LABORATORY SCIENCE

Lipopolysaccharide/interferon- γ and not transforming growth factor β inhibits retinal microglial migration from retinal explant

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Br J Ophthalmol 2003;**87**:481–487

Background/aims: The retina possesses a rich network of CD45⁺ positive myeloid derived cells that both surround inner retinal vessels and lie within the retina (microglia). Microglia migrate and accumulate in response to neurodegeneration and inflammation. Although microglia express MHC class II, their role remains undefined. The aims of this study are to investigate changes in human microglia phenotype, migration, and activation status in response to pro-inflammatory and anti-inflammatory stimulation.

Methods: Donor eyes were obtained from the Bristol Eye Bank with consent and whole retina was removed. 5 mm retinal trephines were cultured in glucose enhanced RPMI on cell culture insert membranes for up to 72 hours. The effects of lipopolysaccharide/interferon- γ (LPS/IFN γ) and transforming growth factor β inhibits (TGF β) stimulation, alone or in combination, on migration, phenotype, and activation status (iNOS expression) of microglia were studied using immunofluorescence and cytokine analysis by ELISA.

Results: CD45⁺ MHC class II⁺ retinal microglia were observed within retinal explants, and in culture microglia readily migrated, adhered to culture membrane, downregulated MHC class II expression, and produced interleukin 12 (IL-12) and tumour necrosis factor α (TNF α). Following LPS/IFN γ stimulation microglia remained MHC class II⁺ iNOS⁺, and secreted IL-10. Migration was suppressed and this could be reversed by neutralising IL-10 activity. TGF β did not affect ability of microglia to migrate and was unable to reverse LPS/IFN γ induced suppression.

Conclusions: Microglia readily migrate from retinal explants and are subsequently MHC class II⁺, iNOS⁺, and generate IL-12. In response to LPS/IFN γ microglia produce IL-10, which inhibits both their migration and activation. TGF β was unable to counter LPS/IFN γ effects. The data infer that microglia respond coordinately, dependent upon initial cytokine stimulation, but paradoxically respond to classic myeloid activation signals.

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Accepted for publication
15 July 2002

Increasingly, it is appreciated that the retina, like the anterior chamber of the eye has active immune regulatory networks,^{1,2} that in combination with the blood-retina barrier, protect against danger signals³ which may lead to immune mediated destruction or degeneration of tissue and thus preserve normal function. Despite such assumed immune privilege, intraocular inflammation is not uncommon clinically and, moreover, with evidence that during retinal degeneration there is a loss of immune regulation,⁴ more understanding is needed of the cellular and molecular mechanisms that confer regulation and how such tolerance is broken.

Within the eye there are cells that are able to initiate and perpetuate inflammatory responses and include professional antigen presenting cells (APC), such as dendritic cells (DC) present in both choroid and iris.^{5,6} However, such cells are notably absent from the retina. In antigen specific posterior segment inflammation, leucocytic infiltration, which includes CD4⁺ T cells, occurs early in disease evolution,^{7,8} alongside the early retinal infiltration of DC.⁹ With the absence of resident DC within the retina, two other potential candidates for local APC within the retina are perivascular macrophage and resident microglia, with distinct phenotypes.^{10–12} In comparison, the CNS is also immune privileged and protected by the blood-brain barrier.¹³ The CNS is also subject to immune mediated attack, such as multiple sclerosis (MS), Creutzfeldt-Jakob disease (CJD), and Alzheimer's disease (AD), despite an immunoregulatory environment. Rodent CNS microglia (isolated by flow cytometric sorting) induce apoptosis of both

antigen specific and non-antigen specific CD4⁺ T cells, in an attempt to regulate immune mediated damage.¹⁴

Functionally, retinal microglia control neuronal growth,¹⁵ and are active phagocytes, clearing dying photoreceptor cells.¹⁶ Responses to photoreceptor degeneration and retinal injury induce microglia migration and accumulation.^{17–19} Microglia readily migrate to areas of damage or pathological disturbance, when changes within the CNS environment occur, either due to immune mediated or non-specific stress.^{20–22} Consequently either retinal or CNS microglia are activated, associated with an upregulation of co-stimulatory molecules (CD86, CD80) and MHC class II expression.^{11,23–26}

