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Radiation-Induced Dermatitis is Mediated by IL17-Expressing γδ T Cells

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Liao, W., Hei, T. K. and Cheng, S. K. Radiation-Induced Dermatitis is Mediated by IL17-Expressing γδ T Cells. *Radiat. Res.* 187, 464–474 (2017).

Radiation dermatitis is a serious cutaneous injury caused by radiation therapy or upon accidental nuclear exposure. However, the pathogenic immune mechanisms underlying this injury are still poorly understood. We seek to discover how the dysregulated immune response after irradiation orchestrates skin inflammation. The skin on the left flank of C57BL/6J wild-type and C57BL/6J *Tcrd*$^{-/-}$ mice, which are deficit in γδ T cells, was exposed to a single X-ray dose of 25 Gy, and the right-flank skin was used as a sham-irradiated control. At 4 weeks postirradiation, the wild-type skin exhibited signs of depilation, erythema and desquamation. Histological analysis showed hyperproliferation of keratinocytes and acanthosis. Dramatic elevation of IL17-expressing T cells was identified from the irradiated skin, which was mainly contributed by γδ T cells and innate lymphoid cells, rather than Th17 cells. Furthermore, protein levels of critical cytokines for IL17-expressing γδ T cell activation, IL1β and IL23 were found markedly upregulated. Lastly, radiation-induced dermatitis was significantly attenuated in γδ T cell knockout mice. *In vitro*, normal human epidermal keratinocytes (NHEKs) could be initiator cells of inflammation by providing a great number of pro-inflammatory mediators upon radiation, and as well as effector cells of epidermal hyperplasia in response to exogenous IL17 and/or IL22 treatment. Our findings implicate a novel role of IL17-expressing γδ T cells in mediating radiation-induced skin inflammation. This study reveals the innate immune response pathway as a potential therapeutic target for radiation skin injury. **© 2017 by Radiation Research Society**

INTRODUCTION

Radiation-induced skin injury, often referred to as radiation dermatitis, can be a serious cutaneous injury caused by radiation therapy or after a nuclear or radiological event (*1*). Radiation causes prompt DNA, lipid and protein damage of keratinocytes and vascular endothelial cells due to generation of free radicals, which results in injury of epidermis (*2*). ''Damaged self'' can be recognized by immune response through damage-associated molecular patterns (DAMPs) or also known as ''alarmins'', which recruits and activates a variety of inflammatory cells to the site of injury to eliminate dead cells and initiate wound repair (*3*). Radiation skin injury occurs in about 95% of patients who receives radiation treatment for cancer (4).

Radiation-induced skin injury is manifested with initial depilation and erythema, followed by dry and moist desquamation of the epidermis, leading to potential ulceration and necrosis (*5, 6*). Histologically irradiated skin exhibits hyperproliferation of the epidermis and thickening of the stratum corneum, infiltration of inflammatory cells (*7*). Radiation induces a cascade of signaling events involving numerous cytokines, chemokines and growth factors from a variety of skin resident and infiltrated immune cells, contributing to the clinical cutaneous symptoms and ultimate structural alteration of the skin (*8, 9*). However, the major cell types and crucial mediators involved in acute radiation dermatitis are still unclear.

Stress-initiated immune responses are mediated by several types of unconventional lymphocytes such as innate lymphoid cells (*10*) and γδ T cells (*11*), which respond more like surveillance innate immune cells. The vast majority of γδ T cells normally reside in mucosal layer of tissues such as skin, lung, gut and reproductive tract as immune sentinel cells (*12*). Skin IL17-expressing γδ cells have been widely studied in TLR7/8 ligand imiquimod-induced psoriasis model, in which γδ-derived IL17 and IL22 have found to play a major role in inflammation and tissue remodeling (*13–15*).

By using a radiation dermatitis murine model in this study, we have identified a significant elevation of pathogenic IL17-expressing γδ T cells which are essential in the pathogenesis of radiation dermatitis as demonstrated

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by mitigation of dermatitis in γδ T-cell knockout mice. Together, our findings reveal that dysregulated innate immune response as represented by IL17-expressing γδ T cells as a potential therapeutic target for radiation-induced skin injury.

MATERIALS AND METHODS

Mice

C57BL/6J WT and C57BL/6J *Tcrd*$^{-/-}$ mice were purchased from Jackson Laboratory (Bar Harbor, MA). All animals were bred and treated in accordance with the institutional guidelines and all animal protocols were approved by Institutional Animal Care and Use Committee of Columbia University Medical Center.

Cell Culture

Adult normal human epidermal keratinocytes (NHEKs; Lonza, Basel, Switzerland) were grown in the optimized KGM-Gold™ growth medium (Lonza) and experiments were performed on cells between passage 3 and 5. All the cell cultures were maintained in a humidified 37°C, 5% CO_2 incubator.

Radiation

Mice were anesthetized and flank skin was stretched out for an approximately 1.2 cm exposure area and was secured with tape to be within the irradiation field. The rest of the mouse body was shielded with a lead block (Supplementary Fig. S1; http://dx.doi.org/10.1667/RR0007CC.1.S1). A single dose of 25 Gy was given at 250 KV with a rate of 90 cGy/min (X-RAD 320, Precision X-Ray, North Branford, CT) using the F2 filter (0.75 mm tin + 0.25 mm copper + 1.5 mm aluminum). Control mice were sham-irradiated and subjected to the same anesthesia and skin stretching as irradiated animals. Cells were also irradiated with the X-RAD 320 with 5 Gy.

Scoring of Skin Injury

To score the severity of skin injury in the mouse model, a modified scoring system was developed based on clinical radiation skin injury symptoms described elsewhere (*6, 16*). Within irradiated skin area, the severity was primarily estimated by three signs: epilation (hair loss), erythema (redness), desquamation (scaling). Severity parameters were measured on a scale of 0–4, from none to maximum. The cumulative score was used as a measure of the severity of skin inflammation (scale 0–12).

Skin Histology and Thickness Scoring

Skin was fixed in 10% formalin (Sigma) for 24 h, followed by embedment and 5 μm sectioning for H&E staining (Richard Allan Scientific). The images H&E-stained sections were captured under the same magnification (20× objective) for analysis and comparison. The skin thickness was measured on the images with a ruler and presented as arbitrary values normalized to the sham-irradiated control.

