Activated CD8+ T-Effector/Memory Cells Eliminate CD4+ CD25+ Foxp3+ T-Suppressor Cells from Tumors via FasL Mediated Apoptosis

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Activated CD8\(^+\) T-Effector/Memory Cells Eliminate CD4\(^+\) CD25\(^+\) Foxp3\(^+\) T-Suppressor Cells from Tumors via FasL Mediated Apoptosis\(^1\)

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Tumor-resident CD8\(^+\) T cells display a quiescent effector/memory phenotype that is maintained in part by infiltrating CD4\(^+\) CD25\(^+\) Foxp3\(^+\) T-suppressor cells. Intratumoral delivery of IL-12, in contrast, can restore cytotoxic function to tumor-associated CD8\(^+\) T cells and induce the apoptotic death of T-suppressor cells. Depletion of CD8\(^+\) T cells from tumors before IL-12 treatment resulted in the abrogation of treatment-mediated T-suppressor cell apoptosis revealing a link between CD8\(^+\) T cell activation and T-suppressor elimination. Furthermore, IL-12 failed to induce T-suppressor cell loss in IFN-\(\gamma\)- or FasL-deficient mice demonstrating a requirement for IFN-\(\gamma\) and FasL in this process. Adoptive transfer of wild-type CD8\(^+\) T cells to FasL-knockout mice restored posttherapy T-suppressor cell elimination from tumors establishing that expression of FasL on CD8\(^+\) T cells was sufficient to promote T-suppressor cell death. IL-12 failed to induce FasL on T-effectors in IFN-\(\gamma\)-knockout mice demonstrating a requirement for IFN-\(\gamma\) in FasL up-regulation. Adoptive transfer of wild-type CD8\(^+\) T cells induced T-suppressor cell death in IFN-\(\gamma\)-knockout mice confirming that autocrine IFN-\(\gamma\) was sufficient for CD8\(^+\) T cell FasL expression. These findings reveal a mechanism by which cytotoxic T cells can abrogate regulatory cell activity. The Journal of Immunology, 2009, 183: 0000 – 0000.

Importantly, activation of CD8\(^+\) T cells was found to be accompanied with elimination of CD4\(^+\) CD25\(^+\) Foxp3\(^+\) T cells from tumors via apoptotic cell death (9). Because CD8\(^+\) T cell activation and T-suppressor cell apoptosis were essentially simultaneous, we hypothesized that these events may be linked. Although several recent studies have revealed that CD4\(^+\) CD25\(^+\) Foxp3\(^+\) T cells can eliminate T-effectors via FasL (10, 11) or cytotoxic granule release (12, 13), the reverse has not been reported. To this end, the cellular and molecular basis of IL-12-mediated T-suppressor cell apoptosis was investigated. Our results establish that post-IL-12 CD4\(^+\) CD25\(^+\) Foxp3\(^+\) T cell apoptosis is mediated by activated CD8\(^+\) T cells via FasL in an IFN-\(\gamma\)-dependent manner.

Materials and Methods

Mice and cell lines

Thy1.2 BALB/c mice were purchased from Taconic Farms. Thy1.1 and perforin-deficient mice in the BALB/c background were maintained in our breeding colony (14). FasL- and IFN-\(\gamma\)-deficient mice in the BALB/c background were purchased from The Jackson Laboratory. Mice were used for experiments at 6–8 wk of age. Line-1, a weakly immunogenic MHc class I-low metastatic lung alveolar carcinoma of the BALB/c mouse, was used to induce tumors in all studies as described (9, 14, 15). In brief, 1 × 10\(^6\) cells were injected in 0.1 ml of DMEM subcutaneously in the subscapular area of the mice and the tumors were allowed to grow to 300–400 mm\(^3\) before treatment.

Microspheres. Poly-lactic acid microspheres with a cytokine loading of 0.025% (weight/weight) were prepared using the phase inversion nanoencapsulation method as described previously (9). Mice were treated with 4 mg of microspheres (1 \(\mu\)g equivalent of cytokine) suspended in 0.1 ml of sterile PBS, via direct injection into the tumor.

