Myeloid-Related Protein 14 Promotes Inflammation and Injury in Meningitis

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Background. Neutrophilic inflammation often persists for days despite effective antibiotic treatment and contributes to brain damage in bacterial meningitis. We propose here that myeloid-related protein 14 (MRP14), an abundant cytosolic protein in myeloid cells, acts as an endogenous danger signal, driving inflammation and aggravating tissue injury.

Methods. The release pattern of MRP14 was analyzed in human and murine cerebrospinal fluid (CSF), as well as in isolated neutrophils. Its functional role was assessed in a mouse meningitis model, using MRP14-deficient mice.

Results. We detected large quantities of MRP14 in CSF specimens from patients and mice with pneumococcal meningitis. Immunohistochemical analyses and a cell-depletion approach indicated neutrophils as the major source of MRP14. In a meningitis model, MRP14-deficient mice showed a better resolution of inflammation during antibiotic therapy, which was accompanied by reduced disease severity. Intrathecal administration of MRP14 before infection reverted the phenotype of MRP14-deficient mice back to wild type. Moreover, intrathecal injection of MRP14 alone was sufficient to induce meningitis in a Toll-like receptor 4 (TLR4)-CXCL2-dependent manner. Finally, treatment with the MRP14 antagonist paquinimod reduced inflammation and disease severity significantly, reaching levels comparable to those achieved after genetic depletion of MRP14.

Conclusions. The present study implicates MRP14 as an essential propagator of inflammation and potential therapeutic target in pneumococcal meningitis.

Keywords. central nervous system infection; danger-associated molecular pattern; innate immune response; resolution of inflammation; calprotectin; S100A8/S100A9.
a nonclassical pathway [7]. Moreover, MRP8/MRP14 can be released from necrotic cells or within neutrophil extracellular traps [8]. Extracellular MRP8/MRP14 can bind to pattern-recognition receptors (PRRs), such as Toll-like receptor 4 (TLR4) [9–11]. Interaction of MRP8/MRP14 with PRRs leads to the production of proinflammatory cytokines [9]. In vitro, MRP8/MRP14 was also reported to be chemotactic for neutrophils [12], to stimulate neutrophil adhesion and migration [13], to increase the bactericidal activity of neutrophils by enhancing phagocytosis [14], and to have direct antimicrobial activity against pathogens [15]. Further support for a proinflammatory activity of MRP8/MRP14 came from in vivo experiments that used mice with a targeted deletion of the gene encoding MRP14 (ie, S100a9), which results in concomitant loss of MRP14 and MRP8 [16, 17]. In a murine model of abdominal sepsis, MRP14 deficiency was associated with diminished bacterial outgrowth and decreased systemic inflammation, resulting in reduced lethality [9, 18]. Mice lacking MRP14 also showed an attenuated neutrophil infiltration in an acute pancreatitis model and in different models of vascular injury [19, 20]. Apart from their immunomodulatory properties, extracellular MRP8/MRP14 can also increase endothelial permeability and induce endothelial cell death [21].

Since MRP8/MRP14 is abundantly present in neutrophils, neutrophils are the predominant cells in the cerebrospinal fluid (CSF) during pneumococcal meningitis and undergo necrosis upon challenge with Streptococcus pneumoniae [22], we hypothesized that MRP14 is released into the CSF and modulates the disease course in experimental meningitis. In this study, we determined the release pattern and mechanism of MRP14 and assessed its functional significance by using a mouse meningitis model, murine neutrophils, and CSF samples from patients with pneumococcal meningitis.

**MATERIALS AND METHODS**

**Ethics Statement**

This study was performed in accordance with the recommendations in the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The study protocol was approved by the local committee on the ethics of animal experiments (permit number 55.2-1-54-2531-31-09).

**Human CSF Samples**

CSF samples were obtained from 20 adult patients with culture-proven pneumococcal meningitis and from 10 age- and sex-matched control patients with noninflammatory neurological disorders (3 with migraine, 2 with headaches of other etiology, and 5 with idiopathic facial palsy). In all patients, lumbar puncture was performed for diagnostic purposes. The use of the samples was approved by the ethic committees of the University of Munich and the University of Copenhagen.

