The role of retinal microglial cells (MCs) in age-related macular degeneration (AMD) is unclear. Here we demonstrated that all retinal MCs express CX3C chemokine receptor 1 (CX3CR1) and that homozygosity for the CX3CR1 M280 allele, which is associated with impaired cell migration, increases the risk of AMD. In humans with AMD, MCs accumulated in the subretinal space at sites of retinal degeneration and choroidal neovascularization (CNV). In CX3CR1-deficient mice, MCs accumulated subretinally with age and albino background and after laser impact preceding retinal degeneration. Raising the albino mice in the dark prevented both events. The appearance of lipid-bloated subretinal MCs was drusen-like on funduscopy of senescent mice, and CX3CR1-dependent MC accumulation was associated with an exacerbation of experimental CNV. These results show that CX3CR1-dependent accumulation of subretinal MCs evokes cardinal features of AMD. These findings reveal what we believe to be a novel pathogenic process with important implications for the development of new therapies for AMD.
CX3CR1-dependent subretinal microglia cell accumulation is associated with cardinal features of age-related macular degeneration

Christophe Combadière,1,2,3 Charles Feumi,4,5,6 William Raoul,4,5,6 Nicole Keller,4,5,6 Mathieu Rodéro,1,2 Adeline Pézard,1 Sophie Lavallette,4,5,6 Marianne Houssier,1,5,6 Laurent Jonet,4,5,6 Emilie Picard,4,5,6 Patrice Debré,1,2,3 Mirna Sirinyan,7 Philippe Deterre,1,2 Tania Ferroukhi,1 Salomon-Yves Cohen,8 Dominique Chauvaud,9 Jean-Claude Jeanny,5,6 Sylvain Chemtob,7 Francine Behar-Cohen,4,5,6,9 and Florian Sennlaub4,5,6

1INSERM U543, Laboratoire d’Immunologie Cellulaire, Paris, France. 2Université Pierre et Marie Curie, Laboratoire d’informatique de Paris 6, Paris, France. 3AP-HP, Groupe Hospitalier Pitié-Salpêtrière, Service d’Immunologie, Paris, France. 4INSERM U872, Paris, France. 5Centre de Recherche des Cordeliers, Université Pierre et Marie Curie, Laboratoire d’informatique de Paris 6, Paris, France. 6Université Paris Descartes, UMR S 872, Paris, France. 7Department of Pediatrics, Ophthalmology and Pharmacology, Research Center, Hôpital Ste Justine, Montréal, Québec, Canada. 8Centre d’Angiographie et de Laser, Paris, France. 9AP-HP, Hôtel Dieu, Service d’Ophthalmologie, Centre de Recherche Ophthalmologique, Paris, France.

Introduction
Age-related macular degeneration (AMD) is the leading cause of vision loss in elderly people in the industrialized countries (1). Its most prominent pathologic features are lesions involving the retinal pigment epithelium (RPE) and Bruch’s membrane (BM), photoreceptor degeneration (2), and, in the most aggressive cases, choroidal neovascularization (CNV). Early AMD is characterized by yellowish-white dots called drusen, located on BM, partially covered by the RPE and clinically visible with funduscopy (3). Composed of lipids and glycoproteins (4), drusen are believed to be formed by extracellular deposit of materials or by transformed degenerating RPE cells (5). Controversy continues to surround their pathogenesis, as well as the causes of AMD.

Epidemiological studies have helped to identify key factors in the pathogenesis of AMD. Age (1) and family history (3) are the principal predisposing factors. Genes with polymorphisms in the pathogenesis of AMD. Age (1) and family history (3) are

Nonstandard abbreviations used: AMD, age-related macular degeneration; BM, Bruch’s membrane; CCL2, CC chemokine ligand 2; CCR2, CC chemokine receptor 2; CNV, choroidal neovascularization; CX3CL1, CX3C chemokine ligand 1; CX3CR1, CX3C chemokine receptor 1; MC, microglial cell; OS, outer segment; RPE, retinal pigment epithelium; SrMC, subretinal MC.

