Interleukin-18 Protects Splenectomized Mice from Lethal *Streptococcus pneumoniae* Sepsis Independent of Interferon-γ by Inducing IgM Production

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The mechanism of the susceptibility of splenectomized mice to *Streptococcus pneumoniae* infection and the therapeutic effect of interleukin (IL)–18 were investigated. We demonstrated that, although *S. pneumoniae* challenge induced IL-12 production, it did not induce either interferon (IFN)–γ or IL-18 production in mice with or without a splenectomy. Liver mononuclear cells stimulated with heat-killed *S. pneumoniae* but not with viable *S. pneumoniae* produced IFN–γ in vitro. However, IL-18 pretreatment recovered the low serum immunoglobulin (Ig) M levels in splenectomized mice and completely inhibited mortality after *S. pneumoniae* infection without any IFN–γ up-regulation. Injection of IgM from noninfected control mice into splenectomized mice before infection confirmed the essential role that IgM plays against *S. pneumoniae* infection. Therefore, low serum IgM levels but not a low IFN–γ response in splenectomized mice cause lethality in *S. pneumoniae* infection, and IL-18 pretreatment protects them from infection by increasing IgM levels before infection.

Despite the development of antibiotics, pneumococcal pneumonia continues to be a major cause of hospitalization and death [1–3], and antibiotic-resistant *Streptococcus pneumoniae* has recently emerged [4]. Splenectomized hosts are extremely susceptible to infection by this bacterium [5–7]. It is widely accepted that the Th1 cytokines interleukin (IL)–12, IL-18, and interferon (IFN)–γ play a crucial role in host defense against various bacterial and viral infections [8–13]. We also previously reported that liver NK cells as well as NK T (NKT) cells stimulated with IL-12 and/or IL-18 play an important role in IFN–γ production and defense against gram-negative bacterial infections [14–16]. However, although one study reported that IFN–γ played a protective role in host response to pneumococcal infection [17], another study reported that IFN–γ did not play a protective role [18]. On the other hand, Lauw et al. reported that IL-18–deficient mice but not IL-12–deficient mice had an increased susceptibility to *S. pneumoniae* infection, even though both cytokines are essential IFN–γ inducers [19]. In addition, IL-12–neutralizing antibody (Ab) did not aggravate pneumococcal infection [19]. Therefore, although IL-12 and IFN–γ play a critical role against bacterial infections, their role in *S. pneumoniae* infection has yet to be elucidated.

Pneumococcal polysaccharides (PPSs) are the main virulence factors that protect pneumococci from the host defense [20, 21]. In humans, vaccination with PPS has been shown to stimulate IgG production [22, 23] and to protect healthy adults from pneumococcal in-
fections [24, 25]. Natural IgM has also been reported to play a crucial role against severe bacterial infections [26]. Brown et al. reported that the natural IgM and the classical complement pathway, partially targeted by the binding of natural IgM to bacteria, is the dominant pathway for activating the complement system in innate immunity to S. pneumoniae [27]. In the present study, we investigated the IFN-γ and IL-18 as well as the IgM responses of mice to S. pneumoniae infection and the therapeutic effects of these molecules in splenectomized mice with pneumococcal sepsis.

MATERIALS AND METHODS

This study was conducted in accordance with the guidelines of the Institutional Review Board for the Care of Animal Subjects at the National Defense Medical College, Tokorozawa, Japan.

Mice and procedure of splenectomy. Male C57BL/6 mice (8 weeks old; Charles River) were studied. Splenectomies were performed using an electric scalpel under deep ether anesthesia through a left-sided lateral 1.0-cm subcostal incision. Sham operations were also performed by the same incision process.

Bacteria and reagents. S. pneumoniae serotype 3 (ATCC 6303; Amco) grown in a brain-heart infusion broth (Difco), recombinant IL-2 (Shionogi Pharmaceutical), mouse recombinant IL-12 (R&D Systems), mouse recombinant IL-18 (MBL), mouse IgM (CHEMICON International), and mouse recombinant IFN-γ (PEPROTECH) were used.

