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# Human T Regulatory Cells Can Use the Perforin Pathway to Cause Autologous Target Cell Death

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## Summary

**Cytotoxic T lymphocytes and natural killer cells use the perforin/granzyme pathway to kill virally infected cells and tumor cells. Mutations in genes important for this pathway are associated with several human diseases. CD4+ T regulatory (Treg) cells have emerged as important in the control of immunopathological processes. We have previously shown that human adaptive Treg cells preferentially express granzyme B and can kill allogeneic target cells in a perforin-dependent manner. Here, we demonstrate that activated human CD4+CD25+ natural Treg cells express granzyme A but very little granzyme B. Furthermore, both Treg subtypes display perforin-dependent cytotoxicity against autologous target cells, including activated CD4+ and CD8+ T cells, CD14+ monocytes, and both immature and mature dendritic cells. This cytotoxicity is dependent on CD18 adhesive interactions but is independent of Fas/FasL. Our findings suggest that the perforin/granzyme pathway is one of the mechanisms that Treg cells can use to control immune responses.**

## Introduction

The innate and adaptive immune systems include cytotoxic T-lymphocytes (CTLs) and natural killer (NK) cells that can identify and kill cells infected with intracellular pathogens, and also tumor cells. Although there are several mechanisms by which these cells kill, the major one is the perforin/granzyme pathway (Lieberman, 2003; Russell and Ley, 2002). Dysregulation of this pathway is associated with several human diseases, including hemophagocytic lymphohistiocytosis, Griscelli's syndrome, and X linked lymphoproliferative disease (Arico et al., 2001; Menasche et al., 2000; Stepp et al., 1999). Although CD4+ T cells have been shown to express perforin and granzymes, the functional importance of the granule exocytosis pathway in these cells is not clear (Grossman et al., 2003; Hanson et al., 1991; Susskind et al., 1996).

CD4+ T cells with regulatory properties (Treg cells) are generally divided into two subtypes, natural and adaptive, based on their ontogeny and mode of action (Bluestone and Abbas, 2003; Maloy and Powrie, 2001; Sakaguchi, 2000). Natural Treg cells are generated in the thymus, constitutively express high levels of CD25, require the transcription factor *FoxP3*, and mediate their suppressive effects in vitro in a cell contact-dependent manner. Adaptive Treg cells are generated in the periphery, require IL-2 for their survival and function, and are believed to suppress immune responses by releasing anti-inflammatory cytokines such as IL-10 and TGF-β. Both subtypes of CD4+ Treg cells control pathological processes, including graft versus host disease, autoimmunity, and infections (Bluestone and Abbas, 2003; Dittmer et al., 2004; Maloy and Powrie, 2001; Sakaguchi et al., 2001). Most of the work defining these regulatory subsets has been carried out in murine models, and studies of human Treg cells are limited.

Several reports have indicated that natural Treg cells exert their antiproliferative effects via a cell contact-dependent mechanism; however, the precise mechanism(s) by which this effect is mediated remains to be determined (Bluestone and Abbas, 2003; Sakaguchi et al., 2001; Shevach, 2002). Since perforin/granzyme-mediated target cell death requires cell contact, we investigated whether Treg cells are armed with cytotoxic granules. We have previously shown that adaptive Treg cells generated from human CD4+ T cells by coligation of the T cell coreceptor CD3 and the complement regulatory protein CD46 in the presence of IL-2 express predominantly granzyme B (Grossman et al., 2004). These adaptive Treg cells demonstrated cytotoxicity against allogeneic tumor cell lines in a perforin-dependent, but MHC/TCR-independent, manner.

In this report, we characterize the granzyme expression pattern in human Treg cells and show that activated natural CD4+CD25+ Treg cells express predominantly granzyme A. We demonstrate that both subtypes of Treg cells exhibit perforin-dependent cytotoxicity against a variety of autologous target cells, including CD4+ and CD8+ T cells, CD14+ monocytes, and dendritic cells. Interestingly, activated CD4+ and CD8+ T cells were preferentially killed over unactivated CD4+ and CD8+ T cells. In addition, immature myeloid dendritic cells were preferentially killed over LPS matured dendritic cells by Treg cells. We found no involvement of the Fas/

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Fas ligand (FasL) pathway in the observed cytotoxicity, but selective inhibitors of perforin (e.g., EGTA and concanamycin A), as well as neutralizing antibodies against the adhesive protein CD18, abrogated killing. These results establish that human natural and adaptive Treg cells can kill autologous target cells in a CD18 and perforin-dependent manner and suggest a role for the perforin/granzyme pathway in immune regulation by Treg cells.

## Results

### Human Adaptive T Regulatory Cells Express Granzyme B upon Activation and Exhibit Perforin-Dependent Cytotoxicity against Autologous Target Cells

We have previously shown that adaptive Treg cells are generated by stimulating $CD4^+$ T cells with antibodies to CD3/CD46 in the presence of IL-2 (Kemper et al., 2003). These adaptive Treg cells are highly proliferative, secrete IL-10, and supernatants from adaptive Treg cells suppress naive T cell proliferation through the production of IL-10. We have recently demonstrated that these cells also express granzyme B and perforin and are able to kill allogeneic tumor cell lines in a perforin-dependent manner (Grossman et al., 2004). An example of the flow cytometric profile of these cells is shown in Figure 1A. These cells are large and granular, as reflected by considerable forward and side scatter, respectively. As previously established, these cells predominantly express granzyme B (Figure 1A). The increase in cell size and granularity of the adaptive Treg cells was confirmed by light microscopy, which also revealed an open nuclear chromatin pattern (Figure 1B). Confocal immunofluorescence confirmed the presence of granzyme B-containing granules (Figure 1C). Granzyme A expression by adaptive Treg cells was not detected by flow cytometry or confocal immunoflourescence (Figures 1A and 1C).

Since adaptive Treg cells kill allogeneic tumor cell lines in a perforin-dependent (but MHC/TCR-independent) manner (Grossman et al., 2004), we tested whether these cells exhibited cytotoxicity against autologous targets. $CD4^+$ T cells were isolated and cultured with IL-2 alone or with antibodies to CD3/CD46 to generate adaptive Treg cells. Target cells consisted of purified $CD4^+$ and $CD8^+$ T cells isolated from the same donor and cultured for 4 days in IL-2 only or with plate bound antibodies to CD3/CD28. Freshly purified autologous $CD14^+$ cells were also used as target cells. Adaptive Treg effector cells were cocultured with CFSE-labeled target T cell subsets or $CD14^+$ monocytes at a 20:1 effector to target ratio. Target cell death was analyzed by flow cytometry by using 7-AAD incorporation and reduction in cell size as surrogate markers of cell death (Dumitriu et al., 2001; Grossman et al., 2004; Lecoeur et al., 2001). $CD4^+$ T cells stimulated with IL-2 alone were inefficient at killing autologous target cells ($CD4^+$, $CD8^+$, and $CD14^+$ cells), consistent with their lack of granzyme expression (data not shown) (Grossman et al., 2004). Adaptive Treg cells induced the death of autologous activated $CD4^+$ and $CD8^+$ T cells, as well as freshly isolated $CD14^+$ monocytes, while resting $CD4^+$ and $CD8^+$ T cell targets were relatively resistant to killing (Figure 2A). The observed killing was perforin dependent, since cytotoxicity was abolished with the perforin inhibitors EGTA and concanamycin A (Figure 2A; data not shown). Blocking antibodies to CD18 were similar to EGTA in their ability to inhibit cytotoxicity (Figure 2B), suggesting that adhesive immunological synapse interactions are required for cytotoxicity. FasL neutralizing antibodies failed to prevent cytotoxicity in these studies but were effective at neutralizing the cytotoxicity by purified FasL against Jurkat cells, (Figures 2C and 2D). Interestingly, the purified FasL was unable to induce significant cytotoxicity against U937 targets, which express high levels of surface Fas, suggesting that additional cellular requirements besides Fas expression are required for Fas/FasL induced cytotoxicity (Figure 2D) (Grossman et al., 2004).

