mation) using anti-CD4, anti-CD25, and anti-CD45RB (Tconvs: CD4+CD45RBhiCD25−; Tregs: CD4+CD45RBloCD25+ in all assays reported). All Abs used were from BioLegend or eBiosciences.

In vitro suppression assays

The assay was performed as previously described (23). Briefly, Tconvs (CD4+CD45RBhiCD25−, 2.5 × 105) were cultured with irradiated splenocytes and anti-CD3 with titrating numbers of Tregs for 60 h. The assay was performed as previously described (26). Briefly, Tconvs (2 × 106) via the tail vein into Rag1−/− mice were sorted and incubated with varying concentrations of purified Tregs, and proliferation was assessed following stimulation by anti-CD3 plus APCs. Tregs from WT mice were able to suppress Tconvs from Bim−/−, Bim−/−Puma−/−, Bcl-2 Tg, and Ripk3−/− mice in a manner indistinguishable from WT Tconvs, as shown by [3H]thymidine uptake (Fig. 1). Tregs were also equally capable of

In vivo homeostasis assays

The assay was performed as previously described (26). Briefly, Tconvs (2 × 106) were injected or not with Treg (0.5 × 106) via the tail vein into Rag1−/− Tconv recipients or Rag1−/− mice (Bcl-2 Tg Tconv recipients). Spleens were analyzed 7 d posttransfer.

Inflammatory bowel disease

The colitis recovery model of inflammatory bowel disease was used, as described previously, with modifications (26). Briefly, 0.4 × 106 purified Tconvs were injected via the tail vein into Rag1−/− mice. Recipients were monitored and, upon clinical signs of disease (5–10% body weight loss, ∼3–4 wk post-
FIGURE 2. Tregs can suppress Tconv with defects in apoptosis via TGF-β and IL-35. A. Tconvs sorted from spleen and lymph nodes of C57BL/6 (WT), Bim−/−, and Ripk3−/− mice were labeled with CFSE and cultured with WT Tregs at a 1:2 ratio for 68 h. Analysis of CFSE dilution was measured by flow cytometry. Data are the mean ± SE of three independent experiments, with each group assayed in triplicate. B. The Tconv populations depicted were stained with fluorochrome-conjugated Abs to CD4 and Annexin V and the viability dye 7AAD, and the percentage of cell death was determined by flow cytometry. Data are the mean ± SE of three independent experiments, with each group assayed in triplicate. C. Tconvs from WT, Bim−/−, and Ripk3−/− mice were cultured with anti-CD3/anti-CD28-coated magnetic beads in the bottom chamber of a 96-Transwell plate. In the top chamber, 1.25 × 10⁶ WT Tregs and WT Tconvs were stimulated with anti-CD3/anti-CD28-coated magnetic beads for 72 h. [Note that WT Tconvs are required in the top chamber to potentiate maximal Treg activity (24).] Cultures were supplemented with 10 μg/ml of isotype control, anti–TGF-β, anti–IL-10, or anti–IL-35 Abs, as indicated. Tconvs in the bottom wells were pulsed with [³H]thymidine for the last 8 h of culture, and proliferation was measured. Data are the mean ± SE of three independent experiments, with each group assayed in triplicate.

Next, we performed in vivo homeostasis assays to determine whether Bim-mediated apoptosis, or mechanisms that can be blocked by Bcl-2, play a role in the control of Tconv homeostatic expansion. Tconvs were purified from Bim−/− and Bcl-2 Tg mice and transferred either alone or with WT Tregs into lymphopenic mice; splenic T cell numbers were assessed 1 wk later. Both Bim−/− and Bcl-2 Tg Tconvs expanded following transfer (Fig. 3A, 3B). Importantly, the capacity of Tregs to suppress the homeostatic proliferation of Bim−/− and Bcl-2 Tg Tconvs was comparable to WT Tconvs.

Lastly, Tregs have been shown to control colitis, a mouse model of inflammatory bowel disease, initiated by the transfer of naive Tconvs into lymphopenic Rag1−/− recipients. We used the recovery method of colitis induction to compare the ability of Tregs to ameliorate disease caused by WT versus Bim−/− Tconvs (29). This method represents a more physiologic environment in which Tregs would act to exert suppression. WT or Bim−/− Tconvs were transferred into Rag1−/− recipients and monitored for clinical signs of sickness (weight loss). At ∼3–4 wk post-Tconv transfer, mice began to lose weight and were injected with Tregs and monitored for clinical recovery (weight gain) and histological recovery (using a semiquantitative grading scheme to score pathology). Control mice that did not receive Tregs continued to lose weight and had a marked increase in large intestine pathology (Fig. 3C, 3D). However, Bim−/− Tconv recipients that received Tregs recovered to the same extent, if not better, than did WT Tconv recipients receiving Tregs. Bim−/− Tconv recipients had restored appetite and resumed weight gain by 2 wk post-Treg transfer and had a marked decrease in large intestine pathology, as shown by reduction in mean pathology score (Fig. 3D) and reduced inflammation and goblet cell destruction (Fig. 3E). These results demonstrated that Tconvs that are resistant to apoptosis induced by suppress WT, Bim−/−, and Ripk3−/− Tconvs, as determined by CFSE dilution (Fig. 2A). Furthermore, no functional defects were observed in the capacity of Tregs from any of the mouse strains used to suppress their corresponding Tconv population or WT Tconvs (Fig. 1). To exclude the possibility that Tconv death was occurring in these Treg cocultures, we stained Tconvs with Annexin V and 7AAD. There was no difference in the percentage of live Tconvs among any of the groups, with an average of just ∼3–8% of Tconvs undergoing cell death in the presence of Tregs (Fig. 2B). These results suggested that in vitro Treg-mediated suppression is not mediated via cell death induced by cytokine or growth factor withdrawal, because these cells are resistant to death by such mechanisms.

