Published in final edited form as:

Int J Radiat Biol. 2016 December ; 92(12): 754–765. doi:10.1080/09553002.2016.1222094.

Effects of IL-4 on Pulmonary Fibrosis and the Accumulation and Phenotype of Macrophage Subpopulations Following Thoracic Irradiation

Angela M. Groves¹, Carl J. Johnston^{1,2}, Ravi S. Misra¹, Jacqueline P. Williams², and Jacob N. Finkelstein^{1,2}

¹Department of Pediatrics M&D Neonatology, University of Rochester Medical Center, Rochester, New York

²Department of Environmental Medicine, University of Rochester Medical Center, Rochester, New York

Abstract

Purpose—Thoracic irradiation injures lung parenchyma, triggering inflammation and immune cell activation, leading to pneumonitis and fibrosis. Macrophage polarization contributes to these processes. Since IL-4 promotes pro-fibrotic macrophage activation, its role in radiation-induced lung injury was investigated.

Materials and methods—Lung macrophage subpopulations were characterized from 3–26 wk following exposure of WT and IL-4^{-/-} mice to 0 or 12.5 Gray single dose thoracic irradiation.

Results—Loss of IL-4 did not prevent fibrosis, but blunted macrophage accumulation within the parenchyma. At 3 wk following exposure, cell numbers and expression of F4/80 and CD206, an alternative activation marker, decreased in alveolar macrophages but increased in infiltrating macrophages in WT mice. Loss of IL-4 impaired recovery of these markers in alveolar macrophages and blunted expansion of these populations in infiltrating macrophages. CD206⁺ cells were evident in fibrotic regions of WT mice only, however Arg-1⁺ cells increased in fibrotic regions in IL-4^{-/-} mice only. Radiation-induced proinflammatory Ly6C expression was more apparent in alveolar and interstitial macrophages from IL-4^{-/-} mice.

Conclusions—IL-4 loss did not prevent alternative macrophage activation and fibrosis in irradiated mice. Instead, a role is indicated for IL-4 in maintenance of macrophage populations in the lung following high single dose thoracic irradiation.

Keywords

radiation; IL-4; macrophage; pulmonary fibrosis

Corresponding author: Angela M. Groves, Ph.D., Department of Pediatrics M&D Neonatology, University of Rochester Medical Center, 601 Elmwood Ave., Box 850, Rochester, NY 14642, angela_groves@urmc.rochester.edu, Phone: (585)275-1495, Fax: (585)756-7780.

Declaration of interest

The authors report no declarations of interest.

Introduction

Injury resulting from the direct exposure of DNA and other cellular components to ionizing radiation generates reactive oxygen species and modifies cellular oxidation/reduction processes, bringing about an inflammatory response in the exposed tissue that perpetuates the further generation of reactive species and drives the production and release of cytokines, chemokines and adhesion molecules that mediate the radiation induced damage responses (Johnston et al., 1996, Johnston et al., 2002, Johnston et al., 1998, Rubin et al., 1995, Schaeue et al., 2015). Following high total (single or fractionated) doses, this results in an accumulation of activated macrophages and lymphocytes in the irradiated tissue that in turn can regulate the inflammatory and wound healing response (Lehnert et al., 1991, Paun et al., 2015, Wesselius and Kimler, 1989). The regulatory mechanisms that mediate this injury response therefore evolve over the various phases of inflammation and repair. Macrophages are highly plastic, multifunctional cells that are adaptable to their microenvironment. In the lung, macrophages have been found to play an important role in the development of the acute and chronic outcomes of exposure to thoracic irradiation, pneumonitis and pulmonary fibrosis, respectively (Buttner et al., 1997, Coates et al., 2008, Rube et al., 2000).

Multiple macrophage populations exist in the lung to fulfill specific functions. Macrophages that exist in the alveolar space (AMs) are a long-lived resident population that is established in the lung during development, and which derives from yolk sac precursors (Yona et al., 2013). This population plays important roles in immune surveillance of the lung for pathogens and particles and is efficient at phagocytosis and antigen presentation (Hussell and Bell, 2014). Macrophages that reside in the interstitium (IMs) by contrast are a shorter lived population that play a role in regulation of immune responses (Schneberger et al., 2011). In addition, monocytes infiltrating into the lung from the circulation mature into macrophages in response to signals they encounter in their microenvironment, and can also contribute to lung macrophage pools during inflammation or after loss of resident populations (Epelman et al., 2014, Hashimoto et al., 2013, Landsman and Jung, 2007, Landsman et al., 2007). Single, high dose thoracic irradiation results in a depletion of local pulmonary macrophage populations over the subsequent days to weeks, that is then followed by their repopulation (Chiang et al., 2005, Hong et al., 2003). Infiltrating monocytes have been found to contribute to the replenishment of lung macrophage populations that have been depleted in response to radiation exposure (Hashimoto et al., 2013). In macrophages repopulating the lung, the manner in which these cells are replaced and the nature of radiation induced alterations in phenotype are not fully understood, but appears to be subpopulation dependent (Groves et al., 2015, Hong et al., 2003, Zhang et al., 2011).

The pleiotropic functions of macrophages reside in their ability to become differentially activated in response to factors they encounter in their local environment (Davies and Taylor, 2015, Guth et al., 2009, Hussell and Bell, 2014). Because their activation state is adaptable, this enables these cells to play different roles as mediators of inflammation and wound healing throughout the progression of the injury response (Italiani and Boraschi, 2014). The wide spectrum of multiple macrophage activation states can be broadly categorized: classical activation describes a phenotype that supports pro-inflammatory functions and alternative activation generates phenotypes with the ability to resolve inflammation and promote wound

healing responses (Mosser and Edwards, 2008). Signals that induce a classically activated phenotype include bacterial components and pro-inflammatory cytokines, such as IFN- γ . Classically activated macrophages are efficient at killing and removing pathogens and clearing infected or damaged cells. Alternatively activated macrophages are generated through stimulation by Th2 type cytokines, such as interleukin (IL)-4 and IL-13. Macrophages activated in this manner show increased production of growth factors and pro-fibrotic factors, important in returning the tissue to homeostasis (Gordon and Martinez, 2010, Murray, 2015).

Increased expression and production of IL-4 has been observed in the lung following thoracic irradiation, with the major source of this cytokine being of macrophage origin (Buttner et al., 1997, Xu et al., 2014). IL-4 not only induces an alternatively activated phenotype in macrophages, but is also a mediator of macrophage proliferation and accumulation within a tissue (Jenkins et al., 2011, Jenkins et al., 2013). Importantly, alternatively activated macrophages have been implicated in pulmonary fibrosis (Barkauskas and Noble, 2014, Gibbons et al., 2011, Lech and Anders, 2013). Therefore, in order to examine the role of IL-4 in the development of the clinical outcomes of radiation exposure, namely pneumonitis and pulmonary fibrosis, mice lacking IL-4 were exposed to 12.5 Gray (Gy) single dose thoracic irradiation and the responses of their pulmonary macrophage populations were examined. We hypothesized that IL-4 signaling in response to radiation exposure would influence the replenishment and maintenance of depleted macrophage populations and affect the development of radiation outcomes.

Materials and methods

Animals

IL-4^{-/-} mice (female, 6–8 wk of age) were generated on a C57BL/6 background and were bred at the University of Rochester. C57BL/6J (WT) mice were obtained from Jackson Laboratory (Bar Harbor, ME). Following arrival, animals were acclimated for one wk prior to experimentation, housed five per cage in microisolator units under pathogen-free conditions. All animals received standard animal care services from the University's Department of Laboratory Animal Medicine and vivarium staff, including standard laboratory diet and water *ad libitum* and a 12 hour light/dark cycle. The University Committee on Animal Resources approved all animal protocols.

