RETINAL MICROGLIA ARE ACTIVATED BY SYSTEMIC FUNGAL
INFECTION.

Victoria Maneu^{1,2,*}, Agustina Noailles³, Javier Megías⁴, Violeta Gómez-Vicente³,
Núria Carpena⁴, M^a Luisa Gil⁴, Daniel Gozalbo⁴, Nicolás Cuenca^{3,5}

¹Departamento de Óptica, Farmacología y Anatomía, Universidad de Alicante,
Spain.

²Instituto Teófilo Hernando de I+D del Medicamento, Universidad Autónoma de
Madrid, Spain.

³Departamento de Fisiología, Genética y Microbiología, Universidad de
Alicante, Spain.

⁴Departamento de Microbiología y Ecología, Universitat de València, Burjassot,
Spain.

⁵Instituto multidisciplinar para el estudio del medio Ramón Margalef.
Universidad de Alicante, Spain.

*Author for correspondence: Victoria Maneu. Departamento de Óptica,
Farmacología y Anatomía. Pabellón 13. Universidad de Alicante. Carretera San
Vicente del Raspeig s/n. 03690 - San Vicente del Raspeig (Alicante).
vmaneu@ua.es

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ABSTRACT

Purpose: To determine whether systemic fungal infection could cause activation of retinal microglia and therefore could be potentially harmful for patients with retinal degenerative diseases.

Methods: Activation of retinal microglia was measured in a model of sublethal invasive candidiasis in C57BL/6J mice by (i) confocal immunofluorescence and (ii) flow cytometry analysis, using anti-CD11b, anti-Iba1, anti-MHCII and anti-CD45 antibodies.

Results: Systemic fungal infection causes activation of retinal microglia, with phenotypic changes in morphology, surface markers expression, and microglial re-location in retinal layers.

Conclusions: As an excessive or prolonged microglial activation may lead to chronic inflammation with severe pathological side effects, causing or worsening the course of retinal dystrophies, a systemic infection may represent a risk factor to be considered in patients with ocular neurodegenerative diseases, such as diabetic retinopathy, glaucoma, age-related macular degeneration or retinitis pigmentosa.

KEY WORDS: microglial activation, retina, infection, candidiasis

1. Introduction

Microglial cells are part of the mononuclear phagocyte population of the central nervous system (CNS) and the retina, and play a relevant role in both, physiological and pathological conditions. In a healthy retina, microglia are in a "resting state", in which, while scanning the environment, they secrete neurotrophic factors that support surrounding neurons survival. After injurious stimuli such as ocular infections, trauma or neurodegeneration processes, microglial cells acquire an activated state, in which they proliferate, migrate to damaged sites, undergo morphological transformation into amoeboidic phagocytes and secrete molecules that initiate tissue repair mechanisms ¹⁻⁴. Hence, at first stages of a neurodegenerative process, microglia initiate repair mechanisms and are considered to have a neuroprotective role. On the other hand, excessive or prolonged microglial activation leads to chronic inflammation, due to the continuous secretion of neurotoxic agents that cause severe pathological side effects, retinal damage and neuronal apoptosis ^{2, 3, 5}.

To date, several CNS injuries, such as infections, neurodegenerative diseases, chemical injury or aging, have been correlated with microglial activation and high levels of pro-inflammatory cytokines ^{3, 4, 6, 7}. In Parkinson's disease, there is an increased number of activated microglia in the brain, accompanied by increased expression of proinflammatory cytokines ^{8, 9}. Also in Alzheimer's disease, activated microglia have been associated to the amyloid plaques in the brain ^{10, 11}. Moreover, microglial activation has been demonstrated in several other neurodegenerative diseases, such as amyotrophic lateral sclerosis or Huntington's disease ³. Retinal neurodegenerative diseases are associated with

chronic microglial activation and neuroinflammation as well, as it is the case for retinitis pigmentosa¹², age-related macular degeneration (AMD)^{13, 14} or glaucoma^{15, 16}. In the degenerating retina, endogenous signals activate microglial cells leading to local proliferation, migration, enhanced phagocytosis, and secretion of cytokines, chemokines, and neurotoxins. These immunological responses and the loss of limiting control mechanisms may contribute significantly to retinal tissue damage and proapoptotic events in retinal degenerations^{1, 2, 12}.

