Immunology and Microbiology

Natural Killer T Cells Contribute to Neutrophil Recruitment and Ocular Tissue Damage in a Model of Intraocular Tumor Rejection

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Purpose. Immune privilege of the eye protects the nonregenerative ocular tissues from innate and adaptive immune-mediated inflammation. In the case of intraocular tumors, immune privilege can be arrested to allow for immune-mediated rejection. Activation of innate immune cells can contribute to necrosis of the intraocular tumor and bystander ocular tissue. Identifying the cellular components of the innate immune system that contribute to ocular destruction, but are not needed for tumor rejection, provides insights into the immunopathological sequelae in intraocular tumor rejection.

Methods. Wild-type (WT), Jα18 knockout (KO) mice lacking type I natural killer T (NKT) cells, and CD1d KO mice lacking all NKT cells, were used to identify the role of type II NKT cells in intraocular tumor rejection immunopathology.

Results. CD1d KO mice had significantly lowered rates of necrotic eye destruction during tumor rejection compared to WT or Jα18 KO mice. Transcriptome and protein analyses revealed that CD1d KO mice had significantly lower expression of CXCL3 compared to WT or Jα18 KO mice, and this was associated with decreased neutrophil recruitment. The presence of type II NKT cells in WT or Jα18 KO mice led to increased CXCL3, which attracted neutrophils to the intraocular tumor and culminated in destruction of the eye.

Conclusions. We found that type II NKT cells are critical in initiating a damaging inflammatory antitumor response involving the recruitment of neutrophils that compromises the integrity of the eye. Loss of type II NKT cells or depleting neutrophils allows for a productive intraocular tumor response that converts the rejection phenotype to preserve the eye.

Keywords: tumor, NKT cells, neutrophils, immune rejection, T cell

The eye is endowed with remarkable immunologic properties including the stringent regulation of immune-based inflammation, a phenomenon termed immune privilege.[1,2] Immune privilege is critical in maintaining visual integrity by preventing immunologic damage to the ocular tissues that cannot be replaced. Four major factors contribute to maintaining and establishing immune privilege: anatomical barriers, cell membrane-bound molecules that disable immune effector elements, soluble anti-inflammatory and immunosuppressive factors, and induction of immune tolerance.[1] The blood–ocular barrier contains nonfenestrated vessels that prevent the extravasation of blood-borne leukocytes into the ocular tissues. Cell membrane-bound molecules present in the cornea, iris, and retina induce apoptosis or prevent the inflammatory activities of leukocytes that come in contact with the ocular barriers. Soluble factors, present especially within the aqueous humor of the anterior chamber (AC), suppress inflammatory responses of leukocytes.

While AC-associated immune deviation (ACAID) is well characterized by the development of antigen-specific T regulatory cells that suppress immune responses to antigens introduced into the AC, it also contributes to global leukocyte suppression including innate immune cells.[2–4] In fact, invariant type I natural killer T cells (NKT cells) contribute to the establishment of ACAID[5–7] by recruiting antigen presenting cells (APCs) and $CD8^+$ T cells to the spleen to promote the generation of antigen-specific T regulatory cells.[8] Neutrophils, NK cells, and macrophages that comprise the innate immune cell population are also suppressed by ocular immune privilege mechanisms. Cell membrane–bound Fas-ligand and tumor necrosis factor related apoptosis inducing ligand (TRAIL) induce apoptosis of activated neutrophils and macrophages.[9–11] ed with soluble factors such as TGF-β, CGRP, α-MSH, and soluble Fas-ligand that contribute to inhibiting innate immune cell activation and functions.[12–15]

Natural killer T cells are a heterogeneous population of cells that bridge the innate and adaptive immune systems with T-cell receptors (TCRs) that recognize mainly lipid antigens in the context of CD1d presented on APCs. Natural killer T cells quickly produce large amounts Th1- and Th2-associated cytokines after TCR recognition of antigen[16] to activate the adaptive immune response. Type I, or invariant NKT cells, are the most abundant NKT cell type; they are better characterized due to their invariant TCR expression utilizing the Vα14 and Jα18 segments and can be studied using the Jα18 knockout (KO) mouse. Furthermore, all type I NKT cells can be activated following TCR binding to α-galactosylceramide.[17] Type II, or variant NKT cells, express variable TCR gene rearrangements; and while a subset react to sulfatide,[18] an activating ligand that identifies and activates the whole cell population has not yet

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been identified. CD1d is essential for positive selection of NKT cells[19] causing CD1d KO mice to lack both type I and type II NKT cells.

Type I and type II NKT cells typically have opposing roles in tissue and diseases. Type I NKT cells can fully respond to both TCR and TLR signals[20] while type II NKT are primarily activated by TCR signals.[21] In murine hepatitis, type II NKT cells stimulate pDC cytokine production and recruit type I NKT cells that are anergized to ameliorate disease.[22] Type II NKT cells have also been implicated in contributing to liver disease in another mouse model[23] and can promote tissue injury in ulcerative colitis.[24] Therefore, NKT cells are diverse and are capable of mediating either pro- or anti-inflammatory effects depending on the tissue and disease. In the context of tumor immunity, type I NKT cells are usually associated with antitumor responses while type II NKT are associated with suppression.[16] We have reported that NKT cells disable NK cell-mediated control of liver metastases from intraocular melanomas in mice.[25,26] However, the role of NKT cells in regulating the immune response and rejection of intraocular tumors has not been explored.

Natural killer T cells influence the expression of immune-based diseases at the ocular surface; they are necessary for maximal pathology of allergic conjunctivitis[27] and influence the pathogenesis of dry eye disease in mice.[28] Type I NKT cells are critically involved in the development of ACAID[5–7] that promotes the survival of corneal allografts.[29] There is a lack of research addressing the role of type II NKT cells in the context of ocular tumor immunology. This study aims to address the role of type II NKT cells in a well-described intraocular tumor model that is dependent on an immune response for its rejection in syngeneic mice.[30–32] The role of type II NKT cells on the resulting immunopathology of bystander ocular tissue destruction normally seen in this model of intraocular tumor rejection was assessed by utilizing three genotypes of mice: C57BL/6J wild-type (WT), Jα18 KO, and CD1d KO mice. The study of type II NKT cells is achieved by comparing WT mice with all NKT cells and Jα18 KO that have type II NKT cells to CD1d KO mice that lack all NKT cells.