Skin Immune Cell Preparation and Stimulation

Mouse hair was shaved off of the skin and followed by removal of subcutaneous fat and connective tissue. The skin was cut into 2–3 mm pieces and incubated in 1× HBSS containing 5% FBS, collagenase (2 μg/ml, Sigma) and DNase I (200 U/ml, Invitrogen) for 90 min to isolate single cell suspension. Skin cell suspension was stimulated in full RPMI medium containing 10% FBS, 12-myristate 13-acetate (PMA; 50 ng/ml, Sigma), ionomycin (1 μg/ml, Sigma), and protein transport inhibitor Brefeldin A (5 μg/ml, eBioscience, San Diego, CA) in a 37°C, 5% CO_2 incubator for 5 h. Alternatively, skin cell suspensions were cultured and re-stimulated with plate-bound mouse anti-CD3 (2 μg/ml, eBiosciences) and anti-CD28 (5 μg/ml, eBiosciences) monoclonal antibodies for 3 days. Culture media were collected for ELISA.

Intracelluar Cytokine Staining and Flow Cytometry Analysis

Anti-mouse CD45, Gr-1, TCRδ, CD3, IL17 were all purchased from eBioscience. Anti-mouse Vγ4 and Vγ5 were obtained from Biolegend (San Diego, CA). After staining of surface markers, cells were fixed and permeabilized with IC fixation buffer (eBioscience) before staining for intracellular cytokines. The corresponding isotype control antibodies were also used. Flow cytometry data were acquired by BD FACS Calibur (San Jose, CA) and analyzed with FlowJo software (TreeStar).

ELISA

Mouse IL17 DuoSet ELISA kits (R&D Systems) were used to measure the IL17 level in cell culture media. Briefly, 96-well BD Falcon ELISA plates were coated overnight at 4°C with anti-mouse IL17 capture antibody. After washing and blocking, culture media or standards were added and incubated for 2 h. The biotinylated anti-mouse IL17 detection antibody, mixed with enzyme reagent streptavidin-HRP conjugate was added and incubated for 1 h at room temperature. The plates were washed and developed with tetramethylbenzidine (TMB) peroxidase EIA substrate kit (BD Biosciences). The reaction was stopped with stopping solution (1.8 *N* H_2SO_4) and the OD was read at 450 nm with a reference of 630 nm on Synergy2 microplate reader (BioTek, Winooski, VT).

Immunohistochemistry Analysis

Paraffin-embedded sections were deparaffinized with xylene/ethanol and rehydrated. Antigen retrieval was made in sodium citrate buffer (10 m*M*, pH 6.0) at a sub-boiling temperature for 10 min, followed by 30 min cooling on bench top. After incubation with 3% hydrogen peroxide to remove endogenous peroxidase activity, the slides were washed with PBS and blocked with 5% normal goat serum for 1 h. The sections were immunostained with anti-IL23p19 (1 μg/ml, Abcam, Cambridge, MA), anti-IL1β (1 μg/ml, Abcam), anti-IL6 (1 μg/ml, Abcam), anti-IL17 (1 μg/ml, Abcam) and anti-IL22 (1 μg/ml, Abcam) at 4°C overnight. After washing with PBST, the slides were incubated with a biotinylated anti-rabbit secondary antibody (Vector Labs, Burlingame, CA), followed by streptavidin-HRP (Vector labs). With another round of washing, bound antibodies were developed by ImmPACT™ DAB peroxidase substrate kit (Vector Labs).

MTT Cell Metabolic Assay

3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT), a tetrazole was used as an indicator of cell viability as determined by its mitochondrial-dependent reduction to formazone. Briefly, 4000 NHEK cells/well were seeded in 96-well plates for 24 h, followed by indicated treatments for 5 days. MTT (0.5 mg/ml diluted with medium) was added to the wells for 4 h. Then, the supernatant was removed, and the formazone crystals were dissolved using dimethyl sulfoxide (DMSO, Sigma). The absorbance was read at 595 nm with Synergy2 microplate reader (BioTek).

RNA Extraction and Quantitative (q)PCR

Total RNA was extracted from NHEK cells or homogenized mouse skin using TRIzol reagent (Life technologies, Carlsbad, CA) according to the manufacturer's instructions. cDNAs were synthesized by SuperScript™ III First-Strand Synthesis System (Life Technologies). All the primer pairs were synthesized by Invitrogen and are listed in

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Supplementary Table S1 (http://dx.doi.org/10.1667/RR0007CC.1.S1). cDNA synthesis was performed in a GeneAmp PCR system 2700 thermal cycler (Life technologies). qPCR was performed with SYBR Green as a detection dye in ABI ViiA™ 7 system presented as fold differences over the controls by the $2^{-\Delta\Delta Ct}$ method with normalization to the housekeeping gene *Actb*.

Statistical Analysis

Graphs were made with GraphPad Prism 5.0. Results are presented as means ± SEM of *n* animals or *n* cultured samples of one experiment, which was representative of at least two independent experiments. Statistical significance was determined by two-tailed unpaired *t* test for two group comparison or one-way analysis of variance by Bonferroni for multiple comparisons with GraphPad Prism. (*$P < 0.05$; **$P < 0.01$).

RESULTS

Murine Radiation-Induced Dermatitis Model

The crucial mechanisms of radiation-induced dermatitis are not well established. In this study, we seek to understand how the dysregulated immune response after irradiation causes the skin injury. A murine radiation-induced dermatitis model was developed by giving a single X-ray dose of 25 Gy to the skin on the left flank of C57BL/6J mice (Supplementary Fig. S1; http://dx.doi.org/10.1667/RR0007CC.1.S1). At two weeks postirradiation, irradiated mice started to exhibit signs of depilation and skin erythema in the exposed field. At four weeks, cutaneous irradiation led to further depilation, erythema and desquamation of the epidermis (Fig. 1A). The independent and cumulative skin injury scores are shown in Fig. 1B. Histological analysis showed acanthosis (Fig. 2A) and approximately a fivefold increase of measured skin thickness (Fig. 2B). Ionizing irradiation resulted in hyperproliferation of keratinocytes as confirmed by staining of proliferation marker Ki67 (Fig. 2C). In addition, cleaved caspase-3 indicative of tissue damage was detected in the irradiated epidermis (Fig. 2D). Accumulation of $Gr1^+$ neutrophils in dermatitis mice was detected by flow cytometry (Fig. 2E), and also IHC staining in the epidermis of irradiated mice (Fig. 2F).

