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B7-H3 Participates in the Development of Experimental Pneumococcal Meningitis by Augmentation of the Inflammatory Response via a TLR2-Dependent Mechanism

Xuqin Chen,*† Edel M. Quinn, † Hong Ni, ‡ Jian Wang § Siobhan Blankson, † H. Paul Redmond, † Jiang Huai Wang, † and Xing Feng‡

In addition to a well-documented role in regulating T cell-mediated immune responses, B7-H3, a newly discovered member of the B7 superfamily, has been recently identified as a costimulator in the innate immunity-mediated inflammatory response. In this study, we further report that B7-H3 participates in the development of pneumococcal meningitis in a murine model. Exogenous administration of B7-H3 strongly amplified the inflammatory response, exacerbated blood–brain barrier disruption, and aggravated the clinical disease status in Streptococcus pneumoniae-infected C3H/HeN wild-type mice. Consistent with the in vivo findings, B7-H3 substantially augmented proinflammatory cytokine and chemokine production, upregulated NF-κB p65 and MAPK p38 phosphorylation, and enhanced the nuclear transactivation of NF-κB p65 at both TNF-α and IL-6 promoters in S. pneumoniae-stimulated primary murine microglia cells. These B7-H3–associated in vitro and in vivo effects appeared to be dependent on TLR2 signaling, as B7-H3 almost completely lost its amplifying actions in both TLR2-deficient microglial cells and TLR2-deficient mice. Furthermore, administration of the anti–B7-H3 mAb (MIH35) attenuated the inflammatory response and ameliorated blood–brain barrier disruption in S. pneumoniae-infected wild-type mice. Collectively, our results indicate that B7-H3 plays a contributory role in the development of S. pneumoniae infection-induced bacterial meningitis. The Journal of Immunology, 2012, 189: 347–355.

Bacterial meningitis caused by Streptococcus pneumoniae, which is the principal etiologic agent in humans, remains a life-threatening infectious disease in both adults and children. It causes death in ∼25% of cases and neurologic sequelae in nearly half of survivors despite targeted antibiotic therapy, adjunctive treatment with steroids, and supportive intensive care (1–4). It has been well established both clinically and neuropathologically that the poor outcome of pneumococcal meningitis is often associated with development of intracranial complications such as brain edema and cerebrovascular damage (5, 6). Several studies have shown that S. pneumoniae–induced bacterial meningitis and its intracranial complications occur not only due to uncontrolled bacterial growth in the CNS, but they also depend on the host innate immunity-initiated inflammatory response to the pathogen via the pattern recognition receptors TLR2 and the adaptor protein MyD88 (7,9). Indeed, an overwhelming inflammatory response evoked by S. pneumoniae infection in the CNS may lead to significant damage to the brain, thus contributing to the frequently unfavorable outcome of pneumococcal meningitis (5, 7, 10, 11).

B7-H3, a newly discovered member of the B7 superfamily of costimulatory proteins, possesses a contrasting role in regulating T cell-mediated immune responses by functioning as both a T cell costimulator and coinhibitor (12–15). Although the definitive counterreceptors for B7-H3 have not yet been identified, previous work revealed that B7-H3 binds to a putative receptor expressed on PHA- or anti-CD3 mAb-activated T cells that is distinct from CD28, CTLA-4, ICOS, and programmed death-1 (12, 16). A recent study identified triggering receptor expressed on myeloid cell-like transcript 2 as a costimulatory receptor for murine B7-H3 (17), but further work on both human and murine B7-H3 did not support this finding (18). Nevertheless, it has been postulated that multiple counterreceptors for B7-H3 may exist on different types of immune cells (17, 19).

In addition to a well-documented role of B7-H3 in regulating T cell-mediated immune responses, it has been recently shown that B7-H3 is also involved in the innate immunity–associated inflammatory response. Although B7-H3 is not expressed in significant amounts on freshly isolated human lymphocytes, it is induced in human monocytes/macrophages and dendritic cells upon inflammatory cytokine stimulation (12–14, 20). A soluble form of B7-H3 (sB7-H3), released from monocytes, dendritic cells, and activated T cells, is detectable in the circulation of healthy humans (21). Our recent work demonstrated that B7-H3 strongly enhanced both the TLR4 agonist LPS- and the TLR2 agonist bacterial lipoprotein (BLP)-stimulated NF-κB activation and proinflammatory cytokine production in monocytes and/or...
macrophages via both a TLR4- and TLR2-dependent manner (22). Furthermore, patients diagnosed with sepsis had significantly elevated levels of plasma sB7-H3, and this level correlated with the clinical outcome and plasma levels of TNF-α and IL-6 in these patients. These results indicate that B7-H3 functions as a co-stimulator of innate immunity by augmenting the inflammatory response, and it thus may contribute to the development of sepsis (22).