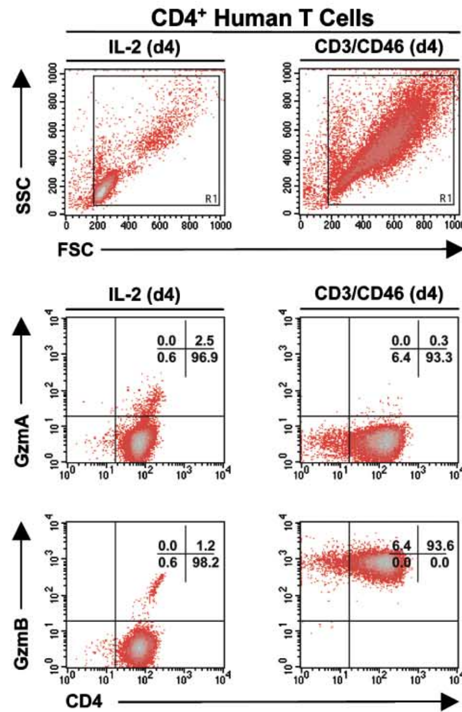
### Human Adaptive T Regulatory Cells Preferentially Kill Autologous Immature Myeloid Dendritic Cells

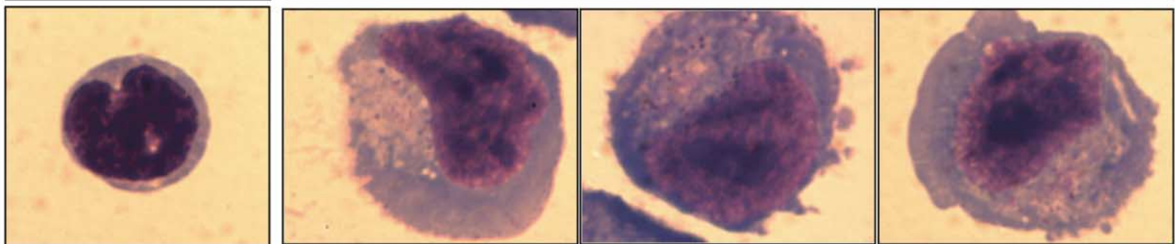
Because of the high level of cytotoxicity observed against freshly isolated autologous $CD14^+$ monocytes (Figure 2A) and myeloid tumor cell lines (Grossman et al., 2004), we tested the ability of adaptive Treg cells to induce the death of myeloid dendritic cells. $CD14^+$ monocytes were isolated from the same donor and cultured in the presence of GM-CSF and IL-4 for 4 days to generate immature myeloid dendritic cells as previously described (Sallusto and Lanzavecchia, 1994). Adaptive Treg cells were efficient at inducing the death of autologous immature myeloid dendritic cells, with $>80\%$ of target cells incorporating 7-AAD after 4 hours of coculture (Figure 3). In contrast, maturation of the myeloid dendritic cells with LPS for 24–36 hr significantly reduced the percentage of 7-AAD positive cells. The observed killing was perforin- and CD18 dependent, since cytotoxicity was abolished by EGTA or blocking antibodies against CD18, while FasL blocking antibodies had no effect (Figure 3). To ensure that the addition of LPS to mature dendritic cells had no effect on the adaptive Treg cells, LPS was added to the killing assay, which caused no reduction in cytotoxicity (data not shown).

### Human $CD4^+CD25^+$ Natural T Regulatory Cells Express Granzyme A after Activation

Since adaptive Treg cells express granzyme B and were cytotoxic against autologous targets, we tested whether natural $CD4^+25^+$ Treg cells expressed a similar granzyme profile under identical modes of stimulation. Human $CD4^+$ T cells were purified from peripheral blood by magnetic isolation, stained with antibodies against CD25, and then sorted to obtain $CD4^+CD25^+$ double-positive natural Treg cells. Only the highest CD25-positive cells ($<2\%$ of all $CD4^+$ cells; Figure 4A) were used in these experiments, since this selective sorting is required to isolate natural Treg cells from human peripheral blood (Baecher-Allan et al., 2001). These flow-purified $CD4^+CD25^{+hi}$ cells yielded a strong PCR signal for the transcription factor FoxP3, while the $CD4^+CD25^-$ cell population yielded a substantially weaker signal (Figure 4B).

To further confirm that these $CD4^+CD25^{+hi}$ cells exhibit the characteristics of natural Treg cells, coculture experiments were performed with autologous $CD4^+CD25^-$



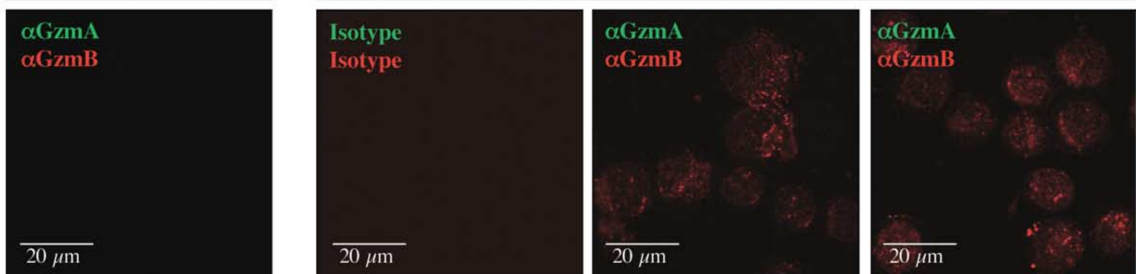




Figure 1. Activation of Human CD4$^+$ T Cells with CD3/CD46 Antibodies to Generate Adaptive Treg Cells Results in the Expression of Granzyme B, but Not Granzyme A

(A) Human CD4$^+$ T cells exhibit a differential increase in side-scatter (SSC)/granularity and forward scatter (FSC)/size when stimulated with CD3/CD46 + IL-2 versus IL-2 alone (top). Increased granularity in CD3/CD46 activated CD4$^+$ T cells corresponded with the expression of granzyme A (middle), but not granzyme B (bottom).

(B) Light microscopic evaluation (1000×) of adaptive Treg cells demonstrating their large size, abundant cytoplasm, cytoplasmic granules, and open nuclear chromatin pattern, compared to CD4$^+$ T cells incubated in IL-2 only.

(C) Confocal immunofluorescence microscopy of adaptive Treg cells using specific monoclonal antibodies shows cytoplasmic granular staining for granzyme B (red), but not granzyme A (green), in adaptive Treg cells, but not resting T cells. Cell size is indicated. Data shown are representative of more than three independent donor samples. The cells shown in (C) are different from those in (A).