Materials and Methods

Mice

C57BL/6 (WT) mice (H-2^b) were obtained from the mouse breeding facility at University of Texas Southwestern Medical Center (UTSWMC, Dallas, TX, USA). CD1d KO mice on the C57BL/6 background breeding pairs were kindly provided by Mark A. Exley (Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA, USA). Jα18 KO mice on the C57BL/6 background breeding pairs were generated and kindly provided by Masaru Taniguchi, RIKEN Research Center for Allergy and Immunology, Yokohama, Japan.[33] All animals were cared for and bred in compliance with animal protocols approved by UTSWMC's institutional animal care and use committee (IACUC) standards. All animal use conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Tumor Cell Growth and Immunization

Toes and colleagues[34] created the murine Ad5E1 tumor cell line by transforming embryonic C57BL/6 mouse cells with the human *Ad5E1* gene. Studies on the original Ad5E1 tumor cell line demonstrated that these tumors undergo spontaneous T-cell-dependent immune rejection in the eyes of syngeneic C57BL/6 mice.[35–37] Rejection of these original intraocular Ad5E1 tumors does not require TNF-α, FasL, TRAIL, perforin, B cells, NK cells, or CD8$^+$ T cells.[31,36–38] Immune rejection of Ad5E1 tumors leaves the eye anatomically intact without inflicting injury to normal ocular tissues.[37] However, during the course of our studies, we discovered that Ad5E1 tumors occasionally undergo a necrotizing form of immune rejection that leads to extensive damage to innocent bystander cells and culminates in phthisis of the tumor-containing eye.[32] Our lab isolated a clone from a subpopulation of the original Ad5E1 tumor cell line that demonstrated a high incidence of necrotizing immune rejection and phthisis of the eyes of C57BL/6 mice, designating this cell line Ad5E1 clone 2.1,[32] and that require both CD4$^+$ and CD8$^+$ T cells for intraocular tumor rejection. The clone 2.1 tumor model was used to evaluate the mechanisms that tilt the intraocular immune response from a nonnecrotizing form of immune rejection occurring in the parental Ad5E1 cell line to a necrotizing pattern of tumor rejection that occurs with clone 2.1 tumors, ridding the eye of the tumor yet culminating in destruction of the eye.

Tumor growth, AC injections, and subcutaneous (SC) injections were performed as previously described.[30]

Delayed Type Hypersensitivity (DTH) Assay

Delayed type hypersensitivity (DTH) was measured utilizing a tumor cell-specific ear swelling assay. Wild-type or CD1d KO mice were AC or SC injected with Ad5E1 tumor cells. Fourteen or 21 days later, the injected and naïve mice were anesthetized, and baseline (0 hour) measurements of both ears were taken using a digital micrometer with 0.0005-inch resolution (Mitutoyo, Kawasaki, Japan). A 20-μL volume of 1×10^5 mitomycin C-treated Ad5E1 tumor cell suspension was injected into the ear pinnae (experimental ear), and 20 μL Hanks' balanced salt solution (HBSS) was injected into the other ear pinnae (negative control ear) of each mouse using a 1-mL tuberculin syringe fitted into a Hamilton delivery apparatus. Twenty-four hours later, the mice were anesthetized and both ears were measured using a digital micrometer. Tumor cell-specific ear swelling was calculated as (24-hour − 0-hour measurement of experimental ear) − (24-hour − 0-hour measurement of negative control ear).

mRNA Sequencing

Wild-type and CD1d KO mice were euthanized 14 days after AC injection with Ad5E1 tumor. The tumor-bearing eyes were extracted and immediately frozen in liquid nitrogen and stored at −80°C. RNA was extracted from the frozen tissue using the Qiagen RNeasy Kit (Hilden, Germany) per manufacturer's recommendation. Quality (RNA quality indicator [RQI] > 8.5) and quantity of the extracted RNA were evaluated using the Experion StdSense RNA chip and regents (BioRad, Hercules, CA, USA). Two pools from four mice were generated for both the WT and the CD1d KO mice and submitted to the UTSW DNA Next Generation Sequencing Core Facility for strand-specific single-end mRNA-Seq. The differential expression analysis of the results was performed by the UTSW Bioinformatics Core utilizing cuffdiff using known genes in igenomes. Qiagen ingenuity pathway analysis (IPA) was also run to identify relevant biological pathways from the statistically significant differentially expressed genes.

CXCL3 ELISA

Wild-type, Jα18 KO, and CD1d KO mice were euthanized 14 days after AC injection with Ad5E1 tumor. The tumor-bearing eyes were extracted into 1× PBS containing protease inhibitors (complete mini; Roche, Basel, Switzerland) and sonicated.

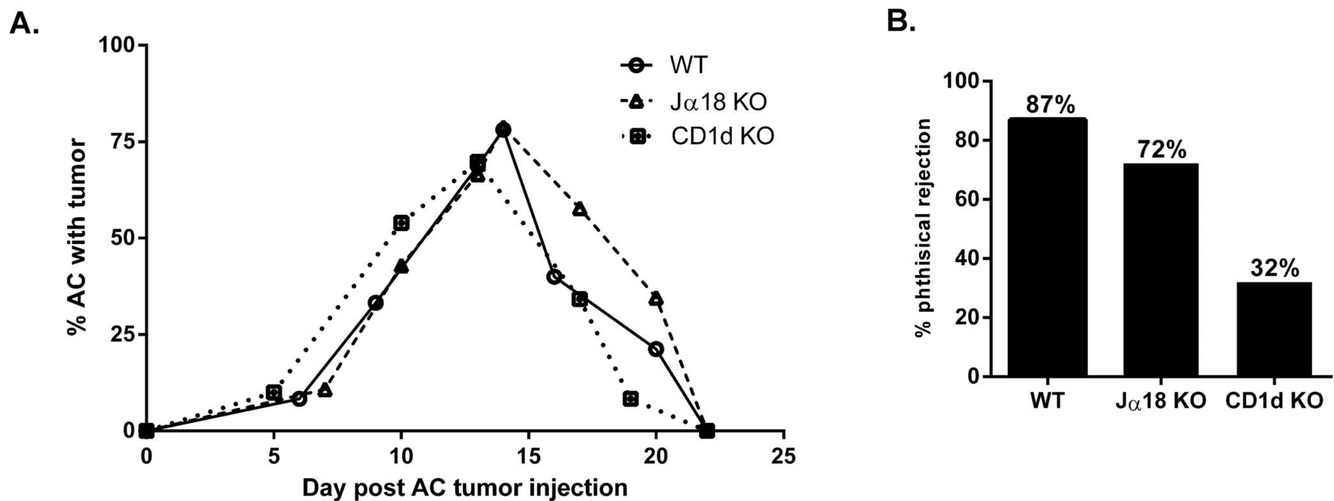

FIGURE 1. Nonnecrotizing intraocular tumor rejection is prevalent in CD1d KO mice lacking both type I and II NKT cells. (**A**) Percent of the AC occupied with tumor. Intraocular tumor grew in the AC and was rejected in 100% of the WT, Jα18 KO, and CD1d KO mice. Data are combined from two independent experiments for each mouse group. (**B**) Percent of mice that rejected the intraocular tumor phthisically. Data are combined from at least two independent experiments for each mouse group. WT ($n = 23$), Jα18 KO ($n = 18$), and CD1d KO ($n = 28$) mice.

