Research Article

Control of Ocular Tumor Growth and Metastatic Spread by Soluble and Membrane Fas Ligand

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Abstract

Fas ligand (FasL) can be either membrane bound, or cleaved by metalloproteinases (MMP) to produce a soluble protein. The two different forms of FasL are reported to have opposite functions—membrane-bound FasL (mFasL) is proinflammatory and soluble FasL (sFasL) is antiinflammatory. We previously showed that, within the immune-privileged eye, tumors expressing high levels of mFasL overcame the suppressive ocular environment, triggered an inflammatory response, and were subsequently rejected. By contrast, eye tumors expressing low levels of mFasL grew progressively. To evaluate the effect of sFasL on the tumor growth and metastatic potential of ocular FasL-expressing tumors, we compared tumor cell clones that expressed equal amounts of (low) mFasL in the presence or absence of sFasL. Tumor cells transfected with a modified FasL gene expressed only mFasL (noncleavable), grew progressively within the eye, and induced systemic protective immunity that prevented metastatic spread of tumor cells to the liver. Unexpectedly, tumors transfected with wild-type FasL (wtFasL; cleavable), which could produce both sFasL and mFasL, elicited considerably more inflammation and grew more slowly within the eye. However, the cleavable wtFasL eye tumors failed to trigger protective immunity and gave rise to liver metastases. Interestingly, exposure to the ocular environment was required for the wtFasL tumors to gain metastatic potential. We conclude that the fate of FasL-expressing tumors is determined by a combination of the following: (*a*) the relative proportion of membrane and sFasL, and (*b*) the local environment that determines the extent of FasL cleavage. [Cancer Res 2007;67(24):11951–8]

Introduction

The eye is an immune-privileged site in which immunogenic tissue transplants survive and escape immune destruction (1). There are several additional anatomic sites that possess immune privilege status, including the eye, brain, testis, and maternal-fetal interface (2–5). These immune-privileged sites use a variety of mechanisms to regulate innate and adaptive immunity (6, 7). Tumors can also acquire immune-privilege status and escape immunologic rejection by actively suppressing the local immune response at the site of tumor growth (8). There are many similarities between the mechanisms used by the eye to maintain immune privilege and the mechanisms used by tumors to establish immune privilege (9). Although much is known about how immune privilege is established and maintained, important questions remain unresolved.

Fas ligand (FasL) is an important molecule involved in maintaining immune privilege in the eye (2). FasL is a type II transmembrane protein belonging to the tumor necrosis factor (TNF) family, originally identified for its ability to induce the rapid cell death of Fas+ target cell populations (10, 11). Similar to other members of the TNF family, membrane-bound FasL (mFasL) can be cleaved from the cell surface by MMP to release a soluble form of FasL (sFasL; refs. 12–14). The two forms of FasL exhibit significant functional differences (15–17). Although mFasL is both proapoptotic and proinflammatory, sFasL fails to induce apoptosis or inflammation and can even block the proinflammatory effects of mFasL (15, 16). Using an ocular tumor model, we previously showed that tumors expressing high levels of membrane-only FasL (*a*) initiated vigorous neutrophil-mediated inflammation, (*b*) terminated ocular immune privilege, and (*c*) the tumors were completely rejected. Moreover, the rejection coincided with activation of both innate and adaptive immunity. By contrast, tumors expressing soluble-only FasL (*a*) failed to trigger inflammation, (*b*) ocular immune privilege remained intact, and (*c*) the tumors grew progressively (17).

Tumor cell lines transfected with vectors corresponding to full-length (wild-type) FasL normally provoke an inflammatory response that leads to tumor rejection. That is not always the case for tumors that are growing within the immune-privileged environment within the eye (17). Tumor cells transfected with cDNA encoding a membrane-only form of FasL [containing a deletion at 115–129 amino acid (AA), which spans two putative cleavage sites at 124/125 AA and 127/128 AA], expressed remarkably high levels of cell surface FasL. These noncleavable FasL (ncFasL) tumors induced a vigorous ocular immune response and were rejected from the immune-privileged anterior chamber of the eye (17). The same number of tumor cells transfected with wild-type FasL (wtFasL; cleavable), and expressing a lower absolute amount of membrane FasL on their cell surface, were not rejected from the eye. These results indicated that the eye was unique in that a higher level of FasL was required to initiate inflammation, presumably due to the presence of transforming growth factor β within the aqueous humor that blocked the proinflammatory action of wtFasL. However, the ability of sFasL (derived from cleavage of wtFasL) to actively oppose the proinflammatory effects of mFasL, and thereby facilitate tumor progression of the ocular wtFasL tumors, could not be discounted.

To further explore the mechanisms responsible for the observed growth properties of mFasL tumors in the eye, we developed clones of either wtFasL- or ncFasL-expressing tumor cells that exhibited equal (low) levels of FasL on the cell surface. The only difference between the clones was the ability or inability to cleave and release sFasL. If the final level of mFasL expression was the key variable

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doi:10.1158/0008-5472.CAN-07-0780

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predicting tumor progression, then both lines would be expected to behave comparably *in vivo*. However, we found that the wtFasL and ncFasL tumor cells exhibited markedly different growth properties and metastatic potential *in vivo*.

Materials and Methods

Animals. Adult female DBA/2 mice were purchased from Taconic. All animals were treated according to the Association for Research in Vision and Ophthalmology Resolution on the Use of Animals in Research. All procedures involving mice were approved by the Schepens Animal Care and Use Committee.

Tumor cell lines. L5178Y-R (L5) tumors expressing no FasL (*FasL negative*), wtFasL (cleavable), or membrane-only (noncleavable) FasL were produced as described previously (15). In addition, green fluorescent protein (GFP)+ L5 tumor cells were generated by cotransfecting L5 tumor cells with the pEBB-mFasL (noncleavable) or pEBB-wtFasL (cleavable) expression vector and cytomegalovirus-pEGFP-1 using the Gene Pulser II (BioRad) with 260 V and 950 uF in OptiMem medium (Life Technologies). P815 mastocytoma cells tumors were used as irrelevant syngeneic tumors. The P815 and L5 tumor cells were grown in suspension cultures in RPMI 1640 (Life Technologies) supplemented with 10% heat-inactivated FCS (Hyclone), 0.01 mol/L HEPES buffer (Life Technologies), 2.0 mmol/L glutamine (Life Technologies), 100 units/mL penicillin G sodium (Life Technologies), 100 μg/mL streptomycin sulfate (Life Technologies), and 2-mercaptoethanol (1× 10–5 mol/L; Sigma). For selection purposes, 800 μg/mL Geneticin (Life Technologies) was added to L5 tumor cultures.

