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Influence of Immune Privilege on Ocular Tumor Development

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Abstract

Mechanisms that maintain ocular immune privilege may contribute to ocular tumor progression by inhibiting tumoricidal immune responses. Consistent with that notion are observations from transplantable tumor models in mice demonstrating that the tumoricidal activity of $CD8^+$ cytolytic T lymphocytes (CTL) may be inhibited directly by interfering with CTL effector function in the eye or indirectly by abrogating the effector function of $CD8^+$ T cell-activated intratumoral macrophages that are critical for ocular tumor rejection. In addition, epigenetic gene regulation by factors within the ocular tumor environment favors the generation of tumor variants that are resistant to $CD8^+$ CTL. Intratumoral macrophages may be essential for eliminating these variants because, unlike CTL, their tumoricidal activity is nonspecific. Hence, the inhibition of macrophage effector function within the eye, presumably to preserve immune privilege by minimizing ocular immunopathology, may hasten the outgrowth of tumor escape variants which contributes to ocular tumor progression.

Keywords

immune privilege; tumor; epigenetic; T cells; macrophage; CD8; CTL

INTRODUCTION

Ocular immune privilege is defined by the observation that foreign tissues transplanted in the anterior chamber (a.c.), vitreous cavity, or subretinal space persist indefinitely whereas the same tissues transplanted in the skin are rejected by the host immune response.[(1,2)] The initial characterization of ocular immune privilege utilized tumor cell lines derived from genetically inbred strains of mice because of their defined major histocompatibility and minor histocompatibility antigens (Table 1). Tumors expressing only minor antigen differences with recipient mice grew progressively and sometimes fatally when transplanted in the a.c. of the eye but were rejected when transplanted in the skin exemplifying immune privilege.[(3)] By contrast, tumors expressing both minor and major antigenic differences were rejected in the a.c. indicating that privilege was not absolute.[(3)]

Ocular immune privilege is clearly not immune ignorance as progressively growing ocular tumors in mice induce detectable tumor-specific antibody,[(4)] and T-cell responses that eliminate extraocular metastases and protect mice from a subsequent tumor challenge in the skin or in the opposite eye.[(5,6)] Moreover, T cells, but not antibody, from ocular tumor-

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bearing mice, conferred immunity against an ocular tumor challenge when adoptively transferred to naïve recipients.[5,7] These data indicate that T-cell responses are somehow inhibited within the established ocular tumor microenvironment, and the mechanisms that inhibit tumoricidal activity of T cells within ocular tumors are not completely understood. In this review, we highlight how ocular immune privilege creates an environment, which is permissive for tumor growth and persistence by directly inhibiting immune responses within the eye and by promoting the generation of tumor escape variants, which are no longer recognized by the immune response.

IMMUNE REGULATION IN THE EYE

The removal of pathogens (bacteria, viruses, parasites, and tumors) from the eye by the host immune response is absolutely critical to maintain vision. However, an immune response also poses a threat if pathogen clearance damages the delicate ocular tissues that are unable to regenerate (e.g. corneal endothelium and neurons). Ocular immune privilege is believed to be an evolutionary adaptation that preserves vision by minimizing immunopathology during pathogen clearance. Correspondingly, anatomical and biochemical features of the eye along with the generation of systemic immune tolerance to ocular antigens have been shown to modulate or inhibit both innate and adaptive immune responses in the eye to preserve immune privilege.

Anatomical features of the eye which contribute to immune privilege

The immune response to an ocular pathogen follows a set program in which pathogen-associated molecular patterns (PAMPs) are engaged by pathogen recognition receptors to induce regional expression of inflammatory mediators. PAMPs also activate resident immune cells that process and present pathogen molecules on their cell surface in context with classical and nonclassical major histocompatibility complexes (MHC). These antigen presenting cells (APC) then migrate via afferent lymphatics and/or blood to secondary lymphoid organs (lymph nodes and spleen) where they interact with antigen-specific T cells and B cells to induce adaptive immune responses that return to the pathogen site via the bloodstream as effector T cells and/or antibodies, respectively.

