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

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Abstract

Aims—This study aimed to isolate and classify by flow cytometry, the cell surface phenotype of microglia in the normal rat retina with a view to identifying putative antigen presenting cells (APC) within the retina, which has to date not been possible by immunohistochemistry.

Methods—Normal rat retinal microglia were isolated and classified using a modification of an isolation technique employing graduated Percoll density gradient cell separation and flow cytometric phenotypic criteria used for CNS microglia.

Results—Retinal microglia can be defined by flow cytometry on the basis of their CD45^{low}CD11b/c⁺CD4^{low} cell surface expression. Constitutive MHC class II expression in the normal rat retina was confined almost exclusively to a very minor population of cells expressing neither low (microglia) nor high levels of CD45. Three colour flow cytometric analysis confirmed that these MHC class II positive cells were ED2⁺.

Conclusions—Using this sensitive isolation technique we have identified the cell surface characteristics of ramified, resident microglia, and found that they do not constitutively express MHC class II. There is, however, constitutive MHC class II expression on a phenotypically distinct population of cells (CD45^{low/high}ED2⁺). We propose these cells are the counterpart of the perivascular macrophages found in the CNS which present antigen to extravasating T cells, although their exact retinal location can only be confirmed by immunohistochemical analysis. The role of parenchymal microglia as APC remains undefined. Future isolation of microglia and putative perivascular cells using this technique will help identify the role these cells play in the initiation and perpetuation of immune responses within the retina.

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Experimental autoimmune uveoretinitis (EAU) is an established model for auto-

immune endogenous posterior uveitis in humans.^{1 2} This model of intraocular inflammation is mediated by MHC class II restricted CD4⁺ T cells which infiltrate the retina early in the course of the disease and, histologically, can be seen entering the retina through both the inner retinal vessels and choroid, ultimately causing destruction of the photoreceptor outer segments where the major uveitogenic antigens are found.^{1–3} Local antigen presentation by MHC class II positive antigen presenting cells (APC) is required to activate systemically primed CD4⁺ T cells and initiate the autoimmune inflammatory response. Recently there have been reports of inducible MHC class II antigen expression on non-haemopoietic structural elements within the eye including retinal pigment epithelium, vascular endothelium, and Müller cells.^{4–6} This is analogous to the situation in the CNS where both astrocytes⁷ and endothelial cells^{8 9} can be induced to express MHC class II molecules and process and present protein antigen to T lymphocytes, at least in vitro (although do not necessarily stimulate the T cells to divide^{8 10 11}). However, there is no evidence that any of these cell types function as APC in vivo.¹² Morphological identification of haemopoietically derived candidate APC within the eye have included the rich network of classic dendritic cells expressing high levels of MHC class II in the choroid¹³ and iris/ciliary body.¹⁴ The presence of perivascular cuffing in uveitis also argues for the existence of retinal vessel associated APC to enable antigen recognition. However, no such APC has been definitively identified within the rat retina. Thus, to date, the major candidate APC within the rat retina are parenchymal ramified microglia, similar to CNS microglia. These express the CD11b/c molecule, are thought to be bone marrow derived,¹⁵ but are not MHC class II positive.¹⁴ In contrast, immunohistochemical analysis of adult human retina has revealed constitutive MHC class II expression on two classes of macrophage lineage cells, expressing low and high levels of the CD45 molecule and representing resident microglia and perivascular macrophage populations respectively,^{16 17} analogous to well defined CNS populations in

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Figure 1 CD45 expression and scatter profile of retinal microglia. Labelling of both retinal and CNS preparations with CD45 mAb subdivides the cells into three main populations (1–3). Number 2 denotes CD45^{low} population (microglia) whose scatter profile seen in the bottom three dot plots is similar for retina (of both LEW and PVG) and CNS preparations.

rat.^{18 19} In the past, not only has immunohistochemistry of the rat retina failed to either distinguish populations of CD45⁺ cells or demonstrate MHC class II expression but there has been no in vitro or in vivo assessment of the putative APC capacity of human retinal microglia/macrophages. Here we analyse the cell surface phenotype of retinal microglia in the rat by flow cytometry in an attempt to classify putative APC. A minority non-microglial population was identified which has a similar phenotype to CNS perivascular macrophages and is the predominant MHC class II antigen expressing cell within the retina.

