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Accumulation of Immunosuppressive CD11b+ Myeloid Cells Correlates with the Failure to Prevent Tumor Growth in the Anterior Chamber of the Eye

Kyle C. McKenna and Judith A. Kapp

The purpose of these studies is to determine why an immunogenic tumor grows unchecked in the anterior chamber (a.c.) of the eye. The OVA-expressing EL4 tumor, E.G7-OVA, was injected into the a.c. or skin of immunocompetent and immunodeficient mice. Tumor growth and tumor-specific immune responses were monitored. Ocular tumor-infiltrating leukocytes were characterized phenotypically and functionally. Growth of E.G7-OVA was inhibited when limiting numbers of cells were injected in the skin but not in the a.c. of C57BL/6 mice, although both routes primed OVA-specific immune responses, which prevented the growth of a subsequent injection with E.G7-OVA in the skin or opposite eye. Tumor regression was OVA-specific because growth of the parental EL-4 tumor was not inhibited in primed mice. E.G7-OVA growth in the skin was not inhibited in immunodeficient Rag-/- or CD8 T cell-deficient mice, suggesting that CD8+ CTLs mediate tumor elimination. CD8+ T cell numbers were significantly increased in eyes of mice primed with E.G7-OVA, but few were detected in primary ocular tumors. Nevertheless, growth of E.G7-OVA was retarded in the a.c. of TCR-transgenic OT-I mice, and CD8+ T cell numbers were increased within eyes, suggesting that tumor-specific CD8+ CTLs migrated into and controlled primary ocular tumor growth. E.G7-OVA did not lose antigenicity or become immunosuppressive after 13 days of growth in the eye. However, CD11b+ cells accumulated in primary ocular tumors and contained potent immunosuppressive activity when assayed in vitro. Thus, CD11b+ cells that accumulate within the eye as tumors develop in the a.c. may contribute to immune evasion by primary ocular tumors by inhibiting CTLs within the eye. The Journal of Immunology, 2006, 177: 1599–1608.

Uveal melanoma is the most common primary malignant intraocular tumor in adults (1). These malignancies arise in pigmented ocular tissues including the iris, ciliary body, and choroid and metastasize hematogenously to the liver (2, 3). Metastatic disease of the liver is the leading cause of death in patients with uveal melanoma, and there is currently no effective treatment (2, 3).

It is now well documented that malignant uveal and cutaneous melanomas express common tumor-specific Ags, e.g., tyrosinase, GP-100, and MART, that are highly immunogenic (4). These Ags are presented on the melanoma cell surface as peptides associated with MHC class I molecules and target malignant cells for recognition by CD8+ CTL via the TCR. Patients with cutaneous melanomas that lose expression of MHC class I have a shorter life span, supporting the idea that CD8+ CTL control tumor growth (5). However, cutaneous melanoma patients vaccinated with melanoma-specific Ags in one study showed no evidence of tumor regression despite increased melanoma-specific CD8+ CTL precursors (6). Thus, melanoma-specific CD8+ T cells were not able to promote melanoma regression when they were induced in patients after tumors had been identified.

In contrast with cutaneous melanoma, uveal melanomas that express MHC class I molecules have been associated with a more aggressive phenotype, which correlates with a poorer prognosis in comparison to uveal melanomas that have lost or decreased MHC class I expression (7). The association of a poor prognosis with MHC class I expression in uveal melanoma is believed to result from the loss of antitumor NK cell activity whose effector function is inhibited by MHC class I molecules (8), but it may also involve inhibition of CD8+ CTLs, which recognize tumor Ags presented by MHC class I on uveal melanomas. Understanding the mechanisms of CTL inhibition to ocular tumors may lead to therapeutic approaches to overcome T cell inhibition and promote ocular tumor elimination.

Ocular immune privilege is exemplified by the observation that immunogenic tumors are eliminated by tumor-specific CD8+ CTL responses when injected in the skin but not in the anterior chamber (a.c.) of the eye (9). Several potential explanations have been put forth to explain the evasion of CD8+ CTL by tumors developing in the eye, including the following: 1) immune ignorance due to sequestration of ocular Ags; 2) systemic tolerance in tumor-specific CTLs; 3) inhibited migration of CTL into the eye; 4) inhibition of CTL activity within the eye; and 5) loss of Ag expression by the tumor. Immune ignorance due to sequestration of ocular Ags was postulated because of the blood-aqueous barrier and the
absence of demonstrable afferent lymphatics draining the a.c. (10). However, many observations indicate that ocular Ags normally escape the eye. For example, a.c. administration of soluble OVA induces T cell expansion in the draining submandibular lymph nodes and spleen (11–13), leading to the induction of tolerance in CD4\(^+\) (12, 14) and CD8\(^+\) (13, 15) T cells. In addition, tumor-specific TCR transgenic CD8\(^+\) T cells proliferate in the submandibular lymph nodes when transferred into mice bearing tumors in the a.c. (16). Moreover, P815 tumor cells injected into the a.c. grow progressively but also induce tumor-specific CD8\(^+\) CTL responses (17–19) that eliminate a subsequent injection with P815 in the opposite eye, a phenomenon termed intracamerally induced, concomitant immunity (20). Thus, sequestration of tumor Ags in the a.c. leads to induction of systemic tumor-specific CTL tolerance, or the concomitant immunity (20).

To determine why an immunogenic tumor grows unchecked in the a.c. of the eye in primary ocular tumor development, we used a model of immune evasion using E.G7-OVA, an EL-4 thymoma transduced to express OVA, a surrogate tumor Ag (21). E.G7-OVA cells can be tracked, enumerated, and isolated in vivo by the a.c. induction of systemic tumor-specific CTL tolerance, or the concomitant immunity (20).

