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Cutting Edge: IL-10-Producing CD4\(^+\) T Cells Mediate Tumor Rejection\(^1\)

Benjamin M. Segal,\(^2\,*\)†‡ Deborah D. Glass,§ and Ethan M. Shevach§

IL-10 has potent immunosuppressive properties, and IL-10-producing CD4\(^+\) Tr1 cells have been characterized as regulators of Th1-mediated immunity. In this study, using a s.c. model of glioma cell growth in mice, we demonstrate that CD4\(^+\), but not CD8\(^+\), T cells play a critical role in tumor rejection following vaccination with irradiated glioma cells. Surprisingly, glioma-specific CD4\(^+\) T cells produce IL-10 but neither IL-4 nor IFN-\(\gamma\), and glioma rejection is compromised in IL-10-/- hosts. Hence, our findings demonstrate that IL-10-producing CD4\(^+\) T cells can manifest antitumor functions and suggest that IL-10 may have proinflammatory effects in disease states. *The Journal of Immunology*, 2002, 168: 1–4.

Traditionally, CD8\(^+\) CTLs and CD4\(^+\) Th1 cells are viewed as the primary mediators of tumor-specific immune responses. According to the current paradigm, CD8\(^+\) T cells act as the final effectors of tumor destruction, directly lysing cancer cells that bear tumor-associated Ag/MHC class I complexes via Fas- or perforin-mediated pathways. CD4\(^+\) Th1 cells, in contrast, are depicted as “helpers” that facilitate CD8\(^+\) T cell activation by secreting cytokines, such as IFN-\(\gamma\) and IL-2 (the “bystander” effect), or by licensing APCs through the CD40R (1, 2). Consequently, experimental tumor vaccines are often directed against MHC class I-restricted epitopes and incorporate Th1-promoting adjuvants (3, 4).

However, there is a growing recognition that CD4\(^+\) T cells may play alternative roles in tumor immunity independent of CD8\(^+\) CTL responses (5). In some instances these antitumor CD4\(^+\) T cells do not fall within the Th1 subset but paradoxically produce cytokines that can act as suppressors of Th1-mediated inflammation. For example, tumor-specific CD4\(^+\) Th2 cells act in collaboration with more traditional Th1 effectors to reject experimental B16 melanomas in vaccinated C57BL/6 mice (6). The development of effective immunotherapies will be facilitated by an understanding of the entire range of T cell phenotypes that can exert tumoricidal effects and could potentially be harnessed for therapeutic purposes.

Gliomas are the most common and most lethal primary brain tumors of adults (7). Immunotherapy offers an attractive experimental approach against these malignancies because it has the potential to selectively target migrating glioma cells while sparing adjacent healthy brain tissue. In this study, using a s.c. model of glioma cell growth in mice, we demonstrate that CD4\(^+\), but not CD8\(^+\), T cells play a critical role in tumor rejection following vaccination with irradiated tumor cells. Surprisingly, glioma-specific CD4\(^+\) T cells produce IL-10, but neither IL-4 nor IFN-\(\gamma\), and glioma rejection is compromised in IL-10-/- hosts. Hence, the types of antitumor CD4\(^+\) T cells should be expanded to include IL-10 producers with a cytokine profile reminiscent of the Tr1 subset (8).

Materials and Methods
Mice
C57BL/6 wild-type mice were obtained from either the National Cancer Institute (Fredrick, MD) or The Jackson Laboratory (Bar Harbor, ME). C57BL/6 mice genetically deficient in either IFN-\(\gamma\), IL-4, IL-12 p40, IL-10, MHC class II, or \(\beta\)-microglobulin were obtained from The Jackson Laboratory. All mice were housed under specific pathogen-free conditions.

**Glioma cell lines**
Cryopreserved GL261 tumor fragments were obtained from the National Cancer Institute Tumor Repository (Fredrick, MD) and maintained in monolayer cultures in RPMI 1640 with 10% FCS and standard supplements. CT-2A cells were obtained from Dr. T. N. Seyfried (Boston College, Boston, MA) and grown in DMEM with 10% FCS and standard supplements. Both cells lines were found to be negative for the presence of indigenous murine viruses (standard panel of 12) by the Mouse Antibody Production test (Charles River Laboratories, Wilmington, MA). Glioma cells were harvested from culture by treatment with trypsin, washed three times, counted by trypan blue exclusion, and resuspended in PBS before s.c. or i.p. inoculation.

