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Neonatal Irradiation Sensitizes Mice to Delayed Pulmonary Challenge

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Abstract

Significant differences exist between the physiology of the immature, neonatal lung compared to that of the adult lung that may affect acute and late responses to irradiation. Identifying these differences is critical to developing successful mitigation strategies for this special population. Our current hypothesis proposes that irradiation during the neonatal period will alter developmental processes, resulting in long-term consequences, including altered susceptibility to challenge with respiratory pathogens. C57BL/6J mice, 4 days of age, received 5 Gy whole-body irradiation. At subsequent time points (12, 26 and 46 weeks postirradiation), mice were intranasally infected with 120 HAU of influenza A virus. Fourteen days later, mice were sacrificed and tissues were collected for examination. Morbidity was monitored following changes in body weight and survival. The magnitude of the pulmonary response was determined by bronchoalveolar lavage, histological examination and gene expression of epithelial and inflammatory markers. Viral clearance was assessed 7 days post-influenza infection. Following influenza infection, irradiated animals that were infected at 26 and 46 weeks postirradiation lost significantly more weight and demonstrated reduced survival compared with those infected at 12 weeks postirradiation, with the greatest deleterious effect seen at the late time point. The results of these experiments suggest that radiation injury during early life may affect the lung's response to a subsequent pathogenic aerial challenge, possibly through a chronic and progressive defect in the immune system. This finding may have implications for the development of countermeasures in the context of systemic radiation exposure.

INTRODUCTION

A mass casualty event involving radiological or nuclear materials will likely affect a broad spectrum of the population. However, the response of children to systemic radiation-induced damage has been a relatively unexplored area. The concept that children (developing

organisms) have altered susceptibility to injury is not a novel one, with the majority of researchers believing that this population may be at greater risk due to increased sensitivity (1, 2). Indeed, we previously reported differential responses to radiation between the adult and neonatal animal with respect to lung response (3). We therefore hypothesized that, as a result of unique windows of developmental sensitivity, the lung may be dramatically affected if injured during maturation, an outcome that we believe extends postnatally. Furthermore, if this specific differential effect were exhibited as an apparent change in radiation sensitivity, this finding would suggest that the pediatric population may be at an altered (likely increased) risk for developing radiation-induced pulmonary late effects. The majority of the supporting data come from therapeutic (i.e., fractionated, localized) radiation fields (4). With respect to whole-body irradiation (WBI), the most likely form of exposure associated with a terrorist event, much of the available human data are limited to small follow-up studies of children who have received a bone marrow transplant. Interestingly, many report late developing pulmonary disease in these patients (5, 6).

We hypothesized that relatively low-level early life WBI exposure will lead to increased sensitivity to environmental challenges during adulthood. To address this hypothesis, we irradiated 4-day-old mice with 5 Gy WBI, followed by infection with influenza virus at later time points. The effect of radiation on morbidity and mortality, innate versus adaptive immune cell recruitment, cytokine expression, and viral clearance were analyzed.

MATERIALS AND METHODS

Animals

C57BL/6J mice (4 days of age) were obtained from an in-house breeding colony. Pups were kept with dams until weaning at 21 days of age, then grouped by sex and maintained in groups of five mice per cage. All animals were kept under filter caps in pathogen-free rooms and were supplied standard laboratory diet and water *ad libitum*. Mice were sacrificed at 14 days post-influenza infection and tissues were collected for analysis. Our Institutional Animal Care and Use Committee approved all treatment protocols.

Radiation

Animals were placed in plastic jigs by litter group and received 5 Gy WBI from a ^{137}Cs γ -ray source operating at a dose rate of approximately 2 Gy/min. Age-matched control animals were sham irradiated and were maintained under identical conditions for the course of the experiment.

Infection

At 12, 26 or 46 weeks postirradiation (PI), sham and irradiated animals were intranasally infected under anesthesia (Avertin, 2,2,2-tribromoethanol; Sigma-Aldrich, St. Louis, MO) with 120 HAU influenza virus in 25 μl sterile PBS. Mock-infected controls received 25 μl of sterile PBS alone. Briefly, on gestational day 10, viable, fertilized chicken eggs were inoculated with 100 μl of sterile Hank's balanced salt solution containing 10 mM Hepes and 0.05 hemagglutinating units (HAU) of influenza virus. Infected eggs were incubated for 48 h at 37°C followed by refrigeration overnight at 4°C. Allantoic fluid was harvested under aseptic conditions, centrifuged and immediately frozen at -80°C until use. The titer of the allantoic fluid was determined by hemagglutination of chicken red blood cells. After infection, animal survival and body weight was monitored for two weeks (7).