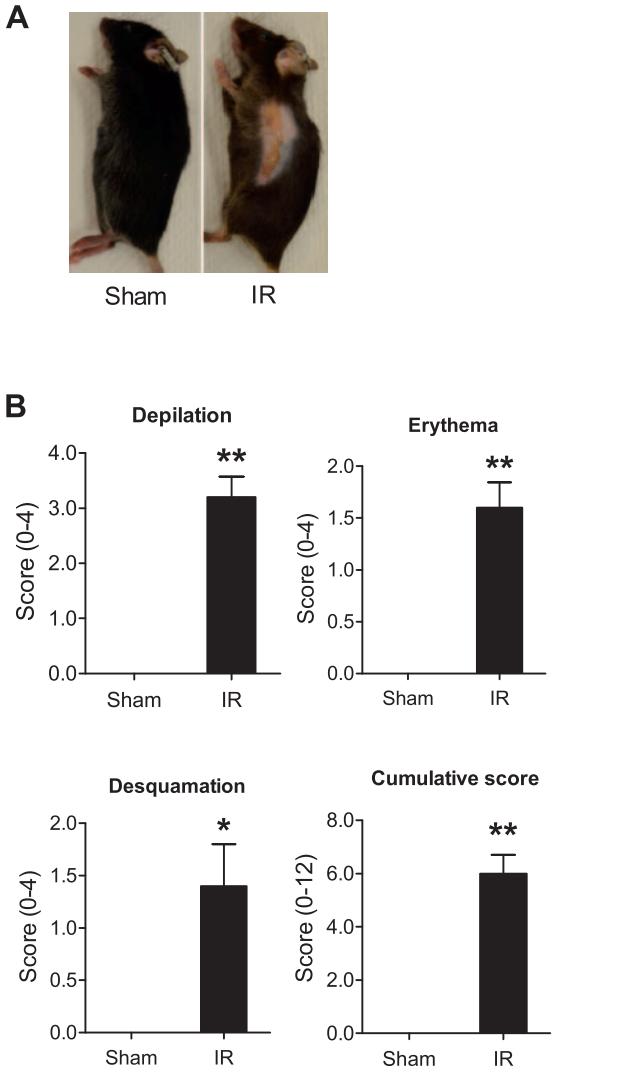


FIG. 1. Manifestation of murine radiation-induced dermatitis. The left-flank skin of C57BL/6J mice was exposed to a single X-ray dose of 25 Gy (IR) or sham irradiated as a control (sham). Panel A: Representative photo images of sham-irradiated and X-ray irradiated skin at 4 weeks postirradiation. Panel B: The severity of depilation, erythema and desquamation were scored on a scale from 0 to 4. In addition, the cumulative score (depilation plus erythema plus desquamation) is depicted. Data are shown as mean ± SEM. n = 5. **$P < 0.01$.

IL17-Expressing γδ T Cells are Elevated in Dermatitis Skin

IL17 is a pro-inflammatory cytokine which is extensively implicated in inflammation, infection and autoimmunity (17). Particularly in murine psoriasis models, the crucial roles of IL17 family members including IL17, IL17F and IL22 have been best characterized and have been shown to be produced by skin γδ T cells (13–15). In our radiation-induced dermatitis model, skin-infiltrating lymphocytes were isolated, re-stimulated *ex vivo* with PMA/ionomycin and analyzed by flow cytometry for IL17 expression, combined with lymphocyte surface marker TCRδ. Interestingly, a substantial elevation of IL17-expressing cells was detected in the irradiated skin, majority of which was contributed by $TCR\gamma\delta^+$ cells (Fig. 3A). Consistently, a significant increase of IL17 protein level was detected from the cultured medium of isolated skin cells when the cells were re-stimulated *ex vivo* with anti-CD3 and anti-CD28 antibodies for 4 days (Fig. 3B). In addition, the IL17-producing cells were also found to co-express IL17 lineage transcription factor RORC (Fig. 3C). In adult mice, the major resident γδ T cells in murine skin are called dendritic epidermal T cells (DETCs) characterized by Vγ5 expression (Heilig & Tonegawa nomenclature) (*18*). However, it has been shown that murine dermis contain another type of resident γδ T cells which express Vγ4, which are highly inducible for IL17 expression (*13*). Analysis of γδ T cells from irradiated skin for Vγ4 and Vγ5 lineage indicated exclusive expression of IL17 by Vγ4 but not Vγ5 (Fig. 3D).

