CTL Induction of Tumoricidal Nitric Oxide Production by Intratumoral Macrophages Is Critical for Tumor Elimination

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To characterize mechanisms of CTL inhibition within an ocular tumor microenvironment, tumor-specific CTLs were transferred into mice with tumors developing within the anterior chamber of the eye or skin. Ocular tumors were resistant to CTL transfer therapy whereas skin tumors were sensitive. CTLs infiltrated ocular tumors at higher CTL/tumor ratios than in skin tumors and demonstrated comparable ex vivo effector function to CTLs within skin tumors indicating that ocular tumor progression was not due to decreased CTL accumulation or inhibited CTL function within the eye. CD11b$^+$Gr-1$^+$F4/80$^+$ cells predominated within ocular tumors, whereas skin tumors were primarily infiltrated by CD11b$^+$Gr-1$^+$F4/80$^+$ macrophages (Mφs), suggesting that myeloid derived suppressor cells may contribute to ocular tumor growth. However, CD11b$^+$ myeloid cells isolated from either tumor site suppressed CTL activity in vitro via NO production. Paradoxically, the regression of skin tumors by CTL transfer therapy required NO production by intratumoral Mφs indicating that NO-producing intratumoral myeloid cells did not suppress the effector phase of CTL. Upon CTL transfer, tumoricidal concentrations of NO were only produced by skin tumor-associated Mφs though ocular tumor-associated Mφs demonstrated comparable expression of inducible NO synthase protein suggesting that NO synthase enzymatic activity was compromised within the eye. Correspondingly, in vitro-activated Mφs limited tumor growth when co-injected with tumor cells in the skin but not in the eye. In conclusion, the decreased capacity of Mφs to produce NO within the ocular microenvironment limits CTL tumoricidal activity allowing ocular tumors to progress. The Journal of Immunology, 2010, 185: 000–000.
CTL responses in vitro (5), MDSCs could play a role in ocular tumor development by suppressing tumor-specific CTLs. Finally, tumoricidal activity of CTLs could be ineffective because of their failure to induce tumoricidal activity in other cells within the tumor microenvironment that are critical for tumor elimination. For example, Hollenbaugh and Dutton (22) demonstrated that elimination of established skin tumors in mice by CTL transfer therapy required NO production by unidentified host cells, which was induced by IFN-γ expressed by CTLs.

To elucidate the mechanisms of CTL inhibition within the ocular tumor microenvironment, we tracked and functionally characterized tumor-specific CTLs transferred into mice with tumors developing in the a.c. of the eye or in skin. We demonstrate that ocular tumors are resistant to CTL transfer therapy whereas skin tumors are exquisitely sensitive. Transferred CTLs infiltrated both tumor sites and surprisingly displayed similar effector function in terms of cytokine production (TNF, IFN-γ) and granule exocytosis, despite the presence of CD11b+ cells, which were suppressive to CTL responses in vitro via NO production. We extend the previous findings of Hollenbaugh and Dutton (22) by showing that transferred CTLs activate skin tumor-associated CD11b+F4/80+ Mφs to produce NO at tumoricidal concentrations. In contrast, ocular tumor-associated myeloid cells, primarily composed of CD11b+Gr-1− cells and few CD11b+F4/80+ Mφs in this model, are profoundly inhibited in their tumoricidal capacity because of their inability to produce tumoricidal levels of NO. We conclude that intratumoral myeloid cells do not suppress the effector phase of CTL responses and that the failure of CTLs to control ocular tumor development is due to inhibited tumoricidal activity of intratumoral Mφs.

Materials and Methods

Experimental animals

Male and female C57BL/6.PL (B6.PL; H-2b, CD90.1+), C57BL/6 (B6; H-2b, CD90.2+), B6.129S7-Rag1tm1Mom/J (RAG1−/−) (23) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). OT-I mice were backcrossed with B6.PL mice to Ot1−/− mice for experiments. Tumor cell lines (3×105 cells) were injected i.v. via the tail vein in 200 μl PBS and were tracked in recipient B6 mice in vivo using OVA257-264 Kb tetramers or CD90.1 as a congenic marker by flow cytometric analysis (Supplemental Fig. 1F–I).

Ocular tumor definitions

The anterior segment of the eye containing ocular tumors was dissected from the posterior of the eye using surgical scissors. The iris was then removed, and the anterior segment was blocked with purified anti-CD16/32 mAb (BD Pharmingen, San Diego, CA), then incubated with PE-labeled anti-CD90.1, Pacific Blue-labeled anti-CD11b, and FITC-labeled anti-CD45, anti-CD124 (IL-4Rα), Gr-1, Ly-6C, Ly-6G, MHC class I (H-2Kd), MHC class II (I-Aε), anti-CD4, anti-CD8, anti-CD11c, anti-CD40, anti-CD45, B220 (CD45RB), CD8, CD86, CD90.1, CD90.2, CD107a, CD115 (M-CSF), CD124 (IL-4Rα), F4/80, FceRI, Gr-1, Ly-6C, Ly-6G, MHC class I (H-2Kd), MHC class II (I-Aε), eBioscience, San Diego, CA), or PE-Cy7−/−C4d−2/− mice (Biolegend, San Diego, CA), or allylphycocyanin-conjugated H-2Kd tetramers containing the C57BL6 immunodominant peptide SIINFEKL (Beckman-Coulter, Miami, FL), and fixable violet dead cell stain (Invitrogen). After incubation, wells were washed and resuspended in 0.2 ml FACS buffer or fixed with Cytofix/Cytofix reagent (BD Pharmingen). When needed, fixable violet dead cell stain was resuspended in Perm/Wash buffer (BD Pharmingen). For intracellular staining of OT-I CTLs, cell suspensions were incubated with allylphycocyanin-conjugated or PE-Cy7−/−C4d−2/− tetramers anti-IFN-γ, anti-granzyme B, and anti-TNF mAbs (BD Pharmingen) or respective labeled
isotype control Abs. For intracellular staining of intratumoral myeloid cells, cells were blocked overnight in Perm/Wash buffer (BDPharmingen) containing 5% donkey serum, then cell suspensions were incubated with unconjugated anti-NO synthase (anti-NOS2) polyclonal rabbit Ab (BD Pharmingen) and mouse anti-arginase 1 (ARG1) mAb (BD Pharmingen), followed by incubation with PE- or AF546-conjugated goat anti-rabbit IgG (Invitrogen) and aliphophycocyanin-conjugated rat anti-mouse IgG1 (BD Pharmingen), respectively. Samples were run on a FACSArray or FACS caliber flow cytometer (Becton Dickinson, San Jose, CA), and data were analyzed using FACS Diva (Becton Dickinson) and FlowJo software (Tree Star, Ashland, OR). For some experiments, the integrated median fluorescence intensity (IMFI) was calculated by multiplying the percentage of positive cells by the median fluorescence intensity as described elsewhere (26).

