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Rejection of Intraocular Tumors by CD4⁺ T Cells Without Induction of Phthisis¹


Immune privilege of the eye protects against sight-threatening inflammatory events, but can also permit outgrowth of otherwise nonlethal immunogenic tumors. Nonetheless, ocular tumor growth can be controlled by cellular immune responses. However, this will normally result in phthisis of the eye, if in case tumor rejection is mediated by a delayed-type hypersensitivity response orchestrated by CD4⁺ T cells. We now show that intraocular tumors can be eradicated by CD4⁺ Th cells without inducing collateral damage of neighboring ocular tissue. Injection of tumor cells transformed by the early region 1 of human adenovirus type 5 in the anterior chamber of the eye leads to intraocular tumor formation. Tumor growth is transient in immunocompetent mice, but lethal in immunodeficient nude mice, indicating that T cell-dependent immunity is responsible for tumor clearance. Tumor rejection has all the characteristics of a CD8⁺ T cell-mediated immune response, as the tumor did not express MHC class II and only tumor tissue was the subject of destruction. However, analysis of the molecular and cellular mechanisms involved in tumor clearance revealed that perforin, TNF-α, Fas ligand, MHC class I, and CD8⁺ T cells did not play a crucial role in tumor eradication. Instead, effective tumor rejection was entirely dependent on CD4⁺ Th cells, as CD4⁺-depleted as well as MHC class II-deficient mice were unable to reject their intraocular tumor. Taken together, these observations demonstrate that CD4⁺ T cells are able to eradicate MHC class II-negative tumors in an immune-privileged site without affecting surrounding tissues or the induction of phthisis. The Journal of Immunology, 2001, 167: 5832–5837.

Maintenance of the precise microanatomy of the eye is absolutely essential for the eye’s physiological function. Small distorting lesions, e.g., caused by inflammation, can have devastating effects on the visual axis, leading to loss of vision and blindness. The eye, an immune-privileged site, displays unique immunological characteristics that influence inflammatory responses in an unusual way. In the past 20 years, several mechanisms that contribute to ocular immune privilege have been elucidated.

In 1980, Streilein et al. (1) described that intracameral injection of Ag can lead to anterior chamber-associated immune deviation (ACAIID)³ (2), which is characterized by impaired cell-mediated immunity. ACAID results primarily in a selective suppression of delayed-type hypersensitivity (DTH) reactions with intact humoral and cytotoxic T cell responses. Other mechanisms that contribute to ocular immune privilege are immunosuppressive cytokines present in the aqueous humor such as TGF-β and expression of CD95 ligand on ocular tissues (2–4). However, these immunologic characteristics may have deleterious consequences both for the eye and for the host, since effective protection against infectious pathogens and immunogenic tumors may be impaired.

Nevertheless, immune privilege is not an absolute and insurmountable barrier that shields intraocular tumors from the systemic immune system, as has been demonstrated in animal models (5, 6). In 1987, Knisely et al. (7) described two rejection patterns that were observed in immunogenic, syngeneic intraocular tumors. Tumor rejection could occur by a process that strongly resembles DTH, a rejection mechanism orchestrated by CD4⁺ Th cells, producing extensive nonspecific damage to normal tissues and resulting in phthisis of the eye. In the other type of immune response, CTL were the main effector cells. The tumor was eradicated without bystander damage to normal ocular host tissue. In the latter case, the role of CD4⁺ Th cells seemed to be restricted to the efficient induction of CTL-effector cells, as depletion of CD4⁺ T cells resulted in a dramatic decrease in tumor-specific CD8⁺ CTL.

We have shown that tumor cells transformed by the human adenovirus type 5 early region 1 (Ad5E1) oncocenes are rapidly rejected when injected s.c., but that they do form tumors when injected into the anterior chamber (AC) of the eye (8). Ad5E1-specific CTL can eradicate these tumors, since adoptive transfer of Ad5E1-specific CTL clones results in a rapid and definite rejection of the intraocular tumor. This rejection did not result in collateral damage to the neighboring ocular tissues, showing that this process is highly tumor-specific.

As the presence of Ag in the ocular environment is associated with immune suppression, we now evaluated the long-term antitumor response in mice bearing intracameral Ad5E1 tumors. Intraocular tumors did not grow progressively, but were ultimately
rejected after several weeks. Unexpectedly, the effector mechanism responsible for rejection of the MHC class II-negative tumors was not dependent on CD8\(^+\) CTLs, but relied entirely on CD4\(^+\) T cells. These data show that CD4\(^+\) T cells can provide effective immune surveillance in an immune-privileged site without disruption of healthy eye tissue.

