Systemic Inflammation Modulates Fc Receptor Expression on Microglia during Chronic Neurodegeneration

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Systemic Inflammation Modulates Fc Receptor Expression on Microglia during Chronic Neurodegeneration

Katie Lunnon,*†,1 Jessica L. Teeling,*†,1 Alison L. Tutt,‡ Mark S. Craggs,‡ Martin J. Glennie,§ and V. Hugh Perry*

Chronic neurodegeneration is a major worldwide health problem, and it has been suggested that systemic inflammation can accelerate the onset and progression of clinical symptoms. A possible explanation is that systemic inflammation “switches” the phenotype of microglia from a relatively benign to a highly aggressive and tissue-damaging phenotype. The current study investigated the molecular mechanism underlying this microglia phenotype “switching.” We show in mice with chronic neurodegeneration (ME7 prion model) that there is increased expression of receptors that have a key role in macrophage activation and associated signaling pathways, including TREM-2, Siglec-F, CD200R, and FcγRs. Systemic inflammation induced by LPS further increased protein levels of the activating FcγRIII and FcγRIV, but not of other microglial receptors, including the inhibitory FcγRII. In addition to these changes in receptor expression, IgG levels in the brain parenchyma were increased during chronic neurodegeneration, and these IgG levels further increased after systemic inflammation. γ-Chain–deficient mice show modified proinflammatory cytokine expression in the brain after systemic inflammation. We conclude that systemic inflammation during chronic neurodegeneration increases the expression levels of activating FcγR on microglia and thereby lowers the signaling threshold for Ah-mediated cell activation. At the same time, IgG influx into the brain could provide a cross-linking ligand resulting in excessive microglia activation that is detrimental to neurons already under threat by misfolded protein. The Journal of Immunology, 2011, 186: 7215–7224.

In chronic neurodegenerative diseases, there is a highly atypical innate immune response within the brain, which is dominated by microglia, the resident macrophages of the brain (1). The microglia adopt an activated morphology that may last for many years prior to the death of the patient. The contribution of this innate inflammatory response to the progression of disease is unclear: epidemiological studies suggest that anti-inflammatory drugs may delay the onset and progression of disease, but clinical trials have yet to show clear benefit (2). Other studies have suggested a neuroprotective role of microglia, mediated by phagocytosis and clearance of toxic protein aggregates and apoptotic cell debris (3). To define better the contribution of the inflammatory response associated with chronic neurodegeneration to disease progression, we have investigated the innate immune response associated with prion disease in mice, a laboratory model of chronic fatal neurodegenerative disease. The disease, initiated by the ME7 prion agent, is associated with the accumulation of misfolded protein, microglial activation, systematic spread of the disease through the brain, and neuronal death and has many features in common with Alzheimer’s disease pathology (4–6).

In murine prion disease, the microglia adopt a highly branched activated morphology and upregulation of F4/80, CD11b, and CD68 but surprisingly do not express a proinflammatory phenotype: instead, the inflammatory mediator milieu is dominated by TGF-β and PGE2 (7–9). This anti-inflammatory profile appears early in the course of disease and persists despite the increasing accumulation of misfolded protein and progressive neuronal loss (7, 10). This profile is akin to that described when macrophages phagocytose apoptotic cells and develop an anti-inflammatory phenotype (11, 12).

It is well established that systemic inflammation communicates with the brain leading to transient behavioral changes and cytokine production (13). We previously showed that a systemic LPS challenge in animals with prion disease leads to a rapid switch in microglia phenotype: from an anti-inflammatory or “primed” phenotype to an aggressive proinflammatory response associated with increased IL-1β, TNF-α, and IL-6 production, exaggerated behavioral changes, neuronal death, and a significantly faster progression of the disease (14, 15). The effect of systemic inflammation on brain pathology and disease progression is not restricted to the prion model and has now been documented in animal models of normal aging (16), acute neurodegeneration (17), motor-neuron disease (18), and ischemia (19, 20). Importantly, the impact of systemic infections on human brain pathology has also been observed in patients with Alzheimer’s disease, stroke, and multiple sclerosis (21–24). These studies not only suggest that microglia priming is a general phenomenon of brain neuropathology but, importantly, that systemic infections can contribute to disease progression in a wide range of diseases by inducing a switch in microglial phenotype.

In this study, we aim to unravel the molecular mechanisms involved in microglial switching to better understand the regulatory...
pathways that control microglia in health and disease. Our results show that IgG FcγR and increased influx of IgG into the brain play an important role in microglial switching that may exacerbate neuronal damage and disease progression after systemic inflammation.