Flow cytometric analysis of FasL. Flow cytometry was used to assess surface expression of FasL on L5 tumor cells expressing no FasL, wtFasL, or ncFasL. Tumor cells (1×10^6 cells) were stained with phycoerythrin-conjugated anti-mouse FasL (MFL3; BD PharMingen) in 50 μL of staining buffer (1× PBS, 1.0 % bovine serum albumin, and 0.02% NaN_3) for 30 min on ice and washed thrice with staining buffer. Cells were resuspended in 1× PBS and then analyzed on a FACScan flow cytometer (BD Biosciences), and the data were analyzed using FlowJo software.

In one set of experiments, single-cell suspensions were prepared from tumor-containing eyes. Eyes were enucleated at 10 days after anterior chamber inoculation, and single-cell suspensions were prepared using the whole eye. FasL expression was determined on eye-derived GFP+ tumor cells immediately after enucleation or after 5 days of culture in complete medium plus 800 μg/mL Geneticin (Life Technologies).

In a second set of experiments, single-cell suspensions were prepared from liver metastases. Large tumor nodules were excised from the liver at 10 to 15 days after anterior chamber inoculation. FasL expression was assessed by flow cytometry immediately after excision or after 5 days of culture in complete medium plus 800 μg/mL Geneticin (Life Technologies).

Setting gates to identify FasL on eye-derived tumor cells. Two-color fluorescence was used to determine the level of membrane FasL expressed on either wtFasL-GFP or ncFasL-GFP tumor cells that grew within the anterior chamber of the eye for 10 days. Because FasL can be expressed not only on tumor cells but also on (*a*) infiltrating inflammatory cells, and/or (*b*) ocular tissue, it was important to rigorously identify and separate the ocular tumor cells from the surrounding normal tissues. Representative clones of wtFasL-GFP and ncFasL-GFP tumors were selected based on comparable GFP and mFasL expression when cultured *in vitro*. When these GFP+ cells were costained with phycoerythrin–anti-FasL, they were also found to express comparable levels of FasL on the cell surface (Fig. 1*A*). Two-color staining was subsequently used to monitor the level of membrane FasL on tumor cells recovered from the eye by first gating on the GFP+ tumor cells and then determining the phycoerythrin intensity of the GFP+ cells using a phycoerythrin-conjugated anti-FasL antibody (MFL3; BD PharMingen).

Western blot analysis. The L5 transfectants (5×10^5 cells/mL) were cultured for 24 h in RPMI 1640 containing 10% FCS. For the last 14 h, phorbol 12-myristate 13-acetate (PMA; Sigma Chemical Co.) and ionomycin (Calbiochem) at a final concentration of 300 and 400 ng/mL, respectively,

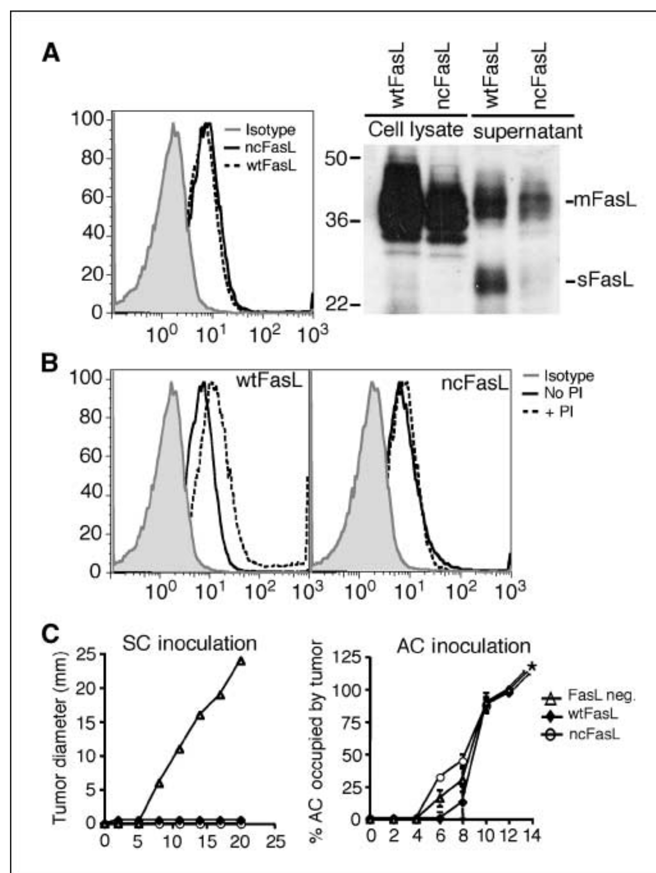

Figure 1. Characterization of L5 thymic lymphoma cell line expressing wtFasL or ncFasL. L5-transfected tumor cells were analyzed by flow cytometry using the anti-FasL monoclonal antibody (mAb) MFL3 to show that both cell lines express equal levels of membrane FasL. *A,* Western blot analysis on cell lysates and 10-fold concentrated culture supernatants from PMA and ionomycin-stimulated cells was performed to show that only wtFasL tumor cells produced sFasL. *B,* the level of mFasL was also analyzed by flow cytometry on tumor cells expressing wtFasL or ncFasL in the presence (*dotted line*) and absence (*black line*) of the protease inhibitor KB8301. *C,* growth and rejection of tumors expressing no FasL, wtFasL, or ncFasL was assessed in the s.c. tissue (2×10^6 tumor cells/mouse) or anterior chamber of the eye (2×10^3 tumor cells/mouse). Data are presented from one experiment (n = 5 per group). Each experiment was repeated thrice. *, extraocular tumor growth; tumors have perforated the cornea and are growing beyond 100% of the anterior chamber. *SC,* s.c.; *AC,* anterior chamber.

were added to each flask. Similarly activated transfectants were previously shown to make higher levels of mFasL and sFasL. Western blots of cell lysates were run using the conditions described previously (15).

