Reversing Tumor Immune Suppression with Intratumoral IL-12: Activation of Tumor-Associated T Effector/Memory Cells, Induction of T Suppressor Apoptosis, and Infiltration of CD8+ T Effectors

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Reversing Tumor Immune Suppression with Intratumoral IL-12: Activation of Tumor-Associated T Effector/Memory Cells, Induction of T Suppressor Apoptosis, and Infiltration of CD8\(^+\) T Effectors\(^1\)

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A single intratumoral injection of IL-12 and GM-CSF-loaded slow-release microspheres induces T cell-dependent eradication of established primary and metastatic tumors in a murine lung tumor model. To determine how the delivery of cytokines directly to the microenvironment of a tumor nodule induces local and systemic antitumor T cell activity, we characterized therapy-induced phenotypic and functional changes in tumor-infiltrating T cell populations. Analysis of pretherapy tumors demonstrated that advanced primary tumors were infiltrated by CD4\(^+\) and CD8\(^+\) T cells with an effector/memory phenotype and CD4\(^+\)CD25\(^-\)Foxp3\(^+\) T suppressor cells. Tumor-associated effector memory CD8\(^+\) T cells displayed impaired cytotoxic function, whereas CD4\(^+\)CD25\(^+\)Foxp3\(^+\) cells effectively inhibited T cell proliferation demonstrating functional integrity. IL-12/GM-CSF treatment promoted a rapid up-regulation of CD43 and CD69 on CD8\(^+\) effector/memory T cells, augmented their ability to produce IFN-\(\gamma\), and restored granzyme B expression. Importantly, treatment also induced a concomitant and progressive loss of T suppressors from the tumor. Further analysis established that activation of pre-existing effector memory T cells was short-lived and that both the effector/memory and the suppressor T cells became apoptotic within 4 days of treatment. Apoptotic death of pre-existing effector/memory and suppressor T cells was followed by infiltration of the tumor with activated, nonapoptotic CD8\(^+\) effector T lymphocytes on day 7 posttherapy. Both CD8\(^+\) T cell activation and T suppressor cell purge were mediated primarily by IL-12 and required IFN-\(\gamma\). This study provides important insight into how local IL-12 therapy alters the immunosuppressive tumor milieu to one that is immunologically active, ultimately resulting in tumor regression. *The Journal of Immunology, 2006, 177: 6962–6973.*

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\(^\text{3}\)Abbreviations used in this paper: LN, lymph node; TIL, tumor-infiltrating lymphocytes; FNA, fine needle aspirate; Ct, threshold cycle; TDJN, tumor-draining LN; AICD, activation-induced cell death; FasL, Fas ligand; wt, wild type; GKO, IFN-\(\gamma\)-knockout.
Subsequently, we demonstrated that a single intratumoral injection of IL-12 and GM-CSF microspheres was superior to either cytokine alone in inducing the development of systemic antitumor T cell immunity and the eradication of disseminated disease in a murine spontaneous lung metastasis model (14). The antitumor activity was found to be mediated by T and NK cells in this model.

Although earlier studies established the antitumor efficacy of IL-12/GM-CSF microsphere therapy, the cellular and molecular basis of the posttreatment immune activity within the tumor microenvironment was not elucidated. The ability of IL-12 to stimulate both innate (NK, NKT) and adaptive (T cell) immunity (15), and that of GM-CSF to augment Ag presentation are well-established (16). Numerous preclinical and clinical studies have demonstrated that IL-12 mediates tumor regression by promoting Th1 responses, by increasing CD8+ T cell, NKT cell, NK cell, and granulocyte cytotoxicity, and by inhibiting angiogenesis (15, 17). GM-CSF, in contrast, augments the generation and recruitment of dendritic cells, macrophages, granulocytes, and NK/T cells (10, 16). In the majority of these studies, monitoring of cytokine-induced immune activity within the tumor microenvironment was limited to histological analyses, providing snapshots of leukocytic infiltrates. A quantitative characterization of the lymphocyte activation kinetics as induced by intratumoral cytokine administration, particularly with specific reference to tumor immune suppression, has not been conducted. To this end, we characterized the long-term kinetics and the phenotypic/functional properties of tumor-associated T cells to determine how intratumoral delivery of IL-12/GM-CSF induces antitumor immunity. The results demonstrate that local release of IL-12/GM-CSF reverses tumor immune suppression via the modulation of a complex multicomponent T cell network, including the activation of pre-existing tumor-associated effector/memory T cells, elimination of T suppressor cells, and the priming of a secondary CD8+ T effector response.

Materials and Methods

**Mice and tumor cells**

Line-1, a BALB/c lung alveolar carcinoma cell line, was maintained in DMEM/F-12 (Invitrogen Life Technologies) supplemented with 10% heat-inactivated FBS (Equitech-bio), 2 mM t-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 0.1 mM nonessential amino acids, and 0.1 mM sodium pyruvate (Mediatech). Female BALB/c mice at 6–8 wk of age were obtained from Taconic Farms. IFN-γ-knockout BALB/c mice were purchased from The Jackson Laboratory. All studies were approved by the Institutional Animal Care and Use Committee of the University of Louisville.

**Cytokines and microspheres**

Recombinant murine IL-12 (2.7 × 10^6 U/mg) was a gift from Wyeth. Recombinant murine GM-CSF (5 × 10^6 U/mg) was purchased from Pepro-Tech. Preparation of cytokine-encapsulated biodegradable polymer microspheres has been described in detail previously (14).

