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Tissue-expressed B7x Affects the Immune Response and Outcome to Lethal Pulmonary Infection

Kimberly A Hofmeyer[*], **Lisa Scandiuzzi**[*], **Kaya Ghosh**[*], **Liise-Anne Pirofski**[†], and **Xingxing Zang**[*]

[*]Department of Microbiology and Immunology, Albert Einstein College of Medicine, Bronx, NY 10461

[†]Division of Infectious Diseases, Departments of Medicine, Microbiology and Immunology, Montefiore Medical Center and Albert Einstein College of Medicine, Bronx, NY 10461

Abstract

B7x (B7-H4 or B7S1), a member of the B7 family, inhibits *in vitro* T cell proliferation and cytokine production by binding to an unidentified receptor on activated T cells, but its *in vivo* function remains largely unclear. We show that B7x protein was expressed in epithelial cells of the lung, but not in lymphoid tissues. To investigate the role of B7x in the lung, we determined the susceptibility of B7x deficient (B7x−/−) mice to a lethal pulmonary infection with *Streptococcus pneumoniae*. B7x−/−, but not B7-H3 deficient, mice were significantly more resistant to *S. pneumoniae* pulmonary infection than their wild-type (Wt) counterparts. B7x−/− mice had significantly lower bacterial burdens and levels of inflammatory cytokines in lungs as early as 12 hours post-infection. They also had milder immunopathology that was localized in alveolar spaces, while Wt mice had severe inflammation that was perivascular. Control of infection in B7x −/− mice was associated with a marked increase in activated CD4 and CD8 T cells and fewer neutrophils in lungs, whereas the susceptible Wt mice had the opposite cellular profile. In B7x−/ −Rag1−/− mice that lack T cells, reduction in bacterial burden was no longer observed. Control of *S. pneumoniae* and the increased survival observed was specific to the lung, as systemically infected B7x−/− mice were not resistant to infection. These data indicate that lung-expressed B7x negatively regulates T cells and that in its absence, in B7x−/− mice, an enhanced T cell response contributed to reduced lethality in a pulmonary infection model with *S. pneumoniae*.

Introduction

The B7/CD28 family is of central importance in regulating the T cell response and affects both activation and the outcome of effector function (1, 2). B7x (B7-H4 or B7S1) is the most recently identified member of this family and *in vitro* studies show that it sends a coinhibitory signal by binding to a currently unknown receptor that is expressed on activated T cells (3–5). Binding of B7x to its putative receptor on both CD4 and CD8 T cells inhibits cell cycle progression, proliferation, and production of associated effector cytokines such as IL-2 and IFN-γ (3–5). B7x mRNA expression is higher in peripheral non-lymphoid tissues, particularly the lung, pancreas, prostate, and testes, whereas in lymphoid tissues such as the spleen it is comparatively low (3, 6). This is in marked contrast to the classical B7 family members, B7-1 and B7-2, which are most highly expressed on professional antigen presenting cells (APCs) in lymphoid tissues (6, 7). The pattern of B7x mRNA expression coupled with its coinhibitory signaling suggests that it may be a potent regulator of the

Address correspondence and reprint requests to Dr. Xingxing Zang, Department of Microbiology and Immunology, Albert Einstein College of Medicine, 1300 Morris Park Avenue, Forchheimer Building 405, Bronx, NY 10461. xingxing.zang@einstein.yu.edu.

immune response in the periphery. Murine studies support this notion as B7x protein expression is detected on the insulin-producing β cells of the pancreas, and while B7x knockout results in exacerbation of diabetes, overexpression of B7x in β cells abrogates development of disease (6). Clinical data also support a coinhibitory role for B7x, as overexpression of the protein has been observed on many types of tumors and is often associated with enhanced disease progression and poor clinical outcome (8–12). These clinical observations lead to a hypothesis that expression of B7x by tumors represents a previously unrecognized mechanism of down-regulating antitumor T cell responses at the level of the effector cell (2, 3, 13).

At present there is very limited data regarding the role of B7x in the immune response to infection, as evidenced by only two publications (14, 15). One study shows that B7x deficient (B7x−/−) mice have a slightly stronger Th1 response and lower parasite burdens following a footpad challenge with *Leishmania major* (14). Another study reveals that B7x −/− mice are more resistant to a lethal intraperitoneal infection with *Listeria monocytogenes*, although this is attributed to an augmented neutrophil, rather than T cell, response (15). Thus, the role of B7x in infection warrants further investigation.

As B7x mRNA expression is highly detected in the lung (3), we decided to investigate the role of this molecule in the immune response to and outcome of pulmonary infection with *Streptococcus pneumoniae*. *S. pneumoniae* is an encapsulated Gram-positive bacterium that colonizes the respiratory tract and can cause pneumonia, otitis, meningitis, and sepsis (16). It primarily affects infants, young children, the elderly, and immunocompromised patients and is a major cause of death in unimmunized children under the age of five years (17). Although the innate immune response and the humoral arm of adaptive immunity are associated with pneumococcal clearance, new data have demonstrated an important role for T cells in the host response to infection. Mice lacking CD8 T cells are more susceptible to pulmonary infection with *S. pneumoniae* (18) while mice lacking CD4 T cells are reported to either be more or less protected (19, 20). In this study, we found that B7x protein was detected in epithelial cells lining the bronchial passages of the lung but not in the spleen. B7x−/−- mice were more resistant to lethal pulmonary, but not systemic, challenge with *S. pneumoniae*, and had lower bacterial burdens in the lung, blood, and spleen than wild-type (Wt) controls. Wt mice had a severe inflammatory response and elevated levels of associated cytokines and chemokines in their lungs, but inflammation was largely controlled in B7x−/− mice. In contrast, mice with deficiency in B7-H3, the closest homolog of B7x (3, 21, 22), were unable to control *S. pneumoniae*-induced lethal lung infection. Since Wt mice succumbed to death within four days post-infection, while a significant proportion of B7x−/−4 mice survived, we decided to evaluate if B7x−/− mice had preexisting immunity to *S. pneumoniae*. However, no difference in natural antibodies to *S. pneumoniae* or *in vivo* phagocytosis by neutrophils or alveolar macrophages was found. Analysis of the immune infiltrate in lungs post-infection revealed that B7x−/− mice had more activated T cells, but fewer neutrophils than Wt mice. In B7x−/−Rag1−/− mice that lack mature T cells, a reduction in bacterial burden was no longer observed and the mice had similar burdens as the susceptible Wt mice. This study suggests that B7x negatively regulates T cells in the lung, which contributes to the lethality of pulmonary *S. pneumoniae* infection.

