Mice Deficient in Ficolin, a Lectin Complement Pathway Recognition Molecule, Are Susceptible to *Streptococcus pneumoniae* Infection


*J Immunol* 2012; 189:5860-5866; Prepublished online 12 November 2012;
doi: 10.4049/jimmunol.1200836
http://www.jimmunol.org/content/189/12/5860

---

**Supplementary Material**

http://www.jimmunol.org/content/suppl/2012/11/12/jimmunol.1200836.DC1

**References**

This article cites 47 articles, 17 of which you can access for free at:
http://www.jimmunol.org/content/189/12/5860.full#ref-list-1

**Subscription**

Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

**Permissions**

Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**

Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Mice Deficient in Ficolin, a Lectin Complement Pathway Recognition Molecule, Are Susceptible to *Streptococcus pneumoniae* Infection

Yuichi Endo,* Minoru Takahashi,* Daisuke Iwaki,* Yumi Ishida,* Naomi Nakazawa,* Toshihisa Kodama,* Tomohiro Matsuazaka,* Kazuko Kanno,* Yu Liu,* Kohsuke Tsuchiya, ‡ Ikuko Kawamura, † Masahito Ikawa, ‡ Satoshi Waguri, § Ikuo Wada, † Misao Matsushita, † Wilhelm J. Schwaeble, † and Teizo Fujita*†

Mannose-binding lectin (MBL) and ficolin are complexed with MBL-associated serine proteases, key enzymes of complement activation via the lectin pathway, and act as soluble pattern recognition molecules in the innate immune system. Although numerous reports have revealed the importance of MBL in infectious diseases and autoimmune disorders, the role of ficolin is still unclear. To define the specific role of ficolin in vivo, we generated model mice deficient in ficolins. The ficolin A (Fcna)-deficient (Fcna−/−) and FcnA/ficolin B double-deficient (Fcna−/−b−/−) mice lacked FcnA-mediated complement activation in the sera, because of the absence of complexes comprising FcnA and MBL-associated serine proteases. When the host defense was evaluated by transnasal infection with a *Streptococcus pneumoniae* strain, which was recognized by ficolins, but not by MBLS, the survival rate was significantly reduced in all three ficolin-deficient (Fcna−/−, Fcnb−/−, and Fcn−/−b−/−) mice compared with wild-type mice. Reconstitution of the FcnA-mediated lectin pathway in vivo improved survival rate in Fcna−/− but not in Fcn−/−b−/− mice, suggesting that both FcnA and ficolin B are essential in defense against *S. pneumoniae*. These results suggest that ficolins play a crucial role in innate immunity against pneumococcal infection through the lectin complement pathway. **The Journal of Immunology**, 2012, 189: 5860–5866.

The complement system plays a crucial role in protecting against invading microorganisms through three activation pathways: the classical, alternative, and lectin pathways. These activation routes focus to activate the central complement component C3, and finally mediate many immune responses including opsonization, phagocytosis, cytokine production, and chemotaxis. Model animals deficient in complement components have provided evidence for the roles of the classical and alterna-

---

*Department of Immunology, Fukushima Medical University School of Medicine, Fukushima 960-1295, Japan; †Department of Microbiology, Kyoto University Graduate School of Medicine, Sakyo-ku, Kyoto 606-8501, Japan; ‡Genome Information Research Center, Osaka University, Suita, Osaka 565-0871, Japan; †Department of Anatomy and Histology, Fukushima Medical University School of Medicine, Fukushima 960-1295, Japan; §Department of Cell Science, Institute of Biomedical Sciences, Fukushima Medical University, Fukushima 960-1295, Japan; †Department of Applied Biochemistry, Tokai University, Hiratsuka, Kanagawa 259-1292, Japan; and ‡Department of Microbiology and Immunology, University of Leicester, Leicester LE1 9HN, United Kingdom

Received for publication March 15, 2012. Accepted for publication October 13, 2012.

