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Regulation of Fcγ Receptors and Immunoglobulin G-Mediated Phagocytosis in Mouse Microglia

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Abstract

As resident macrophages in the CNS, microglia can transform from a surveillance state to an activated phenotype in response to brain injury. During this transition microglia become highly capable phagocytic cells. Invading pathogens undergo opsonization with immunoglobulins and microglia recognize these opsonized pathogens through interaction with their cognate F_c receptors. In mice, both FcγRI and FcγRIIb receptors are involved in IgG-mediated phagocytosis of opsonized pathogens. At sites of inflammation, microglial activity is regulated by T-cell derived cytokines. Here we first investigated the effects of IFN-γ, IL-4, IL-13 and GM-CSF on expression of FcγRI and FcγRIIb mRNA levels in both primary microglia and microglial cell line N9. Using quantitative real-time PCR we show that IFN-γ induced a four-fold increase in the mRNA level of FcγRI but did not induce changes in FcγRIIb expression. IL-4 and IL-13 induced approximately two-fold increases in expression of FcγRIIb mRNA, but had no effect on FcγRI expression. GM-CSF increased both FcγRI and FcγRIIb mRNA expression. We then characterized the ability of these same cytokines to regulate phagocytosis of immune complexes composed of IgG and the bacteria *Staphylococcus aureus*. IFN-γ and GM-CSF both induced approximately two-fold increases in IgG-mediated phagocytosis whereas IL-4 and IL-13 both decreased IgG-mediated phagocytosis by about one-third. None of the cytokines influenced basal levels of phagocytosis. These findings demonstrate a highly selective cytokine-induced regulation of both phagocytosis-related Fcγ receptor subtypes and IgG-mediated phagocytosis itself in microglia. This selective regulation has implications for our understanding of the pathophysiology of CNS infection and autoimmune disease.

Keywords

microglia; IFN-γ; IL-4; IL-13; FcγRI; FcγRIIb

INTRODUCTION

Microglia, the resident tissue macrophages of the CNS, are active sensors and versatile effector cells in the normal and pathologic brain[14]. Microglia shift activity states depending on the surrounding microenvironment. Under normal conditions they are characterized by a small cell body with fine, ramified processes and low expression of surface antigens. In response to brain injury, ischemia and inflammatory stimuli, microglia rapidly transform into an activated

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phenotype associated with proliferation, migration to the site of injury, elaboration of both neurotoxic and neurotrophic factors and phagocytosis of cellular debris[9,14,33].

In the CNS, microglia are the predominant phagocytic cell type[9]. Many different phagocytic receptors with differing modes of action have been described [12]. For example, the macrophage phosphatidylserine receptor specifically targets apoptotic cells by directly recognizing phosphatidylserine at the cell's surface[29]. In contrast, invading pathogens and non-cellular CNS autoimmune antigens (such as myelin basic protein) require opsonization by immunoglobulin (Ig) and/or complement fixation for efficient recognition and phagocytosis by Fc and complement receptors[5,35].

In most mammals, two general classes of Fc receptors for IgG are now recognized: (i) signal transduction activation receptors such as FcγRI (CD64) and FcγRIII (CD16), characterized by the presence of a cytoplasmic immunoreceptor tyrosine-based *activation* motif (ITAM) sequence associated with the receptor, and (ii) inhibitory receptors such as FcγRII (CD32), characterized by the presence of an immunoreceptor tyrosine-based *inhibitory* motif (ITIM) sequence[15,27]. The main function of FcγRIII is to induce killing by NK cells, whereas FcγRI and FcγRII in macrophages mediate phagocytosis of IgG-opsonized invading pathogens [16]. Interestingly, Fcγ receptor-mediated phagocytosis of beta-amyloid by microglia has a protective effect in a mouse model of Alzheimer's disease[2]. In humans, there are three different FcγRI subtypes (FcγRIa, b and c), however, in mice, there is only a single FcγRI species, encoded by a single gene[39]. Similarly, in humans there are three different FcγRII subtypes (FcγRIIa, b and c) but mice express only FcγRIIb[28]. Cellular immunity-related Th1 cells and humoral immunity-related Th2 cells are both important in peripheral immune responses and regulation of microglial cells[18]. IFN-γ is produced by Th1 cells whereas IL-4 and IL-13 are produced by Th2 cells. In many aspects, the Th1 and Th2 response associated cytokines oppose each other's functional effects[23]. In peripheral macrophages Fcγ receptors are differentially regulated by the opposing actions of Th1 or Th2 derived cytokines[4,26, 34], however, little is known about the regulation of phagocytosis-related Fcγ receptors in microglial cells in the CNS. GM-CSF is also produced by T-cells as well as by a number of other immune and neural cell types. GM-CSF is associated with macrophage/microglial differentiation and maturation and has been shown to influence microglial phagocytosis as well [37]. In order to better understand the effects of T-cell-derived cytokines on phagocytosis in microglia, we investigated: (i) the effects of IFN-γ, IL-4, IL-13 and GM-CSF on the mRNA levels of FcγRI and FcγRIIb and (ii) the functional effects of these same four cytokines on IgG-mediated phagocytosis of the bacterial pathogen *Staphylococcus aureus* (*S. aureus*) in mouse primary microglia (pMG) and the mouse microglial cell line N9.