** Priming and challenge of mice with glial tumor cells**
Mice were injected i.p. with three weekly doses of 1 \(\times\) 10\(^6\) irradiated GL261 cells (15,000 rad) in 0.1 ml of PBS. Control mice were injected with vehicle alone according to the same schedule. One week following the final inoculum all mice were anesthetized with Avertin, shaved across the lower back, and challenged s.c. with 4 \(\times\) 10\(^6\) live GL261 cells in 0.1 ml of PBS. In certain experiments, mice were injected i.p. with rat anti-mouse IL-10 (1 mg each of mAbs SXC-1 and SXC-2 per injection; Ref. 9) or control rat IgG (2 mg; Sigma-Aldrich, St. Louis, MO) on days 0, 3, 6, and 9 post challenge. Tumor size was determined as the product of length and width measured by a blinded examiner using calipers.

**Cytokine quantification**
Supernatants of splenocyte cultures were collected at 24-h intervals and subjected to a standard sandwich ELISA technique using noncompeting
pairs of cytokine-specific mAbs as previously described (10). The lower limit of detection of each assay was 30 pg/ml or less.

RT-PCR

Total RNA was isolated from tumor biopsy specimens using RNAzol RNA isolation solvent (Tel-Test, Friendswood, TX) and transcribed into cDNA using reverse transcriptase. RT-PCR was performed with primers specific for cytokines, β-actin, and the CD3ε chain (BD PharMingen, San Diego, CA).

Results

Mice vaccinated with irradiated GL261 cells are protected from tumor growth

We successfully stimulated protective immunity against the murine glioma, GL261, by priming syngeneic C57BL/6 mice with three i.p. injections of irradiated tumor cells in PBS. The vaccine was similarly effective whether GL261 cells were obtained from cell lines propagated in vitro (Fig. 1A) or from s.c. tumors serially passaged in nu/nu mice (data not shown). One hundred percent of GL261-vaccinated mice rejected tumors within 1 wk of challenge. In contrast, control mice inoculated with either vehicle alone or with irradiated cells from an independently derived C57BL/6 glial tumor cell line (CT-2A) experienced progressive GL261 glioma growth up to the time of sacrifice. Furthermore, mice primed with irradiated GL261 cells were not protected against challenge with live syngeneic B16 melanoma cells or CT-2A cells (data not shown). Collectively these results suggest that vaccination with GL261 cells generates an immune response specific for an Ag expressed by the GL261 tumor, and not an Ag universally expressed on gliomas or transformed cells.

Accelerated rejection of tumors in vaccinated mice is CD4+, but not CD8+, T cell dependent

Flow cytometric analysis of the cells contained in GL261 gliomas undergoing rejection revealed infiltration by both CD8+ and CD4+ T cells, as well as CD45+MHC class II+ cells that could serve as APCs (our unpublished observations). We next assessed the relative contributions of CD4+/CD8+ cell subsets to the antitumor response. C57BL/6 wild-type, MHC class II-deficient, and MHC class I (β2-microglobulin)-deficient mice were primed with irradiated GL261 cells or vehicle alone and subsequently challenged with live GL261 cells. In contrast to their wild-type counterparts, none of the MHC class II-deficient mice mounted a protective anti-glioma response, suggesting that tumor-specific CD4+ T cells are required for accelerated glioma rejection (Fig. 1B). In fact, tumor growth rates in GL261-primed as well as mock-primed MHC class II-deficient mice were comparable to those observed in immunodeficient nu/nu and RAG2−/− mice (our unpublished observations). In contrast, 100% of vaccinated β2-microglobulin−/− mice successfully rejected their tumors during a 25-day observation period. Therefore, we concluded that glioma rejection can proceed in the absence of CD8+ T cells. Consistent with the results of these in vivo studies, CD8+ T cells purified from the spleens of GL261-vaccinated immunocompetent mice failed to lyse glioma targets, as opposed to allogeneic lymphoblasts, in 51Cr release assays (data not shown).

GL261-primed CD4+ T cells selectively produce the cytokine IL-10 upon challenge with glioma cells ex vivo

To investigate the mechanism underlying the anti-glioma effects of CD4+ T cells, we harvested spleens from vaccinated mice and measured cytokine production in response to tumor challenge ex vivo. Surprisingly, whole splenocytes as well as highly purified CD4+ T cells cocultured with fresh APCs failed to produce cytokines that have been shown to have antitumor effects in other experimental systems, including IFN-γ, IL-4, and TNF-α (data not shown). In contrast, CD4+ T cells isolated from the spleens of both primed wild-type or β2-microglobulin−/− mice produced large quantities of IL-10 upon stimulation with mitomycin C-treated GL261 cells (Fig. 2A). We did not detect IL-10 in supernatants from GL261 cells cultured either alone or in combination with splenocytes from primed MHC class II-deficient mice (data not shown).

