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Inflammasome-Dependent IFN-γ Drives Pathogenesis in Streptococcus pneumoniae Meningitis

Andrew J. Mitchell,*,†,‡ Belinda Yau,*† James A. McQuillan,* Helen J. Ball,* Lay Khoon Too,* Arby Abtin,‡ Paul Hertzog,‡ Stephen L. Leib,§ Cheryl A. Jones,*⊥ Sebastien K. Gerega,∥ Wolfgang Weninger,⊥ and Nicholas H. Hunt*

The pathology associated with Streptococcus pneumoniae meningitis results largely from activation of immune-associated pathways. We systematically investigated the production of IFN subtypes, as well as their influence on pathology, in a mouse model of S. pneumoniae meningitis. Despite the occurrence of a mixed IFN type I/II gene signature, no evidence for production or involvement of type I IFNs in disease progression was found. In contrast, type II IFN (IFN-γ) was strongly induced, and IFN-γ−/− mice were significantly protected from severe disease. Using intracellular cytokine staining and targeted cell-depletion approaches, NK cells were found to be the dominant source of IFN-γ. Furthermore, production of IFN-γ was found to be dependent upon ASC and IL-18, indicating that an ASC-dependent inflammasome pathway was responsible for mediating IFN-γ induction. The influence of IFN-γ gene deletion on a range of processes known to be involved in bacterial meningitis pathogenesis was examined. Although neutrophil numbers in the brain were similar in infected wild-type and IFN-γ−/− mice, both monocyte recruitment and CCL2 production were less in infected IFN-γ−/− mice compared with infected wild-type controls. Additionally, gene expression of NO synthase was strongly diminished in infected IFN-γ−/− mice compared with infected controls. Finally, bacterial clearance was enhanced in IFN-γ−/− mice, although the underlying mechanism remains unclear. Together, these data suggest that inflammasome-dependent IFN-γ contributes via multiple pathways to pathology during S. pneumoniae meningitis. The Journal of Immunology, 2012, 189: 4970–4980.

Bacterial meningitis (BM) is a major cause of mortality worldwide and is responsible for 340,000 deaths each year (1). The Gram-positive extracellular bacterium Streptococcus pneumoniae (pneumococcus) is the most common pathogen in adults and, despite antibiotic therapy, it has the highest mortality of all meningitis-causing agents: 4–16% in children and up to 60% in adults (2–4). Furthermore, pneumococcal meningitis causes the highest rates of neurologic sequelae (5), with long-term disabilities reported in 27–57% of survivors (6–8). The processes underlying pathogenesis in BM are complex, with contributions from both bacteria and host, but the immune response is believed to be a key factor (9–11).

Immune targeting of S. pneumoniae is essential for clearance of infection; however, within the brain, the attendant inflammatory response contributes to pathogenesis. The presence of S. pneumoniae is initially sensed by resident cells via interaction of microbial products with pattern recognition receptors, including TLR2 and TLR4 (12-14), as well as NOD2 (15). More recently, it was shown that S. pneumoniae may also be recognized by inflammasomes that are dependent upon the adaptor molecule ASC (16–19). Together, these pathways initiate immune activation and lead to production of inflammatory cytokines and chemokines that subsequently mediate the recruitment of leukocytes to the site of infection (20–26).

However, when this occurs within the brain, the ensuing inflammatory response is argued to ultimately cause neuronal damage and/or death (9, 10). Indeed, administration of steroids diminishes inflammation in rodent models of pneumococcal meningitis (27), and it is recommended as adjunct therapy in human disease (28). Most studies investigating the roles of cytokines in BM have focused on those cytokines that are traditionally regarded as mediators of acute inflammation, such as TNF-α and IL-1β (20, 29, 30); however, the contribution of other cytokines, including IFNs, to pathogenesis has not been well studied. Although type I IFNs (IFN-α and IFN-β) historically have been associated with responses to viral infection, it is becoming increasingly clear that they may influence the outcome of responses to other pathogens (31, 32). In contrast, type II IFN (IFN-γ) has a well-described role in protection against infection with intracellular bacteria (33, 34).

Early studies of S. pneumoniae meningitis showed the presence of IFN bioactivity in the cerebrospinal fluid (CSF) of patients with BM (35), but reports on the presence of IFN-γ specifically are inconsistent, with some studies reporting concentrations that are low to undetectable (36–39), whereas others suggested that IFN-γ...
production may be present in at least some patients (40–42). Of particular note is the fact that many of these studies did not distinguish between causative agents when investigating the presence of IFN-γ in CSF. This is significant, because BM caused by *S. pneumoniae* was reported to be specifically associated with increased levels of IFN-γ in CSF (41, 42). Although IFN-γ was reported to be produced in the brain in a rodent model of *S. pneumoniae* meningitis (43), the significance of this finding has not been investigated.