S. pneumoniae challenge and collection of blood samples or tissue homogenates. The mice were intravenously (iv) injected with 1 × 10⁶ cfu of S. pneumoniae at 2 weeks after either a splenectomy or a sham operation. Blood samples were obtained from the retro-orbital plexus, and then the serum samples were stored at −80°C for ELISA. The liver, spleen, and lungs were removed from the mice to produce 1 mL of homogenized PBS suspension. The homogenates were centrifuged at 400 g for 15 min, and then the supernatants were stored at −80°C for ELISA.

IL-18, IgM, and IFN-γ treatments. Multiple IL-18 treatments were performed by intraperitoneal (ip) injections of IL-18 (0.2 µg/0.5 mL/mouse) [16, 28] at days 10, 12, and 14. Two hours after the last IL-18 injection, S. pneumoniae was iv injected into the splenectomized mice. Single IL-18 treatment was performed by an injection of IL-18 at 2 h before the S. pneumoniae challenge. Sham treatments were also performed by ip injections of PBS (0.5 mL). IgM treatment was performed

Figure 1. Effects of splenectomy on mouse survival rate (A) and on serum levels of interleukin (IL)-12 (B) and IL-18 (C) after Streptococcus pneumoniae challenge. Mice were intravenously challenged with S. pneumoniae (1 × 10⁶ cfu) at 2 weeks after either a splenectomy or a sham operation, and survival was monitored. Serum samples were obtained from the mice immediately before the bacterial injections and at 1, 3, 6, 12, 24, and 72 h after the bacterial injections, to measure IL-12 and IL-18 levels. Data are the mean ± SE results from 10 mice in each group. *P<.01, vs. the sham group.
Figure 2. Effects of pneumococcal infection on liver and spleen mononuclear cells (MNCs). A, Flow-cytometric analysis of NK cells and NK T (NKT) cell fractions in liver MNCs at 24 h after intravenous (iv) Streptococcus pneumoniae or PBS challenge. Liver MNCs were stained with fluorescein isothiocyanate–conjugated anti–mouse T cell receptor (TCR) αβ monoclonal antibody (MAb) and phycoerythrin-conjugated anti–mouse NK1.1 MAb. The percentages of lymphocyte subsets shown are the mean ± SE results from 5 mice. B, Expression of interleukin-12 receptor β1 (IL-12Rβ1) and IL-12Rβ2 mRNA, analyzed by reverse-transcriptase polymerase chain reaction. Liver and spleen MNCs were obtained from mice at 3 h after S. pneumoniae or PBS challenge. C, Interferon (IFN)–γ production by liver and spleen MNCs stimulated with IL-2 and IL-12 in vitro. Liver and spleen MNCs were obtained from mice at 2 h after S. pneumoniae or PBS challenge and were then cultured with 10 U/mL IL-2 and 25 ng/mL IL-12. Data are the mean ± SE results from 5 mice in each group. GAPDH, glyceraldehyde-3-phosphate-dehydrogenase. *P < .01, for the indicated comparison.

by an ip injection of IgM (350 μg/0.5 mL/mouse) at 1 h before the S. pneumoniae challenge or at 6 h after the challenge. IFN-γ treatment was performed by an ip injection of IFN-γ (0.5 μg [5000 U]/0.5 mL) [29] immediately before the S. pneumoniae challenge. Sham treatment was also performed by an ip injection of PBS (0.5 mL).

