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Cellular and Molecular Requirements for Rejection of B16 Melanoma in the Setting of Regulatory T Cell Depletion and Homeostatic Proliferation

Justin Kline,* Long Zhang,* Lauren Battaglia,* Kenneth S. Cohen,* and Thomas F. Gajewski*†

We have recently demonstrated that adoptive transfer of regulatory T cell-depleted polyclonal T cells into lymphopenic mice leads to rejection of B16 melanoma, which generated an opportunity to study host requirements for tumor rejection when it effectively occurred. CD8+ T cell priming and tumor rejection required tumor Ag cross-presentation, as evidenced by tumor outgrowth in Kb−/− bone marrow chimeric or B71/2−/− mice. CD4+ T cells were additionally required for optimal tumor control, although not through classical CD4 “help,” as the frequency of primed CD8+ T cells was similar in the absence of CD4+ T cells, and tumor rejection did not depend upon CD40–CD40L interactions or on IL-2 production by CD4+ T cells. Rather, CD4+ T cells appeared to act at the effector phase of tumor rejection and responded to B16-derived Ags in vitro. At the effector phase, IFN-γ production by transferred T cells, but not host cells, was necessary. IFN-γ acted either on host or tumor cells and was associated with reduced tumor vascularity. Finally, tumor rejection occurred after transfer of TNF-α, perforin, or FasL-deficient T cells. However, perforin/FasL double-knockout T cells failed to reject, arguing that the killing of B16 melanoma cells could occur either via the cytotoxic granule or Fas pathways. Collectively, these results support a model in which host tumor Ag cross-presentation primes adoptively transferred T cells, which remain functional in the setting of homeostatic proliferation and regulatory T cell depletion, and which promote tumor rejection via IFN-γ and lysis via cytotoxic granules and/or FasL.

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Recent advances in the efficacy of adoptive T cell therapy have resulted in high rates of objective tumor responses in patients with advanced melanoma. Improvements in the ability to culture and expand tumor-infiltrating lymphocytes (TILs) ex vivo and the addition of chemotherapy or total body irradiation (TBI) conditioning regimens to deplete host T cells and regulatory T cells (Tregs) prior to the adoptive transfer of ex vivo-activated TILs have resulted in objective tumor response rates exceeding 50% in published reports (1). Currently, several groups have attempted to improve upon these observations through transduction of host T cells with TCRs specific for known melanoma-derived Ags with some success (2). Although a thorough investigation of the Ag-specificity, differentiation state, and phenotype of ex vivo-expanded T cells ideal for adoptive transfer has been undertaken, little is known regarding either the host requirements or optimal effector functions of adoptively transferred T cells, which are necessary for tumor regression to occur in vivo.

The tumor microenvironment can exploit a number of mechanisms that can inhibit the execution of effective host antitumor immune responses (3), which has pointed toward strategies to reverse these immune suppressive pathways and restore immune-mediated tumor control. We have previously reported a murine adoptive T cell therapy model in which tumor-induced T cell anergy was reversed by transfer of polyclonal T cells into lymphopenic hosts through the process of homeostatic proliferation (HP) (4). Concomitantly, Tregs were depleted using an anti-CD25 mAb-mediated approach prior to adoptive transfer. When Treg-depleted T cells were transferred into lymphopenic hosts (either RAG−/− or sublethally irradiated C57BL/6 mice), potent rejection of B16 melanoma ensued, which correlated with robust, persistent IFN-γ production by tumor Ag-specific CD8+ T cells (4). This powerful rejection of B16 melanoma provided a rare opportunity to determine the host requirements for rejection of this tumor, which is normally difficult to control in vivo. Although this model does not make use of ex vivo-activated TILs or tumor Ag-specific TCR transgenic T cells, it is an approach that is straightforward and clinically translatable.

Although the major role of HP in this approach appears to be to prevent anergy of antitumor T cells, the process of HP itself has been reported to promote transition of naive T cells into pseudo-memory phenotype cells (5). Therefore, it was not clear if Ag-specific T cell priming might still be required for productive antitumor immunity. Cross-priming via tumor Ag released to host dendritic cells could theoretically still occur and, superimposed upon HP, preferentially expand tumor Ag-specific effector T cells for tumor control.

In addition to priming of CD8+ T cells, which are necessary for tumor eradication, the role of polyclonal CD4+ T cells in responding directly to B16 melanoma Ags has not been well elucidated. Previous work has suggested that CD4+ T cells con-
tribute to the antitumor immune response through several mechanisms, including providing help during the priming phase of CTL via production of IL-2 (6), licensing of APC through CD40–CD40L interactions (7, 8), production of IFN-γ in the tumor microenvironment (9), and direct tumor cell lysis (10–12). More recently, several groups have demonstrated that TCR transgenic CD4+ T cells specific for MHC class II-restricted Ags expressed by B16 melanoma can mediate tumor regression (10, 12, 13). Although these data are important, they may not translate well into the clinical setting in which polyclonal adoptively transferred CD4+ T cells might also be required to provide classical “help” to Ag-specific CD8+ T cells or license APC through CD40L, in addition to directly recognizing and eliminating malignant cells.

Finally, during the effector phase of the antitumor immune response, the mechanism of elimination of B16 melanoma tumors in the setting of adoptive T cell transfer was of interest to elucidate. It was conceivable that production of selected cytokines and cytolytic molecules by adoptively transferred Treg-depleted T cells would be required in order for tumor regression to occur. This could involve actions directly on tumor cells but also indirect effects on stromal cell components. For example, the necessity of IFN-γ production by tumor-specific T cells has been previously shown to be required for tumor rejection in numerous models (14–18). But IFN-γ can act through several, distinct mechanisms, such as inducing MHC upregulation on tumor cells and APCs (14), an antiproliferative effect on tumor cells in combination with TNF-α (19), activation of innate immune cells, such as macrophages (20), or through induction of molecules that inhibit angiogenesis (15, 21, 22). With these questions in mind, the host requirements for rejection of B16 melanoma by adoptively transferred Treg-depleted T cells in lymphopenic recipient mice were explored.

