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Mitigation of Radiation-Induced Epithelial Damage by the TLR5 Agonist Entolimod in a Mouse Model of Fractionated Head and Neck Irradiation

Iliia A. Toshkov^a, Anatoli S. Gleiberman^a, Vadim L. Mett^a, Alan D. Hutson^b, Anurag K. Singh^c, Andrei V. Gudkov^{d,e}, and Lyudmila G. Burdelya^{d,1}

^aBuffalo BioLabs, LLC, Buffalo, New York

^bDepartment of Biostatistics and Bioinformatics, Roswell Park Cancer Institute, Buffalo, New York

^cDepartment of Radiation Medicine, Roswell Park Cancer Institute, Buffalo, New York

^dDepartment of Cell Stress Biology, Roswell Park Cancer Institute, Buffalo, New York

^eCleveland BioLabs, Inc., Buffalo, New York

Abstract

Radiation treatment of head and neck cancer frequently causes severe collateral damage to normal tissues including mouth mucosa, salivary glands and skin. This toxicity limits the radiation dose that can be delivered and affects the patient's quality of life. Previous studies in mice and nonhuman primates showed that entolimod, a toll-like receptor 5 (TLR5) agonist derived from bacterial flagellin, effectively reduced radiation damage to hematopoietic and gastrointestinal tissues in both total-body and local irradiation scenarios, with no protection of tumors. Here, using a mouse model, we analyzed the efficacy of entolimod administered before or after irradiation in reducing damage to normal tissues. Animals received local fractionated radiation to the head and neck area, thus modeling radiotherapy of head and neck cancer. Tissue damage was evaluated through histomorphological examination of samples collected at different time points up to four weeks, mice were exposed locally to five daily fractions of 5, 6 or 7 Gy. A semiquantitative scoring system was used to assess the severity of observed pathomorphological changes. In this model, radiation damage was most severe in the lips, tongue and skin, moderate in the upper esophagus and minor in salivary glands. The kinetics of injury appearance and recovery of normal morphology varied among tissues, with maximal damage to the tongue, esophagus and salivary glands developing at earlier times (days 8–11 postirradiation) relative to that of lip and skin mucosa (days 11–15 postirradiation). While both tested regimens of entolimod significantly reduced the extent of radiation damage and accelerated restoration of normal structure in all tissues analyzed, administration of entolimod 1 h after each irradiation was more effective than treatment 30 min before irradiation. These results support the potential clinical use of entolimod as an adjuvant for improving the therapeutic index of head and neck cancer radiotherapy by reducing the radiation toxicity in normal tissues.

¹Address for correspondence: Department of Cell Stress Biology, Roswell Park Cancer Institute, Elm and Carlton Streets, Buffalo, NY, 14263; Lyudmila.Burdelya@RoswellPark.org.

INTRODUCTION

Local radiotherapy, often combined with chemotherapy, is one of the most widely used and successful strategies for treatment of head and neck cancers. However, the sensitivity of normal, nontumor tissues in the oral cavity and upper gastrointestinal tract to direct (DNA damaging) and indirect (inflammation associated) toxic effects of radiation frequently leads to development of treatment-limiting complications such as mucositis, infection, salivary gland dysfunction, taste impairment and pain (1–4). Local radiation treatment of the head and neck area can irreversibly injure oral mucosa, vasculature, muscle, skin and bone (5, 6). In cases of severe oral morbidity, patients may not be able to continue cancer therapy, which can directly affect patient survival (7).

With increasing use of aggressive combined radiotherapy and chemotherapy regimens in head and neck cancer patients, the incidence of grade 3 or 4 mucositis has risen from 30–40% seen with radiotherapy alone to more than 60% as indicated by several scoring systems (e.g., World Health Organization, Radiation Therapy Oncology Group, European Organization for Research and Treatment of Cancer), based on symptoms such as soreness, erythema, ulceration and hemorrhage (8). Several approaches to mitigate radiation toxicity to normal tissues and its impact on therapeutic outcomes are currently under development or in clinical use (4, 9). These approaches include use of intensity-modulated radiotherapy or co-treatment with radioprotective agents based on natural antioxidants or synthetic thiol compounds, such as amifostine (10–12). However, the radioprotective effects of synthetic thiols are not entirely selective for normal cells and these agents may subvert the effectiveness of radiotherapy by protecting tumor cells (13). In addition, this strategy may have other adverse effects, as demonstrated by amifostine-related epidermal toxicity observed during head and neck radio-therapy (14, 15).