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a sample of elderly individuals with AMD (Genetics of Age-related Macular Degeneration study; GAMD; Table 1 and Supplemental Table 1; supplemental material available online with this article; doi:10.1172/JCI31692DS1). The frequencies of CX3CR1 polymorphisms did not deviate from Hardy-Weinberg equilibrium among AMD cases or subjects from reference studies (data not shown). Table 1 shows the distribution of the CX3CR1 T280M genotypes among the subjects from the DETER study (P. Deterre, unpublished observations) and the previously published Etude Cas-Témoin de l’Infarctus du Myocarde (ECTIM; ref. 16), Offspring Cohort of the Framingham Heart Study (FHS; ref. 17), and Étude du Profil Géné- tique de l’Infarctus Cérébral (GENIC; ref. 18) studies used as references. Homozygosity for the M280 allele was consistently more frequent in studies of AMD than of other diseases. Odds ratios associated with homozygosity for the rare allele were therefore computed with genotypes carrying the more frequent allele as the reference group. M280 homozygosity was associated with an increased risk of AMD, with odds ratios ranging from 1.97 to 2.695 (95% confidence intervals, 1.069–3.652 and 1.268–5.728, respectively). This association was independent of age, and disease severity was not associated with the polymorphism. The similarity of the genotype distribution of the CX3CR1 V249I alleles among AMD patients and subjects from reference studies (Supplemental Table 1) indicated that this polymorphism had no significant effect on AMD risk.

**CX3CR1 expression in AMD.** To understand the mechanism by which CX3CR1 alterations may contribute to AMD development, we analyzed CX3CR1 expression in eyes of healthy donors and subjects with AMD (Figure 1). CX3CR1 positivity (red staining) was found in cells of the inner retina. Double labeling for CX3CR1 (green fluorescence) and the vascular endothelial cell marker ulex (red fluorescence) showed the ramified morphology of cells expressing CX3CR1 and their closeness to the retinal vasculature in the healthy eye (Figure 1A, inset). No CX3CR1 positivity was detected in photoreceptors or RPE cells in healthy donor eyes. In the macula of age-matched patients with AMD, we observed localized RPE disruption and clumping as well as photoreceptor degeneration (Figure 1B). In the affected zones, CX3CR1-positive cells were found ectopically in the outer retina and in the subretinal space, as well as in the perivascular retinal MCS in the inner retina (Figure 1B). Some of these cells in the outer retina were very large and bloated (Figure 1B, inset). All CX3CR1-positive cells (Figure 1C) overlapped precisely with cells expressing the microglial marker CD18 (Figure 1D), and vice versa (Figure 1E). Furthermore, CX3CR1 deposits were found in drusen, specifically in the CD18-positive dendritic cells interposed between the choroidal vessels (Figure 1F). CX3CR1-positive cells were also found in close contact with CNV (Figure 1G).

**Table 1** Distribution of CX3CR1 T280M in GAMD and reference studies

<table>
<thead>
<tr>
<th>Distribution</th>
<th>Study (N)</th>
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<tr>
<td></td>
<td>GAMD (284)</td>
</tr>
<tr>
<td>TT (no.)</td>
<td>64.1% (182)</td>
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<tr>
<td>TM (no.)</td>
<td>29.9% (85)</td>
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<tr>
<td>MM (no.)</td>
<td>6.0% (17)</td>
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<td>P</td>
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<td>Odds ratio</td>
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<td>(95% confidence interval)</td>
<td>(1.268 to 5.728)</td>
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Distribution of patients homozygous for threonine (TT) and methionine (MM), common and rare alleles, respectively, at position 280, as well as patients heterozygous for threonine/methionine (TM) at position 280. N, total number of participants. aAllelic frequency of allele M. P values and odds ratios were determined by comparing the frequency of the MM genotype with the frequency of T carriers. The DETE

(P. Deterre, unpublished observations), ECTIM (16), FHS (17), and GENIC (18) studies were used as references.