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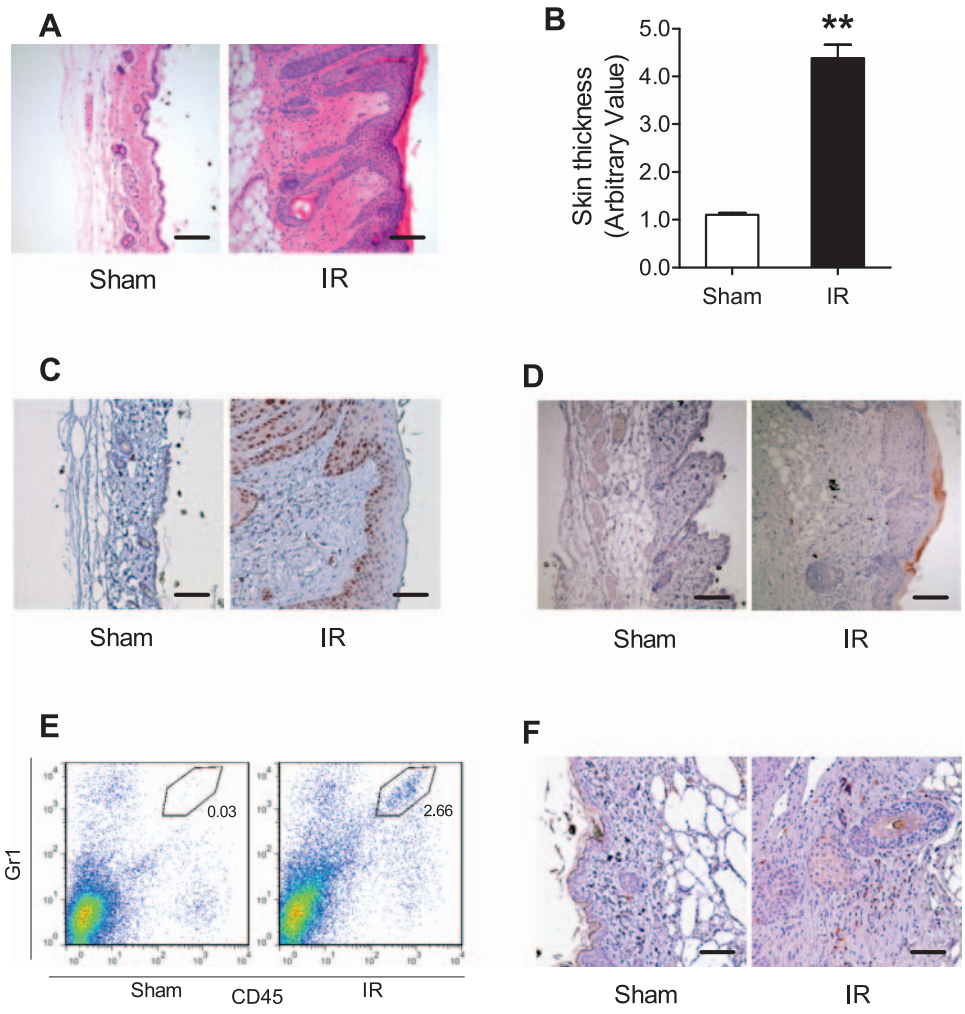


FIG. 2. Radiation induces skin hyperplasia and inflammation. The left-flank skin of C57BL/6J mice was exposed to a single X-ray dose of 25 Gy (IR) or sham irradiated as a control (sham). Panel A: Representative images of H&E staining. Panel B: Skin thickness was measured on the left flank. Immunohistochemistry staining of proliferation marker Ki67 (panel C) and apoptosis marker cleaved caspase-3 (panel D). Panel E: Single cell suspension was prepared from mouse dermis and immunostained with neutrophil markers $CD45^+$ and $Gr1^+$. Analysis of the percentage of $CD45^+Gr1^+$ neutrophils was performed by flow cytometry. Panel F: Immunohistochemistry staining of neutrophil marker $Gr1^+$. Data are shown as mean ± SEM. n = 5. $^{**}P < 0.01$.

Two distinct γδ T-cell populations were also revealed by staining the isolated lymphocytes with anti-CD3 and anti-pan-TCRδ antibody, one with $CD3^{hi}TCR\gamma\delta^{hi}$ and the other with $CD3^{med}TCR\gamma\delta^{med}$, which may correspond to Vγ5 and Vγ4 cells, respectively. Upon radiation dermatitis induction, the $CD3^{med}TCR\delta^{med}$ population produced IL17 whereas none of the $CD3^{hi}TCR\delta^{hi}$ cells expressed IL17, further confirmed the Vγ4 and Vγ5 identities of these two γδ T-cell populations (Supplementary Fig. S2; http://dx.doi.org/10.1667/RR0007CC.1.S1).

Cytokine Microenvironment in the Irradiated Skin Favors γδ T Cell Activation and IL17-Mediated Inflammation

Cytokines IL1β and IL23 have been established to promote IL17-expressing γδ T cell activation in a variety of inflammatory diseases (*11, 19*). We next sought to determine if these cytokine expressions were elevated in irradiated skin, which might be conducive to cutaneous IL17-expressing γδ T cell activation. By using immunohistochemistry staining, the protein levels of IL23 and IL1β, together with acute pro-inflammatory cytokine IL6 and IL17 superfamily member IL22, were confirmed to be elevated in irradiated skin (Fig. 4).

Radiation-Induced Dermatitis is Attenuated in γδ T Cell Knockout Mice

Having established that the increase of IL17-expressing $V\gamma4^+$ γδ T cells well correlated with manifestation of radiation-induced dermatitis, we tried to elucidate whether these cells mediated dermatitis pathogenesis or their accumulation was just a secondary effect to the radiation response. We obtained a γδ T-cell-specific knockout mice ($Tcrd^{-/-}$) which are deficient in γδ T cells but have intact αβ T cells. The skin of the left flank of WT and $Tcrd^{-/-}$ mice