Certain anatomic features of the eye should limit the generation and expression of ocular immune responses. For example, the interior of the eye lacks demonstrable afferent lymphatics,[8,9] the cornea is avascular,[10] and tight junctions between vascular endothelial cells in the iris and retinal vessels create a blood ocular barrier.[11] However, these barriers are clearly not absolute as antigens injected into the a.c. induce T-cell expansion in regional lymph nodes[12–14] via uveal–scleral drainage of antigens in aqueous humor,[8,15] corneal transplant rejection involves lymphangiogenesis/hemangiogenesis,[10] and activated T cells can enter even a noninflamed retina.[16] Therefore, the unique anatomy of the eye may increase the threshold for generation and expression of the immune response but clearly does not prevent it.

Biochemical features of the eye which contribute to immune privilege

T-cell recognition is restricted to processed peptides presented by MHC. As a general rule, $CD8^+$ T cells recognize peptides presented by MHC Class I molecules and $CD4^+$ T cells recognize peptides presented by MHC Class II molecules. Ocular tissues express low levels of MHC Class I and are thus less susceptible to direct lysis by $CD8^+$ cytolytic T lymphocytes (CTL) responses.[17] In addition, human uveal melanomas (UMs) fail to express MHC Class II upon stimulation with interferon gamma (IFN-γ), which minimizes the activation of tumor-specific $CD4^+$ T cells infiltrating ocular tumors.[18,19]

The aqueous humor contains several molecules that are immunosuppressive. Transforming growth factor (TGF-β), α-melanocyte stimulating hormone (α-MSH), and calcitonin gene-related peptide have been shown to inhibit innate immunity by interfering with nitric oxide (NO) production by macrophages.[20] T helper cell differentiation can also be influenced by the aqueous humor as TGF-β, α-MSH, and the vasoactive intestinal peptide inhibit IFN-γ expression by activated $CD4^+$ T cells.[20] In addition, TGF-β modifies APC by inhibiting their expression of interleukin 12 (IL-12) and CD40 and by inducing the expression of active TGF-β[21] through a process which is dependent on expression of thrombospondin.[22] These TGF-β-treated APC promote the generation of regulatory T cells (Treg) that suppress immune responses via production of TGF-β.[23,24] α-MSH also induces Treg generation[25] and synergizes with TGF-β to increase the frequency of Treg by abrogating the anti-proliferative effects of TGF-β on T cells.[26] Another molecule within the aqueous humor, somatostatin, further amplifies immune suppression by inducing α-MSH production.[27]

Pigmented epithelial (PE) cells of the iris, ciliary body, and retina, and corneal endothelial (CE) cells can also convert T cell effectors into Treg *in vitro* via immunosuppressive cell surface molecules. For example, iris/ciliary body PE express CD86 and CE cells express programmed death ligand-1 (PD-L1), which engages cytotoxic T-lymphocyte antigen 4 (CTLA4) or PD-1, respectively on activated T cells to induce the generation of $CD4^+FoxP3^+$Treg.[28] Hence, effector function may be inhibited as activated T cells extravasate from vessels in the iris/ciliary body into the a.c. by conversion of T effectors into Treg. A similar phenomenon may occur in the retina as retinal PE cells express PD-L1 which inhibits T-cell activation.[29]

Ocular cell surface expression of PD-L1/PD-L2[30] and the CD95 ligand (FasL)[31] can also induce apoptosis of the activated T cells. The significance of these death-inducing molecules in maintaining immune privilege is well established in corneal transplantation as mice that are deficient in either of these molecules reject corneal allografts at a higher frequency than their wild-type counterparts.[30,32] Moreover, T-cell apoptosis is demonstrable in accepted corneal allografts whereas rejecting grafts are heavily infiltrated by $CD4^+$ T cells.