**Animal Model of Pneumococcal Meningitis**

A well-characterized mouse model of pneumococcal meningitis was used in this study [23, 24]. Briefly, adult male C57BL/6 or MRP14-deficient mice (backcrossed >10 times to the C57BL6 background) aged 8–16 weeks were clinically examined and scored. Thereafter, meningitis was induced by intracisternal injection of 10⁶ colony-forming units S. pneumoniae type 2 (D39 strain) under short-term anesthesia induced by isoflurane. All animals that were studied for >24 hours after infection received antibiotic therapy with ceftriaxone, starting 24 hours after infection. At the end of each experiment, animals were clinically evaluated. Then, mice were anesthetized with ketamine/xylazine, and a catheter was placed into the cisterna magna. CSF samples were withdrawn for the determination of CSF leukocyte counts and MRP8/MRP14 levels. Intracranial pressure was measured. Subsequently, blood samples were obtained by transcardial puncture for the assessment of bacterial titers and total leukocyte counts. After deep anesthesia induced by thiopental, mice were perfused with ice-cold phosphate-buffered saline containing heparin. Brains were removed and frozen immediately.

**Determination of Bacterial Titers in Blood and Brain**

Cerebella were dissected and homogenized in sterile saline. Blood samples and cerebellar homogenates were diluted serially in sterile saline, plated on blood agar plates, and cultured for 24 hours.

**Analysis of the Blood-Brain Barrier Integrity**

Frozen mouse brain extracts were examined for diffusion of albumin, using an enzyme-linked immunosorbent assay (ELISA) [25].

**Assessment of Hydrocephalus and Intracerebral Hemorrhages**

Mice brains were cut in a frontal plane into 10-μm thick sections. Beginning from the anterior parts of the lateral ventricles, 9 serial sections were photographed with a digital camera in 0.3-mm intervals throughout the ventricle system. The brain and ventricle areas were measured (ImageTool; University of Texas Health Science Center at San Antonio, Texas), and their volumes were estimated. Hemorrhagic spots were counted, and the bleeding area was measured as described previously [24].

**Detection of Apoptotic Cell Death**

Apoptosis was detected using a commercial in situ histochemical assay based on DNA fragment end labeling (FragEL; Klenow FragEL DNA fragmentation detection kit; Calbiochem, Darmstadt, Germany) as described previously [26]. FragEL-positive cells in the ventricular infiltrate and in the hippocampus were counted and expressed as number of FragEL-positive cells per 100 infiltrated leukocytes and as the number of FragEL-positive cells per brain section, respectively.
Immunohistochemical Detection of MRP14 and MRP8

Ten-micrometer-thick coronal brain sections containing the lateral ventricles and hippocampal tissue were stained either with an affinity-purified rabbit antiserum directed against MRP14 or MRP8 [27].

Purification of MRP14 and MRP8

Murine MRP14 and MRP8 were purified in a manner identical to that described earlier for human MRP14 [28].

Measurement of CXCL1, CXCL2, Granulocyte Colony-Stimulating Factor (G-CSF), and MRP8/MRP14 Concentrations

Mouse brain concentrations of CXCL1, CXCL2, and G-CSF were assessed by ELISA (R&D Systems, Wiesbaden-Nordenstadt, Germany) according to the manufacturer’s instructions [24]. MRP8/MRP14 concentrations were determined in CSF and serum samples, as well as in neutrophil and macrophage supernatants, by immunoblotting and ELISA as described previously [9].

Cell Culture Experiments

Bone marrow-derived neutrophils (BMNs) were isolated as described previously [29]. Bone marrow-derived macrophages (BMDMs) were prepared from bone marrow cells obtained from femurs [30].

Determination of Lactate Dehydrogenase (LDH) Activity

LDH activity was determined in centrifuged cell culture supernatants, centrifuged supernatants of control cells after lysis with Triton X-100 (positive control), and control medium (negative control), using a colorimetric assay kit (Biocat, Heidelberg, Germany). Cytotoxicity was calculated as the percentage of LDH activity was determined in centrifuged cell culture supernatants, centrifuged supernatants of control cells after lysis with Triton X-100 (positive control), and control medium (negative control).

Western Blot Analysis of MRP14 and MRP8

Ten-microliter cell culture supernatant or 3 µL of CSF were diluted in sample buffer, heated, loaded on a 4%–12% NuPage Tris-Bis gel (Life Technologies, Darmstadt, Germany), and subjected to electrophoresis. After transfer of proteins on polyvinylidene fluoride membranes (Merck Millipore, Darmstadt, Germany), membranes were incubated with an affinity-purified rabbit antiserum monospecific for MRP14 or MRP8 (1:2000 dilution) for 18 hours at 4°C. Bound primary antibodies were detected using a peroxidase-conjugated antibody against rabbit immunoglobulin G and the FemtoMax chemiluminescence substrate kit (Rockland, Gilbertsville, Pennsylvania). Blots were visualized and digitalized using a Doc-It LS Image analysis system (UVP, Upland, California).

