Human retinal microglia express candidate receptors for HIV-1 infection

V T Pham, L Wen, P McCluskey, M C Madigan, P L Penfold

Background/aims: Microglia are the primary antigen presenting cells in the central nervous system and the retina, and can harbour viral antigens that may damage neural tissue via the release of neurotoxins. All cells bearing CD4 molecules and co-receptors (members of the chemokine receptor and Fcγ receptor families) are potential targets for the human immunodeficiency virus (HIV). In this study, retinal microglia (in vitro and in situ) were investigated for the expression of candidate HIV-1 binding receptors.

Methods: Cultured human retinal microglia and frozen sections of human retinas were used. Immunohistochemistry was used to investigate expression of cell surface receptors necessary for HIV-1 infection: CD4, CC chemokine receptor 5 (CCR5), and Fcγ receptors.

Results: Human retinal microglia expressed detectable levels of CD4, CD16, CD64, and CCR5 in vitro and Fcγ receptor I (CD64) in situ.

Conclusions: Human retinal microglia express several candidate receptors required for viral binding and as such may be a potential reservoir for HIV-1 infection.

Materials and methods

Microglial culture

For tissue culture, adult human eyes from donors aged 10–39 years (n = 7), with 17 (SD 5.5) hours postmortem delay and free from detectable ocular history, were obtained from the Lions NSW Eye Bank and with approval from the human ethics committee, University of Sydney. All studies conformed to the tenets of the Declaration of Helsinki.

Human retinal microglia were cultured as described previously. Briefly, the retina was dissected from the choroid and the retinal pigmented epithelium (RPE), then incubated in 0.05% trypsin/0.02% EDTA (ThermoElectron Pty Ltd, Australia) and 150 μl of DNase (Roche Applied Sciences Australia Pty Ltd) for 10 minutes at 37°C. Digests were double filtered through 70 μm and 44 μm meshes, centrifuged, and the cells resuspended in growth medium (Iscoves Modification of Dubecco’s medium, IMDM, ThermoElectron) supplemented with 10% FBS, 50 IU/ml penicillin, 50 μg/ml streptomycin, and 2 mM glutamine, and cultured in a humidified atmosphere of 5% CO2 and air at 37°C. After 7 days, cultures were washed with HBSS and fresh growth medium was added and cells were cultured for a further 7 days. The supernatant containing predominantly microglia was centrifuged at 400xg for 7 minutes at room temperature (RT). The pellet was then resuspended in growth medium, and cells seeded on plastic and glass coverslips for immunostaining experiments.

Abbreviations: AIDS, acquired immunodeficiency syndrome; BBB, blood-brain barrier; BRB, blood-retinal barrier; CCR5, CC chemokine receptor 5; CAV, cytomegalovirus; HIV, human immunodeficiency virus; IL, interleukin; INL, inner nuclear layer; IPL, inner plexiform layer; PBS, phosphate buffered saline; RPE, retinal pigmented epithelium; TNF-α, tumour necrosis factor alpha
Immunohistochemistry: fluorescence
After 3–5 days in culture, coverslips were fixed in 2% paraformaldehyde, and subsequently rinsed in 0.1 M phosphate buffered saline (PBS). Following incubation in 10% normal sheep serum (NSS) for 15 minutes to reduce non-specific antibody binding, cells were immunolabelled with primary mouse monoclonal antibodies to CD4 (Neomarkers, 1:50), CD16, CD32, CD64, CD45, and CCR5 (Becton-Dickinson Pty Ltd, 1:50) for 1 hour at RT. After rinsing in PBS, coverslips were incubated in biotinylated sheep anti-mouse immunoglobulins (Amersham Pharmacia Biotech Pty Ltd, 1:100) for 1 hour, further rinsed in PBS, and incubated in streptavidin-Cy3 (Zymed Inc, San Francisco, CA, USA) (1:200) for 45 minutes, followed by three washes in PBS. All antibodies were diluted in PBS and 2% NSS. Immunolabelled coverslips were mounted onto glass slides with glycerol.

