Published in final edited form as:

J Mol Med (Berl). 2010 June ; 88(6): 577–588. doi:10.1007/s00109-010-0602-9.

Reciprocal granzyme/perforin-mediated death of human regulatory and responder T cells is regulated by interleukin-2 (IL-2)

Malgorzata Czystowska,

Department of Pathology, University of Pittsburgh School of Medicine, and University of Pittsburgh Cancer Institute, Pittsburgh, PA 15213, USA

Laura Strauss,

Department of Pathology, University of Pittsburgh School of Medicine, and University of Pittsburgh Cancer Institute, Pittsburgh, PA 15213, USA

Christoph Bergmann,

Department of Pathology, University of Pittsburgh School of Medicine, and University of Pittsburgh Cancer Institute, Pittsburgh, PA 15213, USA

Marta Szajnik,

Department of Pathology, University of Pittsburgh School of Medicine, and University of Pittsburgh Cancer Institute, Pittsburgh, PA 15213, USA

Hannah Rabinowich, and

Department of Pathology, University of Pittsburgh School of Medicine, and University of Pittsburgh Cancer Institute, Pittsburgh, PA 15213, USA

Theresa L. Whiteside

Departments of Pathology, Immunology and Otolaryngology, Suite L1.27 at the Hillman Cancer Center, University of Pittsburgh Cancer Institute, 5117 Centre Avenue, Suite 1.27, Pittsburgh, PA 15213-1863, USA, whitesidetl@upmc.edu

Abstract

Human CD4⁺CD25^{high}FOXP3⁺ T regulatory cells (Treg) can suppress responder T cell (RC) functions by various mechanisms. In co-cultures of Treg and autologous activated RC, both cell subsets up-regulate the expression of granzymes and perforin, which might contribute to Treg-mediated suppression. Here, we investigate the sensitivity and resistance of Treg and RC to granzyme/perforin-mediated death. CD4⁺CD25^{neg} RC were single cell-sorted from the peripheral blood of 25 cancer patients and 15 normal controls. These RC were carboxyfluorescein diacetate succinimidyl ester (CFSE) labeled and co-cultured with autologous CD4⁺CD25^{high}FOXP3⁺ Treg ± 150 or $\pm 1,000$ IU/ mL of interleukin-2 (IL-2) to evaluate suppression of RC proliferation. In addition, survival of the cells co-cultured for 24 h and 5 days was measured using a flow-based cytotoxicity assay. Freshly isolated Treg and RC expressed granzyme A (GrA), granzyme B (GrB), and perforin. Percentages of positive cells were higher in cancer patients than controls ($p < 0.01$) and increased upon OKT3 and IL-2 stimulation. Treg, co-cultured with RC at 150 IU/mL

© Springer-Verlag 2010

Correspondence to: Theresa L. Whiteside.

Electronic supplementary material The online version of this article (doi:10.1007/s00109-010-0602-9) contains supplementary material, which is available to authorized users.

Disclosure statement All authors have read the manuscript and concur with its content. The authors do not have any financial/commercial conflict of interest that could be considered to have influenced the content of this paper.

of IL-2, no longer expressed cytotoxins and became susceptible to RC-mediated, granzyme/perforin-dependent death. However, in co-cultures with 1,000 IU/mL of IL-2, Treg became resistant to apoptosis and induced GrB-dependent, perforin-independent death of RC. When the GrB inhibitor I or GrB-specific and GrA-specific small inhibitory ribonucleic acids were used to block the granzyme pathway in Treg, RC death, and Treg-mediated suppression of RC, proliferation were significantly inhibited. Human CD4⁺CD25^{high} Treg and CD4⁺CD25^{neg} RC reciprocally regulate death/growth arrest by differentially utilizing the granzyme–perforin pathway depending on IL-2 concentrations.

Keywords

Human CD4⁺CD25^{high} Treg; Cell death; Granzymes; Perforin; Interleukin-2

Introduction

The mechanisms of human regulatory T cells (Treg) utilized for suppression of responder T-cell (RC) proliferation were previously shown to be mediated by the release of inhibitory cytokines, interleukin-10 (IL-10), and transforming growth factor-beta (TGF- β) [1–3]. In addition, we have recently demonstrated that the Fas/FasL pathway is engaged in elimination by Treg of CD8⁺ RC, but not CD4⁺ RC [4]. The observed relative resistance of CD4⁺ RC to death receptor-mediated apoptosis underscores their sensitivity to other contact-dependent suppressor mechanisms [3, 5, 6]. For example, it is possible that the mitochondrial pathway of death is activated in CD4⁺ RC interacting with Treg [7]. Alternatively, death mechanisms involving granzymes and perforin could be responsible for elimination of CD4⁺ RC by Treg, as has been previously reported in mice [8] and in man [9]. The ability of murine and human Treg to catalyze conversion of adenosine triphosphate to adenosine monophosphate and then to immunosuppressive adenosine have been recently described [10–12]. Thus, several distinct mechanisms could be responsible for Treg-mediated suppression, selectively targeting CD8⁺ or CD4⁺ effector T cells.

Cytolytic T lymphocytes (CTL) and natural killer cells use cytokine-, Fas/FasL-, or granzyme/perforin-mediated pathways to kill their targets and may be eliminated by these mechanisms once their function is completed [12, 13]. The possibility that human CD4⁺CD25^{high}FOXP3⁺ Treg might utilize the same pathways to mediate suppression of other T cells remains a subject of intense interest. In this study, we utilized an in vitro model system consisting of human Treg and autologous RC co-cultured in the presence of T cell receptor (TCR) stimulation and interleukin-2 (IL-2) to evaluate expression and activity of the granzyme/perforin pathway in these cells. We demonstrate that in co-cultures containing elevated IL-2 levels, CD4⁺ RC are eliminated by Treg which utilize a GrB-dependent/perforin-independent death pathway but are themselves resistant to cytotoxin-mediated death. In the presence of low IL-2 concentrations, Treg are no longer resistant and are lysed by RC using the canonical granzyme/perforin pathway. This reciprocal elimination of RC or Treg in the co-cultures is strictly regulated by IL-2 concentrations.

