Sialoadhesin Expression in Intact Degenerating Retinas and Following Transplantation

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Purpose. Resident microglial cells normally do not express sialoadhesin (Sn; a sialic acid–binding receptor), whereas recruited inflammatory macrophages have been shown to do so. The expression of Sn was examined in the course of photoreceptor cell degeneration and after transplantation.

Methods. Sn expression was analyzed in retinas of *rd1* and *rds* mice. For transplantation studies, neonatal (P2) retinal cells derived from GFP mice were injected intraocularly in adult *rd1* mice and control mice. Antibodies recognizing different Sn epitopes, CD11b, and MHC-II were used to identify activated microglial cells in intact retinas and 21 days after transplantation.

Results. In *rd1* mice, a few CD11b-positive cells were observed in the outer nuclear layer in the central retina at postnatal day (P)11 and in increasing numbers between P12 to P21. In *rds* mice, CD11b-expressing cells were found from P16 onward. No Sn-expressing cells were observed within the *rd1* or *rds* mouse retinas at any of the ages examined (up to P150). Specific staining was observed only in cells found in the vitreous margin of the retina and in surrounding tissues (sclera, cornea, ciliary body, choroid). After transplantation to normal and *rd1* mice, a variable number of Sn-positive cells were detected within the grafts, in the graft–host interface, and in the subretinal space.

Conclusions. The significant activation of microglia/macrophages observed in the various stages of degeneration in *rd1* and *rds* mouse retinas is not accompanied by Sn expression. However, Sn-expressing cells are observed after transplantation. The occurrence of such cells could be of significance for the integration and long-term survival of retinal grafts, as the expression of Sn could facilitate other phagocytic receptors.

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Retinitis pigmentosa (RP) is a genetically and phenotypically heterogeneous family of inherited blinding diseases, with a prevalence of 1:3500 to 1:4000.[1,2] It develops as a result of defects in genes responsible for the structural and/or functional integrity of photoreceptor cells (http://www.sph.uth.tmc.edu/Retnet/ RetNet is provided in the public domain by the University of Texas Houston Health Science Center, Houston, TX). The progressive loss of these cells leads to characteristic alterations, such as a reduced ability to dark adapt (night blindness), gradual constriction of the visual field (tunnel vision), accumulation of intraretinal pigment deposits, and eventually loss of central vision.[3]

Earlier studies have established that the primary rod photoreceptor cell loss, observed in most forms of RP, occurs by apoptosis,[4–6] although it appears, at least in some cases, to involve activation of effectors other than caspases.[7] A fundamental question is why cone photoreceptor cells invariably degenerate when mutations occur in rod-specific genes. A lack of rods is likely to lead not only to a lack of rod-derived structural and paracrine support,[8–10] but also to alterations in, for example, retinal oxygen metabolism, as well as to disruption of glutamate and calcium homeostasis, which also affects the rest of the retina.[7,11–13] Accordingly, numerous reports have shown that secondary pathologic changes, such as reactive gliosis, neuronal remodeling, retinal pigment epithelium (RPE) cell proliferation and migration, vascular attenuation, and neovascularization, occur in retinas of several RP animal models and in patients.[11,14–16] It is therefore important to consider that responses elicited in the other retinal cells (neuronal and nonneuronal) are very likely to affect the progression of the disease and ultimately also the outcome of potential treatments.

There is strong evidence that the immune system may play a central role in the pathogenesis of another group of photoreceptor degenerative diseases, age-related macular degeneration (AMD).[17] Several studies have implicated inflammation and immune system activation in the progression of retinal cell loss in other prevalent ocular diseases, as well, such as diabetic retinopathy and glaucoma.[18–21] Although whether inflammation and other immune responses contribute to the progression of photoreceptor cell loss in human retinitis pigmentosa and animal models is still unresolved, it is likely that they play a role in the outcome of cell-based therapies designed to treat photoreceptor degeneration. Several studies have explored the use of various cell types (e.g., neuroretinal cells, retinal pigment epithelial cells, brain and retinal precursors, and Schwann cells) in retinal transplantation approaches aimed at slowing down the progression of the degenerative process or reconstructing the degenerating retina.[22–26] Although intraocular grafts are seen to thrive for relatively long periods, it is clear that host immune responses are triggered,[27–29] ultimately limiting the survival and function of the grafts.

Sialoadhesin (Sn), also known as CD169 or Siglec-1, is the prototypic member of the Siglec (sialic acid binding Ig-like

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lectin) family of cellular interaction molecules. Sn is a cell surface adhesion receptor that binds preferentially to a particular subpopulation of sialic acids, negatively charged carbohydrate residues that are found on the cell surfaces and glycoproteins.[30–32] It is constitutively expressed at high levels by subsets of macrophages, for example, in the perifollicular zones of lymphoid tissues and in the inner marginal zone of the spleen, but can be expressed also by inflammatory macrophages, promoting their adhesion to T cells, to neutrophils, or to other activated macrophages.[33] It has been shown to be expressed by macrophages in the eye after experimental autoimmune uveoretinitis and to contribute to the inflammatory response elicited in this model.[34,35] It has also been reported to be expressed in retinal microglia/macrophages in a model of photoreceptor degeneration, the retina degeneration slow (*rds*) mouse.[36]

The present studies were conducted with the purpose of examining the distribution of Sn in the *rd1* mouse model of human RP,[37,38] a model extensively used to elucidate the mechanisms of photoreceptor cell death, and after transplantation of retinal cells in this same model. Sn expression was examined with several monoclonal antibodies recognizing the different epitopes of Sn.