We have shown that the toll-like receptor 5 (TLR5) agonist entolimod (CBLB502), a recombinant pharmacologically optimized derivative of the bacterial flagellin protein, effectively rescues rodents and nonhuman primates from lethal total-body irradiation (TBI) by preventing damage to radiosensitive hematopoietic and gastrointestinal tissues and promoting their recovery (16–18). Entolimod provides these benefits when administered as a single injection either 30 min before and up to 48 h after TBI. The drug was also shown to protect against cutaneous radiation damage: treatment of mice prior to single or fractionated local exposures reduced the severity of radiation-induced dermatitis and oral mucositis (19). Similar protection of the oral mucosa was observed with entolimod pretreatment of nonhuman primates (Rhesus macaques) exposed to sublethal single-dose TBI (17). These findings suggest that entolimod could be used as an adjuvant in head and neck cancer patients to reduce the adverse effects of radiation and thus broaden the therapeutic window. Importantly, for this application, the radioprotective and radiomitigative effects of entolimod are strictly limited to normal, nontumor cells due to tumor-associated changes in the signaling pathways that lie downstream of TLR5 (19).

Current clinical regimens of head and neck cancer radiation therapy typically involve delivery of a cumulative dose of 70 Gy to the gross tumor and margin area and 56 Gy to at-risk areas. These doses are delivered in 30–35 fractions over 6–7 weeks. In this study, we

tested the ability of entolimod to mitigate the damaging effects of fractionated head and neck irradiation on normal tissues and accelerate their recovery when administered after irradiation. Using nontumor-bearing mice that received five daily fractions of head and neck irradiation with cumulative doses ranging from 20–40 Gy, we compared the effects of the previously described protective entolimod regimen [subcutaneous injection 30 min before each fractionated irradiation (19)] with entolimod injected 1 h after each fractionated irradiation. Efficacy was assessed through histopathological analysis of radiation-induced morphological changes in sections of oral mucosa (lips and tongue), esophagus, skin and salivary gland tissues collected at different time points after the first irradiation. The results obtained confirm that entolimod reduces radiation damage to epithelial tissues and promotes their regeneration when administered to mice either 30 min before or 1 h after head and neck irradiation. Administration of entolimod 1 h after each irradiation was identified as the optimal treatment regimen in this model.

MATERIALS AND METHODS

Mice

NIH Swiss (National Cancer Institute, Frederick, MD) female mice (10–12 weeks old) were used in the study. Mice were housed four per cage in a climate- and light-controlled environment with free access to food and water. All animal experiments were performed at Roswell Park Cancer Institute (RPCI; Buffalo, NY) and followed protocols approved by the RPCI Institutional Animal Care and Use Committee (IACUC).

Irradiation of Mice

Mice were irradiated as described elsewhere (19) using a Philips RT-250 Orthovoltage X-ray Unit (250 kV, 12.1 mA, 1 mm copper filtering) at a dose rate of 0.72 Gy/min while under isoflurane inhalation anesthesia. The mice were positioned such that only their heads and necks were under the beam applicator. Additionally, the rest of the body was protected by a 3-mm-thick lead shield. Radiation doses of 4, 5, 6, 7 and 8 Gy per fraction were given once per day (24 h interval) for five consecutive days. Thus, cumulative doses were 20, 25, 30, 35 and 40 Gy, respectively. Analysis of mice was timed relative to the day that the first fraction was delivered (set as study day 1). Untreated, nonirradiated control mice were used to establish normal tissue morphology. Mice were observed up to study day 29 with survival and body weight recorded daily or every second day. Mice that demonstrated weight loss >25% and limited mobility were euthanized.

Treatment of Mice with Entolimod

A stock solution of entolimod was obtained from Cleveland BioLabs, Inc. (Buffalo, NY) (17). Mice were treated with subcutaneous injection of entolimod (0.3 or 1 µg per mouse per injection in an injection volume of 100 µl) according to schedule. All regimens of entolimod treatment consisted of five injections (one injection for five days at 24 h intervals) starting either 30 min before or 1 h after each irradiation. Control mice were injected with vehicle (PBS/0.1% Tween[®] 80) 1 h after each irradiation.

Histological Analysis

At scheduled times after the start of the radiation cycle (i.e., day 8, 11, 15 and 29 after delivery of the first fraction at day 1), mice from each treatment group were euthanized for histopathological analysis of lips, tongues, the upper section of the esophagus, skin and salivary glands. The collected tissue samples were fixed in 10% formalin, processed and embedded in paraffin blocks following standard procedures. Transverse sections (4- μ m-thick) were prepared, depar-affinized and stained with hematoxylin and eosin (H&E). The stained slides were analyzed for morphological integrity and presence of tissue damage in the lip vermillion, mouth mucosa, dorsal tongue, ventral tongue, upper esophagus, salivary glands and skin from the neck. Evaluation was performed in a “blinded” fashion by a qualified core facility pathologist using a Zeiss Axio Imager A1 microscope equipped with an Axiocam MRc digital camera. The obtained images and final results were examined by two scientists involved in the study who were experienced with histological testing. Stained (H&E) tissue sections from untreated nonirradiated control mice were used to illustrate normal tissue morphology.

