Macrophages and MHC class II positive cells in the choroid during endotoxin induced uveitis

Peizeng Yang, Alex F de Vos, Aize Kijlstra

Abstract

Aims/background—Endotoxin induced uveitis has been regarded as a model for acute anterior uveitis and until now little was known about choroidal involvement. The aim of this study was to investigate changes in macrophages and MHC class II positive cells in the choroid of Lewis rats during endotoxin induced uveitis.

Methods—Choroid-sclera wholemounts were isolated from normal Lewis rats and at different time points—4, 8, 16, 24, 48, 72, and 96 hours, and 7, 10, and 14 days after a footpad injection of 200 μg of lipopolysaccharide (LPS). Immunohistochemistry was performed using the monoclonal antibodies ED1 (monocytes, macrophages, dendritic cells), and OX6 (MHC class II antigen).

Results—In normal rats, two layers of macrophages were identified in the choroid; a layer located immediately beneath the retinal pigment epithelium (RPE) and a layer bordering the sclera. The density of ED1 positive cells in the layer bordering the RPE cells was 902 (SD 132) cells/mm² whereas the scleral layer had a cell density of 389 (73) cells/mm². Based on morphology, positive cells could be divided into two main categories; pleomorphic/round cells and dendritic cells with varying appearances, with the latter being predominant in normal eyes. A network of MHC class II positive dendritic cells was found in the choroid, beneath the RPE, with a density of 659 (96) cells/mm². No MHC class II positive cells were found in the macrophage layer bordering the sclera. LPS injection caused a massive influx of ED1 positive macrophages in the area below the RPE cells but did not result in an influx of macrophages at the scleral side of the choroid. The infiltrate reached a maximum at 16 hours following LPS injection and decreased at 96 hours. The morphology of the infiltrating cells was pleomorphic/round at early stages of inflammation and changed into a dendritic cell population later. The number of MHC class II positive cells on the anterior side of the choroid increased 8 hours after injection and reached a peak at 72–96 hours. MHC class II positive cells were not observed in the vicinity of the sclera at any time after LPS injection. Both resident and MHC class II positive dendritic cell numbers returned to normal values at day 14 following LPS injection.

Conclusions—These results indicate that the choroid is severely inflamed after systemic LPS administration to Lewis rats and suggests that endotoxin induced uveitis may serve as a model for generalised uveitis in humans.
although anterior uveitis is a very prominent aspect of this disorder, EIU may also be used as an experimental model of panuveitis.

Materials and methods
EXPERIMENTAL DESIGN
Fifty inbred male Lewis rats (6–8 weeks of age), weighing 150–200 g, were used in the study. All animals were treated according to the ARVO statement for the use of animals in ophthalmic and vision research. LPS (Salmonella typhimurium, Difco Ltd, Detroit, MI, USA) was dissolved in sterile pyrogen-free saline 0.9% at a concentration of 2 mg/ml. Fifty µl of LPS was injected into each hind footpad of Lewis rats, the total doses per animal being 200 µg. At different time points—for example, before LPS injection (control group), and 4, 8, 16, 24, 48, 72, and 96 hours, and 7, 10, and 14 days after LPS injection, rats were perfused and whole mounts of the choroid-sclera complex were prepared as described below. The number of Lewis rats in each group varied between 2 and 6.

ISOLATION AND PREPARATION OF WHOLE MOUNTS OF THE CHOROID-SCLERA COMPLEX
Rats were anaesthetised by intramuscular injection of 0.5 mg flunisone and 15 µg fentanyl citrate (50 µl of Hypnorm, Janssen, Goirle, the Netherlands) and an intraperitoneal injection of 30 mg sodium pentobarbitone (0.5 ml Nembutal, Sanofi, Maassluis, the Netherlands). Intravascular coagulation was prevented by injection of 0.3 ml of heparin (5000 IU/ml) into the left ventricle. Perfusion through the left ventricle with cold phosphate buffered saline (PBS, pH 7.4) was performed to ensure that blood was completely expelled from the blood vessels. Eyes were immediately enucleated and extraocular tissues were removed. The enucleated eyes were dissected into an anterior and posterior part by an incision behind the ciliary body. Lens and vitreous body were carefully removed from the posterior part. The choroid-sclera complex was gently separated from the retina and subsequently placed in cold 100% ethanol for

