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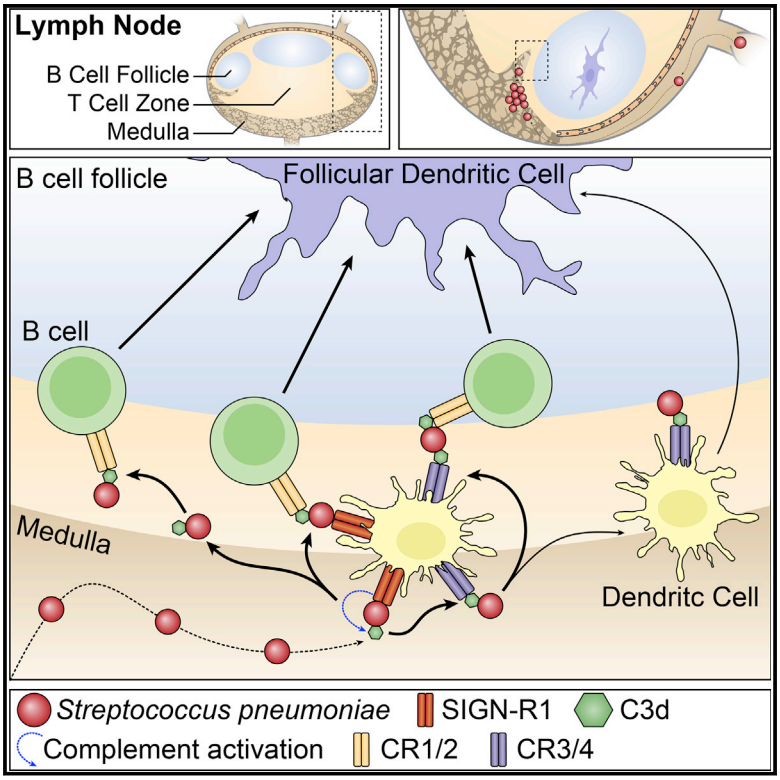
The Role of Dendritic Cells in *S. pneumoniae* Transport to Follicular Dendritic Cells

Graphical Abstract



Highlights

- Subcapsular sinus macrophages do not bind *Streptococcus pneumoniae*
- SIGN-R1-positive dendritic cells bind *Streptococcus pneumoniae*
- Dendritic cells can physically transport antigen to follicular dendritic cells
- Bacteria are transported to follicular dendritic cells intact

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In Brief

Transport of antigen to the stromal follicular dendritic cell (FDC) population is important for antibody production. Heesters and Carroll show that, in addition to B cells, dendritic cells also have the capability to transport antigen to FDCs. Furthermore, they show that intact bacteria (*S. pneumoniae*) are transported to FDCs.

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The Role of Dendritic Cells in *S. pneumoniae* Transport to Follicular Dendritic Cells

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SUMMARY

Affinity-mature B cells require cognate antigen, retained by follicular dendritic cells (FDCs), for clonal selection within germinal centers. Studies on how FDCs in lymphoid tissues acquire antigen have relied primarily on model protein antigens. To examine delivery of intact bacteria to FDCs, we used inactivated *Streptococcus pneumonia* (SP). We found that both medullary macrophages and a subset of SIGN-R1-positive dendritic cells (DCs) in the lymph node capture SP from the draining afferent lymphatics. The presence of DCs is required for initial complement activation, opsonization of the bacteria, and efficient transport of SP to FDCs. Moreover, we observed a major role for transport of bacteria to FDCs by naive B cells via a CD21-dependent pathway. We propose a mechanism by which efficient transport of SP to FDCs is dependent on DCs for initial binding and activation of complement and either direct transport to FDCs or transfer to naive B cells.

INTRODUCTION

The Gram-positive pathogen *Streptococcus pneumoniae* (SP), commonly known as pneumococcus, is the predominant cause of community-acquired pneumonia and causes many cases of Otis media, sinusitis, meningitis, and septicemia (Musher, 1994; Tomasz, 2000; Tuomanen, 2004). SP resides in the nasopharynx as a commensal in up to 20% of healthy adults and up to 50% of healthy children. It is an opportunistic bacterium and infection often occurs after another respiratory tract infection, e.g., during influenza pandemics the leading cause of death is often a secondary infection with SP (Palese, 2004). Invasive pneumococcal disease leads to high mortality and morbidity rates, especially in young, elderly, debilitated, or immunosuppressed individuals (Janoff and Rubins, 1997). Worldwide more than one million people die from pneumococcal infections each year, mostly in the developing world (Klein et al., 1994; Mulholland, 1999). However, the rate at which resistance of SP to antibiotics is increasing in the United States and the rest of the developed world is alarming (Appelbaum, 2002; Klugman, 2004). As of 2013, data show that SP is resistant to one or more antibiotics in 30% of clinical cases (Centers for Disease Control and Prevention, 2013a, 2013b).

Clearance of SP is mediated through opsonization by immunoglobulin (Ig) and complement. In 1969 it was shown that complement is protective in pneumococcal disease by increasing phagocytosis (Johnston et al., 1969). Opsonization of SP with complement C3 and its breakdown product C3d facilitates uptake of immune complexes (ICs) by CD21 (Griffioen et al., 1991). Recognition and binding of SP by the C-type lectin receptor SIGN-R1 activates the classical complement pathway via C1q, leading to opsonization and deposition on the follicular dendritic cell (FDC), which is required for humoral immunity against SP (Brown et al., 2002; Kang et al., 2006).