Therefore, it can be assumed that different stimuli that induce prolonged microglial activation can be potentially harmful for neuronal tissue. In this sense, systemic infections can induce microglial activation in the brain and may be considered as a risk factor in neurodegenerative diseases. It has been described that microglia are the most abundant defense population in the brain, showing a 3-fold increase after 7 days of infection in a model of systemic invasive candidiasis¹⁷. The study of microglial homeostasis in the retina meets some technical limitations, such as the heterogeneous distribution of microglial cells in the retinal tissue and the lack of experimental tools, which prevent an accurate quantification and molecular analysis of this specialized neuronal macrophage population¹. The aim of this work was to determine whether systemic fungal infection might cause activation of retinal microglia in order to discern whether it should be considered as a risk factor for patients affected with ocular neurodegenerative diseases.

2. Material and methods

2.1. Mice

Female C57BL/6J mice (8-10 weeks old) purchased from Harlan Ibérica (Barcelona, Spain) were bred and maintained under specific pathogen-free conditions at the University of Valencia animal facilities. The protocol was approved by the Committee on the Ethics of Animal Experiments of the University of Valencia (Permit Number: A1264596506468). All animals were handled in accordance with current regulations for the use of laboratory animals (NIH, ARVO and European Directive 2010/63/UE) in order to minimize animal suffering and limit the number used for the experiments.

2.2. Yeast strain and infection model

Both, viable and inactivated *Candida albicans* ATCC 26555 yeasts were used. Yeasts were obtained as previously reported^{18, 19}. Briefly, starved yeast cells were inoculated (200 µg, dry weight, of cells per ml) in a minimal synthetic medium and incubated for 3 h at 28° C to obtain yeasts. Only yeast cells without germ tubes were observed at 28 °C. Viable yeasts were resuspended in phosphate-buffered saline (PBS) at the desired concentration and used in infection experiments (see below). For inactivation, yeast cells were resuspended (20×10^6 cells/ml) in 4% (w/v) paraformaldehyde (fixation buffer, eBioscience, San Diego, CA) and incubated for 1 hour at room temperature. After treatment, fungal cells were extensively washed in PBS and brought to the desired cell density in PBS. For infection assays, mice were injected (day 1) with a sublethal dose (6×10^5 viable yeasts in 100 µl of PBS) of *C. albicans*, by intravenous administration, according to the method previously described

by our group^{20, 21}. Alternatively, mice were injected with three doses of 10×10^6 inactivated yeasts on days 1, 2 and 3. In both cases the retinas were analyzed on day 4. Untreated mice were used as controls.

To assess *C. albicans* invasion of retinal tissue in infected animals, retinal cryo-sections were stained with Calcofluor White M2R 0.1% for 15 minutes, coverslipped and observed under a fluorescence microscope. Alternatively, whole retinas were dissociated, resuspended in 100 μ l of PBS, plated in Sabouraud-Dextrose agar and cultured for 48 hours at 37°C to allow colony formation.

2.3. Immunohistochemistry

For immunohistochemistry assays, enucleated eyes were fixed using 4% paraformaldehyde in 0.1M phosphate buffer pH 7.4 (PB), for 1h at room temperature. To maintain retinal orientation, a suture was placed on the superior pole of each eye previously to enucleating. Following several washes with PB, the eyes were sequentially cryo-protected in 15% and 20% (w/v) sucrose for one hour, and 30% sucrose overnight. Next day, the cornea, lens and vitreous were dissected out, and the eyecups were embedded in Tissue-Tek® (Sakura Finetek, Torrance, CA) and frozen in liquid N₂. 16- μ m vertical retinal cryo-sections along the naso-temporal axis were mounted on slides (Superfrost Plus; Menzel GmbH & Co. KG, Braunschweig, Germany), washed and incubated with blocking solution (5% normal donkey serum in PB containing 0.5% Triton X-100) for 1 hour at room temperature. Immunostaining with a cocktail of mouse anti-MHC class-II RT1B monoclonal antibody, 1:50 (clone OX-6, AbD Serotec, Kidlington, United Kingdom) and rabbit anti-Iba1

polyclonal antibody, 1:500 (Wako Chemicals, Neuss, Germany) was performed overnight at room temperature in a wet chamber. After incubation with the primary antibody, retinal sections were washed and incubated in a mix of secondary antibodies: Alexa Fluor 555-conjugated donkey anti-mouse IgG (Molecular Probes, Eugene, OR) and Alexa Fluor 488-conjugated donkey anti-rabbit IgG, both at a 1:100 dilution, for 1 hour. Finally, the sections were washed and mounted with Citifluor (Citifluor Ltd., London, United Kingdom) for visualization under a laser scanning confocal microscope (TCS SP2 Leica Microsystems, Wetzlar, Germany). Immunohistochemical controls were performed by omission of either the primary or the secondary antibodies. Only retinal sections that contained the optic nerve were used for cell scoring.

