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

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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 *Fcna*$^{-/-}$*b*$^{-/-}$) mice compared with wild-type mice. Reconstitution of the FcnA-mediated lectin pathway in vivo improved survival rate in *Fcna*$^{-/-}$ but not in *Fcna*$^{-/-}$*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 alternative pathways in protection against microorganisms (1, 2). The lectin pathway, the third pathway of complement activation, is thought to be working as the first-line host defense in innate immunity (3, 4).

In mammals, three kinds of recognition molecules for the lectin pathway have been identified: mannose-binding lectin (MBL), ficolin, and collectin 11/collectin kidney 1 (5–7). These molecules act as pattern recognition molecules and recognize pathogen-associated molecular patterns on the surface of microorganisms and aberrant carbohydrate structures on the surfaces of apoptotic, necrotic, and malignant cells. Almost all of these molecules form complexes with three MBL-associated serine proteases (MASPs): MASP-1, MASP-2, and MASP-3 (8–10). They also interact with small MBL-associated protein (sMAP)/Map19, which is a truncated splicing product generated by *MASP2* gene and lacks protease activity (11, 12). Targeted recognition of the complexes induces the activation of MASPs, and, in turn, MASP-2 cleaves C4 and C2 to generate C4b2a, a C3 convertase, and MASP-1 and MASP-3 activate factors D and B to initiate the alternative pathway, an amplification loop of C3 activation (13, 14). Although MBL has been intensively investigated by using MBL-deficient and MBL-null mice (4, 15), the in vivo role of ficolin remains unclear, mainly because of the lack of an experimental animal model in combination with a ficolin-specific pathogen.

Ficolin is a unique lectin in that it has a C-terminal fibrinogen-like domain that is responsible for carbohydrate recognition (16, 17). Ficolin–MASPs complexes initiate the ficolin-mediated lectin pathway, which appears to be independent of the MBL-mediated lectin pathway driven by the MBL–MASPs complexes. To date, three and two ficolins have been identified in humans and mice, respectively. Comparing their phylogenetic and biochemical properties, it was found that human ficolins are related to murine

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Abbreviations used in this article: FcnA, ficolin A; FcnB, ficolin B; GlcNAc, *N*-acetylglucosamine; H-FCN, H-ficolin; L-FCN, L-ficolin; MASP, 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 $CaCl_2$; TBS-CaT, TBS-Ca containing 0.05% Tween 20; WT, wild-type.

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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 (*FCN3*) 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 ($Fcna^{-/-}$) and FcnB-deficient ($Fcnb^{-/-}$) mouse lineages were generated according to the standard protocol of gene targeting (Supplemental Fig. 1). Targeted embryonic stem cells (129SVJ) were implanted into mouse C57BL/6J blastocysts to generate chimeric mice. Finally, intercrossing respective F2 heterozygous offspring was used to produce homozygous $Fcna^{-/-}$ and $Fcnb^{-/-}$ mice. These knockout mice were backcrossed to C57BL/6J, and $Fcna^{-/-}$ and $Fcnb^{-/-}$ mice used in this study were in 15th and 10th filial generations, respectively.

Double-heterozygous $Fcna^{+/-}b^{+/-}$ mice carrying the haplotype $Fcna^{-}b^{-}$ were interbred to generate a lineage of FcnA/FcnB double-deficient ($Fcna^{-/-}b^{-/-}$) mice. As previously reported (18), the *Fcna* and *Fcnb* genes are located on the same chromosome (2A3). Therefore, $Fcna^{+/-}b^{+/+}$ and $Fcna^{+/+}b^{+/-}$ mice were initially crossed to generate the offspring carrying haplotype $Fcna^{-}b^{-}$. The obtained double-heterozygous mice carrying genotype $Fcna^{+}b^{+}/a^{-}b^{-}$ were then interbred to generate the $Fcna^{-/-}b^{-/-}$ offspring.

Transiently FcnA-expressing $Fcna^{-/-}$ and $Fcna^{-/-}b^{-/-}$ mice were produced using a *piggyBac* transposon-mediated long-term gene expression system (24). In brief, FcnA cDNA was constructed in a pIRCMV plasmid between inverted terminal repeats. Twenty micrograms of the plasmid was mixed with 40 μg pFerH 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/sMAP complexes 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 previously described (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 pIRCMV plasmid and pFerH 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 $CaCl_2$ (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 mannose (mannose-eluate) and then 0.3 M GlcNAc (GlcNAc-eluate). 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.

