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TNF Plays an Essential Role in Tumor Regression after Adoptive Transfer of Perforin/IFN-γ Double Knockout Effector T Cells

Christian H. Poehlein,* Hong-Ming Hu,*† Jane Yamada,* Ilka Assmann,* W. Gregory Alvord,‖ Walter J. Urba,*‡† and Bernard A. Fox*‡‡§¶

We have recently shown that effector T cells (T\textsubscript{E}) lacking either perforin or IFN-γ are highly effective mediators of tumor regression. To rule out compensation by either mechanism, T\textsubscript{E} deficient in both perforin and IFN-γ (perforin knockout (PKO)/IFN-γ knockout (GKO)) were generated. The adoptive transfer of PKO/GKO T\textsubscript{E} mediated complete tumor regression and cured wild-type animals with established pulmonary metastases of the B16BL6-D5 (D5) melanoma cell line. PKO/GKO T\textsubscript{E} also mediated tumor regression in D5 tumor-bearing PKO, GKO, or PKO/GKO recipients, although in PKO/GKO recipients efficacy was reduced. PKO/GKO T\textsubscript{E} exhibited tumor-specific TNF-α production and cytotoxicity in a 24-h assay, which was blocked by the soluble TNFRII-human IgG fusion protein (TNFRII:Fc). Blocking TNF in vivo by administering soluble TNFR II fusion protein (TNFRII:Fc) significantly reduced the therapeutic efficacy of PKO/GKO, but not wild-type T\textsubscript{E}. This study identifies perforin, IFN-γ, and TNF as a critical triad of effector molecules that characterize therapeutic antitumor T cells. These insights could be used to monitor and potentially tune the immune response to cancer vaccines. The Journal of Immunology, 2003, 170: 2004–2013.

Laboratories of *Molecular and Tumor Immunology and †Clinical Research, Robert W. Franz Cancer Research Center, Earle A. Chiles Research Institute, Providence Portland Medical Center, Portland, OR 97213; Departments of 2Biochemistry and Molecular Biology, Oregon Graduate Institute, and 3Molecular Microbiology and Immunology, and 4Oregon Cancer Center, Oregon Health and Science University, Portland, OR 97202; and 5Data Management Services, Frederick Cancer Research and Development Center, National Cancer Institute, Frederick, MD 21702

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2 Address correspondence and reprint requests to Dr. Bernard A. Fox, Laboratory of Molecular and Tumor Immunology, Earle A. Chiles Research Institute, 4805 NE Glisan Street, Portland, OR 97213. E-mail address: foxb@foxlab.org
3 Abbreviations used in this paper: T\textsubscript{E}, effector T cell; KO, knockout; PKO, perforin KO; FasL, Fas ligand; GKO, IFN-γ KO; GRKO, IFN-γ receptor KO; wt, wild type; CM, complete medium; TVDLN, tumor vaccine draining lymph node; LT, lymphotoxin.
To test directly whether either perforin or IFN-γ were required for T cell-mediated tumor regression, mice deficient in both genes were developed. These mice were unable to produce IFN-γ and failed to generate T cells or lymphokine-activated killer cells that could lyse appropriate targets in 6-h 51Cr-release assays. In this report, we examined whether tumor-specific T cells that lacked both IFN-γ and perforin could exhibit significant antitumor activity in vitro and in vivo.

Materials and Methods

Animals
PKO (C57BL/6-PPm1sDz), GKO (C57BL/6-IFN-γtm1Ts), and wild-type (wt) mice on a C57BL/6 background were purchased from The Jackson Laboratory (Bar Harbor, ME). PKO and GKO mice were used to generate double KO mice. Because both perforin and IFN-γ are on the same chromosome, multiple backcrosses were required to generate a colony of PKO/GKO mice. Mice were screened for deficiency of both genes according to the protocols provided by The Jackson Laboratory (http://www.jax.org). These mice were submitted to The Jackson Laboratory and given the designation C57BL/6-Pip<tm1Dzd>-Ifng<tm1TTS>+. All animal research protocols were approved by the Earle A. Chiles Research Institute animal care and use committee.

Tumor cell lines
B16BL6-D5 (D5) is a poorly immunogenic subclone of the spontaneously arising B16BL6 melanoma derived from C57BL/6 mice (provided by Dr. S. Shu, Cleveland Clinic Foundation, Cleveland, OH) (34, 36). D5-G6 is a stable clone of D5 that was transduced with a murine GM-CSF retroviral MFG vector (provided by Dr. M. Arca, University of Michigan, Ann Arbor, MI) (37). D5-G6 cells secrete ~200 ng/ml/106 cells/24 h GM-CSF. MCA-310 is a methyl-cholanthrene-induced, weakly immunogenic, fibrosarcoma of C57BL/6 mice (20).

