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A subpopulation of activated retinal macrophages selectively migrated to regions of cone photoreceptor stress, but had limited effect on cone death in a mouse model for type 2 Leber congenital amaurosis

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ABSTRACT

Background: Studies of antigen presentation in retina using mice that expressed green fluorescent protein (GFP) from a transgenic CD11c promoter found that retinal GFP^{hi} cells possessed antigen presentation function. Subsequent studies found that these high GFP^{hi} cells preferentially localized to sites of retinal injury, consistent with their APC function. Interest in the roles of macrophages in degenerative CNS diseases led us to study the GFP^{hi} cells in a retinal model of neurodegeneration. We asked if apoptotic cone photoreceptor cell death in *Rpe65*$^{-/-}$ knockout mice induced the GFP^{hi} cells, explored their relationship to resident microglia (MG), and tested their role in cone survival.

Methods: *Rpe65*$^{-/-}$ mice were bred to $CD11c^{GFP}$ mice on the B6/J background. $CD11c^{GFP}$*Rpe65*$^{-/-}$ mice were also backcrossed to $CX3CR1^{YFP\text{-}creER}ROSA^{DTA}$ mice so that $CX3CR1^{+}$ mononuclear cells could be depleted by Tamoxifen. Retinas were analyzed by immunohistochemistry, confocal microscopy, fluorescence fundoscopy and flow cytometry.

Results: Elevated numbers of GFP^{hi} cells were concentrated in photoreceptor cell layers of $CD11c^{GFP}$*Rpe65*$^{-/-}$ mice coinciding with the peak of cone death at 2 to 4 weeks of age, and persisted for at least 14 months. After the initial wave of cone loss, a slow progressive loss of cones was found that continued to retain GFP^{hi} cells in the outer retina. Sustained, four-week Tamoxifen depletions of the GFP^{hi} cells and MG in *Rpe65*$^{-/-}$ mice from day 13 to day 41, and from day 390 to day 420 promoted a small increase in cone survival. We found no evidence that the GFP^{hi} cells were recruited from the circulation; all data pointed to a MG origin. MG and GFP^{hi} cells were well segregated in the dystrophic retina; GFP^{hi} cells were foremost in the photoreceptor cell layer, while MG were concentrated in the inner retina.

Conclusions: The expression of GFP on a subset of retinal mononuclear cells in $CD11c^{GFP}$ mice identified a distinct population of cells performing functions previously attributed to MG. Although GFP^{hi} cells dominated the macrophage response to cone death in the photoreceptor cell layer, their ablation led to only an incremental increase in cone survival. The ability to identify, ablate, and isolate these cells will facilitate analysis of this activated, antigen-presenting subset of MG.

1. Introduction

Rod and cone photoreceptors are the two neural retina cell types that mediate phototransduction, the basis for vision. The visual cycle is a series of enzymatic reactions among photoreceptors, the retinal pigment epithelium (RPE), and the Müller glia to metabolize Vitamin A derivatives (retinoids) for phototransduction (Fig. 1A). The essential product is the 11-*cis* form of the retinal aldehyde (RAL), a chromophore that binds opsin G-protein coupled receptors within the outer segment (OS) of photoreceptors to form photopigment. The RPE65 protein is highly expressed in RPE and cone OS (Tang et al., 2011a; Tang et al., 2011b; Znoiko et al., 2002; Ma et al., 1998), and is essential for the eye to metabolize retinoids (Tang et al., 2012; Moiseyev et al., 2005). Mutation of the human *Rpe65* gene is the underlying defect in Type 2 Leber congenital amaurosis (LCA2), leading to extensive cone loss within the first year of life (Travis et al., 2007; den Hollander et al., 2008). In the *Rpe65*$^{-/-}$ mouse, production of 11-*cis* RAL within the eye was undetectable, and a wave of cone death occurred within the first month of life (Fan et al., 2008). Treating *Rpe65*$^{-/-}$ mouse pups with exogenous 11-*cis* RAL immediately after birth rescued cones,

