Retinal Microenvironment Controls Resident and Infiltrating Macrophage Function during Uveoretinitis

Morag J. Robertson,[1] *Lars P. Erwig,*[2] *Janet Liversidge,*[1] *John V. Forrester,*[1] *Andrew J. Rees,*[2] *and Andrew D. Dick*[3]

Purpose. Macrophages infiltrating an inflamed or injured tissue undergo development of coordinated sets of properties that contribute to tissue damage, repair, and remodeling. The purpose of this study was to determine whether macrophages isolated from normal or inflamed retina are programmed to a distinct set of properties and to examine whether the development of experimental autoimmune uveoretinitis (EAU) affects macrophage function.

Methods. EAU was induced in Lewis rats, and a retina-derived macrophage–enriched population was generated by density centrifugation during the prepeak, peak, and resolution phases of the disease. Cell surface phenotype was assessed by two- and three-color flow cytometry, and function was determined in vitro by nitric oxide (NO) production, with or without further cytokine stimulation or by immunohistochemistry to determine expression of β-glucuronidase, nitric oxide synthase (NOS)-2, and nitrotyrosine.

Results. Myeloid-derived cells from normal retina were programmed similar to TGF-β–stimulated uncommitted bone-marrow–derived macrophages (BMDMs). Contrary to BMDM behavior, retina-isolated macrophages displayed distinct properties and phenotype at different phases of the disease course and remained resistant throughout, to further cytokine challenge in vitro. During peak disease, retina-isolated macrophages had characteristics of IFN-γ/TNF-α primed cells (nitrotyrosine positive and NO producing). Despite equivalent numbers of macrophages during resolution, their function reverted to characteristics of TGF-β primed cells (β-glucuronidase positive).

Conclusions. Resident retinal myeloid-derived cells are primed and are resistant to further cytokine stimulation, and, similar to macrophages derived during EAU recovery, behave operationally as though TGF-β primed. During peak inflammation, infiltrating macrophages adapt to concurrent hierarchical Th1 T-cell response (IFN-γ/TNF-α), generating NO. The results provide evidence of in vivo programming of macrophages within the retina. (*Invest Ophthalmol Vis Sci.* 2002;43: 2250–2257)

There are two discrete populations of myeloid-derived cells in the retina: perivascular cells (PVCs) and microglia (MG).[1] Their roles as antigen-presenting cells (APCs), immune effectors, or immunomodulators remains unconfirmed.[1] One notion, however, akin to resident central nervous system (CNS) macrophages,[2,3] is that resident retinal macrophages are pivotal to restricting and regulating immune responses.[4] Experimental autoimmune uveoretinitis (EAU) is a retinal antigen–specific $CD4^+$ T-cell–mediated inflammation of the retina,[5] although $CD4^+$ T cells alone are insufficient to cause retinal damage.[6,7] During EAU, there is extensive tissue destruction and necrosis of resident ganglion cells and photoreceptors,[8] which in part can be restricted by inhibiting nitric oxide (NO),[9–12] depleting macrophages,[6] or neutralizing TNF-α activity.[13] Thus, understanding and controlling macrophage function in inflammatory conditions such as EAU is of therapeutic importance.

Macrophages are versatile cells that are intimately involved in all aspects of the immune response and the complex process of inflammation. Depending on the circumstances, macrophages increase the intensity of inflammation[14] or promote its resolution.[15] It has long been recognized that resident macrophages isolated from different anatomic sites are heterogeneous and that resident macrophages are adapted to the local microenvironment.[16,17] Injury leads to a rapid increase in the number of macrophages in an inflamed or otherwise damaged tissue, and the infiltrating macrophages also adapt to the local microenvironment by the development of coordinated sets of properties that enable them to perform a particular function.[18] Knowledge of what controls macrophage adaptation and dictates (or limits) the function that develops is essential for understanding how inflammation is regulated.

Macrophage functional development within an inflamed focus must be tightly regulated. It is unknown how that occurs, but in vitro studies suggest it is unlikely to be an aggregated response to all the stimuli to which they are exposed. We[19] and others[20,21] have shown that a number of pro- and anti-inflammatory cytokines influence macrophages toward development of sets of nonoverlapping and mutually exclusive properties or programs. Programming is determined by the first cytokine to which macrophages are exposed, and an essential component of the program is the development of unresponsiveness to other activating cytokines.[19]

Studies of glomerular macrophages isolated from normal and nephritic rats demonstrate the importance of macrophage programming in vivo.[22] Glomerular macrophages from normal rats are uncommitted and can be activated in a variety of ways, whereas macrophages from glomeruli of rats with nephrotoxic nephritis behave operationally as though programmed by IFN-γ.[22] Furthermore, TGF-β_3 infused into normal rats increases β-glucuronidase expression by glomerular macrophages,[23] which is identical with the response of bone-marrow–derived macrophages (BMDMs) to all TGF-β isoforms in vitro.[22] By contrast, glomerular macrophages from nephritic rats infused with TGF-β_3 did not increase β-glucuronidase expression, which demonstrates that their usual activation pathway is dominant.[23] These experiments suggest that high endogenous levels of TGF-β_3 may program resident tissue macrophages while failing to modulate the activities of macrophages infiltrating in response to immunologic injury. Whether

From the Departments of [1]Ophthalmology and [2]Medicine and Therapeutics, University of Aberdeen, Aberdeen, Scotland, United Kingdom; and the [3]Division of Ophthalmology, University of Bristol, Bristol, United Kingdom.

Supported by the Iris Fund, United Kingdom (MJR, LPE, JL, JVF).

Submitted for publication December 27, 2001; revised February 14, 2002; accepted March 1, 2002.

Commercial relationships policy: N.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Corresponding author: Andrew D. Dick, University of Bristol, Bristol Eye Hospital, Lower Maudlin Street, Bristol BS1 2LX, UK; a.dick@bristol.ac.uk.

similar macrophage function and responses are observed within an immunoregulatory environment, such as in either resident retinal myeloid populations or in infiltrating macrophages during retinal inflammation, is not known.[24] Given that there is constitutive expression of TGF-β (particularly TGF-β_2) within the eye[25] and that retinal pigment epithelial cells can produce TGF-β, studies of resident and infiltrating macrophages during an immune response in the retina are likely to provide important information on the mechanism responsible for macrophage activation and programming in vivo.

The purpose of this study was to examine macrophage functional development in the immune-privileged, TGF-β–rich microenvironment of the eye during the evolution of inflammation in EAU. The results show that myeloid-derived cells from normal retina behave like BMDM that have been programmed by TGF-β, whereas macrophages at peak disease have the characteristics of IFN-γ–primed, TNF-α–activated macrophages, which shows that the immune regulatory effects of TGF-β are overcome in the course of EAU. Resolution of inflammation is associated with restoration of macrophage properties to those found in resident retinal macrophages and BMDMs programmed by TGF-β in vitro.