Anterior chamber tumor inoculation and growth. Tumor cells were washed in HBSS and resuspended in HBSS for inoculations. Using a quantitative technique described previously, 2×10^3 cells in 3 μL were injected into the anterior chamber of DBA/2 mouse eyes (18). Daily slit-lamp examinations were used to determine the percentage of the anterior chamber occupied by tumor cells. Tumor growth and ensuing inflammation was also examined histologically. All mice were examined for 21 days to document growth and rejection of the ocular tumors and the development of liver metastases.

I.v. inoculations. Tumor cells were washed in HBSS and resuspended in HBSS for inoculations. Using aseptic technique, DBA/2 mice were anesthetized and the tail veins were warmed under an approved light source and placed within a Plexiglas cylinder to properly position the tail for i.v. injection. Using aseptic techniques, mice received i.v. inoculations (2×10^3 cells in 100 μL HBSS) of the tumor cells into the tail vein using a 25-gauge needle.

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S.c. tumor inoculations. L5 cells (2×10^6 cells) were washed with HBSS and injected s.c. in the rear flank of syngeneic DBA/2 mice (17). Tumor growth was assessed for 3 weeks, using calipers to measure the perpendicular diameter of the tumor.

Results

Expression of membrane FasL on wild-type and mutant tumor cells. Two types of cloned FasL-transfected L5 tumor cells that expressed either wtFasL or ncFasL were established. The ncFasL tumor cells were produced by deleting the MMP cleavage sites from the FasL cDNA as described previously (15). Using fluorescence-activated cell sorting analysis, we identified lines that expressed equal levels of FasL on the cell surface when cultured *in vitro* under normal conditions (Fig. 1*A*). Western blot analysis of cell lysates and supernatants prepared from these lines revealed that the tumor cells expressing ncFasL constitutively expressed only the full-length form of FasL. By contrast, tumor cells expressing wtFasL constitutively produced both full-length FasL and the shorter soluble form, as shown by the presence of the 27 kDa sFasL fragment in the supernatant (Fig. 1*A*). The MMP inhibitor KB8301 increased mFasL on tumor cells expressing wtFasL but had no effect on tumor cells expressing ncFasL (Fig. 1*B*). Taken together, these studies show that both wtFasL and ncFasL tumor lines expressed equal amounts of cell surface FasL under normal culture conditions. However, wtFasL tumor cells constitutively produce sFasL, in addition to cell surface FasL, due to the endogenous MMP activity of the tumor cells.

Tumor cells expressing either wtFasL or ncFasL experience ocular immune privilege. By definition, a tumor benefits from immune privilege within the anterior chamber of the eye if the tumor grows progressively within the eye but is rejected from a nonprivileged s.c. site (19). To determine whether the tumors expressing wtFasL or ncFasL experienced immune privilege, tumor cells were injected into either the nonprivileged s.c. tissue of the flank or the immune-privileged anterior chamber of the eye of syngeneic DBA/2 mice. Tumor diameter was measured at regular intervals to assess s.c. tumor growth (Fig. 1*C*). The percent of the anterior chamber occupied by the anterior chamber–injected tumor was determined by regular slit-lamp examinations. FasL-negative tumor cells (transfected with an empty vector) were used as a positive control and grew progressively in both privileged and nonprivileged sites, demonstrating that FasL-negative L5 tumor cells are not immunogenic. By contrast, tumor cells expressing either wtFasL or ncFasL were rejected from the nonprivileged s.c. tissue of the flank but grew progressively within the immune-privileged eye. Notably, wtFasL tumors consistently grew slower within the eye compared with ncFasL tumors (Fig. 1*C*; days 6 and 8). We conclude that tumor cells expressing either wtFasL or ncFasL experience ocular immune privilege.

Tumor cells expressing wtFasL form metastases in the liver. Although both tumor cell lines experienced immune privilege and grew progressively within the eye, only tumor cells expressing wtFasL metastasized to the liver. A mortality rate of 100% was observed in mice challenged with tumor cells expressing wtFasL, and this was in stark contrast to the 0% mortality observed in mice challenged with tumor cells expressing ncFasL (Fig. 2*A*). Gross clinical examination and histologic studies revealed that the tumor cells expressing wtFasL formed metastases in the liver (Fig. 2*B*). Interestingly, upon completion of the experiment at day 21 after anterior chamber inoculation, none of the mice inoculated with tumor cells expressing ncFasL developed liver metastases. As before, FasL-negative tumor cells were used as a positive control and grew progressively in the eye and metastasized to the liver, resulting in 100% mortality (Fig. 2*A*). These data indicate that the ability to cleave FasL and produce sFasL permits the development of liver metastases.

One explanation for the lack of metastases in mice inoculated with ncFasL tumor cells was a failure to disseminate from the eye into the blood vasculature. To rule out this possibility, peripheral blood was recovered at 3 days after anterior chamber inoculation from mice previously injected in the anterior chamber with either FasL-negative, wtFasL, or ncFasL tumor cells. Peripheral blood mononuclear cells were then cultured *in vitro* to determine whether tumor cells had gained access to the circulation. Tumor cells grew out of five of five cultures taken from each group of mice, demonstrating that tumor cells disseminated from the eye irrespective of the type of FasL expressed (or not expressed) on the cell surface (data not shown).