Semiquantitative Scoring of Tissue Injury

The severity of pathomorphological changes observed in H&E-stained tissue sections was graded according to a semiquantitative scoring system. The scores were based on changes in the epithelium of the dorsal (covering the superior surface) and ventral (covering the under surface) tongue mucosa, lip vermillion (the outer surface bordering the skin) and inner mouth mucosa. The parameters included erosions and ulcerations (partial or complete), thickness of the mucosal layer, keratinization, stroma, transitory and infiltrating lymphoid elements, size, appearance and number of basal and suprabasal cells in lamina propria and bundles of cross-striated muscles and blood vessels. Injury in the esophageal mucosa was evaluated based on the morphology of the stratified squamous epithelium with layers of basal proliferating cells and suprabasal epithelial cells, which under normal conditions contains nonproliferating differentiating cells toward the lumen. The severity of pathomorphological changes in the irradiated skin was assessed by the presence of hemorrhage, erosion, ulceration, hyperplasia, dysplasia, atrophy of the hair follicles and infiltration of the inflammatory cells. Mouse submandibular salivary glands of a branching tubule-alveolar type were examined for morphological changes in mucinous and serous glandular components with parenchymal cells organized in acini (alveoli) and ducts lined with cuboidal epithelium. All analyzed tissue components were graded as follows: 4. severely abnormal; 3. markedly abnormal; 2. moderately abnormal; 1. mildly abnormal; and 0. normal. Intermediate noninteger scores were assigned to view fields displaying degrees of injury falling within each scoring interval based on the evaluating pathologist’s judgment. Over the course of the study, several independent experiments were evaluated in a “blinded” fashion by a highly experienced pathologist and the images and results were reviewed and appraised by two other study participants.

Statistical Analysis

Radiation dose-dependent morphological damage determined by injury scoring was analyzed using a standard generalized linear model with factors for tissue type, dose and

tissue-type dose interaction. Multiple comparisons of the damage among different tissues were performed using Tukey's studentized range approach (HSD test). Differences in tissue injury scores among treatment groups at particular time points and over the entire course of the experiments were analyzed by Student's *t* test. Statistical significance was considered at $P = 0.05$.

RESULTS

Dose-Dependent Radiation-Induced Epithelial Injury in a Mouse Model of Head and Neck Fractionated Irradiation

It has been previously reported that severe damage in oral epithelial tissues develops by day 7–10 after high-dose local irradiation (up to 40 Gy given as a single dose or multiple fractions) (20). To identify fractionated doses of radiation that induce substantial tissue damage without being lethal in our mouse model, NIH Swiss mice received 5 daily fractions of 4, 5, 6, 7 and 8 Gy, resulting in cumulative doses of 20, 25, 30, 35 and 40 Gy, respectively. The head and neck local-irradiation field included the lips, mouth area, salivary glands and upper esophagus (Supplementary Fig. S1A; <http://dx.doi.org/10.1667/RR14514.1.S1>). Based on the pathologist's judgment during daily observation of oral tissue conditions in these mice, it was determined that substantial radiation-associated pathological changes developed by day 8 after cumulative doses of 30, 35 and 40 Gy (days counted relative to delivery of the first fraction at day 1). Therefore, mice were euthanized at day 8 for tissue collection to assess radiation damage to the oral epithelium (tongue, lips), upper esophagus and salivary glands by comparison of H&E-stained tissue sections to corresponding samples from age-matched nonirradiated control mice. A semiquantitative scoring system was used to assess the dose dependence of radiation injury of the oral epithelium in the collected tissue samples.

At day 8 postirradiation, there were no significant pathological changes observed in any of the analyzed tissues associated with the lowest cumulative dose of 20 Gy. In the 25 Gy dose group, there were only minimal degenerative changes in all analyzed tissues compared to nonirradiated controls (mean injury scores are shown in Fig. 1B). The general morphology of the tongue mucosa was preserved with basal and suprabasal layers of epithelial cells, lamina propria, submucosa and muscle layers. More severe degenerative changes in tissue morphology were observed in the higher dose groups (cumulative dose of 30, 35 and 40 Gy), resulting in injury scores that correlated with the applied radiation dose (representative images of dorsal tongue and lip vermillion are shown in Fig. 1A, injury scores are shown in Fig. 1B). With increasing dose, the mucosa of irradiated animals appeared atrophic (thinner) compared to nonirradiated controls, the basal layer of epithelial cells (especially of the dorsal mucosa) was less pronounced with irregular epithelial cell shape and orientation, the suprabasal cells were more pallid and enlarged and the number of epithelial nuclei was reduced. Cumulative doses of 35 and 40 Gy brought about pronounced damage in the submucosa and muscle bundles and the disappearance of epithelial cells in stretches along the tongue sections, leaving only a single layer of altered epithelial cells. In all animals that received 40 Gy, partial or complete erosions with increased edema, inflammatory infiltration

and ulcerations that compromised the structure and function of the tongue mucosa were observed.