Materials and Methods

Antibodies and Cytokines

Rat recombinant (r)IFN-γ was obtained from Bradsure Biologicals, Ltd. (Loughborough, UK) and human rTNF-α and rTGF-β_1 were obtained from Sigma Chemical Co. (Dorset, UK). Rat rIL-4 was produced in-house, as described previously,[26] using a CHO cell line generously donated by Neil Barclay (Medical Research Council [MRC] Cellular Immunology Unit, Oxford, UK). Soluble (s)TNF receptor (R)-IgG was kindly provided by and produced at the Therapeutic Antibody Centre, University of Oxford, as a chimeric molecule containing the soluble domain of the human p55 TNFR coupled to the CH_2 and CH_3 constant domains of human IgG_1. Unless otherwise stated, mouse mAbs specific for rat cell surface markers (Table 1) were obtained from Serotec (Oxford, UK), including mouse anti-rat I-A (OX6)-RPE and mouse anti-rat CD4 (w3/25). Purified anti-rat CD86 (B7-2), biotinylated mouse IgG1κ (negative isotype control), biotinylated mouse anti-rat mononuclear phagocyte mAb (IC7), anti-rat CD11b/c (OX42), and streptavidin-AP complex (SA-APC) were obtained from PharMingen (San Diego, CA). Anti-mouse IgG (whole molecule) FITC conjugate was obtained from Sigma Chemical Co.

Induction and Clinical Assessment of EAU

Female Lewis rats used throughout the study were treated humanely according to the UK Animal License Act and to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. EAU was induced in adult female Lewis rats (6–8 weeks old) by 0.1-mL intradermal immunization into the foot pad of 7 mg/mL retinal extract (RE; vol/vol) in complete Freund's adjuvant, with 1 μg pertussis toxin (CAMR, Wiltshire, UK), intraperitoneally. Soluble bovine RE was prepared by hypotonic lysis of freshly dissected bovine retinas in the dark, as described.[27] Ocular examination was performed daily and EAU graded[27] from day 6 after immunization by slit lamp biomicroscopy.

Immunization with bovine retinal antigens in the Lewis rat causes a self-limiting model of uveitis that, by convention, is segregated into three clinical phases. Prepeak injury starts 8 to 9 days after immunization and is characterized by a modest leukocyte infiltrate with little or no tissue injury. Leukocyte infiltration increases rapidly on about day 9 to 10 and is associated with photoreceptor destruction. This is the phase of peak injury, lasting for 2 to 3 days before injury subsides during the resolution phase. The cellular infiltrate is predominantly mononuclear, with infiltrates of macrophages and T cells within the retina, subretinal exudate, and vitreous.[6]

Table 1. Surface Antigen Targets of mAbs and Their Functions

mAb Clone	Surface Marker	Function
OX1	CD45	Leukocyte common antigen required for T- and B-cell antigen receptor–mediated activation
W3/25	CD4	Expressed by helper T cells, thymocytes, peripheral blood monocyte subsets and macrophages
CD86	CD86 (B7-2)	Expressed on antigen-presenting cells, monocytes, DCs, and activated B cells; accessory molecule that plays an important role in T- and B-cell costimulatory interactions
OX42	CD11b/c	Mac-1 receptor, αM-integrin chain
OX6	I-A (MHC II)	Present on B cells, DCs, some macrophages, and certain epithelial cells
IC7	IC7	Reacts with an antigen present on almost all cells of the mononuclear phagocyte system; detected on macrophages, DCs, and peripheral blood monocytes; not on lymphocytes
ED1	CD68-like glycoprotein	Glycoprotein expressed predominantly on the lysosomal membrane of myeloid cells; detected in the majority of tissue macrophages and weakly on peripheral blood granulocytes

Isolation and Preparation of Macrophage Cultures

Retinal Myeloid-Cells. Animals were killed by CO_2 asphyxiation from days 7 to 16 after immunization. The retinas of each animal were dissected microscopically in cold 1% BSA-PBS and then mechanically disrupted through a sieve (50 μm mesh) to obtain a single-cell suspension. Retinal cells of each animal were kept separate throughout isolation procedures. After further washes in 1% BSA-PBS, cells were subsequently isolated using a graduated gradient (Percoll density gradient; Pharmacia Upjohn, Uppsala, Sweden), as previously described,[5] that enriches leukocytic populations within a mixed population of cells containing resident retinal cells, such as ganglion cells and neuronal elements. Cells were collected from the 1.072 and 1.088 g/mL layers and washed, and viability was determined by trypan blue exclusion (enriched retinal cell population). At this point 1 to 2×10^6 cells were separated for flow cytometric analysis (each animal was individually assessed). For culture, purity of CD11b/c$^+$ macrophage cell numbers was confirmed by single-color flow cytometric analysis, and macrophage-enriched populations were plated at 5×10^5 CD11b/c$^+$ macrophages per milliliter in 24-well tissue culture plates and rested for 1 hour at 37°C. RPMI complete medium was then replaced and the macrophages incubated for a further 24 hours, with or without cytokines. For analysis of normal and prepeak phases, animals were pooled to obtain adequate cell numbers for further analysis (between 4 and 6 retinas were required).

Bone-Marrow–Derived Macrophages. Uncommitted BMDMs were prepared as previously described[19] and used in parallel in vitro assays as a positive control to confirm cytokine-mediated macrophage function. In brief, bone marrow cells were flushed aseptically from the dissected femurs of male Sprague–Dawley rats with complete medium through a 25-gauge needle to form a single-cell suspension. The cells were cultured in 75-mm tissue culture flasks and adhered to plastic in complete medium with L929-conditioned medium as a source of mac-

rophage colony-stimulating factor (M-CSF). After 5 days in culture, macrophages were removed by using trypsin and recultured in 24-well plates at a concentration of 5×10^5 cells per well and rested overnight in M-CSF–free medium before being washed and stimulated (described later).

Cytokine Stimulation of Macrophage Cultures. Assay culture conditions were optimized using BMDMs that had been cultured and stimulated as previously described.[19] Cytokines used were IFN-γ (20 U/mL), TNF-α (5 ng/mL), IL-4 (5 μL), and sTNFR-IgG (13 μg/mL), alone or administered sequentially in combination, each cytokine separated by a 4-hour period. Macrophage function was assessed 24 hours after first cytokine addition. IFN-γ, followed 4 hours later by TNF-α (IFNγ/TNFα), was used as a positive stimulation for NO production, confirming previous in vitro studies.[19] Where stated, cytokine additions were made relative to IFN-γ/TNF-α (positive control) stimulation. Cytokines were not removed before macrophage function was assessed.

