Published in final edited form as:

Adv Exp Med Biol. 2014 ; 801: 339–345. doi:10.1007/978-1-4614-3209-8_43.

Isolation and *Ex Vivo* Characterization of the Immunophenotype and Function of Microglia/Macrophage Populations in Normal Dog Retina

Sem Genini¹, William A. Beltran¹, Veronika M. Stein², and Gustavo D. Aguirre¹

¹Department of Clinical Studies, School of Veterinary Medicine, University of Pennsylvania, Philadelphia, PA 19104, USA

²Department of Small Animal Medicine and Surgery, University of Veterinary Medicine Hannover, 30559 Hannover, Germany

Abstract

Microglia are the primary resident immune cells of the retina and are involved in the pathogenesis of various retinal diseases. In this study, we optimized experimental conditions to isolate microglia from canine retinas and characterized *ex vivo* their immunophenotype and function using flow cytometry (FACS). The most suitable protocol included a mechanical dissociation of the retina and an enzymatic digestion using DNase and collagenase. Extraction was carried out by density gradient centrifugation, and retinal microglia accumulated on distinct interfaces of 1.072 and 1.088 g/mL of a Percoll gradient. Immunophenotypical characterization was performed with monoclonal antibodies CD11b, CD11c, CD18, CD45, CD44, B7-1 (CD80), B7-2 (CD86), CD1c, ICAM-1 (CD54), CD14, MHCI, MHCII, CD68, CD3, CD4, CD8α, and CD21. The most prevalent microglia population in the normal canine retina is CD11b^{high}CD45^{low}. Functionally, retinal microglia exhibited phagocytosis and reactive oxygen species (ROS) generation activities. To conclude, *ex vivo* examinations of retinal microglia are feasible and possibly reflect the *in vivo* conditions, avoiding artifacts observed in tissue culture. The established method will be relevant to examine microglia from diseased canine retinas in order to elucidate their roles in degenerative processes.

Keywords

dog model; retinal microglia; density percoll gradient extraction; *ex vivo* examination; flow cytometry (FACS) analysis; immunophenotype characterization; phagocytosis assay; reactive oxygen species (ROS) generation test

43.1 Introduction

Microglia are important resident immune cells of the retina and central nervous system (CNS). They are particularly sensitive to changes in the surrounding environment, becoming readily activated in host response to infection or injury (reviewed by [1]). Microglia occur in

different isoforms and respond to pathological events by progressing from a resting ramified state to an active state with retraction of processes [2]. In retina, these active sentinels have essential roles in controlling development, aging, and function by secreting growth factors and inflammatory cytokines to promote either neuroprotection or neuronal damage. They also have been implicated in the pathogenesis of various retinal diseases [3-5].

Microglia isolation and purification *ex vivo* is complex; difficulties include contamination with macrophages, a relatively small number of microglia present in tissues, and absence of specific markers differentiating microglia from other blood derived mononuclear cells [6, 7]. However, *ex vivo* analysis has the great advantage to more closely reflect *in vivo* conditions compared to results obtained using cell culture systems. Previous studies established microglia isolation protocols in mouse [6] and rat [8] CNS, canine spinal cord [9], and canine brain that was either normal [7] or infected with canine distemper virus [10]. Retinal microglia have been isolated and characterized in humans [11] and rats [12] using Percoll density gradient centrifugation and FACS analysis, but these experimental tools have not yet been applied to dog retinas.

With the goal of characterizing microglia immunophenotypes and function in different retinal diseases, we have developed a protocol for *ex vivo* isolation of microglia from canine retinas.

43.2 Materials and Methods

43.2.1 Dogs

Normal retinas from mixed-breed dogs were examined to define optimal experimental conditions for microglia isolation and characterization. The ages were 7 (dog #1, female), 20 (dog #2, female), 25 (dog #3, female), and 35 weeks (dog #4 and #5, males). The research was conducted in full compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

