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*J Immunol* published online 14 October 2011
http://www.jimmunol.org/content/early/2011/10/14/jimmunol.1100790

Supplementary Material

http://www.jimmunol.org/content/suppl/2011/10/14/jimmunol.1100790.DC1

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The NLRP3 Inflammasome Contributes to Brain Injury in Pneumococcal Meningitis and Is Activated through ATP-Dependent Lysosomal Cathepsin B Release

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Streptococcus pneumoniae meningitis causes brain damage through inflammation-related pathways whose identity and mechanisms of action are yet unclear. We previously identified caspase-1, which activates precursor IL-1β type cytokines, as a central mediator of inflammation in pneumococcal meningitis. In this study, we demonstrate that lack of the inflammasome components ASC or NLRP3 that are centrally involved in caspase-1 activation decreases scores of clinical and histological disease severity as well as brain inflammation in murine pneumococcal meningitis. Using specific inhibitors (anakinra and rIL-18–binding protein), we further show that ASC- and NLRP3-dependent pathologic alterations are solely related to secretion of both IL-1β and IL-18. Moreover, using differentiated human THP-1 cells, we demonstrate that the pneumococcal pore-forming toxin pneumolysin is a key inducer of IL-1β expression and inflammasome activation upon pneumococcal challenge. The latter depends on the release of ATP, lysosomal destabilization (but not disruption), and cathepsin B activation. The in vivo importance of this pathway is supported by our observation that the lack of pneumolysin and cathepsin B inhibition is associated with a better clinical course and less brain inflammation in murine pneumococcal meningitis. Collectively, our study indicates a central role of the NLRP3 inflammasome in the pathology of pneumococcal meningitis. Thus, interference with inflammasome activation might be a promising target for adjunctive therapy of this disease. The Journal of Immunology, 2011, 187: 000–000.

S. pneumoniae is a leading cause of pneumonia, bacteremia, and meningitis and is responsible for >1.5 million deaths each year worldwide. Meningitis has the worst prognosis of any pneumococcal diseases, with a mortality rate of 15–30%. Moreover, up to half of the survivors are left with long-term sequelae (1). The poor prognosis of meningitis is related to weaknesses of the host’s immune system inside the cerebrospinal fluid (CSF) space, which includes the absence of soluble pattern recognition receptors and the presence of immunosuppressive factors such as TGF-β (2). As a consequence, once having entered the CSF, S. pneumoniae multiplies easily, reaching similar high titers as under bacterial culture conditions (3). As a result of the unrestrained pneumococcal proliferation and autolysis, large quantities of subcapsular bacterial components are released into the CSF (4). Their presence is recognized by resident immunocompetent cells by means of surface and intracellular pattern recognition receptors such as TLR2 and TLR4 (5). TLR activation results in MyD88-dependent production of high levels of cytokines and chemokines (6), which facilitates the accumulation of large amounts of blood-borne leukocytes (predominantly neutrophils) inside the CSF. The resultant excessive neutrophilic inflammation causes collateral damage to the brain, thus contributing substantially to the unfavorable outcome of meningitis (7, 8).

Among the cytokines that induce and perpetuate meningeval inflammation are IL-1 cytokine family members. Concentrations of IL-1β and IL-18 are elevated in CSF samples from patients with bacterial meningitis. Moreover, CSF IL-1β (but not IL-18) levels correlate significantly with CSF leukocyte counts and clinical outcome (9, 10). In rats and rabbits, intracisternal application of rIL-1β was sufficient to induce meningitis (11, 12) and neutralizing Abs directed against IL-1β prevented meningeval inflammation after intracisternal inoculation with S. pneumoniae (13). In line with the latter finding, mice lacking IL-1R1 showed less profound inflammatory infiltrates around the meninges and lower amounts of brain cytokines and chemokines as compared with wild-type mice in a model of hematogenous pneumococcal meningitis (14). Consistently, our group demonstrated that mice lacking caspase-1, which activates pro–IL-1 type cytokines, showed a strongly diminished inflammatory host response to pneumococci in the CSF (15). Accordingly, Braun et al. (16) reported that the pan-caspase inhibitor z-VAD-fmk prevented hippocampal injury...
and leukocyte influx into the CSF compartment of rabbits with pneumococcal meningitis. These data of others and us indicate a key role of the IL-1/caspase-1 pathway in pneumococcal meningitis. However, the molecular mechanisms through which IL-1 is produced during pneumococcal meningitis are still not resolved. In general, IL-1β is produced in a two-step process that first involves generation of the biologically inactive precursor pro–IL-1β, typically in response to TLR activation (17, 18). In a second step, pro–IL-1β is then cleaved by caspase-1 (or further proteases such as neutrophil-derived serine proteases) into an active cytokine and secreted. Activation of caspase-1 is controlled by a large multiprotein complex called the inflammasome. The inflammasome contains a nucleotide-binding domain and leucine-rich repeat containing gene product family receptor (NLR) protein (such as NLRP3) and an adaptor protein called apoptosis-associated speck-like protein (ASC), which links the NLR protein to the proform of caspase-1 (17, 18).

In this study, we analyzed inflammation upon pneumococcal infection by applying a murine meningitis model and differentiated human monocytoid cells, and we identified the NLRP3 inflammasome as central driver of \textit{S. pneumoniae}-induced brain pathology.

Materials and Methods

Ethics statement

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, National Research Council) and with the German Animal Protection Act. The study protocol was approved by the Committee on the Ethics of Animal Experiments of the Government of Upper Bavaria (permit nos. 55.2-1-54-2531-32-04 and 55.2-1-54-2531-47-08). All surgery was performed under ketamine/xylazine anesthesia.

