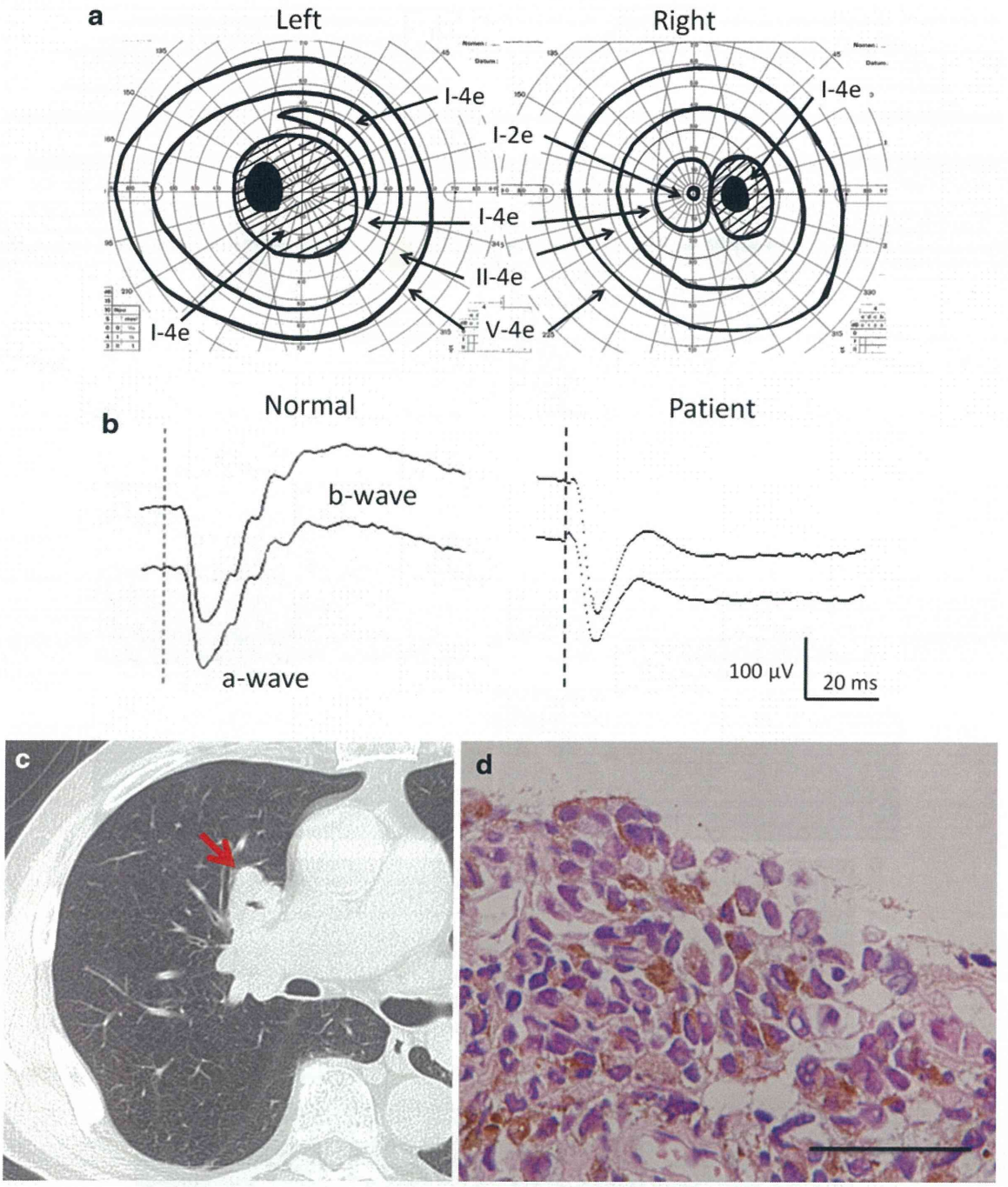

Fig. 2 Goldmann visual fields, full-field ERGs, CT of the right lung, and histology of a specimen obtained from the hilar lymph node of the patient. **a** Goldmann visual fields revealed a relative central scotoma including the blind spot OD and a relative scotoma around the blind spot OS. **b** Full-field electroretinograms (ERGs) with a bright flash stimulus after dark adaptation showed a "negative-type" pattern for the patient (*right*) compared with the normal pattern (*left*). **c** A computed tomography (CT) scan revealed a tumor in the right lung (*red arrow*). **d** Hematoxylin–eosin staining of a biopsy specimen obtained from a hilar lymph node in the right lung revealed spindle-shaped malignant cells. *Scale bar* 50 µm

TRPM1 is a component of the visual transduction cation channel specifically expressed in retinal ON bipolar cells [15–17, 19]. Immunohistology of the adult mouse retina revealed punctate TRPM1 signals at the tips of ON bipolar cell dendrites detected with antibodies to metabotropic glutamate receptor 6 (mGluR6) and to the α subunit of the G_o protein [16]. TRPM1-null mice were also shown to lack ON bipolar cell responses to light [15, 16]. In addition, *TRPM1* mutations have been associated with the complete form of congenital stationary night blindness (CSNB) in

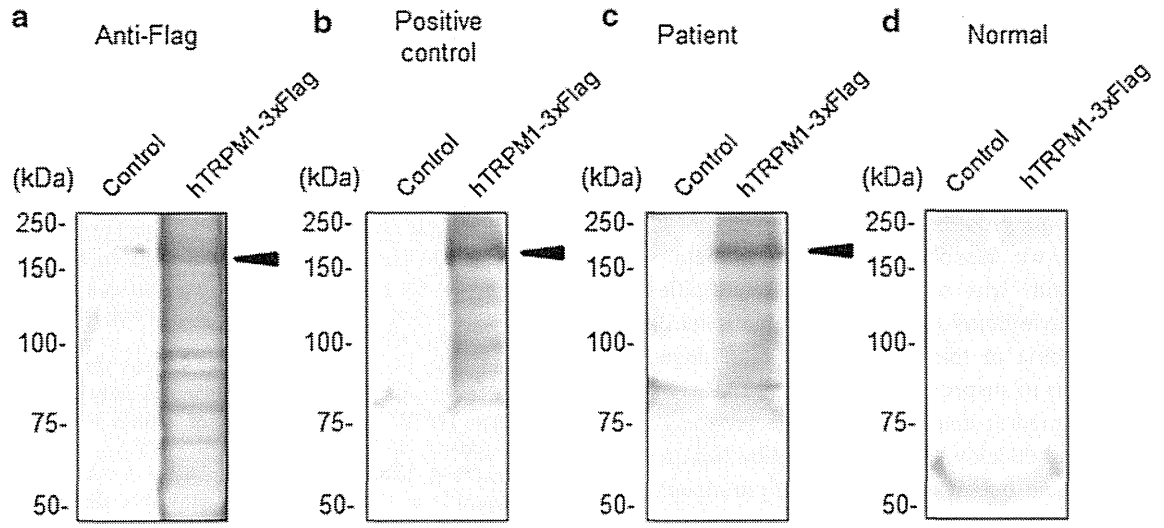

Fig. 3 Immunoblot analysis of serum from the patient for reactivity with TRPM1. HEK293T cells transfected with the expression vector pCAGGS alone (*control*) or with pCAGGS encoding human transient receptor potential cation channel, subfamily M, member 1 (*TRPM1*) tagged with three copies of the flag epitope were subjected to immunoblot analysis with antibodies to the flag tag (**a**), with serum from a patient previously shown to contain autoantibodies to TRPM1 as a positive control (**b**), with serum from the proband (**c**), and with normal control serum (**d**). *Arrowheads* indicate the 3× flag-tagged TRPM1 protein, which showed substantial reactivity with serum from the proband. The positions of molecular size standards (in kilodaltons) are also shown

humans, which is characterized by pronounced dysfunction of the retinal ON pathway [20–23]. These observations suggest that TRPM1 plays a key role in synaptic transmission from photoreceptors to ON bipolar cells. It is possible that ectopic expression of TRPM1 in the malignant melanoma cells of the patient triggered the production of autoantibodies to this protein by B lymphocytes. These antibodies might then have reacted with TRPM1 in retinal ON bipolar cells, resulting in severe dysfunction of the retinal ON pathway.

The average latency from the diagnosis of melanoma to that of MAR was previously found to be 3.6 years, with a range of 2 months–19 years [5]. We diagnosed the present patient with malignant melanoma after only 18 days from the time of his first visit. However, he died 11 months later as a result of metastasis to several organs. In the present case, the visual symptoms preceded the diagnosis of malignant melanoma. The patient complained of blurred vision, night blindness, and photopsia; but Fundus photographs, fluorescein angiograms, and SD-OCT findings were all essentially normal at his initial visit. Perimetry revealed relative scotomas OU. It was his "negative-type" ERGs, indicative of extensive bipolar cell dysfunction, that led us to suspect the patient might have MAR. We, therefore, recommend that ERGs be performed on patients with progressive visual disturbance of unknown origin.

"Negative-type" ERGs are also observed in patients with CSNB. A previous study [5] found that 18 of 27 patients with MAR showed either central or paracentral scotomas or depressions, and 6 of 27 patients had arcuate visual field defects. The present patient had relative central scotomas that included or surrounded the blind spot. In contrast, patients with CSNB manifest a largely intact visual field. MAR patients also show a greater loss of S (blue) cone sensitivity by perimetry than do CSNB patients, and S cone ERGs were not detected in MAR patients [2]. Two subtypes of CAR were previously identified [2]: one in which cone cells are damaged and in which Goldmann perimetry reveals central scotoma, and one in which rod cells are damaged and in which Goldmann perimetry reveals annular scotoma. We speculate that the visual field disturbance of the present patient reflected damage to cone cells.

Drug therapy for MAR is aimed at immunomodulation in order to attenuate the autoimmune attack on the retina before irreversible damage occurs. Treatments include corticosteroid administration, plasmapheresis, intravenous injection of immunoglobulin and immunosuppression. However, the effectiveness of these treatments remains unclear. Oral corticosteroid treatment alone was found to be beneficial in only one of seven patients [5]. In the present study, the patient was treated with oral prednisolone. We believe that this treatment was effective because the vitreous opacity and enlarged blind spots were markedly attenuated after its onset. Further studies are needed to determine the optimal treatment for MAR.

There are several limitations to our study. First, we recorded only bright-flash ERGs after dark adaptation; we did not record rod responses to low-intensity stimuli, photopic responses, or photopic ERGs in response to a

long-duration stimulus in order to determine whether the postreceptor ON pathway was specifically affected. Second, we did not perform immunohistochemical analysis with the serum of the patient to confirm that the autoantibodies recognize retinal bipolar cells. And third, we did not confirm that the autoantibodies actually reacted with malignant melanoma proteins.

In conclusion, we described a Japanese patient with MAR whose serum was positive for autoantibodies to TRPM1. Visual symptoms preceded the identification of malignant melanoma in this patient, and his "negative-type" ERGs led us to suspect a diagnosis of MAR. Further studies are warranted to determine both the proportion of MAR patients who develop TRPM1 autoantibodies as well as the best treatment option for this type of paraneoplastic retinopathy.