43.2.2 Ex vivo isolation of canine retinal microglia

Following an optimized protocol developed for brain [7], dogs were given 12,000 units of heparin intravenously and euthanized by pentobarbital overdose. Immediately after death, perfusion was performed with 1 L of normal saline solution via left ventricle of the heart. Sufficient perfusion was indicated by water-like fluid leaving the right atrium and the absence of blood in the retinal veins, as assessed by indirect ophthalmic examination. Following perfusion, eyes were removed and both neuroretinas separated and pooled. After mincing through a stainless-steel sieve, mechanically dissociated cells were centrifuged and then enzymatically digested for 30 min at 37 °C with type II collagenase (5.7 mg/g retina; Roche Diagnostics) and DNase I (500 units/g retina; Sigma-Aldrich). A Percoll gradient was established in a 15 mL Falcon tube with 2 mL of Percoll (GE-Amersham Biosciences) diluted in Hanks' buffer at 1.124 g/mL, subsequently overlaid with 2 mL Percoll of 1.088 g/mL, 2 mL of 1.072 g/mL, and finally 2 mL of 1.030 g/mL containing the cell solution. After centrifugation microglia were collected from the interfaces of the 1.072 (majority of cells) and 1.088 g/mL (less cells) layers. Microglia were adjusted to a concentration of 2×10^5 cells in 50 mL, immunostained, and analyzed immediately by FACS.

The above-described protocol was applied for dog #1, while for dogs #2 and #3 neither perfusion nor DNase and collagenase digestion were performed. The cells of dogs #4 and #5 were isolated using two successive Percoll gradients as previously done for microglia isolation from rats [8] and dogs [7, 9]; an initial gradient consisting of two densities and a major gradient with five densities, including an additional density of 1.060 g/mL. Microglia from dog #5 were collected separately from Percoll densities 1.060 and 1.072 g/mL and, as the number of cells was lower, no functional analyses were performed.

43.2.3 Monoclonal antibodies (mAb) and immunophenotyping

Microglia characterization was performed with mAbs binding the epitopes B7-1 (CD80), B7-2 (CD86), CD11b, CD11c, CD18, CD1c, ICAM-1, CD3, CD4, CD8 α , CD21, and MHC class II (dilution 1:5; Leukocyte Antigen Biology Laboratory, University of California, Davis), CD14 (1:10; Dako), CD44 (1:10; Serotec), CD45 (1:10; Serotec), MHC class I (1:20; Veterinary Medical Research & Development), and CD68 (1:10; Santa Cruz Biotechnology). Secondary antibodies (dilution 1:100) were goat anti-mouse (Jackson Immuno Research Laboratories), rabbit anti-rat (Serotec) for CD44, and streptavidin-conjugated fluorescein-isothiocyanate (Serotec) for CD45. Cell preparations with secondary Abs only served as negative controls. Cells were measured immediately after incubation and washing using a FACSCalibur™ (BD Biosciences) flow cytometer and analyzed with the CellQuest™ software. Microglia were identified based on the parameters size (FSC), complexity (SSC), and relative expression of CD11b^{high} and CD45^{low}, as previously shown [6, 11, 12].

43.2.4 Phagocytosis assay

Directly FITC-labeled *Staphylococcus aureus* (Life Technologies) were suspended in PBS, adjusted to a concentration of 10⁸ bacteria/mL, and incubated with either PBS (non-opsonized bacteria) or pooled dog serum (opsonized bacteria). Non-opsonized and opsonized bacteria suspensions were added to the microglia (1:100) and incubated for 60 min at 37 °C and 5% CO₂ [7]. PBS served as negative control. Phagocytosis was measured immediately using a FACSCalibur™ flow cytometer.

43.2.5 ROS generation test

Production of reactive oxygen species (ROS) by microglia was measured as previously described [7, 10]. Briefly, 2 × 10⁶ microglia/mL were stimulated with either PBS or phorbol-12-myristate-13-acetate (PMA; Sigma-Aldrich), followed by 15 µg/mL dihydrorhodamine (DHR) 123 (Marker Gene Technologies Inc.). A tube containing only microglia was used as negative reference. ROS generation was measured immediately using a FACSCalibur™ flow cytometer.

43.3 Results

We developed a protocol for *ex vivo* analysis of canine retinal microglia based on previous reports for brain and spinal cord [7, 9]. Five young dogs were examined and retinal cells collected under slightly different conditions to achieve the highest purified microglia populations. Retinal microglia showed a characteristic immunophenotype of

CD11b^{high}CD45^{low} (Fig. 43.1a). In normal dogs, retinal microglia represented a population of relatively small cells with low complexity (Fig. 43.1b, gate R1). All 17 surface markers could be detected in the microglia of the 5 dogs; the % of positive cells is presented in Table 43.1. Dogs #1-3 showed low contamination of the retinal microglia cell yield with lymphocytes (cells positive to CD3, CD4, or CD21 <2.4%), while dogs #4 and #5 (density 1.060) showed low expression of the microglia markers and high expression of the lymphocyte markers.

