Macrophages Are Vital in Spontaneous Intraocular Tumor Eradication

Zita F. H. M. Boonman,[1] *Lucas R. H. M. Schurmans,*[1] *Nico van Rooijen,*[2] *Cornelis J. M. Melief,*[3] *René E. M. Toes,*[3,4] *and Martine J. Jager*[1]

PURPOSE. Injection of tumor cells transformed by the early region 1 of human adenovirus type 5 (Ad5E1) in the anterior chamber (AC) of C57BL/6 mice leads to intraocular tumor formation. This tumor disappears spontaneously 3 to 4 weeks after tumor inoculation without damaging the neighboring ocular tissues. Previous studies have shown that $CD4^+$ T cells, IFNγ, and TRAIL (tumor necrosis factor-related apoptosis-inducing ligand) play a role in the spontaneous eradication of this particular intraocular tumor. This study was conducted to determine whether macrophages are involved in the natural elimination of this intraocular tumor.

METHODS. Ad5E1-expressing tumor cells were inoculated into the AC of syngeneic C57BL/6 mice. Macrophage depletion was obtained by subconjunctival (scj), subcutaneous (sc), or intravenous (iv) injection of clodronate liposomes 2, 8, and 14 days after tumor inoculation. Control C57BL/6 mice received PBS liposomes at similar time points after tumor injection or were left untreated. The presence of macrophages in the AC tumor was determined with the macrophage marker F4/80.

RESULTS. Progressive tumor growth was observed in mice that were subconjunctivally depleted of macrophages, whereas spontaneous tumor eradication occurred in all other groups. F4/80 staining was negative in the AC tumors of mice treated scj with clodronate liposomes in contrast to the positive F4/80 staining in the tumors of the other groups. Ad5E1 tumor antigen still reached the tumor-draining lymph nodes (DLNs) of mice locally depleted for macrophages.

CONCLUSIONS. Local macrophages in the eye are involved in the process of spontaneous AC tumor eradication in mice. However, it is not conclusive from these data exactly how tumor-specific $CD4^+$ T cells and macrophages interact with each other to eliminate the Ad5E1-AC tumor without any collateral eye damage. (*Invest Ophthalmol Vis Sci.* 2006;47:2959–2965) DOI:10.1167/iovs.05-1427

The eye has evolved special immunologic features to protect itself from immune-mediated disease. This concept of immune privilege was suggested during classic studies demonstrating that transplanted allografts survive longer in the AC of the eye than in nonprivileged body sites, such as the skin.[1] The biological significance of immune privilege of the eye seems clear; the visual axis needs to be protected from irreversible injury caused by a severe immune response, as such an evoked immunologic reaction in the delicately structured eye may be more damaging than the initial invading pathogen.

It has traditionally been believed that immune privilege of the eye was due to lack of lymphatic drainage and the presence of blood–ocular barriers. Furthermore, it has been speculated that ocular antigen (Ag) is hidden from the immune system, thus sparing the eye from the destructive effects of inflammatory cells, as they do not reach the eye.[2] More recently, it has been acknowledged that ocular immune privilege is much more multifaceted. It includes the production and action of immunosuppressive cytokines, expression of Fas ligand (FasL), and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL).[3,4] FasL and TRAIL are placed at strategic places in the eye, such as corneal epithelium, corneal endothelium, iris vasculature, and retina to induce apoptosis of invading lymphoid cells and protect ocular tissues from the damaging effects of inflammation.[5,6] This may be why ocular inflammatory disease is relatively rare. However, intraocular tumors are also exceptional. The position of both FasL and TRAIL may contribute to these observations. Another mechanism for limiting intraocular tumor progression is T-cell-dependent immune rejection of intraocular tumors, showing that ocular immune privilege can be circumvented.[7,8] Thus, the eye has both T-cell-dependent and -independent mechanisms for controlling intraocular tumors.

Immune-mediated rejection of intraocular tumors can follow two fundamental patterns.[9,10] The first pattern strongly resembles a delayed-type hypersensitivity (DTH)–mediated process and culminates in extensive collateral damage to all structures of the eye, eventually leading to phthisis. This rejection mechanism is executed by $CD4^+$ T cells. By contrast, the second pattern of intraocular tumor eradication mainly involves tumor-specific cytolytic T lymphocytes (CTLs) and leaves the eye morphologically intact. In recent studies, we report a third pattern of intraocular tumor rejection. Tumor cells transformed by the human adenovirus type 5 early region 1 (Ad5E1) oncogenes are rapidly rejected when injected sc, but form tumors when injected into the anterior chamber (AC) of the eye.[7] These intraocular tumors do not grow progressively but are eventually rejected after several weeks. This tumor elimination process is $CD4^+$ T-cell dependent, but does not require TNFα, FasL, perforin, B cells, NK cells, or $CD8^+$ T cells.[8] However, this nonphthisis form of tumor rejection is IFNγ-dependent and appears to be mediated by TRAIL.[11]

Because Ad5E1-expressing tumor cells are major histocompatibility complex (MHC) class II negative it is unlikely that tumor-specific $CD4^+$ T cells directly recognize and kill the Ad5E1-expressing cells. It is plausible that the effective antitumor response involves communication between tumor-specific $CD4^+$ T cells and MHC class II-positive host cells that cross present tumor-derived material to the $CD4^+$ T cells. Accordingly, we considered the possibility that macrophages may act as additional effector cells for inducing apoptosis of intraocular tumor cells. It is well known that macrophages have a central

From the Departments of [1]Ophthalmology, [3]Immunohematology and Blood Transfusion, and [4]Rheumatology, Leiden University Medical Center, Leiden, The Netherlands; and the [2]Department of Cell Biology, Free University, Amsterdam, The Netherlands.

Submitted for publication November 4, 2005; revised January 26, 2006; accepted May 9, 2006.

