Conditions of Retinal Glial and Inflammatory Cell Activation after Irradiation in a GFP-Chimeric Mouse Model

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PURPOSE. Microglia cells have been associated with immunologic defense and repair. The course of retinal disease after lethal irradiation for bone marrow depletion and substitution was evaluated with respect to macrophage and microglial involvement.

METHODS. Lethal irradiation in C57BL/6 mice was conducted with a low-voltage radiation unit. The animals were randomized to shielded or unshielded radiation and subsequently received transplants of GFP+ bone marrow cells (β-actin promoter). The GFP transformation rate was analyzed by flow cytometry. GFP+ cells in the retina were examined for localization with macrophage and dendritic cell markers at various time points between 1 and 7 months after irradiation. Clodronate liposomes were used to investigate the fate of migrated and resident microglia cells. Pathologic angiogenesis was investigated in laser-induced choroidal neovascularization (CNV) after unshielded and shielded irradiation.

RESULTS. Flow cytometry revealed average transformation rates of 78.2% in unshielded and 64.1% in shielded group. Four weeks after transplantation, perfused flat mounts were virtually free of extravasal GFP+ cells in both groups, whereas 4 months after irradiation, cluster cell infiltrations, preferentially in the peripheral retina, became apparent exclusively in the unshielded group. Cell morphology ranged from oval, to a few extensions, to dendritiform with long-branched extensions. Clodronate treatment resulted in a reduction of GFP+ cells in the retinal tissue when applied 3 months after unshielded irradiation. Although GFP+ cells accumulated in the choroidal scar after laser treatment, in both the shielded and unshielded groups, GFP+ cells in the overlying retina were restricted to the unshielded group.

CONCLUSIONS. Approximately 3 months after lethal full-body irradiation including the eye, bone marrow–derived leukocytes exhibit a wound-healing reaction, and unlike physiologic turnover, infiltrate the retina and form microglial cells. (Invest Ophthalmol Vis Sci. 2010;51:4831–4839) DOI: 10.1167/iovs.09-4923

The retina contains two different populations of monocyte-derived cells: perivascular and parenchymal. Perivascular cells are usually macrophages; the parenchymal cells have been identified as microglia.1,2 Microglial cells have been associated with immunologic defense and repair,3,4 and may also contribute to the onset of neurodegeneration and inflammation by producing various cytokines.5,6 In contrast, they may produce neuroprotective molecules that are important in neuronal survival and retinal vascular repair.7,8

There is increasing evidence that similar mechanisms involving inflammatory cells and microglia contribute to the slow-degenerative diseases of the retina, including diabetic retinopathy.9–13 Still, research on the role of retinal glia cells in these entities has been difficult.

Generation of chimeric GFP+ animals14 has become a powerful tool in the research field of stem cell participation and homing in various models of physiologic development and in diverse models of disease. Mostly after full-body irradiation, recipients undergo transplantation of either whole bone marrow or subpopulations of specific bone marrow–derived progenitor cells. The model is suitable for in vivo noninvasive tracing of cell populations15,16 and for facilitated microscopic detection of GFP+ cells.17 Impressive advances in the research into bone marrow recruitment in various ocular diseases have been made with this model.18–20

However, a follow-up on the putative effects of high-dose irradiation in the GFP-chimeric model has rarely been documented.19 Moreover, most studies published to date on the examination of specific vascular injury or vascular development models did not require long-term chimerism for more than 3 months.18,21–23 Using an extended time span in this model, Xu et al.24 suggested a complete turnover of resident retinal microglial cells within 6 months. After total-body irradiation, the researchers observed an increasing number of GFP+ cells in the retinal tissue and attributed their presence to the normal turnover of microglial cells from bone marrow monocyct precursors. In their study, the effect of irradiation on the retinal tissue was not taken into consideration.

It is well known and we have demonstrated in other studies that in diabetic animals, an increased number of leukocytes can
be found in the retinal vasculature\textsuperscript{9-11, 25, 26} and in the neuronal tissue,\textsuperscript{27} compared with the number in normoglycemic control subjects. Similar mechanisms are likely to be in play after irradiation. The irradiation of the neuroretinal tissue and the retinal vasculature could trigger a similar and even more pronounced inflammatory reaction.