Mouse meningitis model

The model used in this study has been described previously (5, 8). Briefly, mice were weighed and clinically examined. The clinical score used consists of: presence of tremor, piloerection, and seizures; spontaneous motor activity; proprioceptive postural reflex test; and blood agar plates as well as blood bacterial titers. After deep anesthesia with ketamine, animals were perfused transcardially with 15 ml ice-cold PBS containing 10 U/ml heparin. The brain was removed and frozen immediately.

Experimental groups

To analyze the role of the NLRP3 inflammasome, ASC-deficient mice (\textit{n} = 10; ASC–/–; provided by Prof. V.M. Dixit, San Francisco, CA) and NLRP3-deficient mice (\textit{n} = 10; NLRP3–/–; a gift from Prof. J. Tschopp, Lausanne, Switzerland) were infected with \textit{S. pneumoniae} serotype 2 strain D39 and compared with infected wild-type (WT) mice (\textit{n} = 12; C57BL/6). Because receptor-interacting protein (RIP)2 was reported to compete with ASC binding to caspase-1 (20) and contribute to NOD1 and NOD2 signaling, we also studied mice lacking RIP2 (\textit{n} = 10; RIP2–/–; provided by Prof. V.M. Dixit). For the evaluation of the role of IL-1 family cytokines in the immunopathogenesis of pneumococcal meningitis, WT mice were treated either with the IL-1 receptor antagonist anakinra (\textit{n} = 6; from Amgen; 100 mg/kg given i.p. prior to infection) (21) or with anakinra in combination with mouse IL-18–binding protein (mIL-18BP; \textit{n} = 6; from Sino Biological; 5 mg/kg given i.p. prior to infection) (22); controls received 0.5 ml and 1.0 ml PBS i.p., respectively (\textit{n} = 6 in each group). Additionally, we evaluated the role of cathepsin B in \textit{S. pneumoniae}-induced meningeal inflammation. In these series of experiments, WT mice were treated with 5 mg/kg Ca-074Me (diluted in 5% DMSO-containing PBS) (23) or DMSO-PBS (given i.p. immediately before and 6 h postinfection; \textit{n} = 7 in each group). The influence of pneumolysin on the clinical course was assessed by infecting WT and ASC-deficient mice either with the pneumolysin-deficient strain D39ΔPly (\textit{n} = 6 and \textit{n} = 4, respectively) or with the WT D39 strain (\textit{n} = 6, respectively). Finally, the role of granulocytes in pneumococcal-infection inflammation was investigated by rendering mice neutropenic with anti-GR1 Abs prior to infection (\textit{n} = 6) (8).

Determination of bacterial titters 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 h at 37°C under 5% CO2.

Analysis of the blood–brain barrier integrity

For the determination of the blood–brain barrier (BBB) integrity, frozen mouse brain extracts were examined for diffusion of albumin using ELISA as described previously (6).

Analysis of cerebral bleeding

Mice brains were cut in a frontal plane into 10-μm-thick sections. Beginning from the anterior parts of the lateral ventricles, 10 serial sections were photographed with a digital camera in 0.3-mm intervals throughout the ventricular system. Hemorrhagic spots were counted and the bleeding area was measured (ImageTool; University of Texas Health Science Center at San Antonio, San Antonio, TX).

Assessment of brain pathology

For better comparison, the degree of BBB disruption and the number of cerebral hemorrhages were combined in a neuropathological score (neuropsychore). The degree of BBB disruption was scored as follows: 0, 1, and 2 points were given if the brain albumin concentration was <30 ng/μg, between 31 and 90 ng/μg, and >90 ng/μg brain protein, respectively. The number of hemorrhagic spots was scored as follows: a score of 0 indicated 0–1 cerebral bleeding spots, whereas scores of 1 and 2 indicated 2–12 and >12 cerebral bleeding spots per 10 investigated brain sections, respectively. The maximum neuropathological score was 4 and indicated severe brain injury, whereas a score of 0 stood for no pathological alterations.

Measurement of mouse brain IL-1β levels

Mouse brain concentrations of IL-1β were assessed by ELISA (R&D Systems), according to the manufacturer’s instructions.

Cell culture experiments

Human THP-1 cells were maintained in RPMI 1640 supplemented with 10% heat-inactivated low-endotoxin FCS (PAA Laboratories) and 10 μg/ml penicillin/streptomycin. For experiments, cells were plated in 24-well plates (5 × 105 cells/well) and differentiated for 48 h with 100 nM PMA (Sigma-Aldrich) in RPMI 1640 supplemented with FCS. Then, the culture medium was replaced by RPMI 1640 supplemented with 10% normal human serum or 10% C5-depleted human serum (in selected experiments; both from TECOmedical), and cells were exposed to \textit{S. pneumoniae} serotype 2 strain D39 (106, 5 × 105, or 1 × 105 CFU/ml) in selected experiments, to its isogenic pneumolysin mutant D39ΔPly (105 CFU/ml or GFP-expressing strain D39 [105 CFU/ml]) for 24 h, 3, 6, or 18 h. In separate experiments, the following compounds were added to the culture medium: z-VAD-fmk (50 μM; BIO-CAT), Ca-074-Me (50 μM), bafilomycin A1 (250 mM), diphenylene iodonium (DPI, 10 μM; all from Merck Chemicals), cytochalasin D (1 μM), oxidized ATP (ox-ATP, 1 mM) and potassium chloride (65 mM; all from Sigma-Aldrich).