In vivo T cell depletion. In vivo CD8\(^+\) T cell depletion was achieved via administration of anti-CD8 mAb 53.6.72 (BioXCell) or an isotype-matched control Ab as described (15). In brief, mice were injected i.p. with 200 \(\mu\)g mAb in 0.2 ml sterile PBS 1 day before microsphere treatment. Ab injections were performed on days −1 and 2.
Single cell suspensions

Single cell suspensions from tumors and tumor-draining lymph nodes (TDLN) were prepared as described previously (9).

Flow cytometry

Flow cytometric analysis of leukocyte subsets was performed on a 4-color BD FACSCalibur (BD Biosciences) using chromofluor-labeled Abs to surface and intracellular markers as described previously (9, 14). Abs were purchased from BD Pharmingen or eBiosciences.

Adoptive cell transfer model

Adoptive transfer of primed CD8+ T-effector cells isolated from the TDLN of IL-12 plus GM-CSF-treated wild-type Thy1.1 donors to tumor bearing FasL or IFN-γ-deficient recipients was performed as previously described (14).

Statistical analysis

Student’s t test was used to determine the significance of the differences between control and experimental groups with p ≤ 0.05 considered significant.

Results

Tumor-resident CD8+ T cells are required for IL-12-mediated elimination of CD4+ CD25+ Foxp3+ T-suppressor cells from tumors

Intratumoral IL-12 induces CD8+ T cell activation and CD4+ CD25+ Foxp3+ T-suppressor cell apoptosis in established tumors (9). To determine whether these events were linked, the apoptotic profile and the quantity of tumor-associated T-suppressor cells were monitored in CD8+ T cell-depleted mice following IL-12 therapy. BALB/c mice with established Line-1 tumors were administered control or anti-CD8 Abs and were treated with a single intratumoral injection of IL-12 microspheres the next day. Single-cell suspensions prepared from tumors were then analyzed for CD4+ CD25+ Foxp3+ T cell numbers and apoptosis between days 1–4 posttherapy. The results are shown in Fig. 1A. In control mice (no CD8+ T cell depletion) IL-12 microsphere treatment resulted in a 2.4-fold increase in the proportion of Annexin V-positive CD4+ CD25+ T cells in tumors within 3 days of treatment (Fig. 1A, Apoptosis). Importantly, induction of apoptosis was paralleled with a 2.5-fold decline in the numbers of CD4+ CD25+ Foxp3+ T cells by day 3 (Fig. 1A, Cell number). In contrast, depletion of CD8+ T cells before treatment resulted in a complete abrogation of the treatment-mediated increase in apoptosis and the corresponding decline in the numbers of T-suppressor cells. These data demonstrate that CD8+ T cells were critical to treatment-induced elimination of T-suppressor cells from tumors.

T-suppressor cell loss is dependent on IFN-γ and FasL

Activation of CD8+ T cells results in the production of IFN-γ, release of cytotoxic granules and up-regulation of death receptor ligands by CD8+ T cells (9). To determine whether any of these cytotoxic effector mechanisms were involved in the CD8+ T cell-mediated T-suppressor cell apoptosis, posttreatment CD4+ CD25+ Foxp3+ T cell elimination was evaluated in mice deficient in IFN-γ, perforin, or FasL. Treatment of tumors in perforin knockout mice resulted in a 2.4-fold decline in intratumoral CD4+ CD25+ Foxp3+ T cell numbers demonstrating that perforin was not required for T-suppressor cell elimination (Fig. 1B). In contrast, treatment failed to deplete T-suppressor cells in IFN-γ-knockout (GKO) or FasL-knockout (FasLKO) mice, suggesting that both IFN-γ and FasL were required for the observed effect.