**Materials and Methods**

**Animals**

Male C57BL/6 (B6) mice (H-2\(^b\)), between 3 and 6 mo of age, were obtained from Iffa Credo (Brussels, Belgium). C57BL/6 nude mice (H-2\(^b\)) were obtained from Bomholtgard (Ry, Denmark). C57BL/6 perforin\(^{−/−}\) (PKO, H-2\(^b\)), C57BL/6 class II\(^{−/−}\) (class II knockout H-2\(^b\)), C57BL/6 gld/gld (H-2\(^b\)), and TNF-\(α^{−/−}\) (H-2\(^b\)) mice were bred at TNO-PG (Leiden, The Netherlands).

**Cells**

Mouse embryo cells (C57BL/6 origin) transformed by the human Ad5E1 were generated and maintained, as described previously (9, 10). Monocellular suspensions of Ad5E1-induced tumor cells were washed and resuspended in PBS for intracameral injections.

**Intracameral inoculations**

A previously described technique for deposition of a defined number of tumor cells into the AC of the mouse eye was employed (11). Mice were deeply anesthetized with a mixture (ratio 1:1) of xylazine (Rompun 2%; Bayer, Leverkusen, Germany) and ketamine hydrochloride (Aescoket; Aesculaap bv, Boxtel, The Netherlands) given i.p. The eye was viewed by 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 (approximately 80 μm in diameter) was fitted into a sterile infant feeding tube, which was mounted onto a sterile 0.1-ml Hamilton syringe (Hamilton, Whittier, CA). The pipette, loaded with Ad5E1 cell suspension (0.3 \(×\) 10\(^6\) cells/μl), was introduced through the puncture site of the cornea, and 4 μl of the Ad5E1 cell suspension was delivered into the AC. The eyes were examined three times per week with a dissecting microscope to observe and document tumor growth.

**Depletion of CD8\(^+\) and CD4\(^+\) T and NK cells in vivo**

C57BL/6 mice were treated with anti-CD4 (GK1.5) or anti-CD8 (2.43) mAbs before intracameral injection of Ad5E1-transformed tumor cells. Ab treatment leads to selective depletion (>95%) of these T cell subsets. Abs were administered by i.p. injections of 100 μg in 0.2 ml PBS at days −7, −5, −3, and −1, and then injections were continued twice per week. Depletion of the T cell subsets was monitored by FACS analysis of venous blood samples at day −1.

Likewise, C57BL/6 mice were treated with mAb (PK136) directed against NK cells. Abs were administered by i.p. injections of 100 μg in 0.2 ml PBS at days −7, −5, −3, and 1, and then continued twice per week. Before the intracameral inoculation of tumor cells, the effect of Ab treatment was monitored by a \(^{51}\)Cr release assay of spleen cells from NK cell-depleted mice.

**Results**

**Tumor eradication does not lead to disruption of intraocular tissue**

Recently, we reported that injection of 0.3 \(×\) 10\(^6\) Ad5E1-transformed cells into the AC of the eye results in intraocular tumor growth in C57BL/6 mice (8). Adoptively transferred tumor-specific cytotoxic T cells were able to eradicate intraocular tumors without damaging the normal ocular host tissue (8). These data show that CTL can mediate potent antitumor effects against tumors growing in the eye, an immune-privileged site.

Unexpectedly, this intraocular tumor was also eradicated spontaneously 3–6 wk after intracameral inoculation without apparent collateral damage to the adjoining ocular tissues. B6 nude mice were unable to control tumor growth, indicating that T cells play a pivotal role in tumor eradication (Fig. 1).

