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A Dominant Complement Fixation Pathway for Pneumococcal Polysaccharides Initiated by SIGN-R1 Interacting with C1q

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SUMMARY

The intricate system of serum complement proteins provides resistance to infection. A pivotal step in the complement pathway is the assembly of a C3 convertase, which digests the C3 complement component to form microbial binding C3 fragments recognized by leukocytes. The spleen and C3 provide resistance against blood-borne *S. pneumoniae* infection. To better understand the mechanisms involved, we studied SIGN-R1, a lectin that captures microbial polysaccharides in spleen. Surprisingly, conditional SIGN-R1 knockout mice developed deficits in C3 catabolism when given *S. pneumoniae* or its capsular polysaccharide intravenously. There were marked reductions in proteolysis of serum C3, deposition of C3 on organisms within SIGN-R1$^+$ spleen macrophages, and formation of C3 ligands. We found that SIGN-R1 directly bound the complement C1 subcomponent, C1q, and assembled a C3 convertase, but without the traditional requirement for either antibody or factor B. The transmembrane lectin SIGN-R1 therefore contributes to innate resistance by an unusual C3 activation pathway.

INTRODUCTION

The intricate but elegant system of complement proteins is responsible for several innate and adaptive resistance mechanisms. A pivotal step is the formation of a C3 convertase, which catalyzes the proteolysis of complement component, C3. The C3 convertase is formed by (1) a "classical" pathway initiated by the binding of C1q to immunoglobulin (Ig) in immune complexes, (2) an "alternative" pathway triggered directly by certain microbial cell walls and catalyzed by factor B, and (3) a soluble mannose binding lectin pathway with many homologies to the classical C1q based pathway (reviewed in Fearon and Wong, 1983; Walport, 2001a; Walport, 2001b; Fujita, 2002). The C3 convertase first generates C3b that is further processed to C3bi and C3d. C3b, C3bi, and C3d serve as ligands or "opsonins" for cellular receptors on B cells, phagocytes, and follicular dendritic cells (FDCs). Receptor-mediated binding of these C3 opsonins leads to microbial clearance by phagocytes (Fearon and Wong, 1983), stimulation of B cells (Carroll, 1998), and retention of antigens on FDCs (Pozdnyakova et al., 2003). C3 processing also releases the C3a peptide, which is an anaphylatoxin that causes smooth muscle contraction, mast cell histamine release, and local inflammation. Additionally, a lytic membrane attack complex of other complement components C5b, 6, 7, 8 is assembled on cellular targets. Therefore the complement system is a major pathway for resistance and pathology, and the formation of a C3 convertase is pivotal.

The spleen clears microorganisms from the blood stream, with *Streptococcus pneumoniae* representing a major example (Amlot and Hayes, 1985; Zandvoort and Timens, 2002). Individuals lacking a spleen are more susceptible to infection with *S. pneumoniae* (Schutze et al., 2002). Despite the development of effective treatments, this gram-positive coccus has remained a significant cause of morbidity and mortality (Garau, 2002), being one of the most common causes of bacterial pneumonia, otitis media, meningitis, and septicemia (Musher, 1992). Opsonin-dependent phagocytosis via complement plays an important role in host defense against pneumococci (Brown et al., 1982; Brown et al., 1983). Patients with deficiencies of early components of the classical pathway of complement and C3 are at high risk for pneumococcal disease (Picard et al., 2003). In contrast, the soluble mannose binding lectin binds poorly to *S. pneumoniae* (Neth et al., 2000; Krarup et al., 2005), and lower lectin levels are only weakly associated with *S. pneumoniae* infections (Kronborg et al., 2002; Atkinson et al., 2004). Knockout mice have revealed that C3 and C4 are dominantly

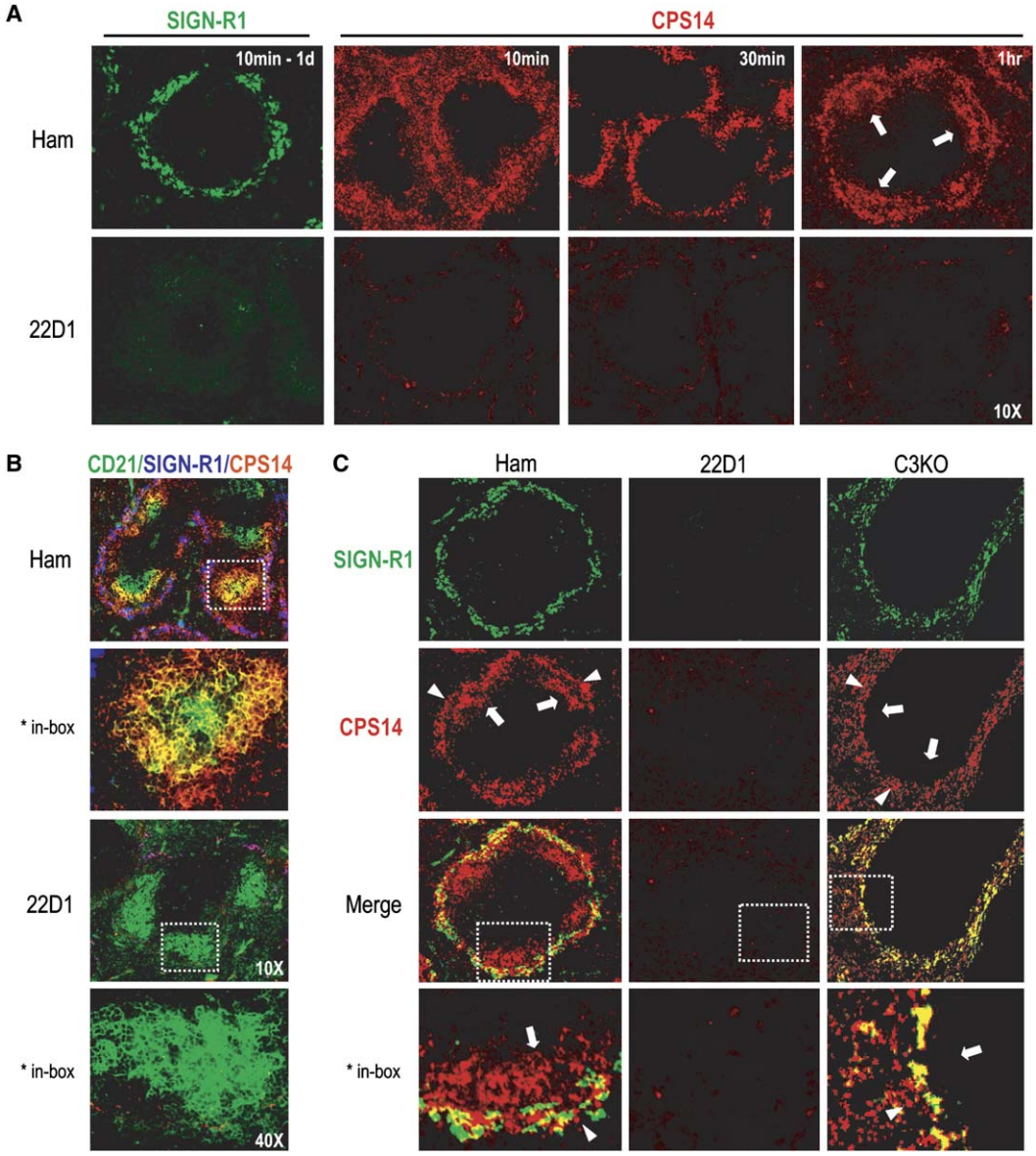

Figure 1. SIGN-R1 Is Required for Binding of CPS to FDCs

(A) CPS14 is not bound in B cell follicles (white arrows) of 22D1 treated mice. Control and 22D1-treated mice were injected i.v. with 100 μg of CPS14 for 10, 30, 60 min. Spleen sections were stained for SIGN-R1 (green, left) and CPS14 (red). Mice were treated with 22D1 anti-SIGN-R1 mAb 24 hr earlier or nonreactive hamster IgG control.

