

Granzyme B and Perforin Are Important for Regulatory T Cell-Mediated Suppression of Tumor Clearance

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SUMMARY

Granzyme B is important for the ability of NK cells and CD8⁺ T cells to kill their targets. However, we showed here that granzyme B-deficient mice clear both allogeneic and syngeneic tumor cell lines more efficiently than do wild-type (WT) mice. To determine whether regulatory T (Treg) cells utilize granzyme B to suppress immune responses against these tumors, we examined the expression and function of granzyme B in Treg cells. Granzyme B was not expressed in naive Treg cells but was highly expressed in 5%–30% of CD4⁺Foxp3⁺ Treg cells in the tumor environment. Adoptive transfer of WT Treg cells, but not granzyme B- or perforin-deficient Treg cells, into granzyme B-deficient mice partially restored susceptibility to tumor growth; Treg cells derived from the tumor environment could induce NK and CD8⁺ T cell death in a granzyme B- and perforin-dependent fashion. Granzyme B and perforin are therefore relevant for Treg cell-mediated suppression of tumor clearance *in vivo*.

INTRODUCTION

Many studies have established that cell-mediated innate and adaptive immunity are essential for preventing primary tumor outgrowth and for rejecting transplanted tumors (Dunn et al., 2004a; Pardoll, 2003). Natural killer (NK) cells are innate immune effectors that can kill MHC class I-deficient (MHC I⁻) tumor cell lines *in vivo* (Karre et al., 1986; Smyth et al., 2000a). Mice genetically deficient for NK cells display severe defects in the clearance of MHC I⁻ tumor cells (Kim et al., 2000). In addition, mice depleted of NK cells are more susceptible to chemically induced tumors (Smyth et al., 2001). Adoptive transfer studies have established that CD8⁺ T cells, as major effectors for antigen-specific antitumor immunity, can recognize and kill malignant cells that present antigen peptides

with MHC I molecules (Gorelik and Flavell, 2001; Okada et al., 1997). At the molecular level, the granule exocytosis pathway and Fas-FasL system account for virtually all of the measurable contact-mediated cytotoxicity delivered by NK and CD8⁺ T cells (Lieberman, 2003; Russell and Ley, 2002). The granule exocytosis pathway utilizes perforin to traffic granzymes appropriately into the cytosol (Keefe et al., 2005; Shi et al., 1997), where granzymes A and B (and orphan granzymes) induce cell death by cleaving critical substrates (Heusel et al., 1994; Lieberman, 2003; Revell et al., 2005; Russell and Ley, 2002). Perforin-deficient (*Prf1*^{-/-}) mice are more susceptible to the development of spontaneous lymphomas (Smyth et al., 2000b), suggesting that the perforin-granzyme pathway is an important component of tumor surveillance.

Despite the fact that activated immune cells exist in cancer patients, the immune system often fails to prevent tumors and limit their spread (Dunn et al., 2004b). Thus, established tumors must have developed strategies to inhibit or evade the immune system. Sakaguchi and colleagues first established the essential role of CD4⁺CD25⁺ regulatory T (Treg) cells in the induction and maintenance of peripheral tolerance to self-antigens (Sakaguchi, 2004; Sakaguchi et al., 1995; Yamaguchi and Sakaguchi, 2006). More recent studies have implicated Treg cells in inducing tolerance to tumors (von Boehmer, 2005; Yamaguchi and Sakaguchi, 2006; Zou, 2006). For example, adoptively transferred Treg cells can inhibit tumor-specific CD8⁺ T cell-mediated immunity (Antony et al., 2005; Turk et al., 2004). Furthermore, Treg cells have been shown to suppress NK cell-mediated rejection of transplanted tumor cells or bone-marrow grafts (Barao et al., 2006; Ghiringhelli et al., 2005).

Treg cells may use the perforin-granzyme pathway as a mechanism to suppress the function of immune cells by killing them. Grossman et al. demonstrate that activated human Treg cells expressed granzyme A and/or B and could kill various autologous immune cells in a perforin-dependent but FasL-independent fashion (Grossman et al., 2004a). In addition, Zhao et al. report that activated murine Treg cells suppressed B cell proliferation in a granzyme B- and perforin-dependent fashion (Zhao et al., 2006), whereas Gondek et al. report that activated

murine Treg cells suppressed CD4⁺CD25⁻ T effector cells via a granzyme B-dependent, but perforin-independent, mechanism (Gondek et al., 2005). Importantly, all of these studies utilized in vitro methods to activate Treg cells. It is not yet clear whether Treg cells activated in vivo express granzymes, and if so, whether these molecules are important for Treg cell-mediated suppression of antitumor immune responses.

In this study, we examined the roles of perforin and granzymes for tumor clearance in congenic 129/SvJ mice, by using RMAS lymphoma and B16 melanoma cell lines (both derived from B6 mice) and an acute myeloid leukemia (AML) cell line developed in the 129/SvJ strain. We have found that these tumor cells induced granzyme B (but not granzyme A) expression in Treg cells, which in turn utilized granzyme B to suppress tumor clearance mediated by NK and/or CD8⁺ T cells. Granzyme B deficiency (*Gzmb*^{-/-}) appears to reduce the function of Treg cells more than that of NK and/or CD8⁺ T cells; these immune effectors can utilize granzyme A and/or other mechanisms to kill tumor cells and are not completely disabled by granzyme B deficiency. As a consequence, *Gzmb*^{-/-} mice clear these tumors more efficiently than do wild-type (WT) mice. A series of experiments demonstrated that this phenotype is Treg cell dependent and that granzyme B and perforin are important for the ability of Treg cells to suppress NK and/or CD8⁺ T cell-mediated antitumor responses.

RESULTS

Gzmb^{-/-} Mice Clear Allogeneic Tumor Cells More Efficiently than Do WT Mice

The RMAS lymphoma and B16 melanoma cell lines were derived from C57BL/6 (B6) mice (H-2^b, I-A^b) and express very low amounts of MHC class I and no detectable MHC class II (Figure S1A in the Supplemental Data available online). NK cell-deficient B6 mice have defects in clearing RMAS and B16 cells (Kim et al., 2000). Because all of our granzyme mutations were made and maintained in congenic 129/SvJ mice (also H-2^b, I-A^b), we first evaluated whether the minor histocompatibility differences between the B6-derived RMAS and B16 cell lines and the 129/SvJ mice would affect the ability of these cell lines to be cleared in an NK cell-dependent fashion. By using a well-established strategy to deplete NK cells by intravenous (i.v.) injection of anti-asialo GM1 antiserum, we determined that clearance of both RMAS and B16 tumor cells in the pure 129/SvJ strain is NK cell dependent (Figures S1B and S1C). However, CD8⁺ T cell-depleted mice also displayed reduced survival after RMAS challenge (Figure S1B), suggesting that minor histocompatibility differences can induce CD8⁺ T cell-mediated clearance of these tumor cells.

To define the roles of perforin and granzymes for the clearance of these cell lines, several doses of RMAS cells were used to challenge the mice. The survival curves after i.v. injection of 1 × 10⁵ and 2 × 10⁵ RMAS cells are shown in Figures 1A and 1B, respectively. *Prf1*^{-/-} mice and gran-