Materials and Methods

Animals

The experiments were conducted with the approval of the local animal experimentation and ethics committee. Animals were handled according to the guidelines on care and use of experimental animals set forth by the Government Committee on Animal Experimentation at the University of Lund and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Retinal degeneration 1 (*rd1*), *rds*, and corresponding control wild-type (wt) mice were used for studies on the expression of retinal CD11b, Sn, and MHC. Animals were bred on a homozygous background (*rd1:* C3H/HeA or C57BL6/129; *rds*: C3H/HeA; and wt: C3H/HeA or C57BL6/129; own colonies), and maintained on a 12-hour light-dark cycle, with free access to food and water. They were killed with carbon dioxide at different ages (*rd1:* postnatal days (P)7–P150, $n = 24$; *rds:* P8–P30, $n = 16$; and wt: P11–P150, $n = 8$). Eyes were thereafter quickly enucleated and immersed in a solution of 4% paraformaldehyde (PFA) in Sørensen's buffer (pH 7.4) for 2 hours at 4°C. The tissue was subsequently rinsed, cryoprotected in the same buffer containing increasing concentrations of sucrose, embedded in an albumin-gelatin medium, frozen, and stored at −20°C. The sections were obtained on a cryostat (12 μm), collected on gelatin/chrome alum-coated glass slides, air-dried, and stored at −20°C until further processing. Some eyes from all groups were enucleated, quickly frozen without prior fixation ($n = 8$), and stored at −80°C. Retinas from wild-type control mice were also dissected from the pigment epithelium under cold Sørensen's buffer and transferred to a 3-μm pore filter (Millipore AB, Solna, Sweden) with the photoreceptor side down. The attached flattened retinas were fixed for 5 minutes with 4% PFA and rinsed thereafter with Sørensen's buffer.

Transplantation

For the transplantation studies, neonatal (P2) retinal tissue derived from transgenic mice expressing green fluorescent protein (GFP, C57BL6) was injected intraocularly in adult (P55 to P70) *rd1* mice and in wild-type control animals (C57BL6/129), as previously described.[39–40] Briefly, the eyes were enucleated from GFP mouse pups and the neural retinas carefully dissected (without RPE or the optic nerve head region). The retinal pieces were kept for up to 20 minutes in Ames' medium (Sigma-Aldrich, St. Louis, MO) at 4°C until transplanted. The recipients were anesthetized with an intraperitoneal injection of xylazine (100 mg/kg; Rompun; Bayer AG, Göteborg, Sweden) and ketamine (100 mg/kg; Ketalar; Parke, Davis & Co., Morris Plains, NJ) and locally anesthetized with 1% amethocaine hydrochloride. Donor tissue was drawn into a plastic (polyethylene) pipette tip (GELoader Tip, Eppendorf, Hamburg, Germany) connected to a precision microsyringe, and injected (1.0 μL total volume) through the sclera into the superior subretinal or epiretinal space of recipients. Twenty-one days after transplantation, the recipients were killed with carbon dioxide and the surgically altered eyes (*rd1*, $n = 7$; wt, $n = 8$) were enucleated and processed as just described. A group of animals received a subretinal injection of 1.0 μL of Ames' medium alone (sham surgery; *rd1*, $n = 3$; wt, $n = 4$). No immunosuppression was used.

Immunohistochemistry

Retinal sections were thawed and air dried before preincubation for 60 to 90 minutes at room temperature in Tris-buffered saline (TBS; pH 7.2) containing 0.25% Triton X-100 (TBS-T), 1% bovine serum albumin (BSA), 50% fetal bovine serum, and 20% normal serum (goat or rabbit). Immunohistochemistry was performed overnight at 4°C, with rat anti-mouse monoclonal antibodies (Abs) that recognize different Sn epitopes: CD169 (four different batches of clone 3D6.112; 1:75; Serotec, Oxford, UK) and SER-4 (1:20; provided by author PRC).[41] MOMA-1 (rat anti-mouse metallophilic macrophage antibody), which has been recently shown to recognize Sn[42] was also used (1:75; BMA Biomedical, Augst, Switzerland). To identify microglia/macrophages, CD11b (monoclonal rat anti-mouse; 1:75; R&D Systems, Abingdon, UK) was used. In addition, a rat anti-mouse I-A/I-E monoclonal antibody (clone M5/114.15.2; Alexa Fluor 647–conjugated; 1:100; BioLegend, San Diego, CA) was used to detect major histocompatibility complex (MHC)-II–expressing cells. All primary antibodies were diluted in TBS-T containing 5% goat or donkey normal serum. The tissue was subsequently rinsed and incubated for 90 minutes with one of the following secondary antibodies: Alexa 594 goat anti-rat, Alexa 488 goat anti-rat (1:200; Invitrogen-Molecular Probes, Leiden, The Netherlands), Texas-red donkey anti-rat (1:200; Jackson ImmunoResearch Laboratories, West Grove, PA), or biotinylated rabbit anti-rat IgG (H+L, 1:200; Vector Laboratories, Burlingame, CA), followed by anti-biotin streptoavidin-cy3 (1:400; Jackson ImmunoResearch Laboratories). The sections were rinsed and mounted (Vectashield; Vector Laboratories). Flat-mounted retinas were processed in a similar manner, except that incubation with the primary antibody was performed for 48 hours at 4°C.

