Nonessential Role of β3 and β5 Integrin Subunits for Efficient Clearance of Cellular Debris after Light-Induced Photoreceptor Degeneration

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Purpose. During light-induced photoreceptor degeneration, large amounts of cellular debris are formed that must be cleared from the subretinal space. The integrins αvβ5 and αvβ3 are involved in the normal physiological process of phagocytosis in the retina. This study was conducted to investigate the question of whether the lack of β5 and/or β3 integrin subunits might influence the course of retinal degeneration and/or clearance of photoreceptor debris induced by acute exposure to light.

Methods. Wild-type, $\beta5^{-/-}$ and $\beta3^{-/-}$ single-knockout, and $\beta3^{-/-}/\beta5^{-/-}$ $Ccl2^{-/-}/\beta5^{-/-}$ double-knockout mice were exposed to 13,000 lux of white light for 2 hours to induce severe photoreceptor degeneration. Real-time PCR and Western blot analysis were used to analyze gene and protein expression, light- and electron microscopy to judge retinal morphology, and immunofluorescence to study retinal distribution of proteins.

Results. Individual or combined deletion of β3 and β5 integrin subunits did not affect the pattern of photoreceptor cell loss or the clearance of photoreceptor debris in mice compared with that in wild-type mice. Invading macrophages may contribute to efficient phagocytosis. However, ablation of the MCP-1 gene did not prevent macrophage recruitment. Several chemokines in addition to MCP-1 were induced after light-induced damage that may have compensated for the deletion of MCP-1.

Conclusions. Acute clearance of a large amount of cellular debris from the subretinal space involves invading macrophages and does not depend on β3 and β5 integrins. (*Invest Ophthalmol Vis Sci.* 2009;50:1423–1432) DOI:10.1167/iovs.08-2432

Age-related macular degeneration (AMD) is one of the most common retinal diseases that affect people older than 60 years.[1] Lipofuscin, a major correlate for the development of AMD, is generated during ageing by the incomplete decomposition of phagocytosed material from photoreceptor outer segments (POS) in the retinal pigment epithelium (RPE).[2] Efficient and complete phagocytosis is therefore necessary to ensure the integrity of a healthy retina. Various receptors on the membrane surface of the phagocyte and the phagocytic substrates mediate phagocytosis.[3] In the retina, these molecules include CD36, MerTK, and integrin receptors. On the apical membrane of the RPE layer, the CD36 scavenger receptor participates in particle recognition and regulates the internalization process of shed POS.[4–6] After POS binding to the RPE, the tyrosine kinase receptor Mer (MerTK), also present on the apical surface of RPE cells,[7] is indirectly activated via phosphorylation by focal adhesion kinase (FAK).[8] MerTK phosphorylation in turn activates the second messenger inositol 1,4,5-trisphosphate (InsP3), which initializes POS internalization and degradation.[8,9] The αvβ5 integrin receptor localizes similarly to the apical membrane of the RPE and is thought to participate in POS binding.[4,10–12] Moreover, the β5 integrin subunit and MerTK cooperate via FAK activation in the initialization step of phagocytosis.[8,11] On the other hand, αvβ3 vitronectin receptor has a crucial role in the recognition of apoptotic neutrophils and lymphocytes by blood-derived macrophages.[13] Since macrophages invade the lesioned retina and the β3 integrin complex is also found in the RPE,[14] β3 integrin may also be involved in the clearance of retinal debris.

AMD can be divided into two developmental stages: (1) the early stage is characterized by the presence of deposits called drusen[15] and the loss of photoreceptor and RPE cells leading to the development of geographic atrophy[16]; and (2) the hallmark of late AMD is choroidal neovascularization with retinal hemorrhages and increased degeneration of visual cells and the RPE.[17] Several mouse models show AMD-like features during ageing: ELOVL4 (elongation of very long-chain fatty acid) mutant transgenic mice accumulate lipofuscin and show progressive photoreceptor degeneration[18]; $Sod1^{-/-}$ (Cu, Zn-superoxide dismutase) mutant mice develop drusen, choroidal neovascularization, and RPE dysfunction[19]; the $ABCR^{-/-}$ mouse shows an enhanced accumulation of A2E, one of the most important and potentially devastating components of lipofuscin[20]; and the *mcd/mcd* mice with a mutated form of cathepsin D (catD), a lysosomal enzyme responsible for opsin degradation, accumulate phagocytosed photoreceptor material in the RPE, leading to the formation of deposits.[21] Based on defects of the phagocytic mechanism, three additional mouse models have been described: (1) ageing retinas of β5 integrin knockout mice ($\beta5^{-/-}$) display functional and histologic alterations linked to lipofuscin deposits and impairment of phagocytosis[22]; (2) apoptotic debris accumulate in the subretinal space of mice with a blocked β3 integrin receptor[23]; (3) accumulation of lipofuscin, formation of drusen, photoreceptor atrophy, and neovascularization have been reported in mice lacking monocyte chemoattractant protein (MCP)-1 (or CCL2).[24]

Since disturbed phagocytosis seems to be a major feature in retinal disease, we studied the role of β3 and β5 integrin subunits in the clearance of photoreceptor debris generated by exposure to high levels of visible light. Our data demonstrate that the individual or combined deletion of β3 and β5 integrin

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subunits does not lead to an accumulation of photoreceptor outer segments or to a delayed clearance of cell debris in the model of acute light-induced damage. Both macrophages and RPE cells efficiently cleared the subretinal space in mice independent of the presence or absence of β3 and/or β5 integrin subunits. Alternative receptors such as MerTK may compensate for the lack of integrin receptors. Additional deletion of MCP-1 did not influence phagocytosis or the recruitment of macrophages to the site of retinal injury. Gene expression studies show that additional chemokines are strongly induced after retinal injury and may thus account for macrophage attraction in the absence of MCP-1.

Materials and Methods

Animals and Exposure to Light

All procedures were conducted in accordance with the ARVO Statement for the use of Animals in Ophthalmic and Vision Research and with the regulations of the Veterinary Authority of Zurich. All animals were raised in cyclic light (12 hours light and 12 hours dark; 60 lux at cage level) and were homozygous for the $Rpe65_{450Leu}$ variant.[25] β3 ($\beta3^{-/-}$) and β5 ($\beta5^{-/-}$) knockout mice (generously provided by Richard O. Hynes, MIT, Cambridge, MA), $Ccl2^{-/-}$ mutant mice (Jackson Laboratory, Bar Harbor, ME), and corresponding wild-type mice (all on a mixed 129/B6 background) were maintained at the University Hospital Zurich. Double-mutant mice ($\beta3^{-/-}/\beta5^{-/-}$ and $Ccl2^{-/-}/\beta5^{-/-}$) were generated by classic breeding schemes.

Adult (8–10 weeks of age) wild-type and mutant mice were dark adapted for 12 hours, and their pupils were dilated with 1% Cyclopentolate (Cyclogyl; Alcon, Cham, Switzerland) and 5% phenylephrine (Ciba Vision, Niederwangen, Switzerland) 30 minutes before exposure to 13,000 lux of white light for 2 hours. Mice were killed at different time points ($n = 3$ for each group) after light offset and the retinas or eyes prepared.

RNA Preparation and Semiquantitative RT-PCR Analysis

Retinas were removed through a slit in the cornea and immediately frozen in liquid nitrogen. The rest of the eye (eye cup) was isolated and also frozen in liquid nitrogen. Total RNA was prepared with an RNA isolation kit (RNeasy; Qiagen, Hilden, Germany), including a digestion of the residual genomic DNA by DNase treatment. Identical amounts of RNA were used for reverse transcription using oligo(dT) and M-MLV reverse transcriptase (Promega, Madison, WI). cDNA amplifications were performed with primer pairs designed to span large intronic sequences or cover exon–intron boundaries (Table 1). Gene expression was analyzed by real-time PCR using a polymerase ready mix (LightCycler 480 SYBR Green I Master Mix; Roche Diagnostics, Indianapolis, IN) in a thermocycler (LightCycler Roche Diagnostics). For relative quantification of gene expression, mRNA levels were normalized to β-actin by using the comparative threshold cycle ($\Delta\Delta^{CT}$) method, and relative values were calculated with a respective control sample used for calibration. For the chemokine amplifications, cDNAs from three different mice were pooled and amplified in duplicate.