Flow Cytometric Phenotypic Analysis of Retinal Macrophages

Immunophenotyping of infiltrating leukocytes was performed using mouse mAbs specific for the rat cell surface markers (listed later). mAbs used were either unconjugated or conjugated to biotin, PE, or FITC for three-color immunofluorescence. Unconjugated mAbs were detected with rat anti-mouse FITC, and biotinylated mAbs were detected with SA-APC. Staining was performed using flow cytometry buffer (PBS-BSA, 10 mM NaN_3) for washes. All reagents, buffers, and incubations were kept and performed at 4°C. Negative isotype control and single positive control experiments were performed to allow accurate breakthrough compensation. Primary unconjugated mAbs included CD4, CD11b/c, and CD86. Cells were incubated sequentially with the primary mAb, anti-mouse IgG (whole molecule) FITC conjugate, in the presence of 10% normal rabbit serum (NRS), blocked with 10% normal mouse serum (NMS)-NRS, biotinylated second antibody (CD45, CD11b/c, or IC7) with PE-conjugated third antibody (mouse anti-I-A mouse/rat), and finally SA-APC. Acquisition was performed by flow cytometry (FACS Calibur; BD Bioscience, Plymouth, UK) and analysis with the accompanying software (CellQuest; BD Bioscience). A total of 10,000 events were collected, and gates and instrument settings were set according to forward- and side-scatter characteristics. Fluorescence analysis was performed after further back gating to exclude dead cells, the majority of neutrophils, and background staining.

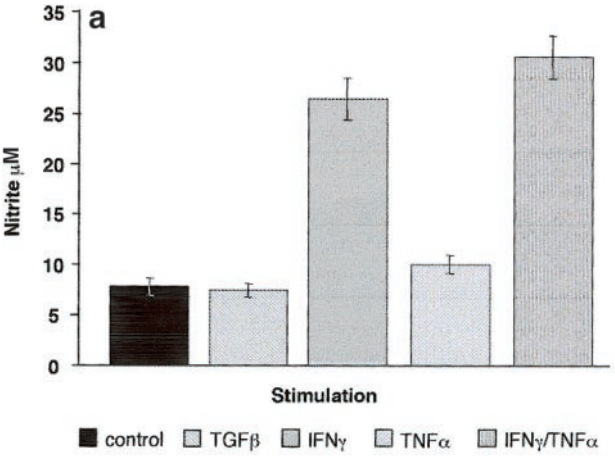
Quantification of NO Synthesis

NO generation was estimated after 24 hours in culture by assaying culture supernatants for the stable reaction product of NO, nitrite, against a sodium nitrite standard on the same plate. Aliquots (200 μL) of each cell-free supernatant were incubated with 50 μL Griess reagent (0.5% sulfanilamide, 0.05% *N*-(1-naphtyl) ethylenediamine dihydrochloride in 2.5% phosphoric acid) in 96-well flat-bottomed plates for 10 minutes at room temperature. The optical densities were measured at 540 and 690 nm to account for background.

Parallel assays with control BMDMs typically produced the following amounts of NO: medium alone, 7.9 ± 0.8 μM; TGF-β, 7.5 ± 0.7 μM; IFN-γ, 26.5 ± 2.1 μM; TNF-α, 10.1 ± 0.9 μM; IFN-γ/TNF-α, 30.6 ± 2.1 μM (Fig. 1a). The control findings illustrate the viability of the assay and permit comparison with stimulation profiles of retinal macrophages.

Quantification of β-Glucuronidase Expression

Expression of β-glucuronidase was determined by an enzymatic staining method in which β-glucuronidase catalyzed the reaction of α-naphthol AS-BI β-D-glucuronide into the red soluble chromogenic naphthol AS-BI-horseradish peroxidase (HPR) complex.[28] All components were obtained from Sigma Chemical Co. Cytospin preparations of macrophages harvested from culture or whole retinal cell populations were fixed in glutaraldehyde-acetone solution and the slides air dried. They



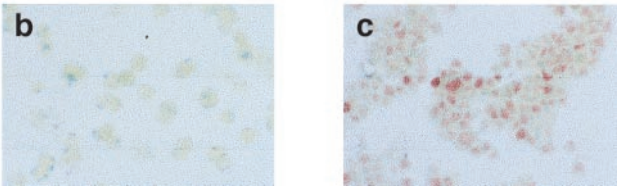


Figure 1. Functional analysis of BMDMs. (**a**) Nitrite production by day 5 in cultured BMDMs. Data are mean ± SEM. (**b**) Control BMDMs did not express β-glucuronidase. Cells were counterstained with methylene blue. (**c**) β-Glucuronidase production (*red* staining) was stimulated by TGF-β in cultured BMDMs at day 5.

were then stained with β-glucuronidase staining solution and counterstained with methylene blue before being mounted in aqueous medium. BMDMs were stimulated, as described, to act as the control for cytokine conditioning and confirming previous data.[19] Control BMDMs did not express β-glucuronidase (Fig. 1b), whereas TGF-β induced increased expression of β-glucuronidase in cultured BMDMs (Fig. 1c).

Immunohistochemistry and Immunocytochemistry

Eyes removed from normal or immunized animals at the various stages of EAU were embedded in optimal cutting temperature (OCT) compound, snap frozen, and stored at −30°C. Serial 8-μm cryostat sections of the eyes were taken onto poly(L-lysine)-coated slides and air dried overnight. Cells were isolated from diseased eyes at various phases of disease and cytospins made with ~5×10^5 cells per slide. Before staining, tissue sections or cytospins were fixed in an acetone-methanol (1:1) mix at −20°C for 5 to 10 minutes and air dried. Sections were stained using either single or dual fluorescence for the following Abs: nitric oxide synthase (NOS)-2 (Clone 6, 1:100; Transduction Laboratories, Nottingham, UK) and infiltrating blood-borne macrophage marker ED1 (1:50, FITC conjugate; Serotec), and also through a standard avidin-biotin (alkaline phosphatase) technique for ED1 (1:100, purified, Serotec). The number of ED1-positive cells infiltrating throughout the retina and subretinal space, were counted throughout the section under a ×20 objective. Three sections per eye were counted. Cytospins were stained through the same technique as was used for mouse monoclonal anti-nitrotyrosine (Clone 1A6, 1:20; Upstate Biotechnology, Lake Placid, NY). All washes and dilutions were in Tris-buffered saline (TBS), unless otherwise stated. For dual fluorescent staining, sections were blocked with NRS (1:5) for 20 minutes, followed by overnight incubation with anti-NOS-2 (1:100) at 4°C. After three 5-minute washes, a secondary biotinylated rabbit anti-mouse Ig (1:100,