Material and Methods

Mice

B7x−/− and B7-H3 deficient (B7-H3−/−) mice on C57BL/6 background were previously described (6, 23). Age and sex-matched wild-type C57BL/6 mice were purchased from National Cancer Institute. Rag1−/− mice were purchased from The Jackson Laboratory, and were crossed with the B7x−/− mice to generate the double knockout B7x−/−Rag1−/−mice.

Mice that were 7−9 weeks of age were used for studies. All mice were maintained under specific pathogen-free conditions at the Albert Einstein College of Medicine following protocols approved by the Institutional Animal Care and Use Committee.

Bacteria and pneumococcal infection models

The *S.pneumoniae* serotype 3 (ST3) strain A66 was used in this study. A66 has been used extensively to study the pathogenesis of pneumococcal pneumonia in mice (18, 24, 25). Bacteria were grown in Bacto™ Tryptic Soy Broth (TSB) (Becton-Dickinson, BD) to mid-log phase at 37°C in 5% CO_2 and stored in TSB + 10% glycerol at −80°C until use as described previously (28). Colony-forming unit (CFU) count of frozen stock was determined by plating of 10-fold serial dilutions on Trypticase soy agar with 5% sheep's blood (Becton Dickinson). Dilutions were grown at 37°C with 5% CO_2 for 18 hours, at which CFU counts were determined.

Intranasal infection was performed as described previously (18). Briefly, prior to infection, frozen bacterial stock was thawed and diluted to the desired dose in TSB media. Mice were minimally anesthetized and intranasally infected with 1×10^5CFU/40uL/per mouse by slowly pipetting 20uL onto each nare. Mice were held upright to allow inhalation of bacteria and effective delivery to the lungs. Dose confirmation was performed by plating bacteria before and after infection on Trypticase soy agar with 5% sheep's blood and were grown at 37°C with 5% CO_2 for 18 hours, at which CFU counts were determined.

Bacterial burden

Tissue bacterial burdens were determined as previously described (18). Mice were deeply anesthetized and blood was collected and kept in eppendorf tubes with heparin to prevent coagulation. Following blood collection, mice were vascularly perfused with PBS through the right ventricle of the heart to remove any remaining blood from the lungs. Whole lungs and spleen were collected and placed in sterile HBSS on ice, and homogenized (Fisher Scientific PowerGen 125) for 20 seconds at maximum speed, 10-fold serial dilutions were plated on Trypticase soy agar with 5% sheep's blood and incubated for CFU determination.

Immunohistochemistry and histopathology

Tissues were collected from mice and fixed in 10% neutral buffered formalin (NBF) (Fisher) prior to paraffin embedding. Following clearing in xylene washes and rehydration, antigen retrieval was performed in a vegetable steamer in Tris/EDTA antigen retrieval solution. Sections were biotin blocked using the ScyTek Biotin Blocking kit and then normal blocking was done using 2% bovine serum albumin/5% donkey serum (Jackson ImmunoResearch). Following blocking sections were incubated with a 1:200 dilution of biotinylated anti-B7-H4 Ab or isotype IgG control (R&D Systems) overnight at 4°C and then blocked for endogenous peroxidase for 10 min in a 1% H_2O_2. Sections were incubated in ABC reagent (Vector Labs) for 20 min, then the tyramide amplification kit (Invitrogen) was used, followed again with the ABC reagent for 20 min. Sections were developed with the DAB kit (Vector Labs), counterstained with hematoxylin, dehydrated, cleared in xylene, and coverslipped.

For histopathological examination, lungs were inflated through the trachea using an 18G needle with 10% NBF until fully expanded, removed, and incubated overnight in 10% NBF. After fixation, lungs were transferred to 70% ethanol for at least one hour prior to paraffin embedding. Sectioned tissues were stained with hematoxylin and eosin (H&E) by the Pathology Core facility and viewed with AxioCam MRc Zeiss microscope. A board certified veterinary pathologist performed histopathological examination.

Cytokine levels in the lung, blood and spleen

Blood, lungs, and spleen were collected as described above. Blood was centrifuged and plasma was collected and stored at −80°C until use. Lungs and spleen were homogenized as described above, centrifuged at 3000 rpm for 30 minutes, and homogenate supernatant was collected and stored at −80°C until use. Cytokine levels were determined using both the mouse inflammation and Th1/Th2/Th17 BD™ Cytometric Bead Array kits (BD Biosciences) according to the manufacturer's instructions. The chemokines KC, MIP-1α MIP-1β, and RANTES were detected with BD™ Cytometric Bead Flex Sets that were combined and analyzed according to the manufacturer's instructions.

Natural antibody detection—Bacteria were heat killed by incubation in a water bath at 65°C for 15 minutes and then adhered to ELISA plates overnight. Sera from naive B7x−/− and Wt mice were incubated with adhered bacteria. Anti-IgM-HRP and anti-IgG3-HRP (SouthernBiotech) were then used separately to detect the presence of any natural antibodies to the bacteria. 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) substrate was added and allowed to develop at room temperature. The plates were read at 405nm using a Synergy H4 Hybrid reader (BioTek). Values for blanks were subtracted from sample values to get the actual OD readings.

Phagocytosis Assay

The phagocytosis assay was modified from an existing protocol (26). Live *S. pnemoniae* ST3 A66 were labeled with 2×10^{-6} M of PKH26 (Sigma-Aldrich). A 1mL frozen stock of A66 was thawed at room temperature, spun down at 13,000rpm for 3 minutes, washed twice in serum-free RPMI medium (Sigma-Aldrich), and resuspended in a final volume of 500μL of Dilutant C from the PKH26 Kit. 500μL of a 4×10^{-6} M solution of PKH26 in Dilutant C was added to the resuspended A66 and incubated for 3 minutes, protected from light, at room temperature. The PKH26-labeled A66 was spun down and washed three times with serum-free RPMI and resuspended in serum-free RPMI to a concentration of 2×10^{7} CFU/50uL dose. B7x−/− and Wt mice were anesthetized as described and the PKH26-labeled bacteria was intranasally delivered intranasally. After a 30 minute incubation mice were euthanized as described and bronchoalveolar lavage fluid (BALF) was collected with five 1mL washes of cold PBS. The BALF macrophages and neutrophils were detected by FACS as described in the flow cytometry section to follow. Phagocytosis by macrophages or neutrophils was determined by fluorescent detection of the PKH26-labeled bacteria that fluoresce at 565nm and can be detected on a 561nm or 488nm laser in the same channel as R-Phycoerythrin (PE).