Materials and Methods

Experimental animals

Male and female C57BL/6J (B6; H-2\(^b\), Thy1.2\(^b\)) mice were purchased from the Jackson Laboratory, C57BL/6J-P2R2-B2Mtm1Unc/J (B2M\(^b\), CD4\(^b\), and CD8\(^b\), Thy1.2\(^b\)) mice were purchased from The Jackson Laboratory, and C57BL/6-TgN (TUG1-1) (22), also referred to as OT-I, were a gift from Dr. M. Bevan (University of Washington, Seattle, WA). B6;P2R2-B2Mtm1Unc/J, CD4\(^b\), and OT-I mice were bred and maintained in the animal facilities at Emory University. B2M\(^b\) and CD4\(^b\) are on a B6 background, and OT-I mice have been backcrossed for >10 generations to B6 mice and are considered congenic with B6 mice. All of the procedures on animals were approved by the Institutional Animal Care and Use Committee at Emory University, conducted according to the principals in the guidelines of the Committee on Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, National Research Council, Washington, DC) and in adherence to the provisions of the Association for Research in Vision and Ophthalmology (ARVO) Statement for the use of Animals in Ophthalmic and Vision Research.

Tumor cell lines and Ag

EL-4 (H-2\(^b\), Thy 1.2\(^b\)) and EL-4 transduced to express OVA (E.G7-OVA) (21), provided by Dr. M. Bevan (University of Washington, Seattle, WA), or human insulin (EL-4 insulin) (27) were grown in standard growth medium (SGM) (RPMI 1640 medium supplemented with 10% FBS, 2 mM L-glutamine, 1 mM sodium pyruvate, 50 \(\mu\)M 2-ME, gentamicin, penicillin, and streptomycin) at 37°C in a 5% CO\(_2\) atmosphere. All cell lines were maintained free of mycoplasma. E.G7-OVA were continuously cultured in 1.0 mg/ml 2-ME to maintain the expression of the transgenic OVA\(^b\) gene. OVA peptide 257–264 (SIINFEKL) was a gift from Dr. B. Evavold (Emory University, Atlanta, GA) and was dissolved in PBS at a concentration of 3 mM.

Tumor cell and Ag administration

For all injections, mice were anesthetized with of a saline solution containing 1–2 mg of ketamine (Sigma-Aldrich) and 0.2–0.4 mg of xylazine (Bayer) delivered i.m. in the thigh or i.p. For intradermal (i.d.) injections, tumors (10\(^4\)–10\(^5\) cells) were injected into the skin of the back in 0.2 ml of PBS. For a.c. injections, the eye was anesthetized by topical drops of proparacaine HCl (Alcon Laboratories). The eye was then probed using forceps, and the cornea was punctured by insertion of a 30-gauge needle directly above the pupil and almost parallel to the iris. Released aqueous humor was removed with an ophthalmic sponge. A 33-gauge beveled needle fitted on a Hamilton 700 series microsyringe (Hamilton) was then inserted briefly into the incision site to wick the remaining aqueous humor from the a.c. and then the a.c. was filled by injection of 1.5 \(\mu\)l of air. Tumor cells (10\(^2\)–2.0 \(\times\) 10\(^4\)) contained in 2 \(\mu\)l of PBS were then injected into the a.c. by pressing a 33-gauge blunt needle fitted on a Hamilton 700 series microsyringe against the incision. The air bubble seals the corneal puncture and prevents leaking. In most experiments, only one eye was treated, and the experiment was terminated before tumor growth ruptured the globe. In experiments where both eyes were treated, one eye was enumerated (see below) before rupture of the globe.

Enucleation

Tumor-bearing eyes were removed to minimize stress from progressive growth of ocular tumors that, during the course of the experiment, would rupture the globe. Mice were anesthetized as described above for a.c. injections. The eye was probed using forceps, and then the conjunctiva, muscles, and optic nerve were cut with microscissors to remove the eye from the orbit. Bleeding, which was minimal, was stopped by applying direct pressure with a surgical sponge. Mice were monitored postoperatively for signs of pain and distress, such as wincing, hunched posture, or ruffled fur. In multiple experiments, no evidence of distress or infection was observed in any mice, indicating that the procedure was well-tolerated.

Collagenase digestion of eyes and flow cytometric enumeration of tumors and tumor-infiltrating leukocytes (TIL)

Mice were euthanized by cervical dislocation, and their eyes were removed, washed in 2 ml of PBS on ice, and then incubated for 1–2 h at 37°C in a 5% CO\(_2\) atmosphere in 2 ml of RPMI 1640 medium supplemented with 58.5 U/ml Collagenase IV (Sigma-Aldrich) and 1% FBS. Eyes were then pressed between frosted glass slides to generate single-cell suspensions. The cell suspension was filtered through a nylon mesh screen to remove debris, washed with PBS, and resuspended in 0.4 ml of FACs buffer (PBS + 1% FBS). Eye suspensions (0.2 ml) were added to individual wells of a 96-well plate for staining. Cells were washed with FACs buffer and incubated for 15 min at 4°C. Fc receptors were blocked by incubation with purified CD16/32 monoclonal antibodies (PE-Cy7; Biolegend) delivered i.m. in the thigh or i.p. For intradermal (i.d.) injections, tumors (10\(^4\)–10\(^5\) cells) were injected into the skin of the back in 0.2 ml of PBS. For a.c. injections, the eye was anesthetized by topical drops of proparacaine HCl (Alcon Laboratories). The eye was then probed using forceps, and the cornea was punctured by insertion of a 30-gauge needle directly above the pupil and almost parallel to the iris. Released aqueous humor was removed with an ophthalmic sponge. A 33-gauge beveled needle fitted on a Hamilton 700 series microsyringe (Hamilton) was then inserted briefly into the incision site to wick the remaining aqueous humor from the a.c. and then the a.c. was filled by injection of 1.5 \(\mu\)l of air. Tumor cells (10\(^2\)–2.0 \(\times\) 10\(^4\)) contained in 2 \(\mu\)l of PBS were then injected into the a.c. by pressing a 33-gauge blunt needle fitted on a Hamilton 700 series microsyringe against the incision. The air bubble seals the corneal puncture and prevents leaking. In most experiments, only one eye was treated, and the experiment was terminated before tumor growth ruptured the globe. In experiments where both eyes were treated, one eye was enumerated (see below) before rupture of the globe.

