Tumor Necrosis Factor Receptor 1 (TNFR1) Mediates the Radioprotective Effects of Lipopolysaccharide in the Mouse Intestine

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Abstract

Lipopolysaccharide (LPS) is radioprotective in the mouse small intestine through a mechanism that includes the synthesis of cyclooxygenase-2 (Cox-2) and prostaglandin (PGE₂). The goal of this study was to identify the intermediate steps in this process. We used wild type (WT) C57BL/6 mice and knockouts for tumor necrosis factor receptors 1 and 2 (TNFR1 -/-, TNFR2-/-) and RAG-1 -/- mice. Mice were given parenteral LPS and then subjected to 12 Gy total body γ-irradiation. The number of surviving intestinal crypts was assessed 3.5 days after irradiation using a clonogenic assay. Crypt cell apoptosis was assessed by histology. Parenteral administration of LPS induced Cox-2 expression, PGE₂ production, and radioprotection in WT and TNFR2/- mice, but not in TNFR1/- mice. TNFR1/- mice were radioprotected by administration of exogenous dimethyl-PGE₂. Immunohistochemical studies localized TNFR1 and Cox-2 expression to subepithelial fibroblasts and villus epithelial cells. Radiation-induced apoptosis was reduced by pretreatment with LPS in WT and TNFR2 -/- mice but not in TNFR1 -/- mice. In the absence of LPS, crypt survival was elevated in TNFR1 -/- when compared with WT mice. These findings demonstrate that TNFR1 function is required for LPS induced radioprotection in C57BL/6 mice, and defines an essential role for TNFR1 function in the induction of Cox-2 expression and PGE₂ production in this process. The immunolocalization of TNFR1 and Cox-2 expression to subepithelial fibroblasts following LPS administration suggests that this cell type plays an intermediate role in LPS induced radioprotection in the intestine.

Keywords: Cyclooxygenase, prostaglandin E₂, gamma radiation, epithelial cell, subepithelial fibroblast
INTRODUCTION

Gamma radiation, an extensively characterized model of intestinal injury, affects rapidly proliferating cell populations (21-23). After total body irradiation, the major sites of injury are the bone marrow and the intestinal epithelium. Exposure of mice to 7-15Gy total body irradiation results in the death of most proliferating cells in the bone marrow, but some stem cells survive and repopulate the marrow (10). With higher doses of radiation the number of surviving bone marrow stem cells is insufficient to repopulate the marrow and the animal dies. Similarly, after 8-14Gy of total body irradiation proliferating transit cells in the intestinal crypt are killed but some stem cells survive (21-23). Surviving stem cells proliferate and give rise to transit cells that form regenerative crypts and eventually repopulate the mucosa. Higher doses of radiation kill more of the stem cells and, consequently, reduce the number of regenerative crypts.

The cellular response to radiation injury in the bone marrow and intestine can be modulated by agents given prior to irradiation. Agents that decrease the amount of radiation-induced injury are said to be radioprotective. Il-1, Il-11, Il-12, TNFα, granulocyte-macrophage colony-stimulating factor, stem cell factor, prostaglandin E2 (PGE2), and lipopolysaccharide (LPS) are radioprotective in the bone marrow (16-18,27,35). In the intestine, Il-1, Il-11, PGE2 and transforming growth factor β (TGFβ) are radioprotective; given prior to radiation they increase the number of surviving crypts after radiation (24,25,33).

We recently demonstrated that LPS is radioprotective in the intestine through a mechanism that involves the induction of cyclooxygenase-2 (Cox-2) and the production of PGE2 (28). LPS-induces Cox-2 expression in villus epithelial cells and subepithelial fibroblasts but not in stem cells or other crypt epithelial cells. LPS is radioprotective in the intestine through a Cox-2 mediated mechanism but Cox-2 was not induced in stem cells. Radioprotective agents act by promoting stem cell survival. The absence of LPS-induced Cox-2 expression in stem cells suggests that the radioprotective effects of LPS are mediated through other cell types in which Cox-2 is induced and PGE2 synthesized. In this study we sought to identify the intermediate steps in LPS-induced radioprotection in the intestine.
MATERIALS AND METHODS