Culture conditions
Lymphocytes and tumor cells were cultured in complete medium (CM), which consisted of RPMI 1640 supplemented with 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 2 mM t-glutamine, 50 μg/ml gentamicin sulfate (all from BioWhittaker, Walkersville, MD), 50 μM 2-ME (Sigma-Aldrich, St. Louis, MO), and 10% FBS (Life Technologies, Grand Island, NY). Tumor cells were harvested two to three times per week by brief trypsinization (Trypsin; BioWhittaker) and maintained in T-75 or T-150 culture flasks.

Vaccination and challenge experiments
PKO/GKO or wt mice were injected s.c. with 10 × 10⁶ irradiated D5-G6 tumor cells (10,000 rad) and challenged 14 days later with 2 × 10⁶ D5-G6 s.c. (10 × TD100 for D5 tumor). The TD100 is the dose at which 100% of the injected animals will develop tumor. In all experiments naive wt and PKO/GKO mice were included as controls and were challenged with the same tumor dose.

Generation of T E and adoptive immunotherapy
One million D5-G6 or MCA-310 tumor cells were injected s.c. in all four flanks of wt and PKO/GKO mice. Eight days following tumor inoculation, the tumor vaccine draining lymph nodes (TVDLN) were harvested, washed, resuspended at 2 × 10⁶ cells per ml in CM and cultured in 24-well plates with anti-CD3 as described previously (20). After 2 days of activation, the T cells were harvested and expanded in CM containing 60 IU rHuL-2/ml (kind gift from Chiron, Emeryville, CA) for 3 additional days. T cells were then harvested, washed twice in HBSS, counted and used in cytotoxicity, intracellular cytokine staining, cytokine release assays, and adoptive transfer studies. For adoptive transfer experiments, wt, PKO, GKO, and PKO/GKO mice bearing 3-day established pulmonary metastases were generated by i.v. inoculation with 0.2 × 10⁶ D5 or 0.3 × 10⁶ MCA-310 tumor cells. T cells were administered i.v. and starting with the day of T cell transfer, animals received 90,000 IU IL-2 i.p. q.d. for 4 days. Where specified, 250 μg of TNFRIL-Fc (38) (Amgen, Seattle, WA) or control human IgG (Sigma-Aldrich) were administered i.p at the time of adoptive transfer and again 2 days later. Mice were sacrificed 14 days following tumor inoculation, lungs were resected (in the case of MCA-310 metastases, lungs would be tracheal-infused with India ink for contrast), fixed in Fekete’s solution, and the number of pulmonary metastases were enumerated. In all experiments, T E used for in vivo studies were analyzed in parallel for their cytolytic capability and/or cytokine profile.

ELISA
PKO/GKO and wt T E were washed, resuspended in CM, and cultured at 4 × 10⁶ cells/well in a 24-well plate. The cells were either left unstimulated (negative control) or stimulated with 2 × 10³ tumor cells (D5, MCA-310) or anti-CD3 (positive control). Supernatants were harvested after 20–24 h and assayed for IFN-γ, TNF-α, IL-4, IL-5, and IL-10 using commercially available reagents (BD Pharmingen, San Diego, CA).

In vitro cytotoxicity—6 and 24 h assay
For 6- and 24-h 51Cr-release assays, D5 or MCA-310 were incubated with 100 μCi Na₂¹⁵CΟ₃ for 90 min, washed and plated into 96-well round bottom plates with 10⁶ tumor target cells/well. Target cells were incubated with 10⁴ T E in triplicate at the indicated E/T ratios in 200 μl of CM at 37°C in a CO₂ incubator. The supernatant was harvested, counted, and the mean percent lysis was determined as previously described (20). For microscopic analysis, long-term (24 h) tumor-specific cytotoxicity assays were performed by culturing 0.2 × 10⁶ D5 or MCA-310 cells in CM for 24 h with 4 × 10⁶ D5-specific T E generated from wt or PKO/GKO mice in a 24-well flat-bottom plate. Tumor cells alone and tumor cells cultured with 4 × 10⁶ T E generated from nonvaccinated naïve wt and PKO/GKO lymph nodes were included as controls. After 24 h, T E and nonadherent cells were washed out and the remaining adherent tumor cell monolayer was fixed and stained with 0.05% crystal violet. The remaining fixed cells were documented using digital microscopic imaging.