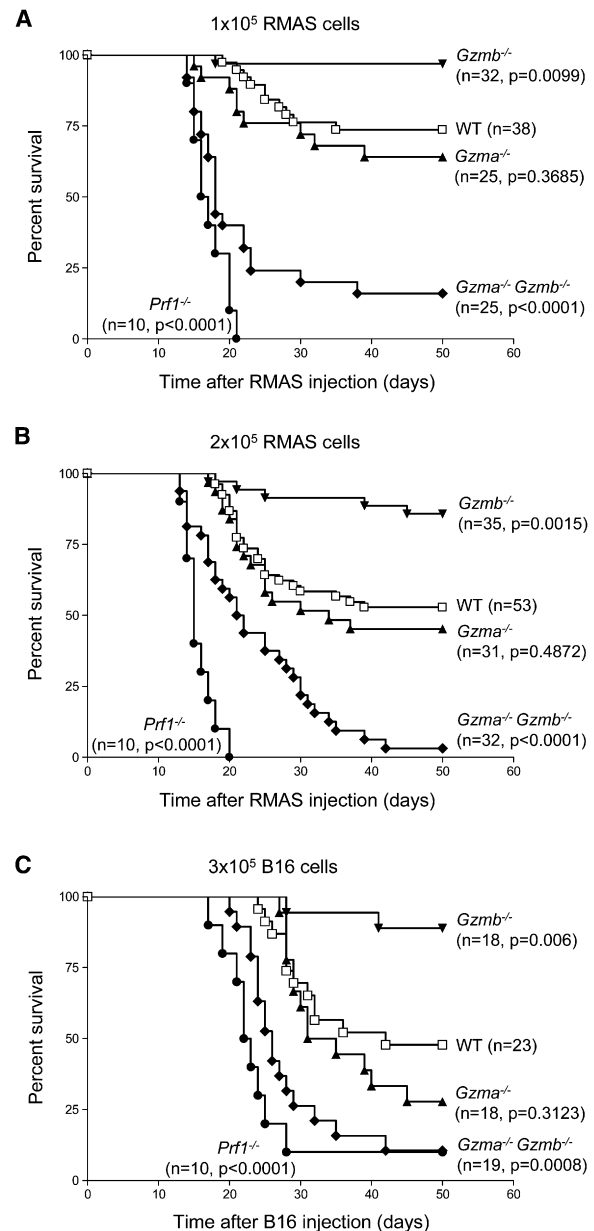


Figure 1. *Gzmb*^{-/-} Mice Have Improved Survival after RMAS or B16 Tumor Challenge

(A) Kaplan-Meier survival curves of 129/SvJ WT mice versus strain-matched mice deficient for the indicated granzymes (*Gzm*) or perforin (*Prf1*), after intravenous injection with 1 × 10⁵ RMAS lymphoma cells. (B) Kaplan-Meier survival curves of 129/SvJ WT mice versus mice deficient for the indicated granzymes or perforin, after intravenous injection with 2 × 10⁵ RMAS lymphoma cells (LD50 for WT mice). (C) Kaplan-Meier survival curves of 129/SvJ WT mice versus mice deficient for the indicated granzymes or perforin, after intravenous injection of 3 × 10⁵ B16 melanoma cells (LD50 for WT mice).

zyme AxB-deficient (*Gzma*^{-/-}*Gzmb*^{-/-}) mice both exhibited significantly reduced survival compared to WT mice (p < 0.0001); the survival of *Prf1*^{-/-} mice was less than that of *Gzma*^{-/-}*Gzmb*^{-/-} mice (p < 0.05 at both doses). The survival of *Gzma*^{-/-} mice was similar to that

of WT mice. Surprisingly, *Gzmb*^{-/-} mice were significantly more resistant to RMA cells than did WT mice ($p < 0.01$; Figures 1A and 1B). Similar patterns were found with mice challenged with 3×10^5 B16 cells (Figure 1C), except that the survival of *Prf1*^{-/-} mice and *Gzma*^{-/-}*Gzmb*^{-/-} mice was not statistically different.

Because RMA clearance in the 129/SvJ strain is influenced by both NK and CD8⁺ T cells (Figure S1), we used flow cytometry to examine granzyme A and B expression in DX5⁺CD3⁻ NK and CD8⁺ T cells isolated from the tumor environments (both ascites fluid after intraperitoneal [i.p.] tumor injection and tumor-infiltrated livers and/or lungs after i.v. tumor injection). Within the NK cell compartment, granzyme A expression was constitutive and did not change after RMA challenge; in contrast, granzyme B was detected in only 1%–5% of resting NK cells from the spleens, livers, or lungs of naive mice (Fehniger et al., 2007). 10%–40% of NK cells in the tumor environments expressed substantial amounts of granzyme B 4–14 days after RMA or B16 tumor challenge (Figure S2 and data not shown). Within the CD8⁺ T cell compartment, neither granzyme A nor B was detected before tumor challenge; both were detected in 10%–50% of CD8⁺ T cells found in the tumor environments 4–14 days after tumor challenge (Figure S3 and data not shown). Because *Gzma*^{-/-}*Gzmb*^{-/-} mice were more susceptible than WT mice to these tumors (Figure 1), these data suggest that NK and/or CD8⁺ T cells utilize granzymes A and B to clear these tumor cells in vivo. Recent data from our laboratory, using in vitro activated NK cells derived from the same mouse strains, are consistent with these findings (Fehniger et al., 2007).

Granzyme B Is Expressed in Allogeneic Tumor-Activated Treg Cells

Previously, our laboratory demonstrated that activated human Treg cells can use the perforin-granzyme pathway to kill a variety of autologous immune cells in vitro (Grossman et al., 2004a). To explain the increased survival of *Gzmb*^{-/-} mice challenged with RMA or B16 tumor cells, we hypothesized that these tumor cells may induce granzyme B expression in Treg cells, which in turn utilize this enzyme to suppress the NK and CD8⁺ T cells that are responsible for clearing these tumors. To test this hypothesis, we assessed the impact of Treg cell depletion with a CD25 antibody (PC61) injected before the tumor challenge. Treg cell-depleted mice cleared RMA cells more efficiently than did mice treated with an isotype control (Figure 2A).

In addition, we used flow cytometry to evaluate granzyme expression in CD4⁺Foxp3⁺ Treg cells (Fontenot et al., 2003; Hori et al., 2003; Khattry et al., 2003) isolated directly from the tumor environments (ascites fluid after i.p. injection or tumor-infiltrated livers and/or lungs after i.v. injection). Granzyme B, but not granzyme A, was detected in 5%–30% of CD4⁺Foxp3⁺ Treg cells found in the ascites fluid of mice bearing RMA tumor cells for 4–14 days (Figures 2B and 2C); very similar granzyme B expression patterns were found in the Treg cells in tumor-in-

filtrated livers and/or lungs after i.v. injection with RMA or B16 cells (data not shown). In contrast, very few granzyme B-expressing Treg cells were found in the spleens (Figure S4) or peripheral lymph nodes of tumor-bearing mice, or in the spleens, livers, or lungs of naive mice (<1%, data not shown). To confirm these flow cytometry results, we purified Treg cells from the ascites fluid of RMA tumor-injected mice and performed immunofluorescence (IF) studies. Granzyme B was detected in the granules of Treg cells derived from the tumor ascites fluid, but not from Treg cells derived from the spleens of naive mice (Figure S5). Perforin was not detected by flow cytometric methods in the same Treg cells and NK cells that were found to express granzyme B (data not shown), by means of an antibody that is capable of detecting perforin in NK cells activated in vitro, and NK cells isolated from MCMV-infected mice (Fehniger et al., 2007). However, the sensitivity of this antibody was relatively low, because we have shown that cells expressing minimal amounts of perforin (as defined by this antibody) are still capable of killing target cells in a perforin-dependent fashion (Fehniger et al., 2007).

Enhanced Clearance of a Syngeneic Tumor Cell Line in Treg Cell-Depleted Mice

To determine whether the enhanced tumor clearance in *Gzmb*^{-/-} mice is also observed with a syngeneic tumor challenge, we developed a tumor cell line from 129/SvJ mice, via an established retroviral transduction strategy (Luo et al., 2005). In brief, bone-marrow cells harvested from two female 129/SvJ mice were transduced with an MSCV-Myc-IRES-Bcl2 construct as described (Luo et al., 2005). Myc-Bcl2 immortalized cells (MB0) were immunophenotyped with flow cytometry (Figure 3A). Compared to primary 129/SvJ splenocytes, MB0 cells expressed relatively low amounts of MHC I (H-2K^b) and were negative for MHC II (I-A^b). MB0 cells were Gr-1 positive and negative for lymphoid markers (CD3, B220). 129/SvJ WT mice injected with MB0 cells developed AML-like histopathology (data not shown). To determine whether Treg cells play a role in the clearance of these tumor cells in 129/SvJ mice, we depleted Treg cells from these animals with a CD25 antibody (PC61), as described above. The Treg cell-depleted mice cleared MB0 cells more efficiently than did mice treated with an isotype control (Figure 3B), suggesting that Treg cells suppress the clearance of these syngeneic tumor cells in vivo.