Animals: Mice were maintained on a 12-hour light/dark schedule and fed standard laboratory mouse chow. Animal procedures and protocols were conducted in accordance with the institutional review board at Washington University School of Medicine (St. Louis, MO). C57BL/6, TNFR1-deficient (13,19), TNFR2-deficient, and recombination-activating gene (RAG)-1-deficient, on the C57BL/6 background were purchased from The Jackson Laboratory (Bar Harbor, ME). Whole body irradiation of mice was carried out in a Gamma Cell 40 $^{137}$Cs irradiator at a dose rate of 80.7 cGy/min. Animals were killed at various times and rapidly dissected as described previously (1). The proximal jejunum was fixed in Bouin’s solution and divided into 5 mm segments before paraffin embedding and immunohistochemical analysis. The distal jejunum was snap-frozen in liquid nitrogen for Western blotting and analysis of TNFα levels. The proximal ileum was snap frozen for measurement of PGE$_2$ levels.

Crypt Survival: Crypt survival was measured in animals killed 3.5 days after irradiation as described previously (28) using a modification of the microcolony assay (23,32), with signal detection using 3, 3’-diaminobenzidine tetrahydrochloride (Sigma).

Immunohistochemistry: For immunohistochemical localization of mouse Cox-2 or TNFR1, deparaffinized sections of Bouin’s fixed tissue were incubated with either a 1:2000 dilution of rabbit anti-mouse Cox-2 (Cayman Chemical, Ann Arbor, MI) (28) or a 1:50 dilution of Armenian hamster anti-mouse TNFR1 monoclonal antibody (BD Pharmingen, San Diego, CA). Endogenous peroxidase activity was quenched with 3% hydrogen peroxide. Nonspecific staining was blocked by incubating sections sequentially in blocking buffer (NEN, Boston, MA), avidin-D solution (Vector Laboratories, Burlingame, CA), biotin-D (Vector Laboratories), and
either normal donkey serum for Cox-2 (Sigma, St. Louis, MO) or normal goat serum for TNFR1 (Sigma) before incubating slides overnight at 4°C with the primary antibody. Sections incubated with preimmune rabbit serum (for Cox-2), preimmune goat serum (for TNFR1) or without primary antibody served as negative controls. A 1:2000 dilution of biotinylated donkey anti-rabbit immunoglobulin G (Jackson Immuno Research Laboratory, West Grove, PA) was used as the secondary antibody for Cox-2, and a 1:2000 dilution of biotinylated goat anti-Armenian hamster immunoglobulin G (Jackson Immunoresearch) was used as the secondary antibody for TNFR1. Signal amplification was carried out with the indirect biotinyl tyramide system (NEN). The amplified antibody signal was detected using 3,3′ diaminobenzidine tetrahydrochloride.

**Sodium Dodecyl Sulfate-Polyacrylimide Gel Electrophoresis and Western blot analysis of Cox-2:** Distal jejunum tissues were assayed for Cox-2 by Western blotting. Samples for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were homogenized in a proteinase inhibitor cocktail as described previously (28). A rabbit polyclonal antibody against mouse Cox-2 (Cayman Chemical) was used to detect bands corresponding to Cox-2. Bound antibody was revealed using a donkey anti-rabbit immunoglobulin G linked to horseradish peroxidase and an ECL kit (Amersham, Arlington Heights, IL) with fluorographic detection on BioMax ML film (Kodak, Rochester, NY).

**Cox-2 Inhibitor:** NS-398 (Biomol, Plymouth Meeting, PA) was reconstituted as a concentrated stock solution in dimethylsulfoxide. Working solutions were made by diluting NS-398 in sterile 5% sodium bicarbonate immediately before use, and administering a dose of 1 mg/kg intraperitoneally (12).

**16, 16-dimethyl PGE2:** 16, 16-dimethyl PGE2 (Sigma, St. Louis, MO) was dissolved in ethanol and diluted into sterile 5% sodium bicarbonate immediately before use. Animals treated with
dmPGE₂ received a single dose (0.5 mg/kg) injected intraperitoneally at 1 hour before irradiation (12 Gy).