Materials and Methods

Mice and tumor cell lines

C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME) or Taconic (Germantown, NY). RAG2−/−, Kb−/−, and Db−/− mice on the C57BL/6 background were purchased from Taconic. RAG1−/−, B7-1−/−, B7-2−/−, CD4−/−, CD8−/−, CD40−/−, CD40L−/−, IL-2−/−, IFN-γ−/−, IFN-γR−/−, TNF-α−/−, perforin−/−, and gld/gld mice, all on the C57BL/6 background, were purchased from The Jackson Laboratory. Perforin−/− × gld/gld mice were interbred in our animal facility. Mice were housed in micro-isolator cages in a specific pathogen-free barrier facility and treated under National Institutes of Health guidelines. Animals were maintained and used according to protocols approved by the Institutional Animal Care and Use Committee of the University of Chicago. RAG1−/− and RAG2−/− mice were used as available by our suppliers, and we have previously found that B16.SIY rejection occurs similarly in either strain after adoptive transfer of Treg-depleted T cells (J. Kline, unpublished observations). Because RAG−/− mice contain no endogenous mature T cells to confound results, they were used as hosts in experiments investigating the role of CD8+ versus conventional CD4+ T cells in tumor rejection and also when T cell-produced effector/lytic molecules (IL-2, IFN-γ, TNF-α, perforin, FasL) were examined. When evaluating host (i.e., non-T cell) factors required for tumor rejection, irradiated, targeted gene knockout mice on the C57BL/6 background (Kb−/−, B7-1−/−, B7-2−/−, CD40−/−, IFN-γ−/−, IFN-γR−/−) were used, as these knockout strains are not commercially available. B16.SIY tumor cell line. A control empty-vector pMY construct (Milltenyi Biotec) was similarly transduced into B16.SIY (SIY.DSRED) cells to generate the B16.SIY.SIGN−/− tumor cell line. A control empty-vector pMY construct was similarly transduced into B16.SIY (SIY.DSRED) cells to generate the B16.SIYEV tumor cell line.

T cell purification and adoptive transfer

Cell suspensions were generated from spleens of indicated donor mice, and total CD4+ and CD8+ T cells were purified by negative selection over magnetic columns using a T cell enrichment kit (Miltenyi Biotechnologies) according to the manufacturer’s protocol. Flow cytometric analysis was periodically performed and routinely showed greater than 95% purity of CD3+ T cells. Purified total splenic CD4+ and CD8+ T cells were additionally depleted of CD25+ T cells (Treg) by negative selection using a magnetic-bead conjugated anti-CD25 Ab (Miltenyi Biotec). CD25 depletion was also confirmed by flow cytometry to eliminate >95% of CD25+ T cells from the total T cell population. For transfer in vivo, purified T cells were washed three times with PBS and then resuspended at a concentration of 106 cells/ml. For adoptive transfer experiments, a volume of 0.1 ml was injected into the lateral tail vein of mice.

Tumor challenge and measurement

After washing B16.SIY tumor cells three times with PBS to remove FCS, they were resuspended in PBS at a concentration of 106 cells/ml. A volume of 106 tumor cells was injected s.c. into the lower right abdomen of each mouse. Tumor measurements were performed two to three times weekly with calipers by a single individual taking the greatest tumor diameter and its perpendicular to determine an average. Mice with mean tumor diameters greater than 20 mm, or those demonstrating ulceration or showing signs of declining health, were euthanized.

Generation of bone marrow chimeras

Cohorts of C57BL/6 mice were lethally irradiated with 1000 rad TBI. Bone marrow cells were harvested from femurs of C57BL/6, Kb−/−, or Db−/− mice, and 106 cells were injected into the tail veins of irradiated C57BL/6 mice 1 d after TBI. After 11 wk, bone marrow chimeric mice received 600 rad TBI to induce host lymphodepletion. Twenty-four hours later, mice received adoptive transfer of 106 CD25-depleted T cells isolated from spleens of C57BL/6 mice, followed the next day by a s.c. challenge with 106 B16.SIY cells.

IFN-γ ELISPOT assay

The IFN-γ ELISPOT assay was conducted with the BD Pharmingen (San Diego, CA) mouse IFN-γ ELISPOT kit according to the provided protocol. Briefly, ELISPOT plates were coated with anti-mouse IFN-γ Ab and stored overnight at 4°C. Plates were then washed and blocked with DMEM supplemented with 10% FCS for 2 h at room temperature. Splenocytes from tumor-challenged mice were harvested at various indicated time points and plated at 105 cells/well. Stimulation was performed with irradiated B16.SIY tumor cells (10,000 rad) at 5 × 105 cells/well, 80 nM SIY peptide, or PMA and ionomycin as a positive control. Plates were stored at 37°C in a 7.5% CO2 incubator overnight, washed, and coated with detection Ab for 2 h at room temperature. They were again washed and coated with avidin-peroxidase for 1 h at room temperature. Plates were then washed and developed by addition of AEC substrate. Developed plates were dried overnight, read using an ImmunoSpot Series 3 Analyzer, and analyzed with ImmunoSpot software.

Tetramer staining and FACS analysis

The SIY (SIYVYGLL) and negative control OVA (SIINFEKL) peptide pentamers were purchased from Proimmune (Oxford, U.K.). For cell staining, the manufacturer’s protocol was followed. Abs against the following molecules coupled to the indicated fluorochromes were purchased from BD Biosciences: FITC-anti-CD3, allophycocyanin-anti-CD8a, PerCP-Cy5.5-anti-CD4, PerCP-Cy5.5-anti-B220, PE-anti-CD25 (PC61), biotin-anti-Kb, and biotin-anti-Dα. Allophycocyanin-anti-FoxP3 (FJK-16s) was purchased from eBioscience (San Diego, CA). FACS analysis was performed on the FACSCanto cytometer using FACSDiva software (Franklin Lakes, NJ). Data analysis was performed using FlowJo software (Tree Star, Ashland, OR).

Tumor vascular density measurement

B16.SIY tumors were established for 2 d in either C57BL/6 or IFN-γ−/− hosts. On day 6, tumor-bearing mice received 600 rad TBI. On day 8, 106 CD25-depleted T cells from C57BL/6 or IFN-γ−/− mice were transferred into tumor-bearing, irradiated C57BL/6 or IFN-γ−/− hosts, respectively.