Flow cytometry

Mice were deeply anesthetized and vascularly perfused with PBS through the right ventricle of the heart to remove blood from the vasculature via a puncture in the inferior vena cava. Whole lungs were collected and placed in cold digestion buffer (5% FCS, collagenase D, and heparin in plain DMEM) and broken apart using lung program 1 of the gentleMACS Dissociator (Miltenyi), incubated with gentle rotation at 37°C for 30 min, and then a single cell suspension was prepared using lung program 2 of the gentleMACS Dissociator. Spleens were collected and placed in 4mL of cold, plain DMEM and a single cell suspension was prepared using spleen program 1 of the gentleMACS Dissociator. Red blood cells were lysed, cells were filtered, and finally resuspended to the desired concentration. Cells were preincubated with anti-CD16/CD32 (eBioscience) to block Fc receptor binding and then stained with combinations of the following antibodies: CD45-PE or PerCP, CD8a-PerCP, B220-FITC, NK1.1-biotin, CD11c-biotin, CD86-PE, CD49d-biotin, CD11b-PE (eBioscience), CD4-alexa 488, CD62L-APC, Ly6G-FITC (clone 1A8, BD), F4/80-alexa

fluor 647 (Abd Serotec), or CD44-pacific blue (Biolegend). For intracellular staining, cells were fixed and permeabilized using the Foxp3 fixation/permeabilization kit (eBioscience) and then stained with anti-Foxp3-PE (eBioscience). Samples were examined with the BD Biosciences LSRII flow cytometer using BD FACSDiva (BD Biosciences) software with subsequent analysis of data in FlowJo (Treestar).

Statistical analyses

Statistical significance was calculated with the unpaired t-test using Prism software version 4.0b (GraphPad). Data for survival were analyzed according to the Kaplan-Meier method, and the univariate comparison of survival for control versus knockout group was tested using a log-rank test. Analysis of CFU data, cytokine/chemokines, and cellular phenotyping analysis was performed using Student t test. A p value of < 0.05 was considered statistically significant.

Results

B7x protein is expressed in the lung, but not in lymphoid tissues

Previously, B7x mRNA expression was detected in both lymphoid and peripheral nonlymphoid tissues (3–5). However, levels in non-lymphoid tissues, such as the lung, were much higher than in lymphoid tissues (3, 6). Here we used immunohistochemistry analysis to determine whether the pattern of B7x protein expression mirrors that of the existing mRNA data. We analyzed the lung and spleen of Wt mice (Fig.1A–C, E, G) and used both the equivalent tissues of B7x−/− mice (Fig. 1D, F, H) and an isotype control Ab (Fig.1B) as negative controls. In Wt mice, B7x protein was detected on the epithelium lining the bronchial passages of the lung, seen as the brown Ab-specific signal (Fig. 1A, C, D). Expression was not observed when an isotype control Ab was used in Wt lung (Fig. 1B) or when an anti-B7x Ab was used in tissues from B7x−/− mice. There was no detectable B7x protein expression in spleen of Wt (Fig. 1G) or B7x−/− (Fig. 1H) mice or in lymph nodes of both mice (data not shown). As in a previous study (6), we detected B7x protein expression specifically in the β cells of the pancreas (data not shown). This immunohistochemical staining is consistent with our recent work where B7x protein was not detected on human or mouse APCs and T cells before or after activation using a panel of monoclonal Abs and a polyclonal Ab (6). Collectively, these results demonstrate that, unlike other B7 molecules, endogenous B7x protein is expressed in the periphery but not in lymphoid tissues.

B7x deficient, but not B7-H3 deficient, mice are significantly more resistant to lethal pulmonary infection with *S. pneumoniae*—Based on our data showing that B7x was expressed in the lungs (Fig. 1E), we examined the outcome of pulmonary infection with *S. pneumoniae* in B7x−/− mice. We performed a dose titration in Wt mice and decided to use an inoculum of 1×10^5 CFU, as it was the lowest dose resulting in 100% lethality (data not shown). At this inoculum, B7x−/− mice were significantly more resistant to *S. pneumoniae* infection than Wt mice ($P < 0.002$); 100% of Wt mice died by day four post-infection, while 44% of B7x−/− mice survived for 30 days at which time the experiment was terminated (Fig. 2A). As the B7 family of proteins is phylogenetically divided into three groups and B7x and B7-H3 make up the group III (2, 3), so we infected B7-H3−/− mice with the same inocula to determine if the resistance phenotype was specific to B7x. Similar to Wt mice, B7-H3−/− mice exhibited a susceptible phenotype and there was no significant difference in survival between B7-H3−/− and Wt mice (Fig. 2B).

To determine whether the enhanced survival in B7x−/− mice was specific to pulmonary infection, we employed a systemic intraperitoneal challenge model (27). When B7x−/− and Wt mice were intraperitoneally infected with 1×10^5 CFU of *S. pneumoniae*, 100% of both

groups died within four days of infection (Fig. 2C), suggesting B7x does not regulate *S. pneumoniae*-induced systemic infection. Taken together, these findings suggest that B7x in the lung, but not B7-H3, plays an important role in the outcome of *S. pneumoniae* pulmonary infection.

B7x deficient mice exhibit better bacterial clearance in pulmonary infection— During pulmonary infection with *S. pneumoniae*, bacteria spread from the lung into blood and then distant organs such as spleen. Therefore, we determined the bacterial burden to assess dissemination in B7x−/− and Wt mice (Fig. 3A–C). At six hours post-infection, both B7x−/− and Wt mice had similar numbers of bacteria in their lungs (Fig. 3A). However by 12 hours post-infection, while Wt mice had a minimal increase in burden, B7x−/− mice had a significantly lower number of lung CFU (Fig. 3A). This difference was also observed at 36 hours post-infection (Fig. 3A). Analysis of blood and spleen of these mice also revealed significantly lower CFU at 36 hours in B7x−/− than Wt mice (Fig. 3B,C). These results reveal that B7x−/− mice are able to better control bacterial burdens as early as 12 hours post-infection.

B7x deficient mice have less immunopathology in lungs following pulmonary infection

We next examined lung histopathology. Both B7x−/− and Wt mice had normal lung tissue before infection (data not shown). However, histological analysis of lungs from B7x−/−and Wt mice intranasally infected with a lethal dose of *S. pneumoniae* showed marked differences in inflammation. At 12 hours post-infection, each strain of mouse had evidence of pneumonia, which was marked by neutrophil infiltration, especially around bronchioles and in alveolar spaces (Fig. 4A and Table 1). By 36 hours post-infection, inflammation in lungs of Wt mice had progressed, exhibiting severe neutrophilic perivascular inflammation with necrosis, edema and hemorrhage, but little to no airway-associated inflammation (Fig. 4B and Table 1). In contrast, lungs of B7x−/− mice exhibited an inflammatory pattern that was similar to that observed 12 hours post-infection, which was minimal and airway centric (Fig. 4C and Table 1). Only one out of the four B7x−/− mice had a similar distribution and severity of inflammation as the Wt mice (Table 1). There were no notable differences at any time points between histopathological findings in the spleen, liver, kidney, and brain (data not shown).