Materials and methods

ANIMALS

Inbred adult rats of the Lewis (LEW) and PVG strains were obtained from the Animal Resources Centre (Perth, Australia) or bred in local facilities (Blackburn Animal House, University of Sydney). Six-week-old (150–200 g) animals were used in the experiments which were conducted according to the code of practice for experiments on animals and guidelines set by the National Health and Medical Research Council (Australia), which adhered to the ARVO statement for the use of animals in ophthalmic and vision research. Each experiment was conducted on two or three rats (four to six retinas) in order to achieve enough cell numbers for flow cytometric analysis and was repeated at least three

times except where indicated (n refers to number of experiments).

PREPARATION OF RETINAL AND CNS MICROGLIA
Animals were killed by carbon dioxide asphyxiation then perfused slowly through the left ventricle by intracardiac catheterisation with 250 ml of cold phosphate buffered saline (PBS) with heparin (250 U) to remove circulating mononuclear cells. The retinas from enucleated eyes were microscopically dissected in cold PBS 0.02% (w/v)/bovine serum albumin (BSA) taking care to remove the retina posterior to the ciliary body/pars plana to exclude ciliary body/choroidal contamination, and then disrupted mechanically through a stainless steel sieve. The dissociated material was washed by centrifugation at $400\times g$ for 10 minutes in 50 ml of PBS/BSA. Subsequent isolation steps were modified from the previously described technique for CNS microglia.^{18 19} In brief, a 2 ml cell suspension of dissociated retinas in PBS/BSA ($4\text{--}8\times 10^5$ cells/retina) was added to a graduated Percoll (Pharmacia) gradient prepared in a 10 ml conical bottomed tube. This consisted of 2 ml steps of Percoll at 1.21 g/ml, 1.088 g/ml, 1.072 g/ml, and 1.03 g/ml. This gradient was centrifuged at $1250\times g$ for 20 minutes at 20°C with slow acceleration time and no brake. Cells were collected from both the 1.072 and 1.088 g/ml layers although the majority of cells were present at 1.072 g/ml. Viability was determined by trypan blue exclusion after a further wash in PBS/BSA. At this stage $1\text{--}2\times 10^5$ cells/retina

Figure 2 CD11b/c⁺ expression on CD45^{low} retinal microglia. Two colour flow cytometric analysis of both retina and CNS showing that the entire CD45^{low} population (8% in retina and 39% in CNS of gated populations, arrow) express the CD11b/c molecule (complement receptor 3/P150-95) known to be present on microglia. This further differentiates the CD45^{high} cell population (population 3, Fig 1) which is split into CD11b/c⁺ and CD11b/c⁻ cells.

were isolated after density centrifugation for analysis. CNS microglia were isolated as previously described,^{18 19} but only from Lewis strain rats. The significant difference between the CNS and retinal procedures is that the former requires collagenase/DNAse digestion to enhance cell yield and a low density gradient to remove myelin. The non-myelinated retina do not require these steps.

MONOCLONAL ANTIBODIES (mAb) AND FLOW CYTOMETRY

Mouse mAb specific for rat cell surface markers were originally generated from hybridomas provided by the MRC, Cellular Immunology Unit, Oxford which include MRC OX1 (anti-CD45), MRC OX42 (anti-CD11b/c), W3/25 (anti-CD4), MRC OX6 (anti-MHC class II, I-A), MRC OX21 (anti-human C3bi and not rat cells), and R73 (anti- $\alpha\beta$ TCR) from Dr Thomas Hünig, Würzburg, Germany (see Sedgwick *et al*¹⁸ and Ford *et al*¹⁹ for cross reference details). ED2 mAb²⁰ was kindly provided by Dr Christine Dijkstra (Amsterdam, the Netherlands). Phycoerythrin (PE) conjugated anti-CD45 was purchased directly from Serotec, UK. mAb MRC OX21 was used as an isotype control for flow cytometry. mAb were prepared as tissue culture supernatant (TCS) and as ascites (ED2). For direct staining, mAb were purified and conjugated to biotin or fluorescein isothiocyanate (FITC). TCS mAb was detected with rat absorbed reagent, FITC conjugated sheep F(ab')₂ anti-mouse Ig (Sigma, USA) and biotinylated mAb were detected either with streptavidin-PE (SA-PE, Caltag, USA) for two colour flow cytometry or streptavidin-Texas Red (SA-TxR, Molecular Probes Inc, USA) for three colour flow cytometry.

