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# Lung B cells promote early pathogen dissemination and hasten death from inhalation anthrax

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## Abstract

Sampling of mucosal antigens regulates immune responses but may also promote dissemination of mucosal pathogens. Lung dendritic cells (LDC) capture antigens and traffic them to lung-draining lymph nodes (LDLNs) dependent on the chemokine receptor CCR7. LDCs also capture lung pathogens such as *Bacillus anthracis* (BA). However, we show here that the initial traffic of BA spores from lungs to LDLN is largely independent of LDCs and CCR7, occurring instead in association with B cells. BA spores rapidly bound B cells in lungs and cultured mouse and human B cells. Binding was independent of the B cell receptor (BCR). B cells instilled in the lungs trafficked to LDLN and BA spore traffic to LDLN was impaired by B cell deficiency. Depletion of B cells also delayed death of mice receiving a lethal BA infection. These results suggest that mucosal B cells traffic BA, and possibly other antigens, from lungs to LDLN.

### Keywords

anthrax; B cells; CCR7; lung; dissemination

## Introduction

Mucosal epithelia serve as barriers to the establishment of systemic bacterial infections. However, many pathogenic bacteria have evolved virulence strategies that enable them to overcome these barriers, disseminate from mucosa to peripheral tissues, and initiate systemic disease[1]. One mechanism for systemic dissemination of bacteria involves their phagocytosis and trafficking by host dendritic cells (DC)[2,3]. However, pathogen trafficking to peripheral lymph nodes can also occur via DC-independent mechanisms[4,5]. It is not clear



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**No conflict**

how such DC-independent dissemination of pathogenic bacteria occurs, or whether it contributes to host susceptibility.

DC migration from peripheral tissues to draining lymphoid tissues occurs when the DCs receive an activation signal, for example from inflammatory stimuli[6]. Activated DCs upregulate expression of the lymph node homing chemokine receptor CCR7, which mediates their traffic to draining lymphatics[7]. CCR7 is critical for migration of skin DCs to draining lymphatics[7], migration of intestinal DCs from the lamina propria to mesenteric lymph nodes[7,8], and for migration of lung DCs (LDCs) from lung alveolae to lung draining lymph nodes (LDLN)[9]. However, CCR7 is not required for the entry of B and T cells into lymph nodes (LN)[10].

*Bacillus anthracis* (BA) is a spore-forming bacterium that infects diverse mucosal tissues. Pulmonary exposure to BA spores causes inhalation anthrax, a rare but highly fatal disease. Data from humans contracting anthrax and from animal models consistently indicate that BA spores disseminate from the lungs to LDLN within five hours of their inhalation[11–13]. It is thought that this rapid dissemination is responsible for the development of lethal systemic disease. In the lung, BA spores are rapidly engulfed by immune cells such as phagocytic LDCs and alveolar macrophages (AM)[14]. It has been suggested that LDCs traffic phagocytosed BA spores away from the lungs[15]. However, trafficking of LDCs to the LDLN reportedly peaks at 24 h after antigen or pathogen exposure[9,16]. Thus, it is not clear whether BA spore trafficking by LDCs is responsible for the very rapid initial spore dissemination. It is also unknown whether trafficking of spores by LDCs is required for establishment of systemic BA infection.

In addition to LDC and alveolar macrophages (AM), the lungs of mice and humans contain a third population of professional APCs: B cells. Lung B cells are essential for maintaining pulmonary homeostasis and actively contribute to immune responses against a variety of pathogens[17,18]. B cells can respond to pathogens through their cell surface B cell immunoglobulin receptor (BCR) as well as a variety of innate receptors that recognize microbial products independent of the BCR[19]. Indeed, B cells have been shown to influence early immune responses to *Mycobacterium tuberculosis*[20] and *Salmonella typhimurium*[21] independent of antibody production.

Here, we employed a mouse model of inhalation anthrax with spores of the attenuated Sterne BA strain to investigate the mechanisms of BA spore traffic from the lungs to the LDLN. Using both Sterne-resistant C57BL/6 and Sterne-sensitive A/J mouse strains, we confirmed previous reports that BA spores traffic to the LDLN within a few hours of exposure. We further showed that such trafficking was independent of LDCs and CCR7 expression. Instead, BA spores rapidly associated with B cells in the lungs of infected mice and soon thereafter were observed in association with B cells in the LDLN. We observed migration of B cells from the lung to the LDLN and found that depletion of B cells from the lungs impaired the initial trafficking of spores to the LDLN. B cell depletion also significantly delayed death of lethally infected A/J mice. These findings suggest B cells contribute to the DC-independent establishment of systemic infections by mucosal pathogens.

## Results

### Rapid traffic of BA spores from the lungs to the LDLNs

To study trafficking of BA spores following pulmonary *in vivo* infection, we fluorescently labeled highly purified spores of Sterne (34F2) strain *B. anthracis* (pXO1+X02−) using DyLight 649 and administered them to Sterne-susceptible A/J mice using a non-surgical intratracheal (i.t.) inoculation procedure[22]. The labeled spores were uniformly bright and readily detected using flow cytometry (Fig. 1a). When the labeled spores were administered to A/J mice, we reproducibly detected a small population of DyLight 649+ host cells in the LDLNs within 6 hrs (Fig.1b). These data indicated that transport of BA spores to the LDLN occurs rapidly and reproducibly in this model infection. Comparison of DyLight 649+ cell numbers from the LDLN with numbers of heat resistant CFUs obtained from lysates of LDLN revealed an average ratio of 0.35 ±0.01 across three independent experiments (Fig. 1c). Thus, the number of DyLight 649+ cells consistently corresponded to ~1/3 the number of live spores. This suggests that each DyLight 649+ cell carried an average of ~3 viable spores. Hereafter, we refer to DyLight 649+ cells as spore+ cells.

Spore+ cells accumulated in the LDLNs as early as 30 min after i.t. inoculation with $10^8$ DyLight 649+ spores (Fig. 1d). The number of spore+ cells detected in the LDLN increased to ~0.015 % of the total cells by 5 hpi (Fig. 1b). No DyLight 649+ cells or BA CFU were observed in the spleens or blood of infected animals at these early time points. These data are consistent with prior histological observations of virulent BA spores in LDLNs within 5 hpi[11,13,15], and suggest that BA spores are rapidly and specifically trafficked from lungs to the LDLN.

### CCR7 is dispensable for trafficking of BA spores to the LDLNs

Active migration of particle or antigen-bearing LDCs to the LDLN in both steady state and inflamed conditions requires the lymph node homing G-protein coupled chemokine receptor, CCR7[9]. Thus, we examined whether CCR7-deficiency prevented accumulation of spore+ cells in the LDLN following i.t. infection. Approximately $3\times10^8$ spores were administered to groups of control B6 and B6.CCR7$^{-/-}$ mice. At 6 hpi, the LDLN were harvested and single cell suspensions analyzed by flow cytometry. A similar frequency and number of spore+ cells was seen in both groups of mice (Fig. 2a–b). Thus, CCR7 deficiency did not significantly impact spore trafficking to LDLN at 6 hpi.

To confirm that LDC migration was defective in the BA-infected CCR7$^{-/-}$ mice, we repeated these studies by analyzing mice co-injected with a mixture (1:1) of FITC+ latex beads and DyLight 649+ spores. Beads or influenza virus entering the lungs are known to be are carried to the LDLN by LDC and this trafficking peaks 24 h after antigen exposure[9,16]. Hence, we analyzed trafficking of spores and beads at 24 h after administering the mixed inocula i.t.. By this time point, both bead+ and spore+ cell populations were readily detected in suspensions of the LDLN from B6 mice (Fig. 2c). The beads and spores largely segregated into different cell populations, with very few cells staining doubly for FITC and DyLight 649 (Fig. 2c). As at 6 hpi, the frequencies of spore+ cells in the LDLNs of B6 and B6.CCR7$^{-/-}$ mice were nearly identical at 24 h (Fig. 2c). In addition, the total number of

spore+ cells seen in the LDLN of the CCR7$^{-/-}$ mice at 24 h was also not significantly reduced (Fig. 2d). There was a modest trend towards reduced numbers of spore+ cells in the CCR7$^{-/-}$ LDLN, which may indicate a minor contribution of the LDCs to BA spore traffic at this latter time point. However, bead+ cells were present only in the LDLN of B6 but not B6.CCR7$^{-/-}$ mice (Fig. 2c & 2e). These data confirmed that trafficking of latex beads requires CCR7 and showed that BA spore trafficking was largely independent of CCR7 until at least 24 hpi.