IL-10 mRNA is up-regulated in tumor beds of vaccinated mice during remission

We next questioned whether IL-10 is expressed within gliomas undergoing rejection in vaccinated hosts. Tumors were removed from GL261- or mock-primed C57BL/6 mice on day 11 following challenge and RNA was isolated for RT-PCR with primers specific for a panel of cytokines and the housekeeping gene, β-actin. CD3ε chain mRNA was also measured in an attempt to assess the extent of T cell infiltration. At the time of harvest, gliomas were beginning to shrink in the vaccinated cohort, whereas they were continuing to grow in the control group. Messenger RNA encoding IL-10 was up-regulated in the gliomas from vaccinated mice during the period of accelerated rejection (Fig. 2B). This was true despite low levels of CD3ε mRNA, suggesting that few infiltrating T cells were responsible for significant intratumor IL-10 expression.

It is likely that the IL-10 is expressed by infiltrating CD4+ T cells, because we detected it in the tumor beds of primed wild-type and β2-microglobulin−/−, but not MHC class II−/−, C57BL/6 mice (Fig. 2C). IL-10 mRNA was also not detected in GL261 cells
that were freshly harvested from in vitro lines or from tumors grown in immunodeficient mice.

**IL-10 plays a physiological role in glioma rejection**

To definitively determine which cytokines actively participate in glioma rejection in vaccinated mice, we primed and challenged C57BL/6 mice deficient in either IL-12, IL-4, IFN-γ, or IL-10 with GL261 cells. By day 10 post challenge, tumors were significantly smaller on vaccinated IL-10−/− mice than the tumors borne by their unvaccinated counterparts \((p < 0.005, \text{ Student's } t \text{ test})\). In fact, vaccinated IL-12−/−, IFN-γ−/−, and IL-4−/− mice rejected GL261 tumors at a comparable rate to identically treated immunocompetent animals (Table I and data not shown). By contrast, glioma rejection was significantly delayed in primed IL-10−/− mice as well as in wild-type mice treated with neutralizing Abs against IL-10.

**Discussion**

IL-10 has been widely characterized as an immunosuppressive cytokine. Its major effects include the down-regulation of costimulatory and MHC molecule expression as well as proinflammatory cytokine production by APCs (11, 12). In addition, IL-10 may exert direct inhibitory effects on T cells (13). Hence, it is not surprising that the IL-10−/− mouse spontaneously develops inflammatory colitis, is more susceptible than IL-10-sufficient mice to experimental Th1-mediated autoimmune diseases and mounts overexuberant, self-destructive Th1 immune responses against pathogens (10, 14, 15). Therefore, one might predict that IL-10 would also play a role in the suppression of antitumor immunity. Indeed, transgenic mice expressing IL-10 under the control of the IL-2 promoter are compromised in their ability to reject experimental lung carcinomas, while IL-10-deficient mice mount more potent Th1 and CTL responses against bladder tumors and melanomas (16, 17). Furthermore, murine plasmacytomas treated with antisense IL-10 are more immunogenic than the parental cell lines (18). IL-10 has been detected in biopsy specimens of human carcinomas and gliomas in association with more aggressive tumor growth and shorter survival times (19, 20).

However, IL-10 is a cytokine with pleiotropic effects and has been shown in some instances to paradoxically augment tumor growth and shorter survival times. Therefore, one might predict that IL-10 would also play a role in the suppression of antitumor immunity. Indeed, transgenic mice expressing IL-10 under the control of the IL-2 promoter are compromised in their ability to reject experimental lung carcinomas, while IL-10-deficient mice mount more potent Th1 and CTL responses against bladder tumors and melanomas (16, 17). Furthermore, murine plasmacytomas treated with antisense IL-10 are more immunogenic than the parental cell lines (18). IL-10 has been detected in biopsy specimens of human carcinomas and gliomas in association with more aggressive tumor growth and shorter survival times (19, 20).