Statistical Analysis

The principal statistical test was 1-way analysis of variance, followed by Bonferroni post hoc testing. Differences were considered statistically significant at P values of <.05. Data are displayed as means ± standard deviation (SD).

RESULTS

MRP8/MRP14 Release Into the CSF During Pneumococcal Meningitis

Using ELISA, we found high concentrations of MRP8/MRP14 in CSF samples from patients with pneumococcal meningitis but not in those from controls. Western blotting using antibodies specific for MRP14 or MRP8 confirmed the ELISA results (Figure 1A–C). Similar to observations in humans, MRP8/MRP14 were found in CSF samples obtained from mice with pneumococcal meningitis. Of note, CSF MRP8/MRP14 concentrations were markedly higher at 18 hours, 24 hours, and 48 hours after infection than at 6 hours after infection, pointing at an eventual role of these proteins in advanced stages of meningitis (Figure 1D and 1E). Immunohistochemical staining of brains obtained from mice with pneumococcal meningitis revealed the presence of MRP14 (and MRP8; Supplementary Figure 1) within the inflammatory mass in the subarachnoid space (Figure 1F–I). To verify the role of leukocytes as a major source of MRP14 in pneumococcal meningitis, we next depleted mice of granulocytes by pretreatment with anti-GR1 antibodies and determined CSF MRP8/MRP14 levels 24 hours after disease induction. Anti-GR1 pretreatment resulted in reduction of CSF granulocyte counts by >90% (mean level, [±SD], 1149 ± 469 cells/µL, compared with 11 872 ± 2603 cells/µL in control antibody–treated mice). This was paralleled by a significant reduction in CSF MRP8/MRP14 levels (Figure 1f). Immunocytochemical staining of CSF cytospins revealed a heterogenous staining pattern for MRP14, ranging from strong staining to an absence of staining (Figure 1K and 1L), suggesting MRP14 liberation from immigrated leukocytes.

To further characterize MRP14 release during pneumococcal infection, we next challenged murine BMNs with live S. pneumoniae. Infection of BMNs with pneumococci led to a significant increase in extracellular MRP14 levels. Accordingly, immunocytochemical staining of BMNs demonstrated loss of

Myeloid-Related Protein 14 in Meningitis • JID 2015:212 (15 July) • 249
staining for MRP14 upon pneumococcal challenge. The release of MRP14 was paralleled by a loss of cell viability, as judged by an elevation of extracellular LDH levels (Figure 2A–C).

**MRP14 Potently Drives Inflammation During Antimicrobial Therapy**

To test the functional significance of MRP14 in pneumococcal meningitis, MRP14-deficient mice and wild-type mice were infected intracisternally with *S. pneumoniae* and examined 24 hours afterward. MRP14 deficiency did not affect the development of meningitis: 24 hours after infection, MRP14-deficient mice showed signs of the disease that were identical to those of wild-type mice (Supplementary Table 1). Compatible with the lack of effect on the clinical status, there were no between-group differences in the degree of intracranial complications, the numbers of CSF leukocytes, as well as pneumococcal titers in the brain and blood. Only the brain CXCL2 concentrations were slightly, albeit significantly lower in MRP14-deficient mice than in wild-type mice. Different results were obtained when MRP14-deficient mice were evaluated at more-advanced stages of the disease (ie, 48 hours after infection). In this series of experiments, mice were treated with ceftriaxone as rescue therapy from overwhelming infection and associated death. MRP14 deficiency did not modulate the killing of *S. pneumoniae* by ceftriaxone (mean levels ± SD, 3.0 ± 1.3 log10 CFU/cerebellum and 2.9 ± 1.2 log10 CFU/cerebellum in MRP14-deficient mice and wild-type mice, respectively). However, compared with wild-type mice, MRP14-deficient mice had significantly lower CSF leukocyte counts (Figure 3A). The less pronounced CSF pleocytosis was paralleled by an 80% reduction of brain levels

