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Migration of Skin Dendritic Cells in Response to Ionizing Radiation Exposure

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Abstract

We describe an imaging assay that monitors the migration of two unique subsets of immune dendritic cells (DC), interstitial dendritic cells (iDC) and Langerhans cells (LC), found in the dermal and epidermal layers of skin, respectively. Using this assay, we study responses of these cells to ionizing radiation. Results obtained using whole-mount histology and fluorescence microscopy suggest that ionizing radiation triggered the migration of both major histocompatibility complex (MHC) class II⁺ iDC and Langerin⁺ LC in a dose- and time-dependent manner. Migration appeared to be limited by local administration of recombinant IL-12, a potent immunostimulatory cytokine known to induce DNA repair. Those findings were extended to an *in vivo* model by injecting fluorescently conjugated anti-MHC class II antibodies intradermally into the ears of live, anesthetized mice and visualizing the DC population in the same ear before and after radiation exposure using confocal microscopy.

INTRODUCTION

The effects of electromagnetic radiation on the immune system have long been an intriguing topic. Whether at the tissue scale or the molecular level, research has shown that radiation can have varying effects depending on dose, the cell/tissue with which it interacts, exposure time and wavelength (1-3). Of great interest are the forms of radiation with wavelengths shorter than 200 nm, including X rays and γ rays (4), collectively referred to as ionizing radiation. The sensitivity of a variety of cell types, including immune cells, to radiological exposure is well documented (5,6). Pioneering cell radiosensitivity studies conducted by Bergonie and Tribondeau in 1906 concluded that rapidly dividing, poorly differentiated cells were highly sensitive to radiation (7). Those findings eventually became the cornerstone of our understanding of cellular radiosensitivity and helped establish the therapeutic use of radiation. In contrast, cutaneous immune dendritic cells (DC), including Langerhans cells (LC) of the epidermis and interstitial dendritic cells (iDC) of the dermis, have been found to be quite radioresistant. Unlike cancerous cell types or stimulated lymphocytes, cutaneous DC do not divide rapidly, and they have been shown to survive several months after whole-body radiation exposure (8,9). These cells are essential for immune surveillance because they serve as antigen presenting cells, constantly sampling the epidermal and dermal layers of the skin where they ingest foreign pathogens. They then migrate to draining lymph nodes where they display the antigens to naive T cells for subsequent activation and clonal expansion (10).

Both Cole *et al.*, who explored the long-term effects after high-dose local irradiation of mouse footpads (11-13), and Groh *et al.*, who investigated clinically relevant doses of local exposure to mouse ears (14), concluded that LC were depleted from the epidermis after radiation exposure. Using the available technology, both groups identified epidermal LC via intracellular ATPase and did not examine the iDC population of the dermis. More importantly, these earlier experiments were *ex vivo* studies and did not involve characterization of cellular migration kinetics using an applicable *in vivo* model.

In the present study, we made an innovative use of fluorescently conjugated antibodies to selectively label cutaneous DC and show that their migration and recovery are dependent on both dose and time after irradiation. We further describe the migration kinetics of both LC and the minimally studied iDC populations after irradiation and show that local administration of recombinant IL-12 (rIL-12), an immunostimulatory cytokine, can prevent the migration triggered by radiation exposure. To our knowledge, this is the first characterization of LC and iDC with unique *ex vivo* as well as *in vivo* models using confocal microscopy after local irradiation.

MATERIALS AND METHODS

Mice

Female BALB/c mice were used for the first seven experiments. The final experiment (see Fig. 8) was carried out using female C57BL/6-IL12a^{tm1Jm} IL-12 receptor-deficient mice, which are of the C57BL/6 strain background. Thus C57BL/6 mice were used for the entire experiment. Mice were purchased from the Jackson Laboratory (Bar Harbor, ME). All animal use protocols were approved by the University of Rochester's University Committee on Animal Resources (UCAR).

Antibodies and Reagents

Antibodies—Fc Block was purchased from BD Biosciences (San Jose, CA). Fluorescein isothiocyanate (FITC)-conjugated anti-mouse MHC class II (I-A/I-E), FITC-conjugated anti-rat IgG_{2b}, phycoerythrin (PE)-conjugated internal anti-mouse Langerin (CD207), allophycocyanin (APC)-conjugated anti-mouse F4/80, and APC-conjugated anti-mouse MHC class II (I-A/I-E) were purchased from eBioscience (San Diego, CA).