In addition to testing different antibodies recognizing Sn, a series of further control experiments were performed by including the following: (1) sections obtained from the spleen of adult normal mice (C3H and C56BL6/129; $n = 2$). Pieces of the spleens were either fixed, cryoprotected, and sectioned as described earlier ($n = 2$) or frozen without prior fixation ($n = 2$); (2) eyes and spleens from Sn-deficient mice[42] (C56BL6; $n = 2$); (3) sections from fresh-frozen retinas ($n = 9$). The sections were thawed, air-dried, fixed in cold acetone for 10 minutes, rinsed and further processed for immunohistochemistry. Immunodetection was also performed with the avidin-biotin complex (ABC) method. Retinal sections were incubated with the primary antibodies as described earlier, followed by a biotinylated rabbit anti-rat secondary antibody (1:75; Vector Laboratories) for 45 minutes. Staining was performed using ABC and diaminobenzidine (DAB) substrate kits (Vectastain ABC Elite; Vector Laboratories), according to the manufacturer's protocol ($n = 3$). Sections were washed, dehydrated, and mounted. An additional control was also performed by omitting the primary antibody and incubating ocular and spleen sections with secondary antibodies alone. Epifluorescence and confocal microscopes were used to examine the sections (Carl Zeiss Meditec, Inc., Oberkochen, Germany). Images were captured with digital cameras and software (Axiovision 4.2 and LSM5 Pascal, respectively; Carl Zeiss Meditec). Image-analysis software (Photoshop; Adobe, San Jose, CA) was used for contrast and brightness adjustment of images.

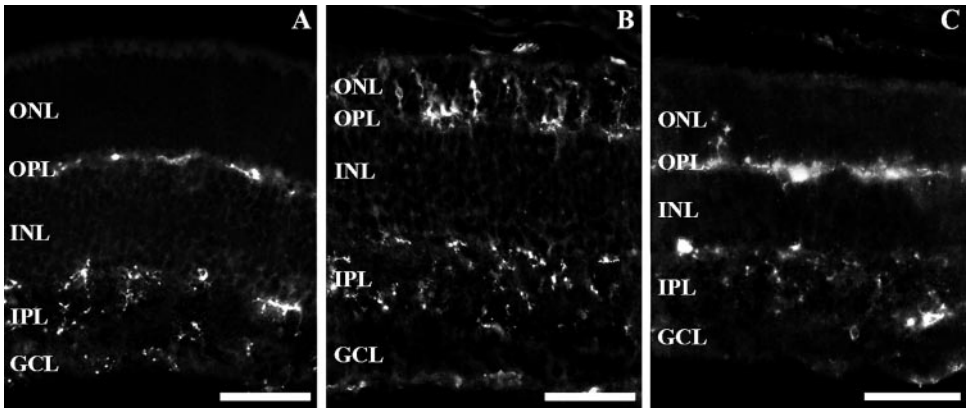

FIGURE 1. CD11b expression. (**A**) Wild-type control mouse retina at P13: labeled cells were seen in the inner retina over the IPL and at the level of the OPL. (**B**) *Rd1* mouse retina at P14: numerous labeled cells were also seen in the ONL and in the subretinal space. (**C**) *Rds* mouse retina at P21: CD11b-positive cells were seen in the inner retina, in the OPL, and in small numbers in the ONL. Scale bars, 50 μm.

RESULTS

Microglial Cell Activation in Intact Retinas

In wild-type control mice, CD11b-positive cells were found mostly in the inner retina, over the inner plexiform (IPL) and ganglion cell (GCL) layers, and at the level of the outer plexiform layer (OPL). The labeled cells exhibited a typical ramified morphology and were observed in both the central and the peripheral retina (Fig. 1A). The morphology and localization of these CD11b-expressing cells indicate that they correspond to microglial cells.

In the *rd1* mouse retina, a large number of labeled cells were also noted in the outer nuclear layer (ONL; Fig. 1B). The first cells were observed in this layer at postnatal day (P11) in the central areas of the retina, but were seen in the whole retina as early as P17 to P18. From P21 onward, when the ONL consists of only about one cell row, it was no longer possible to determine the exact localization of the labeled cells in the outer retina. Most labeled cells were then observed in the OPL and occasionally also in the subretinal space. The same distribution of CD11b-positive cells was seen in all *rd1* mouse retinas, irrespective of the strain examined (C3H/HeA or C57Bl6/129). In the *rds* mouse retina, a few CD11b-positive cells were also observed over the ONL both in the central and peripheral retina from P16 onward (Fig. 1C).

Sn in Intact Retinas

In this study, it was essential to verify the specificity of the Sn-recognizing antibodies, and this was accomplished with several assays. Staining of spleen sections obtained from normal animals revealed the presence of immunoreactive macrophages in the inner marginal zone with all batches of anti-CD169 antibodies examined (Fig. 2A), whereas no specific staining was observed with any of the batches in Sn-deficient mice (Fig. 2B). The same was observed after staining with MOMA-1 (Fig. 2C) and SER-4 (Fig. 2D). No labeling was noted in the ocular tissues of Sn-deficient animals with two of the batches of anti-CD169 tested (hereafter referred to as CD169-specific; Fig. 2E), whereas staining with the two other batches resulted in labeling of some cells in the peripheral retina and choroid (Fig. 2F) and within the degenerating retina (see below). These batches of anti-CD169 are hereafter referred to as nonspecific. The observations were consistent whether using unfixed or fixed spleen and ocular tissues and irrespective of the detection method used.

With the specific CD-169 antibodies, no labeled cells were observed in the retina of wild-type control mice at any of the examined ages (Figs. 3A, 3B). Labeled cells were, however, observed in the ciliary body (Figs. 3C, 3D), in the sclera (Fig. 3E), and between the lens and the retina. Many of the latter appeared to adhere to the vitreous–retinal surface (Fig. 3A). In Sn-deficient mice, no labeled cells were noted in these ocular tissues when the specific CD169 antibodies were used (Fig.

