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J Immunol 2013; 190:2756-2766; Prepublished online 1 February 2013; doi: 10.4049/jimmunol.1202697
http://www.jimmunol.org/content/190/6/2756

Supplementary Material http://www.jimmunol.org/content/suppl/2013/02/01/jimmunol.1202697.DC1

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Print ISSN: 0022-1767 Online ISSN: 1550-6606.
B1a Cells Enhance Susceptibility to Infection with Virulent Francisella tularensis via Modulation of NK/NKT Cell Responses

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B1a cells are an important source of natural Abs, Abs directed against T-independent Ags, and are a primary source of IL-10. Bruton’s tyrosine kinase (btk) is a cytoplasmic kinase that is essential for mediating signals from the BCR and is critical for development of B1a cells. Consequently, animals lacking btk have few B1a cells, minimal Ab responses, and can preferentially generate Th1-type immune responses following infection. B1a cells have been shown to aid in protection against infection with attenuated Francisella tularensis, but their role in infection mediated by fully virulent F. tularensis is not known. Therefore, we used mice with defective btk (CBA/CaHN-Btk<sup>XID</sup>/J [XID mice]) to determine the contribution of B1a cells in defense against the virulent F. tularensis sspp. tularensis strain SchuS4. Surprisingly, XID mice displayed increased resistance to pulmonary infection with F. tularensis. Specifically, XID mice had enhanced clearance of bacteria from the lung and spleen and significantly greater survival of infection compared with wild-type controls. We revealed that resistance to infection in XID mice was associated with decreased numbers of IL-10–producing B1a cells and concomitant increased numbers of IL-12–producing macrophages and IFN-γ–producing NK/NKT cells. Adoptive transfer of wild-type B1a cells into XID mice reversed the control of bacterial replication. Similarly, depletion of NK/NKT cells also increased bacterial burdens in XID mice. Together, our data suggest B cell–NK/NKT cell cross-talk is a critical pivot controlling survival of infection with virulent F. tularensis. The Journal of Immunology, 2013, 190: 2756–2766.

Successful defense against pathogens requires the coordinated function of multiple host cells. Among infections with intracellular bacteria, T cells, macrophages, and dendritic cells (DC) all play well-described, essential roles in the resolution of infection. In contrast, the contribution B cells make toward control of infection with intracellular bacteria is less well understood. The primary function of B cells in infection is generally attributed to their ability to secrete Ab directed against pathogens that allow for neutralization and/or opsonization of the bacteria (1). However, B cells have a multitude of other functions that may aid in control of infection and/or inflammation including Ag presentation, costimulation of T cells, and secretion of both pro- and anti-inflammatory cytokines (as reviewed in Ref. 2). Thus, the relative contribution of B cells in the pathogenesis of disease is complex. Francisella tularensis is a facultative intracellular, Gram-negative bacterium that replicates in the cytoplasm of host cells (3, 4). Virulent subspecies of this bacterium (e.g., F. tularensis ssp. tularensis) cause lethal disease following inoculation of as few as 10 bacteria (5). There are no licensed vaccines for F. tularensis, and recrudescence following treatment with antibiotics has been noted (6). F. tularensis was also developed and deployed as a biological weapon (7). Thus, this pathogen requires manipulation under biosafety level 3 laboratory conditions, is classified as a Category A priority pathogen, and is regulated as a select agent in the United States.

Given the high virulence of F. tularensis ssp. tularensis and restriction regarding its use, many laboratories have turned to using attenuated subspecies and strains of F. tularensis (e.g., F. tularensis ssp. holarctica live vaccine strain [LVS] and F. novicida) as surrogates for their more virulent cousin. Thus, our understanding of the role specific cell types, including B cells, play in F. tularensis infection is largely derived from data generated with attenuated LVS and F. novicida.

Previous work with attenuated strains of F. tularensis has demonstrated that mice completely lacking B cells (μMT<sup>−/−</sup>) exhibit modest increase in susceptibility to primary infection with LVS and poor resistance to secondary infection (8). Similarly, we have established that μMT<sup>−/−</sup> exhibit greater susceptibility to infection with virulent F. tularensis ssp. tularensis strain SchuS4 than wild-type (WT) animals (9). Thus, B cells as a complete cellular compartment are required to resolve F. tularensis infections.

Because these previous data show that B cells are important for control of F. tularensis infection and the fact that Ab production is considered one of the primary functions of B cells, several laboratories have explored the efficacy of immune sera and mAbs to aid in protection against F. tularensis infection. Passive transfer of immune sera or mAbs protects animals against F. tularensis (10–19). Furthermore, passive transfer of hyperimmune serum into humans newly infected with F. tularensis aids in the resolution of infection (20). The specific role of opsonizing IgM and defense against the attenuated F. tularensis vaccine strain (LVS) was
highlights the study by Cole et al. (21). In that study, animals immunized with LPS purified from LVS are protected from infection with LVS, and this protection is largely dependent on Abs secreted by B1a cells (21). In total, these reports show that Abs can mediate protection against F. tularensis infection and that Abs derived specifically from B1a cells are key players in this protection. However, these reports do not address the absolute requirement for Abs in survival of infection with virulent F. tularensis. Furthermore, the contribution made by alternative B cell functions (i.e., cytokine secretion) toward resolution or exacerbation of disease mediated by virulent F. tularensis has not been explored.