2.4. Flow cytometry analysis

First, the eyes were enucleated and the retinas were carefully dissected. Retinas were placed in 1 ml of PBS buffer and were disaggregated by gently pipetting up and down. The cell suspension was filtered through a 30 µm-filter from BD Biosciences to avoid cell clumps. Disaggregated cells were labelled with rat FITC- or APC- conjugated anti-CD11b antibody, at 1:200 dilution (Clone M1/70, eBioscience, San Diego, CA). For the detection of microglial activation, disaggregated retinal cells were triple-stained with a cocktail of rat APC-conjugated anti-CD11b antibody, PE-conjugated anti-MHC class II antibody (clone M5/114.15.2, Milteny Biotec) and FITC-conjugated anti-mouse CD45 (clone 104.2, Milteny Biotec). Labelled cells from each mouse retina were analyzed separately. Analyses were performed in a FACSCanto cytometer (BD Biosciences) and the data were analyzed with FACSDiva

software. To quantify the area corresponding to retinal microglial cells in density plots, CD11b positive cells were represented in forward scatter (FSC)-FITC and the total area was measured for each retinal sample.

2.5. Statistical analysis.

All experiments were performed in at least three animals. Student's t-test was applied using GraphPad software (Graph-Pad Software, Inc., USA). Results are expressed as mean $\pm$ SD and * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ values were considered significant.

3. Results

In control mice, microglial cells labelled with anti-Iba1 antibody appeared with the usual morphology of resting microglia in a healthy retina (Fig. 1A, C, G, I). These quiescent cells had a small, round soma with various branching processes and showed no reactivity against MHC II class antibody (Figure 1B, C, H, I). Regarding cell distribution, microglial cells were located mainly in the inner plexiform layer (IPL) and the outer plexiform layer (OPL) in control mice (Fig. 1A-C, G-I).

In the retinas of infected mice, after three days of infection, ramified cells experienced a transformation through gradations of “activated” states, exhibiting changes in shape and distribution: the cellular soma was enlarged, showing coarse and swollen appearance, processes were shortened and thickened with little branches (Fig. 1D-F, J-L). Some of the microglial cells showed an amoeboid shape (Fig. 2A-C). In these retinas, microglial cells were immunoreactive to anti-MHC class II antibody, which colocalized with Iba1 in most cells (Fig. 1E, F, K, L). Specifically, we found colocalization of Iba1 and MHCII RT1B antibodies in all amoeboid cells, including amoeboid microglial cells surrounding blood vessels (Fig 2A-C).

In infected mice, microglial cells were not only detected in the plexiform layers of the retina but also in the nuclear layers, the outer nuclear layer (ONL) and the inner nuclear layer (INL) (Fig 1D-F, J-L). Amoeboid microglial cells were observed in the ONL, between the outer segments (OS) and the retinal pigment epithelium (RPE), and surrounding blood vessels (Fig 2A-C). We also found

Iba1+/MHCIIRT1B+ amoeboid microglial cells in the IPL (Fig 2D-F) and Iba1+ cells getting out of blood vessels (Fig 2G-I). Cells within the blood vessels appeared as Iba1+/ MHCIIRT1B- while the ones within the retinal tissue were Iba1+/MHCIIRT1B+ (Fig 2D-F). Microglial Iba1+ and MHCIIRT1B+ processes can be observed around the blood vessels in Fig 2G-I and in Fig 2J-L.

In flow cytometry analyses, the whole microglial population of the retina was identified by its immunoreactivity against CD11b antibody in both, control and infected mice (Fig 3A). Triple immunostaining showed a significant increase in the expression of MHCII and CD45 antigens in microglial cells of infected mice (1.91 and 3.22-fold increase in fluorescence mean intensity respectively) (Fig 3A,B), as well as an increase in the CD11b fluorescence mean intensity value (3.13-fold) with respect to the mean value in control mice (Fig. 3B). In infected mice, a CD11b+ CD45++ population was observed. Microglia activated state was also detected by quantifying the area of the retinal microglial cell population identified in forward scatter (FSC)-CD11b-FITC density plots. In control mice, CD11b positive cells appeared as a homogeneous population in FSC-CD11b-FITC dot and density plots, while retinal microglia of infected mice appeared as a much more heterogeneous population. The area value of activated microglia in infected mice was 3.5-fold increased with respect to control (Fig. 3C).