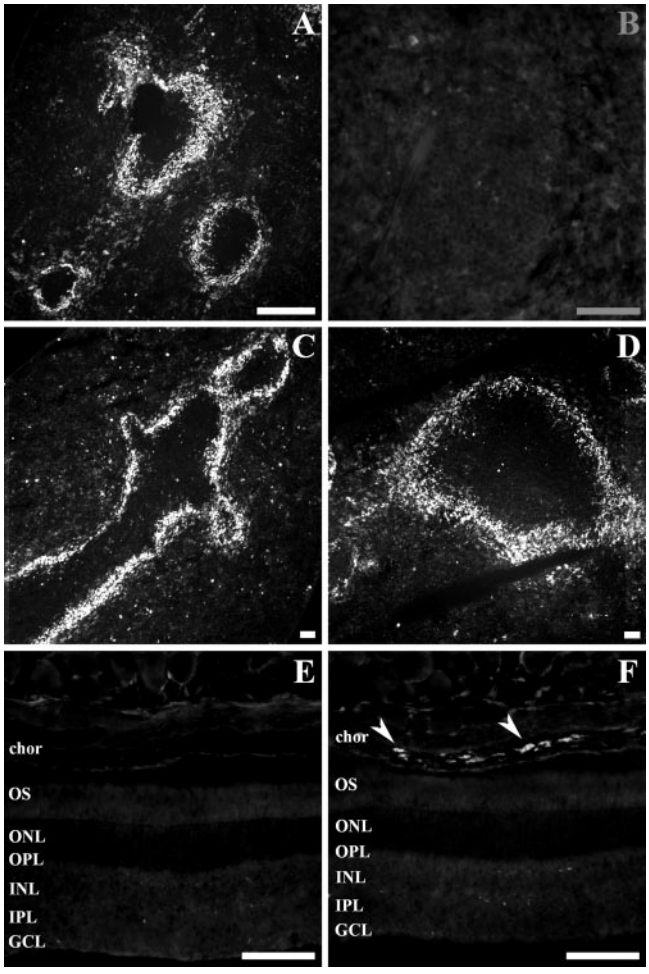

FIGURE 2. Sn expression in the spleen and retina with different antibodies. (**A**) Wild-type mouse spleen: characteristic localization of CD169-labeled macrophages in the marginal zone with all antibodies tested. (**B**) No specific signal was observed in the spleen of Sn-deficient mice. (**C**, **D**) The same distribution of labeled macrophages was seen in the spleen of wild-type mice with MOMA-1 (**C**) and SER-4 (**D**). (**E**) No labeling was observed in ocular tissues in Sn-deficient mice with CD-169 specific antibodies. (**F**) CD-169-positive cells (*arrowheads*) were seen in the choroid in Sn-deficient mice with the nonspecific antibodies. chor, choroid; OS, outer segments. Scale bars, 50 μm.

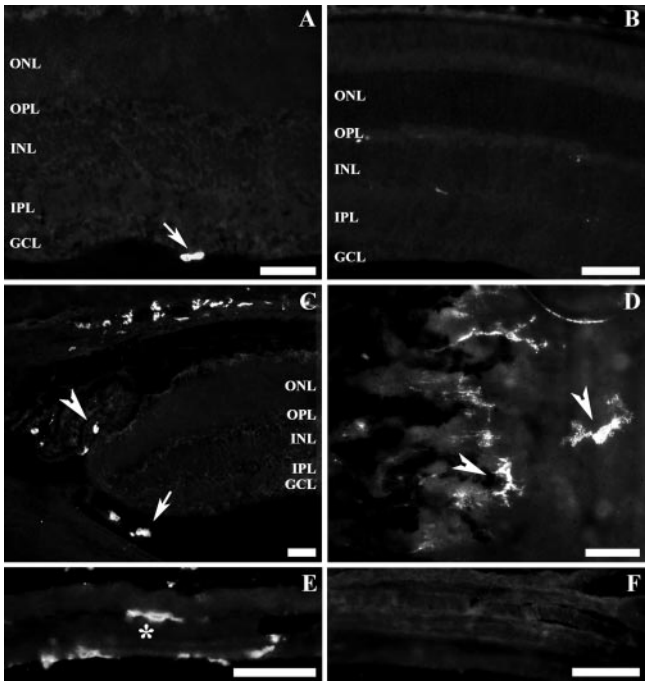

FIGURE 3. Sn expression in the eye detected by specific CD169 antibodies. (**A**, **B**) Wild-type mouse retina at P13 (**A**) and P150 (**B**): staining was not observed within the retina, with occasional labeled cells seen only along the vitreous margin of the retina (*arrow*). (**C–E**) CD169-positive cells are observed in wild-type animals also in the ciliary body and retinal margin (*arrowheads*), in the intravitreous space (*arrow*), and in the sclera (✻). No specific labeling was observed in the sclera of Sn-deficient animals (**F**). The image in (**D**) was taken from a flat-mounted retina. Scale bars, 50 μm.

3F). These antibodies and MOMA-1 and SER-4 antibodies also produced no signal in *rd1* or *rds* mouse degenerating retinas at any of the ages examined (Figs. 4B–J) and detected only occasional cells in the other ocular tissues (Figs. 4D, 4G, 4J).

With the nonspecific CD169 antibody batches, however, several stained cells were noted over the ONL and in the subretinal space in *rd1* mice (Fig. 4A) and in *rds* mice (not shown).

Sn after Transplantation

After subretinal transplantation in wild-type control mice, GFP-positive cells were noted within the ONL, where many of them assumed the position and morphology of photoreceptor cells (Figs. 5A, 6B). Little or no migration of graft cells was noted in transplantation to *rd1* mice (Figs. 5C, 5E, 5G, 5I, 5K).