Exposure to the ocular environment is required for metastatic spread to the liver. To determine if the ability of

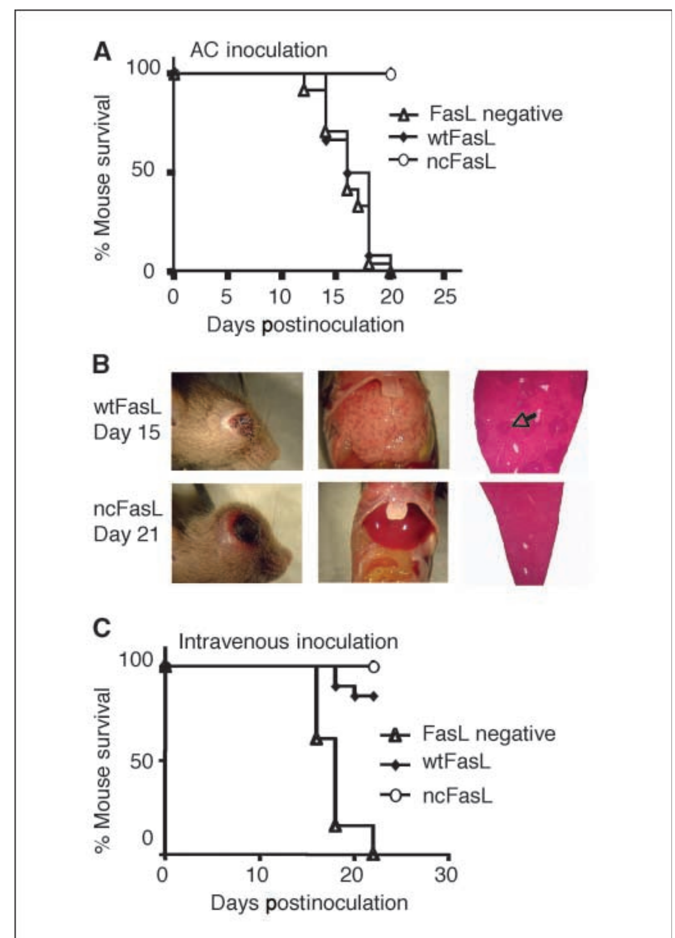

Figure 2. Transfectants expressing wtFasL metastasize from the eye to the liver. *A,* DBA/2 mice received anterior chamber inoculations of 2×10^3 tumor cells expressing either no FasL, wtFasL, or ncFasL. Mouse survival was monitored daily. *B,* photographs of the tumor containing eye and the liver were taken as mice became moribund. All remaining mice were sacrificed at the conclusion of the experiment and histologic analysis of the livers was performed. *C,* A second group of DBA/2 mice received an i.v. inoculation of 2×10^3 tumor cells expressing either no FasL, wtFasL, or ncFasL. Mouse survival was monitored daily. Data are presented from one experiment (n = 5 per group). Each experiment was repeated thrice. Histology: magnification, ×4; *arrow,* tumor foci.

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wtFasL tumors to form liver metastases was independent of tumor growth in the eye, tumor cells were injected directly into the blood vasculature bypassing the eye. Tumor cells (2×10^3) expressing either (*a*) no FasL, (*b*) wtFasL, (*c*) or ncFasL were injected i.v. Surprisingly, only 16% of the mice developed liver metastases after an i.v. inoculation of wtFasL tumor cells, and this was in contrast to the 100% of mice that developed liver metastases after an anterior chamber inoculation of the same tumor cells (Fig. 2*A* and *C*). As expected, none of the mice receiving ncFasL tumor cells developed liver metastases after anterior chamber or i.v. inoculation. FasL-negative tumor cells were used as a positive control, and 100% of these mice succumbed to liver metastases after either anterior chamber or i.v. inoculation. These data reveal that exposure to the ocular environment is required for wtFasL tumor cells to acquire metastatic capability.

WtFasL tumors trigger neutrophil-mediated inflammation in the eye. We originally predicted that wtFasL tumors that could produce sFasL would be less immunogenic, induce less inflammation, and grow faster within the eye than ncFasL tumors. To test this prediction, ocular tumors were examined histologically at 3, 6, and 10 days postinoculation. Three days after injection into the anterior chamber, tumor cells were present within the iridocorneal angle of all three groups of mice (Fig. 3*A–C*). FasL-negative tumors and ncFasL tumors continued to grow progressively within the eye, filling 100% of the anterior chamber by 10 days postinoculation (Fig. 3*A* and *B*). Unexpectedly, although wtFasL tumors also grew progressively within the eye, tumor growth was slower (Fig. 3*C*). This slowed growth rate was only observed *in vivo*, as the wtFasL and ncFasL tumor cell lines grew at the same rate when cultured *in vitro* (data not shown). Furthermore, the slowed growth of wtFasL tumors coincided with a potent inflammatory response within the eye, marked by a significant neutrophil infiltrate within the cornea and anterior chamber at 6 days postinoculation, resulting in significant tumor cell death (Fig. 4*C*). By contrast, mice inoculated with ncFasL tumors developed larger tumors associated with less inflammation within the eye (Fig. 4*B*). Mice inoculated with FasL-negative tumor cells developed large tumors associated with very little inflammation (Fig. 4*A*). We conclude that the growth of wtFasL tumors coincides with considerably more inflammation that slows tumor growth within the eye.

Expression of FasL on eye-derived tumors. One explanation for the different ocular inflammatory response elicited by the FasL-clones was that, within the eye, the clones no longer expressed comparable levels of FasL. To test this possibility, we used flow cytometry to quantitate the level of membrane FasL expressed by tumor cells that had been growing within the anterior chamber for 10 days. Because FasL can be expressed on either (*a*) normal ocular tissues, (*b*) infiltrating inflammatory cells, or (*c*) FasL-positive tumors, we used GFP to differentiate tumor cells from normal cells. The level of FasL was then determined on the tumor cells using a phycoerythrin-conjugated anti-FasL antibody.