MATERIALS AND METHODS

Solutions and Reagents

Fluorescein labeled *S. aureus* was obtained from Invitrogen. Anti-*S. aureus* monoclonal IgG antibody was purchased from QED Bioscience. Isotype control for the anti-*S. aureus* monoclonal antibody was obtained from BD Biosciences. Recombinant mouse interferon-γ (IFN-γ), Interleukin-4 (IL-4), Interleukin-13 (IL-13) and granulocyte macrophage colony stimulating factor (GM-CSF) were purchased from R&D systems. All solutions were freshly prepared from frozen stock solutions or lyophilized preparations. All materials were handled in a sterile manner using endotoxin-free microfuge tubes (Eppendorf/Fisher Scientific), polypropylene tubes, polystyrene culture vessels (Becton Dickinson Labware), serological pipettes (Costar/Corning), precision pipette tips (Rainin Instruments), water (Associates of Cape Cod), and PBS (Gibco/Invitrogen).

Cell Culture

The mouse microglial cell line N9 was a gift of Dr. M. Righi, International School for Advanced Studies, Trieste, Italy, and was cultured in accordance with the original publication[31]. Briefly, cells were cultured in Dulbecco's Modified Eagle's Medium (Gibco/Invitrogen), supplemented with 10% fetal bovine serum (Hyclone) and penicillin/streptomycin (P/S, 50 I.U./50 µg/mL; Mediatech). Cells were passaged weekly with 0.05% trypsin (Gibco/Invitrogen) and serum-starved in macrophage serum-free medium (MSFM, Gibco/Invitrogen) for at least 24 h before each experiment as detailed below.

Primary microglia (pMG) were prepared from cortex of newborn (p4) C57BL/6J mice as described[10,22]. In brief, cortical tissue was freed from blood vessels and meninges, digested with 50 ng/mL DNase, triturated, and washed. Cortical cells were cultured in DMEM/10% FBS with P/S plus 2 ng/ml GM-CSF for 11–50 d (medium change every 3–4 d). Microglia were separated from underlying astrocytic monolayer by gentle agitation and spun down. Cell pellet was resuspended in DMEM/10%FBS with P/S plus 2 ng/ml GM-CSF and plated on Primaria™ culture dishes (BD Biosciences). Non-adherent cells were removed after plating for 30–60 min by changing the medium and adherent microglia were incubated for 24 h in culture medium before serum starving in MSFM plus 0.2 ng/ml GM-CSF for 24 h.

RNA Isolation, Reverse Transcription and Quantitative Real-Time PCR

To quantify mRNA expression of FcγRI and FcγRIIb in microglia after treatment with cytokines for 24 h, RNA isolation and quantitative Real-Time polymerase chain reaction (qRT-PCR) were done as described[38]. In brief, after RNA isolation and reverse transcription, multiplex qRT-PCR was performed using the 7500 Real Time PCR System (Applied Biosystems). HPRT probe sequence is ACC TAG ATT TGT TTT GTA TAC CT and contains VIC at 5' end. HPRT forward primer TCC CAG CGT CGT GAT TAG C and reverse primer TCC AAA TCC TCG GCA TAA TGA. Probe-primer sets for mouse FcγRI and FcγRIIb were generated using ProbeFinder software (Roche). Primers for FcγRI and FcγRIIb were selected: Universal Probe Library probe #56 for FcγRI and #66 for FcγRIIb (Roche). FcγRI forward primer TGC TGG ATT CTA CTG GTG TGA and reverse primer AAA CCA GAC AGG AGC TGA TGA. FcγRIIb forward primer CCA AGC CTG TCA CCA TCA C and reverse primer GAT AAT AAC AAT GGC TGC GAC A.

