CD36 Deficiency Inhibits Retinal Inflammation and Retinal Degeneration in Cx3cr1 Knockout Mice

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Background: CD36, a member of the class B scavenger receptor family, participates in Toll-like receptor signaling on mononuclear phagocytes (MP) and can promote sterile pathogenic inflammation. We here analyzed the effect of CD36 deficiency on retinal inflammation and photoreceptor degeneration, the hallmarks of age-related macular degeneration (AMD), that characterize Cx3cr1−/− mice.

Methods: We analyzed subretinal MP accumulation, and cone- and rod-degeneration in light-challenged and aged, CD36 competent or deficient, hyper-inflammatory Cx3cr1−/− mice, using histology and immune-stained retinal flatmounts. Monocytes (Mo) were subretinally adoptively transferred to evaluate their elimination rate from the subretinal space and Interleukin 6 (IL-6) secretion from cultured Mo-derived cells (MdCs) of the different mouse strains were analyzed.

Results: CD36 deficient Cx3cr1−/− mice were protected against age- and light-induced subretinal inflammation and associated cone and rod degeneration. CD36 deficiency in Cx3cr1−/− MPs inhibited their prolonged survival in the immune-suppressive subretinal space and reduced the exaggerated IL-6 secretion observed in Cx3cr1−/− MPs that we previously showed leads to increased subretinal MP survival.

Conclusion: Cd36 deficiency significantly protected hyperinflammatory Cx3cr1−/− mice against subretinal MP accumulation and associated photoreceptor degeneration. The observed CD36-dependent induction of pro-inflammatory IL-6 might be at least partially responsible for the prolonged MP survival in the immune-suppressive environment and its pathological consequences on photoreceptor homeostasis.

Keywords: age related macular degeneration, mononuclear phagocyte, CX3CR1, CD36, IL-6, photoreceptor
INTRODUCTION

Age-related Macular Degeneration (AMD) is a common (1), highly heritable, neuroinflammatory disorder characterized by central, sizeable deposits under the retinal pigment epithelium (drusen) in its early form and choroidal neovascularisation (CNV, wet AMD) or an extending lesion of the outer central retina (geographic atrophy) in its late form (2).

We (3–6) and others (7, 8) showed that both advanced forms [wet AMD (5) and Geographic Atrophy, GA (3, 4, 6)] are associated with non-resolving accumulation of monocular phagocytes (MP) in the subretinal space. MPs are a family of cells that include monocyte (Mo), resident macrophages (rMo) such as microglial cells (MC), and monocyte-derived inflammatory macrophages/dendritic cells (MdC) that arise during inflammation (9). Physiologically, the subretinal space does not contain blood- and lymphatic-vessels, and is additionally devoid of immune cells, including resident MCs (4, 5, 7, 8, 10). Among others this particularity is due to the potent immunosuppressive, pro-apoptotic factors produced by the RPE that eliminate infiltrating leukocytes (4, 11). While MP accumulation observed around large drusen in early AMD (4, 6) might control debris accumulation, we showed that MdCs invariably participate in the infiltrate in and around GA lesions (6), where they are closely associated with rod and cone degeneration (3). Indeed, MPs, and in particular MdCs, an important source of inflammatory cytokines that play a critical role in animal models of pathological choroidal neovascularisation (CNV) (12, 13) and photoreceptor degeneration (5, 6, 14–18). In particular we showed that the inflammatory cytokines CCL2, IL-6, and IL-1 lead to the excessive MdC accumulation, diminish the subretinal immunosuppressiveness and induce photoreceptor degeneration and CNV, respectively (3, 4, 6, 18, 19).

In the eye, CX3CL1 is constitutively expressed as a transmembrane protein in inner retinal neurons (20, 21) and provides a tonic inhibitory signal to CX3CR1 bearing retinal MCs that keeps these cells in a quiescent surveillance mode under physiological conditions (5, 22). Cx3cr1 deficiency in mice leads to a strong increase of subretinal MP accumulation with age, after light-challenge and laser-injury (5, 23, 24), in diabetes (25), and in a parquat-induced retinopathy model (26).

We showed that Cx3cr1-deficient MPs express high levels of Apolipoprotein E, also observed in humanized transgenic mice expressing the AMD-risk APOE2 isoform (TRE2-mice) (3–6). Of interest, the latter is enriched in the Mac387+ fraction of subretinal Mφ, which is increased in AMD (32), and re-expressed in the choroid of humanized mouse model (33). We previously showed develop pathogenic subretinal MP accumulation in Cx3cr1−/− mice (34). In particular we showed that the inflammatory cytokines CCL2, IL-6, and IL-1 lead to the excessive MdC accumulation, diminish the subretinal immunosuppressiveness and induce photoreceptor degeneration and CNV, respectively (3, 4, 6, 18, 19).