Ex vivo stimulation of transferred OT-I CTLs

Tumors and spleens were harvested 6 d after CTL transfer therapy and were rendered into single-cell suspensions as described earlier. Cells (4 × 10⁶) from skin tumor cell suspensions or 0.2-ml ocular tumor cell suspensions were incubated for 6 h at 37°C in a 5% CO₂ atmosphere with or without SIINFEKL peptide (10 μg/ml) in either SGM containing brefeldin A (BD Biosciences) when cytokines were measured or monensin and anti-CD107a mAb (BD Pharmingen) when degranulation was measured. After stimulation, cell suspensions were stained to determine surface CD107a expression and intracellular expression of granzyme B, TNF, and IFN-γ by viable OT-I CTLs (violet dead cell stain negative, CD8⁺, CD90.2⁻/CD90.1⁺ cells) using flow cytometry.

Flow cytometric cell sorting of tumor-associated myeloid cells

Skin or eye tumors were rendered into single-cell suspensions, then magnetic cell sorting with the EasySep PE selection kit (Stem Cell Technologies, Vancouver, British Columbia, Canada) was performed to exclude CD90.2⁺ tumors from cell suspensions to those flow cytometric cell sorting. Nonselected cells were stained with fluorochrome-conjugated anti-CD45, anti-CD11b, and anti-Gr-1 mAbs, and CD11b⁺Gr-1⁻ cells were then isolated by flow cytometric cell sorting for use in T cell suppression and tumoricidal assays. Purity of sorted populations was always higher than 95% as shown in Supplemental Fig. 2.

T cell suppression assay

Naïve splenocytes from OT-I mice were diluted 1:5 with naïve splenocytes from B6.Pl mice. Cells (4.0 × 10⁶) were added to an individual well of a 24-well flat-bottom plate with or without the addition of CD11b⁺ cells (0.5 × 10⁶) isolated from ocular tumors or skin tumors, with or without SIINFEKL (0.1 μg/ml) and with or without the addition of Nω-nitro-L-arginine (L-NAME, Calbiochem, EMD Biosciences, San Diego, CA) (0.5 mM final concentration) in a total volume of 2 ml SGM. After incubation for 4 d at 37°C in a 5% CO₂ atmosphere, the number of live cells was determined by trypan blue exclusion, and the percentage of live OT-I cells was determined by flow cytometric analysis of cultured cells stained with 7-aminoactinomycin D, FITC-labeled anti-CD8a, and PE- or AF546-conjugated anti-Gr-1 mAbs. All experiments were performed at 7-aminoactinomycin D negative, CD8⁺, and CD90.2⁻. Cultured cells were tested for lytic activity using a standard chromium release assay performed as described earlier.

In vitro tumor growth inhibition assay

E.G7-OVA tumors (5 × 10⁵) were cultured with or without 10⁵ cells of the indicated flow-sorted CD11b⁺ cell subset. Three days later, nitrite was measured to determine the percentage of inhibition of E.G7-OVA tumor growth using nitrite assay. Tumor cells were identified as CD45⁺/CD90.2⁺, CD8⁺/CD11b⁻/Gr-1⁻ cells by viable OT-I CTLs (violet dead cell stain negative, CD8⁺, CD90.2⁻/CD90.1⁺ cells) using flow cytometry.

Statistical analysis

Statistical analyses were performed using Prism 5 software (GraphPad, La Jolla, CA). For each studied variable, the mean, median, and SD were calculated, and to determine normality the D’Agostino–Pearson test was used. Differences in indicated cell numbers or percentages were compared by unpaired Student t test or unpaired Mann-Whitney U tests depending on the normality of the data. For comparison of multiple groups, one-way ANOVA or Kruskal-Wallis test was on with ranks with Tukey’s or Dunn’s multiple comparison post hoc tests to compare the individual groups, respectively, were used depending on the normality of the data. Two-way ANOVA was used for comparison of skin tumor growth over time. Differences in the incidence of measurable tumors were computed with the Kaplan–Meier method, and differences were validated with log-rank test; p values < 0.05 were considered statistically significant.

Results

Established ocular tumors are resistant to CTL transfer therapy

E.G7-OVA tumors grow progressively in the a.c. of the eye despite priming for OVA-specific CD8⁺ T cells responses capable of rejecting a subsequent injection of E.G7-OVA in the skin or opposite eye (5). These data indicate that CTLs can demonstrate effector function within the eye but are inhibited within the established ocular tumor microenvironment. Therefore, to determine the fate of CTLs within ocular tumors, we transferred CD90.1⁺ OVA-specific OT-I CTL effectors into CD90.2 congenic B6 mice before and 3 or 5 d after injection of E.G7-OVA in the a.c. of the eye.