For quantitative analysis of long-term cytotoxicity, 10⁴ D5 melanoma cells were cultured in 200 μl of CM in a 96-well flat-bottom plate alone or with 10⁶ T E in triplicates. After 24 h at 37°C in a CO₂ incubator, plates were gently washed, adherent tumor cells were fixed (3:1 methanol-glacial acid for 5–10 min), rinsed with tap water, and stained with 0.05% crystal violet in 10% ethanol for 15 min. The plate was then washed thoroughly with tap water and stained cells were dissolved in 10% acetic acid/20% ethanol and the OD was determined using a 592-nm filter on a microplate reader (BioTek Instruments, Winooksi, VT). Absorbance of wells with tumor cells alone was used as the reference for no cytotoxicity and buffer alone as reference for 100% lysis.

Intracellular TNF-α staining and flow cytometric analysis
PKO/GKO and wt T E were stimulated for 12 h in the presence of 5 μg/ml brefeldin A (Sigma-Aldrich) in CM only (no stimulation), with specific tumor (D5), unrelated syngeneic tumor (MCA-310), or immobilized anti-CD3. T E were harvested and stained with anti-CD8FITC and anti-CD3εPE mAbs, fixed and permeabilized in Cytotox/Cytoperm and stained intracellularly with anti-TNF-αPE or anti-IFN-γPE mAbs (all from BD Pharmingen). Analysis was performed on 50,000 gated CD8⁺/CD3⁺ T E with a FACSCalibur and CellQuest software (BD Biosciences, San Jose, CA). Data is presented as the percentage of CD8⁺/CD3⁺ T E, or CD8⁺/CD3⁺/IFN-γ⁺ T E over the total number of CD8⁺/CD3⁺ T E.

Statistical analysis
Statistical analysis of the number of pulmonary metastases was performed by Wilcoxon rank sum test and survival analysis was performed using Kaplan-Meier plots and log rank sum tests using S-plus 2000 software (Data Analysis Product Division; Mathsoft, Seattle, WA).

Results
Vaccination of PKO/GKO mice does not induce protective antitumor immunity
After documenting that PKO/GKO T E lacked functional expression of perforin and IFN-γ (data not shown), we investigated whether vaccination with irradiated D5-G6 cells would induce a protective immune response in these double KO mice. Previously we had observed that D5-G6 vaccination in wt or PKO mice was highly effective at protecting from a subsequent tumor challenge, but in GKO mice, vaccination failed to protect from a subsequent tumor challenge (35). To determine whether vaccination with D5-G6 would induce protective immunity in mice lacking both genes, we repeated this experiment in PKO/GKO mice. All vaccinated PKO/GKO mice eventually developed tumors, while 80%
of vaccinated wt mice were immune and resisted tumor challenge (Table I). Thus, in the absence of both perforin and IFN-γ, vaccination with D5-G6 is unable to protect mice from a subsequent tumor challenge.

**PKO/GKO T_E are therapeutic in wt recipients**

We next investigated whether PKO/GKO T_E cells could mediate tumor regression. The adoptive transfer of 35 or 70 × 10⁶ T cells from either wt or PKO/GKO mice significantly (p < 0.05) reduced the number of pulmonary metastases in wt mice. However, PKO/GKO T_E were clearly less effective than wt T_E, as evidenced by the resulting increased number of metastases in mice receiving 35 × 10⁶ in all three experiments (Fig. 1). Although, PKO/GKO T_E were not as therapeutic as wt T_E on a per cell basis, they were capable of mediating complete tumor regression in wt animals with systemic tumor when 70 × 10⁶ T_E were transferred. Furthermore, this finding demonstrates that neither perforin nor IFN-γ was required to prime T_E in response to a GM-CSF-secreting tumor vaccine. Next, we examined whether the antitumor activity of PKO/GKO T_E could improve survival of wt-recipient mice with established systemic tumor. Although all IL-2-treated control animals were dead by 18–20 days, animals treated with the adoptive transfer of 70 × 10⁶ PKO/GKO T_E and IL-2 treatment survived significantly longer (*, p < 0.0001) and 50% of treated animals were cured (Fig. 2).

**PKO/GKO T_E antitumor capacity is not limited to melanoma**

The ability of PKO/GKO T_E to mediate tumor regression was not unique to the D5 melanoma system. The adoptive transfer of T_E from MCA-310-vaccinated PKO/GKO mice were able to mediate regression of established 3-day MCA-310 pulmonary metastases (Fig. 3). The results in this model resembled those seen with D5. The adoptive transfer of PKO/GKO T_E resulted in significant (*, p < 0.01), dose-dependent regression of pulmonary metastases from this weakly immunogenic fibrosarcoma, but on a per-cell basis PKO/GKO T_E were not as efficient as wt T_E.

