Published in final edited form as:

Radiat Res. 2015 January ; 183(1): 72–81. doi:10.1667/RR13802.1.

Interleukin-12 Preserves the Cutaneous Physical and Immunological Barrier after Radiation Exposure

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Abstract

The United States continues to be a prime target for attack by terrorist organizations in which nuclear detonation and dispersal of radiological material are legitimate threats. Such attacks could have devastating consequences to large populations, in the form of radiation injury to various human organ systems. One of these at risk organs is the cutaneous system, which forms both a physical and immunological barrier to the surrounding environment and is particularly sensitive to ionizing radiation. Therefore, increased efforts to develop medical countermeasures for treatment of the deleterious effects of cutaneous radiation exposure are essential. Interleukin-12 (IL-12) was shown to elicit protective effects against radiation injury on radiosensitive systems such as the bone marrow and gastrointestinal tract. In this article, we examined if IL-12 could protect the cutaneous system from a combined radiation injury in the form of sublethal total body irradiation and beta-radiation burn (β -burn) directly to the skin. Combined radiation injury resulted in a breakdown in skin integrity as measured by transepidermal water loss, size of β -burn lesion and an exacerbated loss of surveillant cutaneous dendritic cells. Interestingly, intradermal administration of IL-12 48 h postirradiation reduced transepidermal water loss and burn size, as well as retention of cutaneous dendritic cells. Our data identify IL-12 as a potential mitigator of radiation-induced skin injury and argue for the further development of this cytokine as a radiation countermeasure.

INTRODUCTION

There is a legitimate threat of both intentional (bioterrorism) and/or accidental (industrial catastrophe) dispersal of radiologic material where a significant portion of the population could be exposed to damaging amounts of ionizing radiation (1). This type of radiation

exposure can seriously compromise the wellbeing of individuals depending on their location and radiation dose, and the resulting biological effect is often referred to as acute radiation syndrome (ARS) (2, 3). This syndrome is predominately dependent on four particular organ systems that have the greatest acute sensitivity to radiation: the neurovascular, hematopoietic, gastrointestinal and cutaneous systems (4). The combined radiation effect on these systems can lead to multi-organ failure and/or death (2).

Cutaneous radiation syndrome (CRS) is a general term to describe the biological effects of ionizing radiation on the skin (5–8). CRS has been documented globally in all major radiation accidents over the last 15 years (5), with many deaths resulting from a lack of management of radiation-induced cutaneous damage (7, 9–11). The degree of radiation-mediated skin injury depends on the type, dose and quality of radiation (2, 4, 7). CRS most often presents as distinct stages of acute distress (erythema, blistering, pigmentation changes, dry/moist desquamation) followed by chronic disorders (fibrosis, necrosis, ulceration) (5, 7, 12). In essence, radiation exposure can compromise a hallmark feature of the skin: its protective barrier function.

The skin functions as a physical and immunological barrier against the surrounding environment. It is comprised primarily of two layers: the epidermis and the dermis. The epidermis is made up of stratifying layers of keratinocytes, which undergo differentiation to form the outermost layer called the stratum corneum (8, 13). The epidermis is the primary physical barrier and prevents both water loss and the entry of foreign substances (13). A disruption of the skin's physical barrier can be measured by an increase in transepidermal water loss (TEWL) (14) and can often be indicative of enhanced susceptibility to infection or disease (15–18). In addition to the physical barrier, a distinct subset of immune cells, called cutaneous dendritic cells (cDCs) are located within the skin and form a dense immunological barrier that protects against invasion and dissemination of pathogens (8, 19). The cDCs can be further divided into two populations based on their anatomical location within the skin. Langerhans cells (LCs) are located in the epidermis, whereas interstitial dendritic cells (iDCs) are present in the dermis (8). Collectively, the physical and immunologic barriers contribute to the maintenance of skin integrity. Previous data have demonstrated that radiation exposure results in increased water loss (14) and depletion of cDCs (20) suggesting a breakdown of both the physical and immunological barriers. Because the loss of skin barrier function can have dire consequences, significant effort is being devoted to identify therapeutic mitigators that would preserve these cutaneous barriers from the deleterious effects of radiation exposure.

There is recent evidence suggesting that reversing the damaging effects of radiation exposure within the skin might actually help prevent multiple organ failure (7). One product that may act as a mitigator to radiation injury is interleukin-12 (IL-12) (21–24), which is a cytokine primarily produced by monocytes, macrophages and DCs and classically thought to be involved in the development of cell-mediated immunity (25, 26). IL-12 is composed of two subunits, IL-12p35 and IL-12p40 that associate to form the bioactive molecule IL-12p70, which binds to the heterodimeric receptor made up of IL-12R- β 1 and IL-12R- β 2 (25, 27). Basile *et al.* treated both mice and monkeys with systemic IL-12 24 h after lethal total body irradiation (TBI) and demonstrated enhanced survival compared to untreated

controls (21). In these experiments, IL-12 appeared to preserve radiosensitive organs such as the gastrointestinal and hematopoietic systems. Additionally, Chen *et al.* and Neta *et al.* demonstrated radioprotective and stimulatory effects of systemic IL-12 on bone marrow after irradiation (22, 23). These reports highlight the promising radioprotective properties of IL-12, although the effects of this cytokine on cutaneous radiation injury were not addressed in these reports.

In the current study, we developed a murine model that recapitulates extensive, but potentially survivable exposure to ionizing radiation, which would most likely include a total body dose of gamma irradiation followed by superficial cutaneous beta irradiation as a result of fallout. To model this scenario, mice were subjected to sublethal 6 Gy TBI (Cs137) and beta-radiation burns (β -burns) (Sr-90) of 40 Gy to the skin. This dose causes moist desquamation, which is a condition that resembles radiation exposure from fallout (28). We show that radiation exposure resulted in significant impairment of vital skin parameters suggesting a breakdown in both the physical and immunological skin barriers, and most notably, that intradermal (i.d.) administration of IL-12 after radiation exposure reversed many of the damaging effects on the skin. The data presented here suggest that in addition to the hematopoietic and gastrointestinal systems, the cutaneous system may also benefit from IL-12 therapy after radiation exposure.