In this article, we demonstrate that neither high titers of Ab directed against F. tularensis ssp. tularensis strain SchuS4 nor natural IgM are required for survival of SchuS4 infection. Moreover, we found that B1a cells contribute to the pathogenesis of F. tularensis infection and that this contribution was tightly associated with the interference of early, effective NK/NKT cell responses.

Materials and Methods

**Mice**

Specific pathogen-free, 6–8-wk-old CBA/J (WT) and CBA/CaHN-Btk<sup>(XID)</sup> (XID) (n = 5–10/group) were purchased from The Jackson Laboratory (Bar Harbor, ME). Mice were housed in sterile microisolator cages in the biosafety level 3 facility at the Rocky Mountain Laboratories (RML). All mice were provided sterile water and food ad libitum, all research involving animals was conducted in accordance with Animal Care and Use guidelines, and animal protocols were approved by the Animal Care and Use Committee at RML.

**Bacteria**

Francisella tularensis ssp. tularensis strain SchuS4 was originally provided by Jeannine Peterson (Centers for Disease Control, Fort Collins, CO). SchuS4 was cultured in modified Mueller–Hinton (MMH) broth at 37°C with constant shaking overnight, aliquoted into 1-ml samples, frozen at −80°C, and thawed just prior to use as previously described (9). Frozen stocks were titered by enumerating viable bacteria from serial dilutions plated on MMH agar as previously described (22, 23). The number of viable bacteria in frozen stock vials varied <1% over a 12-mo period.

For generation of killed SchuS4, ~1.5 × 10<sup>9</sup> bacteria were incubated with 50 µg/ml levofloxacin (LVF) overnight at 37°C. Bacteria were washed once and diluted to the equivalent multiplicity of infection of live organisms in PBS immediately prior to use. Confirmation of efficacy of LVF treatment to obtain 100% dead bacteria was confirmed in preliminary experiments by incubating the entire inoculum onto MMH agar and incubating for 96 h at 37°C/7% CO2. After this time, no colonies, representing viable SchuS4, were detected as previously described below (Supplemental Fig. 1). This depletion regimen results in >98% depletion of NK/NKT cells.

**Infection of mice with treatment with antibiotic**

Mice were infected intranasally (i.n.) with F. tularensis SchuS4 as previously described (27). Briefly, bacteria were thawed and diluted in PBS. Mice were anesthetized by i.p. injection of 100 µl 12.5 mg/ml ketamine plus 3.8 mg/ml xylazine. Approximately 50 CFU was administered into the nares of each mouse in a total volume of 25 µl. Actual inoculum concentration was confirmed by plating a portion of the inoculum onto MMH agar plates, incubating plates at 37°C with 5% CO2 and enumerating colonies. As indicated, mice were treated with LVF (Ortho-McNeil Pharmaceutical, Raritan, NJ) as previously described (9). Mice were monitored for 30 d or euthanized at the indicated time points postinfection. Following infection, mice were monitored regularly and euthanized at the first signs of illness. All experiments using animals were performed in accordance with protocols approved by the Animal Care and Use Committee at RML.

**Detection of serum Abs**

Serum was collected from mice at the indicated time points postinfection. Quantification of total serum IgM was performed by ELISA as previously described (28). Presence of serum IgG and IgM Abs that recognize Ags in whole-cell lysate prepared from SchuS4 and agglutinating serum Abs directed against viable SchuS4 were detected as previously described (28).

**Collection of tissue homogenate and enumeration of bacteria**

Bacteria were enumerated from the lungs and spleens as previously described (24, 27). Briefly, organs were aseptically collected and placed in ice-cold homogenization buffer (150 mM Tris-HCl, 5 mM EDTA, and 10 mM Trizma-base) supplemented with a 1:100 dilution of Phosphatase Inhibitor mixture I, Phosphatase Inhibitor mixture II, and Protease Inhibitor mixture III (all from AG Scientific, San Diego, CA). Organs were homogenized by grinding tissues through a sterile SS Type 304 #60 wire-mesh screen (Billeville Wire Cloth, Cedar Grove, NJ) using a 5-ml syringe plunger. A portion of the resulting homogenate was immediately serially diluted in PBS and plated on MMH agar for enumeration of bacterial loads. The remaining homogenate was clarified by centrifuging at 14,000 × g for 30 min at 4°C. The resulting supernatants were sterile-filtered through 0.2-µm syringe filters (Millipore Ireland, Cork, Ireland) and stored at −80°C.

**Isolation of lung and spleen cells**

Lung cells and splenocytes were collected as previously described with the following modifications (9). Lung cells and splenocytes were resuspended in FACs buffer prior to flow cytometric analysis or DMEM supplemented with 10% heat-inactivated FCS, 0.2 mM l-glutamine, 1 mM HEPES

F4/80 as described below. B1a cells (CD5<sup>−</sup>CD19<sup>+</sup>) were collected by FACs using an Aria II and FACs Diva Software (BD Biosciences, San Jose, CA) or cultured immediately after collection. Sorted B1a cells were >98% pure. All cultured cells (sorted populations and total peritoneal exudate cells [PEC]) were adjusted to 1 × 10<sup>6</sup> cells/ml in cDMEM and added to a 96-well tissue-culture plate at 200 µl/well. Cells were incubated with the indicated stimulus overnight. Then, supernatants were collected and immediately analyzed for IL-10 or IL-12p40 by ELISA. As indicated, some cultures were supplemented with either 10 µg/ml rat IgG as an isotype control (Ig) or neutralizing anti–IL-10 Abs (both from R&D Systems).