Bone marrow-derived macrophages (BMDMs; from WT and ASC-deficient mice) were differentiated from bone marrow cells isolated from the femur. Bones were flushed with HBSS and the cell suspension was forced through a 70-μm mesh. Collected cells were resuspended in complete macrophage medium containing DMEM, 50 ng/ml T-chemo, 10% FCS, 10 mM HEPES, 10 mM l-glutamine, and 10 μg/ml penicillin/streptomycin and cultured at 37°C in 5% CO2. After 7 d, virtually 100% of the cells expressed the macrophage marker CD11b. The culture medium was replaced with complete macrophage medium lacking penicillin/streptomycin. Again 24 h later, cells were exposed to \textit{S. pneumoniae} serotype 2 strain D39 (105 CFU/ml) or its isogenic pneumolysin mutant D39ΔPly (105 CFU/ml).
Determination of IL-1β and TNF-α in cell culture supernatants and lysates

IL-1β and TNF-α levels were measured in cell culture supernatants and cell lysates using a commercially available ELISA (R&D Systems) in accordance with the instructions of the manufacturer.

Western blot analysis of IL-1β and caspase-1

THP-1 cells were lysed in a hypotonic buffer containing 10 mM HEPES, 10 mM KCl, 1.5 mM MgCl₂, 2 mM DTT, and a protease and phosphatase inhibitor mixture (consisting of aprotinin, PMSF, leupeptin, pepstatin A). For IL-1β immunoblotting, lysates and precollected supernatants were further processed separately, whereas both samples were mixed and processed together for caspase-1 detection. To concentrate IL-1β and caspase-1, 15 μl StrataClean resin was added to 300 μl sample. The resin-bound proteins were recovered by centrifugation, washed with PBS, separated on a 4–12% NuPage Tris-Bis gel (Invitrogen), transferred to a polyvinylidene difluoride membrane (Millipore), and probed either with a rabbit polyclonal Ab to Casp1 p10 (sc-515, 1:500 dilution) or IL-1β (sc-7884, 1:1000 dilution; both from Santa Cruz Biotechnology). For all blots, bound primary Abs were detected using a peroxidase-conjugated Ab against rabbit IgG (1:2000 dilution; Sigma-Aldrich) and the FemtoMax supersensitive chemiluminescence substrate kit (Rockland Immunochemicals). Blots were visualized and digitalized using a Doc-ITLS image analysis system (UVP).

Immunocytochemical detection of IL-1β, caspase-1, LAMP-2, and cathepsin B

Differentially THP-1 cells adherent to round glass coverslips (2 × 10⁶ cells/well, 24-well plate) were fixed with 4% buffered paraformaldehyde and permeabilized with 0.1% Triton X-100. Then, cells were stained using rabbit anti–IL-1β (1:100), rabbit anti-caspase-1 (1:100, both from Santa Cruz Biotechnology), mouse anti–LAMP-2 (2 μg/ml; BD Pharmingen), or mouse anti-cathepsin B Abs (4 μg/ml; Merck Chemicals), followed by secondary anti-rabbit or anti-mouse Alexa Fluor 546-coupled Abs (Invitrogen) and a DAPI nucleic acid stain. Additionally, caspase-1–like activity was detected using the FAM FLICA caspase-1 kit following the manufacturer’s protocol (AbD Serotec). Pictures were recorded using a cooled, high-resolution Moticam 5000 CCD camera mounted on an Olympus B51 fluorescence microscope.

Labeling of lysosomes with LysoSensor Green DND-189

The evaluation of the stability of lysosomal membranes was performed by means of an acidotropic fluorescent probe LysoSensor Green DND-189, which accumulates in acidic organelles. Briefly, THP-1 cells were loaded with 1 μM LysoSensor Green DND-189 (Molecular Probes/Invitrogen) for 15 min. After replacing the loading medium with fresh medium, cells were monitored on a Leica DM IL inverted fluorescence microscope equipped with a cooled CCD camera (VarioCam; PCO Computer Optics).

Assessment of cellular caspase-1 and cathepsin B activity

Caspase-1 and cathepsin B activities were determined by measuring the cleavage of enzyme-specific, fluorogenic substrates in cell lysates using commercially available assay kits (BioVision).

Determination of ATP and lactate dehydrogenase

Release of ATP from cells into the supernatant was monitored using a biofluorescence assay kit (Molecular Probes). The lactate dehydrogenase (LDH) activity was determined in centrifuged culture supernatants (S) and in cell pellets (P) of THP-1 control wells after lysis with an equal volume of RPMI 1640 containing 0.1% Triton X-100, using a colorimetric assay kit (BioVision). The cytotoxicity was calculated as percentage LDH release by the ratio of P(S + P).

Statistical analysis

SYSTAT 9 (SPSS) was used for statistical analysis. The principal statistical test was an unpaired Student’s t test (combined with Bonferroni α adjustment in case of multiple comparisons) and the Mantel log-rank test for survival. Differences were considered significant at p < 0.05. Data are displayed as means ± SD.

Results

Amelioration of pneumococcal meningitis by ASC and NLRP3 deficiency

Previously, we demonstrated that depletion of caspase-1 improves clinical outcome of pneumococcal meningitis (15). To clarify the mechanisms underlying meningitis-associated caspase-1 activation, we first infected WT (C57BL/6n) and ASC−/− mice with high doses of S. pneumoniae D39 (10⁸ CFU/ml). More than 70% of WT mice (8 of 11 mice) died within 24 h postinfection whereas the death rate of ASC-deficient mice was merely 20% (2 of 10 mice; p = 0.017). We next inoculated mice with lower doses of S. pneumoniae (10⁷ CFU/ml). By 24 h postinfection, all WT mice developed clinical signs of infection, which manifested in an increased clinical score (Fig. 1A), loss of body weight, and hypothermia, but only 2 of 12 mice succumbed within the observation period. Compared to WT mice, ASC−/− mice developed less severe disease. This was reflected by lower clinical scores and a less pronounced loss of body weight and change of temperature (data not shown). Lethality of infected ASC−/− mice was 10% (1 of 10 mice).