Despite these elegant findings on the mechanism of antigen binding and complement activation, it remains unclear how SP is delivered to B cell follicles in draining lymph nodes (LNs) for presentation in germinal centers (GCs). Earlier studies tracking uptake of particulate antigen within skin-draining LNs show that subcapsular sinus macrophages (SCSMs) were essential in the induction of a humoral response and have been referred to as guardians of the LN (Gaya et al., 2015). In similar studies, Cyster and colleagues identified one pathway by which SCSMs bind C3-opsonized IC via complement receptor 3 (CR3) and shuttle it into the underlying B cell compartment, where mature, non-cognate B cells take up the opsonized IC via CD21 (Phan et al., 2007, 2009). More recently, Heesters et al. (2013) reported that naive B cells deliver C3-opsonized IC directly to FDCs, where it is transferred via CD21 and rapidly internalized into a cycling endosomal compartment for long-term retention.

FDCs play a critical role in regulating the architecture of B cell follicles and maintenance of GCs via secretion of chemokines and cytokines and retention of antigen (Tew et al., 1990; Wang et al., 2011; Wu et al., 2009). Studies using model protein antigens demonstrate that B cell activation in the presence of co-stimulation by T follicular helper (TFH) cells leads to the formation of GCs, where B cells undergo class switch recombination and somatic hypermutation, which finally results in differentiation to memory and effector B cells (see Victora et al., 2010 for review) (Victora and Nussenzweig, 2012). FDC retention of antigen within GCs is required for efficient clonal selection and affinity maturation as cognate B cells acquire antigen and present it to TFH cells (Gitlin et al., 2015; Shulman et al., 2013; Suzuki et al., 2009; Victora et al., 2010).

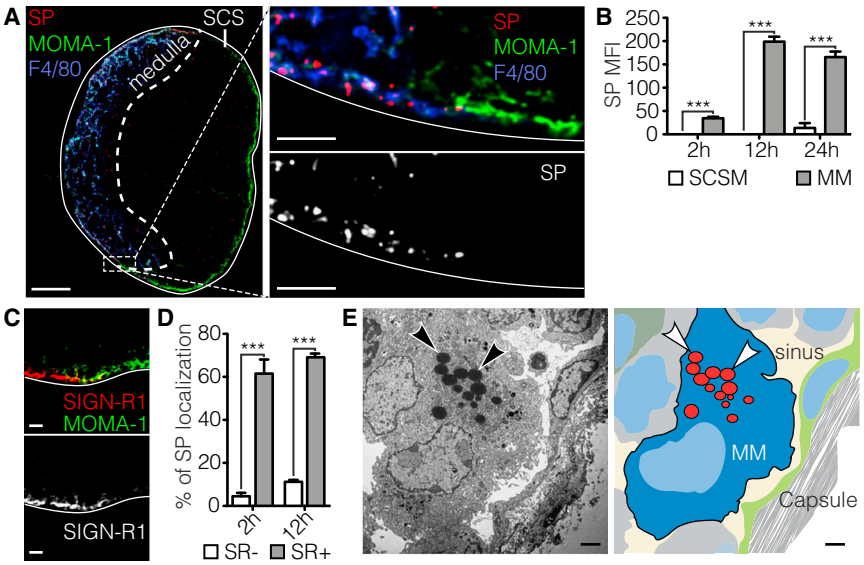

Figure 1. Capture of *Streptococcus pneumonia* Is Exclusive to the Medulla

(A) Left: confocal image of the popliteal lymph node (pLN) in a mouse immunized with *Streptococcus pneumoniae* (SP) (CMTPX, red) 2 hr prior. Subcapsular sinus (SCS) macrophages (MOMA-1, green) mark the SCS area, while medullary macrophages (MMs) (F4/80, blue) mark the medullary region. Scale bar, 100 μm. Right: magnification of the transition from medulla to SCS in which single cocci can be resolved. Gray scale image of the SP channel shows selective binding to the medulla. Scale bar, 10 μm.

(B) Quantification of (A) based on mean fluorescence intensity (MFI) of SP. Arrival and accumulation of SP in the medulla is gradual over the course of 12 hr. At 2 hr SP was detected in the medulla, in contrast to the SCS region were no binding was detected up to 24 hr (***$p < 0.001$, ANOVA with Tukey's multiple comparisons test).

(C) Confocal micrograph of the medulla-SCS interface shows exclusive staining for SIGN-R1 (SR1, red) in the medulla. SCS is marked with MOMA-1 (green). Grayscale image of the SR1 channel is shown. Scale bar, 10 μm.

(D) Quantification of (C) percentage of total identified cocci is shown (***$p < 0.001$, ANOVA with Tukey's multiple comparisons test).

(E) Left: electron micrograph of an MM that has engulfed multiple SP (arrows), adjacent to the medullary sinus, is shown. Scale bar, 2 μm. Right: color-coded overlay is shown to aid in structure identification.