Ab-mediated CD8\(^+\) T cell depletion

Mice were injected with 0.4 mg of anti-CD8 Ab (clone 2.43) (provided by Dr. A. Lukacher, Emory University) i.p. given in daily doses of 0.2 mg, 0.1 mg, and 0.1 mg in a 1 mg/ml solution in sterile PBS before tumor challenge. After tumor challenge, mice were given 0.1 mg of anti-CD8 Ab.
developed a tumor when 10^4 tumor cells were injected into the skin (Fig. 1 and Table I). The failure of E.G7-OVA to form tumors in our hands when injected with ≥10^6 E.G7-OVA cells in the skin, which grew progressively over time, whereas only a rare mouse developed a tumor when 10^5 tumor cells were injected into the skin (Fig. 1 and Table I). The failure of E.G7-OVA to form tumors at low doses was largely the result of tumor elimination by the adaptive immune response because 44% of immune-deficient Rag^−/− mice developed skin tumors when injected with 10^4 E.G7-OVA cells, a difference that was statistically significant (p = 0.04) compared with similarly challenged B6 mice.

To determine whether T cells were responsible for tumor elimination in the skin, CD8 T cell-deficient (B2M^−/−) and CD4 T cell-deficient (CD4^−/−) mice were injected with 10^4 E.G7-OVA cells. B6 and Rag^−/− mice that received a similar tumor challenge were included as controls. The majority of Rag^−/− mice but only a few immune-competent B6 mice developed tumors, and this difference was statistically significant (Table I). The number of CD4^−/− mice that developed tumors was comparable to B6 mice. In contrast, the number of B2M^−/− mice that developed tumors was comparable to Rag^−/− mice and significantly different from B6 mice. These data suggest that CD8^+ T cells are responsible for tumor elimination in the skin and that CD8 T cell induction and expression of effector function is CD4 independent. In addition, 43% of B6 mice in which CD8^+ T cells were depleted by Ab developed skin tumors when injected with 10^4 E.G7-OVA cells, whereas similarly challenged B6 mice given control Ab did not (Table I), further substantiating the contribution of CD8 T cells to tumor regression.

The specificity of tumor immunity in B6 mice was determined in a CTL assay using E.G7-OVA and EL-4-insulin targets. E.G7-OVA was created by transfecting EL-4 with a vector in which OVA and neomycin phosphotransferase (neo) expression, a selectable marker, were expressed under control of ubiquitous promoters (21). A similar expression vector was used to express human insulin and neo genes in EL-4 insulin cells (27). Hence, EL-4 insulin target cells control for CTL activity directed against neo or shared tumor Ags expressed by E.G7-OVA and EL-4. In naive untreated mice, splenic CTL responses were at background levels (Fig. 2). Splenic CTL responses in mice injected in the skin with 10^6 E.G7-OVA cells preferentially lysed E.G7-OVA but not EL-4 insulin targets (data not shown), which likely reflects a limited CTL expansion in the spleen and/or trafficking of the CTL to the tumor site. A larger tumor dose most likely induces sufficient expansion of tumor-specific CTL precursors to reach a detectable level.

### Statistical analysis

Differences in the number of mice developing tumors in the skin were compared by a Fisher exact test. Differences in indicated cell numbers in collagenase-digested eyes or percentage of control in CTL assays were compared by a Student’s t test with equal or unequal variance as determined by a F test, and p values <0.05 were considered statistically significant.

### Results

#### Immunological control of the growth of E.G7-OVA in the skin of B6 mice

Previous studies have shown that E.G7-OVA forms tumors when 10^3 cells are injected in the skin (28) or 4.0 × 10^6 cells are injected in the peritoneal cavity (29) of syngeneic B6 mice. Consistent with these observations, B6 mice uniformly developed skin tumors in our hands when injected with ≥10^6 E.G7-OVA cells in the skin, which grew progressively over time, whereas only a rare mouse developed a tumor when 10^5 tumor cells were injected into the skin (Fig. 1 and Table I). The failure of E.G7-OVA to form tumors
be distinguished from infiltrating leukocytes. We have used an assay using immunofluorescent Abs and flow cytometry of collagenase-digested eyes to enumerate tumor cells and infiltrating leukocytes, simultaneously. Representative results of an experiment (Fig. 3), in which E.G7-OVA cells were added to untreated eye suspensions from B6.PL mice, validate this approach. E.G7-OVA can be easily distinguished from ocular cells because the majority of the E.G7-OVA (>90%) display cell surface expression of CD45 (data not shown), whereas only a minority of cells (<1%) in normal untreated eyes of B6.PL mice are CD45+ (Fig. 3A). The small percentage of CD45+ bone marrow-derived cells in untreated eyes are most likely contained within blood vessels and/or parenchymal tissues of the eye. Although these leukocytes are CD45+ they can be distinguished from tumors because E.G7-OVA expresses the Thy1.2 allotype, whereas no cells in Thy 1.1 congenic B6.PL mice express Thy 1.2 (Fig. 3B). This experiment also demonstrates that E.G7-OVA do not express CD8, which further distinguishes tumors from infiltrating CTL.