**Measurement of TNFα levels:** Extracts from distal jejunum were obtained from supernatants of tissue lysates prepared in proteinase inhibitor cocktail (30). Samples were assayed by a TNFα-specific enzyme-linked immunoassay (B-D Pharmingen, San Diego, CA) according to the manufacturer’s directions.

**Measurement of PGE₂ levels:** PGE₂ concentrations in extracts from proximal ileum were determined by a PGE₂-specific enzyme-linked immunoassay (Cayman Chemical, Ann Arbor, MI) according to the manufacturer’s directions.

**Apoptosis:** The proximal jejunum was obtained from mice 6 hours after of 12 Gy whole body irradiation, fixed overnight in Bouin’s solution, and embedded in paraffin. Sections were stained with hematoxylin and eosin, and the number of apoptotic cells per crypt section was assessed by morphological criteria as described by Potten and Grant (26).

**Lipopolysaccharide:** Lipopolysaccharide (LPS) from Escherichia coli K-235 was purchased from Sigma (St. Louis, MO). Animals treated with LPS received a single dose (0.5 mg/kg) injected intraperitoneally.
RESULTS

We had previously demonstrated that LPS is radioprotective in the small intestine in FVB/N mice (28). Treatment with LPS (0.5 mg/kg given 14 hours prior to irradiation) increases the number of surviving crypts per cross section from 10 to 20. In FVB/N mice the radioprotective effects of LPS in the intestine are mediated through Cox-2 and PGE2. In the current study we used a series of knockout mice on the C57BL/6 background to define the intermediate steps in the induction of radioprotection by LPS in the mouse small intestine. In untreated C57BL/6 mice there were 12 surviving crypts per cross section 3.5 days after $\gamma$-irradiation (12 Gy) (Figure 1). In C57BL/6 mice given 0.5mg/kg of LPS 14 hours prior to radiation there were 26 surviving crypts per cross section. Thus, the fold increase in crypt survival with LPS was similar in FVB/N and C57BL/6 mice although the baseline crypt survival was somewhat higher in the C57BL/6 strain. We utilized RAG1-/- mice to determine if the radioprotective effects of LPS are mediated through T-lymphocytes or B-lymphocytes. Baseline crypt survival in the RAG 1 -/- mice was somewhat lower then in C57BL/6 controls; however, LPS induced a three-fold increase in crypt survival in the RAG1 -/- mice demonstrating that the radioprotective effects of LPS are not mediated through T-lymphocytes or B-lymphocytes.

Tumor necrosis factor $\alpha$ (TNF $\alpha$) is a candidate intermediate molecule for LPS-induced radioprotection, as LPS is known to induce TNF$\alpha$ production, which in turn may induce Cox-2 expression and PGE2 production(9). In addition to these observations, previous studies have demonstrated that TNF$\alpha$ is radioprotective in the bone-marrow (18). The biologic effects of TNF$\alpha$ are mediated through binding to two distinct receptors. It is not known which TNF receptor(s) are required for Cox-2 induction in the intestine in response to LPS. Signaling
through TNFR1 is associated with apoptosis and cytokine production; whereas signaling through TNFR2 is associated with proliferation and enhanced cell survival. To define the role of TNFα in LPS-induced radioprotection in the intestine and to identify the relevant TNF receptor(s) for this process, we measured crypt survival in TNFR1 -/- and TNFR2 -/- mice in the presence and absence of LPS. Baseline crypt survival in TNFR1 -/- mice was more than double that seen in the C57BL/6 WT mice; however, treatment with LPS did not induce a further increase in crypt survival in the TNFR1 -/- mice. In contrast, baseline crypt survival in TNFR2 -/- mice was similar to that seen in WT C57BL/6 mice and administration of LPS-induced a 2.7 fold increase in crypt survival. These data demonstrate that the absence of TNFR1 results in a significant increase in baseline crypt survival and that TNFR1 is essential for LPS-induced radioprotection in the intestine. Conversely, the absence of TNFR2 has no effect on either the baseline response to radiation injury or LPS-induced radioprotection.