**Adaptive transfer of B1a cells and passive transfer of serum**

Freshly isolated B1a cells were washed extensively in PBS and adjusted to 1 × 10<sup>7</sup> ml in PBS. Immediately following resuspension in PBS, XID mice were injected i.p. with 100 µl (1 × 10<sup>6</sup> total B1a cells) of cells 1 d prior to infection. For passive transfer studies, serum was collected from uninfected WT mice or from WT mice 7 d postinfection and passed through a 0.2-µm filter to sterilize. Serum of SchuS4-infected mice was confirmed by plating onto MMH agar plates. XID mice were injected with 200 µl indicated serum i.p. 1 d prior to infection.

**Depletion of NK/NKT cells**

NK/NKT cells were depleted from XID mice using anti-Asialo GM1 Abs (WAKO Chemical, Richmond, VA) as previously described (26). Briefly, 2 d prior to infection, mice were injected i.p. with 50 µl anti-Asialo GM1 or rabbit Ig (Jackson Immunoresearch Laboratories, West Grove, PA) as a negative control. Mice were injected with anti-Asialo GM1 or rabbit Ig again on days 1 and 4 and every third day thereafter following infection. Depletion of NK/NKT cells was confirmed by flow cytometry as described below (Supplemental Fig. 1). This depletion regimen results in >98% depletion of NK/NKT cells.

**Collection of lung and spleen cells**

Lung cells and splenocytes were collected as previously described with the following modifications (9). Lung cells and splenocytes were resuspended in FACs buffer prior to flow cytometric analysis or DMEM supplemented with 10% heat-inactivated FCS, 0.2 mM l-glutamine, 1 mM HEPES
buffer, and 0.1 mM nonessential amino acids (all from Invitrogen, Carlsbad, CA) (cDMEM) prior to addition to tissue cultures. Total live cells from the lungs and spleens were enumerated using trypan blue and a TC10 Automated Cell Counter (Bio-Rad, Hercules, CA). A portion of cells were immediately stained for surface receptors as described above. Additional lung and spleen cells were resuspended in cDMEM at 2 × 10^6/well in 96-well plates in the presence of 10 μg/ml brefeldin A, 10 ng/ml PMA, and 1 μg/ml ionomycin (all from Sigma-Aldrich) at 37°C 1% CO_2 for 4 h. Cells were stained for the indicated surface markers and intracellular cytokines as described below.

**Flow cytometry**

Lung and splenocyte populations were assessed by flow cytometry as previously described (9). Briefly, the following Abs in various combinations were used for flow cytometric analysis: allophycocyanin CD4, PerCP-Cy5.5 CD8, FITC NKp46, PE-Cy7 B220, PerCP-Cy5.5 CD11c, PE-Cy7 CD11b, PE-EcY, FITC Ly6G, allophycocyanin F4/80, PE MHC class II (MHC II), PerCP-Cy5.5 CD19, and allophycocyanin CD5 (all from BD Biosciences, San Jose, CA). Staining was performed in FACS buffer at room temperature. Following staining, cells were washed and fixed in 1% paraformaldehyde for 30 min at 4°C. Cells were washed a final time, resuspended in FACS buffer, and stored at 4°C until analyzed. Samples were collected using an LSR II flow cytometer (BD Biosciences). Analysis gates were set on viable unstained cells and were designed to include all viable cell populations. Approximately 10,000 gated events were analyzed for each sample. Isotype control Abs were included when analyses and panels were first being performed to assure specificity of staining, but were not routinely included with each experiment. Data was analyzed using FlowJo software (Tree Star, Ashland, OR).

Intracellular cytokines were detected by flow cytometry as previously described (9). Following incubation, cells were washed once and resuspended in FACS buffer and stained for CD4, CD8, NKp46, CD19, CD5, F4/80, and CD11b as described above. Then cells were fixed in 2% paraformaldehyde in PBS for 10 min at 37°C and washed twice more in Perm buffer (FACS buffer supplemented with 0.25% saponin [Sigma-Aldrich]). Cells were incubated for 20 min at room temperature with various combinations of PE IFN-γ, allophycocyanin, Alexa Fluor 488 IL-10, and PE IL-12p40 (all from BD Biosciences). Cells were washed twice in Perm buffer, fixed in 1% paraformaldehyde for 30 min, and then resuspended in FACS buffer and stored at 4°C until analysis. Cells were acquired and analyzed using an LSR II flow cytometer (BD Biosciences) and FlowJo software (Tree Star).

**Detection of secreted cytokines**

Concentration of TNF-α present in cell-culture supernatants was determined using commercially available ELISA kits following the manufacturer’s instructions (BD Biosciences).

**Statistical analysis**

Statistical differences between two groups were determined using an unpaired t test with the significance set at p < 0.05. For comparison between more than two groups, analysis was done by nonparametric one-way ANOVA (Kruskal–Wallis Test) followed by Dunn’s comparisons test with significance determined at p < 0.05. Significance in survival between groups was determined using log-rank (Mantel–Cox) test with significance determined at p < 0.05.