Light and Electron Microscopy

Eyes were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.3) at 4°C overnight. For each eye, the superior and inferior retina was prepared, washed in cacodylate buffer, incubated in osmium tetroxide for 1 hour, dehydrated in a series of increasing ethanol concentrations, and embedded in Epon 812. Semithin cross sections of 700 nm in thickness were prepared from the lower central retina, which was the most affected region in our light-induced damage model and counterstained with toluidine blue for light microscopy ($n = 3$ for each group), and 50-nm ultrathin sections were cut for electron microscopy analysis.

Immunohistochemistry

Mice were perfused transcardially with 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB; pH 7.4). After isolation of the eyes and removal of cornea and lens, the remaining ocular tissue was immersed for 2 hours in 4% PFA followed by cryoprotection in 30% sucrose in PBS (pH 7.4) at 4°C. The eyes were then embedded in tissue-freezing medium and frozen in a 2-methylbutane bath cooled by liquid nitrogen. Retinal sections (12 μm thick) were blocked in 3% normal goat serum and 0.3% Triton X-100 in 0.1 M PBS (pH 7.4) for 1 hour at room temperature. The sections were then incubated overnight, at 4°C, in the blocking solution containing one of the following primary antibodies: rabbit polyclonal anti-Mer (generous gift from Greg Lemke, Salk Institute for Biological Studies, La Jolla, CA), guinea pig anti-RPE65, and rat monoclonal anti-F4/80 (BMA Biomedicals, Augst, Switzerland). After they were washed with PBS, the slides were incubated with the appropriate secondary antibody coupled to Cy2 or Cy3 fluorescence for 1 hour at room temperature and mounted with anti-fade medium (10% Mowiol 4-88; vol/vol; Calbiochem, San Diego, CA), in 100 mM Tris (pH 8.5), 25% glycerol (wt/vol) and 0.1% 1,4-diazabicyclo [2.2.2] octane (DABCO). Immunofluorescent stainings were analyzed with a digital microscope (Axiovision; Carl Zeiss Meditec, Inc., Dublin, CA).

Western Blot Analysis

Retinas or eye cups ($n = 3$ per group) were homogenized in 100 mM Tris/HCl (pH 8.0) and protein content was analyzed by using the Bradford reagent. Equivalent amounts of proteins were resolved by electrophoresis on sodium dodecyl sulfate–polyacrylamide gels and transferred to nitrocellulose membranes. Membranes were blocked in 5% milk (Bio-Rad, Hercules, CA) in TBST (10 mM Tris/HCl [pH 8.0], 150 mM NaCl, and 0.05% Tween-20) for 1 hour at room temperature before they were incubated overnight at 4°C in 5% milk (in TBST) containing one of the following primary antibodies: anti-phospho-FAK_{Tyr397} (cat. No. 44-624G; Biosource, Camarillo, CA), anti-FAK (cat No. 610087; BD Transduction Laboratory, Lexington, KY), anti-phospho-$Stat3_{Tyr705}$ (cat. No. 9131; Cell Signaling, Beverly, MA), anti-Stat3 (cat. No. 9132; Cell Signaling, Beverly, MA), anti-cathepsin D (generous gift from Tom Cotter, University College Cork, Ireland), and anti-actin (cat. No. sc-1616; Santa Cruz Biotechnology, Santa Cruz, CA). After they were washed, the blots were incubated at room temperature for 1 hour with HRP-conjugated secondary antibodies. Immunoreactivity was visualized with chemiluminescence detection (Western Lightning Chemiluminescence Reagent Plus kit; Perkin Elmer, Boston, MA). Densitometry analysis was performed with NIH software and by normalizing the band intensities to β-actin values.

Table 1. Primer Pairs for Semiquantitative RT-PCR

Genes	Upstream	Downstream	Product Size (bp)
β-Actin	CAACGGCTCCGGCATGTGC	CTCTTGCTCTGGGCCTCG	153
MCP-1	GGCTCAGCCAGATGCAGTTA	CTGCTGCTGGTGATCCTCTT	108
MerTK	GAGGACTGCTTGGATGAACTGTA	AGGTGGGTCGATCCAAGG	73
CCL4	CAAGCCAGCTGTGGTATTC	AGCTGCTCAGTTCAACTCC	109
CCL5	GCTCCAATCTTGCAGTCGT	CTAGAGCAAGCGATGACAGG	165
CCL9	CAACTGCTCTTGGAATCTGG	AGGCAGCAATCTGAAGAGTC	136
CCL12	CCTCAGGTATTGGCTGGAC	GACACTGGCTGCTTGTGATT	124
CX3CL1	CCGCGTTCTTCCATTTGT	CTGTGCTGTGTCGTCTCCA	175

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RESULTS

Effect of Lack of β3 and/or β5 Integrin Subunits on Photoreceptor Degeneration and the Clearance of Cellular Debris from the Subretinal Space

To determine whether phagocytosis or clearance of photoreceptor debris generated by acute photoreceptor degeneration were affected by the deletion of β3 and β5 integrin subunits, we examined retinal histology after exposure to bright light (Fig. 1). In wild-type mice, exposure to light induced cell death in a large number of photoreceptor cells. As early as 6 hours after light offset, the retinas displayed disorganized and disrupted POS compared with nonexposed control retinas (Fig. 1A). As photoreceptor degeneration progressed, typical pyknotic nuclei with condensed chromatin appeared in the outer nuclear layer after 1 day. After 5 days, the photoreceptor layer