Irradiation

Animals, restrained in plastic jigs, received a 12.5 Gy single dose thoracic exposure from a ¹³⁷Cs γ -ray source operating at a dose rate of approximately 2.2 Gy/min. Age and sex matched 0 Gy control mice were sham irradiated with identical handling. All mice were irradiated at identical time periods of the day to normalize any potential circadian influences. Identical numbers of IL-4^{-/-} and WT mice were exposed and collected at each time point with samples sizes ranging from 3–5 mice for each time point in both control and irradiated groups.

Sample Collection

Mice were euthanized at 3 wk (pre-pneumonitis), 8 wk (beginning of pneumonic period), 16 wk (post-pneumonitis), and 26 wk (beginning of fibrotic period) following irradiation. Lung digests were collected by instilling the lung with 1.8 units/ml dispase (Gibco Life Technologies, Grand Island, NY) in Dulbecco's Modified Eagle Medium (DMEM) (Gibco Life Technologies) and incubated at room temperature for 45 min. Lungs were next disrupted by mincing and the cell solution filtered through 100, 40, and 25 μ m cell strainers (Fisher Scientific, Waltham, MA) using DMEM plus 0.01% DNase-1 (Sigma, St Louis, MO) as a wash buffer, then transferred to DMEM plus 10% fetal bovine serum (FBS) (BD Biosciences, San Jose, CA). Alternatively, the left lobe of the lung was inflation-fixed in 10% zinc buffered formalin (Anatech, Battle Creek, MI) and paraffin embedded. Tissue sections (6 μ m) were prepared and stained with Gomori trichrome or Hematoxylin and Eosin (H and E) and examined by light microscopy. Images were acquired on an Olympus BX51 microscope (Olympus America, Center Valley, PA).

Magnetic Cell Sorting and Flow Cytometry

CD45+ myeloid cells were enriched in lung digest by incubating cells with a biotin anti-CD45 antibody (BD Pharmingen, San Jose, CA) followed by binding to BD IMag Streptavidin Particles (BD Biosciences) and placement on a Magna grIP magnet (Millipore, Billerica, MA). Unbound CD45- cells were then removed and DMEM plus 10% FBS was used to resuspend the CD45+ cells retained on the magnet. CD45+ cells were then counted using a hemocytometer and 1×10^6 cells were stained for flow cytometric analysis. Cells were first transferred to staining buffer (PBS plus 10% FBS). To block non-specific antibody binding, cells were incubated with antimouse CD16/CD32 Fc block (BD Pharmingen) diluted 1:500 in staining buffer for 10 min at 4°C. Surface staining was then performed for 30 min at 4°C using PerCP-Cy5.5-conjugated rat anti-mouse CD-11b (BD Pharmingen), Alexa Fluor 647-conjugated anti-mouse Ly6G, Brilliant Violet 605-conjugated anti-mouse CD206, Brilliant Violet 711-conjugated anti-mouse Ly6C, PE-conjugated anti-mouse CD45 (Biolegend, San Diego, CA), eFluor 450-conjugated antimouse CD11c, and PE/Cy7-conjugated anti-mouse F4/80 (eBioscience, San Diego, CA). Cells were washed and then stained with LIVE/DEAD fixable aqua dead cell stain kit (Life Technologies) for 15 min at 4°C, then washed and resuspended in 2.5% phosphate buffered formalin (Fisher Scientific). Flow cytometry was performed on an 18-paramater LSRII flow cytometer (BD Biosciences). Simply Cellular anti-mouse compensation standard (Bangs Laboratories, Fishers, IN) was incubated with 1 μ l of each detection antibody and used for single color positive controls. Data were analyzed using FlowJo software (FlowJo, Ashland, OR).

Immunohistochemistry

Lung sections were deparaffinized and antigen retrieval was performed by heating sections to 90°C for 20 min in Target Retrieval Solution (DAKO, Carpinteria, CA) using a water bath. Endogenous peroxidases were quenched using Peroxidized-1 (Biocare Medical, Concord, CA). To block non-specific binding, sections were next incubated with Rodent Block M (Biocare Medical) for 20 min at room temperature. Sections were then incubated with antibodies to either Mannose Receptor (CD206) (1:2500, Abcam, Cambridge, UK)

overnight at 4°C, Arginase-1 (Arg-1) (1:300, Proteintech Group, Chicago, IL) for 45 min at room temperature, or with monocyte chemotactic protein-1 (MCP-1) (1:1000, Abcam) overnight at 4°C, all diluted in Renaissance Diluent (Biocare Medical). Sections were then rinsed and Rabbit on Rodent HRP polymer (Biocare Medical) was next applied for 25 min at room temperature and binding was visualized using diaminobenzidine (DAB) (Biocare Medical).

Statistical Analysis

Data, expressed as mean $\pm$ SEM, were analyzed by two-way ANOVA, followed by Tukey's multiple comparisons test. Differences were considered significant at $P < 0.05$.

Results

Pulmonary fibrosis is not prevented but pneumonitis is abrogated in irradiated IL-4^{-/-} mice

In order to assess the effects of loss of IL-4 on thoracic radiation induced fibrosis and pneumonitis, Gomori Trichrome and H and E stained lung sections from WT and IL-4^{-/-} mice were examined for evidence of collagen accumulation and immune cell infiltration, respectively. In accordance with previous studies using this model (Groves et al., 2015, Johnston et al., 1995), fibrotic regions could be observed histologically in trichrome stained lung sections of WT mice by 26 wk following thoracic irradiation (Figure 1). Furthermore, this response was not prevented or delayed with loss of IL-4, as fibrotic regions were also noted in lungs of IL-4^{-/-} mice beginning at the 26 wk time point. In H and E stained lung sections of WT mice, accumulations of macrophages were noted in alveolar spaces in samples collected 8 wk following irradiation, a response also documented in previous studies (Groves et al., 2015) (Figure 2). With loss of IL-4, however macrophage accumulation was not observed, except when associated with fibrotic regions at 26 wk post exposure, suggesting the pneumonitic response is abrogated or delayed in these mice.

Loss of IL-4 affects repopulation of depleted lung macrophages in irradiated mice

Since loss of IL-4 affected macrophage accumulation in response to thoracic radiation, the specific responses of macrophage subpopulations within the lung were further characterized by flow cytometric analysis of the CD45⁺ myeloid population. Lung AMs, IMs and infiltrating macrophages were distinguished as previously described (Groves et al., 2015). Briefly, following doublet and dead cell exclusion, CD45⁺ cells were gated and Ly6G⁺ granulocytes were removed from further analysis. Macrophage subpopulations were distinguished based on autofluorescence and expression of CD11b and CD11c. Within each subpopulation, F4/80 positive and negative subsets were identified and the expression of CD206 and Ly6C was analyzed in each (Figure 3). Thoracic radiation decreased the numbers of AMs recovered from lung digests at 3 wk post exposure in both mouse strains, regardless of IL-4 status (Figure 4a). These values became similar to the numbers recovered in sham irradiated mice by 8 wk post radiation in WT mice, but remained below control values in mice lacking IL-4 until 16 wk post radiation. Interestingly, in WT mice this response was not repeated in either the IMs (Figure 4b) or infiltrating macrophage populations (Figure 4c). Instead, increases in cell number above sham values were observed

through 8 wk post irradiation in both these populations. In IL-4^{-/-} mice, in contrast, decreased numbers of infiltrating macrophages was observed at 3 wk following radiation. In these mice, the numbers of infiltrating macrophages recovered to sham values by 8 wk post radiation, while the IM population became significantly greater than 0 Gy control mice at 16 wk post radiation.

