IMMUNE-MEDIATED MECHANISMS OF PHTHISICAL AND NON-PHTHISICAL INTRAOCULAR TUMOR REJECTION

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# DEDICATIONS

To my grandfather, Glenn Coursey, for instilling in me the importance of hard work, determination, and a love for learning. Without you I would have never accomplished this.

To my wife, Erin Canty Coursey, for being my best friend and my constant source of strength and encouragement. You mean so much to me and in the words of our daughter “if you lined up all people in the world to chose from I’d still chose you”.

To my parents, Terry and Teresa Coursey, for their constant and loving support in everything in my life. I know this makes them very proud.

# IMMUNE-MEDIATED MECHANISMS OF PHTHISICAL AND NON-PHTHISICAL INTRAOCULAR TUMOR REJECTION

by

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The University of Texas Southwestern Medical Center at Dallas, 2011

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Although intraocular tumors reside in an immunoprivileged site where immune responses are suppressed, some tumors are rejected nonetheless. An example of this is the syngeneic adenovirus-induced (Ad5E1) tumor model. Intraocular tumors are rejected in one of two pathways: one that maintains normal architecture and function of the eye and one that causes gross destruction leading to necrosis or phthisis. I created Ad5E1 tumor cell lines from single cell clones that are consistently rejected in a phthisical manner or non-phthisical manner. The three major objectives of this research project were to identify the rejector

mechanisms of; a) non-phthisical intraocular tumor rejection, b) phthisical intraocular rejection, and c) IFN-γ-independent tumor rejection.

The first objective sought to characterize non-phthisical intraocular tumor rejection. I demonstrated that this form of rejection is dependent on T cells; either $CD4^+$ or $CD8^+$ T cells. Rejection was also found to be dependent on M1 macrophages; however, iNOS was not required. Tumor cells were also susceptible to macrophage-mediated killing. However, rejection was not dependent on IFN-γ, TNF-α, TRAIL, or IL-17. An unidentified soluble factor was determined to be responsible for macrophage-mediated killing.

My results indicated that T cells and M1 macrophages were required for phthisical rejection of intraocular Ad5E1 clone 2.1 tumors. In addition, *in vitro* inhibition of iNOS abolished most of the macrophage-mediated killing of the tumor cells, and *in vivo* results indicated that iNOS was essential for controlling the growth of the intraocular tumors. Studies in tumor necrosis factor (TNF)-deficient mice revealed that although TNF-α was not necessary for tumor rejection, it was required for phthisis of the tumor-containing eye. Thus, this model demonstrates that it is possible to modify the host's response such that the immune system eliminates the intraocular tumor while preserving the integrity of the eye.

The last aim sought to determine the mechanisms of IFN-γ-independent tumor rejection. Although phthisically-rejected Ad5E1 tumors were not rejected

when transplanted into the eyes of IFN-γ KO mice, they were rejected following subcutaneous transplantation (SC). Thus, outside of the eye, Ad5E1 tumors elicit a form of tumor immunity that is IFN-γ-independent. I demonstrated that IFN-γ-independent SC rejection required both $CD4^+$ and $CD8^+$ T cells. Furthermore, SC tumor rejection required IL-17, which was produced by IFN-γ-deficient $CD4^+$ T cells in response to tumor antigens (TAs). Additionally, depletion of IL-17 decreased CTL activity against Ad5E1 tumor cells. In this model it is proposed that in the absence of IFN-γ, IL-17 produced by $CD4^+$ T cells in response to TAs increases CTL activity, which mediates tumor rejection. However, this does not occur in the eye. IL-6 production within the eye is severely reduced, which is consistent with the failure to induce Th17 cells within the intraocular tumors. Therefore, IFN-γ-independent tumor rejection is excluded from the eye and may represent a newly recognized form of ocular immune privilege.

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PRIOR PUBLICATIONS

Halcomb K.E., Contreras C.M., Hinman RM, Coursey TG, Wright HL, Satterthwaite AB. (2007) Btk and phospholipase C gamma 2 can function independently during B cell development. *European Journal of Immunology* 37(4):1033-42

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Coursey, T. G., Chen, P.W. and Niederkorn, J.Y. (2011) IFN-γ-Independent Non-Destructive Intraocular Tumor Rejection is Mediated by Macrophages (manuscript in preparation).

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LIST OF ABBREVIATIONS

Ab – Antibody

AC – Anterior chamber

ACAID – Anterior chamber-associated immune deviation

Ad5E1 – Adenovirus type 5 early region 1

AqH – Aqueous humor

AMD – Age-related macular degeneration

APC – Antigen presenting cell

BMDM- Bone marrow-derived macrophage

BSA – Bovine serum albumin

CGRP – Calcitonin gene-related protein

CIA – Collagen-induced arthritis

CNS – Central nervous system

CPM – Counts per minute

CRPs – Complement regulatory proteins

CTLs – Cytotoxic T lymphocytes

DAMP - Damage-associated molecular pattern

DCs – Dendritic cells

DMEM – Dulbecco's modified Eagle's medium

DNA – Deoxyribonucleic acid

D-NAME - N $_{\omega}$-Nitro-D-arginine methyl ester hydrochloride

DTH – Delayed-type hypersensitivity

E1 – Early region 1

EAE – Experimental autoimmune encephalitis

ELISA – Enzyme-linked immunosorbent assay

FasL – Fas ligand

HBSS – Hanks' balanced salt solution

HMGB-1 - High mobility group box protein B1

IBD – Inflammatory bowel disease

IDO – Indoleamine dioxygenase

IFNs – Interferons

IFN-$\alpha$ – Interferon-alpha

IFN-$\beta$ – Interferon-beta

IFN-$\gamma$ – Interferon-gamma

iNOS – Inducible nitric oxide synthase

IP – Intraperitoneally

IV – Intravenously

KO – Knockout

LPS - lipopolysaccharide

L-NAME - $N_{\omega}$-Nitro-L-arginine methyl ester hydrochloride

MEC – Mouse embryo cells

MEF – Mouse embryonic fibroblasts

MHC – Major histocompatability complex

MLTC – Mixed lymphocyte tumor culture

MIF – Macrophage migration inhibitory factor

MMP-9 - Matrix metalloprotease 9

MyD88 – Myeloid differentiation factor 88

NK – Natural killer cells

PAMPs – Pathogen-associated molecular patterns

PECs – Peritoneal exudate cells

PBS – Phosphate buffered saline

PMN - Polymorphonuclear leukocytes

PRPs – Pattern-recognition receptors

RAG – Recombinase-activating gene

RNA – Ribonucleic acid

ROI - Reactive oxygen intermediates

RPMI – Roswell Park Memorial Institute medium

SC – Subcutaneous

SCJ – Subconjunctival

SO - Sympathetic ophthalmia

sFasL – Soluble Fas ligand

TAM – Tumor associated macrophages

TGF-β – Transforming growth factor-beta

TDLN - Tumor draining lymph node

TIL – Tumor infiltrating lymphocyte

TNF – Tumor necrosis factor

TLR - Toll-like receptor

TRAIL – Tumor necrosis factor-related apoptosis-inducing ligand

Treg - Regulatory T cells

VEGF - Vascular endothelial growth factor

VIP – Vasoactive intestinal peptide

α-MSH – alpha melanocyte-stimulating hormone

ºC – Degrees Celsius

$^{3}H$ – $^{3}$Hydrogen (tritium)

$^{51}Cr$ – $^{51}$Chromium

# CHAPTER ONE
# INTRODUCTION AND LITERATURE REVIEW

## THE EYE

The sole function of the eye is transmission of visual stimuli to the brain. When light enters the eye it is directed by the cornea through a watery substance in the anterior chamber (AC), called the aqueous humor (AqH), then through the lens (Figure 1). The iris is responsible for controlling the diameter of the pupil and thus the amount of light reaching the retina. The lens is a transparent structure that, along with cornea, refracts light that is focused on the retina. By changing its shape, the lens functions to change the focal distance so the eye can focus on objects at various distances, allowing a sharp image of an object to be formed on the retina. This adjustment of the lens is known as accommodation. The image formed on the retina is sent to the brain via the optic nerve.

The eye is an extension of the central nervous system (CNS) and, like other components of the CNS, the eye has limited regenerative properties. Thus, damage to ocular tissues can lead to disruption of light transmission from the cornea to the retina, and ultimately lead to blindness. Tissue damage can occur through a variety of insults including the body's own immune system. Obviously, a strict regulation of immune response in the eye is needed to maintain homeostasis and preserve vision.

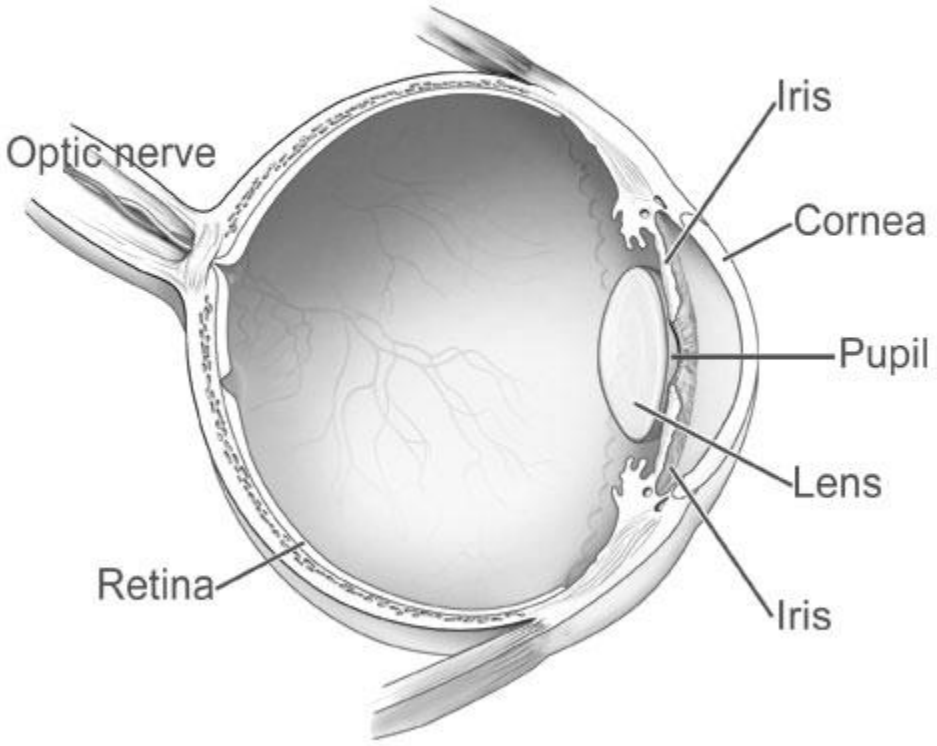



**Figure 1.** Cross section of the eye. The anterior segment of the eye includes the cornea, anterior chamber (filled with aqueous humor), and the iris and ciliary body. The posterior segment of the eye is composed of the vitreous humor and is bound posteriorly by the retina. The lens separates both chambers. Image from National Eye Institute, National Institutes of Health: http://www.nei.nih.gov.

## OCULAR IMMUNE PRIVELEGE

The earliest studies of ocular immune privilege were conducted over 130 years ago by the Dutch ophthalmologist J.C. van Dooremaal, who demonstrated the prolonged survival of mouse skin grafts and other tissues when implanted in the anterior chamber of dog eyes (1). In the 1930s, H. Greene confirmed van Dooremaal's observations by demonstrating the growth of human and rabbit tumors in the eyes of different animal species (2, 3). P. Medawar soon followed with studies that demonstrated the long term survival of foreign skin grafts in the anterior chamber and brain. To describe this phenomenon he coined the term "immune privilege" and attributed this to the lack of lymphatic drainage from the eye and brain, thus keeping antigens sequestered from the systemic immune system (4). Sites of immune privilege include the brain, hair follicles, testes, the pregnant uterus, the anterior chamber of the eye, and the cornea (5). About 30 years ago, Streilein and others challenged the explanation of "immunological ignorance" as the source of immune privilege (6). In fact, there are multiple mechanisms that are employed by the eye to maintain an immunologically quiescent environment (5). There are thought to be three major mechanisms of maintaining immune privilege in the eye: (a) anatomical and cellular barriers; (b) immunosuppressive factors in the ocular environment; and (c) eye-derived systemic tolerance of ocular antigens called anterior chamber-associated immune deviation (ACAID).

*Anatomical and cellular features that contribute to immune privilege*

An example of an anatomical barrier is the ocular blood barrier which is created by tight junctions of the endothelium of capillaries of the retina and iris, ciliary epithelium and retinal pigment epithelium. This physical barrier between the local blood vessels and most parts of the eye itself restricts the access immune cells have to the eye. There is also a decrease of lymphatic drainage from the AC which restricts ocular antigens from reaching the lymphoid tissues for antigen presentation to lymphocytes (7). An example of a cellular feature that contributes to immune privilege is the absence of MHC class II molecule expression on corneal epithelial cells, keratocytes, and endothelial cells. These cells also express only low levels of MHC class I molecules. This makes ocular cells less susceptible targets for $CD4^+$ and $CD8^+$ T cells.

*The immunosuppressive microenvironment of the AC*

Another important facet of ocular immune privilege is the immunosuppressive intraocular microenvironment. The AqH contains molecules that suppress the activation of $CD4^+$ T cells, polymorphonuclear lymphocytes (PMN), M1 macrophages, and natural killer (NK) cells (7). Transforming growth factor-β2 (TGF-β2) was one of the first immunomodulatory molecules identified in the AqH and has multiple immunosuppressive properties. TGF-β2 has been shown to inhibit the cytolytic activity of CTL, B cells and NK cells (8). The AqH

contains many other factors that contribute to the immune privilege of the eye. One of these is the neuropeptide alpha-melanocyte stimulating hormone (α-MSH), which inhibits T cells, macrophages, and neutrophils. Somatostatin, another neuropeptide present in the AqH, contributes to immunoregulation by stimulating the production of α-MSH and activation of Tregs (9). The AqH also contains vasoactive intestinal peptide (VIP), which suppresses IFN-γ production and T cell activation and activated Tregs. (10, 11).

Calcitonin gene-related peptide (CGRP) is another neuropeptide which suppresses both nitric oxide and peroxide generation by macrophages (12). Macrophage migration inhibitory factor (MIF) is soluble factor that inhibits NK cell activity (13). In other tissue sites, inhibitory KIR molecules bind target cell MHC class I molecules to prevent NK cell attack on normal cells (14). Due to the low level of MHC class I expression, ocular cells are susceptible to NK cell attack. However, the function of MIF prevents NK cell-mediated attack of ocular cells. Soluble Fas ligand (sFasL), also present in the AqH, mediates apoptosis of T cells and suppresses neutrophil recruitment and activation (5, 15). The immunosuppressive factors of the AqH are summarized in Table 1.

| Factor | Function in immune privilege |
|---|---|
| α-MSH | suppresses T cells, macrophages, and neutrophils |
| VIP | inhibits T cell activation and proliferation |
| Somatostatin | suppresses production of IFN-γ by T cells |
| CGRP | suppresses the production of inflammatory molecules by macrophages |
| TGF-β2 | suppresses activation of T cells, macrophages, and NK cells |
| MIF | suppresses NK cell activity |
| sFasL | suppresses neutrophil recruitment and activation |
| complement regulatory proteins | inhibits the complement cascade |

**Table 1. Selected soluble factors in the AqH that support immune privilege.**

*ACAID*

Work by Kaplan and Streilein showed that the immune system was not ignorant of alloantigens placed in the AC, but manifested a different type of immune response (6). ACAID is an important aspect of immune privilege that not only affects the local environment of the eye, but acts systemically. The term ACAID was coined by Streilein and Niederkorn to identify this form of peripheral tolerance that is induced via the AC of the eye (16). Their studies demonstrated that DBA/2 mastocytoma cells (P815) survive and grow in the AC of BALB/c mice, but are rejected if transplanted to other sites. When AC-injected mice were subsequently grafted with skin from the same DBA/2 donors, mice primed in the AC with P815 cells failed to reject the DBA/2 skin grafts. However, these mice were capable of rejecting skin grafts from non-related donors, suggesting that the immune suppression was antigen-specific. This antigen-specific delay in graft rejection was associated with a systemic down-regulation of DTH (16-18). The transplantation of foreign antigens in the AC induces suppressive systemic immunity.

ACAID is an amazingly complex immunoregulatory process that involves at least four different organ systems: the eye, the thymus, the spleen, and the sympathetic nervous system (5). A simplified summary of ACAID is as follows. Antigen is induced into the AC of eye. Antigen-bearing ocular APCs ($F4/80^{+}$ macrophages) migrate from the eye to the thymus and then to the spleen. In the

thymus ocular APCs elicit the generation of $CD4^-$ $CD8^-$ NK T cells, which transfer the immunoregulatory activity from the thymus to the spleen (5). In the spleen, $CD4^-$ $CD8^-$ NK T cells contribute to the generation of splenic $CD8^+$ Tregs. Antigen-bearing ocular APCs are also thought to migrate directly from the eye to the spleen where they interact, either directly or indirectly, with $CD4^+$ T cells, $CD8^+$ T cells, B cells, NK T cells, and γδ T cells. A complex set of processes leads to the generation of these $CD8^+$ Tregs that suppress T helper type 1 (Th1) and T helper type 2 (Th2)- mediated immune responses (5, 15).

## CIRCUMVENTION OF IMMUNE PRIVILEGE AND INTRAOCULAR TUMOR REJECTION

### *Intraocular Tumors*

Conceptually, it would seem that the immune privileged eye would be an ideal site for tumor growth. However, uveal melanoma, the most common intraocular tumor in adults, represents less than 1% of the annual cancer registrations (19). Retinoblastoma, a neuroblastic tumor, is the most common primary intraocular malignancy of childhood with an incidence of only one in 20,000 children (20). Although these tumors arise in an immune privileged environment, immune privilege can be circumvented, as spontaneous rejection occasionally occurs with both of these intraocular tumors (21, 22).

*Immune surveillance of tumors*

In the early 1900s, Paul Ehrlich proposed the notion that the immune system can be employed as a therapeutic agent to treat established tumors (23). In the late 1950s, Burnet and Thomas expanded this concept and hypothesized that the immune system may protect against nascent cancers by killing malignant cells before they develop into detectable tumors. This concept has become known as "cancer immune surveillance" (24, 25). However, this idea was nearly abandoned in the 1970s. Several different groups had conflicting results using neonatal thymectomy versus heterologous anti-lymphocytes serum to induce immune suppression. The overall conclusion was that immunodeficient animals had a high susceptibility to virally-induced tumors and increased tendency to develop spontaneous lymphomas compared to wild-type mice (26). For example, the work of Osisas Stutman demonstrated that although athymic nude mice had an increased incidence of lymphomas and virally induced tumors, there was not an increased incidence of spontaneous or carcinogen-induced tumors (27). Based on the limited understanding of the immunologic defects in the athymic nude mice available at the time, this work was convincing enough to lead to the abandonment of the immunosurveillance hypothesis. There are several problems with the use of this model. First, athymic nude mice are not fully immunocompromised; T cells are not absent but only present in fewer numbers than wild-type animals, as there is a detectable population of functional αβ T cells

(28, 29) . Second, athymic nude mice have normal populations of NK cells and γδ T cells which can develop extrathymically (30).

In the mid-1990s with a new understanding of mouse models the validity of this concept was confirmed. Studies showed that IFN-γ was shown to prevent both chemically induced and spontaneous tumors (31). Recombinase-activating gene (RAG) KO mice, which lack functional T and B cells, and perforin KO mice were shown to develop chemically induced tumors more frequently than wild-type mice (32, 33). Other studies demonstrated that both arms of the immune system, innate and adaptive immune responses, were involved in immune surveillance. Mice deficient in NK cells, T cells, NKT cells, IFN-γ, or IL-12 had increased susceptibilities to tumors (34, 35). In humans, non-viral cancer incidence is dramatically increased (10-25 fold) in immunosuppressed transplant patients (36, 37). Clearly these studies demonstrate the importance of the immune system in the rejection of tumors.

*Immune-mediated rejection of intraocular tumors*

As mentioned above, immune privilege of the eye is not an insurmountable barrier preventing the rejection of intraocular tumors by the systemic immune system. However, immune-mediated rejection of intraocular tumors requires the circumvention of ocular immune privilege. There is compelling evidence from experimental animal models indicating that ocular

immune privilege can be circumvented, resulting in the emergence of effector T cells that mediate the immune rejection of intraocular tumors (38, 39). These studies have revealed two fundamental patterns by which intraocular tumors can undergo T cell-dependent immune rejection. The first pattern is characterized by piecemeal necrosis of intraocular tumor cells and preservation of the architecture of the eye (40, 41). Animal studies using UV-induced fibrosarcomas and SV40 large T antigen-induced retinal pigment epithelial carcinomas have revealed evidence suggesting that piecemeal necrosis of intraocular tumors is mediated by tumor-specific $CD8^+$ CTLs (42, 43). In addition, murine embryonic tumors induced with the adenovirus gene (Ad5E1) undergo spontaneous immune rejection in the eyes of syngeneic C57BL/6 mice without damaging the ocular architecture (44). However, rejection of Ad5E1 tumors occurs in CD8 knockout (KO) and perforin KO mice, suggesting ocular tumor rejection that leaves the eye anatomically intact can be mediated by a CTL-independent process.

A second pattern of T cell-dependent rejection of murine intraocular tumors involves ischemic necrosis and extensive damage to both the tumor and innocent bystander cells within the eye (45). This pattern of rejection results in atrophy of the eye, a condition called phthisis (40). This form of immune rejection was first revealed in studies using a highly immunogenic clone of P815 mastocytoma (P91), which underwent an ischemic necrotizing form of T cell-dependent immune rejection in the eyes of syngeneic DBA/2 mice (46). The

histopathological and immunological features of phthisical rejection of P91 tumors were reminiscent of a delay-type hypersensitivity (DTH)-mediated process based on a characteristic histopathology involving: a) damage to microvascular endothelium; b) ischemic bulk necrosis; c) innocent bystander destruction of normal host tissues; and d) the absence of host effector cell-to-tumor cell contact in tumor death (40)**.** Obviously, this pattern of rejection leads to blindness.

*Phthisis in other models*

Phthisis bulbi, the clinical term of phthisis of the eye, is the end stage appearance of the eye after a variety of insults (injury or disease). In experimental models the eye is shrunken and anatomically disfigured. Phthisis bulbi has varied etiologies from ocular injury, radiation, infection, and inflammation (specifically autoimmune disease). Phthisis bulbi is the end result of some cases of ocular human cancers. Phthisis bulbi results in a small percentage of patients with retinoblastoma (47). There are also rare cases in which uveal melanoma results in phthisis bulbi (48-50).

Another disease that typically results in phthisis is sympathetic ophthalmia (SO). SO is a rare, bilateral, granulomatous uveitis that occurs after ocular trauma or surgical procedures to one eye that threatens the sight of the other eye (51). The pathophysiology is not fully understood, but it appears to be the result of the disrupted integrity of the inciting eye that leads to an autoimmune response

against ocular antigens of the injured eye as well as the sympathizing eye. The eye sustaining the injury is referred to as the inciting eye and the other eye is called the sympathizing eye. The time between ocular injury of the inciting eye and the development of SO is varied from 2 weeks to 50 years, but typically occurs within 3 months (52). It is estimated that about 80% of patients who sustained open globe trauma developed SO within 3 months and 90% in l year (53). Clinical presentation is acute anterior uveitis with mutton-fat keratic precipitates. The posterior segment manifests moderate to severe vitritis and is usually accompanied by multiple yellowish-white choroidal lesions. Evidence suggests that SO represents an autoimmune inflammatory response against choroidal melanocytes mediated by T cells. Treatment of sympathetic ophthalmia consists of systemic anti-inflammatory agents, such as oral corticosteroids. However, if the inflammation cannot be controlled, topical cyclosporine is then used. Other immunosuppressive agents, such as chlorambucil, cyclophosphamide or azathioprine, are also used to control inflammation (54).

*Ad5E1 tumor model*

The adenovirus-induced tumor Ad5E1 (adenovirus type 5 early region 1) has been extensively used to characterize the circumvention of immune privilege and to analyze the immune mechanisms that lead to non-phthisical rejection of intraocular tumors. The Ad5E1 tumor was created by the transfection of C57BL/6

mouse embryo cells (MEC) with a plasmid encoding the early region 1 (E1) genes of the human adenovirus type 5 (55, 56). Ad5E1 tumor cells express viral antigens that induce T cell-mediated tumor-specific immune responses (57, 58). The E1 region of human adenoviruses consists of two transcriptional units, E1A and E1B, which have been shown to be highly immunogenic as specific epitopes for recognition by tumor-specific T cells (56).

Early studies with this tumor model sought to understand CTL responses against Ad5E1 tumor cells and develop peptide-based tumor vaccines that elicit CTL responses against tumors (56, 59-61). In these studies it was shown that rejection of SC Ad5E1 tumors grow progressively in syngeneic naïve C57BL/6 nude mice, but are rejected in wild-type C57BL/6 mice and determined CTL efficacy and specificity in SC rejection. Schurmans et al. (62) were the first to employ this model to study the rejection of intraocular tumors. Their results showed that tumor growth did not induce tumor-specific tolerance (ACAID) as 70% of mice rejected tumor spontaneously after 5 weeks. However, no tumor growth was observed at extraocular sites even if the tumor inoculum was increased 30 times. This study demonstrated that ocular immune privilege delayed, but did not prevent, an anti-tumor immune response that mediated non-phthisical rejection of the intraocular tumors. Furthermore, adoptive transfer of an Ad5E1-specific CTL clone accelerated tumor rejection in the eye indicating that CTLs contributed to anti-tumor immunity.

This group published a follow-up study that indicated that T cells were required for intraocular rejection of Ad5E1 as tumors grew progressively in immunodeficient nude mice (63). Additionally, they demonstrated that $CD4^+$ T cells were specifically required for rejection as progressive growth occurs in CD4-depleted mice as well as MHC class II-deficient mice. The authors noted that this form of rejection had the characteristics of a $CD8^+$ T cell-mediated response since tumors were MHC class II negative and non-phthisical rejection was the pattern of rejection observed. However, further analysis indicated that $CD8^+$ T cells were not required for rejection, although it is mentioned that both $CD4^+$ and $CD8^+$ T cells are present in the tumor. Additional experiments examining the cellular mechanisms involved in tumor rejection demonstrated that Ad5E1 tumor rejection occurred in the eyes of perforin KO mice, FasL deficient (*gld/gld*) mice, TNF-α KO mice, B cell deficient (IgM KO) mice, and NK cell-depleted mice. This indicated that none of these immune components were necessary for intraocular tumor rejection.

Another study by Wang et al. (64) confirmed these results and demonstrated that $CD4^+$ T cell-dependent tumor rejection requires IFN-γ. In addition Ad5E1 tumor cells expressed DR5 receptor for TNF-related apoptosis-inducing ligand (TRAIL) and were susceptible to TRAIL-induced apoptosis. Further analysis determined that IFN-γ enhanced TRAIL expression on $CD4^+$ T cells and increased susceptibility of tumor cells to TRAIL-induced apoptosis.

Thus the expression of TRAIL on $CD4^+$ T cells and corneal endothelial cells was sufficient to induce apoptosis of tumor cells. This study suggested that the effect of IFN-γ was not directly on Ad5E1 tumors cells, but was required for the increase of TRAIL expression that mediates apoptosis of the tumor. Thus, TRAIL was believed to be the primary mediator of non-phthisical rejection (64).

A more recent study has demonstrated that IFN-γ was required for $CD4^+$ T cell-dependent rejection of Ad5E1 but TRAIL independent (65). IFN-γ has a direct effect on Ad5E1 tumor cells by: a) inhibiting tumor cell proliferation; b) inducing tumor cell apoptosis; and c) downregulating pro-angiogeneic genes and upregulating anti-angiogeneic genes in the tumors (64, 65). Interestingly, this study showed that IFN-γ was only required for rejection of intraocular tumors, as SC tumors were rejected in an IFN-γ-independent manner. This observation represents a newly recognized form of immune deviation in which IFN-γ-independent immune processes can mediate tumor rejection at SC tumor sites but cannot operate in the eye.

Another recent study examined the role of $CD8^+$ T cells in intraocular Ad5E1 tumor rejection (66). Flow cytometry analysis confirmed the presence of $CD8^+$ T cells within intraocular Ad5E1 tumors; however, they were not conventional CTLs as there was an absence of CTL activity against Ad5E1 target cells. However, this was remedied by increasing the number of tumor cells used in the mixed lymphocyte tumor culture (MLTC) prior to measuring CTL activity.

Adoptive transfer of $CD8^+$ T cells from tumor rejector mice to SCID mice indicated that $CD8^+$ T cells are sufficient to mediate tumor rejection. Further experiments showed that $CD8^+$ T cells did not mediate rejection via IFN-γ, FasL, perforin, or TRAIL, as $CD8^+$ T cells did not produce IFN-γ in response to tumor antigens and tumor cells did not express Fas. $CD8^+$ T cells from rejector TRAIL KO or perforin KO mice were able to mediate rejection when adoptively transferred to SCID mice indicating that TRAIL and perforin were not used by $CD8^+$ T cells to mediate rejection. However, $CD8^+$ T cells from rejector TNF-α KO mice adoptively transferred to SCID mice could not mediate intraocular Ad5E1 tumor rejection. In agreement with these results Ad5E1 tumors cells were shown to be sensitive to TNF-α-induced apoptosis.

Two studies have explored the role of macrophages in the rejection of intraocular Ad5E1 tumors (67, 68). In the first study, local depletion of ocular macrophages by the subconjunctival (SCJ) injection of liposomes containing clodronate prevented rejection of intraocular tumors (67). Clodronate liposomes have been shown to selectively deplete macrophages, and are not toxic to other phagocytic cells (69, 70). By contrast, intravenous (IV) injection of clodronate liposomes had no effect on intraocular tumor growth as tumors were rejected. When mice were treated with a SC injection of clodronate liposomes in the area of the tumor-draining lymph node (TDLN), approximately 50% of the mice had progressively growing intraocular tumors at day 34 post-tumor injection.

Immunohistochemical analysis revealed a lack of F4/80$^+$ macrophages in the intraocular tumors in mice treated with SCJ injections of clodronate liposomes. In order to determine at which phase of the immune response macrophage-mediate rejection occurred, clodronate liposome injections were delayed until days 8 and 14 post-tumor injection. Intraocular Ad5E1 tumors underwent rejection suggesting that macrophages were required in the induction of the anti-tumor immune response but not in the effector phase of tumor rejection (67).

A recent study has confirmed that macrophages were necessary for intraocular Ad5E1 tumor rejection and has further demonstrated that macrophages were required for CD4$^+$ T cell function (68). CD4$^+$ T cells from clodronate liposome-treated mice produced significantly less IFN-γ compared to PBS liposome-treated mice. Furthermore, rejector CD4$^+$ T cells adoptively transferred to clodronate liposome-treated SCID mice were unable to mediate rejection, whereas rejector CD4$^+$ T cells were able to mediate rejection in PBS-liposome-treated SCID mice. This demonstrated that macrophage and CD4$^+$ T cell cooperation was needed for non-phthisical intraocular rejection of Ad5E1 tumors.

## IMMUNE COMPONENTS OF TUMOR REJECTION

### *CD4$^+$ T cells: Th1, Th2, and Th17 cells*

$CD4^+$ T helper cells are important mediators of immune responses functioning to coordinate other components of the immune system. Adaptive immune responses are induced by the presentation of antigenic peptides loaded on MHC molecules expressed by APCs and presented to naïve T cells. The class of MHC molecules expressed determines which type of T cells will be activated. $CD4^+$ T cells are activated by APCs expressing MHC class II molecules, and $CD8^+$ T cells are activated by APCs expressing MHC class I molecules. MHC class II molecules are restricted to expression on "professional" APCs such as dendritic cells (DC), macrophages, and B cells; however, MHC class I molecules are expressed on almost all nucleated cells. The activation of T cells requires two signals. The first signal, which is antigen-specific, is provided through the TCR which interacts with peptide-MHC molecules on the membrane of APCs. The second signal, a co-stimulatory signal, is antigen nonspecific and occurs via the interaction between co-stimulatory molecules expressed on the membrane of APCs and the T cell. The best characterized co-stimulatory molecule expressed by T cells is CD28, which interacts with CD80 and CD86 on the membrane of APC. There are other co-stimulatory molecules, such as ICOS. T cell co-stimulation is necessary for proliferation, differentiation and survival.

In 1989, Mosmann et al., described two distinct subsets of $CD4^+$ T cells, Th1 cells and Th2 cells (71). Until recently $CD4^+$ T cells were thought to consist

of only these two subsets. Today, four distinct subsets have been described: Th1, Th2, Th17, and regulatory T cells (Treg). The three effector cell populations that have been shown to play a role in anti-tumor immunity will be discussed below.

*Th1 cells*

Naïve $CD4^+$ T cells are differentiated into Th1 cells by IL-12, which is produced by activated macrophages and DCs (72). Activated Th1 cells secrete IL-1, IL-2, TNF-α, TNF-β, and GM-CSF (73). However, the prototypical Th1 effector cytokine is IFN-γ. IFN-γ has two key functions in Th1 immune responses. First, IFN-γ (along with lipopolysaccharide; LPS) activates macrophages, enhancing their antimicrobial and anti-tumor actions (72, 74). Second, IFN-γ stimulates the production of IgG antibodies that bind to high-affinity Fc receptors and complement proteins, inducing opsonization and phagocytosis by phagocytic cells (72). IFN-γ also continues to perpetuate the induction of Th1 cells by enhancing IL-12 secretion by macrophages (75) and maintaining the expression of IL-12 receptors on $CD4^+$ T cells (76). Th1-dominant immune responses are often associated with inflammation and tissue injury. TNF-α and IFN-γ recruit and activate inflammatory cells such as M1 macrophages. Importantly, Th1 derived IFN-γ, in combination with IL-2, promotes the differentiation, activation and cytolytic function of $CD8^+$ T

lymphocytes into active CTLs (72, 77). The effect of Th1 cells in anti-tumor immunity is clear; however, the mechanisms of action are not. Some reports argue that Th1 cells are only needed for the generation of tumor-specific $CD8^+$ CTLs and do not directly mediate cytolysis of tumor cells (78-84). Other studies demonstrate that Th1 cells can orchestrate effector anti-tumor mechanisms that are independent of $CD8^+$ T cells (85-87).

*Th2 cells*

Naïve T cells are induced to become Th2 cells by signaling through the IL-4R. IL-4 is the prototypical Th2 cytokine, but Th2 cells also make IL-5, IL-10 and IL-13. IL-4 is the major inducer of B-cell switching to IgE production, and is the key initiator of IgE-dependent, mast-cell-mediated reactions (88). IL-5 functions to activate eosinophils (89). Due to these functions, IL-4 and IL-5 are the dominant cytokines that mediate Th2 dominated responses, such as in allergies and helminthic infections (72). Th2 cytokines crossregulate Th1 immune reactions (72). IL-4 and IL-13 antagonize the macrophage-activating action of IFN-γ, and IL-10 suppresses numerous macrophage responses (72) favoring growth of tumors. Generally, it is thought that Th1 cell dominated responses are optimal for anti-tumor immunity; however, there several reports of Th2 cell involvement in anti-tumor immunity.

The anti-tumor activity of IL-4, first reported by Tepper et al., was shown to be dependent on infiltrating eosinophils and macrophages (90, 91). IL-4 also has anti-angiogenic properties. IL-13 can have both a positive and negative effect on tumor growth. IL-13 also induces granulocyte-mediated tumor clearance (92, 93), but also inhibits CTL activation by inducing TGF-β production by macrophages (94). IL-10 in some models can promote tumor clearance by promoting NK cell activity (95, 96), however, in most cases IL-10 has a pro-tumor role by inhibiting CTL activity and activating Tregs (97-99).

*Th17 cells*

IL-17 was originally described and cloned by Rouvier et al. in 1993 (100). It was originally recognized as an inflammatory cytokine that acted on myeloid cells and mesenchymal cells to induce the expression of granulocyte colony-stimulating factor (G-CSF) and IL-6, which increase granulopoiesis and recruitment of neutrophils to the site of infection (101, 102). However, it was originally thought that IL-17 had minimal effects on T and B cells. The IL-17 family of cytokines has six members - IL-17A-F. IL-17A is the prototypic family member and was initially described at the mRNA level as a product of activated $CD4^+$ T memory cells and $CD8^+$ T memory cells (103). For the sake of simplicity, Il-17A will be referred to as simply IL-17 for the remainder of this discussion. IL-17 binds to and signals through the IL-17 receptor RA (which consists of IL-

17RA and IL-17RC). IL-17RA is widely expressed by mesenchymal cells such as epithelial cells, endothelial cells and fibroblasts (104).

It has only been recently determined that a distinct linage of $CD4^+$ T cells that produces IL-17, Th17 cells, has been described (105, 106). Th17 cells are thought to function to clear extracellular pathogens not effectively handled by Th1 and Th2 cells by the recruitment of neutrophils and macrophages to infected tissues (107). This was first studied in a respiratory *Klebsiella pneumonia* model which demonstrated that mice deficient in IL-17 signaling had impaired ability to recruit neutrophils and overall had reduced bacterial clearance in the lung (108).

IL-17 plays a prominent role in the pathogenesis of many autoimmune diseases. The first evidence for the role of IL-17 in autoimmune disease came from the mouse models experimental autoimmune encephalitis (EAE) and collagen-induced arthritis (CIA). It was thought that these diseases were exclusively mediated by Th1 cells. However, these studies revealed that mice deficient in IFN-γ and IFN-γ receptor signaling developed more severe disease (109-111). Consistent with this observation, a deficiency in the IL-12p35 subunit (specific for IL-12) does not change the progression of EAE, however, a deficiency in either p40 or p19 (which form IL-23R) results in a decrease in the number of Th17 cells and protection from EAE and CIA (112, 113). Confirming this, IL-17-deficient mice develop attenuated EAE (114) and CIA (115). In humans, patients with rheumatoid arthritis (116), multiple sclerosis (117),

inflammatory bowel disease (118), and psoriasis (119) have increased levels of IL-17.