It has not been demonstrated to date whether B7-H3 participates in the development of pneumococcal meningitis and its intracranial complications. Our recent clinical study did show significantly elevated levels of sB7-H3 in the circulation and cerebrospinal fluid of patients diagnosed with bacterial meningitis (23). Levels of cerebrospinal fluid and plasma sB7-H3 in these patients correlate with the intensity of their infectious inflammatory process and may also act as a useful marker for distinguishing bacterial from aseptic meningitis. In this study, we further identify that B7-H3 substantially augments the S. pneumoniae-stimulated inflammatory response, exacerbates S. pneumoniae-induced damage to blood–brain barrier (BBB) integrity, and aggravates the clinical disease status in a murine model of pneumococcal meningitis. B7-H3 also significantly enhances proinflammatory cytokine and chemokine production in primary murine microglial cells challenged with S. pneumoniae, which is associated with B7-H3–upregulated TLR2 downstream signaling pathways, including increased NF-κB p65 and MAPK p38 phosphorylation and enhanced binding of NF-κB p65 to the TNF-α and IL-6 promoters. Administration of an anti–B7-H3 mAb (MIH35) in vivo markedly attenuates the S. pneumoniae–induced inflammatory response and BBB disruption, indicating that B7-H3 contributes to the development of pneumococcal meningitis. Furthermore, B7-H3 is unable to augment proinflammatory cytokine and chemokine production or to exacerbate BBB disruption in S. pneumoniae-challenged, TLR2-deficient microglial cells and TLR2-deficient mice, suggesting that these observed in vitro and in vivo augmentations by B7-H3 on S. pneumoniae–induced bacterial meningitis is specific, wild-type mice also received an intracerebral ventricular injection with 7.5 μl PBS containing 0.75 × 10⁶ CFU/ml S. pneumoniae and 7.5 μl PBS containing 2.5 μg B7-H3; 4) mice in the S. pneumoniae plus an anti-B7-H3 mAb (MIH35) (a gift from Dr. M. Azuma, Tokyo Medical and Dental University, Tokyo, Japan) group injected with 7.5 μl PBS containing 15 μg MIH35 mAb and 7.5 μl PBS containing 0.75 × 10⁶ CFU/ml S. pneumoniae; and 5) mice in the S. pneumoniae plus the isotype-matched control IgG (MP Biomedicals, Solon, OH) group injected with 7.5 μl PBS containing 15 μg control IgG and 7.5 μl PBS containing 0.75 × 10⁶ CFU/ml S. pneumoniae. To distinguish whether B7-H3 on S. pneumoniae–induced bacterial meningitis is specific, wild-type mice also received an intracerebral ventricular injection with 7.5 μl PBS containing 0.75 × 10⁶ CFU/ml S. pneumoniae and 7.5 μl PBS containing 2.5 μg recombinant mouse B7-H4 (R&D Systems).

Mice were weighed, allowed to wake up, and evaluated clinically at 6, 18, and 30 h after S. pneumoniae infection. The severity of disease status was examined by body weight loss and spontaneous motor activity. The following scales were used to assess spontaneous motor activity of mice as described previously (25, 26): 1, normal motor activity and turned upright in <5 s when put on back; 2, reduced spontaneous motor activity, but still turned up in <5 s; 3, turned up in >5 s; 4, did not turn up; 5, did not move at all. At 6, 18, and 30 h after S. pneumoniae infection, mice were sacrificed by CO₂ inhalation. The brain of each animal was removed, and half of the brain was frozen immediately in liquid nitrogen and stored at −80°C for measurement of proinflammatory cytokines and chemokines by quantitative real-time PCR and ELISA.

Isolation and culture of primary murine microglial cells

All culture media and reagents used for culture of primary murine microglial cells were purchased from Invitrogen Life Technologies (Paisley, U.K.). DMEM-F12 was supplemented with 10% heat-inactivated FCS, glutamine (2.0 mM), penicillin (100 U/ml), and streptomycin sulfate (100 μg/ml) for mixed glial cultures. DMEM was supplemented with 20% heat-inactivated FCS, glutamine (2.0 mM), penicillin (100 U/ml), and streptomycin sulfate (100 μg/ml) for microglial cell cultures.