These data suggest that TNFα production after LPS administration and subsequent ligation of the TNFR1 are essential for LPS-mediated radioprotection. To support this observation, we measured TNFα levels in the intestines of control mice and mice treated with parenteral LPS. TNFα protein levels increase 5-6 fold in response to LPS in both WT and TNFR1 -/- mice (Figure 2).

We previously demonstrated that the radioprotective effects of LPS in the small intestine are mediated through the induction of Cox-2 and increased production of PGE2 (28). To better define the roles TNFα and TNFR1 play in LPS-induced radioprotection in the intestine, we measured PGE2 levels in the intestine of WT, RAG1 -/-, TNFR1 -/-, and TNFR2 -/- mice given LPS (Figure 3). Administration of LPS resulted in an increase in intestinal PGE2 levels in WT, RAG 1 -/-, and TNFR2 -/- mice. In TNFR1 -/- mice baseline intestinal PGE2 levels were similar to those in WT mice; however, administration of LPS did not increase PGE2 levels. In other
mouse strains LPS administration induced the expression of Cox-2 in the intestine (13). We observed that LPS administration also induces Cox-2 protein expression in the intestine of C57BL/6, RAG-1 -/- and TNFR2-/- (Figure 4). However, in TNFR1 -/- mice there is little expression of Cox-2 in the intestine at baseline and no increase with LPS. These data suggest that the failure of LPS to induce radioprotection in TNFR1 -/- mice is due to the inability of LPS to induce increased PGE₂ production in these mice, and demonstrate an essential role for TNFR1 in the induction of Cox-2 expression and PGE₂ production in the intestine in response to LPS.

To identify the cell types involved in the intermediate steps of LPS-induced radioprotection in the intestine, we performed immunohistochemistry for Cox-2 and TNFR1. Untreated WT intestine shows little Cox-2 staining (Figure 5A); whereas after treatment with parenteral LPS, Cox-2 expression is seen in villus epithelial cells and subepithelial fibroblasts (Figure 5B). At higher power (Figure 5C) staining of fibroblasts immediately adjacent to crypt epithelial cells is seen. In TNFR1 -/- mice there is little Cox-2 expression either at baseline (Figure 5D) or after treatment with LPS (Figure 5E). We observed TNFR1 expression in crypt and villus epithelial cells and in subepithelial fibroblasts (Figure 6). Villus epithelial cells and subepithelial fibroblasts both express TNFR1 at baseline and express Cox-2 after treatment of with LPS, however given the proximity of subepithelial fibroblasts to the crypt epithelial cells, we favor this cell type for playing a role in the intermediate steps of LPS-induced radioprotection in the intestine.

To further support the position of TNFR1 as upstream of PGE₂ mediated events in LPS-induced radioprotection in the intestine, we examined the ability of TNFR1-/- mice to be protected from radiation injury by the administration of exogenous dimethyl-PGE₂. In both WT and TNFR1-/- mice the administration of dimethylPGE₂ resulted in a 2-3 fold increase in the number of surviving crypts per cross section (Figure 7), demonstrating that PGE₂ is
radioprotective in TNFR1 -/- mice and the failure of LPS to induce a radioprotective response in TNFR1 -/- mice is not due to the inability to respond to PGE2. These observations place TNFR1 mediated events upstream of Cox-2 induction and PGE2 production in the cascade of events leading to LPS-induced radioprotection in the intestine.

To address the mechanism of the increased crypt survival in TNFR1 -/- mice and determine if the increased baseline crypt survival in TNFR1 -/- mice was Cox-2 dependent, TNFR1 -/- mice and WT mice were treated with a specific Cox-2 inhibitor NS-398 and irradiated. Administration of NS-398 had no effect on the baseline crypt survival in either wild type or TNFR-1 -/- mice indicating that the increased crypt survival in TNFR1 -/- mice is not mediated through events dependent upon Cox-2 (Figure 7).