The percentage of CD11b cells was increased in the retinas of infected mice. As deduced by the flow cytometry analysis, the microglial population in control mice constituted 0.10 ± 0.07 % of total cells analyzed, whereas in infected mice the CD11b positive population raised to 0.61 ± 0.01 % (Fig. 3A). In agreement

with the flow cytometry data, the immunohistochemistry also revealed an increase in microglial cell number, as the number of Iba1⁺ cells per retinal section was 10.1 ± 1.4 % in control mice vs 13.9 ± 0.9 % in infected mice (Fig. 4A). A quantitative analysis of immunohistochemical sections showed that the number of Iba1⁺ cells associated with blood vessels was 26,7 % in infected mice, while in control mice no Iba1⁺ cells were associated with vessels (Fig. 4B).

In the ciliary body of control mice, we found Iba1⁺/MHCII⁻ cells with ramified morphology (Fig. 5A-B), while in infected mice some Iba1⁺/MHCII⁺ cells with a typical activated amoeboid morphology were found (Fig 5C-D). Also, in the ciliary body of infected mice, blood borne monocytes were found within blood vessels (Fig. 5E-F). A significant increase in the number of Iba1⁺ cells was observed in the ciliary body of infected animals (12.9 ± 0.1 cells/ciliary body section) compared to untreated controls (8.8 ± 0.8 cells/ciliary body section) (Fig. 4C).

The injection of three doses of 10×10^6 inactivated yeasts on days 1-3, did not elicit a significant increase in the retinal microglia population. FITC-CD11b fluorescence mean value appeared similar to the one in control, uninjected mice (not shown).

In our conditions, we could not detect *C. albicans* invasion of retinal tissue neither by Calcofluor White M2R staining of retinal sections nor by incubating retinal tissue of infected mice in Sabouraud-Dextrose plates for 48 hours.

4. Discussion

We studied the effect of a systemic fungal infection on retinal microglia in a mouse model of systemic candidiasis previously used in our laboratory^{20, 21}. The microglia population was identified by its immunoreactivity against Iba1 protein, as it is specifically localized in microglia and not found in neurons, astrocytes or oligodendroglia^{22, 23}. MHC class II antigens and the leukocyte common antigen CD45 were used to detect microglial activated states, as their upregulation is well known to occur in an activated state²⁴⁻²⁷. In control mice, microglial cells appeared with the characteristic morphology, reactivity and distribution of microglial cells in a healthy state², while microglia in the retinas of infected mice appeared in a greater number and with different activated states, from cells with enlarged soma, shortened and thickened processes to cells with a real amoeboid shape, thus in accordance with an advanced activation stage, in which microglia turn into phagocytic microglia, morphologically defined by the complete absence of cellular processes^{1, 2}.

Hence, our observations indicate that a systemic fungal infection causes retinal microglia activation and an increase of microglial cells in the retina. Our result is in agreement with a recent publication by Zinkernagel et al., which found that systemic cytomegalovirus infection caused migration movements of the microglial cells in the retina, also showing signs of morphological activation²⁸.

The increased microglial cell number in infected mice can be due to the proliferation of retinal microglia and/or the arrival of infiltrating macrophages, as

both, proliferation and the arrival of new blood-derived precursors that transform into phagocytes, have been described in pathological situations^{1, 29-38}. Zinkernagel et al. conclude that the increased number of retinal microglia cells after a systemic cytomegalovirus infection, can be attributable just to proliferation *in situ*²⁸. Although the increase in the CD11b+, MHCII+ and CD45+ populations we observed in infected mice could be also consistent with microglial proliferation, the appearance of a high-expressing CD45 population in infected mice favors the arrival of macrophages or infiltrating perivascular microglial cells^{25, 39}. This is further supported by immunohistochemical analyses, as some of Iba1-expressing cells appeared associated with blood vessels and a number of these seemed to be getting out of the vessels to reach the retinal tissue. In some cases, Iba1 and MHCII double positive cells were observed around blood vessels, probably corresponding to perivascular macrophage cells^{24, 25, 39}. Moreover, we observed a statistically significant increased Iba1+ population in the ciliary body of infected mice, some of them within blood vessels, which supports the idea of the presence of blood borne macrophages³¹.

The fact that inactivated yeasts did not elicit a significant increase in the retinal microglia population showed that only viable microorganisms and not inactivated yeasts cause microglial activation, probably due to a rapid clearance of inactivated yeasts by phagocytotic cells.