Reagents—Recombinant mouse IL-12 was purchased from BD Biosciences and injected intradermally using a 3/10cc insulin syringe. The tribromoethanol anesthesia stock solution was prepared by dissolving 5 g of 2,2,2-tribromoethanol in 5 ml of 2-methyl-2-butanol (Sigma, St. Louis, MO) with gentle heating. The anesthesia was diluted 1:40 in PBS, and mice were injected intraperitoneally with 300 µl of this dilution.

Irradiation Protocol

The 3200 Ci sealed ¹³⁷Cs source operated at roughly 1.90 Gy/min was used for all irradiated samples. Gamma radiation was administered at doses ranging from 1–25 Gy. Once under anesthesia, mice were placed into specially made jigs allowing for radiation exposure to one ear only while the rest of the mouse, including the contralateral control ear, remained shielded and unexposed to the radiation.

Tissue Removal and Processing

Mice were killed humanely according to a UCAR-approved protocol. Ear hair was removed with a chemical depilatory agent (Nair, Church & Dwight Co., Princeton, NJ) applied for 3 min followed by gentle washing with water and wiping down with surgical gauze. The ears

were then removed, split into dorsal and ventral halves with the aid of forceps, and placed into dishes of HBSS (Sigma) for dermal layer staining.

Epidermal Sheet Processing

After ear splitting, halves were floated in 0.5 M ammonium thiocyanate (Sigma) at 37°C for 20 min to separate epidermal from dermal layers. The epidermal layer was then fixed in 2% paraformaldehyde (J. T. Baker, Phillipsburg, NJ) at room temperature for 15 min to permeabilize cells for 2 h of intracellular staining with PE-conjugated internal anti-Langerin.

Whole-Mount Analysis and Conventional Fluorescence Microscopy

Whole-mount histology was performed on split ear samples as described previously (15). Briefly, samples roughly 12 mm × 10 mm × 0.5 mm in size were placed in 6-ml polypropylene tubes (Falcon, BD Biosciences). Ear tissues were blocked with Fc Block (BD Biosciences) at 10 µg/ml in 200 µl of phosphate-bovine-azide solution (PBA) (PBS with 1% bovine serum albumin and 0.1% sodium azide) (Sigma). After the Fc Block, fluorescently conjugated antibodies were added at 7.5 µg/ml and gently rocked at 4°C for 2 h. Samples were washed once by adding 4 ml of PBA with gentle rotation at 4°C for 45 min. To control for nonspecific binding, FITC-conjugated anti-rat IgG_{2b} isotype control was used, and staining was nonexistent.

Conventional Fluorescence Microscopy

Whole-mount analysis was performed using the Olympus BX40 conventional fluorescence microscope (Olympus America Inc., Center Valley, PA), and images were acquired using the Retiga 1300 camera (QImaging, Tucson, AZ). Stained samples were placed on a glass slide, and a cover slip and 20 µl PBA were added to wet the slide. The split ear sample selected for imaging was the ventral/inner ear half, because it had fewer densely packed cartilage cells within the hypodermal layer. The epidermal side was placed facing toward the cover slip and microscope objective. Ear tissue was then viewed using three fluorescence filter cubes (Chroma Technology Corp, Rockingham, VT); green fluorescent protein Endow cube: excitation filter HQ470/40 and emission filter HQ525/50 m; PE Hg light source cube: excitation filter D546/10 and emission filter D580/30 m; APC cube: excitation filter D595/40 and emission filter D660/40 m. Images were obtained at a 10× magnification 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 antibody edge effect. Bright-field images were acquired first followed by fluorescence images. Images obtained under fluorescence were created using the extended depth of field tool within the Image Pro Software (Media Cybernetics Inc., Bethesda, MD) and consisted of three to six images at adjacent axial locations to create one final composite. A minimum of 10 images were 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 a 20× magnification with the extended depth of field tool using the same criteria as for the dermis.

Cell Density Calculations

The densities of positively stained MHC class 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 approximately 10–22 µm. Using those diameters, an area filter ranging from 100 µm² to 480 µm² was used to select for positively stained 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 non-specific antibody labeling. Finally, those values were used as a baseline to distinguish positively stained events from any

background/nonspecifically stained events. Densities were then calculated in cells/mm². For epidermal analysis, a 50-μm² to 625-μm² area filter was used to quantify Langerhans cells.