B7x deficient mice produce less inflammatory cytokines and chemokines after pulmonary infection

It is reported that increased levels of IL-6 and CCL2 (MCP-1) are associated with *S. pneumoniae*-induced death (28). Therefore we compared production of inflammatory cytokines and chemokines between B7x−/− and Wt mice. The reduced inflammation observed in lungs of B7x−/− compared to Wt mice (Fig. 4) was accompanied by lower levels of proinflammatory cytokines and chemokines (Fig. 5). There was no significant difference in basal levels of the examined cytokines and chemokines in the lungs or serum between naive B7x−/− and Wt mice (Fig. 5 and 6). However, at 12 hours post-infection, IL-6, TNF-α, and CCL2 were significantly lower in lungs of B7x−/− than Wt mice. There were no significant differences in IL-10 and IFN-γ (Fig. 5). B7x−/− mice also had significantly lower levels of the chemokines MCP-1, KC, MIP-1α, and MIP-1β, but not RANTES (Fig. 5). Although there was not as marked a difference in these cytokines and chemokines in the blood, at all points of statistical significance B7x−/− mice had lower levels than Wt mice (Fig. 6). These results demonstrate that B7x−/− mice had lower levels of inflammatory cytokines and chemokines in lungs and blood than Wt mice as early at 12 hours post-infection.

Enhanced survival in B7x deficient mice is not due to higher levels of natural antibodies or phagocytosis—Wt mice succumbed to intranasal infection within four days post-infection (Fig. 2A), whereas B7x−/− mice survived significantly beyond this point and had lower lung CFU as early as 12 hours post-infection (Fig. 3A). Since the control of infection in B7x−/− mice occurred at such an early time we decided to assess the innate immune response to *S. pneumoniae* by evaluating natural antibodies and phagocytosis. Natural antibody levels are critical for survival of *S. pneumoniae*-infected mice and facilitate opsonization and phagocytic killing of bacteria by macrophages and neutrophils (29–31). To determine whether the enhanced survival of B7x−/− mice was due to a difference in their level of natural antibodies, we analyzed anti-*S. pneumoniae* IgM and IgG3 by ELISA. There was no significant difference in the level of these antibodies (Fig. 7A). We then performed an *in vivo* phagocytosis assay in the lungs of B7x−/− and Wt mice. *S. pneumoniae* was labeled with the fluorescent cell dye PKH26, delivered intranasally into the lung, and following 30 minutes BALF was collected for analysis by FACS for phagocytosed bacteria. There was no statistically significant difference in *in vivo* phagocytosis by either alveolar macrophages (Fig. 7B) or neutrophils (Fig. 7C). Therefore, neither natural antibodies to *S. pneumoniae* nor serum-mediated phagocytosis contributes to the observed resistant phenotype of B7x−/− mice.

Enhanced survival in B7x deficient mice after pulmonary infection with *S. pneumoniae* is associated with an increase in activated T cells in lungs

Given that the enhanced bacterial clearance and survival of *S. pneumoniae*-infected B7x−/− mice could not be attributed to enhanced natural antibody or phagocytosis, we analyzed cellular infiltrates in the lung and spleen at 12 hours post-infection, a time when a difference in CFU between B7x−/− and Wt mice was evident (Fig. 3A). As compared to Wt mice, naïve B7x−/− mice have no significant differences in their immune cell populations, no immunopathology, no elevation of autoantibodies, and no spontaneous autoimmune disease (6, 14, 15). Following infection, both strains had similar numbers of CD45-positive cells in their lungs (Fig. 8A), which were B7x protein negative (data not shown). However, B7x−/− mice had significantly more CD4 (Fig. 8B) and CD8 (Fig. 8C) T cells and fewer neutrophils (Fig. 8D) in their lungs than Wt mice. This mirrored the immunopathology analysis that revealed lowered neutrophilic inflammation in lungs of B7x−/− mice (Fig. 4). No significant difference in the number of these cells was observed in the spleen (Fig. 8E–G). We also analyzed foxp3+CD4+ regulatory T cells (Treg), B cells, NK cells, dendritic cells, and macrophages in the lung (Fig. 8H) and spleen (Fig. 8I), but there were no differences in the number of these cells between strains. In addition, at 6 hours post-infection no significant difference in the total number of neutrophils in the lungs of B7x−/− and Wt mice was observed (data not shown).

As CD8 T cells have been shown to be required for resistance to *S. pneumoniae* A66 in naïve C57BL/6 mice (18) and CD4 and CD8 T cells are recruited to the lung in immunized mice that are protected against a lethal challenge with A66 (24), we determined the activation state of T cells in lungs by detecting CD62L and CD44 surface markers in order to distinguish between naive ($CD62L^{hi}CD44^{lo}$) and activated ($CD62L^{lo}CD44^{hi}$) T cells. No significant difference in the absolute number of naive CD4 (Fig. 9A) or CD8 (Fig. 9C) T cells was observed in the lung. However, there were significantly more activated CD4 (Fig. 9B) and CD8 (Fig. 9D) T cells in the lung of B7x−/− than Wt mice, with B7x−/− mice having more than two times the number of cells. We also analyzed cytokines in the whole lung and found a significant, although slight, increase of IL-2 in the whole lung of B7x−/− mice at 12 hours post-infection (Fig. 9E), but there was no difference in IL-4, IL-17A (Fig. 9E), or IFN-γ (Fig. 5). To support the importance of the role of T cells in controlling infection in our B7x−/− mice, we used B7x−/−Rag1−/− mice that lack mature T cells. B7x−/

−Rag1−/−, B7x−/−, and Wt mice were challenged with lethal *S. pneumoniae* and at 12 hours post-infection, as seen in prior analyses (Fig. 3A), B7x−/− mice had significantly reduced lung bacterial burden by this time point (Fig. 9F). However, in the B7x−/−Rag1−/− mice this reduction was no longer observed and the mice had significantly higher bacterial burdens than the B7x−/− that were comparable to those in the susceptible Wt mice (Fig. 9F). These results suggest that the resistance of B7x−/− mice to lethal pulmonary infection may be due to an increase in activated T cells in the lung at early time points post-infection.