ACAID

Mice harboring progressively growing ocular tumors expressing minor MHC antigen differences with their host display prolonged acceptance of skin grafts sharing the same haplotype as ocular tumors, whereas major and minor MHC antigen-disparate skin grafts are rejected with normal kinetics.[33] Tolerance to these semi-allogeneic skin grafts was associated with inhibited $CD4^+$ T-cell-mediated delayed type hypersensitivity (DTH) responses specific for minor antigens[34] while tumor-specific $CD8^+$ CTL responses[35] and antibody production[4] were unimpaired. These data indicate that the immune system responds to ocular antigens but is clearly deviated from the response evoked by the same antigens encountered at extraocular sites. Hence, Streilein and Niederkorn proposed the term a.c. associated immune deviation (ACAID) to describe this phenomenon.[36]

ACAID has been primarily characterized by the suppression of $CD4^+$ T-cell mediated DTH responses to ocular antigens and is a complex process involving the spleen, thymus, and the sympathetic nervous system.[37] The current paradigm suggests that $F4/80^+$ APC from the eye traffic via the bloodstream to the thymus and the marginal zone of the spleen where they directly present antigens as well as indirectly present antigens to B cells that function as APC for thymus-derived NK T cells and γδ T cells via nonclassical MHC molecules. Coordinate interactions of these cell populations along with the expression of interleukin-10

(IL-10) and inhibited IL-12 production culminate in the generation of $CD4^+$ and $CD8^+$ Treg, which inhibit the induction and expression of DTH responses.

IMMUNE PRIVILEGE AND OCULAR TUMOR DEVELOPMENT

Immune suppressive mechanisms, which maintain ocular immune privilege, should favor ocular tumor development and persistence. However, ocular tumors are very rare. The prevalence of the most common intraocular malignancy, UMs, is over 30 times lower than cutaneous melanoma.[38,39] One explanation for this paradox is that the eye compensates for an absence of immune surveillance by expressing death receptors that target transformed cells for apoptosis. For example, tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) targets several different transformed cell lines for apoptosis,[40] and P815 tumor cells transduced to express TRAIL receptor DR5 failed to develop into ocular tumors when injected into the a.c. of mice where TRAIL is constitutively expressed.[41] Moreover, UM cell lines that express Fas[42] and retinoblastoma cell lines that express Fas and TRAIL receptors (DR4 and DR5)[43] are resistant to apoptosis induced by respective death receptors, which is consistent with the hypothesis that apoptosis induction in the eye and in general must be prevented for ocular tumors to develop.

Immunosurveillance and immunoediting: shaping tumor phenotype through antitumor immunity

In the early 20th century, Paul Erlich proposed that a major function of the immune system was to detect and eliminate tumors from the host.[44] Thomas and Burnet formulated the theory of immune surveillance, which stated that transformed cells expressed immunogenic antigens that induced tumor-specific immune responses which eliminated transformed cells before tumor formation.[45] Studies in the 1960s by Old, Boyse, and Klein identified several tumor antigens on experimentally transformed cells and reported that the injection of these tumor cells into syngeneic mice resulted in rapid immune-mediated tumor rejection which bolstered support for the immune surveillance theory.[46,47] However, spontaneous tumors do develop in immunocompetent hosts indicating that although tumors have the capacity to elicit specific antitumor immune responses, tumors develop mechanisms to escape immune surveillance. The immunoediting hypothesis developed by Schreiber and co-workers[48] describes this complex and dynamic interaction between tumors and the immune system.

Immunoediting comprises three events: elimination, equilibrium, and escape.[49,50] In the elimination phase, which is similar to the immune surveillance hypothesis, tumors are eradicated by the innate and adaptive immune system. Tumor cells that survive the elimination phase undergo an equilibrium phase where the immune system functions to control tumor growth but fails to promote sterile elimination. Tumors in the equilibrium phase are subjected to constant selection pressure provided by the immune system, which promotes random genetic mutations and epigenetic changes. Ultimately, subpopulations of tumor cells become resistant to immune attack, culminating in the escape phase where these tumor escape variants develop and disseminate systemically.