This work was supported by grants from the Ministry of Education, Science, Sports and Technology of Japan (to Y.E. and T.F.), MRC Grant G0801952 (to W.J.S.), and the Core Research for Evolutional Science and Technology, the Japan Science and Technology Agency (to T.F.).

Address correspondence and reprint requests to Dr. Yuichi Endo, Department of Immunology, Fukushima Medical University School of Medicine, 1-Hikarigaoka, Fukushima 960-1295, Japan. E-mail address: yendo@fmu.ac.jp

The online version of this article contains supplemental material.

Abbreviations used in this article: FcnA, ficolin A; FcnB, ficolin B; GlcNAc, N-acetylglucosamine; H-FCN, H-ficolin; L-FCN, L-ficolin; MAP, mannose-binding lectin-associated serine protease; MBL, mannose-binding lectin; M-FCN, M-ficolin; rFcna, recombinant FcnA; rFcnb, recombinant FcnB; sMAP, small MBL-associated protein; TBS-Ca, TBS containing 2.5 mM CaCl₂; TBS-CaT, TBS-Ca containing 0.05% Tween 20; WT, wild-type.

Copyright © 2012 by The American Association of Immunologists, Inc. 0022-1767/12S16.00

www.jimmunol.org/cgi/doi/10.4049/jimmunol.1200836
ficolins. More specifically, human M-ficolin (M-FCN, FCN-1) is the ortholog of murine ficolin B (FcnB); Human L-ficolin (L-FCN, FCN-2) is closely related to murine ficolin A (Fcna), although the genes encoding these ficolins are suggested to have evolved independently in each murine and primate lineage, respectively (18). The H-ficolin (H-FCN, FCN-3) gene (FCNJ) is a pseudogene in the murine lineage. Accumulating data indicate that each ficolin recognizes an overlapping spectrum of microorganisms (5, 6, 19, 20), and that the deficiency and/or low level of human ficolins has been associated with specific infectious diseases (21–23).

To evaluate the contribution of ficolins in innate immunity, we generated three mouse lineages deficient in ficolins in this study. Based on the observations of their in vitro and in vivo phenotypes, we provide in this study the first evidence, to our knowledge, that the ficolin-mediated lectin pathway plays an essential role in protection against Streptococcus pneumoniae infection that is a major cause of pneumonia, septicemia, otitis media sinusitis, and meningitis.

**Materials and Methods**

**Mice**

Fcna-deficient (Fcna<sup>−/−</sup>) and FcnB-deficient (Fcnb<sup>−/−</sup>) mouse lineages were generated according to the standard protocol of gene targeting (Supplemental Fig. 1). Targeted embryonic stem cells (129SV) were implanted into mouse C57BL/6J blastocysts to generate chimeric mice. Finally, intercrossing respective F2 heterozygous offspring was used to produce homozygous Fcna<sup>−/−</sup> and Fcnb<sup>−/−</sup> mice. These knockout mice were backcrossed to C57BL/6J, and Fcna<sup>−/−</sup> and Fcnb<sup>−/−</sup> mice used in this study were in 15th and 10th filial generations, respectively.

Double-heterozygous Fcna<sup>−/−</sup> Fcnb<sup>+/−</sup> mice carrying the haplotype Fcna<sup>b+</sup> were interbred to generate a lineage of Fcna<sup>b−</sup>Cnb<sup>b−</sup> double-deficient (Fcna<sup>−/−</sup>Fcnb<sup>−/−</sup>) mice. As previously reported (18), the Fcna and Fcnb genes are located on the same chromosome (2A3). Therefore, Fcna<sup>+/−</sup> Fcnb<sup>−/−</sup> and Fcna<sup>−/−</sup> Fcnb<sup>+/−</sup> mice were initially crossed to generate the offspring carrying haplotype Fcna<sup>b−</sup>. The obtained double-heterozygous mice carrying genotype Fcna<sup>−/−</sup>Fcnb<sup>−/−</sup> were then interbred to generate the Fcna<sup>−/−</sup>Fcnb<sup>−/−</sup> offspring.