Disclosure: **Z.F.H.M. Boonman**, None; **L.R.H.M. Schurmans**, None; **N. van Rooijen**, Roche Diagnostics GmbH (F); **C.J.M. Melief**, None; **R.E.M. Toes**, None; **M.J. Jager**, None

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Corresponding author: Martine J. Jager, Department of Ophthalmology J3-S, Leiden University Medical Center, Albinusdreef 2, 2300 RC, Leiden, The Netherlands; m.j.jager@lumc.nl.

Copyright © Association for Research in Vision and Ophthalmology

Downloaded from iovs.arvojournals.org on 04/24/2024

role in immune responses, also in antitumor immunity.[12] Activated macrophages are potent producers of IFNγ and stimulate $CD4^+$ T cells.[13] In addition, Ag-presenting macrophages can be eliminated by activated $CD4^+$ T cells.[14] It is also conceivable that macrophages act as ancillary effector cells for inducing apoptosis of intraocular Ad5E1-expressing tumor cells. Hence, we investigated the role of macrophages in this nonphthisis intraocular tumor-rejection process.

Methods

Mice

Male C57BL/6 mice (H-$2D^b$) were obtained from Jackson Laboratory Iffa Credo (Brussels, Belgium). Strain 42 (Thy 1.2) mice, bred at TNO-PG (Leiden, The Netherlands), are T-cell receptor (TCR) transgenic mice expressing the TCRα and β chains derived from the H-$2D^b$-restricted, Ad5E1A234-243-specific CTL clone 5.[15] All animals were housed and cared for in accordance with the guidelines of the University Committee for the Humane Care of Laboratory Animals, NIH Guidelines on laboratory animal welfare, and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Murine Tumor Cell Line

The characterization of murine embryo cells transfected with the early region 1 of human adenovirus type 5 (Ad5E1) used in this study have been described previously.[16] Cell lines were maintained in Iscove's modified Dulbecco's medium (IMDM; Invitrogen, Rockville, MD) supplemented with 8% fetal calf serum (FCS), 50 μM 2-mercaptoethanol, glutamine, and penicillin.

Clodronate-PBS–Containing Liposomes

The drug dichloromethylene diphosphonate (clodronic-acid disodium salt tetrahydrate; Cl_2MDP) was a gift from Roche Diagnostics GmbH (Mannheim, Germany). Preparation of multilamellar phosphatidyl-choline (PC) liposomes containing Cl_2MDP or PBS as a control were prepared as described previously.[17]

Intracameral Inoculations and Clinical Evaluation

A previously described technique for deposition of a definite number of tumor cells into the AC of the mouse was used.[7] Mice were anesthetized with a mixture (ratio 1:1) of xylazine (Rompun 2%; Bayer, Leverkusen, Germany) and ketamine hydrochloride (Aescoket; Aesculaap bv, Boxtel, The Netherlands) given intraperitoneally. The eye was viewed at low power (8×) under a dissecting microscope, and a sterile 30-gauge needle was used to puncture the cornea at the corneoscleral junction, parallel and anterior to the iris. A glass micropipette (80 μm in diameter) was fitted into a sterile infant feeding tube, which was mounted onto a sterile 0.1-mL syringe (Hamilton Co., Inc., Reno, NV). The pipette, loaded with Ad5E1-transformed tumor cells ($0.3 \cdot 10^6$ cells/4 μL) was introduced through the puncture site of the cornea, and 4 μL of the tumor cell was delivered into the AC. The eyes were examined three times a week with a dissecting microscope to observe and document tumor growth. Tumor volume was recorded as the percentage of AC occupied with tumor.[8]

In Vivo Macrophage Depletion

For in vivo macrophage depletion, mice (each group consisting of 10 mice) were injected either subconjunctivally (scj), subcutaneously (sc), or intravenously (iv) with Cl_2MDP liposomes or PBS liposomes as a control (sham depletion) on days 2, 8, and 14 post-Ad5E1-tumor inoculation. In short: for the scj liposome injections, animals were anesthetized with a mixture (ratio 1:1) of xylazine (Rompun 2%; Bayer) and ketamine hydrochloride (Aescoket; Aesculaap bv) given intraperitoneally. Under a dissecting microscope, the conjunctiva was lifted and a 30-gauge needle was used to puncture the conjunctiva at four different locations around the AC and limbus. A glass micropipette (80 μm in diameter) was fitted into a sterile infant feeding tube, which was mounted onto a sterile 0.1 mL syringe (Hamilton Co., Inc). The pipette, loaded with liposomes was introduced through the four different puncture sites of the conjunctiva, and at each puncture site 4 μL of the liposome suspension was delivered. In total, 16 μL of Cl_2MDP or PBS liposomes were injected into the bulbar conjunctiva, resulting in an equally distributed bleb surrounding the four injection sites around the AC and limbus. Local subcutaneous macrophage depletion was obtained by injecting 50 μL liposome suspension under the skin of the cheek ipsilateral to the intraocular tumor. For systemic in vivo macrophage depletion 200 μL of Cl_2MDP liposome suspension (or PBS liposomes as control) was injected into the tail vein of tumor-bearing mice. Control groups, in addition to the animals treated with PBS-containing liposomes, were intraocular tumor-bearing mice without any additional treatment.

Histology

Eyes and submandibular lymph nodes (LNs) were collected from two to three mice from each group (each group contained 10 mice in each experiment) for histology on days 14, 17, 23, and 28 after tumor inoculation. Eyes were fixed in paraformaldehyde, progressively dehydrated to 99% ethanol and embedded in paraffin. Serial paraffin-embedded 4-μm sections of a murine (tumor-bearing) eye were stained with hematoxylin and eosin (HE).