To analyze the role of IL-4 on pulmonary macrophage development and maturation, expression of F4/80, present on mature macrophages, was assessed in the pulmonary macrophage subsets repopulating the lung following radiation exposure (Hirsch et al., 1981, Lee et al., 1985, Leenen et al., 1986, van den Berg and Kraal, 2005). By 3 wk post irradiation, the percentage of F4/80⁺ AMs was significantly decreased in both WT and IL-4^{-/-} mice (Figure 4d). In WT mice, the proportions of these AMs returned to sham values by 16 wk post irradiation, but the F4/80⁺ AMs from IL-4^{-/-} mice did not recover to 0 Gy values during the time course of the study. In contrast to AMs, the proportion of F4/80⁺ IMs (Figure 4e) and infiltrating macrophages (Figure 4f) were increased by 3 wk post radiation in WT mice. The proportions of F4/80⁺ infiltrating macrophages (Figure 4f) became greater than sham values at the 16 wk and 26 wk time points in these mice as well. This response was not observed in IL-4^{-/-} mice where, instead, the percentage of F4/80⁺ IMs (Figure 4e) were decreased compared to sham values throughout the course of the study, reaching significance at 3 wk and 26 wk post radiation, while the percentage of F4/80⁺ cells in the infiltrating population (Figure 4f) remained un-altered from the age-matched 0 Gy control values.

Loss of IL-4 affects lung macrophage phenotype following irradiation

To assess the influence of IL-4 on the phenotype of macrophages at different maturation states within each subpopulation, CD206 and Ly6C expression was analyzed in both F4/80 positive and negative macrophages in each subpopulation. CD206, expressed by alternatively activated and resident macrophage subpopulations in the lung was first evaluated (Stein et al., 1992, Zhang et al., 2005). In both WT and IL-4^{-/-} mice, the majority of F4/80⁺ AMs expressed CD206 under control conditions (Figure 5a), whereas this proportion was smaller in F4/80⁻ AMs (Figure 5d). Within both the F4/80 positive and negative AM populations (Figure 5a and 5d), radiation resulted in a decrease in the percentage of cells expressing CD206 at 3 wk post exposure in both WT and IL-4^{-/-} mice. In WT mice, the proportion of CD206⁺ F4/80⁺ AMs (Figure 5a) recovered and surpassed 0 Gy values by 8 wk post exposure, while in IL-4^{-/-} mice, the percentage of CD206⁺ macrophages in this population did not reach 0 Gy control values until the 16 wk time point. In the F4/80⁻ AM subpopulation (Figure 5d), the proportion of CD206⁺ cells was increased above control values at 8 wk post exposure in WT mice; however this response did not occur in mice lacking IL-4. In unirradiated control IM (Figure 5b and 5e) and infiltrating macrophage (Figure 5c and 5f) subpopulations of WT mice, less than half and less than a fourth of the population, respectively, expressed CD206 in both F4/80 positive and negative subsets. In mice lacking IL-4, baseline proportions of F4/80⁺ macrophages expressing CD206 was increased compared to WT mice in these subpopulations. In WT mice, radiation exposure resulted in decreased CD206 expression by 3 wk in the F4/80⁻ IM subpopulation (Figure 5e), but not the F4/80⁺ subset (Figure 5b). In mice lacking IL-4 however, this

decrease in CD206+ IMs was observed in both F4/80 positive and negative subsets at 3 wk. By 16 wk post exposure, CD206 expression was decreased in both F4/80 positive and negative subsets of IMs in WT mice, a response that was not present in IL-4^{-/-} mice, which instead achieved baseline values in both populations by 8 wk following irradiation. In the infiltrating macrophage subpopulations, radiation increased CD206 expression in F4/80+ cells in both WT and IL-4^{-/-} mice (Figure 5c); however this response occurred to a greater extent and persisted for a longer period of time in WT mice compared to mice lacking IL-4. Additionally, in WT mice, an increase in the proportion of CD206+ cells was observed in the F4/80- population (Figure 5f) at 8 wk post exposure that did not occur in mice lacking IL-4.

Ly6C, expressed by inflammatory monocytes and macrophages, was assessed in each pulmonary macrophage subpopulation (Sunderkotter et al., 2004, Swirski et al., 2007). In AMs (Figure 6a and 6d) and IMs (Figure 6b and 6e) of control WT mice, less than 20 percent expressed Ly6C, regardless of F4/80 expression, while loss of IL-4 increased this proportion. In contrast, over 80 percent of F4/80+ infiltrating macrophages (Figure 6c) expressed Ly6C in both strains of mice, and this was also true of the F4/80- subset (Figure 6f) in IL-4^{-/-} mice. In F4/80+AMs (Figure 6a) and IMs (Figure 6b) of both WT and IL-4^{-/-} mice, thoracic radiation increased the proportion of cells expressing of Ly6C by 3 wk post exposure. In contrast, in F4/80+ infiltrating macrophages (Figure 6c), the percentage of Ly6C expressing cells was decreased at 3 wk following irradiation in WT mice and was not significantly altered from 0 Gy control values in mice lacking IL-4. In F4/80- AMs (Figure 6d) and infiltrating macrophages (Figure 6f) of WT mice, radiation resulted in a decrease in the proportion of Ly6C+ cells at this time. This response was mirrored in F4/80- IMs (Figure 6e) but did not reach significance and did not occur in any subset with loss of IL-4. In WT mice, Ly6C expression was significantly increased by 26 wk following irradiation compared to sham controls in F4/80+ infiltrating macrophages (Figure 6c). This increased expression at late time points was also observed in WT mice at the 16 wk following exposure in F4/80+ AMs (Figure 6a) and at 16 and 26 wk post exposure in F4/80- IMs (Figure 6e). In IL-4^{-/-} mice, Ly6C expression reached sham values by 8 wk in F4/80+ IMs (Figure 6b) and by 16 wk in F4/80+ AMs (Figure 6a). In F4/80- cells of the AM and IM subpopulations (Figure 6d and 6e) of IL-4^{-/-} mice, however, the percentage of Ly6C expressing cells was not significantly altered compared to sham controls at any of the time points analyzed following exposure. This was also true of both F4/80 positive and negative cells in the infiltrating macrophage subsets (Figure 6c and 6f) of these mice.

Irradiation-induced alterations in macrophage phenotype become localized within the lung

In all pulmonary macrophages collected from whole lung digests, the differences between irradiated and control mice diminished as they aged. Since irradiation-induced alterations in macrophage phenotype may have become more localized within the lung to areas of inflammation and fibrosis, immunohistochemistry on paraffin embedded lung sections from irradiated and sham control mice was used to test this hypothesis, using markers of macrophage activation and polarization. CD206 staining was performed on lung sections collected from WT and IL-4^{-/-} mice at 16 and 26 wk post irradiation only to analyze the localization within the lung of cells expressing this cytokine during the development of

fibrotic outcomes (Figure 7). In all 0 Gy control slides of WT mice, macrophages within the alveolar space stained positively for CD206. When compared to WT mice, loss of IL-4 did not alter the appearance of CD206 staining in control mice at either time point. In WT mice, irradiation resulted in little change in macrophage staining in the parenchyma of CD206 at these late time points, in a trend similar to that observed by flow cytometry; however in the fibrotic regions detected at 26 wk post exposure, many of the cells stained positively for CD206. In contrast to this response, although irradiation again did not alter CD206 macrophage staining in the parenchyma of IL-4^{-/-} mice, the presence of positively stained cells within the fibrotic regions was not observed.

