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Low doses of radiation therapy increase the immunosuppressive profile of lung macrophages via IL-10 production and IFNγ/IL-6 suppression: a therapeutic strategy to counteract lung inflammation?

Lydia Meziani[1,2], Charlotte Robert[1,2,3], Pierre Mordant[4] & Eric Deutsch[1,2,3*]

[1] INSERM U1030, Radiothérapie Moléculaire et Innovations Thérapetuiques, Gustave Roussy Cancer Campus, Université Paris-Saclay, Villejuif, France

[2] Labex LERMIT, DHU TORINO, SIRIC SOCRATE

[3] Department of Radiation Oncology, Gustave Roussy Cancer Campus, Villejuif, France

[4] Department of vascular surgery, thoracic surgery, and lung transplantation, Bichat Hospital, Assistance Publique – Hôpitaux de Paris, INSERM U1152, Université de Paris, Paris, France

Corresponding authors: Eric Deutsch and Lydia Meziani, Gustave Roussy, INSERM U1030, 114 rue Edouard Vaillant; 94805 Villejuif, France; eric.deutsch@gustaveroussy.fr, lydia.meziani@gustaveroussy.fr

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Abstract

Patients with severe COVID-19 have a marked inflammatory state characterized by a cytokine storm syndrome. Severe pneumonia and acute respiratory distress syndrome (ARDS) have been described in 14% of the reported cases. Several therapeutic approaches are under investigation but safety and potential efficacy remain to be determined. Chest irradiation at equivalent doses below 1 Gy has been used successfully in the past to treat pneumonia**.** However, after the onset of effective antimicrobial agents, the use of low doses in the treatment of patients has been discontinued and the involved mechanism remained unknown. The objective of this study was to determine the mechanism by which low doses of radiation therapy (RT) protect the lung from inflammation**.** Nerve- and airway-associated macrophages (NAMs) and the pro- versus anti-inflammatory cytokine balance (IL6-IFNγ/IL-10) were recently shown to regulate lung inflammation. Here, we used Toll-like receptor 3 (TLR3) ligand polyinosinic:polycytidylic acid [Poly(I:C)] and lipopolysaccharide (LPS) in human lung macrophages and murine model of lung inflammation for functional assays. Our results show that irradiation of Poly(I:C)-stimulated human lung macrophages *in vitro* using low doses of RT (0.5-1 Gy) increased IL-10 secretion and decreased IFNγ production in the culture supernatant. The percentage of human lung macrophages producing IL-6 was also decreased *in vitro* with low doses of RT. Furthermore, using experimental models (LPS- and Poly(I :C)-induced lung inflammation), we showed that chest irradiation using low doses of RT significantly increased the percentage of NAMs producing IL-10, leading to lung protection from inflammation. Altogether, our data highlight one of the mechanisms by which low doses of RT regulate lung inflammation and positively favor anti-inflammatory cytokine secretion by lung macrophages. These data strongly suggest that low doses of RT could be used in order to mitigate lung inflammatory processes in situations such as COVID-19-induced ARDS.

Key words: Low dose, radiation, lung inflammation, macrophage.

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Introduction

The COVID-19 pandemic is responsible for more than 240,000 deaths and 3,4M cases worldwide as of May 2nd, 2020. The responsible agent, SARS-CoV-2, is an enveloped RNA virus of the Coronaviridae virus family. Human-to-human transmission occurs through respiratory droplets or contaminated surfaces (1). The average incubation period is 5 days, but ranges from 1–14 days. Most patients present with mild respiratory tract infection, most commonly characterized by fever (82%) and cough (81%). Severe pneumonia and acute respiratory distress syndrome (ARDS) have been described in 14% of the reported cases, and the overall mortality is around 1-2% (2). Current therapeutic approaches involve mechanical ventilation, acute supportive care, management of organ failure and antiviral therapies such as remdesivir, lopinavir–ritonavir and interferon beta-1, which are currently under investigation. The FDA has recently approved the use of hydroxychloroquine and remdesivir in COVID-19 patients but the efficacy remains disputed. Growing information also suggests that patients with severe COVID-19 have a marked inflammatory state characterized by a cytokine storm syndrome, for which anti-inflammatory agents such as anti-IL-6 receptor tocilizumab and the IL-1 receptor antagonist anakira are currently used or under investigation (3). Chest irradiation at equivalent doses below 1 Gy has been used successfully in the past to treat pneumonia, especially before the onset of effective antimicrobial agents. Clinical reports are suggestive of an early improvement of breathing difficulties within hours and a reduction of mortality (4–7). However, the involved mechanism remains unknown. Radiation therapy (RT) exerts well-known anti-inflammatory properties when used at doses up to 1 Gy (8) while exerting pro-inflammatory effects at higher doses (9), highlighting the complexity of the immunological mechanisms and the interrelationship between ionizing radiation (IR) and inflammation.