Transiently FcnA-expressing Fcna<sup>−/−</sup> and Fcnb<sup>−/−</sup> mice were produced using a piggyBac transposon-mediated long-term gene expression system (24). In brief, Fcna cDNA was constructed in a piggyBac plasmid between inverted terminal repeats. Twenty micrograms of the plasmid was mixed with 40 μg pFRh plasmid encoding a transposase in 2.6–2.9 ml of a hydrodynamic delivery solution (Mirus Bio LLC, Madison, WI), and the mixture was injected into the mice via the tail vein according to the manufacturer’s instruction. FcnA expression was monitored using Western blotting of the serum, and the formation of Fcna/MASP-2/5sMAP complex was assessed by N-acetylglucosamine (GlcNAc)-agarose chromatography followed by Western blotting.

All DNA recombination and animal studies were conducted according to the guidelines of Fukushima Medical University and Osaka University.

**Recombinant lectins**

Recombinant Fcna (rFcna) was prepared by using a Drosophila expression system with the pMT/Bip/V5-His A vector (Invitrogen, Carlsbad, CA) as described previously (25). Recombinant FcnB (rFcnb) was produced in the Chinese hamster ovary cells using a piggyBac transposon-mediated gene expression system: cotransfection with mouse FcnB cDNA-containing pRCMV plasmid and pFRh plasmid. The rFcnb secreted into the culture media (CHO-S-SFM; Life Technologies, Grand Island, NY) was purified by affinity chromatography with a GlcNAc-agarose column. The protein concentrations were determined using a BCA protein assay kit (Pierce, Rockford, IL). Recombinant mouse MBL-A (rMBL-A) and MBL-C (rMBL-C) were obtained from R&D Systems (Minneapolis, MN).

**Affinity chromatography**

Affinity chromatography with GlcNAc-agarose (Sigma-Aldrich, St. Louis, MO) was performed to isolate ficolins in the mouse serum separately from MBLs (MBL-A and MBL-C). In brief, the pooled serum was incubated with a 0.4 vol of 50% GlcNAc-agarose slurry in TBS containing 2.5 mM CaCl2 (TBS-Ca) at 4˚C for 3 h, and the bound fraction after washing with TBS-Ca containing 0.05% Tween 20 (TBS-CaT) was sequentially eluted with 0.3 M mannosse (mannose-eluate) and then 0.3 M GlcNAc (GlcNAc-eluate).

**Western blotting**

Western blotting was performed under reducing conditions using the primary Abs against Fcna and Fcnb (25), MASP-1 (26), MASP-2 (27), MBL-A, and MBL-C (HyCult Biotechnology, Uden, The Netherlands) as described previously. Signals were detected by further incubation with HRP-conjugated secondary Abs (DakoCytomation, Glostrup, Denmark), avidin-biotinylated HRP complex (Vector Laboratories, Burlingame, CA). Enzymatic activity was determined by incubation with 3,3’,5,5’-tetramethylbenzidine (KPL, Gaithersburg, MD) and H2O2. After termination of the reaction with 0.5 M H2PO4, color was monitored at 450 nm as described earlier.

**ELISA**

To quantify FcnA in the mouse serum, we incubated 10 μl serum on a rabbit anti-FcnA IgG-coated microtiter plate in 100 μl PBS at room temperature for 1 h. Fcna<sup>−/−</sup> serum containing known amounts of rFcna was used as a standard. Bound FcnA was detected with HRP-conjugated rabbit anti-Fcna Fab’. Color was developed using ABTS (Zymed Laboratories, South San Francisco, CA) and H2O2, and monitored in a MultiMode Detector (Beckman Coulter, Fullerton, CA) at 405 nm.