Microglial cells were isolated from neonatal wild-type and TLR2-deficient mice as described previously (27). Briefly, brains were removed from 1- to 3-d-old neonatal mice. After removal of the meninges, the left and right hemispheres of the brain were gently dissociated and the resulting cell suspension was passed through a 70-μm nylon cell strainer (BD Biosciences, Franklin Lakes, NJ). The single-cell suspension was resuspended in complete DMEM-F12 medium, seeded in poly-D-lysine (10 μg/ml) (Sigma-Aldrich, St. Louis, MO) precoated tissue culture flasks (BD Biosciences) and incubated at 37°C in a humidified 5% CO₂ atmosphere for 10–14 d. Microglial cells were then removed from the astroglial layer by shaking microglial cells for 2 h. The primary microglial cells were removed from the flask, resuspended in complete DMEM, and seeded in poly-D-lysine– precoated 24-well tissue culture plates (BD Biosciences).

Primary microglial cells plated in 24-well tissue culture plates at a density of 7.5 × 10⁵ cells/well were exposed to culture medium as the control, live S. pneumoniae (1 × 10⁹ CFU/ml), or live S. pneumoniae (1 × 10⁹ CFU/ml) plus recombinant mouse B7-H3 (2.5 μg/ml) for 6, 24, and 48 h. Ceftriaxone (100 μg/ml) (Sigma-Aldrich) was added to the plates for restraining S. pneumoniae overgrowth throughout the experiment. Cell-free supernatants were collected and stored at −80°C until analysis.

Determination of the BBB integrity

Two endogenous serum-abundant proteins albumin and IgG are normally excluded from the brain by the intact BBB. To assess the BBB integrity, albumin and IgG levels in mouse brain homogenates collected at 6, 18, and 30 h after S. pneumoniae infection were assessed by ELISA (RD Biotech, Besancon, France, and Abnova, Walnut, CA, respectively) according to the manufacturers’ instructions.

Quantitative real-time PCR

Total RNA from frozen brain sections was extracted with a GeneElute mammalian total RNA purification kit (Sigma-Aldrich) and reverse-transcribed with the SuperScript first-strand synthesis system (Invitrogen). The cDNA was amplified by quantitative real-time PCR with the following gene-specific primers: mouse TNF-α (sense, 5′-CATCTTCTCAAAATT- CGAGTGACA-3′ and antisense, 5′-TGGGAGTAGACAAGGTACAAC- CTGG-3′); mouse IL-1β (sense, 5′-CAACCAACAGTATCTTCTTG- CAG-3′ and antisense, 5′-GATCCACCTTCTCAGCTCA-3′); mouse IL-6 (sense, 5′-CCACCGACCTAAGCCAGCC-3′ and antisense, 5′-ATCCATGTGTTCATCA-3′); mouse MCP-1 (sense, 5′-CTTCGAGGCTCTGCTGCTCA-3′ and antisense, 5′-CCGCTCACTTACCTGGAGATC-3′); mouse β-actin (sense, 5′-AGAGGGAAATCTGGTGCT-3′ and
antisense, 5'-CAATTAGTATGACCTTGCCGT-3') on a LightCycler system (Roche Molecular Biochemicals, Indianapolis, IN) according to the manufacturer's instructions. The cycling conditions were 95°C for 15 min as an initial step of denaturation, followed by 40 cycles at 95°C for 10 s, 55°C for 25 s, and 72°C for 20 s. At the end of the last cycle, the temperature was increased from 65 to 95°C at 1°C/s with continuous fluorescence monitoring to produce a melting curve. The specificity of amplification was assessed for each sample by melting curve analysis, and each PCR product showed a single peak. The size of the PCR product was identified by 2% agarose gel electrophoresis. The data were analyzed with QGene software (28), and the target gene mRNA expression was normalized with the housekeeping gene β-actin.

**ELISA**

Brain samples collected at various time periods from the in vivo experiments were homogenized and centrifuged. Cell-free supernatants were collected at different time points from the in vitro experiments. The concentrations of proinflammatory cytokines TNF-α, IL-1β, IL-6, and chemokine MCP-1 in brain homogenates and cell-free supernatants were assessed by ELISA (eBioscience, Hatfield, U.K.) according to the manufacturer's instructions.

**Assays for NF-κB p65 and MAPK p38 phosphorylation**

Primary microglial cells isolated from wild-type and TLR2-deficient mice were incubated with culture medium as the control, live *S. pneumoniae* (1 × 10^6 CFU/ml), or live *S. pneumoniae* (1 × 10^6 CFU/ml) plus recombinant mouse B7-H3 (2.5 μg/ml) for different time periods as mentioned above. Phosphorylation of NF-κB p65 in microglial cells was assessed by a phospho-RelA/NF-κB p65 ELISA kit (R&D Systems) according to the manufacturer's instructions. For determination of intracellular MAPK p38 phosphorylation, microglial cells fixed and permeabilized with Phosflow Perm Buffer (BD Biosciences) were stained with a PE-conjugated anti-phospho p38 mAb (BD Biosciences). PE-conjugated isotype-matched mAb was used as the control. FACScan analysis was performed from at least 10,000 events for detecting the intracellular staining of phospho-p38 using CellQuest software (BD Biosciences).