As T cell-mediated intraocular antitumor responses might culminate in extensive injury to normal ocular tissues (6, 12, 13), we evaluated the consequences of tumor rejection on the integrity of the eye. At the time at which tumor rejection started, tumor-bearing eyes were examined by immunohistological procedures as a means of identifying the effector lymphocytes infiltrating the intraocular tumors. CD11c\(^+\) cells, N4/18\(^+\) cells (myeloid cells), and both CD8\(^+\) as well as CD4\(^+\) T cells were observed (data not shown). MHC class II expression was expressed on the infiltrating cells during tumor resolution, but not on Ad5E1 tumor cells at any stage of tumor growth or resolution (not shown). The rejection did not occur as an acute reaction, but instead showed a gradual decrease in the size of the tumor without evidence of bulk necrosis. We analyzed tumors at macro- and microscopic levels and were unable to find any damage during tumor growth and resolution (not shown). Infiltrating vessels could be found in the tumor, and the vascular endothelium appeared normal throughout tumor resolution (data not shown). After rejection of the intraocular tumors, there were no indications of an ongoing immune response, as no immune cells were detected anymore after tumor rejection in the AC. Together, these observations reveal that the spontaneous resolution of the Ad5E1-positive tumor cells was not associated with bulk tumor necrosis or damage to the normal ocular tissues (Fig. 2).

**Tumor eradication is independent of perforin, CD95 ligand, and TNF**

The findings described above indicate that intraocular tumor growth is controlled in a highly specific T cell-dependent manner, as no bystander damage could be observed during or after tumor clearance. These observations suggest that direct target cell lysis by cognate T cell-tumor cell interaction is responsible for tumor clearance. The most prominent molecular mechanisms mediating T cell-dependent cytolysis are perforin-dependent granule exocytosis and engagement of target Fas with its ligand CD95L (14–16). The use of perforin-deficient (PKO) and B6 gld/gld mice (which lack functional CD95 ligand) allows the assessment of the relative contribution of each of these two major T cell-mediated cytotoxic pathways. To obtain more insight in the molecular mechanism leading to intraocular resolution of Ad5E1-positive tumors, B6 PKO and gld/gld mice were injected intraocularly with Ad5E1-transformed tumor cells. As shown in Fig. 3, both perforin\(^{−/−}\) as
well as B6 gld/gld mice were able to resolve the intraocular tumors, indicating that neither perforin-dependent cytotoxicity nor CD95 ligand-dependent cytotoxicity is crucial for tumor clearance.

Another important effector molecule produced by Ag-specific T cells known to possess antitumor activity is TNF-α. Therefore, we wished to study whether TNF-α plays a role in tumor resolution. Fig. 3B shows that intraocular tumors in TNF-α−/− mice are still eradicated, demonstrating also that the TNF-α pathway does not play a major role in tumor rejection. Taken together, these findings indicate that the most prominent known effector molecules employed by T cells in immune surveillance against tumors are not crucially involved in eradication of Ad5E1-expressing intraocular tumors.

**Antitumor immunity against Ad5E1 tumor cells requires CD4+ T cells**

Several studies reported that intraocular immune attack leading to massive destruction of the eye is the result of CD4+ T cell-mediated responses, whereas CD8+ T cell immunity can be tumor-specific without inflicting damage (7, 17). These observations and our finding that the Ad5E1-transformed tumor cells do not express MHC class II molecules (data not shown) support the notion that CD8+ T cell-dependent effector mechanisms play a crucial role in tumor rejection. However, the observation that neither PKO nor gld/gld mice were susceptible to progressive tumor growth argues against a role for CD8+ T cells, as perforin and CD95 ligand are pivotal constituents of the major cytolytic pathways of CD8+ CTL.

To study the contribution of several cellular subsets responsible for tumor clearance in more detail, we wished to analyze the role of B cells, NK cells, CD8+ T cells, and CD4+ T cells in tumor rejection. Ad5E1-transformed tumor cells were injected into the AC of B cell-deficient mice, or mice depleted for NK cells by injection of a depleting NK1.1-specific Ab. B cell-deficient mice as well as mice depleted for NK cells were still able to reject their tumor with the same efficiency as immunocompetent C57BL/6 mice. These results indicate that neither B cells nor NK cells play a pivotal role in tumor resolution (Fig. 4).

To investigate the role of CD4+ and CD8+ T cell subsets in the tumor resolution, we examined the effect of CD4+ and CD8+ T cell depletion on tumor growth. Panels of C57BL/6 mice were treated with anti-CD4 or anti-CD8 mAbs. Ab treatment resulted in...
depletion of $\geq 95\%$ of the respective T cell subsets, as monitored by FACScan analysis of venous blood samples (data not shown). Fig. 5A shows that the tumors in the CD8$^+$ T cell-depleted mice disappeared with the same kinetics as in the control mice. Similar findings were obtained when tumor growth was monitored in TAP-deficient mice in which the mature CD8$^+$ T cell repertoire is severely compromised (data not shown). In contrast, CD4$^+$ T cell-depleted mice were not able to eradicate intraocular tumors (Fig. 4B), indicating that CD4$^+$ T cells play a critical role in the tumor rejection. These findings were confirmed when tumor growth was followed in MHC class II-deficient mice that lack functional mature CD4$^+$ T cells (Fig. 5). MHC class II-deficient animals were not able to resist intraocular tumor growth and died as a consequence of progressive tumor growth (Fig. 5). These data demonstrate that CD4$^+$ T cells, but not CD8$^+$ T cells, play a critical role in the eradication of these intraocular tumors.