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. $Fcna^{-/-}$ 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 H_2O_2, and monitored in a Multimode Detector DTX880 (Beckman Coulter, Fullerton, CA) at 405 nm.

To quantify FcnB in the $Fcna^{-/-}$ sera, we incubated 15 μl serum on a rabbit anti-FcnB IgG-coated microtiter plate. Pooled serum of $Fcna^{-/-}b^{-/-}$ 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 H_2O_2. After termination of the reaction with 0.5 M H_3PO_4, color was monitored at 450 nm as described earlier.

Western blotting

Western blotting was performed under reducing conditions using the primary Abs against FcnA and FcnB (25), MASP-1 (26), MASP-2/sMAP (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 and a chemiluminescence substrate (ECL; Amersham Biosciences, Buckinghamshire, U.K.), or with biotinylated secondary Abs (DakoCytomation, Glostrup, Denmark), avidin-biotinylated HRP complex, and ECL. Chemiluminescence image was observed in an LAS-3000 (Fujifilm, Tokyo, Japan).

Binding assay

Binding of the recombinant lectins (rFcnA, rFcnB, rMBL-A, and rMBL-C) to *S. pneumoniae* D39 strain (NCTC 7466) were assessed as follows: heat-killed bacteria (1×10^6 cells) were incubated with 0.5 μg of the recombinants in 300 μl TBS-CaT containing 3% BSA at 4˚C for 1 h. The bacteria were then washed extensively with TBS-CaT and subjected to Western blotting. Binding specificity was confirmed by inhibition with 150 mM GlcNAc.

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, mannose-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-sheep anti-human C4 Ab (Biogenesis, Poole, U.K.). Color was developed by incubation with 3,3′,5,5′-tetramethylbenzidine, and H_2O_2 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 1×10^6 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) Abs, 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_2 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 1×10^6 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 (3.3×10^6 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 with transiently FcnA-expressing $Fcna^{-/-}$ and $FcnA^{-/-}B^{-/-}$ mice prepared as described earlier, 2 d after plasmid injection, the mice were infected with 3.3×10^6 CFU. Mouse survival was counted every 24 h for >7 d.

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 χ^2 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-eluate of WT, whereas only trace amounts were recovered in the GlcNAc-eluate (Fig. 2C). They were not detected in the GlcNAc-eluate 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 rFcnA 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 MASP-2 and sMAP was confirmed by a pull-down of the complex after the addition of rFcnB to $Fcna^{-/-}b^{-/-}$ serum (Fig. 2G). This result was further confirmed by our recent study, where rFcnB formed the complexes with the recombinant MASP-2 and recombinant sMAP, and the formed rFcnB–recombinant MASP-2 complex activated C4 on GlcNAc-coated plates (28). A similar result was recently reported in which the rat rFcnB 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.

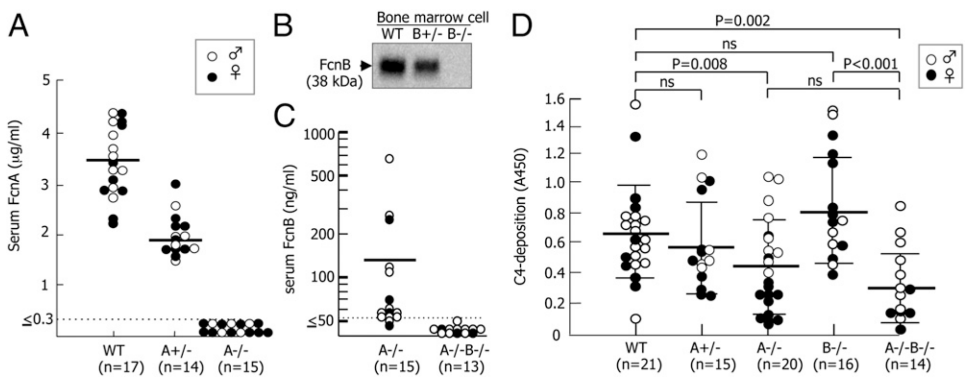

FIGURE 1. Ficolin levels and C4-deposition activities in ficolin-deficient mice. (**A**) FcnA levels estimated by ELISA in WT, $Fcna^{+/-}$ (A+/−), and $Fcna^{-/-}$ (A−/−) mouse sera. Horizontal and dotted lines depict the mean level and threshold of the detectable level, respectively. Open and closed 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 an 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.

