The Development of Classically and Alternatively Activated Macrophages Has Different Effects on The Varied Stages of Radiation-induced Pulmonary Injury in Mice

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Macrophage/Radiation-induced pulmonary injury/Classical activation/Alternative activation.

The classical and alternative activation of macrophages has been proposed to play a role in radiation-induced pneumonitis and fibrosis, respectively. To test this hypothesis, the thoraces of C57BL/6 mice were irradiated with 12 Gy X-rays, and irradiated and control mice were euthanized at 1, 8, 12, 24 and 72 hours, and 2, 4, 8, 16 and 24 weeks after irradiation. The expression of inducible nitric oxide synthase (iNOS) and arginase type 1 (Arg-1) was evaluated at the mRNA and protein levels at different stages post-irradiation. We demonstrated that the enhanced mRNA and protein expression of iNOS occurred within the pneumatic stage, whereas the high levels of Arg-1 expression occurred within the fibrotic phase. Immunohistochemistry revealed that iNOS and Arg-1 were mainly expressed in macrophages. The expression of iNOS and Arg-1 may be associated with acute radiation pneumonitis and the development of radiation fibrosis, respectively. Although the function of macrophages cannot explain the whole process of radiation-induced pulmonary injury development, it may play an important regulatory role during this process.

INTRODUCTION

Radiation is one of the most frequently administered forms of therapy for thoracic tumors. However, radiation-induced pulmonary injury (RIP) to adjacent normal lung tissue is one of the dose-limiting factors. This complication reduces the survival rates and influences the quality of life of these patients. RIP clinically manifests as radiation interstitial pneumonitis and subsequent radiation pulmonary fibrosis.1,2 Although much research has been carried out in this area, the molecular mechanism behind this pathological process remains to be fully elucidated. Several possible mechanisms have been proposed, of which a perpetual cytokine cascade is the universally accepted mechanism.3 Parenchymal cells in lung and circulating immune cells may be stimulated to produce pro-inflammatory cytokines and profibrotic cytokines.

The classical activation of macrophages plays an important part in inducing radiation pneumonitis, while the alternative activation is associated with radiation fibrosis. It was reported that macrophages are one of the dominantly recruited cell types post-irradiation (p.i.), with deformed macrophages appearing during pneumatic and fibrotic phases,4 and nitric oxide (NO) induced by inducible nitric oxide synthase (iNOS) in macrophages after radiation was thought as an important mediator of radiation pneumonitis.5 Macrophages play an important role in alveolar physiology and have specialized functions in response to different microenvironments.6 Activated macrophages display two main phenotypes, classical and alternative activation.7,8 Classically activated macrophages (caMΦ), which are mainly induced by interferon (IFN)-γ, play an important role in protecting against intracellular pathogens and increase the production of iNOS, and simultaneously produce pro-inflammatory factors such as tumor necrosis factor (TNF), interleukin (IL)-12 and reactive nitrogen intermediates (RNI).5,10 Whereas IL-13/IL-4-induced alternatively activated macrophages (aaMΦ), are indispensable for tissue remodeling and collagen production and enhance the expression of arginase type 1 (Arg-1).11 The difference between the two activation pathways is whether L-arginine metabolism is catalyzed by iNOS or Arg-1.12 L-arginine is the substrate for both of these enzymes. iNOS is important in the protection against intracellular pathogens.
because it hydrolyzes L-arginine to L-citrulline and NO. Arg-1 metabolizes L-arginine to urea and L-ornithine, which is further metabolized by ornithine decarboxylase (ODC) and ornithine amino transferase (OAT), and produces polyamines and L-proline, which control cell growth and collagen synthesis, respectively. Arg-1 is therefore required for tissue remodeling and fibrosis. The characteristic features of differentially activated macrophages are therefore mainly based on differential arginine metabolism, via either iNOS or Arg-1.13–16 So we used iNOS and Arg-1 as prototypic markers of classical and alternative macrophage activation, respectively. The relative ratio of iNOS to Arg-1, which exhibited a marked increase in caMΦ and a marked decrease in aaMΦ, is frequently used as a functional readout to differentiate between these two activated forms of macrophages.

Our preliminary studies in vitro showed that the macrophage cell line RAW 264.7 exhibited increased urea levels in the presence of IL-13, and γ-rays stimulated increased production of NO and iNOS in macrophages in response to IFN-γ. From these observations, we addressed the question of whether classical activation of macrophages plays a role in radiation-induced pneumonitis, and whether the development of lung fibrosis is due to alternative activation of macrophages using a mouse model of RIP.