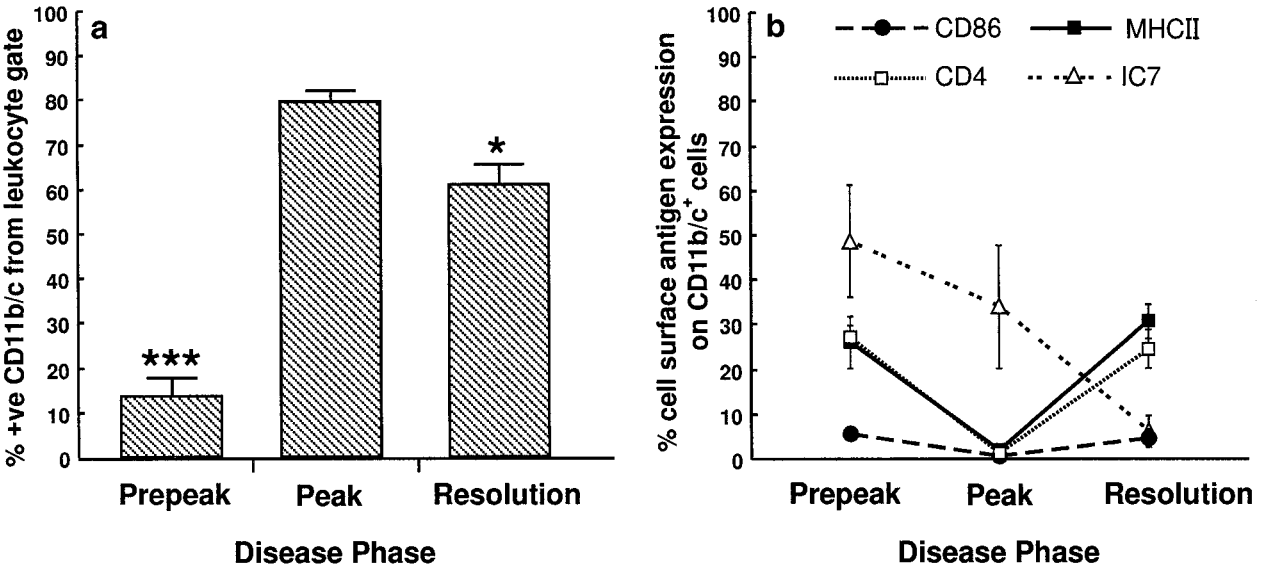


FIGURE 2. Cell surface phenotypic analysis of infiltrating leukocytes during EAU. (**a**) The proportion of CD11b/c$^+$ cells within the enriched retinal cell isolate increased throughout the course of disease (***$P < 0.001$), concomitant with increasing numbers of infiltrating leukocytes (Table 2). There was a significant reduction in the proportion of CD11b/c$^+$ cells from the peak to the resolution phase of disease (*$P < 0.05$), although actual numbers remained constant (Table 2). (**b**) Cell surface markers on CD11b/c$^+$ cells varied, depending on disease phase. IC7 expression was maximal during prepeak and peak phase EAU, followed by a significant reduction during the resolution phase. At prepeak disease 26.0% ± 5.8% and 27.2% ± 2.5% of CD11b/c$^+$ macrophages expressed MHC II and CD4, respectively, which decreased significantly during the peak phase EAU (**$P < 0.01$) before increase during the resolution phase. CD86 expression remained minimal throughout. Results are representative of repeated experiments. Data are the mean ± SEM of three to four animals per disease phase.

10% NRS; Dako, Glostrup, Denmark) was added for 30 minutes, followed by further washes. Sections were then incubated with streptavidin-Texas red (1:50) for 30 minutes, washed, and incubated in ED1-FITC–conjugated mAb alone for 1 hour. Washed sections were then mounted (Vectashield; Vector Laboratories, Burlingame, CA) and viewed under the appropriate excitation filters of a microscope, according to the manufacturer's instructions (BH2-RFC; Olympus, Tokyo, Japan).

Statistical Analysis

Differences between groups of NO production were analyzed by Kruskal-Wallis analysis of variance (ANOVA) and Dunnett posttest. Differences between groups for phenotypic analysis were analyzed by a one-way ANOVA and Tukey-Kramer posttest. All results are expressed as the mean ± SEM.

RESULTS

Macrophage Infiltration in EAU

Injury in experimental rats followed exactly the same course as previously described and was graded on a severity scale from 1 to 5.[29,12] For this study, disease progression was classified as prepeak (grades 1–2), peak (grades 3–5), postpeak (grades 2–3), and resolution (grades 1–0) phases on clinical grounds. Phenotypic analysis of retinal inflammatory cells was performed. A liberal leukocyte gate applied on the scatterplot during flow cytometric analysis demonstrated that CD11b/c$^+$ myeloid cells, although representing a low proportion of the enriched retinal cell population (13.6%) at prepeak phase, rapidly increased to comprise 79.6% ($P < 0.001$) during peak phase of disease, followed by only a small decrease in number during the resolution phase (61.0%, $P < 0.05$; Fig. 2a). Further analysis using immunohistochemistry confirmed significant differences in macrophage phenotype during the individual phases of EAU. ED1$^+$ monocyte/macrophages were absent from the normal retina but were present in the earliest inflammatory infiltrates with 90.9 ± 23.2 increasing to 282.7 ± 14.0 ($P < 0.001$) ED1$^+$ cells per section at the peak phase of disease (Table 2), reflecting the large number of blood monocyte-derived macrophages infiltrating the retina in this model. The number of ED1$^+$ macrophages remained high throughout the resolution phase (192.0 ± 10.7).

The specific configuration of surface receptors during different phases of the disease is likely to reflect differences in macrophage function. To address this question more precisely we isolated macrophages from the inflamed retina and analyzed their repertoire of surface receptors using flow cytom-

TABLE 2. Numbers of Infiltrating Cells during Phases of EAU

Phase of EAU	Leukocyte Number (CD45$^+$) (×10^5)	Macrophage Number (CD11b/c$^+$) (×10^5)	Number of ED1$^+$ Cells per Section
Prepeak	2.8 ± 1.0	2.0 ± 0.6	90.9 ± 23.2
Peak	20.1 ± 1.2*	18.1 ± 1.2*	282.7 ± 14.0*
Resolution	26.1 ± 1.2*	18.7 ± 0.5*	192.0 ± 10.7*

Data are mean ± SEM of individual animals (three to four animals per disease phase). CD45$^+$ and CD11b/c$^+$ cell numbers are derived from two retina/animal. ED1 is an immunohistochemical comparison of cell count per section per animal.

* $P < 0.001$.

