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CD4⁺CD25⁺ T Regulatory Cells Suppress NK Cell-Mediated Immunotherapy of Cancer

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CD4⁺CD25⁺ regulatory T cells (Treg) that suppress T cell-mediated immune responses may also regulate other arms of an effective immune response. In particular, in this study we show that Treg directly inhibit NKG2D-mediated NK cell cytotoxicity in vitro and in vivo, effectively suppressing NK cell-mediated tumor rejection. In vitro, Treg were shown to inhibit NKG2D-mediated cytolysis largely by a TGF-β-dependent mechanism and independently of IL-10. Adoptively transferred Treg suppressed NK cell antitumor function in RAG-1-deficient mice. Depletion of Treg before NK cell activation via NKG2D and the activating IL-12 cytokine, dramatically enhanced NK cell-mediated suppression of tumor growth and metastases. Our data illustrate at least one mechanism by which Treg can suppress NK cell antitumor activity and highlight the effectiveness of combining Treg inhibition with subsequent NK cell activation to promote strong innate antitumor immunity. The Journal of Immunology, 2006, 176: 1582–1587.

CD4⁺CD25⁺ regulatory T cells (Treg) are a unique population of T cells that maintain immune tolerance and are critical in host suppression of organ-specific autoimmune diseases (1, 2). Treg also contribute to elicit a dominant tolerance during infections (3, 4) and allogeneic transplantation (5) and suppress immune responses to tumors at both the priming and effector phases (6–9). Depletion of Treg in mice with anti-IL-2 receptor (PC61) Ab (7) or low dose cyclophosphamide depletion of Treg (9) improves T cell-based tumor clearance. In addition, cancer patients have increased numbers of peripheral and tumor-infiltrating Treg that functionally inhibit tumor-specific T cells and predict poor survival (10, 11).

Tumor immunity is not only T cell dependent; recently, a number of studies in leukemia (12), lymphoma (13), and gastrointestinal stromal tumors (14) revealed that NK cell activation and cytotoxicity influence patient outcome. NK cells are regulated by cytokines in the environment, and when interacting with tumor directly, there is a delicate balance between inhibitory signals mediated by MHC class I molecules and activating signals triggered by specific ligands (15–17). In particular, the activating NKG2D receptor expressed by NK cells is an important mechanism of tumor recognition and suppression (18–20). Tumors possess many mechanisms by which they might evade NK cell-mediated suppression (16), such as shedding of soluble ligands for activation (21, 22) and secretion of inhibitory cytokines such as TGF-β (23–25); however, to date, the role that Treg might play in hampering NK cell activation has been largely ignored. In this study we present a series of experiments that convincingly demonstrate that Treg directly suppress NK cell cytotoxicity in vitro and in vivo, thus providing a novel rational approach to stimulating more powerful innate antitumor immunity.

Materials and Methods

Mice

Inbred C57BL/6 (B6) and BALB/c wild-type (WT) mice were purchased from the Walter and Eliza Hall Institute of Medical Research. The B6 RAG-1-gene-targeted mice were 10 generations backcrossed to B6 and were bred at the Peter MacCallum Cancer Center. Mice, 6–12 wk of age, were used in all experiments that were performed according to animal experimental ethics committee guidelines.

Isolation of spleen NK cells, Treg, and cytotoxicity assay

Spleen cells were depleted of B cells by labeling with anti-B220 (clone RA3-6B2), followed by incubation with sheep anti-rat IgG-conjugated immunomagnetic Dynabeads M-450 (Dynal Biotech). Bead-labeled cells were magnetically removed using a Dynal MPC-1 magnetic particle concentrator. Remaining cells were subsequently labeled with anti-FeR mAb (clone 2.4G2 grown in-house), CD4-FTC (clone RM4-5), anti-CD25-PE (clone PC61), anti-NK1.1-PE-Cy7 (clone PK136), and anti-TCRβ-APC (clone H57-597; all purchased from BD Pharmingen), and specific cell populations were sorted using a FACSAria (BD Biosciences). Spleen NK cells were sorted as NK1.1⁺CD90⁺ cells, Treg were sorted as TCRαβ⁺CD4⁺CD25high cells, and conventional CD4 T cells were sorted as TCRαβ⁺CD4⁺CD25high. The cytolytic activity of NK cells from PC61- or control Ig-treated mice was tested against tumor target cells at a number of E:T cell ratios against NK cell-sensitive targets in a standard 6-h ⁵¹Cr release assay as previously described (26). Where indicated, NK cells were cocultured at a 3:1, 1:1, or 1:3 ratio with CD4⁺CD25high or CD4⁺CD25low cells. Before coculture, in some groups Treg were preactivated with anti-CD3 (clone 145-2C11) and anti-CD28 on plastic for 48 h with 500 U/ml IL-2 as previously described (27). In some experiments NK cells and activated CD4⁺CD25high T cells were cocultured or separated by Transwells, with targets and NK cells incubated together. In the same experiments the supernatants from 48-h activated T cells were cocultured with NK cells and tumor target cells. The coculture assay was performed in the presence of neutralizing anti-mouse...
Tumor cell lines