MATERIALS AND METHODS

Mice and Irradiation

The University of Rochester's Committee on Animal Resources approved all animal use protocols. C57BL6/hairless mice are used in this study. These immunocompetent mice lack hair, which allows for optimal skin studies (20, 29, 30), but are similar to C57BL/6 mice in all other tested parameters. C57BL6/hairless mice were exposed to 6 Gy TBI using a 3200 Curie sealed ^{137}Cs source that operates at 1.90 Gy/min. Mice were placed into a Plexiglas[®] box designed to prevent vertical movement away from the radiological source atop a collimator designed specifically for TBI. The source and collimators are calibrated periodically to test for uniform distribution of radiation. Immediately after TBI, three 40 Gy β -burns (on both ears and one on the back) were performed on anesthetized mice using a 7.5 mm diameter circular strontium-90 applicator, which delivers 25.21 Gy/ min at the skin surface. This dose results in a visible wound 5–7 days (raised red swelling) after irradiation (characterized by focal edema and erythema, followed by crusting then an atrophic scar). This combined radiation injury in these mice did not result in lethality.

IL-12

Mouse recombinant IL-12 (PeproTech[®] Inc., Rocky Hill, NJ) was reconstituted in sterile PBS/1% BSA (Sigma-Aldrich[®], St. Louis, MO). All dilutions were performed in PBS/1% BSA. Mice were initially exposed to TBI and β -burns as described above and 48 h later each β -burn site was treated with a single intradermal (i.d.) injection of 100 ng rIL-12 (total of 300 ng). Vehicle control consisted of PBS/1% BSA.

Blood Analyses

Whole blood was collected at the time of sacrifice by drawing blood from the descending aorta into a 1 cc heparinized syringe with a 25 gauge needle (Fisher Scientific, Boston, MA). Blood was transferred to a K₂ EDTA collection tube (Fisher Scientific). HemaTrue™ Hematology Analyzer (Heska Inc., Loveland, CO) was used to collect complete blood cell counts (CBCs) from whole blood. Whole blood was spun down at 2,500 rpms for 5 min to collect plasma and stored at -80°C. Plasma was analyzed for various cytokines and chemokines using a Mouse Cytokine Magnetic 20-plex Panel Luminex® kit (Invitrogen™, Carlsbad, CA) according to manufacturer's instructions.

Quantification of LC and iDC Density

Mice were exposed to TBI + β -burns and treated with or without IL-12 as described above. At various time points, one ear was removed and split into dorsal and ventral halves. The epidermis was separated from dermal layers by floating each half in 0.5 M ammonium thiocyanate (Sigma-Aldrich) for 20 min at 37°C followed by fixation/permeabilization in 2% paraformaldehyde (J.T. Baker®, Center Valley, PA) for 15 min at room temperature. The epidermis was stained for LCs by incubating tissue in 200 μ l of PBS/1% BSA/ 0.1% azide (PBA) +Fc block [anti-CD16/CD14 (BD Biosciences, San Jose, CA)] (10 μ g/ml) +anti-CD207-PE (eBioscience, San Diego, CA) (4 μ g /ml) for 1.5 h gently rocking at 4°C. The other ear was split into dorsal and ventral halves and each half was stained for iDCs by incubating tissue in 200 μ l PBA +Fc block (anti-CD16/ CD14) (10 μ g/ml) +anti-MHC II-allophycocyanin (eBioscience) (3 μ g/ml) for 1.5 h gently rocking at 4°C. Both the epidermis and dermal samples were washed once by rotating each tube in 3 ml of PBA for 30 min at 4°C.

Conventional whole mount microscopy was performed as previously described (20). Briefly, ear tissue was mounted on a slide along with 20 μ l of PBA and cover slipped. Fluorescent images of fields of view devoid of debris or autofluorescent proteins were taken using a BX40 Olympus fluorescence microscope and a Retiga™ 1300 camera (QImaging®, Surrey, BC, Canada). A minimum of 10 fields of view were acquired per ear. Control ear images (no irradiation or IL-12 administration) were taken in similar fashion.

Image analysis was performed on acquired images using Image-Pro® Plus software (MediaCybernetics®, Warrendale, PA) as previously described (20). Briefly, the densities of positively labeled CD207⁺(LCs) and MHC class II⁺(iDCs) were determined by creating a mask of the original image and applying area and intensity filters to specifically enumerate DCs only. Positively labeled cells were expressed as percentage positive in a known area. Densities were calculated and reported as cells/mm² for control and experimental samples.

Transepidermal Water Loss

At various time points after irradiation, β -burn sites on the back were measured for TEWL using a Tewameter® (TM 210, Acaderm Inc., Menlo Park, CA). To obtain a measurement, the Tewameter probe was gently placed on the back β -burn of nonanesthetized mice for ~60 s.

β -Burn Area Measurements

Mice were exposed to TBI and β -burns as described above and the area of the β -burn on the back was measured by acquiring digital images using a Canon® Powershot camera at a fixed distance above the mouse. A scale bar was included in each image and the area was calculated using Image-Pro Plus software. β -burns were scored daily for acute skin toxicity using a scale from 0.0–4.0 (with 0.5 increments) based on the following clinical score criteria (31, 32): 0.0 =normal; 0.5 =slight erythema; 1.0 =definite erythema; 1.5 =some dry desquamation and thickening; 2.0 =dry desquamation and mild edema; 2.5 =dry desquamation with early moist desquamation; 3.0 =moderate moist desquamation (<50%); 3.5 =moist desquamation (>50%) $\pm$ necrosis; 4.0 =ulceration, necrosis, loss of dermis.

Histology

Hematoxylin and eosin (H&E) staining was performed on formalin-fixed, paraffin-embedded, sections of back skin (5 μ thick) from the TBI + β -burn site from untreated and IL-12-treated mice. All stained sections were imaged using 10 $\times$ Plane F1/0.3 N.A. using a Nuance™ Fx Multispectral camera (CRI, Perkin-Elmer, Waltham, MA) mounted on an Olympus BX40 microscope. Epidermal thickness and dermal cellularity densities were quantified using Nuance multispectral imaging, InForm™ pattern recognition and customized Image-Pro Plus 7.0 image processing.

Statistical Analysis

Results are presented as means $\pm$ standard error from replicates listed in the figure legends. Significance ($P < 0.05$) was determined by GraphPad Prism using a one-way analysis of variance (ANOVA) followed by a Bonferroni or Tukey's post hoc test.