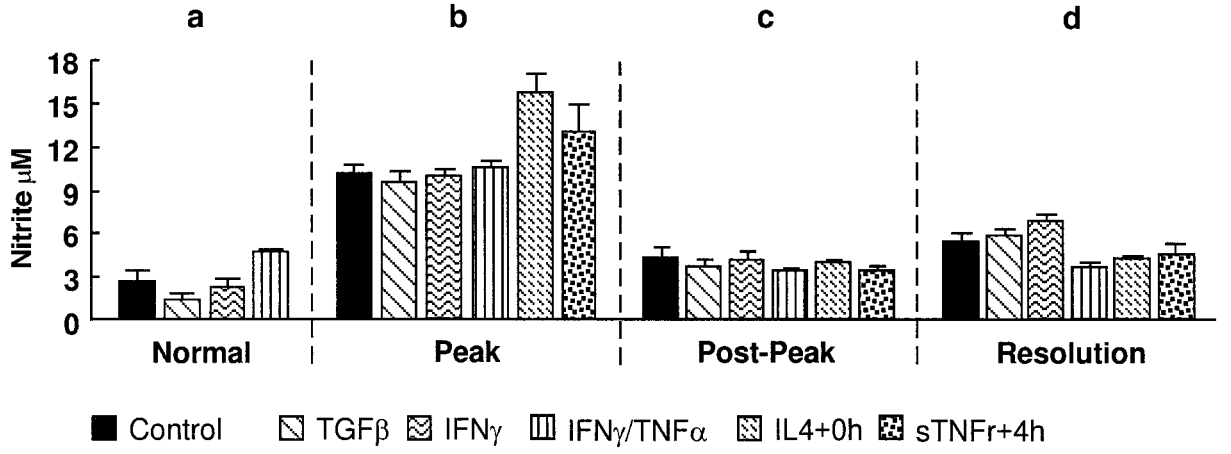


FIGURE 3. Quantification of NO production by infiltrating macrophages during EAU. Histogram bars display NO production by retinal macrophages incubated for 24 hours with medium alone (Control) or in the presence of IFN-γ , IFN-γ/TNF-α, sTNFR-IgG, TGFβ, or IL-4. Addition of sTNFR-IgG and IL-4 were relative to stimulation with IFN-γ/TNF-α. (**a**) Stimulation with cytokines had no effect on NO production by resident retinal macrophages, which are constitutively low NO producers and similar to macrophages isolated at prepeak, postpeak (**c**), and resolution (**d**) phases of disease. (**b**) During peak disease, NO production was maximal ($P < 0.001$), but macrophages were again unresponsive to further NO production after stimulation with cytokine or cytokine cocktail. For normal retina and resolution phases, animals were pooled to obtain enough retinal macrophages for analysis, and each test condition was analyzed in triplicate. During peak disease, animals were individually assessed by each test condition, in duplicate. Data are the mean ± SEM (n = 3–12 test wells).

etry. The total number of leukocytes infiltrating the retina during EAU is shown in Table 2. The number of $CD45^+$ cells within the enriched retinal cell population (see the Methods section) increased from $2.8 \pm 1 \times 10^5$ at prepeak to $20.1 \pm 1.2 \times 10^5$ at the peak phase of disease ($P < 0.001$) and $26.1 \pm 1.2 \times 10^5$ at resolution ($P < 0.001$). Accordingly, the number of $CD11b/c^+$ macrophages was $2.0 \pm 0.6 \times 10^5$, $18.1 \pm 1.2 \times 10^5$, and $18.7 \pm 0.5 \times 10^5$, respectively, at the prepeak, peak, and resolution phases of disease (Table 2). Analysis of the surface receptor expression of the $CD11b/c^+$ macrophages clearly demonstrated distinct receptor expression profiles for each phase of the disease (Fig. 2b). Prepeak macrophage infiltrates contained a substantial proportion of $IC7^+$ macrophages (48.7% ± 12.2%), but this proportion decreased to only 6.4% ± 3.0% $IC7^+$ macrophages during the resolution phase of disease. MHC class II^+ and $CD4^+$ macrophages followed a different pattern. At prepeak the proportion of macrophages that expressed major histocompatibility complex (MHC) class II and CD4 were 26.0% ± 5.8% and 27.2% ± 2.5%, respectively; the proportions then declined to background levels (2.1% ± 0.5% and 1.4% ± 0.7%, respectively) at peak-phase disease and increased again to 24.4% ± 4.3% $CD4^+$ and 30.6% ± 3.9% MHC class II^+ at resolution phase. These data infer that macrophages may possess antigen-presenting properties in early and late stages of disease, whereas at peak disease their surface receptor profile suggests that they exhibit distinctly different functions. This is supported by the complete absence of CD86 expression at peak disease (Fig. 2b).

Macrophage Function at Different Phases of EAU

NO generation by macrophages purified from uveitic eyes at different stages of the disease was chosen as a reliable indicator of macrophage programming by TGF-β and IFN-γ in BMDMs and macrophages isolated from inflamed tissue.[19,22] We ran parallel cultures of BMDMs with cytokine stimulation as the culture control for both IFN-γ–mediated NO production and additionally TGF-β–mediated β-glucuronidase expression, thus allowing interpretation of retinal macrophage behavior. Macrophages isolated from normal retina (consisting of perivascular $ED2^+$ macrophages and MG^1) generated little NO spontaneously (2.8 ± 0.6 μM; Fig. 3a) and was not induced to do so by incubation with IFN-γ (Fig. 3a). This supports the hypothesis that TGF-β, known to be present abundantly in the eye[25] programs the resident macrophage population and renders the cells unresponsive to alternative activation by IFN-γ. These results are similar to those from resident glomerular macrophages from rats subjected to systemic administration of TGF-β_1,[23] and contrast with those from normal rats that behave as though uncommitted and can be induced to generate NO by IFN-γ.[19]

Macrophages isolated from prepeak phase disease also generated little NO spontaneously (3.0 ± 0.5 μM), which was not augmented by activation with IFN-γ and TNF-α (data not shown). However, macrophages purified from uveitic eyes at peak disease (corresponding to maximum macrophage infiltration in the retina) spontaneously produced significant amounts of NO (9.0 ± 0.5 μM, $P < 0.001$; Fig. 3b), and this production was not influenced by ex vivo incubation with the anti-inflammatory mediators IL-4[19] or sTNFR-IgG.

Because ED1 positivity remained high throughout the later stages of disease, we further divided the resolution phase into postpeak (days 13–15) and resolution (days 15–17 and onward), to dissect and assess kinetics of any change in macrophage function. During these phases, infiltrating retinal macrophages again produced little NO (3.9 ± 0.6 and 5.2 ± 0.5 μM, respectively) even after activation with IFN-γ and TNF-α (Figs. 3c, 3d).