Pulmonary macrophages have been implicated in maintaining lung homeostasis by immune surveillance and clearance of dead cells, debris, and invading pathogens. The lung harbors two distinct populations of macrophages, alveolar macrophages (AMs) and interstitial macrophages (IMs) (10,11). IMs are located in the interstitium, along with dendritic cells and lymphocytes. Recently, a population of nerve- and airway-associated macrophages (NAMs) has been characterized (12). NAMs are distinct from other lung-resident macrophage subsets and highly express immunoregulatory genes. NAMs proliferated robustly after influenza infection and activation with the polyinosinic:polycytidylic acid [Poly(I:C)], and in their absence, the inflammatory response was augmented, resulting in excessive production of inflammatory cytokines and innate immune cell infiltration. NAMs function to maintain

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immune and tissue homeostasis, and regulate the infection-induced inflammation through the secretion of immunosuppressive factors such as IL-10. AMs seem to play a direct antiviral role since AM depletion yields higher viral loads. In contrast, viral infection in the absence of NAMs is associated with an excess of proinflammatory cytokines and chemokines such as IL-6, CCL2, CCL3, and CCL5.

Irradiation has a direct role in macrophage activation, depending on time, dose, and subpopulations. In the tumor stroma, high doses of IR (> 8 Gy) promote anti-inflammatory activation of macrophages (13) and low doses (< 2 Gy) alone or combined with immunotherapy induce proinflammatory activation of macrophages that favor tumor elimination (14,15). Of note, these previous studies focused on tumor associated macrophages. In the lung, irradiation at high dose impacts differentially the phenotype of alveolar and interstitial macrophages resulting in distinct local cytokine and chemokine microenvironments in the tissue and alveoli. Interestingly, a difference between lung subcompartments in the response to fibrogenic irradiation dose (16 Gy) has been reported with an immune response first in the parenchyma and then in the alveolar compartment (16).

In the present study, using Toll-like receptor 3 (TLR3) ligand Poly(I:C) or Lipopolysacharide (LPS) as inductor of inflammation (17), we investigated whether lung macrophages could be involved in counteracting lung inflammation.

Materials and methods

Human tissue samples

All patients signed an informed consent allowing the use of their surgical specimen for research purposes. The database was declared to the National Board for Informatics and Freedom (Commission Nationale Informatique et Liberté, CNIL, authorization #2217874) and to the National Institute for Heath Data (Institut National des Données de Santé, INDS, authorization #MR4316030520). Immediately after anatomical lung resection for lung cancer (n =2) and benign disease (n=1), a 2-cm wide peripheral wedge of macroscopically normal lung parenchyma was harvested on the surgical specimen and kept in sterile saline solution at 4°C.

Animals

Animal procedures were performed according to protocols approved by the Ethical Committee CEEA 26 and in accordance with recommendations for the proper use and care of laboratory animals. For the pneumonia model, female C57BL/6 mice (10 weeks old) were

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purchased from Janvier Laboratories (France) and housed in the Gustave Roussy animal facility.

LPS and Poly(I:C) administration

For the pneumonia model, mice were anesthetized (isoflurane), and either LPS (O55:B5) or Poly(I:C) in 50 µl sterile phosphate-buffered saline (PBS), or PBS alone (as control), were administered intratracheally. Mice received two doses (100 µg and 50 µg) of LPS or Poly(I:C) with a 24-hour rest period between each administration.

Irradiation procedure

Six hours after the second administration of LPS or Poly(I:C), the mice were immobilized by anesthesia (2% isoflurane) and locally irradiated at the thorax using a Varian Tube NDI 226 (X-ray machine; 250 Kev, tube current:15 mA, beam filter: 0.2 mm Cu), with a dose rate of 1.08 Gy·min−1. A single dose of 0.5 or 1 Gy was locally administered to the whole thorax.

Spectral computed tomography (CT) imaging

Mice underwent CT scan at lung level at several time points after lung irradiation. During scanning, mice were immobilized by anesthesia (2% isoflurane). The scan was operated on 3 windows that displayed axial, sagittal and coronal slices. ImageJ was used to quantify lung density on coronal slice using Hounsfield Unit (HU).

Lung tissue dissociation

Human and mouse lung tissues were digested using the Tumor Dissociation Kit (Miltenyi Biotec) for 30 minutes at 37°C and 1,500 rpm. The cells from the digested lung tissues were filtered using cell strainers (70 µm, Miltenyi Biotech) and used for subsequent experiments.

Cell culture and irradiation procedure

After washing with PBS and centrifugation (300 g, 4°C, 5 minutes), the human lung cells were suspended and cultured in DMEM-F12 supplemented with both fetal bovine serum (FBS; 10%) and penicillin/streptomycin (1%). Human lung cells were incubated in the indicated medium at 37°C and 5% CO_2 for 30 minutes. Then, the adherent cells (macrophages and monocytes) were washed using PBS, and the nonadherent cells were discarded. The adherent cells were incubated in fresh medium (DMEM-F12 containing 10% FBS and 1% penicillin/streptomycin) and stimulated with either Poly(I:C) at 1ug/mL or PBS (as control).