Confocal Analysis of the Ears In Vivo

In vivo imaging was performed using a custom-built inverted laser scanning confocal fluorescence microscope (16). Using methods described previously (17), APC-conjugated antibodies against MHC class II were injected intradermally in a volume of 40 μl. Mice were placed on the microscope stage in the supine position with the ventral side of the ear facing downward toward the objective lens. A 639-nm diode laser (Power Technology Inc., Alexander, AR) was used to excite APC fluorescence, which was detected using a combination of 645 LP and 655 LP filters (Chroma). To visualize the spatial depth distribution of the LC and iDC populations, a series of sequential optical sections was obtained. Adjacent images were separated by 3 μm, and the first image in the stack was obtained at a depth of approximately 45 μm from the ventral surface of the ear. Images were acquired using a 10×, 0.45 NA objective that gave an optical section thickness of approximately 6 μm.

RESULTS

Examination of Normal Cutaneous Dendritic Cells using Whole-Mount Histology Ex Vivo

Previous *ex vivo* analysis of the B16 mouse melanoma tumor microenvironment by Gerber *et al.* (15) has shown that it is possible to visualize fluorescently conjugated antibodies specific for antigens expressed by a wide array of cell types using whole-mount histology techniques. This technique, however, had been used previously only on solid tumors and not on cutaneous tissue samples. To visualize normal cutaneous DC, BALB/c mice were killed, and hair on the ears was removed with a chemical depilatory agent. Ears were then removed and gently split by methods described previously (18). Samples were stained with fluorescein isothiocyanate (FITC)-conjugated anti-MHC class II, a prominent marker on cutaneous DC (19), and allophycocyanin (APC)-conjugated anti-F4/80, a macrophage marker, and processed further using the whole-mount histology approach (15). Figure 1A shows a bright-field image of the dorsal half of a cartilage-free ear with blood and lymphatic vasculature running throughout the ear and several black hair follicles amidst a few small, round, adipose cells. Using conventional fluorescence microscopy, the FITC-conjugated anti-MHC class II-positive cells could be seen with short dendritic-like projections stretching out from an irregularly shaped cell body, features characteristic of iDC (20) (Fig. 1B). The same image viewed for APC fluorescence revealed very little positive staining for F4/80, suggesting that a majority of the MHC class II-positive cells were iDC and not macrophages (10) (data not shown). When stained with fluorescent isotype controls, the images were negative (data not shown).

Depletion of Cutaneous Dendritic Cells as a Result of Irradiation

These initial experiments confirmed the utility of the whole-mount technique for analysis of cutaneous tissue samples and led to further investigation of the effects of radiation on these immune cells. Studies conducted using ultraviolet (UV) radiation on these cutaneous antigen-presenting cells showed a marked migration from the exposed site toward the auricular draining lymph node after UV-radiation exposures (9). Merad *et al.* also noted these monocyte derived antigen presenting cells to be highly radioresistant in the event of whole-body exposure to radiation (9). To determine the effect of radiation on cutaneous DC, we used a ¹³⁷Cs γ-radiation source with a single beam collimator to focus and channel the radiation. Mice were anesthetized and placed in a specially constructed jig positioning only the right ear over the collimator while the rest of the body remained shielded. The right ear was then exposed to a variety of doses ranging from 1–25 Gy.

In the initial experiments, an exposure of 15 Gy was used. Three days later, mice were killed and the ears were prepared and stained with FITC-conjugated anti-MHC class II for whole-mount processing. Although 15 Gy was a relatively high dose, we chose to examine an extreme case to determine whether any changes in cell and skin morphologies were visible. Gross examination of the ear tissue 3 days after exposure to radiation revealed no noticeable reddening or thickening of the skin, nor were there necrotic lesions. Compared to the unirradiated control ear (Fig. 2A), a marked decrease in the density of MHC class II positively stained cells was clearly visible in the exposed ear (Fig. 2B). These data suggest that like UV radiation (9), ionizing radiation can also cause marked decreases in cutaneous DC densities of the ears, which are detectable by staining with anti-MHC class II antibody.

Quantification of Cutaneous Dendritic Cells at Various Doses

Quantification of anti-MHC class II positively stained cells was performed using Image Pro analysis software. A “mask” based on cell size and fluorescence intensity (see the Materials and Methods) (Fig. 2C, D) was made of each image, which selectively gated out non-specific staining. Densities of fluorescent cells per square millimeter in each image were then calculated and averaged. The average irradiated cell densities were then divided by the average unirradiated control cell density, which was approximately 86.5 ± 2.8 cells/mm² (mean $\pm$ SEM) (Fig. 3). The fastest and most dramatic changes in density were seen after exposure to 25 Gy. Three days after this dose, a 70% decrease in cell density relative to the control was evident. After exposure to 15 Gy, it was not until day 10 that a decrease of similar magnitude could be seen. Importantly, this assay was sensitive enough to detect doses as low as 1 Gy, although there were no detectable differences in response between 1 and 5 Gy. In response to all doses, the initial depletion in cell density was followed by repopulation. Finally, the greatest uncertainties in normalized data points were seen on day 14 after exposure, suggesting that the rate of recovery and extent of MHC class II positive cell populations vary among individual mice (Fig. 3).