**METHODS AND MATERIALS**

**Mice and radiation schedule**

Adult, female, specific-pathogen-free (SPF) C57BL/6 mice, of about eight-weeks-old, were purchased from Vital River Laboratory (Beijing, China). Our animal experimental protocols were approved by the Medical Sciences Animal Care Committee of Hubei province, China. The mice were divided into two groups: no treatment (28 mice) and radiation (70 mice). Mice were then housed under SPF conditions and supplied with standard laboratory food and water, and allowed to acclimatize for one week prior to treatment. The radiation schedule was referred to some articles4,17 and was similar to our previously published model.21 Two mice were irradiated simultaneously, a dose of 12 Gy to the whole thorax was delivered in a single fraction at the posterior field using a linear accelerator (Siemens Primus-Hi, Germany). Plastic jigs were used to restrain the mice without anesthesia. The radiation parameters were as follows: beam energy, 6 MV X-rays; dose rate, 188.61 cGy/min; source–surface distance (SSD), 100 cm; and the field size (2,600 × 38,000 cm) was set to provide adequate coverage of the whole lung. The head, neck, abdomen, pelvis and legs were out of the treatment field. Plexiglass, 1 cm thick, was placed above the animal to obtain even dose distribution. After radiation, the mice were maintained in SPF rooms. Radiated and control mice were euthanized at 1, 8, 12, 24 and 72 h, and 2, 4, 8, 16 and 24 weeks p.i.

**Histological examination**

Parts of the right lungs were fixed with 4% paraformaldehyde for 24 h and paraffin-embedded. Sections of 4-μm thickness were cut. For routine histological analysis, the sections were stained with hematoxylin and eosin (HE). Masson staining was also used to delineate collagen, which indicated the severity of lung fibrosis, and stained sections were examined by light microscopy.

**Hydroxyproline assay**

A hydroxyproline assay was performed to determine the hydroxyproline content of the lung (wet weight) as a quantitative measure of collagen deposition and fibrosis. An alkaline hydrolysis kit (Nanjing Jiancheng Biotechne Company, Nanjing, China) was used according to the manufacturer’s instructions. Individual lung tissues were weighed before alkaline hydrolysis, and the absorbance was measured at 550 nm using a Beckman DU 640 spectrophotometer (Beckman Coulter, Inc, Los Angeles, California, America). A standard sample with a hydroxyproline content of 5 μg/mg was used for calibration. The hydroxyproline content of lung samples was determined using the following equation: hydroxyproline (μg/mg) = (sample absorbance – control absorbance) × total lysate volume (10 ml) × standard concentration (5 μg/mg)/(standard absorbance – control absorbance)/tissue weight (mg).

**mRNA expression of iNOS and Arg-1**

The left lungs were flash frozen in liquid nitrogen after removal, and homogenized in TRIzol reagent (Invitrogen, Carlsbad, CA, USA) to extract total RNA. First-strand cDNA was synthesized using the EnergeticScript® cDNA Synthesis Kit for RT-PCR (Invitrogen, Carlsbad, CA, USA). PCR primers and probes for murine iNOS, Arg-1 and the housekeeping gene GAPDH are shown in Table 1. Real-time PCR amplifications were performed using the ShineProbe® Real Time qPCR MasterMix Kit (Shinegene, Shanghai, China) in the FTC-2000 (Funglyn Biotech Inc, Toronto, Canada) sequence detection system. For reproducibility within and between PCR amplifications, we used a standard cDNA in PCR amplification. Relative mRNA expression of the test cDNA samples was compared with that of the standard cDNA and expressed as a ratio calculated using the following equation: relative mRNA expression (E) = 2−ΔΔCt, where Ct represents the cycle threshold in the PCR and ΔCt = Ct of the test Ct of the GAPDH gene.