**Chromatin immunoprecipitation assay**

ChIP assay was performed using the ChIP-IT Express kit (Active Motif, Carlsbad, CA) according to the manufacturer’s instructions. Briefly, isolated primary murine microglial cells were stimulated, washed with PBS, and fixed with 1% formaldehyde at room temperature for 10 min. The cells were lysed in ice-cold lysis buffer and sheared by sonication to generate 200- to 1000-bp DNA fragments. Immunoprecipitation was carried out by incubation of the diluted sonicates with protein G magnetic beads and a specific anti–NF-κB p65 Ab (Sc-372; Santa Cruz Biotechnology, Santa Cruz, CA) with rotation overnight. Protein G magnetic beads were collected with a magnetic stand (Active Motif) and washed extensively. Protein/DNA complexes were eluted, the cross-link was reversed, and proteins were digested with proteinase K. Immunoprecipitated DNA and nonimmunoprecipitated DNA (input control) were amplified by quantitative PCR using the following promoter-specific primers: mouse TNF-α (sense, 5'-TCCCTTGATCGCTGGTTCGCC-3' and antisense, 5'-GCAGACGGCCGCTTTATAGC-3') and mouse IL-6 (sense, 5'-TCCAATCA-GCCACCCACACCTC-3' and antisense, 5'-GGTGGGCTCCAGACAGA-ATG-3').

**Statistical analysis**

All data are presented as the means ± SD. Statistical analysis was performed using the Kruskal–Wallis test for clinical status and body weight loss between different groups, and the Mann–Whitney U test for all others, with GraphPad Prism software version 5.01 (GraphPad Software, La Jolla, CA). A p value < 0.05 was judged statistically significant.

**Results**

**B7-H3 amplifies S. pneumoniae-stimulated inflammatory response and exacerbes S. pneumoniae-induced damage in BBB integrity during pneumococcal meningitis**

Having considered the major contribution of inflammatory cytokines in the development of pneumococcal meningitis (5, 7, 10, 11), we first assessed the expression of proinflammatory cytokines TNF-α, IL-1β, IL-6, and chemokine MCP-1 in wild-type mice infected with *S. pneumoniae* via intracerebral ventricular inoculation. Significantly increased protein expression of TNF-α, IL-1β, IL-6, and MCP-1 in brain homogenates was observed at 6, 18, and 30 h after infection in *S. pneumoniae*-challenged mice when compared with that in PBS-treated control mice (Fig. 1). Notably, a substantial further accumulation of TNF-α, IL-1β, IL-6, and MCP-1 proteins in the brain was evident in mice challenged with a combination of *S. pneumoniae* and B7-H3 at almost all time points assessed (p < 0.05 versus mice challenged with *S. pneumoniae* alone) (Fig. 1), whereas treatment of wild-type mice with B7-H3 alone had no stimulatory effect on proinflammatory cytokine and chemokine production (data not shown). We also measured the mRNA expression of all those cytokines in mice challenged with *S. pneumoniae* alone or a combination of *S. pneumoniae* and B7-H3. *S. pneumoniae* infection led to increased mRNA expression in brain TNF-α, IL-1β, IL-6, and MCP-1; however, a combined treatment of *S. pneumoniae* and B7-H3 caused a more pronounced increase in TNF-α, IL-1β, IL-6, and MCP-1 mRNA expression at 6 and 18 h after *S. pneumoniae* and

![FIGURE 1](http://www.jimmunol.org/Downloadedfrom/FIGURE1.png)
B7-H3 treatment ($p < 0.05$ versus mice challenged with *S. pneumoniae* alone) (Supplemental Fig. 1). These results indicate that B7-H3 augments *S. pneumoniae* infection-induced inflammatory response during the acute stage of pneumococcal meningitis by enhancing proinflammatory cytokine and chemokine production.