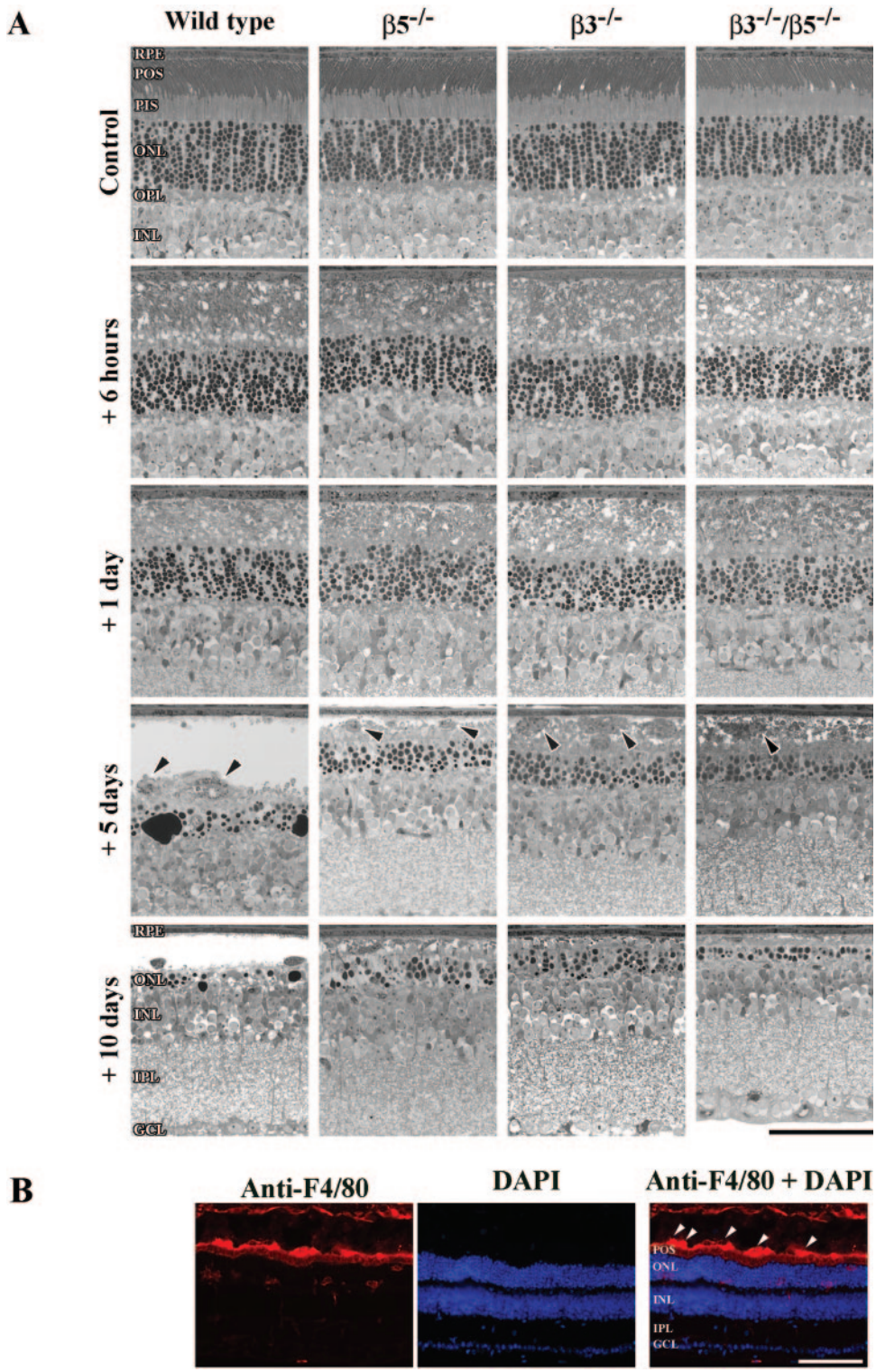

FIGURE 1. Acute clearance of photoreceptor debris from the subretinal space did not depend on β3 and β5 integrins. (**A**) Representative photomicrographs of retinas of wild-type, $\beta5^{-/-}$, $\beta3^{-/-}$, and $\beta3^{-/-}/\beta5^{-/-}$ mutant mice as indicated. Control mice were reared in a normal cyclic light environment (12 hours dark; 12 hours light) and not exposed to light. For light-induced damage, the mice were exposed to 13,000 lux for 2 hours and were killed after 6 hours, 1 day, 5 days, or 10 days, as indicated. Strong vesiculation and degradation of POS was detected already at 6 hours after light offset in all strains. The subretinal space of all strains was invaded by macrophages after 5 days (*arrowheads*). At 10 days, the photoreceptor layer (POS, PIS, and ONL) almost completely disappeared in all groups tested. (**B**) Activated macrophages, detected by the monoclonal antibody anti-F4/80, infiltrated the subretinal space of wild-type mice 3 days after exposure to light (*arrowheads*). Nuclei were stained with DAPI. RPE, retinal pigment epithelium; POS, photoreceptor outer segments; PIS, photoreceptor inner segments; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer. Scale bar: (**A**) 50 μm; (**B**) 100 μm.

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was severely degraded, whereas many macrophages colonized the subretinal space in the injured region of the retina (Fig. 1A, 1B, arrowheads). Recruitment of macrophages and their invasion of the outer retina have already been documented after light-induced damage[26,27] and in a model of chemical-induced injury.[28] Nonexposed (control) $\beta3^{-/-}$, $\beta5^{-/-}$, and $\beta3^{-/-}/\beta5^{-/-}$ mutant mice exhibited normal retinal morphology. The same pattern of light-induced photoreceptor degeneration and macrophage invasion was observed in single $\beta5^{-/-}$ and $\beta3^{-/-}$ mutants, as well as in $\beta3^{-/-}/\beta5^{-/-}$ double-mutant mice (Fig. 1). Although $\beta5$ is involved in phagocytosis and the clearance of photoreceptor tips during the physiological process of photoreceptor shedding[14] and $\beta3$ may be relevant for phagocytosis by macrophages[14,29] and/or RPE,[14] our data show that a lack of these integrin subunits, individual or combined, did not influence efficient clearance of the large amount of photoreceptor debris generated by acute exposure to light.

Moreover, evaluation of the RPE and the subretinal space in the region of photoreceptor degeneration by electron microscopy did not reveal any accumulation of photoreceptor debris (Fig. 2A). At 6 hours after exposure to light, the time of severe POS damage (Fig. 1), distorted outer segments showed strong vesiculation in all mice (Fig. 2A, middle row, arrows) instead of the regular stacks of disks apposed to the RPE in control mice (Fig. 2A, top row). In addition, POS material was observed in RPE cells suggesting ongoing phagocytosis in all the mouse strains (Fig. 2A, middle row, arrowheads). After 10 days, most of the debris in the RPE was digested, and cells of the RPE appeared normal in all the mouse strains. We did not observe accumulation of POS debris in the RPE or in the subretinal space (Fig. 2A, bottom row).

As a molecular measure of phagocytosis, protein levels of pro-cathepsin D (pro-catD) and mature cathepsin D (mature catD) were determined by Western blot analysis in eye cups of wild-type and of $\beta5^{-/-}$ mice (Fig. 2B). CatD is one of the key enzymes involved in lysosomal digestion of POS.[21,30] Levels of the pro form were comparable in the two mouse strains and seemed to increase slightly with time after exposure to light. Mature catD was also present in both mice with a slight tendency of higher levels 1 to 5 days after exposure in wild-type mice. Levels in the knockout mice remained constant throughout the period of analysis.

In conclusion, our results suggest that the time course for the clearance of photoreceptor debris from the subretinal space after acute exposure to light was not severely disturbed by the deletion of $\beta3$ and of $\beta5$ integrins. In addition, the presence of similar catD protein levels in the two mouse strains suggests no major differences in the lysosomal activity of the RPE. However, subtle differences may still exist as may be suspected in light of the minor increase in the levels of mature catD (Fig. 2B) in wild-type mice.

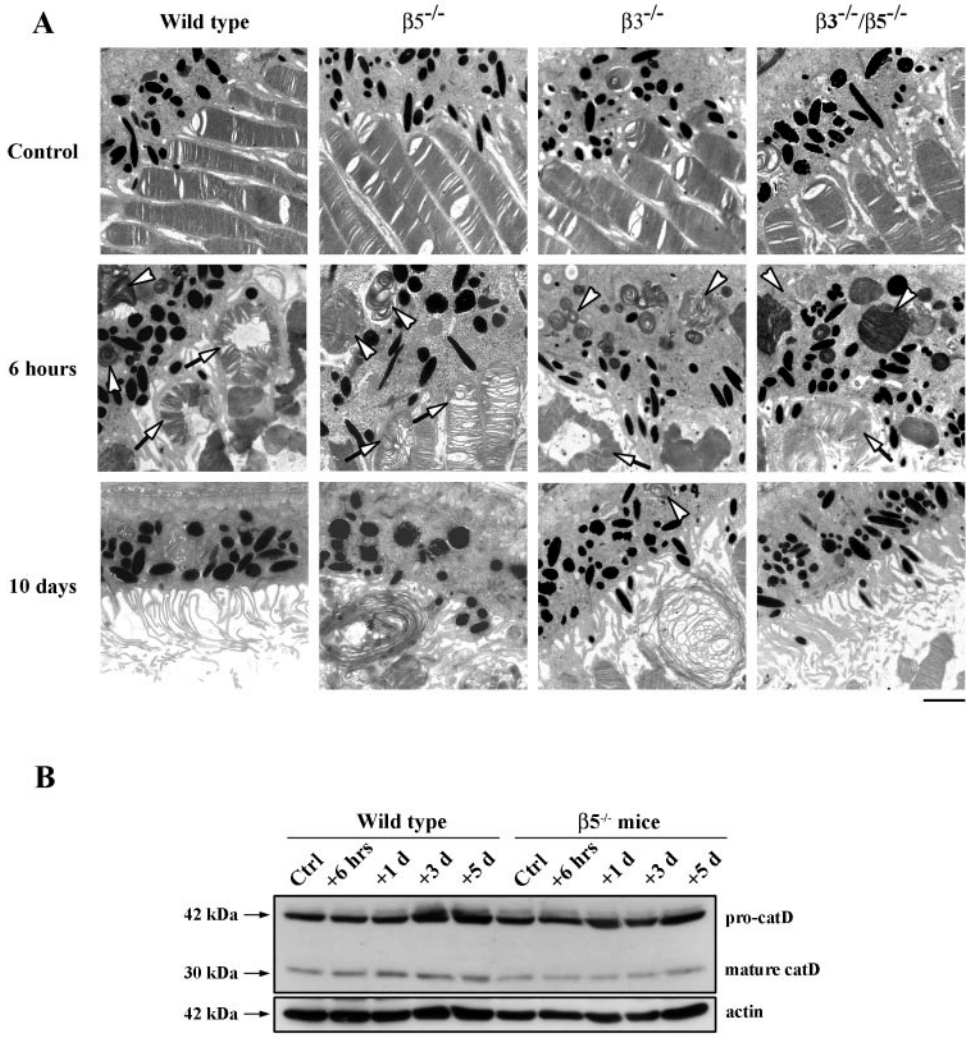

FIGURE 2. Lack of $\beta5$ and/or $\beta3$ integrins did not lead to an accumulation of photoreceptor debris after light-induced damage. (**A**) A normal ultrastructure of the RPE and POS was detected by electron microscopy in unexposed (control) wild-type, $\beta5^{-/-}$, $\beta3^{-/-}$ and $\beta3^{-/-}/\beta5^{-/-}$ mice. Six hours after exposure to light, the POS showed severe vesiculation (*arrows*), and still undigested POS were present within the RPE of all strains tested (*arrowheads*). At 10 days after exposure, no abnormal debris accumulation was detected in the RPE or the subretinal space of all mice tested. Scale bar: 2 μm. (**B**) Western blot for the pro and mature form of cathepsin D in the RPE at different time points after exposure to light as indicated. Retinas of nonexposed mice served as the control (Ctrl). Equal amounts of protein extracts from the RPE of three animals were pooled and tested for the levels of cathepsin D. Actin served as loading control.