RESULTS

Development of a TBI + β -Burn Model and Administration of IL-12

A catastrophic radiation event would likely result in total body exposure to gamma radiation along with potential cutaneous β -burns as a result of fallout (4). To model this, we utilized hairless mice on the C57BL/6 background (80% C57BL/6; 20% SKH1) to facilitate our analysis and skin imaging without the complications of fur removal (30). The mice were exposed to sublethal (6 Gy) TBI immediately followed by three 40 Gy β -burns (one on each ear and one on the upper back). This treatment did not result in mortality in any mice. We first assessed a well established systemic parameter to determine what effect this exposure would have on radiosensitive circulating immune cells. As anticipated, TBI + β -burns resulted in a significant reduction of white blood cells (WBC) in blood, which reached a nadir on day 7 postirradiation and recovered to levels of the control mice by day 60 postirradiation (Fig. 1A). This model is utilized throughout this article to assess the effects of cutaneous radiation exposure.

We further developed this model to examine whether local administration of IL-12 could mitigate the deleterious effects of radiation exposure in skin. C57BL6/hairless mice were administered three i.d. 100 ng injections of IL-12 (one injection at each β -burn site) 48 h postirradiation. This dose of IL-12 did not have an effect on WBC levels in the blood in

irradiated mice as assessed by complete blood counts (Fig. 1A). To determine whether an i.d. injection of IL-12 had systemic effects, we monitored a biological mediator of IL-12 in plasma from mice isolated at various time points postirradiation. CXCL9, a downstream chemokine induced by IFN γ , which is induced by IL-12 (26, 33), was measured by Luminox. Although CXCL9 could be detected in plasma two days after IL-12 administration (i.e., day 4 postirradiation), levels were undetectable in plasma five days after injection (Fig. 1B). Unirradiated controls, as well as TBI + β -burns +vehicle mice, had no significant induction of CXCL9 in plasma. These data demonstrate that a single administration of i.d. IL-12 (total dose =300 ng) had an initial detectable systemic biological activity, however these effects were not sustained.

IL-12 Improved the Cutaneous Physical Barrier after TBI + β -Burn

To assess what effect TBI + β -burns had on the integrity of the cutaneous physical barrier and whether IL-12 could mitigate skin injury, we examined two established parameters that monitor skin barrier function. First, since an important role of the skin is to maintain the cutaneous barrier to prevent abnormal water loss, we assessed TEWL from the epidermis into the atmosphere which has been shown to be increased by disruption of the physical integrity of the skin. Mice were exposed to TBI + β -burns as described above and TEWL was measured using a Tewameter at the burn site on the back at various time points over 60 days. Radiation exposure resulted in increased TEWL levels, which peaked at day 11 postirradiation and gradually returned to control levels by day 30 (Fig. 2A). IL-12 administration on day 2 postirradiation significantly reduced TEWL levels during the time points when water loss was at the maximum (Fig. 2A).

Second, we employed a clinical scoring system (as described in the Materials and Methods section) to assess acute radiation skin toxicity after irradiation. TBI + β -burns alone resulted in higher acute skin toxicity scores, which again peaked at day 11 and returned to control levels by day 25 (Fig. 2B). Unexpectedly, IL-12 administration significantly improved the clinical skin toxicity scores at early time points and resulted in a faster recovery time by day 21 (Fig. 2B). These data demonstrate that a single administration of IL-12 on day 2 postirradiation reduced radiation damage to the cutaneous physical barrier as assessed by TEWL and acute clinical skin toxicity scores.

Intradermal IL-12 Accelerated Burn Healing after TBI + β -Burns

The fundamental ability of skin to heal from wounds and the length of time this process takes can be used to assess function. A 40 Gy β -burn on the back presented as a visible burn approximately day 7 postirradiation characterized by edema and erythema. We monitored the burn area to determine if IL-12 administration reduced burn size and improved healing over time. Figure 3A contains representative images of the TBI + β -burns at day 11 postirradiation from mice without or with IL-12. A measurable burn was observed postirradiation in the untreated TBI + β -burn mice, which slowly healed over time until around day 18 postirradiation (Fig. 3B). Although these wounds decreased in size, visible atrophic scarring was evident and persisted for months. In contrast, IL-12-treated mice had a significantly reduced initial burn size, which slowly decreased over time (Fig. 3B). Furthermore, the majority of β -burns in IL-12 treated mice were not visible until day 8

postirradiation. Additionally, we examined the cellular response to IL-12 in the β -burn site by histology (Fig. 4A). Although no differences in epidermal thickening were observed between IL-12-treated and untreated β -burns (data not shown), IL-12 did significantly reduce the dermal cellularity at day 7 and 14 postirradiation (Fig. 4A and B). These data suggest that administration of IL-12 shortly after radiation exposure may minimize the initial tissue damage elicited by beta radiation by modulating the cutaneous microenvironment resulting in smaller, less damaging insults.

IL-12 Mitigates the Depletion of Cutaneous DCs Induced by TBI + β -Burns

The skin is comprised of an immunological barrier made up of numerous immune cells, including Langerhans cells in the epidermis and interstitial dendritic cells in the dermis. To examine what effect radiation exposure had on these cells specifically and the immunological barrier as a whole, mice were exposed to TBI + β -burns and ears were processed and imaged by fluorescence whole mount microscopy to determine the density of both LCs and iDCs as described in the Materials and Methods. Radiation exposure resulted in a decrease of both LCs and iDCs in the epidermis and dermis, respectively. LCs were maximally decreased on day 7 postirradiation and did not return to basal levels by day 60 postirradiation (Fig. 5A). In contrast, the iDC density reached the lowest point by day 5 postirradiation, however these cells gradually returned to normal levels by day 60 postirradiation (Fig. 5B). The combination of both TBI + β -burns exacerbated the loss of LCs and iDCs when compared to TBI alone (20). Interestingly, i.d. IL-12 administration on day 2 postirradiation resulted in a modest retention of LCs at early time points, but failed to sustain LC density at later time points (Fig. 5A). However, IL-12 treatment did increase the iDC density at most time points when compared to animals not treated with the mitigator. Additionally, IL-12 maintained iDC density near unirradiated control levels and returned iDC numbers back to normal values by day 14 postirradiation compared to day 60 in animals not treated with the IL-12 mitigator (Fig. 5B). These data demonstrated that IL-12 maintained cutaneous DC numbers (especially iDCs), which may contribute to the preservation of the immunological and physical barrier after radiation exposure.

