

Inflammatory Mediators Induced by Amyloid-Beta in the Retina and RPE In Vivo: Implications for Inflammasome Activation in Age-Related Macular Degeneration

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Abstract

Purpose—Drusen are hallmarks of age-related macular degeneration (AMD). Amyloid-beta 1-40 (A β 1-40), a constituent of drusen, is known to stimulate inflammatory pathways in RPE; however, its effect in vivo is not known. The purpose of this study was to examine the effect of A β 1-40 on cytokine expression and inflammasome activation relevant to AMD in an animal model.

Methods—Wild-type rats received intravitreal injections of A β 1-40, and eyes were taken at days 1, 4, 14, and 49 postinjection. The RPE, neuroretina, and vitreous were analyzed for cytokine expression, inflammasome activation, and microglial response via RT-PCR, immunohistochemistry, and suspension array assay. Retinal cell loss was assessed via apoptotic markers and retinal thickness.

Results—A β 1-40 stimulated upregulation of *IL-6*, *TNF- α* , *IL-1 β* , *IL-18*, *caspase-1*, *NLRP3*, and *XAF1* genes in the RPE/choroid and the neuroretina. Increased IL-1 β and IL-6 immunoreactivity was found in retinal sections, and elevated levels of IL-1 β and IL-18 were found in the vitreous of A β -injected eyes. A β 1-40 induced a moderate increase in CD11b/c-reactive cells on day 1 postinjection only. No evidence of the proapoptotic XAF1 protein, p53, TUNEL immunoreactivity, or retinal thinning was observed.

Conclusions—These results confirm earlier in vitro work and support the proinflammatory role of drusen component A β 1-40 in the RPE and retina. Inflammasome activation may be responsible for this effect in vivo. This model is useful for understanding cellular triggers of inflammasome activation and proposed early inflammatory events in the outer retina associated with the etiology of AMD.

Age-related macular degeneration (AMD) is a multifactorial disease that is responsible for a significant proportion of visual impairment in the elderly in Western society.^{1,2} Early AMD

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is characterized by the presence of drusen and abnormal pigmentation of retinal pigment epithelium (RPE)^{3,4} and is associated with gradual vision loss.⁵ In some cases early AMD will progress to a late, more advanced form that causes profound loss of central vision.⁶ Little is known about the etiology of this disease or the processes that occur in the eye during the early stages of AMD. Identifying the molecular and cellular events that lead to symptomatic AMD and the development of treatment strategies that act at the early stages of the disease are therefore of high priority.⁷

One of the strongest predictors of AMD is the number and size of drusen.⁵ Amyloid-beta ($A\beta$), the peptide associated with neurodegenerative events in the brain in Alzheimer's disease (AD), is an important constituent of drusen (Fig. 1).^{5,8} By analogy, the presence of $A\beta$ in drusen raises the possibility that it may also contribute to neurodegenerative events in the retina in AMD.^{9,10} A study on donor eye tissue established a correlation between $A\beta$ and geographic atrophy (GA).¹¹ A recent clinical trial addressing the safety and efficacy of anti- $A\beta$ treatment for AMD has begun, further emphasizing the need to understand the specific mechanisms underlying the effect of $A\beta$ in the eye.⁷

In contrast to past studies that used the more AD-specific, $A\beta$ 1-42 peptide, we focused on the structurally similar $A\beta$ 1-40 peptide in this study.¹² $A\beta$ 1-40, the more common and less toxic form, is present in drusen^{13,14} and is thus a relevant candidate to consider for AMD (Fig. 1). Using microarray analysis, Kurji et al.¹⁵ identified genes in the immune response and inflammation pathways as being highly upregulated by RPE cells in response to $A\beta$ 1-40 stimulation in vitro. There is emerging evidence that these pathways may be mediated by inflammasome activation.¹⁶ Recent studies on AMD pathogenesis have concentrated on NLR family, pyrin domain containing 3 (NLRP3) inflammasome activation in the RPE. The inflammasome NLRP3 is an intracellular multiprotein complex that recruits caspase-1 to catalyze the formation of IL-1 β and IL-18,^{17,18} along with other cytokines and growth factors such as IL-33 and fibroblast growth factor.¹⁹ However, important questions remain, such as whether inflammasome activation is involved in the retina's response to $A\beta$ and whether exposure to inflammatory mediators such as IL-1 β and IL-18 leads to AMD-like pathology.

To elucidate the effect of $A\beta$ 1-40 in vivo and investigate the sequelae of inflammatory mediators on the retina and RPE in the context of early AMD, we performed intravitreal injection of $A\beta$ 1-40 in a rodent model and studied the expression profile of key genes involved in the putative pathways of AMD pathogenesis, with a focus on inflammation and apoptosis. Intravitreal injection was chosen because it achieves the same effect of delivering peptide to outer retina/RPE as subretinal injections without the associated retinal damage or detachment, which may itself cause inflammation and confound the results.^{20,21} We hypothesized that $A\beta$ would stimulate inflammasome activation and overexpression of inflammatory mediators, including cytokines, in the retina and RPE; such a response to $A\beta$ may occur in the outer retina of presymptomatic AMD patients. Thus, treatments to minimize $A\beta$'s effect in the eye may slow the progression of AMD, a strategy being investigated in clinical trials.^{22,23}

Methods

Ethics

The postmortem tissue study was approved by the Clinical Research Ethics Board at the University of British Columbia. Methods for securing human tissue were in compliance with the Declaration of Helsinki. The animal procedures were carried out according to the protocol reviewed and approved by the University of British Columbia Animal Care Committee and conformed to the Canadian Council on Animal Care guidelines. All animal studies were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Donor Eye Tissue Immunohistochemistry

Human eyes were obtained from the Eye Bank of British Columbia. Eye tissues were fixed in 10% formalin and embedded in paraffin to obtain 6 μm sections through the pupil and optic nerve axis. Sections were deparaffinized and rehydrated by standard procedures. After antigen retrieval in 70% formic acid for 18 minutes at room temperature (RT), sections were blocked with 3% H_2O_2 for 15 minutes and 3% goat serum for 40 minutes. Sections were incubated in primary antibody specifically against $\text{A}\beta$ 1-40 (Cell Signaling Technology, Beverly, MA; Table 1) overnight at 4°C. Primary antibody omission or nonimmune isotype antibodies were used as negative controls. Sections were then incubated in appropriate secondary antibodies and developed in the ABC–AEC system (Vector Laboratories, Burlingame, CA).

$\text{A}\beta$ Oligomerization

$\text{A}\beta$ 1-40 (American Peptide, Sunnyvale, CA) was prepared as previously described.¹⁵ Briefly, lyophilized $\text{A}\beta$ peptide was dissolved in hexa-fluoroisopropanol and agitated at RT for 48 hours. Oligomerization was confirmed with atomic force microscopy and dot blot assay. Stock $\text{A}\beta$ was diluted in phosphate-buffered saline (PBS, pH 7.4) to yield a final concentration of 1.4 $\mu\text{g}/\mu\text{L}$. Aliquots of this solution were kept at -80°C until use. Human reverse peptide $\text{A}\beta$ 40-1 (American Peptide) was prepared in an identical manner.

