Splenectomy Promotes Indirect Elimination of Intraocular Tumors by CD8\(^+\) T Cells That Is Associated with IFN\(\gamma\)- and Fas/FasL-Dependent Activation of Intratumoral Macrophages

Maxine R. Miller\(^1\), Jonathan B. Mandell\(^1\), Kelly M. Beatty\(^1\), Stephen A.K. Harvey\(^1\), Michael J. Rizzo\(^1,2\), Dana M. Previte\(^1,2\), Stephen H. Thorne\(^3,4,5\), and Kyle C. McKenna\(^1,3,5\)

Abstract

Ocular immune privilege (IP) limits the immune surveillance of intraocular tumors as certain immunogenic tumor cell lines (P815, E.G7-OVA) that are rejected when transplanted in the skin grow progressively when placed in the anterior chamber of the eye. As splenectomy (SPLNX) is known to terminate ocular IP, we characterized the immune mechanisms responsible for rejection of intraocular tumors in SPLNX mice as a first step toward identifying how to restore tumoricidal activity within the eye. CD8\(^+\) T cells, IFN\(\gamma\), and FasL, but not perforin, or TNF\(\alpha\) were required for the elimination of intraocular E.G7-OVA tumors that culminated in destruction of the eye (ocular phthisis). IFN\(\gamma\) and FasL did not target tumor cells directly as the majority of SPLNX IFN\(\gamma\)R1\(^-/-\) mice and Fas-defective lpr mice failed to eliminate intraocular E.G7-OVA tumors that expressed Fas and IFN\(\gamma\)R1. Bone marrow chimeras revealed that IFN\(\gamma\)R1 and Fas expression on immune cells was most critical for rejection, and SPLNX increased the frequency of activated macrophages (M\(\phi\)) within intraocular tumors in an IFN\(\gamma\)- and Fas/FasL-dependent manner, suggesting an immune cell target of IFN\(\gamma\) and Fas. As depletion of M\(\phi\)s limited CD8 T cell–mediated rejection of intraocular tumors in SPLNX mice, our data support a model in which IFN\(\gamma\)- and Fas/FasL-dependent activation of intratumoral M\(\phi\)s by CD8\(^+\) T cells promotes severe intraocular inflammation that indirectly eliminates intraocular tumors by inducing phthisis, and suggests that immunosuppressive mechanisms that maintain ocular IP interfere with the interaction between CD8\(^+\) T cells and M\(\phi\)s to limit the immunesurveillance of intraocular tumors. Cancer Immunol Res; 2(12): 1175–85. ©2014 AACR.

Introduction

Ocular ‘immune privilege’ (IP) limits the immune surveillance of intraocular tumors as certain immunogenic tumor cell lines (P815 and E.G7-OVA), which are rejected by host immune responses when transplanted in the skin, grow progressively when placed into the anterior chamber (a.c.) of the eye (1). IP is not immune ignorance, as several studies have shown that intraocular tumor growth primes systemic immune responses to tumor antigens (2–4). Rather, ocular immune responses are very tightly regulated to limit inflammation during pathogen removal so that certain intraocular tissues, which do not regenerate and are essential for vision, are not damaged (5, 6).

Ocular IP is maintained by anatomic and biochemical barriers to host immune responses along with the generation of systemic tolerance to antigens encountered within the eye (reviewed in ref. 7). Splenectomy (SPLNX) terminates ocular IP and promotes rejection of immunogenic tumors transplanted in the a.c. of the eye (8). However, the mechanism of tumor elimination has not been defined. Herein, we characterize the requirements for elimination of intraocular tumors in SPLNX mice as a first step toward identifying how to overcome IP and restore tumoricidal activity within intraocular tumors. We demonstrate that CD8\(^+\) T cells, macrophages (M\(\phi\)), IFN\(\gamma\), and FasL, but not perforin, or TNF\(\alpha\) were necessary for intraocular tumor elimination. Although tumors expressed IFN\(\gamma\)R1 and Fas, IFN\(\gamma\) and FasL did not directly target tumors. Rather, IFN\(\gamma\) and Fas/FasL interactions were required for intratumoral M\(\phi\) activation that was associated with severe ocular inflammation, which indirectly eliminated intraocular tumors by inducing complete destruction of the eye (ocular phthisis). Our data suggest that immunosuppressive mechanisms that preserve ocular IP interfere with the complex interplay between CD8\(^+\)
T cells and intratumoral Mφs necessary to eliminate intraocular tumors.

Materials and Methods

Tumor cell lines

P815, provided by Dr. Judith A. Kapp (University of Alabama, Birmingham, AL) in 2006, and E.G7-OVA (ATCC CRL-2113), obtained from the ATCC in 2008, were used. E.G7-OVA were transduced to express firefly luciferase (Luc-E.G7) using ViraPower (Invitrogen). Luc-E.G7 cultures initiated from frozen stocks were cultured monthly with 1.0 mg/mL G418 sulfate to maintain the expression of OVA and used in experimentation for no longer than 3 months, at which time a new Luc.E.G7 culture was initiated. Luc-E.G7 cultures were authenticated every three months, in comparison with ATCC CRL-2113 by evaluating bioluminescence and sensitivity to lysis by OVA-specific CD8+ OT-I CTL. P815 was authenticated by IDEXX RADIL in 2014 by direct comparison with P815 obtained from the ATCC (ATCC TIB-64) in 2014 (Supplementary Table S1).