Since CD206 can be expressed by macrophages resident in the lung as well as those that have been alternatively activated, an additional marker of alternative activation, Arg-1, was also assessed (Gordon and Martinez, 2010). In the control lungs of WT mice taken from 3 wk to 26 wk post sham irradiation, light Arg-1 staining was observed in interstitial cells throughout the lung parenchyma (Figure 8). Macrophages in the alveolar space did not stain for Arg-1. Similarly to WT mice, in the lungs of 0 Gy exposed IL-4^{-/-} mice, scarce Arg-1 staining was observed in cells in the interstitial spaces. In both WT and IL-4^{-/-} mice, little Arg-1 staining could be detected in the lung at 3 wk following irradiation. In WT mice, by 8 wk post exposure Arg-1 staining was observed to be greater than in sham controls. The majority of the Arg-1⁺ cells were observed in the interstitium, while the macrophages in the alveolar space remained Arg-1⁻. This response was maintained through 26 wk following irradiation. In mice lacking IL-4 however, apparent Arg-1 staining was not observed until 26 wk post exposure. In WT mice, some Arg-1⁺ cells could be observed within the fibrotic regions of the lung that have developed by 26 wk following irradiation. In contrast to the sparse appearance of Arg-1⁺ cells in the fibrotic regions of irradiated WT mice, in mice lacking IL-4, positively staining cells were frequently observed in these regions. Large, foamy macrophages present in the fibrotic area were largely not Arg-1⁺.

MCP-1, an inflammatory chemokine important for the infiltration of monocytes and macrophages into tissues, was analyzed at 3 wk and 8 wk post exposure to examine the role of IL-4 on MCP-1 mediated repopulation of macrophage populations following acute depletion (Deshmane et al., 2009). Scarce MCP-1 staining was detected on macrophages residing in the alveolar space in sham irradiated WT and IL-4^{-/-} mice at both time points (Figure 9). In WT mice, irradiation increased MCP-1 staining in lung macrophages at the 3 wk time point, returning to sham control levels by 8 wk post exposure. This response did not occur in mice lacking IL-4; irradiation did not affect MCP-1 staining intensity or prevalence relative to control levels at either the 3 wk or 8 wk time points.

Discussion

Exposure of the lung to single, high doses of ionizing radiation initiates the expression of a multitude of cytokines, chemokines, and adhesion molecules as part of the tissue response to injury-induced extensive cell killing and, importantly, cellular damage. Failure to fully resolve these injuries leads to a progression of events, including the perpetuation of inflammatory signaling and alterations to repair processes that drive the development of the clinical outcomes of pneumonitis and pulmonary fibrosis (Spitz et al., 2014, Johnston et al.,

1998, Rubin et al., 1995, Schaeue et al., 2012). Previous studies from our lab have determined that lung exposure of C57BL/6J mice to irradiation results in dose-dependent increases in immune regulators, chemotactic proteins and inflammatory cytokines, such as IL-1, KC, and TNF- α , as well as repair factors such as collagen and TGF- β in the immediate days following exposure. Following a 12.5 Gy exposure, cyclical and time-dependent increases are also evident during the pneumonitic and fibrotic phases of the radiation response; while late changes in expression levels do not occur at late time points in mice exposed to lower radiation doses (0.5 Gy to 10 Gy) and are not associated with overt changes in pathophysiological endpoints (Johnston et al., 1995, Johnston et al., 1996, Johnston et al., 1998, Johnston et al., 2010, Johnston et al., 2011, Rubin et al., 1995, Manning et al., 2013). Furthermore, we have demonstrated that 12.5 Gy thoracic irradiation depletes resident pulmonary macrophages populations (Groves et al., 2015); their repopulation occurs in a subpopulation specific manner and with altered phenotypes from those originally contained within the lung (Groves et al., 2015, Hong et al., 2003).

The delayed changes in cytokine expression levels following radiation exposure are associated with an accumulation of activated macrophages within the lung (Schaeue et al., 2015), and because these highly plastic cells are responsive to signals encountered in their local environment, their phenotypes evolve over the course of injury progression and wound healing and, in turn, act to mediate the development of its outcomes (Mantovani et al., 2013). Although alternative activation of macrophages produces a phenotype that supports the resolution of inflammation and the promotion of wound healing, when this process is dysregulated, over-activation of these responses can result in the development of fibrosis (Gordon and Martinez, 2010, Lech and Anders, 2013). As such, alternatively activated macrophages have been implicated in the development of pulmonary fibrosis in humans as well as in multiple experimental animal models (He et al., 2013, Kolodnick et al., 2004, Mariangela et al., 2015, Murray et al., 2010, Stahl et al., 2013, Zhang et al., 2011). IL-4 is a potent inducer of alternatively activated phenotypes in macrophages (Mosser and Edwards, 2008), and has also been found to play an important role in the development and maintenance of macrophage populations within a tissue (Jenkins et al., 2013). We have yet to fully understand how pulmonary macrophage populations are affected by exposure of the lung to radiation. In particular, the role of IL-4 in the progression of the radiation response is largely unknown and was the focus of these studies.

Interestingly, the development of radiation-induced fibrosis was not prevented or delayed with loss of IL-4. Although multiple studies have implicated IL-4 in the development of fibrosis, and have found that its activities can promote collagen synthesis by fibroblasts, as well as regulating their chemotaxis and proliferation (Postlethwaite et al., 1992, Sempowski et al., 1994), the complete removal of IL-4 using knockout mice has shown varied effects on fibrogenesis. For example, one study using a model of FITC-induced pulmonary fibrosis found that loss of IL-4 did not confer protection from fibrosis development (Kolodnick et al., 2004), while a study on bleomycin-induced pulmonary fibrosis found that the severity of fibrosis was decreased in IL-4 $^{-/-}$ mice (Huaux et al., 2003). It is possible that IL-13, which binds shared receptor components with IL-4 and whose signaling is mediated through a variety of similarly interacting pathways (Jiang et al., 2000), could be stimulating fibrotic processes in a compensatory manner. Indeed, IL-13 has been found to play a crucial role in

the development of bleomycin-induced fibrosis, (Fichtner-Feigl et al., 2006). These findings underscore our contention that alternative activation of macrophages depends on the integration of multiple activation signals, since they reveal that loss of IL-4 did not prevent the presence of alternatively activated macrophages in the lung, as CD206+ and Arg-1+ macrophages were evident in both 0 Gy control mice and after radiation exposure. Further studies characterizing the altered cytokine microenvironment in the lungs of IL-4^{-/-} mice, both at baseline and following thoracic radiation exposure, would increase our understanding of the manner in which pulmonary macrophages are activated in the setting of pulmonary fibrosis, and whether alternative activation and pro-fibrotic factors, such as IL-13 and TGF- β , may compensate for loss of IL-4 signaling. Moreover, these results emphasize the need to more fully investigate the role of IL-4 in fibrosis development.