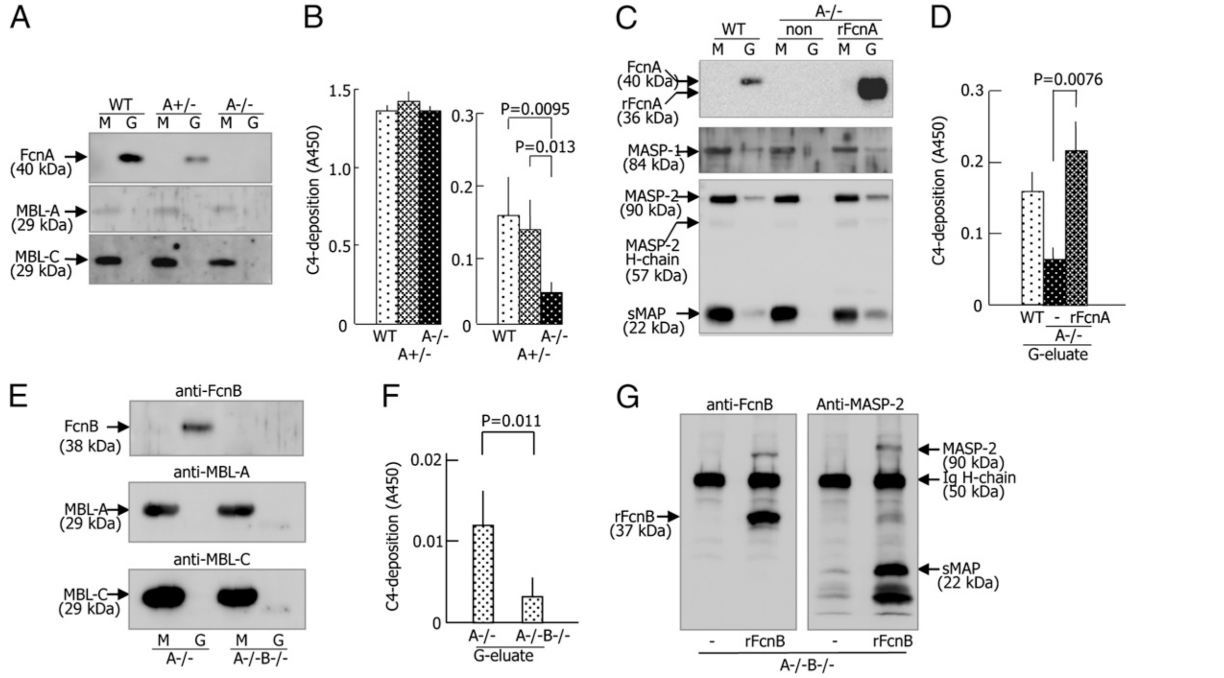

FIGURE 2. Deficiency and reconstitution of the ficolin-mediated lectin pathway in sera. (**A**) Western blotting of FcnA and MBLs in 25 μl serum-equivalent amounts of the mannose (M)-eluate and GlcNAc (G)-eluate from WT, $Fcna^{+/-}$ (A+/−), and $Fcna^{-/-}$ (A−/−) mice. (**B**) C4 deposition on a GlcNAc-coated microtiter plate by the M-eluate (*left*) and G-eluate (*right*). C4-deposition activity was determined using 5 and 20 μl serum-equivalent amounts of M-eluate and G-eluate, respectively. The mean + SD of five determinations is depicted by a column and vertical bar. (**C** and **D**) Reconstitution of the FcnA-mediated lectin pathway in the $Fcna^{-/-}$ serum. rFcnA was added to $Fcna^{-/-}$ serum at an ~25-fold excess amount of the original concentration of native FcnA. M-eluate and G-eluate prepared were subjected to Western blotting (C) and the G-eluate was to C4-deposition assay (D). In Western blotting, added rFcnA was distinguishable from the native FcnA (40 kDa) by its slightly lower m.w. (36 kDa) under reducing conditions. Anti–MASP-2/sMAP Ab recognizes the N-terminal region common to MASP-2 and sMAP. (**E**) Western blotting of FcnB and MBLs in the G-eluates of the sera from $Fcna^{-/-}$ (A−/−) and $Fcna^{-/-}b^{-/-}$ (A−/−B−/−) mice. Pooled serum of 1.8 ml was applied to a GlcNAc-agarose column, and the bound fraction was eluted with 0.3 M mannose and then 0.3 M GlcNAc. Eluates equivalent to 200 μl serum were subjected to Western blotting. (**F**) C4-deposition activity of the same G-eluate as in (E). Activity