DISCUSSION

The threat of harmful radiation exposure either by means of a device that disperses radiologic material (e.g., dirty bomb), or a catastrophic malfunction similar to the reactor meltdown at the Fukushima Daiichi Nuclear Power Plant has intensified efforts to identify agents that reduce the damaging effects of ionizing radiation on organ systems. IL-12 has been shown to be efficacious in alleviating the effects of ionizing radiation in the hematopoietic system, and at low doses, in the gastrointestinal tract (21, 22). Here, we described the use of IL-12 to minimize damage and improve conditions in the skin after radiation exposure. Our model, mimicking a combined injury of TBI and β -burns, resulted in significant TEWL, burn injury and a decrease of cutaneous immune cells, all of which are risk factors that could significantly impair the host's ability to maintain critical skin homeostasis and eliminate invading cutaneous pathogens (32). IL-12 reduced TEWL and severity of β -burns, suggesting an overall improvement of the physical barrier and repair function in the skin. Additionally, IL-12 prevented the radiation-induced depletion of cDCs,

which are the immune sentinels of the skin that are an important component of the cutaneous immunological barrier. Overall, our studies highlight the use of IL-12 as a therapeutic mitigator against cutaneous radiation injury.

A catastrophic radiation event, such as detonation of an explosive radiologic device or nuclear power plant meltdown, is likely to cause initial confusion and panic, thereby delaying mobilization of medical personnel and first responders to afflicted individuals. This in turn would likely delay the administration of potential medical countermeasures for individuals exposed to radiation, which in a perfect scenario would be available to the affected population shortly after exposure (11). However, it is more realistic to assume that these mitigators would not be available until at least 24–48 h after the emergency (3, 21). In fact, a recent statement from the Centers for Medical Countermeasures against Radiation (CMCR) concerning potential mitigators of radiation-induced sickness has recommended that priority be given to “products that show efficacy when administered 24 h or later after radiation exposure”. In this work, we administered a single dose of IL-12 48 h postirradiation and still were able to demonstrate a significant improvement in multiple parameters associated with skin homeostasis. Our initial goal was to specifically target the skin with an intradermal injection of IL-12, however, our Luminex data clearly demonstrated that this route of injection had detectable systemic effects as reflected by elevated CXCL9 levels in the blood on day 4 postirradiation (Fig. 1B). Systemic levels of CXCL9 are undetectable at day 7 (five days post injection), although it is possible that IL-12 may still be having a local cutaneous effect at this time point. This pattern suggests that IL-12, although effective when administered as a single dose, may be even more efficacious if cytokine levels could be sustained. This could occur through multiple injections of IL-12, or possibly through the utilization of technology in the form of IL-12-encapsulated polylactic acid microspheres, which have the ability to provide a local and sustained release of a protein over time (34–36). Further examination into different scheduling and dose schemes of IL-12 are warranted. Nevertheless, our data identify IL-12 as a promising treatment for the mitigation of radiation-induced cutaneous injury.

IL-12 is currently being tested in multiple clinical trials as a promising antitumor therapy (37). Early trials utilizing IL-12 identified regimens that resulted in toxicity (38–42). However, subsequent studies used refined dosing and delivery schedules to minimize safety concerns (37, 43–46). Interestingly, intravenous administration of IL-12 often resulted in a higher rate of toxicity (40–42, 47). In contrast, nonsystemic routes such as intraperitoneal and especially subcutaneous injection were well tolerated (43–45). In our study, an i.d. route of administration was chosen not only to target IL-12 to the skin, but also to minimize the safety concerns associated with systemic delivery. For example, systemic delivery of IL-12 occasionally resulted in neutropenia, lymphopenia and/or anemia (37, 41, 47), however, as shown in Fig. 1A, i.d. IL-12 therapy did not exacerbate white or red blood cell (data not shown) counts. Additionally, no visible signs of toxicity (e.g., lethargy, GI distress, anorexia, etc.) were observed in animals that received IL-12.

The mode of action of IL-12 in minimizing radiation-induced cutaneous damage is still unknown. It may involve the crosstalk between cytokines and skin cells. Cytokines are biological mediators that facilitate communication between immune and nonimmune cells.

CD4⁺T helper cells orchestrate a controlled release of specific cytokines that polarize the immune response to specific pathogens. For example CD4⁺T helper cells can be divided further into Th1 and Th2 promoting cells (48). Th1 T cells promote cell-mediated immunity through the secretion of cytokines such as IFN γ and IL-12, whereas Th2 T cells induce potent antibody and allergy-like responses by the secretion of IL-4, IL-5 and IL-10 (48). Often, the cytokine milieu present within a tissue microenvironment will govern subsequent immune responses and pathophysiology (49). Of importance, exposure to radiation promotes the release of cytokines in the skin as early as a few hours after the exposure (dose dependent) (49). More importantly, exposure to radiation often results in a Th1/Th2 imbalance within most tissues, including skin, favoring a Th2-like microenvironment (32, 50–53). Th2-like responses in the skin have been implicated in the exacerbation of a number of disorders. Bao *et al.* have suggested that atopic dermatitis, a chronic inflammatory skin disease, is dominated by a strong Th2 type response (54), and Wise *et al.* demonstrated impaired sinusoidal epithelial wound healing (albeit not cutaneous epithelium) by Th2 cytokines (55). Tredget *et al.* reported that Th2 responses correlated with the development of skin fibrosis in patients with thermal injury (56). Furthermore, ultraviolet (UV)-induced cutaneous immunosuppression, a condition that promotes infection and cancer formation, was mediated by the induction of Th2 cytokines, which interestingly, was reversed with IL-12 treatment (57). Although Th2 responses have been shown to be beneficial in wound healing in a nonirradiated setting (58), radiation exposure appears to set up a chronic condition where Th2 responses are undesirable and promote a rapid acceleration of impairments often associated with aged skin (50). Studies have demonstrated that radiation exposure lowers systemic levels of Th1 cytokines including IL-12 (50, 51), and some preliminary data from our laboratory suggest that basal levels of IL-12 mRNA are reduced after TBI + β -burns in the skin (data not shown). IL-12 is a potent inducer of Th1-like responses and strikingly counteracts the formation of Th2 responses (25, 26). Perhaps IL-12 administration in our model is skewing the cytokine milieu in the skin towards less of a Th2-like response or by restoring the balance between Th1 and Th2 responses in the cutaneous microenvironment after radiation exposure.

