OCULAR INFLAMMATION IN AGE-RELATED EYE DISEASES

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‘The way to love anything is to realize it might be lost’

***G.K. Chesterton**, English writer and Christian apologist, 1921*

voor mijn ouders

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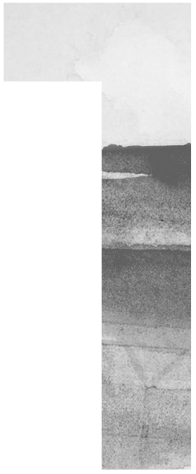

GENERAL INTRODUCTION

AGING AND THE MANIFESTATION OF AGE-RELATED EYE DISEASES

Aging has been a worldwide phenomenon since the mid-twentieth century. Aging is defined as the progressive accumulation of changes with time that are associated with or responsible for an increasing susceptibility to disease.[2] The percentage of the elderly, defined as age 60 and over, in the world population increased from 9.2% in 1990 to 11.7% in 2013 and is expected to continue to grow, reaching 21% by 2050.[1] Among the diseases associated with aging, ocular changes are prevalent. The most well-known ocular condition related to aging is cataract, but many other eye diseases may occur as a result of aging, such as tumors and degenerative diseases such as age-related macular degeneration and glaucoma. As the eyes age, they undergo a number of structural and functional changes that are associated with degeneration including the loss of cells in the ganglion cell layer, loss of retinal pigment epithelial (RPE) cells and photoreceptors, decreased retinal thickness, changes in the optic nerve and the ocular vasculature.[3-8] In parallel, age-related alterations in visual function have been described, as retinal and cortical processing of visual information is significantly different in healthy elderly individuals compared to young adults.[9]

Age-related Macular Degeneration

A classical example of an ocular condition that is typically found in the elderly is age-related macular degeneration (AMD). Among those aged 50-54, there is a 0.4% prevalence of advanced AMD, which increases dramatically to 11.8% in those over 80.[10] AMD is the leading cause of permanent, irreversible, central blindness in patients over the age of 50 in Europe and North America.[11] Age-related changes in the RPE are a central element in the pathogenesis of AMD. One of the most important functions of the RPE is the renewal of photoreceptors through phagocytosis of metabolic waste products and cellular debris.[12] As the number of RPE cells diminishes with age, phototoxic waste products and debris accumulate in the RPE, which imposes an ever-increasing burden on the remaining RPE cells. Further impairment with age causes the accumulation of debris in the cytosol of these cells, and in individuals over 80, this debris can occupy one fifth of the total RPE cell volume.[3,12,13] These byproducts damage DNA and cell membranes and cause chronic inflammation and apoptosis of RPE cells, which is the first step in the pathogenesis of AMD. According to these findings, the presence of inflammation is an essential component, with a detrimental effect.

Primary Open Angle Glaucoma

Another ocular age-related condition is primary open angle glaucoma (POAG). Similar to the trend seen in AMD, there is a 0.7% prevalence of glaucoma in individuals 40-49 years of age, which increases to 7.9% in individuals over 80.[14] In 2020, an estimated 79.6 million people worldwide will be affected by glaucoma, and of these, 74% will have POAG and 5.9 million patients with POAG will present with bilateral blindness.[15] Glaucoma, the second leading cause of blindness in the world, is a group of ocular diseases characterized by damage to the optic nerve and degeneration of retinal ganglion cells (RGCs), which leads to progressive and permanent vision loss.[15,16] Elevation of intraocular pressure (IOP) and older age are considered major risk factors for the onset and development of POAG.[17,18] It has been reported that the mean reduction in retinal nerve fiber layer thickness is 3 μm per decade, corresponding with a loss of approximately 60.000 RGCs.[19] The significant loss of neurons in the inner retina during normal aging might explain why age is a significant factor for the development of visual defects associated with glaucomatous neuropathy.[20,21] For example, on average the number of RGCs in the retina of a 25-year old is twice that of a 95-year old.[19] This large neural reserve observed in young individuals could explain why the aging population is more susceptible to glaucomatous damage. It is possible that clinically significant visual field defects occur when the sum of pathologic (e.g. elevated IOP, ischemia, etc.) and non-pathologic (e.g. aging) neuronal losses reaches a critical level. Age-related changes in neuronal susceptibility to damage have indeed been reported, in which older animals show faster loss of RGCs following axonal damage compared to younger animals.[22] Other structural changes seen with aging are a decrease in the mechanical compliance of the lamina cribrosa[4,23] and increased resistance in the trabecular outflow tract[24] which is in part due to alterations in the distribution and amount of connective tissue and extracellular matrix components.[23,24] In addition, vessel density and diameters of the choriocapillaries and retinal arterioles and venules decrease with increasing age, independent of other risk factors.[25] These changes are consistent with a reduction in ocular blood flow with advancing age in retrobulbar, choroidal and optic nerve vascular beds, leading to impairment of oxygen supply and metabolic exchange, and ultimately to ischemic conditions of the eye.[7,26] Burgoyne and Downs[27] have described that age-related alterations in the biomechanics of the optic nerve head (ONH) underlie the susceptibility of the aged ONH to glaucomatous damage. They suggest, that non-IOP related insults, such as inflammation or reduced ocular blood flow, damage the connective tissue and

axons of the ONH, making the optic nerve vulnerable to secondary damage, e.g. through elevated IOP.[7,27]

Uveal Melanoma

Not only are neurodegenerative diseases more prevalent in the aging eye, but also ocular malignancies demonstrate a strong correlation with the aging process. Uveal melanoma is a tumor that can develop in the iris, the ciliary body, or the choroid and the most common primary intraocular malignancy in adults with an estimated annual incidence of 6 to 10 cases per million per year in Caucasian populations, with a median age at diagnosis of 62 years.[28,30,31] The incidence rate of uveal melanomas progressively increases from 3.9 cases per million in males and 2.4 cases per million in females aged 40-44 years to a peak of 24.5 cases per million in males and 17.8 cases per million in females aged 70-74.[28-30]. Up to 50% of patients with uveal melanoma may die from metastatic disease, and metastasis may still develop after 10-15 years, and even 35 years after diagnosis.[32,33] Over the past decades, patient survival has not improved, despite progress in the diagnosis of melanocytic lesions and successful treatment of the intraocular melanoma.[28,34,35]

The term "inflammatory phenotype" has been proposed to describe a combination of inflammatory markers in uveal melanoma with an poor prognosis, including high numbers of tumor-associated macrophages and lymphocytes, and high levels of HLA class I and II expression. These tumors are further characterized by the presence of epithelioid cells and a high vascular density, as well as an association with the loss of one copy of chromosome 3.[33,36] Loss of expression of a specific gene on chromosome 3, BAP1, is strongly correlated with the influx of lymphocytes in primary uveal melanoma and metastasis. [37-40]

THE IMMUNE SYSTEM: UNDERSTANDING THE M1/M2 - T_H1/T_H2 PARADIGM

M1 and M2 macrophages

Although specific pathogenic mechanisms are involved in the initiation and progression of different types of age-related eye diseases, it is believed that inflammation is an important component which contributes to the pathogenesis of many of these diseases.[41] However, to fully understand and elucidate the altered inflammatory responses seen in aged individuals,

it is important to first realize that there are functionally different types of macrophages and immune effector cells. Mantovani described two subsets of macrophages, the classically-activated (M1) and the alternatively-activated (M2) macrophage.[42-44] Bacterial products such as lipopolysaccharide (LPS) and IFN-γ produced by other immune cells polarize macrophages toward the M1 phenotype, which gain immunostimulatory functions, anti-bactericidal activity and release pro-inflammatory (e.g. IL-12, TNF-α) cytokines. These cells have a higher expression of major histocompatibility (MHC) class II and costimulatory molecules, and function as antigen-presenting cells (APCs) to stimulate T and B cell-adaptive immune responses against infectious agents.[42,43] In contrast, M2 polarization occurs when other immune cells produce IL-4 and IL-13.[45,46] M2 macrophages are mainly involved in the dampening of inflammation, the promotion of tissue remodeling and repair, angiogenesis, tumor progression and immunoregulatory functions.[42,47]

T_H1 and T_H2 cells

Mirroring M1 and M2 polarization, $CD4^+$T helper cells can also differentiate into one of the two divergent pathways, resulting in functionally polarized T_H1 and T_H2 cells.[48,49] T_H1 cells secrete IL-2, IFN-γ and lymphotoxin, which are potent pro-inflammatory cytokines while T_H2 cells secrete IL-4, IL-5, IL-6, and IL-10.[48,49] In addition, there are T suppressor (regulatory) cells (Treg), which can regulate other T cells, macrophages and APCs.[48] M1 or M2- dominant macrophage responses can influence whether an T_H1 or T_H2 response occurs via their expression of certain cytokines and chemokines.[50] In response, T_H1, T_H2 or Treg cells can integrate M1 and M2 macrophages in circuits of amplification and regulation of polarized T-cell responses.[42,43] Vice versa, T cells can also control the type of immune responses generated by the profile of cytokines they secrete. In particular, immune responses induced by T_H1 cell-derived IFN-γcause macrophage activation, whereas IL-4, IL-5, and IL-10 produced by T_H2 cells inhibit macrophage activation.[50]

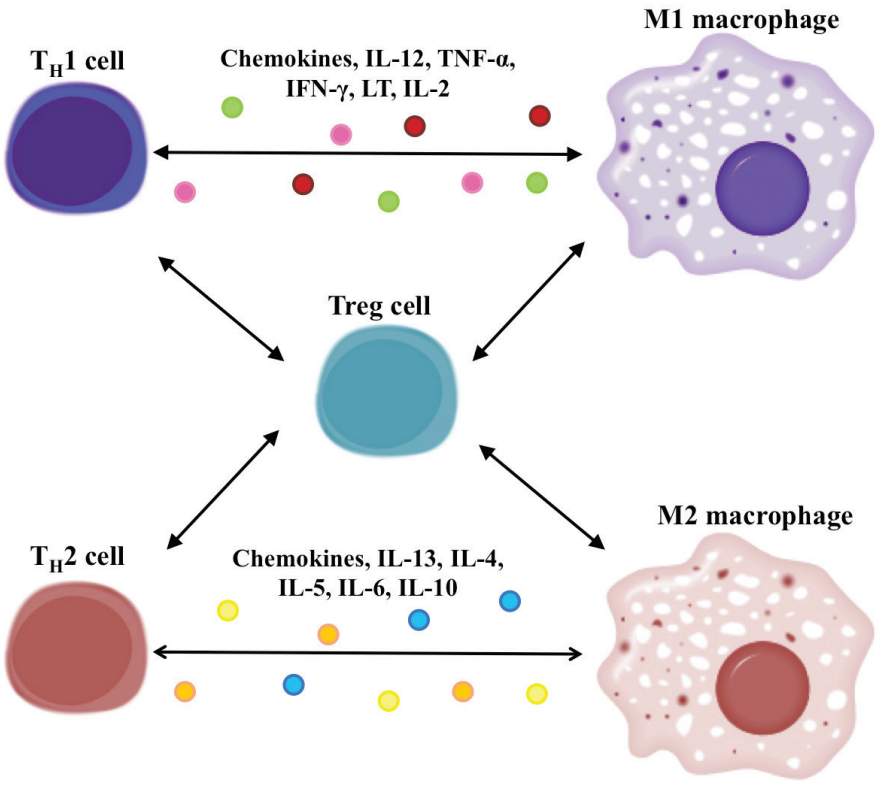

Figure 1. A simplified view of the orchestration of macrophage-T cell activation and polarization.

INFLAMMATION IN THE AGING EYE

An immune-privileged site

Traditionally, the eye has been considered an immune-privileged site[48,51], which assumedly allows it to be protected from the potentially destructive effects of a local inflammatory immune response. This protection is created by passive and active contributors. Passive contributors include the blood-ocular barrier and the presence of specific immunosuppressive cytokines, neuropeptides and growth factors in the ocular fluids.[48] Dysfunction of the blood-ocular barrier due to injury or disease can cause loss of immune privilege which in turn results in destruction of neural tissue in the eye.[52] Active contributors to the ocular eye-induced immunosuppression have been identified through the study

of ACAID (anterior chamber associated immune deviation). Uptake of an antigen in the anterior chamber by an APC that was simultaneously incubated in immunosuppressive cytokines such as TGF-β, and subsequent transport to the spleen leads to a systemic downregulation of antigen-specific immune responses.[48]

Currently, the retina is known to have an endogenous immune system that is coordinated by immunocompetent cells, such as microglia and dendritic cells.[53,54] In addition, the pigment epithelial cells lining the iris, ciliary body, and retina exhibit immunomodulatory functions through the production of cell-surface and soluble inhibitory molecules.[55-58] More importantly, resident retinal microglia, RPE cells, and choroidal macrophages/dendritic cells are major players in mounting the immune response which deals with stress or malfunction of the retina in several pathologic conditions.[41] In fact, an immune response, when controlled, is an adaptive response to restore tissue homeostasis and to monitor tissue malfunction. However, when alterations occur secondary to aging, metabolic abnormalities, altered vascular perfusion or degenerative conditions may initiate various inflammatory cascades leading to a prolonged, deregulated and pathogenic immune response.[59]

Para-inflammation

Medzhitov[60] first introduced a concept called "para-inflammation," later supported by Forrester[41]. The physiological purpose of para-inflammation is to restore tissue functionality and homeostasis. This type of inflammatory response is likely to be more common but of lower magnitude than the classic inflammatory response induced by infection or injury. Aging is one of the main factors that can lead to chronic para-inflammation and increased tissue stress and malfunction.[2,61] Retinal cells encounter progressive amounts of oxidative and metabolic stress during the aging process. "The free radical theory of aging" described by Harman[2] implicates that reactive free radicals are formed endogenously via normal oxygen-utilizing metabolic processes and that tissue damage induced by free-radicals is progressively accumulated during aging.[2,41,61] As a result, the magnitude of this (low-grade) para-inflammation may increase and evolve into a chronic (maladaptive) classical inflammatory response which contributes to the initiation and progression of age-related diseases.[41]

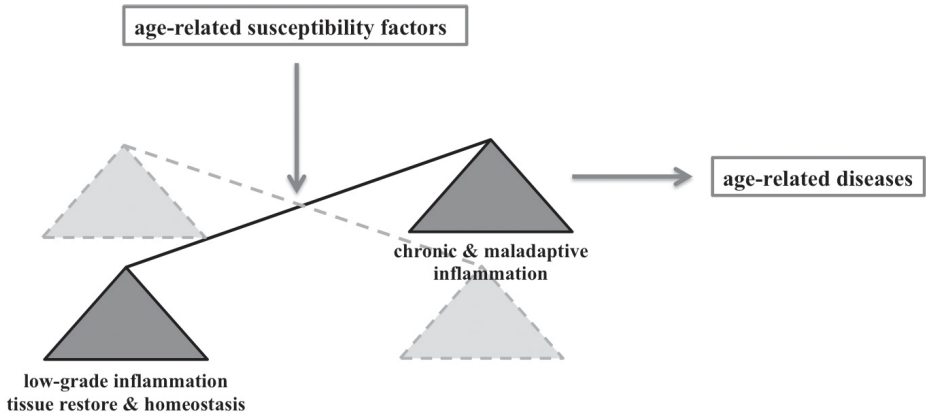

Figure 2. The premise of inflammation in age-related diseases.

Aging and inflammation in animal models

Gene expression studies in animals have shown that retinal aging is accompanied by activation of genes linked to immune responses and to tissue stress/injury responses, which mainly involves the innate immune system.[62] More interestingly, animal studies indicate an age-related decline in the secretion of macrophage-derived pro-inflammatory cytokines and chemokines.[63-66] Renshaw et al.[66] have shown that splenic and activated peritoneal macrophages from aged mice express significantly lower levels of all Toll-like receptors (TLRs). Moreover, macrophages from aged mice secrete significantly lower levels of pro-inflammatory cytokines (IL-6 and TNF-α) and chemokines (MIP-1α and CCL5) when stimulated with known ligands for TLR2, TLR4, TLR5 and TLR9 compared with those from young mice.[66]

In the normal aging retina and choroid of rats, the level of prostaglandin E2 (PGE2) is highly increased accompanied by decreased protein levels of TNF-α.[64] It appears that activated macrophages from old mice produce more PGE2 than those from young mice.[67] PGE2 has several effects on the immune system and contributes to dysfunctional immune responses in the elderly. It suppresses IL-12 secretion, decreases MHC class II expression on APCs and enhances IL-10 secretion, resulting in diminished activated T cell-function.[41,65,67]

Aging also results in altered expression of growth factors, including increased expression of vascular endothelial growth factor (VEGF, which mediates vascular remodeling),

and decreased expression of pigment epithelium-derived factor (PEDF, an inhibitor of angiogenesis) in the choroid of aged rats.[68] The above findings of decreased secretion of macrophage pro-inflammatory cytokines/chemokines (IL-6, IL-12, TNF-α, MIP-1α, CCL5) and increased proangiogenic factors (VEGF) suggest an anti-inflammatory and a proangiogenic profile, which contributes to dysfunctional immune responses in the elderly.

With regard to the different types of macrophage responses, the eyes of young and old naïve mice have been analyzed for a range of inflammation-associated markers.[69] The common macrophage marker F4/80 was expressed at a higher level in the eyes of old mice compared to young mice: especially M2 markers (CD163, PPARG) were highly increased in aged mice. Immunohistological analysis showed large amounts of M2 type macrophages ($F4/80^+$ and $CD163^+$ cells) in the anterior eye of old mice, with fewer cells seen in eyes of young mice.[69] In addition, the proangiogenic genes VEGF and TIE-2, which are associated with the angiogenic effector functions of M2 macrophages, were expressed at higher levels in aged mice compared to young mice.[69] These findings suggest that normal eyes in old animals show an increased basal level of inflammation and repair processes that will more easily give rise to angiogenesis and chronic inflammation.

Although the pathogenesis of AMD, uveal melanoma, and glaucoma differ and various genetic and environmental factors are involved, low-grade chronic inflammation (para-inflammation) is a common process which is involved in all three of these age-related eye diseases in the elderly population.

INFLAMMATION IN AGE-RELATED EYE DISEASES

Age-related Macular Degeneration

With aging, oxidative stress in retinal and choroidal tissues may trigger a tissue-adaptive response, in which cells of the innate immune system mount a low-grade inflammatory response in order to restore tissue homeostasis.[6,12,41] In AMD, however the balance between the stress-induced damage and para-inflammation-related tissue repair is disturbed due to either sustained/increased injury (aging, smoking), or an altered/decreased repair ability of the immune system (aging, genetic susceptibility).[41] Injured RPE cells release cytokines and chemokines that recruit and activate macrophages and dendritic cells, which

in turn may amplify the inflammatory process via cell-to-cell contact, immune complex formation, and complement activation, leading to additional RPE cell damage.[70] Kelly et al. have shown that the function of choroidal macrophages changes with age.[71] Following laser injury to the retina, IL-10 was upregulated and Fas ligand (FasL), IL-12, and TNF-α were downregulated in ocular macrophages of old mice, suggesting an alternatively-activated macrophage phenotype.[44,71] These IL-10 producing M2 type macrophages of old mice alter the growth of abnormal blood vessels, and therefore the initiation and progression of choroidal neovascularization (CNV), the major vision-threatening complication of AMD.[71] Others have also reported the role of macrophages in angiogenesis in models of intraocular CNV.[72,73] After laser treatment of the retina in mice, CNV develops, which is accompanied by massive infiltration of macrophages. More importantly, laser treatment in young mice leads to limited neoangiogenesis, while in old mice, massive neovascularization develops.[71,72] Depletion of macrophages or inhibition of their effector functions diminishes CNV in old mice.[73] Thus, in AMD, M2 type macrophages play an essential role in regulating angiogenesis at the site of tissue injury, thereby determining the outcome of disease progression.

Uveal Melanoma

As discussed above, the development of uveal melanoma is also related to age. Important clinical prognostic factors are tumor size and location of the tumor (e.g. involvement of the ciliary body) in the eye, whereas others are related to the tumor characteristics (cell type, antigen expression, and karyotype).[35,36] One of the most important factors that correlates strongly with metastatic disease and survival in uveal melanoma is the loss of one copy of chromosome 3 (i.e. monosomy 3), which occurs in more than 50% of all uveal melanoma.[35,74-78] Other important parameters associated with prognosis include immunological determinants such as increased HLA class I and II expression, and infiltration of macrophages and lymphocytes into the tumor.[79-84] Maat et al.[36] have shown that tumors with a poor prognosis do not only exhibit monosomy of chromosome 3, but also the inflammatory phenotype as well. In addition, others have also shown that a high number of tumor-infiltrating $CD68^+$ macrophages are related to a poor prognosis and are associated with an increased microvascular density.[83,85] As mentioned before, the role of macrophages in angiogenesis has been observed previously in models of intraocular choroidal neovascularization after laser treatment of the retina. These combined findings demonstrate that age influences blood vessel growth, which has

considerable consequence for diseases, such as AMD and cancer, in which angiogenesis plays an essential role.[33] Tumor-associated macrophages can stimulate the formation of new vessels, helping tumors to survive by supplementing nutrients and creating a route for cancer cells to disseminate hematogenously.[86-88] This may clarify why an increased presence of tumor-infiltrating macrophages in unfavorable uveal melanoma is associated with a high microvascular density. Based on these findings, Ly et al.[69] studied whether macrophages are similarly involved in intraocular melanoma, as they are in laser-induced neovascularization. As already mentioned above, eyes of old mice expressed higher levels of macrophages and angiogenesis markers than eyes of young mice, corresponding to the phenomenon known as para-inflammation in the elderly.[41] Furthermore, in aged mice, tumor progression depended on the presence of macrophages, as local depletion of these cells prevented tumor outgrowth, indicating that macrophages in old mice have a strong tumor-promoting role. Further analysis showed that these macrophages in tumors of aged mice carried M2-type characteristics such as increased expression of CD163, PPARG, and angiogenic genes (VEGF, TIE-2).[69] Thus, naïve as well as tumor-containing eyes of old mice have more proangiogenic and tumor-promoting M2-type macrophages than comparable eyes of young mice, which carry macrophages that are probably polarized toward an immunostimulatory, tumor-suppressing M1-type macrophage.

In order to gain more profound insights into the interplay between the different subsets of immune cells in uveal melanoma and their influence on the clinical outcome of disease, we studied the presence of different functional phenotypes of tumor-infiltrating macrophages and lymphocytes, and compared them with the tumor characteristics and genetic background of 43 primary uveal melanomas (Chapter 2). Traditional treatment of uveal melanoma was enucleation, however, a shift to more eye-saving approaches occurred as the COMS study showed that outcomes were similar in small to medium-sized tumors for both therapies.[89] Local radiotherapy is often able to preserve eye and sight, and is less mutilating than enucleation. In spite of that, secondary enucleation may be required when tumor recurrence occurs. Because primarily-enucleated uveal melanoma that have monosomy 3 contain more inflammatory cells, and tumor recurrence may perhaps especially occur in tumors that have similarly lost one copy of chromosome 3, we also analyzed the presence of inflammatory cells in tumors enucleated after prior irradiation (Chapter 3).

Glaucoma

As mentioned before, glaucoma is a highly prevalent ocular degenerative disease in which age is one of the main risk factors.[18] The current prevailing view is that glaucoma pathogenesis is multifactorial with a complex interplay of IOP-induced events and genetic/epigenetic/aging-related susceptibility factors which contribute to neurodegeneration.[90] The increased age-related oxidative stress occurs especially at the level of the neuroretina leading to para-inflammatory responses, which includes microglial activation and chemokine/cytokine production.[41] Microglial cells are specialized tissue macrophages in the brain and retina, and are the main cells responsible for immune surveillance involved in clearing and shielding of stressed or injured tissue.[91] Due to their extreme plasticity, microglia can respond to tissue injury/stress in a short period of time without causing immunological imbalance under normal aging conditions.[41,92] Under normal physiological circumstances, the immune-regulatory functions of microglia do not cause immunological imbalance, and promote immune privilege rather than neurodegenerative immune responses. However, in the presence of accumulating tissue stress/injury and risk factors associated with aging, chronic microglial activation in the neuroretina may result in dysfunction of their regulatory activity leading to innate and adaptive cytotoxicity.[93]

In addition to having innate immune activities, microglial cells also play a role in the initiation of adaptive immune responses in glaucoma and other neurodegenerative diseases.[94,95] For example, Tezel et al.[95] have shown that following exposure to reactive oxygen species/oxidative stress, microglial cells, derived from the rat retina and ONH, upregulated MHC class II molecules, and became potent inducers of T cell activation, as assessed by T cell proliferation and TNF-α secretion. An increasing number of studies have provided clinical evidence of an abnormal activity of the adaptive immune system in glaucoma patients.[96] For example, abnormal T-cell subsets and increased production of serum autoantibodies against optic nerve and retinal antigens were observed in many glaucoma patients.[97-103] Increased autoantibodies in the serum of glaucoma patients include heat shock proteins (HSPs), such as HSP27 and HSP60[104], which are highly expressed in the glaucomatous retina and optic nerve head[105]. Furthermore, significant alterations of serum T_H1 and T_H2 cytokines are associated with glaucoma.[106] It is possible that peripapillary chorioretinal atrophy areas and splinter hemorrhages seen in glaucoma patients represent the areas of breakdown of the blood ocular barrier, thereby providing contact with the systemic

immune system, facilitating access of T cells and autoantibodies into the retina and optic nerve head.[107,108]

There is no doubt that the immune response is initially beneficial and necessary to maintain neuronal homeostasis and promote tissue repair (para-inflammation) without causing an autoimmune neurodegenerative disease. However, alterations in the immune response due to accumulating risk factors (e.g. high IOP, ischemia, trauma) along with age-related oxidative stress may impair the physiological equilibrium, and switch the protective immunity to a neuroinflammatory degenerative process.[41,109]

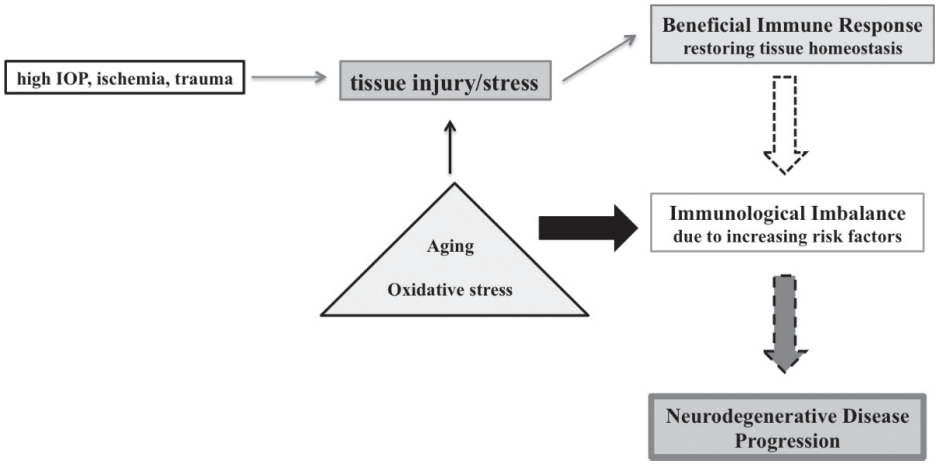

Figure 3. Switching from protective immunity to neurodegenerative immunity

In Chapter 4, we summarize the current concepts and insights in the role of the host immune response in glaucomatous neurodegeneration, proposing a unifying scheme of cellular processes at the level of the neuroretina, integrating risk factors, antigenic stimuli, glial activation responses, and T cell participation with the altered regulation of immune responses in glaucoma.

Although there is increasing interest in the possibility that glaucoma may act as an autoimmune disease or has an autoinflammatory component, the evidence supporting this assumption is not conclusive. While it can be imagined that inflammation plays an

important role in the damaging effects of high IOP or any type of tissue insult (e.g. ischemia, traumatic optic nerve injury), the definite evidence to support a role for autoimmune pathogenesis in glaucoma is currently lacking. Therefore, in Chapter 5 we describe a study which examines the role of CD4+ T cell responses in inducible and genetic mouse models of glaucoma in combination with immunodeficient or germ-free mice and adoptive T cell transfer. Furthermore, in Chapter 6 we describe how immune responses play a role in degenerative conditions, such as acutely elevated IOP leading to ischemia of the retina. We focus especially on the role of the adaptive immune system after the initial tissue insult has occurred. In addition, we studied the effect of suppressing CD4+T cell-mediated immune response in ischemic eyes.

IN SHORT

Although the pathogenesis of macular degeneration, uveal melanoma and glaucoma differs and various genetic and environmental factors are involved, it is believed that these three ocular diseases share two unifying elements: 1) they are diseases of the aging population; and 2) they involve low-grade chronic inflammation (para-inflammation) in the aging eye. Therefore, the main focus of this thesis is to describe the role of the host immune system in the initiation and progression of age-related eye disease, focusing on uveal melanoma and glaucoma.

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DIFFERENT SUBSETS OF TUMOR-INFILTRATING LYMPHOCYTES CORRELATE WITH MACROPHAGE INFLUX AND MONOSOMY 3 IN UVEAL MELANOMA

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ABSTRACT

Purpose

In contrast to many other malignancies, in uveal melanoma (UM) the presence of an immune infiltrate is associated with a bad prognosis. An analysis of the different functional phenotypes of tumor-infiltrating leukocytes (TIL) and a comparison with the genetic background of the tumors may help to explain this apparent anomaly.

Methods

We performed a comprehensive immunohistochemical study by evaluating the density of $CD8^+$ and $CD4^+$ T lymphocytes, forkhead box p3 ($Foxp3^+$) regulatory T cells (Tregs), and $CD68^+$ and $CD68^+CD163^+$ macrophages in 43 cases of UM in relation to tumor characteristics. Expression of the chemokines CCL2, CCL17, and CCL22 in cultured human UM cells and peripheral blood monocytes was analyzed by quantitative PCR (qPCR).

Results

The presence of TILs was highly variable between tumors and was dominated by $CD8^+$ T cells with fewer $CD4^+$ T cells and Tregs. When tumors were infiltrated by immune cells, the infiltrate generally comprised all different subsets of lymphocytes ($P < 0.001$) and M2 macrophages ($P < 0.001$). Different T-cell ratios did not influence clinical outcome. In addition, the presence of TIL correlated with the loss of one chromosome 3 ($P < 0.04$). UM cells express CCL2 and CCL22, two chemokines known to mediate trafficking of immune cells to the tumor.

Conclusion

All studied subtypes of tumor-infiltrating immune cells were collectively increased and showed an association with monosomy of chromosome 3 suggesting that tumor intrinsic factors control the leukocyte influx, possibly through local chemokine secretion.

INTRODUCTION

Uveal melanoma (UM) is the most common form of cancer in the eye of adults, and is a highly malignant disease. Regarding prognosis, Callender's [1] description revealed that an epithelioid cell type is associated with an unfavorable outcome. Over time, different molecular techniques to determine prognosis of uveal melanocytic neoplasms have been developed [2]. A specific cytogenetic profile (i.e., the presence of monosomy of chromosome 3 and other cytogenetic markers) is related with a poor prognosis [3]. Furthermore, a specific gene expression profile, based on a molecular characterization of primary UM, can also be used to determine prognosis [4]. All these (intrinsic) properties help to differentiate between UM with a more or less favorable clinical course of disease.

Evaluation of the prognostic significance of tumor-infiltrating leukocytes (TIL) in other human cancers revealed that increased numbers of TIL are often associated with a better prognosis. [5] In UM, the presence of substantial numbers of leukocytes does not represent an effective immunological antitumor response. Rather, a pronounced infiltration of UM by, for example, lymphocytes, is associated with a poor prognosis. [6, 7] This may be related to the immunoregulatory influence of the intraocular microenvironment. [8] For instance, similar to the situation in experimental anterior chamber associated immune deviation (ACAID), human ocular tumor cells may escape from the eye and induce regulatory T cells (Tregs) that subsequently suppress the local and systemic immune response against the tumor cells. Tregs are a subset of $CD4^+$ T cells and are required for the maintenance of self-tolerance, and they are broadly characterized by their expression of the nuclear transcription factor forkhead box p3 (Foxp3). Increased numbers of these immunosuppressive lymphocytes have been detected in a variety of malignant tumors. [9] In UM, Tregs have been described in 12%–24% of cases. [10, 11] In these studies, the presence of monosomy 3 was not analyzed, or extrapolation of trends was limited due to the small number of tumors found to contain Tregs. However, in 50 COX-2-positive tumors, the presence of $Foxp3^+$cells was an independent predictive factor for worse overall survival [11].

Another nonmutually exclusive option might be the regulation of local immune responses by macrophages. In UM, an unfavorable prognosis is also associated with an increased density of $CD68^+$ macrophages. [12] Tumor-associated macrophages (TAMs) are involved in the regulation of angiogenesis, which can promote tumor growth. [13] On the other

hand, macrophages that have less immune-stimulating molecules can induce Tregs, thereby suppressing tumor immunity. Vice versa, Tregs may support the differentiation of monocytes to tumor-promoting M2 macrophages. [14] In humans, M2 macrophages are characterized by a higher expression of CD163 and the majority of macrophages in human UM carry this characteristic. [15] In a murine ocular tumor model, intratumoral accumulation of M2 macrophages was shown to foster tumor growth in elderly mice. [16]

In order to gain a more profound insight in the interplay between the different immune cells in UM and their influence on the clinical outcome of disease, we determined the presence of different infiltrating immune cells, by counting the number of subtypes of intratumoral T cells and macrophages in a cohort of 43 primary tumors, which were characterized for chromosome 3 status. Our data indicate that all different types of immune cells studied were collectively increased in the presence of monosomy 3, leading to the conclusion that, specifically tumors with poor prognosis, support the influx of immune cells, including those with an immunosuppressive function such as Foxp3$^+$ Tregs and M2 macrophages.

METHODS

Study Population

Tissue specimens were obtained from 43 patients who had undergone a primary enucleation for UM between the years of 1999 and 2004 at the Leiden University Medical Center (LUMC), Leiden, The Netherlands. Patient data and survival were updated until December 2010 from the patients' charts and from the database of the Integral Cancer Center West. Survival was termed the interval between enucleation and death from UM or the interval between enucleation and the last observation for surviving patients. The research protocol followed the current revision of the tenets of the Declaration of Helsinki. The present study was performed with the same group of tumors as described previously.[15]

Immunohistology

Enucleated eyes were fixed in 4% buffered neutralized formalin for 48 hours. After embedding in paraffin, 4-µm serial sections were made and mounted on a slide. Hematoxylin-eosin–stained 4-µm sections were reviewed by one ocular pathologist for pathological diagnosis and evaluated for histologic parameters, which included largest

basal diameter (in millimeters), prominence (in millimeters), cell type according to the modified Callender classification [17] , ciliary body involvement, and intrascleral tumor growth. These parameters were used for classification in the TNM category/stage. [18]

For HLA class I staining, we used the mouse monoclonal antibodies HCA2 and HC10 (anti–HLA-A and anti–HLA-B/C, respectively), while for class II/ HLA-DR we used HLA-DR (Tal.1B5) antibody, as described previously. [19] The number of HLA-positive cells was estimated at 100× magnification and expressed as a percentage of the total number of tumor cells.

Fluorescent Immunostaining of Tumor-Infiltrating Leukocytes

Phenotypic characterization of lymphocytes was performed using triple fluorescent immunostaining. A previously developed technique for simultaneous immunofluorescence (IF) staining of different epitopes was applied to 4-µm formalin-fixed, paraffin-embedded tissue sections [20] . In brief, deparaffinized and citrate antigen retrieval-treated sections were stained by a mixture of the antibodies ab828 (rabbit polyclonal, anti-CD3; Abcam, Cambridge, MA), 4B11 (mouse monoclonal IgG2b, anti-CD8; Novocastra, Valkenswaard, The Netherlands), and the anti-FoxP3 antibody (IgG1, clone 236A/E7; Abcam) for the detection of Tregs. As secondary antibodies to visualize the lymphocytes, we used a combination of fluorescent antibody conjugates (goat anti-rabbit IgG-Alexa Fluor 546, goat anti-mouse IgG2b-Alexa Fluor 647, and goat anti-mouse IgG1-Alexa Fluor 488; Molecular Probes, Invitrogen, Breda, the Netherlands). Counting the number of $CD3^+CD8^-$ cells by this technique is a proven good marker for the presence of $CD4^+$ T cells. [21] We did not stain for NK cells as they are rare in UM. [22] Images were captured with a confocal laser scanning microscope (LSM510; Carl Zeiss Meditec, Jena, Germany) in a multitrack setting. All images were stack size 368.5 × 368.5 µm. A microscope objective (PH2 Plan-NEOFluar 25x/0.80 Imm Korr; Zeiss) was used. Ten images were scanned per slide, and each scan represented one square optical field (area, 0.137 mm^2): positive cells were counted in these randomly selected, high-power (250×) fields by two of the authors (IHGB and THKV). The mean was calculated and the SDs were analyzed; these showed no outliers. Counts of intratumoral infiltrating lymphocytes were represented as the number of cells per millimeters squared. Macrophages in these tumors were identified and measured using double IF staining, with mAbs directed against CD68 and for the M2 type, CD163, as performed and described previously [15] . The amount of staining was objectively determined in pixels per millimeters

squared by the image analysis software program, Stacks (Department of Molecular Cell Biology, LUMC, Leiden, The Netherlands).

Chromosome 3 Status

Analysis of chromosome 3 status (standard cytogenetic analysis and fluorescence in situ hybridization on isolated nuclei) of this patient material was described previously. [19]

Quantitative PCR of CCL2, CCL17, and CCL22

Tregs (and other immune cells) are known to be recruited into the tumor site through certain chemokines released by tumor and surrounding cells. Therefore, we analyzed expression of chemokines CCL2, CCL17, and CCL22 in UM cell cultures and monocytes, as extracting macrophages from fresh UM tissue has proven to be a technical challenge. Fresh tissue from four tumors, obtained immediately after enucleation, was placed in Amniochrome Pro Medium (Lonza Group Ltd., Basel, Switzerland) to develop a primary cell culture. Peripheral blood mononuclear cells (PBMC) were isolated from peripheral blood of three control subjects using ficoll-amidotrizoaat. $CD14^+$ cells were magnetically labeled with CD14 MicroBeads and separated according to the manufacturer's protocol (Miltenyi Biotec, Auburn, CA). RNA was extracted with an RNeasy Mini Kit (Qiagen, Valencia, CA). Primers were designed with Beacon Designer (Biosoft, Palo Alto, CA). Quantitative PCR (qPCR) was performed in duplicate according to our standard laboratory protocol, as described previously.[23] Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), β-actin, ribosomal protein (*RPL*) 13, and ribosomal protein S (*RPS*)11 were initially included, and *RPL13* and *RPS11*, as determined with the geNorm software (qBase, Bio-Rad Laboratories, Inc., the Netherlands), were selected as suitable reference genes. The calculated values were the normalized values of each sample.

Statistical Analysis

All statistical analyses were performed with a statistical software program (PASW Statistics 17.0; SPSS Inc., Chicago, IL, US). Wilcoxon rank sum test (Mann-Whitney*U* test) for nonparametric analysis was used for determining associations of the number of TILs with clinical variables. In statistical correlation analysis, the Spearman's rank test was used. Cumulative survival rate was calculated by the Kaplan-Meier method and analyzed by the logrank test. Univariate Cox proportional models were used to determine the hazard ratio (HR).

RESULTS

Patients

At the time of enucleation, the mean age of the 43 patients within our study group was 60 years (range 27–88 years). At the end of the follow-up period, 17 patients had died from UM metastases, 23 were alive, and two died of causes unrelated to the primary disease but showed no evidence of metastases; in one case, the cause of death was unknown. The mean follow-up at the time of analysis was 92 months (range 62–133 months).