Materials and Methods

Animals and stereotaxic surgery
Female C57BL/6J mice (Harlan, Bicester, U.K.) were bred and maintained in local facilities. Fcγ-chain–deficient mice were maintained in-house (25). Mice were housed in groups of 4 to 10, under a 12-h light/12-h dark cycle at 21˚C, with food and water ad libitum. To induce prion disease, mice were anesthetized with Avertin (2,2,2-tribromoethanol in tertiary amyl alcohol), and 1 μl of either ME7-derived brain homogenate (ME7 animals) (10% w/v) or normal brain homogenate (NBH animals) was injected stereotaxically and bilaterally at the coordinates from bregma: anteroposterior, −2.0 mm; lateral, −1.7 mm; depth, 1.6 mm. All procedures were performed in accordance with U.K. Home Office licensing.

LPS challenges
At 18 wk postinoculation, ME7 animals and control NBH animals were injected i.p. with 500 μg/kg LPS (Salmonella abortus; Sigma, Dorset, U.K.) or an equivalent volume of sterile saline as a control.

Tissue collection
Mice were terminally anesthetized and transcardially perfused with 0.9% saline. For microarray and quantitative PCR studies, tissue was collected at 6 and 24 h after LPS or saline treatment. The left dorsal hippocampus and underlying thalamic region (~20 mg) was punched out, placed in RNAlater (Qiagen, Crawley, U.K.), and stored at 4˚C prior to RNA isolation. For immunocytochemistry, unfixed brain or spleen tissue was collected 24 h after LPS or saline treatment and directly embedded and frozen in OCT compound (Sakura Finetek Europe B.V, Zoeterwoude, The Netherlands). For collection of fixed tissue, mice were further perfused with periodate-lysine-paraformaldehyde (PLP) and tissue postfixed in PLP for 4–6 h, transferred to 30% sucrose in 0.1 M phosphate buffer, and embedded in OCT compound. All tissue was kept at −20˚C until further use.

Table I. Differential gene expression during ME7 prion disease before and after systemic LPS challenge

<table>
<thead>
<tr>
<th>Category</th>
<th>ME7 versus NBH</th>
<th>ME7 + LPS versus ME7</th>
<th>NBH + LPS versus NBH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immune response inflammation</td>
<td>13.7</td>
<td>26.6</td>
<td>28.2</td>
</tr>
<tr>
<td>Signal transduction</td>
<td>27.3</td>
<td>31.6</td>
<td>25.1</td>
</tr>
<tr>
<td>Cellular adhesion</td>
<td>3.7</td>
<td>3.8</td>
<td>3.5</td>
</tr>
<tr>
<td>Cell death</td>
<td>3.4</td>
<td>3.9</td>
<td>3.5</td>
</tr>
<tr>
<td>Protein turnover</td>
<td>14.7</td>
<td>11.6</td>
<td>11.1</td>
</tr>
<tr>
<td>Transcription translation</td>
<td>6.25</td>
<td>6.57</td>
<td>8.37</td>
</tr>
<tr>
<td>Mitochondrial</td>
<td>4.7</td>
<td>1.93</td>
<td>3.08</td>
</tr>
<tr>
<td>Neuronal</td>
<td>15.9</td>
<td>6.95</td>
<td>8.36</td>
</tr>
<tr>
<td>Other</td>
<td>10.98</td>
<td>6.95</td>
<td>8.8</td>
</tr>
</tbody>
</table>

The percentage (%) of genes that are differentially expressed in different categories is shown for ME7 versus NBH animals, ME7 + LPS versus ME7 animals (i.e., the effect of LPS in ME7 animals), and NBH + LPS versus NBH animals (i.e., the effect of LPS in NBH animals).

Microarray analysis
The quality of RNA for microarray analysis was determined on an Agilent BioAnalyzer 2100 (Agilent Technologies), and samples with a 28S/18S ratio of 2.0 were used for microarray analysis. RNA was reverse transcribed to cDNA and amplified using a WT ovation kit (Nugen Technologies), and cDNA was hybridized to Mouse 430.2.0 Affymetrix chips. Rosetta Resolver Gene Expression Data was analyzed by the Rosetta Resolver Gene Expression Analysis System and Bioconductor Open Source software for bioinformatics. All analysis was performed using a cutoff p value <0.01. The p value was generated by a modified t test called local pooled error, which is frequently used for identifying differentially expressed genes with a small number of replicated microarrays (26). The microarray data were deposited in the National Center for Biotechnology Information Gene Expression Omnibus database (http://www.ncbi.nlm.nih.gov/geo/) under GEO Series accession number GSE213182.