Exposure to radiation often culminates in apoptosis and cell death causing uncontrolled local inflammation and tissue damage. In our previous studies, a 6 Gy total body irradiation did not result in significant TUNEL⁺ cells in the skin (20), however, the addition of the β -burns is likely to elicit more targeted damage to skin cells/cDCs resulting in increased cell death. Perhaps IL-12 is “protecting” skin cells from radiation-induced death. Schwartz *et al.* demonstrated that IL-12 minimized damage and thus retained LCs in the skin through an induction of nucleotide excision repair (NER) genes required to fix the cyclobutane pyrimidine dimers (CPD) caused by UVB radiation (not ionizing radiation) (59, 60). In this model, IL-12 retention of LCs was abrogated in NER deficient mice. Although NER genes are more commonly associated with DNA repair resulting from UV damage, they are also induced by ionizing radiation to assist in repair (61–64). If IL-12 is directly acting on radiation-damaged cells in a radioprotectant fashion, this in turn could minimize apoptosis and necrotic cell death either directly on cDCs (thereby maintaining their cell density in the skin) or other skin cells, ultimately resulting in less tissue damage (as is evident in the reduced severity of β -burns in Fig. 3).

As for our cDC data, we previously demonstrated that TBI alone (6 Gy) resulted in a migration of cDCs out of the skin to DLN, rather than induction of apoptosis (20). More specifically, our findings indicated that a migratory phenotype was induced by TBI as chemokine CCL21 on lymphatics and its cognate receptor CCR7 on cDCs were upregulated within the cutaneous microenvironment, which appeared to facilitate cDC migration to the DLN in a dose- and time-dependent manner. It is likely that the combined injury model described here also results in a similar mechanism that governs cDC migration out of the skin. Therefore, it is possible that IL-12 administration suppresses the expression of CCR7 or CCL21 causing cDCs to remain in the skin. A number of reports indirectly support this statement. For example, as mentioned above, many of the biological effects of IL-12 are mediated by IFN γ (26, 33). It was recently reported that IFN γ diminished the expression of CCR7 mRNA in murine bone marrow-derived DCs despite the presence of a pro-migratory cytokine milieu (65). Additionally, IFN γ modulated human monocyte-derived DCs as expression of CCR7 was reduced and chemotaxis towards CCL21 was impaired (66). We were able to detect IFN γ in the plasma of naïve mice treated with 300 ng IL-12 i.d. at 6 and 24 h post injection, however, the amount of IFN γ fell below levels of detection at 72 h (data not shown). Furthermore, the IL-12-induced retention of cDCs was *not* observed in IFN $\gamma^{-/-}$ mice in a separate model where mice were treated with a 25 Gy local dose to the ear (24). This dose also results in cDC migration to the DLN, and this migration is similarly reduced by IL-12 treatment in wild-type mice (24). Overall, these results suggest that IFN γ , a downstream mediator of IL-12, may be an important part of the radioprotective mechanism of action of IL-12.

Currently, the Food and Drug Administration has not approved any drugs to counteract the effects of acute radiation exposure. This is unfortunate especially in light of an ever-increasing risk of harmful radiation exposure to the mass public. IL-12 has been shown to be efficacious in protecting the bone marrow, gastro-intestinal tract and now, the skin against radiation-induced damage. Although more mechanistic studies are certainly required to ascertain the exact mode of action of this cytokine in response to ionizing radiation, our findings presented here argue for the further development of IL-12 as a potential countermeasure against harmful radiation exposure.

Acknowledgments

This work was supported by a grant from the National Institute of Allergy and Infectious Diseases (U19-AI091036) and National Institutes of Health (R01CA28332).

References

1. Wolbarst AB, Wiley AL Jr, Nemhauser JB, Christensen DM, Hendee WR. Medical response to a major radiologic emergency: a primer for medical and public health practitioners. *Radiology*. 2010; 254:660–77. [PubMed: 20177084]
2. Dorr H, Meineke V. Acute radiation syndrome caused by accidental radiation exposure - therapeutic principles. *BMC Med*. 2011; 9:126. [PubMed: 22114866]
3. Arora R, Chawla R, Marwah R, Kumar V, Goel R, Arora P, et al. Medical radiation countermeasures for nuclear and radiological emergencies: Current status and future perspectives. *J Pharm Bioallied Sci*. 2010; 2:202–12. [PubMed: 21829316]