Immunohistochemistry: immunoperoxidase
After blocking in 10% NSS and incubating in primary and biotinylated secondary antibodies, coverslips were incubated in ExtrAvidin peroxidase (Sigma Pty Ltd) (1:200 in PBS) for 1 hour and antibody binding visualised with 3-3’ diaminobenzidine (DAB) (Sigma). Coverslips were rinsed several times in PBS, counterstained in haematoxylin for 2–3 minutes and dehydrated through alcohols and xylene and mounted in DePeX.

Human retinal sections
Adult human eyes from donors aged 35–70 years (n = 7), less than 24 hours postmortem delay and free from detectable ocular history, were obtained with consent from the Lions NSW Eye Bank and approval from the human ethics committee, University of Sydney. The anterior segments and vitreous were removed and the eye cups fixed in 2% paraformaldehyde/PBS for more than 24 hours. Retinal and vitreous were removed and the eye cups fixed in 2% paraformaldehyde/PBS for more than 24 hours. Retinal sections were cut using a Leitz Cryostat. The sections from human adult retinas (not shown).

RESULTS
Microglia in culture
Within 3–5 days of seeding, three separate microglial cultures grew on coverslips. Cultured retinal microglia displayed an opalescent colour and veiled cell morphology, and remained adherent to the coverslips, helping to isolate them from the mixed retinal glial cells. All immunolabelling experiments were done after 14 days’ culture, yielding increased numbers of resting microglial cells with typical ramified morphology.

CD4 expression
In culture, ramified retinal microglia expressed detectable levels of CD4 immunostaining. Immunoperoxidase positive granules were localised within the cell bodies while the cell processes stained weakly (fig 1B and C, table 1). IgG non-specific isotype controls were negative (fig 1A, 2A) and CD45 immunolabelled positive controls showed strong staining of microglia (fig 1D, 2B). No obvious CD4 immunoreactivity was observed in any of the sections from human retinas (not shown).

CCR5 expression
Cultured retinal microglia expressed CCR5 receptors (fig 2C and D, table 1). Microglial cells with bipolar or ramified morphology were selected for microscopic examination. Bipolar ramified microglia displayed dense staining of the cell body but not the processes, in contrast with CD45 immunostaining where both cell bodies and processes were visible (fig 2B). Overall, fewer CCR5 positive cells were observed compared to CD45 positive cells in microglial culture. CCR5 immunoreactivity could not be detected in sections from human adult retinas (not shown).

Fcγ receptor expression
In culture, retinal microglia expressed immunoreactivity for Fcγ receptor III (CD16) (fig 2E) and Fcγ receptor I (CD64) (fig 2F). Retinal sections from all specimens showed microglial cells immunostained for anti-Fcγ receptor I (CD64) antibody (fig 3C). The CD64 immunoreactive cells displayed different morphologies depending on their location within the retinal layers, similar to the patterns of CD45 immunostaining of microglia (fig 3B). Microglial cells in the nerve fibre layer displayed an elongated shape, while microglial cells in the inner plexiform layer (IPL) and the inner nuclear layer (INL) had highly branched dendritiform morphology, although less ramified in the INL (fig 3C, table 1). Vessel associated microglia were CD45 and CD64 immunoreactive. IgG non-specific isotype controls were negative (fig 3A), and no obvious immunostaining was seen for antibodies to Fcγ receptors II or III (CD32 or CD16 respectively) (not shown).