Animal Model and Treatment

Five-month-old male Long-Evans rats (Charles River Laboratory, Wilmington, MA) were raised on standard rodent diet and kept in an enclosure with environmental enrichment and a 12-hour light cycle. On day 0, under inhalational anesthesia, intravitreal injections were performed under a dissecting microscope (Stereo dissection microscope, SMZ 1000; Nikon, Tokyo, Japan) using a 32-gauge Hamilton needle and syringe (Hamilton, Reno, NV) to deliver 5 μL oligomeric $\text{A}\beta$ 1-40 peptide (7 μg) with 0.1 μL 10% sodium fluorescein as dye (Akorn, Buffalo Grove, IL). This dosage yields an $\text{A}\beta$ intravitreal concentration of approximately 30 μM , higher than that used in the in vitro experiment¹⁵ and comparable to in vivo doses in other studies.^{21,24,25} Age-matched control animals received 5 μL intravitreal injection of reverse peptide $\text{A}\beta$ 40-1 (1.4 $\mu\text{g}/\mu\text{L}$) or vehicle (PBS). After the procedure, animals were given oxygen and were monitored in a recovery enclosure until they resumed baseline activity level. Animals were kept for 1, 4, 14, and 49 days postinjection ($n = 7$ per

treatment group), and euthanasia was performed with CO₂ inhalation. Eyes were immediately enucleated and frozen or preserved in 4% paraformaldehyde in Dulbecco's phosphate-buffered saline (DPBS; Invitrogen, Carlsbad, CA).

Real-Time PCR

The anterior segment of the frozen eye was removed to expose the posterior eye cup. Separate samples of neuroretina or RPE/choroid were obtained from the eye cup by surgical dissection under a dissecting microscope (Stereo dissection microscope, SMZ 1000; Nikon). Total RNA was isolated from tissue using the RNAqueous-4PCR Kit (Ambion, Austin, TX). RNA quantity and quality were assessed using the Nanodrop 2000c spectrophotometer (Fisher Thermo Scientific, Wilmington, DE). RNA (750 ng) from each tissue was reverse transcribed into cDNA using the High Capacity RNA-to-cDNA Master Mix (Invitrogen). PCR primer sequences are listed in Table 2. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as endogenous control. All reactions were carried out on the Applied Biosystems 7500 Fast SDS (Applied Biosystems, Carlsbad, CA) using Power SYBR Green (Applied Biosystems). Each sample was repeated in triplicate with near-identical results. Cycling conditions were as follows: 95°C for 15 seconds, 58°C for 30 seconds, 60°C for 45 seconds, 40 cycles. Melting curve analysis was automatically performed immediately after amplification. Gene product was quantified relative to *GAPDH* using the 2^{-CT} method.

Retinal Tissue Immunohistochemistry

Rat paraffin tissue sections cut at a thickness of 4 μm were prepared through standard procedures as described in our previous publication.²⁶ Sections of retina within 200 μm from the optic disc were known to be of even thickness regardless of embedding orientation²⁷ and thus were selected for processing.

To identify the localization of intravitreally injected Aβ 1-40 peptide, anti-Aβ antibody (4G8; Covance, Princeton, NJ; Table 1) was applied to tissue sections for 1 hour at RT and subsequently left overnight at 4°C, following antigen retrieval in 70% formic acid and blocking steps (3% H₂O₂, 5% goat serum). Incubation in primary antibody was followed by standard biotinylated secondary antibody incubation. These sections were developed with VIP chromogen (Vector Laboratories) and counterstained with methyl green.

Immunohistochemistry was also performed to evaluate the product of selected upregulated genes in the retina. Sections first underwent antigen retrieval, removal of endogenous peroxidase, and normal serum blocking by sequential incubation in protease K (20 μg/mL, pH 8.0; Sigma-Aldrich, St. Louis, MO), 0.3% H₂O₂, and 3% normal horse serum. Sections were then incubated overnight at 4°C with monoclonal antibodies including interleukin-6 (IL-6), interleukin-1 beta (IL-1β), X-linked inhibitor of apoptosis protein-associated factor 1 (XAF1), and CD11b/c, a cell surface marker for microglial cells (Table 1). After development with VIP chromogen, sections were photographed at ×20 and ×60 magnification using a brightfield microscope (Eclipse Ni U; Nikon) with a digital camera attachment (DS-Fi2 camera and DS-L3 monitor; Nikon). Microscopic scoring was conducted by scanning whole retinal sections under ×20 magnification in 1000 μm increments (diameter of the field) and averaging the number of immunoreactive cells per

increment. The final mean immunoreactive cell count was the averaged score of a minimum of two to four retinal sections per animal at each time point.

Retinal thickness was measured from the internal limiting membrane to the photoreceptor outer segments/RPE junction, and the mean value was derived from measurements of two to four retinal sections per animal at each time point.

Suspension Array Assay for Secreted Cytokines

Rat vitreous was collected and aliquoted for cytokine analysis using suspension 23-plex cytokine array plate (Bio-Rad Laboratories, Hercules, CA) as described by the manufacturer. Fifty microliters each of cytokine standards, samples (pooled vitreous fluids), and blanks (water only) was incubated with 25 μ L anticytokine conjugated beads in 96-well filter plates for 30 minutes at RT with agitation (1100 rpm for 30 seconds, then 300 rpm for 30 minutes). Plates were subsequently washed three times by vacuum filtration with 100 μ L Bio-Plex (Bio-Rad Laboratories) wash buffer per well using the Bio-Plex Pro Wash Station (Bio-Rad Laboratories). This was followed by the addition of 25 μ L diluted biotinylated detection antibody and further incubation with agitation at RT. After three filter washes (described above), 25 μ L streptavidin-phycoerythrin (SAPE) was added, and the plates were incubated for 10 minutes at RT with agitation. Finally, plates were washed by vacuum filtration three times. Beads were subsequently resuspended in 125 μ L Bio-Plex assay buffer, vortexed for 30 seconds at 1100 rpm, and further incubated for 2 minutes at 300 rpm. Standards, samples, and blanks were analyzed using the Bio-Plex 200 Suspension Array System, and subsequent raw median fluorescent intensity (MFI) data were captured and analyzed using Bio-Plex Manager software 4.1 (Bio-Rad Laboratories) at a standard high photomultiplier tube (PMT) setting.

Statistical Analysis

Statistical analyses on RT-PCR and immunohistochemical studies were performed between the A β 1-40 group and the reverse peptide group and/or the vehicle control group using Student's *t*-test with unequal variance. Suspension array data statistics were performed using Bio-Plex Manager software 4.1 (Bio-Rad Laboratories). Standard curves were developed using Brendon's Five Point logistic regression analysis, and a recovery range of 70% to 130% was established for the determination of a statistically valid standard curve.²⁸ RT-PCR and suspension array data are expressed as mean \pm SE; immunohistochemical data are expressed as mean \pm SD with *P* < 0.05 set as threshold for statistical significance.

Results

A β 1-40 Is a Component of Drusen

We first used a specific antibody to verify that A β 1-40, the more prevalent but less toxic form of A β peptide, is a component of human drusen (Fig. 1A). Using nonimmune isotype antibody yielded no A β 1-40 immunoreactivity (Fig. 1B). This is consistent with previous studies of drusen composition.⁸

Presence of A β 1-40 in All Retinal Layers after Intravitreal Injection

To confirm the effectiveness of intravitreal injection in delivering peptide to the outer retina, we probed the retinal tissue sections with 4G8 antibody that binds residues 17 to 24 in the human A β peptide. In A β 1-40-injected eyes, A β immunoreactivity was detected throughout retinal layers on day 1; the greatest intensity was localized to the photoreceptor outer segment (OS), while the RPE also demonstrated marked immunoreactivity (Figs. 2A, 2G). This immunoreactive pattern was less intense on day 4 (Figs. 2D, 2I). Preferential accumulation of A β in the OS has been previously reported in both mice and humans.²⁹ Our results were in keeping with previous reports²¹ and verified the penetration of intravitreally injected peptide through the retina and reaching the RPE. The reverse peptide A β 40-1 sections showed significantly less 4G8 immunoreactivity in the retina (Figs. 2B, 2E, 2H, 2J); this was expected as the 4G8 antibody is specific to the forward (1-40) peptide. We observed background levels of immunoreactivity in vehicle-injected eyes, confirming minimal nonspecific binding of the antibody (Figs. 2C, 2F).