We next sought to define the biologic mechanism for LPS-induced radioprotection. The epithelial response to radiation injury includes cell cycle arrest, DNA repair and apoptosis (23). We had previously demonstrated that prostaglandins influence the apoptotic response to radiation in epithelial cells (4). We next sought to determine if the radioprotective effects of LPS are mediated through effects on apoptosis. WT, RAG-1-/-, TNFR1 -/- and TNFR2-/- mice were given either LPS or saline; fourteen hours later all the mice received 12 Gy γ-irradiation. At six and twenty-four hours after irradiation the animals were sacrificed and the number of apoptotic epithelial cells per small intestinal crypt was assessed. The number of apoptotic cells in irradiated WT, RAG-1-/- and TNFR2-/- mice were similar, but the number of apoptotic cells in irradiated TNFR1 -/- mice was significantly lower (Figure 8). The 50% reduction in radiation-induced apoptosis in the TNFR1 -/- mice compared with WT mice is consistent with the 2.5 fold increase in crypt survival. These data are consistent with the suggestion that the increased baseline crypt survival in TNFR1 -/- mice is the result of decreased radiation-induced apoptosis, and provide support for a role of TNFR1 in radiation-induced apoptosis. Administration of LPS
dramatically reduced the number of apoptotic cells per crypt in WT, RAG-1-/- and TNFR2-/- mice but had no effect on the number of apoptotic cells in the TNFR1 -/- mice. Results at six and twenty-four hours were similar. The number of apoptotic cells at 6 hours after irradiation is shown in Figure 8, similar results were seen at 24 hours. These data demonstrate that in WT mice the radioprotective effects of LPS are paralleled by a decrease in radiation-induced apoptosis; moreover, the failure of LPS to induce radioprotection in TNFR1 -/- mice is paralleled by a failure to decrease radiation-induced apoptosis. One potential interpretation of these data is that TNFR1 is essential for LPS-induced Cox-2 expression and PGE₂ synthesis reducing radiation-induced apoptosis and, as a result, increasing crypt survival.
DISCUSSION

We previously demonstrated that parenteral LPS is radioprotective in the mouse intestine through a mechanism mediated by the induction of Cox-2 and the production of PGE₂ (28). LPS-induced Cox-2 expression in villus epithelial cells and subepithelial fibroblasts but not in crypt epithelial cells. Radioprotection reflects the survival and proliferation of epithelial stem cells after radiation injury; therefore the absence of LPS-induced Cox-2 expression in epithelial stem cells suggested that the radioprotective effects of LPS were mediated through other cell types in which Cox-2 was induced and PGE₂ synthesized.

In this study, WT C57BL/6 mice and knockout mice on the C57BL/6 background were used to explore the intermediates in LPS-induced radioprotection. We found that LPS is not radioprotective in TNFR1 -/- mice. There are two ligands for TNFR1, TNFα and TNFβ, or soluble lymphotoxin (LT). In adult mice LT is expressed exclusively by lymphocytes. We favor TNFα as the relevant ligand for TNFR1 in LPS-induced radioprotection as both RAG1-/− mice (lacking T-lymphocytes and B-lymphocytes) and LT deficient mice are protected from radiation induced injury following LPS administration (data not shown). Consistent with TNFα being the relevant TNFR1 ligand for this process, LPS administration increases TNFα expression in the intestine of both WT and TNFR1 -/- mice.

To determine the sequence of events in the cascade leading to protection from radiation injury following LPS administration, we examined Cox-2 expression and PGE₂ production in the intestine of TNFR1-/− mice following LPS administration. LPS administration does not enhance Cox-2 expression or increase PGE₂ production in the intestine of TNFR1-/− mice. To further support the role of TNFR1 as being upstream of PGE₂ in this cascade, we observed that TNFR1-/− mice are protected from radiation-induced injury by the administration of exogenous dmPGE₂. Although these studies define the molecular intermediates involved in LPS-induced
radioprotection in the mouse intestine they do not absolutely define the cell types involved. Currently we favor TNFR1 expressing subepithelial fibroblasts as performing an intermediate role in this LPS-induced radioprotection cascade. We have not identified the relevant cell(s) producing TNFα in this cascade. Macrophages are attractive candidates for TNFα production in response to LPS, as they respond to LPS through CD14 and toll like receptors and produce TNFα in response to LPS (9, 34). Intestinal macrophages are known to express CD14 although the level of CD14 expression on intestinal macrophages is lower than that of peripheral blood monocytes (2).