MO. The IFN-γ−/− tumor cell line was generated by adoptively transferring Treg-depleted T cells into irradiated B16.SIY (SIY.DSRED) cells to generate the B16.SIY.SIGN−/− tumor cell line. A control empty-vector pMY construct was similarly transduced into B16.SIY (SIY.DSRED) cells to generate the B16.SIYEV tumor cell line.
Approximately 2 wk later, mice were injected with 100 μg biotinylated tomato lectin (Vector Laboratories, Burlingame, CA) and euthanized 5 min later. Tumors were surgically excised and placed in 4% paraformaldehyde at 4°C for 4 h, and then in 30% sucrose/PBS overnight at 4°C. Tumor specimens were embedded in 7.5% gelatin/15% sucrose/PBS and flash frozen in liquid nitrogen-cooled isopentane at −80°C. Eight-micrometer sections were cut and stored at −80°C until use. For microvessel density analyses, tumor sections were warmed to room temperature and gelatin removed by incubation in PBS at 37°C. Sections were washed twice with room temperature PBS, stained with SA–Alexa fluor 594 (Molecular Probes) for 20 min at room temperature, and then counterstained with Vectashield with DAPI (Vector Laboratories). Stained sections were imaged using an Axiovert 200 (Zeiss) microscope. Vessel density was measured using ImageJ (National Institutes of Health).

Statistical analysis

The Student t test was used to compare differences in tumor size between mice in various treatment arms at the indicated time points and also to analyze differences in numbers of IFN-γ spot-forming cells and SIY/Kb tetramer-reactive CD8+ cells in individual mice assigned to various treatment groups. A p value ≤ 0.05 between groups was considered to be statistically significant.

Results

SIY Ag cross-presentation by host hematopoietic cells is necessary for B16.SIY tumor rejection after adoptive transfer of CD25-depleted T cells

Our previous results have demonstrated that adoptive transfer of polyclonal CD4+ and CD8+ T cells depleted of CD25+ Tregs (hereafter referred to as CD25-depleted T cells) into lymphopenic hosts [either RAG2−/− or sublethally irradiated (600 rad) C57BL/6 mice] resulted in robust homeostatic T cell expansion, which resulted in equivalently potent rejection of B16.SIY tumors (4), although homeostatic T cell proliferation occurred somewhat more slowly in irradiated C57BL/6 versus RAG2−/− hosts. Low-dose TBI has been used as a lymphodepletion strategy to mimic what might be used for clinical translation (4). After adoptive transfer of CD25-depleted T cells into lymphopenic recipients, tumor rejection occurred in both the prophylactic setting (100% rejection) and also when B16.SIY tumors were established to ~5–6 mm in mean tumor diameter prior to transfer of CD25-depleted T cells (Supplemental Fig. 1).

Because the process of HP can drive naive T cells to acquire a partially activated phenotype (5), it was of interest to determine whether the activation of Ag-specific CD8+ T cells in this context would still require tumor Ag cross-presentation by host APCs. To this end, we generated radiation bone marrow chimeric mice using Kb-deficient donor bone marrow cells, as Kb is the restricting element for the dominant SIY Ag. Eleven weeks later, chimeric mice received 600 rad TBI for lymphodepletion and adoptive transfer of CD25-depleted T cells, followed by B16.SIY cell challenge. Db−/− bone marrow chimeras were included as a control to ensure that any differences in tumor rejection observed were not due to reduced total expression of class I MHC molecules in Kb−/− chimeric hosts, for example to support homeostatic T cell proliferation. As shown in Fig. 1A, Kb and Db expression was not detected on WBCs from Kb−/− → C57BL/6 and Db−/− → C57BL/6 chimeric mice, respectively. Similar numbers of CD3+ T cells were present in spleens of all groups of chimeric mice, suggesting that T cell expansion was supported in all cases (data not shown). As shown in Fig. 1B, B16.SIY tumors were rejected, as expected, in lymphopenic C57BL/6 → C57BL/6 and Db−/− → C57BL/6 chimeras after adoptive T cell transfer. In contrast, in Kb−/− → C57BL/6 chimeras, tumors grew progressively, though slightly more slowly than in chimeric mice that did not receive...
a T cell transfer, suggesting that some degree of Ag-specific immunity was generated in the K<sup>b</sup>-/- → C57BL/6 chimeras. This possibly occurred through recognition of other melanoma Ags on B16 cells, as we and others have previously demonstrated (4, 23).

Overall, these results indicate that cross-presentation of Ag on host K<sup>b</sup>-expressing hematopoietic cells is necessary for B16.SIY rejection by CD25-depleted T cells in the context of HP.

**T cell costimulation through B7-1/B7-2 on host cells is required for tumor rejection by CD25-depleted T cells in the context of HP**

If encounter of transferred T cells with host APCs was necessary for Ag presentation, then it seemed likely that costimulatory signals from host APCs might also be required for T cell priming and tumor rejection. To test this notion, C57BL/6 or B7-1/B7-2<sup>+</sup> mice were lymphodepleted with 600 rad TBI followed by transfer of CD25-depleted T cells from C57BL/6 donor spleens and B16.SIY tumor cell challenge. Whereas B16.SIY tumors were completely rejected in irradiated C57BL/6 mice after a CD25-depleted T cell transfer, they grew progressively in irradiated B7-1/B7-2<sup>-/-</sup> hosts (Fig. 2A). To assess whether T cell priming paralleled tumor control, SIY/R<sup>k</sup> pentamer staining and IFN-γ ELISPOT assay were performed on spleens harvested ~24 d after B16.SIY tumor cell inoculation (Fig. 2B, 2C). Consistent with the differences in tumor growth, numbers of SIY-specific CD8<sup>+</sup> T cells were ~2-fold higher in irradiated C57BL/6 versus irradiated B7-1/B7-2<sup>-/-</sup> hosts after transfer of CD25-depleted T cells (p = 0.05) (Fig. 2B). More strikingly, a 7-fold increase in the number of IFN-γ-producing, SIY-specific CD8<sup>+</sup> T cells was observed in irradiated C57BL/6 compared with irradiated B7-1/B7-2<sup>-/-</sup> mice after T cell transfer (p < 0.001) (Fig. 2C). Collectively, these results demonstrate the importance of host B7-1 and/or B7-2 costimulation in promoting the priming and subsequent effector function of tumor Ag-specific CD8<sup>+</sup> T cells in this system.