The differentiation of Th17 cells requires two cytokines, IL-6 and TGF-β (120-122). Interestingly, these are two cytokines with opposing effects. IL-6 is a proinflammatory cytokine that is strongly induced in cells of the innate immune system on stimulation of pattern-recognition receptors (PRP) such as Toll-like receptors (TLRs). Infection or local inflammation induces large amounts of IL-6. However, TGF-β is an anti-inflammatory cytokine and functions by blocking the activation of lymphocytes and monocyte-derived phagocytes. Several studies, again in the EAE model, have shown that TGF-β plus IL-6 are the differentiating factors for Th17 cells. Mice with a defect in TGF-β responsiveness in T cells are protected from EAE and have a lack of Th17 cells (123). Also, IL-6-deficient mice fail to develop Th17 responses and are resistant to EAE (122). Although not needed for Th17 cell differentiation, IL-23 is required for maintenance of Th17 cells *in vitro.* Little is known about the role of IL-23 *in vivo,* however, mice lacking the IL-23p19 subunit do not develop EAE and have a lack of Th17 cells (113). It has been shown that IL-21, which is produced by NK cells and NKT cells, could induce the differentiation of Th17 cells in the absence of IL-6 (124). IL-21 is also produced in large amounts by Th17 cells and functions in amplification of Th17 cells (124, 125). Interestingly, IL-21 is a potent positive regulator of $CD8^+$ T cell proliferation and function (126-128). Thus, it is thought

that full Th17 cell differentiation requires three steps: induction by TGF-β and IL-6, amplification by IL-21, and stabilization by IL-23 (107).

Transcriptional control of Th17 differentiation is controlled by the master regulator retinoic-acid-receptor-related orphan receptor γt (RORγt). RORγt is induced by TGF-β and IL-6 (129). Studies have demonstrated that overexpression of RORγt promoted Th17 cell differentiation when both Th1 and Th2 cell differentiation was blocked. In agreement with the role of master regulator of Th17 cell differentiation, mice deficient in RORγt have attenuated EAE and lack Th17 cells (129). Th17 cell differentiation is negatively regulated by IFN-γ, IL-4 (105, 106), and IL-2 (through promotion of FOXP3 expression) (130).

The role of Th17 cells in tumor immunity is controversial. Increased levels of Th17 cells have been observed in many mouse models and patients with various cancer types. These include lymphoma, ovarian cancer, breast cancer, colorectal cancer, lung cancer, myeloma, renal cell carcinoma, cervical carcinoma, fibrosarcoma, gastric cancer, hepatocellular carcinoma, acute myeloid leukemia, prostate cancer and melanoma (131). The role of Th17 cells, whether pro-tumor or anti-tumor, depends on the model or cancer type. For example, the overexpression of IL-17 in murine fibrosarcoma or colon adenocarcinoma cell lines significantly enhance *in vivo* tumor growth and angiogenesis (132). Another example of the protumor function of IL-17 is that in the MB49 bladder

adenocarcinoma and B16 melanoma model there is decreased tumor growth in IL-17 KO and IL-17R KO mice (133, 134).

Although IL-17 may have a minor direct effect on the proliferation or survival of tumor cells (135), the major protumor role of IL-17 is the induction of angiogenesis in endothelial cells and fibroblasts. For example, human cervical cancer cells that were induced to overexpress IL-17 had increased growth in immunocompromised mice compared to control tumor cells (136). IL-17 is able to up-regulate vascular endothelial growth factor (VEGF) production by fibroblasts to promote new vessel formation (137). IL-17 can also stimulate the production of IL-8 which further promotes angiogenesis in endothelial cells, increases proliferation in some tumor cells, and potentiates the migration of neutrophils (138). In addition, IL-23 further upregulates IL-17 and matrix metalloprotease 9 (MMP-9), thus increasing angiogenesis and extracellular matrix remodeling and ultimately leading to metastasis (139).

Th17 cells have also played a role in anti-tumor immunity. The anti-tumor effects of Th17 cells appear to depend on the immune status of the host, the immunogenicity of the tumor, and phase of disease. In human ovarian cancer and prostate cancer increased Th17 cells levels positively predicts survival (140-142). Th17 polarized cells were found to be more effective than Th1 cells in eliminating large established melanoma in mouse models (143). In another mouse model, the colon cancer cell line MC38 had accelerated growth in IL-17 KO mice (144).

There are several mechanisms that have been suggested to explain the anti-tumor properties of Th17 cells. Th17 cells are negatively correlated with the presence of Tregs and positively correlated with effector immune cells such as Th1 cells, CTLs, and NK cells (140, 145). Thus the role Th17 cells play in anti-tumor activity is indirect and functions via recruitment of effector cells into the tumor microenvironment. Benchetrit et al. demonstrated that IL-17 inhibits growth of the mastocytoma P815 by enhancing CTL activity (146). Martin-Orozco et al. confirmed these results and described the mechanism of the action. Th17 cells induce expression of chemokines at tumor sites to recruit $CD8\alpha^+$ DCs, which generate tumor-specific CTLs (147). Tumor-specific CTLs then mediate destruction of the tumor.

Studies that examined the location of Th17 cells in cancer patients and in mouse models have indicated that Th17 cells are present predominately at the tumor site. Th17 cells constitute only a minor population in human and mouse peripheral blood and TDLN with no major frequency changes compared to healthy patients and non-tumor bearing mice (134, 140, 148). Thus, Th17 cells appear to be primarily induced at the tumor microenvironment and not in peripheral lymphoid tissues.

*$CD8^+$ T cells: CTLs*

$CD8^+$ T cells are significant contributors to the adaptive immune system. Activation of $CD8^+$ T cells occurs by recognition of antigens presented in MHC class I complexes. MHC class I molecules, unlike MHC class II molecules, is almost ubiquitously expressed by all cells. Although recognition of antigens by $CD8^+$ T cells does not require presentation by APCs, cross-priming is an important mechanism to activate CTLs. This occurs by the uptake of antigens by several types of DCs that present antigen to CTLs on MHC class I molecules. Only DCs expressing the surface molecules CD24, CD8α and CD103 are able to cross-present antigen (149).

Helper $CD4^+$ T cells are required for the generation of $CD8^+$ CTL responses (150, 151). As discussed above, effector $CD4^+$ T cells are polarized into Th1, Th2, or Th17 cells. Th1 cells are the most effective in anti-tumor immunity and support this by promoting the generation of $CD8^+$ T cells that mediated killing of tumor cells (152, 153). According to the classic model of $CD4^+$ T cell help, the Th1 cytokines (IFN-γ, IL-2, etc.) are released when APCs directly present antigen via MHC class II molecules to responding $CD4^+$ T cells. APCs can also present antigen bound to MHC class I to $CD8^+$ T cells. Due to the close proximity to one another, the cytokines released by the $CD4^+$ T cells facilitate activation of antigen-specific $CD8^+$ T cells (150).

Once activated, CTLs utilize numerous mechanisms to induce cytolysis of pathogens and tumor cells. There are three distinct pathways of killing target

cells. The first pathway is contact-dependent and is mediated by CTL expression of members of the tumor necrosis factor family, such as TNF-α, FasL, and TRAIL, which selectively bind to their specific receptors and induce the caspase cascade of proteins leading to the apoptosis of the tumor cell (154). The second pathway is also contact-dependent and occurs through the release of perforin/granzyme granules by CTLs. After recognition of the target cell, cytotoxic granules move along microtubules to the plasma membrane where they are secreted into the immunological synapse between the two cells. The major constituents of cytotoxic granules are peforin and granzymes, which combine to induce rapid death of the target cell (155). It was originally thought that perforin simply facilitated the entry of granzyme into target cells by physically forming holes in the cell membrane. Recent studies have shown that perforin also has a crucial role in the function of granzyme (155-157). The third pathway is cell-contact independent and is mediated by CTL-secreted TNF-α and IFN-γ (158). The functions of TNF-α and IFN-γ in anti-tumor immunity are discussed below.

*In vitro* studies have shown that $CD8^+$ T cells alone can reject many different tumors (56, 159), however, the generation of tumor-specific CTLs *in vivo* requires the presence of Th1 cells. Alternatively, Th17 cells are able to promote the activation of CTLs through increasing the antigen presentation ability of DCs in the tumor microenvironment (146, 147).

*IFN-γ*

Interferons (IFNs) were first characterized as a group of related molecules that "interfere" with viral replication and infection. IFNs are divided into two groups: type I and type II IFNs. Type I IFNs (IFN-α and IFN-β) are secreted only by virally-infected cells. Type II IFN, or IFN-γ, was initially identified as antigen-specific factor induced during viral infections (160). Today it is known that IFN-γ has a more important role as a proinflammatory molecule modulating many aspects of the immune response (161). IFN-γ is only superficially similar to type I IFNs, and its actions on cells are mediated by a receptor different from that used by type I IFNs (162). IFN-γ receptor is ubiquitously expressed on all nucleated cells (163). T cells are the most abundant producers of IFN-γ, although NK cells also produce it. As discussed above, IFN-γ is the prototypic cytokine of Th1 cells and inhibits the differentiation of Th2 and Th17 cells.

In addition to modulating the immune system and promotion of inflammation, IFN-γ has a critical role in many tumor models. During the anti-tumor response IFN-γ can either target the host cells or tumor cells. Many studies have demonstrated that is the host cell responsiveness to IFN-γ is needed for an effective anti-tumor response. Such responses include the activation of cells of the innate immune response, such as macrophages. IFN-γ also induces the upregulation of pro-apoptotic molecules on the host cell surface, such as TNF-α,

TRAIL, or FasL. IFN-γ also has direct effects as it can bind to tumor cells to facilitate rejection. Upon binding to the IFN-γR on tumor cells, IFN-γ can activate anti-proliferative, pro-apoptotic, and anti-angiogenic signaling cascades that ultimately leads to death of the tumor cell (164). Indirectly, IFN-γ can induce the upregulation of MHC class I and II molecules on the surface of tumor cells, facilitating tumor antigen presentation and recognition by $CD8^+$ T cells.

*TNF-α*

TNF-α is a pleiotropic cytokine with a central role in immune homeostasis, inflammation, and host defense (165).TNF-α is a trimeric type II transmembrane protein but also exists as a soluble protein when cleaved by the TNF-α converting enzyme (TACE). Both membrane-bound and soluble TNF-α interact with two distinct receptors, TNFR1 and TNFR2. Binding of transmembrane TNF-α triggers activation of both receptors, while soluble TNF-α only triggers activation of TNFR1 (165). Depending on the cellular context, binding TNFR can induce diverse effects such as apoptosis, necrosis, angiogenesis, immune cell activation, differentiation, and cell migration (165).

Originally called cachectin, TNF-α was once believed to be strictly a product of macrophages; however, TNF-α is also produced by Th1 cells and in some cases NK cells, neutrophils and mast cells (166). Tumor necrosis factor-α

was first identified as an anti-tumor cytokine that inflicted serious toxicity to tumor cells, hence its name (167). However, the role of TNF-α in tumor immunity is not as simple as its name implies, as TNF-α has anti-tumor and pro-tumor effects. Anti-tumor effects of TNF-α include the ability to trigger apoptotic and necrotic signaling pathways, but it also affects endothelial cell permeability, inducing hyperpermeability in tumor-associated vessels, facilitating the entry of immune cells into the tumor (168). Muller-Hermelink et al. showed that TNF-α in conjunction with IFN-γ was able to inhibit tumor growth and angiogenesis in the RIP-Tag2 tumor model with a mechanism that was not related to apoptosis (169).

It has been recognized for several years that the constitutive production of TNF-α from the tumor microenvironment is a characteristic of many malignant tumors and is often associated with poor prognosis (170). There is evidence that TNF-α plays a role in tumor proliferation, migration, invasion, and angiogenesis. TNF-α increases tumor initiation by the activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) which is essential for transformation of tumor cells (171). TNF-α can also promote tumor cell survival inducing antiapoptotic genes (172). In some cases TNF-α enhances angiogenesis by increasing the angiogeneic factors such as IL-8 and VEGF (173). TNF-α also can enhance invasiveness of tumor cells by inducing MMPs (174).

It has been recently demonstrated that the ubiquitination status of receptor interacting protein serinetheronin kinase 1 (RIP1) determines whether TNF-α will function as a pro-survival (pro-tumor) or a pro-death (anti-tumor) molecule. What determines the state of RIP1 is currently unknown and may prove to be a powerful tool to control the diverse biological activity of TNF-α (175).

TNF-α plays a major role in autoimmune diseases. The anti-TNF-α monoclonal antibody, infliximab, and the soluble TNF receptor, etanercept, have been widely used to treat rheumatoid arthritis, juvenile idiopathic arthritis, spondyloarthropathies, Crohn's disease, and psoriasis (176). Several lines of evidence derived from experimental models have suggested a role for TNF-α in ocular inflammation. For example, it has been shown that TNF-α plays a major role in the pathogenesis of EAU (177-179). In support of this, patients with uveitis have higher than normal TNF-α in their serum and AqH (176). Increased levels have also been found in patients with retinal vasculitis and SO (180). Thus, there is great interest in a drug that specifically targets TNF-α and may represent a therapeutic approach for treating ocular inflammation.

*Macrophages*

Macrophages are widely distributed immune cells that play important roles in both innate and adaptive immunity. Macrophages originate as monocytes,

and monocytes are known originate in the bone marrow from a common myeloid progenitor that is shared with neutrophils. When released from the bone marrow into the peripheral blood, monocytes circulate for several days before entering tissues where they mature into macrophages (181). Macrophages initiate innate immune responses against microbes by recognition of pathogen-associated molecular patterns (PAMPs) by pattern-recognition receptors (PRPs) (182). Macrophages contribute to the balance between antigen presentation to T cells and clearance of pathogens through phagocytosis (183). Macrophage heterogeneity has been well documented and is determined by the genetic background as well as by specific tissue-related and immune related stimuli (181, 184).

Akin to the Th1/Th2 paradigm, macrophages are polarized into two distinct populations. The two major subsets are classically (M1) or alternatively (M2) activated macrophages. Macrophages are polarized into a M1 phenotype in response to IFN-γ or microbial products such as LPS (185). M1 macrophages vary in morphology depending on their tissue location but generally are able to produce significant amounts of proinflammatory cytokines, such as IL-1β, IL-15, IL-18, TNF-α, IL-23, IL-6 and IL-12 (186). They also produce chemokines such as CCL15, CCL20, and CXCL11 which coordinate NK and Th1 cell recruitment (187). M1 macrophages have the ability to kill intracellular pathogens and tumors. This microbial killing and tumorcidial ability is mediated by different

mechanisms including the restriction of iron (and other nutrients) from microorganisms, acidification of the phagosome, production of TNF-α, synthesis of reactive oxygen intermediates (ROI) and release of nitric oxide (NO) (74, 188). Factors such as ROI and NO mediate tumor destruction and cause extensive damage to surrounding tissues (184). To produce NO, M1 macrophages use a macrophage specific IFN-γ-inducible enzyme called inducible nitric oxide synthase (iNOS) which catalyzes L-arginine into NO. The gene that encodes iNOS is *NOS2* and is only expressed by M1 macrophages (189). NO produces cell death by biochemical changes to DNA and nuclear fragmentation, cell shrinkage, membrane blebbing and apoptotic body formation (190). Tumor cells are diverse in their response to NO. Administration of iNOS inhibitors, such as $N_{\omega}$-Nitro-L-arginine methyl ester hydrochloride (L-NAME), to mice promotes the growth of several transplantable tumors (191, 192). By contrast, the production of NO in the human colon adenocarcinoma enhances vascularization and growth in immunodeficient hosts (193). The reason for such diversity in tumor responses to NO lies in the tumor microenvironment. Tumor microenvironments rich in TGF-β and macrophage suppressive factors suppress NOS2 expression (194). Even systemically, macrophages from tumor-bearing mice have lower expression of NOS2 and cytotoxic activity (189, 195).

M1 macrophages also have efficient antigen presentation capacity displaying elevated levels of MHC class II and costimulatory molecules, CD80

and CD86 (74). Due to this increased antigen presentation capacity and the production of IL-12, M1 macrophages effectively drive the polarization and recruitment of Th1 cells and amplify a type I response (181).

M2 is a generic name for various forms of macrophage activation other than M1. M2 macrophages have been described in a number of variants depending on the stimuli used to generate them. All M2 groups are similar in their function and play a critical role in type II immune responses. The various versions of M2 macrophages generally share a IL-$12^{low}$, IL-$23^{low}$, and IL-$10^{high}$ phenotype (186). They generally have high levels of mannose and galactose-type receptors; and metabolize arginine to ornithnine and polyamine which promotes growth. Three subgroups have been described: M2a, M2b, and M2c (186). M2a macrophages are generally thought of as the macrophage population that makes up a majority of tumor associated macrophages (TAM) (196). For simplicity, throughout the remainder of this discussion M2a macrophages will be referred to as M2 macrophages.

M2 activation occurs through stimulation with IL-4, IL-10 and IL-13, which is mainly made by Th2 cells, mast cells, and basophils (197). M2 macrophages play a role in allergy and responses to encapsulated parasites (74). M2 macrophages are conspicuously pro-tumor and produce IL-10, TGF-β, and indoleamine 2,3-dioxigenase (IDO) which suppresses anti-tumor Th1 cells responses and promotes the induction of Treg (198). The production of MMPs

and TGF-β function in matrix remodeling and metastasis. M2 macrophages also produce VEGF, epidermal growth factor (EGF), and platelet derived growth factor (PDGF) to promote angiogenesis (198).

Unlike M1 macrophages, M2 macrophages do not express iNOS, but express high levels of arginase 1 (Arg1), which skews the metabolic pathway of NO to the production of proline (74, 184). This significantly reduces the ability of M2 macrophages to have anti-microbial function (197, 198). There are several molecules that are useful for the identification of M2 macrophages. *ARG1* and the chitinase-like protein, Ym1, are the most strongly up-regulated genes in polarized M2 macrophages (184). Chitinase-like protein are a novel class of cytokines that are soluble mediators of cell differentiation, proliferation, activation, migration and adhesion (184). The role of macrophages in tumor immunity is extremely complicated and deserves considerable future research.

## OBJECTIVES AND RATIONALE FOR RESEARCH

Previous work in the Ad5E1 tumor model determined the mechanism of non-phthisical intraocular tumor rejection (65, 66, 68). An initial observation that a proportion of animals rejected Ad5E1 in a phthisical manner allowed for the characterization of tumor clones that consistently undergo resolution in a phthisical or non-phthisical manner. One of my research objectives is to characterize the immune mechanisms required to elicit phthisical rejection of intraocular Ad5E1 tumors. As mentioned above, other ocular inflammatory diseases, such as uveitis and SO, can result in phthisis. Understanding the immune mechanisms of phthisis may lead to the development of therapies that allow for treatment of the disease or resolution of the tumor without destruction of the eye.

Another objective is to determine the immune mechanisms that lead to non-phthisical rejection in a model that consistently rejects in a non-phthisical manner. Discovering how tumors are rejected in this manner may also have important implications on future treatments for the rejection of intraocular tumors. As mentioned earlier, uveal melanoma is the most common intraocular tumor in adults. Although uveal melanoma is relatively rare compared to other cancer types, it is one of the most lethal. Nearly half of uveal melanoma patients die within 15 years after initial diagnosis due to the high metastatic nature of the disease. Understanding the mechanisms of non-phthisical intraocular tumor

rejection in mouse models may provide the foundation for the development strategies to intraocular tumors in humans while preserving vision.

Another objective of this research project is to understand a new form of ocular immune privilege that allows for the rejection of SC tumors but not AC tumors in the IFN-γ-deficient environment. This form of immunity is dependent upon IL-17-dependent CTLs. However, this cannot occur in the eye due to the immunosuppressive environment of the AC that precludes the generation of the Th17 cells needed for induction of a CTL response in IFN-γ- deficient environments. IL-17 plays an important role in the recruitment of cytotoxic neutrophils. These cells are known to produce a variety of molecules, such as reactive oxygen species, nitric oxide, superoxide, etc, that are destructive to the normal architecture of the eye. This phenomenon may represent a new form of ocular immune privilege where the generation of cytotoxic neutrophils is prevented.

# CHAPTER TWO
# MATERIALS AND METHODS

*Animals*

C57BL/6 (H-$2^b$) mice, CD4 KO mice (B6.129S2-$CD4^{tm1Mak}$/J), IFN-γ KO mice (B6.129S7-$Ifng^{tm1Ts}$/J), severe combined immune deficiency mutation (SCID) (B6.CB17-*Prkdc*$^{scid}$/SzJ) , TNF-α KO mice (B6.129S6-$TNF^{tm1Gk1}$/J), tumor necrosis factor receptor-1 (TNFR1) KO mice (B6.129-$Tnfsf1a^{tm1Mak}$/J), and tumor necrosis factor receptor-2 (TNFR2) KO (B6.129S2.$Tnfr2^{tm1Mwm}$/J) were obtained from The Jackson Laboratory (Bar Harbor, ME). TRAIL KO breeding pairs were kindly provided by Dr. Thomas Griffith (University of Iowa). C57BL/6 Jα$18^{-/-}$ mice were generated as previously described and kindly provided by Masaru Taniguchi, (RIKEN) Research Center for Allergy and Immunology, Yokohama, Japan (199) . C57BL/6 CD1d KO mice were kindly provided by Mark A. Exley, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA, USA. (55) All animals were housed and cared for in accordance with the guidelines of the University of Texas Southwestern Medical Center Committee for the Humane Care of Laboratory Animals, National Institutes of Health Guidelines on Laboratory Animal Welfare, and the Association for Research in Vision and Ophthalmology statement about the Use of Animals in Ophthalmic and Vision Research. For CD4-depleted mice,

C57BL/6 mice were injected i.p. with 500 μg of rat anti-mouse CD4 (GK1.5) on day –2 and the day of injection, and then twice weekly throughout the course of tumor growth. For CD8-depleted mice, C57BL/6 mice were injected i.p. with 500 μg of rat anti-mouse CD8 (YTS169.4) on day –2 and the day of injection, and then twice weekly throughout the course of tumor growth. For IFN-γ depletion, C57BL/6 mice were injected i.p. with 500 μg of rat anti-mouse IFN-γ (HB170) on day –2 and the day of injection, and then twice weekly throughout the course of tumor growth. For IL-17A depletion, C57BL/6 mice were injected i.p. with 500 μg of rat anti-mouse monclonal IL-17A on day –2 and the day of injection, and then twice weekly throughout the course of tumor growth. Monoclonal Abs were isolated from hybridoma cultures and affinity purified. Rat IgG was purchased from Sigma-Aldrich (St. Louis, MO).

*Tumor cells*

Ad5E1 tumor cells were kindly provided by Dr. Rene E.M.Toes (Leiden University Medical Center). The tumor cells were generated by the transformation of C57BL/6 mouse embryo cells with a plasmid encoding the human adenovirus type 5 early region 1 (Ad5E1) and propagated as previously described (55). Single-cell suspensions of Ad5E1 tumor cells were washed in Hanks' Balanced Salt Solution (HBSS; Cambrex, East Rutherford, NJ) and

suspended in HBSS for anterior chamber (AC) injections. Tumor cells were cultured in Dulbecco's modified Eagle's medium (DMEM; GibcoBRL, Grand Island, NY) containing 10% heat-inactivated fetal calf serum, 1% L-glutamine, 1% sodium pyruvate, 1% nonessential amino acids, 1% HEPES buffer, and 1% antibiotic–antimycotic solution (GibcoBRL, Grand Island, NY).

*Identification of tumor clones that undergo non-phthisical and phthisical rejection*

I suspected that the original Ad5E1 tumor cultures contained subpopulations that underwent phthisical, T cell-dependent immune rejection in C57BL/6 mice. Accordingly, monoclonal cell cultures were established from the parental Ad5E1 tumor cells by isolating single cells from bulk cultures using a MoFlo XDP cell sorter (Beckman Coulter, Fullerton, CA). Monoclonal cell cultures of Ad5E1 were established, expanded, and were screened for their rejection following transplantation into the AC of normal C57BL/6 mice. Several clones were found to consistently undergo non-phthisical or phthisical rejection in C57BL/6 mice. One clone, designated as clone 2.1 of Ad5E1, was used to study phthisical rejection. Another clone, Ad5E1 clone 4, was chosen for the study of non-phthisical rejection.

*Ocular cells*

A C57BL/6 corneal endothelial cell line was established and immortalized with human papilloma virus genes E6 and E7 using the disabled recombinant retroviral vector pLXSN16E6/E7 as previously described (200). Corneal endothelial cells were cultured in DMEM as described above. Iris/ciliary body (I/CB) cells were isolated from the eyes of C57BL/6 mice and cultures were established as previously described (201). I/CB cells were not immortalized and were cultured in complete RPMI 1640 medium containing the same additives described above for DMEM.

*Intraocular tumor cell injections*

Tumor cell suspensions were injected into the AC as previously described (17). Mice were anesthetized with 0.66 mg/kg of ketamine hydrochloride (Vetalar; Parke-Davis and Co., Detroit, MI) given i.p. The eye was viewed by low power (x8) 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 (diameter ~80 microns) was fitted onto a sterile infant feeding tube (5 French; Tyco Healthcare Group, Mansfield, MA) and mounted onto a 0.1-ml Hamilton syringe (Hamilton, Whittier, CA). A Hamilton automatic dispensing apparatus was used to inject 6 μl of a monocellular suspension of Ad5E1 tumor cells ($3 \times 10^5$ cells/6 μl or $5 \times 10^4$ cells/6 μl). Eyes

were examined three times per week, and the tumor volume was recorded as the percentage of AC occupied with tumor (17).

*Histology*

Tumor-containing eyes were removed from euthanized mice, fixed in formalin, embedded in paraffin, and cut into 5-μm sections. Eye sections were stained with H&E to examine tumor pathology.

*Tumor cells proliferation assay*

Ad5E1 tumor clone cells ($1x10^5$ cells/ml) were added to 24-well plates (3526; Corning Inc.). Cells were incubated for 48 h or 72 h at 37ºC, then pulsed with 2 μCi of $^3$H-thymidine (PerkinElmer, Boston MA) and incubated for an additional 12 h. Wells were washed with phosphate-buffered saline (PBS) and the contents were then solubilized with 10% sodium dodecyl sulfate and the radioactivity counted in a liquid scintillation counter (LS 6500 multi-purpose scintillation counter; Beckman Coulter, Fullerton, CA). All tests were performed in triplicate.

*Flow cytometric analysis*

Surface expression of IFN-γR, TNFR, TRAILR, and Fas were assessed by flow cytometry. Intracellular expression of HMGB-1 was also assessed by flow

cytomtery. After washing 3 x with HBSS, cells were maintained in HBSS containing 0.3% BSA. Cells ($1x10^6$) were incubated with 1 μg/ml of purified anti-mouse molecule-specific antibody for 30 min. at 4ºC. Cells were washed 3 x with HBSS containing 0.3% BSA. Cells for HMGB-1 staining were fixed and permeablized prior to staining. After washing, cells were resuspended in 0.5 ml PBS and assessed for fluorescence in a FACScan flow cytometer (BD Biosciences, Palo Alto, CA), and the results were analyzed using CellQuest version 3.1f software (BD Biosciences).

*Adoptive transfer experiments*

C57BL/6 mice were injected in the AC with Ad5E1 tumors as described above. Upon rejection of intraocular tumors in wild-type or TNF-α KO mice, animals were euthanized, splenocytes collected, and erythrocytes were lysed. T cells isolated by incubation with $CD4^+$ T cell-specific microbeads (10-μl beads/$10^7$ cells) or a Pan T cell isolation kit in 0.5% BSA in PBS for 15 min. in the refrigerator. The cells were washed with 0.5% BSA in PBS followed by magnetic separation using LS+ columns as described by the manufacturer. The retained cells were eluted from the column. Cells were washed 3x and resuspended in HBSS, and injected IV into SCID mice using a 1:1 donor recipient ratio (~$1x10^7$ cells/mouse). Following adoptive cell transfer, recipient mice were injected in the AC with Ad5E1 tumors as described above.

*Liposome-encapsulated dichloromethylene diphosphonate (clodronate)*

Multilamellar liposomes were prepared as described earlier (202). Briefly, 8 mg cholesterol and 86 mg phosphatidylcholine (Sigma) were dissolved in 10 ml of chloroform (Sigma) in a round-bottomed flask. After low-vacuum rotary evaporation at 37°C, a thin film was formed on the inner surface of the flask. This film was then dispersed by gentle rotation for 10 minutes in PBS for the preparation of PBS-containing liposomes (PBS-LIP). For clodronate liposomes (C12MDP-LIP), 2.5 g clodronate (C12MDP; Roche, Mannheim, Germany) was dissolved in 10 ml PBS. The suspension was kept for 2 hours at room temperature and sonicated for 3 minutes at 20°C. To remove free C12MDP, the liposomes were washed twice by centrifugation in PBS at 100,000g for 30 minutes and resuspended in 4 ml of PBS that contained approximately 20 mg of C12MDP. Each 100 μl of C12MDP-LIP suspension contained 1 mg of C12MDP. The cytotoxicity of C12MDP-LIP and PBS-LIP was tested using an *in vitro* toxicity assay with RAW 264.7 macrophages as target cells. C12MDP-LIP (100 μl) typically induced 85-90% cytotoxicity of $1x10^5$ RAW 264.7 cells within 24 hours. Liposomes were used immediately, and were stored at 4°C for up to one month, after which liposomes were discarded.

*Macrophage Depletion*

Previous studies have shown that subconjunctival injection of C12MDP-LIP (clodronate-containing liposomes) (Sigma) induces the elimination of >95% of the conjunctival macrophages (203) and >99% depletion of $F4/80^{+}$ macrophages that infiltrate intraocular Ad5E1 tumors (15). Depletion of macrophages with C12MDP-12 has demonstrated the role of ocular macrophages in intraocular tumor rejection (67, 68) and corneal allograft rejection (204, 205). Multilamellar liposomes were prepared as described earlier (202). Under an operating microscope, the conjunctiva was lifted and the C12MDP-LIP suspension (8 μL) was injected into the bulbar conjunctiva using a 30-gauge needle mounted on a 1-ml tuberculin syringe. Injection of the C12MDP-LIP suspension resulted in a bleb around the injection site. To obtain a more equal distribution of the suspension around the limbus, the dose was divided by injecting at four different sites 90º apart around the limbus until a circular conjunctival bleb was obtained. PBS-LIP was used as a negative control for macrophage depletion. Liposome injections were performed on the day of tumor injection and repeated every 3-4 days throughout tumor observation (68).

*Quantitative Real-time PCR*

Expression of NOS2, Ym1, Arg1, F4/80, CD11b, TNF-α, HMGB-1, IL-6, IL-17, CD8, and perforin mRNA was assessed by quantitative real-time PCR by

MyiQ Single-Color Real-Time PCR Detection system (Bio-Rad). Briefly, 1 μg of total RNA was converted into first-strand cDNA using $RT^2$ First Strand Kit (SA Biosciences) according to the manufacturer's conditions. The PCR amplification reactions contained 1.0 μl of first-strand cDNA mixed with 12.5 μl of $RT^2$ qPCR Master Mix (SA Biosciences), 10.5 μl $ddH_2O$, and $RT^2$ qPCR primers (SA Biosciences) in a final reaction volume of 25 μl. All reactions were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and performed in duplicate. The PCR profile consisted of an initial denaturation of 10 min. at 95 °C, 40 cycles of 15 s at 95 °C denaturing, and 60 s at 60 °C annealing. A dissociation (melting) curve was performed to insure proper quality control for each sample.

*Isolation of Bone Marrow-Derived Macrophages (BMDMs)*

BMDMs were isolated as described previously (206). Bone marrow cells were obtained from the femurs of C57BL/6 mice and cultured with recombinant murine macrophage-colony stimulating factor (rm-M-CSF; 10 ng/ml; R&D Systems) for 7 days.

*Bone Marrow- Derived Macrophage-Mediated Cytotoxicity Assay*

A macrophage-mediated cytotoxicity assay utilizing BMDMs was performed as previously described (207). Briefly, BMDMs were plated 1 x

$10^5$/well in 96-well flat bottom plates and incubated with medium alone or medium containing rmIFN-γ (10U/ml; R&D Systems) plus LPS (10 ng/ml; R&D Systems) for 24 hours. Ad5E1 tumor cells, iris and ciliary body cells, or corneal endothelial cells (target cells) were incubated with 0.2 μCi/ml of $^3$H-thymidine (MP Biomedicals) for 24 hours at an effector to target (E:T) ratio of 10:1. Corneal endothelial cells were prepared as previously described (208). Iris and ciliary body cells were isolated from the irises of C57BL/6 mice and cultured in complete RPMI. Target cells were plated with 1 x $10^5$ resting or activated macrophages (1:10). Cells were incubated for 48 or 72 hours and cultures were washed twice with PBS. Cells were harvested with a Combi Cell Harvester (SKATRON, Lier, Norway) according to manufacturer's instructions. Radioactivity was measured in a liquid scintillation counter. Cytotoxicity was calculated use the following formula: percent cytotoxicity = ((A-B)/A) x 100, where A represents CPM of tumor cells cultured alone, and B represents the CPM in test cultures.

*Inhibition of iNOS with L-NAME*

To inhibit iNOS, the compound L-$N^G$-nitroarginine methyl ester (hydrochloride) (L-NAME) (Cayman Chemical, Ann Arbor, MI) was used in both *in vitro* and *in vivo* assays. Final concentrations of 1 mM and 5 mM L-NAME were used in the *in vitro* BMDM-mediated tumor killing assays (209, 210). As a

control, the biologically inactive isomer, D-$N^G$-nitroarginine methyl ester (hydrochloride) (D-NAME) (Sigma), was used at the same concentrations as L-NAME. To inhibit iNOS *in vivo*, C57BL/6 mice were treated with 50 mg/kg L-NAME daily given i.p. (211, 212). D-NAME was used as a negative control in these *in vivo* studies.

*TNF-α cytotoxicity assay*

TNF-α-induced cytotoxicity was evaluated using the CytoTox96 (Promega). Single-cell suspensions of Ad5E1 clone 2.1 tumor cells ($1x10^5$ cells/ml), I/CB cells, or corneal endothelial cells were added to 24-well plates (3526; Corning Inc., Corning, NY). Cells were suspended in either medium alone or medium containing various concentrations of murine TNF-α (1, 10, or 100 ng/ml). Cultures were incubated for 48h at 37ºC. Following incubation, culture medium was removed and LDH was measured according to manufacturer's instructions.

*Antigen presenting cell (APC) isolation*

APCs were obtained by mincing spleens from naïve animals and incubating them with 1mg/ml collagenase D (Roche, Indianapolis, IN) at 37ºC for 30 min. Cells were plated on Primaria tissue culture dishes (BD Biosciences) and incubated for 2 hours at 37ºC. Non-adherent cells were aspirated and the plates

were vigorously washed, leaving APCs (adherent macrophages and dendritic cells). APCs were incubated with tumor antigens (TA) from freeze-thawed and sonicated Ad5E1 tumor cells for 24 hours and used as stimulator cells for *in vitro* assays.

*In vitro stimulation of T cells to produce TNF-α*

T cells were isolated from draining lymph nodes of Ad5E1 clone 2.1 tumor rejector mice using CD4 (L3T4) MicroBeads (Miltenyi Biotec) and incubated with either medium alone, tumor antigen–pulsed antigen-presenting cells (APC) or anti-CD3/ CD28 beads (25 μl/ml; Invitrogen) for 5 days at 37° C as previously described. Supernatants from T-cell cultures were harvested and the concentration of TNF-α was determined using a mouse TNF-α Quantikine ELISA kit (R&D Systems).

*Microarray analysis*

Ad5E1 clone 2.1 or clone 4 tumor cells were incubated for 24 hr in medium alone or in medium containing either 20 U/ml recombinant murine IFN-γ or in medium containing 1 ng/ml recombinant murine TNF-α. RNA was harvested and submitted to the UT Southwestern Medical Center Microarray Core Facility. Preparation of cDNA, and subsequent procedures, including sample hybridization and scanning of the MouseWG-6 v2.0 Array (Illumina, Inc., San

Diego, CA), were performed by the UT Southwestern Medical Center Microarray Core Facility. Analysis was done by the Gene Set Enrichment Analysis (GSEA) program developed by the Broad Institute of MIT and Harvard University (213, 214).

*Subcutaneous tumor cell injections*

Tumor cell suspensions ($5 \times 10^4$/100 μl) were injected subcutaneously in flank. Inoculations sites were palpated three times per week to assess SC tumor growth. Tumor size was measured using calipers and tumor volumes ($mm^3$) were estimated from the dimensions of the tumor with the following formula: volume = $AB^2/2$, in which A was the length and B the width of the tumor.

*IL-17 cytotoxicity assay*

The possibility of IL-17-induced cytotoxicity was evaluated using the CytoTox96 (Promega). Single-cell suspensions of Ad5E1 clone 2.1 tumor cells ($1 \times 10^5$ cells/ml) were added to 24-well plates (3526; Corning Inc., Corning, NY). Cells were suspended in either medium alone or medium containing various concentrations of murine IL-17 (10, 100, 500 or 1000 ng/ml). Cultures were incubated for 48h at 37ºC. Following incubation, culture medium was removed and LDH was measured according to manufacturer's instructions.

*Mixed lymphocyte-tumor cell (MLTC) culture*

Splenocytes (30 x $10^6$) from either immunized or naïve mice were stimulated with 5 x$10^6$ mitomycin-C treated tumor cells in complete RPMI 1640 medium (BioWhittaker) containing 10% heat-inactivated FBS (HyClone), as previously described (215). Cells were cultured for 5 days at 37°C. Restimulated lymphocytes were harvested, washed twice, and tested for cytolytic activity.

*CTL assay*

A standard 4-h $^{51}$Cr release assay, as previously described (216), was used to measure CTL activity *in vitro*. Briefly, single-cell suspensions of lymphocytes in complete RPMI 1640 medium (BioWhittaker) containing 10% heat-inactivated FBS (HyClone) were prepared from various spleens and used as effector cells. Experimental and control effector lymphocytes were boosted *in vitro* for 120 h at 37°C with mitomycin C-treated Ad5E1 tumor cells or mitomycin C-treated P815 tumor cells. The *in vitro* boosted effector cells were washed and resuspended in complete RPMI medium. Effector cells were dispensed along with 2 x $10^4$ $^{51}$Cr-labeled Ad5E1 or P815 cells/well in triplicate at several E:T ratios (100:1, 50:1, 25:1, and 12.5:1) in a 96-well U-bottom microtiter plate (Corning), in a total volume of 200 µl/well. Tumor cells were also incubated alone (spontaneous release) or with 50 µl of Zapoglobin (Beckman Coulter) lytic reagent (total release). The plate was incubated at 37°C for 4 h. The plate was then centrifuged

at 800 rpm for 6 min. before harvesting 100 µl of the supernatant from each well and counting on a gamma counter. Cytotoxicity was determined by the amount of $^{51}Cr$ released by the target cells, and the specific lysis was calculated as follows: [(experimental cpm) – (spontaneous release cpm)] ÷ [(maximum release cpm) – (spontaneous release cpm)] x 100%.

*In vitro stimulation of T cells and IL-17 ELISA*

Tumor-bearing CD4-depleted IFN-γ animals and non-tumor-bearing isotype IFN-γ mice were killed and spleens were obtained. $CD4^+$ T cells were isolated from spleens using mouse CD4 microbeads and magnetic cell sorting. T cells ($1 \times 10^6$) were incubated alone (negative control) or with 10 µl of Dynabeads Mouse T-Activator CD3/CD28 (Invitrogen). Cells were incubated for 120 h at 37°C. Supernatants were harvested and levels of IL-17 in cell supernatants were determined using a mouse IL-17 Quantikine ELISA kit (R&D Systems).