In this study, total-body irradiation that includes the eye and retina triggered enhanced recruitment of blood monocytes precursor cells to the retina. In contrast, sparing of the retinal tissue from direct irradiation does not result in a similar invasion of microglia. This finding suggests that retinal microglial proliferation from bone marrow-derived cells and their attraction to the retina is low in steady state conditions. Along these lines, macrophage depletion by clodronic acid can effectively inhibit the recruitment of perivascular and microglial cells after irradiation of the retina. Of interest, recruitment of microglia cells to the retinal tissue can also be initiated by induction of inflammatory angiogenesis, such as laser-induced choroidal neovascularization (CNV). Thus, normal turnover of resident retinal microglia cells in the retina is low but can be accelerated by activation of glia cells via irradiation of the eye.

**MATERIALS AND METHODS**

**Animals**

Female C57BL/6 mice 8 weeks of age, weighing 20 to 25 g, were purchased from Jackson Laboratories (Bar Harbor, ME). Bone marrow was obtained from C57BL/6 donor mice (C57BL/6-Tg(ACTB-EYFP)1Osbi/J), which are transgenic for the chicken β-actin promoter GFP and the cytomegalovirus enhancer (kindly provided by Bernd K. Fleischmann, Institute of Physiology, University of Bonn, Germany). In this transgenic mouse line with enhanced GFP (EGFP) cDNA under the control of the chicken β-actin promoter, all tissues, except for erythrocytes and hair, appear green under excitation.

All animal experiments complied with the guidelines of the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the Animal Care and Use Committee of the Medical Faculty of the University of Cologne (Regierungspraesidium Köln), Germany. All surgical procedures were performed with the animals under general anesthesia with 10 mg/kg xylazine hydrochloride (Riemser Arzneimittel AG, Riems, Germany) and 50 mg/kg ketamine hydrochloride (Ketanest; Parke-Davis, Berlin, Germany). Irradiation of the retina. Of interest, recruitment of microglia lines, macrophage depletion by clodronic acid can effectively inhibit the recruitment of perivascular and microglial cells after irradiation of the retina.

**Irradiation Procedure**

C57BL/6 mice were randomized to an unshielded and a shielded group. They were irradiated while under general anesthesia in an acrylic, custom-made case with venting holes. After induction of anesthesia, they were lined up so that the neck of each animal was positioned on the same line. The cage was closed with a slide-on lid. Irradiation was conducted with a low-voltage radiation unit (U = 120 kV, I = 25 mA). The irradiation source was adjusted to achieve total-body irradiation. For shielded irradiation, a 3-mm lead plate was used to protect the animals' heads. A radiation probe was positioned between the animals' heads to register the remaining radiation under the lead shield. Irradiation was fractionated to 5.5 Gy twice (total dose, 11 Gy), with a time interval of 8 hours for full-body treatment, and fractionated to 8 Gy (total dose, 16 Gy) twice for head-shielded treatment. Irradiated mice in which venipuncture for transplantation was unsuccessful served as the control for the lethal irradiation dose.

**Bone Marrow Transplantation**

Transplantation was performed 24 hours after irradiation. Reconstitution of lethally irradiated C57BL/6 mice with HSCs from GFP\textsuperscript{+} donors was performed according to methods described by Grant et al.\textsuperscript{18} Sengupta et al.,\textsuperscript{20} and Espinosa-Heidmann et al.\textsuperscript{19}

The donor mice were killed by cervicopatinal dislocation while under CO\textsubscript{2} anesthesia and were cleaned in 70% ethanol for disinfection. The hind legs were dissected and submerged in DMEM/Ham's F12 culture medium with L-glutamine (PAA Laboratories, Pasching, Germany) at 4°C. Bone marrow was extracted by preparing and flushing the bones with DMEM and 10% FCS (PAA Laboratories). The cells were washed twice with PBS and centrifuged at 500g for 5 minutes. Freshly made red blood cell (RBC) lysis buffer (10 mM KHCO\textsubscript{3}, 150 mM NaCl, and 0.1 mM NaEDTA in 1 L distilled H\textsubscript{2}O, adjusted to pH 7.5) was applied for 2 minutes, and the reaction was stopped with PBS and 10% FCS. After centrifugation, the cells were resuspended in PBS and filtered through a 0.22-µm mesh (BD Biosciences, Erembodegem, Belgium). A cell count was performed with a Neubauer chamber after the cells were stained with trypan blue solution 0.4% (Sigma-Aldrich, Steinheim, Germany) to exclude the dead cells. The concentration of the cell suspension was adjusted to 1 × 10\textsuperscript{6} cells/100 µL. All procedures were performed on ice, to avoid cell activation. A GFP\textsuperscript{+} cell suspension of 150 µL equaling 1.5 × 10\textsuperscript{6} cells, was injected into the tail vein. Animals with unsuccessful injections served as the control for lethal dose evaluation.