Pronounced dose-dependent tissue damage was also observed in lip sections (Fig. 1A and B), including atrophic and degenerative changes in the mouth mucosa, submucosa and adnexia. There was progressive loss of the basal layer of epithelial cells in the lip vermillion region and the hyperplastic overlying cells were reduced in number and enlarged with irregular shape and orientation. The morphological changes were progressively more severe with increasing doses up to 40 Gy, at which point there was disruption of the covering mucosal layer leading to erosions and ulcerations, degeneration of hair follicles and disappearance of sebaceous glands in the submucosa (Fig. 1A). The relationship between radiation dose and morphological change is reflected in the dose dependent increase of injury scores (Fig. 1B). As in the tongue and lips, substantial dose-dependent injury was seen in the upper portion of the esophagus isolated at day 8 from mice exposed to a cumulative dose of 25, 30, 35 and 40 Gy (Fig. 1B). However, in all dose groups, pathological changes in the lip vermillion and upper esophagus were less severe than those observed in lip mucosa and dorsal and ventral tongue sections. There were only minor morphological changes indicative of tissue damage found in the salivary glands of mice at day 8 regardless of the dose (maximum injury score, <1.0, data not shown). A gradual dose-dependent increase in the radiation injury of the glandular epithelium was more visible in the serous region and less so in the mucinous region of the glands. Interstitial edema and perivascular inflammatory infiltration indicative of fine vasculature injury with increased permeability, enlargement of cells alongside with atrophic and degenerative changes in the parenchyma resulted in vagueness of acinar outlines, pale staining of the acini and reduction of zymogen granules. Interstitial edema and perivascular inflammatory infiltration resulting from the increased permeability indicative of injury of the fine vasculature along with enlargement of cells with atrophic and degenerative changes in the parenchyma caused vagueness of acinar outlines, pale staining of the acini and reduction of zymogen granules. The epithelium of the ducts was less affected than the acini. The mucinous glands appeared less radiosensitive, but displayed atrophic and degenerative changes such as enlarged and paler acini, mucin degranulation and disappearance, changes in the nuclei and loss of acini.

Overall, these data show a dose-dependent severity of radiation-induced morphological damage observed at day 8 after exposure to 25–40 Gy in all evaluated tissues with the exception of the salivary glands.

Comparison of the Kinetics of Radiation-Induced Epithelial Damage after Head and Neck Local-Fractionated Irradiation

Based on the findings described above, we selected two nonlethal head and neck fractionated irradiation regimens that induced severe oral tissue damage, 30 Gy (6 Gy $\times$ 5) and 35 Gy (7 Gy $\times$ 5), for subsequent experiments assessing the kinetics of radiation damage and regeneration in the oral epithelium (tongue, lips), upper esophagus, skin from the neck and salivary glands. Histological evaluation was performed at day 8, 11, 15 and 29 after the first fraction. Selection of these time points was based on body weight change data (Supplementary Fig. S1B; <http://dx.doi.org/10.1667/RR14514.1.S1>), observed pathological

changes in the skin and mouth area and previous experience indicating that such time points would allow detection of significant oral epithelial damage, signs of regeneration and advanced restoration of tissue integrity. The head and neck area of the animals were exposed to five daily fractions of 6 or 7 Gy, and euthanized at each time point for collection of tissues to assess radiation damage by comparing H&E-stained tissue sections to corresponding samples from age-matched nonirradiated control mice. Semiquantitative scoring of injury induced by the 30 and 35 Gy cumulative doses revealed differences in the kinetics of tissue damage and regeneration between the two doses.

After 30 Gy irradiation, the most pronounced pathological changes to the dorsal and ventral tongue and upper esophageal tissues were observed at day 8 and 11 with significant improvement at day 15 and 29 (Fig. 1C). The lip vermillion and mouth mucosa showed substantial patho-morphological changes at day 8 postirradiation, which became more severe at day 11 and 15 (Fig. 1C). This damage was made evident by distention of vessels and hemorrhages, cells in the basal layer having irregular orientation and a thicker expanded cornified layer above them and cell atrophy and necrosis in the adnexia. In a similar pattern, the irradiated skin demonstrated significant morphological changes at day 8 with maximum atrophic and degenerative changes in all skin components at day 15. These changes were characterized by reduced mitosis and increased necrosis of the sensitive germinal cells in the epidermis, irregular epidermal cells, thinning of the epidermis, hyperkeratinization, damage to the dermis, adnexia and blood vessels (edema, congestion, hemorrhage) and inflammatory infiltration. All three components (lip vermillion, mouth mucosa and skin) showed partial tissue regeneration at day 29 postirradiation.