Because disruption of BBB integrity is an important hallmark among pathological alterations during pneumococcal meningitis and is often related to an unfavorable clinical outcome of this disease (5, 6, 29), we next examined whether B7-H3 contributes positively to *S. pneumoniae* infection-induced damage in BBB integrity by determining the endogenous serum albumin and IgG in brain homogenates. As shown in Fig. 1, there were very little amounts of albumin and IgG present in brain homogenates from PBS-treated control mice; in contrast, intracerebral infection with *S. pneumoniae* caused extensive extravasation of serum albumin and IgG into the brain ($p < 0.05$ versus mice that received PBS). Moreover, the addition of B7-H3 further aggravated *S. pneumoniae* infection-induced BBB permeability, as evidenced by more profound levels of both albumin and IgG in brain homogenates from mice challenged with both *S. pneumoniae* and B7-H3 ($p < 0.05$ versus mice challenged with *S. pneumoniae* alone) (Fig. 1). Thus, in addition to amplifying *S. pneumoniae* infection-induced inflammatory response, B7-H3 exacerbates BBB disruption during pneumococcal meningitis. In contrast, B7-H4, another member of the B7 superfamily of costimulatory proteins, neither augmented *S. pneumoniae*-stimulated TNF-α and IL-6 production nor aggravated *S. pneumoniae*-induced BBB disruption as represented by the accumulation of serum albumin and IgG in the brain (Supplemental Fig. 2A).

**FIGURE 2.** B7-H3 augments *S. pneumoniae*-stimulated proinflammatory cytokine and chemokine expression in primary murine microglial cells. Primary microglial cells isolated from C3H/HeN (wild-type) mice were exposed to culture medium (CM), live *S. pneumoniae* (SP), or live *S. pneumoniae* plus B7-H3 (SP+B7H3) for various time periods as described in Materials and Methods. The expression of TNF-α, IL-1β, IL-6, and MCP-1 at the protein level in the culture supernatants (A) and at the mRNA level in the cell lysates (B) was assessed by ELISA and quantitative real-time PCR, respectively. Data are expressed as means ± SD of six to eight independent experiments, and each experiment was conducted in duplicate. *$p < 0.05$ compared with cells exposed to CM, **$p < 0.05$ compared with cells exposed to *S. pneumoniae* alone.

To further clarify the impact of B7-H3 on *S. pneumoniae*-induced inflammatory response during pneumococcal meningitis, we examined whether B7-H3 is capable of enhancing proinflammatory cytokine and chemokine production in *S. pneumoniae*-stimulated primary microglial cells, the resident macrophage-like population of the CNS, representing the first line of CNS defense against invading pathogens (27, 30, 31). Stimulation of primary murine microglial cells with live *S. pneumoniae* at 1 × 10⁶ CFU/ml led to a significant release of TNF-α, IL-1β, IL-6, and MCP-1 at 6, 24, and 48 h after *S. pneumoniae* challenge when compared with culture medium-treated microglial cells ($p < 0.05$) (Fig. 2A). In keeping with the in vivo finding, B7-H3 alone did not stimulate any of these cytokines to release (data not shown); however, a combined treatment of microglial cells with both *S. pneumoniae* and B7-H3 resulted in much higher levels of the released TNF-α, IL-1β, IL-6, and MCP-1 than those observed in microglial cells challenged with *S. pneumoniae* alone ($p < 0.05$) (Fig. 2A). In
contrast, costimulation with B7-H4 did not lead to a further release of TNF-α and IL-6 from the S. pneumoniae-treated microglial cells (Supplemental Fig. 2B). The amplifying effect of B7-H3 on proinflammatory cytokine and chemokine release from S. pneumoniae-stimulated microglial cells was further supported by B7-H3–induced upregulation of those cytokines at the mRNA level, where B7-H3 substantially enhanced the mRNA expression of TNF-α, IL-1β, IL-6, and MCP-1 at 6 and 24 h after stimulation in S. pneumoniae-challenged microglial cells (p < 0.05 versus cells stimulated with S. pneumoniae alone) (Fig. 2B).

To elucidate the underlying mechanism by which B7-H3 amplifies S. pneumoniae-stimulated inflammatory response, we determined whether B7-H3 has a positive effect on S. pneumoniae-induced activation of the TLR2 downstream signaling pathway. S. pneumoniae stimulation induced significantly increased phosphorylation of NF-κB p65 and MAPK p38 (Fig. 3A) and promoted the recruitment and binding of p65 to NF-κB sites in the promoter region of TNF-α and IL-6 genes (Fig. 3B) in primary murine microglial cells (p < 0.05 versus cells incubated with culture medium). In keeping with an amplifying effect B7-H3 has on proinflammatory cytokine and chemokine release from S. pneumoniae-stimulated microglial cells, the additional treatment with B7-H3 led to further increases in phosphorylated p65 and p38 (Fig. 3A) and strongly enhanced the nuclear transactivation of NF-κB p65 at both TNF-α and IL-6 promoters (Fig. 3B) in S. pneumoniae-stimulated microglial cells (p < 0.05 versus cells treated with S. pneumoniae alone).