To track delivery of SP to the FDCs and its subsequent acquisition by cognate B cells, we developed a model in which mice were immunized subcutaneous (s.q.) with fluorescently labeled inactivated SP. We found, unexpectedly, that SCSMs did not bind SP and that macrophages were not required for humoral immunity to SP. Instead, bacteria captured and opsonized by LN-resident dendritic cells (LNDCs) were either handed off to naive B cells in the nearby follicles or, although less common, transported directly to the FDCs. We propose a mechanism in which B cells require dendritic cell (DC)-mediated opsonization of SP (presumably through SIGN-R1) and in which both B cells and DCs have the capacity to transport opsonized SP to the FDCs within the LN follicles.

RESULTS

Medullary Macrophages Capture Lymph-Borne SP

To identify the mechanism underlying the capture and transport of lymph-borne SP in the LN, mice were injected in the footpad with fluorescent-labeled, heat-inactivated SP (strain D39, serotype 2). This results in passive drainage of the bacteria to the popliteal LN (pLN) (Gonzalez et al., 2011; Roozendaal et al., 2009). Imaging of cryosections of pLNs early after injection indicates localization of SP in the medullary region and not in the subcapsular region (Figure 1A). Capture is apparent at 2 hr after injection, and quantification of the data shows an increase in binding in the medullary region at 12 hr but negligible binding in the subcapsular region over a 24-hr period (Figure 1B). This was unexpected as earlier studies had identified the SCSMs as required for humoral immunity to particulate antigens (Carrasco and Batista, 2007; Gaya et al., 2015; Iannacone et al., 2010; Junt et al., 2007). One difference from the earlier studies is that here we immunized with intact bacteria. For example, influenza virus, which is taken up by SCSMs, is opsonized by serum complement component mannan-binding lectin (MBL) whereas SP is not (Figure S1A).

Medullary macrophages (MMs), which mark the medullary region, are distinguished by staining with antibody to SIGN-R1, whereas SCSMs are identified with anti-MOMA-1 and a lack of SIGN-R1 staining (Gonzalez et al., 2010; Phan et al., 2007). To further identify cell types involved in the uptake of labeled SP, cryosections of pLN isolated at 2 and 12 hr post-injection in the footpad were stained with antibody to SIGN-R1 and MOMA-1. Quantitation of the results identified a high percentage of the SP co-localized with SIGN-R1+ MMs, whereas negligible co-localization was observed with the MOMA-1+, SIGN-R1− SCSMs (Figures 1C and 1D).

Qualitative analysis of pLNs by electron microscopy (EM) following injection of SP further confirmed internalization of the bacteria by macrophages within the medullary, but not the subcapsular, region (Figure 1E). At later time points, SP was observed in the follicular region, co-localizing with the FDC marker 8C12; this is discussed in more detail below.

LNDCs and Macrophages Bind SP

Flow cytometric analysis of single-cell suspensions prepared from pLN was performed to determine which cell types are responsible for the binding and possible transport of SP to the FDCs. Heat-inactivated fluorescent-labeled SP was injected in the footpad and the pLN was harvested at 2 hr, after which a single-cell suspension was prepared that was stained with antibodies for CD11b, CD11c, CD4, CD8, and SIGN-R1. Three distinct populations of CD11c+ DCs were identified as SIGN-R1+ (i.e., CD11b-int SIGN-R1-int, CD11b-hi SIGN-R1-int, and

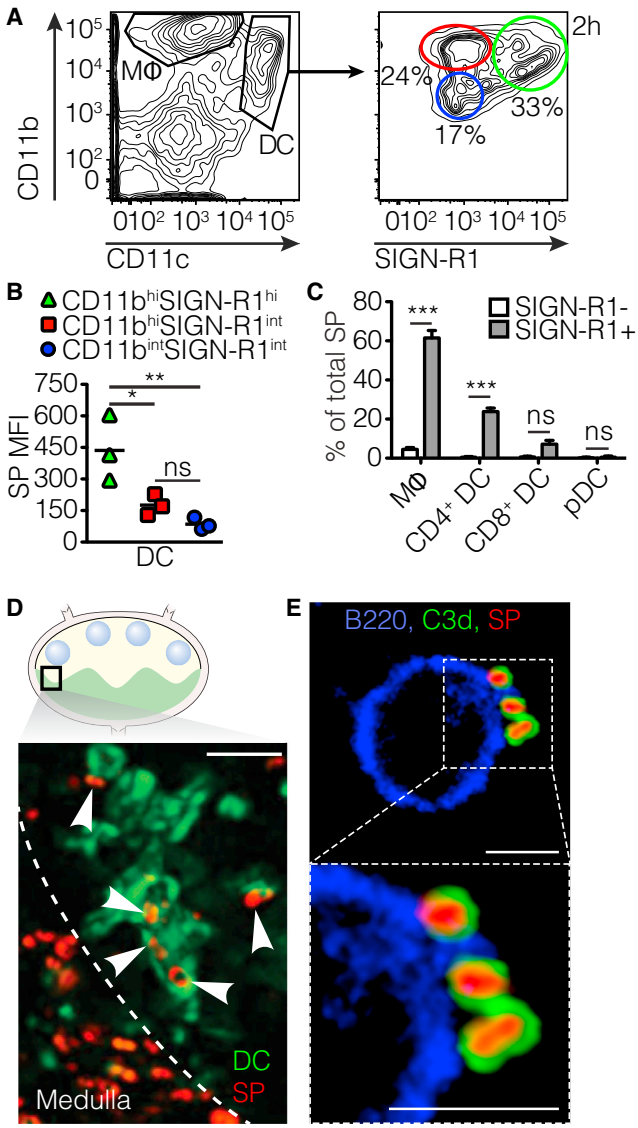

Figure 2. SIGN-R1+ Dendritic Cells and Macrophages Bind SP

All experiments were performed 12 hr after injection of fluorescently labeled heat-inactivated SP in the footpad.