Expression of FasL on CD8+ T cells is necessary and sufficient for T-suppressor cell depletion

The finding that both CD8+ T cells and FasL were required for T-suppressor cell loss raised the possibility that CD8+ T cells mediated T-suppressor cell apoptosis directly via the extrinsic death receptor pathway. However, expression of FasL is not limited to T cells and other effector cells found in the tumor microenvironment, including macrophages, dendritic cells, and NK cells, can up-regulate FasL upon activation (16–18). Whether FasL-dependent T-suppressor death was mediated directly by CD8+ T cells, via IFN-γ-activated secondary effectors (19), or both, was not clear. To address this question, we first monitored posttherapy intratumoral leukocyte populations for expression of FasL. The results are shown in Fig. 2A. These data demonstrate that treatment resulted in the up-regulation of FasL on CD8+ T-effector and CD4+ Th cells but not on any of the other subsets tested, suggesting that posttherapy CD4+ CD25+ Foxp3+ T cell elimination was mediated directly by activated T-effectors. Because depletion studies demonstrated an absolute requirement for intratumoral CD8+ T cells in this process we next examined whether expression of FasL by CD8+ T cells alone was sufficient to induce T-suppressor cell

FIGURE 1. Requirement for tumor-resident CD8+ T cells in CD4+ CD25+ Foxp3+ T cell apoptosis. A, CD8+ T cells are central to IL-12-mediated elimination of T-suppressor cells. Tumor-bearing mice were treated with anti-CD8 or control Ab 1 day before IL-12 microsphere treatment. Intratumoral T-suppressor cells were analyzed for AnnexinV binding (Apoptosis) and quantified (Cell number) on day 0 (pretreatment) and on days 1–4 posttherapy. The differences between control and CD8+ T cell-depleted mice were significant on days 3 and 4 (p ≤ 0.013). Error bars = SE, n = 3–5 mice per timepoint. B, Effector mechanisms involved in IL-12-mediated T-suppressor cell apoptosis. Tumors were induced in knockout mice and CD4+ CD25+ Foxp3+ T cells were quantified on days 0 (pretreatment) and 4 (posttherapy). The difference between days 0 and 4 was significant in the perforin knockout mice (p = 0.008). Error bars = SE, n = 4–9 mice per group. The above experiments were repeated three times with similar results.

Abbreviations used in this paper: TDLN, tumor-draining lymph node; GKO, IFN-γ-knockout; FasLKO, FasL-knockout.
apoptosis. This notion was tested in an adoptive cell transfer experiment (Fig. 2B). In this model, treatment is administered to tumor-bearing donors to induce antitumor CD8+ T-effector cell priming in the TDLN (14). The newly primed cells are then isolated from the TDLN and transferred to recipient mice, in which they preferentially home to tumors (14). In the current study, CD8+ T-effector cells were isolated from wild-type donors and transferred to FasLKO recipients to create FasL-deficient mice with tumors that are infiltrated by CD8+ T-effectors capable of expressing FasL. Consequently, elimination of the T-suppressor cells from such mice following IL-12 delivery would suggest that expression of FasL on the donor CD8+ T cells alone is sufficient to induce T-suppressor cell apoptosis. The results from one such experiment are shown in Fig. 2B. These data demonstrate that treatment resulted in a 6-fold decrease in CD4+ CD25+ Foxp3+ T-suppressor cells (19) and sensitizes target cells to apoptosis (20–22). Consistent with these findings IL-12 treatment enhanced FasL expression on CD8+ T cells in wild-type but not in GKO mice in our model (Fig. 3A). In contrast, treatment

