Total-Body Irradiation Exacerbates Dissemination of Cutaneous *Candida Albicans* Infection

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Exposure to radiation, particularly a large or total-body dose, weakens the immune system through loss of bone marrow precursor cells, as well as diminished populations of circulating and tissue-resident immune cells. One such population is the skin-resident immune cells. Changes in the skin environment can be of particular importance as the skin is also host to a number of commensal organisms, including *Candida albicans*, a species of fungus that causes opportunistic infections in immunocompromised patients. In a previous study, we found that a 6 Gy sublethal dose of radiation in mice caused a reduction of cutaneous dendritic cells, indicating that the skin may have a poorer response to infection after irradiation. In this study, the same 6 Gy sublethal radiation dose led to a weakened response to a *C. albicans* cutaneous infection, which resulted in systemic dissemination from the ear skin to the kidneys. However, this impaired response was mitigated through the use of interleukin-12 (IL-12) administered to the skin after irradiation. Concomitantly with this loss of local control of infection, we also observed a reduction of CD4⁺ and CD8⁺ T cells in the skin, as well as the reduced expression of IFN- γ , CXCL9 and IL-9, which influence T-cell infiltration and function in infected skin. These changes suggest a mechanism by which an impaired immune environment in the skin after a sublethal dose of radiation increases susceptibility to an opportunistic fungal infection. Thus, in the event of radiation exposure, it is important to include antifungal agents, or possibly IL-12, in the treatment regimen, particularly if wounds are involved that result in loss of the skin's physical barrier function. © 2016 by Radiation Research Society

INTRODUCTION

Radiation exposure is a potent health hazard. Sources include controlled exposure through radiation therapy, accidental high-dose exposure through nuclear power plant disasters and deliberate dispersal through dirty bombs (1). Sources such as nuclear power plants and dirty bombs are of particular concern as they potentially expose individuals to large doses in which the entire body absorbs the dose in the form of total-body irradiation (TBI). Additionally, while the risk of a high-dose exposure with a dirty bomb is limited to the immediate area surrounding the detonation, populations in the vicinity will also likely suffer physical injuries, further complicating their condition (1).

Combined radiation and physical injury is of serious concern for several reasons. Radiation alone is capable of damaging multiple organs and tissues, including bone marrow (2, 3). Since the bone marrow produces the precursor cells of the immune system, production of new immune cells can be impaired. Other immune cell populations may also be affected, as was the case in atomic bomb survivors from World War II, who were found to have fewer naïve T cells and T cells capable of producing IL-2 than age-matched individuals who were not exposed (4). This exposure leaves an individual in an immunologically weakened, or immunocompromised state and at a higher risk of infection.

While skin tissue acts as a physical barrier to invading organisms, it is easily damaged by external sources of radiation. The skin is likely the first organ to come in contact with radiation, especially in cases of power plant disasters and dirty bombs, with TBI resulting in the entirety of the skin absorbing a dose. After a high-dose exposure, the symptoms of cutaneous radiation syndrome (CRS) will occur within 24–72 h, while late-stage symptoms, such as fibrosis, may not arise until years after exposure (5). Initially, CRS is largely a physical disruption of the skin, with symptoms that include burns, blisters and other breaks in the skin, which result in an increased risk of infection as a secondary complication after exposure. Additionally, CRS also impairs the immunological barrier function of the skin,

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resulting in a suppressed immune environment. Most studies pertaining to the effects of radiation on the skin focus on ultraviolet (UV) exposure where the initial inflammatory state is followed by a loss of Langerhans cells, a subpopulation of dendritic cells (DCs) that can serve as antigen-presenting cells (APCs), only to be replaced by other APCs that preferentially activate suppressive regulatory T cells (T_{Reg}) (6). Much less is known about the effect of ionizing radiation on the skin, however, in a few reported studies, an increase in the ratio of T_{Reg} to T effector (T_{Eff}) cells was observed (7, 8) in irradiated skin. These data suggest that an increase in the ratio of T_{Reg} cells, combined with loss of precursor cells, alterations to other immune populations and physical breaks due to CRS or injury may leave the skin vulnerable to infection after exposure.

In cases of infection in immunocompromised hosts, the invading organism is often a member of the resident flora that is normally controlled by the immune system. One such organism commonly responsible for these opportunistic infections is the fungus *Candida albicans* (*C. albicans*), which colonizes the skin and is one of the most commonly isolated fungi in cases of these infections (9–12). Often these cases occur in patients with other complications, such as invasive procedures and, very commonly, patients with head and neck cancers who are undergoing radiation therapy (10, 13, 14). Upon infection with *C. albicans*, the primary effector cells are neutrophils of the innate immune system (15, 16). If the infection persists, $CD4^+$ T-helper cells are recruited to the infection site (17, 18). When this infection occurs in the skin, cutaneous DCs are necessary for priming and recruiting these T-helper cells (19). The cutaneous DCs also produce several cytokines, such as IL-6 and interferon gamma ($IFN-\gamma$), to aid in the response. Once recruited, the T-helper cells can then further increase recruitment of neutrophils and induce keratinocytes to increase production of antimicrobial peptides (AMPs) through the production of IL-17 (20, 21). The T-helper cells will also secrete IL-2 and $IFN-\gamma$ to increase fungicidal activity of phagocytic cells (20, 22). However, if radiation interferes in this response, then the infection may disseminate beyond the skin, resulting in a more lethal form of disease. Upon spreading to the blood stream, *C. albicans* may infect the kidneys, which can result in renal failure (23). Despite this, the topic of potential infections after irradiation has largely focused on models with bacteria species (24, 25). Here, we investigate the potential risk of a serious *C. albicans* infection after sublethal TBI.