(A) Left: flow cytometry gating strategy to distinguish dendritic cells (DCs) and macrophages. Right: based on SIGN-R1(SR1) and CD11b expression, three DC subsets were identified.

(B) In the three groups defined, the MFI of SP was determined as a correlate of binding (**$p < 0.01$ and *$p < 0.05$, ANOVA with Tukey's multiple comparisons test).

(C) Macrophages, plasmacytoid DCs, CD4+ DCs, and CD8+ DCs were divided into SR1-positive (green) and -negative (blue and red) groups (negative group comprised of both CD11b^{hi} and CD11b^{int}, SR1^{int} groups), and the percentage of total SP bound by these groups was determined (***$p < 0.001$; ns, not significant; ANOVA with Tukey's multiple comparisons test).

(D) Snapshot was taken from multiphoton intravital imaging of the pLN in a CD11c-eYFP (green) mouse immunized with SP (red, white arrows) near the medullary border. Scale bar, 10 μm.

(E) High-magnification image of an isolated B cell binding complement C3d-coated SP on its surface. Structures are marked as follows: CD45R/B220, blue; SP, red; and C3d, green. Scale bar, 5 μm.

CD11b-hi SIGN-R1-hi) (Figure 2A). Among the three populations, CD11b-hi SIGN-R1-hi bound SP with the highest mean fluorescence intensity (MFI) (Figure 2B). Further analysis of this SP+ population revealed a specificity for SIGN-R1+ macrophages and SIGN-R1+ CD4+ DCs (Figure 2C). One explanation is opsonization and subsequent stabilization through complement receptors CD11b and CD11c, also known as complement receptor (CR) 3 and 4, respectively. The critical role of SIGN-R1 and complement in SP opsonization and subsequent binding by DCs was further emphasized by the lack of stable SP binding by DCs in C3$^{-/-}$ and C1q$^{-/-}$ mice (Figure S1B). Opsonization through SIGN-R1 is mediated by C1q (Kang et al., 2006). These data suggest that stable binding of SP by DCs requires complement opsonization and possibly subsequent binding of either CR3 or CR4 (CD11b or CD11c).

To follow the capture in the LN of SP by CD11c+ DCs in real time, mice expressing enhanced yellow fluorescent protein (eYFP) under the CD11c promoter were immunized with labeled, inactivated SP, and uptake was visualized by multiphoton intravital imaging (MP-IVM) of the pLN at 2 hr post-injection (Gonzalez et al., 2010; Lindquist et al., 2004) (Figure 2D). Imaging of the interfollicular region adjacent to the B cell follicles identified eYFP+ DCs in close proximity of the medullary sinus and loaded with bacteria. DCs captured bacteria in close proximity to the medullary sinus, possibly via SIGN-R1 (Figure 2D). Notably, a similar uptake location and involvement of SIGN-R1+ LNDCs were reported earlier by Gonzalez et al. (2010) following footpad injection of inactivated influenza A (IAV) viral particles. Interestingly, complement C3-coated, labeled SP also was observed on the surface of B220+ cells (B cells) (Figures 2E and S2A–S2D).

LNDCs Are Required for Immune Response against SP

To assess whether DCs, macrophages, or both are required for an immune response against SP, chimeric mice were constructed in which bone marrow (BM) from CD11c-diphtheria toxin receptor (DTR) transgenic mice was transferred into lethally irradiated wild-type (WT) C57BL/6 recipients. In this model, BM-derived cells within the CD11c-DTR>>WT chimeric mice express the DTR under control of the CD11c promoter, making DCs susceptible to diphtheria toxin (DTx). As controls, irradiated WT recipients, reconstituted with either WT BM (WT BM>>WT) or BM isolated from mice deficient in the alpha chain of T cell receptor (TCR$\alpha^{-/-}$ BM>>WT), were used. After 6–8 weeks recovery, the three groups were treated with 100 ng (~5 ng/g body weight) DTx and immunized with heat-inactivated SP in the footpad 48 hr later. IgM titers were assessed by ELISA at day 10 post-immunization (Figures S3A–S3D). As expected, WT BM>>WT and TCR$\alpha^{-/-}$ BM>>WT chimeric mice treated with DTx responded with a robust IgM titer. By contrast, the IgM response was severely diminished in CD11c-DTR BM>>WT chimeras when administered DTx. These results support the requirement of DCs in the initiation of a T-independent humoral immune response against SP, and they suggest that DCs may participate in the transport of SP to the follicle.