**Results**

**XID mice show greater survival and control of bacterial replication than WT mice**

To determine the requirement for high titers of anti-SchuS4 Abs and B1a cells in resolution of pulmonary SchuS4 infection, we first determined if mice lacking B1a cells exhibited differences in survival compared with WT mice. Infection of mice with SchuS4 typically results in death within 5 d of infection (9, 27). This rapid mean time to death often precludes the ability of the host to mount an immune response. Thus, determination of the role-specific host cell components make in the pathogenesis of SchuS4 infection among untreated mice is typically not fruitful. Indeed, there was no statistically significant difference among untreated WT and XID mice infected with SchuS4 (Fig. 1A). We recently developed a model in which WT mice are treated with low doses of LVF following SchuS4 infection that results in ~50% survival of WT mice, thus allowing comparisons of host cell components required for either exacerbation or resolution of infection (9). Using this model, we made the surprising observation that, following treatment with LVF, XID mice exhibited significantly greater survival of SchuS4 infection than WT controls (p = 0.034) (Fig. 1A). Consistent with our previous observations in C57BL/6J mice, animals that survived to 30 d after SchuS4 infection did not have detectable numbers of SchuS4 in the lung or spleen (data not shown).

WT mice that succumb to SchuS4 infection following treatment with antibiotic fail to control bacterial replication after therapy with LVF has ended (9). Thus, we hypothesized that the increased survival of XID mice treated with LVF would correlate with better control of bacterial replication compared with similarly treated WT animals. We first assessed control of bacterial burdens in among SchuS4-infected animals that had not received LVF. Interestingly, despite no difference in mean time to death, untreated XID mice had significantly fewer bacteria in the lungs (days 1 and 3) and spleen (days 2 and 3) postinfection compared with untreated WT animals (Fig. 1B). However, by day 4 postinfection, there were no significant differences in bacterial loads among untreated animals, which is consistent with the similar mean time to death observed in these groups. In contrast, following administration of LVF XID mice had significantly fewer bacteria in the lung on days 7, 10, and 14 postinfection compared with LVF-treated WT animals (Fig. 1B). LVF-treated XID mice also had significantly fewer SchuS4 organisms in the spleen on days 7 and 10 postinfection compared with WT mice at these time points (Fig. 1B). Following cessation of LVF therapy, and 21 d postinfection, three out of five WT mice had detectable bacteria in the lungs, and all WT animals had detectable bacteria in the spleen (Fig. 1C). In contrast, only one out of five XID mice had detectable bacteria in the lung and spleen at this time point postinfection (Fig. 1C). The control of recrudescence of infection among LVF-treated XID mice correlated with enhanced survival of these animals following infection with SchuS4 and treatment with LVF. Thus, the absence of functional Bruton’s tyrosine kinase (btk) in XID mice is associated with enhanced clearance of bacterial burdens and increased survival of SchuS4 infection.

**XID mice have low concentrations of circulating Abs directed against SchuS4**

The immune defect in XID mice that results in attrition of B1a cells and hampered production of IgM and IgG Abs by conventional B cells is mutation of btk. As discussed above, B1a cells contribute to the humoral response via secretion of natural and T-independent IgM Abs. Furthermore, B1a cells have been shown to be an important source of protective IgM directed against F. tularensis LVS LPS, in that XID mice vaccinated with LVS LPS fail to survive lethal LVS infection (21). Because Abs, and specifically Abs derived from B1a cells, contribute to protection against attenuated strains of F. tularensis, we next determined if XID mice truly exhibited differences in their ability to secrete Abs that recognize SchuS4. As previously established, XID mice had lower titers of total serum IgM Abs compared with WT controls prior to infection (29) (data not shown). Following exposure to SchuS4 and treatment with LVF, XID mice mounted a delayed and weaker IgM and IgG response directed at SchuS4 compared with similarly treated WT controls (Fig. 2A, 2B). Moreover, XID mice also had a delayed and lower titer of serum Abs capable of agglutinating viable SchuS4 compared with WT mice (Fig. 2C). Thus, our observation of attenuated Ab production in XID mice following SchuS4 infection and LVF therapy is consistent with previously published reports examining Ab production in these animals follow.
lowing infection with unrelated microorganisms (29–31). Because XID mice have lower levels of Abs directed against SchuS4 yet exhibit better survival against infection with this bacterium, our data suggest that high, sustained titers of anti-SchuS4 Abs are not required to survive SchuS4 infection.

**XID macrophages are equally susceptible to infection with SchuS4 compared with WT cells in vitro**

The dampened Ab response in XID mice is attributed to the lack of functional btk in B cells and the absence of B1a cells in these animals. However, in addition to B cells, btk is also expressed in a variety of myeloid cells including macrophages (32). Among macrophages, btk participates in mediating signals from TLRs, cell-death pathways, and production of NO (33–35). Macrophages represent one of the primary cell types targeted by *F. tularensis* for intracellular replication. Thus, it was possible that the differences in bacterial burden we observed in vivo were due to impaired replication of SchuS4 in XID macrophages. To test this hypothesis, we compared infection and replication of SchuS4 in freshly isolated alveolar macrophages and BMM from XID and WT mice. We did not observe any statistically significant differences in uptake or replication of SchuS4 in XID macrophages compared with WT cells (Fig. 3A).