Quantitative PCR
Total RNA was extracted from hippocampus/thalamus tissue using RNeasy mini kits (Qiagen). Any contaminating genomic DNA was degraded using a DNase I enzyme (Qiagen). cDNA was synthesized using RT-gold reagents (Applied Biosystems, Warrington, U.K.). Samples were quantified against a standard curve derived from normal mouse spleen or brain tissue of ME7 animals that received an intracerebral injection of LPS. All results were normalized to the measurement for GAPDH. Primers and probes were designed using Primer Express software and purchased from Sigma Genosys: FcγR: forward 5′-GGGATGCATGGTGGCCTTCAACA-3′, reverse 5′-GGCTTTGGGCAATACAA-3′, probe 5′-CACCACGGTACCAAAATCCAC-3′; FcγRII: forward 5′-CATGCAATTCAATTTTATGGCA-3′, reverse 5′-AGGACTGCCACTCTGCTTCT-3′, probe 5′-AACCCCGCTATGCTGACTG-3′; FcγRIII: forward 5′-CAATGGATATTTTCTACCTG-3′, reverse 5′-AGGACTGCTCCTGCTTCT-3′, probe 5′-AAAGCCTGCTATTGTAAGAGACGGTG-3′; Kinases: forward 5′-ATGCTCCTGCTTCTCTGCT-3′, reverse 5′-AGGACTGCTCCTGCTTCT-3′, probe 5′-CAGGGCCGCGCTTGGTG-3′. Probes were labeled at the 5′ end with a 6′-carboxyfluorescein reporter dye (FAM) and at the 3′ end with a 6′-carboxyfluorescein reporter dye (FAM) and at the 3′ end with a 6′-carboxyfluorescein reporter dye (FAM).
Table III. Fold change in gene expression for receptors and adapter molecules involved in ITAM and ITIM signaling between treatment groups.

<table>
<thead>
<tr>
<th>Signaling</th>
<th>ME7 versus NBH</th>
<th>ME7 + LPS versus ME7</th>
<th>NBH + LPS versus NBH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Activation (ITAM)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD200R4</td>
<td>7.2</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>TREM-2</td>
<td>20.9</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Gp49a</td>
<td>21.5</td>
<td>2.3</td>
<td>4.6</td>
</tr>
<tr>
<td>Dectin-1</td>
<td>91</td>
<td>−1.5</td>
<td></td>
</tr>
<tr>
<td>DAP12</td>
<td>11.8</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>CD300D</td>
<td>7.4</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>FcRI</td>
<td>4.3</td>
<td>—</td>
<td>2.5</td>
</tr>
<tr>
<td>FcRIII</td>
<td>6.0</td>
<td>2.5</td>
<td>3.5</td>
</tr>
<tr>
<td>FcRIIV</td>
<td>9.4</td>
<td>2.4</td>
<td>−</td>
</tr>
<tr>
<td>γ-Chain</td>
<td>8.5</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Inhibitory (ITIM)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Siglec-3</td>
<td>2.8</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Siglec-F</td>
<td>26.7</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Lair</td>
<td>3.8</td>
<td>−1.6</td>
<td>—</td>
</tr>
<tr>
<td>CD300A</td>
<td>4.1</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>CD72</td>
<td>14</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>FcRI</td>
<td>11.7</td>
<td>2.5</td>
<td>3.5</td>
</tr>
</tbody>
</table>

The fold change in gene expression for various receptors that use ITAM or ITIM for their cell signaling is shown for ME7 versus NBH animals, ME7 + LPS versus ME7 animals (i.e., the effect of LPS in ME7 animals), and NBH + LPS versus NBH animals (i.e., the effect of LPS in NBH animals).

carboxy-tetramethyl rhodamine quencher dye (TAMRA). Primers and probe for the housekeeping gene GAPDH were purchased from Applied Biosystems. To determine the effect of LPS on gene expression, we calculated the fold-difference in mRNA transcripts between the LPS-treated groups of ME7 or NBH animals and those without LPS challenge.

Immunocytochemistry

Coronal sections (10 μm) were cut on a cryostat. mAbs against mouse FcγRI (IgG2b, clone 290305; R&D Systems) and Siglec-F (a kind gift from Prof. P. Crocker, University of Dundee) were used on PLP fixed tissue, whereas mAbs against FcγRII (AT130-2; in-house), FcγRIII (FCR4G8; Serotec), FcγRIV (AT137; in-house), CD68 (FA11; Serotec), CD11b (5C6; Serotec), F4/80 (rabbit polyclonal, a kind gift from Prof. P. Crocker, University of Dundee) were used on alcohol-fixed, fresh-frozen tissue. Sections were blocked with 10% rabbit serum/1% BSA, incubated with primary Abs for 1 h, followed by biotinylated secondary Abs (Vector Lab) for 1 h at room temperature. Staining was either visualized by the ABC method (Vector Lab) and diaminobenzidine (DAB) as chromagen or by the fluorescently labeled secondary Abs, goat anti-rabbit Alexa Fluor 546 or rabbit anti-Alexa Fluor 488 (Invitrogen). Expression of F4/80, Siglec-F, and FcγR was quantified using Leica software. Two images per brain were obtained at ×20 magnification and the number of pixels/field of DAB staining of cells and their processes measured. Data were analyzed by two-way ANOVA followed by Bonferroni’s post hoc test, and p < 0.05 was considered significantly different.

Behavioral changes

Changes in species-specific behaviors (open field activity, burrowing, and glucose consumption) were assessed as previously described (15, 27).

