Flagellin or Lipopolysaccharide Treatment Modified Macrophage Populations after Colorectal Radiation of Rats

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Received February 20, 2013; accepted April 16, 2013

ABSTRACT

Radiation-induced acute intestinal toxicity remains a major limitation to the delivery of tumoricidal doses of colorectal irradiation. Recent reports indicate that Toll-like receptor (TLR) agonists TLR4 and TLR5 protect against toxicity due to intestinal irradiation. The phenotype (M1 or M2) of macrophages expressing TLRs may play a role in tissue repair. The aim was to investigate whether administration of TLR4 agonist lipopolysaccharide (LPS) or TLR5 agonist flagellin after irradiation modified the recruitment and phenotype of colonic macrophages and improved tissue damage. Rats were exposed to single 20- or 27-Gy doses of colorectal irradiation. TLR4 agonist LPS or TLR5 agonist flagellin (at 50 or 200 μg/rat) was administered i.p. 3 days after irradiation. Flow cytometric analysis, immunostaining, and real-time polymerase chain reaction analysis were used to assess the M1/M2 phenotype and crypt cell proliferation 7 days after irradiation. Irradiation (20 and 27 Gy) increased TLR4+ and TLR5+ macrophage frequency in the mucosa. LPS or flagellin administration maintained this elevated frequency after the 27-Gy irradiation. LPS and flagellin drove macrophages toward the anti-inflammatory M2 phenotype by increasing Arg1 and CD163 expression and microenvironmetal effector molecules (C-C motif chemokine 22, transforming growth factor-β1, and interleukin-10). Proliferating cell nuclear antigen immunostaining, Ki67 expression, and antimicrobial growth factor Reg3γ showed that the M2 shift correlated with epithelial regeneration. In conclusion, administration of either LPS or flagellin after colorectal irradiation may provide effective protection against epithelial remodeling. This tissue repair was associated with an M2 macrophage shift. Using TLR agonists to moderately activate innate immunity should be considered as a strategy for protecting healthy tissue from irradiation.

Introduction

Radiotherapy in the pelvic area is associated with a high incidence of acute and chronic intestinal complications because of this organ’s low tolerance of radiation (O’Brien, 2001). Radiation-induced acute intestinal toxicity remains a major limitation to the delivery of tumoricidal doses of colorectal irradiation. The different forms of acute toxicity are associated with the inflammatory and immune responses of the tissue. Previous studies have demonstrated that irradiation induces the preferential differentiation of T helper (Th) cells into Th2 cells (Linard et al., 2012). A potential strategy to reduce the harmful side effects of radiotherapy is to manipulate innate immunity to protect against them.

An important trigger of the innate immune response is the engagement of Toll-like receptors (TLRs), which recognize both pathogen-associated molecular patterns and endogenous ligands. TLR4, for example, is a specific receptor for lipopolysaccharide (LPS), a major cell wall component of Gram-negative bacteria, and TLR5 selectively recognizes bacterial flagellin. TLRs are important in maintaining tissue homeostasis through their regulation of tissue repair and regeneration. Stimulation of TLR signaling by commensal microflora (such as LPS and flagellin) plays a protective role in the gut by decreasing the sensitivity of cells to radiation-induced apoptosis (Riehl et al., 2000; Jones et al., 2011). Notably, flagellin administered before total body irradiation protects against lethal irradiation toxicity and prevents host cell apoptosis, as illustrated by preservation of crypt stem cells (Burdelya et al., 2008; Vijay-Kumar et al., 2008). However, studies are required to determine whether the radioprotective effects of TLR ligands are mediated by direct interactions with epithelial cells (including stem cells) or via other cells in the intestinal mucosa.