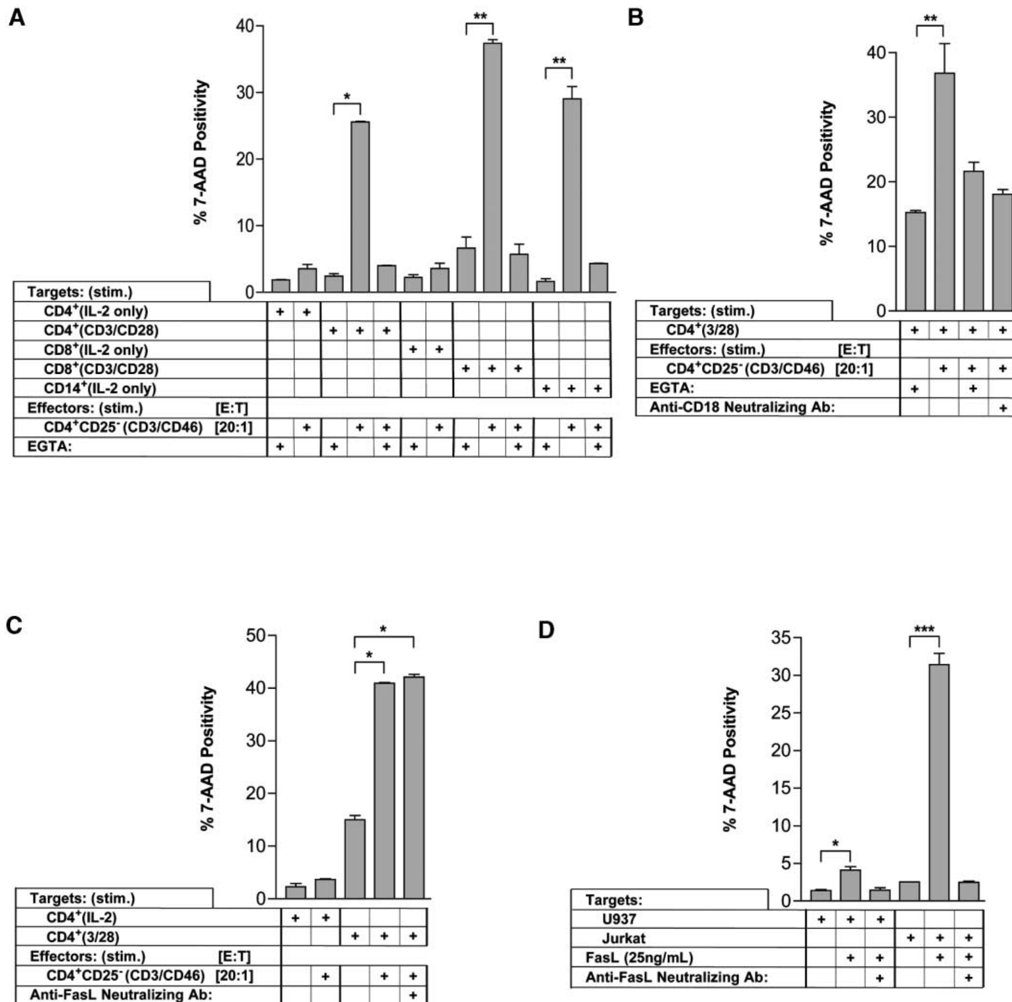



Figure 2. Human Adaptive Treg Cells Exhibit Perforin-Dependent Cytotoxicity against Autologous T Cells and Monocytes

(A) Representative example of adaptive Treg cell cytotoxicity against autologous target cells. Adaptive Treg effector cells were cultured with different autologous target cells at a 20:1 ratio for 4 hr, and the percentage of 7-AAD positive cells was measured. EGTA (4mM) was added to target cells alone to control for EGTA toxicity.

(B) Neutralizing antibodies against CD18 abrogated adaptive Treg cell cytotoxicity similar to EGTA inhibition.

(C) FasL neutralizing antibodies failed to inhibit adaptive Treg cell cytotoxicity.

(D) Neutralizing FasL antibody abrogates cytotoxicity induced by recombinant human soluble FasL in a cell line susceptible to Fas/FasL induced cytotoxicity (Jurkat). Also shown is the relative resistance of the U937 cell line to cytotoxicity by soluble FasL. Data shown is representative of more than three independent donor samples. Values shown are the mean ± SD. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

cells. Stimulation of $CD4^+CD25^-$ cells with either CD3/CD28 or CD3/CD46 in the presence of IL-2 (10–50 IU/mL rhIL-2) resulted in a strong proliferative responses (Figure 4C). In contrast, $CD4^+CD25^{+hi}$ cells stimulated under identical conditions showed little proliferation (Figure 4C). However, inclusion of a larger percentage of $CD25^+$ cells (i.e., top 3%–5% of $CD4^+CD25^+$ cells) resulted in moderate proliferation (data not shown). When $CD4^+CD25^{+hi}$ cells were cocultured with $CD4^+CD25^-$ cells, the proliferative response of $CD4^+CD25^-$ cells to plate bound CD3/CD28 or CD3/CD46 was abolished. Neutralizing antibodies to IL-10 had no effect on the inhibition of proliferation by the $CD4^+CD25^{+hi}$ cells (Figure 4C). Finally, flow cytometry of the isolated $CD4^+CD25^{+hi}$ cells showed them to express high levels of L-selectin and CD45RO, consistent with prior reports (data not shown) (Baecher-Allan et al., 2001). These results confirm that these $CD4^+CD25^{+hi}$ cells exhibit the characteristics of natural Treg cells (Figure 4C).

We next stained these cells with antibodies to granzyme A and B as previously described (Grossman et al., 2004). Unstimulated natural Treg cells showed minimal granularity and no expression of either granzymes A or B (Figure 5A). In contrast to the flow cytometry profile of CD3/CD46 generated adaptive Treg cells, stimulation of natural Treg cells with plate bound antibodies to CD3 and CD46 in the presence of IL-2 induced a population of cells that showed increased granularity (side scatter) but no significant increase in cell size (forward scatter). This granular population also corresponded to a new population of $CD4^{+lo}$ cells (Figure 5A). Staining with granzyme-specific antibodies established that these granu-

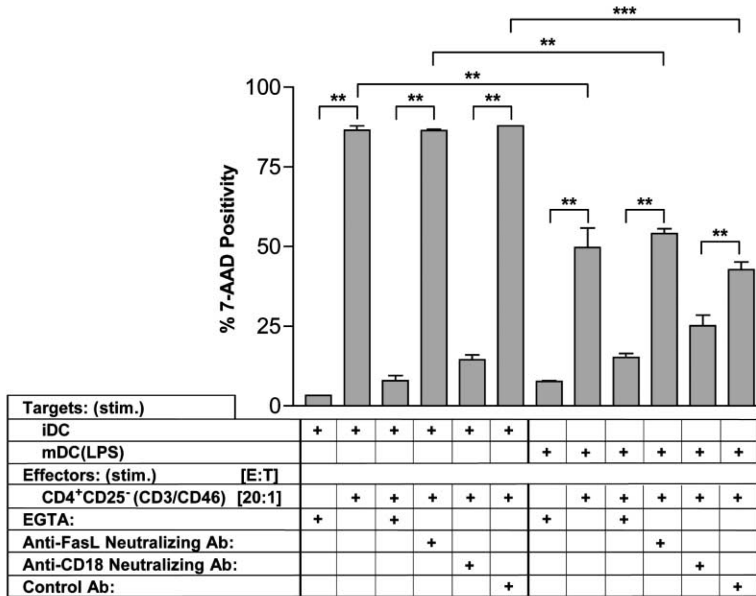



**Figure 3. Human Adaptive Treg Cells Exhibit Perforin-Dependent Cytotoxicity against Autologous Myeloid Dendritic Cell Targets**

Representative example of adaptive Treg cell cytotoxicity against immature (iDC) and LPS matured (mDC) autologous myeloid dendritic cells showing resistance to cytotoxicity by mDCs. mDCs were generated by culture of iDCs with LPS for 36 hr prior to the killing assay. Adaptive Treg cells and autologous dendritic cells were incubated for 4 hr and 7-AAD incorporation measured. EGTA (4 mM), neutralizing antibodies to FasL and CD18 were added to the killing assay. Effector to target (E:T) ratios are shown. Data shown are representative of more than three independent donor samples. Values shown are the mean ± SD. **$p < 0.01$, ***$p < 0.001$.

lar, $CD4^{+lo}$ cells expressed predominantly granzyme A (gate R2, Figure 5A). Similar results were seen when these cells were stimulated with CD3/CD28 in the presence of IL-2, although the percentage of $CD4^{+lo}$ cells expressing granzyme A was reduced compared with CD3/CD46 stimulated cells (21% versus 85%, respectively; Figure 5A). Light microscopic examination of CD3/CD46 stimulated natural Treg cells showed a slight increase in cytoplasm, while the nuclear chromatin pattern remained condensed, similar to natural Treg cells cultured in IL-2 alone (Figure 5B). Confocal immunofluorescence microscopy confirmed the presence of granules containing granzyme A, but not granzyme B (Figure 5C). Furthermore, these cells were shown to be viable (i.e, they excluded 7-AAD and were not apoptotic by morphological criteria; data not shown; Figure 5B).