Cytokine measurement

Hippocampal tissue was collected and homogenized in ice-cold buffer (150 mM NaCl, 25 mM Tris, 1% Triton, pH 7.4) containing a protease inhibitor mixture (Roche Diagnostics, Mannheim, Germany). Homogenates were centrifuged for 30 min at 14,000 rpm, and supernatants were assayed for total protein using a Bio-Rad DC protein assay (Bio-Rad, Hertfordshire, U.K.). Cytokine levels were measured using MSD multiplex for mouse proinflammatory cytokines (Mesoscale Discovery, Gaithersburg, MD) according to the manufacturer’s instructions.

Statistical analysis

Data were analyzed by two-way ANOVA. Post hoc one-tailed t tests (Bonferroni) were performed to analyze individual comparisons. Results are reported as means ± SEM.

Results

Upregulation of innate immunity-associated genes in chronic neurodegeneration

To find candidate genes involved in microglia phenotype switching, we performed microarray analysis on brain tissue from control (NBH animals) or prion diseased (ME7 animals) animals before and after i.p. LPS challenge and compared the gene expression profile in the hippocampal and thalamic regions. We chose to study the dorsal hippocampus/thalamic region at 18 wk into disease because previous characterization of the pathology at this stage demonstrated significant neuronal degeneration and extensive microgliosis (10, 15). In this model, behavioral deficits in burrowing and open field activity are observed after systemic LPS challenge peaking at 6 h and returning to baseline levels at 48 h (14). Tissue for microarray was taken at 6 h. Quantification of the microarray data showed that 2336 genes (5.8% of total of 40,000 genes) were differentially expressed in ME7 animals compared with the expression of genes in NBH animals. Analysis of the data

![FIGURE 1. Heat map of fluorescent intensity of Affymetrix FcγR probe IDs. The fluorescent intensity measured in each sample (n = 3) for each of the probes corresponding with the four murine FcγRs and the common Fcγ-chain is displayed. Green color in the heat map indicates relatively low transcript expression, and red color indicates relatively high transcript expression.](image-url)
showed that 41% of the genes that were upregulated are linked to the immune response or cell signaling (Table I). A systemic LPS challenge induced a further 1118 genes in ME7 animals of which a large proportion have a function in immune responses or signaling (58.2%; Table I) with many genes (28.9%) encoding soluble mediators/receptors (13.1%) and cytokines (15.8%) (Table II). A selection of genes that were upregulated in ME7 animals is shown in Table III. We found significant upregulation of receptors that signal through an ITAM, including TREM-2 (21-fold), gp49a (22-fold), Dectin-1 (91-fold), FcγRI (4-fold), FcγRIII (6-fold), and FcγRIV (3.4-fold), and FcγRs, apart from FcγRI, whereas the majority of other immunoregulatory receptors did not change or showed a downregulated expression pattern. A heat map displaying the individual fluorescent intensities for the Affymetrix probe set IDs corresponding with each of the four murine FcγRs and the common Fcγ-chain is shown in Fig. 1, demonstrating the transcription of genes encoding all FcγRs was higher in brain tissue from ME7 animals than in control NBH animals and verifying further increase of FcγRI, FcγRII, and FcγRIV after a systemic LPS challenge. The data discussed in this article have been deposited in the National Center for Biotechnology Information Gene Expression Omnibus database and are accessible through GEO Series accession number GSE23182 (http://www.ncbi.nlm.nih.gov/geo/).

We used quantitative PCR to measure FcγR gene expression before and after LPS challenge and compared this with TREM-2 and Siglec-F gene expression. Tissue taken from the dorsal hippocampus/thalamus after 6 h (data not shown) or 24 h (Fig. 2) was analyzed. At 24 h, ME7 animals showed a significant upregulation of FcγRI mRNA levels [F(1,24) = 13.77, p = 0.01], and systemic administration of LPS induced a small but significant further increase [F(1,24) = 6.208, p = 0.02]. There was no significant interaction between disease and LPS treatment [F(1,24) = 2.357, p = 0.138], suggesting that LPS treatment increased FcγRI in brains of control NBH animals and ME7 animals. FcγRII was significantly upregulated in ME7 animals [F(1,24) = 26.49, p < 0.0001] and further increased after a systemic LPS challenge [F(1,24) = 20.29, p = 0.0001]. Similar observations were made for FcγRIII and FcγRIV. ME7 animals showed increased FcγRIII [F(1,24) = 7.692, p = 0.01] and FcγRIV mRNA expression [F(1,24) = 5.785, p = 0.024], and expression further increased after LPS challenge [FcγRIII: F(1,24) = 7.813, p = 0.01; FcγRIV: F(1,24) = 7.563, p = 0.011]. All three low-affinity FcγRs had a significant disease and LPS interaction [FcγRII: F(1,24) = 18.86, p = 0.0002; FcγRII: F(1,24) = 7.121, p = 0.013; FcγRIV: F(1,24) = 4.386, p = 0.047], indicating that ME7 animals have a heightened central response to LPS challenge. Finally, analysis of the neonatal Fc receptor, FcRn, also showed increased expression during ME7 prion disease [F(1,22) = 11.90, p = 0.0023], and further