Using pattern recognition receptors such as TLRs, macrophages continually probe their surrounding environment. They are a functionally and phenotypically diverse cell population with a crucial role in innate and adaptive immune reactions, and they contribute to the initiation and resolution of inflammation, coordination of tissue repair (Daley et al., 2010), and maintenance of local tissue homeostasis (Murray and Wynn, 2011). This multifunctionality arises from their intrinsic plasticity, which enables them to polarize into distinct functional phenotypes. Depending on the stimulus, macrophage activation states have historically been designated in several different ways: classically activated (or M1),...
that is, stimulated with LPS and interferon-γ, or alternatively activated macrophages (M2), further subdivided in M2a [after exposure to interleukins (ILs) 4 or 13] or M2b [immune complexes combined with IL-1β or LPS] and M2c [IL-10, transforming growth factor-β (TGF-β), or glucocorticoids]. Classically activated macrophages (M1) have been associated with production of antipathogen and inflammatory products such as tumor necrosis factor-α (TNF-α) and inducible nitric oxide synthase (iNOS) (Mosser, 2003). In contrast, alternative activated macrophages (M2) inhibit the production of proinflammatory cytokines by classically activated macrophages and contribute to wound healing and tissue repair; they have been associated with high arginase 1 (Arg1) expression (Menzies et al., 2010; Zhang et al., 2011). The typical expression of membrane receptors, cytokines, and chemokines also differs for M1 and M2 macrophages: the M1 type expresses CD197 (CCR7), whereas the M2 type expresses CD163, IL-10, and C-C motif chemokine 22 (CCL22) (Nomura et al., 2009). Currently, too little is known about the roles and characterization of M1 and M2 macrophages in vivo after irradiation.

Several approaches to mitigating the limiting irradiation dose problem are currently under development or in clinical use. Previous reports that TLR ligands protect against irradiation toxicity (Vijay-Kumar et al., 2008) and initiate tissue repair suggested to us that administration of TLR ligands might enable tissue repair by triggering a shift to M2 polarization. To test this hypothesis, we used a rat model of colorectal irradiation with a single dose of 20 Gy known to produce inflammatory reactions and denudation of the mucosa that increase in severity with dose and become ulcerative at a dose of 27 Gy (O’Brien, 2001) and examined the effects of administering the TLR4 ligand LPS and the TLR5 ligand flagellin. Our data suggest that this promoted the transition of macrophages from a primarily proinflammatory M1 to a more anti-inflammatory M2 phenotype and resulted in the proliferation of intestinal crypt cells.

### Materials and Methods

#### Animals and Treatment Procedure

All experiments and procedures were carried out in accordance with the Guide for the Care and Use of Laboratory Animals as published by the French regulations for animal experiments (Ministry of Agriculture Order No. B92-032-01, 2006) with European Directives (86/609/CEE), and were approved by the local ethical committee of the Institute for Radiologic Protection and Nuclear Safety (permit no. P12-01). Six-week-old male pathogen-free Sprague Dawley rats (200–220 g; n = 72) were purchased from Charles River (L’Abreux, France) and divided into 12 groups (n = 6 rats in each group). Two groups consisted of nonirradiated rats. Five groups were irradiated at 20 Gy and injected with 50 or 200 μg/rat sterile phosphate-buffered saline (PBS) of flagellin (Bacillus subtilis flagellin; InvivoGen, Toulouse, France) or LPS (derived from Escherichia coli 0111:B4; InvivoGen) or vehicle (PBS in a fixed volume of 5 ml). Five groups were irradiated at 27 Gy and injected with flagellin or LPS (at 50 or 200 μg/rat) or vehicle PBS. Flagellin and LPS were administered i.p. 3 days after irradiation. Irradiation was performed using an external cobalt-60 source emitting γ radiation at 1 Gy/min. Rats were anesthetized with isoflurane (Aerane; Baxter, Maurepas, France) and the radiation field was confined to a 2 × 3 cm surface of the colorectum, with a lead shield protecting the rest of the animal. This model of localized single-dose radiation exposure does not directly stimulate fractionation treatment, but generates histopathological lesions similar to those seen clinically at the 27 Gy 100% incidence of obstruction by 6 months, as previously shown (Skwarchuk and Travis, 1998). Each group was killed humanely 7 days after irradiation (4 days after flagellin or LPS treatment) and colorectal segments from nonirradiated, irradiated, and irradiated–treated rats were harvested for analysis.

#### Isolation of Leukocytes from the Lamina Propria

Leukocytes from the lamina propria were generated from the rat colons. Each colon was washed in calcium- and magnesium-free Hank’s balanced salt solution (HBSS), cut into 5-mm pieces and digested 4 times in HBSS (Invitrogen, Saint Aubin, France) containing 0.05 mg/ml Liberase and 0.1 mg/ml DNase (Roche, Boulogne-Billancourt, France) in a shaking incubator at 37°C for 40 minutes, then 20, 20, and 40 minutes. The cells were washed and resuspended in HBSS supplemented with 0.1 mg/ml DNase (Roche) and 10% fetal bovine serum (Invitrogen). The cells were washed, resuspended in 40% Percoll solution, and layered on a 100% Percoll solution (Sigma-Aldrich, St. Quentin Fallavier, France). The leukocytes were pelleted after 20 minutes of centrifugation at 1000g.