Given the involvement of btk in mediating production of inflammatory cytokines following engagement of TLRs, we also tested whether XID macrophages mounted a different inflammatory response following SchuS4 infection compared with WT controls. In agreement with our previous reports, SchuS4 failed to elicit production of TNF-α, IL-6, and IL-12p40 from WT or XID macrophages (Fig. 3B and data not shown). SchuS4 also readily inhibits production of inflammatory cytokines and NO among infected macrophages (36–38). Therefore, an inability of SchuS4 to inhibit this process in XID macrophages may explain the reduced ability to replicate. However, we did not observe any differences in the inhibition of proinflammatory cytokines or induction of NO among XID macrophages compared with WT cells or in tissues of infected animals (Fig. 3B and data not shown). Together, these data suggest that the impaired control of SchuS4 infection in vivo is not likely due to altered interactions specifically between the bacteria and macrophages alone.

*XID and WT mice have similar populations of inflammatory cells following SchuS4 infection*

Because there were no observable differences in the interaction between SchuS4 and cells typically targeted for replication (i.e., macrophages) in vitro, we next evaluated changes in populations of inflammatory cells following in vivo SchuS4 infection. We and others have previously shown that monocytes are capable of producing inflammatory cytokines associated with protection against various subspecies of *Francisella* (27, 39, 40). Recruitment of granulocytes (i.e., neutrophils) have also been associated with survival of infection with attenuated subspecies of *Francisella* (41). Thus, enhanced control of bacterial burdens among XID mice may be attributed to increased numbers of monocytes and/or granulocytes in target organs. However, we did not observe significant differences in the overall cellularity nor specific monocyte (CD11b+/Ly6C+/MHC II⁺) or granulocyte (Ly6G+/MHC II⁺) populations in the lung or spleen on days 4, 7, or 10 post-infection (Fig. 4 and data not shown).

*Decreased IL-10–producing B1a cells and increased IL-12–producing macrophages in XID mice in vivo*

The greater control of SchuS4 infection we observed in XID mice, compared with WT animals, could not be attributed to differences in the primary interaction of bacteria with target macrophages or recruitment of specific inflammatory cells. This suggested the presence or absence of other cell types contribute to control of SchuS4 infection in XID animals. XID mice lack B1a cells that act as an important source of IL-10 (42). It is well established that IL-10 can downmodulate Th1 responses via inhibition of IL-12 production from macrophages, which, in turn, results in weakened IFN-γ production from NK/NKT and T cells (43). IL-12 is essential for survival of SchuS4 infection (9). Given the counterbalance between IL-10 and IL-12 and reported absence of B1a cells capable of producing IL-10 in XID mice, we determined if there were differences in populations of IL-10– and/or IL-12–producing cells in XID and WT mice following SchuS4 infection.
In agreement with previous reports, uninfected WT mice had significantly higher numbers of B1a cells in the lung and spleen compared with XID mice (Fig. 5A) (44). Following SchuS4 infection, we did not observe significant changes in B1a populations in the lungs of WT mice, nor did we observe significant differences in B1a IL-10+ cells in this organ (Fig. 5A). In contrast, WT mice had a significant increase in B1a IL-10+ cells in the spleen following infection. Furthermore, WT animals also had significantly greater numbers of IL-10+ B1a cells in the spleen before and postinfection compared with XID mice (Fig. 5A).

IL-12 is a critical cytokine for protection against SchuS4 infection (9, 45). It is well established that IL-10 downregulates IL-12 production from macrophages (43). Thus, we also assessed the ability of macrophages (identified as CD11b+/F480+/Ly6G<sup>+</sup>) in the lungs and spleens of WT and XID mice to produce IL-10 and IL-12. WT and XID mice had similar numbers of macrophages in the lung and spleen regardless of their infection status (Fig. 5B).

Furthermore, unlike B1a cells, we did not detect significant changes in IL-10+ macrophages among WT and XID mice following SchuS4 infection. Interestingly, and in correlation with decreased numbers of IL-10–producing B1a cells, XID mice had significantly higher numbers of IL-12+ macrophages in the lung and spleen following SchuS4 infection compared with WT animals (Fig. 5B). Together, these data suggest that decreased IL-10+ B1a cells and increased IL-12–producing macrophages in vivo correlate with enhanced control of SchuS4 infection in XID mice.

SchuS4 induces IL-10 production by B1a cells to inhibit IL-12

To determine if B1a cells could produce IL-10 in response to SchuS4, we isolated B1a, B1b and macrophages from the peritoneal cavity of resting mice and cultured each population of cells in the presence of live or dead SchuS4. In agreement with previous work assessing activity of B1a cells in models of unrelated infectious disease (46–50), B1a cells were the primary source of IL-10 in the peritoneum following coculture with either live or dead SchuS4 (Supplemental Fig. 2 and data not shown). We also confirmed that XID mice lacked B1a cells in the peritoneum, and, as expected, PEC from XID mice produced significantly less IL-10 in response to SchuS4 than PEC isolated from WT animals (Supplemental Fig. 2).

IL-10 is a potent inhibitor of IL-12 responses (43). Thus, we hypothesized that the IL-10 produced by B1a cells exposed to SchuS4 would inhibit IL-12 production. Because the peritoneal cavity is comprised primarily of B cells and macrophages, we took advantage of this compartment to test our hypothesis. Neutralization of IL-10 in PEC cultures treated with killed SchuS4 significantly enhanced production of IL-12p40 (Fig. 6). Interestingly, although live SchuS4 can provoke production of IL-10 from B1a cells, neutralization of IL-10 in those cultures does not significantly increase production of IL-12 (data not shown). However, it is well established that viable SchuS4 readily inhibits production of IL-12. Thus, the absence of IL-12 in cultures stimulated with
live SchuS4 in which 1a cell–derived IL-10 is neutralized is likely due to the suppressive effect viable SchuS4 is exerting on transcription and translation of IL-12. Together, our data demonstrate that B1a cell–derived IL-10 can effectively inhibit the ability of macrophages to produce IL-12 in response to dead SchuS4.

