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Exposure to Ionizing Radiation Induces the Migration of Cutaneous Dendritic Cells by a CCR7-Dependent Mechanism

Ryan J. Cummings,* Scott A. Gerber,* Jennifer L. Judge,† Julie L. Ryan,† Alice P. Pentland,# and Edith M. Lord*

In the event of a deliberate or accidental radiological emergency, the skin would likely receive substantial ionizing radiation (IR) poisoning, which could negatively impact cellular proliferation, communication, and immune regulation within the cutaneous microenvironment. Indeed, as we have previously shown, local IR exposure to the murine ear causes a reduction of two types of cutaneous dendritic cells (cDC), including interstitial dendritic cells of the dermis and Langerhans cells of the epidermis, in a dose- and time-dependent manner. These APCs are critical regulators of skin homeostasis, immunosurveillance, and the induction of T and B cell-mediated immunity, as previously demonstrated using conditional cDC knockout mice. To mimic a radiological emergency, we developed a murine model of sublethal total body irradiation (TBI). Our data would suggest that TBI results in the reduction of cDC from the murine ear that was not due to a systemic response to IR, as a loss was not observed in shielded ears. We further determined that this reduction was due, in part, to the upregulation of the chemoattractant CCL21 on lymphatic vessels as well as CCR7 expressed on cDC. Migration as a potential mechanism was confirmed using CCR7 knockout mice in which cDC were not depleted following TBI. Finally, we demonstrated that the loss of cDC following TBI results in an impaired contact hypersensitivity response to hapten by using a modified contact hypersensitivity protocol. Taken together, these data suggest that IR exposure may result in diminished immunosurveillance in the skin, which could render the host more susceptible to pathogens. The Journal of Immunology, 2012, 189: 4247–4257.

As evident by the recent nuclear disaster in Japan and threats of radiological warfare through the use of nuclear weapons or attack on nuclear power plants, it is imperative that effective medical countermeasures and emergency strategies be implemented to triage victims exposed to ionizing radiation (IR) (1, 2). In these instances, the skin would most likely receive substantial IR poisoning that, over the course of several days, could manifest into a form of cutaneous radiation syndrome (3) and lead to multiorgan failure (4). As skin is the largest organ of the human body and first line of defense against a hostile environment of biological, chemical, and physical traumas, further investigation into the mechanisms regulating IR-induced skin injury is needed so that we can properly mitigate the risk of cutaneous infection and subsequent damage to other organ systems.

Cutaneous dendritic cells (cDC), including interstitial dendritic cells (iDC) of the dermis and Langerhans cells (LC) of the epidermis, are members of a highly specialized, immunosurveillance family known as APCs that are critical for tissue homeostasis, the induction of immune tolerance, and the elicitation of protective immunity (5). Within the dermis, iDC can be distinguished from dermal macrophages by surface CD11c, CD205, and elevated MHC class II (MHC II) expression (6), whereas LC are the only dendritic cells (DC) present in the epidermis and therefore can be identified by CD207 alone (7). It is well established that, in addition to steady state migration (8), cDC are mobilized and depleted from the skin in response to Ag acquisition as well as a variety of other proinflammatory stimuli (9, 10). Following signaling, cDC enter lymphatics and traffic to the draining lymph nodes to further initiate the appropriate adaptive immune response. Of critical importance for cDC migration and entry into draining lymphatics are the chemotactic ligand, CCL21, made by lymphatic endothelial cells (11), and the corresponding receptor, CCR7, which is expressed on activated DC in the skin (12). Whereas mice deficient in CCL21 exhibit impaired cDC migration to draining lymph nodes (13), deletion of CCR7 completely abolishes cDC migration from the skin (12).

Similar to nonionizing, UV radiation (14), we have previously shown that local IR exposure causes a dose- and time-dependent reduction of cDC from the mouse ear (15). Despite extensive studies investigating the UV-induced depletion of cDC and subsequent suppression of cell-mediated immunity within the skin, there are little data describing the immunomodulatory effects of IR within the cutaneous microenvironment. Many of the existing references are largely descriptive and solely focus on the signs and symptoms following IR exposure without addressing the consequences on the immune system aside from complete blood count analyses. In addition, few studies have examined the cDC compartment and the immune response following sublethal total body irradiation (TBI), which would most likely result after a radiological emergency (3). Although eloquent studies conducted by Merad et al. (8) examining the long-term maintenance of cDC populations after complete bone marrow chimera suggest that
both LC and, to a degree, iDC (6) were repopulated in situ by radioreistant progenitor cells, little is known of the fate or functional relevance of cDC immediately after sublethal TBI. Depending on mouse strain, the lethal dose at which 50% of the animals die over the course of 30 d following TBI (LD$_{50/30}$) is ~9 Gy, whereas for humans the LD$_{50/30}$ is believed to be between 3 and 4 Gy (16). In this study, we describe a mouse model of sublethal, 6 Gy TBI that mimics skin exposure following a radiological emergency to examine the mechanism of the IR-induced partial depletion of cDC. Similar to local IR exposure of mouse ear, cDC were reduced following TBI and accumulated in the auricular draining lymph node (ADLN) over time. Our data would suggest that this TBI-induced migration is driven, in part, by CCL21 and CCR7, as mRNA expression for each was upregulated after TBI. In addition, we detected a significant increase in CCL21 protein associated with LYVE-1–positive lymphatic endothelial cells following TBI relative to unirradiated controls. Importantly, the requirement for CCR7 in this model was further supported by the retention of both iDC and LC populations within the dermis and epidermis of TBI-exposed CCR7$^{-/-}$ mice, respectively. Finally, TBI results in an impaired cDC response to hapten challenge, as measured by a modified contact hypersensitivity (CHS) assay. Taken together, IR exposure appears to induce the migration of cDC, which may limit skin immunosurveillance and render the host more susceptible to pathogen entry.