**Assessment of the Bone Marrow Conversion Rate by Flow Cytometry**

The survival rate of the mice receiving exogenous bone marrow transplants was 100%. Blood components were allowed to reconstitute for 1 month. Peripheral blood samples from the tail vein were used for flow cytometry. Blood taken from the tail vein was diluted in 200 µL PBS + 5 µL heparin-Na (25,000 U; Roche Diagnostics GmbH, Mannheim, Germany). After centrifugation at 500g for 5 minutes, the cells were resuspended in 500 µL RBC buffer for 2 minutes. The reaction was stopped with an equal amount of PBS with 10% FCS. After centrifugation, the cells were resuspended in PBS for flow cytometry. Dead cells were excluded from the analysis by gating of the live cells in the forward–sideward scatterplot. The number of GFP\textsuperscript{+} cells in the peripheral blood is depicted in Figure 2. By contrast, irradiated mice without exogenous bone marrow transplantation died between 7 and 14 days after irradiation.

**Determination of Retinal Apoptosis by TUNEL Assay**

The amount of cellular apoptosis was examined by TUNEL assay (In Situ Cell Death Detection Kit; Roche Diagnostics GmbH), according to the manufacturer’s instructions, and the results were analyzed by fluorescence microscopy. The specificity of the TUNEL assay was tested by staining the sections with labeling solution without terminal transferase (negative control) and, as expected, no apoptotic nuclei were observed. In addition, a positive control was prepared by treatment of the sections with DNase (Sigma-Aldrich). TUNEL\textsuperscript{+} cells were observed in all retinal nuclear layers as well as in the choroid and sclera. All sections were counterstained with 4',6-diamidino-2-phenylindole (DAPI) after the TUNEL-reaction, to verify that the TUNEL staining was localized in the nucleus. Two images of each retinal part (central, middle, and peripheral) were captured from each retinal section. The total number of TUNEL\textsuperscript{+} nuclei in the ganglion cell (GCL) and inner nuclear (INL) layers were counted in each retina (n = 4 per group head-shielded and open-irradiated).

**Clodronic Acid Depletion of Circulating Macrophages**

Clodronate (dichloromethylene diphosphonate; CL2MDP) liposomes were received from Nico van Rooijen, Department of Molecular Cell Biology (University of Amsterdam). Briefly, 86 mg phosphatidylcholine (Lipoid EPC, Lipoid KG, Ludwigshafen, Germany) and 8 mg cholesterol (Sigma-Aldrich) were combined with 10 mL of a clodronate (0.7 M)
solution and sonicated gently. Subsequently, the created liposomes were washed to eliminate free drug. Empty liposomes were prepared in the same way by using PBS instead of the clodronate solution. Mice received two intraperitoneal injections of 200 μL CL2MDP-LIP (n = 3 mice) or PBS-LIP (n = 3 mice) at 12 and 14 weeks after transplantation. Blood counts were performed at the laboratory of animal clinical diagnostics (Laboklin, Bad Kissingen, Germany).

### Induction of Experimental CNV

Eyes of C57BL/6 /C57BL/6-Tg(ACCT-EGFP)1Osb/J chimaeras after shielded and unshielded irradiation were exposed to laser photococulation for induction of experimental CNV, as described previously. Briefly, laser photococulation was performed with a diode-pumped, frequency-doubled, 532-nm laser (Coherent Novus 2000; Carl Zeiss Meditec, Oberkochen, Germany). Five lesions were induced, with a power of 120 mW, a spot size of 50 μm, and a duration of 100 ms. Laser-induced rupture of Bruch’s membrane was identified by the appearance of a bubble at the site of photococulation. CNV formation was assessed 14 days after photococulation in a standardized manner.

