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Regulation of Fc receptor and major histocompatibility complex antigen expression on isolated rat microglia by tumour necrosis factor, interleukin-1 and lipopolysaccharide: effects on interferon-gamma induced activation

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SUMMARY

Isolated rat brain microglia display enhanced expression of Fc receptors on treatment with interferon-gamma (IFN-γ), tumour necrosis factor-alpha (TNF-α), interleukin-1 (IL-1) and lipopolysaccharide (LPS), whereas major histocompatibility complex (MHC) antigen expression is enhanced only by IFN-γ. Although TNF and LPS individually have no effect on MHC expression by microglia, they both antagonize IFN-γ-induced expression. The enhanced expression of Fc receptors observed in the presence of IFN-γ, TNF or LPS is significantly inhibited by the combination of IFN-γ with either LPS or TNF. IL-1α has little effect on IFN-γ-induced MHC or Fc receptor expression by microglia. Peritoneal macrophages behave similarly to microglia, with the notable exception that IL-1α enhances IFN-γ-induced FcR expression. These observations suggest that the functional activity of microglia during inflammation or demyelination in the central nervous system can be influenced by the changing profile of cytokines present during lesion development.

INTRODUCTION

Microglia, the resident mononuclear phagocytes of the central nervous system (CNS), respond to immunological insult or direct mechanical injury to the brain with an increase in the expression of macrophage markers and production of cytokines. Microglia produce interleukin-1 (IL-1), interleukin-6 (IL-6) and tumour necrosis factor (TNF) on stimulation by lipopolysaccharide (LPS) *in vitro*, and immunocytochemical evidence has implicated microglia as a source of cytokines detected *in vivo*.[1,2]

Isolated microglia have a similar phenotype to macrophages and demonstrate many of the same functional properties *in vitro*.[3–5] When cultured in the presence of interferon-gamma (IFN-γ), rat microglia display increased expression of MHC antigens[6] and Fc receptors and produce reactive oxygen intermediates.[7]

Increased expression of MHC class II on microglia in white matter of multiple sclerosis (MS) brain tissue suggests that they may function as antigen-presenting cells during T-cell activation.[8] Electron microscopic evidence indicates that under certain circumstances microglia are also capable of the ingestion and subsequent destruction of myelin.[9] Cytokines crossing a leaky blood–brain barrier or produced by both infiltrating and resident cells, including activated microglia, at sites of inflammation may influence the rate of lesion growth in MS. The complex nature of the cytokine network would result in cells and myelin present at the lesion site being exposed to different combinations of cytokines at different times.

In view of the wide spectrum of cytokine mediators released at sites of inflammation, our earlier study on the effect of IFN-γ on microglia *in vitro*[7] has been extended to include a wide range of cytokines. The effects of individual cytokines and combinations of cytokines on the potential for antigen presentation and phagocytosis by microglia may provide insight into mechanisms of activation of inflammatory demyelination and suggest possible means for down-regulating the process.

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MATERIALS AND METHODS

CNS tissue

After decapitation of 30-day-old Sprague–Dawley rats of both sexes (Charles River, U.K.) cerebra were dissected out and the meninges removed as described previously.[13]

Media

Cell dissociation and separation were carried out in Earle's balanced salt solution (EBSS; Gibco Ltd, Paisley, Renfrewshire, U.K.). Resident peritoneal cells were collected in EBSS without calcium and magnesium (EBSS-Ca^{2+}, Mg^{2+}; Gibco Ltd). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco Ltd) supplemented with 10% foetal calf serum (FCS) (ICN, Flow Laboratories, High Wycombe, Bucks, U.K), 1 mM

sodium pyruvate, 100 IU/ml penicillin and 100 μg/ml streptomycin solution (Gibco Ltd) (DMEM + 10% FCS).