**Protein expression of Arg-1 and iNOS**

Protein extracts from lung tissue samples were prepared using cold cell lysates in RIPA (50 mM Tris–HCl [pH 7.4], 150 mM NaCl, 1% Nonidet-P [NP-40], 0.1% SDS), containing 1 mM PMSF and a protease inhibitor cocktail. The protein concentration was determined using a Bradford kit (Applygen, Beijing, China). The extracted proteins were
Table 1. Primers and probes used for Real-Time TaqMan PCR

| Gene product | Sequences (5’→3’) | Direction |
|--------------|-------------------|-----------|
| iNOS         | ACTCAGCCAAGCCCTCAC    | forward   |
|              | GCCTATCGTCTCGTCCGT   | reverse   |
|              | fam + ACCTCGACATACGCACCCCTCC + Tamra | probe |
| Arg-1        | TTGATTCCAACGACATACCA | forward   |
|              | CGTTTTCCATTAGCTCTTCAT | reverse   |
|              | fam + CTCGCCCTGCCTGGCTCTTA + Tamra | probe |
| GAPDH        | TGTGTCCGTGCTGGATCTGA  | forward   |
|              | CCTGCTTACCCACTTCTTGA  | reverse   |
|              | fam + CGCCTGGAGAAACTGCACTAG + Tamra | probe |

Fig. 1. HE staining for histological features in C57BL/6 mice following thoracic irradiation. Six representative slides were for control (A), 72 h p.i. (B), 2 w.p.i. (C), 4 w.p.i. (D), 16 w.p.i. (E) and 24 w.p.i. (F). In control mouse, the alveoli septa are normal and tissue architecture is intact. In the irradiated mouse, however, at 72 h after radiation, lungs of the radiated mice represented acute inflammation, including edema of the alveolar walls, intra-alveolar hemorrhage, alveoli exudation, neutrophil infiltration and interstitial edema, these inflammatory changes were over the first month. At 16 and 24 w.p.i., there was a decreasing in neutrophil, the alveolar septa become thickened and the alveolar space becomes small. All figures were taken at ×400.
electrophoresed on 8–10% SDS-PAGE gels and then electro-transferred to polyvinylidene fluoride (PVDF, Millipore company, America) membranes. The blots were blocked with 5% non-fat dry milk, and then incubated overnight at 4°C with primary antibodies raised against Arg-1 (1:500) and iNOS (1:500). β-actin (1:1000) was used as an internal control. Horseradish peroxidase-conjugated anti-rabbit IgG (1:3000 in TBS-T) was used as a secondary antibody. Finally, the reaction was visualized with an ECL reagent kit (Biotime, Shanghai, China) and exposed to Kodak Scientific Imaging Systems film. All primary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Immunohistochemistry
Slides were deparaffinized, rehydrated though a graded series of ethanol and treated with 3% H2O2 in H2O to quench endogenous peroxidase activity. The specimens were incubated overnight at 4°C with 100-fold-diluted rabbit polyclonal antibody against iNOS or Arg-1 (1:100; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and goat anti-rabbit secondary antibody (Maixin Biotechnology, Fuzhou, China), then were detected with HRP system, Diaminobenzidine (DAB) (Maixin Biotechnology, Fuzhou, China) was used as chromogen. All micrographs were obtained using a light microscope with a digital camera system.

Statistical processing
Statistical analysis was performed with the SPSS version 13.0 software package. All data were expressed as the mean ± standard deviation (SD). Comparison of the data between groups was performed using the Mann–Whitney U test, and the data for different time points were analyzed using the independent sample T-test. Two-sided tests were used, and a value of P < 0.05 was considered statistically significant.

RESULTS
Histologic and hydroxyproline content changes
Figure 1 shows the pathological features of RIP. Normal lung morphology was observed in non-irradiated mice (Fig. 1A). The initial injury included edema of the alveolar walls, intra-alveolar hemorrhage, alveoli exudation, neutrophil infiltration and interstitial edema, with these inflammatory changes occurring over the first month (Fig. 1B, C and D). Then, the alveolar septa became thickened and the alveolar space became smaller. In the later phase, from 16 weeks onwards, fibrosis developed together with loss of capillaries, the thickness of the alveolar septa increased and obliteration of the alveolar space was evident (Fig. 1E, F). Figure 2 shows Masson staining for collagen in mouse lung tissues. Light blue stain is for collagen staining. Four representative slides are for control (A), 2 w p.i. (B), 16 w p.i. (C) and 24 w p.i. (D). In control mouse, the alveolar septa are normal and without obvious collagen deposition. In the irradiated mouse, however, significant thickening is seen for the alveolar septa, large amounts of collagen deposits can be observed in the alveolar septa and the bronchiolar area at 16 w and 24 w p.i. Original magnification 400×.
shows collagen expression in normal lung tissues and irradiated lung tissues. Fibrosis began at week 8 (data not shown) and increased progressively, and marked fibrotic changes were observed at 24 weeks (Fig. 2D). At different time points after irradiation, the hydroxyproline content of the lung tissue was measured to monitor fibrosis develop-