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**Figure 1.** Expression and release of myeloid-related protein 8 (MRP8)–MRP14 complex (MRP8/MRP14) in pneumococcal meningitis. MRP8/MRP14 levels, as determined by enzyme-linked immunosorbent assay (A, E, and J) and Western blot analysis (B–D) in cerebrospinal fluid (CSF) samples obtained from two representative patients with noninflammatory neurological disorders (NINDs; 1 and 2) and 2 with pneumococcal meningitis (PM; 3 and 4; A–C), as well as from wild-type or MRP14-deficient (knockout [KO]) mice subjected to pneumococcal meningitis (D and E). Values on the x-axis indicate hours after infection. *P < .05, compared to patients with NIND, using unpaired Student t test. **P < .05, compared to CSF samples obtained before or 6 hours after infection, using 1-way analysis of variance and the Bonferroni test for post hoc analysis. F–I, Immunohistochemical analysis of murine brain sections with antibodies to MRP14 for an uninfected control mouse (F) and wild-type mice 6 hours (G), 24 hours (H), and 48 hours (I) after induction of meningitis. Black and open arrows point to MRP14-positive leukocytes in the subarachnoid space and superficial cortex, respectively. The scale bar indicates 100 µm. J, CSF samples from infected mice treated with neutrophil-depleting monoclonal antibody (Ab; anti-Gr1) or isotype control Abs (isotype). ***P < .05, compared to isotype control Ab–treated mice, by an unpaired Student t test. K and L, Immunocytochemical analysis of CSF cytospins with antibodies to MRP14 (red; green, nucleus), revealing granulocytes showing patchy, minimal, or no immunostaining for MRP14 (mean percentage of granulocytes ± SD, 34.5% ± 6.4%; a minimum of 100 cells on cytospins from 3 mice subjected to meningitis for 24 hours were scored; L) and a granulocyte with an intensely stained cytoplasm (normal appearance; L). Abbreviation: SD, standard deviation.
of CXCL2 (but not of CXCL1 and G-CSF); serum G-CSF concentrations were even higher in MRP14-deficient mice than in wild-type mice (mean levels [±SD], 15 272 ± 11 533 pg/mL vs 4030 ± 1982 pg/mL; \( P = .013 \)). The reduction in meningial inflammation was associated with a significant amelioration of disease severity, as evidenced by lower losses of body weight (data not shown) and lower clinical scores (Figure 3B–E). The alleviation of disease was accompanied by lower intracranial pressure, regression of meningitis-induced hydrocephalus, and fewer FragEL-positive apoptotic neurons in the hippocampus (Figure 3F–I). These data suggest a major role of MRP14 in sustaining leukocyte infiltration in antibiotic-treated meningitis, thereby possibly contributing to persisting hydrocephalus by interference with normal CSF flow. This suggestion is supported by our observation that CSF leukocyte counts correlated positively with ventricle volumes (\( r^2 = 0.472; \ P = .002 \)).

Extracellular MRP14 Promotes Leukocyte Infiltration by Enhancing CXCL2 Production