Subtypes of Tumor-Infiltrating Lymphocytes

For a comprehensive analysis of TILs, we performed triple IF staining, using anti-CD3 (red), anti-CD8 (blue), and anti-Foxp3 (green) monoclonal antibodies. We identified and measured the number of $CD8^+$ cytotoxic T cells ($CD3^+CD8^+$T cells), $CD4^+$ helper T cells ($CD3^+CD8^-$ $Foxp3^-$T cells), and Treg ($CD3^+CD8^-Foxp3^+$) cells on sections from 43 primary UMs. In Figure 1, an example of the analysis of $CD3^+$ (red), $CD3^+CD8^+$ (purple) T-cell, and $CD3^+Foxp3^+$ (red with green nucleus) infiltration by confocal microscopy is shown. $CD8^+$ $Foxp3^+$ T cells were rarely present, and were therefore not enumerated. In general, all tumors contained all the different subtypes studied, but the number of infiltrating T cells varied enormously between tumors (range, 1–1834 per mm^2) (Table 1). $CD3^+CD8^+$ cells were identified in all samples with a mean score of 163 per mm^2 (range 1–1566 positive cells). $CD3^+CD8^-$ ($CD4^+$) cells were identified in 39 samples (91%) with a mean score of 42 per mm^2 (range 1–268 positive cells). Naïve CD4 T cells may differentiate into one of several lineages of T helper (Th) cells (including Th1, Th2, Th17), and into Tregs, as defined by their pattern of cytokine production and function. These cells can influence their environment. By combining three antibodies in one staining experiment, we were able to ensure the Tregs were not missed. Separated on the basis of their Foxp3 expression level, the $CD4^+$ cells consisted of $Foxp3^+$ cells, identified in 26 samples (61%), with a mean score of 20 per mm^2 (range, 1–158), and $Foxp3^-$ cells (defined by exclusion), which were identified in 39 samples (91%), with a mean score of 22 per mm^2 (range, 1–151). Infiltration by Tregs was paralleled by other types of immune cells in the tumor; Spearman rank analysis revealed significant correlations between the number of Tregs and the number of $CD4^+$Th cells (Spearman correlation coefficient (r) = 0.82, $P < 0.001$), as well as with the number of $CD8^+$T cells ($r = 0.89$, $P < 0.001$). The number of Tregs was also associated with the number of macrophages detected by CD68 and CD163. Interestingly, the number of Tregs showed a stronger correlation with the number

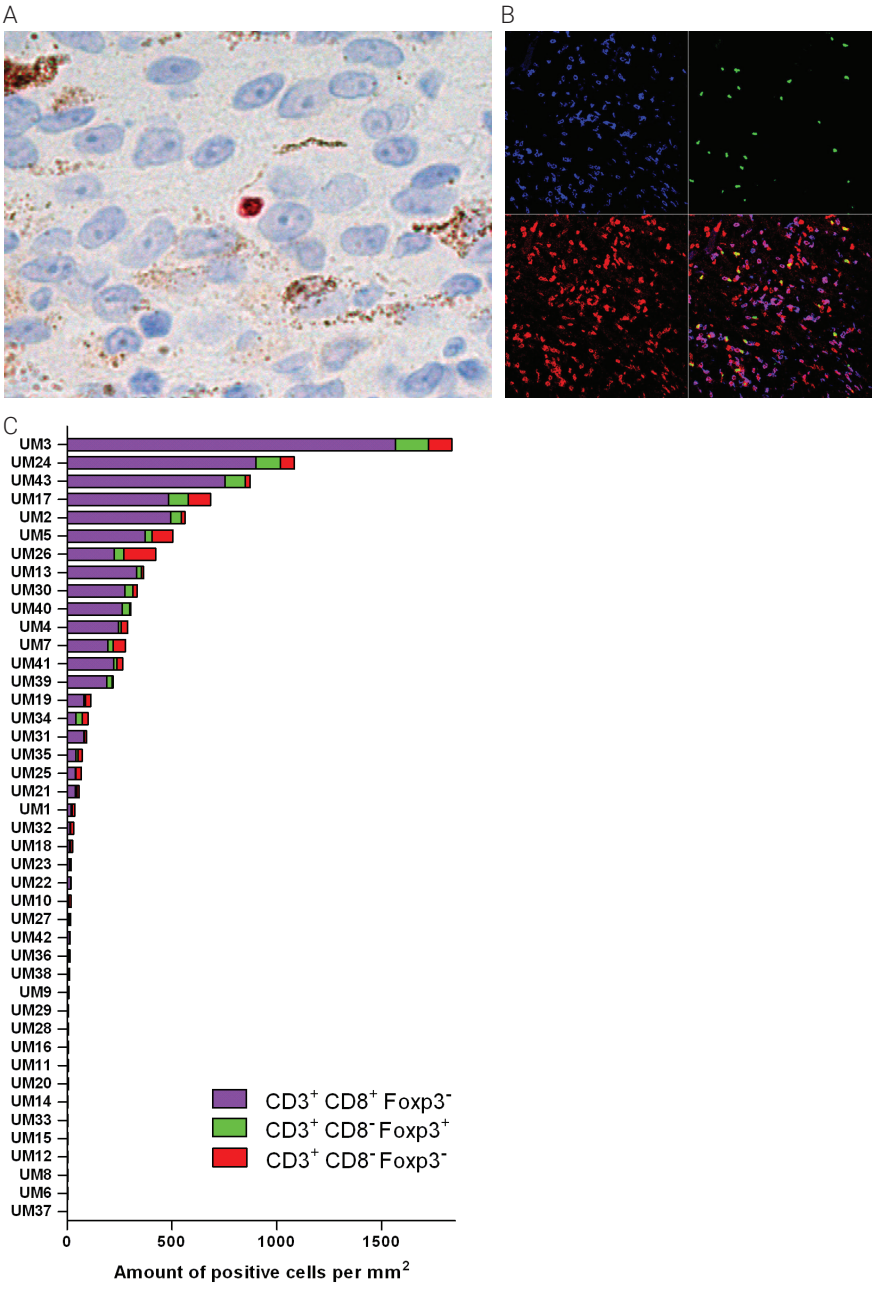

Figure 1. Analysis of T cell subsets in human UM. (**A**) Immunostaining with anti-Foxp3: intratumoral infiltration of a Foxp3+ cell (red nuclear staining) (original magnification, ×200). (**B**) Immunofluorescence staining using three antibodies directed against CD3 (*red*), CD8 (*blue*), or Foxp3 (*green*). Merged image: $CD3^+CD8^+$ $Foxp3^-$ T cells are purple, $CD3^+CD8^-Foxp3^+$ regulatory T cells show a nuclear green Foxp3 staining accompanied with surface red CD3 staining, and $CD3^+CD8^-Foxp3^-$ helper T cells are red. (original magnification, ×250). (**C**) Distribution of CD3, CD8, and Foxp3 IF staining in 43 UMs. Infiltrating T cells were counted in 10 representative fields per tissue section and the number of cells per millimeters squared was calculated.

Table 1. Baseline Characteristics of Patients and Histological Data of Primarily Enucleated Eyes

		Baseline data		Associations (P-values)				
Categorial variables		**N=43**	**%**	**CD3⁺ total**	**CD8⁺**	**CD4⁺⁻ total**	**CD4 Th**	**Foxp3⁺**
Gender	Male	23	53%	.65	.73	.61	.56	.44
	Female	20	47%					
Prognostic groups	Stage I	3	7%	.86	.84	.64	.20	.89
	Stage IIA	9	21%					
	Stage IIB	13	30%					
	Stage IIIA	15	35%					
	Stage IIIB	3	7%					
Cell type	Spindle	10	23%	*.03*	*.03*	*.04*	*.09*	*.02*
	Mixed+epithelioid	33	77%					
Ciliary body involvement	Not present	25	58%	.19	.32	.11	.16	.046
	Present	18	42%					

	Baseline data		Correlations (P-values)				
Numerical variables	**Mean**	**±SD**	**CD3⁺ total**	**CD8⁺**	**CD4⁺ total**	**CD4 Th**	**Foxp3⁺**
Age at enucleation (years)	60	(±15)	.60	.86	.39	.71	.26
Diameter (in mm)	13	(±3)	.12	.08	.16	.16	.07
Prominence (in mm)	8	(±2)	.57	.46	.72	.97	.44
$CD68^+$ (pixels$*10^3$ /mm^2)	125	(±76)					
$CD68^+CD163^+$ (pixels$*10^3$ /mm^2)	97	(±56)					
$CD3^+$total (cells/mm^2)	205	(±358)					
$CD8^+$ T cells	163	(±301)					
$CD3^+CD8^-(CD4^+)$ T cells	42	(±64)					
$CD3^+CD8^-Foxp3^-$Th cells	22	(±34)					
$CD3^+CD8^-Foxp3^+$Tregs	20	(±35)					

The numbers positively for $CD3^+$ lymphocytes, consisting of $CD8^+$ T cells, $CD4^+$ Th cells, and $CD4^{+-}$ $Foxp3^+$ Tregs compared with other parameters. Only the ***P*** values are shown. ***P*** values for the comparison with categorical data were obtained by Wilcoxon rank sum test (Mann-Whitney ***U*** test), and for the numerical data by Spearman's correlation (two-tailed). $P \leq 0.05$ are shown in italics.

of $CD68^+CD163^+$ M2 macrophages ($r = 0.81$, $P < 0.001$) (Fig. 2) than with the total amount of $CD68^+$ macrophages ($r = 0.76$, $P < 0.001$). All correlation coefficients are shown in Table 2.

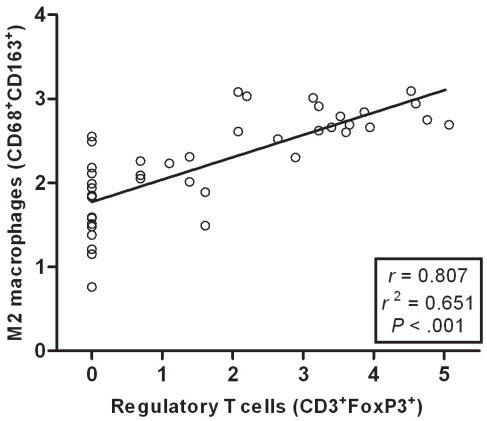

Figure 2. A comparison between the number of infiltrating regulatory T cells and M2 macrophages in 43 observations (logarithmic graph). A significant *r* value of 0.81 was seen, and the variability of M2 macrophages can be explained in approximately 65% (r^2) by the variability of the regulatory T cells.

Table 2. Correlation between Different Infiltrating Immune Cells (T Cells and Macrophages) as well as with HLA Expression

		CD8	CD4	CD4 Th	CD4Foxp3	CD68	CD68CD163	HC10	HCA2	HLA-DR
CD3 total	*r*	.984	.959	.890	.917	.692	.769	.775	.440	.493
	P-value	<.001	<.001	<.001	<.001	<.001	<.001	<.001	.003	.001
CD8	*r*		.914	.839	.894	.663	.742	.758	.400	.467
	P-value		<.001	<.001	<.001	<.001	<.001	<.001	.008	.002
CD4 total	*r*			.956	.929	.697	.765	.755	.451	.495
	P-value			<.001	<.001	<.001	<.001	<.001	.002	.001
CD4 Th	*r*				.824	.607	.689	.665	.365	.405
	P-value				<.001	<.001	<.001	<.001	.016	.007
CD4Foxp3	*r*					.760	.807	.701	.435	.519
	P-value					<.001	<.001	<.001	.004	<.001
CD68	*r*						.958	.639	.363	.517
	P-value						<.001	<.001	.017	<.001
CD68CD163	*r*							.652	.342	.528
	P-value							<.001	.025	<.001
HC10	*r*								.746	.456
	P-value								<.001	.002
HCA2	*r*									.403
	P-value									.007

r = two-tailed Spearman correlation coefficient, with 43 observations.

Associations with Variables

There were significantly higher amounts of TILs in tumors containing epithelioid cells compared with spindle cells. The mean numbers of different TILs and associations with clinical parameters are described in Table 1.

In the current group, on average, 35% of the tumor cells stained positive for HLA-B/C (HC10; range, 0%–100%), 42% for HLA-A (HCA2; range, 0%–100%) and 21% for HLA-DR (range, 5%–100%). A significant positive correlation was observed between the expression of HLA class I and II on the tumor cells and the numbers of infiltrating cells of all TIL subsets (r = 0.37–0.78; $P < 0.02$, Table 2).

Loss of a copy of chromosome 3 was present in 26 of 43 tumors. This analysis revealed that tumors with monosomy 3 contained higher numbers of intratumoral $CD3^+$ lymphocytes ($P = 0.006$), $CD8^+$ T cells ($P = 0.01$), $CD4^+$ T cells ($P = 0.006$), and its subtypes $CD4^+Foxp3^-$ Th cells ($P = 0.04$) and $CD4^+Foxp3^+$ Tregs ($P = 0.004$) (Wilcoxon rank sum test), while TILs were sparingly detected in the tumors without loss of one chromosome 3 (Fig. 3). The mean numbers of different TIL in the different prognostic groups are described in Table 3.

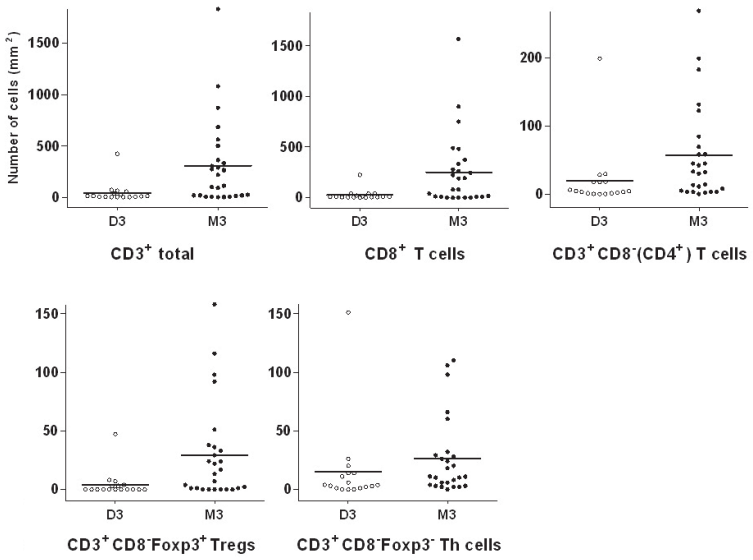

Figure 3. Tumors characterized by detrimental monosomy 3 contain significantly (all P < 0.05) higher numbers of different subtypes of intratumoral T cells (D3 = disomy for chromosome 3, and M3 = monosomy 3).

Table 3. UM Groups Based on Presence or Absence of Monosomy 3 Were Compared Regarding Different Types of TILs and Ratios between These Immune Cells

	Chromosome 3 status				
	Normal (n=17)		Monosomy (n=26)		
	Mean	SD	Mean	SD	P-value
Immunofluorescencestaining					
$CD3^+$ total (cells/mm^2)	46	100	309	425	*.006*
$CD8^+$ T cells	26	53	252	360	*.01*
$CD3^+CD8^-(CD4^+)$ T cells	20	47	56	70	*.006*
$CD3^+CD8^-Foxp3^+$ Tregs	4	11	30	42	*.004*
$CD3^+CD8^-Foxp3^-$ Th cells	15	36	27	33	*.04*
$CD8^+$/ regulatory T-cell ratio	6,2	3,4	9,2	7,8	.22
$CD4^+$/ regulatory T-cell ratio	3,4	2,3	3,2	2,3	.85
$CD4^+$Foxp3-Th/ Treg-cell ratio	3,0	2,1	2,4	2,5	.11
$CD8^+/CD4^+$ T-cell ratio	2,7	2,4	3,6	2,7	.33
$CD8^+/CD4^+Foxp3^-$ Th T-cell ratio	2,9	2,4	10,2	12,5	.10
$CD68^+$ macrophages (pixels $*10^3$)	83	82	153	58	*.001*
$CD68^+CD163^+$ M2 (pixels $* 10^3$)	68	61	116	44	*.002*
Immunohistochemicalstaining					
HLA-DR (%)	10	7	28	26	*.002*
HC10 (%)	16	22	48	33	*.001*
HCA2 (%)	22	26	54	25	*.001*

For the numerical parameters we show mean ± SD. ***P*** values for numerical data were obtained by Wilcoxon rank sum test (Mann-Whitney ***U***). $P \leq 0.05$ are shown in italics.

Survival Analysis

The Kaplan-Meier method and the logrank test were used to analyze the correlation between TIL subtypes, HLA class I and II expression, and patient survival. For logrank testing and Cox regression analysis, appropriate parameters were divided into categorical variables: TIL and macrophage groups were based on the median (50th percentile) of positive immune cells per millimeters squared; 25% was used as a cutoff point to dichotomize the HLA expression variable. A significantly worse patient survival was associated with a high total number of macrophages as M2 macrophages ($P = 0.01$), but not with increased numbers of Tregs. A trend towards significance for worse survival was seen with a high number of total $CD3^+$ T cells ($P = 0.07$) and $CD3^+CD8^+$ T cells ($P = 0.07$).

Previous studies in other malignancies show the relevance of the ratios between different immune cells for survival [21, 24-26], indicating that especially the proportion between the different subtypes of immune cells within the tumor of each individual patient is important. When we evaluated the ratios between different TILs, no significant difference in survival was seen (data not shown).

No significant association between HLA expression and survival was found.

A significant association was seen between decreased survival and monosomy 3 ($P < 0.001$, logrank testing). *P* values and HRs are shown in Table 4.

Table 4. Associations between Different Inflammatory Markers, Tumor Intrinsic Properties, and Survival in 43 Cases of UM

	Kaplan-Meier (Log-Rank Test)		**Cox Univariate**		
	χ^2	*P*-value	*P*-value	Hazard Ratio	95% Confidence Interval
Immunologic determinants					
Immunofluorescence staining					
$CD3^+$total	3.3	.07	.08	2.4	0.9-6.6
$CD8^+$cells	3.3	.07	.08	2.4	0.9-6.6
$CD4^+$ ($CD3^+CD8^-$)	1.2	.28	.29	1.7	0.6-4.5
$CD4^+Foxp3^+$Tregs	0.7	.41	.41	1.5	0.6-3.9
$CD4^+Foxp3^-$ Th	3.1	.08	.09	2.4	0.9-6.4
$CD68^+$macrophages	6.0	.01	.03	5.2	1.2-22.9
$CD68^+CD163^+$ M2 macrophages	6.4	.01	.03	5.5	1.2-24.0
Tumorcel properties					
Immunohistochemical staining					
HCA2	1.9	.17	.19	2.1	0.7-6.6
HC10	1.5	.22	.23	1.9	0.7-5.0
HLA-DR	0.1	.73	.73	1.2	0.4-3.8
Monosomy of chromosome 3	13.5	<.001	.007	16.5	2.2-125.5

Expression of CCL22, CCL17, and CCL2

We wondered what determined the influx of specific TIL and, therefore, investigated the production of different chemokines, such as macrophage-derived chemokine (MDC/CCL22) and thymus- and activation-regulated chemokine (TARC/CCL17), both of which

play a role in Treg migration, [27] and monocyte chemoattractant protein-1 (MCP-1/CCL2), [28] a chemokine that attracts and activates mononuclear cells and can be produced by the M2 macrophages themselves. We measured the expression of these chemokines in primary tumor cultures and monocytes by real-time PCR. Both CCL22 and CCL2 were expressed in all freshly cultured UM cells as well as in monocytes. Expression of CCL22 was higher in monocytes than in any of the UM cultures, while CCL2 showed the opposite result (Fig. 4). CCL17 showed extremely low expression in all samples tested (data not shown).

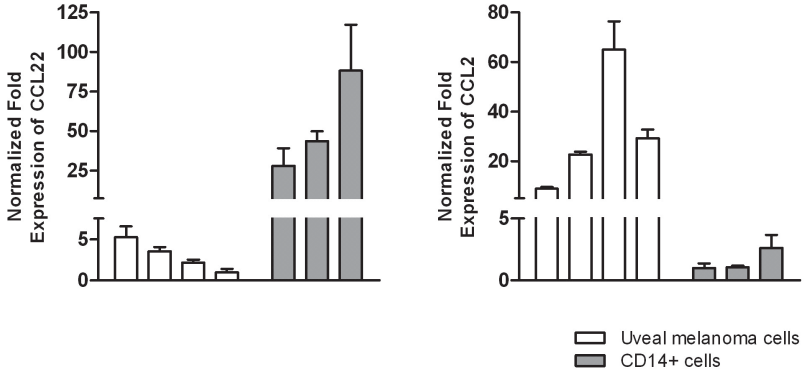

Figure 4. Quantitative PCR shows CCL22 and CCL2 expression in UM cells and CD14+ monocytes. Especially primary UM cultures (*n* = 4) expressed CCL2 mRNA, while CCL22 was more strongly expressed in monocytes (*n* = 3).

DISCUSSION

Previous studies have observed associations between the presence of a leukocytic infiltrate and a poor prognosis in UM, but such studies did not look at the complex diversity and balance of the infiltrating leukocytes. Recent reports suggested that thymic-derived $CD4^+CD25^+$ Treg ($Foxp3^+$ lymphocytes) participate in the control of tumor immunity, and the presence of these cells in UM has been reported, but the relationship between the Treg population and other immune cell types or with important prognostic parameters, such as monosomy 3, has not yet been clarified in this malignancy. In order to understand whether the presence of TIL may be associated with nonimmunological tumor characteristics,

we grouped the UMs according to their chromosome 3 status. Our study shows a clear association between monosomy 3, a highly reliable indicator of poor prognosis in UM, [29] and the presence of a complex immune infiltrate, including $CD8^+$ and $CD4^+$ lymphocytes, $Foxp3^+$ Tregs, and the pro-angiogenic and immunosuppressive M2 macrophages [15].

UM cells, both primary and metastatic, are poor stimulators of proliferative responses by allogeneic lymphocytes [30], while by using autologous skin melanoma cell lines, generation of tumor-specific T cells from autologous mixed lymphocyte tumor cell cultures was possible [31]. This suggests that UM cells hamper the expansion of tumor-specific T cells either via direct interactions or by secretion of soluble factors. In a study by Ksander et al. [32], TILs were recovered from a series of human choroidal melanomas and expanded in culture media containing IL-2. In these experiments, tumor-specific T cells were detected among TILs from some tumors, but proliferation of TILs was limited. Furthermore, to function optimally, $CD8^+$ T cells usually require $CD4^+$ Th1 cells [33]. Although in our study the number of $CD4^+$ TILs correlated significantly with the presence of $CD8^+$ T cells, we counted a relatively lower number of $CD4^+$ than $CD8^+$ TILs, similar to other tumors [21,25]. The inability to proliferate or to exert other effector functions may be limited due to presence of immunosuppressive cells (e.g., Tregs and M2 macrophages).

The increase of infiltrating Tregs in UM seems due to an increase in the total number of T lymphocytes. Using our immunofluorescent technique, we see more Treg-positive samples than in previously published studies using immunohistochemical stainings[10,11]. When looking at our numbers of $Foxp3^+$cells, we frequently found only a few cells: in eight tumors there were less than five $Foxp3^+$ cells per millimeter squared. It is possible that using our technique, the cells could be more easily detected. In contrast, in another Foxp3 study (Khatib et al. 2011; ARVO Meeting Abstract 1452), the authors were able to detect $Foxp3^+$ Tregs in approximately 77% of posterior UM samples. As Tregs comprise less than 10% of the T-cell population within the UMs, one may think that $Foxp3^+$infiltrating cells would not play a dominant role. In addition, we did not find a correlation between Foxp3 status and TNM classification, suggesting that Tregs do not influence the progression of cancer. However, in an experimental corneal transplantation study it was demonstrated that the functional status of Tregs is more related to allograft outcome than their numbers. [34] The essential role of Foxp3 in regulating the suppressor function of Tregs has been well documented, [35] and all our counted intratumoral Tregs express Foxp3, indicating that part

of the tumor-infiltrating $CD4^+$ T cells may well be able to suppress local immunity and, thus, have immunosuppressive properties. However, to adequately evaluate antitumor immune functions, multiple factors should be analyzed simultaneously. [36] The purpose of this study, therefore, was to determine if there is an association between Tregs, $CD4^+$ and $CD8^+$ T cells, and macrophages in UM. HLA peptide complexes at the cell surface of tumor cells allow for recognition by and interactions with T cells. This is the first report that has clarified the correlation of all these different leukocytic subsets and HLA expression in UM, and indicates that this infiltration by subtypes of lymphocytes and macrophages increased simultaneously in the inflamed tumors.

Excessive inflammatory responses may be dampened by adaptive immune responses. Suppression of melanoma-specific $CD8^+$ T-cell responses by ocular TAMs would provide another explanation of the association between a poor prognosis and the presence of a macrophage infiltrate [12] . Two different types of macrophages can be identified, of which the M1 phenotype is pro-inflammatory and the M2 phenotype is associated with tissue repair and the production of anti-inflammatory cytokines. [38] M2 macrophages are known to be able to directly induce Tregs, resulting in suppression of tumor-specific cytotoxic T-cells [39] . We have previously shown that the majority of macrophages in UM belong to the M2 type [15] and now show a strong correlation between the presence of Tregs and M2 macrophages. This correlation has also been observed in intrahepatic cholangiocarcinoma [40] and gastrointestinal stromal tumors [41] .

The selective accumulation of Tregs and macrophages in the tumor microenvironment suggests that this process is tumor driven, and it may be that TIL influx is controlled by local chemokine secretion. CCL22 secreted by ovarian cancer cells and macrophages in the tumor microenvironment is known to induce selective migration of Treg [27] . In a mouse skin melanoma transplantation model, Treg migration towards melanomas was related to secretion of CCL2, and not CCL22 [42] . CCR4, the chemokine receptor that recognizes CCL22 and its alternative ligand CCL2, is broadly expressed on other immune cells that, therefore, can be attracted to the tumor. As UMs, as well as monocytes, produce these chemokines, the tumor as well as its TAM may be the source of these chemokines and may induce immune cell trafficking to tumors. The possibility that chemokine-mediated immune cell migration to tumor tissue may be blocked by targeting of either the chemokines or

their specific receptors, provides opportunities to prevent TIL accumulation in the tumor microenvironment and suppression of tumor development [43] .

In thyroid tumors, where many of the genetic tumor-initiating events have been identified, the oncogenes that drive tumorigenesis were proven to be able to induce an inflammatory program. [44] We did not find an association between mutations in *GNAQ* and *GNA11* and infiltrate (data not reported), which was to be expected, as the presence of these mutations is not associated with monosomy 3, [45] and the molecular pathways by which monosomy 3 induces an inflammatory program remains, therefore, as yet, largely unknown. However, some oncogenes may directly activate inflammatory pathways in tumor cells, [44] and have been studied in UM as, for example, the *MYC* oncogene (located on 8q24.1) [46] : only tumors with monosomy 3 showed amplification of *c-myc* and this suggests a unique pathway of genetic progression, possibly involving attraction of inflammatory cells. *RAS* and *BRAF* mutations have been described as oncogene-induced promoters of an inflammatory microenvironment, but mutations in these genes are usually absent in UM. [47] Our data suggest that loss of chromosome 3 may be associated with the initiation of inflammation. Moreover, infiltrating immune cells, such as macrophages, can increase the genomic instability of malignant cells as well as promote tissue remodeling and angiogenesis, through secretion of effector molecules, [48] suggesting a self-reinforcing mechanism.

In our study of the tumor microenvironment, UMs with poor prognosis are characterized by a brisk inflammatory infiltrate containing $CD8^+$ and $CD4^+$ T cells, $Foxp3^+$ regulatory T cells, as well as macrophages, in an environment with an increased HLA class I and II expression. The phenotypic analysis of the TIL cell population and macrophages show that this infiltration is collectively increased, and that the balance of different immune cells is of no relevance. In addition, the presence of an immune infiltrate is associated with monosomy of chromosome 3. Therefore, intrinsic malignant properties of the tumor may lead to the production of leukocyte-attracting chemokines. However, the causal relationship between these processes remains elusive.

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FOOTNOTES

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ANALYSIS OF INFLAMMATORY CELLS IN UVEAL MELANOMA AFTER PRIOR IRRADIATION

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ABSTRACT

Purpose

Primary uveal melanoma with a poor prognosis contain high numbers of infiltrating macrophages, especially of the M2 phenotype, as well as lymphocytes. We wondered whether local inflammatory responses were affected by irradiation and therefore determined the presence of inflammatory cells in uveal melanoma enucleated after prior irradiation.

Methods

We analyzed 46 uveal melanoma-containing eyes that had to be enucleated due to non-responsiveness, tumor recurrence, or complications. Immunofluorescent staining was performed to determine the presence of CD68+ and CD68+CD163+ macrophages, and of CD4+, CD8+ and Foxp3+ -regulatory T lymphocytes. Outcomes were compared with clinical and histological parameters.

Results

Numbers of CD68+ and CD68+CD163+ macrophages in secondarily-enucleated eyes varied widely, but did not differ from primarily-enucleated eyes and were not related to the reason for enucleation. Similarly, the number of CD4+, CD8+ and Foxp3+ T lymphocytes showed great variability. Tumors with epithelioid cells showed significantly more lymphocytes than spindle cell tumors. In the first two years after enucleation, previously irradiated tumors showed increased numbers of lymphocytes compared to primarily-enucleated eyes.

Conclusions

Numbers of infiltrating T lymphocytes and macrophages varied widely between tumors, but tumors with high numbers of macrophages also contained more lymphocytes. Irradiation had no effect on the number and type of macrophages, but led to an increased amount of T lymphocytes up to 24 months post-irradiation. As the presence of infiltrating cells was related to the tumor cell type, it is conceivable that the presence of an infiltrate is especially a consequence of the primary tumor characteristics before irradiation.

INTRODUCTION

Malignant melanoma of the uvea is the most common primary intraocular neoplasm, with an annual incidence varying from 2 to 8 cases per million per year in European countries[1]. Over the past decades, patient survival has not improved, despite progress in the diagnosis of melanocytic lesions and successful treatment of the intraocular melanoma.[2-4] The 5-year mortality-rate after diagnosis of an uveal melanoma is approximately 30% due to metastatic disease.[2,5]

Until the 1970s, the traditional treatment of uveal melanoma was enucleation.[6] However, when eye-preserving techniques became available, and after the COMS study revealed no survival difference in medium-sized melanoma after irradiation versus enucleation,[2-4,6-8] a shift to more eye-saving approaches occurred.[2-5,7,9] Local radiotherapy is often able to preserve the eye and sight, and is less mutilating than enucleation.[6,10] While eye retention following local treatment is usually achieved in more than 80% of cases after 5 years,[8,10,11] secondary enucleation may be required when failure of local tumor control occurs. This may be inadequate tumor regression or local (contiguous and noncontiguous) recurrence.[12-17] In addition, secondary enucleation is sometimes necessary in case of radiation-related ocular side effects, such as neovascular glaucoma, persistent hemorrhage, or exudative retinal detachment.[14]

The histopathological findings of uveal melanoma previously treated with different eye-conserving therapies are quite similar, regardless of the difference in physical properties of the various radiation sources employed.[15-17] These findings include: vacuolization with balloon cell degeneration, tumor cell necrosis surrounding vascular sclerosis, vascular damage, and fibrosis of the tumor stroma with frequent accumulation of pigmented macrophages.[15-17]

Several studies have revealed the presence of tumor-infiltrating macrophages and lymphocytes in primarily-enucleated as well as irradiated and secondarily-enucleated eyes with uveal melanoma.[18-25] High numbers of tumor-infiltrating macrophages in primarily-enucleated eyes are related to an unfavorable prognosis and are associated with the presence of epithelioid cells, increased microvascular density, and monosomy of chromosome 3.[18,21] Such intra-tumoral macrophages were mainly of the tumor-

promoting M2 phenotype, harboring anti-inflammatory and pro-angiogenic functions.[26] Moreover, tumors containing higher numbers of lymphocytes were associated with a bad prognosis.[25,27] Histological studies after transpupillary thermotherapy (TTT) or transscleral thermotherapy (TSTT) showed an influx of macrophages after local therapy.[28,29] It is feasible that macrophages play an important scavenger role in removing debris after irradiation. Based on these findings, we hypothesize that the combination of irradiation and thermotherapy will induce an influx of macrophages that should be noticeable in uveal melanoma enucleated after prior radiotherapy. In addition, as primarily-enucleated uveal melanoma that have monosomy 3 contain more inflammatory infiltrate,[20,21,27] and tumor recurrence may perhaps especially occur in tumors that have similarly lost one copy of chromosome 3, irradiated uveal melanoma enucleated due to failure of local tumor control can be expected to contain more inflammatory cells. We therefore determined the number and subtype of tumor-infiltrating macrophages and lymphocytes in uveal melanoma-containing eyes enucleated after irradiation and compared results to prior studies on primarily-enucleated eyes.

MATERIAL AND METHODS

Eye-preserving radiotherapy

The primary choice of conservative treatment at the Department of Ophthalmology of the Leiden University Medical Center (LUMC), the Netherlands, is brachytherapy, delivered with ruthenium-106 applicators. Between 1995 and 2008, plaque treatment was frequently combined with adjuvant TTT.[30] Proton beam radiotherapy was indicated for large and highly prominent melanoma, or in tumors located close to or in contact with the optic disc.[6,11] For this treatment, patients were referred to the Hôpital Ophthalmique Jules Gonin, Lausanne, Switzerland.

Patients and specimens

Tissue specimens were obtained from 69 consecutive eyes with uveal melanoma that had undergone a secondary enucleation after prior irradiation between 1996 and 2010 at the LUMC. Only formalin-fixed paraffin-embedded specimens from which enough tumor material was present for histopathological analysis were selected from the archives of the Pathology and Ophthalmology Departments, leaving 46 eligible specimens for inclusion.

Reasons for enucleation included the following: failure of local tumor control, defined as 1. contiguous tumor progression after partial regression due to non-responsiveness to prior treatment; 2. intraocular tumor recurrence after total tumor regression; or 3. radiation-related ocular complications, such as persistent hemorrhage, exudative retinal detachment, or a blind painful eye due to neovascular glaucoma. Clinical histories were analyzed by a clinician, using fluorescein angiographic and ultrasonographic (including A-and B-scan) images, to accurately divide eyes into three subgroups based on the reason for enucleation. Clinical data from the 46 enrolled cases were collected from the clinical records.

Clinical, histopathological, and inflammatory parameters on a set of 43 primarily- enucleated non-irradiated tumors have been described previously[20,21,27] and were used for the comparison with irradiated, secondarily-enucleated eyes.

All patients were informed regarding the use of their eye for research purposes and signed an informed consent form. The use of tumor material for research followed the current revision of the tenets of the Declaration of Helsinki (World Medical Association Declaration of Helsinki 1964; Ethical principles for medical research involving human subjects).

Histopathological data

After enucleation, eyes were fixed in 4% neutral-buffered formalin for 48 hours and embedded in paraffin. Two ocular pathologists analyzed the hematoxylin-eosin-stained (H&E) 4μm sections for the pathological diagnosis, tumor location, ciliary body involvement (presence or absence), tumor cell type (spindle or non-spindle), largest basal diameter (LBD in millimeters), prominence (in millimeters), and the presence of necrosis.

Immunostaining protocol for tumor-infiltrating macrophages and lymphocytes

Immunostaining was performed using double and triple-immunofluorescence (IF) staining as described earlier,[20,31] to identify the different subsets of tumor-infiltrating leucocytes. In short, 4 μm sections were cut from paraffin-embedded tumor blocks and deparaffinized. Antigen retrieval was performed by a 10-minute incubation in boiling Tris- EDTA buffer, at pH 9.0.

The primary antibodies used to characterize the macrophage phenotype were mouse anti-human CD68 mAb (1:50, clone 514H12; ab49777; Abcam, Cambridge, UK) as a marker for

total macrophages, and mouse anti-human CD163 mAb (1:100, clone 10D6, Novocastra NCL- CD163, Newcastle upon Tyne, UK.) as a marker for M2 macrophages. The rabbit anti-human CD3 pAb (1:100, IgG, clone ab828, Abcam, Cambridge, UK) was used to label CD3+ T cells, and the mouse anti-human CD8 mAb (1:100, IgG2b, clone 4B11, Novocastra, Newcastle-upon-Tyne, UK) was used to label CD8+ T cells. The mouse anti-human Foxp3 (forkhead box p3) mAb (1:200, IgG1, clone 236A/E7, Abcam, Cambridge, UK) was used as a nuclear marker for regulatory T cells (Tregs). Secondary antibodies were AlexaFluor IgG2a (488) goat-anti-mouse for CD68, IgG1 (546) goat-anti-mouse for CD163, IgG (546) goat-anti-rabbit for CD3, IgG2b (647) goat-anti-mouse for CD8, and IgG1 (488) goat-anti-mouse for Foxp3 (Invitrogen- Molecular Probes, Eugene, Oregon, USA).

Detection of immunostaining

Images of the stained sections were captured with a confocal laser-scanning microscope (LSM510; Carl Zeis Meditec, Jena, Germany) in a multitrack setting and H&E-stained sections were used for orientation and location of the scans. Each scan represented one square optical field (area, 0.137 mm2). All images for the tumor-infiltrating lymphocytes were 1,024 x 1,024 pixels and for the macrophages 512 x 512 pixels with stack size 368.5µm x 368.5µm. A PH2 Plan- NEOFluar 25x/0.80 Imm Korr objective (Zeiss) was used. The fluorochrome signals in the slides were visualized with an artificial color: red for both AlexaFluor-546 antibodies (CD163, CD3), blue for AlexaFluor-647 (CD8) and green for both AlexaFluor-488 antibodies (CD68, Foxp3). Images were viewed as an overlay, as a set of two (for macrophages) or three divided panels (for lymphocytes).

Assessment of immunostaining

For characterization of the macrophage subtypes, five representative high-power fields (x250 magnification) per slide were randomly selected. Because of the polymorphic appearance of macrophages, it was difficult to count them. Instead, we calculated the amount of staining in pixels per mm2 by using an image-analysis software program, Stacks (Department of Molecular Cell Biology, LUMC, Leiden, the Netherlands). In the resulting binary images, the green and red pixels corresponded to the anti-CD68 and anti-CD163 staining, respectively. The overlay of both colors was expressed as yellow pixels.

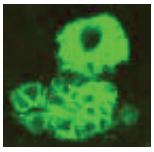
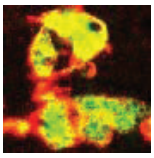
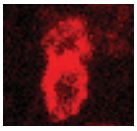
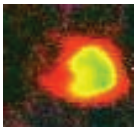
Cell type	*Epitopes*	*Phenotype*
Double-IF staining		
Macrophages	$CD68^+$	**Macrophages** 
	$CD68^+CD163^+$	**M2-type macrophages** 
Triple-IF staining		
Lymphocytes	$CD3^+CD8^+$	**Cytotoxic T cells** 
	$CD3^+CD8^-Foxp3^-$	**Helper T cells** 
	$CD3^+CD8^-Foxp3^+$	**Regulatory T cells** 

Figure 1. Detection of macrophages and lymphocytes in uveal melanomas by double- and triple-IF staining with antibodies directed against specific immune cells.

CD3+CD8+ cells (purple signal) were considered to be CD8+ T cells, CD3+CD8- cells (red signal) CD4+ helper T cells, and CD3+CD8-FoxP3+ cells (red signal with a green center) Foxp3+ Tregs (Figure 1). Positive cells were counted in ten randomly taken high-power fields (x250 magnification) by two independent observers masked to the clinical outcome and the reason for enucleation. The mean of the two observers was calculated for each tumor and tumor-infiltrating lymphocyte cell counts were presented as the number of cells per mm2. Necrotic areas were not analyzed.

Statistical analyses

All analyses were performed with a statistical software program (SPSS for Windows, version 17.0; SPSS inc., Chicago, IL). The performed tests were two-sided and a p-value of <.05 was considered as statistically significant. For the comparison of categorical data between two or more independent groups, the Chi-squared test was performed, and for numerical data the non-parametric Wilcoxon rank sum test and the Kruskal-Wallis test. Spearman's rank correlation analysis (two-sided) was performed to assess correlations between the different types of immune cells, and with time between irradiation and enucleation.

RESULTS

The numbers of infiltrating immune cells were determined in 46 uveal melanoma that had been irradiated previously, and often treated by TTT. We compared the cell counts with demographic and histopathological patient and tumor characteristics, as summarized in Table 1.

