A Novel Treatment for Ocular Tumors Using Membrane FasL Vesicles to Activate Innate Immunity and Terminate Immune Privilege

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PURPOSE. Ocular immune privilege promotes tumor growth by hindering the development of innate and adaptive immunity. A prior study showed that ocular tumors expressing the membrane-only form of Fas ligand (FasL) termite immune privilege, induce vigorous inflammation, undergo rejection, and induce systemic protective immunity. In these previous experiments the tumor cells used were genetically engineered to express membrane FasL. As an initial step toward developing an immunotherapy for intraocular tumors, the present study was conducted to examine whether injection of microvesicles expressing membrane FasL into ocular tumors (that are FasL negative) would have a similar effect.

METHODS. Microvesicles expressing either no FasL or membrane-only Fas ligand were coinjected with L5178Y-R lymphoma cells into the anterior chambers (AC) of DBA/2 mice.

RESULTS. Tumor cells coinjected with control vesicles grew progressively in the AC, and all mice died of metastatic disease by day 15. By contrast, a single injection of membrane FasL vesicles induced a potent inflammatory response characterized by GR1+ neutrophils and F4/80+ macrophages and significantly improved survival from 0% in untreated mice to 58% in mFasL-treated mice. Among the surviving mice, the ocular tumor was eliminated in 55%, and the mice exhibited systemic protection from a second tumor challenge. In the remaining 45%, the ocular tumor was not eliminated, but the mice were protected from liver metastases.

CONCLUSIONS. Bioactive membrane FasL microvesicles coinjected with tumor cells induce a potent inflammatory response that terminates immune privilege, eliminates ocular tumors, and prevents metastatic disease. (Invest Ophthalmol Vis Sci. 2005;46:2495–2502) DOI:10.1167/iovs.05-0048

A perennial goal of cancer researchers is to develop effective cancer immunotherapies that eliminate the primary tumor and prevent the development of metastases. Immunotherapies typically target antigens that are found on tumor cells, but are absent from normal cells, and immunity against these tumor-specific antigens is the basis for a protective therapy.1–4 Ideally, antigen-specific tumor immunity would eliminate only the malignant tumor cells without harming the surrounding normal tissue. An important barrier to the success of these cancer immunotherapies is the immunosuppressive environment that surrounds the site of an established tumor.5–7 The immunosuppressive environment within the eye is similar to the immunosuppressive environment that surrounds these established tumors, even when the tumors develop at nonocular anatomic sites.8,9 The fact that the eye and tumors are identified as immune-privileged sites, in which innate and adaptive immunity is downregulated, has been attributed to their unique local environments.10 Although there are multiple mechanisms used to establish immune privilege, those that block the development of innate immunity are believed to have a central role.5,6,11 Therefore, the development of an effective immunomodulating therapy to eliminate tumors must include a strategy that activates innate immunity and terminates immune privilege.

Although it is clear that immunosuppression coincides with the more advanced stages of disease progression in cancer patients, it is unclear when an immunosuppressive environment and immune privilege are first established at the initial site where a tumor forms. It seems unlikely that these events occur at the earliest stages of malignant transformation. This is an important question in relation to tumors that develop within the eye, since these tumors form within a site that already possesses immune privilege. In other words, ocular tumors do not have to convert a normal environment into an immunosuppressive environment. This makes ocular tumors a particularly attractive model for studying methods of activating innate immunity and terminating immune privilege, since this anatomic site is especially hostile to the development of an effective antitumor immune response.

Studies performed in our laboratory have revealed that expression of the cell-surface protein membrane Fas ligand (FasL) terminates ocular immune privilege.12 FasL is a type II transmembrane protein that can be cleaved from the cell surface to generate a soluble form.13–15 The two different forms of FasL (membrane-bound and soluble) exhibit opposite effects on inflammation; membrane FasL is proinflammatory, whereas soluble FasL is anti-inflammatory.16,17 Using a murine ocular tumor model, we demonstrated that ocular tumors expressing the membrane-only form of FasL terminate immune privilege, induce vigorous inflammation, are eliminated from the eye, and induce systemic protective immunity.12 Moreover, the data indicate that this was achieved when mFasL triggered Fas receptor-positive neutrophils and/or macrophages within the tumor to release proinflammatory cytokines.