Tumor escape mechanisms: tumors as immune-privileged tissues

There are multiple mechanisms that tumors employ to establish tumor escape variants including loss of tumor antigen expression by downregulation of (i) MHC class I genes,[51] (ii) antigen processing genes such as the peptide transporter genes TAP1 and TAP2,[52,53] (iii) immunoproteasome genes LMP-2 and LMP-7,[52] or (iv) loss of tumor antigen gene expression.[54–56] Tumors may also directly inhibit immune responses by producing immunosuppressive factors such as IL-10 and TGF-β[57–59] or by the expression of CD95L (FasL) which promotes tumor-specific CD95 (Fas)+ immune cells to undergo

apoptosis.[60,61] Therefore, the plasticity of tumor cells allows for the generation of tumor variants, some of which may express the phenotype of an immune-privileged tissue.

As mentioned previously, the historical definition of an immune-privileged site is an anatomical site where the transplanted foreign tissue survives for an extended period of time in an immunocompetent host. Immune-privileged tissues are also capable of sustaining an immune-privileged microenvironment in non-privileged sites. For example, corneal allografts depleted of epithelium persist indefinitely when transplanted to the non-privileged kidney capsule[62] due to the expression of FasL on the corneal endothelium.[63] Therefore, tumors that possess properties of an immune-privileged tissue would enjoy a distinct survival advantage irrespective of their ultimate invasive destination. A vast number of tumor progression of studies focus on investigating genetic changes responsible for the generation of tumor escape variants. However, there is also growing evidence that tumors undergo phenotypic changes that are not attributed to genetic alterations, but are termed an epigenetic phenomenon.

Regulation of gene expression by genomic modification

Regulation of gene expression by genomic modification involves a permanent and irreversible physical alteration of genes at the nucleotide level. Genomic modifications induce either initiation or silencing of gene expression through (i) point mutations, (ii) frameshift mutations, (iii) genomic translocations, (iv) insertions, and (v) deletions.[64–66] Examples of genes that undergo genetic mutation include the BRCA-1 gene in breast cancer,[67] the epidermal growth factor receptor gene in non-small cell lung cancer[68] and recently, GNAQ, a stimulatory α_q subunit of heterodimeric G-protein in UM.[69,70] Studies by Feinberg and Vogelstein demonstrated that genomic DNA from malignant cells was hypomethylated when compared with genomic DNA from nonmalignant cells from the same organ.[71] Hypomethylation in malignant cells destabilizes chromatin structure resulting in the expansion of DNA strands that are normally condensed around histones leading to the expression of genes not normally transcribed by normal cells.[72] Moreover, aberrant genomic DNA expansion promotes a higher incidence of genomic modifications due to the exposure of novel transcriptional open reading frames that are normally silenced.[71,72] Studies analyzing hypomethylation in tumors of various origins demonstrated that tumor progression coincided with an increase in genomic DNA hypomethylation.[73] However, genomic modification alone cannot explain the diverse mutations observed during tumor progression.

Epigenetic gene regulation

Epigenetics is defined as a change in gene expression which is not attributed to physical alterations within the DNA sequence for a particular gene (e.g. mutation), but is heritable to successive generations and the phenotype is reversible. Hypermethylation and/or acetylation of genomic DNA are catalysts that initiate the modulation of gene expression and are associated with certain malignancies.[74] Specifically, CpG islands, regions of DNA composed of clusters of cytosine and guanine nucleotides are hypermethylated in a number of tumors[75] resulting in the downregulation of tumor suppressor genes including the retinoblastoma suppressor gene (rb), p16ink4a, and p53.[76–78] The mechanisms involved in determining how and which CpG islands for specific genes are methylated remain unknown. However, it has been proposed that certain CpG islands are located in the regions of chromatin that are susceptible to hypermethylation. Moreover, histone methylation can be flagged by epigenetic “marks” by a methytransferase-enhancing protein, EZH2, a member of the polycomb gene-silencing protein to histones.[79] Therefore, gene regulation by DNA hypermethylation is very complex and may occur globally and/or locally depending on

whether the methylation occurs on the histones binding the genomic DNA or on the CpG islands within the genome, respectively.