In contrast to macrophages isolated from the postpeak phase, those isolated from peak phase did not express β-glucuronidase (Fig. 4a). Thus, they behaved operationally as though activated by IFN-γ. A result identical with macrophages from inflamed glomeruli of rats at peak disease in nephrotoxic nephritis.[22] This clearly demonstrates that the presumed immunoregulatory effects of TGF-β are overcome in the posterior chamber of the eye during the course of EAU and that the macrophages infiltrating the retina are programmed by IFN-γ, known to be present in the retina at peak disease.[4,30] Macrophages isolated from postpeak phase EAU expressed β-glucuronidase; 21% showed strong β-glucuronidase expression (Fig. 4b). However, by the resolution phase, this expression was absent (Fig. 4c). Such heterogeneity raises an important question: Either this is the first demonstration of macrophage re-

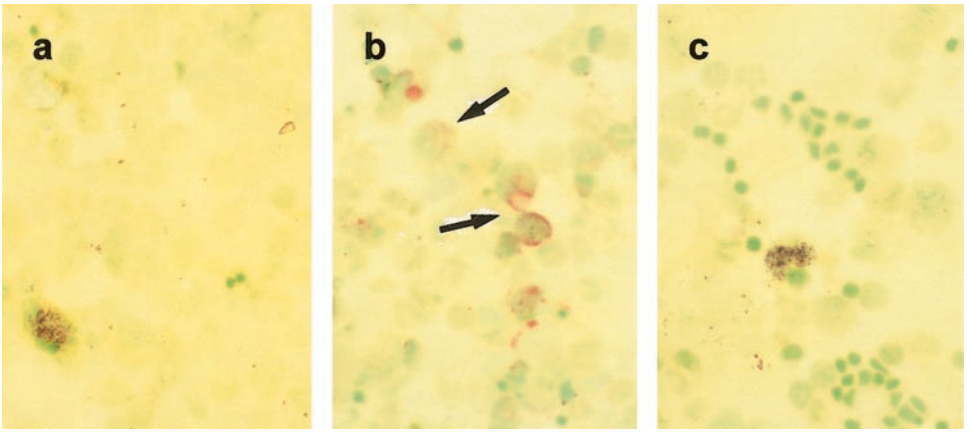


FIGURE 4. Macrophage β-glucuronidase expression during EAU. β-Glucuronidase was expressed only by retinal macrophages during late EAU. (**a**) Peak-phase EAU macrophages were negative for β-glucuronidase. (**b**) During the postpeak phase of EAU, macrophages were increasingly β-glucuronidase positive (*arrows*), with approximately 21% of macrophages staining positive. (**c**) During the resolution phase, macrophages were again negative for β-glucuronidase expression.

programming within an inflamed focus, or it is a reflection of the replacement of IFN-γ programmed peak disease macrophages by differentially activated macrophages infiltrating the retina during the resolution phase.

Macrophage Expression of NOS-2 and Nitrotyrosine in EAU

Dual immunohistochemistry was performed using ED1 to identify macrophages together with antibodies to NOS-2. The results confirmed previous reports[12,31] that NOS-2 was expressed during the prepeak phase with colocalization with ED1 staining within the retina (Fig. 5a). As previously reported,[12] macrophages did not express NOS-2 at peak disease (Fig. 5b), despite our demonstration that they produced NO spontaneously ex vivo. NOS-2 was not expressed during the resolution phase. Nitrotyrosine was also detected in retinal macrophages at peak disease through to the resolution phase (Fig. 5c), demonstrating that some of the macrophages found in the eye during these times had previously been exposed to substantial amounts of NO and superoxide anion, although they no longer produced NO. Given the heterogeneity of macrophages within the eye during disease, the proportion of macrophages that stained positive for nitrotyrosine was not high enough for us to be confident that they also expressed β-glucuronidase.

DISCUSSION

Macrophages infiltrating an inflamed site enter an environment where they are exposed to many different signals, some with opposing effects.[18] Understanding how macrophages process multiple and often conflicting signals and yet undergo development of functions appropriate to different stages of injury is an important question. EAU in rats provides an exceptional opportunity to study these processes, because it is an example of macrophage-dependent T-cell–mediated immunity[5] occurring in an environment rich in the anti-inflammatory cytokine TGF-β.[25] The ability to purify retinal macrophages from rats with EAU has enabled us to address the specific question of how macrophages respond to opposing signals in vivo and also to examine how macrophage properties change during the evolution of the inflammation. The results demonstrate clearly that resident retinal macrophages (and MG) and those from the recovery phase of EAU behave operationally as though conditioned by anti-inflammatory cytokines such as TGF-β, whereas those harvested at the height of the disease are indistinguishable from macrophages activated by proinflammatory cytokines, such as IFN-γ and TNF-α. Thus, inflammatory macrophages adapt to the local environment in a hierarchical way with the IFN-γ response predominating over that of TGF-β at the height of EAU.

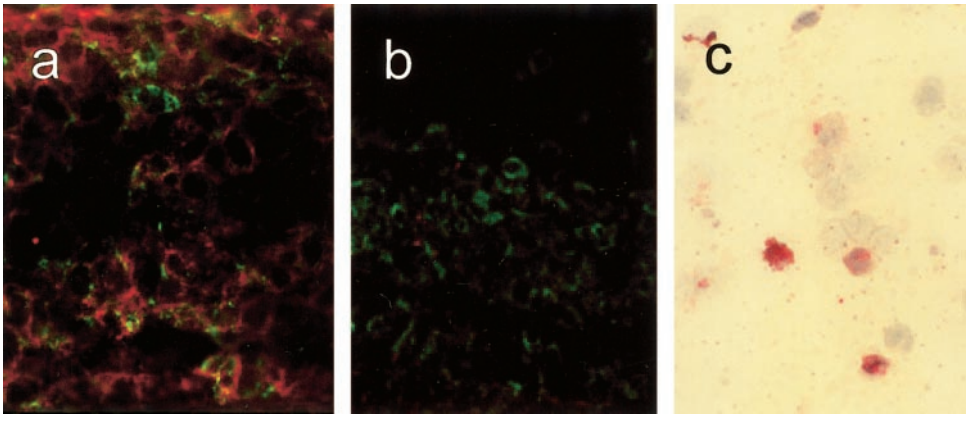


FIGURE 5. Immunohistochemical analysis of macrophage NOS-2 and nitrotyrosine expression during EAU. Two-color immunofluorescence was performed, with ED1 (FITC) and NOS-2 (Texas red). Nitrotyrosine expression was determined on cytospin columns by the alkaline phosphatase-antialkaline phosphatase (APAAP) method. (**a**) An increased number of $ED1^+$ $NOS\text{-}2^+$ macrophages were found during prepeak phase EAU only. (**b**) NOS-2 expression was absent in $ED1^+$ macrophages during peak phase EAU. (**c**) Nitrotyrosine was expressed from peak disease to resolution as represented by the nitrotyrosine-positive expression during postpeak phase EAU.

Resident retinal macrophages and those from the earliest phase of EAU generated little NO spontaneously, a characteristic feature of TGF-β–conditioned BMDMs in vitro.[19] They were also unable to generate NO in response to IFN-γ and TNF-α in vitro—that is, they had been programmed not to respond to these cytokines. By contrast, macrophages from the height of disease spontaneously generated NO while displaying little β-glucuronidase activity and were unable to increase expression in vitro—that is, they had programmed unresponsiveness to TGF-β. These results are consistent with those in murine[20] and rat[19] BMDMs studied in vitro, in that prior exposure to TGF-β abrogates responses to IFN-γ, whereas the IFN-γ response predominates when macrophages are stimulated with both cytokines at the same time. This is not simply the result of loss of cytokine receptors from the cell surface but reflects cross-talk between intracellular signaling pathways. For example, in some cell types,[32] IFN-γ increases expression of the inhibitory Smad7 which abrogates TGF-β signaling through the Smad pathway. Macrophages purified from eyes during the resolution phase of EAU again behaved as though conditioned by TGF-β, presumably reflecting the reduction of proinflammatory stimuli at later stages. Regardless of the precise mechanisms involved, programming in vivo provided a mechanism for macrophages to promote repair after the need for them to cause tissue destruction had passed.