Isolation of liver, spleen, and bone marrow (BM) mononuclear cells (MNCs). Liver and spleen MNCs were obtained as described elsewhere [15, 30, 31]. Briefly, each liver was minced, suspended in RPMI 1640 medium containing 0.05% collagenase (Wako), and shaken for 20 min in a 37°C water bath. The liver specimen was then passed through a 200-gauge
Figure 3. In vitro interferon (IFN)–γ production by liver and spleen mononuclear cells (MNCs) from nontreated control mice stimulated with viable or heat-killed *Streptococcus pneumoniae*. A, Stimulation of liver and spleen MNCs (2.5 × 10⁶ cells/ml) with viable or heat-killed *S. pneumoniae* (5 × 10⁵, 5 × 10⁴, or 5 × 10³ cfu/ml) for 24 h. Supernatants were subjected to ELISA. Data are mean ± SE results from 3 independent experiments with 1 or 2 mice in each experiment. B, Stimulation of liver MNCs with viable *S. pneumoniae* (5 × 10⁶ cfu/ml), heat-killed *S. pneumoniae* (5 × 10⁵ cfu/ml), or a combination of viable *S. pneumoniae* (5 × 10⁵ cfu/ml) and heat-killed *S. pneumoniae* (5 × 10⁵ cfu/ml) for 24 h. Supernatants were subjected to ELISA. Data are mean ± SE results from 5 mice in 2 independent experiments. *P < .01, for the indicated comparison.

Stainless steel mesh. After washing, cells were obtained by use of 33% Percoll solution. Splenocytes were also passed through the stainless steel mesh and then were treated with red blood cell (RBC) lysis solution. BM MNCs were obtained from the right femoral bone by an injection of 2 mL of RPMI 1640 and then were treated with the RBC lysis solution.

**Cell cultures.** After the cells were counted, 5 × 10⁵ of the liver, spleen, or BM MNCs in 200 μL of 10% fetal bovine serum/RPMI 1640 medium were cultured in 96-well, flat-bottomed plates in 5% CO₂ at 37°C for 24 h, and then the culture supernatants were stored at −80°C until the assays were performed.

**Measurement of cytokine and immunoglobulin levels in serum samples, tissue homogenates, and culture supernatants.** The IFN-γ and IL-12 levels in the serum samples, tissue homogenates, and culture supernatants were measured by use of cytokine-specific ELISA kits (Endogen). The IL-18 levels in the serum samples were measured by use of a mouse IL-18 ELISA kit (MBL). The serum samples were usually 10-fold diluted by the assay buffer included in the respective ELISA kits and were used to perform the measurements. The IgM, IgG1, and IgG2a levels in the serum samples and the IgM levels in the culture supernatants were measured using a mouse IgM, IgG1, or IgG2 ELISA quantitation kit (Bethyl Laboratories).

**Phenotypical analysis of liver MNCs.** The liver MNCs extracted from the mice at 24 h after the *S. pneumoniae* challenge were characterized by 2-color flow-cytometry analysis by use of fluo escein isothiocyanate–conjugated anti–mouse T cell receptor αβ monoclonal Ab (MAb) (BD PharMingen) and phycoerythrin-conjugated anti-NK1.1 MAb (BD PharMingen).

**Reverse-transcriptase polymerase chain reaction (RT-PCR) analysis.** The sequences of oligonucleotide primers for RT-PCR analysis were as follows: For mouse IL-12 receptor β1 (IL-
12Rβ1), 5′-AACCTTGATCATCAGGGGTG-3′ (antisense) and 5′-GAACCAACACACTGCTACCTG-3′ (sense) were used [32]. For mouse IL-12Rβ2, 5′-AATCTTGATCATCAGGGGTG-3′ (antisense) and 5′-GAGTACATAGTTGAAATGGAAGAGG-3′ (sense) were used. For mouse glyceraldehyde-3-phosphate-dehydrogenase, 5′-TTCAGGATTTCTTATCCTCCTG-3′ (antisense) and 5′-ATGACCAAGTTCCATGCAAT-3′ (sense) were used.

**Statistical analysis.** Data are presented as mean ± SE values. Statistical analyses were performed using an iMac computer (Apple) and the Stat View software package (version 4.02); Abacus Concepts. Survival rates were compared using the Wilcoxon rank test, whereas other statistical evaluations were done using standard 1-way analysis of variance followed by Student’s *t* test.