Samples were centrifuged at 650*g* for 20 minutes at 4°C and the supernatant was collected and stored at −80°C. These supernatants were analyzed for CXCL3 content using the GRO gamma (CXCL3) Mouse SimpleStep ELISA Kit (Abcam, Cambridge, UK) according to manufacturer's recommendations. Linear regression analyses were done using the standard curve to identify the concentration of CXCL3 in each sample.

Flow Cytometry

Mice were euthanized 14 days after AC injection with Ad5E1 tumor, and their tumor-bearing eyes were harvested and single cell suspensions generated. The eye was digested and prepared for flow cytometry as previously described[30] and incubated with a master mix containing the following fluorescent antibodies: CD45 rF710 (clone 30-F11), Ly6G PE (clone 1A8). Flow cytometry data were acquired using the Attune NxT acoustic focusing cytometer (Applied Biosystems by Life Technologies, Carlsbad, CA, USA) and analyzed using FlowJo v10 software (Tree Star, Ashland, OR, USA).

Immunohistochemistry

Mice were euthanized 14 days after AC injection with Ad5E1 tumor, and their tumor-bearing eyes were processed for immunohistochemistry as previously described.[30] The eye sections were incubated with a 1:500 dilution of primary rat antibody to Ly6G (clone 1A8; Biolegend, San Diego, CA, USA) and developed using biotinylated secondary antibody (Vectastain Elite ABC Kit; Vector Laboratories; Burlingame, CA, USA) and 3, 3′-diaminobenzidine (DAB) substrate solution (Vector Laboratories) and counterstained with hematoxylin QS (Vector Laboratories). Stained eyes were imaged using differential interference contrast (DIC) microscopy with a ×40 brightfield lens on the Zeiss Observer.D1 microscope with AxioVision Imaging System software (Carl Zeiss, Jena, Germany).

Neutrophil Depletion

Wild-type mice were depleted of neutrophils via intraperitoneal (IP) injections of 500 μg anti-mouse Ly6G monoclonal antibody (clone 1A8; Bio X Cell, Lebanon, NH, USA) every 3 days starting one day previous to the AC injection of Ad5E1 tumor cells. Treatment control WT mice received IP injections of 500 μg rat IgG at the same intervals. Single cell suspensions of splenocytes were generated from WT mice to confirm neutrophil depletion by flow cytometry comparing 1A8 treatment to rat IgG control using methodology similar to that outlined in the flow cytometry section above.

Statistical Analyses

All statistical analyses were performed using GraphPad Prism software (San Diego, CA, USA). Mann-Whitney U tests were performed for ELISA data; all other statistical analyses were done using Student's t-tests assuming equal variance of SD, and P values less than 0.05 were considered significant.

RESULTS

Type II NKT Cells Contribute to Phthisical Necrotic Intraocular Tumor Rejection

We have previously shown that the Ad5E1 intraocular tumor undergoes a necrotizing form of immune rejection in the eyes of WT mice.[30,32] To determine if NKT cells influenced the immune rejection of Ad5E1 tumors we compared the pattern of rejection in mice with or without an intact NKT cell repertoire. In WT mice with intact NKT cell populations, the tumors grew in the AC and subsequently underwent rejection by 3 weeks (Fig. 1A) and culminated in phthisis in 87% of the mice (Fig. 1B). We next evaluated the phenotype of intraocular tumor rejection in two strains of mice with different NKT cell deficiencies. Jα18 KO mice lack type I invariant NKT cells but still retain their type II NKT cell population. Intraocular tumors grew transiently and underwent rejection in all the Jα18 KO mice, with a similar incidence of phthisis (72%) found in WT mice (Fig. 1). CD1d KO mice lack both type I invariant NKT cells and type II NKT cells. Ad5E1 tumors injected into the AC grew and were rejected in all of the CD1d KO mice (Fig. 1A). However, the pattern of immune rejection in CD1d KO mice was strikingly different than in either WT or Jα18 KO mice and was characterized by a steep reduction in the incidence of