To quantify FcnB in the Fcna<sup>−/−</sup> sera, we incubated 15 μl serum on a rabbit anti-FcnB IgG-coated microtiter plate. Pooled serum of Fcna<sup>−/−</sup>Fcnb<sup>−/−</sup> mice containing known amounts of rFcnb was used as a standard. Bound FcnB was detected with biotin-labeled anti-FcnB Ab and avidin-biotinylated HRP complex (Vector Laboratories, Burlingame, CA). Enzymatic activity was determined by incubation with 3,3’,5,5’-tetramethylbenzidine (KPL, Gaithersburg, MD) and H2O2. After termination of the reaction with 0.5 M H2PO4, color was monitored at 450 nm as described earlier.

**Complement activation assay**

C4-deposition assay was used to evaluate complement activation via the lectin pathway as previously described (25, 27). In brief, mouse serum, mannosse-eluate, or GlcNAc-eluate was incubated on a GlcNAc-BSA-coated microtiter plate in 100 μl TBS-Ca at 37˚C for 10 min. Plates were further incubated with human C4 at 4˚C for 30 min, and the bound C4b was detected with HRP-avidin human C4 Ab (Biogenesis, Poole, U.K.). C4b deposited was developed by incubation with 3,3’,5,5’-tetramethylbenzidine and H2O2 for 5–60 min at room temperature and monitored as described earlier. The activity was expressed as absorbance at 450 nm at 5-min incubation.

C3 deposition was assessed by incubating 1 × 10<sup>5</sup> cells of heat-killed S. pneumoniae D39 strain with 10 μl mouse serum in 45 μl HBSS at 37˚C for 2 min. The reaction was terminated by the addition of 1 ml chilled HBSS. C3b on the bacteria was quantified by FACS using rat anti-mouse C3b (HyCult Biotechnology and FITC-conjugated anti-rat IgG (DakoCytomation) Ab, in a FACScan flow cytometer (BD Biosciences, Franklin Lakes, NJ), and the levels were quantified as a mean intensity of fluorescence.

**Infection**

S. pneumoniae D39 strain was inoculated onto blood agar plates for 20 h in a CO<sub>2</sub> incubator, and the colonies were collected and suspended in brain–heart infusion broth (Nikken Biomedical Laboratory, Kyoto, Japan). The number of bacteria in the solution was calculated as 1 × 10<sup>8</sup> CFU/μl at an OD of 38 at 600 nm. Male mice (12–13 wk-old) were anesthetized with pentobarbital. After 20 min, 20 μl bacteria solution (3.3 × 10<sup>5</sup> CFU) in brain–heart infusion broth was pipetted onto the nose of each mouse. In a preliminary experiment, the survival of wild-type (WT) mice was assessed using various doses of pathogen (Supplemental Fig. 2). In another experiment, mice expressing FcnA<sup>−/−</sup> or FcnB<sup>−/−</sup> mice prepared as described earlier, 2 d after plasmid injection, the mice were infected with 3.3 × 10<sup>5</sup> CFU. Mouse survival was counted every 24 h for >7 d.

The eluates were dialyzed against TBS-Ca and concentrated in a centrifugal filter (Amicon Ultra-4; Millipore, Billerica, MA). The eluate volume used in subsequent studies was represented as an equivalent to the original serum volume.
Viable bacteria counts in the lungs were determined by sacrificing the mice 3 d postinfection. After drawing blood from heart of anesthetized mouse, the lungs were collected, weighted, and homogenized in 4 vol PBS in a tissue homogenizer (TH115; Omni International, Kennesaw, GA). Viable bacteria counts were determined by inoculating the serial dilutions of the lung homogenate on blood agar plates.

Statistics
The difference in C4- and C3-deposition level was evaluated by Student t test. The difference in survival rate in pneumococcal infection was evaluated by Pearson's χ² test.

Results
General phenotypes of ficolin-deficient mice
No abnormality was observed in three ficolin-deficient mouse lineages, Fcna−/−, Fcnb−/−, and Fcna−/− b−− mice, in their appearance, body weights, and reproductive fitness (data not shown). Specific abnormalities were also not observed in the tissues from the adult mice of these lineages, including the liver, spleen, lung, and bone marrow. In addition, no significant difference was observed in the peripheral blood cell counts and coagulation time between these knockout and WT mice.