### Visualization of GFP+ Cells in the Retina

To localize and quantify bone marrow-derived GFP+ cells within the retina in relation to the retinal vasculature, we performed transcardial perfusion with rhodamine-coupled concanavalin A (conA) lectin. Animals were perfused at 1, 4, and 7 months after transplantation. In summary, transplant recipients were given deep general anesthesia and subsequently perfused with conA lectin, as described elsewhere. In short, after dissection of the stroma, the left ventricle was punctured, followed by catheter insertion. Transcardial perfusion was performed with 5 mL each of PBS, 1% paraformaldehyde, and rhodamine-labeled conA lectin (200 μg/mL, diluted in PBS; Alexis, Grünberg, Germany) via the left ventricle and finally with 0.9% NaCl solution. The eyes were enucleated; the cornea, lens, and iris were dissected; and the posterior cup was fixed in 4% paraformaldehyde for 5 minutes. Four radial incisions were cut into the remaining retina/choroid/sclera complex, to enable flat mounting of the tissue. The retina was carefully dissected from the choroid with a scalpel. The preparations were mounted in fluorescence mounting medium (DakoCytomation, Hamburg, Germany) for fluorescence microscopy. Alternatively, 1 week after the last clodronate injection, mice were killed for detection and counting of the GFP+ labeled cells that had migrated into the retina. At this point in time, the general condition of the clodronate-treated animals was poor (loss of body weight, immobility). Transcardial perfusion with lectin conA for visualization of the blood vessels was impossible. Therefore, in this set of experiments, the retinal vessels were visualized on retinal flat mounts by immunocytochemistry, as just described, and immunohistocytochemistry was performed with isoleucin B4-conjugated biotin (1:200; Invitrogen, Paisley, UK). The secondary Cy3-conjugated streptavidin (1:500; Sigma-Aldrich) was used as the detection reagent.

### Morphologic Assessment and Quantification of Retinal GFP+ Cells

For quantification of the number of the GFP+ cells on retinal flat mounts, all images of the flat mounts were captured with a digital camera (ORCA ER; Hamamatsu, Hamamatsu City, Japan) attached to a fluorescence imaging microscope (Axioscan 2; Carl Zeiss Meditec). The images were captured on computer (Power Mac G4; Apple, Cupertino, CA) and analyzed (OpenLab software; ImproVision Inc., Lexington, MA). The images were resolved at 1344 × 1022 pixels and converted to tagged information file format (.tif). Retinal overview images were used for cell counting of extravasal GFP+ cells. For evaluation of cell morphology, high-magnification images were used. Images for colocalization studies were taken with FITC/GFP and rhodamine fluorescence filters and merged (OpenLab software; ImproVision, Inc.). Confocal microscopy with a confocal laser-scanning microscope (TCS SL; Leica, Wetzlar, Germany) was used for morphology studies.

### Macrophage and Dendritic Cell Staining

For visualization and colocalization studies of immunocompetent retinal cells, retinal flat mounts were stained with F4/80 and CD11c antibodies (for labeling of macrophages and dendritic cells, respectively) and evaluated with confocal and fluorescence microscopy. For F4/80 macrophage staining and CD11c dendritic cell staining, retinal preparation was conducted with the perfusion procedure described earlier. After fixation and isolation, the retinas were permeabilized in 1% Triton X-100 (Sigma-Aldrich) in PBS for 10 minutes, followed by five rinses in fresh PBS. The tissue was blocked in 5% bovine serum albumin (BSA; Sigma-Aldrich) in PBS at 4°C for 1 hour. Primary antibody staining was performed with F4/80 (1:100; AbD Serotec, Düsseldorf, Germany) or CD11c (1:100; BD Pharmening, Heidelberg, Germany) in 2% BSA in PBS at 4°C overnight, followed by five washes in fresh PBS. Incubation was performed with a secondary antibody for F4/80 (anti-rat, 1:200; Invitrogen), or for CD11c (streptavidin; 1:100; Sigma Aldrich) in 2% BSA in PBS at 4°C for 2 hours, followed by five washes in fresh PBS. Background binding was excluded by secondary antibody application alone. Flat mounts were mounted with fluorescence mounting medium (DakoCytomation) and analyzed by fluorescence microscopy.