Cycling conditions were as follows: (i) 95°C for 10 min; (ii) 40 cycles, with each cycle consisting of 95°C for 15 s and 60°C for 1 min; (iii) Fluorescent data was acquired at 60°C step. All experiments had “no template” negative controls and only intron-spanning primers were used. Data was analyzed using Sequence 7 Detection Software v1.3 (Applied Biosystems) as described[38].

Phagocytosis Assay

After reconstitution, Fluorescein labeled *S. aureus* were pre-incubated with equimolar concentrations of either anti-*S. aureus* IgG or isotype control IgG (20–40µg IgG/mg bacteria). Antibody coated *S. aureus* were washed, spun down and resuspended in MSFM. Suspensions were then incubated with serum-starved mouse pMG for 30 min. Extracellular fluorescein was quenched with 0.1% trypan blue and pMG were dislodged with PBS containing 0.25% trypsin plus 2 mM EDTA (30 min at 37°C). Analysis was performed on a FACScan2 flow cytometer (BD Biosciences) using Flojo software (Treestar).

Statistic Analysis

Statistical evaluation was carried out using PRISM software (GraphPad). Multiple comparisons were made using one-way ANOVA as appropriate with Bonferroni post-test. $P < 0.05$ was considered to be significant. Data are given as mean $\pm$ S.E.M.

RESULTS

Regulation of Fc γ RI mRNA expression in mouse microglia

In N9 cells, both IFN- γ and GM-CSF enhanced the expression of Fc γ RI mRNA. Twenty-four hour treatment with either IFN- γ (10 U/mL) or GM-CSF (10 ng/mL) induced 3.7- and 2.6-fold increases, respectively in Fc γ RI mRNA levels compared with unstimulated control (Fig. 1A). Neither IL-4 nor IL-13 (both at 10 ng/mL) had a significant effect on expression of Fc γ RI mRNA following 24 hour treatment.

In pMG, twenty-four hour treatment with IFN- γ induced a robust 4.6-fold increase in the levels of Fc γ RI mRNA (Fig.1B). GM-CSF induced a modest 1.4-fold increase in Fc γ RI mRNA expression, but this change did not reach statistical significance. Neither IL-4 nor IL-13 induced significant changes in Fc γ RI mRNA expression at 24 hours.

Regulation of Fc γ RIIb mRNA expression in mouse microglia

In N9 cells, GM-CSF induced a 2.8-fold increase in Fc γ RIIb mRNA expression whereas IL-4 and IL-13 induced 1.7- and 1.8-fold increases respectively in Fc γ RIIb mRNA (Fig.2A). IFN- γ appeared to modestly down-regulate Fc γ RIIb mRNA expression, but this change did not reach statistical significance.

In pMG, IL-4, IL-13 and GM-CSF all induced between 2- and 3-fold increases in the levels of Fc γ RIIb mRNA compared with unstimulated control (Fig.2B). IFN- γ showed no effect on the expression of Fc γ RIIb mRNA.

Regulation of the IgG-mediated phagocytosis by T-cell-derived cytokines

We quantified the effects of IFN- γ , IL-4, IL-13, and GM-CSF on IgG-mediated phagocytosis. IFN- γ induced an almost two-fold increase in IgG-mediated phagocytosis of *S. aureus* whereas both IL-4 and IL-13 decreased IgG-mediated phagocytosis by about one-third compared with control (Fig.3). GM-CSF increased IgG-mediated phagocytosis approximately two-fold. None of these cytokines influenced basal levels of *S. aureus* phagocytosis (in the absence of antibody) although some small changes in phagocytosis were seen in the presence of isotype control IgG.