OT-I CTL transfer before a.c. injection of 10⁴ E.G7-OVA delayed the kinetics of ocular tumor growth reducing tumor burden (17-fold) on day 7 in comparison with that in non-transferred control mice (Fig. 1A). Over time, however, tumor growth was uncontrolled. Delaying the time of CTL transfer after tumor challenge minimized the effectiveness of CTLs to control ocular tumor growth. For example, when CTLs were transferred 3 d after tumor challenge, tumor burden was reduced by 31-fold on day 11, whereas only a 4-fold reduction was observed when CTL transfer occurred on day 5 (Fig. 1B). As in our previous experiment, ocular tumor growth ultimately became uncontrolled. Increasing tumor burden over time could not entirely explain the failure of CTLs to eliminate established ocular tumors because an equivalent CTL transfer before (Fig. 1C) or 10 d after (Fig. 1D) injection of a much larger tumor challenge (10⁶) in the skin mediated rejection of intradermal tumors in the majority of mice, which reproduces previously published observations (22, 27). These data indicated that established ocular tumors were resistant to CTL transfer therapy whereas skin tumors were sensitive.

In vitro tumor growth inhibition assay

E.G7-OVA tumors (5 × 10⁵) were cultured with or without 10⁵ cells of the indicated flow-sorted CD11b⁺ cell subset. Three days later, nitrite concentration was determined in culture supernatants using the Griess assay (Promega, Madison, WI), and cells were harvested to determine the percentage of inhibition of E.G7-OVA tumor growth using flow cytometry. Tumor cells were identified as CD45⁺/CD90.2⁺, CD8⁺/CD11b⁻/Gr-1⁻ cells by high forward scatter events, and Count Bright absolute counting beads (Invitrogen) were used to collect half of each total cell sample to determine the number of tumor cells per sample. Percentage growth inhibition was determined as follows: 100 × (1 – [number of E.G7-OVA in experimental sample/number of E.G7 in control sample]).

Winn-type assays

Møs were harvested from the peritoneal cavities of B6 mice that had been injected with 2 ml thioglycolate 4 d previously, and Møs were activated in vitro with a combination of IFN-γ (200 U/ml) and TNF (100 U/ml) for 48 h. The activated Møs were mixed with E.G7-OVA tumor cells to obtain a 30:1 or 100:1 ratio with a final concentration of 5 × 10⁴ E.G7-OVA/ml, immediately before tumor injection of RAG1⁻/⁻ mice. Two microliters or 200 μl of these cell suspensions was injected into the a.c. (10⁴ tumor cells) or intradermally (10⁵ tumor cells) to produce ocular or skin tumors, respectively. Tumor growth was then monitored as described earlier.

Real-time quantitative RT-PCR

RNA was isolated using the RNA easy kit (Qiagen, Germantown, MD) from whole tumor bearing eyes or skin tumor biopsies. cDNA was then prepared using a high-capacity cDNA reverse transcription kit (Applied Biosystems, Carlsbad, CA), and expression of CXCL1 (primer Mm00433859_m1), CXCL2 (primer Mm00434645_m1), CSF-1 (primer Mm00432688), CSF-2 (primer Mm00432688), CSF-3 (primer Mm00438334_m1), and the housekeeping gene PCX (primer Mm00500992_m1) was determined by quantitative real-time PCR using the TaqMan PCR universal mix (Applied Biosystems) and a Step One Plus real-time PCR thermocycler (Applied Biosystems). All primers were obtained from Applied Biosystems. Relative expression was determined by normalizing gene expression (40 – Ct) to the expression of PCX gene in each sample.

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Tumor-specific CTLs efficiently infiltrate both tumor sites

Failure to control ocular E.G7-OVA tumor growth could be due to limited accumulation of CTLs within ocular tumors or to inhibited
CTL migration into the tumor site. Therefore, flow cytometry and confocal microscopy were used to evaluate infiltration of CD90.1+ OT-I CTLs into skin and eye tumors. Five days after injection of E.G7-OVA tumor cells in the a.c. (10^6) (A, B) or intradermally in the flank (10^5) (C, D) at the indicated time points. Ocular tumor burden was measured by flow cytometric analysis, and skin tumor measurements were made with a caliper. Each symbol or line indicates the measurement from an individual mouse. For A and B, lines are medians. The p values for A were calculated by Kruskal-Wallis test on ranks with Dunn’s comparison post test and for B by one-way ANOVA and Tukey’s multiple comparison post test (data were pooled from at least two independent experiments). **p < 0.01; ***p < 0.001. For C, p < 0.01 on day 9, and p < 0.001 on the subsequent days; for D, p < 0.001 on days 7 and 9, by two-way ANOVA and Tukey’s multiple comparison post test (one experiment shown, representative results from two independent experiments).

ctl migration into the tumor site. Therefore, flow cytometry and confocal microscopy were used to evaluate infiltration of CD90.1+ OT-I CTLs into skin and eye tumors. Five days after injection of E.G7-OVA in the a.c. or 10 d after tumor injection in the skin, mice received an i.v. OT-I CTL transfer. Six days later, flow cytometric analysis was performed and demonstrated that intratumoral CD90.1+ OT-I CTLs were present in both tumor sites and were easily distinguished from CD90.2+ tumor cells and endogenous CD90.2+ T cells (Fig. 2A), which were very few in number (Supplemental Fig. 3). Importantly, though ocular tumors were resistant and skin tumors sensitive to CTL transfer therapy, the ratio of OT-I CTLs to tumor cells in ocular tumors was more than 50 times higher than that in skin tumors (Fig. 2B). However, our flow cytometric analysis of whole collagenase-digested tumor-bearing eyes did not allow us to discern whether CTLs were present within the ocular tumor or in other areas of the eye. Therefore, confocal microscopy of ocular tumor wholemounts stained with fluorescently labeled Abs was performed. This analysis demonstrated that CD90.1+ OT-I CTLs extensively infiltrated ocular tumors and were in close proximity to CD90.2+ tumor cells and infiltrating CD11b+ myeloid cells (Fig. 2C). The migration of tumor-specific CTLs into ocular tumors was regulated by IFN-γ, as IFN-γR1−/− mice demonstrated reduced numbers of infiltrating OT-I CTLs compared with that in wild-type mice (Supplemental Fig. 4). These data indicated that the failure of CTLs to control ocular tumors was not due to impaired infiltration of ocular tumors.