Figure 1 Immunohistochemical staining of choroidal whole mounts with monoclonal antibody ED1. Whole mounts were obtained from normal Lewis rats (A, B) and at different time points after footpad lipopolysaccharide injection: 16 hours (C), 96 hours (D), day 7 (E), and day 14 (F). In normal rats, ED1 positive cells were present in the inner ‘RPE bordering’ layer (A) and an outer layer adjacent to the sclera (B). Note the presence of ED1 positive cells after the RPE layer was brushed off (E); the remaining RPE is still detectable as faintly stained hexagonal cells in the right hand bottom corner (E). Magnification bar in (A) equals 250 µm and refers to all parts of the figure.
fixation for 5 minutes. The fixed choroid-sclera complex was stored at 4°C until use.

### IMMUNOHISTOCHEMISTRY

Monoclonal antibody ED1 (recognising a cytoplasmic antigen in rat monocytes, macrophages, and 90% of dendritic cells) was kindly provided by Dr C D Dijkstra, Free University, Amsterdam. Monoclonal antibody OX6 (recognising rat MHC class II antigen) was purchased from Sera-Lab (Sussex).

Other reagents used were biotinylated sheep anti-mouse antibody (Amersham Life Science, Amersham), streptavidin-biotin-peroxidase complex (Dako, Glostrup, Denmark) and 3,3-diaminobenzidine tetrahydrochloride (DAB; Sigma, St Louis, MO, USA). A standard ABC technique was performed on the whole mounts of the choroid-sclera complex. Endogenous peroxidase activity was eliminated by incubating the tissue in 1% hydrogen peroxide in PBS for 20 minutes. Whole mounts were incubated with monoclonal antibodies at 4°C overnight. All other procedures were performed at room temperature. Stained wholemounts were placed on gelatin coated glass slides with the inner side facing up, dried at room temperature, and embedded in Entellan.

### QUANTITATIVE ANALYSIS

Quantitative analysis of ED1 and OX6 positive cells was carried out on normal choroid wholemounts using a calibrated eyepiece graticule under a microscope with 40× object lens. Different layers of stained cells could be distinguished in separate focal planes of the whole mounts. Immunostained cells were counted in three separate fields per wholemount and the mean number of cells/mm² of each rat was calculated. In total, six eyes from six rats were examined and the mean (SEM) determined for the whole group. The data were analysed using Student’s t test.

Table 1 Differences in tissue resident macrophages and MHC class II positive cells between the inner ‘RPE bordering’ layer and the outer layer adjacent to the sclera. (Data represent the mean (SEM))

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Inner layer</th>
<th>Outer layer</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>ED1 positive cells (cells/mm²)</td>
<td>902 (132)</td>
<td>389 (73)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Dendritiform (%)</td>
<td>74 (6)</td>
<td>93 (11)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>MHC II positive cells (cells/mm²)</td>
<td>659 (96)</td>
<td>Absent</td>
<td></td>
</tr>
<tr>
<td>Dendritiform (%)</td>
<td>83 (10)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### RESULTS

**MACROPHAGES AND MHC CLASS II POSITIVE CELLS IN THE NORMAL CHOROID**

Analysis of choroidal wholemounts of normal Lewis rats revealed a network of ED1 stained macrophages as well as MHC class II positive cells (ED1, Fig 1: MHC class II, Fig 2). Most of the cells had a dendritic appearance although approximately 25% had a round or pleomorphic shape. Macrophages were observed in two distinct planes in the whole mounts. One layer was located immediately beneath the RPE, and the other bordered the sclera. Both layers contained ED1 positive macrophages, but the layer in the vicinity of the sclera lacked MHC class II positive cells. The location of these cells beneath the RPE, is illustrated in wholemounts stained with OX6, where part of the RPE cell layer was brushed off (Fig 2A).

Quantitative analysis was performed for ED1 positive cells and showed that the RPE bordering layer (902 (132) cells per mm²) contained significantly more macrophages than the scleral side (389 (73) cells per mm²; p < 0.0001) (Table 1).

The number of MHC class II positive cells just below the RPE was 659 (96) cells per mm², and was significantly lower than the number of ED1 positive cells in this layer of the choroid.