Gene Expression Changes after A β 1-40 Intravitreal Injections

To assess whether the proinflammatory effect of A β on RPE extrapolates to an in vivo setting, we first screened for expression of inflammatory mediators by examining two cytokines. IL-6 is a known inducer of acute-phase protein production, and its serum level has been associated with progression of AMD.³⁰ In the A β group, there was significant upregulation of *IL-6* gene on days 1 and 4 compared to reverse peptide (Figs. 3A, 3B). The RPE/choroid exhibited a greater fold change than the neuroretina. No significant *IL-6* upregulation was found in either tissue at the later time points.

TNF- α is a prototypic cytokine with a potent effect on the retina and RPE.^{20,31} The A β group showed significantly higher levels of *TNF- α* mRNA than the reverse peptide group in the neuroretina at all time points, with a relatively greater response on days 1 and 14 (Fig. 3C). In contrast, *TNF- α* in the RPE/choroid from the A β group was significantly upregulated on day 1 and increased further on day 4, but was no longer significant at days 14 and 49 (Fig. 3D).

Apoptosis is a putative mechanism of RPE and photoreceptor loss in AMD, but its exact trigger and onset remain unknown. *XAF1*, a proapoptotic gene, was markedly overexpressed in RPE stimulated by A β 1-40¹⁵; therefore it was examined here. Compared to reverse peptide and vehicle, A β caused significantly higher fold change in *XAF1* expression in the neuroretina and RPE/choroid on days 1 and 4 (Figs. 3E, 3F). The magnitude of change was slightly greater in the RPE/choroid than in the neuroretina. No significant fold change in *XAF1* expression was found on days 14 and 49 in either tissue.

Given the central role of vascular endothelial growth factor (VEGF) in stimulating choroidal neovascularization (CNV) in late AMD, we assessed the expression profile of *VEGF*. *VEGF* upregulation has been observed in RPE cells treated with A β 1-40³² and in vivo after an intravitreal injection with A β 1-42.²⁴ Our results, however, showed no significant upregulation of *VEGF* in either neuroretina or RPE/choroid in the A β 1-40 group compared

to the reverse peptide group throughout the time course of the study (Figs. 3G, 3H). This is consistent with our earlier in vitro observations using A β 1-40.¹⁵

Recently, inflammasome NLRP3 activation has been proposed as a potential mechanism promoting inflammation in AMD.¹⁷ The increased levels of inflammatory cytokines in response to A β in our animals raised the possibility that NLRP3 could mediate cytokine upregulation in this setting. The expression profiles of *IL-6* and *TNF- α* indicated that the strongest inflammatory response occurred on days 1 and 4 postinjection; therefore we sought evidence of inflammasome activation at these time points using *IL-1 β* , *IL-18*, *caspase-1*, and *NLRP3* genes. IL-1 β and IL-18, two proinflammatory cytokines, are products of NLRP3 inflammasome processing,³³ and IL-1 β was markedly elevated in RPE upon A β 1-40 stimulation in vitro.¹⁵ Caspase-1 is part of the NLRP3 inflammasome complex and is required for the cleavage of IL-1 β and IL-18 propeptides.¹⁶ In our study, *IL-1 β* transcription was significantly elevated in the A β group on day 1; the neuroretina showed a greater fold change than the RPE/choroid (Figs. 4A, 4B). In contrast, *IL-18* was not significantly upregulated on day 1 in the A β group. *Caspase-1* expression was significantly higher in the neuroretina but not RPE/choroid on day 1 (Fig. 4A). *NLRP3* was markedly upregulated in the A β group, with neuroretina showing a greater response than RPE/choroid (Figs. 4A, 4B). The trend for inflammasome marker was similar on day 4. Of note is the significant upregulation of *IL-18* at this later time point, with the RPE/choroid showing the largest fold increase (Figs. 4C, 4D).

Cytokine Levels in the Retina and Vitreous following A β 1-40 Intravitreal Injection

Based on the overexpression of inflammatory mediator genes in both neuroretina and RPE/choroid, we performed immunohistochemistry on retinal sections to further study the distribution of selected mediators. IL-6 immunoreactivity in the A β -injected eyes was distributed predominantly in the ganglion cell layer (GCL) and inner nuclear layer (INL) (Figs. 5A–C), but the RPE also demonstrated significantly greater immunoreactivity compared to that in the reverse peptide group (Figs. 5D, 5E). The overall number of IL-6-immunoreactive cells was greater in the A β group than in the reverse peptide or vehicle control group on day 4 (Fig. 5F), corresponding with the peak in *IL-6* mRNA level (Figs. 3A, 3B). This difference was diminished on day 14 but still significant. By day 49, however, there was no significant difference between the groups.

IL-1 β immunoreactivity was primarily detected in the GCL and INL in the A β group, with sparse immunoreactivity in the reverse peptide and vehicle control groups (Figs. 6A–C). The RPE also demonstrated greater IL-1 β immunoreactivity in the A β group than in the reverse peptide group (Figs. 6D, 6E). Comparing the cell counts among the groups, we noted more IL-1 β -immunoreactive cells in both the A β and reverse peptide groups than in vehicle control on days 1 and 4, but these differences did not reach significance threshold (Fig. 6F). On days 14 and 49, the A β group, compared to the reverse peptide and vehicle control groups, had significantly more IL-1 β -immunoreactive cells.

Increased vitreal levels of inflammatory cytokines are present in patients with retinal pathologies including proliferative diabetic retinopathy³⁴ and central retinal vein occlusion.³⁵ Few studies have assessed the cytokines in the vitreous of AMD patients. In our study, the

elevated amount of IL-1 β immunoreactivity in the retina exposed to A β prompted us to look further for evidence of cytokines in the vitreous in this model. Using ELISA-based assay, we found significantly higher levels of IL-1 β and IL-18 in the vitreous of A β -injected eyes (13,920 \pm 433 pg/mL and 1126 \pm 264 pg/mL, respectively) than in reverse peptide controls (9803 \pm 280 pg/mL and 440 \pm 56 pg/mL, respectively) (Fig. 7). Of special note, Macrophage Inflammatory Protein 3 alpha (MIP-3 α), a lymphocyte and dendritic cell chemokine, showed the greatest increase in vitreal concentration in the A β group (759 \pm 54 pg/mL) compared to the reverse peptide group (199 \pm 21 pg/mL) (Fig. 7).

Microglia Activation Was Significant at Day 1

Microglia are known to take up A β and generate an inflammatory response that affects RPE function^{36,37}; this has been proposed as a possible mechanism of AMD pathogenesis. To test this hypothesis we evaluated the microglial response to A β 1-40 using OX-42 antibody against CD11b/c antigen, a cell surface marker representing total microglia (active and resting) (Figs. 8A–C). The majority of CD11b/c-positive cells were localized to the GCL and INL, and some demonstrated evidence of activation based on their rounded morphology (Fig. 8A, inset). We observed a statistically significant but marginal increase in CD11b/c-positive cells in the A β group compared to reverse peptide and vehicle control on day 1 postinjection (Fig. 8D). No significant difference in microglial count was observed among the groups at other time points.