Materials and Methods

Mice

Male and female C57BL/6 albino hairless mice (hairless mice) were used for experiments described in Figs. 1–5 as well as Fig. 7 and were provided by A. Pentland (Department of Dermatology, University of Rochester Medical Center). This colony was bred and maintained by our laboratory. The experiment in Fig. 6 was carried out using female B6.129P2(C)-Ccr7$^{tm1Rfor/J}$ (CCR7$^{−/−}$) mice, which are also of the C57BL/6 strain background. These mice were purchased from The Jackson Laboratory. All animal use protocols were approved by University of Rochester’s University Committee on Animal Resources.

Abs and reagents

Antibodies. Fc Block was purchased from BD Biosciences. Allophycocyanin-conjugated anti-mouse MHC II (I-A/I-E), PE-conjugated internal anti-mouse langerin (CD207), PE-CY7−conjugated anti-mouse CD11c, allophycocyanin-Cy7−conjugated anti-mouse CD45, PE-conjugated anti- mouse Ly-6G, and FITC-conjugated CD31 were purchased from eBioscience.

Reagents. The tribromoethanol anesthesia stock solution was prepared by mixing 3 g 2,2,2-tribromoethanol in 5 ml 2-methyl-2-butanol (Sigma-Aldrich) with gentle heating. The anesthesia was diluted 1:40 in PBS, and mice were injected i.p. with 300 μl of this dilution. Tetramethylrhodamine-5-(and-6)-isothiocyanate (TRITC) was prepared and applied to mouse ears, as previously described (17) (Life Technologies).

Irradiation protocol

The 3200 Curie-sealed $^{137}$Cs source operated at ~1.90 Gy/min was used for all irradiated samples. The gamma radiation was administered at doses ranging from 1 to 25 Gy, and both TBI and single-beam collimators were used for these studies. Mice receiving a TBI dose of 6 Gy were placed into a Plexiglas box designed to prevent vertical movement away from the radiological source, and the TBI collimator was used. For those mice receiving local radiation, the single-beam collimator was used, and, once under anesthesia, mice were placed into specially made jigs allowing for radiation exposure to one ear, whereas the rest of the mouse (including the contralateral ear) remained shielded and unexposed to the radiation. The TBI-ear studies were performed in a similar fashion; however, for these experiments, the TBI collimator was used, and the entire body, except for one ear, was exposed to radiation, whereas the single ear remained shielded and unexposed.

Tissue removal and processing

Ear removal. Mice were sacrificed according to University of Rochester’s University Committee on Animal Resources-approved protocol. The ears were then removed, split into dorsal and ventral halves with the aid of forceps, and placed into dishes of HBSS (Sigma-Aldrich) for dermal layer labeling.

Epidermal sheet processing. Following ear splitting, halves were floated in 0.5 M ammonium thiocyanate (Sigma-Aldrich) at 37°C for 20 min to separate epidermal from dermal layers. The epidermal layer was then fixed in 2% paraformaldehyde (J.T. Baker) at room temperature for 15 min to permeabilize cells for 1.5 h of intracellular labeling with PE-conjugated internal antilangerin (eBioscience), at a concentration of 4 μg/ml.

Whole-mount analysis and conventional fluorescence microscopy

Fluorescence labeling. Whole-mount histology was performed on split ear samples, as previously described (15). Briefly, samples ~12 × 10 mm × 0.5 mm in size were placed in 6 ml polypropylene tubes (BD Biosciences). Ear tissues were incubated with Fc Block (BD Biosciences) at 10 μg/ml in 200 μl phosphate-bolevine-azide solution (PBA; PBS with 1% BSA and 0.1% sodium azide; Sigma-Aldrich) for 45 min. Following Fc Block, dermal samples were labeled with anti-MHC II-allophycocyanin (eBioscience) at 3 μg/ml and gently rocked at 4°C for 1.5 h. Samples were washed once by adding 3 ml PBA with gentle rotation at 4°C for 30 min. To control for nonspecific binding, anti-rat Ig isotype controls were used, and labeling was nonexistent. For anti-CCL21 labeling, a modified protocol was followed. In brief, mouse ears were excised, split, and fixed in 1% paraformaldehyde for 2 h at 4°C with gentle rocking. Next, samples were labeled with anti–CCL21 primary Ab (R&D Systems) at 0.5 μg/ml and goat-anti-rat AlexaFluor 488 secondary Ab (Life Technologies) at 0.5 μg/ml and gently rocked for 2 h at 4°C with gentle rocking. Finally, samples were labeled with CCL21 secondary Ab, a Cy3-conjugated donkey anti-goat IgG, at 5 μg/ml for 2 h at 4°C with gentle rocking. All samples were labeled and blocked in PBS Triton X-100 (0.3% v/v) supplemented with 5% (v/v) normal donkey serum.

Conventional fluorescence microscopy. Whole-mount analysis was performed using an Olympus BX40 conventional fluorescence microscope (Olympus America), and images were acquired using a Retiga 1300 camera (QImaging). Labeled samples were placed on a glass slide with a coverslip and 20 μl PBA added to wet the slide. The split ear sample selected for imaging was the ventral/inner ear half, as it had fewer densely packed cartilage cells within the hypodermal layer. The epidermal side was placed facing toward the coverslip and microscope objective. Ear tissue was then viewed using three fluorescence filter cubes (Chroma Technology); allophycocyanin cube-excitation filter D598/40× and emission filter D660/40; PE Hg light source cube-excitation filter D546/10× and emission filter D580/30m. Images were obtained at an original magnification ×10 of the entire ear only if they met the following criteria: no cartilage cells, stray hairs, or debris within the field of view, and a full field of view away from the edges to avoid the nonspecific Ab-edge effect. Bright field images were acquired first, followed by fluorescent images and ghost images obtained under identical exposure were combined to evaluate the extended depth of field tool within the Image Pro Plus Software (Media Cybernetics) and consisted of three to six images at adjacent axial locations to create one final composite. A minimum of 10 images was taken, spanning the entire ear for accurate statistical analysis. Images of the control samples were obtained in the same fashion. The epidermal layer was imaged at an original magnification ×20 with the extended depth of field tool using the same criteria as for the dermis.