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References

1. Berson EL, Lessell S. Paraneoplastic night blindness with malignant melanoma. Am J Ophthalmol. 1988;106:307–11.
2. Milam AH, Saari JC, Jacobson SG, Lubinski WP, Feun LG, Alexander KR. Autoantibodies against retinal bipolar cells in cutaneous melanoma-associated retinopathy. Invest Ophthalmol Vis Sci. 1993;34:91–100.
3. Potter MJ, Thirkill CE, Dam OM, Lee AS, Milam AH. Clinical and immunocytochemical findings in a case of melanoma-associated retinopathy. Ophthalmology. 1999;106:2121–5.
4. Lei B, Bush RA, Milam AH, Sieving PA. Human melanoma-associated retinopathy (MAR) antibodies alter the retinal ON response of the monkey ERG in vivo. Invest Ophthalmol Vis Sci. 2000;41:262–6.
5. Keltner JL, Thirkill CE, Yip PT. Clinical and immunologic characteristics of melanoma-associated retinopathy syndrome: eleven new cases and a review of 51 previously published cases. J Neuroophthalmol. 2001;21:173–87.
6. Alexander KR, Barnes CS, Fishman GA, Milam AH. Nature of the cone ON pathway dysfunction in melanoma-associated retinopathy. Invest Ophthalmol Vis Sci. 2002;43:1189–97.
7. Chan JW. Paraneoplastic retinopathies and optic neuropathies. Surv Ophthalmol. 2003;48:12–38.
8. Adamus G. Autoantibody targets and their cancer relationship in the pathogenicity of paraneoplastic retinopathy. Autoimmun Rev. 2009;8:410–4.
9. Potter MJ, Adamus G, Szabo SM, Lee R, Mohaseb K, Behn D. Autoantibodies to transducin in a patient with melanoma-associated retinopathy. Am J Ophthalmol. 2002;134:128–30.
10. Hartmann TB, Bazhin AV, Schadendorf D, Eichmuller SB. SEREX identification of new tumor antigens linked to melanoma-associated retinopathy. Int J Cancer. 2005;114:88–93.
11. Lu Y, Jia L, He S, Hurley MC, Leys MJ, Jayasundera T, et al. Melanoma-associated retinopathy: a paraneoplastic autoimmune complication. Arch Ophthalmol. 2009;127:1572–80.
12. Bazhin AV, Dalke C, Willner N, Abschütz O, Wildberger HGH, Philippov PP, et al. Cancer-retina antigens as potential paraneoplastic antigens in melanoma-associated retinopathy. Int J Cancer. 2009;124:140–9.
13. Dhingra A, Fina ME, Neinstein A, Ramsey DJ, Xu Y, Fishman GA, et al. Autoantibodies in melanoma-associated retinopathy target TRPM1 cation channels of retinal ON bipolar cells. J Neurosci. 2011;31:3962–7.
14. Kondo M, Sanuki R, Ueno S, Nishizawa Y, Hashimoto N, Ohguro H, et al. Identification of autoantibodies against TRPM1 in patients with paraneoplastic retinopathy associated with ON bipolar cell dysfunction. PLoS One. 2011;6:e19911.
15. Morgans CW, Zhang J, Jeffrey BG, Nelson SM, Burke NS, Duvoisin RM, et al. TRPM1 is required for the depolarizing light response in retinal ON bipolar cells. Proc Natl Acad Sci USA. 2009;106:19174–8.
16. Koike C, Obara T, Uriu Y, Numata T, Sanuki R, Miyata K, et al. TRPM1 is a component of the retinal ON bipolar cell transduction channel in the mGluR6 cascade. Proc Natl Acad Sci USA. 2010;107:332–7.
17. Koike C, Numata T, Ueda H, Mori Y, Furukawa T. TRPM1: a vertebrate TRP channel responsible for retinal ON bipolar function. Cell Calcium. 2010;48:95–101.
18. Loduca AL, Zhang C, Zelkha R, Shahidi M. Thickness mapping of retinal layers by spectral-domain optical coherence tomography. Am J Ophthalmol. 2010;150:849–55.
19. Klooster J, Blokker J, Ten Brink JB, Unmehopa U, Fluiter K, Bergen AA. Ultrastructural localization and expression of TRPM1 in the human retina. Invest Ophthalmol Vis Sci. 2011;52:8356–62.
20. Li Z, Sergouniotis PI, Michaelides M, Mackay DS, Wright GA, Devery S, et al. Recessive mutations of the gene TRPM1 abrogate ON bipolar cell function and cause complete congenital stationary night blindness in humans. Am J Hum Genet. 2009;85:711–9.
21. van Genderen MM, Bijveld MM, Claassen YB, Florijn RJ, Pearring JN, Meire FM, et al. Mutations in TRPM1 are a common cause of complete congenital stationary night blindness. Am J Hum Genet. 2009;85:730–6.
22. Audo I, Kohl S, Leroy BP, Munier FL, Guillonneau X, Mohand-Saïd S, et al. TRPM1 is mutated in patients with autosomal-recessive complete congenital stationary night blindness. Am J Hum Genet. 2009;85:720–9.
23. Nakamura M, Sanuki R, Yasuma TR, Onishi A, Nishiguchi KM, Koike C, et al. TRPM1 mutations are associated with the complete form of congenital stationary night blindness. Mol Vis. 2010;16:425–37.

Special Issue: Inflammation in Ophthalmology

Mini Review

Regulation of Th1 and Th17 cell differentiation in uveitis

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Noninfectious uveitis is one of the sight-threatening disorders that are associated with systemic autoimmune diseases, such as Behçet's disease. Uveitis is often recurrent and causes subsequent tissue destruction and scarring, especially in the retina and uvea, leading to permanent loss of vision. Early studies have shown that T-helper (Th) 1 cells are the major effector cells and are critical for the development of uveitis. Recently, Th17 cells, a newly defined effector T-helper lineage that is distinct from Th1 and Th2 cell lineages, were also shown to play a pivotal role in the pathogenesis of uveitis. Furthermore, several clinical studies have reported that biological agents targeting Th17-related cytokines, such as IL-6, IL-23, and TNF-α, induced and maintained remission in human autoimmune diseases, including rheumatoid arthritis, Crohn's disease, psoriasis, and noninfectious uveitis. In this mini-review, we focus on the roles of proinflammatory cytokines in the regulation of Th1 and Th17 cell responses in uveitis, both experimentally and clinically. A deeper understanding of the underlying mechanisms will provide new insights into the development of new therapies for refractory human noninfectious uveitis.

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Key words cytokine, eye, Th1, Th17, uveitis

Introduction

Ocular inflammation, which is termed generally as uveitis, leads to vision loss as a result of the destruction and scarring of delicate tissue along the visual axis, especially in the retina and uvea. Thus, understanding the pathophysiology of uveitis, especially that of autoimmune origin, is important for therapy. Experimental autoimmune uveoretinitis (EAU) is a well-established animal disease model that resembles human uveitis and serves as a model for investigating the mechanism of human uveitis[1]. EAU is an organ-specific T cell-mediated autoimmune disease that can be induced in several susceptible animal species by immunization with retinal self-antigens, such as interphotoreceptor retinoid-binding protein (IRBP) and the retinal soluble antigen (S-Ag),

emulsified with complete Freund's adjuvant (CFA). During EAU, the integrity of the blood-retinal barrier is compromised, and infiltration of monocyte/macrophages and antigen-specific CD4+ T lymphocytes into the retina causes tissue damage. The adoptive transfer of retinal antigen-specific CD4+ T cells into naïve syngenetic recipients induces EAU[2]. In humans, several lines of evidence have revealed that activated T cells, especially CD4+ T cells, play pivotal roles in the pathogenesis of autoimmune uveitis[3-5]. In previous studies, Mossman, Coffman, and their colleagues advocated the T helper (Th) 1/Th2 paradigm to explain the immune responses involved in infection, autoimmunity, and allergy[6]. When naïve CD4+ T cells encounter antigens, effector naïve CD4+ T cells begin to differentiate into interferon (IFN)-γ-producing Th1 or Th2 cells, depending on the cytokine milieu; this is considered to be a critical process in the control of autoimmunity or allergy[7]. Recently, a third lineage of newly recognized interleukin (IL)-17-producing CD4+ T cells, called Th17 cells, were found to a crucial role in several autoimmune diseases by mediating tissue inflammation[8]. Transforming growth factor-β1 (TGF-β1) and IL-6 have been found to initiate Th17 cell differentiation, while IL-23 was found to enhance the expansion or activation of Th17 cells[9]. Th17 cells produce proinflammatory cytokines, such as IL-17A, IL-21, and tissue necrosis factor (TNF)-α. Recently, several biological agents that target proinflammatory cytokines, including IL-6, IL-17A, TNF-α, and IL-23, have been developed for the treatment of autoimmune diseases, such as rheumatoid arthritis, Crohn's disease, psoriasis, and noninfectious uveitis[10]. Anti-TNF-α therapy with infliximab, which is a chimeric monoclonal antibody against TNF-α, has been validated in patients with refractory uveoretinitis in Behçet's disease in Japan[11]. At the 12-month follow-up, uveitis improved in more than 90% of the patients and worsened in none. Thus, the elucidation of particular CD4+ T cell subsets for uveitis has been a key to understanding the pathogenesis of uveitis and developing new effective treatments. This review focuses on some roles of several proinflammatory cytokines in the differentiation of naïve CD4+ T cells into Th1 and Th17 cells in uveitis.

Th1 and uveitis

Th1 cells, which secrete IL-2, IFN-γ, and lymphotoxin, are critical for macrophage activation and nitric oxide production, which is required for eliminating intracellular pathogens and cell-mediated and delayed-type hypersensitivity

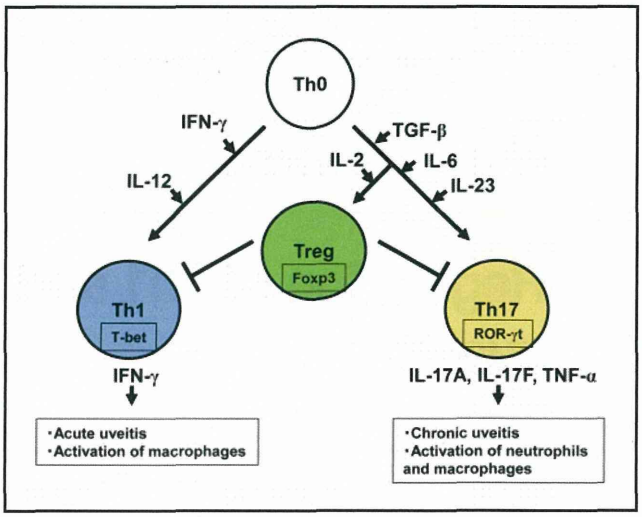

Fig. 1 Scheme of Th1 and Th17 cells differentiation

Differentiation of T helper (Th) 1, Th17, and iTreg cells from naïve CD4+ T cells. Designated cytokines promote differentiation of naïve CD4+ T cells into Th1, Th17, or iTreg cells after antigen presentation. T-bet, ROR-γt, and Foxp3 are the key transcriptional factors for the differentiation of Th1, Th17, and iTreg cells. Th1 cells play central roles in early/acute phase of uveitis, whereas Th17 cells act in the late/choronic phase of uveitis. iTreg cells suppress both Th1 and Th17 cell responses.

IFN, interferon; IL, interleukin; Foxp, forkhead box protein; ROR-γt, retinoid-related orphan receptor-γt; T-bet, T box expressed in T cells; TGF, transforming growth factor

(Fig. 1), whereas Th2 cells, which secrete IL-4, IL-5, IL-6, IL-9, and IL-13, are necessary for promoting the humoral immunity that underlies responses to helminthic infections and allergies[12]. In early studies, Th1 cell responses have been reported to play important roles in the induction of autoimmune diseases such as experimental autoimmune encephalomyelitis (EAE) and collagen-induced arthritis (CIA)[13], whereas Th2 cell responses were found to suppress these diseases. In Th1 cell differentiation, signal transducer and activator of transcription (STAT) 1, which functions downstream of IFN-γ, is an important transcriptional factor for inducing T-bet, a master transcription factor for Th1 cells, and subsequent expression of IL-12 receptor (R) β2 chain, in order to respond to IL-12 stimulation[14]. IL-12, which is a heterodimeric cytokine composed of p40 and p35, transmits signals through STAT4, leading to the expansion of Th1 cells and enhancement of IFN-γ production[15]. As in EAE, several studies have revealed that autoreactive Th1 cells mediate EAU and that their induction correlates with the production of IFN-γ by T cells[16]. Furthermore, it has been reported

that augmented Th1 cell responses result in high susceptibility to ocular autoimmunity[17]. As seen in patients with uveitis, such as Behçet's disease, Th1 cell responses are also associated with disease activity[18]. Recently, several IL-12-related cytokines have been identified, and it has been revealed that the action of these cytokines is related to the function and differentiation of $CD4^+$ T cells in autoimmunity. Among them, IL-27, which is a heterodimeric cytokine composed of Epstein-Barr virus-induced gene 3 (EBI3) and IL-27p28, engages a receptor composed of gp130 and IL-27Rα[19]. Initially, IL-27 was reported to be a proinflammatory cytokine that induces Th1 cell responses. However, IL-27 is now widely regarded as a regulatory cytokine of activated T cells, such as Th17 cells, as discussed later in this mini-review. In an analysis of IL-27Rα-deficient mice, IL-27Rα was found to be required for the normal production of IFN-γ by naïve $CD4^+$ T cells[20]. Subsequently, we and others demonstrated that IL-27 does not directly induce IFN-γ production, but rather induces IL-12-dependent IFN-γ production by the augmentation of T-bet and subsequent IL-12Rβ2 expression in naïve $CD4^+$ T cells[21-23]. Consistent with these results, we found that IL-27 participates in the development of Th1 cell responses and invasion of inflammatory cells in the eyes, especially in the early phase of EAU[24]. Taken together, these results suggest that Th1 cell responses are important for the initiation of EAU.

However, neutralization of IFN-γ, which is a hallmark cytokine for Th1 cells, is highly susceptible to EAU[25]. Similarly, mice deficient in IL-12p35 are susceptible to EAU by enhanced induction of proinflammatory cytokines other than IFN-γ, even though mice deficient in IL-12p40 protected against EAU[26, 27]. Oppmann et al. found that IL-12 shares the p40 subunit with IL-23, which activates $CD4^+$ T cells distinctly from IL-12[28]; thus, the protective role of deletion or neutralization of p40 in EAU reflects the function of IL-23, but not that of IL-12. Furthermore, when we neutralized regulated on activation, normal T cell expressed and secreted (RANTES), a Th1-related chemokine, cellular infiltration into the eyes and the disease severity of EAU were exacerbated in the late phase[29]. These results suggested that Th1 cell responses play not only a proinflammatory role, but also a protective role in EAU. Alternatively, there may be another T cell lineage that can promote autoimmunity independently of both Th1 and Th2 cell lineages, including in EAU.