Adoptive transfer of B1a cells exacerbates SchuS4 infection

The data presented above suggested that increased presence of serum Abs directed against SchuS4 or IL-10–producing B1a cells contributed to exacerbation of SchuS4 infection in CBA/J mice. To determine if this was the case, we first examined the effect of passively transferred serum collected from SchuS4-infected WT mice or uninfected controls on the replication of SchuS4 in XID mice. Passive transfer of serum, regardless of the source, had no effect on the burden of SchuS4 in the lungs and spleens of XID mice (data not shown). We then examined the effect adoptive transfer of B1a cells into XID mice would have on tissue burden of SchuS4. One day prior to infection with SchuS4, XID mice received B1a cells enriched from the peritoneal cavities of WT mice. We confirmed that B1a cells obtained from the peritoneal cavity were a primary source of IL-10 in additional in vitro assays (Supplemental Fig. 2). XID mice that received WT B1a cells had significantly higher numbers of SchuS4 in the lung and spleen compared with sham-inoculated controls (Fig. 7A). Although adoptive transfer of B1a cells into XID mice did not reduce the surviving number of animals to the same percent observed in WT mice, adoptive transfer of B1a cells into XID mice decreased survival of XID mice infected with SchuS4 compared with untreated controls (Fig. 7B). Moreover, unlike untreated XID mice, survival of XID mice receiving B1a cells was not significantly different from WT animals. Thus, absence of IL-10–producing B1a cells is one facet to the enhanced control of SchuS4 infection among XID mice compared with WT animals.

Increased IFN-γ–producing NK/NKT cells in XID mice

IL-10 derived from B1a cells can act on a variety of cell types. Our data suggest that presence of IL-10–producing B1a cells is inversely correlated with IL-12+ macrophages and contributes toward exacerbated SchuS4 infection. Furthermore, we established that IL-10 produced by B1a cells in response to SchuS4 inhibits the ability of cells to produce IL-12. As discussed above, IL-12 is an essential cytokine for the propagation of Th1 responses, namely through its regulation of Th1 cytokine production.
ability to induce production of IFN-γ from NK/NKT and T cells. In addition to IL-12, we have also shown that IFN-γ is critical for survival of SchuS4 infection (9). Thus, we postulated that the increase in IL-12+ macrophages observed in XID mice after SchuS4 infection (Fig. 4B) would correlate with increased numbers of IFN-γ+ lymphocytic cells in the lung and spleen. We first determined if there was an increase in the specific lymphocyte populations in the organs of WT and XID mice after SchuS4 infection. We detected expansion of CD4+, CD8+, and Nkp46+ cells in the lung and spleen of both WT and XID mice (Fig. 8). XID mice had significantly higher numbers of each of these cell types compared with WT controls in the lung and significantly higher numbers of NK/NKT cells in the spleen (Fig. 8A). However, despite the overall increase in CD4+ and CD8+ cells observed in XID mice, there were no differences in the number of CD4+IFN-γ+ or CD8+IFN-γ+ compared with WT controls (Fig. 8B). In contrast, XID mice had significantly more IFN-γ+NKp46+ cells compared with WT mice at this time point postinfection (Fig. 8B).

NK/NKT cells contribute toward resolution of SchuS4 infection in XID mice

Given the significantly higher numbers of IFN-γ+NK/NKT cells observed in both the lungs and spleens of XID mice following SchuS4 infection and the critical role IFN-γ plays in resolution of tularemia (Fig. 8) (9), we next assessed the contribution NK/NKT cells made in control of SchuS4 infection in XID mice. XID mice depleted of NK/NKT cells had significantly higher numbers of bacteria in the spleen compared with animals treated with rabbit Ig on day 7 postinfection (Fig. 9). Although not significantly different, depletion of NK/NKT cells in XID mice also increased bacterial burdens in the lungs on day 7 and 21 postinfection (Fig. 9). Notably, depletion of NK/NKT cells reversed the clearance of SchuS4 in the lungs of XID mice when assessed 21 d postinfection (Fig. 9). Thus, NK/NKT cells contribute to the control of SchuS4 replication in XID mice.

Discussion

B cells have a variety of functions during development of immune responses to infection. In addition to serving as Ab-producing cells, B cells also act as APC, participate in activation of T cells, and secrete cytokines that may contribute to the control or exacerbation of infection. There is growing evidence and understanding that these functions are often associated with specific subsets of B cells. For example, B1a cells are an important subset B cells because they are the primary producers of natural IgM, IgM Abs directed against T-independent Ags, and are a central source of IL-10 (42, 51, 52). Thus, B1a cells can act in a protective role during infection via their secretion of IgM that aids in opsonization and killing of pathogens or in a negative role via their production of IL-10 that largely functions to dampen Th1 responses.