We could not detect *C. albicans* invasion of retinal tissue, which supports the idea that the activation of retinal microglial cells is due to a massive cytokine

production that occurs during systemic infections. This finding is in agreement with Gallego et al.¹⁶ who showed in a mouse model of glaucoma that, after inducing ocular hypertension with laser in one eye, the glial cells in the contralateral eye also appeared activated, maybe by immunologically mediated processes. However, a possible direct interaction of microglia with circulating soluble fungal ligands such as β -glucan and mannan⁴⁰ may also contribute to their activation.

As it is well known, toll-like receptors (TLRs), mainly TLR2 and TLR4, are critical for cytokine production in response to *C. albicans*, and Dectin-1, the β -glucan receptor, is required for phagocytosis of fungal cells, and also collaborates with TLR2 in cytokine production^{41, 42}. Retinal microglia, like microglia of the CNS and other immune cells, probably recognize Pathogen Associated Molecular Patterns (PAMPs) through TLRs and Dectin-1. Several studies have shown that TLRs and Dectin-1 play an important role in microglial-mediated neuroinflammation and CNS injury following infection; β -glucan activates CNS microglia in a Dectin-1-dependent manner, suggesting that this receptor may play an important role in antifungal immunity in the CNS^{43, 44}. Moreover, we have previously demonstrated that both, TLR2 and Dectin-1 are expressed in mouse retinal microglia and that Dectin-1 is involved in the phagocytosis of *C. albicans*⁴⁵. Therefore, the possibility that fungal-derived ligands cause microglia activation following direct recognition by Pattern Recognition Receptors should not be ruled out.

Although microglial activation is commonly associated with neuroprotection, excessive or prolonged activation may lead to chronic inflammation, with severe pathological side effects^{5, 46}. In the retina, chronic microglia activation has been related to various neurodegenerative diseases, likely contributing to retinal tissue damage and proapoptotic events^{1, 2}. Despite its relevance, the study of microglial homeostasis in the retina has the challenge of the lack of experimental tools to analyze its activation in a simple and rapid way. In this work, we describe the retinal microglial activation induced by systemic infection using both, immunohistochemistry and flow cytometry analysis. Previous reports have shown that microglia in the brain can be activated by systemic fungal infections¹⁷, as well as by systemically administered LPS^{47, 48} and systemic viral infections²⁸. Our results show that a systemic infection by *C. albicans* greatly activates microglia in the retina, as indicated by: (a) the changes in microglial cell morphology and distribution, (b) the upregulation of MHC class II and CD45, as well as CD11b antigens, and (c) the increase of the microglial population. Furthermore, our results demonstrate that quantifying the area corresponding to retinal microglial cells in FSC-CD11b-FITC density plots constitutes a useful tool to detect microglial activation.

In conclusion, we have demonstrated that a systemic fungal infection activates the microglia in the retina, indicating that it may be considered as a risk factor for patients affected with ocular neurodegenerative diseases such as diabetic retinopathy, glaucoma, AMD or retinitis pigmentosa.

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LEGENDS TO FIGURES

Figure 1. Confocal immunofluorescence images of retinal microglial cells from C57BL/6J mice from a control mouse showing typical ramified morphology (A-C, G-I) and a mouse infected with viable yeasts of *C. albicans* ATCC26555 by intravenous administration showing activated microglia with enlarged soma and short, thick processes (D-F, J-L). Retinal sections were stained with anti-Iba1 antibody (A, D, G, J), anti-MHC class-II RT1B antibody (B, E, H, K) or both (C, F, I, L) showing the coexpression of MHC class-II RT1B and Iba1 in microglial cells of infected retinas. Arrows indicate microglial cell soma. Arrowheads point non-specific immunostaining of vessels with the secondary antibody. IS: inner segments, ONL: outer nuclear layer, OPL: outer plexiform layer, INL: inner nuclear layer, IPL: inner plexiform layer, GCL: ganglion cell layer. Scale bar 20 μm (A-F) and 10 μm (G-L).