OT-I CTLs demonstrate effector function in the ocular tumor microenvironment

Previous studies have demonstrated that IFN-γ produced by transferred OT-I CTLs is required to promote skin tumor rejection, whereas CTL-expressed perforin, FasL, and TNF are dispensable (22, 27). Therefore, we tested whether differences in IFN-γ production or in other canonical effector functions performed by CTLs infiltrating skin or ocular tumors accounted for the failure of transferred CTLs to control ocular tumor growth. B6 mice were challenged with tumors in the eye (10^5) or skin (10^6). Five days after eye tumor challenge or 10 d after skin tumor challenge, mice received an OT-I CTL transfer. Tumors and spleens from both groups were then harvested and processed independently into single-cell suspensions 6 d after CTL transfer and stimulated ex vivo with SIINFEKL peptide. OT-I CTLs

FIGURE 1. Ocular tumors are resistant to tumor-specific CTL transfer therapy. OT-I CTLs were i.v. transferred before (A, C) or after (B, D) the injection of E.G7-OVA tumor cells in the a.c. (10^6) (A, B) or intradermally in the flank (10^5) (C, D) at the indicated time points. Ocular tumor burden was measured by flow cytometric analysis, and skin tumor measurements were made with a caliper. Each symbol or line indicates the measurement from an individual mouse. For A and B, lines are medians. The p values for A were calculated by Kruskal-Wallis test on ranks with Dunn’s comparison post test and for B by one-way ANOVA and Tukey’s multiple comparison post test (data were pooled from at least two independent experiments). **p < 0.01; ***p < 0.001. For C, p < 0.01 on day 9, and p < 0.001 on the subsequent days; for D, p < 0.001 on days 7 and 9, by two-way ANOVA and Tukey’s multiple comparison post test (one experiment shown, representative results from two independent experiments).
infiltrating both tumor sites exhibited equivalent production of IFN-γ, TNF, and granzyme B and comparable release of lytic granules, as measured by CD107a expression (Fig. 3). Notably, tumor-infiltrating OT-I CTL expression of both IFN-γ and TNF was markedly reduced compared with that of splenic CTLs at both tumor sites (Fig. 3). These data indicated that canonical CTL effector functions were not compromised within the ocular tumor microenvironment.

FIGURE 3. Tumor-infiltrating OT-I CTLs exhibit similar effector function in skin and ocular tumor microenvironments. Cytokine production and granule exocytosis by tumor-infiltrating CD90.1+ OT-I CTLs was determined by flow cytometric analysis. Contour plots display expression of IFN-γ and TNF (A) or granzyme B (Gzm B) and CD107a (C) by gated transferred OT-I CTLs, respectively. Data shown are from a representative mouse of each group (n = 4 per group); numbers indicate the percentage of cells within each quadrant. Mean percentages of OT-I CTLs positive only for one marker or for both from all mice within each group (n = 12 per group) are shown in B and D. Data shown were pooled from three independent experiments; scale bars indicate mean ± SD.
Characterization of intratumoral myeloid cell populations in skin and ocular tumors

We previously demonstrated that ocular tumors were heavily infiltrated by CD11b+Gr-1+ cells and that CD11b+ cells isolated from ocular tumors inhibited CTL activity in vitro (5). Therefore, we characterized the CD45+ infiltrate of both skin and eye tumors by flow cytometry to determine whether differences in myeloid cell subsets may be associated with sensitivity or resistance to CTL transfer therapy. Greater than 95% of CD45+ cells within ocular and skin tumors of C57BL/6 mice were composed of CD90.2+ cells and CD11b+ cells (Fig. 4A). In non-CTL-transferred mice, 99% of these CD90.2+ cells within ocular tumors were E.G7-OVA tumor cells and the remainder was endogenous tumor-infiltrating CD3ε+ cells as shown by the characterization of the cellularity of ocular tumors in CD90.1 congenic B6.PL mice and RAG1−/− mice (Supplemental Fig. 3). This characterization of ocular tumors also indicated that the small percentage of CD45+ cells (∼3−5%) that were not tumors or CD11b+ cells were primarily CD4+ CD8+ CD3ε+ T cells, B220+ cells, and NK 1.1+ cells (Supplemental Fig. 3B). FcεRI+ mast cells were not observed (Supplemental Fig. 3B). The number of tumors was comparable in B6.PL and RAG-1−/− mice, whereas a modest (2-fold) reduction was observed in CD11b numbers in RAG1−/− mice indicating that T cells were largely not required for CD11b+ cell infiltration (Supplemental Fig. 3).

Ocular tumors were composed of a greater percentage of CD11b+ cells in comparison with skin tumors (Fig. 4A). The proportion of tumor cells to CD11b+ cells in ocular and skin tumors that received a CTL transfer or were untreated did not differ significantly at the examined time point (Fig. 4A, 4B). However, striking differences in the composition of intratumoral CD11b+ myeloid cell subsets were observed, as CD11b+Gr-1+F4/80+ cells predominated in ocular tumors (63.81 ± 9.6% of CD11b+ cells), reproducing our previous results (5), whereas CD11b+Gr-1−F4/80+ cells predominated in skin tumors (76.48 ± 4.1% of CD11b+ events) (Fig. 4C, 4D). Upon OT-I CTL transfer therapy of day 5 ocular E.G7-OVA tumors, the percentage of intratumoral CD11b+Gr-1−F4/80+ Mφs doubled, whereas within E.G7-OVA skin tumors of CTL transferred mice, the percentage was unchanged (Fig. 4C, 4D). These data indicated that increased percentages of intratumoral F4/80+ cells were associated with tumor regression.