Reduced activity of the lectin pathway in Fcna−/− and Fcna−/− b−− mice
Fcna levels were estimated to be 3.50 ± 0.58 and 1.77 ± 0.24 μg/ml (mean ± SD) in the sera from WT and heterozygous Fcna+/− mice, respectively, whereas no FcnA was detected in the homozygous Fcna−/− mice (Fig. 1A). FcnB was detected in the bone marrow, a major expression site in mice, of WT but not of Fcnb−/− mice (Fig. 1B). FcnB was also detected in the circulation of Fcna−/− mice at a low average concentration of 130 ng/ml serum, although it was not detected in the Fcna−/− b−/− sera (Fig. 1C). To avoid complications resulting from the copresence of large amounts of FcnA, we assessed Fcna−/− and Fcna−/− b−/− sera instead of WT and Fcnb−/− sera, respectively, for the FcnB ELISA. C4-deposition activities of Fcna−/− and Fcna−/− b−/− sera were significantly lower than that of WT sera (Fig. 1D), suggesting a deficiency in the activity driven by FcnA. In contrast, the activity of Fcnb−/− sera was comparable with WT, suggesting that the activity of FcnB was too low to contribute to the lectin pathway in the circulation, at least under normal conditions.

To further characterize complement activation by FcnA, we subjected mouse serum to GlcNAc-agarose affinity chromatography to separate ficolins from MBLs. FcnA was recovered in the GlcNAc-eluates of WT and heterozygous Fcna+/− mice, whereas MBLs were in the mannose-eluates of WT, Fcna+/−, and Fcna−/− mice at similar levels (Fig. 2A). The substantial amounts of MASP-1, MASP-2, and sMAP were recovered in the mannose-elute of WT, whereas only trace amounts were recovered in the GlcNAc-eluate (Fig. 2C). They were not detected in the GlcNAc-elute from Fcna−/− mice. Consistently, C4-deposition activity of the GlcNAc-eluate was significantly lower in Fcna−/− than in WT mice, whereas the activity of mannose-eluates was not different among the three genotypes (Fig. 2B). These results indicate that Fcna−/− mice lack FcnA-mediated C4 deposition because of the lack of FcnA-MASPs-sMAP complexes; however, they have a normal MBL-mediated C4 deposition in the sera. As shown in Fig. 2C, addition of an excess amount of FcnA into the Fcna−/− serum recovered MASP-1, MASP-2, and sMAP in the GlcNAc-eluate. This eluate exhibited a comparable or rather higher C4-deposition activity than that of the WT (Fig. 2D). Thus, Fcna−/− and Fcna−/− b−/−, but not Fcnb−/− mice demonstrated reduced activities of C4 deposition in the sera, because of the lack of complexes comprising FcnA and MASPs.

Role of FcnB in complement activation
GlcNAc-agarose chromatography of the sera revealed that FcnB was recovered in the GlcNAc-eluate of Fcna−/−, but not in Fcna−/− b−/−, confirming that FcnB is present in the circulation (Fig. 2E). Consistently, C4-deposition activity was significantly higher in the GlcNAc-eluate from Fcna−/− than that from Fcna−/− b−/−, although the observed activity was on the border of detectable level (Fig. 2F). Complex formation of FcnB with MASPs and sMAP was confirmed by a pull-down of the complex after the addition of FcnB to Fcna−/− b−/− serum (Fig. 2G). This result was further confirmed by our recent study, where FcnB formed the complexes with the recombinant MASP-2 and recombinant sMAP, and the formed FcnB–recombinant MASP-2 complex activated C4 on GlcNAc-coated plates (28). A similar result was recently reported in which the rat rFcna activated MASP-2 on the immobilized GlcNAc (29). These results suggest that FcnB is capable of forming complexes with MASP-2 and sMAP, and that the FcnB-mediated complement activation might occur at the inflammatory sites rather than in the normal circulation.