### Statistical Analysis

All results are presented as the mean (±SD). Analysis of variance (ANOVA) and Student’s t-test were used to assess statistical significance in all experiments (SPSS Software, Munich, Germany). Differences were significant at P < 0.05.

### RESULTS

**Radiation and Transplantation**

Lethal radiation required 11.0 Gy for full-body radiation and 16.0 Gy for head-shielded radiation, fractionated to 5.5 Gy twice with a time interval of 8 hours for full-body treatment, and fractionated to 8 Gy twice for head-shielded treatment. Tail vein injection was difficult in some animals, leading to the unintended creation of control animals for the lethal radiation dose. The full-body radiation dose >15.0 Gy was lethal, notwithstanding a successful transplantation procedure, with 18.0 Gy for the head-shielded group constituting the maximum tolerable dose for survival. The residual radiation dose to the head detected in the shielded group was 0.6 Gy. Decolorized fur became apparent in both groups of radiated animals, commencing after 2 months, with nonhomogenous gray fur becoming white within 10 months; however, the head remained black in the shielded group (Fig. 1).

Peripheral vein blood flow cytometry was used for verification of stable chimerism. Nonirradiated GFP heterozygous mice (n = 4) had 92.2% ± 1.3% GFP+ cells and female C57BL/6 mice (n = 5) 1.1% ± 0.7%. After open irradiation, peripheral GFP+ cells amounted to 74.9% ± 6.1%, 1 month after transplantation (n = 16) increasing to 75.7% ± 13.2% and 83.9% ± 8.5% after 4 (n = 7) and 7 (n = 5) months, respectively. Shielded animals showed an increase in GFP+ cells to 49.8% ± 13.8% and 63.3% ± 6.2% after 1 (n = 12) and 4 (n = 4) months (Fig. 2). There was no statistically significant difference between the two groups from 4 months after irradiation.

**Visualization and Quantification of Retinal GFP+ Cells**

To localize and quantify bone marrow-derived GFP+ cells within the retina in relationship to the retinal vasculature, we performed transcardial perfusion with rhodamine-coupled
versus 5 examination time points. Almost no co-localization of GFP with macrophages and dendritic cells in the retina was found at all fluorescence microscopy. In shielded animals, even distribution of dritic cells, respectively) and evaluated by confocal and fluo-
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Immunohistochemical Analysis of Macrophage
and Dendritiform Cells in the Retina
For visualization and colocalization studies of immunocompetent retinal cells, retinal flat mounts were stained with F4/80 and CD11c antibodies (for labeling of macrophages and dendritic cells, respectively) and evaluated by confocal and fluorescence microscopy. In shielded animals, even distribution of macrophages and dendritic cells in the retina was found at all examination time points. Almost no co-localization of GFP with F4/80 or CD11c was seen (Fig. 5). After unshielded irradiation, as expected, the density of F4/80 + and CD11c + cells increased over time. GFP − cells stained with F4/80 or CD11c, as well as double-stained GFP +/F4/80 + or GFP +/CD11c + cells, were apparent 4 months after irradiation. Approximately half of the cells stained with F4/80 and CD11c were GFP +. These cells were found both spread within the retinal tissue and surrounding the vessels (Fig. 5). There were CD11c + and F4/80 + elongated cells in clusters surrounding the large vessels, similar to perivascular cells. Furthermore, there were round, dendritiform CD11c + and F4/80 + cells throughout the retinal tissue in the unshielded group.

Detection of Apoptosis in the Retina
after Irradiation
A TUNEL assay was performed on paraffin-embedded sections 4 months after irradiation. There were 26.25 ± 7.9 (n = 4) TUNEL + cells in the GCL after shielded irradiation (Fig. 6, left), compared with 170.5 ± 36 (n = 4, P < 0.001) in the unshielded group (Fig. 6, right). Similarly, there were 11 ± 4.2 (n = 4) TUNEL + cells in the INL after shielded irradiation, compared with 47.5 ± 7.9 (n = 4, P < 0.001) after unshielded irradiation.