Immunohistochemistry

Macrophages were visualized by immunohistochemistry using the rat anti-mouse macrophage mAb F4/80 (clone C1:A3-1, IgG2b; Serotec, Oxford, UK).[18] Eyes were enucleated, and the submandibular LNs were dissected from cervically dislocated mice with AC tumors untreated or treated scj, sc, or iv with Cl_2MDP or PBS liposomes from day 3 and further on after the last liposome injections. Paraffin-embedded 4-μm sections, mounted on slides coated with aminopropyltriethoxy silane (APES; Sigma-Aldrich, St. Louis, MO), were deparaffinized in xylene (two times for 10 minutes) and ethanol 99% (two times for 5 minutes), rehydratated followed by Ag retrieval in a 37°C trypsin water bath for 30 minutes. Subsequent incubations of the sections were performed sequentially for 60 minutes, with 5-minute washings in PBS between each step.[19] Unspecific antibody binding was blocked by incubation with PBS containing 1% bovine serum albumin for 10 minutes. Incubation with the monoclonal antibody F4/80 was followed by biotinylated rabbit anti-rat IgG antibody (code no. E0467; Dako, Glostrup, Denmark), diluted 1:300 in PBS containing 1% bovine serum albumin. After a final incubation with biotinylated alkaline phosphatase-streptavidin (code no. K0391; Dako), the alkaline phosphatase reaction was developed using Fast Red (Scytek, Logan, Utah) in a naphthol-phosphate buffer (Scytek) with levamisole 50 mM. After 20 minutes, this reaction was blocked in distilled water. The slides were counterstained with Mayer's hematoxylin and mounted in Kaiser's glycerin. Control sections were incubated with isotype-matched primary Ab or with secondary Abs alone. In addition, lung sections were used as the positive control. The microscopic slides containing F4/80-positive staining were evaluated with the reader unaware of the treatment given.

CFSE Labeling and Adoptive Transfer of Transgenic T Cells

CFSE (carboxyfluorescein diacetate succinimidyl ester) labeling was performed as previously described.[20] Cells in peripheral LNs and spleens from TCR-transgenic mice (strain 42) were resuspended in PBS at 1×10^7 cells/mL and incubated with 0.5 μM CFSE (Invitrogen, Eugene, OR) for 30 minutes at 37°C. FCS was added in a concentration of 5%, and the cells were washed in PBS. CFSE labeled TCR-transgenic $CD8^+$ T cells (3×10^6) were injected into the tail vein of (tumor-bearing) mice in 200 μL of PBS.

Downloaded from iovs.arvojournals.org on 04/24/2024

Flow Cytometry Analysis

CFSE-labeled transgenic E1A-specific T cells from draining and nondraining LNs and spleens were stained with APC-labeled anti-CD8 and PerCp-conjugated propidium iodide. Data acquisition and analysis were performed on a flow cytometer (FACScan with CellQuest software; BD Biosciences, Franklin Lakes, NJ).

Experimental Design

To observe whether macrophages play an important role in the specific eradication of Ad5E1-expressing tumors in the AC of the eye, we performed the following experiments. Any difference in intraocular Ad5E1 tumor growth behavior was monitored in the several experimental setups. In particular, attention was paid to the rate of tumor development, (maximal) percentage of AC occupied with tumor cells and pace of tumor eradication. Seventy mice inoculated with Ad5E1-expressing tumor cells were divided into seven groups of 10 mice each. Group 1 was untreated; group 2 received scj Cl_2MDP liposomes; group 3 had Cl_2MDP liposomes injected sc in the region of the tumor-draining lymph node (DLN), the submandibular LN,[21] ipsilateral to the tumor-bearing eye; group 4 was injected iv with Cl_2MDP liposomes in the tail vein; and groups 5, 6, and 7 received PBS liposomes scj, sc, or iv, respectively, and served as control groups. This process was repeated three times with similar results.

Observing Tumor Growth. Both Cl_2MDP and PBS liposomes were deposited according to the mode of administration just described at days 2, 8, and 14 after Ad5E1 tumor cell injection into the AC of the eye. Intraocular tumor growth was observed by using a dissecting microscope in which tumor volume was recorded as the percentage of AC occupied by the tumor.[8] This experiment was repeated two more times.

Histology. The experimental setup was as described earlier, with the exception that a few hours after the last injection of either clodronate or PBS liposomes (day 14 after tumor injection), two to three mice in each group were killed for the histologic workup of the enucleated tumor-containing eye and the resected submandibular LN. This was repeated on days 17, 23, and 28 after tumor inoculation. Serial paraffin-embedded 4-μm sections of a murine (tumor-bearing) eye or of a submandibular LN were stained with HE or macrophages were visualized by immunohistochemistry using the monoclonal antibody F4/80.

Timing of Macrophage Depletion. Ten tumor-bearing mice were treated with scj Cl_2MDP liposomes (group 2). Ten mice were injected with scj PBS liposomes (group 5), and another 10 mice were not treated (group 1). To find out whether the timing of clodronate injections mattered, we started the first injection 8 days after tumor inoculation. We repeated the procedure at day 14 after tumor injection. This experiment was repeated once.

Intraocular Ag Drainage and T-Cell Priming in Tumor-DLNs. Recently, we described that intraocular tumor Ags are presented to tumor-specific CTLs in tumor-DLNs.[21] This Ag is likely to be derived from the ocular growing tumor, as previous control experiments have shown that leakage of tumor cell suspension into the conjunctival sac during the inoculation into the AC does not lead to T-cell priming in the DLNs.[21] Twenty-one mice with intraocular Ad5E1-expressing tumors were divided in three groups of seven mice each. Group 1 was not treated, group 2 received scj clodronate deposits, and group 3 was injected sc with clodronate liposomes in the region of the submandibular LN at days 2, 8, and 14 after tumor injection. Group 0 consisted of seven naïve mice having neither tumor nor liposome injections. Two days after the last clodronate liposome injection (16 days after tumor inoculation), we adoptively transferred CFSE-labeled E1A-specific TCR-transgenic T cells into tumor-bearing mice, which had received local clodronate liposome injections scj (group 2) or sc (group 3), into nontreated tumor-bearing mice (group 1) and into naïve mice (group 0). Three days after adoptive transfer of the TCR transgenic cells (day 19 after tumor injection), lymphoid organs of tumor-bearing recipients were removed and prepared for flow cytometry. The experiment was then repeated.