Viral Titer

Madin-Darby Canine Kidney (MDCK) cells were used for the viral titer assay. MDCK cells were maintained in MDCK media [MEM media with Earle's salts and L-glutamine

(Invitrogen, Carlsbad, CA) supplemented with 100 U/ml penicillin/100 µg/ml streptomycin (Invitrogen), 0.1 mM nonessential amino acids (Invitrogen), 1 mM sodium pyruvate (Invitrogen), and 10% fetal bovine serum. For the assay, MDCK cells were plated in flat-bottomed 96-well plates at a density of 5×10^4 /well in 100 µl of MDCK media containing 0.01% bovine serum albumin (BSA). Plated cells were placed in a 37°C incubator until ready for use. For sample preparation, the entire left lung lobe was homogenized in 1.2 ml cold MDCK media containing 0.1% BSA. Homogenates were centrifuged for 10 min at 2,100 rpm. Supernatants were diluted from 1:25 to 1:51,200 in MDCK media containing 0.01% BSA. One hundred microliters of each dilution was transferred onto the MDCK cells. Cultures were incubated overnight at 37°C, 5% CO₂. On the following day, 50 µl of MDCK media containing 0.01% BSA and 50 µg/ml trypsin was added to each well. The cultures were incubated for an additional 2 days. After the incubation period, flat-bottomed plates were centrifuged at 1,000 rpm for 2 min. One hundred microliters of supernatant from each well was transferred to a round-bottomed 96-well plate, and 100 µl of a 1% red blood cell suspension in phosphate buffered saline (PBS) (v/v) was transferred onto the cells in the round-bottomed plate. Hemagglutination of red blood cells was assessed after 45 min. Virus concentration was calculated, as described previously (8). The assay end point was taken as the dilution that would contain 1 HAU and is the dilution factor halfway between the two dilutions in which agglutination was lost. Virus concentration was calculated in HAU/ml by dividing the appropriate dilution factor by the volume of sample in the well.

Histology

After sacrifice, the animals' lungs were inflated using zinc-buffered formalin (Z-fix; Anatech LTD, Battle Creek, MI) with a gravity perfusion apparatus. Tissues were post-fixed for 16–24 h in Z-fix. Tissues were dehydrated through graded alcohols, cleared through several changes of xylene and infiltrated with paraffin.

Immunohistochemistry

Five micrometer paraffin lung sections were incubated overnight at 60°C and then quickly transferred to xylene. Lung sections were progressively hydrated then transferred to DAKO antigen retrieval solution (Dakocytomation) and heated at 96°C for 30 min. Slides were cooled for 20 min at room temperature, rinsed several times with deionized water, and transferred to PBS. To prevent nonspecific binding, lung sections were blocked with 5% normal donkey serum (Jackson ImmunoResearch Laboratories) and 10 µg/ml Fc block (anti-mouse CD16-CD32, 24G2) for 30 min in a humid chamber. Then, without washing, primary antibodies were added to the slides and incubated overnight at room temperature. Goat anti-mouse proliferating cell nuclear antigen (PCNA, clone C-20; Santa Cruz Biotechnology, Santa Cruz, CA) was used to detect proliferating cells, peanut agglutinin-FITC (PNA-FITC, L7381; Sigma,) to detect special carbohydrates expressed characteristically by germinal center B cells, and B220-APC (RA3-6B2, BD-Pharmingen, San Jose, CA) to detect B cells. Primary antibodies were visualized with donkey anti-goat and conjugated to alexa fluor 568 (A11057; Invitrogen Life Sciences) to detect PCNA-expressing cells. Rabbit anti-FITC, conjugated to alexa fluor 488 (A11090; Invitrogen Life Sciences) was added to amplify the signal of PNA-FITC, and no secondary antibody was required to amplify B220-APC. After incubating slides with secondary antibodies for 2 h at room temperature, they were washed several times in PBS and mounted with Slow Gold antifade with DAPI (4,6-diamidino-2-phenylindole, S36938; Invitrogen Life Sciences). Pictures were taken with a 20× lens from a Zeiss Axioplan 2 microscope (Carl Zeiss Microscopy, North America, Peabody, MA) and were recorded with a Zeiss AxioCam digital camera.