Transplantation resulted in a variable number of Sn expressing cells within the grafts and at the graft-host interface, both in transplantation into wild-type (Fig. 5B) and *rd1* mice (Figs. 5D, 5F). In addition, labeled cells were found in the subretinal space in the vicinity of the grafts, even after intravitreous grafting (Figs. 5F, 5H). The same observations were made with the specific CD169 antibodies, MOMA-1, and Ser-4. The extent of the detachment produced in the host retina by the surgery and the size of the grafts varied considerably between the different specimens, precluding a reliable quantitative analysis. However, there was no indication that the number of Sn-expressing cells was higher in transplantation into *rd1* mice than in transplantation into wild-type mice.

MHC-II Expression

No MHC-II-labeled cells were detected within the retina of unoperated, normal or *rd1* mice. However, a highly variable number of MHC-II-expressing cells were observed in both

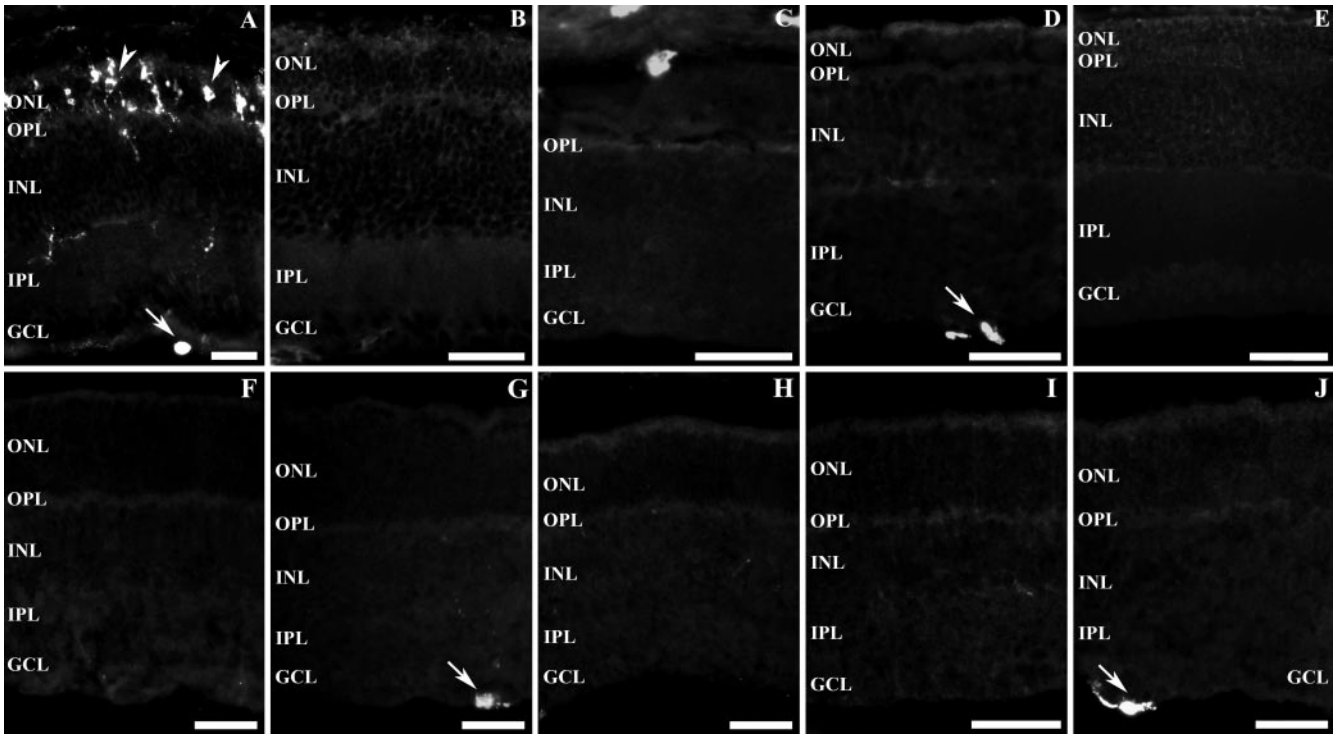

FIGURE 4. Sn expression in the retina of *rd1* (**A–E**) and *rds* (**F–J**) mouse retinas. (**A**) A large number of stained cells (*arrowheads*) over the ONL in *rd1* mouse retina at P14 detected by a nonspecific CD169 antibody. No staining was observed within the retina with specific antibodies at P14 (**B**; CD169), P150 (**C**, CD169), P15 (**D**, MOMA-1), or P15 (**E**, Ser-4). The same was observed in *rds* mouse retinas with specific CD169 antibodies: P8 (**F**), P16 (**G**), P18 (**H**), P21 (**I**), and P30 (**J**). A few cells found next to the vitreous margin of the retina were labeled with all antibodies (**A**, **D**, **G**, **J**, *arrows*). Scale bars, 50 μm.