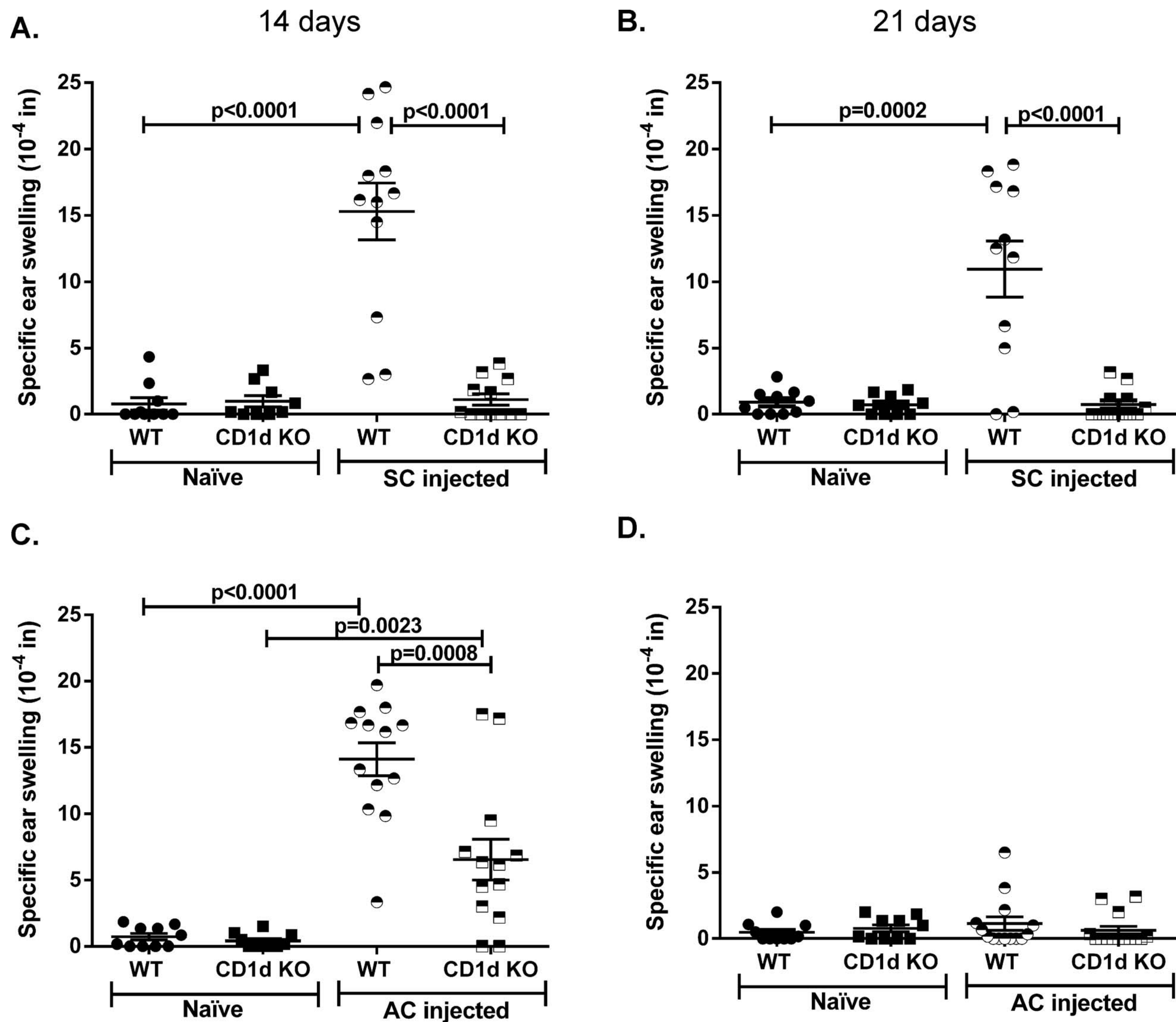

Figure 2. CD1d KO mice have reduced tumor antigen-specific ear swelling measured by delayed type hypersensitivity (DTH) after AC or SC injection routes compared to WT mice. Specific ear swelling (**A**) 14 days and (**B**) 21 days after SC injection. Specific ear swelling (**C**) 14 days and (**D**) 21 days after AC injection. Data are combined from two independent experiments for (**A–D**). Mean and SEM are shown for each group.

phthisis (32%) (Fig. 1B). Thus, the absence of an intact type II NKT cell repertoire profoundly affects the immunopathological phenotype of intraocular tumor rejection and as a result, preserves the integrity of the eye.

Absence of NKT Cells Results in Reduced DTH Responses to Intraocular Tumors

Due to the different intraocular rejection phenotypes in WT mice compared to CD1d KO mice, we tested if the loss of NKT cells would lead to reduced inflammatory responses after AC tumor injection. Delayed type hypersensitivity responses are characterized by an immune response culminating in high amounts of inflammatory mediators that can result in ischemic necrosis, culminating in damage to innocent bystander cells. Therefore, an increased DTH response is associated with an exacerbated inflammatory response and necrotic phthisical tumor rejection. We hypothesized that CD1d KO mice would have reduced DTH responses after AC tumor injection compared to WT mice, which would correlate with the observed reduction in the incidence of phthisis in CD1d KO mice.

Tumor-specific DTH responses were tested in WT and CD1d KO mice 14 and 21 days after tumor injection. As expected, naïve WT and CD1d KO mice did not produce a DTH response specific for the Ad5E1 tumor (Fig. 2). Subcutaneously (SC) injected tumor cells injected in WT mice induced robust tumor-specific DTH responses 14 and 21 days after injection (Figs. 2A, 2B). By contrast, SC tumor injections did not elicit positive DTH responses in CD1d KO mice (Figs. 2A, 2B). Thus, the absence of an intact NKT cell repertoire prevents the generation of DTH in intraocular tumor-bearing mice. By contrast, WT and CD1d KO mice mounted DTH responses at the peak of intraocular tumor growth (i.e., 14 days after AC injection) (Fig. 2C). However, the responses in CD1d KO mice were significantly reduced compared to their WT counterparts ($P = 0.0008$; Fig. 2C). Tumor resolution in both WT and CD1d KO mice was complete by day 21 and was accompanied by a loss of DTH responses (Fig. 2D). Interestingly, CD1d KO mice had significantly reduced tumor-specific DTH responses compared to WT mice after AC injection, which corresponds with the reduced incidence of necrotizing tumor rejection (i.e., phthisis).

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Table. Immune System-Associated Differential Gene Expression From WT Tumor-Bearing Eyes Compared to CD1d KO Tumor-Bearing Eyes Isolated 14 Days After AC Injection

Gene	Protein Name	Fold Change: WT vs. CD1d KO	*P* Value	Biological Activities
Cxcl3	Chemokine ligand 3; macrophage inflammatory protein 2-beta—MIP-2β	9.45	5×10^{-5}, 0.00005	Chemotactic activity for neutrophils; granulocyte adhesion and diapedisis
Mmp13	Matrix metallopeptidase 13—collagenase-3	5.54	5×10^{-5}	Degrades collagen type 2; granulocyte adhesion and diapedisis; leukocyte extravasation signaling
S100a4	S100 calcium binding protein A4—metastasin	4.99	5×10^{-5}	Chemotactic activity for macrophages; cell cycle progression and differentiation
CD68	Macrophage antigen CD68	3.92	5×10^{-5}	Marker for cells in the macrophage lineage
Lyz2	Lysozyme 2	3.76	5×10^{-5}	Bacteriolytic and cytolysis in macrophages
Rac2	Ras-related C3 botulinum toxin substrate 2—rho family, small GTP binding protein	3.68	1×10^{-4}, 0.0001	Augments the production of reactive oxygen species, ROS; leukocyte migration; Fcγ receptor-mediated phagocytosis in macrophages

Reduced Necrotizing Inflammation Correlates With Diminished Expression of Proinflammatory Gene Products

Since CD1d KO mice have a lower rate of phthisis and tumor-specific DTH responses, we hypothesized that there were fundamental differences in the inflammatory environment present in the intraocular tumors in WT and CD1d KO mice. This was assessed by removing tumor-bearing eyes from WT and CD1d KO mice at the peak of AC tumor growth and immune rejection (i.e., 14 days after AC injection) and isolating the RNA for mRNA sequencing analysis to determine the transcriptome of the ocular tumor environment. Of the differentially expressed genes, we were interested in those involved in the immune response as identified by relevant biological pathway analysis. The Table shows the immune response genes that were significantly upregulated in WT mice compared to CD1d KO mice. The genes that were most noteworthy included those associated with granulocyte adhesion, diapedesis, and effector functions, which were enriched in the WT mice compared to the CD1d KO mouse tumor-bearing eyes (Table). The six most significantly upregulated genes associated with the immune system were *Cxcl3*, *Mmp13*, *S100a4*, *CD68*, *Lyz2*, and *Rac2*.