Staining was performed as previously detailed^{18 19} using FACS buffer (PBS/BSA/10 mM NaN₃) for washes. All reagents, buffers, and incubations were kept and performed at 4°C. In brief, for two colour labelling a cell suspension of between 10⁴ and 10⁵ cells was incubated sequentially with TCS mAb or a 1:50 dilution of ED2 ascites, FITC conjugated sheep F(ab')₂ anti-mouse Ig in the

presence of 10% normal rat serum (NRS), blocked with 10% NRS/normal mouse serum (NMS), biotinylated second mAb in the presence of both NMS and NRS and finally SA-PE. For three colour labelling, steps were the same except the second mAb was directly conjugated to PE (OX1-PE) and biotinylated mAb were detected with SA-TxR. Cells were fixed with a final 1% (v/v) formalin solution and stored at 4°C until analysis was performed. Negative controls (identical species and isotype) and single fluorochrome controls were performed to allow accurate breakthrough compensation. Two colour phenotypic analysis of both retina and CNS preparations were performed using FACScan (Becton Dickinson, USA). Three colour phenotypic analysis was performed using a FACStar^{Plus} sorter (Becton Dickinson, USA) and LYSIS II acquisition and analysis software. A total of 10 000 events were collected and both microglial gates and instrument variables were set according to their forward and side scatter characteristics. Analysis of fluorescence was performed after further back-gating to exclude dead cells and aggregates. Thus, in most cases, profiles represent less than 50% of collected events.

Results

SCATTER PROFILES AND CD45 EXPRESSION

Cells were isolated from the retina of two rat strains, the albino LEW and the PVG. Staining of retinal cells as well as those isolated from the normal LEW rat CNS with an antibody specific for the leucocyte common antigen (CD45) found only on cells of haemopoietic origin, revealed three major populations (top, Fig 1). The characteristic cell surface phenotype of microglia is based on their CD45 expression.^{18 19} There are two populations of CD45 expressing retinal cells; CD45^{low} microglia (population 2), accounting for 6.2–9% (n=4) of gated cells and CD45^{high} cells (population 3) accounting for 0.6–1.9% of gated cells (n=4). In CNS both these populations are present at a higher percentage of the total and this can be attributed

Figure 3 CNS derived CD45^{high} cells can be proportionally reduced by removal of meninges and choroid plexus. (A) Two colour labelling with CD45 and CD11b/c of whole CNS preparation reveals a characteristic four population profile (see Fig 2). (B) Analysis of tissue after removal of meninges, tissue dissection away from the ventricles, and then needle biopsies taken from the remaining tissue shows an increase in the microglial yield (population 2) with a corresponding reduction in CD45^{high} cells (populations 3 and 4).

to the significantly lower proportion of non-haemopoietic (CD45⁻, population 1) cells recovered in the CNS compared with the retina. In retinas, population 1 is most likely to be neural retinal elements and bipolar and ganglion cells because of their scatter profile and lack of CD45 expression. Comparable cells are not recovered with similar efficiency from the CNS and we believe this is due to the fact that many would be lost during the initial low density purification step to remove myelin (see methods). Cell size may also be a relevant factor. Forward and side scatter (Fig 1, bottom) are a measure of cell size and heterogeneity respectively, and illustrate the relatively small size of population 2 (microglia). They also show population 2 in all preparations has very similar characteristics. Additionally, when analysing cells by this technique, electronic forward and side scatter gates are placed around the major population of cells to exclude dead (small) or aggregated (large) cells and population 1 cells in the retina obviously fall into a gate size that is not significantly different from microglia, whereas larger motor neurons in the CNS, for example, may be excluded. It is not possible, nor is it the intention of this study to compare precisely the populations isolated from retinas and CNS, but the latter was included here in view of the substantial characterisation of microglia and other associated macrophages already documented for this tissue.^{18 19} In particular, the whole CNS preparation contains relatively higher numbers of CD45^{high} cells (Fig 1). In both CNS and retinas, this population can be further subdivided into CD11b/c positive (other macrophages) and negative populations (Fig 2). If the CNS is prepared by dissecting frontal lobe away from the ventricles, followed by removal of meninges, including dura mater, arachnoid, and pia mater, and needle biopsy of the remaining tissue the CD45^{high} population is substantially reduced (Fig 3). Whether the remaining CD45^{high} cells are exclusively perivascular/parenchymal and a parallel

population to the CD45^{high} cells of the retina is difficult to determine and for this reason we have not further characterised their phenotype. Figure 2 also shows that all the CD45^{low} microglial population are CD11b/c⁺ (arrow), a monocyte/granulocyte marker which is known to be expressed by both CNS and retinal microglia.^{14 18 19} There was no apparent species difference between LEW and PVG with respect to proportion of cell populations and cell surface phenotype. The slightly different levels of CD45 expression in LEW and PVG retinal microglia represents the use of altered fluorescence gain settings only.