Figure 2. Distribution and morphology of microglial cells in the retinas of infected mice. Retinal sections were immunostained with anti-Iba1 antibody (A, D, G, J), anti-MHC class-II RT1B antibody (B, E, H, K) or both (C, F, I, L). Arrowheads (A-C) and arrows (A-C) detail the characteristic morphology of Iba1 and MHCII double positive microglial cells in infected animals, with amoeboid or ovoid forms that were widely distributed in all retinal layers. Small arrows (A-C) point Iba1+ cells that surrounded blood vessels. Small arrows (D-F) point Iba1 + cells leaving blood vessels and getting into the retinal tissue. Arrows (D-F) mark the non-specific immunostaining of blood vessels with the secondary antibody. The arrowhead (D-F) points an Iba1+/MHCII+ microglial cell with a typical activated morphology. The arrowhead (G-I) points an Iba1+ cell within a blood

vessel observed in a transversal section. Small arrows (G-I) point immunoreactive cells that seem to be perivascular macrophages as described by Guillemin & Brew, 2004. Arrows (G-I) mark the non-specific immunostaining of blood vessels with the secondary antibody. The small arrow (J-L) details the part of an immunopositive Iba1 microglial cell that was leaving a blood vessel. The arrowhead (J-L) points the part of the same microglial Iba1+ cell that was still into the blood vessel. Arrows (J-L) mark the non-specific immunostaining of blood vessels with the secondary antibody. GCL, ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer; OPL, outer plexiform layer; ONL, outer nuclear layer; IS, inner segments; OS, outer segments. Scale bar 20 μm (A-C) and 10 μm (D-L).

Figure 3. Effect of fungal systemic infection on mouse retinal microglia, assessed by flow cytometry. (A) Retinal cells from C57BL/6J control and infected mice were triple labelled with a cocktail of APC-conjugated anti-CD11b, PE-conjugated anti-MHC class-II and FITC-conjugated anti-mouse CD45 antibodies and analyzed by flow cytometry (10^6 cells were analyzed in each assay). CD11b positive cells were gated (the percentage of gated cells is indicated). Double plots of CD11b+ cells presenting CD11b/MHCII and CD11b/CD45 double staining are shown in each case. Results show data from a single experiment representative of eight independent assays. (B) Histograms showing mean fluorescence intensity values of microglial cells (CD11b positive population) labeled with anti-CD11b, anti-MHCII and anti-CD45 antibodies (10^6 cells were analyzed in each assay) from control and infected mice. (C) Contour plots representing forward scatter (FSC) against CD11b expression of gated

cells. Results show data from a single representative experiment. Histograms show mean values $\pm$ SD of the CD11b positive population area delimited in FSC-CD11b contour plots. **p<0.01, ***p<0.001, Student's t-test.

Figure 4. Histograms showing (A) Iba1+ cells associated with vessels in infected and control animals, (B) Iba1+ cells per mm² of retina from retinal sections around the optic nerve head and (C) Total Iba1+ cell number in the ciliary body of control and infected mice. **p<0.01, Student's t-test.

Figure 5. Confocal immunofluorescence images of Iba1+ cells in the ciliary body from a C57BL/6J control mouse (A-B) and a mouse infected with viable yeasts of *C. albicans* ATCC26555 (C-F). Sections were stained with anti-Iba1 and anti-MHCII RT1B antibodies (A, C, E), or anti-MHCII RT1B antibody (B, D, F). Arrows (C-F) point MHCII+ microglial cells. (E-F) Detail of a ciliary body of an infected mouse. Arrows point Iba1+/MHCII+ cells within blood vessels. Scale bar 20 μ m.