Th17 and uveitis

Th17 cells constitute a Th cell lineage distinct from Th1 and Th2 cells[30]. The generation of Th17 cells is enhanced by the blockade of IL-4 and IFN-γ and is independent of Th1- or Th2-related transcriptional factors. Th17 cells produce proinflammatory cytokines, such as IL-17A, IL-17F, IL-21, IL-22, TNF-α, and granulocyte macrophage-colony stimulating factor (GM-CSF)[8]. IL-17A induces inflammation mainly through release of pro-inflammatory and neutrophil-mobilizing cytokines/chemokines, leading to neutrophil trafficking to the site of inflammation. Enhanced expression of IL-17A is also observed in human peripheral blood mononuclear cells from patients with various autoimmune diseases, including active uveitis in VKH and Behçet's disease[31, 32].

By analyzing Th17 cell responses in EAU in IL-17A-deficient mice, we and others found that mice deficient in IL-17A are susceptible to EAU because of the induction of Th1 cell responses[27, 33, 34]. In addition, we found that IL-17 deficiency is dispensable for the induction of EAU and diminishes the severity of EAU only in the late phase[33]. Furthermore, the augmentation of Th17 cell responses by the systemic administration of both anti-IFN-γ and anti-IL-4 neutralizing antibodies exacerbated EAU in the late phase. Thus, these results suggest that Th17 cell responses contribute to the disease severity, not in the initial phase, but particularly in the late/chronic phase of EAU (Fig. 1). On the other hand, analysis of mice deficient in IL-17A revealed that Th1 cell responses and subsequent invasion of inflammatory cells into the eyes occurs in the early phase of EAU. Amadi-obi et al. also demonstrated that expression of IL-17 in the retina is very low before cellular infiltration starts and that elevation of IL-17 expression in the retina is observed after cellular invasion into the eyes[35]. Thus, it is reasonable to assume that Th1 cells, rather than Th17 cells, play some roles in the initial phase of EAU. Recently, Tang et al. revealed that uveitis could not be induced in mice deficient in IFN-γ despite enhancement of Th17 cell responses in their Th1-dominant antigen-pulsed dendritic cell-induced EAU, which is different from conventional EAU, suggesting that Th17 cell responses are not essential for the induction of EAU[36]. Furthermore, we and others have shown that IFN-γ increases in the aqueous humor of acute human uveitis patients, but not in the vitreous humor of patients with chronic uveitis[37, 38]. Taken together, we speculate that Th1 cell responses may be important in the acute or initial phase, but not in the late phase of ocular inflammation in uveitis (Fig. 1).

The roles of IL-6 and IL-23 in uveitis

Initially, IL-23, a member of the IL-12-related cytokine family, which contains the p40 subunit common to IL-12 and IL-23 as well as a unique p19 subunit, was reported to be responsible for Th17 cell expansion and to be critical for the development of autoimmune diseases, such as EAE and CIA[13]. IL-23 transmits its signaling via IL-23R and STAT3[42]. However, since naïve CD4+ T cells express very low levels of IL-23R, this process produces only small fractions of IL-17-secreting T cells[39]. Subsequently, TGF-β, in combination with IL-6, IL-21, or IL-1β, initiates Th17 cell differentiation, leading to the enhanced expression of IL-23R and retinoid acid-related orphan receptor γ thymus (ROR-γt), which is a master transcriptional factor for Th17 cells, and resulting in the expansion of Th17 cells by IL-2[38]. On the other hand, TGF-β promotes the generation of inducible forkhead box protein 3 (Foxp3)-positive regulatory T (iTreg) cells, which are essential for maintaining peripheral tolerance, preventing autoimmune disease, and limiting chronic inflammatory diseases. However, IL-6 abolishes the inducible effect of TGF-β on iTreg cells[40]. Thus, the differentiation of Th17 and iTreg cells by TGF-β is reciprocally related through the action of IL-6.

In our analysis of mice deficient in IL-6 or IL-23p19, we demonstrated that lack of either IL-6 or IL-23 diminishes systemic induction of Th17 cell responses and suppression of EAU during the entire phase[38]. Several studies have provided evidence that Th17 cells promote the expression of IL-17A, IL-6, and TNF-α, resulting in the activation of fibroblasts, vascular endothelial cells, epithelial cells, and macrophages. These cells then produce chemokines to recruit neutrophils and macrophages into the retina, leading to the induction of regional inflammation[41]. We also found that the blockade of Th17 cell development reduces chemokine expression, leading to amelioration of both macrophage and neutrophil infiltration into the retina during EAU[38].

Recently, tocilizumab, a humanized anti-IL-6R monoclonal Ab, has been found to be effective for the treatment of RA patients[42]. To address the therapeutic efficacy of IL-6 in EAU, we utilized MR16-1, which is rat anti-mouse IL-6Rα neutralizing antibody, and found that systemic administration of MR16-1, but not regional treatment with this antibody, ameliorates EAU[38]. Interestingly, the blockade of IL-6 decreased Th1 cell responses as well as Th17 cell responses. Haruta et al. also showed that blockade of IL-6 signaling inhibits Th1 cell responses, but that this inhibition is abrogated by depletion of Foxp3+ Treg cells in EAU[43], suggesting that Foxp3+ Treg cells can inhibit Th1 cell responses in EAU. Thus, the targeting of IL-6 signaling is attractive for the treatment of uveitis by direct inhibition of Th17 cell development and indirect inhibition of Th1 cell responses.

Furthermore, several studies have revealed that IL-27 directly acts as a negative regulator of fully activated CD4+ T cells, including Th17 cells and that IL-27 also induces the differentiation of IL-10-producing type-1 regulatory T (Tr1) cells, which suppress Th17 cell responses[44]. In addition, we and others found that IL-27 can suppress IL-17 production by activated CD4+ T cells, thereby counteracting IL-23[35, 45]. In EAU, Amadi-Obi et al. found that IL-27 is constitutively expressed in the retina and that IL-27 can suppress IL-23-dependent production of IL-17 by uveitogenic CD4+ T cells[35]. In addition, Wang et al. reported that IL-27 was downregulated in active uveitis in patients with VKH and that an increase in IL-27 and a decrease in IL-17 were observed upon resolution of the disease by systemic administration of immunosuppressive drugs[31]. Thus, these results suggested that IL-27 is a therapeutic target for chronic inflammation in uveitis.

Anti-TNF-α therapy and uveitis

Based on the previous studies on autoimmunity in uveitis, uveitis patients are currently being treated with several immunosuppressive drugs that possess strong T-cell-suppressive effects, such as glucocorticoids, tacrolimus, and cyclosporine, to control inflammation in the eyes[10]. However, in patients with Behçet's disease, these drugs often fail to maintain remission of ocular inflammation on a long-term basis[46]. In addition, the long-term use of these drugs is unacceptable due to their severe systemic adverse side effects. Recently, several biological agents, such as monoclonal antibodies and a recombinant form of natural inhibitory molecules against proinflammatory cytokines or receptors, have been developed for the treatment of refractory immune disorders[10]. For instance, anti-TNF agents, such as infliximab, have been successfully applied to the treatment of refractory uveitis, including in Behçet's disease[10]. TNF-α is a soluble potent proinflammatory cytokine exerting pleiotrophic effects on various cell types and plays important roles in choronic inflammatory disorders[47]. It has been reported that Th17 cells as well as Th1 cells induce TNF-α in the active uveitis seen in Behçet's disease[48]. In addition, transmembrane TNF-α, the precursor form of TNF-α, also acts as a bipolar molecule that transmits signals as a ligand

as well as a receptor. Soluble TNF-α acts apart from TNF-α-bearing cells, whereas transmembrane TNF-α on TNF-α-bearing cells binds to TNF-α receptors on the target cells and transmits not only signals to the target cells as a ligand, but also reverse signals back to the TNF-α-bearing cells as a receptor, in a cell-to-cell contact manner[47]. Thus, transmembrane TNF-α is considered to play important roles in local inflammation. Since infliximab is a chimeric monoclonal antibody designed against the soluble and membrane-bound forms of TNF-α, the suppressive mechanism of infliximab involves inhibition not only of the proinflammatory roles of soluble TNF-α, but also of the action of TNF-α-producing cells, such as Th1 and Th17 cells.

Recently, Th22 cells, a Th cell lineage distinct from Th1, Th2, and Th17 cells, were found to secrete IL-22 and TNF-α and to be increased in active uveitis in Behçet's disease[49]. Th22 cell clones, which could be differentiated from $CD4^+$ T cells in patients with active Behçet's disease in the presence of IL-6 and TNF-α, secreted IL-22 and TNF-α, but not IFN-γ and IL-17. In addition, the Th22 cell clones failed to produce IL-22 after treatment with anti-TNF-α neutralizing Abs, anti-IL-6 neutralizing Abs, and infliximab[49]. Thus, an inhibitory effect of infliximab on ocular inflammation in patients with Behçet's disease may reflect the suppression of Th22 cell responses, as well as that of Th1 and Th17 cell responses. The potential mechanism of the effect of Th22 cell responses on the pathogenesis of other forms of uveitis may warrant further research in this area.

In conclusion

In the last decade, numerous studies on Th17 cells and Th17 cell responses have led to remarkable progress in the understanding of the pathophysiology of uveitis. In this review, we demonstrated that both Th1 and Th17 cell responses are responsible for the pathogenesis of uveitis, but that they act at different time points. However, in human uveitis, we cannot exclude that the relative importance of Th1 and Th17 cell responses differs depending on the circumstances, including the types of innate receptors and/or antigen-stimulating cells. A more profound understanding of the intricacies of immune responses in autoimmunity will improve innovation in approaches for the management and treatment of uveitis.

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Conflicts of interest

None

Reference

1) Kerr EC, Copland DA, Dick AD, Nicholson LB: The dynamics of leukocyte infiltration in experimental autoimmune uveitis. Prog Retin Eye Res. 2008; 27: 527-535.
2) Mochizuki M, Kuwabara T, McAllister C, Nussenblatt RB, Gery I: Adoptive transfer of experimental autoimmune uveoretinitis in rats. Invest Ophthalmol Vis Sci. 1985; 26: 1-9.
3) Ohta K, Norose K, Wang XC, Ito S, Yano A, Segawa K: Apoptosis-related fas antigen on memory T cells in aqueous humor of uveitis patients. Cur Eye Res. 1996; 15: 299-306.
4) Sugita S, Takase H, Taguchi C, Imai Y, Kamoi K, Kawaguchi T, Sugamoto Y, Futagami Y, Itoh K, Mochizuki M: Ocular infiltrating CD4+ T cells from patients with Vogt-Koyanagi-Harada disease recognize human melanocyte antigens. Invest Ophthalmol Vis Sci. 2006; 47: 2547-2554.
5) Kojima K, Maruyama K, Inaba T, Nagata K, Yasuhara T, Yoneda K, Sugita S, Mochizuki M, Kinoshita S: The CD4/CD8 ratio in vitreous fluid is of high diagnostic value in sarcoidosis. Ophthalmology. 2012; 119: 2386-2392.
6) Mosmann TR, Cherwinski H, Bond MW, Giedlin MA, Coffman RL: Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. J Immunol. 1986; 136: 2348-2357.
7) Romagnani S: Immunol Today. 1997; 18: 263-266.
8) Korn T, Bettelli E, Oukka M, Kuchroo VK: IL-17 and Th17 cells. Annu Rev Immunol. 2009; 27: 485-517.
9) Veldhoen M, Hocking RJ, Atkins CJ, Locksley RM, Stockinger B: TGF-β in the context of an inflammatory cytokine milieu supports de novo differentiation of IL-17-producing T cells. Immunity. 2006; 24: 179-189.
10) Mesquida M, Molins B, Llorenc V, Hernández MV, Espinosa G, Dick AD, Adán A: Current and future treatments for Behçet's uveitis: road to remission. Int Ophthalmol. 2013; Jun 1. [Epub ahead of print]
11) Okada AA, Goto H, Ohno S, Mochizuki M: Multicenter study of infliximab for refractory uveoretinitis in Behcet