Morphometric Analysis of Lymphoid Follicles

All compact inflammatory cell infiltrates containing predominantly lymphocytes were outlined with an automated tool of the Axioplan Zeiss microscope in each lobe. Lymphoid follicles of 4–5 lobes, collected from different mice were measured in each group. Individual values were used to calculate the average size of lymphoid follicles and area was expressed in squared microns. Morphometric analysis was performed in a blinded fashion. Representative pictures of lymphoid follicles were taken at 200× magnification.

Lavage Cell Analysis

Bronchoalveolar lavage (BAL) was performed at 7 days post-exposure to infection (or saline). Pooled lavages were centrifuged at 400g for 10 min and the cell pellet was used to determine total lavagable cell numbers, cell viability (trypan blue exclusion) and cell differential. BAL fluid cells were quantified by hemocytometer counting and cell viability was determined by the exclusion of trypan blue dye. Cell differentials were performed on cytocentrifuge preparations that were fixed with methanol and stained with Diff Quik (Sigma).

Quantitative Real-Time Polymerase Chain Reaction (QRT-PCR)

Total RNA was isolated from lung tissue using TRIzol Reagent (Life Technologies, Grand Island, NY) according to the manufacturer's instructions. Each frozen lung lobe (50–100 mg) was homogenized in 1 ml of TRIzol Reagent. Each final RNA pellet was resuspended in 50 µl of diethylpyrocarbonate-treated water. The RNA concentration and purity was quantified using the Gene Quant RNA/ DNA calculator (Pharmacia Biotech, Piscataway, NJ). Total mouse lung mRNA (1 µg) was used to synthesize first strand cDNA following the GeneAMP (Applied Biosystems, Carlsbad, CA) kit protocol. QRT-PCR was set up using Assays-on-Demand Primer/ MGB Probes (Applied Biosystems) for MCP1, CCSP and L32. Standard curves were made by making a mixed pool of all of the cDNA samples and making dilutions. All values for chemokines were normalized to the L32 for each sample.

Statistics

Results of experiments were evaluated by ANOVA for independent measures, followed by Fisher's test of significance in multiple group comparisons. The two-tailed level of significance for all analyses was set at $P < 0.05$.

RESULTS

Body Weight and Survival

The severity of influenza A virus infection was assessed by monitoring weight loss and mortality after infection. For mice infected at 12 weeks PI, loss in body weight (BW) was observed shortly after infection, with no differences observed between irradiated and sham groups with respect to either degree of weight loss or kinetics of recovery, and no mortality was observed in any group (data not shown). In mice infected at 26 weeks PI, both sham and irradiated influenza A virus-infected animals exhibited significant weight loss compared to the saline-treated, noninfected control groups beginning at day 3 post-infection. Over the course of the infection, the unirradiated but infected animals lost 20% of their initial BW, began to regain weight after day 7 post-infection, and recovered to near control levels by day 14 (Fig. 1A). In contrast, irradiated and infected animals lost 30% of their initial BW, began to regain weight by day 8, but only regained approximately 10% BW by 14 days post-infection. Consistent with this exacerbated morbidity, decreased survival (80%) was observed in the infected irradiated mice (Fig. 1B).

For mice infected at 46 weeks PI, both sham and irradiated animals had significant weight loss by day 2 post-infection compared to the noninfected control groups. Over the course of the infection, irradiated mice lost 35% of their baseline BW, whereas unirradiated animals lost only 25% BW (Fig. 2A). Unirradiated animals began to regain weight after day 8, and recovered to approximately 85% BW of noninfected controls. In contrast, the irradiated animals continued to lose weight through day 9 post-infection and had not regained BW by day 14. At this time point, mortality in the irradiation-infection group was approximately 50% (Fig. 2B).

Assessment of Innate Immune System and Inflammatory Response

Due to the known acute and chronic inflammatory effects of radiation, we assessed whether neonatal radiation resulted in changes in the accumulation of innate inflammatory cells in the adult mouse lungs. Mice that were infected at 26 weeks PI exhibited no significant differences in the numbers of macrophage polymorphonuclear neutrophil (PMN) or lymphocytes in the BAL of sham and infected versus irradiated plus infected animals measured at 7 days post-infection. However, for mice that were infected at 46 weeks PI, lymphocyte and macrophage BAL cell numbers were slightly lower in the irradiated and infected animals compared to sham-infected mice, and PMN numbers were significantly reduced (Fig. 3).