References

1. Karlstetter M, Ebert S, Langmann T. Microglia in the healthy and degenerating retina: insights from novel mouse models. *Immunobiology* 2010;215:685-691.
2. Langmann T. Microglia activation in retinal degeneration. *J Leukoc Biol* 2007;81:1345-1351.
3. Polazzi E, Monti B. Microglia and neuroprotection: from in vitro studies to therapeutic applications. *Prog Neurobiol* 2010;92:293-315.
4. Harry GJ. Microglia during development and aging. *Pharmacol Ther* 2013;139:313-326.
5. Czeh M, Gressens P, Kaindl AM. The yin and yang of microglia. *Dev Neurosci* 2011;33:199-209.
6. Benveniste EN. Inflammatory cytokines within the central nervous system: sources, function, and mechanism of action. *Am J Physiol* 1992;263:C1-16.
7. Lucin KM, Wyss-Coray T. Immune activation in brain aging and neurodegeneration: too much or too little? *Neuron* 2009;64:110-122.
8. Hirsch EC, Hunot S. Neuroinflammation in Parkinson's disease: a target for neuroprotection? *Lancet Neurol* 2009;8:382-397.
9. Tansey MG, McCoy MK, Frank-Cannon TC. Neuroinflammatory mechanisms in Parkinson's disease: potential environmental triggers, pathways, and targets for early therapeutic intervention. *Exp Neurol* 2007;208:1-25.
10. Bornemann KD, Wiederhold KH, Pauli C, et al. Abeta-induced inflammatory processes in microglia cells of APP23 transgenic mice. *Am J Pathol* 2001;158:63-73.
11. Griffin WS, Liu L, Li Y, Mrak RE, Barger SW. Interleukin-1 mediates Alzheimer and Lewy body pathologies. *J Neuroinflammation* 2006;3:5.
12. Gupta N, Brown KE, Milam AH. Activated microglia in human retinitis pigmentosa, late-onset retinal degeneration, and age-related macular degeneration. *Exp Eye Res* 2003;76:463-471.
13. Ardeljan D, Chan CC. Aging is not a disease: Distinguishing age-related macular degeneration from aging. *Prog Retin Eye Res* 2013;37:68-89.
14. Penfold PL, Madigan MC, Gillies MC, Provis JM. Immunological and aetiological aspects of macular degeneration. *Prog Retin Eye Res* 2001;20:385-414.
15. Bosco A, Crish SD, Steele MR, et al. Early reduction of microglia activation by irradiation in a model of chronic glaucoma. *PLoS One* 2012;7:e43602.
16. Gallego BI, Salazar JJ, de Hoz R, et al. IOP induces upregulation of GFAP and MHC-II and microglia reactivity in mice retina contralateral to experimental glaucoma. *J Neuroinflammation* 2012;9:92.
17. Lionakis MS, Lim JK, Lee CC, Murphy PM. Organ-specific innate immune responses in a mouse model of invasive candidiasis. *J Innate Immun* 2011;3:180-199.
18. Yanez A, Murciano C, O'Connor JE, Gozalbo D, Gil ML. Candida albicans triggers proliferation and differentiation of hematopoietic stem and progenitor cells by a MyD88-dependent signaling. *Microbes Infect* 2009;11:531-535.

19. Murciano C, Yanez A, Gil ML, Gozalbo D. Both viable and killed *Candida albicans* cells induce in vitro production of TNF-alpha and IFN-gamma in murine cells through a TLR2-dependent signalling. *Eur Cytokine Netw* 2007;18:38-43.
20. Villamon E, Gozalbo D, Roig P, O'Connor JE, Fradelizi D, Gil ML. Toll-like receptor-2 is essential in murine defenses against *Candida albicans* infections. *Microbes Infect* 2004;6:1-7.
21. Murciano C, Villamon E, Gozalbo D, Roig P, O'Connor JE, Gil ML. Toll-like receptor 4 defective mice carrying point or null mutations do not show increased susceptibility to *Candida albicans* in a model of hematogenously disseminated infection. *Med Mycol* 2006;44:149-157.
22. Imai Y, Ibata I, Ito D, Ohsawa K, Kohsaka S. A novel gene *iba1* in the major histocompatibility complex class III region encoding an EF hand protein expressed in a monocytic lineage. *Biochem Biophys Res Commun* 1996;224:855-862.
23. Ito D, Imai Y, Ohsawa K, Nakajima K, Fukuuchi Y, Kohsaka S. Microglia-specific localisation of a novel calcium binding protein, *Iba1*. *Brain Res Mol Brain Res* 1998;57:1-9.
24. Dick AD, Ford AL, Forrester JV, Sedgwick JD. Flow cytometric identification of a minority population of MHC class II positive cells in the normal rat retina distinct from CD45^{low}CD11b/c+CD4^{low} parenchymal microglia. *Br J Ophthalmol* 1995;79:834-840.
25. Guillemin GJ, Brew BJ. Microglia, macrophages, perivascular macrophages, and pericytes: a review of function and identification. *J Leukoc Biol* 2004;75:388-397.
26. Kreutzberg GW. Microglia: a sensor for pathological events in the CNS. *Trends Neurosci* 1996;19:312-318.
27. Sedgwick JD, Schwender S, Imrich H, Dorries R, Butcher GW, ter Meulen V. Isolation and direct characterization of resident microglial cells from the normal and inflamed central nervous system. *Proc Natl Acad Sci U S A* 1991;88:7438-7442.
28. Zinkernagel MS, Chinnery HR, Ong ML, et al. Interferon gamma-dependent migration of microglial cells in the retina after systemic cytomegalovirus infection. *Am J Pathol* 2013;182:875-885.
29. Ajami B, Bennett JL, Krieger C, McNagny KM, Rossi FM. Infiltrating monocytes trigger EAE progression, but do not contribute to the resident microglia pool. *Nat Neurosci* 2011;14:1142-1149.
30. Sasahara M, Otani A, Oishi A, et al. Activation of bone marrow-derived microglia promotes photoreceptor survival in inherited retinal degeneration. *Am J Pathol* 2008;172:1693-1703.
31. Joly S, Francke M, Ulbricht E, et al. Cooperative phagocytes: resident microglia and bone marrow immigrants remove dead photoreceptors in retinal lesions. *Am J Pathol* 2009;174:2310-2323.
32. Ritter MR, Banin E, Moreno SK, Aguilar E, Dorrell MI, Friedlander M. Myeloid progenitors differentiate into microglia and promote vascular repair in a model of ischemic retinopathy. *J Clin Invest* 2006;116:3266-3276.
33. Simard AR, Soulet D, Gowing G, Julien JP, Rivest S. Bone marrow-derived microglia play a critical role in restricting senile plaque formation in Alzheimer's disease. *Neuron* 2006;49:489-502.
34. Kezic J, Xu H, Chinnery HR, Murphy CC, McMenamin PG. Retinal microglia and uveal tract dendritic cells and macrophages are not CX3CR1