Cell density calculations. The densities of positively labeled MHC II cells of the dermis were calculated using Image Pro and the masking application, by which a mask was generated using area and threshold filters. The area filter was created based on prior characterization of iDC diameter, which is ~10–22 μm. Using those diameters, an area filter ranging from 100 to 480 μm$^2$ was used to select for positively labeled events. The threshold filter was generated by first measuring the resultant intensity ranges due to autofluorescence from the cutaneous ear microenvironment. Next, intensity ranges were measured from nonspecific Ab labeling. Finally, those values were used as a baseline to distinguish positively labeled events from any background/nonspecifically labeled events. Densities were then calculated in cells/mm$^2$. For epidermal analysis, a 50–625 μm$^2$ area filter was used to determine LC. To measure changes in LC morphology, such as cellular swelling and dendrite extension, the feret diameter filter was applied to the same images used to calculate cell density (Image Pro Plus). Data acquired from the feret diameter filter measurements for each ear were pooled to generate a frequency distribution across the specified bin size ranges for each mouse, and then values from each mouse were pooled and expressed as frequency per bin size range ± SEM.

Immunohistochemistry. Ear tissue was isolated and fixed in 10% neutral buffered formalin (Thermo Fisher Scientific) for 24 h at room temperature.
Samples were then put in 70% ethanol at 4°C before paraffin embedding. Paraffin-embedded ear sections were cut 4 μm thick and deparaffinized for TUNEL using the ApopTag Plus Peroxidase In Situ Apoptosis Detection Kit (Millipore), as per manufacturer’s directions, and for H&E staining.

Flow cytometric analysis. ADLN and spleen were isolated and digested with 2 mg/ml collagenase for 20 min at 37°C with rotation (Sigma-Aldrich). Following collagenase digestion, single-cell suspensions were prepared by gently pressing the tissues over a 40 μm nylon cell strainer (BD Biosciences) and then washed, pelleted, and resuspended with PBA. Samples were then pretreated with Fc Block for 10 min at 4°C, followed by gentle rocking at 4°C for 45 min with an Ab mixture, including anti-CD45, anti-MHC II, and anti-CD11c. Propidium iodide (BD Biosciences) was used for live-dead discrimination. Compensation, using single-color anti-CD45 splenocytes, and sample acquisition were performed using the FACSCantoII (BD Biosciences). FlowJo analytical software (Tree Star) was used to analyze the data.

mRNA isolation and real-time quantitative RT-PCR

Total RNA was isolated from mouse ears using a RNasey mini kit (Qiagen), per the manufacturer’s protocol, and quantified by a spectrophotometer. First-strand cDNA was synthesized using equal amounts of input RNA, the iScript cDNA synthesis kit (Bio-Rad), and the PTC-100 Programmable Thermal Controller (MJ Research). Real-time quantitative RT-PCR (qRT-PCR) was performed on 5 μg cDNA using forward and reverse primers designed to span introns and SYBR Green (Bio-Rad) master mix for a total volume of 25 μl. A list of the forward and reverse primers (5'-3') is as follows: GAPDH, forward, 5'-CTTGGCTCT-CATTGACCAACT-3', and reverse, 5'-GGGTTTCTTACTCCTTGGAG-3'; CCR7, forward, 5'-GGTGGCTCTCCTGTGATT-3', and reverse, 5'-ACATACTTCCTGAAGCACAC-3'; CCL21, forward, 5'-CTATAGGAAGCCA-GAACAAAGT-3', and reverse, 5'-TTCTTCAAGGTTTGGACACATA-3'. Samples were run on the iCycler (Bio-Rad), and data were normalized using GAPDH as a reference value. Data are presented as fold increase over unirradiated samples.

Contact hypersensitivity

CHS was induced in mice, as previously described (19). In brief, mice were sensitized on the back with 30 μl 0.5% 2,4-dinitro-1-fluorobenzene (DNFB) in a mixture of acetone and olive oil (4:1). Four days later, mice were exposed to radiation and, after another 4 d, challenged with 10 μl 0.2% DNFB applied to both the dorsal and ventral sides of the ear. Ear thickness was measured 24 and 48 h following challenge using a spring-loaded caliper (Mitutoyo), and data are expressed as follows: change in ear thickness = ear thickness − baseline thickness.

Results

TBI results in the partial depletion of both iDC and LC in a time-dependent manner

Data from our previous studies investigating the response of both mouse iDC and LC (collectively cDC) to local, IR exposure suggest that these cells were depleted from the skin in a dose- and time-dependent fashion (15). Therefore, to examine whether cDC were depleted in a manner similar to local IR exposure, we total body irradiated mice with a sublethal dose of 6 Gy and quantified both the iDC and LC densities over time using whole-mount fluorescence microscopy, as described in Materials and Methods. In contrast to dermal macrophages, which are F4/80+, CD11b+, CD11c−/−, and MHC IIlow, iDC were CD11c+ and MHC IIhigh, as we (15) and others (6) have previously shown. Dermal (Fig. 1A) and epidermal (Fig. 1B) ear tissues from unirradiated, control mice show densely populated anti-MHC II-labeled iDC and antilangerin-labeled LC, respectively. However, 7 d following TBI exposure, both iDC (Fig. 1C) and LC (Fig. 1D) were depleted, resulting in areas of tissue lacking labeled cells. In addition, the LC exhibited unique morphological changes, including cellular swelling and dendrite extension following TBI (Fig. 1D), and this increase in size was quantified by image analysis and determined to be significant relative to unirradiated controls (Supplemental Fig. 1A). Similar to local IR exposure, both iDC (Fig. 1E) and LC (Fig. 1F) were depleted over time following TBI. With respect to the iDC population, the loss of cells was rapid, reaching the lowest point 4 d after TBI exposure, whereas the LC population had slower reduction rates, gradually reaching the lowest point ~day 14 post-TBI. Interestingly, whereas the iDC population appeared to fully repopulate the dermis by day 60 post-TBI, the LC compartment was only partially repopulated.