To test whether changes in leukocyte recruitment could be traced to the altered production of inflammatory mediators, we next evaluated the expression of monocyte chemo-attractant protein (MCP)-1/CCL2 mRNA, a chemotactic signal for monocytes associated with inflammatory disorders in the lung. When mice were infected at 26 or 46 weeks PI, significant increases in MCP-1 mRNA abundance were detected in lungs from irradiated and infected mice compared to the sham and infected mice (Fig. 4A). Interestingly, both infected groups demonstrated significant age-related increases in levels of MCP-1 mRNA abundance relative to noninfected controls when infected at the later time points (26 and 46 weeks).

Analysis of Clara cell specific protein (CCSP/CC16) mRNA (the major secretory protein of the Clara cell) demonstrated a differential response compared to MCP-1. No significant changes in message abundance were observed in any group infected at 12 weeks PI. However, for mice infected at 26 and 46 weeks PI, a significant decrease in CCSP mRNA abundance was detected in infected groups relative to noninfected controls, with a greater and significant decrease seen in the irradiated and infected mice compared to the sham and infected mice (Fig. 4B).

Assessment of Adaptive Immune System

To assess whether neonatal irradiation had altered the adult animals' ability to respond to pathogenic challenge, the viral load in the lungs of infected mice was measured on day 7 post-infection by hemagglutination assay. An age-associated upward trend in viral load was detected in all groups, with slightly greater, though not significant, increases measured in the infected and irradiated animals (Fig. 5A). Assessment of the relative ability of these animals to generate specific antibodies indicated that the sham-irradiated mice showed a progressive reduction in influenza-specific IgG titers in the serum. However, this decline was not as drastic as was seen in the irradiated mice (Fig. 5B).

To further assess the impact of neonatal irradiation on the adaptive immune system, we evaluated the formation and organization of inducible Bronchus Associated Lymphoid Tissue (iBALT), since this tissue is known to be associated with local immune response in the lung (8). Lung sections stained with antibodies against PCNA, PNA and B220 were

examined for detecting active germinal centers containing proliferating B blasts (PCNA+PNA+B220+) and evaluating the stability of B cell follicles. In general, the sections from the noninfected mice, both sham and irradiated, demonstrated minimal B cell infiltration (white cells), preferential binding of PNA to lung parenchyma (green signal), and similar levels of homeostatic proliferation (red nuclei) (Fig. 6A–D). At 26 weeks PI, sham-infected mice had B cell follicles containing proliferating B cells (PCNA+PNA-B220+) (Fig. 6E), although there was no evidence of germinal center formation. In contrast, the lungs from irradiated and infected mice had bigger B cell follicles containing large proliferating B blasts, which were triple positive stained for PCNA, PNA and B220 (Fig. 6F). Mice that were sham-infected at 46 weeks PI exhibited small B cell follicles containing exclusively proliferating B cells, although germinal center B cells were no longer present (Fig. 6G). In contrast, lungs from irradiated and infected mice lacked B cells and contained big clusters of B220-PNA-PCNA+ proliferating cells (Fig. 6H).

Characteristically, iBALT is composed predominantly by lymphocytes that accumulate in the lungs as distinctive concentric and compact cell clusters. To assess the impact of neonatal irradiation on the average size of lymphoid follicles after influenza infection, we look for lymphocytic aggregates in the influenza-infected lung by using bright field microscopy. Although, at 26 weeks irradiated mice had bigger lymphoid follicles than did sham-irradiated mice, the lymphoid follicles had the smallest size in the group of irradiated mice infected with influenza at an older age (46 weeks) (Fig 7A). To confirm our visual findings, we measured the size of the lymphoid follicles and corroborated that lymphoid follicle size was considerably affected in the group of irradiated mice when they were infected with influenza at 46 weeks (Fig 7B).

Given that B lymphocytes are predominantly populating the iBALT structure, they are constantly exposed to pathogen stimulation that facilitates their cell proliferation and differentiation. To assess the activation and differentiation of B lymphocytes after influenza infection, lung sections were evaluated with antibodies against PCNA.

DISCUSSION

The results generated from this study demonstrate that WBI during the postnatal period causes the development of chronic and progressive subclinical damage in the lung. Furthermore, our data indicate that altered pulmonary response to a late-occurring influenza infection is due in part to a defective immune response operating within a chronically injured microenvironment. Taken in conjunction with our work in an adult model, these data show that irradiation differentially affects the developing lung relative to that of the adult, indicating that alternative strategies and mitigation treatments will need to be developed for this special population.