In our previous studies, we used tumor cells that were transfected in vitro with membrane-FasL cDNA and then in-

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jected into the anterior chamber (AC) of the eye. Because it is technically difficult to transfect tumor cells in situ, a strategy was devised to deliver membrane-bound Fasl directly into the tumor site without transfecting the tumor cells. This was accomplished through the use of microvesicles that express high levels of membrane Fasl. We hypothesize that mFasL vesicles will trigger inflammation and overcome the immunosuppressive environment within the eye, even though mFasL is not expressed on the surface of the tumor cells. We predicted that mFasL vesicles would trigger Fas-receptor–positive neutrophils and/or macrophages within the tumor site to secrete proinflammatory cytokines, with resultant amplification of the innate immune response causing vigorous inflammation, tumor rejection, and long-term protective immunity.

**Materials and Methods**

**Animals**

Adult female DBA/2 mice (6–8 weeks) were purchased from Taconic Farms (Germantown, NY). All animals were treated according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

**Cell Lines and Reagents**

L5178Y-R lymphoma tumors (L5) expressing no Fasl and Neuro2a (AJ)-derived Neuro2a-mFasL and Neuro2a-neo (FasL negative) cells were established as previously described. L5 tumor cells were grown in suspension cultures in RPMI 1640 (Invitrogen-Life Technologies, Rockville, MD) supplemented with 10% heat-inactivated FCS (HyClone, Logan, UT), 0.01 M HEPES buffer, 2.0 mM glutamine (Invitrogen-Life Technologies), 100 U/mL penicillin G sodium (Invitrogen-Life Technologies), 100 g/mL streptomycin sulfate (Invitrogen-Life Technologies), and 2-mercaptoethanol (1 × 10−3 M; Sigma-Aldrich, St. Louis, MO). Neuro2a cells were maintained in 10% FCS-DMEM supplemented with 1× penicillin-streptomycin-glutamine, 1× nonessential amino acids, and 1 mg/mL genetin-selective antibiotic (G418; Invitrogen-Life Technologies).

**Preparation of Microvesicles**

Vesicles expressing mFasL or no FasL were produced as previously described. Briefly, Neuro2a-mFasL and Neuro2a-neo cells were grown in G418-containing medium to 70% confluence. The medium was then replaced with G418-free medium, and the culture supernatants were collected 24 hours later and centrifuged at 250g for 10 minutes at 4°C to remove any cellular debris. Residual cell debris in the culture supernatant was removed by further centrifugation at 20,000 rpm for 30 minutes at 4°C. The cell-free supernatant was then centrifuged at 90,000g for 3 hours at 4°C. The resultant vesicle pellet was resuspended in serum-free RPMI medium to 10% of the original volume and passed through a 0.45-μm sterile filter. The expression of membrane Fasl was assessed by Western blot analysis, with a polyclonal rabbit anti-mouse Fasl antibody, as described previously.