Global regulation occurs when histones are methylated by DNA methyltransferases (DNMTs). Methylated histones attract methyl-binding proteins (e.g. Methyl CpG binding Protein 2 (MECP2)), which, in turn, recruit histone deacetylases (HDACs). Deacetylation causes histones to contract around the nucleosomes resulting in chromatin condensation, which restricts transcription factors from recognizing DNA-binding sites thereby inhibiting gene expression.[80] Global epigenetic modification is the prevalent mechanism of regulating gene expression during embryogenesis and development[81] and is responsible for chromatin remodeling.[82]

Local epigenetic modification regulates the expression of specific genes on euchromatin by methylating CpG islands located at the transcription-binding sites in the gene promoter region. Local regulation also involves methylation of CpG islands found in regions of DNA that encode for enhancer and suppressor elements in the gene[83] and within gene exons. Active transcription of these methylated exons results in nonfunctional RNA transcripts.[84]

DNA methylation patterns are established and maintained by four DNMTs: DNMT1, DNMT2, DNMT3a, and DNMT3b.[85] DNMT1 has traditionally been characterized as a maintenance methyltransferase specialized for copying DNA methylation patterns following DNA replication. DNMT2 exhibits methyltransferase activity, but its function is still under investigation. The exact mechanisms for targeting DNA methylation remain largely unknown, however, studies on chromatin remodeling have shown that the histone H3 and H4 N-terminal tails play a role in determining which regions of the genome are targeted for methylation.[86]

DNMT3a and DNMT3b are primarily involved with *de novo* methylation, particularly during embryonic development.[81] There is evidence that DNMT3a and DNMT3b *de novo* methyltransferases can also directly bind to HDACs (HDAC1 and HDAC2)[82] and in particular when DNMT3b is associated with HDAC1, it co-localizes with enzymes (SIN3A, hSNF2H, KIF4A) known as a condensin complex that is involved in chromatin condensation.[82] Recently, there have been studies demonstrating that DNMT3b is unique in that it can be expressed as alternatively spliced variants that differ in their catalytic domains.[80] The role of the alternatively spliced DNMT3b isoforms remains largely unknown. However, it is interesting to note that the DNMT3b isoforms are overexpressed in a number of tumors suggesting that epigenetic gene regulation by *de novo* methylation plays a role in tumor progression.[87]

Epigenetic gene regulation in ocular tumors

Recent studies have investigated whether epigenetic gene regulation plays a role in the tumorigenesis of UM. The promoter region of two tumor suppressor genes, ras-association domain family 1 and human telomerase reverse transcriptase, contain CpG islands that are hypermethylated resulting in cell cycle disruption.[87] In addition, UM are resistant to IFN-γ-induced upregulation of MHC class II molecules due to the epigenetic suppression of the MHC class II transactivator Class II Transactivator (CIITA).[18,19] Further studies demonstrated that the methyltransferase-enhancing protein EZH2 is responsible for methylation of histone lysine residues, which suppresses CIITA expression.[88]

Establishment of tumor escape mutants in the immune-privileged ocular microenvironment

One of our laboratories (P.W.C.) demonstrated that mice immunized against P815 tumor cells were protected against a subsequent P815 tumor challenge in the flank but were not

protected against an identical tumor challenge in the immune-privileged a.c. of the eye.[89] Ocular tumor growth eventually destroyed the eye, which was intriguing because destruction of the eye should have terminated immune privilege[90] allowing systemic immunity generated by immunization to reject the ocular tumor. These data suggested that factors in the ocular microenvironment induced the generation of tumor escape mutants. To test this hypothesis, P815 tumors were isolated after 10 days of growth in the a.c and then injected in the flank of immunized mice. Wild-type, but not eye-derived, P815 tumor cells were rejected in the flank indicating that tumor escape variants had developed within the immune-privileged ocular environment. Further studies indicated that the escape phenotype did not require T and/or B cells for establishment, and was not due to either downregulation of MHC class I or the cell adhesion molecule intercellular adhesion molecule 1.