#### Flow Cytometry

Flow cytometry analysis was performed on a FACSCanto II and analyzed with DIVA software (BD Biosciences, Le Pont de Claix, France). The following antibodies were used: anti-TLR4-Alexa 647 (Imgenex, Nanterre, France), anti-TLR5-Alexa 405 (Imgenex), and macrophage–PE (Clone HIB36; BD Biosciences, San Jose, CA). Appropriate isotype controls (Alexa or PE conjugated rat IgG2; BD Biosciences) were matched with each antibody.

#### Immunohistochemical Analysis

Freshly isolated tissue was fixed in 4% paraformaldehyde and embedded in paraffin. Sections 5 μm in thickness were deparaffinized and immunostained for CD68 (Serotec, Düsseldorf, Allemagne, Germany). Sections were pretreated with trypsin and incubated with CD163 antibody (MCA342GA; Serotec) for CD163 detection. The EnVision™ System HRP Kit (Dako, Trappes, France) was used as the secondary reagent. Detection of proliferating cell nuclear antigen (PCNA) requires pretreatment by the heat-induced epitope retrieval method and mouse monoclonal PCNA antibody (Dako), followed by the LSAB2-HRP Kit (Dako). The color reaction was developed with the NovaRED Kit (Vector Laboratories Inc, Burlingame, CA) and counterstained with Mayer's hemalum.

#### Real-Time Quantitative Polymerase Chain Reaction

Total RNA was extracted from rat colons with the RNeasy Mini Kit (Qiagen, Courtaboeuf, France) and cDNA was prepared with the SuperScript RT Reagent Kit (Applied Biosystems, Thiais, France). Real-time polymerase chain reaction (PCR) was performed on an ABI Prism 7000 Sequence Detection System (Applied Biosystems), with SYBR Green PCR Master Mix (Applied Biosystems). The primer sequences (Invitrogen) are listed in Table 1. TaqMan primers and probes for Arg1, CCR7, CD163, Ki67, IL-22, and Reg3γ were purchased from Applied Biosystems. Relative quantification of target gene mRNA used the comparative ΔΔCt-method, normalized to an endogenous reference (hypoxyantine phosphoribosyl transferase) and a relevant nonirradiated-untreated control equal to 2−ΔΔCt.

#### Result Expression and Statistical Analysis

All data are expressed as the mean ± S.E.M. for six animals. Data were analyzed by one-way analysis of variance followed by a Bonferroni test to determine the significance of the differences.

### Results

#### Administration of TLR Ligands Modified the Irradiation-Induced Macrophage Recruitment

TLRs are involved in controlling intestinal homeostasis, and macrophages play an important role in wound healing; they may also contribute to TLR ligand signaling (Murray and Wynn, 2011). Accordingly, we first examined whether TLR4 and TLR5 stimulation, by their respective specific ligands, LPS...
and flagellin, modulated macrophage recruitment in the lamina propria 7 days after colonic irradiation at the moderate (20 Gy) and high (27 Gy) doses. At 7 days after 20 and 27 Gy, we have 100% of survival animals. Flow cytometric analysis showed that at 7 days after irradiation, the macrophage population in the lamina propria was significantly higher ($P < 0.01$) than in nonirradiated control rats, with the increase most marked at the 20-Gy dose (Fig. 1A). Similarly, administration of flagellin and LPS 3 days after irradiation at this dose significantly decreased the macrophage population; in the high-dose group, however, administration of both LPS and flagellin tended to result in higher levels of macrophage infiltration than in irradiated untreated rats. Real-time PCR analysis showed no variation in macrophage/monocyte chemotactic protein-1 expression after a 20-Gy dose of irradiation and ligand administration, compared with nonirradiated, untreated controls (Fig. 1B). After a 27-Gy irradiation dose and flagellin/LPS injection, however, macrophage/monocyte chemotactic protein-1 was overexpressed ($P < 0.001$), at a level correlated with that of macrophage infiltration. Immunostaining analysis confirmed the increase of CD68-positive macrophages in the mucosa and submucosa after irradiation (both doses) and flagellin/LPS injection (Fig. 1C). In addition, histologic analysis of colons showed severe erosive lesions and ulceration after the 27-Gy dose, whereas a 20-Gy dose produced more moderate structural changes characterized by an inflammatory infiltrate, compared with nonirradiated rats. Flagellin and LPS each independently considerably reduced mucosal damage from the 27-Gy dose. These results indicate that after a nonulcerative irradiation dose (20 Gy), injection of either flagellin or LPS decreases irradiation-induced mucosal infiltration of macrophages. On the other hand, after an ulcerative irradiation (27 Gy) in which macrophage infiltration was more moderate, administration of either flagellin or LPS tended to enhance this infiltration.