The following standard experimental mouse tumor cell lines were used in vitro and in vivo: matched B16 melanoma (H-2b) and B16-Rae-1 melanoma (provided by Dr. L. Lanier, University of California, San Francisco, CA), matched RMA-S lymphoma (H-2b) and RMA-S-Rae-1B lymphoma, and the 3LL Lewis lung carcinoma (H-2b). The maintenance of all tumor cell lines has been previously described (28).

Tumor models in vivo

3LL, B16, and B16-Rae-1 tumor cells were inoculated i.v. at the doses indicated, and on day 14, the surface metastases were counted with the aid of a dissecting microscope. Recombinant mouse IL-12 was provided by Genetics Institute, and the preparation of IL-12 was diluted in PBS immediately before use. RMA-S-Rae-1B and RMA-S were inoculated s.c. at the doses indicated. Tumor growth was measured every second day with a caliper square as the product of two diameters.

NK and CD8 cell depletion

To determine the effector subsets mediating tumor suppression, some groups of B6 and BALB/c mice were additionally depleted of NK cells or CD8\(^+\) cells using 100 \(\mu\)g of rabbit anti-asialoGM1 Ab i.p. (Wako Chemicals) or anti-CD8 (53-6.7) on days 0, 1, and 7 (after tumor inoculation), respectively, or both together as described previously (29). Specific NK cell and CD8\(^+\) T cell depletion was validated as previously described (29, 30).

Statistical analysis

Significant differences in the number of metastases and tumor size were determined by unpaired Mann-Whitney \(U\) test, whereas significant differences in NK cell cytotoxicity were determined by Student’s \(t\) test. A value of \(p < 0.05\) was considered significant.

Results

Enhanced NK cell-mediated suppression of tumors expressing NKG2D ligands in the absence of Treg

To examine the importance of Treg in controlling NK cell-mediated immunity to tumors, we initially chose to examine the Rae-1\(^+\) 3LL experimental metastases model (31). Using a standard protocol for effectively depleting Treg using the anti-CD25 mAb (data not shown), we demonstrated that at lower tumor doses inoculated, groups of mice depleted of Treg displayed significantly lower numbers of 3LL lung metastases than mice receiving a control Ig (Fig. 1A). At the highest dose of 3LL inoculated, Treg depletion alone was without effect, suggesting that the natural host response was completely overwhelmed at this point. The enhanced rejection associated with Treg depletion was NK cell dependent, as illustrated by the loss of all host control of metastases with concomitant depletion of NK cells using either anti-asialoGM1 (Fig. 1A) or anti-NK1.1 (data not shown).

To specifically examine the effects of Treg depletion on NKG2D-mediated tumor suppression, B16-Rae-1 tumor cells were injected at two different doses that grew effectively in mice receiving control Ig (Fig. 1B). Depletion of Treg cells strikingly enabled host immunity to control and in some mice completely suppress the lung metastases of B16-Rae-1 tumor cells (Fig. 1B). Again, this suppression enabled by Treg depletion was NK cell dependent (data not shown). Suppression required NKG2D ligand on the tumor cells, because at these doses B16 tumor cells grew in B6 mice regardless of whether Treg had been depleted (Fig. 1C).

Data presented are representative of two independent experiments for \(C\).
To examine the effect of Treg depletion on tumor growth in a s.c. site and in a model system where tumor cells lack MHC class I expression and are rejected by NK cells using perforin, we used the RMA-S-Rae-1β tumor model (19). To examine the effects of Treg depletion, RMA-S-Rae1β tumor cells were injected at two different high doses that grew effectively in mice receiving control Ig (Fig. 1C). By contrast, depletion Treg cells strikingly enabled host immunity to control in some mice reject the growth of large numbers of RMA-S-Rae1β tumor cells (Fig. 1C). At least some of the suppression was mediated by NKG2D ligand on the tumor cells at these doses, because RMA-S tumor cells grew just as effectively in B6 mice regardless of whether Treg cells had been depleted (data not shown).