Granzyme B Expression in Treg Cells Activated by a Syngeneic Tumor Cell Line

We used flow cytometry to evaluate granzyme expression in CD4⁺Foxp3⁺ Treg cells isolated directly from the MB0 tumor environment (ascites fluid after i.p. injection). Granzyme B, but not granzyme A, was detected in CD4⁺Foxp3⁺ Treg cells in the tumor ascites fluid (Figures 4A and 4B). However, MB0 cells induced granzyme B expression in Treg cells with delayed kinetics compared to that of RMA cells, suggesting that the allogeneic signals

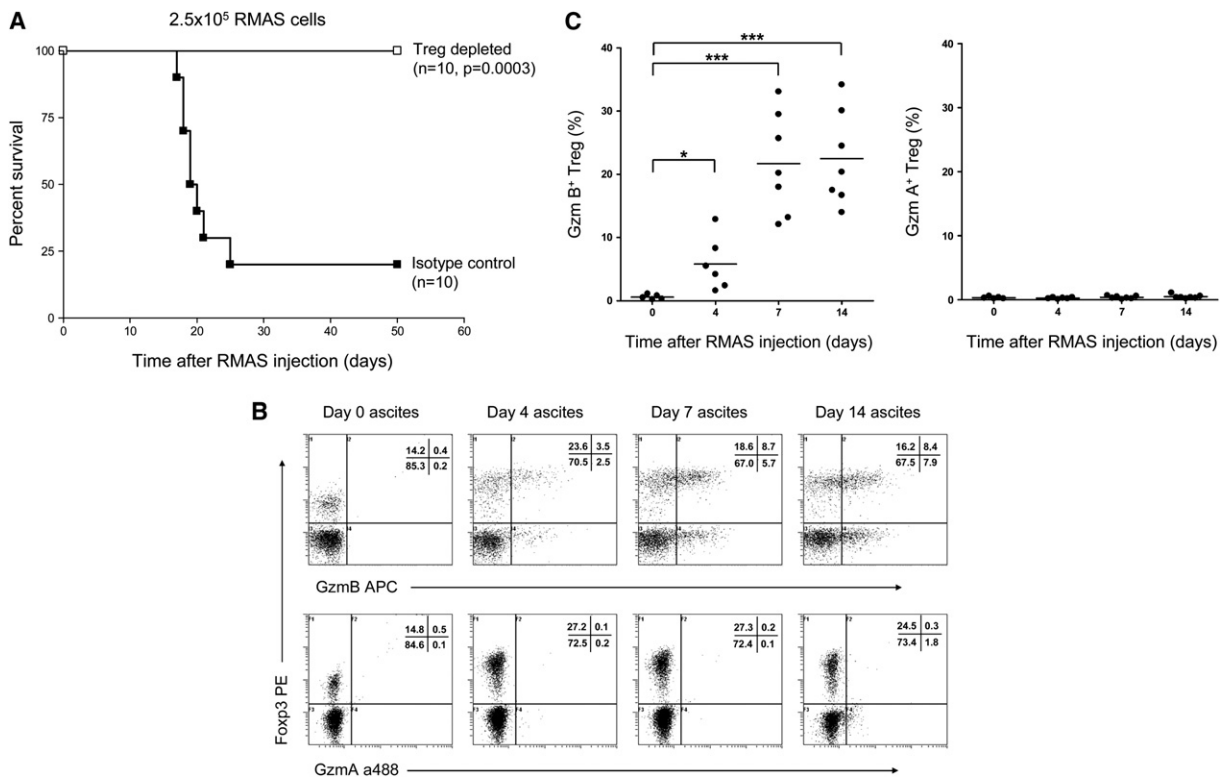


Figure 2. Treg Cell-Depleted Mice Have Improved Survival after RMAS Tumor Challenge, and Granzyme B Is Induced in Tumor-Associated Treg Cells

(A) 129/SvJ WT mice were depleted of Treg cells by PC61 antibody injection as described in the [Experimental Procedures](#). Kaplan-Meier survival curves of Treg cell-depleted mice versus mice treated with an isotype control antibody, after intravenous injection with 2.5×10^5 RMAS cells.

(B) 1×10^6 RMAS cells were injected intraperitoneally, and ascites fluid was collected at the indicated times and analyzed by flow cytometry. CD4⁺Foxp3⁺ cells were examined for the expression of granzymes B and A. Representative flow cytometry plots of cells derived from tumor ascites of individual mice are shown.

(C) Summary scatter plots of the percentage of CD4⁺Foxp3⁺ Treg cells positive for granzyme B or A in the tumor ascites are shown, with each data point representing an individual mouse. Horizontal bars represent the average percentage of granzyme B- or A-positive Treg cells. Two-tailed t tests were used to determine statistical significance between day 0 and the indicated time points (*p < 0.05, **p < 0.01, ***p < 0.001).

caused by minor histocompatibility differences induce more rapid activation of Treg cells.

Because MB0 cells overexpress Bcl-2, and because granzyme B-induced target cell death is inhibited by Bcl-2 (Goping et al., 2003; Jans et al., 1999; Sutton et al., 2003), this cell line is expected to be relatively resistant to granzyme B-mediated killing. We would predict that if granzyme B was relevant only in the NK and/or CD8⁺ T cell effector arm, granzyme B deficiency would have a minimal impact on the clearance of MB0 cells in vivo. Instead, we found that *Gzmb*^{-/-} mice cleared MB0 cells more efficiently than did WT mice, consistent with a predominant effect of granzyme B in the Treg cell compartment (Figures 4C and 4D).

Adoptively Transferred Treg Cells Suppress Tumor Clearance in a Granzyme B- and Perforin-Dependent Fashion

To directly monitor tumor cell clearance in vivo, we transduced RMAS cells with a retroviral vector containing a click beetle red (CBR) luciferase-GFP fusion cDNA,

and we used bioluminescence imaging to serially monitor mice injected intraperitoneally with these cells (Figure 5A). With this method, we quantified the clearance of RMAS cells in vivo and found that *Gzmb*^{-/-} mice cleared RMAS cells more rapidly than did WT mice. As predicted, *Prf1*^{-/-} mice and *Gzma*^{-/-}*Gzmb*^{-/-} mice exhibited a trend toward reduced tumor clearance (Figure 5B; Figure S6). Three independent luciferase-expressing RMAS clones were completely cleared in WT mice by day 14, even when $2\text{--}8 \times 10^6$ cells were injected (data not shown); the transduced RMAS clones expressed high amounts of CBR luciferase and GFP, which presumably induced more potent rejection after the first week of tumor growth in the mice. However, the evaluation of tumor clearance during the early phase (days 0–7) of the antitumor response (Figure 5B; Figure S6) in the various knockout strains correlated well with the survival curves of the mice receiving the nontransduced RMAS cells (Figures 1A and 1B). MB0 cells were resistant to secondary viral transductions with the luciferase-GFP retroviral vector and could not be further evaluated with this assay.

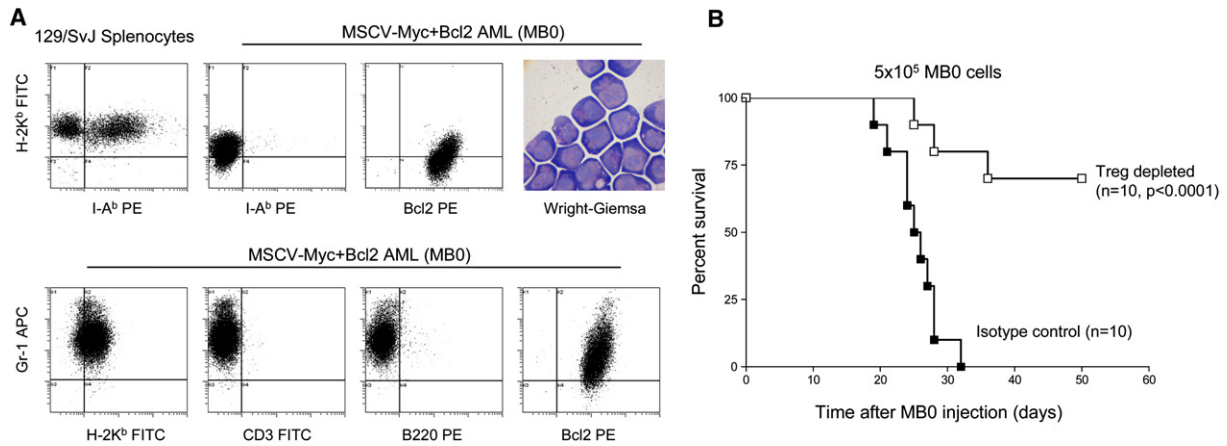


Figure 3. Treg Cell-Depleted Mice Have Improved Survival after Injection of a Syngeneic AML Cell Line

(A) 129/SvJ bone-marrow cells were transduced with an MSCV-Myc-IRES-Bcl2 construct as described in the *Experimental Procedures*, and the cell line MB0 was generated by serial passaging in vitro. MB0 cells were characterized by flow cytometry for the expression of Bcl2, myeloid (Gr-1), and lymphoid markers (CD3, B220), and MHC class I and II (H-2K^b, I-A^b). Wright-Giemsa-stained MB0 cells are also shown.