As noted previously, little research has been carried out in the field of pediatric lung response to systemic irradiation (5, 6). A 5 Gy WBI model was selected since recent studies from our group, using this regimen in the adult mouse, had indicated that there was minimal induction of histological alterations over the subsequent life span in the C57BL/6 strain (9). However, the same studies demonstrated that, following similar sublethal WBI doses, animals developed an exacerbated response to a delayed pulmonary challenge despite these doses being below the threshold for the development of radiation-induced lung late effects (9). This observation supported an earlier hypothesis proposed by Rubin and Casarett (10) that low radiation doses may induce chronic subclinical damage that can be revealed by subsequent injury. We therefore chose to challenge our mice with influenza A, a pulmonary pathogen, at delayed time points to assess any long-term susceptibility of the irradiated neonate lung to complications. Interestingly, we not only demonstrated an exacerbated

response to influenza that was characterized by sustained loss of body weight and increased mortality, but also demonstrated that this increased sensitivity appeared to be progressive.

Under normal conditions following infection with influenza, viral clearance is mediated by both innate and adaptive immune responses, which, if fully effective and functional, will lead to complete viral clearance from the lung. The results of these studies indicated that there were age-related increases in viral titers, but that these were further exacerbated in the irradiated groups at all three time points. Furthermore, our data demonstrated that there were defects in both arms of the immune system that worsened over time. For example, the cell differential counts were the same or lower for irradiated mice infected with influenza at 26 and 46 weeks compared to the sham group, despite increased levels of expression of MCP-1 mRNA. This suggests a reduction in the ability of inflammatory cells to recognize and/or respond to the infection-induced chemotatic signal, a possible defect that may be partly or wholly due to the systemic component of the WBI affecting the bone marrow. Indeed, although the time points used were outside the acute period classically associated with radiation-induced bone marrow effects, there is now growing evidence that bone marrow may exhibit long-term changes in functionality following WBI (11, 12).

With respect to the observed changes in the adaptive immune system, one explanation is that radiation accelerates immunosenescence, leading to age-associated changes with features that include a substantial decrease in the number of naïve lymphocytes as a result of a reduction in thymic output of T cells (13), fewer early progenitor B cells, and functionally incompetent memory lymphocytes (14). Indeed, many investigators have proposed that radiation accelerates aging (15, 16). A major immune defect in aged animals is the progressive decline in the function of CD4⁺ T cells (17), which leads to poor antibody responses due to impairment in the acquisition of a T follicular helper (TFH) phenotype. Thus, it is possible that radiation accelerates the aging process in T cells, alters the microenvironment of the lung or lymphoid organs, and ultimately leads to poor antibody responses.

This concept led us to further examine B cell physiology in our study, since we were unaware of any work describing either the effects of neonatal irradiation on the formation of pulmonary lymphoid structures after influenza infection or its impact on the generation of influenza-specific humoral immunity. Our data indicated that, although neonatal irradiation facilitates iBALT formation early in life, which results in higher antibody titers in the serum of irradiated mice infected at 12 weeks PI, it compromises the organization of iBALT later in life, which may correlate with the rapid and progressive decline in influenza-specific antibody titers of mice infected at 26 and 46 weeks PI. These observations are consistent with the proposed link between iBALT formation and local antibody production (18). However, the effects of radiation on the systemic immune response do not explain the specific sensitivity of the neonatal lung to radiation. It certainly has been shown by many investigators that the saccular stage of human lung development is vulnerable to inflammation-mediated injury, such as radiation, and that the lungs of a 4-day-old mouse neonate are indeed in the late saccular to early alveolar stage (19). In addition, our data clearly shows declining levels of CCSP mRNA. Clara cells are located principally in the epithelium of the proximal or central portions of the pulmonary acinus where their function is oriented toward protection of the respiratory tract. Of note is that the influenza virus has been shown to preferentially target epithelial cells (20), whereas the effects of radiation on this population are relatively unknown. Currently, it is unclear whether neonatal irradiation has reduced the Clara cell population *per se* or has affected their expression of CCSP. Interestingly, the biological function of CCSP is incompletely understood, although it has been shown to interact with multiple components of the inflammatory and coagulation cascades, with a possible attenuating role associated with inflammation (21). Moreover,

subsets of CCSP-expressing cells have been identified in both lung and bone marrow compartments and have been described as a progenitor/stem cell pool involved in airway regeneration and alveolar homeostasis. Radiation-induced damage within this compartment may, therefore, have profound downstream long-term effects on lung development. These findings are now being investigated further.