was determined using 20 μl serum-equivalent of the eluate in quadruplicate (mean + SD). (**G**) Western blotting of FcnB and MASP-2/sMAP in the pull-down sample prepared from $Fcna^{-/-}b^{-/-}$ serum supplied with rFcnB. rFcnB (75 ng) was incubated with 100 μl $Fcna^{-/-}b^{-/-}$ serum on ice overnight and then pulled down with anti-FcnB–coupled Sepharose 4B. The gel was washed, mixed with 0.1% SDS, and the supernatant was subjected to Western blotting at the equivalent volume of 30 μl serum/lane.

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. $Fcna^{-/-}$ and $Fcna^{-/-}b^{-/-}$, but not $Fcnb^{-/-}$, 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. pneumonia* D39 strain, where the nasal dose was fixed at 3.3×10^6 CFU to achieve ~80% survival rate of WT mice (Supplemental Fig. 2). Knockout and some WT mice died within 3–5 d postinfection (Fig. 3C). The survival rate was significantly lower than the WT in all three ficolin-deficient ($Fcna^{-/-}$, $Fcnb^{-/-}$, $Fcna^{-/-}b^{-/-}$) lineages. Viable counts of bacteria in the lung homogenates were widely ranged up to $>2 \times 10^3$ 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 $Fcna^{-/-}$ mice (Fig. 3D).

FcnA was transiently expressed in vivo in $Fcna^{-/-}$ and $Fcna^{-/-}b^{-/-}$ 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. $Fcna^{-/-}$ 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, $Fcna^{-/-}b^{-/-}$ mice injected with both plasmids did not show a significantly improved survival rate as compared with the mice injected 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