![Hydroxyproline graph](image)

**Fig. 3.** Time-course of hydroxyproline expression in mouse lung tissues. At 1, 8, 12, 24, 72 h, 2, 4, 8, 16 and 24 w after radiation, hydroxyproline levels were measured in the lung tissue obtained from control or radiation-treated C57BL/6 mice. The results are expressed as the amount of hydroxyproline (μg) per mg lung tissue for each mouse. The lines represent means ± SD. The results of statistical comparisons were obtained as follows: irradiation and blank at 4 weeks p.i. (P < 0.05) and at 16 (0.708 ± 0.081) and 24 (0.744 ± 0.098) weeks p.i. (P < 0.01).

![iNOS expression graph](image)

**Fig. 4.** The relative mRNA expression for iNOS. The vertical bars represent means ± SD. The expression of mRNA was determined from 1 h until 24 w. Compared with control group, the irradiated group showed a significant increased from 8 h (0.073 ± 0.018, P = 0.007) and reached maximal at 24 h (0.082 ± 0.020, P = 0.005), and then gradually reduced to normal.

![Arg-1 expression graph](image)

**Fig. 5.** The relative mRNA expression for Arg-1. The vertical bars represent means ± SD. It showed that Arg-1 mRNA was low expressed before 16 w p.i., especially at 24 h and 72 h p.i. (P < 0.05), but increased suddenly at 16 weeks p.i. and maintained high level to 24 weeks p.i. (P < 0.01).
ment. As shown in Fig. 3, the increased hydroxyproline content indicated the progressive establishment of lung fibrosis in radiation-treated mice. This increase started at week 8 and was clearly evident from week 16 onwards after irradiation.

Expression of iNOS and Arg-1 mRNA and protein in C57BL/6 mice after irradiation

C57BL/6 mice were analyzed for mRNA expression of iNOS and at 8, 12, 24 and 72 h, and 2, 4, 8, 16 and 24 weeks p.i. The results of the quantitative assessment of iNOS and Arg-1 mRNA expression in lung tissue after irradiation are shown in Fig. 4 and Fig. 5. iNOS mRNA expression was clearly induced in the lungs of irradiated mice at 8 h (0.073 ± 0.018, \( P < 0.05 \)) and reached maximal levels at 24 h (0.082 ± 0.020, \( P < 0.05 \); Fig. 4), then decreased to normal levels. By contrast, Arg-1 mRNA levels did not increase until week 16 (0.056 ± 0.004, \( P < 0.01 \)) and were maintained at a high level until week 24 (0.047 ± 0.002, \( P < 0.01 \); Fig. 5). Figure 6 shows that the iNOS/Arg-1 mRNA expression ratio increased nearly 50-fold at 24 h, but decreased by 0.3–0.5-fold at weeks 16 and 24. Western blot analysis confirmed these results showing that iNOS protein expression was easily detected during the first two weeks p.i. (Fig. 7).

![Fig. 6. The ratio of iNOS/Arg-1 mRNA expression. The vertical bars represent the mean ratio of iNOS/Arg-1 at every time point in radiation group. The ratio reached 46.863 at 24 h, and reduced to very low levels at 16 w and 24 w, 0.226 and 0.463 respectively.](image)

![Fig. 7. The protein expression for iNOS and Arg-1. The control group expressed little iNOS and Arg-1 proteins. The protein expression for iNOS could be detected within the first 2 w p.i., but iNOS hardly expressed at 16 w and 24 w p.i.. While Arg-1 expression has different characteristic, it gradually increased with the time, and reach the maximal at 24 w p.i..](image)
The protein level of Arg-1 in lung tissue increased with time post-irradiation (p.i.), as shown in Fig. 7.

**Immunohistochemistry**

We analyzed which cells expressed iNOS and Arg-1 in the lung tissue of mice by immunohistochemistry. iNOS and Arg-1 were seldom detectable on non-irradiated slides (Fig. 8A, D). Sections from irradiated mice at 2 weeks p.i. showed positive staining for iNOS (Fig. 8B) but little staining for Arg-1 (Fig. 8E), while positive staining for Arg-1 was easily detected at 24 weeks (Fig. 8F) but minor for iNOS (Fig. 8C). The expression of Arg-1 and iNOS was mainly localized to the cytoplasm of macrophages (indicated by arrows in Fig. 8B, F).