Administration of the anti-B7-H3 mAb attenuates S. pneumoniae-stimulated inflammatory response and ameliorates S. pneumoniae-induced BBB disruption in vivo

Because the exogenous addition of B7-H3 amplified S. pneumoniae-stimulated inflammatory response and aggravated S. pneumoniae-induced BBB permeability during pneumococcal meningitis, we next addressed the fundamental question of whether B7-H3 contributes to the development of S. pneumoniae infection-induced bacterial meningitis by using the anti-B7-H3 mAb (MIIH35). Mice receiving MIIH35 mAb displayed a significant reduction in their brain TNF-α, IL-1β, and IL-6 expression at both the protein level (Fig. 4) and mRNA level (data not shown) at 18 and 30 h after S. pneumoniae infection (p < 0.05 versus mice challenged with S. pneumoniae alone). An even earlier reduction in chemokine MCP-1 at 6 and 18 h after S. pneumoniae infection was also observed in brain homogenates from MIF35 mAb-treated mice (p < 0.05 versus mice challenged with S. pneumoniae alone) (Fig. 4).

Furthermore, treatment with MIF35 mAb markedly ameliorated S. pneumoniae infection-induced BBB disruption, with significantly reduced extravasation of albumin and IgG into the brain in MIF35 mAb-treated mice (p < 0.05 versus mice challenged with S. pneumoniae alone) (Fig. 4). However, administration of an isotype-matched control IgG affected neither proinflammatory cytokine production nor BBB permeability in S. pneumoniae-infected mice (Fig. 4), demonstrating the specific and direct effect MIF35 mAb has on B7-H3. These results indicate that B7-H3 contributes positively to the development of S. pneumoniae infection-associated meningitis by amplifying the inflammatory response and exaggerating the damage to BBB integrity.

Augmentation of S. pneumoniae-induced inflammatory response and BBB disruption by B7-H3 occur via a TLR2-dependent mechanism

To ascertain whether TLR2 is involved in the B7-H3–amplified inflammatory response to S. pneumoniae infection, we repeated our in vivo experiments in TLR2-deficient mice. Proinflammatory cytokine and chemokine production and BBB integrity in B7-H3-treated, S. pneumoniae-infected TLR2-deficient mice were assessed and compared with those we previously obtained from wild-type mice. TLR2-deficient mice displayed slight to moderate protein expression of TNF-α, IL-1β, IL-6, and MCP-1 in the brain at 6, 18, and 30 h after S. pneumoniae inoculation (Fig. 5), which is in contrast to the much higher levels of these cytokines observed in wild-type mice infected with the same amount of S. pneumoniae (Fig. 1). Consistent with a reduced inflammatory response, S. pneumoniae infection also caused less damage to BBB integrity in TLR2-deficient mice (Fig. 5) than it did in wild-type mice (Fig. 1). More importantly, B7-H3 almost completely lost its amplifying action in TLR2-deficient mice, as administration of B7-H3 failed to enhance TNF-α, IL-1β, IL-6, and MCP-1 expression at both the protein level (Fig. 5) and mRNA level (data not shown) in the brain and was unable to exacerbate BBB disruption (Fig. 5) in S. pneumoniae-infected, TLR2-deficient mice. This demonstrates an involvement of the TLR2 signaling in B7-H3 action during S. pneumoniae infection-induced bacterial meningitis. To further confirm the above in vivo finding, we stimulated primary microglia cells isolated from TLR2-deficient mice with either S. pneumoniae alone or a combination of S. pneumoniae and B7-H3. Consistent with the in vivo data from TLR2-deficient mice, the

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**FIGURE 3.** B7-H3 enhances S. pneumoniae-induced NF-κB p65 and MAPK p38 phosphorylation and p65 binding to the TNF-α and IL-6 promoters in microglial cells. Primary microglial cells isolated from C3H/HeN (wild-type) mice were incubated with culture medium (CM), live S. pneumoniae (SP), or live S. pneumoniae plus B7-H3 (SP+B7H3) for various time periods as described in Materials and Methods.

(A) Expression of phosphorylated p65 and p38 was assessed by ELISA and FACSscan analysis, and expressed as relative fluorescence units (RFU) and mean channel fluorescence per cell (MCF/cell), respectively.