## B cells are the primary cell type associated with BA spores in the LDLNs at early times after infection

Given the initial trafficking of BA spores was largely CCR7-independent, we asked if a cell population other than LDC might be responsible for the initial wave of BA spore dissemination. Consistent with traffic of BA spores by professional APCs, spore+ cells from LDLN of A/J mice at 6 hpi showed strong cell surface staining for class II MHC (MHCII) antigens (Fig. 3a). Yet, very few of these spore+ cells co-stained positive for the DC marker CD11c (Figs. 3a–b). These results suggested that most of the spore+ cells were not LDCs or that uptake of spores might cause LDCs to down regulate cell surface CD11c. Three lines of evidence excluded the latter explanation: (1) cell surface CD11c expression was similar in spore-positive and spore-negative LDCs at 6h after *in vivo* infection (Fig. 3c); (2) addition of BA spores to bone marrow-derived DCs cultured in GM-CSF did not alter CD11c expression (data not shown); (3) on both a percentage and numerical basis over 3/4 of the spore+ cells isolated from LDLN stained positive for the B cell surface markers IgM and B220 (Fig. 3a–b). BA spores also rapidly reached the LDLN and associated with B cells following an intranasal (i.n.) route of infection, and when using GFP-expressing or fixed spores (Supplementary Figure 1). Thus, spore association with B cells was not an artifact of germination or labeling. Moreover, DyLight 649+ BA spores were visible at the surface of B220+ cells sorted from the LDLNs of i.t. infected mice (Fig. 3d). Sorted spore+ cells had large nuclei and the small cytoplasm typical of lymphocytes. Spore+ cells from LDLNs of infected mice also expressed CD19, IgD, CD21, and CD23 (Fig. 3e). B cells were also the predominant spore+ cell type in the LDLN of B6.CCR7$^{-/-}$ mice (Fig. 3f). These data confirmed that the B cells are the APC type predominantly associated with BA spores in the LDLN early after intratracheal or intranasal infection, and suggested B cells might play an important role in the early dissemination of BA spores.

## BA spores associate with both LDC and B cells soon after entering the lungs

We next asked whether BA spores associate with B cells in the lungs prior to trafficking to the LDLN. Not surprisingly, we found that alveolar macrophages and LDCs were the major spore+ cell types recovered from single cell suspensions of collagenase digested infected lungs (Fig. 4a). However, BA spores were also associated with B cells in these lung digests. Roughly $10^4$ (~1%) of the ~$10^6$ recovered B cells stained positive for BA spores (Fig. 4a). To further characterize spore+ cells *in situ*, cryosections were prepared from infected lungs and stained using antibodies to IgM and CD11c (Fig. 4b & 4c). In both uninfected and BA infected lungs, IgM+ B cells were seen in proximity to both airway (AW) and alveolar epithelia (yellow arrows in Fig. 4b). DyLight 649+ spores frequently colocalized with AW-lining epithelium and with the lung B cells and LDCs located beneath the epithelium by 1 h

after pulmonary challenge (Fig. 4b & 4c). Thus, BA spores associated with lung B cells prior to the observed association with B cells in the LDLN. Moreover, such associations were readily observed *in situ* within lungs of infected mice.

## B cells of diverse origin bind *Bacillus* spores independent of complement receptors, IgM expression, and BCR specificity

BA spores rapidly bound to B cells when added to single cell suspensions of cells from mouse spleens (Fig. 5a), lymph nodes and lungs (Fig. 5b). Indeed, in these cell culture experiments, which used suspensions from uninfected tissues, IgM+B220+ B cells were the dominant cell type found to associate with the BA spores. These *ex vivo* cultures contained only heat-inactivated serum. Thus, the ability of BA spores to associate with lung B cells was not dependent on complement deposition on the BA spore surface.

Following their incubation with heat-inactivated mouse serum, the BA spores stained positive with an anti-IgM antibody (Fig. 5c), suggesting they might interact with IgM on the B cell surface. This finding was consistent with a prior study that showed single chain IgM fragments bound with high affinity to BA spores selectively when a κ light chain was used in the antibody fragment[23]. However, pre-treatment of BA spores with heat-inactivated mouse serum or purified mouse IgM failed to reduce the binding of BA spores to B cells *in vitro* (Fig. 5d & data not shown). Moreover, spores also bound to IgM negative A20 B cells (Fig. 5e). Finally, the BA spores bound to intact B cells expressing Igκ+ or Igλ+ with a similar frequency (Figs. 5f & 5g). Thus, the association of BA spores with intact B cells occurred independent of cell surface IgM and independent of light chain and BCR specificity. BA spores also bound immortalized human B cell lines (Fig. 5h). Thus, the receptor for the BA spores on the B cell surface is conserved in humans and mice, but appears to only be expressed only by a subset of B cells.

## Depletion of B cells significantly impairs BA spore traffic to LDLN and increases survival of mice during a lethal pulmonary infection

Although DCs traffic antigens from peripheral to lymphoid tissues, it is less clear whether B cells routinely traffic from the lung mucosa to LDLN. To test whether B cells might traffic BA spores or other antigens from the lungs to the LDLN, we isolated B cells from spleens of Ub-gfp mice and administered them i.t. into naïve B6 mice. Twenty-four hours after transfer, $1–2 \times 10^6$ could be recovered from lung digests and 100–200 gfp+ B cells were detected in the LDLNs (Fig. 6a). These data demonstrate that a proportion of the B cells present in the lung traffic to the LDLNs in the absence of any infectious stimuli. Such traffic appears to primarily occur via the lymphatics rather than the bloodstream, since we failed to detect gfp + B cells in the blood or non-draining (inguinal) lymph nodes of the recipient mice (data not shown). In a related experiment, both wt and $CCR7^{-/-}$ B cells (CD45.2) trafficked to LDLN in congenic CD45.1 recipient mice (Fig. 6b). This confirmed that B cells accessed the LDLN independent of CCR7 expression.

We next evaluated how depletion of lung B cells prior to infection impacted trafficking of BA spores to the LDLN. To deplete B cells, A/J mice were given a single i.p. injection of 250 μg anti-CD20 or an isotype control antibody. Analysis of lung digests revealed extensive

B cell depletion 7–8 days after the anti-CD20 treatment, with B cell numbers in LDLN and lungs reduced by 85–90% (Fig. 6c). Thus, each organ contained only ~1–2 × $10^5$ residual B cells. Mice were infected on day 8 with an i.t. dose of 1×$10^8$ unlabeled BA spores. At 6 hpi, LDLN were harvested and lysates were plated to determine the number of heat resistant CFUs (spores). Despite considerable variation in the recovery of CFU from LDLN of individual mice, the results indicated that B cell depletion significantly impaired transport of viable spores to the LDLN (Fig. 6d). The average number of spores trafficked to LDLN was also reduced from ~4500 to ~600 in two experiments comparing BALB/c mice and B cell deficient mb-1 null mice (Fig. 6e). By contrast, B cell depletion had no effect on the total numbers of CFU recovered from lungs of the infected mice (Fig. 6f). B cell depletion did not significantly alter numbers of CD11b+Ly6G+ neutrophils, CD11c+SigF+MHCII−SSChi alveolar macrophages, CD11c+MHCII+SSClo lung dendritic cells, CD4+ or CD8+ T cells, or Ly6G−Ly6C+CD11b+ monocytes recovered from digests of BA infected animals (data not shown). Thus, the presence of B cells had no effect on the numbers of live spores in the lungs or on numbers of phagocytes or other immune cells available to capture spores in the lungs, yet B cells increased traffic of live spores to the LDLN.