Cytokines

Stock solutions of all cytokines were prepared under sterile conditions at the following concentrations and stored as described. Recombinant rat IFN-γ [$2{\cdot}5 \times 10^5$ U/ml in 1% bovine serum albumin (BSA)/phosphate-buffered saline (PBS); Holland Biotechnology, The Netherlands] and recombinant mouse IL-1α (10 ng/ml in 0·1% BSA/PBS; New Brunswick Biologicals, Hatfield, Herts, U.K.) were stored at −70°. Recombinant mouse IL-1β (5 μg/ml in 1% BSA/PBS; British Biotechnology, Abingdon, Oxon, U.K.), recombinant mouse IL-3 (10^5 U/ml in 1% BSA/PBS; Gibco BRL, Middlesex, U.K.) and recombinant mouse granulocyte–macrophage colony-stimulating factor (GM-CSF) (5×10^3 U/ml in 1% BSA/PBS; New Brunswick Biologicals) were stored at −20°. Recombinant human TNF-α (2×10^4 U/ml in 1% BSA/PBS, British Biotechnology Ltd, Cowley, Oxford, U.K.) was stored at 4°. LPS (1 mg/ml in EBSS; Sigma Chemical Co., Dorset, U.K.) was stored at −20°.

Cell culture

Microglia were isolated from a mixed glial suspension by rosette formation with opsonized erythrocytes via the Fc receptor, and subsequent separation of rosetted cells on a density gradient was performed as described in ref. 3 with modifications as in ref. 2.

Briefly, approximately 30–35 g of tissue (from 30 rats) was processed at a time in three batches of 10–15 g. An average yield of $0{\cdot}8 \times 10^6$ cells per g of brain was obtained. Cells were plated into 96-well flat-bottomed plates at 10^5 cells per well in 150 μl of DMEM + 10% FCS and maintained at 37° in a 95% air/5% CO_2 incubator. The medium was changed after 1 and 2 days and cells were maintained in culture for 5 days prior to cytokine treatment.

Peritoneal macrophages (Mϕ) were obtained by peritoneal lavage with 50 ml of ice-cold EBSS-Ca/Mg, washed in EBSS-Ca/Mg, resuspended in DMEM + 10% FCS and plated out as for the microglia.

Incubation of cells with cytokines

On the fifth day after plating, the cells (microglia and Mϕ) were treated with varying concentrations of cytokine in DMEM + 10% FCS which included indomethacin (1 μg/ml). Assays were performed in triplicate wells except when two cytokines were used in combination, in which case duplicate wells were used. All cytokines were incubated with the cells for 72 hr before assaying.

Enzyme-linked immunosorbant assay (ELISA) for MHC class II

ELISA was performed as described previously[7] using OX6 (Serotec Ltd, Oxford, U.K.) ascites fluid, specific for MHC class II, as the first antibody and mouse IgG1 kappa (MOPC 21) myeloma protein (Sigma Chemical Co.) as a subclass-specific control. Results were analysed by computer (ELISA + software, Meddata Inc., NY) and expressed as the mean optical density at 405 nm (OD_{405}) of duplicate or triplicate wells.

Fc receptor assay

The pseudoperoxidase activity of opsonized erythrocytes (EA) bound and ingested by cells in culture was used as a measure of FcR expression,[10] as a good correlation has been observed between the relative number of Fc receptors expressed and phagocytosis of EA by macrophages following cytokine stimulation.[11] The procedure was as detailed in ref. 7. As a control for non-specific binding, non-opsonized erythrocytes (E) were substituted for EA. The absorbance of the pseudoperoxidase-catalysed product was read at a noted time (0–15 min) and results were analysed by computer (ELISA +). The mean number of red cells in duplicate or triplicate wells was calculated. Results are calculated as number of red cells bound and ingested via FcR per 10^5 cells plated by subtraction of E binding (non-specific) from EA binding (total specific and non-specific) (EA − E).

MTT assay

Cell viability was assessed by the capacity of the cells to convert the tetrazolium salt, MTT [3-(4,5-dimethylthiazol-2,yl)-2,5-diphenyltetrazolium bromide] to a coloured product as described in ref. 2.

Statistical analyses

Statistical significance was determined using Wilcoxon one- and two-sample tests.

RESULTS

In view of the large number of cells required for this study, the protocol for microglial cell isolation described in ref. 3 was scaled up, and the conditions for rosetting microglia in a mixed glial suspension were further optimized.[2] Cell density, as assayed at the end of the incubation period, was found to be unaffected by cytokines at the concentrations used in this investigation. For 10^5 cells seeded per well the same proportions of microglia and macrophages were adherent, as measured by OD_{620} using the MTT assay. The mean OD_{620} for microglia was $0{\cdot}15 \pm 0{\cdot}01$ ($n = 16$) and for Mϕ was $0{\cdot}16 \pm 0{\cdot}01$ ($n = 50$). The absolute number of viable cells assayed was not determined; results are therefore expressed per 10^5 cells plated.