MRP14 is a multifunctional protein, operating intracellularly and extracellularly [6]. Our finding that MRP14 is released in large quantities into the CSF pointed toward an extracellular function in meningitis. To evaluate this possibility, we performed reconstitution experiments, using purified MRP14. MRP14-deficient mice were injected intracisternally prior to infection with MRP14 in a dose equivalent to that found in infected wild-type mice and were evaluated 48 hours afterward. Reconstitution with MRP14 almost completely reverted the phenotype of MRP14-deficient mice back to wild type. This effect was abolished by heat inactivation of MRP14 (Figure 4A–C). Extracellular MRP14 can modulate inflammatory responses in multiple ways [9, 12, 31, 32]. Our observation that MRP14 deficiency affected neither pneumococcal outgrowth in CSF and blood nor ceftriaxone-induced bacterial killing argues against an antimicrobial activity of MRP14 against S. pneumoniae in our model system. To rule out the possibility that the persistence of inflammation is related to MRP14-mediated inhibition of leukocyte apoptosis, we performed FragEL assays on cryosections from mouse brains. At 48 hours after infection, the mean percentage (±SD) of FragEL-positive leukocytes in the ventricular infiltrate of wild-type mice was 8.6% ± 2.6%. A similar frequency of apoptotic cells (9.3% ± 1.8%) was detected in leukocyte infiltrates from MRP14-deficient mice. Thus, the lower CSF leukocyte numbers in MRP14-deficient mice are unlikely due to enhanced leukocyte apoptosis. We next tried to clarify whether MRP14 may propagate leukocyte recruitment directly, by acting as a chemoattractant, or indirectly, by stimulating chemokine production. We injected purified MRP14 (and MRP8; Supplementary Figure 2) into the CSF of mice and determined CSF leukocyte counts 6 hours later. This led to a significant increase in CSF leukocyte counts, compared with counts in mice that received phosphate-buffered saline or heat-inactivated MRP14 (Figure 5). To elucidate the mechanism underlying MRP14-induced leukocyte infiltration, we further administered MRP14 to mice treated with either blocking antibodies to murine CXCL2 or TLR4, as well as to TLR4-deficient mice, because TLR4 is a putative PRR for MRP14, and MRP14 deficiency was accompanied by reduced CXCL2 production in our model. MRP14 inoculation led to significantly lower CSF leukocyte counts in anti-CXCL2–treated mice, anti-TLR4–treated mice, and TLR4-deficient mice, compared with respective controls. We next assessed the influence of MRPs on CXCL2 production in vitro. MRP14 (and also MRP8; Supplementary Figure 2) was able to induce CXCL2 expression in murine macrophages in a dose-dependent manner. This effect could be abolished by anti-TLR4 treatment or TLR4 deficiency (Figure 6A). A similar, even more pronounced release of CXCL2 was observed when macrophages were challenged with pneumococci that were freshly killed (by exposure to antibiotic treatment for 2
Figure 3. Myeloid-related protein 14 (MRP14) deficiency is protective in antibiotic-treated meningitis. Pneumococcal meningitis was induced by intracisternal injection of *Streptococcus pneumoniae*. Twenty-four hours later, mice were treated with ceftriaxone (10 mice per group). Mice that were injected with phosphate-buffered saline into the cisterna magna served as negative controls (n = 4). Twenty-four hours after the start of antibiotic therapy (48 hours after infection), animals were evaluated. A, MRP14-deficient mice showed significantly lower white blood cell (WBC) counts in the cerebrospinal fluid (CSF) than infected wild-type mice. B–I, The reduction in CSF pleocytosis was associated with a significantly lower concentration of CXCL2 (B, but not CXCL1 [C] and granulocyte colony-stimulating factor [G-CSF, D]) in the brain and with significant amelioration of brain pathology and disease severity, as evidenced by lower clinical scores (E), lower intracranial pressures (ICPs; F), smaller ventricle volumes (G and H), and fewer DNA fragment end labeling (FragEL)-positive cells in the dentate gyrus (I). J and K, There were no between-group differences in brain albumin concentrations (J) and numbers of cerebral hemorrhages (K). G, Representative brain sections from 3 randomly selected mice per group. Dotted lines indicate lateral ventricles. Arrowheads point to cerebral hemorrhages. Data are given as means ± SD. *P < .05, compared to uninfected wild-type mice; and **P < .05, compared to infected wild-type mice, using analysis of variance and the Bonferroni test for post hoc analysis. Abbreviation: SD, standard deviation.
hours; Figure 6B). This stimulatory activity was nearly completely lost when pneumococci were incubated with antibiotics for 20 hours. Coadministration of MRP14 with this pneumococcal preparation resulted in 3.4-fold and 7.5-fold increases in CXCL2 production, compared with macrophages exposed to bacteria or MRP14 alone, respectively, arguing for a synergistic effect of MRP14 with dead pneumococci on chemokine release.

MRP14 May Represent a Target for Adjunctive Therapy in Pneumococcal Meningitis

Since MRP14 deficiency was protective in our meningitis model, we wondered whether antagonizing MRP14 activity might be a promising strategy for adjunctive therapy. Recently, paquinimod (ABR-215757) was reported to inhibit MRP14 interactions with TLR4 and RAGE [10]. These interactions showed all of the features of highly specific interactions. With regard to off-target effects, it is very hard to exclude such effects. However, to date, no additional molecular targets for paquinimod have been identified.

To test its therapeutic potential, we infected wild-type mice with S. pneumoniae and treated them with ceftriaxone either in combination with paquinimod or its respective vehicle. Treatment with paquinimod did not modulate the killing of S. pneumoniae by ceftriaxone (data not shown), but it significantly attenuated CSF pleocytosis and brain CXCL2 levels (Figure 7A and 7B). This antiinflammatory effect was associated with a significant amelioration of disease severity, as evidenced by lower intracranial pressure, less hydrocephalus, less hypothermia (data not shown), and lower clinical scores (Figure 7C–E).