Using Sterne-susceptible A/J mice, we next investigated the effects of B cell depletion on susceptibility to lethal BA infection (Fig. 7). Challenge of control mice with 2–3 × $10^6$ purified, unlabeled BA spores caused 100% lethality within 65 h of infection. By contrast, ~20% of the mice depleted of B cells remained alive at this time point (Fig. 7B). In three independent survival experiments the median time to death for mice receiving this dose of BA spores was significantly higher for B cell depleted versus control mice (59 versus 48 h). When a lower dose ($10^5$) of BA spores was given, the mice depleted of B cells also showed an increased survival rate (~40% vs 20% in Fig. 7C). B cell depletion also increased the mean survival time from ~3 to ~9 d in these experiments. Thus, depletion of B cells significantly delayed in the progression of inhalation anthrax and improved survival following lung exposure to BA spores.

## Discussion

Our studies have revealed novel aspects of lung B cell biology and a novel role for B cells during lung infection by *Bacillus anthracis* (BA). We found that a subset of B cells from lungs and other tissues are capable of binding BA spores with strong affinity or avidity. In addition, we found that some of the B cells present in the lung can traffic to the lung draining lymph nodes (LDLN). Thus, when BA spores interact with lung B cells following i.t. or i.n. spore inoculation, they are rapidly transported to the LDLN. Reductions in the numbers of lung and LDLN B cells reduced this rapid spore transport to the LDLN and significantly delayed death of A/J mice from a lethal inhalation anthrax infection using the attenuated Sterne strain. These results suggest that early, B cell-dependent traffic of BA spores from the lung mucosa to the LDLN (or possibly other sites) exacerbates host susceptibility to BA by providing this pathogen an early foothold to establish lethal systemic infection.

The rapid transport of *Bacillus anthracis* spores from the lungs to the lung draining lymph nodes was first observed over fifty years ago[11]. Yet, mechanisms for such transport have

remained enigmatic. Consistent with findings from other infection models[2,5], prior data indicated that LDCs associate with BA spores in the lungs and thus might promote their dissemination[3]. However, trafficking of LDCs to the LDLN requires CCR7[9], and we observed equivalent BA spore traffic from lungs to LDLNs in wildtype and CCR7-deficient mice. Using the minimal doses of labeled spores that permitted reproducible detection of *in vivo* spore transport using flow cytometry (~$10^8$), we also found that the spores accumulating in the LDLN early after infection were almost exclusively associated with B cells. Since both DCs and B cells in the lung efficiently bind the labeled spores, this result indicates enrichment for spore+ B cells in the lungs. We considered whether spores might reach the LDLN through passive transport in the lymph then bind B or other LDLN cells *in situ*. However, 1 μm particles cannot access draining LNs without the aid of migratory cells and BA spores are larger than this (0.86 × 1.4 μm)[24–26]. Moreover, we failed to observe CCR7-independent transport of even 0.5 μm latex beads to the LDLN when given alone or co-inoculated with BA spores. Instead, we observed constitutive traffic of GFP+ wt B cells and CD45.1− wt and CCR7$^{-/-}$ B cells from the lung to the LDLN and found that depletion or genetic ablation of B cells significantly reduced early spore dissemination to the LDLN. These data support the interpretation that B cells carry spores to the LDLN in a CCR7-independent manner. We do not yet know why only a subset of the total B cell population seems competent for spore binding. Although spore binding to IgM was observed, B cells also bound to spores independently of IgM. This IgM-independent binding may be due to expression by a of a unique spore-binding protein or lectin by a thus far undefined B cell subset.

We also observed that B cell depletion partially ameliorated disease progression in Sterne-susceptible A/J mice. Effects of B cell depletion on host susceptibility were seen with inocula as low as $10^5$ spores, and were not attributable to reduced spore uptake in the lungs, or an altered recruitment of inflammatory cells in the lungs. Moreover, accumulation of spores in the LDLN of the B cell depleted mice was significantly reduced. These data suggest that the presence of B cells exacerbates lethal BA infection due to their ability to promote rapid dissemination of BA spores from the lung to distal tissues. It is not clear why depletion of B cells failed to completely prevent spore trafficking to the LDLN and failed to more completely block death of A/J mice challenged with Sterne strain BA spores. However, the residual B cell populations in the lungs and LDLN may be responsible for this residual spore dissemination, and/or such dissemination may be mediated by other means. For example, the small population of spore+ non-B cells observed in our studies (some of which were CD11c+) may traffic sufficient spores to establish a lethal systemic infection in these susceptible mice.

Recently, CCR7 expression was also found to be dispensable for systemic dissemination of the bacterial pathogens *Yersinia* and *Salmonella* from lung and intestinal mucosae[4,5,27]. In one of these studies, it was reported that systemic dissemination of *Yersinia pseudotuberculosis* involved multiple waves of pathogen traffic from the intestine, the first of which was not seen in mice lacking B cells[27]. The absence of B cells was also previously shown to delay dissemination of *M. tuberculosis* from the lungs[20]. The role played by B cells in these other models was not determined. However, in light of our data these findings suggest that mucosal B cells might directly traffic these bacterial pathogens to lymphoid

tissues. B cells might also conceivably deliver pathogens to other, non-lymphoid systemic tissues. However, in our studies we failed to observe GFP+ B cells, spore+ cells, or *B. anthracis* CFU in the blood or other organs within the first 24 hpi.

In summary, our findings have revealed a previously unappreciated role for B cells in a mouse model of inhalation anthrax. Our data suggest that B cells participate directly in the trafficking of BA spores to lymphoid tissues and thereby hasten the systemic dissemination of anthrax bacteria. Since we also found that BA spores bound to human B cells, it is possible that B cells also promote pathogen dissemination in humans. Moreover, the data from our studies suggest that, like DCs, B cells may routinely participate in antigen transport from mucosal to lymphatic tissues. A refined understanding of how B cells promote pathogen/antigen trafficking may reveal new therapies to delay progression of inhalation anthrax and other diseases.

## Materials and Methods

### Cell lines

Namalwa, A20, and Ramos B cells were from Drs. James Hagman and Jing Wang (National Jewish Health).

### Mice

Six to eight week old C57BL/6, BALB/c, A/J, B6.CCR7$^{-/-}$, and B6.CD45.2+ mice were purchased (Jackson Laboratories, Bar Harbor, ME). BALB.mb-1 null mice were from Dr. Roberta Pelanda, (National Jewish Health). Animals were housed in the National Jewish Health Biological Resource Center under veterinary care. All studies were approved by National Jewish Health Institutional Animal Care and Use Committee and Institutional Biosafety Committee.

### *Bacillus anthracis* spores

*Bacillus anthracis* strain 34F2 (pXO1$^{+}$, pXO2$^{-}$) was obtained from the Colorado Serum Company, Denver, CO. GFP expressing *B. anthracis* (7702) (GFP-BA) was a kind gift from Dr. Timothy Hoover (USAMRIID). Sporulation was induced in Difco Sporulation Medium. Cultures were centrifuged to pellet spores when >90% sporulation was achieved as verified under a light microscope. Pellets were washed 3× with cold $dH_20$, heated to 70°C for 30 min, and stored 4°C overnight before passage through Renografin (Renografin-60, Bracco Diagnostics, Princeton, NJ) as previously described[23]. After labeling of purified spores with Dylight 649 (Thermo Scientific, Waltham, MA) according to the manufacturer's protocol, they were washed 5 times with ice-cold water to remove excess dye and analyzed on an LSRII flow cytometer to confirm efficient and uniform labeling prior to use in experiments. Pure spores were stored at 4°C for no more than 1 month before use.

### Bacterial infections and B cell depletion

Mice were anesthetized with an i.p. injection of xylazine (2 mg/ml) plus ketamine (10 mg/ml). Intratracheal injections were given in 50 μl volumes using a laryngoscope as we

previously detailed[22]. For intranasal challenge, 30 μl was pipetted into the left nostril and mice were held upright for 30 seconds to ensure delivery of full inoculum.

For B cell depletion studies, 250 μg of anti-CD20 antibody was injected i.p. 7 days prior to infection. Purified anti-CD20 monoclonal (18B12; IgG2a) was a gift of Robert Dunn and Marilyn Kehry (Biogen Idec, Weston, MA). Purified IgG2a isotype control antibody (C1.18) was purchased from BioXCell (West Lebanon, NH).