The percentage of CD11b+Gr-1+ cells within ocular tumors of B6.PL mice and RAG1−/− mice was equivalent indicating that T cells were not required for Gr-1+ cell accumulation though T cells partially contributed to F4/80+ cell infiltration (Supplemental Fig. 3). The unique accumulation of Gr-1+ cells within ocular tumors may have been due to differences in the expression of chemokines that induce their recruitment, as a 3-fold increase in CXCL2 expression was observed within ocular tumors (Supplemental Fig. 5A). However, Gr-1+ cell accumulation was not common to all tumors developing in the eye, as P815 tumors injected into the a.c. of BALB/cJ mice were infiltrated primarily by CD11b+Gr-1−F4/80+ Mφs (Supplemental Fig. 5B).

Further characterization of the phenotype of Gr-1− and Gr-1+ myeloid cell subpopulations was also performed (Supplemental Fig. 6). These myeloid cell subpopulations differed in their expression of Ly-6G and Ly-6C, with Gr-1− cells being completely negative for Ly-6G but partially positive for Ly-6C, whereas Gr-1+ cells expressed high levels of both markers (Supplemental Fig. 6A). In addition, Gr-1− cells expressed high levels of F4/80 and CD68 in both tumor sites, whereas Gr-1+ cells expressed lower levels of these markers. A greater percentage of Gr-1− cells expressed CD11c, whereas comparable expression of CD124 and CD115 was observed between Gr-1+ and Gr-1− cells in ocular tumors. CD115 expression was greater in Gr-1− cells than in Gr-1+ cells of skin tumors (Supplemental Fig. 6A). These data indicated that Gr-1− cells were essentially composed of Mφ. This
conclusion was further supported by the almost exclusive expression of high levels of MHC class I, MHC class II, and co-stimulatory molecules (CD40, CD80, CD86) in the Gr-1+ sub-population (Supplemental Fig. 6B). CD40 expression was reduced in ocular tumor-associated Mϕs, which is consistent with other published observations (28). Notably, the levels of MHC class I, MHC class II, and CD86 appear to be regulated by IFN-γ as these molecules were downregulated in IFN-γR1−/− mice whereas the levels of CD40 and CD80 were essentially IFN-γ independent in our model system (Supplemental Fig. 6B).

**Immunosuppressive and tumoricidal activities of intratumoral CD11b+ cells are mediated by NO**

To determine whether myeloid cells isolated from eye but not skin tumors were suppressive to T cell responses, OT-I T cells were stimulated with cognate Ag in the presence of CD11b+ cells isolated from eye (Fig. 5A–C) and skin tumors (Fig. 5D–F) of non-CTL-transferred mice. Myeloid cells isolated from both tumor sites suppressed the lytic activity of CTLs primarily by inhibiting CTL expansion. This suppression by tumor-associated CD11b+ cells was mediated by NO, as nitrite levels, an indirect measure of NO production, were elevated in supernatants of cultures containing tumor-associated CD11b+ cells, and the addition of NMMA, an inhibitory l-arginine analogue, inhibited NO production and restored lytic activity.

Paradoxically, Hollenbaugh and Dutton (22) demonstrated that the regression of established E.G7-OVA skin tumors by transferred OT-I CTLs required IFN-γ expression by CTLs and IFN-γR expression and NO production by host cells. As CD11b+ F4/80+ Mϕs produce tumoricidal NO when stimulated with IFN-γ and TNF, we hypothesized that interactions between CTLs and intratumoral F4/80+ Mϕs to induce tumoricidal concentrations of NO were critical for skin tumor elimination and that reduced production of NO by F4/80+ Mϕs within the ocular tumor microenvironment could explain CTL evasion by ocular tumors.

To determine the tumoricidal activity of intratumoral myeloid cell populations from both tumor sites, flow cytometric cell sorting was used to isolate CD11b+Gr-1+ and CD11b+Gr-1− populations (Supplemental Fig. 2), the latter being primarily composed of F4/80+ cells as shown in Fig. 4C. In vitro tumor growth was markedly inhibited by F4/80+ Mϕs (96% inhibition) and to a lesser extent by Gr-1+ myeloid cells (44% inhibition) isolated from skin tumors of CTL transferred mice but not by myeloid cells isolated from non-transferred mice (Fig. 6A). In contrast, F4/80+ Mϕs and Gr-1+ myeloid cells isolated from ocular tumors of CTL transferred mice failed to inhibit in vitro tumor growth (Fig. 6A). Tumor growth inhibition by skin tumor-associated F4/80+ Mϕs was associated with increased nitrite in culture supernatants (Fig. 6A). To evaluate NO production in vivo, nitrotyrosine (NT) levels were measured in skin and ocular tumors of B6 mice, IFN-γR1−/− mice, and NOS2−/− mice (negative control). CTL transfer increased NT levels in skin but not eye tumors, which showed NT levels equivalent to tumors in NOS2−/− mice. These data confirm in vivo production of NO within skin but not ocular tumor microenvironments (Fig. 6B). NT levels were reduced in skin tumors of CTL transferred IFN-γR1−/− mice indicating the contribution of IFN-γ toward inducing NO production by intratumoral Mϕs. Furthermore, skin tumor regression was not observed in CTL transferred NOS2−/− mice (Fig. 6C), reproducing previously published results (22), and the transient regression of ocular tumors upon CTL transfer was unchanged in NOS2−/− mice (Fig. 6D). These data indicate that direct CTL activity remains in NOS2−/− mice and is responsible for only a temporary reduction in ocular or skin tumor burden. Compromised tumoricidal activity in intratumoral Mϕs thereby results in progressive tumor growth.