The altered response to infection supports our hypothesis that sublethal doses of systemic radiation delivered during early childhood can result in subclinical pulmonary injury. Furthermore, these observations suggest that an individual receiving radiation during postnatal lung growth will continue to be at risk for complications from pulmonary infections and that these risks will increase with age. However, our use of a 4-day-old mouse neonate, the lungs of which are equivalent to an immediately postnatal human, limits the applicability of this model. Further work is in progress to assess the effects of postnatal irradiation at later pre-weaning time points to fully characterize this model and allow for better extrapolation to the full range of developmental stages seen in the human pediatric population.

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FIG. 1.

Twenty-six week postirradiation percent survival and body weight. Line plots show the change in body weight (panel A) and survival (panel B) in the first 14 days following challenge with influenza A (or saline) at 26 weeks PI. Error bars indicate the standard error of the mean (SEM). * $P < 0.05$, $n = 13-16$ for virus infected groups, $n = 6$ for mock-infected groups.

FIG. 2.

Forty-six week postirradiation percent survival and body weight. Line plots show the change in body weight (panel A) and survival (panel B) in the first 14 days following challenge with influenza A (or saline) at 46 weeks PI. Error bars indicate the SEM. * $P < 0.05$, $n = 7-8$ for virus infected groups, $n = 3$ for mock-infected groups.

FIG. 3.

Cell differential counts in BAL at day 7 post-infection. Changes in macrophage, PMN and lymphocyte cell counts were measured in bronchoalveolar lavage fluid in the lungs of mice at 12, 26 and 46 weeks PI and 7 days post-infection. $n=3-7$.

FIG. 4. mRNA abundance of MCP-1 (panel A) and CCSP (panel B). QRT-PCR measuring CCSP or MCP-1 mRNA abundance in the lungs of mice at 12, 26 and 46 weeks PI at 14 days post-infection. All values were normalized to the constitutively expressed mRNA encoding L32. Each bar represents the mean for a minimum of three mice. * $P < 0.05$, $n = 3-7$.

FIG. 5.

Lung viral titer at 12, 26 and 46 weeks PI after infection in sham and irradiated mice (panel A) and accelerated decline in systemic levels of influenza-specific IgG in irradiated mice (panel B). Panel A: Titers were measured in the lung by hemagglutination of the blood cells. Limit of detection of the assay was 250 HAU/ml. Each point represents one animal within the group. Line represents the mean value for the group. $*P < 0.05$. Panel B: Serum from sham and irradiated mice was collected at 12, 26 and 46 weeks PI at 7 days post-infection. Influenza-specific IgG titers are expressed as an inverse of the dilution at a cutoff value of 1.5 (optical density) ($n = 3-7$, $P < 0.05$). Graph from one of two experiments with similar results is shown.

FIG. 6.

Neonatal irradiation compromises the stability of iBALT. Paraffin lung sections, collected at 26 weeks (panels A, B, E, F) and 46 weeks (panels C, D, G, H) PI, were stained with antibodies against PCNA (red), PNA (green) and B220 (white). Proliferating cells (PCNA +PNA-B220-) had an intense PCNA signal in the nuclei (red), infiltrating B lymphocytes exhibited a membrane white signal (PCNA-PNA-B220+), carbohydrates in the lung parenchyma strongly bound PNA (green) and large germinal center B cells (GCB) showed a triple positive stain for PCNA, PNA and B220. Lung sections from noninfected mice, both sham (panels A and C) and irradiated (panels B and D). Lung sections from infected mice, both sham (panels E and G) and irradiated (panels F and H). Representative images of two independent experiments are shown (200× magnification).

FIG. 7.

Impact of neonatal exposure to irradiation on the size of pulmonary lymphoid follicles formed during Influenza infection. Panel A: 5 μm formalin-fixed paraffin embedded lung sections were stained with H&E and pictures were taken with an Axioplan Zeiss microscope. Representative pictures are shown (200 $\times$ magnification). Subpanel a: 26 weeks sham + flu; subpanel b: 26 weeks irradiated + flu; subpanel c: 46 weeks sham + flu; and subpanel d: 46 weeks irradiated + flu. Panel B: Compact accumulations of lymphocytes were outlined with an automated tool of the Axioplan Zeiss microscope and results are shown as average size of lymphoid follicle expressed in squared microns. Representative pictures and results from two independent experiments are shown. $n=4-5$ mice per group, * $P=0.05$, ** $P=0.005$, *** $P=0.0005$.