RESULTS

CD36 Deficiency Prevents Age-Related, Chronic, Pathogenic Subretinal Inflammation in Cx3cr1-Knockout Mice

We first sought to determine whether Cd36 deficiency would alter disease onset and progression in Cx3cr1−/− mice. Abundance of subretinal IBA-1+ MPs on retinal and RPE/choroidal flatmounts of 2–3 month and 12 month old animals showed that the age-related increase in subretinal MP accumulation with age, after light-challenge and laser-injury (5, 23, 24), in diabetes (25), and in a parquat-induced retinopathy model (26).

We showed that Cx3cr1-deficient MPs express high levels of Apolipoprotein E, also observed in humanized transgenic mice expressing the AMD-risk APOE2 isoform (TRE2-mice) and subretinal MPs of AMD patients (4, 6, 27). The excessive expression of APOE in turn can activate the CD14/TLR2/TLR4-dependent innate immunity receptor complex (IIRC) on MPs (4, 27), likely by modifying the cholesterol content of the lipid raft, in which the complex is located (28). Its activation in subretinal MPs induces CCL2 (27), which increases Mo recruitment (6), but also IL-6, which we show reduces RPE FasL expression and MP elimination (4). The age-dependent accumulation of subretinal Cx3cr1-deficient MPs is associated with a significant degeneration of rods and cones (5, 29, 30), but not with RPE atrophy. They therefore quite accurately model the transitional zone of GA and a subtype of GA called incomplete outer retinal atrophy [iORA; (31)].

CD36, a member of the class B scavenger receptor family, is expressed in a variety of cell types and binds a diverse array of ligands (32). It is expressed on Mφ, MC, RPE cells, and microvascular endothelial cells (32), all cell types potentially involved in AMD. On vascular endothelial cells CD36 has been shown to mediate the antiproliferative effect of the thrombospondins (33, 34). Its expression on RPE cells has been shown to influence the phagocytosis of rod outer segments (ROS) (35, 36), in particular oxidized ROS (37, 38), but also oxidized lipoproteins (39), whose CD36-mediated uptake activates the NLRP3 inflammasome in the RPE (40). Finally, it is expressed on MC and Mφ where it is a co-receptor of the toll-like receptor 2 (TLR2) and activates a proinflammatory signaling cascade and the release of inflammatory cytokines (41, 42). On the other hand, internalization of apoptotic bodies via CD36 can inhibit proinflammatory pathways (43, 44).

We have previously shown that CD36 expression regulates COX-2 and VEGF expression in RPE cells. Its deficiency leads to a slow, age-related choroidal involution and mild retinal degeneration (45). On the other hand deficiency of CD36 protected against subretinal inflammation and photoreceptor degeneration in an acute model of light toxicity, as it inhibited the release of neurotrophic IL-1β from subretinal MPs (42).

Here we investigated the influence of CD36 on inflammation and photoreceptor degeneration in Cx3cr1-deficient mice that we previously showed develop pathogenic subretinal MP accumulation with age and after a non-toxic light-challenge.
chronic, age-related subretinal MP accumulation and associated photoreceptor degeneration observed in Cx3cr1−/− mice.

CD36 Deficiency Prevents Pathogenic Subretinal Inflammation in Acute, Light-Induced Cx3cr1-Knockout Mice

Next, we evaluated its impact on acute light-induced stress. The intensity of the light-challenge model used herein was calibrated to induce substantial subretinal MP infiltration in AMD-prone Cx3cr1−/−-mice but not in C57BL6/J controls (6). Quantification of subretinal IBA-1+ MPs on retinal and RPE/choroidal flattmounts of 2–3 month old animals that were exposed to 4500 lux of green light with fully dilated pupils for 96h, followed by 10 days of housing in the normal animal quarters, revealed an increase in subretinal MPs in Cx3cr1−/− mice, which was significantly inhibited in the absence of CD36 (Figures 3A,B). To quantify photoreceptor apoptosis in light-challenged mice we prepared TUNEL stained retinal flattmounts as previously described (5, 18). Flatmounts from Cx3cr1−/− mice displayed TUNEL positive cells in the outer nuclear layer, where the photoreceptor nuclei are located, to a much greater extent than C57BL6/J controls as previously reported (6). Importantly, Cx3cr1−/− Cd36−/− mice were protected against the photoreceptor apoptosis observed in Cx3cr1−/− mice (Figure 3C).