Of the 46 irradiated eyes with uveal melanoma, 12 had been treated with proton beam radiotherapy, 4 with ruthenium monotherapy, and 30 with sandwich therapy (ruthenium-106 brachytherapy with transpupillary thermotherapy TTT). Given the fact that only foureyes received ruthenium-106 as monotherapy and no conclusion can be drawn from such a small sample size, these cases were combined with the sandwich therapy group for statistical analysis. The median interval between irradiation and enucleation was 14 months (range 4-146) for patients who received proton beam irradiation, and 22 months (range 3-125) for ruthenium-106 brachytherapy (with or without TTT).

Table 1. Comparison of clinical and histological data of non-irradiated and irradiated eyes with uveal melanoma

	Non-irradiated eyes	Irradiated eyes			
		Total	Non-responsiveness	Recurrence	Complications
Subjects (N)	43	46	15	14	17
Gender (male/female)	23/20	31/15	8/7	9/5	14/3
Eye (right/left)	23/20	25/21	8/7	8/6	9/8
Prognostic groups					
Stage I	3	17*	6	3	8
Stage IIA	9	18	7	4	7
Stage IIB	13	4	1	1	2
Stage IIIA	15	5	1	4	0
Stage IIIB	3	2	0	2	0
Cell type					
Spindle	11	10	5	1	4
Mixed + Epithelioid	32	36	10	13	13
CB involvement					
Not present	25	41*	15	10	16
Present	18	5	0	4	1†
Bruch's membrane					
Broken	33	23*	6	5	12
Intact	4	18	9	5	4
Not clear	6	5	0	4	1
Necrosis					
Not present	36	22*	8	9	5
Present	7	22	7	3	12
Unclear	0	2	0	2	0
Median prominence, mm (range)	8 (2-12)	4 (1-14)*	3 (1-8)	4 (1-14)	4 (1-11)
Median LBD, mm (range)	13 (8-18)	12 (4-21)*	12 (6-20)	12 (4-21)	10 (5-15)
Median age at irradiation (range)	-	60 (32-84)	56 (42-84)	61 (32-78)	60 (42-70)
Median age at enucleation (range)	63 (27-88)	63 (38-85)	57 (42-85)	69 (38-80)	61 (43-77)

Prominence and largest basal diameter (LBD) measurements were obtained from histological examinations.
Prognostic groups based on the 7th edition AJCC-UICC criteria (American Joint Committee on Cancer-International Union on Cancer) for T staging, including the anatomical extent of the tumor based on involvement of the ciliary body and extrascleral tissues.

P-values for categorical parameters were obtained by the Chi-Square test, for the numerical data by Wilcoxon rank sum test and Kruskal-Wallis test; all statistical tests were two-tailed.
* Significant at P≤0.05 between irradiated and non-irradiated eyes
† Significant at P≤0.05 between secondarily-enucleated eyes: non-responsiveness, recurrence and complications

Causes for enucleation were: non-responsiveness to prior irradiation in 15 (33%) cases, tumor recurrence after prior total regression in 14 (30%) cases, and radiation-related ocular side-effects in 17 (37%) cases. Of the 12 eyes that had to be enucleated following proton beam irradiation, one (8%) was due to non-responsiveness, two (17%) showed a tumor recurrence, and nine (75%) had radiation-related complications (especially neovascular glaucoma). Of the 34 eyes treated with ruthenium-106 brachytherapy, 14 (41%) had to be enucleated due to non-responsiveness, 12 (35%) due to tumor recurrence, and 8 (24%) due to complications.

At the time of irradiation, the median age of the patients (31 males, 15 females) was 60 years (range 32-84), and by the time of enucleation it was 63 years (range 38-85).

The primarily-enucleated eyes and the irradiated eyes differed significantly with regard to the AJCC-UICC prognostic stage groups (p<.001), tumor prominence (p<.001), LBD (p=.014), involvement of the ciliary body (p=.001), break through Bruch's membrane (p=.005), and the presence of necrosis (p=.001): these variables, except for necrosis, were seen more often in non-irradiated eyes.

Tumor-infiltrating macrophages

Double-IF was performed on tumor sections of 46 irradiated eyes to analyze the amount and phenotype of tumor-infiltrating macrophages. In cases of severe tumor pigmentation, positive cells were easily recognized by IF staining with the confocal microscope.

As most of the CD68-positive cells were also CD163-positive, macrophages in irradiated uveal melanoma belonged mainly to the M2 phenotype. The amount of CD68+ (p=.80) and CD68+CD163+ (p=.44) staining was similar in irradiated and primarily-enucleated uveal melanoma (Table 2 and Figure 2A). In addition, there was no relation between the amount of CD68+ (p=.16) and CD68+CD163+ (p=.34) staining and the cause of enucleation in irradiated eyes (Table 2 and Figure 2A) or with the type of irradiation (data not shown).

However, the frequency of macrophages in secondarily-enucleated uveal melanoma seemed to decrease slightly with increase in time interval between irradiation and enucleation ((*r*): <-0.402, p<.03, Figure 2B). As the time between irradiation and enucleation varied broadly between patients, the data was normalized to account for time after irradiation by dividing the secondarily-enucleated eyes into four time-interval categories: 0-12, 13-24, 25-36, and >36 months, and then to compare these categories with the primarily-enucleated eyes. The amount of CD68+ (p=.11, p=.71, p=.91, p=.12) and CD68+CD163+ staining (p=.37, p=.66, p=.49, p=.06) was not significantly different between the four categories compared to non-irradiated uveal melanoma (Figure 2C, D). Moreover, the phenotype did not change over time, as the M2 macrophages stayed dominant.

Subtypes of tumor-infiltrating lymphocytes

Triple-IF staining was performed on tumor sections of 46 irradiated eyes to assess the number of intratumoral CD8+ cytotoxic T cells, CD4+ helper T cells, and Foxp3+ Tregs. CD8+Foxp3+ T cells were seldom observed and therefore excluded from analysis. Generally, all tumors displayed the whole spectrum of different subtypes of lymphocytes, but the number varied widely between the non-irradiated and irradiated uveal melanoma, and also between the tumors of each category (Table 2).

Irradiated uveal melanoma contained more lymphocytes of all subtypes than uveal melanoma from primarily-enucleated eyes (Table 2, p- values <.02, Figure 3A), but this was not related to the reason for secondary enucleation (Table 2, Figure 3A) or the type of irradiation (data not shown).

However, the total number of lymphocytes decreased slightly with increasing time between irradiation and enucleation ((*r*): <-0.422, p<.055, Figure 3B). In order to normalize the data to account for time after irradiation, the secondarily-enucleated eyes were divided into four categories: 0-12, 13-24, 25-36, and >36 months, and were then compared to primarily-enucleated eyes. Significant differences were observed in the numbers of total intratumoral CD3+ T cells between non-irradiated and irradiated eyes enucleated 0-12 months (p=.001) and 13-24 months (p=.014) after radiotherapy, but not at 25-36 months and >36 months (Figure 3C).

Table 2. Tumor-infiltrating lymphocytes and macrophages in non-irradiated and irradiated eyes, and in relationship with the different reasons of secondary enucleation.

Immunofluorescence staining	Non-irradiated eyes (n=43)		Irradiated eyes (n=46)			Non-responsiveness (n=15)		Recurrence (n=14)		Complications (n=17)		
	Median	Range	Median	Range	P-value	Median	Range	Median	Range	Median	Range	P-value
CD3+ total cells	31	1-1834	164	6-2526	*.001*	127	23-1684	152	13-1988	247	6-2526	.26
CD8+ T cells	16	1-1566	99	2-1956	*.002*	59	15-1336	62	11-1017	179	2-1956	.12
CD3+CD8- (CD4+) T cells	14	0-268	64	1-971	*<.001*	49	8-530	69	1-971	65	4-570	.60
CD3+CD8- FoxP3+ Tregs	3	0-158	9	0-226	*.02*	14	0-75	9	0-226	8	0-63	.74
CD3+CD8- FoxP3- Th cells	10	0-151	55	1-745	*<.001*	40	4-467	65	1-745	60	4-523	.36
CD68+ macrophages	125	13-290	119	13-301	.80	119	21-248	94	36-222	149	13-301	.16
CD68+CD163+ macrophages	86	11-211	82	9-230	.44	90	11-225	55	22-162	83	9-230	.34

The amount of intratumoral lymphocytes is represented as the number of cells per mm2. The amount of staining for tumor-infiltrating macrophages is represented as pixels per mm2. For the numerical parameters, the median and range are shown. For the comparison between non-irradiated and irradiated eyes, P-values were obtained by the Wilcoxon rank sum test (Mann-Whitney U). For the comparison between the three secondary enucleation reasons, P-values were obtained by the Kruskal-Wallis test. P-values ≤0.05 are shown in italics.

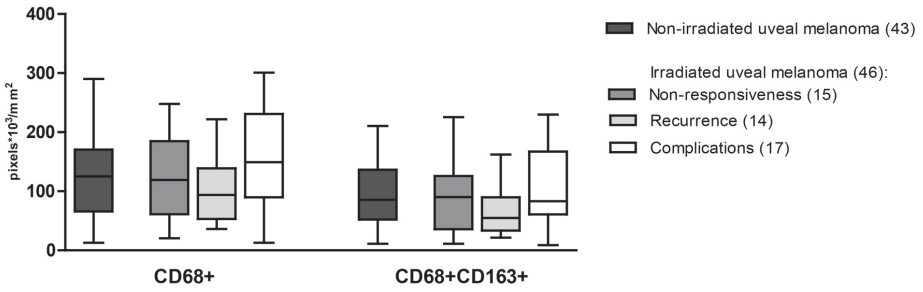


Figure 2A. Subtypes of tumor-infiltrating macrophages in uveal melanoma. Comparison in the amount of CD68+ and CD68CD163+ staining in non-irradiated and irradiated uveal melanoma, in which the latter group is divided into three subgroups based on the cause of secondary enucleation. The box-and-whisker plots represent the 25th and 75th percentile with the median and the minimum and maximum values. No significant differences were observed between the irradiated and non-irradiated uveal melanoma or subgroups of secondary enucleation.

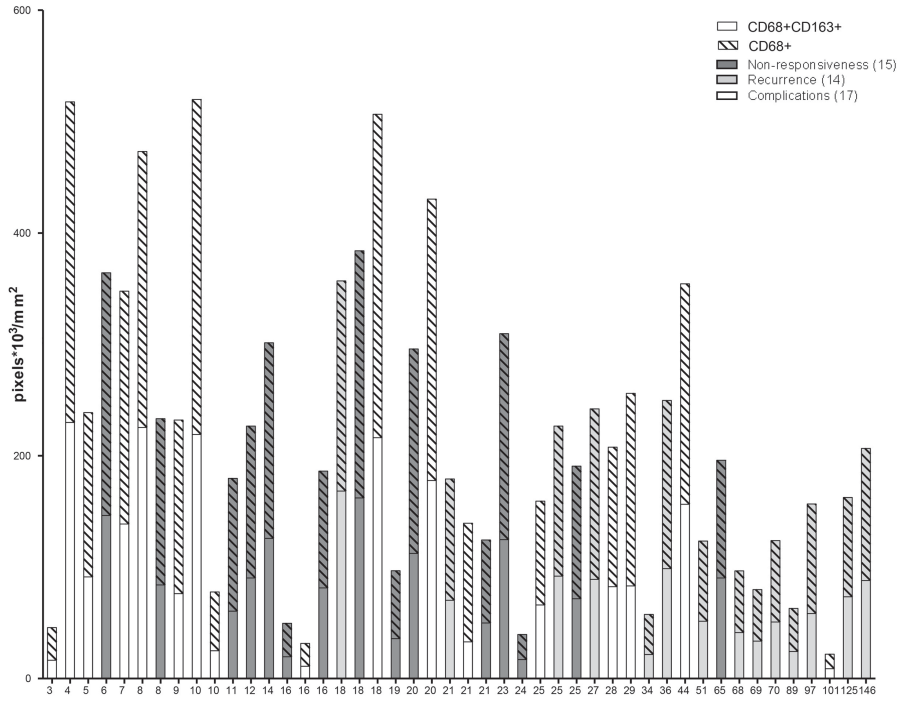


Figure 2B. Tumor-infiltrating macrophages in uveal melanoma post-irradiation. Variation in the amount of CD68+ and CD68+CD163+ staining in irradiated uveal melanoma with time. The stacked bars represent the total amount of CD68+ and CD68+CD163+ staining at a specific time point. The 46 time points represent the time interval between irradiation and enucleation for each of the 46 irradiated, secondarily-enucleated eyes.

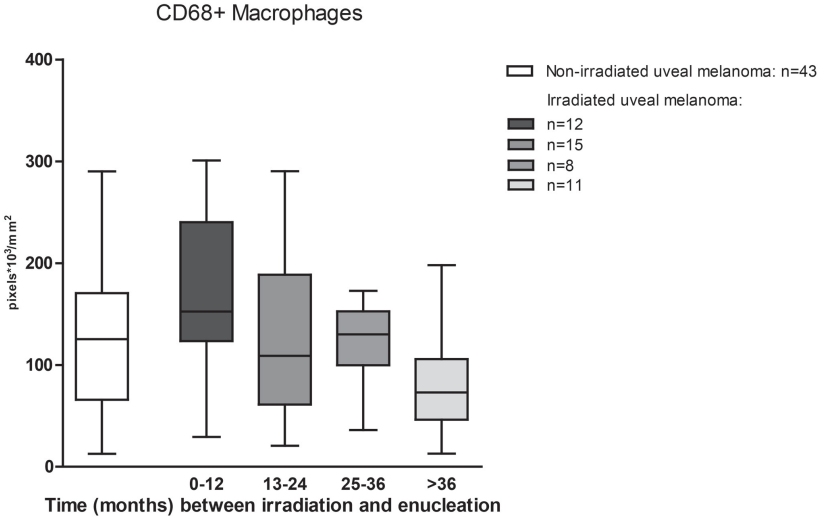


Figure 2C. Tumor-infiltrating CD68+macrophages in uveal melanoma post-irradiation. Comparison in the amount of CD68+staining in non-irradiated and irradiated uveal melanoma, in which the latter group is

divided into 4 subgroups based on the time (in months) between irradiation and enucleation. The box-and-whisker plots represent the 25th and 75th percentile with the median and the minimum and maximum values. No significant differences were observed in the amount of CD68+ staining in irradiated uveal melanoma at different time periods after irradiation and the non-irradiated uveal melanoma.

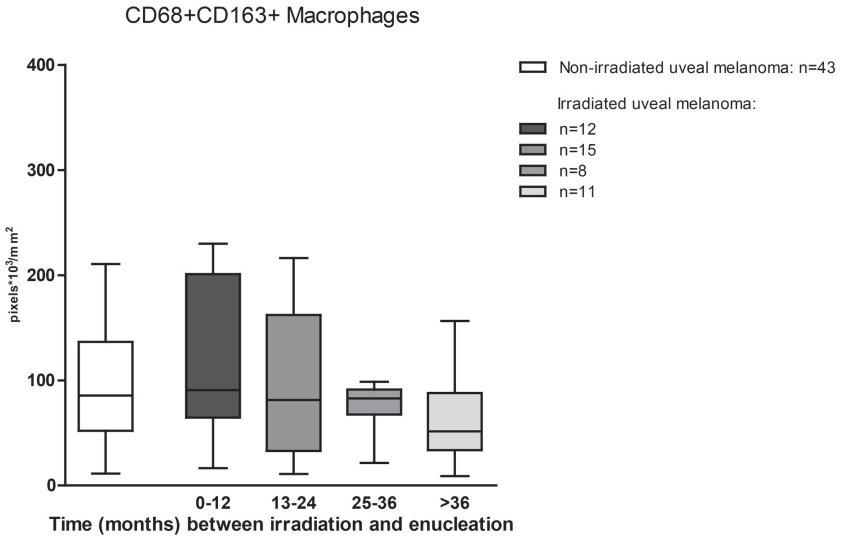


Figure 2D. Tumor-infiltrating CD68+CD163+macrophages in uveal melanoma post-irradiation. Comparison in the amount of CD68+CD163+staining in non-irradiated and irradiated uveal melanoma, in which the latter group is divided into 4 subgroups based on the time (in months) between irradiation and enucleation. The box-and-whisker plots represent the 25th and 75th percentile with the median and the minimum and maximum values. No significant differences were observed in the amount of CD68+CD163+staining in irradiated uveal melanoma at different time periods after irradiation and non-irradiated uveal melanoma.

Correlations between subtypes of immune cells

Infiltration of the tumor by one subtype of immune cell was accompanied by other subtypes. Spearman rank analysis showed significant correlations between the numbers of CD8+ T cells, CD4+ T cells, Foxp3+ Tregs, and the amount of CD68+ and CD68+CD163+ macrophages (Table 3, Spearman correlation coefficient (*r*) range = 0.379 to 0.983, p< .009). These observations are similar to the situation in non-irradiated uveal melanoma where tumors with high numbers of Tregs contained more of any other type of tumor-infiltrating immune cell[27]; however, the effect and correlations are less strong in irradiated eyes.

Relation with clinical and histological parameters

The amount of tumor-infiltrating immune cells (macrophages or lymphocytes) in irradiated uveal melanoma was not associated with gender, AJCC-UICC prognostic groups, or ciliary

body involvement. Tumors with necrosis showed a trend towards more IF-CD68+ and CD68+CD163+ staining (data not shown). Irradiated uveal melanoma with epithelioid cells contained significantly more intra-tumoral CD3+ lymphocytes (p=.011), CD8+ T cells (p=.009), CD4+ T cells (p=.018), and Foxp3+ Tregs (p=.036) than pure spindle cell tumors (Figure 4). Previously, for non-irradiated uveal melanoma, it had been shown that tumors with an increased LBD contained more CD68+ and CD68+CD163+ macrophages.[20] In irradiated eyes, however, no correlation was found between the amount of tumor-infiltrating leucocytes and LBD or tumor prominence (data not shown).

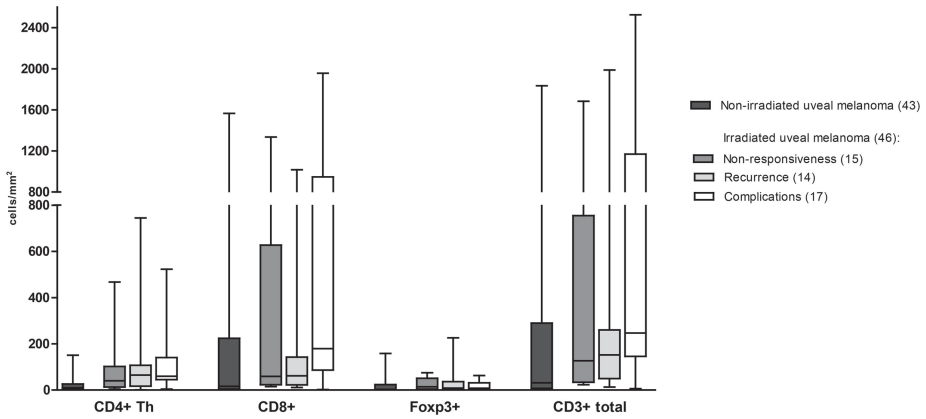


Figure 3A. Subtypes of tumor-infiltrating lymphocytes in uveal melanoma. Comparison of the number of intratumoral CD4+ helper T cells, CD8+ T cells, Foxp3+ Tregs and total T cells (CD3+) in non-irradiated and irradiated uveal melanoma, in which the latter group is divided into three subgroups based on the cause of secondary enucleation. The box-and-whisker plots represent the 25th and 75th percentile with the median and the minimum and maximum values. Significant differences were observed between non-irradiated and irradiated eyes for CD4+ helper T cells (p<.001), CD8+ T cells (p=.002), Foxp3+ Tregs (p=.02), and the total amount of CD3+ T cells (p=.001). No significant differences were observed between the subgroups of secondary enucleation.

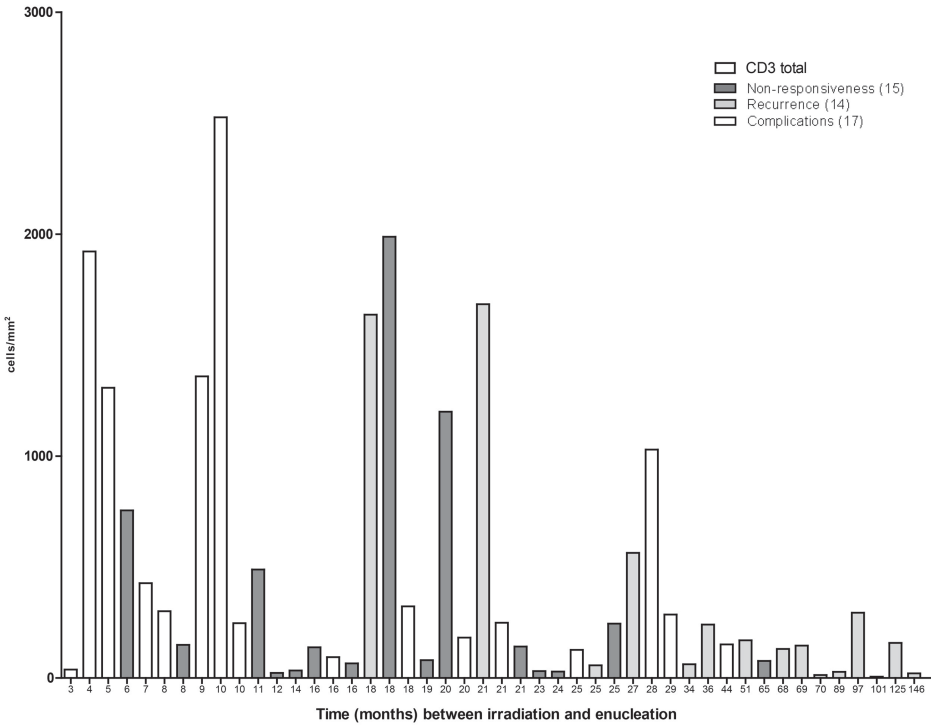


Figure 3B. Tumor-infiltrating lymphoyctes in uveal melanoma post-irradiation. Variation in the total number of intratumoral T cells in irradiated uveal melanoma with time. The columns represent the total number of CD3+ T cells at a specific time point. The 46 time points represent the time interval between irradiation and enucleation for each of the 46 irradiated, secondarily-enucleated eyes.

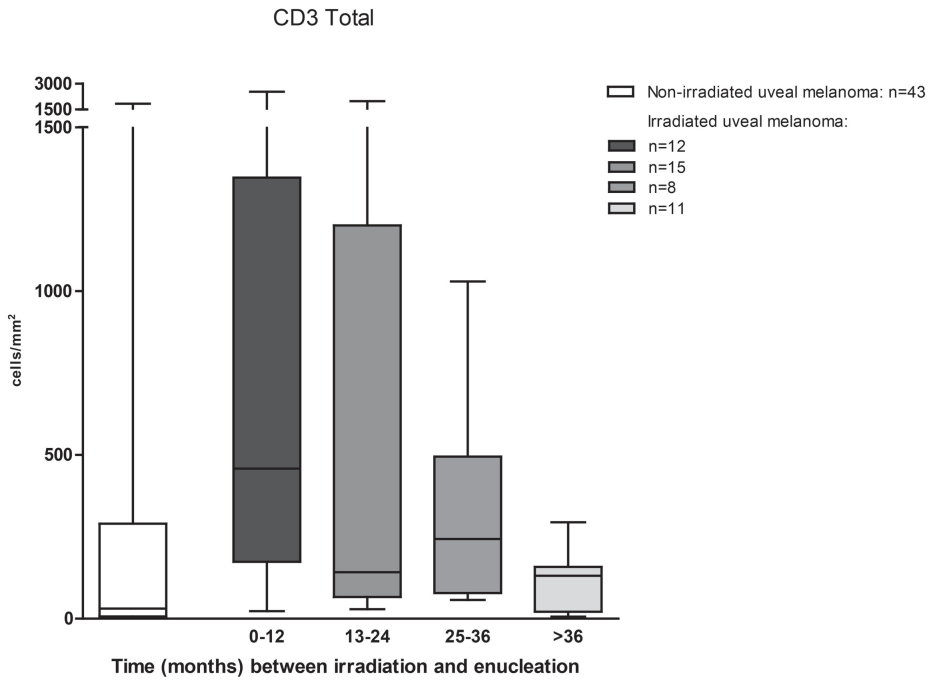


Figure 3C. Tumor-infiltrating CD3+ lymphocytes in uveal melanoma post-irradiation. Comparison in the number of total T cells (CD3+) in irradiated uveal melanoma, in which the latter group is divided into 4 subgroups based on the time (in months) between irradiation and enucleation. The box-and-whisker plots represent the 25th and 75th percentile with the median and the minimum and maximum values. Significant differences were observed in the total number of intratumoral CD3+ T cells between non-irradiated and irradiated eyes enucleated 0-12 months (p=.001) and 13-24 months (p=.014) after irradiation, but not at 25-36 months and >36 months.

Table 3. Correlations between different infiltrating immune cells (lymphocytes and macrophages) in irradiated, secondarily-enucleated eyes.

Immunofluorescence Staining	CD8	CD4 total	CD4 Th	CD4FoxP3	CD68	CD68CD163
CD3 r	0.980	0.939	0.916	0.818	0.607	0.497
	<.001	*<.001*	*<.001*	*<.001*	*<.001*	*<.001*
CD8 r		0.873	0.851	0.766	0.616	0.501
		<.001	*<.001*	*<.001*	*<.001*	*<.001*
CD4 total r			0.983	0.833	0.470	0.379
			<.001	*<.001*	*.001*	*.009*
CD4 Th r				0.746	0.467	0.380
				<.001	*.001*	*.009*
CD4FoxP3 r					0.450	0.387
					.002	*.008*
CD68 r						0.956
						<.001

r= rho, two-tailed Spearman correlation coefficient. ***P*** values ≤0.05 are shown in italic

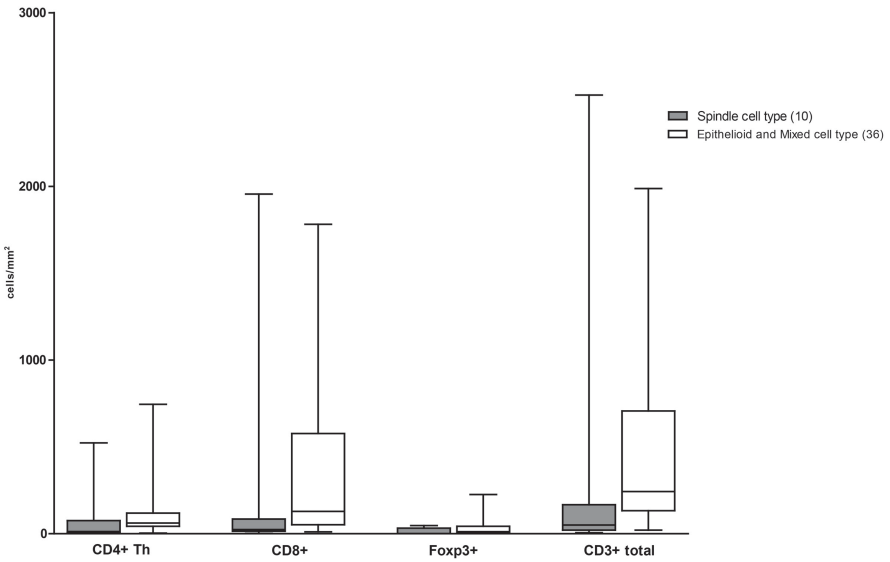


Figure 4. Subtypes of tumor-infiltrating lymphocytes in uveal melanoma and tumor cell type. Comparison of the number of intratumoral CD4+ helper T cells, CD8+ T cells, Foxp3+ Tregs and total T cells (CD3+) in irradiated uveal melanoma containing spindle or epithelioid (mixed) cells. The box-and-whisker plots represent the 25th and 75th percentile with the median and the minimum and maximum values. Significantly more intratumoral total CD3+ T cells (p=.011), CD8+ T cells (p=.009), CD4+ helper T cells (p=.018), and Foxp3+ Tregs (p=.036) were seen in tumors containing epithelioid cells compared to spindle cells.

DISCUSSION

As the local immune response may play a role in removing tumor debris or in either stimulating or suppressing anti-tumor immune responses, we determined the quantity of infiltrating leucocytes in uveal melanoma previously treated with ruthenium-106 brachytherapy (with or without adjuvant TTT) or proton beam radiotherapy. These eyes had been enucleated after failure of local tumor control or radiation-related complications, and were studied by IF-staining of intratumoral immune cells. We compared the characteristics of local inflammation in these uveal melanoma with primarily-enucleated eyes. We found that prior irradiation had no effect on the number and type of tumor-infiltrating macrophages, which showed a similar variability as previously observed in primarily-enucleated eyes,[20] but led to an increased T lymphocytic infiltrate up to 24 months post-irradiation. The reason to perform a secondary enucleation was not related to the amount and type of tumor-infiltrating leucocytes.

In a previous study, significantly more necrosis and a lower microvascular density were present in previously-irradiated eyes, but the number of macrophages was not affected.[19] In our study, we were able to assess the macrophage phenotype by determining the presence of the alternatively-activated (M2) macrophage. M2 macrophages show more phagocytic activity, promote tissue remodeling, tumor progression, and angiogenesis, and have immunoregulatory functions.[26] In both of our study populations, CD68+CD163+ immunopositive cells constituted the majority of CD68+ cells, showing that in irradiated as well as in non-irradiated uveal melanoma, the tumor-promoting M2 macrophage is the main type. In addition, we observed no increase in the amount of macrophages in non-necrotic areas of irradiated compared to primarily-enucleated eyes, even when normalized for time after irradiation, despite more extensive necrosis in the former group. An explanation may be that obliteration of the vascular supply prevented influx of leucocytes to the tumor and clearance of necrotic tumor debris by macrophages, leading to more necrosis.[15-17,19] Toivonen et al. described that along with melanoma cells, resident macrophages may have been sterilized by irradiation. This is supported by the finding that necrotic, irradiated macrophages lacking vimentin filaments were immunopositive for CD68.[19] They may therefore not be functional.

Interestingly, in contrast with our findings, previous studies with TTT and TSTT demonstrated an increased amount of tumor-infiltrating macrophages, especially at the borders of the TSTT-treated areas, with predominantly M2 macrophages.[28,29] These tumors had only been treated one or several weeks before enucleation. Their presence may reflect direct phagocytosis, i.e. removal of dead tumor cells, by a macrophage- mediated repair mechanism.[28,29] Infiltration of the tumor by M2 macrophages instead of M1 macrophages would be unfavorable for the stimulation of a specific immune response, but it may be that in this post-treatment stage, macrophages may only function in an innate immune response, clearing tumor debris, instead of exploiting their tumor- promoting functions.[26,29]

After irradiation, increased numbers of tumor-infiltrating lymphocytes were observed up to 24 months post-irradiation, which included the whole spectrum of CD8+ T cells, CD4+ T cells, and Foxp3+ Tregs. However, a previous study of nine enucleated eyes with uveal melanoma managed by pre-enucleation electron beam radiation showed a lower expression of HLA class II antigens and a lack of lymphocytic infiltrate in comparison with non-irradiated tumors.[32] It is uncertain why we observed an increased influx of lymphocytes

after irradiation. The differences in the time and type of treatment may be important, as in the afore-mentioned study, irradiation took place one week prior to enucleation, in otherwise quiet eyes, while the cases studied here had to be enucleated following failure of local tumor control or complications, at a median time after irradiation of 21 months (range 3-146). Moreover radiotherapy may induce intratumoral expression of chemokines that favors the recruitment of T cells, in the same manner as chemotherapy-induced chemokines correlate with T cell infiltration in mouse and human melanoma tumors.[33] This effect might dampen as we could not see any difference between irradiated eyes enucleated after >24 months post-irradiation and primarily-enucleated eyes.

As most of the irradiated eyes were enucleated due to failure of local tumor control, the presence of M2 macrophages and Tregs in these eyes would be detrimental for the induction of an effective immunological anti-tumor response, thereby promoting tumor growth in an immunosuppressive environment.[26] Unfortunately, we were not able to assess the functional activity of these tumor-infiltrating immune cells, as functional immunological studies were not possible on our sections. Another limitation of our study is its cross sectional design, as it only provides a snapshot of the moment of enucleation and does not reflect potential changes in infiltrating leucocytes over time. Thus it remains unclear whether this inflammatory infiltrate is a consequence of the characteristics of the primary tumor before irradiation, or is due to irradiation. Besides, eyes that had to be enucleated following radiation represent biased material and therefore do not allow us to draw definite conclusions regarding the effect of therapy. A logical limitation is the fact that the largest group of irradiated tumors, i.e. those that were successfully managed without local tumor control failure or complication, were not available for analysis, as such eyes are not enucleated.

An association exists between the presence of epithelioid cells and increased numbers of intratumoral lymphocytes in irradiated eyes. Epithelioid cells may be responsible for providing a tumor microenvironment with different chemokines and cytokines, thereby attracting a different composition of immune cells. A previous study at our department showed that prognostically bad tumors, i.e. those with loss of one chromosome 3, in primarily-enucleated eyes contained high numbers of tumor-infiltrating macrophages and lymphocytes.[20,21,27] One might thus expect that uveal melanoma enucleated due to local recurrence and non- responsiveness would contain more inflammatory cells than those

enucleated due to complications. Nonetheless, no significant differences were found between the subgroups of secondary enucleation. This is quite surprising, as several studies showed an increased tumor cell proliferative activity in uveal melanoma in case of recurrent tumor growth, in comparison with irradiated uveal melanoma enucleated due to complications.[34-36] However, radiosensitive uveal melanoma, displaying monosomy of chromosome 3, greater tumor height, and an epithelioid cell type, regressed more rapidly post-irradiation and were associated with a worse prognosis. [15,37,38] It may be that especially these melanoma led to locally-uncontrolled tumors and ended up in our study. Therefore, we will in the future analyze tumor intrinsic properties such as monosomy of chromosome 3, to evaluate whether such tumors are especially present in our secondary-enucleation group, and are associated with an infiltrate.

In conclusion, the present study showed that prior irradiation leads to an increased amount of T lymphocytes, but not of macrophages. Moreover, the inflammatory infiltrate was associated with tumor characteristics, but not with the cause of secondary enucleation. Future studies involving information on biopsies obtained prior to irradiation and analysis of tissues after secondary enucleation may help to understand the changes in tumor behavior in locally-uncontrolled uveal melanoma.

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THE IMMUNOLOGY OF GLAUCOMA

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ABSTRACT

The presence of specific antibodies and T cells that are specific in patients with glaucoma supports the idea that the immune system may play an important role in the initiation and/or sustainment of glaucomatous optic neuropathy, at least in some patients. At present, our understanding regarding immunological mechanisms associated with glaucomatous optic neuropathy is far from satisfactory. In this review, we examined evidence suggesting involvement of autoimmune responses in the pathogenesis of glaucoma. These include detection of auto-antibodies and -T cells and expression of cytokines and stress proteins in patients with glaucoma. While immune responses are thought to be detrimental, some responses may exert a protective effect against neurodegenerative damage. Likely, the balance between positive and negative regulators determines the survival or demise of cells. It is vital that research continues to elucidate the roles of the immune system in glaucomatous neurodegeneration and the possibility of alternative modalities of treatment. These studies may also provide valuable molecular biomarkers for the diagnosis and identification of a specific cohort of patients with glaucoma, i.e. those with normal tension glaucoma.

Glaucoma is a global unmet medical challenge because of its prevalence, debilitating consequences and lack of effective treatment. Affecting 70 million people worldwide, it is a group of ocular diseases characterized by damage to the optic nerve and degeneration of retinal ganglion cells (RGCs) that leads to progressive and permanent loss of vision. Although elevation of intraocular pressure (IOP) is considered a major risk factor associated with optic nerve damage, glaucoma is no longer viewed simply as a neurodegenerative condition caused by elevated IOP. A conservative estimation is that 20% – 30% of patients with glaucoma exhibit a normal IOP range, known as normal tension glaucoma.[1]

To date, the precise mechanisms of the pathogenesis of glaucoma are not fully resolved. Clinical theories attribute optic nerve damage from elevated IOP either to mechanical trauma at the cribriform plate or to alterations in vascular perfusion and subsequent ischemia.[2,3] Evidence is rapidly accumulating that damage to the optic nerve may be initiated or sustained not only by a high IOP, but by a number of factors, including ischemia, glutamate excitotoxicity, a specific genetic background, and immunological factors that have not yet been properly defined.[3-5] Recently, it has been suggested that autoimmune responses directed against retinal proteins may be related to the development of glaucomatous optic neuropathy.[5] These insults likely act through common final pathways that eventually activate cellular proteases and neuronal programmed cell death. The current prevailing view is that glaucoma pathogenesis is multifactorial, with a complex interplay of elevated IOP-induced events and genetic/epigenetic/ageing-related susceptibility factors contributing to neurodegeneration.[4] At present, most therapeutic approaches aim towards lowering IOP, which slows down the disease progress but does not cure the disease. Therefore, more insights into the pathogenesis might lead to earlier treatment and new therapeutic options.

An association of autoimmunity with glaucoma

The eye is an immune-privileged site so that its delicate structures are protected from dangerous immune reactions and pathogens. The immune-privilege of the eye is anatomically established through the blood-aqueous barrier and the blood-retina barrier and partially maintained by local production and release of immune-suppressive cytokines and neuropeptides.[6] The eye's own system blocks activated immune cells that might invade the eye and destroy vision by reacting with important structures such as the

retina.[7] However, disease or injury may interfere with the eye's immune privilege due to the breakdown of the blood-retina barrier or changes in cytokine production.

In the past years, growing evidence obtained from clinical and experimental studies supports a prominent immune response in the pathogenesis of glaucoma, at least in a subset of patients.[8] While glaucoma typically is associated with chronic elevation in intraocular pressure, in many individuals, glaucomatous damage occurs without the eye pressure exceeding the normal range, namely normal tension glaucoma (NTG). While current glaucoma therapy is directed specifically at lowering the IOP, it does not always stop the progression of the disease. The actual mechanisms causing RGC death are still unclear. It has been evident that glaucomatous pathology in some of these cases arises from an autoimmune response and thus comprises autoimmune neuropathy.[8-10] This may partly be associated with an increased expression and exposure of neuronal and stress antigens as a result of neuronal injury.[5] Oxidative stress caused by an increased generation of reactive oxygen species (ROS) and nitric oxide (NO)-induced damage has been implicated as a major force causing antigen-specific immune activation.[4,11] In addition, an increased prevalence of monoclonal gammopathy and elevated serum antibodies to retinal antigens has been reported in patients with NTG.[5]

It emerges that glaucoma could be a disease with an autoimmune component;[12-14] however, there is an opposing view that immune responses in glaucoma may be neuroprotective or neural destructive. For example, T-cell mediated immune responses may initially be beneficial to limit neurodegeneration.[15-17] The recruitment of T cells allows early communication of the immune system with cellular debris, destruction of damaged cells, and removal of pathogenic agents.[18] Subsequently, T cells mediate the protection of neurons against degenerative conditions, which is referred as 'protective immunity'.[15,16,18] This response is suggested to reduce the secondary degeneration of RGCs when exposed to various noxious stimuli. Schwartz and her colleagues reported that T-cell mediated autoimmunity plays an important role in the progression of glaucomatous optic neuropathy, as they induce protective effects in rodents by active or passive immunization with self-antigens.[18-20]

Although an initial immune response may be beneficial and necessary to limit neurodegeneration, expansion and secondary recruitment of circulating T cells through

an antigen-mediated process is known to shift the protective immunity into chronic autoimmune neurodegeneration. This is usually associated with a failure to control aberrant and stress-induced immune-response.[18] The premise that neurodegeneration in glaucoma contains an autoimmune component is supported by the presence of abnormal T-cell subsets[21,22] and increased production of serum autoantibodies to optic nerve and retina antigens in many glaucoma patients.[13,23,24] An increased glial production of neurotoxic cytokines,[25] T cell[26] and autoantibody-mediated cytotoxicity,[27] and uncontrolled complement attacks[28] may all cause destruction of RGCs and other neurodegenerative consequences. Thus, a failure to properly control aberrant, stress-induced immune responses likely converts the protective immunity to an autoimmune neurodegenerative process that can facilitate the progression of neurodegeneration in some, if not all, glaucoma patients.