Depletion of Macrophages after Unshielded Irradiation
The causality of macrophages and dendritic cells in the cell invasion after irradiation was determined by depleting macrophage-like cells with clodronic acid and evaluating the counts 4 months after irradiation. Four weeks after clodronate treatment, blood counts demonstrated a reduction of monocytes from 14% in control animals to 2% in clodronate-treated animals, whereas the remaining blood counts were stable (Table 1). Four months after total-body irradiation, extravasal GFP + cells were found in the retinal periphery and around the optic nerve head (Fig. 7).

Four months after irradiation, GFP + cells were counted in the retinal tissue. The number of GFP + cells in the retinal tissue was 188.2 ± 35.1 (n = 6) in the control group after unshielded irradiation. In contrast, after unshielded irradiation and treatment with clodronic acid, 48.5 ± 8.3 GFP + cells (n = 4, P < 0.001) were found in the retinal tissue after 4 months (Fig. 3, right).

Reaction of GFP + Cells to Experimentally
Induced CNV
The integration of GFP + cells to pathologic neovascularization was assessed in a laser-induced CNV model. Integration

![Image](https://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/932963/ on 07/22/2018)
of GFP+/H11001 cells in the choroid and the overlying retina was assessed in animals 4 months after unshielded and shielded irradiation.

After both shielded and unshielded irradiation, there was a massive recruitment of GFP+/H11001 cells to the sites of choroidal damage after laser photocoagulation. Retinal recruitment of GFP+ cells was clearly seen in the areas above the laser scars in the unshielded group, whereas there was almost no invasion of GFP+ cells into the retina in the shielded group (Fig. 8).

**DISCUSSION**

In our study, irradiation led to the invasion of the eye by inflammatory cells. These cells transformed to a glialike CD11c+ cell type and F4/80-expressing retinal macrophages. The recruitment of blood-derived inflammatory cells into the eye after irradiation was inhibited both by shielding of the animal’s head, thereby excluding the retinal tissue from irradiation, and by using clodronic acid to eliminate the macrophages. An increased recruitment of blood-derived glial and inflammatory cells to the overlying retina was observed only after unshielded irradiation. In an interesting finding, when the retina and head were shielded, attraction of inflammatory cells from the blood was not observed after induction of pathologic angiogenesis in the choroid. This finding demonstrates that the physiological turnover of retinal glia cells is largely accelerated and activated after irradiation of the retina.

Classification and statistical assessment of the severity of radiation retinopathy remain difficult, in part because of the...
prolonged quiescence between application of the radiation and the manifestation of deterioration. Most of the data available are from clinical observations, comprising a vast compendium of effects on the retinal vasculature due to different doses of radiation. Chacko suggested that 3500 cGy is the maximum tolerable dose for the retina when given as a single fraction. Radiation retinopathy does not occur at doses below 3.5 Gy. However, there are other reports indicating that radiation retinopathy occurs after a minimal dose to the retina of 11 Gy.

In our experiments, lethal treatment required 11.0 Gy (2/5.5 Gy) for unshielded, full-body radiation and 16.0 Gy (2 × 8 Gy) for head-shielded irradiation. In other studies, the investigators used 9.5 Gy each for total-body irradiation; others used fractionated doses of 2 × 5.0 Gy or 2 × 6.0 Gy. Several reports did not specify irradiation doses. The dose used in our experiments is within this range.

Using that dose, we achieved transduction rates of more than 50% in all groups, starting 1 month after irradiation. Transduction rates increased with time. Control animals without GFP transplantation showed the insufficient regeneration ability of the remaining bone marrow.

Doses for lethal irradiation may differ, depending on the mouse strain, as they do for different species. Even doses of 16 Gy, which were necessary to generate a lethal dose in the shielded animals, did not result in an invasion of the retinal tissue by GFP cells.

Most published papers allow for a 3-month time period to establish stable chimerism (e.g., Ref. 19). In agreement with

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such as retinopathy of prematurity,40 diabetes,9,10 and CNV.29,41 Of alterations in entities primarily not considered inflammatory diseases, are known to play a major role in the development of vascular interest, these inflammatory diseases seem to ameliorate radiation retinal tissue increase in the unshielded radiated animals. Months after irradiation, extravasal GFP increased from 75% after 4 months to up to 83.6% after 7 experiments, the transformation rate in the unshielded group after shielding is related to a lesser transformation rate. In our section. Thus, it can be assumed that the invasion of GFP cells, notwithstanding reception of a residual dose of 600 cGy, did not show any GFP notwithstanding a shielded control in their experiments.