**RESULTS**

No increase in either serum IL-18 or IFN-γ levels and a decrease in survival rate in splenectomized mice after *S. pneumoniae* challenge, despite the splenectomized mice showing increased serum IL-12 levels. Mice were iv injected with 1×10^6 cfu of *S. pneumoniae* at 2 weeks after either a splenectomy or a sham operation. Serum samples were obtained from the mice at the time points indicated in figure 1 after *S. pneumoniae* challenge. The splenectomized mice showed a significantly decreased survival rate after *S. pneumoniae* challenge (figure e 1A). Although the serum IL-12 levels increased similarly in both mouse groups (figure e 1B), the serum IL-12 levels did not increase (figure e 1C), and serum IFN-γ levels were undetectable during the course of *S. pneumoniae* infection in both mouse groups (data not shown).

No significant change in the populations of NK and NKT cells and the expression of IL-12 mRNA in liver and spleen MNCs, and the potential of liver MNCs to respond to IL-12 in vitro by producing IFN-γ, after pneumococcal challenge. Next, we examined the proportions of NK and NKT cells and IL-12R mRNA expression in liver MNCs after *S. pneumoniae* challenge in the nontreated normal mice, because liver MNCs (especially NKT cells) express IL-12Rβ1 and IL-12Rβ2 [33] and because NKT cells are the main IL-12γ producers in response to IL-12 [15, 32, 34]. Liver and spleen MNCs were obtained at 24 h after *S. pneumoniae* or PBS injection. The population of either NK or NKT cells in the liver (n = 5) did not significantly differ between the mice challenged with *S. pneumoniae* and PBS (figure e 2A). The total number of liver MNCs tended to increase in mice at 24 h after *S. pneumoniae* challenge (3.2×10^6 ± 0.5×10^6 cells/mL) (n = 5) in comparison with that in PBS-injected mice (2.5×10^6 ± 0.3×10^6 cells/mL) (n = 5), but the difference was not statistically significant IL-12Rβ1 and IL-12Rβ2 mRNA expression in liver MNCs at 3 h after *S. pneumoniae* challenge did not decrease (figure e 2B). In contrast, splenocytes of both mice did not express IL-12Rβ1 probably because the spleen has few NKT cells. When the liver and spleen MNCs were isolated from the mice at 2 h after *S. pneumoniae* or PBS challenge and were then cultured with IL-2 and IL-12 for 24 h, the liver MNCs from mice challenged with *S. pneumoniae* increased IFN-γ production remarkably. In contrast, the spleen MNCs produced small amounts of IFN-γ (figure e 2C). These results suggest that, although the MNCs of *S. pneumoniae*-infected mice have a capacity to produce a large amount of IFN-γ in response to IL-12 in vitro, IFN-γ production by MNCs (especially NKT cells) in response to endogenous IL-12 in vivo is somehow inhibited.

*Stimulated production of IFN-γ by liver and spleen MNCs with heat-killed *S. pneumoniae* but not with viable *S. pneumoniae* in vitro.* We also investigated the potential of liver and spleen MNCs to produce IFN-γ after in vitro stimulation with viable or heat-killed *S. pneumoniae*. IFN-γ production by the liver and spleen MNCs (2.5×10^6 cells/mL) stimulated with 5×10^6 cfu/mL heat-killed *S. pneumoniae* increased remarkably, compared with that by MNCs stimulated with viable *S. pneumoniae* (5×10^6 cfu/mL) (figure e 3A). Furthermore, when liver and spleen MNCs were stimulated simultaneously with both viable (5×10^6 cfu/mL) and heat-killed (5×10^6 cfu/mL) *S. pneumoniae*, the production of IFN-γ by the spleen MNCs was remarkably decreases. This decrease was reversed by incubation with IL-12 (figure e 3B). In contrast, no increases in IFN-γ production by liver MNCs were observed in response to *S. pneumoniae* stimulation.
pneumoniae, they did not increase IFN-γ production (fig. e 3B). These results, shown in fig. es 2 and 3, thus suggest that liver and spleen MNCs of S. pneumoniae–infected mice are potentially activated but that some components or factors of viable S. pneumoniae may inactivate MNCs. Indeed, although MNCs cultured with heat-killed S. pneumoniae made clusters and appeared to vigorously proliferate, MNCs cultured with viable S. pneumoniae did not (data not shown).