**TLR Ligands Modulated the Frequency of TLR4**

**and TLR5**

**Macrophages.** Macrophage expression of TLR4 and TLR5 and responsiveness to TLR ligands were tested by flow cytometry analysis. Figure 2 shows that compared with nonirradiated rats, the number of TLR4$^+$ and TLR5$^+$ macrophages among the macrophage population increased significantly ($P < 0.001$) after a 20- and 27-Gy irradiation dose. At the lower dose, flagellin normalized the number of TLR4$^+$ and TLR5$^+$ macrophages and LPS reduced only the TLR5$^+$ macrophage number. At the 27-Gy dose, the frequency of TLR4$^+$ and TLR5$^+$ macrophages remained elevated after flagellin and LPS administration. Together, these results show that irradiation enhanced the proportion of TLR4$^+$ and TLR5$^+$ macrophages and that flagellin and LPS maintained their high frequency in ulcerative irradiation, whereas these ligands decreased in frequency in nonulcerative irradiation.

**The M1/M2 Shift Was Associated with Gene Expression.** To determine whether the macrophages present after flagellin or LPS administration differed phenotypically from those present after irradiation, we used real-time PCR to quantitatively assess the expression of iNOS (marker of the M1 proinflammatory macrophages) and Arg1 (expressed by the M2 alternatively activated macrophages). As Fig. 3A shows, at 7 days after irradiation, iNOS expression increased by a factor of 3 ($P < 0.05$) after the 20-Gy dose and by a factor of 14 ($P < 0.001$) after the 27-Gy dose. Arg1 expression increased significantly, by a factor of 5, at a 27-Gy dose ($P < 0.001$). LPS or flagellin treatment normalized iNOS expression after the 20-Gy dose; however, after the 27-Gy dose, treatment resulted in overexpression of iNOS and Arg1 (only with a moderate effect at 200 μg LPS) compared with irradiated untreated rats. Analysis of the ratio of iNOS/Arg1 mRNA levels as an indicator of the M1/M2 activity balance (Zhang et al., 2011) shows that both 20- and 27-Gy doses of irradiation skewed this ratio toward iNOS expression. Treatment by LPS (50 and 200 μg) or flagellin (200 μg) normalized this ratio for both irradiation doses, except at the low (50 μg) dose of flagellin, which tended to express more Arg1. Similarly, when we assessed the ratio of mRNA levels of CCR7 and CD163 as an index of M1/M2 surface marker balance (Brown et al., 2009), we found that irradiation (both doses) skewed this ratio toward CCR7 expression, especially at the 20-Gy dose (Fig. 3B). After either flagellin or LPS treatment, this ratio was markedly decreased and skewed back toward CD163 expression at both irradiation doses, compared with the irradiated but untreated rats.

Immunostaining of CD163$^+$ cells showed that the number of M2 macrophages in nonirradiated colon tissue was low and that these were localized in the subepithelial region of the lamina propria mucosae (Fig. 3C). The irradiation dose did not alter their localization. After flagellin or LPS administration, however, numerous CD163$^+$ cells were found in the submucosa. They were particularly abundant after flagellin treatment at either dose (50 and 200 μg) and did not differ according to irradiation dose.