Association between glaucoma prevalence and autoimmune disease

Cartwright and colleagues reported that 30% of patients with NTG showed evidence of immune-related disease.[29] Individuals with a tendency toward the development of autoimmune disease, may develop a cross reaction between the antigenic stimuli related to a systemic autoimmune disorder and antigens in the optic nerve or its vessels. For example anti-myelin basic protein antibodies, which can also be detected in patients with multiple sclerosis, can be found in the sera of glaucoma patients.[30]

Thyrotoxicosis (Graves' disease) is an autoimmune disorder and patients with this disorder are known to have several kinds of autoantibodies.[31] It might be possible that some of these antibodies are associated in the development of glaucoma associated Graves's disease. Thyroid eye disease (TED) is an organ-specific autoimmune reaction affecting extraocular muscles and intra-orbital content and occurs in 25-50% of patients with Graves' disease.[31] Visual field loss in patients with Graves' disease can be caused by glaucoma as well as compressive optic neuropathy.[32] In TED, a humoral agent (IgG) induces cellular inflammation with production of glycosaminoglycans and edema in both extraocular muscles and intra-orbital content.[33] The extraocular muscles may be so enlarged that they compress the optic nerve and eventually lead to blindness. In addition, the intraorbital content volume may be so increased that it can lead to a secondary elevation of the intraocular pressure. In TED, the short-term IOP increase observed when looking up is caused by eyeball compression due to enlarged and infiltrated extra-ocular muscles. The

long-term IOP increase seen in TED is caused by episcleral venous pressure elevation secondary to the increase in intra-orbital content and pressure.[31] Ohtsuka et al. showed that the prevalence of primary open angle glaucoma (POAG) and NTG was higher among patients with Graves' disease than in the normal population.[32] However, other studies did not find a significant increase in glaucoma prevalence in patients with TED, and from a pathophysiological standpoint, the long-term IOP increase is essentially due to episcleral venous pressure elevation.[31] Still the importance of these mechanical findings does not exclude a contribution of autoimmune disease to RGC and optic nerve damage.

Another disease where an autoimmune component might play a role is diabetes.[34,35] Diabetes and glaucoma are two of the leading causes of blindness worldwide.[36] The possible interaction between diabetes and glaucoma is not well established and up till now evidence regarding a clinical or an epidemiological association between the two diseases remains contradictive.[36,37] Several population-based studies have shown weak associations between occurrence of diabetes and an increased risk for development and severity of glaucoma, for example in the Beaver Dam Study[38] and in the Blue Mountain Eye Study.[39] However, in large-scale epidemiological studies such as the Framingham study,[40] the Barbados[41] and the Baltimore Eye studies,[42] no relationship was found. Both glaucoma and diabetes are diseases with vascular components, which may be the common denominator to ocular damage in patients with both conditions.[36] Abnormalities in blood flow in the retrobulbar and retinal microcirculation have been identified in both diabetes and glaucoma.[36,43,44] There is increasing evidence that hyperglycemia causes crosslinking and is associated with increased central corneal thickness, which results in augmented stiffness of the cornea, leading to overestimation of the actual IOP.[37,45,46] On the contrary, one study even showed that the optic nerve and RGCs are partially protected by short-term hyperglycemia in a rat model of experimental glaucoma.[37] As yet, no evidence has yet been established of one similar immune response underlying both diseases.

However, an association between the presence of Alzheimer's disease and glaucoma was seen.[47] Considerable evidence supports the presence of the characteristic pathological mechanisms of Alzheimer's disease, of amyloid accumulation, neuronal apoptosis and cell loss, in glaucoma.[48]

One might think that when increased serum immunoreactivity to retinal proteins may play a role in glaucomatous damage, immunosuppressive treatment might have a protective effect. A case report described a patient with NTG and rheumatoid disease whose serum immunoreactivity (antibodies) to retinal proteins regressed following methotrexate treatment for rheumatoid disease. During a three-year treatment, the patients' visual fields appeared to have improved, while during a period without treatment, signs of clinical deterioration occurred. Although this is only a report of one case, it suggests a potential role for immune-based intervention.[49]

Association of autoantibodies with glaucomatous neural damage

Autoantibody-based immune processes have long been suspected to be involved in the pathogenesis of glaucoma. Studies reported an increase in autoantibodies against ocular and optic nerve head (ONH) antigens in patients with glaucoma. These included antibodies directed against rhodopsin,[50] γ-enolase, heat shock proteins,[13,51,52] gluthatione-S-transferase,[24] α-fodrin[53], and glycosaminoglycans.[51] Using an antibody-profiling technique based on western blot and digital image analysis combined with multivariate statistics and artificial neural networks, Grus' group were able to analyze complex antibody patterns and demonstrated selective up-regulation of autoantibody repertoires against ocular antigens, including cellular retinaldehyde-binding protein, retinal S-antigen, in the sera of patients with POAG or NTG.[52,54-56]

Many glaucoma patients typically reveal a prominent and progressive atrophy of the retinal pigment epithelium close to the ONH in these eyes that is associated with disease progression. Wax et al. proposed that these parapapillary defects of the outer blood-retina barrier permit communication and access of circulating reactive antibodies to the retina, thereby binding to specific antigens in retinal neural tissue and retinal vasculature leading to injury.[14] Furthermore, Maruyama et al. studied the pathogenic role of serum autoantibodies against RGC in patients with glaucoma.[57] Immunohistochemical labeling revealed that most of the glaucoma patients' sera specifically reacted with the ganglion cell layer, possibly causing apoptosis of the RGCs, in the presence of additional unknown factors that breakdown the blood-retinal barrier to facilitate the antibody to access to the target antigens.

It is possible that autoantibodies are the result of a stress response of the RGCs.[24,58] This stress may be caused by ischemia, mechanical stress from high IOP, a high level of amino acids, or toxic products from high nitric oxide synthethase production in neurons.[54,59] Epitope mapping of anti-rhodopsin antibodies from patients with NTG revealed epitopic specificity of the patients' antibody profile, which suggests that a common mechanism underlies their generation.[60] A potential explanation is molecular mimicry, in that immune responses to infectious agents may generalize to native cellular proteins with similar epitope homology, resulting in serum antibodies that recognize these proteins.[60,61] An infection may thus initiate aberrant autoimmune tissue-specific responses due to cross-reactions between the infectious agent and native tissue proteins.[62,63] This mechanism has been implicated in the development of several organ-specific autoimmune diseases.[64] It has been shown that the C terminus of rhodopsin shares sequence identical to the C termini of numerous proteins from pathogenic bacteria and viruses.[60]

Apart from the serum, abnormalities in antibody patterns have also been detected in the aqueous humor of glaucoma patients.[65,66] Despite the immune privilege of the eye, autoantibody reactivity in the aqueous humor is nearly similar to those in serum of the same patients.[67] This concordance underlines the specificity and utilization of detected changes in sera of glaucoma patients, and suggests changes in systemic immunity in at least some patients. While these findings are consistent with the possibility that aberrant autoimmunity directed toward antigens in the retina or optic nerve is present in some patients with glaucoma, specific roles of these autoantibodies in the pathogenesis of the disease remains to be determined. It is not known whether these antibody profiles are an epiphenomenon of the disease, causative for the development of the disease, or if they occur as a result of glaucoma.

Accumulating evidence supports that the autoimmune mechanisms play a role in NTG, and it points to new approaches to the investigation, diagnosis, and treatment of this devastating disorder. In the future, it is important to compare autoantibody concentrations with optic nerve and RGC damage to establish the relevance of autoantibodies in the pathogenesis of NTG. There is also the possibility that the stage of glaucoma plays a role in the antibody profile composition.[30] However, we should remember that antibodies not only can be destructive, but can also be regulatory as they may help to modulate the activity of target molecules and influence their physiological functions, as natural autoantibodies

even occur in the sera of healthy subjects.[68-71] Loss of endogenous naturally-occurring and maybe protective autoantibodies in glaucoma may lead to a loss of immune protection, or to an increased risk to develop the disease.[30]

If autoantibodies detected in the sera of glaucoma patients are not directly responsible for the manifestation of glaucoma, they may still serve as diagnostic markers.[72] Finding a specific antigen or antibody repertoire may also provide a possibility to evaluate progression of the disease and treatment efficacy in a glaucoma patient. Glaucoma is sometimes called the "silent blinder" because the patients have no noticeable symptoms before they lose their central vision.[73] A screening method in which glaucoma can be detected prior to vision loss is therefore needed. In clinical practice, screening for specific autoantibodies or antibody patterns is performed for neurological disease screening or diagnosis.[74] This might also be possible for glaucoma. However, looking at only one specific marker may not be sufficient; a complex pattern of biomarkers is probably needed, as is seen by the complex antibodies patterns in glaucoma patients. [53,56,66,75,76] In fact, complex IgG antibody patterns against optic nerve and retinal antigens can be reproducibly identified in the serum of a study population from the United States and Germany, and autoantibodies to α-fodrin found in other neurodegenerative diseases such as Alzheimer's, further imply a role for autoimmunity and the neurodegenerative processes in glaucoma.[53] The high correspondence of autoantibody patterns found in the study populations from different continents supports the idea that serum autoantibody patterns may be useful as biomarkers for glaucoma detection or for determining prognosis.[53,77]

T cell participation in glaucomatous neural damage

Despite the assumption that the CNS is an immune-privileged site, T cells are able to enter normal, uninjured brain[78] as part of constitutive immune surveillance.[78,79] The site-specific parenchymal recruitment of T cells may initially play a beneficial role, as mentioned above.

According to Schwartz and colleagues, 'protective autoimmunity' refers to the situation in which immune cells recognize self-antigens and act as the body's defense mechanism against various injury, including high elevated IOP as seen in glaucoma.[80] They also suggest the existence of a mechanism, called secondary degeneration, that may explain why glaucomatous neuropathy continues to progress even after the primary cause (e.g. high IOP), has been removed. Secondary degeneration refers to the spread of degeneration

to healthy neurons that survived the primary insult.[81] Likely, cells undergoing secondary degeneration are adjacent to the damaged neurons and therefore are exposed to the degenerative environment. The degenerating neurons create a hostile milieu due to physiological factors that emerge in toxic amounts from the injured neurons, such as excessive glutamate and NO.[80,82] The first observation of 'protective autoimmunity' was seen in rodents where passive transfer of T cells specific to myelin basic protein (MBP) reduces the loss of RGC after traumatic optic nerve injury in rodents.[4] The spontaneous protective T-cell response may not be sufficient in its natural state or in case of severe insult, and may not be properly controlled, leading to chronic glaucomatous neurodegeneration.[80,82] For this reason Schwartz and colleagues suggested boosting of this protective autoimmune response by either passive transfer of self-reactive T cells or active immunization using self-antigens.[16,82-84] Once activated by self-reactive antigens, the protective T cells create a neuroprotective milieu that prevents or attenuates the secondary degeneration by affecting the local innate immune response. T cells facilitate recruitment of monocytes from the blood to the site of injury and properly activate resident microglia, which contribute in the regulation of the local inflammation and restoration of the homeostasis.[82] However, careful use of self-antigens is recommended, as the antigen should not induce an autoimmune response leading to further destruction.[15,80,85] Interestingly, in rats with different strains, the same IOP leads to different quantities of RGC loss, due to their difference in immune potency. The number of surviving RGCs in Sprague-Dawley (SPD) rats, a strain in which a beneficial autoimmune can be evoked spontaneously, was higher than in Lewis rats. In addition, in thymectomized SPD rats, the number of surviving RGCs after IOP increase was decreased.[16]

T cells can also initiate an immune response leading to chronic autoimmune neurodegeneration, particularly if they are presented with retinal or ONH antigens, due to failure to control an aberrant, stress-induced immune-response. Co-cultures of RGC-5 cell line with T cells isolated from immunized rats revealed that T cells activated by heat shock protein (HSP) 27 and HSP60 immunization in rats induced RGC degeneration and axon loss via the release of the inflammatory cytokine Fas-Ligand (FasL), accompanied by an up-regulation of the FasL receptor in RGCs. ***In vivo***, this resulted in a transient infiltration of T cells into the retina of HSP-immunized rats.[26] T-cell mediated neurodegeneration does not depend only on aberrant activation of autoreactive T cells, but maybe also reflect a dysfunction in the termination of T cell responses in this immune-privileged site by

apoptosis.[18,26] It is widely accepted that chronic activation of glial cells and accompanying increases in the production of proinflammatory cytokines, primarily TNF-α, are hallmarks of inflammation or parainflammation in the glaucomatous eyes. TNF-α through binding to TNFR1, a death receptor, elicits RGC death and inflammation during neurodegenerative injury in glaucoma.[86,87]

Activation of an adaptive immune response, which requires antigen presentation, is also evident in glaucoma patients.[8] For example, abnormal T-cell subsets[22] as well as elevated titers of serum autoantibodies to ONH and retina antigens are detected in glaucoma patients.[58] In order to examine whether the cellular immune system may play a role in the initiation/and or sustainment of glaucomatous optic neuropathy, T lymphocyte subsets in peripheral blood from patients with NTG or POAG were assessed and compared to age-matched control subjects.[22] In this study, the frequency of CD8+HLA-DR+ lymphocytes were seen to increase in NTG patients, and CD3+CD8+ lymphocytes in both patients with NTG and POAG, as compared to healthy subjects. An increased expression of HLA-DR molecules on lymphocytes has also been found in several other organ-specific autoimmune diseases, such as autoimmune thyroid disease, rheumatoid arthritis and in newly manifested type I diabetes.[88-92] This might suggest that autoantigens originating from the retina or optic nerve may be present in glaucoma patients in a similar way as in the aforementioned autoimmune-related diseases.[22]

Heat shock proteins

Heat shock proteins, also called stress proteins, are among the most highly-conserved and abundant proteins found in nature that are constitutively expressed in most cells under normal physiological condition.[93,94] They are thought to play a vital role in the cell in the presence of stress. One of the main roles that HSPs play is that of molecular chaperones. Particularly, they have been shown to function in protein maturation events, such as protein folding, unfolding, and translocation. In response to environmental stresses, such as heat, anoxia, and mechanical stress, HSPs accumulate to very high levels in stressed cells. Increased expression of HSPs is involved in protecting cells from the deleterious effects of heat and other stresses and helps them survive or promotes recovery from stress.[93,95,96] The expression of HSPs in neuronal cells suggests that they may require constitutive levels of these proteins for survival against various stresses. Furthermore, the accumulation

of HSPs in these cells during acute toxic states and in a variety of degenerative and inflammatory neurological diseases suggests their role for neuronal survival.[97-99]

Stress proteins have been shown to be among the dominant antigens recognized in immune responses. On the one hand, immune responses against HSPs can be highly cross-reactive and can even evolve anti-self antibody; at the other hand, they may lead to elimination of the bodies' own cells when the cells are affected, transformed, or otherwise stressed.[93] By serving as a danger signal, up-regulation of HSP27 may facilitate detection and elimination of stressed RGCs by the immune system. Uncontrolled immune-mediated cytotoxicity to RGCs and their axons may eventually facilitate the progression of neurodegeneration.

HSPs have been involved in the innate immune response and in the anti-apoptosis process.[100] They can bind to the CD14-toll like receptor 4 (TLR4) complex of the APCs and produce an inflammatory effect.[101] Furthermore, HSPs are highly antigenic, and immune responses to HSPs are implicated in the development of a number of human autoimmune diseases.[64] An activated immune response, such as increased autoantibodies to HSP27, 60 and 70 have been found in many patients with glaucoma.[51,61,66,75,77] The elevated titers of HSP27 autoantibodies in glaucoma patients are thought to induce neuronal apoptosis through attenuation of the ability to stabilize retinal actin cytoskeleton and activation of caspases.[27] Tezel et al. observed that exogenously applied anti-HSP27 antibodies enter neuronal cells in the human retina by an endocytotic mechanism. Subsequent to internalization, the anti-HSP27 antibodies facilitate apoptotic cell death. Anti-HSP27-antibody binding to actin results in depolymerization and proteolytic cleavage of actin, which leads to a decreased ability of endogenous HSP27 to stabilize actin cytoskeleton, thereby facilitating neuronal cell death. This may clarify why anti-HSP27 autoantibodies underlie glaucomatous optic neuropathy. [27]

Anti-HSPs may be generated through molecular mimicry, as described above: immune responses to infectious agents may generalize to native tissue proteins that share similar protein sequences, resulting in serum antibodies that recognize both HSP of infectious agents as well as native tissue proteins.[61,77] Mycobacterial stress proteins are recognized by human antibodies and T lymphocytes, and the evidence suggests that these proteins are among the major targets of the human cell-mediated immune response. The major stress protein antigen recognized by antibodies in bacterial infection is HSP60.[93] Therefore,

invasion of a host by a pathogenic organism such as a bacteria or virus is misinterpreted by the immune system in such a way that a response is mounted not only to the invading agent, but to other body proteins that share the same protein sequence to the invading agent.[77]

Increased expression of HSP27 and HSP60 was seen in postmortem eyes from glaucoma patients. Retinal immunostaining showed that HSP60 was prominent in RGCs and photoreceptors, whereas HSP27 was prominent in the nerve fiber layer and the RGCs as well as in the retinal vessels.[94] Although increased expression of HSPs in glaucomatous eyes may serve initially to protect cells from further destruction and facilitate repair,[94] they subsequently may recruit immune responses that contribute to the progression of disease.[64]

Glial cell contribution to immune responses associated with glaucoma

During the development and maintenance of the central nervous system (CNS), there exists a complex partnership between neurons and glial cells. Glial cells maintain the normal function of the CNS both by controlling the extracellular environment and by supplying metabolites and growth factors.[102] In addition, glial cells play an important role in maintaining perivascular barriers and securing immune privilege to protect neurons from potentially damaging effects of an inflammatory immune response.[4,102] Both microglial and macroglial cells exhibit important immunoregulatory functions in the brain as well as in the ONH and retina.[103-105] The immune privilege nature of the CNS makes it crucial that glial cells are capable of responding rapidly to any injurious condition or damage. Activation of glial cells can be brought about either through mechanical or ischemic stress or any other type of injury.[3]

Progressive degeneration of RGCs and their axons in human glaucoma and in animal models is accompanied by a chronic activation of glial cells,[3,4] which is usually displayed by a change in cell morphology and gene expression profile and upregulation of glial fibrillary acidic protein (GFAP).[3,4,102] After becoming reactive in glaucoma, stressed glial cells may be insufficient and/or dysfunctional in their ability to support neurons, which may facilitate RGC and axonal injury.[4,102] Recent evidence supports the view that glial cells may not be purely neuroprotective but may participate in the pathological process of neuronal damage.[4,102] Glial cells in the glaucomatous human retina and ONH rapidly respond to

tissue disturbance by exhibiting a reactivate phenotype.[106,107] This may lead to increased production of cytokines, immunomediators, cell death inducers (e.g. Tumor necrosis factor-α (TNF-α) and NO) and complement components, extracellular matrix remodeling of the ONH, and increased antigen-presenting ability of glial cells.[4,102] In agreement with this notion, a study by Tezel et al. demonstrated that following exposure to different stress conditions, such as ischemia and elevated hydrostatic pressure, glial cells secreted TNF-α and other noxious agents, such as NO, into the culture media and facilitated the apoptotic death of RGCs. Moreover, RGC apoptosis was attenuated by a neutralizing antibody against TNF-α and by a selective inhibitor of inducible NO synthase.[108]

Microglia, which are derived from the monocyte/macrophage lineage, play a crucial role in the regulation of the immune response.[109] As in other types of neurodegeneration,[110,111] resident microglial cells with increased MHC expression in glaucomatous eyes[112] likely participate in the immune-mediated process by functioning as antigen-presenting cells (APCs) in innate immune responses. Resident microglia also regulate the initiation and perpetuation of adaptive immune responses mediated by both T cells (the cellular immune response) and B cells (the humoral immune response).[109,113-115] It has been shown that following exposure to ROS/oxygen stress, microglial cells derived from the rat retina and ONH upregulated MHC class II molecules and became potent inducers of T cell activation as assessed by T-cell proliferation and TNF-α secretion, when were compared with non-treated microglial cells.[11]

Considerable evidence indicates that astrocytes, the most numerous glial cells in the CNS, are also capable of regulating immune responses. Similar to microglia, human lamina cribrosa astrocytes can be induced to express MHC molecules and acquire the ability of APCs. Yang et al. studied the expression of HLA-DR (a human MHC class II molecule) in astrocytes and found an increased expression of HLA-DR in ONH astrocytes of glaucomatous eyes secondary to exposure with elevated serum cytokines (IFN-γ, IL-10) or ischemia as compared to those of age-matched normal donors.[106] As a result, human lamina cribrosa astrocytes acquire the potential of APCs and may contribute to immunoregulatory events participating in the neurodegeneration process in glaucoma.

The finding that both microglial cells[112] and GFAP-positive astrocytes exhibit increased HLA-DR expression in glaucomatous human eyes suggests the presence of auto-immunity.

The fundamental basis of cellular immune recognition demands that T cells recognize antigens in the form of small peptides bound to MHC class II molecules displayed on the surfaces of APCs.[116] Consequently, upregulation of MHC class II molecules may help stimulate detrimental adaptive immune responses and lead to T cell activation and expansion.[107] These observations further support the likelihood of autoimmune injury in glaucoma.

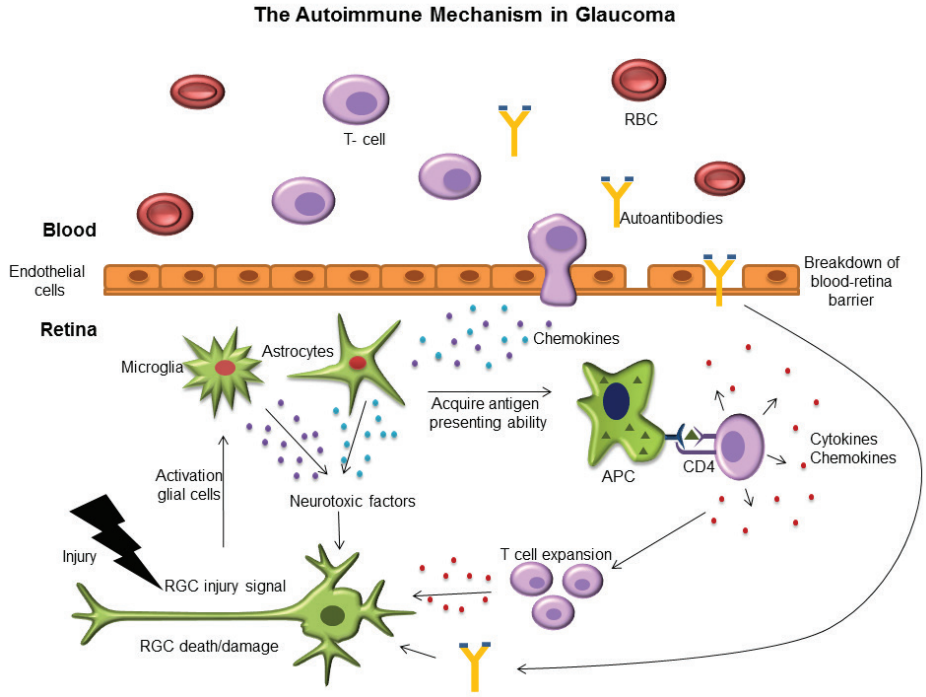

Figure 1. The Autoimmune Mechanism in Glaucoma This figure represents an oversimplified summary of the autoimmune mechanism in glaucoma. Mechanical or ischemic stress injury to RGCs and their axons causes chronic activation of glial cells (microglia and astrocytes), thereby exhibiting a reactivate phenotype. After becoming reactive in glaucoma, stressed glia cells produce cytokines, immunomediators, cell death inducers (e.g. TNF-α, NO) and complement component, resulting in death or damage of the RGCs and their axons. In addition, glial cells acquire and increase their antigen-presenting ability by expressing increased HLA-DR (a human MHC class II molecule) on the surface. Subsequently, the antigen-presenting cells present retinal or optic nerve head antigens to the T cells, which induce T cell activation and expansion. T cells in turn, produce and secrete pro-inflammatory cytokines (e.g. TNF-α, FasL) and chemokines, which results in RGC and axon damage and T cell recruitment into the retina, respectively. In glaucoma, breakdown of the blood-retina barrier facilitate communication and access of circulating autoantibodies and autoreactive T cells to the retina, thereby binding to retinal neural tissue leading to injury.

CONCLUSION

Evidence suggests that glaucoma may (in part) be a consequence of an immunological process. Findings supporting this statement include: 1) Insufficiency and/or dysfunctions of glial neurosupportive abilities in glaucomatous conditions; 2) abnormal T-cell subsets as well as elevated titers of serum autoantibodies to ONH and retinal antigens; 3) elevated serum levels of autoantibodies to small heat shock proteins in animal models and patients with glaucoma; and 4) possible comorbidity of immune-related disease in glaucoma patients.

Alterations of the cellular and humoral immune system in patients with glaucoma support that the immune system may play an important role in the initiation and/or sustainment of glaucomatous optic neuropathy.[22] The outcome of complex interactions between the immune system and retinal resident immune cells appears to be critical for the development of autoimmune RGC degeneration in animals as well as in humans. Preventing migration of activated T cells into the retina could be an important neuroprotective strategy, as these cells can recruit and activate other immune cells and initiate an autoimmune process.[26] On the other hand, it has been suggested that the involvement of T cell-mediated cellular immunity plays a neuroprotective role against glaucomatous optic neuropathy.

Despite the strength of these associations, there is presently no evidence to confirm that RGC loss occurs as the direct result of aberrant cellular and humoral immunity in these patients. Therefore, current understanding of the mechanisms underlying the complex interplay between immune-privilege, protective immunity, and autoimmune neurodegenerative disease is not satisfactory yet. An improved understanding of how tissue stress, neuronal injury, and glial responses during glaucomatous neurodegeneration orchestrate individual components of immunity can help designing immunomodulation-based treatment strategies.[117] It is vital that research continues to elucidate the potential role of the immune system in glaucomatous neurodegeneration and the possibility of alternative treatment. In addition, such studies may lead to valuable molecular biomarkers for the diagnosis and identification of patients in whom we suspect that aberrant immunity is directly relevant to glaucomatous neurodegeneration. To date, more extensive studies are needed to improve our knowledge about the role of autoimmune mechanisms and the autoantibodies in glaucoma.

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COMMENSAL MICROFLORA-INDUCED T CELL RESPONSES MEDIATE PROGRESSIVE NEURODEGENERATION IN GLAUCOMA

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ABSTRACT

Glaucoma is the most prevalent neurodegenerative disease and a leading cause of blindness worldwide. The mechanisms causing glaucomatous neurodegeneration are not fully understood. Here we show, using mice deficient in T and/or B cells and adoptive cell transfer, that transient elevation of intraocular pressure (IOP) is sufficient to induce T cell infiltration into the retina. This T cell infiltration leads to a prolonged phase of retinal ganglion cell degeneration that persists after IOP returns to a normal level. Heat shock proteins (HSPs) are identified as target antigens of T cell responses in glaucomatous mice and human glaucoma patients. Furthermore, retina-infiltrating T cells cross-react to human and bacterial HSPs; mice raised in the absence of commensal microflora do not develop glaucomatous T cell responses or the associated neurodegeneration. These results provide compelling evidence that glaucomatous neurodegeneration is mediated in part by T cells that are pre-sensitized by exposure to commensal microflora.

INTRODUCTION

Glaucoma affects 70 million people worldwide[1], making it the most prevalent neurodegenerative disease and a leading cause of irreversible blindness. The disease is characterized by progressive degeneration of retinal ganglion cells (RGCs) and axons. The most important risk factor for glaucoma is elevated intraocular pressure (IOP), which is thought to directly cause damage to neurons and the optic nerve. However, glaucomatous RGC and axon loss also occur in individuals with normal IOP, and patients whose IOP is effectively controlled by medical treatment often continue to suffer progressive neuron loss and visual field deterioration[2,3], suggesting mechanisms beyond pressure-mediated damage in neurodegeneration. One possibility is that pathophysiological stress, such as that induced by elevated IOP, triggers secondary immune or autoimmune responses, leading to RGC and axon damage after the initial insult is gone. To date, this remains as a hypothesis, as the molecular and cellular events underlying glaucomatous neural damage have not been identified.

Evidence suggests an autoimmune component in glaucoma[4]. Among the most direct evidence, a wide range of serum autoantibodies particularly those against heat shock proteins (HSPs) and retinal deposits of immunoglobulins, were found in glaucoma patients and animal models of glaucoma[5,6]. Moreover, inoculation of rats with human HSP27 and HSP60 induced an optic neuropathy that resembles glaucomatous neural damage[7], and elevated IOP has been reported to induce expression of HSPs in the retina, particularly RGCs[8,9]. Thus, a link among IOP elevation, HSP up-regulation, and induction of anti-HSP autoimmune responses in glaucoma has been suggested; however, the roles of these events in the disease pathogenesis remain unknown.

Because the eye is an immune-privileged site, a critical question is how autoimmune responses, such as those against HSPs, are induced in glaucoma. As HSPs are among the most highly-conserved proteins from bacteria to mice to humans (up to 60% identity)[10], a possibility is that the anti-HSP immune responses are induced originally by bacterial HSPs, and are reactivated by host HSPs during glaucoma. The facts that glaucoma patients exhibit increased titers of antibodies against *Helicobacter pylori* and that immunization with HSPs in rats induces glaucomatous neural damage are in line with this possibility[11]. Currently, little direct evidence is available to testify this hypothesis.

Here we show that: 1) a transient elevation of IOP is sufficient to induce $CD4^+$ T cell infiltration into the retina; 2) T cell responses are essential in the development of progressive glaucomatous neurodegeneration following IOP elevation; 3) both bacterial and human HSPs are target antigens of these T cells; and 4) HSP-specific $CD4^+$ T cell responses and glaucomatous neurodegeneration are both abolished in mice raised in the absence of commensal microbial flora (germ-free (GF) mice), supporting a mechanism of bacteria sensitized T cell responses underlying the pathogenesis of glaucoma. These observations identify a sequence of events that contribute to progressive neurodegeneration in glaucoma, and may lead to a paradigm shift for the diagnosis, prevention, and treatment of glaucoma.

MATERIALS AND METHODS

Mice

Adult (10 - 16 weeks old) male and female C57BL/6J (B6), *Rag1*$^{-/-}$, *TCRβ*$^{-/-}$, *Igh6*$^{-/-}$, and GFP transgenic mice (all on a B6 background) and 3 - 12 months old DBA/2J mice were purchased from the Jackson Laboratory. Mice of both sexes were evenly randomized into control and experimental groups. All experimental procedures and use of animals were approved and monitored by the Institute's Animal Care Committee and conform to the standards of the National Institute of Health and the Association for Research in Vision and Ophthalmology. GF and ASF colonized Swiss Webster mice of both sexes (10 – 16 weeks old) were originally purchased from Taconic Biosciences (Germantown, NY) and maintained at MIT in a genotobiotic core. DBA/2J mice were re-derived by embryo transfer into the germfree health status using GF Swiss Webster recipient mothers. These mice were maintained in sterile plastic film isolators in open-top polycarbonate cages on autoclaved hardwood bedding and fed autoclaved water and diet (ProLab 2000, Purina Mills, St. Louis, MO) *ad libitum*. Macroenvironmental conditions included a 14:10 light/dark cycle and temperature maintenance at 68 ± 2ºF. The sterile plastic film isolators were confirmed to be maintained free of all aerobic and anaerobic bacteria, and mold by bimonthly microbiologic monitoring of feces from each cage mixed with drinking water, interior swabs of the isolator walls, and a sampling of wet food left exposed to trap molds. These composite samples were screened for contaminants using aerobic and anaerobic culture, PCR, and fecal Gram stains. Control specific-pathogen-free mice (free of all

murine bacterial pathogens, adventitious viruses and parasites) were maintained under barrier conditions in standard microisolator cages under similar environmental conditions, including the same autoclaved diet and reverse osmosis purified water. The ASF was originally developed for colonizing GF rodents with a defined microbiota, consisting of 8 murine bacterial species: 2 aerotolerant lactobacillus strains, ASF360 and ASF361; 2 *Clostridium* sp. strains, ASF356 and ASF502; *Eubacterium* sp. strain ASF492; *Bacteroides* sp. strain ASF519; low-GpC-content Gram-positive bacterial strain ASF500; and strain *Mucispirillum schaedleri* strain ASF457[22,41]. A cohort of DBA/2J and Swiss-Webster mice was maintained under specific pathogen free (SPF) conditions to serve as controls. Unless specified, 6 to 8 mice were used per group in all experiments.

Induction of IOP elevation in mice

IOP was induced in adult mice (10 - 12 weeks old) as described previously[12]. Briefly, mice were anesthetized, and supplemented with topical proparacaine HCl (0.5%; Bausch & Lomb Incorporated, Tampa, FL). Elevation of IOP was induced unilaterally in adult mice by an anterior chamber injection of polystyrene microbeads having a uniform diameter of 15 µm (Invitrogen), which were re-suspended in sterile PBS at a final concentration of 5.0 x 10^6 MB/ml. The control group received an injection of sterile saline (2 µl) into the anterior chamber. Briefly, the right cornea was gently punctured near the center using a 30-gauge needle to generate an easy entry for injection. Following this entry wound, 2 µl of MB were injected into the right anterior chamber using a glass micropipette connected with a Hamilton syringe. Polystyrene microbeads are widely used in the clinic and have proven to be safe without inducing significant levels of inflammation[42,43]. No clinical signs of anterior segment inflammation, including corneal ectasia or neovascularization, inflammatory cell infiltration into the anterior chamber, or synechiae and fibrosis in the angle area, were observed. The morphology of the anterior chamber and Schlemm's canal appeared normal in MB-injected mice, consistent with previous reports[44-46]. In all experimental groups, IOP was measured every other day in both eyes using a TonoLab tonometer (Colonial Medical Supply) as previously described[12]. The measurement of IOP was conducted consistently at the same time in the morning. Mice were anesthetized by isoflurane inhalation (2-4%; Webster Veterinary, Sterling, MA) delivered in 100% Oxygen with a precision Vaporizer.

Elevation of IOP in adult GF and ASF mice (16-20 weeks old) was performed in a sterile laminar flow hood under aseptic conditions. All equipment was gas sterilized with ethylene

oxide before entering the hood. Sterile drug vials and bottles were only used once, and discarded after the end of each experiment. IOP was induced unilaterally in the right eye, by anterior chamber injection of MB as described; control animals received an injection of sterile saline. For MB injection and periodic measurement of IOP, GF and ASF mice were exited from their home isolators in a sealed sterile container and transported to a Clidox (Pharmacal, Naugatuck, CT) sterilized hood. All manipulations of the mice were performed in the hood using strict aseptic technique. The mice were returned to separate, dedicated sterile isolators that were monitored closely for inadvertent contamination. Since retrieving mice out of the sterile isolators and any intervention on them increases the risk of contamination, IOP was measured shortly before anterior chamber injection, and subsequently on only 1, 2 and 8 weeks post injection. IOPs of GF DBA-2J mice were measured at 3, 8, 10 and 12 months of age.

Immunohistochemistry and quantification of RGC and axon loss

For immunohistochemistry, mice were sacrificed and perfused with saline for 5 min to remove all blood cells from their vessels followed by 4% paraformaldehyde for 15 min, and eyes and optic nerves were dissected. Retinal flat-mounts or transverse sections were incubated with a primary antibody against CD3, CD4, CD11b, Tuj1, Brn3a, and RECA1 (all from Invitrogen) followed by an Alexa Fluor 488-conjugated secondary antibody (Invitrogen). Quantification of RGC axon loss in optic nerve sections was as described[47], with minor modifications. All of the quantification procedures were routinely conducted under a masked fashion. Briefly, the optic nerve cross sections were traced using NIH Image J software, and the total area of each cross section was measured. For axon counts, 5 rectangular regions (64 μm x 85 μm) covering the entire optic nerve cross areas were photographed at 1000x magnification (Nikon Eclipse E800), and 4 rectangular regions (30 μm x 30 μm) were cropped from each of the 5 rectangular areas. Axons were identified by the microbules and axolemma surrounded by the electron-dense myelin sheath, and all axons in the 20 selected regions, covering approximately 50% of the total optic nerve area, were counted. The percentage of axon loss was determined by dividing the axonal density calculated from the optic nerve with high IOP to that of the contralateral control optic nerve of the same mouse in B6 mice, or to that of 3 month-old (normal IOP) DBA/2J mice. RGC loss was assessed quantitatively in retinal flat-mounts using a standard protocol described previously[12] with minor modifications. In brief, retinal flat-mounts were incubated with a primary antibody against a RGC marker, β-III-tubulin[48] (Tuj-1; Sigma-Aldrich, St.), followed by

a Cy3-conjugated secondary antibody. Immuno-positive cells in 24 areas from each retina (400x; 12 areas taken from the peripheral retina, 8 from the intermediate regions, and 4 from the central retina), each covering 0.09 mm^2, were counted[12]. The numbers were averaged to calculate the RGC densities, and the percentage of RGC loss was determined by dividing the RGC density calculated from the retina with high IOP by that of the contralateral control retina of the same mouse in B6 mice or by that of 3 month-old (normal IOP) DBA/2J mice.

Isolation and adoptive transfer of $CD4^+$ T cells

Single-cell suspensions were prepared from spleens in RPMI-1640 media (Sigma) containing 10% fetal bovine serum (FBS), 1% penicillin/streptomycin, and 1% L-glutamine after lysis of red blood cells with red blood cell lysis buffer (Sigma). $CD4^+$ T cells were purified using an auto MACS Separator and a $CD4^+$ T Cell Isolation Kit (MiltenyiBiotec) according to the manufacturer's protocol. Briefly, $CD4^+$ T cells were negatively selected from splenocytes of Hsp27-immunized mice, or from mice with high IOP, by depletion with a mixture of lineage-specific biotin-conjugated antibodies against CD8 (Ly-2), CD11b (Mac-1), CD45R (B220), CD49b (DX5), Ter-119, and antibiotin microbeads. The procedure yielded purity higher than 90% $CD4^+$ T cells, as assessed by flow cytometry. These cells (2 x 10^8 cells per mouse in 200 µl PBS) were adoptively transferred into recipient mice via tail vein injection. Control animals received the same numbers of $CD4^+$ T cells, isolated from mice with normal IOP or from OVA-immunized mice.

ELISA and ELISPOT Assays

Sera were collected from mice injected with MB or saline. Ninety-six-well plates (Nunc) were pre-coated with recombinant human HSP27 or HSP60 protein (1 µg/ml) blocked with with 10% normal goat serum, followed by addition of diluted serum samples (1:10) and anti-HSP27 or anti-HSP60 antibody (1:10 as positive controls) and incubation for 2 hours at room temperature. Horseradish peroxidase (HRP)-conjugated anti-mouse (or human) IgG (1:1,000) were added and incubated for 45 min at room temperature. TMP substrate (Sigma) was added and optical density (OD) was measured at excitation wavelength 405 nm using XFlour4 software. Each sample was processed and analyzed in triplicate.

A mouse IFN-γ ELISPOT assay (eBioscience) was used to determine the frequency of IFNγ–producing T cells in response to HSP27 or HSP60. ELISPOT plates (Multiscreen-MAIPS4510), pre-coated with 100 µl/well of capture antibody, were blocked with 200 µl/

well of complete RPMI-1640 medium. Mouse splenocytes (1 x 10^6 cells/well) were added and incubated with antigens, including HSP27 or HSP60 (Sigma Aldrich), *E. Coli* HSP24 or HSP60 (EnzoLifesciences), IRBP and MBP (Invitrogen), at a final concentration of 10 µg/ml, for 48 hours. Cell cultures incubated alone were used as controls. Results are shown as the mean number of antigen-specific spot forming cells (SFC) after background subtraction from the control wells containing no antigen.