In both FVB/N and C57Bl/6 mice parenteral LPS induces Cox-2 expression in villus epithelial cells and subepithelial fibroblasts. TNFR1 is expressed in the plasma membranes of many mammalian cells including intestinal epithelial cells and fibroblasts. Incubation of either intestinal epithelial cells or fibroblasts with TNFα results in increased expression of Cox-2 (9). Thus, it is possible that TNFα secreted by macrophages binds to TNFR1 on fibroblasts and villus epithelial cells resulting in the induction of COX-2. Why TNFα secretion does not also induce Cox-2 in other cell types which also express TNFR1, for example crypt epithelial cells, is less clear. Previous studies have demonstrated that TNFR1 signals through TRAF-2 and NIK leading to NFKB activation, and through the activation of MAP kinases both of which may lead to Cox-2 expression (3, 6, 11). It may be that some cell types, such as crypt epithelial cells, lack the intracellular signaling mechanisms required for the induction of Cox-2 expression by TNFα.

Prostaglandins are rapidly metabolized; as a consequence they usually act on cells immediately adjacent to the cells in which they are produced. Villus epithelial cells are physically far removed from crypt epithelial stem cells and it is unlikely that prostaglandins made by villus cells would affect crypt stem cells. However, subepithelial fibroblasts are immediately adjacent to stem cells and prostaglandins produced by Cox-2-expressing
subepithelial fibroblasts could affect the stem cell response to radiation injury. Cox-2 expression in gastrointestinal myofibroblasts is found in murine colitis (29) and human gastric ulcers (8). Thus one potential sequence of events is that LPS induces TNFα production by intestinal macrophages. The binding of TNFα to TNFR1 on subepithelial myofibroblast results in the induction of Cox-2 and the production of PGE2, which in turn, binds to receptors on stem cells and thus affects the epithelial response to radiation injury. We have developed data elsewhere demonstrating that the effects of PGE2 on the stem cell response to radiation are mediated through the EP2 receptor (5).

TNFα is radioprotective in the bone marrow (18); whether or not TNF α is radioprotective in the intestine had not previously been addressed. The data presented here suggest that exogenous TNFα is likely to be radioprotective in the intestine through binding to TNFR1 with the induction of Cox-2 and PGE2 production. This study also raises the possibility that the radioprotective effects of LPS and TNFα in the bone marrow are mediated through TNFR1, Cox-2 and PGE2.

The epithelial response to radiation injury reflects a complex series of biologic events. γ-radiation damages DNA resulting in cellular responses including cell cycle arrest, DNA repair and a choice between apoptosis and survival (23). Radioprotective agents could act through effects on any of these responses. Radioprotection could be induced by agents that protect against DNA damage, that shorten cell cycle arrest, that promote DNA repair or diminish apoptosis. TNFα induces epithelial apoptosis (20) and PGE2 affects radiation-induced apoptosis (4); therefore we sought to determine if the radioprotective effects of LPS or TNFα are the products of effects on apoptosis. Treatment with parenteral LPS reduced radiation-induced intestinal epithelial apoptosis at six and twenty-four hours after 12 Gy γ-radiation. LPS induces
Cox-2 expression and PGE₂ production and we have previously demonstrated that PGE₂ diminishes radiation-induced apoptosis in the intestinal epithelium (4). Irradiated cells that sustain significant DNA damage accumulate at the G2 phase of the cell cycle. One potential model for the LPS-induced decrease in apoptosis and increase in crypt survival is that PGE₂, synthesized in response to LPS, allows injured epithelial cells to escape from G2 arrest or to otherwise avoid apoptosis and go on to generate viable crypts.