Table IV. Fold change in gene expression for Fcγ receptors

<table>
<thead>
<tr>
<th>Receptor</th>
<th>ME7 Versus NBH</th>
<th>ME7 + LPS versus ME7 6 h</th>
<th>NBH + LPS versus NBH 6 h</th>
<th>ME7 + LPS versus ME7 24 h</th>
<th>NBH + LPS versus NBH 24 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>FcγRI</td>
<td>3.6</td>
<td>1.8</td>
<td>3.1</td>
<td>2.4</td>
<td>2.2</td>
</tr>
<tr>
<td>FcγRII</td>
<td>10.8</td>
<td>2.3</td>
<td>2.3</td>
<td>10.9</td>
<td>3.0</td>
</tr>
<tr>
<td>FcγRIII</td>
<td>11</td>
<td>5</td>
<td>7.8</td>
<td>48.2</td>
<td>13.2</td>
</tr>
<tr>
<td>FcγRIV</td>
<td>3.4</td>
<td>5.1</td>
<td>—</td>
<td>13.8</td>
<td>10.8</td>
</tr>
</tbody>
</table>

Data show the fold change in gene expression for FcγRs between ME7 and NBH animals; the effect of LPS in ME7 or NBH animals after 6 h; and the effect of LPS in ME7 and NBH animals after 24 h. The effect of LPS is expressed as fold change of ME7 or NBH alone.
upregulation after LPS \( [F_{1.22} = 10.55, p = 0.0037] \). Analysis of TREM-2 and Siglec-F mRNA expression levels confirmed the microarray data: an increase in TREM-2 expression was found in ME7 animals \( [F_{1.18} = 4.353, p < 0.0514] \), but there was no further increase after LPS challenge \( [F_{1.18} = 0.421, p = 0.525] \). Similarly, Siglec-F was significantly upregulated in ME7-infected mice \( [F_{1.17} = 72.53, p < 0.0001] \), and no further increase was seen after LPS challenge \( [F_{1.17} = 0.09, p = 0.77] \).

The neuropathology in the hippocampus and thalamus of ME7 animals is characterized by extensive microgliosis, and therefore changes in FcγR mRNA levels may be simply due to increased number of microglia. We quantified CD68 gene expression before and after LPS challenge: ME7 animals showed a significant increase in CD68 compared with control NBH animals \( [F_{1.24} = 22.30, p < 0.0001] \), with no further changes in CD68 mRNA levels in ME7 animals after LPS challenge \( [F_{1.24} = 0.09, p = 0.77] \). Therefore, changes in FcγR mRNAs after systemic LPS challenge are not explained by more microglial cells alone. In summary, Table IV shows that mRNA levels of all four FcγRs increase during chronic neurodegeneration, and, with the exception of FcγRI, these changes are markedly increased 6 and 24 h after a systemic LPS challenge.

**FIGURE 3.** Expression of F4/80 in the thalamus of NBH animals and ME7 animals before and after i.p. LPS challenge. F4/80 protein expression was visualized by immunohistochemistry in NBH animals (A), ME7 animals (B), NBH + LPS animals (C), and ME7 + LPS animals (D). Mice were treated with 500 µg/kg LPS and F4/80 expression levels assessed 24 h later as described in Materials and Methods. A representative example of one independent experiment with \( n = 3 \) mice per group is shown. Expression levels (DAB staining/field) were quantified and analyzed as described in Materials and Methods \( (n = 3) \). **p < 0.01 (two-way ANOVA, followed by Bonferroni posttest).

**FIGURE 4.** Characterization of novel mouse FcγR Abs. A, Specificity of anti-FcγRII mAb AT130-2. FcγRII−/− mice were immunized with an FcγRII–CD4 fusion protein and the resulting hybridomas tested by flow cytometry. AT130-2 was assessed for binding to lymphocytes from WT, FcγRII−/−, or FcγRII−/− (TKO) mice. B–D, Specificity of anti-FcγRIV mAb AT137. Rats were immunized with an FcγRIV–CD4 fusion protein and the resulting hybridomas tested by ELISA and flow cytometry. Binding of AT137 to various concentrations of plate-bound CD4 fusion proteins (0.5, 1, and 2 µg/ml) displaying FcγRI, FcγRII, FcγRIII, or FcγRIV extracellular domains (B). Costaining of peripheral blood monocytes from WT or FcγRI, II, III−/− (TKO) mice with CD11b–PE and either AT137, 2.4G2, or an irrelevant isotype matched mAb (C). Staining of thioglycolate-elicited peritoneal macrophages from WT or γ-chain−/− mice with either AT137, 2.4G2, or an irrelevant isotype matched mAb (D). E, Light microscopy images of spleen sections stained for FcγRs. Fresh-frozen spleen sections (10 µm) from naïve mice were stained for FcγRI, FcγRII, FcγRII/III, and FcγRIV expression using immunohistochemistry. Double fluorescence images of spleen sections stained for FcγRs (green) and F4/80 (red) show colocalization of FcγRI and FcγRIV with macrophages/monocytes. A representative example of one independent experiment with \( n = 3 \) mice per group is shown. Scale bars, 20 µm.
Protein levels of FcγRIII and FcγRIV are increased in chronic neurodegeneration and further upregulated after a systemic LPS challenge