## Flow cytometry

Brochoalveolar lavage (BAL) was performed as described[9]. Where indicated, lungs were perfused with 10 ml of ice cold PBS. Our protocol for obtaining lungs and LDLNs is described[22]. Numbers of cells in LDLN analyses represent cells obtained from the same 3 LDLNs (two cranial mediastinal and one tracheaobronchial) per animal. For cell isolation, lungs were finely chopped and digested with 1 mg/ml collagenase (Sigma, Ronkonkoma, NY) for 30 min at 37°C. EDTA was added to a final concentration of 10 mM, then digest was passed through an 18G needle and 70 μm cell strainer (BD Biosciences, Bedford, MA). LDLN (two cranial mediastinal and one tracheaobronchial) were harvested from left thoracic cavity and digested 20 min with 1 ml of 0.125% collagenase type IV (Worthington, Lakewood, NJ) at 37°C in HBSS plus cations before strainer. Red bloods cells were lysed using ACK lysis buffer. Single cells suspensions for flow cytometry were treated with anti-CD16/32 (2.4G2) to block Fc receptors and stained as described[28]. Antibodies were obtained from eBioscience (San Diego, CA): CD45 (30-F11), IgM (II/41), B220 (RA3-6B2), CD11c (N418), CD11b (M1/70); BD BioSciences: Ly6G (1A8), Igκ (187.1); BioLegend (San Diego, CA): MHCII (14-4-4S); or SouthernBiotech (Birmingham, AL): Igλ (JC5-1). Samples fixed with 4% paraformaldehyde overnight were run on an LSRII (BD Biosciences) and analyzed using FlowJo software (Treestar, Ashland, OR).

## CFU analysis

Lungs harvested into 10 ml of 0.02% NP40 were homogenized and incubated at room temperature (RT) for 15 min before plating serial dilutions. Single cell suspensions of LDLNs prepared as above were pelleted, lysed in 500 μl 0.02% NP40, and incubated at RT for 15 min before plating. For heat resistant CFUs, aliquots were incubated at 70°C for 30 min before plating.

## Isolation of B cells and adoptive transfer

Splenic B cells were isolated by negative selection using anti-CD43 microbeads according to the manufacturer's protocol (Miltenyi Biotec, Auburn, CA). For adoptive transfers, $15 \times 10^6$ B cells were resuspended in PBS and injected i.t. or i.n. into anesthetized naïve mice.

## Immunofluorescence microscopy of lung sections

Infected mice were sacrificed and lungs were prepared as described elsewhere[29]. Briefly, lungs were perfused with 10 ml ice cold PBS. The trachea was then cannulated and 1 ml of optimum cutting temperature (OCT) compound freezing medium was injected into the lung. The trachea was tied to prevent leakage of OCT and lungs were harvested into ice cold PBS. Lobes were separated and placed, convex side down, in plastic molds half-filled with OCT.

Molds were transferred onto dry ice, filled with OCT, and stored at −80°C. Cryosections were cut on a cryostat and collected onto charged microscope slides (Thermo Scientific). Slides were stored at −80°C. Prior to staining, slides were air-dried at room temperature and rehydrated with PBS for 15 min. Sections were blocked with anti-FcR (2.4G2) for 30 min then stained with directly conjugated fluorophore labeled antibodies specific for CD11c (N418) and Fab fragments specific for IgM (μ chain specific; Jackson Immunoresearch, West Grove, PA). After 1 h at room temperature, slides were washed in 3× in PBS (5 min each) and counterstained stained with DAPI. After three additional washes in PBS (5 min each),Stained 5–7 μm cryosections were imaged using a Leica DMRXMA upright fluorescence microscope (Knowhill, UK). Images were analyzed using Slidebook 5.0 (3I Inc, Atlanta GA).

### Cytospin

B cells from LDLNs of infected mice were enriched with anti-B220 MACS microbeads (Miltenyi), then cytocentrifuged onto glass slides, fixed with 4% paraformaldehyde, and stained for imaging and analysis as described above.

### Statistical analysis

Data were processed and plotted in GraphPad PRISM (Graph-Pad Software Inc., San Diego, CA). All error bars indicate SEM. Two-tailed unpaired t-tests were used for statistical analyses except for survival. For survival, curves were Kaplan-Meier, and p-values were calculated by Log-rank (Mantel-Cox) test. Throughout: * indicates $p<0.05$, ** $p<0.005$ and *** $p<0.0005$.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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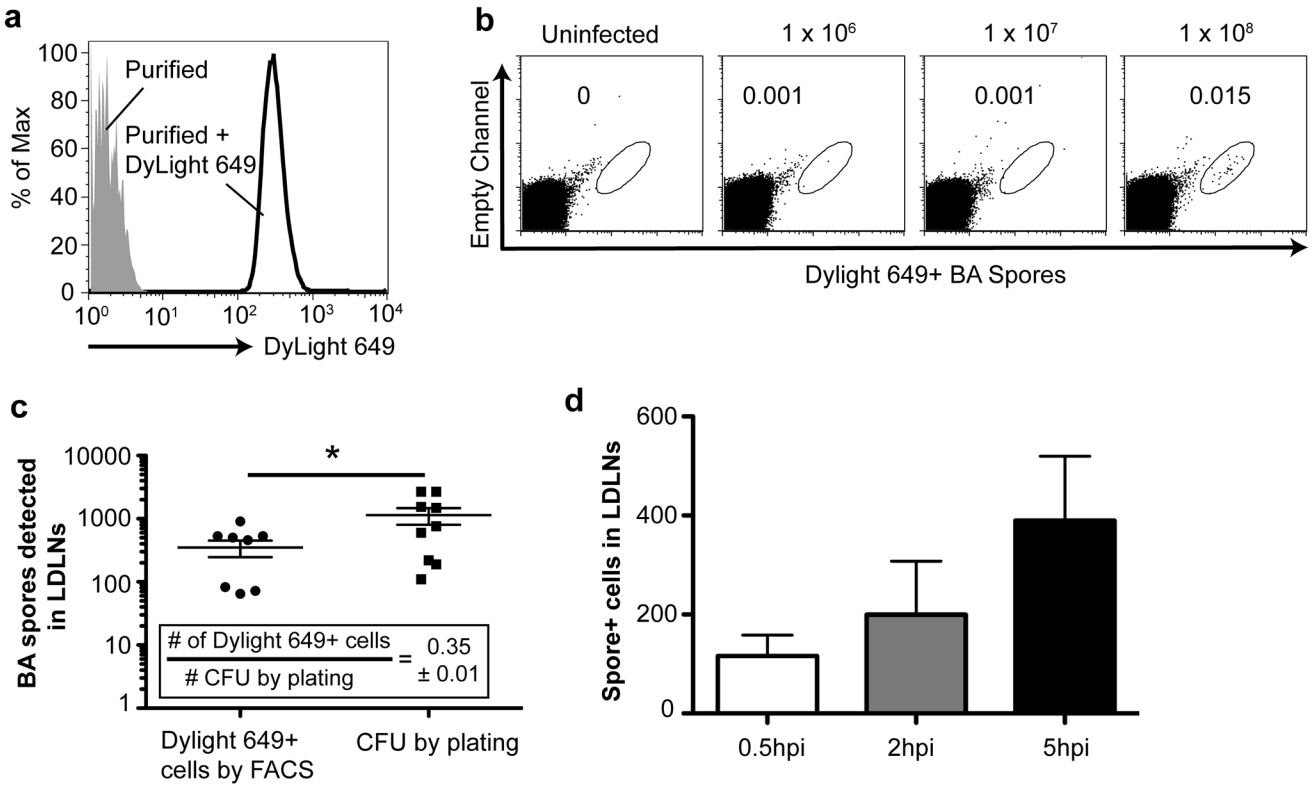


**Figure 1.**

Labeled *B. anthracis* (BA) spores are transported to the lung draining lymph nodes (LDLNs) early after i.t infection. (**a**) Representative histogram demonstrating uniform labeling of BA spores with DyLight 649. Data were collected on an LSRII. Histogram shows labeled BA spores (black) compared to unlabeled BA spores (solid grey). Dylight649 labeled spores were always checked for uniform labeling before using for an experiment. (**b**) Mice were infected i.t with $1\times10^6$, $1\times10^7$ or $1\times10^8$ BA spores or given PBS alone. LDLNs were analyzed by FACS at 6 hpi. Fluorescence from BA spores is plotted against an empty channel to show percentage of spore+ cells in the LDLNs. (**c**) LDLNs of infected A/J mice were analyzed for spore+ cells by FACS and plated for heat resistant CFUs. (**d**) A/J mice were given $1\times10^8$ BA spores i.t and single cell suspensions from LDLNs were analyzed by flow cytometry at 30 min, 2 h and 5 h post infection for total number of spore+ cells. Data in (c) is pooled from 3 independent experiments with n=2–3 per group. Asterisk (*) indicates $p<0.05$. Experiments in (b) and (b) are representative of 2 independent experiments with n=2–3 each.