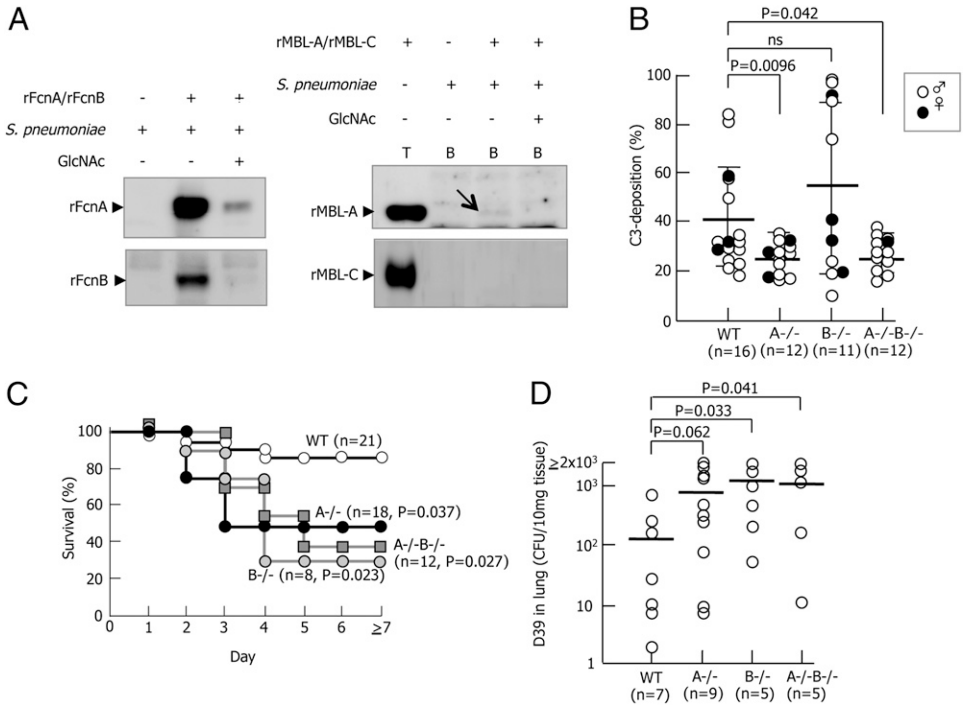

FIGURE 3. Reduced survival rates of ficolin-deficient mice infected with *S. pneumoniae* D39 strain. (**A**) Binding of recombinant lectins to *S. pneumoniae* D39 strain. Binding specificity was tested in the presence of 0.15 M GlcNAc. (*Left panel*) rFcnA and rFcnB; (*right panel*) rMBL-A and rMBL-C. Arrow denotes the very weak binding of rMBL-A. (**B**) C3-deposition activities of the sera from WT, *Fcna*$^{-/-}$ (A−/−), *Fcnb*$^{-/-}$ (B−/−), and *Fcna*$^{-/-}$*b*$^{-/-}$ (A−/−B−/−) mice on *S. pneumoniae* D39 strain. (**C**) Survival rates (%) of WT, *Fcna*$^{-/-}$, *Fcmb*$^{-/-}$, and *Fcna*$^{-/-}$*b*$^{-/-}$ mice infected with *S. pneumoniae* D39 strain. Open circle, WT; closed circle, *Fcna*$^{-/-}$; gray circle, *Fcnb*$^{-/-}$; gray square, *Fcna*$^{-/-}$*b*$^{-/-}$. The *p* values calculated were 0.037, 0.023, and 0.027, between WT and *Fcna*$^{-/-}$, *Fcmb*$^{-/-}$, and *Fcna*$^{-/-}$*b*$^{-/-}$ mice, respectively. (**D**) Viable counts of *S. pneumoniae* D39 in the lung homogenates. CFU level in each mouse was expressed as the count per 10 mg original lung tissue. B, bound MBLs; T, total amount of the recombinant MBLs added.

lectin 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). No association was reported between human *FCN2* gene polymorphisms leading to low serum levels of L-FCN (human counterpart of FcnA) and pneumococcal infectious disorders (31). This suggests that half of the ficolin levels observed in the heterozygous state might be sufficient to fight *S. pneumoniae*. Taken together, it is suggested that the FcnA-mediated lectin pathway specifically serves as a surveillance system against pneumococcal infection. Recently, it was reported that MASP-2–deficient mice failed to opsonize *S. pneumoniae*, suggesting that in addition to FcnA, MASP-2 is also essential for defense against *S. pneumoniae* (32). It was also reported that the *Fcna*$^{-/-}$ mice were susceptible to infection with another microbe, influenza A virus (33).

FcnA is a plasma/serum-type ficolin, which is mainly expressed in the liver, suggesting that FcnA plays its defensive role in the circulation. It is possible that FcnA has a latent defensive ability in the lung, because the *Fcna* gene is expressed at low level in the lung (34). Expression of the *Fcna* gene in tissues overlaps to that of the *FCN2* gene (35). This study confirmed that the serum concentration of FcnA was also similar to that of L-FCN, which was reported to be an average of 3.7 μg/ml (36). These results predict that the deficiency of L-FCN would result in increased susceptibility to pneumococcal infection in humans. No case with complete L-FCN deficiency has been reported so far.