disease. Arch Ophthalmol. 2012; 130: 592-598.
12) Mosmann TR, Sad S: The expanding universe of T-cell subsets: Th1, Th2 and more. Immunol Today. 1996; 17: 138-146.
13) Cua DJ, Sherlock J, Chen Y, Murphy CA, Joyce B, Seymour B, Lucian L, To W, Kwan S, Churakova T, Zurawski S, Wiekowski M, Lira SA, Gorman D, Kastelein RA, Sedgwich JD: Interleukin-23 rather than interleukin-12 is the critical cytokine for autoimmune inflammation of the brain. Nature. 2003; 421: 744-748.
14) Robinson DS, O'Garra A: Further checkpoints in Th1 development. Immunity. 2002; 16: 755-758.
15) Murphy KM, Ouyang W, Farrar JD, Yang J, Ranganath S, Asnagli H, Afkarian M, Murphy TL: Signaling and transcription in T helper development. Annu Rev Immunol. 2000; 18: 451-494.
16) Sun B, Sun SH, Chan CC, Wiggert B, Caspi RR: Autoimmunity to a pathogenic retinal antigen begins as a balanced cytokine response that polarizes towards type 1 in a disease-susceptible and towards type 2 in a disease-resistant genotype. Int Immunol. 1999; 11: 1307-1312.
17) Xu H, Rizzo LV, Silver PB, Caspi RR: Uveitogenicity is associated with a Th1-like lymphokine profile: cytokine-dependent modulation of early and committed effector T cells in experimental autoimmune uveitis. Cell Immunol. 1997; 178: 69-78.
18) Koarada S, Haruta Y, Tada Y, Ushiyama O, Morito F, Ohta A, Nagasawa K: Increased entry of CD4+ T cells into the Th1 cytokine effector pathway during T-cell division following stimulation in Behçet's disease. Rheumatology. 2004; 43: 843-851.
19) Pflanz S, Hibbert L, Mattson J, Rosales R, Vaisberg E, Bazan JF, Phillips JH, McClanahan TK, de Waal Malefyt R, Kastelein RA: WSX-1 and glycoprotein 130 constitute a signal-transducing receptor for IL-27. J Immunol. 2004; 172: 2225-2231.
20) Yoshida H, Hamano S, Senaldi G, Covey T, Faggioni R, Mu S, Xia M, Wakeham AC, Nishina H, Potter J, Saris CJ, Mak TW: WSX-1 is required for the initiation of Th1 responses and resistance to L. major infection. Immunity. 2001; 15: 569-578.
21) Takeda A, Hamano S, Yamanaka A, Hanada T, Ishibashi T, Mak TW, Yoshimura A, Yoshida H: Cutting edge: role of IL-27/WSX-1 signaling for induction of T-bet through activation of STAT1 during initial Th1 commitment. J Immunol. 2003; 170: 4886-4890.
22) Hibbert L, Pflanz S, de Waal Malefyt R, Kastelein RA: IL-27 and IFN-α signal via Stat1 and Stat3 and induce T-bet and IL-12Rβ2 in naïve T cells. J interferon Cytokine Res. 2003; 23: 513-522.
23) Lucas, S, Ghilardi N, Li J, de Sauvage FJ: IL-27 regulates IL-12 responsiveness of naïve CD4$^+$ T cells through STAT1-dependent and --independent mechanisms. Proc Natl Acad Sci U S A. 2003; 100: 15047-15052.
24) Sonoda KH, Yoshimura T, Takeda A, Ishibashi T, Hamano S, Yoshida H: WSX-1 plays a significant role for the initiation of experimental autoimmune uveitis. Int Immunol. 2007; 19: 93-98.
25) Caspi RR, Chan CC, Grubb BG, Silver PB, Wiggert B, Parsa CF, Bahmanyar S, Billiau A, Heremans H: Endogenous systemic IFN-gamma has a protective role against ocular autoimmunity in mice. J Immunol. 1994; 152: 890-899.
26) Gran B, Zhang GX, Yu S, Li J, Chen XH, Ventura ES, Kamoun M, Rostami A: IL-12p35-deficient mice are susceptible to experimental autoimmune encephalomyelitis: evidence for redundancy in the IL-12 system in the induction of central nervous system autoimmune demyelination. J Immunol. 2002; 169: 7104-7110.
27) Luger D, Silver PB, Tang J, Cua D, Chen Z, Iwakura Y, Bowman EP, Sgambellone NM, Chan CC, Caspi RR: Either a Th17 or Th1 effector response can drive autoimmunity: conditions of disease induction affect dominant effector category. J Exp Med. 2008; 205: 799-810.
28) Oppmann B, Lesley R, Blom B, Timans JC, Xu Y, Hunte B, Vega F, Yu N, Wang J, Singh K, Zonin F, Vaisberg E, Churakova T, Liu M, Gorman D, Wagner J, Zurawski S, Liu Y, Abrams JS, Moore KW, Rennick D, de Waal-Malefyt R, Hannum C, Bazan JF, Kastelein RA: Novel p19 protein engages IL-12p40 to form a cytokine, IL-23, with biological activities similar as well as distinct from IL-12. Immunity. 2000; 13: 715-725.
29) Sonoda KH, Sasa Y, Qiao H, Tsutsumi C, Hisatomi T, Komiyama S, Kubota T, Sakamoto T, Kawano Y, Ishibashi T: Immunoregulatory role of ocular macrophages: the macrophages produce RANTES to suppress experimental autoimmune uveitis. J Immunol. 2003; 171: 2652-2659.
30) Harrington LE, Hatton RD, Mangan PR, Turner H, Murphy TL, Murphy KM, Weaver CT: Interleukin 17-pro-

ducing CD4+ effector T cells develop via a lineage distinct from the T helper type 1 and 2 lineages. Nat Immunol. 2005; 6: 1123-1132.

31) Wang C, Tian Y, Lei B, Xiao X, Ye Z, Li F, Kijlstra A, Yang P: Decreased IL-27 expression in association with an increased Th17 response in Vogt-Koyanagi-Harada disease. Invest Ophthalmol Vis Sci. 2012; 53: 4668-4675.

32) Sugita S, Kawazoe Y, Imai A, Yamada Y, Horie S, Mochizuki M: Inhibition of Th17 differentiation by anti-TNF-alpha therapy in uveitis patients with Behcet's disease. Arthritis Res Ther. 2012; 14: R99.

33) Yoshimura T, Sonoda KH, Miyazaki Y, Iwakura Y, Ishibashi T: Differential roles for IFN-γ and IL-17 in experimental autoimmune uveoretinitis. Int Immunol. 2008; 20: 209-214.

34) Hohki S, Ohgura N, Haruta H, Nakai K, Terabe F, Serada S, Fujimoto M, Nomura S, Kawahata H, Kishimoto T, Naka T: Blockade of interleukin-6 signaling suppresses experimental autoimmune uveoretinitis by the inhibition of inflammatory Th17 responses. Exp Eye Res. 2010; 91: 162-170.

35) Amadi-Obi A, Yu CR, Liu X, Mahdi RM, Clarke GL, Nussenblatt RB, Gery I, Lee YS, Egwuagu CE: Th17 cells contribute to uveitis and scleritis and are expanded by IL-2 and inhibited by IL-27/STAT1. Nat Med. 2007; 13: 711-718.

36) Tang J, Zhu W, Silver PB, Su SB, Chan CC, Caspi RR: Autoimmune uveitis elicited with antigen-pulsed dendritic cells has a distinct clinical signature and is driven by unique effector mechanisms: initial encounter with autoantigen defines disease phenotype. J Immunol. 2007; 178: 5578-5587.

37) Takase H, Futagami Y, Yoshida T, Kamoi K, Sugita S, Imai Y, Mochizuki M: Cytokine profile in aqueous humor and sera of patients with infectious or noninfectious uveitis. Invest Ophthalmol Vis Sci. 2006; 47: 1557-1561.

38) Yoshimura T, Sonoda KH, Ohguro N, Ohsugi Y, Ishibashi T, Cua DJ, Kobayashi T, Yoshida H, Yoshimura A: Involvement of Th17 cells and the effect of anti-IL-6 therapy in autoimmune uveitis. Rheumatology. 2009; 48: 347-354.

39) Ghoreschi K, Laurence A, Yang XP, Tato CM, McGeachy MJ, Konkel JE, Ramos HL, Wei L, Davidson TS, Bouladoux N, Grainger JR, Chen Q, Kanno Y, Watford WT, Sun HW, Eberl G, Shevach EM, Belkaid Y, Cua DJ, Chen W, O'Shea JJ: Generation of pathogenic T(H)17 cells in the absence of TGF-β signaling. Nature. 2010; 467: 967-971.

40) Korn T, Mitsdoerffer M, Croxford AL, Awasthi A, Dardalhon VA, Galileos G, Vollmar P, Stritesky GL, Kaplan MH, Waisman A, Kuchroo VK, Oukka M: IL-6 controls Th17 immunity in vivo by inhibiting the conversion of conventional T cells into Foxp3+ regulatory T cells. Proc Natl Acad Sci U S A. 2008; 105: 18460-18465.

41) Park H, Li Z, Yang XO, Chang SH, Nurvieva R, Wang YH, Wang Y, Hood L, Zhu Z, Tian Q, Dong C: A distinct lineage of CD4 T cells regulates tissue inflammation by producing interleukin 17. Nat Immunol. 2005; 6: 1133-1141.

42) Nishimoto N, Yoshizaki K, Miyasaka N, Yamamoto K, Kawai S, Takeuchi T, Hashimoto J, Azuma J, Kishimoto T: Treatment of rheumatoid arthritis with humanized anti-interleukin-6 receptor antibody: a multicenter, double-blind, placebo-controlled trial. Arthritis Rheum. 2004; 50: 1761-1769.

43) Haruta H, Ohguro N, Fujimoto M, Hohki S, Terabe F, Serada S, Nomura S, Nishida K, Kishimoto T, Naka T: Blockade of interleukin-6 signaling suppresses not only Th17 but also interphotoreceptor retinoid binding protein-specific Th1 by promoting regulatory T cells in experimental autoimmune uveoretinitis. Invest Ophthalmol Vis Sci. 2011; 52: 3264-3271.

44) Hunter CA, Kastelein R. Interleukin-27: Balancing protective and pathological immunity. Immunity. 2012; 37: 960-969.

45) Yoshimura T, Takeda A, Hamano S, Miyazaki Y, Kinjyo I, Ishibashi T, Yoshimura A, Yoshida H. Two-sided roles of IL-27; Induction of Th1 differentiation on naïve CD4+ T cells versus suppression of proinflammatory cytokine production including IL-23-induced IL-17 on activated CD4+ T cells partially through STAT3-dependent mechanism. J Immunol. 2006; 177: 5377-5385.

46) Sfikakis PP, Markomichelakis N, Alpsoy E, Assaad-Khalil S, Bodaghi B, Gul A, Ohno S, Pipitone N, Schirmer M, Stanford M, Wechsler B, Zouboulis C, Kaklamanis P, Yazici H: Anti-TNF therapy in the management of Behçet's disease-review and basis for recommendations. Rheumatology. 2007; 46: 736-741.

47) Horiuchi T, Mitoma H, Harashima S, Tsukamoto H, Shimoda T: Transmembrane TNF-α: structure, function and interaction with anti-TNF agents. Rheumatology. 2010; 49: 1215-1218.

48) Hamazaoui K: Th17 cells in Behçet's disease: a new immunoregulatory axis. Clin Exp Rheumatol. 2011; 29: S71-S76.
49) Sugita S, Kawazoe Y, Imai A, Kawaguchi T, Horie S, Keino H, Takahashi M, Mochizuki M: Role of Il-22- and TNF-α-producing Th22 cells in uveitis patients with Behçet's disease. J Immunol. 2013; 190: 5799-5808.

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IL-10 Is Significantly Involved in HSP70-Regulation of Experimental Subretinal Fibrosis

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Abstract

Subretinal fibrosis is directly related to severe visual loss, especially if occurs in the macula, and is frequently observed in advanced age-related macular degeneration and other refractory eye disorders such as diabetic retinopathy and uveitis. In this study, we analyzed the immunosuppressive mechanism of subretinal fibrosis using the novel animal model recently demonstrated. Both TLR2 and TLR4 deficient mice showed significant enlargement of subretinal fibrotic area as compared with wild-type mice. A single intraocular administration of heat shock protein 70 (HSP70), which is an endogenous ligand for TLR2 and TLR4, inhibited subretinal fibrosis in wild-type mice but not in TLR2 and TLR4-deficient mice. Additionally, HSP70 induced IL-10 production in eyes from wild-type mice but was impaired in both TLR2- and TLR4-deficient mice, indicating that HSP70-TLR2/TLR4 axis plays an immunomodulatory role in subretinal fibrosis. Thus, these results suggest that HSP70-TLR2/TLR4 axis is a new therapeutic target for subretinal fibrosis due to prognostic CNV.

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Introduction

Age-related macular degeneration (AMD) is the leading cause of irreversible blindness, which is estimated to affect more than 8 million individuals in the USA, and the advanced form of the disease affects more than 1.75 million individuals [1]. The neovascular form of the disease is characterized by the invasion of new pathological vessels under the macula (choroidal neovascularization, CNV) and it is associated with a rapid and severe decrease of vision. Numerous studies about the mechanism of CNV formation have been reported, many of which resulted in the initiation of clinical trials. The accumulated knowledge has led to the development of several therapeutic strategies for AMD, such as verteporfin photodynamic therapy (PDT), anti-vascular endothelial growth factor (VEGF) therapy, and combined therapy [2]. In contrast, little is known regarding the molecular mechanism(s) of tissue scar formation in CNV. Since fibrotic changes in the foveal CNV lesion frequently result in severe, permanent visual impairment in patients with wet AMD, the treatment of tissue fibrosis in the late stage of AMD is of great interest.

Fibrosis is a common pathophysiological response of many tissues to chronic injury, which can be considered wound repair, mostly associated with robust inflammatory response [3]. Recruitment of inflammatory cells and the subsequent laying down of extracellular matrix during wound repair is a healthy response to tissue damage. However, this evolutionary adaptation comes at the cost of an excessive and poorly ordered matrix deposition and fibrosis, which affects normal tissue architecture and ultimately can disable proper functioning of tissues.

Toll-like receptors (TLRs) are germline-encoded pattern recognition receptors that are important in the innate immune system involved in initial step of host defense against microorganisms. Accumulated lines of evidence indicate that TLRs are also activated by endogenous ligands such as high mobility group box 1 (HMGB1), hyaluronan, and heat shock proteins released from damaged tissues, termed damaged associated molecular patterns (DAMPs). Such innate immune responses contribute not only to inflammation, but also to physiological and pathological repair processes including fibrosis.