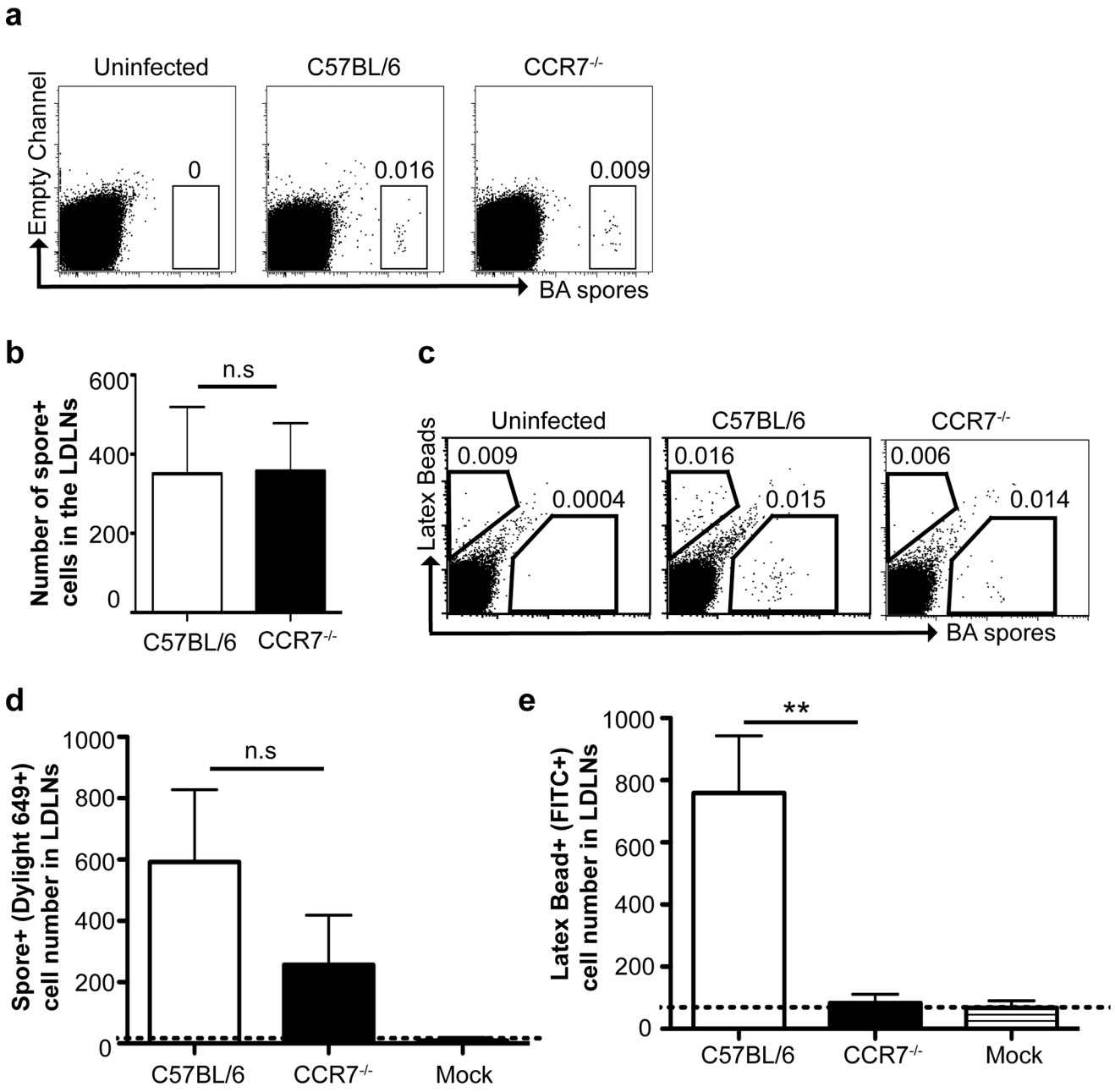


**Figure 2.**

Rapid transport of BA spores to the LDLN is independent of CCR7. (**a**) C57BL/6 (n=4) or B6.CCR7$^{-/-}$ (n=4) mice were infected i.t. with $3\times10^8$ DyLight 649 labeled BA spores. LDLNs were analyzed for spore+ cells at 6 hpi. Data from representative mice are plotted with gated frequencies. The y-axis represents an empty channel. (**b**) Calculated mean number of spore+ cells from each group in (a). (**c**) C57BL/6 (n=3) or B6.CCR7$^{-/-}$ (n=4) mice were inoculated i.t with a mixture of $1\times10^8$ BA spores and $1\times10^8$ FITC+ latex beads. LDLNs were analyzed by flow cytometry at 24 hpi. Spore+ (x-axis) and bead+ (y-axis) cells from representative mice are plotted with gated frequencies. Data in (a) (b) and (c) are representative of two independent experiments. (**d**) Calculated mean number of spore+

(Dylight 649+) cells from each group in (c). (**e**) Calculated mean number of Latex bead (FITC+) cells from each group in (c). Data in (d) and (e) are pooled from 2 independent experiments with n=3–4 per group.