Using hairless mice, we found previously that sublethal 6 Gy TBI resulted in a decrease in skin resident dendritic cells (DCs) over time (26), similar to what was observed after UV exposure. We also observed that administration of recombinant interleukin-12 (IL-12) either before or after TBI resulted in retention of some, although not all, of these cells (27). DCs are proficient APCs, linking the innate and adaptive arms of the immune system. It is possible that a reduction in these cells could result in a poorer immune

response upon fungal infection, since DCs are able to present *C. albicans* antigen to prime an adaptive response (28). Interestingly, we have previously observed a decrease in DC function after irradiation, as the DCs failed to elicit a contact hypersensitivity reaction (CHS) (27). We hypothesized that sublethal TBI will result in a breakdown of the cutaneous immunological barrier. If this impairment should occur with a break in the physical integrity of the skin, such as through an injury that may be sustained during or after exposure, the skin may not be able to contain an infection.

In our current study, we modeled this infection after a combined immunological and physical breakdown of the skin by injecting hairless mice intradermally (i.d.) in the ear with *C. albicans* one week postirradiation (29). Our previous study demonstrated the decrease in DCs was maximal at this point after irradiation, and may be when the skin is at greatest risk of infection (27). In this model, we observed not only an increase in dissemination of *C. albicans* after irradiation, but also significant deficiencies in the host response against the microbe including changes in the cutaneous immune cells and cytokine environment, as well as a general reduction of circulating immune cells. Lastly, we examined the ability of IL-12 to mitigate this loss of infection control.

MATERIALS AND METHODS

Animals

SKH-1 (hr/hr) hairless mice, partially backcrossed with C57BL/6 to approximately 60% genetic similarity, were used in all experiments to facilitate studies in skin. All experiments were conducted using male mice only. All protocols using mice were approved by the University of Rochester's University Committee on Animal Resources (UCAR).

Animal Irradiations

Adult mice (9–10 weeks old) received 6 Gy TBI using a 3,200 Curie cesium-137 source, at a rate of 1.9 Gy/min. All exposed mice were placed in a plexiglass box on top of a collimator for the duration of irradiation, allowing for uniform exposure for each mouse.

Candida Albicans Infection

Candida albicans strain SC5314 was used for all infections (30). Cultures were grown overnight in a shaking incubator in 10 ml 1% yeast extract, 2% peptone, 2% dextrose (YPD) broth (Fisher Scientific, Pittsburgh, PA). Organisms used were washed twice in PBS and adjusted to 2.5×10^8 cells/ml in phosphate buffered saline (PBS). Anesthetized mice were given an i.d. injection of 20 μ l of yeast suspension (5×10^6 cells) using an insulin syringe with an ultra-fine 31-gauge needle (BD Biosciences, San Jose, CA). For short-term studies, mice were injected in either one ear pinna or the upper dorsal skin at 6–8 weeks of age. For the day 60 experiment, anesthetized mice were injected 60 days after TBI in one ear pinna.

Colony-Forming Assay

For organism burden assessment, mice were sacrificed 4 days after start of infection, or 21 days after start of infection for the long-term infection study. Whole ears or approximately 10 mm² of back skin (epidermis and dermis) and both whole kidneys were excised and homogenized using a PRO200® tissue homogenizer (PRO Scientific

TABLE 1
Flow Cytometry Antibodies

Antibody	Source
Anti-CD4 (GK1.5)	BD Biosciences
Anti-CD19 (C1-1D3)	BD Biosciences
Anti-gamma delta T-cell receptor (GL3)	BD Biosciences
Anti-CD3 (145-2C11)	BD Biosciences
NK1.1 (PK136)	BD Biosciences
CD11c (C1-HL3)	BD Biosciences
Anti-Ly6C (AL-21)	BD Biosciences
Anti-Ly6G (1A8)	BD Biosciences
Anti-Gr-1 (RB6-865)	BD Biosciences
Anti-CD45 (30-F11)	BD Biosciences
Anti-CD8 (53-6.7)	eBiosciences
Anti-CD11b (M1170)	eBiosciences
F4/80 (1BM8)	eBiosciences

Inc., Oxford, CT) in 0.5% trypsin ethylenediaminetetraacetic acid (EDTA) (Gibco®, Grand Island, NY). Homogenates were serially diluted and plated on YPD agar (Fisher Scientific) media plates containing 1 µg/ml of chloramphenicol (Roche, New York, NY) and 30 µg/ml of gentamicin (Sigma-Aldrich®, St. Louis, MO). Plates were incubated at 37°C overnight, colony forming units (CFU) determined and data expressed as CFU/tissue sample.

Complete Blood Counts

Blood samples were collected from mice at day 11 postirradiation (4 days after *C. albicans* injection) for short-term studies. For the day 60 experiment, blood samples were collected four days after *C. albicans* injection. Blood samples were acquired after mice were sacrificed via the descending aorta using a heparinized syringe with a 25-gauge needle (BD Biosciences), and then transferred into 1 ml BD Microtainer® tubes with EDTA (BD Biosciences). Blood samples were analyzed on a HemaTrue™ Hematology Analyzer (Heska, Loveland, CO).

Flow Cytometry

Infected and noninfected adult mice were sacrificed at day 11 postirradiation and the whole ears were excised. Ears were split into dorsal and ventral halves and digested in collagenase (0.1%) for 35 min at 37°C. Ears were mechanically disrupted against a wire mesh strainer using a 10 ml syringe plunger (BD Biosciences), filtered using 100-micron nylon mesh cell strainers (Fisher Scientific), then washed in balanced salt solution (BSS) with 1% fetal bovine serum (FBS), and washed again in 1× PBS. Samples were stained with Live/Dead® Aqua (Life Technologies, Carlsbad, CA) for 30 min in the dark at room temperature. Samples were then washed in PBS, and stained using fluorescently conjugated antibodies (Table 1). Samples were washed again in PBS, fixed with 150 µl of BD Cytofix/Cytoperm™ (BD Biosciences) and stored at 4°C until analysis on a FACSCanto™ II flow cytometry instrument. Gating on CD45⁺ live cells, we were able to account for an average of 80–90% of the CD45⁺ cells.