Because intracranial complications are major determinants of an unfavorable clinical outcome in meningitis (25), we next investigated the impact of ASC deficiency on meningitis-associated brain pathology. Intrathecal infection with S. pneumoniae D39 significantly increased ICP in WT mice. At 24 h after pneumococcal inoculation, ASC-deficient mice had significantly lower ICP values than did infected WT mice (Fig. 1B). Additionally, BBB breaching and cerebral bleeding were less pronounced in brains of infected ASC−/− mice than in those of WT mice, as indicated by a significantly reduced neuropathologic score (Fig. 1C, 1D). The reduction in brain pathology correlated with an attenuated accumulation of neutrophils, major contributors to meningitis-associated brain damage, in the CSF of ASC−/− mice as compared with infected WT mice (Fig. 1E). In contrast, pneumococcal outgrowth within the brain and blood was not significantly altered in ASC−/− mice compared with WT mice (Fig. 1F, 1G).

Caspase-1 activation by bacterial muramyl dipeptide was reported to require the NLRs NOD2 and NLRP3, which recruit RIP2 and ASC upon their activation (26). Experimental work also demonstrated that 1) NOD2–RIP2 signaling contributes to pneumococci-induced cell activation (27), and 2) the pneumococcal toxin pneumolysin promotes caspase-1 activation in an NLRP3-dependent manner (28). We thus used mice lacking either NLRP3 or RIP2. Similar to ASC−/− mice, infected NLRP3−/− mice showed statistically significant amelioration of both disease severity and brain pathology, as evidenced by lower clinical scores and ICP values as well as less hemorrhagic spots and lower neuropathologic scores (Fig. 1A–D). The alleviation of disease was again associated with a reduction in CSF pleocytosis (Fig. 1E). In contrast to the NLRP3 deficiency, the lack of RIP2 had no impact on the clinical course, meningitis-associated brain pathology, and meningeal leukocyte infiltration (Fig. 1A–E). Neither the genetic depletion of RIP2 nor that of NLRP3 resulted in significant alterations of pneumococcal titers in the brain and the blood (Fig. 1F, 1G). Lethality of infected RIP2 and NLRP3−/− mice was 20% (2 of 10 mice) and 10% (1 of 10 mice). Our data assign a central role to the NLRP3 inflammasome, but not to NOD/RIP2 signaling, in the pathogenesis of pneumococcal meningitis.

Blockade of IL-1 family cytokine signaling is protective in pneumococcal meningitis

To determine the contribution of NLRP3 inflammasome-dependent IL-1 signaling to pneumococcal meningitis, infected WT mice were pretreated with the rIL-1R antagonist anakinra (shown to be
effective in mice; Refs. 21, 29) alone or in combination with mouse rIL-1BP (22). IL-1R blockade significantly attenuated the meningitis-induced rise in intracranial pressure as well as meningeal inflammation. However, the improvement of the clinical status and brain pathology was not significant (Fig. 2). The combination of anakinra with rIL-1BP resulted in a more pronounced reduction of CSF pleocytosis (by 64.5%, compared with 47.9% in anakinra-treated mice), which was also paralleled by a significant amelioration of the disease, as evidenced by significantly lower clinical and neuropathological scores (Fig. 2 C, D). As in mice lacking ASC or NLRP3 expression, mice receiving IL-1R competitor or the combination of the competitor with rIL-18BP showed nearly identical bacterial titers in the brain as compared with PBS-treated mice (data not shown). Thus, we conclude that the ASC<sup>−/−</sup> and NLRP3<sup>−/−</sup> phenotypes are related to impaired IL-1β and IL-18 signaling.

**Cathepsin B activity is required for S. pneumoniae–induced caspase activation and IL-1β release by differentiated THP-1 macrophages**

To further investigate S. pneumoniae-induced caspase-1 activation and IL-1β production, we performed experiments in differentiated THP-1, as macrophages are the predominant source of IL-1β in pneumococcal meningitis (30). First, we challenged the cells with increasing amounts of live S. pneumoniae D39. Infection with 10<sup>7</sup> CFU/ml (but not with 10<sup>6</sup> or 5 × 10<sup>5</sup> CFU/ml) pneumococci markedly elevated IL-1β concentrations in cell culture supernatants 6 h later (data not shown). Next, we characterized the impact of pneumococcal challenge on the release of IL-1β and ATP (a well-known stimulator of IL-1β production), on the activation of caspase-1 and cathepsin B (a potential caspase-1 activator), as well as on LDH release (a widely used cell death indicator) over time (Supplemental Fig. 1). Significantly elevated IL-1β levels in cell culture supernatants were found 6 and 18 h after pneumococcal stimulation. Prior to the secretion of IL-1β, increases in both caspase-1 and cathepsin B activities (10- and 5-fold, respectively) were detectable in cell lysates. The time kinetic of caspase-1 activity equalled that of the release of ATP into the supernatant, suggesting involvement of both ATP release and cathepsin B activation in pneumococci-induced pro–IL-1β processing. Raised LDH concentrations in the supernatant were seen at late time points during infection (18 h after challenge), and no temporal relationships were found between LDH levels and caspase-1 activity (Supplemental Fig. 1). These data argue against
a significant role of caspase-1–dependent inflammatory cell death (31, 32) in pneumococcal infection.