What the control of microglia activation is and what role microglia have in regulating inflammatory or degenerative responses remains unanswered. Resting microglia express low levels of CD45 and co-stimulatory molecules have minimal migration and phagocytic activity, all of which may facilitate the maintenance of immune privilege.^{24,27} One notion is that the retinal microenvironment regulates microglia behaviour—for example, by the modulation of macrophage (including microglia) APC and T cell function by IL-10,^{28–33} and by cognate interaction of microglia or any infiltrating macrophage with neuronal CD200.^{34,35} We have recently shown in human retina microglia isolates and in retinal explants that following stimulation with IFN γ and LPS, microglia reduce their capacity to migrate and display an IL-10 dependent downregulation of both MHC class II and co-accessory molecules.²⁶ Therefore, despite microglia phenotypic and behavioural characteristics

that on the one hand supports an antigen processing and presenting capability at least in vitro, their function in vivo mediated by the microenvironment is downregulatory.

Based on these observations, this study was undertaken to obtain a more complete understanding of microglia behaviour; specifically to determine how microglia migration is affected by pro-inflammatory stimulation and microglia response to changing the homeostatic balance of regulatory cytokines (TGF β , IL-10).

METHODS

Human research material

Donor tissue was obtained from the Bristol Eye Bank with research consent after the removal of the cornea for transplant. Postmortem times varied between 20 and 80 hours. Retinas removed from eyes with longer postmortem times were unable to be used for explant culture owing to poor tissue viability.

Retinal explants

One pair of eyes per experiment were initially dissected to obtain the retina by first removing the iris and the lens. The vitreous was then removed including stripping of the hyaloid face with pipette if required. The retina was removed as a whole by cutting free at the optic nerve. The retina was placed in a petri dish containing RPMI and 5% fetal calf serum (FCS). Using a 5 mm sterile biopsy punch, sections were taken from central and peripheral regions of the retina and placed on the membrane of a 0.4 μ m cell culture insert (Falcon, UK), and placed in individual wells of a 12 well plate. In each well approximately 1 ml of glucose enhanced tissue culture medium (TCM) was added containing RPMI (Gibco, UK), 5% FCS, 50 U/ml penicillin-streptomycin (Gibco, UK), and 5 ng/ml of GM-CSF (Sigma, UK). Plates were incubated at 35°C, in 5% carbon dioxide, for 24, 48, and 72 hours. Approximately 24 5 mm sections were obtained from one eye allowing a total of four 12 well plates run in parallel. For activation studies 5 μ g/ml of LPS (Sigma, UK) and 100 U/ml of IFN γ (R&D systems, UK) were added to the TCM. In experiments for co-stimulation, 5 ng/ml of human recombinant TGF β 2 (expressed in NSO murine myeloma cells Sigma, St Louis, MO, USA) were added to the TCM and 5 ng/ml to the activation TCM (tissue culture medium plus 5 μ g/ml LPS and 100 U/ml IFN γ). Tissue explants were cultured as above. Tissue explant supernatants were removed after 24, 48, and 72 hours and frozen at -20°C for analysis of cytokine production. Both the retina and culture insert membrane were removed and processed for immunohistochemistry.

IL-10 neutralisation assay

Following our previous observations,²⁶ we wished to further assess the role of IL-10. Therefore in subsequent experiments, neutralisation of IL-10 was performed by incubating 5 mm retina tissue explants (cultured as described above on cell culture insert membranes) with the addition of saturating dose of 5 ng/ml of rat IgG_{2a} anti-IL-10 mAb (JES3-19F1, Pharmingen, USA).

Immunohistochemistry

The following mouse anti-human primary mAb were used at optimal concentrations to label microglia within retinal explants and adherent microglia on cell culture insert membranes: monoclonal mouse IgG1 anti-human HLA-DP, DQ, DR antigen (CR3/43 Dako, Denmark 1:50), mouse IgG1 anti-human CD45 (2B11 +PD7/26, Dako, Denmark, 1:50), mouse IgG2a iNOS/NOS 2 (6 BD Transduction Laboratories, Lexington 1:50), mouse IgG1, ki67 (B56, BD Pharmingen, Europe 1:50), mouse IgG1 anti-human CD40 (5C3, BD Pharmingen, Europe, 1:10), mouse IgG1 anti-human CD80 (L307.4, BD Pharmingen, Europe, 1:50), mouse IgG_{2b} (IT2.2, BD