Cell Death and Neovascularization Were Not Associated with A β 1-40

Geographic atrophy represents a late stage of AMD and is characterized by extensive RPE and photoreceptor loss that is thought to occur via apoptosis. Cytokines including TNF- α have been linked to photoreceptor apoptosis²⁰; therefore it is important to establish whether apoptosis is a feature in this model of early AMD. We first looked for protein expression of the proapoptotic factor XAF1. Despite upregulation of *XAF1* gene, we did not detect higher XAF1 immunoreactivity in the retina in the A β group compared to controls (data not shown). Additional immunohistochemical analyses of the tumor suppressor gene p53, as well as terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) staining, were undertaken, but no significant differences were observed between the groups (data not shown). To further evaluate retinal degeneration and preclude cell loss via nonapoptotic mechanisms,³⁸ we compared the neuroretinal thickness between A β -injected and control eyes. Neuroretinal thickness was preserved at all time points (Fig. 9), and we did not observe any evidence of Bruch's membrane disruption or angiogenic activities.

Discussion

A compelling role for local, chronic inflammation in the pathogenesis of AMD has been established by studies on drusen^{5,39} and gene polymorphisms.⁴⁰ Cytokines, key drivers of inflammation, have gained much interest for their potential role in AMD pathophysiology; for instance, high serum IL-6 level has been found to be an independent predictor for the progression of AMD.³⁰ Analysis of postmortem human eyes from AMD patients revealed elevated eotaxin and IP-10,⁴¹ as well as increased IL-1 β immunoreactivity in association with large drusen.⁴² Finding a trigger for cytokine expression is clearly important for

targeted intervention, and we hypothesized that A β 1-40, the principal form of the A β peptide found in the eye⁴³ and in drusen,¹³ may be responsible for promoting cytokine production and inflammatory pathways.¹⁵ Our results demonstrated that intravitreal injection of A β 1-40 caused marked upregulation of cytokines in the RPE and neuroretina. This model is consistent with the early phases of age-related maculopathy according to the hypothesis of Hageman et al.⁵ proposing that RPE dysfunction generates a proinflammatory milieu in early AMD pathogenesis.

RPE Plays a Major Role in Responding to A β

Our data suggest that both neuroretina and RPE/choroid upregulate inflammatory pathway genes in response to A β stimulation. Relative to the neuroretina, the RPE demonstrated a comparable magnitude of gene expression changes despite fewer total cells, indicating a high sensitivity to A β stimulation. The RPE is chiefly responsible for the maintenance and homeostasis of the outer retina and photoreceptor, but it is also known to actively participate in immune response via cytokine production.^{44,45} In contrast, it is mostly glial cells,⁴⁶ and principally microglia, that secrete cytokines in the neuroretinal layers.^{10,37} Based on immunohistochemistry data, the microglial response was minimal and transient in the A β group. Previous studies have induced marked microglial activation using intravitreal A β 1-42,^{10,21} likely due to the greater neurotoxicity associated with A β 1-42 compared to A β 1-40.¹⁴ The increase in microglia we observed on day 1 did not correlate with cytokine gene expression at other time points, nor was the magnitude of change large enough to convincingly demonstrate an association with the differential expression of inflammatory mediators seen. Combined with the fact that A β induces RPE to upregulate inflammatory pathways in vitro,¹⁵ it is likely that the RPE, not microglia, was the predominant source of cytokine in this study. The choroid was considered as a potential portal for systemic cytokines entering the retina; however, given that the blood-retinal barrier was intact after intravitreal injections, it is unlikely that A β oligomers (dimer: 8.6 kDa) and cytokines (all larger than A β) were exchanged between the choroidal vasculature and the retina.

A Role for Inflammasome NLRP3 Activation?

We observed a time-dependent overexpression of IL-6 and TNF- α in eyes treated with A β 1-40. IL-1 β , a cytokine product of inflammasome NLRP3, was concurrently upregulated on days 1 and 4 in the neuroretina and RPE. IL-18 was also upregulated on day 4. The associations between A β , inflammasome NLRP3, and RPE have been described separately: Halle et al.¹⁶ reported A β -induced NLRP3 activation in microglia while Kauppinen et al.¹⁸ showed NLRP3-dependent cytokine synthesis in human RPE under oxidative stress in vitro. The temporal pattern that emerged from our data is strongly suggestive of a role for NLRP3 in response to A β in the eye, a novel finding. We propose that, upon A β exposure, NLRP3 inflammasome is initially activated in the RPE and neuroretina, as illustrated by the overexpression of NLRP3 gene on day 1. The activation of NLRP3 recruits caspase-1 and catalyzes the formation of IL-1 β initially, followed shortly by IL-18. IL-1 β in turn stimulates the RPE in an autocrine manner,⁴⁷ causing overexpression of TNF- α and IL-6 to further propagate a local proinflammatory state.⁴⁸ This novel association highlights the key function of NLRP3 inflammasome in coordinating cytokine expression in the eye. In our model, cytokine expression mostly diminished to baseline levels by 14 days postinjection. This

observation presumably reflects clearance of A β , as microglia are known to remove A β from the retina.¹⁰ Interestingly, we observed high TNF- α mRNA levels and IL-1 β immunoreactivity on days 14 and 49 that might indicate some degree of persistent inflammatory pathway activation in the eye. Whether this represents the establishment of chronic inflammation is unclear and should be investigated in a future study that extends the observation period beyond the 49 days studied here. We demonstrated that A β 1-40 stimulates cytokine production in healthy RPE and retina and establishes a proinflammatory environment in the posterior segment of the rodent eye. It can be inferred that accumulation of A β in drusen may generate an increasing level of cytokines that primes the outer retina for subsequent damage, prior to the onset of clinical evidence of AMD.

Cytokines are integral to the host's immune defense, but their imbalances are often implicated in diseases. TNF- α , IL-6, and IL-1 β are also elevated in retinal detachment,²⁰ proliferative vitreoretinopathy,⁴⁷ and ischemia.⁴⁹ These cytokines also play an important part in the pathophysiology of AMD based on evidence from clinical and epidemiologic studies.³⁰ For instance, cytokines are known to stimulate chemotaxis, and our vitreous assay revealed an elevated level of chemokine MIP-3 α (Fig. 7). MIP-3 α mediates migration of dendritic cells and lymphocytes via CCR6 receptor as part of mucosal defense against microbes.⁵⁰ Human ARPE-19 produces MIP-3 α in response to IL-17A.⁵¹ RPE has also been known to increase production of IL-8, another chemokine, when stimulated by A β 1-40.¹⁵ In light of these observations, chemotaxis may constitute an important part of the response to A β in the eye and warrants further investigation.

To the best of our knowledge, our study showed, for the first time, that IL-18 is upregulated in the retina, RPE, and vitreous following A β stimulation. IL-18 is thought to be involved in late stages of AMD. Doyle et al.⁵² showed that IL-18 inhibits angiogenesis in CNV, while Tarallo et al.¹⁷ reported that IL-18 promotes RPE death in dry AMD. Neither event was observed in this study, but our model assessed the effects only up to 49 days following a single A β injection. It is likely that multiple A β injections and follow-up beyond 49 days may better simulate the chronic, local proinflammatory environment thought to be present in the retina of patients with early AMD and thus may be helpful to define the role of IL-18 in AMD.