To assess the contribution of sinus-lining macrophages in the immune response against SP, clodronate liposomes (CLLs) were used to deplete phagocytic macrophages. Earlier studies have used this approach to eliminate sinus-lining macrophages in

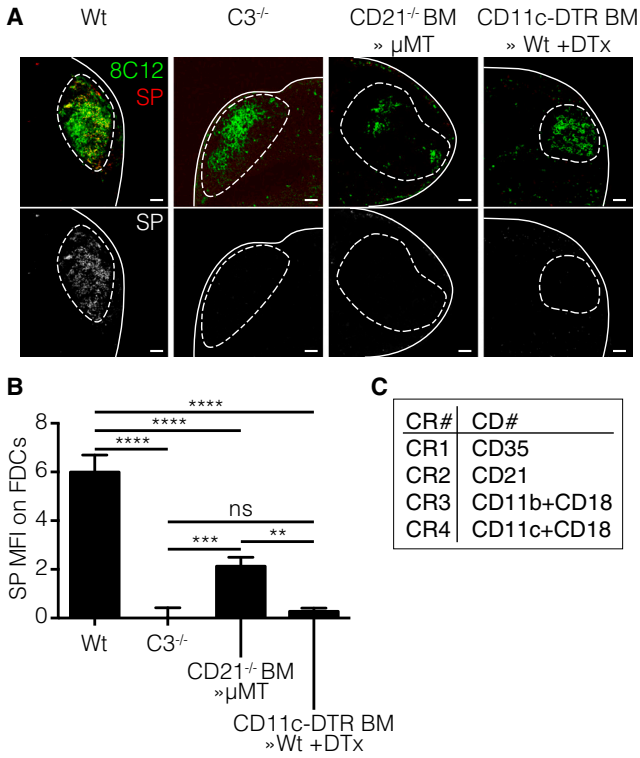

Figure 3. DCs and B Cells Collaborate in the Transport of SP to the FDCs

Mice were injected with fluorescently labeled SP (red) in the footpad as described earlier. Four different mouse strains were used: wild-type (WT), complement C3 knockout ($C3^{-/-}$), complement receptor 2 (CD21) knockout bone marrow (BM) in µMT B cell-deficient recipients ($CD21^{-/-}$ BM>>µMT), and CD11c-dtr BM in WT recipients (CD11c-dtr BM>>WT) injected with DTx 48 hr prior to immunization. The FDC network was stained using 8C12 (green).

(A) Confocal micrographs of pLN 12 hr after immunization with SP. Line indicates LN capsule and dotted line indicates follicle area. Bottom row: gray-scale image of the SP channel is shown. Scale bars, 100 µm.

(B) Quantification of confocal images by Cell Profiler software. Only co-localized signal was measured (****$p < 0.0001$, ***$p < 0.001$, and **$p < 0.01$; ns, not significant; ANOVA with Tukey's multiple comparisons test).

(C) Overview shows complement receptor (CR) nomenclature and their respective cluster of differentiation (CD) numbers.

skin-draining LNs without affecting LNDCs (Gonzalez et al., 2010; Junt et al., 2007). Importantly, under the conditions used in this study, CLLs did not deplete LNDCs but did deplete CD11b+ macrophages (Figures S3F and S3H). Subsequently, the CLL-treated group and controls (PBS only or empty clodronate liposomes [ECLLs]) were immunized with SP, and their humoral response was assayed by quantitating number of antibody-secreting cells (ASCs) in the pLN and serum IgM titers determined by ELISA. Remarkably, the CLL treatment had a negligible effect on the IgM or total Ig response relative to the control groups, and it did not affect the number of ASCs in the LN (Figures S3C–S3E). By contrast, a knockdown of SIGN-R1 with anti-SIGN-R1 (clone 22D1), as described by Kang et al. (2003), diminished the number of ASCs in the LN (Figure S4). Taken together, LNDCs, but not sinus-lining macrophages, are required for an immune response against SP.

DCs and B Cells Collaborate to Transport SP to FDCs

As discussed above, naive mature B cells take up C3-opsonized ICs via CD21 within the follicles and deliver them to FDCs (Heesters et al., 2013, 2014; Phan et al., 2007, 2009). To examine whether transport of C3d-opsonized SP is also dependent on CD21 expression in B cells, BM chimeras were constructed in which lethally irradiated µMT mice, which lack mature B cells, were reconstituted with BM from $CD21^{-/-}$ mice. Thus, in the chimeric mice, the only source of mature B cells was from the donor-derived $CD21^{-/-}$ BM, whereas the FDCs were CD21 sufficient. C3-deficient mice ($C3^{-/-}$) and CD11c-DTR BM chimeric mice (CD11c-DTR BM>>WT) were used as a negative control and WT mice were used as a positive control. The four groups of mice were injected in the hock with labeled SP. Visualization and quantification of cryosections prepared from draining LNs identified the deposition of SP on FDCs in the WT mice, but not in the C3-deficient and CD11c-DTR BM>>WT control mice, as expected. Strikingly, a significant reduction in antigen deposition was observed on FDCs in pLNs of the $CD21^{-/-}$ µMT BM chimeras (Figures 3A and 3B). Interestingly, the phenotype observed in the $CD21^{-/-}$ µMT BM chimeras was not as severe as in $C3^{-/-}$ or CD11c-DTR BM>>WT mice, suggesting other C3-dependent pathways (such as LNDCs) may be involved in SP transport (Figures 3A and 3B).