To further examine the TBI-induced partial depletion of cDC and explore whether this was a systemic response to IR, we modified our local IR protocol. For this experiment, mice were anesthetized and placed into specially constructed jigs so that the entire body was exposed to TBI, whereas the right ear was pulled out of the jig and shielded from IR (termed TBI − ear). We hypothesized that if the TBI-induced partial depletion was a systemic response, then there would be a loss of cDC in both the ear exposed to radiation (TBI + ear) and the contralateral ear that was shielded (TBI − ear). However, 7 d following a TBI − ear exposure, both the iDC (Fig. 1G) and LC (Fig. 1H) density from the TBI − ear samples remained unchanged from that of the unirradiated controls. Importantly, the ear exposed to radiation (TBI + ear) exhibited a partial depletion of cDC, as follows: ~30% for iDC and 40% for LC, which was comparable to that observed on day 7 post-full TBI exposure. Taken together, these data suggest that, like local IR exposure, TBI causes a partial depletion of cDC that is not a systemic response and only occurs when tissue/cells are directly exposed to IR.

Partial depletion of cDC following TBI is not mediated by DNA fragmentation-induced apoptosis

To address the mechanism of the TBI-induced partial depletion of cDC, we first examined the possibility that it was a result of cell death. For these experiments, paraffin-embedded ear sections from mice exposed/unexposed to IR were labeled with TUNEL to assess the degree of DNA fragmentation and cell death. As a positive control, mouse ears were locally exposed to 0 Gy (Fig. 2A) and 25 Gy (Fig. 2B) because our earlier studies confirmed this dose caused the most significant reduction of cDC from the ear. Six hours following the local, 25 Gy exposure, there was a statistically significant increase in the number of TUNEL-positive cells relative to the unirradiated contralateral control ear. TUNEL labeling was also examined 6 h after a local dose of 6 Gy, which was a comparable dose to TBI (Fig. 2C). Unlike the local 25 Gy exposure, 6 Gy did not cause a statistically significant increase in the number of TUNEL-positive cells compared with the unirradiated, contralateral control ear (Fig. 2D). Therefore, our data suggest that only high doses of IR (25 Gy) are capable of causing significant cell death in the ear at the 6-h time point.

We next wanted to assess the degree of cell death in ear skin caused by TBI. For these experiments, we investigated TUNEL labeling on days 4 and 7 post-TBI, as these were the most active time points of cDC loss (Fig. 1). However, relative to the unirradiated mice (Fig. 2E), there was not a statistically significant increase in TUNEL labeling on day 4 (Fig. 2F) or 7 (Fig. 2G) post-TBI (Fig. 2H). Together, these data suggest that IR-induced apoptosis is not the primary mechanism responsible for the observed cDC reduction following TBI exposure.

Migratory cDC from the ear can be detected in the draining lymph node following local radiation exposure

Our data suggest that apoptosis was not the primary mechanism responsible for the IR-induced cDC reduction; therefore, we next hypothesized that perhaps the cells were actively migrating to the draining lymph nodes. Therefore, to address whether cDC actively migrate from the ear following IR exposure, we locally irradiated.
mouse ears with 25 Gy, leaving the contralateral ear unirradiated for an internal control, and harvested the ADLN for flow cytometric analysis. Migratory cDC, which are known to traffic to draining lymph nodes during steady state and inflammatory conditions, were identified as propidium iodide negative, CD45+, MHC II⁺, and CD11c⁺, as previously described (Fig. 3) (20). Local radiation of the ear, rather than TBI, was used in these experiments to directly compare the irradiated ear/ADLN with the unirradiated contralateral control ear. Ten days following a local dose of 25 Gy to the ear, there was a greater frequency of migratory cDC in the ADLN draining the irradiated ear relative to the unirradiated, contralateral control (Fig. 3A). Migratory cDC in the ADLN increased in both frequency (Fig. 3B) and absolute number (Fig. 3C) over time following local IR exposure. This accumulation of migratory cDC correlated with both the TBI-induced (Fig. 1) and local IR-induced reduction of cDC from the skin, as our previous studies would support. Taken together, and in accordance with the limited TUNEL labeling (Fig. 2), these data suggest that the IR-induced cDC reduction mechanism is most likely due to an active migration of these cells from the ear to the ADLN.

**FIGURE 1.** Exposure to TBI results in a partial depletion of iDC and LC in a time-dependent manner. Fluorescence whole-mount microscopy images from unirradiated mouse ear dermis (A) and epidermis (B) labeled with anti-MHC II and anti-langerin, respectively. Dermal and epidermal images from day 7 post-TBI are shown in images (C) and (D), respectively, and were labeled with the same aforementioned Abs. Using these images, irradiated ear densities were calculated, divided by nonirradiated control ear densities, and multiplied by 100 to obtain normalized points for the MHC II⁺ (E) and langerin⁺ cells (F). (G) and (H) Quantification of MHC II⁺ and langerin⁺ cells in mouse ear dermis and epithelium, respectively, from unirradiated mice (black bar) or mice receiving TBI (TBI + ear) (white bar) and the shielded contralateral ear (TBI – ear) (gray bar). Images and data in (G) and (H) are from day 7 post-TBI. Percent depletion was calculated using the following equation: % depletion = 100 – ([TBI + ear/TBI – ear] × 100). n = 6 mice per time point ± SEM, and statistical analyses were performed using a one-way ANOVA with a Tukey post hoc test. Scale bars, 100 and 50 μm for dermal and epidermal images, respectively.