The phenotype of pathologic changes in unshielded animals including macrophage and dendritic cell invasion seems to resemble that of inflammatory diseases.36,39 Macrophages and inflammatory cells are known to play a major role in the development of vascular alterations in entities primarily not considered inflammatory diseases, such as retinopathy of prematurity,40 diabetes,9,10 and CNV.29,41 Of interest, these inflammatory diseases seem to ameliorate radiation retinopathy in humans37 as well as in experimental animals.32 Nevertheless, there are studies indicating that irritation alone is sufficient to cause macrophage invasion of the retina.38,43

This notion is in accordance with our data demonstrating that irradiation of the retinal tissue was responsible for microglia and macrophage activation and invasion of the retinal tissue. Similarly, Xu et al.24 showed an increased number of GFP myeloid cells in the retina in a mouse model of bone marrow transplantation after total-body irradiation with 8 or 10 Gy. Unfortunately, they did not have a shielded control in their experiments.

Six months after irradiation, Xu et al.24 indicated that all retinal myeloid cells were GFP+. In contrast, we found that about half of the GFP+ cells showed co-localized staining with CD11c or F4/80 markers 4 months after irradiation, and the ratio was constant for up to 7 months. Our results indicate that, besides the GFP+ cells that are derived from the bone marrow, residential microglia cells and macrophages (that stained positive for CD11c and F4/80, but were negative for GFP) were present. These residential cells, as well as blood-derived cells, became activated and increased in number.

The normal distribution of immunocompetent cells in the human retina has been described by Yang et al.39 Before irradiation, a regular distribution of F4/80 and CD11c was present in the mouse retina. After head-shielding, there was neither increased recruitment of bone-marrow-derived cells nor visible changes in the distribution of residential cells, despite higher irradiation doses. The data indicate that irradiation of the retinal tissue causes activation of resident macrophages and microglia. Activated macrophages and microglia may in turn release attractant signals and further increase the recruitment and homing of circulating monocytes into the retina. Further experiments are needed to investigate the mechanism by which these residential macrophages and glia cells become activated and increase in number after irradiation.

Figure 7. Spatial arrangement of GFP+ cells after clodronic acid treatment. Four months after unshielded irradiation, extravasal GFP+ cells were found in the retinal periphery and around the optic nerve head. The number of these cells largely diminished in the group receiving treatment with clodronic acid 4 weeks before death. The quantification is shown in Figure 5, right.
Our data show that the GFP cell invasion after long-term transplantation cannot be explained by physiological cell turnover, as indicated by Xu et al.\textsuperscript{24} Contrasting previous data with those on HSCs\textsuperscript{18,20,44} produced the interesting result that the number of extravasal GFP\textsuperscript{+} microglial cells and macrophages in our experiments exceeded by far the number of elongated GFP\textsuperscript{+} perivascular cells. Endothelial progenitor cells resemble a small fraction of the bone marrow and possibly require specific homing factors for incorporation into the vessel wall.\textsuperscript{45} In the current experiments, we used whole bone marrow for transplantation. We did not observe integration of GFP\textsuperscript{+} cells into the endothelial cell wall, even 7 months after unshielded irradiation when plenty of microglia cells were found in the retinal tissue. There were, however, few perivascular CD11c/GFP\textsuperscript{+} cells.

Our data further suggest that the primary site of injury is the retinal tissue, as shielded animals did not demonstrate microglia invasion. If activation of the vascular endothelial cells plays a major role, one would expect, to a greater extent, the adhesion of GFP\textsuperscript{+} cells to the retinal endothelial cells.

Systemic depletion of inflammatory cells by clodronic acid significantly reduced the inflammatory response; however, it did not completely inhibit GFP\textsuperscript{+} cells from invading the retina. Inhibition of bone marrow macrophage extravasation by clodronic acid results in a reduction of inflammatory neovascularization in different models.\textsuperscript{46-49} Clodronate liposomes elicit selective depletion of macrophages by apoptosis\textsuperscript{48,49} and have been studied in systems demonstrating their insensitivity toward other cell types.\textsuperscript{49-51} In our experiments, clodronic acid in liposomes were applied systemically. When applied 3 months after total-body irradiation, clodronate led to a significant inhibition of microglial and perivascular recruitment of GFP\textsuperscript{+} cells, compared with that in control animals treated with empty liposomes. This result supports the theory that irradiation of the retinal tissue leads to signals that result in an accelerated turnover of retinal microglial cells and an attraction of inflammatory cells to the retinal tissue.