Discussion

One of the most intriguing characteristics of B7x is that its mRNA expression is much higher in peripheral non-lymphoid tissues than lymphoid tissues (3, 6). In previous studies, we could not detect B7x protein expression on any cells of hematopoietic origin under a variety of *in vitro* stimulatory conditions or in various *in vivo* disease models (6). Our previous study revealed that lungs have the highest level of B7x mRNA (3) and we now extended this finding by confirming B7x protein was expressed on the bronchial epithelium of the lung, but not in the spleen. These results are consistent with clinical observations that B7x protein is expressed in human lung tissue (10) and abundantly expressed by non-small-cell lung cancer (32). Interestingly, patients with B7x positive non-small-cell lung tumors have fewer infiltrating lymphocytes and increased lymph node metastasis (32). Taken together, these results suggest lung-expressed B7x may contribute to disease progression by attenuating T cell function in the lung. To examine this question further, we investigated the effect of B7x on the outcome of a lethal pulmonary infection.

We found that B7x−/− mice were significantly more resistant to a lethal pulmonary infection with *S. pneumoniae* (ST3, strain A66) than Wt mice and that this was accompanied by lower bacterial burdens in the lung, blood, and spleen and markedly less lung pathology. All Wt mice died within four days following infection, while a significant proportion of B7x−/− mice survived indefinitely. To determine if the absence of B7x conferred resistance to systemic infection as well, we infected mice intraperitoneally with a lethal dose of *S. pneumoniae*. However, we found that in this scenario, B7x−/− mice were no longer resistant to infection. Based on phylogenetic analysis, B7 proteins can be divided into three groups and group III includes B7-H3 and B7x (3). Like B7x, B7-H3 mRNA is expressed in the lung (21, 22). However, the physiological function of B7-H3 on T cell proliferation and cytokine production remains controversial (33, 34). B7-H3 binds activated T cells, leading to costimulation in some cases and to coinhibition in others. Similarly, studies in B7-H3−/− mice support a costimulatory role in some disease models and a coinhibitory role in others (23, 35). We infected B7-H3−/− mice intranasally with the same lethal dose of *S. pneumoniae*, but found the mice were not resistant. Therefore, B7x, but not its closest homolog B7-H3, plays an important role in disease progression in a lethal pulmonary infection.

When *S. pneumoniae* infects the lung the first immune cells it encounters are the resident alveolar macrophages, with subsequent recruitment of neutrophils that help phagocytose the bacterium and clear infection (16). Although recruited neutrophils can enhance bacterial clearance, an overly exuberant inflammatory response can damage host tissue, with increasing morbidity and mortality (18, 25, 28, 31). A damaging neutrophil response has been associated with lethality in murine *S. pneumoniae* infection, which was accompanied by an increase in proinflammatory factors, particularly IL-6 (18, 28, 31). Similarly, Wt mice in our model exhibited elevated levels of proinflammatory cytokines and chemokines and succumbed rapidly to pulmonary infection. These mice also had severe necrotizing neutrophilic inflammation that was located in perivascular spaces and associated with tissue damage in the lung and dissemination of bacteria to the blood and spleen. In contrast, B7x−/

− mice had lower levels of cytokines and mild lung inflammation that was limited to the alveolar space and no significant bloodstream dissemination. Consistent with their severe lung pathology, Wt mice had significantly more neutrophils in their lungs at 12 hours post-infection. While our data show that *S. pneumoniae*-infected B7x−/− mice exhibited a resistance phenotype and had a reduced neutrophil response in lungs, another study shows that B7x−/− mice infected intraperitoneally with *L. monocytogenes* exhibit enhanced survival and an increased neutrophil response (15). In this infection model, B7x inhibits development of neutrophils from bone marrow progenitors and upon challenge with *L. monocytogenes* the absence of B7x allows for a more numerous protective neutrophil response, which controls bacterial growth and leads to enhanced survival in their B7x−/− mice (15). However, at 12-hours post-infection in our *S. pneumoniae* model neutrophils appear to be detrimental to the host and at 6 hours post-infection, before a difference in lung bacterial burden is observed between B7x−/− and Wt mice, we saw no difference in the neutrophil response. This could be due to the different immune responses initiated by *S. pneumoniae* and *L. monocytogenes* and the sight of infection.

Following lung infection with *S. pneumoniae* in mice, the bacterial burden usually stagnates until approximately 12 hours post-infection after which it increases (16). We found that B7x −/− mice had markedly lower bacterial burdens in their lungs than Wt mice. At six hours post-infection B7x−/− and Wt mice had comparable CFU in the lungs. By 12 hours Wt mice maintained a similar CFU that was significantly elevated over the following 24 hours. While CFU in Wt mice at 12 post-infection hours were comparable to those at 6 hours, B7x−/− mice had significantly fewer lung CFU at this time. Natural antibodies to *S. pneumoniae* are important for resistance to infection (29, 30, 36). Despite early control of infection in B7x−/ mice, these mice did not have elevated levels of natural antibody to *S. pneumoniae* or an increase in serum-mediated phagocytosis by alveolar macrophages or neutrophils.

Analysis of the immune infiltrates in lungs of intranasally infected mice revealed different cellular profiles. B7x−/− mice, which exhibited a resistance phenotype, had fewer neutrophils and more activated T cells, whereas Wt mice, which exhibited a susceptible phenotype, had more neutrophils and fewer T cells. We supposed that the control of infection in the B7x−/− mice may be due to the enhanced T cell response, and this appears to be supported by the inability of the B7x−/−Rag1−/− mice, which lack mature T cells, to reduce bacterial burdens by 12 hours post-infection as the B7x−/− mice do. T cells have been shown to be important for bacterial clearance in colonization models (37–40), but their role in pneumonia is only recently emerging (18, 19). HIV-infected individuals are more susceptible to *S. pneumoniae* pneumonia, which is associated with decreased (41) or defective (42) T cells. Murine studies show that CD4 T cells accumulate in the lung following infection with *S. pneumoniae* in a manner dependent on pneumolysin, an important virulence factor of the bacterium (19, 43). Several studies have demonstrated a role for CD4 T cells in murine models as mice lacking these cells are more susceptible to pneumonia (19) or colonization with *S. pneumoniae* (40) and are not protected against colonization when challenged following vaccination (37, 38, 44). However, CD4 T cells are also shown to be detrimental or dispensable in other pneumococcal pneumonia models (18, 20). In addition, CD8 T cells are shown to be critical for resistance to ST3 *S. pneumoniae* pulmonary infection as mice lacking these cells are more susceptible to death and are not protected in a vaccine challenge model (18, 45). It is currently unclear how CD4 or CD8 T cells protect mice from respiratory infection with *S. pneumoniae*. In the case of CD4 T cells it appears that the mechanism of protection may be through production of IL-17, which recruits neutrophils, as mice lacking IL-17 are more susceptible to infection with ST6B (46) and colonization with ST23F (47), although it is dispensable in a ST3 pneumonia model (18). In the ST3 model in which CD8 T cells are required, IFN-γ (18, 48) and perforin (18) are each also required for the resistance phenotype. While IFN-γ could work by activating