DISCUSSION

In this study, we determined the effects of T-cell-derived cytokines on (i) expression of phagocytic Fc γ receptors and (ii) IgG-mediated phagocytosis in microglia. We investigated the effects of four specific cytokines associated with three distinct types of immune response: (i) IFN- γ ; associated with Th1 cellular immune response and classical macrophage/microglial activation[11], (ii) IL-4 and IL-13; associated with Th2 adaptive responses and alternative activation[11] and (iii) GM-CSF, associated with macrophage/microglial differentiation and maturation[13]. We found that IFN- γ induced increases in microglial expression of Fc γ RI, but not Fc γ RIIb mRNA. In contrast, IL-4 and IL-13 up-regulated the expression of Fc γ RIIb, but not Fc γ RI mRNA. GM-CSF increased mRNA expression of both Fc γ receptors. In functional analyses, both IFN- γ and GM-CSF induced significant increases in IgG-mediated phagocytosis of *S. aureus*, whereas IL-4 and IL-13 both significantly reduced IgG-mediated phagocytosis of *S. aureus*.

The balance between activating Fc γ RI and inhibitory Fc γ RIIb may influence the quality and magnitude of antibody-based immune responses[4]. For example, activation of Fc γ RI receptors on macrophages/microglia by the Fc portion of IgG typically leads to induction of cytolytic activity, elaboration of pro-inflammatory cytokines and phagocytosis[3,27]. However, binding of the Fc portion of IgG to Fc γ RIIb receptors suppresses cytolytic activity, cytokine elaboration and phagocytosis[6,27]. Interestingly, antagonism of inhibitory Fc γ RIIb receptors with blocking antibodies can lead to an “activating” response similar to that elicited by Fc γ RI agonists[7]. A previous study reported that in human dendritic cells, IFN- γ induced an increase in Fc γ RI and a decrease in Fc γ RIIb protein expression, respectively[4]. Other reports have shown that in human monocytes, IL-4 induced an increase in Fc γ RIIb mRNA and protein expression while IFN- γ down-regulated Fc γ RIIb expression[26,34]. Our results are consistent with those of the above reports with the exception being that in pMG we did not see an IFN- γ -induced down-regulation of Fc γ RIIb. This discrepancy may be secondary to differences in the responses of varying myeloid cells types. Alternatively, it may be due to species differences in cellular responses between humans and mice.

Our functional data on regulation of microglial IgG-mediated phagocytosis of *S. aureus* by the T-cell derived cytokines are consistent with our Fc γ receptor mRNA regulation data. For example, based on the IFN- γ -induced up-regulation of Fc γ RI and minimal effect on Fc γ RIIb mRNAs in pMG, one would predict that IFN- γ would induce a net increase in IgG-mediated phagocytosis as we see in Fig. 3. Similarly, the fact that both IL-4 and IL-13 significantly down-regulated IgG-mediated phagocytosis (Fig. 3) would be predicted by their induction of significant increases in Fc γ RIIb mRNA expression (combined with their lack of significant effect on Fc γ RI expression). GM-CSF increased expression of both Fc γ RI and Fc γ RIIb mRNAs (Figs. 1-2), so it would be difficult to predict its functional effect on IgG-mediated phagocytosis with expression data alone. However, we demonstrated a substantial increase in IgG-mediated phagocytosis induced by GM-CSF (Fig.3). This suggests that the increase in Fc γ RI mRNA expression may predominate functionally over the (larger) increase in Fc γ RIIb mRNA expression. This could be explained by: (i) differences in either efficiency of translation/post-translational processing of the two transcripts, (ii) a net summation of effects generated by simultaneous activation of conflicting signaling pathways (ITAM vs. ITIM) or (iii) GM-CSF-induced changes in expression or function of other (non-Fc γ R) components of the phagocytic pathway.

IFN- γ and IL-4/IL-13, are pleiotropic modulators of macrophage activation and can each induce distinctive programs of altered gene expression after engagement of their specific receptors[1,24]. Classical activation of macrophages is mediated by the priming stimulus IFN- γ , followed by a microbial-associated trigger such as lipopolysaccharide (LPS). Initiation of this pathway results in production of pro-inflammatory cytokines, NO synthesis and up-regulation of immunomodulatory surface antigens including MHC class II[9]. IFN- γ -induced activation of microglia has been implicated in the pathophysiology of experimental autoimmune encephalomyelitis[20] and in lesional models of hippocampal neuronal injury [17]. Phagocytosis of myelin *in vitro* down-regulates production of pro-inflammatory cytokines such as TNF- α and IL-1 β in IFN- γ -stimulated microglia[19]. Thus, the IFN- γ -induced alteration in the balance of phagocytic Fc γ receptors (Figs.1-2), as well as the IFN- γ -induced increase in phagocytic activity (Fig.3), may serve as a negative feedback mechanism to limit classical pro-inflammatory microglial activation. In contrast, alternative activation is mediated by IL-4 or IL-13, acting through a common receptor (IL-4R α) and results in increased cell fusion and endocytosis [11]. Alternative activation of microglia induced by IL-4 in the CNS plays an important role in tissue repair and is essential for controlling autoimmune inflammation[25]. IL-4 may also provide a significant immunomodulatory signal that could protect against microglia-mediated motoneuron toxicity [40]. The effects of IL-4 and IL-13 on phagocytosis of *S. aureus* by microglia seen here are consistent with those of a previous study