**Preparation of single-cell suspensions from tumors**

Tumors were induced by s.c. injection of 1 × 10^6 viable tumor cells in 0.1 ml of sterile PBS behind the neck just above the scapula (14). Thirteen to 15 days later, when tumors reached 400–500 mm^3 in size, mice were treated with cytokine-encapsulated microspheres as described previously (14). Experimental groups received IL-12/GM-CSF-encapsulated microspheres (1.5 μg of each cytokine in 12 μg of particles suspended in 50 μl PBS), whereas control mice received blank microspheres. At selected time-points after microsphere treatment, tumors were removed from mice and single-cell suspensions were prepared by enzymatic digestion. Resected tumors were weighed, minced into small (1–2 mm^3) pieces with a scalpel, and immersed in 10 ml of digestion mixture (5% FBS in RPMI 1640, 0.5 mg/ml collagenase A (Roche Diagnostic), 0.2 mg/ml hylauronidase, type V (Sigma-Aldrich), and 0.02 mg/ml DNase I (Sigma-Aldrich)) per 0.25 g of tumor tissue. This mixture was incubated at 37°C for 45 min on a rotating platform. The resulting cell suspensions were filtered sequentially through 70- and 40-μm cell strainers (BD Falcon) and washed with 5% FBS in RPMI 1640. RBC were lysed by brief incubation in 0.15 M ammonium chloride solution, and cell debris/dead cells were removed by centrifugation on Lymphocyte-M gradients as recommended by the manufacturer (Cedarlane).

**Flow cytometry**

Single-cell suspensions obtained from samples were labeled with Abs to various T cell markers using standard staining methods (18) and were analyzed on a four-color FACSCalibur flow cytometer (BD Biosciences). The following panel of commercially available and directly fluorochrome-conjugated anti-mouse mAbs was included in this study: CD3 (clone 17A2), CD4 (clone GK1.5, RM4-5), CD8 (clone 53-6.7), CD25 (clone PC61), CD69 (clone H1.2F3), CD43 (clone B11), CD44 (clone IM7), CD49b/pan-NK (clone DX5b), CD62L (clone MEL-14), CD95 (clone Jo2), CD95 ligand (clone MFL3), and TCR β-chain (clone H57-597). To evaluate membrane TGFβ expression, the staining was performed with biotinylated anti-TGFβ1 mAb (clone A7S-5) followed by streptavidin-PE labeling. All Abs and mouse Fc block (clone 2.4G2) were purchased from BD Pharmingen. Flow cytometry data were analyzed using CellQuest software (BD Biosciences).

**Intracellular cytokine staining**

Single-cell suspensions of primary tumors were prepared as described above. For intracellular staining of IFN-γ or IL-10, tumor-infiltrating lymphocytes (TIL) were stimulated with PMA (5 ng/ml) and ionomycin (0.5 μg/ml) for 5 h. Two hours before harvesting, 0.5 μl of BD Golgistop (BD Pharmingen) was added to every 1 ml of cell culture (1 × 10^6 live cells/ml). After two washes, intracellular-IL-10 and IFN-γ staining was performed according to the manufacturer’s instructions using the BD Cytofix/Cytoperm Plus kit and PE-conjugated anti-mouse IL-10 or IFN-γ (BD Pharmingen). For granzyme B, cells were directly pretreated with FcR block and stained with Abs targeting cell surface markers (CD4 and CD25 or CD8). Granzyme B staining was performed using eBioscence Fixation and Permeabilization kit and PE anti-mouse granzyme B (eBioscience). For detection of apoptosis, cells were first stained for the expression of the respective surface markers and then with anti-Annexin V-allophycocyanin Ab according to the manufacturer’s protocol (Annexin V apoptosis detection kit; BD Pharmingen). For detection of anti- and proapoptotic proteins, PE-labeled anti-Bcl-xL (clone H-5) was purchased from Santa Cruz Bio-technology. Active caspase-3 (C92-605) and Bcl-2 (S911) detection was performed using the BD Pharmingen kits according to the manufacturer’s protocol. Foxp3 expression was evaluated using an anti-mouse Foxp3 PE staining kit according to the manufacturer’s protocol (eBioscience).

**In vitro T suppressor assay**

Total CD4+ TIL were purified from single-cell suspensions using magnetic cell sorting. Cells were incubated with anti-CD4 microbeads (Miltenyi Biotec) and passed through the autoMACS separator according to the manufacturer’s instructions. Tumor-infiltrating CD4+CD25+ and CD4+CD25- T cells were isolated from the enriched population via FACS sorting. Responder cells were obtained from LN and spleens of naïve BALB/c mice, and irradiated as described previously (19). CD4+ T cells were enriched on a CD4 cell-enrichment column (R&D Systems), then labeled with PE-anti-CD25 Ab and incubated with anti-PE beads (Miltenyi Biotec). CD4+CD25- T cell purity was consistently >90%. Subsequently, CD4+CD25- responder T cells (1 × 10^5/well in 96-well round-bottom plates) were cultured for 3 days at 37°C in 5% CO2 in the presence of irradiated spleen cells as APC (1 × 10^5/well), anti-CD3 Ab at 0.5 μg/ml, with or without CD4+CD25+ T cells at a ratio of four CD4+CD25+ T cells to one responder cell. The cell cultures were pulsed on day 3 with 0.5 μCi of [3H]thymidine for the last 18 h.