Although the Fc receptor is utilized during microglial cell isolation, the response of peritoneal macrophages to IFN-γ did not alter with regard to Fc receptor or MHC class II expression, following isolation by the same procedure.[7]

Fc receptor expression

Microglia and Mϕ were cultured for 72 hr in the presence of cytokines. The increase in specific binding (EA − E) at indicated cytokine concentrations over that observed in the absence of cytokine is shown in Fig. 1. Although basal levels of expression of Fc receptors varied between cell preparations, the increases observed upon treatment with cytokines were consistent. Constitutive FcR expression (EA − E) in the absence of cytokine was in general higher in microglia than in Mϕ ($100 \pm 14 \times 10^3$ compared with $37 \pm 5 \times 10^3$ RBC per 10^5 cells plated).

Background non-specific binding of uncoated erythrocytes (E) to microglia was very low at all concentrations of the cytokines tested. However, Mϕ showed considerable increases in non-specific binding, particularly on treatment with IFN-γ, TNF and LPS (results not shown). IFN-γ induced a much greater increase in FcR expression in microglia than in Mϕ, and in both cases the increases were significant ($P < 0{\cdot}05$). TNF significantly increased FcR expression by both cell types,

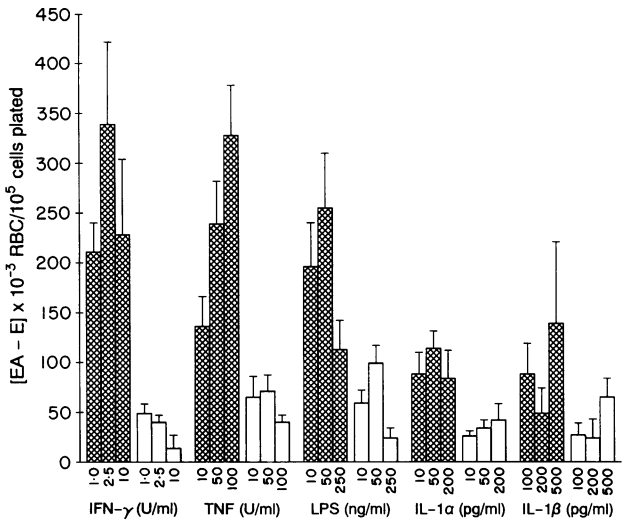

Figure 1. Increase in specific Fc receptor-mediated binding of opsonized erythrocytes (EA – E) by microglia (cross-hatched) and Mϕ (plain) after cytokine treatment. Cells were plated at 10^5 cells per well and incubated in the presence of cytokine for 72 hr before assay. Values represent mean increases over basal levels ± SEM ($n > 4$ in all cases). $P < 0.05$ for all except Mϕ with IFN-γ at 10 U/ml; Mϕ with TNF at 100 U/ml; and Mϕ with IL-1β (insufficient data to test significance).

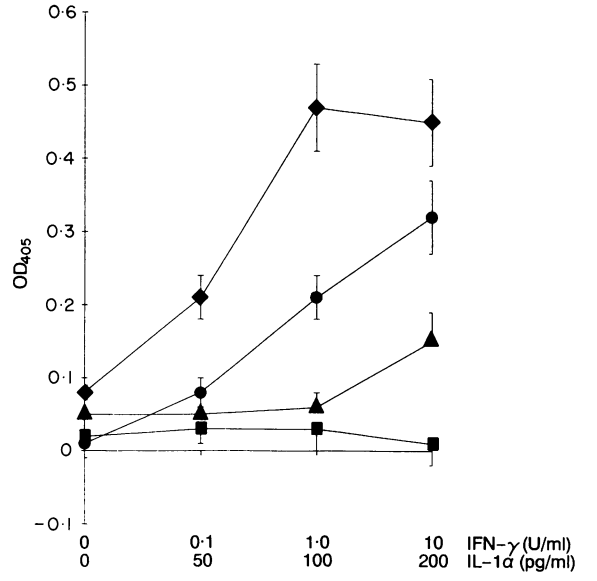

Figure 2. Effect of IFN-γ and IL-1α on MHC class II expression by microglia and Mϕ. Cells were plated at 10^5 cells per well and incubated in the presence of cytokine for 72 hr before assay. Values are means of absorbance at 405 nm ± SEM. (●) Microglia with IFN-γ ($n > 14$); (◆) Mϕ with IFN-γ ($n > 27$); (■) microglia with IL-1α ($n > 3$); (▲) Mϕ with IL-1α ($n > 6$).

although the effect was more pronounced with microglia than with Mϕ. FcR expression in microglia decreased significantly at 300 U/ml TNF compared with 100 U/ml ($P < 0.05$) (J. Loughlin, unpublished observation).