not distinguish between the 2 monocyte populations. To gain insight into the ability of cells expressing different variants of CX3CR1 to migrate in response to chemotactic factors present in the retina, we studied the in vitro chemotaxis of monocytes in response to CCL2 through porous membranes with and without a CX3CL1 coating. Monocytes from individuals homozygous for the CX3CR1-VM or CX3CR1-IM isoforms migrated equally well to CCL2 (Figure 2A). CCL2-specific chemotactic responses of monocytes from individuals homozygous for the VT variant were similar whether or not the filter-bound CX3CR1 was present (Figure 2A), with chemotactic index ratios (index without CX3CL1/index with CX3CL1) of 1.1 ± 0.1. In contrast, monocytes from individuals homozygous for CX3CR1-IM variants migrated in smaller numbers to the lower chamber in response to CCL2 when anchored CX3CL1 was present than when it was not (Figure 2A). This inhibition reached 48% ± 11% (Figure 2B). These data are consistent with our previous work (19) and indicate that chemotaxis of monocytes from individuals with IM genotypes is impaired in the presence of bound CX3CL1.

**SrMCs accumulate in CX3CR1−/− mice with age and develop retinal degeneration.** Because risk CX3CR1 was expressed on human MC (Figure 1) and because a CX3CR1 polymorphism that impairs cell migration was associated with risk of AMD (Table 1 and ref. 10), we explored the role of CX3CR1 in retinal homeostasis using mice deficient in CX3CR1 gene (20). We first identified the site of CX3CR1 expression by using CX3CR1<sup>GFP/GFP</sup> (CX3CR1-deficient with GFP driven by CX3CR1 promoter) and CX3CR1<sup>+/GFP</sup> mice of different ages. In 18-month-old CX3CR1<sup>+/GFP</sup> and CX3CR1<sup>GFP/GFP</sup> mice, GFP-positive cells abutted retinal endothelial cells (red fluorescence, BSA-1; Figure 3, A and B). Confocal images of the outer plexiform layer of retinal flatmounts displayed dense networks of ramified MCS of similar density in CX3CR1<sup>+/GFP</sup> and CX3CR1<sup>GFP/GFP</sup> animals (266 ± 19 and 305 ± 27 MCSs/mm<sup>2</sup>; n = 4 per group; Figure 3, C and D), indicating that all MCSs expressed CX3CR1 and that homing of retinal MCSs was CX3CR1 independent. The expression of the reporter gene in individual MCSs was similar in CX3CR1<sup>+/GFP</sup> and CX3CR1<sup>GFP/GFP</sup> mice. The absence of CX3CR1 expression in neurons, astrocytes, Müller cells, and RPE demonstrated that MCS were the only CX3CR1-positive cells in the retina.

**The M280 polymorphism leads to migrational defect.** We previously showed that only the membrane-anchored form of CX3CL1 functionally discriminates between the 2 allelic isoforms of CX3CR1 (19): monocytes bearing the CX3CR1-IM allele adhere more strongly than cells expressing only the CX3CR1-VM variant. In contrast, their chemotactic response to CX3CL1 does not distinguish between the 2 monocyte populations. To gain insight into the ability of cells expressing different variants of CX3CR1 to migrate in response to chemotactic factors present in the retina, we studied the in vitro chemotaxis of monocytes in response to CCL2 through porous membranes with and without a CX3CL1 coating. Monocytes from individuals homozygous for the CX3CR1-VM or CX3CR1-IM isoforms migrated equally well to CCL2 (Figure 2A). CCL2-specific chemotactic responses of monocytes from individuals homozygous for the VT variant were similar whether or not the filter-bound CX3CR1 was present (Figure 2A), with chemotactic index ratios (index without CX3CL1/index with CX3CL1) of 1.1 ± 0.1. In contrast, monocytes from individuals homozygous for CX3CR1-IM variants migrated in smaller numbers to the lower chamber in response to CCL2 when anchored CX3CL1 was present than when it was not (Figure 2A). This inhibition reached 48% ± 11% (Figure 2B). These data are consistent with our previous work (19) and indicate that chemotaxis of monocytes from individuals with IM genotypes is impaired in the presence of bound CX3CL1.