Mice

Male and female mouse strains older than 7 weeks from The Jackson Laboratory, detailed in Supplementary Table SII, were used. OT-I mice (9) were crossed with gld or IFNγR1−/− mice to generate OT-I mice deicient in FasL or IFNγR1. All experimentation was approved by the IACUC at the University of Pittsburgh.

Splenectomy

Mice were anesthetized with a solution of ketamine (100 mg/kg) and xylazine (10 mg/kg). The skin above the spleen was prepared for surgery by depilation and contact sterilization. Sterile scissors were used to open the skin and fascia to visualize the spleen. The spleen was gently held with sterile forceps, splenic vessels cauterized, and the spleen removed. The incision site was closed with sterile surgical staples (Roboz) that were removed 10 to 14 days after surgery.

Ocular injections and intraocular tumor growth measurements

Tumor cell lines (10^5 cells) were injected into the a.c. of the eye as previously described (4). P815 tumor growth was monitored by visual inspection (Supplementary Fig. S1), and scored as either progressive or phthisical at least 17 days after tumor challenge. A very significant correlation was observed between bioluminescence measurements and Luc-E.G7 numbers in vitro (Supplementary Fig. S1C). Hence, the sequential growth in Luc-E.G7 in the a.c. of the eye of live mice was monitored by bioluminescent imaging (BLI) using an IVIS imager (Caliper Life Sciences) following sedation of mice with isoflurane and within 15 minutes after intraperitoneal injection of 6 mg r-luciferin salt (Gold Biotechnology; Supplementary Fig. S1D). Background bioluminescence was defined at 10^3 photons/second (Supplementary Fig. S1E). Rejection of Luc-E.G7 tumors was scored in individual mice as a two-log decrease in tumor bioluminescence that was maintained for at least two successive measurements.

In vivo depletion of immune cell subsets and Fas/FasL neutralization

To selectively remove CD4+ or CD8+ T cells, mice were given intraperitoneal injections of anti-CD4 (clone GK1.5) or anti-CD8 (clone 2.43) antibodies from BioXCell. Antibody treatment (0.2–0.4 mg) began 3 days before or 7 days after ocular tumor injections and continued every 3 to 4 days thereafter (0.1 mg injections). Depletion was greater than 96%, as determined by the flow cytometric analysis of peripheral blood (data not shown). Macrophages were depleted by subconjunctival (scon.) injections (10 μL) or, scon. and intravenous (i.v.) injections (100–200 μL) as indicated. Neutralization of Fas/Fasl interactions was accomplished by 0.1 mg intraperitoneal (i.p.) injections of Ultra-LEAF anti-mouse CD178/Fasl antibodies (BioLegend) that were given before tumor challenge and every 3 to 4 days thereafter. Equivalent injections of Hamster IgG (BioXCell) were given to control for antibody injection.

Flow cytometric analysis

Fifteen to 16 days after tumor challenge, single-cell suspensions of whole tumor–bearing eyes were prepared as previously described (10). Fc receptors were blocked and then stained with combinations of fluorescently conjugated antibodies from BD Pharmingen to the following cell surface molecules: CD45, CD11b, Thyl.2, Gr-1, and/or F4/80 in FACS buffer (PBS + 2% fetal bovine serum). Cells were then washed and fixed in Cytofix/Cytopermin (BD Pharmingen) and in some experiments incubated with PE-conjugated anti-CD68, or polyclonal rabbit anti-mouse NOS2 antibody (BD Pharmingen) in Perm/Wash buffer (BD Pharmingen). Cells treated with polyclonal anti-mouse NOS2 were then stained with AlexaFluor 546 anti-rabbit IgG (R&D Systems). Events were collected using a FACS Diva Flow Cytometer (Becton Dickinson) and analyzed using FACS Diva (Becton Dickinson) and FlowJo (TreeStar) software.

Generation of bone marrow chimeric mice

Mice were irradiated (10 Gy) in a Cs source irradiator (Nordion) and then injected i.v. with bone marrow (4.5 × 10^6 cells) isolated from femurs, and tibias of B6, lpr, or IFNγR1−/− mice. Experimentation was performed 8 weeks later after reconstitution of the immune system.