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Six hours after stimulation with the Poly(I:C), macrophages were irradiated using X-RAD320 (X-ray machine; 320 Kev, 4 mA) at a single dose of 0.5 or 1 Gy.

Flow cytometry

For cultured human lung macrophages staining: anti-CD169 (7-239) and anti-CD11c (REA618, Miltenyi Biotec) were used for membrane staining. Anti-IL-10 (REA842) and anti-IL-6 (REA1037, Miltenyi Biotec) were used for intracellular staining.

For mouse lung cell staining: cell suspensions were incubated with purified anti-mouse CD16/32 (clone 93, BioLegend) for 10 minutes at 4°C. For membrane staining, anti-Ly6G (REA526), anti-CD169 (REA197), anti-CD11c (REA754, Miltenyi Biotec), anti-CD11b (M1/70, BD Horizon™), anti-Ly6C (HK 1.4) and anti-D64 (X54-5/7.1, BioLegend) antibodies were used to identify NAMs ($CD11b^{+}$ $Ly6G^{-}$ $Ly6C^{-/low}$ $CD64^{+}$, $CD11c^{-}$, $CD169^{+}$). Anti-IL-10 (REA1008) was used for intracellular staining. For membrane staining, cells were incubated with the antibody panel at the adapted concentrations for 20 minutes at 4°C. Then, cells were fixed using 4% PFA for 15 minutes at 4°C and permeabilized for intracellular cytokine staining using Perm/Wash Buffer (BD Perm/Wash™). For intracellular staining, cells were pre-activated before membrane staining using Cell Activation Cocktail (with Brefeldin A, Biolegend) for 2 hours at 37°C. Samples were acquired on an LSR Fortessa X20 (BD, Franklin Lakes, NJ) with FACSDiva software, and data were analyzed with FlowJo 10.0.7 software (Tree Star, Inc., Ashland, OR).

Cytokine analysis

Cytokine concentrations in culture supernatants from *in vitro*-activated human macrophage samples were profiled. The proteins in the supernatant were diluted to 4 mg/mL and analyzed using MACSplex Human cytokine (Miltenyi Biotec) and data were analyzed with FlowLogic 7.3 software.

Statistical analysis

Statistical analyses were performed using GraphPad Prism 7. One-way ANOVA was used to detect differences among multiple treatment groups. A p value equal to or less than 0.05 was considered significant (*$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$). Data are expressed as the mean±standard error of mean (SEM).

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Results

Low doses of RT increase IL-10 production and decrease IFNγ secretion by human lung macrophages *in vitro*

Sixteen hours after human macrophage irradiation (**figure 1A**), culture supernatants were analyzed for cytokine secretion and human macrophage activation was analyzed by flow cytometry. The quantification of the supernatants showed that Poly(I:C) treatment increased IFNγ production by stimulated human lung macrophages compared to the PBS control (**figure 1B**). Interestingly, low doses of 0.5 and 1 Gy significantly decreased IFNγ secretion and increased IL-10 secretion by Poly(I:C)-stimulated human lung macrophages compared to non-irradiated Poly(I:C)-stimulated ones (**figure 1B**).

Subsequently, we performed flow cytometry analysis for human lung macrophages and observed an increase in the percentage of human lung macrophages producing IL-10 after low-dose irradiation of 0.5 Gy compared to other culture conditions (**figure 1C**). Interestingly, the percentage of human lung macrophages producing IL-6 decreased after low-dose irradiation of 0.5 and 1 Gy compared to non-irradiated human lung macrophages (**figure 1D**).

Our results show that low doses of RT are able to reprogram the proinflammatory human lung macrophages into anti-inflammatory ones and suggest that macrophage reprogramming is one of the mechanisms by which low doses of RT protect the lung from pneumonia.

Low doses of RT increase IL-10 production by NAMs in preclinical pneumonia models

Macrophages are key players in immune defenses and lung homeostasis (10). A subset of resident murine lung macrophages with an immunosuppressive role, NAMs, has been described. Authors reported that NAMs activate early after infection-induced inflammatory process and act as a main player to counteract lung inflammation via IL-10 secretion (12). We therefore hypothesized that, in the preclinical pneumonia model, low doses of RT could further stimulate IL-10 secretion by the NAMs. To evaluate this hypothesis, we treated mice with either Poly(I:C) or LPS by intratracheal administration for two consecutive days. Six hours after the second intratracheal administration, mice were irradiated at the whole thorax at 0.5 or 1 Gy. Eighteen hours after irradiation, we analyzed IL-10 production by the NAMs. Our results showed that induction of lung inflammation with either Poly(I:C) or LPS increased the percentage of NAMs producing IL-10 compared to control PBS (**figure 2**). Interestingly, low doses of RT induced the highest percentage of NAMs producing IL-10

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compared to the non-irradiated group. In the Poly(I:C)-induced infection model, 0.5 Gy seemed to induce a greater increase of IL-10$^+$ NAMs percentage compared to the 1 Gy-irradiated group (**figure 2A**), in contrast to LPS-induced infection model where 1 Gy seemed to induce a greater increase in the percentage of IL-10$^+$ NAMs (**figure 2B**) compared to 0.5 Gy.