DISCUSSION
Infection of monocytes/macrophages with HIV-1 occurs through direct interaction of the viral envelope glycoprotein gp120 with the CD4 receptor.23 The lack of evidence confirming expression of this surface marker on certain cell types including microglia and astrocytes has prompted research into CD4 independent binding sites. Several earlier studies have suggested that viral infection may also occur

| Table 1 | Expression of immunoreactivity for viral binding receptors on cultured and in situ human retinal microglia |
|---|---|---|---|---|---|---|
| CD4 | CD16 (FcγRIII) | CD32 (FcγRII) | CD64 (FcγRI) | CD45 | CCR5 |
| Cultured microglia | + | + | -- | + | + | + |
| In situ microglia | -- | -- | -- | + | + | -- |

+ detected; – not detected.
through the interaction of antibody opsonised HIV-1 with the FcγRs on mononuclear phagocyte series cells or through chemokine receptor molecules. More recently, the presence of CD4 has been shown to be essential for viral binding. Additionally, chemokine receptors—that is, CCR3, CXCR4, and CCR5, function as co-receptors necessary for viral internalisation and FcγRs enhance viral stability at the cell surface, allowing infectivity through high affinity CD4 interactions.

**Microglia and CD4 expression**

CD4 normally has an important role in activating T lymphocytes by interacting with major histocompatibility complex class II (MHC-II) molecules on the surface of local macrophages during antigen presentation to T cells. During HIV-1 infection, the presence of CD4 is crucial for the efficient binding of virions to the plasma membrane.

CD4 has been found to be highly expressed on Langerhans cells, U937 monocytc cells, mouse brain microglia and more recently on resting human retinal microglia. However, many studies have been unable to detect CD4 on human brain microglia or have observed only very low CD4 levels in situ on activated human brain microglia.

In the present study, we were unable to detect CD4 immunoreactivity on human retinal sections. In contrast, cultured retinal microglia expressed detectable levels of CD4 and this may be related to activation of microglia in culture or isolation of microglia from the normal immunosuppressive retinal environment.

**Microglia and Fc receptor (FcR) expression**

In this study, we have shown that cultured retinal microglial cells expressed FcγRI (CD64) and FcγRIII (CD16). In situ microglial cells expressed FcγRI (CD64) but not FcγRII (CD32) or FcγRIII (CD16) in normal retina. The presence of Fcγ receptors—that is, FcγRI (CD64), FcγRII (CD32), and FcγRIII (CD16) on human microglia has been reported in brain tissues from patients with multiple sclerosis.

FcγR expression primarily serves to trigger the lysis of extracellular antibody coated targets. However, some pathogens may exploit the process of FcγR binding to facilitate penetration into FcγR expressing cells. FcγRII has been reported to be absent on U937 cells and in such cells, CD4 in addition to Fcγ1 and FcγII receptors has been demonstrated to be necessary for any infection via the antibody dependent enhancement (ADE) process.
occurs at the level of the third variable loop, more precisely next to the CD4 inducible epitopes on gp120. The presence of soluble CD4 and preservation of the third loop are critical for viral binding to CCR5. Deletion of this loop preserves binding of CD4 to gp120 but ablates binding to CCR5, although the deletion of both first and second loops does not alter the binding of gp120/CD4 complex to CCR5. Moreover, the addition of gp120 from macrophage but not T cell tropic isolates may inhibit the binding of chemokines to CD4 and CCR5 expressing cells. In situ, CD4 is more important for the interaction between viral envelope and the chemokine receptors and the addition of soluble CD4 can abolish HIV-1 infection.

The expression of chemokine receptors has not been reported previously on human retinal microglia. However, U937 monocytic cells and human brain microglia have been shown to express CCR5, the co-receptor most commonly used by viral isolates; these cells can be infected with many macrophage tropic HIV-1 strains. Moreover, antibodies against CCR5 can inhibit microglial infection with HIV-1, while anti-CCR3 and anti-CXCR4 antibodies have little or no effect.

The present study shows that retinal microglia can express immunoreactivity for viral binding receptors and as such, may be involved in the pathogenesis of viral retinopathies including HIV-1/AIDS retinopathy.

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Authors’ affiliations
V T Pham, L Wen, M C Madigan, P L Penfold, Save Sight Institute, University of Sydney, Australia
P McCluskey, Ophthalmology Department, Royal Prince Alfred Hospital, Sydney, Australia

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