HSPs are a family of highly conserved proteins found in all eukaryotes and prokaryotes. The HSP70 family, located in the cytosol and the nucleus of various kinds of cells, is released in response to cellular stress such as UV light, trace metals, and xenobitics. Several studies have shown that extracellular HSPs have important immunomodulatory functions [4]. Induction of HSP70 is protective in animal models of various diseases, such as inflammatory bowel diseases [5], ultraviolet light-induced skin damage [6] and Alzheimer's disease [7]. Furthermore, several studies have reported that inflammation, fibrosis and dysfunction are suppressed in transgenic mice expressing HSP70 [8]. HSP70 specifically binds both TLR2 and 4 [9]. Dybdahl et al. reported that autologous release of HSP70 after open heart surgery induces a proinflammatory response in innate immune cells potentially mediated via TLR2 and TLR4 [1,10].

In the present study, we investigated the involvement of the HSP70 and its downstream TLR2 and TLR4 signalings in the formation of subretinal fibrosis with animal model which we recently introduced. Moreover, the mechanisms underlining the HSP70-regulation of on subretinal fibrosis were examined. Here we show that exogenous HSP70, by inducing anti-inflammatory cytokine IL-10, ameliorates experimental subretinal fibrosis

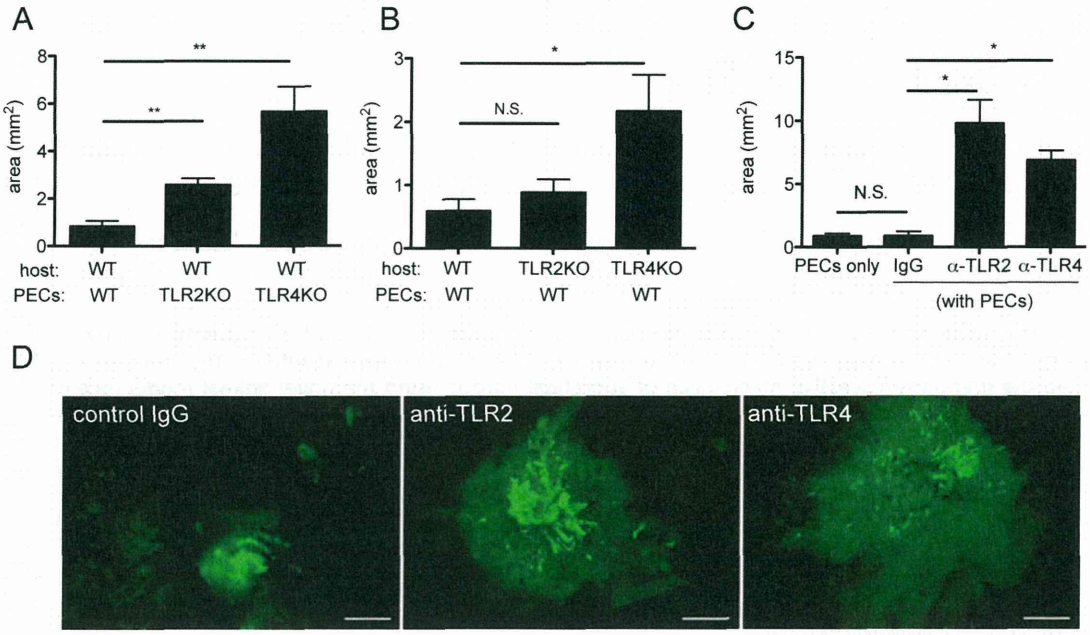

Figure 1. Both TLR2 and TLR4 signaling pathways are significantly involved in the formation of subretinal fibrosis. Subretinal fibrotic models were generated as previously described [11]. Briefly, laser photocoagulation (wave length 532 nm, 0.1 s, spot size 75 um, power 200 mW) was performed to the retina to make subretinal bubble and rupture Bruch membrane in WT mice. PECs (4×10^4) from indicated mice collected as Materials and Methods without any stimulation were inoculated into the subretinal space of indicated mice. (A) PECs from each WT, TLR2KO and TLR4KO mice were inoculated into subretinal space of WT mice (n = 5). (B) PECs from WT mice were inoculated into subretinal space of WT, TLR2KO and TLR4KO mice. (n = 5) (C) With neutralizing antibody anti-TLR2, anti-TLR4 antibody, control IgG or without any reagent (naïve), PECs from WT mice were inoculated into subretinal space of WT mice. After 7 days, eyes were enucleated, and choroidal flatmounts were prepared and stained with anti-GFAP antibody. The areas of subretinal fibrosis were measured by ImageJ. *$p<0.05$ versus control; double asterisks, **$p<0.005$ versus control. (n = 5) Data represents mean ± SEM. (D) Histological cross sections from (C) were stained with anti-GFAP antibody. Representative images were shown. Scale bars, 500 μm. Results are represents as mean ± SEM.
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formation through both TLR2- and TLR4-dependent mechanisms.

Materials and Methods

Ethics Statement

This study was carried out in strict accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The protocol was approved by the Committee on the Ethics of Animal Experiments of Kyushu University (Permit Number: A21-147-1, A23-088-0 and A23-220-0). All surgery was performed under sodium pentobarbital anesthesia, and all efforts were made to minimize suffering.

Mice

C56BL/6 (WT) mice were obtained from SLC Japan (Shizuoka, Japan). TLR2-deficient (TLR2KO) and TLR4-deficient (TLR4KO) mice were obtained from WPI Immunology Frontier Research Center, Osaka University and backcrossed to WT mice for more than 10 times. All animals were housed in specific pathogen free conditions at Kyushu University and treated humanely. Experiments conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Preparation of PECs

Macrophage-rich (peritoneal exudate cells) PECs were obtained from WT, TLRKO and TLR4KO mice that received 2.5 ml of 3% aged thioglycolate solution (Difco, Detroit, MI) 3 days before sacrifice, plated in 10 cm dish, from which nonadherent cells were washed out with PBS. The remaining adherent cells were incubated with 0.02% EDTA and collected by vigorous pipetting. Cells were counted and resuspended with PBS.

Subretinal Fibrotic Model

Subretinal fibrotic models were generated as previously described [11]. Briefly, laser photocoagulation (wave length 532 nm, 0.1 s, spot size 75 um, power 200 mW) was performed to the retina to make subretinal bubble and rupture Bruch membrane in WT, TLR2 KO, or TLR4 KO mice. PECs (4×10^4) collected as above without any stimulation were inoculated into the subretinal space. On day 7 after PEC-inoculation, mice were sacrificed and eyes were enucleated, which were then fixed in 4% paraformaldehyde. Choroidal flatmounts were prepared and stained with anti-glial fibriary acidic protein (GFAP) antibody conjugated with FITC.

Vitreous Cavity Injection

1 ng of anti-TLR2 or TLR4 neutralizing antibodies or PBS was injected into vitreous cavity with subretinal inoculation of PECs in WT mice. 2 hours after PEC inoculation, 50 ng (25 μg/ml, 2 μl) of recombinant human heat shock protein 70 (HSP70) (Assay designs, # NSP-555) or control PBS was administered into vitreous cavity in WT or TLR4 KO mice. 2 hours after PECs-inoculation, 1 μg of lipopolysaccharide (LPS) or PBS was administered into vitreous cavity in WT mice.

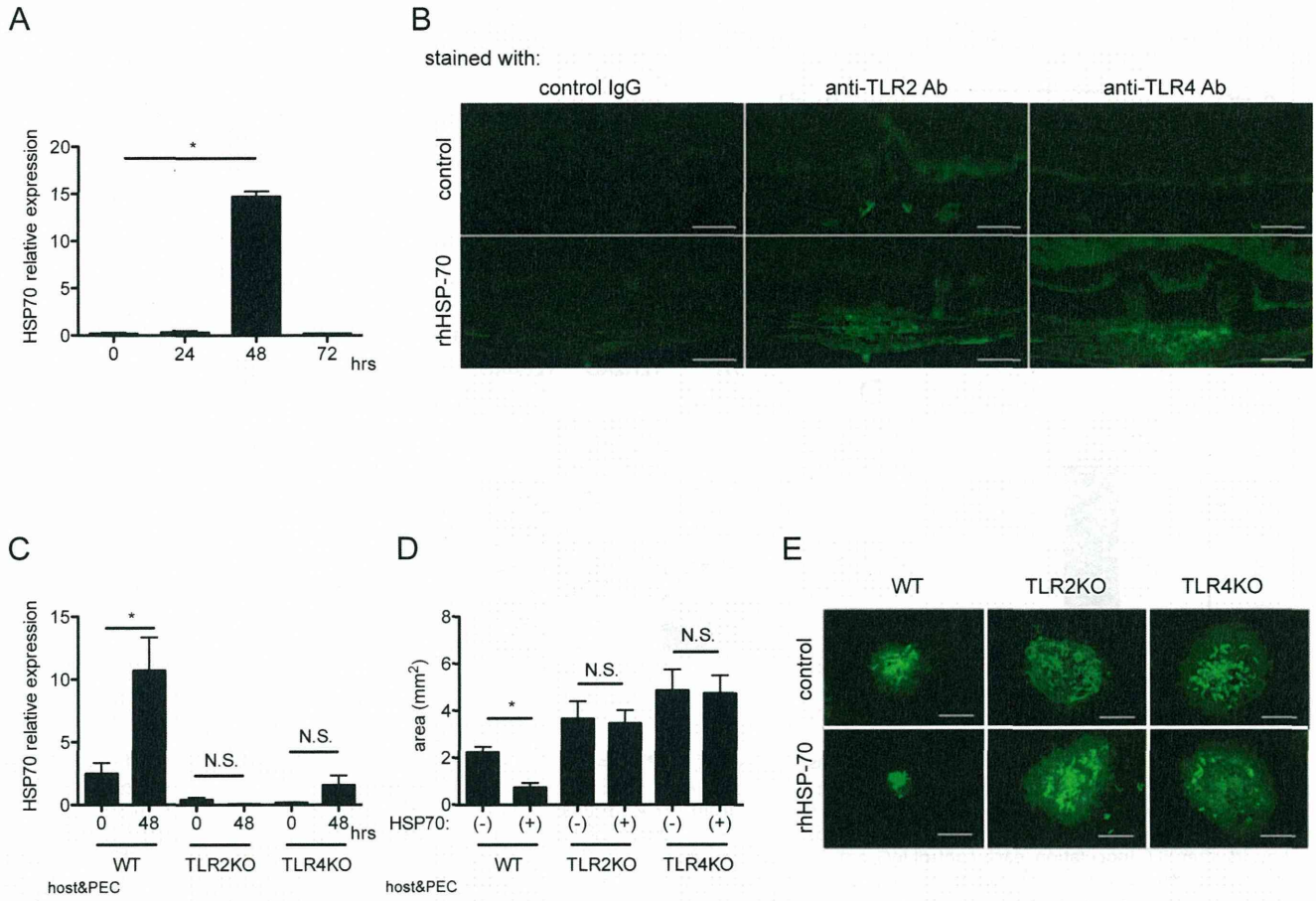

Figure 2. Role of HSP70 in the formation of subretinal fibrosis. (A) 0, 24, 48 and 72 hours after PEC inoculation, total RNA was extracted from the eyes and the amounts of HSP70 mRNA were assessed by quantitative real-time RT-PCR. (B) 72 hours after PEC inoculation following intravitreal HSP70 injection, eyes were enucleated, then histological cross sections at the site of subretinal fibrosis were stained with control IgG, anti-TLR2 and anti-TLR4 antibody, respectively. Representative images were shown. Scale bars, 100 μm. (C) Indicated hours after PECs inoculation, total RNA was extracted from eyes of each WT (WT PEC into lasered WT mice), TLR2KO (TLR2KO PEC into lasered TLR2KO) and TLR4KO (TLR4KO PEC into lasered TLR4KO) mice. The amount of HSP70 mRNA were evaluated by quantitative real-time PCR (n = 9). (D) Recombinant human HSP70 or control PBS was injected into vitreous cavity of WT, TLR2KO and TLR4KO mice 2 hours after PEC inoculation. After 7 days, eyes were enucleated, and choroidal flatmounts were prepared and stained with anti-GFAP antibody. The area of subretinal fibrosis were measured by ImageJ. (n = 5) (E) Representative images of choroidal flatmount stained with anti-GFAP antibody. Scale bars, 500 μm. Results are represents as mean ± SEM.
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Quantitative Real-time Reverse Transcriptase (RT)-PCR

0, 24, 48 or 72 h after PEC inoculation, eyes were enucleated under deep anesthesia, the conjunctival tissue was removed, and the remaining eye tissues (cornea, iris, vitreous body, retina, choroids and sclera) were homogenized using a Biomasher (Nippi Inc., Tokyo, Japan). Then homogenized tissues were immersed in TRIzol reagent (Invitrogen Corp., Carlsbad, CA) and processed for RNA isolation. The reverse-transcriptase cDNAs were then subjected to real-time PCR using SYBR Premix Ex Taq (Takara Bio Inc., Otsu, Japan) and a Light Cycler (Roche Diagnostics GmbH, Mannheim, Germany). The primers were as follows; 5′-AACTGCACCCACTTCCCAGTC-3′ and 5′-CATTAAG-GAGTCGGTTAGCAG3′for IL-10, 5′-GGCTGATCGGCCG-CAAGTT-3′ and 5′-AACTGCACCCACTTCCCAGTC-3′ for HSP70, 5′-GATGACCCAGATCATGTTTGA-3′ and 5′-GGA-GAGCATAGCCCTCGTAG-3′ for beta-actin. All estimated mRNA values were normalized to beta-actin mRNA levels. Each experiment was carried out at least twice and representative data are shown.