To enumerate tumors or infiltrating leukocytes in collagenase-digested eyes, the percentage of CD45+ cells that were tumors or respective TIL was multiplied by the absolute number of CD45+ cells within samples, which was determined by flow cytometric cell counts. To validate this assay, 5-fold dilutions, beginning at 10⁶ E.G7-OVA cells, were added to collagenase-digested eyes, and the number of E.G7-OVA cells (CD45+, Thy1.2+, CD8 negative) was determined. The number of E.G7-OVA cells significantly correlated (r² = 0.99; slope = 1) with the number of E.G7-OVA cells added to collagenase-digested eyes (Fig. 3C). When 5.0 × 10⁴ total events were collected, the threshold of E.G7-OVA detection was 100 cells.

Using this flow cytometry assay, tumor cell numbers were determined at several time points following injection of various numbers of E.G7-OVA into the a.c. of B6.PL or B6.Rag⁻/⁻ mice (Fig. 4A). E.G7-OVA grew progressively after injection with 10⁴ tumor cells in all B6.PL mice and in immunodeficient B6.Rag⁻/⁻ mice. Tumor growth was confined to the anterior and posterior compartments of the a.c. (compare Fig. 4, B and C). Decreasing the number of cells injected into the eye by 10- to 100-fold delayed the kinetics of tumor cell growth and the length of time for tumors to completely fill the a.c., as has been previously reported (31). However, all of the mice injected with 10² E.G7-OVA developed ocular tumors. These data indicate that tumor-specific CD8+ CTL did not eliminate doses ≥10⁴ tumor cells injected in the a.c. of B6 mice, whereas doses as high as 10⁴ were rejected when injected into the skin.

**Induction of systemic OVA-specific tumor immunity by E.G7-OVA**

One potential explanation for the growth of E.G7-OVA in the eye but not the skin is that tumor Ags are sequestered in the eye, and...
hence the immune system is ignorant of ocular tumors. However, previous studies have shown that mice injected with P815 cells in the a.c. generated tumor-specific CTL responses (19) and were immune to a secondary tumor challenge with P815 cells in the skin or the opposite eye (20). To determine whether a.c. administration of E.G7-OVA induced tumor immunity, B6 mice were injected with E.G7-OVA (5.0 \times 10^5 cells) in the a.c. of eye. Eleven days later, the tumor containing eyes were removed, mice were challenged with 5.0 \times 10^4 E.G7-OVA or EL-4 cells in the skin, and tumor growth was monitored. All of the control mice, which were not exposed to E.G7-OVA in the a.c., developed tumors when challenged with E.G7-OVA or EL-4 in the skin (Table II). By contrast, \( >90\% \) of the mice previously injected with E.G7-OVA in the a.c. rejected a subsequent challenge with E.G7-OVA. Rejection was OVA specific because E.G7-OVA regressed, but the parental EL-4 cells grew into tumors in most of the mice. E.G7-OVA and EL-4 grew with similar kinetics in the skin of control mice (Fig. 5). However, EL-4 tumors developing in the skin of mice previously primed with E.G7-OVA a.c. were slightly smaller than EL-4 tumors in control mice (Fig. 5). This difference was statistically significant at day 14 (\( p = 0.05 \)) and day 20 (\( p = 0.03 \)) and may indicate that a.c. administration of E.G7-OVA also induced weak immune responses to tumor Ags, other than OVA, that are shared by E.G7-OVA and EL-4. However, in another experiment, EL-4 tumors in E.G7-OVA-primed mice were not significantly different in size from EL-4 tumors in control mice (data not shown). Only one mouse that was given E.G7-OVA via the a.c. developed a tumor after challenge with E.G7-OVA in the skin, and this tumor was markedly smaller than E.G7-OVA tumors in control mice (Fig. 5). These data indicate that the administration of E.G7-OVA in the a.c. induced a systemic tumor-specific immune response, demonstrating that tumor Ags were not sequestered in the eye in this model.

**CD11b\(^{+} \)** myeloid cells accumulate in primary ocular tumors

Another potential explanation for immune evasion of E.G7-OVA tumors developing in the a.c. is that tumor-specific CD8\(^{+} \) CTLs may fail to migrate into the eye. To address this possibility, CD45\(^{+} \) leukocytes infiltrating primary ocular tumors were analyzed for CD8, CD11b, and Gr-1 expression at multiple time points after a.c. administration of E.G7-OVA. Ten days after injection of 10^4 E.G7-OVA cells into the a.c., 62 \( \pm \) 6% of live CD45\(^{+} \) cells were Thy1.2\(^{+} \). E.G7-OVA tumors, \(<1\% \) were CD8\(^{+} \), and 38 \( \pm \) 6% were Thy 1.2 negative and CD8 negative (Fig. 6A), suggesting that CD8\(^{+} \) T cells did not accumulate in the primary ocular tumor. Alternatively, CD8 T cells may have migrated into the eye, but the CD8 molecules down modulated on CTL that were activated in the eye. However, 38 \( \pm \) 8% of CD45\(^{+} \) cells within the eye were CD11b\(^{+} \) (Fig. 6B), which accounts for both CD8-negative and Thy 1.2-negative populations. Roughly half of the CD45\(^{+} \) cells that expressed CD11b coexpressed Gr-1, a marker expressed by monocytes (32), neutrophils (32), and MSC (33). The number of CD8\(^{+} \) T cells in tumor-containing eyes on day 10 was increased 3-fold from untreated control eyes, but this difference was not statistically significant (\( p = 0.14 \)), whereas CD11b\(^{+} \) cells were significantly increased at all time points tested and accumulated to almost equal numbers with tumor cells as the tumors grew progressively (Fig. 6C). By day 10, CD11b\(^{+} \) cells that did not express Gr-1 increased 9-fold, and CD11b\(^{+} \) cells that expressed Gr-1 increased 145-fold.