Gene Expression Studies

Mice were sacrificed at day 11 postirradiation and the whole ears were excised. Skin samples were placed into RNAlater (QIAGEN®, Hilden, Germany) and stored at 4°C. RNA was extracted from samples using the QIAGEN Fibrous Tissue Kit (QIAGEN) following the manufacturer's instructions. cDNA was synthesized using iScript™ cDNA Synthesis Kit (Bio-Rad Laboratories Inc., Hercules, CA). RT-PCR was performed using Cytochemokines Tier 1 M96 Mouse gene array plates (Bio-Rad) with SYBR® Green fluorescent dye. Expression

was normalized using expression of GAPDH and TBP (TATA-box-binding protein) as housekeeping genes. Data from the array plates were analyzed and plotted using CFX Manager software (Bio-Rad). Significance was considered at $P < 0.05$ and was determined using the Student's *t* test.

IL-12 Mitigation

Mice were treated with either a single i.d. injection in the ear of 300 ng of recombinant IL-12 in PBS with 0.1% BSA or vehicle only in a volume of 60 µl. Injection was given at day 2 postirradiation. Mice were infected day 7 postirradiation in the same ear that was treated with IL-12.

Data Analysis

A Student's *t* test with Welch's correction was performed for colony forming assays and flow cytometry data. Where appropriate, a Student's *t* test without Welch's correction was performed. A one-way analysis of variance (ANOVA) was performed for the IL-12 mitigation colony forming assays. In each case, $P < 0.05$ was considered significant.

RESULTS

Exposure to Radiation Leads to Increased C. Albicans Dissemination from the Ear Skin to the Kidneys

To determine whether radiation exposure worsens the ability of the skin to contain a subsequent cutaneous infection, hairless mice received sublethal 6 Gy TBI. We used a 6 Gy dose since it has been previously shown that at this dose cutaneous DCs were depleted, while no significant damage of other tissues, such as the GI tract, was observed (27). At day 7 postirradiation, mice were i.d. injected in one ear with of 5×10^6 CFU *C. albicans*. This time point was chosen since, in our previously reported study, a reduction in DCs after 6 Gy irradiation was observed (27). Four days after infection (11 days postirradiation) mice were sacrificed, and the infected ear and both kidneys were excised and homogenized, and homogenates were plated (Fig. 1A). Organism burden was assessed at the initial site of infection as CFU per whole infected ear (Fig. 1B). There was a trend of increased organism burden at the local infection site in irradiated mice compared to nonirradiated mice, while this difference was not significant. Although local infections are often controlled, dissemination of *C. albicans* may be life threatening. Therefore, we assessed organism burden in the kidney, which is a sensitive target organ (31). Importantly, there was a significant increase in fungal burden in the kidneys of irradiated mice compared to nonirradiated mice, suggesting a loss of local control of the infection (Fig. 1C).

To test a second skin site, a similar experiment was performed using the upper dorsal skin as the initial site of infection (Supplementary Fig. S1A; <http://dx.doi.org/10.1667/RR14295.1.S1>). As with the ear site, the back skin showed similar burdens between irradiated and nonirradiated mice (Supplementary Fig. S1B). However, unlike the

FIG. 1. Total-body irradiation of hairless mice results in increased *C. albicans* dissemination from the ear to kidneys. Panel A: Radiation and acute ear infection model protocol. Adult hairless mice received 6 Gy TBI. One week postirradiation, mice were injected with 5×10^6 CFU of *C. albicans* intradermally in one ear. At day 11 postirradiation, ear and kidneys were excised and processed for a colony forming assay. Colony forming assay results are shown for infected ear (panel B) and kidneys (panel C). Values are pooled from 14 nonirradiated or 18 irradiated animals. Error bars are SD. * $P < 0.05$.

ear site, there was no increase in dissemination to the kidneys (Supplementary Fig. S1C). Therefore, this loss of control occurs in the ear but not the back skin.

Increased Dissemination of C. Albicans Infection Due to Radiation Exposure is Limited to the Acute Infection Model

To determine whether the radiation-induced effects on *C. albicans* dissemination were chronic, hairless mice were injected with *C. albicans* at the increased time interval of 60 days after 6 Gy TBI. The 60-day time point was chosen based on our observation that the number of cutaneous DCs recovers by approximately this time (27), and therefore, the cutaneous immunological barrier could have recovered by this time as well. Mice were infected with 5×10^6 CFU of *C. albicans* i.d. in one ear as before, and were sacrificed four days after the start of infection. The ear tissue and kidneys were used to determine organism burden at both the local and distal site of infection, respectively (Fig. 2A). Interestingly, there was a decrease in fungal burden at the initial site of infection (Fig. 2B). Additionally, contrary to what we observed in the acute model (Fig. 1A), there was no increase in dissemination, as the kidneys showed similar yeast burdens between irradiated and nonirradiated mice (Fig. 2C). The decreased fungal burdens at both sites are consistent with a full recovery of the cells responsible for local control between day 7 and 60 postirradiation, as evident with the recovery of cutaneous DCs in a previously published study (27).

We also investigated a later time point of 28 days after TBI (21 days after infection) to ensure that the infection did

not worsen after day 11 postirradiation. Mice were irradiated and infected as shown in Fig. 1A, and were weighed daily starting from day 0 (Fig. 2D) to monitor response to infection. After a slight initial drop in weight after irradiation and infection, all of the nonirradiated mice began to gain weight four days after infection (Fig. 2E). Four of the six irradiated mice began to gain weight within a week after infection and showed no signs of overt infection as far as three weeks after infection. When assayed, all of the mice that had gained weight in both the irradiated and nonirradiated groups had no *C. albicans* present in either the ear or kidney. Interestingly, one of the two that did demonstrate weight loss early succumbed to infection having 7.5×10^5 CFU/total kidneys (the other mouse was excluded due to being found several hours after death and after decomposition had begun). More importantly, these mice demonstrated weight loss before day 11 postirradiation end point described in this study. In view of these data, we concluded that the end point of 11 days after TBI was optimal, because no mice developed infection after this time point and all significantly infected mice demonstrated signs of infection (weight loss) before this end point.