Because tissue from ME7 animals analyzed 24 h after LPS challenge revealed further increased mRNA levels in FcγR, we analyzed protein expression levels at the same time point. Brain sections from NBH animals and ME7 animals were first stained for well-known microglial activation markers. The characteristic morphology of quiescent microglia and perivascular macrophages with low a level of F4/80 expression was observed in NBH animals, whereas ME7 animals showed an increase in both cell number and F4/80 expression levels (Fig. 3A, 3B). Quantification revealed that F4/80 immunoreactivity was significantly higher in ME7 animals than that in NBH animals \[F_{(1,8)} = 18.8, p = 0.003\], with no further significant increase after LPS (Fig. 3). Similar findings were obtained when brain sections were stained for CD11b or CD68 (data not shown). These data confirm an increase in both microglial number and/or activation in ME7 animals.

To analyze FcγR protein expression, we used a panel of anti-mouse FcγR Abs, including in-house–generated anti-FcγRII (AT130-2) and FcγRIV (AT137), and tested their specificity in a number of cell-based assays (Fig. 4). FcγRII−/− mice were immunized with an FcγRII–CD4 fusion protein and the resulting hybridomas tested by flow cytometry for reactivity against lymphocytes from wild-type (WT), FcγRII−/−, or FcγRII−/− mice. Fig. 4A shows loss of binding of AT130-2 to lymphocytes from FcγRII−/− but not FcγRII−/− mice. To generate FcγRIV-specific Abs, rats were immunized with an FcγRIV–CD4 fusion protein and the resulting hybridomas tested by ELISA and flow cytometry. Fig. 4B shows that AT137 specifically binds to FcγRIV.

Peripheral blood monocytes from WT or FcγRII, II, III−/− (triple knockout; TKO) mice were stained with CD11b–PE and either an irrelevant isotype matched mAb, 2.4G2, or AT137 (Fig. 4C). These data demonstrate that the dual (FcγRIII and II) specificity mAb 2.4G2 loses all binding in the TKO mouse as expected, whereas AT137 retains binding on these cells, indicating its binding to an FcγR other than I, II, or III. Thioglycolate-elicited peritoneal macrophages from WT or γ-chain−/− mice were stained with either AT137, 2.4G2, or an irrelevant isotype matched mAb. All surface binding of AT137 is lost on cells from the γ-chain−/−, unlike 2.4G2, which retains binding because of its ability to bind FcγRII (Fig. 4D). Finally, immunocytochemistry indicated that FcγR expression pattern in the spleen was as expected, with FcγRI and FcγRIV colocalizing with F4/80-positive macrophages (Fig. 4E, lower panel). Altogether, these data demonstrate specificity of AT137 for FcγRIV.

Next, we stained brain tissue from NBH animals and ME7 animals for FcγR expression before and after systemic LPS challenge. FcγR expression was low or undetectable in the brains of control NBH animals, and no changes were observed after LPS, with the exception of FcγRI, which modestly increased 24 h after LPS (Fig. 5). In contrast, ME7 animals show high levels of FcγRII \[F_{(1,8)} = 35.6, p = 0.0003\] and FcγRIII on cells with characteristic morphology of “activated” microglia (Figs. 5, 6) with FcγRII being further increased 24 h after LPS (Fig. 6C, 6D). ME7 animals showed detectable but very low expression levels of both FcγRII and FcγRIV, but FcγRIV levels notably increased after systemic LPS challenge (Fig. 6C, 6D). Dual-labeling immunofluorescence identified these cells as F4/80-positive microglia (Fig. 6D). Siglec-F protein levels were not detectable in brains of NBH animals,
whereas ME7 animals in both the hippocampus (data not shown) and thalamus (Fig. 7) showed increased expression of Siglec-F \( [F(1,8) = 208.9, \ p < 0.0001] \). LPS did not significantly increase Siglec-F expression \( [F(1,8) = 1.575, \ p = 0.14] \).