Our data clearly show that low doses of RT further stimulate the murine NAMs to produce IL-10, suggesting that low doses of RT could counterbalance the inflammatory process in the lung.

Low doses of RT protect mice from LPS-induced pneumonia

To investigate whether low doses of RT protect mice from lung inflammation, we treated mice with LPS by intratracheal administration for two consecutive days. Six hours after the second intratracheal administration, mice were irradiated at the whole thorax at 0.5 or 1 Gy and underwent CT imaging at lung level at several time points after whole thorax irradiation. Our results showed that LPS administration increased lung density 96 hours (post administration of the first dose of LPS) compared to PBS control, suggesting the development of an inflammatory process in the lung (**figure 3**). Interestingly, irradiated lungs at 1 Gy presented less tissue density at 96 hours (post administration of the first dose of LPS) compared to other groups, suggesting that a low dose of 1 Gy protects mice from LPS-induced lung inflammation.

Altogether, our results provide a rationale for the use of a low dose of RT to treat refractory pneumonia in humans.

Discussion

In the present study, we described the major role of low-dose RT in: 1-counterbalancing the effect of the proinflammatory stimuli *in vitro* on human macrophages from the lung; 2-further stimulating the production of anti-inflammatory cytokine IL-10 by NAMs *in vivo*; and 3-protecting mouse lungs from inflammation.

The effect of low doses of RT on bone marrow-derived macrophage phenotype and on macrophage cell lines (RAW264.7 and THP-1) has already been reported elsewhere. Interestingly, a low dose of RT (0.5 Gy) affects the bone marrow-derived macrophage phenotype. However, this strongly depends on the microenvironment (18). Further, a decrease in IL-1β secretion by macrophage cell lines after low doses of RT (0.5 and 0.7 Gy) has been reported and LPS-induced TNFα production was suppressed in 0.5 Gy-irradiated RAW264.7

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(19,20). Accordingly, our results showed that human lung macrophages are significantly affected by low doses of RT (0.5 and 1 Gy) *in vitro*. Interestingly, irradiated human lung macrophages decreased IFNγ production and increased IL-10 secretion. Furthermore, the percentage of human lung macrophages producing IL-10 increased after low doses of RT, while the percentage of human lung macrophages producing IL-6 decreased. Our data clearly demonstrate that low doses of RT are directly involved in disrupting the balance of the inflammatory response in favor of an anti-inflammatory response. In preclinical pneumonia model, tissue resident NAMs have been reported to robustly respond to inflammatory stimuli early during infection and to be the main regulators of the inflammation in the lung *via* IL-10 production as a potential mechanism (12). Similarly, our data confirmed the production of IL-10 by NAMs after Poly(I:C) and LPS stimuli. Interestingly, our data showed that low doses of RT exert a marked effect on NAMs. Furthermore, we showed that a low dose of RT (1 Gy) protect mice from LPS-induce lung inflammation, suggesting that NAMs could exert a pivotal role by which low doses of RT protect the mice from lung inflammation. Whether the same NAMs described in mice exist in human lungs with the same immunosuppressive function is not yet defined. Our *in vitro* culture is not sufficient to answer this question and further immunohistological analyses of human lung tissues are required.

In human pneumonia, several old clinical studies have reported the effectiveness of low doses of RT (4–6). However, after the onset of effective antimicrobial agents, the use of low doses in the treatment of patients has been discontinued and the involved mechanism remained unknown. In the present study, we suggest that human lung macrophage reprogramming, in particular the production of the immunosuppressive IL-10 cytokines and suppression of the inflammatory signals (IL-6 and IFNγ), could be a mechanism by which low doses of RT protect from human pneumonia (**figure 4**).

Conclusion

In summary, our data highlight the effect of low doses of RT on both human and murine macrophage reprogramming and the positive regulation of the lung inflammation. The present study suggests that a single low-dose chest irradiation of 0.5-1 Gy could be an efficient strategy to resolve human lung inflammation in severe conditions such as COVID-19-induced ARDS.