Lower serum IgM levels in splenectomized mice than in sham mice, both before and after S. pneumoniae challenge. We next examined serum immunoglobulin levels after S. pneumoniae challenge, because not only cellular immunity but also humoral immunity is crucial for host defense against bacterial infections [26, 35]. Interestingly, at 2 weeks after splenectomy, splenectomized mice showed a significant lower serum IgM level than did sham mice (fig. e 4A, day 0), whereas the serum IgG1 level in both mouse groups did not significantly differ (fig. e 4B, day 0). Furthermore, serum IgM levels did not effectively increase after S. pneumoniae challenge in splenectomized mice (fig. e 4A). Serum IgG1 levels did not increase within 7 days after the S. pneumoniae challenge in both mouse groups (fig. e 4B). These results suggest that the splenectomized mice have decreased IgM production.

Multiple IL-18 treatments increase mouse survival rate after S. pneumoniae challenge without up-regulation of IFN-γ but induce elevation of serum IgM levels. Because we previously reported that IL-18 treatment increases serum or tissue IFN-γ levels and improves the survival rate of mice infected with either Escherichia coli or Listeria monocytogenes by up-regulating IFN-γ [16, 28, 36], the splenectomized mice were thus ip injected with IL-18 at 10, 12, and 14 days after the splenectomy. At 2 h after the last IL-18 injection, the mice were challenged with S. pneumoniae (1 × 10^6 cfu). Splenectomized mice were also injected with IL-18 once at 2 h before S. pneumoniae challenge as a single IL-18 injection group. The multiple IL-18 injections but not the single IL-18 injection increased the mouse survival rate remarkably (fig. e 5A). However, unexpectedly, even the multiple IL-18 injections did not induce serum IFN-γ levels (undetectable) after S. pneumoniae challenge in the splenectomized mice. Consistently, IFN-γ levels in the liver, spleen (data not shown), and lung tissue homogenates did not significantly increase at 24 and 72 h after S. pneumoniae challenge in either the sham or the splenectomized mice with or without multiple IL-18 injections (table 1).

We next examined serum immunoglobulin levels after S. pneumoniae challenge in the IL-18–treated splenectomized

Figure 5. Effects of single or multiple interleukin (IL)–18 treatments on mouse survival rate (A) and on serum levels of IgM (B) and IgG1 (C) after Streptococcus pneumoniae challenge. Splenectomized mice were intraperitoneally (ip) injected with IL-18 (0.2 μg) or PBS at 10, 12, and 14 days after the splenectomy. At 2 h after the last IL-18 injection, the mice were intravenously (iv) challenged with S. pneumoniae (1 × 10^6 cfu), and then serum IgM and IgG1 levels and survival were monitored. The single IL-18 treatment was performed by an ip injection at 2 h before the S. pneumoniae iv challenge. Serum samples were obtained from the mice immediately before the bacterial injections and at 1, 3, 5, and 7 days after the bacterial injections, to measure immunoglobulin levels. Data are the mean ± SE results from 10 mice in each group. *P < .05, vs. the other group; **P < .05, vs. the PBS group.
treatments. MNCs were isolated from the liver, spleen, or not shown). A splenectomy or a sham operation. The splenectomized mice were intraperitoneally (IP) injected with IL-18 (0.2 μg) at 4 days, 2 days, and immediately before they were killed (multiple IL-18 IP treatment) or only immediately before they were killed (single IL-18 IP treatment). The proportion of B cells (CD5−CD11b+CD23−) was detected using flow-cytometric analysis. The proportion of B cells was counted in the lymphocyte gate. MNCs were cultured for 24 h to measure the IgM levels in the supernatants.