Cell Loss Is Not a Feature of This Model of Early AMD

There is an established connection between inflammation and apoptosis. Both IL-1 β and TNF- α are known to increase expression of apoptotic factors in the RPE.⁵³⁻⁵⁵ Apoptosis has been proposed as a mechanism contributing to degenerative events in AMD.⁵⁶ The present study saw a significant upregulation of the *XAF1* gene by RPE/choroid in A β -injected eyes (Figs. 3E, 3F), in keeping with our previous in vitro observations.¹⁵ XAF1 directly activate caspase-dependent apoptosis in conjunction with TNF- α .^{56,57} Surprisingly, there was no significant change in the immunoreactivity of XAF1 in the retinal tissue sections. Such mismatch between XAF1 mRNA and protein was previously reported in other tissue types as well.⁵⁸ We suspect that antiapoptotic processes might have interfered with the synthesis of XAF1 protein. Absence of significant p53 or TUNEL immunoreactivity and preservation of retinal thickness in A β -injected eyes strongly suggest that cell death was not a feature of

the present study. This is in contrast to intravitreal A β 1-42-induced retinal neuron degeneration in AD models.^{24,25} The difference is likely due to the inherently greater neurotoxicity of A β 1-42 compared to A β 1-40.¹⁴ On the other hand, existing animal models of AMD capture many features of late disease: Malek et al.⁵⁹ demonstrated drusen-like deposits, RPE changes, and CNV in the apoE4-HFC diet mouse model. Both the Ccl2/Cx3cr1 double-knockout mouse model and the Ccl2- or Ccr2- knockout mouse model produced subretinal deposits and RPE/photoreceptor atrophy.^{60–62} However, these models do not directly or independently rely on the local proinflammatory retinal environment thought to be present in presymptomatic AMD. Using intravitreal A β 1-40 injection, we induced a proinflammatory response in the healthy retina without causing cell loss. This model creates the opportunity to study how inflammatory cytokines affect the homeostasis of the outer retina and to evaluate the effectiveness of interventions that control the proinflammatory environment, hypothesized to be one of the earliest changes associated with the etiology of AMD.

In conclusion, the present study verified the proinflammatory effects of drusen component A β 1-40 in vivo in the rat retina. Cytokine genes including IL-6, TNF- α , IL-1 β , and IL-18 are upregulated by the RPE and neuroretina after A β stimulation. The activation of NLRP3 inflammasome may play a central part in mediating this response and is thus a potential novel target for AMD treatment. A β 1-40 is likely to be an important contributor to AMD by promoting the release of cytokines and establishing a background level of chronic, local inflammation that promotes dysfunction of cells of the outer retina including the RPE. The absence of retinal cell apoptosis in this model makes it suitable for studying the early proinflammatory events that may prime the retina toward AMD development. Our results are useful for future studies requiring an in vivo platform for testing therapeutics that target and suppress inflammasome activation and cytokine production in the retina. Current literature on the use of anti-inflammatory agents in AMD derives from studies that focus on late stages of the disease⁶³; however, our study prompts a closer look at the possible benefit of such an approach in early AMD with the goal to minimize disease progression and prevent vision loss.

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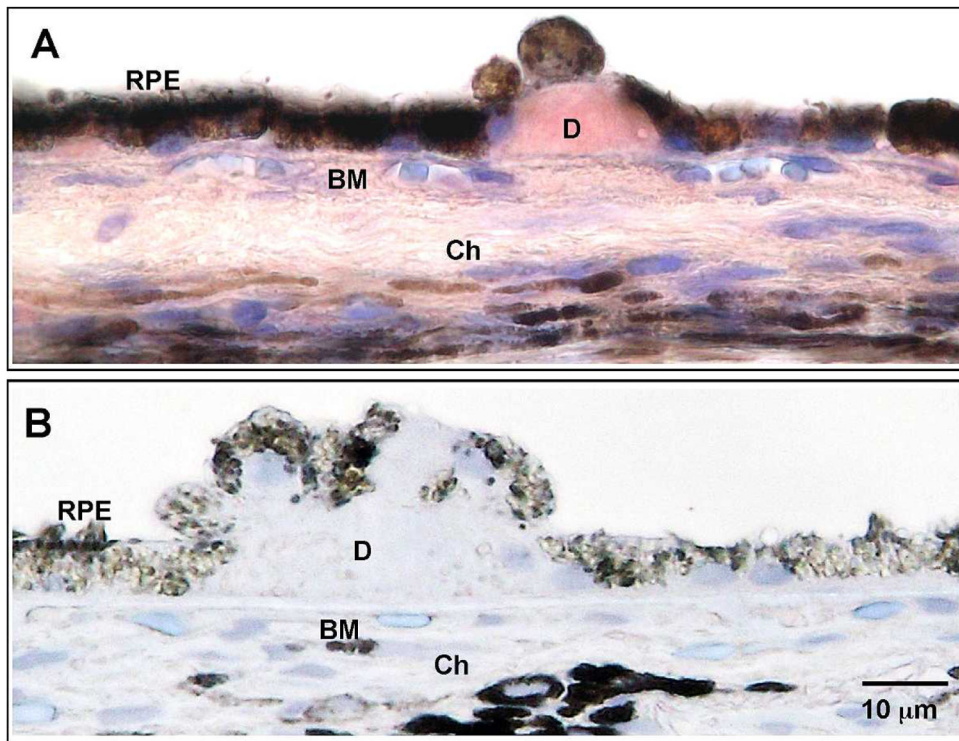


Figure 1. A β 1-40 in drusen. Immunohistochemistry for A β 1-40 was developed in AEC (*red*) and counterstained with Mayer's hematoxylin (*blue*). (A) A β 1-40 in drusen of a 72-year-old non-GA female postmortem donor eye. (B) Negative control using nonimmune rabbit IgG isotype antibody on the same donor eye section. RPE, retinal pigment epithelium; D, drusen; BM, Bruch's membrane; Ch, choroid. *Scale bar*: 10 μ m.