Statistics

Statistical analysis was based on three similar experimental setups, every experiment consisting of 70 mice with intraocular tumors, divided over seven groups of 10 animals each (look at experimental design). The χ^2 statistic test was used to compare proportions between the groups. Because multiple χ^2 tests were performed (one for the whole group and one for each comparison of a treatment group with the controls), the α level was divided by seven (tests in total) to correct for multiple comparisons problem (Bonferroni correction, $P = 0.05/n = 7$ $P = 0.007$). Group 1: control group, untreated animals with transient intraocular tumor growth; group 2: animals with intraocular tumors treated with scj Cl_2MDP liposome injections: χ^2=52.33 (df = 1, $P \ll 0.007$) with Yates continuity correction; group 3: animals with intraocular tumors treated with sc Cl_2MDP liposome injections: χ^2=9.12 (*di* f= 1, $P = 0.02$, not significant [ns]) with Yates continuity correction; group 4: animals with intraocular tumors treated with iv Cl_2MDP liposome injections: ns; groups 5, 6, and 7: animals with intraocular tumors treated with scj, sc, and iv PBS liposome injections respectively: ns.

RESULTS

Progressive Intraocular Tumor Growth in Mice Locally Depleted of Macrophages

In prior studies, it has been shown that Ad5E1 intraocular tumors undergo $CD4^+$ T cell-dependent rejection without collateral eye damage. We considered the possibility that macrophages might act as additional effector cells for inducing apoptosis of these intraocular tumor cells. This hypothesis was tested by transplanting Ad5E1 tumor cells into the AC of syngeneic C57BL/6 mice. Macrophages and myeloid dendritic cells (DCs) were depleted locally or systemically by clodronate (Cl_2MDP) liposomes 2, 8, and 14 days after tumor inoculation. Control C57BL/6 mice received PBS liposomes at similar time points after tumor injection or were left untreated. The results of a representative experiment are shown in Figure 1 and demonstrate that intraocular tumors grew in both macrophage depleted and undepleted mice for approximately 3 weeks. However, the intraocular tumors disappeared by 4 weeks in the untreated mice, in mice treated systemically (iv) either with clodronate or PBS liposomes and in all the mice treated scj and sc with PBS liposomes. In repeated experiments, most of the mice treated with sc clodronate liposome injections in the area of the tumor-draining lymph node rejected the tumor, although some mice had progressive intraocular tumor growth that was not significant compared with the untreated control group ($P = 0.02$; $P > 0.007$, not significant after Bonferroni correction). However, intraocular tumor rejection never occurred in mice treated subconjunctivally with clodronate liposomes compared with the untreated tumor-bearing mice ($P < 0.007$, significant after Bonferroni correction). Thus, local subconjunctival depletion of phagocytic cells in the eye significantly inhibited intraocular tumor clearance, and the mice died as a consequence of progressive tumor growth.

In the repetitive experiments, it was noteworthy that almost all tumor-bearing mice treated with local (scj or sc) clodronate liposome injections showed a faster and more aggressive intraocular tumor growth in the AC of the eye. For example, 2 weeks after tumor injection more than 80% of the AC was filled with tumor in scj clodronate liposome-treated animals compared with only 30% to 45% tumor filling in the AC of the eyes in mice treated with PBS liposomes or nontreated mice. The results of a typical experiment are shown in Figure 2.

Downloaded from iovs.arvojournals.org on 04/24/2024

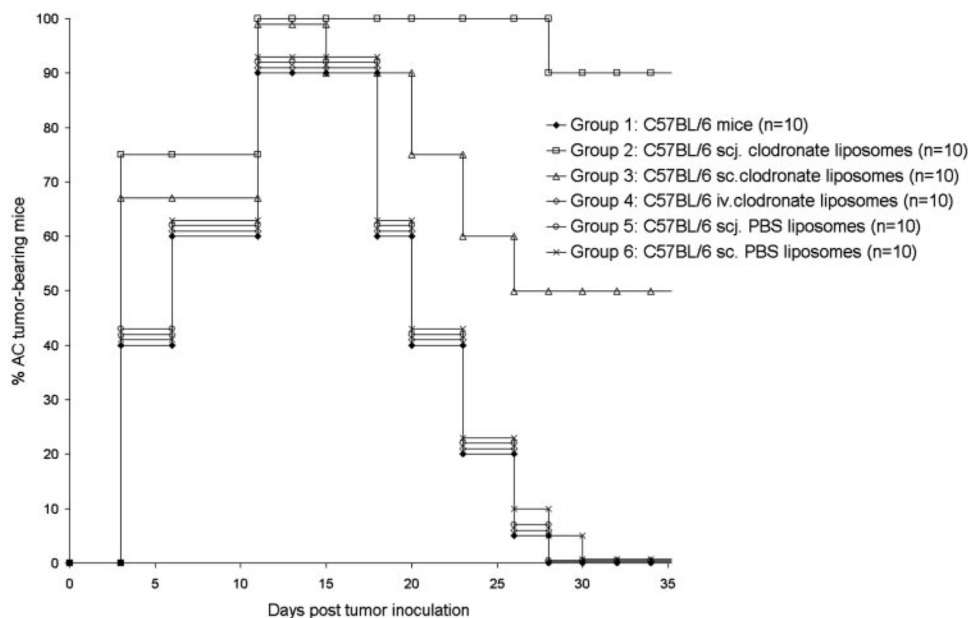

FIGURE 1. Progressive intraocular tumor growth in mice locally depleted of macrophages. Macrophages were depleted scj and sc in the region of the tumor-DLN or iv with clodronate liposomes 2, 8, and 14 days after tumor inoculation. Control C57BL/6 mice received PBS liposomes scj, sc, or iv (data not shown) at similar time points after tumor injection or were untreated. Only mice treated with scj injections of clodronate liposomes did not reject their intraocular tumors, which is statistically significant compared with the untreated tumor-bearing mice ($P < 0.007$ after Bonferroni correction). This experiment was performed two more times, with similar results.