The involvement of IFN-γ in the development of systemic and pulmonary pneumococcal disease has been studied in detail and has typically been associated with enhanced clearance of bacteria. In patients with *S. pneumoniae* sepsis, plasma IFN-γ levels were reported to be elevated, and circulating levels of the cytokine correlated with mortality (44). In murine models, IFN-γ inhibition/ablation was reported to lead to decreased survival (25, 26, 45) or to have no effect on mortality (23, 46). Furthermore, in the absence of IFN-γ, decreased levels of chemokines involved in neutrophil recruitment, as well as neutrophils themselves, were reported by some groups (23, 26), and this was associated with a lower bacterial load (23). More recently, and in contrast, IFN-γ–deficient mice were argued to have higher levels of neutrophils and chemokines associated with their recruitment, as well as increased bacterial load (25). Alternatively, IFN-γ may influence pneumococcal clearance via modulation of macrophage scavenger receptors. These pattern recognition receptors directly mediate phagocytosis of *S. pneumoniae* and are critical for clearance both within the lung (47–49) and during systemic infection (50). Expression of scavenger receptors was shown to be modulated by IFN-γ (51), and high levels of IFN-γ, resulting from viral infection, for example, can lead to drastically reduced pneumococcal load (48).

In addition to its influence on bacterial clearance, IFN-γ has the potential to exacerbate inflammation and subsequent pathology, primarily through its ability to modulate the functions of neutrophils and monocyte/macrophages. Tight control of neutrophil function is particularly relevant during pneumococcal meningitis, because these cells may contribute both to protection via clearance of bacteria (22) and, if their apoptosis is delayed, to tissue damage and pathology (52). In conjunction with stimulation through pattern recognition receptors, IFN-γ activates macrophages and polarizes them toward an M1 phenotype, which is characterized by upregulation of proinflammatory factors (reviewed in Ref. 53). In particular, IFN-γ induces NO synthase-2 (NOS2) (54), and increased NO production secondary to NOS2 induction was argued to contribute to pathology during pneumococcal meningitis (55).

In this study, we systematically investigated the production of both type I and type II IFNs in a murine model of *S. pneumoniae* BM. We find that IFN-γ, but not type I IFNs, is produced and drives pathology during pneumococcal meningitis. This IFN-γ is made by NK cells and is dependent upon inflammasome activation and IL-18. Protection is associated with decreased bacterial load, but only a limited number of inflammatory factors was IFN-γ dependent.

**Materials and Methods**

**Mice**

Mice of mixed sex, aged 6–14 wk at the commencement of experimentation, were used. Wild-type (WT; C57BL/6) mice were sourced from the Animal Resources Centre (Canning Vale, WA, Australia), IL-18−/− (56), IFN-γ−/− (57), CXCR3−/− (58), and CD1d−/− (59) mice were maintained in the Medical Foundation Building Animal House (The University of Sydney). IFNAR1−/− (60) and IFNAR2−/− (61) mice were from the Monash Institute of Medical Research, ASC−/− mice (62) were maintained in the Centenary Institute Animal Facility. All mice were on a C57BL/6 background. They were housed in the Medical Foundation Building Animal House or Centenary Institute Animal Facility in group cages under a 12-h light–dark cycle, with food and water ad libitum. All procedures adhered to the Australian National Health and Medical Research Council guidelines for animal research and were approved by the University of Sydney Animal Care and Ethics Committee.

**Model**

*S. pneumoniae*, strain U2, serotype 3 (courtesy of Prof. J. Paton, University of Adelaide, Adelaide, SA, Australia) was stored at −80°C. Aliquots were thawed and cultured overnight in 5% CO₂ at 37°C in brain heart infusion broth (Oxoid, Adelaide, SA, Australia). Bacteria were then subcultured into brain heart infusion broth and grown to late log phase. After harvesting, bacteria were washed in PBS and adjusted to ~5 × 10⁷ CFU/ml based on OD at 570 nm. Exact inoculum concentration (CFU/ml) was determined by serial dilution and plating on horse blood agar plates (Oxoid), followed by overnight incubation in 5% CO₂ at 37°C. For induction of meningitis, animals were lightly anesthetized with isoflurane, the head was swabbed with 70% ethanol, and a 29G needle was inserted between the hemispheres to a depth of 3–4 mm. Ten microliters of inoculum (~5 × 10⁶ CFU) was injected into the third ventricle. Sham-infected mice received 10 μl PBS. In this model, infected mice begin to develop severe disease at ~48 h postinoculation (p.i.). In some experiments, IFN-γ was neutralized by i.p. administration of anti–IFN-γ (500 μg 2 h prior to infection, then 250 μg at 48 h and 96 h postinfection), or by intra-cerebroventricular (icv) administration of 20 μg Ab mixed with bacterial inoculum. Animals were euthanized if they showed overtly hunched posture, gait disturbances, fitting, or difficulty righting.