Flow cytometry assays

The mouse cervical lymph nodes, spleen, or retina were freshly isolated. Lymph nodes (LNs) and spleens were dissociated using forceps; the cell mixtures were filtered through a 70 um nylon mesh. For the neuroretina, 2-3 retinas were combined for each test and dissociated using Papain Dissociation system (Worthington Biochemical Product). Dissociated cells taken from the cervical LNs, spleen, or retina were immunolabeled with fluorescein isothiocyanate- (Biolegend) or PerCP/Cy5.5- (BD Bioscience) conjugated anti-CD4 antibody. The cells were then permeabilized with permeabilizing/washing buffer (Biolegend), followed by immunolabeling for cytokines with AlexaFluor488- (BD Bioscience) or phycoerythrin-conjugated primary antibodies against IFN-γ, IL-4, IL-17 or TGFβ (BioLegend) for 30 min at 4ºC in dark. Corresponding isotype antibodies were used as controls. Data were collected with BD LSR II Flow Cytometer as 20,000 – 30,000 events and analyzed by FlowJo (Tree Star).

Preparation of human blood samples and T cell assays

Patients (40 – 60 years) who had been diagnosed with POAG with unambiguous clinical evidence of pathological "cupping" of the optic nerve head and documentation of visual field loss, were recruited for this study. Recruited control subjects showed no evidence of optic nerve or CNS damage from any cause, and were found to have no significant visual or neurological disorder. In addition, patients with retinal detachment as well as those suffering from skin injuries were examined to exclude the possibility that the increase in HSP27- and Hsp60-responsive T cells in POAG was a result of stress responses to inflammation or disease conditions independent of POAG. All participants were provided with informed consent to participate in the relevant research resources. The study was approved by the Institutional Review Board (IRB) of the Massachusetts Eye and Ear. Sixteen milliliters of venous blood was drawn from each volunteer into a vacutainer CPT tube (Becton Dickinson) with sodium citrate and processed according to the manufacturer's

instructions. The peripheral blood mononuclear cells were resuspended at 1.0 x 10^7 cells/ml in RPMI containing 20% heat-inactivated fetal bovine serum. ELISPOT and ELISA assays were performed as described above. The measurements were performed in a masked fashion.

Delayed-type hypersensitivity assay

The thickness of the mouse ear was measured using a micrometer prior to antigen stimulation. The dorsal side of the ear was injected intradermally with 10 µl of human recombinant HSP27 (1 µg/µl; Enzo Life Science) or with a control antigen – either MBP or IRBP (1 µg/µl; Invitrogen). The thickness of the injected ear was measured again after 24 and 48 hours, respectively, and change in thickness was calculated. We did not find significant differences between the results obtained at 24 and 48 hours; thus, only results obtained at 24 hours were reported.

HSP27 inoculation

To immunize mice, 50 µl of human recombinant HSP27 (50 µg; Enzo Life Science) was emulsified with 50 µl incomplete Freund's adjuvant (IFA) emulsion and injected subcutaneously in adult B6 mice. Two to three weeks later, immune responses to HSP27 were analyzed by DTH and ELISPOT assays.

Statistical Analysis

Based on the 90% statistical power analysis done with our previous data in glaucoma studies, 6 mice per group were shown to yield over 95% significance, 2-sided test[12,49-51]. This is also the group size commonly used in other reports seeking statistical significance in rodent models of experimental glaucoma[52,53]. Thus, most of our experiments used a group size of 6-8 animals, unless otherwise indicated. Scatter and bar plots are presented as mean ± s.e.m., Box-and-whisker plots are presented as minimum-maxium, and a paired analysis of variance was used for statistical analysis.

Data availability

The authors declare that the data supporting the findings of this study are available within the article and its supplementary information files, or are available upon reasonable requests to the authors.

RESULTS

Elevation of IOP induces retinal T-cell infiltration

To investigate if a high IOP evokes retinal immune responses, we induced IOP elevation in mice by microbead (MB) injection. As shown previously[12], a single MB injection into the anterior chamber of adult C57BL/6 (B6) mice led to a transient 3-week elevation of IOP (Fig. 1a), whereas injection of saline did not induce any significant change in IOP (Supplementary Fig. 1a). Using immunostaining of retinal flat-mounts for general T-cell marker CD3 and RGC marker Tuj1, we detected T cell infiltration into the ganglion cell layer (GCL) of MB-injected, but not of uninjected or saline-injected mice (Fig. 1b). Infiltrating T cells were noted at 2 weeks after MB-injection (Fig. 1c), scattered throughout the retina without apparent clustering or preference to any specific quadrant. The number of T cells had declined by 4 weeks. To define the subpopulations of infiltrating T cells, we performed triple-immunolabeling with antibodies specific for CD4 or CD8 T cells, RECA1 (for blood vessels) and Tuj1. CD4+, but not CD8+, T cells were detected in the GCL of glaucomatous retina (Supplementary Fig. 1b). To verify T-cell retinal infiltration and define the subsets of CD4+ T cells, we examined T-cell cytokine secretion profiles, including IFN-γ (T_H1), IL-17 (T_H17), IL-4 (T_H2), and TGF-β (Treg). We detected IFN-γ secreting, but no other subsets of, CD4+ T cells in the retina. The number of infiltrating CD4+ T cells was significantly increased in MB-injected retina compared to saline-injected controls (Fig. 1d,e). As with T-cell infiltration, significantly more CD11b+ microglia and macrophages were detected in the retinas of mice 2-4 weeks after MB, but not after saline-injection (Fig. 1f,g). These immune responses were a result of the elevated IOP because injection of fewer MB (2.0 × 106 beads/eye), which did not elevate IOP, induced neither T cell infiltration (Fig. 1h) nor an increase in CD11b+ cells (Supplementary Fig. 1c) in the retina compared to saline-injected group. In contrast, correlating with IOP elevation, injection of more MB (5.0×10^6 beads/eye) induced both T-cell infiltration (Fig. 1h) and an increase in CD11b+ cells (Supplementary Fig. 1c). Induction of immune responses by elevated IOP was also confirmed using DBA/2J mice, which spontaneously develop increased IOP and glaucoma by 6-8 months of age[13] (Supplementary Fig. 1d,e). T-cell infiltration was detected in the retinas of 8 month-old DBA/2J mice, but not in 3 month-old mice (Fig. 1c). No B cell infiltration was detected in the retinas in either MB-injected B6 mice or 8 month-old DBA/2J mice. Thus, elevation of IOP is followed by T-cell infiltration into the retinas.

To assess retinal neurodegeneration, we quantified RGC and axon loss in mice. As shown by RGC quantification with Tuj1 labeling, percentages of RGC and axon losses gradually increased from ~17% at 2 weeks post MB-injection to ~35% by 8 weeks (Fig. 1i-k). Accordingly, RGC densities decreased significantly from ~3,800/mm^2 in saline-injected mice to ~3,300/mm^2 at 2 weeks after MB-injection, and to ~2,500/mm^2 at 8 weeks after MB-injection. This result was further verified by immunolabeling for Brn3a (Supplementary Fig. 1f,g). Notably, although elevation of IOP lasted only transiently for 3 weeks in the MB-injected mice, RGC and axon loss continued to 8 weeks after MB-injection, the longest time point that the mice were monitored. These results show that a transient elevation of IOP induces T-cell infiltration into the retina and a prolonged period of retinal neurodegeneration, even after the IOP has returned to the normal level.

T cells mediate prolonged retinal neurodegeneration

To determine the role of T cells in elevated IOP-induced retinal neurodegeneration, we used mice deficient in both T and B cells (*Rag1*$^{-/-}$ mice), only T cells (*TCR*β$^{-/-}$ mice), or only B cells (*Igh6*$^{-/-}$ mice)[14-16]. Injection of MB into these immune-deficient mice resulted in a transient elevation of IOP that had the same magnitude and kinetics as that observed in B6 mice (Supplementary Fig. 2a) and a similar loss of RGCs and axons at 2 weeks as seen in B6 mice (Fig. 2a,b). However, glaucomatous *Rag1*$^{-/-}$ mice did not exhibit continued loss of RGCs or axons from 2 to 8 weeks post MB injection when IOP had returned to the normal range, whereas B6 and *Igh6*$^{-/-}$ mice did. In *TCRβ*$^{-/-}$ mice, which lack CD4$^+$ αβT-cells but are compensated by expansion of γδ and natural killer T cells and B cells, no significant further loss of RGCs was detected between 2 and 8 weeks after MB injection; whereas, an attenuated albeit significant loss of axons was observed at 8 weeks. The data suggest a primary role for CD4$^+$ αβT-cells, with a possible involvement of other immune cells, in late glaucomatous axon damage. These findings support a two-phase neurodegeneration in glaucoma: the initial phase when IOP is elevated and the prolonged (progressive) phase after IOP has returned to the normal range. Neither T- nor B-cell deficiency attenuates the initial phase of neural damage, but T cells are an essential player in the prolonged phase of glaucomatous RGC and axon degeneration.

To further define the subsets of systemic CD4$^+$ T cells that were activated under elevated IOP, we examined cytokine secretion profiles of CD4$^+$ T cells in the spleens and eye draining (cervical) lymph nodes (LN) by flow cytometry. Unlike that seen in the retina, the frequencies

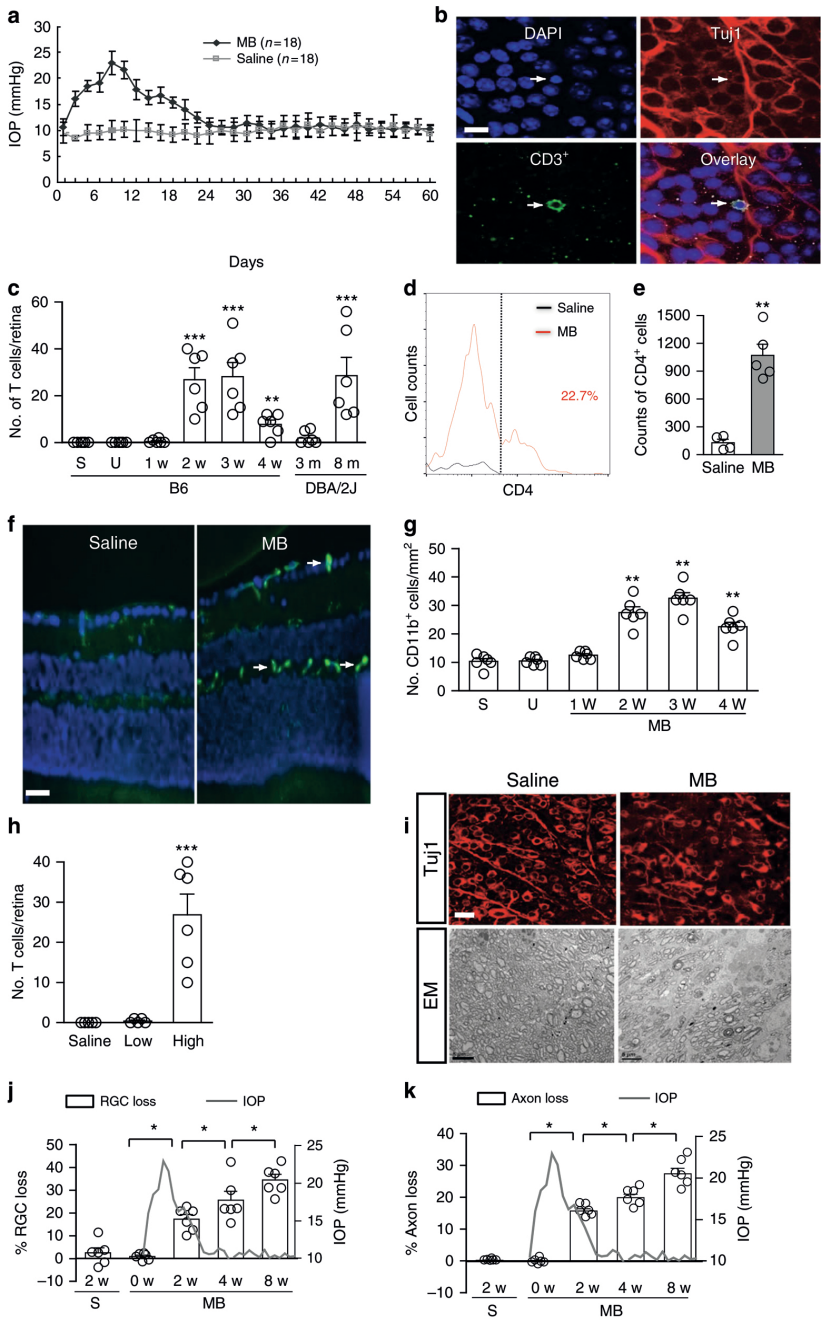


Figure 1. A Transient IOP elevation induces T-cell infiltration and progressive RGC and axon loss. (**a**) IOP levels in MB- or saline-injected B6 mice (n=18/group). (**b**) Retinal T cell infiltration post-MB injection. Retinal flat-mounts from mice 3 weeks after MB injection were stained with Tuj-1 (red), an anti-CD3 antibody (green;

Figure 1 (continued) arrow) and DAPI (blue). Scale bar: 10µm. (**c**) Quantification of $CD3^+$ T cells in retinas of uninjected mice (U), mice 3 weeks after saline-injection (S) or 1-4 weeks (w) after MB-injection (n≥6/group), and DBA/2J mice at 3 (n=6) or 8 (n=8) months (m) old. ***P*<0.01, ****P*<0.001 as compared to saline-injected group or between 3 and 8 months-old DBA/2J mice. **(d)** Flow cytometry plot of retinal cells double-immunolabeled for IFN-γ and CD4; cell count was gated for IFN-γ secreting cells. (**e**) Quantification of $CD4^+$ T cells by flow cytometry 2 weeks post saline- or MB-injection (n=5/group). Mice were perfused with saline. $CD4^+$ cell counts were obtained by multiplying total cells recovered from the retina with percentage of $CD4^+$ cells. ***P*<0.01 by *t*-test (n=6/group). (**f**) Representative photomicrographs of retinal sections labeled with anti-CD11b (green; arrow) and counter-stained with DAPI (blue). Scale bar: 20µm. (**g**) Quantification of $CD11b^+$ cells at 1-4 weeks post-MB injection, 3 weeks post-saline (S) injection, and in uninjected (U) mice. ***P*<0.01 as compared to saline-injected group (n=6/group). (**h**) Quantification of infiltrated T cells in the retinas of mice 2 weeks after a saline, low- (Low; 2.0×10^6 beads/eye) or high- (High; 5.0×10^6 beads/eye) dose of MB injection. ****P*<0.001 as compared to saline injected group (n=6/group). (**i**) Representative Tuj1-stained epifluorescence photomicrographs of retinal flat-mounts (Tuj1) and electron microscopy (EM) of optic nerve cross-sections from mice 8 weeks after saline or MB injection. Scale bar for Tuj1 and EM: 10 and 50 µm, respectively. (**j,k**) Progressive loss of RGCs and axons in MB-injected mice. RGCs were counted in Tuj1-stained retinal flat-mounts and axons in optic nerve cross sections. Shown are percentage of RGC (**h**) and axon (**i**) loss. The latter is overlaid with changes in IOP levels in MB-injected eyes. **P*<0.05, ***P*<0.01, ****P*<0.001 by ANOVA as compared to saline injected mice (n=6/group).

of $CD4^+$ T cells that expressed IFN-γ (T_H1), IL-17 (T_H17), IL-4 (T_H2), or TGF-β (Treg) were all significantly increased in both the spleen and the cervical LN of mice at 2 weeks post MB injection compared to saline-injected mice (Supplementary Fig. 2b,c). Thus, elevated IOP induced enhanced systemic reactivity of T cells, without any bias towards a specific subset of $CD4^+$ T cells.

We investigated if $CD4^+$ T cells play a causal role in progressive neurodegeneration in glaucoma by adoptively transferring T cells from glaucomatous B6 mice into *Rag1*$^{-/-}$ mice. As all subtypes of $CD4^+$ T cells were activated following IOP elevation, total $CD4^+$ T cell populations from the spleens of B6 mice, which had received MB (glaucomatous) injection 14 days earlier, were transferred; $CD4^+$ T cells isolated from naïve or saline-injected mice served as controls. Cells were injected via the tail vein into *Rag1*$^{-/-}$ mice that had received MB injection 14 days earlier (on the same day when the donor mice had received MB injections) (Fig. 2c). Two weeks after $CD4^+$ T-cell transfer (or 4 weeks after MB injection), MB-injected *Rag1*$^{-/-}$ recipients, which had not received any $CD4^+$ T cell transfer (U) or that received $CD4^+$ T cells from saline-injected (S) normal IOP B6 mice showed no retinal T-cell infiltration (Fig. 2d,f). As expected, these *Rag1*$^{-/-}$ mice exhibited ~15% RGC and axon loss as a result of the elevated IOP-induced initial phase of neural damage (Fig. 2g,h). In contrast, MB-injected *Rag1*$^{-/-}$ recipients that had received a $CD4^+$ T-cell transfer from glaucomatous (MB-injected) B6 mice showed retinal T-cell infiltration (Fig. 2f) and a significant further increase in RGC and axon loss compared to the uninjected (U) and saline-injected (S) control mice (Fig. 2d-h). Induction of IOP elevation in recipient mice was required for T-

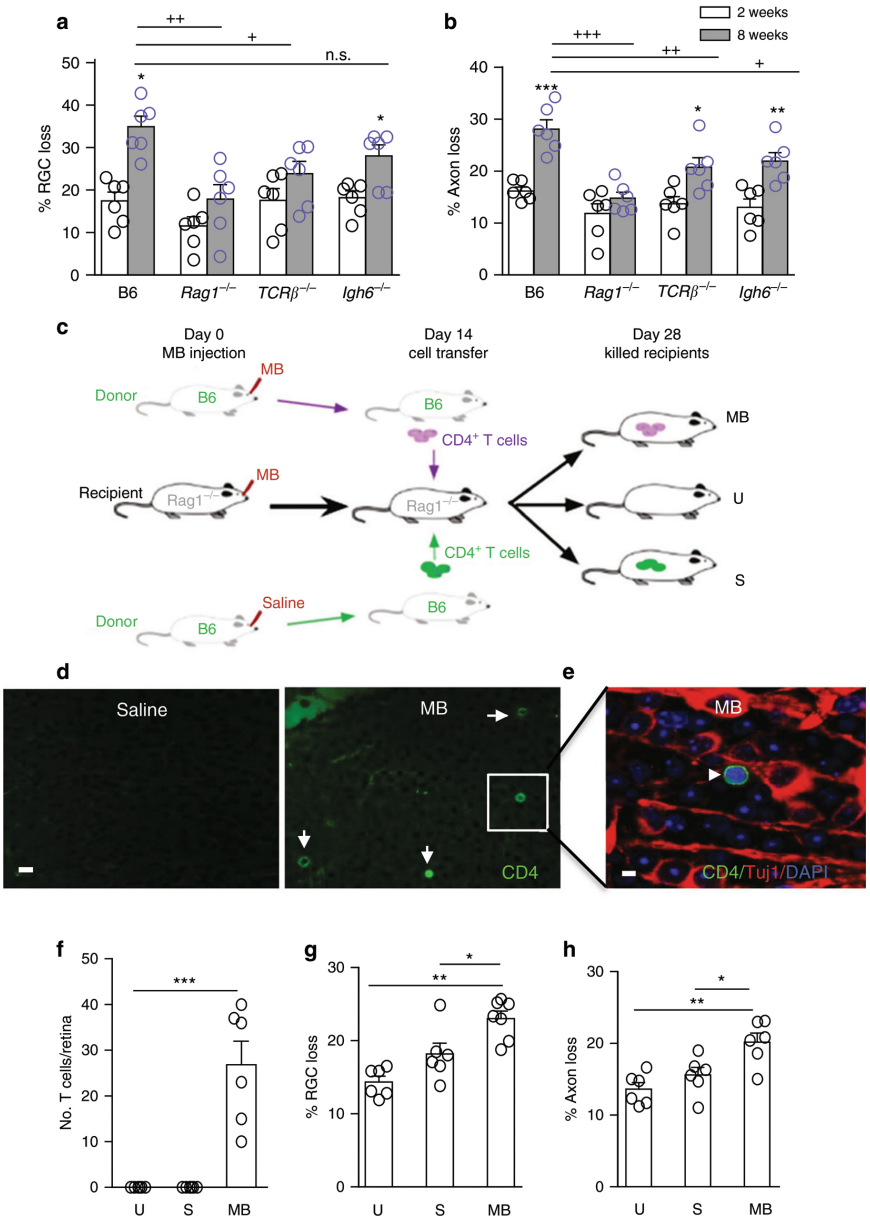


Figure 2. T cells are required for the prolonged retinal neurodegeneration. (**a**,**b**) Quantification of RGC (**a**) and axon (**b**) loss in B6, *Rag1*$^{-/-}$, *TCRα*$^{-/-}$, and *Igh6*$^{-/-}$ mice at 2 (white box) and 8 (grey box) weeks after anterior chamber MB injection. NS, not significant, **P* < 0.05, ***P* < 0.01, ****P* < 0.001 by ANOVA comparing between B6 and indicated mutant mice at 8 weeks post MB injection; ^{+}P<0.05, ^{++}P<0.01, ^{+++}P<0.001 by ANOVA comparing between 2 and 8 weeks post MB injection of mice with the same genotype (n=8/group). (**c**) Scheme of adoptive CD4^{+} T cell transfer. Both donor B6 and recipient *Rag1*$^{-/-}$ (Rag1$^{-/-}$) mice were injected with MB or saline into the anterior chamber. Fourteen days later, CD4^{+} T cells were isolated from the spleens of donor mice and injected via tail vein into Rag1$^{-/-}$ mice that had received MB injection 14 days

Figure 2 (continued) earlier. (**d-h**) Fourteen days post cell transfer, T cell infiltration and RGC and axon loss in *Rag1*$^{-/-}$ mice were analyzed. Retinal flat-mounts were stained with an anti-CD4 antibody (green), Tuj-1 (red) and DAPI (blue). Shown are anti-CD4-stained (**d**) and overlay (**e,** from inset of **d)** images of retinal flat-mounts taken from *Rag1*$^{-/-}$ recipient mice receiving CD4^{+} T cells from saline- or MB-injected B6 mice. Arrows point to CD4^{+} cells. Scale bar: 25 μm (**d**) and 10 μm (**e**). Quantification of infiltrated T cells (**f**), and RGC (**g**) and axon (**h**) loss in glaucomatous *Rag1*$^{-/-}$ recipient mice that received no CD4^{+} T cells (U - uninjected) or donor CD4^{+} T cells from saline-injected (S or normal IOP donor) and MB-injected (MB or glaucomatous donor) B6 mice two weeks post cell transfer. **P*<0.05; ***P*<0.01 by ANOVA (n=6/group).

cell transfer-induced neurodegeneration because injection of T cells from glaucomatous mice into *Rag1*$^{-/-}$ mice with normal IOP did not cause further RGC and axon degeneration after week 2 (Supplementary Fig. 2d,e). Injection of total IgG from the MB-injected B6 mice induced no significant increase in RGC and axon loss in MB-injected *Rag1*$^{-/-}$ mice (Supplementary Fig. 2f,g). These results indicate that conditioned CD4^{+} T cells are sufficient to drive the prolonged phase of retinal neurodegeneration in glaucomatous mice.

IOP Elevation activates T-cell responses to HSPs

To define the autoantigens that stimulate T-cell activation in glaucoma, we searched for proteins that are expressed or upregulated in RGCs following IOP elevation. Since autoantibodies to HSPs, particularly HSP27 and HSP60, had previously been detected in patients and animal models of glaucoma[8,17], we speculated that these HSPs are involved as pathogenic autoantigens, which may be expressed at a low level under the normal condition but are upregulated in RGCs under glaucoma. The levels of HSP27 and HSP60 proteins were low in the retinas of B6 mice with normal IOP, but were upregulated three-to fourfold 1-8 weeks after MB injection (Fig. 3a; Supplementary Fig. 3a-c). HSP27 upregulation was found primarily in the GCL (Supplementary Fig. 3d), both associated with RGC membranes and the outside of RGC bodies (Fig. 3b). This is consistent with the report that HSP27 is also upregulated in astrocytes following elevated IOP and be released extracellularly under stress[18,19]. HSP60 signal was seen to be scattered between the GCL and INL in MB-injected retina (Supplementary Fig. 3d). Significantly higher levels of anti-HSPs, particularly anti-HSP27 autoantibody, were detected in the sera of MB-injected B6 mice than in the serum of normal IOP mice (Fig. 3c).

To test if HSP27 acts as an antigen participating in elevated IOP-induced T cell responses, we assessed Delayed-Type Hypersensitivity (DTH) responses in mice. Significant induction of a HSP27-specific DTH was observed 2 and 8 weeks after MB injection in B6 mice when compared to saline-injected mice (Fig. 3d,e). More CD4^{+} T-cell infiltration was detected in the ear sections of MB-injected than in saline-injected B6 mice (Supplementary Fig. 4a).

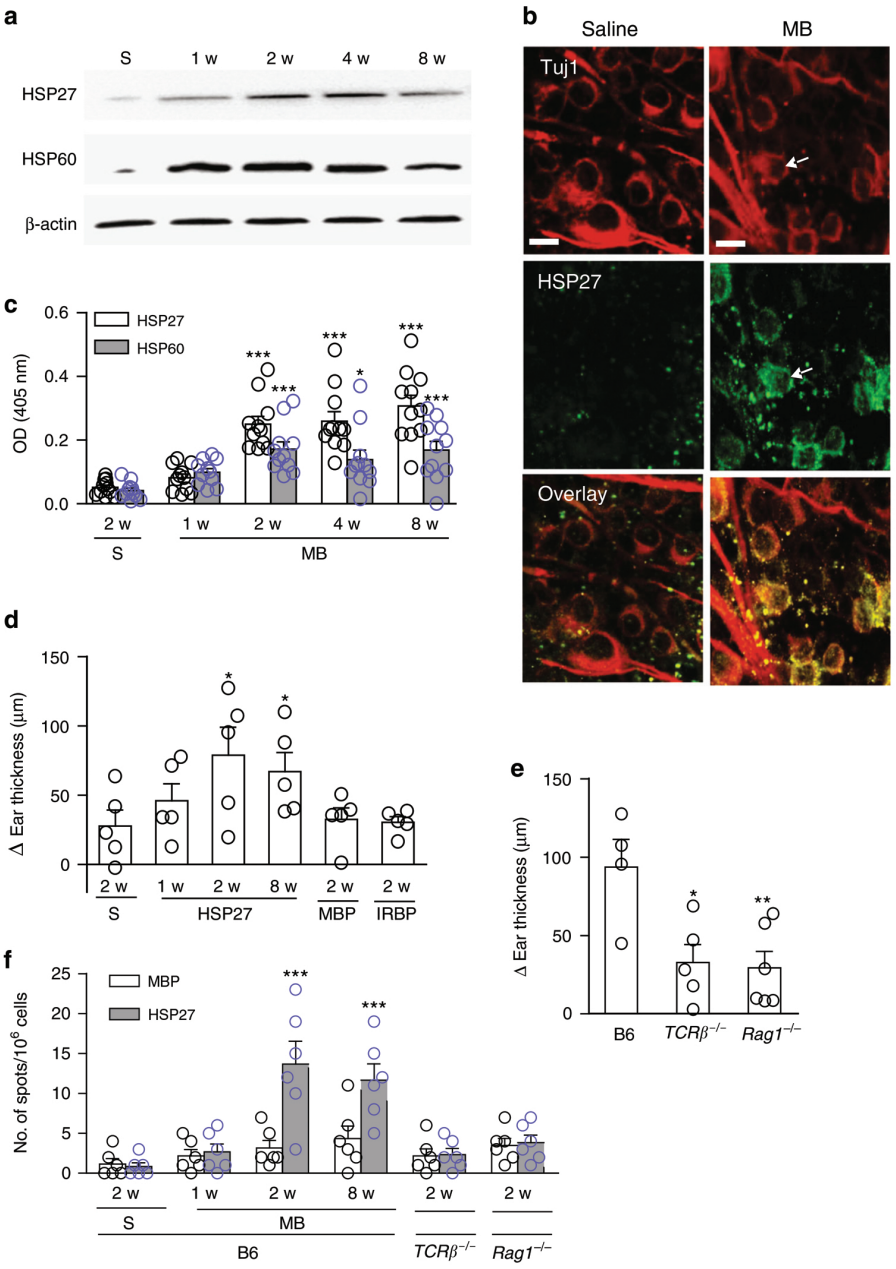


Figure 3. Elevated IOP stimulates T-cell responses to HSPs. (**a**) Induction of HSP27 and HSP60 expression in the retinas following MB injection. B6 mice were injected with MB or saline and 1, 2, 4 and 8 weeks later retinas were harvested and homogenized. Cell homogenates were fractionated on SDS-PAGE and blotted with anti-HSP27, anti-HSP60 and anti-β-actin. Shown are representative Western blots. (**b**) Upregulation of HSP27 in the glaucomatous retina. Retinal flat-mounts were stained with Tuj-1 (red) and anti-HSP27 (green). Shown are representative epifluorescence photomicrographs taken from mice 4 weeks post injection of

Figure 3 (continued) saline or MB. Note the extracellular and membrane associated HSP27 signals in the MB-injected retina. Scale bar: 10µm. (**c**) ELISA quantification of serum levels of antibodies specific for HSP27 or HSP60 in B6 mice 1, 2, 3 and 4 weeks after MB injection or 2 weeks after saline (S) injection. **P*<0.05, ****P*<0.001 by ANOVA comparing to saline injected group (n=10/group). (**d,e**) DTH assays for T cell responses to HSP27. Shown are ear thickness among B6 mice under indicated conditions (**d**) or among B6, *Rag1*$^{-/-}$ and *TCRβ*$^{-/-}$ mice 2 weeks after MB injection were compared (**e**). B6, *Rag1*$^{-/-}$ and *TCRβ*$^{-/-}$ mice were injected with saline or MB in the anterior chamber of the eye. One, 2 and 8 weeks later mice were challenged with saline, HSP27, MBP or IRBP and ear thickness was measured 24 hours later. **P*<0.05 by ANOVA compared to saline (**d**) or to HSP27 challenged B6 mice (**e**) (n≥4/group). (**f**) Frequencies of HSP27-specific T cell responses. B6, *Rag1*$^{-/-}$ and *TCRβ*$^{-/-}$ mice were injected with saline or MB in the anterior chamber of the eye. One, 2 and 8 weeks later splenocytes were stimulated with HSP27 or MBP and the frequencies of IFN-γ-secreting cells were measured by ELISPOT. ****P*<0.001 by ANOVA as compared to saline and MB-injected mice (n≥6/group).

In agreement with the absence of elevated IOP-induced T cell responses in *Rag1*$^{-/-}$ and *TCRα*$^{-/-}$ mice, HSP27-specific DTH was not evoked in these mice (Fig. 3d). The DTH response was specific to HSP27 because challenge with control antigens, such as human myelin basic protein (MBP) or interphotoreceptor retinoid-binding protein (IRBP), did not induce a DTH in MB-injected B6 mice. Induction of HSP27-specific T cell responses by elevated IOP was also verified using an Enzyme-Linked ImmunoSpot (ELISPOT) assay: HSP27 stimulation of splenocytes from mice at 2 and 8 weeks after MB-injection induced a ~10-times higher frequency of IFN-γ secreting T cells than the splenocytes of saline-injected B6 mice; in contrast, the frequencies of IFN-γ secreting T cells in MB-injected *Rag1*$^{-/-}$ and *TCRβ*$^{-/-}$ mice were comparable with those of saline-injected B6 mice (Fig. 3f). Stimulation with control antigen MBP did not increase the frequency of IFN-γ secreting T cells, further corroborating an induction of HSP27-specific T-cell responses. These T-cell responses were induced by elevated IOP, but not by the presence of MBs, as injection of fewer MB, which did not cause IOP elevation did not lead to an increased frequency in IFN-γ secreting cells (Supplementary Fig. 4b). Moreover, a similar increase in the frequencies of IFN-γ secreting T cells was observed in splenocytes taken from 10 month-old DBA/2J mice compared to those from 3 month-old DBA/2J mice or age-matched (10-month old) control B6 mice (Supplementary Fig. 4c). Thus, elevated IOP-induced T cell responses are specific to HSPs.

HSP-specific T cells augment glaucomatous neurodegeneration

To examine if HSP27-specific IFN-γ secreting T cells infiltrate the retina and contribute to glaucomatous pathogenesis, we performed fluorescence-activated cell sorting analysis (FACS) for HSP27-stimulated retinal cell cultures following a gating strategy shown in Supplementary Fig. 5. At 2 weeks post MB injection, ~1,000 $CD4^+$ T cells were noted in a

glaucomatous mouse retina (Fig. 1e). Supporting retinal infiltration of HSP27-specific IFN-γ secreting T cells, HSP27 stimulation induced an approximately threefold higher frequency of IFN-γ$^+$CD4$^+$ T cells from the retinal cultures of B6 mice than from saline-injected mice (Fig. 4a,b; Supplementary Fig. 5); whereas, the other three subtypes of CD4$^+$ T cells (T_H2, T_H17, and Treg) were not detectable. Results of quantitative PCR also detected the induction of IFN-γ expression, but a lack of TGF-β, IL-17, and IL-4 transcripts, in the glaucomatous retinas at 1, 2 and 4 weeks after MB injection (Fig. 4c). To corroborate the above finding, GFP transgenic mice were immunized with HSP27 or ovalbumin (OVA), and CD4$^+$ T cells from immunized mice were adoptively transferred into glaucomatous *Rag1*$^{-/-}$ mice (which had received an MB injection two weeks earlier). Approximately 15-fold more GFP$^+$ T cells were detected in the retinas of recipient *Rag1*$^{-/-}$ mice after T-cell transfer from HSP27- than OVA-immunized donors (Fig. 4d), further demonstrating HSP27-specific T cell infiltration into the glaucomatous retinas.

We then tested if HSP27-specific T cells can induce progressive glaucomatous neural damage. B6 mice were immunized with HSP27 or OVA. Successful immunization was confirmed by DTH and ELISPOT assays (Fig. 4e,f). CD4$^+$ T cells were then isolated from spleens of the immunized or unimmunized mice and adoptively transferred into glaucomatous B6 mice (injected with MB 2 weeks earlier). Recipient B6 mice that received CD4$^+$ T cells from the HSP27-immunized mice exhibited a significantly greater loss of RGCs and axons compared to mice that received T cells from unimmunized or OVA-immunized mice (Fig. 4g). Thus, HSP27-specific CD4$^+$ T cells mediate the prolonged phase of RGC and axon degeneration.

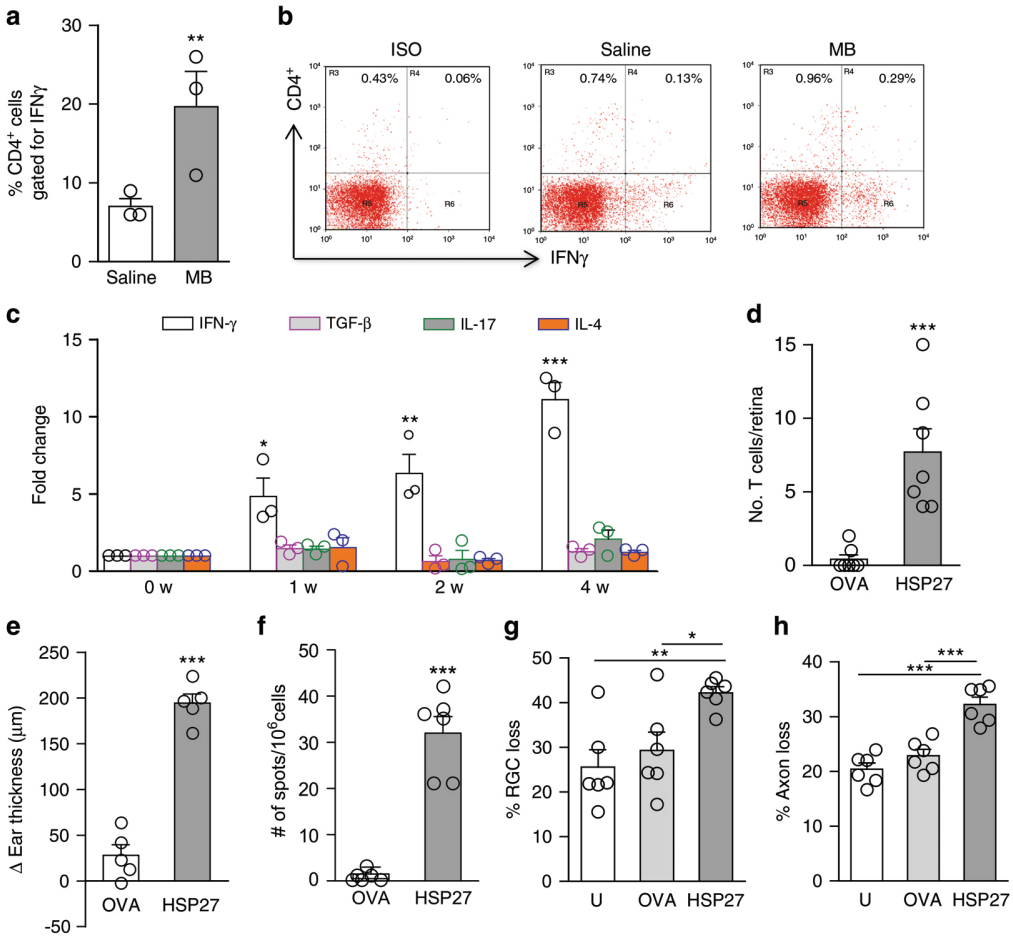


Figure 4. HSP-specific T cells infiltrate the retinas and augment glaucomatous neurodegeneration. (**a,b**) Infiltration of HSP27-specific T cells into the glaucomatous retina. Retinal cells from mice at 2 weeks post saline- or MB-injection were stimulated by HSP27 in culture, stained for CD4 and IFN-γ, and assayed by flow cytometry. Shown are frequencies of INF-γ+ cells in CD4+ gated cells (**a**) and representative isotype control (Iso) and anti-CD4 vs. anti-IFN-γ staining profiles (**b**) from the retina of saline- and MB-injected mice. The numbers indicate percentages of cells in the gated regions. Note the increased number of INF-γ+ cells among CD4+ gated cells in MB-injected retina compared to saline-injected retina. $^{**}P<0.01$ (n=3/group; each group was pooled from 5 mice). **(c)** qPCR quantification of IFN-γ, TGF-β, IL-17, and IL-4 transcripts in the mouse retinas before (0) and at 1, 2 and 4 weeks post MB- or saline-injection. $^{**}P<0.01$, $^{***}P<0.001$ by ANOVA (n=3/group). (**d**) Quantification of CD4+ T cell numbers in retinal flat-mounts of glaucomatous *Rag1*$^{-/-}$ mice that received an injection of GFP+/CD4+ T cells from HSP27- or OVA-immunized mice. $^{***}P<0.001$ (n=6/group). (**e,f**) T cell responses in ovalbumin (OVA)- or HSP27-immunized mice. B6 mice were immunized with HSP27 or OVA, and two weeks later, mice were challenged with HSP27 by intradermal injection in the ear followed by measurement of ear thickness 24 hours later (**e**) or mouse splenocytes were isolated, stimulated with HSP27 and then IFN-γ-secreting cells were detected by ELISPOT (**f**). $^{***}P<0.001$ (n=6/group). (**g, h**) Greater loss of RGCs (**g**) and axons (**h**) in WT recipient mice received adoptive T cell transfer from HSP27 immunized mice. B6 mice were immunized with HSP27 or ovalbumin (OVA) and two weeks later CD4+ T cells were isolated from spleens of immunized mice and unimmunized (U) mice, and adoptively transferred into B6 mice that had been injected with MB 2 weeks earlier. Two weeks after cell transfer, retinal flat-mounts of recipient mice were stained with Tuj-1, and RGCs were counted. $^{**}P<0.01$, $^{***}P<0.001$ by ANOVA (n=6/group).

Absence of glaucomatous neural damage in germ-free mice

A key remaining question here is how T cell responses to HSPs are induced by elevated IOP, especially given that the retina is an immune-privileged site. HSPs are highly conserved from bacteria to mice to humans[20,21]; mice and humans are exposed to commensal microflora after birth and occasionally are infected by pathogenic bacteria. We hypothesized that mice harbor memory T cells to bacterial HSPs that can be activated by host HSPs through molecular mimicry when the blood-retinal barrier is compromised by elevated IOP. If this is the case, we expected that T cells from glaucomatous mice would react to both bacterial and human HSPs. Indeed, following stimulation with either human HSP27 and HSP60 or *Escherichia coli* HSP24 and HSP60, we observed similar increases in the frequencies of IFN-γ secreting cells in splenocyte cultures of MB-injected mice as compared to saline-injected mice (Fig. 5a).