**Cytotoxicity Assays**

The Fas-Fasl specific cytotoxicity of Neuro2a-mFasL vesicles and Neuro2a-neo vesicles was evaluated with a standardized 51Cr-release assay, as previously described. Briefly, Fas+ A20 target cells were incubated with 100 μCi of 51Cr sodium chromate in 200 μL of RPMI 1640 containing 10% FCS for 1 hour in a 37°C water bath. Labeled targets were added to a 96-well round-bottomed plate (Falcon 3077; BD Biosciences, Franklin Lakes, NJ) at a concentration of 3 × 104 cells per well. The Neuro2a vesicle preparations were diluted 1:25, and increasing amounts of the diluted vesicles were added to the appropriate wells in a total volume of 200 μL RPMI 1640 with 10% FCS. The plates were incubated at 37°C in a 5% CO2 incubator for 6 hours. At 6 hours, the radioactivity of 25 μL of supernatant was counted in a scintillation counter (1205 Betaplate; Amersham Pharmacia Biotech, Piscataway, NJ). Spontaneous release of 51Cr was determined by incubating targets with medium alone. The maximum release was determined by incubating target cells with 5% HCl. Data are expressed as the percent specific lysis calculated as follows: 100 × (experimental release − background release)/(total release − background release). The specific lysis for each vesicle concentration is displayed as the mean specific lysis for triplicate wells ± SEM. One unit of activity was determined as the amount of neuro2a-mFasL vesicles to achieve 50% maximum cell death of A20 cells. In each experiment described herein, control groups were treated with the Neuro2a-neo vesicles at a volume equal to that of the Neuro2a-mFasL vesicle group.

**Anterior Chamber Inoculations**

L5 tumor cells were washed in Hanks’ balanced salt solution (HBSS) and resuspended in HBSS for inoculations. With a quantitative technique that has been described previously, 2 × 105 tumor cells and control or mFasL vesicles were coinjected (total volume 3 μL HBSS) into the AC of DBA/2 mice. In control experiments, 3 μL of control or mFasL vesicles were injected alone. Tumor growth and rejection was assessed by slit lamp examination. In addition, corneal neovascularization (NV) was monitored using the following scoring system: 0, no NV; 1, NV in one or two quadrants of the periphery only; 2, NV in three or four quadrants of the periphery only; 3, NV one half the distance to the corneal center in one or two quadrants; 4, NV one half the distance to the corneal center in three or four quadrants; 5, NV three fourths the distance to the corneal center in one or two quadrants; 6, NV three fourths the distance to the corneal center in three or four quadrants; 7, NV to the corneal center in one or two quadrants; and 8, NV to the corneal center in three or four quadrants. Representative eyes (n = 3 for each time point) were examined histologically at days 3, 6, or 10 after AC inoculation. The eyes were enucleated and livers were excised to examine for tumor metastases. The tissues were fixed in 10% paraformaldehyde, embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H&E).

**Immunohistochemistry and Confocal Microscopy**

To examine the inflammatory infiltrate, we euthanatized the mice at days 3, 6, and 10 after AC inoculation of control vesicles only, mFasL vesicles only, tumor cells plus control vesicles, or tumor cells plus mFasL vesicles. Eyes were enucleated and snap frozen in optimal cutting temperature (OCT) compound (Tissue-Tek; Sakura Finetek, USA, Torrance, CA). The frozen eyes were sectioned at 12-μm increments and stored at −20°C until used. The tissue sections were immunostained for macrophages with rat anti-mouse F4/80 (Caltag, Burlingame, CA) and for neutrophils with rat anti-mouse Ly-6 (GR1 neutrophil; BD-PharMingen, San Diego, CA) followed by a biotinylated mouse anti-rat IgG2bK (BD-PharMingen). Rat IgG2bK was used as an isotype control. A cyanine nucleic acid stain (To-Pro-3; Molecular Probes, Eugene, OR) was used to stain all cells. After the immunostaining, tissue sections were mounted with antifade medium (VectaShield; Vector Laboratory, Burlingame CA), protected with coverslips, and stored at 4°C until analyzed by confocal microscopy. DBA2 (2%) was used to block nonspecific staining. Immunostained tissue sections were analyzed with a confocal laser scanning microscope (TCS 4D; Leica, Deerfield, IL).