Liver

<table>
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<tr>
<th>Study group, tissue</th>
<th>IFN-γ level, pg/mL</th>
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<th>24 h after challenge</th>
<th>72 h after challenge</th>
<th>P</th>
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<td>Splenectomy</td>
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<tr>
<td>Liver</td>
<td>498 ± 23 508 ± 24 523 ± 36 NS</td>
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<td>Lungs</td>
<td>547 ± 47 640 ± 15 638 ± 16 NS</td>
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<td>Sham</td>
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<tr>
<td>Liver</td>
<td>517 ± 14 528 ± 10 502 ± 33 NS</td>
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<tr>
<td>Lungs</td>
<td>656 ± 88 660 ± 36 408 ± 163 NS</td>
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<td>Splenectomy and multiple IL-18 IP treatments</td>
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<tr>
<td>Liver</td>
<td>507 ± 26 518 ± 25 495 ± 11 NS</td>
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<td>Lungs</td>
<td>552 ± 74 626 ± 58 621 ± 56 NS</td>
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NOTE. Data are the mean ± SE results from 5 mice in each group. Mice were intravenously challenged with S. pneumoniae (1 × 10^9 CFU) at 2 weeks after either a splenectomy or a sham operation. The splenectomized mice were obtained from the mice either immediately before the bacterial injections or at 24 or 72 h after the bacterial injections, to measure IFN-γ levels in tissue homogenates. IL, interleukin; IP, intraperitoneal; NS, not significant.

Increased IgM production in liver MNCs after multiple IL-18 treatments. MNCs were isolated from the liver, spleen, or BM at 2 weeks after the splenectomy or sham operation and then were cultured for 24 h. MNCs were also isolated from the splenectomized mice that received the multiple IL-18 treatments. In the sham mice, the spleen MNCs actively produced more IgM (50.4 ± 4.1 μg/mL) (n = 5) than did either the liver or BM MNCs (table 2). On the other hand, the BM MNCs of splenectomized mice showed increased IgM production (table 2). The multiple IL-18 treatments markedly increased IgM production by the liver MNCs of splenectomized mice, but the single injection of IL-18 did not (table 2). The multiple IL-18 treatments significantly decreased IgM production by the BM MNCs of splenectomized mice (table 2). However, the proportion of surface IgM–expressing B cells in the liver MNCs of splenectomized mice was 43% (n = 5) and did not increase by the multiple IL-18 treatments (table 2). We also did not find any significant increase of B1 cells (CD5−CD11b−CD23−) in the liver of the IL-18–treated mice (data not shown).

Mouse survival rate improved by IgM injection either before or after S. pneumoniae challenge but by IFN-γ injection only before S. pneumoniae challenge. We next examined the effect that IgM (CHEMICON International) or IFN-γ injection has on survival rate after S. pneumoniae challenge in splenectomized mice. The splenectomized mice were IP injected with mouse IgM (350 μg/mouse) or PBS at 1 h before the S. pneumoniae challenge. This dose of IgM was estimated to increase 15–20 μg of IgM/20 g of mouse body weight, and serum IgM levels in the splenectomized mice 1 h after IgM injection, just before the S. pneumoniae challenge, ranged between 40 and 60 μg/mL. No IgM-treated mice died after the S. pneumoniae challenge, whereas most PBS-treated mice died within 8 days (n = 10/group) (figure 6A). On the other hand, mouse IgG1 treatment could not save the mice with S. pneumoniae infection (data not shown). Furthermore, when IgM was injected at 6 h