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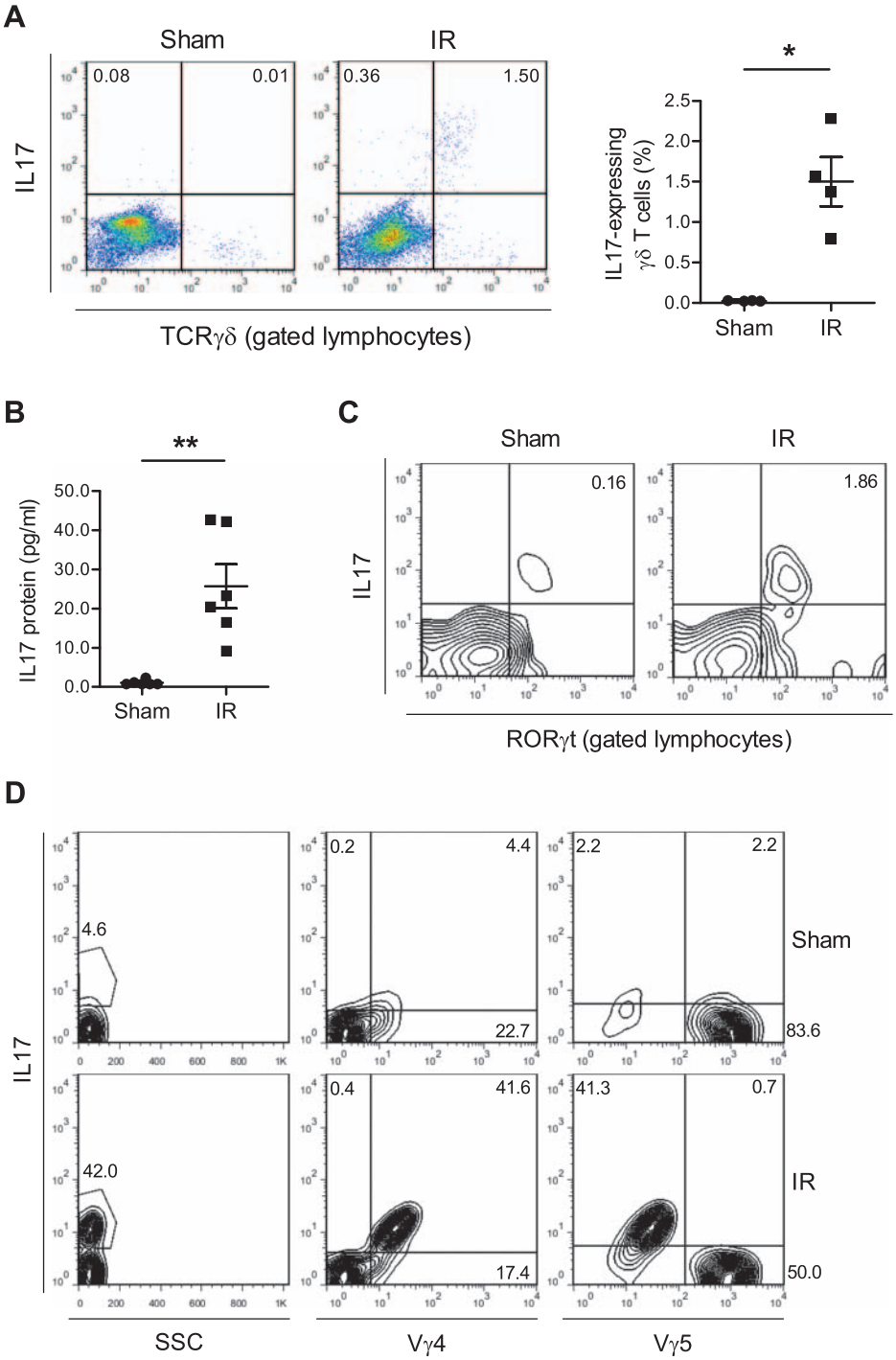


FIG. 3. Elevation of IL17-expressing Vγ4$^+$ γδ T cells in dermatitis skin. Mouse skin was enzymatically digested to isolate single cell suspension. Panel A: IL17-expressing and IL17-expressing γδ T cell frequency from digested skin cell suspension was analyzed by flow cytometry after re-stimulation with PMA/Ionomycin for 5 h. Panel B: Skin single cell suspension was re-stimulated with anti-CD3 and anti-CD28 antibodies for 3 days, and IL17 protein level in the culture media was measured by ELISA. Panel C: Intracellular cytokine staining of IL17 and transcription factor Rorc was analyzed by flow cytometry after re-stimulation with PMA/Ionomycin for 5 h. Panel D: γδ T cell subsets Vγ4$^+$ and Vγ5$^+$ were examined for IL17 expression by flow cytometry. Data are shown as mean ± SEM. n = 5. *$P < 0.05$.

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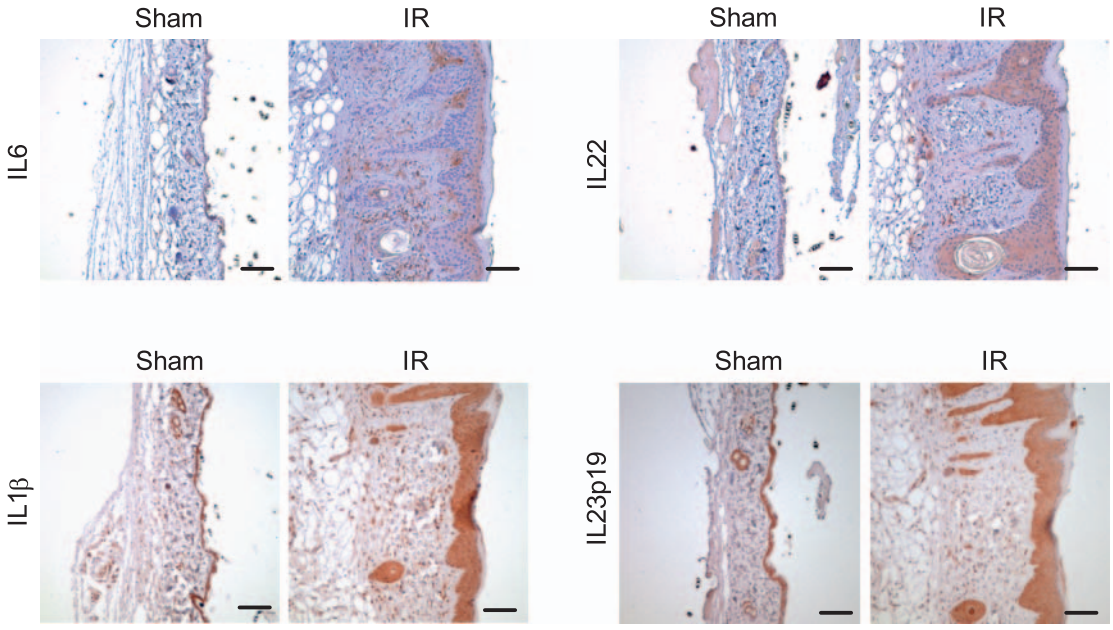


FIG. 4. Elevation of cytokines crucial for IL17-expressing γδ T cell activation. Representative images of immunohistochemistry staining of IL22, IL6, IL23p19 and IL1β in sham- or X-ray-irradiated mouse torso skin.

were irradiated with a 25 Gy single fraction. At 4 weeks postirradiation, the irradiated skin of wild-type mice showed significant dermatitis including depilation, erythema and desquamation, which was partially alleviated in the irradiated *Tcrd*$^{-/-}$ mice (Fig. 5A) with a significantly reduced depilation and desquamation resulting in a reduced cumulative skin injury score (Fig. 5C). In addition, acanthosis with skin thickness (Fig. 5B and D) and keratincyte hyperproliferation (Fig. 5E) observed in irradiated wild-type skin was significantly mitigated in irradiated *Tcrd*$^{-/-}$ mice. Furthermore, the absence of γδ T cells markedly reduced Gr1^{+} neutrophil infiltration into the skin (Fig. 5F). The irradiated *Tcrd*$^{-/-}$ mice, which are deficit in both Vγ4^{+} and Vγ5^{+} DETCs, show a substantial depletion of IL17-expressing cells suggesting that these cells are the main source of IL17 in irradiated skin (Fig. 5G). The residual IL17 expression may be contributed by the innate lymphoid cells (*10*).