The following observations suggest the involvement of epigenetic changes in the escape phenotype. First, the escape phenotype was established reproducibly in a time span of 10 days and the escape variant cells were phenotypically stable. Therefore, it is unlikely that this phenotype was the result of random genomic mutation events that would require more time to develop. Next, we have recently demonstrated that mice immunized against P815 tumor cells reject a tumor challenge of eye-derived P815 tumor cells treated with the demethylating agent 5-aza-2-deoxycytidine (PWC, submitted for publication). In addition, our studies demonstrate that P815 tumor cells exposed to the ocular environment have upregulated DNMT3a and DNMT3b *de novo* methyltransferase genes that are capable of methylating CpG islands present in the genome. Eye-derived P815 cells also exhibit increased genomic DNA methylation compared with wild-type P815 cells. Moreover, purified histone preparations from eye-derived P815 cells demonstrate increases in dimethylated and trimethylated H3 histones on lysine 27 (K27) residues, an epigenetic mark compared to wild-type P815 cells, suggesting that factors in the immune-privileged ocular environment induced epigenetic gene regulation locally by gene-specific methylation as well as globally by affecting chromatin structure conformation.

IMMUNE RESPONSE AGAINST OCULAR TUMORS

Several lines of evidence indicate that ocular tumors are subject to immune surveillance. $CD8^+$ T-cell responses to UM antigens are detected in the peripheral blood and within primary tumors of UM patients,[91,92] and immunogenic tumors injected in the a.c. of the eye induce CTL responses that are equivalent to CTL responses induced by skin tumor development.[35] These data indicate that the induction of ocular tumor-specific CTL is not inhibited by Treg generated by ACAID induction which inhibit $CD4^+$ DTH responses.[34,93]

Direct inhibition of $CD8^+$ CTL effector function

A simple explanation for ocular tumor progression despite priming of tumor-specific $CD8^+$ CTL is that the tumoricidal activity of $CD8^+$ T cells may be inhibited within the a.c. of the eye. In support of this explanation, Ksander et al. demonstrated that the lytic activity of tumor infiltrating lymphocytes (TIL) was greater in P815 tumor cultures that were ultimately rejected in the conjunctiva when compared with similarly cultured P815 tumors that grew progressively in the a.c. even though a higher frequency of precursor cytotoxic T cells was observed in a.c. tumors.[94] A caveat to this interpretation, however, was that higher numbers of contaminating tumor cells in a.c. tumors could have acted as cold target inhibitors in their assay which measured ^{51}Cr release of radiolabeled tumors added to the cultures that could obscure the true measure of lytic activity within these TILs.[94] In fact, the observation of intraocular concomitant immunity by Niederkorn and Streilein[5] wherein mice primed by P815 tumor development in the a.c. of one eye rejected a subsequent P815 tumor challenge in the opposite eye suggests that $CD8^+$ CTL precursors can differentiate into competent effector CTL within the eye.

Systemic inhibition of T-cell responses against ocular tumors may also occur under certain circumstances. For example, one of our laboratories (K.C.M.) recently observed that CD3 zeta chain expression was reduced on all T cells within blood and primary tumors of UM patients that had increased percentages of activated $CD11b^+$ $CD15^+$ granulocytes in the blood.[95] These granulocytes appear to be analogous to myeloid derived suppressor cells which, in other malignancies, promote systemic immunosuppression by directly inhibiting T-cell activation through down regulation of the CD3 zeta chain resulting in T-cell signaling defects.[96] The reduced signaling capacity of T cells would explain why T cells isolated from primary UMs were generally nonresponsive, proliferating poorly after stimulation.[92]

Influence of efferent $CD8^+$ Treg on DTH responses within the eye

Ocular tumor growth induces immunosuppressive $CD8^+$ Treg that inhibit DTH responses.[34] These cells are analogous to efferent $CD8^+$ Treg generated during ACAID induction to soluble antigens which inhibit the expression of DTH responses. As spontaneous rejection of p91 tumors in the a.c. involves intraocular expression of a DTH response,[97–99] it is tempting to speculate that P91 tumor growth failed to induce efferent Treg that normally inhibit DTH responses within the eye or that the strength of the tumor-specific immune response overcame this immune regulation. P815 tumors are rejected when Treg-mediated ACAID is abrogated by splenectomy,[36] which is consistent with this notion. However, to date, the identification and characterization of efferent $CD8^+$ Treg within ocular tumors has not been described. Indeed, the role of these ACAID-induced Treg in promoting ocular tumor development by inhibiting intraocular DTH responses is questionable because cyclophosphamide-treated mice do not reject intraocular B16F10 melanomas despite abrogation of Treg-mediated ACAID (i.e. systemic DTH responses to tumor antigens were restored).[100] Moreover, if these intraocular Treg exist, they could only be active in established ocular tumors and not systemically because, as described above, mice bearing ocular tumors reject subsequent challenges with the same tumor line in the skin or in the contralateral eye.[5,6]