**RT-quantitative PCR**

Tissues were homogenized in RLT buffer (Qiagen, Valencia, CA), using 1-mm zirconium beads (Biospec Products) in a FastPrep FP120 bead mill (Thermo Fisher, Waltham, MA). After storage at ~80°C, samples were thawed, and RNA was extracted using an RNeasy miniprep kit (Qiagen), following the manufacturer’s instructions. RNA was reverse-transcribed using Moloney murine leukemia virus-RT (Ambion) primed with random hexamers (GeneWorks, Hindmarsh, VIC, Australia). Quantitative PCR (qPCR) was performed, using the primers listed in Table 1, on a Corbett Research Rotor Gene 3000 using platinum SYBR Green Supermix (Invitrogen, Carlsbad, CA) with cycling conditions as follows: 2 min of initial denaturation (95˚C for 15 s and 60˚C for 45 s). Following amplification, product purity was determined by gel electrophoresis. Quantitative PCR (qPCR) was performed, using the primers listed in Table 1, on a Corbett Research Rotor Gene 3000 using platinum SYBR Green Supermix (Invitrogen, Carlsbad, CA) with cycling conditions as follows: 2 min of initial denaturation (95˚C for 15 s and 60˚C for 45 s). Following amplification, product purity was assessed by melt-curve analysis. RPL13a was used as a reference gene, and the gene-expression levels in pooled samples were normalized against the brains of uninfected animals using the ΔΔCt method (63). The primers used for RT-qPCR had similar amplification efficiencies to the reference gene.

**Bacterial load estimation**

Half brains from infected mice were removed aseptically and homogenized in a 1.5 (w/v) dilution in PBS using a TissueRuptor (QIAGEN). Bacterial load was determined by serial dilution and plating on horse blood agar plates, followed by overnight incubation in 5% CO₂ at 37°C.

**Array analysis**

For array analysis, RNA from brains of infected and sham-infected mice (n = 4/group) was pooled and analyzed using an Affymetrix Mouse Gene 1.0-ST array. Arrays were normalized using the robust multi-array average algorithm (64) implemented in BioConductor, and differentially expressed genes with fold change >2.0 were identified. Array data have been sub-
1% FCS, 5 mM EDTA, 0.05% sodium azide, and BFA. Thereafter, intracellular lysis. Fc-blocked cells were stained for surface markers in PBS, containing BFA, and centrifuged at 500 g. Experiments with the modification that volumes of all reagents and samples were either 50% or 10% of those in the original protocol. Data were collected using an FC500 Flow Cytometer (Beckman Coulter, Gladesville, NSW, Australia) and analyzed with FlowJo software (Tree Star, Ashland, OR). Concentrations of cytokines in tissue homogenates were normalized to total protein content for each sample.

Flow cytometry
Quantification of leukocyte subsets in the brains of infected animals was performed as described previously (65, 66), with minor modifications. Briefly, brains were mashed between frosted glass slides in RPMI 1640, digested with collagenase/DNase for 20 min at room temperature, and triturated before a second 20-min collagenase/DNase step. Low-density dead cells/debris/myelin were removed by resuspension in 30% Percoll and centrifugation at 500 × g for 10 min at 4˚C. Following Percoll isolation, contaminating RBCs were lysed by Tris- ammonium chloride lysis. Prior to staining, cells were incubated with anti-CD16/32 (Fc block). Ab staining for flow cytometry was performed by standard methods, and dead cells were excluded using propidium iodide. Samples were analyzed on a five-laser LSR II (Becton Dickinson Biosciences), and data were analyzed using FlowJo software (Tree Star). Cells for intracellular cytokine staining were prepared using a modified isolation and staining procedure, as follows. Animals received an i.p. injection of 250 μg brefeldin A (BFA; Sigma) at 42 h p.i. Following euthanasia, brains were placed into ice cold RPMI 1640 containing 10 μg/ml BFA and disaggregated immediately by pressing through a 100-μm mesh. Cells were pelleted (300 × g, 5 min, 4˚C), resuspended in 30% Percoll containing BFA, and centrifuged at 500 × g for 5 min at 4˚C. Following Percoll isolation, contaminating RBCs were removed by a 10-s distilled water lysis. Fc-blocked cells were stained for surface markers in PBS, 1% FCS, 5 mM EDTA, 0.05% sodium azide, and BFA. Thereafter, intracellular IFN-γ was stained using an intracellular cytokine staining kit (BioLegend), as per the manufacturer’s instructions. Staining controls included cells preincubated with either an excess of unlabeled anti–IFN-γ (isoclonic control) or with a molar excess of rIFN-γ. Additionally, brain leukocytes isolated from S. pneumoniae-infected IFN-γ−/− mice showed no significant IFN-γ staining.