**Quantitative real-time-PCR**

Fine-needle aspirates (FNA) were obtained by aspirating four quadrants of each tumor with a 23-gauge needle attached to a 1.0-ml syringe. Tissue samples were disinfected into TRIzol reagent (Invitrogen Life Technologies), total RNA was isolated and was reverse-transcribed to cDNA in a 25-μl reaction mixture containing 12.5 U M-MLV reverse transcriptase (Applied Biosystems), 100 ng of cDNA template, and selected primers (200 nM) using the recommended cycling conditions (denaturation at 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 1 min). The primer sequences, designed with Primer Express software (Applied Biosystems), were as follows:
**Results**

**Advanced primary tumors are infiltrated by CD8+ and CD4+ CD25+ effector/memory T cells and CD4+ CD25+ suppressor T cells**

In initial studies, TIL populations from untreated tumors were analyzed to determine the extent of pre-existing T cell infiltration. Tumors were induced and allowed to grow to ∼400–500 mm3 in size (13–15 days), representing a well-established tumor. Flow cytometric analysis of single-cell suspensions prepared from these tumors revealed a substantial (51%) T cell (TCRβ+ CD3+) component within the TIL, with an overall CD4:CD8 ratio of 5:1 (data not shown). All intratumoral CD8+ T cells expressed high levels of CD44, demonstrating Ag experience (Fig. 1A). To determine whether these cells displayed an effector or memory phenotype, CD43 expression was evaluated. CD43 is up-regulated rapidly on effector T cells upon activation (similar to CD44); however, its expression declines (unlike CD44) as effector T cells develop into memory cells (21). The great majority of the CD8+ T cells were positive for CD43, consistent with an effector phenotype (Fig. 1A). In contrast, early activation markers CD25 and CD69, which are expressed transiently by fully activated effector T cells (22), were found to be expressed at low levels, indicating late- or postpeak effector status. The presence of low levels of CD127, which is expressed by either naive or central memory but not by effector cells (22), also suggested a late effector phenotype. Finally, low CD62L expression, a marker that is up-regulated on either naive or central memory T cells, was again consistent with a T effector/memory phenotype.

Analysis of tumor-associated CD4+ T cells demonstrated that tumors were infiltrated with two distinct populations, i.e., CD25+ and CD25- subsets, corresponding to 40 and 60% of the CD4+ T cells, respectively (Fig. 1B, top panel). The CD4+CD25- and CD4+CD25+ populations were analyzed separately with regard to their CD44, CD43, CD69, CD127, and CD62L expression. This approach established that the CD4+CD25- T cells had a phenotype that was similar to that of the CD8+ T effector/memory cells (Fig. 1B). CD4+CD25+ T cells, in contrast, expressed significantly higher levels of CD43 and CD69, consistent with an activated effector cell phenotype. CD62L expression was mixed, suggesting that these cells were likely circulating between the tumor and the tumor-draining LN (TDLN).

Next, we undertook functional analysis of tumor-associated CD8+ and CD4+ T cell populations. CD8+ T cells were analyzed with respect to their ability to secrete IFN-γ and granzyme B to determine whether they were active and displayed cytotoxic function. Stimulation with PMA and ionomycin resulted in IFN-γ production by more than half of the CD8+ T cells, suggesting that a significant portion of the CD8+ T cells were functional with regard to cytokine secretion (Fig. 2A). In contrast, these cells did not produce granzyme B, demonstrating that they were impaired in cytotoxic function. Similar to the CD8+ T cells, in vitro stimulation of CD4+ T cells resulted in the production of IFN-γ but not IL-10 by a significant number of CD4+CD25- T cells consistent with a type 1 Th (Th1) cell phenotype (Fig. 2B). CD4+CD25+ T cells did not produce IFN-γ upon stimulation, suggesting that they represented either activated type 2 Th (Th2) cells or suppressor T cells (Fig. 2B). To determine whether the CD4+CD25+ T cells represented a T suppressor population, they were analyzed for expression of Foxp3 and TGFβ. The results demonstrated that ≥75% of CD4+CD25+ T cells were positive for Foxp3 as well as TGFβ, thus confirming their suppressor phenotype. In contrast, CD4+CD25- cells did not express significant levels of either protein. To determine whether tumor-associated CD4+CD25+ T cells were functionally suppressive, their activity was tested in a coculture assay (Fig. 2C). Tumor-associated CD4+CD25+ T cells effectively suppressed the proliferation of responder T cells, whereas the CD4+CD25- subset did not. Collectively, the above data establish that advanced tumors were infiltrated by three major T cell subsets. These included CD8+ T cells that displayed an effector/memory phenotype and impaired cytolytic function, a CD4+CD25- subset that primarily showed effector/memory Th1 characteristics and a CD4+CD25+ subset that displayed T suppressor phenotype and function.

**Statistical analysis**

All data were analyzed using unpaired Student’s t test analysis. A p value of 0.05 or less was considered significant.