*Statistics*

Student's *t*-test or a $\chi^2$ test was used to assess the statistical significance of the differences between experimental and control groups. A *P*-value of $<0.05$ was considered significant.

# CHAPTER THREE

# RESULTS

## I. IDENTIFICATION AND CHARACTERIZATION OF AD5E1 TUMOR CLONES THAT CONSISTENTLY UNDERGO REJECTION WITH A PHTHISICAL OR NON-PHTHSICAL PHENOTYPE

In order to study immune-mediated rejection of intraocular Ad5E1 tumors, tumor cell clones were generated that consistently underwent rejection in a phthisical or non-phthisical manner. Tumor cell clones were selected based on the phenotype of the intraocular rejection. This was accomplished by establishing monoclonal cell cultures from the parental Ad5E1 tumor cells by isolating single cells from bulk cultures using cell sorting. Monoclonal cell cultures of Ad5E1 were established, expanded, and were screened for their rejection following transplantation into the AC of normal C57BL/6 mice. Animals were screened by the injection of $3 \times 10^5$ tumor cells in AC and the pattern of rejection was observed. Several clones were found to consistently undergo phthisical and non-phthisical rejection in C57BL/6 mice. One of these, designated as clone 2.1 of Ad5E1, consistently underwent phthisical rejection in approximately 80% of animals observed. Ad5E1 clone 2.1 typically occupies approximately 60-75% of the AC before undergoing phthisical rejection which, as mentioned above,

destroys the eye (Figure 2 and Figure 3). Phthisical rejection produced extensive necrosis that culminated in atrophy of the eye. In these eyes the anterior chamber was compressed, the corneal was opaque and vascularized, and only the lens remained intact. The clone chosen to study non-phthisical rejection was designated as Ad5E1 clone 4 and underwent non-phthisical rejection in all eyes observed. Growth of Ad5E1 4 tumors typically occupied 35- 40% of the AC before undergoing non-phthisical rejection, in which the eye has normal architecture and is nearly identical to an untouched eye (Figure 2 and 3).

**Figure 2. Intraocular tumor growth in C57BL/6 of Ad5E1 clones 2.1 and 4.** Ad5E1 tumor cells of each clone ($3x10^5$cells/6μl) were injected into the AC on day 0. Tumor growth was scored as the percentage of AC occupied by tumor. Clones were initially screened in 10 mice; however, this has been repeated approximately 20 times with consistent results.

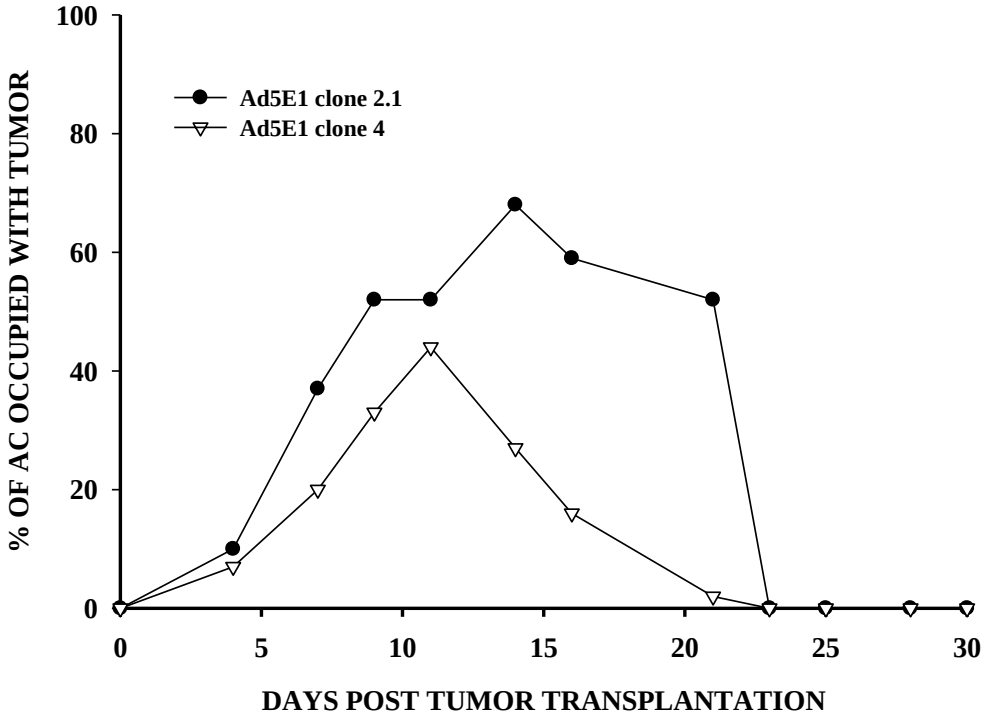
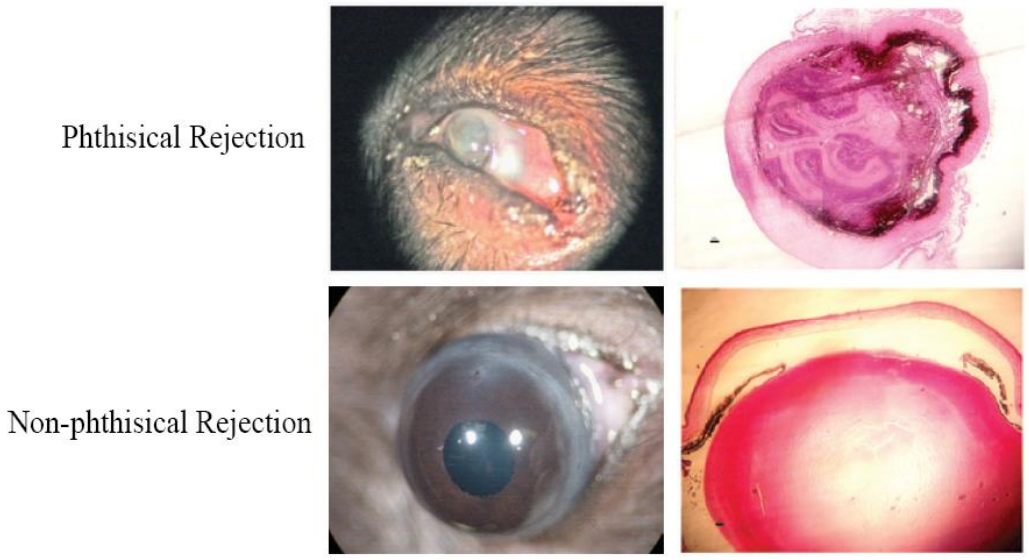


**Figure 3. Phenotype of phthisical and non-phthisical rejection.** Clinical photos (left) were taken of the phthisical rejection of Ad5E1 clone 2.1 (upper) or non-phthisical rejection of Ad5E1 clone 4 tumors (lower). Histology photos (right) were also taken of phthisical and non-phthisical rejection.



*In vitro growth of Ad5E1 tumor clones*

Due to the difference in the percentage of AC occupied during the tumor growth, I sought to determine if the difference in AC tumor size could be accounted for by increased cell proliferation of Ad5E1 clone 2.1. To determine this, single-cell suspensions of Ad5E1 clone 2.1 and Ad5E1 clone 4 cells ($1x10^5$ cells/ml) were added to 24-well plates. Cells were incubated for 24 h or 48 h with the addition of 100 µCi of $^3$H-thymidine during the last 12 h of incubation. Wells were washed with phosphate-buffered saline (PBS) and the contents were then solubilized with 10% sodium dodecyl sulfate and the radioactivity counted in a liquid scintillation counter. There was not a significant difference in cell proliferation at either 24 h or 48 h (Figure 4), thus increased cell proliferation does not account for the increased growth of Ad5E1 clone 2.1 in the eye.

*T cells are required for phthisical and non-phthisical rejection*

In order to address the question that tumor rejection is immune-mediated and not a byproduct of physiological constraints such as inadequate nutrient supply, Ad5E1 tumor clones were transplanted into the eyes of T cell-deficient SCID mice. The inability of immune deficient SCID mice to reject either Ad5E1 tumor clones demonstrates that rejection was an immune-mediated process that required T cells (Figure 5).

**Figure 4. Both Ad5E1 clone 2.1 and clone 4 tumor cells proliferate at similar rates *in vitro*.** Ad5E1 clone 2.1 and 4 cells ($1x10^5$ cells/ml) were added to 24-well plates. Cells were incubated for 24 h or 48 h with the addition of 100 µCi of $^3$H-thymidine during the last 12 h of incubation. Radioactivity (CPM) was counted in a liquid scintillation counter. The differences in growth between Ad5E1 clone 2.1 and clone 4 tumor cells were not significantly different at either time point ($P > 0.05$).

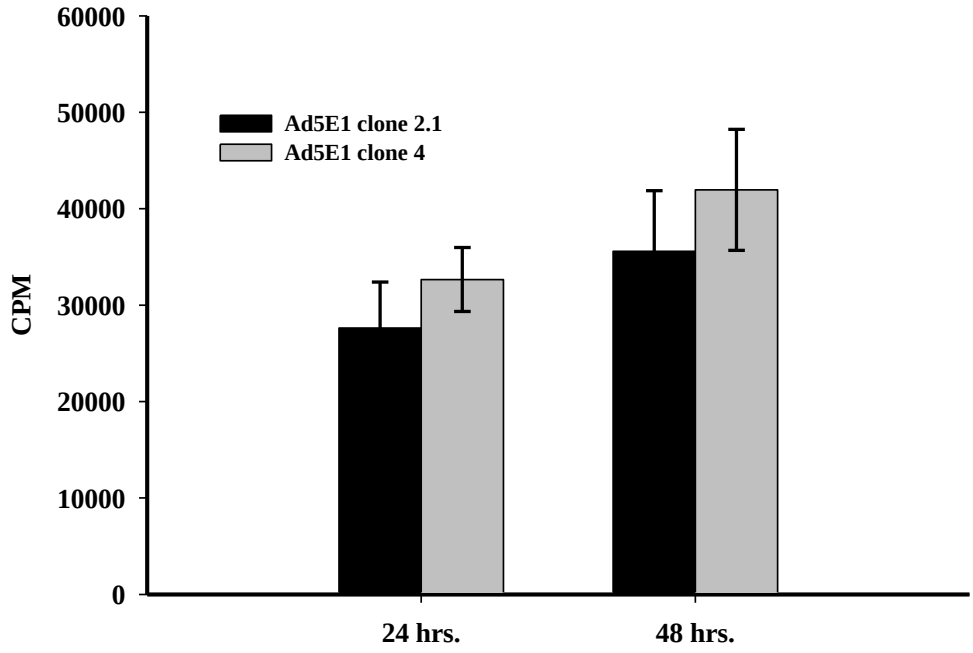
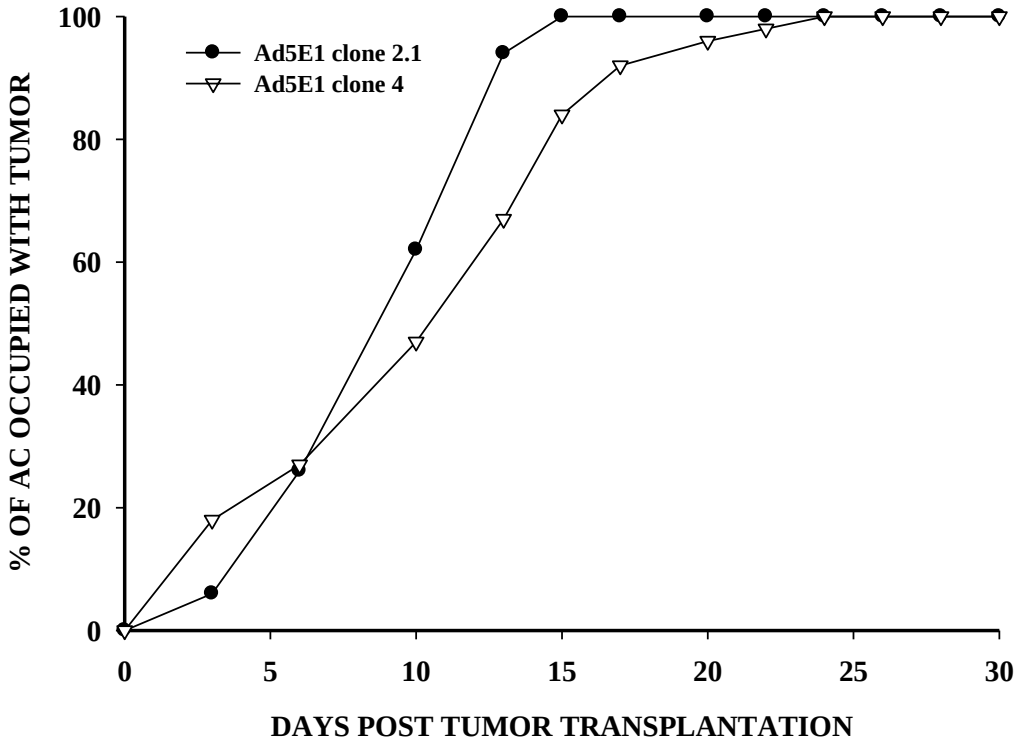


**Figure 5. Rejection of both Ad5E1 clone 2.1 and clone 4 tumors is immune-mediated and requires T cells.** Ad5E1 clone 2.1 or clone 4 cells ($3 \times 10^5$ cells/6μl) were injected into the AC on day 0. Tumor growth was scored as the percentage of AC occupied by tumor. Graph represents the combined results of three independent experiments (N=5/ group/ experiment for a total number of 15 mice per group).

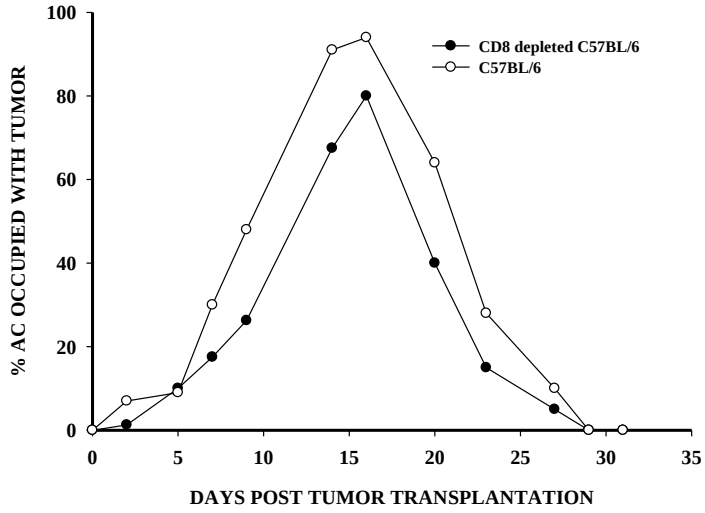


*Rejection of Ad5E1 tumor clones can be mediated by either $CD4^+$ or $CD8^+$ T cells*

The requirement of T cells led me to determine the type of T cells that were necessary for rejection. It would make sense that such different patterns of rejection may be mediated by different types of T cells. For example, in many non-phthisical models, the predominant T effector cells are $CD8^+$ T cells (42, 217). This brought up the hypothesis that non-phthisical rejection may require $CD8^+$ T cells and phthisical rejection may rely primarily on $CD4^+$ T cells that may mediate rejection by recruitment of proinflammatory cells such as M1 macrophages. To test the hypothesis that rejection requires $CD8^+$ T cells, C57BL/6 mice were depleted of $CD8^+$ T cells using anti-CD8 antibody prior to AC injection of the tumor. Similarly, to test the requirement of $CD4^+$ T cells for rejection, tumor clones were injected into the AC of CD4 KO C57BL/6 mice. Rejection of Ad5E1 clone 2.1 tumors did not exclusively rely on either $CD8^+$ or $CD4^+$ T cells as AC tumors were rejected in a phthisical manner in both CD8-depleted and CD4 KO mice (Figure 6). However, Ad5E1 clone 2.1 tumors failed to reject in CD8-depleted CD4 KO mice (data not shown), demonstrating that either T cell type was sufficient for rejection. In a similar fashion, rejection of Ad5E1 clone 4 tumors also did not exclusively depend on either $CD8^+$ or $CD4^+$ T cells, as either population were sufficient to mediate rejection (Figure 7). Ad5E1

**Figure 6. Rejection of Ad5E1 clone 2.1 intraocular tumors can be mediated by either $CD8^+$ or $CD4^+$ T cells.** A. C57BL/6 mice were depleted of $CD8^+$ T cells or were untreated prior to AC injection of 3 x $10^5$ Ad5E1 clone 2.1 tumor cells. B. C57BL/6 CD4 KO or WT mice were injected with 3 x $10^5$ Ad5E1 clone 2.1 tumor cells. Tumor growth was scored as the percentage of AC occupied by tumor. N = 15, combined results of three independent experiments.

A.




B.

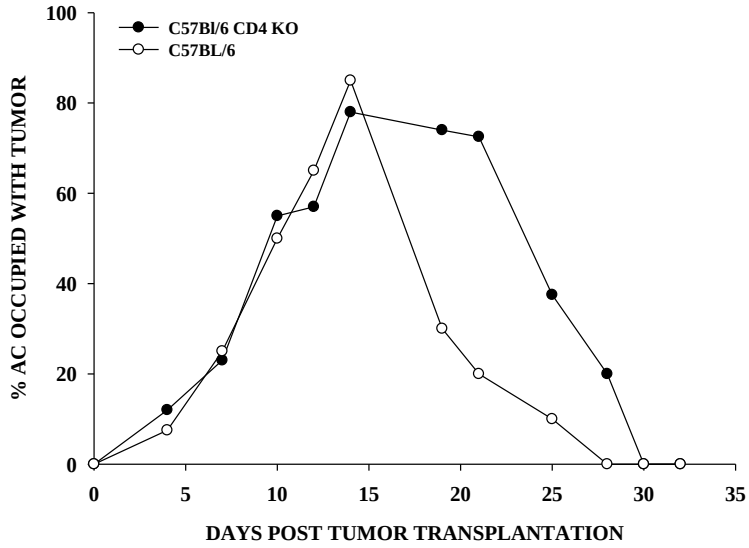
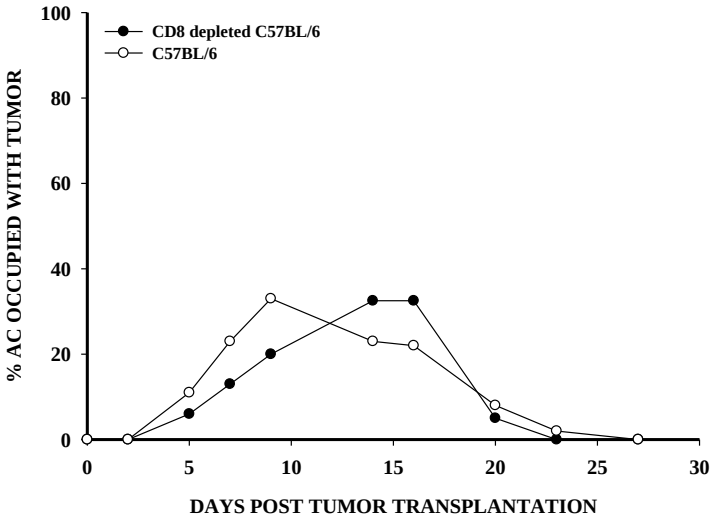
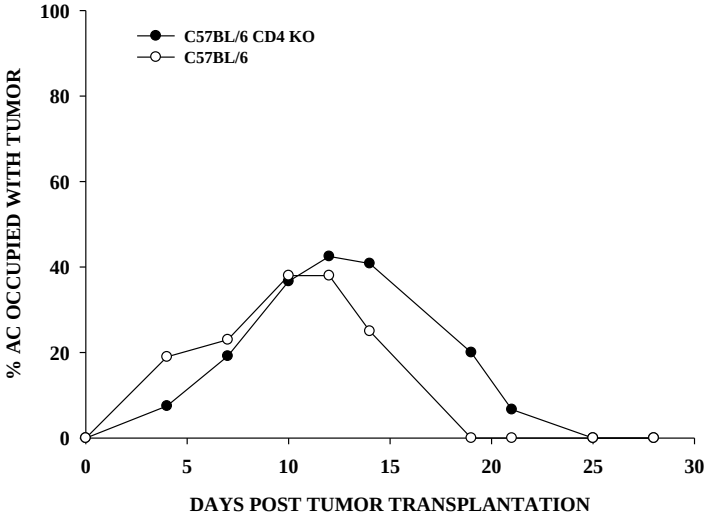


**Figure 7. Rejection of Ad5E1 clone 4 tumors can be mediated by either $CD8^+$ or $CD4^+$ T cells.** A. C57BL/6 mice were depleted of $CD8^+$ T cells or were untreated prior to AC injection of 3 x $10^5$ Ad5E1 clone 4 cells. B. C57BL/6 CD4 KO or WT mice were injected with 3 x $10^5$ Ad5E1 clone 4 tumors cells. Tumor growth was scored as the percentage of AC occupied by tumor. N = 15, combined results of three independent experiments.

A.




B.



clone 4 tumors, however, were not rejected in CD8-depleted CD4 KO mice (data not shown).

*Rejection of Ad5E1 clone 2.1 tumors requires IFN-γ, however, rejection of Ad5E1 clone 4 tumors is IFN-γ-independent*

Previous results indicated that rejection of the parental Ad5E1 tumor line required IFN-γ (64). In order to establish the requirement of IFN-γ for rejection of Ad5E1 clone 2.1 and clone 4 tumors, IFN-γ KO mice were injected in the AC with each tumor clone cell line. The requirement of IFN-γ was confirmed by progressive intraocular growth of Ad5E1 clone 2.1 tumors in IFN-γ KO mice (Figure 8). However, Ad5E1 clone 4 tumors are rejected in the same manner and tempo in IFN-γ KO mice as in WT C57BL/6 mice (Figure 9). Thus, the rejection of Ad5E1 clone 2.1 tumors requires IFN-γ but rejection of Ad5E1 clone 4 tumors does not. I then hypothesized that altered requirement of IFN-γ for rejection of Ad5E1 clone 4 tumors may be due to a differential expression of the IFN-γR. To test this hypothesis both Ad5E1 tumor clones were assessed for their level of IFN-γR expression by flow cytometric analysis. Both tumor clones expressed similar levels of IFN-γR (Figure 10).

*Both phthisical and non-phthisical intraocular Ad5E1 tumor rejection requires macrophages*

Although a recent emphasis has been placed on tumor promoting macrophages, the role of macrophages in tumor rejection has been firmly established (218). Previous studies have reported that rejection of parental Ad5E1 intraocular tumors requires macrophages (67, 68); however, the role of macrophages in phthisical and non-phthisical intraocular tumor rejection has not been examined. To determine whether macrophages were required for phthisical and non-phthisical rejection of Ad5E1 tumor clones macrophages were depleted locally through the subconjunctival injections of clodronate liposomes prior to AC tumor injection. C57BL/6 mice were injected in the AC with $3 \times 10^5$ Ad5E1 clone 2.1 or clone 4 tumor cells. Following tumor injection, two groups of mice were injected subconjunctivally with either clodronate-containing or PBS-containing liposomes, respectively. Liposome injection was repeated every 3-4 days. Depletion of macrophages in eyes injected with Ad5E1 clone 2.1 tumor cells prevented tumor rejection, indicating that macrophages were associated with phthisical rejection of intraocular Ad5E1 clone 2.1 tumors (Figure 11). By contrast, tumor rejection proceeded unabatedly in mice treated with PBS-containing liposomes.

Similarly, depletion of macrophages also prevented the rejection of Ad5E1 clone 4 tumors (Figure 12). As before, tumor rejection occurred in mice treated with PBS-containing liposomes. Thus macrophages are associated with both phthisical and non-phthisical intraocular tumor rejection.

**Figure 8. Rejection of Ad5E1 clone 2.1 tumors requires IFN-γ.** C57BL/6 IFN-γ KO or WT mice were AC injected with 3 x $10^5$ Ad5E1 clone 2.1 tumor cells. Tumor growth was scored as the percentage of AC occupied by tumor. N = 15 per group, combined results of three independent experiments.

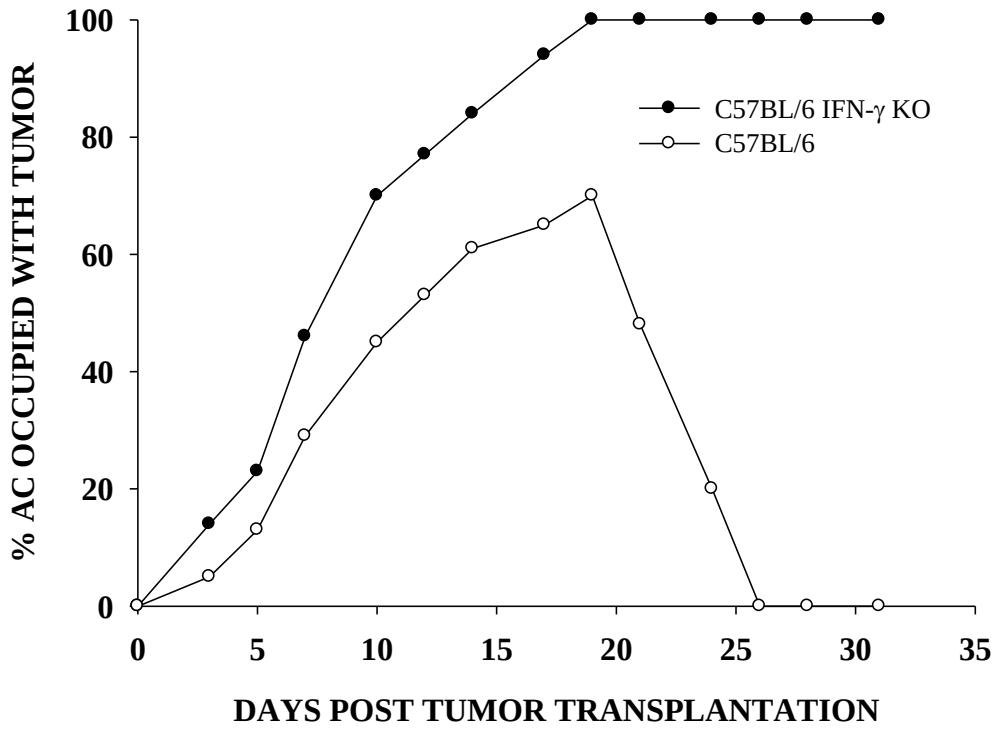


**Figure 9. Rejection of Ad5E1 clone 4 tumors does not require IFN-γ.** C57BL/6 IFN-γ KO or WT mice were AC injected with 3 x $10^5$ Ad5E1 clone 4 tumor cells. Tumor growth was scored as the percentage of AC occupied by tumor. N = 15/ group; combined results of three independent experiments.

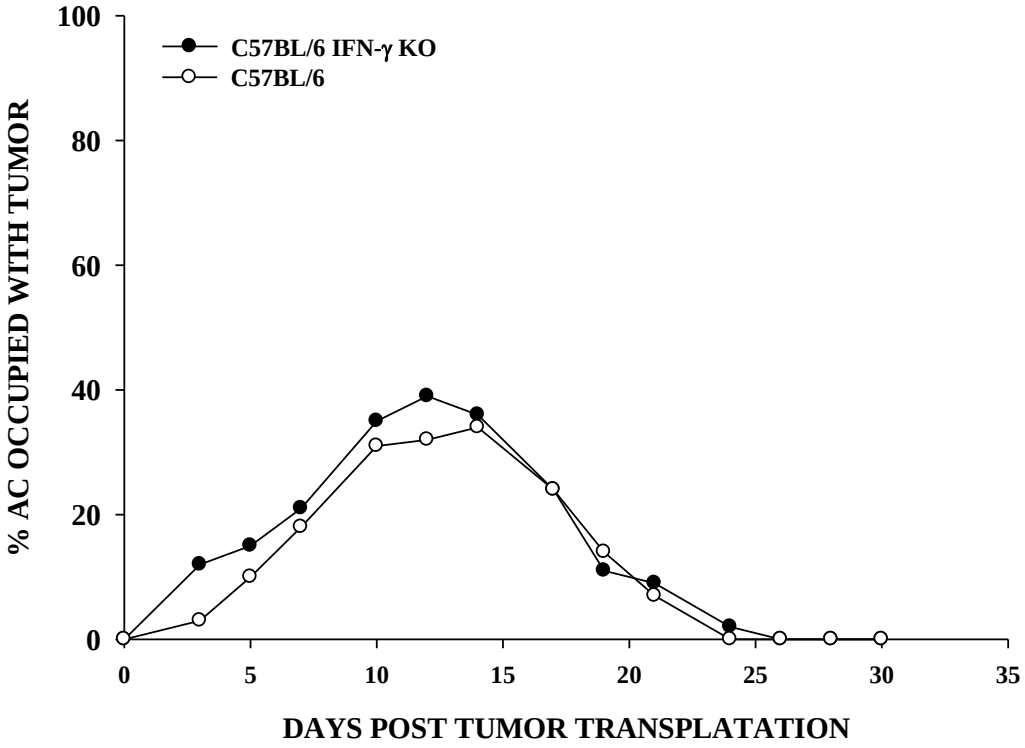


**Figure 10. Murine IFN-γ receptor expression on Ad5E1 tumor cells.** Ad5E1 clone 2.1 and clone 4 tumor cells were stained with either anti-IFN-γ receptor (open histogram) or an isotype control Ab (shaded histogram) and evaluated by flow cytometry.

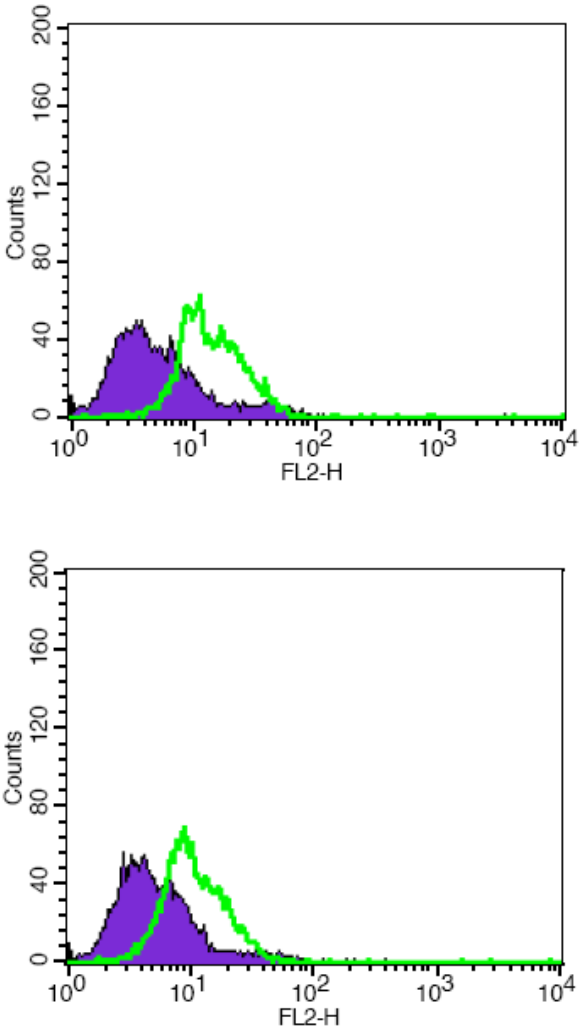


**Figure 11. Macrophages are necessary for rejection of intraocular Ad5E1 clone 2.1 tumors.** C57BL/6 mice were injected in the AC with $3x10^5$ Ad5E1 clone 2.1 tumor cells. Following tumor injection, some mice were injected SCJ with clodronate liposomes or PBS liposomes every 3-4 days. Ad5E1 tumors were rejected in naïve (N = 10) and PBS liposome-treated mice (N = 10), but grew progressively in clodronate liposome-treated mice (N = 10). This experiment was repeated with similar results.

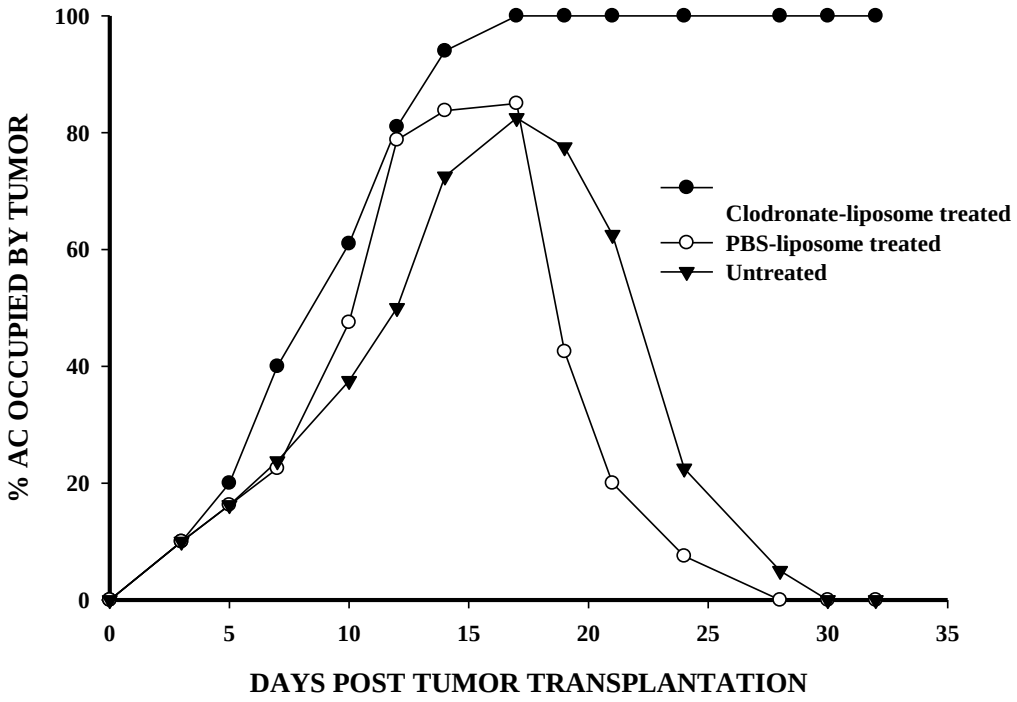
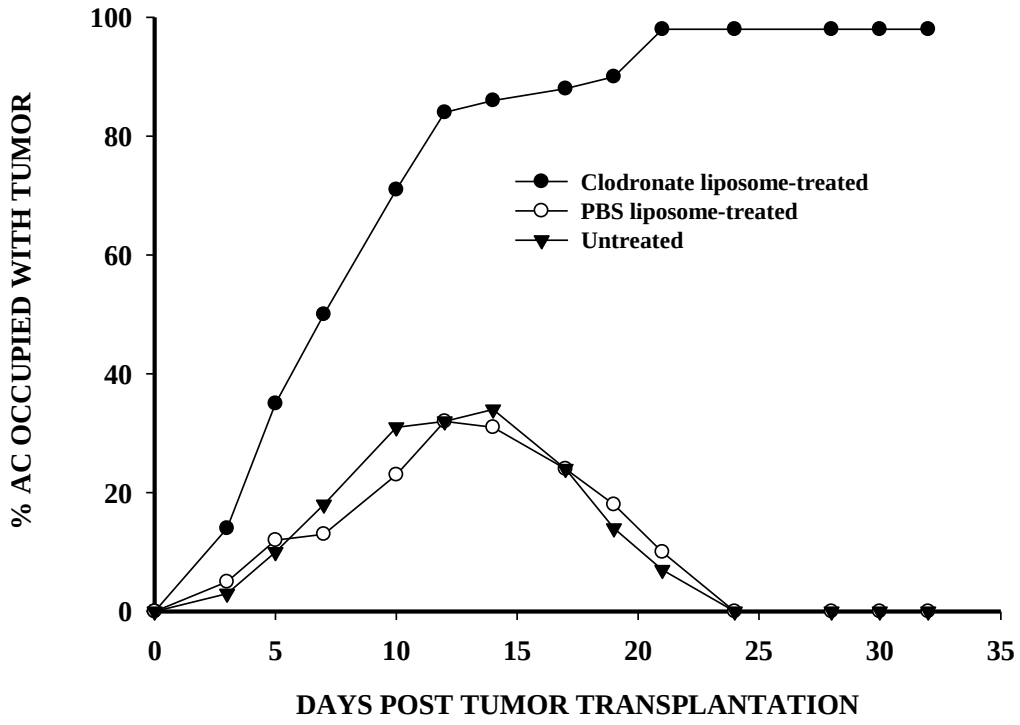


**Figure 12. Macrophages are necessary for rejection of intraocular Ad5E1 clone 4 tumors.** C57BL/6 mice were injected in the AC with $3x10^5$ Ad5E1 clone 4 tumor cells. Following tumor injection, some mice were injected SCJ with clodronate liposomes or PBS liposomes every 3-4 days. Ad5E1 tumors were rejected in naïve (N = 10) and PBS liposome-treated mice (N = 10), but grew progressively in clodronate liposome-treated mice (N = 10). This experiment was repeated with similar results.



*Ad5E1 tumors are susceptible to macrophage-mediated killing*

Direct *in vitro* killing of tumor cells by M1 macrophages has been reported by other investigators (31). To determine whether Ad5E1 tumor cells were susceptible to direct macrophage-mediated killing, *in vitro* cytotoxicity assays were performed. BMDMs were untreated or activated with IFN-γ plus LPS. BMDMs were then cocultured with $^{3}$H thymidine– labeled Ad5E1 clone 2.1 or clone 4 tumors or B16F10 melanoma cells at an E: T ratio of 10:1 for 48 or 72 h. Activated BMDMs killed Ad5E1 clone 2.1 tumor cells (75%–80% cytotoxicity) and Ad5E1 clone 4 tumor cells (75-80%) at both 48 and 72 h time points (Figure 13 and data not shown), indicating that Ad5E1 clone 2.1 and clone 4 tumor cells are highly susceptible to macrophage-mediated killing.

*Macrophage-mediated killing is partially contact-dependent*

In order to investigate the mechanism(s) of macrophage-mediated killing, contact- dependency was tested using a transwell culture system. The transwell system allows for populations of cells to be separated, eliminating contact-dependent mechanisms but allowing soluble factors to mediate their function. $^{3}$H thymidine–labeled Ad5E1 clone 2.1 or clone 4 tumors ($1 \times 10^{4}$) cells were plated on the bottom chamber of the transwell apparatus. Activated BMDMs ($1 \times 10^{5}$) were seeded in the upper chamber. Cells were incubated for 48 h and the percent of cytotoxicity of tumor cells was determined as detailed above. Macrophage-

mediated cytotoxicity of both Ad5E1 clone 2.1 and clone 4 tumor cells were partially contact-dependent (Figure 14). The elimination of contact-dependent mechanism(s) reduced cytotoxicity of Ad5E1 clone 2.1 tumor cells from 60% to 33% (Figure 14A) and cytotoxicity of Ad5E1 clone 4 tumor cells from 83% to 52% (Figure 14B). These results indicate that macrophage-mediated cytotoxicity is mediated by two mechanisms, one contact-dependent and one contact-independent.