Recent studies suggested that extracellular ATP induces cathepsin B activation (33) and accelerates caspase-1–dependent pro–IL-1β processing (34). Cathepsin B activity, in turn, might contribute to NLRP3 inflammasome activation or circumvent it by direct IL-1 family proform cytokine cleavage (35). We therefore examined the effect of inhibiting ATP signaling and cathepsin B on pneumococci-induced caspase-1 activation and IL-1β release. Moreover, we comparatively analyzed the caspase-1 antagonist z-YVAD-fmk and purposeful increase of extracellular potassium concentration for their potential to interfere with IL-1 activation (36). The addition of potassium chloride to the culture medium inhibited pneumococci-induced caspase-1 activation and IL-1β release (Fig. 3A–D) to a similar degree as did the caspase-1 inhibitor z-YVAD-fmk. Both treatments had no effect on ATP liberation, cathepsin B activation, as well as the production of the inflammasome-independent cytokine TNF-α (Fig. 3E–G). Additionally, neither z-YVAD nor the increase in extracellular potassium affected macrophage cell death in response to pneumococcal infection (Fig. 3H).

Next, we treated THP-1 macrophages either with ox-ATP, a P2X7 purinoreceptor antagonist (37), or the cathepsin B inhibitor Ca-074-Me (38), and exposed them to S. pneumoniae. Supplementation of the culture medium with ox-ATP resulted in a significant inhibition of caspase-1 activation and IL-1β release. In contrast to potassium chloride substitution, however, ox-ATP also blocked cathepsin B activation (Fig. 3A–F). Similarly, S. pneumoniae-induced cathepsin B activation, caspase-1 activation, and IL-1β secretion were blunted in THP-1 pretreated with Ca-074-Me (Fig. 3A–F), suggesting a dominant role of cathepsin B in pneumococci-induced IL-1β release by macrophages. Cathepsin B inhibition was also associated with an attenuation of TNF-α and LDH release into the cell supernatant (Fig. 3G, 3H). These findings are in line with recent studies that demonstrated an involvement of cathepsin B in caspase-1–independent necrosis as well as in the trafficking of TNF-α containing vesicles to the plasma membrane and its subsequent liberation (38, 39).

It has been proposed that the NLRP3 inflammasome is activated either by lysosomal damage induced by the cargo taken up by phagocytosis and the subsequent release of cathepsin B (35) or by reactive oxygen species (ROS) generated by a NADPH oxidase upon particle phagocytosis (40). Thus, we assessed whether treatment of cells with 1) cytochalasin D, which impairs actin filament assembly and thus prevents phagocytosis, 2) bafilomycin A, which blocks the vacuolar H+-ATPase system required for lysosomal acidification, or 3) DPI, a well characterized NADPH oxidase inhibitor, interferes with IL-1β release. Neither the addition of cytochalasin D nor that of bafilomycin A or DPI to the culture medium resulted in a significant inhibition of pneumococci-induced caspase-1 and cathepsin B activation as well as IL-1β and ATP liberation (Fig. 3A–F). Furthermore, immunohistochemistry of differentiated THP-1 macrophages using an Ab against LAMP-2, which is known to stain late endosomes as well as lysosomes (38), showed a splotchy staining that did not recede after exposure to S. pneumoniae. This indicated that the lysosomal membrane compartment remained intact upon infection. Moreover, bacteria that constitutively express GFP were virtually exclusively present in the extracellular space, but hardly inside the lysosomes (Fig. 4A). Using an anti-cathepsin B Ab, we found a splotchy staining in unchallenged THP-1 macrophages similar to the LAMP-2 staining, which is in agreement with its known lysosomal localization. However, translocation of cathepsin B to the cytoplasm was observed 3 h after S. pneumoniae stimulation. At later times (6 h) the staining seemed to be entirely cytoplasmic (Fig. 4B). Similarly, when cells were preloaded with LysoSensor Green D-189, we found a splotchy staining that disappeared after pneumococcal challenge, which is suggestive of lysosomal leakage (Fig. 4C) and release of the lysosomal content such as cathepsin B. This was paralleled by an increase in caspase-1 activity and also an increased intracellular staining for IL-1β and caspase-1, reflecting induction of protein expression by pneumococcal challenge (Fig. 4D).
Taken together, our data provide evidence for a pneumococci-induced ATP-dependent lysosomal release of cathepsin B in the absence of bacterial phagocytosis and subsequent lysosomal rupture, which is at least partly dependent on ATP liberation and leads to caspase-1-dependent IL-1β release.

S. pneumoniae-induced IL-1β release by THP-1 macrophages depends on the presence of terminal complement factors and pneumolysin

Recent studies demonstrated that the pore-forming pneumococcal toxin pneumolysin induces caspase-1 activation and IL-1β secretion in murine peritoneal macrophages (41, 42) through activation of the NLRP3 inflammasome (28). Moreover, we observed in a previous study that complement activation is a key factor in IL-1β production during pneumococcal meningitis (43). Both the anaphylatoxin C5a and the terminal complement complex C5b-9, which can form pores in host membranes (44), were also reported to stimulate IL-1β release by human mononuclear cells (45, 46).

In this study, we contribute to clarification of the role of pneumolysin and complement in pneumococci-induced IL-1β release by exposing cells to a pneumolysin-deficient strain and challenging cells in the absence of C5.