Statistical analysis
Experimental group size giving 90% statistical power was estimated based on preliminary data using PS Power and Sample Size Calculation software (67). Because large group sizes were often required to attain this level of power, data from multiple independent experiments were typically pooled for analysis, as described in the figure legends. Data were analyzed using GraphPad Prism version 5.01 for Windows (GraphPad Software, La Jolla, CA). For two groups, statistical significance was determined using a t test, whereas one-way ANOVA with the Tukey posttest was used for multiple-group comparisons. Quantitative PCR and bacterial load data were log transformed prior to analysis. Survival experiments were analyzed using a log-rank test.

Results
Type II IFN, but not type I IFNs, contributes to disease progression in pneumococcal meningitis
The potential for IFN subtypes to contribute to the immune response to S. pneumoniae during meningitis was initially examined by investigating the overall patterns of gene expression by gene microarray, followed by analysis of IFN response signatures (68). In infected mice, the majority of differentially expressed genes during BM were identified as IFN-regulated genes that were capable of being regulated by either type I or type II IFNs. Of the top 200 differentially expressed genes, 132 were classified as IFN regulated. Of these 132 genes, 130 were associated with type I

**FIGURE 1.** IFN responses in S. pneumoniae meningitis and effect of subtypes on development of severe disease. (A) Classification of top 200 differentially expressed (DE) genes into type I and type II IFN regulated. Brains were collected from S. pneumoniae-infected (~5 × 103 CFU, icv) mice at 48 h p.i. Venn diagram shows IFN type I- and type II-dependent genes (68). Values indicate the number of genes in each category. Data are from a single experiment with samples pooled from n = 5 animals/group. (B) Time course of gene expression, relative to expression in noninfected animals, of IFN subsets. Changes in mRNA for IFN-α4, IFN-αN (IFN-α1, IFN-α2, IFN-α7, IFN-α11, and IFN-α12), IFN-β, and IFN-γ were determined by RT-qPCR in the brains of infected mice. Symbols represent individual animals, and horizontal lines represent the geometric mean of each group. **p < 0.01, one-way ANOVA with Tukey posttest on log-transformed data. Data are representative of two independent experiments with similar, but nonidentical, collection times. (C) Contribution of IFN subtypes to severe disease. Upper panels, WT and IFN-γ−/− or WT and IFN-αR1−/− or IFN-αR2−/− mice were infected with S. pneumoniae (~5 × 103 CFU, icv), and survival to a predefined clinical end point was determined (Materials and Methods). Lower panels, WT mice were treated with neutralizing anti–IFN-γ Ab via either i.p. or icv routes at the time of infection, and survival to a predefined clinical end point was determined. Control animals received an isotype control Ab (i.c.). Data sets shown for each strain/treatment are pooled from a minimum of two independent experiments, with a minimum of n = 16/condition. **p < 0.01, versus WT/isotype control-treated group, log-rank test.
IFN-stimulated responses, and 65 were associated with type II IFN responses, with an intersection of 63 genes (Fig. 1A). These data suggested that a mixed type I and type II IFN response occurs following infection with *S. pneumoniae*.

Because the array data suggested that both type I and type II IFN-regulated genes were upregulated following infection, the induction of mRNA for IFN subtypes, at 24 and 48 h p.i., was examined by RT-qPCR in Table I. In contrast to the apparent strong type I IFN gene signature suggested by array, when changes in the expression of type I IFN genes (IFNα1, IFNα2, IFNα4, IFNα7, IFNα11, IFNα12, IFNβ) themselves were examined, no significant induction was seen over the course of 48 h (Fig. 1B). This was in marked contrast to changes seen in IFN-γ mRNA levels, with IFN-γ message increasing by ~10-fold over uninfected control at 24 h and ~100-fold by 48 h p.i. (Fig. 1B).

The contribution of IFN subtypes to the development of severe pathology was investigated by following disease progression in mice deficient either in type I IFN signaling (IFNAR1−/− or IFNAR2−/− mice) or in the gene for IFN-γ. Consistent with the apparent absence of either IFN-α or IFN-β production following infection, mice that lacked functional type I IFN signaling by disruption of either the IFNAR1 or IFNAR2 chain showed no significant differences in survival compared with infected WT mice (Fig. 1C). This was in distinct contrast to the outcome in IFN-γ–deficient mice, which were significantly protected from the development of severe disease; 82% of IFN-γ–deficient animals survived to 200 h p.i., whereas only 37% of WT animals did so (Fig. 1C). Confirmation of the involvement of IFN-γ that was produced within the brain, in the development of pathology, was determined by Ab-mediated neutralization of IFN-γ. When IFN-γ was neutralized by systemic (i.p.) administration of Ab, there was no protection; however, when anti–IFN-γ was administered icv at the time of infection, mice were significantly protected from severe disease (Fig. 1C).