**PKO/GKO T_E mediate tumor regression in PKO- and GKO-recipient animals**

The adoptive transfer studies noted above used wt mice as the tumor-bearing recipient animals. To determine whether the production of perforin or IFN-γ by the tumor-bearing recipient animal was responsible for the antitumor effects mediated by the PKO/GKO T_E, 70 × 10⁶ PKO/GKO T_E were adoptively transferred into wt, PKO, and GKO mice bearing 3-day established D5 pulmonary metastases. The lack of either perforin or IFN-γ in the recipient animal did not compromise the efficacy of this high dose (70 × 10⁶) of PKO/GKO T_E; the antitumor effects were comparable regardless of the recipient animals phenotype (Table II). Thus, PKO/GKO T_E can mediate effective tumor regression even when the recipient animal cannot produce either perforin or IFN-γ.

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**Table I. Vaccination of PKO/GKO mice does not induce protective antitumor immunity**

<table>
<thead>
<tr>
<th>Mouse</th>
<th>Vaccinea</th>
<th>Number of Mice with Tumor/Total Number of Miceb</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt</td>
<td>None</td>
<td>10/10</td>
</tr>
<tr>
<td>PKO/GKO</td>
<td>None</td>
<td>10/10</td>
</tr>
<tr>
<td>wt</td>
<td>D5-G6</td>
<td>2/10</td>
</tr>
<tr>
<td>PKO/GKO</td>
<td>D5-G6</td>
<td>10/10</td>
</tr>
</tbody>
</table>

*a Ten wt and 10 PKO/GKO mice were injected s.c. with 10⁷ irradiated D5-G6 cells (10⁴ rad) into the left hind flank. Fourteen days later, 10 naive wt and PKO/GKO mice, or vaccinated wt and PKO/GKO mice were challenged with 2 × 10⁴ viable D5 tumor cells in the right hind flank.

*b Tumor size was assessed 5 days after D5 challenge and every second day until animals were sacrificed or 40 days following tumor challenge.
survival advantage (*p < 0.0001) and wt (T E) were adoptively transferred into wt mice. Animals receiving T E was significantly improved compared with mice treated with IL-2 alone. PKO/GKO T E mediate tumor regression and cure PKO/GKO recipients To rule out the possibility that therapeutic activity of PKO/GKO T E depends on the expression of either IFN-γ or perforin by the tumor-bearing recipients, we examined their ability to mediate tumor regression in PKO/GKO recipients. Although PKO/GKO T E were less effective than wt T E (†, p < 0.05), they still mediated significant (‡, p < 0.01) tumor regression (Fig. 4), and provided a survival advantage (§, p < 0.0001) compared with treatment with IL-2 alone (Fig. 5) in PKO/GKO-recipient animals. However, only 12% of animals that received 7 × 10⁶ PKO/GKO T E survived >120 days. The survival benefit was improved significantly to 40% (§, p < 0.01) by doubling the number of PKO/GKO T E transferred (Fig. 5). This shows that T E can use mechanism(s), other than perforin or IFN-γ, to destroy tumor cells, extend survival, and cure animals with systemic disease. At the same time it is also clear that the therapeutic efficacy of PKO/GKO T E is significantly (†, p < 0.01) reduced compared with wt T E (Fig. 5). Because the transfer of PKO/GKO T E into wt, PKO, and GKO recipients bearing 3-day established D5 pulmonary metastases was significantly (*, p < 0.0001) improved compared with mice treated with IL-2 alone.

PKO/GKO T E produce tumor-specific TNF-α Because PKO/GKO T E have significant antitumor activity, we sought to characterize the effector mechanism(s) in these cells. Because IFN-γ is a type 1 cytokine and plays a role in attenuating type 2 cytokine responses, PKO/GKO T E might exhibit a tumor-specific type 2 cytokine profile. Therefore, simultaneous with the adoptive transfer studies, PKO/GKO T E were also examined for their capacity to release cytokines in response to tumor cells. Although PKO/GKO T E released IL-4, IL-5, and IL-10 when stimulated following tumor inoculation and the number of pulmonary metastases. Data are representative of two experiments.

**FIGURE 3.** PKO/GKO T E also mediate tumor regression in a fibrosarcoma model. PKO/GKO and wt T E generated from MCA-310 TVDLN mediated regression of 3-day established MCA-310 pulmonary metastases (*, p < 0.01) compared with the IL-2 control. All groups received 90,000 IU IL-2 i.p. for four consecutive days beginning at the time of adoptive transfer. The number of transferred T E is presented in the figure. Mice were sacrificed 14 days following tumor inoculation and the number of pulmonary metastases. Data are representative of two experiments.