In contrast to fibrogenesis, the pneumonitic response appeared to be somewhat abrogated in irradiated IL-4^{-/-} mice when compared to WT mice, raising the question of how IL-4 may influence the development and maintenance of macrophage accumulation in the lung. Our results suggest an involvement of IL-4 in this process and are in line with studies showing that IL-4 can enable macrophage colonization in tissues during inflammation (Jenkins et al., 2013). Furthermore, IL-4 administration has been shown to not only induce the proliferation and accumulation of macrophages in multiple tissues in the mouse, but also promote survival in lethally irradiated mice (Jenkins et al., 2011, Van der Meeren et al., 1999). To further investigate the influence of IL-4 on macrophage depletion and repopulation following thoracic irradiation, individual pulmonary macrophage subsets were distinguished and analyzed by flow cytometry.

Pulmonary macrophages are susceptible to radiation-induced damage and, following exposure, the resident populations become depleted (Groves et al., 2015, Hong et al., 2003). Hashimoto et al. has suggested that infiltrating macrophages significantly contribute to repopulating and maintaining AM pools following irradiation, while the contribution from local resident cell proliferation is minimal (Hashimoto et al., 2013). Therefore the contribution of IL-4 in these processes was further investigated. Compared to WT mice, the nature of macrophage repopulation of the irradiation depleted lung was altered in IL-4^{-/-} mice, as well as their phenotype. Although loss of IL-4 did not affect the intensity of radiation-induced AM depletion evident at 3 wk following exposure, the rate of recovery of this subpopulation to control values was impaired. That the magnitude of this depletion was not significantly different between WT and IL-4^{-/-} mice suggests that this effect was dependent on radiation exposure rather than some intrinsic difference in the sensitivity of the IL-4^{-/-} mouse. The fact that the infiltrating macrophage subpopulation, and to a lesser extent IMs, were increased at 3 and 8 wk following exposure in WT mice supports the suggestion that infiltrating populations are contributing to the lung macrophage pool, while in contrast, this response did not occur in infiltrating macrophages of IL-4^{-/-} mice and was delayed in their IM population. Furthermore, MCP-1, a molecule important for monocyte and macrophage recruitment, was not observed following irradiation in IL-4^{-/-} mice, but was evident at the early 3 wk time point in WT mice, further implicating a role for IL-4 in this process. Finally, since IL-4 may also contribute to maturation of lung macrophages, assessment of F4/80, which is expressed by macrophages that are in a mature activation state, (Hirsch et al., 1981, Leenen et al., 1986) showed that although irradiation decreased

proportions of F4/80+ AMs regardless of loss of IL-4, this molecule recovered to baseline values in WT mice only. In IMs, which have a more rapid turnover rate, increases in the proportions of F4/80+ cells in this population above control values was delayed until the 16 wk time point in IL-4^{-/-} mice.

Loss of IL-4 also had an effect on radiation-induced alterations in macrophage phenotype. Although the decreased proportions of CD206+ AMs as well as less mature F4/80- IMs at 3 wk do not appear to be dependent on IL-4, since it occurred in both C57 and IL-4^{-/-} mice, recovery of CD206 to 0 Gy control values was affected by loss of IL-4, in that this was attenuated in both F4/80 positive and negative AMs of IL-4^{-/-} mice. Furthermore, the greater proportion of CD206+ infiltrating cells in WT mice vs. IL-4^{-/-} mice may explain not only the more rapid recovery of this marker in AMs, but also why CD206 is not depleted in IMs at 3wk in WT mice, as the recruited cells may contribute to maintaining resident populations. Since fewer infiltrating cells express CD206 in IL-4^{-/-} mice, this may explain the impaired ability of AMs to express this marker at baseline proportions following exposure. This is supported by work from Stein et al. showing that IL-4 can enhance mannose receptor activity in macrophages (Stein et al., 1992). Interestingly, by the 26 wk time point, the proportions of CD206+ cells reached sham levels in all macrophage subpopulations in both strains of mice. To further investigate whether this was the result of CD206 expressing macrophages becoming more localized within the irradiated tissue over time, immunohistological analysis of this marker was also performed. That AM staining for CD206 in the parenchyma was not different between sham and irradiated mice at 26 wk, regardless of IL-4 status, is in agreement with the results generated in whole lung digests by flow cytometry. However, increased CD206 staining in the fibrotic regions of WT mice demonstrates that its expression had indeed become localized within the tissue and also revealed that this response was abrogated with loss of IL-4.

To determine if phenotypic alterations following radiation exposure were specific to individual types of alternatively activated macrophages, Arg-1 staining was also performed. This was indeed true, since the presence of Arg-1+ interstitial cells was increased following radiation exposure in WT mice, a response not observed in CD206 stained lungs and abrogated by the loss of IL-4. Within the fibrotic areas of the lung, effects of IL-4 on the presence of Arg-1+ cells was not similar to CD206 in that very few positively stained cells were observed in these regions in WT mice whereas incorporation of these cells into fibrotic tissue was increased with loss of IL-4, highlighting the phenotypic differences between these two alternatively activated populations.

In contrast to the effects of loss of IL-4 on CD206+ macrophages, the effects of irradiation on the proportion of resident macrophages expressing Ly6C were amplified in IL-4^{-/-} mice. Specifically, the greater proportions of mature Ly6C+ AMs and IMs were observed at the first time point in mice lacking IL-4, whereas in the less mature F4/80- subsets of these populations, the proportions of these cells were maintained. In WT mice, however, this more inflammatory phenotype was observed only in mature resident cells and was decreased in the less mature F4/80- subsets. Additionally, in both the F4/80 positive and negative subsets of infiltrating populations, the radiation-induced decreases in proportions of Ly6C+ cells observed at early time points in WT mice were prevented in the IL-4^{-/-} mice. Taken

together, these results demonstrate enhanced pro-inflammatory macrophage phenotypes in response to irradiation in lung tissues lacking IL-4.

These findings reveal that IL-4 is not required for the development of radiation-induced pulmonary fibrosis, and instead these results indicate that IL-4 may be playing an important role in the restoration and maintenance of pulmonary macrophage populations in the weeks following lung radiation exposure. Moreover, the phenotypic polarization of macrophages throughout the inflammatory and wound healing processes appears also to be affected by the actions of IL-4, since the phenotypes of these populations were altered with the loss of this cytokine and can differ amongst multiple phenotypes of alternatively activated macrophages. That alterations in macrophage phenotype can become localized within the lung to regions experiencing fibrogenesis provides additional insight into the manner in which macrophages respond to radiation-induced injury, illustrating that their responses evolve both temporally and spatially within the lung. The failure of the irradiated lung to return to homeostasis and the perpetuation of the injury responses appears likely dependent, at least in part, on the actions of pulmonary macrophages, whose phenotypes remained altered relative to those of unexposed mice throughout the multiple phases of the lung's response to radiation. Given their pleiotropic nature, the functions of these cells also likely affect multiple components of the immune response, such as the actions of T cells, which accumulate in the lung following exposure and are also regulators of radiation-induced injury. For example, T regulatory cells, which are important in the control of inflammatory responses, are increased in mediastinal lymph nodes of mice that had received thoracic irradiation (Misra et al., 2015). The interaction between these cell types in the context of radiation responses is not well known and warrants further investigation. Because these pulmonary macrophage populations are likely critical mediators of the chronic inflammation that occurs in the lung following irradiation and may contribute to the development of pulmonary fibrosis, a more complete understanding of their contribution to these processes will aid in the development of mitigators to the clinical outcomes of pneumonitis and pulmonary fibrosis.