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Participation of Alternative Receptors in the Removal of Damaged Photoreceptors

A variety of membrane receptors localized on the surface of phagocytes can contribute to the elimination of apoptotic or damaged cells.[3] Among them, the tyrosine kinase receptor Mer is present on the apical surface of the RPE membrane (Fig. 3A) and on the microvilli of RPE cells[7] and thus is a salient candidate for retinal phagocytosis.[8] Since activation of MerTK protein needs the β5 integrin,[22] we first analyzed gene expression of MerTK in eye cups of the wild-type and $\beta5^{-/-}$ animals. Although the lack of β5 downregulated basal expression levels of MerTK to 43% of wild-type (not shown), exposure to light induced the expression of MerTK in eye cups of the $\beta5^{-/-}$ but not in the wild-type animals (Fig. 3B). The increased expression of MerTK in the $\beta5^{-/-}$ animals did not detectably alter the localization of the protein (data not shown). Altogether, these results suggest that RPE cells may compensate for the absence of β5 integrin by the upregulation of molecules such as MerTK to efficiently clear the subretinal space from photoreceptor debris.

Role of Integrin β5 in the Activation of Focal Adhesion Kinase after Light-Induced Damage

It has been demonstrated in vitro that phospho-FAK_{Tyr397} is activated 2 to 3 hours after challenging RPE-J cells with photoreceptor outer segments.[8] Thus, we examined phospho-FAK_{Tyr397} levels in eye cups of the wild-type mice and of the $\beta5^{-/-}$ mice (Figs. 4A–D). Light-induced damage slightly activated FAK in the RPE of the wild-type mice (Figs. 4A, 4B). In contrast, FAK was not upregulated in the RPE of the $\beta5^{-/-}$ mice (Figs. 4C, 4D) supporting a direct role of FAK in β5-mediated phagocytosis.[8] Nevertheless, a comparable increase in retinal phospho-$Stat3_{Tyr705}$ 6 hours after exposure to light showed that the retinas of both wild-type and $\beta5^{-/-}$ mice had experienced similar levels of retinal stress (Fig. 4E).

Upregulation of MCP-1 after Exposure to Light in the Retinas of All Mice

The presence of macrophages in the subretinal space of wild-type but also of the $\beta3^{-/-}$ and $\beta5^{-/-}$ mice (Fig. 1) led us to consider the possibility that these phagocytic cells compensate for a potential phagocytic defect in mice deficient of β3 and/or β5 integrins. MCP-1 (or CCL-2) has already been identified as an important factor for monocyte/macrophage recruitment in pathologic conditions such as experimental autoimmune encephalomyelitis (EAE)[31] and atherosclerosis.[32] Ablation of the MCP-1 gene also decreases microglial activation in the thalamus[33] whereas in the retina, MCP-1 deficiency may result in the formation of drusen-like deposits and other retinal features similar to AMD.[24]

MCP-1 gene expression was highly induced in the neuronal retina at 6 hours after acute exposure to light in the wild-type mice as well as in the single- and double-integrin knockout mice (Fig. 5). Of note, gene expression returned to basal or even dropped below basal levels faster in the mice lacking β5 integrin, but not in those with deleted β3 integrin. The strong activation of MCP-1 combined with the presence of macrophages in the subretinal space of retinas exposed to light supports the hypothesis that invading phagocytic cells compensated for integrin deficiency.

To address the role of MCP-1 in macrophage attraction and thus in the clearance of photoreceptor debris in the retina after light-induced damage, we generated double-mutant mice lacking both MCP-1 and β5 integrin ($Ccl\text{-}2^{-/-}/\beta5^{-/-}$). However, the time course of light-induced photoreceptor degeneration in these double-mutant mice was similar to that in the wild-type and single $Ccl2^{-/-}$ mice (Fig. 6A). In addition, phagocytic

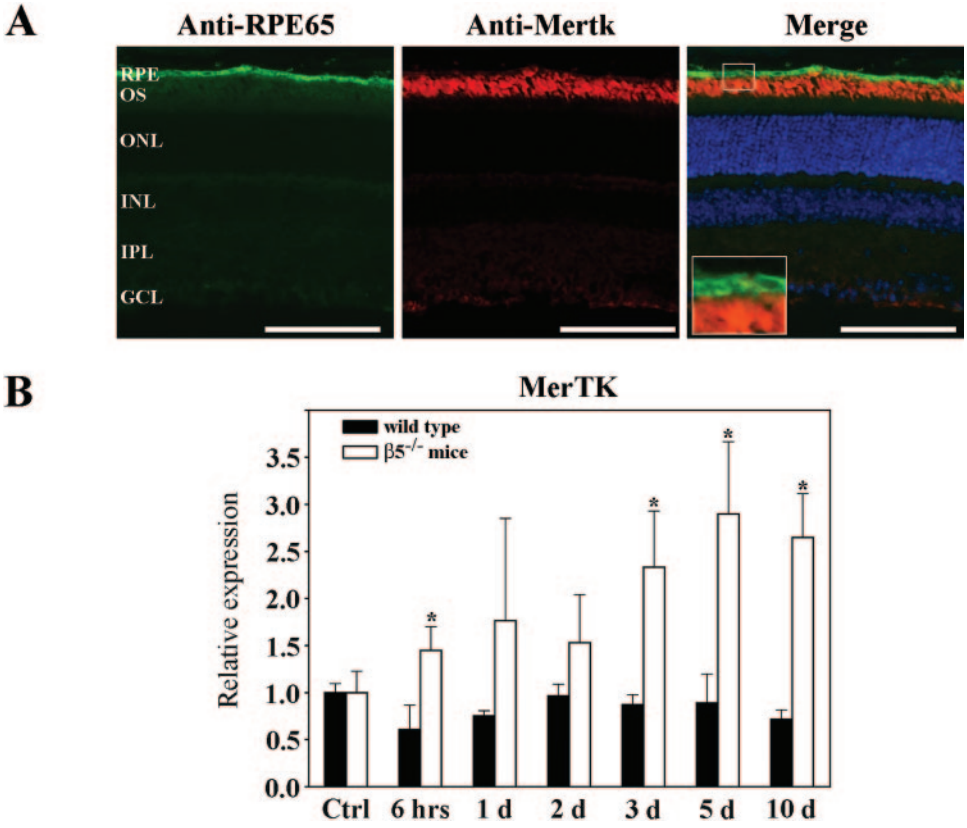

FIGURE 3. Expression of MerTK was increased in the eye cup of β5 knockout mice after exposure to light. (**A**) Localization of MerTK receptor in unexposed wild-type retinas was tested by immunofluorescence using normal fluorescence microscopy. RPE65 specifically localized to the RPE layer (*green*), whereas MerTK staining (*red*) was on the apical surface of RPE cells and on microvilli, as reported by others.[7] Nuclei have been stained with DAPI. Scale bar: 100 μm; *inset*, 33.3 μm. (**B**) Gene expression of MerTK in eye cups of wild-type and $\beta5^{-/-}$ mice as indicated. Expression was analyzed in nonexposed eye cups (Ctrl) or at different time points after exposure to light as indicated. Values were normalized to β-actin, and levels in control eye cups were set to 1 for each strain analyzed. Statistical analysis was performed with the unpaired Student's *t*-test (*$P < 0.05$) to compare MerTK retinal levels between wild-type and $\beta5^{-/-}$ mice. Amplification reactions were performed in triplicate and the results are shown as the mean ± SD ($n = 3$).