### Human $CD4^+CD25^+$ Natural T Regulatory Cells Exhibit Perforin-Dependent Cytotoxicity against Autologous Target Cells

Given the induction of granzyme A in stimulated natural Treg cells, we tested their ability to kill a variety of autologous target cells. Natural Treg cells were isolated by flow cytometry and cultured for 4 days with IL-2 only or with plate bound antibodies to CD3/CD46, then used as effector cells in a flow-based killing assay. Although unstimulated natural Treg cells exhibited minimal cytotoxicity (Figure 6A), stimulated natural Treg cells were capable of killing purified autologous $CD4^+$, $CD8^+$, and $CD14^+$ target cells (Figure 6A). This killing was shown to be perforin dependent, since the perforin inhibitors EGTA and concanamycin A abrogated nearly all cytotoxicity (Figure 6A; data not shown). CD3/CD28 activated autologous $CD4^+$ and $CD8^+$ T cell targets were more efficiently killed than target cells cultured in IL-2 alone (Figure 6A). Neutralizing antibodies to FasL failed to inhibit cytotoxicity (Figure 6B). However, neutralizing antibodies to CD18 blocked cytotoxicity as effectively as EGTA (Figure 6A; data not shown). Interestingly, significant target cell death occurred at very low effector:target ratios (i.e., 1:1), indicating potent cytotoxicity by the activated natural Treg cells (Figures 6A and 6B). In contrast, when adaptive Treg cells were utilized in the killing assay at a 1:1 ratio, no significant cytotoxicity was observed (data not shown). CD3/CD28 stimulated natural Treg cells similarly killed autologous target cells but less than that of CD3/CD46 stimulated natural Treg cells (data not shown).

Autologous dendritic cells were also tested as targets of natural Treg cell killing. Both immature and mature dendritic cells were incubated with natural Treg cells as described above, and cytotoxicity was measured. Interestingly, natural Treg cells exhibited cytotoxicity against both immature and mature dendritic cells, and similar to adaptive Treg cell killing, immature dendritic cells were more susceptible to cytotoxicity than mature dendritic cells (Figure 6C). However, the effectiveness of dendritic cell killing was much lower by natural Treg cells compared to adaptive Treg cell killing (35% versus versus 75%, respectively; Figures 6C and 3). Both EGTA and neutralizing antibodies to CD18 were able to abrogate the observed cytotoxicity to near background levels (Figure 6C). Finally, CD3/CD28-stimulated natural Treg cells were significantly less effective at killing autologous immature dendritic cells than CD3/CD46-stimulated natural Treg cells (Figure 6C; different donor than depicted in Figure 5A).

## Discussion

In this report we demonstrate different patterns of granzyme A and B expression in human Treg cell subsets. Upon activation, $CD4^+CD25^+$ natural Treg cells predominantly express granzyme A, while adaptive Treg cells express granzyme B. Both Treg cell populations are highly granular, as determined by flow cytometry (side scatter) and confocal immunofluorescence studies. Adaptive Treg cells proliferated vigorously and exhibited

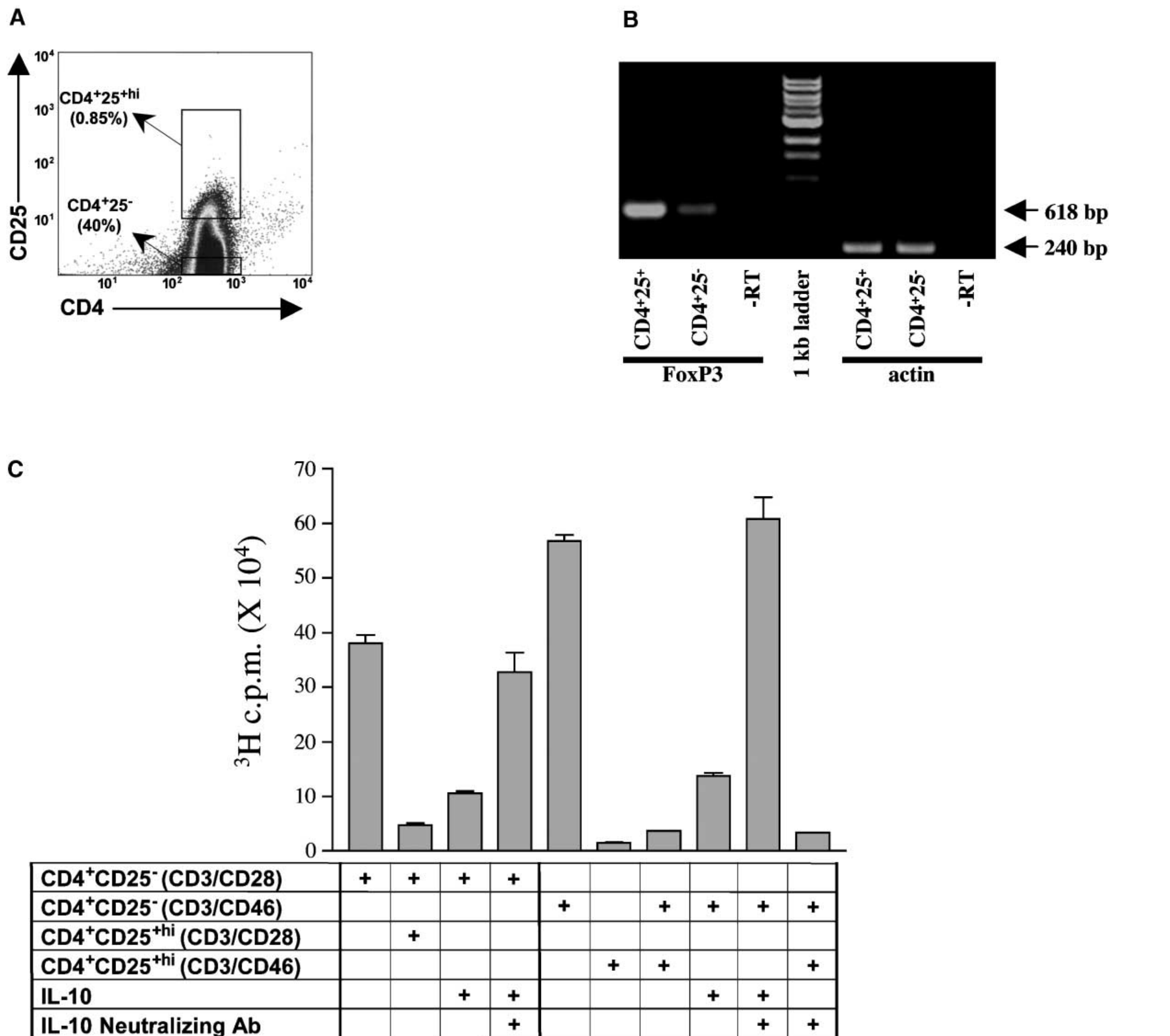



Figure 4. Isolation and Characterization of Human Natural T Tegulatory Cells

(A) Representative FACS plot of purified human CD4$^+$ T cells demonstrating the gate used to sort CD4$^+$CD25$^+$ natural Treg cells and CD4$^+$CD25$^-$ T cells. All experiments were performed by using $<$2% of the highest CD25$^+$ expressing CD4$^+$ T cells.

(B) RT-PCR analysis for FoxP3 expression in flow-sorted CD4$^+$CD25$^+$ natural Treg cells and CD4$^+$CD25$^-$ T. β-actin control lanes are shown to the right for each sample to control for cDNA input, and −RT indicates cDNA samples generated without reverse transcriptase.

(C) Coculture $^3$H-thymidine proliferation assay demonstrating suppression of autologous CD4$^+$CD25$^-$ T cell proliferation by CD4$^+$CD25$^+$ natural Treg cells. Ratios of CD4$^+$CD25$^+$ natural Treg cells to CD4$^+$CD25$^-$ responder T cells were at 1:2 for all data points shown. Individual and coculture samples were performed in the presence of plate bound anti-CD3/CD28 or anti-CD3/CD46 in the presence of IL-2 (10 U/mL). Recombinant IL-10 (10 ng/mL) was able to inhibit CD4$^+$25$^-$ T cell proliferation under both stimulation conditions, and neutralizing antibody to IL-10 was able to abrogate the effect of exogenous IL-10. CD4$^+$CD25$^+$ natural Treg cells exhibit low proliferative capacity but are able to inhibit the proliferation of CD4$^+$CD25$^-$ T cells. Neutralizing IL-10 antibodies had no effect on the suppression of CD4$^+$CD25$^-$ cells by CD4$^+$CD25$^+$ natural Treg cells.

a "blast-like" morphology, whereas activated natural Treg cells did not proliferate or increase in size, and their chromatin pattern remained condensed. We also demonstrate that both activated natural and adaptive Treg cells exhibit perforin-dependent cytotoxicity against autologous targets, including activated CD4$^+$ and CD8$^+$ T cells and dendritic cells.

