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The role of immune regulator IRAK-M in myeloid cell activation in light-challenged mouse retina

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Abstract:

Oxidative stress is one of the pathogenic factors involved in the mediation of age-related macular degeneration (AMD) and is associated with immune cells infiltrating to subretinal space and changes in the retinal pigmented epithelium, i.e. autophagy impairment. Retinal inflammation perturbs homeostatic para-inflammatory response via release of certain cytokines and chemokines. IRAK-M is recognised as one of the intracellular regulators responsible for negatively regulating inflammatory responses.

Methods: Genetically altered IRAK-M knock out (KO) mice and wild type (WT) C57BL/6J mice aged 8-10 weeks were subjected to fundus camera-delivered light-induction for retinal degeneration. 3-days post light induction, eyes were enucleated and cryosections prepared for fluorescent staining of CD11b+ cells.

Results: IRAK-M KO-mice with light-induction show increased number of CD11b+ cells in comparison to WT-mice with light-induction. In KO-mice, light induced infiltration of CD11b+ myeloid cells were largely seen in subretinal space but not in WT-mice.

Conclusion: IRAK-M deficient mice's retina is susceptible to light-induced CD11b+ cell infiltration and particularly accumulation within the subretinal space. The data suggest a role of IRAK-M in regulating myeloid cell -mediated tissue inflammation under oxidative stress.

Key words: Age-related macular degeneration, Retinal Pigmented Epithelium, IRAK-M, Oxidative-stress, Myeloid cells

Introduction:

AMD is a progressive ocular neurodegenerative disorder and results in severe loss of central vision (1). AMD is the most common cause of visual loss in western countries. Approximately, 50 million of the elderly population worldwide is affected by AMD and this figure is expected to rise by 3-folds in the coming 20 years, making AMD a major public health issue (2). The initial stages of AMD are characterized by drusen formation and abnormalities to the retinal pigmented epithelial (RPE) cells (1). Drusen formation contains immunologically active compounds such as lipids and other immune activating components (3). These form deposits that primarily accumulate between RPE layer and Bruch's membrane. Drusen mainly affects the macula, which is responsible for central and precise vision (4). Progressively, this may either lead to geographic atrophy (GA) or neurovascular AMD (nAMD).

Progression of AMD is based on many factors and triggers including degeneration of RPE cells (2), the altered immune response within the retinal layers (1) and oxidative stress. The dysregulation in the

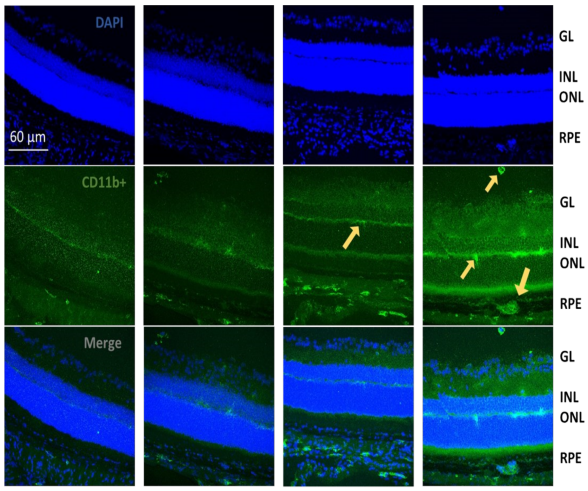

Figure 1: *Images of stained retinal sections taken by confocal microscope. The representative images showing difference in number of CD11b+ stained cells in the retina. The top row of pictures is stained with DAPI and all nuclei are stained blue. Green staining shows the CD11b+ expressed cells. The yellow arrows pointing at the brighter areas represent the presence of activated myeloid cells within different layers of retina.*

homeostatic para-inflammatory response leading to a harmful chronic inflammation is likely a result of persistent lifetime oxidative stress of RPE (1). RPE is a monolayer consisting of hexanocuboidal epithelial cells (2), situated between the neural retina and choroid (5). The RPE forms the part of the outer blood retinal barrier where the apical membrane envelops the neural retina, and the basal membrane lines the Bruch's membrane (6). The essential function of the RPE layer is to maintain the survival of overlying photoreceptor cells by performing critical functions, i.e. a) formation of outer blood retinal barrier, b) secretion of growth factors, c) regulation of ions, water and nutrients between the basal and apical membrane, d) phagocytosis and degradation of the tips of outer segments of the photoreceptors (2,5,6,7). If RPE function fails, this leads to build up of oxidative stress (7).