**Increased IgG levels in ME7-infected brain during systemic inflammation**

IgG levels are very low in the normal, healthy brain, due to an intact blood–brain barrier. Immunocytochemistry demonstrated that IgG was undetectable in the brain parenchyma of saline-treated NBH animals, and the very low levels that were detected were restricted to the cerebral blood vessels (Fig. 8A). Increased levels of IgG were found in the cerebral vasculature and on cells in the parenchyma of ME7 animals (Fig. 8B), consistent with previous data showing increased permeability in the blood–brain barrier in advanced prion disease (28). Notably, after a systemic LPS challenge in ME7 animals, IgG was no longer confined to the blood vessels, and 6 h later increased levels of IgG were found diffusely throughout the brain parenchyma (Fig. 8D). IgG levels were still increased 24 h later (Fig. 8F), and this was associated with microglia cells. These results suggest that the blood–brain barrier is compromised in ME7 animals with increased influx of IgG during periods of systemic inflammation.

**A role for FcγR in microglial switching?**

Thus far, our data are consistent with the observation that increased IgG and expression of activating FcγRs during chronic neurodegeneration play a role in microglial phenotype switching after systemic infection. To investigate this further, we inoculated WT and γ-chain–deficient mice with ME7 prion agent. To test if onset and disease progression of ME7 prion disease depends on the Fcγ-chain, we weekly assessed open field activity, burrowing, and glucose consumption. No major differences were found between the two mouse strains, and the increase in open field activity, decline in burrowing, and a decline in glucose consumption after 12 wk is as previously described (29) (Fig. 9A). We collected brain tissue from ME7-infected WT and ME7-infected γ-chain–deficient mice and analyzed the tissue for region-specific microgliosis typical of the late-stage ME7-induced prion disease. No differences in CD68 expression levels between ME7-infected WT and ME7-infected γ-chain–deficient mice were found (Fig. 9B), suggesting that onset and progression of prion disease does not depend on the Fcγ-chain. We next investigated if LPS-induced microglial switching depends on FcγR interaction. At 18 wk postinoculation, we systemically challenged NBH animals, ME7-infected WT animals, and ME7-infected γ-chain–deficient mice with LPS and measured proinflammatory cytokine levels in the brain as a measure of microglial switching. We found that LPS results in increased levels of IL-1β and IL-12 \( [F(2,8) = 8.00, \ p = 0.012] \) in WT ME7 animals (Fig. 10), confirming microglial switching in the experiment. In contrast, ME7-infected γ-chain–deficient mice do not increase expression of proinflammatory cytokine IL-1β after systemic infection, and levels are comparable with those of NBH animals receiving the same dose of LPS (Fig. 10). In addition, ME7-infected γ-chain–deficient mice show altered levels of IL-10 and IL-12 compared with those of ME7-infected WT animals. These differences in cytokine responses in the brains of γ-chain–deficient mice are not due to lack of systemic response to the LPS challenge, as serum levels of IL-1β were comparable between the two mouse strains (Fig. 10A).
In our study, increased levels of IgG were found on CD68+ cells in the parenchyma of WT ME7 animals, whereas IgG levels remained largely associated with blood vessels in γ-chain–deficient mice without colocalization of CD68+ cells after LPS challenge (Fig. 10B). These results further suggest a role of activating FcγR in microglial switching during chronic neurodegeneration.

Discussion
The neuropathology of murine prion disease is characterized by extensive neuronal degeneration, the accumulation of misfolded protein, an increase in the number of morphologically activated or “primed” microglia and astrocytes, but no typical proinflammatory cytokines. Systemic inflammation “switches” this benign phenotype to an aggressive proinflammatory phenotype resulting in increased proinflammatory cytokine production and irreversible neuronal damage (15, 30). It is now clear that “primed” microglia are seen in a variety of neurologic diseases, all of which are exaggerated by systemic inflammation, but the mechanism underlying these changes remains unknown (24).

In chronic neurodegeneration, microglia have the capacity to clear cell debris and apoptotic cells (31) and become vulnerable to activation to an innate immune challenge due to increased expression of phagocytic receptors (11, 12, 17, 31). The current study is consistent with the hypothesis that microglial phenotype switching is initiated by immune receptors expressed on macrophages, and their subsequent activation depends on the signaling cascades engaged. In this study, we focused on well-described receptors that potently regulate macrophage function and, depending on their cytoplasmic domains, either activate or inhibit macrophage function.
through phosphorylation of their receptors bearing ITAM or ITIM motifs. Various cellular activities are controlled by ITAM and ITIM signaling pathways (32), and the balance between these opposing motifs determines whether and to what extent cells become activated. For example, proinflammatory cytokines can significantly increase the expression of receptors that signal through ITAM motifs, lowering the threshold for activation (33). Microglia express several members of this family, including FcγRI, TREM-2, SIRPβ1, DAP12, and CD200R (34–36), and there is evidence that the loss of inhibitory signals results in microglial priming, making them more prone to activation (37). The current study shows increased expression of both ITAM- and ITIM-bearing receptors in ME7 animals at the mRNA level and on microglia at the protein level. Systemic inflammation induced further changes in the expression levels of ITAM-associated FcγRs and gp49a expression, whereas other receptors and adapter molecules remained unaffected or downregulated. Gp49a, a type I transmembrane glycoprotein, does not have its own signaling motif and is strongly associated with the ITAM-bearing γ-chain for its function. It is expressed on the surfaces of mast cells, NK cells, and macrophages. Cross-linking of gp49a results in calcium mobilization, eicosanoid production, and cytokine gene transcription, induced by signaling through the γ-chain, similar to activating FcγRs (38).