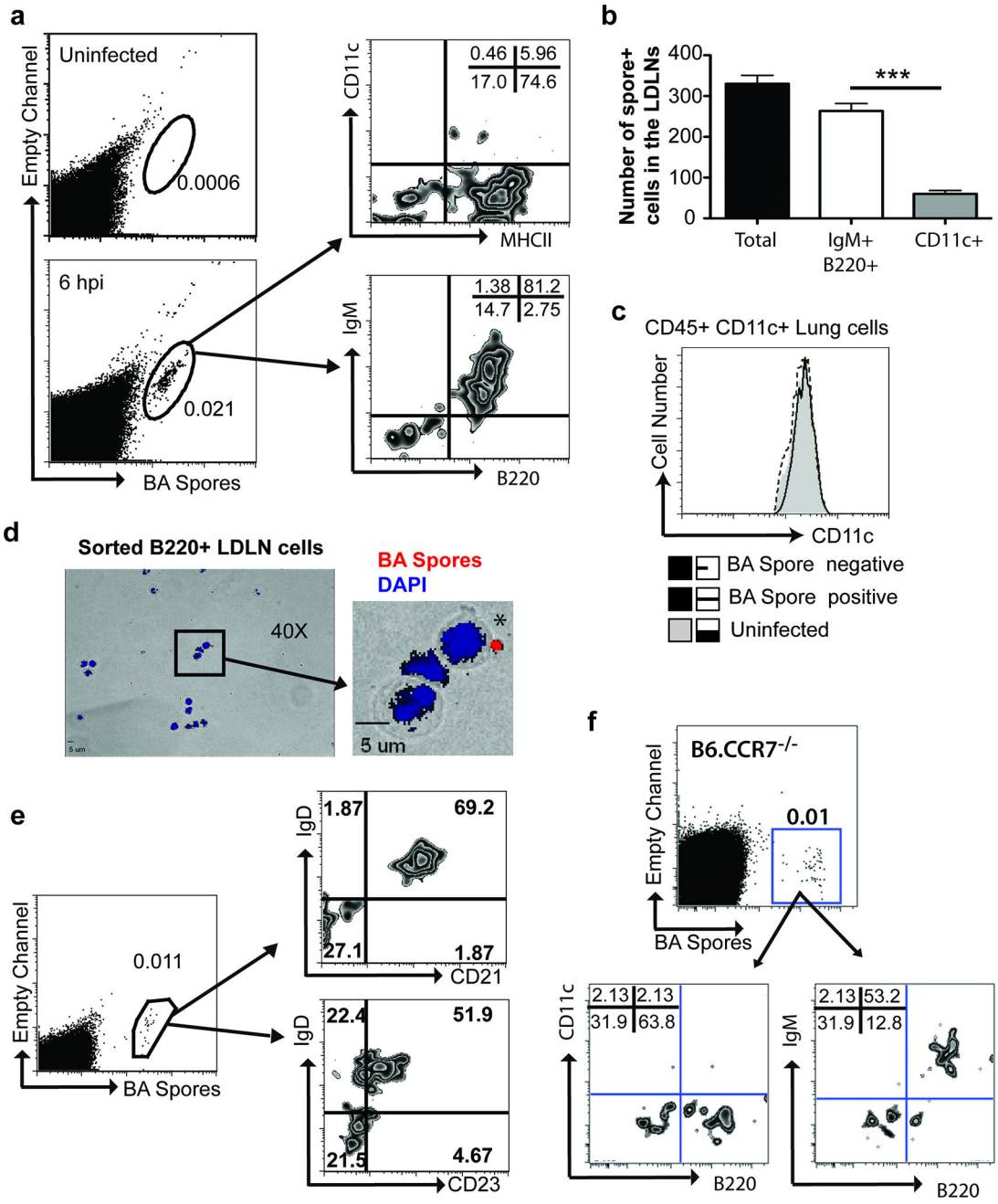


**Figure 3.**

BA spores that rapidly reach the LDLNs are primarily associated with B cells. (**a**) At 6 hpi with $1\times10^8$ BA spores i.t., single cell suspensions were pooled from LDLNs of A/J mice (n=4) and analyzed by flow cytometry. Left panels show the gating strategy for spore+ cells. Panels on the right depict the expression of B cell markers (B220, IgM, and MHCII) and dendritic cell markers (CD11c and MHCII) on spore+ cells. (**b**) Quantification of spore+ DCs and B cells from panel (a). Data in (a) and (b) are representative of 3 independent observations with n=3 per group. Asterisk (***) indicates $p<0.0005$. (**c**) A/J mice were infected i.t. with $10^8$ BA spores. At 6 hpi, lungs were harvested and processed into a single

cell suspension. Lung cells were stained and CD11c expression levels were assessed on CD45+ CD11c+ cells that were either DyLight 649+ (solid black line) or DyLight 649− (dashed line). Staining of CD11c on gated CD45+ CD11c+ cells from an uninfected mouse (filled gray histogram) is shown as a control. (**d**) A/J mice (n=4) were infected with $1\times10^8$ DyLight 649-labeled BA spores intranasally (i.n.). At 12 hpi single cell suspensions of the LDLN were pooled and B220+ cells were positively sorted using MACs columns. Sorted cells were cytospun onto glass slides and stained for DAPI. Shown is a representative merged image of spores (red) and nuclei (blue) using bright field and fluorescence. (**e**) A/J mice were infected with $1\times10^8$ fluorescent BA spores i.t. LDLNs were harvested at 6 hpi and single cell suspensions were analyzed for the expression of B cell markers IgD, CD21 and CD23 (bottom panels) on gated spore+ cells. (**f**) Gated, spore+ cells from LDLN of B6 and B6.CCR7$^{-/-}$ mice were analyzed for DC and B cell surface markers. Data in (c) is representative of 3 independent experiments. (d–f) are results from 2 independent experiments with n=3–4 per group.

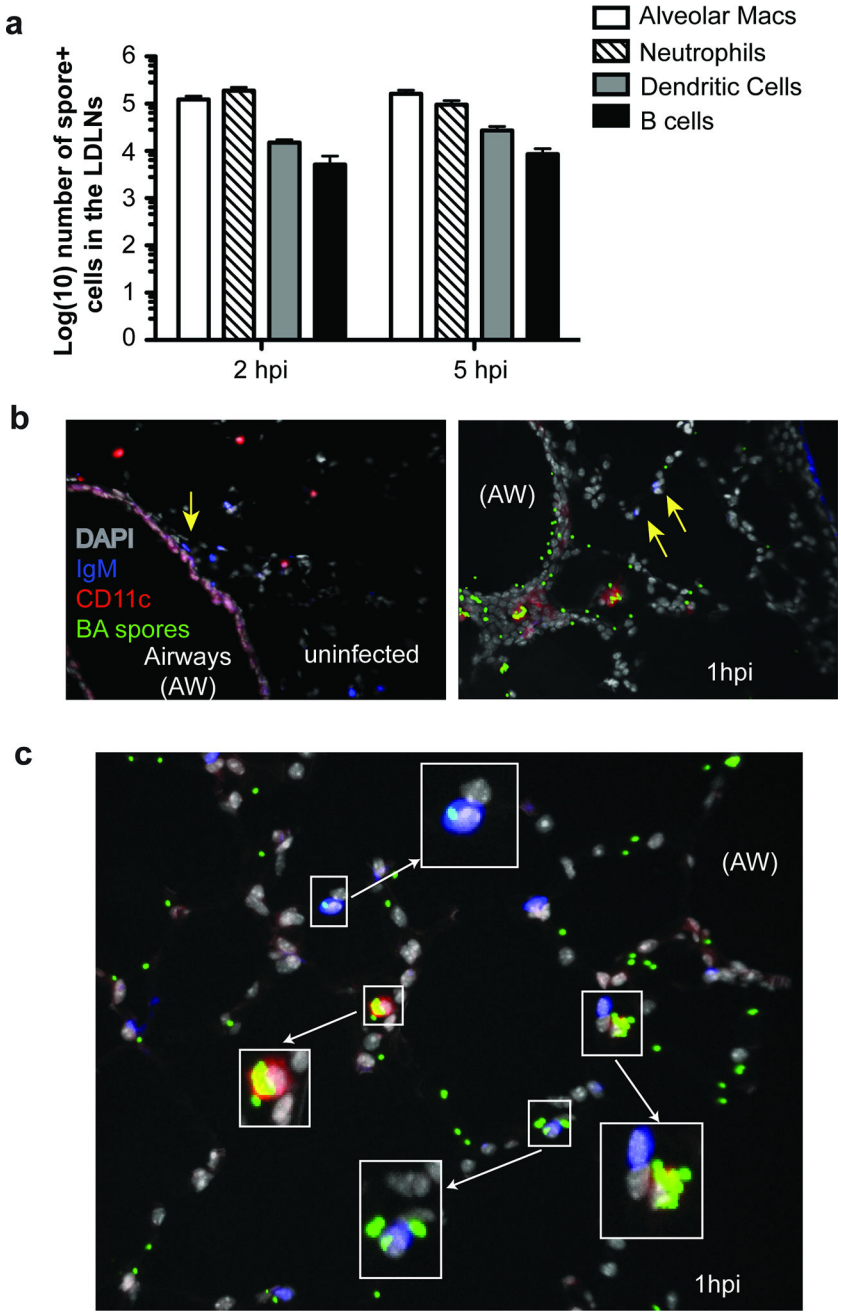


**Figure 4.**

BA spores rapidly associate with B cells in lungs of infected mice. (**a**) A/J mice were infected i.t. with $1\times10^8$ DyLight 649 labeled BA spores. At 2 and 5 hpi, bronchioalveolar lavage (BAL) was performed. Lavaged lungs were perfused, excised, and digested with collagenase to prepare single cell suspensions. Lung digests were stained and analyzed by FACS. CD45+ cells were selected from the live gate and analyzed for cell populations as previously reported[30], including B cells (IgM+ CD19+), alveolar macrophages (highly autofluorescent, CD11c+, Side scatter$^{hi}$ and MHCII$^{lo}$), lung dendritic cells (CD11c+, SSC$^{lo}$, MHCII+), and neutrophils (Ly6G+). The number of each gated population that was spore+

was calculated and graphed (mean ± SEM). Data representative of 3 independent experiments with n=3 per group. (**b–c**) Cryosections of lower right lobes from lungs of naïve (left panel) and infected (right panel) mice were analyzed for the distribution of BA spores (green), IgM (blue), CD11c (red) and DAPI (grey). Airways (AW) are labeled and insets show spore association with either IgM+ cells or CD11c+ cells. Arrows in (b) highlight B cell locations. All images were taken at 25×. Similar observations were made in 2 independent experiments with n=2–3 per group.