The pathomorphological changes induced by the cumulative dose of 35 Gy were more severe than those induced by exposure to 30 Gy in all evaluated tissues (Supplementary Fig. S2; <http://dx.doi.org/10.1667/RR14514.1.S1>) except for salivary glands, which showed a similar low level of morphological damage after either dose (maximum injury score <1.0). While the extent of tissue damage increased along with radiation dose (except for salivary glands), the kinetics of damage and recovery were similar to those observed with the cumulative dose of 30 Gy for all analyzed tissues. Substantial damage observed in lip tissue samples collected at day 8 became more pronounced at day 15 after 35 Gy exposure with increased erosion and ulceration. In contrast, maximum damage of the tongue mucosa, esophagus and salivary glands was observed at day 8 with substantial recovery apparent by day 15. At day 29 after initial irradiation, morphology in all evaluated tissues was substantially improved compared to earlier time points. However, while tongue and esophagus samples showed near-complete restoration of normal morphology by day 29, lip vermillion and mucosa samples still displayed mild-to-moderate radiation-induced injury.

The dynamics of radiation-induced weight loss (Supplementary Fig. S1B; <http://dx.doi.org/10.1667/RR14514.1.S1>) were consistent with those describing the appearance of pathomorphological changes in the mouse tongue and esophagus epithelium, while significant lip (both vermillion and mouth mucosa) and skin injury remained for a longer period of time (day 15 and 29, respectively) with body weight already restored.

Mitigation of Radiation-Induced Epithelial Damage in the Head and Neck Area by Entolimod Treatment

Our previously reported work demonstrated that mice treated with entolimod 30 min before single or fractionated irradiation reduced damage to the tongue mucosa and dermal epithelium (19). To evaluate the efficacy of entolimod in reducing the damaging effects of head and neck irradiation in different compartments of the mouth epithelium and skin, the animals received five daily fraction of 6 Gy (cumulative dose of 30 Gy) in combination with a daily injection of entolimod either 1 h after each fraction (mitigative regimen) or 30 min before each fraction [protective regimen as in our previous study (19)]. Additional groups of mice were irradiated in a similar manner with five daily fractions of 7 Gy (cumulative dose of 35 Gy) in combination with either a daily injection of entolimod or vehicle 1 h after each fraction. Mice from each treatment group were euthanized for tissue collection at day 8, 15 and 29 after the first dose. Two more groups of mice were injected with entolimod 1 h after each fraction for a cumulative dose of 25 Gy and sacrificed at day 11. H&E-stained sections of lips, tongue, upper esophagus, skin from the irradiated neck area and salivary glands were examined for radiation-induced pathological changes in tissue morphology and compared to corresponding sections from nonirradiated control mice.

Tongue—Radiation-induced damage to tongue tissue in 30 Gy irradiated animals was apparent in the basal and suprabasal layers of epithelial cells, lamina propria, submucosa and muscle layers at day 8 postirradiation (Fig. 2). The mucosa showed some atrophic and degenerative changes appearing thinner than in nonirradiated controls. The basal layer of epithelial cells in both the dorsal and ventral mucosa was thinner and discontinuous, suprabasal cells were enlarged and the number of epithelial nuclei was reduced. Maximum damage was seen at day 8 after 30 Gy irradiation with partial recovery apparent by day 15 and full restoration of normal morphology by day 29 (Fig. 3). Injection of entolimod 1 h postirradiation reduced damage to both the dorsal and ventral tongue mucosa, resulting in average injury scores significantly lower than those of the vehicle-treated control groups at day 8 and 15. Entolimod treatment 30 min before each fraction only showed a statistically significant benefit at day 15 for the ventral tongue; thus, in terms of damage to the tongue, this treatment regimen was clearly less effective than entolimod treatment injected 1 h postirradiation. The examination of tongue tissue at day 11 after 25 Gy irradiation demonstrated significant mitigation of radiation injury with entolimod injected 1 h postirradiation (Supplementary Fig. S3; <http://dx.doi.org/10.1667/RR14514.1.S1>). As expected, 35 Gy irradiation induced degenerative changes in the tongue mucosa that were more severe than those observed with 30 Gy (Supplementary Fig. S4). In both the dorsal and ventral mucosa, necrosis of epithelial cells in stretches along the tongue sections resulted in a single row of altered enlarged epithelial cells lacking any orientation and with vesicular or hyperchromatic nuclei and, in some cases, fenestrations with no epithelial cells and detachment of the submucosa from the keratinized superficial layer. There was also muscle damage, increased edema and inflammatory infiltration in the submucosa of the tongue. As with 30 Gy irradiation, administration of entolimod in conjunction with 35 Gy irradiation ameliorated radiation damage to the tongue.