The role of FcγRs in chronic neurodegeneration

FcγRs can be divided into two types, and mice express four different classes: the activating receptors FcγRI, FcγRIII, and FcγRIV, which signal through an ITAM, and an inhibitory receptor FcγRIIB, which signals through an ITIM (39, 40). The inhibitory FcγRIIB is an important modulator of inflammatory effector cells, and FcγRIIB deficiency leads to amplified immune responses and autoimmunity (41). Cross-linking of activating FcγR results in a range of downstream effector functions, including cytokine release, whereas coaggregation of inhibiting FcγR inhibits these functions (39). In the healthy brain, FcγR expression is very low and restricted to microglia (42). Enhanced expression is observed after treatment with IFN-γ, TNF-α, and LPS in vitro (43) and after stereotaxic injection of LPS in vivo (44). It was also shown that transient opening of the blood–brain barrier by adrenaline in otherwise healthy rats increased FcγRI expression on microglia (45), and multiple sclerosis lesions have an elevated expression of various activating FcγRs (46, 47). These studies suggest that FcγRs can be induced in the brain where they can play an important role in controlling Ab-mediated neuroinflammation. However, some studies do not support a key effector function of FcγRs in neuroinflammation and suggest a key role of complement activation instead (48).

In contrast to studies in peripheral tissues, the differential expression of activating versus inhibitory FcγR has not been studied in the brain. Furthermore, to our knowledge, this is the first time FcγRIV has been directly studied in a mouse model of neurologic disease. The role of FcγR and complement in the latency of prion disease has been previously studied (49). In this study, Fcγ-chain-deficient mice showed an increased survival time after intracerebral inoculation of the prion agent. Notably, survival times of FcγRII- and FcγRII-deficient mice were not different from those of WT mice (49), suggesting a possible role for FcγRIV in disease onset in this mouse model. Using a unique panel of anti-FcγR Abs, we confirmed our microarray and quantitative PCR results and showed strong increase in FcγRIII and FcγRIV protein levels in the thalamus of LPS-challenged ME7 animals. No changes in protein levels of the inhibitory FcγRII were observed, although the Ab used for histological analysis showed clear staining of immune cells in the spleen. This was surprising, as FcγRII mRNA was found to be increased in ME7 brains and further increased after systemic LPS. A previous in vitro study showed that macrophages cultured in the presence of TNF-α show a reduced surface expression of FcγRII, while at the same time the mRNA of this receptor was strongly increased as a result of increased mRNA stability. It is possible that a similar post-transcriptional regulation of FcγRII explains the lack of differential protein expression in our model (50). To study if activating FcγRs have a functional role in switching microglial phenotype, we induced prion disease in γ-chain-deficient mice. We show that systemic inflammation no longer induces expression of IL-1β in the brain, strongly suggesting a lack of microglial switching in γ-chain–deficient mice in response to a systemic LPS challenge. The IL-10 levels were increased to a greater extent in γ-chain–deficient mice compared with WT ME7 animals, but without information on baseline levels of this cytokine, it is unclear if this observation is functionally relevant. Serum levels of IL-1β were comparable between WT and γ-chain–deficient mice, ruling out a lack of systemic response to LPS. Furthermore, a study from Le et al. (51) shows that microglia from WT or Fcγγ−/− mice release similar levels of TNF-α after LPS stimulation, whereas IgG immune complexes activate WT cells only. We observed reduced IgG deposition on parenchymal cells in γ-chain–deficient mice, likely due to lack of FcγR expression. These results suggest a role for activating FcγR in LPS-induced microglial switching; however, as IL-12 and IL-10 are still induced in γ-chain–deficient mice, there may be additional mechanisms that underlie microglial switching.

Implications for immunotherapy

There is growing academic and commercial interest in using the power of Ab-mediated responses to treat neurodegenerative disease by vaccination strategies, including Alzheimer’s disease and prion diseases, but recent studies suggest that immunotherapy targeting brain Ags is not without risks (52, 53). Increased expression of FcγR on microglia as a consequence of neurodegeneration may partly explain the inflammatory reactions within the brain observed after immunotherapy in animal models (54, 55). Further understanding of the role of activating and inhibiting FcγR in these processes and controlling FcγR function is likely to increase the success of immunotherapy for neurodegenerative diseases and avoid the clinical setbacks experienced to date.

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Disclosures

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References