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<th>MNC type, measurement</th>
<th>Splenectomy and multiple IL-18 IP treatments</th>
<th>Splenectomy and single IL-18 IP treatment</th>
<th>Splenectomy</th>
<th>Sham</th>
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<td>IgM level, μg/mL</td>
<td>23.5 ± 3.8^a</td>
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<td>41.7 ± 3.2</td>
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<td>Bone marrow</td>
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<tr>
<td>IgM level, μg/mL</td>
<td>12.4 ± 2.5</td>
<td>22.0 ± 2.9^b</td>
<td>22.6 ± 2.4^b</td>
<td>13.9 ± 2.9</td>
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<tr>
<td>B cells, % of MNCs^c</td>
<td>35.7 ± 1.1 (11.5 ± 1.1)</td>
<td>34.7 ± 0.5 (12.4 ± 1.8)</td>
<td>35.6 ± 0.1 (13.6 ± 3.4)</td>
<td>24.9 ± 0.4 (11.0 ± 0.5)</td>
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NOTE. Data are the mean ± SE results from 5 mice in each group. Liver, spleen, and bone marrow MNCs were obtained from mice at 2 weeks after either a splenectomy or a sham operation. The splenectomized mice were intraperitoneally (IP) injected with IL-18 (0.2 μg) at 4 days, 2 days, and immediately before they were killed (multiple IL-18 IP treatment) or only immediately before they were killed (single IL-18 IP treatment). The proportion of B cells (B220−IgM−cells) was detected using flow-cytometric analysis. The proportion of B cells was counted in the lymphocyte gate. MNCs were cultured for 24 h to measure the IgM levels in the supernatants.

^a P < .01, vs. other mouse groups.

^b P < .05, vs. splenectomy and multiple IL-18 IP and sham.

^c Values in parentheses indicate the percentage of B cells in the total bone marrow cells.
after the *S. pneumoniae* challenge, 8 of 10 mice survived (figure e 6B). On the other hand, when IFN-γ (0.5 μg; half-life of 20 min) [37] was injected immediately before the *S. pneumoniae* challenge, 8 of 12 mice survived (figure e 6C), whereas IFN-γ injection did not increase mouse survival rate when it was injected at 6 h after the infection (data not shown). Consistently, *S. pneumoniae* preincubated with mouse serum and IgM for 3 h showed a much smaller number of colony-forming units than did *S. pneumoniae* preincubated with mouse serum alone (figure e 6D). These results confirm the protective effect of natural IgM induced by IL-18 and suggest that IFN-γ may exert a substantial protective effect against *S. pneumoniae* infection if it is properly produced.

**DISCUSSION**

*S. pneumoniae* induced an elevation in serum IL-12 levels, but it did not induce an elevation in either IFN-γ or IL-12 level in mice with or without a splenectomy. However, the in vitro IFN-γ production by the liver MNCs obtained from the mice after *S. pneumoniae* challenge had significantly increased by the subsequent stimulation of IL-2 and IL-12 in vitro. Furthermore, liver and spleen MNCs stimulated with heat-killed *S. pneumoniae* but not with viable *S. pneumoniae* in vitro produced a large amount of IFN-γ. Repeated IL-18 pretreatments of splenectomized mice increased IgM production by liver B cells and improved mouse survival rate after pneumococcal infection without any up-regulation of IFN-γ. Furthermore, the IgM transfer from normal mice before *S. pneumoniae* challenge completely inhibited mouse mortality, and the IgM transfer after the infection still reduced mouse mortality.

These results suggest that *S. pneumoniae* may escape the host defense by inhibiting both IFN-γ and IL-12 production by liver and spleen MNCs. *S. pneumoniae* infection may render NK cells and NKT cells unresponsive to endogenous IL-12 even though they have a potential capacity to produce IFN-γ in vitro in response to IL-12 and heat-killed *S. pneumoniae*, which may explain why IL-12-deficient mice or mice treated with anti–IL-12 Ab did not increase susceptibility to *S. pneumoniae* infection [19]. Our finding also account for the fact that susceptibility to *S. pneumoniae* infection did not increase