**FIGURE 1.** Analysis of membrane phenotype markers on tumor-infiltrating CD8+ and CD4+ T cells. A, CD8+ T cells. Single-cell suspensions prepared from tumors were analyzed after gating on the mononuclear lymphocyte population. CD8+ T cells were gated on and analyzed for the expression of CD25, CD44, CD69, and CD127 (solid lines). Filled histograms represent isotype controls. B, CD4+ T cells. Top panel, CD25+ and CD25- subpopulations after gating on CD4+ T cells. Lower panels, CD4+CD25+ or CD4+CD25- cells were gated on separately and analyzed for the expression of CD43, CD69, CD127, and CD62L. Filled histograms, CD4+CD25+ cells; solid lines, CD4+CD25- cells.
FIGURE 2. Functional analysis of tumor-infiltrating CD8+ and CD4+ T cells. A, IFN-γ and granzyme B production by CD8+ T cells. TIL were stimulated with PMA/ionomycin, and CD8+ T cells were gated on and analyzed for intracellular IFN-γ. Granzyme B production was analyzed by direct ex vivo staining of TIL for intracellular granzyme B. B, IFN-γ, IL-10, Foxp3, and TGFβ expression by CD4+ T cells. All cells were stained for membrane CD4 and CD25. They were then stained for intracellular IFN-γ, IL-10, membrane-bound TGFβ, or intracellular Foxp3 as described in Methods. CD4+ T cells were gated on and analyzed. C, In vitro T cell suppression assay. The ability of tumor-derived CD4+CD25+ T cells to suppress the proliferation of naive CD4+CD25− responder cells was determined as described in Methods. *, The differences between responder cells alone and groups including CD4+CD25+ cells were highly significant (p < 0.00006). Error bars, SD.
In situ vaccination with IL-12/GM-CSF microspheres induces T effector/memory activation, T suppressor purge, and CD8\(^+\) T effector cell infiltration

The next series of experiments were designed to address how delivery of IL-12 and GM-CSF affected the quantity, phenotype, and function of tumor-associated T cells. Initially, quantitative analysis of T cell subsets was performed to establish the posttreatment T cell kinetics. For this purpose, established tumors were injected either with control (blank) or IL-12/GM-CSF-loaded microspheres, and the numbers of tumor-infiltrating CD8\(^+\) and CD4\(^+\)CD25\(^+\) T cells were monitored for a 2-wk period. The results are summarized in Fig. 3. Intratumoral T cell numbers remained unchanged after treatment with control microspheres (Fig. 3A, inset). In contrast, IL-12/GM-CSF microsphere treatment induced significant quantitative changes in both CD8\(^+\) and CD4\(^+\)CD25\(^+\) T cell populations (Fig. 3A). The most dramatic changes involved a 3-fold decrease in the number of tumor-associated CD4\(^+\)CD25\(^+\) T cells between days 0 and 14 and a 2.5-fold increase in the number of CD8\(^+\) T cells on day 7. Although the decrease in CD4\(^+\)CD25\(^+\) T cells was progressive after day 1, there was no significant change in the number of tumor-infiltrating CD8\(^+\) T cells during the first 4 days. The transient increase in CD4\(^+\)CD25\(^+\) T cells on day 1 was most likely due to CD4\(^+\)CD25\(^+\) Th cell activation because the number of CD4\(^+\)CD25\(^+\) T cells decreased by 1.8-fold between days 0–2 and remained unchanged thereafter (data not shown). The increase in the quantity of intratumoral CD8\(^+\) T cells on day 7 and the progressive decline in CD4\(^+\) T cell numbers were confirmed by real-time PCR-based quantification of CD8 and CD4 mRNAs following serial FNA of tumors (Fig. 3B). These data demonstrate that whereas the levels of CD8 and CD4 remained constant between days 0 and 2, CD8 transcript levels increased by almost 10-fold on day 7 and intratumoral CD4 mRNA levels decreased progressively, up to 4-fold by day 10. Intratumoral IFN-\(\gamma\) levels followed a pattern similar to that observed for CD8\(^+\) T cells, suggesting that treatment induced a CD8\(^+\) T cell-associated production of IFN-\(\gamma\) in vivo. Overall, CD8/CD4 kinetics obtained by real-time PCR analysis confirmed the flow cytometry data.

Whereas the data shown in Fig. 3A established that CD4\(^+\)CD25\(^+\) T cell numbers declined between days 0 and 14, it was not clear whether this decline was specifically associated with T suppressors because the CD4\(^+\)CD25\(^+\) T cell population can include both Foxp3\(^+\) T suppressors and Foxp3- activated Th. To determine whether the reduction in CD4\(^+\)CD25\(^+\) T cells reflected a loss of bona fide T suppressors, Foxp3\(^+\)CD4\(^+\)CD25\(^+\) T cells were quantified following intracelullar staining. The results shown in Fig. 3C confirm that treatment induced a rapid, progressive loss of T suppressor cells from the tumor, with the absolute numbers of CD4\(^+\)CD25\(^+\)Foxp3\(^+\) cells decreasing by 6-fold between days 1 and 14. At the same time, analysis of the CD4\(^+\)CD25\(^+\)Foxp3\(^-\) Th cells demonstrated that treatment induced a brief but significant increase in the numbers of activated Th cells between days 0 and 1 (most likely due to the activation of preexisting CD4\(^+\)CD25\(^+\) Th cells), which did not persist beyond day 2 (Fig. 3D, inset). The overall pattern that emerges from this analysis is a rapid and effective reversal of the immune-suppressive characteristics of the tumor microenvironment, which is then maintained for at least 2 wk.