Similar Migration Kinetics in the Two Distinct Cutaneous Cell Populations

Although anti-MHC class II whole-mount staining selectively targeted cutaneous DC, it did not specifically distinguish the epidermal LC population, which is also MHC class II positive (19). To distinguish between these cell types, we targeted the internal Langerin epitope by permeabilizing the ear tissue and performing whole-mount staining with a phycoerythrin (PE)-conjugated internal anti-Langerin (CD207) antibody (18). Langerin is a specific C-type lectin unique to LC found within the cell's Birbeck granules and embedded within the transmembrane domain of the cell (21,22). In brief, the excised ear was split into dorsal and ventral halves, which were further floated in 0.5 M ammonium thiocyanate for 20 min to separate the epidermis from the dermis. The epidermis was then floated in 2% paraformaldehyde for 15 min to permeabilize and fix the cells. After permeabilization/fixation, we stained the epidermis with both FITC-conjugated anti-MHC class II and PE-conjugated internal anti-Langerin. Images from epidermal samples stained in this way were positive for anti-MHC class II (Fig. 4A) and internal anti-Langerin (Fig. 4B). An overlay (Fig. 4C) revealed the double positively stained LC of the epidermis with a density of approximately 745 ± 11 cells/mm² (mean $\pm$ SEM). Figure 4D illustrates the double-positive epidermal cell population imaged at a 40 $\times$ magnification, revealing the multiple dendritic projections stretching out from the cell body that are characteristic of LC (10).

Successful identification of LC using the anti-Langerin now allowed us to accurately measure the kinetics of their migration after irradiation and compare it to that of the iDC. Thus mice were exposed to various doses of radiation, epidermal sheets were prepared as described previously, images were acquired, cell number densities were calculated (using a similar masking procedure), and data were normalized against the control unirradiated ear. Figure 5A

shows the densely populated LC of the control unirradiated ear with the corresponding mask shown in Fig. 5C. The LC span the entire field of view and have short but much more noticeable dendritic projections than their iDC counterparts. However, 3 days after exposure to 15 Gy, the LC become quite depleted, leaving large areas of epidermal tissue devoid of stained cells (Fig. 5B). When we applied the masking function on the irradiated sample (Fig. 5D), it appeared that the overall length of the dendritic projections was slightly longer than that of the unirradiated counterparts (Fig. 5C). Average irradiated cell densities were normalized to unirradiated control densities, and the results are plotted in Fig. 6. Interestingly, though tenfold greater in number than iDC, LC followed similar migration kinetics and appeared to be depleted in a similar dose- and time-dependent fashion. Thus *ex vivo* analysis of two unique cell types, iDC and LC residing in the dermis and epidermis, respectively, shows that these cells share similar migratory kinetics days after local radiation exposure to the ear.

In Vivo Confocal Microscopy Analysis of Migratory Langerhans and Interstitial Dendritic Cells as a Result of Exposure to Ionizing Radiation

After successful *ex vivo* analysis of cutaneous samples, we shifted our emphasis toward establishing an *in vivo* model to accurately monitor changes in DC density over time. To this end, we attempted to image cutaneous DC populations by directly injecting fluorescently conjugated antibodies into the skin of live anesthetized mice, a technique recently developed and demonstrated in our laboratories (17). In brief, mice were anesthetized, ear hair was removed with a chemical depilatory agent, and the ventral side of the ear was injected intradermally (i.d.) with a cocktail containing APC-conjugated anti-MHC class II and Fc Block in PBS. Two hours after the i.d. injection, mice were again anesthetized and positioned on the stage of a custom confocal microscope with the ventral side of the ear facing downward toward the microscope objective. On day 0, a series of 15 to 20 sequential optical sections was then acquired every 3 μm beginning at a depth of roughly 45 μm from the ventral surface of the ear. Langerhans cells appeared roughly 55 μm from this surface (Fig. 7A, top two panels) and formed a mesh network similar to that seen in the *ex vivo* studies (Fig. 4A). Beyond the epidermal layers at a depth of roughly 70 μm from the surface, the LC were no longer present. Upon entry into the dermis, a larger, stouter iDC population was seen (Fig. 7A, bottom two panels). *In vivo* images of both iDC and LC were very similar to those taken from *ex vivo* processed ears, which reinforces the findings in our live mouse model.