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Declarations:

Competing interests: The authors declare that they have no potential conflicts of interest related to this work. E. DEUTSCH reports grants and personal fees from ROCHE GENENTECH, grants from SERVIER, grants from ASTRAZENECA, grants and personal fees from MERCK SERONO, grants from BMS, grants from MSD, outside the submitted work

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Figure legends

Figure 1. Low doses of RT increase IL-10 production and decrease IFNγ secretion by human lung macrophages *in vitro*. (**A**) Human lung macrophages were stimulated *in vitro* using the TLR3 ligand polyinosinic:polycytidylic acid [Poly(I:C)], or treated with PBS (as control). After 6 hours, stimulated macrophages were irradiated at 0.5 Gy or 1 Gy. Culture supernatants were analyzed for cytokine secretion and human macrophage activation was analyzed by flow cytometry. (**B**) Sixteen hours after human macrophage irradiation supernatants from cultured macrophages were analyzed for cytokine secretion. Data were obtained from 3 independent experiments and are represented as the mean±SEM. n=6-9, *p<0.05, **p<0.01 (one-way ANOVA). (**C** left) The percentages of IL-10$^+$ human macrophages are presented for each treatment group. (**C** right) Gating strategy to identify human lung macrophages CD169$^+$ producing IL-10. Data were obtained from one experiment and are represented as the mean±SEM. n=2. (**D** left) The percentages of IL-6$^+$ human macrophages are presented for each treatment group. (**D** right) Gating strategy to identify human lung macrophages CD169$^+$ producing IL-6. Data were obtained from one experiment and are represented as the mean±SEM. n=2. Data information: human lung macrophages were obtained from healthy lung biopsies.

Figure 2. Low doses of RT increase IL-10 production by NAMs in preclinical pneumonia models. Mice were treated with either Poly(I:C) or lipopolysccharide (LPS) by intratracheal administration for two consecutive days. Six hours after the second intratracheal administration, mice were irradiated at the whole thorax at 0.5 Gy or 1 Gy. (**A** and **B** left) Eighteen hours after irradiation, the percentages of IL-10$^+$ NAMs are presented for each treatment group. (**A** and **B** middle) Gating strategy to identify NAMs producing IL-10. (**A** and **B** right) Representative histograms of IL-10$^+$ NAMs are shown for each treatment group. Data were obtained from 2 independent experiments and are represented as the mean±SEM. n=7-8, *p<0.05, **p<0.01, ****p<0.0001 (one-way ANOVA).

Figure 3. Low doses of RT protect mice from LPS-induced pneumonia. Mice were treated with LPS by intratracheal administration for two consecutive days. Six hours after the second intratracheal administration, mice were irradiated at the whole thorax at 0.5 Gy or 1 Gy and underwent computed tomography (CT) imaging at lung level. (**A**) CT scans of lung density in different treatment groups 24 hours and 96 hours after the first dose of LPS. (**B)** Lung density quantification in different treatment groups 96 hours after the first dose of LPS. Data are

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representatives of two independent experiments and are represented as the mean±SEM. n=4, **p<0.01, ***p<0.001 (one-way ANOVA).

Figure 4. Lung macrophage reprogramming by low doses of RT during pneumonia. Low doses of RT induced the immunosuppressive profile of lung macrophages by increasing the IL-10 production and decreasing the proinflammatory cytokines such as IFNγ leading to the lung inflammation resolution.

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Figure1. Low doses of RT increase IL-10 production and decrease IFNγ secretion by human lung macrophages *in vitro*