Pharmingen, Europe, 1:50). To demonstrate other glial cells rabbit IgG anti-human GFAP (Sigma, St Louis, MO, USA 1:80) was used and for endothelial cells mouse IgG1 anti-human CD31 (WM-59 BD Pharmingen, Europe 1:50). Primary antibodies were visualised using goat IgG anti-mouse FITC conjugated Fab fragment (Sigma, St Louis, MO, USA, 1:250), and anti mouse IgG R-phycoerythrin Fab fragment (PE Sigma, St Louis, MO, USA, 1:200), anti-mouse secondary antibodies. Non-specific binding was eliminated by diluting the primary antibody in 1% BSA/PBS. Negative control experiments eliminated autofluorescence by incubating sections and membranes in antibody diluant (1%BSA/PBS) followed by fluorescent secondary antibodies. Apoptosis was detected using an Apoptag in situ apoptosis detection kit as per manufacturer's instructions (Intergen Company, USA) but adjusting incubation times to 2 hours.³⁶ Both retinal tissue and membrane insert were first removed and fixed in 1% paraformaldehyde for 30 minutes followed by washing three times in PBS. Primary antibody (diluted in 1% BSA/PBS) incubation with MHC class II (1 hour room temperature) was followed by several washing steps and incubation with the secondary antibody FITC (1 hour RT). In double labelling studies secondary primary antibodies were incubated overnight at 4°C followed by washing and incubation with labelling antibody PE (2 hours RT). Tissue and culture insert membrane were mounted using anti-fading fluorescent mount (Vector and Sigma, UK) and viewed under a Leitz (Dialux 22EB) fluorescent microscope at $\times$ 40 magnification. Positive cells were counted in 12 random fields of view within the tissue; at the tissue edge and on the cell culture insert membrane by starting top left of slide and moving in a clockwise direction. Pictures were taken using a Nikon E990 digital camera and analysed using Microsoft Photo Editor.

β -Glucuronidase assay

Macrophages that are activated with TGF- β exhibit increased phagocytic activity, which is represented by the extent of β -glucuronidase expression. Unfixed sections of tissue and cell culture insert membranes were stained for β -glucuronidase expression using an enzymatic staining method. β -Glucuronidase catalysed α -naphthol AS-BI β -D-glucuronide into a red reaction product naphthol AS-BI-HPR complex.³⁷ Sections and membrane were then counterstained in methyl green and visualised with a light microscope under oil at $\times$ 100.

ELISA

Production of IL-10, IL-12p40, and TNF α were analysed, from a minimum of triplicate experiments using supernatants removed from retina explant cultures in which one pair of eyes was used, using human cytokine capture ELISA kits (OptEIA, Pharmingen, USA). In brief, each cytokine capture plate contained a standard, which was serially diluted down the plate to determine cytokine concentration. Using undiluted supernatants in duplicate from each retinal explant culture condition, absorbance was read within 30 minutes of stopping the reaction (1 M H₃PO₄) at 450 nm and corrected for 570 nm. Cytokine concentration was determined from the standard curve, using computer software Excel (Microsoft).

Statistics

Differences between cell culture conditions for microglia migration and cytokine production was analysed using a one way analysis of variance and to distinguish the significantly different pairs of means, the F test was applied; $p < 0.05$ was considered to be statistically significant.

RESULTS

Microglia migrate from retinal explants

Previous reports have documented the use of an explant culture system that maintains retinal architecture and enables