Lips—Analysis of lip sections containing mucosa proper (vermillion) and mouth mucosa collected from mice at day 8 after 30 Gy irradiation demonstrated that the severity of tissue damage was clearly greater in vehicle-injected groups than in entolimod-treated groups (Fig. 2). The epithelium of the mucosa proper was irregular and atrophic in PBS-treated irradiated mice compared to that in nonirradiated controls. Each regimen of entolimod treatment applied to 30 Gy irradiated mice (30 min before or 1 h after each fraction) ameliorated radiation toxicity to the lips. This was observed in all structural components of the lips at day 8 and 15, resulting in a statistically significant reduction of the average injury scores relative to those seen in the corresponding vehicle-injected group (Fig. 3). A similar protective effect of entolimod treatment 1 h after each fraction in the 25 Gy irradiated group was observed in lip tissue at day 11 postirradiation (Supplementary Fig. S3; <http://dx.doi.org/10.1667/RR14514.1.S1>). Moreover, while both components of the lips (vermillion and mouth mucosa) showed partial restoration of normal morphology at day 30 after 30 Gy irradiation without entolimod treatment, recovery was close to complete in the entolimod-treated mice.

After 35 Gy irradiation, pathomorphological changes in the lip tissues were more pronounced than those observed after 30 Gy irradiation. The most severe damage was noted at day 15 and was followed by partial tissue regeneration by day 29 (Supplementary Fig. S4; <http://dx.doi.org/10.1667/RR14514.1.S1>). Animals exposed to a 35 Gy cumulative dose that were administered entolimod 1 h after each irradiation had a relatively minor ameliorative effect on radiotoxicity to the lips. The reduction in average injury scores in the entolimod- vs. vehicle-treated mice was only statistically significant at some time points, not including day 15.

Esophagus—The esophageal epithelium at day 8 and 15 after 30 Gy irradiation, radiation-induced atrophy and degenerative changes were observed in the mucosa with damage to the basal layer cells and suprabasal cells (Fig. 1C). Esophageal damage was more severe after 35 Gy irradiation (Supplementary Fig. S2; <http://dx.doi.org/10.1667/RR14514.1.S1>). With either radiation dose, these pathological changes were less pronounced in entolimod-injected mice compared to vehicle-injected mice (Fig. 3 and Supplementary Fig. S4). Furthermore, observation at day 8 after 30 Gy irradiation revealed that administration of entolimod 30 min before irradiation was less effective in mitigating esophageal damage than administration 1 h after irradiation. Entolimod treatment also significantly improved the morphology of the esophageal epithelium, as noted at day 15 when partial regeneration was apparent in all groups, while at day 29 the mucosa of the proximal esophagus was almost completely regenerated in all groups.

Skin—Substantial damage to the skin was observed at day 8 after 30 Gy irradiation with a further increase in morphological changes at day 15 and significant recovery by day 29 (Figs. 1C and 2). Entolimod treatment reduced the extent of dermal epithelial damage at every time point analyzed and led to more rapid regeneration compared to vehicle-injected mice (Fig. 3, Supplementary Fig. S3; <http://dx.doi.org/10.1667/RR14514.1.S1>). Although a trend indicating faster skin regeneration was observed in mice injected with entolimod 1 h

after irradiation compared to mice injected 30 min before irradiation, the statistically significant difference was only reached at day 29 postirradiation.

Salivary glands—The morphological radiation-induced damage in the salivary glands during the first four weeks after a cumulative dose of 30 Gy was much less severe (maximum average injury score in vehicle-treated irradiated mice was ~0.7) than observed in the other tissues evaluated (Supplementary Fig. S5; <http://dx.doi.org/10.1667/RR14514.1.S1>). Nevertheless, as observed at day 8 and 15, entolimod treatment led to noticeable mitigation of damage in this tissue, with treatment 1 h after irradiation being more effective than 30 min before irradiation. The effect of entolimod at day 29 postirradiation could not be assessed for this dose since the salivary glands had completely recovered in both the vehicle- and entolimod-treated groups. After the cumulative dose of 35 Gy, the degenerative changes in the salivary glands were more severe than after the 30 Gy dose, but showed the same kinetics of tissue damage/regeneration. Administration of entolimod in conjunction with the 35 Gy dose also ameliorated radiation damage. As expected, treatment 1 h after, relative to 30 min before irradiation was more favorable.