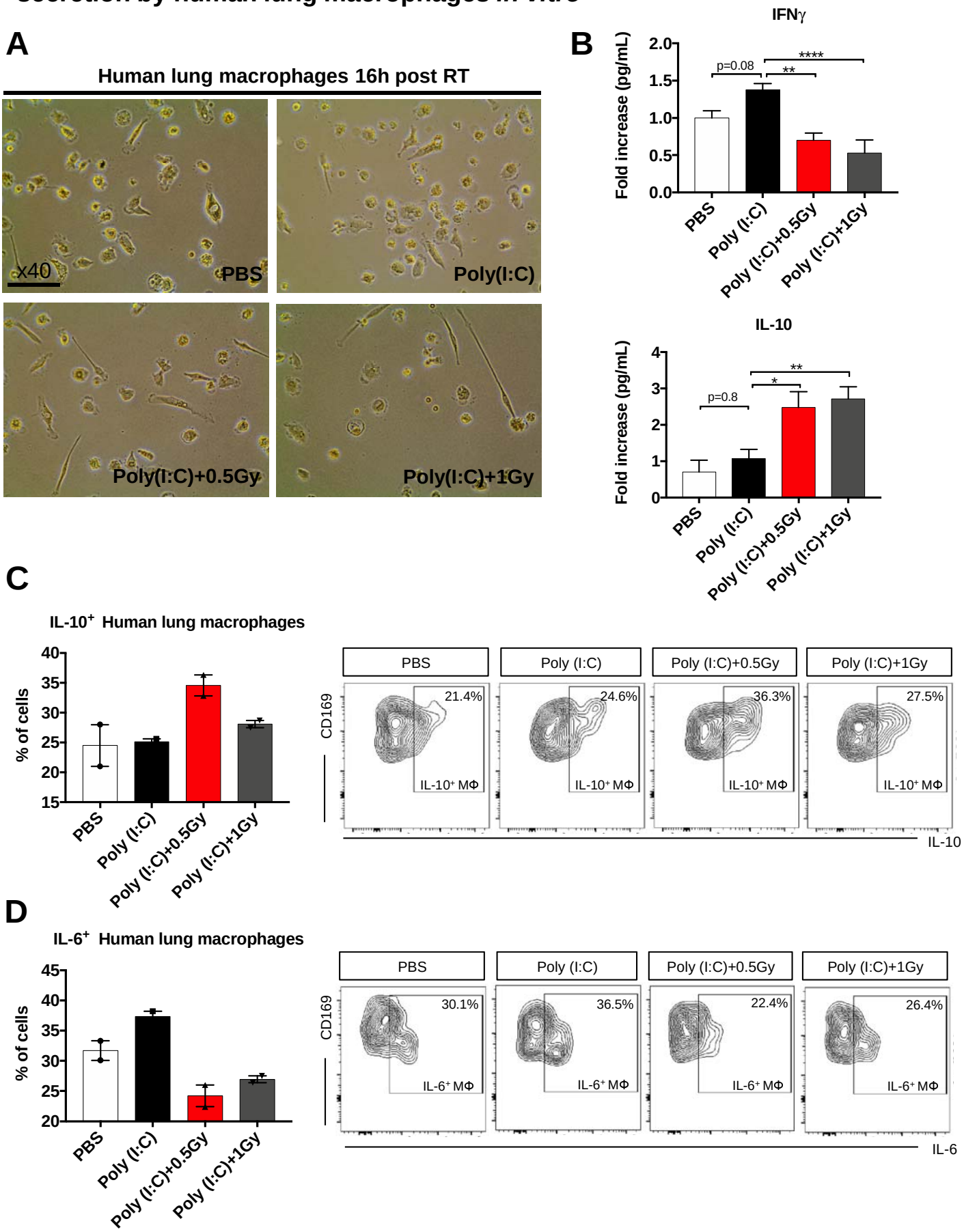

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Figure2. Low doses of RT increase IL-10 production by NAMs in preclinical pneumonia models