Circulating Leukocyte Populations Remain Decreased Systemically 11 Days after TBI in Infected and Noninfected Mice

To assess possible systemic effects of radiation during infection, whole blood samples were used to determine leukocyte counts. Noninfected control mice received 6 Gy TBI then sacrificed 11 days later and blood samples

FIG. 2. Radiation-induced risk of increased *C. albicans* dissemination from ear to kidney is acute. Panel A: Radiation and 60-day infection model protocol. Mice were infected 60 days post-TBI. Colony forming assay results are shown for infected ear (panel B) and kidneys (panel C). Values were pooled from six animals. Panel D: Long-term infection protocol. End point was changed to 28 days after TBI, and mice were weighed daily starting from day 0. Panel E: Weights of individual nonirradiated mice (black lines) and irradiated mice (gray-dashed lines). Colony forming assay results are shown for infected ear (panel F) and kidneys (panel G). Values were pooled from six nonirradiated or five irradiated animals. One mouse died and was excluded from the tissue burdens. Error bars are SD. * $P < 0.05$.

collected and analyzed. As expected, total leukocyte counts, as well as the individual subpopulations of lymphocytes, monocytes and granulocytes, were all significantly reduced at day 11 postirradiation (Fig. 3), similar to what we had previously observed at earlier time points after irradiation (data not shown). Blood samples were also collected from mice infected (see Fig. 1A) at day 11 post-TBI. As with the noninfected mice, the irradiated/infected mice demonstrated a significant decrease in circulating monocytes and granulocytes, as well as decreased white blood cells collectively (Fig. 3). Unlike the noninfected mice, there was not a significant decrease in circulating lymphocytes.

TBI Alters the Relative Abundance and Proportions of T Cells and Granulocytes in the Local Site of Cutaneous Infection

To assess the immune response at the site of infection, mice were treated as shown in Fig. 1A. At day 11 post-TBI, infected whole-ear tissue was processed for analysis by flow cytometry, and cell populations analyzed using the gating strategy shown in Supplementary Fig. S2 (<http://dx.doi.org/10.1667/RR14295.1.S1>). The proportions of CD45⁺ immune cells in the ears of noninfected mice were relatively similar among irradiated and nonirradiated mice (Fig. 4A). As expected, upon infection, the majority of CD45⁺ cells in

FIG. 3. Concentrations of certain white blood cells in the peripheral circulation remain decreased during *C. albicans* infection after irradiation. Adult hairless mice were treated as in the acute infection model. At day 11 postirradiation, mice were sacrificed and blood samples collected and used for complete blood counts for both infected and noninfected animals. Values were pooled from four nonirradiated or five irradiated animals. Error bars are SD. * $P < 0.05$.

the skin of the ear were granulocytes (Fig. 4B). However, there was a noticeable decrease in the percentage of lymphocytes in the irradiated/infected ear, particularly $CD4^+$ and $CD8^+$ T cells. Additionally, we observed an increase in the proportion of monocytes and granulocytes in the irradiated/infected ear when compared to the nonirradiated/infected ear. The absolute cell numbers/g of tissue in the irradiated/infected ears were significantly decreased for $CD4^+$ and $CD8^+$ T-cells populations, whereas we observed a significant increase in granulocytes relative to the nonirradiated/infected ear (Fig. 4C). This decrease of $CD4^+$ and $CD8^+$ lymphocytes in the irradiated/infected ears correlates with irradiated mice having increased dissemination. Additionally, there were no observable changes in noninfected mice (Supplementary Fig. S3).

Expression of Several Immune Cell Chemotactic Factors is Reduced During C. Albicans Infection after Irradiation

Several cytokines and chemokines are necessary for initiating and maintaining immune responses to infection. In our previous study, we observed a radiation-induced change in expression of the chemokine CCR7, which we hypothesized to be the reason for a reduction of cutaneous DCs after irradiation (32). Radiation exposure in this infection model resulted in altered cutaneous T-cell and granulocyte populations, we tested for another possible radiation-induced change in cytokine and chemokine expression by

measuring the relative gene expression of an array of these molecules. Mice were treated as shown in Fig. 1A, and infected whole ears were excised at day 11 post-TBI. Total RNA was extracted from infected ear tissue and gene expression was assessed by RT-PCR. Collectively, radiation exposure of mouse skin resulted in more downregulated than upregulated genes when compared to nonirradiated controls (Fig. 5, Table 2). Additionally, there was a noticeable cluster of downregulated genes predominately involved in T-cell function and chemotaxis (circled genes). CXCL9, a chemokine for activated T cells and NK cells during an inflammatory response in the skin, was significantly downregulated (33–36). IFN- γ , which induces CXCL9, was also significantly downregulated along with the cytokine IL-9. Interestingly, IL-9 can induce a Th17 T-cell response conducive to clearing *C. albicans* infections (37). CXCL10, another chemokine induced by IFN- γ (33) that strongly attracts activated T cells into inflamed skin, was decreased in irradiated/infected skin. The lowered expression of this gene was associated with reduced T-cell populations observed in irradiated mice (Fig. 4C).

IL-12 Administered after TBI Results in Decreased Dissemination of C. Albicans to the Kidneys

In a previously reported study, we observed that IL-12 administration after local irradiation to the ear was able to retain some of the cutaneous dendritic cells and thus

FIG. 4. Radiation exposure decreases certain immune cell populations in the ear during *C. albicans* infection. Adult hairless mice were treated as in the acute infection model. At day 11 post-TBI, mice were sacrificed and ear tissue was excised, processed and analyzed by flow cytometry. Panel A: Percentage of live CD45⁺ cells in the ear. Data were pooled from five animals. Panel B: Percentage of live CD45⁺ cells in the skin of infected mice. Data were pooled from four animals (irradiated/noninfected mice) or five animals (irradiated/infected mice). Panel C: Live cells/g of infected ear skin from *C. albicans*-infected mice. Data were pooled from four nonirradiated or five irradiated animals. Error bars are SD. **P* < 0.05.

maintain the immunological barrier (26). IL-12 has numerous immunostimulatory effects, such as an increase in the phagocytic activity of neutrophils through IFN- γ (38). Considering this cytokine's ability to both protect the skin after irradiation and create an immunostimulatory environment, we investigated the use of IL-12 as a potential mitigator of *C. albicans* infection after TBI.