macrophages and increasing their killing functions (49), perforin might act by enhancing extracellular killing of *S. pneumoniae* as it, in addition to another cytotoxic factor produced by CD8 T cells, granzyme B, is capable of killing extracellular *Mycobacterium tuberculosis* (50, 51). The enhanced T cell response and survival of B7x−/− mice provides *in vivo* support that B7x inhibits T cell responses, specifically in the lung, which contributes to the lethality of pulmonary *S. pneumoniae* infection.

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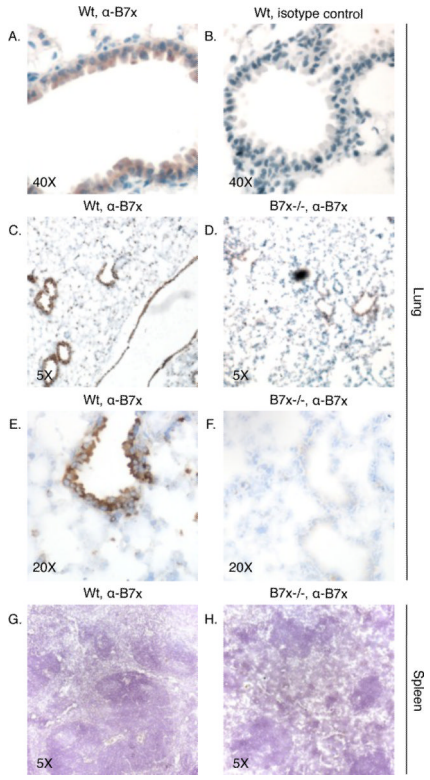

Figure 1. B7x protein is expressed in lung tissue

Lung tissue from Wt (A–C, E) and B7x−/− mice (D, F) was stained with anti-B7x (A, C–F) or isotype IgG control (B) to detect B7x protein expression. In lung, B7x protein expression was localized to bronchial epithelial cells. Wt (G) and B7x−/− (H) spleen was also stained for B7x, but no expression was detected. Hematoxylin staining of nuclei in images is blue, Ab-specific staining of B7x is brown. Representative of two experiments.

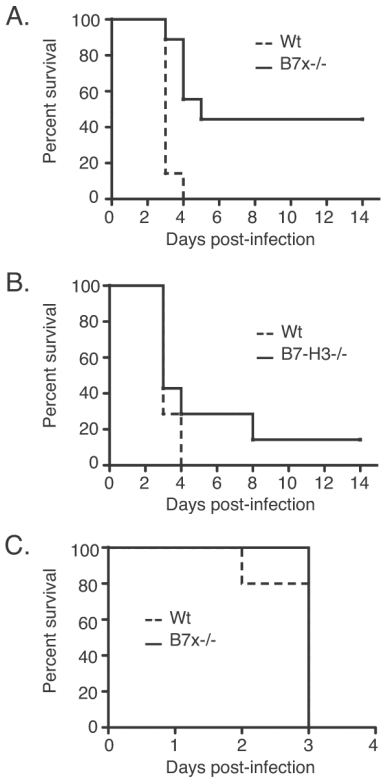

Figure 2. B7x deficient mice are more resistant to lethal pulmonary infection

(A,B) Mice were infected intranasally with 1×10^5 CFU of *S. pneumoniae* serotype 3 strain A66 and survival was monitored daily. (A) B7x−/− C57BL/6 (n = 9) and C57BL/6 Wt (n = 7) survival curve, (B) B7-H3−/− C57BL/6 (n = 7) and C57BL/6 Wt (n = 7) survival curve. (C) B7x−/− C57BL/6 and C57BL/6 Wt mice were infected intraperitoneally with 1×10^5 CFU and survival was monitored daily. **$p < 0.01$. B7x−/− intranasal infection survival representative of four separate experiments. B7x−/− intraperitoneal and B7-H3 intranasal infections representative of two separate experiments.

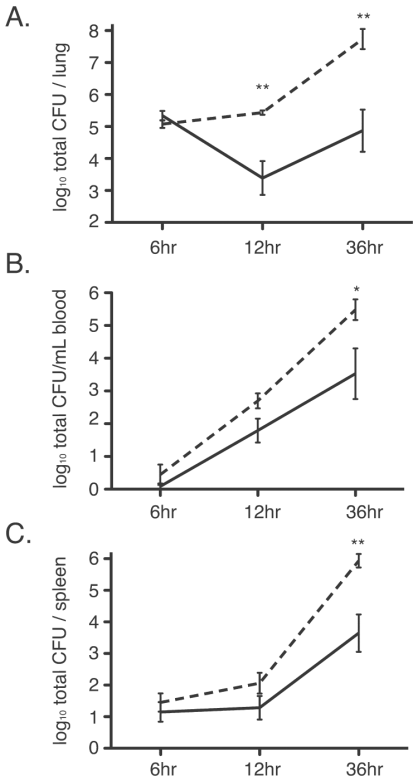

Figure 3. B7x deficient mice have reduced bacterial burdens

B7x−/− (n = 6) and Wt (n = 6) mice were infected with 1×10^5 CFU of *S. pneumoniae* serotype 3 strain A66 and bacterial burdens were analyzed in homogenized whole tissues. (A) In lungs, B7x−/− mice had significantly lower bacterial burdens at 12 hours post-infection, which continued to 36 hours post-infection. B7x−/− mice also had lower bacterial dissemination into and burden in the (B) blood and (C) spleen, which reached statistical significance at 36 hours post-infection. $^{**}p < 0.01$ $^{*}p < 0.05$. Error bars represent SEM. Representative of three separate experiments.