Alternatively, the mechanisms that reject established eye tumors may be different from those that reject subsequent tumor challenges in the skin or in the contralateral eye of ocular tumor-bearing mice and thus, may be subject to different immune regulation. For example, an adenovirus-transformed tumor, Ad5E1, is spontaneously rejected in the a.c. via an IFN-γ-dependent process requiring $CD4^+$ T cells and macrophages.[101–104] However, IFN-γ-deficient mice harboring progressively growing Ad5E1 tumors in one eye reject a subsequent Ad5E1 challenge in the contralateral eye.[104] Therefore, a regulatory cell, which abrogated macrophage activity within ocular tumors either directly or indirectly by inhibiting IFN-γ production by tumor infiltrating T cells would prevent immune elimination of the established ocular tumors. By contrast, metastases from ocular tumors or a contralateral a.c. challenge of tumor cells in ocular tumor-bearing mice may not require macrophages but instead, tumor eradication is dependent on other effector cells, for example, $CD8^+$ T cells, which are not subject to regulation by $CD8^+$Treg.[105] Why macrophages are required to eliminate established ocular tumors but not subsequent tumor challenges is not understood. However, differences in tumor burden or establishment of an immune suppressive microenvironment may be involved.

Indirect inhibition of tumoricidal activity of $CD8^+$ T cells

The T-cell induction of tumoricidal activity in other intratumoral immune cells appears to be most critical for promoting regression of established ocular tumors because as previously described, two different spontaneous ocular tumor rejection models (Ad5E1 tumors in C57Bl/6 mice and P91 tumors in Balb/C mice) require infiltrating $CD4^+$[101] or $CD8^+$ T cells,[100] respectively, to activate intratumoral macrophages to become tumoricidal or to

induce a DTH response. Direct tumoricidal activity by $CD8^+$ T cells is also not required for the elimination of certain established subcutaneous murine tumors. For example, the intravenous transfer of *in vitro*-generated tumor-specific CTL deficient in direct killing mechanisms (perforin, FasL, or TNFα) promoted a complete regression of the established E.G7-OVA skin tumors in the majority of mice.[106] However, skin tumors were not eliminated if transferred CTL were deficient in IFN-γ, or host cells were deficient in IFN-γ receptor or inducible nitric oxide synthase 2.[107] E.G7-OVA tumor cells are not sensitive to IFN-γ *in vitro*.[107] Hence, these data indicate that regression of the established E.G7-OVA skin tumors by transferred CTL is indirect, involving the activation of other tumoricidal effectors by IFN-γ. Recent work from one of our laboratories (K.C.M.) has extended these observations by demonstrating that skin tumor-associated $CD11b^+$ $F4/80^+$ macrophages are induced by transferred $CD8^+$ CTL to produce NO, which is tumoricidal *in vitro* (KCM, submitted for publication), suggesting that intratumoral macrophages are the critical tumoricidal effectors induced by CTL to eliminate established E.G7-OVA skin tumors. Therefore, it is plausible that a spontaneous rejection of P91 tumors in the a.c. of Balb/C mice and P815 tumors in splenectomized Balb/C mice is also macrophage-mediated because the histopathology of regressing ocular tumors is consistent with a DTH response *in situ* and macrophages are a critical effector of DTH responses.