Figure 1 Immunohistochemical analysis of retinal microglia and migrated microglia that have adhered to a cell culture membrane. (A–D) represent CD45 visualised with PE (red/left panels) and MHC class II with FITC (green/right panels). (A) A small group of adherent CD45⁺ microglia that have migrated from the retina. Cells are elongated (closed arrows) and have small branches (open arrows). Magnification $\times 400$. (B) Representation of dual stained CD45⁺ MHC class II⁺ adherent microglia. CD45⁺ MHC class II⁺ expression was only found on approximately 7% of adherent CD45⁺ microglia population. Arrow represents faint MHC class II expression. Magnification $\times 400$. (C) Dual stained ramified microglia within the retinal explant immediately post mortem, where majority express CD45 and MHC class II. Microglia display their usual resting morphology of small cell bodies and have many long extending branched processes (arrows). Magnification $\times 400$. (D) Dual stained amoeboid microglia within the retinal explant, after 72 hours in culture without activation. Within explant microglia maintained both CD45 and MHC class II expression. Microglia now show activated morphology of enlarged cell bodies and have few branched processes. There was variability in extent of MHC class II staining (intracellular staining, closed arrows; weak staining, open arrows). Magnification $\times 560$. (E) β -Glucuronidase positive expression within the retinal explant demonstrated as a red/dark brown reaction product formed when β -glucuronidase catalyses α -naphthol AS-BI β -D glucuronide (arrows). Magnification $\times 1000$. (F) Only 8% of migrated CD45⁺ microglia from culture were activated, expressing iNOS visualised with FITC (green). Magnification $\times 400$.

Figure 2 Mean number of CD45⁺ adherent microglia migrated from a 5mm retinal explant. CD45⁺ microglia migrated from 5 mm retinal explants and adhered to cell culture membrane inserts. Maximal migration was observed by 72 hours in culture. CD45⁺ microglia were counted in 12 fields of view under $\times 40$ objective. Data are represented as mean (SD) of eight experiments.

microglia migration,^{26, 38} as visualised immunohistochemically by CD45⁺ expression. Initially, these current experiments were designed to examine any difference in microglial migration between central and peripheral regions of the retina. There was no difference between regions of the retina and the results to follow are therefore representative of central retina. Migratory CD45⁺ microglia adhere (adherent microglia) to the cell culture insert membrane when cultured in medium alone (Fig 1A). Microglial migration increased over 72 hours (9 (SD 13) CD45⁺ cells at 24 hours, compared to 64 (36) CD45⁺ cells at 72 hours; Fig 2). Only 7% of adherent CD45⁺ microglia were CD45⁺ MHC class II⁺ by 72 hours (Fig 1B). There was no GFAP⁺ or CD31⁺ adherent cells throughout the time course of the experiment. Initially, CD45⁺ microglia remaining within the tissue explant maintained a ramified morphology, but over 72 hours became increasingly amoeboid in appearance and to varying extent, all expressed MHC class II (Fig 1C and D). Neither microglia within the explant nor adherent microglia expressed CD86, CD80, or CD40 and there was no evidence of proliferation as determined by Ki67 expression (data not shown). Within retinal explant β -glucuronidase production was observed (Fig 1E). There was sparse iNOS expression around blood vessels but not within retinal tissue. However in adherent CD45⁺ microglia, iNOS expression was seen in 8% of cells (4 (2) iNOS⁺ adherent cells) (Fig 1F), irrespective of culture conditions by 72 hours. No β -glucuronidase production was observed in migrated cells in any of the culture conditions.

Activation causes a suppression of microglial migration with a concomitant increase in IL-10 and TNF α

Previous studies have shown that upon activation microglia migration is suppressed,²⁶ and these current experiments confirmed previous data. When retinal explants were treated with LPS/IFN γ a reduction in microglia migration was observed by 72 hours, in comparison with microglia migrating from retinal explants cultured in medium alone (15 (12) CD45⁺ adherent cells, 64 (36) CD45⁺ adherent cells respectively $p = 0.0005$; Fig 3). Only 14% of adherent CD45⁺ microglia following LPS/IFN γ activation were CD45⁺ MHC class II⁺ and 7% of adherent CD45⁺ microglia cultured in medium alone. There was no evidence of microglia apoptosis either within explant or in an adherent cell population, as a consequence of activation with LPS/IFN γ , determined by enzymatic labelling of the free 3' OH terminal end of DNA fragments.³⁶ Commercially available ELISA kits with a minimum detection level of 7.8 pg/ml and a maximum detection level of 500 pg/ml were used to assay supernatants of retinal explants. LPS/IFN γ activation of retinal explants resulted in a significant increase in IL-10 and TNF α production compared to medium alone (Table 1). By 48 hours IL-10 levels increased from 16.7 (10.3) pg/ml to 167 (54.4)

Table 1 IL-10, IL-12, TNF α levels in explant culture supernatants, with and without IL-10 neutralisation