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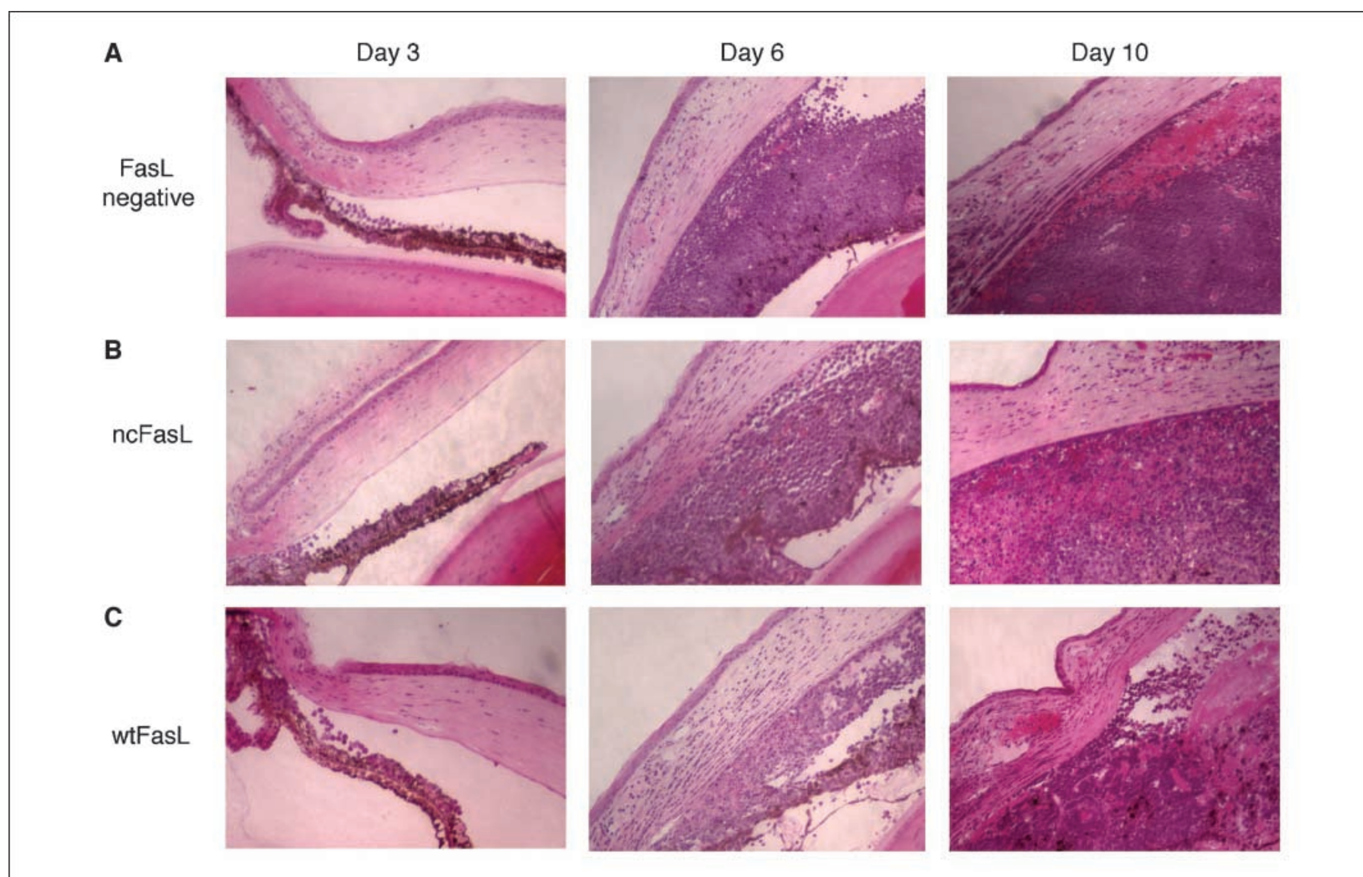

Figure 3. Tumors expressing wtFasL grow slower than ncFasL tumors in the eye. DBA/2 mice received anterior chamber inoculations of 2×10^3 tumor cells expressing either no FasL (*A*), ncFasL (*B*), or wtFasL (*C*). Histologic analysis was performed on days 3, 6, and 10 postinoculation. *C,* cornea; *T,* tumor; *I,* iris; *L,* lens. Magnification, ×10.

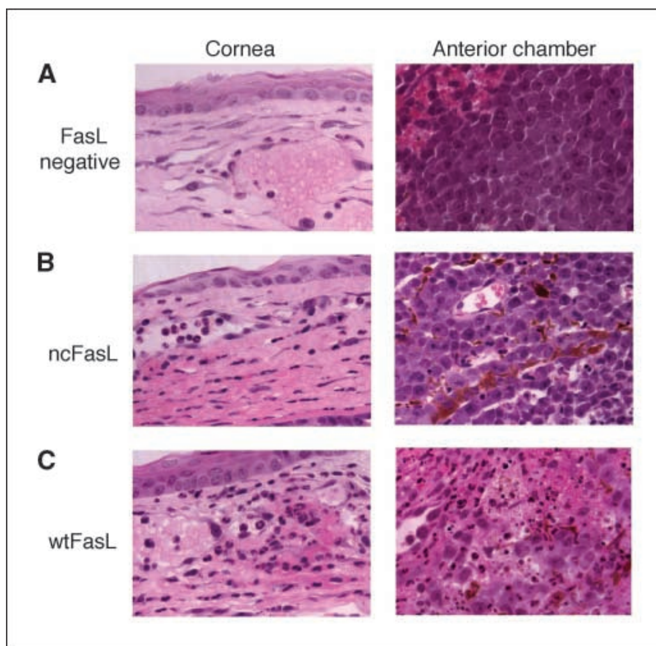

Figure 4. Tumors expressing wtFasL trigger potent neutrophil-mediated inflammation within the eye. DBA/2 mice received anterior chamber inoculations of 2×10^3 tumor cells expressing either no FasL (*A*), ncFasL (*B*), or wtFasL (*C*). Histologic analysis was performed on day 6 postinoculation. Pictures were taken of the cornea and the anterior chamber. Magnification, ×40.

Ten days after anterior chamber injection of GFP+wtFasL or GFP+ncFasL tumor cells, the tumor-containing eyes were removed and a single-cell suspension was prepared from four individual mice per group. The percentage of GFP+ tumor cells recovered from four separate eyes containing wtFasL tumors was not significantly different from eyes containing ncFasL tumors (data not shown). However, the level of FasL was decreased on ncFasL tumors obtained from tumor-containing eyes and assayed immediately. This was evident by the drop in median channel fluorescence from eye-derived tumors compared with ncFasL tumor cells cultured *in vitro* (Fig. 5*A*). The decrease in FasL on eye-derived tumors was transient and returned to the original level after 5 days of culture *in vitro*. By contrast, there was no decrease in FasL on wtFasL eye-derived tumors compared with wtFasL tumors cultured *in vitro* (Fig. 5*A*). We conclude that the lower levels of inflammation observed in eyes containing ncFasL tumors coincided with decreased levels of membrane FasL, whereas higher level of inflammation observed in eyes containing wtFasL tumors coincided with higher levels of membrane FasL.