*Macrophages in Ad5E1 tumors express characteristics of classically-activated (M1) macrophages*

Many studies have reported that classically-activated (M1) macrophages are able to kill tumor cells (186). The observation that Ad5E1 tumor rejection requires macrophages led me to hypothesize that the predominant population of macrophages involved in the rejection of Ad5E1 tumors was the M1 phenotype. Intraocular tumors were removed from the AC on day 14 (i.e., the peak time of intraocular tumor growth) in WT C57BL/6 mice and homogenized. RNA was immediately isolated and quantitative PCR was performed to determine the expression of NOS2 (M1 marker), Arg 1, and YM1 (M2 markers). The expression of two macrophage markers, F4/80 and CD11b, was determined by qPCR as a means of confirming that the isolated cells were indeed macrophages. As a control, the RAW 264.7 macrophage cell line was polarized into

**Figure 13. Ad5E1 clone 2.1 and clone 4 tumor cells are susceptible to macrophage-mediated killing**. BMDMs were untreated or treated with IFN-γ and activated with LPS. BMDMs were then cocultured with $^{3}H$ thymidine–labeled Ad5E1 clone 2.1, clone 4 tumor cells or B16F10 melanoma cells at a 10:1 E:T ratio for 48 h.

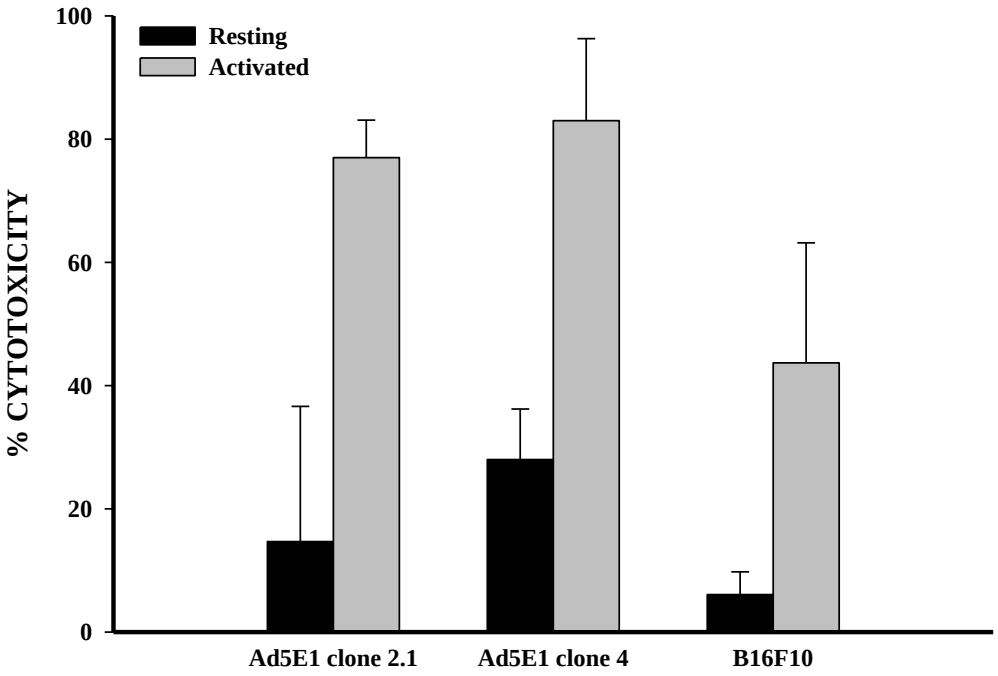
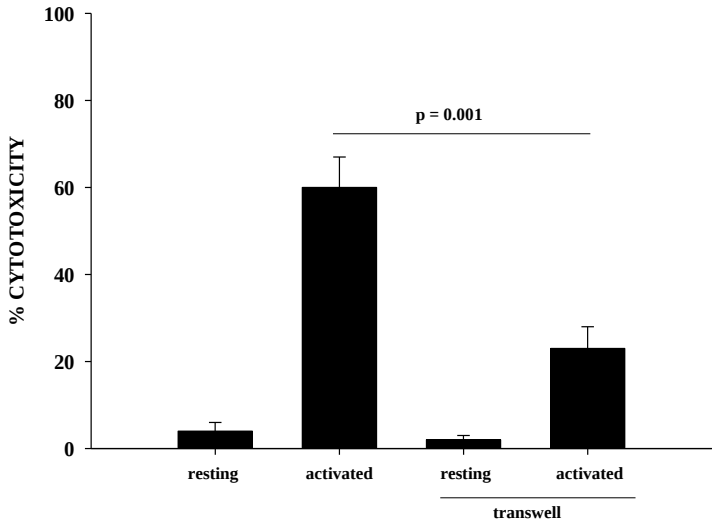
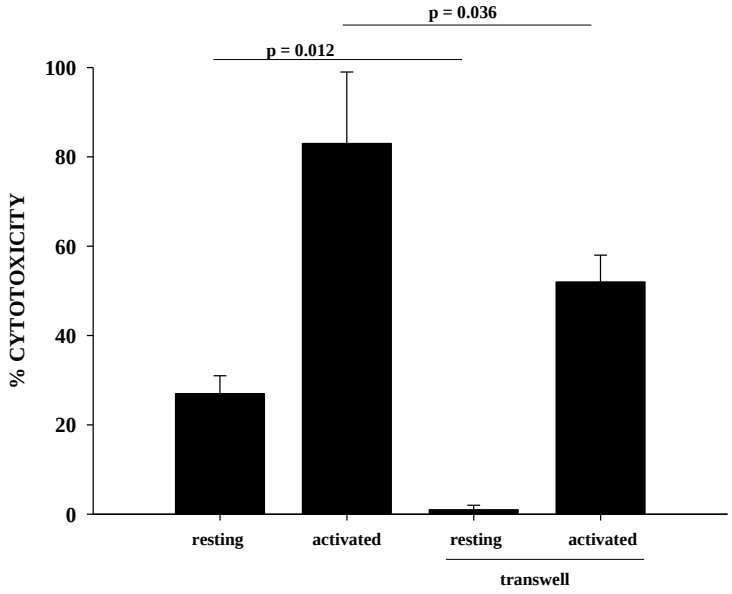


**Figure 14. Macrophage-mediated cytotoxicity is partially contact-dependent**. Ad5E1 clone 2.1 (A) or Ad5E1 clone 4 (B) tumor cells (1 x $10^4$) were plated in the bottom chamber of a transwell culture apparatus. Resting or activated BMDMs (1 x $10^5$) were seeded in the upper chamber. Cells were cultured for 48 h and the percent of cytotoxicity of tumor cell was determined.

**A**.




**B**.



either an M1 or M2 phenotype by culturing with IFN-γ/LPS or IL- 4/IL-10/IL-13, respectively. All samples were compared to naïve eyes and normalized to GAPDH expression.

Classically activated M1 macrophages are induced in "proinflammatory" environments (IFN-γ/LPS) and strongly promote anti-tumor Th1 responses, which can lead to tumor suppression. The dominant population of macrophages in intraocular Ad5E1 clone 2.1 tumors was of the M1 phenotype as shown by the 36-fold increase in the expression of the M1-associated NOS2 gene (Figure 15A). This is consistent with previous findings that rejection of the original Ad5E1 tumor cell line was dependent on IFN-γ. By contrast, there were very few M2 macrophages as noted by the base line expression of the arginase-1 and YM1 gene (Figure 15B and data not shown), both of which are the classic markers for M2 macrophages.

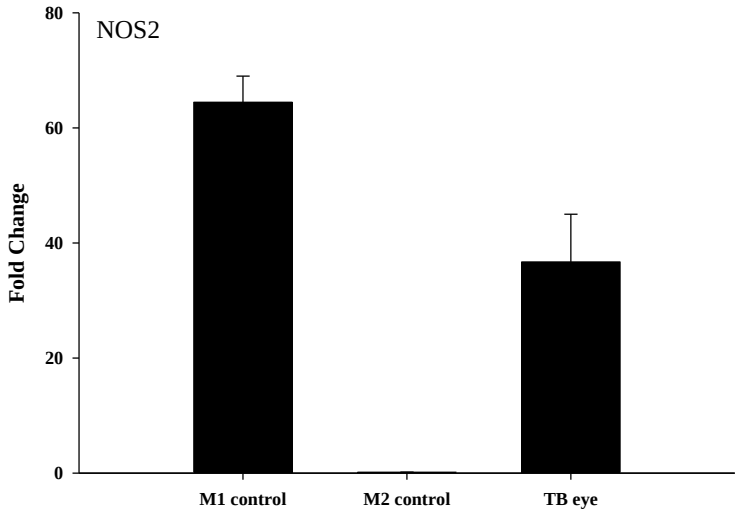
Intraocular Ad5E1 clone 4 tumors also expressed increased levels of NOS2 (approximately 14 fold) compared to normal eyes (Figure 16A). Interestingly, Ad5E1 tumors also expressed increased levels of Arg-1 (approximately 11 fold) compared to normal eyes, providing evidence that M2 macrophages may also be present in the Ad5E1 clone 4 tumor microenvironment (Figure 16B).

*Normal ocular cells are susceptible to BMDM killing*

As described earlier, phthisical rejection of intraocular tumors results in the destruction of innocent bystander cells and ischemic necrosis of the eye. Therefore, M1 macrophages may kill normal ocular cells in addition to killing tumor cells. To determine if activated M1 macrophages contribute to the phthisical rejection of intraocular Ad5E1 clone 2.1 tumors, C57BL/6 corneal endothelial and iris and ciliary body cells were labeled with 0.2 μCi/ml of $^{3}$H-thymidine for 24 hours and cultured with BMDMs.

**Figure 15. The dominant macrophage population in intraocular Ad5E1 clone 2.1 tumors is the M1 phenotype.** C57BL/6 mice bearing Ad5E1 clone 2.1 tumors were sacrificed on day 14 post-tumor injection, and tumor-bearing eyes and naïve eyes were harvested and homogenized. RNA was isolated and qPCR was performed to determine the expression of NOS2 (A) or Arg1 (B). Samples were compared to naïve eye and normalized to GAPDH. TB = tumor bearing.

**A.**




**B.**

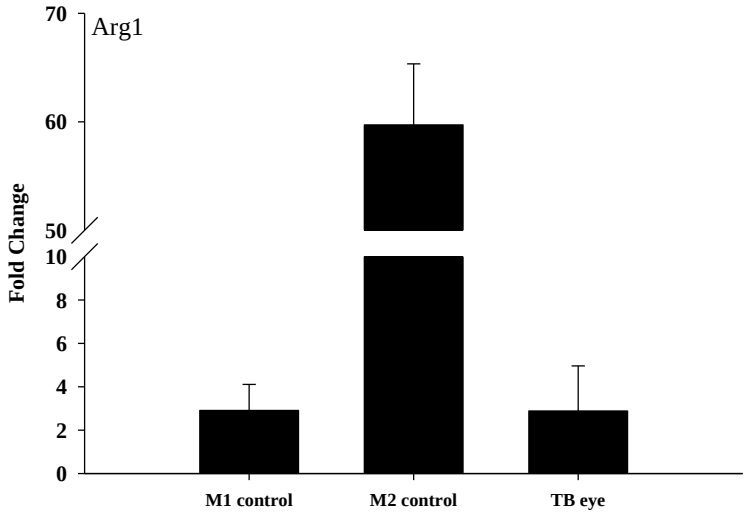
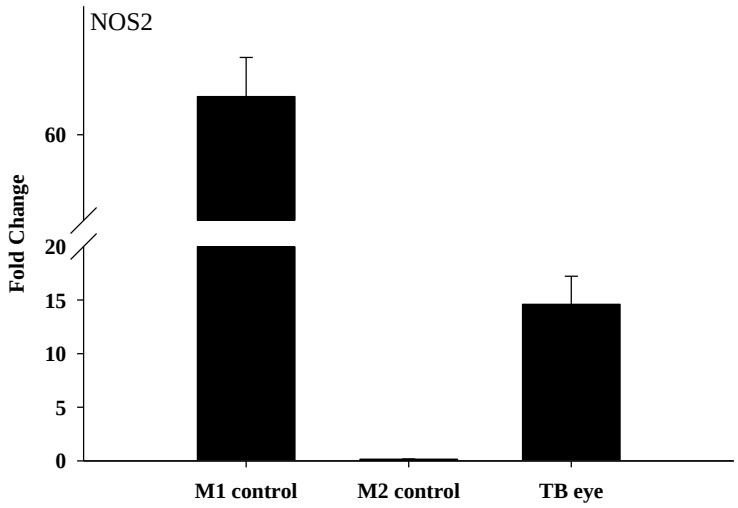


**Figure 16. Ad5E1 clone 4 tumors have both M1 and M2 macrophages.** C57BL/6 mice bearing Ad5E1 clone 4 tumors were sacrificed on day 14 post-tumor injection, and tumor-bearing eyes and naïve eyes were harvested and homogenized. RNA was isolated and qPCR was performed to determine the expression of NOS2 (A) or Arg1 (B). Samples were compared to naïve eye and normalized to GAPDH. TB = tumor bearing

**A.**

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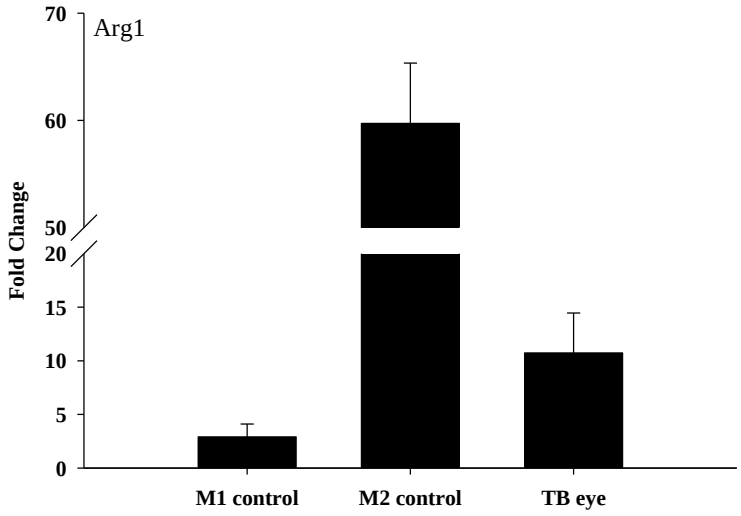


Cells were cultured for 72 h, and cytotoxicity was addressed as previously described. As shown in Figure 17, activated macrophages killed 40-50% of I/CB cells and 30-40% of corneal endothelial cells. This clearly demonstrates that classically activated M1 macrophages are able to kill normal ocular cells *in vitro* and in addition to rejecting intraocular tumors, M1 macrophages might contribute to the phthisis that occurs in intraocular tumor rejection.

*Macrophage-mediated killing of Ad5E1 clone 2.1 tumor cells is iNOS-dependent*

Macrophage-mediated cytotoxicity involves several diverse mechanisms, such as reactive nitrogen intermediates and members of the TNF receptor family (CD40, Fas, etc.). Nitric oxide (NO) is a major molecule employed by M1 macrophages to mediate tumor cytotoxicity (189, 219, 220). In order to examine macrophage-mediated tumor cytotoxicity of Ad5E1 clone 2.1 or clone 4 tumor cells, BMDMs were cultured from the bone marrow of C57BL/6 mice. NO production assays indicated that BMDMs activated with IFN-γ and LPS produced NO, which was blocked by the specific NO synthase inhibitor, L-NAME (data not shown). To determine if BMDMs use NO to kill Ad5E1 2.1 tumor cells, *in vitro* cytotoxicity assays were performed in either the presence or absence of L-NAME.

**Figure 17. Normal ocular cells are susceptible to macrophage-mediated cytotoxicity.** BMDMs were untreated or activated with IFN-γ plus LPS. BMDMs were then cocultured with $^{3}H$ thymidine–labeled corneal endothelial cells or iris/ ciliary cells at a 10:1 E:T ratio for 48 h.

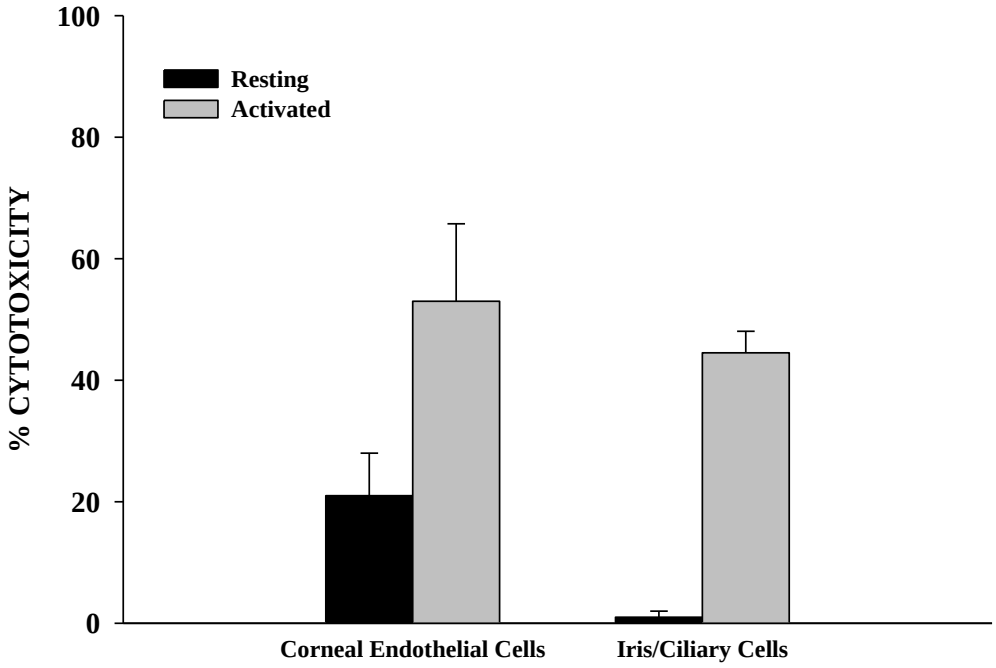
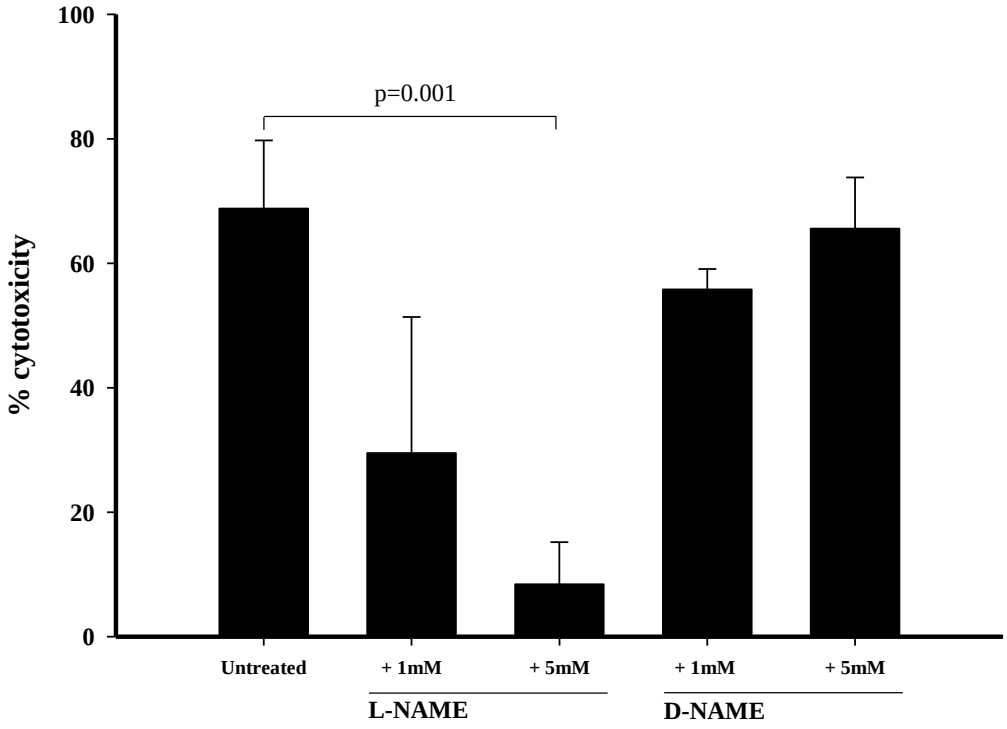


**Figure 18. Inhibition of iNOS reduced macrophage-mediated cytotoxicity of Ad5E1 clone 2.1 tumor cells.** BMDMs were activated with rmIFN-γ plus LPS and used as effector cells *in vitro*. Cultures were incubated for 48 hours and then harvested. iNOS was inhibited by the addition of 1 or 5 mmol/L L-NAME to activated BMDMs. D-NAME, a biologically inactive isomer of L-NAME, was used as a control.



As a control, the biologically inactive isomer of L-NAME, D-NAME, was also used. Inhibition of iNOS by L-NAME significantly reduced the ability of BMDMs to kill Ad5E1 tumor cells (Figure 18). However, the presence of D-NAME did not affect macrophage-mediated killing, indicating that the inhibition of BMDM-mediated killing was NO-specific (Figure 18). The addition of L-NAME or D-NAME was not toxic to the tumor cells at the doses used in the *in vitro* assays (data not shown). These results are consistent with the previous findings indicating that M1 macrophages are the dominant macrophage population present in intraocular tumors undergoing phthisical rejection.

By contrast, the addition of L-NAME did not significantly reduce the ability of BMDMs to kill Ad5E1 clone 4 tumor cells (Figure 19). Thus, the primary mechanism of macrophage-mediated killing of Ad5E1 clone 4 tumor cells is not NO. It is possible that in this system macrophages utilize a different mechanism of rejection of Ad5E1 clone 4 tumor killing prior to the induction of NO.

*Phthisical intraocular tumor rejection is iNOS-dependent*

To determine the role of iNOS in phthisical rejection *in vivo*, NO synthesis was blocked by injecting L-NAME (50mg/kg/day) in 250 μl PBS i.p. into C57BL/6 mice prior to AC injection of Ad5E1 clone 2.1 tumor cells. As a control, a group of untreated mice and a group of mice treated with D-NAME

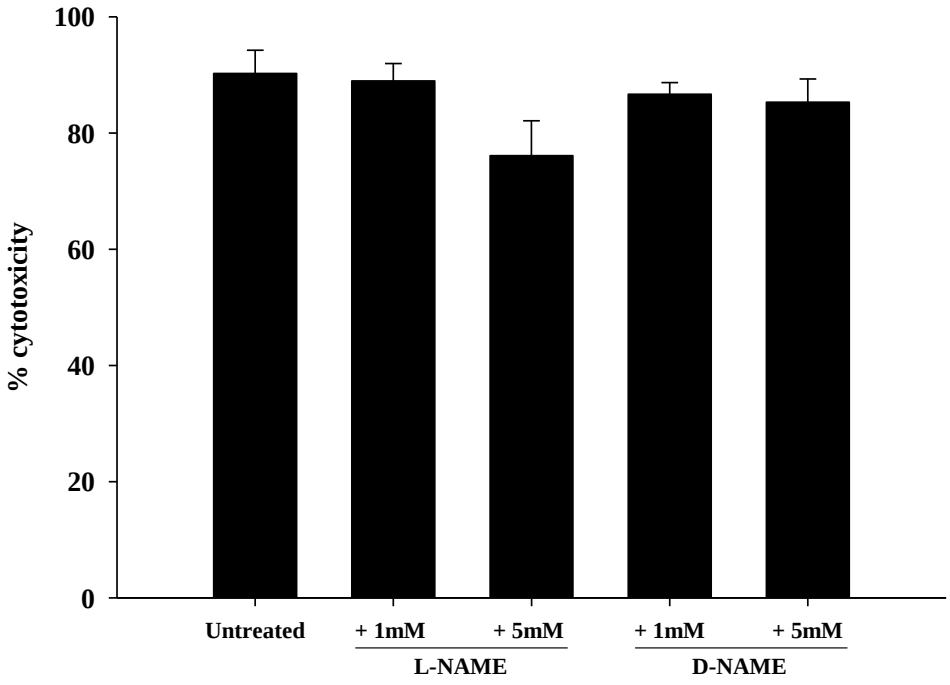
were also challenged with AC injections of Ad5E1 tumor cells. Inhibition of NO synthesis prevented the phthisical rejection of Ad5E1 tumors (Figure 20). By contrast, naïve mice and mice treated with D-NAME rejected their intraocular tumors in a phthisical manner that was indistinguishable from untreated controls (Figure 20). These results indicate that the phthisical rejection of Ad5E1 clone 2.1 tumors is NO-dependent.

Consistent with the *in vitro* data previously shown, inhibition of iNOS by L-NAME did not affect rejection of Ad5E1 clone 4 tumors (Figure 21). Like in the *in vitro* assay, *in vivo* macrophages utilized another method of mediating tumor rejection that is independent of NO. This is consistent with a non-phthisical rejection phenotype as NO would also cause extensive bystander damage to ocular cells.

*IFN-γ is required for phthisical rejection of Ad5E1 clone 2.1 intraocular tumors*

The recruitment of M1 macrophages into inflammatory sites often requires IFN-γ , IL-12, and TNF-α (186, 221). As shown before, the rejection of Ad5E1 clone 2.1 tumors requires IFN-γ and macrophages (Figure 8). This suggests that IFN-γ might function to recruit and activate M1 macrophages. To address this, Ad5E1 tumor cells were injected into the AC of SCID, IFN-γ KO, and WT C57BL/6 mice. Eyes were collected at day 14 and RNA was isolated. The expression of NOS2, CD11b, and F4/80 was examined by qPCR. As a positive

**Figure 19. Inhibition of iNOS does not reduce macrophage-mediated cytotoxicity of Ad5E1 clone 4 tumor cells.** BMDMs were activated with rmIFN-γ plus LPS and used as effector cells *in vitro*. Cultures were incubated for 48 h and then harvested. iNOS was inhibited by the addition of 1 or 5 mmol/L L-NAME to activated BMDMs. D-NAME, a biologically inactive isomer of L-NAME, was used as a control.



qPCR control, M1 polarized RAW 264.7 cells were used. Tumors isolated from animals deficient in IFN-γ had significantly fewer M1 macrophages than WT C57BL/6, as noted by decreased expression of CD11b and F4/80 genes and the reduction in NOS2 expression (Figure 22). SCID mice also had decreased numbers of macrophages suggesting that T cells were the source of IFN-γ involved in generating M1 macrophages in WT C57BL/6 mice. (Figure 22**)** These results suggest that IFN-γ is essential for macrophage-dependent rejection of Ad5E1 clone 2.1 intraocular tumors.

Although IFN-γ is not required for non-phthisical rejection of Ad5E1 clone 4 tumors, IFN-γ plays a role in the recruitment of macrophages, as IFN-γ KO mice and SCID mice have reduced mRNA signal for macrophage makers F4/80 and CD11b (Figure 23). Thus, IFN-γ clearly plays a role in the infiltration of macrophages into eyes containing Ad5E1 clone 4 tumors but is not required for rejection, as Ad5E1 clone 4 tumors are rejected in IFN-γ KO mice but are not rejected in macrophage-depleted mice.

**Figure 20. iNOS is required for rejection of intraocular Ad5E1 clone 2.1 tumors.** WT mice were injected i.p. with L-NAME daily. Controls consisted of C57BL/6 mice injected i.p. with D-NAME daily or left untreated. All C57BL/6 mice were injected in the AC with clone 2.1 tumor cells. N = 10/ group/ experiment in two independent experiments.

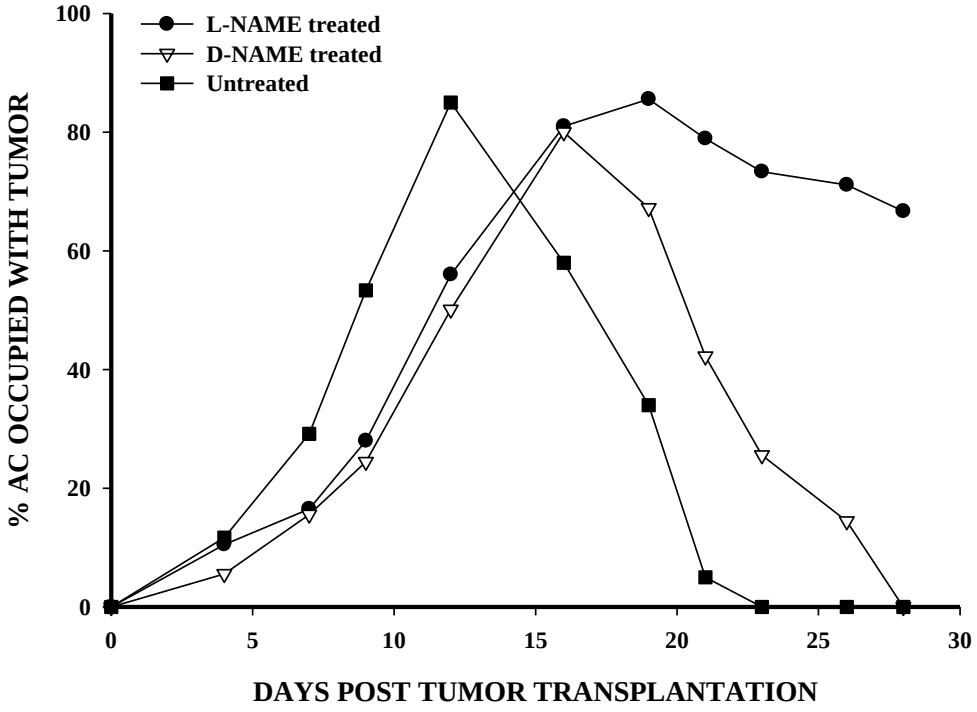


**Figure 21. iNOS is not required for rejection of intraocular Ad5E1 clone 4 tumors.** WT mice were injected i.p. with L-NAME daily. Controls consisted of C57BL/6 mice injected i.p. with D-NAME daily or left untreated. All C57BL/6 mice were injected in the AC with clone 4 tumor cells.

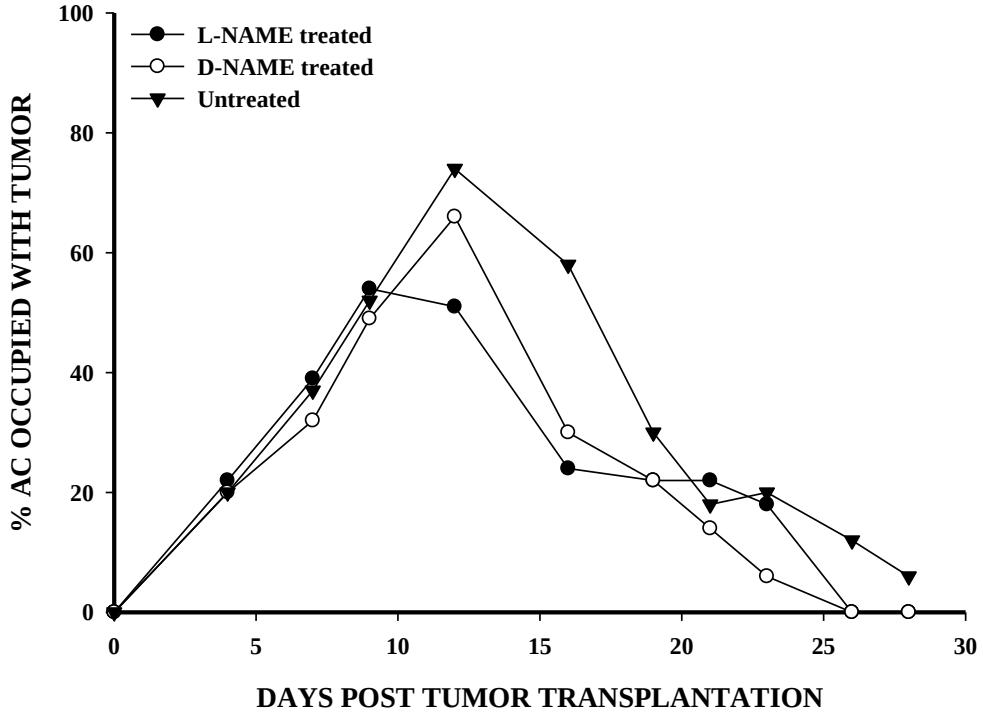
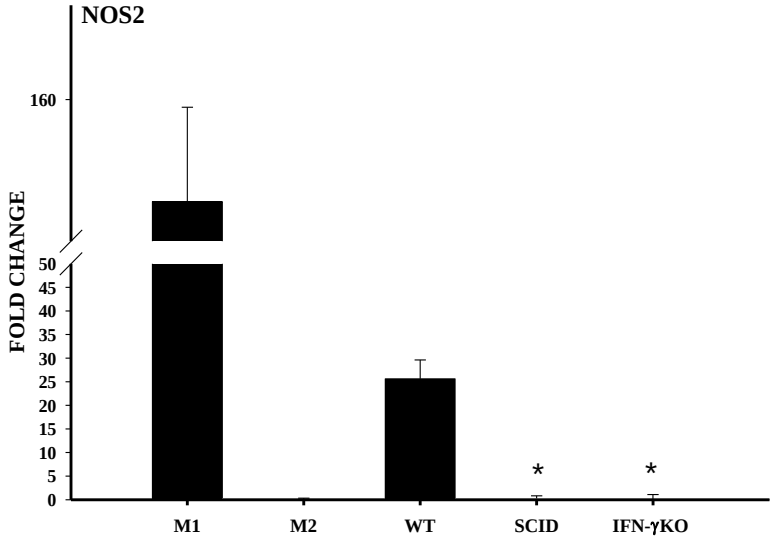
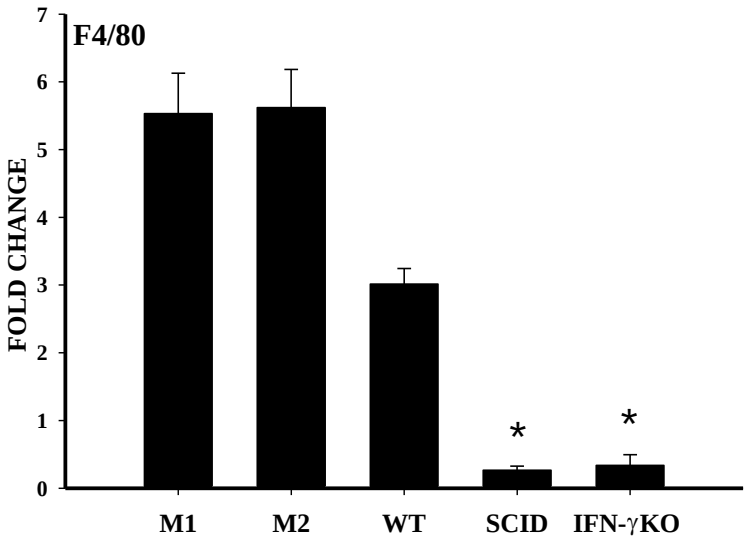


**Figure 22. IFN-γ is required for M1 macrophage infiltration into eyes containing Ad5E1 clone 2.1 tumors**. Tumor-bearing eyes from C57BL/6, SCID, and IFN-γ KO mice were collected at day 14 and real-time qPCR was used to detect NOS2 (A), F4/80 (B), and CD11b (C). All samples were normalized to GAPDH and compared with non–tumor bearing eyes in two independent experiments. M1- and M2-polarized RAW 264.7 cells served as controls. Mean ± SD. *, $P < 0.05$.

**A**.




**B**.



**C.**

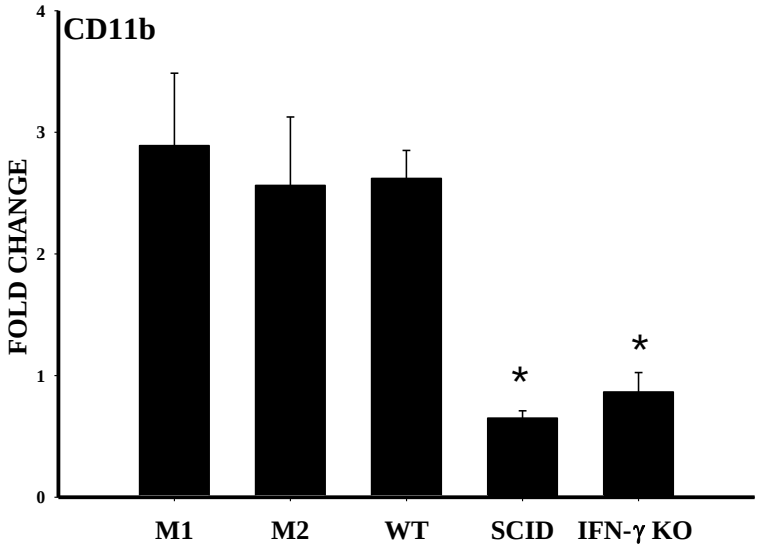
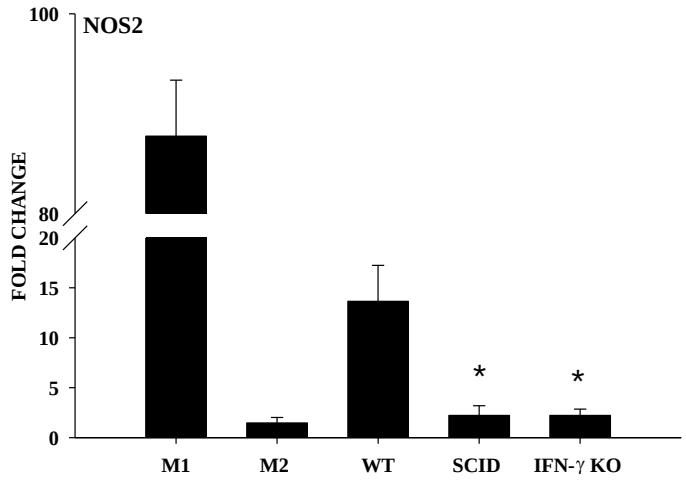


**Figure 23. IFN-γ is required for M1 macrophage infiltration into eyes containing Ad5E1 clone 4 tumors**. Tumor-bearing eyes from C57BL/6, SCID, and IFN-γ KO mice were collected at day 14 and real-time qPCR was used to detect NOS2 (A), F4/80 (B), and CD11b (C). All samples were normalized to GAPDH and compared with non–tumor bearing eyes in two independent experiments. M1- and M2-polarized RAW 264.7 cells served as controls. Mean ± SD. *, $P < 0.05$.

A.