Keratinocytes Play Active Roles in Radiation-Induced Dermatitis

Besides its major function as the physical barrier of the skin to foreign invaders, skin keratinocytes modulate skin inflammatory and remodeling processes by secreting a large number of mediators upon external stimuli (*20*). By irradiating normal human epidermal keratinocytes (NHEKs) *in vitro*, we found upregulation of the mRNA levels of epithelium-derived pro-inflammatory cytokines such as TNF-α, IL6, IL1β and IL17C (Fig. 6A). IL17C is a newly characterized IL17 family member produced mainly by epithelial cells, including epidermal keratinocytes (*21, 22*), bronchial (*23*)and intestinal epithelial cells (*24*), which helps promote inflammation. By treating the keratinocytes with human recombinant IL17C protein, we detected increased mRNA levels of pro-inflammatory cytokine TNF-α, IL1β, IL6, antimicrobial gene S100A8, γδ T-cell chemokine ligand CCL20 and interestingly, IL17C itself (Fig. 6B). Skin disorders such as psoriasis and rheumatoid arthritis are manifested with keratinocyte hyperplasia, where IL17 and IL22 are shown to play important roles in inducing proliferation of keratinocytes (*25, 26*). Notably, keratinocyte hyperproliferation has also been observed in our murine radiation dermatitis model. By using MTT metabolic assay, we further confirmed there is increased keratinocyte cell viability in response to either IL17 or IL22 or synergistically IL17 and IL22 combination.

DISCUSSION

Radiation-induced skin injury is not only a side effect for cancer patients receiving radiation therapy but as the largest organ exposed to the environment, it is one of the subsyndromes of Acute Radiation Syndrome. However, there is no effective treatment to prevent or mitigate this injury as the crucial inflammatory pathways are still elusive. Our results demonstrate that ionizing radiation-treated murine skin closely resembles human radiation dermatitis symptoms ranging from mild depilation to severe desquamation, epidermal damage and hyperplasia, as well as inflammatory cell infiltration. Mechanistically, a significant increase in cutaneous IL17-expressing γδ T cells that express Vγ4 correlates with radiation dermatitis *in vivo*. In addition, cytokines for IL17-expressing γδ T cell activation, IL1β and IL23, are markedly upregulated in the skin of irradiated mice. Significantly, depletion of γδ T cells in

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Tcrd$^{-/-}$ mice substantially attenuates radiation dermatitis, indicating a pathogenic function of these cells in radiation-induced skin injury. *In vitro* culture of human keratinocytes further corroborates an active role of epidermal cells in initiating inflammation at early time and as an effector of skin structural alteration at a later phase. In summary, our *in vivo* murine genetic model and *in vitro* cellular studies have provided direct evidence that the γδ T-cell-mediated innate immune response is an essential contributor of radiation dermatitis.

Compared with the more extensively studied mechanisms of radiation-induced lung injury, particularly pulmonary fibrosis where TGF-β signaling pathway plays a critical role (*27*), very little is known about the crucial mechanisms of radiation-induced skin injury. Ionizing radiation triggers the activation of skin resident cells between epidermis and dermis including keratinocytes, fibroblasts and endothelial cells to produce a large amount of acute phase pro-inflammatory cytokines such as IL6, IL1β and TNF-α (*28*). Recruitment of various immune cells including Langerhans cells, dendritic cells, mast cells, macrophages, lymphocytes and neutrophils, which are associated with elevated levels of cytokines, chemokines and adhesion molecules such as IL1α, IL8, CCL4, CCL2, CXCL10, ICAM-1 and VCAM-1 has been observed in the skin of irradiated mice (*29, 30*). In a murine radiation-induced lung injury model, Th17-associated cytokines like IL17 and IL23, and lymphocytes $CD4^+CD25^+IL17^+$ and $CD4^+CD25^+Foxp3^+$, to the best of our knowledge, for the first time, have been described in tissue injury after irradiation (*31*). We have also demonstrated an essential role of IL17-expressing γδ T cells in a murine radiation pneumonitis model (data not shown). In skin disorder such as psoriasis, IL17-expressing γδ T cells and $Rorc^+$ innate lymphocytes have been shown to be necessary and sufficient for disease progression (*11, 13*). Our analysis of T-cell subtypes expressing IL17 has identified a substantially increased population of $IL17^+$ cells which are dominated by γδ T cells that express Vγ4 in radiation dermatitis. In contrast, none of the $V\gamma5^+$ resident dendritic epidermal cells (DETCs) produced IL17, consistent with a previous report (*13*). The pathogenic role of γδ T cells has further been demonstrated in our *Tcrd*$^{-/-}$ mice, which exhibit decreased levels of IL17 expression upon irradiation, with subsequent decreased signs of dermatitis including less epidermal hyperplasia and reduced neutrophil infiltration. These results indicate radiation dermatitis is an IL17-producing innate lymphocyte Vγ4-expressing γδ cell-mediated inflammatory response. In our irradiated *Tcrd*$^{-/-}$ mice, there is residual expression of IL17. Though its etiology is unclear, innate lymphoid cells such as NK cells has been shown to express IL17 in other injury models (*32*). It would be interesting to examine how the skin of IL17 knockout mice respond to radiation.