By giving clodronate systemically, we reduced the number of systemic macrophages, in contrast to the role of resident microglia in the growing retinal vasculature: Checchin et al.\textsuperscript{52} distinguished the role of systemic macrophages from that of resident retinal microglia by administering clodronate liposomes, either intraperitoneally or intravitreally. In accordance with our experiments, intraperitoneal clodronate liposomes diminished systemic macrophages by approximately 70%. Of note, the retinal vasculivity was indistinguishable between animals injected intraperitoneally with clodronate liposomes or PBS. The depletion of resident retinal microglia by intravitreal injection, however, reduced developmental vessel growth and density. This effect was restored by intravitreal injection of microglial cells, which indicates a prominent role for resident retinal microglia, as opposed to systemic macrophages in normal retinal blood vessel formation.\textsuperscript{52} In our experiments, systemic depletion of macrophages and microglia cells was effective because of the retinal damage by irradiation that attracts systemic macrophages and facilitates migration of circulating mononcytic cells across the blood-retinal barrier.

Along these lines, we found increased TUNEL staining in the retinal tissue after irradiation. One could suggest that the increase in the number of ganglion cells and neuronal cells in the INL that undergo apoptosis may play a role in the recruitment of macrophages and microglia cells and vice versa. Similar effects have been discussed for diabetic retinopathy.\textsuperscript{53} Shielding of the head and thus of the retinal tissue from irradiation led to a diminution of neuronal apoptosis.

Several studies have focused on the role of macrophages in regulating the growth of pathologic new vessels underneath the retina, so-called CNV (e.g., Ref. 47). Nevertheless, no research has been performed to evaluate the role of inflammation as a mechanism of vision loss and retinal degeneration in the retina overlying the CNV. We combined laser-induced CNV in mice and bone marrow transplantation with GFP\textsuperscript{+} cells to determine the relative role of recruited blood-derived macrophages versus resident microglia in the retina overlying the CNV lesions. In a similar chimeric model, after laser photocoagulation, Caicedo et al.\textsuperscript{54} showed that infiltration with blood-derived macrophages precedes pathologic changes in the retina, causing endogenous glial cell (Müller cell) activation. In accordance with our data, they found a response localized to the retina overlying the neovascular lesion in the absence of generalized inflammation of the eye. In contrast, the density of resident microglia did not increase.\textsuperscript{51} Our data further supplement these findings by demonstrating that recruitment of microglial cells to the retina is dependent on preconditioning of an inflammatory state of the retina by irradiation. We were able to demonstrate that shielding of the mouse heads similarly resulted in a lack of GFP\textsuperscript{+} cells in the retinal vasculature overlying the CNV lesions; however, it did not affect recruitment of GFP\textsuperscript{+} cells to the CNV lesions. Thus, irradiation of the retinal tissue results in an accelerated by prerequisite of activated circulating macrophages to the damaged retina after irradiation. This result is in accordance with the data by Caicedo et al. showing that depleting circulating macrophages with clodronic acid diminishes the density of F4/80-immunoreactive cells as well as the density of pERK-immunoreactive Müller cells in the retina under CNV.\textsuperscript{54} Resident endogenous glia of the retina, however, activated via irradiation, may send signals that further increase inflammatory cell recruitment and homing within the eye. In conclusion, the CNV-induced retinal damage is associated with recruitment of blood-derived macrophages rather than resident retinal microglia.

Taken together, the evidence shows that irradiation of retinal tissue causes increased apoptosis of the neuronal cells and is associated with an increased attraction of blood-derived macrophages and increased microglial activation and turnover. Shielding of the head can prevent these mechanisms and should be considered for long-term experiments in chimeric mice. Lethal unshielded radiation combined with GFP bone marrow transplantation could serve as a long-term animal model for inflammatory retinopathy providing a straightforward means of microglial tracing that may improve our knowledge of inflammatory entities, including diabetic retinopathy.

References


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