**FIGURE 1.** Ficolin levels and C4-deposition activities in ficolin-deficient mice. (A) Fcna levels estimated by ELISA in WT, Fcna+/− (A+/−), and Fcna−/− (A−/−) mouse sera. Horizontal and dotted lines depict the mean level and threshold of the detectable level, respectively. Open and closed circles denote male and female individuals, respectively. (B) FcnB levels estimated by Western blotting in the bone marrow cells from WT, Fcnb+/− (B+/−), and Fcnb−/− (B−/−) mice. Bone marrow cells sonicated in 10-fold volume of PBS containing 1% Tween X-100 and 5% protease inhibitor were centrifuged at 12,000 rpm, and 25 μl of the supernatant was subjected to Western blotting. (C) FcnB levels in the sera from Fcna−/− (A−/−) and Fcna−/− b−/− (A−/− B−/−) mice as estimated by ELISA. Horizontal and dotted lines depict the mean level and threshold of the detectable level, respectively. (D) C4-deposition activities of 0.75 μl sera from WT, Fcna+/+, Fcna−/−, Fcnb−/−, and Fcna−/− b−/− mice on GlcNAc-coated plates.
Defensive role of ficolins against *S. pneumoniae* infection

The D39 strain of *S. pneumoniae* was recognized by rFcNA and rFcNB, and very weakly recognized by rMBL-A, but not by rMBL-C (Fig. 3A). Binding of rFcNA and rFcNB was inhibited, in part, by the presence of GlcNAc, suggesting specificity via their fibrinogen domains. The activity of mouse sera to opsonize this bacterium was determined using the C3-deposition assay. *Fcna<sup>−/−</sup>* and *Fcna<sup>−/−</sup>* *b<sup>−/−</sup>* sera showed significantly lower activities than WT sera (Fig. 3B), which was consistent with the C4-deposition activity results (Fig. 1D). Based on these results, we next established an experimental infection with *S. pneumoniae* D39 strain, where the nasal dose was fixed at 3.3 × 10<sup>6</sup> CFU to achieve ~80% survival rate of WT mice (Supplemental Fig. 2). Knockout and some WT mice died within 3–5 d after infection (Fig. 3C). The survival rate was significantly lower than the WT in all three ficolin-deficient (*Fcna<sup>−/−</sup>*; *Fcnb<sup>−/−</sup>*; *Fcna<sup>−/−</sup>* *b<sup>−/−</sup>*<sup>−/−</sup>) lineages. Viable counts of bacteria in the lung homogenates were widely ranged up to >2 × 10<sup>7</sup> CFU per 10 mg lung tissue in the 3 ficolin-deficient mice. The average counts were much higher by one order magnitude than that in WT mice, although the statistics did not reach significance in the *Fcna<sup>−/−</sup>* mice (Fig. 3D).

FcNA was transiently expressed in vivo in *Fcna<sup>−/−</sup>* and *Fcna<sup>−/−</sup>* *b<sup>−/−</sup>* mice by i.v. injecting FcNA-encoding pIRCMV plasmid before the mice were infected with *S. pneumoniae* to further confirm the defensive role of ficolins. FcNA was produced at significant amounts in the sera for at least 6 d after injection (Fig. 4A). GlcNAc-agarose chromatography revealed that the transiently expressed FcNA was recovered in the GlcNAc-eluate, together with MASP-2 and sMAP, suggesting reconstruction of the FcNA–MASPs–sMAP complexes in the sera (Fig. 4B). The same GlcNAc-eluate showed a comparable C4-deposition activity with that of the WT (Fig. 4C).

Finally, the survival against infection was comparatively evaluated between the mice injected with both pIRCMV and pFerH plasmids and the mice infected with pFerH alone. *Fcna<sup>−/−</sup>* mice injected with both plasmids exhibited a significantly higher survival rate, which was comparable with that of the WT (Fig. 4D). As shown in Fig. 4E, however, *Fcna<sup>−/−</sup>* *b<sup>−/−</sup>* mice injected with both plasmids did not show a significantly improved survival rate as compared with the mice infected only with pFerH plasmid. These results suggest that both FcNA and FcNB are essential for defense against *S. pneumoniae* D39 infection.