Recent studies have revealed that IL1β and IL23 are crucial cytokines to regulate γδ T cells and γδ T cell derived IL17 production *in vivo* and *in vitro* (*19, 33*). IL23 has been shown to predominantly stimulate dermal γδ T cell expansion and IL17 production with the help of IL1β, and endogenous IL1β is required in this process as skin γδ T cells isolated from IL1R1$^{-/-}$ mice or in the presence of IL1β neutralizing antibody could not secrete IL17 upon stimulation with IL23 alone (*11*). In addition, IL1β pathway has been shown to play a significant role in the development of radiation fibrosis (*34*). The mice that are deficit in either IL1 or the IL1 receptor have been reported to develop less inflammation and less severe pathological changes in their skin (*35*). Likewise, in irradiated skin, we detected elevated protein levels of IL1β and IL23 by immunohistochemistry. Collectively, these results suggest that elevated IL1β and IL23 in the skin, probably secreted by resident and innate immune cells such as keratinocytes, Langerhans cells, dendritic cells and macrophages after irradiation, could activate resident γδ T cells and induce IL17 expression, leading to a pathogenic inflammatory response.

IL17C is a functionally distinct member of the IL17 family that is exclusively produced by epithelial cells and regulate innate defense. Increased expression of IL17C has been observed from inflamed bronchial epithelia, colonic epithelia and psoriatic keratinocytes IL-17C is a mediator of respiratory epithelial innate immune response (*21, 24*). Mice genetically engineered to overexpress IL17C in keratinocytes develop obvious psoriasiform dermatitis (*21*). We have detected elevated mRNA level of IL17C as well as other inflammatory mediators in NHEKs after irradiation. Additionally, augmented mRNAs of pro-inflammatory mediator TNF-α, IL1β, IL6, CCL20 and interestingly IL17C itself have been identified from NHEKs when these cells were treated with human recombinant IL17C protein. These *in vitro* findings indicate a potential pathogenic function of IL17C in initiating skin inflammation after irradiation in autocrine and paracrine manners. An analysis of the function of IL17C in radiation dermatitis *in vivo* by employing IL17C transgenic mice might warrant further study in the future.

Recent studies have shown that instead of conventional αβ Th17 cells, innate immune cells like γδ and invariant NK cells are dominant source of IL17 and IL22 expression upon PAMPs and DAMPs activation (*32*). IL17 receptors are universally expressed and IL17 pathway is essential for neutrophil recruitment through adaptor Act1 and transcription factor NF-κB (36). In contrast to IL17, IL22 receptors are restricted to barrier surface but not immune cells, indicating this cytokine might be important for tissue repair (*37*). Keratinocyte dysregulation and hyperproliferation are hallmarks of skin disorders like psoriasis, RA as well as radiation dermatitis as demonstrated in our study. We, in this study, and others have shown that NHEK cells propagate upon IL17 or IL22 and more potently in combination treatment *in vitro* (*25*). These data suggest that the keratinocyte hyperproliferation in radiation dermatitis may be the result of induction by IL17 and IL22, derived from γδ T cells.

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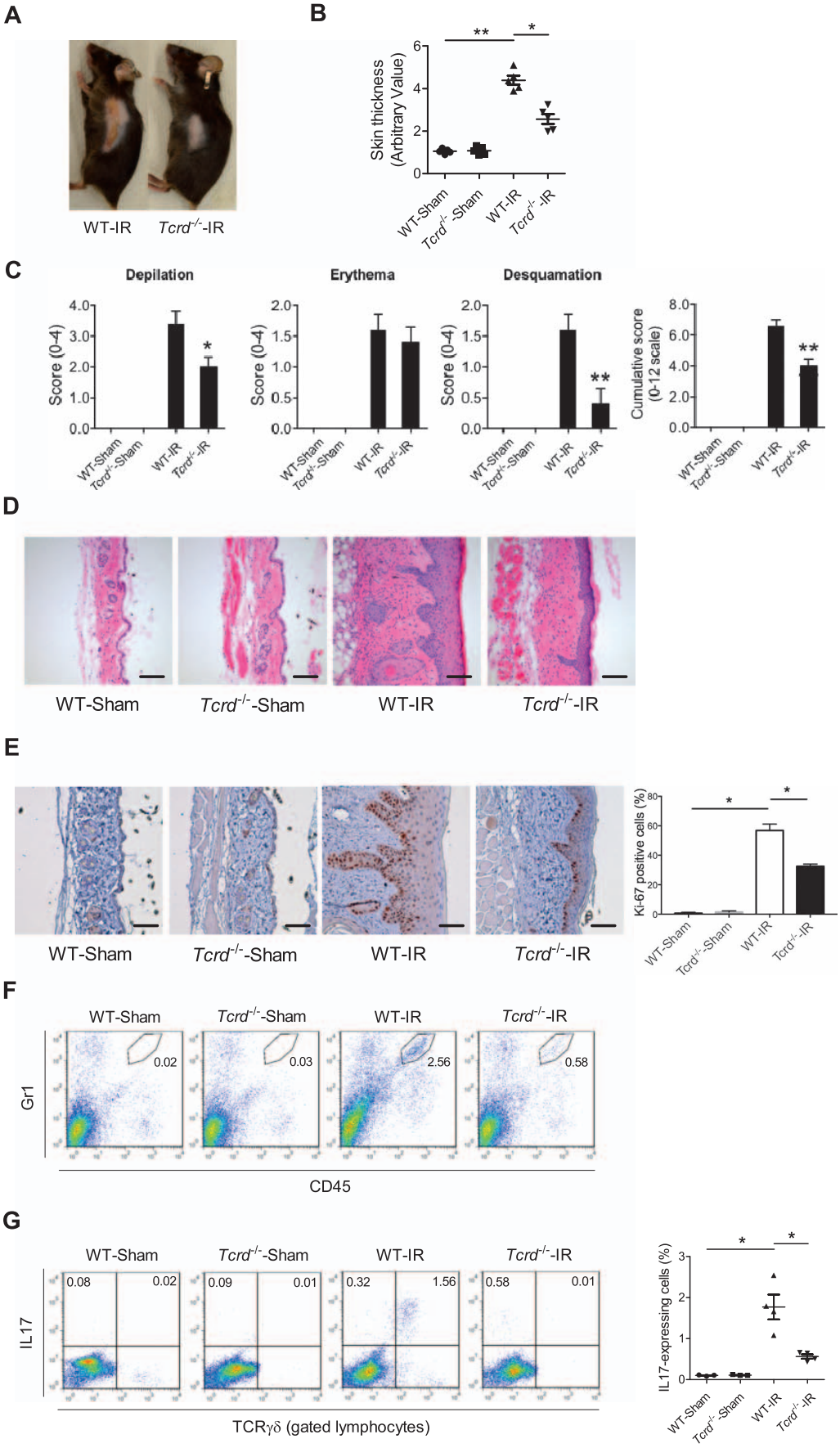