### CHANGES IN THE CHOROID AFTER LPS INJECTION

Systemic LPS administration resulted in a dramatic change in the macrophage and dendritic cell population in the choroid (Figs 1 and 2). The changes occurred mainly in the inner layer at an early stage after LPS injection. ED1

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**Figure 2** Immunohistochemical staining of choroidal wholemounts with monoclonal antibody OX6 (A, B). Wholemounts were obtained from a normal Lewis rat (A) and 96 hours after footpad LPS injection (B). Note the presence of OX6 positive cells after partial removal of the RPE layer (A); the RPE layer is still visible as faintly stained hexagonal cells in the right, third, part of (A). Magnification bar in (A) equals 250 µm and refers to both parts of the figure.
cell population increased in number at 4 hours in some areas in the choroid and involved large areas of the choroid at 8 hours (Table 2).

The whole area of the choroid was infiltrated with a large number of round immunopositive (ED1) cells at 16 hours. At 24 hours after LPS injection, the round cells began to decrease in number, whereas the number of irregular cells and dendritiform cells increased. A striking feature, observed between 48 to 96 hours after LPS, was a large number of dendritiform cells, with more and longer dendrites than seen in untreated animals. A gradual decrease of both dendritiform cells and pleomorphic cells was noted at the later time points.

On day 14, the ED1 positive cells in the choroid returned to the preinjection levels both in number and in morphology (Table 2). The layer of macrophages bordering the sclera was not affected except for the occasional presence of a few round cells in some areas. Figure 3 shows the dynamics of ED1 positive cells in the choroid after LPS injection. The influx of pleomorphic cells was maximal at 16 hours after LPS injection, whereas the dendritiform cell number reached a maximum between 24 and 96 hours.

Staining for MHC class II showed an influx of immunostained round cells in the choroid at 8 hours. Subsequently, the number of these cells gradually increased and reached a peak at 72 and 96 hours (Fig 2). MHC class II positive dendritiform cells with longer and more dendrites compared with the cells observed in the normal choroid were detected at 72 and 96 hours. A gradual decrease of the MHC class II positive cells was noted at the later time points, returning to the preinjection level on day 14.

MHC class II positive cells were only found just beneath the RPE layer and LPS administration did not induce the appearance of these cells on the scleral side of the choroid.

### Table 2  Changes in ED1 positive cells in the choroid following systemic lipopolysaccharide injection

<table>
<thead>
<tr>
<th>Hours after LPS injection</th>
<th>Changes in ED1 positive cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>4 and 8 hours</td>
<td>Increased number of round cells, occasionally accompanied by an increase of dendritiform cells</td>
</tr>
<tr>
<td>16 hours</td>
<td>A tremendous number of round cells, diffusely present in the whole area of the choroid with focal accumulation</td>
</tr>
<tr>
<td>24 hours</td>
<td>Round cells decreased in number, dendritiform cells numbers increased</td>
</tr>
<tr>
<td>48, 72, and 96 hours</td>
<td>A large number of dendritiform cells with more and longer dendrites and a larger but vague cell body was present, accompanied by a decrease of pleomorphic cells</td>
</tr>
<tr>
<td>7, 10, and 14 days</td>
<td>Both dendritiform cell and pleomorphic cell numbers decreased and gradually returned to preinjection level</td>
</tr>
</tbody>
</table>

**Discussion**

The present study confirms the presence of a network of tissue resident macrophages and MHC class II positive dendritic cells in the choroid of normal Lewis rats and shows that systemic LPS administration induces a marked temporary increase in both cell populations.

Immunohistological analysis of wholemounts, obtained from normal rats, revealed the presence of macrophages in a layer immediately below the RPE and in a layer bordering the sclera.

To our knowledge, the network of tissue resident macrophages on the outer surface of the choroid bordering the sclera has not yet been described. Our study revealed that the cells in this network are predominantly dendritiform cells and lack MHC class II membrane markers.

Previous studies using tissue sections showed the infiltration of the posterior segment of the eye by polymorphonuclear cells and mononuclear cells after LPS injection. The studies did not determine the phenotype of the infiltrating cells, nor did they evaluate the morphological features of the cells involved. The present study shows that systemic LPS injection induces a massive influx of macrophages into the choroid and emphasises the severe involvement of this tissue in EIU. The layer of macrophages bordering the sclera was not affected by LPS injection, a finding that was also confirmed by analysing tissue sections of this area (data not shown). It is not clear why the inner and outer layer of the choroid are not equally affected by LPS. It may be possible that LPS directly or indirectly stimulates RPE cells to release chemotactic cytokines which results in a preferential migration of inflammatory cells to the anterior side of the choroid. The migration of inflammatory cells into the choroid was seen as early as 4 hours after LPS injection and reached a maximum at 16 hours, which is similar to the invasion of the iris (unpublished own data). Moreover, a dramatic increase of dendritiform cells was noticed in the choroid between 48 to 96 hours.
after injection. Since there is no difference between the choroid and the anterior uvea with regard to the time course of the inflammatory response, it is reasonable to presume that the whole uvea is directly affected by LPS. Recently, we demonstrated a massive influx of ED1 positive cells into the retina during EIU but were not able to detect MHC class II positive cells, either before or after systemic LPS administration. Taking together these findings may also shed doubt on the concept that the EIU model is so ‘eye’ specific.