**Table I.** The role of cytokines in mediating tumor rejection in GL261-vaccinated mice

<table>
<thead>
<tr>
<th>Groups</th>
<th>Mean Tumor Size (mm²)</th>
<th>Tumor-Free Survivors</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT control</td>
<td>52 ± 4</td>
<td>0/10 (0%)</td>
</tr>
<tr>
<td>WT primed</td>
<td>8 ± 4b</td>
<td>4/9 (44%)</td>
</tr>
<tr>
<td>IL-12−/− control</td>
<td>59 ± 5</td>
<td>0/10 (0%)</td>
</tr>
<tr>
<td>IL-12−/− primed</td>
<td>7 ± 3b</td>
<td>4/8 (50%)</td>
</tr>
<tr>
<td>IFN-γ−/− control</td>
<td>48 ± 6</td>
<td>0/11 (0%)</td>
</tr>
<tr>
<td>IFN-γ−/− primed</td>
<td>6 ± 3b</td>
<td>5/8 (63%)</td>
</tr>
<tr>
<td>IL-4−/− control</td>
<td>34 ± 5</td>
<td>0/8 (0%)</td>
</tr>
<tr>
<td>IL-4−/− primed</td>
<td>6 ± 2b</td>
<td>4/8 (50%)</td>
</tr>
<tr>
<td>IL-10−/− control</td>
<td>55 ± 7</td>
<td>0/6 (0%)</td>
</tr>
<tr>
<td>IL-10−/− primed</td>
<td>38 ± 2</td>
<td>0/6 (0%)</td>
</tr>
<tr>
<td>WT primed/rat IgG</td>
<td>10 ± 5b</td>
<td>2/4 (50%)</td>
</tr>
<tr>
<td>WT primed/oIL-10</td>
<td>32 ± 4</td>
<td>0/5 (0%)</td>
</tr>
</tbody>
</table>

\(^{a}\) C57BL/6 wild-type (WT) and cytokine −/− mice received three i.p. injections of irradiated GL261 cells (primed) or PBS alone (control) prior to s.c. challenge with live GL261 cells \((4 × 10^6 \text{ cells}/\text{mouse})\). Results are presented as mean tumor size ± SE as well as the number of tumor-free survivors over the total number per group on day 10 post challenge.

\(^{b}\) Mean tumor size was significantly lower in primed compared to control mice \((p < 0.005, \text{ Student’s } t \text{ test})\).

\(^{c}\) Mice were injected with either rat anti-mouse IL-10 or control rat IgG on days 0, 3, 6, and 9 postchallenge as described in Materials and Methods.
immunity. For example, introduction of the IL-10 gene into several rodent tumor cell lines, including mammary and pulmonary adenocarcinomas, melanomas, and lymphomas, unexpectedly increased their immunogenicity and rendered them highly susceptible to rejection (21–24). Book et al. (25) reported that rat 9L gliomas transfected with IL-10 cDNA are heavily infiltrated with inflammatory cells and grow at a significantly slower rate than control gliomas (transfected with empty vector) following intracranial implantation. In addition, transgenic mice expressing IL-10 under the control of an MHC class II (as opposed to IL-2) promoter manifest enhanced resistance to the growth of a mastocytoma cell line that is tumorigenic in wild-type mice (26). Systemic IL-10 acts as an adjuvant when administered in conjunction with experimental tumor vaccines against melanomas, sarcomas, and colorectal and lung carcinomas in animals (21, 24, 27).

In all of the above instances the tumoricidal effects of IL-10 appeared to be T cell and/or NK cell dependent because they are abrogated in immunodeficient (T cell and/or NK cell depleted) mice. In fact, a number of recent studies demonstrate that IL-10 directly enhances NK cell, as well as CD8+ T cell, cytotoxicity in vitro. NK cells pretreated with IL-10, either alone or in combination with other cytokines such as IL-2, IL-18, and IL-12, are more efficient at lysing YAC-1 cells and tumor targets (28, 29). IL-10 might play a similar role in our model because GL261 gliomas are susceptible to lysis by activated NK cells ex vivo (data not shown). IL-10 may also exert indirect effects on tumor growth by down-regulating MHC expression on the tumor cells, modulating apoptotic pathways, stimulating the production of chemokines, and/or suppressing angiogenesis (23, 30–32).

Most of the previous reports demonstrating a contributory role of IL-10 in tumor rejection used highly artificial systems in which pharmacological doses of the cytokine were administered either systemically or locally via genetically engineered tumor cells. To our knowledge, this study represents the first time that IL-10-producing CD4+ T cells have been demonstrated to exert antitumor effects. Most importantly, the anti-glioma CD4+ T cells do not appear to be typical Th2 cells, because production of IL-4 could not be detected in the spleen or in tumor-infiltrating lymphocytes from vaccinated animals. Thus the IL-10-producing CD4+ T cells that we have described have certain properties in common with Th1 cells that have been generated in vivo in the presence of IL-10 and are generally characterized as regulatory cells that dampen inflammation and cooperation with IL-2.

References