Gene array analysis and RT-PCR

Fifteen to 16 days after ocular tumor challenge, eyes were removed, homogenized in RLT buffer (from the RNAeasy Kit; Qiagen) in a Tenbroeck frosted glass tissue grinder, then stored at −80°C until isolation of total RNA using QiaShredder and RNAeasy kits (Qiagen). cDNA was synthesized using a High Capacity cDNA Reverse Transcription Kit and quantitative RT-PCR was performed using a StepOne Plus instrument with commercially available TaqMan primer probe sets (Applied Biosystems). Pyruvate decarboxylase (Pcx) was used as the normalizing (housekeeping) gene. Extracted RNA (~500 ng) was also processed using a 3’ RT Express Kit to yield amplified RNA (~20 μg), which was hybridized to M430 2.0 microarrays; the microarrays were scanned using a GeneChip 3000 Array scanner (Affymetrix Inc.). Raw data were processed using
Affymetrix GCOS v.1.4 software with default settings, then exported to Microsoft Excel. The ratio (mean SPLNX:mean control) was calculated for each microarray panel, with candidate panels having ratios ≥ 1.5 or ≤ 0.67. Valid candidates showed detectable (i.e., called Present by GCOS) transcript in both samples of the higher-expressing group, and no overlap of values was found between groups. The dataset is available from the NCBI GEO database (www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE57101).

Adaptive transfer of OT-I T cells
In some experiments, naïve CD8+ T cells were isolated from spleens of OT-I, OT-I X gld, or OT-I X IFNγR1−/− mice using mouse CD8 T-cell enrichment kits (Stemcell Technologies) and then injected i.v. via tail-vein injection (3.0 × 10⁶ cells). To generate OT-I T-cell effectors, splenocytes from OT-I mice were stimulated in vitro for 3 days with 0.1 μg/mL SIINFEKL peptide, and dead cells were removed by centrifugation over Fico/Lite LM (mouse; Atlanta Biologicals). OT-I T-cell effectors (1.0–3.0 × 10⁶ cells) were injected i.v.

Statistical analysis
Prism (GraphPad) software was used for statistical analysis.

Results
SPLNX promotes CD8 T cell–mediated rejection of intraocular tumors
Preliminary studies showed that P815 tumors that normally grew progressively when transplanted in the a.c. of the eye were rejected in SPLNX mice by a process that culminated in ocular phthisis (8). To characterize the requirements for elimination of intraocular tumors, we monitored intraocular tumor growth after injection of 10⁴ P815 tumor cells (H-2d) into the a.c. of SPLNX Balb/C J mice or Balb/C J mice that were not surgically manipulated (hereafter referred to as controls). The majority of SPLNX mice rejected P815 tumors with phthisis beginning 10 to 14 days after tumor challenge, whereas progressive intraocular tumor growth was observed in controls (Table 1), confirming the previous observations (8). This delayed immune response suggested the contribution of adaptive immunity. Therefore, intraocular tumor growth was monitored in SPLNX mice that received depleting anti-CD4 or anti-CD8 antibodies. Removal of CD4+ T cells before (Table 1, Exp. 2) or at 7 days after tumor challenge (Table 1, Exp. 4) did not influence the rejection of intraocular tumors in SPLNX mice. In contrast, SPLNX mice depleted of CD8+ T cells (Table 1, Exp. 3 and Exp. 4) did not reject intraocular tumors. Intraocular growth of transplanted bioluminescent Luc-E.G7 tumors (H-2b) that expressed OVA as a defined tumor antigen (11) was also monitored by BLI in SPLNX and control C57Bl/6 J (B6) mice that received a mock surgery (Fig. 1A–C). During the first 14 days after tumor challenge, equivalent tumor growth was observed (Fig. 1A and B). However, by day 21, the majority of SPLNX mice eliminated these established intraocular tumors (Fig. 1B and C) resulting in ocular phthisis. The rejection of intraocular Luc-E.G7 tumors required CD8+ T cells as progressive intraocular tumor growth was observed in SPLNX mice deficient in CD8+ T cells (CD8−/− mice) and in SPLNX B6 mice given depleting anti-CD8 antibodies (Fig. 1D).

Table 1. Splenectomy promotes CD8 T cell–mediated rejection of intraocular P815 mastocytomas in Balb/C mice

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Surgery</th>
<th>Antibody treatment</th>
<th>Number of mice with tumor/total, number of mice (percentage with tumor)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>None</td>
<td>None</td>
<td>5/8 (63%)</td>
</tr>
<tr>
<td></td>
<td>Splenectomy</td>
<td>None</td>
<td>0/7 (0%)</td>
</tr>
<tr>
<td>2</td>
<td>Splenectomy</td>
<td>Rat IgG</td>
<td>0/4 (0%)</td>
</tr>
<tr>
<td></td>
<td>Splenectomy</td>
<td>Anti-CD4</td>
<td>1/4 (25%)</td>
</tr>
<tr>
<td>3</td>
<td>None</td>
<td>None</td>
<td>6/7 (86%)</td>
</tr>
<tr>
<td></td>
<td>Splenectomy</td>
<td>Rat IgG</td>
<td>0/8 (0%)</td>
</tr>
<tr>
<td></td>
<td>Splenectomy</td>
<td>Anti-CD8</td>
<td>8/8 (100%)</td>
</tr>
<tr>
<td>4</td>
<td>None</td>
<td>Rat IgG</td>
<td>6/6 (100%)</td>
</tr>
<tr>
<td></td>
<td>Splenectomy</td>
<td>Rat IgG</td>
<td>0/6 (0%)</td>
</tr>
<tr>
<td></td>
<td>None</td>
<td>Anti-CD4</td>
<td>5/5 (100%)</td>
</tr>
<tr>
<td></td>
<td>Splenectomy</td>
<td>Anti-CD4</td>
<td>0/5 (0%)</td>
</tr>
<tr>
<td></td>
<td>None</td>
<td>Anti-CD8</td>
<td>5/5 (100%)</td>
</tr>
<tr>
<td></td>
<td>Splenectomy</td>
<td>Anti-CD8</td>
<td>4/5 (80%)</td>
</tr>
</tbody>
</table>