Using our acute model, we administered 300 ng of recombinant IL-12 in one ear at day 2 post-TBI (Fig. 6A). Control mice received vehicle control. Mice were infected in the same ear at day 7 post-TBI. At the local infection site, there was no change in *C. albicans* burden among treatment groups (Fig. 6B). However, there was a decrease in fungus burden in the kidneys of irradiated IL-12-treated mice

FIG. 5. Radiation exposure results in downregulation of several cytokines in *C. albicans*-infected ear skin. Volcano plot of gene expressions in irradiated compared with nonirradiated mice. Adult hairless mice were treated as in the acute infection model. At day 11 postirradiation mice were sacrificed and ear tissue excised and processed for RT-PCR. Genes to the left of the green line showed a greater than twofold decrease in expression. Genes to the right of the red line showed a twofold or greater increase in expression. Genes above the blue line have a statistically significant expression change ($P < 0.05$). Data were combined from four animals.

compared to irradiated vehicle-treated mice (Fig. 6C). This suggests that IL-12 restores the ability of the skin to contain the *C. albicans* infection.

DISCUSSION

Candida albicans opportunistic infections pose a serious problem in immunocompromised patients, including patients exposed to radiation (39). As we observed in our model, mice exposed to sublethal doses of ionizing gamma radiation exhibited an increase in *C. albicans* burden in the kidneys, indicating the skin in these irradiated mice was less able to contain infection compared to nonirradiated mice. This may have been due to several of the changes in the local cutaneous environment that we also observed.

The gene array data showed changes in expression that may ultimately modulate the type and function of immune

TABLE 2
Genes Measured in Gene Array

Gene	Fold change	P value	Gene	Fold change	P value
Adipoq	1.65	0.48	Ifnb1	-2.99	0.37
Areg	1.52	0.44	Ifng	-16.03	0.03
Bmp2	-1.21	N/A	Il-10	-1.04	0.72
Bmp4	1.99	0.67	Il11	-2.75	0.40
Bmp7	5.27	0.55	Il-12a	-1.04	0.57
Ccl11	-1.03	0.60	Il-13	-2.13	0.17
Ccl12	1.14	0.97	Il-15	-1.36	0.21
Ccl17	-3.04	0.17	Il-17a	-4.39	0.95
Ccl20	-1.6	0.37	Il-17f	-4.78	0.73
Ccl21c	-1.25	0.96	Il-18	1.29	0.50
Ccl22	1.91	0.82	Il-1a	3.47	0.87
Ccl4	-3.01	0.37	Il-1b	-2.77	0.40
Ccl5	-1.28	0.59	Il-2	-5.5	0.15
Cd40lg	-2.77	0.11	Il-21	-22.7	N/A
Cntf	-1.99	0.37	Il-23a	-4.72	0.34
Csf1	1.38	0.82	Il3	-11.7	0.33
Csf2	-4.5	0.29	Il4	-6.01	0.17
Csf3	-4.12	0.58	Il5	-1.28	0.41
Cx3cl1	2.58	0.58	Il6	-3.32	0.33
Cxcl1	-1.87	0.74	Il7	-1.06	0.88
Cxcl10	-2.99	0.09	Il9	-24.02	0.05
Cxcl12	1.51	0.57	Kitl	1.69	0.58
Cxcl3	-2.16	0.83	Lep	1.37	0.81
Cxcl9	-28.15	0.03	Lif	-3.61	0.37
Edn1	1.56	0.52	Lta	-3.62	0.25
Fasl	-3.56	0.26	Mif	1.18	0.79
Fgf2	1.95	0.68	Mstn	1.35	0.69
Gapdh ^a	N/A	0.59	Nrg1	-1.21	0.85
Gdf10	1.6	0.75	Osm	-1.73	0.48
Gdf15	-1.27	0.45	PCR	1.08	0.39
gDNA	-5.71	0.34	Pf4	1.35	0.73
Gm12597	-4.17	0.35	Ppbp	-1.54	0.58
Gm13280	-5.34	0.35	Sectm1a	1.02	0.37
Gpi1	1.26	0.51	Slurp1	-1.39	0.32
Grn	1.1	0.38	Spp1	-1.46	0.84
Hmgbl	-1.57	0.25	Tbp ^a	N/A	0.86
Hprt	-1.33	0.19	Tgfb2	1.26	0.98
Ifna1	-6.31	0.35	Thpo	-3.5	0.36
Ifna12	-4.27	0.35	Tnf	-3.07	0.25
Ifna13	-4.05	0.36	Tnfrsf11b	-1.06	0.74
Ifna14	-3.57	0.36	Tnfsf10	-1.4	0.66
Ifna2	-4.25	0.36	Tnfsf11	1.1	0.65
Ifna6	-3.1	0.36	Tnfsf13b	-1.15	0.62
Ifna7	-50.77	N/A	Vegfa	-1.05	0.81
Ifna9	-5.99	0.35	Wnt1	-4.84	0.36
Ifnab	-4.97	0.35	Wnt5a	2.35	0.77

^a Genes were used as housekeeping genes.