In irradiated TNFR1 -/- mice baseline intestinal crypt survival is increased compared to that in wild type mice. To address the mechanism of the increased crypt survival in TNFR1 -/- mice we measured radiation-induced apoptosis and found that the number of apoptotic cells per crypt was significantly less in TNFR1 -/- mice compared with wild type mice. This suggests that in wild type mice the binding of TNFα to TNFR1 mediates radiation-induced apoptosis. An association between the apoptotic effects of TNFα and signaling through TNFR1 has been demonstrated; parenteral injection of TNFα in mice induced enterocyte apoptosis through TNFR1 (20). TNFR1 -/- mice had also been demonstrated to have diminished intestinal epithelial apoptosis four hours after exposure to 5 Gy (γ-radiation (7). Thus, in this system TNFα has two conflicting effects on radiation-induced apoptosis. In the absence of LPS, TNFα binding to TNFR1 mediates radiation-induced apoptosis. However, in the presence of LPS the production of TNFα is increased and TNFα binding to TNFR1 results in Cox-2 induction, PGE₂ synthesis and a reduction in radiation-induced apoptosis. In LPS-treated mice the anti-apoptotic effects of PGE₂ outweigh the direct pro-apoptotic effects of TNFα. The explanation for this distinction may relate to the cells producing the TNFα and their anatomic position in the intestinal mucosa. LPS stimulates macrophages to release TNFα which binds to TNFR-1 on the adjacent subepithelial myofibroblasts. This results in Cox-2 expression and PGE₂ production. In
contrast, the TNFα produced in response to radiation appears to bind to TNFR-1 on epithelial cells resulting in apoptosis. The cellular source of the TNFα produced in response to radiation is not clear but it may be the epithelial cells themselves.

LPS protected against radiation-induced injury in TNFR2-/- mice, and in fact we observed an augmented crypt survival in TNFR2-/- mice after LPS administration when compared to wild type mice. Previous studies have demonstrated that LPS-induced TNFα production in TNFR2-/- mice remains intact (19). The augmented crypt survival in TNFR2-/- mice following LPS administration could be explained by the increased availability of ligand for binding TNFR1 in the absence of TNFR2, or by the loss of TNFR2 effects which normally oppose those of TNFR1 as previously demonstrated in other animal models (19). TNFα binding to TNFR2 modulates both the level of inflammation and epithelial proliferation in response to injury in mouse models of colitis (15). The importance of signaling through TNFR2 in epithelial proliferation in response to inflammation is consistent with the established role of TNFR2 signaling in cell proliferation and cell survival. Similarly the importance of TNFR1 signaling in inducing Cox-2 expression is consistent with the established role of TNFR1 signaling in gene transcription.

In this study we have demonstrated that TNFα and TNFR1 mediate the induction of Cox-2 by LPS. Moreover we have found that the LPS-induced increase in radioprotection is paralleled by a decrease in radiation-induced apoptosis. These findings suggest this sequence of events: LPS stimulates CD14 and toll like receptor bearing cells inducing the secretion of TNFα which binds to TNFR1 on subepithelial fibroblasts inducing the synthesis of Cox-2 and the production of PGE2. PGE2 produced by subepithelial fibroblasts acts locally and binds to EP receptors on adjacent epithelial stem cells reducing radiation-induced apoptosis resulting in increased stem cell survival and increased crypt survival. We have demonstrated that TNFR1 is essential for the
radioprotective effects of LPS in the intestine. The TNFR1 dependent events occurring in the
cascade leading to LPS-induced radioprotection occur upstream from Cox-2 induction and PGE₂
production. Our observations suggest that LPS or other agents that induce TNFα production
may be useful in protecting the intestinal epithelium in patients receiving radiation therapy.
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Figure 1: LPS treatment before radiation increases crypt survival in C57BL/6 WT, RAG1 -/-, and TNFR2 -/-, but not in TNFR1 -/- mice. LPS (0.5mg/kg) was given intraperitoneally 14 hours before irradiation (12Gy). Mice were killed 3.5 days after irradiation, and the number of surviving crypts per cross section was determined. Data are the mean ± SEM for 4-12 animals. *p<0.001 compared with WT irradiated control; **p<0.001 compared with RAG1 -/- irradiated control; ***p<0.002 compared with TNFR2 -/- irradiated control.

Figure 2: LPS treatment increases TNFα production in the small intestines of WT and TNFR1 -/- mice. Mice were treated with vehicle (control) or LPS (0.5mg/kg). After 1 hour mice were killed and the TNFα content of the small intestine was determined by enzyme immunoassay. Data are the mean ± SEM of 4 animals. *p<0.001 compared with WT control; **p<0.001 compared with TNFR1 -/- control.