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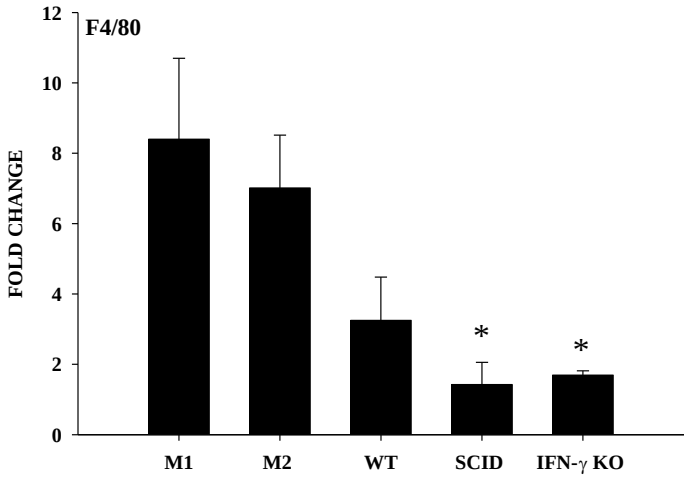

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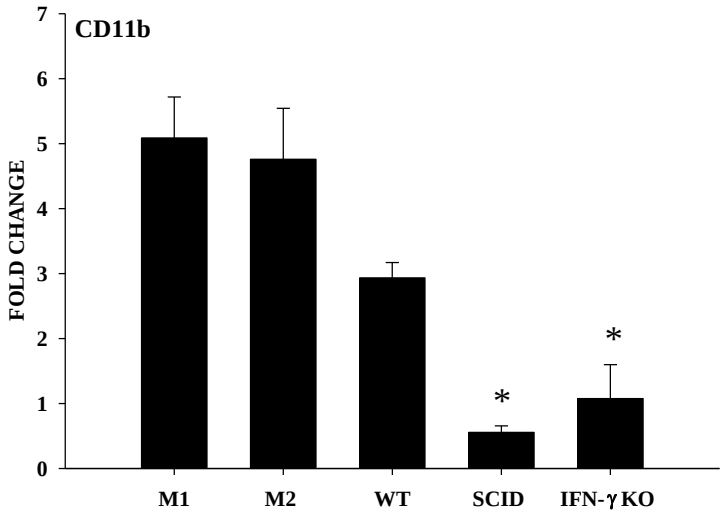


*Phthisical destruction of the tumor-containing eye requires TNF-α but rejection is TNF-α-independent*

Although the intraocular tumor rejection of wild-type parental Ad5E1 tumor cell line occurred in TNF-α KO mice (17), parental Ad5E1 tumor cells were found to be highly sensitive to TNF-α-mediated cytolysis (66). Therefore, I considered the hypothesis that TNF-α elaborated by T cells during the rejection of intraocular Ad5E1 clone 2.1 and clone 4 tumors contributed to tumor rejection. To address the role that TNF-α contributes to rejection, *in vitro* assays were performed to confirm that TNF-α was capable of mediating killing of Ad5E1 tumor clones. Indeed, like the parental Ad5E1 tumor cell line, Ad5E1 clone 2.1 and clone 4 cells were susceptible to TNF-α-mediated killing (Figure 24). Consistent with this, both Ad5E1 tumor clones expressed both TNFR1 and TNFR2 (data not shown).

The sensitivity of Ad5E1 clone 2.1 and clone 4 tumor cells to TNF-α brought up the question of whether TNF-α was required for intraocular tumor rejection. Accordingly, Ad5E1 clone 2.1 or clone 4 cells were injected into the AC of TNF-α KO and wild-type mice and the eyes were observed for tumor growth and resolution. As previously reported with the original Ad5E1 tumor cell line, Ad5E1 clone 2.1 and clone 4 tumors underwent rejection in TNF-α KO mice and wild-type mice (Figure 25). However, rejection of Ad5E1 clone 2.1 tumors in TNF-α KO mice did not culminate in phthisis (Table 2).

I considered the hypothesis that TNF-α contributed to phthisis by killing normal ocular cells that line the AC of the eye. This was addressed by incubating corneal endothelial cells and I/CB cells with various concentrations of TNF-α and assessing cell death. The results indicated normal ocular cells were susceptible to TNF-α-induced apoptosis, albeit less than tumor cells (Figure 24).

To further address the hypothesis that the effect of TNF-α on the development of phthisis was through toxic effects of TNF-α on the host's cells, Ad5E1 clone 2.1 tumor cells were injected into the AC of TNFR1 KO and TNFR2 KO mice. Contrary to my initial hypothesis, phthisical tumor rejection occurred in TNFR1 KO, TNFR2 KO and WT mice (Figure 26 and Table 2). This observation was confirmed in TNFR double KO in which all tumors were rejected in a phthisical manner (data not shown). This provided evidence that the rejection phenotype was determined by the tumor cells and not by host cells.

**Figure 24. Ad5E1 tumor cells and normal ocular cells are susceptible to TNF-α-mediated cytotoxicity.** Ad5E1 tumor cells and normal ocular cells (corneal endothelial cells and iris/ciliary body cells) were incubated with increasing doses of TNF-α and cell death was assessed by LDH release.

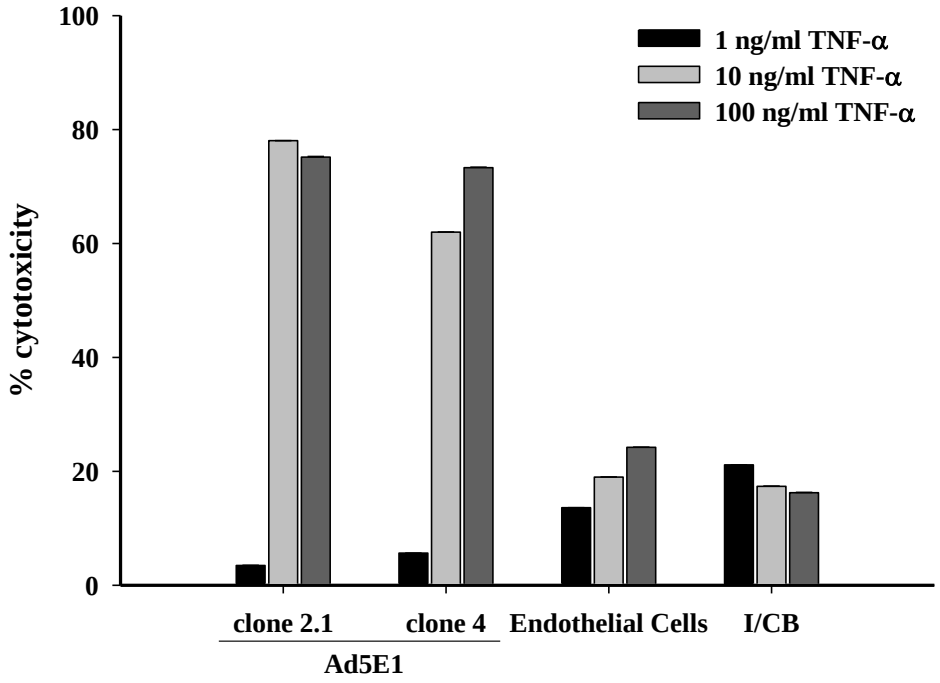
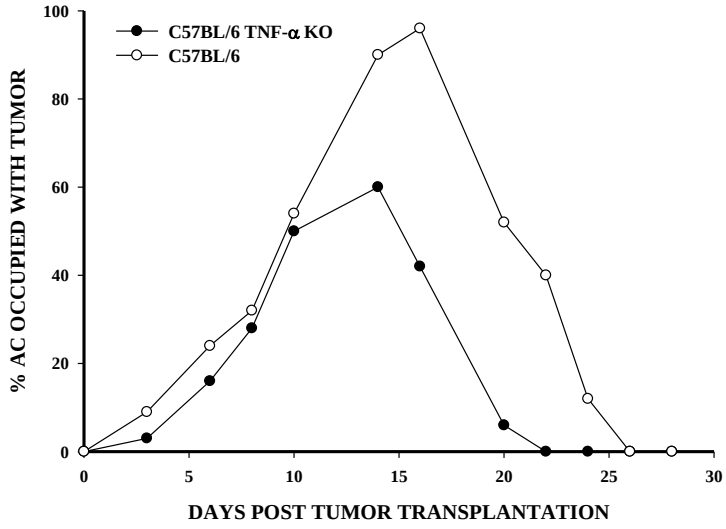


**Figure 25. TNF-α is not required for intraocular rejection of either Ad5E1 clone 2.1 or clone 4 tumors.** Ad5E1 clone 2.1 (A) or clone 4 (B) cells (3 x $10^5$ cells/6μl) were injected into the AC of C57BL/6 TNF-α KO on day 0. Tumor growth was scored as the percentage of AC occupied by tumor. Graph represents the combined results of three independent experiments; N = 10 group/experiment for Ad5E1 clone 2.1 tumors. Two independent experiments were performed with Ad5E1 clone 4 tumors; N = 5 mice/ group/ experiment.

A.

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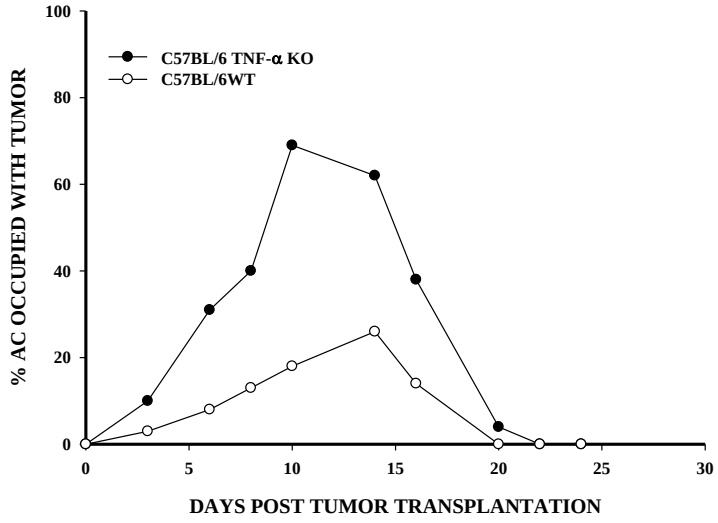
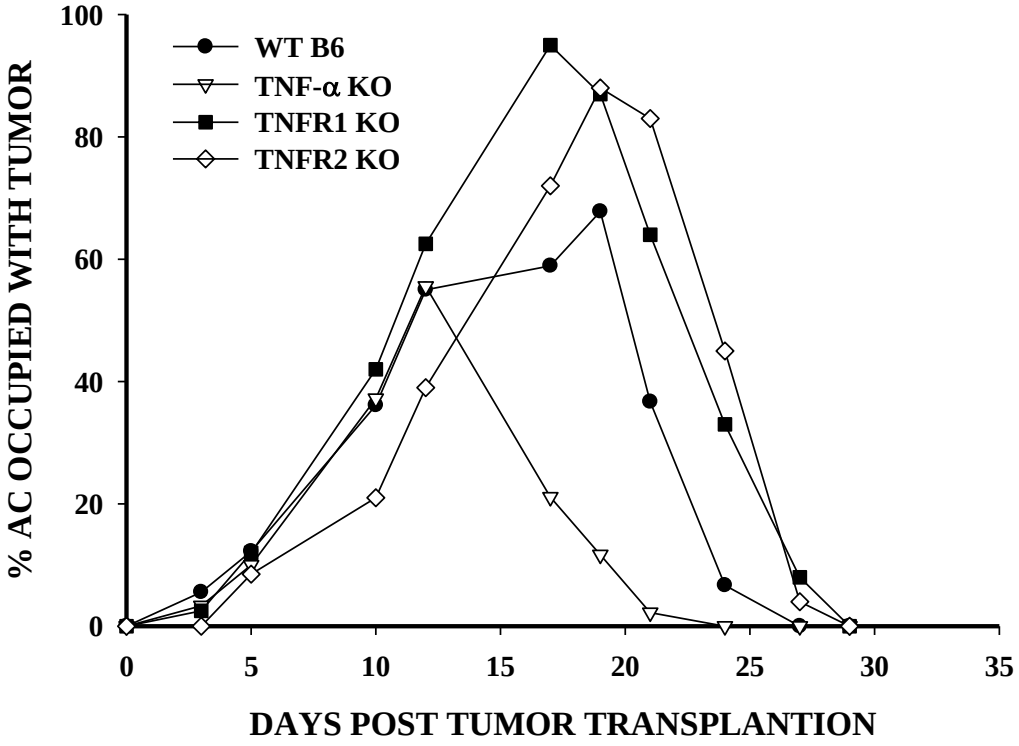


**Table 2. Phthisical rejection of intraocular Ad5E1 clone 2.1 tumors requires TNF-α.**

| Host | Non-phthisical, % | Phthisical, % |
|---|---|---|
| C57BL/6 (N = 34) | 21 | 79 |
| TNF-α KO (N = 33) | 88 | 12 |
| TNFR1 (N = 10) | 10 | 90 |
| TNFR2 (N = 10) | 0 | 100 |

**Figure 26. Phthisical rejection of Ad5E1 clone 2.1 tumors does not require signaling through TNFR1 and TNFR2**. Ad5E1 clone 2.1 tumor cells ($3 \times 10^5$) were AC injected in WT, TNF-α KO, TNFR1KO, and TNFR2 KO mice. Tumor growth was scored as the percentage of AC occupied by tumor. Experiments were performed twice with similar results (N = 10 mice per group).



*Phthisical tumor rejection requires T cell-produced TNF-α*

Tumor cells were injected into the AC of SCID, IFN-γ KO, and WT C57BL/6 mice and the expression of TNF-α was examined by qPCR. Intraocular tumors from mice deficient in either T cells or IFN-γ expressed no TNF-α (compared with WT mice), thereby suggesting that T cells are a major source of TNF-α (Figure 27). To further examine the hypothesis that T cells produce TNF-α in response to intraocular tumors, $CD4^+$ T cells were isolated from draining lymph nodes of mice that had rejected intraocular clone 2.1 tumors and were stimulated *in vitro* with APCs pulsed with clone 2.1 tumor antigens or stimulated with anti-CD3/CD28 beads. Rejector T cells secreted TNF-α in response to either tumor antigen or anti-CD3/CD28 stimulation (Figure 28).

Although T cells are a major source of TNF-α, M1 macrophages can also produce this cytokine. To demonstrate that T cells were the source of TNF-α, purified T cells from rejector WT and TNF-α KO mice were transferred to naive SCID mice. Mice were immediately challenged with clone 2.1 tumor cells. As expected, SCID mice that received T cells from rejector mice eliminated intraocular tumors in a phthisical manner. In contrast, SCID mice that received T cells from TNF-α KO mice failed to develop phthisical rejection (Figure 29). As a control, C57BL/6 mice were injected with clone 2.1 cells and, as expected, the tumors underwent phthisical rejection.

**Figure 27. The source of TNF-α is T cells.** The expression of TNF-α mRNA from tumor-bearing WT and IFN-γ KO eyes was determined by qPCR and compared with a non–tumor bearing eye. Tumor bearing eyes were harvested on day 14 after tumor transplantation. RNA was isolated and mRNA expression was compared to naïve eye. All samples were normalized to GAPDH. Graph depicts the combined results of two independent experiments.

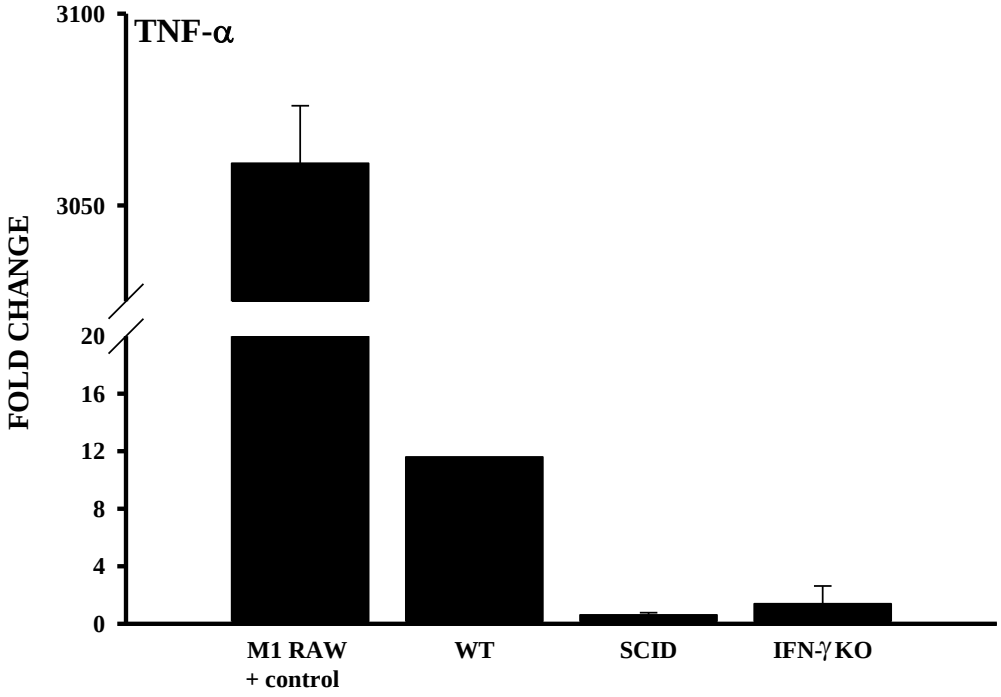


**Figure 28. T cells from Ad5E1 clone 2.1 tumor rejector mice produce TNF-α in response to tumor antigens**. $CD4^+$ T cells ($1 \times 10^5$) were isolated from draining lymph nodes of tumor rejector mice and WT untreated or were stimulated with tumor antigen (TA)-pulsed APCs or with anti-CD3/CD28 beads for 5 days at 37 °C. Cells were isolated after tumor rejected (around day 21). Secretion of TNF-α was determined by ELISA. n.d. = not detected

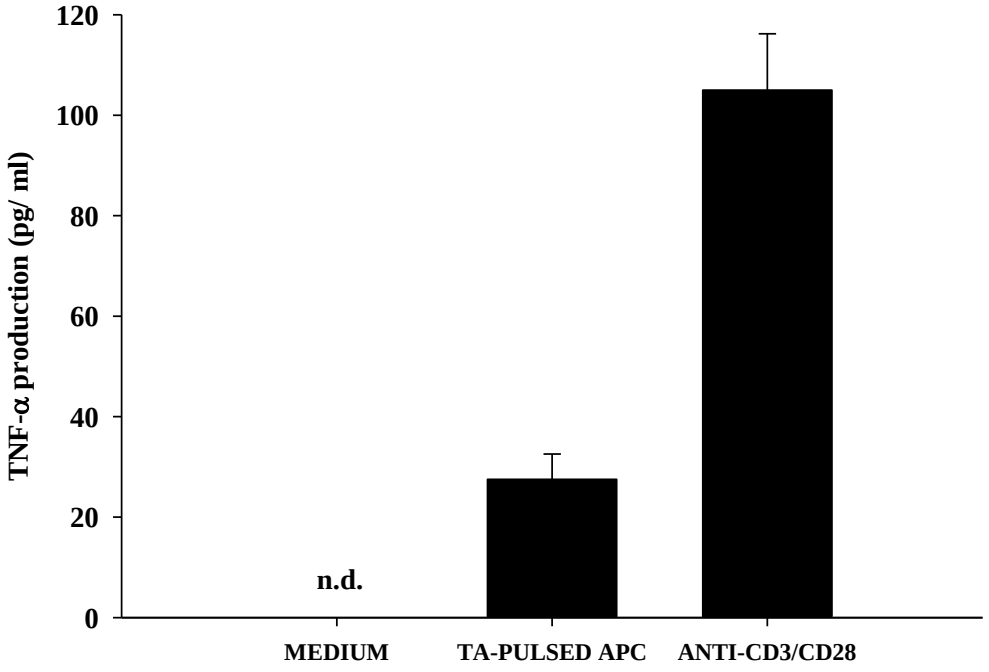
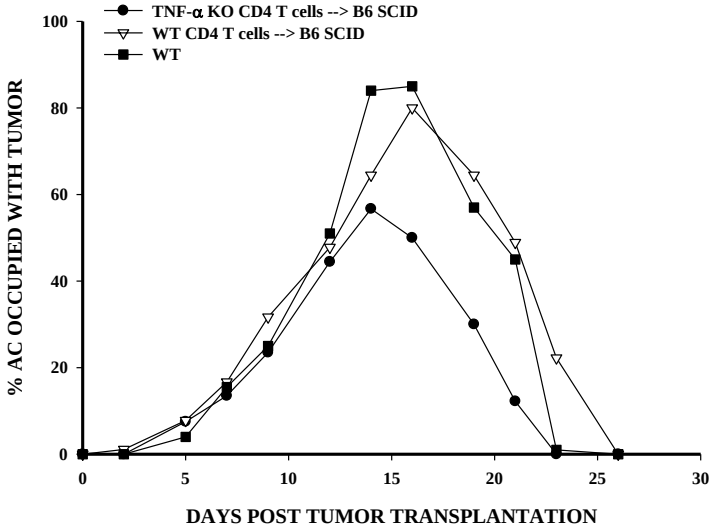
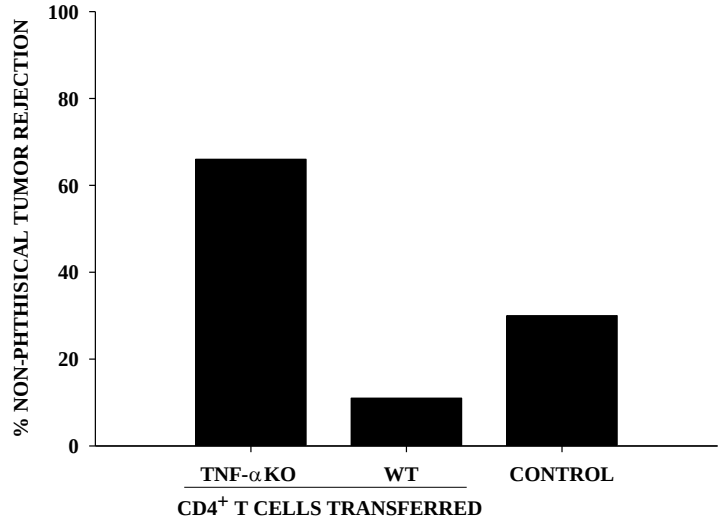


**Figure 29. T cells are the sole source of TNF-α in phthisical rejection of Ad5E1 clone 2.1 intraocular tumors.** (A) $CD4^+$ T cells from C57BL/6 and TNF-α KO tumor rejector mice were adoptively transferred to SCID mice and recipient mice were injected with Ad5E1 clone 2.1 tumor cells ($3 \times 10^5$) in the AC. (N = 9 or 10 mice in each group) Tumor growth was scored as the percentage of AC occupied by tumor. (B) Percentage of animals that underwent non-phthisical intraocular tumor rejection in A.

A.




B.



*Other possible effector mechanisms for Ad5E1 clone 4 tumor rejection*

As noted earlier, the rejection of Ad5E1 clone 4 tumors, unlike Ad5E1 clone 2.1 tumor rejection, does not require IFN-γ, TNF-α, or NO. However, rejection does require T cells and macrophages, and macrophages are able to mediate cytotoxicity of Ad5E1 clone 4 tumor cells *in vitro*. I sought to determine what molecules are required for the rejection of Ad5E1 clone 4 tumors. Accordingly, the requirement of TRAIL, IL-17, γδ T cells, NKT cells, and Fas for rejection was examined. This was addressed using TRAIL KO, γδ T cell KO, NKT cell KO and Fas deficient mice. None of these molecules were found to be required for rejection of either Ad5E1 tumor clone (Table 3).

*Macrophage-mediated killing of Ad5E1 clone 4 tumor cells*

As shown above, Ad5E1 clone 4 tumor cells are susceptible to macrophage-mediated killing, which is only partially contact-dependent. In order to determine what soluble factors mediate rejection, resting and activated macrophages were cultured with tumor cells for 48 h. Supernatants from these cultures were filter-sterilized to remove cell debris and added to $^{3}$H-thymdine-labeled Ad5E1 tumor cell cultures. After an additional 48 h, the level of tumor cytotoxicity was determined. Consistent with the transwell data, Ad5E1 clone 4 tumors cells were effectively killed by supernatants collected from macrophage plus tumor cultures (Figure 30). As a control for the possible cytotoxic affects of

lysed tumors cells in macrophage-tumor cultures, tumor cells cultured alone were lysed by repeated freeze-thaw cycles, filtered, and added to $^{3}$H-thymidine- labeled tumor cells. This did not induce cytotoxicity of tumor cells (data not shown). To ensure this factor was produced in an antigen-specific manner, supernatants from activated macrophages cultured alone were also added to labeled tumor cells. Again, this did not affect tumor cytotoxicity. Similarly, supernatants from tumor cells cultured alone did not induce cytotoxicity of tumor cells (data not shown).

In order to determine if this factor is a protein, supernatants were treated with proteinase K for 1 h at 37ºC. This did not induce cytotoxicity of Ad5E1 clone 4 tumor cells confirming that cytotoxicity was mediated by a protein. In order to determine the molecular weight of the cytotoxic molecule(s) in supernatants from activated macrophages cultured with tumor cells were separated by centrifugal filters. Proteins were separated in 3, 10, 30, 50 and 100 kDa fractions. All fractions were able to mediate tumor cytotoxicity except the 100 kDa fraction (Figure 30). Thus, one or more soluble factors that mediates cytotoxicity of Ad5E1 clone 4 tumor cells is a protein with a molecular weight between 50 and 100 kDa.

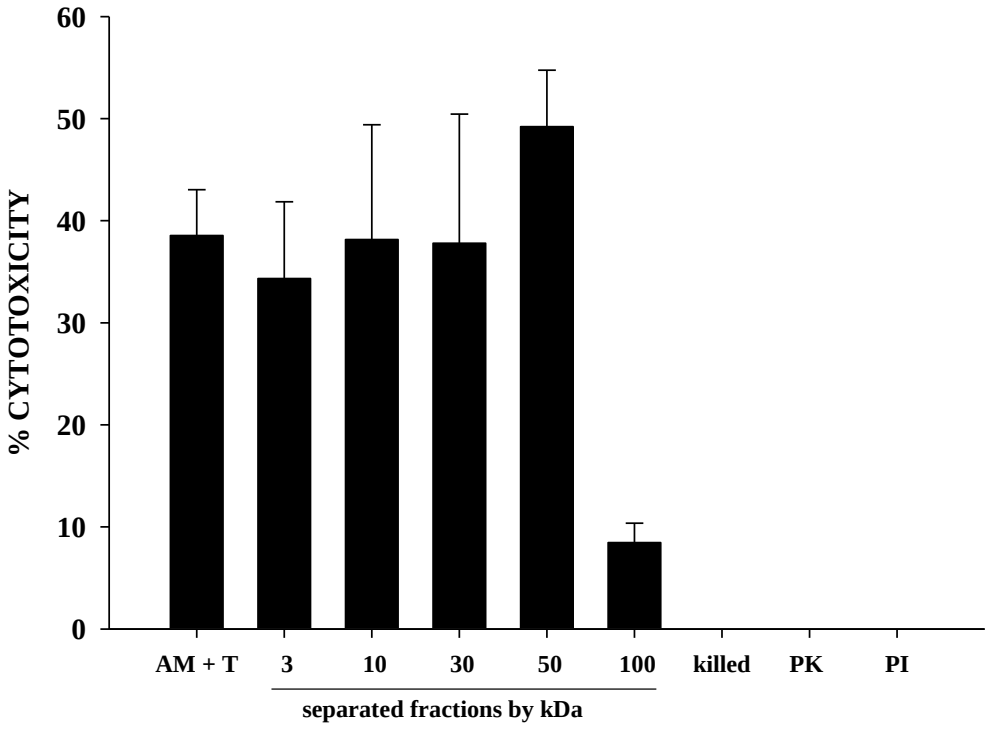
In order to determine if the unknown protein was a protease, supernatants (from activated macrophages cultured with tumors) were treated with a broad spectrum protease inhibitor cocktail. This cocktail inhibits proteases in extracts from almost any tissue or cell and avoids hazardous compounds by using a non-

toxic inhibitor cocktail. Treatment with this protease inhibitor cocktail completely abolished the cytotoxic effect of supernatants, providing evidence that the unknown soluble protein is a protease (Figure 30). Treatment of tumor cells with the protease inhibitor cocktail or proteinase K did not induce tumor cytotoxicity (data not shown).

**Table 3. Other possible mechanisms of Ad5E1 tumor rejection**

| Host | Ad5E1 clone 2.1 | Ad5E1 clone 4 |
|---|---|---|
| **TRAIL KO (N = 10)** | Rejected | Rejected |
| **IL-17 depleted (N = 10)** | Rejected | Rejected |
| **γδ KO (N=10)** | Rejected | Rejected |
| **CD1d KO (N = 10) (NKT cells)** | Rejected | Rejected |
| **Jα18 KO (N = 10) (NKTcells)** | Rejected | Rejected |
| **FasL defective (gld/gld) (N = 10)** | Rejected | Rejected |

**Figure 30. Macrophage-mediated killing of Ad5E1 clone 4 tumor cells is mediated by a soluble factor between 50 and 100 kDa.** Supernatants from activated macrophages cultured with Ad5E1 tumor cells for 48 h were added to $^{3}$H-labeled thymidine Ad5E1 clone 4 tumor cells. Supernatants were fractionated by molecular weight and added to tumor cells. AM + T = supernatants from macrophages plus tumor cells co-cultures; killed AM + T = macrophages plus tumor cells co-culture supernatants incubated for 10 mins. at 100 °C; PK = proteinase K; PI = proteinase inhibitor treated AM + T.



## II. Differential Gene Expression of Ad5E1 Tumor Clones

*The phenotype of tumor rejection is determined by the tumor clone, not the immune response*

Previous results presented above demonstrated that phthisical rejection occurs even in TNFR KO mice, suggesting that phthisis was not a result of the immune response of the host but it was TNFR signaling on the tumor cells that started the process that culminated in phthisis. To follow up on this, studies were done to determine if the tumor cells or the host's immune response dictated the phenotype of intraocular tumor rejection. To test this hypothesis T cells (using a pan T cell isolation kit) were isolated from the spleens of mice rejecting either Ad5E1 clone 2.1 or clone 4 tumors. One donor T cell equivalent (~ $5 \times 10^6$ cells) was then adoptively transferred to SCID mice. One day later, mice were AC injected with either Ad5E1 clone 2.1 or clone 4 tumor cells ($3 \times 10^5$/ 6 μl). Regardless of the donor source of T cells (whether from clone 2.1 or clone 4 tumor rejector mice), intraocular tumors were rejected according to the phenotype of rejection normally observed (Table 4). Thus, it is the tumor itself that determines the rejection phenotype and not the host's immune response.

**Table 4: The phenotype of intraocular tumor rejection is determined by the tumor cells and not the host's immune response.** Pan T cells were isolated from the spleens of mice that rejected Ad5E1 clone 2.1 or clone 4 tumors. One donor equivalent (~5 x $10^6$ cells) of T cells was adoptively transferred to SCID mice. One day later, SCID recipient mice were AC injected with either Ad5E1 clone 2.1 or clone 4 tumor cells (3 x $10^5$/ 6 μl). Tumor rejection was scored as either phthisical or non-phthisical.

| **Rejector T cells from** | **Tumor challenge in SCID mice** | **Rejection Phenotype** |
|---|---|---|
| Ad5E1 clone 4 | Ad5E1 clone 4 | non-phthisical (10/10) |
| Ad5E1 clone 4 | Ad5E1 clone 2.1 | phthisical (9/10) |
| Ad5E1 clone 2.1 | Ad5E1 clone 2.1 | phthisical (9/10) |
| Ad5E1 clone 2.1 | Ad5E1 clone 4 | non-phthisical (10/10) |

*Differential gene expression of Ad5E1 clone 2.1 and Ad5E1 clone 4 tumors*

In light of the understanding that it is the tumor that determines the rejection phenotype, I sought to determine if Ad5E1 clone 2.1 and clone 4 tumors have differential gene expression. To accomplish this gene expression of the tumor clones was compared by microarray analysis. As shown above, IFN-γ and TNF-α are critically important for the induction of phthisis. Accordingly, both Ad5E1 clone 2.1 and Ad5E1 clone 4 tumor cells were treated with either IFN-γ or TNF-α for 24 h and gene expression was compared by microarray analysis. Samples were run on the Mouse WG-6 v2.0 BeadChip from Illumina, Inc. The content on this chip is derived from the National Center for Biotechnology Information Reference Sequence (NCBI RefSeq) database and supplemented with probes derived from the Mouse Exonic Evidence Based Oligonucliotide (MEEBO). Analysis was done by the Gene Set Enrichment Analysis (GSEA) program developed by the Broad Institute of MIT and Harvard University (213, 214). This program assigns significantly upregulated genes into gene sets based upon phenotypes submitted by various researchers. All genes listed were significantly upregulated compared to the other cell type.

When gene expression of Ad5E1 clone 2.1 genes was compared to Ad5E1 clone 4 genes, 892 genes were upregulated in Ad5E1 clone 2.1 tumors with a p value of less than 0.01. Genes were then assigned to pathways known to be involved in biological processes. Many of these pathways are important for

embryonic developement. The GSEA program lists genes in the 20 most upregulated pathways. Table 5 shows selected genes found in these gene sets that were upregulated in Ad5E1 clone 2.1 tumor cells. Hypotheses discussing the significance of many of these genes are discussed in the Discussion section.

When gene expression of Ad5E1 clone 4 tumor cells was compared to Ad5E1 clone 2.1 tumor cells, 123 genes were found to upregulated in Ad5E1 clone 4 with a p value of less than 0.01. Selected genes are found in Table 6. Hypotheses considering the role differentially expressed genes have in non-phthisical are stated in the Discussion section.

Gene expression of Ad5E1 clone 2.1 tumor cells treated with IFN-γ or with TNF-α was compared to untreated Ad5E1 clone 2.1 tumor cells. This analysis indentified 507 and 187 genes that were upregulated with a p value of less than 0.01 in Ad5E1 clone 2.1tumor cells treated with IFN-γ or TNF-α, respectively. Selected genes are found in Tables 7 and 8.

Lastly, gene expression of Ad5E1 clone 4 tumor cells treated with IFN-γ or treated with TNF-α was compared to untreated Ad5E1 clone 4 tumor cells. There were 150 and 172 genes upregulated with a p value of less than 0.01 in Ad5E1 clone 4 tumor cells treated with IFN-γ or TNF-α, respectively. Selected genes are found in Tables 9 and 10.

**Table 5: Genes upregulated in Ad5E1 clone 2.1 versus Ad5E1 clone 4.**
Selected genes upregulated in Ad5E1 clone 2.1 versus Ad5E1 clone 4.

| Pathway | Selected Genes Upregulated |
|---|---|
| Proinflammatory Genes | HMGB-1, MMP13, CXCL5 |
| Toll-like receptor signaling pathway | TLR2, TLR4,TLR6, TLR9, FOS, IRAK1, TRAF3, TRAF6, MAP3K7, TOLLIP and others |
| Mitogen-activated protein kinase (MAPK) pathway | MAP2K1, MAPK1, MAPK6, MAPK8, MAP3K1, MAPK14, CREB1, and others |
| Regulation of apoptosis | CASP7, BAD, BIRC2, BCL2L1, AKT3, IKK1 |

**Table 6: Genes upregulated in Ad5E1 clone 4 versus Ad5E1 clone 2.1.**
Selected genes upregulated in Ad5E1 clone 4 versus Ad5E1 clone 2.1.

| Pathway | Selected Genes Upregulated |
|---|---|
| Genes in apoptotic signaling | CASP8, CASP1, CASP3, BCL2, BIRC3, BOK |
| Genes involved antigen presentation | PSMB8, PSMA9, PSMA10, PSMB7, PSMA7, PSMA5 |
| Genes related to IL-7 signaling; IL-7 increases proliferation of certain subsets of T and B cells | IL7, IL7R, STAT5A, STAT5B |

**Table 7: Genes upregulated in Ad5E1 clone 2.1 + IFN-γ versus untreated Ad5E1 clone 2.1. S**elected genes upregulated in Ad5E1 clone 2.1 + IFN-γ versus Ad5E1 clone 2.1

| Pathway | Selected Genes Upregulated |
|---|---|
| Genes related to immune responses | CCL5, CCL6, CXCL9, TAP1, TAP2, LCK, CXCL10, LY6A, SOCS1 and others |
| Genes related to IL-7 signaling; IL-7 increases proliferation of certain subsets of T and B cells | IL2RG, IL7R, STAT5A, STAT5B |
| Toll-like receptor signaling pathway | TLR2, TLR4, TLR9, IL12A, IL13, IL4 and others |
| Regulation of apoptosis | CFLAR, CASP9, BIRC3, CASP7, TNFSF10, MAP3K14, RIPK1, BID |

**Table 8: Genes upregulated in Ad5E1 clone 2.1 + TNF-α versus untreated Ad5E1 clone 2.1.** Selected genes upregulated in Ad5E1 clone 2.1 + TNF-α versus Ad5E1 clone 2.1

| Pathway | Selected Genes Upregulated |
|---|---|
| Genes related to apoptosis and regulation of apoptosis | FADD. DFFB, BIRC3, CASP8, BID, CHUK, CFLAR, GAS2, CASP1, CASP3 |
| Genes involved in TNF-α signaling | TRAF2, MAPK8, TRAF6, TRAF5, TRAF3, RELA, CHUK, DUSP1, CASP3, TNFSF5, NFKB1, TNFSF13C, TNFRSF17, CD40L |
| Genes upregulated in response to stress | PSME4, NEK4, TFAM, RRM2 |

**Table 9: Genes upregulated in Ad5E1 clone 4 + IFN-γ versus untreated Ad5E1 clone 4.** Selected genes upregulated in Ad5E1 clone 4 + IFN-γ versus Ad5E1 clone 4.

| Pathway | Selected Genes Upregulated |
|---|---|
| Genes related to antigen presentation | CCL5, CCL7, CD53, TAP1, TAP2, FAS, IL6ST, CCR1, IL18R1, CXCL9, VEGFB, CXCL10 |
| Genes involved in various aspects of immune regulation | CD72, CD22, IL13, IL4, CD19, NKTR, CD8A, CD4, IFNGR1, CD69, IL15, IL18R1, CD37 |
| Proteasome genes | PSMB8, PSMA9, PSMA10, PSMB7, PSMA7, PSMA5 |
| Genes in apoptotic signaling | CASP8, CASP3, BAD, IL1A, BFAR |
| Genes involved in regulation of the inflammatory process | CXCL10, IL1R1, CXCR4, IL8RB, IL1A |

**Table 10: Genes upregulated in Ad5E1 clone 4 + TNF-α versus untreated Ad5E1 clone 4.** Selected genes upregulated in Ad5E1 clone 4 + TNF-α versus Ad5E1 clone 4.

| Pathway | Selected Genes Upregulated |
|---|---|
| Genes in apoptotic signaling | CASP1, BCL2, CXCR4, BIRC3 |
| Proteasome genes | PSMB8, PSMA9, PSMA10, PSMB7, PSMA7, PSMA5 |

*Expression of HMGB-1 of Ad5E1 clone 2.1 and Ad5E1 clone 4 tumor cells*

In order to confirm the results obtained from the microarray analysis above, the mRNA and protein expression of HMGB-1 of Ad5E1 clone 2.1 and Ad5E1 clone 4 tumor cells was examined. To address the hypothesis that IFN-γ and/or TNF-α induces the expression of HMGB-1, Ad5E1 clone 2.1 tumor cells were untreated, treated with IFN-γ, TNF-α, or IFN-γ + TNF-α. Cells were treated with 10 ng/ml IFN-γ or 5 ng/ ml TNF-α for 24 hours. Cells were then collected and qPCR was performed to examine HMGB-1 expression. All samples were compared to mouse embryonic fibroblasts (MEF). Ad5E1 clone 2.1 tumor cells had a seven-fold increase of HMGB-1 expression compared to MEF (Figure 31). Treatment with TNF-α alone decreased expression (from 7-fold to 4-fold increase) compared to untreated Ad5E1 clone 2.1tumor cells. However, treatment with IFN-γ increased expression from seven-fold to over nine-fold. Treatment with IFN-γ and TNF-α increased HMGB-1 expression from seven-fold to ten-fold compared to MEF cells (Figure 31). Both Ad5E1 clone 2.1 and Ad5E1 clone 4 tumor cells expressed similar levels of HMGB-1 (Figure 31). Treatment of Ad5E1 clone 4 tumor cells with IFN-γ did not increase expression of HMGB-1 (data not shown).