Stimulation of microglia and Mϕ with LPS produced a similar pattern, with significant increases in FcR expression, and in both cases the decrease in expression between 50 ng/ml and 250 ng/ml LPS was significant. Treatment of microglia with 50 pg/ml IL-1α produced an increase in FcR expression approximately three times the increase observed in Mϕ under similar conditions. At the higher concentration of 200 pg/ml IL-1α, the number of FcR increased further in Mϕ. Stimulation with 500 pg/ml IL/1β produced a significant increase in FcR expression in microglia ($P < 0.01$), approximately twice that found in Mϕ (insufficient data to test significance).

Microglia therefore proved more responsive than Mϕ to IFN-γ, TNF, LPS, IL-1α and IL-1β with respect to FcR expression. Also tested, but not shown in Fig. 1, were IL-3 and GM-CSF, both of which at 1 U/ml induced comparable increases in FcR number in microglia (46×10^3 and 44×10^3 RBC per 10^5 cells, respectively) and Mϕ (52×10^3 and 45×10^3 RBC per 10^5 cells, respectively), decreasing at higher concentrations.

MHC class II expression

The effect of IFN-γ and IL-1α on MHC class II expression on microglia and Mϕ is shown in Fig. 2. Microglia consistently had less constitutive MHC class II expression than Mϕ. After 7–8 days in culture (the last 72 hr including 1 μg/ml indomethacin in the culture medium), microglia expressed a level of MHC class II fourfold less than in Mϕ [0·02 ± 0·01 and 0·08 ± 0·01 OD units, respectively ($P < 0.05$)]. In agreement with previous reports, treatment with IFN-γ produced significant increases in MHC class II expression ($P < 0.01$ at all concentrations) in both microglia and Mϕ, with a greater response in Mϕ than in microglia. There was a small but significant increase ($P < 0.05$) in MHC class II expression in Mϕ on treatment with 200 pg/ml IL-1α, but no increase was observed in microglia (Fig. 2). TNF (1–300 U/ml), LPS (1–250 ng/ml), IL-1β (10–500 pg/ml) and GM-CSF (1–50 U/ml) had no significant effect on MHC class II expression in either microglia or Mϕ (results not shown).

Simultaneous treatment of cells with two cytokines

Figure 3a shows the effect of IFN-γ on FcR expression by Mϕ in combination with either 50 U/ml TNF, 50 ng/ml LPS or 10 pg/ml IL-1α. Individually IFN-γ and IL-1α produce increases in FcR expression, and in combination these doses have an additive effect on FcR expression. Co-treatment of Mϕ with IFN-γ plus TNF or LPS produces net reductions in FcR expression by Mϕ. In contrast to the additive effect of IFN-γ and IL-1α on Mϕ, similar treatment of microglia had no effect on the level of expression induced by IFN-γ, although the response of microglia to 10 pg/ml IL-1α alone was comparatively low (Fig. 3b). Microglia responded similarly to Mϕ on co-treatment with IFN-γ and TNF or LPS. Individually these cytokines all stimulate Fc receptor expression in microglia, but the combination of IFN-γ with TNF or LPS produced net decreases in expression.

As described earlier, IL-1α, TNF and LPS have no effect on MHC class II expression by Mϕ at the concentrations used here (10 pg/ml, 50 U/ml and 50 ng/ml, respectively). However, both TNF and LPS depress the response of Mϕ to IFN-γ with respect to MHC class II expression (Fig. 4a). IL-1α appears to give a small increase in the response of Mϕ to IFN-γ, but this increase is not significant. Similarly, microglial class II expression as induced by 10 U/ml IFN-γ is reduced when 50 U/ml TNF or 50 ng/ml LPS is included, as shown in Fig. 4b. As with Mϕ,