IL-12/GM-CSF delivery restores effector function to tumor-associated CD8\(^+\) T effector/memory cells

Phenotypic and functional analyses of CD8 and CD4 T cell populations were then performed to determine whether the observed quantitative changes correlated with functional activation. These studies demonstrated that following treatment, CD8\(^+\) T cells rapidly up-regulated CD43 and CD69 expression, with the proportion of CD43/CD69 double-positive cells increasing from an average of 17% on day 0 to 60% on day 2 and then to 65% on day 4 (Fig. 4, A and C). Because there was no increase in cell numbers between days 0 and 4, these findings supported the notion that treatment induced a rapid conversion of pre-existing quiescent CD8\(^+\) T
effector/memory cells to activated effector cells in the absence of proliferation. The activation level of day 7 CD8<sup>+</sup> T cells was similar to that of day 4 cells with 70% of CD8<sup>+</sup> T cells demonstrating a CD43/CD69 double-positive phenotype. In contrast to the day 2 cells, however, there was a dramatic increase in the number of CD8<sup>+</sup> T cells on day 7, suggesting that day 7 CD8<sup>+</sup> T effectors were distinct from day 2 cells (Fig. 3A). Both the activation level (Fig. 4, A and C) and the absolute number (Fig. 3A) of T effectors declined after day 7, approaching pretherapy levels by day 14.

Functional analysis of intratumoral CD8<sup>+</sup> T cells demonstrated that treatment enhanced both the proportion of IFN-γ-secreting cells (from an average of 58 to 80%) and the amount of IFN-γ produced per cell (average mean fluorescence intensity increasing from 230 ± 30 to 510 ± 60) between days 0 and 2 (Fig. 4, B and C). More importantly, the cytotoxic activity of CD8<sup>+</sup> T cells showed a dramatic change, with the percentage of granzyme B-positive cells increasing from 5% on day 0 to 80% on day 2 (Fig. 4C). The activated effector characteristics of CD8<sup>+</sup> T cells were maintained through day 7 with regard to both IFN-γ production and granzyme B expression (Fig. 4C), except that on a per cell basis, day 7 cells demonstrated much higher production of IFN-γ compared with day 2 cells (average mean fluorescence intensity of 510 ± 60 vs 1300 ± 200 on days 2 and 7, respectively). This finding again suggested that day 7 CD8<sup>+</sup> T cells were functionally different from day 2 cells. By day 14, CD8<sup>+</sup> T cell activity decreased significantly with the proportion of IFN-γ and granzyme B-positive cells retreating to pretherapy levels.

Treatment-induced activation of CD8<sup>+</sup> T effector/memory cells is followed by rapid apoptotic cell death, the pattern of which supports two different phases of CD8<sup>+</sup> T cell activity within the tumor microenvironment.

The above studies demonstrated that conversion of pre-existing CD8<sup>+</sup> T effector/memory to a T effector phenotype on day 2 was followed by the expansion of the CD8<sup>+</sup> T effector cell population on day 7. However, it was not clear whether day 7 expansion was due to delayed proliferation of pre-existing T cells or involved the infiltration of tumor with a distinct, exogenous CD8<sup>+</sup> effector T cell population, possibly migrating from the TDLN. CD8<sup>+</sup> TIL with a quiescent nonapoptotic phenotype have been shown to apoptosis rapidly after activation due to activation-induced cell death (AICD) (23, 24), a finding that is inconsistent with the notion that treatment resulted in the proliferation of pre-existing CD8<sup>+</sup> T effector/memory cells. We thus hypothesized that analysis of post-therapy CD8<sup>+</sup> T cell apoptosis may provide additional clues as to whether day 2 and day 7 T effector populations were distinct. To this end, the apoptotic profile of tumor-associated CD8<sup>+</sup> T cells was determined between days 0–7. The results established that the percentage of Annexin V<sup>+</sup> CD8<sup>+</sup> T cells increased rapidly from 8% on day 0 to 45% on day 4 following IL-12/GM-CSF delivery, demonstrating the induction of apoptosis (Fig. 5A). Further analysis of downstream apoptotic events, i.e., expression of antiapoptotic molecules Bcl-x<sub>L</sub>/Bcl-2 and activation of proapoptotic caspase-3, demonstrated that whereas the expression of antiapoptotic proteins remained stable between days 0 and 4 (except for A...
s slight decrease in Bcl-2), activated caspase-3 levels increased significantly (Fig. 5B). The increase in caspase-3 activity on day 4 confirmed the Annexin V data and further supported the notion that CD8+ T effector/memory activation was followed by irreversible apoptotic cell death. In addition, enhancement of caspase-3 activity in the absence of a substantial decline in Bcl-xL/Bcl-2 levels suggested that the increase in Bcl-xL expression between days 4 and 7 was significant (p = 0.013). For caspase-3, the differences between day 4 and days 0 or 7 were significant (p = 0.027). Error bars, SD.

**FIGURE 5.** Posttherapy CD8+ T cell apoptosis. A, Annexin V binding. CD8+ T cells were stained for Annexin V binding. Annexin V− gate was determined based on Annexin V− staining of naive LN lymphocytes (>98% live; data not shown). The line graph demonstrates average percentage of Annexin V− positive cells from three independent experiments (n = 3/experiment). The differences between day 4 and days 1, 2, or 7 were significant (p = 0.037). Error bars, SD. B, Bcl-xL/Bcl-2 expression and caspase-3 activation. CD8+ T cells were stained for intracellular Bcl-2, Bcl-xL, and activated caspase-3 as described in Materials and Methods (n = 3/group). The increase in Bcl-xL expression between days 4 and 7 was significant (p = 0.013). For caspase-3, the differences between day 4 and days 0 or 7 were significant (p = 0.027). Error bars, SD.