Materials and methods

Cancer patients and healthy volunteers

Blood samples were obtained from 25 patients with head and neck squamous cell carcinoma (HNSCC) and 15 age-matched healthy volunteers (NC). All subjects signed an informed consent approved by the Institutional Review Board (IRB) of the University of Pittsburgh. All patients were seen at the Outpatient Otolaryngology Clinic between January 2006 and April 2007. The patient cohort included 17 males and 8 females (mean age 60 years; range

23–83 years). The NC group included 10 males and 5 females with a mean age of 60 years. Among the patients, 22 had primary and 3 recurrent disease. At the time of blood draws, 7 patients were untreated, and 18 had no evident disease after receiving various forms of oncologic therapy, which was terminated from 3 weeks to 12 months before the time of phlebotomy for this study. Fifteen patients had oral cavity tumors, while the remaining patients included 4 with laryngeal, 2 with oropharyngeal, 1 with hypopharyngeal, 2 with the nasal cavity tumors, and 1 with an undetermined tumor site. Fifteen tumors were moderately, 5 poorly, and 3 well-differentiated, and in 2, differentiation was not determined. Eight tumors were T1 or T2, 15 were T3 or T4, and 2 were unstaged. The nodal status was 12 N₀ and 13 N₁–N₃. The patient cohort was the same as described in a greater detail previously [4].

Collection of PBMC, isolation of cell subsets, and co-culture conditions

Peripheral venous blood (20–30 mL) was drawn into heparinized tubes. Peripheral blood mononuclear cells (PBMC) were separated by centrifugation on Ficoll-Hypaque, washed in AIM-V medium (Invitrogen, Carlsbad, CA), counted in a trypan blue dye and immediately used for experiments. CD4⁺CD25^{high} and CD4⁺CD25^{neg} T cell subsets were single-cell sorted using a high speed MoFlo cell sorter (Dako, Ft. Collins, CO) as previously described [4, 6]. The purity of each fraction was >95%, and the cell viability was >98%. The sorted CD4⁺CD25^{high} and CD4⁺CD25^{neg} cells were phenotyped to determine FOXP3 and CTLA-4 (CD152) expression and were immediately used for establishing co-cultures containing soluble OKT3 and anti-CD28 Ab as well as IL-2 at 150 or 1,000 IU/mL.

Antibodies

The following anti-human monoclonal antibodies (mAbs) were used for flow cytometry: anti-CD3-ECD (clone UCHT1), anti-CD4-PC5 (clone 13B8.2), anti-CD8-PC5 (clone B9.11), anti-CD25-FITC (clone B1.49.9), anti-FOXP3-FITC (clone PCH101), anti-CD45RA-FITC (clone 2H4LDHILDB9), anti-CD45RO-FITC (clone UCHL1), anti-CD25-PE (clone B1.49.9), anti-CD152-PE (CTLA-4) (clone BNI3), anti-FasL (clone NOK1), anti-GrB-PE (clone GBIII), anti-GrA-PE (clone CB9), and anti-perforin-FITC (clone dG9). Antibodies and their respective isotypes used as negative controls for surface and intracellular staining were purchased from Beckman Coulter (Miami, FL), except for anti-GrA-PE (BD Pharmingen, San Jose, CA), anti-FOXP3-FITC clone PCH101 (eBioscience, San Diego, CA), anti-perforin-FITC, anti-FasL-PE (BioLegend, San Diego, CA), and anti-GrB-PE (PeliCluster Inc, the Netherlands, clone GB111). Prior to use, all mAbs were titrated using normal resting or activated PBMC to establish optimal staining dilutions.

Surface and intracellular staining

To determine the frequency of CD4⁺CD25^{high} and CD4⁺CD25^{neg} T cells and the Treg marker and cytotoxin expression, PBMC (at least 2×10⁵ cells/tube) were stained with pre-titrated mAbs for 15 min at 4°C. Isotype Ab controls were used in all experiments. Cells were washed and examined by four-color flow cytometry [6].

Intracellular staining for FOXP3, CD152 (CTLA-4), granzyme A (GrA), granzyme B (GrB), and perforin was performed as described [4, 6]. Briefly, cells incubated with mAbs against surface markers (CD4, CD8, CD3, and CD25) were washed, fixed with 4% (v/v) paraformaldehyde in phosphate buffered saline (PBS) for 20 min at room temperature (RT), washed once with PBS containing 0.5% (v/v) bovine serum albumen (BSA) (w/v) and 2 nM ethylene diamine triacetic acid, permeabilized with PBS containing 0.5% BSA and 0.1% (v/v) saponin and stained with pretitrated anti-CTLA-4-PE, anti-FOXP3-FITC, anti-GrA-PE, anti-GrB-PE, or anti-perforin-FITC for 30 min at RT. Cells were further washed twice with PBS containing 0.5% (w/v) BSA and 0.2% (v/v) saponin, resuspended in FACS flow

solution and immediately analyzed by flow cytometry. Appropriate isotype controls were included for each sample.

Detection of AnxV⁺ cells

Freshly isolated PBMC and PBMC activated *ex vivo* with OKT3 and/or IL-2 were analyzed for AnnexinV binding (percentage AnxV⁺ PI^{neg} cells) using flow cytometry as described [1, 4].

Flow cytometry

Flow cytometry was performed on Beckman Coulter equipped with Expo32 software. The acquisition and analysis gates were restricted to the lymphocyte gate as determined by the characteristic forward and side-scatter properties of the cells. At least 1×10^5 lymphocytes were acquired for analysis. The analysis gates were restricted to the CD3⁺CD4⁺, CD4⁺CD25^{neg}, or CD4⁺CD25^{high} T cell subsets, as appropriate. Cells expressing Treg markers, apoptosis-related proteins, or death molecules were acquired and analyzed in the FL1 or FL2 logarithmic scale using the set gates.

Flow-based cytotoxicity assay

To determine the ability of Treg to suppress RC proliferation and death or to measure the capability of activated RC to induce death of Treg, the flow-based cytotoxicity assay (FLOCA) was employed, as previously described [4]. In FLOCA, 7-amino-actinomycin D (AAD) incorporation is used as a surrogate marker for cell death, and RC are labeled with CFSE to evaluate their proliferation. All CFSE data were analyzed using the ModFit software (Verity Software House, Topsham, ME), and the percentages of suppression were calculated based on the proliferation index (PI) of RC alone compared with the PI of cultures containing RC plus Treg. The PI of RC was defined by the ModFit program as described previously [14]. The program determines the percent of cells within each division peak, and the sum of all peaks in the control culture (RC, alone) is taken as 100% proliferation while the sum of all peaks in the co-culture (RC+Treg) represents proliferation suppression.