**NK cells are the major source of IFN-γ in pneumococcal meningitis**

Because a wide variety of cells is capable of producing IFN-γ, the cellular source of IFN-γ during *S. pneumoniae* meningitis was investigated by intracellular cytokine staining and cell-specific depletion approaches. The inflammatory infiltrate occurring in response to *S. pneumoniae* infection was dominated by neutrophils and inflammatory monocytes, although appreciable numbers of CD4+ and CD8+ βT cells, as well as γδ T cells and NK cells, were also recruited to the brains of infected mice (Fig. 2A), whereas significant numbers of NKT cells were not detected (data not shown). Although a small percentage of CD3+ T cells expressed IFN-γ protein, a much larger number of NK1.1+, NKP46+ NK cells expressed high levels of IFN-γ (Fig. 2A). In contrast, myeloid cells, which included microglia (CD45int, CD11bhi), monocytes (CD45hi, CD11bhi, Ly6G−), and neutrophils (CD45hi, CD11bhi, Ly6Cint, Ly6G−), showed no staining for IFN-γ (data not shown). The dominant contribution of NK cells to IFN-γ production was confirmed in animals depleted of NK cells by administration of anti–asialo-GM1 Ab. High levels of IFN-γ protein (8810 ± 2890 pg/g of total protein) were detected in infected control mice, whereas IFN-γ protein was below the limit of assay detection (<400 pg/g) in the majority of infected, NK-depleted animals (Fig. 2B). Furthermore, IFN-γ mRNA was decreased >10-fold by NK depletion (Fig. 2C). The absence of a major contribution of T cell populations and, in particular, CD8+ T cells, to IFN-γ production was confirmed by infection of

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Sequence</th>
</tr>
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<tbody>
<tr>
<td>RPL13a</td>
<td>5′-CTTAGGCACTGTTCCTGGAAT-3′ (sense)</td>
</tr>
<tr>
<td>IFN-α</td>
<td>5′-GGTCGCCGTCTACCTCTTCTAA-3′ (antisense)</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>5′-AGGCTTCGAGTCTTGTGCCG-3′ (antisense)</td>
</tr>
<tr>
<td>IFN-β</td>
<td>5′-TCACCTCGGAGCCTCA-3′ (sense)</td>
</tr>
<tr>
<td>OAS1b</td>
<td>5′-TGAGCAATCCTCCCACTGCA-3′ (antisense)</td>
</tr>
<tr>
<td>MARCO</td>
<td>5′-CCCTCCTCCTCTGATCTTCCG-3′ (sense)</td>
</tr>
<tr>
<td>SRA1</td>
<td>5′-GAGGCATCCTGGAGGCTTGC-3′ (sense)</td>
</tr>
<tr>
<td>CCL2</td>
<td>5′-GAGGCATCCTGGAGGCTTGC-3′ (sense)</td>
</tr>
<tr>
<td>CCL4</td>
<td>5′-GAGGCATCCTGGAGGCTTGC-3′ (sense)</td>
</tr>
<tr>
<td>CXCL2</td>
<td>5′-GAGGCATCCTGGAGGCTTGC-3′ (sense)</td>
</tr>
<tr>
<td>CXCL9</td>
<td>5′-GAGGCATCCTGGAGGCTTGC-3′ (sense)</td>
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<td>CXCL10</td>
<td>5′-GAGGCATCCTGGAGGCTTGC-3′ (sense)</td>
</tr>
<tr>
<td>IL-1β</td>
<td>5′-CTTAGGCACTGTTCCTGGAAT-3′ (antisense)</td>
</tr>
<tr>
<td>IL-10</td>
<td>5′-CTTAGGCACTGTTCCTGGAAT-3′ (antisense)</td>
</tr>
<tr>
<td>TNF-α</td>
<td>5′-CTTAGGCACTGTTCCTGGAAT-3′ (antisense)</td>
</tr>
<tr>
<td>GFAP</td>
<td>5′-CTTAGGCACTGTTCCTGGAAT-3′ (antisense)</td>
</tr>
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</table>
Using this approach, it was found that, under resting conditions, microglia expressed comparatively high levels of pro–IL-18 mRNA (Fig. 3D).