**Conventional CD4<sup>+</sup> T cells are required for complete rejection of B16.SIY tumors in lymphopenic mice after CD25-depleted T cell transfer**

CD4<sup>+</sup> T cells have been reported to contribute to antitumor immune responses through several mechanisms. CD4<sup>+</sup> T cells have been shown to provide “help” for priming of tumor Ag-specific CD8<sup>+</sup> effector T cells, either through production of IL-2 or via licensing of APCs through CD40-CD40L interactions (6, 7). They also have been shown to promote memory CD8<sup>+</sup> T cell development (24, 25). However, recent evidence has indicated that CD4<sup>+</sup> T cells can also act at the effector phase (13) and in some cases directly recognize and kill tumor cells (10, 11). In the human setting, adoptive transfer of tumor Ag-specific CD4<sup>+</sup> T cell clones can mediate complete tumor rejection in at least a subset of melanoma patients (26). Thus, the role of conventional CD4<sup>+</sup> T cells in supporting B16.SIY rejection by CD25-depleted T cells was examined. Several T cell populations were prepared prior to adoptive transfer into RAG1<sup>-/-</sup> hosts: 1) CD25-depleted T cells, consisting of CD8<sup>+</sup>/CD25<sup>−</sup> and CD4<sup>+</sup>/CD25<sup>−</sup> T cells from spleens of C57BL/6 donor mice, 2) CD8<sup>+</sup> T cells from spleens of C57BL/6 donor mice, and 3) CD4<sup>+</sup>/CD25<sup>−</sup> T cells from spleens of C57BL/6 donor mice. CD4<sup>+</sup>/CD25<sup>-/-</sup> rather than wild-type C57BL/6 mice were chosen for these experiments because the isolation of CD4<sup>+</sup> or CD8<sup>+</sup> T cells from wild-type mice is imperfect, and even a few contaminating T cells can undergo robust homeostatic expansion after adoptive transfer into lymphopenic hosts and potentially confound results. As demonstrated in Fig. 3A, RAG1<sup>-/-</sup> mice that received only CD8<sup>+</sup> T cells temporarily controlled B16.SIY outgrowth but failed to achieve complete tumor rejection, suggesting an important role for conventional CD4<sup>+</sup> T cells in this model. Notably, transfer of CD4<sup>+</sup>/CD25<sup>-/-</sup> T cells in the absence of CD8<sup>+</sup> T cells resulted in a modest delay in the outgrowth of B16.SIY tumors, pointing toward a potential role for conventional CD4<sup>+</sup> T cells in the direct recognition and control of B16.SIY tumors.

The mechanism by which CD4<sup>+</sup> T cells might be contributing to tumor control was then investigated. To determine whether the presence of conventional CD4<sup>+</sup> T cells provided help for the priming of SIY-specific CD8<sup>+</sup> T cells in B16.SIY tumor-challenged mice, spleen cells were stained with SIY/R<sup>k</sup> pentamers (Fig. 3B, 3C) and analyzed by IFN-γ ELISPOT assay (Fig. 3D, 3E). Surprisingly, similar percentages of SIY<sup>+</sup>CD8<sup>+</sup> T cells with equivalent IFN-γ production after SIY peptides restimulation were observed in spleens of tumor-challenged RAG1<sup>-/-</sup> mice that had received either CD8<sup>+</sup> or CD25-depleted T cells, arguing that the effect of CD4<sup>+</sup>/CD25<sup>-/-</sup> T cells on B16.SIY rejection was not primarily through provision of classical “help” to CD8<sup>+</sup> T cells (Fig. 3B, 3C). Furthermore, a significant number of IFN-γ spot-forming cells were detected in spleens of RAG1<sup>-/-</sup> mice that received only CD4<sup>+</sup>/CD25<sup>-/-</sup> T cells after restimulation with irradiated B16.SIY cells (Fig. 3E), most likely through cross-presentation of MHC class II-restricted Ags derived from dying B16.SIY cells to CD4<sup>+</sup> T cells, as B16.SIY cells do not express MHC class II molecules in vitro. This result suggests that CD4<sup>+</sup> T cells were naturally primed in vivo and could recognize Ags derived from B16.SIY tumors.

**IL-2 production and provision of CD40L by CD4<sup>+</sup>/CD25<sup>-/-</sup> T cells are dispensable for B16.SIY rejection**

Although the presence of conventional CD4<sup>+</sup> T cells did not appear to significantly increase the priming of SIY-specific CD8<sup>+</sup> T cells, the results presented earlier did not formally exclude the possibility that classical mechanisms of CD4<sup>+</sup> T cell help might contribute to tumor rejection. Therefore, the key defined mechanisms of CD4<sup>+</sup> T cell help, such as IL-2 production, and provision of CD40L were directly interrogated. First, the role of secreted IL-2 was examined. Wild-type CD8<sup>+</sup> T cells (3.5 × 10<sup>6</sup>) were combined with CD4<sup>+</sup>/CD25<sup>-/-</sup> T cells (6.5 × 10<sup>5</sup>) from either wild-type or IL-2<sup>-/-</sup> mice and adoptively transferred into RAG2<sup>-/-</sup> hosts, followed by tumor challenge. Surprisingly, B16.SIY tumors were rejected whether or not the transferred CD4<sup>+</sup> T cells were capable of IL-2 production (Fig. 4A). At the same time, we examined whether IL-2 by transferred T cells was required at all for tumor rejection in this model. Notably, when CD25-depleted total T cells from IL-2<sup>-/-</sup> mice were used for adoptive transfer, no effective tumor control was observed (Fig. 4B) and resulted in a marked reduction in SIY-specific T cell responses (Supplemental Fig. 2A, 2B). Together, these data imply that although IL-2 is important for optimal priming of CD8<sup>+</sup> tumor Ag-specific T cells in this model, it does not need to be provided by CD4<sup>+</sup> T cells and presumably can be sufficient when produced by the CD8<sup>+</sup> T cells themselves.