To find out whether timing of macrophage depletion make a difference in tumor development, the starting point of subconjunctival clodronate liposome injection was delayed until days 8 and 14 after tumor inoculation. In these experiments, the intraocular tumor vanished after several weeks, showing that timing of macrophage and myeloid DC depletion matters. Thus, the eradication process in which macrophages are involved starts very early. In conclusion, these results indicate that phagocytic cells are necessary for the $CD4^+$ T cell-dependent rejection of Ad5E1 tumors.

F4/80 Immunoreactive Cells in Diminishing Intraocular Tumors

It was important to determine the local depleting effect of Cl_2MDP on macrophages and myeloid DCs in tumor-bearing mice. Accordingly, the tumor-bearing eyes were enucleated, and the submandibular LNs were resected in untreated animals and in mice locally or systemically treated with Cl_2MDP or PBS liposome injections. Tissue was examined for the presence of macrophages by using the monoclonal antibody F4/80, a marker present on the cell surface of most macrophages and many myeloid DCs.[18] Figure 3 shows that no $F4/80^+$ cells were visible in the intraocular tumor of a mouse injected with Cl_2MDP liposomes subconjunctivally. In contrast, F4/80 immunoreactive cells were observed in the deteriorating tumor of a nontreated animal. In fact, at any time point, $F4/80^+$ cells were detected in the intraocular tumors of mice either treated sc or iv with clodronate liposomes, as in the tumors of mice treated scj, sc, or iv with PBS liposomes. Moreover, harvested submandibular LNs showed F4/80-immunoreactive cells in all groups, even in the mice treated with scj Cl_2MDP liposomes (data not shown). Thus, subconjunctival Cl_2MDP liposome injections

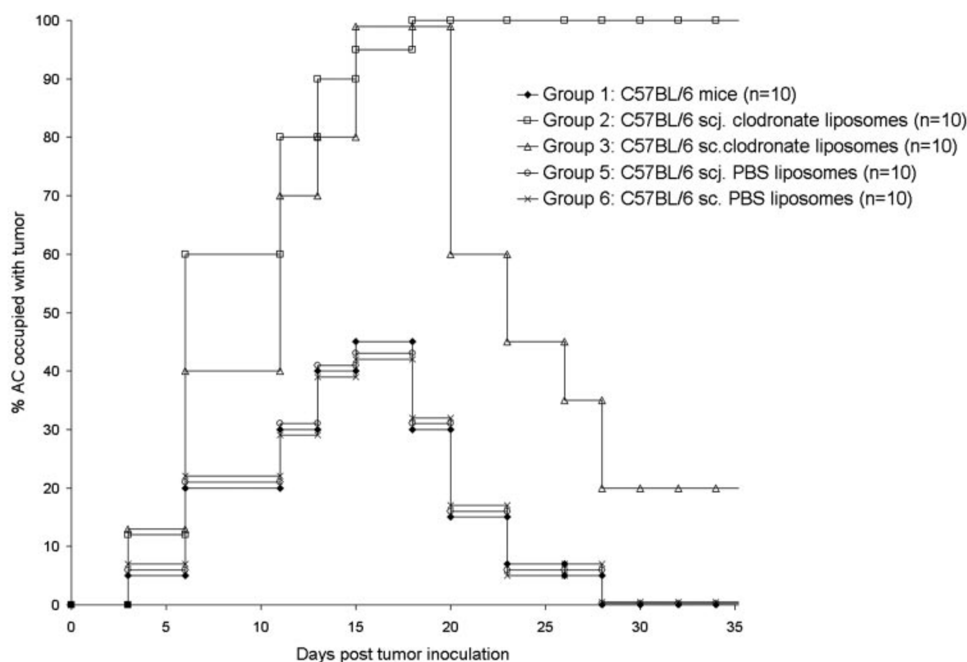

FIGURE 2. Faster and more aggressive intraocular tumor growth in mice locally injected with clodronate liposomes. Mice received 2, 8, and 14 days after tumor inoculation either scj and sc in the region of the tumor-DLN, or iv (group 4, not shown) injections with clodronate liposomes. Control mice received PBS liposomes scj, sc, or iv (group 7, not shown) at similar time points after tumor injection or were untreated. Both groups 2 and 3 treated with scj or sc clodronate liposome injections, respectively, showed faster and more aggressive tumor growth. This experiment was performed two more times, with similar results.