Loss of CD4+CD25+ T suppressor cells from the tumor microenvironment is due to apoptotic cell death

Because treatment resulted in the rapid apoptosis of pre-existing CD8+ T effector/memory cells, we hypothesized that loss of CD4+CD25+ T suppressors from the tumor microenvironment could also be due to apoptotic cell death. To this end, CD4+CD25+ T cells were evaluated for Annexin V binding (Fig. 6A). The results demonstrated a significant and progressive increase in the proportion of Annexin V+CD4+CD25+ cells between days 0 and 4 (from an average of 18 to 45%), establishing that treatment induced CD4+CD25+ T cell apoptosis. Cell death declined between days 4 and 7 and leveled off thereafter, a pattern that correlated well with the kinetics of Foxp3− T cell loss from the tumor (Fig. 6A). The possibility that apoptosis was associated with activated CD4+CD25+ Th cells rather than bona fide T suppressors was ruled out by further analysis of CD4+CD25+ T suppressors for Annexin V-binding, which demonstrated that treatment specifically induced apoptosis within this subset (data not shown).

Analysis of activated caspase-3 levels in posttherapy CD4+CD25+ T cells established that treatment induced caspase-3 activation between days 0–4, which then subsided on day 7, consistent with the Annexin V data. Similar to that observed with CD8+ T cells, Bcl-xL/Bcl-2 levels did not change between days 0–4 (Fig. 6B). In contrast, Bcl-xL/Bcl-2 levels declined between days 4 and 7 in CD4+CD25+ cells, suggesting a partial involvement of the intrinsic mitochondrial pathway during later stages of apoptosis in CD4+CD25+ T cells.

**FIGURE 6.** Analysis of posttherapy CD4+CD25+ T cell apoptosis. A, Changes in the proportion of Annexin V− T cells. The dot plot demonstrates Annexin V− gating of CD8+ T cells from day 0 to day 7. The line graph demonstrates average percentage of Annexin V− positive cells from three independent experiments (n = 3/group). The differences between day 4 and days 1, 2, or 7 were significant (p ≤ 0.037). Error bars, SD. B, Analysis of activated caspase-3 levels in posttherapy T effector/memory and CD4+CD25+ Th cells.

Posttherapy T cell apoptosis was not simply a byproduct of enhanced cytotoxic activity within the tumor microenvironment because analysis of other leukocyte subsets, i.e., monocytes and granulocytes, did not show increased apoptosis between days 0–4 (data not shown). Both the activation kinetics and the pro-/anti-apoptotic protein expression patterns of intratumoral CD8+ T cells suggested that the observed events were more consistent with death receptor-mediated AICD (25, 26). To this end, membrane expression of Fas and FasL on the T effector/memory and T suppressor subsets was monitored before and after therapy. The results are shown in Fig. 6C. Analysis of pretreatment cells demonstrated that whereas all three T cell subsets expressed Fas, none expressed FasL. Upon treatment, Fas expression did not change significantly (data not shown). In contrast, FasL expression increased on CD8+ and CD4+CD25+ T effector/memory cells but not on CD4+CD25+ T suppressors on day 4 (Fig. 6D). Analysis of the cells on day 7 showed that FasL expression on CD8+ T cells decreased back to day 0 levels, a finding consistent with the notion that CD8+ T cells are distinct from the day 0–4 populations. A similar change was not observed for the CD4+CD25+ cells that remained FasL negative throughout (Fig. 6D). These data are consistent with the hypothesis that posttherapy apoptosis of tumor-associated T effector/memory cells was likely due to Fas/FasL-mediated AICD.

Both CD8+ T effector cell activation and T suppressor apoptosis are mediated by IL-12 in an IFN-γ-dependent manner

The above studies were performed with IL-12/GM-CSF-encapsulated microspheres because these cytokines were previously shown to be synergistic in the long-term eradication of metastatic lung tumors in the Line-1-BALB/c model (14). It was of interest to
determine the specific contribution of each cytokine to the therapy-induced changes in tumor-associated T cell subsets. To this end, we compared the effect of IL-12 and GM-CSF administered individually or together on T effector/memory cell activation, T suppressor cell loss, and T effector cell infiltration. The results are shown in Fig. 7. These data demonstrate that both the initial CD8⁺ T effector/memory cell activation as determined by CD43/CD69 expression, as well as the increase in CD8⁺ T effector cell numbers on day 7 were primarily mediated by IL-12 and not GM-CSF (Fig. 7A and B). Similarly, posttherapy T suppressor apoptosis (Fig. 7C and D) and up-regulation of FasL on CD8⁺ T cells (Fig. 7E) were also dependent on IL-12 and not GM-CSF.

Numerous studies, including ours, have demonstrated that IL-12-mediated tumor regression is IFN-γ dependent (14, 15). Others showed that IFN-γ is also required for death receptor-mediated apoptosis (27–30). This raised the question whether the CD8⁺ T effector cell activation and the CD4⁺CD25⁺ T suppressor cell apoptosis observed in this study were mediated directly by IL-12 or required the induction of downstream effectors, i.e., IFN-γ. To this end, therapy-induced changes in tumor-infiltrating CD8⁺ and CD4⁺CD25⁺ T cell populations were monitored in wild-type (wt) and IFN-γ-knockout (GKO) mice. The results are shown in Table I. These data demonstrate that both CD8⁺ T effector cell expansion and CD4⁺CD25⁺ T suppressor cell loss/apoptosis required IFN-γ. We therefore conclude that IFN-γ is central to the IL-12-induced changes in intratumoral T cell populations.