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On RPE flatmounts of 18-month-old CX3CR1+/GFP mice (Figure 3E), GFP-positive SrMCs were seen occasionally, as previously reported (21), whereas in CX3CR1GFP/GFP mice, SrMCs juxtaposed to RPE (Figure 3B, arrows) accumulated markedly (Figure 3F) as a function of age (Figure 3G). In CX3CR1−/− mice without the GFP transgene, CD11b-positive SrMCs accumulated in a manner similar to that of the analogous CX3CR1GFP/GFP animals (data not shown). Histological sections of 18-month-old CX3CR1+/+ (Figure 3H) and CX3CR1−/− (Figure 3I) mice revealed substantial degeneration of the outer retina in the knockout mice. Measurements of the photoreceptor cell layer showed significant thinning (40%) in CX3CR1−/− mice (Figure 3J).

SrMC accumulation is associated with retinal degeneration in albino CX3CR1−/− mice. Light has been suggested to be a contributing factor in the genesis of AMD (22). In conventional light conditions in wild-type albino animals, SrMCs accumulate as a function of ambient light intensity; this accretion is inhibited by light restriction (21). Accordingly, we found that 1-month-old CX3CR1+/− albino mice raised in normal light conditions (12-h light/12-h dark cycle) exhibited some CD11b-positive SrMCs (green fluorescence) adjacent to the RPE (phalloidin red fluorescence; Figure 4A), but these were significantly more abundant in age-matched CX3CR1+/− mice (Figure 4B). The number of SrMC increased as a function of age in wild-type and knockout mice, but the accumulation was accentuated in the latter (Figure 4C). Moreover, the CX3CR1−/− BALB/c mice housed in complete darkness throughout the experiment accumulated significantly fewer SrMCs at 2 months than did the CX3CR1−/− mice raised in ambient light (Figure 4C).

Histological sections of 4-month-old CX3CR1+/+ (Figure 4D) and CX3CR1−/− (Figure 4E) mice showed marked degeneration of the outer retina in the latter; this degeneration was not observed in the dark-raised knockout mice (Figure 4F). Quantification of photoreceptor cell layer thickness showed a slight (20%) but significant thinning at 2 months and complete degeneration at 4 months in the knockout mice, both of which were prevented by light restriction (Figure 4G).
Drusen observed in CX3CR1 knockout mice are lipid-bloated SrMCs. Subretinal deposits are characteristic in AMD. Funduscopy of senescent CX3CR1+/− (Figure 5A) and CX3CR1−/− (Figure 5B) mice showed that all the CX3CR1+/− mice had numerous drusen-like yellow nodular deposits in the deep layer of the retina that were not present in age-matched CX3CR1+/+ or 2-month-old CX3CR1+/− mice (data not shown). Similar features were observed in a second strain of CX3CR1 knockout mouse, CX3CR1GFP/GFP (23). To characterize in more detail the anatomical structure that produced this drusen-like appearance under funduscopy in CX3CR1+/− mice, we flatmounted the eyes after removal of the neuroretina, thus exposing the RPE monolayer. The RPE monolayer of CX3CR1+/− animals, observed in tangential white light, appeared regular and smooth (Figure 5C). In contrast, in knockout mice, examination of the monolayer showed it was abutted by multiple elevated white spots (Figure 5D). Higher magnification revealed the intracellular location of this white material: bloated cells adjacent to the RPE cell monolayer. These intracellular yellow-white deposits were present only in GFP-positive cells (Figure 5, E–G). Further confocal microscopy of these GFP-positive ramified cells (Figure 5H) used the specific MC marker 5D4 (22) (Figure 5I) and CD11b or F4/80 (data not shown) to identify these cells as activated SrMCs. Not all SrMCs were bloated, but only the bloated cells appeared drusen-like on RPE flatmounts.