Downloaded from iovs.arvojournals.org on 04/24/2024

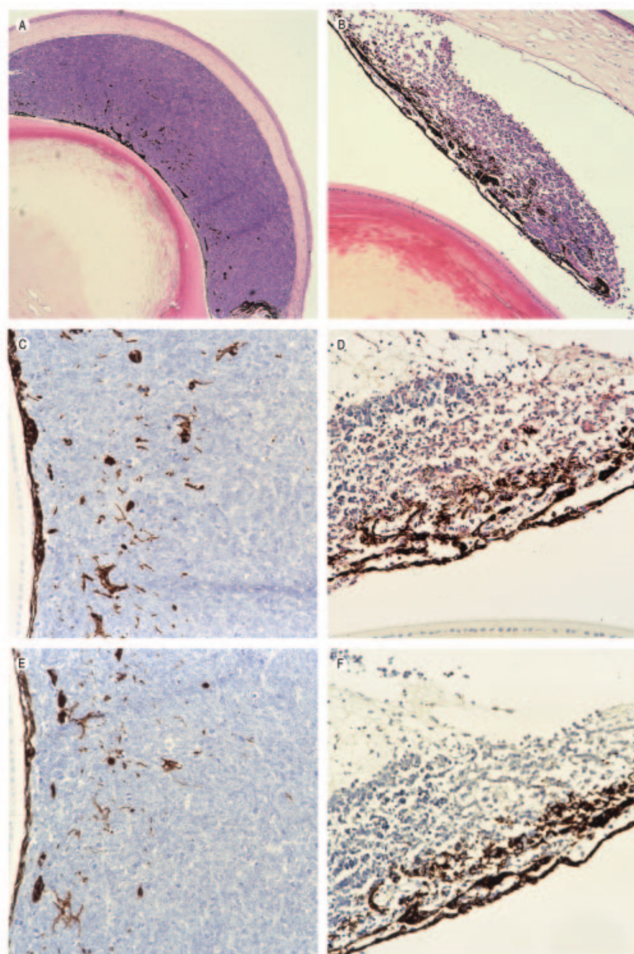

FIGURE 3. Regressing intraocular tumor contained F4/80$^+$ immunoreactive cells in contrast to eye tumor locally depleted of macrophages by clodronate liposomes. Tumor-bearing eyes of treated and untreated mice were enucleated at several time points and stained with HE and rat anti-mouse macrophage mAb F4/80. The HE-stained tumor-containing eye of a mouse treated scj with Cl_2MDP liposomes (**A**) did not show any signs of regression 21 days after tumor injection and did not contain any F4/80$^+$ cells (**C**). The HE-stained AC tumor of an untreated mouse (**B**) was almost eliminated after 21 days and contained F4/80$^+$ cells (**D**). Control sections were incubated with isotype-matched primary Ab (**E**, **F**).

suppressed macrophages and/or myeloid DCs in the tumor-bearing eye resulting in very few, if any, F4/80$^+$ cells at each time point examined. In addition, the macrophage or myeloid DC content was not affected in the submandibular LN, the tumor-DLNs.[21] In conclusion, the absence of or presence of very few F4/80$^+$ cells detected in mice treated with subconjunctival Cl_2MDP injections correlated strongly with the observed progressive intraocular tumor growth.

Maintaining Tumor Ag Presentation in Local Draining Lymph Nodes after sc Macrophage Depletion with Clodronate Liposomes

Both macrophages, myeloid DCs and macrophage precursors can be depleted by clodronate liposomes.[22] These cells, but especially DCs, are able to present (tumor) Ags to T cells in secondary lymph tissues.[23] Even though F4/80$^+$ cells were observed in the submandibular LN of all treated groups, we set out to determine whether treatment with clodronate liposomes affect Ag presentation and/or T-cell priming in the tumor-draining submandibular LNs.[21] We studied whether tumor-specific Ags (E1A-epitope) were still presented in the tumor-DLNs in mice treated locally with clodronate liposomes either sc or scj compared with nontreated animals. Figure 4 shows that intraocular tumor-specific E1A reached the tumor-DLNs in all groups, resulting in clonal tumor-specific CD8$^+$ T-cell expansion. Subcutaneous injection of clodronate liposomes affected presentation of tumor-derived Ag in the DLNs, since division of tumor-specific CD8$^+$ T cells was reduced compared with that in the control group. In contrast, no such effect was observed after scj clodronate injection. In conclusion, subconjunctival clodronate injection led to progressive intraocular tumor growth in tumor-bearing hosts but did not reduce tumor Ag presentation to tumor-specific CD8$^+$ T cells in the local tumor-draining submandibular LNs.

DISCUSSION

The present study was designed to investigate whether phagocytic cells, especially macrophages, play a role in nonphthisis intraocular tumor eradication. The results presented in this work identify a principal role for local macrophages in ocular antitumor immunity. By injecting Cl_2MDP liposomes sc, which leads to local depletion of macrophages in the tumor-bearing eye, we showed that local macrophages are vital in the rejection phase of intraocular tumors. Previous work has already shown that CD4$^+$ T cells are crucial in the eradication of these intraocular tumors. Another study showed that this nonphthisis intraocular tumor rejection is dependent on IFNγ and appears to be mediated by TRAIL.[11] It is peculiar that CD4$^+$ T cells are crucial in this nondestructive intraocular tumor eradication process, as tumor-specific CTLs are mostly associated with this clean form of tumor elimination without collateral damage in contrast to intraocular tumor-directed CD4$^+$ T cells, which are in general associated with massive destruction of the eye.[9]

These results make it conceivable that the effective antitumor response involves communication between tumor-reactive CD4$^+$ T cells and MHC class II–positive host cells, possibly DCs and macrophages, that cross present tumor Ags with the CD4$^+$ T cells. It is unlikely that CD4$^+$ T cells recognize intraocular Ad5E1-expressing tumor cells directly, as these cells are MHC class II negative. Furthermore, the data presented in this study show that protection against tumor challenge was strongly associated with the presence of macrophages in the dissolving AC tumor of the eye. However, this does not demonstrate direct tumor cell killing by CD4$^+$ T cell-activated innate macrophages in vivo. In addition, the release of cell death-inducing molecules, such as oxygen radicals, by CD4$^+$ T cell-activated macrophages is likely to result in phthisis, as these molecules cannot discriminate between tumor and normal ocular tissues, as described for many CD4$^+$ T-cell-mediated intraocular DTH reactions.

Because we did not observe damage of normal ocular tissues after tumor clearance, we consider it more likely that tumor eradication does not rely on cytolytic molecules that act directly on tumor cells. An attractive hypothesis is that tumor-reactive CD4$^+$ T cells either directly or indirectly inhibit tumor-induced angiogenesis, a process in which macrophages also participate.[24] In this way, formation of new tissues that still rely on the development of blood vessels will be prevented, whereas the pre-existing tissues will not be affected. It has been described that production of IFNγ by tumor-reactive CD4$^+$ T cells is an essential requirement for CD4$^+$ T-cell-mediated tumor immunity, resulting in inhibition of tumor-induced angiogenesis in developing tumors leading to tumor clearance.[25]