Although the mechanism(s) by which the Treg cell subsets recognize their targets is unclear, several lines of evidence suggest that it is an MHC/TCR-independent process, akin to NK cell target recognition. First, we have previously shown that adaptive Treg cells kill the allogeneic tumor cell line K562 (Grossman et al., 2004), which lacks both MHC class I and class II expression (Day et al., 2003; Grossman et al., 2004). Second, the effector Treg cells were not primed against their target cells prior to the killing assay. Third, both Treg cell subtypes effectively killed autologous target cells. Interestingly, Rabinovich et al. (2003) recently reported that lymphokine-activated killer cells preferentially kill activated autologous CD4$^+$ and CD8$^+$ T cells through a perforin/NKG2D-dependent pathway. In addition, we demonstrated that neutralizing antibodies against CD18, a critical component of immune synapses for both CD8$^+$ and

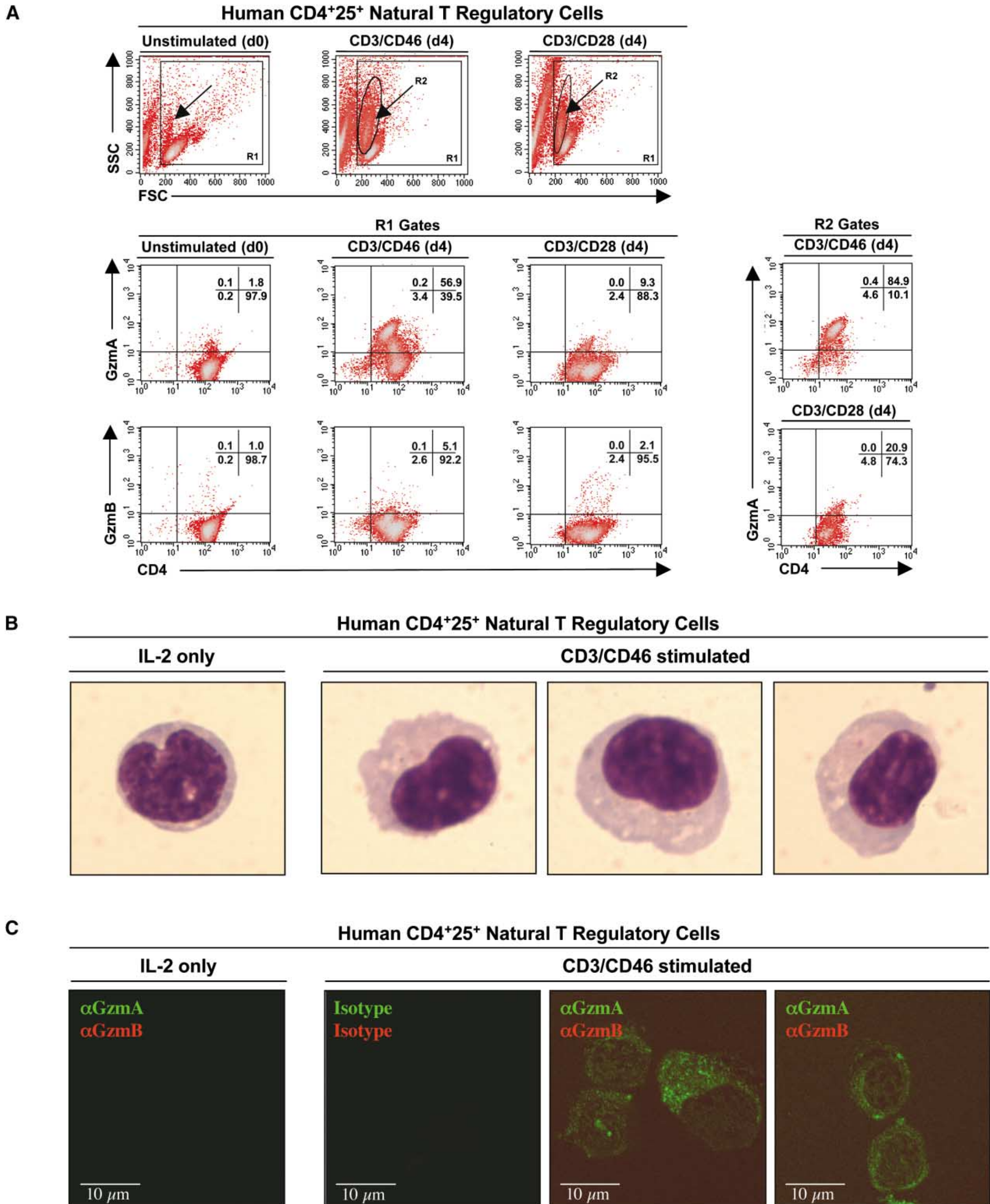



Figure 5. Activation of Human Natural Treg Cells Results in the Expression of Granzyme A, but Not Granzyme B

(A) Natural Treg cells show an increase in granularity (SSC), but not size (FSC), when stimulated through CD3/CD46 (arrow). The granular population (R2 gate) corresponds to a cell population expressing low surface CD4 and granzyme A, but not granzyme B (bottom/side panels). CD3/CD28 stimulation of natural Treg cells results in a similar granular $CD4^{lo}$ population but with fewer granzyme A-expressing cells (bottom/side panels).

(B) Light microscopic evaluation of $CD4^+$ T cells stimulated with IL-2 only and natural Treg cells stimulated with CD3/CD46. Pictures are shown at 1000× magnification.

(C) Confocal immunofluorescence of CD3/CD46-activated natural Treg cells with specific monoclonal antibodies against granzyme A or granzyme B shows cytoplasmic granular staining for granzyme A (green), but not granzyme B (red). Isotype antibodies controls and cell size are indicated. Data shown are representative of more than three independent donor samples. The cells shown in (C) are different from those in (B).