Analysis of suppression mechanisms in co-cultures of CD4⁺ or CD8⁺ RC with Treg

To determine whether the granzyme/perforin pathway is involved in Treg-mediated suppression of CD4⁺ or CD8⁺ RC proliferation, the FLOCA was performed under the following conditions:

- A. Perforin activation within the lytic granules was blocked in Treg or CD4⁺ RC with concanamycin A (Sigma Aldrich, St. Louis, MO) before co-culture. Treg or CD4⁺ RC were incubated +/- concanamycin A (100 nM) for 2 h at 37°C. The ability of perforin to induce cell death was measured by the FLOCA after 24 h or 5 days of co-incubation.
- B. GrB activity was neutralized in Treg or CD4⁺ RC before co-culture using GrB inhibitor I, Z-AAD-CMK (C₁₉H₂₄CIN₃O₇; Calbiochem, San Diego, CA). Cells were co-incubated +/- Z-AAD-CMK (250 μM/mL) for 2 h at 37°C. The ability of GrB to induce apoptosis was measured in co-cultures by the FLOCA on days 1, 3, and 5. In the above experiments, the optimal concentrations of concanamycin A or GrB inhibitor I were predetermined in titration assays using OKT3 and IL-2 activated-PBMC. To exclude direct cytotoxic effects of concanamycin A or Z-AAD-CMK, the 7-AAD uptake or AnxV binding by Treg or RC incubated with these reagents for 3–5 days was determined.

- C. GrA or GrB were silenced at the messenger ribonucleic acid level in Treg or CD4⁺ RC before co-cultures. The cells were transfected with small inhibitory ribonucleic acid (siRNA) specific for GrA or GrB (siGENOME SMART pool purchased from Dharmacon, Chicago, IL) or with scrambled siRNA which had no homology to any known human sequences (non-targeting 20–25 nucleotide siRNA). Human T-Cell Nucleofector Kit (Amaxa Biosystems, Gaithersburg, MD), was used. Aliquots (100 μ L) of freshly isolated cells (5×10^6 cells/mL) were mixed with 5 μ g siRNA and nucleofected as recommended by the manufacturer. After nucleofection, the cells were transferred to wells of the 12-well plate filled with 1.5 mL of pre-warmed culture medium and maintained at 37°C overnight prior to testing for GrA or GrB expression by flow cytometry. The nucleofected cells were immediately set up in co-cultures, and cell death was measured in the FLOCA on day 5 of co-culture.

Statistical analysis

Data were summarized by descriptive statistics as means $\pm$ SD for continuous variables and the frequency and percentage for categorical variables. The paired student's t test was used to evaluate differences between treated vs. untreated groups.

For FLOCA and CFSE experiments performed with cells of three different individuals, results are presented for one representative donor of three donors for each treatment or condition tested. *P* values 0.05 were considered significant.

Results

Purity and activity of sorted human CD4⁺CD25^{high} Treg

Treg were isolated from the PBMC of NC or patients with HNSCC using immunobeads followed by flow cytometry-based sorting of CD4⁺CD25^{high} T cells as previously described [4, 6, 12]. These cells were routinely tested for expression of FOXP3, CTLA-4, and CD39 markers and, in some experiments, for suppressor function with CFSE-labeled autologous CD4⁺CD25^{neg} T cells as described by us [4, 6, 12]. We have confirmed in this study that the CD4⁺CD25^{high} T cell fraction we refer to as Treg contained 90 $\pm$ 5% of FOXP3⁺ cells, 87 $\pm$ 7% of CD39⁺ cells, and 60 $\pm$ 8% of CTLA-4⁺ cells which mediated suppression of RC proliferation [4, 6, 12].

Granzyme and perforin expression by CD4⁺CD25^{high} and CD4⁺CD25^{neg} T cells in NC or cancer patients

CD4⁺CD25^{high} (Treg) and CD4⁺CD25^{neg} (RC) were examined for expression of GrA, GrB, and perforin by flow cytometry. As shown in Fig. 1, significantly higher proportions of freshly isolated Treg and RC cells were positive for cytotoxins in cancer patients than in NC, e.g., >90% of fresh Treg expressed GrB in patients vs. <50% in NC. Following 24-h stimulation with anti-CD3/anti-CD28 Abs and IL-2 (150 IU/mL), the percentages of Treg positive for GrB and perforin increased in NC but not in cancer patients. These data suggested that fresh Treg are activated in cancer patients and that further stimulation does not substantially up-regulate cytotoxin expression.

Cell death in co-cultures of Treg and RC

When Treg or RC cells were incubated alone in the presence of 150 or 1,000 IU/mL of IL-2 for 24 h or 3–5 days, only few 7-AAD⁺ cells were present in these cultures, as measured in FLOCA (Fig. 2a, b). However, upon co-incubation of Treg and OKT3-activated CFSE-labeled RC at the 1:1, 1:5 or 1:10 ratios, the proportion of 7-AAD⁺ cells was increased to over 50% in the co-cultures containing 150 IU/mL of IL-2 and to about 85% in the co-cultures containing 1,000 IU/mL of IL-2 after 24 h of co-incubation (Fig. 2a). These data

suggested that co-incubation promoted early cell death in both subsets of lymphocytes. However, upon longer co-incubation (e.g., 3–5 days), it became apparent that T cell death/survival depended on the IL-2 concentration. Thus, at 150 IU/mL of IL-2, nearly all Treg were dead, and there was no suppression of RC proliferation in day 5 co-cultures (Fig. 2b, c). At 1,000 IU/mL of IL-2, all Treg survived and the RC were dead (Fig. 2b). Consistently, complete suppression of RC proliferation was observed in the 5-day co-cultures (Fig. 2c). These data suggested that both T-cell populations expressing cytotoxins after activation were potentially capable of reciprocally killing each other and did so early, within 24 h of being placed in a co-culture. At low IL-2 concentrations, Treg did not survive and RC proliferated normally in 5-day co-cultures. At high IL-2 concentrations, Treg were resistant to death and suppressed RC proliferation (Fig. 2c).