The pneumolysin-deficient mutant failed to induce IL-1β release by both murine BMDMs and THP-1 macrophages (Fig. 5A, 5B, 5K). In BMDMs, IL-1β production was dependent on the presence of ASC. In THP-1 cells, the impairment of IL-1β secretion...
but it did not affect pneumococci-induced ATP liberation as well as these data suggest that pneumolysin contributes to IL-1β when C5 was depleted. Additionally, the absence of C5 in the niae S. pneumoniae-dampened in THP-1 macrophages challenged with WT deficient mutant was less potent in inducing TNF-α was also evident, suggesting a cytokine-inducing potency of pneumolysin-deficient mutant was associated with a reduction in CSF pleocytosis (by ∼50%; Fig. 6C) as well as in blood bacterial titers (3.06 ± 0.69 and 2.02 ± 0.51 log_{10} CFU/ml in mutant strain-infected and WT strain-infected mice, respectively; p = 0.007). Bacterial outgrowth in the brain, however, was equal in mice infected either with the mutant or the WT strain. To investigate the significance of pneumolysin-induced inflammasome activation in vivo, we next infected ASC-deficient mice with both pneumococcal strains. ASC-deficient mice developed milder clinical signs of meningitis, irrespective of the bacterial strain inoculated. Moreover, ICP values and CSF leukocyte numbers were quite similar in ASC-deficient mice infected with the pneumolysin-deficient mutant and the WT strain (Fig. 6). Thus, the inflammasome appears to play a critical role in mediating immune responses to pneumolysin-expressing S. pneumoniae.

In a next series of experiments, we inhibited cathepsin B in vivo. The administration of Ca-074-Me prior to pneumococcal infection resulted in a reduction of disease severity, ICP, as well as CSF pleocytosis without altering brain and blood bacterial titers (Fig. 6).

To extend our analysis of pneumolysin and cathepsin B in S. pneumoniae infection, we next assessed their role in murine pneumococcal meningitis. Intracisternal inoculation of the pneumolysin-deficient mutant caused less severe disease than did the WT strain. This was reflected by lower clinical score values (Fig. 6A) as well as a less pronounced loss of body weight and temperature (data not shown). The amelioration of disease was accompanied by a reduction in intracranial complications as exemplified by lower ICP values in mutant strain-infected than in WT strain-infected mice (Fig. 6B). Additionally, infection with the pneumolysin-deficient mutant was associated with a reduction in CSF pleocytosis (by ∼50%; Fig. 6C) as well as in blood bacterial titers (3.06 ± 0.69 and 2.02 ± 0.51 log_{10} CFU/ml in mutant strain-infected and WT strain-infected mice, respectively; p = 0.007). Bacterial outgrowth in the brain, however, was equal in mice infected either with the mutant or the WT strain. To investigate the significance of pneumolysin-induced inflammasome activation in vivo, we next infected ASC-deficient mice with both pneumococcal strains. ASC-deficient mice developed milder clinical signs of meningitis, irrespective of the bacterial strain inoculated. Moreover, ICP values and CSF leukocyte numbers were quite similar in ASC-deficient mice infected with the pneumolysin-deficient mutant and the WT strain (Fig. 6). Thus, the inflammasome appears to play a critical role in mediating immune responses to pneumolysin-expressing S. pneumoniae.

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In an attempt to gain insight into the role of pneumolysin and cathepsin B in pneumococcal-induced IL-1β production in vivo, we also measured IL-1β concentrations in brain homogenates by ELISA. We observed a strong induction of IL-1β expression in brains obtained from WT strain-infected mice (97 ± 34 pg/mg protein versus not detectable in PBS-injected mice). Brain IL-1β levels were significantly lower in mice infected with the pneumolysin-deficient mutant (33 ± 42 pg/mg brain protein; p = 0.017) and in mice infected with the WT strain and treated with Ca-074-Me (44 ± 16 pg/mg brain protein; p = 0.012). Thus, the amelioration of disease observed in mice infected with the pneumolysin-deficient strain or treated with the cathepsin B inhibitor might be attributable to an attenuated IL-1β generation.

Observing that the effects of pneumolysin deficiency or cathepsin B inhibition on IL-1β production were weaker in vivo than in vitro, we further assessed the role of neutrophils in meningitis-induced IL-1β generation. Neutrophils are the predominant cell population within the meningeal infiltrate (8) and can produce IL-1β in a caspase-1–independent manner (49). Granulocyte depletion by pretreatment with a monoclonal anti-GR1 Ab led to a dramatic reduction in neutrophil counts in the blood (52 ± 14 cells/μl, compared with 2196 ± 501 cells/μl in isotype control Ab-treated mice) and CSF at 24 h postinfection (Supplemental Fig. 2). The elimination of neutrophils was paralleled by a significantly attenuated brain pathology (Supplemental Fig. 2), but increased bacterial titers in the blood (data not shown) and brain (Supplemental Fig. 2). Additionally, granulocyte depletion resulted in a significant reduction in brain IL-1β levels (41 ± 26 and

![Image](https://example.com/image.png)
91 ± 25 pg/mg brain protein in anti–GR1-treated and isotype Ab-treated mice, respectively; p = 0.034), suggesting a possible contribution of neutrophil-dependent (caspase-1–independent) pathways to meningitis-induced IL-1β production.

Discussion
Data from patients (9, 10) and animal experiments (13, 14) indicate that excessive production of IL-1β plays a key role in the pathogenesis of pneumococcal meningitis. Furthermore, we previously demonstrated that caspase-1 is an essential mediator in this disease (15). Our present data provide evidence that S. pneumoniae induces caspase-1 activation via the NLRP3 inflammasome and that NLRP3 and ASC deficiencies are associated with significantly decreased clinical and histological disease severity as well as brain inflammation. This observation is in line with the concept that meningitis-related brain damage is largely due to a massive neutrophilic inflammatory reaction and the concomitant release of cytotoxic host factors (2). Initial evidence was raised with blockade of adhesion-promoting receptors of neutrophils. For instance, i.v. injection of anti-CD18 Abs effectively protected against meningitis-related brain damage and death (7, 16). This finding was strengthened by results of recent mouse studies that demonstrated a nearly complete abrogation of brain tissue injury when neutrophils were depleted by using neutrophil-specific Abs (8). In addition to the toxic effects of the host
response, direct bacterial toxicity was implicated as an additional factor driving brain damage in pneumococcal meningitis. However, this aspect is of minor relevance for the protective phenotype associated with the lack of NLRP3 or ASC, as neither was associated with different bacterial loads of the brain as compared with WT controls.