Discussion

The studies described here establish that delivery of IL-12 to tumors promotes an effective reversal of tumor immune suppression via the activation of tumor-associated T effector/memory cells, the elimination of intratumoral CD4⁺CD25⁺ T suppressor cells, and...
FIGURE 7. Specific roles of IL-12 and GM-CSF in T cell activation and apoptosis. A, CD8⁺ T cell activation. CD8⁺ T cells were gated on and analyzed for double-positive (CD43/CD69) cells on days 0 (untreated tumor) or days 4 and 7 following treatment with IL-12, GM-CSF, or IL-12 + GM-CSF microspheres (n = 3/group). The differences between day 0 and days 4 or 7 were significant (p ≤ 0.05) for the IL-12 + GM-CSF or IL-12 alone groups. The differences between day 0 and days 4 or 7 were not significant (p > 0.10) for GM-CSF alone. Error bars, SD.

B, CD8⁺ T cell proliferation kinetics. Absolute numbers of CD8⁺ T cells in tumors were determined on day 0 (untreated tumor) or days 4 and 7 following treatment in each group (n = 3/group). The differences between day 0 and day 7 were significant (p ≤ 0.03) for IL-12 + GM-CSF or IL-12 alone. The difference between day 0 and day 7 was not significant (p = 0.57) for GM-CSF alone. Error bars, SD.

C, T suppressor cell kinetics. Absolute numbers of tumor-infiltrating CD4⁺CD25⁺ T cells were determined on day 0 (no treatment) and 4 days after treatment in each group (n = 3/group). The differences between no treatment and IL-12 + GM-CSF or IL-12 alone groups were significant (p ≤ 0.019). The difference between no treatment and GM-CSF alone
the infiltration of CD8⁺ T effectors. This reversal is maintained for at least 2 wk after treatment due to continued loss of CD4⁺ CD25⁻ T suppressors from the tumor. These data demonstrate the highly complex nature of post-IL-12 therapy T cell activity in the tumor microenvironment and provide a detailed understanding of how intratumoral delivery of IL-12 microspheres promotes tumor regression.

Characterization of tumor-associated T lymphocytes before treatment identified three distinct T cell subsets. These subsets included functionally impaired CD8⁺ and CD4⁺ CD25⁻ T cells with an effector/memory phenotype, and a CD4⁺ CD25⁺Foxp3⁺ TGFβ⁺ T cell population that demonstrated suppressive function. These data are consistent with the findings of previous studies demonstrating the quiescent nature of tumor-associated CD8⁻/CD4⁺ T cells (6, 31–33), and more recently the presence of T suppressor cell infiltrates in murine and human tumors (34). Evidence from other studies suggests that the dysfunctional state of tumor-associated CD8⁻/CD4⁺ T cells is, at least in part, due to T suppressor cell activity, establishing a functional link between the two populations (7–9). To this end, it was recently demonstrated that in vivo, T suppressor cells modulate anti-tumor T effector activity via selective inhibition of cytotoxic function, and that TGFβ is critical to inhibition (35). Our findings that pretherapy T effector/memory cells were impaired in granzyme B production, and that the tumor-infiltrating CD4⁺ CD25⁺ T suppressor cells expressed membrane-bound TGFβ, are consistent with the notion that T suppressor cells are responsible for T effector/memory cell dysfunction in our model.

Intratumoral delivery of IL-12 resulted in a rapid and dramatic reversal of the anergic phenotype and function of pre-existing T effector/memory cells. Several studies have shown that whereas TIL are dysfunctional in situ, purified CD8⁺ T cells can be reactivated in vitro upon stimulation with cytokines (6). This study demonstrates a highly effective rescue of both the phenotypic and functional characteristics of tumor-associated T cells in situ. Others recently reported that local delivery of IL-12 induced the activation of tumor-associated CD4⁺ T cells, resulting in an IFN-γ-dependent eradication of tumor xenografts in a human tumor/SCID mouse xenograft model (36). Our data establish that treatment resulted in the activation of both CD4⁺ and CD8⁺ T effector/memory cells and that in addition to enhanced IFN-γ production, a concurrent recovery of CD8⁺ T cell cytolytic function was achieved. More importantly, we also found that activation of pre-existing CD8⁺ T effector/memory cells did not result in their proliferation, but led to apoptotic death within 4 days of treatment. This finding is consistent with others’ observations that anergic, tumor-associated T cells are predisposed to AICD upon in vitro activation (23) and that IL-12-mediated activation enhances Fas/FasL-dependent AICD of T cells (37, 38). To this end, analysis of FasL expression on tumor-associated T effector/memory cells in our model demonstrated that treatment resulted in the up-regulation of FasL on CD8⁺ T cells and CD4⁺ Th cells, consistent with the onset of AICD.

An unexpected finding in this study was that, concurrent with the activation of CD8⁺ T effector/memory cells, intratumoral delivery of IL-12 induced a rapid quantitative decline in tumor-infiltrating T suppressor cells. Further analysis established that the loss of T suppressors from tumors was due to apoptotic cell death, which was detectable within 24 h of treatment and peaked on day 4 posttherapy. CD4⁺ CD25⁺ T cells displayed Fas on their cell surface, suggesting that the Fas/FasL pathway could be involved in therapy-induced apoptosis. Because treatment did not induce FasL expression on T suppressors, the death signal (FasL) was likely provided by activated T effector/memory cells. Current evidence for the role of Fas/FasL-mediated apoptosis in T suppressor homeostasis is inconclusive. Although T suppressor cells express Fas constitutively (39, 40), they are resistant to anti-Fas Ab-mediated apoptosis (41). In contrast, it was recently reported that whereas CD4⁺ CD25⁺ T suppressors were resistant to AICD via TCR-stimulation, they were uniquely sensitive to soluble CD95 ligand-mediated apoptosis in vitro (42). Similarly, overexpression of Foxp3 in CD4⁺CD25⁺ T cells of Foxp3 transgenic mice has been shown to result in hypersensitivity to Fas/FasL-mediated apoptosis (43). These studies support the hypothesis that the Fas/FasL pathway may be involved in T suppressor homeostasis. Whether elimination of T suppressor cells from immunologically active environments via death receptor-mediated apoptosis is a general mechanism for overcoming regulation remains to be shown. In this case, T suppressor loss from tumors continued for at least 2 wk, providing a relatively broad window for the effector responses to occur. Whether T suppressor cells eventually reinfiltrated persisting tumors was not determined.