Taken together, CD36-deficiency very significantly inhibited the acute subretinal inflammation and associated photoreceptor apoptosis induced in the light-challenged Cx3cr1−/− mice, similar to our observations in chronic age-related inflammation.

CD36 Deficiency Normalizes the Resistance to Subretinal Elimination of Cx3cr1-Deficient Monocytes and Reduces Their IL-6 Production in vitro

We have previously shown that subretinal MPs in aged- and light-challenges Cx3cr1−/− mice are in part derived from infiltrating Mo (6). We showed that subretinally injected WT Mos, MCs or Mϕs quickly undergo apoptosis and are eliminated (4), and that such clearance was significantly delayed when MPs lacked Cx3CR1 (4). We demonstrated that this resistance was at least in part due to the fact that Cx3cr1-deficient MPs overexpress interleukin 6 (IL-6), which downregulates Fasl expression the RPE that normally induces subretinal MP elimination (4).

To evaluate whether CD36 expression on MPs influences their susceptibility to be eliminated by the RPE, we adoptively transferred CFSE-labeled Mos from the different mouse strains into the subretinal space of wildtype mice. The surviving CFSE+ Mos were then enumerated 24h after injection (Figure 4A). As previously shown, Cx3cr1-deficient Mo were significantly more numerous than subretinally injected wildtype Mos. Importantly, the deletion of Cd36 in Cx3cr1-deficient Mo completely reversed this effect (Figure 4B).

Cx3cr1-deficient MPs express increased levels of APOE, which we showed activates the TLR2-CD14-dependent innate immunity receptor cluster and induces IL-6 (4). CD36 is a co-receptor of the toll-like receptor 2 (TLR2) and involved in the proinflammatory signaling cascade and the release of inflammatory cytokines (41, 42). Indeed, IL-6 transcription measured by RT-PCR (Figure 4C) and IL-6 secretion evaluated by ELISA (Figure 4D) was significantly increased in bone marrow derived Mo from Cx3cr1-deficient mice compared to control, but the deletion of Cd36 in Cx3cr1-deficient Mo prevented this increase of expression.

Taken together this data demonstrates that Cd36 deletion in Cx3cr1-deficient Mos inhibits the over-expression of IL-6 in Cx3cr1-deficient Mos and reverses their ability to withstand the immunosuppressive environment of the subretinal space.

DISCUSSION

We previously showed that the deletion of Cx3cr1, a gene that is exclusively expressed on MPs in the adult eye, is sufficient to trigger an age-related or light-induced pathogenic non-resolving subretinal inflammation. We here demonstrate that the deletion of CD36 in Cx3cr1-deficient mice prevented the age- and light-challenge-dependent accumulation of subretinal MPs and protected against the associated photoreceptor cell death. Our data demonstrates that CD36 expression on Mo is necessary for the over-expression of IL-6 that we observe in Cx3cr1-deficient MDCs. We previously showed that IL-6 expression in Cx3cr1-deficient MPs is necessary for the accumulation of subretinal MPs in Cx3cr1-deficient mice, as it induces the deregulation of Fasl expression the RPE that induces subretinal MP elimination in wildtype animals (4). Indeed, when we subretinally adoptively transferred Cx3cr1−/− and Cd36−/− Cx3cr1−/−-Mo to wildtype recipients, the deletion...
FIGURE 2 | CD36 deficiency prevents age-related rod and cone degeneration in Cx3cr1-knockout mice. (A) Micrographs, taken 1,000 µm from the optic nerve, of 12 month-old Cx3cr1−/− and Cx3cr1−/−Cd36−/− mice. (B) Photoreceptor nuclei rows at increasing distances (−3,000 µm: inferior pole, +3,000 µm: superior pole) from the optic nerve (0 µm) in 12 month-old mice. (C) Quantification of the area under the curve of photoreceptor nuclei row counts of 12 month-old mice of the indicated transgenic mouse strains (Mann Whitney wildtype vs. Cx3cr1−/− mice *p = 0.0024; Mann Whitney Cx3cr1−/− vs. Cx3cr1−/−Cd36−/− mice $p = 0.0024$). (D) Micrographs, taken in the superior periphery of peanut agglutinin (staining cone segments, red) stained 12 month-old Cx3cr1−/− and Cx3cr1−/−Cd36−/− mice. (E) Cone density quantification on retinal flatmounts in peripheral and central, inferior and superior retina (−3,000 µm: inferior pole, +3,000 µm: superior pole, optic nerve: 0 µm) and their average (F) in 12 month-old mice of the indicated mouse strains (Mann Whitney wildtype vs. Cx3cr1−/− mice *p = 0.0022; Mann Whitney Cx3cr1−/− vs. Cx3cr1−/−Cd36−/− mice $p = 0.0022$). ONL, outer nuclear layer; PNA, peanut agglutinin Scale bar (A,D) = 50 µm. n = number of replicates indicated in the graphs; replicates represent quantification of eyes from different mice of at least three different cages.