**Discussion**

In this study, we found that the circulating FcNA works to protect against *S. pneumoniae* infection via the lectin pathway, because the deficiency of the FcNA-mediated lectin pathway resulted in a reduced survival rate of infected animals, and its in vivo reconstitution resulted in the improved survival. The FcNA-mediated lectin pathway appears to be independent of the MBL-mediated...
lecitin pathway, because MBL-mediated C4 deposition driven by MBL–MASPs–sMAP complexes was detected at similar levels in Fcna−/− and WT mice (Fig. 2B). Therefore, it is clear that FcnA predominantly plays a defensive role in protection against S. pneumoniae. Although MBLs are the major initiators of the lectin pathway, our data suggest that they are not involved in pneumococcal infection. This is also supported by a limited role of MBL in pneumococcal pneumonia in humans (30). MBL–MASPs–sMAP complexes was detected at similar levels in complete L-FCN deficiency has been reported so far. This suggests that in addition to FcnA, MASP-2 is also essential for defense against pneumococcal infection. In contrast, the complement activation activity showed a lower survival rate as described earlier. The defensive role of FcnB was confirmed by no significant improvement of survival rate in the transiently FcnA-expressing Fcna−/− mice. In comparison with the full improvement of survival in the transiently FcnA-expressed Fcna−/− mice, this result clearly suggests that FcnB is also essential for defense against pneumococcal infection. In contrast, the complement activation activity of the Fcnb−/− sera was comparable with the WT sera (Figs. 1D, 3B). This result appears to be reasonable, because FcnB is a nonplasma/serum-type ficolin and detected in the serum at a trace amount (Fig. 1B). Several explanations are possible to explain the discrepancy between low survival rate in the Fcnb−/− mice and normal complement activation activity in their sera. First, it is known that FcnB expression is upregulated upon macrophage activation (37), and that the expression of M-FCN (human ortholog of FcnB) is induced several times in monocyte-derived macrophages after treatment with TLR2 and TLR4 ligands (38). Second, FcnB might execute its defensive function at the local site of lung rather than in the circulation. It is important to note that FcnB is produced in the myeloid cell lineage or in granulocytes (39, 40), and M-FCN is produced in and secreted from peripheral monocytes, macrophages, and neutrophils (6, 41). To date, there is no evidence that the Fcnb gene is expressed in the lung cells, although it is known that the FCN1 gene is expressed in the lung at a significant level (36, 42). Although the origin of FcnB in the circulation is unclear, it is possible that FcnB is produced in the infiltrated macrophages and granulocytes, and thereby explores its function at the local site of lung. Third, FcnB partially executes its function without complement activation via the lectin pathway. It is noteworthy that rFcnB produced in Drosophila S2 cells associated.
to a lesser extent with MASPs, exhibited a strong activity to aggregate *Staphylococcus aureus*, and enhanced phagocytosis by phagocytes (43). This suggests that FcnB can potentially work more effectively via primitive opsonophagocytosis. This speculation might be supported by the observation that FcnB was colocalized with Lamp-1, a marker for lysosomes and late endosomes in macrophages (37). The orthology between FcnB and M-FCN predicts that M-FCN deficiency would result in the increased susceptibility to pneumococcal infection in humans. This study confirmed that the serum concentration of mouse FcnB was comparable with that of M-FCN, which was reported to be an average of 60.5 ng/ml (41).