Tumor cells that metastasize to the liver down-regulate FasL. To determine the level of cell surface FasL expressed on liver metastases, FasL-negative and wtFasL tumor cells were injected into the anterior chamber, and at 10 to 15 days postinoculation, metastatic tumor cells were isolated from the liver. Single-cell suspensions were prepared and FasL expression was assessed by flow cytometry. As expected, no FasL was detected on the FasL-negative tumor cell line or on FasL-negative tumor cells recovered from the liver (Fig. 5*B*). As previously shown, the wtFasL tumor cell line expressed high levels of FasL when cultured *in vitro* (Fig. 5*B*). However, the level of FasL was decreased significantly on metastatic wtFasL tumor cells directly isolated from the liver. Similar to the eye-derived tumor cells, the change in FasL expression was transient and the level of mFasL on metastatic wtFasL tumors returned to normal when cultured for 5 days *in vitro* (Fig. 5*B*). These data suggest that the decreased expression of mFasL on liver metastases is due to a local source of liver-derived proteases. Once the metastases are removed from the local source of proteases and placed in culture, the mFasL is no longer cleaved from the surface and the levels of mFasL return to normal. We conclude that the level of membrane FasL is reduced on metastatic wtFasL tumor cells that grow progressively in the liver, and this decrease is dependent on the proteases present in the local environment.

Tumors expressing ncFasL grow progressively within the eye but confer systemic concomitant immunity. As described above, both wtFasL and ncFasL tumor cells grew progressively within the eye, but only mice inoculated with wtFasL tumor cells succumbed to metastatic disease. To determine if the failure to form metastatic tumors coincides with systemic protective immunity, DBA/2 mice received an anterior chamber inoculation of tumor cells expressing either (*a*) no FasL, (*b*) wtFasL, or (*c*) ncFasL. At 7 days after anterior chamber inoculation, the same mice received a s.c. tumor challenge of FasL-negative tumor cells. As expected, mice challenged with FasL-negative tumor cells in the anterior chamber exhibited no signs of systemic protection as evidenced by the progressively growing flank tumors (Fig. 6*A*). Interestingly, although mice challenged with wtFasL tumor cells in the anterior chamber exhibited no systemic protection, mice challenged with ncFasL tumor cells in the anterior chamber exhibited strong systemic protection as shown by the rejection of the secondary flank tumor. Furthermore, the systemic protection induced by ncFasL tumor cells was tumor specific, as mice challenged with ncFasL tumor cells in the anterior chamber exhibited no systemic protection against an irrelevant syngeneic tumor (P815; Fig. 6*B*). These data show that although wtFasL and ncFasL tumors grew progressively within the eye, only mice challenged with ncFasL tumor cells were immunized effectively against a second challenge. Our previous data showed that systemic protection induced by ncFasL tumor cells was absent in SCID/beige mice, indicating antitumor immunity was mediated by T and/or B cells (17).

Discussion

Previously, we showed that the form and level of FasL were critical in the growth and rejection of tumors within the immune-privileged eye (15). In these earlier experiments, we examined the growth of ocular tumors that expressed (*a*) high levels of noncleavable membrane-only FasL, (*b*) low levels of noncleavable membrane-only FasL, or (*c*) soluble-only FasL (15). Only tumors that expressed high levels of mFasL were capable of terminating ocular immune privilege, resulting in rejection of the tumor and triggering of systemic protective immunity. For tumors expressing either a low level of mFasL or sFasL, immune privilege remained intact and the tumors grew progressively within the eye. However, only the tumor cells that produced sFasL metastasized to the liver. Although these earlier studies showed the importance of the level of ncFasL on the tumor, they did not directly compare tumors that expressed a noncleavable form of FasL with tumors that expressed a cleavable form of FasL that could be influenced by factors that either increased or decreased the extent of MMP cleavage. Cleavage of mFasL could influence tumor growth by both reducing the level of FasL