**Tumor-specific CD8\(^{+} \) CTL can migrate into the eye**

The low frequency of CD8\(^{+} \) T cells in primary ocular tumors could result from the failure of large numbers of CD8\(^{+} \) CTL to migrate into the eye or the death of CD8\(^{+} \) CTL within the eye. To determine whether tumor-specific CTL could migrate into the eye, we tested whether tumor-specific immunity induced by administration of E.G7-OVA via the a.c. could cause rejection of a subsequent challenge with E.G7-OVA in the opposite eye. B6.PL mice were given PBS or injected with E.G7-OVA (2.0 \( \times \) 10^4 cells) in the a.c. of the right eye. Seven days later, treated eyes were removed, and mice were challenged with E.G7-OVA in the a.c. of the left eye. E.G7-OVA tumors and CD8\(^{+} \) cells were enumerated in the left eyes 4 days after challenge, and representative flow cytometry plots are shown in Fig. 7. The percentage of Thy 1.2\(^{+} \) E.G7-OVA cells was significantly reduced in E.G7-OVA-primed mice compared with mice injected with PBS, indicating that tumor burden was significantly reduced in the opposite eye as the result of priming with E.G7-OVA via the a.c. In contrast, the percentage of CD8\(^{+} \) cells increased dramatically in eyes of mice previously injected with E.G7-OVA in the opposite eye compared with un.injected mice. Similar results were observed when the absolute number of cells in the eyes was determined in multiple mice (Table III). These data further substantiate the interpretation that a.c. administration of E.G7-OVA induced tumor-specific immune responses and demonstrate that tumor-specific CD8\(^{+} \) CTL can migrate into the eye, where they prevented the growth of the tumor presumably by lysing the E.G7-OVA.

The association of tumor rejection in the eye with increased CD8\(^{+} \) T cell numbers suggests that increasing the frequency of tumor-specific T cells may overcome regional inhibition of CTL.

### Table II. a.c. administration of E.G7-OVA induces OVA-specific immune responses

<table>
<thead>
<tr>
<th>Tumor</th>
<th>Mouse with Tumors/Total Mice</th>
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<tbody>
<tr>
<td></td>
<td>a.c. Primary challenge</td>
</tr>
<tr>
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<td>E.G7</td>
</tr>
<tr>
<td>E.G7</td>
<td>E.G7</td>
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<tr>
<td>None</td>
<td>EL-4</td>
</tr>
<tr>
<td>E.G7</td>
<td>EL-4</td>
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\(^{a}\) Skin tumors were defined by a mass > 25 mm\(^2\) and were scored 20–22 days after the injection of tumors (5.0 \( \times \) 10^5 cells) in the skin. p values were calculated by a Fisher exact test comparing indicated groups of mice with unprimed control mice given the same tumor in the skin.
which 5.0
the a.c. of OT-I mice because injection of E.G7-OVA, gated CD45 Thy1.2, anti-CD8, anti-CD11b, and anti-Gr-1 Abs. Ten days after the a.c. injection of E.G7-OVA, equivalent CD45^+ events in the eye were evaluated for expression of Thy1.2 (E.G7-OVA) and CD8 (A) and CD11b and Gr-1 (B). Plots shown are from one mouse, which is representative of three mice in each treatment group. Absolute numbers of tumors and indicated leukocyte populations within tumor-bearing eyes were determined at indicated time points (C). Equivalent sample volumes containing varying numbers of events (range, 5.0 × 10^3–37.3 × 10^5 events) were collected, with the exception of one day-3 mouse challenged with 10^4 E.G7-OVA cells in which 5.0 × 10^5 cells were collected. Collagenase-digested eye suspensions used to determine tumor numbers in mice injected with 10^4 E.G7-OVA via the a.c. in Fig. 4 were also used to determine the absolute number of indicated TIL in this figure. E.G7-OVA tumor numbers from Fig. 4 are shown for comparison. Each symbol represents the mean measurement ± 1 SD of indicated cell populations from three to seven individual mice. Day 0 values are from eight untreated eyes of B6.PL mice in which 2.0 × 10^5 events were collected for analysis.

FIGURE 6. CD11b^+ cells accumulate in the a.c. as tumors develop. B6.PL mice were injected with E.G7-OVA (10^6 cells) in the a.c. of the eye. Tumor growth and leukocyte infiltration were monitored by flow cytometric analysis of collagenase-digested eyes labeled with anti-CD45, anti-Thy1.2, anti-CD8, anti-CD11b, and anti-Gr-1 Abs. Ten days after the a.c. injection of E.G7-OVA, equivalent CD45^+ events in the eye were evaluated for the expression of Thy1.2 (E.G7-OVA) and CD8 (A) and CD11b and Gr-1 (B). Plots shown are from one mouse, which is representative of three mice in each treatment group. Absolute numbers of tumors and indicated leukocyte populations within tumor-bearing eyes were determined at indicated time points (C). Equivalent sample volumes containing varying numbers of events (range, 5.0 × 10^3–37.3 × 10^5 events) were collected, with the exception of one day-3 mouse challenged with 10^4 E.G7-OVA cells in which 5.0 × 10^5 cells were collected. Collagenase-digested eye suspensions used to determine tumor numbers in mice injected with 10^4 E.G7-OVA via the a.c. in Fig. 4 were also used to determine the absolute number of indicated TIL in this figure. E.G7-OVA tumor numbers from Fig. 4 are shown for comparison. Each symbol represents the mean measurement ± 1 SD of indicated cell populations from three to seven individual mice. Day 0 values are from eight untreated eyes of B6.PL mice in which 2.0 × 10^5 events were collected for analysis.