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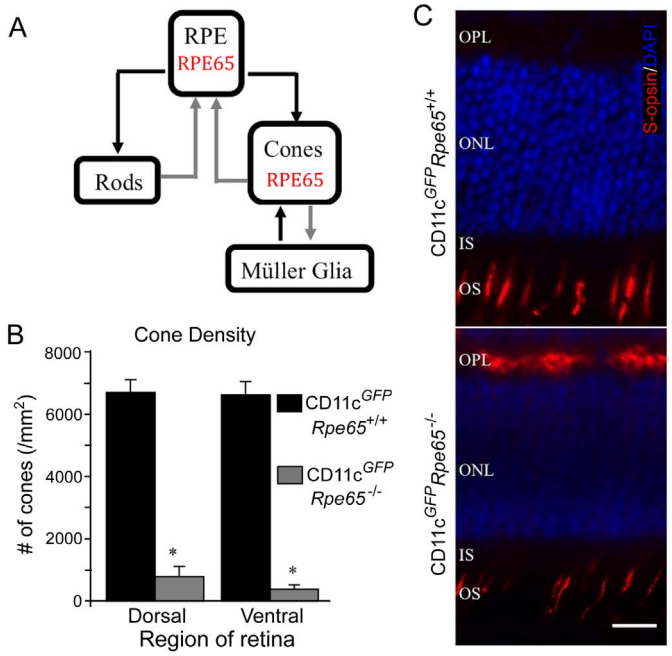

Fig. 1. Retinoid visual cycle and cone death. (A) Schematic diagram depicts trafficking of retinoids between cell types in mammalian retina. Retinoids destined for photo-transduction are synthesized by retinal pigment epithelium (RPE) and Müller glia, and then trafficked to photoreceptors (black arrows). Spent retinoids from phototransduction events are trafficked back to the RPE and Müller cells for recycling (grey arrows). RPE65 protein is localized within the RPE and cone outer segments to support the retinoid visual cycle. (B) Cone survival at P28 was determined from cone densities in retinal flatmounts by counting S-opsin^{+} cells from dorsal and ventral regions adjacent to the optic nerve head. CD11c^{GFP}*Rpe65*$^{+/+}$ mice (black bars) exhibited significantly more ($P < 0.05$ indicated by *; error bars show SEM) cone survival at P28 compared to age-matched CD11c^{GFP}*Rpe65*$^{-/-}$ mice (grey bars). (C) Cone stress in P21 CD11c^{GFP}*Rpe65*$^{-/-}$ retina leads to characteristic mislocalization of cone opsin to the OPL. Scale bar = 15 μm. Blue, DAPI; Red, S-opsin.

reaffirming that an active visual cycle is essential for cone health (Tang et al., 2010).

Our previous studies of retinal mononuclear cells began with the search for cells in the retina with antigen presenting cell function (APC) that might support the T cell responses associated with retinal autoimmunity. The study of APC's in retina was facilitated by use of transgenic mice whose dendritic cells (DCs) express a chimeric protein containing green fluorescent protein (GFP) using a transgenic CD11c promoter (CD11c^{GFP}) (Jung et al., 2002). We previously showed that the level of CD11c (ITGAX protein) from the endogenous promoter in retinal macrophages from CD11c^{GFP} mice does not correlate with the expression of DTR/GFP from the transgenic promoter (Lehmann et al., 2010), and led us to describe the cells as high GFP-expressing (GFP^{hi}), rather than CD11c^{hi}. We found that the GFP^{hi} cells in retina had APC function based on *in vivo* and *in vitro* studies revealing that antigen-specific retinal T cell responses in the retina were dependent on antigen processing and presentation by local APC that could be visualized by their expression of GFP in CD11c^{GFP} mice (McPherson et al., 2013; McPherson et al., 2014; Heuss et al., 2012). Conversely, low GFP-expressing cells (GFP^{lo}) cells bearing markers of microglia (MG) lacked APC function for naïve, antigen-specific CD4 T cells (McPherson et al., 2014). In studies designed to examine the influence of the retinal environment on the APC function of GFP^{hi} cells, an optic nerve crush (ONC) was performed. This injury to the axons of the retinal ganglion cells (RGC) stimulated an increase in the number of retinal GFP^{hi} cells, promoted their close association with the retinal ganglion cells (RGC) and nerve fibers, and showed that they engulfed the RGC post-ONC (Heuss et al., 2014). With respect to their APC function, the ONC reduced production of regulatory T cells (T_{regs}) by the retinal GFP^{hi} cells and increased the number of effector T cells in the retina. Another test of the significance of the GFP reporter in the CD11c-DTR/GFP mice was done by crossing them onto MyD88 and TRIF double knockout mice (Heuss et al., 2014). When crossed to TRIF/MyD88 deficient mice, the appearance of GFP^{hi} cells was dramatically reduced post-ONC. The absence of GFP^{hi} cells led to the phagocytosis of RGC debris by GFP^{lo} MG. Depletion of GFP^{hi} cells by diphtheria toxin (DTx) ablation was followed by GFP^{lo} MG replacing GFP^{hi} cells in close contact with injured neurons.