To confirm that Ad5E1 clones express HMGB-1 protein, intracellular staining of Ad5E1 clone 2.1 and Ad5E1 clone 4 tumor cells was performed with an anti-HMGB-1 antibody. As a positive control HeLa cells were examined for

expression of HMGB-1. As a negative control HeLa and Ad5E1 tumor cells were staining with an isotype antibody. Both Ad5E1 tumor clones express HMGB-1 protein at similar levels in the cytoplasm (Figure 32).

**Figure 31: Expression of HMGB-1 of Ad5E1 clone 2.1 and clone 4 tumor cells.** Ad5E1 clone 2.1 tumor cells were untreated or treated with TNF-α, or IFN-γ, or TNF-α + IFN-γ for 24 hours. Tumor cells were treated with 5 ng/ ml of TNF-α or 10 ng/ ml of IFN-γ. RNA was isolated and mRNA expression was compared to mouse embryonic fibroblasts (MEF). All samples were normalized to GAPDH.

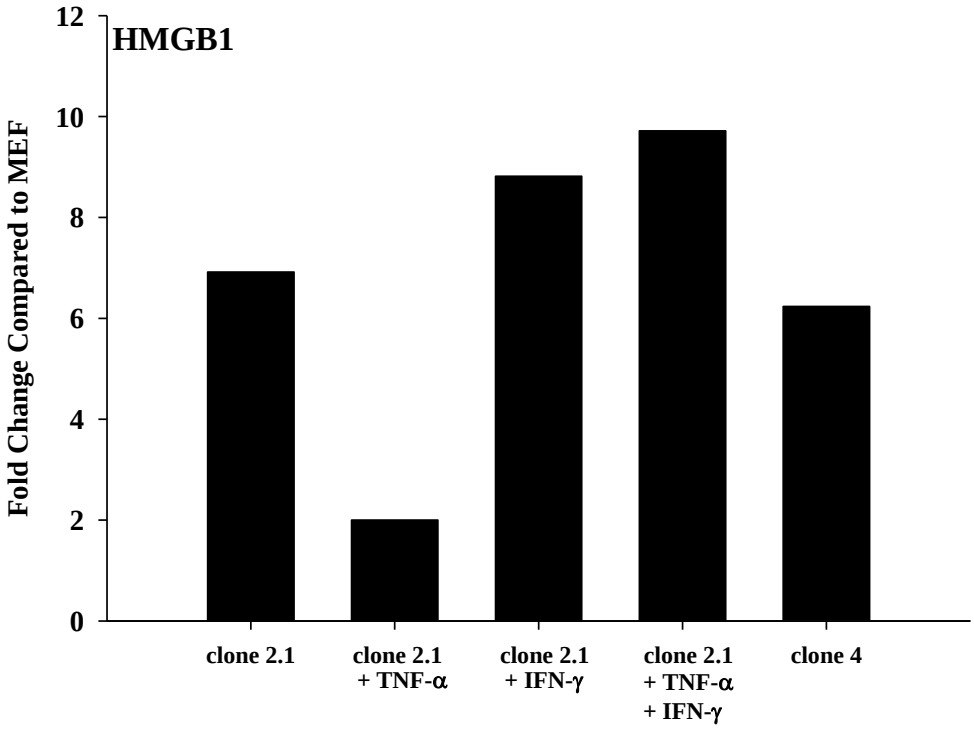
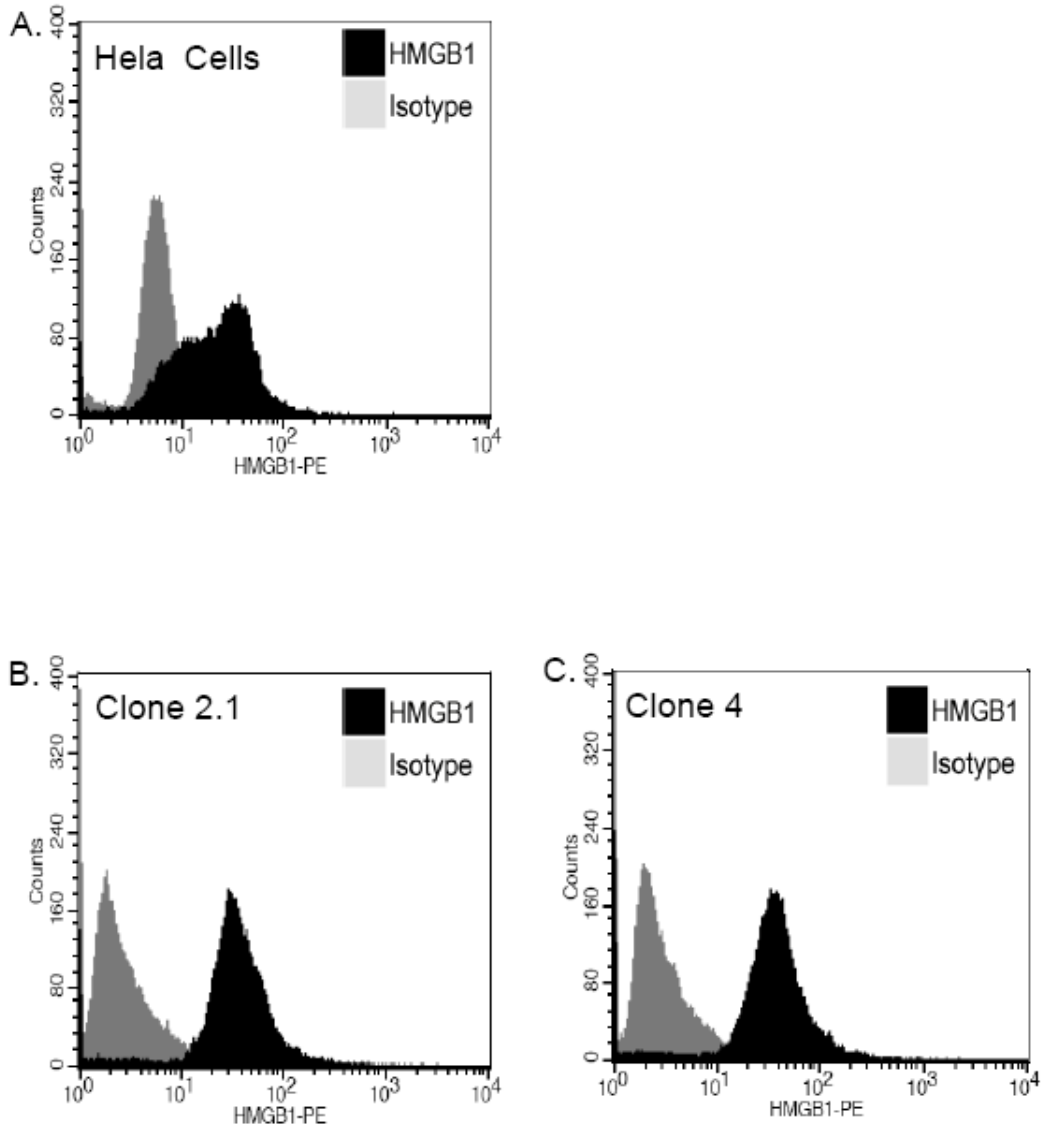


**Figure 32: Intracellular expression of HMGB-1 of Ad5E1 clone 2.1 and clone 4 tumor cells.** Ad5E1 clone 2.1 or Ad5E1 clone 4 tumor cells were examined for intracellular expression of HMGB-1 protein. A. HeLa cells were stained with anti-HMGB-1 antibody or isotype control. B-C Intracellular expression of HMGB-1 was examined in Ad5E1 clone 2.1 and Ad5E1 clone 4 tumor cells, respectively.



## III. IL-17-Dependent, IFN-γ-Independent Tumor Rejection is Mediated by CTLs and Occurs at Extraocular Sites, but is Excluded from the Eye

*Progressive growth of intraocular tumors in IFN-γ-deficient mice*

Previous studies showed that the parental Ad5E1 tumor cell line grew progressively in the eyes of IFN-γ deficient mice (64). To confirm that IFN-γ was also required for intraocular rejection of Ad5E1 clone 2.1 tumors, which undergo phthisical rejection in wild-type C57BL/6 mice, Ad5E1 clone 2.1 ($5 \times 10^4$) tumor cells were injected into the AC of wild-type C57BL/6, IFN-γ KO and SCID mice. Whereas Ad5E1 clone 2.1 tumors were rejected in C57BL/6 mice, tumors grew progressively in IFN-γ KO mice (Figure 33). To confirm that this form of immunity was dependent on T cells, SCID mice were injected in the AC. Indeed, AC tumors grew progressively in T cell-deficient mice (Table 11). Thus, like the parental Ad5E1 tumor line, clone 2.1 tumor rejection in the eye requires IFN-γ produced by T cells.

*IFN-γ-independent tumor rejection can be induced by subcutaneous tumors, but not by intraocular tumors*

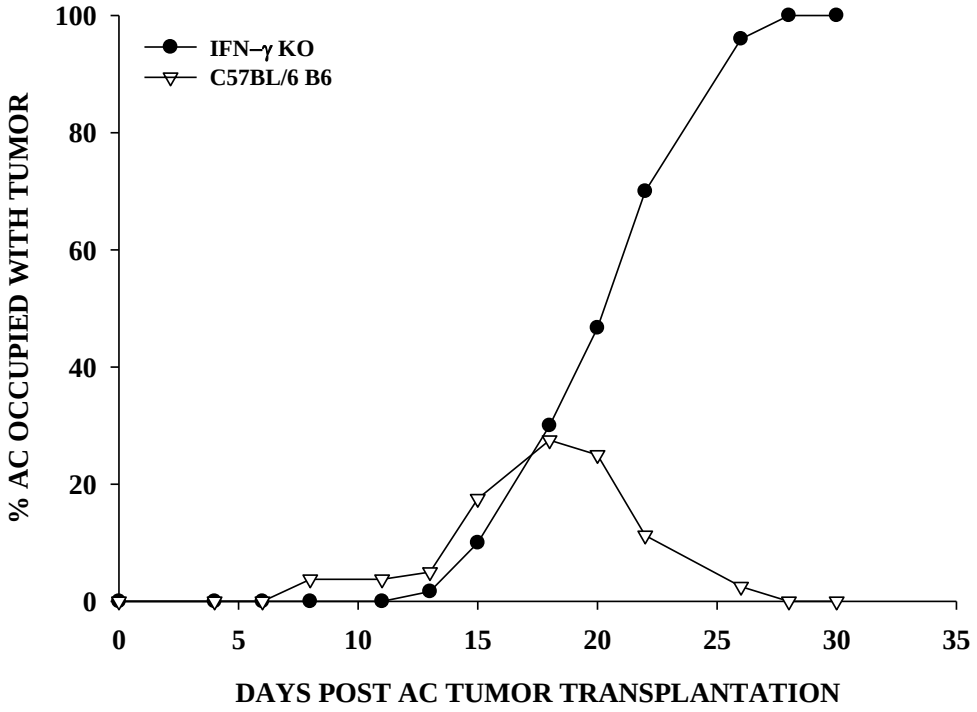
The requirement of IFN-γ for rejection of intraocular Ad5E1 clone 2.1 tumors raised the question as to whether IFN-γ is required for rejection of the

tumor implanted at extraocular sites, or if this form of immunity is unique to the eye. Accordingly, panels of C57BL/6 and IFN-γ KO mice were injected with Ad5E1 clone 2.1 tumor cells ($5 \times 10^4$) either SC or into the AC. As previously shown in Figure 33, Ad5E1 clone 2.1 tumor cells grew progressively in the eyes of IFN-γ KO mice. However, tumors were rejected when injected SC (Table 11). Thus, SC injection of Ad5E1 clone 2.1 tumor cells elicits a form of immunity that is IFN-γ-independent. In order to establish that this form of immunity is T cell-mediated, C57BL/6 SCID mice were injected either SC or in the AC with $5 \times 10^4$ Ad5E1 clone 2.1 tumor cells. Indeed, rejection of both AC and SC tumors required T cells (Table 11).

*SC immunization does not protect against AC tumor challenge*

Previous results demonstrated that SC immunization with parental Ad5E1 tumor cells prevents tumor growth after AC challenge with tumor cells in IFN-γ KO mice (68). However, SC immunization with Ad5E1 clone 2.1 tumor cells does not protect against AC challenge, as the tumors are not rejected and grow progressively in the AC (data not shown). Additionally, attempts to reproduce these results with the parental Ad5E1 tumor line were unsuccessful, as intraocular tumors grew progressively in IFN-γ KO mice regardless of prior SC immunization (data not shown).

**Figure 33. IFN-γ is required for intraocular tumor rejection.** Ad5E1 clone 2.1 tumor cells (5 x $10^4$) were injected into the AC of WT C57BL/6 (∇) and IFN-γ KO (•) mice. Two independent experiments were performed (N = 10/group/experiment).



*IFN-γ-independent tumor rejection requires both $CD4^+$ and $CD8^+$ T cells*

The requirement of T cells for IFN-γ-independent tumor rejection raised the question as to whether $CD4^+$ or $CD8^+$ T cells were required for SC tumor rejection. The T cell population that mediates IFN-γ-independent rejection was determined by depleting IFN-γ KO mice of either $CD4^+$ or $CD8^+$ T cells using monoclonal antibodies. Depletion of $CD4^+$ T cells led to increased SC tumor incidence in anti-CD4 treated IFN-γ KO mice injected with Ad5E1 clone 2.1 tumor cells (80%) compared to the tumor incidence in IFN-γ KO mice treated with the isotype antibody and injected SC with Ad5E1 clone 2.1 tumor cells (30%) (Figure 34). Similarly, depletion of $CD8^+$ T cells led to increased Ad5E1 clone 2.1 SC tumor incidence. SC tumors grew in 90% of CD8-depleted IFN-γ KO animals compared to 30% in isotype control - treated IFN-γ KO mice (Figure 34). Thus, SC Ad5E1 clone 2.1 tumor rejection requires both $CD4^+$ and $CD8^+$ T cells.

*IFN-γ-independent tumor rejection requires IL-17*

Reports by other investigators demonstrated that IFN-γ produced by Th1 T cells cross-regulates the development of Th17 cells that secrete IL-17 (106, 222, 223). Therefore, I hypothesized that the absence of IFN-γ favors the development of Th17 cells that elaborate IL-17, which is necessary for IFN-γ-independent rejection of SC-injected Ad5E1 clone 2.1 tumor cells.

**Table 11: IFN-γ-independent tumor rejection occurs at extraocular sites but is excluded from the eye.** Ad5E1 clone 2.1 tumor cells ($5 \times 10^4$) were injected in the AC or SC. Tumor growth was observed three times/week. Results shown below were recorded on day 30 post tumor transplantation.

| **Host** | **Anterior Chamber** | **Subcutaneous** |
|---|---|---|
| C57BL/6 | Rejected (15/15) | Rejected (10/10) |
| IFN-γ KO | Not Rejected (0/20) | Rejected (22/25) |
| SCID | Not Rejected (0/10) | Not Rejected (1/10) |

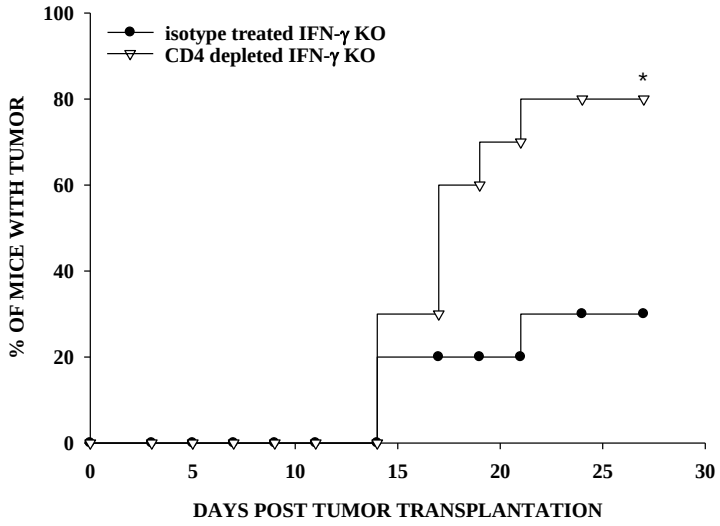
To address this hypothesis IFN-γ KO mice were treated with either anti-IL-17 antibody or an isotype control antibody and injected SC with 5 x $10^4$ Ad5E1 clone 2.1 tumor cells. The majority of the animals treated with anti-IL-17 had SC tumors three weeks after tumor injection (12/15) (Figure 35). By contrast, none of the mice (0/15) treated with the isotype antibody developed tumors, and all remained tumor-free for the duration of the experiment. Although IL-17 was required for tumor rejection in the absence of IFN-γ, it was not required in wild-type hosts that could produce IFN-γ; AC and SC tumor rejection remained unchanged in C57BL/6 mice depleted of IL-17 (Table 3 and data not shown).

*$CD4^+$ T cells from SC tumor rejector IFN-γ KO mice produce IL-17 in response to tumor antigens*

I hypothesized that $CD4^+$ T cells produce IL-17 in response to Ad5E1 clone 2.1 tumor antigens. In order to address this hypothesis, $CD4^+$ T cells were isolated from spleens of IFN-γ KO mice that had rejected SC inoculations of 5 x$10^4$ Ad5E1 clone 2.1 tumor cells two weeks earlier. $CD4^+$ T cells from IFN-γ KO SC tumor rejectors were incubated with Ad5E1 tumor antigen-pulsed APCs, mitomycin-C-treated tumor cells, or medium alone.

**Figure 34. IFN-γ-independent SC tumor rejection requires $CD4^+$ T and $CD8^+$ T cells.** (A) IFN-γ KO mice were treated with either isotype control antibody (rat IgG) or anti-CD4 antibody twice per week. Ad5E1 clone 2.1 tumor cells (5 x $10^4$) were SC injected in the right flank. (B) Animals were treated with either isotype antibody or anti-CD8 antibody twice per week. Ad5E1 clone 2.1 tumor cells (5 x $10^4$) were SC injected in the right flank. Tumor growth was observed three times per week. Each graph represents the combined results of two independent experiments (N = 10/group/experiment). * = $p < .01$, as determine by $\chi^2$ test.

A.



B.

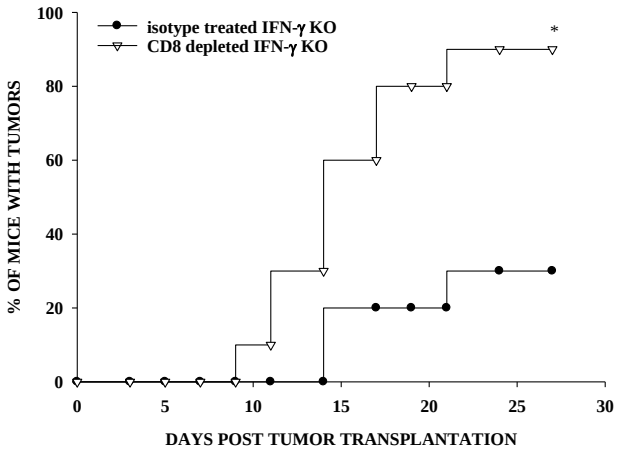
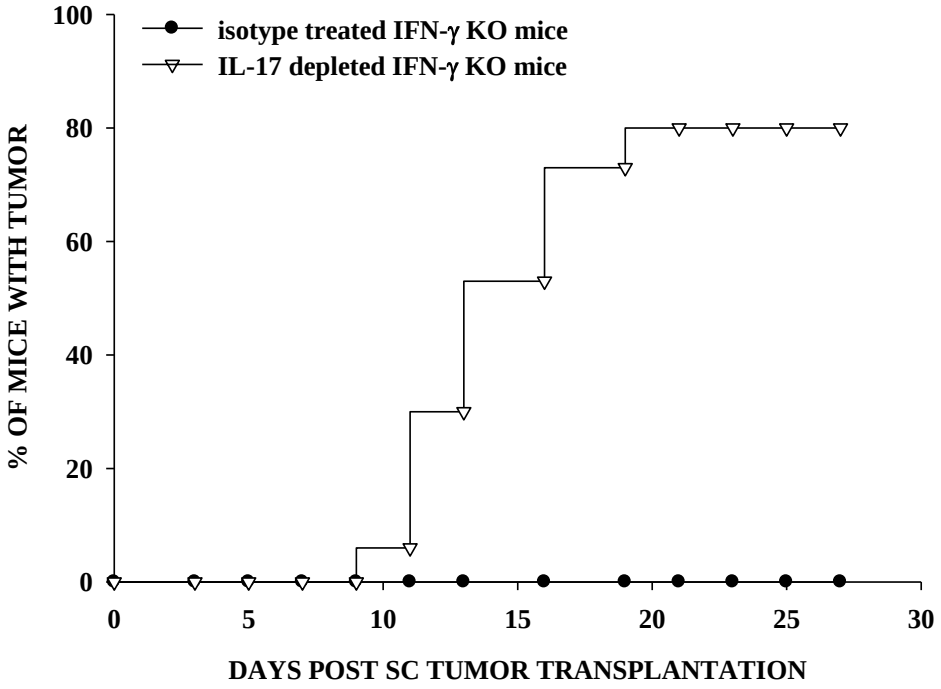

100
80
60
40
20
0
% OF MICE WITH TUMORS
isotype treated IFN-γ KO
CD8 depleted IFN-γ KO
*
0
5
10
15
20
25
30
DAYS POST TUMOR TRANSPLANTATION

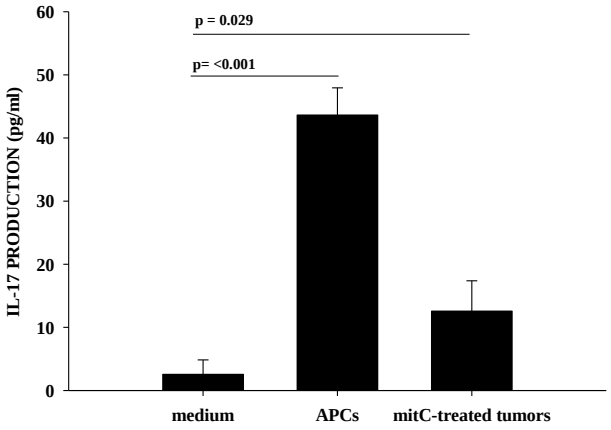
**Figure 35. IFN-γ-independent SC tumor rejection requires IL-17.** IFN-γ KO mice were treated with either isotype control antibody or anti-IL-17 antibody twice per week. Ad5E1 clone 2.1 tumor cells ($5 \times 10^4$) were SC injected in the right flank. Tumor growth was observed three times per week. This graph represents the combined results of three independent experiments (N = 15 mice/group/experiment).



As a positive control, all splenocytes and T cells were found to produce IL-17 when incubated with anti-CD3/CD28 beads (data not shown). After five days, supernatants were harvested and IL-17 concentration was measured by ELISA. IFN-γ- deficient $CD4^+$ T cells produced IL-17 at significantly higher levels when stimulated with Ad5E1 tumor antigen-pulsed APCs or mitomycin-C-treated tumor cells than $CD4^+$ T cells incubated in medium alone (Figure 36A). To confirm that $CD4^+$ T cells from IFN-γ KO mice produced IL-17, bulk splenocyte suspensions were isolated from $CD4^+$ T cell-depleted or rat IgG isotype-treated IFN-γ KO mice and stimulated under the same conditions as described above. As a control, WT C57BL/6 splenocytes were harvested and cultured under the same conditions. Splenocytes from isotype-treated IFN-γ KO mice produced significantly more IL-17 than splenocytes from $CD4^+$ T cell-depleted IFN-γ KO mice in response to Ad5E1 tumor cell lysate (Figure 36B). By contrast, splenocytes from wild-type C57BL/6 mice produced IL-17 at similar levels as $CD4^+$ T cell-depleted IFN-γ KO mice and significantly less than isotype control antibody-treated IFN-γ KO mice (Figure 36B). Together, these results provide evidence that $CD4^+$ T cells are responsible for IL-17 production in the absence of IFN-γ, and the presence of IFN-γ suppresses IL-17-dependent immunity against SC Ad5E1 tumors.

**Figure 36. Rejector CD4$^+$ T cells produce IL-17 following stimulation with tumor antigens.** T cells from SC tumor rejector IFN-γ KO mice were cultured with medium alone, tumor antigen-pulsed APCs, mitomycin-C treated tumor cells (mitC), or anti-CD3/CD28 dynabeads for 5 days at 37 ºC. (A) CD4$^+$ T cells were harvested from isotype antibody treated IFN-γ KO mice. (B) Splenocytes were harvested from isotype antibody-treated IFN-γ KO mice, from anti-CD4-treated IFN-γ KO mice or from WT C57BL/6 mice. Production of IL-17 was determined by ELISA. Each graph is a representative of two independent experiments. P values < 0.05 were considered significant as determined by student's *t* test.

A.




B.

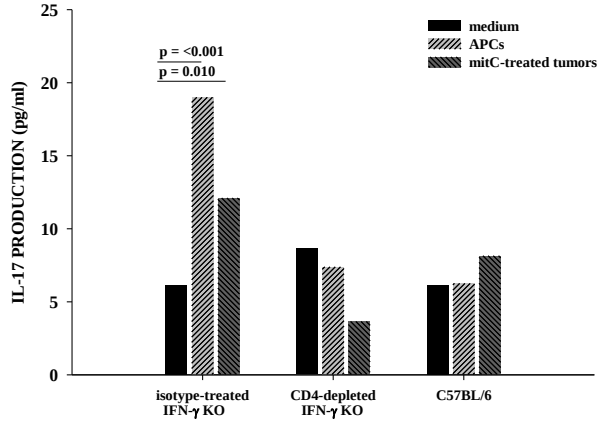
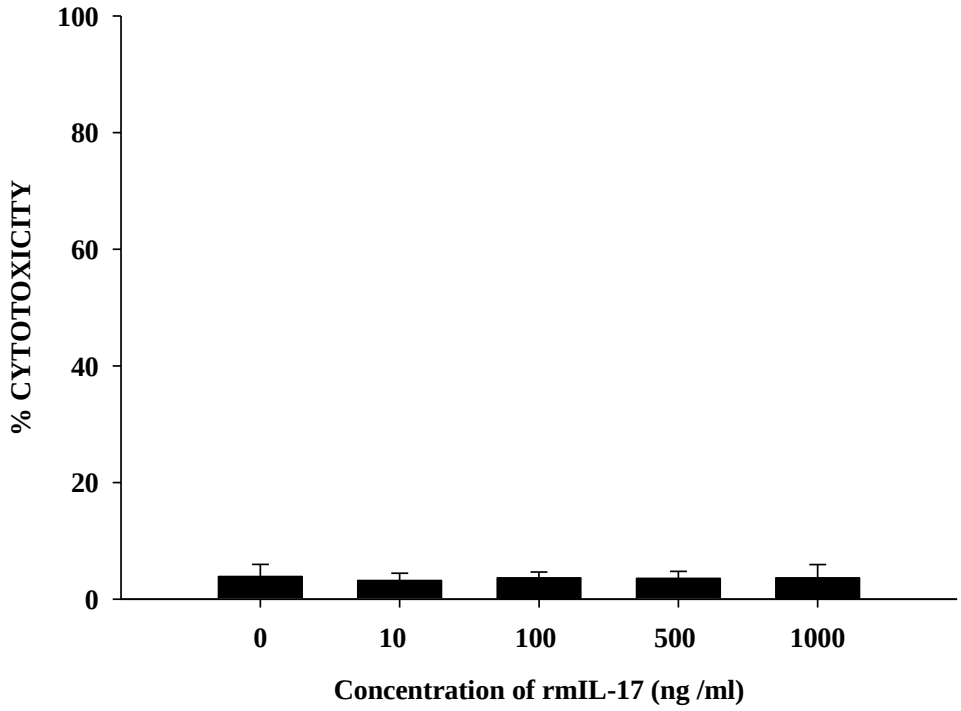


**Figure 37. IL-17 is not toxic to Ad5E1 clone 2.1 tumor cells.** Ad5E1 clone 2.1 cells (1 x $10^5$) were incubated with increasing concentrations of rmIL-17 for 24 h and cytotoxicity was measure by release of LDH.



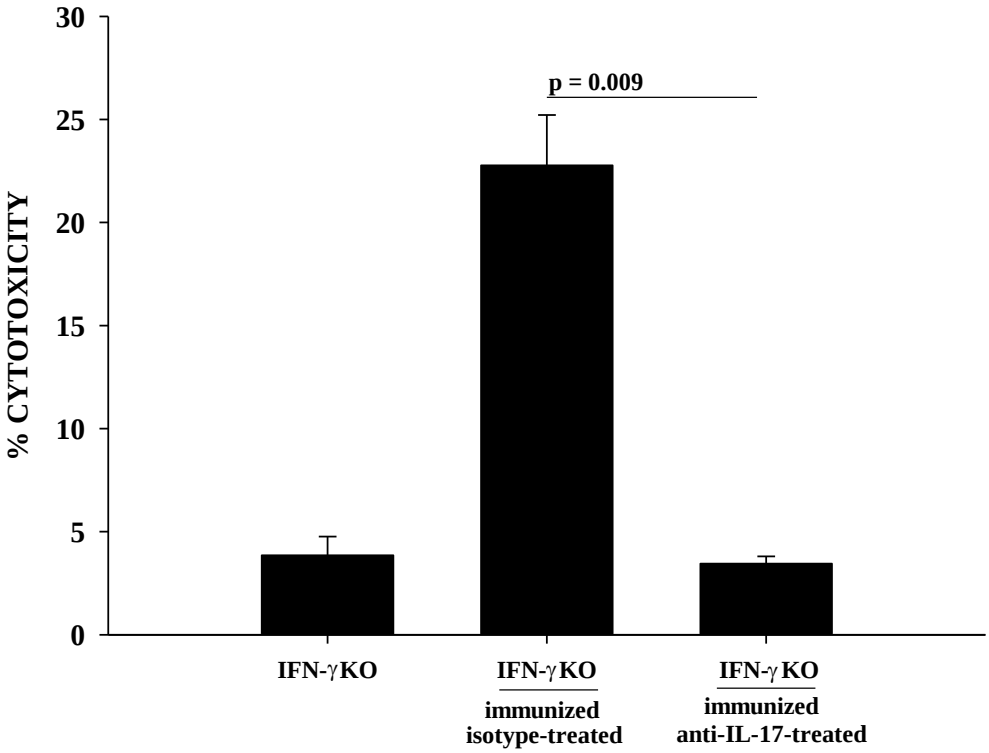
*Depletion of IL-17 reduces CTL activity against Ad5E1 clone 2.1 tumors in IFN-γ-deficient environments*

The next experiments examined the role of IL-17 in the rejection of extraocular Ad5E1 clone 2.1 tumors. First, I tested whether IL-17 was cytotoxic to Ad5E1 clone 2.1 tumor cells. Tumor cells were treated *in vitro* with rmIL-17 at varying concentrations (10 – 1000 ng/ml) for 24 and 48 h. No cytotoxicity was observed at any IL-17 doses at either time point (Figure 37), and thus suggested that IL-17 does not act directly on tumor cells. As presented above, IFN-γ-independent tumor rejection requires $CD8^+$ T cells. The ability of $CD8^+$ CTL to mediate tumor rejection is reflected by their ability to lyse tumor targets. Accordingly, I confirmed $CD8^+$ CTL killing of Ad5E1 clone 2.1 tumor cells using a standard *in vitro* $^{51}Cr$ release CTL assay. Spleen cells from Ad5E1 clone 2.1 SC immunized IL-17-depleted or isotype-treated IFN-γ KO mice were used as tumor-specific effector cells against Ad5E1 clone 2.1 tumor target cells. Spleen cells from C57BL/6 mice injected SC with allogenic P815 mastocytoma cells were used as effector cells against P815 tumor cell targets and served as a positive CTL control (data not shown). Depletion of IL-17 greatly reduced the ability of CTLs to kill Ad5E1 clone 2.1 tumor targets, compared to CTL killing by effector cells from isotype-treated IFN-γ KO mice (4% killing versus 22% killing, respectively) (Figure 38).

*Putative lack of IL-6 in the ocular environment prevents Th17 induction of CTL*

Kryczek et al. (134) demonstrated that Th17 cells predominantly accumulate and differentiate in the tumor microenvironment and not in the tumor draining lymph node. Th17 cell differentiation requires TGF-β and IL-6 (120-122). Notably, the normal ocular environment is devoid of IL-6 unless there is ocular inflammation (224). The presence of IL-6 in the eye has been shown to abolish ocular immune privilege by inhibiting apoptosis of T cells entering the aqueous humor (AqH) or by antagonizing the effects of TGF-β (225). Therefore, the absence of detectable IL-6 in the AqH creates an intraocular environment that dramatically reduces the likelihood of Th17 cell generation in eyes bearing Ad5E1 tumors. The finding that Ad5E1 clone 2.1 tumors grew progressively in the eyes of IFN-γ KO mice but were rejected at extraocular sites led me to hypothesize that the absence of IL-6 within the eye prevents the induction of Th17 cells within the intraocular tumors. Accordingly, Ad5E1 clone 2.1 tumor-bearing eyes and non-tumor bearing eyes were collected from WT C57BL/6, IFN-γ KO, and SCID mice on day 14 post AC tumor injection (i.e., the peak time of intraocular tumor growth in WT C57BL/6 mice) and homogenized. To assess the IL-17 gene transcription levels in the eye, RNA was immediately isolated and quantitative PCR was performed to determine the expression of IL-17.

**Figure 38. Depletion of IL-17 results in the reduction of CTL activity against Ad5E1 clone 2.1 tumors in IFN-γ KO mice.** Splenocytes from naïve or Ad5E1 clone 2.1 immunized C57BL/6 and IFN-γ KO mice (either treated with isotype-antibody or anti-IL-17 antibody) were harvested and cultured with mitomycin-C-treated Ad5E1 clone 2.1 tumor cells for 5 days at 37 ºC. Cytotoxicity was measured by a standard $^{51}Cr$ release assay at a 100:1 E: T ratio. Graph is the combined results of two independent experiments. P values < 0.05 were considered significant as determined by student's *t* test.

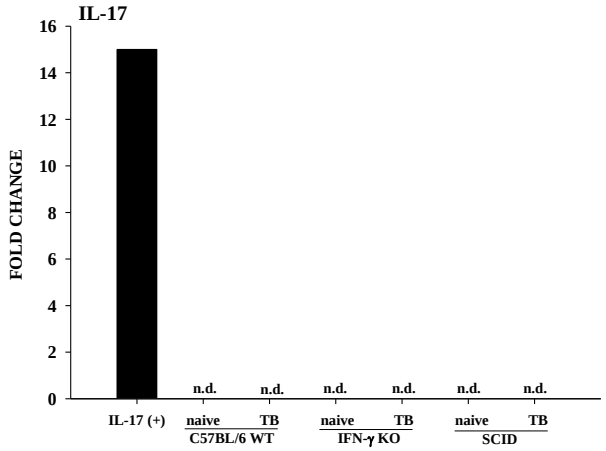


All samples were compared to IL-6 KO eyes because it has been reported that IL-6 KO mice do not generate Th17 cells (226). IL-17 expression was not observed in normal eyes or tumor-bearing eyes of WT mice, IL-6 KO mice, IFN-γ KO mice, or SCID mice (Figure 39). As a positive IL-17 control, $CD4^+$ T cells were polarized *in vitro* into a Th17 phenotype and demonstrated an elevated level of IL-17 mRNA expression. qPCR was also performed to assess the level of intraocular IL-6 expression. IL-6 mRNA expression was not detected in either non-tumor-bearing or tumor bearing-eyes of C57BL/6, IFN-γ KO or SCID mice (Figure 39B). As a positive IL-6 control, RAW 264.7 cells were polarized into a M1 macrophage phenotype and displayed >4 fold increase in IL-6 expression.

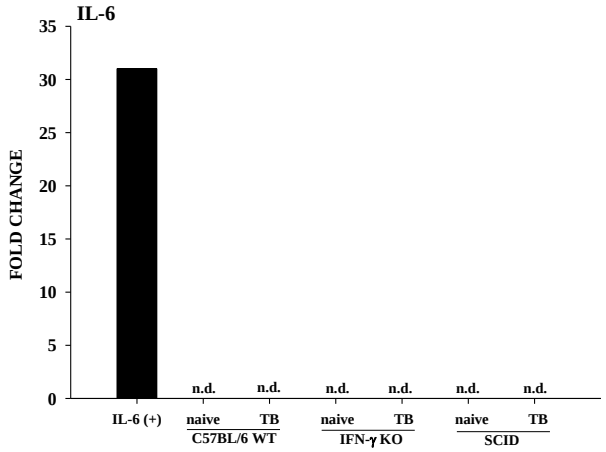
To further address the hypothesis that CTLs are not generated in the eyes of IFN-γ KO mice, the expression of CD8 and perforin was examined by qPCR. Expression of CD8 and perforin was not detected in naïve or tumor-bearing eyes of IFN-γ KO or SCID mice (Figure 39C and D). As expected, CD8 and perforin was observed in tumor-bearing eyes of WT IFN-γ competent C57BL/6 mice.