(B) CPS14 is fixed to $CD21^{+}$ FDCs in control (yellow) but not 22D1 treated mice. 90 min after 100 μg CPS 14 i.v., spleen sections were stained for CPS14 (red), CD21/35 (green), and SIGN-R1 (blue).

(C) Uptake of CPS (red) into MZ macrophages (white arrowheads; yellow in merge) in control and $C3^{-/-}$ mice, but not in 22D1 treated animals, and binding of CPS to FDCs in control mice (white arrows).

required for innate resistance to *S. pneumoniae,* but surprisingly, there is only a small or partial need for the traditional mediators of C3 fixation like factor B, mannose binding lectin, and immunoglobulin (Ig) (Brown et al., 2002; Kars et al., 2005). Together, these findings indicate that there is likely to be an unusual complement fixation pathway that is independent of factor B, Ig, and mannose binding lectin but that nevertheless forms C3 opsonins for resistance to pneumococci by phagocytes and B cells.

The spleen contains specialized regions called marginal zones (MZ), which lie at the junction of each white pulp nodule with the red pulp (Mebius and Kraal, 2005). The MZ contains unique MZ B cells (Martin and Kearney, 2002) enmeshed with MZ macrophages in a reticular cell

network (Kraal and Janse, 1986). In the MZ, there is a strongly reduced blood flow allowing intimate contact between antigens and effector cells (Guinamard et al., 2000). The MZ thus has several functions, including the generation of antigen-specific B cell responses to T cell-independent antigens (Humphrey, 1985) and the capture of blood-borne pathogens (Humphrey and Grennan, 1981; Kang et al., 2004) such as *S. pneumoniae* (Lanoue et al., 2004).

SIGN-R1, a C-type lectin related to DC-SIGN (Park et al., 2001), is an uptake receptor expressed at high levels by spleen MZ and lymph node macrophages (Geijtenbeek et al., 2002; Kang et al., 2003). SIGN-R1 is the principal receptor for bacterial dextrans as well as the capsular pneumococcal polysaccharide (CPS) of *S. pneumoniae*, so that deletion of SIGN-R1 ablates clearance of these polysaccharides by MZ macrophages (Geijtenbeek et al., 2002; Kang et al., 2003; Kang et al., 2004; Lanoue et al., 2004). Our approach to conditional deletion of SIGN-R1 involves administration of the monoclonal antibody 22D1. Treatment with this mAb selectively ablates SIGN-R1 expression, but not MZ macrophages, as monitored with a combination of antibodies to a distinct epitope to SIGN-R1 and other MZ receptors (Kang et al., 2004). Furthermore, Lanoue et al. used genetic deletion of SIGN-R1 to demonstrate the contribution of this lectin to protection of mice against pneumococcal septicemia (Lanoue et al., 2004). In our continuing studies of 22D1 anti-SIGN-R1-treated mice, we found surprisingly that, in addition to a lack of CPS and dextran uptake in SIGN-R1$^+$ MZ macrophages, the polysaccharides were no longer deposited on SIGN-R1$^-$ spleen FDCs. This suggested a link between SIGN-R1 and the formation of C3 opsonins for FDCs. We will show that this membrane bound lectin initiates a classical but Ig-independent pathway for C3 fixation by binding C1q and depositing C3 on bound polysaccharides and *S. pneumoniae*. We suggest that this new pathway for C3 fixation by SIGN-R1 bound polysaccharides contributes to the role of the spleen in resistance to certain encapsulated organisms.

RESULTS

Initial Clue that SIGN-R1 Lectin Is Involved in C3 Fixation

To study the previously defined interaction of capsular pneumococcal polysaccharide (CPS) with the SIGN-R1 lectin on marginal zone (MZ) macrophages of spleen (Kang et al., 2004), we injected mice with 100 μg of CPS14 i.v. and localized it with anti-CPS14 antibody in sections of spleen 10 min to 1 hr later. Within minutes, the CPS localized to SIGN-R1$^+$ MZ macrophages, which were identified with anti-SIGN-R1 antibodies (Kang et al., 2003; Kang et al., 2004). However, by 1 hr the CPS was also found in the B cell areas or follicles of the white pulp (Figure 1A, white arrows). The follicle-associated CPS colocalized to follicular dendritic cells (FDCs), which are known to express CD21/CD35 complement receptors but not SIGN-R1 (Figure 1B, yellow staining). In SIGN-R1-transient knockout mice generated by mAb 22D1 treatment (Kang et al., 2004), CPS14 was not taken up by MZ macrophages as expected, but in addition the CPS did not localize to follicles (Figure 1A) or FDCs (Figure 1B). This surprising finding suggested that SIGN-R1 on MZ macrophages is needed for the deposition of CPS14 on SIGN-R1$^-$ FDCs.

Since prior results (Harms et al., 1996) showed that CPS binding to the MZ and FDCs was dependent on complement, probably a C3 fragment, we compared C3$^{-/-}$ and wild-type mice. We found that CPS14 was taken up by SIGN-R1$^+$ MZ macrophages in wild-type and C3$^{-/-}$ mice (Figure 1C). However, CPS14 did not bind to FDCs in C3$^{-/-}$ mice (Figure 1C). Thus SIGN-R1 and C3 are somehow both needed for CPS binding to FDCs, suggesting that this lectin might contribute to C3 fixation.

SIGN-R1 Mediates Systemic Activation of the Classical Complement Pathway

To pursue the suggestion that SIGN-R1 was required for the formation of C3 ligands, we injected CPS or mitomycin-C treated (growth inactivated) *S. pneumoniae* into control and 22D1-treated mice (controls included mice given hamster Ig, N418 anti-CD11c, and anti-Marco, a scavenger receptor on MZ macrophages). Over the ensuing hour, we monitored C3 processing in blood by immunoblotting serum samples with polyclonal anti-C3 antibody. This antibody reacts with the two components of native C3, αC3, and βC3 as well as the fragments of αC3 that are generated during C3 processing by C3 convertases (Figure 2A shows information for human C3) (Law and Dodds, 1997). In the steady state, C3 convertase is active so that two C3 fragments (iC3b; 70 kDa and 43 kDa) are always evident in serum. However, when pneumococci or CPS were given i.v., there was rapid processing of αC3 (but not βC3, which serves as a loading control for the immunoblotting), such that the serum sample lost most of the detectable αC3 as well as the 70 kDa iC3b but accumulated the 43 kDa iC3b (see the two left lanes of Figures 2B and 2C). These data in vivo are consistent with previous results in vitro (Campbell et al., 1991). Pneumococci, but not several other bacteria that we tested, rapidly activated C3 in vivo (Figure S1). The processing of C3 was more active with CPS from type 14 *S. pneumoniae* than CPS23 and CPS26 (data not shown), and it is known that CPS14 binds with higher affinity to SIGN-R1 relative to CPS23 and CPS26 (Kang et al., 2004). Importantly, the activation of C3 in response to bacteria (Figure 2B) or CPS (Figure 2C) was blocked in 22D1-treated mice, indicating that SIGN-R1 is in large part responsible for rapid C3 convertase formation induced by *S. pneumoniae* in mice.