Figure 4. Extracellular myeloid-related protein 14 (MRP14) is responsible for its activity in pneumococcal meningitis. MRP14-deficient mice were injected intracisternally with MRP14 (10 µg/mouse; n = 6) 1 hour prior to infection and evaluated 48 hours afterward. Control mice received an injection of MRP14 inactivated by exposure to 80°C for 30 minutes (hiMRP14; n = 6). Intracisternal reconstitution with MRP14 reverted the phenotype of MRP14-deficient mice back to wild type, as evidenced by quite similar clinical scores (A), intracranial pressures (ICPs; B), and white blood cell (WBC) counts in the cerebrospinal fluid (CSF; C). The left 2 bars are the same as those in Figure 3A, 3E, and 3F. *P < .05, compared to hiMRP14-injected MRP14-deficient mice, using the unpaired Student t test.

Figure 5. Myeloid-related protein 14 (MRP14) induces cerebrospinal fluid (CSF) pleocytosis. To investigate the inflammatory activity of MRP14 in vivo, 1 µg of MRP14 was injected into the CSF via the cisterna magna (n = 6 mice per group). Six hours later, the number of white blood cells (WBCs) in CSF was determined. All experiments but 1 were conducted on C57BL/6 mice; the exception involved Toll-like receptor 4 (TLR4)-deficient mice (TLR4<sup>−/−</sup>; backcrossed 10 times onto the B6 background). MRP14 in TLR4<sup>−/−</sup> = MRP14 was injected in TLR4-deficient mice. Data are given as means ± SD. *P < .05, compared to heat-inactivated MRP14 (hiMRP14); and **P < .05, compared to mice injected with MRP14, irrespective of whether mice received isotype control monoclonal antibody (immunoglobulin G2b [IgG2b]) or not, by analysis of variance and the Bonferroni test for post hoc analysis. Abbreviations: PBS, phosphate-buffered saline; SD, standard deviation.
DISCUSSION

In this study, we demonstrated that MRP14 is released in large quantities into the CSF of patients and mice with pneumococcal meningitis, resulting in aggravation of disease severity.

Up to now, nothing was known about the role of released MRP14 in acute central nervous system disorders. Here, we showed that MRP14 (and MRP8/MRP14) is detectable in very high concentrations (>100 µg/mL) in the CSF during pneumococcal meningitis. Our finding of high MRP14 (and MRP8/MRP14) levels at the primary site of infection is consistent with previous observations in patients and/or animal models of Klebsiella pneumonia [33], Escherichia coli peritonitis [18], and pneumococcal pneumonia [34]. In these infections, the elevations in local MRP8/MRP14 levels have been explained by active nonclassical secretion of these proteins from activated resident macrophages and/or immigrated monocytes and granulocytes [9, 18]. Our immunohistochemical investigations suggested that granulocytes, which account for the vast majority of cells within the leukocytic infiltrate [35], are the primary source of MRP14. This idea is supported by the results of our granulocyte depletion experiments, in which granulopenic mice displayed markedly lower MRp8/MRP14 levels in CSF specimens, compared with control animals. Noteworthy, the anti-GR1 antibody we used can also cause a loss of other GR1-expressing cells, particularly GR1-positive monocytes [36]. In this study, we assessed total CSF leukocyte counts but not the numbers of monocytes or their subsets. The difference in CSF nongranulocyte numbers between control antibody–treated and anti-GR1–treated mice (1180 cells/µL and 490 cells/µL, respectively) was suggestive for a side effect of the anti-GR1 antibody on leukocyte subsets other than granulocytes. This side effect, however, may have only modest influence on our MRP8/MRP14 measurement results, because in CSF specimens from mice subjected to pneumococcal meningitis, nongranulocytes are present at much lower numbers than granulocytes [35] and because nongranulocytes contain no or considerably less MRP14 than granulocytes [37].
Once released into the extracellular space, MRP14 has been proposed to act like a DAMP, exerting proinflammatory effects. In this study, we found that MRP14 deficiency did not modulate the induction but did modulate the progress of inflammation during antibiotic therapy. The functional importance of MRP14 in established meningitis is consistent with the time course of its release. High quantities of MRP14 were not detected until ≥18 hours after infection. Along with previous studies that demonstrated pneumococcal PAMPs as sufficient to induce meningitis [38], these findings support the following scenario of immune regulation in meningitis. Once in the CSF, S. pneumoniae grows easily owing to local immunodeficiency [3]. Pneumococcal outgrowth results in CSF acidosis, which causes some bacteria to undergo autolysis [39], triggering the liberation of PAMPs into the CSF. Their presence is sensed by host PRRs such as TLR2, ultimately leading to the recruitment of neutrophils into the CSF [23]. The neutrophils may not be able to limit pneumococcal outgrowth, allowing bacterial titers to reach levels that cause damage to host cells, including neutrophils themselves [22]. Damaged neutrophils release MRP14 into the CSF, where it can act as a contributing driving force of inflammation. This activity seems to be particularly important after the start of antibiotic therapy, which results in rapid reduction of PAMP concentrations in the CSF [4]. In this situation, MRP14 may overtake the role of PAMPs, thereby counteracting resolution of inflammation.