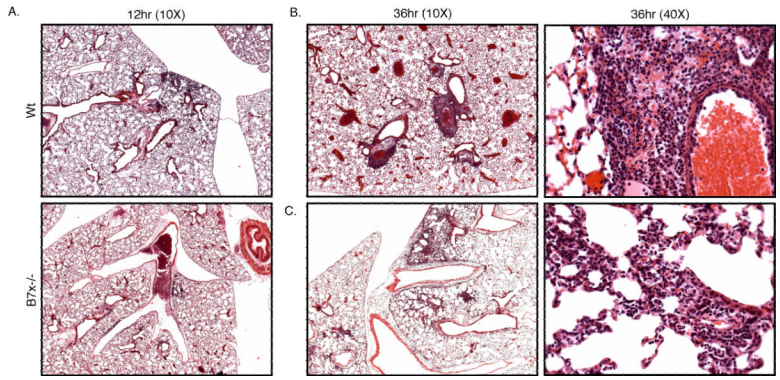

Figure 4. B7x deficient mice have less severe histopathology following lethal pulmonary infection

B7x−/− (n = 4) and Wt (n = 4) mice were infected with 1×10^5 CFU of *S. pneumoniae* ST3 strain A66 and lung histopathology was analyzed at (A) 12 and (B,C) 36 hours post-infection. H&E stained sections were imaged at 10× and 40× and inflammation was scored. (A) At 12 hours, both groups of mice had mild inflammation that was focused in the peribronchial and alveolar spaces of the lung. At 36 hours, (B) Wt mice had progressed to a severe inflammation that was centered around perivascular spaces, while (C) B7x−/− mice continued to have mild inflammation in the alveolar spaces. Representative images of four mice from each group shown. Representative of two separate experiments.

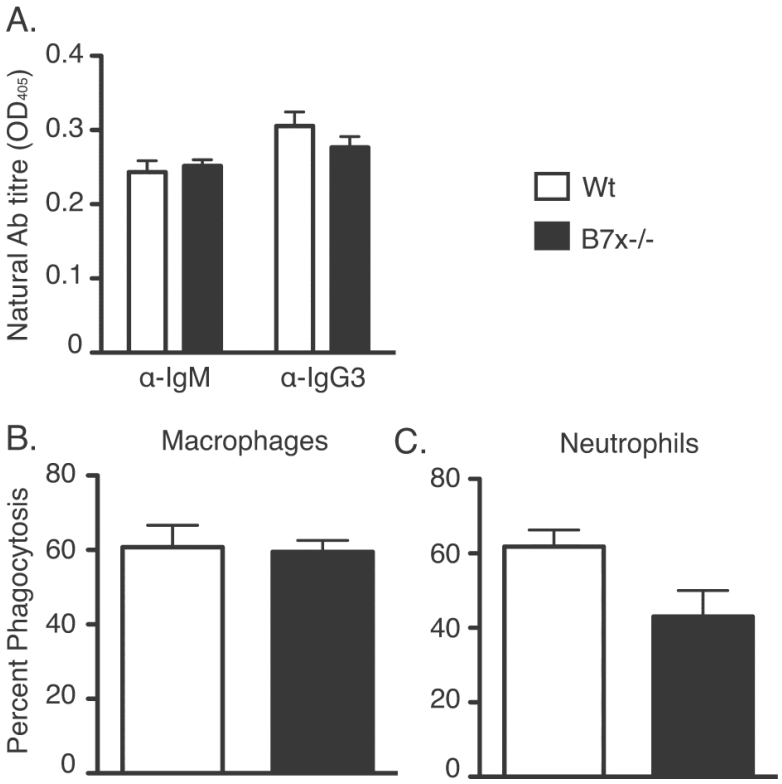

Figure 5. B7x deficient mice have reduced lung inflammatory cytokines and chemokines

B7x−/− (n = 6) and Wt (n = 6) mice were infected with 1×10^5 CFU of *S pneumoniae* ST3 strain A66 and cytokines and chemokines were analyzed in homogenized whole lung. For naive analysis, n = 4. Cytokine levels were analyzed in the lung in naive B7x−/− and Wt mice and at 12 and 36 hours post-infection. B7x−/− mice had a reduced inflammatory response in the lung, as indicated by significantly lower amounts of IL-6 and TNF-α; there was no significant difference in IL-10 and IFN-γ. The lungs were also analyzed for levels of the chemokines MCP-1, KC, MIP-1α, and MIP-1β, which were all significantly reduced in the B7x−/− mice; there was so significant difference for RANTES. $^{**}p < 0.01$ $^{*}p < 0.05$. Error bars represent SEM. For analysis of naive mice four mice were used per group. Representative of three separate experiments.

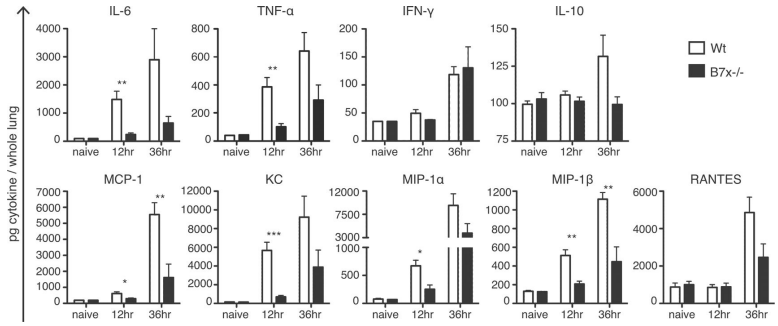

Figure 6. B7x deficient mice have reduced serum inflammatory cytokines and chemokines

B7x−/− (n = 6) and Wt (n = 6) mice were infected with 1×10^5 CFU of *S pneumoniae* ST3 strain A66 and cytokines and chemokines were analyzed in the serum. For naive analysis, n = 4. Cytokine levels were analyzed in the serum of naive B7x−/− and Wt mice and at 12 and 36 hours post-infection. B7x−/− have reduced serum cytokines following infection and significantly lower levels of IL-6 at 12 hours, TNF-α at 36 hours, IFN-γ at 12 hours, and IL-10 at 36 hours. B7x−/− mice also have reduced levels of the chemokines KC, MIP-1α, and MIP-1β, and RANTES, but not MCP-1. $^{**}p < 0.01$ $^{*}p < 0.05$. Error bars represent SEM. For analysis of naive mice four mice were used per group. Representative of three separate experiments.