These results were unexpected because in vitro, C3 can be activated via the alternative pathway when *S. pneumoniae* (but not CPS) is added to serum (Winkelstein and Tomasz, 1977), as we confirmed (Figure 2D, left; data for CPS not shown). We also verified that C3 processing in vitro in response to bacteria occurred similarly when

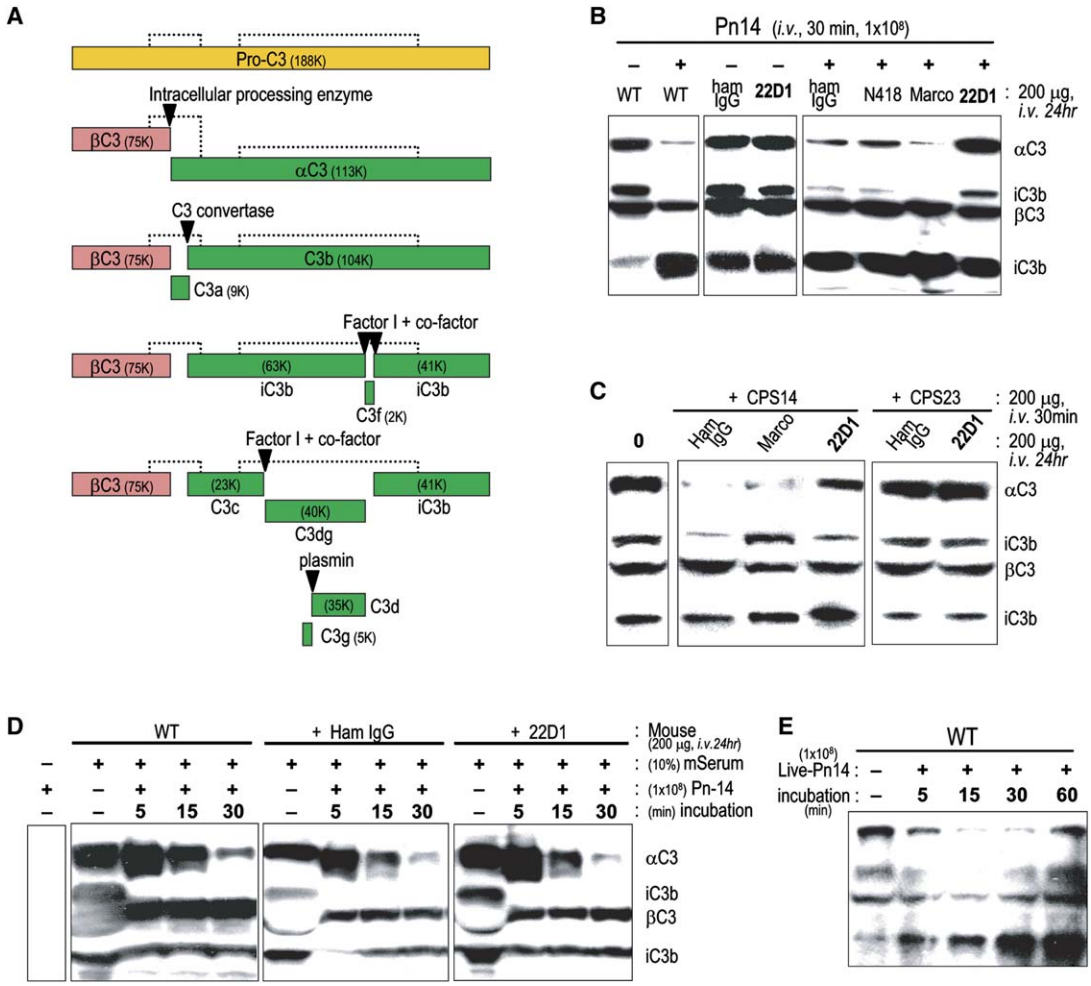

Figure 2. SIGN-R1 Dominates C3 Activation by *S. pneumoniae* and CPS14

(A) Diagram of C3 processing by C3 convertases (MW are for human C3, but MW for rat are 120 for αC3, 70 and 43 for iC3b fragments, and 65 for βC3 (Sawant-Mane et al., 1996; Cowell et al., 2003).

(B) Analysis of C3 processing by immunoblot of serum from mice without (−) or with (+) 1×10^8 live type 14 *S. pneumoniae* i.v. 30 min earlier (left lanes), and in mice injected i.v. 24 hr earlier with hamster IgG, N418 hamster anti-CD11c, ED31 rat anti MARCO scavenger receptor on MZ macrophages, or 22D1 hamster anti-SIGN-R1.

(C) As in (B), but the indicated CPS were given i.v., where CPS14 binds more vigorously to SIGN-R1.

(D) Relatively slow C3 catabolism by live *S. pneumoniae* type 14 added to serum from wild-type mice, or serum from mice treated 24 hr earlier with 200 μg hamster Ig or 22D1 mAb.

(E) More rapid C3 catabolism, relative to (D), when organisms were given in vivo prior to the immunoblot assay.

we compared serum from wild-type mice or from mice treated with hamster Ig or 22D1 anti-SIGN-R1 mAb 24 hr earlier (Figure 2D). In other words, 22D1 treatment was not consuming C3 and not preventing direct fixation of C3 from serum by *S. pneumoniae*. However, the kinetics of C3 processing was much more rapid when *S. pneumoniae* was administered in vivo, being detectable within 5 min (Figure 2E), as opposed to adding organisms to serum in vitro, where 30 min were required for significant C3 catabolism (Figure 2D). Thus CPS and *S. pneumoniae* are both captured within minutes from the blood by SIGN-R1$^+$ MZ macrophages, and the latter dominate the early processing of C3 in vivo.

SIGN-R1-Dependent C3 Fixation by *S. pneumoniae* Type 14 In Vivo

We next wanted to verify a role for SIGN-R1 during C3 fixation by *S. pneumoniae* directly in spleen sections in vivo. We injected i.v. 10^8 CFU (colony-forming units), mitomycin C-treated type 14 (Pn14) organisms that were also labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE). 90 min later, to allow for uptake by MZ macrophages and deposition of CPS on FDCs, the spleens were sectioned and examined by deconvolution microscopy. The Pn14 were taken up in the MZ in control and C3 deficient (C3$^{-/-}$) mice, but there were diminished numbers of organisms in the MZ of mice treated beforehand

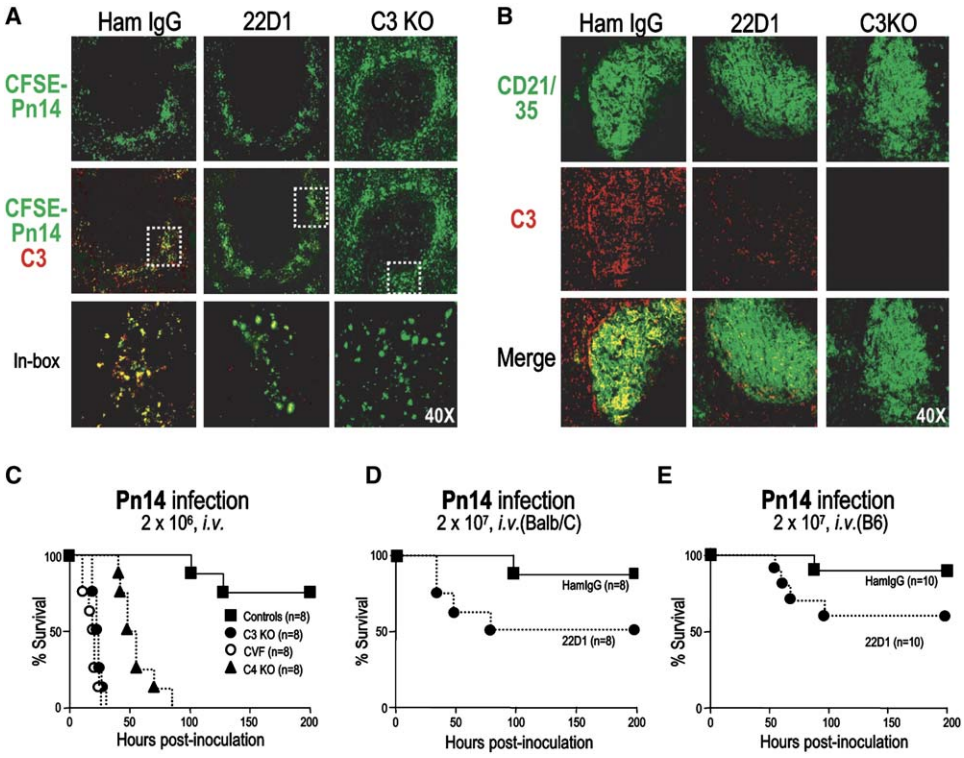

Figure 3. SIGN-R1 Mediates C3 Fixation to *S. pneumoniae* in Spleen and Provides Resistance to Pneumococcal Infection

(A) 10^8 mitomycin C-treated and CFSE-labeled *S. pneumoniae* type 14 were injected i.v. into isotype control, 22D1 treated, and $C3^{-/-}$ mice. 90 min later, spleens were sectioned to show uptake of organisms into MZ (top row) and labeling for C3 (red) to show SIGN-R1-dependent C3 fixation by bacteria as in the enlargements (yellow, bottom row). For the 22D1 treated panel, uptake into MZ macrophages is reduced, and a field is selected to show many organisms.