With a working *in vivo* model to image cutaneous DC, we next explored the migratory kinetics of both LC and iDC after irradiation. Using our i.d. antibody labeling technique, we were able to visualize the loss of cutaneous DC from the ears of individual mice over a period of several days. To assess the degree to which the previously imaged MHC class II-positive cells migrated as a result of irradiation *in vivo*, the right ear was exposed to 25 Gy, and 3 days later the same ear was injected i.d. with an APC-conjugated anti-MHC class II cocktail. Two hours after injection, the ear was reimaged at the same site and depths as it was on day 0. As shown in Fig. 7B from top to bottom, the LC were clearly depleted after ionizing radiation exposure, leaving several voids in the mesh network of cells. Deeper within the dermis, a noticeable decrease in the density of iDC could also be seen 3 days after irradiation; these findings also mirrored those observed in *ex vivo* tissues (Fig. 3).

Intradermal Administration of rIL-12 Limits Migration of MHC Class II Positive Cells after Ionizing Radiation Exposure

The immunostimulatory cytokine interleukin 12 (IL-12) has been shown to have profound effects in both innate and adaptive immune responses (23). Produced primarily by phagocytic cells including DC and human LC through stimulation of Toll-like receptors, IL-12 is critical for the development of both Th1 and NK cell populations (23). In addition to its large role in a variety of immune responses, studies conducted by Schwarz *et al.* have suggested that the

pro-inflammatory cytokine is also pivotal at the DNA level, inducing the nucleotide excision repair (NER) pathway required to repair mutations to DNA, such as cyclobutane pyrimidine dimers (CPD), as a result of exposure to ultraviolet B (UVB) radiation (24,25). With a similar rationale in mind, we sought to explore whether IL-12 had comparable effects on cutaneous DC populations exposed to radiation using both wild-type and IL-12 receptor knockout (IL-12RKO) mice of C57BL/6 background. In our first experiments, we addressed the role of rIL-12 administration *before* irradiation using two groups: the untreated/unirradiated control mice and the treated/irradiated mice. Treated mice received an i.d. injection of 40 μ l of PBS in the left ear and 100 ng of rIL-12 in 40 μ l in the right ear. Three hours later, each ear was irradiated separately with a dose of 25 Gy. Three days after exposure, mice were killed and prepared for *ex vivo* whole-mount analysis. Compared to the untreated/unirradiated group, a significant ($P < 0.0134$; unpaired t test) decrease in the density of MHC class II-positive cells was seen after exposure to 25 Gy in mice pretreated with PBS (Fig. 8A). These data suggest that the decrease of MHC class II-positive cells from mouse dermis after radiation exposure is not strain specific because it is observed in both BALB/c and C57BL/6 mice. When the C57BL/6 mice were pretreated with rIL-12, the density of MHC class II-positive cells after exposure to 25 Gy also decreased; however, this density change, compared to untreated/unirradiated samples, was not significant ($P < 0.1041$; unpaired t test) (Fig. 8A), possibly suggesting a causative effect from the cytokine treatment. The likelihood that rIL-12 treatment may have an effect on the dermal microenvironment and retention of MHC class II-positive cells is further supported when PBS- and rIL-12-treated ears are compared directly using the paired t test. Statistical analysis using the paired t test on the measure of pooled mouse average cell densities in the PBS- and the rIL-12-treated ears revealed a significant difference ($P < 0.0065$) between samples, again suggesting a causative effect from the cytokine treatment before radiation exposure. Next, we investigated rIL-12 treatment 3 h *after* radiation exposure. Similar results were observed: a significant ($P < 0.0001$; unpaired t test) decrease of MHC class II⁺ cells in PBS-treated ears relative to the untreated/unirradiated mice and a marginal decrease in those treated with rIL-12 compared to the untreated/unirradiated ears ($P < 0.1546$) (Fig. 8B). Using the paired t test to compare the PBS- and rIL-12-treated ears, a significant difference is again observed ($P < 0.0068$), suggesting that cytokine treatment after radiation exposure also limits the depletion of MHC class II-positive cells from the dermis. As an additional test, IL-12 receptor knockout mice were also examined. Interestingly, baseline densities of MHC class II cells were slightly lower than in wild-type mice, and a significant ($P < 0.001$; unpaired t test) decrease in cell density was seen for both PBS and rIL-12 treatments relative to the untreated/unirradiated mice (Fig. 8C). Examination of the PBS- and rIL-12-treated groups using the paired t test revealed no significant difference between the groups ($P < 0.0615$), confirming that without a functional IL-12 receptor the cytokine treatment does not affect the dermal microenvironment. Thus, while the IL-12 mechanism and action on cutaneous dendritic cells remains to be elucidated, these data suggest a possible role in the retention and/or recruitment of MHC class II-positive cells both before and after radiation exposure.