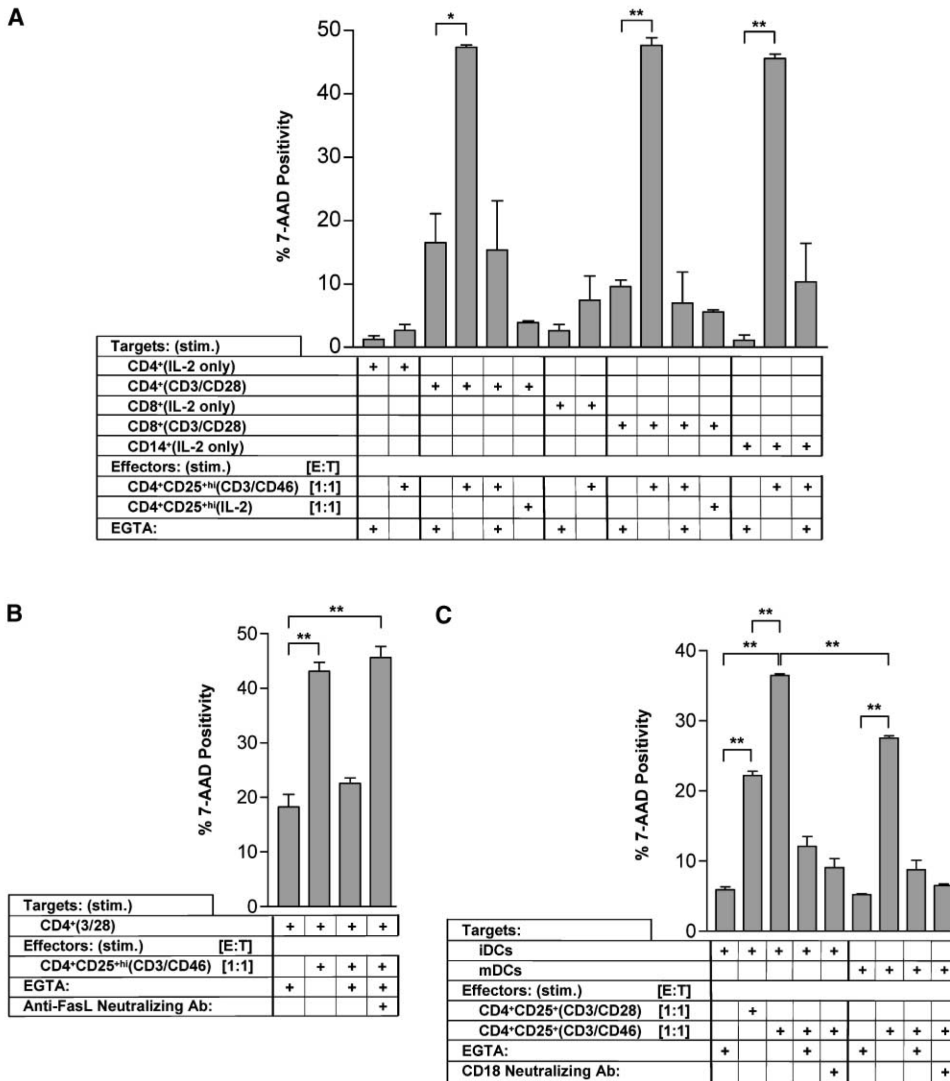


Figure 6. Human Natural Treg Cells Exhibit Perforin-Dependent Cytotoxicity against Autologous T Cells and Monocytes

(A) Representative example of natural Treg cell cytotoxicity against different autologous target cells at 4 hr. Cytotoxicity was preferential against CD3/CD28-activated $CD4^+$ and $CD8^+$ target cells, as well as freshly isolated $CD14^+$ monocytes, and required activation of natural Treg cells for cytotoxicity. EGTA (4 mM) added to target cells inhibited cytotoxicity by natural Treg cells.

(B) Neutralizing antibodies against FasL failed to block natural Treg cell cytotoxicity.

(C) Natural Treg cells exhibit cytotoxicity against autologous immature (iDC) and mature (mDC) dendritic cells, with iDC showing greater susceptibility than mDCs to cytotoxicity. CD3/46 stimulation of natural Treg cells also demonstrated a significantly higher level of cytotoxicity against target cells than CD3/CD28 stimulated natural Treg cells. Neutralizing antibodies against CD18 abrogated activated natural Treg cell cytotoxicity similar to EGTA inhibition. Effector to target (E:T) ratios are shown. Data shown are representative of more than three independent donor samples. Values shown are the mean ± SD. *$p < 0.05$, **$p < 0.01$.

$CD4^+$ T cells (Somersalo et al., 2004), effectively abrogated cytotoxicity.

Target cell activation status and cell lineage also appear to be important determinants of susceptibility to killing, as demonstrated by our data which showed that activated $CD4^+$ and $CD8^+$ T cells and immature myeloid dendritic cells, were preferentially killed. Mature dendritic cells and resting lymphocytes may lack surface recognition receptors or, alternatively, may express inhibitors of the perforin/granzyme pathway to account for their increased resistance to cytotoxicity (Figure 7). It is known that mature dendritic cells express the endogenous granzyme B inhibitor, PI-9 (Bladergroen et al., 2001; Hirst et al., 2003). PI-9 is also expressed in immune-privileged sites such as the placenta, testis, ovary, and eye, which may offer these sites protection from CTL induced damage (Bladergroen et al., 2001; Buzza et al., 2001; Hirst et al., 2001). While adaptive Treg cells were very effective at causing the death of dendritic cells, natural Treg cells were much less effective. Although different E:T ratios were utilized, these results indicate that the killing of target cells by different Treg cell populations does not represent nonspecific cytotoxicity but, rather, suggests that Treg subsets may have differing potentials to kill specific target cell populations.

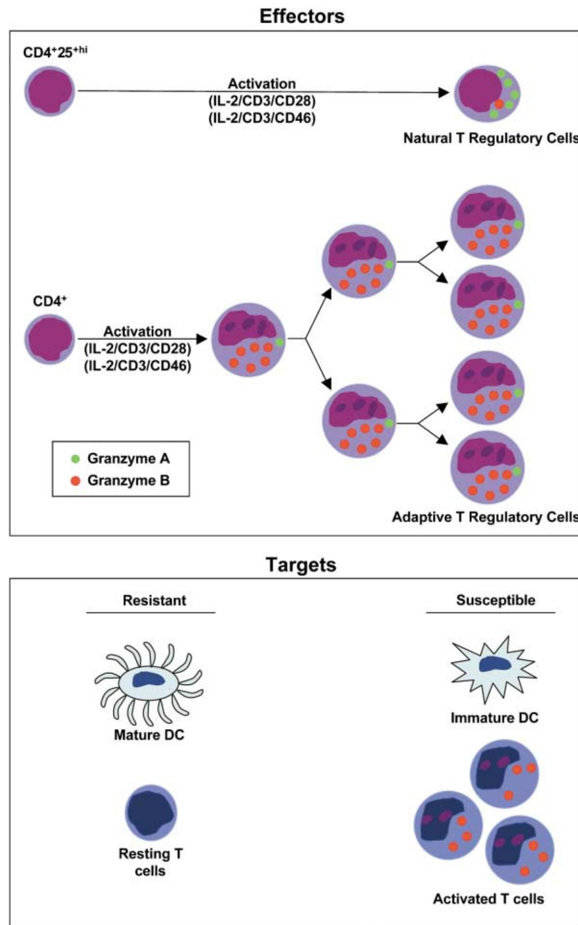



Figure 7. Summary of Data Demonstrating the Differential Granzyme Expression in Activated Human Natural and Adaptive Treg Cells and Their Cytotoxicity against Autologous Target Cells

Upon activation, natural Treg cells express primarily granzyme A, whereas adaptive Treg cells express primarily granzyme B. Natural Treg cells demonstrate a low proliferative capacity, while adaptive Treg cells exhibit significant proliferation after stimulation. Autologous activated $CD4^+$ and $CD8^+$ T cells are preferentially killed over unactivated T cells. Autologous immature myeloid dendritic cells are also preferentially killed over matured myeloid dendritic cells.

$CD14^+$ cells were also effectively killed in our studies, providing evidence that Treg cells can kill autologous cells of the myeloid lineage. In support of this, we and others have previously shown that Jak3-deficient mice, which lack $CD4^+CD25^+$ natural Treg cells, develop an expansion of the myeloid compartment with age (Antov et al., 2003; Grossman et al., 1999). In addition, FoxP3-deficient mice, which lack natural Treg cells, exhibit a similar expansion of myeloid cells (Talal Chatila, personal communication). Misra et al. (2004) showed that human $CD4^+25^+$ T cells inhibit the maturation and function of immature myeloid dendritic cells through an unidentified mechanism that was largely independent of IL-10 and TGF-β. Although not specifically examined, their data are consistent with a cytotoxic effect of Treg cells on immature dendritic cells. Further experiments will be required to determine if there are different effects of natural versus adaptive Treg cells on dendritic cell development and function.

The differential expression of granzymes A and B in Treg subsets suggests that effector cell subtypes may be armed with different granzymes to kill their target cells. Our data also indicate that granzymes may possess different potencies in cytotoxicity, since granzyme A expressing natural Treg cells were able to kill at a very low E:T ratio (1:1), while granzyme B expressing adaptive Treg cells exhibited cytotoxicity only at higher E:T ratios (20:1). Since natural Treg cells preferentially express granzyme A after stimulation, these cells may be required to suppress/kill activated autologous targets cells that express endogenous inhibitors of granzyme B, such as PI-9 (Figure 7) (Bird et al., 1998; Bird, 1998). Initial proteomic data from our laboratory has shown that there is minimal overlap between the cellular protein targets of murine granzymes A and B, suggesting that granzymes A and B induce target cell death by cleaving non-overlapping sets of substrates (Bredemeyer et al., 2004).