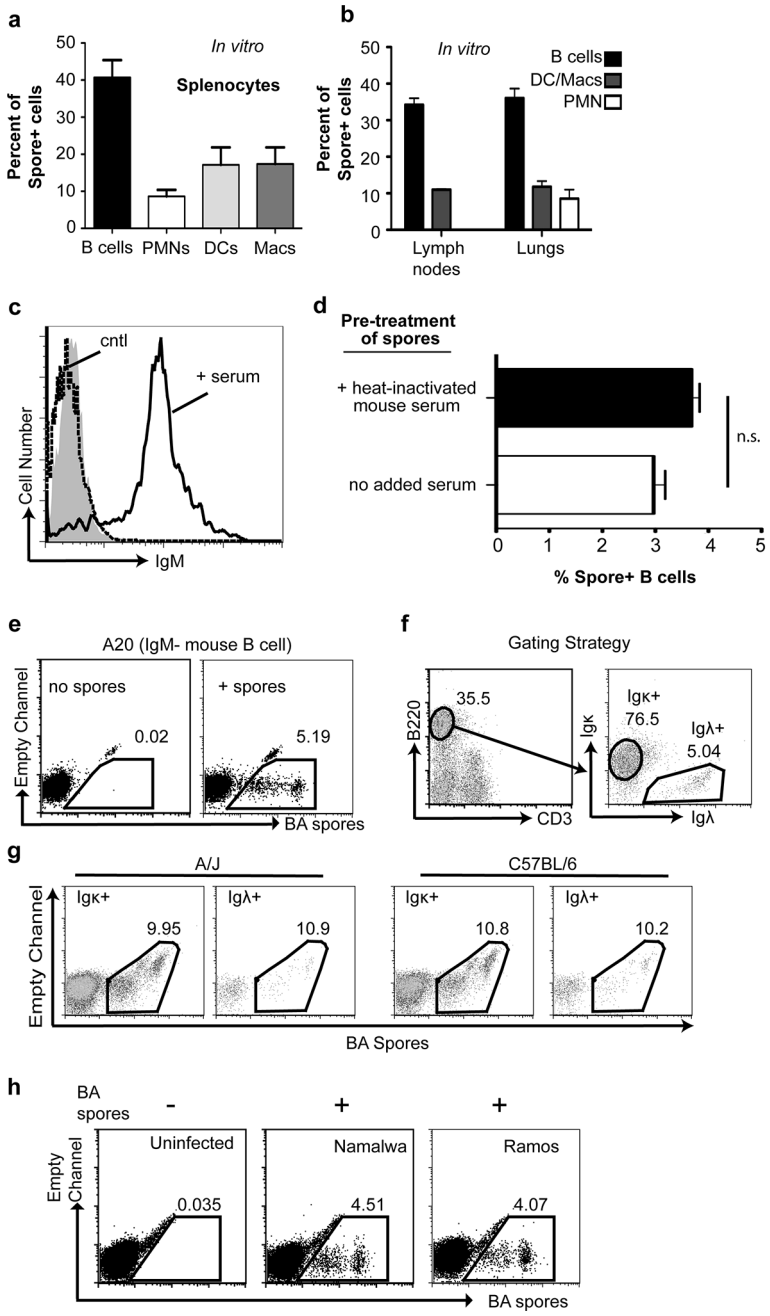


**Figure 5.**

BA spores bind mouse and human B cells independent of complement, IgM, and BCR specificity. (**a**) Single cell suspensions from spleens of naïve A/J mice were co-incubated with Dylight 649 labeled BA spores (MOI=5) for 30 min at 37°C in a tissue culture dish. Cells were washed and stained for B cells (IgM+B220+), neutrophils (Ly6G+CD11b+), macrophages (CD11b+) and DCs (Cd11c+). (**b**) Cells from the lungs and lymph nodes of naïve mice were co-incubated with BA spores and analyzed for B cells, neutrophils and DCs +Macs as in (a). Ly6G-CD11b+ and Ly6G-CD11c+ populations are pooled here and percentages of respective cell types associated with DyLight 649+ BA spores are shown.

Data in (a) and (b) were pooled from 4 and 2 independent experiments respectively. (**c**) $1\times10^8$ BA spores were incubated with PBS or 100 μl of serum from a naïve C57BL/6 mouse for 1 hr at 4°C then washed and stained with Alexa488 conugated anti-mouse IgM($F_{ab}$) (Jackson Immunoresearch). Histograms show IgM staining on BA spores. (**d**) BA spores ($1\times10^8$) were incubated were 100 μl of heat inactivated serum (30 min at 56°C) from naïve C57BL/6 mice for 1 h at 4°C. (**e**) Mouse B cell line, A20 was co-incubated with BA spores as in (d) and analyzed. Numbers in dot plots indicate percentage of total cells gated positive for BA spores. (**f**) Splenocytes from naïve A/J and C57BL/6 were co-incubated with BA spores at MOI=5 for 30 min at 37°C and stained for B220, CD3, Igκ and Igλ. Shown is the gating strategy to identify CD3−B220+ B cells expressing Igκ and Igλ light chains. (**g**) Plots indicate percentages of Igκ+ or Igλ+ B cells from A/J (left panels) and C57BL/6 (right panels) that bind BA spores. Gated as in panel (f). (**h**) Human B cell lines Ramos (top panel) and Namalwa (middle panel) were co-incubated with DyLight 649 labeled BA spores as in (d) and analyzed by flow cytometry. Data in (d) – (h) are representative of 2 independent experiments done in triplicates.