**NKG2D-mediated NK cell cytotoxicity is suppressed by Treg cells**

We next examined whether Treg may directly inhibit NK cell-mediated cytotoxicity. We first compared the abilities of naive and activated CD4+CD25high (Treg) and conventional CD4+CD25− T cells to affect NK cell-mediated cytotoxicity in vitro. By sorting both NK and T cells from B6 mice and coculturing them at a ratio of 1:1, we were able to demonstrate the specific ability of in vitro 3-h activated Treg to suppress NK cell-mediated cytolytic activity against RMA-S-Rae1β target cells (Fig. 2A). Similar data were obtained at a ratio of 3:1, and consistent with the importance of cell density, at a 1:3 ratio of Treg:NK cells, the suppression of Treg began to diminish (data not shown). Neither unstimulated Treg nor unstimulated or stimulated conventional CD4+ T cells had any effect on NK cell-mediated cytotoxicity in this assay (Fig. 2A). Surprisingly, 3-h activated Treg did not suppress NK cell-mediated cytotoxicity of RMA-S target cells to any detectable extent (Fig. 2B). In support of a specific effect of 3-h activated Treg on the NKG2D-mediated cytotoxicity of target cells, comparative data were obtained using B16-Rae1e and B16 target cells (Fig. 2, C and D). In both RMA-S-Rae1β and B16-Rae-1e experiments, the release from suppression of the NKG2D-mediated NK cell cytotoxicity was almost complete, given the residual non-NKG2D-mediated cytosis (i.e., vs RMA-S or B16) displayed by NK cells against these targets. To examine whether additional activation of Treg might enhance and broaden Treg suppression of NK cell cytotoxicity, Treg were activated with anti-CD3/CD28 and IL-2 for 48 h before coculture (Fig. 2, E and F). Very similar data were obtained with Treg able to significantly and specifically suppress NK cell-mediated cytolytic activity of RMA-S-Rae1β target cells.

**Treg suppression of NKG2D-mediated NK cell cytotoxicity is contact and TGF-β dependent**

Given the reported ability of TGF-β to inhibit lymphocyte cytotoxicity (23, 24), we next examined whether TGF-β produced by
coclulturing NK cells and 48-h activated Treg might be responsible for the suppression of NKG2D-mediated NK cell cytotoxicity of ligand-expressing tumor targets (Fig. 3A). Clearly, an anti-TGF-β mAb was able to restore NK cell-mediated cytotoxicity of RMA-S-Rae-1β tumor cells, whereas IL-10, which is also produced by activated Treg (32), was without effect (Fig. 3A). Importantly, neutralization of TGF-β did not increase the NK cell cytotoxicity of these target cells in the absence of activated Treg or in the presence of activated conventional CD4+ T cells (Fig. 3B), suggesting that neither TGF-β production by NK cells or tumor cells was contributing to the suppression of NK cell-mediated cytotoxicity. Attempts to transfer suppression on NK cell cytotoxicity by using the supernatants of activated Treg were unsuccessful, suggesting that the functional TGF-β may be surface bound, rather than secreted by Treg (Fig. 3C). In concert with this supposition, when activated Treg were separated by Transwells from NK cells and tumor targets, suppression was not detected (Fig. 3C). Consistent with a need for continual direct exposure of Treg to NK cells to mediate suppression of cytotoxicity, spleen cells examined ex vivo from Treg-depleted and clg-treated B6 mice displayed equivalent levels of cytotoxicity toward each of the target cells, RMA-S-Rae-1β and RMA-S (Fig. 3D), B16-Rae-1e, and B16 (data not shown). We did not detect any other alterations in spleen, liver, bone marrow, peripheral blood, or lymph node NK cell numbers, activation status (as determined by Mac-1 and CD69), or homeostatic proliferative capacity in naïve B6 mice depleted of Treg (data not shown).

