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Effects of lipopolysaccharide on the response of C57BL/6J mice to whole thorax irradiation

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ABSTRACT

Background and purpose: Inflammatory and fibrogenic processes play a crucial role in the radiation-induced injury in the lung. The aim of the present study was to examine whether additive LPS exposure in the lung (to simulate respiratory infection) would affect pneumonitis or fibrosis associated with lung irradiation.

Material and methods: Wildtype C57Bl/6J (WT-C57) and TNFα, TNFR1 and TNFR2 knockout ($^{-/-}$) mice, in C57Bl/6J background, were given whole thorax irradiation (10 Gy) with or without post-irradiation intratracheal administration of LPS (50 μg/mice). Functional deficit was examined by measuring breathing rate at various times after treatment. Real-time Reverse Transcription–Polymerase Chain Reaction (RT–PCR) and immunohistochemistry were used to analyze the protein expression and m-RNA of Interleukin-1 alpha (IL-1α), Interleukin-1 beta (IL-1β), Interleukin-6 (IL-6), Tumour Necrosis Factor alpha (TNFα) and Transforming Growth Factor beta (TGFβ) in the lung at various times after treatment. Inflammatory cells were detected by Mac-3 (macrophages) and Toluidine Blue (mast cells) staining. Collagen content was estimated by hydroxyproline (total collagen) and Sircol assay (soluble collagen). Levels of oxidative damage were assessed by 8-hydroxy-2-deoxyguanosine (8-OHdG) staining.

Results: LPS exposure significantly attenuated the breathing rate increases following irradiation of WT-C57, TNFR1$^{-/-}$ and TNFR2$^{-/-}$mice and to a lesser extent in TNFα$^{-/-}$ mice. Collagen content was significantly reduced after LPS treatment in WT-C57, TNFR1$^{-/-}$ and TNFα$^{-/-}$ mice and there was a trend in TNFR2$^{-/-}$ mice. Similarly there were lower levels of inflammatory cells and cytokines in the LPS treated mice.

Conclusions: This study reveals a mitigating effect of early exposure to LPS on injury caused by irradiation on lungs of C57Bl mice. The results suggest that immediate infection post irradiation may not impact lung response negatively in radiation-accident victims, however, further studies are required in different animal models, and with specific infectious agents, to confirm and extend our findings.

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Lung is one of the more sensitive organs to irradiation and recent concerns for the accidental or deliberate exposure of the general population to irradiation due to terrorism have resulted in studies of agents to mitigate or treat the symptoms of pneumonitis or fibrosis [1–3]. Radiation-induced pneumonitis and fibrosis are distinguished by their time of expression after irradiation and characteristic histologic changes [4]. Although the exact mechanisms involved in tissue response after lung exposure to irradiation remain uncertain, there is evidence for increased expression of inflammatory cytokines in lung taking place within hours to days to weeks after irradiation, consistent with a prolonged inflammatory response [5–10].

The aim of the present study was to examine whether additive LPS exposure in the lung (to simulate respiratory infection) would affect pneumonitis or fibrosis associated with lung irradiation. LPS, a glycolipid, is the only lipid present in the outer membrane of gram-negative bacteria. On release of LPS into the circulation, a series of tissue responses are activated that may trigger severe reactions resulting in septic shock and death. Major events that lead to LPS-induced pathogenesis include inflammatory responses via NF-kB activation and TNF signaling. The effects of TNFα are mediated through two distinct cell surface receptors, the 55-kDa type 1 TNFR1 and the 75-kDa type 2 TNFR2. TNFR1 mediates most pro-inflammatory and cytotoxic effects of TNFα, including shock and tissue injury induced by endotoxins such as LPS [11–15]. The role of TNFR2 is indirect. It aids in the recruitment of TNF to the cell membrane and passes the signal to TNFR1 or regulates the amount of TNF which is accessible to TNFR1 [16,17]. Expression of TNFR1 has been

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reported to be increased in various models of inflammatory lung injury and disease, suggesting that TNFα may be a major mediator of the pathogenic response to toxicants [18–21]. This is supported by findings that mice lacking TNFR1 are protected from lung injury induced by pulmonary irritants such as ozone, silica, bleomycin and radiation [22–24]. Furthermore, TNFR1 knockout mice fail to develop fibroproliferative lesions in lung after asbestos exposure [25].

In this study we explored the effects of LPS on pneumonitis and fibrosis and investigated the pro- and anti-inflammatory cytokine and fibrotic response produced by radiation-induced lung injury in C57Bl/6J wild type and TNFα knockout ($^{-/-}$), TNFR1$^{-/-}$and TNFR2$^{-/-}$ mice. Our previous studies had found that TNFα ($^{-/-}$) mice had reduced sensitivity to lung irradiation [10].

Methods

Mice

Eight-week old female C57-WT, TNFα$^{-/-}$, TNFR1$^{-/-}$ and TNFR2$^{-/-}$, mice were housed at the Ontario Cancer Institute/Princess Margaret Hospital small animal facility accredited by the Canadian Council on Animal Care and were treated in accordance with approved protocols. The W/T mice were purchased from JAX Laboratories. The knockout mice were bred in house from stock obtained from the laboratories of Drs. Khokha and Mak. They had been backcrossed at least 10 generations into the C57BL/6 background.Mice from each strain were divided into three experimental groups: control, radiation, radiation plus LPS. Mice were sacrificed at 12, 20 and 24 weeks post irradiation for subsequent analysis.