As mentioned above, macrophages are also required for spontaneous rejection of Ad5E1 tumors developing in the a.c. Tumor regression of Ad5E1 or P91 tumors is not evident until 16 days after tumor challenge suggesting that the immune response eliminates an established ocular tumor. We favor an interpretation that $CD8^+$ CTL are capable of directly eliminating a limited number of tumor cells within the established tumor microenvironment and must induce tumoricidal activity in intratumoral macrophages to facilitate regression of established tumors. Therefore, $CD8^+$ CTL may be able to directly control the growth of metastases or subsequent tumor challenges because the tumor burden is relatively smaller or an immunosuppressive tumor microenvironment has not yet been established.

The aqueous humor has been shown to inhibit NO production by macrophages,[108] which may contribute to the inability of T-cell responses to control the growth of ocular tumors. Consistent with this notion, we have recently demonstrated that macrophages isolated from established and progressively growing eye tumors of mice transferred with *in vitro*-generated CTL failed to produce NO or demonstrate tumoricidal activity *in vitro* (KCM, submitted for publication). Transferred CTL that infiltrate eye tumors expressed IFN-γ and CD107a, a marker of granule exocytosis, after *ex vivo* stimulation that was comparable to transferred CTL infiltrating skin tumors which were ultimately rejected, indicating that the CTL effector function was not compromised within the eye in this model. Therefore, suppression of NO production by eye tumor-associated macrophages appears critical for ocular tumor progression and may be due to immunosuppressive factors produced within the eye or by immunosuppressive regulatory cells within the ocular tumor microenvironment. An area of future experimentation is to determine whether some infiltrating CTL are converted into $CD8^+$ efferent Treg.

CONCLUSIONS

Immune suppressive mechanisms, which maintain ocular immune privilege, protect the eye from potentially sight destroying inflammations. However, this evolutionary compromise comes at the price of impaired intraocular tumoricidal immune responses because factors within the ocular microenvironment inhibit critical effector functions of infiltrating immune cells and promote the generation of tumor escape variants that escape detection by the host immune response. Our present efforts are focused on characterizing the mechanisms of immune suppression within ocular tumors and on identifying epigenetic mechanisms

involved in conferring the tumor escape phenotype. The discovery that factors within the ocular microenvironment induce epigenetic changes may represent a novel molecular mechanism the eye utilizes to maintain immune privilege. Experiments in murine tumor models also suggest that direct tumor elimination by $CD8^+$ T cells may be less important than CD8 T-cell induction of tumoricidal activity in intratumoral macrophages when eliminating established ocular tumors. Intratumoral macrophages may also be essential for eliminating tumor variants that are no longer recognized by CTL because their tumoricidal activity is nonspecific. Hence, the tipping point of established ocular tumor regression may be the activation of intratumoral macrophages. Effective ocular tumor immunotherapies may, therefore, require restoration of function in both infiltrating T cells and macrophages while managing the confounding effects of epigenetic gene regulation.

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TABLE 1

Tumor rejection in the immune privileged ocular microenvironment

Tumor	Mouse strain of derived tumor	Recipient mouse strain	Antigens on tumor	Immunogenicity of tumor antigens	Influence of ocular immune privilege	Intraocular tumor rejection	Reference
P815	DBA/2	DBA/2	Tumor antigens	Weak	Tumor protection	No	3
P815	DBA/2	Balb/C	Minor MHC	Weak	Tumor protection	No	3
B16F10	C57BL/6	A/J	Tumor antigens	Weak	Tumor protection	No	5
D5.164	C57BL/6	C57BL/6	Tumor antigens	Weak	Tumor protection	No	109
E.G7-OVA	C57BL/6	C57BL/6	OVA peptide	Strong	Tumor protection	No	6
P815	DBA/2	A/J	Major MHC	Strong	Transient protection	Yes	3
P815	DBA/2	C57BL/6	Major + Minor MHC	Strong	Transient protection	Yes	3
P91	DBA/2	DBA/2	Mutated tumor ag	Strong	Transient protection	Yes	97
UV-5C25	Balb/C	Balb/C	UV-induced tumor ag	Strong	Transient protection	Yes	98
SV40FVN	FVB/n	FVB/n	SV40 T ag	Strong	No tumor protection	Yes	110
Ad5E1	C57BL/6	C57BL/6	Adenovirus	Strong	No tumor protection	Yes	101

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