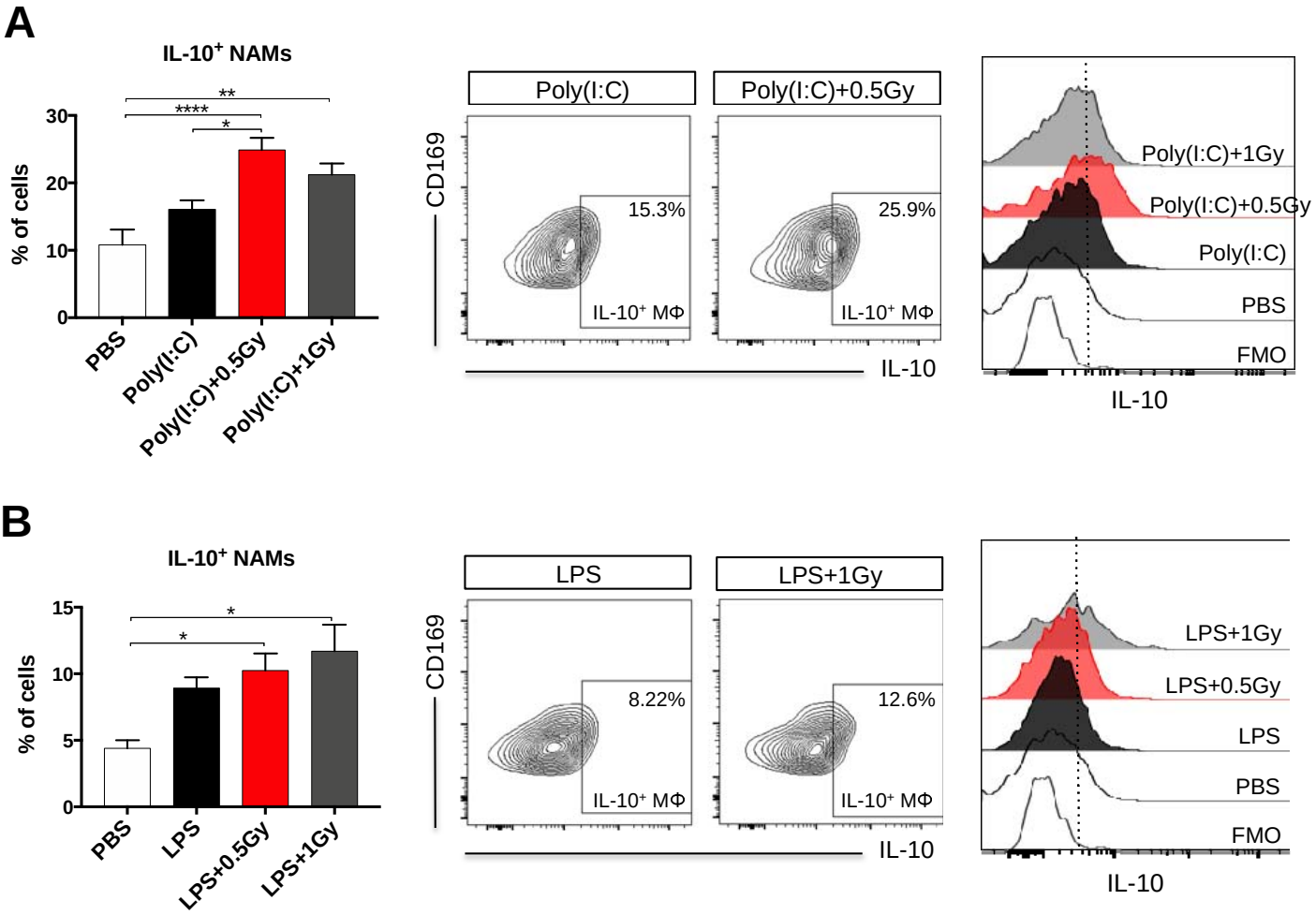

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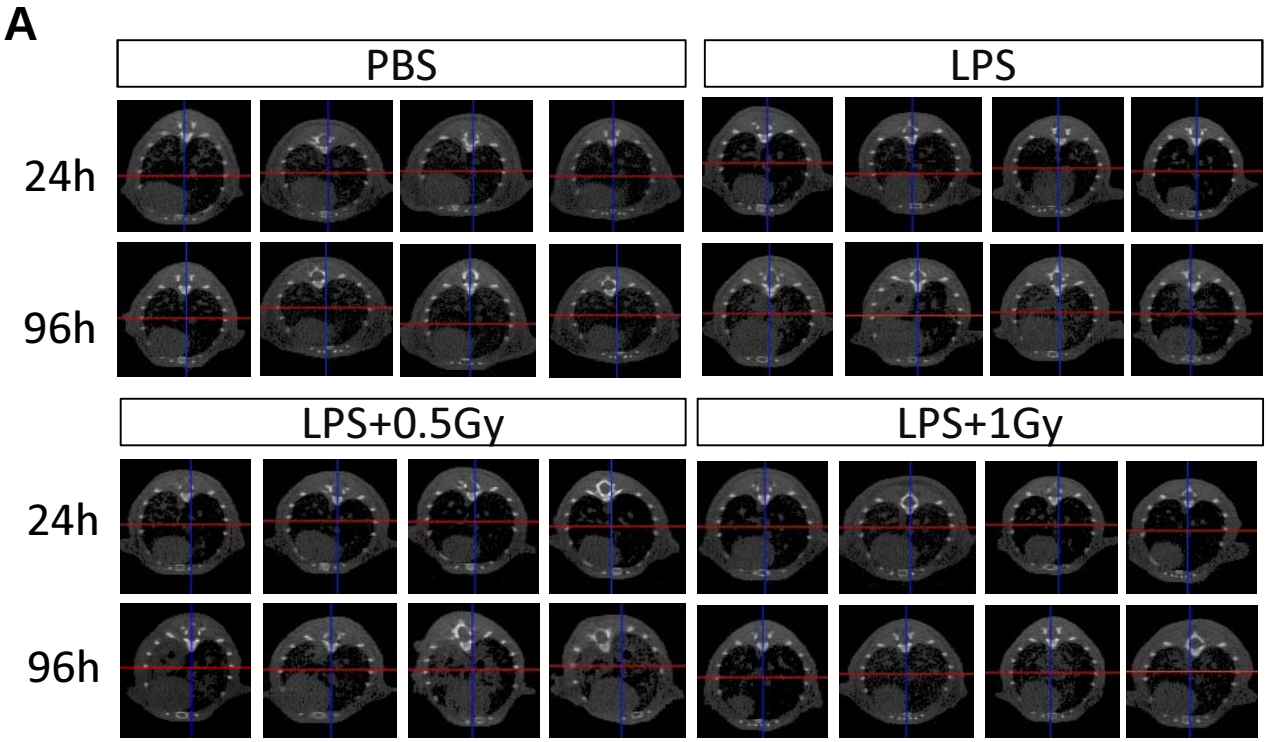
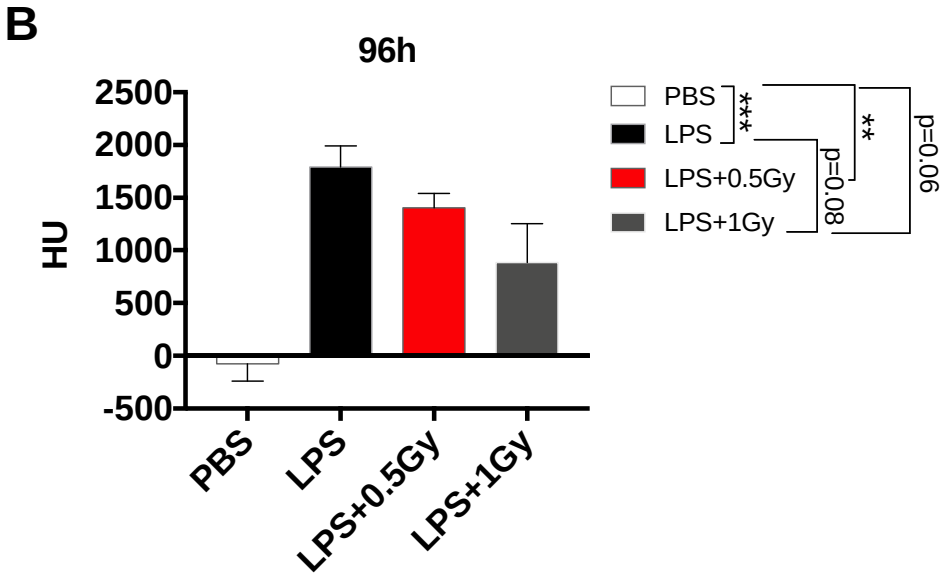
Figure 3. Low doses of RT protect mice from LPS-induced pneumonia