**IFN-γ-dependent effects on pathology**

Following infection of the CSF with *S. pneumoniae*, numerous processes may contribute to pathology and, ultimately, death, in particular, the development of edema, enhancement of inflammation, as well as inhibition of clearance of bacteria; therefore, the influence of IFN-γ on these processes was assessed. An initial time point of 48 h p.i. was chosen for examination, because differences in clinical severity between WT and gene-deficient animals first become apparent at this time. An additional time point of 72 h p.i. was investigated because, although ∼10–15% of WT animals had developed severe disease and had been euthanized by this stage (Fig. 1C), there was a clear difference in clinical signs between infected WT and IFN-γ−/− mice (data not shown). Because the initial investigation of the role of edema in the development of pathology found that there was no statistically significant difference in brain water content between infected WT or gene-deficient animals at either 48 or 72 h p.i. (Supplemental Fig. 2), alternative pathways were focused upon.

**IFN-γ modulates inflammation**

Because inflammation is critical in the development of pathology during pneumococcal meningitis and because IFN-γ can enhance many inflammatory processes, modulation of inflammation might explain the protection of IFN-γ−/− mice from mortality. Therefore, the influence of IFN-γ on a range of inflammatory parameters, including leukocyte recruitment, as well as cytokine, chemokine, and enzyme gene expression, was determined.

At both 48 and 72 h p.i., neutrophils and inflammatory monocytes were the dominant cell populations recruited to the brains of both WT and IFN-γ−/− animals following infection, whereas smaller numbers of lymphocytes (T and NK cells) were present...
CD8+ T cells in infected gene-deficient animals (Fig. 4A). Although similar total numbers of neutrophils were seen in both infected WT and IFN-γ−/− mice, whereas IL-10 mRNA abundance in infected animals remained similar to that seen in sham-infected animals at 48 h but was induced in infected WT mice at 72 h p.i. (Fig. 5A, 5B). Although CCL4, CXCL1, and CXCL2 mRNA were induced following infection at both time points, there was no effect of IFN-γ gene deletion on their abundance at 48 or 72 h p.i. (Fig. 5A, 5B). Consistent with the gene-expression data, protein levels of TNF-α and IL-1β in brain homogenates were significantly induced in both WT and IFN-γ−/− mice compared with sham-infected controls (Fig. 5C, 5D), whereas IL-10 protein was not detected in either strain following infection (data not shown). In contrast, infection led to an induction of CCL2 mRNA in both WT and IFN-γ−/− mice, but infected gene-deficient mice showed a diminished level of expression at 72 h p.i. compared with infected WT controls (Fig. 5A, 5B). A similar diminution in CCL2 protein in homogenates of infected IFN-γ−/− mice, compared with infected controls, was seen at 48 h, but not at 72 h, p.i. (Fig. 5C, 5D). In addition to chemokines that act primarily on myeloid cells, the influence of IFN-γ on the expression of the known IFN-γ-dependent CXCR3 ligands CXCL9 and CXCL10 was examined. CXCL9 mRNA was significantly lower in infected IFN-γ−/− mice compared with infected WT mice. However, the CXCR3 system did not make a major contribution to pathology, because CXCR3−/− mice were not protected from severe disease (data not shown).

Because components of inflammation, including recruitment of myeloid cells, appeared to be downmodulated following infection in IFN-γ−/− mice compared with WT mice and because IFN-γ influences the activation state of monocyte/macrophages, we investigated the expression of proinflammatory enzymes that may be produced by these cells. NOS2 and cyclooxygenase-2 (COX2) are IFN-γ-inducible enzymes that were shown to contribute to the development of neuropathology in a range of diseases. NOS2, in particular, was implicated in the development of pathology in pneumococcal meningitis (70, 71). At both 48 and 72 h p.i., NOS2 and COX2 were significantly induced in the brains of infected WT animals compared with uninfected controls. Although there was no statistically different in the induction of COX2 between infected WT and IFN-γ−/− animals, induction of NOS2 was significantly decreased in infected IFN-γ−/− mice compared with infected WT animals at both time points (p < 0.05, Fig. 5A, 5B).