NOTE: Balb/C J mice were used in these experiments and 10⁴ P815 tumor cells were injected in the a.c. of the eye. In experiments 2 and 3, antibody treatments began 1 day before tumor challenge and continued every 3 to 4 days thereafter. In experiment 4, antibody treatments began 7 days after tumor challenge. Splenectomized mice were compared with control mice by the Fisher exact test. P values are shown.

Abbreviation: ns, not statistically significant.
but not in SPLNX B6 mice given Rat IgG as a control for antibody administration. Combined with our equivalent observations in the P815/Balb/cJ intracocular tumor model (Table 1), these data identified a common requirement for CD8\(^+\) T cells in phagistical rejection of intracocular tumors in SPLNX mice.

**Rejection of intraocular tumors requires FasL and IFN\(\gamma\)**

CD8\(^+\) T cells can directly eliminate tumors by perforin-dependent cytolytic activity, FasL-induced apoptosis, or via the production of tumoricidal cytokines, including TNF\(\alpha\) and IFN\(\gamma\) (12). Therefore, to determine the contribution of these molecules in the control of intracocular tumors, Luc-E.G7 intracellular tumor growth was monitored in SPLNX and control mice with genetic deficiencies in perforin (pfp\(^{-/-}\)), FasL (gld), TNF\(\alpha\) (TNF\(\alpha\)-/-), or IFN\(\gamma\) (IFN\(\gamma\)-/-). The majority of controls in these immunodeficient strains showed progressive intracocular tumor growth (Fig. 2). SPLNX pfp\(^{-/-}\) mice rejected established intracocular Luc-E.G7, which indicated that lytic activity of CD8\(^+\) T cells was not required to eliminate these intracocular tumors. Similarly, P815 tumors that grew progressively in pfp\(^{-/-}\) control mice on a Balb/c ByJ background were rejected when pfp\(^{-/-}\) mice were SPLNX before tumor challenge (Supplementary Table SIII). Hence, perforin was dispensable for rejection of intracocular tumors. TNF\(\alpha\) was also not required for rejection of Luc-E.G7 tumors in SPLNX mice (Fig. 2C). However, FasL and IFN\(\gamma\) were necessary as intracocular Luc-E.G7 tumors grew progressively in SPLNX gld and IFN\(\gamma\)-/- mice (Fig. 2B and D). A greater frequency of SPLNX Balb/c ByJ mice given anti-FasL antibodies showed progressive intracocular P815 tumor growth in comparison with SPLNX mice given control antibodies (Supplementary Table SIII, Exp. 3), which indicated that Fas/FasL interactions also contributed to elimination of P815 tumors in SPLNX Balb/c J mice.

**IFN\(\gamma\)R1 and Fas expression on hematopoietic cells is critical for intracocular tumor rejection**

Flow cytometric analysis indicated that Luc-E.G7 tumors expressed both IFN\(\gamma\)R1 (Fig. 3A) and Fas (Fig. 3D), suggesting that IFN\(\gamma\) and FasL, expressed by CD8\(^+\) T cells, could target tumor cells directly. However, 3-day culture of Luc-E.G7 or P815 tumors with IFN\(\gamma\) at concentrations ranging from 12.5 to 5,000 U/mL did not affect tumor viability and had only a modest effect on growth (Supplementary Fig. S2). In addition, EL-4 cells, the parental cell line of Luc-E.G7, are resistant to FasL-induced apoptosis due to overexpression of cellular FLICE inhibitory protein (c-FLIP; ref. 13). Hence, we tested the hypothesis that IFN\(\gamma\) and FasL acted on nontumor (stromal) cells within the tumor microenvironment to eliminate intracocular tumors by monitoring intracocular Luc-E.G7 growth in SPLNX and control IFN\(\gamma\)R1/-/- and Fas-defective lpr mice. Luc-E.G7 tumors grew progressively in the majority of controls of both the strains. Despite tumor expression of IFN\(\gamma\)R1 and Fas, the majority of SPLNX IFN\(\gamma\)R1/-/- (Fig. 3B) and SPLNX lpr (Fig. 3E) failed to eliminate intracocular Luc-E.G7 tumors. Similarly, progressive intracocular P815 tumor growth was observed in SPLNX and control IFN\(\gamma\)R1/-/- on a Balb/c ByJ background.
These data indicated that IFNγ and FasL indirectly eliminated intraocular tumors by targeting stromal cells within the tumor microenvironment.