This latter finding contrasts with the choroid which contains a rich network of MHC class II positive cells. Our study using choroidal wholemounts, confirms earlier observations using cryostat sections whereby MHC class II positive dendritic cells were readily found in the rat choroid but not in the retina and sclera. Analysis of wholemounts revealed that the MHC class II positive dendritic cells were located in the choroid in close association with the RPE, which confirms earlier immunoelectron microscopic findings of Forrester et al. The number of MHC class II positive cells was significantly lower than that of ED1 positive cells. The exact nature of the infiltrating macrophages and dendritic cells was not analysed in our study. Whether LPS injection induces an influx in both macrophages and dendritic cells or whether only macrophages increase in number but now transiently express class II MHC warrants further study employing double label techniques. Enumeration of these cells first showed a peak of cells with a ‘rounded pleomorphic’ appearance at approximately 16 hours after LPS, followed by a rise in number of dendritiform cells occurring between 24 and 96 hours. These data strongly suggest an influx of blood borne round cells into the extravascular choroidal space followed by a transition of these cells into a dendritiform shape.

Double label experiments performed earlier by Forrester et al on choroidal sections revealed two distinct populations of cells since no double stained cells were observed when using OX-6 and ED2 monoclonal antibodies. Forrester et al found a smaller number of MHC class II positive cells in the choroid compared with the present data (373 versus 659 cells/mm²). The reason for the discrepancy in these studies is unclear, but may be due to differences in the animals used, as has been pointed out earlier by McMenamin et al. In this study we focused on markers for dendritic cells and macrophages. EIU is also characterised by a heavy influx of polymorphonuclear cells whereas a role for T lymphocytes is still a controversial issue. (A de Vos et al, unpublished observations.)

MHC class II positive dendritic cells may play an important role in antigen presentation and initiation of an inflammatory response in the posterior segment of the eye. Isolation of MHC class II positive cells from the rat choroid and in vitro testing has confirmed that these cells have functional capacities in common with lymphoid dendritic cells with regard to T cell responses following mitogen stimulation or mixed lymphocyte reactions. The location of the choroidal MHC class II positive cells gives them ample opportunity to continuously sample antigens from the choroidal vasculature or from the neighbouring RPE cells. Presentation of these antigens to T cells may initiate choroidal granulomatous disease as often seen in various uveitis entities and clinically represented by the appearance of fundal white dots. The concomitant presence of tissue resident macrophages, MHC class II positive dendritic cells, and RPE cells, each in close contact with each other, may represent a unique immunological microenvironment whereby the various cell types interact with each other to mediate overt choroidal inflammation. Disruption of this balance by, for instance, the release of bacterial endotoxin from sites elsewhere in the body may alter this balance. The consequences of an altered balance on the local control of immune responses are not yet known and deserves further investigation. The interaction of macrophages and dendritic cells has been extensively studied by Holt and co-workers, who have shown a suppressive effect of resident macrophages in the lung on antigen presentation by dendritic cells. The suppressive role of macrophages in the lung refers to body cavity macrophages and it may well be that interstitial macrophages behave differently, owing to the fact that these latter cells may be proinflammatory by acting as a rich source of cytokines.

The short lived inflammation in the choroid as well as in the other ocular tissues, after systemic LPS injection, suggests that there may be potent suppressive mechanisms following the inflammatory response. Analysis of cytokine expression during EIU has shown a rapid induction of the interleukin-1 receptor antagonist, which is an important natural regulator of the cytokine network. Further elucidation of these and other anti-inflammatory mechanisms may provide a new understanding of the pathogenesis and treatment of human uveitis.

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6 McMenamin PG, Crewe J, Morrison S, Holt PG. Immunomorphologic studies of macrophages and MHC


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