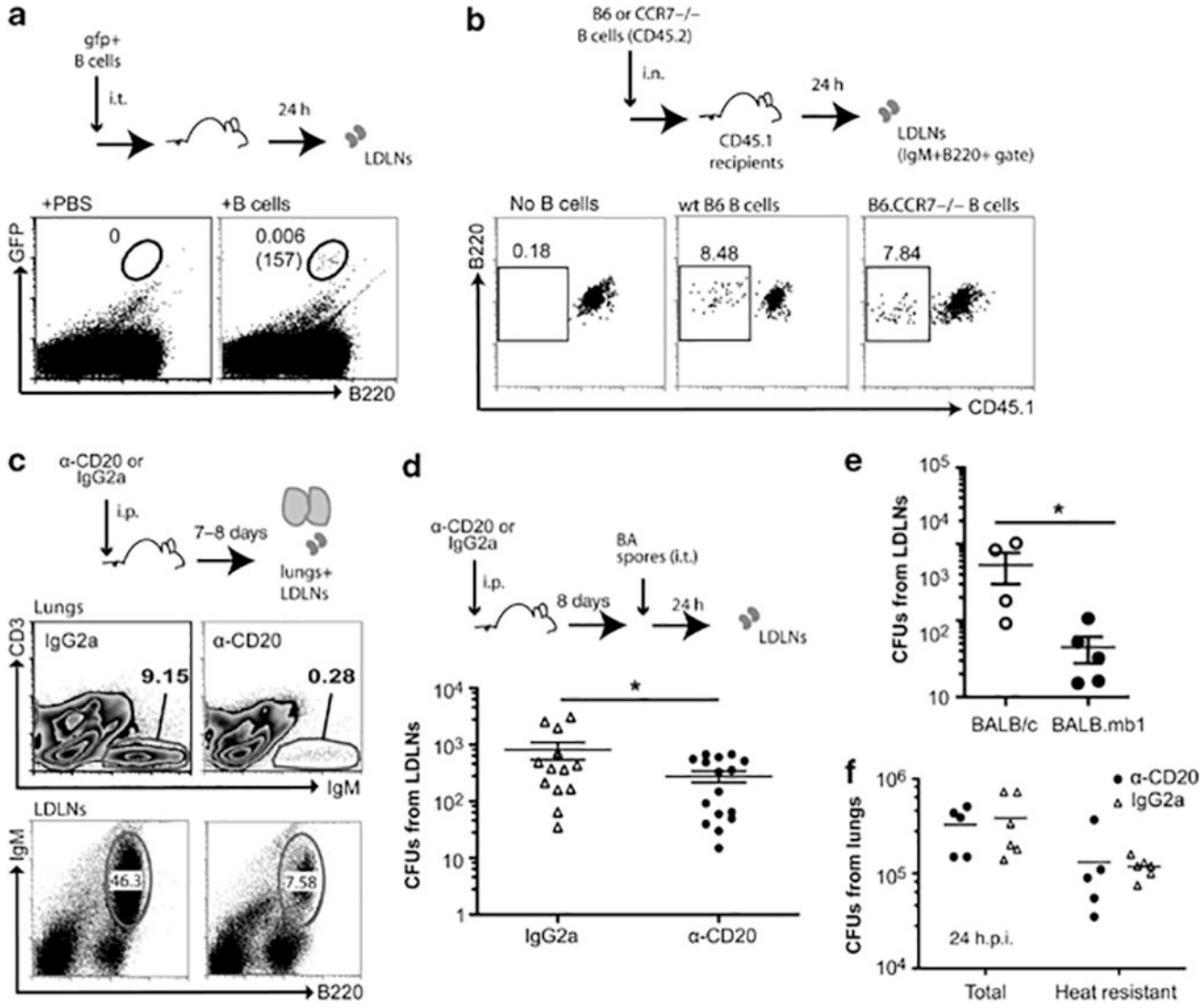


**Figure 6.**

B cells traffic BA spores to the LDLN. (**a**) B cells from the spleen of C57BL/6-Ub-GFP mice were isolated using MACS columns by negative selection (>95% CD43−). $15 \times 10^6$ of the purified CD43− cells were transferred i.t. into each of three naïve C57BL/6 recipients. LDLNs of recipients were analyzed at 24 hpi for expression of GFP, B220, CD11c, and IgM. Left panel shows B220 and GFP staining in control mice and right panel is a representative plot from a recipient mouse. Numbers represent percentage of total cells positive for GFP and B220 and numbers in parenthesis indicate the absolute number of cells in the gate. The gated GFP+ cells were IgM+ and CD11c−. Data representative of 2 independent experiments with n=3 per group. (b) CD43− cells were purified from age and sex matched wt B6 or B6.CCR7$^{-/-}$ mice (both CD45.2+ CD45.1−). $15 \times 10^6$ of the resulting B cells (92–94% purity) were administered i.n. to congenic CD45.2+ B6 mice and LDLN were harvested and analyzed 24 h later. Plots depict IgM+B220+ B cells that are gated to indicate the donor-derived (CD45.1+) population. Results are representative of two experiments using two recipient mice per group. (**b**) A/J mice were injected i.p. with 250 μg of α-CD20

antibody or an isotype control IgG2a antibody. Lungs and LDLNs were analyzed for B cells on d 7. Plots show CD3 (y-axis) and IgM (x-axis) on live gated cells from lungs (top panels) or IgM (y-axis) and B220 (x-axis) staining on live LDLN cells. Numbers indicate percentage of cells in the gate. Dot plots are indicative of 3 independent experiments with n=3 per group. (**c**) A/J mice were i.p. injected with α-CD20 Ab or isotype control (IgG2a). On day 8, mice where challenged i.t. with $1\times10^8$ BA spores. At 6 hpi, lung draining lymph nodes were harvested and processed into single cell suspensions, then lysed and heat treated at 70°C for 30 min to kill vegetative or geminated spores. Shown are numbers of recovered heat resistant CFU pooled from 4 independent experiments with n=3–5 mice per group. (**d**) BALB/c and BALB.mb1 mutant mice were infected i.t. with $2–3\times10^6$ BA spores. At 24 hpi, LDLN were isolated and lysed and plated to determine the number of BA CFU. Data are pooled from 2 independent experiments. (**e**) A/J mice received an i.p. injection of α-CD20 or isotype control (IgG2a). On day 8, mice were infected i.t. with $2–3 \times 10^6$ BA spores. At 24 hpi, homogenized lung lysates were plated for total and heat resistant CFUs. Shown are data pooled from 2 independent experiments.

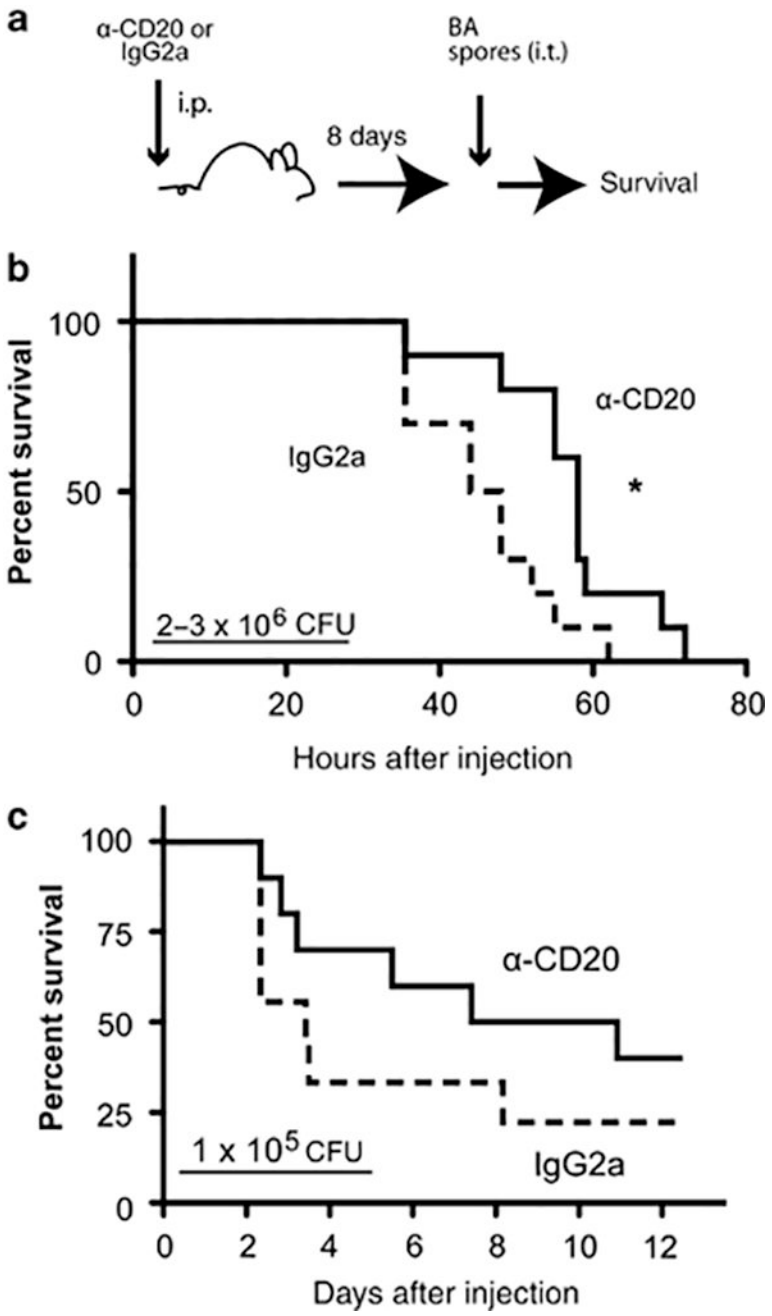


**Figure 7.**

Depletion of B cells delays death of A/J mice following lethal infection of the lungs with the Sterne strain of BA. (**a**) A/J mice were injected i.p. with 250 μg of α-CD20 or isotype control (IgG2a) antibody. Day 8 post injection, both groups were challenged with unlabeled BA spores and survival was monitored. (**b**) Survival of A/J mice given 2–3 $\times 10^6$ BA spores. Data are representative of 3 independent experiments using 10 mice per group. (**c**) Survival of mice given $1 \times 10^5$ BA spores. Data are representative of 2 independent experiments using 10 mice per group.