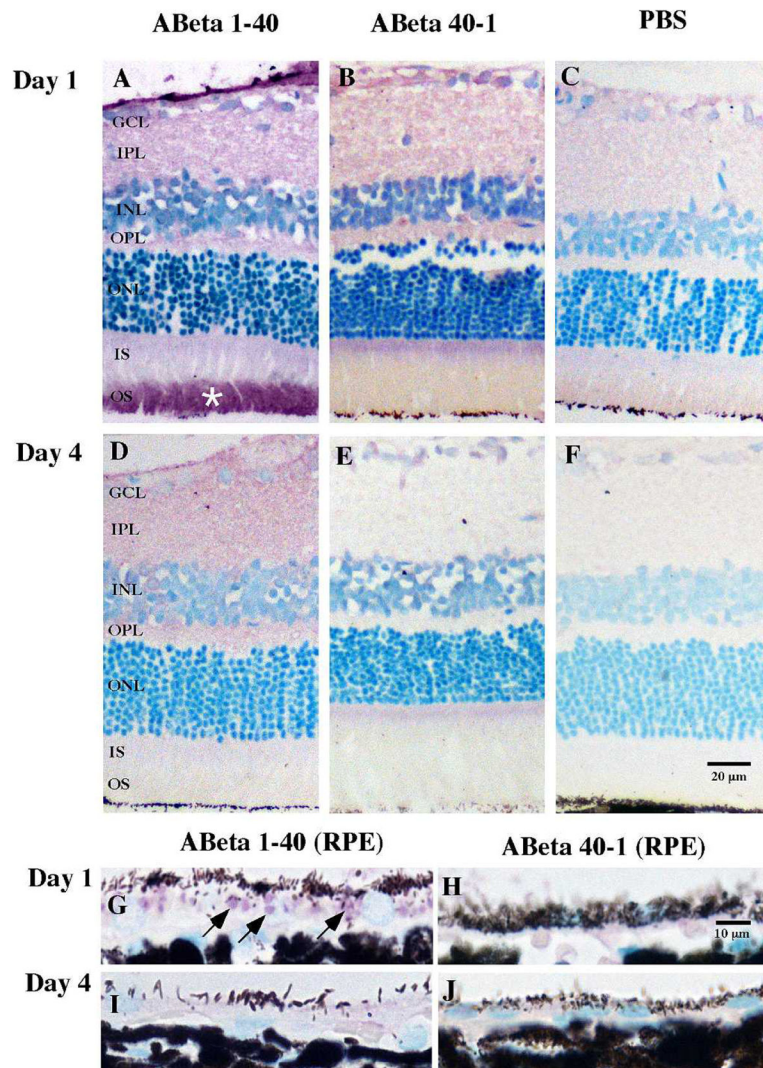


Figure 2. Localization of A β 1-40 or reverse peptide A β 40-1 demonstrated in retinal tissue on days 1 and 4 following intravitreal injections. Immunoreactivity for A β 1-40 and A β 40-1 was undertaken with 4G8 antibody, visualized with VIP chromogen (*pink/purple*), and counterstained with methyl green. VIP chromogen revealed immunoreactivity in the neuropil and extracellular compartments throughout all retinal layers on day 1 and less on day 4 in the A β group (**A**, **D**). Strong immunoreactivity was detected in the OS (*star*) in A β -injected eyes. Tissues from eyes receiving reverse peptide injection demonstrated significantly less immunoreactivity than the forward peptide group (**B**, **E**), as did the vehicle-injected eyes (**C**, **F**). Focusing on the RPE, there are discrete intracellular vesicles (*arrows*) possibly representing phagocytosed outer segment discs in the A β group compared to the reverse peptide group in day 1 sections (**G**, **H**). Day 4 sections show no vesicles but rather a diffuse and faint layer of VIP chromogen at the RPE in the A β group compared to the reverse peptide group (**I**, **J**). GCL, ganglion cell layer; IPL, inner plexiform layer; INL,

inner nuclear layer; OPL, outer plexiform layer; ONL, outer nuclear layer; IS, inner segment; OS, outer segment. *Scale bar:* 20 μm (A–F), 10 μm (G–J).

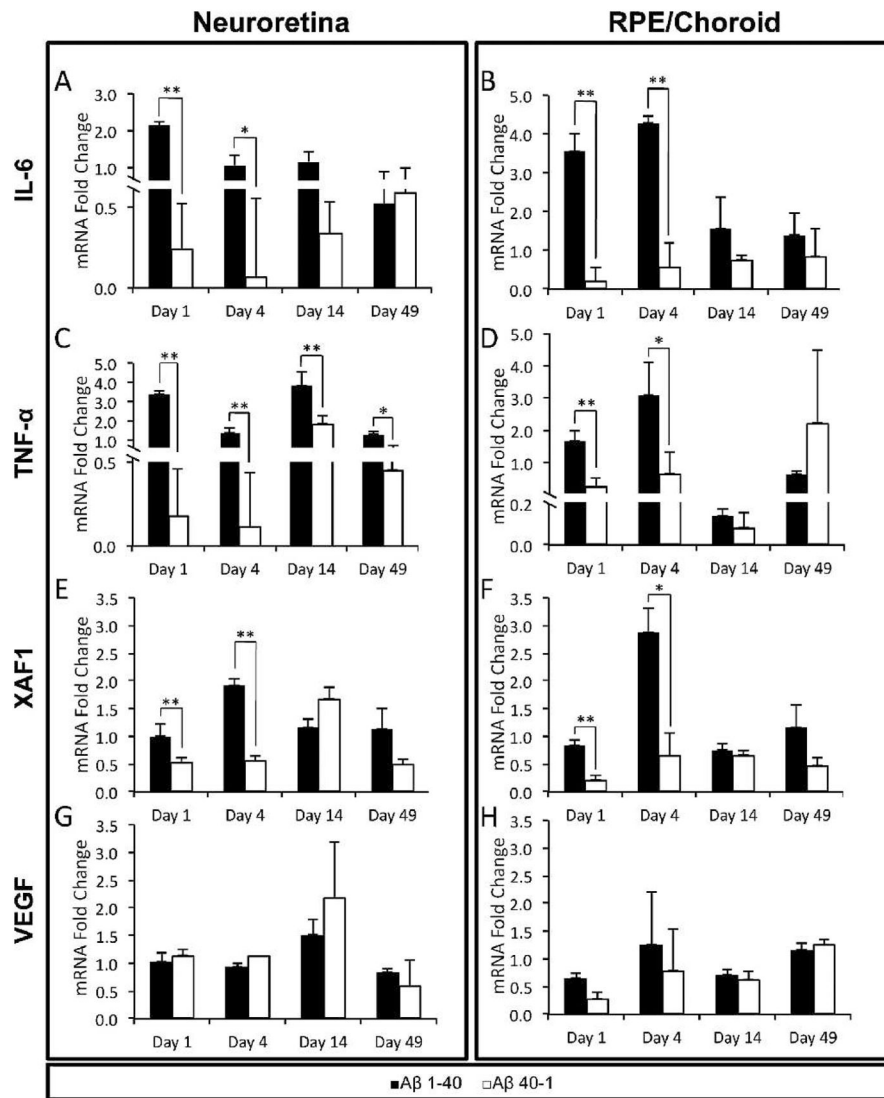


Figure 3. RT-PCR of selected genes in the RPE/choroid and neuroretina. The relative mRNA quantity (normalized to endogenous control GAPDH and expressed as fold change over vehicle control PBS) in the A β 1-40 group was compared to that in the reverse peptide group. Genes were examined on days 1, 4, 14, and 49 to capture both immediate and later changes after intravitreal injection at day 0: (A, B) IL-6, (C, D) TNF- α , (E, F) XAF1, (G, H) VEGF. Error bars denote standard error. * $P < 0.05$; ** $P < 0.01$.

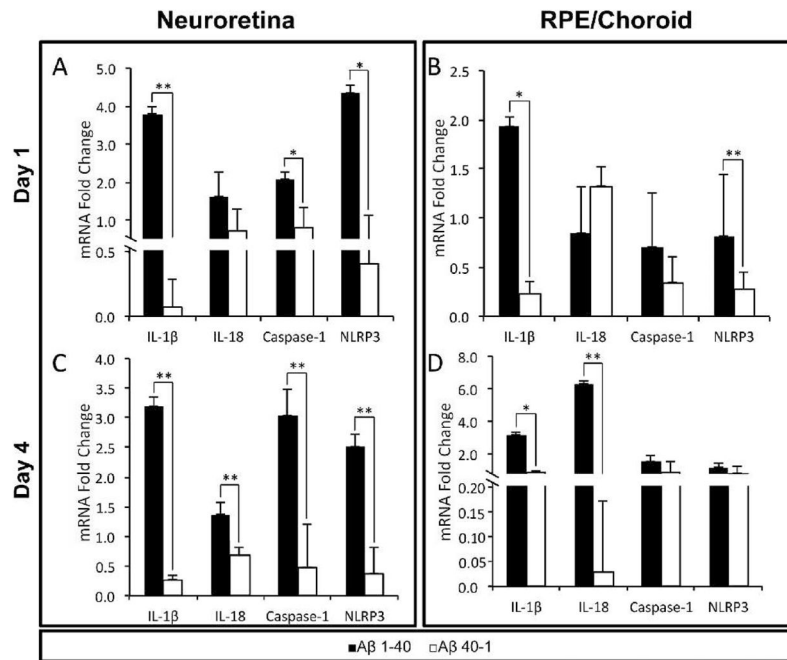


Figure 4. Inflammation gene RT-PCR data. The relative mRNA quantity (normalized to endogenous control GAPDH and expressed as fold change over vehicle control PBS) in the Aβ 1-40 group was compared to that in the reverse peptide group. (A, B) Day 1, (C, D) day 4. Error bars denote standard error. * $P < 0.05$; ** $P < 0.01$.