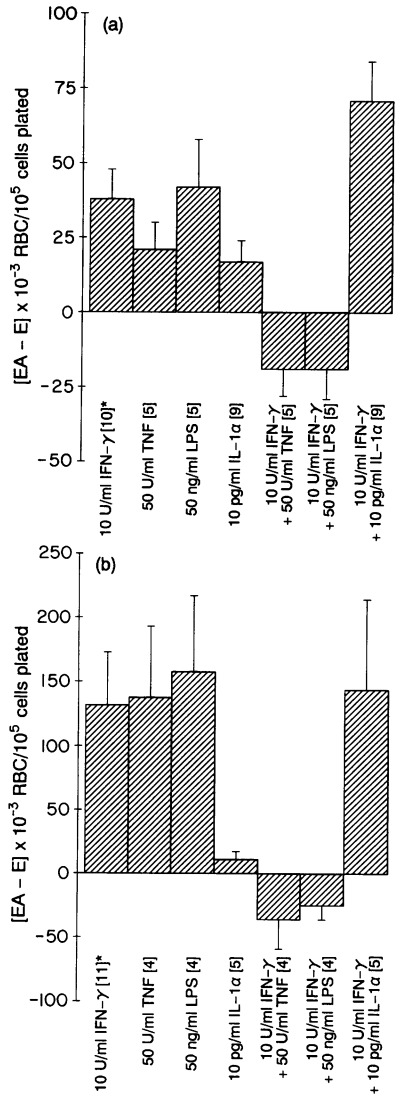

Figure 3. Effect of TNF (50 U/ml), LPS (50 ng/ml) or IL-1α (10 pg/ml) on IFN-γ-induced FcR expression by Mϕ (a) and by microglia (b). Cells were plated at 10^5 cells per well and incubated in the presence of cytokines for 72 hr before assay. Values are mean increases in specific binding (EA − E) over basal levels ± SEM. *n values.

microglia showed no significant change in class II expression when IL-1α was included with IFN-γ.

The effect of IL-1β on Fc receptor expression by microglia in combination with TNF is shown in Fig. 5. Individually IL-1β (500 pg/ml) and TNF (50 U/ml) increase FcR expression, and in combination these doses result in an additive response. Neither IL-1β nor TNF affected MHC class II expression on microglia individually, and co-treatment with IL-1β and TNF had no effect on basal levels of MHC class II.

DISCUSSION

The present study demonstrates that LPS and a panel of cytokines, including IFN-γ, TNF, IL-1α and β, IL-3 and GM-CSF, enhance the binding of opsonized erythrocytes to macrophages and microglia, to varying degrees, whereas stimulation of MHC class II antigen expression is restricted to IFN-γ, a T-cell-derived cytokine. MHC class II expression on macrophages thus appears to be tightly regulated and dependent on a product of antigen-stimulated T cells, whereas a wider range of cytokines such as TNF, IL-1 and IL-6, products of Mϕ or activated T cells, are able to influence functional properties, including the level of Fc receptor expression. As Fc receptors mediate both phagocytosis of immune complexes and antibody-dependent cellular cytotoxicity, an increase in their expression on microglia in response to locally produced cytokines such as IL-1 and TNF could contribute to the breakdown of myelin in demyelinating diseases. It is interesting to note that microglia have a lower level of constitutive and IFN-γ-stimulated class II expression than do peritoneal macrophages. In contrast, constitutive expression of FcR as well as stimulated increases with TNF, LPS, IL-1 and IFN-γ are higher in microglia than in Mϕ.