**TBI causes an upregulation of the migratory receptor CCR7 and the corresponding chemotactic ligand CCL21**

To more closely examine the mechanism of the TBI-induced migration of cDC, we next focused on the migratory receptor, CCR7, and the cognate ligand, CCL21. Changes in mRNA expression of CCR7 and CCL21 as a result of TBI were examined by qRT-PCR (Fig. 4). As early as 12 h following TBI exposure, there is an upregulation of CCR7 (Fig. 4A) above unirradiated controls; by 24 h, CCL21 (Fig. 4B) was also upregulated. However, by day 4 post-TBI, the expressions of CCR7 and CCL21 were both dramatically increased by nearly 25- and 15-fold over unirradiated controls; by 24 h, CCL21 (Fig. 4B) was also upregulated. However, by day 4 post-TBI, the expressions of CCR7 and CCL21 were both dramatically increased by nearly 25- and 15-fold over unirradiated controls, respectively, and remained elevated to day 7 post-TBI before returning to baseline on day 10. Importantly, these temporal genetic patterns correlate with both of the following: 1) the mobilization of each cDC population from the skin (Fig. 1), and 2)
the accumulation of cDC in the ADLN (Fig. 3B), as days 4–7 post-TBI appear to be the most active periods of cDC migration.

**TBI causes an upregulation of the CCL21 protein in LVYE-1 lymphatic vessels**

We next examined whether TBI induced the upregulation of CCL21 protein using whole-mount fluorescence microscopy. For these studies, we investigated day 5 post-TBI because we had previously identified an increase in CCL21 mRNA on day 4 following TBI (Fig. 4B). In unirradiated mice, punctate CCL21 labeling (Fig. 5A) was observed in LVYE-1–positive lymphatic vessels (Fig. 5B) from the mouse ear (overlaid in Fig. 5C). However, on day 5 following TBI exposure, the CCL21-labeling pattern changed and was much larger, brighter (Fig. 5D), and covered a greater area of LVYE-1 lymphatic vessels (Fig. 5E) relative to unirradiated control mice (overlaid in Fig. 5F). The changes in CCL21 labeling were then quantified and expressed as the percentage of LVYE-1 vessels that were CCL21 positive (Fig. 5G). Relative to the unirradiated mice, there was a statistically significant 7.5-fold increase in CCL21 expression on lymphatic vessels from the day 5 post-TBI mice. This increase in CCL21 protein on day 5 following TBI correlates with both the increase in CCL21 mRNA (Fig. 4B) and the rapid mobilization of cDC from the ear (Fig. 1). Taken together, these data suggest that CCL21 is upregulated on lymphatic endothelial cells following TBI exposure indicative of a promigratory microenvironment for cDC.

**CCR7 is required for the TBI-induced migration of cDC**

To determine whether CCR7 was required for the TBI-induced migration of cDC from the skin, we used CCR7−/− knockout mice. Because CCR7−/− cDC are unable to migrate from the skin (12), we hypothesized that these cells would remain in the ear skin and not be depleted following TBI. To test this, CCR7−/− mice were TBI and, 7 d later, sacrificed, and ears were prepared for whole-mount microscopy, as before. Compared with unirradiated dermal (Fig. 6A) and epidermal (Fig. 6B) controls, the densities of iDC and LC on day 7 post-TBI exposure (Fig. 6C and 6D, respectively) were not significantly changed (Fig. 6E and 6F, respectively). Unlike TBI of wild-type mice (Fig. 1), there were no areas within the dermis or epidermis that were devoid of cDC in the CCR7−/− mice following TBI. In addition, the cellular swelling and extension of dendrites that were observed in the LC population following TBI of wild-type mice (Fig. 1D) were not observed in LC from TBI CCR7−/− mice (Fig. 6D), as these cells were not significantly changed in size relative to unirradiated controls (Supplemental Fig. 1B). Taken together, these data suggest that CCR7 is required for the migration of cDC following TBI exposure.

**Mobilization of cDC from the skin following TBI impairs the CHS response to hapten**

Finally, to determine whether there was a functional impairment of cDC to process and present Ag, we employed a model of CHS. To determine the role of cDC after TBI exposure, mice were sensitized on the back at day 0, exposed to TBI or TBI+ear on day 4, and then challenged on the ear at day 8 (Fig. 7A). Relative to the DNFB-sensitized, unirradiated, vehicle-challenged ears (Fig. 7B), extensive swelling and cellular infiltrate were seen 48 h after DNFB challenge in the positive control ear (Fig. 7C). However, following TBI treatment, both the vehicle-challenged ear (Fig. 7D) and the DNFB-challenged ear (Fig. 7E) did not swell or display cellular infiltrates. These data would suggest that TBI and perhaps the subsequent reduction of cDC during the time of challenge greatly impair the CHS response to hapten.
To further confirm the requirement for cDC during the challenge phase and rule out the possibility that TBI was eliminating the antihapten-specific effector cell pool, we used the TBI + ear treatment. For these studies, mice were sensitized, exposed to the TBI + ear treatment, and then challenged with DNFB on both ears. We hypothesized the following: 1) the unirradiated ear would swell because cDC are not depleted following TBI + ear treatment, and 2) the ear exposed to radiation (TBI + ear) would have minimal swelling due to the loss of cDC. As expected, unexposed ears had comparable swelling to positive controls that persisted for 2 d (Fig. 7G), whereas ears exposed to IR did not swell (Fig. 7F, 7G). Taken together, these data suggest that, due to the TBI-induced mobilization of cDC from the skin, the CHS response to hapten is greatly impaired despite the fact that antihapten-specific effector cells are present to mount an inflammatory ear-swelling response.