Tumor stroma comprised hematopoietic and nonhematopoietic cells (14). To determine the critical cell populations that expressed IFNγR1 and FasL, bone marrow chimeras were generated with selective deficiencies in IFNγR1 or Fas in hematopoietic cells, nonhematopoietic cells, or both cell populations. IFNγR1 expression on hematopoietic cells was critical for intraocular tumor rejection in SPLNX mice as a lesser frequency of B6 recipients of IFNγR1−/− bone marrow rejected Luc-E.G7 intraocular tumors in comparison with B6 recipients of B6 bone marrow (Fig. 3C). Indeed, the percentage of B6 recipients of IFNγR1−/− bone marrow was statistically comparable with IFNγR1−/− recipients of IFNγR1−/− bone marrow. SPLNX IFNγR1−/− recipients of B6 bone marrow rejected intraocular Luc-E.G7 tumors confirming the necessity for IFNγR1 on hematopoietic cells. Fas expression on hematopoietic cells was also required for intraocular tumor elimination as all SPLNX B6 recipients of lpr bone marrow demonstrated progressive intraocular Luc-E.G7 tumor growth (Fig. 3F), whereas SPLNX lpr recipients of B6 bone marrow rejected intraocular Luc-E.G7 tumors (Fig. 3F).

IFNγR1 and FasL on CD8+ T cells are not required for intraocular tumor elimination

One explanation for impaired intraocular tumor rejection in SPLNX IFNγR1−/−, gld, and lpr mice is that priming of tumor-specific CD8+ T cells is impaired in these strains. Therefore, to address the functionality of tumor-specific CD8+ T cells in...
tumor-speciﬁc activation and markers of ocular inflammation. Intraocular Luc-E.G7 tumor burden in SPLNX or control CD8$^+$ H-2$^b$ mice that received naïve OT-I, OT-I X IFNγR1$^−/−$, or OT-I X gld CD8$^+$ T cells before intraocular tumor challenge. This experiment was performed twice with similar results.

Intraocular tumor regression in SPLNX mice is associated with IFNγ- and Fas/Fasl-dependent Mφ activation and markers of ocular inﬁammation

Previously, we and others demonstrated that rejection of established E.G7 skin tumors required IFNγ expression by tumor-speciﬁc CD8$^+$ T cells to activate tumoricidal nitric oxide (NO) production by intratumoral Mφs (10, 15, 16). NO is synthesized in activated Mφs by the enzyme NO synthase 2 (NOS2; ref. 17). Therefore, to determine whether a similar mechanism was involved in rejection of intraocular tumors in SPLNX mice, we compared the frequency of NOS2$^+$ Mφs by ﬂow cytometry in day 16 Luc-E.G7 intraocular tumors of SPLNX and control B6, gld, lpr, and IFNγR1$^−/−$ mice. This time point, immediately before Luc-E.G7 intraocular tumor regression in SPLNX B6 mice, was chosen as tumor burden measured by BLI was statistically comparable in both the treatment groups. Some intraocular tumors in SPLNX mice did, however, show lower tumor burden, indicating the beginning of tumor regression.

In all strains of mice tested and regardless of surgical treatment, intraocular Luc-E.G7 tumors were inﬁltrated by CD45$^+$ CD11b$^+$ myeloid cells composed of Gr-1$^+$ and Gr-1$^-$ cells (Supplementary Fig. S3). Gr-1$^-$ cells also expressed F4/80 and CD68, indicating that they were Mφs (Supplementary Fig. S3). The number of Gr-1$^+$ cells was statistically equivalent between SPLNX and control mice of each strain although a trend of lower or higher Gr-1$^+$ cell numbers was observed in SPLNX B6 and SPLNX lpr mice, respectively (Fig. 5A). The percentage of NOS2$^+$ Gr-1$^+$ cells in intraocular tumors was very low (<5%) (Supplementary Fig. S3B) and their numbers were equivalent between BALB/c and control mice in all the strains (Fig. 5B). Mφ numbers were comparable between SPLNX and control B6 or gld mice, whereas the number of Mφs increased in SPLNX lpr and SPLNX IFNγR1$^−/−$ in comparison with their controls (Fig. 5C). A statistically signiﬁcant 2.4-fold increase in the number of NOS2$^+$ Mφs was observed in SPLNX B6 mice in comparison with control B6 mice, whereas no differences in NOS2$^+$ Mφs were observed between SPLNX and control gld, lpr, or IFNγR1$^−/−$ mice (Fig. 5D). These data indicated that both IFNγ and Fas/Fasl interactions were required for Mφ activation and that increased Mφ expression of NOS2 correlated with intraocular tumor regression.

To determine whether NO production was required for elimination of intraocular tumors in SPLNX mice, Luc-E.G7 tumor growth was monitored in SPLNX and control NOS2$^+$-deﬁcient B6 mice. Luc-E.G7 grew progressively in all control NOS2$^−/−$ mice, whereas the majority of SPLNX NOS2$^−/−$ mice rejected intraocular Luc-E.G7 tumors (Fig. 5F). P815 tumors were also rejected in SPLNX NOS2$^−/−$ mice on a Balb/C background (Supplementary Table SIII, Exp. 4), which indicated that NO production was dispensable for intraocular tumor elimination in SPLNX mice. Nevertheless, NOS2 expression remained a very sensitive marker of Mφ activation.