Recently, at least two pathways have been proposed for the activation of the NLRP3 inflammasome (50). According to the first hypothesis, NLRP3 activators induce lysosomal damage, which leads to the release of lysosomal proteases such as cathepsin B into the cytosol. The lysosomal proteases, in turn, could either degrade a putative NLRP3 inhibitor or cleave a substrate in the cytosol that would generate a NLRP3 ligand (53). This model has been consolidated by the demonstration that diverse crystals and protein aggregates disrupt lysosomal integrity and activate NLRP3 (35, 54). Accordingly, IL-1β secretion upon infection with the intracellular pathogen Listeria monocytogenes was found to be largely dependent on phagolysosomal rupture (induced by the pore-forming bacterial toxin listeriolysin O) and cathepsin B release (55). Moreover, nigericin, thought to activate the NLRP3 inflammasome solely through acting as a potassium ionophore, has been demonstrated to induce lysosomal leakage of cathepsin B and subsequent caspase-1 activation, noteworthy in the absence of obvious lysosomal desintegration (38). In line with the latter finding is our observation that pneumococcal challenge does not lead to disruption of the lysosomal membrane compartment, but to lysosomal deacidification and cathepsin B release into the cytosol, and that cathepsin B inhibition prevents pneumococci-induced IL-1β secretion.

We further observed an increase in ATP levels in the supernatant of THP-1 cells upon exposure to S. pneumoniae. Moreover, pretreatment with the purinoreceptor P2X7 antagonist oxidized ATP resulted in an attenuated activation of cathepsin B and caspase-1 as well as in reduced IL-1β generation. Thus, pneumococcal challenge might result in secretion of ATP that activates P2X7 receptors through an autocrine loop and triggers a series of signaling events culminating in IL-1β release. This is in line with a previous study demonstrating endogenous ATP release from monocytes as an early step in the inflammasome activation cascade induced by a variety of pathogen- or danger-associated molecular patterns including muramyl dipeptide or monosodium urate (56). Moreover, hyperoxia exposure has also been reported to lead to ATP release, which further stimulates P2X7-mediated potassium efflux and inflammasome activation (57). In both studies, the ATP concentrations measured in the cell culture supernatants were quite similar to that we found in our experiments, but well below the threshold required to stimulate P2X7 receptors. This discrepancy may be explained by a significant underestimation of the ATP amount released at the cell surface, due to a fast diffusion and rapid hydrolysis of cell-derived ATP by ectonucleotidases expressed by host cells and/or bacteria (58).

Similar to the mode of action to the potassium ionophore nigericin, a major event triggered by extracellular ATP is the release of cathepsin B (33). The ATP-induced protease release was not preventable by high extracellular potassium concentrations, suggesting its independence of potassium ion efflux (33). Accordingly, we did not detect a significant reduction in pneumococci-induced cathepsin B activity in THP-1 cells in the presence of high potassium levels. Instead of the potassium efflux, a rise in lysosomal pH has been proposed as the trigger of the lysosomal secretion of cathepsin B. This hypothesis is based on the observation that compounds capable of increasing lysosomal pH, including ammonium chloride, hydrogen ionophores, or vacuolar H+-ATPase inhibitors such as bafilomycin A, can induce the release of lysosomal content (59). This concept may also explain the lack of effect of bafilomycin A pretreatment on pneumococci-induced cathepsin B and caspase-1 activation by us.
Furthermore, we demonstrated in this study that differentiated THP-1 cells release significantly less ATP into cell culture supernatants upon challenge with a pneumolysin-deficient as compared to a pneumolysin-producing pneumococcal strain. This was paralleled by reduced cathepsin B and caspase-1 activity as well as IL-1β release. Our data are in agreement with a recent study (28) that showed an amplification of TLR agonist-induced IL-1β secretion from murine dendritic cells by pneumolysin (28). This effect was dependent on cathepsin B, NLRP3, and caspase-1. Moreover, live *S. pneumoniae* was found to promote IL-1β release. This release also required NLRP3 and pneumolysin (28). Similarly, Shoma et al. (41) reported a critical involvement of pneumolysin in IL-1β generation by murine peritoneal macrophages after exposure to *S. pneumoniae*. Besides activating caspase-1, pneumolysin was shown to induce the cellular production of IL-1 family cytokines in a TLR4-dependent manner. This observation is in line with the study of Malley et al. (48) who first described a role of TLR4 in pneumolysin recognition, but it contrasts with the results from studies of McNeela et al. (28) and Witzenthurn et al. (42). In the latter study, stimulation of IL-1β production in *S. pneumoniae*-infected human monocytes and murine BMDMs involved signals dependent on TLR2 (but not TLR4) and the NLRP3 inflammasome (42). In the study by McNeela et al. (28), pneumolysin was not capable of inducing cytokine secretion by murine dendritic cells. By using live pneumolysin-producing and pneumolysin-deficient bacteria, we demonstrated in this study that pneumolysin is required for both induction of cytokine expression (such as IL-1β and TNF-α) and activation of caspase-1. The latter was evidenced by 1) the repression of caspase-1 activation and IL-1β release by human THP-1 cells upon exposure to the pneumolysin-deficient strain, and 2) the secretion of similar amounts of IL-1β from WT and ASC-deficient BMDMs following challenge with pneumolysin-deficient and WT bacteria. The activation of caspase-1 seems to be at least partly related to the pneumolysin-triggered liberation of ATP from infected THP-1 cells. However, further studies are necessary to identify which pattern recognition receptors are involved in sensing pneumolysin-producing versus pneumolysin-deficient pneumococcal strains. Collectively, our experiments suggest the following model of inflammasome activation by *S. pneumoniae*: the pneumococcal toxin pneumolysin may induce the release of ATP. Extracellular ATP, in turn, might be the major trigger of the lysosomal secretion of cathepsin B as activator of the NLRP3 inflammasome upon *S. pneumoniae* challenge.