cells that respond to a local cutaneous infection. Typically, both the innate and adaptive arms of the immune system are used to clear a *C. albicans* infection. Inflammatory cytokines such as IL-1 β and IL-6 initiate the response, resulting in recruitment of neutrophils to the infection site. These innate cells are known to be highly efficient in killing *C. albicans* cells and hyphae (16). Additionally, IFN- γ is produced, which can further enhance fungicidal activity of both neutrophils and macrophages (20, 22, 40). Shortly afterward, the adaptive immune system responds with CD4⁺ T cells trafficking to the infection site. Ideally, they differentiate into Th17 cells, which further recruit neutro-

FIG. 6. IL-12 given after TBI results in less dissemination to the kidneys. Panel A: IL-12 mitigation protocol. Adult hairless mice were treated as in the acute infection model, only with 300 ng of rIL-12 given at day 2 post-TBI. Colony forming assay results are shown for infected ear (panel B) and kidneys (panel C). Values were combined from nine animals (nonirradiated + vehicle, nonirradiated + IL-12 and irradiated + IL-12) or 12 animals (irradiated + vehicle). Error bars are SD. * $P < 0.05$. Notably, the irradiated + vehicle and nonirradiated + vehicle groups were also shown in Fig. 1 as some of the data points in those groups.

phils to the infection site (17, 22). However, Th1 cells are also able to respond to a *C. albicans* infection, and are induced by certain DCs, as further production of IFN- γ and IL-2 can continue to promote killing of yeast cells by phagocytic cells (19). Some studies have also shown that CD8 $^{+}$ T cells may play a role in responding to *C. albicans* infections, with the capability to impair hyphal growth and to lyse macrophages that have ingested yeast cells (41). While CD8 $^{+}$ T cells are not the most efficient at clearing a *C. albicans* infection, they may serve as a secondary defense. Our data demonstrate that TBI results in a reduction of these important immune cells at the local site of infection. In essence, it is possible that radiation exposure results in a cutaneous environment with fewer cells capable of responding to and eliminating the fungus.

Of note, a significant reduction of DCs was not observed after irradiation only (Supplementary Fig. S3; <http://dx.doi.org/10.1667/RR14295.1.S1>), unlike in our previous study (27). It is possible that our tissue dissociation technique did not isolate all of the cutaneous DCs, as these cells are more difficult to remove from tissue. Our previous imaging studies therefore may have been a more accurate method of determining DC density after irradiation. Imaging studies may also show a similar reduction of DCs during infection after irradiation.

After irradiation there was a change in the cytokine environment, with many genes downregulated, and those that decreased most significantly belong to a family of genes related to T-cell function and chemotaxis. For example, IFN- γ was one of these cytokines, which is

normally produced upon *C. albicans* infection (22, 40). This cytokine not only boosts killing of *C. albicans* by innate cells, but can also induce CXCL9, CXCL10 and IL-9 (33, 42). CXCL9 and CXCL10 are both capable of stimulating T-cell chemotaxis. Therefore, we hypothesize that the decrease of CXCL9 and CXCL10 could be one reason for fewer CD4 $^{+}$ and CD8 $^{+}$ T cells seen in irradiated/infected skin. This may have also been compounded by the decrease in circulating lymphocytes in irradiated mice, resulting in the ability of fewer lymphocytes to respond to the infection site (Fig. 3). In addition, notably, both CXCL9 and CXCL10 have shown some antimicrobial properties (43, 44). While CXCL9 has antibacterial properties, CXCL10 has shown some antifungal properties (43). The reduction of these chemokines, and IFN- γ , may also indicate that the cutaneous environment's innate defenses are impaired. This could have contributed to the poorer containment of infection in the skin in irradiated mice.

In addition, IL-9 is important in responding to *C. albicans* infection. It has been previously reported that IL-9, together with TGF- β , induced Th17 differentiation (45). Interestingly, there was no change in TGF- β expression (Fig. 4), and there was a slight (not significant) decrease in IL-17. It is therefore possible that not only did nonirradiated/infected mice have more cutaneous T cells, but that those T cells could have been more skewed toward a Th17 response. This could have helped provide more protection for the nonirradiated mice. It would therefore be important to characterize the immune cells that were present in the irradiated/infected skin. Knowing what types of T-helper cells are present could help assess the extent of protection these T cells provide.

With this decrease in T cells and T-cell cytokines, there was an increase in granulocytes. In previous studies, these cells were shown to be protective early during *C. albicans* infections, and responded to bacterial infections after irradiation (25, 46). However, increased dissemination was observed in mice with this increase in neutrophils. The neutrophils may have been less efficient in killing *C. albicans* cells, possibly due to the decreased CD4 $^{+}$ T-helper cell population or the reduced amount of IFN- γ in the skin. Testing the fungicidal activities of the neutrophils, as well as other innate cells capable of killing *C. albicans*, after irradiation, would help determine how capable the irradiated/infected skin was of responding to *C. albicans*.

While these alterations in the cutaneous environment after irradiation impair the skin's response to *C. albicans*, a potential means of countering this impairment is the use of IL-12. This cytokine may have boosted the skin's immune response in several ways. Based on our previously reported findings, there were likely more cutaneous DCs present at the time of infection in irradiated, IL-12-treated mice. As IL-12 induces IFN- γ , the IL-12 may have also boosted the response of the otherwise impaired cutaneous T cells, as well as improved activity of the cDCs. An IL-12-induced increase in IFN- γ would have also increased phagocytic

activity of neutrophils (37, 38), potentially allowing for improved killing of the fungi. Therefore, further investigation into the mechanisms of the IL-12 mitigation would be useful in understanding how best to treat a *C. albicans* infection after irradiation.

It should be noted that the increase in dissemination was not seen in the back skin, the second skin site we tested. This may be due to several factors. The proportions of immune cells in the back skin differ from the proportions of those same cells in the ear, with the back having a larger population of T cells (47). The ear skin is also much more vascularized, similar to mucosal tissue. This may also allow for easier access to the kidneys via the bloodstream, allowing for the *C. albicans* to disseminate more easily from the ear.