To evaluate additional genes associated with intraocular tumor rejection, oligonucleotide microarrays were used to identify gene expression differences between whole tumor-bearing eyes (d15) from control or SPLNX mice (2 mice per group). Relative to controls, SPLNX mice showed increased (>1.5-fold) expression of 1,488 genes and decreased expression of 523. Canonical pathways analysis (IPA) parsed 397 of these genes into 88 signiﬁcantly enriched pathways (Supplementary Table SIV), of which the most signiﬁcant were inﬂammation pathways. As IFNγ mRNA increased in intraocular Luc-E.G7 tumors of SPLNX mice, a group of approximately 600 IFN-stimulated genes was manually assembled from the NCBI Gene database, the Interferome.org database, and Schoggins and colleagues (18); the microarrays surveyed 408 of these genes. In whole tumor-bearing eyes from SPLNX mice, 127 IFNγ-stimulated genes (31.1%) showed increased expression, whereas only nine (2.2%) showed decreased expression.

Only four of the 88 canonical pathways identiﬁed (Cell Cycle Control of Chromosomal Replication, Estrogen-mediated S-phase entry, Role of CHK Proteins in Cell Cycle Checkpoint Control, and Role of BRCA1 in DNA Damage Response) contained more genes showing decreased than increased expression. A net decrease in cell-cycle activity and cell proliferation in the tumor-bearing eyes of SPLNX mice was conﬁrmed by examining 192 cell-cycle (19) genes present on the microarrays: 40 (20.8%) were downregulated, whereas only four (<1%) were upregulated. Of note was Ki67, a marker of cell proliferation, with a valid decrease to 71% of control tumor-bearing eyes.
which was consistent with the lower tumor burden seen in some SPLNX mice at this time.

The expression of inflammatory genes in tumor-bearing eyes of SPLNX and control mice (day 16) was confirmed by RT-PCR (Supplementary Fig. S4). FasL, CXCL2, NOS2, and Tbet expression was significantly greater in tumor-bearing eyes of SPLNX mice. IFNγ expression was also greater in tumors of SPLNX mice, but the increase was not statistically significant. Fas expression was comparable between SPLNX and control tumors. Combined, these data indicated that rejection of intraocular tumors in SPLNX mice was associated with increased expression of genes associated with ocular inflammation.

**Mφs are required for elimination of intraocular Luc-E.G7 tumors in SPLNX mice**

To determine the contribution of Mφs in the elimination of Luc-E.G7 intraocular tumors in SPLNX B6 mice, clodronate liposomes were used to deplete Mφs in vivo according to established protocols (20). In comparison with SPLNX mice that received PBS liposomes, a significantly greater percentage of SPLNX mice given clodronate liposomes showed progressive intraocular tumor growth (Fig. 6A).

To further evaluate the role of Mφs in the elimination of intraocular tumors, we developed a system in which in vitro primed tumor-specific OT-I CD8+ T-cell effectors controlled growth of established intraocular Luc-E.G7 tumors in SPLNX CD8-/- mice. As shown in Fig. 6B and C, Luc-E.G7 tumors grew progressively in control and SPLNX CD8-/- mice reproducing our previous observations. Control (Fig. 6B) and SPLNX (Fig. 6C) CD8-/- mice that were given OT-I CD8 T effectors 10 days after tumor challenge in the a.c. of the eye both showed reduced tumor burden in comparison with that in nontransferred mice. However, the magnitude of tumor regression (fold change in tumor burden between nontransferred and transferred mice) was significantly greater in SPLNX mice than in controls 5 days after transfer (day 15). Hence, despite an equivalent T-cell transfer, the tumoricidal activity of transferred CD8+ T effectors was much greater in SPLNX mice.

To determine whether the interaction between CD8+ T effectors and Mφs contributed to the observed increased tumoricidal activity in OT-I effector-transferred SPLNX CD8-/- mice, SPLNX CD8-/- mice were given PBS or clodronate liposome treatments before CD8+ T-effector cell transfer given 10 days after intraocular Luc-E.G7 tumor challenge. It is important to note that day 10 tumor burden was lower in mice given clodronate liposomes, and this was a consistent observation in multiple experiments. CD8+ T effector transfer at day 10 caused a significant reduction in tumor burden in SPLNX CD8-/- mice treated with PBS liposomes.
In contrast, the same effector T-cell transfer did not cause tumor regression in SPLNX CD8−/− mice treated with clodronate liposomes (Fig. 6F) despite lower tumor burden at the time of transfer. These data confirmed the critical role of Mφs at the effector phase of CD8 T cell–mediated elimination of intraocular tumors in SPLNX mice.