Downloaded from iovs.arvojournals.org on 04/24/2024

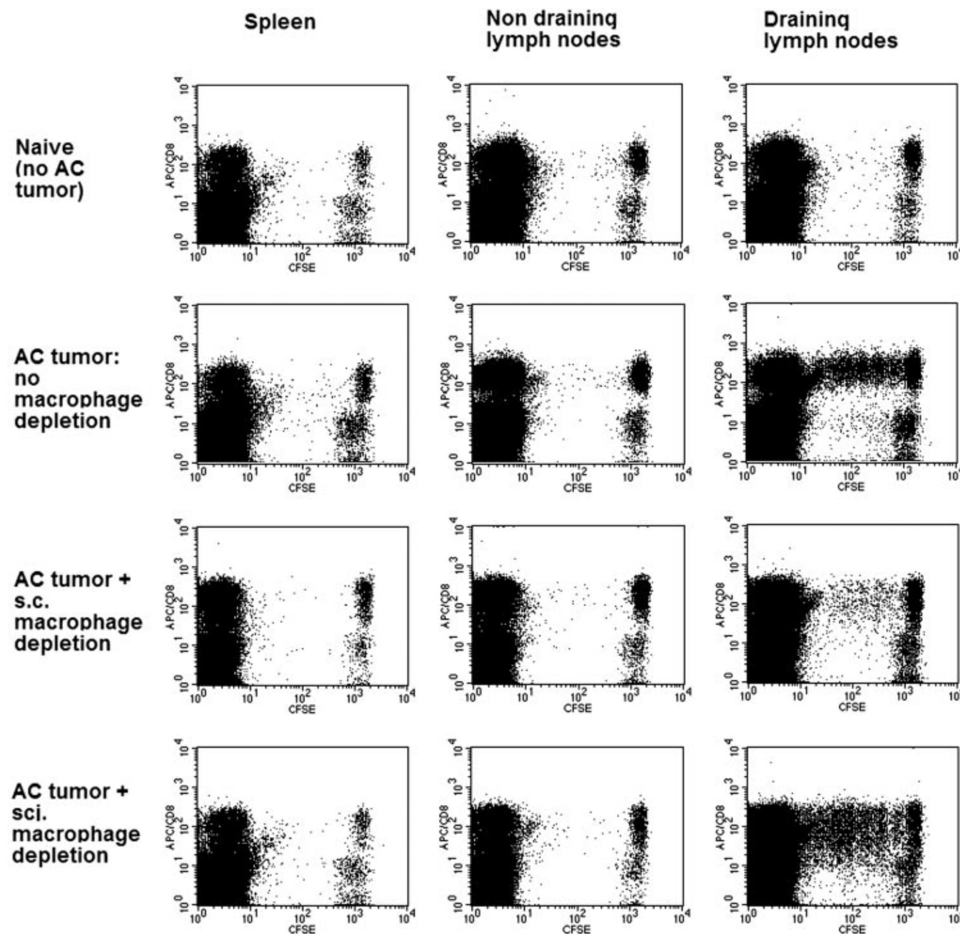

FIGURE 4. Tumor-Ag presentation in local draining lymph nodes was still present after local macrophage depletion with clodronate liposomes. Two days after the last clodronate liposome injection (16 days after tumor inoculation), we adoptively transferred CFSE-labeled E1A-specific $CD8^+$ T cells into tumor-bearing mice with local clodronate liposome injections sc or scj, in nontreated tumor-bearing mice and in naïve mice (no tumor). Three days after transfer (day 19 after tumor injection), proliferation of adoptively transferred E1A-specific T cells in DLN, non-DLN and spleen were analyzed by flow cytometry (x-axis = CFSE, y-axis = E1A-specific $CD8^+$ T cells). Proliferation of tumor-specific T cells persisted in the tumor DLNs of all tumor-bearing mice either locally depleted of macrophages with Cl_2MDP liposomes or untreated. This experiment was repeated once.

Indeed, Ad5E1 intraocular tumor growth was unhampered in IFNγ KO mice.[11] IFNγ can either be produced by tumor-specific $CD4^+$ T cells or macrophages.[13,26] However, IFNγ does not directly induce Ad5E-tumor cell death in vitro, which makes it conceivable that IFNγ acts in vivo through an indirect pathway. For example, IFNγ may restrain tumor-induced angiogenesis.[25,27] Another possibility may be that IFNγ provokes TRAIL–TRAIL-R2 interactions as previously suggested by us and others.[11,28] TRAIL is expressed on the surface of activated $CD4^+$ T cells and ocular cells[6] whereas TRAIL-R2 is detected on Ad5E1 tumor cells.[11,29] IFNγ enhances TRAIL expression on ocular cells and $CD4^+$ T cells followed by increased susceptibility of tumor cells to TRAIL-induced apoptosis in vitro.[11] In light of the present study, it may be that macrophages are also induced to express TRAIL[30,31] and act as supplementary effector cells for inducing intraocular tumor cell apoptosis.

This intraocular tumor model is illustrative of the fact that ocular immune privilege is not absolute or undefeatable. Most tumors, especially in other more “conventional” body parts, create their own immunosuppressive environment.[32] Understanding the mechanism behind the circumvented ocular immune privilege in this tumor model may take us nearer to finding new tools for developing effective immune intervention strategies against cancer in general. In conclusion, our results identify a crucial role of $F4/80^+$ cells in the eradication of intraocular tumors devoid of detectable bystander-damage to neighboring cells, yet the precise interaction between macrophages, IFNγ, and $CD4^+$ T cells in this intraocular tumor-eradication model needs to be defined in more detail.

Acknowledgments

The authors thank Ed R. Barthen and Christa van den Bergh for the histologic workup.

References

1. Medawar P. Immunity to homologous grafted skin. III. The fate of skin homografts transplanted to the brain, to subcutaneous tissue, and to the anterior chamber of the eye. *Br J Exp Pathol.* 1948;29:58–69.
2. Streilein JW. Immune privilege as the result of local tissue barriers and immunosuppressive microenvironments. *Curr Opin Immunol.* 1993;5:428–432.
3. Ferguson TA, Green DR, Griffith TS. Cell death and immune privilege. *Int Rev Immunol.* 2002;21:153–172.
4. Streilein JW. Ocular immune privilege: therapeutic opportunities from an experiment of nature. *Nat Rev Immunol.* 2003;3:879–889.
5. Kezuka T, Streilein JW. Evidence for multiple CD95-CD95 ligand interactions in anteriorchamber-associated immune deviation induced by soluble protein antigen. *Immunology.* 2000;99:451–457.
6. Lee HO, Herndon JM, Barreiro R, Griffith TS, Ferguson TA. TRAIL: a mechanism of tumor surveillance in an immune privileged site. *J Immunol.* 2002;169:4739–4744.
7. Schurmans LR, den Boer AT, Diehl L, et al. Successful immunotherapy of an intraocular tumor in mice. *Cancer Res.* 1999;59:5250–5254.
8. Schurmans LR, Diehl L, den Boer AT, et al. Rejection of intraocular tumors by CD4(+) T cells without induction of phthisis. *J Immunol.* 2001;167:5832–5837.