that reported IL-4-induced inhibition of microglial phagocytosis of apoptotic T-cells. The IL-4- and IL-13-induced reductions in phagocytosis may in turn modulate the microglial cytokine production profile as has been seen in macrophages[36]. Recent studies have suggested that microglia in culture are not terminally differentiated and that exposure to high concentrations of GM-CSF (in the 10 ng/mL range) can drive them toward a macrophage or dendritic cell phenotype[13,30]. However, low concentrations of GM-CSF are widely used as a culture medium supplement to allow for generation of sufficient numbers of microglia *in vitro* [8]. In our study, we used 2 ng/mL GM-CSF for initial culturing. This was reduced to 0.2 ng/mL during serum starvation. For stimulation, we used a high concentration (10 ng/mL). While we cannot exclude an effect of the low GM-CSF concentrations used in our baseline culture conditions on Fc γ receptor expression, the step-up increase induced a robust enhancement in expression of both Fc γ receptors investigated. Terminally differentiated macrophages express both of the Fc γ receptors examined here[16]. Thus, the GM-CSF-induced increase in Fc γ receptor expression in microglia seen here could reflect an overall transition toward a more macrophage-like cell lineage. The GM-CSF-derived changes in Fc γ receptors described here serve as a cautionary reminder that routine addition of high concentrations of this growth factor to microglial culture medium may be artifactually altering the phenotype. Experiments performed under these baseline conditions, especially ones focused on phagocytosis, will need to take this phenomenon into account.

Blocking monoclonal antibodies targeting Fc γ receptors, or more commonly intravenous immunoglobulins (IV Ig) that bind Fc receptors have been used in the treatment of autoimmune diseases in the CNS including Multiple Sclerosis[32]. Changes in expression of phagocytic Fc γ receptors are likely to alter baseline Fc γ receptor functional balance. This in turn may affect both the pathophysiology of these disorders and their responsiveness to antibody based therapies. Changes in the Fc γ receptor balance has been implicated in both the pathophysiology and therapy response of a number of non-CNS autoimmune diseases [21]. Therefore, by modulating the expression of phagocytosis-related Fc γ receptors in microglia, T-cell derived factors could influence both phagocytic and immune activation responses in CNS. These findings may help refine and target pharmacological approaches for modulating CNS immune responses to infection and autoimmune diseases.

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Figure 1. Regulation of Fc γ RI mRNA expression in mouse microglia. Serum starved N9 (A) and pMG (B) were treated for 24 h with 10 U/mL IFN- γ or 10 ng/mL of IL-4, IL-13 or GM-CSF and Fc γ RI expression was assessed by qRT-PCR. Data are mean $\pm$ SEM of n=4 from each group presented as fold increase in expression. **: P<0.01 vs. unstimulated.

Figure 2. Regulation of Fc γ RIIb mRNA expression in mouse microglia. Serum starved N9 (A) and pMG (B) were treated for 24 h with 10 U/mL IFN- γ or 10 ng/mL of IL-4, IL-13 or GM-CSF and Fc γ RIIb expression was assessed by qRT-PCR. Data are mean $\pm$ SEM of n=4 from each group presented as fold increase in expression. *: P<0.05; **: P<0.01 vs. unstimulated.

Figure 3.

Regulation of IgG-induced phagocytosis by cytokines in mouse microglia. Serum-starved pMG were treated for 24 h with 10 U/mL IFN- γ or 10 ng/mL IL-4, IL-13 or GM-CSF. Cells were incubated 30 min with iso IgG or anti-*S. aureus* IgG opsonized *S. aureus*. Data are mean $\pm$ SEM of n=4 from each group presented as fold increase in phagocytosis. *: P<0.05 vs iso IgG in unstimulated group. #: P<0.05, ##: P<0.01, ###: P<0.001 vs. anti-*S. aureus* IgG in unstimulated group.