**Cytokines and Chemokines Associated with Macrophage Phenotypes.** M1 and M2 polarization were each associated with expression of a particular set of cytokines and chemokines (Mantovani et al., 2005). Mucosal real-time PCR analysis showed that the M1-associated C-X-C motif chemokine 10 was highly expressed (increased by a factor of roughly 3; $P < 0.01$) after both irradiation doses; its expression fell after

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**TABLE 1**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
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<tr>
<td>MCP-1</td>
<td>CAGCGAGATCAGTTATGCCC</td>
<td>AGGCGACTGTTGAGATCAT</td>
</tr>
<tr>
<td>INOS</td>
<td>GATTTTTCAGACACCCCT</td>
<td>GCTCCCTGCTGCAAACCT</td>
</tr>
<tr>
<td>IL-10</td>
<td>GTCTCCAGAAGCTTGGAGAAA</td>
<td>TTTGCGGCAAGTCTTTT</td>
</tr>
<tr>
<td>TGF-β1</td>
<td>TCCCACACCTGCGAGTGACC</td>
<td>AGGGTTTGGACCCCTTCCA</td>
</tr>
<tr>
<td>CXCL10</td>
<td>CAGAGGACACATGAAACCAGG</td>
<td>TCAACATGCCGCAAGTGA</td>
</tr>
<tr>
<td>CCL22</td>
<td>CTCGGTGCCGCTCTGFCCTT</td>
<td>TCTTCACCTGUGCCACATAGG</td>
</tr>
<tr>
<td>HPRT</td>
<td>GCTGAGATCTCAGAAGGAGA</td>
<td>TCAACGCTTTTATGTAATCAGC</td>
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CXCL10, C-X-C motif chemokine 10; HPRT, hypoxanthine phosphoribosyl transferase; MCP-1, macrophage/monocyte chemotactic protein-1.
flagellin or LPS administration (Fig. 4A). On the other hand, neither dose of irradiation modified the expression of the M2-associated chemokine CCL22, but flagellin administration significantly enhanced it. Specifically, after an irradiation dose of 20 Gy, a 200-µg dose of flagellin increased CCL22 expression by a factor of 10 ($P < 0.001$). After an irradiation dose of 27 Gy, flagellin increased this expression by a factor of approximately 4 after a 50-µg dose and by a factor of 10 after a 200-µg dose. LPS had no effect on CCL22 expression after a 20-Gy irradiation dose, but induced CCL22 overexpression after the higher irradiation dose. These results confirm the M2 shift induced by TLR ligands.

A previous report indicated that M2 macrophages are associated with production of TGF-β1 and IL-10 production and M2 macrophages promote IL-10 production in the presence of LPS (Gordon, 2003; Wu et al., 2010). Inversely, by inhibiting C-X-C motif chemokine 10 production, IL-10 induces differentiation into M2 macrophages (Park-Min et al., 2005) and modulates the resultant inflammatory response (Lang et al., 2002). In our model, real-time PCR analysis
showed that a 20-Gy irradiation dose did not modify TGF-β1 expression, although the latter decreased by one third after the 27-Gy dose (Fig. 4B). The level of TGF-β mRNA was significantly higher after irradiation by a 20-Gy dose; it returned to normal after the 27-Gy dose and flagellin or LPS administration. Similarly, a 20-Gy dose of irradiation did not modify IL-10 expression, but it was cut in half after a 27-Gy dose. IL-10 expression did, however, increase significantly after the high irradiation dose when LPS (200 μg) or either dose of flagellin (50 and 200 μg) was administered.

Analysis of proinflammatory cytokine expression showed that a 20-Gy irradiation induced moderate TNF-α and IL-6 expression; LPS and flagellin administration did not modify cytokine expression (Fig. 4C). Conversely, the overexpression of IL-1β and TNF-α induced after 27-Gy dose was significantly but not totally reduced by LPS and flagellin administration.

**TLR Stimulation Enhanced Proliferation and Expression of Antimicrobial Proteins.** Previous reports indicate that macrophage phenotype transitions from proinflammatory M1 to alternatively activated M2 macrophages promote repair and proliferation of host tissue after injury (Gordon, 2003) and that TLR ligands upregulate genes associated with cell proliferation and expression of antimicrobial proteins (Jones et al., 2011). They led us to examine how cell proliferation responded to TLR bonding after irradiation. Irradiation at doses of both 20 and 27 Gy cut Ki67 expression by approximately one third (P < 0.05) compared with control rats (Fig. 5A). Administration of flagellin or LPS normalized the Ki67 level after a 20-Gy irradiation dose and resulted in its overexpression after the 27-Gy dose. Immunostaining of PCNA, the proliferation marker, confirmed a marked reduction of cell proliferation after both irradiation doses (Fig. 5B). Administration of flagellin or LPS increased the number of PCNA + cells, in comparison with irradiated (both doses) untreated rats.