The presence of yellowish-white deposits in 5D4+, CD11b- and F4/80-positive SrMCs was corroborated in CX3CR1+/− eyes (data not shown). Histological sections of 12-month-old CX3CR1+/− (Figure 3J) and CX3CR1−/− (Figure 5K) mice showed subretinal cells adhering to the pigmented monolayer of the RPE in CX3CR1+/− but not CX3CR1−/− mice. Ultrastructural analysis of senescent CX3CR1+/− eyes by transmission electron microscopy confirmed the presence of subretinal cells between the outer segment (OS) and the RPE cells (Figure 5L), which contained intracellular lipid deposits and remnants of OS (Figure 5M), as typically observed in RPE cells (24). Rhodopsin immunoreactivity in CD11b-positive SrMCs of CX3CR1−/− RPE flatmounts (Figure 5N) was consistent with ingestion of OS by SrMCs.

SrMC accumulation is associated with CNV and retinal degeneration in CX3CR1 knockout mice. Laser coagulation of the mouse choroid induces a local inflammatory reaction composed of blood-derived macrophages and activated MCs (25). After detecting SrMC accumulation in senescent and ambient light–exposed CX3CR1-deficient mice, we studied the influence of CX3CR1 deficiency in laser injury. Fourteen days after a laser injury, CX3CR1+/GFP mice showed some GFP-positive SrMCs adjacent to the choroid scar (Figure 6A), but many more such cells had accumulated in the CX3CR1−/−GFP mice (Figure 6B) at this stage. The cell counts after laser impact (Figure 6C) showed a transient increase in the CX3CR1−/−GFP mice, peaking at day 7; again, the increase in CX3CR1GFP/GFP mice was significantly higher and continued to rise. Immunohistochemistry at day 7 in CX3CR1−/− mice (Figure 6, D–F) revealed that the proangiogenic factor VEGF (Figure 6D) was produced by activated SrMCs (25). After detecting SrMC accumulation in senescent and ambient light–exposed CX3CR1-deficient mice, we studied the influence of CX3CR1 deficiency in laser injury. Fourteen days after a laser injury, CX3CR1+/GFP mice showed some GFP-positive SrMCs adjacent to the choroid scar (Figure 6A), but many more such cells had accumulated in the CX3CR1−/−GFP mice (Figure 6B) at this stage. The cell counts after laser impact (Figure 6C) showed a transient increase in the CX3CR1−/−GFP mice, peaking at day 7; again, the increase in CX3CR1GFP/GFP mice was significantly higher and continued to rise. Immunohistochemistry at day 7 in CX3CR1−/− mice (Figure 6, D–F) revealed that the proangiogenic factor VEGF (Figure 6D) was produced by activated SrMCs that expressed F4/80 (Figure 6, E and F).

At 14 days after laser injury, there was significantly less CNV in CX3CR1−/− mice (Figure 6G) than in their CX3CR1+/− counterparts (Figure 6H). Quantification of the surface covered by the endothelial cells showed twice as much neovascularization in the CX3CR1−/− and CX3CR1GFP/GFP knockout strains (Figure 6I) than their controls. Finally, because retinal degeneration worsens secondary to subretinal neovascularization in AMD, we tested whether the increased MC recruitment in the vicinity of the subretinal neovascularization...
was associated with an aggravation of the retinal degeneration. In 12-month-old animals, in which a 30% reduction in outer nuclear layer thickness was observed in CX3CR1+/− mice compared with CX3CR1+/+ mice, we analyzed the photoreceptor cell layer at 21 days after the laser treatment. In CX3CR1+/− mice, photoreceptor cell layer thickness adjacent (200 μm) to the laser-induced retinal destruction did not differ significantly from that in the nonimpacted retina (Figure 6J). In contrast, under these conditions, the photoreceptor layer had thinned substantially in CX3CR1−/− mice (Figure 6K). This difference may result from the combination of age- and laser-induced degeneration. To evaluate the laser-induced alteration, the relative retinal thicknesses of injured and uninjured retina were compared at different distances from the laser impact (ranging 100–1,000 μm). Laser-dependent retinal degeneration was significantly more pronounced in CX3CR1−/− mice than in CX3CR1+/− mice at distances of 100 and 200 μm but not at 300 and 400 μm (Figure 6L).