FIGURE 3 | CD36 deficiency prevents pathogenic subretinal inflammation in acute, light-induced Cx3cr1-knockout mice. (A) Schematic representation of the light-challenge model. (B) Representative images and quantification of IBA-1 (green) stained RPE flatmounts of 2 month-old mice of the indicated strains after 10 days after the light-challenge (‘Mann & Whitney test C57BL/6J control mice vs. Cx3cr1−/− mice p = 0.0014; ‘Mann & Whitney test Cx3cr1−/− vs. Cx3cr1−/−Cd36−/− mice p = 0.02). (C) Representative images and quantification of TUNEL+ cells in the outer nuclear layer of TUNEL-stained retinal flatmounts of d14 light-challenged mice of the indicated strains (‘Mann & Whitney test C57BL/6J control mice vs. Cx3cr1−/− mice p = 0.0005; ‘Mann & Whitney test Cx3cr1−/− vs. Cx3cr1−/−Cd36−/− mice p = 0.0012), n, indicated for each bar; Scale bar (A) = 50 µm.

of Cd36 on Cx3cr1−/−-monocytes restored the susceptible to subretinal elimination to the level observed in wildtype monocytes. Taken together our data demonstrates that Cd36 deficiency inhibits retinal inflammation and retinal degeneration in Cx3cr1 knockout mice.

We previously showed that Cx3cr1-deficient MPs are characterized by an over-expression of APOE that triggers NFκB activation and cytokine secretion. ApoE-deletion in Cx3cr1-deficient mice significantly prevents the accumulation of pathogenic subretinal MPs (4, 6, 27), similar to Cd36 deficiency.
CD36 is also part of the innate immunity receptor complex (IIRC) on MPs (47, 48) and an obligate co-receptor of the toll-like receptor 2 (TLR2). Its inhibition blocks the TLR2-dependent NFκB activation and pro-inflammatory signaling cascade and the release of inflammatory cytokines such as IL-6 (41, 42). In Cx3cr1-deficient mice the deletion of CD36 therefore likely counters the APOE-induced activation of the IIRC, activation of NFκB and IL-6 secretion, which show reduces RPE FasL expression and MP elimination (4). As a result, the CD36-deficient MPs are quickly eliminated from the subretinal space, preventing pathogenic subretinal inflammation. Interestingly, it has recently been demonstrated that Cd36 deletion and pharmacological inhibition of CD36 also inhibits pathogenic inflammation in an acute model of light-induced degeneration in Cx3cr1-competent mice (42). The authors demonstrated that the protective effect is likely due to the inhibition of IL-1 that we showed also plays an important role in photoreceptor degeneration in Cx3cr1-deficient mice (3, 18).

In summary, the inhibition of CD36 on MPs in diseases that are characterized by pathogenic subretinal inflammation such as AMD, holds the promise to reduce MP accumulation and their production of pathogenic cytokines. On the other hand CD36 expressed by RPE cells has an important role in the maintenance of the chorio-capillaries and the elimination of lipids from Bruchs membrane (39, 45). Ideally, future therapies for AMD might therefore want to specifically target CD36 on MPs.

MATERIALS AND METHODS

Animals

Cx3cr1+/− CD36−/− mouse strains on C57BL/6 background were generated from Cx3cr1+/− mice and CD36−/− mouse strains, which were generated as previously described (49, 50). All mice were negative for the Crb1<sup>Δαβ</sup>, Pde6b<sup>Δαβ</sup>, and Gnat2<sup>Δβδ</sup> mutations. Mice were housed in the animal facility under specific pathogen-free condition, in a 12/12 h light/dark (100–500 lux) cycle with...
water and normal diet food available ad libitum. All experimental protocols and procedures were approved by the local animal care ethics committee “Comité d’Éthique en Expérimentation Animale Charles Darwin” (N° p3/2008/54).