**FIGURE 3.** Treg suppression of NKG2D-mediated NK cell cytotoxicity is contact and TGF-β dependent. A and B, Activated CD4+CD25high or CD4+CD25− T cells were cocultured with NK cells at a 1:1 ratio in a 6-h 51Cr release assay using RMA-S-Rae-1β target cells (at the indicated E:T cell ratios: 20:1, 5:1, and 1:1) in the presence of neutralizing anti-mTGF-β, anti-IL-10, or control Ig (30 μg/ml). In some wells (B; E:T cell ratio of 20:1), NK cells were alternatively cocultured with activated CD4+CD25− conventional T cells (Tcon). In following experiments (C), NK cells alone (NK) or cocultured with activated Treg (NK + Treg) or activated Treg supernatant (NK + Treg sup) were tested for cytotoxicity against RMA-S-Rae-1β tumor cells as described above. In the same experiment, NK cells and tumor cells were coincubated across Transwells from activated Treg (NK + Treg TW). D, Spleen cells harvested from mice treated with clg or anti-CD25 mAb 24 h previously were examined in a 6-h 51Cr release assay against RMA-S or RMA-S-Rae-1β target cells at the E:T cell ratio shown. In all experiments (A–D) data are shown as the mean ± SEM of triplicate samples. The significance of suppression by activated Treg was determined by Student’s t test (*, p < 0.05).
NKG2D ligand-expressing tumors in a setting where Treg were depleted. Thus, we examined the effect of Treg depletion on IL-12 therapy at a dose of 3LL tumor cells at which Treg depletion alone was of no effect and the efficacy of IL-12 was limited. A combination of IL-12 and Treg depletion significantly reduced 3LL lung metastases compared with Treg depletion or IL-12 therapy alone (Fig. 5A). The combined effect of IL-12 and Treg depletion was completely dependent on NK cells and was independent of CD8+/H11001 T cells (Fig. 5B). Some groups of mice were additionally depleted of NK cells using anti-NK1.1 mAb. Lungs were removed from mice on day 14, and metastatic nodules were quantified. Data are recorded as the mean ± SEM, with the significance of Treg transfer or NK cell depletion determined by Mann-Whitney U test (*, p < 0.05). The results presented in A are representative of two independent experiments.

NK cells to the therapeutic activity of the NK cell-activating cytokine, IL-12.

Discussion

Although earlier studies have suggested a role for Treg in suppressing NK cell effector functions in vitro (6, 32, 33), we have defined at least one mechanism by which activated Treg can directly suppress NK cell function via the NKG2D pathways in vivo. We have extended the implications further by illustrating that relief of that Treg suppression can greatly enhance the functional activity of NK cells responding to the activating IL-12 cytokine (31). The suppressive effect of Treg-expressed TGF-β on NK cell cytotoxicity is not surprising given the previously reported ability of soluble TGF-β to reduce NK cell cytotoxicity and perforin gene transcription (24) and the role of perforin downstream of NKG2D receptor function (31). However, most consideration has previously been given to a likely scenario where tumor secretes TGF-β to suppress NK cell activation and effector functions, rather than a direct effect of Treg on NK cell cytotoxicity. Although we were unable to detect surface TGF-β on activated Treg...
We do not want to suggest that the effect of Treg on NK cell function may be simplistic; indeed, to the contrary, Treg may suppress NK cell function by a number of different mechanisms. Interestingly, we were unable to detect any down-regulation of NKG2D expression on human NK cells was down-regulated by soluble TGF-β produced by tumor cells (25, 34). It remains to be determined whether Treg-expressed TGF-β might interfere with NKG2D signaling or NKG2D ligand expression on tumor cells. Treg may have direct, and possibly indirect, effects on NK cell homeostasis, activation, proliferation, migration, and function in the context of tumor challenge, and many additional experiments will be required to explore the consequences of depleting Treg cells on NK cell antitumor activity in vivo. Recent data also suggest that functional TGF-β can be provided by a non-Treg source (35), and thus, additional cell types that regulate NK cell antitumor activity must also be considered carefully when designing effective immunotherapies. NK cell function in cancer patients is often severely impaired, and it will be of interest to determine whether an inverse relationship between NK cell activation and cytotoxicity and Treg expansion is detected in these patients.

Acknowledgments

We thank Mark Shannon for reagent acquisition, Shannon Griffiths for maintaining the mice, Nadeen Zerafa and Jonathan Coquet for producing and purifying mAb, and Dr. Lewis Lanier for kindly providing the B16 and B16-Rae1 tumor cells.

Disclosures

The authors have no financial interest of interest.

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