We also demonstrated in CD11c^{GFP} mice that GFP^{hi} cells also appeared at sites of stress associated with light-induced photoreceptor injury (Lehmann et al., 2010). The Saban lab has also observed GFP^{hi} cells in the retina of this particular strain of CD11c-GFP reporter mice (O'Koren et al., 2016). Studies to further test the hypothesis that GFP^{hi} cells preferentially respond to stressed or injured cells led to this study of *Rpe65*$^{-/-}$ mice, in which a defect in retinoid metabolism leads to apoptotic death of the small population of cones found in murine retina. The question of their effect on the survival of stressed cones was also of interest. To this end, *Rpe65*$^{-/-}$ mice were bred onto the CD11c^{GFP} background to generate a mouse model that exhibited RPE65 dysfunction and GFP expression from a CD11c promoter (CD11c^{GFP}*Rpe65*$^{-/-}$ mice). In addition, CD11c^{GFP}*Rpe65*$^{-/-}$ mice were backcrossed to CX3CR1$^{YFP\text{-}creER}$ROSA^{DTA} mice so that all retinal microglial-like cells could be tracked and ablated.

Our results showed that GFP^{hi} cells were preferentially attracted to stressed and dying cones in the outer retina of CD11c^{GFP}*Rpe65*$^{-/-}$ mice, while the GFP^{lo} MG remained in the inner retina. We found no evidence of mononuclear cell recruitment from the circulation in the *Rpe65*$^{-/-}$ mouse model, which has minimal inflammation. The GFP^{hi} cells represented the fraction of MG that responded to the stimulus provided by cone degeneration, and allowed their distinction from MG. Ablation of CX3CR1^{+} cells by Tamoxifen (TAM) also ablated GFP^{hi} cells associated with the cones, and gave a small, but significant increase in the number of surviving cones. Use of this reporter will enable more specific studies of the pathways to MG activation and APC function in the retina. In the absence of this GFP reporter, all resting and activated retinal macrophages appeared as MG. Previous studies lack the ability to make this distinction, which has limited specific analysis of the responding cells. While GFP^{hi} cells possess the functions, and many of the markers of CD11b^{+} DCs, their origin in retinas with minimal inflammation suggests they are an activation state of MG that supports immune surveillance without loss of immune privilege.

2. Materials and methods

2.1. Animals

Rpe65$^{-/-}$ mice were a generous gift of T.M. Redmond (National Eye Institute, National Institutes of Health, Bethesda, MD) (Redmond et al., 1998). CD11c^{GFP} mice have been previously described and express GFP as a chimeric cell surface protein comprised of the diphtheria toxin receptor (DTR) and GFP, under control of a transgenic CD11c promoter (Jung et al., 2002). The *Rpe65*$^{-/-}$ mice and CD11c^{GFP} mice were bred to produce the CD11c^{GFP}*Rpe65*$^{-/-}$ mice used for this study, and genotypes were confirmed by PCR (data not shown). CD11c^{GFP}*Rpe65*$^{-/-}$ mice were crossed with CX3CR1$^{YFP\text{-}creER}$ROSA^{DTA} mice so that MG and GFP^{hi} cells could be depleted with TAM treatment and distinguished by expression of YFP in all mononuclear cells, and expression of YFP in the GFP^{hi} cells. Mice were confirmed to be *rd8*-negative (Mattapallil et al., 2012). All mice were reared under cyclic light under specific pathogen-free conditions. Mice were sacrificed by CO_2 exposure.

2.2. Ethics approval

All experiments were performed in accordance with the Association