We reasoned that mice raised in the absence of microbial colonization would exhibit attenuated or absent HSP-specific T-cell responses and retinal neurodegeneration following IOP elevation. We therefore examined T-cell responses and glaucomatous neurodegeneration in germ-free (GF) Swiss Webster mice, which were readily available from a commercial source. Anterior chamber injection of MB in GF Swiss-Webster mice induced IOP elevation with similar kinetics and magnitude to conventionally-colonized (specific pathogen-free [SPF]) Swiss-Webster mice (Supplementary Fig. 6a). HSP27 upregulation in the GCL of both SPF and GF mice following IOP elevation was verified by immunolabeling, but no apparent differences between the SPF and GF groups, before or after IOP elevation, were noted (Supplementary Fig. 6b). Elevated IOP-induced retinal T-cell infiltration (Fig. 5b) and HSP27- and HSP60-specific T-cell responses (Fig. 5c,d) were detected in MB injected SPF but not GF mice. Correspondingly, glaucomatous RGC and axon damage was observed in SPF but not in GF mice, when examined either 4 or 8 weeks following MB injection (Fig. 5e,f). We then examined "altered Schaedler flora" (ASF) Swiss-Webster mice, which were colonized with eight defined bacterial species[22]. We noted significant RGC and axon loss following MB-injection in ASF mice, although the magnitude of loss was significantly attenuated compared to MB-injected SPF mice (Fig. 5e,f). These results suggest that induction of a full spectrum of HSP-specific T-cell responses and retinal neurodegeneration requires pre-exposure to diverse microbial flora, rather than a specific bacterial species.

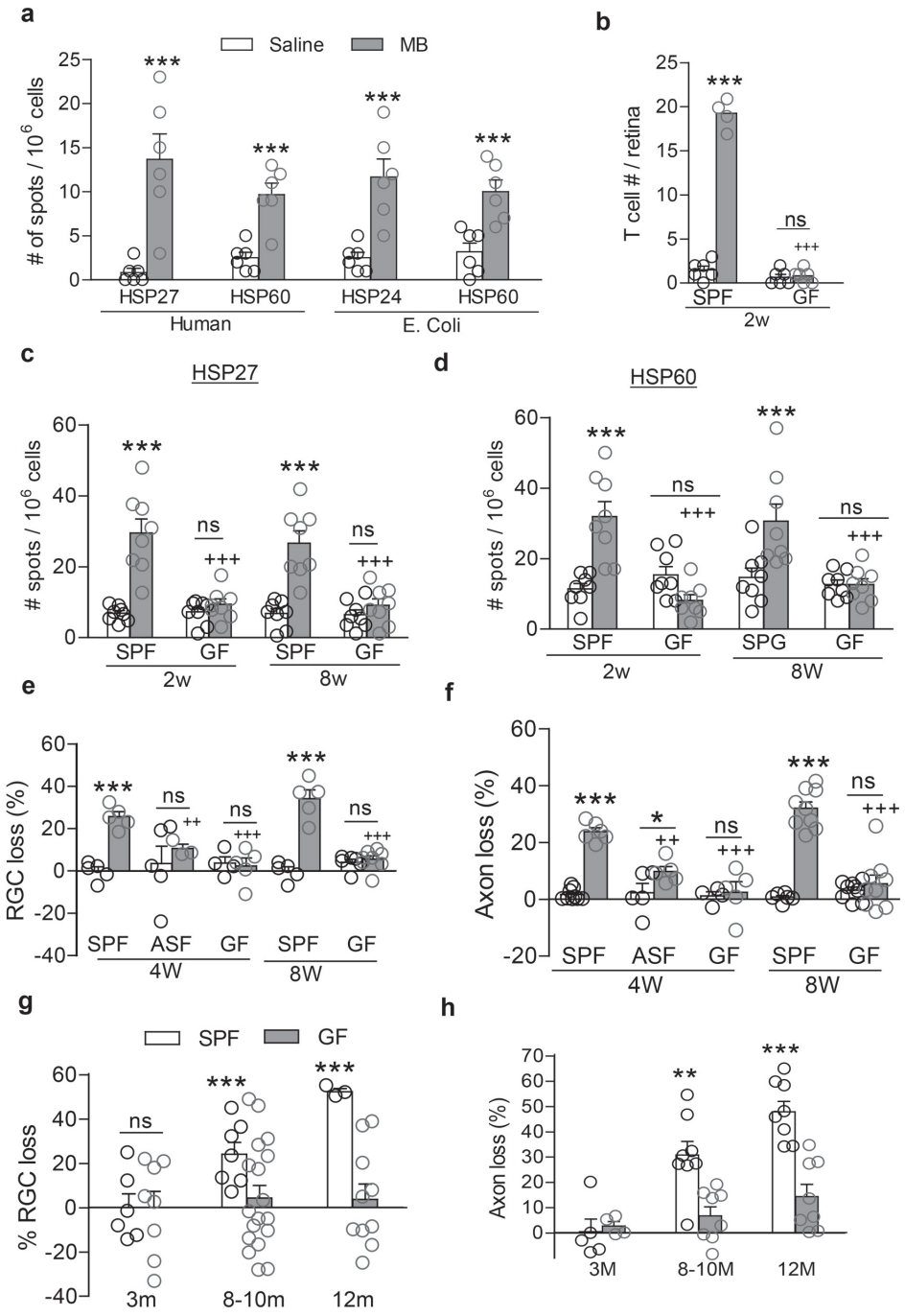


Figure 5. HSP-specific T-cell responses and retinal damage are absent in germ-free mice (**a**) Frequencies of T cell responses to human and bacterial HSPs. Splenocytes from mice at 2 weeks post saline- or MB-injection were stimulated with human HSP27 and HSP60 or *E. Coli* HSP24 and HSP60, and the frequencies

of IFN-γ-secreting cells were assayed by ELISPOT (n=6/group). (**b**) Quantification of infiltrated T cells in the retinas of SPF and GF SW mice at 2 weeks after saline- or MB-injection (n = 6/group). (**c,d**) Frequencies of HSP27- (**c**) and HSP60- (**d**) specific T cells in GF and SPF Swiss-Webster mice (n≥8/group). Splenocytes from GF and SPF Swiss-Webster mice at 2 and 8 weeks post MB- or saline-injection were stimulated with human HSP27 or HSP60, and the frequencies of IFN-γ-secreting cells were assayed by ELISPOT. *NS*: not significant. (**e,f**) Comparison of RGC (**e**) and axon (**f**) loss at 4 and 8 weeks after MB injection in SPF, ASF and GF Swiss-Webster mice. *P<0.05; ***P<0.001 over saline-injected group; ++P<0.01; +++P<0.001 over MB-injected SPF mice at the indicated time points. *NS*: non-significant (n=8/group). (**g,h**) Comparison of RGC (**g**) and axon (**h**) loss in SPF, and GF DBA/2J mice at 3 (n = 16), 8-10 (n=24) and 12 (n=14) months of age. NS: not significant; ***P*<0.01, ****P*<0.001 by ANOVA.

To verify the above finding using the inherited mouse model of glaucoma, we derived/created GF DBA/2J mice. DBA/2J mice housed under SPF or GF conditions both naturally developed high IOP with a peak of ~22 mmHg by 8 to 10 months of age (Supplementary Fig. 6c), consistent with previous reports[23]. The SPF DBA/2J mice underwent the expected progressive RGC and axon loss: ~25% by 8-10 months and ~50% by 12 months of age, whereas no neural loss was detected in GF DBA/2J mice as late as 12 months of age (Supplementary Fig. 6d; Fig. 5g,h). In agreement with the above finding, both RGC and axon counts remained stable from 3 to 12 months of age, showing the absence of neurodegeneration in GF DBA/2J mice. Collectively, these results indicate a need for prior exposure to commensal microbial flora in the induction of both HSP-specific T cell responses as well as RGC and axon loss following IOP elevation.

HSP-specific T cells are increased in patients with glaucoma

To investigate the possible involvement of HSP-specific T-cell responses in human glaucoma, we compared the frequencies of HSP27- and HSP60-specific T cells and the levels of autoantibodies between normal subjects and patients with primary open angle glaucoma (POAG) or normal tension glaucoma (NTG). The frequencies of HSP27- and HSP60-responsive T cells were over fivefold higher in both POAG and NTG patients than in age-matched healthy individuals (Fig. 6a,b). Patients with retinal detachment or traumatic skin injuries did not have any significant increase in HSP27- or HSP60-reactive T cells. In addition, the titers of HSP27- and HSP60-specific IgGs were approximately twofold higher in both POAG and NTG patients than in healthy individuals (Fig. 6c,d), which was consistent with a previous report[24]. Thus, the levels of HSP27- and HSP60-reactive T cells and antibodies are also elevated in patients with POAG and NTG.

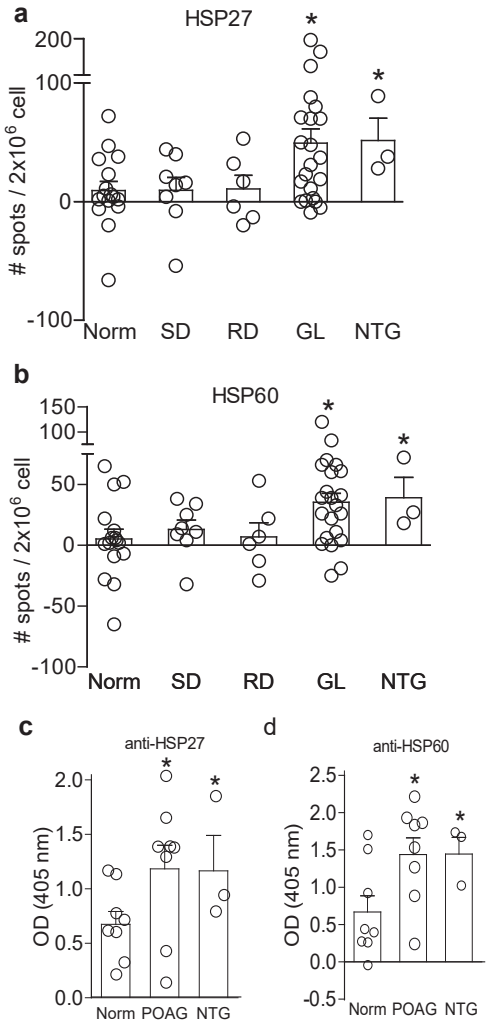


Figure 6. T cells are involved in retinal neurodegeneration in human glaucoma patients. (**a**,**b**) Comparison of frequencies of HSP27- and HSP60-specific T cells among healthy individuals and patients with POAG or other diseases. Peripheral blood mononuclear cells from patients with POAG (n=18), NTG (n=3), retinal detachment (RD; n=6), skin injuries (SD; n=8), and age-matched healthy controls (Norm; n=16) were stimulated with HSP27 (**a**) or HSP60 (**b**). Two days later, the frequencies of IFN-γ-secreting cells were determined by ELISPOT. **P*<0.05 by ANOVA. (**c**,**d**) Comparison of serum levels of HSP27- and HSP60-specific IgGs between healthy individuals and patients with POAG or NTG. Sera from POAG (n=18), NTG (n=3), and age-matched healthy individuals (Norm, n=16) were assayed for HSP27- and HSP60-specific IgG by ELISA. **P*<0.05 by ANOVA.

DISCUSSION

This is the first report that, to our knowledge, describes an unexpected link and the sequential roles of elevated IOP, intact commensal microflora, and activation of T-cell responses in the pathogenesis of glaucoma. Our comprehensive studies employed both inducible and genetic mouse models of glaucoma, immunodeficient mice, GF mice, clinical samples, and adoptive T-cell transfer, and assessed T-cell infiltration and retinal neurodegeneration. We showed that 1) a transient elevation of IOP by MB injection is sufficient to induce T-cell infiltration into the retina and a prolonged phase of retinal neurodegeneration, 2) T cells are required for mediating the prolonged retinal neurodegeneration and are specific for HSPs, and 3) induction of HSP-specific T cells and glaucomatous retinal neurodegeneration by elevated IOP require exposure to commensal microflora. Together, these findings are compelling as they suggest the essential involvement of a T cell-mediated mechanism in the pathogenesis of glaucoma. A similar mechanism likely operates during the development of glaucoma in humans as HSP-specific T cells are more than fivefold higher in POAG and NTG patients than age-matched healthy individuals.

Our study is the first demonstration of direct induction of HSP-specific T-cell responses by an elevation of IOP. HSP-specific T-cell responses have been proposed to contribute to NTG as HSP immunization elicits glaucomatous RGC loss in rats[7]. Here we show that elevated IOP induces two phases of retinal damage: the acute phase correlates with the IOP elevation, likely mediated by physical stress; the prolonged retinal degeneration, which continues even after IOP has returned to normal, is mediated by T cells. Supporting this notion, mice deficient in T cells, but not B cells, displayed a dramatically attenuated RGC and axon damage. Adoptive transfer of $CD4^+$ T cells, but not total IgG antibodies, from diseased mice restored the progressive retinal neurodegeneration in *Rag1*$^{-/-}$ recipients. Adoptive transfer of $CD4^+$ T cells from HSP27- but not OVA-immunized mice augmented the progressive retinal neurodegeneration in MB-injected B6 recipients. Similarly, adoptive transfer of $CD4^+$ T cells from HSP27-immunized mice into MB-injected *Rag1*$^{-/-}$ recipient mice induced 16 ± 2.1% (n = 5/group) more RGC loss 2 weeks after cell transfer as compared to those receiving T cells from OVA-immunized mice. The kinetics of T cell infiltration into the retinas and their specificity for HSPs lends further support for their involvement in the progressive retinal degeneration. Furthermore, accumulating evidence indicates an essential role for commensal microflora in T cell activation. Lack of HSP-specific T-cell responses and neural

damage in GF mice offers the first and compelling evidence supporting that mere elevation of IOP does not directly attribute to progressive neurodegeneration. It is the subsequent event, involving T-cell responses, which have been pre-sensitized by commensal microflora, that mediates progressive glaucomatous neurodegeneration. Finally, we show that HSP-specific T-cell responses occur not only in glaucomatous mice but also in patients with glaucoma. The induction of HSP-specific T cells is not a result of general stress responses as patients with retinal detachment or traumatic skin diseases did not induce HSP-specific T-cell responses. These findings show that IOP elevation triggers secondary anti-HSP $CD4^+$ T cell responses that mediate a prolonged retinal neurodegeneration, providing an explanation for continued neurodegeneration in patients with normal or perfectly controlled IOP.

Previous studies have speculated a connection between glaucoma and bacterial infections, such as by *Helicobacter pylori*[25]. The notion that a process of microbiota-dependent activation of T cells may precede the clinical onset of a disease in the eye is supported by a recent study in a uveitis animal model[26]. Using transgenic mice expressing a T cell receptor (TCR) specific to IRBP in the retina, the authors showed that commensal microbiota is required for the activation of transgenic T cells to cause uveitis. Although both our and the previous studies demonstrated the requirement of commensal microflora and autoreactive T cells in inflammatory diseases in the eye, the two studies differ in several aspects. The previous study examined how autoreactive transgenic T cells were activated to cause uveitis – a model of experimental encephalomyelitis; whereas, our study examined how endogenous T cells could be activated to perpetuate glaucomatous retinal neurodegeneration. Furthermore, our study identified both bacterial and host HSPs as likely key natural antigens, and showed that commensal microflora induces HSP-specific memory T cells, which are then activated by host HSPs induced in the retina after IOP elevation. Our study also showed that elevated IOP-induced degeneration of RGCs and axons was partially attenuated in ASF mice with eight defined bacteria, suggesting that induction of $CD4^+$ T-cell responses by HSPs requires a diverse flora rather than a specific bacterium. Thus, our study shows how physical stress, HSPs, microbial flora, and T cells interact in the pathogenesis of glaucomatous neurodegeneration.

Induction of HSP expression has been documented to associate with various pathological conditions in the retina and brain[27,28], and similar mechanisms may underly other causes

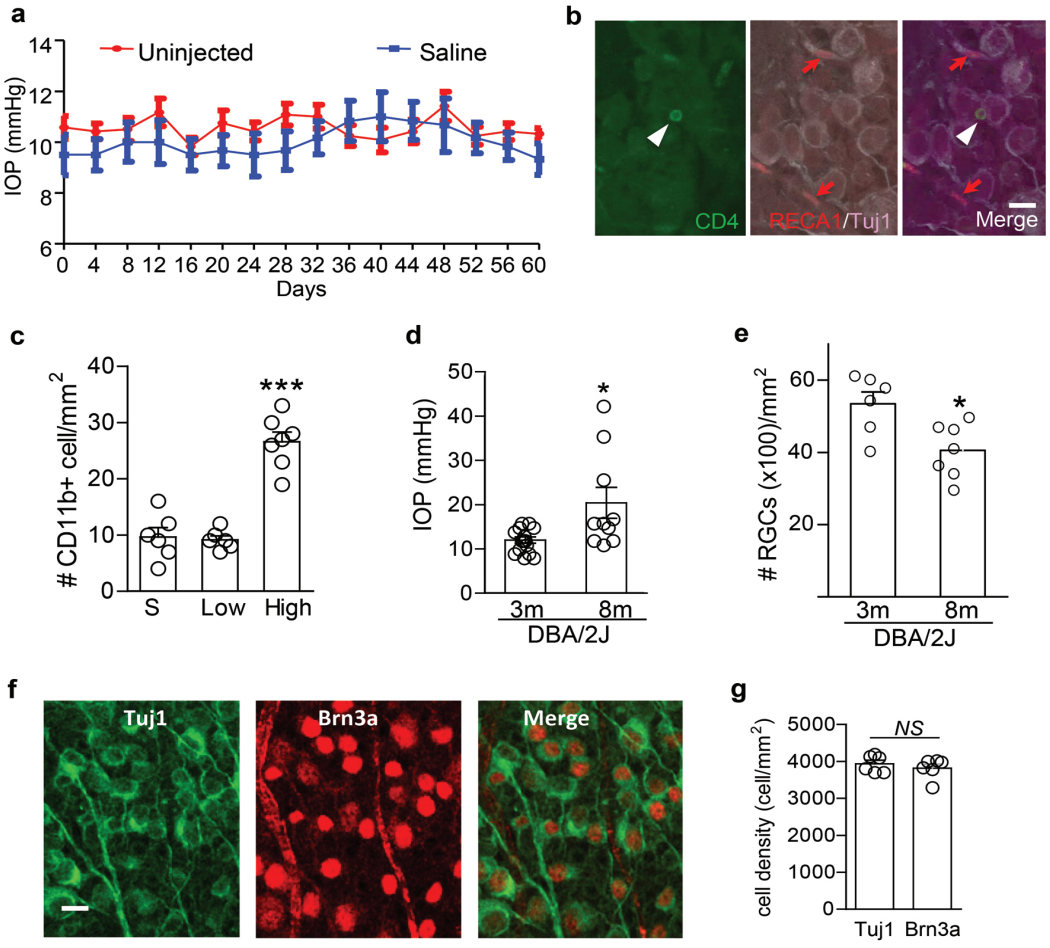
of neurodegeneration. Intracellular expression of HSPs is thought to protect neurons and axons from stress-induced damage[29,30] and promotes nerve regeneration[31]. However, it has been reported that membrane-bound and extracellular HSPs, which are likely to be "seen" by antigen-presenting cells and T cells, elicit immune responses of the adaptive or innate immune system[32]. Our results show that elevation of IOP upregulates membrane-bound and extracellular HSPs in the GCL, subsequently leading to immune-mediated neural damage through activating HSP-specific $CD4^+$ T cells, which are originally induced by microbial HSPs. As microbes and humans share significant homologies in many other proteins, it is likely that HSPs are not the only cross-reactive antigens involved. The same mechanism of molecular mimicry may activate T cells which are originally induced by other microbial proteins. Consequently, this may lead to induction of autoimmune responses under stress conditions. T-cell sensitization and progressive neurodegeneration were also noted in other conditions involving HSP upregulation, including retinal ischemia and optic nerve trauma (unpublished data). Identification of $CD4^+$ T cell responses in glaucoma opens the possibility of targeting T cells in the retina as a treatment to halt the progressive RGC and axon degeneration and vision loss.

Induction of retinal damage by adoptive transfer of $CD4^+$ T cells is associated with local injury/inflammation, such as that induced by elevated IOP. We noted that neurodegeneration was observed following T-cell transfer only into high IOP mice, but not those with a normal IOP, suggesting that local inflammation is required for T-cell infiltration into the retina. This offers an explanation for why glaucomatous neural damage is limited to the retina and optic nerve as elevated IOP selectively causes inflammation in these areas. This notion is supported by the observation that peripheral administration of lipopolysaccharide (LPS) exacerbated neuron loss in glaucoma[33]. Another report, however, showed that neurodegeneration could be induced in mice even with a normal IOP 8 weeks or longer after adoptive transfer of T cells isolated from genetic mouse models of glaucoma[34]. It suggests that activated T cells of glaucomatous mice are also capable of entering the retina with an intact blood-retinal barrier, although probably at a much slower rate or under certain conditions.

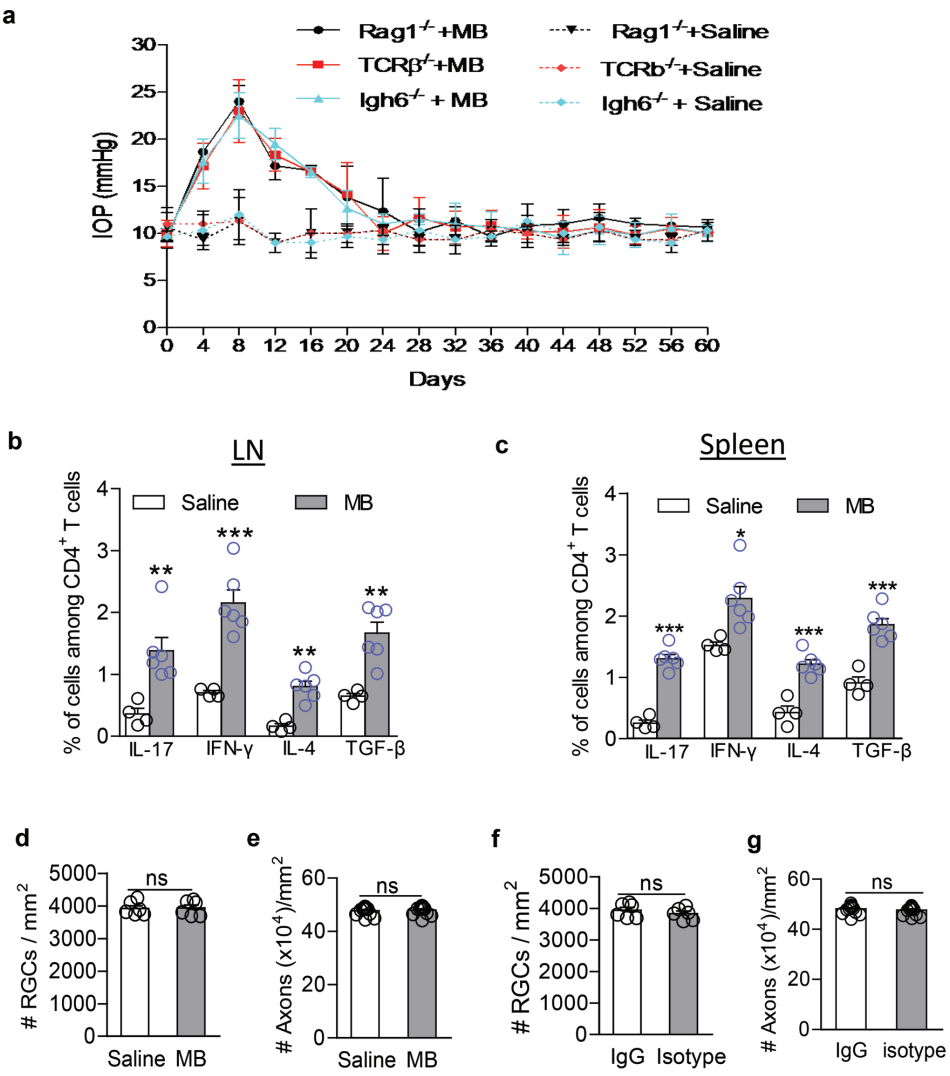
Presently, it is unclear which subsets of T cells serve as effector cells or act as initiators of glaucomatous neurodegeneration. T_H cells are characterized by different cytokine profiles, which are key contributors to immune diseases and commonly used to define the

functional subsets of T-cells[35]. Thus, in the pesent study, we investigated cytokine secretion profiles of $CD4^+$ T cells, including IFN-γ (T_{H1}), IL-4 (T_{H2}), IL-17 (T_{H17}) and TGF-β (T cell subsets with regulatory functions). We found that T cells from glaucomatous retina secreted IFN-γ, suggesting they are T_{H1} cells, which are known to mediate inflammation and DTH reactions. T_{H2} cells predominantly secrete IL-4 and stimulate the proliferation of B cells and production of antibodies; T_{H17} cells produce proinflammatory cytokine IL-17 and play a major role in autoimmunity; Treg and those with a regulatory function, in contrast, are important for the control of immune responses to self-antigens, preventing autoimmunity and maintaining self-tolerance. While our data suggest that T_{H1} cells are the predominant subset infiltrating the glaucomatous retina, it is increasingly recognized that "one pathogenic T_H cell" and "one cytokine" does not fit the bill of autoimmune pathology anymore. Involvement of multiple different effector T-cell subsets in experimental autoimmune encephalomyelitis, via overlapping or distinct mechanisms, has been documented. Similar mechanisms can also be involved in glaucomatous neurodegeneration considering that IOP elevation induced activation of all four subsets of $CD4^+$ T cells in the spleen and draining LN. Autoimmune responses in the retina usually start with the activation of microglia, which can function as antigen-presenting cells to capture and present antigens[36,37], such as HSPs. This in turn triggers HSP-specific T-cell responses in the draining LN and retinal production of inflammatory cytokines, such as TNF-α and IL-1β, that can continue after the IOP has returned to a normal range. These cytokines are known to weaken the blood-retinal barrier, allowing or facilitating T cell infiltration into the retina[38,39]. Disruption of the blood-retina barrier has been reported in patients with POAG and in DBA/2J mice, correlating with the IOP elevation[40]. While we did not detect apparent vessel leakage in microbead-induced glaucomatous mice using fluorescein angiography, we did observe T-cell infiltration in the retina of MB-injected eyes. Activated CD4+ T cells can cause neuronal damage directly as seen in multiple sclerosis or by activating other immune cell types, such as microglia, through secreted cytokines such as IFN-γ. Localized cytokine induction and microglia activation have likely limited the neural damage to the eye rather then spreading the damage to other sites of the central nervous system. Future examination of cytokine production and T-cell behavior in the retina of GF mice with elevated IOP will further help elucidate the cellular and molecular pathways underlying T cell-mediated neurodegeneration to develop therapies. Although these questions remain to be addressed, our current study provides compelling evidence for commensal microbial flora-induced HSP-specific $CD4^+$ T cells in the pathogenesis of chronic neurodegeneration in the eye.

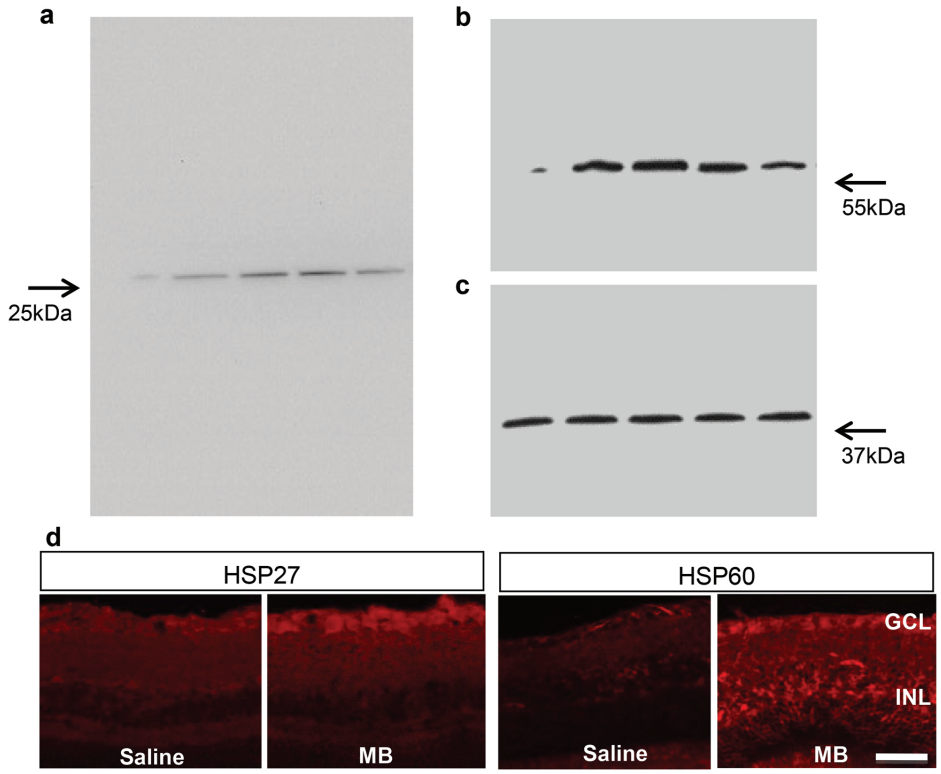
SUPPLEMENTARY INFORMATION



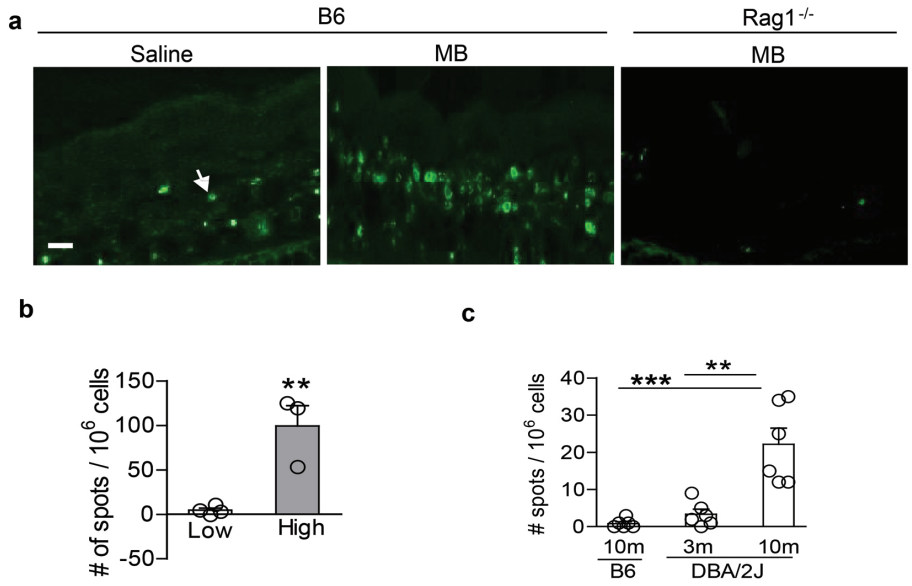
Supplementary Fig. 1. Comparison of IOP kinetics and quantification of CD11b⁺ cells and infiltrated T cells in saline- and MB-injected mice. (**a**) IOP levels in saline-injected (blue) or un-injected (red) mouse eyes. (**b**) Representative images taken from a retinal of a MB-injected eye, which was triple-stained with antibodies for CD4 (green), RECA1 (red) and Tuj1 (purple). Arrowhead points to a CD4⁺ T cell; arrows point to RECA1⁺ blood vessel cells. Scale bar: 10μm. (**c**) Quantification of CD11b⁺ cells in the retinas of mice 2 weeks after saline, low- (Low; 2.0×10^6 beads/eye) or high- (High; 5.0×10^6 beads/eye) dose injection of MB. ***P<0.001 as compared to saline injected group (n=6/group). (**d,e**) Quantification of IOP levels (**d**) and RGC densities (**e**) in 3 (n=6) and 8 (n=7) month old DBA/2J mice. *P<0.05 by ANOVA. (**f,g**) Representative images of retinal flat-mount double-immunolabeled with Tuj1 (green) and anti-Brn3a (red) (**f**) and quantification of Tuj1⁺ and Brn3a⁺ cell density in B6 mice (**g**). Scale bar: 10μm. NS: P>0.05 by ANOVA (n=6/group).



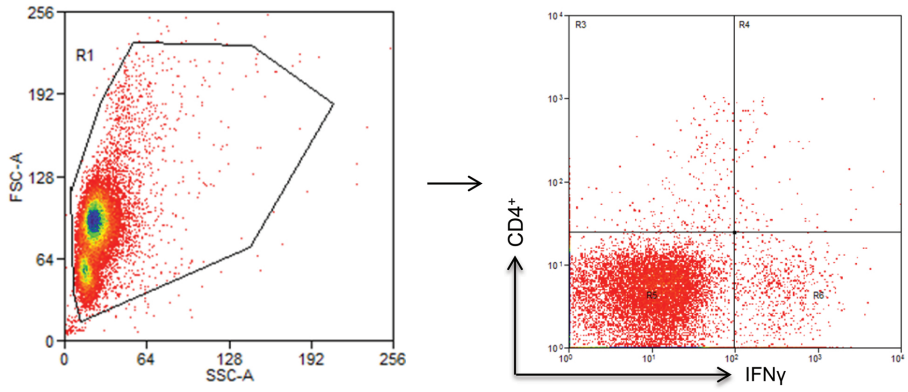
Supplementary Fig. 2. IOP kinetics in genetic mouse lines, systemic T-cell profiling, and RGC and axon counts in retinas of control *Rag1*$^{-/-}$ mice. (**a**) IOP levels over time in *Rag1*$^{-/-}$, *TCRβ*$^{-/-}$ and *Igh6*$^{-/-}$ mice following anterior chamber injection of saline or MB (n≥6/group). (**b,c**) Flow cytometry analysis of systemic CD4^{+} T cell profile in the cervical LN (**b**) and spleen (**c**) of saline- (white bars) and MB-injected (black bars) mice. Mice were sacrificed 2 weeks post injection (n≥4/group). Note the significant increase in frequencies of all 4 subtypes of CD4^{+} T cells expressing IFN-γ, IL-17, IL-4, or TGF-β, in MB injected mice when were compared with control mice. **P*<0.05, ***P*<0.01, ****P*<0.001 by ANOVA. (**d,e**) Quantification of RGC (**d**) and axon (**e**) density in the retinas of *Rag1*$^{-/-}$ mice 2 weeks following adoptive transfer of CD4^{+} T cells isolated from saline- (Saline) or MB- (MB) injected B6 mice. *P*>0.05 by ANOVA (n=6/group). (**f,g**) Quantification of RGC (**f**) and axon (**g**) density in *Rag1*$^{-/-}$ mice that received intravitreal injection of isotype (Isotype) or total IgGs isolated from MB-injected B6 mice (IgG). *P*>0.05 by ANOVA (n=6/group).



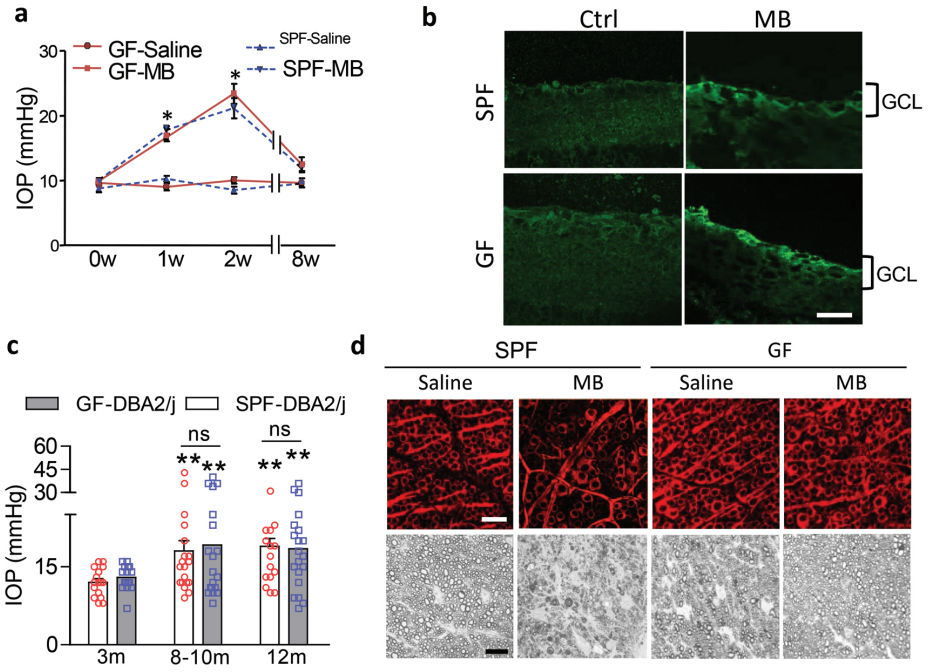
Supplementary Fig. 3. Western blot analysis and immunofluorescence detection of HSP induction following elevated IOP. (**a**-**c**) Representative images of Western blots detecting HSP27 and HSP60 expression in the retinas of saline and MB injected mice (original uncropped scans of Fig. 3a). The position of the molecular size marker is indicated. (**d**) Photomocrographs of HSP27 and HSP60 immunolabeled retinal sections taken from saline- or MB-injected mice. Note the GCL localization of HSP27 and the wider distribution of HSP60 labeling in MB-injected retinas. Bar: 40 µm.



Supplementary Fig. 4. Elevated IOP-induced T-cell responses to HSPs. (**a**) Induction of HSP27-specific T cell responses in MB-injected B6 mice, but not in *Rag1*$^{-/-}$ mice. B6 and *Rag1*$^{-/-}$ mice were injected with saline or MB. Two weeks later, mice were challenged with Hsp27 in the ears and sacrificed 24 hours later. Ear sections were immunolabeled with anti-CD4 (green) and representative epifluorescence photomicrographs are shown. (**b**) Comparison of frequencies of HSP27-specific T cells in mice received a low- or high-dose MB injection: Adult B6 mice were injected with a low- (Low; 2.0x10^6) or high- (High; 5.0×10^6) dose of MB in the anterior chamber of the eye. Two weeks later splenocytes were isolated and stimulated with HSP27 in culture; IFN-γ-secreting cells were detected by ELISPOT after 3 days of incubation. ***P*<0.01 by ANOVA (n≥3/group). (**c**) Frequencies of HSP27-specific T cell responses in DBA/2J mice. Splenocytes were harvested from naïve B6 (n=8) and 3 months (n=8) and 8 months old (n=6) DBA/2J mice and assayed for frequencies of IFN-g-secreting cells by ELISPOT following Hsp27 stimulation. Bar: 10μm. ****P*<0.001 by ANOVA.



Supplementary Fig. 5. Gating strategy for CD4⁺ T-cell quantification. Representative flow cytometry dot plots and the employed gating strategy for the quantification of CD4⁺/IFNγ⁺ T cells in HSP27-stimulated retinal cells after culturing.



Supplementary Fig. 6. IOP kinetics, T-cell infiltration, and RGC degeneration in SPF and GF mice. (**a**) IOP levels in SFP and GF Swiss-Webster mice before (0w) and 1, 2, and 8 weeks after anterior chamber injection of MB or saline. **P*<0.001 as compared to saline injected eyes (n≥10/group). (**b**) Up-regulation of HSP27 in the retinas of SPF and GF SW mice at 1 week after MB injection. Retinal sections were stained with anti-HSP27 (green) (n=5/group). Scale bar: 15 µm. (**c**) IOP levels in SPF and GF DBA/2J mice at 3, 8-10, and 12 months of age. ***P*<0.01 as compared to 3 months old mice by ANOVA (n≥10/group). (**d**) Retinal flat-mounts immunolabeled with Tuj-1 (red) and electron microscopy of optic nerve cross sections from SPF and GF Swiss-Webster mice at 8 weeks after saline or MB injection. Bar: 50µm .