(B) As in (A), but the sections were stained for C3 (red) and CD21/35 (green) to show a C3-labeled FDC network at high power (lower left).

(C) Survival times after 2×10^6 *S. pneumoniae* were injected i.v. into $C4^{-/-}$, $C3^{-/-}$, or CVF-treated C57BL/6 mice.

(D and E) Survival times after 2×10^7 live type14 *S. pneumoniae* were injected i.v. into C57BL/6 (B6) or BALB/c mice that were treated with hamster Ig or 22D1 anti-SIGN-R1.

with anti-SIGN-R1 mAb 22D1, possibly because there were residual SIGN-R1 molecules and/or receptors for the organisms in addition to SIGN-R1 (Figure 3A). C3 was deposited on the organisms in control mice in the MZ when mice were given pneumococci but not in $C3^{-/-}$ mice, as expected (Figure 3A, yellow labeling). In 22D1 treated mice, when we selected regions of the MZ where there was some uptake of CFSE-labeled *S. pneumoniae*, C3 was minimally fixed to the individual organisms (Figure 3A). C3 likewise was clearly deposited on $CD21/35^+$ FDCs in control mice given the CFSE labeled organisms, very little in the 22D1 treated animals, and not at all in $C3^{-/-}$ mice (Figure 3B). Likewise, when we injected either CPS (or dextran, data not shown) i.v. in control mice, the polysaccharides were taken up in MZ macrophages and C3 was deposited there, but again not in 22D1 treated animals (Supplemental Figure S2). Therefore when pneumococci or polysaccharides are injected i.v., SIGN-R1 dominates the fixation of C3 to MZ macrophages and subsequently, FDCs.

Because we were using treatment with mAb 22D1 to selectively deplete SIGN-R1, we needed to verify that this approach was relevant to the resistance of mice to infection with *S. pneumoniae*, as described by Lanoue et al., who showed that genetic deletion of SIGN-R1 decreased innate resistance (Lanoue et al., 2004). First, we repeated the experiments of J.S. Brown et al. (Brown et al., 2002) on the heightened susceptibility of $C3^{-/-}$ mice, as well as mice treated with cobra venom factor (CVF) to deplete C3 (Figure 3C). $C4^{-/-}$ mice were also very susceptible to infection, although somewhat less than the C3 deficient mice (Figure 3C), again confirming prior results that a classical C4 dependent pathway is the dominant pathway for C3 fixation during innate resistance (Brown et al., 2002). Splenectomized mice were likewise very sensitive to i.v. infection (Figure S3). 22D1 treated mice also showed a decrease in resistance to live *S. pneumoniae* in BALB/c and C57BL/6 strains (Figures 3D,E), comparable to genetic knockouts, but this pathway is only one of several that contribute to C3 fixation. For example, other contributions arise from the alternative pathway in serum (Figure 2D) (Brown et al., 2002), natural antibody (Koppel et al., 2005), and perhaps other lectins in the SIGN-R family such as the related SIGN-R3 lectin (Takahara et al.,

2004). Thus SIGN-R1 is one pathway for resistance to *S. pneumoniae*, most likely contributing to the early phase of C3 fixation (Figure 2B,E).

The Lectin SIGN-R1 Binds C1q from the Classical Complement Pathway

To understand the linkage between SIGN-R1 and C3 processing, we searched for SIGN-R1 binding proteins in the spleen. We biotinylated the soluble extracellular domain of SIGN-R1, which was expressed by CHO cells, to allow for binding to streptavidin beads for 2 hr at 4°C. Binding was verified by the reactivity of the SIGN-R1-coated beads with mAb 22D1. We then added 10 mg of spleen or lymph node extracts to the SIGN-R1 coated beads, or beads coated with transferrin as control, for 12 hr at 4°C. The bound proteins were eluted and separated on a 4%–15% gradient SDS page gel and developed with silver staining. Since the transmembrane lectin SIGN-R1 is a mannose binding lectin (Galustian et al., 2004), we expected SIGN-R1 to retrieve proteases called MASPs, which are soluble, mannose lectin-associated, serine proteases (Fujita, 2002). Instead, the 30 kDa band that was selectively bound to SIGN-R1-coated beads was repeatedly isolated and identified by MALDI-TOF peptide sequence analysis to be C1q, a key early component of the classical pathway for complement fixation. Less frequently, we also obtained peptide sequences for C1r and C1s, two proteases that bind to aggregated C1q and initiate proteolysis of the classical complement components C2 and C4. Since SIGN-R1 is known to exist primarily in an aggregated form in spleen and lymph node (Kang et al., 2003), we reasoned that SIGN-R1 would aggregate C1q, much like IgM or immune complexes, and allow for the initiation of the classical complement pathway.

To verify the finding of C1q binding to SIGN-R1, we determined whether SIGN-R1 beads would bind C1q from either mouse or human serum. We added 50 μl of mouse (Figure 4A) or human serum (Figure 4B) to the SIGN-R1-coated beads for 12 hr at 4°C and identified C1q by immunoblotting with chicken anti-C1q serum, whereas no C1q was bound to the beads coated with bovine serum albumin, ovalbumin, or transferrin. To clarify the mechanism of C1q binding to SIGN-R1, the same assays were performed with mouse sera genetically deficient in C1q ($C1q^{-/-}$), Ig ($J_H^{-/-}$), and C3 ($C3^{-/-}$), as well as C1q-depleted human serum. As expected, C1q binding was not found in the SIGN-R1 eluate when we applied C1q deficient mouse or human serum, but C1q binding was evident with sera of $J_H^{-/-}$ and $C3^{-/-}$ mice (Figure 4C, left lanes), or if purified C1q was added back to the C1q deficient serum (Figure 4C, right lanes). To verify direct binding of C1q to SIGN-R1, e.g., to rule out the possible effects of C1q binding proteins such as CRP or C1 inhibitors in mouse or human sera, we showed that purified human C1q bound to SIGN-R1 beads (Figure 4D). To determine if C1q bound Ig and SIGN-R1 at similar sites, we assessed in vitro competition for C1q binding to biotinylated SIGN-R1 by human IgG. Human IgG blocked C1q binding to SIGN-R1 as efficiently as unlabeled SIGN-R1 (Figure 4E), implying that SIGN-R1 binds the same globular region of C1q as Ig.