IL-22 has properties that regenerate epithelial tissue, increase intestinal epithelial cell proliferation, and regulate expression of the antimicrobial protein Reg3γ, thereby ensuring innate immune defense (Hanash et al., 2012;
Kinnebrew et al., 2012). Analysis of IL-22 expression showed that it was significantly reduced (P < 0.05) only after irradiation with the 27-Gy dose (Fig. 6A). After the 20-Gy dose of irradiation, flagellin upregulated IL-22 expression (by a factor of approximately 30 with a 50-µg dose and by a factor of 40 with a 200-µg dose), compared with controls in which LPS had a lesser effect (by a factor of approximately 10 with 50 µg LPS and by a factor of 4 with 200 µg). Similarly, after a 27-Gy dose of irradiation, IL-22 expression was normalized after administration of 50 µg flagellin and overexpressed by a factor of 8 after a 200-µg dose. LPS administration induced a more moderate effect (approximately 3-fold at doses of 50 and 200 µg). Although irradiation (at either dose) did not significantly modify Reg3g expression, subsequent flagellin and LPS administration (50 and 200 µg) induced its overexpression after a 20-Gy irradiation dose, compared with nonirradiated control rats (Fig. 6B). Only flagellin administration induced a marked increase of the level of Reg3g mRNA after 27 Gy of irradiation, compared with nonirradiated control rats. Under normal conditions, immunohistochemical analysis of Reg3g protein expression revealed staining in the base of the crypts and in epithelial cell of the top of crypts (Fig. 6C). Absence of Reg3g staining was observed at the high dose (27 Gy) of irradiation when it was present in epithelial cells at the moderate dose of 20 Gy. Flagellin restored the Reg3g staining in the base and the top of the crypts at 20 and 27 Gy, whereas this staining was compared with nonirradiated controls and was only observed in the base of the crypts with LPS treatment at the moderate and high dose of irradiation, respectively.
Discussion

TLR activation by bacterial products induces the expression of cytoprotective or antimicrobial genes that decrease cell sensitivity to irradiation-induced apoptosis (Gewirtz, 2003; Riehl et al., 2004; Alderson et al., 2006; Burdelya et al., 2008; Vijay-Kumar et al., 2008). A new concept has emerged regarding the possibility of combining radiation and immune-based therapies to achieve better microenvironmental protection against radiation toxicity. These reports led us to administer the TLR4 and TLR5 ligands to rats after two different doses of colon irradiation and to assess the recruitment of intestinal macrophages and their consequence on cell proliferation. Microscopic observations showing that moderate denudation of mucosa occurs at a single dose of 20 Gy and that inflammatory reaction in the mucosa and edema of the submucosa both increase in severity at a dose on the order of 27 Gy were parallel, in many ways, with the histologic changes in rectal toxicity described in humans (O’Brien, 2001).

Macrophages can be involved in both pro- and anti-inflammatory responses and in tissue destruction as well as restoration (Pollard, 2009). Indeed, their microenvironment polarizes macrophages to mount specific functional activities relevant to different phases of inflammation (Stout et al.,