Activation and subsequent apoptotic death of pre-existing CD8⁺ T effector/memory cells between days 0–4 was followed by infiltration of tumors with nonapoptotic CD8⁺ T cells displaying full effector phenotype on day 7. Apoptosis did not result in a

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**Table I. IFN-γ is required for IL-12-induced T effector cell expansion and T suppressor cell apoptosis**

<table>
<thead>
<tr>
<th>Day</th>
<th>CD8⁺ T Cells</th>
<th>CD4⁺ CD25⁺ T Suppressor Cells</th>
<th>Percentage of caspase-3-positive cells*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>wt / GKO</td>
<td>wt / GKO</td>
<td>wt / GKO</td>
</tr>
<tr>
<td>0</td>
<td>33.3 ± 3.5 / 18.1 ± 3.4</td>
<td>52.1 ± 8.3 / 66 ± 19</td>
<td>10.5 ± 2.1 / 10.5 ± 0.7</td>
</tr>
<tr>
<td>4</td>
<td>29 ± 3.5 / 12.8 ± 1.8</td>
<td>13.5 ± 1.2 / 63.5 ± 7.2</td>
<td>22.3 ± 3.6 / 9.8 ± 0.9</td>
</tr>
<tr>
<td>7</td>
<td>83.5 ± 11.2 / 11.6 ± 1.7</td>
<td>ND / ND</td>
<td>ND / ND</td>
</tr>
</tbody>
</table>

*Values shown are averages of three mice per group. Error, SD. ND, Not done.

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*p Differences between day 7 and days 0/4 were significant (p ≤ 0.002).

*p Differences in cell numbers and caspase-3 activity between days 0–4 were significant in wt mice (p ≤ 0.015).
detectable reduction in intratumoral CD8\(^+\) T cell numbers between days 0–4 (as seen with T suppressor cells), due possibly to the compensatory infiltration/expansion of CD8\(^+\) T effectors. Whether day 7 CD8\(^+\) T effectors expanded from pre-existing T effector/memory cells or represented a secondary wave of newly primed CD8\(^+\) T cells arriving from the TDLN remains equivocal. The significant differences in the intensity of IFN-\(\gamma\) production by day 2 vs day 7 CD8\(^+\) T cells, the sudden switch in the apoptotic phenotype of day 7 CD8\(^+\) T cells, as well as the long interval between IL-12 delivery and the actual expansion collectively suggest that day 7 population was distinct from pre-existing CD8\(^+\) T effector/memory cells and likely represented newly primed effectors arriving from the TDLN. In contrast, the current data cannot rule out the possibility that a subset of pre-existing T effector/memory cells escaped/reversed AICD and expanded on day 7. Studies addressing this possibility are currently underway. Regardless of source, the secondary T effector response contracted rapidly by day 10, consistent with the established activation kinetics of cytotoxic T cell responses (23, 44).

Analysis of TIL from mice treated either with IL-12 or GM-CSF alone demonstrated that essentially all posttreatment T cell activity described in this study was mediated by IL-12. The ability of IL-12 to directly enhance the activity and proliferation of Ag-experienced T cells is well-established (15). Therefore, the dominant role of IL-12 in mediating the early posttherapy changes within tumor-infiltrating T cell populations is not surprising. To this end, our earlier studies demonstrated synergy between these cytokines in the long-term eradication of systemic disease, but not necessarily in short-term regression of primary tumors (Ref. 14 and M. O. Kilinc and N. K. Eglímez, unpublished data). Because GM-CSF has been shown to augment tumor vaccines primarily via the induction of APC generation (16), its synergistic properties are likely associated with a qualitative enhancement of long-term T cell memory.

The observation that the secondary CD8\(^+\) T effector response contracted rapidly between days 7–14, even in the absence of T suppressor cells, has important clinical implications. Although treatment resulted in an effective reversal of immune suppression within the tumor microenvironment and enhanced the intensity of T cell activity, cumulative effector activity was still transient, providing a cytotoxic window between days 1 and 10. T effector cell activation/contraction kinetics has been shown to be independent of both Ag load and persistence (44) and, as the above results suggest, possibly of T suppressor activity. Whereas manipulation of Ag dose, use of inflammatory adjuvants, and/or blocking of regulation enhances the intensity of T cell responses, the contraction kinetics of T effector cells remain unaltered (44, 45). Therefore, long-term efficacy of vaccine-based approaches may be limited by tumor burden and/or persistence even when immune regulatory mechanisms are successfully blocked. Accordingly, vaccines designed to induce antitumor T cells would be more likely to achieve complete tumor eradication and disease-free survival in patients with limited tumor burden. To this end, therapeutic tumor vaccines have been significantly more successful when administered in the minimal residual disease setting in both preclinical and clinical studies (14, 46–48).

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Disclosures

N. K. Eglímez has partial ownership in Therapayx, Incorporated, which is currently developing the microsphere technology for tumor vaccination.

References


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