**Figure 39. IFN-γ-deficient naïve and Ad5E1 clone 2.1 tumor-bearing eyes do not express IL-17, IL-6, CD8, or perforin.** Non-tumor-bearing and Ad5E1 clone 2.1 tumor-bearing eyes of C57BL/6, IFN-γ KO and SCID mice were harvested. RNA was isolated and converted to cDNA. mRNA expression of IL-17 (A), IL-6 (B), CD8 (C) and perforin (D) was determined by qPCR. Samples were compared to naïve IL-6 KO eye. As positive controls for IL-17 and IL-6, $CD4^+$ T cells and RAW 264.6 cells were polarized into a Th17 and M1 phenotype, respectively. Each graph is a representative of two independent experiments. TB = tumor-bearing; n.d. = not detected.

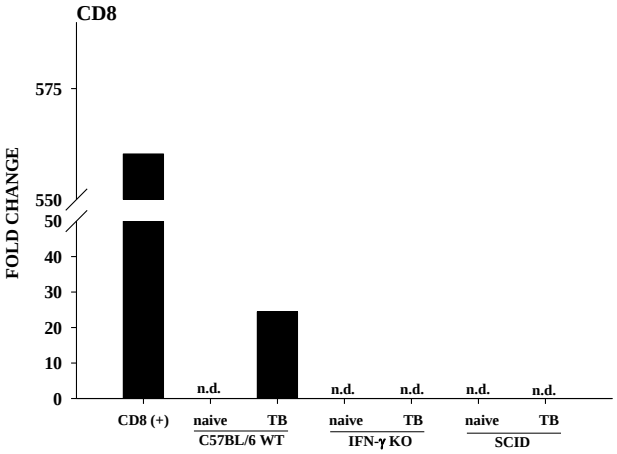
A.



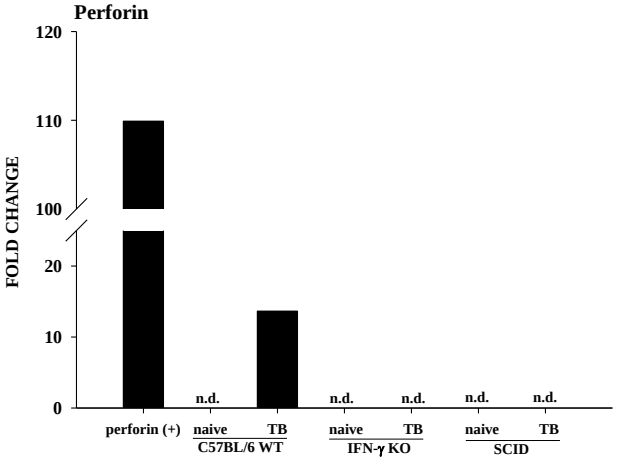

B.



C.


CD8
FOLD CHANGE
575
550
50
40
30
20
10
0
n.d.
n.d.
n.d.
n.d.
n.d.
n.d.
CD8 (+)
naive
TB
C57BL/6 WT
naive
TB
IFN-γ KO
naive
TB
SCID

D.


Perforin
FOLD CHANGE
120
110
100
20
10
0
n.d.
n.d.
n.d.
n.d.
n.d.
n.d.
perforin (+)
naive
TB
C57BL/6 WT
naive
TB
IFN-γ KO
naive
TB
SCID

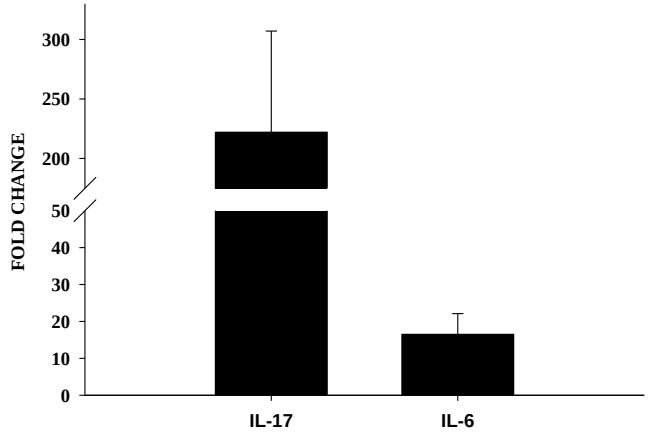
Additional experiments assessed the expression of IL-6 and IL-17 within the SC tumor site. Ad5E1 clone 2.1 tumor cells ($5 \times 10^4$) were injected SC in the right flank of IFN-γ KO mice. After 7 days, skin surrounding the tumor injection site or from the contralateral flank (non tumor bearing) was excised and homogenized. RNA was isolated from skin samples and qPCR was performed to detect IL-6 and IL-17 mRNA expression. Skin samples from the Ad5E1 clone 2.1 tumor site expressed a 200-fold and a 10-fold increase in IL-17 and IL-6 expression, respectively, compared to tumor-free contralateral skin samples from the same mouse. (Figure 40A). The finding that extraocular (SC) tissues injected with tumor cells express IL-6 and IL-17, and that these cytokines are absent in tumor-bearing eyes, suggests that IL-17 is required for IFN-γ-independent tumor rejection at extraocular sites.

In order to confirm the role of CTLs in rejection of SC tumors the expression of CD8 and perforin was examined. mRNA expression of CD8 and perforin was increased 15-fold and 5-fold in tumor-bearing skin compared to normal skin of IFN-γ KO mice (Figure 40B). By contrast, neither CD8 nor perforin expression was observed in tumor-bearing eyes of IFN-γ KO mice (Figure 39C and D). Thus, CTL activity only occurs in extraocular sites that are replete with IL-6.

Lastly, I hypothesized that SC immunization of IFN-γ KO mice with Ad5E1 clone 2.1 tumor cells may allow for the generation of Th17 cells that migrate to the eye to induce CTL-mediated tumor rejection. In order to test this hypothesis, IFN-γ KO mice were immunized with 5 x $10^4$ Ad5E1 clone 2.1 tumor cells two weeks prior to AC injection of tumor. As controls, non-immunized IFN-γ KO and WT mice were also AC injected with Ad5E1 clone 2.1 tumor cells. Tumor-bearing eyes were harvested at days 12, 14, and 17 post tumor injection. Expression levels of IL-17, IL-6, CD8, and perforin were assessed by qPCR. No expression of IL-17, IL-6, CD8, or perforin was observed in tumor-bearing eyes of immunized or non-immunized IFN-γ KO mice (Figure 39). However, expression of CD8 and perforin was observed in tumor-bearing eyes of WT mice, but not IL-6 or IL-17 (Figure 39). Thus, SC immunization does not induce the generation of Th17 cells or $CD8^+$ CTLs that can mediate tumor rejection in the eye.

**Figure 40. SC tumor sites express IL-17, IL-6, CD8, and perforin**. To determine the expression of IL-17, IL-6, CD8, and perforin in SC tumors Ad5E1 clone 2.1 tumor cells (5 x $10^4$) were injected in the skin of IFN-γ KO mice. After 7 days, skin samples were excised and RNA was isolated. The same qPCR procedure was done as described above. mRNA expression of IL-17 and IL-6 (A), CD8 and perforin (B) was observed in skin samples injected with tumor cells. Samples were normalized to GAPDH and compared to normal (naïve) skin. Each graph is the combined results of eight individual mice.

A.

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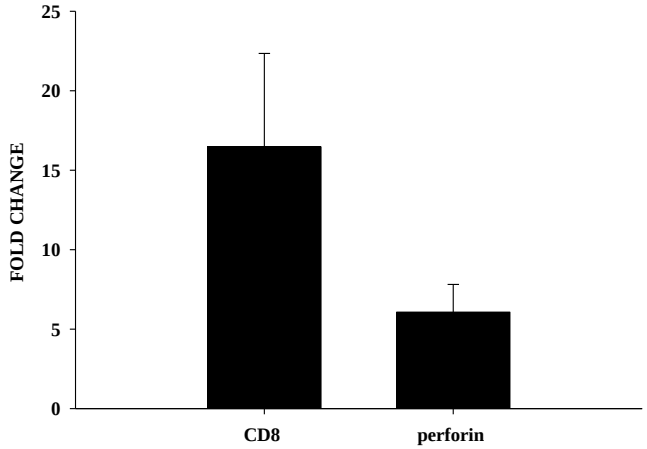


# CHAPTER FOUR
# DISCUSSION AND FUTURE RESEARCH

## Non-Phthisical versus Phthisical Rejection

Immune-mediated rejection of intraocular tumors can follow two different pathways (40). The first pathway involves piecemeal necrosis and eradication of the tumor without damage to innocent bystander ocular cells. The second pathway involves rejection of intraocular tumors and culminates in extensive damage to innocent bystander cells and phthisis of the eye (40, 41). Obviously, phthisical rejection of intraocular tumors is detrimental, as it leads to blindness. The hallmarks of phthisis are generally known, however, the mechanisms of phthisical rejection are not. Previous studies described phthisical intraocular rejection in gross anatomical terms, but have not identified the distinct immune mechanisms that culminate in this rejection phenotype. The presence of infiltrating mononuclear cells is not a hallmark restricted to either of these pathways, as both patterns of rejection are observed to have mononuclear infiltrates; however, the outcome of rejection is dramatically different. The objective of my work is to characterize the immune mechanisms that are involved in each rejection phenotype. Although previous studies have characterized immune mechanisms of non-phthisical rejection (65, 66, 68), to my knowledge, this is the first in depth

study that has examined the immune mechanisms that mediate phthisical rejection of intraocular tumors.

### *Identification of phthisical and non-phthisical clones*

In some studies using the parental Ad5E1 tumor line, it was observed that approximately 20% of animals receiving intraocular tumor cells rejected tumors in a phthisical manner. I used this heterogeneity to my advantage and created Ad5E1 clones that consistently underwent rejection either with a non-phthisical or phthisical rejection phenotype. Accordingly, I identified tumor clones that consistently underwent rejection in a phthisical or non-phthisical manner, Ad5E1 clone 2.1 or Ad5E1 clone 4, respectively. It was observed that Ad5E1 clone 2.1 tumors had increased growth i*n vivo* and occupied much of the AC. I hypothesized that phthisis may occur with Ad5E1 clone 2.1 tumors because the tumor size is so large that it results in necrosis, and increased growth is the result of an increased proliferation rate of Ad5E1 clone 2.1 tumor cells. However, *in vitro* proliferation studies showed that there was not a significant difference in cell proliferation, indicating that increased *in vivo* growth of Ad5E1 clone 2.1 tumors is not the result of an increased ability to proliferate, but is due to an aberrant immune response.

### *The role of IFN-γ in phthisical and non-phthisical intraocular tumor rejection*

Although rejection of both Ad5E1 clone 2.1 and Ad5E1 clone 4 tumors can be mediated by either $CD4^+$ or $CD8^+$ T cells, these divergent patterns of rejection have a dissimilar requirement of IFN-γ for rejection. Dace et al. previously described mechanisms of non-phthisical rejection and showed that rejection of intraocular Ad5E1 tumors requires IFN-γ, which can act directly on tumor cells by: (a) inhibiting tumor cell proliferation, (b) inducing tumor cell apoptosis, and (c) simultaneously downregulating proangiogenic genes and upregulating antiangiogenic genes in the tumors (40, 41, 65, 66, 68). In contrast with these results, I have demonstrated that rejection of Ad5E1 clone 2.1 tumors requires IFN-γ; however, rejection of Ad5E1 clone 4 tumors does not. This points to the pleiotropic nature of IFN-γ in intraocular tumor rejection of Ad5E1 tumors. The observation that tumors that are rejected in a phthisical manner grow substantially larger than non-phthisically rejected tumors prior to rejection is evidence that the role of IFN-γ in phthisical rejection does not function the same as in non-phthisical rejection. The role of IFN-γ in non-phthisical rejection is to limit the size of intraocular tumors. If IFN-γ functioned the same in phthisical rejection, it would have a negative effect on angiogenesis, limiting the size of the tumor. Thus, the tumor could not develop the necessary blood vessels to maintain growth. Once the tumor reached a certain size, it could not grow any larger due to limitations in nutrition. The inhibition of tumor cell proliferation, increased apoptosis, and decreased angiogenesis would lead to a smaller tumor mass and

may prohibit phthisical rejection. Although not required for non-phthisical rejection, IFN-γ probably acts directly on Ad5E1 clone 4 tumor cells as previously described above. The role of IFN-γ in phthisical rejection is related to its essential function in the activation of the innate immune system. IFN-γ is important for activation of macrophages that kill microbial pathogens and tumor cells (227-229).

Previous studies with the parental Ad5E1 tumor cell line indicated that there was spontaneous rejection of ocular tumors in a non-phthisical manner, which required IFN-γ and $CD4^+$, but not $CD8^+$ T cells (65, 68). What changed about the Ad5E1 clone 4 tumor cell line that allowed rejection by both $CD4^+$ and $CD8^+$ T cells in an IFN-γ-independent manner? Studies have shown that the immune response is tumor specific between Ad5E1 clone 2.1 and Ad5E1 clone 4 tumors. It is possible that Ad5E1 clone 4 tumor cells are more sensitive to $CD8^+$ T cells than the parental Ad5E1 tumor line because Ad5E1 clone 4 tumors express more receptors or produce factors that disable the $CD8^+$ T cells. However, the mechanisms of rejection appear to be highly redundant as rejection of Ad5E1 clone 4 tumors can be mediated by either $CD4^+$ or $CD8^+$ T cells.

***The role of macrophages in non-phthisical and phthisical intraocular tumor rejection***

Macrophages are crucial for the non-phthisical and phthisical rejection of intraocular Ad5E1 tumors. Like in the case of IFN-γ, macrophages have different functions in phthisical rejection than in non-phthisical rejection. As discussed above, there are two basic subsets of macrophages: (a) M1 macrophages, polarized by IFN-γ and microbial products and (b) M2 macrophages, polarized by IL-4, IL-10, and IL-13. M2 macrophages are typically associated with Th2 responses and tend to reduce tissue destruction through their elaboration of immunosuppressive and anti-inflammatory cytokines such as TGF-β and IL-10.

*The role of macrophages in phthisical rejection*

The requirement of macrophages for rejection of Ad5E1 clone 2.1 tumors led to the hypothesis that macrophages are intimately involved in the necrosis that culminates in phthisis. Although I found evidence of M2 macrophage-related molecules in Ad5E1 clone 2.1 tumor-bearing eyes, M2 cytokine expression was only slightly elevated above background levels. In contrast, the same tumor-bearing eyes expressed a 30-fold increase in M1 macrophage–associated NOS2. This supports the conclusion that M1 macrophages were involved in the pathologic sequelae leading to phthisis. M1 macrophages produce large amounts of proinflammatory and Th1-polarizing cytokines including IL-12, IL-6, IL-23 and TNF-α. M1 macrophages also contribute to tissue damage through their production of toxic intermediates such as NO and ROI.

My findings indicate that macrophages can directly kill Ad5E1 clone 2.1 tumor cells *in vitro*, supporting the hypothesis that tumor infiltrating macrophages in Ad5E1 intraocular tumors are of the M1 subset that can directly mediate tumor rejection via secretion of TNF-α and NO and ROI (230-232). This is in contrast to previous work done with the Ad5E1 tumor model. Dace et al. showed that macrophages predominately function as APCs in the afferent phase of tumor rejection as mice depleted of macrophages developed $CD4^+$ T cells that were unable to produce IFN-γ in response to Ad5E1 tumors (68). However, this study also showed a role for $CD4^+$ T cells in the effector phase of tumor rejection as $CD4^+$ T cells isolated from rejector mice were unable to mediate rejection when adoptively transferred to macrophage-depleted SCID mice (68), but direct macrophage killing was not observed using macrophages isolated from the peritoneum. Peritoneal macrophages were induced by injection of the sterile, inflammatory agent, thioglycollate. Concerns have been raised as to how inflammatory agents affect the activation state and contribute to the heterogeneity of peritoneal macrophages. For this reason I chose to assess the ability of macrophages to mediate rejection of Ad5E1 tumor with bone marrow-derived macrophages (BMDMs). Thus, studies done with BMDMs may prove that the parental Ad5E1 tumor line is also susceptible to macrophage-mediated killing.

Similar to previous studies, Ad5E1 tumor clone 2.1 tumor cells are highly susceptible to macrophage-mediated killing *in vitro* and presumably *in vivo*, based

on the progressive intraocular tumor growth in mice whose periocular macrophage population has been deleted by subconjunctival injection of clodronate-containing liposomes. This conclusion is further supported by the observation that inhibition of the M1 macrophage mediator, NO, prevents tumor rejection. Nitric oxide was one of the first macrophage killing mechanisms that was identified and has pleiotropic tumorigenic properties from promotion of angiogenesis to enhancement of immunosuppression by tumors (233, 234). Paradoxically, the most recognized effector molecule employed by M1 macrophages to kill tumors is NO, and it has been well documented that NO produced by M1 macrophages effectively kills tumor cells (233-236). My results show that inhibition of iNOS reduces macrophage-mediated cytotoxicity *in vitro* and prevents tumor rejection.

In addition to direct killing of tumor cells, a decrease in iNOS could reduce the angiogenic and immunosuppressive properties of tumor cells that are resistant to cytotoxicity, thus further reducing tumor growth. The role that NO plays in Ad5E1 tumor rejection is complex as it may have different functions depending on the stage of tumor growth or rejection. Early on in tumor growth NO, which induces expression of VEGF, may increase angiogenesis allowing increased growth of Ad5E1 clone 2.1tumors. Low levels of NO have also been shown to prevent apoptosis in several cell lines (237, 238) and also possibly allow for increased initial growth of Ad5E1 clone 2.1tumors. However, when the tumor

grows larger and more M1 macrophages are induced, NO may then become cytotoxic. The cytotoxic nature of NO is dependent on a high concentration of NO in the tumor microenvironment (235). In other words, NO has a protumor function until reaching a certain concentration threshold, whereby it becomes cytotoxic.

Interestingly, several studies in pancreatic cancer, breast cancer, and colon cancer have indicated that NO-mediated apoptosis is correlated with increased levels of IL-1, TNF-α, and IFN-γ in the tumor microenvironment (239-241). This may explain the requirement of IFN-γ and TNF-α for phthisical rejection but not for non-phthisical rejection.

Consistent with this idea, IFN-γ plays a pivotal role in recruitment of M1 macrophages into the intraocular tumor microenvironment. Progressive growth of Ad5E1 clone 2.1 tumors in SCID mice and the failure of these hosts to recruit macrophages into the intraocular tumors suggest that T cells produce IFN-γ that is required for recruitment and activation of M1 macrophages. More specifically, in IFN-γ deficient mice there are fewer $F4/80^+$ and $CD11b^+$ macrophages and less $NOS2^+$ M1 macrophages. Thus, in the absence of IFN-γ, M1 macrophages are not present to produce NO and induce phthisical rejection. The production of IFN-γ polarizes macrophages to an M1 phenotype. M1 macrophages produce NO that effectively destroys the tumor, but also causes bystander damage to normal ocular cells.

Evidence that further supports the hypothesis that M1 macrophages are intimately involved in phthisical rejection is that normal ocular cells, such as corneal endothelial cells and iris and ciliary body cells, are highly susceptible to macrophage-mediated cytotoxicity. The corneal endothelium, which is a single layer of cell on the inner surface of the cornea, is in direct contact with tumors residing in the AC. Like other structures of the eye, the corneal endothelium does not have regenerative properties and there are currently no medical treatments that can promote wound healing or regeneration of the corneal endothelium.

*The role of macrophages in non-phthisical rejection*

The function of macrophages in non-phthisical rejection is not as straight forward. As in rejection of Ad5E1 clone 2.1 tumors, the rejection of Ad5E1 clone 4 tumors also requires macrophages as depletion of macrophages leads to progressive growth of intraocular tumors. Ad5E1 clone 4 tumor-bearing eyes also contain M1 macrophages, but also may contain a population of M2 macrophages; as tumor-bearing eyes containing Ad5E1 clone 4 tumors have increased expression of the M2 macrophage markers, Arg1 and Ym1, compared to naïve eyes. The function of these M2 macrophages in this model has not been examined. M2 macrophages, typically called TAMs, are protumor and suppress anti-tumor responses, specifically iNOS production, by producing IL-10, TGF-β, and other suppressive cytokines. It is possible that M2 macrophages are charged

with the responsibility of keeping M1 macrophages in check. Thus, M2 macrophages may suppress the production of NO by M1 macrophages, preventing phthisis but still allowing tumor rejection by other means.

However, Ad5E1 clone 4 tumor cells are highly susceptible to macrophage-mediated killing *in vitro*. Unlike the macrophage-mediated killing of Ad5E1 clone 2.1 tumor cells, the inhibition of iNOS does not reduce macrophage-mediated cytotoxicity of Ad5E1 clone 4 tumor cells. Consistent with these results, *in vivo* inhibition of iNOS does not affect the rejection of Ad5E1 clone 4 tumors, suggesting that rejection of Ad5E1 clone 4 tumors is independent of NO. Thus, it is unlikely that macrophages mediate Ad5E1 tumor rejection by the expression of NO, as this would induce significant damage to the normal ocular tissue, whereas elimination of Ad5E1 clone 4 tumors results in a pristine tumor rejection that results in no damage to normal ocular tissue. Although not required for rejection, it is possible that M1 macrophages are producing low levels of NO that mediate Ad5E1 clone 4 tumor rejection. Damage to normal ocular tissue could be prevented by the presence of antioxidants in the ocular environment. For example, the AqH has abundant levels of ascorbic acid, a known antioxidant, which can lead to decreased levels of nitrite in the eye (242). NO sensitivity varies among different tumor lines and cell types (243). Another explanation for different requirement of NO for phthisical rejection versus non-phthisical rejection is that Ad5E1 clone 2.1 tumor cells are less sensitive to NO-mediated

cytotoxicity compared to Ad5E1 clone 4 tumor cells. Very low levels of NO mediate rejection of Ad5E1 clone 4 tumors and higher concentrations are required for rejection of Ad5E1 clone 2.1tumors. A higher concentration of NO mediates destruction of normal ocular cells and further increases the production of proinflammatory cytokines.

*In vitro* studies have indicated that macrophage-mediated killing occurs via a least two mechanisms. Work done with the transwell culture system has demonstrated that there is a contact-dependent and contact-independent mechanism of rejection. I have identified that macrophages use a soluble protein with a molecular weight between 50 and 100 kDa. The discovery of this soluble factor may give new insights into the role of macrophages in non-phthisical rejection and describe the mechanism macrophages could use without inducing phthisical rejection via NO.

As mentioned earlier, previous studies of non-phthisical Ad5E1 tumor rejection have shown that macrophages have an important role in antigen presentation to T cells. It is possible that the major *in vivo* function of macrophages in non-phthisical rejection is to act as APCs. Thus, the role of macrophages in non-phthisical rejection of Ad5E1 clone 4 tumors is probably multifunctional and has important duties in both the afferent and efferent arms of the immune response.

***The role of TNF-α in non-phthisical and phthisical intraocular tumor rejection***

*The role of TNF-α in phthisical rejection*

Previous studies demonstrated that TNF-α, although not required for tumor rejection, was produced by $CD8^+$ T cells in response to parental Ad5E1 tumor antigens to mediate tumor rejection (66). My initial hypothesis was that TNF-α, like in the rejection of the parental Ad5E1, mediated non-phthisical rejection. However, TNF-α was not found to be required for the rejection of either tumor clone. Interestingly, in TNF-α- deficient mice, rejection of Ad5E1 clone 2.1 tumors was found to be non-phthisical. Although the generation of TNF-α is not required for tumor rejection, it is essential for the development of phthisis. Like NO, TNF-α kills normal ocular cells *in vitro* and plays a crucial role in the extensive injury to innocent bystander cells in the eye, which culminates in phthisis. Both Ad5E1 clones are sensitive to TNF-α-mediated cyotoxicity; however, binding of TNF-α on TNFRs (or IFN-γR) may have a different response on Ad5E1 clone 2.1 tumor cells compared to Ad5E1 clone 4 tumor cells. Muller-Hermelink et al. showed that in T-antigen (Tag)-induced multistage carcinogenesis in pancreatic islet, combined TNFR1 signaling and IFN-γ signaling on tumor cells prevented tumor angiogenesis and proliferation without further destruction of islet cells. In the absence of either TNFR signaling or IFN-γ signaling, the same immune response promoted angiogenesis and multistage

carcinogenesis (169). By similar means, dual signaling of TNF-α and IFN-γ may lead to a cascade of events that culminate in phthisis. In the absence of TNF-α this does not occur, and the tumor is rejected in a non-phthisical manner.

Ad5E1 clone 2.1 tumors have decreased growth in the AC of TNF-α KO mice compared to WT mice and resemble the growth pattern of Ad5E1 clone 4 intraocular tumors in WT mice. The question remains whether this is due to less immune cell infiltration or to differences in tumor growth in TNF-α deficient environments. This could be addressed by examining the percentage of tumor cells present in intraocular tumors compared to the total number of cells present in the tumor mass. It is possible that TNF-α promotes a strong pro-inflammatory environment that induces the recruitment of lymphocytes contributing to the increased size of the tumor within the AC resulting in an increase in tumor mass size, intraocular damage and culminating in phthisis.

This hypothesis is supported by experiments conducted in TNFR KO mice. Intraocular tumors in mice lacking either TNFR1 or TNFR2 still underwent phthisical rejection. In these experiments the only cells capable of responding to TNF-α were the tumor cells. Thus, the required TNF-α signaling needed for phthisis occurs within the tumor cells themselves and not the host cells. Although host cells do not need to respond to TNF-α for phthisis to occur, TNF-α does promote cytotoxicity of normal ocular cells such as corneal endothelium and iris and ciliary body cells contributing to phthisis.

Further studies showed that T cells were the major source of TNF-α, as mRNA levels of TNF-α were significantly reduced in SCID and IFN-γ KO mice. Additionally, $CD4^+$ T cells make TNF-α in response to tumor antigens, and TNF-α KO T cells mediate non-phthisical rejection in a majority of animals compared to WT T cells that mediate phthisical rejection.

Thus, this model demonstrates that it is possible to modify the host's immune response against tumors such that the immune system eliminates the intraocular tumor while preserving the integrity of the eye. Immune-mediated phthisis underscores the importance of immune privilege in restraining intraocular inflammation and preserving the integrity of ocular tissues, many of which are incapable of regeneration. Understanding the mechanisms that circumvent immune privilege and culminate in phthisis may facilitate the development of immunotherapy that promotes tumor rejection while preserving vision. This may shape the nature of therapies invoked for the treatment of other inflammatory eye diseases such as sympathetic ophthalmia and uveitis.

*The role of TNF-α in non-phthisical rejection*

In contrast to phthisical rejection, TNF-α is not required for non-phthisical rejection. As mentioned above, previous studies with the parental Ad5E1 tumor model indicated that TNF-α produced by $CD8^+$ T cells, although not required,

was sufficient to mediate tumor rejection. Thus, TNF-α may play a similar role in rejection of Ad5E1 clone 4 tumors. The role of TNF-α in tumor rejection may be dependent upon which TNFR is bound by TNF-α. Although TNFR1 is sufficient to induce the cytotoxicity and proinflammatory response of TNF-α, TNFR2 contributes to the TNFR1 responses at low concentrations of TNF-α. TNFRs are thought to have opposing actions as TNFR1-dominant responses support inflammation and TNFR2 responses promote apoptosis and have a protective role (170, 244, 245). Therefore, it is possible that the effect of TNF-α-mediated responses is dependent on preferential binding to specific TNFRs. At high concentrations, TNF-α may predominantly bind TNFR1 on normal ocular cells and Ad5E1 clone 2.1 tumor cells and induce inflammation and perpetuating phthisis. The increased inflammatory response may lead to the production of more TNF-α, thereby amplifying the response. A caveat to this hypothesis is the fact that Ad5E1 clone 2.1 tumors are rejected phthisically in both TNFR1 and TNFR2 KO mice. Nevertheless, at low concentrations TNF-α may dominantly bind TNFR2 on normal ocular cells and Ad5E1 clone 4 tumor cells leading to apoptosis and prevention of further inflammation, allowing for non-phthisical rejection.

## A MODEL FOR PHTHISICAL AD5E1 TUMOR REJECTION

Following injection of Ad5E1 clone 2.1 tumor cells into the eye, I hypothesize that innate immune cells migrate to the tumor-bearing eye, specifically dendritic cells (DCs). DCs, as antigen-presenting cells, present Ad5E1 tumor antigens to both $CD4^+$ T cells and $CD8^+$ T cells. These T cells produce molecules, specifically IFN-γ, that polarize monocytes into M1 macrophages. $CD4^+$ T cells also produce TNF-α that further polarizes macrophages into a M1 phenotype. Activated M1 macrophages produce NO that mediates rejection of the tumor and results in phthisis of the eye. Also, ocular macrophages might possibly present antigen to $CD4^+$ T cells in the eye, keeping the $CD4^+$ T cells in an activated state and increasing their IFN-γ production in response to Ad5E1 clone 2.1 tumor antigens. These components are essential for phthisical rejection as depletion of macrophages, T cells, and IFN-γ prevents rejection of Ad5E1 clone 2.1 tumors. Although TNF-α is not required for rejection, it is required for the phthisis of the eye after rejection. My working model (described above) of intraocular Ad5E1 clone 2.1 tumor rejection is illustrated in Figure 41. Further studies remain to determine the exact role of TNF-α in phthisical rejection. This model is only my hypotheses of how $CD4^+$ T cells, macrophages (NO), IFN-γ, and TNF-α contribute to rejection, and is not to be interpreted as the definitive description of phthisical Ad5E1 tumor rejection.

My study is unique because, to the best of my knowledge, it is the first study to examine the mechanisms of phthisical intraocular tumor rejection. This

work may enhance our scientific knowledge of how to combat tumors in an immune privileged environment without inducing damage to normal tissue. My work demonstrates that phthisical intraocular tumor rejection can be prevented, and identifies TNF-α as the key cytokine in phthisical intraocular tumor rejection. This may have important implications in treatments of other diseases such as uveitis or SO.

## A MODEL OF NON-PHTHISICAL AD5E1 TUMOR REJECTION

Following injection of Ad5E1 clone 4 tumor cells into the eye, I hypothesize that innate immune cells migrate to the tumor-bearing eye, specifically DCs. DCs, as antigen-presenting cells, present Ad5E1 tumor antigens to both $CD4^+$ T cells and $CD8^+$ T cells. These T cells produce molecules, specifically IFN-γ, that polarize monocytes into M1 macrophages. Activated M1 macrophages produce an unknown factor that mediates rejection of the tumor and allows for non-phthisical rejection. Although ocular macrophages are capable of mediating cytotoxicity of Ad5E1 clone 4 tumor cells *in vitro*, the major function of macrophages may possibly be to present antigen to $CD4^+$ T cells in the eye. Interestingly, Ad5E1 clone 4 tumor-bearing eyes have elevated mRNA expression of M2 macrophage markers, such as Arg1 and Ym1. The role these macrophages play in non-phthisical rejection has not been explored. However, it is possible that M2 macrophages may function to dampen the inflammatory response of M1

macrophages. This may be done through the production of suppressive cytokines such as IL-10 and TGF-β.

Thus, in this model $CD4^+$ T cells or $CD8^+$ T cells may be the major effector cells in non-phthisical rejection. Immune components that are essential for non-phthisical rejection are macrophages and T cells (either $CD4^+$ or $CD8^+$ T cells). Although $CD8^+$ T cells are not required for rejection, they are possibly the major effector cells in non-phthisical rejection, a type of rejection that is consistent with $CD8^+$ CTL mediated rejection. Supporting this hypothesis, Ad5E1 clone 4 tumors are susceptible to CTL-mediated killing *in vitro.* My working model of intraocular Ad5E1 clone 4 tumor rejection is illustrated in Figure 42. This model is based only my hypotheses of how $CD4^+$ and $CD8^+$ T cells, macrophages, and possibly IFN-γ contribute to non-phthisical rejection, and, again, this is not to be interpreted as the definitive description of non-phthisical Ad5E1 tumor rejection.

This work builds on previous knowledge of how tumors in an immune privileged environment are rejected without inducing damage to normal tissue. As stated earlier, uveal melanoma is the most common intraocular malignancy in adults, and approximately half of uveal melanoma patients will die within 10 to 15 years of diagnosis of the primary tumor. In addition to understanding the rejection of intraocular tumors, this knowledge may provide insights into

understanding tumors in other immune privileged sites, such as the brain and testes.

**Figure 41. A model of phthisical intraocular Ad5E1 tumor rejection.**

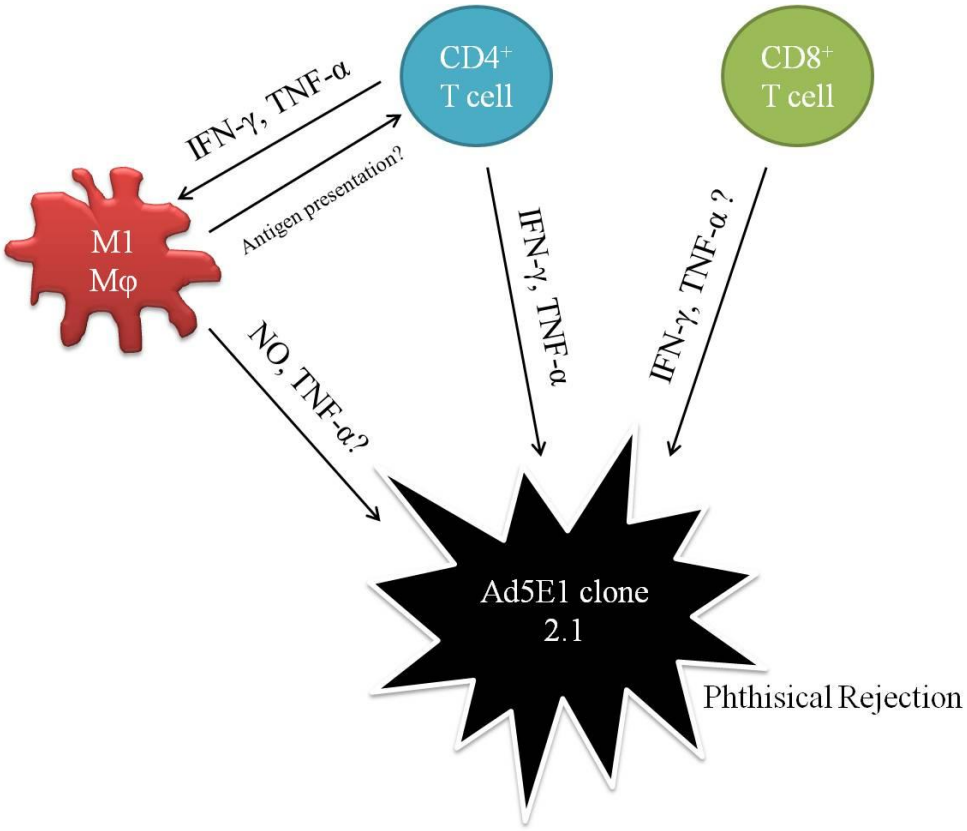
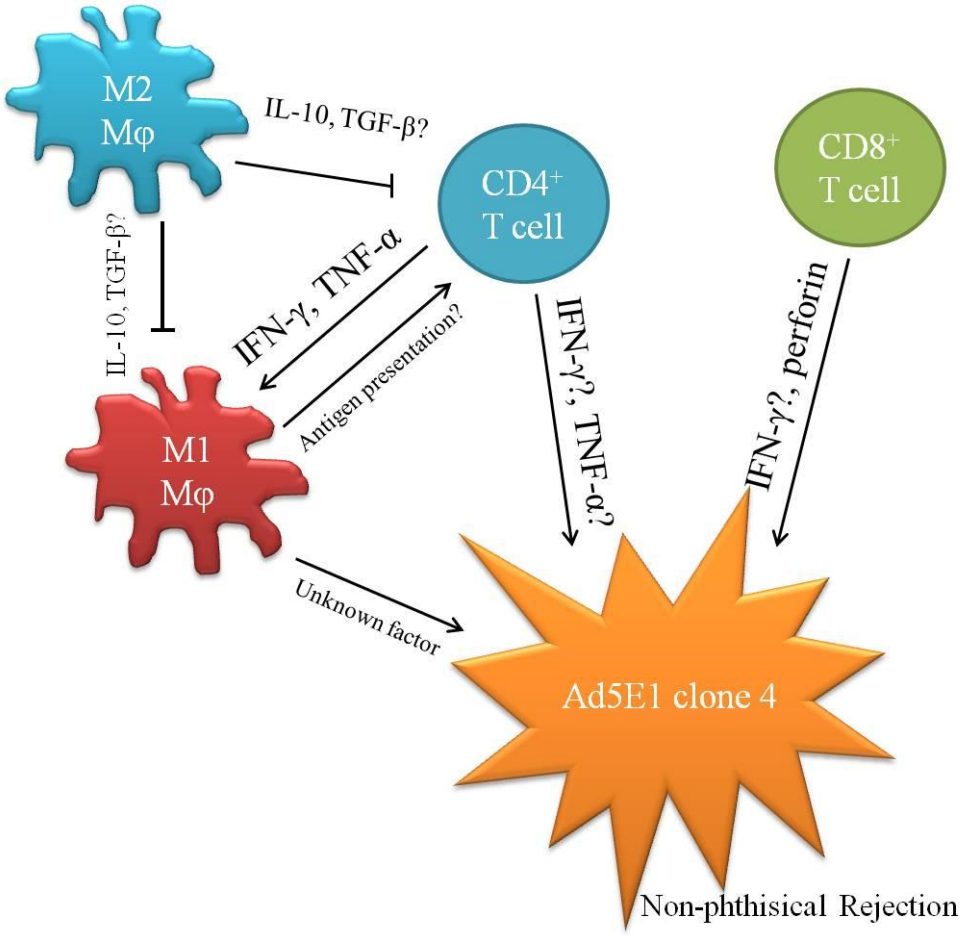


**Figure 42. A model of non-phthisical intraocular Ad5E1 tumor rejection.**



## Differential Gene Expression of Ad5E1 clone 2.1 and clone 4 Tumor Cells

Current microarray technology has both advantages and limitations. The advantages are obvious. Microarray assays are a powerful molecular technology that allows the simultaneous study of the expression of thousands of genes or their RNA products. This gives an accurate picture of gene expression in the cell or the sample at the time of the study. The limitation of this technology is a major issue when conducting microarray assays. The quality and amount of RNA remains a major challenge in the microarray experiments. Processing of tissue must be done rapidly to maintain RNA integrity. False microarray data can be generated from degraded mRNA. Due to these concerns, the experiments need to be replicated in order to eliminate sources of error. The data presented below is from one assay and undoubtedly, needs to be repeated to have reliable results. Another major obstacle to the success of this microarray assay was data analysis. With one set of samples, statistical significance cannot be reached with this analysis and these microarray assays must be repeated.