**Table II.** Efficacy of PKO/GKO T E is not impaired after adoptive transfer into PKO- and GKO-recipient mice

<table>
<thead>
<tr>
<th>Adoptive Immunotherapy</th>
<th>Mean Number of Pulmonary Metastases§ (SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>T E Donor</td>
</tr>
<tr>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>PKO/GKO</td>
<td>None</td>
</tr>
<tr>
<td>PKO/GKO</td>
<td>70 × 10⁶</td>
</tr>
<tr>
<td>PKO/GKO</td>
<td>None</td>
</tr>
<tr>
<td>PKO/GKO</td>
<td>70 × 10⁶</td>
</tr>
</tbody>
</table>

*Mice were vaccinated s.c. with D5-G6 tumor cells and TVDLN were harvested 8 days later. Lymph node cells were stimulated in vitro with anti-CD3 for 2 days and then were expanded for 3 days in 60 IU/ml IL-2. Effector cells were harvested and 70 × 10⁶ T cells were adoptively transferred into animals with established 3-day D5 pulmonary metastases.

^IL-2 (90,000 IU) was administered daily i.p. for 4 days following adoptive transfer.

^Mice were sacrificed on day 14 following i.v. inoculation of tumor and the number of pulmonary metastases was enumerated in a blinded fashion. Results presented for each experiment are the mean of five animals.

^x, p < 0.01 compared to IL-2 alone treated controls.
with anti-CD3 in 24-h assays, there was no release of these type 2 cytokines when they were stimulated with D5 or control tumor cells (data not shown). Thus, the priming of an immune response in the absence of perforin and IFN-γ did not lead to the development of a tumor-specific type 2 cytokine response. Therefore, it is unlikely that tumor regression was mediated by these cytokines.

Because Barth et al. (17) reported that both IFN-γ and TNF-α had a key role in T cell-mediated tumor regression, we examined whether PKO/GKO T_E produced TNF-α in response to stimulation with specific tumor. When PKO/GKO T_E, generated from D5-G6 TVDLN were stimulated with D5 tumor, no TNF-α was detected in the culture supernatant in our standard 24-h ELISA (data not shown). Thinking that we might have missed the period when TNF-α was secreted, a time course was performed and PKO/GKO T_E assayed for TNF-α secretion 6, 12, and 24 h following stimulation. Again, although TNF-α was secreted in response to stimulation with anti-CD3, no secretion was detected following stimulation with D5 or MCA-310 (data not shown). However, interrogation by RT-PCR of the same PKO/GKO T_E revealed tumor-specific up-regulation of message for TNF-α (data not shown). We considered that TNF-α produced in response to stimulation with specific tumor might be membrane-bound, consumed or bound by tumor stimulators and not be available for detection in the culture supernatant. To test this possibility we used intracellular cytokine analysis to assay PKO/GKO T_E. Results of these studies identified a population of CD8+ T cells that up-regulated expression of TNF-α in response to stimulation with D5 (Fig. 6A). In four consecutive experiments, a similar pattern of tumor-specific TNF-α expression was seen for both PKO/GKO and wt T_E (Fig. 6B). Although PKO/GKO T_E appeared to have a lower frequency of tumor-specific TNF-α-expressing T cells than wt T_E, the difference was not statistically significant. Simultaneously with the monitoring of TNF-α production, the frequency of tumor-specific IFN-γ-producing cells was also assessed. Fig. 6B shows that the frequency of tumor-specific TNF-α- and IFN-γ-producing wt T_E was similar. As expected, there was no detection of IFN-γ by PKO/GKO T_E. These studies suggest that tumor-specific expression of TNF-α provides a potential effector molecule that may be responsible for the antitumor effects mediated by PKO/GKO T_E.

PKO/GKO T_E-mediated tumor-specific cytotoxicity is blocked by TNFR1:Fc

Although PKO/GKO T_E are not cytolytic in 6-h 31Cr-release assays, long-term 24-h cytotoxicity assays were developed to investigate whether PKO/GKO T_E might mediate TNF-dependent D5-specific killing. Microscopic evaluation of tumor cell-T_E cultures.

**FIGURE 4.** PKO/GKO T_E mediate tumor regression in PKO/GKO recipients. T_E from PKO/GKO or wt mice (35 or 70 × 10^6) were transferred into PKO/GKO-recipient mice with 3-day established pulmonary D5 metastases. All animals received 90,000 IU IL-2 i.p. for four consecutive days beginning at the time of adoptive transfer. Animals were sacrificed on day 14 after tumor inoculation and the number of pulmonary metastases was determined. Although PKO/GKO and wt T_E mediated significant (*, p < 0.01) tumor regression, the transfer of wt T_E cells was significantly more therapeutic (†, p < 0.05) than the transfer of PKO/GKO T_E. Three consecutive experiments are presented.