**Discussion**

We have previously shown that local IR exposure to the mouse ear results in the partial depletion of cDC (15). In this study, we report a similar observation using a sublethal TBI dose of 6 Gy and identify the mechanism responsible for the IR-induced mobilization of cDC. Our TBI studies confirm that, like local IR exposure, there is an initial mobilization of cDC (days 0–14), followed by a gradual repopulation over time (days 14–60) that we could monitor using whole-mount fluorescence microscopy. During the mobilization phase, the iDC appeared to be the first to respond to the TBI-induced trauma signals, followed by the slower trafficking LC. The loss of both iDC and LC left noticeable vacancies within the dermis and epidermis, respectfully, that became larger and more prevalent over time. Interestingly, we observed unique morphological changes in the LC population following TBI, including cellular swelling and dendrite outgrowth, which are a suggestive phenotype for migratory LC. In support of these findings, the same morphological characteristics have also been observed in several well-established methods to trigger cDC migration such as tape stripping of the ear (17), injection of TNF-α (21), or topical application of DNFB (22). Importantly, cDC were not depleted from the shielded ears of mice exposed to TBI (TBI + ear), nor were the aforementioned morphological changes in the LC population observed in those shielded ears, suggesting a local mechanism for the TBI-induced partial depletion of cDC.

In our models of IR exposure, the lack of TUNEL labeling during both fast and late apoptosis would suggest that there is limited DNA fragmentation-induced cell death of both cDC and other cell types (23). Therefore, our data would support an alternative cDC reduction mechanism that is likely centered on the induction of migration, which has been well characterized during steady state...
and inflammatory conditions (9). However, to our knowledge, we are the first to describe an active migratory response of cDC following TBI. Our kinetics studies quantifying the accumulation of migratory cDC in the ADLN draining locally irradiated ears correlate with previous observations made by Kissenpfennig et al. (17). In these studies, TRITC was applied to the ears of enhanced

**FIGURE 5.** TBI causes an upregulation of the CCL21 protein in LVYE-1 lymphatic vessels. (A–C) Fluorescence whole-mount images from an unirradiated mouse ear dermis labeled with CCL21 (A) and LYVE-1 lymphatic vessels (B) and overlaid in (C). (D–F) Fluorescence whole-mount images from a day 5 post-TBI mouse ear dermis labeled with CCL21 (D) and LYVE-1 (E) and overlaid in (F). (G) The percentage of CCL21 area on LYVE-1 lymphatic vessels is shown. n = 6 mice per group ± SEM, and statistical analyses were performed using an unpaired t test with Welch correction. Scale bars, 25 μm. *p < 0.01.

**FIGURE 6.** CCR7 is required for TBI-induced migration of cDC. Fluorescence whole-mount microscopy images from unirradiated CCR7<sup>−/−</sup> mouse ear dermis (A) and epidermis (B) labeled with anti-MHC II and anti-langerin, respectively. Dermal and epidermal images from day 7 post-TBI are shown in images (C) and (D), respectively, and were labeled with the same aforementioned Abs. Using these images, MHC II<sup>+</sup> (E) and langerin<sup>+</sup> cellular densities (F) were quantified from unirradiated (black bars) and irradiated (gray bars) CCR7<sup>−/−</sup> mice. n = 6 mice per group ± SEM, and statistical analyses were performed using an unpaired t test with Welch correction. Scale bars, 100 μm (A, C) and 50 μm (B, D).
GFP-langerin mice to track cDC migration to the ADLN, and it was shown that iDC were the first to arrive in the ADLN, followed by the LC (17). Using a similar protocol (17), we also identified the migratory cDC population within the ADLN (Supplemental Fig. 2A) by painting the mouse ears with TRITC (Supplemental Fig. 2B). On day 4 postpainting, the only TRITC-positive cells were those that were in the cDC migratory gate (Supplemental Fig. 2C), whereas B, T, and resident DC (collectively remaining ADLN cells) were TRITC negative (Supplemental Fig. 2D). More importantly, when both mouse ears were painted with TRITC, but only one ear was irradiated (6 Gy), while the contralateral ear remained unirradiated, we observed an increase in the frequency (Supplemental Fig. 2E) and total number of TRITC+ migratory cDC (Supplemental Fig. 2F) in the ADLN from the irradiated ear relative to the ADLN from the unirradiated control ears on day 4 postirradiation. Based on these findings and our model of TBI, we can draw parallels linking the reduction kinetics of cDC from the ear with the subsequent accumulation of cDC in the ADLN following IR exposure. iDC, with a density of ∼110 cells/mm² in the dermis (Fig. 1G), were rapidly depleted from the ear between days 1 and 4 following both local (15) and TBI exposure; however, it was not until day 4 that we observed a reduction of LC that have a density of ∼720 cells/mm² in the epidermis (Fig. 1H). Interestingly, it was not until after day 4 following a local 25 Gy IR exposure that an increase in both frequency and absolute number of migratory cDC was observed. These increases are most likely contributed by the influx of slower trafficking LC rather than iDC because these cells reached maximal reduction on day 4 post-IR exposure. Importantly, we did not detect an increase in frequency or absolute number of migratory cDC in the ADLN from the unirradiated contralateral control ear, which confirms our conclusions that cDC are only depleted in skin sites exposed to IR. Not surprisingly, we also saw increases in migratory cDC frequency (Supplemental Fig. 3A) and absolute number (Supplemental Fig. 3B) over time following a local 6 Gy exposure that was similar to the local 25 Gy exposure used in Fig. 3. This coincides with our previous studies examining a dose-dependent migration of local IR exposure by which 25 Gy resulted in the greatest mobilization of cDC from the ear (15). It is possible that detection of migratory cDC following IR exposure may be dependent on the relatively short t1/2 of migratory cDC in the ADLN, which is ∼2–3 d (24). Although it has been suggested that migratory DC bearing certain foreign Ags can persist in draining lymph nodes for an extended duration (25) and that those Ag-bearing DC may exhibit an extended t1/2 due to specific T cell interactions (26, 27), the antigenic profile for TBI-induced migratory cDC remains unclear.