(B) The binding of NF-κB p65 to the TNF-α and IL-6 promoters was assessed by ChIP analysis and presented as percentage of input. Data are expressed as means ± SD of three to four independent experiments, and each experiment was conducted in duplicate. *p < 0.05 compared with cells exposed to CM. **p < 0.05 compared with cells exposed to S. pneumoniae alone.
additional treatment with B7-H3 had no effect on either *S. pneumoniae*-stimulated proinflammatory cytokine and chemokine production (Fig. 6) or *S. pneumoniae*-induced NF-κB p65 and MAPK p38 phosphorylation (Supplemental Fig. 3) in TLR2-deficient microglial cells.

**B7-H3 exaggerates the clinical disease status in *S. pneumoniae*-infected mice**

Within 30 h after *S. pneumoniae* inoculation, all infected wild-type mice displayed moderate signs of disease as assessed by spontaneous motor activity and body weight loss. However, combined treatment of mice with *S. pneumoniae* and B7-H3 further exaggerated the severity of disease with significantly decreased spontaneous motor activity and increased body weight loss (*p* < 0.05 versus mice challenged with *S. pneumoniae* alone) (Fig. 7). Moreover, administration of the anti–B7-H3 mAb improved the clinical disease status in *S. pneumoniae*-infected, wild-type mice (*p* < 0.05 versus mice challenged with *S. pneumoniae* alone) (Fig. 7). Consistent with the previous finding that B7-H3 was unable to amplify *S. pneumoniae*-induced inflammatory response and BBB interruption in TLR2-deficient mice, no significant difference in the clinical status was observed in TLR2-deficient mice challenged with either *S. pneumoniae* alone or a combination of *S. pneumoniae* and B7-H3 (data not shown).

**Discussion**

Invasive pneumococcal disease caused by *S. pneumoniae* is a leading cause of morbidity and mortality worldwide, particularly in developing countries where this illness continues to be a major burden in hospitalized patients (32, 33). Pneumococcal meningitis occurs when bacteria invade into the brain, and it remains a serious life-threatening condition with an overall case fatality rate of up to 20–30% and a 25–45% risk of neurologic sequelae, including hearing loss, neurologic deficits, and long-term learning and behavioral disabilities in the survivors (1–4, 29, 34). Bacterial factors and the host innate immunity-initiated inflammatory response both contribute to the development of pneumococcal meningitis and its intracranial complications (7–10, 35). Although...
the inflammatory response triggered by the invaded S. pneumoniae normally helps to eradicate pathogens from the CNS, a persistent and/or amplified activation of this response with the excessive proinflammatory cytokine production in the CNS may cause severe damage to the brain and thus be predominantly responsible for unfavorable outcomes during the development of pneumococcal meningitis (5, 7, 10, 11).

It has been well documented that B7-H3, a newly discovered member of the B7 superfamily, acts as both a T cell costimulator and coinhibitor, and thus plays an important role in regulating T cell-mediated immune responses (12–15). Our recent work has identified that B7-H3, in addition to its impact on T cell activation, also functions as a costimulator of host innate immunity by augmenting proinflammatory cytokine release from bacterial cell wall components LPS- and BLP-stimulated monocytes/macrophages (22). In this study, we found that the exogenous administration of B7-H3 via intracerebral ventricular inoculation in S. pneumoniae-infected wild-type mice strongly amplified the S. pneumoniae-stimulated inflammatory response as demonstrated by a substantial further accumulation of proinflammatory cytokines TNF-α, IL-1β, IL-6, and chemokine MCP-1 in the brain at both the protein level and mRNA level. Our in vitro experiments where B7-H3 stimulation led to a significantly increased release of TNF-α, IL-1β, IL-6, and MCP-1 from S. pneumoniae-challenged primary murine microglial cells further clarify that microglial cells, the resident macrophage-like population of the CNS, may be responsible, at least in part, for B7-H3–augmented proinflammatory cytokine and chemokine production seen in S. pneumoniae-infected wild-type mice. Furthermore, B7-H3 displayed an augmentative effect on S. pneumoniae-induced activation of the TLR2 downstream signaling pathway, as the addition of B7-H3 significantly increased NF-κB p65 and MAPK p38 phosphorylation and substantially enhanced the nuclear transactivation of NF-κB p65 at both TNF-α and IL-6 promoters in S. pneumoniae-stimulated microglial cells. Thus, the upregulated activation of TLR2 signaling by B7-H3 may be one of the underlying mechanisms responsible for the B7-H3–amplified inflammatory response observed in S. pneumoniae-infected mice.