(B) Kaplan-Meier survival curves of 129/SvJ WT mice depleted of Treg cells versus mice treated with an isotype control antibody, after intraperitoneal injection with 5×10^5 MB0 AML cells.

To determine whether Treg cells can suppress tumor clearance in vivo, we adoptively transferred naive WT Treg cells and luciferase-expressing RMAS cells into *Gzmb*^{-/-} mice. A series of imaging studies with luciferase-expressing RMAS cells (at doses of 3×10^5 , 6×10^5 , 1×10^6 , 2×10^6 , 4×10^6 , and 8×10^6 cells) indicated that a dose of 2×10^6 cells reproducibly established the difference in tumor clearance between WT and *Gzmb*^{-/-} mice (Figures 5B and 5C; Figure S6; and data not shown). By using MACS purification techniques (Miltenyi Biotec), we isolated CD4⁺CD25⁺ Treg cells from the spleens of naive mice for adoptive transfer experiments. The purity of these cells (as determined by post-sort FACS analysis) was consistently above 95%; more than 90% of the purified CD4⁺CD25⁺ T cells were shown to be Foxp3⁺ (Figure S7). To determine the optimal Treg cell dose for adoptive transfer, we simultaneously injected 5×10^5 , 1×10^6 , or 2×10^6 WT Treg cells with 2×10^6 luciferase-expressing RMAS cells into the peritoneal cavity of *Gzmb*^{-/-} mice. Tumor clearance was assessed sequentially by bioluminescence imaging. Compared to *Gzmb*^{-/-} mice that received RMAS cells only, adoptive transfer of 5×10^5 Treg cells showed no effect on clearance, 1×10^6 Treg cells exhibited slightly delayed clearance (data not shown), and 2×10^6 Treg cells significantly suppressed RMAS clearance (Figure 5C). We performed three independent adoptive transfer experiments with identical conditions and pooled the data for analysis (Figure 5C). Adoptive transfer of WT Treg cells into *Gzmb*^{-/-} mice restored tumor growth to about 40% of that observed in WT mice. Transfer of equal doses of *Gzmb*^{-/-} Treg cells (Figure 5C) or *Prf1*^{-/-} Treg cells (Figure 5D) had no significant effect on tumor growth, suggesting that granzyme B and perforin are both required for Treg cell-mediated suppression of tumor clearance in this model system.

The adoptive transfer of 2×10^6 naive WT Treg cells showed no effect on the survival of *Gzmb*^{-/-} mice; however, this dose of Treg cells produced only partial restoration of tumor growth. Because only small numbers of Treg cells could be purified from naive spleens (about 7×10^5 cells per spleen), we were not able to purify adequate numbers of cells for a large study designed to assess survival outcomes.

NK and CD8⁺ T Cell Death in Tumor Ascites Is Treg Cell Dependent

We hypothesized that NK and CD8⁺ T cells might be “suppressed” by being killed by Treg cells in the tumor environment. To test this hypothesis, we performed ex vivo experiments to assess NK and T cell death in tumor ascites fluid. We collected tumor ascites fluid from WT mice, *Prf1*^{-/-} mice, and *Gzmb*^{-/-} mice 5 days after i.p. injection of RMAS cells and immediately stained the cells with 7-AAD to assess cell death. Fewer than 5% of DX5⁺CD3⁻ NK cells or CD8⁺ T cells in the tumor ascites fluid of all three genotypes were 7-AAD positive (data not shown). Because the ingestion of apoptotic cells by peritoneal macrophages could potentially mask our ability to detect apoptotic cells in vivo, we decided to first deplete adherent cells by incubating the tumor ascites fluid on tissue culture plates for 40 min at 37°C (Kobayashi et al., 1990; Law et al., 1989). The nonadherent cells were either directly stained with 7-AAD at time 0 or incubated for 3 hr at 37°C before 7-AAD staining. Immediately after depletion of adherent cells, fewer than 5% of NK or T cells in the tumor ascites of all three genotypes were 7-AAD positive (data not shown); after the 3 hr incubation, however, about 30% of NK and CD8⁺ T cells in the tumor ascites of WT mice became 7-AAD positive (Figures 6A–6C). Importantly, NK and CD8⁺ T cell death was

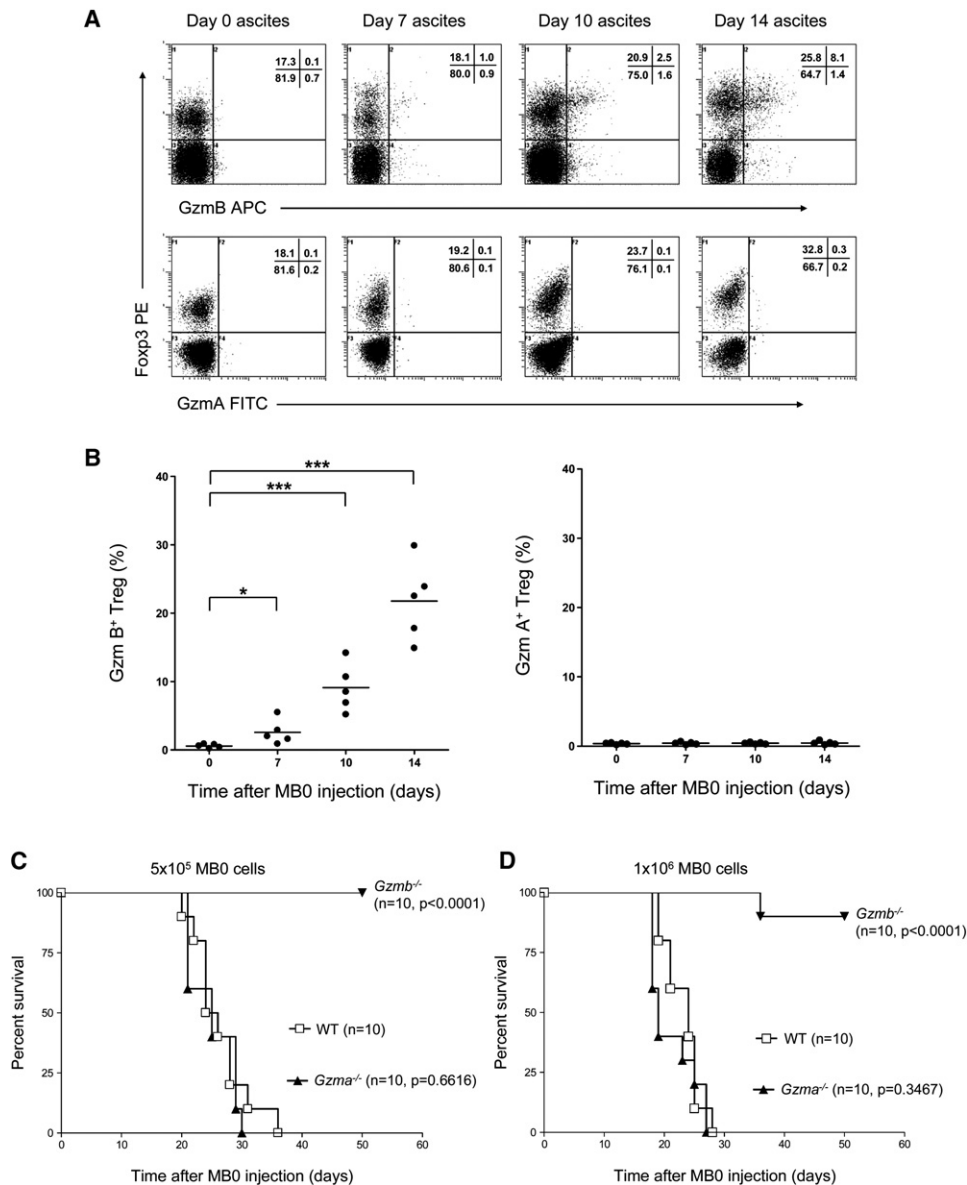


Figure 4. Syngeneic AML Cells Induce Granzyme B Expression in Treg Cells

(A) 1×10^6 MB0 AML cells were injected intraperitoneally, and ascites fluid was collected at the indicated times and analyzed by flow cytometry. CD4⁺Foxp3⁺ cells were examined for the expression of granzymes B and A. Representative flow cytometry plots of cells derived from MB0 AML tumor ascites of individual mice are shown.