The large number of macrophages in the eye from the height of disease onward provides further evidence that macrophages have twin roles in injury and repair in uveitis, and this conclusion is supported by the changes that take place in the molecules they express on the cell surface. Roughly half the retinal macrophages purified from rats before peak injury reacted with the monoclonal antibody IC7, which recognizes an antigen found on mononuclear phagocytic cells of normal rats with a cellular distribution similar to CD68 and ED1.[33] However in EAU the proportion of IC7-expressing macrophages decreased substantially throughout the course of the disease to 6% at the resolution phase, although it is worth noting that the greater number of retinal macrophages during this phase means that there were equivalent IC7-expressing retinal macrophages during both phases of the disease. Given that macrophages were predominantly $ED1^+$ throughout the course of disease, the functional significance of IC7 expression remains undetermined. It is apparent that $ED1^+$ cells can be either $IC7^+$ or not at various phases of disease, and as we have shown herein, display varying activation profiles, implying that IC7 expression confers functional macrophage properties that differ from those conferred by ED1.

The change in the nature of the macrophages in the eye from prepeak disease to resolution was even more striking with regard to expression of CD4 and MHC class II. Both were expressed by roughly a quarter of macrophages from prepeak disease (by an average of 1000 macrophages per animal). By contrast, only 1% to 2% of macrophages at peak phase of disease expressed these markers, but the rapid increase in number of macrophages at this stage means that this fraction represents an almost identical number of positive macrophages. In other words, there was a substantial influx of CD4- and MHC class II–negative cells without reduction in the number of CD4- and MHC class II–positive cells. However, the absolute number of positive cells increased by about 10-fold during the resolution phase of disease. Whether the macrophages mature from CD4- and MHC class II-negative to CD4- and MHC class II–positive cells during the evolution of EAU or whether traffic of macrophages through the inflamed site means that negative cells are replaced by positive cells later in the course of the disease remains undetermined. Whatever the explanation, our data clearly show that the observed phenotypic differences in macrophages corresponding to changes in activation status had functional significance in vivo.

Classic and alternative macrophage activation has been proposed as a parallel to the Th1-Th2 paradigm.[34] Classically, activated macrophages (effector macrophages) are characterized after exposure to IFN-γ and TNF-α by their high NO production.[19] However, macrophages, if conditioned by IL-10 or -4, have been proposed to downregulate inflammation and act to balance proinflammatory and anti-inflammatory immune reactions.[35] Alternatively, activated macrophages exhibit broad specificity for foreign antigen and anti-inflammatory cytokines, as well as chemokines.[36] We were able to confirm that resting-state resident macrophages, which are mainly MGs, exhibit responses corresponding to TGF-β–mediated conditioning, in that macrophages were constitutively low NO producers (Fig. 2a). We chose NO generation as a measure of macrophage activation because, first, such functions have been used to characterize macrophage programming in vitro,[19] and, second, the cytokines that induce such responses are present not only in normal retina but during EAU.[37] Our results did not support evidence that once activated, macrophage function could be alternatively activated, despite previous in vitro studies that showed that macrophages can be reprogrammed when rested before alternative activation.[18] For example, resident retinal macrophages including MGs displayed TGF-β conditioning, with no increase in NO production after IFN-γ/TNF-α stimulation, which was also observed in infiltrating macrophages during disease resolution. Because there was no correlation of the number of persistently nitrotyrosine-positive cells from peak to resolution and β-glucuronidase–positive cells during the resolution phase, we have no evidence to support the notion that macrophages can change function in situ. To resolve this, we are currently investigating ex vivo macrophages in longer-term culture with respect to NOS-2 and β-glucuronidase expression.

During peak and prepeak phases of disease, the dominant response in the rat is a Th1 IFN-γ response,[38] and this corresponds with our findings that macrophages are activated, similar to BMDMs in vitro when stimulated with IFN-γ/TNF-α. If TNF-α activity is neutralized systemically through sTNFR-IgG, then retinal tissue damage is significantly reduced, despite continued infiltration of macrophages.[13] Changes in phenotype have been shown during EAU, in that oxidative damage and peroxynitrite is principally restricted to the photoreceptors concomitant with $ED1^+$/MHC class II^+ mononuclear cell infiltration.[31] In context with the present results, MHC class II and CD4 appear to be poor indicators of macrophages that cause tissue injury; we showed that during maximal NO production MHC class II expression was lowest, which is in keeping with reports that only NOS-2 relates directly to activation of macrophages to destroy tissue.[39] The exact function of the MHC class II–positive macrophage remains to be determined. However, BMDMs, when stimulated through uptake of necrotic cells present antigen to T cells with greater efficiency and therefore may propagate or even initiate immune responses dependent on the signals they receive.[40]

In conclusion, therefore, our studies emphasize the distinct roles for macrophages at different stages during the evolution of EAU, and the potential importance for macrophage programming in determining which role they adopt. This regulation may permit the downregulation of macrophage function and allow, with the subsequent change in microenvironment during disease course, a new influx of macrophages to be alternatively programmed and provide different functions.