Cytotoxin expression in co-cultured Treg and RC

To further study the interactions between Treg and RC, we measured cytotoxin expression by these cell subsets after 24-h or 5-day co-culture (Fig. 3). After 24 h of co-incubation in 150 IU/mL of IL-2, half of Treg were 7-AAD⁺ (Fig. 2a), the other half expressed GrA and GrB, but fewer cells contained perforin (Fig. 3a). By day 5, almost no Treg expressing cytotoxins were detectable, consistent with their demise (Fig. 2b). In contrast, CD4⁺CD25^{neg} RC were fully viable (Fig. 2b), with almost all cells expressing cytotoxins (Fig. 3b).

In co-cultures containing 1,000 IU/mL of IL-2, close to 80% of Treg expressed cytotoxins at 24 h, and by day 5, all Treg were positive for GrB, GrA, and perforin (Fig. 3a). However, RC began losing expression of cytotoxins, especially of perforin, at 24 h of co-incubation and by day 5 became negative for GrA and perforin, still retaining expression of GrB (Fig. 3b).

Perforin involvement in reciprocal Treg/RC killing

To evaluate effects of the individual cytotoxins on T-cell responses in 5-day co-cultures of Treg with RC, we pre-treated either Treg or RC with concanamycin A, which blocks perforin activation. In co-cultures with 150 IU/mL of IL-2, perforin was expressed in RC but not in Treg (Fig. 3). Therefore, RC were pre-treated with concanamycin A to inactivate perforin prior to co-cultures. Conversely, in co-cultures with 1,000 IU/mL of IL-2, perforin was expressed in Treg but not in RC (Fig. 3). Therefore, Treg were pre-treated with concanamycin A prior to their co-incubation with RC at 1,000 IU/mL of IL-2.

In the absence of concanamycin A and at high IL-2 concentrations, Treg mediated effective suppression of RC proliferation in the co-cultures (81–95%, Table 1) as previously shown (Fig. 2a, b). Concanamycin A-pre-treated Treg also survived well in the co-cultures supplemented with 1,000 IU/mL of IL-2 (few 7-AAD⁺ Treg), and these Treg mediated high levels of suppression (80–95%, Table 1). These data suggested that perforin activation in Treg was not necessary for Treg-mediated suppression of RC proliferation at high IL-2 concentrations. Thus, Treg-mediated demise of RC was perforin independent.

At 150 IU/mL of IL-2 in the absence of concanamycin A, 50–60% of Treg but few RC were 7-AAD⁺, and RC proliferation was not suppressed, as expected. Upon pre-treatment with concanamycin A, RC proliferation was strongly suppressed, although no 7-AAD⁺ RC were evident. Importantly, few Treg were 7-AAD⁺ in these co-cultures, indicating that inhibition of perforin activation in RC protected Treg from death. At low IL-2 concentrations, death of Treg was dependent on perforin activation in RC.

GrB involvement in reciprocal Treg and RC killing

To neutralize GrB activity in Treg or RC prior to their co-cultures, we used GrB inhibitor I. The pre-treatment of RC with this inhibitor prevented Treg death in co-cultures with 150 IU/mL of IL-2 (Table 2), allowing Treg to mediate suppression of RC proliferation (>80%). The pre-treatment of Treg with the inhibitor prevented the death of RC in co-cultures with 1,000 IU/mL of IL-2 and significantly inhibited suppression of RC proliferation (Table 2). Thus, neutralization of GrB activity in RC had no effect on suppression mediated by Treg, while neutralization of GrB activity in Treg was effective in reducing suppression of RC proliferation. These results implicate GrB activity in Treg-mediated death of co-cultured RC.

Silencing of GrB or GrA expression using siRNA

To further confirm the key role of GrB and probe the GrA involvement in Treg–RC interactions, expression of the granzymes in T cells was blocked using GrB- or GrA-specific siRNAs. All siRNA experiments were performed with cells of patients with cancer in which granzyme expression was elevated (Fig. 1). Nucleofection with GrA- or GrB-specific siRNA reduced expression of granzymes in Treg stimulated with OKT3 and IL-2 (1,000 IU/mL, Fig. 4a). The ability of these nucleofected Treg to suppress proliferation of CFSE-labeled autologous RC in 5-day co-cultures in the presence of 1,000 IU/mL of IL-2 was also significantly reduced (by $75\pm 20\%$ in three independent experiments), although silencing of GrA was less effective than that of GrB (Fig. 4b). Concomitantly, RC death (i.e., the percent of 7-AAD⁺ RC) was decreased (by $80\pm 14\%$) following silencing of GrB in Treg, while silencing of GrA had a less pronounced effect on RC death (Fig. 4c). In control co-cultures, RC were incubated with non-silenced Treg or Treg nucleofected with scrambled siRNA.

Our earlier experiments showed that in co-cultures with 150 IU/mL of IL-2, RC were resistant to death, while Treg were sensitive. When GrB siRNA-nucleofected RC were co-cultured with autologous Treg, no 7-AAD⁺ Treg were observed in the co-cultures (Fig. 5b), as few RC expressed GrB and Treg became completely resistant to RC-mediated death. In control co-cultures (Fig. 5a), nearly all RC were GrB⁺ and 65% of Treg were 7-AAD⁺, consistent with the data shown in Fig. 2b. The silencing of GrB in RC gave results similar to those obtained when GrB expression in RC was inhibited using GrB inhibitor I (see Table 2). In aggregate, our data confirm that silencing of the granzyme genes in Treg blocks the suppression of RC proliferation and RC death. Conversely, silencing of the granzyme genes in RC protects Treg from killing by activated RC.

Discussion

Peripheral T-cell tolerance mediated by CD4⁺CD25^{high} Treg has recently emerged as one of the key mechanisms of control in healthy, balanced immune responses to infectious agents and self antigens [3, 15–19]. Mechanisms used by human Treg to mediate suppression of autologous CD4⁺ or CD8⁺ RC might vary depending on the environmental context and may or may not require direct cell-to-cell contact [15]. Beyond this, little is known about molecular pathways that regulate Treg functions in health or disease.