**Figure 6.** Survival rates of splenectomized mice treated with IgM or interferon (IFN)–γ after *Streptococcus pneumoniae* infection and in vitro effect of IgM on the no. of colony-forming units of cultured *S. pneumoniae*. At 2 weeks after splenectomy, mice were intraperitoneally (ip) injected with mouse IgM (350 μg) or PBS at 1 h before or at 6 h after intravenous challenge with *S. pneumoniae* 1 × 10⁶ cfu/mL or PBS, and then survival was monitored (A and B). The splenectomized mice were also ip injected with IFN-γ (0.5 μg) or PBS immediately before the *S. pneumoniae* challenge, and then survival was monitored (C). Data are the mean ± SE results from 10 or 12 mice in each group. *S. pneumoniae* (1 × 10⁷ cfu) was incubated with 100 μL of normal mouse serum together with 10 μg/10 μL IgM or 10 μL of PBS for 3 h at 37°C and then was cultured on agar plates for an additional 24 h, and colony-forming units were counted (D). Data are the mean ± SE results from 3 independent experiments. *P < .01, vs. the PBS group; **P < .05, for the indicated comparison.
in IFN-γ deficiency or IFN-γ receptor deficiency mice [18], because IFN-γ production in response to IL-12 per se is inhibited in normal mice with *S. pneumoniae* infection.

IgM acts as a potent complement activator during the early stage of infections. Binding IgM with bacteria and the activation of the complement system by IgM can either induce the lysis of bacteria or enhance the opsonization of bacteria for efficient phagocytosis by macrophages and neutrophils [26, 38]. The action of IgM is thus an important antibacterial defense mechanism that takes effect before the IgG-mediated defense begins. IgM induced by IL-18 is not likely *S. pneumoniae*-specific Ab, because the level of this Ab increased before the *S. pneumoniae* infection, thus suggesting that elevated IgM levels include natural IgM Ab. In fact, the results of the present study showed that incubation of *S. pneumoniae* with serum and IgM from noninfected mice suppressed the subsequent growth of *S. pneumoniae*. The important role that natural IgM and the complement system play in pneumococcal and other bacterial infections has also been reported elsewhere [26, 27]. It is noteworthy, however, that IL-18 treatments did not increase serum IgM levels in normal control mice ([39] and authors’ unpublished data), thus suggesting that IL-18 may stimulate IgM production in the livers of splenectomized mice to compensate for the IgM production in the spleens.

The adoptive transfer of IgM from other noninfected mice confirmed that IgM indeed protected splenectomized mice from otherwise lethal pneumococcal infection. Moreover, IgM also significantly inhibited mouse mortality even when it was injected after *S. pneumoniae* challenge. These findings suggest that, in addition to vaccination, IL-18 or IgM can be used as a new therapeutic strategy against pneumococcal infection.

The liver is considered to be an important organ in the Th1 immune response and in cellular immunity against bacterial infections and tumors because it contains abundant NK cells and NKT cells with a potent IFN-γ-producing capacity [14, 16, 28, 32, 40] and because >70% of the bacteria that enters the bloodstream accumulates in the liver and thus becomes entrapped by Kupffer cells and hepatocytes [14]. In addition, hepatocytes produce acute-phase proteins and complements [14]. In splenectomized mice that do not receive multiple IL-18 injections, BM may compensate for the role of the spleen in IgM production, whereas IL-18 treatment may shift such IgM production to liver B cells. The finding of the present study also show that the liver plays an important role in innate humoral immunity under certain inflammatory conditions.

It should be noted that the administration of exogenous IFN-γ before pneumococcal infection partially protected the mice regardless of the mice not being able to produce IFN-γ in response to *S. pneumoniae* infection. In addition, both mice that did or did not undergo a splenectomy did not effectively produce IL-18. This is in marked contrast to mice with *E. coli* infection [28] or mice injected with lipopolysaccharide [16], which produced large amounts of IL-12, IL-18, and IFN-γ. These findings indicate that *S. pneumoniae* escapes the host-defense mechanism (both cellular immunity and humoral immunity) by inhibiting IFN-γ production by NK cells and NKT cells as well as IL-18 production by Kupffer cells/macrophages via mutually independent mechanisms. We have recently reported that *L. monocytogenes* uses Th-1–defective spleen for its initial growth and, in contrast to encapsulated bacterial infection, splenectomy thereby inhibits *Listeria* infection [36]. Taken together with the results of the present study, it appears that each bacterium may have means to escape the host-defense mechanism.

References


15. Ogasawara K, Takeda K, Hashimoto W, et al. Involvement of NK1+