Fig. 4. Effect of flagellin and LPS on expression of chemokines and cytokines associated with M1 and M2 macrophage activation induced by irradiation in the colonic mucosa. (A) Real-time PCR analysis of C-X-C motif chemokine 10 (CXCL10) and CCL22 expression showing a decrease in CXCL10 expression associated to M1 polarization and inversely an increase in CCL22 expression associated with M2 polarization after flagellin or LPS administration. (B) Microenvironment real-time PCR analysis of IL-10 and TGF-β genes responsible for macrophage polarization and (C) IL-1β, TNF-α, and IL-6 in control rats, and in rats 7 days after irradiation (20 and 27 Gy) and 7 days after irradiation and 4 days after flagellin (50 and 200 μg) and LPS (50 and 200 μg) administration. Results are expressed as means ± S.E.M. P values were calculated by analysis of variance with Bonferroni correction. *P < 0.05; **P < 0.01; ***P < 0.001 compared with controls; #P < 0.05; ##P < 0.01; ###P < 0.001 compared with irradiated controls. C, control; FLAG, flagellin; Irr, irradiated.
Immunohistochemistry combined with flow cytometric analysis showed a major infiltration of macrophages into the colonic mucosa and submucosa 7 days after irradiation, particularly at 20 Gy. The dose-dependent microvasculature apoptosis induced by irradiation may contribute to alter the transendothelial migration and in fine the monocytes-macrophages recruitment notably at the high dose of 27 Gy (Abderrahmani et al., 2012). Immunophenotyping demonstrated that these macrophages expressed TLR4 and TLR5 and that these specific TLR4\textsuperscript{+} and TLR5\textsuperscript{+} macrophage subsets were most frequent after irradiation. Our observation was consistent with the intense infiltration by macrophages expressing high levels of TLRs in the inflamed mucosa observed in human inflammatory bowel disease (Frolova et al., 2008). After 20 Gy irradiation the LPS or flagellin administration significantly decreased as well as macrophages number than TLR4\textsuperscript{+} and TLR5\textsuperscript{+} expression on the macrophage surface. The authors argued that downregulation of the TLR4\textsuperscript{+} macrophage could be responsible for the LPS tolerance demonstrated in mouse models and in patients with inflammatory bowel disease (Frolova et al., 2008). Our results may indicate a potent form of tolerance...
developed in moderate tissue alteration after TLR ligand administration. Conversely, after 27 Gy, the frequency and macrophages expressing TLR4 and TLR5 were similar to that in irradiated rats. Increased TLR signaling can upregulate antiapoptotic, cytoprotective, or antibacterial gene expression in altered tissue such as irradiated colonic mucosa (Vijay-Kumar and Gewirtz, 2009).

The ratios of markers, chosen for their ability to distinguish M1 and M2 macrophages, showed a decrease in inflammatory M1 in favor of an increase in anti-inflammatory M2 induced by flagellin and LPS. A recent report described the surprising entry of macrophages into the cell cycle and their local proliferation, which was previously considered improbable (Jenkins et al., 2011). Thus far, such in situ proliferation has been found only for Th2-type responses to insult (Murray and Wynn, 2011). Abdominal irradiation promoted a Th2-dominant inflammation and thus accelerated the onset of fibrosis (Linard et al., 2012). In Th2 responses, Arg1-expressing macrophages were found to be involved in the repair of irradiated tissue.

![Figure 6](image-url)
alternatively activated macrophages contribute to the resolution of chronic Th2-driven inflammation and fibrosis by functioning as suppressor cells (Pescce et al., 2009). It is now known that the functional plasticity of macrophages can change progressively as the signaling milieu of their microenvironment changes. LPS and flagellin can each promote an anti-inflammatory M2-like phenotype in macrophages that were in a Th2 microenvironment before activation (Stout et al., 2005).

One common functional outcome after macrophage exposure to strong inflammatory stimuli such as TLR ligands, including LPS, appears to be the development of the tolerant state that begins approximately 6 hours after stimulation and lasts for several days, characterized by induction of inflammatory genes refractory to activation by subsequent challenge by TLR ligands (Biswas and Lopez-Collazo, 2009). LPS-tolerized macrophages express aspects of alternative activation and may resemble a tolerant subtype of M2 macrophages, expressing CCL22, Arg1, and high levels of IL-10. The ligand challenge in our model does not rule out the presence of this initial M2 macrophage state, especially given that LPS and flagellin were administered 3 days after irradiation and genes related to M2-like phenotype were analyzed 4 days later (7 days after irradiation). The experiment kinetics covered the period of time during which TLR-tolerized macrophages might be induced.