Skin vascularization could have also been increased as part of an inflammatory response to radiation, potentially providing an easier route of dissemination for the fungus in irradiated mice. To account for this, we used confocal imaging to measure the density of CD31⁺ blood vessels in the ear one week after 6 Gy TBI. Among the five irradiated and five nonirradiated mice, we observed no significant changes in blood vessel density (data not shown).

Interestingly, while many of the irradiated/infected mice had high kidney burdens, the majority of these mice survived and appeared healthy. It would therefore be important to monitor the kidney burden of irradiated/infected mice later than four days after infection, since the infection may worsen in those mice that are less capable of clearing the infection. Additionally, we have seen that nonirradiated mice infected with this dose of *C. albicans* are able to clear the infection within two weeks (data not shown). If the irradiated/infected mice are also able to clear the infection, this may be due to the impairment from radiation recovering over time. However, these mice may also take longer to clear the infection, as they have fewer cells capable of initially responding to it. Regardless, it was intriguing that we did not observe more severe infections in irradiated mice especially after observing the significant decrease in systemic immune cells. This may attest to an undetermined antimicrobial mechanism inherent to the skin that is unaffected by radiation exposure.

In conclusion, radiation alters the ear skin environment during acute infection, resulting in a diminished local response. This may be due to a loss of T-cell cytokines and chemokines, as well as T cells themselves, which would otherwise help to promote an immune environment capable of clearing yeast. This impairment may worsen depending on radiation dose, which could further alter the immune cell density or cytokine and chemokine production. Future therapies, such as IL-12, administered to irradiated patients, could potentially be adjusted to account for these changes. For example, our data demonstrates that the cutaneous immunological barrier can be breached shortly after irradiation, but recovers over time. This key finding suggests that acute rather than long-term mitigation/

treatment for infection would be more advantageous in individuals exposed to sublethal radiation. As such, antifungal therapy immediately after irradiation may help compensate for the weakened cutaneous immune system and control *C. albicans* infections.

SUPPLEMENTARY INFORMATION

Figure S1. No increased *C. albicans* dissemination from back skin after irradiation.

Figure S2. Flow cytometry gating strategy for immune cell phenotyping.

Figure S3. Radiation exposure alone does not result in significant changes in ear immune cell population at day 7 postirradiation.

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REFERENCES

1. Vogel H. Rays as weapons. *Eur J Radiol* 2007; 63:167–77.
2. Senn JS, McCulloch EA. Radiation sensitivity of human bone marrow cells measured by a cell culture method. *Blood* 1970; 35:56–60.
3. Shadad AK, Sullivan FJ, Martin JD, Egan LJ. Gastrointestinal radiation injury: symptoms, risk factors and mechanisms. *World J Gastroenterol* 2013; 19:185–98.
4. Kusunoki Y, Hayashi T. Long-lasting alterations of the immune system by ionizing radiation exposure: implications for disease development among atomic bomb survivors. *Int J Radiat Biol* 2008; 84:1–14.
5. Peter RU. Cutaneous radiation syndrome in multi-organ failure. *BJR Suppl* 2005; 27:180–4.
6. Ruocco E, Di Maio R, Caccavale S, Siano M, Lo Schiavo A. Radiation dermatitis, burns, and recall phenomena: Meaningful instances of immunocompromised district. *Clin Dermatol* 2014; 32:660–9.
7. McFarland HI, Puig M, Grajkowska LT, Tsuji K, Lee JP, Mason KP, et al. Regulatory T cells in gamma irradiation-induced immune suppression. *PLoS One* 2012; 7:e39092.
8. Zhou Y, Ni H, Balint K, Sanzari JK, Dentchev T, Diffenderfer ES, et al. Ionizing radiation selectively reduces skin regulatory T cells and alters immune function. *PLoS One* 2014; 9:e100800.
9. Netea MG, Brown GD, Kullberg BJ, Gow NA. An integrated model of the recognition of *Candida albicans* by the innate immune system. *Nat Rev Microbiol* 2008; 6:67–78.
10. Bassetti M, Merelli M, Ansaldi F, de Florentiis D, Sartor A, Scarparo C, et al. Clinical and therapeutic aspects of candidemia: a five year single centre study. *PLoS One* 2015; 10:e0127534.
11. Odds FC, Hanson MF, Davidson AD, Jacobsen MD, Wright P, Whyte JA, et al. One year prospective survey of *Candida* bloodstream infections in Scotland. *J Med Microbiol* 2007; 56:1066–75.