**DISCUSSION**

The pathogenesis of RIP has not been fully elucidated. Macrophages are one of the target cells p.i. and deformed macrophages appear at the pneumonic and fibrotic phases. Ultrastructure changes have also been observed in macrophages following irradiation in vivo, with the number and size of macrophages increasing and lysosome numbers also increasing in irradiated tissues. Moreover, the ability...
of macrophages to produce cytokines and matrix proteins has been found to change after irradiation. It can therefore be concluded that macrophages play a role in RIP, but further research is required to determine the exact mechanism. To date, few studies have specifically studied the relationship between macrophages and RIP. We focused our attention on macrophages and used the C57BL/6 mouse model. We delivered 12 Gy X-rays on the thoraces of the mice and studied the correlation between the classical and alternative activation of macrophages and RIP.

Macrophages regulate immune responses in lungs by expressing mRNA which encodes a range of pro-inflammatory and anti-inflammatory factors under different stimuli. Macrophages can be differentiated into two functional types depending on their micro-environment: caMΦ that are activated by IFN-γ and TNF (type 1 cytokines), and aaMΦ that are activated by type 2 cytokines. An important difference between caMΦ and aaMΦ is the different pathways they use to metabolize L-arginine. caMΦs undergo a respiratory burst and express iNOS, whereas aaMΦs metabolize arginine through Arg-1. Both Arg-1 and iNOS use L-arginine as a substrate. However, while iNOS generates reactive NO species with micobidal and pro-inflammatory effects, Arg-1 competes with iNOS to generate L-ornithine, an important precursor for proline that enhances collagen biosynthesis, promoting cell growth and tissue repair. This pattern of arginine metabolism is important for the balance between pro-inflammatory and anti-inflammatory responses. The induction of iNOS is usually associated with suppression of Arg-1, and vice versa, indicating the competitive nature of these alternative states of macrophage metabolism. Thus, the pathway macrophages use to metabolize arginine could alter the outcome of inflammation, resulting in acute inflammation or chronic fibrosis.

Classically activated macrophages play a leading role in acute inflammation following irradiation. In this study, we explored the induction of iNOS and Arg-1 in the lungs of mice exposed to radiation for a period of 24 weeks after treatment. We observed that the mRNA expression of iNOS was significantly enhanced within 24 h after irradiation, with the corresponding protein levels increasing during the first two weeks p.i., but then decreased to almost undetectable levels. The simplest explanation for these findings was that caMΦ become activated at this time but aaMΦ do not. iNOS is one of the characteristic markers of caMΦ. Hesse and colleagues showed, using their granuloma model, that acute inflammation was dependent on iNOS. iNOS is important not only for its antimicrobial activities, but also because it can serve as a potent anti-fibrotic mediator. Our data regarding iNOS expression were consistent with the radiation-induced iNOS expression described by Lorimore et al., in which macrophages were activated after irradiation and expressed iNOS. iNOS hydrolyzes arginine to NO, and NO damages the parenchymal and mesenchymal cells leading to exaggerated production of cytokines and interstitial edema. Based on these facts, we proposed that iNOS may be involved in radiation-induced acute pneumonitis, inducing the production of NO, which damages tissues and initiates a perpetual reaction.

The development of fibrosis has been linked to alternative macrophage activation. The term “alternative activated macrophages” was described by Gordon and colleagues in the 1990s after they noticed that an efficient way to induce mannose receptor expression was to treat macrophages with IL-4. The concept of an alternative pathway of macrophage activation by the type 2 cytokines, IL-4 and IL-13, has gained credence in the past decade and this distinct macrophage phenotype is now accepted and thought to be involved in tissue repair. Markers used to detect alternative activation include high levels of Arg-1, low levels of nitrate, and high levels of IL-4/IL-13 and IL-10 in cell culture or plasma. FIZZ1 and YM1/2 were also recently detected in aaMΦ. When macrophages are exposed to type 2 cytokines, Arg-1 is induced and degrades L-arginine to produce ornithine, a precursor of polyamines and proline, ultimately leading to collagen deposition and fibrosis. It was shown that proline was produced by aaMΦ under strict Arg-1 control in vitro. In this respect, aaMΦ have the potential to support fibrosis and tissue repair, and there is therefore growing interest in Arg-1. As our data clearly demonstrated, mRNA and protein expression of Arg-1 were significantly induced at later time points when fibrosis appeared. The induced Arg-1 probably decreased arginine availability for the iNOS reaction in activated macrophages and may down-regulate the overproduction of NO. Fibrosis is characterized by the accumulation of excessive numbers of fibroblasts, deposition of extracellular matrix proteins such as collagen, and distortion of normal tissue architecture, with accompanying evidence of injury and inflammation. Immune and cytokine mechanisms may play a role in the genesis of pulmonary fibrosis. It is interesting that type 2 cytokines, such as IL-4 and IL-13, which allow for alternative macrophage activation, have been shown to be strongly involved in the pathogenesis of lung fibrosis, supporting the hypothesis of a link between cytokine expression, alternative activation of macrophages and fibrosis development.