Downloaded from iovs.arvojournals.org on 04/24/2024

9. Knisely TL, Luckenbach MW, Fischer BJ, Niederkorn JY. Destructive and nondestructive patterns of immune rejection of syngeneic intraocular tumors. *J Immunol*. 1987;138:4515–4523.
10. Knisely TL, Niederkorn JY. Immunologic evaluation of spontaneous regression of an intraocular murine melanoma. *Invest Ophthalmol Vis Sci*. 1990;31:247–257.
11. Wang S, Boonman ZF, Li HC, et al. Role of TRAIL and IFN-gamma in CD4+ T cell-dependent tumor rejection in the anterior chamber of the eye. *J Immunol*. 2003;171:2789–2796.
12. Whitworth PW, Pak CC, Esgro J, Kleinerman ES, Fidler IJ. Macrophages and cancer. *Cancer Metastasis Rev*. 1990;8:319–351.
13. Munder M, Mallo M, Eichmann K, Modolell M. Murine macrophages secrete interferon gamma upon combined stimulation with interleukin (IL)-12 and IL-18: a novel pathway of autocrine macrophage activation. *J Exp Med*. 1998;187:2103–2108.
14. Kaplan MJ, Ray D, Mo RR, Yung RL, Richardson BC. TRAIL (Apo2 ligand) and TWEAK (Apo3 ligand) mediate CD4+ T cell killing of antigen-presenting macrophages. *J Immunol*. 2000;164:2897–2904.
15. Toes RE, Offringa R, Blom RJ, Melief CJ, Kast WM. Peptide vaccination can lead to enhanced tumor growth through specific T-cell tolerance induction. *Proc Natl Acad Sci USA*. 1996;93:7855–7860.
16. Toes RE, Offringa R, Blom RJ, et al. An adenovirus type 5 early region 1B-encoded CTL epitope-mediating tumor eradication by CTL clones is down-modulated by an activated ras oncogene. *J Immunol*. 1995;154:3396–33405.
17. Van Rooijen N, Sanders A. Liposome mediated depletion of macrophages: mechanism of action, preparation of liposomes and applications. *J Immunol Methods*. 1994;174:83–93.
18. Austyn JM, Gordon S. F4/80, a monoclonal antibody directed specifically against the mouse macrophage. *Eur J Immunol*. 1981; 11:805–815.
19. Wickramasinghe SN, Mawas F, Hasan R, Brown IN, Goldin RD. Macrophages are a major source of acetaldehyde in circulating acetaldehyde-albumin complexes formed after exposure of mice to ethanol. *Alcohol Clin Exp Res*. 1994;18:1463–1467.
20. Lyons AB, Parish CR. Determination of lymphocyte division by flow cytometry. *J Immunol Methods*. 1994;171:131–137.
21. Boonman ZF, van Mierlo GJ, Fransen MF, et al. Intraocular tumor antigen drains specifically to submandibular lymph nodes, resulting in an abortive cytotoxic T cell reaction. *J Immunol*. 2004;172: 1567–1574.
22. Leenen PJ, Radosevic K, Voerman JS, et al. Heterogeneity of mouse spleen dendritic cells: in vivo phagocytic activity, expression of macrophage markers, and subpopulation turnover. *J Immunol*. 1998;160:2166–2173.
23. Melief CJ, Schoenberger S, Toes R, Offringa R. Cytotoxic T lymphocyte priming versus cytotoxic T lymphocyte tolerance induction: a delicate balancing act involving dendritic cells. *Haematologica.* 1999;84(suppl EHA-4):26–27.
24. Lobov IB, Rao S, Carroll TJ, et al. WNT7b mediates macrophage-induced programmed cell death in patterning of the vasculature. *Nature*. 2005;437:417–421.
25. Qin Z, Blankenstein T. CD4+ T cell–mediated tumor rejection involves inhibition of angiogenesis that is dependent on IFN gamma receptor expression by nonhematopoietic cells. *Immunity*. 2000;12:677–686.
26. Blankenstein T, Qin Z. The role of IFN-gamma in tumor transplantation immunity and inhibition of chemical carcinogenesis. *Curr Opin Immunol*. 2003;15:148–154.
27. Qin Z, Schwartzkopff J, Pradera F, et al. A critical requirement of interferon gamma-mediated angiostasis for tumor rejection by CD8+ T cells. *Cancer Res*. 2003;63:4095–4100.
28. Smyth MJ, Cretney E, Takeda K, et al. Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) contributes to interferon gamma-dependent natural killer cell protection from tumor metastasis. *J Exp Med*. 2001;193:661–660.
29. Routes JM, Ryan S, Clase A, et al. Adenovirus E1A oncogene expression in tumor cells enhances killing by TNF-related apoptosis-inducing ligand (TRAIL). *J Immunol*. 2000;165:4522–4527.
30. Griffith TS, Wiley SR, Kubin MZ, et al. Monocyte-mediated tumoricidal activity via the tumor necrosis factor-related cytokine, TRAIL. *J Exp Med*. 1999;189:1343–1354.
31. Takeda K, Hayakawa Y, Smyth MJ, et al. Involvement of tumor necrosis factor-related apoptosis-inducing ligand in surveillance of tumor metastasis by liver natural killer cells. *Nat Med*. 2001;7:94–100.
32. Zou W. Immunosuppressive networks in the tumour environment and their therapeutic relevance. *Nat Rev Cancer*. 2005;5:263–274.

Downloaded from iovs.arvojournals.org on 04/24/2024