It was also of interest to determine whether CD4<sup>+</sup> T cells were providing help through the alternative mechanism of CD40L-mediated activation of host APCs. To explore this possibility, CD25-depleted T cells were transferred into irradiated (600 rad TBI) C57BL/6 or CD40<sup>-/-</sup> mice followed by B16.SIY tumor challenge. In fact, tumors were similarly rejected in C57BL/6 or CD40<sup>-/-</sup> lymphopenic hosts (Fig. 4C). As expected, SIY-specific immune responses were similar in the two groups, based on SIY/R<sup>k</sup> pentamer staining and IFN-γ ELISPOT analysis (data not shown). These data argue against a significant role of the CD40-CD40L pathway in controlling B16.SIY rejection.

Taken together with the observation that CD4<sup>+</sup> T cell recognition of Ags derived from B16.SIY tumor cells occurred and that partial
Tumor control was achieved with transfer of conventional CD4+ T cells alone, these data argue that the major role of CD4+ conventional T cells in this model is to contribute to direct tumor control in vivo. Consistent with this notion, CD4+ T cells were regularly identified within the tumor microenvironment in vivo (Fig. 4D). Additionally, only \( \sim 1\% \) of the tumor-infiltrating CD4+ T cells were Tregs (Fig. 4D), in stark contrast to what we have observed in the B16 tumor microenvironment of unmanipulated, wild-type mice in which between 20 and 30% of CD4+ T cells express Foxp3 (4). Further, we repeatedly observed significant upregulation of MHC class II (I-A\(^{b}\)) molecules on B16.

SIY tumor cells when examined directly ex vivo (Fig. 4E), which occurred in a TBI-dependent and IFN-\( \gamma \)-dependent manner (Supplemental Fig. 2C). Together, these observations provide additional evidence in favor of direct antitumor control by conventional CD4+ T cells in this model.

### Tumor rejection by CD25-depleted T cells plus HP requires either the cytotoxic granule pathway or FasL, but not TNF-\( \alpha \)

We next turned to investigate the effector mechanisms involved in rejection of B16.SIY tumors by CD25-depleted T cells in the setting of HP. Two major cytolytic mechanisms of CTL have been...
FIGURE 3. Complete rejection of B16.SIY is dependent upon conventional CD4+ T cells. (A) T cells were purified from spleens of C57BL/6 mice (CD25-depleted T cell group), CD4+ mice (CD8+ T cell group), or CD8+ mice (CD4+25 T cell group). CD25+ T cells were subsequently depleted using anti-CD25 microbeads in the CD25-depleted T cell and CD4+25 T cell groups. CD25-depleted T cells, CD8+ T cells, or CD4+25 T cells (10^7) were adoptively transferred into cohorts of RAG1-/- mice. Twenty-four hours later, mice were challenged with 10^6 B16.SIY cells s.c., and tumor growth was monitored. *p < 0.01 (comparison of tumor size at day 21 between RAG1-/- mice after adoptive transfer of CD25-depleted T cells versus both CD8+ and CD4+25 T cells); #p = 0.02 (comparison of tumor size at day 21 between RAG1-/- mice after adoptive transfer of CD8+ and CD4+25 T cells). (B) Twenty-five days after B16.SIY inoculation, spleens were harvested from tumor-challenged mice and analyzed by flow cytometry for the percentage of CD8+SIY+ cells among the CD8+ T cell population present in individual mice. p = n.s. (comparison of SIY-reactive CD8+ T cells in RAG1-/- mice receiving CD25-depleted T cells versus RAG1-/- mice receiving CD8+ T cells). (C) SIY and negative control OVA pentamer (Figure legend continues)
described: release of granules containing perforin and granzymes (27), and engagement of Fas on target cells through surface expression of FasL (28, 29). In Addition, TNF-α produced by T cells is necessary for tumor rejection in some models (18). To address the role of each of these pathways in rejection of B16.SIY in vivo, T cells from various gene knockout mice were used.

To investigate whether TNF-α production was necessary for B16.SIY rejection, CD25-depleted T cells were isolated from spleens of C57BL/6 or TNF-α−/− donor mice and transferred into RAG2−/− mice. As with wild-type T cells, B16.SIY tumors were completely rejected by TNF-α−/− CD25-depleted T cells, indicating that TNF-α production by transferred T cells is not necessary for tumor rejection (Fig. 5A). Surprisingly, B16.SIY tumors were rejected similarly after transfer of either perforin−/− or gld/gld CD25-depleted T cells into RAG2−/− mice, suggesting that expression of neither perforin nor FasL was required for tumor rejection (Fig. 5B, 5C). However, it was conceivable that either pathway might be sufficient and that functional compensation could occur. Therefore, perforin−/− × gld/gld double-mutant mice were generated. Notably, CD25-depleted T cells isolated from perforin−/− × gld/gld mice and transferred into RAG2−/− mice failed to reject B16.SIY tumors (Fig. 5D), although partial tumor control was seen. These results argue that adoptively transferred CD25-depleted T cell-mediated killing of tumor cells involves both the perforin and FasL pathways but that either is sufficient to drive tumor rejection.

IFN-γ production by transferred T cells but not by host cells is required for tumor rejection

It was also of interest to clarify whether IFN-γ played a role in promoting B16.SIY rejection in the current model. To determine whether IFN-γ produced by adoptively transferred T cells was required, CD25-depleted T cells from C57BL/6 or IFN-γ−/− donor mice were transferred into RAG2−/− mice challenged with B16.SIY cells. As shown in Fig. 6A, tumor rejection was significantly impaired with IFN-γ−/− T cells, arguing for a critical importance of this cytokine in regulating B16.SIY rejection. The failure of IFN-γ−/− CD25-depleted T cells to eliminate B16.SIY tumors was not due to decreased numbers of SIY Ag-specific CD8+ IFN-γ−/− T cells in lymphopenic recipients, as significantly higher numbers of SIY-specific CD8+ IFN-γ−/− versus wild-type T cells were routinely identified in spleens of RAG2−/− hosts (Supplemental Fig. 3A, 3B).