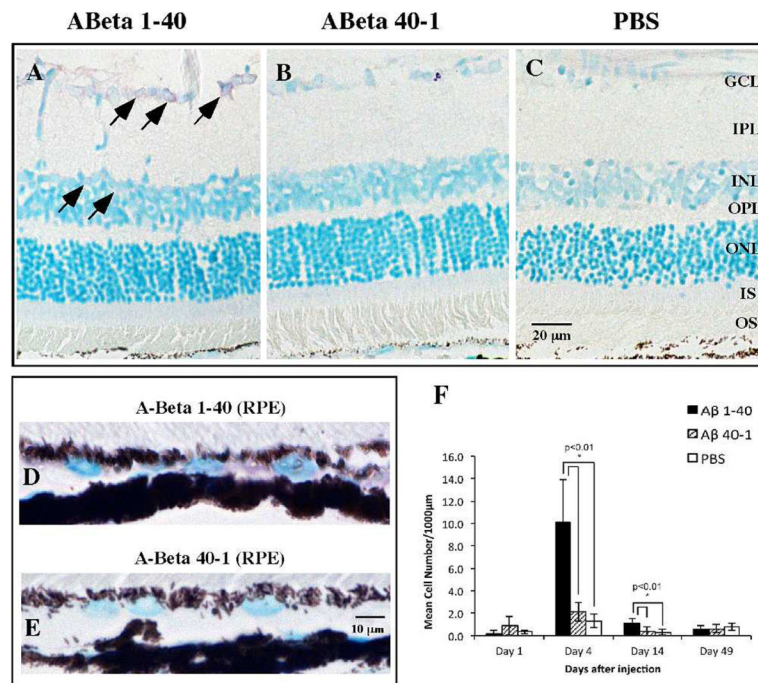


Figure 5.

IL-6 immunoreactivity in the retina and RPE. (A–C) Representative retinal micrographs of IL-6 immunoreactivity on day 4. In the Aβ group, IL-6 immunoreactivity was mostly detected in cellular profiles in the GCL and INL (*arrows*). In contrast, the reverse peptide group shows very sparse IL-6 immunoreactivity, while the vehicle injection group identifies background level of IL-6 immunoreactivity. (D, E) The Aβ group also demonstrates more intense IL-6 immunoreactivity in the RPE than the reverse peptide group. (F) *Bar graph* of mean cell count of IL-6-immunoreactive cells at four time points, showing significantly greater IL-6 immunoreactivity in the Aβ group on days 4 and 14 than in the reverse peptide or vehicle control group. Micrographs were examined at ×20 and ×60 magnification. *Scale bar*: 20 μm (A–C), 10 μm (D, E). *Error bars* denote standard deviation. **P* < 0.01.

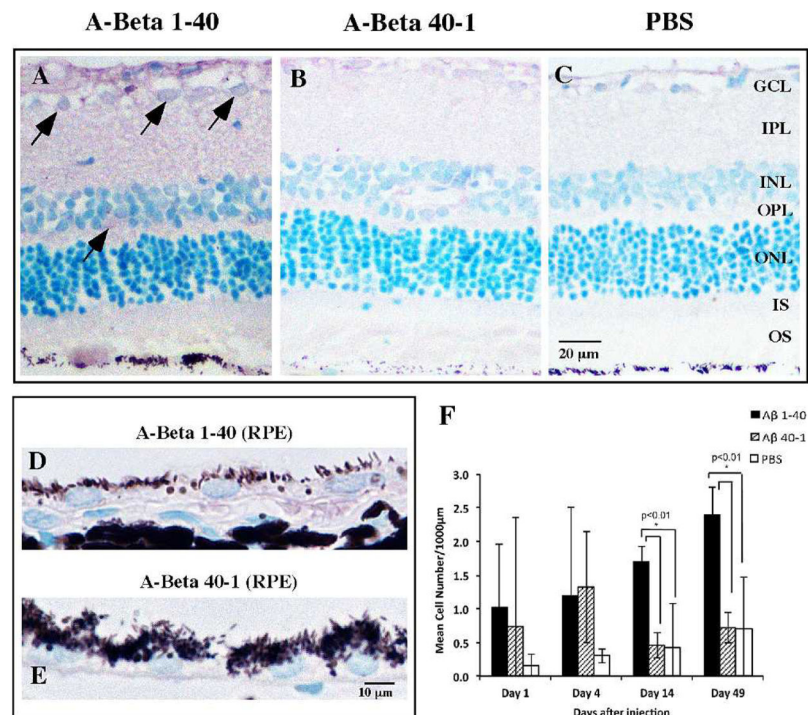


Figure 6. IL-1 β immunoreactivity in the retina and RPE. (A–C) Representative retinal micrographs of IL-1 β immunoreactivity on day 14. Marked IL-1 β immunoreactivity is observed in the A β group and is predominantly localized to cellular profiles in GCL and INL (*arrows*) and in the neuropil IPL and OPL. Reverse peptide group demonstrates weak IL-1 β immunoreactivity without any foci of distribution. Vehicle injection group demonstrates background IL-1 β immunoreactivity. (D, E) A β group shows greater IL-1 β immunoreactivity in the RPE than the reverse peptide group. (F) *Bar graph* of mean cell count of IL-1 β -immunoreactive cells at four time points, showing significantly more IL-1 β immunoreactivity in the A β group on days 14 and 49 compared to the reverse peptide or control group. Micrographs were examined at $\times 20$ and $\times 60$ magnification. *Scale bar*: 20 μm (A–C), 10 μm (D, E). *Error bars* denote standard deviation. * $P < 0.01$.

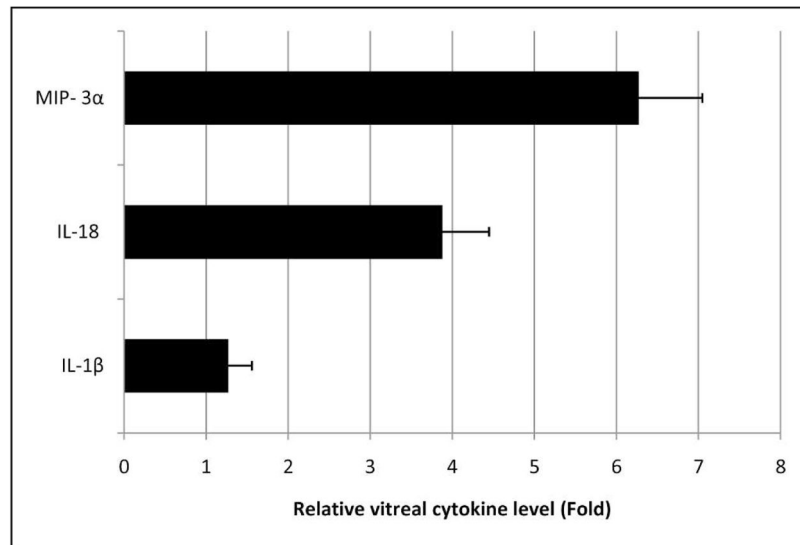


Figure 7.

Day 14 vitreal cytokine suspension array assay. Only statistically significant results ($P < 0.05$) are shown. Inflammasome product IL-1 β was significantly elevated in A β -injected vitreal ($13,920 \pm 433$ pg/mL) compared to reverse peptide-injected vitreal (9803 ± 280 pg/mL). Similarly, IL-18 showed significantly higher concentration in A β -injected vitreal (1126 ± 264 pg/mL) than in reverse peptide-injected vitreal (440 ± 56 pg/mL). MIP-3 α showed the greatest difference between the A β group (759 ± 54 pg/mL) and the reverse peptide group (199 ± 21 pg/mL). Other cytokines including IL-6 and TNF- α did not reach threshold of significance ($P < 0.05$). *Error bars* denote standard error.