In the report presented in this paper, we demonstrate that B1a cells exacerbate infections mediated by virulent F. tularensis strain SchuS4. Specifically, mice largely deficient for B1a cells and signaling via the BCR (i.e., XID mice) exhibit better survival following intranasal infection with SchuS4. This survival was correlated with superior control of bacterial loads in the lung and spleen. Additionally, adoptive transfer of B1a cells from WT animals into XID mice resulted in increased bacterial loads and reduced survival following SchuS4 infection (Fig. 7). Furthermore, our data also suggest that the increased resistance to SchuS4 infection observed in XID mice is not correlated with intrinsic defect in macrophages found in these mice. For example, both BMM and freshly isolated alveolar macrophages obtained from XID mice phagocytosed and supported SchuS4 replication similarly to that observed in WT macrophages. Although we did not
We directly tested the ability of DC and neutrophils from XID mice to respond to SchuS4 infection, we failed to observe differences in WT and XID mice that could be associated with defects in responses of other cell types in these animals (e.g., there were no differences in the generation of NO, recruitment of inflammatory cells, or ability of SchuS4 to suppress production of inflammatory cytokines). Thus, the primary difference in XID mice that is associated with survival of SchuS4 infection is the absence of B1a cells. Moreover, our findings point toward an indirect function of B1a cells on other cells via their secretion of IL-10.

Initially, our finding that XID mice were more resistant to infection with SchuS4 was surprising. In our previous study using μMT−/− mice, we established that B cells are critical for survival of SchuS4 infection (9). Thus, we predicted that XID mice with their inherent defects in BCR signaling and inability to mount high titers against SchuS4 would be equally susceptible to SchuS4 infection as observed in μMT−/− animals. Furthermore, it has been repeatedly shown that XID mice have increased susceptibility against a wide range of pathogens, including intracellular bacteria (46–50). With regard to infection mediated by attenuated *F. tularensis* strain LVS, immunization with LVS LPS failed to protect XID mice from infection with LVS (21). The inability of XID mice to develop protective immunity in this report was specifically correlated with the absence of Ab directed against LVS LPS produced by B1a cells. A potential role for natural Ab produced by B1a cells in protection against *F. tularensis* was also suggested in a recent report (53). Together, these data suggested that B1a cells would play a critical role in the early control of *F. tularensis* infections via their ability to produce Abs directed against surface Ags.

However, our data suggest that Abs derived from B1a cells do not play a significant role in the resolution or exacerbation of infection mediated by virulent *F. tularensis* SchuS4. XID mice have nearly undetectable levels of natural IgM Abs and develop very poor titers of Abs directed against SchuS4. Furthermore, passive transfer of serum Abs from infected WT mice had no impact on bacterial burdens in XID mice (data not shown). Thus, the enhanced clearance of SchuS4 observed in XID mice and the im-

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**FIGURE 8.** XID mice have significantly more IFN-γ+ NK/NKT cells after SchuS4 infection compared with WT mice. Mice (n = 4 to 5/group) were i.n. infected with 50 CFU *F. tularensis* strain SchuS4. As indicated, animals received 5 mg/kg LVF on days 3–6 of infection. Animals were euthanized on day 7 of infection, and single-cell suspensions of the lung and spleen were generated. Cells were incubated with PMA, ionomycin, and brefeldin A for 4 h, stained for specific surface receptors (A), permeabilized, stained for IFN-γ (B), and assessed for specific cell populations by flow cytometry. Error bars represent SEM. Data are representative of two experiments of similar design. *Significantly greater than uninfected WT (p < 0.05), **significantly greater than all other groups (p < 0.05).
**FIGURE 9.** NK/NKT cells contribute to control of SchuS4 infection in XID mice. Mice were treated with anti-asialo GM1 (anti-AGM1) Abs to deplete NK/NKT cells or rabbit Ig as a negative control on days -2, 1, 4, 7, 10, 13, 16, and 19 of infection. Mice were i.n. infected with 50 CFU *F. tularensis* strain SchuS4. Animals received 5 mg/kg LVF on days 3–6 of infection. All mice were euthanized on days 7 and 21 of infection, and bacteria were enumerated from the lung and spleen. Error bars represent SEM. Data are representative of two experiments of similar design. *Significantly different Ig-treated controls (*p* < 0.05).

proved survival of these animals compared with WT controls suggests that neither natural IgM Abs nor Abs that recognize SchuS4 are required for survival of tularemia.

In contrast to a protective function of B1a cells in tularemia, our data clearly demonstrate that presence of these cells exacerbates infection with *F. tularensis* SchuS4 resulting in increased mortality under the setting of antibiotic therapy. The importance of B1a cells in mediating inhibitory effects on protection against SchuS4 infection, rather than other cells types affected by the defect in btk in XID mice, was confirmed in two ways. First, we established that there were no detectable differences in the ability of macrophages derived from XID mice to support SchuS4 infection or replication compared with WT cells (Fig. 3). Second, adoptive transfer of WT B1a cells into XID mice resulted in significant increase in replication of SchuS4 in the lung and spleen (Fig. 7). Thus, B1a cells (that are largely absent in XID mice) negatively impact the outcome of SchuS4 infection. Initially, these data appear to be in conflict with our previous observation that the presence of an intact B cell compartment is required to survive SchuS4 infection (9). However, it should be noted that XID mice still retain conventional B cells. Although these cells would be poor producers of Ab in XID mice (due to the lack of signaling through the BCR), these conventional B cells may have other functions that support resolution of SchuS4 infection (e.g., Ag presentation and secretion of protective cytokines) (2).