Previous studies reported that deposition of C3-opsonized SP on FDCs correlates with the B cell memory response (Kang et al., 2006). To determine if DCs (CD11c+) can transport SP directly to the FDCs, MP-IVM was used in combination with the CD11c-eYFP reporter mice. Visualization of early events following subcutaneous injection of labeled SP identified direct transfer of SP from the LNDCs to the FDCs (Figure S3G; Movie S1). However, given the limits of real-time imaging of the draining LN, quantitation of the event was not possible by MP-IVM. Therefore, an ex vivo FDC approach was established. Cd11c-eYFP+ cells were sorted by fluorescence-activated cell sorting (FACS) and loaded with complement-opsonized SP. Subsequently, the cells were magnetically sorted with anti-CD45 beads to remove unbound SP and co-cultured with FDCs previously isolated and plated on coverslips (Heesters et al., 2013) (Figures 4A and 4B). After washing, co-cultures were imaged and SP binding to FDCs was quantified (Figures 4C and 4D). Results show that DCs are capable of transfer of SP to FDCs.

Our combined results suggest a model in which both LNDCs and B cells are necessary for the efficient binding and transport of C3-opsonized SP to the FDCs. Although naive non-cognate B cells provide the major pathway for transport of opsonized SP to the FDCs, LNDCs are crucial in the initial binding of SP that drains into the LN and subsequently either transports the bacteria directly to the FDCs or transfers opsonized bacteria to naive B cells in a CD21-dependent mechanism. In our model SP enters the LN through the afferent lymphatics after passive drainage from the injection site. Due to its thick glycan capsule, SP has evaded complement so far and thus does not bind CR3/CD11b on SCSMs. Cells in the medulla are capable of binding SP through the SIGN-R1 receptor, which recruits C1q and activates the classical complement pathway. Macrophages can then endocytose and digest the bacterium, while DCs can use CR3 (or CR4/CD11c) to stabilize SP. Then there are multiple

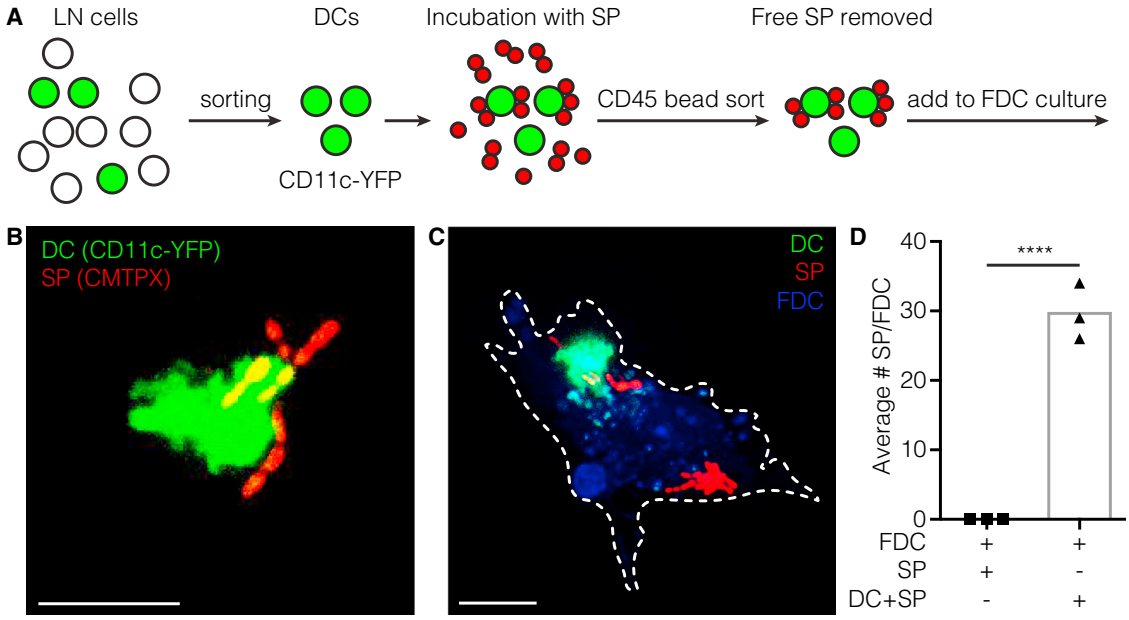

Figure 4. DCs Can Transfer SP Directly to FDCs

(A) Schematic of experimental setup. LN single-cell suspension from CD11c-YFP mouse is made. YFP-positive DCs are sorted via FACS. Sorted DCs are incubated with SP in the presence of complement components. Then DCs are sorted again using CD45 magnetic beads to remove unbound SP. DCs carrying SP are added to FDC cultures isolated 3 days prior.

(B) Sorted CD11c-YFP DC multiple carrying SP (CMTPX) on its surface is shown. Scale bar, 10 μm.

(C) FDC (blue) loaded with SP (CMTPX) and a DC (YFP) transferring SP is shown. Scale bar, 10 μm.