**Assay for Protective Antitumor Immunity**

DBA/2 mice that had been injected in the AC with 2 × 105 L5 tumor cells plus control or mFasL vesicles were subsequently challenged (10 days after the initial AC injection) with a subcutaneous injection of 2 × 105 L5 tumor cells into the rear flank. As a negative control, naive DBA/2 mice (no previous AC injection) received an equivalent subcutaneous tumor-cell challenge. Subcutaneous tumor growth was fol-
Characterization of Microvesicles Expressing mFasL

Bioactive vesicles expressing membrane-only FasL or control vesicles (no FasL) were isolated from the supernatants of Neuro-2a cells by ultracentrifugation. The expression of mFasL on the vesicles was assessed by Western Blot analysis (Fig. 1A). A cell lysate prepared from L5 tumor cells that overexpress mFasL, was used as a positive control for mFasL. As expected, a high level of mFasL could be detected in mFasL-expressing vesicles, and no FasL was detected in the control vesicles (FasL negative). A standard chromium release assay was performed to determine the bioactivity of the mFasL vesicles, by using Fas+ A20 cells as targets. Increasing amounts of mFasL vesicles were cultured with 51Cr-labeled A20 target cells (Fig. 1B). The vesicles expressing mFasL induced significant cytotoxicity against the Fas+ A20 targets in a dose-dependent manner. By contrast, the control vesicles (FasL negative) were unable to induce cytotoxicity in the Fas+ A20 targets. These results confirm that the microvesicles isolated from Neuro2a-mFasL cells express high levels of mFasL, and the mFasL is functional.

mFasL-Vesicle-Induced Transient Corneal Inflammation

To determine whether mFasL vesicles alone induce nonspecific tissue damage, either locally within the eye or systemically within the liver, we injected the mFasL vesicles (10–12 units) or equivalent volumes of the control vesicles into the AC of DBA/2 mice. Daily slit lamp examinations and histologic analysis were performed to monitor ocular inflammation. At the conclusion of the study (day 21), all the mice were euthanized, and the livers were examined grossly and histologically for evidence of tissue damage.

A single injection of control vesicles (FasL negative) failed to induce either corneal inflammation, as determined by slit lamp examination, or liver damage (Fig. 2). Histologic studies of the cornea on days 3, 6, and 10 after inoculation did not reveal any signs of inflammation (Fig. 3A). This observation was further supported by fluorescence confocal microscopy, which revealed the presence of a few macrophages only at day 3 after inoculation and a complete absence of neutrophils at all three time points examined (Fig. 3A). These data indicate that vesicles alone do not induce any significant nonspecific inflammation.

By contrast, a single AC injection of mFasL vesicles triggered an early inflammatory response characterized by corneal NV and corneal opacification that peaked on day 3 and resolved completely by day 7 after inoculation (Fig. 2). Histologic analysis revealed a mild inflammatory infiltrate throughout the corneal stroma and to a lesser extent within the AC on day 3 after inoculation (Fig. 3B). By day 10, the cornea appeared normal, with no evidence of tissue damage or scarring. Furthermore, the liver showed no signs of tissue damage on gross and histologic analyses (Fig. 2). To examine further the transient corneal infiltrate induced by the mFasL vesicles, we performed fluorescence confocal microscopy with antibodies specific for macrophages (F4/80) and neutrophils (GR1), key cell types involved in the early innate immune response. Confocal microscopy revealed a moderate corneal infiltration of macrophages and neutrophils at day 3 after inoculation (Fig. 3B). The early neutrophil infiltrate diminished to undetectable levels by day 10. The early macrophage infiltrate remained steady, and a small number of macrophages were still observed on day 10. Taken together, these data demonstrate that mFasL vesicles alone induced a mild corneal inflammatory response marked by an early infiltration of macrophages and neutrophils. How-
ever, the inflammatory response was transient and did not result in long-term tissue damage, either locally or systemically.