C1q Binds to SIGN-R1 on the Cell Surface of SIGN-R1 Transfectants

To extend the studies of C1q binding to SIGN-R1, we examined cell-associated SIGN-R1, first using DCEK cell lines stably expressing SIGN-R1 (D-SR1 in the figures) as well as SIGN-R1 lacking its cytosolic domain (termed D-48), the latter to help restrict binding to cell-surface (versus endocytosed) SIGN-R1. Following incubation in 10% mouse or human serum for 1 hr at 37°C, the cells were washed and stained with 22D1 anti-SIGN-R1 mAb and chicken anti-C1q polyclonal antibody. C1q was clearly bound by both DCEK-SR1 and DCEK-48 but not to wild-type DCEK cells (Figure 4F). Bound C1q was not observed with $C1q^{-/-}$ mouse serum, whereas C1q binding from $Ig^{-/-}$ ($J_H^{-/-}$) serum was similar to controls (Figure S4A). To gain more quantitative evidence, we did FACS studies. Some C1q binding above background was noted when mouse serum was added to wild-type DCEK cells, but binding was increased to SIGN-R1 transfectants (Figure S4B, bottom left). Likewise, when we tested normal human serum (NHS), we again observed increased human C1q binding to the SIGN-R1 transfectants relative to wild-type (Figure S4B, bottom right). We also expressed the full-length cDNAs for mouse C1q in HEK293 cells but inserted a myc tag at the carboxyl terminus of C1q A chain. The protein was expressed, as evidenced by immunoblotting with polyclonal anti-C1q or monoclonal anti-myc. The expressed C1q was again bound by SIGN-R1 transfectants but not untransfected DCEK cells (Figure 4G, yellow labeling in overlay of green myc and red SIGN-R1). Also, when we incubated biotin conjugated-purified C1q with DCEK cell lines without the addition of serum, only SIGN-R1 transfectants showed binding of C1q (Figure 4H). We also assessed C1q binding to different CHO transfectants, including CHO transfected with DEC-205 and SIGN-R3, which are other types of lectins. Only the SIGN-R1 transfectant showed increased C1q binding (Figure 4I). These results indicate that SIGN-R1 on transfected cell lines binds both mouse and human C1q.

SIGN-R1 on Marginal Zone Macrophages Mediates C1q Binding In Vivo

To move these findings with C1q into the mouse, we first evaluated whether mouse C1q binds to SIGN-R1 in tissue sections. In both control and $C3^{-/-}$ mice, we observed overlapping labeling for C1q and SIGN-R1 (Figure 5A). We also noted staining for C1q in the white pulp, in the follicles, and in the red pulp, as reported (Schwaeble et al., 1995). To assess whether SIGN-R1 was required for C1q localization, we treated mice 24 hr beforehand with 100 μg of 22D1 anti-SIGN-R1 mAb i.v. or with ED31 anti-MARCO mAb as control. The 22D1-treated spleen no longer showed staining for C1q in the MZ, although some C1q was noted on FDCs as in untreated mice

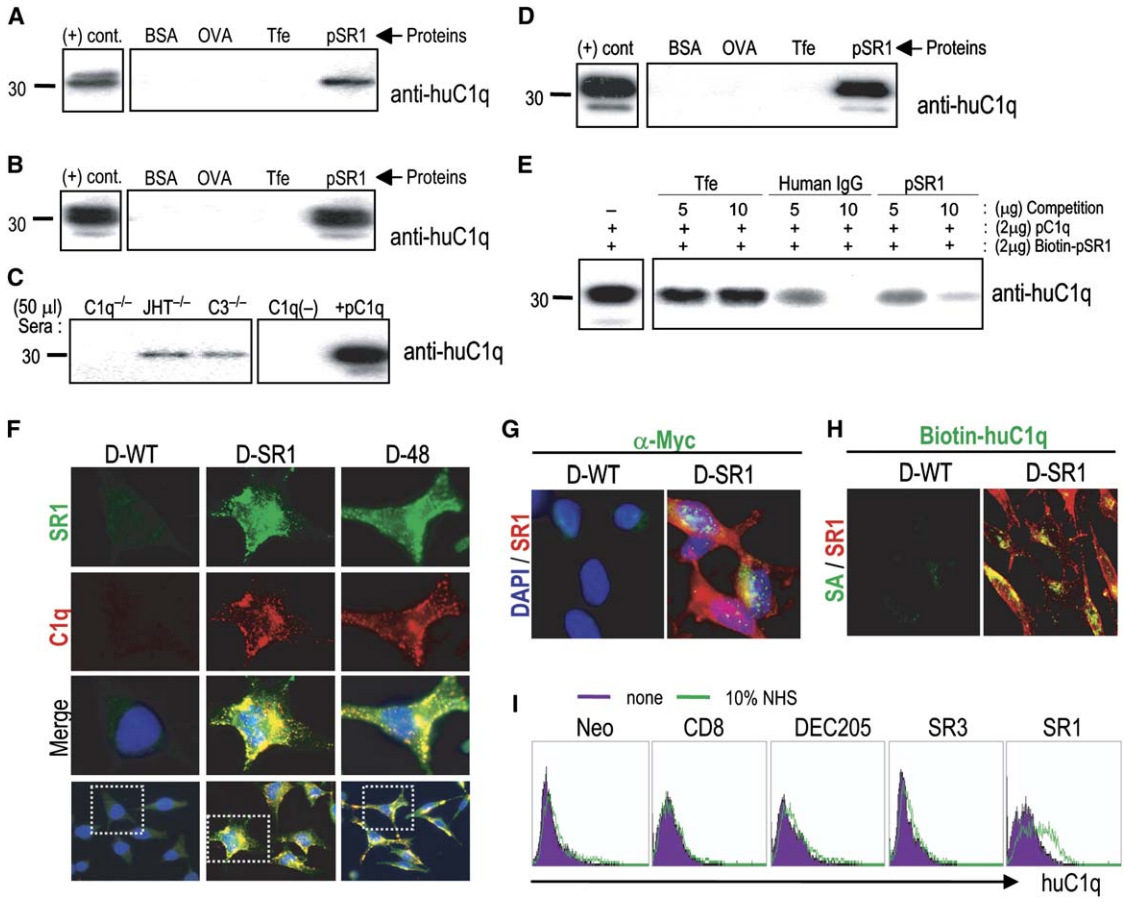

Figure 4. Binding of C1q to SIGN-R1 Attached to Beads or SIGN-R1-Transfected Cell Lines

(A, B, and D) 50 µl of fresh mouse serum (A), normal human serum (B), or purified C1q (D) were incubated for 2 hr at 4°C with 5 µg of different biotin-proteins (BSA, ovalbumin, transferrin, purified SIGN-R1) and added to 40 µl of streptavidin beads for 12 hr at 4°C in cold PBS. Bead bound proteins were blotted with polyclonal chicken anti-human C1q, which was detected with HRP conjugated anti-chicken IgY.

(C) As in (B), but the 50 µl of sera were from different knockout mice ($C1q^{-/-}$, $J_H^{-/-}$, and $C3^{-/-}$; left) or from C1q depleted human serum to which 1 µg of purified C1q was added (right).

(E) As in (B), but increasing doses (5, 10 µg) of transferrin (Tfe; control), human IgG, or purified SIGN-R1 were added in the above assay to test for competition for C1q binding.

(F) 10% fresh mouse serum was incubated with DCEK cells transfected with nothing (D-WT), SIGN-R1 (D-SR1), or SIGN-R1 lacking a cytosolic domain (D-48) for 1 hr at 37°C. Each cell line was stained with mAb α-SIGN-R1 (green), polyclonal anti-human C1q (red), and DAPI (nuclei, blue).

(G) Mouse C1q, expressed with Myc tag at the carboxyl terminus of A chain, was applied to DCEK-WT and DCEK-SR1 transfectants for 2 hr at 37°C. The cells were then stained with anti-Myc (green) or control serum (not shown), anti-SIGNR1 (red), and DAPI (blue).

(H) As in (G), but 2 µg of biotin-human C1q was added for 1 hr in serum-free RPMI media prior to staining with α-SIGN-R1 (red) and strepavidin (green).

(I) C1q binding from 10% human serum to the indicated CHO transfectants by FACS analysis.

(Figure 5B, arrow), presumably because other C1q receptor(s) can also bind C1q. Thus, SIGN-R1 normally is required for C1q deposition in MZ macrophages in vivo.