responses in the eye. To determine whether increasing the frequency of tumor-specific CD8^+ CTL would promote elimination of primary ocular tumors, E.G7-OVA (10^6 cells) were injected into the a.c. of OT-1 mice because >90% of CD8^+ T cells in OT-1 mice express a TCR that recognizes OVA peptide 257–264 presented by MHC class I H-2 K^b (13). Furthermore, the E.G7-OVA used in this experiment was isolated from collagenase-digested eyes of B6.PL mice 10–13 days after a.c. administration of tumors to simultaneously evaluate whether any changes in the antigenicity of E.G7-OVA were induced by growing in the eye. The number of E.G7-OVA tumors in eyes of OT-1 mice was 86-fold less (Experiment 1) and 54-fold less (Experiment 2) than similarly challenged B6.PL mice (Fig. 8). It is important to note that E.G7-OVA was defined in OT-1 mice as Thy 1.2^+CD8^- cells, and OT-1 mice express Thy 1.2. Therefore, the number of E.G7-OVA in these experiments potentially includes a background number of Thy 1.2^+, CD8-negative cells that are not tumors. However, the size and granularity of Thy 1.2^+, CD8^- cells in tumor-challenged OT-1 mice was equivalent to cultured E.G7-OVA tumor cells and distinct from leukocytes (data not shown), suggesting that Thy 1.2^+ tumor cells rather than leukocytes were being measured in the OT-1 eyes. Our data suggest that CD8^- CTL infiltrated primary ocular tumors and that increasing the frequency of tumor-specific CD8^- T cells inhibited the growth of primary ocular tumor cells. These data also demonstrate that E.G7-OVA did not lose antigenicity or become immune-suppressive after 13 days of growth in the eye.

The next experiment was designed to determine whether the decreased frequency of E.G7-OVA in the eyes of OT-1, compared with B6.PL, mice correlated with an increase in the number of CD8^- T cells and an increase or decrease in the number of CD11b^+ cells infiltrating the eye. The number of CD8^- T cells detected in the eyes of OT-1 mice on day 0 was 15-fold greater (Fig. 9B) than that in B6.PL mice (Fig. 6C). Seven days after injection of 10^4 E.G7-OVA, 33-fold more CD8^- T cells were detected in OT-1 than B6.PL mice, whereas 45-fold more E.G7-OVA cells were detected in B6.PL than OT-1 eyes (Fig. 9A), which confirms our previous experiments. This suggests that increasing the number of tumor-specific CD8^- T cells within the eye contributes to reducing the number of tumor cells. At day 7, the number of CD11b^-Gr-1^- and CD11b^-Gr-1^+ cells were significantly less in OT-1 than B6.PL recipients. Similar results were observed in OT-1 mice on day 7 in a second experiment (Fig. 9B). At this point, the eyes of OT-1 mice were almost indistinguishable from naive unchallenged mice by visual inspection, and only a few mice displayed very slight a.c. cloudiness. E.G7-OVA tumor cells increased 11-fold from day 7 to day 9 (Fig. 9A) and 49-fold on day 13 in comparison to background numbers of Thy 1.2^+, CD8^- T cells in OT-1 T cells at day 0 (Fig. 9B), whereas the number of CD8^- T cells remained fairly constant and similar to the number observed in OT-1 mice before tumor cells were injected. By day 13, the number of tumor cells in the OT-1 recipients was similar to that observed in B6.PL mice on day 7, which indicates that increased numbers of CD8^- T cells in OT-1 mice delayed, but did not prevent, the growth of E.G7-OVA. Both CD11b^+Gr-1^- and CD11b^+Gr-1^+ cells increased in parallel with the increasing growth of E.G7-OVA, but the number of CD11b^+Gr-1^- cells was virtually the same as the number of tumor cells in both strains of mice at all times after tumor injection, suggesting that there might be a causal relationship between these cells.
Myeloid cells within primary ocular tumors suppress CD8\(^+\) CTL responses

CD11b\(^+\) cells have been shown to inhibit tumor-specific CTL responses in other murine tumor models (33–37), raising the possibility that CD11b\(^+\) cells may be inhibiting tumor-specific CD8\(^+\) CTL responses. To test this hypothesis, CD11b\(^+\) cells were sorted from tumor-bearing eyes or normal spleen cells by flow cytometry and then added to cultures of spleen cells from naive OT-I mice that were stimulated with the cognate SINFEKL peptide. CTL responses were measured 4 days later (Fig. 10A). CTL cultures that were stimulated with SINFEKL developed CTL activity against OVA-expressing E.G7-OVA targets, but not against the parental cell line EL-4 (data not shown), in comparison to unstimulated cultures. In five independent experiments (Fig. 10B), CD11b\(^+\) cells isolated from ocular tumors significantly inhibited the lytic activity of peptide-stimulated cultures (37 ± 27% of control). Lytic activity was also significantly reduced after the addition of naive splenic CD11b\(^+\) cells (68 ± 29% of control), but their specific activity was slightly, albeit not significantly (\(p = 0.12\)), less than that of the CD11b\(^+\) cells from the eye of tumor-bearing mice. These data suggest that myeloid cells infiltrating primary ocular tumors may suppress CD8\(^+\) CTL responses, thereby contributing to primary ocular tumor growth.