Another important finding is that infected *Fcnb*$^{-/-}$ mice also 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*$^{-/-}$*b*$^{-/-}$ 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

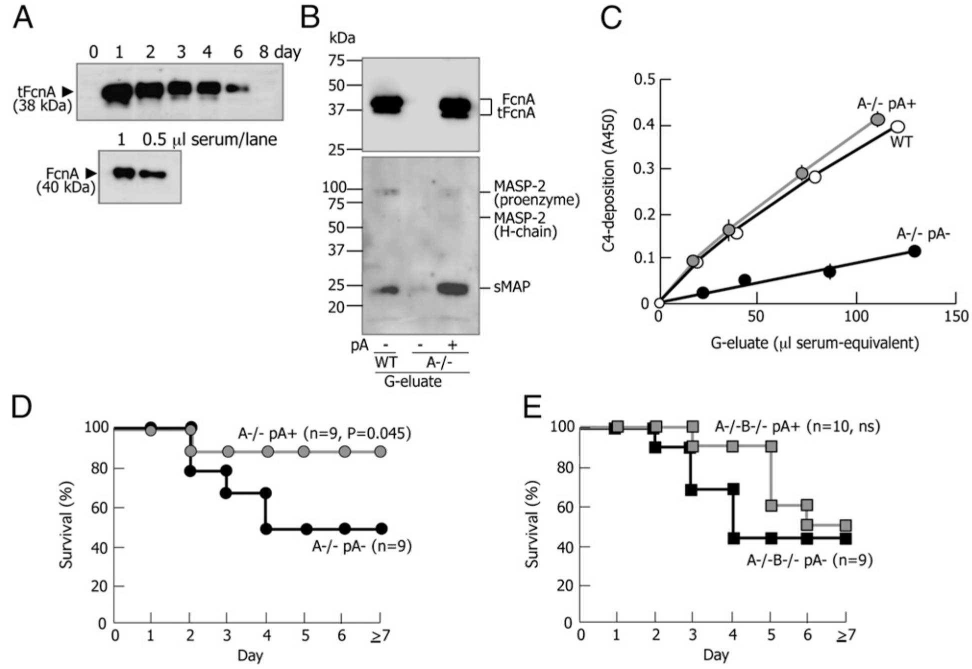

FIGURE 4. Improved survival of transiently FcnA-expressing *Fcna*$^{-/-}$ mice infected with *S. pneumoniae* D39 strain. (**A**) Western blotting of FcnA in the *Fcna*$^{-/-}$ sera on 1–8 d after plasmid injection (*upper panel*). One microliter of serum per lane was run under reducing conditions. Transiently expressed FcnA in *Fcna*$^{-/-}$ sera (tFcnA) was slightly smaller than the native FcnA in molecular size (38 kDa). Serum sample before plasmid injection is depicted by day 0. (*Lower panel*) Western blotting of the native FcnA in 1 or 0.5 μl WT serum on the same membrane filter as described earlier. (**B**) Western blotting of FcnA and MASP-2/sMAP in the G-eluates of the sera from the WT, *Fcna*$^{-/-}$ injected with pIRCMV and pFerH plasmids (A−/− pA+), and *Fcna*$^{-/-}$ injected with pFerH alone (A−/− pA−). Sera were collected 2 d after plasmid injection. (**C**) C4-deposition activities of the G-eluates from WT (open circle), *Fcna*$^{-/-}$ injected with the two plasmids (A−/− pA+; gray circle), and *Fcna*$^{-/-}$ with pFerH alone (A−/− pA−; closed circle). G-eluates were the same as in (B). C4-deposition activity was determined in quadruplicate using the indicated amounts (serum-equivalent), and represented as the mean ± SD. (**D**) Comparison in survival rates of *Fcna*$^{-/-}$ mice injected with two plasmids (gray circle) and with pFerH alone (closed circle; p = 0.045). (**E**) Survival rate of *Fcna*$^{-/-}$*b*$^{-/-}$ mice injected with two plasmids (gray square) in comparison with pFerH alone (closed square).

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 *Fcna*$^{-/-}$*b*$^{-/-}$ 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 MBL (45), whereas in mice, ficolin concentrations (mainly FcnA) are lower 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*$^{-/-}$ and *Fcna*$^{-/-}$*b*$^{-/-}$ mice exhibited reduced survival rates when infected transnasally with *S. pneumoniae* D39 strain, and reconstitution of the ficolin-mediated lectin pathway in infected *Fcna*$^{-/-}$ mice resulted in improved survival rate. *Fcnb*$^{-/-}$ 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 *Fcna*$^{-/-}$*b*$^{-/-}$ 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.

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Disclosures

The authors have no financial conflicts of interest.

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