Whether IFN-γ production was required by transferred CD8+ T cells, conventional CD4+ T cells, or both was investigated by transferring permutations of CD8+ (3.5 × 10^5) and CD4+25− T cells (6.5 × 10^5) from C57BL/6 or IFN-γ−/− donor mice into RAG2−/− mice (such that each mouse received a total of 10^7 T cells: 3.5 × 10^6 CD8+ and 6.5 × 10^6 CD4+25− T cells). As shown in Fig. 6B, IFN-γ production by either CD8+ or CD4+25− T cells was sufficient to support B16.SIY rejection, whereas tumors grew progressively after transfer of combined IFN-γ−/− CD8+ and IFN-γ−/− CD4+25− T cells. Therefore, either CD4+ or CD8+ T cells must produce IFN-γ for tumor rejection to occur. These results provide additional support for the participation of CD4+ T cells at the effector phase of the antitumor T cell response.

To determine whether IFN-γ produced by host cells was also important, C57BL/6 or IFN-γ−/− mice received 600 rad TBI, followed by adoptive transfer of CD25-depleted T cells from C57BL/6 or IFN-γ−/− donor mice. As before, IFN-γ−/− CD25-depleted T cells failed to reject B16.SIY tumors in either irradiated C57BL/6 or IFN-γ−/− mice. However, complete tumor rejection was observed in irradiated IFN-γ−/− mice receiving C57BL/6 CD25-depleted T cells (Fig. 6C). Therefore, production of IFN-γ by host cells (e.g., by NK cells) was not required for B16.SIY rejection after adoptive transfer of CD25-depleted T cells into lymphopenic hosts.

IFN-γ can act either upon host cells or tumor cells to mediate tumor rejection

IFN-γ can have pleiotropic effects on target cells and may contribute to antitumor immunity at multiple levels, mediating potential effects on T cells, tumor cells, and host stromal cells. To define the cellular targets for IFN-γ-mediated tumor rejection, IFN-γR−/− mice were used. First, to investigate whether host cells or transferred CD25-depleted T cells themselves were responding to IFN-γ to attain tumor rejection, irradiated C57BL/6 or IFN-γR−/− mice were lymphodepleted prior to transfer of CD25-depleted T cells from either C57BL/6 or IFN-γR−/− mice and B16.SIY tumor cell challenge (Fig. 7A). Surprisingly, all cohorts that received adoptive transfer of CD25-depleted T cells showed effective tumor control, including irradiated IFN-γR−/− hosts receiving transfer of IFN-γR−/− T cells, suggesting that responsiveness to IFN-γ was not absolutely required on transferred T cells or on any host cell.

It was therefore conceivable that IFN-γ produced by adoptively transferred CD25-depleted T cells might act directly on the implanted tumor cells through upregulation of class I MHC molecules, increased expression of Ag processing machinery, or induced production of antiangiogenic factors, such as the chemokines CXCL9 or CXCL10 (14, 15). We confirmed that IFN-γ induced a substantial increase of K b expression on B16.SIY implanted tumor cells through upregulation of class I MHC molecules, increased expression of Ag processing machinery, or induced production of antiangiogenic factors, such as the chemokines CXCL9 or CXCL10 (14, 15). We confirmed that IFN-γ induced a substantial increase of K b expression on B16.SIY implanted tumor cells through upregulation of class I MHC molecules, increased expression of Ag processing machinery, or induced production of antiangiogenic factors, such as the chemokines CXCL9 or CXCL10 (14, 15).

To determine whether IFN-γ signaling at the level of the tumor cells was required for rejection in this model, B16.SIY cells were transduced with either an empty vector retroviral construct (B16.SIY.EV) or one containing the cDNA for a truncated, nonsignaling form of the IFN-γR (dominant-negative IFN-γR; B16.SIY.DIFN-γR). B16.SIY.DIFN-γR cells failed to upregulate K b expression in vitro expression to IFN-γ (Fig. 7B, right panel). CD25-depleted T cells from C57BL/6 donor mice were adoptively transferred into RAG2−/− mice, followed by challenge with either B16.SIY.EV or B16.SIY.DIFN-γR cells. Surprisingly, potent tumor control was still observed against B16.SIY.DIFN-γR tumors, al-

analysis of spleen cells from individual mice in (B). FACS plots demonstrate the percentage of SIY or OVA-reactive CD8+ T cells after gating on CD4+ expression and CD8+ cells. (D and E) On day 25 after B16.SIY implantation, spleens from tumor-challenged mice were harvested, and 10^6 spleen cells from individual mice in each group were restimulated in vitro overnight with media, SIY peptide (80 nM), or 5 × 10^5 irradiated B16.SIY cells (10,000 rad). An IFN-γ ELISPOT assay was performed, and results are recorded as spot-forming cells per 10^6 total cells in individual mice (three wells per mouse per condition) after media, SIY peptide (D), or irradiated B16.SIY cell (E) restimulation. *p < 0.001 (comparison of IFN-γ spot-forming cells in RAG1−/− mice receiving CD8+ or CD4+25− T cells when restimulated with irradiated B16.SIY cells); **p = n.s. (comparison of IFN-γ spot-forming cells in RAG1−/− mice receiving CD8+ versus CD4+25− T cells when restimulated with irradiated B16.SIY cells); ***p = n.s. (comparison of IFN-γ spot-forming cells in RAG1−/− mice receiving CD8+ versus CD25-depleted T cells when restimulated with SIY peptide). These results are representative of two independent experiments with four to five mice per group.
though a partial detriment was observed in some experiments (Fig. 7B, left panel). We therefore reasoned that IFN-γ signaling might be required on both host and tumor cells, and only when both were insensitive to IFN-γ would tumor rejection fail. To test this notion, C57BL/6 or IFN-γR2−/− mice received 600 rad TBI, followed by adoptive transfer of CD25-depleted T cells from C57BL/6 mice. Subsequently, mice were challenged with B16.SIY.EV or B16.SIY.ΔIFN-γR cells. In fact, we observed that tumor rejection often failed when B16.SIY.ΔIFN-γR tumor cells were implanted into IFN-γR−/− mice (50–75% of B16.SIY.ΔIFN-γR tumors were not rejected after implantation into irradiated IFN-γR−/− hosts; p = 0.18 for comparison of tumor size at day 28 between irradiated C57BL/6 hosts receiving transfer of B16.SIY.EV versus irradiated IFN-γR−/− mice challenged with B16.SIY.ΔIFN-γR) (Fig. 7C, 7D).
Therefore, either host cell or B16.SIY cell responsiveness to IFN-γ was generally sufficient for tumor rejection to occur in response to CD25-depleted T cell transfer and HP, suggesting that both host and tumor cells are the targets of IFN-γ produced by transferred CD25-depleted T cells in this system.