Discussion

Administration of E.G7-OVA via the a.c. generated OVA-specific CD8\(^+\) CTL responses systemically that eliminated a subsequent E.G7-OVA challenge in the skin and opposite eye but failed to eliminate the primary ocular tumor. The generation of CTL effectors after the administration of tumors in the eye is thought to be a two-step process involving expansion of CTL precursors in secondary lymphoid organs that differentiate into lytic effectors upon subsequent encounter with Ag at the site of tumor injection (38, 39). We observed a slight increase in CD8\(^+\) T cells within primary ocular tumors, and E.G7-OVA tumor growth was retarded in the a.c. of OT-I mice in which the majority of CD8\(^+\) T cells express an OVA-specific TCR. Delayed kinetics of primary ocular tumor growth in OT-I mice correlated with increased numbers of CD8\(^+\) T cells within the eye, suggesting that tumor-specific CTL can infiltrate primary ocular tumors. Because the frequency of OVA-specific CTL precursors in OT-I mice is markedly increased in comparison to B6 mice, these data suggest that the tumor cell: effector CTL ratio is critical for tumor elimination in the eye. This is compatible with the observation that P815 cells injected into the a.c. were completely eliminated when activated P815-specific CD8\(^+\) CTL effectors were coinjected at a 1:1, 3:1, or 8:1 tumor cell:effector T cell ratios (31) but not when the ratio exceeded 60:1 (31). Therefore, the growth of E.G7-OVA tumors in the eye and not the skin may be the result of fewer CD8\(^+\) CTL within primary ocular tumors compared with the numbers in primary skin tumors. Alternatively, preventing the growth of tumors in the eye may require a higher effector:tumor ratio than the skin because the various factors that maintain ocular immune privilege (reviewed in Refs. 40, 41) interfere with the lytic activity of the CD8\(^+\) T cells on a per cell basis (13, 39). In our experiments, primary ocular tumor growth was equivalent in B6 and in Rag\(^{-/-}\), suggesting that the few CD8\(^+\) T cells within primary ocular tumors of B6 mice were functionally inhibited. However, a.c. administration of E.G7-OVA did not induce systemic CD8\(^+\) T cell tolerance but rather primed for CD8\(^+\) CTL responses, as has been previously reported for P815 mastocytes that had been injected into the a.c. (19).

E.G7-OVA tumors grew progressively in the a.c. of B6.PL mice and with delayed kinetics in OT-I mice despite the infiltration of CD11b\(^+\) cells that accumulated at numbers almost equal to the tumor cells. These data suggest that CD11b\(^+\) cells within primary ocular tumors are not tumoricidal, although CD11b\(^+\) cells are known to have tumoricidal properties under other circumstances. In addition, the CD11b\(^+\) cells within primary ocular tumors were particularly efficient suppressors of CD8\(^+\) CTL responses in vitro. Therefore, these CD11b\(^+\) cells may be analogous to MSC, which have been shown to promote tumor progression by suppressing CD8\(^+\) CTL responses systemically and regionally within the tumor microenvironment (reviewed by Serafini et al. (42)).

MSC accumulate in peripheral blood of patients with malignancies (43, 44) and accumulate in the spleen (33–37), and within tumors (45) in several different murine tumor models. The mechanism by which tumor-derived CD11b\(^+\) T cells suppress CTL responses in our system is not currently understood. However, MSC might inhibit tumor-specific CD8\(^+\) CTL activity by altering l-arginine metabolism (reviewed by Bronte et al. (46)) via inducible NO synthase or arginase I, which promotes CTL apoptosis (45, 47).

**Table III. Intracameraly induced concomitant immunity**

<table>
<thead>
<tr>
<th>Treatments</th>
<th>E.G7-OVA (Thy 1.2(^+))</th>
<th>Thy 1.2/CD8(^+)</th>
<th>Thy 1.2(^+)/CD8(^+)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Right eye</td>
<td>Left eye</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PBS</td>
<td>E.G7</td>
<td>43.8 ± 17.0</td>
<td>1.7 ± 1.1</td>
</tr>
<tr>
<td>E.G7</td>
<td>E.G7</td>
<td>0.2 ± 0.1</td>
<td>0.04 ± 0.04</td>
</tr>
<tr>
<td>p value</td>
<td></td>
<td>0.01</td>
<td>0.06</td>
</tr>
</tbody>
</table>

* Indicated cell populations were enumerated by flow cytometric analysis of collagenase-digested left eyes. A total of 10\(^5\) events was collected for analysis. \(p\) values were determined by a Student’s \(t\) test comparing mice given PBS or primed with E.G7-OVA in the right eye before E.G7-OVA challenge in the left eye.

**FIGURE 8.** E.G7-OVA numbers are reduced in the a.c. of OT-I mice. B6.PL and OT-I mice (5 mice/group) were injected with E.G7-OVA (10\(^4\) cells) that was isolated after 10 (Experiment 1) or 13 (Experiment 2) days of growth in the a.c. of B6.PL mice. Ten (Experiment 1) or 14 days (Experiment 2) later, E.G7-OVA cells were enumerated in collagenase-digested eyes. A total of 5.0 \(\times\) 10\(^5\) cells was collected for analysis. E.G7-OVA were defined as CD45\(^-\)/Thy1.2\(^+\)/CD8-negative cells in OT-I mice that express the Thy 1.2 allotype. The number of CD45\(^-\)/Thy1.2\(^+\)/CD8-negative cells in collagenase-digested eyes (\(n = 6\)) from three naive OT-I mice was 3.2 ± 0.8 \(\times\) 10\(^5\) cells. A total of 10\(^5\) events was collected for analysis.

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**References**

- 40, 41
- 42
- 43, 44
- 33–37
- 38, 39
- 45
- 46
- 45, 47
or interferes with CTL signal transduction (48) in other murine tumor models. The low frequency of CD8+ T cells within primary ocular tumors would be consistent with CD8+ T cell apoptosis within the eye mediated via NO production by CD11b+ cells. This interpretation is supported by the observation that inhibition of NO production, by administration of N\textsuperscript{-}nitro-L-arginine methyl ester in an experimental model of autoimmune uveoretinitis, decreased T cell apoptosis in the retina (49).