Figure 3: LPS treatment increases PGE2 production in WT, RAG1 -/-, and TNFR2 -/- mice, but not in TNFR1 -/- mice. Mice were treated with vehicle (control) or LPS (0.5mg/kg). After 1 hour mice were killed and the PGE2 content of the small intestine was determined by enzyme immunoassay. Data are the mean ± SEM of 4-8 animals. *p<0.001 compared with WT control; **p<0.001 compared with RAG1 -/- control; ***p<0.001 compared with TNFR2 -/- control.

Figure 4: Detection of COX-2 in the small intestines of control and LPS-treated WT, RAG-1-/-, TNFR-1-/-, and TNFR-2/- mice by Western blotting. LPS (0.5mg/kg) was given to mice 1 hour before death, intestinal tissue was harvested, and the expression of COX-2 protein in tissue lysates was determined by Western blotting using rabbit anti-mouse antibody. A mouse macrophage cell line treated with LPS was used as positive control.

Figure 5: Immunohistochemical localization of COX-2 in the proximal jejunum of control and LPS-treated WT and TNFR1 -/- mice. (A) Control WT mice showed COX-2 staining in a few villus epithelial cells. (B) One hour after LPS (0.5mg/kg intraperitoneally) treatment, there was COX-2 staining of villous epithelial cells and subepithelial fibroblasts. (C) Higher power view of intestine from LPS-treated mouse showed staining of fibroblasts between crypts. Control (D) and LPS-treated (E) TNFR1 -/- mice demonstrated COX-2 staining in scattered villus epithelial cells. (original magnification A, B, D, E, 400X; C, 1000X).
Figure 6: Immunohistochemical localization of TNFR1 in the proximal jejunum of control C57BL/6 WT mice shows TNFR1 staining in villus and crypt epithelial cells and in subepithelial fibroblasts. (B) Higher power view of intestine shows TNFR1 staining of a crypt epithelial cell (arrow) and a fibroblast between crypts. (C) Negative staining with isotype control antibody. (original magnification A, 400X; B, 1000X; C, 400X).

Figure 7: Exogenous dmPGE-2 is radioprotective in WT and TNFR1 -/- mice, but the elevated level of baseline crypt survival in irradiated control TNFR1 -/- mice is not mediated through COX-2. Mice received vehicle, (irradiated control), 16,16-dimethyl PGE2 (0.5mg/kg) or NS-398 (1mg/kg) 1 hour before irradiation (12 Gy). Mice were killed 3.5 days after irradiation, and the number of surviving crypts per cross section was determined. Data are the mean + SEM for 3 animals. *p<0.001 compared with WT irradiated control; **p<0.002 compared with TNFR1 -/- irradiated control.

Figure 8: LPS treatment reduces the number of apoptotic cells per crypt in WT, RAG-1-/-, and TNFR-2-/- mice, but not in TNFR-1-/- mice. Irradiated control TNFR-1-/- mice have significantly less epithelial apoptosis than WT mice. Irradiated control RAG-1-/- and TNFR-2-/- mice had significantly more epithelial apoptosis than WT mice. Mice received vehicle or LPS (0.5 mg/kg) 14 hours before irradiation (12 Gy). Mice were killed 6 hours after radiation, and the number of apoptotic epithelial cells per crypt was determined. Data are mean + SEM for 4 animals. * P < 0.001 compared with WT unirradiated control; ** p <.002 compared with WT unirradiated control; *** p <.001 compared with RAG-1-/- irradiated control; **** p <.001 compared with TNFR-2-/- irradiated control. WT and TNFR-1-/- mice were also killed 24 hours after radiation and had the same number of apoptotic cells per crypt as mice killed at 6 hours after radiation.
Figure 1
Figure 2
Figure 3
Figure 4
Figure 5
Figure 6
Figure 7

- WT
- TNFR1-/-

Surviving Crypts/ Cross Section

- Irrad Control
- NS-398 + 12Gy
- dmPGE2 + 12Gy
**Figure 8**