The NLRP3 inflammasome complex acts as a sensor that monitors cellular stress such as oxidative stress via Toll-like receptors (TLRs). Activation of NLRP3 leads to caspase-1-mediation that release proinflammatory cytokines IL-1β and IL-18. Oxidative stress evokes an inflammatory response within the RPE cells due to increased levels of IL-1β and IL-18 gene expression (3). Activated TLRs recruit myeloid differentiation primary response gene 88 (MyD88) to form the Myddosome complex, where interleukin -1 receptor-associated kinase (IRAK) binds. This adaptor complex then activates the signalling pathway for the gene transcription for the production of IL-1β and IL-18 (11). Excessive or dysregulated inflammation is dangerous for healthy cells and pro-inflammatory mediators must be tightly regulated.

IRAK-M is responsible for negatively regulating TLRs/IL-1 induced inflammatory cascade. It inhibits the production of Nuclear Factor kappa-light-chain-enhancer of activated B cells (NF-KB) and activator protein-1 (AP-1). Both are responsible for the regulation of gene transcription in response to inflammatory stimulus, ceasing the production of pro-inflammatory cytokines and chemokines (11,12,13). These cytokines and chemokines are responsible for attracting monocytes from blood and retina that differentiate into infiltrating macrophages (1).

Methods:

Mice were kept in the animal house facilities of the University of Bristol in accordance to Home regulation office. Animals were treated according to the Association for Research in Vision and Ophthalmology (ARVO). The experiment was complied with the approved University of Bristol institutional guidelines under a Home Office Project Licence 30/2745 and by University of Bristol Ethical Review Group. Genetically altered IRAK-K KO mice along with WT C57BL/6J mice (Jackson Laboratories) aged 8-10 weeks were used for the experiment.

Fundus camera-delivered light-induced retinal degeneration was performed according to previous studies (14). In brief, mice were anesthetized, and pupils of left eyes were dilated. The eyes were centred in observation of fundus camera and focused on the RPE layer. The light was applied to the retina at an intensity of 100 klux for 20 min. Right eyes were kept exposed to normal light condition as control. Post light induction, mice were kept under background light for three days.

Three days post light induction, eyes were enucleated and preserved in OCT and frozen immediately. Preserved eyes were sectioned using cryosection

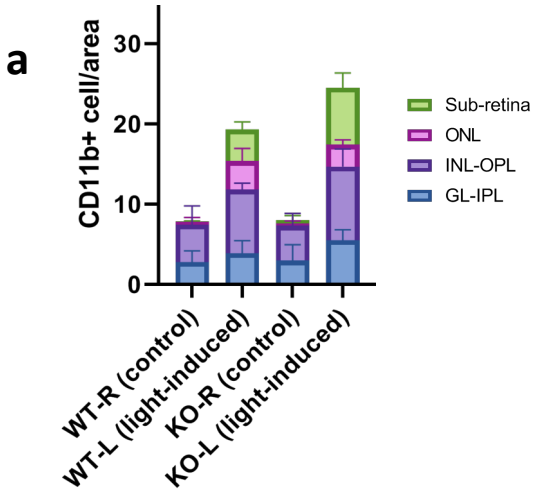

Figure 2(a): Bar chart showing the quantification for Figure 1. Confocal images of 10 retinal sections across the entire retina were taken. Numbers of CD11b+ expressed cells were counted in each section and were compartmentalised within the 4 layers of the retina. This data was then used to generate the bar chart using GraphPad Prism. The figure represents an overall increased the number of CD11b+ cells in KO light induced eye compared to WT. However, both controls had similar number of CD11b+ cells.

technique to prepare slides of 16 µm in thickness.

All slides were stored at -20°C until immunohistochemistry analysis. Prior to immune-fluorescence staining, slides were bought down to room temperature for 10-15 minutes. On the slide, all three sections were circled with an IMMedge hydrophobic pen and allowed to dry. Following tissue fixation with 2% paraformaldehyde and permeabilization with 0.1% Triton X-100, sections were blocked with 5% bovine serum albumin before incubation with a rat anti-CD11b mAb (clone M1/70, 1:100, BD Biosciences) overnight at 4 °C. After wash, sections were further incubated with goat anti-rat conjugated with Alexa Fluor 488 (1:400, ThermoFisher Scientific). DAPI counterstain was used to show nuclei in sections. The slides were then analysed using confocal immunofluorescence microscopy. Figures and statistics were generated using Graphpad 8 Prism. Two-way ANOVA were used to compare differences between the genotypes.