Discussion

Here we show that retinal MCs all and invariably expressed CX3CR1, that these cells accumulated subretinally in AMD, and that the T280M polymorphism of CX3CR1 was associated with both impaired cell migration and AMD. MCs accumulated under the retinas of CX3CR1-deficient mice with age, after laser injury, or with albino background. This accumulation elicits photoreceptor degeneration and exacerbated neovascularization. Moreover, the resulting prolonged contact of MC with lipid-rich OS was associated with intracellular lipid accumulation in the MCs. Surprisingly, these subretinal microglial foam cells turned out to be the origin of drusen-like deposits in mice. Our results show that accumulation of MCs in the subretinal space may be a driving force in the pathogenesis of AMD, rather than a secondary occurrence.

Our study of patients with AMD showed that homozygosity for the M280 allele was consistently more frequent in groups with AMD than in studies of other populations and confirmed a previous report (10) of a dominant association between the CX3CR1 M280 allele and AMD risk. Subjects in the control studies were not checked for AMD, and some certainly had or will develop the disease. The effect of this bias, however, should be an underestimation of the impact of CX3CR1 polymorphisms on AMD risk. These data indicate that the chemokine receptor CX3CR1 may play an important role in AMD pathogenesis.

To understand how CX3CR1 polymorphisms contribute to AMD pathogenesis, it is essential to identify the types of cells expressing CX3CR1 in the retina. Our immunohistochemical analysis in humans showed that CX3CR1 was expressed on all retinal MCs, which were identified by their morphology and CD18 expression. In addition, experiments using CX3CR1+/GFP mice and the murine MC markers CD11b, F4/80, and 5D4 confirmed that only MCs express CX3CR1. Using both techniques in 2 different species, we found no CX3CR1 expression in neurons, astrocytes or glial cells: as a previous report showed for the CNS (14), MCs are the only cells in the retina that express CX3CR1. Contrary to a previous report (10), when we used the CX3CR1-specific antibody revealed by the fast red system to circumvent autofluorescence interference and conducted experiments with a reporter gene, we failed to find significant CX3CR1 expression in RPE cells. In AMD, CX3CR1-positive MCs accumulated subretinally in affected areas of the macula. Our data are consistent with the infiltration of the subretinal space by activated MCs previously described in AMD (15) and indicate that a specific inflammatory environment characterized by production of specific cytokines and chemokines may determine particular pathological conditions.

Cells of the CNS and of the retina express CX3CL1 abundantly (26, 27). On the other hand, RPE cells under oxidative stress express other monocyte chemoattractant chemokines such as CCL2 (28), which may be responsible for the recruitment of MCs in the subretinal space. To evaluate the effect of the M280 polymorphism on cell migration in a CX3CL1-rich environment, we tested M280 CX3CR1 monocyte migration through a CX3CL1-coated porous membrane in response to CCL2. Previous studies show that the M280 polymorphism provokes loss of chemotaxis (17) or increases adherence to its ligand (19). Here, the presence of bound CX3CL1 significantly inhibited the CCL2-dependent chemotaxis of monocytes expressing the CX3CR1 M280 variant (Figure 2). If similar alterations occur in vivo to retinal MCs, the M280 polymorphism may cause excessive MC adherence to membrane-anchored CX3CL1 and reduce migration in response to other inflammatory chemokine receptors. Over the years, in subjects with the M280 polymorphism, clearance of MCs from the subretinal space (in response to...
to soluble CX3CL1 or other chemoattractants) would thereby be inhibited, and SrMC accumulation might occur.