(Fig. 6E). In contrast, the same effector T-cell transfer did not cause tumor regression in SPLNX CD8−/− mice treated with clodronate liposomes (Fig. 6F) despite lower tumor burden at the time of transfer. These data confirmed the critical role of Mφs at the effector phase of CD8 T cell–mediated elimination of intraocular tumors in SPLNX mice.
Discussion

Niederkorn and colleagues (21) reported more than 30 years ago that immune responses directed against tumors transplanted in the a.c. of the eye were different from those induced by transplantation of the same tumors in the skin. For example, P815 tumors that were rejected when placed in the skin of Balb/C mice induced robust DTH and CTL responses directed against minor alloantigens expressed by tumors (2). In contrast, P815 tumors transplanted in the a.c. of the eye grew progressively, did not induce DTH responses, but interestingly, demonstrated tumor-specific CTL responses equivalent to those observed in mice that rejected P815 in the skin (2). The term "anterior chamber–associated immune deviation" (ACAID) was used to describe this unique immune response.

SPLNX terminated ACAID as DTH responses were restored in mice injected with P815 in the a.c. and these intraocular tumors were eliminated (8). The logical interpretation of these data, that CD4 T cell–dependent DTH responses mediated intraocular tumor rejection in SPLNX mice, was further supported by the destructive nature of tumor elimination, which caused fulminant inflammation that culminated in ocular phthisis (8). In contrast, CD8 T cell–mediated rejection of other intraocular tumors left the eye intact (22). However, we definitively demonstrate that CD4+ T-cell responses were not required for rejection of P815 tumors in SPLNX Balb/CJ mice. Rather, CD8+ T cells were necessary. These data are consistent with another study describing a P815 variant, P91, which was spontaneously rejected in the a.c. of non–SPLNX DBA/2 mice (23, 24). In these studies, the administration of depleting anti-CD4 antibodies abrogated DTH responses to P91 antigens measured in the skin but failed to prevent phthisical rejection of intraocular tumors that demonstrated histopathologic features of a DTH response in the eye (23, 24). In contrast, progressive intraocular P91 tumor growth was observed in mice given depleting anti-CD8 antibodies although DTH responses in the skin were manifested (23, 24). Hence, CD8+ T cells are also capable of inducing destructive inflammatory responses resembling DTH responses within the eye that eliminate intraocular tumors by inducing ocular phthisis.

In our study, CD8+ T cells, Mφs, and IFNγ were critical for phthisical elimination of intraocular Luc-E.G7 tumors. These data suggest that SPLNX restored the DTH response (25) within intraocular tumors and support a model in which CD8+ T cells expressed IFNγ to activate Mφs, which induced intraocular inflammation. In further support of that interpretation, gene array analysis indicated increased expression of IFNγ, IFNγ-inducible genes, and several other inflammatory genes in intraocular Luc-E.G7 tumor-bearing eyes of SPLNX mice.

IFNγ and Mφs were also necessary for elimination of another immunogenic tumor cell line, Ad5E1, transplanted in the a.c. (20, 26–29) However, there are several differences between elimination of intraocular Ad5E1 and intraocular P815 or Luc-E.G7 that bear noting. Rejection of intraocular Ad5E1 tumors was spontaneous and did not require SPLNX. In addition, Ad5E1 tumor rejection did not induce ocular phthisis, and required CD4+ but not CD8+ T cells (26–28). The cellular target of IFNγ was also different between the experimental models. IFNγ targeted Ad5E1 tumors directly as intraocular Ad5E1 tumors were rejected in IFNγR1−/− mice (28) and in vitro addition of IFNγ induced Ad5E1 apoptosis via increased expression of TRAIL (27). As Ad5E1 is MHC class II negative, these data suggest that cross-presentation of Ad5E1 tumor antigens to CD4+ T cells by intratumoral Mφs promoted T-cell expression of IFNγ that induced tumor cell apoptosis via TRAIL interactions. IFNγ also appeared to limit tumor vascularization by inducing Ad5E1 expression of antiangiogenic chemokines (28). In contrast, rejection of P815 and Luc-E.G7 in SPLNX mice required that IFNγ-targeted immune cells, including Mφs, within the tumor.

The extent of Mφ activation may determine whether intraocular tumors are eliminated in a sight-preserving or blinding (phthisical) fashion. For example, Coursey and colleagues recently generated Ad5E1 variants that were rejected in a nonphthisical (clone 4) or phthisical (clone 2.1) manner by a process that required Mφs and IFNγ (30). Phthisical rejection was dictated by tumor sensitivity to TNFα, as clone 2.1 tumors were rejected without phthisis in TNFα-deficient mice, whereas TNF receptor 1–deficient mice rejected clone 2.1 tumors in a phthisical manner. These data suggested that a second signal released from necrotic tumor cells in combination with IFNγ induced stronger Mφ activation, thereby causing greater intraocular inflammation that eliminated intraocular tumors by destroying the eye.