Accumulated data have demonstrated that a consequence of meningeal inflammation during S. pneumoniae-induced bacterial meningitis is an alteration in BBB integrity, and the resultant BBB disruption contributes substantially to the development of CNS complications (5, 6, 29, 36). Consistent with this, the B7-H3–amplified inflammatory response in the CNS seen in the present study is also associated with increased disruption of BBB integrity and deteriorated clinical disease status. Challenge with B7-H3 caused a further increase in BBB permeability with extensive extravasation of serum albumin and IgG into the brain and exaggerated the severity of disease with significantly impaired spontaneous motor activity and increased body weight loss in S. pneumoniae-infected wild-type mice. Thus, our in vivo data indicate that B7-H3 not only amplifies S. pneumoniae infection-initiated inflammatory response in the CNS, but also results in aggravated damage to BBB integrity, suggesting that B7-H3 plays a detrimental role in the development of pneumococcal meningitis and its intracranial complications. In contrast, another member of the B7 costimulatory superfamily B7-H4 showed no augmentative effect on either S. pneumoniae-stimulated proinflammatory cytokine production or S. pneumoniae-induced BBB disruption, demonstrating that in comparison with S. pneumoniae alone-treated mice, the observed amplified inflammatory response and exacerbated BBB disruption in S. pneumoniae plus B7-H3–treated mice is B7-H3 specific.

Although administration of exogenous B7-H3 led to a substantially enhanced inflammatory response and exacerbated BBB disruption in S. pneumoniae-infected wild-type mice in this study, a fundamental question that has to be addressed is whether B7-H3 contributes to the development of S. pneumoniae infection-induced bacterial meningitis. To test this, we treated S. pneumoniae-infected wild-type mice with the anti–B7-H3 mAb, MIH35. Administration of MIH35 mAb significantly attenuated proinfla-
flamatory cytokine and chemokine production, markedly alleviated BBB disruption, and improved clinical disease status, demonstrating a contributory role of B7-H3 in the development of *S. pneumoniae* infection-associated meningitis. Although we did not measure the level of B7-H3 in *S. pneumoniae*-infected mice in the present study, our previous work has shown that significantly elevated levels of sB7-H3 are detected in the serum and cerebrospinal fluid samples from patients diagnosed with bacterial meningitis and correlate closely with the severity of disease (23), thus further supporting our hypothesis that B7-H3 participates actively in the pathogenesis of bacterial meningitis.

The definitive costimulatory receptors on monocytes/macrophages responsible for B7-H3–induced augmentation of innate immunity-initiated inflammatory responses have not yet been identified; however, our recent work revealed that both TLR4 signaling and TLR2 signaling were involved in B7-H3–amplified NF-κB activation in response to either the TLR4 agonist LPS or the TLR2 agonist BLP (22). To further clarify whether the B7-H3–mediated effects observed in the present study are also dependent on TLR2 signaling, we repeated our in vitro and in vivo experiments in TLR2-deficient microglial cells and TLR2-deficient mice. In contrast to an augmentative effect on *S. pneumoniae*-stimulated, wild-type microglial cells, B7-H3 was unable to enhance proinflammatory cytokine TNF-α, IL-1β, IL-6, and chemokine MCP-1 release from *S. pneumoniae*-stimulated TLR2-deficient microglial cells and to upregulate phosphorylated NF-κB p65 and MAPK p38 expression in *S. pneumoniae*-stimulated TLR2-deficient microglial cells. Consistent with the in vitro finding in TLR2-deficient microglial cells, B7-H3 almost completely lost its amplifying action in TLR2-deficient mice, as the additional challenge with B7-H3 failed to augment proinflammatory cytokine and chemokine production and to aggravate BBB disruption in *S. pneumoniae*-infected, TLR2-deficient mice. These in vivo and in vitro data clearly indicate that TLR2 signaling is essential for the observed B7-H3 action during *S. pneumoniae* infection-induced bacterial meningitis.

TLR2 acts primarily as an innate sensor of Gram-positive bacteria by detecting their cell wall components including BLP, peptidoglycan, and lipoteichoic acid, and triggers the transcriptional inflammatory response through activation of NF-κB, leading to the production of proinflammatory cytokines (37–40). Accordingly, TLR2-deficient cells are unable to respond to Gram-positive bacterial cell wall components (41, 42) and mice with targeted disruption of *tlr2* gene are hyporesponsive to peptidoglycan stimulation but are susceptible to *Staphylococcus aureus* infection (41, 43). In supporting this, we also observed in the present study that TLR2-deficient mice in response to intracerebral *S. pneumoniae* infection exhibited less meningeal inflammation and disease severity, indicating that B7-H3 may act as a potential target candidate for altering the effects of pneumococcal meningitis.

### Disclosures

The authors have no financial conflicts of interest.

### References