Culture conditions	IL-10	IL-12	TNF α
Medium alone			
- anti IL-10 mAb	16.7 (10.3)*	327.9 (45.4)†	14.5 (4.4)‡
+ anti IL-10 mAb	4.8 (4.1)	489.7 (314.1)	196.1 (111.6)
Activated			
- anti IL-10 mAb	167.0 (54.4)*	150.8 (42.4)†	57.5 (12.2)‡
+ anti IL-10 mAb	3.1 (2.7)	211.7 (211.7)	273.0 (138.8)
TGF β			
- anti IL-10 mAb	44.44 (12.1)	95.6 (66.3)	65 (30)
+ anti IL-10 mAb	4.3 (3.7)	86.3 (99)	240.4 (284.7)
Activated + TGF β			
- anti IL-10 mAb	197.7 (39.9)	13.6 (10.6)	84.9 (15.4)
+ anti IL-10 mAb	15.6 (13.4)	42.9 (42.9)	227.0 (169.0)

Data represented are mean (SEM) of cytokine levels obtained after 48 hours of culture from a minimum of three experiments. Culture conditions include medium alone, IFN γ /LPS (activated), TGF β , IFN γ /LPS + TGF β , with and without anti IL-10 mAb. (Cytokine concentrations are pg/ml). Statistical comparisons were made following one way analysis of variance with an F test to allow multiple comparisons.

*IL-10 in medium alone versus activated; $p = 0.0053$.

†IL-12 in medium alone versus TGF β ; $p = 0.016$, IL-12 in medium alone versus TGF β (+anti IL-10); $p = 0.03$.

‡TNF α in medium alone versus activated; $p = 0.029$, TNF α in medium alone versus activated (+anti IL-10); $p = 0.003$.

pg/ml ($p = 0.0053$) and TNF α increased from 14.5 (4.4) pg/ml to 57.5 (12.2) pg/ml with activation ($p = 0.029$). There was no further significant increase in IL-10 concentration over time and there was no further significant change in any other cytokine levels at 72 hours.

The addition of TGF β did not overcome LPS/IFN γ induced suppression of microglial migration

TGF β , an abundant anti-inflammatory cytokine found within the eye, is central to both the induction of ACAID^{29, 39, 40} and regulating macrophage function.^{37, 41} We therefore aimed to assess the effects of TGF β on microglia migration in the presence or not of LPS/IFN γ . The addition of TGF β alone did not prevent microglia migration (43 (23) CD45⁺ microglia compared to medium alone 64 (36); Fig 3). Moreover, addition of TGF β did not prevent suppression of microglia migration following LPS/IFN γ activation (Fig 3). IL-10 and TNF α cytokine concentrations were not significantly affected by addition of TGF β . Contrary to LPS/IFN γ activation, which showed no significant decrease in IL-12p40 there was a

Figure 3 Mean number of CD45⁺ adherent microglia migrated from a 5 mm retinal explant following cytokine activation. Explant culture membranes were removed and fixed in 1% paraformaldehyde and subsequently stained for CD45. CD45⁺ microglia were counted in 12 fields of view under $\times 40$ objective. LPS/IFN γ activation suppresses migration from retina and TGF β is unable to prevent this suppression. Data are represented as mean (SD) of eight individual experiments.

Figure 4 Mean number of CD45⁺ adherent microglia migrated from a 5 mm retinal explant in the presence of activating medium and with or without anti-IL-10 mAb. IL-10 was neutralised by saturating doses of anti IL-10 mAb added to retinal explant culture medium. Adherent microglia were labelled with CD45 and CD45⁺ microglia were counted in 12 fields of view under $\times 40$ objective. Neutralising IL-10 activity, microglia migrated despite LPS/IFN γ activation. No statistical difference in migration was found between the different activation culture conditions in the presence of anti-IL-10 and medium alone and medium alone + irrelevant mAb. Data are represented as mean (SD) of four experiments.

significant suppression of IL-12p40 following TGF β activation (at 48 hours medium alone 327.9 (45.4); TGF β 95.6 (66.3) p = 0.016, Table 1).