4. DiCarlo AL, Maher C, Hick JL, Hanfling D, Dainiak N, Chao N, et al. Radiation injury after a nuclear detonation: medical consequences and the need for scarce resources allocation. *Disaster Med Public Health Prep.* 2011; 5 (Suppl 1):S32–44. [PubMed: 21402810]
5. Peter RU. Cutaneous radiation syndrome in multi-organ failure. *BJR Suppl.* 2005; 27:180–4.
6. Porock D, Nikolett S, Kristjanson L. Management of radiation skin reactions: literature review and clinical application. *Plast Surg Nurs.* 1999; 19:185–92. 223. quiz 191–2. [PubMed: 12024597]
7. Meineke V. The role of damage to the cutaneous system in radiation-induced multi-organ failure. *BJR Suppl.* 2005; 27:85–99.
8. Valladeau J, Saeland S. Cutaneous dendritic cells. *Semin Immunol.* 2005; 17:273–83. [PubMed: 15953735]
9. International Atomic Energy Agency. IAEA reports on Tokaimura accident. *Health Phys.* 2000; 78:231. [PubMed: 10647991]
10. Ishii T, Futami S, Nishida M, Suzuki T, Sakamoto T, Suzuki N, et al. Brief note and evaluation of acute-radiation syndrome and treatment of a Tokaimura criticality accident patient. *J Radiat Res.* 2001; 42 (Suppl):S167–82. [PubMed: 11791750]
11. Meineke V, van Beuningen D, Sohns T, Flidner TM. Medical management principles for radiation accidents. *Mil Med.* 2003; 168:219–22. [PubMed: 12685687]
12. Gottlober P, Steinert M, Weiss M, Bebesko V, Belyi D, Nadejina N, et al. The outcome of local radiation injuries: 14 years of follow-up after the Chernobyl accident. *Radiat Res.* 2001; 155:409–16. [PubMed: 11182791]
13. Callard RE, Harper JI. The skin barrier, atopic dermatitis and allergy: a role for Langerhans cells? *Trends Immunol.* 2007; 28:294–8. [PubMed: 17544846]
14. Schmuth M, Sztankay A, Weinlich G, Linder DM, Wimmer MA, Fritsch PO, et al. Permeability barrier function of skin exposed to ionizing radiation. *Arch Dermatol.* 2001; 137:1019–23. [PubMed: 11493094]
15. Kedzierska A, Kapinska-Mrowiecka M, Czubak-Macugowska M, Wojcik K, Kedzierska J. Susceptibility testing and resistance phenotype detection in *Staphylococcus aureus* strains isolated from patients with atopic dermatitis, with apparent and recurrent skin colonization. *Br J Dermatol.* 2008; 159:1290–9. [PubMed: 18795934]
16. Lebre MC, van Capel TM, Bos JD, Knol EF, Kapsenberg ML, de Jong EC. Aberrant function of peripheral blood myeloid and plasmacytoid dendritic cells in atopic dermatitis patients. *J Allergy Clin Immunol.* 2008; 122:969–76. e5. [PubMed: 18845325]
17. Niebuhr M, Mai U, Kapp A, Werfel T. Antibiotic treatment of cutaneous infections with *Staphylococcus aureus* in patients with atopic dermatitis: current antimicrobial resistances and susceptibilities. *Exp Dermatol.* 2008; 17:953–7. [PubMed: 18557929]
18. Scott JE, ElKhal A, Freyschmidt EJ, MacArthur DH, McDonald D, Howell MD, et al. Impaired immune response to vaccinia virus inoculated at the site of cutaneous allergic inflammation. *J Allergy Clin Immunol.* 2007; 120:1382–8. [PubMed: 17889291]
19. Kupper TS, Fuhlbrigge RC. Immune surveillance in the skin: mechanisms and clinical consequences. *Nat Rev Immunol.* 2004; 4:211–22. [PubMed: 15039758]
20. Cummings RJ, Gerber SA, Judge JL, Ryan JL, Pentland AP, Lord EM. Exposure to ionizing radiation induces the migration of cutaneous dendritic cells by a CCR7-dependent mechanism. *J Immunol.* 2012; 189:4247–57. [PubMed: 23002435]
21. Basile LA, Ellefson D, Gluzman-Poltorak Z, Junes-Gill K, Mar V, Mendonca S, et al. HemaMax, a recombinant human interleukin-12, is a potent mitigator of acute radiation injury in mice and non-human primates. *PLoS One.* 2012; 7:e30434. [PubMed: 22383962]
22. Chen T, Burke KA, Zhan Y, Wang X, Shibata D, Zhao Y. IL-12 facilitates both the recovery of endogenous hematopoiesis and the engraftment of stem cells after ionizing radiation. *Exp Hematol.* 2007; 35:203–13. [PubMed: 17258069]
23. Neta R, Stiefel SM, Finkelman F, Herrmann S, Ali N. IL-12 protects bone marrow from and sensitizes intestinal tract to ionizing radiation. *J Immunol.* 1994; 153:4230–7. [PubMed: 7930625]
24. Cummings RJ, Mitra S, Foster TH, Lord EM. Migration of skin dendritic cells in response to ionizing radiation exposure. *Radiat Res.* 2009; 171:687–97. [PubMed: 19580475]

25. Gee K, Guzzo C, Che Mat NF, Ma W, Kumar A. The IL-12 family of cytokines in infection, inflammation and autoimmune disorders. *Inflamm Allergy Drug Targets*. 2009; 8:40–52. [PubMed: 19275692]
26. O'Garra A, Murphy KM. From IL-10 to IL-12: how pathogens and their products stimulate APCs to induce T(H)1 development. *Nat Immunol*. 2009; 10:929–32. [PubMed: 19692989]
27. Naeger LK, McKinney J, Salvekar A, Hoey T. Identification of a STAT4 binding site in the interleukin-12 receptor required for signaling. *J Biol Chem*. 1999; 274:1875–8. [PubMed: 9890938]
28. Williams JP, Coggle JE, Charles MW, Wells J. Skin carcinogenesis in the mouse following uniform and non-uniform beta irradiation. *Br J Radiol Suppl*. 1986; 19:61–4. [PubMed: 3480030]
29. Benavides F, Oberyszyn TM, VanBuskirk AM, Reeve VE, Kusewitt DF. The hairless mouse in skin research. *J Dermatol Sci*. 2009; 53:10–8. [PubMed: 18938063]
30. Pentland AP, Schoggins JW, Scott GA, Khan KN, Han R. Reduction of UV-induced skin tumors in hairless mice by selective COX-2 inhibition. *Carcinogenesis*. 1999; 20:1939–44. [PubMed: 10506108]
31. Okunieff P, Xu J, Hu D, Liu W, Zhang L, Morrow G, et al. Curcumin protects against radiation-induced acute and chronic cutaneous toxicity in mice and decreases mRNA expression of inflammatory and fibrogenic cytokines. *Int J Radiat Oncol Biol Phys*. 2006; 65:890–8. [PubMed: 16751071]
32. Ryan JL. Ionizing radiation: the good, the bad, and the ugly. *J Invest Dermatol*. 2012; 132:985–93. [PubMed: 22217743]
33. Seder RA, Gazzinelli R, Sher A, Paul WE. Interleukin 12 acts directly on CD4+T cells to enhance priming for interferon gamma production and diminishes interleukin 4 inhibition of such priming. *Proc Natl Acad Sci U S A*. 1993; 90:10188–92. [PubMed: 7901851]
34. Egilmez NK, Jong YS, Mathiowitz E, Bankert RB. Tumor vaccination with cytokine-encapsulated microspheres. *Methods Mol Med*. 2003; 75:687–96. [PubMed: 12407772]
35. Gerber SA, Lim JY, Connolly KA, Sedlacek AL, Barlow ML, Murphy SP, et al. Radio-responsive tumors exhibit greater intratumoral immune activity than nonresponsive tumors. *Int J Cancer*. 2014; 134:2383–92. [PubMed: 24154990]
36. Egilmez NK, Jong YS, Sabel MS, Jacob JS, Mathiowitz E, Bankert RB. In situ tumor vaccination with interleukin-12-encapsulated biodegradable microspheres: induction of tumor regression and potent antitumor immunity. *Cancer Res*. 2000; 60:3832–7. [PubMed: 10919657]
37. Pegram, HJ.; Chekmasova, AA.; Imperato, GH.; Brentjens, RJ. Interleukin 12: Stumbling blocks and stepping stones to effective ant-tumor therapy. In: Arnouk, DH., editor. *Advancemments in tumor immunotherapy and cancer vaccines*. Vol. Chapter 10. InTech; 2012. (<http://bit.ly/15p4c3u>)
38. Sykes M, Pearson DA, Taylor PA, Szot GL, Goldman SJ, Blazar BR. Dose and timing of interleukin (IL)-12 and timing and type of total-body irradiation: effects on graft-vs-host disease inhibition and toxicity of exogenous IL-12 in murine bone marrow transplant recipients. *Biol Blood Marrow Transplant*. 1999; 5:277–84. [PubMed: 10534057]
39. Robertson MJ, Cameron C, Atkins MB, Gordon MS, Lotze MT, Sherman ML, et al. Immunological effects of interleukin 12 administered by bolus intravenous injection to patients with cancer. *Clin Cancer Res*. 1999; 5:9–16. [PubMed: 9918197]
40. Lacy MQ, Jacobus S, Blood EA, Kay NE, Rajkumar SV, Greipp PR. Phase II study of interleukin-12 for treatment of plateau phase multiple myeloma (E1A96): a trial of the Eastern Cooperative Oncology Group. *Leuk Res*. 2009; 33:1485–9. [PubMed: 19243818]
41. Atkins MB, Robertson MJ, Gordon M, Lotze MT, DeCoste M, DuBois JS, et al. Phase I evaluation of intravenous recombinant human interleukin 12 in patients with advanced malignancies. *Clin Cancer Res*. 1997; 3:409–17. [PubMed: 9815699]
42. Leonard JP, Sherman ML, Fisher GL, Buchanan LJ, Larsen G, Atkins MB, et al. Effects of single-dose interleukin-12 exposure on interleukin-12-associated toxicity and interferon-gamma production. *Blood*. 1997; 90:2541–8. [PubMed: 9326219]
43. Lenzi R, Edwards R, June C, Seiden MV, Garcia ME, Rosenblum M, et al. Phase II study of intraperitoneal recombinant interleukin-12 (rhIL-12) in patients with peritoneal carcinomatosis