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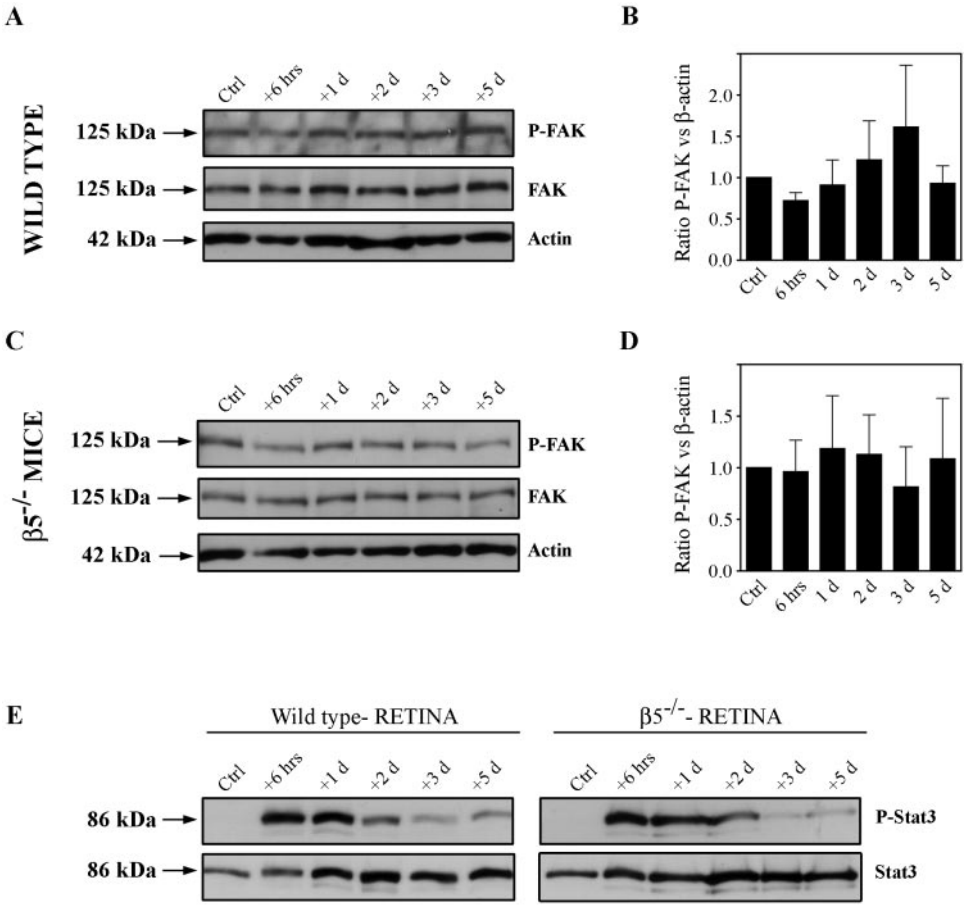

FIGURE 4. Analysis of phospho-FAK and phospho-Stat3 protein levels after exposure to light. (**A–D**) Levels of phospho-FAK$_{Tyr397}$ and total FAK protein in the eye cups of wild-type (**A**) and $\beta5^{-/-}$ mice (**C**). For protein quantifications (**B**, **D**), phospho-FAK levels were normalized to β-actin. Shown are the mean ± SEM of three independent experiments. (**E**) Levels of phospho-Stat3 protein in retinas of wild-type (*left*) and $\beta5^{-/-}$ mice (*right*). Animals were killed at the indicated time points after light offset. Ctrl: nonexposed mice. Shown are representative blots from three independent experiments.

cells appeared in the subretinal space after 5 days in all mice and by 10 days, most of the debris was cleared (Fig. 6A). It has been reported that the absence of MCP-1 is protective in a model of retinal detachment.[34] We noticed that the retinas of the Ccl-2$^{-/-}$ mice seemed to retain more photoreceptor nuclei at 5 and 10 days after exposure to light, suggesting that the absence of MCP-1 may render the retinas also less susceptible to light (Fig. 6A). However, the biochemical quantification of apoptosis did not result in a significantly different rate of cell death between the strains (data not shown).

The identity of these phagocytic cells as macrophages was verified by staining for F4/80, a specific marker for activated macrophages.[35] Three days after light offset, retinas from the Ccl2$^{-/-}$/$\beta5^{-/-}$ mice displayed F4-80-positive cells, showing that activated macrophages invaded the subretinal space of the lesioned area (Fig. 6B). These results suggest that recruitment of macrophages to injured photoreceptors does not depend on functional MCP-1 protein.

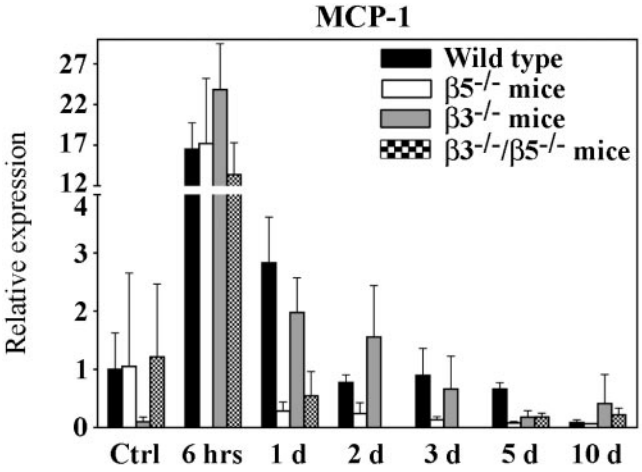

FIGURE 5. MCP-1 gene expression was strongly upregulated 6 hours after light offset in all strains. MCP-1 RNA levels were determined in retinas of wild-type, $\beta5^{-/-}$, $\beta3^{-/-}$, and $\beta3^{-/-}$ /$\beta5^{-/-}$ mice which were or were not (Ctrl) exposed to light. Analysis after exposure was at time points as indicated. Expression is shown relative to levels of control wild-type mice. Amplifications were performed in triplicates (mean ± SD; $n = 3$) and were normalized to actin values.

Role of Induced Expression of Chemokines in Macrophage Recruitment after Light-Induced Stress

Since MCP-1 was not crucial for macrophage recruitment, we hypothesized that other chemokines[36] might be able to compensate for the lack of MCP-1 and may thus be involved in the activation and attraction of macrophages. To test this possibility, we analyzed the expression of five different chemokines after exposure to light in retinas of wild-type and single- and double-knockout mice. The tested chemokines are members of the same CC chemokine family as MCP-1 except fractalkine (CX3CL1) that belongs to the CX3C family.[37] At 6 hours after light offset, CCL4 (also called MIP-1β, Fig. 7A), CCL5 (or RANTES, Fig. 7B), and CCL12 (or MCP-5, Fig. 7C) were highly induced in the neuronal retina in all mouse strains tested. Except for the Ccl2$^{-/-}$/$\beta5^{-/-}$ mutant mice in which levels were close to those of the wild-type, the $\beta3^{-/-}$, $\beta5^{-/-}$, and $\beta3^{-/-}$/$\beta5^{-/-}$ mice presented a 3.5- to 4-fold CCL4 mRNA