DISCUSSION

Using antibody-labeled fluorescence imaging techniques, we have shown in both *ex vivo* and *in vivo* models that ionizing radiation induces migration of two cutaneous dendritic cell populations, specifically epidermal Langerhans cells (LC) and dermal interstitial dendritic cells (iDC). Not only have we characterized and quantified the cellular migration of each population as a result of radiation exposure in a dose- and time-dependent manner, we have shown through intradermal injection of fluorescently conjugated antibodies that similar results could be achieved in live mice, suggesting a possible assay to monitor and diagnose degrees of radiation exposure. We also discovered an additional role for the immunostimulatory cytokine IL-12 and have shown that it can modify the migration induced by radiation when injected at the site of exposure. Taken together, these results suggest a novel *in vivo* approach to analyze cutaneous

DC after radiation exposure, which may be useful in the field as a biomarker to aid in the triage of potential victims after a radiation event.

Although the depletion of LC as a result of radiation exposure has been documented previously (11-14), improved visualization techniques, more specific antibodies, and further elucidation of the relationships between cutaneous immune cells and radiation provide motivation for further investigation. Using a specially constructed mouse jig, we were able to irradiate one ear while the remainder of the mouse including the contralateral ear remained shielded and acted as an internal self control. After radiation exposure, we saw a marked decrease in the density of iDC and LC via anti-MHC class II and anti-internal-Langerin staining, respectively, using fluorescence microscopy. Though the kinetics of the iDC and LC was qualitatively similar, there were subtle interesting differences that may reflect the degree of radioresistance between the cells. Regarding dose, it is clear for each cell type that the larger the dose, the more dramatic the loss in cells; however, the magnitude and initial rate of loss appears to differ between the populations (Figs. 3 and 6). Initially, the iDC have a more dramatic drop in overall density than the LC. These differences may be attributed to several possible factors: (a) LC must down-regulate E-cadherin mediated attachment to keratinocytes, whereas iDC do not, thus slowing the migration process (26); (b) LC must traverse greater distances and navigate through the basement membrane zone that separates epidermis from dermis (27); and finally (c) LC may be more radioresistant than iDC, as is evident from bone marrow chimeras and whole-body radiation experiments (9). While each factor suggests interesting possible interpretations, additional studies to further elucidate the mechanism responsible for the differences in migratory rates between each DC population after irradiation need to be conducted before firm conclusions can be drawn. These studies are currently under way.

Upon examination of each population after radiation exposure, distinct morphological differences were evident. After irradiation, the remaining iDC appeared rounded with shorter projections than their unirradiated counterparts. These morphological changes are reasonable from a sterics standpoint, because they permit efficient trafficking through thick extracellular matrices toward draining lymphatics (27). In contrast, LC were slightly larger with longer dendritic projections, which may seem counterintuitive but actually reflects similar morphological changes seen during contact hypersensitivity reactions triggered by the topical application of a reactive hapten such as dinitrofluorobenzene (28). Similar to hapten-treated ears, irradiated ears also had gaps within the network of cells where LC were not present. The remaining LC appeared larger and with longer projections that extended over the gaps devoid of cells. Taken together, these morphological changes suggest an exodus from the cutaneous environment toward draining lymphatics as a result of radiation exposure similar to the migration seen from documented hypersensitivity experiments.

In addition to migration as a plausible explanation for the observed depletion of cutaneous DC after radiation exposure, it is reasonable to infer that some fraction of the cells may be dying as a result of the higher doses (15 Gy and 25 Gy). The iDC in particular (Fig. 3) appear to be more radiosensitive, suffering greater initial decreases in overall density 2 and 4 days after exposure to 25 Gy and 15 Gy, respectively. However, the rates of depletion for the LC population (Fig. 6) at days 2 and 4 do not appear to be as dramatic as for the iDC counterparts, suggesting again that LC are more radioresistant. Currently, we are experimenting with a modified TUNEL assay (29), used to label and detect DSBs within DNA, to assess apoptosis in both dermal and epidermal tissues.