**FIGURE 5.** Tumor-associated CD11b+ cells inhibit T cell proliferation by NO production. Splenocytes from OT-I mice were untreated or stimulated with cognate Ag (SIINFEKL) in the presence or absence of tumor-associated CD11b+ cells isolated from the eye (A–C) or skin (D–F), and with and without L-NMMA, an inhibitor of NOS2 activity. After 4 d of incubation, lytic activity against chromium-labeled E.G7-OVA cells was measured at a 67:1 effector/target ratio (A, D), the number of OT-I T cells within each culture was quantified by flow cytometry (B, E), and nitrite concentrations in culture supernatants were measured using the Griess assay (C, F). A–C and D–F display results from a single experiment that is representative of four independent experiments for ocular tumor-associated myeloid cells and two independent experiments for skin tumor-associated myeloid cells. Nitrite was measured in one experiment for ocular tumor-associated myeloid cells and in both experiments for skin tumor-associated myeloid cells. Dashed line in C and F indicates the threshold of detection for the measurement of nitrite by the Griess assay. *p < 0.5; **p < 0.01, ***p < 0.001, one-way ANOVA and Tukey’s multiple comparison post-test.
Inhibited NO production within ocular tumor-associated Mφs is not associated with decreased NOS2 protein expression

To identify potential mechanisms responsible for decreased NO production in ocular tumor-associated myeloid cells, the expression of NOS2 and ARG1 proteins in ocular or skin tumor-associated CD11b+ cells was evaluated by flow cytometric analysis (Fig. 7). Gr-1+ myeloid cells within both tumor microenvironments were primarily negative for NOS2 and ARG1 regardless of whether mice received a CTL transfer (Fig. 7A). In contrast, the majority of ocular and skin tumor-associated F4/80+ Mφs expressed ARG1 but only low levels of NOS2 in non-transferred mice. Upon CTL transfer, ARG1 expression was maintained, but the expression of NOS2 increased 10-fold in both skin and eye tumors (Fig. 7B) confirming the observations of Hu et al. (29).

Tumoricidal activity of activated F4/80+ Mφs is inhibited within the eye

Numerous molecules, including TGF-β, α-melanocyte-stimulating hormone, and calcitonin gene-related peptide, present in normal aqueous humor (AqH), the fluid filling the a.c., are capable of suppressing various aspects of Mφ activation (30). To explore the possibility that the normal a.c. microenvironment was capable of suppressing activated Mφs, we tested whether in vitro activated Mφs displaying tumoricidal activity could be inactivated in vivo by placement into the a.c. of the eye using Winn-type assays (31). Thiglycolate-elicited F4/80+ Mφs were harvested from the peritoneal cavities of B6 mice and then activated in vitro to induce tumoricidal activity. Activated F4/80+ Mφs were then mixed with E.G7-OVA cells immediately before a.c. or intradermal injection of RAG1−/− mice. RAG1−/− mice were used in these experiments to remove the contribution of endogenous tumor-specific CD8+ T cell responses, which inhibit the development of skin tumors when mice are injected with limited numbers of tumor cells intradermally (5).
The a.c. microenvironment suppressed the tumoricidal activity of activated F4/80+ Mφs as evidenced by equivalent ocular tumor numbers in mice injected with tumors alone or with tumors and activated F4/80+ Mφs (Fig. 8A). In contrast, activated F4/80+ Mφs reduced the initial tumor burden in the skin delaying the kinetics of skin tumor growth (Fig. 8B) and reducing the incidence of measurable skin tumors (Fig. 8C). OT-I CTLs co-injected with E.G7-OVA cells into the a.c. transiently reduced the number of tumor cells in the eye, confirming that CTL activity was not inhibited in the eye (Supplemental Fig. 8). Therefore, the ocular microenvironment alone is capable of completely suppressing the effector function of activated F4/80+ Mφs but not that of CTLs.

**Discussion**

The mechanisms that inhibit tumoricidal activity of CTLs within an ocular tumor microenvironment are not fully understood. Miki et al. (32) demonstrated that tumor-specific CTLs injected intratumorally completely eliminated ocular tumors in mice when the CTL/tumor cell ratio was 1:6, whereas a ratio of 1:50 failed to cure. Therefore, one proposed explanation is that primary ocular tumor growth outpaces priming and expansion of CTLs in secondary lymphoid organs. Hence, primed CTL effectors infiltrating primary ocular tumors could encounter an unmanageable number of tumor cells. However, our data indicate that this simplistic explanation cannot explain the failure of CTLs to control ocular
tumors because a CTL transfer that is unable to control ocular tumor growth eliminates a much larger tumor burden (100-fold) in the skin (Fig. 1).

CTLs were not excluded from ocular tumors, rather, the CTL/tumor cell ratio was greater in ocular tumors than in skin tumors (Fig. 2). This result was intriguing because the tissues lining the a.c. of the eye express CD95L (FasL) (13), TRAIL (14), and PD-L1/PD-L2 (15), which are capable of inducing apoptosis of activated CTLs that express CD95 (Fas), TRAIL-R2 (DR5), and PD-1. However, our studies cannot exclude the possibility that ocular tumor growth decreased expression of these death-inducing molecules on ocular tissues allowing CTLs to accumulate within ocular tumors. Regardless, CTLs infiltrating ocular tumors were ineffective at controlling progressive tumor growth, and lower CTL/tumor ratios promoted rejection of skin tumors in the majority of mice.

We anticipated that CTLs infiltrating ocular tumors would be functionally inhibited within the eye because PD-L1, a molecule that can induce CTL exhaustion (33, 34), is expressed in the a.c. Moreover, normal AqH contains immunosuppressive molecules including TGF-β2, vasoactive intestinal peptide, somatostatin, and α-melanocyte-stimulating hormone, which can inhibit T cell effector function and/or promote conversion to FoxP3+ T regulatory cells (Tregs) (30). However, we observed no differences in cytokine production or granule exocytosis between PD-1+ CTLs (data not shown) infiltrating tumors developing in the skin and those infiltrating tumors developing in the eye (Fig. 3). One explanation for these results is that CTL effectors that were generated in vitro were less susceptible to immunosuppressive molecules expressed within the eye. However, endogenous CD8+ T cells primed by ocular tumor growth in one eye were shown to differentiate into CTL effectors that rejected a subsequent challenge with the original tumor in the opposite eye (5, 8). These data indicate that endogenous CTL effectors generated by ocular tumor growth in vivo are also functional within the eye. In addition, UVSC25, an ultraviolet-induced fibrosarcoma, is spontaneously rejected by tumor-specific CD8+ T cells in the eye (35) providing further support that CTL effector function is not compromised within the a.c. of normal or tumor-bearing eyes. Therefore, our data clearly indicate that in vitro-differentiated CTLs are not inhibited in the ocular tumor microenvironment, suggesting another critical effector cell being dysfunctional.