The mode of stimulation of natural Treg cells also appears to affect their cytotoxic potential. As demonstrated, CD3/CD46 stimulation was able to induce significantly more autologous target cell death than CD3/CD28 stimulation, and the cytotoxic potential correlated with the percentage of granzyme A-expressing natural Treg cells. Examination of multiple donors has consistently demonstrated significantly less cytotoxicity and granzyme A expression by using natural Treg cells stimulated with CD3/CD28 versus CD3/CD46. Although the reasons are unclear, preliminary data suggests that in the presence of plate bound CD46 antibodies, 10- to 100-fold less CD3 ligation is required to give similar proliferative responses as seen with plate bound CD28 antibodies (J.W.V. and J.P.A., unpublished data). These data suggest that CD46 lowers the threshold of T cell activation, which may explain the greater levels of granzyme A expression and cytotoxicity observed in natural Treg cells with CD3/CD46 compared to CD3/CD28 coligation. These data indicate that the full cytotoxic potential of natural Treg cells may not be obtained until the height of an immune response, when the highest level of immunoglobulin and complement deposition is observed. It is at such a time that complement (C3b and C4b) attached to immune complexes would be able to activate CD46 on natural Treg cells (Atkinson et al., 1994; Kemper et al., 2003; Lindahl et al., 2000; Marie et al., 2002; Riley-Vargus et al., 2004; Song, 2004).

The lack of detectable proliferation of $CD4^+CD25^+$ natural Treg cells in the presence of exogenous IL-2 stands in contrast to reports that showed partial restoration of proliferation with exogenous IL-2 (200 and 500 IU/mL) (Dieckmann et al., 2001; Jonuleit et al., 2001), while other reports show no effect with exogenous IL-2 (100 IU/mL) (Levings et al., 2001). The reasons for the discrepancies between these studies and our report are not clear but may reflect the lower dose of IL-2 used in our studies (10–50 IU/mL versus 200–500 IU/mL). In addition, the natural Treg cells used in our studies are likely to represent a very pure population of these cells, since only the cells with the highest CD25 expression were used. Indeed, preliminary results from our laboratories indicate that as a larger percentage of $CD25^+$ cells are included (i.e., 3%–5%), more proliferation is observed under similar stimulatory conditions (data not

shown). By using these less-stringent conditions of purification, we have noticed that the relative number of natural Treg cells (exhibiting low surface $CD4^+$ and granzyme A expression) decreases, and suppression is overcome by the larger number of "nonnatural" Treg cells (data not shown). One reason for these differences in proliferation may be the higher basal expression of the transcriptional factor FoxP3 in natural Treg cells, which has been shown to prevent cytokine production and proliferation of T cells (Ramsdell, 2003; Schubert et al., 2001).

A potential role of the perforin/granzyme pathway in the regulation of immune responses was first suggested by the discovery of perforin mutations in patients with hemophagocytic lymphohistiocytosis (HLH) (Stepp et al., 1999). HLH is a disease in which the immune system becomes overactive due to its inability to effectively clear infections and/or shutdown the immune response to such infections. Untreated HLH patients develop end-organ damage from lymphocyte infiltration and macrophage activation. All of the known mutations associated with HLH (e.g., *Perforin*, *SAP*, *Munc13-4*, *Rab27a*, *CHS-1*, *RGGT*, and others) affect genes that are important in the granule exocytosis pathway, implicating its importance for controlling immune responses (Allen et al., 2001; Feldman et al., 2003; Menasche et al., 2000; Russell and Ley, 2002; Stepp et al., 1999; Stinchcombe et al., 2004). Perforin-deficient mice develop a disease that is similar to HLH if challenged with viruses. An overactive virus-specific T cell response leads to death within weeks of viral challenge (Badovinac et al., 2003; Clark et al., 1995; Jordan et al., 2004; Matloubian et al., 1999). Depletion of $CD8^+$ T cells reduces their mortality, suggesting that antigen-activated $CD8^+$ T cells cause tissue damage and death (Badovinac et al., 2003). Similarly, Murakami et al. (2002) have shown that natural Treg cells help control the expansion of $CD8^+$ cells in mice. Excessive immune suppression by natural Treg cells may also have deleterious consequences, since Treg cells have been recently shown to contribute to viral persistence by suppressing virus-specific $CD8^+$ T cells in a mouse model (Dittmer et al., 2004).

The perforin/granzyme pathway may also be relevant for the prevention of autoimmunity, as supported by evidence from the experimental autoimmune encephalomyelitis (EAE) model in which the absence of perforin (but not Fas/Fas ligand) led to a more severe form of EAE in several murine strains (Malipiero et al., 1997). We have extended these observations with granzyme B knockout mice, which also display a more severe form of EAE (J. Russell and T.J.L., unpublished data). These in vivo data suggest a role of the perforin/granzyme pathway in controlling immune responses through the killing of multiple types of autologous immune cells. In addition, although natural Treg cells may have a relatively low abundance in human peripheral blood (<1%) and exhibit a low proliferative capacity after stimulation, the fact that these cells have been found to be dysfunctional in the life-threatening autoimmune disorder IPEX (Immunodysregulation/Polyendocrinopathy/Enteropathy/X linked; a syndrome lacking natural Treg cells due to a deficiency in *FoxP3*) suggests that they are clearly important for the control of normal immune responses (Chatila et al., 2000).

Natural Treg cells have previously been shown to utilize TGF-β and CTLA-4 to mediate their suppressive effects in in vitro coculture experiments, although the results are conflicting (Gavin and Rudensky, 2003; Kingsley et al., 2002; Levings et al., 2002; Nakamura et al., 2001; Piccirillo et al., 2002; Shevach, 2002). In addition, recent data has shown that a $CD4^+CD25^+$ cell line is able to exhibit cytotoxicity against B cells in an antigen-/MHC class II-specific manner that is partly mediated through FasL (Janssens et al., 2003). While these mechanisms of suppression are likely to be important, we propose that an additional mechanism of suppression by natural Treg cells is mediated by the killing of autologous cellular targets through a perforin/granzyme-dependent pathway.

## Experimental Procedures

### Cell Isolation and Stimulation

Human peripheral blood samples were collected from healthy donors in accordance with protocols approved by the Washington University School of Medicine Human Studies Committee and Internal Review Board. Peripheral blood mononuclear cells (PBMCs) were obtained by using Ficoll-Paque (Amersham). For preparation of adaptive Treg cells, human PBMCs were positively selected with anti-CD4 antibodies (OKT4; ATCC), then isolated with magnetically labeled goat-anti-mouse antisera according to manufacturer's instructions (Miltenyi Biotec). $CD4^+$ lymphocytes were routinely >98% pure as determined by flow cytometry. Similar results were obtained with positive selection by using anti-CD4 microbeads (Miltenyi Biotec). Purified $CD4^+$ lymphocytes were cultured on plates coated with antibodies to CD3 (OKT3 or Hit3a, Pharmingen) or CD46 (TRA-2-10) in complete RPMI containing 10 U/mL rhIL-2 as previously described (Kemper et al., 2003). Purified autologous target cells were isolated in a similar fashion utilizing antibodies against CD4 (OKT4, ATCC; or anti-CD4 microbeads, Miltenyi Biotec), CD8 (OKT8; ATCC), and CD14 (60bca; ATCC). Target cells were cultured in IL-2 with or without plate bound CD3/CD28 as described above for the same period as effector cells.

### Intracellular Granzyme Staining and Flow Cytometry

$1 \times 10^6$ cells were washed and resuspended in staining buffer (PBS, 1% human albumin [Aventis Behring L.L.C.], 0.2 μg/μL human immunoglobulin [Gammaguard; Baxter Healthcare]. Samples were labeled with antibodies (Per-CP- and/or FITC-conjugated antibodies against cell surface markers (anti-human CD4 [Becton Dickinson-Pharmingen]). Samples were fixed, permeabilized (Cytofix/Cytoperm containing 0.2 μg/μL of human immunoglobulin; Pharmingen), and stained with primary-conjugated anti-granzyme A antibody (CB9; Becton Dickinson-Pharmingen) diluted at 1:100 in staining buffer and/or primary-conjugated anti-granzyme B antibody (GB12; Caltag) diluted at 1:400 in staining buffer. Samples were analyzed on a FACScan (Becton Dickinson). All FACS scans depicted are representative of three or more donors. Anti-granzyme A and anti-granzyme B antibodies were analyzed for antigen specificity by competitive assays by using recombinant granzyme A (Kamiya Biomedical Co.) and/or granzyme B proteins (Pham et al., 1998) (10:1 molar ratio), as well as knockout mouse staining assays (Grossman et al., 2004).