**FIGURE 5.** Adoptive transfer of PKO/GKO T_E can cure PKO/GKO mice bearing systemic tumor. PKO/GKO mice bearing 3-day established D5 pulmonary metastases were treated with 70 × 10^6 wt T_E (●, n = 25), 70 × 10^6 PKO/GKO T_E (▲, n = 26), or 140 × 10^6 PKO/GKO T_E (○, n = 10). All groups received IL-2 as described before, the control group received IL-2 i.p. alone (■, n = 26). All groups receiving T_E showed significantly increased (+, p < 0.0001) survival compared with the IL-2 alone control group. Survival was significantly improved (†, p < 0.01) by transferring 140 × 10^6 PKO/GKO T_E as compared with 70 × 10^6 PKO/GKO T_E. But survival mediated by adoptive transfer of both PKO/GKO T_E doses was still significantly decreased (§, p < 0.01) as compared with the survival after adoptive transfer of wt T_E. Statistical differences were assessed via the log rank sum test.
revealed tumor-specific killing in 24-h assays (Fig. 7A). As an additional specificity control, naive wt and PKO/GKO lymph nodes were harvested, activated with anti-CD3 mAb, expanded in IL-2, and used in parallel with the D5-specific T_E generated from D5-G6 TVDLN. Anti-CD3/IL-2-activated “effector” T cells from naive lymph nodes (not tumor-primed) did not exhibit cytotoxicity for D5 or MCA-310, further supporting the specificity of T_E generated from TVDLN of PKO/GKO mice. Although it is possible that several TNF family members might be responsible for the cytotoxicity seen in these assays, previous studies with the D5 melanoma document its resistance to killing by FasL, anti-Fas, or TRAIL (20), (H. Winter, unpublished observation). To directly address whether TNF was responsible for the tumor lysis seen in this assay, TNF was neutralized by adding soluble TNFRII:Fc to the cytotoxicity assays. PKO/GKO T_E appeared less cytotoxic than wt T_E, but both specifically killed D5 tumor cells (*, p < 0.05) and not the MCA-310 control tumor cells (Fig. 7B). There was in fact no significant difference in the level of D5-specific cytotoxicity mediated by PKO/GKO and wt T_E. The addition of soluble TNFRII:Fc to these assays diminished significantly (*, p < 0.05) the killing of D5 by PKO/GKO T_E, but failed to inhibit the tumor-specific killing of wt T_E (Fig. 7B). Because soluble TNFRII:Fc can bind both TNF-α and lymphotoxin (LT)-α (TNF-β) we were unable to confirm which molecule was responsible. Nonetheless, these results document that tumor-specific T_E generated from PKO/GKO mice maintain an in vitro cytolytic function that is detectable at 24 h and is mediated by TNF.
PKO/GKO T_E-mediated tumor regression is blocked by TNFRII:Fc administration

To determine whether TNF played a critical role in tumor regression mediated by PKO/GKO T_E, soluble TNFRII:Fc (14) was administered i.p. to mice following the adoptive transfer of wt or PKO/GKO T_E. In three of three experiments, treatment with soluble TNFRII:Fc significantly (*, p < 0.01) reduced the therapeutic efficacy of PKO/GKO T_E, while administration of human IgG (control) had no effect on T_E function (two of two experiments). In contrast, treatment with TNFRII:Fc did not reduce the efficacy of wt T_E (Fig. 8). These results imply that in the absence of perforin and IFN-γ, TNF, either α and/or β, is the major mechanism for T cell-mediated tumor regression. The undisturbed efficacy of wt T_E in animals treated with TNFRII:Fc also demonstrates the importance of perforin and/or IFN-γ in wt animals and further illustrates a redundant, but limited, repertoire of effector mechanisms available for T cells to eliminate established pulmonary metastases.

Discussion

The adoptive transfer of tumor-specific CD8^+ T cells has been shown to mediate significant antitumor effects and cure animals of established metastases in numerous murine models. Considerable effort has been directed at dissecting the mechanisms of T cell-mediated tumor regression in these models in the hope that the information will lead to improved treatment for patients with cancer. Recent studies have documented that established pulmonary and intracranial metastases can be treated effectively by T cells that lack either perforin, FasL, or IFN-γ (20–22, 35). Furthermore, most studies reported efficacy in host animals that were deficient in the same molecule, thereby eliminating a requirement for that effector mechanism in T cell-mediated destruction of pulmonary or intracranial tumor. Because these studies demonstrated that there was not an absolute requirement for either perforin, FasL, or IFN-γ, three dominant effector systems of CD8^+ T cells, it suggests that there is redundancy in the effector mechanisms that can be mobilized by CD8^+ T cells to destroy tumor cells in vivo.