Hollenbaugh et al. (27) showed that direct lysis of E.G7-OVA tumor cells by CTLs is not required to promote skin tumor rejection, as transferred CTLs deficient in perforin, TNF, or FasL eliminated established skin tumors. CTL expression of IFN-γ, however, was critical for tumor regression and acted indirectly by induction of NOS2-mediated tumoricidal activity in another cell population, as IFN-γ alone or in combination with TNF did not affect E.G7-OVA growth in vitro, and CTL transfer failed to eliminate E.G7-OVA skin tumors in IFNγR1−/− and NOS2−/− mice (22). We have reproduced and extended these observations by identifying intratumoral CD11b+F4/80+ Møs as the critical NO-producing tumoricidal effectors induced by transferred CTLs (Fig. 6A and Supplemental Fig. 7). Most importantly, we demonstrate that ocular tumor-associated Møs fail to produce tumoricidal concentrations of NO despite NOS2 protein expression, which is induced by IFN-γ expressed by tumor-infiltrating CTLs (Fig. 7). These data suggested that NOS2 enzymatic activity was compromised within the ocular tumor microenvironment, which was supported by the observation that NO-mediated tumoricidal activity was inhibited when activated NOS2+ Møs were injected in the a.c. (Fig. 8A). Therefore, immunosuppressive mechanisms within the eye that inhibit tumoricidal activity of Møs by inhibiting NO production contribute to the failure to control ocular tumors by CTLs.

Taylor et al. (36) showed that Møs stimulated in the presence of AqH failed to produce NO despite expression of NOS2, which supports our observations that the ocular microenvironment is suppressive to Mø function via posttranslational regulation of NOS2. Posttranslational regulation of NOS2 could be mediated by limiting l-arginine entry into the cell through modulation of the cationic amino acid transporter 2b (37), by limiting production of the cofactor tetrahydrobiopterin by guanosine triphosphate cyclohydrolase 1 (38), or by a competition for limited l-arginine by ARG1 or ARG2, which have Vmax/Km values similar to those of NOS2 (39). Our data indicate that competition for l-arginine between NOS2 and ARG1 does not explain limited NO production by ocular tumor-associated Møs because our assays to evaluate tumoricidal or immunosuppressive activity of myeloid

**FIGURE 8.** The ocular microenvironment suppresses the tumoricidal activity of Møs activated in vitro. A, Thioglycolate-elicited peritoneal Møs were harvested from the peritoneal cavities of B6 mice, activated in vitro, recovered, mixed with E.G7-OVA tumor cells to obtain a 30:1 and 100:1 Mø to tumor cell ratio, and then injected into the a.c. of RAG1−/− mice. RAG1−/− mice that received the same number of tumor cells alone were used as controls. Data are representative of results from two independent experiments. Lines displayed are medians. B, Cellular suspensions at a 100:1 Mø to tumor cell ratio were injected into the skin of RAG1−/− mice. Control mice received the same number of tumor cells alone. Tumor growth was then monitored by caliper measurements. Each point represents mean ± SD. **p < 0.01; ***p < 0.001. two-way ANOVA and Tukey’s multiple comparison post test. C, The incidence of measurable tumors in these mice was also determined. Data shown represent results from one of two independent experiments. p = 0.0135, log-rank test.
cells were performed in RPMI 1640 medium, which contains a high concentration of L-arginine (1.15 mM) capable of overcoming substrate competition between NO2 and ARG1 or ARG2 (40). Therefore, future experimentation will focus on cofactor availability and regulatory proteins as they relate to regulation of homodimerization of NO2, which is a requirement for its activity (41).

We observed a dramatic difference in CD11b+ myeloid cell subsets within skin and ocular tumors suggesting that factors that are unique to each tumor microenvironment shape components of the tumor stroma. We do not fully understand why Gr-1+ myeloid cells accumulated to such a greater extent in ocular tumors. However, CD11b+ subsets were equivalent in ocular tumors developing within immunodeficient RAG1−/− mice indicating that tumor-derived factors and not infiltrating T cells dictated myeloid cell accumulation in the eye. In addition, we demonstrated that CXCL2, which is chemotactic for Gr-1+ cells, was increased in ocular tumors, which could suggest a possible mechanism for the accumulation of Gr-1+ cells in ocular E.G7-OVA tumors. Eye-derived F4/80+ Mϕs have been shown to express CXCL2, which is critical for the recruitment of NKT cells to the spleen that contribute to the generation of systemic tolerance to ocular Ags (42). Therefore, these data could suggest that the ocular microenvironment may selectively upregulate CXCL2 in activated Mϕs, which results in Gr-1+ cell accumulation. However, pronounced Gr-1 accumulation was not observed when a different tumor cell line, P815, was injected into the a.c. indicating that Gr-1 accumulation was specific to E.G7-OVA tumors and not the ocular microenvironment (Supplemental Fig. 5B). The unique factors that regulate chemokine expression within ocular E.G7-OVA tumors will require further experimentation, and our data cannot exclude that differences in the differentiation of Gr-1+ cells into F4/80+ Mϕs within the tumor microenvironment, which has been demonstrated in EL-4 skin tumors (43), may also be involved.