SIGN-R1 Mediates C3 Fixation via the Classical Pathway to SIGN-R1 Transfectants

To determine if C1q on SIGN-R1 transfectants could fix C3, we added 3% normal or heat inactivated (HI) mouse serum, where C3 is known to be heat-labile (Caraher et al., 1999), and we stained the cells for mouse C3, IgM, or IgG. The SIGN-R1 transfectants fixed C3 but not Ig from serum, but only with normal and not HI serum (compare green and red tracings in left panels, Figure 6A). In dose response studies, some C3 fixation was noted in DCEK wild-type cells when the concentration of serum was 10% of the culture medium, but SIGN-R1 transfectants fixed C3 more efficiently (Figure S5, left). No C3 fixation was observed when we tested $C4^{-/-}$ and $C3^{-/-}$ sera (Figure S5, right), consistent with the idea that the classical pathway was involved. Likewise, C3 fixation from serum by mouse SIGN-R1 transfectants required C1q (Figure 6B) but not factor B or Ig (the latter from $J_H^{-/-}$ and RAG-$1^{-/-}$ mice). These findings were confirmed with human serum, where again C1q and C4 were required for C3 fixation by

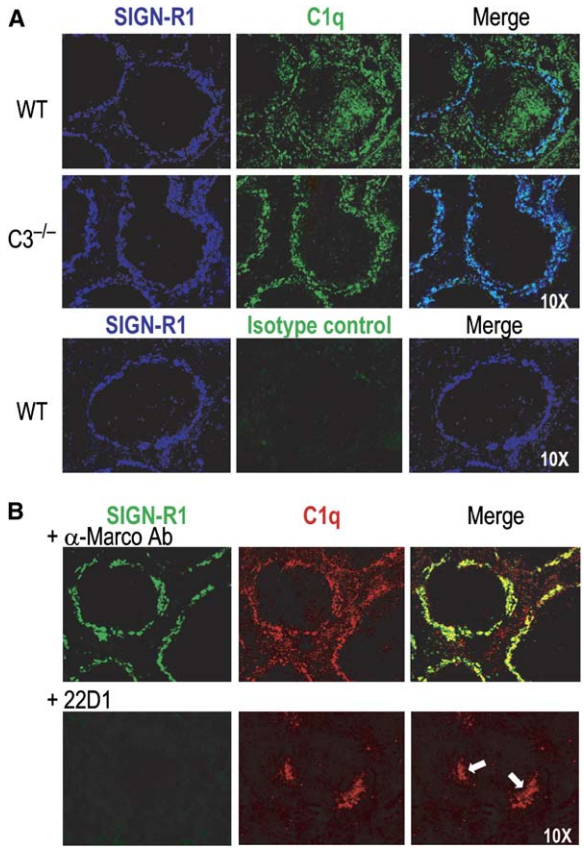

Figure 5. SIGN-R1-Dependent C1q Accumulation in Mouse Spleen

(A) Sections from control and $C3^{-/-}$ spleens were stained for SIGN-R1 (blue) and C1q (green).

(B) In parallel with (A), mice were injected with 100 μg of 22D1 α-SIGN-R1 or with ED31 mAb to another MZ macrophage receptor, MARCO. Sections were stained 24 hr later for SIGN-R1 (green) and C1q (red). Arrows indicate binding of C1q to follicles.

SIGN-R1 transfectants (Figure S6). When we examined the cells fixing C3 by microscopy, we observed considerable overlap in the deposits of C1q and C3 on the cell surface (Figure 6C, yellow arrows). Also, when we tested CHO cells transfected with other membrane proteins, C3 fixation only was noted with SIGN-R1 transfectants (Figure 6D). Thus cell associated SIGN-R1 fixes C3 from mouse and human serum via the classical pathway but independent of antibodies.

DISCUSSION

Complement-mediated opsonophagocytosis is essential for innate immunity against invasive pneumococci. Initially, E.J. Brown et al. (Brown et al., 1983) suggested that the alternative complement pathway is crucial. More recently, J.S. Brown et al. (Brown et al., 2002) found that C1q and C4 were dominant for innate resistance, rather than factor B, while natural IgM mediated a smaller portion of the innate resistance to *S. pneumoniae*. In contrast, IgM is typically the major route to the initiation of the complement pathway via C1q. Therefore, an unusual innate mechanism must exist that mediates the classical C1q-dependent pathway of complement fixation against *S. pneumoniae*.

We believe that our findings identify this missing mechanism. It involves a transmembrane C-type lectin, SIGN-R1, that is expressed on splenic MZ macrophages and binds C1q as well as polysaccharides. Since SIGN-R1 is itself aggregated in vivo (Kang et al., 2003) and is required for C1q accumulation in MZ macrophages, we suggest that C1q binding to SIGN-R1 is analogous to the binding of C1q multimers to IgM or immune complexes in the usual classical pathway for complement activation (Figure 7). In the SIGN-R1 pathway, we propose that the lectin captures C1q, which is bound to C1r and C1s in serum, and this in turn leads to the capture and proteolysis of C2 and C4 to form a C3 convertase. Indeed we found that cell-associated SIGN-R1 leads to C4-dependent but Ig- and factor B-independent fixation of C3. When SIGN-R1 lectin binds polysaccharides, we suggest that this or adjacent molecules are able to form a C3 convertase that fixes C3 from the fluid phase (Figure 7). This might occur at the cell surface or possibly within intracellular vesicles, since SIGN-R1 is an endocytic receptor (Kang et al., 2003; Kang et al., 2004). We further suspect that the processing of C3 to form ligands for C3 receptors (opsonins) allows for the release of opsonized polysaccharide or organisms from MZ macrophages. These can then bind to phagocytes, B cells, and FDCs, the latter emphasized in this paper.

The ability of the membrane bound lectin SIGN-R1 to bind C1q and lead to C3 fixation is homologous to the soluble mannose binding lectin pathway for C3 fixation (Fujita, 2002). In that pathway, the lectin forms multimers analogous to C1q, and then MASP proteases are captured that are analogous to C1r and C1s, allowing for C2 and C4 binding and C3 convertase activity. However, there is evidence that the soluble mannose binding lectin is not a critical player in the recognition of *S. pneumoniae* (Neth et al., 2000; Krarup et al., 2005) or in defense against this pathogen (Atkinson et al., 2004).

Instead, the membrane bound lectin SIGN-R1 seems to be dominantly required at the level of the whole animal for the immediate activation of C3 by *S. pneumoniae* upon entry into the blood stream (Figure 2). In contrast, the direct fixation of C3 from serum through the alternative pathway is much slower and is observed with *S. pneumoniae* but not with CPS (Figure 2D). Because polysaccharides can be recognized by the abundant SIGN-R1 on MZ macrophages, both CPS and *S. pneumoniae* are within minutes of i.v. injection sequestered in the MZ and much of the C3 in serum is catabolized (Figure 2E). The consequences of this unusual pathway for C3 fixation, unusual because it is a classical pathway that does not require Ig, could include lysis of the organisms,