Interestingly, in a manner similar to UV radiation, LC as well as iDC migrate from the site of exposure to draining lymph nodes (in this case the auricular lymph node) after exposure to ionizing radiation (24). The reasons for this migration and a plausible mechanism remain unknown; however, we are currently exploring a variety of assays to further elucidate this

phenomenon. One explanation could be the induction of apoptosis of keratinocytes resulting from a variety of DNA lesions such as double-strand breaks (DSBs) caused by radiation exposure (5). In the case of UVB radiation, keratinocytes develop cyclobutane pyrimidine dimers (CPD) within their DNA, which triggers death receptors that further activate the pathways to apoptosis in an effort to protect the body from potentially malignant mutations (25). This process is known as sunburn cell (SBC) formation (30,31), and the same damage has been reported within LC (25). In an effort to save some of these SBC, the nucleotide excision repair (NER) pathway is activated to remove CPD and repair the DNA. Schwarz *et al.* have shown that NER can be induced by administration of the immunostimulatory cytokine IL-12 (25) and that it can further limit the number of SBC (24) as well as LC with CPD in draining lymph nodes (25). Perhaps the same can be inferred for ionizing radiation, which does not create CPD but rather makes DSBs within DNA.

Our studies with rIL-12 administration would suggest that not only does the cytokine trigger NER, it may play a role in initiating DSB repair pathways, which could explain the recovery seen when compared to PBS-treated/irradiated mice. In addition to the possible retention induced by rIL-12, cells deemed *unsalvageable* by repair mechanisms as a result of radiation exposure might trigger LC to engulf the apoptotic bodies and migrate to draining lymph nodes, as is the case for UVB-radiation-induced damage (32). Although it is well established that apoptotic bodies are engulfed and cleared by phagocytic cells such as LC and iDC (33), it remains unclear what “self” signals are modified by UV radiation and, in this case, ionizing radiation to cause such a phenomenon (6,34).

Another explanation for the migration of LC may be attributed to cytokines and cadherins that control and regulate mobilization of LC. Upon antigen recognition by LC, IL-1 β is up-regulated internally and acts in an autocrine fashion through the IL-1 receptor on both LC as well as neighboring keratinocytes (35). The cytokine TNF- α is then secreted by keratinocytes and acts back on the LC as a secondary messenger to cause down-regulation of E-cadherin and other adhesion molecules (35). Thus mobilization is initiated, and the LC are permitted to traffic to the draining lymph node. In the event of exposure to radiation, perhaps trauma induced upon neighboring keratinocytes causes a faulty release of TNF- α or simply down-regulates the strong adhesive cadherins, permitting LC to traffic. Additional analysis of secreted IL-1 β and TNF- α as well as their expression patterns from both LC and keratinocytes after radiation exposure need to be conducted to elucidate the role of cytokines in inducing this migration. Studies aimed toward exploring E-cadherin and other adhesion molecules would also be relevant for this proposed mechanism.

Although the ability to monitor changes in cutaneous dendritic cell populations for use as a potential biomarker appears promising, additional research needs to be conducted, namely in the area of local compared to whole-body ionizing radiation exposure. Whereas the extremities can withstand over 20 Gy, there have been no reported survivors of victims exposed to 10 Gy whole body (4). Therefore, we are expanding our research to further elucidate the mechanisms and signaling pathways involved in this marked migration as a result of low-dose exposure. Currently, we are attempting to delineate among five low-dose groupings of radiation exposure including 1–2, 2–4, 4–6, 6–8 and 8–10 Gy, where well-defined clinical symptoms and manifestations have been reported (4,36,37).

In summary, we have demonstrated a novel approach using fluorescently conjugated antibodies to monitor a unique cellular response to ionizing radiation. We have also shown that cutaneous tissue samples can be fluorescently stained *in vivo* to monitor changes in the density of two morphologically distinct antigen-presenting cells including Langerhans cells and interstitial dendritic cells. These findings were confirmed by *ex vivo* analysis using whole-mount histology, further showing the usefulness and range of the application.

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FIG. 1. Whole-mount histology on excised and split mouse ears is effective for labeling cutaneous dendritic cells. The dorsal, cartilage-free half of a mouse ear under bright field (panel A) and stained with FITC conjugated anti-MHC class II (panel B). Scale bars represent 100 μ m.