Neutralising IL-10 activity overrides LPS/IFN γ mediated suppression of microglia migration

Owing to the association between increased IL-10 production and suppressed microglia migration, IL-10 was neutralised using an anti-IL-10 monoclonal antibody and effect of LPS/IFN γ activation was observed. We found in the absence or very low levels of IL-10 (Table 1) microglia migrated even when activated with LPS/IFN γ (Fig 4). By 72 hours microglia migration in the presence of LPS/IFN γ showed no statistical difference from migration observed in medium alone, TGF β , or LPS/IFN γ + TGF β . However, following LPS/IFN γ activation, migration was significantly increased compared to migration in the absence of IL-10 neutralisation with anti-IL-10 mAb (at 72 hours: with anti-IL-10 mAb 48 (18); no anti-IL-10 mAb 12 (8); p = 0.003, Fig 4).

As we had previously noted, the degree and extent of MHC class II expression was variable. Between 7–15% of CD45⁺ adherent microglia expressed MHC class II irrespective of culture conditions or presence or not of anti-IL-10 mAb (data not show). As a result of neutralising IL-10 activity by the addition of anti-IL-10 mAb, TNF α levels increased significantly by 48 hours (Table 1: 14.5 (4.4) in medium alone; 196.1 (111.6) in the presence of anti-IL-10 mAb, p = 0.003). There was no significant increase in IL-12p40 concentration in the presence of anti-IL-10 mAb (Table 1). However IL-12p40 concentration was significantly suppressed following stimulation with TGF β in the presence of anti-IL-10 mAb (p = 0.03; Table 1).

DISCUSSION

In response to degeneration within the CNS, microglia transform from their “resting” ramified state, retracting their long branched processes and assume an activated amoeboid form and migrate.⁴² Activated microglia upregulate cell surface markers including MHC class II, CD40, CD86, secrete cytokine, and have the ability to phagocytose apoptotic cells in areas of inflammation and/or damage within the CNS.⁴³ Similarly, within the retina, as seen in ganglion cell and nerve fibre loss

in primary open angled glaucoma or during retinal degeneration in retinitis pigmentosa,⁴⁴ microglia are activated and exhibit phagocytic activity clearing neuronal debris.⁴⁵ However, these processes of injury related remodelling and regeneration may generate signals that are perceived by microglia as danger,³ leading to presentation of antigen and following systemic T cell priming, facilitating tissue destruction via tissue specific immune responses as well as non-specific production of free radicals such as NO, cytokines TNF- α and IL-1, and enzymes.^{22–45–46} Myeloid cells are conditioned and responses are controlled via the microenvironment and cytokine milieu.^{35–37–47} How microglia respond to injury and cytokine signals is pivotal to how retinal tissue maintains structure and function following insult. Here we further our previous findings²⁶ and demonstrate in an ex vivo retinal explant model of microglia behaviour that classic myeloid activation signals result in an IL-10 dependent suppression of microglia migration.

Previous reports have highlighted a central role of IL-10 in the maintenance of ocular immune regulation.³⁰ IL-10 opposes Th1 cell activation^{48–49} and IL-10 suppresses experimental autoimmune uveoretinitis (EAU) via the downregulation of Th1 responses.⁵⁰ However, during uveitis, IL-10 expression in aqueous humour and vitreous was detectable in only very few cases where Th1 cell responses predominated.⁵¹ These data confirm modulation of microglia behaviour during regulation of inflammatory responses and support previous reports that showed that LPS/IFN γ activation of microglia resulted in an increased concentration of this anti-inflammatory cytokine.^{26–52} Here, activation with LPS/IFN γ prevented microglia migration and caused an increase in IL-10 concentration in the culture supernatant. The reduction of microglia migration was not found to be associated with apoptosis in contrast with reports of IFN γ increasing susceptibility of microglia to apoptosis via upregulation of FasL.⁵³ Additionally, we showed downregulation of MHC class II expression on microglia that had migrated following cell activation with LPS/IFN γ and we were unable to confirm previous reports, which had shown that TGF β enhanced LPS/IFN γ induced MHC class II expression.⁵⁴ This may illustrate the differences observed between directly ex vivo isolated microglia and cultured microglia or microglia cell lines. The absence of co-stimulatory molecule expression (CD80, CD86) on retinal microglia within retinal explants or on adherent microglia, further demonstrates the downregulation of microglia. Despite activation via LPS/IFN γ and the production of pro inflammatory cytokines IL-12 and TNF α , detection of iNOS was negligible. This is in contrast with reports that implicate microglia as producers of NO in inflammatory diseases such as multiple sclerosis (MS) or experimental correlate, experimental allergic encephalomyelitis (EAE),^{55–56} although differentiation between resident microglia and infiltrating myeloid cells was not made. With reference to the effects of TGF β induction of β -glucuronidase expression in macrophages,³⁷ we noted within retinal explants that there were positive areas of β -glucuronidase but expression was absent in microglia that had migrated. Expression may have been inhibited by the concomitant negative effect of IL-10.³⁷ These data support our recent experimental data in rats, which show that microglia behave similar to TGF β conditioned bone marrow derived macrophages, and do not secrete significant amounts of NO.⁴⁷