Mechanisms responsible for control of T-cell survival/ death include death receptor-mediated apoptosis, granzyme/ perforin-mediated cytotoxicity, and cytokine-induced suppression [7, 15, 20, 21]. Sensitivity/resistance of distinct T-cell subsets to these mechanisms is not entirely clear, except for a well-documented phenomenon of activation-induced cell death, which is Fas/FasL mediated, IL-2 dependent, and preferentially eliminates activated CD8⁺ effector cells [22]. We have previously shown that Treg use this pathway to mediate suppression of RC proliferation [4]. Treg, especially those present in

human tumors, also suppress RC proliferation using IL-10 and TGF- β_1 [1, 2]. Here, we show for the first time that human Treg and RC can reciprocally regulate death and expansion arrest by differentially utilizing the granzyme–perforin pathway in an IL-2 dose-dependent manner.

Treg, freshly isolated from human PBMC express granzymes A and B and perforin, and in patients with cancer, nearly all CD4⁺ T cells are positive for cytotoxins. Upon co-culture of RC with Treg and TCR-mediated activation in the presence of IL-2, CD4⁺ T cells further up-regulated cytotoxin expression. We have consistently observed that Treg obtained from patients with cancer mediated significantly higher immunosuppressive activities than Treg of NC. Possibly, this is due to their ability to utilize three distinct cytolytic mechanisms: inhibitory cytokines [1, 2, 15], death receptor mediated apoptosis [4], and the granzyme/perforin pathway [9, 23]. While Treg obtained from NC have the potential to use the same mechanisms, they express lower levels of Fas and FasL [4, 6] and, as shown here, significantly lower levels of GrA, GrB, and perforin relative to Treg of cancer patients. Activation and expansion of death-resistant Treg with potent suppressor function in patients with cancer might play a key role in down-regulation of anti-tumor immune responses.

Our data suggested that upon direct cell-to-cell contact of RC with Treg, a release of accumulated cytotoxins resulted in an immediate demise of a considerable proportion of the interacting T cells and that IL-2 concentrations regulated this process. The potential for reciprocal killing raises questions regarding granzyme vs. perforin involvement in Treg interactions with RC and whether this killing is responsible for suppression of RC proliferation by Treg. Using an autologous co-culture system to simultaneously measure Treg and RC death (7-AAD⁺ cells), expression of cytotoxins by these cells and suppression of proliferation in CFSE-labeled RC, we studied the fate of the interacting T-cell subsets and mechanisms responsible for suppression. The reciprocal and early demise of Treg and RC in co-cultures was reflected by changes in RC proliferation measured on day 5. The co-culture system was manipulated to include low or high IL-2 concentrations. Our data showed that IL-2 concentrations determined the outcome of Treg/RC interactions. In co-cultures containing 150 IU/mL of IL-2, Treg completely lost the cytotoxin expression, so that after 5 days of co-culture, they expressed no GrB, GrA, or perforin and nearly all were dying (i.e., were 7-AAD⁺). In contrast, all RC remained viable, expressed GrB, GrA, and perforin, and induced GrB-dependent as well as perforin dependent death in Treg. Blocking of GrB by siRNA or GrB inhibitor I in RC significantly inhibited Treg death and restored RC suppression, as did concanamycin A-mediated inhibition of perforin activation in RC. Our data suggest that RC use the canonical GrB/perforin pathway to eliminate Treg in co-cultures containing low IL-2 concentrations. When, however, the co-cultures contained 1,000 IU/mL of IL-2, the surviving, perforin⁺, and GrB⁺ Treg induced death of RC, remaining fully viable themselves. The obligatory involvement of GrB in Treg-mediated killing of RC was shown by using GrB inhibitor I or silencing of GrB and GrA genes with siRNAs, which abrogated Treg-mediated suppression as well as death of RC. This mechanism of suppression was perforin independent, as in the presence of concanamycin A added to Treg to inhibit perforin activation, Treg were still able to kill RC and completely suppress RC proliferation. The work of Grondek et al [8, 24] confirms that in mice, GrB activity is independent of perforin and represents one of the key mechanisms through which CD4CD25⁺ Treg induce cell contact-mediated suppression. It has been suggested in the literature that at high GrB concentrations, the need for perforin to release GrB from the endosomal compartment and facilitate its entry into a target cell may be circumvented [25, 26]. As Treg interacting with RC in the presence of 1,000 IU/mL of IL-2 contain abundant GrB, Treg-derived perforin may not be necessary to facilitate GrB activity. The role of GrA, which is expressed in activated Treg and RC in Treg-mediated suppression, is less clear,

although in our hands, silencing of the GrA gene also partially abrogated suppression of RC proliferation and their apoptosis.

The biologic significance of our in vitro co-culture system using human T cells is speculative. Nevertheless, it is likely that the divergent, IL-2-dependent susceptibility or resistance of Treg and RC to the GrB/perforin-mediated death might represent a regulatory mechanism relevant to Treg/RC interactions taking place in vivo. T lymphocytes have evolved mechanisms for down-regulation of their own numbers by suicidal (“self-killing”) or fratricidal (“killing of each other”) mechanisms that appear to be hard-wired into the T-cell basic biology [22]. Distorted lymphocyte homeostasis is associated with human disease [27–29], and regulation of lymphocyte activation/expansion characterizes a normal immune response. It is likely that IL-2 concentrations vary from low to high between the early (proliferative) and late (contraction) phases of an immune response, creating a microenvironment that shapes Treg–RC interactions [30]. At the level of a developing immune response, the divergent susceptibility of Treg and RC to GrB-mediated lysis might be an advantage. Thus, T cells responding to an initial antigenic signal (when IL-2 levels are low) are able to suppress the expression of effector molecules in Treg and even induce death of Treg to protect themselves from suppression and death. Alternatively, Treg could use perforin and GrB to induce their own demise at low IL-2 concentrations. However, in the contraction phase of the immune response (when IL-2 levels are high), Treg activity is essential for the arrest of excessive expansion of RC subsets. Therefore, survival and suppressor activity of Treg are enhanced and maintained at high IL-2 concentrations, as also confirmed by reports of elevated frequency of Treg and Treg-mediated suppression in patients with cancer treated with high-dose IL-2 therapy [31, 32].