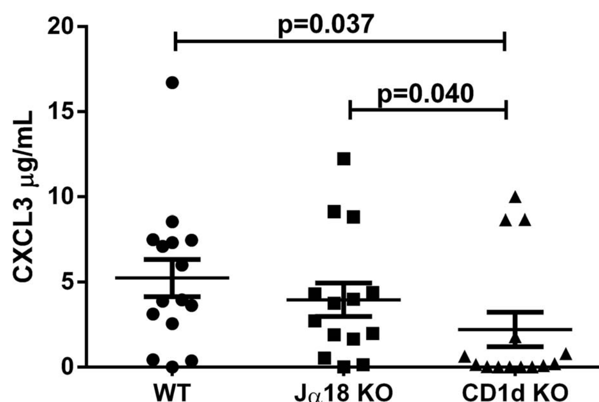

Figure 3. Protein levels of CXCL3 are reduced in CD1dKO mice compared to either WT or Jα18KO mice. Tumor-bearing eye lysates were generated 14 days after AC injections and assayed by ELISA. The mean and SEM are shown for each group of mice combined from three independent AC injection experiments and assayed over three independent ELISA runs. WT ($n = 15$), Jα18 KO ($n = 14$), and CD1d KO ($n = 14$) mice.

Cxcl3 was the most dramatically upregulated gene (9.45-fold higher) in WT mice (Table). CXCL3 is also known as MIP-2β, the mouse homolog of human IL-8, and is an important chemoattractant for neutrophils. This bears noting as neutrophils are known to cause bystander tissue damage in other tissues such as the heart,[39] peritoneal toxic shock,[40,41] pancreas,[42] kidney,[43] and liver,[44] as well as in ocular infections.[45–48] Since phthisical tumor rejection involves the destruction of bystander ocular tissues, we focused our attention on the role of neutrophils in Ad5E1 intraocular tumor rejection.

In order to confirm the transcriptome difference of *Cxcl3* mRNA expression to the protein levels of CXCL3, tumor-bearing eyes were evaluated by ELISA 14 days after AC tumor injection. Jα18 KO mice were also included in this analysis to distinguish between the presence of type II NKT cells to the absence of both type I and II NKT cells present in CD1d KO mice. Consistent with the reduced expression of CXCL3 mRNA, protein levels of CXCL3 were significantly reduced in CD1d KO mouse tumor-bearing eyes compared to tumor-bearing eyes from either WT (2.2 vs. 5.2 ug/mL; $P = 0.037$) or Jα18 KO (2.2 vs. 4.0 ug/mL; $P = 0.040$) mice (Fig. 3). Furthermore, there was no significant difference in CXCL3 protein expression in the tumor-bearing eyes of WT mice or the Jα18 KO mice (Fig. 3).

Presence of a Type II NKT Cell Repertoire Is Associated With Neutrophil-Dependent Inflammation and Phthisis of Tumor-Bearing Eyes

The reduced level of CXCL3 protein and mRNA in tumor-bearing eyes from CD1d KO mice compared to WT mice suggests that a reduced influx of neutrophils would be present in the intraocular tumor environment. In order to confirm that neutrophils infiltrate intraocular tumors, tumor-bearing eyes were interrogated by immunohistochemistry 14 days after AC tumor injection using anti-Ly6G antibody, which is a specific marker for neutrophils. Immunohistochemical analysis revealed that Ly6G$^+$ neutrophils were within the intraocular tumor mass in all three mouse strains, but were most pronounced in WT and Jα18 KO mice (Fig. 4).

Based on the association between phthisical rejection and the elevated expression of CXCL3, we hypothesized that CD1d KO mice would have significantly reduced numbers of infiltrating neutrophils within the intraocular tumors compared to either WT or Jα18 KO mice. Flow cytometry was used to assess the percent of Ly6G$^+$ neutrophils present in the

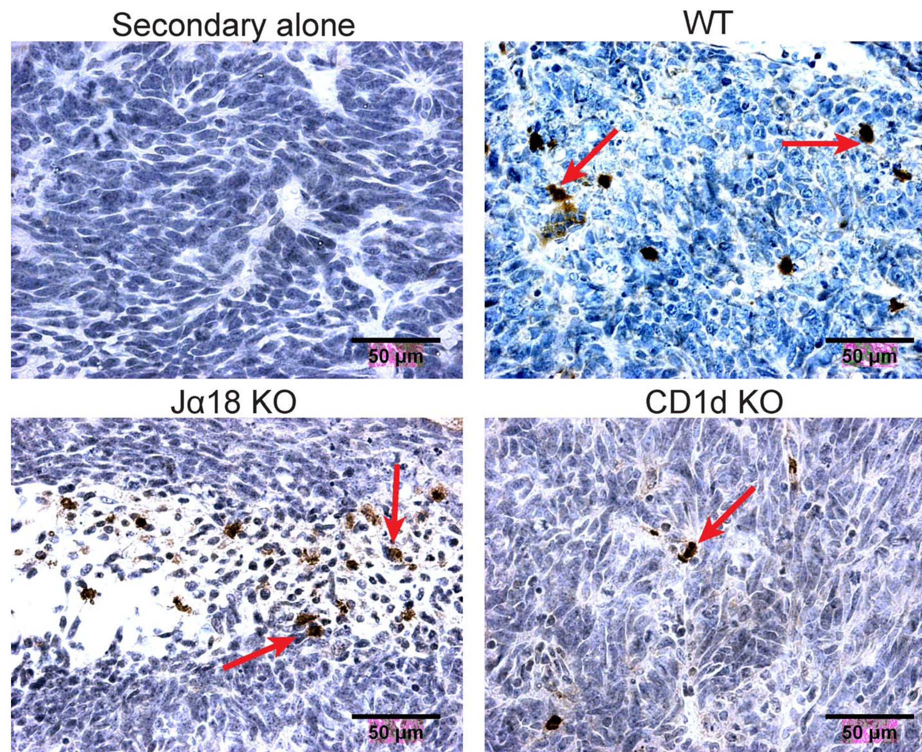

Figure 4. Neutrophils migrate to the AC during intraocular challenge. Staining of Ly6G+ neutrophils are visualized by the brown DAB stain (*red arrows*) and counterstained with blue hematoxylin QS. Tumor-bearing eyes were harvested 14 days after AC tumor injection from WT, Jα18 KO, and CD1d KO mice, and representative images are shown at ×40 magnification with a 50-μm *scale bar*.