Furthermore, our data show involvement of pneumolysin and cathepsin B in the immunopathogenesis of pneumococcal meningitis. Both inoculation of pneumolysin-deficient pneumococci and inhibition of cathepsin B were associated with a reduction in brain IL-1β levels, CSF leukocyte counts, and disease severity. In line with our results are reports on the protective effects of cathepsin B inhibition in animal models of acute and chronic neurodegenerative disorders, including cerebral ischemia and Alzheimer’s disease (60, 61). Moreover, high cathepsin B activity was detected in CSF samples from patients with neuroinflammatory diseases such as multiple sclerosis (62). Inflammasome activation plays an important role in the three diseases listed above by exacerbating brain inflammation (54, 63, 64). Therefore, it is conceivable that cathepsin B release and activation may represent a more widespread mechanism for the propagation of inflammasome-dependent immune responses in the brain.

The role of pneumolysin in meningitis has been evaluated in previous studies, which, however, gave inconclusive results with regard to its impact on clinical outcome, bacterial outgrowth, as well as meningeal inflammation (65–68). By using an adult mouse model, in this study we found that pneumolysin-deficient *S. pneumoniae* evoked merely ameliorated disease. The milder clinical course was paralleled by lower bacterial numbers in the blood, suggesting that the effect on the clinical picture is partly due to less severe sepsis, a typical systemic complication of pneumococcal meningitis (1, 6). This observation is in agreement with previous studies in which adult rodents were applied (66, 67). Additionally, we observed less pronounced neuropathological alterations in adult mice infected with the mutant strain, which was also reported for adult rats (67) and neonatal rats (68). This might be owing to the absence of a direct toxic effect of the bacterial cytolsin on brain cells. Also, it is conceivable that a milder inflammatory reaction upon confrontation with pneumolysin-deficient bacteria contributes to the observed reduction in brain injury. This statement is supported by our observation of significantly lower CSF leukocyte counts and brain IL-1β levels in mice subjected to meningitis with pneumolysin-deficient pneumococci. This reduction might be related to the lack of the cytokine-inducing and cytokine-activating potency of pneumolysin because bacterial outgrowth in the brain (and thus the amounts of other proinflammatory pneumococcal molecules) was similar postinfection with the pneumolysin-deficient and the WT strain. In accordance with our observation, a reduction in CSF neutrophil/monocyte counts in adult rats challenged with pneumolysin-deficient bacteria has been reported (67). Results from application of mouse models of pneumococcal bacteremia or pneumonia in which pneumolysin-deficient bacteria were applied lend further support to this idea (69, 70). However, in none of the three other studies (65, 66, 68) did pneumolysin deficiency result in significantly lower meningeal inflammation. The causes for these differing results are unclear, but they might be attributable to different experimental setups (e.g., animal species used).

Meningitis due to *S. pneumoniae* is a serious disease with high mortality and morbidity rates. Therefore, new therapies based on an understanding of pathophysiology are warranted. In this study, we show that pneumococcal infection of the CSF leads to inflammasome activation that enhances the inflammatory reaction and contributes to brain injury and adverse outcome. We also demonstrate that pneumolysin is a key player in meningitis-induced IL-1β generation by both inducing IL-1β protein expression and inflammasome activation. Inflammasome activation upon *S. pneumoniae* challenge depends on the release of ATP, lysosomal destabilization, and cathepsin B activation. We conclude that interference with inflammasome activation might be a promising target for adjunctive therapy in pneumococcal meningitis.

**Disclosures**

The authors have no financial conflicts of interest.

**References**


Suppl. Figure 2

A) CSF WBC [x1000 cells/μl]

B) Neuro score

C) Brain titer [log_{10} cfu/organ]

- Infected, IgG-treated
- Infected, anti-GR1-treated

* Statistical significance
Figure S1: Effect of pneumococcal challenge on IL-1ß release, caspase-1 and cathepsin B activity, as well as ATP and LDH liberation by THP-1 cells. Human THP-1 cells (5x10^5 cells/well; differentiated for 48 h with 100 nM PMA) were exposed to 10^7 cfu/ml of *Streptococcus pneumoniae* (D39 strain). At 1, 3, 6, and 18 h after stimulation, IL-1ß (A), ATP (D), and LDH (E) release were monitored using ELISA, bioluminescence, and colorimetric assay kits, respectively. Caspase-1 (B) and cathepsin B (C) activity were determined by measuring the cleavage of enzyme-specific, fluorogenic substrates in cell lysates using commercially available assay kits. All data are presented as means ± SD for at least two independent experiments performed in triplicate. * P < 0.05, compared to medium-stimulated cells using paired Student’s t test and Bonferroni correction for multiple comparisons.

Figure S2: Neutrophil depletion is protective in pneumococcal meningitis. Granulocyte depletion by pretreatment with the monoclonal anti-GR1 antibody (n=6 for each group) led to a dramatic reduction in neutrophil counts in the CSF (A). The elimination of neutrophils was paralleled by an attenuated brain pathology (as indicated by lower neuroscore values; B), but increased bacterial titers in the brain (C). Data are given as means ± SD. * P < 0.05, compared to infected mice treated with mouse immunoglobulins.