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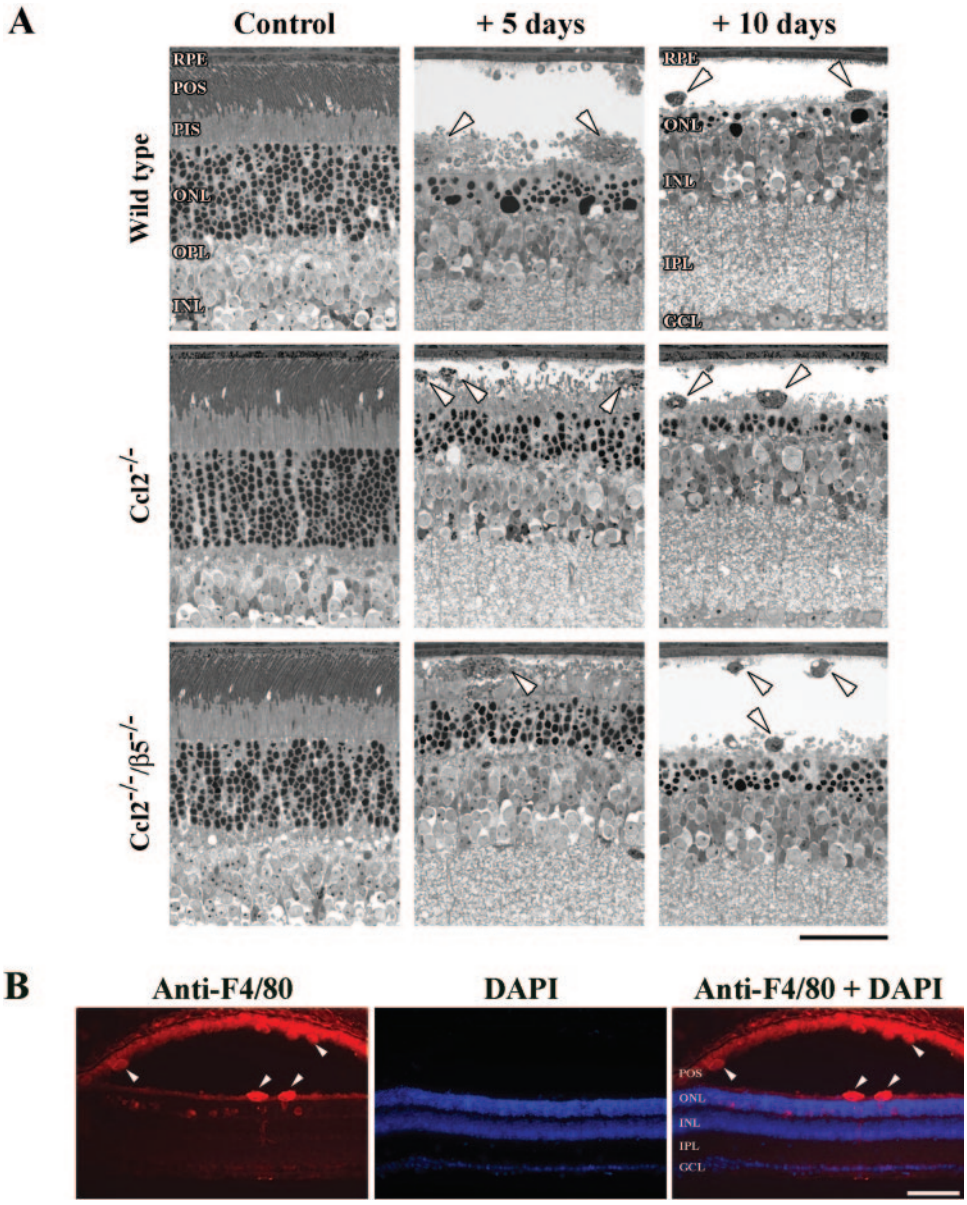

FIGURE 6. Lack of MCP-1 did not prevent infiltration of the subretinal space by macrophages after light-induced stress. (**A**) Representative photomicrographs of retinas from wild-type, $Ccl2^{-/-}$, and $Ccl2^{-/-}/\beta5^{-/-}$ mice not exposed to light (Control) showed regular morphology in all animals. At 5 and 10 days after exposure to light, the subretinal space of all mice was infiltrated by phagocytic cells (*arrowheads*) (**B**) Activated macrophages, detected by the monoclonal antibody anti-F4/80, infiltrated the subretinal space of $Ccl2^{-/-}/\beta5^{-/-}$ knockout mice 3 days after exposure to light (*arrowheads*). Nuclei were stained with DAPI. Layer abbreviations are as in Figure 1. Scale bar: (**A**) 50 μm; (**B**) 100 μm.

superinduction (over wild-type) after 6 hours (Fig. 7A). Whereas CCL4 and CCL12 mRNA levels steadily declined after the initial peak of expression, the levels of CCL5 mRNA remained high or increased even further at 2 and 3 days after exposure. In contrast, expression of CCL9 (or MIP-1γ, Fig. 7D) was regulated with different kinetics and to different levels. The mRNA levels increased later after exposure and stayed slightly elevated for up to 10 days. Of interest, mice lacking β3 or MCP-1 (with or without simultaneous lack of β5) but not those lacking only β5 exhibited increased CCL9 expression after 5 and 10 days compared with the wild-type. Expression of CX3CL1 mRNA (Fig. 7E) was not affected by the light treatment. Our data show that some chemokines were overexpressed early after exposure to light (CCL4, CCL5, and CCL12), whereas others demonstrated a delayed increase (CCL9) or were not differentially regulated at all (CX3CL1). The increased expression of some of the tested chemokines may significantly influence the migration and the recruitment of macrophages to the injured photoreceptors and may thus substitute for MCP-1 in the $Ccl2^{-/-}$ knockout mouse.

DISCUSSION

The β3 and β5 integrins were reported to have crucial roles in particle recognition/internalization[4] and in the binding of shed POS,[11,14] respectively, under physiological conditions. However, both types of integrins were not essential for the clearance of the subretinal space from debris generated after light-induced photoreceptor degeneration. The increased expression of alternate receptors present on the RPE membrane such as MerTK suggests the existence of mechanisms that can compensate for the lack of β5 and/or β3 integrins in acute situations such as light-induced damage. An important part of such mechanisms may be invading macrophages that are attracted and activated by the action of several chemokines. Since the deletion of MCP-1 ($Ccl2^{-/-}$) did not dramatically reduce macrophage infiltration, our results suggest a functional redundancy among chemokines in the retina. Expression of chemokines other than MCP-1 seems to be sufficient to attract a large number of macrophages to support efficient clearance of photoreceptor debris after

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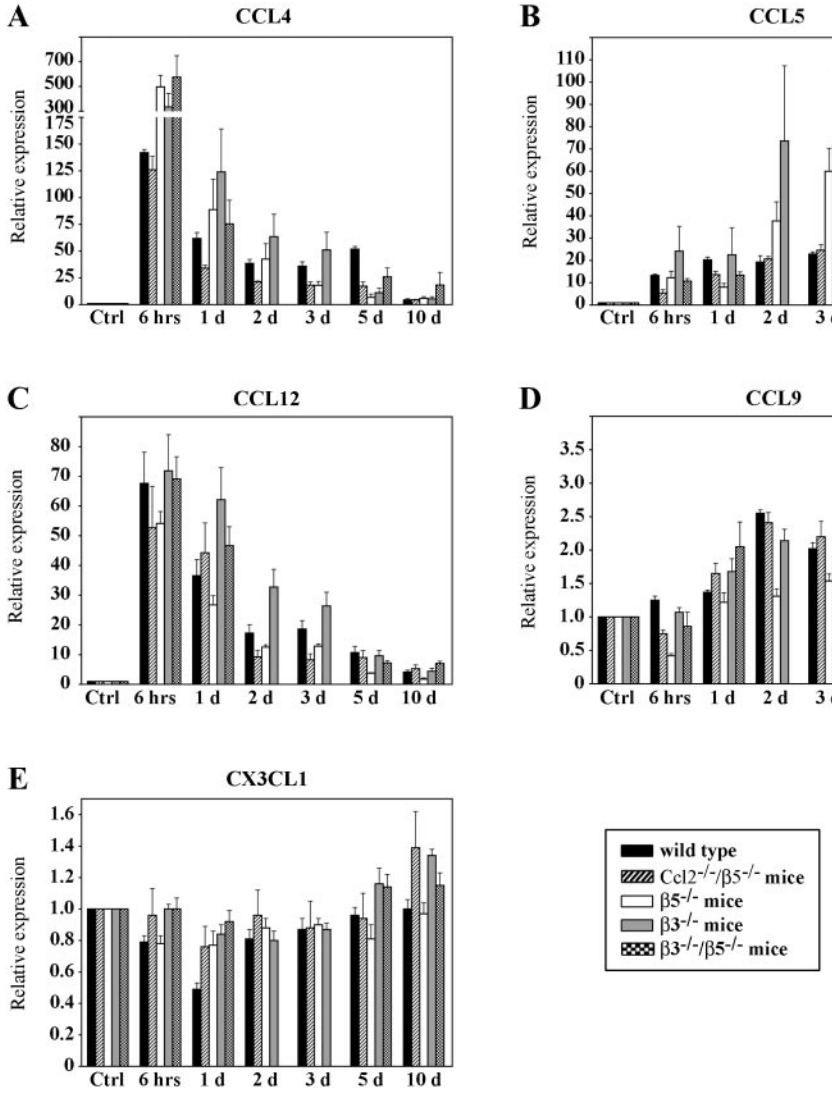

FIGURE 7. Specific regulation of chemokine expression after exposure to light. Relative RNA levels of CCL4 (**A**), CCL5 (**B**), CCL12 (**C**), CCL9 (**D**), and CX3CL1 (**E**) in retinas of wild-type, $Ccl2^{-/-}/\beta5^{-/-}$, $\beta5^{-/-}$, $\beta3^{-/-}$, and $\beta3^{-/-}/\beta5^{-/-}$ mice at various time points after exposure to light as indicated. For each strain, expression is shown relative to the levels of nonexposed mice that were set to 1. Pooled RNA of three different mice was amplified in duplicates. Results are expressed as the mean ± SD and are normalized to actin values.

light-induced damage, even in the absence of β3 and/or β5 integrin.