The in vitro model of Treg/RC interactions we have described allows us to conclude that IL-2-dependent, reciprocal granzyme/perforin-mediated killing is one of the mechanisms used by human T cells to regulate the outcome of Treg/RC interactions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Supported in part by National Institutes of Health (NIH) grants PO1 CA109688, PO1 DE12321 and RO1 DE13918 to TLW. Dr. Bergmann was supported in part by Philip Morris USA, Inc. and Philip Morris International.

References

1. Strauss L, Bergmann C, Szczepanski M, Gooding W, Johnson JT, Whiteside TL. A unique subset of CD4+CD25^{high}Foxp3+ T cells secreting IL-10 and TGF-beta1 mediates suppression in the tumor microenvironment. *Clin Cancer Res.* 2007; 13:4345–4354. [PubMed: 17671115]
2. Levings MK, Sangregorio R, Sartirana C, Moschin AL, Battaglia M, Orban PC, Roncarolo MG. Human CD25+CD4+ T suppressor cell clones produce transforming growth factor beta, but not interleukin 10, and are distinct from type 1 T regulatory cells. *J Exp Med.* 2002; 196:1335–1346. [PubMed: 12438424]
3. Miyara M, Sakaguchi S. Natural regulatory T cells: mechanisms of suppression. *Trends Mol Med.* 2007; 13:108–116. [PubMed: 17257897]
4. Strauss L, Bergmann C, Whiteside TL. Human circulating CD4+CD25^{high}Foxp3+ Treg kill autologous CD8+ but not CD4+ responder cells by Fas-mediated apoptosis. *J Immunol.* 2009; 182:1469–1480. [PubMed: 19155494]

5. Levings MK, Sangregorio R, Roncarolo MG. Human CD25(+)CD4(+) T regulatory cells suppress naïve and memory T cell proliferation and can be expanded in vitro without loss of function. *J Exp Med.* 2001; 193:1295–1302. [PubMed: 11390436]
6. Strauss L, Whiteside TL, Knights A, Bergmann C, Knuth A, Zippelius A. Selective survival of naturally occurring human CD4+CD25+Foxp3+ regulatory T cells cultured with rapamycin. *J Immunol.* 2007; 178:320–329. [PubMed: 17182569]
7. Krammer PH, Arnold R, Laurik IN. Life and death in peripheral T cells. *Nat Rev Immunol.* 2007; 7:532–542. [PubMed: 17589543]
8. Gondek DC, Lu LF, Quezada SA, Sakaguchi S, Noelle RJ. Cutting edge: contact-mediated suppression by CD4+CD25+ regulatory cells involves a granzyme B-dependent, perforin-independent mechanism. *J Immunol.* 2005; 174:1783–1786. [PubMed: 15699103]
9. Grossman WJ, Verbsky JW, Barchet W, Colonna M, Atkinson JP, Ley TJ. Human T regulatory cells can use the perforin pathway to cause autologous target cell death. *Immunity.* 2004; 21:589–601. [PubMed: 15485635]
10. Deaglio S, Dwyer KM, Gao W, Friedman D, Usheva A, Erat A, Chen JF, Enjoji K, Linden J, Oukka M, et al. Adenosine generation catalyzed by CD39 and CD73 expressed on regulatory T cells mediates immune suppression. *J Exp Med.* 2007; 204:1257–1265. [PubMed: 17502665]
11. Borsellino G, Kleinewietfeld M, DiMitri D, Sternjak A, Diamantini A, Giometto R, Hopner S, Centonze D, Bernardi G, Dell'Acqua ML, et al. Expression of ectonucleotidase CD39 by Foxp3+ Treg cells: hydrolysis of extracellular ATP and immune suppression. *Blood.* 2007; 110:1225–1232. [PubMed: 17449799]
12. Mandapathil M, Szczepanski MJ, Szajnik M, Ren J, Lenzner DE, Jackson EK, Gorelik E, Lang S, Johnson JT, Whiteside TL. Increased ectonucleotidase expression and activity in Treg of patients with head and neck cancer. *Clin Cancer Res.* 2009; 15(20):6348–6357. [PubMed: 19825957]
13. Davis JE, Sutton VR, Browne KA, Trapani JZ. Purification of natural killer cell cytotoxic granules for assaying target cell apoptosis. *J Immunol Meth.* 2003; 276:59–68.
14. Givan AL, Fisher JL, Waugh M, Ernstoff MS, Wallace PK. A flow cytometric method to estimate the precursor frequencies of cells proliferating in response to specific antigens. *J Immunol Meth.* 1999; 230:99–112.
15. Strauss L, Bergmann C, Gooding W, Johnson JT, Whiteside TL. The frequency and suppressor function of CD4+CD25^{high}Foxp3+ T cells in the circulation of patients with squamous cell carcinoma of the head and neck. *Clin Cancer Res.* 2007; 13:6301–6311. [PubMed: 17975141]
16. Wang RF. Immune suppression by tumor specific CD4(+) regulatory T cells in cancer. *Semin Cancer Biol.* 2006; 16:73–79. [PubMed: 16140545]
17. Zou W. Immunosuppressive networks in the tumor environment and their therapeutic relevance. *Nat Rev Cancer.* 2005; 5:263–274. [PubMed: 15776005]
18. Wei WZ, Morris GP, Kong YC. Anti-tumor immunity and autoimmunity: a balancing act of regulatory T cells. *Cancer Immunol Immunother.* 2004; 53:73–78. [PubMed: 14610619]
19. Shevach EM. CD4+CD25+ suppressor T cells: more questions than answers. *Nat Rev Immunol.* 2002; 2:389–400. [PubMed: 12093005]
20. Rafaei Y, Van Parijs L, London CA, Tschopp J, Abbas AK. Biochemical mechanisms of IL-2-regulated Fas-mediated apoptosis. *Immunity.* 1998; 8:615–623. [PubMed: 9620682]
21. Trapani JA, Sutton VR. Granzyme B: pro-apoptotic, antiviral and antitumor functions. *Curr Opin Immunol.* 2003; 15:533–543. [PubMed: 14499262]
22. Lenardo M, Chan KM, Hornung F, McFarland H, Siegel R, Wang J, Zheng L. Mature T lymphocyte apoptosis-immune regulation in a dynamic and unpredictable antigenic environment. *Annu Rev Immunol.* 1999; 17:221–253.
23. Cao X, Cai SF, Fehniger TA, Song J, Collins LI, Piwnica-Worms DR, Ley TJ. Granzyme B and perforin are important for regulatory T cell-mediated suppression of tumor clearance. *Immunity.* 2007; 27:635–646. [PubMed: 17919943]
24. Gondek DC, Devries V, Nowak EC, Lu LF, Bennett KA, Scott ZA, Noelle RJ. Transplantation survival is maintained by granzyme B+ regulatory cells and adaptive regulatory T cells. *J Immunol.* 2008; 181:4752–4760. [PubMed: 18802078]