We previously demonstrated that CD11b+ cells isolated from ocular tumors suppressed the generation of CTLs in in vitro cocultures (5) suggesting that these cells might be MDSCs that could promote ocular tumor growth by inhibiting CTL responses. In this study, we have reproduced and expanded that observation by identifying NO as the mediator of this suppression. Notably, as shown in Fig. 7, the cell type that produces NO within nontransferred tumors was exclusively CD11b+Gr-1+ F4/80+ Mϕs, suggesting that these cells were solely responsible for inhibiting T cell proliferation in T cell/intratumoral CD11b+ cell cocultures. These results are consistent with observations made by other researchers that indicate that intratumoral CD11b+Gr-1+ F4/80+ Mϕs (43) or splenic CD11b+Gr-1lowF4/80+ Mϕ-like cells (44) are the main cell type responsible for T cell suppression in these in vitro coculture assays. Moreover, we showed that the concentration of NO necessary to inhibit T cell responses in vitro was much lower than the concentration required for tumoricidal activity, highlighting the different roles NO can play in the interactions between tumors and the immune system depending on its local concentration. However, despite demonstrating clear suppressive activity of CD11b+ myeloid cells isolated from skin and ocular tumors toward T cells in vitro, CTL effector function was preserved in both tumor sites in vivo, and in the case of CTL transferred skin tumors, the presence of these NO-producing myeloid cells with suppressive activity toward T cells in vitro were required for CTL tumoricidal activity in vivo. These data indicate that myeloid cells displaying immunosuppressive activity in vitro do not inhibit the effector phase of CTL responses in vivo. Indeed, other studies have reported that splenic myeloid cells from tumor-bearing mice suppress T cell proliferation but not early effector function, such as IFN-γ expression (44, 45). Therefore, MDSCs may primarily act during the priming phase to limit tumor-specific T cell expansion. On the other hand, Mϕs cocultured with MDSCs have been reported to be impaired in their ability to secrete IL-12 suggesting that MDSCs can inhibit Mϕ function (46). However, Mϕ NO production was not reduced by MDSCs in this study, which minimizes the relevance of these observations to immune evasion by ocular tumors in our model. It is very clear, however, that tumor growth induces the expansion of CD11b+Gr-1+ cells (47). Future experiments in which Gr-1+ cells are eliminated in ocular tumor-bearing mice will be necessary to better define their contribution to promoting ocular tumor growth.

In our previous study, we also showed that after 7 d of E.G7-OVA growth in the a.c., mice were protected from a subsequent injection of E.G7-OVA in the skin or opposite eye (5) reproducing previous observations by Niederkorn and Streinlein (8) using P815 mastocytomas. However, in this study we observed that CTL transfer before tumor challenge in the a.c. only delayed the kinetics of ocular tumor growth. The differing results may have been due to a smaller tumor challenge (4 × 105) injected into the opposite eyes of mice with established ocular tumors and to the measurement of tumor burden only 3 d after the second eye challenge. Even though these measurements of tumor burden were below the limit of detection in the opposite eye, it is possible that tumors may have grown out over time. Our data suggest that CTLs alone directly eliminate a very limited number of E.G7-OVA tumors and require additional immune cell populations, for example Mϕs, to eliminate larger tumor burdens. Smaller tumor burdens may be controlled without the need for Mϕs. However, in larger tumor burdens, selective pressure by CTL responses in the absence of other immune effector mechanisms, such as the tumoricidal activity of activated F4/80+ Mϕs, may favor the generation of tumor escape variants resistant to CTL responses, which could explain why CTL transfer on the same day as ocular tumor challenge failed to eliminate ocular tumors completely (Fig. 1).

Ocular tumor growth has been shown to induce the generation of CD8+ Tregs, which inhibit the expression of delayed-type hypersensitivity responses (48). As ocular tumors ultimately completely fill the a.c. displacing the immune-suppressive AqH, another explanation for progressive ocular tumor growth is that CD8+ Tregs infiltrate established ocular tumors and suppress Mϕ tumoricidal activity when AqH is lost. Additional experimentation will be required to determine whether endogenous CD8+FoxP3+ Tregs infiltrate ocular tumors and contribute to ocular tumor progression.

Not all tumor cell lines grow unabated in the a.c. of the eye, and destructive and nondestructive patterns of immune rejection of ocular tumors have been observed (35). Destructive immune responses culminating in ocular phthisis were observed when P91 tumors, a variant of P815 mastocytomas, were injected in the a.c. of BALB/c mice. This antitumor response was mediated by a T cell-mediated type IV delayed-type hypersensitivity response (49). In contrast, UV5C25 (35) and an adenovirus virus-induced tumor (Ad5E1) (50) are spontaneously eliminated from the a.c. of mice with pristine preservation of delicate ocular microanatomy. UV5C25 and Ad5E1 induce CD8+ (35) and CD4+ (51) T cell responses, respectively, which are required for tumor elimination. Indeed, CD4+ T cell-expressed IFN-γ acts directly on Ad5E1 tumors to upregulate TRAIL-R2 to induce apoptosis (51). However, Mϕs are also required for rejection (52, 53), which is consistent with our results. The requirement for Mϕs in CD8+ T cell-mediated killing of UV5C25 tumors has not been explored, but...
based on our results a critical role for Mφs seems plausible. Notably, although these data suggest that UV5C25 and AdSEI break ocular immune privilege, some immune regulation within the eye is not lost because Mφs selectively destroy tumor cells without causing damage to innocent bystander cells. Understanding the critical factors that promote tumoricidal activity in Mφs while controlling deleterious nonspecific tissue damage will be an exciting area of future study.

In conclusion, though CTLs can directly eliminate some tumor cells via apoptosis, their induction of tumoricidal activity in Mφs is critical for complete elimination of established tumors. In addition to contributing to reducing tumor burden, Mφs may eliminate tumor variants that escape detection by CTLs. Impaired effector function of Mφs within the eye may be an evolutionary compromise to preserve vision by protecting the delicate micro-anatomy of the eye, which fails to regenerate from injury due to nonspecific effector mechanisms of Mφs. However, this compromise comes at the price of impaired tumoricidal immune responses.

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