It was previously reported that MBLs-null (MBL-A/MBL-C double-deficient) mice were susceptible to *S. aureus* infection (4). As described earlier, ficolins also recognized this pathogen and led to its opsonization with C3b (44). These results suggest that ficolins cooperate together with MBLs as defense molecules against *S. aureus*, which is not the case for *S. pneumoniae* infection. This study showed that FcnA and FcnB have a similar defensive role against the same pathogen, suggesting that the two ficolins work cooperatively, at least not competitively, in protecting against pneumococcal infection. This is supported by insufficient improvement in survival rate of the transiently FcnA-expressing mice. Furthermore, the difference between human and mouse in the lectin pathways should be noted: in humans, the serum concentrations of ficolins (mainly L-FCN plus H-FCN) are several times greater than MBLs (MBL-A plus MBL-C) (46). In addition, primates including humans have an additional ficolin, H-FCN, within their circulatory system. Although H-FCN recognizes a limited spectrum of bacteria (47), it was reported that an H-FCN-deficient patient suffered from recurrent infections (21). These evidences suggest that the ficolin-mediated lectin pathway is more active and more important in humans than in mice. Taking this into consideration, we propose that ficolins play the comparable roles with MBL in the lectin pathway, and that these roles are shared among ficolins themselves and between ficolin and MBL.

In conclusion, FcnA-deficient mice exhibited reduced survival rates when infected transnasally with *S. pneumoniae* D39 strain, and reconstitution of the ficolin-mediated lectin pathway in infected FcnA-deficient mice resulted in improved survival rate. FcnB-deficient mice also demonstrated reduced survival against the same bacterial infection. The defensive role of FcnB was confirmed by insufficient improvement of survival in the transiently FcnA-expressing mice. The defense mechanism of FcnB remains to be clarified. The susceptibility of the three ficolin-deficient mice against *S. pneumoniae* D39 was supported by higher viable counts of bacteria in their lungs. These results suggested that ficolins play a pivotal role in the protection against *S. pneumoniae*, which is the most common cause of bacterial pneumonia in children worldwide. It is noteworthy that ficolins are the predominant initiators of the lectin pathway activation and, therefore, the potential key molecules for pneumococcal infection.

**Acknowledgments**

We thank Y. Maruyama and A. Kawai of the Genome Information Research Center, Osaka University, for technical assistance.
Disclosures
The authors have no financial conflicts of interest.

References
Supplemental data (two figures)

Figure Legends

Figure S1 Strategy for generating *Fcna*<sup>−/−</sup> and *Fcnb*<sup>−/−</sup> mice, and ficolin-deficiency in targeted mice. A, Schematic representation of the *Fcna* gene WT allele (upper), pBS plasmid construct for targeting (middle) and the targeted allele (lower). The promoter and the first to third exons of the *Fcna* gene were replaced with a *Neo<sup>R</sup>* (neomycin-resistant gene) cassette by homologous recombination. Arrowheads denote the PCR primer sites used for genotyping. *DT-A*, diphtheria toxin fragment A gene; B, BamHI restriction enzyme site. B, Genotyping of *Fcna*<sup>−/−</sup> by PCR with mouse tail DNA. PCR products with the sizes of 1.9 Kb (U1971-L3865) and 2.3 Kb (U1971-NeoU1) were derived from the WT and targeted alleles, respectively. C, Northern blot hybridization for FcnA mRNA in the livers from the littermates. One μg of liver Poly(A)<sup>+</sup> mRNA was probed with <sup>32</sup>P-labeled cDNA. GAPDH, glyceraldehyde dehydrogenase. D, Schematic representation of the *Fcnb* gene WT allele (upper), pNT1.1 plasmid construct for targeting (middle) and the targeted allele (lower). The promoter and the first to fourth exons were replaced with *Neo<sup>R</sup>*. *TK*, thymidine kinase gene; X, XbaI restriction enzyme site. E, Genotyping for *Fcnb*<sup>−/−</sup> by PCR. The products with sizes of 1.7 Kb (U1-L1) and 2.3 Kb (U1-NeoU781) are derived from the WT and targeted alleles, respectively. F, RT-PCR of FcnB mRNA in the bone marrows and spleens from the littermates.
Figure S2 Survival rate of WT mice infected with various doses of *S. pneumoniae* D39 strain. Male WT mice were infected with fresh bacteria in 20 μl of brain-heart infusion broth at doses ranging from 0 to 3.3x10^7 CFU/mouse. Numbers in parentheses represent the numbers of mice used in infection at the indicated dose.
Fig. S1
Fig. S2