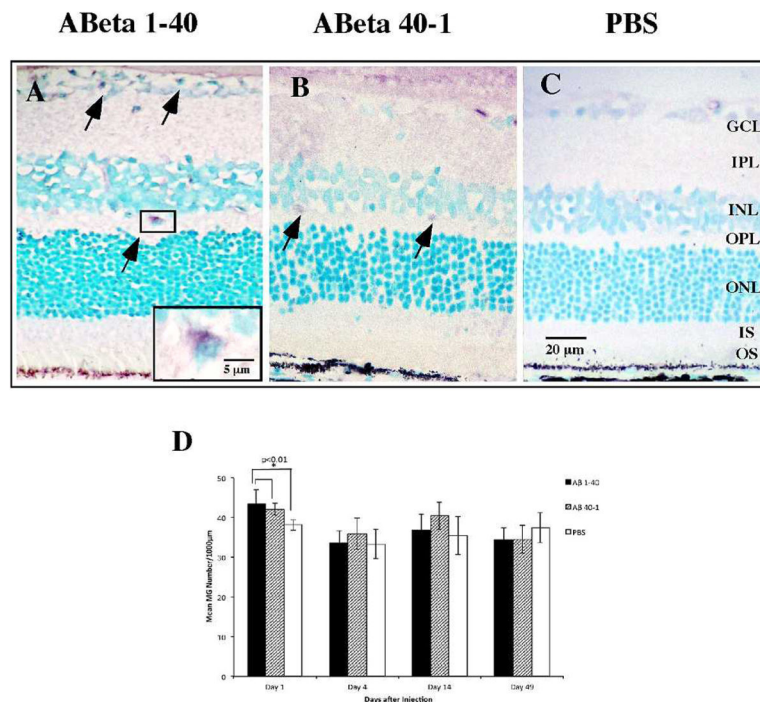


Figure 8. Microglia response to A β 1-40 stimulation. Retinal sections were reacted with OX-42 antibody against cell surface marker CD11b/c, representative of total microglia. (A–C) Representative immunoreactivity pattern of CD11b/c labeling in the retinal sections on day 1. Note that the A β section and the reverse peptide section both contain CD11b/c-positive cells within the GCL and INL (*arrows*), with the vehicle group demonstrating significantly lower CD11b/c immunoreactivity. Some CD11b/c-positive cells demonstrate rounded morphology and are intimately associated with cell bodies of retinal neurons (*inset*). (D) *Bar graph* of mean CD11b/c immunoreactivity profiles in three groups, with significance demonstrated at day 1 between the A β 1-40 forward peptide group and the reverse peptide and vehicle control groups. Micrographs were examined at $\times 20$ and $\times 60$ magnification. *Scale bar*: 20 μm ; 5 μm (*inset*). *Error bars*: standard deviation. * $P < 0.01$.

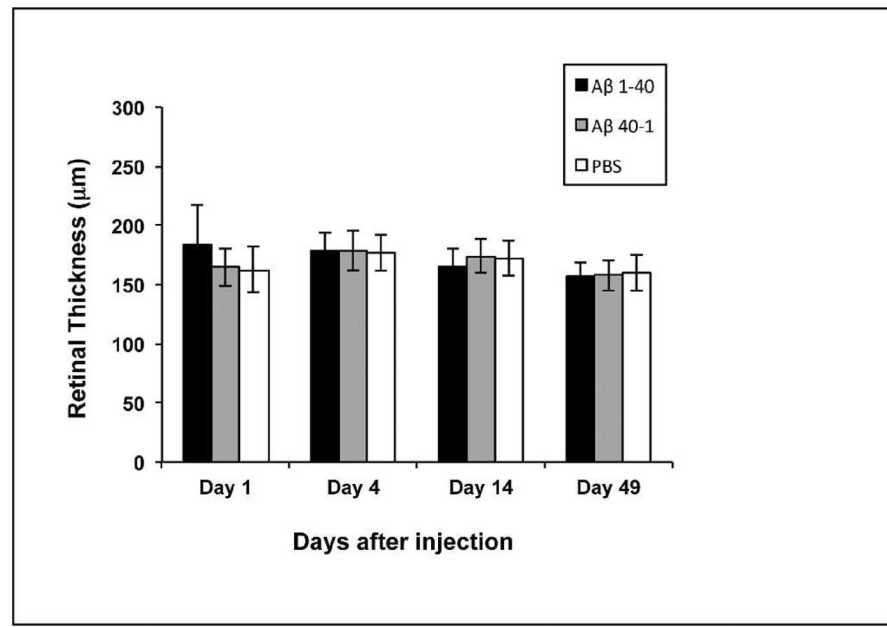


Figure 9. Retinal thickness measurements. Retinal thickness was measured from internal limiting membrane to photoreceptor outer segment/RPE junction in parafoveal sections. No significant difference in retinal thickness was observed in any group throughout the experiment. *Error bars* denote standard deviation.

Table 1

Primary Antibodies Used in Immunohistochemistry

Antigen	Primary Antibody	Type	Dilution	Specificity	Source and Catalog No. (Clone No.)
Amyloid-beta (A β) 1-40	Rabbit antihuman	Polyclonal	1:200	Recognizes human A β 1-40 isoform and crossreacts with mouse, rat, and monkey isoforms	Cell Signaling Technology, Beverly, MA; 9682
Amyloid-beta (A β)	Mouse antihuman	Monoclonal	1:500	Recognizes amino acid residues 17 to 24 of A β , crossreacts with mouse isoform	Covance, Princeton, NJ; SIG-39220 (4G8)
Interleukin-1 beta (IL-1 β)	Goat antirat	Polyclonal	1:400	Detects rat IL-1 β /IL-1F2 in cultured cells or tissue sections	R&D Systems, Inc., Minneapolis, MN; AF-501-NA
Interleukin-6 (IL-6)	Rabbit antirat	Polyclonal	1:400	Detects recombinant and native IL-6 secreted into body fluids and/or cell supernatants in rabbits, humans, mice, rats, and pigs	Abcam, Cambridge, UK; ab6672
OX-42 (CD11b/c equivalent antibody)	Mouse antirat	Monoclonal	1:300	Detects rat CD11 b/c in macrophages/dendritic cells with morphology of microglia in the brain	Cedarlane, Hornby, ON; CL042AP (MRC OX-42)
X-linked inhibitor of apoptosis protein-associated factor 1 (XAF1)	Rabbit antirat	Polyclonal	1:500	Detects cytoplasmic, mitochondrial, and nuclear XAF1 in rabbits, humans, mice, and rats	Abcam, Cambridge, UK; ab81353

Table 2

RT-PCR Primer Sequences

Gene	Forward Primer	Reverse Primer	Reference
<i>IL-6</i>	TCAACTCCATCTGCCCTTCAG	AAGGCAACTGGCTGGAAGTCT	Sanchez et al., 2003 ⁶⁴
<i>TNF-α</i>	AAATGGGCTCCCTCTCATCAGTTC	GCTTGGTGGTTTGCTACGAC	Peinnequin et al., 2004 ⁶⁵
<i>IL-1β</i>	CACCTCTCAAGCAGAGCACAG	GGGTCCATGGTGAAGTCAAC	Peinnequin et al., 2004 ⁶⁵
<i>IL-18</i>	AGAAGGCTCTTGTGTCAAC	CTTCCTTTTGGCAAGCTAG	
<i>Caspase-1</i>	AGAGAAGAGAGTCCTGAAC	TCTCTGAGGTCAACATCAG	
<i>NLRP3</i>	CAGAAGGCATGTGAGAAG	ACAGGATCTTGCAACTG	
<i>XAF1</i>	GGAGAGGAGACAGCCTATG	CCTGGTGCTCATTAGAA	
<i>VEGF</i>	TGCAGACCAAAGAAAGATAGAAC	GGATCTTGGACAAACAAATGC	Mor et al., 2004 ⁶⁶
<i>GAPDH</i>	GAACATCATCCCTGCATCCA	CCAGTGAGCTTCCCGTTCA	Medhurst et al., 1999 ⁶⁷