Phagocytosis assay and ROS generation test were performed in dogs #1-4. As duplicates showed high consistency, the median was used for analysis. Canine retinal microglia performed phagocytosis after adding both, opsonized ($\bar{x} = 64\%$) and non-opsonized ($\bar{x} = 60\%$) *S. aureus* (Fig. 43.2). In all dogs, the % of phagocytosing microglia increased with opsonized compared to non-opsonized bacteria.

ROS generation test demonstrated that canine retinal microglia were able to produce ROS. However, no increase in intensity and % of ROS generating microglia was noted after addition of PMA compared to PBS.

43.4 Discussion and Conclusions

In the present study, we developed an *ex vivo* extraction protocol to examine the microglia cell population from dog retinas. A total of 13 different markers known to be expressed on microglia (CD11b, CD11c, CD18, CD45, CD44, B7-1, B7-2, CD1c, ICAM-1, CD14, MHCI, MHCII, CD68) were analyzed by FACS. Additionally, CD3, CD4, CD21, and CD8 α were tested to verify contamination with lymphocytes although CD8 α has been shown to also be expressed by mouse microglia [13, 14]. Our data showed CD8 α -positive microglia and suggested that this marker is present in canine retinal microglia.

The use of two successive Percoll gradients (dogs #4 and #5) considerably decreased the number of isolated microglia and did not result in an increased purity of the cell yield. Isolation of microglia from canine brain and spinal cord required an initial gradient in particular to remove myelin [7, 9]. As the mammalian retina is devoid of oligodendrocytes and the axons of retinal ganglion cells are not myelinated where they course through the retina, an initial gradient was not necessary. Cells from the Percoll densities 1.088 and 1.072 g/mL were highly positive to microglia markers, while cells from other interfaces (i.e. 1.060 of dog #5) were predominantly lymphocytes.

Based on these results, the optimal experimental conditions for microglia extraction and testing *ex vivo* seem to include perfusion, mechanical dissociation and enzymatic (DNase and collagenase) digestion, separation with one Percoll gradient, and collection from the Percoll interfaces 1.072 and 1.088 g/mL.

Functionally, canine retinal microglia from all tested dogs exhibited phagocytosis activities, and had the capability to generate ROS. They could be triggered to ingest *S. aureus* bacteria, but not to produce more ROS with the trigger PMA. ROS generation has also been described in macrophages [15] and brain microglia [7, 10].

This method to analyze microglia *ex vivo* will be useful in future studies to evaluate and compare the immunophenotype and the function of different microglia populations associated with photoreceptor degeneration in canine models, e.g. rcd1, XLPRA1, XLPRA2, which carry mutations in genes known to cause human inherited blindness. Indeed, this *ex vivo* method will be valuable in examining the effects of therapeutic strategies on microglia populations, as it might better reflect *in vivo* conditions compared to tissue cultures, where artifacts are often observed.

Acknowledgments

This study was supported by NIH Grants EY06855 and EY017549, the Foundation Fighting Blindness (FFB), the Van Sloun Fund for Canine Genetic Research, and Hope for Vision. Many thanks to the National Eye Institute, NIH, for funding a travel award to SG. VMS was supported by the German Research Foundation (STE 1069/2-1).