RPE and PEC Cell Culture

Retinal pigment epithelial (RPE) cells were prepared from eyes of WT, TLR4KO and TLR2KO mice as previously described, [2,12] and then incubated until almost confluent in DMEM supplemented with 20% heat-inactivated fetal calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin, 1% L-glutamine, and 0.1 mM non-essential amino acids at 37°C in 5% CO_2. RPE cells or PECs were plated in six-well dishes (Collagen-Coated Microplate 6 Well with Lid Collagen Typel, IWAKI, Chiba, Japan), and then the media was replaced to 1 ml of media. Cells were stimulated at doses of 0, 0.1, 0.3 and 1 ng/ml recombinant human Hsp70 for 48 h. RPE cells were stimulated with 0, 0.1,1.0 and 10 μg/ml LPS for 48 h.

Cytokine ELISA

Following stimulation of primary RPE cells and PECs by recombinant human Hsp70 (0, 0.1, 0.3 and 1 ng/ml) or LPS (0, 0.1, 1.0 and 10 μg/ml) for 48 h, supernatants were harvested. IL-10 levels were measured by enzyme-linked immunosorbent assay

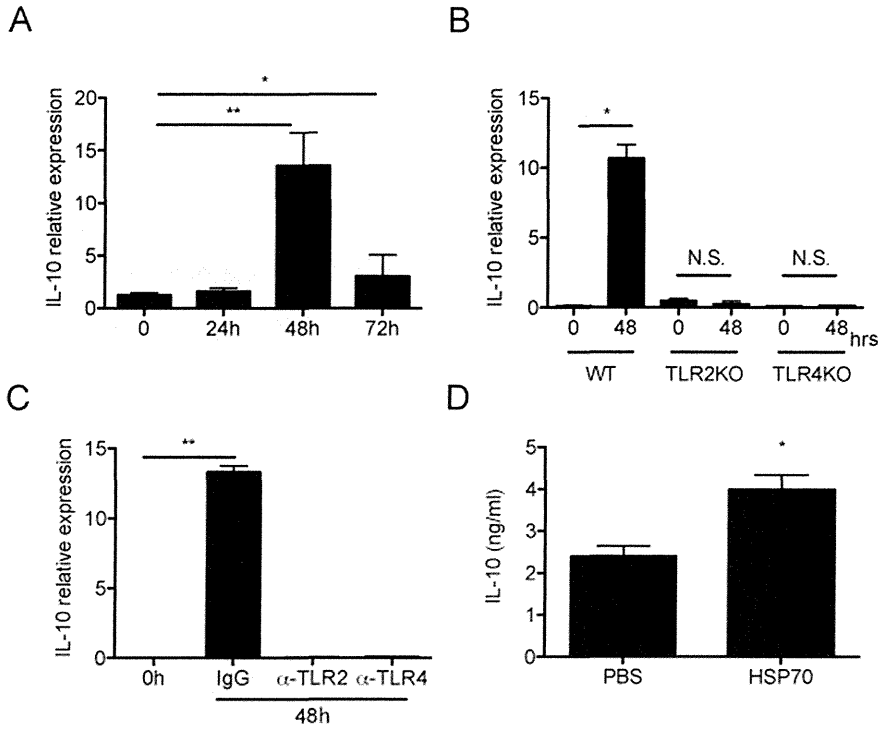

Figure 3. Implications of IL-10 and its possible association of HSP70 in the formation of subretinal fibrosis. (A) PECs from WT mice were inoculated into the subretinal space of WT mice. 0, 24, 72 hours after, total RNA was extracted from the eyes and the amounts of IL-10 mRNA were assessed by quantitative real-time RT-PCR. (n = 6) (B) PECs from WT were inoculated to each WT, TLR2 and TLR4 mice. 48 hours after PEC inoculation, total RNA was extracted from the eyes of each mice and the amount of IL-10 mRNA was assessed by quantitative real-time RT-PCR. (n = 6) (C) 2 hours after PEC inoculation, each control IgG, anti-TLR2 and anti-TLR4 neutralizing antibody was injected into vitreous cavity of WT mice. After 48 hours, total RNA was extracted from the eyes and the amounts of IL-10 mRNA were asessed by quantitative real-time RT-PCR. (n = 5) (D) 2 hours after PEC inoculation into subretinal space of WT mice, recombinant human HSP70 or control PBS was injected into vitreous cavity. After 48 hours, eyes were enucleated, total protein was extracted from the retina-choroid-RPEs and amounts of IL-10 were quantified by ELISA (n = 12). Results are represents as mean ± SEM.
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(ELISA) following the manufacturer's instructions (eBioscience, Cat# 88-7104-88).

For protein analysis, the retina-RPE-choroid complex were isolated from eyes of WT mice 48 hours after PBS or HSP70 inoculation, pooled, lysed, homogenized, centrifuged, then assessed by ELISA. The total protein concentration was determined with Bio-Rad protein assay reagent kit (Bio-Rad Laboratories, Inc, Hercules, CA).

Immunohistochemistry

72 h after PEC inoculation following intravitreal injection of recombinant HSP70 or control PBS, eyes were enucleated and immediately frozen (−80°C) in tetrafluorethane overnight. Then 6 μm transverse sections were cut on a Leica Microtome (Leica Cryostat model CM 1800, Germany) and placed on silanised glass slides. Sections were stained with hematoxylin-eosin (HE) for general morphology. Dehydrated sections were blocked with H_2O_2 (0.3% in methanol) and skim milk (2% in PBS) for 30 minutes, respectively. Then sections were incubated overnight with primary antibodies (Rabbit anti-mouse IgG, 1:200; and TLR4, 1:50; and TLR2, 1:200; and HSP70, 1:50) at 4°C in moist chambers, followed by incubation with the secondary antibody (Goat anti-rabbit IgG, 1:200) for 30 minutes at room temperature. Finally, the signals were detected using the fluorescein streptavidin (1:50). The reaction was allowed to develop for approximately 10 minutes and staining was halted by washing in PBS/Tween20 3 times for 5 minutes each.

Primary RPE cells from mice were treated with recombinant human HSP70 (1 ng/ml) for 48 h. PECs were added to the primary culture of RPEs some samples in the absence or presence of HSP70 (1 ng/ml), IL-10 (10 ng/ml). Cells were washed with PBS 5 times, fixed with cold methanol (1:1) for 1 min and blocked with 20% Blocking One (Nacalai Tesque, #03953-95) for 30 minutes, then incubated with the following primary antibodies (Rabbit anti-mouse IgG, 1:200; and TLR4, 1:50; and TLR2, 1:200; and HSP70, 1:50) at 4°C overnight, followed by incubation with the secondary antibody (Goat anti-rabbit IgG, 1:200) for 60 minutes at room temperature. The nuclei of cells were counterstained with Hoechst 33342 (1:400, Molecular Probes) for 10 minutes.

Statistics

Data were analyzed for significant differences between experimental groups with either ANOVA/Scheffe's test (more than three groups) or Student's t-test (two groups). P values<0.05 were considered significant.

Results

Augmented Subretinal Fibrosis by Blockade of TLR2 and TLR4 Signaling

Subretinal fibrosis is a common outcome after subretinal hemorrhage in advanced age-related macular degeneration. Several studies suggested that both TLR2 and TLR4 are

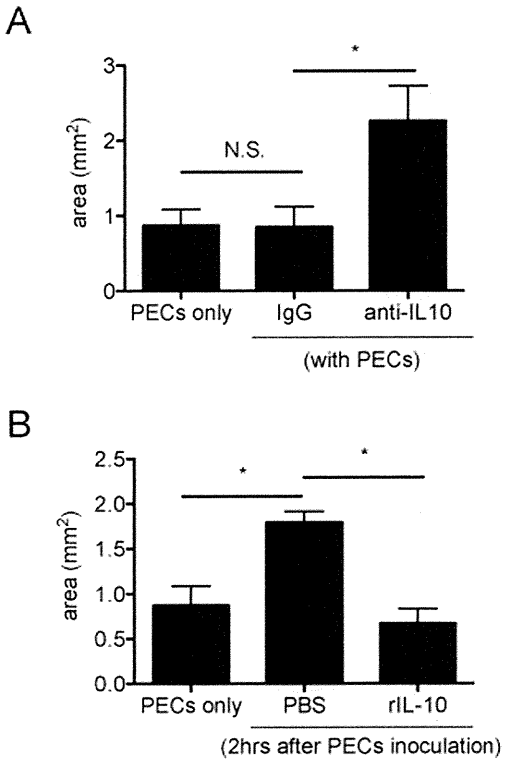

Figure 4. IL-10 is responsible for subretinal formation. (A) Neutralizing anti-IL-10 antibodies were inoculated with PECs from WT to subretinal space of WT mice. (B) 2 hours after PEC inoculation, recombinant IL-10 was intravitreally injected. After 7 days, eyes were enucleated, and choroidal flatmounts were prepared and stained with anti-GFAP antibody. The areas of subretinal fibrosis were measured by ImageJ. Results are represents as mean ± SEM.
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implicated in the formation of fibrosis [3,4,13,14]. These observation prompted us to examine the role of TLR2 and TLR4 signaling in subretinal formation. PECs from WT, TLR2KO and TLR4KO were inoculated into subretinal space of WT mice as described in *Materials and Methods* section. 7 days after PEC inoculation, eyes were enucleated and choroidal flatmount were prepared. Then the area of subretinal fibrosis was evaluated by staining with FITC-conjugated GFAP antibody. As shown in Figure 1A, the area of subretinal fibrosis was exacerbated by PECs in both TLR2KO and TLR4KO mice compared to WT C57BL/6 mice. In the previous report, we show that endogenous macrophages are recruited to form focal fibrosis with inoculated exogenous macrophages [11]. To analyze the environmental effects of TLR2 and TLR4 signaling in response to inoculated PECs, PECs from WT were inoculated in WT, TLR2KO and TLR4KO mice. As shown in Figure 1B, the exacerbated area of subretinal fibrosis was revealed in TLR4KO mice, whereas comparable area in TLR2KO mice. To further show the effect of TLR2 and TLR4 signaling in experimental subretinal fibrosis, a neutralizing antibody against TLR2 (1 ng/ml, 0.25 μl), TLR4 (1 ng/ml, 0.25 μl) with WT mice, or isotype IgG (1 ng/ml, 0.25 μl) was mixed with PECs, then inoculated into subretinal space. As shown in Figure 1C, a significant increase of fibrosis area was seen in both groups with the neutralizing antibody against TLR2 and TLR4, compared with the control group (Figure 1C and Figure 1D). These data clearly show that TLR2 and TLR4 signaling plays a significant role in subretinal fibrosis formation.

Amelioration of Subretinal Fibrosis by HSP70 via TLR2 and TLR4

Heat shock proteins have been known to protect against various stressors and have anti-inflammatory activity, which can be mediated through both TLR2 and TLR4 activation [6,15]. To examine the possible immunoregulatory role of HSP70, the kinetics of HSP70 expression during subretinal fibrosis were examined. 0, 24, 48 and 72 hours after WT PEC inoculation into subretinal space of WT mice, mRNA from eyes were extracted, and were subjected to quantitative real-time PCR. As shown in Figure 2A, HSP70 mRNA expression was significantly elevated 48 h after PEC inoculation. Whereas IL-6 was only detected at day 2, IL-10 was increased as day 2 and reduced at day 5 and 7. HSP70 levels increased at days 2, 3 and 5, and returned back to basal levels at day 7 after PEC inoculation (Figure S1). To confirm TLR2 and TLR4 activation in response to HSP70 in vivo, recombinant HSP-70 (25 ng/ml, 2 μl) was injected into vitreous cavity at the time of WT PEC inoculation. 72 hours after PEC inoculation following intravitreal HSP70 injection, eyes were enucleated, then histological cross sections at the site of subretinal fibrosis were stained with control IgG, anti-TLR2 and anti-TLR4 antibody, respectively. As shown in Figure 2B, HSP70 protein itself have revealed to be express both TLR2 and TLR4 in retinal tissue (Figure 2B). To further examine the role of HSP70 via activation of TLR2 and TLR4, we took advantage to use TLR2KO and TLR4KO mice. As shown in Figure 2C, HSP70 expression at 48 h after PEC inoculation was significantly lower in eyes from both TLR2 and TLR4KO mice compared with WT mice. To further examine the role of HSP70 in the formation of subretinal fibrosis, exogenous recombinant HSP70 (25 ng/ml, 2 μl) was injected into vitreous cavity at the time of PEC inoculation. As shown in Figure 2D, the area of subretinal fibrosis was significantly lower in the HSP70-injected group, as compared with control group. Following HSP70 intravitreal injection, however, no significant changes were observed in both TLR2 and TLR4 mice (Figure 2D, E). Taken together, these results show that HSP70 plays a significant role in ameliorating subretinal fibrosis formation through activation of TLR2 and TLR4.