FIG. 2.

Cutaneous MHC class II⁺ cells are lost after exposure to ionizing radiation. Fluorescence microscopy imaging reveals that FITC-conjugated MHC class II⁺ cells are more dense in the unirradiated control ear (panel A) than in the ear on day 3 after 25 Gy irradiation (panel B). Using the Image Pro “masking” function (panels C, D) on the images (panels A and B), positively stained cells that fit within the specified 100–480-μm² area range can be quantified. Scale bar represents 100 μm.

FIG. 3.

Ionizing radiation induces migration of MHC class II⁺ cells in a dose- and time-dependent manner. On day 0, mice received a single dose to the right ear of 1 (◆), 5 (■), 15 (●) or 25 (▲) Gy. At various times after irradiation, ears were removed, split and stained with FITC-conjugated anti-MHC class II. Irradiated ear densities (cells/mm²) were calculated, divided by nonirradiated ear densities, and multiplied by 100 to obtain normalized percentages. Each point represents the mean of five mice; each mouse represents the average density from at least 10 random fields of view $\pm$ SEM.

FIG. 4.

Intracellular staining with Langerhans cell-specific anti-Langerin confirms MHC class II labeling in the epidermal layer. FITC-conjugated MHC class II⁺ cells (panel A) also stain positively with Langerhans cell-specific PE-conjugated anti-Langerin (panel B) as shown in the image overlay (panel C). Anti-Langerin staining at a 40× magnification (panel D). Scale bar represents 50 μm in panels A—C and 25 μm in panel D.

FIG. 5.

Epidermal Langerhans cells are depleted after exposure to ionizing radiation. Fluorescence microscopy imaging on epidermal sheets reveals that PE-conjugated anti-Langerin⁺ cells are more dense in the unirradiated ear control (panel A) than in the ear on day 3 after 15 Gy irradiation (panel B). Using the Image Pro “masking” function (panels C, D) on the above images in panels A, B, positively stained cells that fit within the specified 50–625 μm^2 area range can be quantified. Scale bar represents 50 μm .

FIG. 6.

Ionizing radiation induced migration of Langerhans cells in a dose- and time-dependent manner. On day 0, mice received a single dose to the right ear of 1 (◆), 5 (▼), 15 (●) or 25 (▲) Gy. At various times after irradiation, ears were removed and split, and epidermal sheets were prepared and stained with PE-conjugated anti-Langerin. Irradiated ear densities (cells/mm²) were calculated, divided by nonirradiated ear densities, and multiplied by 100 to obtain normalized percentages. Each point represents the mean of five mice; each mouse represents the average density from at least 10 random fields of view $\pm$ SEM.

FIG. 7.

Confocal microscopy of a live anesthetized mouse intradermally injected in the ear with anti-MHC class II reveals a significant decrease in the density of MHC class II⁺ cells when locally irradiated. Two hours after intradermal injection of APC-conjugated anti-MHC class II into the right ear of an anesthetized mouse, sequential optical sections (panel A) were acquired every 3 μm beginning at a depth of 45 μm from the ventral surface of the ear. The right ear was then selectively irradiated with 25 Gy and 3 days later was reinjected with anti-MHC class II and imaged (panel B) as in panel A. Scale bar represents 50 μm, and approximately 10 mice were imaged.

FIG. 8.

IL-12 may limit DC migration in response to ionizing radiation. C57BL/6 mice were intradermally injected with 100 ng of recombinant IL-12 in 40 μ l in the right ear and 40 μ l of PBS in the left ear 3 h *before* 25 Gy radiation exposure to both ears. Three days later, mice were killed, ears were excised, split and stained with FITC-conjugated anti-MHC class II, and the average density per mouse was calculated and pooled (panel A). In panel B, the experiment was repeated; however, mice were intradermally injected with recombinant IL-12 3 h *after* 25 Gy radiation exposure; the MHC class II⁺ cellular density of each mouse was again calculated and pooled. To confirm a functional role of recombinant IL-12, IL-12 receptor deficient mice were treated in a similar fashion 3 h *before* 25 Gy radiation exposure (panel C). Significance in panel A (** $P < 0.0065$), panel B (** $P < 0.0068$) and panel C (n.s. $P < 0.0615$) was determined by the paired t test. Four or five mice were used for each grouping, and approximately 10 fields of view were taken per mouse to obtain an average MHC class II⁺ cell count/mm². A standard error of the mean bar for each grouping is reported.