infiltrating CD45+ leukocyte population in the intraocular tumors at the onset of immune rejection (i.e., day 14) (Fig. 5A). The percent of Ly6G+ neutrophils was similar in WT and Jα18 KO tumor-bearing eyes and was consistent with the high incidence of phthisical tumor rejection in both of these mouse strains (Fig. 5A). Therefore, the loss of type I invariant NKT cells that characterizes the Jα18 KO mouse strain does not impact the recruitment of neutrophils into the intraocular tumors. However, the CD1d KO tumor-bearing eyes had a significant reduction in the percent of Ly6G+ neutrophils compared to WT mice (4.71% vs. 15.50%, $P = 0.0007$) and Jα18 KO mice (4.71% vs. 18.32%, $P = 0.0013$) (Fig. 5B). Thus, the absence of type II NKT cells in CD1d KO mice results in a decreased recruitment of neutrophils into the intraocular tumor environment.

Neutrophil Depletion Abrogates Phthisical Tumor Rejection and Converts the WT Mice Rejection Phenotype to One Similar to NKT Cell-Deficient CD1d KO Mice

CD1d KO mice had significantly reduced neutrophils entering the intraocular tumor-bearing eye compared to either WT or Jα18 KO mice. We hypothesized that removal of neutrophils would lead to a reduced rate of phthisis that would resemble the low rate of phthisis seen in CD1d KO mice. We tested this by depleting neutrophils in vivo using anti-Ly6G monoclonal antibody in WT mice. Two groups of WT mice received AC tumor injections and were also injected IP every 3 days with either anti-Ly6G or a rat IgG control antibody. We confirmed that this treatment reduced neutrophils by flow cytometry of splenocytes from mice that received anti-Ly6G antibody compared to those that received rat IgG control antibody (Fig. 6A). The intraocular tumors grew and were eventually rejected in all of the mice, demonstrating that neutrophils are not necessary for the rejection of the Ad5E1 intraocular tumors (Fig. 6A). However, there was a striking difference in the rejection phenotype, with the anti-Ly6G neutrophil-depleted group having only 27% phthisical rejection compared to 80% in the control group (Fig. 6B). Therefore, neutrophils contribute to the pathological processes leading to phthisis during intraocular tumor rejection. The depletion of neutrophils led to a recapitulation of the low phthisical rejection rate of 32% found in CD1d KO mice (Fig. 1B). The absence of type II NKT cells led to a reduction in CXCL3 production, causing a reduced neutrophil infiltration, and ultimately a lowered rate of damaging inflammation that would culminate in phthisical rejection.

Discussion

We have utilized both Jα18 KO and CD1d KO mice to study the pathology of intraocular tumor rejection in comparison to WT mice. The immune-mediated rejection of the intraocular tumor in WT mice culminates in phthisis, characterized by necrosis and destruction to bystander ocular tissue damage compromising the eye. We found that the absence of type I NKT cells in Jα18 KO mice had no impact on the immunopathology generated during the intraocular tumor rejection and resembled that in WT mice with high rates of phthisis. However, the additional absence of type II NKT cells in the CD1d KO mice had a profound impact on the immunopathology, with the majority of intraocular tumors rejecting in a pristine manner that preserved the eye. This decrease in phthisis in the CD1d KO mice was associated with a reduced inflammatory response to tumor antigens measured by a reduction in DTH responses.

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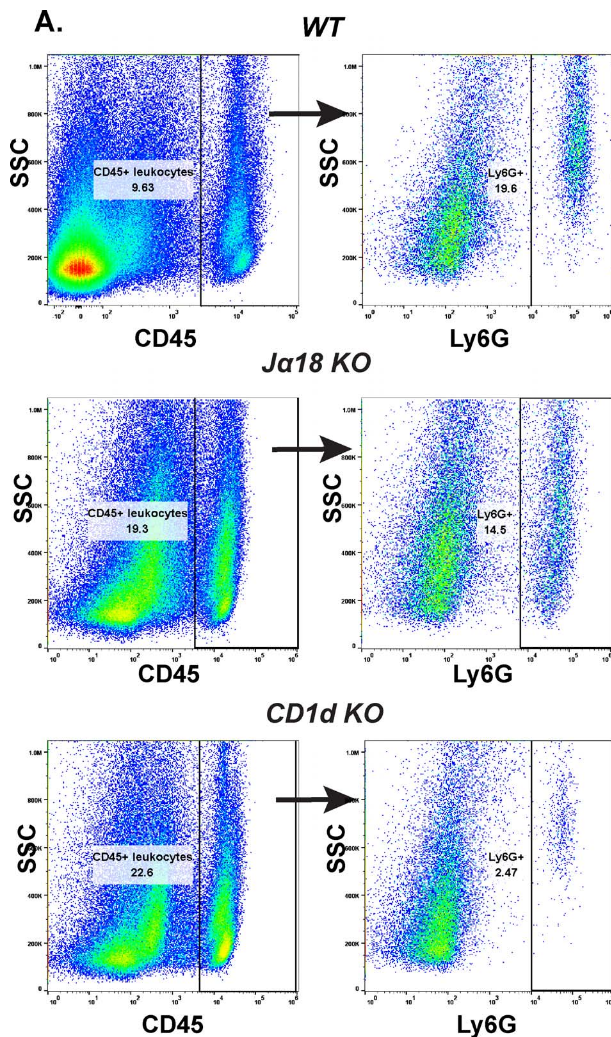

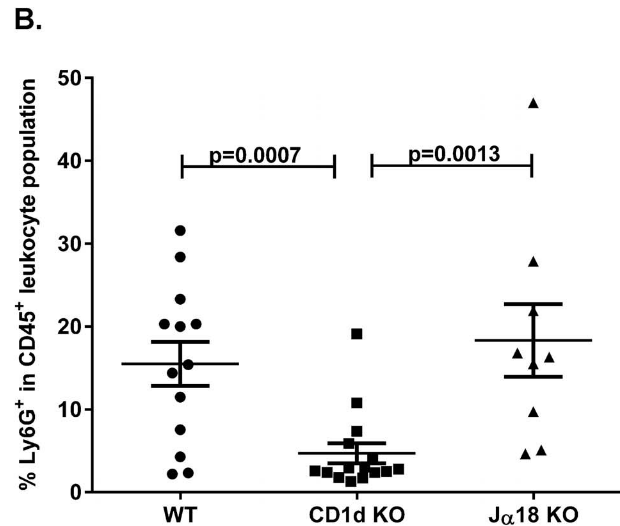

FIGURE 5. CD1d KO mice have reduced percentage of Ly6G$^+$ neutrophils in the CD45$^+$ leukocyte population compared with either WT or Jα18 KO mice. Tumor-bearing eyes were assayed by flow cytometry 14 days after AC injections. (**A**) Suspensions from tumor-bearing eyes were gated on single cells, and the representative gating strategy from CD45$^+$ leukocytes is shown. (**B**) Percent of the CD45$^+$ leukocyte population that are Ly6G$^+$ shown as combined data from four independent experiments for WT ($n = 13$), CD1d KO ($n = 15$), and Jα18 KO ($n = 9$) with the mean and SEM.