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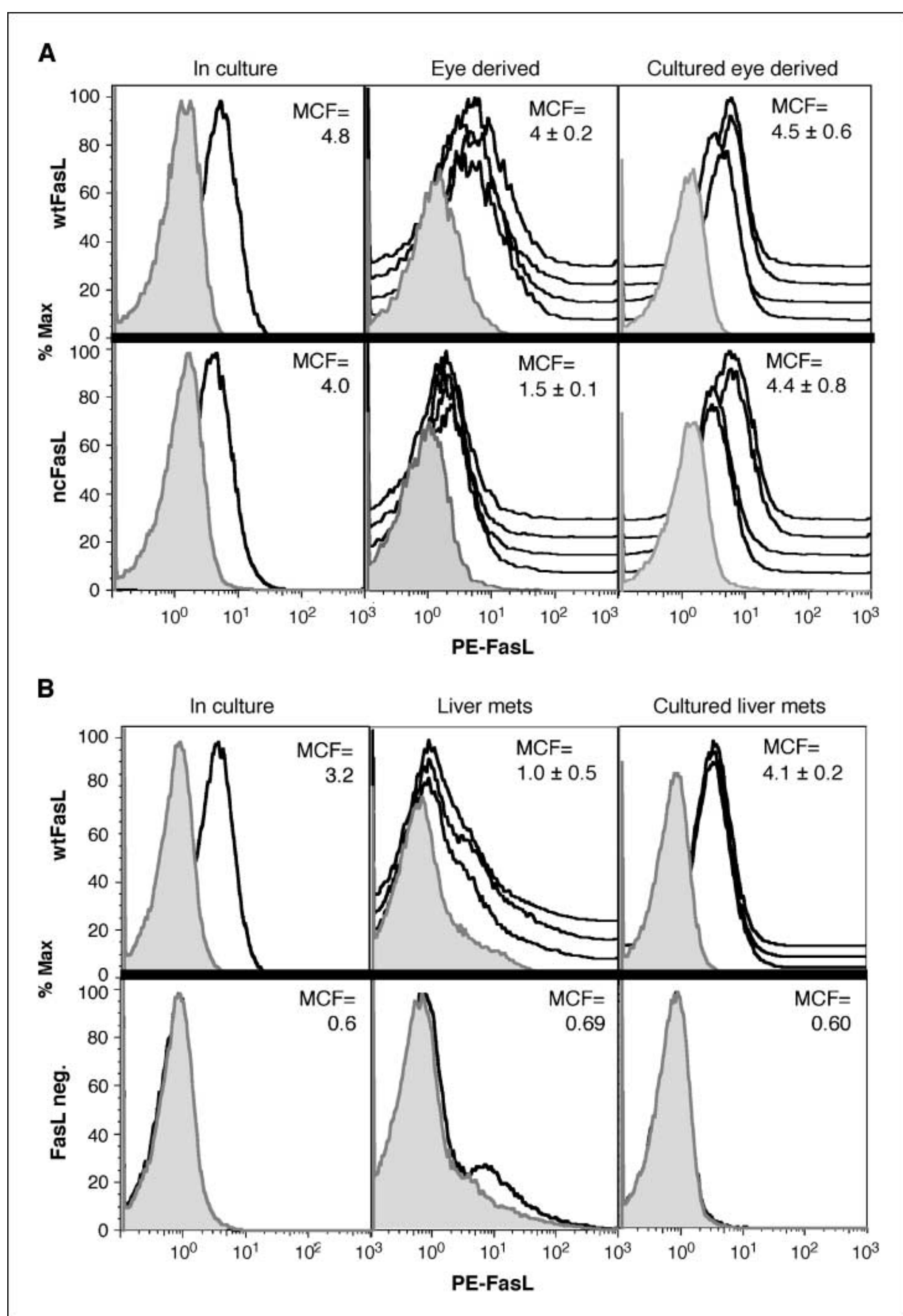

Figure 5. Expression of membrane FasL on eye-derived and liver-derived tumors. *A,* DBA/2 mice received anterior chamber inoculations of 2×10^3 tumor cells expressing either no FasL, wtFasL, or ncFasL. Eyes were enucleated on day 10 after anterior chamber inoculation and single-cell suspensions were prepared using the whole eye. Flow cytometry was used to measure mFasL on the GFP+ eye-derived tumor cells harvested either immediately or after 5 d of culture *in vitro* compared with normal tumor cells cultured *in vitro. B,* in a second group of mice, liver metastases were isolated on day 13 postinoculation. Single-cell suspensions of liver metastases were immediately stained with anti-FasL mAb MFL3 or cultured *in vitro* for 5 d. Flow cytometry was used to measure mFasL compared with mFasL expressed on normal tumor cells cultured *in vitro*. Data are presented from one experiment (n = 3–4 per group). Each experiment was repeated twice. *MCF,* median channel fluorescence for phycoerythrin ± SE. *Grey,* isotype control; *black lines,* results from individual mice.

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expressed by the tumor and also releasing potentially antagonistic sFasL.

In the current study, we have extended our analysis to include tumor cells that have the capacity to produce both the membrane and soluble forms of FasL. For this purpose, we selected tumor cell clones that, in culture, expressed comparably (low) levels of mFasL. The only difference between the two clones was that one expressed membrane-only ncFasL in which the MMP cleavage site was deleted, whereas the second expressed wtFasL and, therefore, could potentially express both mFasL and sFasL. *In vitro*, the two different tumor cell clones expressed equivalent amounts of membrane FasL. However, the relative level of membrane and sFasL produced by the tumors *in vivo* may vary depending upon the microenvironment in which the tumor is growing and the local concentrations MMP and MMP inhibitors.

MMPs mediate the release of sFasL from the cell surface (12–14). MMPs were first discovered as enzymes that degraded extracellular matrix components and played an important role in wound healing and tissue remodeling (20–22). To date, only MMP3 (stromelysin) and MMP7 (matrilysin) have been shown to cleave FasL (23). However, additional cleavage sites have been reported for FasL that are not recognized by MMP3 or MMP7, suggesting that additional MMPs may also cleave FasL (23). MMP7 and MMP3 are expressed at low levels within the eye and can be rapidly induced by a variety of growth factors and cytokines (24). In addition, the tumor cells used in our studies express a protease(s) that cleaves FasL, as

shown by the release of sFasL into the supernatant of *in vitro*, propagated tumor cells. Many laboratories have shown that MMPs are overexpressed in cancers and play an important role in cancer invasion and metastases (25–27). In particular, MMP7 is up-regulated in endometrial, gastric, and colorectal cancers, and the MMP7 activity correlates with vascular invasion and metastases (28–30). Thus, MMPs that mediate the cleaving of FasL could be expressed on the tumor cells and/or present within the ocular and hepatic environment. Our data presented herein shows that the mFasL expressed on wtFasL transfectants can be cleaved by MMPs, and treatment with a protease inhibitor prevents cleavage and increases the level of mFasL. By contrast, Matrisian and colleagues (31) showed that increased protease activity coincides with increased sFasL and decreased mFasL. Therefore, the significant decrease in the level of mFasL on wtFasL liver metastases suggests