**Coinjection of mFasL Vesicles and Tumor Cells**

To determine whether mFasL vesicles would induce an inflammatory response strong enough to terminate ocular immune privilege and reject a FasL-negative tumor within the eye, we coinjected vesicles expressing mFasL (10–12 units) or control vesicles (FasL negative) with $2 \times 10^3$ FasL-negative L5 tumor cells into the AC of DBA/2 mice. Mice were monitored daily for ocular tumor growth, development of liver metastases, and survival. Our prior study demonstrated that L5 tumor cells grow progressively within the eye and metastasize to the liver, resulting in 100% mortality. As expected, the L5 tumor cells injected with the control vesicles grew progressively within the eye (Fig. 4A), resulting in 100% mortality by day 15 after inoculation (Fig. 4B). On further analysis, we determined that 100% of mice treated with control vesicles died of liver metastases (Fig. 4C). By contrast, the mice treated with mFasL vesicles exhibited a delay in tumor growth within the eye (Fig. 4A) and only 42% of the mice died of liver metastases (Figs. 4B, 4C). The treatment with vesicles significantly increased the survival rate from 0% in mice treated with control vesicles to 58% in mice treated with mFasL vesicles (Fig. 4B). Among the mice that survived, 55% completely rejected the ocular tumor, resulting in phthisis (Fig. 4A). These mice displayed a much earlier and more potent inflammatory response characterized by corneal edema and infiltration and NV that coincided with delayed tumor growth. The remaining 45% of the mice that survived presented with progressively growing ocular tumors, but were protected from the development of liver metastases when examined either grossly or histologically (Fig. 4C). These data imply that the mFasL vesicles induce both a local inflammatory response capable of rejecting the ocular tumor and a systemic immune response that protects the mice from the development of liver metastases.

Our initial slit lamp examinations suggested there was an increase in corneal NV in mFasL-vesicle–treated tumors. To
quantitate NV in mice treated with either mFasL vesicles or control vesicles, we used the scoring system described in the Materials and Methods section. Mice treated with control vesicles displayed progressive tumor growth and moderate corneal NV that increased with tumor growth (Fig. 5). By contrast, mice treated with mFasL vesicles displayed a significant increase in corneal NV. These data indicate that mFasL vesicle treatment and tumor regression coincide with an increase in NV, as well as an increase in inflammation.

mFasL-Vesicle–Induced Infiltration of Macrophages and Neutrophils

Histologic analysis and fluorescence confocal microscopy was performed to characterize the inflammation induced in tumor-containing eyes treated with mFasL vesicles. Tumor-containing eyes treated with control vesicles or mFasL vesicles were enucleated at 3, 6, or 10 days after AC inoculation. The eyes were prepared for H&E staining and fluorescence confocal microscopy. H&E-stained slides confirmed the delay in tumor growth observed in the slit lamp examinations (Fig. 6). At day 3 after AC inoculation, tumor cells are observed in the angle of the iris and cornea. By day 10, the tumors treated with control vesicles had completely filled the AC, and the tumor growth was not accompanied by significant inflammation within the cornea or the AC. By contrast, the mice treated with mFasL vesicles exhibited early and prolonged corneal inflammation and significant corneal NV in the periphery at day 3 and throughout the entire cornea by day 10. Furthermore, the increased inflammation coincided with a delay in tumor growth when compared with the control-vesicle–treated group.

We have demonstrated that rejection of ocular tumors genetically modified to express mFasL coincide with an intense neutrophil-mediated inflammation.12 We proposed that mFasL is primarily acting by inducing an early innate immune response. Fluorescence confocal microscopy was performed with neutrophil-specific (GR1) and macrophage-specific (F4/80) antibodies at different time points. Injection of tumor cells with control vesicles showed minimal neutrophil infiltrate on day 3, gradually increasing by days 6 and 10 (Fig. 6A). Macrophage infiltration was undetectable at all time points. The injection of tumor cells with mFasL vesicles induced massive neutrophil infiltration on day 3 that decreased to levels observed in the control-treated group on day 6, but returned with great potency on day 10 (Fig. 6B). Moderate macrophage infiltration was observed through day 6, but was undetectable by day 10 after AC inoculation. Both immune cell types were located in the corneal stroma and iridocorneal angle.