Furthermore, CD1d KO tumor-bearing eyes express significantly lower levels of CXCL3, an IL-8 homolog, resulting in reduced levels of infiltrating neutrophils in the intraocular tumor compared to Jα18 KO and WT mice. Depletion of neutrophils in WT mice converted the intraocular tumor phenotype to a pristine rejection resembling that in CD1d KO mice. In summation, type II NKT cells promote the production of CXCL3 to attract neutrophils into the intraocular tumor site, which results in significant bystander destruction to ocular tissue and ultimately phthisical loss of the eye.

Type II NKT cells are a diverse population that exert varied functions based on tissue location and target antigen. The phthisical environment is characterized by necrosis and ischemia of the tissues. Early innate immune cell activation of antigen-dependent NKT cells and antigen-independent neutrophils can contribute to an inflammatory tissue-destructive process. In peripheral tumor models, type I NKT cells typically promote antitumor responses while type II NKT cells promote tumor growth.[16] We found a distinctly different outcome in our intraocular tumor model, where the loss of type II NKT cells in CD1d KO mice, compared to only the loss of type I NKT cells in Jα18 KO mice, led to a more efficient antitumor response that also spared bystander ocular tissue from destruction. This suggests that type II NKT cells do not function as regulatory cells in the intraocular tumor environment, but instead contribute to the damaging proinflammatory immune response that culminates in phthisis. This is reminiscent of the results reported for a model of ischemia–reperfusion injury of the kidney in which inhibition of both neutrophil infiltration mitigated kidney damage in CD1d-depleted mice compared to Jα18 KO mice.[49] Type II NKT cells are increased in human ulcerative colitis tissue and are cytotoxic to endothelial cells[24]; they are implicated in hepatitis liver destruction[50] and in high fat-induced liver and adipose inflammation.[51] Collectively, these findings suggest that type II NKT cells can initiate inflammation and immunopathology in peripheral tissues similar to what we demonstrate in this current study of ocular inflammation.

CD1d KO mice had reduced Ad5E1 tumor cell-specific DTH responses when compared to WT mice in both AC and SC injected routes. Kinetic studies of DTH responses have demonstrated that neutrophils infiltrate SC reaction sites first, followed by lymphocytes and monocytes.[52] Furthermore, IL-8, the homolog of CXCL3, is required for the early accumulation