Given that tumor rejection required intact IFN-γ signaling in host tissues and that IFN-γ-stimulated tumor cells elaborated the chemokine CXCL10, which has known antiangiogenic properties, we hypothesized that IFN-γ-dependent tumor rejection might involve antiangiogenic mechanisms. Therefore, the tumor vasculature was analyzed in the presence or absence of IFN-γ. For these studies, the experimental design was altered to analyze preestablished tumors to facilitate analysis of vascular density in tumors in a more reproducible manner.

To this end, C57BL/6 or IFN-γ−/− mice were challenged with B16.SIY cells. Six days later, mice received 600 rad TBI. On day 8, irradiated C57BL/6 mice received transfer of CD25-depleted T cells from C57BL/6 mice, and irradiated IFN-γ−/− mice received CD25-depleted T cells from IFN-γ−/− mice. This experimental design was chosen to facilitate an analysis of tumor angiogenesis in a completely IFN-γ-deficient host so as not to require the use of combinations of IFN-γR−/− donor T cells, IFN-γR−/− irradiated hosts, and B16.SIY.DIFN-γR tumor cells, which would render such an analysis impractical.

As expected, established B16.SIY tumors were partially controlled in irradiated C57BL/6 after transfer of wild-type CD25-depleted T cells but grew progressively in irradiated IFN-γ−/− mice after IFN-γ−/− CD25-depleted T cell transfer (Fig. 8A). Consistent with our in vitro results, CXCL10 levels were significantly higher in B16.SIY tumors (Fig. 8B), and Kb expression was upregulated on B16.SIY cells isolated from irradiated C57BL/6 hosts after wild-type CD25-depleted T cell transfer (Supplemental Fig. 4C). When these tumors were analyzed for microvessel density at day 22 after tumor challenge, a significantly higher number of blood vessels both by CD34 expression and by lectin staining was observed in B16.SIY tumors isolated from irradiated IFN-γ−/− mice versus wild-type mice (Fig. 8C, 8D). These observations support the notion that a major effect of IFN-γ produced by T cells in this model may be to inhibit angiogenesis within the B16 tumor microenvironment.

Discussion
A growing body of evidence has suggested that specific immune inhibitory pathways, acting largely at the level of the tumor mi-
FIGURE 6. IFN-γ production by donor T cells, but not host cells, is necessary for B16.SIY rejection. (A) CD25-depleted T cells were purified from spleens of C57BL/6 or IFN-γ−/− mice and adoptively transferred into groups of five RAG2−/− recipient mice. Twenty-four hours later, mice were challenged with 10⁶ B16.SIY cells, and tumor growth was monitored. *p < 0.01 (comparison of tumor size on day 20 between RAG2−/− mice after adoptive transfer of wild-type versus IFN-γ−/− CD25-depleted T cells). (B) CD8− and CD4− cells were isolated separately from spleens of C57BL/6 and IFN-γ−/− mice. Purified CD4− cells were further depleted of CD25− cells, and permutations of C57BL/6 or IFN-γ−/− CD8− and CD4− cells (3.5 × 10⁶ CD8− T cells, and 6.5 × 10⁶ CD4− T cells) were adoptively transferred into groups of five RAG2−/− recipient mice. Mice were subsequently challenged with B16.SIY cells as in (A). *p < 0.01 (comparison of tumor size on day 33 between RAG2−/− mice after adoptive transfer of wild-type CD25-depleted T cells, wild-type CD8− plus IFN-γ−/− CD4− T cells, or IFN-γ−/− CD8− plus wild-type CD4− T cells versus IFN-γ−/− CD8− plus IFN-γ−/− CD4− T cells). (C) Groups of five C57BL/6 or IFN-γ−/− mice received 600 rad TBI, followed by adoptive transfer of 10⁷ CD25-depleted T cells from C57BL/6 or IFN-γ−/− donor mice. Mice were subsequently challenged with B16.SIY cells as in (A). *p = n.s. (comparison of tumor size on day 24 between irradiated IFN-γ−/− and irradiated C57BL/6 mice after adoptive transfer of wild-type CD25-depleted T cells); *p < 0.01 (comparison of tumor size on day 24 between irradiated IFN-γ−/− and C57BL/6 mice after adoptive transfer of IFN-γ−/− CD25-depleted T cells versus irradiated IFN-γ−/− and C57BL/6 mice after adoptive transfer of wild-type CD25-depleted T cells). The tumor growth curves in (A)–(C) are representative of two independent experiments each with five mice per group.

croenvironment, can blunt the effector function of antitumor T cells and thus allow tumor outgrowth. These include metabolic inhibition of T cells by indoleamine-2,3-dioxygenase or arginase (30, 31); expression of ligands for inhibitory receptors, such as PD-L1 (32); extrinsic suppression by CD4−CD25−Foxp3+ Tregs (33); or T cell anergy, resulting from TCR ligation in the absence of adequate costimulation (34). Blockade or elimination of such immune-inhibitory pathways has become an attractive strategy for cancer immunotherapy, and numerous approaches have entered clinical trial testing. Understanding the mechanisms by which tumor control occurs when such pathways are uncoupled is therefore vital for elucidating reasons for success versus failure as data unfold during clinical investigation.