PHENOTYPIC CHARACTERISATION OF RETINAL MICROGLIA

Two colour flow cytometric analysis was used to assess CD4, MHC class II, and ED2 cell surface expression on isolated retinal CD45^{low} (microglial) and CD45^{high} (mixed) populations. CD4/CD45 staining (Fig 4A) subdivides the populations into four parts (1–4). Population 4 (CD45⁻) are CD4⁻ as expected. Populations 1–3 which are all CD45⁺ are a complex mixture of cells. Microglia (CD45^{low}, population 3) are CD4^{low} with average CD4 levels around two times above background. Population 2 is clearly CD4⁺ although levels vary, particularly in the CNS, where there is a mixture of CD4⁺ T lymphocytes (CD4^{high}, around 20 times above background, arrow) with the remainder being macrophages which express slightly less CD4 (5–6 times above background, arrowhead). Most of population 2 in the retina are also macrophage lineage cells on this basis and αβ T cell receptor positive cells are difficult to detect in retinal preparations because of their very low numbers (data not shown). Population 1 is CD4⁻ which would include some CD8⁺ T lymphocytes particularly in the CNS. Flow cytometric analysis reveals a very small population of MHC class II⁺ cells in the retina (0.36–0.42% of gated cells, n=3; R1, Fig 4B) which is substantially less than the equivalent population of cells seen in the whole CNS (6.3% of gated cells). In both retina and CNS, MHC class II expressing cells are positioned midway between CD45^{high} and CD45^{low} populations. In a similar location, a small population of ED2⁺ cells (0.35–0.41% of gated cells, n=3) have been identified in the retina (R1; Fig 4C) and in the CNS (around 1% of total).

RETINAL MHC CLASS II EXPRESSION IS CONFINED TO ED2⁺ CELLS

Constitutive MHC class II expression in the CNS cannot be accounted for by ED2⁺ cells alone (Figs 4B and C) and in view of the whole CNS preparations used here (see above), this is not surprising. Moreover, published data examining MHC class II expression in the rat CNS at the immunohistological level, indicate that not all MHC class II⁺ cells are ED2⁺.²² However, the similarity in percentage positive MHC class II and ED2 expressing cells in the retina implied these were the same population.

Figure 4 CD4, MHC class II, and ED2 expression on CD45⁺ cells. (A) Population 3 (CD45^{low} microglia) are CD4^{low}. The true negative control for this population is exhibited by the negative ED2 staining in (C). Both anti-CD4 and anti-ED2 mAb are of the mouse IgG1 isotype. Thus the shift of population 3 to the right in (A) is real. Population 2 contains some CD4⁺ T cells in the CNS (arrow) with the remainder being macrophages (arrowhead). The CD45⁺ population 4 are CD4⁺ (B) and (C). Minor populations of cells (R1) which are CD45⁺ MHC class II⁺ and CD45⁺ ED2⁺ respectively.

To confirm this, three colour flow cytometry was required and the results of this are shown in Figure 5. By gating around CD45⁺ED2⁺ or CD45⁺ED2⁻ populations and then assessing these for MHC class II expression it is clear that the majority (>85%) of ED2⁺ cells are MHC class II⁺ while few, if any of the ED2⁻ cells are. The latter includes all microglia.

Substantial MHC class II expression is found in the uvea of the eye.^{13 14} Contamination of retinal preparations by this tissue can be excluded as follows: (1) retina was carefully dissected posterior to the ciliary body and also a frill of retina was left around the optic nerve head preventing choroidal seeding when the retina was removed. (2) The retina was easily identified, particularly in the pigmented

PVG, as separate from the choroid because of the presence of retinal pigment epithelium. (3) Choroidal MHC class II⁺ cells are, in the majority ED2 negative,¹³ a phenotype we *did not* identify in the pure retinal preparations.

Discussion

The eye has been considered an immune privileged site because of the lack of dedicated lymphatics and the absence of professional APC. With the recent evidence that the uvea has cells which at least morphologically and phenotypically resemble cells known to act as competent APC,^{13 14 21} the question arises as to whether the retina itself contains any immuno-competent cells which may play a role in the inflamed retina. We have defined by flow cytometric analysis, rat retinal microglia which have markers in common with haemopoietic derived cells, in particular, CD45 and CD11b/c. This resident microglial population has a similar phenotype (CD45^{low}CD11b/c⁺CD4^{low}) to CNS microglia.^{18 19} Retinal microglia can be distinguished from another minor population of cells within the rat which have not been previously described and these express the ED2 marker²⁰ and intermediate levels of CD45 (CD45^{low/high}). Most significantly, three colour flow cytometric analysis provided unequivocal evidence that the majority of retinal ED2⁺ cells are MHC class II⁺, clearly distinguishing them from ED2⁺ cells in the adjacent choroid where most are MHC class II negative.¹³ Retinal derived ED2⁺ cells account for almost all the MHC class II staining in the normal rat retina.