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CD4+ T CELL RESPONSES MEDIATE PROGRESSIVE NEURODEGENERATION IN EXPERIMENTAL ISCHEMIC RETINOPATHY

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ABSTRACT

Retinal ischemic events as a result of occlusion of the ocular vasculature share similar etiologies of central nervous system (CNS) stroke and are among the most common cause of acute and irreversible vision loss in elderly patients. Currently, there is no established treatment, and the condition often leaves patients with seriously impaired vision or blindness. The immune system, particularly T cell-mediated responses, is known to be intricately involved, but its exact roles remain elusive. Here we showed that acute ischemia/reperfusion injury to the retina induced a prolonged phase of retinal ganglion cell (RGC) loss that continued to progress over 8 weeks post procedure. This was accompanied by microglial activation and T cell infiltration into the retina. Adoptive transfer of T cells isolated from diseased mice exacerbated RGC loss in mice with retinal reperfusion damage. Whereas, T cell deficiency or administration of T cell or interferon-γ neutralizing antibody attenuated RGC degeneration and retinal function loss after injury. These findings demonstrate a crucial role for T cell-mediated responses in the pathogenesis of neural ischemia. They point to novel therapeutic strategies of limiting or preventing neuron and function loss for currently untreatable conditions of optic neuropathy and/or CNS ischemic stroke.

INTRODUCTION

Ischemia, broadly defined as the loss of blood supply to tissues, leads to energy depletion and cell death. It is one of the key contributing factors to the pathophysiology of a variety of brain and retinal diseases, such as stroke[1], acute coronary syndrome[2], diabetic retinopathy, and central retinal artery occlusion[3, 4]. Acute retinal ischemia/reperfusion injury, which results in permanent loss of retinal ganglion cells (RGCs), is a common cause of severe impairment of vision and blindness in middle-aged and elderly patients.[5] Recent guidelines of the American Heart Association and American Stroke Association, as well as American Academy of Ophthalmology, have recognized acute retinal ischemia as a stroke equivalent and recommend urgent etiologic work-up, including brain imaging.[6, 7] However, no effective treatment is currently available for acute retinal ischemia, and the optimal management remains unknown because the underlying causes of neuron loss have not been fully understood.

Recent investigations reveal that the eye, while has been known for a long time as an immune-privileged site[8, 9], elicits immunological responses under pathophysiological stress. It was reported that ischemia-reperfusion injury results in sequestration of immune cells, including T cells and macrophages[10], and inflammatory mediators to the ischemic region, which in turn induces local inflammatory responses.[4, 11, 12] A previous study showed that $CD4^+$ T helper cells participate in ischemic neurodegeneration and that severe combined immune-deficient (SCID) mice lacking T and B lymphocytes developed less RGC death after retinal ischemic injury than wild-type (WT) mice.[13] In line with these observations, we showed recently that pathological stress such as that induced by elevated intraocular pressure (IOP) in glaucoma is sufficient to trigger $CD4^+$ T cell infiltration into the retina.[14] Heat shock proteins (HSP) were identified as pathogenic antigens of these T cells. Importantly, these T cells attacked RGCs by recognizing the surface HSPs that were induced following IOP elevation and contributed critically to the development of a prolonged phase of RGC and axon loss in glaucoma.[14] These findings suggest a critical involvement of adaptive immune responses in perpetuating neural damage following neural stress or injury.[15, 16]

As ischemic insult is reported to recruit T cells into the retina and upregulate HSPs in RGCs, we hypothesized that $CD4^+$ T cell-mediated responses also play an important role

in perpetuating retinal neurodegeneration in ischemic/reperfusion injury. In the present study, we sought to test this hypothesis by employing T cell deficient mice and adoptive T cell transfer and assessing T cell responses. Our study provided compelling evidence indicating that an acute ischemic event in the retina induced IFN-γ-secreting $CD4^+$ T helper cell infiltration and a prolonged phase of neurodegeneration over 8 weeks while administration of T cell blocking antibodies attenuated RGC and retinal function loss in an experimental model of retinal reperfusion injury. Our results suggest the existence of a therapeutic window and novel strategies for saving vision in retinal ischemia. Likely, a similar mechanism may be involved in ischemic stroke of the CNS.

METHODS

Mice

C57BL/6J wild-type (B6) mice and mice deficient for Rag1 ($Rag1^{-/-}$) or T cell receptor ($TCR^{-/-}$) between 12-16 weeks old were purchased from Jackson Laboratories, Bar Harbor, Maine. Animals were housed under a 12 h light/dark cycle and kept under pathogen-free conditions. All experimental procedures and the use of animals were approved and monitored by the Animal Care Committee of the Schepens Eye Research Institute/Massachusetts Eye and Ear, and performed according to the standards of the National Institute of Health and the Association for Research in Vision and Ophthalmology.

Acute retinal reperfusion injury

Retinal ischemia was induced in B6, $Rag1^{-/-}$, and $TCR^{-/-}$ mice as previously described.[17, 18] Mice were anesthetized with a mixture of 120 mg/kg Ketamine and 20 mg/kg Xylazine in sterile saline (1:1:6). Retinal ischemia was induced unilaterally in the right eye, while the contralateral eye served as a non-ischemic control. The pupil was dilated with 1% tropicamide (Bausch & Lomb Inc., Tampa, FL), and 0.5% Proparacaine Hydrochloride (Bausch & Lomb Inc., Tampa, FL) was applied topically onto the cornea. The cornea was gently punctured near the center using a 30.5-gauge needle to generate an easy entry for a glass micropipette, which was connected by polyethylene tubing and an intravenous tube set (Abbott Laboratories, North Chicago, IL) to a sterile physiological (0.9% sodium chloride) bag (Hospira, Inc., Lake Forest, IL). By elevating the saline bag up to 120 cm above the eye level, the intraocular pressure (IOP) was raised acutely to 90 mmHg. Whitening of the

fundus was observed to ensure the induction of retinal ischemia, followed by observation of corneal edema. After 60 minutes of a highly-elevated IOP, the saline bag was slowly lowered to the eye level, and the needle was withdrawn from the anterior chamber. Reappearance of vessels in the fundus was confirmed as a sign of reperfusion of the retina. In sham-operated mice, the right cornea was punctured near the center to generate an entry for the glass micropipette, but the saline bag was not raised above eye level so that no IOP elevation was generated in these mice. Mice were sacrificed at day 3, week 1, 2, 4, or 8 after injury.

Adoptive transfer of CD4⁺ T cells

Mouse spleens were dissected and mechanically homogenized, and cells were suspended in RPMI media (Sigma-Aldrich, St. Louis, MO) containing 10% fetal bovine serum (FBS), 1% penicillin/streptomycin, and 1% L-glutamine. Red blood cells (RBC) were lysed with RBC lysis buffer (Sigma-Aldrich, St. Louis, MO). $CD4^+$ T cells were purified using an automated MACS Separator and a $CD4^+$ T cell Isolation Kit (Miltenyi Biotec, Auburn, CA) according to the manufacturer's protocol. Briefly, $CD4^+$ T cells were negatively selected from splenocytes of B6 mice with induced retinal ischemia or sham-operated mice at 2 weeks post procedure by depletion with a mixture of lineage-specific biotin-conjugated antibodies against CD8 (Ly-2), CD11b (Mac-1), CD45R (B220), CD49b (DX5), Ter-119, and antibiotin-conjugated microbeads. The procedure yielded purity of over 90% $CD4^+$ T cells, as assessed by flow cytometry. The donor cells (2×10^8 cells in a volume of 200 µL sterile saline) were adoptively transferred into recipient $Rag1^{-/-}$ mice 2 weeks after the induction of retinal ischemia via tail vein injection. Same numbers of $CD4^+$ T cells isolated from sham-operated mice were injected to the control group of recipient $Rag1^{-/-}$ mice 2 weeks after the induction of retinal ischemia. All recipient mice were sacrificed 2 weeks after adoptive T cell transfer and quantified for RGC loss.

Immunohistochemistry and cell counts

As previously described,[19] mouse eyeballs were dissected and fixed in 4% paraformaldehyde (PFA) overnight, transferred to 20% sucrose for 2 h before embedded in Tissue-Tek (Sakura Finetek Inc., Torrance, CA). Transverse retinal sections (10 µm) or retinal flat-mounts were stained with a primary antibody against CD11b (Invitrogen) or CD4 (clone GK1.5, Abcam, Cambridge), followed by reaction with an Alexa Fluor 488-conjugated secondary antibody (Jackson ImmunoResearch Inc, West Grove, PA), and counterstained with the

nuclear marker 4', 6-diamidino-2-phenylindole (DAPI, Vector laboratories, Burlingame, CA). The numbers of $CD11b^+$ cells and $CD4^+$ T cells were counted under direct fluorescence microscopy (Olympus IX51). RGC loss was assessed quantitatively in retinal flat-mounts using a standard protocol as previously described by our laboratory[20] with minor modifications. In brief, eyeballs were fixed in 4% PFA for 3 hours at room temperature. Retinal flat-mounts were incubated with a primary antibody against an RGC specific-marker, β-III-tubulin[21, 22] (Tuj1; MAB5564, Millipore, Darmstadt, Germany), followed by a Cy3-conjugated secondary antibody (Jackson ImmunoResearch Inc, West Grove, PA). Retinal flat-mounts were divided into quadrants: superior, temporal, nasal and inferior. Using the optic nerve head as the origin, four standard regions were selected from each quadrant: one peripheral, two intermediate, and one central (Fig. 1A). In total, 16 rectangular regions (each 193 mm x 193 mm) of each retinal flat-mount were photographed at 400x magnification with a confocal microscope (Leica TCS-SP5). The degree of RGC loss was assessed as previously described[20]. RGC densities were calculated, and the percentage of RGC loss was determined by dividing the RGC density from the retina with ischemic injury by that of the contralateral control retina of the same mouse. All quantification procedures were conducted by 2 investigators under a masked fashion.

RT-PCR to detect cytokine expression in ischemic retinas

Total RNA was extracted from mouse retina using RNAeasy Plus Kit (Qiagen) according to the manufacturer's protocol. cDNA was synthesized from total RNA using Supercript III First Strand Kit (Invitrogen, Carlsbad, CA). Reaction mixture of RT-PCR contained cDNA, 2x Master Mix from KAPA SYBR Fast qPCR kit and 10 mM of specific primers. Quantitative detection of specific mRNA transcript was carried out by RT-PCR using the Mastercycler ep realplex real-time PCR system (Eppendorf, Westbury, NY). The sequences of all primers are listed in Table 1. Relative amount of specific mRNA transcript was presented in fold changes by normalization to the expression level of the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

Table 1. List of primer sequences used in real-time PCR

Gene	Foward	Backward
IFN-γ	ATGAACGCTACACACTGCATC	CCATCCTTTTGCCAGTTCCTC
IL-4	GGTCTCAACCCCCAGCTAGT	GCCGATGATCTCTCTCAAGTGAT
IL-17	TCAGCGTGTCCAAACACTGAG	TCTCGACCCTGAAAGTGAAGG
TGF-β	CTCCCGTGGCTTCTAGTGC	GCCTTAGTTTGGACAGGATCTG
GAPDH	AACTTTGGCATTGTGGAAGG	ACACATTGGGGGTAGGAACA

Intravitreal administration of antibodies

Intravitreal injection procedure was as previously described.[23] Mice received intravitreal injections of antibodies on days 3, 7, 10, and 14 after induction of acute retinal ischemia. Control mice received intravitreal injections of sterile saline or isotype IgG. To control the small volume (2 µL) of intravitreal injection, a glass micropipette was connected to a Hamilton syringe. The right eye was gently punctured posterior to the limbus using a 30.5-gauge needle to generate access for the glass micropipette. Using this entry wound, 2 µL of Ultra-LEAF (Low Endotoxin, Azide-Free) purified anti-mouse CD4 (IgG2b, clone GK1.5, Biolegend, San Diego, CA), Ultra-LEAF purified anti-mouse interferon (IFN-γ) (IgG1, clone XMG1.2, Biolegend, San Diego, CA), Isotype IgG (Biolegend, San Diego, CA), or sterile saline was given intravitreally using a glass micropipette. Reports have shown that Ultra-LEAF anti-mouse CD4 blocked CD4-mediated cell adhesion and $CD4^+$ T cell activation, causing ***in vivo*** depletion of $CD4^+$ T cells.[24-29] Ultra-LEAF anti-mouse IFN-γ neutralizes the bioactivity of natural or recombinant IFN-γ.[30, 31]

Flow cytometry analysis for identifying $CD4^+$ T cell subsets

To define the subsets of T cells involved in the pathological process following acute retinal ischemia, we analyzed cytokine expression by T cells in the eye draining (superior cervical) lymph nodes (LNs). Superior cervical LNs were dissected, and cells were mechanically dissociated using two forceps. Cell aggregates were separated by filtration through a 70 µm nylon cell strainer (BD Falcon, San Jose, CA). For analyzing the frequencies of $CD4^+$ T cells that expressed IFN-γ (T_H1), IL-17 (T_H17), IL-4 (T_H2), or TGF-β (Treg), isolated lymphocytes were stimulated for 4 hours with phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich, St. Louis, MO) and ionomycine (Sigma-Aldrich, St. Louis, MO) in the presence of monensin (Biolegend, San Diego, CA). Cells were washed in IsoFlow (Beckman Coulter Inc, Brea, CA) and stained with surface FITC-conjugated anti-mouse CD4 antibody (IgG2b, clone GK1.5, Biolegend, San Diego, CA). Thereafter, cells were permeabilized with Perm/Wash buffer (BD biosciences, Frankin Lakes, NJ), and stained with PE-labeled anti-mouse IFN-γ antibody (IgG1, clone XMG1.2, Biolegend, San Diego, CA), PE-labeled anti-mouse IL-4 antibody (IgG1, clone 11B11, Biolegend), PE-labeled anti-mouse TH17A (IgG1, clone TC11-18H10.1, Biolegend), or PE-labeled anti-mouse TGF-β1 antibody (IgG1, clone TW7-20B9), detecting T_H1, T_H2, T_H17, and Treg cells, respectively. The antibody-stained cells were analyzed with BD LSR II Flow Cytometer (BD Biosciences), and data were analyzed using Summit Software v4.3 (Beckman Coulter Inc, Brea, CA).

Electroretinography

Animals were dark adapted for 5 hours prior to electroretinogram (ERG) recordings. All procedures were performed in a dark room under the dim red safety light. Mice were anesthetized with 120 mg/kg Ketamine and 20 mg/kg Xylazine, and the pupils were dilated with 1% tropicamide. Mice were placed in the sternal-abdominal position within the Ganzfield bowl. During recording, mouse body temperature was maintained at 37°C using a heating pad to prevent hypothermia. Recording gold lens electrodes were placed on both corneas; the reference and ground electrodes were placed subcutaneously in the mid-frontal head area and caudal area near the tail, respectively. Light stimulations were delivered with a Xenon lamp at 0.0002, 0.02, 2, 200, 600 cd·s/m^2 for dark-adapted tests. Thereafter, animals were subjected to 7-minute light adaptation with a light intensity of 50 cd·s/m^2 before initiating the light-adapted tests. The light-adapted tests were conducted by Xenon light at 600 cd·s/m^2, green light at 13 cd·s/m^2, and blue light at 1 cd·s/m^2 sequentially. Flickr test were executed with 6,500 K white light at 15 cd·s/m^2 and 3 different frequencies of 3, 10 and 15 Hz. Data were processed by the software included in the ERG recorder (Espion Electroretinography System; Diagnosys LLC, Lowell, MA). ERG a-waves were measured from the baseline to the cornea-negative peak, and b-waves from the cornea-negative peak to the major cornea-positive peak.

Statistical analysis

All statistical analyses were performed using GrapPad Prism for Windows, version 5.0 (GraphPad Software Inc, La Jolla, CA). The performed tests were two-sided and a value of $P < 0.05$ was considered as statistically significant. At least six animals were used for each experimental or control group. For the comparison between two groups, the Mann-Whitney test was performed, and for three or more groups the non-parametric Kruskal-Wallis test was employed.

RESULTS

Acute retinal ischemia induces a prolonged phase of progressive neurodegeneration

To investigate if acute ischemic injury in the retina induces a prolonged phase of neurodegeneration, transient retinal reperfusion injury was induced in adult B6 mice by

raising IOP to 90 mmHg for 60 minutes. RGC loss was quantified at day 3, and weeks 1, 4, and 8 after ischemic injury or at 4 weeks post procedure in sham-operated mice, using Tuj1 labeling in retinal flat-mounts as previously described (Fig. 1B)[20]. As expected, no significant difference of RGC counts was noted in uninjured contralateral eyes at all time points post procedure or in sham-operated control eyes (Supplementary Fig. 1). In contrast, starting from 3 days post injury, retinas subjected to reperfusion injury displayed progressive RGC loss (Fig. 1C, D and Supplementary Fig. 1). Significant less RGCs in the ischemic retinas (3,250 ± 87 cells/mm^2) was counted than that in sham-operated retina (3,831 ± 78 Cells/mm^2) at as early as 3 days post-procedure (Fig. 1C,D). Although the ischemic injury lasted for only 60 minutes, in the absence of any sustained injury progressive RGC loss continued to occur and 1,737 ± 94 cells/mm^2 were counted by 8 weeks post injury. This was equivalent to a 17.2% ± 1.6% RGC loss at day 3 to 54.8% ± 2.6% RGC loss by 8 weeks post-ischemic injury (Fig. 1D). Whereas, RGC counts in retinas contralateral to the injury remained constant through the period and were comparable to sham-operated controls (Supplementary Fig. 1). Thus, acute retinal ischemia triggers a prolonged phase of progressive RGC degeneration in the absence of a sustained insult.

T cells infiltrate into the retina after ischemic injury

Local inflammation represented by microglia/macrophage activation is a common event occurring after retinal injury. We thus performed CD11b immunolabeling to detect activated microglia/macrophage in retinal sections. At as early as 3 days post injury, significantly increased numbers of CD11b+ microglia/macrophages were noted in the ischemic retina compared to sham-operated controls (Fig. 2). Moreover activated microglia showed shortened dendritic processes and enlarged round cell bodies (Fig. 2A). Next, we asked if the adaptive immune system or T cells participate in ischemia-induced responses in the retina by double-immunostaining of CD4$^+$ T cell and RGC marker, anti-CD4 and β-III-tubulin (Tuj1) (Fig. 3A). While no T cells were detected in the uninjured contralateral retinas throughout the period (data not shown), infiltrated CD4$^+$ T cells were found in a close proximity of RGCs in the retinas subjected to reperfusion injury. The number of T cells counted in the flat-mounted retinas of sham-operated mice was minimal when examined at 2 weeks post operation (0.2 ± 0.2 cells/retina); whereas, a significant influx of T cells into the ischemic retina was detected from 1 to 4 weeks, reaching the peak at 2 weeks, after acute reperfusion injury (Fig. 3B). To exploit the subsets of T cells that infiltrated the ischemic retina, we assessed with qPCR the levels of cytokines, which

are hallmarks of different subsets of T_H cells: IFN-γ (T_H1), IL-4 (T_H2), IL-17 (T_H17), and TGF-β (T_{reg}),[14] in the retinas subjected to ischemic injury. Significant increases of T_H1 cytokine marker IFN-γ, but not other cytokines (TGF-β, IL-17, and IL-4), were detected in the ischemic retina at 2 and 4 weeks post injury (Fig. 3C). The subsets of infiltrated T cells were further verified by flow cytometry. The data support induction of local inflammation and CD4+ T_H1 cell infiltration following retinal ischemic/reperfusion injury.

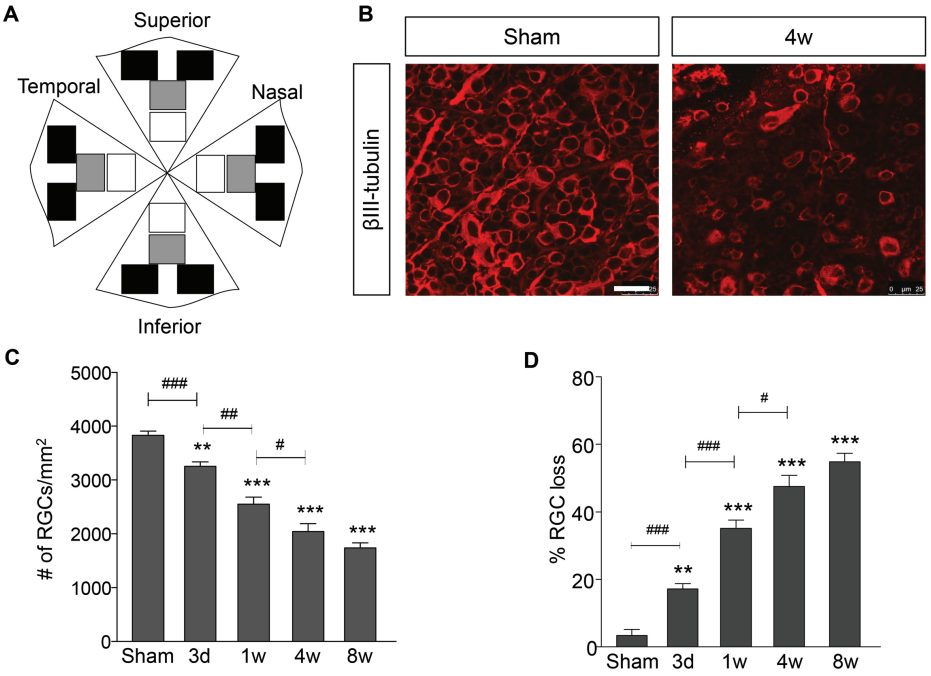
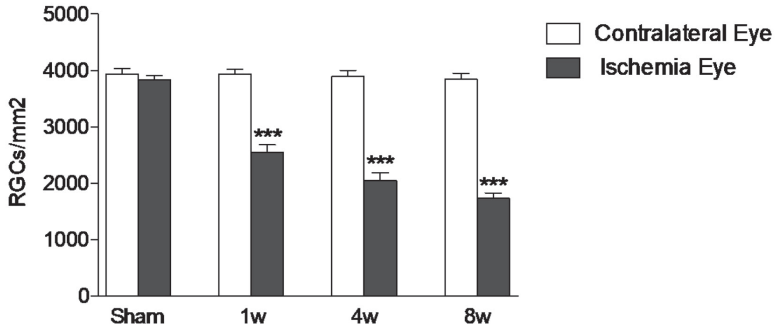


Figure 1. Acute retinal ischemia induces progressive neurodegeneration. A. Schematic illustration of retina sampling for RGC counts in a retinal flat-mount. **B.** Representative epifluorescent photomicrographs of β-III-tubulin (red) immunolabeled retinal flat-mounts taken from a sham-operated B6 mouse (Sham) and a mouse at 4 weeks after retinal ischemic injury (4w). Scale bar: 25 μm. **C.** Quantification of RGC densities in retinal flat-mounts at various time points after ischemic injury or at 4 weeks after sham operation (n = 6/group). **D.** Percentage of RGC loss over that of the uninjured contralateral eye in mice at various time points after retinal ischemia or sham operation. Value = mean ± S.E.M. $^{**}P < 0.01$, $^{***}P < 0.001$ compared to the sham group or $\#P < 0.05$, $\#\#P < 0.01$, $\#\#\#P < 0.001$ as indicated, by one-way ANOVA.



Supplementary Figure 1. Acute retinal ischemia induces progressive neurodegeneration in the ischemic eye. Quantification of RGC densities in retinal flat-mounts taken from the eyes ipsilateral and contralateral to the injury at 1w, 4w, and 8w – post procedure or from sham-operated mice (n = 6/group). Value = mean ± S.E.M. ***P* < 0.01, ****P* < 0.001 compared to the sham group.

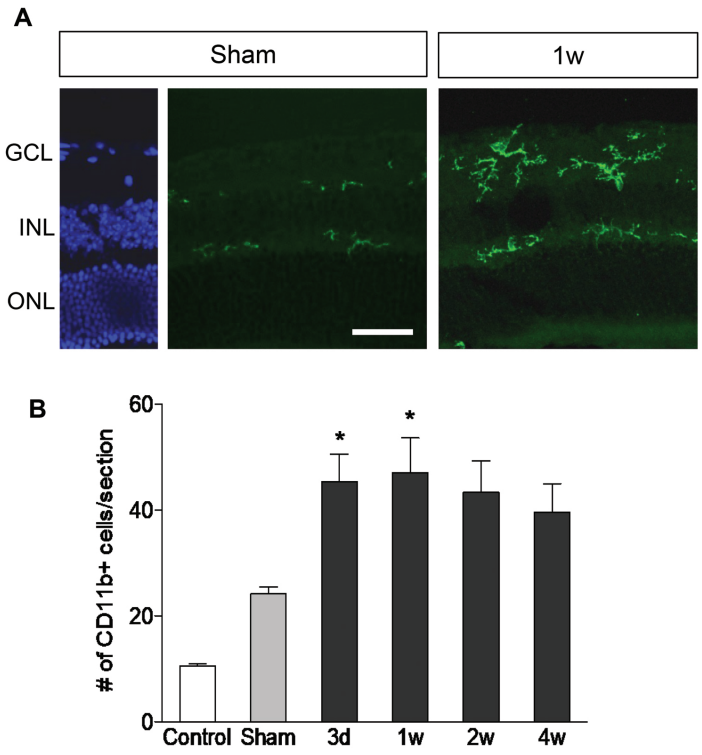


Figure 2: Acute retinal ischemia induces microglia/macrophage activation. A. Epifluorescent photomicrographs of CD11b immunolabeling (green) retinal sections taken from a sham-operated B6 mouse or at 1 (1w) and 4 weeks (4w) after ischemic injury. Scale bar: 10 µm. **B.** Quantification of $CD11b^+$ cells in retinal sections taken from uninjured eyes (control), mice at 4 weeks after sham-operation (Sham), or at 3 days (3d), 1 (1w), 2 (2w), and 4 weeks (4w) after acute ischemic injury (n = 6/group). Value = mean ± S.E.M. **P* < 0.05 compared to sham-operated mice by one-way ANOVA.

Priming of T cell responses usually occurs first in the secondary lymphoid tissues, such as LNs, where naïve T cells become activated and respond to pathogenic antigens presented by their antigen presenting cells.[32] To assess whether transient ischemia leads to activation of immune responses and T cell activations in the eye-draining LNs, functional subsets of CD4+ T cells in the superior cervical LNs were analyzed with flow cytometry. T cell subsets, T_H1, T_H2, T_H17, and Treg cells, again were divided based on the expression profile of hallmark cytokines: IFN-γ, IL-4, IL-17, and TGF-β.[14] Superior cervical LNs were dissected from mice with retinal ischemia at day 3 and 1 – 4 weeks post injury, sham-operated mice at 2 weeks. Correlating with T cell infiltration into the ischemic retina, the frequencies of 3 subsets of $CD4^+$ T cells expressing IFN-γ (T_H1), IL-17 (T_H17) and TGF-β (T_{reg}) were significantly increased at as early as 1 week after ischemic injury (Fig. 3D). The increases of T_H1 and T_{reg} cell frequencies peaked at 2 weeks post injury and remained elevated by 4 weeks after retinal ischemia (Fig. 3D). Thus, acute retinal ischemia induced CD4+ T cell responses, particularly, that involved IFN-γ expressing T_H1 cells, and likely also Treg cells, in the retina and their draining LNs.

T cells mediate the prolonged phase of RGC degeneration following retinal ischemia

To determine if the T cell responses participate in ischemia-induced retinal neurodegeneration, we examined mice deficient in both T and B cells ($Rag1^{-/-}$ mice) or only T cells ($TCR\beta^{-/-}$).[33] While acute ischemic injury to the retina induced sustained RGC degeneration that progressed over 8 weeks, RGC loss in $Rag1^{-/-}$ and $TCR\beta^{-/-}$ mice was significantly attenuated compared to B6 mice (Fig. 4A, B). At 1 week post injury, $Rag1^{-/-}$ and $TCR\beta^{-/-}$ mice showed a similar rate of RGC loss at 23.9% ± 2.8% and 24.0% ± 2.7%, respectively, as compared to 35.2% ± 2.4% RGC loss in B6 mice. No significant further loss of RGCs was detected in $Rag1^{-/-}$ and $TCR\beta^{-/-}$ mice after week 1; by 4 weeks post injury, loss of RGCs counted from $Rag1^{-/-}$ and $TCR\beta^{-/-}$ mice was not significantly increased and remained at 28.2% ± 1.9% and 30.2% ± 2.9%, respectively. In contrast, loss of RGCs in B6 mice had significantly progressed to 47.6% ± 3.3% by week 4 post injury. These results indicate that T cells are essentially involved in perpetuating progressive neurodegeneration in retinal ischemia. The similar extents of RGC loss in $Rag1^{-/-}$ and $TCR\beta^{-/-}$ mice suggest a primary role for T cells, but not B cells, in mediating neural damage following transient reperfusion injury.

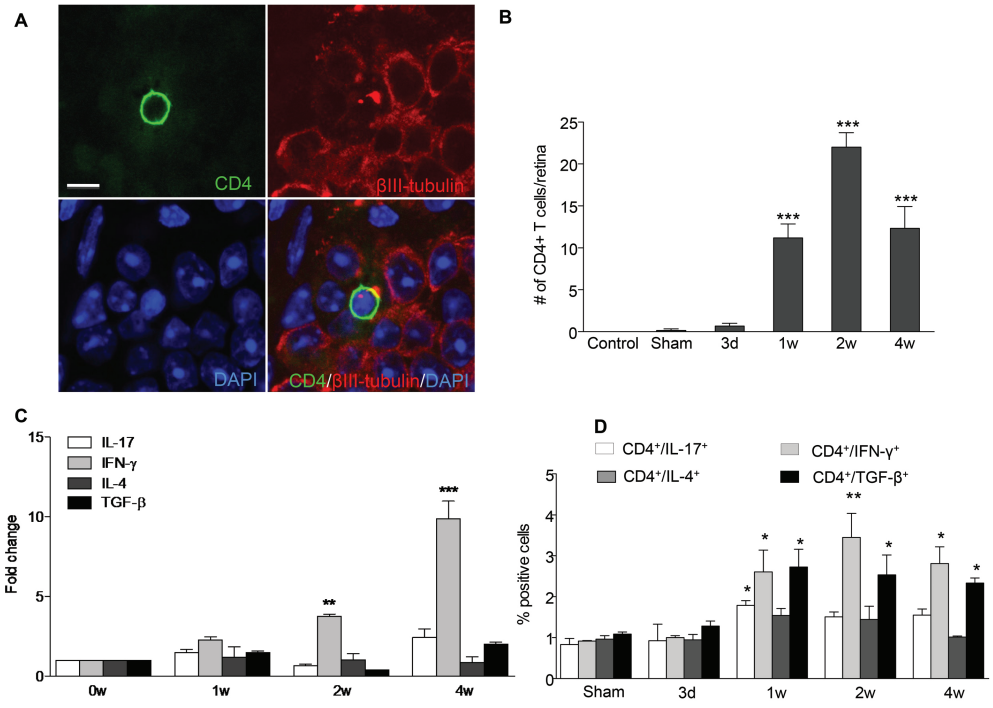


Figure 3. Acute retinal ischemic injury induces T cell infiltration into the retina. A. Epifluorescent photomicrographs of a retina flat-mount image taken from a mouse at 2 weeks after ischemic injury that was double-immunolabeled for CD4 (green) and β-III-tubulin (red) and counter-stained by a nuclear marker DAPI (blue). Scale bar: 10 μm. **B.** $CD4^+$ T cell counts in retina flat-mounts of an uninjured eye (control) and mice at 4 weeks after sham-operation (Sham) or at 3 days (3d) and 1 (1w), 2 (2w), and 4 weeks (4w) after acute ischemic injury (n = 6/group). **C.** Results of qPCR revealing the fold changes in expression of hallmark cytokines of T_H cells in the retina of mice at day 0 (0w as a baseline), 1, 2, and 4 weeks after acute ischemia (n = 6/group). **D.** Flow cytometry quantification of frequencies of subsets of CD4+ T cells in the draining LNs of the eye taken from mice at 2 weeks after sham-operation (Sham) or after 3 days (3d), 1 (1w), 2 (2w), and 4 weeks (4w) after acute ischemic injury. Shown were percentages of $CD4^+$ T cells that expressed IL-17, IFN-γ, Il-4, or TGF-β among freshly-isolated total LN lymphocytes (n = 6/group). *P < 0.05, **P < 0.01, ***P<0.001 compared to sham-operated mice by one-way ANOVA.

To investigate if T cells play a causative role in inducing RGC damage, $CD4^+$ T cells were isolated from the splenocytes of ischemia- or sham-operated B6 mice 2 weeks post procedure and adoptively transferred into $Rag1^{-/-}$ mice which had been subjected to retinal reperfusion injury 2 weeks earlier. Recipient $Rag1^{-/-}$ mice were sacrificed 2 weeks after adoptive T cell transfer. $Rag1^{-/-}$ recipient mice that were subjected to T cell injection from sham-operated mice showed a RGC loss (29.9% ± 1.5%) similar to that was seen in $Rag1^{-/-}$ mice without receiving a T cell transfer (28.2% ± 1.9%) (Fig. 4C, D). In contrast, $Rag1^{-/-}$ mice

that received T cell transfer from ischemic B6 mice showed a significantly increased loss of RGCs (44.4% ± 4.0%) compared to Rag1$^{-/-}$ mice without receiving T cell transfer or those who received T cells from sham-operated B6 mice. Together, these findings demonstrate that diseased CD4^{+} T cells from mice with retinal ischemic injury are sufficient to induce RGC damage.

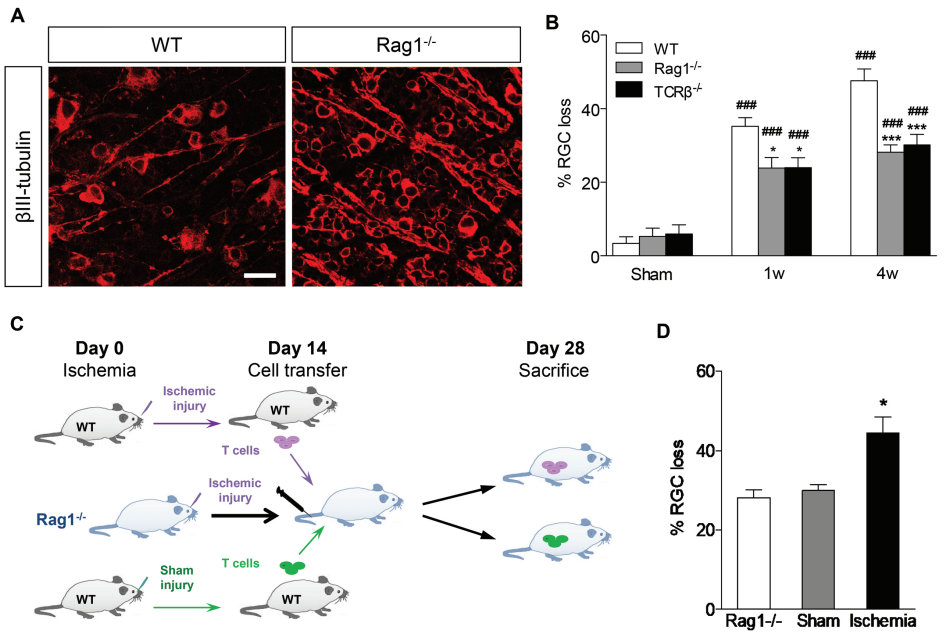


Figure 4. CD4+ T cell-mediated responses are essential and causative contributing factors to the progressive RGC loss after retinal ischemic injury. A. Representative epifluorescent photomicrographs of retinal flat-mounts of B6 and Rag1$^{-/-}$ mice at 4 weeks after retinal ischemia. Scale bar: 25 μm. **B.** Quantification of RGC loss in B6, Rag1$^{-/-}$ and TCRβ$^{-/-}$ mice at 1 and 4 weeks post-ischemic injury or at 4 weeks after sham operation (n = 6/group). Value = mean ± S.E.M.. *P < 0.05, ***P < 0.001 compared to B6 mice taken at the same time point; ###P < 0.001 compared to sham-operated group of mice within the same genotype group, by one-way ANOVA. **C.** Schematic illustration of adoptive T cell transfer: CD4+ T cells were isolated from the spleens of B6 mice 2 weeks after ischemic injury or sham-operation; the donor T cells were injected into the tail veil of recipient Rag1$^{-/-}$ mice that also received retinal ischemic injury 2 weeks prior to the cell transfer. Recipient mice were sacrificed 2 weeks after cell transfer. **D.** Percentage of RGC loss presented as RGC counts relative to that of the unoperated contralateral eyes (n = 6/group). Value = mean ± S.E.M.. *P < 0.05 by two-tailed student *t* test.

Anti-CD4 and IFN-γ antibodies attenuate retinal ischemia-induced RGC loss and improve retinal function

The findings prompted us to investigate the neuroprotective effect and therapeutic potential for retinal ischemic injury by local administration of blocking antibodies against

CD4+ T cells or IFN-γ. It has been shown that Ultra-LEAF anti-mouse CD4 blocked CD4-mediated cell adhesion and CD4+ T cell activation, causing in vivo depletion of CD4+ T cells.[24-29] Ultra-LEAF anti-mouse IFN-γ neutralizes the bioactivity of natural or recombinant IFN-γ.[30, 31] We found that intravitreal injections of anti-CD4 or anti-IFN-γ neutralizing antibodies significantly attenuated RGC loss compared to injections with IgG Isotype antibodies or sterile saline (Fig. 5A, B); whereas, the extent of RGC loss was comparable between saline- and IgG Isotype antibody-treated groups. Acute retinal ischemic injury is reported to induce ERG changes that are associated with functional impairment,[34] so we also evaluated ERG responses at 4 weeks post injury as a readout for their retinal functions. Vehicle treated ischemic retina showed significantly decreased a- and b-wave amplitudes in ERG scotopic-200 or flicker responses compared to sham-operated mice. In contrast, administration of anti-CD4 blocking antibody, but not anti-IFN-γ, prevented the reduction of a- and b-wave amplitudes, under both dark- and light-adapted conditions, following retinal ischemic injury in mice (Fig. 6A-C). These results strongly suggest that local administration of CD4+ T cell blocking antibody in the eye protects against secondary retinal neuron and function loss following reperfusion injury.

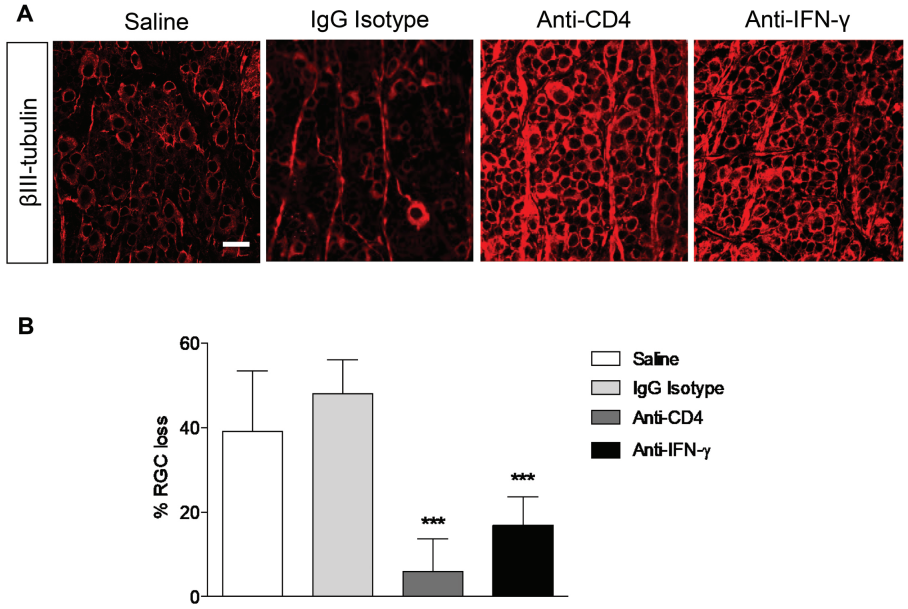


Figure 5. Local administration of anti-CD4+ or anti-IFN-γ antibody attenuates RGC loss after acute retinal ischemia. A. Epifluorescent images of βIII-tubulin-immunolabeled retinal flat-mounts taken from ischemic eye that received an intravitreal injection of saline-, IgG Isotype-, anti-CD4, or anti-IFN-γ. Scale bar: 25 μm. **B.** Percentage of RGC loss at 4 weeks after acute retinal ischemia. Value = mean ± S.E.M. (n > 6/group). ****P*<0.001 compared to the saline-injected group by one-way ANOVA.