- (residual disease < 1 cm) associated with ovarian cancer or primary peritoneal carcinoma. *J Transl Med.* 2007; 5:66. [PubMed: 18076766]
44. Portielje JE, Kruit WH, Schuler M, Beck J, Lamers CH, Stoter G, et al. Phase I study of subcutaneously administered recombinant human interleukin 12 in patients with advanced renal cell cancer. *Clin Cancer Res.* 1999; 5:3983–9. [PubMed: 10632329]
 45. Rook AH, Wood GS, Yoo EK, Elenitsas R, Kao DM, Sherman ML, et al. Interleukin-12 therapy of cutaneous T-cell lymphoma induces lesion regression and cytotoxic T-cell responses. *Blood.* 1999; 94:902–8. [PubMed: 10419880]
 46. Wadler S, Levy D, Frederickson HL, Falkson CI, Wang Y, Weller E, et al. A phase II trial of interleukin-12 in patients with advanced cervical cancer: clinical and immunologic correlates. Eastern Cooperative Oncology Group study E1E96. *Gynecol Oncol.* 2004; 92:957–64. [PubMed: 14984966]
 47. Gollob JA, Veenstra KG, Mier JW, Atkins MB. Agranulocytosis and hemolytic anemia in patients with renal cell cancer treated with interleukin-12. *J Immunother.* 2001; 24:91–8. [PubMed: 11211153]
 48. Mosmann TR, Sad S. The expanding universe of T-cell subsets: Th1, Th2 and more. *Immunol Today.* 1996; 17:138–46. [PubMed: 8820272]
 49. Muller K, Meineke V. Radiation-induced alterations in cytokine production by skin cells. *Exp Hematol.* 2007; 35:96–104. [PubMed: 17379094]
 50. Park HR, Jo SK. Lasting effects of an impairment of Th1-like immune response in gamma-irradiated mice: A resemblance between irradiated mice and aged mice. *Cell Immunol.* 2011; 267:1–8. [PubMed: 21092942]
 51. Park HR, Jung U, Jo SK. Impairment of natural killer (NK) cells is an important factor in a weak Th1-like response in irradiated mice. *Radiat Res.* 2007; 168:446–52. [PubMed: 17903039]
 52. Bass H, Mosmann T, Strober S. Evidence for mouse Th1- and Th2-like helper T cells in vivo. Selective reduction of Th1-like cells after total lymphoid irradiation. *J Exp Med.* 1989; 170:1495–511. [PubMed: 2572669]
 53. Tanay A, Strober S. Opposite effects of total lymphoid irradiation on T cell-dependent and T cell-independent antibody responses. *J Immunol.* 1984; 132:979–84. [PubMed: 6361136]
 54. Bao L, Zhang H, Chan LS. The involvement of the JAK-STAT signaling pathway in chronic inflammatory skin disease atopic dermatitis. *JAKSTAT.* 2013; 2:e24137. [PubMed: 24069552]
 55. Wise SK, Den Beste KA, Hodgeson EK, Parkos CA, Nusrat A. Sinonasal epithelial wound resealing in an in vitro model: inhibition of wound closure with IL-4 exposure. *Int Forum Allergy Rhinol.* 2013; 3:439–49. [PubMed: 23468432]
 56. Tredget EE, Yang L, Delehanty M, Shankowsky H, Scott PG. Polarized Th2 cytokine production in patients with hypertrophic scar following thermal injury. *J Interferon Cytokine Res.* 2006; 26:179–89. [PubMed: 16542140]
 57. Ullrich SE. Does exposure to UV radiation induce a shift to a Th-2-like immune reaction? *Photochem Photobiol.* 1996; 64:254–8. [PubMed: 8760564]
 58. Maeda S, Fujimoto M, Matsushita T, Hamaguchi Y, Takehara K, Hasegawa M. Inducible costimulator (ICOS) and ICOS ligand signaling has pivotal roles in skin wound healing via cytokine production. *Am J Pathol.* 2011; 179:2360–9. [PubMed: 21925472]
 59. Schwarz A, Maeda A, Kernebeck K, van Steeg H, Beissert S, Schwarz T. Prevention of UV radiation-induced immunosuppression by IL-12 is dependent on DNA repair. *J Exp Med.* 2005; 201:173–9. [PubMed: 15657287]
 60. Schwarz A, Stander S, Berneburg M, Bohm M, Kulms D, van Steeg H, et al. Interleukin-12 suppresses ultraviolet radiation-induced apoptosis by inducing DNA repair. *Nat Cell Biol.* 2002; 4:26–31. [PubMed: 11780128]
 61. Amundson SA, Do KT, Shahab S, Bittner M, Meltzer P, Trent J, et al. Identification of potential mRNA biomarkers in peripheral blood lymphocytes for human exposure to ionizing radiation. *Radiat Res.* 2000; 154:342–6. [PubMed: 11012342]
 62. Cramers P, Filon AR, Pines A, Kleinjans JC, Mullenders LH, van Zeeland AA. Enhanced nucleotide excision repair in human fibroblasts pre-exposed to ionizing radiation. *Photochem Photobiol.* 2012; 88:147–53. [PubMed: 22017241]