Hsp70 Induces Intraocular Production of IL-10 in Response to PEC Inoculation

Although the anti-inflammatory property of heat shock proteins (HSP) has been demonstrated in various animal models of inflammatory diseases [7,16,17], the mechanisms underlying these are not fully understood. A potential mechanism could be mediated by induction of anti-inflammatory cytokine IL-10 since the significance of IL-10 in dampening the inflammation is extensively described [8,18]. To examine the possible role of IL-10 in the formation of subretinal fibrosis, the kinetics of intraocular production of IL-10 after PEC inoculation were examined. 0, 24, 48 and 72 hours after PEC inoculation, total mRNAs were extracted from each eye, subjected to quantitative real-time PCR. As shown in Figure 3A, intraocular IL-10 expression was greatly upregulated at 48 h after PEC inoculation. Both TLR2 and TLR4KO failed to induce intraocular IL-10 expression at 48 h after PEC inoculation (Figure 3B). Moreover, the neutralizing antibody against both TLR2 and TLR4 failed to induce intraocular IL-10 production (Figure 3C). Protein levels of IL-10 were detected from eyes at 72 h after PEC inoculation that had received exogenous HSP70 (Figure 3D). These results demonstrate

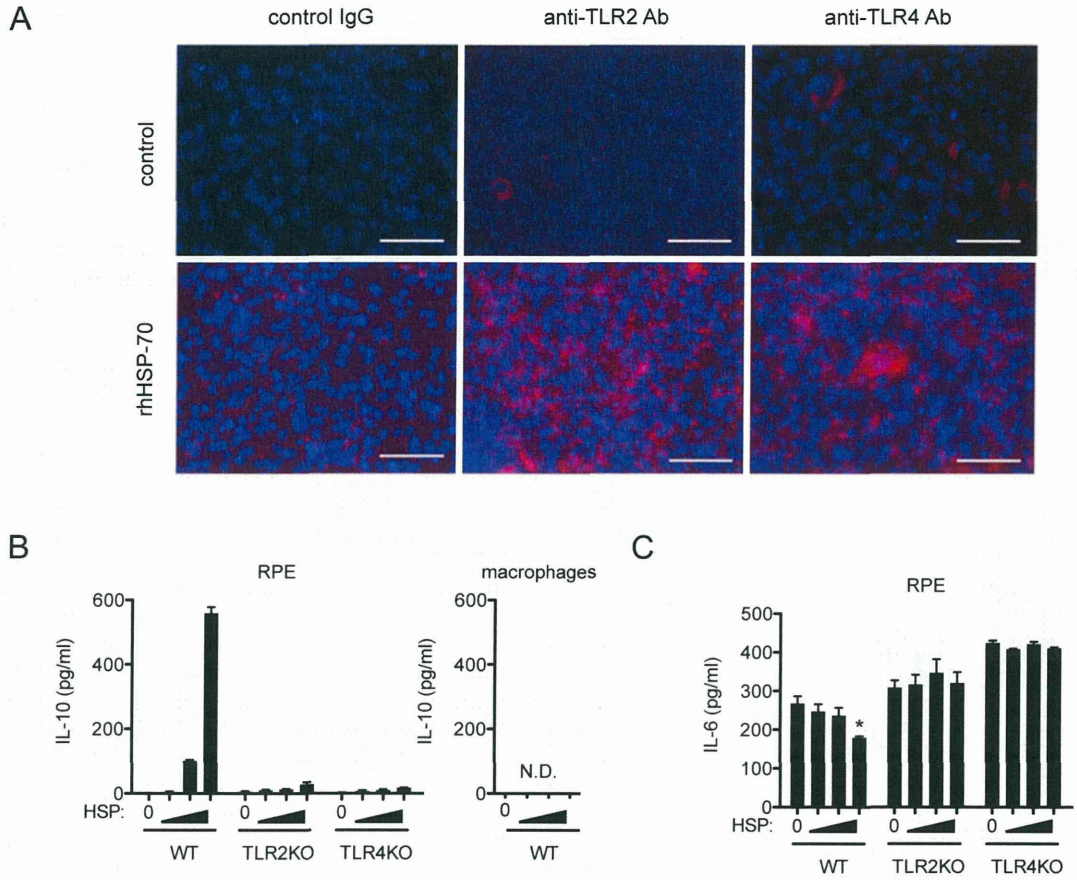

Figure 5. HSP70 induces IL-10 production by cultured RPEs but not by macrophages. (A) Cultured RPE cells in the presence or absence of rhHSP70 were stained with WT, anti-TLR2 and anti-TLR4 antibody, respectively. Representative images were shown. Scale bars, 100 μm. (B) RPE cells from each WT, TLR2 and TLR4 (*left*) and macrophages (*right*) were stimulated with 0.1, 0.3 and 1 ng/ml of recombinant human Hsp70 for 48hours, then supernatants were harvested and the levels of IL-10 were quantified by ELISA. (C) IL-6 was quantified by ELISA from same supernatants from (Figure B, *right*) RPE cells. Each experiment was representative of at least two experiments with similar results. Results are represents as mean ± SEM.
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that both TLR2 and TLR4 signaling have significant role in the production of intraocular IL-10 in response to PEC inoculation to subretinal space.

IL-10 is Responsible for Suppressing Subretinal Fibrosis Formation

Next, the direct effects of IL-10 on subretinal fibrosis were evaluated. A neutralizing antibody against IL-10 (1 μg/ml, 0.25 μl) or isotype control IgG was mixed with PEC and inoculated into subretinal space. The area of fibrosis was then assessed by immunohistochemistry. As shown in Figure 4A, the area of the group that was injected with the TLR2 and TLR4 neutralizing antibody was significantly larger compared with the control group. Next, recombinant IL-10 (0.6 ng/ml, 2 μl) or control PBS was administered intravitreally 2 hours after PEC inoculation. The area of subretinal fibrosis in the group that received IL-10 was significantly suppressed compared with the control group (Figure 4B). These results clearly demonstrated that IL-10 alone could play significant role in suppressing subretinal fibrosis formation.

HSP70 Augmented IL-10 Production by RPE Cells via TLR2 and TLR4 Activation

Retinal pigment cells produce IL-10 and are responsible for intraocular immunosuppressive mechanism [9,19]. To examine whether cultured RPEs can augment IL-10 in response to HSP70, RPE cells were stimulated in the presence or absence of recombinant HSP70 for 48 hours. The expression of both TLR2 and TLR4 were clearly detected by immunohistochemistry (Figure 5A). Culture supernatants were collected, then subjected to ELISA. As shown in Figure 5B, exogenous HSP70 augmented IL-10 production from cultured RPE cells from WT mice in a dose-dependent manner, whereas RPE cells from both TLR2 and TLR4 did not produce any detectable IL-10. We then postulated that macrophages would produce IL-10 by HSP70 stimulation as well. However, cultured macrophages failed to produce IL-10 in response to HSP70; instead, the blockade of proinflammatory cytokine IL-6 modulates experimental subretinal formation (manuscript in preparation). We therefore postulated that HSP70 would suppress IL-6 production. As shown in Figure 5C, a high dose (1 ng/ml) of HSP70 suppressed IL-6 production, whereas RPEs from both TLR2 and TLR4 deficient mice produced comparable levels of IL-6 in response to HSP70 stimulation.

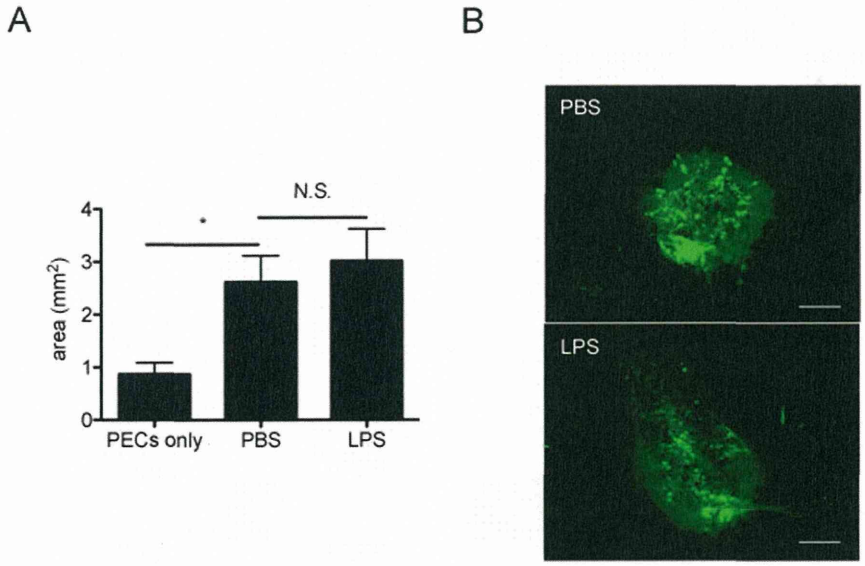

Figure 6. The effects of human Hsp70 on IL-10 production in subretinal fibrotic model are not due to the contamination of LPS. (A) 2 hours after PECs-inoculation, 50 ng (25 μg/ml, 2 μl) of lipopolysaccharides (LPS) or PBS was administered into vitreous cavity in WT mice. After 7 days, eyes were enucleated, choroidal flatmounts were prepared and stained with anti-GFAP antibody. The areas of subretinal fibrosis were measured by ImageJ. Results are represents as mean ± SEM. (B) Histological cross sections from (A) were stained with anti-GFAP antibody. Representative images were shown. Scale bars, 100 μm.
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Contamination of LPS Does Not Affect the Immunomodulatory Role of HSP70

Recent studies have shown that contamination of Hsp70 with LPS might be responsible for its stimulatory activation on macrophages and dendritic cells [20]. To exclude this possibility, we first confirmed that recombinant human Hsp70 used in this study contained <0.05 EU/μg protein (5 pg/μg) of bacterial endotoxin. Then an equal amount of LPS (25 μg/ml, 2 μl) was intravitreally injected following PEC inoculation. According to previous studies [21], it was lower than the dose which would cause uveitis. As shown in Figure 6, the size of subretinal fibrotic area was comparable between control PBS and LPS-injected eyes. These data clearly confirmed that LPS contamination in the recombinant HSP70 protein was minimal.

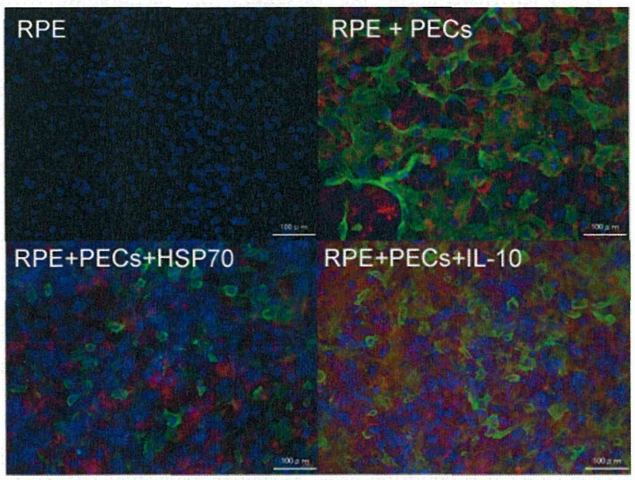

Figure 7. Suppressive effect of HSP70 and IL-10 on α-SMA expression in the RPE cells cultured with PECs. RPE cells were prepared from C57BL/6 mice and cultured approximately 2 weeks until becoming confluent in a 24 well plate. PECs were added to the primary culture in the absence or presence of HSP70 (1 ng/ml) or IL-10 (10 ng/ml). 48 hours later, cells were stained by FITC-conjugated α-SMA and PE-conjugated anti-F4/80 antibody at 4°C for 24 hours. All samples were counterstained with DAPI, mounted, and subjected to fluorescence microscopy. Representative images of α-SMA-stained RPE cells (green) and F4/80-stained macrophages (red) on the dish were shown.
doi:10.1371/journal.pone.0080288.g007

Possible Direct Effect on HSP70 and IL-10 in the Formation of Subretinal Fibrosis

In this model, the formation of fibrosis relays on laser-induced CNV formation. IL-10 has been shown to be anti-angiogenic in the mouse model of CNV [22]. The reduction in fibrosis in TLR2/4 KO and in HSP70/IL-10 treated mice could be the indirect results from the reduced CNV formation, but not the direct effect of the treatment on fibrosis. This possibility attempted us to assess direct effect of HSP70 and IL-10 in the formation of subretinal fibrosis. PECs were added to primary cultured RPE cells from C57BL/6 in the absence or presence of HSP70 or IL-10. 48hours later, cells were stained with FITC-conjugated α-SMA and anti-F4/80. As shown in Figure 7, each HSP70 and IL-10 treatment clearly suppressed α-SMA expression on RPE cells. Intracellular flow cytometry analysis revealed that HSP70 treatment suppresses intracellular expression of α-SMA (data not shown) on RPE cells. These data implicated the direct suppressive effect of HSP70/IL-10 on experimental subretinal formation.