These TLR-tolerized macrophages expressed genes encoding for cell proliferation, antimicrobial peptides, and macrophages that can participate in host defense while avoiding the toxicity associated with inflammatory response (Ivashkiv, 2011). Inversely, intestinal cell types (epithelial cells, myofibroblasts, and regulatory T cell lymphocytes) can produce soluble factors, particularly TGF-β1 and IL-10, which may affect the phenotypic and functional properties of macrophages and promote M2 polarization. M2 macrophages then produce more TGF-β and IL-10 and increase their reparative and regenerative activities (Gordon, 2003). Our study showed that in this particular microenvironment, flagellin and LPS each separately upregulated TGF-β by a factor of 2 to 3 after irradiation doses of 20 Gy, whereas IL-10 was overexpressed only when flagellin or LPS was administered after the 27-Gy dose. Sun and Shi (2001) reported that the phagocytosis of apoptotic cells by macrophages plays an important role in stimulating IL-10 expression. Their argument suggests that this expression results from the difference between the 20- and 27-Gy doses of irradiation, with the latter inducing more drastic mucosal damage. The repair mechanisms induced by TLR ligands may be different after 20 and 27 Gy. Notably, in the moderate inflammatory context and epithelial damage (observed in the 20-Gy model) the release of TGF-β1 after LPS and flagellin administration can have a protective role facilitating the noninflammatory clearance of dead cells by resident macrophages. Although at 27 Gy, TLR treatment decrease significantly IL-1β and TNF-α, their expression rest high. In this inflammatory context, recruited monocytes can repopulate intestinal macrophages and these newly macrophages may drive local inflammation and contributed to repair by promoting the effector T cell function (Sun and Shi, 2001). In early injury, TGF-β1 was described as an immunosuppressor and contributed to tissue regeneration (Gordon, 2003). Although the increase of TGF-β1 expression may be a predictor of radiation-induced complications such as in the progressive fibrosis, any correlation has been established that patients with high TGF-β1 levels at the end of radiotherapy were more likely to develop radiotherapy toxicity (De Jaeger et al., 2004). Additional long-term studies are required to assess the potential side effects of radiation-induced colitis.

Macrophages are important participants in tissue remodeling in which wound macrophages resemble M2, expressing high levels of arginase contributing to cell proliferation. In our study, both flagellin and LPS restored epithelial cell proliferation with a marked effect at 27 Gy. This epithelial cell proliferation was associated with overexpression of IL-22, an IL-10–related cytokine that regulates the expression of antimicrobial Reg3γ protein and promotes epithelial cell proliferation (Kinnebrew et al., 2012). We showed that LPS and flagellin enhance IL-22 expression after 20 and 27 Gy. After flagellin administration, this overexpression was also correlated with Reg3γ expression. Although enhanced infection resistance by LPS-induced Reg3γ was previously reported (Kinnebrew et al., 2012), we did not find an increase in Reg3γ expression after LPS administration following 27-Gy irradiation. The protective effect of flagellin against high doses of irradiation is well known (Burdelya et al., 2008; Vijay-Kumar et al., 2008), but the mechanism of this TLR5-mediated protection remains undefined. TLR signaling in immune cells stimulates Reg3γ expression in intestinal epithelial cells through multiple indirect mechanisms in which TLR5-stimulated dendritic cells produce IL-23 and epithelial cells respond by secreting IL-22 (Kinnebrew et al., 2012). We hypothesized that in a drastic mucosal damage (observed after 27-Gy exposure) one of these mechanisms can be altered and induced only a moderate Reg3γ expression. Further investigation is required to understand why the TLR4 ligand LPS did not induce Reg3γ expression to protect the mucosa at the 27-Gy dose.

M1 to M2 phenotype transition on radiation colitis is currently unknown. A recent in vitro report showed that the macrophage microenvironment determines their ability to interact with apoptotic cells induced by irradiation and that M1 macrophage interaction with apoptotic cells leads to enhanced inflammatory properties, whereas M2 macrophages produce an anti-inflammatory response after the engulfment of apoptotic cells (Rastogi et al., 2013). Although a protective role has been described for M1 macrophages in tumorogenesis, the tumor-associated macrophages promoting tumor growth may express a M2 phenotype, which might be a bad prognosis in a radiotherapy context. However, these cells can exhibit the same plasticity as other macrophages—namely, a mixed M1/M2 gene profile depending on the microenvironment and T cell influence, which remains to be determined (Murray and Wynn, 2011).

This study added credence to the possibility of TLR activation to decrease the acute effect of radiation notably by reducing the inflammatory response and enhancing epithelial repair. The mechanism of such protection is not well determined but may involve immune cell recruitment such as the M2 macrophage. The ability to reduce susceptibility to normal tissue complications could allow oncologists to increase radiation doses, which could in turn enhance cancer cure rates.
References


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