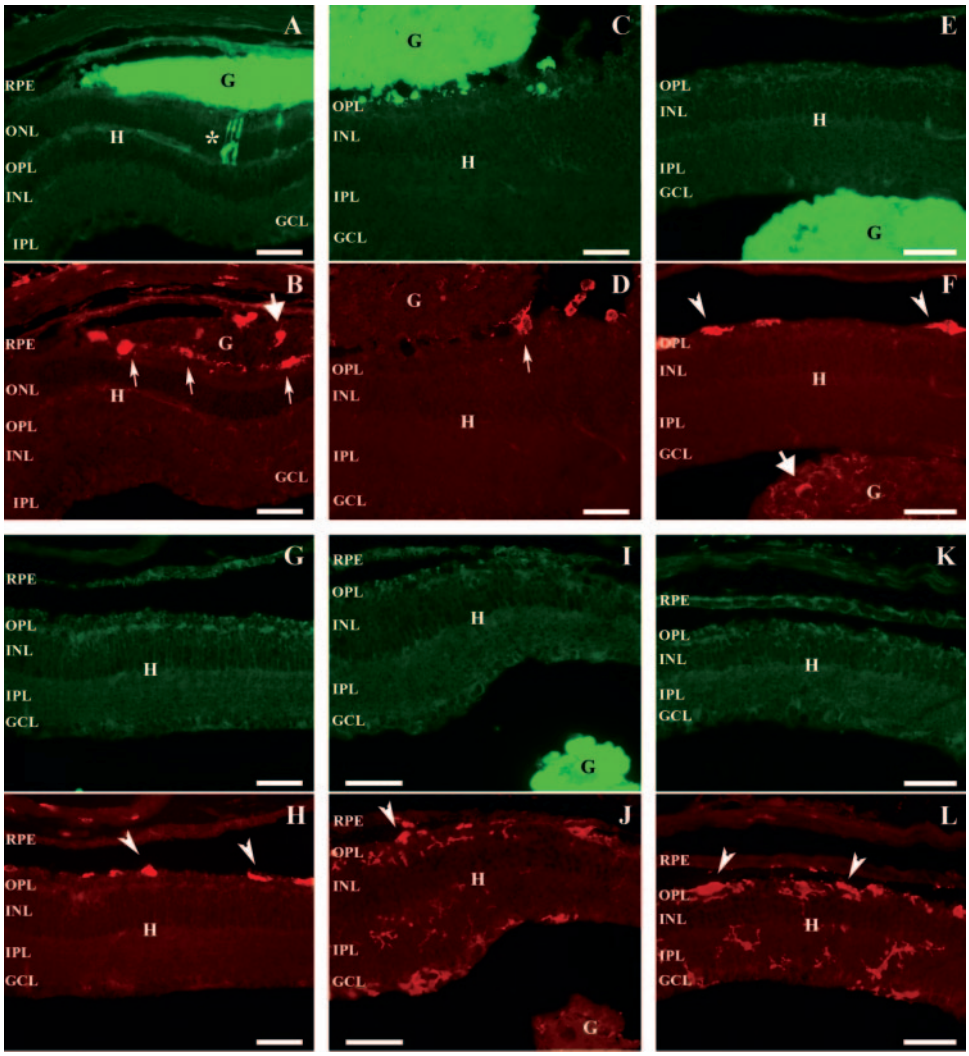

FIGURE 5. Transplantation to normal (**A**, **B**) and *rd1* mice (**C**–**L**). Subretinal (**A**–**D**) and epiretinal (**E**, **F**, **I**, **J**) grafts (G) are shown: (**A**) GFP-expressing cells were observed within the ONL of the host (H) wild-type mouse retina (✻). (**B, D, F, H**) Sn-expressing cells were seen in the graft–host interface (**B**, **D**, *arrows*) and within the grafts (**B**, **F**, *short arrows*). Labeled cells (*arrowheads*) were also found in the subretinal space near (**F**) and away (**H**) from the grafts. (**J**, **L**). The localization of CD11b-positive cells within the host retina and in the subretinal space (*arrowheads*) near an epiretinal graft (**I**, **J**) and away from a subretinal graft (**K**, **L**). Scale bars, 50 μm.

groups after transplantation. Positive cells were mostly found within the grafts and in the graft–host interface (Figs. 6A, 6C), but were occasionally observed also in the subretinal space. In three cases (of nine), a large number of labeled dendriform cells were seen near the host ciliary body (Figs. 6D, 6F). These cells were observed after transplantation in both *rd1* ($n = 2$) and wild-type ($n = 1$) mice. As observed with Sn, most MHC-II-positive cells did not coexpress GFP (Figs. 6C, 6F), suggesting that some of them should be of host origin. No Sn or MHC-II expression was noted within the retina or subretinal space in sham-surgery animals (wild type and *rd1* mice; not shown).

DISCUSSION

The present study confirms previous reports showing microglial cell activation in different models of photoreceptor degeneration.[43–49] CD11b-positive cells were visible in the ONL in *rd1* and *rds* mouse retinas in the early stages of degeneration, which corresponds to observations made using this and other markers.[36,47–50]

Sn and Degenerating Retinas

Sn is expressed by activated microglia and macrophages within the parenchyma in brain tissue only after exposure to plasma proteins, which occurs as a consequence of damage to the blood–brain barrier.[51] In experimental autoimmune uveoretinitis (EAU), a marked increase in the number of Sn-expressing cells has been observed in the retina and in other inflammatory areas in the eye.[34,35] It was found in this model that Sn-expressing cells constitute a subset of activated macrophages that do not coexpress CD11b or MHC class II, and it was suggested that they are not involved in antigen presentation, but correspond rather to phagocytic macrophages.

A slow, but constant migration of blood-borne monocytes into the retina has been shown to occur even under physiological conditions.[52] In addition, changes associated with the vasculature, such as vascular attenuation and neovascularization, and blood–retina barrier breakdown are observed in the course of photoreceptor degeneration in several animal models and in patients.[14,15,53–55] Such changes are generally considered to be relatively late events, although we have also detected an alteration of the matrix composition surrounding the intraretinal vessels in another model of photoreceptor degeneration, the Royal College of Surgeons (RCS) rat, at a very early stage of degeneration.[56] In the *rd1* mouse retina, increased levels of monocyte chemoattractant protein (MCP)-1, MCP-3, macrophage inflammatory protein (MIP)-1α, MIP-1β, regulated on activation normal *t*-cell expressed and **s**ecreted (RANTES), and TNF-α have been detected as photoreceptor cell loss progresses.[48,57,58] The expression of aquaporin-4, essential for the maturation and maintenance of the blood–brain barrier,[59]