FIGURE 2. Direct killing of intratumoral T-suppressor cells by FasL-expressing CD8+ T cells. A, Effect of IL-12 microspheres on FasL expression in different leukocyte subsets. Single-cell suspensions prepared from tumors were analyzed for FasL expression on days 0 (pretreatment) and 2 (posttreatment). CD8+ T cells (CD45+ CD3+ CD8+), CD4+ Th cells (CD3+ CD4+), CD4+ T-suppressor cells (CD3+ CD4+ Foxp3+), CD4+Foxp3+), NK-cells (CD45+ CD5+ DX5+), MDSC (CD45+ CD11b+ Gr-1+), dendritic cells (CD45+ CD11c+ MHC class II+), and macrophages (CD11b+ GR-1+, MHC class II+) were analyzed. The differences between days 0 and 4 were significant for CD8+ T cells and CD4+ Th cells (p = 0.014). Error bars = SE, n = 4 per group. B, Posttherapy T-suppressor cell depletion following adoptive transfer of wild-type CD8+ T cells to FasLKO mice. The schematic depicts the adoptive cell transfer protocol. Briefly, tumors were induced in both wild-type (Thy1.1) and FasLKO (Thy1.2) mice. Wild-type donor mice were then treated with a single intratumoral injection of IL-12 plus GM-CSF microspheres. Combination cytokine treatment was used to induce donor T-effectors because GM-CSF significantly augments IL-12-mediated CD8+ T-effector priming in the TDLN (J. L. Harden and N. K. Egilmez, unpublished observations). Newly primed CD8+ T cells were isolated from the TDLN of donors by magnetic bead sorting 4 days after treatment and were adoptively transferred (3 x 106 cells/mouse) to tumor-bearing FasLKO recipients. Two days after transfer recipients were treated with IL-12 microspheres and T-suppressor cell numbers in recipient tumors were quantified on day 3 posttreatment. Control groups consisted of FasLKO mice with no adoptive transfer, FasLKO mice that received CD8+ T cells from FasLKO donors plus treatment and FasLKO mice that received wild-type CD8+ T cells with no treatment. Error bars = SE, n = 4 mice per group. The above data are representative of two independent studies.

FIGURE 3. Role of IFN-γ in therapy-induced T-suppressor cell death. A, Posttherapy FasL and Fas expression in wild-type vs GKO mice. Single cell suspensions prepared from pre- (day 0) and post-IL-12 therapy (day 2) tumors of wild-type and GKO mice were analyzed for expression of FasL (CD8+ T cells) and Fas (CD4+ Foxp3+ T-suppressor cells). The difference in the proportion of FasL-positive CD8+ T cells between wild-type and GKO mice on day 2 was significant (p = 0.0017). Error bars = SE, n = 3–8 per group. B, Adoptive transfer of wild-type CD8+ T cells into GKO mice. The same approach described in Fig. 2B was used except that wild-type CD8+ T cells were adoptively transferred to tumor-bearing GKO recipients. Error bars = SE, n = 4 per group. Representative results from two independent studies are shown.

Autoimmune IFN-γ drives CD8+ T cell FasL expression and T-suppressor cell death

Whereas the above studies established the direct role of FasL in T-suppressor cell elimination, the mechanistic basis of the requirement for IFN-γ in this process was not determined. IFN-γ induces FasL in a STAT-1-dependent manner (19) and sensitizes target cells to apoptosis (20–22). Consistent with these findings IL-12 treatment enhanced FasL expression on CD8+ T cells in wild-type but not in GKO mice in our model (Fig. 3A). In contrast, treatment
did not result in an appreciable increase in the proportion of Fas-positive CD4<sup>+</sup> CD25<sup>+</sup> Foxp3<sup>+</sup> T cells or the amount of Fas per cell (no change in average MFI, data not shown), which was already expressed at high levels in both wild-type or GKO mice before treatment (Fig. 3A, Ref. 9).

Because IL-12 induces IFN-γ secretion from numerous leukocyte subsets (23), we next wanted to determine whether production of IFN-γ by CD8<sup>+</sup> T cells alone was sufficient to achieve T-suppressor cell elimination. To this end, the adoptive cell transfer model was used (Fig. 2B). Primed CD8<sup>+</sup> T-effectors isolated from the TDLN of treated wild-type donors were transferred to tumor-bearing GKO recipients and tested for their ability to mediate T-suppressor cell killing following IL-12 microsphere treatment. The results are summarized in Fig. 3B. These data demonstrate that reconstitution of tumors in GKO mice with wild-type CD8<sup>+</sup> T cells resulted in the restoration of treatment-induced T-suppressor cell elimination. In contrast, intratumoral CD4<sup>+</sup> CD25<sup>-</sup> Foxp3<sup>+</sup> T cell numbers remained unchanged in treated GKO controls and untreated GKO mice that were reconstituted with wild-type CD8<sup>+</sup> T cells. These findings are consistent with the notion that autocrine IFN-γ production by CD8<sup>+</sup> T cells was sufficient to induce effective T-suppressor cell depletion, further emphasizing the central role of tumor-resident CD8<sup>+</sup> T cells in this process.