IFN-γ inhibits bacterial clearance

Effective bacterial clearance is necessary for the ultimate resolution of pneumococcal meningitis. Indeed, S. pneumoniae clearance in meningitis was reported to be IL-18 dependent (72), at least in part, and because we previously showed that IFN-γ production was dependent upon IL-18 (Fig. 3C), we hypothesized that IL-18 might mediate this effect via induction of IFN-γ. Therefore, the effect of IFN-γ gene deletion on bacterial load in infected animals was determined. Although there was a trend toward decreased bacterial load within the brains of infected IFN-γ−/− mice at 48 h p.i., this was not significant (Fig. 6A). However, at 72 h p.i., there was an ~10-fold decrease in the mean CFU in the brains of infected gene-deficient animals compared with infected control mice (Fig. 6B). The mechanisms by which the immune system clears S. pneumoniae are multiple; however, key among these are
the macrophage scavenger receptors MARCO, SRA1, and SIGNR1, which bind either to nonopsonized particles or to components of the polysaccharide capsule and mediate phagocytosis (47, 49, 73). The influence of IFN-γ on scavenger receptor expression was examined by quantifying mRNA for scavenger receptors within the brains of infected animals at 48 and 72 h p.i. (Supplemental Fig. 3A, 3B), as well as by determining the expression of protein on recruited monocytes and resident microglia by flow cytometry at 72 h p.i. (Supplemental Fig. 3C). Despite the clear difference in bacterial load at this time, and although gene expression of both MARCO and SRA1 was significantly induced following infection, there were no differences in the expression levels of any scavenger receptor between infected WT and IFN-γ−/− animals.

Discussion

Key drivers of pathology in pneumococcal meningitis are the immune processes in response to the bacterium; however, these immune responses are a double-edged sword: they enhance pathology but are also essential for clearance of the organism. It is against this background that we investigated the involvement of IFNs in both pathology and immunity in pneumococcal meningitis.

Initial analysis of gene-array data revealed an apparent overall pattern of upregulation of IFN-responsive genes. The majority of

![FIGURE 4](image)

Influence of IFN-γ gene deletion on leukocyte recruitment. WT or IFN-γ−/− (KO) mice were inoculated with PBS or S. pneumoniae (5 × 10^5 CFU; S.p.) icv, brains were collected at 48 h (A) or 72 h (B) p.i., and recruited leukocytes were quantified by flow cytometry. Data shown are from four (A) or two (B) pooled experiments. Symbols represent individual animals, and horizontal lines represent the arithmetic means of groups. Total n = 12–25/group (48 h p.i.) or n = 5–8/group (72 h p.i.). *p < 0.05, one-way ANOVA with Tukey posttest. ns, Not significant.

![FIGURE 5](image)

Effect of IFN-γ gene deletion on inflammatory mediators. WT or IFN-γ−/− (KO) mice were inoculated with PBS or S. pneumoniae (5 × 10^5 CFU; S.p.) icv, and mRNA levels of the indicated genes were quantified by RT-qPCR in brains from mice at 48 h (A) or 72 h (B) p.i. Levels of cytokine proteins, normalized to total protein, were quantified in brain homogenates from mice at 48 h (C) or 72 h (D) p.i. Symbols represent individual animals, and horizontal lines represent the geometric means of groups. Data shown are from four independent experiments with total n = 10–19/group (A), two independent experiments with n = 6–20/group (B), or a single independent experiment with total n = 6–14/group (48 h p.i.) or n = 3–7/group (72 h p.i.) (C, D). *p < 0.05, **p < 0.01, one-way ANOVA with Tukey posttest on log-transformed data. ns, Not significant.
these were able to be classified as responsive to type I IFNs, and approximately one third of the 200 most differentially expressed genes were classified as type II dependent. Although type I IFNs historically have been associated with rapid innate protection in viral infection, it has recently become clear that they can play critical roles in developing immune responses; thus, a potential role for these was investigated more fully. Despite the initial results of the gene-microarray analysis, a contribution of type I IFNs to pathology was ruled out, because none of the type I genes tested had upregulated expression following infection. The most likely explanation for this discrepancy is that IFN-regulated genes can be activated independently of IFNs. In particular, this can occur in a primary pattern recognition response by activation of IRFs, typically IRF3 or IRF7 (reviewed in Ref. 74). Furthermore, mice rendered incapable of responding to type I IFNs through genetic ablation of either IFNAR1 or IFNAR2 developed severe disease, with similar kinetics to WT animals.

In contrast to the absence of upregulation seen with type I IFN genes, both IFN-γ mRNA and protein were strongly induced following infection with *S. pneumoniae*. More importantly, IFN-γ contributed to the development of pathology, because IFN-γ−/− mice were significantly protected from lethality. Evidence for the production of IFN-γ in experimental models of *S. pneumoniae* BM is conflicting. Production of both IFN-γ and protein was shown in the rat (75). In contrast, although IFN-γ protein and mRNA within the brain, whereas no such decrease was seen in animals deficient or depleted of T cell subsets (RAG1−/−, CD1d−/−, or CD8 depleted).