Extracellular MRP14 can modulate inflammatory responses in multiple ways. MRP8/MRP14 was reported to possess antimicrobial activity against diverse bacteria under in vitro conditions [15, 32]. Accordingly, MRP14-deficient mice showed higher bacterial loads after intravenous Staphylococcus aureus injection [32] and intranasal or intravenous Klebsiella pneumoniae infection [33]. Contrarily, MRP14 deficiency was associated with reduced bacterial outgrowth in experimental murine E. coli sepsis [18] and S. pneumoniae pneumonia models [40]. In our pneumococcal meningitis model, we did not detect any differences in brain bacterial titers between wild-type and MRP14-deficient mice. Combined, the antimicrobial activity of MRP14 in vivo seems to depend on the bacterial strain and the setting in which MRP14 challenges the bacterial strain.

Another mechanism by which MRP14 can contribute to the persistence of inflammation is by facilitating the recruitment of leukocytes to the site of infection. Previous studies reported a direct chemotactic activity of MRP14 on neutrophils in vitro and in vivo [12]. MRP14 can also indirectly mediate leukocyte infiltration into inflamed tissue through the induction of cytokine and chemokine production. This effect requires interaction with TLR4 [9]. In line with this, MRP14 deficiency was associated with attenuated leukocyte infiltrates and reduced cytokine/chemokine concentrations in mouse models of sepsis and vascular injury [18, 20]. Moreover, MRP14 has been reported to modulate neutrophil infiltration into lungs by increasing circulating neutrophil numbers via G-CSF production [41]. In our study, the reduction of CSF pleocytosis in MRP14-deficient mice was accompanied by a decrease in brain CXCL2, suggesting that CXCL2 is a mediator of MRP14 inflammatory activity.

**Figure 7.** Adjuvant treatment with paquinimod is protective in pneumococcal meningitis. Twenty-four hours after pneumococcal induction, mice were treated with a combination of ceftriaxone (100 mg/kg) and paquinimod (10 mg/kg; n = 9) or its vehicle (phosphate-buffered saline containing 25% dimethyl sulfoxide; n = 9). Twenty-four hours after the start of treatment, animals were evaluated. Adjuvant therapy with paquinimod led to a reduction in cerebrospinal fluid (CSF) pleocytosis (A) and brain CXCL2 concentrations (B), as well as an amelioration of brain pathology and disease severity, as evidenced by lower intracranial pressures (ICPs; C), less hydrocephalus (D), and lower clinical scores (E). Data are given as means ± SD. *P < .05, compared to vehicle-treated mice, using the unpaired Student t test. Abbreviations: SD, standard deviation; WBC, white blood cell.
Injection of CXCL2 into the CSF induced meningitis [42]. Additionally, we previously showed that CXCL2 neutralization attenuates neutrophil recruitment in pneumococcal meningitis [24]. A potential role of CXCL2 in MRPI4-dependent leukocyte infiltration into the CSF is further supported by our observations that macrophages released large amounts of CXCL2 upon exposure to MRPI4 and that intracisternal injection of MRPI4 induced CSF pleocytosis in a CXCL2-dependent manner.

In conclusion, the present study demonstrated that, in pneumococcal meningitis, neutrophil-derived MRPI4 can counteract the resolution of inflammation after the start of antibiotic therapy, thereby exacerbating disease severity. Pharmacological interference with MRPI4 activity ameliorated disease outcome, indicating a potential strategy for adjuvant therapy.

Supplementary Data

Supplementary materials are available at The Journal of Infectious Diseases online (http://jid.oxfordjournals.org). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary materials are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

Notes

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