(B) Summary scatter plots of the percentage of CD4⁺Foxp3⁺ Treg cells positive for granzyme B or A in the MB0 tumor ascites are shown, with each data point representing an individual mouse. Horizontal bars represent the average percentage of granzyme B- or A-positive Treg cells. Two-tailed t tests were used to determine statistical significance between day 0 and the indicated time points (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

(C) Kaplan-Meier survival curves of 129/SvJ WT mice versus *Gzmb*^{-/-} mice or *Gzma*^{-/-} mice, after intraperitoneal injection with 5×10^5 MB0 AML cells.

(D) Kaplan-Meier survival curves of 129/SvJ WT mice versus *Gzmb*^{-/-} mice or *Gzma*^{-/-} mice, after intraperitoneal injection with 1×10^6 MB0 AML cells.

significantly reduced in the tumor ascites of *Gzmb*^{-/-} mice and *Prf1*^{-/-} mice. In contrast, less than 5% of CD4⁺CD25⁺ Treg cells in the same ascites samples of all three genotypes were 7-AAD positive at time 0 (data not shown) or after 3 hr incubation (Figures 6B and 6C). To assess background cell death in this system, we isolated

control peritoneal fluids from naive, noninjected WT mice ($n = 3$), *Gzmb*^{-/-} mice ($n = 3$), and *Prf1*^{-/-} mice ($n = 3$); spontaneous 7-AAD positivity in the control peritoneal fluids (for all three genotypes) was consistently low for NK cells ($6.8\% \pm 1\%$, $n = 9$), CD8⁺ T cells ($6.3\% \pm 1.6\%$, $n = 9$), and Treg cells ($4.8\% \pm 1.5\%$, $n = 9$). To

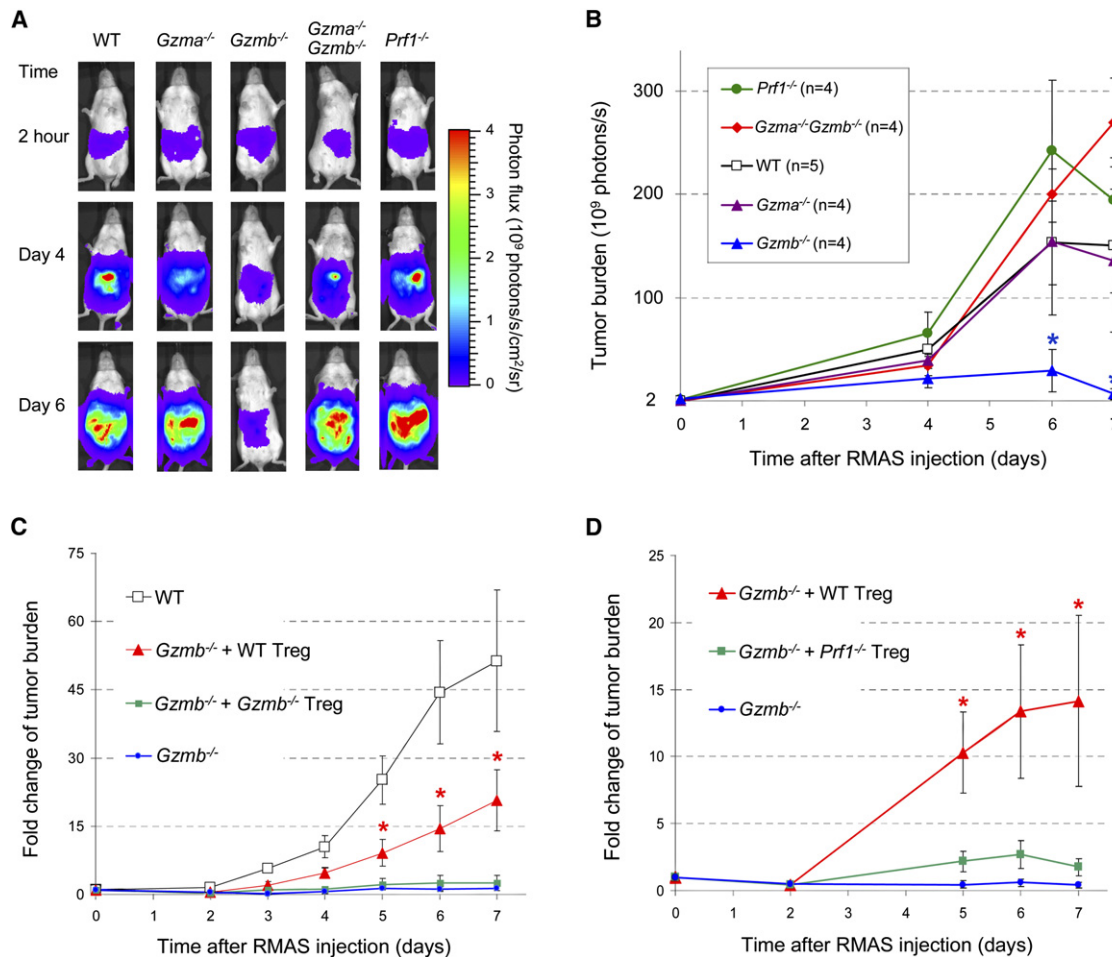


Figure 5. Adoptively Transferred WT, but Not *Gzmb*^{-/-} or *Prf1*^{-/-}, Treg Cells Suppress RMAS Tumor Clearance in *Gzmb*^{-/-} Mice (A) 2×10^6 click beetle red luciferase-expressing RMAS cells were injected intraperitoneally into WT mice, various *Gzm*^{-/-} mice, and *Prf1*^{-/-} mice. Bioluminescence imaging was performed to serially assess whole-body tumor clearance in each mouse, with luciferase activity as a measure for tumor burden. Representative imaging time course of individual mice from each genotype is shown, with the same scale of photon flux indicating luciferase activity.

(B) Summaries of tumor burdens for all genotypes are shown as mean \pm SEM, after i.p. injection of 2×10^6 luciferase-expressing RMAS cells, with 4–5 mice assessed in each genotype. Data points are shown as mean \pm SEM, and two-tailed t tests were used to determine statistical significance between WT mice and mice deficient for the indicated granzymes or perforin ($*p < 0.05$).

(C) A summary of three independent adoptive transfer experiments, with nine mice assessed in each group. Abbreviations: WT, WT mice receiving only tumor cells, as a control for tumor outgrowth in these mice; *Gzmb*^{-/-}, *Gzmb*^{-/-} mice receiving only tumor cells, as a control to demonstrate the reduced tumor growth in these animals; *Gzmb*^{-/-} + WT Treg, *Gzmb*^{-/-} mice adoptively transferred with WT Treg cells, in addition to tumor cells; *Gzmb*^{-/-} + *Gzmb*^{-/-} Treg, *Gzmb*^{-/-} mice adoptively transferred with *Gzmb*^{-/-} Treg cells, in addition to tumor cells.