In summary, using a head and neck area local-fractionated mouse model, we have shown that radiation-induced toxicity, as indicated by semiquantitative scoring of morphological changes in H&E-stained sections of five different epithelial tissues of the head and neck region, was significantly reduced by entolimod treatment at all analyzed time points (day 8, 11, 15 and 29). In addition, administration of entolimod 1 h after irradiation proved more effective than pretreatment 30 min before irradiation. Unfortunately, the tissue-protective effects observed with entolimod treatment were not accompanied by improved radiation-induced body weight loss in this model (Supplementary Fig. S6; <http://dx.doi.org/10.1667/RR14514.1.S1>). However, the histological results provide proof of principle for entolimod application as a safe and effective mitigator of radiation-induced epithelial damage commonly associated with clinical radiotherapy approaches.

DISCUSSION

Previously reported studies of mice and nonhuman primates have clearly established that entolimod treatment before irradiation reduces radiation-induced damage to all three of the major types of radiosensitive tissues: hemato-poietic, gastrointestinal and skin (17, 19). It has also been reported that pretreatment of mice with entolimod before thoracic irradiation reduced lung inflammation and prevented radiation-induced fibrosis (21). In addition, improved survival and accelerated morphological recovery of hema-topoietic and gastrointestinal tissues were observed in nonhuman primates when entolimod was delivered up to 48 h after lethal-dose TBI (22). To mimic the clinical setting for cancer patients undergoing head and neck radiation therapy, we used a mouse model of local fractionated irradiation. We evaluated the effect of entolimod on mucosal and dermal epithelial radiation injury and tissue regeneration over four weeks postirradiation. We demonstrated that administration of entolimod after irradiation leads to effective mitigation of adverse effects on normal tissues in the irradiated area (e.g., lips, tongue, esophagus, skin and salivary glands). Entolimod treatment 1 h after 5 Gy irradiation was identified as a more effective regimen for limiting tissue damage and promoting tissue recovery compared to treatment 30

min before irradiation. The data suggest involvement of different tissue-protective mechanisms dependent upon whether entolimod is injected immediately prior to or shortly after irradiation. The body weight loss assessment in mice exposed to five daily fractions of 6 Gy showed that entolimod treatment yielded no improvement compared to vehicle-treated control mice (Supplementary Fig. S6; <http://dx.doi.org/10.1667/RR14514.1.S1>). This assessment does not support the previously published study in which entolimod treatment mitigated radiation-induced weight loss after 20 Gy irradiation, as well as in mice that received three 10 Gy daily head and upper neck irradiations (19). This indicates that in the current radiation model, using five fractions instead of three, a longer exposure time and a radiation field extended to the upper esophageal area, body weight does not reflect the histopathological state of the analyzed tissues. This may be due to conditions not examined in this study such as additional radiation-induced tissue damage and/or functional impairment of the salivary glands. Xerostomia is the most common injury that can occur due to acute and long-term salivary gland dysfunction associated with fractionated radiation therapy (23). It has been shown that the salivary glands in mice that received 15 Gy irradiation produce less saliva (24, 25). In the current study, our semiquantitative injury scoring system enabled us to only demonstrate mild morphological damage in the salivary glands of animals that received 30 and 35 Gy irradiation (below 1, Supplementary Fig. S5), suggesting that more extensive histological and functional assessment of entolimod's effect on the salivary glands after irradiation is needed.

Because the scope of radiation damage to mucosal and dermal epithelium includes early effects such as erythema, swelling, blistering, ulceration and necrosis in addition to chronic inflammation, fibrosis and sclerosis associated with the onset of delayed effect of acute radiation syndrome (26–29), investigating the beneficial effects of entolimod treatment on radiation-induced epithelial damage and salivary gland function and the mechanisms of its radioprotective activity can be clinically important for both mitigation of early damaging effects of radiation and possible prevention of delayed radiation toxicity.

Radiation toxicity involves both acute direct tissue injury due in large part to induction of apoptosis within radiosensitive tissues and indirect effects of inflammation, which commonly develop after radiation treatment (30, 31). The biological effects of entolimod are known to result, at least in part, from activation of the pro-survival NF- κ B and STAT3 signaling pathways in TLR5-responsive organs (e.g., liver and intestine), which leads to expression of multiple intracellular and secreted factors with reactive oxygen species (ROS) scavenging, anti-apoptotic, anti-infective, tissue regenerating and anti-inflammatory functions (17, 32–34). The known functions of many of these factors support their possible roles in entolimod-mediated protection/mitigation of epithelial radiation damage. It is noteworthy that the radioprotective activity of entolimod involves both direct and indirect mechanisms. As we previously demonstrated, activation of TLR5 signaling in the liver is critical for entolimod-mediated protection of bone marrow hematopoietic stem cells in animals receiving TBI (16). Increased expression of anti-apoptotic proteins and intracellular antioxidants (e.g., BCL2 family members and superoxide dismutase) after entolimod treatment can protect cells from direct radiotoxicity by reducing the extent of apoptotic cell death. These mechanisms likely play important roles in entolimod's efficacy when it is administered before irradiation. The radiomitigative effects of entolimod when administered

after irradiation appear to be more powerful than its protective effects and can be mediated by induced secreted antioxidants (e.g., hepcidin), anti-inflammatory cytokines [i.e., IL-10 and IL-22 (35, 36)], cytokines that promote wound healing by recruiting macrophages to the affected area [i.e., KC, MCP-1, MIP-1 α and IP-10 (37–39)] and other cytokines with radioprotective and anti-inflammatory properties (40–43).