25. Kurschus FC, Kleinschmidt M, Fellows E, Dornmair K, Rudolph R, Lilie H, Jenne DE. Killing of target cells by redirected granzyme B in the absence of perforin. *FEBS let.* 2004; 562:87–92. [PubMed: 15044006]
26. Smyth MJ, Kelly JM, Sutton VR, Davis JE, Browne KA, Sayers TJ, Trapani JA. Unlocking the secrets of cytotoxic granule proteins. *J Leukoc Biol.* 2001; 170:18–21. [PubMed: 11435481]
27. Hellerstein MK, Hoh RA, Hanley MB, Cesar D, Lee D, Neese RA, McCune JM. Subpopulations of long-lived and short-lived T cells in advanced HIV infection. *J Clin Invest.* 2003; 112:821–824. [PubMed: 12975464]
28. Kuss I, Schaefer C, Godfrey TE, Ferris RL, Harris JM, Gooding W, Whiteside TL. Recent thymic emigrants and subsets of naïve and memory T cells in the circulation of patients with head and neck cancer. *Clin Immunol.* 2005; 116:27–36. [PubMed: 15925829]
29. Hoyer KK, Doms H, Barron L, Abbas AK. Interleukin-2 in the development and control of inflammatory disease. *Immunol Rev.* 2008; 226:19–28. [PubMed: 19161413]
30. Josefowicz SZ, Rudensky A. Control of regulatory T cell lineage commitment and maintenance. *Immunity.* 2009; 30:616–625. [PubMed: 19464984]
31. Zhang H, Chua KS, Guimond M, Kapoor V, Brown MV, Fleisher TA, Long LM, Bernstein D, Hill BJ, Douek DC, et al. Lymphopenia and interleukin-2 therapy alter homeostasis of CD4+CD25+ regulatory T cells. *Nat Med.* 2005; 11:1238–1243. [PubMed: 16227988]
32. Cesana GC, DeRaffele G, Cohen S, Moroziewicz D, Mitcham J. Characterization of CD4+CD25+ regulatory T cells in patients treated with high-dose interleukin-2 for metastatic melanoma or renal cell carcinoma. *J Clin Oncol.* 2006; 24:1169–1177. [PubMed: 16505437]

Fig. 1.

Expression of GrA, GrB, and perforin in CD4⁺ CD25^{high} Treg and CD4⁺ CD25^{neg} RC.

a Representative dot blots showing the expression of cytotoxins in unstimulated and stimulated Treg and RC obtained from the peripheral blood of a normal control (NC) and a patient with head and neck squamous cell carcinoma (HNSCC). Percentages of positive cells are indicated. **b** Mean percentages of positive cells $\pm$ SD for GrB, GrA, and perforin in Treg or RC of all NC and HNSCC patients enrolled in this study. Freshly harvested, single cell-sorted CD4⁺ CD25^{neg} or CD4⁺ CD25^{high} T cells were examined immediately after isolation or were activated with OKT3 and IL-2 (150 IU/mL) for 24 h prior to staining with the labeled cytotoxin-specific Abs. Stained cells were studied by flow cytometry. Gates were set on CD4⁺ CD25^{high} or CD4⁺ CD25^{neg} T cells. The asterisks in **b** indicate significant differences ($p < 0.01$) in the percentage of positive cells between fresh vs. stimulated T cell subsets

Fig. 2.

Cell death and suppression function in early and late co-cultures of Treg with RC. CD4⁺CD25^{high} Treg and CD4⁺CD25^{neg} cells obtained from a HNSCC patient were either incubated separately or in co-cultures (1S:1RC ratios) for 24 h (a) or for 5 days (b) in the presence of either 150 IU/mL of IL-2 (left panels) or 1000 IU/mL of IL-2 (right panels). At the dose of 150 IU/mL of IL-2, Treg induced suppression of RC proliferation (C, left panel) but then underwent apoptosis, while RC remained viable in these co-cultures (B, left panel). At the doses of 1,000 IU/mL of IL-2, Treg were resistant to death and mediated apoptosis of RC (B, right panel), suppressing their proliferation (C, right panel). After 5-day co-culture (c), no suppression of RC proliferation is evident at the low IL-2 dose (left), while at the

high IL-2 dose (*right*) a complete suppression of RC proliferation occurs. Results of a representative FLOCA of three independent experiments are shown

Fig. 3.

Expression of GrA, GrB, or perforin measured in early (24 h) and late (5 days) co-cultures of Treg (S) with RC. CD4⁺ CD25^{high} Treg (a) and CD4⁺ CD25^{neg} RC (b) were either incubated separately or in co-cultures (1S:1RC ratios) in the presence of either 150 IU/mL of IL-2 (*left panels*) or 1000 IU/mL of IL-2 (*right panels*) for 24 h or 5 days. Cells were obtained from a patient with HNSCC. When cells were cultured alone, high dose of IL-2 increased the expression of granzymes and perforin both in Treg and RC. Treg had a higher overall expression of granzymes and perforin compared to RC. After the first 24 h of co-culture, dramatic changes in the expression of granzymes and perforin were observed and they intensified over time, depending on the IL-2 concentration. At 150 IU/mL of IL-2, Treg were losing the granule expression, while RC were increasing their expression. At 1000 IU/

mL of IL-2, the situation was reversed. Representative data are shown out of three independent experiments performed

Fig. 4.