References

1. 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;79:834–840.
2. Sedgwick JD, Schwender S, Imrich H, Dörries R, Butcher GW, ter Meulen V. Isolation and direct characterisation of resident microglial cells from the normal and inflamed central nervous system. *Proc Natl Acad Sci USA.* 1991;88:7438–7442.
3. 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;154:4309–4321.
4. Xiao B-G, Link H. Is there a balance between microglia and astrocytes in regulating Th1/Th2 responses and neuropathologies? *Immunol Today.* 1999;20:477–479.
5. Caspi RR, Roberge FG, McAllister CG, et al. T cell lines mediating experimental autoimmune uveoretinitis (EAU) in rats. *J Immunol.* 1986;136:928–933.
6. Forrester JV, Huitinga I, Lumsden L, Dijkstra CD. Marrow-derived activated macrophages are required during the effector phase of experimental autoimmune uveoretinitis in rats. *Curr Eye Res.* 1998;17:426–437.
7. Caspi RR, Chan CC, Fujino Y, et al. Recruitment of antigen-nonspecific cells plays a pivotal role in the pathogenesis of a T cell-mediated organ-specific autoimmune disease, experimental autoimmune uveoretinitis. *J Neuroimmunol.* 1993;47:177–188.
8. Forrester JV, Liversidge J, Dua HS, Towler H, McMenamin PG. Comparison of clinical and experimental uveitis. *Curr Eye Res.* 1990;9:75–84.
9. Thillaye-Goldenberg B, Goureau O, Naud MC, de Kozak Y. Delayed onset and decreased severity of experimental autoimmune uveoretinitis in mice lacking nitric oxide synthase type 2. *J Neuroimmunol.* 2000;110:31–44.
10. Tilton RG, Chang K, Corbett JA, et al. Endotoxin-induced uveitis in the rat is attenuated by inhibition of nitric oxide production. *Invest Ophthalmol Vis Sci.* 1994;35:3278–3288.
11. Mandai M, Yoshimura N, Yoshida M, Iwaki M, Honda Y. The role of nitric oxide synthase in endotoxin-induced uveitis: effects of N^{G}-nitro L-arginine. *Invest Ophthalmol Vis Sci.* 1994;35:3673–3680.
12. Hoey S, Grabowski PS, Ralston SH, Forrester JV, Liversidge J. Nitric oxide accelerates the onset and increases the severity of experimental autoimmune uveoretinitis through an IFN-γ–dependent mechanism. *J Immunol.* 1997;159:5132–5142.
13. Dick AD, Duncan L, Hale G, Waldmann H, Isaacs J. Neutralising TNF-alpha activity modulates T-cell phenotype and function in experimental autoimmune uveoretinitis. *J Autoimmunity.* 1998;11:255–264.
14. Cattell V. Macrophages in acute glomerular inflammation. *Kidney Int.* 1994;45:945–952.
15. Di Pietro LA, Burdick M, Low QE, Kunkel SL, Strieter RM. MIP-1alpha as a critical macrophage chemoattractant in murine wound repair. *J Clin Invest.* 1998;101:1693–1698.
16. Forrester JV, McMenamin PG. Immunopathogenic mechanisms in intraocular inflammation. *Chem Immunol.* 1999;73:159–185.
17. Holt PG, Warner LA, Papadimitriou JM. Alveolar macrophages: functional heterogeneity within macrophage populations from rat lung. *Aust J Exp Biol Med Sci.* 1982;60:607–618.
18. Laszlo DJ, Henson PM, Remigio LK, et al. Development of functional diversity in mouse macrophages: mutual exclusion of two phenotypic states. *Am J Pathol.* 1993;143:587–597.
19. Erwig L-P, Kluth DC, Walsh GM, Rees AJ. Initial cytokine exposure determines function of macrophages and renders them unresponsive to other cytokines. *J Immunol.* 1998;161:1983–1988.
20. Lake FR, Noble PW, Henson PM, H. Riches DW. Functional switching of macrophage responses to tumor necrosis factor-alpha (TNF-alpha) by interferons: implications for the pleiotropic activities of TNFalpha. *J Clin Invest.* 1994;93:1661–1669.
21. Shnyra A, Brewington R, Alipio A, Amura C, Morrison DC. Reprogramming of lipopolysaccharide-primed macrophages is controlled by a counterbalanced production of IL-10 and IL-12. *J Immunol.* 1998;160:3729–3736.
22. Erwig L-P, Stewart K, Rees AJ. Macrophages from inflamed but not normal glomeruli are unresponsive to anti-inflammatory cytokines. *Am J Pathol.* 2000;156:295–301.
23. Wilson HM, Minto AWM, Brown PAJ, Erwig L-P, Rees AJ. Transforming growth factor-β isoforms and glomerular injury in nephrotoxic nephritis. *Kidney Int.* 2000;57:2434–2444.
24. Forrester JV. Duke-Elder Lecture: new concepts on the role of autoimmunity in the pathogenesis of uveitis. *Eye.* 1992;6:433–446.
25. Streilein JW, Ksander BR, Taylor AW. Immune deviation in relation to ocular immune privilege. *J Immunol.* 1997;158:3557–3560.
26. Tam FWK, Smith J, Karkar AM, Pusey CD, Rees AJ. Interleukin-4 ameliorates experimental glomerulonephritis and up-regulates glomerular gene expression of IL-4 decoy receptor. *Kidney Int.* 1997;52:1224–1231.
27. Dick AD, Cheng YF, Liversidge J, Forrester JV. Immunomodulation of experimental autoimmune uveoretinitis: a model of tolerance induction with retinal antigens. *Eye.* 1994;8:52–59.
28. Machin GA, Halper JP, Knowles II DM. Cytochemical demonstrable β-glucuronidase activity in normal and neoplastic human lymphoid cells. *Blood.* 1980;59:1111–1119.
29. Laliotou B, Liversidge J, Forrester JV, Dick AD. Interphotoreceptor retinoid binding protein is a potent tolerogen in Lewis rat: suppression of experimental autoimmune uveoretinitis is retinal antigen specific. *Br J Ophthalmol.* 1997;81:61–67.
30. Barton K, McLauchlan MT, Calder VL, Lightman S. The kinetics of cytokine mRNA expression in the retina during experimental autoimmune uveoretinitis. *Cell Immunol.* 1995;164:133–140.
31. Zhang J, Wu L-Y, Wu G-S, Rao NA. Differential expression of nitric oxide synthase in experimental uveoretinitis. *Invest Ophthalmol Vis Sci.* 1999;40:1899–1905.
32. Stopa M, Benes, V, Ansorge W, Gressner AM, Dooley S. Genomic locus and promoter region of rat Smad7, an important antagonist of TGFbeta signaling. *Mamm Genome.* 2000;11:169–176.
33. van Goor H, Harms G, Gerrits PO, Kroese FGM, Poppema S, Grond J. Immunohistochemical antigen demonstration in plastic-embedded lymphoid tissue. *J Histochem Cytochem.* 1988;36:115–120.
34. Goerdt S, Orfanos CE. Other functions, other genes: alternative activation of antigen-presenting cells. *Immunity.* 1999;10:137–142.
35. Stumpo R, Kauer M, Martin S, Kolb H. Alternative activation of macrophages by IL-10. *Pathobiology.* 1999;67:245–248.
36. Goerdt S, Politz O, Schledzewnski K, et al. Alternative versus classical activation of macrophages. *Pathobiology.* 1999;67:222–226.
37. Okada AA, Sakai J, Usui M, Mizuguchi J. Intraocular cytokine quantification of experimental autoimmune uveoretinitis in rats. *Ocul Immunol Inflamm.* 1998;6:111–120.
38. Caspi RR, Sun B, Agarwal RK, et al. T cell mechanisms in experimental autoimmune uveoretinitis: susceptibility is a function of the cytokine response profile. *Eye.* 1997;11:209–212.
39. MacMicking J, Xie Q, Nathan C. Nitric oxide and macrophage function. *Ann Rev Immunol.* 1997;15:323–350.
40. Barker RN, Erwig L, Pearce WP, Devine A, Rees AJ. Differential effects of necrotic or apoptotic cell uptake on antigen presentation by macrophages. *Pathobiology.* 1999;67:302–305.