AC Tumor Inoculations and Systemic Tumor-Specific Immunity

To determine whether treatment of ocular tumors with mFasL vesicles induces long-term systemic protective immunity, DBA/2 mice were given an AC inoculation of L5 tumors and mFasL vesicles followed by a second tumor challenge (10 days later) in the flank with L5 tumor cells only. Because the L5
tumors are FasL negative, only mice with systemic protective antitumor immunity rejected the flank tumor. As a negative control, naïve mice (without a previous ocular tumor) received a similar tumor challenge in the flank. As expected, tumors grew progressively within the flank of naïve mice. By contrast, mice that were successfully treated with an AC inoculation of tumors and mFasL vesicles were protected completely, and the second L5 tumors in the flank were eliminated (Fig. 7). We conclude that the mFasL vesicles are capable of terminating ocular immune privilege and inducing systemic protective immunity that will eliminate secondary tumors.

**DISCUSSION**

The ultimate goal of our studies is to develop an immunotherapy for the treatment of intraocular tumors. After the identification of tumor-specific antigens, immunotherapies focused on the activation of tumor-specific cytotoxic T cells by using vaccines and gene therapy.1–4 Unfortunately, once these therapies were translated to patients with cancer, they showed limited ability to activate a sustained tumor-specific T cell response.21–25 In a “normal” immune response, an early non-specific inflammatory response (innate immunity) precedes the development of an antigen-specific response (adaptive immunity).24–25 Most of the current immunotherapies focused on the activation of tumor-specific T cells, without much attention to early innate immunity.6,26 Thus, the limited success of the current therapies may lie in their failure to induce an early nonspecific inflammatory response. Therefore, an important question now facing tumor immunologists is how to stimulate and regulate inflammation in a way that leads to antitumor immunity.

An additional complication in the treatment of ocular tumors is the immune privilege that is present in the normal eye even before malignant transformation begins. Until recently, the studies of immune privilege focused mainly on the mechanisms used to prevent inflammation secondary to adaptive immunity. It is now known that the eye possesses many mechanisms that also prevent inflammation secondary to innate immunity.27–30 Thus, the eye possesses immune privilege that regulates both innate and adaptive immunity.11 Therefore, to induce an inflammatory response strong enough to reject ocular tumors and induce systemic tumor-specific immunity, innate and adaptive immune privilege must be terminated.

We propose that the membrane form of FasL is capable of terminating innate immune privilege. Data from several laboratories indicate that FasL expressed on ocular tissues induces apoptosis in infiltrating Fas-receptor-positive lymphocytes.31,32 In this way, it is believed to contribute to adaptive immune privilege by eliminating effector T cells that infiltrate the eye. However, the exact role of FasL in establishing and maintaining immune privilege remains an active subject of study. We propose an alternative hypothesis for the function of FasL within the eye, in which FasL regulates the activation and duration of innate immunity. This is accomplished by the two forms of FasL. Membrane-bound FasL activates innate immunity, whereas soluble FasL inhibits innate immunity. Therefore, the ratio of membrane to soluble FasL is critical in determining the level of activation and the duration of innate immunity within the eye.

Using an ocular tumor model, we previously demonstrated that the membrane form of FasL triggers a potent inflammatory response within the eye.12 Tumor cells transfected with high levels of membrane-only FasL (where the metalloproteinase cleavage site was deleted) induced potent neutrophil-mediated inflammation within the eye, resulting in tumor rejection and the development of tumor-specific T cells that mediated long-term protective immunity from secondary tumors. The data presented herein extend these findings and demonstrate that mFasL vesicles delivered at the site of tumor growth increase the survival of mice significantly, from 0% in mice treated with control vesicles to 58% in mice treated with mFasL vesicles. Among the mice that survived, ocular tumors were eliminated completely in 55%, and more important, long-term protection from a second tumor challenge developed. In the remaining 45% of the mice that survived, the tumors were not eliminated, but the mice were protected from liver metastases. This phenomenon is known as concomitant immunity, in which a primary tumor grows progressively and induces systemic anti-tumor immunity that prevents metastases, but is unable to eliminate the primary tumor.33,34 Neiderkorn and Streilein34 demonstrated that highly immunogenic tumors that express minor H alloantigens grow progressively within the AC and induce concomitant immunity. In these experiments, the tumor cells were sufficiently immunogenic to induce an immune response when they migrated out of the eye, since minor H-disparate tumor cells induce protective immunity in non-immune-privileged sites. In our experiments, the tumor cells express nonimmunogenic tumor antigens that cannot induce an immune response in either privileged or nonprivileged sites. Therefore, our data indicate that mFasL vesicles are capable of inducing complete tumor rejection and/or concomitant immunity in nonimmunogenic tumors growing within an immune-privileged site.