(D) A summary of two independent adoptive transfer experiments is shown (n = 6 in each group). Abbreviation: *Gzmb*^{-/-} + *Prf1*^{-/-} Treg, *Gzmb*^{-/-} mice adoptively transferred with *Prf1*^{-/-} Treg cells, in addition to tumor cells. Data points are shown as mean \pm SEM, and two-tailed t tests were used to determine statistical significance ($*p < 0.05$).

determine whether Treg cells are relevant for the death of NK and CD8⁺ T cells in the tumor ascites fluid, we depleted Treg cells from mice by injecting PC61 CD25 antibody 4 and 2 days prior to injection with tumor cells. This strategy depleted the majority of CD4⁺Foxp3⁺ Treg cells (72% \pm 7%, n = 18) in the tumor ascites fluid (data not shown); NK and CD8⁺ T cell death in the Treg cell-depleted tumor ascites of WT mice was reduced by more than 70% ($p < 0.01$, n = 6) (Figures 6A and 6C). Taken together, these data suggest that Treg cells can induce effector cell death in the tumor environment by a granzyme

B- and perforin-dependent mechanism. Additionally, NK and CD8⁺ T cell death in the tumor ascites of *Gzmb*^{-/-} mice and *Prf1*^{-/-} mice was further reduced after Treg cell depletion (Figures 6A and 6C), suggesting that additional mechanisms may contribute to Treg cell-mediated suppression of NK and CD8⁺ T cells. Absolute NK and CD8⁺ T cell numbers in the ascites fluid of the WT mice, *Gzmb*^{-/-} mice, and *Prf1*^{-/-} mice were difficult to compare at any given time, because the numbers of tumor cells in the ascites fluid of these mice were so drastically different (Figures 5B and 6A; Figure S6).

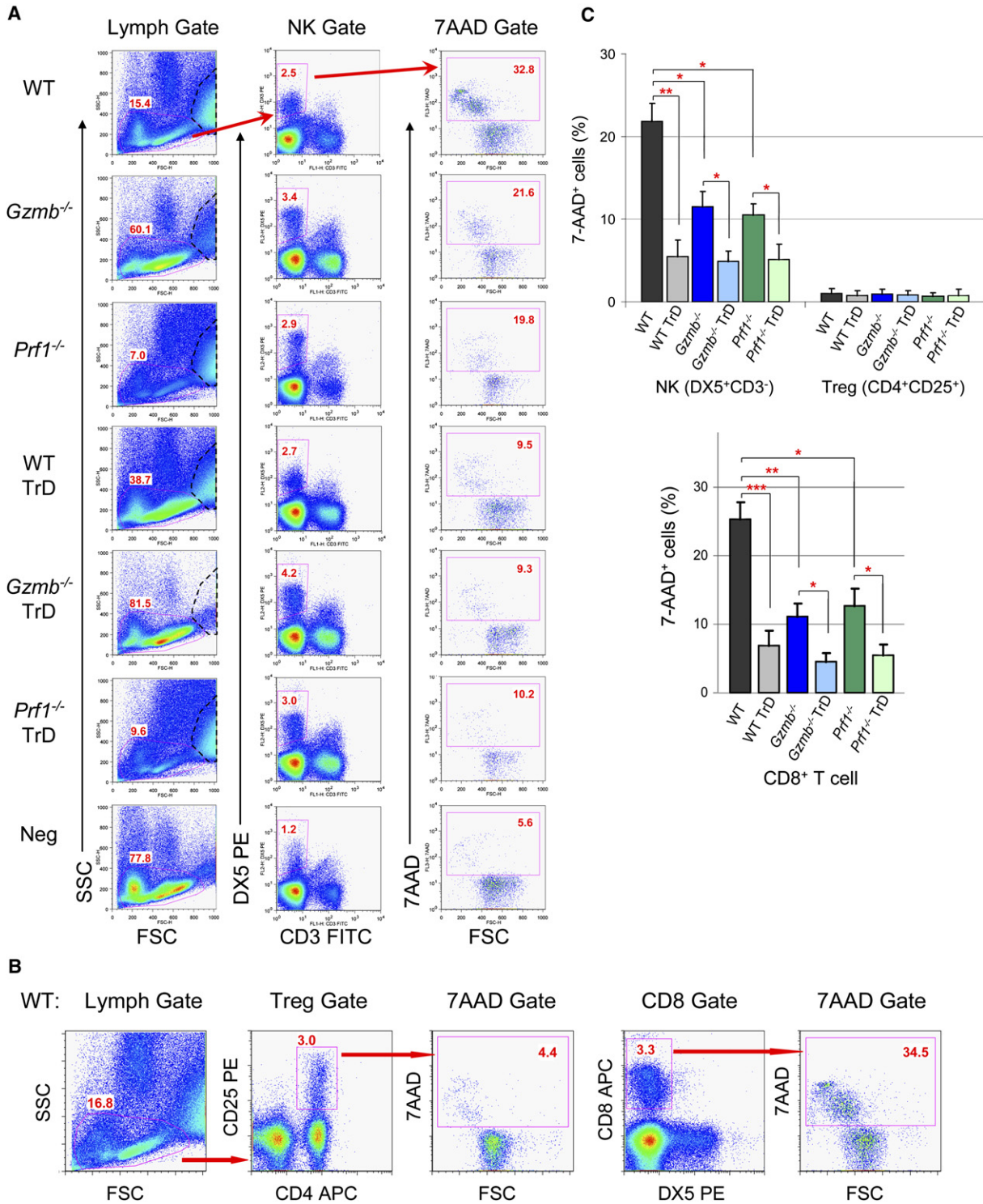
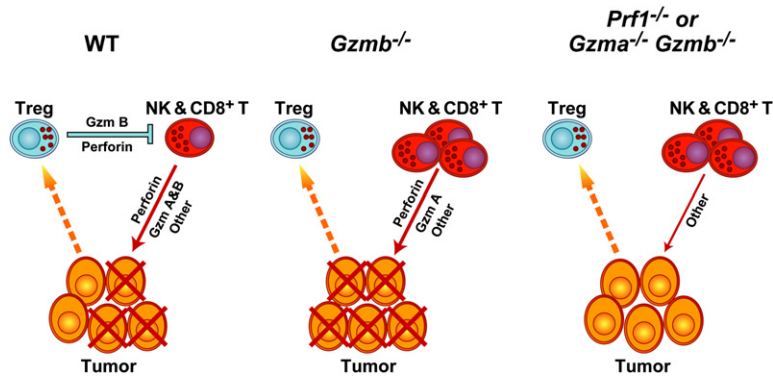


Figure 6. NK and CD8⁺ T Cell Death in Tumor Ascites Is Treg Cell Dependent and Is Reduced in *Gzmb*^{-/-} Mice and *Prf1*^{-/-} Mice

WT mice, *Gzmb*^{-/-} mice, and *Prf1*^{-/-} mice were treated with intraperitoneal injections of 500 μg of PC61 CD25 monoclonal antibody to deplete Treg cells (TrD), or rat IgG1 as a control, on days -4 and -2 prior to intraperitoneal injection of 1 × 10⁶ RMAS cells. On day 5 after tumor injection, tumor ascites was collected and depleted of adherent cells. As a negative control (Neg), peritoneal fluid was isolated from naive, noninjected WT mice (n = 3), *Gzmb*^{-/-} mice (n = 3), and *Prf1*^{-/-} mice (n = 3) and treated identically. Nonadherent cells were incubated in V-bottom 96-well plates for 3 hr, followed by staining with cell-surface markers and 7-AAD to assess cell death in the NK, CD8⁺ T, and Treg compartments by flow cytometry.



necessary to suppress the function of NK cells and CD8⁺ T cells. However, the effector cells can use alternative mechanisms to kill the tumors, and therefore these mice are relatively resistant to tumor outgrowth.

Right: In mice deficient for perforin or granzymes A and B, both regulatory and effector cells are functionally disabled. Tumor cells can proliferate because they cannot be controlled by the alternative killing mechanisms of the effectors, even though they are not suppressed by Treg cells.