Effects of GrB-specific or GrA-specific siRNAs on Treg suppressor function. **a** Treg (CD4⁺ CD25^{high}) were transfected with GrB-specific siRNA, GrA-specific siRNA, or irrelevant siRNA. Transfected Treg were stimulated with OKT3 and IL-2 as described in “Materials and methods” section, and incubated for 4 days. Expression of GrA and GrB, respectively, was determined by flow cytometry in fresh, non-transfected vs. siRNA-transfected Treg. The frequency of GrA⁺ or GrB⁺ cells is indicated in the *upper right quadrants*. **b** Suppression of RC proliferation after co-culture of non-transfected or transfected Treg with RC+1,000 IU/mL of IL-2 at different S:RC ratios. Note decreased levels of suppression mediated by Treg transfected with GrA- or GrB-specific siRNA. **c** Apoptosis of RC (percentage of 7-AAD⁺ RC) after co-culture of siRNA-transfected Treg with RC+1,000 IU/mL of IL-2. Co-cultures were established at different S:RC ratios. Note the decreased percent of 7-AAD⁺ RC in the presence of siRNA. The data are from a representative FLOCA of three independent experiments performed with T-cell subsets obtained from a HNSCC patient

Fig. 5.

Effects of GrB-specific siRNAs on RC-mediated killing of Treg. **a** After co-culture of non-transfected RC with Treg+150 IU/mL of IL-2, 97% of RC expressed GrB and these RC were fully viable, while nearly all Treg were 7-AAD⁺. RC proliferation was not inhibited. **b** After transfection of GrB siRNA, only 4% of CD4⁺ CD25^{neg} RC expressed GrB. When these GrB-silenced RC were co-incubated with Treg +150 IU/mL of IL-2, both Treg and RC were 7-AAD^{neg}. These Treg completely suppressed RC proliferation (98%)

Table 1

Cell death and suppressor function in co-cultures of concanamycin A pre-treated Treg (CD4⁺CD25^{high}) or concanamycin A pre-treated RC (CD4⁺CD25^{neg})

	150IU/mL of IL-2		1,000IU/mL of IL-2	
	7-AAD ⁺ cells Percentage (%)	Suppression of RC	7-AAD ⁺ cells	Suppression of RC
(A) Cells incubated alone				
CD4 ⁺ CD25 ^{high} +concanamycin A	3±1	N/A	5±3	N/A
CD4 ⁺ CD25 ^{neg} +concanamycin A	2±0.7	N/A	17±6	N/A
(B) Cells after co-culture				
	No GrB inhibitor I		No GrB inhibitor I	
CD4 ⁺ CD25 ^{high}	50±9		4±3	
CD4 ⁺ CD25 ^{neg}	5±2	None	48±5	95±2
(C) Cells after co-culture				
	RC+GrB inhibitor I		Treg+GrB inhibitor I	
CD4 ⁺ CD25 ^{high}	7±3 *		1 ±0.3	
CD4 ⁺ CD25 ^{neg}	1 ±0.6	82±7	78±6 ***	92±4

CD4⁺ CD25^{high} Treg or autologous CD4⁺ CD25^{neg} RC were first pre-treated with concanamycin A (100 nM) and then incubated alone or in co-cultures (at the ratio of 1S:1RC) for 5 days in the presence of 150 or 1,000 IU/mL of IL-2. The frequency of 7-AAD⁺ cells and the inhibition of RC proliferation were determined by FLOCA. Results are mean percentages±SD obtained in three independent experiments with cells of three different donors

N/A not applicable

* $p < 0.001$ for differences between the percentage of 7-AAD⁺ Treg (CD4⁺ CD25^{high}) in co-cultures with RC not pre-treated with concanamycin A (control) vs. RC+concanamycin A in the presence of 150 IU/mL of IL-2;

** $p < 0.001$ for difference between the five of 7-AAD⁺ RC (CD4⁺ CD25^{neg}) in co-cultures with Treg not pre-treated with concanamycin A vs. Treg+concanamycin A in the presence of 1,000 IU/mL of IL-2

Table 2

Cell death and suppressor function in co-cultures of Treg (CD4⁺ CD25^{high}) and autologous CD4⁺ RC after blocking of granzyme B activity using inhibitor I

	150IU/mL of IL-2		1,000IU/mL of IL-2	
	7-AAD⁺ cells Percentage (%)	Suppression of RC	7-AAD⁺ cells	Suppression of RC
(A) Cells incubated alone				
CD4 ⁺ CD25 ^{high} +concanamycin A	2.3±0.5	N/A	4.5±1	N/A
CD4 ⁺ CD25 ^{neg} +concanamycin A	1.5±0.2	N/A	3.5±2	N/A
(B) Cells after co-culture				
	No GrB inhibitor I		No GrB inhibitor I	
CD4 ⁺ CD25 ^{high}	94±2		3±2	
CD4 ⁺ CD25 ^{neg}	1 ±0.7	94±3	82±6	96±2
(C) Cells after co-culture				
	RC+GrB inhibitor I		Treg+GrB inhibitor I	
CD4 ⁺ CD25 ^{high}	1 ±0.6 *		1 ±0.3	
CD4 ⁺ CD25 ^{neg}	1 ±0.8	80±9	1 ±0.7 **	13±4 **

Co-cultures of CD4⁺ CD25^{high} and autologous CD4⁺ CD25^{neg} RC were left untreated (A) or were pre-treated with GrB inhibitor I at a concentration of 250 µM (B and C) prior to co-cultures. The cells were co-incubated for 5 days in the presence of 150 or 1,000 IU/mL of IL-2. The frequency of 7-AAD⁺ cells and the inhibition of proliferation of RC were determined by FLOCA. Results are mean percentages ± SD obtained in three independent experiments with cells of three different donors

N/A not applicable

* *p* 0.001 for differences between the percentage of 7-AAD⁺ Treg (CD4⁺ CD25^{high}) in co-cultures with RC not pre-treated with GrB inhibitor I (controls) vs. RC+GrB inhibitor I in the presence of 150 IU/mL of IL-2;

** *p* 0.001 for differences between the percentage of 7-AAD⁺ RC (CD4⁺ CD25^{neg}) or percentage suppression levels mediated by Treg not pre-treated with GrB inhibitor vs. Treg+GrB inhibitor I in the presence of 1,000 IU/mL of IL-2