Discussion

The above data reveal a previously unknown role for CD8<sup>+</sup> T cells in the regulation of CD4<sup>+</sup> CD25<sup>-</sup> Foxp3<sup>+</sup> T-suppressor cell homeostasis. More specifically, our results demonstrate that upon activation with IL-12, tumor-associated CD8<sup>+</sup> T cells can directly mediate rapid T-suppressor apoptosis via FasL. We also found that IFN-γ is necessary for CD8<sup>+</sup> Tem-mediated T-suppressor cell death and that this requirement is associated, at least in part, with the ability to induce FasL in an autocrine manner.

Role of Fas/FasL death receptor pathway in T-effector cytotoxicity and homeostasis is well-defined (24). Recently, T-suppressor cells were found to promote T-effector cell elimination via FasL (10, 11). Conversely, others showed that activated T-suppressor cells themselves express Fas and are sensitive to FasL-mediated apoptosis (25–27). However, a direct role for CD8<sup>+</sup> T-effectors in T-suppressor cell killing had not been demonstrated previously. Our findings reveal a mechanism by which CD8<sup>+</sup> T cells not only override T-suppressor control, but in fact actively eliminate pre-existing T-suppressors from inflammatory milieu. Whether the CD8<sup>+</sup> T cell counterattack is limited to T-suppressor cells or extends to other Fas<sup>+</sup> cell subsets found in the tumor microenvironment was not examined. To this end, it is interesting to note that intratumoral CD11b<sup>+</sup> Gr-1<sup>+</sup> myeloid-derived suppressor cells were also found to express Fas, making them potential targets (data not shown). Finally, whether the above findings are unique to the post-IL-12 tumor microenvironment or represent a universal mechanism that allows pre-existing CD8<sup>+</sup> T cells to overcome T-suppressor cell control in acute inflammatory microenvironments remains to be determined.

The role of IFN-γ in the extrinsic death receptor pathway-mediated apoptosis is multifaceted (19–22). This pleiotropic cytokine has been shown to induce both FasL and Fas up-regulation (19, 20) and sensitize target cells to apoptosis by enhancing DISC formation as well as caspase-8 activation (20–22). In our studies, the IL-12-IFN-γ axis promoted a rapid increase of FasL on CD8<sup>+</sup> T cells but did not significantly alter Fas levels on T-suppressors. Activated T-suppressor cells can constitutively express high levels of Fas (25, 26) and >80% of tumor-associated T-suppressors were found to be Fas-positive in our model. It is thus likely that treatment had minimal effect on the already high levels of Fas expressed on these cells. Whether IFN-γ played an additional role in sensitizing CD4<sup>+</sup> Foxp3<sup>+</sup> T cells to apoptotic signals via enhanced DISC formation and/or caspase-8 activation was not investigated in this study.

We previously established that tumor-resident CD8<sup>+</sup> T-effector/memory cells were central to the initiation of a complex antitumor effector response following sustained delivery of IL-12 to the tumor microenvironment. More specifically, in addition to mediating direct tumor cytotoxicity (9), CD8<sup>+</sup> T cell activation was required for NK-cell recruitment (15), the priming of a secondary de novo CD8<sup>+</sup> T-effector response (14) and the induction of long-term T cell memory (28). The current results further identify pre-existing tumor-associated CD8<sup>+</sup> T cells as the direct mediator of the post-IL-12 T-suppressor cell elimination from tumors. Collectively, these findings reveal the importance of intratumoral CD8<sup>+</sup> T cells as a unique in situ resource for inducing effective reversal of tumor-mediated immune dysfunction.

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Disclosures

N. K. E. has ownership interest in Therapy X, Inc., which is developing sustained-release cytokine formulations for therapeutic purposes.

References