To simulate CX3CR1 dysfunction in an animal model, we used 2 independently generated CX3CR1 knockout mouse strains. Our data demonstrated strong and persistent SrMC accumulation in CX3CR1-deficient mice in 3 experimental conditions: laser injury, aging, and albino animals. In contrast, control mice with laser injury only transiently amassed ramified F4/80- and GFP-positive cells, and aging and albino control animals slowly or steadily accumulated SrMCs. We and others have previously demonstrated that CX3CR1 deficiency reduces macrophage aggregation in atherosclerotic lesions (20, 29), probably by limiting monocyte recruitment from the blood. In view of the role of CX3CR1 in monocyte recruitment, the excessive number of MCs observed in CX3CR1-deficient mice is unlikely to be the result of an excessive influx of monocytes.

Unlike the monocytes in atherosclerotic lesions, MCs can proliferate in situ (30). Their accumulation may be the result of excessive influx of monocytes.

Figure 5
Drusen observed in CX3CR1 knockout animals are bloated SrMCs. (A–D) Comparison of fundus photos and micrographs of a RPE flatmount in tangential light of 1-year-old CX3CR1+/+ (A and C) and CX3CR1−/− mice (B and D) revealed multiple drusen in CX3CR1−/− mice. (E–G) Higher-magnification image of a drusen (arrow) in a CX3CR1GFP/GFP mouse in tangential light (E) superimposed with GFP-positive (green; red, Phalloidin; blue, DAPI) subretinal ramified cells (F) on RPE flatmounts. Merge of tangential light and DAPI is shown in G. (H and I) GFP-positive subretinal ramified cells (H) on CX3CR1GFP/GFP RPE flatmounts were positive for the MC marker 5D4 (red, I). (J and K) Histological sections of historessin-embedded 12-month-old CX3CR1−/− (J) and CX3CR1−/− (K) eyes showed subretinal cells abutting the RPE in CX3CR1−/− (K, arrow) but not in CX3CR1−/− mice. (L) Electron microscopy of the RPE/OS interface in CX3CR1−/− mice revealed subretinal cells (asterisk) with multiple phagosomes juxtaposed to the RPE cell layer. (M) Higher magnification of these subretinal cells revealed multiple lipid deposits and typical remnants of rod OSs in phagosomes (arrow). (N) Subretinal CD11b-positive (green) dendritic cells contained rhodopsin-positive inclusions (red) in confocal micrographs of RPE flatmounts from CX3CR1−/− mice. Results are representative of at least 3 independent experiments. Scale bars: 500 μm (A–D), 50 μm (E–G), 20 μm (H–K and N), 1 μm (L and M).
that can cause the oxidative protein and lipid modifications associated with drusen. The inflammatory proteins found in drusen may therefore be of retinal MC origin, at least in part.

Another consequence of the prolonged presence of MCs in the subretinal space may be photoreceptor cell death. In CX3CR1<sup>−/−</sup> mice, retinal degeneration was observed in senescent mice, in albino mice, and in the vicinity of subretinal neovascularization. The retinal degeneration in CX3CR1<sup>−/−</sup> mice was always preceded by SrMC accumulation and could be averted by limiting MC accumulation in the subretinal space, as seen in CX3CR1<sup>−/−</sup> albino mice raised in the dark (Figure 4). SrMC accumulation was observed as early as 6 months of age in pigmented and 1 month in albino CX3CR1-deficient mice, before any significant retinal degeneration (data not shown). There are no naturally occurring mutations known to cause retinal degeneration in BALB/c mice, and the time course of the degeneration, starting at 2 months, did not match any known retinal degeneration caused by photoreceptor or RPE gene defects. We further note that neither photoreceptors nor RPE cells contained express CX3CR1. Activated MCs participate in ganglion cell death in vivo (42) and can induce photoreceptor cell death in vitro (43). Neuronal cell toxicity caused by the prolonged presence of MCs (CX3CR1<sup>GFP/GFP</sup>) in the brain has previously been described as a mechanism of neurodegenerative diseases (14), and similar mechanisms may cause the degeneration observed in the CX3CR1<sup>−/−</sup> BALB/c mice, in the senescent CX3CR1<sup>−/−</sup> C57BL/6 mice, and in the vicinity of subretinal neovascularization after laser injury. To recapitulate, SrMC accumulation in CX3CR1-deficient mice is associated with photoreceptor degeneration in the absence of any primary photoreceptor or RPE disease. To our knowledge, no such mechanism of retinal degeneration has previously been described.