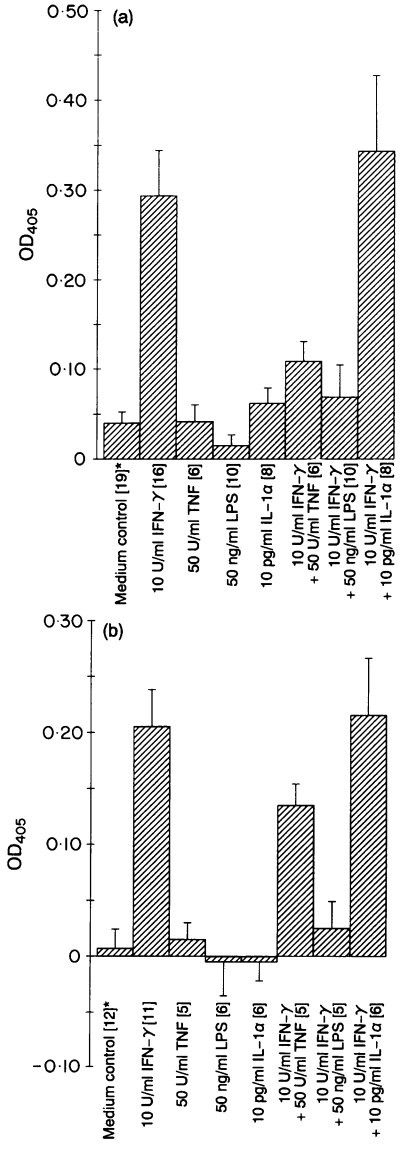

Figure 4. Effect of TNF (50 U/ml), IL-1α (10 pg/ml) and LPS (50 ng/ml) on IFN-γ-induced MHC class II expression by Mϕ (a) and by microglia (b). Cells were plated at 10^5 cells per well and incubated in the presence of cytokines for 72 hr before assay. Values are expressed as mean of absorbance at 405 nm ± SEM. *n values.

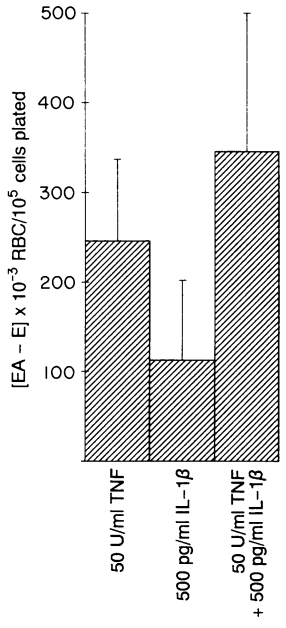

Figure 5. Effect of co-stimulation of microglia with TNF (50 U/ml) and IL-1β (500 pg/ml) on FcR expression. Values are mean increases in specific binding (EA − E) over basal levels ± SEM ($n = 5$).

These observations may reflect a readiness or inducibility of microglia for phagocytic activity *in vivo*.

The restriction of MHC class II antigen induction on microglia to IFN-γ is in line with studies on other macrophage populations.[12,13] Interleukin-1 does not appear to influence MHC class II expression,[14] although in this study a small but statistically significant increase in class II expression was observed on peritoneal macrophages, but not on microglia. There is conflicting evidence regarding the effect of TNF on MHC antigen expression, with reports of induction of MHC class II on macrophage cell lines[15] but not on peritoneal macrophages.[16] TNF had no effect on class II expression on either of the cell populations in the present study. Our results are in general agreement with those of others, who have found that only IFN-γ induces class II expression on astrocytes and synoviocytes whereas other cytokines are ineffective.[17,18] Frendl and Beller[19] demonstrated that GM-CSF, IL-1α, IL-1β and TNF do not up-regulate MHC class II expression on murine peritoneal exudate cells.

LPS has been extensively studied as an activating signal, inducing effector functions and production of IL-1 and TNF by macrophages. IL-1 and TNF are produced by microglia *in vivo* following mechanical injury to the CNS[2] and, as observed in this study, both these cytokines increase the expression of Fc receptors, TNF being the more potent inducer. Microglia have a very low level of non-specific binding, which is unaffected by cytokine treatment, whereas peritoneal macrophages respond to IFN-γ, TNF and LPS stimulation with increases in non-specific as well as FcR-mediated binding and ingestion of red cells. This may reflect a difference in the number and/or variety of cell-surface adhesion molecules expressed on peritoneal macrophages and microglia.

Most studies on the effects of cytokines have been concerned with defining actions of individual factors. As these mediators function in concert in inflammatory lesions, the effect of cytokine interactions on cell function requires definition. Although IL-1 and TNF have many overlapping biological activities,[14] we found that only TNF had any significant effect on FcR and class II expression in the presence of IFN-γ. Both TNF and LPS markedly inhibit the stimulatory effects of IFN-γ on Mϕ and microglia. Co-stimulation of microglia with TNF and IL-1β produced an additive increase in FcR expression and did not affect Ia expression, suggesting that both cytokines may act by a similar mechanism, possibly at a transcriptional or translational level. The effects of individual cytokines and combinations of cytokines on class II and FcR expression in both Mϕ and microglia are summarized in Table 1.