The proposed mechanisms leading to pathology in pneumococcal meningitis are multiple and include development of edema, inflammation, and the generation of reactive oxygen and nitrogen species (reviewed in Refs. 9, 84). A critical role for IFN-γ in edema formation was discounted, because we did not find any effect of IFN-γ on brain water content in infected animals. Perhaps more surprisingly, although some modulation of leukocyte and chemokine expression was seen, leukocyte recruitment was not dramatically affected by IFN-γ gene deletion. Neutrophils are a likely candidate target cell for IFN-γ action. It was reported that, during *S. pneumoniae* infection in the lung, inhibition/genetic ablation of IFN-γ leads to increased mortality, which is typically associated with decreased neutrophil influx and activation, as
well as increased bacterial load (25, 26, 45, 83). However, in meningitis, the influence of neutrophil recruitment, activation, and survival appears to be more complex, perhaps because the brain is likely to be more susceptible to damage caused by inflammation. Neutrophil depletions lead to increased mortality and higher bacterial loads (22), whereas, alternatively, the prolonged presence of activated neutrophils, secondary to inhibition of apoptosis, contributes to pathology (52). Although IFN-γ was shown to have an anti-apoptotic effect on neutrophils (85), it appears that in the model used in the current study, at least, the contribution of IFN-γ to neutrophil survival is minimal and this is not a dominant pathway leading to pathogenesis. The absence of an effect of IFN-γ on neutrophil recruitment contrasts with its apparent role in recruiting monocytes. At 48 h p.i., a time when clear differences were noted in the clinical scores of infected WT and IFN-γ−/− mice, numbers of recruited inflammatory monocytes were significantly lower in the brains of infected IFN-γ−/− mice compared with infected controls. This observed decrease in monocyte numbers was consistent with a decrease in CCL2 protein in brain homogenates at 48 h p.i., supporting a major role for this chemokine in monocyte recruitment. Despite this clear effect on monocyte recruitment, it is difficult to attribute the dramatic increase in the survival of IFN-γ−/− animals to this alone, because the magnitude was relatively modest, and it was reported that blockade of CCL2-mediated inflammatory monocyte recruitment does not influence disease progression (22).

In addition to gross effects on the recruitment and survival of inflammatory cells, IFN-γ has the potential to drive tissue damage through excessive myeloid activation, which may be mediated through a combination of increased oxidative burst and formation of NO and proteinoidats. Indeed, IFN-γ is a potent stimulator of these functions in both neutrophils (reviewed in Ref. 86) and macrophages (reviewed in Ref. 53). NO2 induction, and the subsequent generation of NO, in particular, were argued to contribute to pathology in S. pneumoniae BM (55, 87). We found that induction of mRNA for NO2, but not COX2, was strongly diminished in infected IFN-γ gene-deficient animals at both 48 and 72 h p.i. compared with infected WT controls, suggesting that IFN-γ-dependent induction of NO production via NO2 does indeed contribute to pathology. Because monocytes do not appear to be critical in pathology, it is likely that CNS-resident myeloid populations, such as microglia or meningeal macrophages, may be the source of the induced NO2.

A final, potentially key pathway by which IFN-γ could mediate severe meningitic disease is by inhibiting clearance of bacteria. Indeed, bacterial loads within the brains of infected IFN-γ−/− mice were decreased ~10-fold compared with infected WT mice at 72 h p.i. Previously, evidence for a role of IFN-γ in modulating clearance of bacteria came primarily from lung infection models. In contrast to our data, in the lung, IFN-γ typically has been reported to enhance, rather than inhibit, bacterial clearance, with protection being attributed to increased IFN-γ-dependent chemokine production leading to neutrophil recruitment (46, 83). Based on the previously noted lack of influence of IFN-γ on chemokine expression or neutrophil recruitment, such mechanisms are unlikely to be driving bacterial clearance in our model. Alternatively, in coinfection studies with influenza virus, it was shown that virus-dependent production of IFN-γ by T cells inhibits the expression of the macrophage scavenger receptor MARCO, and this leads to increased susceptibility to secondary infection with S. pneumoniae (48). However, we were unable to demonstrate any effect of IFN-γ on the expression of scavenger receptors in our model. Taken together, this suggests the existence of unidentified, IFN-γ-inhibited pneumococcal clearance mechanism(s).

In conclusion, this study showed that IFN-γ is a major driver of pathology during pneumococcal meningitis. The cytokine is produced following activation of inflammasome pathways and stimulation of NK cells by IL-18. Subsequently, IFN-γ contributes to pathology by modulating a range of processes, including myeloid recruitment and activation, as well as by inhibiting bacterial clearance. These findings reinforce that dysregulated immune processes contribute to morbidity and mortality in meningitis and suggest that targeting of IFN-γ or related pathways may be a feasible approach for adjunctive therapy.

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Disclosures
The authors have no financial conflicts of interest.

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