Similarity of Physiological Phagocytosis and Clearance of Large Amounts of Photoreceptor Debris Generated by Acute Exposure to Light

The β5 integrin receptor, localized on the apical surface of the RPE, is known to be crucial for recognition and binding of POS to the RPE membrane during physiological phagocytosis of shed photoreceptor tips.[14] It has been reported that loss of β5 integrin leads to a desynchronization of retinal phagocytosis and that during ageing, it induced retinal features similar to human AMD including decreased retinal function.[22] These functional defects were associated with morphologic impairments such as vesicular autofluorescent storage bodies and inclusion bodies in the RPE of 12-months-old $\beta5^{-/-}$ mice. In contrast, our results did not show any dysfunction in the clearance of cellular debris after acute exposure to light. This disparity may be due to the age of animals used and/or to the level of cellular stress. Whereas β5 integrin seems to be crucial for the daily task of phagocytosing shed photoreceptor segments, β5 may be dispensable in pathologic situations in which a large amount of cellular debris is generated (as after light-induced damage). The overload of material may trigger additional and β5-independent mechanisms to ensure an efficient clearing of the subretinal space. An important and integral part of such mechanisms may be the recruitment of macrophages—the classic phagocytes—to the lesion site. Thus, lack of β5 may cause more severe alterations in situations where macrophages are not recruited (as in the daily phagocytosis of shed POS) and may be less deleterious in acute situations where additional clearing mechanisms are activated.

Role of the MerTK Receptor in the Removal of Debris after Light-Induced Photoreceptor Degeneration

In vitro blockade of β5 integrin receptor with specific antibodies resulted in an 84% decrease in the uptake of photoreceptor outer segments after 2 hours, but only a 19% decrease after 5 hours,[14] suggesting that RPE cells can activate alternative routes of phagocytosis if the normal pathway has acutely been inactivated.

Many receptors are localized on the apical membrane of the RPE such as MerTK[7] and CD36.[4–6] MerTK is a tyrosine kinase that plays a critical role in the engulfment and clearance of apoptotic cells.[38] It has been demonstrated recently that MerTK cooperates with β5 integrin via FAK phosphorylation.[39,40] Another study reported that initial activation of MerTK by Gas6 ligand (growth arrest-specific gene 6) leads to phosphorylation of FAK and to a secondary activation of the β5

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receptor, suggesting bidirectional interactions between MerTK and β5.[41]

Our data show that total MerTK gene expression was significantly induced in eye cups after exposure to light in $\beta5^{-/-}$ deficient mice but not in wild-type mice (Fig. 3D), suggesting that this receptor may contribute to the removal of photoreceptor debris in the absence of β5 integrin. Moreover, MerTK receptor is also expressed at the surface of macrophages,[42] which could be relevant as we observed a massive invasion of macrophages to the subretinal lesion site (Figs. 1, 6). Whether MerTK stimulates an intracellular signaling pathway in the absence of β5 integrin remains to be determined.

Involvement of Chemokines Other Than MCP-1 in Attracting and Recruiting Macrophages to the Injured Photoreceptors

Chemokines are chemotactic cytokines involved in a variety of physiological activities. They are also considered to be important mediators of pathologic responses such as leukocyte recruitment and activation, viral infections, neurodegenerative diseases,[43] tumor progression[44] and angiogenesis.[45] So far, 50 chemokines and 20 chemokine receptors have been identified indicating the existence of redundant mechanisms since each chemokine has specificity for more than one receptor and each receptor can be activated by a range of chemokines.[37]

MCP-1 has been shown to attract monocytes, activated T-cells, and natural killer cells, but not leukocytes.[46] MCP-1 is the ligand for CCR2 receptor, but it can also act via the alternative receptor CCR11.[47] Investigators in several studies have reported that MCP-1 knockout mice present a reduction in the number of recruited macrophages[24] after retinal detachment[34] and in a model of experimental autoimmune encephalomyelitis (EAE).[31] After acute light-induced damage, however, single $Ccl2^{-/-}$ and double $Ccl2^{-/-}/\beta5^{-/-}$ mouse retinas displayed many activated macrophages in the subretinal space, suggesting that MCP-1 was dispensable for the attraction of these cells to the site of injury (Fig. 6). The strong induction of other chemokines suggests that other chemotactic factors can replace MCP-1 function (Fig. 7). This is consistent with other studies that have reported such redundant mechanisms in vitro[48] and in vivo,[49] where a correlation between MCP-1, CCL3, CCL4, and CCL5 mRNA levels and the intensity of inflammation was found in autoimmune anterior uveitis associated with EAE.

In summary, our data show that β5 and/or β3 integrins do not have an essential role in the clearance of cellular debris from the subretinal space after photoreceptor degeneration induced by acute exposure to light. This finding suggests that phagocytic mechanisms during normal physiological processes such as phagocytosis of shed outer segments differ from mechanisms that are activated in situations of acute overload of cellular debris.