Table 1. Summary of effect of cytokines on FcR and MHC class II expression on microglia (MG) and peritoneal macrophages (Mϕ)

	FcR		MHC class II	
Cytokine	Mϕ	MG	Mϕ	MG
IFN-γ	+	+++	+++	++
TNF	+	+++	0	0
LPS	+	+++	0	0
IL-1	+	++	+	0
IFN-γ + TNF	−	−	+	+
IFN-γ + LPS	−	−	+	0
IFN-γ + IL-1	++	+++	+++	++

+ Denotes an increase above basal levels, − denotes a decrease below basal levels, 0 denotes no change observed.

Conflicting reports regarding the effect of combinations of modulatory cytokines demonstrate the importance of considering the state of differentiation and maturation of cells and the length of time they are exposed to cytokines when making comparisons. For example, Watanabe and Jacob[20] observed that TNF enhances IFN-γ-induced MHC class II expression in undifferentiated cells, whereas in mature macrophage populations MHC class II expression is reduced. Zimmer and Jones[16] found that the effect of TNF and IFN-γ co-treatment depends on how long the cells are exposed to the cytokines. They observed that TNF blocks the increase in Ia and FcR expression as a result of the effect of IFN-γ on peritoneal macrophages after 72 hr, which is consistent with our findings. TNF and IL-1 both inhibit Ia induction by IFN-γ on cerebral endothelial cells,[21] which contrasts with our observation that only TNF markedly inhibits IFN-γ activation of microglia.

Our results show that LPS also antagonizes IFN-γ-induced effects on microglia and macrophages. Arend *et al.*[22] have suggested that the LPS inhibition of IFN-γ-induced FcR expression in human monocytes is primarily caused by IL-1 produced in response to LPS stimulation and, to a lesser extent, by TNF, having observed that IL-1 and TNF are both inhibitory. We have found no evidence that IL-1α inhibits IFN-γ-induced FcR expression and suggest that the inhibitory effect of LPS is due to TNF rather than IL-1. Inconsistencies between results are possibly due to different differentiation states of macrophage populations. A mutual antagonism between TNF and IFN-γ has been reported by Alvaro-Gracia *et al.*[18] As the simultaneous presence of both TNF and IFN-γ was necessary to

block the class II induction, it would appear that the inhibitory mechanism does not involve modulation of IFN-γ receptor expression. Furthermore, inclusion of indomethacin in the culture medium eliminates prostaglandins as mediators of TNF effects. Our results, therefore, are in general agreement with the observations of others and further illustrate that the effects of cytokines depend on cell type, the state of differentiation of the cells and the period of incubation of cells with cytokines.

The mechanisms whereby such a variety of cytokines alter Fc receptor expression remain unclear, as does the mechanism by which TNF and IFN-γ interact. It is interesting to note that TNF and LPS have a more dramatic effect on FcR expression than on Ia expression in IFN-γ-treated microglia or Mϕ, reflecting perhaps a more critical control of phagocytic rather than antigen-presenting capability *in vivo*. This may be significant in diseases such as MS, in which the functional deficit is caused by demyelination. The *in vitro* antagonism between TNF and IFN-γ observed here suggests that, *in vivo*, TNF produced subsequent to IFN-γ induction of MHC class II expression on macrophages may act as a control mechanism to attenuate class II expression and down-regulate the capacity of these cells to present antigen. TNF might induce phagocytic properties in microglia when IFN-γ levels are low and vice versa; however when both cytokines are present the result may be down-regulation of these properties.

Interestingly, IL-1α has comparatively little effect on the IFN-γ stimulation of functional properties in microglia, although it additively increases IFN-γ-induced FcR expression in Mϕ. LPS, which induces TNF and IL-1 production by both cell populations, has similar effects in combination with IFN-γ as TNF, suggesting that *in vivo* TNF may override IFN-γ and/or IL-1, preventing an increase in antigen-presenting capacity or phagocytic properties.

IFN-γ, TNF and IL-1 have all been identified in MS brain tissue (ref. 23 and K. W. Wucherpffenig and J. Newcombe, personal communication). The local environment, the time-course of inflammatory events and the changing profile of cytokines present during the course of the inflammation and demyelination will tightly control cellular events and lesion development as evidenced by the diverse effects of cytokines on microglia and Mϕ function.

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