**Discussion**

The present study was designed to investigate the molecular and cellular mechanisms of intraocular tumor eradication. The results presented in this work identify a principal role for CD4$^+$ T cells in ocular antitumor immunity. Using CD4$^+$ and CD8$^+$ depleting Abs as well as different gene-deficient mice, we show that CD4$^+$ T cells were crucial in the rejection phase. The role of CD4$^+$ Th cells did not involve provision of helper activity for CTL priming, as tumors were still eradicated in the absence of CD8$^+$ T cells. This important role of CD4$^+$ T cells in intraocular tumor eradication without inducing collateral damage has not been recognized previously. In general, intraocular tumor eradication without destruction of the visual axis is associated with tumor-specific CTL-mediated immunity (7, 17). Indeed, we have shown previously that an i.v. injection of an Ad5E1-specific CTL clone leads to the eradication of established intraocular Ad5E1-transformed tumors, while the anatomy of the eye remains intact (8).

The current observation that CD4$^+$ Th cells were both required and sufficient to resolve intraocular tumors is unexpected, as intraocular tumor-directed CD4$^+$ responses are, in general, associated with massive destruction of healthy tissues leading to sight-threatening tissue damage. For example, spontaneous rejection of an intraocular mastocytoma P91, which is characterized by a DTH response, also results in extensive innocent bystander damage to normal ocular tissues and phthisis of the eye (13). In case no tissue damage is induced, CD4$^+$ Th responses directed against Ags present in the AC of the eye are often manifested by deviant immune responsiveness.

The most prominent example of such immune deviation is ACAID. Although ACAID might not directly affect antitumor immunity, it is a phenomenon that is marked by a reduced DTH reaction to specific Ag after intraocular injection of Ag (11).

**FIGURE 4.** B cells or NK cells are not crucially involved in tumor rejection. A total of $0.3 \times 10^6$ Ad5E1-transformed tumor cells injected into the AC of C57BL/6 mice (●) ($n = 10$); A, B cell-deficient IgM knockout mice (□) ($n = 8$); or B, C57BL/6 mice (○) ($n = 8$) depleted for NK cells by i.p. administration of $100 \mu g$ anti-NK1.1 Ab PK136 at days $\sim 7$, $\sim 5$, $\sim 3$, and $\sim 1$, and sustained twice weekly. Depicted is the percentage of mice with an intraocular tumor.

**FIGURE 5.** Intracocular eradication of Ad5E1-transformed tumors is dependent on CD4$^+$, but not CD8$^+$ T cells. C57BL/6 mice were depleted for CD8$^-$ T cells ($n = 7$) or CD4$^-$ T cells ($n = 6$) by i.p. injections of $100 \mu g$ anti-CD8 Ab 2.43 or anti-CD4 Ab GK1.5, respectively. After three injections of Ab, $0.3 \times 10^6$ Ad5E1-transformed tumor cells were injected into the AC of untreated C57BL/6 mice (●) ($n = 10$); A, CD8-depleted C57BL/6 mice (△); B, CD4-depleted C57BL/6 mice (□); or C, MHC class II-deficient mice ($n = 10$) (○). The percentage of mice with an intraocular tumor is depicted.
Although not studied extensively, we have no indications that intraocular Ad5E1 tumors induce systemic Ag-specific immune suppression or ACAID, as mice that have cleared the intraocular tumor are protected against a subsequent tumor challenge (not shown), arguing that an effective memory response was generated, instead of tolerance. Recently, it has been shown in some tumor models that NKT cells are crucially involved in suppression of systemic antitumor responses (18, 19), as well as in the induction of ACAID (20, 21). In these studies, it was shown that depletion of CD4+ (NKT) cells and/or NK1.1+ cells resulted in abrogation of immune suppression leading to enhanced tumor-specific immunity. However, in case of intraocular Ad5E1-transformed tumors, depletion of NK1.1+ NKT cells did not affect tumor growth, whereas depletion for CD4+ cells resulted in the inability to control tumor growth. Together, these findings argue against a crucial role for NKT cells or the induction of ACAID in the outcome of immune responsiveness against intraocular Ad5E1 tumors.