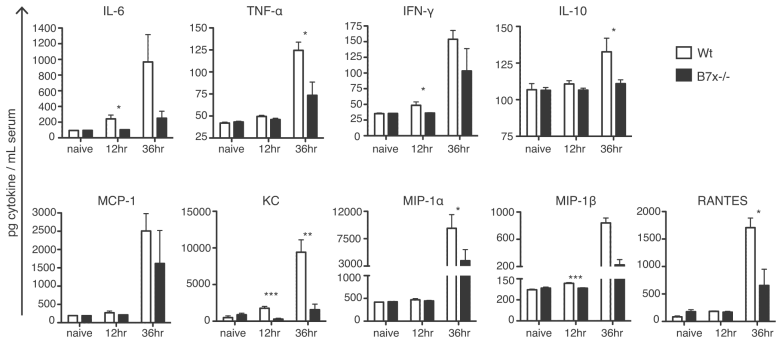

Figure 7. Absence of B7x does not affect natural antibody levels or phagocytosis by neutrophils or alveolar macrophages

(A) Sera from naive B7x−/− and Wt mice were analyzed for natural antibodies, IgM and IgG3, against whole, heat killed *S. pneumoniae* by ELISA. (B) Alveolar macrophage and (C) neutrophil mediated phagocytosis was tested against *S. pneumoniae* in an *in vivo* phagocytosis assay in B7x−/− and Wt mice. PKH-26-labeled *S. pneumoniae* was delivered intranasally and BALF was collected after 30 minutes and analyzed by FACS. No pair of values were significant for the phagocytosis experiments. Data representative of three separate experiments for natural antibodies and one of two experiments for phagocytosis.

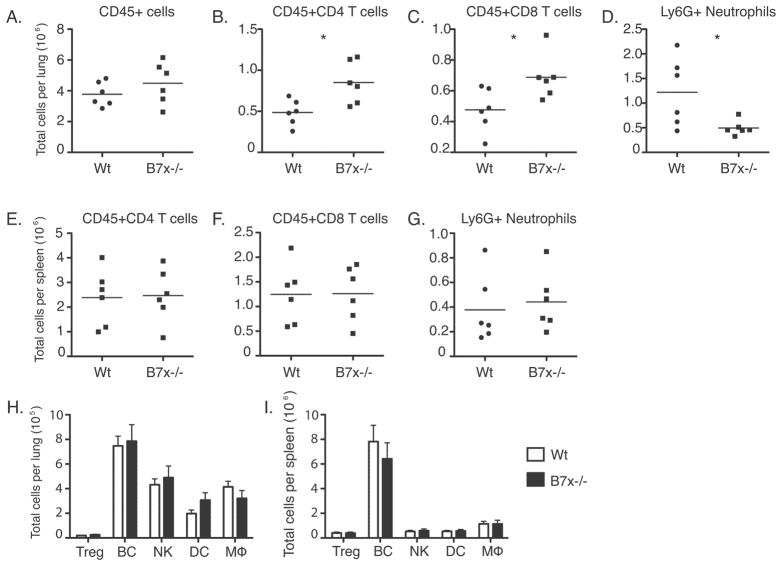

Figure 8. B7x deficient mice have more T cells and less neutrophils in their lungs at 12 hours post-infection

B7x−/− C57BL/6 (n = 6) and C57BL/6 WT (n = 6) mice were infected with 1×10^5 CFU of *S. pneumoniae* serotype 3 strain A66 and immune cell infiltration was analyzed by FACS at 12 hours post-infection. B7x−/− mice had significantly higher numbers of (B) CD4+ and (C) CD8+ T cells in their lungs, but significantly lower numbers of (D) neutrophils. (E–G) There was no significant difference for these cell types in the spleen. There was no significant difference in the numbers of Tregs, B cells, NK cells, dendritic cells, or macrophages in either the (H) lung or the (I) spleen. *$p < 0.05$. Error bars represent SEM. Representative of six separate experiments.

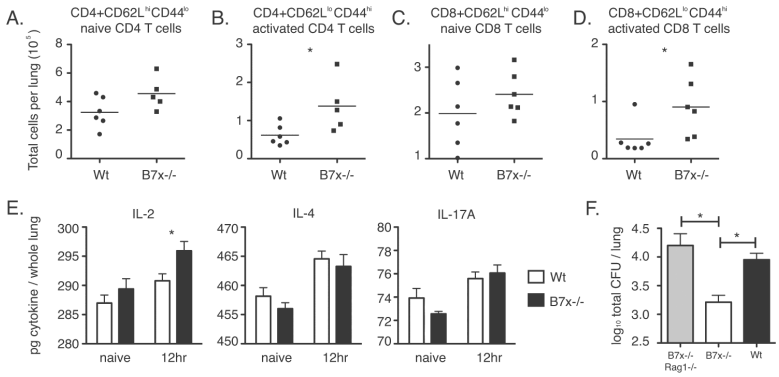

Figure 9. B7x deficient mice have more activated T cells at 12 hours post-infection

B7x−/− (n = 6) and Wt (n = 6) mice were infected with 1×10^5 CFU of *S. pneumoniae* ST3 strain A66 and (A–B) CD4 and (C–D) CD8 T cell populations were analyzed by FACS for an activation phenotype. Both (B) CD4 and (D) CD8 T cell populations had significantly higher numbers of activated cells ($CD62L^{lo}CD44^{hi}$) in their lungs. (E) B7x−/− mice had significantly higher IL-2 in whole lung, but no difference was seen in levels of IL-4 or IL-17A. 12 hours following infection, lung bacterial burdens were analyzed in B7x−/−Rag1−/−, B7x−/−, and Wt. As previously found (Figure 3A), B7x−/− had significantly lower CFU however in the B7x−/−RAG1−/− that lack T cells this reduced burden is no longer observed and these mice have significantly higher CFU than B7x−/− that is similar to the Wt mice. *$p < 0.05$. Error bars represent SEM. FACS data representative of three separate experiments, cytokine data representative of two separate experiments, CFU quantification representative of two separate experiments.

Table 1

Histopathological scoring of lungs from infected Wt and B7x−/− mice at 12 and 36 hours post-infection.

	12 hours							
	Wt				B7x −/−			
Mouse →	1	2	3	4	1	2	3	4
pneumonia, neutrophilic	1	2	1	1	1	1	1	1
Infiltrate, perivascular, neutrophilic	0	1	0	1	1	1	0	0

	36 hours							
	Wt				B7x −/−			
Mouse →	1	2	3	4	1	2	3	4
Inflammation, perivacular, neutrophilic & necrotizing	2	4	4	2	0	0	0	3
pneumonia, neutrophilic	0	0	0	0	0	1	1	1
Pleuritis, neutrophilic & necrotizing	2	1	1	0	0	0	0	0

The scoring refers to the images in Figure 4. Grading: 0 = no finding, 1 = minimal lesion, 2 = mild lesion, 3 = moderate lesion, 4 = marked lesion, 5 = severe lesion. Four mice were scored per group for each time point. Representative of two separate experiments.