Individual application of several growth factors (e.g., EGF, KGF and FGF) has been demonstrated to improve mucosal wound healing, including radiotherapy or chemotherapy induced oral mucositis (44–47). However, since the mechanism of action of these types of agents typically involves stimulating proliferation of both normal and tumor epithelial cells, they are of limited usefulness in cancer patients and are mainly considered for topical application to improve wound healing (45). In contrast, the radioprotective effects of TLR5 activation are strictly limited to normal tissues. It has been shown in numerous animal tumor models that entolimod and its parental bacterial flagellin not only fail to protect tumor cells from radiation, but actually provide substantial antitumor effect apparently through induction of antitumor immune responses (16, 17, 19, 48–51). This body of evidence, together with the results reported here, provides strong support for the rational combination of entolimod treatment with radiotherapy for head and neck cancers to reduce adverse radiation-associated side effects and increase its therapeutic index. Following its proof of concept in head and neck irradiation with further additional functional validation, such a strategy could be applied to many different scenarios of conventional cancer treatment to eliminate one of the major obstacles in achieving positive patient outcomes.

Supplementary Material

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FIG. 1.

Dose-dependent radiation damage to local tissues in mice after fractionated head and neck irradiation. Panel A: Representative images of H&E-stained sections of the dorsal tongue (left-side column) and lip vermilion (right-side column) from nonirradiated control mice and mice exposed to five daily fractions of 6, 7 and 8 Gy (cumulative doses of 30, 35 and 40 Gy, respectively). Samples were obtained at day 8 after irradiation. Yellow arrows indicate the mucosa layer, asterisks indicate irregular epithelium and discontinuous lining, red arrows indicate inflammatory infiltration and green arrows indicate submucosa with hair follicles and sebaceous glands. Panels B and C: Semiquantitative scoring of radiation-induced tissue damage. Scores were obtained through blinded evaluation of H&E-stained tissue sections isolated from irradiated mice with comparison to nonirradiated control mice (the injury score in control tissue is shown as 0). The average morphological injury score per group (4 mice) per time point is shown ($\pm$ SEM). The statistical significance of differences in injury scores was analyzed by Tukey's studentized range (HSD) test. Panel B shows the dose-dependent injury induced by five daily fractions of 5, 6, 7 and 8 Gy (cumulative dose of 25, 30, 35 and 40 Gy, respectively) in the indicated tissues at day 8 after the first dose. NS = not significant; * P 0.0001; ** P 0.05. Panel C: Kinetics of injury and recovery in the head and neck tissues of mice receiving five daily 6 Gy fractions (30 Gy cumulative). Tissues were collected at day 8, 11, 15 and 29 after the first irradiation. Scores were obtained

through evaluation of H&E-stained tissue sections in comparison to sections from nonirradiated control mice (shown as day 1). *Significant difference ($P = 0.05$) in kinetics of lip vermillion, mouth mucosa and skin tissue injury compared to any other analyzed tissue.

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FIG. 2.

Representative images of H&E-stained sections of the dorsal and ventral tongue, lip vermilion, mouth mucosa and neck skin obtained from mice at day 8 after five daily fraction of 6 Gy (cumulative dose of 30 Gy) and treated with vehicle control or entolimod (1 µg/mouse, 30 min before or 1 h after irradiation). Yellow arrows indicate mucosa and epidermal layers, green arrows indicate submucosa with hair follicles and sebaceous glands.

FIG. 3.

Semiquantitative scoring of radiation-induced tissue damage. Injury scores were assigned to H&E-stained sections of the dorsal and ventral tongue, lip vermillion, mouth mucosa, upper esophagus and neck skin collected on the indicated days after delivery of the first irradiation (mean $\pm$ SEM, $n = 4$). Numbers above brackets connecting bars are P values indicating statistical significance of the difference in average morphology scores at a given time point between corresponding groups of irradiated mice with or without entolimod treatment (by Student's t test). Where no P value is shown, the difference between groups irradiated with or without entolimod treatment was statistically nonsignificant ($P > 0.05$). *Statistically significant ($P \leq 0.05$) difference in average morphology scores between groups injected with entolimod before and after irradiation (Student's t test). #No change in morphology in the analyzed tissues (injury score 0).