References

- Barkauskas CE, Noble PW. Cellular Mechanisms of Tissue Fibrosis. 7. New insights into the cellular mechanisms of pulmonary fibrosis. *Am J Physiol Cell Physiol*. 2014; 306:C987–C996. [PubMed: 24740535]
- Buttner C, Skupin A, Reimann T, Rieber EP, Unteregger G, Geyer P, Frank KH. Local production of interleukin-4 during radiation-induced pneumonitis and pulmonary fibrosis in rats: macrophages as a prominent source of interleukin-4. *Am J Respir Cell Mol Biol*. 1997; 17:315–25. [PubMed: 9308918]
- Chiang CS, Liu WC, Jung SM, Chen FH, Wu CR, McBride WH, Lee CC, Hong JH. Compartmental responses after thoracic irradiation of mice: strain differences. *Int J Radiat Oncol Biol Phys*. 2005; 62:862–71. [PubMed: 15936571]
- Coates PJ, Rundle JK, Lorimore SA, Wright EG. Indirect macrophage responses to ionizing radiation: implications for genotype-dependent bystander signaling. *Cancer Res*. 2008; 68:450–6. [PubMed: 18199539]
- Davies LC, Taylor PR. Tissue-resident macrophages: then and now. *Immunology*. 2015; 144:541–8. [PubMed: 25684236]
- Deshmane SL, Kremlev S, Amini S, Sawaya BE. Monocyte chemoattractant protein-1 (MCP-1): an overview. *J Interferon Cytokine Res*. 2009; 29:313–26. [PubMed: 19441883]

- Epelman S, Lavine KJ, Randolph GJ. Origin and functions of tissue macrophages. *Immunity*. 2014; 41:21–35. [PubMed: 25035951]
- Fichtner-Feigl S, Strober W, Kawakami K, Puri RK, Kitani A. IL-13 signaling through the IL-13[alpha]2 receptor is involved in induction of TGF-[beta]1 production and fibrosis. *Nat Med*. 2006; 12:99–106. [PubMed: 16327802]
- Gibbons MA, Mackinnon AC, Ramachandran P, Dhaliwal K, Duffin R, Phythian-Adams AT, Van Rooijen N, Haslett C, Howie SE, Simpson AJ, Hirani N, Gauldie J, Iredale JP, Sethi T, Forbes SJ. Ly6C(hi) Monocytes Direct Alternatively Activated Profibrotic Macrophage Regulation of Lung Fibrosis. *Am J Respir Crit Care Med*. 2011; 184:569–581. [PubMed: 21680953]
- Gordon S, Martinez FO. Alternative Activation of Macrophages: Mechanism and Functions. *Immunity*. 2010; 32:593–604. [PubMed: 20510870]
- Groves AM, Johnston CJ, Misra RS, Williams JP, Finkelstein JN. Whole-Lung Irradiation Results in Pulmonary Macrophage Alterations that are Subpopulation and Strain Specific. *Radiat Res*. 2015; 184:639–49. [PubMed: 26632857]
- Guth AM, Janssen WJ, Bosio CM, Crouch EC, Henson PM, Dow SW. Lung environment determines unique phenotype of alveolar macrophages. *Am J Physiol Lung Cell Molec Physiol*. 2009; 296:L936–L946.
- Hashimoto D, Chow A, Noizat C, Teo P, Beasley MB, Leboeuf M, Becker CD, See P, Price J, Lucas D, Greter M, Mortha A, Boyer SW, Forsberg EC, Tanaka M, Van Rooijen N, Garcia-Sastre A, Stanley ER, Ginhoux F, Frenette PS, Merad M. Tissue-resident macrophages self-maintain locally throughout adult life with minimal contribution from circulating monocytes. *Immunity*. 2013; 38:792–804. [PubMed: 23601688]
- He C, Ryan AJ, Murthy S, Carter AB. Accelerated development of pulmonary fibrosis via Cu, Zn-superoxide dismutase-induced alternative activation of macrophages. *J Biol Chem*. 2013; 288:20745–57. [PubMed: 23720777]
- Hirsch S, Austyn JM, Gordon S. Expression of the macrophage-specific antigen F4/80 during differentiation of mouse bone marrow cells in culture. *J Exp Med*. 1981; 154:713–25. [PubMed: 7276827]
- Hong JH, Jung SM, Tsao TC, Wu CJ, Lee CY, Chen FH, Hsu CH, McBride WH, Chiang CS. Bronchoalveolar lavage and interstitial cells have different roles in radiation-induced lung injury. *Int J Radiat Biol*. 2003; 79:159–67. [PubMed: 12745880]
- Huax F, Liu T, McGarry B, Ullenbruch M, Phan SH. Dual roles of IL-4 in lung injury and fibrosis. *J Immunol*. 2003; 170:2083–92. [PubMed: 12574379]
- Hussell T, Bell TJ. Alveolar macrophages: plasticity in a tissue-specific context. *Nat Rev Immunol*. 2014; 14:81–93. [PubMed: 24445666]
- Italiani P, Boraschi D. From Monocytes to M1/M2 Macrophages: Phenotypical vs. Functional Differentiation. *Front Immunol*. 2014; 5:514. [PubMed: 25368618]
- Jenkins SJ, Ruckerl D, Cook PC, Jones LH, Finkelman FD, Van Rooijen N, Macdonald AS, Allen JE. Local macrophage proliferation, rather than recruitment from the blood, is a signature of TH2 inflammation. *Science*. 2011; 332:1284–8. [PubMed: 21566158]
- Jenkins SJ, Ruckerl D, Thomas GD, Hewitson JP, Duncan S, Brombacher F, Maizels RM, Hume DA, Allen JE. IL-4 directly signals tissue-resident macrophages to proliferate beyond homeostatic levels controlled by CSF-1. *J Exp Med*. 2013; 210:2477–91. [PubMed: 24101381]
- Jiang H, Harris MB, Rothman P. IL-4/IL-13 signaling beyond JAK/STAT. *J Allergy Clin Immunol*. 2000; 105:1063–70. [PubMed: 10856136]
- Johnston CJ, Piedboeuf B, Baggs R, Rubin P, Finkelstein JN. Differences in correlation of mRNA gene expression in mice sensitive and resistant to radiation-induced pulmonary fibrosis. *Radiat Res*. 1995; 142:197–203. [PubMed: 7724735]
- Johnston CJ, Piedboeuf B, Rubin P, Williams JP, Baggs R, Finkelstein JN. Early and persistent alterations in the expression of interleukin-1 alpha, interleukin-1 beta and tumor necrosis factor alpha mRNA levels in fibrosis-resistant and sensitive mice after thoracic irradiation. *Radiat Res*. 1996; 145:762–7. [PubMed: 8643837]