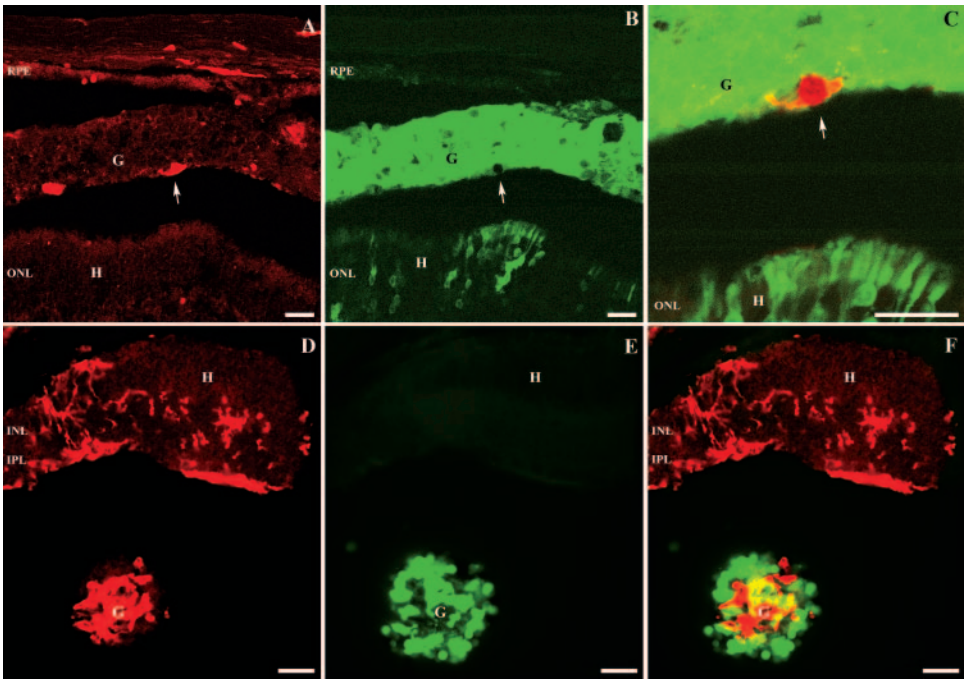

FIGURE 6. Transplantation in normal (**A–C**) and *rd1* mice (**D–F**). Subretinal (**A–C**) and epiretinal (**D–F**) grafts (G) are shown: (**A**) MHC-II-expressing cells were seen in the graft–host interface (*arrow*) and within the graft. (**C**) Merged image, showing in higher magnification the GFP-expressing cells seen in (**B**), within the graft and within the ONL of the host retina (H), and a cell expressing MHC-II (*red, arrow*). A large number of MHC-expressing cells were seen in the host *rd1* mouse retina (**D**), exhibiting a dendritic shape. Numerous MHC-II-positive cells (also GFP-negative) were observed within the epiretinal graft (**D–F**). Scale bars, 20 μm.

is also elevated in older *rd1* mouse retinas.[58] It is therefore reasonable to assume that activated blood-borne macrophages may be recruited in the process of photoreceptor cell loss, at least in some types of degeneration.

Yet, our results showed that no Sn expression could be observed in the *rd1* mouse retina in areas of microglial activation at any stage of the degeneration (0–7 weeks old) or at 5 months of age, when significant vascular alterations are normally observed.[15] It was also not possible to confirm the observations made previously in *rds* mouse retinas.[36]

The observations made in the present study were obtained with antibodies recognizing different Sn epitopes: clone 3D6.112 (four batches available through Serotec), SER-4, and MOMA-1. Two batches of 3D6.112 produced distinct labeling of microglia/macrophages over the ONL in *rd1* mice and of occasional cells in the subretinal space in *rds* mouse retinas. On the other hand, none of the remaining four batches of monoclonal antibodies was seen to produce a specific signal in the retina of any of these animals. The *rds* mice examined in the previous study[36] were on a different genetic background (CBA). However, the possibility that strain differences or method of tissue fixation account for the lack of signal in retinal macrophages in *rd1* and *rds* mouse retinas in the present study appears unlikely. Specific signal was obtained in the present study with the four remaining antibody preparations in spleens of all mice tested irrespective of the strain or method of fixation and was absent in spleens and eyes of Sn-deficient mice.

Using the specific antibodies, signal was detected in the present study in subpopulations of macrophages located in other ocular tissues, as previously found in different mouse strains.[34,60,61] Sn-positive cells exhibiting an elongated or spherical shape were also observed near the optic nerve head and scattered in the vitreous cavity, sometimes next to the inner surface of the retina. In addition, labeling was seen in some large cells with irregular shape in the retinal margin, next to and within the ciliary body. Many, if not all of these cells found in the vitreous and in association with the ciliary body are likely to correspond to hyalocytes, which also belong to the monocyte/macrophage lineage.[62,63]

Retinal Transplantation

The transplantation model used in the present study is well established and has been used in studies on graft–host integration.[39,64] Limited graft–host integration was observed, with only a few cells and fibers bridging the graft–host interface, also confirming previous observations.[39,40] After subretinal transplantation to normal animals, most of the cells that migrated into the host retina assumed the morphology and position of photoreceptor cells. This observation agrees with what has been recently shown in transplantation of dissociated retinal cells obtained from GFP donor mice of the same age as used in the present study.[24] A certain degree of graft–host integration was also observed after subretinal transplantation in *rd1* mice and in epiretinal transplantation in both wild-type and *rd1* mice. In *rd1* mice, most host photoreceptors had degenerated at the time of transplantation, reducing the ONL to a thin layer of sporadic cones. Cells with typical photoreceptor morphology were no longer identifiable, and integrated cells were occasionally seen in the inner host layers (not shown).