Results and Discussion:

Difference in myeloid cell immunostaining between WT and IRAK-M-depleted mouse retina:

CD11b+ myeloid cells in retinal sections after immunofluorescent staining (figure 1) were analysed to investigate the effects of light-induced oxidative stress within the RPE layer of IRAK-M depleted and WT mice. The naïve WT group had a small number of CD11b+ myeloid cells in the ganglion layer (GL) and inner nuclear layer (INL). There was no CD11b+ myeloid cells detected in the subretinal space of the naïve WT mice retina. In contrast, the light-induced WT retina had CD11 b+ myeloid cells in all four retinal layers; GL, INL, outer nuclear layer (ONL) and subretinal layer. We also found that the number of CD11b+ myeloid cells were largely increased in the INL and ONL in comparison to the normal WT retina.

In the navie KO mice retina we observed the presence of CD11b+ myeloid cells in all retinal. CD11b+ myeloid cells were present within the subretinal space even without any light induction. Conversely, in the light-induced KO mice retina we spotted a high number of CD11b+ myeloid cells in all the retinal layers. We also noticed that the myeloid cells within the KO light-induced retina were relatively larger then those in WT retina (figure 1).

Comparison of myeloid cell number between WT and IRAK-M-depleted mouse retina:

Statistical analysis on the number of CD11b+ myeloid cells in WT control and KO control retina, showed that both retinas had equal number of activated myeloid cells. Moreover, the number of CD11b+ myeloid cells in GL and in INL were identical between control WT and control KO. However, we spotted a few CD11b+ myeloid cells within the subretinal space of control KO mice retina, whereas, non were detected in the subretinal space of control WT mice retina.

CD11b+ myeloid cells within the light-induced oxidative stress retina were elevated in both genotypes as expected hypothesized. In order to analyse the increase of CD11b+ myeloid cells among the four layers of retina, we compartmentalised and quantified the data into a bar chart using Graphpad 8 Prism as shown in Figure 2. The number of CD11b+ myeloid cells was highly increased within the light induced KO mice retina compared to the light induced WT retina, particularly in the subretinal space.

Given the different pattern of retinal CD11b+ myeloid cell activation between WT and KO mice, our

b

Tukey's multiple comparisons test								
	WT-R vs WT-L		KO-R vs KO-L		WT-L vs KO-L		WT-R vs KO-R	
	P-value	significance	P-value	significance	P-value	Significance	P-value	Significance
Subretinal	0.0081	**	<0.0001	****	0.0152	*	0.9843	NS
ONL	0.0379	*	0.0203	*	0.8570	NS	>0.9999	NS
INL-OPL	0.0335	*	<0.0001	****	0.6389	NS	0.9876	NS
GL-IPL	0.7755	NS	0.0156	*	0.3757	NS	0.9974	NS

*Figure 2(b): Tukey's multiple comparisons test shows the result of statistical analysis of Figure 2. This statistical data is generated using GraphPad 8 Prism using two way ANOVA. *P<0.05, **P<0.01, ****P<0.0001.*

results support that the decreased expression of IRAK-M is linked with chronic inflammation and with desregulated autophagy (3) of the RPE cells. Here we show an overall rise in the total number of CD11b+ myeloid cells in the oxidative stress-induced retina of IRAK-M KO mice in comparison to the WT mice.

Conclusion:

To find an effective treatment for neuro-degenerative disorders such as AMD, it is crucial to elucidate the mechanisms that are involved in the pathogenesis of the disease. In this study we showed that IRAK-M deficient mice's retina is susceptible to light-induced CD11b+ cell infiltration and subretinal accumulation in comparison to the WT mice's retina. The data suggests a role of IRAK-M in regulating myeloid cell-mediated tissue inflammation under oxidative stress. These results with further experiments underway will provide us with a potential therapeutic target for AMD treatment. If we can induce or activate more IRAK-M within the oxidatively stressed RPE cells, there is a potential possibility to combat high sensitivity of RPE layer to pro-inflammatory cytokines and chemokines. As a result, RPE layer will be able to maintain its physiological functions.

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