**FIGURE 7.** Partial depletion of cDC following TBI impairs the CHS response to hapten, whereas mice exposed to TBI−ear respond similar to positive controls. (A) Experimental scheme for the TBI-induced reduction of cDC in a CHS assay. Mice were sensitized, irradiated, and challenged, as depicted above. (B–E) Unirradiated (B, C) and TBI mouse ear sections (D, E) 48 h following vehicle (B, D) and DNFB (C, E) challenge. All ear sections were stained with H&E. (F) Ear swelling 24 h following challenge (F) and over time (G) was measured in the following groups: positive control (black bar, heavy black line); negative control (white bar, dotted black line); TBI + ear (gray bar, hashed line); and TBI − ear (charcoal bar, thin black line). Change in ear thickness = (X − X₀), where X₀ is the prechallenge measurement and X is the postchallenge measurement. n = 4 mice per group and statistical analyses were performed using a one-way ANOVA with a Tukey post hoc test. Scale bars, 50 μm. *p < 0.01.
To further investigate the migration of cDC, we explored the expression of CCR7 and CCL21 in the mouse ear following TBI, as each of these proteins is required for efficient cDC chemotaxis to ADLN (12). Relative to unirradiated controls, we observed an increase in both CCR7 and CCL21 mRNA in the ears of mice treated with TBI that was highest on day 4 and then returned to baseline 10 d postexposure. It is possible that this temporal expression pattern of CCR7 may correlate with the maturation and mobilization of each cDC subset. In this scenario, the early expression of CCR7 observed 12 h following TBI may be due to the iDC population, as these cells were the first to leave the skin, which is supported by current literature (17). However, on day 4 post-TBI, we observed a 25-fold increase in CCR7 expression that was most likely due to receptor upregulation on the more numerous LC population preparing to exit the epidermis, as these cells require additional time to detach from neighboring keratinocytes and navigate through the cutaneous microenvironment. Importantly, as cDC (namely LC) left the skin after day 4 post-TBI, the expression of CCR7 decreased until it returned to unirradiated levels, at which point migration was no longer observed. These data, and the requirement for CCR7 during the TBI-induced migration of cDC, are further supported by our studies using CCR7−/− mice, as iDC and LC from these animals were not depleted from the dermis and epidermis, respectively, following IR exposure, nor was an accumulation of cDC detected in the ADLN (data not shown) (12). Although it has been suggested that CCR7 is not required for the initial exit of LC from the epidermis, these studies were conducted using the application of hapten (12) and an ear tissue explant model, which is significantly different from the IR-exposure model presented in this work. Therefore, it appears that, in our model of TBI, CCR7 is required for efficient LC exit from the epidermis, suggesting that IR might regulate cDC migration, and possibly maturation, in a unique way. It will be interesting to further characterize the maturation status of cDC by exploring surface phenotype and cytokine production following TBI, as these traits distinguish semimature from fully mature DC (28). Although both semimature and fully mature cDC express CCR7 and share similar costimulatory proteins, they differ in the ability to induce tolerance and immunity, respectively, in the lymph node and in situ (28). The extracellular stimuli and signaling pathways that initiate this spontaneous TBI-induced migration of cDC as well as the expression of CCR7 and maturation status of cDC, however, remain unresolved (29, 30).

CCL21 mRNA also reached maximal expression on day 4 post-TBI and had a similar temporal pattern as the CCR7 profile. Recently, Johnson and Jackson (11) demonstrated that CCL21 was stored within intracellular compartments (stores) of human dermal lymphatic endothelial cells and expression could be increased following TNF-α treatment. In addition, Shakhar and colleagues (31) also identified small, punctate CCL21 labeling in steady state mouse lymphatic vessels within the ear. It is possible that, immediately following TBI, CCL21 protein is actively released from these stores into the cutaneous microenvironment that further induces iDC maturation and facilitates mobilization. The subsequent emptying of these stores might then trigger the production of CCL21 mRNA, which was seen as early as day 1 and was most significantly increased on day 4 post-TBI relative to unirradiated controls. Indeed, as our intracellular staining would suggest, it would appear that, on day 5 post-TBI, fluorescent labeling for CCL21 protein within LYVE-1+ vessels was brighter and covered a larger area relative to unirradiated controls. These data are in agreement with previous findings suggesting that immobilized CCL21 on mouse lymphatic vessels has an equally important role as soluble CCL21 and is essential for cDC docking and entry into draining lymphatics (31). Unexpectedly, we also observed an upregulation of LYVE-1 on some portions of lymphatic vessels from mice exposed to TBI. We are currently investigating these findings, as little is known of the IR-induced effects on lymphatic endothelial cells. Recently, it was shown, by using LYVE-1−/− mice, that LYVE-1 was dispensable for cDC migration (32), whereas others have suggested that cDC enter lymphatic vessels at sites of discontinuous LYVE-1 expression (33). Importantly, although the upregulation of CCL21 was observed on all TBI lymphatics, LYVE-1 upregulation was heterogeneous on these vessels.