Although it is clear that WT and knockout Tconvs are being suppressed without undergoing cell death, it is possible that different suppressive mechanisms are being used. To probe this issue, we restricted analysis to contact-independent Treg-mediated suppression via inhibitory cytokines by using Transwell plates in which the Tregs and Tconv targets are separated by a permeable membrane. We previously showed that maximal Treg activity in this setting requires contact with Tconvs (24), which were included in the top chamber with Tregs, whereas the WT and mutant target Tconvs were placed in the bottom chamber. The contribution of TGF-β, IL-10, and IL-35 (25, 28) was assessed by the inclusion of neutralizing Abs. Comparable Treg-mediated suppression of WT, Bim−/−, and Ripk3−/− Tconvs was observed across the permeable Transwell membrane (Fig. 2C). Furthermore, suppression of all three Tconv populations was mediated comparably by TGF-β and IL-35, but not by IL-10, suggesting that genetically induced resistance to apoptosis or RIPK-dependent necrosis did not affect the sensitivity of Tconvs to inhibitory cytokines and, thus, the mechanisms used by Tregs to mediate suppression.
Tregs can control the homeostatic expansion and colitis caused by Tconvs with defects in apoptosis. Splenic T cells were sorted from C57BL/6 (WT), Bim\textsuperscript{−/−} (A), and Bcl-2 Tg (B) mice. Tconvs were injected into Rag1\textsuperscript{−/−} CD3\textsuperscript{+}CD4\textsuperscript{+}CD8\textsuperscript{+} (A) or Rag1\textsuperscript{−/−} (B) mice, with or without Tregs. Spleens from recipients were analyzed 7 d later. Average percentage of suppression of Tconvs is depicted above each group. Each graph is representative of two experiments, with four to six mice/group. C through E, Tconvs were sorted from the spleen and lymph nodes of C57BL/6 (WT) or Bim\textsuperscript{−/−} mice and injected i.v. into the tail vein of Rag1\textsuperscript{−/−} recipients. Loss of body weight was monitored on a weekly and biweekly basis. Approximately 3–4 wk post-Tconv transfer, upon clinical signs of sickness, Tregs were injected i.p. into the Tconv recipients. C, Body weight was monitored for an additional 4 wk. Scores (D) and representative histology (E) of the large intestine of the T cell recipients –8 wk post-Tconv transfer and 4 wk post-Treg transfer. Original magnification \( \times 10 \). Data are the mean \( \pm \) SE of three independent experiments, with 13–15 (Treg recipients) and 4–6 (controls, no Tregs) mice/group. * \( p < 0.05 \), ** \( p < 0.005 \).

Given a report suggesting that Bim\textsuperscript{−/−} Tconvs cannot be suppressed, implying that Tregs function in a Bim-dependent manner (4). It is unclear why our data and the published observations differ, but this could be due to differences in the experimental systems used. Differences in the Treg/Tconv purification (FACS versus MACS; cell markers used), media selection, type of stimulation, and cell numbers used may have contributed to the discrepancies observed, particularly with the in vitro studies. Nevertheless, it is clear that there is suppression in our study without cell death. A strength of our study is the use of multiple genetic models with deficiencies in cell death pathways that collectively demonstrated in two in vivo systems that Tregs are able to suppress proliferation and pathogenicity in the absence of Tconv death. Susceptibility to and recovery from colitis are greatly affected by the recipient’s microbiota, which may also contribute to discrepancies between our observations and those from the Lenardo group (4).

Tregs use multiple mechanisms to mediate suppression (1). Thus, systems that do not present a challenging environment for Treg function can be of limited value in evaluating the contribution of a specific suppressive mechanism. The colitis-recovery model that we used is a more robust method for analyzing Treg suppression during colitis and, thus, is an effective approach for revealing critical mechanisms used by Tregs. For instance, although a role for IL-10 and IL-35 in Treg suppression is only marginally revealed by in vitro assays, this recovery model clearly demonstrated a key role for these cytokines in Treg function (1). Thus, we would argue that if Bim-mediated apoptosis contributed significantly to Treg function, it would have been revealed in this assay. Nevertheless, we cannot rule out the possibility that this mechanism is used by Tregs but only under unique circumstances or disease scenarios. Furthermore, although Bim and Puma are primary mediators of apoptosis, we cannot rule out the possibility that Tregs use mechanisms of cell death that are Bim, Puma, and Ripk3 independent and not blocked by Bcl-2. Several studies showed that Tregs do not simply suppress T cells but also convert them to an induced regulatory population via suppressive cytokines, such as TGF-β, IL-10, and IL-35 (1). Such mechanisms allow Tregs to expand their regulatory control and potency. In contrast, lytic or apoptotic mechanisms might limit the capacity of Tregs to mediate their suppressive capacity. Further analysis will be required to fully assess the relative contribution of the diverse mechanisms used by Tregs to mediate immune control.

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Disclosures
The authors have no financial conflicts of interest.

References
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