### Isolation of Natural ($CD4^+CD25^+$) Treg Cells

$CD4^+$ T cells were isolated as described above and then stained with anti-CD4-PE and anti-CD25-APC antibodies (Becton Dickinson-Pharmingen). Natural $CD4^+CD25^+$ circulatory Treg cells were isolated on a MoFlo cytometer (Cytomation, Inc.). Only high $CD25^+$-expressing cells (<2% total $CD4^+$ T lymphocytes) were used for analysis. $CD4^+CD25^{+hi}$ Treg cells were used immediately (unstimulated) or were stimulated with anti-CD3/CD46 coated plates for 4 days before analysis of intracellular Granzyme A and B expression.

**RT-PCR Analysis for FoxP3 Expression**

CD4$^{+}$CD25$^{+hi}$ T cells or CD4$^{+}$25$^{-}$ T cells were isolated by cell sorting, and total RNA was isolated from equal number of cells (Qiagen). Reverse transcription of total RNA was performed by using the IMPROM RT system (Promega). PCR analysis was performed with the following FoxP3 specific primers: 5′-AGGAVTTCVTCAAGCACT GCCAG-3′ and 5′-TCCGTTTCTTGCGGAACTCC-3′. Human β-actin specific primers were as follows: 5′-TGGCATCGTGATGGACTCT GGT-3′ and 5′-ATGGCCATCTCCTGCTCGGAGT-3′. PCR conditions included a 94°C hot start for 2 min, followed by 40 cycles as follows: denaturing at 94°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 2 min. Samples were run on a 1% agarose gel with a 1 kb molecular weight marker (New England Biolabs). Negative controls consisted of RNA from CD4$^{+}$ T cells without RT enzyme. PCR products were sequenced and shown to be specific for FoxP3 (data not shown).

**$^{3}$H-Thymidine Proliferation/Coculture Assays**

Proliferation assays were performed as previously described (Geppert and Lipsky, 1988). Briefly, CD4$^{+}$CD25$^{-}$ T cells and CD4$^{+}$CD25$^{+}$ Treg cells were isolated as described above. Cell samples (1 $\times$ 10$^{5}$) were seeded into flat-bottom, 96-well plates (Corning) that were coated with anti-CD3 antibodies with either anti-CD28 or anti-CD46 antibodies, or vehicle only (see above). Cocultures of CD4$^{+}$CD25$^{-}$ T cells and CD4$^{+}$CD25$^{+}$ Treg cells were performed at 2:1 ratios for all experiments shown. IL-10 (10 ng/mL) was added as a control for inhibition of naive T cell proliferation. Control neutralizing IL-10 antibody (10 μg/mL, JES3-9D7, BD-Pharmingen) was added to assess the role of IL-10 production in the observed suppression. All samples were incubated in RPMI containing IL-2 for 4 days, at which time $^{3}$H-thymidine (1 μCi total; MP Biomedicals) was added to each well and allowed to incubate for an additional 16 hr before cells were isolated on glass filters (Baxter) and analyzed on a scintillation counter. All samples were performed in duplicate or triplicate.

**Production of Immature and Matured Myeloid Dendritic Cells**

Immature and matured myeloid dendritic cells were isolated and grown as previously described (Sallusto and Lanzavecchia, 1994). Briefly, CD14$^{+}$ cells were positively selected from autologous normal donor PBMCs by using magnetic bead isolation (Miltenyi Biotec) and then were cultured for 4–5 days in RPMI-1640 supplemented with 10% FBS (Hyclone, 50 ng/mL GM-CSF and 1000 U/mL IL-4). Immature myeloid dendritic cells were then used as targets in the flow-based killing assay (see below). Matured myeloid dendritic cells were obtained by adding 300 ng/mL LPS (from Salmonella abortus equi; Sigma) for 24–36 hr before analysis.

**Flow-Based Killing Assay (FloKA)**

A flow-based killing assay was developed to measure in vitro cellular cytotoxicity of autologous targets in a manner previously described (Grossman et al., 2004; Lecoeur et al., 2001). Purified autologous target cells (see above) were washed with PBS, resuspended at 1 $\times$ 10$^{6}$ cell/mL, and then labeled at 37°C for 15 min with 125 nM of 5-(and-6)-carboxyfluorescein diacetate succinimydyl ester (CFSE; Molecular Probes). Labeling reactions were stopped with RPMI media containing 10% FBS. Labeled target cells (1 $\times$ 10$^{5}$) were added to 96-well plates along with indicated effector cells in complete RPMI media containing 50 U/mL of rhIL-2. The effector:target (E:T) ratio was 20:1 for all time points shown for adaptive Treg cells. Natural (CD4$^{+}$25$^{+}$) Treg cells (stimulated with ConA/IL-2) were used at a 1:1 E:T ratio. Immediately before analysis, 1 μg/mL (final concentration) of 7-amino-actinomycin D (7-AAD; Calbiochem) was added to each sample. 7-AAD incorporation is used as a surrogate marker for late cell death/apoptosis as it intercalates with DNA in cells that have lost membrane integrity. To analyze the role of the perforin/granzyme pathway in killing, EGTA (4mM; Calbiochem) was added to inhibit calcium-dependent perforin polymerization. All target only samples were incubated with EGTA to control for any EGTA-associated toxicity over the indicated coculture period. Similar viability was seen without EGTA. Antibody inhibition studies employed the use of purified FasL neutralizing antibody (40 μg/mL; NOK1, eBiosciences), CD18 (TS1/18, ATCC) neutralizing antibody supernatants (diluted 1:4), or irrelevant antibody supernatants as a control (diluted 1:4). FloKA assays were compared to standard chromium release assays and gave similar results (data not shown) (Lecoeur et al., 2001). To control for the ability of the FasL neutralizing antibody to inhibit Fas/FasL-induced cytotoxicity, recombinant purified soluble FasL (PeproTech, NJ, USA; 25 ng/mL) with or without neutralizing FasL antibody (40 μg/mL; NOK1, Beckton Dickinson-Pharmingen), was used against a known susceptible cell line (Jurkat; ATCC) or a human cell line expressing high levels of surface Fas (U937; ATCC) (Grossman et al., 2004). All cytotoxicity assays were performed in duplicate. Data presented are representative of three or more individual cytotoxicity experiments. Statistical analyses were performed by one-way ANOVA with Bonferroni posttest analysis, or Student's t test by using GraphPad Prism version 3.0a for Macintosh (GraphPad Software, San Diego).

**Light Microscopy and Confocal Immunofluorescence**

Unstimulated (IL-2 only) and stimulated (CD3/CD46) sorter-purified CD4$^{+}$CD25$^{-}$ or CD4$^{+}$CD25$^{+}$ T cells were cytospun (300 rpm for 3 min; Shandon) onto glass slides. Cells were then stained with standard May-Grünwald/Giemsa stain (Sigma). Light microscopy pictures were taken with a Nikon microscope (original magnification for all light microscopy pictures, 1000×). For immunofluorescence of different cell types, cells were cytospun onto slides as indicated above. Samples were fixed, permeabilized (Cytofix/Cytoperm Becton Dickinson-Pharmingen), and costained for 30 min with FITC-conjugated anti-granzyme A antibody (CB9; Becton Dickinson-Pharmingen) diluted at 1:100, along with RPE-conjugated anti-granzyme B antibody (GB12; Caltag) diluted at 1:200 in permeabilization buffer (containing 0.2 μg/μL of human immunoglobulin; CytoPerm Becton Dickinson-Pharmingen). Isotype FITC- and RPE-conjugated control antibodies were used in a similar manner. Confocal immunofluorescence pictures were taken on a Zeiss LSM510 microscope (63× oil lens, NA1.4), with scales depicted in each picture.

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