(D) Quantification of SP transferred to FDCs through DCs is shown (****$p < 0.0001$, Student's t test).

possible pathways: (1) opsonized SP can be released and bound by B cells through CR2, which transports SP to the FDC; (2) the DC can transfer the opsonized SP from SIGN-R1 or CR3 or CR4 to CR2 on a B cell, which transports SP to the FDC; or (3) the DC can transport opsonized SP to the FDC by itself and transfer it to CR1/CR2 on the FDC. The FDC then incorporates SP into a non-degradative cycling endosomal compartment, periodically displaying the antigen to cognate B cells in the GC reaction.

DISCUSSION

Early studies with SP reported that efficient clearance and host protection to the encapsulated bacteria were dependent on complement C3 and Ig (Brown et al., 2002; Griffioen et al., 1991; Neufield and Rimpau, 1904; Ward and Enders, 1933). Kang et al. (2004) made the unexpected observation that C3 opsonization of SP by LN macrophages was dependent on recognition and binding of mannan residues on the capsular surface by the C-type lectin SIGN-RI. Moreover, they demonstrated that SP deposition on FDCs within the follicles of draining LNs was C3 dependent and correlated with protective humoral immunity. These elegant studies raised the general question of the underlying mechanism for transport of C3-opsonized SP from the afferent lymphatics and delivery to the FDCs.

To address this important question, we developed a model in which heat-inactivated SP was fluorescently labeled and injected into the hind limb (hock region) of WT and reporter mice. Labeled SP was tracked both in real-time in CD11c-eYFP reporter mice using MP-IVM and by confocal imaging of cryosections prepared from the pLNs at varying time points. Strikingly, we found that the bacteria drained passively via afferent lymphatics into the medullary sinus, where they were rapidly taken up by MMs and underlying LNDCs. Unexpectedly, we observed that LNDCs migrated into the B cell follicles and directly transferred labeled SP to FDCs. Delivery was impaired by selective elimination of LNDCs in CD11c-DTR BM chimeras with DTx ablation. A limitation of this study is that DTx depletion also affects a small subset of macrophages and neutrophils (Bennett and Clausen, 2007; Tittel et al., 2012). As a control, WT mice also were treated with DTx. Further support for the importance of LNDCs came from our results showing that humoral immunity to SP immunization was significantly reduced following DTx ablation. Notably, BM chimeras ($CD21^{-/-}$ BM>>μMT), in which mature B cells were deficient in the CD21 receptor, also showed a significant reduction in the deposition of SP on FDCs following SP immunization.

Based on the importance of both LNDCs and CD21+ B cells in the delivery of SP to FDCs, we propose a mechanism by which lymph-borne SP is bound by SIGN-R1+ LNDC and opsonized with C3. CR3 and CR4 expressed on LNDCs may stabilize retention of the C3-opsonized complexes. Subsequently, opsonized SP is either delivered directly to FDCs or transferred to naive B cells, which then deliver the complexes to FDCs in a CD21-dependent pathway. These results do not rule out other pathways of delivery. For example, at later time points, it seems probable that SP at the site of injection is phagocytosed by migratory

DCs that deliver the bacteria into the draining LNs. Although, recent studies from our lab, using the UV-inactivated IAV (UV-IAV) model, found that passive drainage of the antigen into the LN was sufficient to support a protective IgG memory response (Woodruff et al., 2014).

The findings that sinus-lining macrophages are not required to support humoral immunity to SP and that SCSMs do not appear to take up the bacteria were unexpected based on earlier studies (Carrasco and Batista, 2007; Gaya et al., 2015; Iannacone et al., 2010; Junt et al., 2007). One explanation for the difference is that the nature and state of opsonization of the particulate antigen dictates in part the pathway for uptake and processing. SP is recognized by SIGN-R1, which is expressed by MMs and LNDCs, but not SCSMs, and earlier opsonization is unlikely due to SP's thick glycocoat. In an earlier study, we found that UV-inactive IAV, which also is decorated with mannan oligosaccharides, was bound by both SCSMs and MMs (as well as LNDCs). Notably in the IAV model, SCSM binding was not essential in the humoral response, whereas LNDC uptake was required (Gonzalez et al., 2010). This is consistent with recent findings by Gaya et al. (2015) where only live virus, bacteria, or Toll-like receptor (TLR) ligands disrupted the SCSM layer, but UV-inactive IAV did not. Interestingly, we did not observe any interaction with SCSMs, nor did depletion with liposomes disrupt the immune response. Our data emphasize that the mechanism of antigen capture in the LN might be more complex than earlier reported. We hypothesize that differences between pathogens, mainly their ability to evade complement fixation, dictate how they are processed in the LN.

Transport of SP to FDCs was shown to be a collaborative effort between DCs and B cells, which depended on C3 opsonization most likely mediated by SIGN-R1 on DCs. We hypothesize that SP is bound by SIGN-R1 and then opsonized with C3d, after which CD11b or CD11c (CR3 or CR4) mediates stabilization of the immune complex. Recent structural studies identified the site on C3d that is bound by CR3 and found that it did not interfere with the known site bound by CD21 (Bajic et al., 2013; van den Elsen et al., 2002). The CR4-binding site does not seem to interfere with CD21 (CR2) binding (Bajic et al., 2013). Thus, SP opsonized with C3d can be recognized by both CR3/CR4 and CD21 concurrently, and this could explain how the bacteria can be transferred from LNDCs to CD21+ B cells or CD21+ FDCs. Although the impaired humoral response to SP seems due to a defect in deposition on the FDCs, we do not exclude a role for TFH cells, which might function as a regulatory agent.