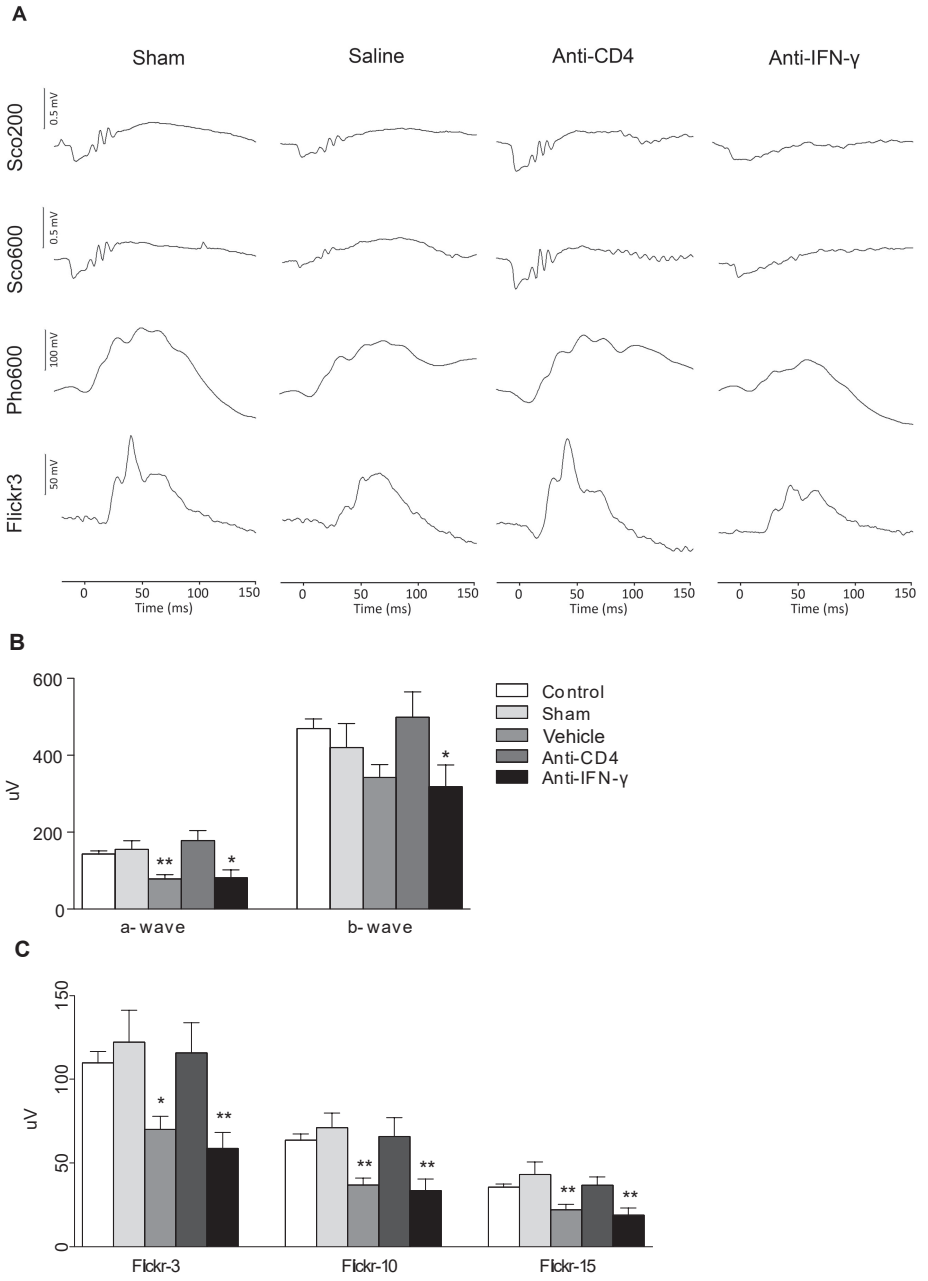


Figure 6. Local administration of anti-CD4 antibody preserves retinal function after acute retinal ischemia. A. Representative ERG waveforms from mice at 4 weeks after receiving sham operation or ischemic injury plus saline (vehicle)-, anti-CD4, or anti-IFN-γ intravitreal injection. **B, C.** Amplitudes of scotopic-200 a- and b-waves (B) or 3, 10, 15 Hz Flickr b-waves assessed in mice at 4 weeks after receiving no injury (control), sham operation (Sham), or ischemic injury plus intravitreal injections of saline- (vehicle), anti-CD4 (anti-CD4), or anti-IFN-γ (anti-IFN-γ). Value = mean ± S.E.M. (n > 6/group). *P<0.05, **P<0.01 compared to control eyes by one-way ANOVA.

DISCUSSION

In this study, we reported a prolonged phase of progressive RGC loss following acute retinal reperfusion injury and a role for CD4+ T cell-mediated responses in the etiology of neurodegeneration in ischemic retinopathy. We showed that transient retinal reperfusion injury led to microglia/macrophage activation and T cell infiltration. Moreover, T cell-mediated responses are responsible for progressive degeneration of RGCs despite of the absence of sustained insults. Adoptive transfer of T cells isolated from mice subjected to acute retinal ischemia was sufficient to drive progressive RGC damage, while ectopic suppression of $CD4^+$ T cell responses protected RGCs against ischemic insult-induced damage and preserved retinal function after injury. These results reveal a therapeutic window as well as a potential novel therapeutic strategy for limiting retinal neuron and function loss in the currently untreatable conditions of ischemic retinopathy or optic neuropathy.

Acute ischemic injury to the retina is accompanied by early activation of innate immune cascades, disruption of the blood retinal barrier, and leukocyte infiltration.[35, 36] However, little is known about the long-term impact of these immune responses on the retina. Here we discovered CD4+ T cell infiltration that was peaked around day 14 and persisted over 4 weeks after reperfusion injury. In addition, we observed the increase in $CD11b^+$ cells in the retina, followed by $CD4^+$ T cell infiltration and activation in the eye's draining LNs. In agreement with that was seen in ischemic stroke model of the brain, CD4+ T cells involved in retinal ischemia were primarily composed of INF-γ+ T_H1 and Treg cells.[37] Antigen-presenting cells in the retina and LNs, including microglia and macrophages, likely present retinal antigens from stressed or damaged RGCs to naïve T cells which further recruit T cells into the retina under a compromised blood retinal barrier.[9, 32]

Previous studies have shown that adaptive immune responses play an essential role in the pathogenesis of many neurodegenerative processes, such as ischemic stroke and traumatic brain injury.[13, 35, 38-40] Induction of ischemic stroke or traumatic brain injury in immunodeficient mice, including SCID, $Rag1^{-/-}$ mice, and IFN-$\gamma^{-/-}$ mice, resulted in attenuated CNS injury and reduced infarct size relative to immunologically-intact mice.[38-40] Adoptive transfer of activated/effector $CD4^+$T cells from ischemic or traumatic injured mice into immunodeficient mice significantly increased the size of traumatic brain injury and the number of apoptotic cells in the CNS.[39, 40] These data are in agreement with ours seeing

in the retinal ischemic model and support that activated CD4+ T cells are highly injurious. Specifically, we demonstrated a role for $CD4^+$ T cells in perpetuating a progressive loss of RGCs after ischemic injury, which is significantly attenuated in $Rag1^{-/-}$ and $TCR\beta^{-/-}$ mice that lack T and B cells or only T cells. In addition, adoptive transfer of $CD4^+$ T cells from ischemia-induced B6 mice to $Rag1^{-/-}$ mice compromised the resistance of RGCs to ischemic injury and resumed the secondary damage in these mice. The fact that RGC loss was not detected in the contralateral eye of mice with ischemic injury suggests the prerequisite for local injury/inflammation or compromised blood-retina barrier to enable T cell-mediated neural damage. A similar observation was found in the mouse model of glaucoma, in which adoptive transfer of diseased $CD4^+$ T cells exacerbated RGC loss in $Rag1^{-/-}$ mice with elevated IOP, but not in naïve mice.[14] Correspondingly, pro-inflammatory T cells, such as IFN-γ- and IL-17-secreting $CD4^+$ T cells, were found to be the primary subsets that infiltrated the retina after ischemic-injury, as that was seen in glaucomatous mice.[14] Collectively, our data support that acute ischemic injury led to $CD4^+$T cell-mediated responses that involve particularly T_H1 type cells in the retina and eye-draining LNs, which contribute to a prolonged phase of RGC degeneration and/or retinal neural damage.

Our results indicate a therapeutic window and opportunity for currently untreatable conditions of ischemic retinopathy, such as that caused by central retinal artery occlusion and non-arteritic anterior ischemic optic neuropathy. We showed that local inhibition of $CD4^+$ T cell activities by intravitreal administration of CD4-blocking antibody protected RGCs against ischemic damage and preserved retinal function as assessed by ERG compared to the non-treated control group. As compared with anti-CD4 antibodies, IFN-γ antibodies were less effective, suggesting the involvement of not only T_H1 cell subset in the pathogenesis of ischemic injury. Future characterization of effector T cells that enter the retina and mediate retinal neuron damage will be necessary. In line with our finding, a study in a traumatic brain injury model showed similar benefit in attenuating acute injury-induced neuron tissue damage when mice were intravenously treated with immunosuppressants and T cell-inhibitory agents, such as cyclosporine A or FK506.[39] Antibodies against α4 integrin that prevent lymphocyte infiltration into post-ischemia brain injury, and methylprednisolone—an agent with inflammation-inhibitory effects and T cell suppressant, also reduced neural damage[41, 42] and promoted tissue healing.[43] In these studies, broader spectrum immunosuppressants and systematic administration via intravenous or intraperitoneal injection were employed. As the eye is more accessible

than the brain, it enables local administration of antibodies, thereby prevents systematic adverse effects. Our study demonstrates that intravitreal injection of antibodies specifically targeting CD4+ T cells is sufficient to prevent RGC and retinal function loss after ischemic optic neuropathy.

In summary, our study has provided novel evidence showing previously unappreciated roles for CD4+ T cells in post-ischemic retinal injury. Local administration of $CD4^+$ T cell blocking antibodies may present an effective therapeutic strategy for preventing RGC death and preserving retinal functions. This finding is in line with our previous report that CD4+ T cell responses are critically involved in propagating progressive neurodegeneration after retinal neuron insults, such as in glaucoma mouse models.[14] These findings point to novel therapeutic strategies of limiting or preventing neuron loss and preserving retinal function for currently untreatable conditions of ischemic retinopathy or optic neuropathy, which may be extended to treat CNS ischemic stroke.

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SUMMARY AND GENERAL DISCUSSION

INFLAMMATION IN AGE-RELATED EYE DISEASES

The subject of this thesis concerns the role of inflammation in the pathogenesis of age-related eye diseases, most notably uveal melanoma and glaucoma. Aging is inevitable and refers to a multidimensional process of physical, psychological, and social changes. With an ever-increasing growth of the proportion of population aged 60 years or older worldwide, we are confronted with an ever increasing number of elderly people suffering from age-related diseases.[1] As we age, the cells in our body endure progressive amounts of oxidative and metabolic stress, which eventually leads to increasing amounts of tissue stress and damage.[2-4] Low-grade inflammatory responses (para-inflammation) are then required at these local sites of injury in order to restore tissue functionality and homeostasis. Due to the plasticity of our immune system, we are able to respond to tissue injury/stress in a short period of time without causing immunological imbalance. This is the case under normal aging conditions. However, changes secondary to aging, such as metabolic and genetic abnormalities, altered vascular perfusion or degenerative conditions, may result in dysfunction of the immune system's regulatory activity and lead to a chronic and dysregulated inflammatory state.[5] Although specific and different genetic and environmental factors are involved, I argue that inflammation is an important unifying component that contributes to the pathogenesis of age-related eye diseases, such as uveal melanoma and glaucoma.

INFLAMMATION IN UVEAL MELANOMA

Primary uveal melanoma

It has been shown that macrophages and lymphocytes are present in uveal melanoma and that they are related to an unfavorable prognosis and decreased survival.[6-9] Increased inflammation is not present in all uveal melanoma, but specifically in those with a high risk for metastases formation. High risk tumors carry specific tumor characteristics such as the presence of epithelioid cells and high vascular density, associated with the presence of monosomy 3 and the loss of BAP1 expression. [10-13] In a murine intraocular melanoma model, intratumoral accumulation of M2 type macrophages was shown particularly to foster tumor growth in elderly mice, indicating that macrophages in aged mice have a strong tumor-promoting role.[14] Tumor-associated macrophages are directly involved in the

formation of new vessels making it possible for tumors to survive by supplying nutrients and creating a route for cancer cells to disseminate hematogenously.[15-17] In humans (Chapter 2), pro-angiogenic and anti-inflammatory M2-type macrophages are characterized by a higher expression of CD163, and our lab found that the majority of macrophages present in human uveal melanoma carry this phenotype.[18] Interestingly, tumors with monosomy 3 exhibit increased number of M2-type macrophages and more robust angiogenesis than tumors with disomy 3.[18] In addition, infiltratration by lymphocytes is also seen in uveal melanoma; in Chapter 2 we examined the subtypes of intratumoral T cells which are present in these tumors and we concluded that the infiltrate is pluriform. Tumors can evade effective antitumor immune responses by the recruitment of Tregs into the tumor. Infiltration of Tregs under physiologic conditions is necessary in order to dampen local inflammatory responses by regulating and suppressing other T cells, macrophages and APCs.[19] Mougiakakos et al.[20] have shown that intratumoral FoxP3$^+$ Tregs are an independent prognostic marker for poor clinical prognosis in COX-2-positive uveal melanoma. Other tumor-specific T cell subsets were not analyzed. Our data indicates that the number of CD163$^+$ M2-type macrophages correlates with CD3$^+$ FoxP3$^+$ regulatory T cells while CD8$^+$ T cells were the most abundant T lymphocytes. Moreover, both the presence of intratumoral macrophages as well as of lymphocytes was associated with monosomy of chromosome 3. More recently, our lab showed that this inflammation is related to the loss of BAP1 expression, a gene present on chromosome 3.[11] We believe that the intrinsic malignant properties of the tumor may lead to mixed inflammatory responses that support a pro-tumorigenic microenvironment. The recruitment of immunosuppressive immune cells into the tumor, such as M2-type macrophages and FoxP3$^+$ T regs, may explain the paradoxical finding of increased numbers of cytotoxic CD8$^+$ T cells and ineffective antitumor activity.

Irradiated uveal melanoma

Until the 1970s, the traditional treatment of uveal melanoma was enucleation, which was subsequently followed by a shift toward more eye-salvaging approaches.[21] While eye retention following local irradiation is achieved in more than 80% of cases after 5 years[22-24], secondary enucleation may be required when tumor recurrence or when radiation-related ocular side effects occur. At present, not much has been documented regarding inflammation after local irradiation of uveal melanoma. Because local immune responses may play a role in removing tumor debris or in either stimulating or suppressing antitumor immune responses, we analyzed the presence of inflammatory cells in uveal melanoma

containing-eyes, which had either been enucleated after prior irradiation or had undergone primary enucleation (Chapter 3). We observed that prior irradiation has no effect on the number and type of tumor-infiltrating macrophages, which showed a similar variability to uveal melanoma in primarily-enucleated eyes. This corresponds to a previous case-control analysis of matched pairs of irradiated and non-irradiated uveal melanoma.[25] Surprisingly, previously-irradiated uveal melanoma contained more lymphocytes than non-irradiated tumors. As the numbers of infiltrating cells were higher in epitheloid tumors, one possible explanation is that primary tumor characteristics such as the genetic status and not the treatment itself determine cellular infiltration. Due to limited non-necrotic tumor material in secondarily enucleated eyes, we were unfortunately unable to determine the chromosomal status of these tumors.

Experimental and future considerations

The inflammatory infiltrate in uveal melanoma is a complex phenomenon which raises many questions. One of the major pitfalls of working with human uveal melanoma-containing eyes is the fact that we were unable to assess the functional activity of these tumor-infiltrating leucocytes, as functional immunologic studies were not possible on our tumor sections. However, it has in the meantime been possible (G. Gezgin, unpublished results) to grow T cells from uveal melanoma, which were able to respond with IFN-γ production when exposed to autologous tumor cells. However, we do not know the specificity of these tumor-infiltrating T cells. Furthermore, the outgrowth of the tumors shows that the T cells were not efficient in removing the tumor in vivo. Although we have shown that the number of infiltrating macrophages is correlated to the number of (regulatory) T cells, it remains unclear whether these cells actually interact and what the outcome of these interactions would be on macrophage and T cell function. Another limitation of our study is its cross-sectional design because it provides only a "snapshot" of the moment of enucleation of the tumor-containing eye and does not reflect potential changes in infiltrating immune cells over time. Gezgin[11] and Robertson[26] recently showed that in uveal melanoma, the presence and type of leukocyte infiltrate depends on the developmental stage of the tumor: tumors with an extra 8q exhibited an influx of macrophages, while the loss of one chromosome 3/BAP1 expression was associated with a T cell infiltrate as well. With regard to irradiated uveal melanoma, it remains unclear whether the inflammatory infiltrate is a consequence of the characteristics of the primary tumor before irradiation or due to irradiation. Future studies involving information on biopsies obtained prior to irradiation

could give us more insight into the intrinsic properties of the tumor, and analysis of these tumors after secondary enucleation may help us to understand the changes in tumor behavior in locally uncontrolled uveal melanomas.

INFLAMMATION IN GLAUCOMA

Glaucoma is a global unmet medical challenge because of its prevalence in especially the elderly population, its debilitating consequences, and lack of effective treatment. Currently, the best and most advanced treatments only work to delay loss of retinal function. Although elevation of IOP is considered a major risk factor associated with optic nerve damage, glaucoma is no longer viewed simply as a neurodegenerative condition caused by elevated IOP. A conservative estimation is that 20-30% of patients with glaucoma exhibit a normal IOP range, and progressive visual field deterioration is observed in patients with perfectly controlled IOP levels.[27] To date, the precise pathogenesis of glaucoma is not fully understood. Recently, it has been suggested that inflammatory responses directed towards retinal and optic nerve proteins may be related to the development of glaucomatous optic neuropathy.[28]

The role of the immune system in glaucoma has been described as either neuroprotective or neurodestructive. For example, T cell-mediated immune responses may initially be beneficial to limit neurodegeneration.[29-31] The recruitment of macrophages and T cells allows early communication of the immune system with cellular debris, destruction of damaged cells, and removal of pathogenic agents. Subsequently, these immune cells mediate the protection of neurons against degenerative conditions in the aging eye, which is referred to as "protective immunity".[32] An initial immune response may be beneficial and necessary to promote tissue repair and limit neurodegeneration. However, failure to control aberrant, stress-induced immune responses due to accumulating risk factors (e.g. IOP, ischemia) along with aging-related oxidative stress likely switches the immune response from a neuroprotective response into a neuroinflammatory degenerative process that facilitates the progression of glaucoma. In Chapter 4, we extensively review the role of host immune responses in relation to RGC and axon survival following glaucomatous tissue stress.

Inflammation in experimental glaucoma models

The possibility that inflammation plays an important role in the damaging effects of high IOP or any type of neural insult (e.g. ischemia, traumatic optic nerve injury) has garnered much interest, however, definitive evidence to support a role for an autoimmune pathogenesis in glaucoma is lacking. We determined (Chapter 5 and 6) whether elevated IOP triggers secondary events, which could lead to prolonged or progressive neuronal damage even when the IOP has subsequently returned to a normal range. An elevated IOP is known to induce expression of Hsps in the retina.[33] Hsps are highly conserved proteins that can potently stimulate immune responses and are implicated in the autoimmune responses of rheumatoid arthritis, atherosclerosis, and type I diabetes.[34-37] Autoantibodies against Hsps were detected in the serum of some glaucoma patients[38], which further supports the involvement of autoimmune responses to Hsps in glaucoma. Here, we report (Chapter 5) that a 3-week transient elevation of IOP induces a high expression of Hsp27 in RGCs, which is consistent with what has been reported in human patients and experimental models of glaucoma.[28,33,39,40] Elevation of IOP induces activation of $CD11b^+$ microglia/ macrophages and infiltration of T cells into the retina. More importantly, this leads to $CD4^+$ T cell responses specific to Hsp27 that were both required and sufficient for propagating a prolonged and progressive phase of glaucomatous neurodegeneration after the IOP had already returned to normal ranges. When ocular hypertension was induced in immunodeficient mice, the progressive loss of RGCs and axons did not occur. However, when we transferred diseased T cells isolated from glaucomatous wild type mice to immunodeficient mice with induced high IOP, we observed exacerbated RGC and axon neurodegeneration. Remarkably, acute retinal or optic nerve damage caused by ischemic (acutely elevated IOP) or traumatic injury also induced T-cell mediated autoimmune responses, leading to chronic RGC and axon loss that continued to occur long after the period of initial injury. Suppression of these $CD4^+$ T cell-mediated immune responses by injecting neutralizing and blocking antibodies into the vitreous cavity of the eye led to a drastic decrease in RGC death against acute ischemic injury. We are the first to show that blocking local $CD4^+$ T cell-activity is not only neuroprotective, but also preserves the retinal function by protecting other retinal cell types against the secondary effects of ischemia (Chapter 6).

To determine whether Hsp-specific T cell responses also play an important role in the pathogenesis of glaucomatous neurodegeneration in humans, we examined Hsp-responsive T cells and autoantibodies in POAG patients. We noticed a significant increase

in the numbers of Hsp27- but also of Hsp60-reactive T cells and autoantibodies in the serum of POAG patients compared to age-matched healthy volunteers.

The neuroprotective effect of a germfree environment in experimental glaucoma models

So far, the role of environmental factors and their contribution to the development of glaucoma are not yet known. As the eye is an immunologically-privileged site, a critical gap in our knowledge are the factors which lead to the induction of destructive inflammatory cells. Because HSPs are highly conserved in sequences from bacteria to humans[41], we hypothesized that these damaging inflammatory reactions in the human eye may develop following induction of HSP-specific T cells by previous exposure to bacteria. These T cells would subsequently recognize damaged RGCs and neuronal cells, leading to the autoimmune attack of RGCs, resulting in optic neuropathy. In our hypothesis, the crucial environmental factor contributing to glaucoma development is the patient's exposure to specific bacterial strains. In Chapter 4, we compared the development and progression of glaucoma between DBA/2J mice housed under pathogen-free and completely germfree conditions. DBA/2J mice are genetically predisposed to develop spontaneous glaucoma in an age-related manner. Interestingly, a germfree environment compromised the development of glaucomatous neurodegeneration in DBA/2J mice despite persistent ocular hypertension in old germfree DBA/2J mice. Similar results were observed when we experimentally induced glaucoma in mice using the microbead model. It seems that mice that have never been exposed to any bacterial stimuli harbor more tolerant and "untrained" microglia/macrophages compared to conventionally-raised mice, as measured by their activation status and functional activity (unpublished data).

Experimental and future considerations

We believe that our study provides evidence that involvement of T cells requires local inflammation in the retina and a disruption in the blood-ocular barrier that occurs after injury. We propose that activated microglia/macrophages function as APCs that capture retinal antigens from damaged or injured RGCs and stimulate an autoimmune attack against the retina and optic nerve through activation and recruitment of $CD4^+$ T cells in the absence of sustained elevated IOP, ischemic or traumatic injury. These microglia/macrophages can also secrete inflammatory cytokines, such as TNF-α and IL-1β, both of which may further impair the integrity of the blood-retina barrier, allowing T cells to infiltrate into the retina.[42-44] Identification of a key role of autoreactive $CD4^+$ T cells in

glaucomatous and acute (ischemic, traumatic) injury-induced neurodegeneration may hold larger implications and contribute to our understanding of the molecular mechanisms underlying chronic neurodegeneration in the CNS in general. Our findings may provide a possible connection between acute CNS injury, such as ischemic stroke and traumatic brain injury, to Alzheimer's disease later in life.[45-47] Furthermore, it may also explain why current glaucoma therapies solely directed at lowering IOP are not always effective in the long-term. Our findings give way to a novel theory in this field: that we are able to prevent and treat neurodegenerative eye conditions through immunomodulation. The eye is more easily accessible compared to the CNS, and by treating it locally, we can reduce systemic side effects and have fewer problems with insufficient penetration of drugs in targeted sites.

Finally, we need to remember that glaucoma pathogenesis is multifactorial which involves a complex interplay of elevated IOP-induced events and genetic/epigenetic/age-related susceptibility factors that contribute to neurodegeneration. Our comprehensive study contributes only one piece to the larger meshwork of complex pathogenic mechanisms underlying glaucoma.

CONCLUDING REMARKS

Although uveal melanoma and glaucoma differ in their pathogenesis, it is believed that these diseases share two unifying elements: 1) both are diseases of the elderly; and 2) both involve inflammation. Due to the extreme plasticity of our immune system, we can respond to tissue injury/stress in a short period of time without causing immunological responses with damaging effects, especially during aging. The immune system is however not a mere bystander in the process of neurodegeneration, protecting our body from harmful injury or infections, but can also be involved in detrimental and chronic immune responses leading to neurodegenerative diseases, such as glaucoma. On the other hand, due to the immune-privileged property of the eye, tumors like uveal melanoma are permitted to grow quietly and unhampered by escaping immune detection and attack. Therefore, understanding the mechanisms underlying the mosaic of mechanisms which link immune privilege, protective immunity, and autoimmune diseases is crucial to understand the pathogenesis of age-related diseases. Further studies can shed light on elusive aspects of the role of inflammation in age-related eye diseases.

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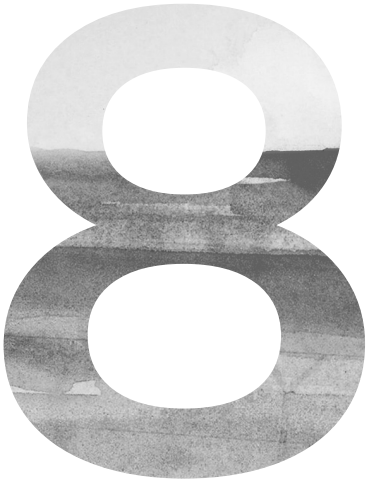

DUTCH SUMMARY

ACKNOWLEDGEMENTS

CURRICULUM VITAE

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DUTCH SUMMARY

NEDERLANDSE SAMENVATTING

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Het onderwerp van dit proefschrift is de rol van ontstekingen in de pathogenese van leeftijdsgebonden oogziekten, zoals maculadegeneratie, uvea melanoom en glaucoom. Veroudering is onvermijdelijk en is een multidimensionaal proces van fysieke, psychologische en sociale veranderingen. Met een toename van de oudere populatie (60 jaar en ouder) wereldwijd zullen we te maken krijgen met een toenemende hoeveelheid mensen met leeftijdsgebonden oogziekten. Naarmate we ouder worden, worden onze lichaamscellen steeds meer blootgesteld aan oxidatieve en metabole stress, hetgeen uiteindelijk leidt tot weefselschade. Laaggradige ontstekingsreacties, ook wel *para-inflammatie* genoemd, proberen de weefselfunctionaliteit en homeostase te herstellen. Dit heeft geen invloed op het goed functioneren van ons beschermende immuunsysteem. Secundaire veranderingen die optreden bij veroudering, zoals metabole en genetische afwijkingen, veranderde vaatdoorbloeding of degeneratieve aandoeningen, kunnen echter leiden tot een minder goed functioneren van het immuunsysteem, hetgeen uiteindelijk kan leiden tot een chronische en niet meer onderdrukte ontstekingsreactie. Ondanks dat bij verschillende ziekten specifieke genetische en omgevingsfactoren betrokken zijn, laat ik in dit proefschrift zien dat weefselontsteking een belangrijke en gemeenschappelijke component is die bijdraagt aan de pathogenese van meerdere leeftijdsgebonden oogziekten, zoals het uvea melanoom en glaucoom.

Uvea melanoom

Het is bekend dat ontstekingscellen zoals macrofagen en lymfocyten aanwezig zijn in uvea melanomen en dat hun aanwezigheid gepaard gaat met een slechte prognose. Niet alle uvea melanomen tonen een ontstekingsreactie, maar specifiek diegene met een genetisch verhoogd risico op metastasering. Deze hoog-risico tumoren bevatten prognostisch ongunstige tumorkarakteristieken zoals de aanwezigheid van een ontstekingsinfiltraat, epithelioide cellen en een verhoogde vaatdichtheid, geassocieerd met verlies van een chromosoom 3 en verlies van een specifiek eiwit, BAP1. In een muizenmodel van intraoculaire melanomen bleek dat de aanwezigheid van pro-angiogene M2 type macrofagen de tumorgroei bevordert in oudere muizen: macrofagen in oudere muizen hebben een sterk tumor-bevorderende rol. Tumor-geassocieerde macrofagen zijn direct betrokken bij de vorming van nieuwe bloedvaten die de tumor voorzien van voedingsstoffen: ze creëren een route voor verspreiding van tumorcellen via de bloedbaan.

Bij mensen (Hoofdstuk 2) worden pro-angiogene en anti-inflammatoire M2-type macrofagen gekarakteriseerd door een verhoogde expressie van CD163, en wij laten zien dat het merendeel van de macrofagen in uvea melanoom bij mensen dit fenotype bevatten. Opvallend genoeg bevatten tumoren met monosomie 3 meer M2-type macrofagen en meer bloedvaten dan tumoren met disomie van chromosoom 3. In Hoofdstuk 2 hebben we ook bepaald welke subtypen T cellen aanwezig zijn in deze tumoren. De aanwezigheid van regulatoire T cellen (Tregs) kan tumoren helpen om een effectieve anti-tumor immuun respons te ontwijken. Onder fysiologische omstandigheden worden Tregs gebruikt om lokale ontstekingsreacties te remmen middels onderdrukking van andere T cellen, macrofagen en antigeen-presenterende cellen. Onze data laat zien dat het aantal $CD163^+$ M2-type macrofagen gecorreleerd is aan het aantal $CD3^+$ $FoxP3^+$ regulatoire T cellen, terwijl het merendeel van de T lymfocyten bestaat uit $CD8^+$ T cellen. Behalve het aantal macrofagen was ook het aantal lymfocyten in de tumor gerelateerd aan monosomie 3. Daarom denken wij dat de intrinsieke maligne eigenschappen van de tumor leiden tot een gemixte ontstekingsreactie die vervolgens tumorgroei stimuleert. De aanwezigheid van immunosuppressieve immuuncellen in de tumor, zoals M2-type macrofagen en $FoxP3^+$ T regs, kan verklaren waarom de aanwezigheid van grote hoeveelheden cytotoxische $CD8^+$ T cellen niet leidt tot een effectieve anti-tumor activiteit.

Tot 1970 was enucleatie de traditionele behandeling van uvea melanomen, daarna ontstond een shift naar oogsparende technieken. Een bestraald oog kan in meer dan 80% van de gevallen worden behouden. Secundaire enucleatie is vereist bij tumor recidief of bij bestralingscomplicaties. Aangezien lokale immuunreacties een rol kunnen spelen in het verwijderen van tumor debris of in het stimuleren of onderdrukken van anti-tumor immuun reacties, hebben wij in Hoofdstuk 3 de aanwezigheid van ontstekingscellen vergeleken tussen uvea melanomen na primaire enucleatie en na bestraling (secundaire enucleatie). We hebben laten zien dat bestraling geen effect heeft op de hoeveelheid en het type tumor-infiltrerende macrofagen en dat deze tumoren een vergelijkbare variabiliteit aan infiltraat tonen als tumoren in primair geënucleerde ogen. Verder bleek dat bestraalde uvea melanomen meer lymfocyten bevatten dan niet-bestraalde ogen. Aangezien het aantal infiltrerende cellen hoger is in epithelioide tumoren dan in spindle cell tumoren, en het epithelioid type geassocieerd is met monosomie 3, kan het zijn dat primaire tumor karakteristieken, zoals de genetische status, en niet de behandeling zelf de celinfiltratie bepaalt. Omdat er echter weinig niet-necrotisch tumor materiaal te vinden was in secundair

geënucleerde ogen, waren wij helaas niet in staat om de chromosale status van deze bestraalde tumoren te bepalen.

Glaucoom

Glaucoom is wereldwijd een medische uitdaging vanwege de prevalentie in met name de oudere populatie, de ernstige gevolgen en een gebrek aan effectieve behandelingen. Ondanks dat een verhoogde oogdruk een belangrijke risicofactor is voor het ontwikkelen van glaucoom, wordt glaucoom niet langer gezien als een neurodegeneratieve aandoening, enkel en alleen veroorzaakt door een verhoogde oogdruk. Ongeveer 20-30% van de glaucoompatiënten hebben een normale oogdruk, en progressieve gezichtsvelduitval kan ook voorkomen in patiënten met goed gecontroleerde oogdrukken. Tot op heden is het exacte mechanisme van de pathogenese van glaucoom niet volledig bekend. Het immuunsysteem kan bij glaucoom zowel beschermend als destructief werken. De aanwezigheid van macrofagen en T cellen in het netvlies maakt het mogelijk dat het immuunsysteem vroeg in contact komt met cel débris door destructie van beschadigde cellen. Een initiële immuunreactie kan weefselherstel bevorderen, zoals boven beschreven voor parainflammatie. De infiltrerende immuuncellen beschermen de neuronen tegen degeneratieve condities in het verouderende oog, hetgeen ook wel 'beschermende immuniteit' wordt genoemd. Wanneer er echter andere factoren zoals verhoogde oogdruk, zuurstoftekort of te veel ontstekingsfactoren bij komen, kan een beschermende immuniteit veranderen in een neuroinflammatoir degeneratief proces dat zenuwverval stimuleert. In Hoofdstuk 4 beschrijven we uitgebreid de rol van het immuun systeem bij de ontwikkeling van glaucoomschade.

In Hoofdstuk 5 en 6 laten we zien dat een verhoogde oogdruk secundaire reacties veroorzaakt die leiden tot langdurige en progressieve zenuwschade, zelfs wanneer de oogdruk vervolgens terugkeert naar een normale waarde. Eerder werd beschreven dat auto-immuun reacties tegen heat-shock eiwitten (Hsps) mogelijk een rol spelen bij glaucoom, aangezien auto-antilichamen tegen Hsps werden gevonden in het serum van glaucoompatiënten. In Hoofdstuk 5 laten we zien dat een 3-week durende drukstijging leidt tot een verhoogde expressie van Hsp27 in retinale ganglioncellen (RGCs). Daarnaast induceert een verhoogde oogdruk een activatie van $CD11b^+$ microglia/ macrofagen en infiltratie van T cellen in de retina. Dit leidt tot een $CD4^+$ T cel reactie die specifiek is gericht tegen Hsp27 met als gevolg langdurige en progressieve glaucomateuze neurodegeneratie,

die nog steeds door bleek te gaan nadat de oogdruk al genormaliseerd was. Wanneer we oculaire hypertensie induceerden in immunodeficiënte muizen zagen we geen progressieve schade van de RGCs en axonen. Wanneer echter reactieve T cellen werden geïsoleerd uit muizen met glaucoom en de cellen werden overgebracht naar immunodeficiënte muizen met verhoogde oogdruk, zagen we alsnog RGC en axon neurodegeneratie ontstaan.

Hetzelfde fenomeen zagen wij wanneer retinale en nervus opticus schade werd veroorzaakt door ischemie (tgv. acuut verhoogde oogdruk) of traumatische schade. Ook hier leidden T cel-gemedieerde auto-immuun reacties tot chronisch RGC en axon verlies, terwijl de initiële periode van schade (ischemie) al voorbij was. Door het in het oog injecteren van neutraliserende en blokkerende antilichamen konden deze $CD4^+$ T cel-gemedieerde immuunreacties worden onderdrukt. Dit leidde tot een drastische daling in het RCG verlies na ischemische schade. Het blokkeren van lokale $CD4^+$ T cel activiteit leidt niet alleen tot neuroprotectie, maar zorgt ook voor het behoud van de retinale functie door het beschermen van andere retinale cellen tegen ischemie (Hoofdstuk 6). We hebben ook laten zien dat Hsp27-specifieke T cel reacties een belangrijke rol spelen in de pathogenese van glaucomateuze neurodegeneratie bij mensen, waarbij niet alleen tegen Hsp-gerichte auto-antilichamen maar ook een significant verhoogd aantal Hsp27 en Hsp60-reactieve T cellen werden gezien in het bloed van glaucoompatiënten.

Aangezien Hsps sterk geconserveerd zijn van bacteriën tot mensen, veronderstellen wij dat de schadelijke ontstekingsreacties in het menselijke oog ontstaan nadat zich eerder Hsp-specifieke T cellen hebben gevormd door blootstelling aan bacteriën. Deze T cellen zullen vervolgens beschadigde RGCs en axonen herkennen, omdat zij door de beschadiging Hsp's tot expressie brengen, wat uiteindelijk leidt tot een auto-immuun reactie tegen RGCs en daarmee tot optische neuropathie. In Hoofdstuk 5 laten we zien dat muizen die zijn grootgebracht in een germfree omgeving geen glaucomateuze schade ontwikkelen ondanks een persisterende verhoogde oogdruk. Het blijkt dat muizen die nooit blootgesteld zijn aan bacteriële stimuli een meer tolerant en een 'ongetraind' immuunsysteem hebben vergeleken met conventioneel grootgebrachte muizen.

Concluderend: Ondanks dat de pathogenese van uvea melanoom en glaucoom verschilt, delen deze oogziekten twee gemeenschappelijke elementen: 1) beiden zijn typisch een ziekte van de oudere mens 2) overdreven ontstekingsreacties spelen een belangrijke rol in

beiden. Door de extreme plasticiteit van ons immuunsysteem kunnen we snel en adequaat reageren op weefselschade-en stress zonder een schadelijke immunologische respons te veroorzaken. Het immuunsysteem dient echter niet alleen om ons te beschermen tegen schade of infecties, maar het kan ook betrokken zijn bij schadelijke en chronische immuunreacties die leiden tot neurodegeneratieve ziekten, zoals glaucoom. Aan de andere kant kunnen door het 'immuun-privilege' van het oog, tumoren zoals uvea melanomen rustig en onbelemmerd groeien door te ontsnappen aan afstoting door het immuunsysteem. Het is daarom van belang om de mechanismen te begrijpen die ten grondslag liggen aan de complexe wisselwerking tussen 'immuun-privilege', beschermende immuniteit, ontstekingsreacties en auto-immuunziekten om de pathogenese van leeftijdsgebonden ziekten beter te begrijpen. Toekomstige studies zullen meer licht werpen op onbegrepen aspecten van de rol van de verschillende typen ontstekingscellen bij leeftijdsgebonden oogziekten.

Dutch summary

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ACKNOWLEDGEMENTS

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CURRICULUM VITAE

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Khanh Vu was born on the January 7, 1987 in Ho Chi Minh City, Vietnam. She moved to The Netherlands at the age of three and was raised in Helmond. After graduating cum laude from the Jan van Brabant College in Helmond in 2005, she started medical school at Leiden University. In her last year of medical school, she started a six-month long scientific project on inflammation in uveal melanoma at the Department of Ophthalmology of the Leiden University Medical Center under the supervision of Prof. Dr. Martine J. Jager. After obtaining her M.D. and graduating cum laude in 2011, she continued on to pursue a Ph.D., focusing on the role of the immune system in glaucoma at the Schepens Eye Research Institute/Massachusetts Eye and Ear Infirmary, Harvard Medical School in Boston under the supervision of Dr. Dong Feng Chen. During her Ph.D. training, the author had the opportunity to present her work at various meetings and conferences. In 2012, she received a grant from the Royal Young Talent Research Award from the Prins Bernhard Cultuurfonds. At the annual meeting of the Association for Research in Vision and Ophthalmology in 2014, the author's poster was selected as top five in her section and competed for the Members-in-Training outstanding Poster Award. In February 2015, the author started her residency training in Ophthalmology under the supervision of Prof. Dr. Gré P.M. Luyten.

Curriculum Vitae

LIST OF PUBLICATIONS

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