- Johnston CJ, Wright TW, Rubin P, Finkelstein JN. Alterations in the expression of chemokine mRNA levels in fibrosis-resistant and -sensitive mice after thoracic irradiation. *Exp Lung Res*. 1998; 24:321–37. [PubMed: 9635254]
- Johnston CJ, Williams JP, Okunieff P, Finkelstein JN. Radiation-induced pulmonary fibrosis: examination of chemokine and chemokine receptor families. *Radiat Res*. 2002; 157:256–65. [PubMed: 11839087]
- Johnston CJ, Hernady E, Reed C, Thurston SW, Finkelstein JN, Williams JP. Early alterations in cytokine expression in adult compared to developing lung in mice after radiation exposure. *Radiat Res*. 2010; 173:522–535. [PubMed: 20334525]
- Johnston CJ, Manning C, Hernady E, Reed C, Thurston SW, Finkelstein JN, Williams JP. Effect of total body irradiation on late lung effects: hidden dangers. *Int J Radiat Biol*. 2011; 87:902–913. [PubMed: 21574903]
- Kolodnick JE, Toews GB, Jakubick C, Hogaboam C, Moore TA, McKenzie A, Wilke CA, Chrisman CJ, Moore BB. Protection from fluorescein isothiocyanate-induced fibrosis in IL-13-deficient, but not IL-4-deficient, mice results from impaired collagen synthesis by fibroblasts. *J Immunol*. 2004; 172:4068–76. [PubMed: 15034018]
- Landsman L, Jung S. Lung macrophages serve as obligatory intermediate between blood monocytes and alveolar macrophages. *J Immunol*. 2007; 179:3488–94. [PubMed: 17785782]
- Landsman L, Varol C, Jung S. Distinct differentiation potential of blood monocyte subsets in the lung. *J Immunol*. 2007; 178:2000–7. [PubMed: 17277103]
- Lech M, Anders HJ. Macrophages and fibrosis: How resident and infiltrating mononuclear phagocytes orchestrate all phases of tissue injury and repair. *Biochim Biophys Acta*. 2013; 1832:989–997. [PubMed: 23246690]
- Lee SH, Starkey PM, Gordon S. Quantitative analysis of total macrophage content in adult mouse tissues. Immunochemical studies with monoclonal antibody F4/80. *J Exp Med*. 1985; 161:475–89. [PubMed: 3973536]
- Leenen PJ, Jansen AM, Van Ewijk W. Murine macrophage cell lines can be ordered in a linear differentiation sequence. *Differentiation*. 1986; 32:157–64. [PubMed: 3792703]
- Lehnert BE, Dethloff LA, Finkelstein JN, Van Der Kogel AJ. Temporal sequence of early alterations in rat lung following thoracic X-irradiation. *Int J Radiat Biol*. 1991; 60:657–75. [PubMed: 1680146]
- Manning CM, Johnston CJ, Hernady E, Miller JN, Reed CK, Lawrence BP, Williams JP, Finkelstein JN. Exacerbation of lung radiation injury by viral infection: the role of Clara cells and Clara cell secretory protein. *Radiat Research*. 2013; 179:617–629.
- Mantovani A, Biswas SK, Galdiero MR, Sica A, Locati M. Macrophage plasticity and polarization in tissue repair and remodelling. *J Pathol*. 2013; 229:176–85. [PubMed: 23096265]
- Mariangela S, Monica M, Francesca T, Laura T, Mauro L, Sabrina C, Lorenzo L. Biological basis of radiation-induced pulmonary fibrosis. *Journal of Tumor*. 2015; 3:325–331.
- Misra RS, Johnston CJ, Groves AM, Dediego ML, St Martin J, Reed C, Hernady E, Miller JN, Love T, Finkelstein JN, Williams JP. Examining the Effects of External or Internal Radiation Exposure of Juvenile Mice on Late Morbidity after Infection with Influenza A. *Radiat Res*. 2015; 184:3–13. [PubMed: 26114328]
- Mosser DM, Edwards JP. Exploring the full spectrum of macrophage activation. *Nat Rev Immunol*. 2008; 8:958–969. [PubMed: 19029990]
- Murray LA, Rosada R, Moreira AP, Joshi A, Kramer MS, Hesson DP, Argentieri RL, Mathai S, Gulati M, Herzog EL, Hogaboam CM. Serum amyloid P therapeutically attenuates murine bleomycin-induced pulmonary fibrosis via its effects on macrophages. *PLoS One*. 2010; 5:e9683. [PubMed: 20300636]
- Murray PJ. Macrophage activation and polarization. *Semin Immunol*. 2015; 27:235–236. [PubMed: 26615009]
- Paun A, Kunwar A, Haston CK. Acute adaptive immune response correlates with late radiation-induced pulmonary fibrosis in mice. *Radiat Oncol*. 2015; 10:45. [PubMed: 25889053]
- Postlethwaite AE, Holness MA, Katai H, Raghov R. Human fibroblasts synthesize elevated levels of extracellular matrix proteins in response to interleukin 4. *J Clin Invest*. 1992; 90:1479–85. [PubMed: 1401080]

- Rube CE, Uthe D, Schmid KW, Richter KD, Wessel J, Schuck A, Willich N, Rube C. Dose-dependent induction of transforming growth factor beta (TGF-beta) in the lung tissue of fibrosis-prone mice after thoracic irradiation. *Int J Radiat Oncol Biol Phys.* 2000; 47:1033–42. [PubMed: 10863076]
- Rubin P, Johnston CJ, Williams JP, McDonald S, Finkelstein JN. A perpetual cascade of cytokines postirradiation leads to pulmonary fibrosis. *Int J Radiat Oncol Biol Phys.* 1995; 33:99–109. [PubMed: 7642437]
- Schaue D, Kachikwu EL, McBride WH. Cytokines in radiobiological responses: a review. *Radiat Res.* 2012; 178:505–23. [PubMed: 23106210]
- Schaue D, Micewicz ED, Ratikan JA, Xie MW, Cheng GH, McBride WH. Radiation and Inflammation. *Semin Radiat Oncol.* 2015; 25:4–10. [PubMed: 25481260]
- Schneberger D, Aharonson-Raz K, Singh B. Monocyte and macrophage heterogeneity and Toll-like receptors in the lung. *Cell Tissue Res.* 2011; 343:97–106. [PubMed: 20824285]
- Sempowski GD, Beckmann MP, Derdak S, Phipps RP. Subsets of murine lung fibroblasts express membrane-bound and soluble IL-4 receptors. Role of IL-4 in enhancing fibroblast proliferation and collagen synthesis. *J Immunol.* 1994; 152:3606–14. [PubMed: 7908305]
- Spitz DR, Azzam EI, Li JJ, Gius D. Metabolic oxidation/reduction reactions and cellular responses to ionizing radiation: a unifying concept in stress response biology. *Cancer Metastasis Rev.* 2004; 23:311–322. [PubMed: 15197331]
- Stahl M, Schupp J, Jager B, Schmid M, Zissel G, Muller-Quernheim J, Prasse A. Lung collagens perpetuate pulmonary fibrosis via CD204 and M2 macrophage activation. *PLoS One.* 2013; 8:e81382. [PubMed: 24278429]
- Stein M, Keshav S, Harris N, Gordon S. Interleukin-4 Potently Enhances Murine Macrophage Mannose Receptor Activity - a Marker of Alternative Immunological Macrophage Activation. *J Exp Med.* 1992; 176:287–292. [PubMed: 1613462]
- Sunderkotter C, Nikolic T, Dillon MJ, Van Rooijen N, Stehling M, Drevets DA, Leenen PJ. Subpopulations of mouse blood monocytes differ in maturation stage and inflammatory response. *J Immunol.* 2004; 172:4410–7. [PubMed: 15034056]
- Swirski FK, Libby P, Aikawa E, Alcaide P, Luscinskas FW, Weissleder R, Pittet MJ. Ly-6Chi monocytes dominate hypercholesterolemia-associated monocytoysis and give rise to macrophages in atherosclerosis. *J Clin Invest.* 2007; 117:195–205. [PubMed: 17200719]
- Van Den Berg TK, Kraal G. A function for the macrophage F4/80 molecule in tolerance induction. *Trends Immunol.* 2005; 26:506–9. [PubMed: 16087400]
- Van Der Meeren A, Gaugler MH, Mouthon MA, Squiban C, Gourmelon P. Interleukin 4 promotes survival of lethally irradiated mice in the absence of hematopoietic efficacy. *Radiat Res.* 1999; 152:629–36. [PubMed: 10581533]
- Wesseliuss LJ, Kimler BF. Alveolar macrophage proliferation in situ after thoracic irradiation of rats. *Am Rev Respir Dis.* 1989; 139:221–5. [PubMed: 2912342]
- Xu L, Xiong S, Guo R, Yang Z, Wang Q, Xiao F, Wang H, Pan X, Zhu M. Transforming growth factor beta3 attenuates the development of radiation-induced pulmonary fibrosis in mice by decreasing fibrocyte recruitment and regulating IFN-gamma/IL-4 balance. *Immunol Lett.* 2014; 162:27–33. [PubMed: 24996042]
- Yona S, Kim KW, Wolf Y, Mildner A, Varol D, Breker M, Strauss-Ayali D, Viukov S, Guillemins M, Misharin A, Hume DA, Perlman H, Malissen B, Zelzer E, Jung S. Fate mapping reveals origins and dynamics of monocytes and tissue macrophages under homeostasis. *Immunity.* 2013; 38:79–91. [PubMed: 23273845]
- Zhang H, Han G, Liu H, Chen J, Ji X, Zhou F, Zhou Y, Xie C. The development of classically and alternatively activated macrophages has different effects on the varied stages of radiation-induced pulmonary injury in mice. *J Radiat Res.* 2011; 52:717–26. [PubMed: 22104268]
- Zhang J, Tachado SD, Patel N, Zhu J, Imrich A, Manfrulli P, Cushion M, Kinane TB, Koziel H. Negative regulatory role of mannose receptors on human alveolar macrophage proinflammatory cytokine release in vitro. *J Leukoc Biol.* 2005; 78:665–74. [PubMed: 16000387]