Furthermore, we showed that activated SrMCs adjacent to the laser injury site express VEGF. In CX3CR1-deficient mice, the increased accumulation of SrMCs and subsequent secretion of VEGF may be responsible for the development of the exaggerated neovascularization. This is consistent with our previous work, which showed that MCs can develop proangiogenic properties in the retina (44). Moreover, CX3CR1-positive MCs were found in human CNV, which indicates that SrMCs are a possible cause or aggravating factor for CNV in AMD.

In summary, our results suggest that MC accumulation in the subretinal space may be a driving force in the pathogenesis of AMD and not a mere consequence of primary RPE or photoreceptor disease. Our study challenges the long-standing paradigm of drusen genesis as the gradual accumulation of extracellular debris from the RPE and choroid. We propose that SrMC accumulation, resulting from a migratory defect associated with CX3CR1, plays a key role in drusen formation, CNV, and retinal degeneration, the main features of AMD.

**Methods**

**Study participants and AMD assessment.** The GAMD study included 285 participants (29% men) recruited in the “Centre d’angiographie et de laser” during the second trimester of 2006. AMD was diagnosed in all participants by 2 independent ophthalmologists. The DETER study included 520 blood donors (56% men) recruited during 2003–2005 at the blood bank of the...
were fully dilated with 1% tropicamide or 1% atropin. Coverslips positioned in triplicate samples was determined, and a chemotactic index — or ratio of cells in the lower chamber with and without CCL2 — was calculated for each sample.

Histology and electron microscopy. For histology, eyes were fixed in 0.5% glutaraldehyde, 4% PFA PBS for 2 h, dehydrated, and mounted in historesin. Sections (5 μm) were cut and stained with eosin. For electron microscopy and semithin sections, eyes were fixed in 2.5% glutaraldehyde of cacodylate buffer (0.1 M, pH 7.4). After 1 h, eyeballs were dissected, fixed for another 3 hours, postfixed in 1% osmium tetroxide in cacodylate buffer, and dehydrated in graduated ethanol solution. The samples were included in epoxy resin and oriented. Semithin sections (1 μm) were sliced with an ultramicrotome Reichert Ultracut E (Leica), stained by toluidine blue, and examined with a light microscope to measure the photoreceptor layer thickness. Multiple measurements of the photoreceptor cell layer of injured and uninjured retina were used to calculate the relative retinal thicknesses at sites 100–1,000 μm from the site of laser impact. Ultrathin sections (80 nm) were stained for contrast with uranyl acetate and lead citrate and were observed in a JEOL 100 CX II electron microscope (JEOL) with 80 kV. The absolute values of outer nuclear layer thickness of epoxy-embedded semithin and historesin-embedded sections (see below) that crossed the center of the laser impact.

Statistics. Allelic frequencies were calculated by gene counting. Hardy-Weinberg equilibrium was tested with the χ² test with 1 degree of freedom. We used the χ² test to compare genotype distributions and allele frequencies in participants. Statistical comparisons used unpaired 2-sample t tests for means and Mann-Whitney U tests for medians. All analyses and graphical representation were performed with Prism software (version 4.0c; GraphPad Software), and values are reported as mean ± SEM. A P value less than 0.05 was considered significant.
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Address correspondence to: Florian Sennlaub, INSERM U872, Centre de recherche des Cordeliers, 15 rue de l’École de Médecine, 75006 Paris, France. Phone: 33-1-40-46-78-63; Fax: 33-1-40-46-78-65; E-mail: sennlaub@ifid.inserm.fr. Or to: Christophe Combadière, INSERM U543, Faculté de Médecine Pitie-Salpêtrière, 91 Boulevard de l’Hôpital, 75013 Paris, France. Phone: 33-1-40-77-98-92; Fax: 33-1-40-77-97-34; E-mail: combad@ccr.jussieu.fr.

Christophe Combadière, Charles Feumi, and William Raoul contributed equally to this work.