Because FasL induces apoptosis in Fas+ cells, one simple interpretation of our data is that the mFasL vesicles are directly...
killing the tumor cells. However, this cannot occur in our model, because L5 tumor cells are Fas-receptor–deficient, and mFasL vesicles cannot induce apoptosis of L5 tumor cells in vitro. A more plausible explanation is that mFasL vesicles amplify and sustain an innate immune response within the eye through the activation of resident antigen-presenting cells and/or infiltrating neutrophils. This sustained inflammation alters the local environment within the eye so that immune privilege is terminated, and antigen-presenting cells can trigger a protective systemic adaptive immune response. Our previous study, in which we used mFasL-transfected tumor cells, demonstrated that both an innate and adaptive immune response were necessary for complete tumor rejection.

The presence of mFasL vesicles within the ocular tumor successfully triggered an early and potent inflammatory response consisting of macrophages and neutrophils. L5 tumor cells injected with mFasL vesicles induced a much stronger and more persistent macrophage and neutrophil infiltration, compared with the injection of mFasL vesicles alone. The macrophage infiltration remained prominent through day 6 and became undetectable by day 10. Previously, we observed that intraperitoneal injection of mFasL vesicles trigger not only apoptosis of peritoneal macrophages, but also induce them to produce the proinflammatory cytokines IL-1β, MIP-2, MIP-1α, and MIP-1β. In vitro experiments indicated that macrophages are the major cytokine–chemokine producers in response to mFasL vesicles. Moreover, purified macrophages stimulated in vitro with mFasL vesicles can restore the ability of Fas-deficient mice to mount an inflammatory response.

These data suggest that within the eye, mFasL vesicles may trigger the release of proinflammatory cytokines that sustain local inflammation, even within an immune-privileged site. Vesicle treatment induced a significant increase in the infiltration of neutrophils. The neutrophil infiltration was biphasic, peaking at day 3 and again on day 10 after tumor inoculation. This biphasic infiltration was similar to that observed after Mycobacterium bovis BCG (bacillus of Calmette and Guérin) infection, in which the first wave of neutrophils was dependent on activated resident macrophages and the second wave of neutrophils was dependent on activated T cells. Our previous tumor studies revealed a similar pattern in which tumors cells expressing membrane-only FasL induce an early infiltration of neutrophils, followed later by a more sustained influx of neutrophils that remain present until the tumor is rejected completely. Complete tumor rejection is ultimately dependent on T cells, suggesting T cells may be important in maintaining the influx of neutrophils. Taken together, these data imply that one injection of mFasL vesicles is capable of inducing a strong and sustained innate immune response followed by the development of an adaptive immune response. Whether rejection of the ocular tumor occurs, or concomitant immunity develops, may depend on whether the immune-privileged environment within the eye is restored before tumor rejection is completed.

Other components of innate immunity (such as complement, and NK cells) that we have not yet examined may also play an important role in terminating privilege, triggering adaptive immunity, and eliminating tumor cells. Complement activation and NK cells are normally inhibited within the privileged eye, preventing them from directly lysing tumor cells. However, this inhibition can be overwhelmed during ocular inflammation, freeing them to participate in shaping adaptive immunity and controlling metastases. Therefore, it is important to examine these other components of innate immunity to understand fully the protective mechanisms triggered by mFasL vesicle treatment. Future studies will focus on identifying the cells that are activated by the mFasL-vesicles, how they alter the ocular environment, and how they contribute to the development of long-term protective immunity.

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