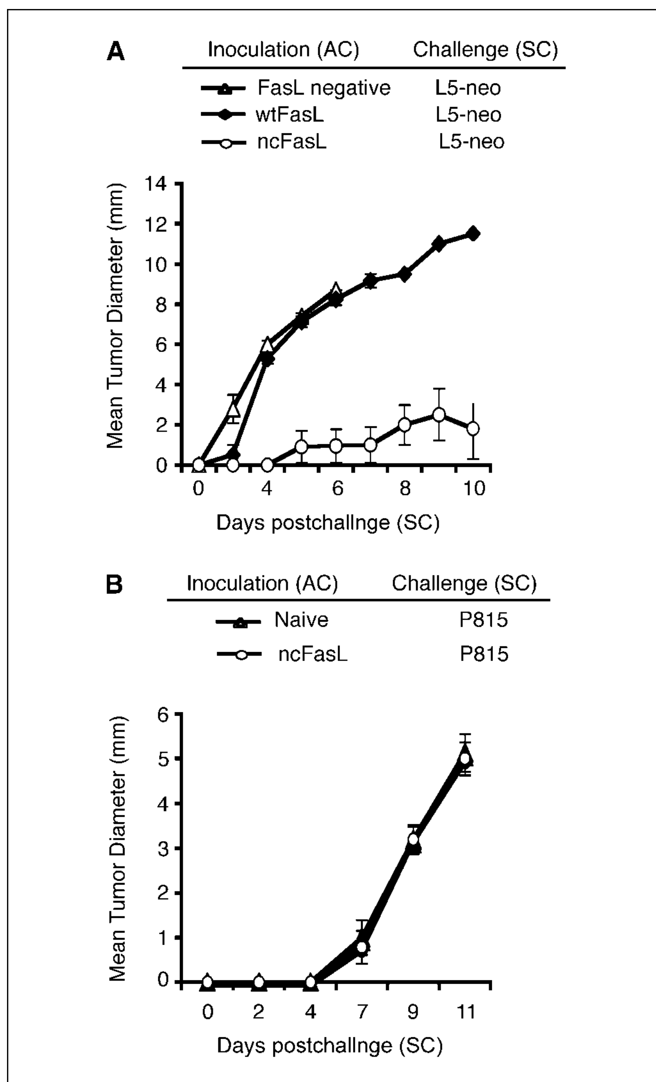

Figure 6. Ocular tumors expressing ncFasL confer protection from a secondary s.c. challenge of L5-neo tumor cells. DBA/2 mice received anterior chamber inoculations of 2×10^3 tumor cells expressing either no FasL, wtFasL, or ncFasL. On day 7 postinoculation, the same mice received a second tumor challenge of FasL-negative tumor cells (L5 neo; *A*), or an irrelevant syngeneic tumor (P815; *B*) into the s.c. tissue of the flank. Mean tumor diameter was measured every 2 d. Data are presented from one experiment (n = 5 per group). Each experiment was repeated twice.

that active MMPs are present in the liver that are cleaving mFasL from the surface of tumor cells. Furthermore, flow cytometric analysis reveals that when removed from the liver and placed in culture, the level of mFasL on the metastatic tumor cells returns to levels observed on the original transfectants. Taken together, these data indicate that liver-derived proteases are responsible for the increased cleavage of FasL, resulting in a significant decrease in the level of mFasL. Future studies will identify the proteases present within the liver responsible for FasL cleavage.

Another factor that potentially regulates the cleavage of FasL is the presence of tissue inhibitors of MMPs (TIMP), which are expressed within the eye and are the major endogenous regulators of MMP activity (24, 32, 33). Taken together with the data presented herein, we propose that the cleaving of FasL from the cell surface of tumor cells plays a critical role in the development of metastases. Furthermore, the activity of the MMPs and TIMPs expressed on the tumor cells and the surrounding tissue is likely to be determined by the environment in which the primary tumor develops.

Our current study shows that, in the eye, the wtFasL tumors displayed higher levels of membrane FasL than the ncFasL tumors. Moreover, consistent with our previous data, the levels of inflammation induced by the ocular tumors coincided with the level of membrane FasL, and the wtFasL tumors induced a stronger inflammatory response. One explanation for the relative increase of mFasL on the wtFasL tumor cells is that TIMPs present in the ocular environment block MMP cleavage of FasL, leading to increased levels of mFasL and the ensuing increased inflammation. Exactly why the level of mFasL decreases on the ncFasL tumors in the eye is less clear. Although the tumor cell lines were originally clones, the expanded population displays a range of FasL in which some of the tumor cells express higher levels, whereas others express lower levels. It is possible that the cells with the highest levels of mFasL are more immunogenic and, therefore, selectively targeted by the resulting inflammatory response, leaving the cells with lower levels of mFasL to expand within the eye.

Another important question is why only the ncFasL ocular tumors, which cannot express sFasL and express lower levels of membrane FasL in the eye, activate systemic protective immunity that prevents the formation of metastatic liver tumors, whereas wtFasL ocular tumors that express sFasL fail to activate systemic protective immunity. One possibility is that the sFasL produced from wtFasL tumors blocks the immunogenic effects of mFasL and prevents wtFasL tumors from activating protective systemic immunity, thereby permitting the formation of liver metastases. The presence of MMPs and TIMPs and other factors that modulate FasL transcription within the eye and liver makes the regulation of membrane and sFasL very complex. Therefore, it is difficult to know how much sFasL is actually produced by the tumors within the eye and the liver. However, although our data suggest that cleavage of FasL from wtFasL tumors is inhibited in the eye, MMPs present outside the eye and/or in the liver may increase the cleavage of FasL, resulting in the decreased levels of mFasL detected on wtFasL liver metastases. The same MMPs would have no effect on the level of mFasL and sFasL expressed by ncFasL tumor cells.

Overall, the data point to an important role for sFasL in determining the metastatic potential of tumors. The level of membrane FasL increases on primary ocular tumors that express wtFasL. However, these same tumors form metastases in the liver, where the expression of membrane FasL is drastically reduced. After 5 days of culture *in vitro*, the level of mFasL returns to the

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level of the original clone, suggesting that the change in mFasL expression is dependent on the local environment. Future experiments will focus on understanding (*a*) how the local environment within different anatomic sites regulates MMP and TIMP activity, (*b*) how this activity affects the level of membrane and sFasL produced by the tumor, and (*c*) how the increase in sFasL promotes the development of metastases.

Acknowledgments

Received 2/25/2007; revised 7/27/2007; accepted 10/8/2007.

Grant support: NIH grants F32-EY13664, RO1-EY016145 (M.S. Gregory), RO1-EY08122 (B.R. Ksander), and RO1-CA90691 (A.M. Rothstein).

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We thank Dr. Parisa Zamiri for her technical assistance with the i.v. injections and Randy Huang for technical assistance with the flow cytometric analysis.

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