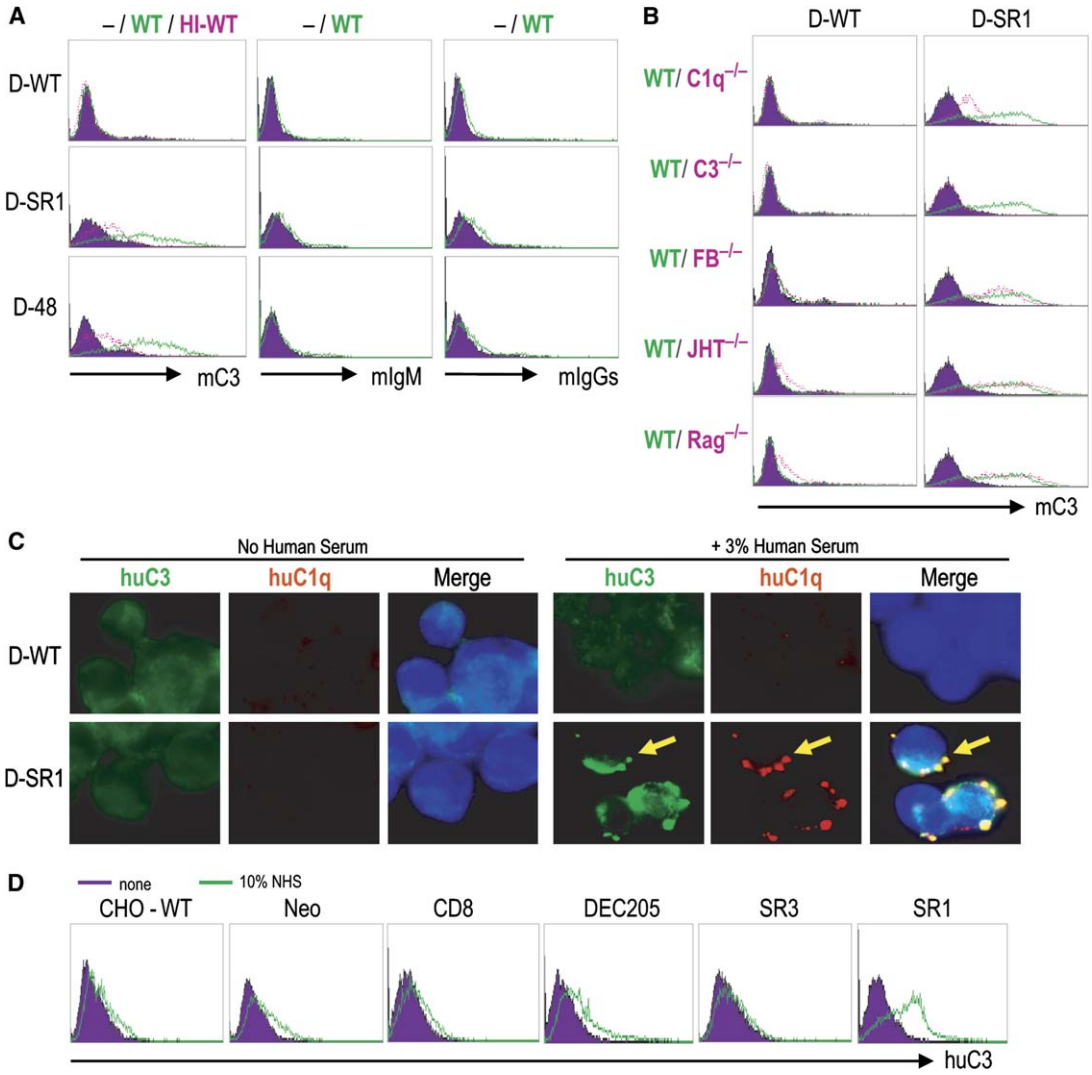

Figure 6. C3 Fixation via SIGN-R1 by the Classical Complement Pathway In Vitro

(A) DCEK transfectants were detached with EDTA and incubated with 3% fresh mouse serum for 30 min at 37°C. Cells were assessed by FACS for mouse C3, IgM, and total IgG with FITC-conjugated antibodies (all green lines). Heat-inactivated human serum (HI) was a negative control for C3 fixation (red dotted line on left).

(B) Binding of C3 to DCEK-WT and SR1 transfectants, incubated 1 hr with 3% of the indicated sera from knockout mice (red) or control wild-type (WT, green) in TC buffer.

(C) After incubating both DCEK-SIGN-R1 transfectants and DCEK wild-type cells with 3% NHS in TC buffer, C1q (red) and C3 (green) binding was assessed by staining at 4°C for 30 min (blue is actin). Arrows indicate colocalization of C3 and C1q.

(D) C3 fixation from 10% normal human serum was assessed by FACS in the indicated CHO transfectants.

capture by phagocytes, and eventual binding to C3 receptors on B cells and FDCs (Figure 7), the latter emphasized here.

C3 is known to be critical for innate resistance to *S. pneumoniae* (Brown et al., 2002). We confirmed prior research showing increased susceptibility to i.v. infection in $C3^{-/-}$ mice and following C3 depletion with CVF (Figure 3D). SIGN-R1 is a major pathway for early C3 catabolism in response to i.v. injection of *S. pneumoniae* and CPS (Figure 2), and SIGN-R1 contributes to innate resistance (Figures 3D and 3E). However, the effect of SIGN-R1 removal on resistance to i.v. infection is not as large as C3 removal. This implies that there are additional C3-dependent pathways for resistance. While SIGN-R1 contributes to the classical C4 dependent pathway, the latter also receives input via natural antibody (Brown et al., 2002; Koppel et al., 2005) and perhaps other lectins related to SIGN-R1, with SIGN-R3 being the best possibility (Takahara et al., 2004). The alternative factor B-dependent pathway to C3 fixation also contributes, although it is

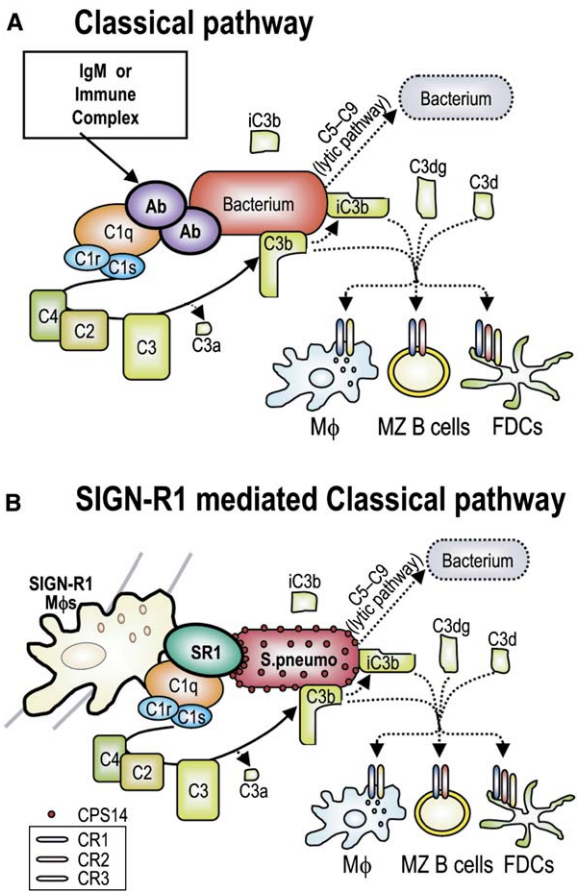

Figure 7. A Proposal for the Ig-Independent Role of SIGN-R1 in Activating the Classical Complement Pathway In Vivo

SIGN-R1 on macrophages, which binds polysaccharides and pneumococci within minutes following i.v. injection, replaces the Ig that is usually used in the classical pathway to bind C1q and then assemble a C4 C2 convertase for C3. Limited proteolysis of C3 leads to the formation of opsonins (C3b, iC3b, C3d) that fix to bacteria or to CPS, which likewise have been bound to SIGN-R1 on macrophages. The C3 opsonins in turn allow for binding to complement receptors on macrophages, B cells and FDCs, which are termed CD35, CD21, CD11b or CR1, CR2, CR3 respectively.

subdominant to C1q and C4 pathways in mice (Brown et al., 2002).

Recent evidence suggests that complement and cellular-complement receptors also play important roles in the localization and retention of nonbacterial pathogens to FDCs, especially HIV-1 (Stoiber et al., 2001; Banki et al., 2005) and PrP^{Sc}, a prion protein (Heppner et al., 2001; Mabbott and Bruce, 2001). Temporary depletion of C3 or genetic deficiency of C1q significantly delays the onset of scrapie following peripheral infection and reduces the early accumulation of PrP^{Sc} in the spleen, implying that in the early stages of infection, C3 and C1q contribute to the localization of scrapie infectivity in lymphoid tissue, i.e., a classical pathway for complement fixation (Heppner et al., 2001; Mabbott and Bruce, 2001). Interestingly, SIGN-R1 binds viral glycoproteins, as do human DC-SIGN and L-SIGN (Marzi et al., 2004). While we have emphasized the role of SIGN-R1 during complement-mediated innate resistance to pneumococci, some viruses and prions may use this new splenic pathway for complement fixation.