63. Rajaraman P, Bhatti P, Doody MM, Simon SL, Weinstock RM, Linet MS, et al. Nucleotide excision repair polymorphisms may modify ionizing radiation-related breast cancer risk in US radiologic technologists. *Int J Cancer*. 2008; 123:2713–6. [PubMed: 18767034]
64. Rieger KE, Chu G. Portrait of transcriptional responses to ultraviolet and ionizing radiation in human cells. *Nucleic Acids Res*. 2004; 32:4786–803. [PubMed: 15356296]
65. Yen JH, Kong W, Ganea D. IFN-beta inhibits dendritic cell migration through STAT-1-mediated transcriptional suppression of CCR7 and matrix metalloproteinase 9. *J Immunol*. 2010; 184:3478–86. [PubMed: 20190134]
66. Alder J, Hahn-Zoric M, Andersson BA, Karlsson-Parra A. Interferon-gamma dose-dependently inhibits prostaglandin E2-mediated dendritic-cell-migration towards secondary lymphoid organ chemokines. *Vaccine*. 2006; 24:7087–94. [PubMed: 16889876]

FIG. 1.

Analyses of blood after radiation exposure. C57BL/6 hairless mice were exposed to 6 Gy TBI followed by a total of three 40 Gy β -burns (1 on each ear; 1 on back). Half of the mice were treated with a single administration of three 100 ng injections of rIL-12 to each β -burn site (300 ng total/mouse) 48 h postirradiation. Control mice were left unirradiated. Panel A: Complete blood counts were performed on blood and white blood cell (WBC) number reported. The dotted rectangle represents the expected range of WBC number in normal unirradiated mice. Panel B: Plasma was isolated from the remainder of the blood and levels of CXCL9 protein to assess IL-12 bioactivity were determined by Luminex. $**P < 0.001$ and $*P < 0.05$ represent significance as determined by ANOVA followed by a Bonferroni post hoc test. $n = 4-6$ for all groups at each time point.

FIG. 2.

IL-12 inhibits water loss and improves skin scores after TBI + β -burns. C57BL6/hairless mice exposed to 6 Gy TBI followed by a total of three 40 Gy β -burns (1 on each ear; 1 on back). Half of the mice were treated with a single administration of three 100 ng injections of rIL-12 to each β -burn site (300 ng total/mouse) 48 h postirradiation. Panel A: TEWL was measured by placing a Tewameter probe on the back β -burn wound site at various time points over 60 days. Panel B: Skin scores for radiation toxicity were assessed as described in the Materials and Methods section. * $P < 0.05$ represents significance (between IL-12 and no IL-12 groups) as determined by ANOVA followed by a Bonferroni post hoc test. $n = 4-6$ for all groups at each time point.

FIG. 3.

IL-12 treatment reduces severity of wounds after TBI + β -burns. C57BL6/hairless mice were exposed to 6 Gy TBI followed by a total of three 40 Gy β -burns (1 on each ear; 1 on back). Half of the mice were treated with a single administration of three 100 ng injections of rIL-12 to each β -burn site (300 ng total/mouse) 48 h postirradiation. Panel A: Digital images from representative samples 11 days postirradiation were taken from a fixed distance above the mouse. Both lines represent 5 mm. Panel B: The area of the β -burn wound on the back was determined as described in the Materials and Methods section from day 7–20. * $P < 0.05$ represents significance (between IL-12 and no IL-12 groups) as determined by ANOVA followed by a Bonferroni post hoc test. $n = 4$ –6 for all groups at each time point.

FIG. 4.

IL-12 reduces dermal cellularity in the β -burn site. C57BL6/hairless mice were exposed to 6 Gy TBI followed by a total of three 40 Gy β -burns (1 on each ear; 1 on back). Half of the mice were treated with a single administration of three 100 ng injections of rIL-12 to each β -burn site (300 ng total/mouse) 48 h postirradiation. Panel A: Hematoxylin & eosin (H&E) staining of TBI + β -burn skin at day 7 and 14 postirradiation with or without intradermal IL-12 administration. Black bar on images represents 75 microns. Panel B: Dermal cellular density was quantitated through imaging analysis of the H&E stained TBI + β -burn skin as described in the Materials and Methods section. * $P < 0.05$ represents significance (between IL-12 and no IL-12 groups) as determined by ANOVA followed by a Tukey's post hoc test. $n = 4-6$ for all groups at each time point.

FIG. 5.

IL-12 retains cutaneous dendritic cells after TBI + β -burns. C57BL6/hairless mice exposed to 6 Gy TBI followed by a total of three 40 Gy β -burns (1 on each ear; 1 on back). Half of the mice were treated with a single administration of three 100 ng injections of rIL-12 to each β -burn site (300 ng total/mouse) 48 h postirradiation. Ears were removed and separated into epidermal and dermal sheets and stained for LCs using anti-CD205 or iDCs using anti-MHC class II. Density of LCs in epidermis (panel A), or iDCs in dermis (panel B) was determined by whole mount fluorescence microscopy as described in the Materials and Methods section. * $P < 0.05$ represents significance (between IL-12 and no IL-12 groups) as determined by ANOVA followed by a Bonferroni post hoc test. $n = 4-6$ for all groups at each time point.