It will be important in future studies to confirm the importance of CR3/CR4 in LNDC transport of SP and gain a better understanding of the relative importance of direct transport of the bacteria by LNDCs versus handoff to naive B cells.

EXPERIMENTAL PROCEDURES

Mice

Mice were bred in house or were from Jackson ImmunoResearch Laboratories or Charles River Laboratory. The following strains were used: WT (C57BL/6), CD11c-eYFP, C3$^{-/-}$, C1q$^{-/-}$, CD21$^{-/-}$, TCR$\alpha^{-/-}$, μMT, and CD11c-DTR (Gack et al., 2007; Lindquist et al., 2004). All mice were on the C56BL/6 background. Mice were maintained in specific pathogen-free facilities at Immune Disease Institute, Dana Farber Cancer Institute, or Harvard Medical School. All animal experiments were in accordance with protocols approved by the Subcommittee on Research Animals Care at Harvard Medical School and The Immune Disease Institute, and they were in accordance with guidelines set by the NIH.

Bacterial Strains and Growth Conditions

SP strain D39 was grown in Todd Hewitt broth supplemented with yeast extracts and horse blood until log phase (OD_{650} = 1.5) (Restrepo et al., 2005). SP was then labeled by incorporation of CMTPX dye and heat inactivated at 65°C for 30 min.

Antibodies

The following antibodies were purchased from BioLegend: anti-B220 (RA3-6B2), anti-CD11c (N418), and anti-CD11b (M1/70). CD169 (36.112 and MOMA-1) was acquired from AbD Serotec. Anti-CD35 (8C12), anti-SIGN-R1 (22D1 and ERTR-9), anti-CD11b (M1/70), and anti-F4/80 (HB-198; American Type Culture Collection) were produced in house and were purified by affinity chromatography. Secondary antibodies, including streptavidin-Alexa Fluor 488 (S11223), streptavidin-Alexa Fluor 568 (S11226), streptavidin-Alexa Fluor 633 (S21375), and Alexa Fluor 633-conjugated anti-rat (A21094) were all from Invitrogen. Purified antibodies were labeled with Alexa Fluor 488 (A10235), Alexa Fluor 568 (A10238), Alexa Fluor 633 (A20170), or Pacific blue (P30012), according to the manufacturer's instructions (Invitrogen).

Mouse Pretreatment

For labeling of FDCs, mice received 5 μg 8C12 antibody intraperitoneally 24 hr before MP-IVM. For labeling of SCSMs in vivo, 1 μg fluorescence-labeled CD169 was injected into the footpad 3–5 hr before MP-IVM.

ELISA and Enzyme-Linked Immunospot Assay

Mice were immunized with 1 × 10^5 bacteria subcutaneously. At day 10, blood was collected and serum was obtained. ELISAs were done as described (Fernandez Gonzalez et al., 2008). For enzyme-linked immunospot assays, pLNs and spleens were removed aseptically and disrupted by passage through 70-μm mesh, and ASCs were quantified as described (Barrington et al., 2006).

Flow Cytometry and Data Analysis

A FACSCanto II (BD Biosciences) was used for flow cytometry. Dead cells were excluded by sytox blue (Invitrogen). Data were analyzed with FlowJo software version 9 (Tree Star).

Statistical Analysis

Samples were analyzed with two-tailed Student's t test or two-way ANOVA followed by Tukey corrections to correct for multiple comparisons; $p < 0.05$ between groups was considered significant. Statistical analysis was performed using GraphPad Prism version 6. Data are presented as data points with lines representing mean values ± SD or as bar graphs with mean ± SD.

Bacteria Injection

Anesthetized mice were injected with 1 × 10^5 heat-inactivated CMTPX-labeled bacteria in the hock (in a volume of 10 μl). At various time points, draining and non-draining LNs were isolated for analysis of bacterial trafficking and SP strain D39-specific immune responses.

Histology and Microscopy

Cryosections of LNs embedded in optimal cutting temperature compound (TissueTek) were prepared, then sections were washed with Hank's balanced salt solution and incubated with anti-FcR (2.4G2) and 2% BSA before incubation with antibody as described (Roozendaal et al., 2009). Transmission EM of LNs injected with SP was performed as described (Roozendaal et al., 2009). MP-IVM was performed as described (Gonzalez et al., 2010). For all mouse pretreatments, the hocks were injected with a volume of no more than 10 μl. Data were analyzed by the open source Fiji and Cell profiler software packages.

In Vivo Imaging

Mice were pretreated by intravenous injection with fluorescent anti-CR2 to label the FDCs in vivo. After 24 hr, fluorescent SP was injected subcutaneously into the footpad. The pLN was surgically exposed and MP-IVM was performed as described (Miller et al., 2002). Data were analyzed by the open source Fiji software package.

SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures and one movie and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2016.08.049.

AUTHOR CONTRIBUTIONS

B.A.H. and M.C.C. conceived and designed the experiments. B.A.H. performed the experiments. B.A.H. analyzed the data. B.A.H. and M.C.C. wrote the paper.

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