DISCUSSION

By using congenic 129/SvJ mice deficient for perforin or granzymes as hosts, we have studied the clearance of three independent tumor cell lines in vivo. RMAS and B16 cells (derived from B6 mice) share the same MHC I and II as 129/SvJ mice but have minor histocompatibility differences. In contrast, the MB0 cell line was derived from the bone-marrow cells of 129/SvJ mice and is therefore syngeneic with the hosts. Surprisingly, *Gzmb*^{-/-} mice cleared all of these tumors more efficiently than did WT mice; Treg cell-depleted mice also displayed enhanced tumor clearance. These results suggested that Treg cells might suppress antitumor immune responses via a granzyme B-dependent mechanism. We indeed found that granzyme B (but not granzyme A) was highly induced in Treg cells in the tumor environment. Adoptive transfer of purified Treg cells directly demonstrated that granzyme B and perforin are required for Treg cell-mediated suppression of the antitumor response against RMAS cells. Ex vivo evaluation of in vivo activated NK and T cells suggested that tumor-activated Treg cells can cause the death of NK and CD8⁺ T cells in a granzyme B- and perforin-dependent fashion. Taken together, these results suggest that these tumors can induce granzyme B expression in Treg cells, which then utilize this protease to induce the death of NK and/or CD8⁺ T cells, thereby suppressing antitumor immunity (Figure 7). By activating granzyme B in Treg cells, these tumor cells gain a survival advantage,

but this advantage is lost in the setting of granzyme B deficiency.

The studies described here suggest that Treg cells can suppress the ability of NK and CD8⁺ T cells to clear tumors in vivo. Recent reports from two other groups have also shown that Treg cells can suppress NK cell-mediated immune responses (Ghiringhelli et al., 2005; Barao et al., 2006). In one model, Treg cells were shown to suppress NK cell proliferation and function via membrane-bound TGF- β ; TGF- β -deficient Treg cells failed to suppress (Ghiringhelli et al., 2005). In a bone-marrow transplant model, Treg cells were likewise shown to suppress NK cell-mediated rejection of transplanted BM cells via a TGF- β -dependent mechanism; neutralizing TGF- β antibodies increased NK cell-mediated rejection of BM grafts (Barao et al., 2006). Neither of these studies specifically addressed the role of the perforin-granzyme pathway for Treg cell-mediated NK suppression in these experimental settings. However, it is important to note that TGF- β can influence the expression of perforin and granzymes, thereby altering the function of cytotoxic lymphocytes (Thomas and Massague, 2005). The effect of TGF- β on perforin and granzyme expression in Treg cells has not yet been defined but is under investigation.

In the model systems described in this study, granzyme B and perforin were shown to be important for the ability of Treg cells to suppress NK and CD8⁺ T cell activity. Recent studies from our laboratory and others have suggested that human and mouse Treg cells can use the

Figure 7. Proposed Model for the Role of Granzyme B and Perforin in Treg Cell-Mediated Suppression of Antitumor NK and CD8⁺ T Cells

Left: In wild-type mice, RMAS, B16, and MB0 AML tumors induce granzyme B expression in Treg cells by an unknown mechanism (indicated by the dashed line). Activated Treg cells utilize granzyme B and perforin to suppress NK cell and/or CD8⁺ T cell-mediated antitumor immunity in vivo. NK cells and CD8⁺ T cells use the perforin-granzyme pathway, as well as perforin- and granzyme-independent mechanisms, to kill tumor cells in vivo.

Center: In granzyme B-deficient mice, Treg cells are missing a key molecule that is

(A) Representative flow cytometry plots showing the percentage of 7-AAD-positive DX5⁺CD3⁻ NK cells in the tumor ascites fluid from individual mice with the indicated genotypes, with or without in vivo depletion of Treg cells (TrD). The black dashed lines (on the right side the lymphocyte gates) indicate the position of the RMAS tumor cells.

(B) Representative flow cytometry plots showing the percentage of 7-AAD-positive CD4⁺CD25⁺ Treg cells and CD8⁺ T cells in the tumor ascites fluid from an individual WT mouse without in vivo depletion of Treg cells. CD4 and CD25 were used as Treg surface markers because intracellular staining (with Foxp3) precludes live-cell analysis with 7-AAD.

(C) A summary of pooled data from three independent experiments is shown (n = 2 in each experimental group, 6 total mice). Spontaneous death of NK cells (6.8% \pm 1%, n = 9), of Treg cells (4.8% \pm 1.5%, n = 9), and of CD8⁺ T cells (6.3% \pm 1.6%, n = 9) in the noninjected control peritoneal fluid was subtracted to provide specific cytotoxicity data. Data points are shown as mean \pm SEM, and two-tailed t tests were used to determine statistical significance (*p < 0.05, **p < 0.01, ***p < 0.001).

perforin-granzyme pathway to kill target cells as a mechanism of suppression (Grossman et al., 2004a; Zhao et al., 2006). Previous reports also demonstrated the expression of granzyme B in purified murine CD4⁺CD25⁺ Treg cells that were activated in vitro by TCR ligation and IL-2 (Gondek et al., 2005; Zhao et al., 2006). However, as noted above, several different mechanisms are potentially relevant for Treg cell-mediated immune suppression, including cytokine-mediated (e.g., TGF- β , IL-10) as well as cell-surface molecule-dependent inhibition of T cells and APCs (e.g., GITR and CTLA4) (von Boehmer, 2005; Zou, 2006). The exact contribution of each pathway to overall Treg suppressive activity may be context and/or model dependent; however, it is also possible that Treg cells may use several mechanisms simultaneously to provide "fail-safe" suppression of activated immune cells, which can damage the host if uncontrolled.

Our studies suggest that Treg cells may suppress NK and CD8⁺ T cells by killing them. The system that we used to demonstrate this finding relies upon the activation of T cells and NK cells in vivo, followed by depletion of adherent cells (to prevent the ingestion of newly apoptotic cells), followed by ex vivo incubation to allow the activated cells to interact. The day 5 ascites fluid contains a complex mixture of cells, but it is probable that NK and CD8⁺ T cell death in this setting is Treg cell dependent (because depletion of Treg cells with the PC61 CD25 antibody reduces NK and CD8⁺ T cell death). Further, our data suggest that both granzyme B and perforin are relevant for this phenotype, because deficiency of either reduces NK and CD8⁺ T cell death. However, the reduction in NK and CD8⁺ T cell death was not as pronounced with perforin or granzyme B deficiency as it was with Treg cell depletion, suggesting that Treg cells must use additional pathways to cause suppression and/or death to NK and CD8⁺ T cells. Because these experiments involved a complex mixture of peritoneal cells, we do not know whether Treg cells induce NK and CD8⁺ T cell death via a direct or an indirect mechanism. To answer this question definitively, we will need to define the precise signals that tumor cells use to activate Treg cells, NK cells, and CD8⁺ T cells in vivo and use highly purified populations of these activated cells to explore a direct killing mechanism. However, because the perforin-granzyme system is contact dependent and capable of directly causing target cell death, it seems possible to us that Treg cells may suppress NK and CD8⁺ T cell function in this setting by directly causing their death. However, our data suggests that activated Treg cells do not kill tumor cells, because depletion of Treg cells improved the survival of tumor-bearing mice.

Granzyme B deficiency decreases Treg cell function more than it reduces the function of antitumor NK and CD8⁺ T cells in vivo. Recent studies from our lab have shown that granzyme B plays a major role in the ability of IL-15-activated NK cells to kill their targets (Fehniger et al., 2007); however, the in vivo activation of NK and CD8⁺ T cells by tumors may invoke additional killing pathways. Indeed, granzyme B-independent mechanisms are clearly capable of providing NK cell-mediated tumor

clearance (Grundy et al., 2007; Rosen et al., 2000; Screpanti et al., 2001).

In summary, the perforin-granzyme pathway is important not only for the function of NK and CD8⁺ T cells, but it may also be utilized by Treg cells to suppress the activity of these same cells (Figure 7). It will be important to determine whether Treg cells can use the perforin-granzyme B pathway to suppress other immune cells in vivo, such as CD4⁺CD25⁻ T cells and B cells; alternative model systems will be required to address these questions in the future.