Figure 1.

Lung histology from WT and IL-4^{-/-} mice. Gomori Trichrome stained lung sections were prepared at 16 and 26 wk following exposure to 0 or 12.5 Gy whole lung irradiation (n = 3–4 mice/treatment group). Collagen staining can be observed in fibrotic regions in irradiated WT and IL-4^{-/-} mice at 26 wk following irradiation. Original magnification $\times 200$.

Figure 2.

Lung histology from WT and IL-4^{-/-} mice. Hematoxylin and Eosin stained lung sections were prepared at 16 and 26 wk following exposure to 0 or 12.5 Gy whole lung irradiation (n = 3–4 mice/treatment group). Immune cell infiltrates are observed in irradiated mice, with this response being more prominent in WT mice. Arrows indicate macrophages. Original magnification $\times 200$.

Figure 3.

Gating scheme for characterization of pulmonary macrophages. In WT and IL-4^{-/-} mice, between 3–26 wk following exposure to 0 Gy or 12.5 Gy thoracic irradiation, CD45⁺ cells were collected from lung digests and enriched using MACS, and were analyzed by flow cytometry. One representative sample is shown for illustrative purposes. Singlet and live cell discrimination was first performed. Next, CD45⁺ Ly6G⁻ cells were gated to identify myeloid cells and exclude Ly6G⁺ granulocytes from further analysis. Pulmonary macrophage subpopulations were distinguished based on autofluorescence and CD11b and CD11c expression. Within each subpopulation F4/80 positive and negative subsets were next identified and then expression of CD206 and Ly6C was analyzed in each subset.

Figure 4.

Characterization of pulmonary macrophages. In WT and IL-4^{-/-} mice, between 3–26 wk following exposure to 0 Gy or 12.5 Gy thoracic irradiation, CD45⁺ cells were collected from lung digests and enriched using MACS, and were analyzed by flow cytometry. 4(a–c): Irradiation, as well as loss of IL-4, altered the numbers of AMs, IMs and infiltrating macrophages. 4(d–f): Lung macrophage subpopulations were next analyzed for their expression of F4/80. Irradiation and loss of IL-4 induced alterations in the proportion of F4/80⁺ cells in each population. Each point represents mean $\pm$ SEM (n = 3–4 mice/treatment group). ^a Significantly different (P < 0.05) from age-matched 0 Gy controls. ^b Significantly different (P < 0.05) from 3 wk time point. ^c Significantly different (P < 0.05) from WT mice.

Figure 5.

Characterization of pulmonary macrophage phenotype. In WT and IL-4^{-/-} mice, between 3–26 wk following exposure to 0 Gy or 12.5 Gy thoracic irradiation, CD45⁺ cells were collected from lung digests and enriched using MACS, and were analyzed by flow cytometry. 5(a–c): Irradiation, as well as loss of IL-4, altered the proportion of CD206⁺ cells in the F4/80⁺ subsets of the AMs, IMs and infiltrating macrophage populations. 5(d–f): Irradiation, as well as loss of IL-4, altered the proportion of CD206⁺ cells in the F4/80⁻ subsets of the AMs, IMs and infiltrating macrophage populations. Each bar represents mean $\pm$ SEM (n = 3–4 mice/treatment group). ^a Significantly different (P < 0.05) from 0 Gy age-matched controls. ^b Significantly different (P < 0.05) from 3 wk time point. ^c Significantly different (P < 0.05) from WT mice.

Figure 6.

Characterization of pulmonary macrophage phenotype. In WT and IL-4^{-/-} mice, between 3–26 wk following exposure to 0 Gy or 12.5 Gy thoracic irradiation, CD45⁺ cells were collected from lung digests and enriched using MACS, and were analyzed by flow cytometry. 6(a–c): Irradiation, as well as loss of IL-4, altered the proportion of Ly6C⁺ cells in the F4/80⁺ subsets of the AMs, IMs and infiltrating macrophage populations. 6(d–f): Irradiation, as well as loss of IL-4, altered the proportion of Ly6C⁺ cells in the F4/80⁻ subsets of the AMs, IMs and infiltrating macrophage populations. Each bar represents mean $\pm$ SEM (n = 3–4 mice/treatment group). ^a Significantly different (P < 0.05) from 0 Gy age-matched controls. ^b Significantly different (P < 0.05) from 3 wk time point. ^c Significantly different (P < 0.05) from WT mice.

Figure 7.

Effects of thoracic radiation on lung expression of CD206. In WT and IL-4^{-/-} mice, lung sections were prepared between 16–26 wk following exposure to 0 Gy or 12.5 Gy thoracic irradiation. Immunohistochemistry was performed using antibody to CD206 and visualized using DAB. Arrows indicate CD206+ cells. Original magnification, 200x. Representative sections are shown from n = 3–4 mice/treatment group.

Figure 8.

Effects of thoracic radiation on lung expression of Arg-1. In WT and IL-4^{-/-} mice, lung sections were prepared between 3–26 wk following exposure to 0 Gy or 12.5 Gy thoracic irradiation. Immunohistochemistry was performed using antibody to Arg-1 and visualized using DAB. Arrows indicate Arg-1⁺ cells. Arrowheads indicate Arg-1⁻ macrophages. Original magnification, 200x. Representative sections are shown from n = 3–4 mice/treatment group.

Figure 9.

Effects of thoracic radiation on lung expression of MCP-1. In WT and IL-4^{-/-} mice, lung sections were prepared between 3–8 wk following exposure to 0 Gy or 12.5 Gy thoracic irradiation. Immunohistochemistry was performed using antibody to MCP-1 and visualized using DAB. Arrows indicate MCP-1+ macrophages. Original magnification, 200x. Representative sections are shown from n = 3–4 mice/treatment group.