*Differential gene expression of Ad5E1 clone 2.1 tumor cells compared to Ad5E1 clone 4 tumor cells*

As seen in Table 5, a gene called high mobility group box 1 (HMGB-1) is upregulated in Ad5E1 clone 2.1 tumor cells. HMGB-1 is a DNA-binding nuclear protein and is released only during necrotic cell death (246, 247). HMGB-1 is the prototypic damage-associated molecular pattern (DAMP) molecule and is released by several types of solid tumors, including melanoma, colon cancer, prostate cancer, pancreatic cancer, and breast cancer (248). Interestingly, the form of HMGB-1 released from necrotic mouse embryonic fibroblasts can stimulate monocytes to produce TNF-α (247). The secretion of HMGB-1 can be triggered by different stimuli. For example, IFN-γ can induce HMGB-1 release from macrophages and requires induction and signaling by TNF-α (249). Supporting this hypothesis in my model was increased HMGB-1 expression in Ad5E1 clone 2.1 tumor cells treated with IFN-γ plus TNF-α. HMGB-1 can also associate with other molecules, including TLR ligands and can activate cells through the engagement of multiple surface receptors, such as TLR2 and TLR4 (248). In the context of pththisical rejection, HMGB-1 may play the most important role in activation of maturation of immature DCs (iDCs) with upregulation of costimulatory molecules and secretion of proinflammatory cytokines, including IL-12, IFN-γ, and TNF-α (250). Thus, in this circumstance, release of HMGB-1 induces an effective anti-tumor response, inducing the maturation of iDCs that promote the activation of M1 macrophages. M1 macrophages induce tumor destruction (and phthisis) by release of NO and TNF-α. TNF-α (together with

IFN-γ) might be essential to induce the release of HMGB-1, and thus ablation of TNF-α prevents the induction of phthisis.

Another gene found in the same pathway as HMGB-1 and upregulated in Ad5E1 clone 2.1 tumor cells is matrix metallopeptidase 13 (MMP13). MMP13 plays a crucial role in promoting angiogenesis (251) and allows for increased growth of Ad5E1 clone 2.1 tumors and increased access to the tumor by immune cells. Also in this pathway, CXCL5 is upregulated in Ad5E1 clone 2.1tumor cells. CXCL5, which promotes inflammation, has been shown in a recent study to be increased synergistically in response to IL-17 and TNF-α (252). Together these molecules may function to promote chronic inflammation that ultimately leads to phthisis.

Interestingly, several genes that are important in promotion of the innate immune response were upregulated in Ad5E1 clone 2.1 tumor cells. These genes include TLR2, TLR4, TLR6, and TLR9. Recent studies have shown that not only immune cells can express toll-like receptors (TLRs), but tumor cells can as well (253). TLRs are expressed on many kinds of cancer including gastric cancer, colorectal cancer, ovarian cancer, cervical cancer, lung cancer, prostate cancer, melanoma, brain cancer, and breast cancer (253). Activated TLR signals on cancer cells may promote cancer progression, anti-apoptotic activity and resistance to host immune response (254). As mentioned above, HMGB-1 is a

ligand for TLR4 which is possibly expressed on Ad5E1 clone 2.1 tumor cells, thus the release of HMGB-1 may trigger TLR4 signaling on other tumor cells perpetuating a cascade of events that leads to phthisis.

TLR signaling promotes chronic inflammation (253) and may increase infiltration of infammatory cells that promote phthisical intraocular tumor rejection. It has been demonstrated that the stimulation of specific TLRs in melanoma cell lines significantly upregulated proinflammatory cytokines and chemokines. Other protumor factors were also upregulated, such as IL-8, CXCR4, and VEGF (253). Cytokine-activated immune cells may contribute further by increasing inflammation. Signaling through TLRs also promotes tumorigenesis through NF-κB upregulation and subsequent production of antiapoptotic factors (255). Thus, increase expression of TLRs on Ad5E1 clone 2.1 tumor cells would contribute to increased inflammation and ultimately to phthisis.

Ad5E1 clone 2.1 tumor cells also had increased expression of genes involved in the mitogen-activated protein kinase signaling pathway. These genes include MAPK1, MAPK6, MAPK8, and MAPK8. Mitogen-activated protein (MAP) kinases are serine/threonine-specific protein kinases that respond to extracellular stimuli (mitogens, osmotic stress, heat shock and proinflammatory cytokines) and regulate various cellular activities, such as gene expression, mitosis, differentiation, proliferation, and cell survival/apoptosis (256). The role

of MAPKs in cancer is pleiotropic and, often there are contradictory findings (256). However, the increased MAPK signaling (and signaling in general) possibly promoting inflammation is consistent with the phenotype of phthisical rejection.

Multicellular organisms employ two main mechanisms for the elimination of cells: necrosis and apoptosis. As mentioned above, necrosis triggered by the rupture of the plasmatic membrane is accompanied by formation of an inflammatory process (257). If the predominate form of cell death of Ad5E1 clone 2.1 tumors is necrosis there would be fewer upregulatated genes involved in apoptosis. Consistent with this hypothesis, few genes in the apoptosis pathway are upregulated. There is only one caspase gene upregulated (Caspase 7). There were also several anti-apoptotic genes, such as BIRC2 and BLCL2L, that were upregulated in Ad5E1 clone 2.1 tumor cells compared to clone 4 tumor cells. This is in contrast to many more pro-apoptotic genes that are upregulated in Ad5E1 clone 4 tumor cells (discussed below) compared to Ad5E1 clone 2.1 tumor cells.

Overall, a working model of phthisical rejection is as follows: I hypothesize that innate immune cells migrate to the tumor-bearing eye, specifically DCs. DCs, as antigen-presenting cells, present Ad5E1 clone 2.1 tumor antigens to both $CD4^+$ T cells and $CD8^+$ T cells. These T cells produce molecules, specifically IFN-γ, that polarize monocytes into M1 macrophages.

$CD4^+$ T cells also produce TNF-α and IFN-γ that further polarizes macrophages into a M1 phenotype. The presence of TNF-α and IFN-γ facilitate the production of HMGB-1 that is released after cell damage by NO produced by activated M1 macrophages. HMGB-1 binds TLR4 on the tumor cells, inducing the release of more HMGB-1. HMGB-1 also may bind TLR4 on DCs and macrophages, inducing the production of more TNF-α. This overall chronic inflammatory environment culminates in phthisis.

*Differential gene expression of Ad5E1 clone 4 tumor cells compared to Ad5E1 clone 2.1tumor cells*

Apoptosis involves a ‘‘cleaner’’ type of death, in which the chromatin is condensed. This leads to formation of vesicles known as ‘‘apoptotic bodies’’. These are rapidly phagocytosed by macrophages without eliciting an inflammatory response (258). Apoptosis induction is achieved by promoting the expression of pro-apoptotic factors while reducing the expression of anti-apoptotic factors in the tumor cells. As seen in Table 6, in the apoptosis pathway Ad5E1 clone 4 tumor cells have the pro-apoptotic genes CASP8, CASP1, CASP3, BCL2, and BOK and others upregulated compared to Ad5E1 clone 2.1 tumor cells. However, several anti-apoptotic genes, such as BIRC3, are upregulated in

Ad5E1 clone 4 tumor cells. Overall, from this analysis Ad5E1 clone 4 tumor cells expressed more pro-apoptotic genes than anti-apoptotic genes. By contrast, Ad5E1 clone 2.1 tumor cells expresses more anti-apoptotic genes than pro-apoptotic genes. Thus, consistent with the rejection phenotype of each tumor clone it is possible that Ad5E1 tumors die by different mechanisms, which highly influences the anti-tumor immune response.

Consistent with decreased *in vivo* growth of Ad5E1 clone 4 tumors, there is an upregulation of PLG, the gene that encodes plasminogen. Angiostatin, a proteolytic fragment of plasminogen, is a potent antiangiogenic and pro-apoptotic agent (259). These genes may serve to limit the growth of Ad5E1 clone 4 tumors *in vivo* by preventing angiogenesis and increasing apoptosis.

In the proteasome pathway, PSMB8 (LMP7), PSMB9 (LMP2), and PSMB10 (MECL-1) are upregulated in Ad5E1 clone 4 tumor cells compared to Ad5E1 clone 2.1 tumor cells. These genes transcribe the subunits of the immunoproteasome. Increased immunoproteasome expression may alter the peptide repertoire presented by MHC class I molecules, making Ad5E1 clone 4 tumors more immunogenic (260). Consistent with the non-phthisical rejection phenotype of Ad5E1 clone 4 tumors, increased antigen presentation (whether quantitative or qualitative) may make Ad5E1 clone 4 cells more susceptible to CTL-mediated killing than Ad5E1 clone 2.1 tumor cells *in vivo.* A caveat to this hypothesis is that both Ad5E1 clones express similar levels of MHC class I and

are equally susceptible to CTL-mediated cytotoxicity. Thus, is possible that increased expression of immunoproteasome genes contribute to non-phthisical rejection of Ad5E1 clone 4 tumor cells. The role of immunoproteases in increased tumor rejection of Ad5E1 clone 4 tumors is unlikely based on the experiment described on Table 4. This experiment demonstrated that it is a tumor intrinsic factor that determines the phenotype of rejection. T cells that mediate phthisical rejection of Ad5E1 clone 2.1 tumors can also mediate non-phthisical rejection of Ad5E1 clone 4 tumors. Thus, it is not the display or supply of TAs that determines the phenotype of Ad5E1 tumor rejection.

Genes in the IL-7 pathway are also upregulated in Ad5E1 clone 4 tumor cells. Two of these genes are IL-7 and IL-7R. IL-7 stimulates the differentiation of pluripotent hematopoietic stem cells into lymphoid progenitor cells. It also stimulates proliferation of all cells in the lymphoid lineage (B cells, T cells and NK cells) (261). IL-7 has been demonstrated to augment the cytolytic activity of CTLs and NK cells against various tumor targets (262). IL-7 binds to the IL-7 receptor (IL7R), resulting in a cascade of signals important for T-cell development within the thymus and survival within the periphery (261). The production of IL-7 by Ad5E1 clone 4 tumors may facilitate the survival of T cells in the tumor microenvironment and enhance the anti-tumor activity of CTLs.

*Differential gene expression of IFN-γ treated Ad5E1 clone 2.1tumor cells compared to untreated Ad5E1 clone 2.1tumor cells*

After IFN-γ treatment of Ad5E1 clone 2.1 tumor cells, the expected pathways were upregulated. As expected, genes involved with antigen presentation are further upregulated by IFN-γ. These genes include TAP1, TAP2, and SOCS1. In the apoptosis pathway the genes encoding the pro-apoptotic genes CASP7 and CASP9 were upregulated, as well as anti-apoptotic BIRC3 and CFLAR. Thus, IFN-γ signaling induces transcription of genes increasing apoptosis and preventing apoptosis, leading to a tug of war of sorts. TLR genes are further upregulated with the addition of IFN-γ. It is possible that overall function of IFN-γ may not be to interact with the tumor cells themselves, but rather to promote the recruitment and activation of M1 macrophages and Th1 cells.

*Differential gene expression of TNF-α- treated Ad5E1 clone 2.1 tumor cells compared to untreated Ad5E1 clone 2.1tumor cells*

Like after treatment with IFN-γ, treatment with TNF-α did not dramatically change the gene transcriptional activity of Ad5E1 clone 2.1 tumor cells. Many of the same pathways and genes were upregulated after treatment

with TNF-α, as with IFN-γ. As expected, several genes involved in TNF-α-signaling and TNF-α-induced apoptosis were upregulated with the addition of TNF-α. As hypothesized above, TNF-α may be required for initiating the release of HMGB-1, which may set off a cascade of events leading to phthisis. It is also possible, as hypothesized for IFN-γ, that TNF-α may play a more prominent role in regulating the immune response to Ad5E1 clone 2.1 tumors than directly influencing gene expression of tumor cells.

*Differential gene expression of IFN-γ-treated Ad5E1 clone 4 tumor cells compared to untreated Ad5E1 clone 4 tumor cells*

Treatment with IFN-γ did not dramatically affect the gene expression of Ad5E1 clone 4 tumor cells. As with Ad5E1 clone 2.1 tumor cells, treatment with IFN-γ of Ad5E1 clone 4 tumor cells upregulated many of the same genes as seen with Ad5E1 clone 4 tumor cells. As expected, genes related to antigen presentation, such as TAP1 and TAP2, were upregulated after treatment with IFN-γ. Interestingly, the genes for CCL5 (chemokine for T cells) and CCL7 (chemokine for monocytes and macrophages) were upregulated. Thus, IFN-γ treatment of Ad5E1 clone 4 tumors may increase recruitment of T cells and macrophages to mediate tumor rejection. Genes related to antigen processing (in

the proteasome pathway) and apoptosis were further upregulated after IFN-γ treatment. Thus, IFN-γ may have a more proapoptotic effect on Ad5E1 clone 4 tumors than Ad5E1 clone 2.1tumors.

*Differential gene expression of TNF-α-treated Ad5E1 clone 4 tumor cells compared to untreated Ad5E1 clone clone 4 tumor cells*

Treatment of Ad5E1 clone 4 tumor cells with TNF-α did not change gene expression considerably. Like with IFN-γ, treatment with TNF-α further upregulated genes involved in the apoptosis and antigen processing (in the proteasome pathway) pathways. IFN-γ and TNF-α may play a more prominent role in regulating genes involved in presenting antigens and regulating the immune response to Ad5E1 clone 4 tumors.

## IFN-γ INDEPENDENT TUMOR REJECTION OF AD5E1 CLONE 2.1 TUMORS

Although IFN-γ is necessary for Ad5E1 clone 2.1 tumor rejection in the eye, it is not needed for the elimination of the tumor at extraocular sites, as IFN-γ KO mice reject SC tumors. Rejection of Ad5E1 tumors is T cell-dependent, as SCID mice fail to reject either SC or AC injected tumors. These findings show that $CD4^+$ T cells are required for rejection, as tumors grow in the majority of CD4-depleted IFN-γ KO mice. By definition, IFN-γ KO mice do not develop

conventional Th1 cells. This leaves either Th2 or Th17 cells as the possible candidates for $CD4^+$ T cell-mediated tumor rejection. There are several studies that implicate Th2-mediated anti-tumor immunity as the primary effectors for tumor rejection (90, 263, 264). These studies demonstrate that anti-tumor activity of Th2 cells is in collaboration with tumor-infiltrating granulocytes, such as eosinophils (265, 266). Moreover, Th2 cells are present in the eye during allergic conjunctivitis (267) and corneal graft rejection (268) and could be employed to reject tumors. This form of immunity appears to be dependent on the production of IL-4 (263) and recruitment of eosinophils (265, 266). However, I did not observe the presence of eosinophils in the tumor rejecting eyes.

This led me to test the hypothesis that Th17 cells are involved in IFN-γ-independent SC Ad5E1 clone 2.1 tumor rejection. The weight of evidence from these studies indicates that IL-17 is required for rejection of SC Ad5E1 clone 2.1 tumors, as IL-17-depleted IFN-γ KO mice fail to reject SC tumors. It is important to note that this IL-17-mediated rejection only occurs in IFN-γ KO mice. Several *in vitro* studies have shown that treatment with IFN-γ neutralizing antibody during the course of Th17 cell differentiation leads to increased frequency of Th17 cells, whereas exogenous IFN-γ reduces the Th17 population (105). *In vivo* studies of IFN-γ-deficient mice exhibit enhanced Th17 responses in several disease models including mycobacterial infection and CIA (269). Aside from its effects on Th17 cell development, IFN-γ inhibits effector functions of Th17 cells

(270). Although this cross-regulation is fairly established the mechanisms of this inhibition are not clear. Thus, this form of immunity is not only IFN-γ-independent but almost certainly is greatly inhibited by IFN-γ.

I sought to determine the mechanism that IL-17 utilizes to mediate tumor rejection of SC tumors. To address this I tested the simplest and most direct hypothesis, that IL-17 is cytotoxic to Ad5E1 clone 2.1 tumor cells. High concentrations of rmIL-17 had no toxic effects when added to *in vitro* cultures of Ad5E1 clone 2.1 tumor cells. Since both $CD4^+$ and $CD8^+$ T cells are capable of producing IL-17 (129, 271, 272), I examined which T cell subset produced IL-17 in this tumor model. The data support the hypothesis that $CD4^+$ T cells were the primary source of IL-17 associated with Ad5E1 clone 2.1 tumor rejection, as $CD4^+$ T cells isolated from SC tumor immunized IFN-γ KO mice produced IL-17 when confronted with Ad5E1 clone 2.1 tumor antigens *in vitro*. Moreover, splenocytes from $CD4^+$ T cell-depleted IFN-γ KO mice produced significantly less IL-17 than $CD4^+$ T cells from isotype-treated IFN-γ KO mice. Interestingly, $CD4^+$ T cells and bulk splenocytes from wild-type C57BL/6 mice made significantly less IL-17 in response to tumor antigens than IFN-γ KO mice. Thus, IL-17-associated tumor rejection is strongly inhibited by IFN-γ and probably does not occur in wild-type IFN-γ-competent mice. In WT C57BL/6 mice, Ad5E1 clone 2.1 tumor rejection (both intraocular and subcutaneous) occurs through IFN-γ-dependent Th1 responses (65).

Many studies have implicated IL-17 in the pathogenesis of autoimmunity (226, 273-275). However, the role of IL-17 in tumor immunity remains controversial. The response to IL-17 varies according to tumors originating from different tissue types and animal models. Reports have indicated that the population of Th17 cells increases within the tumor microenvironment in many animal models and in patients with melanoma, breast cancer, colon cancer, and ovarian cancer (140, 148, 276, 277). Some studies demonstrate that IL-17 plays a pro-tumorigenic role by increasing angiogenesis and promoting metastasis (132, 133, 278). However, in other studies, Th17 cells have been shown to have anti-tumor properties (143, 144, 146). IL-17 might promote antitumor immunity by several mechanisms including: a) stimulating macrophages to produce IL-1β and TNF-α (279, 280); b) recruiting eosinophils and neutrophils (281, 282) and c) increasing the expression of costimulatory molecules on maturing dendritic cells (283).

There is growing evidence that IL-17 promotes tumor rejection by increasing the induction and function of CTLs. Recent work by Martin-Orozco et al. has shown that Th17 cells participate in anti-tumor immunity by facilitating DC recruitment into tumor tissues and draining lymph nodes where they promote the activation of CTLs that eliminate the tumor (147). Benchetrit et al. reported that IL-17 increased generation of tumor-specific CTLs directed against several different immunodominant antigens of P815 mastocytoma (146). My findings

show that in the absence of IFN-γ, both $CD8^+$ T cells and $CD4^+$ T cells are required for rejection of SC Ad5E1 clone 2.1 tumors. This led to the hypothesis that IL-17 increases CTL activity against Ad5E1 clone 2.1 tumors in the SC environment. Indeed, CTLs isolated from IL-17 antibody-depleted IFN-γ KO mice displayed decreased cytotoxicity against Ad5E1 clone 2.1 tumor cells compared to CTLs isolated from isotype antibody-treated IFN-γ KO mice. Increased expression of CD8 and perforin in the SC tumor environment further supports the notion that IFN-γ-independent rejection is CTL-mediated.

Recent work by Bos et al. reported that $CD4^+$ T cells in the tumor microenvironment produce IL-2 and IFN-γ, which promote the recruitment and cytolytic function of tumor- specific CTLs (77). Similarly, I determined that $CD4^+$ Th17 cells produce IL-17 in the local tumor environment, which is important for rejection of extraocular Ad5E1 clone 2.1 tumors. In the absence of IFN-γ, tumor-specific Th17 cells are induced by the presence of IL-6 and TGF-β1 in the tumor microenvironment and promote the activation and/or function of CTLs. Gene expression analysis confirmed that the SC environment was replete with IL-6, which is necessary for IL-17-dependent generation of tumor-specific CTLs. This conclusion is further supported by the findings of Kryczek et al. who reported that Th17 differentiation occurs primarily in the tumor microenvironment and not in the tumor-draining lymph node (134, 140). While the exact mechanism remains to be elucidated, it is possible that enhancement of

CTL activity by Th17 cells is due to an increased expansion of DCs or, alternatively, by enhanced presentation of tumor antigens by individual DCs (147).

As mentioned, IFN-γ KO mice SC immunized with Ad5E1 clone 2.1 tumor cells are not protected from a subsequent ocular tumor challenge. There may be several explanations for this observation. The first is that Ad5E1 clone 2.1 cells are not susceptible to tumor killing by $CD8^+$ CTL. However, *in vitro* assays demonstrated that Ad5E1 clone 2.1 tumors are susceptible to CTL killing. Another possibility is that without IFN-γ, CTLs that function in the skin cannot function in the immunosuppressive environment of the eye in spite of induction by IL-17. Thus, there may be a qualitative difference in the induction of CTLs by IFN-γ and IL-17. Another possibility is that $CD8^+$ T cells cannot differentiate in the AC without IFN-γ, however precursors are recruited and infiltrated Ad5E1 clone 2.1 tumors. Ksander et al. demonstrated that precursors of cytotoxicity T cells (pTc) fail to become mature differentiated CTLs in intraocular P815 tumors (284).

My results show that there is an absence of IL-17 and IL-6 expression in normal eyes and tumor-bearing eyes of WT C57BL/6 and IFN-γ KO mice. However, IL-6 and IL-17 are both detected in SC tumors. Accordingly, a possible explanation as to why there is no IL-17 in the ocular tumor microenvironment is

because IL-6, a crucial cytokine needed for induction and maintenance of Th17 cells (24), is normally absent in the ocular microenvironment, and subsequently, Th17 cells cannot differentiate in the IL-6-deficient intraocular environment. Other investigators have reported that the absence of IL-6 in the aqueous humor is required to maintain ocular immune privilege (224, 285). Moreover, several studies have confirmed that blocking IL-6 suppresses the inflammatory response of Th17 cells in autoimmune arthritis and uveoretinitis (275, 286, 287) and interferes with antigen-specific Th17 differentiation/expansion (226, 286). In order to confirm these results, IFN-γ-depleted IL-6 KO mice were injected SC with Ad5E1 clone 2.1 tumor cells. I expected that mice deficient in IL-6 could not generate Th17 cells and SC tumors would grow progressively. However, IFN-γ-depleted IL-6 KO mice rejected SC tumors (data not shown). These results can be explained by the studies showing that, although Th17 cell numbers are greatly reduced in IL-6 KO mice, IL-6 KO mice can generate small numbers of Th17 cells (288). A small percentage of Th17 cells may be enough to generate a CTL response in the absence of IFN-γ.

Recent unpublished results by the C. Pasare lab at UT Southwestern have shown that *in vivo* differentiation of Th17 cells does not require IL-6 but IL-1β in most tissues. IL-1β, like IL-6, is a proinflammatory cytokine and, like IL-6, may be excluded from the eye. Thus, depletion of IL-1β and IFN-γ may prevent SC

rejection of Ad5E1 clone 2.1 tumors. The Pasare group has also shown that induction of Th17 cells by IL-6 and TGF-β occurs primarily in the gut and requires $CD103^+$ DCs. $CD103^+$ DCs are found primarily in the lamina propria of the gut. In this context, $CD103^+$ DCs perform a regulatory role, which induce mucosal Tregs in order to maintain mucosal tolerance to gut biota. Similarly, the eye must maintain immune privilege. Keino et al. showed that *in vivo*-generated ACAID $CD8^+$ T regulatory cells use CD103 to suppress Th1 immune responses in the AC (289). Thus, it is possible that in the eye the induction of Th17 cells requires IL-6 due to the regulatory nature of $CD103^+$ cells. However, at extraocular sites such as the subcutaneous environment, IL-1β is sufficient to induce Th17 cells and IL-6 is not needed.

An alternative hypothesis is that exclusion of Th17 cell generation in the eye is independent of IL-6. High levels of TGF-β (or other suppressive molecules), as found in the AqH, may prevent the induction of Th17 cells regardless of the presence of IL-6 (or IL-1β).

Overall, the results from my study demonstrate two novel and important findings. First, I present further evidence for a role of Th17 cells in tumor rejection, particularly in the absence of IFN-γ-dependent anti-tumor responses. Second, the abrogation of a Th17 immune response pathway may represent a new mechanism that promotes ocular immune privilege in which IL-17-dependent

immune responses are blocked within the intraocular milieu. Neutrophils have been generally considered to be major effector cells in Th17-cell driven responses. Neutrophils produce a variety of toxic products, such as NO, superoxide anion and hydrogen peroxide, which directly damage the eye. This blockage may be a means of dampening immune responses, such as neutrophil recruitment and activation, that damage the eye (290, 291).

## FUTURE RESEARCH

*The death of Ad5E1 clones: Necrosis or Apoptosis?*

A central question in future studies comparing phthisically rejected Ad5E1 clone 2.1 tumors and non-phthisically rejected Ad5E1 clone 4 tumors would be: Is the inflammation induced in phthisical rejection the result of tumor-intrinsic process initiated by the tumor cells or the result of an adaptive immune response to the tumor? My studies have established that it is the tumor that determines the phenotype of rejection and not the immune response. Thus, the induction of inflammation (i.e. NO, TNF-α) is initiated by tumor-intrinsic process, and is not initiated by the antitumor immune response. A study by Soudja et al. reported two melanoma types that produce tumor-intrinsic factors that control the initiation of inflammation independently of the adaptive immune response (292). Similarly, Ad5E1 clone 2.1 tumors may produce a tumor-intrinsic factor that has qualitative or quantitative effect on the activation of M1 macrophages leading to increased production of NO or TNF-α. Alternatively, Ad5E1 clone 4 tumors may produce a factor that increases the polarization of M2 macrophages limiting the inflammatory anti-tumor response.

Alternatively, it may be the manner in which tumor cells die that determines whether a proinflammatory response will occur. Ad5E1 clone 4 tumor cells may predominately die by apoptosis. There is evidence that cells undergoing

apoptosis release cytokines, such as IL-10 and TGF-β, that inhibit immune responses (293). This immunosuppressive response may prevent a robust immune response that results in phthisis. On the other hand, Ad5E1 clone 2.1 tumor cells may predominantly die by necrosis. Cells undergoing necrotic death are known to be immunostimulatory by allowing the leakage of proinflammatory cellular components (294). The inflammatory molecule, HMGB-1, has been reported to be preferentially released from necrotic cells but not apoptotic cells (246). HMGB-1 acts on DCs to promote immunity (release of TNF-α, IL-6, and IL-1β) (246). Other mediators, such as uric acid, calreticulin, and HSP70, are involved in immunological response to necrotic cells. Thus, necrotic death of Ad5E1 clone 2.1 tumors may induce an immunostimulatory response that results in phthisis of the eye.

Evidence for the possible role of HMGB-1 in phthisical rejection is supported by microarray analysis that showed that the gene encoding this molecule is upregulated in Ad5E1 clone 2.1tumor cells compared to Ad5E1 clone 4 tumor cells. Further evidence supporting this hypothesis is that Ad5E1 clone 4 tumor cells have an upregulation of genes involved in apoptosis, such as CASP1, and CASP3. These initial findings must be confirmed by qPCR. Once this gene upregulation is confirmed the following experiments can be done.

This hypothesis could be tested by labeling tumor cells (both Ad5E1 clone 2.1 and clone 4) with CMPTX prior to AC injection. CMPTX is a "cell tracker

probe" used for the long-term tracing of living cells. Tumors would then be removed from the eye (at several time points) and stained with the apoptotic markers, caspase 3 and annexin V. CMPTX-positive tumor cells positive for caspase 3 and annexin V will be called apoptotic cells. Intracellular staining for HMGB-1 will also be done. If this hypothesis is correct, I would expect eyes bearing Ad5E1 clone 4 tumors to have apoptotic cells, whereas eyes containing Ad5E1 clone 2.1 cells will not be apoptotic, but necrotic. As a control, tumor cells will be induced to undergo apoptosis by irradiation. Necrotic cells could be induced by repeated freeze-thawing. These experiments would allow for increased understanding of the tumor intrinsic nature of phthisical and non-phthisical intraocular tumor rejection.

Contrary to my initial hypothesis, intracellular staining indicated equivalent levels of intracellular HMGB-1 in both Ad5E1 clone 2.1 and clone 4 tumor cells. However, the redox status of HMGB-1 is critical to its function in promotion an inflammatory response. It has been shown that the activation of caspases 3 and 7 induced the production of ROS which causes HMGB-1 to be oxidized thus neutralizing its function (295). In spite of similar levels of intracellular HMGB-1, the oxidation of HMGB-1 may be altered in Ad5E1 clone 4 tumor cells compared to Ad5E1 clone 2.1 tumor cells. My microarray analysis suggested that increased caspase activity may exist in Ad5E1 clone 4 tumor cells, which may lead to greater oxidization of HMGB-1 in Ad5E1 clone 4 tumor cells.

This hypothesis can be addressed by examining the oxidization of state of HMGB-1 by western blot. Data demonstrating increased oxidized HMGB-1 in Ad5E1 clone 4 tumor cells compared to Ad5E1 clone 2.1 tumor cells would support this hypothesis.

Many other interesting questions about the role of HMGB-1 in rejection of Ad5E1 tumor clones remain. For example; "Do Ad5E1 clones secrete HMGB-1?" A comparison of the amount the amount of secreted HMGB-1 between Ad5E1 clones could be done. Another question would be; "Does the secretion of HMGB-1 change in response to TNF-α and/or IFN-γ treatment?" This could be addressed by cytokine ELISA. Another interesting question could address; "Does the neutralization of HMGB-1 (via shRNA or other means) in Ad5E1 clone 2.1 tumor cells prevent phthisical rejection?"

*Confirmation of microarray analysis*

Additional microarray assays need to be completed in order to obtain reliable and reproducible data to lay the foundation for further studies comparing the differential gene expression of Ad5E1 clone 2.1 and clone 4 tumor cells. For example, results in the microarray assay indicated that Ad5E1 clone 2.1 tumor cells had upregulated gene expression of HMGB-1; however, qPCR analysis demonstrated similar mRNA expression levels for HMGB-1 between Ad5E1 clone 4 and clone 2.1 tumor cells. As mentioned above, my experiments must be

repeated with at least two replicates. Additional comparisons should be completed between tumor clones treated with IFN-γ, TNF-α, or IFN-γ + TNF-α (i.e. Ad5E1 clone 2.1 tumor cells + TNF-α versus Ad5E1 clone 4 tumor cells + TNF-α). In order to address the hypothesis that the secretion of HMGB-1 is induced by the combination of IFN-γ + TNF-α, it would be important to include these sample groups in future microarray experiments. It would also be interesting to determine whether genes involved in nitric oxide signaling are differentially expressed in Ad5E1 clone 2.1 tumor cells and clone 4 tumor cells. Such differential gene expression may provide insight into understanding why NO is required for rejection of Ad5E1 clone 2.1 cells and not Ad5E1 clone 4 cells.

*Is nitric oxide required for non-phthisical rejection in TNF-α deficient mice?*

My results presented above demonstrated that phthisical intraocular rejection of Ad5E1 clone 2.1 tumors requires NO. However, TNF-α deficient mice reject tumors in a non-phthisical manner. Although I did not determine whether NO was required for non-phthisical rejection in TNF-α deficient environments in my dissertation, this question would be addressed by treating TNF-α KO mice with L-NAME prior to intraocular tumor rejection. If Ad5E1 clone 2.1 tumors are no longer rejected in L-NAME treated mice, this would indicate that NO is required for tumor rejection, but it would not be necessary for phthisical rejection.

*What is the role of Toll-like receptors in phthisical rejection?*

As discussed above, genes that encode for TLRs (specifically TLR2 and TLR4) are upregulated in Ad5E1 clone 2.1 tumor cells compared to Ad5E1 clone 4 tumor cells. TLRs are expressed by a variety of cancer cell types and upregulate the NF-κB cascade in cancer cells to produce anti-apoptotic proteins and release of cytokines and chemokines that recruit immune cells to enhance anti-tumor immunity. This could be addressed by creating an Ad5E1 clone 2.1 cell line that is defective in TLR signaling, such as inserting a deletion of myeloid differentiation factor 88 (MyD88). If signaling through the TLR pathway on tumor cells is essential for phthisical rejection, MyD88 KO Ad5E1 clone 2.1 tumor cells would be rejected in a non-phthisical manner. This work may give further insight into the induction of phthisical intraocular tumor rejection.

*What is the role of M2 macrophages in non-phthisical rejection?*

As shown in Figure 16, Ad5E1 clone 4 tumor-bearing eyes express both NOS2 and Arg1, suggesting that both M1 and M2 macrophages are present. Again, M2 macrophages promote tumor growth by releasing suppressive cytokines. Thus, I hypothesize that M2 macrophages function to suppress, or dampen, the immune response to intraocular tumors. Further experiments would examine the role these M2 macrophages play in rejection. The following questions would be addressed: Is there an increase in expression of IL-10 or TGF-

β in Ad5E1 clone 4 tumor bearing eyes? Does depletion of IL-10 or TGF-β allow for phthisical rejection of Ad5E1 clone 4?

I made an attempt to deplete M2 macrophages with an anti-IL-4 antibody. Depletion of IL-4 did not affect tumor rejection. However, M2 macrophages can also be induced by other Th2 cytokines, such as IL-13, and removal of IL-4 may not have depleted M2 macrophages. Alternatively, STAT6 KO or IL-4R KO mice could be used; however, they are currently only commercially available on a BALB/c background and not on the needed C57BL/6 background. These experiments would be important for understanding the interactions that occur between different macrophage subpopulations and other immune cells in the tumor microenvironment.

*Do macrophages function in the afferent arm of the immune response to Ad5E1 tumor clones?*

My data indicate that macrophages are required for spontaneous ocular tumor rejection of both Ad5E1 clones. However, depletion of macrophages before and throughout tumor development did not address whether macrophages were required for priming of T cells or were effector cells themselves.

Dace et al. demonstrated that macrophages were necessary for intraocular parental Ad5E1 tumor rejection and further demonstrated that macrophages were

required for $CD4^+$ T cell function, as $CD4^+$ T cells from clodronate liposome-treated mice produced significantly less IFN-γ compared to PBS liposome-treated mice (68). Furthermore, rejector $CD4^+$ T cells adoptively transferred to clodronate liposome-treated SCID mice were unable to mediate rejection, whereas rejector $CD4^+$ T cells were able to mediate rejection in PBS-liposome-treated SCID mice. This demonstrated that macrophage and $CD4^+$ T cell cooperation was needed for non-phthisical intraocular rejection of Ad5E1 tumors (68). Similar experiments could be performed with each Ad5E1 tumor clone to assess if macrophages are required for the activation of T cells. Briefly, T cells could be isolated from animals treated with clodronate liposomes and their activation status could be assessed by their ability to produce IFN-γ.

Boonman et al. demonstrated that macrophages are needed in the afferent phase of the immune response to parental Ad5E1 tumor cells (67). This was accomplished by depleting macrophages at different times after intraocular injection of parental Ad5E1. Depletion of macrophages at day 2 post tumor injection prevents the rejection of intraocular tumors. However, depletion during the effector phase (day 8 -14 post tumor injection) did not prevent tumor rejection. Thus, macrophages in this model play a role in the afferent phase, probably as APCs, but are not essential in the effector phase of rejection. These studies could be repeated with each Ad5E1 tumor clone to better determine the

role of macrophages in Ad5E1 tumor phthisical and non-phthisical rejection. Briefly, macrophages could be depleted in either the afferent phase of rejection (0-2 days post tumor transplantation) or during the effector phase (7-14 days after tumor transplantation) to determine in which phase of the immune macrophages are essential for tumor rejection.

*Are Ad5E1 clone 4 tumors resistant to NO-mediated cytotoxicity?*

The rejection of Ad5E1 clone 4 tumors by activated macrophages in the presence of L-NAME indicates that another tumoricidal mechanism is employed by macrophages. Thus, it is possible that Ad5E1 clone 4 tumors are NO resistant. In order to address the hypothesis that Ad5E1 clone 4 tumors are resistant to NO-mediated cytotoxicity, a simple experiment would be performed by treating Ad5E1 clone 4 tumor cells with increasing concentrations of NO. If Ad5E1 clone 4 tumor cells are resistant to NO, the addition of NO to *in vitro* tumor cultures would not induce cell death. Alternatively, Ad5E1 clone 4 tumor cells may produce a factor which decreases NO production by macrophages. This hypothesis could be addressed by measuring nitrite levels in supernatants of Ad5E1 clone 4 tumor /macrophage cocultures (compared to Ad5E1 clone 2.1/macrophages cocultures). Cultures containing Ad5E1 clone 4 tumor cells/ macrophages would contain lower levels of nitrite than cultures containing Ad5E1 clone 2.1/ macrophages. Other studies showed the *in vivo* treatment of mice with

L-NAME that harbor intraocular Ad5E1 clone 4 tumors does not prevent tumor rejection. To address if NO is produced *in vivo*, the presence of nitrotyrosine could be determined by immunohistochemistry. The expression of nitrotyrosine in tumor-bearing eyes containing Ad5E1 clone 4 tumor cells would be compared to Ad5E1 clone 2.1 tumor bearing eyes.

*What macrophage-produced molecule is responsible for in vitro macrophage-mediated killing of Ad5E1 clone 4 tumor cells?*

As seen in Figure 14, a soluble factor is produced by macrophages that mediate rejection of Ad5E1 clone 4 intraocular tumors. In Figure 30, this factor was shown to be a protein with a molecular weight between 50 kDa and 100 kDa. This protein could be identified by isolating different candidate proteins by SDS-PAGE and having each protein sequenced by mass-spectrometry. Initial experiments have demonstrated that this factor may be a protease. A broad spectrum protease inhibitor completely abolished cytotoxicity. Current experiments using protease inhibitors seek to determine which class of protease this factor belongs. These results could identify a novel protein that macrophages produce in response to tumor antigens and leads to tumor rejection.

*Do neutrophils play a role in IFN-γ-independent IL-17-induced tumor rejection?*

Throughout the literature of autoimmune diseases, such as EAU, EAE, CIA, and IBD, IL-17 has been shown to play a prominent role. In most of these disease models, IL-17 functions in pathogenesis by recruiting and activating neutrophils (114, 118, 270). Neutrophils, which provide protection from extracellular bacteria and fungi, produce NO, $O_2$, and $H_2O_2$, which could mediate tumor rejection. The role that neutrophils play in this form of immunity could be addressed by depletion of neutrophils prior to SC Ad5E1 clone 2.1 tumor injection. The proinflammatory nature of neutrophils may explain why Th17 cell induction is excluded from the ocular environment.

Overall, these experiments would hopefully increase the understanding of immune-mediated rejection of tumors in immune privileged environments without inducing damage to normal tissues, and potentially provide a building block in the creation of possible treatments of such tumors.

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