- dependent in their recruitment and distribution in the young mouse eye. *Invest Ophthalmol Vis Sci* 2008;49:1599-1608.
35. Xu H, Chen M, Mayer EJ, Forrester JV, Dick AD. Turnover of resident retinal microglia in the normal adult mouse. *Glia* 2007;55:1189-1198.
36. Kaneko H, Nishiguchi KM, Nakamura M, Kachi S, Terasaki H. Characteristics of bone marrow-derived microglia in the normal and injured retina. *Invest Ophthalmol Vis Sci* 2008;49:4162-4168.
37. Graeber MB, Streit WJ. Microglia: biology and pathology. *Acta Neuropathol* 2010;119:89-105.
38. Ransohoff RM, Perry VH. Microglial physiology: unique stimuli, specialized responses. *Annu Rev Immunol* 2009;27:119-145.
39. Gregerson DS, Yang J. CD45-positive cells of the retina and their responsiveness to in vivo and in vitro treatment with IFN-gamma or anti-CD40. *Invest Ophthalmol Vis Sci* 2003;44:3083-3093.
40. Held J, Kohlberger I, Rappold E, Busse Grawitz A, Hacker G. Comparison of (1->3)-beta-D-glucan, mannan/anti-mannan antibodies, and Cand-Tec Candida antigen as serum biomarkers for candidemia. *J Clin Microbiol* 2013;51:1158-1164.
41. Gil ML, Gozalbo D. Role of Toll-like receptors in systemic *Candida albicans* infections. *Front Biosci (Landmark Ed)* 2009;14:570-582.
42. Goodridge HS, Underhill DM. Fungal Recognition by TLR2 and Dectin-1. *Handb Exp Pharmacol* 2008;87:109.
43. Shah VB, Huang Y, Keshwara R, Ozment-Skelton T, Williams DL, Keshvara L. Beta-glucan activates microglia without inducing cytokine production in Dectin-1-dependent manner. *J Immunol* 2008;180:2777-2785.
44. Shah VB, Williams DL, Keshvara L. beta-Glucan attenuates TLR2- and TLR4-mediated cytokine production by microglia. *Neurosci Lett* 2009;458:111-115.
45. Maneu V, Yanez A, Murciano C, Molina A, Gil ML, Gozalbo D. Dectin-1 mediates in vitro phagocytosis of *Candida albicans* yeast cells by retinal microglia. *FEMS Immunol Med Microbiol* 2011;63:148-150.
46. Magrone T, Marzulli G, Jirillo E. Immunopathogenesis of neurodegenerative diseases: current therapeutic models of neuroprotection with special reference to natural products. *Curr Pharm Des* 2012;18:34-42.
47. Halder SK, Matsunaga H, Ishii KJ, Akira S, Miyake K, Ueda H. Retinal cell type-specific prevention of ischemia-induced damages by LPS-TLR4 signaling through microglia. *J Neurochem* 2013;126:243-260.
48. Tremblay S, Miloudi K, Chaychi S, et al. Systemic inflammation perturbs developmental retinal angiogenesis and neuroretinal function. *Invest Ophthalmol Vis Sci* 2013;54:8125-8139.

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