Irradiation

All mice were irradiated in an image-guided small animal irradiator (X-Rad 225Cx, Precision X-ray, North Branford, CT, USA). The X-ray tube in this unit is mounted on a rotating C arm with a flat panel detector opposite for image-guided set-up. The imaging characteristics of the unit have been described previously [26]. The X-ray tube was calibrated at 100 kVp, 30 mA following the AAPM TG-61 protocol [27] for radiation treatments. Specifically for this study, a 2.2 cm diameter surface collimator was used for targeting the whole lung. Further dosimetry on this collimator was done by using EBT Gaf chromic films in solid water at the depth of 0.5 cm. The dose rate at 100kVp, 30 mA (HVL: 2.95 mm Al, added filtration: 2 mm Al) was estimated as 3.13 Gy/min. Each animal was anesthetized by isoflurane inhalation and immobilized supine in a Lucite jig. Two circular lead surface collimators (OD: 4.9 cm and ID: 2.2 cm) were inserted on the surfaces of the jig to further facilitate targeting of the lung volume with minimized diaphragm in the radiation field. Animals were imaged and adjusted inside the jig for targeting the whole lung volume inside the lead surface collimators. Anatomical features were used for targeting this volume. The integrated targeting software was used to locate the mid plane of the animal, at a depth of 0.5 cm, on the iso-center of the unit. All animals were irradiated with two beams anterior-posterior (a-p) and p-a to a total dose of 10 Gy. We have found previously that this dose leads to some lethality in C57Bl mice at about 26 weeks after irradiation (data not shown). The imaging dose was estimated to be less than 1 cGy.

LPS treatment

LPS, 50 μg/mice (*Escherichia coli*, 026-B6, Sigma–Aldrich) was administered intratracheally approximately one hour after 10 Gy irradiation. Injections were performed in mice anesthetised by halothane inhalation. After an incision was made in the soft tissue overlying the trachea, 100 μl of LPS was injected via a 27 gauge needle into the visualized trachea. We found that this dose of LPS caused an early increase in cytokine levels in the normal lung of C57Bl W/T mice (data not shown).

Breathing rate

The breathing rate of mice was measured weekly starting at Day 0 up to week 24 post-irradiation using a whole body plethysmograph (Columbus Instruments, Columbus, Ohio, USA). Mice were allowed to acclimatize for one minute before each measurement. The reading for each mouse was taken for one minute and the data of at least three readings were selected manually from regions free of noise. Due to movement it was not always possible to obtain data at each time point for every animal. A minimum of 15 animals contributed to each data point for the C57-WT mice (out of a total of 30/treatment gp) and a minimum of 5 for the knockout mice (out of a total of 10/treatment gp). Data are represented as the mean ± SE.

Lung extraction

For lung extraction, mice were lethally anaesthetized and the lungs removed. The left lung was inflated with 1–2 ml of 10% formalin. The right lobes were frozen and used for the other assays involving digestion and analysis of the lung tissue. The left lobes were placed in 10% formalin and afterward embedded in paraffin, and sectioned at an average thickness of 5 μm for subsequent histological and immunohistochemical analyses. There were a minimum of six mice per treatment group at each time point.

PCR analysis

RNA extraction: Total RNA preparations were performed following manufacturers' instructions with minimal modifications, using the RNeasy® Mini Kit (Qiagen, Mississauga, Ontario, Canada). Briefly, 60 mg of tissue was removed from RNA later solution and homogenized using a rotor–stator homogenizer (IKA, Wilmington, NC, USA). The resulting RNA sample was stored at −80 °C until needed. RNA concentration was determined by spectrophotometer absorption at 260 nm and the integrity of RNA was assessed by running mini agarose gel electrophoresis.

First-strand complementary DNA (cDNA) synthesis: Reverse Transcription was carried out using an Omniscript Reverse Transcription Kit (Qiagen, Mississauga, Canada) according to the manufacturer's protocol. Oligo-dT primers were used for generating first-strand cDNA in a final reaction mix of 20 μl. Samples were stored at −80 °C for no longer than 1 week.

Real-Time RT–PCR: A master mix using the QuantiTectTM SYBR1 Green PCR kit (Qiagen, Mississauga, Ontario, Canada) was prepared according to the manufacturer's protocol. GAPDH was used as a control since we have previously shown that expression of this gene in lung is not affected by irradiation [9]. The expression of IL-1α, IL-1β, IL-6, and TNFα and TGFβ was quantified by RT-PCR (ABI Prism77001 Sequence Detection System, Applied Biosystems, and Foster City, CA, USA).

Immunohistochemistry

Tissue sections (5 μm thick) were stained with Haematoxylin and Eosin (H & E), Toluidine Blue and antibodies to detect 8-hydroxy-2′-deoxyguanosine (JaICA Cat #MOG-100P), MAC3 (BD Pharmingen Cat #550292), IL-1α (Santa Cruz #sc-9983, Santa Cruz Biotechnology), IL-1β (Santa Cruz #sc-7884), IL-6 (Santa Cruz #sc-1265), TNFα (SantaCruz #sc-1348) and TGFβ (Santa Cruz #sc-146). Immunohistochemistry was performed in the Pathology core facility of the Toronto General Hospital. Following staining, the slides were scanned using the ScanScope XT (Aperio Technologies, Vista, CA, USA). This is a brightfield scanner that digitizes the whole