Phthisical rejection of intraocular Luc-E.G7 or P815 tumors in SPLNX mice was also associated with Mφ activation that required two signals (IFNγ and Fas/FasL interactions) presumably delivered by CD8+ T cells that were also indispensable. Interestingly, FasL expression by CD8+ T cells was not necessary for regression of Luc-E.G7 intraocular tumors. However, Mφs (31, 32) and CD8+ T cells express both Fas and FasL. Therefore, Fas expression by CD8+ T cells could engage FasL on tumor-associated Mφs to induce their activation. Although Fas/FasL interactions have traditionally been considered proapoptotic, accumulating evidence indicates that Fas/FasL interactions induce Mφ activation (31–34) without apoptosis induction, which supports this model.

We do not fully understand how splenectomy restored tumoricidal activity of CD8+ T cells and Mφs within the eye. One potential explanation is that SPLNX influences tumor-specific CD8+ T-cell numbers within intraocular tumors. Consistent with that notion, Boonman and colleagues demonstrated that another tumor cell line (Ad5E1 plus EJ-ras) transplanted in the a.c. of the eye formed progressively growing tumors in 40% of Balb mice, whereas the remaining mice spontaneously eliminated tumors by a CD8 T cell–dependent process that culminated in phthisis (35, 36). Although tumor growth in both progressor and regressor mice induced expansion of tumor-specific CD8+ T cells in draining lymph nodes, only regressor mice showed systemic dissemination of these effector CD8+ T cells. Hence, these data could suggest that SPLNX restored tumor-specific CD8+ T-cell migration into intraocular Luc-E.G7 tumors.

It is also well appreciated that intraocular tumor growth induces the generation of tumor antigen–specific CD8+ Tregs that mediate suppression of DTH responses to tumor antigens (7). SPLNX prevents the generation of these CD8+ Tregs, suggesting that the splenic microenvironment is critical for...
The role of Mϕs in tumor growth is complex as we demonstrate that intraocular Luc-E.G7 tumors were smaller in B6 mice depleted of Mϕs by clodronate liposome injections (Fig. 6). These data suggest that Mϕs contributed to intraocular tumor growth, which is consistent with the observations of Ly and colleagues (40). Therefore, tumor regression, which also required Mϕs, may be due to the conversion of intratumoral Mϕs from a protumor to an antitumor phenotype. However, it is important to note that direct tumoricidal activity by Mϕs may not be required to eliminate intraocular tumors as phlisis can result from destruction of the ciliary body of the eye (36). Therefore, SPLNX may remove an immunosuppressive mechanism that normally protects the ciliary body from destructive inflammatory mediators expressed by Mϕs.

In conclusion, SPLNX promotes indirect elimination of intraocular tumors by CD8+ T cells that is associated with IFNγ- and Fas/FasL-dependent activation of intratumoral Mϕs. Therefore, mechanisms that maintain ocular IP may interfere with the interaction between CD8+ T cells and Mϕs to limit the immunosurveillance of intraocular tumors.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: K.C. McKenna
Development of methodology: K.M. Beatty, S.H. Thorne, K.C. McKenna
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): M.R. Miller, J.B. Mandell, S.A.K. Harvey, M.J. Rizzo, D.M. Previte, K.C. McKenna
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): M.R. Miller, J.B. Mandell, K.M. Beatty, S.A.K. Harvey, D.M. Previte, S.H. Thorne, K.C. McKenna
Writing, review, and/or revision of the manuscript: M.R. Miller, J.B. Mandell, S.A.K. Harvey, K.C. McKenna
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): M.R. Miller, D.M. Previte, S.H. Thorne, K.C. McKenna
Study supervision: K.C. McKenna

Acknowledgments
The authors thank Nancy Zarowski for excellent technical assistance in flow cytometry; Robert Hendricks, Walter Storkus, and Pawel Kalinski for critical review of this article; Richard Bilionick for statistical analysis; Joseph Brown and Jessamee Mellon (University of Texas Southwestern Medical School) for preparation of liposomes; and Rachel Sikorski for transduction of E.G7-OVA to express firefly luciferase. The authors regret to announce Rachel’s untimely death and dedicate this article to her memory.

Grant Support
This work was supported by NIH grants R01 EY018355 (to K.C. McKenna, Principal Investigator), P30 EY00809, and F30 CA047904, The Eye and Ear Foundation of Pittsburgh, and by an unrestricted grant from Research to Prevent Blindness, Inc.

Received May 13, 2014; revised September 5, 2014; accepted September 14, 2014; published OnlineFirst September 23, 2014.

References
Mechanisms of Intraocular Tumor Elimination


Cancer Immunology Research

Splenectomy Promotes Indirect Elimination of Intraocular Tumors by CD8+ T Cells That Is Associated with IFNγ- and Fas/FasL-Dependent Activation of Intratumoral Macrophages

Maxine R. Miller, Jonathan B. Mandell, Kelly M. Beatty, et al.


Updated version
Access the most recent version of this article at:
doi:10.1158/2326-6066.CIR-14-0093-T

Supplementary Material
Access the most recent supplemental material at:
http://cancerimmunolres.aacrjournals.org/content/suppl/2014/09/23/2326-6066.CIR-14-0093-T.DC1.html

Cited articles
This article cites 40 articles, 25 of which you can access for free at:
http://cancerimmunolres.aacrjournals.org/content/2/12/1175.full.html#ref-list-1

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.