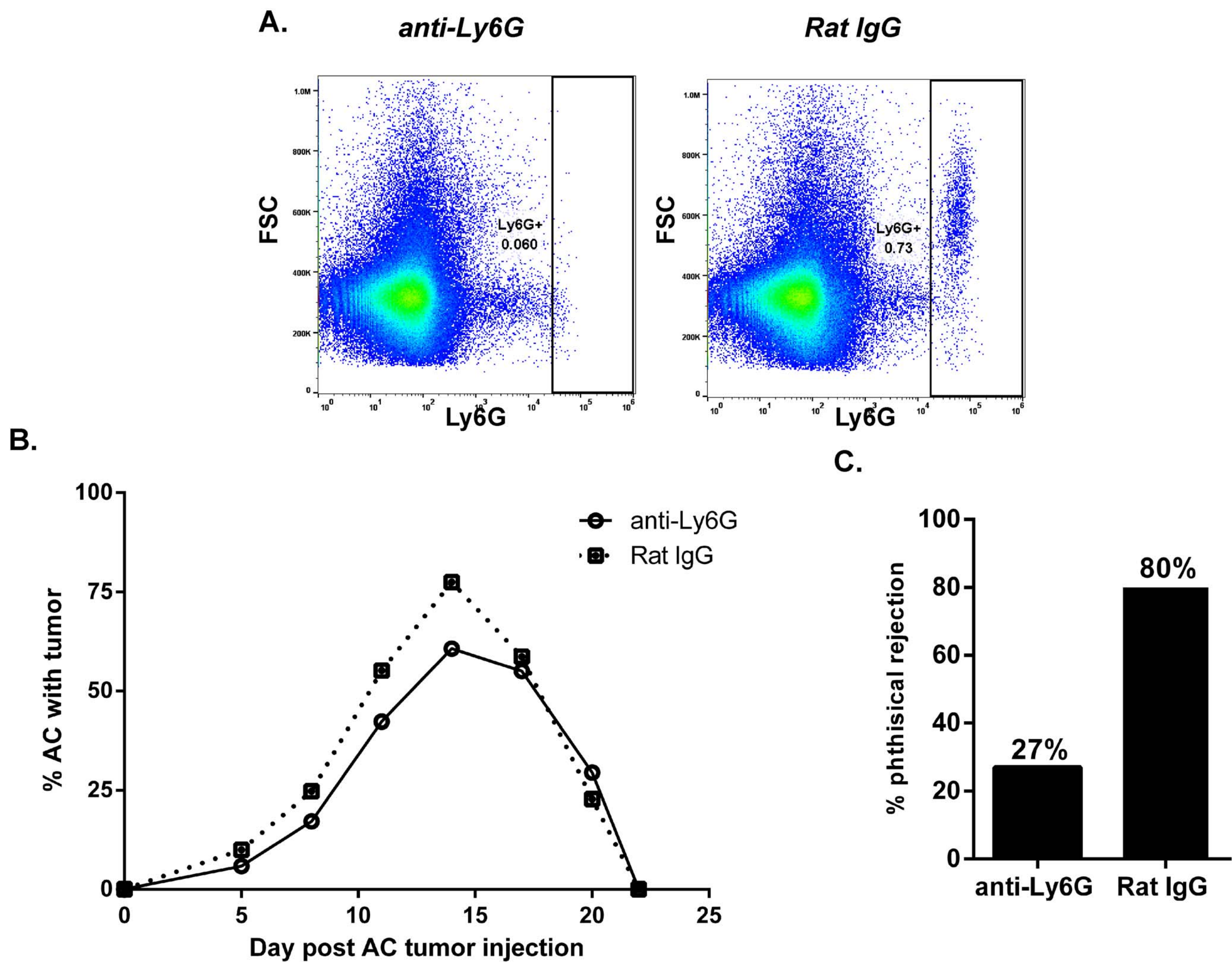

FIGURE 6. Depletion of neutrophils in WT mice results in reduced rates of necrotizing phthisical rejection after intraocular tumor rejection. WT mice were injected IP every 3 days with either the anti-Ly6G neutrophil-depleting antibody (clone 1A8; $n = 15$) or rat IgG as a control ($n = 30$). (**A**) CD45$^+$ splenocytes were tested by flow cytometry to confirm the reduction of neutrophils using this treatment. (**B**) The intraocular tumor growth shown as percent of the AC occupied with tumor. Both groups of mice were able to reject their intraocular tumor. (**C**) Percent of mice that rejected their intraocular tumor phthisically. Data are combined from two independent experiments.

of leukocytes and for a robust DTH response in rabbits.[53] While inflammatory DTH responses are classically CD4$^+$ T-cell mediated, neutrophils can link the innate and adaptive immune response to promote a DTH response through CD4$^+$ T-cell recruitment.[54] In the present study, the absence of type II NKT cells in the CD1d KO mice leads to reduced CXCL3 levels and thus reduces neutrophil recruitment that dampens the full development of a DTH response in these mice. This reduction in DTH responses could be an important contributing factor to the reduction of phthisis after intraocular tumor rejection in CD1d KO mice that is not observed in the WT or Jα18 KO mice.

The absence of NKT cells has been shown to contribute to a decreased neutrophil infiltration in other peripheral tissues. Lungs of CD1d KO mice with *Pseudomonas aeruginosa*-induced pneumonia had lower numbers of neutrophils and MIP-2 levels compared to WT mice.[55] This is similar to our present study demonstrating that CD1d KO mice with intraocular tumors express decreased levels of CXCL3 leading to decreased neutrophil recruitment within the eye. Increased amounts of IL-8 or CXCL3 not only recruit neutrophils, but also augment their degradation of collagen,[56] contributing to destruction of ocular tissue. Mice with induced corneal human IL-8 expression developed corneal ulcers, angiogenesis, and significant endothelial cell disruption[45] permitting increased immune cell infiltration. In humans, elevated ocular levels of IL-8 are found in inflammatory conditions of the conjunctiva, cornea, uvea, retina, and vitreous.[57] We found that WT and Jα18 KO mice had significantly higher levels of CXCL3 and neutrophil recruitment associated with the formation of phthisis during the intraocular tumor inflammatory response. The loss of type II NKT cell-dependent neutrophil recruitment in CD1d KO mice or the depletion of neutrophils in WT mice reduced the immunopathology and protected the eye during the intraocular tumor rejection, with the majority of mice retaining their eyes.

There is growing appreciation for the complexity of neutrophils beyond antimicrobial functions including their interactions with other cells of the innate and adaptive immune systems.[58] They are critical mediators in tissue destruction in ocular infections[45–48,59] and ischemic tissue damage in many other tissues.[39–44] Neutrophils accumulate in a wounded cornea and participate in the breakdown of extracellular

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matrix through release of proteolytic enzymes and reactive oxygen species.[60,61] Superoxide dismutase treatment, to reduce superoxide radicals, significantly reduced the neutrophil-mediated ocular tissue necrosis in *P. aeruginosa*-injected eyes.[62] The ability of neutrophils to secrete proteolytic enzymes compromises endothelium barriers, thus facilitating the entry of other leukocytes into the tissue.[63] Blocking CD11b, an adhesion molecule used by monocytes and neutrophils, reduced retinal pathology in diabetic mice,[64] suggesting that blocking the early innate inflammatory reaction can prevent an immunologic cascade leading to tissue destruction. Depleting neutrophils in WT mice significantly reduced the rates of phthisical rejection of the intraocular tumor, recapitulating what was observed in CD1d KO mice that have reduced levels of neutrophil recruitment. The neutrophils were not necessary for a productive antitumor response; their presence and activity were critical for development of ischemic necrotic bystander tissue destruction.

A focused adaptive immune response that limits the participation of neutrophils provides the optimal conditions for both tumor rejection and preservation of the eye. Our lab has previously reported that intraocular tumor rejection involving another innate immune cell, the macrophage, can contribute to the formation of phthisis through the production of inflammatory mediators such as TNFα and iNOS.[32] Both macrophages and the neutrophils can secrete potent inflammatory cytokines, chemokines, and enzymes into the ocular environment, culminating in irreversible ocular tissue damage. Moreover, we have recently shown that enhancing a targeted antitumor cytotoxic T-lymphocyte response provides an effective anti-intraocular tumor response without phthisis.[30] In the context of the ocular environment, the most advantageous antitumor response involves a targeted adaptive immune response that minimizes bystander damage that can occur with a robust innate immune response. Dampening the initial proinflammatory innate immune responses generated by type II NKT cells, which then promote the activity and recruitment of neutrophils, generates a productive antitumor response with minimal ischemia and necrosis.

The present findings indicate that NKT cells have a profound influence in shaping the adaptive immune response in the eye and in influencing the immunopathological consequences of intraocular tumor rejection. Natural killer T cells also modulate the innate immune responses to metastases arising from intraocular melanomas.[25,26] That is, deletion of NKT cells results in enhanced NK cell-mediated cytolysis of murine melanoma cells and a steep reduction in the development of liver metastases arising from intraocular melanomas in mice.[65,66] The latter finding is noteworthy as there is an expanding body of data indicating that NK cells are extraordinarily important in controlling the development of liver metastases in both human uveal melanoma patients and in mouse models of uveal melanoma.[25,26,67–72] Although uveal melanomas are susceptible to NK cell-mediated lysis in vitro, their susceptibility to NK cell-mediated killing is inversely correlated with the expression of major histocompatibility complex (MHC) class I molecules on the uveal melanoma cells, which transmit an "off" signal to NK cells and inhibit NK cell activity.[73–75] The correlation between MHC class I expression and resistance to NK cell-mediated cytolysis is further supported by studies from Jager and colleagues,[67,68,71,76] and Ericsson et al.[77] who reported that patients expressing high levels of MHC class I on their primary uveal melanomas experienced a poorer prognosis than patients expressing lower levels of MHC class I molecules on their uveal melanomas. Thus, the sphere of NKT cell influence extends beyond the eye and also affects innate immune responses.

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