Thus, until now, CD4+ T cell responsiveness toward Ags expressed in the eye has been described to be involved in active down-regulation of systemic immune responses directed against the same Ag, or has been associated with massive and irreversible destruction of the eye. We now have shown that CD4+ T cell responses are also able to eradicate intraocular tumors without inducing apparent sight-threatening side effects.

The mechanisms used by CD4+ T cells to mediate tumor rejection are ill defined, but we consider it unlikely that the antitumor response involves tumor-reactive CD4+ T cells that recognize intraocular Ad5E1-expressing tumor cells directly, as these cells are MHC class II negative. Moreover, the most prominent effector molecules capable of inducing target cell lysis after cognate interaction with effector cells, CD95 ligand, and perforin were not required for tumor rejection. It is more conceivable that the effective antitumor response involves communication between tumor-reactive CD4+ T cells and MHC class II-positive host cells that cross-present tumor-derived material to the CD4+ T cells. Evidence that CD4+ T cells can eradicate MHC class II-negative tumors without commitment of CD8+ CTL came from studies in a murine leukemia virus-induced tumor model in which adoptively transferred tumor-specific CD4+ T cells are implicated in the activation of tumoricidal macrophages (22). More recently, it was demonstrated in a model involving vaccination with irradiated tumor cells transduced to secrete GM-CSF that cytokines produced by CD4+ T cells can recruit and activate macrophages and eosinophils (23). Protection against tumor challenge was strongly associated with the presence of eosinophils at the tumor challenge site as well as with the production of oxygen radicals by tumoricidal macrophages, since genetically modified mice disabled to produce these radicals were severely hampered in their ability to resist tumor challenge. However, direct tumor cell killing by CD4+ T cell-activated innate effector cells in vivo has not been demonstrated.

Although we cannot exclude that these mechanisms also contributed to the ocular tumor clearance described in this work, we regard it more likely that other mechanisms play a more prominent role. Although ocular tissues might be protected against death-inducing molecules by up-regulation of, for example, anti-apoptotic molecules, the release of cell death-inducing molecules such as oxygen radicals by CD4+ T cell-activated phagocytes is likely to result in phthisis, as these molecules cannot discriminate between tumor and normal ocular tissue. Likewise, release of cytolytic cytokines by either CD4+ T cells or innate effector cells in the tumor will disrupt the integrity of the surrounding normal tissues, as described for many CD4+ T cell-mediated intraocular DTH responses. Indeed, when the role of TNF-α, the most prominent cytokine capable of inducing target cell death, was studied, we found that tumor eradication was TNF-α independent (Fig. 3B).

Because of these considerations and the fact that we did not observe damage of normal ocular tissue 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. In this way, formation of new tissues that still rely on the development of blood vessels will be prevented, whereas the preexisting tissues will not be affected. Recently, it has been described that production of IFN-γ by tumor-reactive CD4+ Th cells is an essential requirement for CD4+ T cell-mediated tumor immunity. The IFN-γ produced most likely has an effect on nonhemopoietic cells and results in the inhibition of tumor-induced angiogenesis. As a consequence, tumors were not able to reach a certain critical size and will eventually be cleared from the site of injection (24). Although we did not observe differences in growth kinetics or clearance of Ad5E1 tumors in mice treated with anti-IFN-γ Abs (not shown), the effects of cytokines such as IFN-γ on tumor development warrant further investigation. Many organisms are critically dependent on the visual axis for survival. However, the eye is a very delicate organ, and the quality of vision is extremely dependent on the microanatomy of the eye. Moreover, many of the tissues within the eye are incapable of regeneration. Therefore, small distortions of the ocular tissue induced by immune responses, which would pass unnoticed in other tissues, could have life-threatening consequences. As a result, the eye has evolved a number of overlapping mechanisms that constitute a unique immune privilege. Nonetheless, the immune-privileged state may be more subtle, as previously proposed, as our results clearly show that eradication of immunogenic tumors by CD4+ T cells is allowed in the eye, despite local immune privilege.

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References