And others have shown that Th1 type immune responses and IFN-γ are required for survival of *F. tularensis* infection (9, 54, 55). IL-10 derived from B1a cells is well established for its ability to interfere with Th1-type immune cells. Indeed, there are examples of B1a cell IL-10 indirectly limiting IFN-γ-mediated protective immune responses against a variety of infections (56–58). Thus, we next addressed if B1a cells may function similarly in our model to inhibit protective immune responses during SchuS4 infection. Our data suggest that the negative role B1a cells play in SchuS4 infection is via indirect modulation of IFN-γ response by NK/NKT via secretion of IL-10. It is important to emphasize that the role for B1a cell–derived IL-10 in modulating IFN-γ production is indirect. IL-10 is a potent anti-inflammatory cytokine that can exert its activity on a wide range of host cells including macrophages and DC (43), one of which is to limit production of IL-12. IL-12 is an important cytokine for induction of IFN-γ responses in NK/NKT cells. Thus, IL-10 indirectly affects IFN-γ production via its ability to downregulate IL-12 production in macrophages and DC. For this paradigm to be true in our system, one would predict that the absence of IL-10–producing B1a cells should lead to an increase in IL-12 production by macrophages, resulting in an increase in IFN-γ–producing NK/NKT and/or T cells. In support of this hypothesis, we first demonstrated that B1a cells respond to killed SchuS4 via production of IL-10. Neutralization of B1a cell–derived IL-10 in this in vitro system resulted in increased secretion of IL-12. Similarly, in vivo, we found that the absence of IL-10–producing B1a cells in XID mice was inversely correlated with the presence of IL-12+ macrophages in the spleens and lungs of infected animals (Fig. 5) and that this increase in IL-12+ macrophages was also correlated with significantly higher numbers of IFN-γ–producing NK/NKT cells in XID mice (Fig. 8).

The presence of protective and inhibitory cytokines in this model is unlike what occurs in cases of untreated tularemia. When antibiotic treatment is withheld, there is a striking absence of cytokines until the last 24–36 h of infection, at which time one observes a cytokine storm (59–61). The lack of cytokine production during early, critical stages of disease is associated with the impressive ability of virulent strains of SchuS4 to evade and inhibit inflammatory responses in the host (24, 62, 63). How then does one elicit IL-10 and IL-12 following infection with SchuS4 during treatment with antibiotic? We propose that induction of host cytokines after day 4 of infection is a result of both the release of damage-associated molecular pattern molecules from dying host cells and accumulation of lysed and/or dying bacteria. In support of this hypothesis, exposure of B1a cells to SchuS4 killed with LVF readily induces production of IL-10 (Fig. 6, **D**).
Supplemental Fig. 2) by these host cells. The specific host cell receptors and mechanism by which IL-10 is liberated by B1a cells is under intense study in our laboratory.

Together, our data suggest the following model (Fig. 10). Dead and/or lysing SchuS4 provokes IL-12 from macrophages, which then acts on NK/NKT cells to produce IFN-γ. IFN-γ activates newly infected macrophages to kill SchuS4. However, in the presence of B1a cells, the dead bacteria provoke a potent IL-10 response. This IL-10 significantly limits IL-12 production from macrophages, resulting in suboptimal production of IFN-γ from local NK/NKT cells. Thus, induction or absence of IL-10 by B1a cells serves as a key pivot point for early control of SchuS4 burdens in target organs and a deciding factor for survival of tularemia infection. Finally, our data highlight an important mechanism for cross-talk among a specific subset of B cells, macrophages, and NK/NKT cells that significantly impacts the outcome of infection with a highly virulent pathogen.

Acknowledgments
We thank Aaron Carmody for assistance in the FACS isolation of B1a cells, Anita Mora and Heather Murphy for generation of the model cartoon, and Dr. Jay Carroll for critical assessment of the manuscript and helpful suggestions.

Disclosures
The authors have no financial conflicts of interest.

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Supplemental Figure Legends

**Supplemental Figure 1. Confirmation of depletion of NK/NKT cells using anti-Asialo GM1 antibodies.** Mice were injected with anti-Asialo GM1 twice, three days apart. Two days after the last injection mice were euthanized and the spleens and lung were evaluated for the presence of NK/NKT cells by flow cytometry.

**Supplemental Figure 2. B1a cells are the primary producers of IL-10 following exposure to SchuS4.** (A) Peritoneal cells (PEC) were collected from resting wild type or XID mice and analyzed for populations of macrophages and B cells by flow cytometry. (B) Each cell population from wild type mice depicted in (A) was isolated by FACS and incubated with killed SchuS4 overnight. IL-10 present in supernatants was measured by ELISA. (C) Total PEC from resting wild type (WT) or XID mice were incubated with killed SchuS4 overnight, then supernatants were collected and assessed for IL-10 by ELISA. BLD = below level of detection. * = significantly less than B1a cells (B) or WT PEC (C) (p<0.05). Error bars represent SEM. Data is representative of three experiments of similar design.
Crane D, et al, Supplemental Figure 1
**A**

Wild Type

Total PEC

- CD5
- F4/80

- B1a cells (57.8%)
- B1b cells (26.7%)

- Macrophages (11.2%)

**B**

IL-10

- B1a
- B1b
- Macrophage

Bar graph showing IL-10 levels in untreated, vehicle control, and killed SchuS4 conditions.

**C**

IL-10 (pg/mL)

- WT
- XID

- Killed SchuS4

Bar graph showing IL-10 levels in WT and XID conditions after killed SchuS4 treatment.

Crane D et al, Supplemental Figure 2