EXPERIMENTAL PROCEDURES

Animals and Tumor Cell Lines

WT 129/SvJ mice were obtained from the Jackson Laboratory. *Gzma*^{-/-} mice were generated as described (Shrestha et al., 1997). *Gzmb*^{-/-} mice were generated as described (Heusel et al., 1994; Revell et al., 2005). Both *Gzma*^{-/-} mice and *Gzmb*^{-/-} mice were generated and maintained in congenic 129/SvJ mice (H-2^b, I-A^b). *Gzma*^{-/-}*Gzmb*^{-/-} mice were generated by crossing *Gzma*^{-/-} mice with *Gzmb*^{-/-} mice, followed by genotyping with Southern and PCR strategies. *Prf1*^{-/-} mice were developed by a speed congenic strategy to backcross B6J mice containing the "Kagi" mutation to 129/SvJ mice for eight generations; >98% of the congenic markers tested were from the 129/SvJ strain in the mice that were used to generate the breeding colony of these mice. RMAS lymphoma and B16 melanoma cell lines, which were originally derived from C57BL/6 mice (H-2^b, I-A^b), were obtained from W. Yokoyama and maintained in complete K10 medium (Fehniger et al., 2007). The MB0 acute myeloid leukemia (AML) cell line was developed from 129/SvJ mice by an established retroviral transduction strategy (Luo et al., 2005). In brief, bone-marrow cells harvested from two female 129/SvJ mice were transduced with an MSCV-Myc-IRES-Bcl2 construct as described (Luo et al., 2005). Myc-Bcl2 immortalized cells (MB0) were passaged in regular K10 medium (without additional growth factors) for 3 months before injection for survival and granzyme expression studies. All mice were bred and maintained in SPF housing, and all experiments were conducted in accordance with Washington University School of Medicine animal care guidelines, via protocols approved by the animal studies committee.

Reagents and Antibodies

The anti-asialo GM1 antiserum was purchased from Wako Chemicals. The purified CD25 antibody (PC61) and rat IgG1 isotype control (HRPN), CD8 antibody (53-6.7), and rat IgG2a isotype control (C1.18) were purchased from Bio Express. The CD4⁺CD25⁺ Treg isolation kits were purchased from Miltenyi Biotec. Antibodies used include anti-mouse CD3 (145-2C11), CD4 (RM4-5), CD8 (53-6.7), CD25 (7D4, PC61), CD49b (DX5), CD16/32 (2.4G2), Bcl-2 (3F11) (BD Biosciences), Foxp3 (eBioscience, FJK-16 s), and granzyme B (Caltag, GB12). The anti-mouse granzyme A monoclonal antibody was produced in our laboratory and is now available from Santa Cruz (3G8.5).

Tumor Challenge and Survival Study

Tumor cells were washed twice in RPMI 1640 by centrifuging for 5 min at 300 \times g and were then resuspended in RPMI 1640 at appropriate concentrations. For survival studies, all WT mice, *Gzmb*^{-/-} mice, and *Prf1*^{-/-} mice were injected in the lateral tail vein with 1×10^5 , 2×10^5 , or 2.5×10^5 RMAS lymphoma cells, or 2×10^5 or 3×10^5 B16 melanoma cells in 200 μ l RPMI 1640 on day 0. All mice were monitored over a 60 day period. For the MB0 tumor challenge, mice were injected i.p. with 5×10^5 or 1×10^6 cells. Mice were sacrificed when they gained more than 25% body weight and/or become moribund. To determine the impact of Treg cell depletion on survival, 500 μ g of purified PC61 CD25 monoclonal antibody (or rat IgG1 isotype control) was administered by intraperitoneal injection 2 days prior to tumor injection

and readministered weekly during the course of the experiment. CD8 monoclonal antibody (53-6.7) or rat IgG2a isotype control was administered similarly to determine its effect on the survival of mice challenged with RMAS cells. Kaplan-Meier survival analysis was performed with Prism software (Graph Pad, San Diego, CA).

Cell Isolation

Lymphocytes were isolated from the tumor environment, which includes ascites fluid after i.p. tumor injection or tumor-infiltrated livers and/or lungs after i.v. tumor injection and the spleens and peripheral lymph nodes of tumor-bearing mice (which were not infiltrated by tumor cells). As controls, the peritoneal fluid, spleens, livers, and lungs of naive mice were also collected as described (Grossman et al., 2004b; Heusel et al., 1994; Revell et al., 2005).

Intracellular Staining and Flow Cytometry

1×10^6 cells were washed and resuspended in staining buffer (PBS, 0.5% bovine serum albumin, 0.5 mM EDTA). Samples were labeled with primary-conjugated antibodies against cell-surface markers (anti-mouse CD3, CD4, CD8, CD25, DX5) (BD Biosciences). Samples were fixed, permeabilized (Foxp3 staining kit, eBioscience), and stained with primary-conjugated anti-granzyme A antibody (3G8.5; Santa Cruz), and/or primary-conjugated anti-granzyme B antibody (GB12; Caltag), and/or primary-conjugated anti-Foxp3 antibody (FJK-16 s; eBioscience) diluted at 1:400 in staining buffer. Sample data were acquired on a Cytex-modified FACScan (Becton Dickinson) or FC500 (Coulter) flow cytometer and analyzed with CXP (Coulter) or FlowJo (TreeStar) software. Isotype controls were used to set quadrant and regional gates. All flow cytometric analyses depicted are representative of five or more mice. Anti-granzyme A and anti-granzyme B antibodies were analyzed for antigen specificity by knockout mouse staining assays as described (Fehniger et al., 2007; Grossman et al., 2004b).

Bioluminescence Imaging of In Vivo Tumor Clearance

RMAS cells were transduced with a Δ U3 retroviral construct that drives the expression of a fusion cDNA encoding click beetle red luciferase (CBR) and green fluorescence protein (GFP). Individual GFP-positive RMAS cells were MoFlo sorted into single wells of a 96-well plate. The expanded clones were further screened for high luciferase expression. CBR luciferase-expressing RMAS clones were injected i.p. into WT mice, *Gzm*^{-/-} mice, and *Prf1*^{-/-} mice in 200 μ l RPMI 1640 on day 0, and bioluminescence imaging was performed 2 hr later and variously on days 2 through 21 after injection. For imaging, mice were injected i.p. with D-luciferin (150 μ g/g body weight), anesthetized (2% isoflurane), and placed in an IVIS 50 imaging system (Xenogen) (exposure time, 1–30 s; binning, 2–8; no filter; f/stop, 1; FOV, 12 cm) (Gross and Piwnicka-Worms, 2005). Regions of interest (ROI) were defined manually over the whole body with Living-Image software (Igor Wave-metrics) for determining tumor burden signal intensities. Data were expressed as photon flux (photons/s) or change from initial (post injection image/2 hr image) across all mice receiving the same treatment.

Adoptive Transfer of CD4⁺CD25⁺ Treg Cells

CD4⁺CD25⁺ Treg cells were isolated from the resting spleens of WT mice, *Prf1*^{-/-} mice, and *Gzm*^{-/-} mice with the CD4⁺CD25⁺ Treg isolation kit, according to the manufacturer's instructions (Miltenyi Biotec). 2×10^6 CD4⁺CD25⁺ Treg cells (>95% pure) were injected i.p. with 2×10^6 luciferase-expressing RMAS cells into each *Gzm*^{-/-} host mouse. Bioluminescence imaging was performed to serially assess tumor clearance.

Flow-Based Ex Vivo NK and CD8⁺ T Cell Death Assay

WT mice, *Gzm*^{-/-} mice, and *Prf1*^{-/-} mice were depleted of Treg cells by intraperitoneal injection of 500 μ g of PC61 CD25 monoclonal antibody (or rat IgG1 control antibody) on day 4 and day 2 prior to intraperitoneal injection of 1×10^6 RMAS cells. On day 5 after tumor injection, tumor ascites fluid was harvested. To prevent the phagocytosis of ap-

optotic cells, the ascites fluid was depleted of adherent cells by incubating total ascites fluid on plastic tissue-culture plates for 40 min (Kobayashi et al., 1990; Law et al., 1989). As a negative control, peritoneal fluid was isolated from naive, noninjected WT mice, *Gzm*^{-/-} mice, and *Prf1*^{-/-} mice, and treated identically. Nonadherent cells were harvested and incubated in V-bottom 96-well plates for 3 hr, followed by staining with primary-conjugated antibodies against cell-surface markers (CD3, CD4, CD8, CD25 [7D4], DX5) and 7-AAD to assess cell death in the NK, CD8⁺ T, and Treg compartments by flow cytometry.

Supplemental Data

Seven figures are available at <http://www.immunity.com/cgi/content/full/27/4/635/DC1/>.

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