Discussion

Substantial progress has been made towards the elucidation of the pathophysiology of angiogenesis and the role of anti-angiogenic therapies in the treatment of pathophysiological neovascularization [23]. To date, inhibition of angiogenesis is one of the leading therapeutic approaches in neovascular diseases such as diabetic retinopathy and AMD. However, focal subretinal fibrosis following the infiltration of exudative leukocytes would be

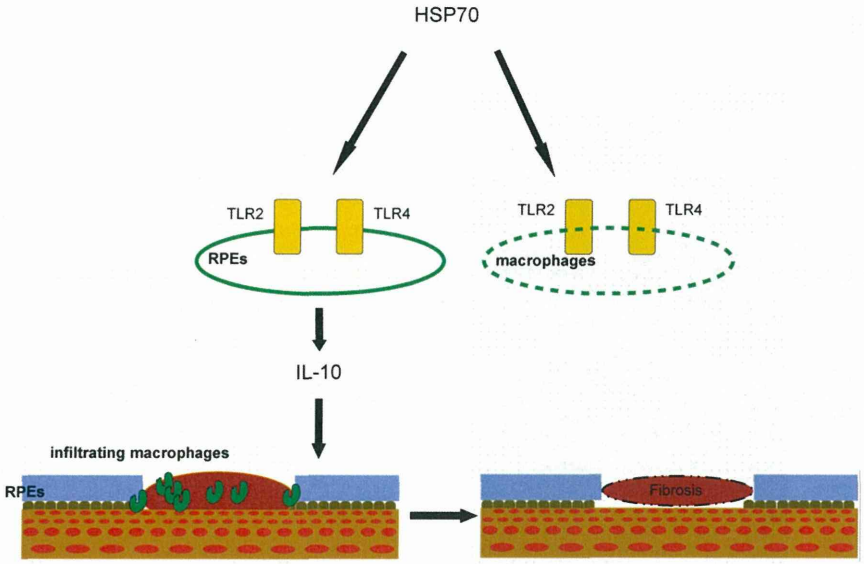

Figure 8. Possible mechanism of HSP70 in the regulation of subretinal formation. Intraocular expression of HSP70 activates TLR2 and TLR4 signaling cascade in RPEs but not in macrophages, results in the regulation of subretinal formation via production of IL-10.
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an important cause of severe visual loss. In this study, we show the importance of HSP70 in subretinal formation by inducing immunomodulatory cytokine IL-10. Blockade TLR2 and TLR4 resulted in exacerbating subretinal fibrosis.

The anti-inflammatory properties of HSPs have been shown in several studies in both animal models and in patients suffering from inflammatory diseases [16]. However, for further development of HSPs for therapeutic application, it is essential to understand the mechanisms by which HSPs affect inflammatory disease in more detail. In this study we analyzed the effect of HSP70 on focal subretinal fibrosis. Our data clearly show that injection of exogenous HSP70 dramatically reduced the area of subretinal fibrosis. In TLR2- and TLR4-deficient mice, exogenous HSP70 administration fail to suppress fibrosis, illustrating the TLR2- and TLR4-dependent mechanism of HSP70 induced immunoregulation. In addition, in vitro data show that extracellular HSP70 can induce anti-inflammatory cytokine IL-10 following the inoculation of PECs into subretinal space possibly through autocrine or paracrine stimulation of TLR2 and TLR4-mediated interaction.

Although HSPs were once regarded as intracellular proteins, there is growing evidence suggesting that HSPs can also be expressed on cellular membranes and even released into the extracellular environment after stress. HSPs have been found in the circulation of both healthy individuals and those suffering from autoimmune diseases and inflammatory conditions [10] [24]. In earlier studies, it was suggested that cellular Hsp70 release may be the result of cell lysis, but it is now recognized that elevated Hsp70 may be found in the absence of necrosis. In fact, Hsp70 could be actively released from glial cells [25], epithelial cells [26], tumor cells [27], PBMC, [28] and importantly, human eyes [29].

Extracellular HSPs have previously been thought of as initiators of a pro-inflammatory response within the innate immune system. It has been shown that extracellular Hsp70 and Hsp60 can induce the production of pro-inflammatory cytokines including TNF-α, IL-1, and IL-6 in monocytes and macrophages [30] [31]. Recently, there is evidence that, in contrast to the pro-inflammatory response, some extracellular HSPs, including human Hsp60 [32], human Hsp27 [33], and human Hsp10, [34] induce a strong anti-inflammatory response with sustained production of IL-10 in vitro and in vivo. Human Hsp60 treatment of T cells in vitro was found to inhibit the production of pro-inflammatory cytokine TNF-α and IFN-γ, and trigger the production of anti-inflammatory cytokines IL-10 [32]. TGFβ stimulation considered to cause much higher levels of IL-10 secretion, however, pretreatment of TGFβ did not alter subretinal fibrosis formation (Figure S2). Although further study needed to clarify the precise mechanism of intraocular inflammatory balance to form subretinal formation, these findings suggest that, rather than being proinflammatory, self HSP reactivity might be a physiological mechanism for regulation of pro-inflammatory responses and inflammatory disease. It should therefore not be surprising if self Hsp70 is found to have an anti-inflammatory effect in this model of subretinal fibrosis. Indeed, our studies showed that human Hsp70 significantly induced the production of anti-inflammatory cytokine IL-10 but failed to induce the production of pro-inflammatory cytokines including IL-6 in RPE cells (Figure 5), suggesting that extracellular Hsp70 have an anti-inflammatory property in the formation of subretinal fibrosis. Thus, it is conceivable that in subretinal fibrosis which is due to CNV, inflammatory stress contributes to the expression and release of Hsp70 and the extracellular Hsp70 may act as natural dimmers of inflammation by inducing RPEs to produce IL-10, which is a part of the normal mechanism to down-regulate an inflammatory response.

The TLRs are a family of pattern recognition receptors, through which cells of the innate immune system, including macrophages and dendritic cells, recognize microbial pathogens [35]. So far, ten different proteins have been defined as members of the human TLR family. Pathogen-associated molecular patterns such as LPS are recognized by TLR4 and TLR2. TLRs have been shown to mediate activation of NF-κB and mitogen-activated protein kinase signaling pathways, resulting in the production of pro-inflammatory mediators such as IL-1, IL-6, IL-8, or TNF-α from the innate immune cells [35] [36,37]. Hsp70 has been reported to be recognized by TLR2 and TLR4 [9], which are abundantly expressed on innate immune cells such as macrophages and dendritic cells [38]. Zhou et al. reported that heat

shock upregulates expression of TLR2 and TLR4 in human monocytes via p38 kinase signal pathway [39]. Although several studies have demonstrated that TLR2 and TLR4 are also expressed in a variety of non-immune cells, their function is less well understood. Our results demonstrated that fibrotic areas increased without TLR2 and TLR4 signaling (Figure 1), which in turn suggests that Hsp70 might affect the formation of subretinal fibrosis by its binding to TLR2 and TLR4. As we have shown here, TLR2 and TLR4 deficient mice and a specific monoclonal antibody (mAb) to TLR2 and TLR4 suppressed the production of intraocular IL-10 after PEC inoculation, indicating that the effect of Hsp70 on fibrosis would depend on TLR2 and TLR4 signaling pathways in the IL-10 production.

There are limitations of this study. Kelly et al. [40] demonstrated that IL-10 augment laser-induced CNV size. In this present study, IL-10 suppressed the formation of subretinal fibrosis, but this could be due to reduced CNV but not fibrosis per se. As described in elsewhere [41], M1 macrophage secrete TNF-α, IL-1, IL-6, and be implicated in the formation of choroidal neovascularization. Kleinman et al. [42] described the role of proinflammatory cytokine IL-12 and IFN-γ as anti-angiogenic agent. IL-10 has been reported to be accelerating CNV [43]. There are still numerous discussions whether proinflammatory or anti-inflammatory cytokine has pro-angiogenic or anti-angiogenic nature. As we have previously reported [11], not only exogenous macrophages but also intrinsic macrophages are activated to form subretinal fibrosis. Although we provided the possible evidence that exogenous HSP and IL-10 directly suppress the fibrosis formation in vitro (Figure 7), further study needed to clarify this point.

Other experimental limitations should be discussed. For example, as shown in Figure 1C, 2D, 4A and 4B, the subretinal lesion size in different control groups varies significantly in different studies. These may be due to the difference of experimental settings in each group; to be much concentrated in subretinal space, neutralizing antibody was mixed with PEC suspension and then inoculated into subretinal space (single injection). However, not worked well with same protocol above, recombinant proteins were intravitreally injected 2 hours after PEC injection (twice injection, Figure 4B and 6). The latter surgical setting might reflect undefined inflammation which may alter subretinal lesion size, resulted in varied basal levels. Moreover, Figure 3A shows that IL-10 mRNA was upregulated at 72 h, whereas in Figure S1 it shows that IL-10 protein levels was normal at 72 h and lower at day5 (than day 0). At day 0 in Figure 3A and Figure S1, eyes were enucleated just after PECs inoculation, which detect IL-10 from combination of exogenous PECs and naive eye. Each base line IL-10 mRNA and protein level at Figure 3A and Figure S1, respectively, is that from activated macrophages and naïve eyes, then mRNA and transcript protein levels alter as inflammation proceed. In Figure S1 we extracted protein from retina-RPE-choroid complex that contains activated macrophages; it could lead to overdetection of both temporary released IL-10 and already existed intracellular IL-10. Therefore it could be reasonable to detect lower levels of IL-10 protein in late phase (day 3 and 5). Overall, this experimental system still has room for improvement in the future.

In summary, we show that exogenous Hsp70 can be a major paracrine/autocrine inducer of IL-10 production in RPE cells via TLR2 and TLR4, resulted in reduced subretinal fibrosis (schema in Figure 8). The results of this study indicate that exogenous Hsp70 can be ideal candidates for immunotherapy against chronic inflammatory diseases, which can form subretinal fibrosis such as AMD.

Supporting Information

Figure S1 Kinetics of intraocular IL-6, IL-10 and HSP70 expression after PEC inoculation. On day 0, 2, 3, 5, 7 after PEC inoculation, protein from retina-RPE-choroid complex (n = 5 each) were subjected to ELISA (IL-6, IL-10 and HSP70). Results are represents as mean ± SEM.
(TIF)

Figure S2 Pretreatment of exogenous macrophage with LPS and TGFβ do not alter subretinal fibrosis formation. (A) PECs were cultured with serum-free medium (complete medium except for FCS) that was supplemented with 0.1% BSA and with 0.2% insulin, transferrin serenium (ITS)+culture supplement (Collaborative Biochemical Products, Bedford, MA). Each cells were stimulated with LPS (1 μg/ml) or or TGFβ (2 ng/ml) for 24hours. Culture supernatants were subjected to ELISA (IL-6 and IL-10). (B) Collected, resuspended PECs were inoculated into subretinal space of WT mice (n = 5). After 7 days, eyes were enucleated, and choroidal flatmounts were prepared and stained with anti-GFAP antibody. The area of subretinal fibrosis were measured by ImageJ. (n = 5) (C) Representative images of choroidal flatmount stained with anti-GFAP antibody (bar = 100 μm). Results are represents as mean ± SEM.
(TIF)

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Author Contributions

Conceived and designed the experiments: YY AT TY KHS. Performed the experiments: YY AT TY. Analyzed the data: YY AT TY YO KHS TI. Wrote the paper: YY TY TI.

References

1. Bressler NM (2004) Age-related macular degeneration is the leading cause of blindness... JAMA 291: 1900–1901.
2. Miller JW (2011) Treatment of age-related macular degeneration: Beyond VEGF. Jpn J Ophthalmol 54: 523–528.
3. Stramer BM, Mori R, Martin P (2007) The inflammation-fibrosis link? A Jekyll and Hyde role for blood cells during wound repair. J Invest Dermatol 127: 1009–1017.
4. van Eden W, van der Zee R, Prakken B (2005) Heat-shock proteins induce T-cell regulation of chronic inflammation. Nat Rev Immunol 5: 318–330.
5. Ludwig D, Stahl M, Ibrahim ET, Wenzel BE, Drabicki D, et al. (1999) Enhanced intestinal expression of heat shock protein 70 in patients with inflammatory bowel diseases. Dig Dis Sci 44: 1440–1447.
6. Simon MM, Reikerstorfer A, Schwarz A, Krone C, Luger TA, et al. (1995) Heat shock protein 70 overexpression affects the response to ultraviolet light in murine fibroblasts. Evidence for increased cell viability and suppression of cytokine release. J Clin Invest 95: 926–933.
7. Yoo BC, Seidl R, Cairns N, Lubec G (1999) Heat-shock protein 70 levels in brain of patients with Down syndrome and Alzheimer's disease. J Neural Transm Suppl 57: 315–322.
8. Tanaka K-I, Tanaka Y, Namba T, Azuma A, Mizushima T (2010) Heat shock protein 70 protects against bleomycin-induced pulmonary fibrosis in mice. Biochem Pharmacol 80: 920–931.
9. Asea A, Rehli M, Kabingu E, Boch JA, Bare O, et al. (2002) Novel signal transduction pathway utilized by extracellular HSP70: role of toll-like receptor (TLR) 2 and TLR4. J Biol Chem 277: 15028–15034.
10. Dybdahl B, Wahba A, Lien E, Flo TH, Waage A, et al. (2002) Inflammatory response after open heart surgery: release of heat-shock protein 70 and signaling through toll-like receptor-4. Circulation 105: 685–690.