Two aspects are of note. Firstly, the ED2⁺ cell population is present at a rate of approximately 1:250 cells, and this assumes an equal efficiency of isolation of all cell types. In fact, it is likely that highly processed cells including Müller cells and many neuronal elements may be isolated with lower efficiency, so the 1:250 frequency is probably an overestimate. Thus, the paucity of ED2⁺ cells may account for the documented absence of MHC class II staining in retina by immunohistochemistry, a technique^{13 14} which is unable to sample entire tissue as we have done here. Secondly, previous reports on isolation, phenotype, and culture of microglia from the CNS and retina which have proposed the microglia as a candidate APC within these tissues have used markers to define the microglial population (for example, CD11b) which are also shared by some peripheral blood leucocytes as well as the minority perivascular cell population. It is clearly difficult to distinguish between resident microglial cells (parenchymal), perivascular cells, other associated macrophages, and those cells infiltrating from the blood by means other than flow cytometry.

The animal model of posterior uveitis, EAU, demonstrates cells which enter the retina from the choroid, where a large inflammatory response is seen and also the inner retinal vessels where large perivascular cuffs develop. The severity of choroiditis and inner retinal vasculitis varies with type and dose of

Figure 5 Most constitutive MHC class II expression in the normal retina is on ED2⁺ cells. Retinal cells were stained for three colour flow cytometry for the markers CD45, ED2, and MHC class II. The dot plot (left) is a representative example of expression of ED2 versus CD45 by two colour flow cytometry. The MHC class II expression of two populations, R1 (CD45⁺ED2⁻) and R2 (CD45⁺ED2⁺) was assessed by forming analysis regions as shown.

antigen given¹² and between actively and passively induced disease.³ As we have mentioned, it has been suggested that the choroidal MHC class II⁺ cells act as local APC for the circulating autoreactive T cells which reach the choroid, but as yet no cell has been identified which may act as an APC for T cells which have entered the retina through the inner retinal vessels. We have shown that a small population of MHC class II⁺ cells are present in perfused retinal preparations of both LEW and PVG rats and that these cells in the retina are confined to the ED2⁺ phenotype. We propose these are the counterpart of the perivascular cell (or perivascular macrophage) found in the CNS,^{12,22} although their location can only be confirmed by immunohistochemical analysis. Studies in irradiation chimeric rats have shown that such perivascular cells can present autoantigen to T cells *in vivo*,²³ and it is likely the ED2⁺ cells detected here may similarly act as APC within the retina. Nevertheless it remains for us to isolate ED2⁺ cells as well as retinal microglia to establish their APC role. Such studies are obviously difficult given the small numbers of cells involved but are currently being attempted.

Our findings are at least in part, consistent with previous immunohistochemical studies in adult human retina^{16,17} which have demonstrated MHC class II⁺ perivascular cells. Unlike human retina, however, we could not identify in rat, any pronounced MHC class II expression on resident microglia. Retinal microglia have been implicated in inducing phagocytic, cellular, and vascular changes in dystrophic retina and also have phagocytic capacity within the subretinal space during development.^{24,25} Their role as professional APC and in immunoregulation has yet to be defined but flow cytometric analysis can identify and has the potential to then isolate cell populations for further study. Although upregulation of MHC class II expression on microglia occurs in the inflamed rat retina (J V Forrester, unpublished data) as it does in the CNS,¹⁸ our recent studies with freshly isolated microglia from rat CNS indicate these

cells are relatively poor APC for secondary T cell responses compared with other non-microglial CNS macrophages.¹⁹ The latter may include at least some of the perivascular cell populations. Finally, the range of CD4 expressing cells in rats and humans appears comparable, unlike mice, in that both express the CD4 antigen on cells of the macrophage monocyte lineage.^{26,27} Thus the finding of CD4 expression in both the perivascular and microglial populations of the retina, though at a lower level than CD4 expression of T cells (Fig 4), suggests that comparable cells in humans may be targets of human immunodeficiency virus (HIV) infection in the retina, similar to the CD4 dependent HIV infection on CNS microglia.²⁸

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