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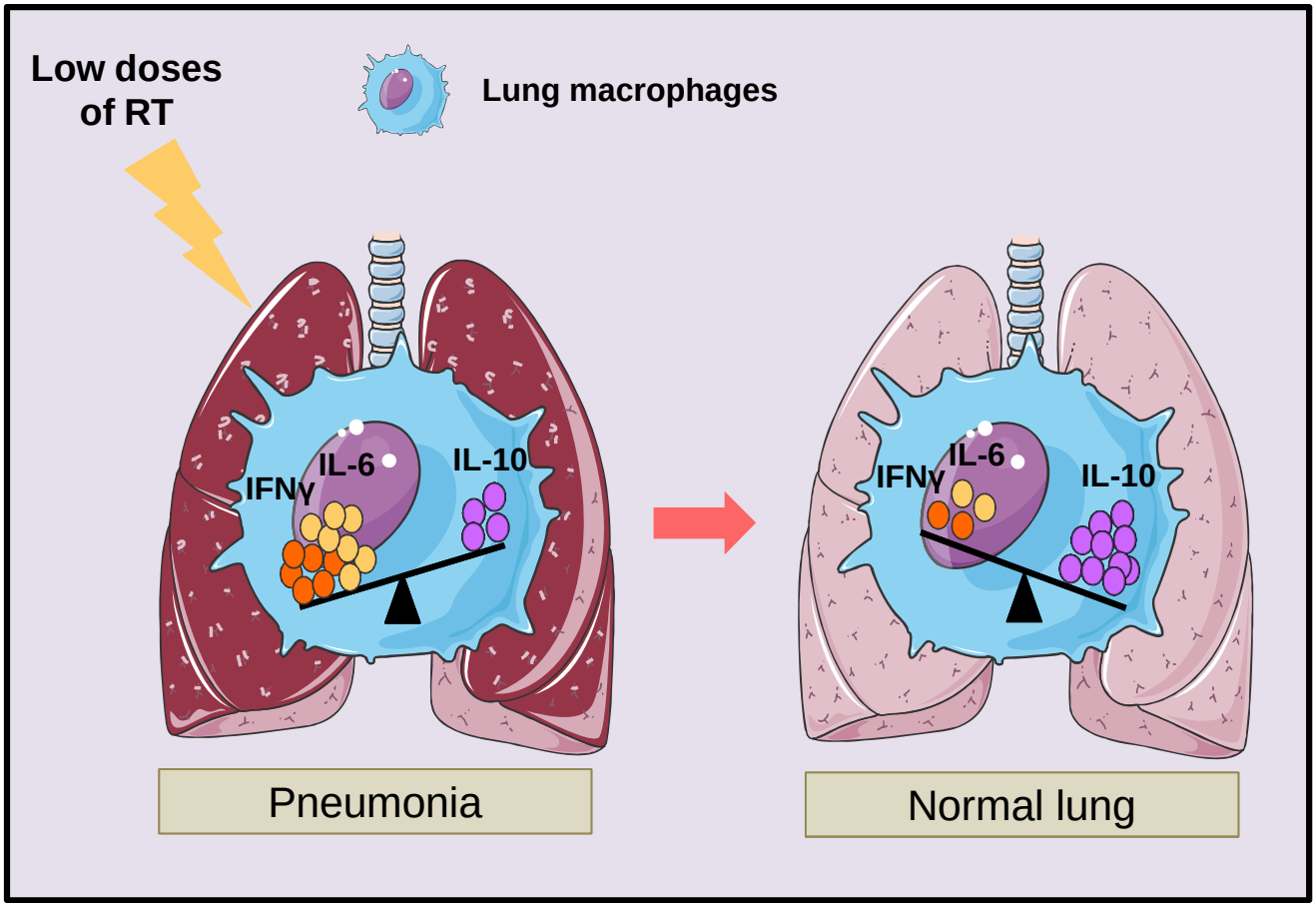
Figure 4. Lung macrophage reprogramming by low doses of RT during pneumonia