This study also identified the importance of perforin or IFN-γ expression by cells of the tumor-bearing recipient in the antitumor effect mediated by adoptively transferred PKO/GKO T_E. Although 70 × 10^6 D5-specific PKO/GKO T_E were highly effective at eliminating pulmonary metastases in tumor-bearing wt, PKO, or GKO mice (Table I), the transfer of the same number of PKO/GKO T_E into tumor-bearing PKO/GKO mice rarely provided complete clearing of the lungs (1 of 15 mice, Fig. 4, as compared with 11 of 15 mice, Fig. 1). Similarly, in survival studies, the transfer of 140 × 10^6 D5-specific PKO/GKO T_E was required to cure 40% of day 3 tumor-bearing PKO/GKO recipients, compared with 84% survival when 70 × 10^6 wt T_E were used to treat the same recipients. In contrast, 70 × 10^6 PKO/GKO T_E were about twice as effective in tumor-bearing wt mice, mediating long-term survival and curing 50% of treated animals (Fig. 2). These results document that in addition to the effector mechanism(s) supplied by the transferred T_E, the recipient mice still use either perforin or IFN-γ-based mechanisms to amplify the effects of the tumor-specific T cells. The observation that IFN-γ plays a role when perforin is absent (PKO/GKO T_E in PKO tumor-bearing recipients, Table II) is not unexpected given its direct antitumor effects, its function in amplifying the innate immune response and in augmenting Ag processing (30, 39). However, the significant role for perforin, when IFN-γ is absent (PKO/GKO T_E in GKO tumor-bearing recipients), is somewhat surprising given that we have not observed
frequency of T cells exhibiting multiple destructive mechanisms would be expected to mediate tumor regression in the absence of a host component, whereas T<sub>E</sub> populations with a low frequency of tumor-reactive cells and/or those lacking robust effector mechanisms would require the “support” of host cells (46). This reasoning may also explain some of the differences reported by investigators studying antitumor properties of T cells in gene KO mice. For instance, Prevost-Blondel et al. (47) showed that IFN-γ was essential in their model, as TCR-transgenic T cells from IFN-γ single KO mice are unable to mediate regression in IFN-γ KO mice. Although we observed that adoptive transfer of in vitro-activated and -expanded GKO T<sub>E</sub> cured GKO mice bearing established pulmonary metastases and subsequently protected them from a subsequent s.c. tumor challenge (35).

One explanation for this difference may be the vaccination and activation process used to generate T<sub>E</sub> in our studies. We have previously shown that vaccination with a GM-CSF-secreting tumor vaccine effectively primes therapeutic CD8<sup>+</sup> T<sub>E</sub> in CD4-depleted and MHC class II KO animals (48). Thus, it seems possible that such a strong vaccine induces a cytokine/costimulatory milieu that drives T cells more effectively and/or with a broader spectrum of effector mechanisms. Hence, the failure of tumor-specific GKO T cells to mediate tumor regression may have more to do with the frequency of primed T<sub>E</sub> cells, their environment during activation and expansion, and/or their available repertoire of effector mechanisms than with an inherent deficiency associated with the loss of a single effector mechanism.

In contrast to our results, which demonstrate that PKO/GKO mice generate a reduced therapeutic antitumor response to vaccination with D5-G6, Badovinac and Harty (49) showed that the immune response to vaccination with <i>Listeria</i> was stronger and more therapeutic in PKO/GKO mice (49, 50). In their study, the significantly heightened protection against a <i>Listeria</i> challenge correlated with the frequency of <i>Listeria monocytogenes</i> Ag-specific T cells, which was up to 4.5-fold higher in PKO/GKO than in wt animals. Consistent with our observation of a reduced therapeutic response, D5-G6-vaccinated PKO/GKO mice had a lower frequency of tumor-specific CD8<sup>+</sup> TNF-α<sup>-</sup> T<sub>E</sub> than vaccinated wt mice, but this difference was not significant (Fig. 6B). Given that both attenuated <i>Listeria</i> and GM-CSF-secreting tumor vaccines are highly effective at priming protective immune responses in their respective models, it is unclear why there is such a striking difference in the frequency of responding T cells in these two models. Particularly, why does the TNF-α response of PKO/GKO cells increase for <i>Listeria</i> and not for D5-G6? Is this an inherent advantage of using a <i>Listeria</i>-based vector to prime an immune response? The difference may be due to <i>L. monocytogenes</i> being a foreign bacterial Ag or contributing some adjuvant property that augments the TNF-α response. Then again, the inherent difference between PKO/GKO mice on a C57BL/6 vs a BALB/c background may explain the observed differences. Alternatively, the difference between these two models may be related to the in vitro activation step that is required to mature fresh TVDLN to functional T<sub>E</sub>. In this case, the activation process may lead to an increased activation-induced cell death and loss of some of the tumor-specific T cells. Although we did not observe decreased recovery of PKO/GKO T<sub>E</sub> following activation with anti-CD3 and IL-2 it is still possible that there was a selective loss of Ag-specific T<sub>E</sub> during the activation step.