Fig. 5 legend is found on the next page

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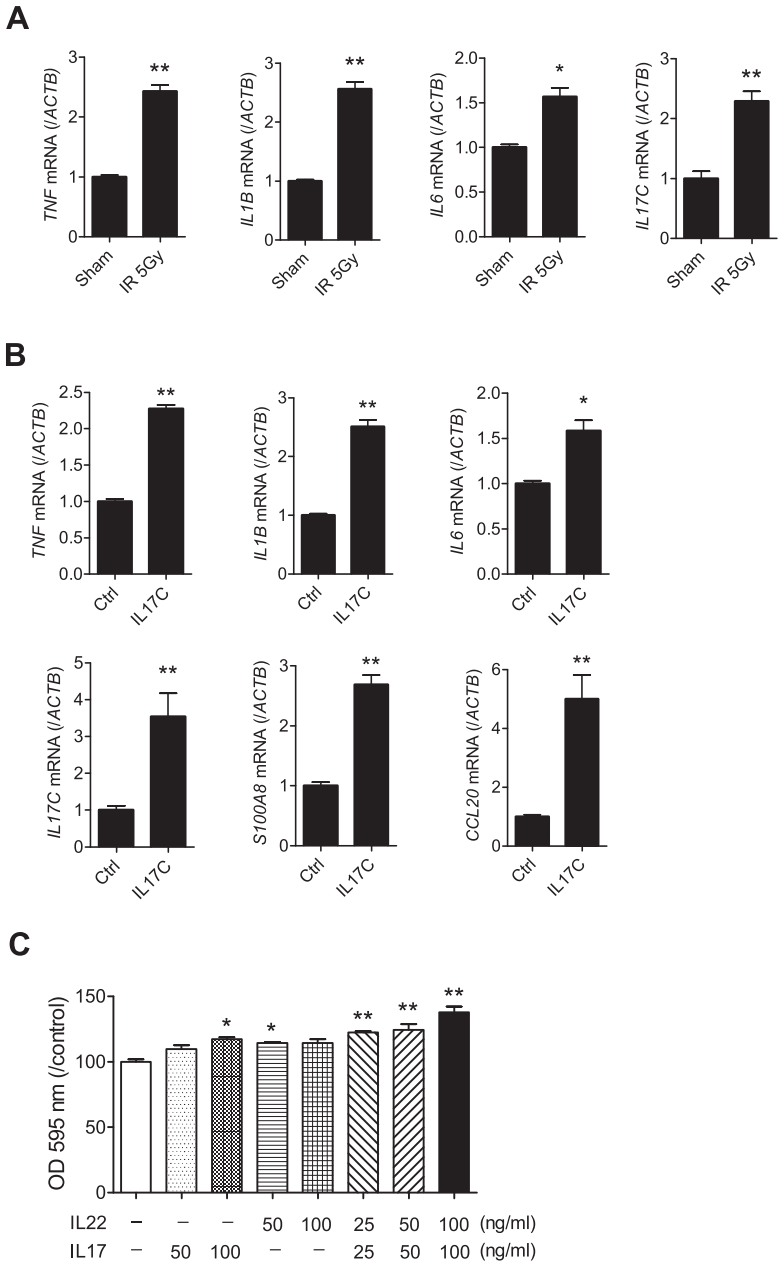


FIG. 6. Keratinocytes express pro-inflammatory mediators after irradiation and hyperproliferate in response to IL17 and/or IL22 *in vitro*. Human normal epithelial keratinocytes (NHEK) were irradiated with 5 Gy of X rays (panel A) or treated with human recombinant IL17C (100 ng/ml) (panel B). Epithelia-derived cytokine S100A4, IL6, IL1β, TNF-α and IL17C mRNAs were analyzed by qPCR. NHEK cells/well were seeded in 96-well plates for 24 h, followed by indicated doses of IL17 and/or IL22 treatments for 5 days. Cell proliferation was measured by MTT assay. Panel C: Relative proliferation rate of NHEK cells in response to IL17 and/or IL22 over untreated control is shown. Data are shown as mean ± SEM. n = 5. $*P < 0.05$, $**P < 0.01$.

←

FIG. 5. Radiation-induced dermatitis is attenuated in γδ T-cell knockout mice. The left-flank skin of WT and γδ T-cell-deficient *Tcrd*$^{-/-}$ C57BL/6J mice were exposed to 25 Gy X-ray or sham irradiated as a control. Panel A: Representative photo images irradiated flank skin of WT and *Tcrd*$^{-/-}$ mice. Skin thickness (panel B) and cumulative skin injury score including depilation, erythema and desquamation scores (panel C) are described as before. Representative images of H&E staining (panel D) and immunohistochemistry staining of proliferation marker Ki67 (panel E) are shown. Panel F: Analysis of CD45^{+}Gr-1^{+} neutrophils by flow cytometry. Panel G: IL17-expressing γδ T cells and IL17-expressing lymphoid cells were analyzed by flow cytometry after re-stimulation with PMA/Ionomycin. Data are shown as mean ± SEM. n = 4 or 5. $*P < 0.05$, $**P < 0.01$.

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Taken together, our results emphasize the importance of cutaneous IL17-expressing γδ T cells in mediating the radiation-induced inflammatory response and suggest that the innate immune response pathway as a potential target for therapeutic intervention in radiation-induced skin injury.

SUPPLEMENTARY MATERIALS

Fig. S1., Fig. S2 and Table S1 are available online.

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