EXPERIMENTAL PROCEDURES

Mice, Cells, and Reagents

Six- to ten-week-old BALB/c or C57BL/6 mice of both sexes (The Jackson Laboratory) were kept in specific pathogen-free conditions and studied according to institutional guidelines. Factor $B^{-/-}$ mice were kindly provided by Dr. Rick Wetsel (University of Texas). Splenectomy was conducted according to institutional guidelines on Biomethodology and Surgical Techniques. Briefly, mice were anesthetized with avertin. A 1 cm midline incision through the skin and musculature was made just below the sternum, followed by exposure of the spleen. The spleen was gently retracted, freed from surrounding tissue, and removed by blunt dissection with mosquito forceps. The musculature and subcutaneous tissues were closed by absorbable sutures and metal wound clips, respectively. Sham control mice underwent this operative procedure, only with spleens remaining intact. Mouse sera were obtained from $C1q^{-/-}$, $J_H^{-/-}$, RAG-$1^{-/-}$, factor $B^{-/-}$, $C4^{-/-}$, and $C3^{-/-}$ mice and stored at −70°C until use as fresh or heat-inactivated (56°C, 30 min) sera. Chinese Hamster Ovary cells (CHO), human embryonic kidney (HEK293) cells, and mouse fibroblast (DCEK) cells were cultured in Dulbecco's Modified Eagle Medium (for CHO and HEK293) or RPMI 1640 (for DCEK) supplemented with 10% FCS, 100 units/ml penicillin G, and 100 μg/ml streptomycin. As described previously (Kang et al., 2003; Kang et al., 2004), we used stable CHO transfectants expressing cDNAs of mouse SIGN-R1, SIGN-R3, DEC205, and human CD8 and DCEK transfectants expressing SIGN-R1 and SIGN–R1-#48 (SIGN-R1 lacking its cytosolic domain). We purchased: purified pneumococcal polysaccharides of type 14, 23, and 26 (American Type Culture Collection, Manassas, VA), streptavidin beads (SA-beads, Amersham Biosciences, Piscataway, NJ), transferrin and DAPI (Sigma-Aldrich, St. Louis, MO), endotoxin free OVA (efOVA, SEKAGAKU Corp. Tokyo, Japan). To produce soluble SIGN-R1 protein, we fused the sequence of a signal peptide from mouse IgG1 to the extracellular portion of SIGN-R1 (GenBank, accession number DQ119139), stably transfected this construct into CHO cells, and purified SIGN-R1 from the culture supernatant by affinity to a 22D1 anti-SIGN-R1 mAb column. The full-length cDNAs for mouse C1q A, B, C chains were cloned by PCR and sequenced. The A chain was modified to carry a myc tag at the carboxyl terminus. All 3 chains of C1q were expressed together in HEK293 cells by transient transfection. We purchased human C1q as well as normal human serum (Gemini Bio-Products) and sera depleted of C3, C4, C1q, factor B (Quidel Corp). CVF (Quidel Corp) was injected i.p. 60U/kg 1 day prior to pneumococcal challenge.

Antibodies and Microscopy

The generation of rabbit polyclonal antibody against the C-terminal 13 amino acid peptide of SIGN-R1 (PAb-C13) was described previously (Kang et al., 2003). 22D1 (anti-SIGN-R1) and N418 (anti-CD11c) hamster mAbs were purified from hybridoma culture supernatants. We purchased antibodies to mouse C3 (FITC- or HRP-conjugated, ICN Pharmaceuticals), human C3 (Abcam), total hamster or human IgG and IgM (Jackson ImmunoResearch Laboratories), MARCO (ED31 rat IgG1 mAb, SeroTec), dendritic cells (CD11c, BD Biosciences Pharmingen), serotype 14 pneumococci (Statens Serum Institute, Denmark), actin (Abcam, Cambridge, MA), and tubulin (SIGMA, St. Louis, MO). Polyclonal anti-human C1q and monoclonal anti-mouse C1q (7H8) were purchased from Abcam and HyCult biotechnology. As secondary reagents, we used HRP-, FITC -, PE -, AMCA-, or

Alexafluore-conjugated donkey anti-chicken IgY, goat anti-hamster IgG, donkey anti-rabbit IgG, goat anti-rat IgG, and HRP-conjugated streptavidin from Abcam (Jackson ImmunoResearch Laboratories), and Molecular Probes. Transfected cells and spleen sections were examined for fluoresence with a deconvolution microscope (Olympus, Melville, NY).

Binding of C1q from Tissue Lysates and Serum to Purified Soluble SIGN-R1

Purified soluble SIGN-R1 and control proteins were biotinylated with EZ-Link NHS-Biotin (PIERCE) for 2 hr at 4°C, and 5 μg of biotinylated proteins were applied to 40 μl of streptavidin beads (SA-beads, Amersham Biosciences) for 2 hr at 4°C in PBS. Spleen and lymph node were lysed in RIPA buffer (150 mM NaCl/50 mM Tris•HCl, pH 8.0/1% Nonidet P-40/0.5% sodium deoxycholate/0.1% SDS) supplemented with protease inhibitor cocktails (Sigma-Aldrich, St. Louis, MO) and stored at −80°C. 10 mg of lysates was incubated for 12 hr at 4°C with SIGN-R1 or control protein coated beads. Bound proteins were eluted and separated on a 4%–15% gradient SDS PAGE gel and developed with silver staining. A specific band that was selectively bound to SIGN-R1 coated beads was identified as C1q by MALDI-TOF peptide sequence analysis (Rockefeller University Proteomics Resource Center). In addition, to demonstrate C1q binding to SIGN-R1, 50 μl of human and mouse sera were incubated for 12 hr at 4°C with SIGN-R1 or control protein coated beads. Bound proteins were eluted and separated on a 4%–15% gradient SDS PAGE gel followed by immunoblotting with chicken anti-human C1q.

S. pneumoniae Strains and Fluorescent Labeling

The capsular serotype 14 (DCC1490) of *S. pneumoniae* was grown in Brain Heart Infusion broth (DIFCO) to mid-log phase, inactivated with 50 μg/ml of Mitomycin-C (SIGMA, St. Louis, MO) for 1 hr, and labeled at 10^9 bacteria/ml with 5 μM of CFSE (Sigma-Aldrich, St. Louis, MO) for10 min at 37°C. Excess CFSE was removed by extensive washing in PBS. 1×10^8 fluorescent bacteria were given i.v. and spleen sections examined by deconvolution fluorescence microscopy.

Assay for In Vivo and In Vitro C3 Processing in Serum

To quantify C3 processing in serum in vivo, 1×10^8 *S. pneumoniae* or 200 μg CPS14 were injected i.v., and 40 μl of blood was collected at 0, 5, 15, and 30 min. The serum (separated at 14,000 rpm 10 min at 4°C) was diluted 1 to 25 with 2 × SDS sample buffer with 2-mercaptoethanol, boiled at 95°C for 10 min, separated on SDS PAGE, and immunoblotted with polyclonal anti-mouse C3. For in vitro C3 processing, 1×10^8 *S. pneumoniae* were incubated with 10% mouse serum in TC buffer (140 mM NaCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM Tris, pH 7.5, supplemented with 1% BSA) for 30 min at 37°C. The bacteria were washed, mixed with 20μl of 2 × SDS sample buffer as above for separation on SDS-PAGE, and immunoblotted with anti-mouse C3.

Complement Binding to Cells Using FACS Analysis

After detaching with 1 mM EDTA in PBS for 10 min, cells were incubated with 1%–10% mouse or human sera in TC buffer for 30 min at 37°C. Cells were washed, incubated with antibodies for 30 min at 4°C, and analyzed for C1q and C3 binding with a FACSCalibur flow cytometer (Becton Dickinson).

Supplemental Data

Supplemental Data include six figures and can be found with this article online at http://www.cell.com/cgi/content/full/125/1/47/DC1/.

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