Light-Challenge Model
Two- to four-month-old mice were adapted to darkness for 6 h and pupils were fully dilated with 1% Atropine (Novartis). Animals were then exposed to green LED light (4500 Lux, JP Vezon équipements) for 4 days and subsequently kept in cyclic 12/12 h normal animal facility conditions. MP accumulation and retinal degeneration were assessed at 10 days after light exposure (6).

Immunohistochemistry and TUNEL Staining of Retinal Flatmounts
Eyes were enucleated, fixed in 4% paraformaldehyde for 20 min at room temperature and sectioned at the limbus; the cornea and lens were discarded. The retinas were peeled from the RPE/choroid/sclera and incubated overnight at 4°C in PBS-1% triton with the following primary antibodies: peanut agglutinin Alexa fluor® 594 (Thermo Fisher Scientific; 1/100), and goat polyclonal anti-IBA1 (1/100, Wako). After few washes, the retinas were incubated for 2 h at room temperature with appropriate Alexa Fluor® conjugated secondary antibodies (Thermo Fisher Scientific; 1:500) in PBS-1% triton and nuclei were counterstained with Hoechst (1:1,000, Sigma Aldrich). The retinas were flatmounted and viewed with a fluorescence microscope (DM5500, Leica). Images centered on the area with the lowest number of PNA+ cone arrestin+ cells were captured with a confocal laser-scanning microscope (FV1000, Olympus) using a 40X lens. Each cell population was manually counted in a masked fashion. IBA-1+ cells were quantified on flatmounts on the outer segment side of the detached retina while PNA+ cone arrestin+ cells were counted on confocal microscopy Z-stacks using ImageJ software.

For histology, eyes were fixed in 0.5% glutaraldehyde, 4% PFA for 2 h, dehydrated and mounted in Historesin (Leica). Five millimeters of oriented sections crossing inferior pole, optic nerve and superior pole were cut and stained with toluidin blue. Rows of nuclei in the ONL were counted at different distances from the optic nerve (6).

For Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL), 4% PFA fixed retinal flatmounts were pretreated with frozen methanol for 30 min and then frozen methanol/acetoc acid (2:1) for another 30 min. After washing with PBS, flatmounts were incubated overnight at 4°C with the reaction mixture as described by manufacturer’s protocol (In Situ Cell Death Detection Kit, Roche Diagnostics) and then for 90 min at 37°C. After reaction was stopped by washing with PBS at RT, nuclei were counterstained with Hoechst (Sigma–Aldrich).

Cell Preparations and Cell Culture
Bone marrow-derived monocytes (in serum-free X-Vivo 15 medium) were performed as previously described (6). RT-PCRs using Sybr Green (Life Technologies) and ELISAs using mouse IL-6 DuoSet (R&D Systems) were performed as previously described (6).

Subretinal Mononuclear Phagocyte Cell Clearance
Bone marrow-derived monocytes (~95% pure) were labeled in 10 μM CFSE (Life technologies). Cells were washed and resuspended in PBS. Twelve thousand cells (4 μl) were injected in the subretinal space of anesthetized 2-month-old mice using a microinjector and glass microcapillaries (Eppendorf). A hole was pierced with the glass capillary prior to the subretinal injection to avoid intra-ocular pressure increase and to allow retinal detachment with 4 μl of solution. The subretinal injection was verified by fundoscopy. Eyes were enucleated after 24 h, fixed in 4% PFA, and flatmounted. CFSE+ transplanted cells were counted on the subretinal aspect of the retinal flatmount and the RPE/choroid flatmount of each eye. Eyes with subretinal hemorrhages were discarded.

Statistical Analysis
Sample sizes for our experiments were determined according to our previous studies Graph Pad Prism 6 (GraphPad Software) was used for data analysis and graphic representation. All values are reported as mean ± SEM. Statistical analysis was performed by Mann–Whitney U-test (2-group comparisons). The n and p-values are indicated in the figure legends.

DATA AVAILABILITY STATEMENT
All datasets generated for this study are included in the article.

ETHICS STATEMENT
The animal study was reviewed and approved by Comité d’Éthique en Expérimentation Animale Charles Darwin.

AUTHOR CONTRIBUTIONS
SL, J-BC, ST, CR, MH, SA, and WR conducted the experiments. CC, MF, HO, SC, J-AS, CD, XG, and FS designed and analyzed the experiments.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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