12. Brown GD, Denning DW, Gow N, Levitz SM, Netea MG, White TC. Hidden killers: human fungal infections. *Med Mycol* 2012; 4.
13. Redding SW, Zellars RC, Kirkpatrick WR, McAtee RK, Caceres MA, Fothergill AW, et al. Epidemiology of oropharyngeal *Candida* colonization and infection in patients receiving radiation for head and neck cancer. *J Clin Microbiol* 1999; 37:3896–900.
14. Grotz KA, Genitsariotis S, Vehling D, Al-Nawas B. Long-term oral *Candida* colonization, mucositis and salivary function after head and neck radiotherapy. *Support Care Cancer* 2003; 11:717–21.
15. Fradin C, De Groot P, MacCallum D, Schaller M, Klis F, Odds FC, et al. Granulocytes govern the transcriptional response, morphology and proliferation of *Candida albicans* in human blood. *Mol Microbiol* 2005; 56:397–415.
16. Urban CF, Reichard U, Brinkmann V, Zychlinsky A. Neutrophil extracellular traps capture and kill *Candida albicans* yeast and hyphal forms. *Cell Microbiol* 2006; 8:668–76.
17. Hernandez-Santos N, Gaffen SL. Th17 cells in immunity to *Candida albicans*. *Cell Host Microbe* 2012; 11:425–35.
18. Conti HR, Peterson AC, Brane L, Huppler AR, Hernandez-Santos N, Whibley N, et al. Oral-resident natural Th17 cells and gammadelta T cells control opportunistic *Candida albicans* infections. *J Exp Med* 2014; 211:2075–84.
19. Igyarto BZ, Haley K, Ortner D, Bobr A, Gerami-Nejad M, Edelson BT, et al. Skin-resident murine dendritic cell subsets promote distinct and opposing antigen-specific T helper cell responses. *Immunity* 2011; 35:260–72.
20. Romani L. Innate and adaptive immunity in *Candida albicans* infections and saprophytism. *J Leukoc Biol* 2000; 68:175–9.
21. Conti HR, Gaffen SL. IL-17-Mediated immunity to the opportunistic fungal pathogen *Candida albicans*. *J Immunol* 2015; 195:780–8.
22. van de Veerdonk FL, Joosten LA, Shaw PJ, Smeekens SP, Malireddi RK, van der Meer JW, et al. The inflammasome drives protective Th1 and Th17 cellular responses in disseminated candidiasis. *Eur J Immunol* 2011; 41:2260–8.
23. Spellberg B, Ibrahim AS, Edwards J, Filler SG. Mice with disseminated candidiasis die of progressive sepsis. *J Infect Dis* 2005; 192:336–43.
24. Elliot TB, Ledney GD, Shoemaker MO, Knudson GB. Management of postirradiation infection: lessons from animal models. *Mil Med* 2004; 169:194–7.
25. Cronkite EP, Brecher G. The protective effect of granulocytes in radiation injury. *Ann N Y Acad Sci* 1955; 59:815–33.
26. 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.
27. 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.
28. D'Ostiani CF, Del Sero G, Bacci A, Montagnoli C, Spreca A, Mencacci A, et al. Dendritic cells discriminate between yeasts and hyphae of the fungus *Candida albicans*: implications for initiation of T helper cell immunity in vitro and in vivo. *J Exp Med* 2000; 191:1661–73.
29. Mitra S, Dolan K, Foster TH, Wellington M. Imaging morphogenesis of *Candida albicans* during infection in a live animal. *J Biomed Opt* 2010; 15:010504.
30. Fonzi WA, Irwin MY. Isogenic strain construction and gene mapping in *Candida albicans*. *Genetics* 1993; 134:717–28.
31. Jacobsen ID, Luttich A, Kurzai O, Hube B, Brock M. In vivo imaging of disseminated murine *Candida albicans* infection reveals unexpected host sites of fungal persistence during antifungal therapy. *J Antimicrob Chemother* 2014; 69:2785–96.
32. 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.
33. Carter SL, Müller M, Manders PM, Campbell IL. Induction of the genes for Cxcl9 and Cxcl10 is dependent on IFN-gamma but shows differential cellular expression in experimental autoimmune encephalomyelitis and by astrocytes and microglia in vitro. *Glia* 2007; 55:1728–39.
34. Farber JM. HuMIG: A new human member of the chemokine family of cytokines. *Biochem Biophys Res Commun* 1993; 192:223–30.
35. Groom JR, Luster AD. CXCR3 in T cell function. *Exp Cell Res* 2011; 317, 620–31.
36. Loetscher M, Gerber B, Loetscher P, Jones SA, Piali L, Clark-Lewis I, et al. Chemokine receptor specific for IP10 and Mig: structure, function, and expression in activated T-lymphocytes. *J Exp Med* 1996; 184:963–9.
37. Djeu JY, Blanchard DK, Halkais D, Friedman H. Growth inhibition of *Candida albicans* by human polymorphonuclear neutrophils: activation by interferon-gamma and tumor necrosis factor. *J Immunol* 1986; 137:2980–4.
38. Moreno SE, Alves-Filho JC, Alfaya TM, da Silva JS, Ferreira SH, Liew FY. IL-12, but Not IL-18, is critical to neutrophil activation and resistance to polymicrobial sepsis induced by cecal ligation and puncture. *J Immunol* 2006; 177:3218–24.
39. Martin MV, Al-Tikriti U, Bramley PA. Yeast flora of the mouth and skin during and after irradiation for oral and laryngeal cancer. *J Med Microbiol* 1981; 14:457–67.
40. Romani L, Mocci S, Bietta C, Lanfaloni L, Puccetti P, Bistoni F. Th1 and Th2 cytokine secretion patterns in murine candidiasis: association of Th1 responses with acquired resistance. *Infect Immun* 1991; 59:4647–54.
41. Marquis M, Lewandowski D, Dugas V, Aumont F, Senechal S, Jolicoeur P, et al. CD8+ T cells but not polymorphonuclear leukocytes are required to limit chronic oral carriage of *Candida albicans* in transgenic mice expressing human immunodeficiency virus type 1. *Infect Immun* 2006; 74:2382–91.
42. Beno DWA, Stover AG, Matthews HL. Growth inhibition of *Candida albicans* hyphae by CD8+ lymphocytes. *J Immunol* 1995; 154:5273–81.
43. Yang D. Many chemokines including CCL20/MIP-3 display antimicrobial activity. *J Leukoc Biol* 2003; 74:448–55.
44. Cole AM, Ganz T, Liese AM, Burdick MD, Liu L, Strieter RM. Cutting edge: IFN-inducible ELR- CXC chemokines display defensin-like antimicrobial activity. *J Immunol* 2001; 167:623–7.
45. Elyaman W, Bradshaw EM, Uyttenhove C, Dardalhon V, Awasthi A, Imitola J, et al. IL-9 induces differentiation of TH17 cells and enhances function of FoxP3+ natural regulatory T cells. *Proc Natl Acad Sci U S A* 2009; 106:12885–90.
46. Romani L, Mencacci A, Cenci E, Del Sero G, Bistoni F, Puccetti P. An immunoregulatory role for neutrophils in CD4+ T helper subset selection in mice with candidiasis. *J Immunol* 1997; 158:2356–62.
47. Tong PL, Roediger B, Kolesnikoff N, Biro M, Tay SS, Jain R, et al. The skin immune atlas: three-dimensional analysis of cutaneous leukocyte subsets by multiphoton microscopy. *J Invest Dermatol* 2015; 135:84–93.