Our in vitro experiments imply that TNF-α played an important role in the tumor-specific cytotoxicity of PKO/GKO T<sub>E</sub>. This observation is consistent with reports of others showing that effector...
cells that lack perforin and/or FasL mediate potent Ag-specific function in vitro via TNF-α. Lee et al. (51) reported that lymphokine-activated killer cells generated from PKO/gld double KO mice mediated cytotoxic activity that could be blocked by anti-TNF Ab. Similarly, Prevost-Blondel et al. (47) recently showed a crucial role for TNF-α in a gp33-transfected 3LL-A9 lung cancer model. In their studies, perforin-deficient TCR transgenic T cells that recognize a lymphocytic choriomeningitis virus peptide Ag stably expressed on a transfected Lewis lung carcinoma cell line (3LL), mediate tumor cell lysis and tumor regression, but loose efficacy in vivo in the absence of TNF-α. Additionally, Seki et al. (22) demonstrated efficient perforin-independent antitumor efficacy of adoptively transferred CTL. In their model, blocking TNF-α reduced CTL-mediated cytotoxicity in vitro.

A critical role for TNF in animal models of autoimmune and infectious disease has also been identified: T cell-induced acute diabetes occurs independently of either perforin or functional FasL, while animals deficient in TNFRII expression do not develop diabetes (52). Furthermore, in studies using perforin/IFN-γ double KO BALB/c mice, Harty and colleague (53) identified an important role for TNF in T cell-mediated immune responses to L. monocytogenes. They also showed that the immune response to vaccination with Listeria was stronger and more therapeutic in perforin/IFN-γ double KO compared with wt mice (49, 50). This is in contrast to our results, where the absence of perforin and IFN-γ resulted in T cells with reduced capacity to mediate tumor regression in both active specific and adoptive immunotherapy studies. Additional studies identified that perforin-independent immunity to L. monocytogenes requires CD8+ T cell-derived TNF-α (53) and is independent of Fas/FasL (53, 54). Although we cannot directly exclude Fas/FasL (CD95/CD95L) interactions in our PKO/GKO model, we have previously demonstrated that D5 tumor cells are resistant to apoptosis induced by anti-Fas mAb in vitro and that Tg with a functional deficiency of FasL can still effectively cure animals with established tumors (20).

Our hypothesis that TNF plays a critical role in the tumor regression mediated by PKO/GKO TCR is consistent with our data and the in vitro findings of others. However, a number of questions remain: although TNF-α is essential in this model, is it TNF-α and/or TNF-β (LT-α)? Because both molecules are bound by the soluble TNFRII:Fc, it is not possible to determine the answer from these experiments. Additionally, while Tg-mediated in vitro cytotoxicity is effectively blocked by soluble TNFRII:Fc, the D5 tumor is resistant to cytotoxicity by rTNF-α (H. Winter, unpublished observation). It is unknown whether this difference is related to the relative effectiveness of membrane bound vs soluble TNF-α or whether LT-α (55) is the mediator. Second, is it the TNF produced by the transferred Tg and/or TNF from the host that is essential for T cell-mediated regression of pulmonary metastases? The answer to these questions requires the generation of mice deficient in perforin, IFN-γ, and TNF-α or LT-α. Current efforts are directed toward addressing these questions.

Our current study clearly identifies TNF as one of three primary effector mechanisms, together with perforin and IFN-γ, for T cell-mediated tumor regression. Although these results do not exclude participation of additional effector molecules, in the absence of perforin, IFN-γ, and TNF-α, they are insufficient to mediate effective tumor regression in our model. The identification of these three central effector mechanisms provides an important characterization of T cells with therapeutic capacity and has repercussions for immunological monitoring and development of surrogate endpoints for patients on cancer vaccine trials.

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