The inhibition of iNOS and Arg-1 may be potential ways to decrease radiation-induced pneumonitis and fibrosis. iNOS inhibitors include the non-selective inhibitors N-nitro-L-arginine methyl ester (L-NNAME) and Nω-monomethyl-L-arginine (L-NMMA), and selective inhibitors amino-guanidine (AG) and N-iminoethyl-L-lysine (L-NIL). Both non-selective and selective inhibitors attenuated acute inflammatory response. 26-28 Giliano et al. demonstrated that iNOS inhibitors AG and L-NNAME decreased intracellular NO level, and L-NNAME significantly reduced the unstable chromosome aberrations induced by radiation. NOS inhibitors exhibit their anti-inflammatory effects through...
inhibiting neutrophil infiltration, prostaglandins (PG), NO production, and the generation of peroxynitrite. NO activates cyclo-oxygenase-1 (COX-1) and COX-2, resulting in an increase in PG production, and the inhibition of NO by NOS inhibitors reduces the PG level, which attenuates inflammatory response. Nozaki Y et al. had found that treating irradiated rats with NOS inhibitor L-NAME reduced thickening of the alveolar wall and interstitial inflammatory changes on both the irradiated and nonirradiated side, and inhibited the increase in procollagen-α1 type III mRNA expression. L-NAME may be a promising way to treat irradiation induced lung injury, it deserves further studies. Another therapeutic approach is to regulate the endogenous NOS inhibitors, those are the free methylarginines. Methylarginines are metabolized to citrulline by dimethylarginine dimethylaminohydrolase (DDAH). So DDAH can be the target of intervention, and the inhibition of DDAH decreases the metabolism of methylarginines to citrulline, so indirectly block the generation of NO. There are also many arginase inhibitors, such as N-omega-Hydroxy-L-arginine (L-NOHA), N-omega-Hydroxy-nor-L-arginine (nor-NOHA), (s)-(2-boronethyl)-L-cysteine HCl (BEC) and 2 (s)-amino-6-boronohexanoic acid (ABH). Bec may be the most potent arginase inhibitor, but L-NOHA and BEC will increase NO level, it is the complications with the use of L-NOHA and BEC. So the selectivity and toxicity must be considered when administrating iNOS or Arg-1 inhibitors. And we should consider whether inhibit one of iNOS or Arg-1 will induce the production of the other, and aggravate the pneumonitis or fibrosis. Inhibitors, which can not only inhibit both iNOS and Arg-1 but also safety, will have perspective to treat radiation induced pulmonary injury.

Although iNOS and Arg-1 are the most commonly used markers for the identification of caMΦ and aaMΦ, it is reported that iNOS and Arg-1 are not solely expressed in macrophages, other cells such as dendritic cells, fibroblasts and epithelial cells were also found to secrete the two enzymes under the appropriate stimulus. The distinction between caMΦ and aaMΦ based on the iNOS–Arg-1 balance may therefore be questioned. To explore whether iNOS and Arg-1 were produced by macrophages, immunohistochemistry was performed. We observed that iNOS and Arg-1 were indeed mainly expressed in macrophages.

In summary, although the detailed mechanisms underlying the evolution and resolution of RIP have not yet been resolved, our data indicated that in our model of RIP orchestrated by macrophages, the modifications in iNOS/Arg-1 expression paralleled radiation-induced pneumonia/fibrosis. We are aware that these studies may have limitations with respect to investigating the process of RIP due to its complex pathology. However, we believe that our findings may provide important insight into potential therapeutic intervention strategies for the treatment of RIP.

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