CD11b+ cells infiltrating primary ocular tumors in our experiments were both Gr-1+ and Gr-1− cells, which were also reported to accumulate in the eyes of mice injected in the a.c. with tumors that express membrane-bound Fas ligand, where they are associated with inflammatory responses that eliminated the tumor (50). In addition, Ab-mediated depletion of CD11b+ cells mitigated endotoxin-induced uveitis, suggesting that CD11b− cells contribute to the pathology of this disease (51). These data indicate that not all Gr-1+, CD11b−, or CD11b/Gr-1 double-positive cells are MSC. In our experiments, CD11b+ cells from naïve spleens also suppressed CTL responses, suggesting that a suppressor phenotype may be the default pathway of CD11b+ myeloid cells. Environmental signals associated with inflammation may promote differentiation of CD11b+Gr-1− cells into mature neutrophils, which are tumoricidal. For example, Gr-1− MSC lost suppressive activity when cultured with all trans-retinoic acid, a regulator of cell differentiation that induces promyelocytes to differentiate into mature neutrophils (52). Recently, Gregory et al. (53) have presented a novel treatment for ocular tumors involving the injection of membrane Fas ligand vesicles into the eye, which induced neutrophil infiltration and promoted ocular tumor elimination. This treatment might also promote the differentiation of MSC within ocular tumors into neutrophils that are tumoricidal.

In our experiments, a.c. administration of tumors induced tumor-specific immunity that eliminated tumors subsequently injected in the opposite eye, suggesting that these protective tumor-specific CD8+ CTL responses may be inhibited by MSC within primary ocular tumors. MSC cells accumulate after immunization (54) and in chronic infection (55). Therefore, the normal physiological function of these cells may be immune suppression to prevent immunopathology. Consistent with this hypothesis, a large number of CD45+ cells that were not tumors and not CD8+ CTL accumulated in the eyes of mice previously primed with E.G7-OVA via the a.c., which displayed reduced tumor burden after a subsequent injection of E.G7-OVA in the opposite eye in comparison to unprimed mice (Fig. 7 and Table III). In preliminary experiments, we have found that CD11b+/Gr-1+ cells accumulate in these eyes but have not determined whether they are MSC or tumoricidal neutrophils. Hence, the ratio of MSC:effector CD8+ CTL may be critical in determining whether CTL are effective at eliminating tumors. CD11b+ cells accumulated with early kinetics in primary ocular tumors (Fig. 6) at a time when CD8+ CTL precursors were most likely expanding and differentiating in secondary lymphoid organs. Therefore, when tumor-specific CD8+ CTL

![FIGURE 9.](image-url) Increased numbers of CD8+ T cells and decreased numbers of CD11b+ myeloid cells are associated with delayed kinetics of tumor growth in the a.c. of OT-I mice. B6.PL and OT-I mice (5 mice/group) were injected with 10^6 E.G7-OVA in the a.c. Seven and 9 days later, tumors and indicated leukocyte populations were enumerated in collagenase-digested eyes (A). The kinetics of tumor growth and leukocyte infiltration in OT-I mice injected with 10^7 E.G7-OVA in the a.c. are shown (B). Each symbol represents the mean measurement ± 1 SD of indicated cell populations from four to nine individual mice. Day 0 values are from six untreated eyes of three B6.PL mice in which 10^5 events were collected for analysis.

![FIGURE 10.](image-url) CD11b+ cells isolated from ocular tumors suppress CTL responses. OT-I splenocytes (4.0 × 10^6 cells) were stimulated with or without SIINFEKL peptide (0.1 μg/ml) and with or without the addition of CD11b+ regulators (1.0 × 10^6 cells) that were isolated by flow cytometric cell sorting from spleens of naïve B6 mice or from collagenase-digested eyes of B6.PL mice injected with E.G7-OVA (5.0 × 10^7) cells in the a.c. 10 days earlier. CTL responses were assayed with E.G7-OVA targets at the indicated E:T ratios 4 days later (A). The lytic activity in OT-I cultures with the addition of CD11b+ regulators (0.5–1.0 × 10^6 cells) from the spleen or eye was normalized to the lytic activity of OT-I cultures without regulators at the same E:T ratio (50:1–67:1) within the same experiment and is presented as percentage of control (B). Each symbol represents an independent experiment.
migrated into primary ocular tumors, they encountered a very high frequency of CD11b+MSC. In contrast, CD8+CTL may outnumber CD11b+MSC after a secondary challenge with tumors in the opposite eye of primed mice because CD8+CTL precursors have already expanded to the first tumor challenge. In support of this hypothesis, primary ocular tumors grew with delayed kinetics in OT-1 mice, which was associated with increased numbers of CD8+ T cells within the eye in comparison to B6.Pi mice. Seven days after a.c. administration of E.G7-OVA to OT-1 mice, the number of CD11b+/Gr-1- cells and CD8+ T cells within eyes was equivalent, and tumor burden was low. However, on day 13, tumor burden increased and CD11b+/Gr-1+ cells outnumbered CD8+ T cells. The observation that CD11b+ cells isolated from tumor-containing eyes of B6.Pi mice inhibited CD8+ CTL responses in vitro suggests that CD11b+ cells may inhibit tumor-specific CD8+ T cell within the primary ocular tumor microenvironment in vivo.

Accumulation of MSC within human primary ocular tumors has not been reported. However, patients with uveal melanomas containing tumor-associated macrophages (TAM) were associated with larger tumors of the more aggressive epithelioid type and a poor prognosis in one study (56). These TAM may be immune suppressive because CD11b+/Gr-1+ MSC have been shown to differentiate into F4/80+ TAM after transfer into tumor-bearing mice, which suppress tumor-specific CD8+ CTL, and thereby promote tumor growth (45). Chemotherapeutic treatments that inhibit or delete MSC, such as Gemcitabine (35), may promote primary ocular tumor elimination by restoring tumor-specific CD8+ CTL activity within the eye.

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References