Sn and Retinal Transplantation

A highly variable number of Sn-positive cells were observed after intraocular grafting of retinal cells in both wild-type and *rd1* mice. Overall, only a relatively small number of labeled cells were seen after transplantation, and these were most often seen in the graft–host interface and in the subretinal space in the vicinity of the grafts, indicating that Sn induction was a localized response. In transplantation to the *rd1* mice, which was performed on adult animals, one would have expected elevated levels of at least some immune-related genes to be present at the time of transplantation, due to the ongoing degeneration. Induction of Sn was not observed during the process of photoreceptor degeneration as such (the present study), which may explain why we could not detect any obvious differences between transplantation to normal and *rd1* mice.

The subretinal and epiretinal spaces have been considered to be immune privileged sites, although this appears not to be

absolute.[65,66] An intrinsic problem associated with sub- and epiretinal transplantation in rodents stems from the fact that grafts need to be delivered through the sclera due to the size and anatomy of the rodent eye. It has been argued that this transscleral approach may disrupt the cellular and molecular bases of immune privilege.[66,67] Furthermore, subretinal transplantation induces a localized, permanent detachment of the host retina, a condition that has been shown to lead to microglial activation and to increased levels of several cytokines and chemokines.[19,68] We observed an increase in the number of Sn-positive cells after transplantation of tissue, but not after sham operations. It is not possible to determine in transplantation to the mouse eye, whether Sn expression would have been triggered without the damage to the choroid and associated structures. It is clear, however, that the surgical trauma alone is not sufficient to elicit expression of Sn, as it was observed only in specimens containing a graft. The latter (perhaps in conjunction with the trauma) is in some way capable of triggering or exacerbating a localized immune response.

Allogeneic neuroretinal transplants can survive for several months without immunosuppression.[69,70] However, upregulation of MHC class II expression on donor microglial cells[27,28] and induction of potent T-cell responses to donor antigens[29] have been observed. We observed MHC-II expression on transplantation, but neither after sham surgery nor within the intact retinas of wild-type and *rd1* mice. It was not possible to establish the origin of all the MHC-II-expressing cells found within the grafts (subretinal or epiretinal), but those found in the subretinal space did not coexpress GFP, indicating that they are most likely derived from the host. The same was observed for Sn-expressing cells.

The donor neuroretina, even if derived from neonatal stages, is considered to be an only partially immune privileged tissue.[27,71] A small population of dendritic cells constitutively expressing MHC class II has been observed in normal, intact mice in the retinal margin and in the juxtapapillary region.[72] Strain differences were noted, but these cells were clearly identifiable in C57BL/6 mice. Such cells were not observed until 2 weeks of age,[72] and should thus not have been included in our donor tissue (obtained from 2-day-old C57BL/6 mice), unless the cells are present at P2 but only mature enough to express MHC II at approximately 2 weeks. Microglial cells are also observed in the normal mouse retina as early as embryonic day (E)11.5, and although their density is reduced after E18.5, a significant number of cells are found also in the neonatal retina.[73] Further, although attempts were made in the present study to dissect the donor neuroretina from the RPE, grafts may contain a small but variable number of these potential antigen-presenting cells. We can therefore not exclude the possibility that a fraction of MHC-II (and perhaps also some Sn)-positive cells were derived from the donor.

It should be noted also that in some specimens, in which the grafts were placed near the ciliary body, a very large number of MHC-II-positive cells of host origin were seen in the peripheral retina and within the graft itself. The proximity to the peripheral retina, where subpopulations of dendritic cells have been shown to express MHC-II even in normal retinas,[72] may have facilitated the infiltration of these cells into the host and the graft. These observations point to the fact that the level of expression of MHC-II, and possibly also that of Sn, is determined not only by the presence of a graft, but also by the position of the graft.

It was not determined in the present study whether and to what extent Sn and MHC-II were expressed in the same cells after transplantation. However, there seemed to be no direct correlation between the number of Sn- and MHC-II-expressing cells after retinal transplantation. Moreover, in a model of EAU, it was shown that MHC class II/Cd11b and Sn are in fact expressed in different subsets of activated macrophages.[34]

In conclusion, the present study showed that Sn is expressed by host-activated microglia/macrophages after retinal transplantation in the mouse. Cotransplantation of neonatal retinal cells with host-strain–derived immature dendritic cells, which can induce immune tolerance, was recently shown to lead to a lower number of activated $CD8^+$ T cells and to a better graft survival rate.[74] Thus, although allogeneic grafts are seen to thrive, expression of Sn (and of MHC-II) by donor- and host-derived macrophages potentially facilitate other phagocytic receptors, making the grafts susceptible to long-term immune responses. Whether transplantation-induced Sn expression occurs in other species and what implications it might have must be verified.

The study also shows that Sn is not expressed in *rd1* (or *rds*) mouse retinas and that its expression is also not significantly altered in the other ocular tissues in these models. It appears that cross-reactivity with an unknown protein may have produced the positive labeling observed with two of the batches of 3D6.112 tested in this study, which may also explain the results previously obtained in the *rds* mouse retina.[36] It has been shown in genetic studies that lack of functional B and T cells or of complement factor C1qα has no impact on the progression of photoreceptor cell loss in *rd1* mice, suggesting that the classic complement system of innate immunity and acquired immune responses may not be implicated.[75] Yet, these observations and the fact that Sn is not expressed in retinal macrophages in the two models studied should not be taken as definitive indication that immune-related events do not contribute to photoreceptor cell loss.

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