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References

1. Fine SL, Berger JW, Maguire MG, Ho AC. Age-related macular degeneration. *N Engl J Med.* 2000;342:483–492.
2. de Jong PT. Age-related macular degeneration. *N Engl J Med.* 2006;355:1474–1485.
3. Lauber K, Blumenthal SG, Waibel M, Wesselborg S. Clearance of apoptotic cells: getting rid of the corpses. *Mol Cell.* 2004;14:277–287.
4. Finnemann SC, Silverstein RL. Differential roles of CD36 and alphavbeta5 integrin in photoreceptor phagocytosis by the retinal pigment epithelium. *J Exp Med.* 2001;194:1289–1298.
5. Ryeom SW, Sparrow JR, Silverstein RL. CD36 participates in the phagocytosis of rod outer segments by retinal pigment epithelium. *J Cell Sci.* 1996;109:387–395.
6. Sparrow JR, Ryeom SW, Abumrad NA, Ibrahimi A, Silverstein RL. CD36 expression is altered in retinal pigment epithelial cells of the RCS rat. *Exp Eye Res.* 1997;64:45–56.
7. Prasad D, Rothlin CV, Burrola P, et al. TAM receptor function in the retinal pigment epithelium. *Mol Cell Neurosci.* 2006;33:96–108.
8. Finnemann SC. Focal adhesion kinase signaling promotes phagocytosis of integrin-bound photoreceptors. *EMBO J.* 2003;22:4143–4154.
9. Strauss O. The retinal pigment epithelium in visual function. *Physiol Rev.* 2005;85:845–881.
10. Finnemann SC, Leung LW, Rodriguez-Boulan E. The lipofuscin component A2E selectively inhibits phagolysosomal degradation of photoreceptor phospholipid by the retinal pigment epithelium. *Proc Natl Acad Sci U S A.* 2002;99:3842–3847.
11. Finnemann SC. Role of alphavbeta5 integrin in regulating phagocytosis by the retinal pigment epithelium. *Adv Exp Med Biol.* 2003;533:337–342.
12. Miceli MV, Newsome DA, Tate DJ Jr. Vitronectin is responsible for serum-stimulated uptake of rod outer segments by cultured retinal pigment epithelial cells. *Invest Ophthalmol Vis Sci.* 1997;38:1588–1597.
13. Savill J, Dransfield I, Hogg N, Haslett C. Vitronectin receptor-mediated phagocytosis of cells undergoing apoptosis. *Nature.* 1990;343:170–173.
14. Finnemann SC, Bonilha VL, Marmorstein AD, Rodriguez-Boulan E. Phagocytosis of rod outer segments by retinal pigment epithelial cells requires alpha(v)beta5 integrin for binding but not for internalization. *Proc Natl Acad Sci U S A.* 1997;94:12932–12937.
15. Sarks SH, Arnold JJ, Killingsworth MC, Sarks JP. Early drusen formation in the normal and aging eye and their relation to age related maculopathy: a clinicopathological study. *Br J Ophthalmol.* 1999;83:358–368.
16. Curcio CA, Medeiros NE, Millican CL. Photoreceptor loss in age-related macular degeneration. *Invest Ophthalmol Vis Sci.* 1996;37:1236–1249.
17. Maguire MG. In: Berger JW, Fine SL, Maguire MG, eds. *Age-Related Macular Degeneration*. St. Louis: Mosby; 1999:17–30.
18. Karan G, Lillo C, Yang Z, et al. Lipofuscin accumulation, abnormal electrophysiology, and photoreceptor degeneration in mutant ELOVL4 transgenic mice: a model for macular degeneration. *Proc Natl Acad Sci U S A.* 2005;102:4164–4169.
19. Imamura Y, Noda S, Hashizume K, et al. Drusen, choroidal neovascularization, and retinal pigment epithelium dysfunction in SOD1-deficient mice: a model of age-related macular degeneration. *Proc Natl Acad Sci U S A.* 2006;103:11282–11287.
20. Mata NL, Tzekov RT, Liu X, Weng J, Birch DG, Travis GH. Delayed dark-adaptation and lipofuscin accumulation in $abcr^{+/-}$ mice: implications for involvement of ABCR in age-related macular degeneration. *Invest Ophthalmol Vis Sci.* 2001;42:1685–1690.
21. Rakoczy PE, Zhang D, Robertson T, et al. Progressive age-related changes similar to age-related macular degeneration in a transgenic mouse model. *Am J Pathol.* 2002;161:1515–1524.
22. Nandrot EF, Kim Y, Brodie SE, Huang X, Sheppard D, Finnemann SC. Loss of synchronized retinal phagocytosis and age-related blindness in mice lacking alphavbeta5 integrin. *J Exp Med.* 2004;200:1539–1545.
23. Hisatomi T, Sakamoto T, Sonoda KH, et al. Clearance of apoptotic photoreceptors: elimination of apoptotic debris into the subretinal space and macrophage-mediated phagocytosis via phosphatidylserine receptor and integrin alphavbeta3. *Am J Pathol.* 2003;162:1869–1879.
24. Ambati J, Anand A, Fernandez S, et al. An animal model of age-related macular degeneration in senescent Ccl-2- or Ccr-2-deficient mice. *Nat Med.* 2003;9:1390–1397.
25. Wenzel A, Grimm C, Samardzija M, Reme CE. The genetic modifier Rpe65Leu(450): effect on light damage susceptibility in c-Fos-deficient mice. *Invest Ophthalmol Vis Sci.* 2003;44:2798–2802.

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26. Hoppeler T, Hendrickson P, Dietrich C, Reme C. Morphology and time-course of defined photochemical lesions in the rabbit retina. *Curr Eye Res.* 1988;7:849–860.
27. Gordon WC, Casey DM, Lukiw WJ, Bazan NG. DNA damage and repair in light-induced photoreceptor degeneration. *Invest Ophthalmol Vis Sci.* 2002;43:3511–3521.
28. Kaneko H, Nishiguchi KM, Nakamura M, Kachi S, Terasaki H. Characteristics of bone marrow-derived microglia in the normal and injured retina. *Invest Ophthalmol Vis Sci.* 2008;49:4162–4168.
29. Fadok VA, Savill JS, Haslett C, et al. Different populations of macrophages use either the vitronectin receptor or the phosphatidylserine receptor to recognize and remove apoptotic cells. *J Immunol.* 1992;149:4029–4035.
30. Rakoczy PE, Lai CM, Baines M, Di Grandi S, Fitton JH, Constable IJ. Modulation of cathepsin D activity in retinal pigment epithelial cells. *Biochem J.* 1997;324:935–940.
31. Huang DR, Wang J, Kivisakk P, Rollins BJ, Ransohoff RM. Absence of monocyte chemoattractant protein 1 in mice leads to decreased local macrophage recruitment and antigen-specific T helper cell type 1 immune response in experimental autoimmune encephalomyelitis. *J Exp Med.* 2001;193:713–726.
32. Braunersreuther V, Mach F, Steffens S. The specific role of chemokines in atherosclerosis. *Thromb Haemost.* 2007;97:714–721.
33. Muessel MJ, Klein RM, Wilson AM, Berman NE. Ablation of the chemokine monocyte chemoattractant protein-1 delays retrograde neuronal degeneration, attenuates microglial activation, and alters expression of cell death molecules. *Brain Res Mol Brain Res.* 2002;103:12–27.
34. Nakazawa T, Hisatomi T, Nakazawa C, et al. Monocyte chemoattractant protein 1 mediates retinal detachment-induced photoreceptor apoptosis. *Proc Natl Acad Sci U S A.* 2007;104:2425–2430.
35. Austyn JM, Gordon S. F4/80, a monoclonal antibody directed specifically against the mouse macrophage. *Eur J Immunol.* 1981;11:805–815.
36. Bajetto A, Bonavia R, Barbero S, Florio T, Schettini G. Chemokines and their receptors in the central nervous system. *Front Neuroendocrinol.* 2001;22:147–184.
37. Savarin-Vuaillat C, Ransohoff RM. Chemokines and chemokine receptors in neurological disease: raise, retain, or reduce? *Neurotherapeutics.* 2007;4:590–601.
38. Scott RS, McMahon EJ, Pop SM, et al. Phagocytosis and clearance of apoptotic cells is mediated by MER. *Nature.* 2001;411:207–211.
39. Finnemann SC, Nandrot EF. MerTK activation during RPE phagocytosis in vivo requires alphaVbeta5 integrin. *Adv Exp Med Biol.* 2006;572:499–503.
40. Nandrot EF, Finnemann S. Roles of integrin receptors in the daily phagocytosis of photoreceptor outer segment fragments by the RPE. In: Williams DS, ed. *Photoreceptor Cell Biology and Inherited Retinal Degenerations.* Mountain View, CA: World Scientific Publishing Co.; 2004:371–395. *Recent Advances in Human Biology*; vol. 10
41. Wu Y, Singh S, Georgescu MM, Birge RB. A role for Mer tyrosine kinase in alphavbeta5 integrin-mediated phagocytosis of apoptotic cells. *J Cell Sci.* 2005;118:539–553.
42. Seitz HM, Camenisch TD, Lemke G, Earp HS, Matsushima GK. Macrophages and dendritic cells use different Axl/Mertk/Tyro3 receptors in clearance of apoptotic cells. *J Immunol.* 2007;178:5635–5642.
43. Cartier L, Hartley O, Dubois-Dauphin M, Krause KH. Chemokine receptors in the central nervous system: role in brain inflammation and neurodegenerative diseases. *Brain Res Brain Res Rev.* 2005;48:16–42.
44. O'Hayre M, Salanga CL, Handel TM, Allen SJ. Chemokines and cancer: migration, intracellular signalling and intercellular communication in the microenvironment. *Biochem J.* 2008;409:635–649.
45. Yoshida S, Yoshida A, Ishibashi T, Elner SG, Elner VM. Role of MCP-1 and MIP-1alpha in retinal neovascularization during postischemic inflammation in a mouse model of retinal neovascularization. *J Leukoc Biol.* 2003;73:137–144.
46. Luster AD. Chemokines-chemotactic cytokines that mediate inflammation. *N Engl J Med.* 1998;338:436–445.
47. Schweickart VL, Epp A, Raport CJ, Gray PW. CCR11 is a functional receptor for the monocyte chemoattractant protein family of chemokines. *J Biol Chem.* 2000;275:9550–9556.
48. Lu B, Rutledge BJ, Gu L, et al. Abnormalities in monocyte recruitment and cytokine expression in monocyte chemoattractant protein 1-deficient mice. *J Exp Med.* 1998;187:601–608.
49. Adamus G, Manczak M, Machnicki M. Expression of CC chemokines and their receptors in the eye in autoimmune anterior uveitis associated with EAE. *Invest Ophthalmol Vis Sci.* 2001;42:2894–2903.

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