References

1. Garden GA, Moller T. Microglia biology in health and disease. *J Neuroimmune Pharmacol.* 2006; 1:127–37. [PubMed: 18040779]
2. Kettenmann H. Triggering the brain's pathology sensor. *Nat Neurosci.* 2006; 9:1463–4. [PubMed: 17128280]
3. Langmann T. Microglia activation in retinal degeneration. *J Leukoc Biol.* 2007; 81:1345–51. [PubMed: 17405851]
4. Sasahara M, Otani A, Oishi A, Kojima H, Yodoi Y, Kameda T, et al. Activation of bone marrow-derived microglia promotes photoreceptor survival in inherited retinal degeneration. *Am J Pathol.* 2008; 172:1693–703. [PubMed: 18483210]
5. Karlstetter M, Ebert S, Langmann T. Microglia in the healthy and degenerating retina: Insights from novel mouse models. *Immunobiology.* 2010; 215:685–91. [PubMed: 20573418]
6. de Haas AH, Boddeke HW, Brouwer N, Biber K. Optimized isolation enables *ex vivo* analysis of microglia from various central nervous system regions. *Glia.* 2007; 55:1374–84. [PubMed: 17661344]
7. Stein VM, Czub M, Hansen R, Leibold W, Moore PF, Zurbriggen A, et al. Characterization of canine microglial cells isolated *ex vivo*. *Vet Immunol Immunopathol.* 2004; 99:73–85. [PubMed: 15113656]
8. Ford AL, Goodsall AL, Hickey WF, Sedgwick JD. Normal adult ramified microglia separated from other central nervous system macrophages by flow cytometric sorting. Phenotypic differences defined and direct *ex vivo* antigen presentation to myelin basic protein-reactive CD4+ T cells compared. *J Immunol.* 1995; 154:4309–21. [PubMed: 7722289]
9. Ensinger EM, Boekhoff TM, Carlson R, Beineke A, Rohn K, Tipold A, et al. Regional topographical differences of canine microglial immunophenotype and function in the healthy spinal cord. *J Neuroimmunol.* 2010; 227:144–52. [PubMed: 20728950]
10. Stein VM, Czub M, Schreiner N, Moore PF, Vandevelde M, Zurbriggen A, et al. Microglial cell activation in demyelinating canine distemper lesions. *J Neuroimmunol.* 2004; 153:122–31. [PubMed: 15265670]
11. Broderick C, Duncan L, Taylor N, Dick AD. IFN-gamma and LPS-mediated IL-10-dependent suppression of retinal microglial activation. *Invest Ophthalmol Vis Sci.* 2000; 41:2613–22. [PubMed: 10937574]
12. 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; 79:834–40. [PubMed: 7488603]
13. 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; 44:3083–93. [PubMed: 12824255]

14. Dimayuga FO, Reed JL, Carnero GA, Wang C, Dimayuga ER, Dimayuga VM, et al. Estrogen and brain inflammation: effects on microglial expression of MHC, costimulatory molecules and cytokines. *J Neuroimmunol.* 2005; 161:123–36. [PubMed: 15748951]
15. Tipold A, Zurbriggen A, Moore P, Schijns V, Jungi TW. Generation and functional characterisation of canine bone marrow-derived macrophages. *Res Vet Sci.* 1998; 64:125–32. [PubMed: 9625468]

Fig. 43.1.

Retinal microglia have a characteristic immunophenotype of CD11b^{high}CD45^{low}. The histograms show expression intensities (mean fluorescent channel numbers) of CD11b (**a1**) and CD45 (**a2**) measured in two fluorescence channels (FL-H) compared to negative controls. (**b**) The isolated retinal microglia form a population of relatively small cells (forward scatter, FSC-H) with low complexity (side scatter, SSC-H). Only cells within the gate R1 were further analyzed.

Fig. 43.2.

Retinal microglial phagocytosis after incubation with (a) PBS (negative control), (b) non-opsonized, and (c) opsonized FITC-labeled *S. aureus*. The x-axis shows fluorescence intensity and the y-axis cell complexity.

Table 43.1
Retinal microglia expression (in %) of 14 microglia and 3 (CD3, CD4, CD21) lymphocyte surface molecules. Values of 5 normal dogs are shown; in dog #5 cells were collected from Percoll densities 1.060* and 1.072 g/mL and evaluated separately**

Antibody	Dog					Mean
	#1	#2	#3	#4	#5*	
CD11b	7.3	24.5	11.5	3.1	6.6	18.5
CD11c	6.8	23.3	12.7	2.4	6.7	39
CD18	10.8	26.5	24.9	3.2	3	26.9
CD45	6.9	3	8.1	1.5	4.5	26.7
CD44	55.8	69.6	93.4	47.8	70.8	52.4
B7-1	11.6	30.8	10	5.9	3.7	13
B7-2	13.0	6.9	8.9	4.6	3.8	23.3
CD1c	31.5	37.3	9.7	3.7	3.9	33.5
ICAM-1	34.2	39.3	33.2	6.5	2.7	36.4
CD14	24.5	19.1	11.6	1.8	3	27.4
MHCI	67.1	12.6	24.2	33	23.8	35.1
MHCII	9.1	32.1	23.2	3.8	2	26.1
CD68	3.3	0.3	1.9	2.3	1.9	17.8
CD8a	7.4	21.2	31.5	1.8	4.9	25
CD3	0.6	1.4	0.9	2.6	5.6	1.3
CD4	1.1	1.1	1.4	2.2	19.1	1.7
CD21	1.3	0.8	2.5	1.7	5.6	2.2