IL-12 is a pro-inflammatory cytokine orchestrating Th1 cytokine production. High levels of the active component of IL-12 (IL-12p40), found in supernatants removed from retina cultured in medium alone, reflect the ability to influence Th1 cell mediated inflammatory responses. Patients with MS have higher levels of IL-12p40 in the CNS compared to control groups.²⁵ Within the eye, retinal pigment epithelium secretes TGF β ,⁵⁷ which in turn may modulate antigen presentation of resident myeloid cells, skewing the response towards Th2 cytokine production.³⁰ Although TGF β did not override

LPS/IFN γ suppression of microglia migration, we noted that IL-12p40 concentration was reduced significantly with the addition of TGF β . Such immunomodulatory effects of TGF β have also been described when its addition suppresses experimental colitis in SJL/J mice but only in the presence of IL-10, required to downregulate Th1 cell cytokines that would otherwise inhibit production of TGF β .³⁸ Consequently, we found the greatest suppression of IL-12p40 production was observed when microglia were activated in the presence of TGF β with a high IL-10 concentration. This infers that the immune regulatory effects of IL-10 and TGF β may act synergistically. In all our experiments we cannot exclude the possible influence of ischaemia and microglia activation in postmortem samples.

Maintaining immune regulation in the steady state may in part be a result of concomitant TNF α production. TNF α promotes cell mobility by cytoskeleton reorganisation via depolymerisation of F-actin and enhancing cytokine responses that promote migration.^{59–60} The low levels of TNF α and IL-10 we observed in culture conditions of medium alone may be acting in concert to allow microglia to migrate in the steady state. Migration may allow efficient removal of apoptotic bodies and in doing so it would be important not to activate microglia and potentially promote inflammation. It has been demonstrated that following phagocytosis of apoptotic cells, microglia suppress pro-inflammatory cytokine production, thereby maintaining immune privilege.⁶¹ However, during powerful inflammatory signals—for example, following LPS/IFN γ activation, the generation of IL-10 regulates the ability of microglia to migrate despite high TNF α levels. This inhibitory effect on microglia migration occurs even when exogenous TGF β is added to activation media. Macrophages and myeloid derived cells respond to cytokine signals hierarchically^{37–47} and similarly in these current experiments microglia responses to LPS/IFN γ activation override that of TGF β , which has an opposing/competing intracellular signalling mechanism.³⁷ The result, however, is potentially to limit microglia activation and tissue destruction by reducing the ability of microglia to migrate and as we have previously shown, downregulate potential antigen presenting function.²⁶ Further indication of the central role of IL-10 was observed when its activity was neutralised and microglia migration was restored. Similar behaviour has been noted with Langerhan cell (LC) migration during contact hypersensitivity. In IL-10 knockout mice a significant increase in LC migration to lymph node was noted and TNF α and IL-1 production was suppressed when exogenous IL-10 was administered.⁶⁰ Although we did not assay IL-1 levels, we did document a significant TNF α rise after neutralising IL-10 activity. Such changes in cytokine profiles may account for the restored microglia migration, but without the immunosuppressive effects of IL-10 can activated microglia promote repair without inducing inflammation? Further work will improve our understanding of microglia responses to various stimuli, their role during inflammation and neurodegenerative diseases, and thus avenues that can be explored to modulate their behaviour and redress retinal homeostasis.

ACKNOWLEDGEMENT

This work was supported by Guide Dogs for the Blind Association. It was presented as an abstract and poster at Association for Research in Vision and Ophthalmology (ARVO), 2002.

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