Interestingly, the mRNA expression of both CCR7 and CCL21 was dramatically upregulated on day 4 post-TBI relative to unirradiated controls and the additional time points examined. Although it is possible that TBI might act as a classic proinflammatory stimulus by inducing granulocyte extravasation into irradiated tissues and subsequent upregulation of CCR7 and CCL21 mRNA, this seems unlikely because of the following: 1) WBC counts (Supplemental Fig. 4A), including Ly-6G+ granulocytes (Supplemental Fig. 4B), from TBI-exposed mice were significantly reduced in peripheral blood samples collected between days 1 and 21 post-IR exposure, and 2) we did not observe an increase in Ly-6G+ granulocytes (Supplemental Fig. 4C) within the ear tissue derived from locally irradiated (Supplemental Fig. 4D) or TBI-exposed mice (Supplemental Fig. 4E), as these cells remained colocalized with the CD31+ vasculature. We are currently examining the early role of inflammatory cytokines, including TNF-α and IL-1β, because these are also produced by nonimmune cells, as well as the nuclear transcription factor NF-κB, which has been shown to augment CCL21 production (34). However, it is also possible that these findings could be explained by the activation of NF-κB via an inflammatory cytokine-independent mechanism. Previous studies in the field have demonstrated that IR alone can activate NF-κB via direct dsDNA damage or indirect DNA damage by the generation of reactive oxygen species (35). In this pathway, IR-induced DNA damage upregulates ataxia telangiectasia mutated, and, following its translocation to the cytoplasm, activates the inhibitor of NF-κB kinase, which liberates NF-κB from the inhibitor of NF-κB complex. Therefore, it is possible in our model of TBI that as DNA damage and/or reactive oxygen species accumulate over time, NF-κB is activated, which causes the release and production of CCL21 protein and mRNA, respectively, from lymphatic endothelial cells as well as inducing the upregulation of CCR7 mRNA in cDC.

The partial depletion of cDC after local and TBI could have a significant impact on the successful maintenance of immune surveillance within the cutaneous microenvironment for individuals exposed to IR. Regarding cDC function, CHS to epicutaneously applied hapten has been one of the gold standards to examine the role of cDC in the elicitation of an adaptive immune response (36). Initial experiments using conditional and inducible mouse models to selectively deplete LC and iDC before sensitization suggested that iDC were essential for the T cell-mediated ear-swelling response, whereas LC were thought to have a tolerogenic role by limiting ear swelling (37). However, this functional dichotomy between iDC and LC has been recently challenged, suggesting that these cells share roles during CHS. In addition, the magnitude of ear swelling was largely determined by the amount of hapten and the number of cDC that activate the antihapten effector T cells (38, 39). Therefore, rather than examining the role of cDC before sensitization (37), we asked whether there was a functional impairment of cDC during the challenge phase following TBI. In our model of CHS, the impetus was to examine the functional response of cDC after IR exposure,
as this would aid in the development of future treatment protocols for patients undergoing radiation therapy or victims of accidental/deliberate radiological disasters. Contrary to other CHS models (37), both the iDC and LC populations remained intact during the sensitization phase of our CHS experiments, which enabled us to examine the functional relevance of cDC during the challenge phase. Our data would suggest that there may be a correlation between the TBI-induced mobilization of cDC upon challenge and subsequent impairment of CHS to epicutaneously applied hapten.

Although we observed a reduction in circulating T cell numbers in the blood and ADLN following TBI (data not shown), our TBI–ear studies would suggest that there is a sufficient number of T cells to elicit an ear-swelling response as long as cDC are present in the skin upon challenge. As an additional control to demonstrate reduced ear swelling on irradiated skin, we locally exposed one ear with a dose of 6 Gy, whereas the contralateral ear remained unexposed. Following DNFB challenge to both ears, we observed reduced swelling on the irradiated ear and normal swelling on the unirradiated ear (data not shown). Taken together, these data confirm the requirement for unirradiated cDC and/or an unirradiated cutaneous microenvironment during the challenge phase of CHS to elicit a normal swelling response and may further suggest that the number of cDC upon challenge could dictate the swelling response (38–40).

We are currently investigating the cytokine milieu within the cutaneous microenvironment following TBI, as the balance between type 1 and type 2 cytokines may further advance our understanding of the TBI-induced migration of cDC as well as the induction of both CCR7 and CCL21. Depending on how this TBI-induced cytokine microenvironment is skewed, it could greatly impact the conditioning of cDC within the skin and dictate how these cells interact with T cells in draining lymph nodes (10). Recently, it has been suggested that atopic dermatitis (AD), a chronic inflammatory skin disease, is, in part, initiated and driven by cDC (41), and patients who suffer from AD often exhibit a type 2 cytokine profile within the skin. However, despite an increased understanding in the immunopathology, AD still remains an idiopathic disease that manifests in individuals with a predetermined genetic background or in those exposed to a variety of environmental stimuli (42). Interestingly, patients undergoing radiotherapy often develop radiation-induced dermatitis (radiodermatitis) and express many of the same clinical signs and symptoms as those observed with AD. It is possible that the IR-induced migration of cDC coupled with a newly generated cytokine microenvironment might elicit an adaptive response that manifests into radiodermatitis. However, further investigation into the function and phenotype of these IR-induced migratory cDC is required before we can make these conclusions.

In summary, we have identified a CCR7-dependent mechanism that is responsible for the TBI-induced migration of cDC. The upregulation of CCL21 following TBI would also suggest that migration is directing these cells toward the lymphatics and ultimately the ADLN. As a result of the cDC mobilization from the skin, we believe the host is more susceptible to pathogen entry as the impaired CHS response would suggest. Indeed, iDC are required for efficient cross-presentation of HSV-1 Ags to naive CD8 T cells (43), and the surveillance of both iDC and LC is needed to dictate distinct humoral responses to epicutaneously applied Ags (44). Importantly, in our model of sublethal TBI, cDC are not completely depleted from the skin, as a fraction of both iDC and LC persists following IR exposure. Therefore, it is possible that the maturation status of cDC and/or newly generated cytokine milieu following TBI may dictate altered skin homeostasis and immune surveillance over time after IR exposure. We are currently investigating how a reduction of cDC impacts immune regulation in the skin following TBI, as these studies will aid in the development of mitigating agents that may alleviate radiodermatitis and onset of cutaneous radiation syndrome.

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Disclosures

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