The overall goal of this work was to understand the cellular and molecular mechanisms underlying B16 tumor rejection upon elimination of two key immune suppressive pathways, Tregs and T cell anergy, in a clinically relevant model. At the priming phase, we determined that cross-presentation of Ag and B7 costimulation by host APCs was required. This is despite the ability of HP to be capable of inducing a “pseudo-memory” T cell phenotype (5) and argues that Ag-specific priming is still required to expand the relevant subset of tumor-reactive T cells from among the adaptively transferred polyclonal T cell population. Notably, we found no evidence that CD4+ T cells were involved in providing “help” for the generation of tumor Ag-specific CD8+ effector cells in this system. Rather, accumulated evidence supported the participation of CD4+ T cells in the effector phase of the antitumor T cell response, which is supported by several other recent publications (10–13). It is interesting to consider that depletion of CD25+ cells from the polyclonal T cells prior to adoptive transfer may have revealed the activity of a CD4+ effector population. It might be of interest in future studies to investigate a role of CD4+ T cells in supporting CD8+ T cell memory in this model, as has been demonstrated in models of antiviral immunity (25).

It is noteworthy that the final event of tumor control by effector T cells in our model could occur similarly with the individual absence of TNF-α, perforin, or FasL. This result is similar to what has been observed in rejection of allogeneic solid organs or in
Twenty-four hours later, mice were challenged with either $10^6$ B16.SIY.EV or B16.SIY.D mice. Cohorts of irradiated C57BL/6 and IFN-$\gamma$-R$^-/-$ mice were cultured in the presence or absence of IFN-$\gamma$ (20 ng/ml) for 48 h and analyzed for cell surface Kb expression. Shaded histograms represent Kb expression on B16.SIY.EV and B16.SIY.DIFN-$\gamma$ cells cultured in the absence of IFN-$\gamma$. Dotted and solid line histograms represent Kb expression on IFN-$\gamma$-treated B16.SIY.DIFN-$\gamma$ and B16.SIY.EV cells, respectively.

For clarity, tumor growth curves from the control groups are presented in (C), and tumor growth curves from experimental groups (those receiving CD25-depleted T cell transfer) are presented in (D). $p = 0.18$ (comparison of tumor size on day 26 between irradiated C57BL/6 mice challenged with B16.SIY.EV and irradiated IFN-$\gamma$-R$^-/-$ mice challenged with B16.SIY.DIFN-$\gamma$). Tumor growth curves in (A)–(D) are representative of two independent experiments, each with four to five mice per group.

T cell-dependent models of autoimmunity (35, 36), but contrary to published results in which TNF-$\alpha$ and/or perforin have been shown to be absolutely required for T cell-mediated tumor rejection (18). It appears that redundant cytolytic mechanisms are in place to minimize opportunity for immune escape from this process. However, combined elimination of both perforin and FasL in transferred T cells allowed for tumor outgrowth, suggesting that either cytotoxic pathway can be sufficient for tumor control. Whereas in other model systems a mandatory role for the perforin pathway has been reported (18, 37–39), differences likely have to do with the specific tumor models and T cell populations involved.

IFN-$\gamma$ production by antitumor T cells has been reported to be vital for tumor elimination in many experimental models (14–18). In the current work, our results have also identified IFN-$\gamma$ as a central mediator of tumor rejection. Through dissection of the contribution of specific cell populations, we have observed that adoptively transferred T cells and not cells derived from the recipient mice must produce IFN-$\gamma$ for tumor rejection to occur. Conventional CD4$^+$ and CD8$^+$ T cells produced IFN-$\gamma$ after adoptive transfer in response to tumor Ags, and IFN-$\gamma$ generated by either CD4$^+$ or CD8$^+$ T cells was sufficient to support tumor rejection. Further, IFN-$\gamma$-R$^-/-$ recipient mice, and dominant-negative IFN-$\gamma$-R B16.SIY cells were used to facilitate a careful dissection into the cellular compartment(s) in which IFN-$\gamma$ responsiveness was necessary for tumor rejection to ensue. Notably, the action of IFN-$\gamma$ could be either on host cells or on B16.SIY tumor cells, and tumor rejection was lost only when both were insensitive to IFN-$\gamma$. Although it was not surprising that IFN-$\gamma$ was absolutely required for tumor elimination in our model, our results clearly define the cell types necessary for both IFN-$\gamma$
production and responsiveness in order for tumor rejection to ensue.

Although IFN-γ has pleiotropic effects and could exert a multitude of influences, the apparent redundant role on either host cells or tumor cells suggested a possible effect on angiogenesis, which is one mechanism that could be shared by these two cellular compartments. Previous studies have suggested that IFN-γ induces expression of CXCL10, which has previously been associated with an antiangiogenic effect (15). Indeed, we observed CXCL10 production in the tumor microenvironment upon T cell infiltration in our model, and microvessel density was found to be reduced. Alternatively, IFN-γ might directly impact host stromal cells leading to impaired blood vessel development. Our experiments have demonstrated high levels of CXCL10 production by B16 cells in vitro after exposure to IFN-γ. CXCL10 production within B16 tumors in vivo was also observed, but only when T cells capable of IFN-γ production were transferred. It is likely that both host tumor stromal cells and B16 cells themselves were secreting CXCL10 in response to T cell-produced IFN-γ in vivo, even though insensitivity to IFN-γ in either compartment alone did not routinely lead to failure of tumor rejection, possibly as a result of compensation.

However, additional roles for IFN-γ are also conceivable, as class I and II MHC upregulation on B16 cells in vivo was also observed, which presumably led to their increased recognition and elimination by Ag-specific T cells. It is important to keep in mind...
that IFN-γ also induces upregulation of immune inhibitory molecules, including IDO and PD-L1 (32, 40). Therefore, an optimal amount and timing of IFN-γ production are likely required to ensure maximal antitumor effects.

In summary, our results suggest a model in which tumor Ag cross-presentation by host cells to adoptively transferred CD25-depleted polyclonal T cells promotes tumor rejection, which is dependent upon both CD8+ and CD4+ cells, IFN-γ, and either the perforin or Fasl pathways of cytotoxicity. The elimination of Tregs combined with HP maintains persistent T cell function in the context of a growing tumor. Combination with inhibition of other immune evasion pathways may also be of interest to investigate. Strategies to reproduce this approach for clinical application in cancer patients are available and will be of great interest to pursue in future studies. Ideally, such clinical/translational investigations should include interrogation of both the priming and effector phases of the antitumor T cell response.

Disclosures

The authors have no financial conflicts of interest.

References

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