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Early Stress-Response Gene REDD1 Controls Oxazolone-Induced Allergic Contact Dermatitis

Salida Mirzoeva,* Yuchen Yang,* Anna Klopot,[†] Irina Budunova,[†] and Melissa A. Brown*

REDD1 is an energy sensor and stress-induced mTOR inhibitor. Recently, its novel role in linking metabolism and inflammation/immune responses has emerged. In this study, we assessed the role of REDD1 in murine oxazolone-induced allergic contact dermatitis (ACD), a T cell–dependent model with features of human ACD. A variety of immune indices, including edema, cellular infiltration, inflammatory gene expression, and glucocorticoid response, were compared in *Redd1* knockout (KO) and isogenic (C57BL/6 × 129)F1 wild-type mice after sensitization and subsequent ear challenge with oxazolone. Despite relatively normal thymic profiles and similar T cell populations in the lymph nodes of naive *Redd1* KO mice, early T cell expansion and cytokine production were profoundly impaired after sensitization. Surprisingly, higher steady-state populations of CD4⁺ and CD8⁺ T cells, as well as macrophages (CD45⁺/Ly-6G[−]/CD11b⁺), dendritic cells (CD45⁺/Ly-6G[−]/CD11c⁺), neutrophils (CD45⁺/Ly-6G⁺/CD11b⁺), and innate lymphoid cells (CD45⁺/Lineage[−]/IL-7Ra⁺/ST2⁺/c-Kit⁺), were observed in the ears of naive *Redd1* KO mice. Upon challenge, ear edema, T cell, macrophage, neutrophil, and dendritic cell infiltration into the ear was significantly reduced in *Redd1* KO animals. Accordingly, we observed significantly lower induction of IFN-γ, IL-4, and other cytokines as well as proinflammatory factors, including TSLP, IL-33, IL-1β, IL-6, and TNF-α, in challenged ears of *Redd1* KO mice. The response to glucocorticoid treatment was also diminished. Taken together, these data establish REDD1 as an essential immune modulator that influences both the initiation of ACD disease, by driving naive T cell activation, and the effector phase, by promoting immune cell trafficking in T cell–mediated skin inflammation. *The Journal of Immunology*, 2021, 207: 1–8.

Regulated in development and DNA damage response 1 (REDD1), also known as DNA damage–inducible transcript 4 (DDIT4), is an early response gene that regulates the response to a variety of cellular stresses, including deficits of cellular energy/nutrients, hypoxia, DNA damage, endoplasmic reticulum stress, and viral infection (1). The best-known REDD1 function is its ability to negatively regulate mammalian target of rapamycin 1 (mTORC1), a signaling pathway that integrates cell growth and survival. It acts by stabilizing tuberous sclerosis complex 1 (TSC1)–TSC2 inhibitory complex (2, 3) or, as shown more recently, via control of AktThr³⁰⁸ dephosphorylation (4). Yet some REDD1 effects—for example, modulation of NF-κB and hypoxia-inducible factor-1α (HIF-1α)—appear to be mTOR independent (5).

Most early studies focused on the role of REDD1 in the control of cell growth, apoptosis, and autophagy, as well as on cancer development (1, 6). However, more recent data suggest that REDD1 is also implicated in the regulation of immune responses, suggesting a contribution to the development of inflammatory diseases. Indeed, REDD1 is overexpressed in immune cells of patients with ulcerative colitis and systemic lupus erythematosus and in the lungs of patients with emphysema (7–10). In animal studies, REDD1 was shown to be induced in immune and endothelial cells after exposure to

inflammatory stimuli, such as LPS or cigarette smoke condensate (11–13). Moreover, *Redd1* knockout animals appear to be more resistant to excessive and damaging inflammatory responses in these settings. Skin inflammatory diseases are among the most common diseases in the world. Yet there have been no systematic analyses of the effect of REDD1 on specific immune cell responses in a skin disease model.

Allergic contact dermatitis (ACD) is a common dermatological condition that affects 15–20% of the world’s population and is characterized by local edema, rash, and itch (14). Two discrete phases are required for disease manifestations: sensitization and elicitation. Sensitization occurs upon initial skin exposure to small chemical compounds called haptens. During this phase, APCs, including resident dermal dendritic cells (DCs) as well as epidermal Langerhans cells, take up hapten-modified proteins and migrate to the draining lymph nodes, where they present these complexes and activate hapten-peptide–specific naive T cells to proliferate and undergo differentiation to effector T cells. Upon challenge, T cells migrate to the affected tissues and are reactivated by local exposure to hapten complexes, resulting in the generation of memory T cells, cytokine and chemokine production, and recruitment of additional inflammatory cells to the sites of allergen contact (14, 15). Although ACD is

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Abbreviations used in this article: ACD, allergic contact dermatitis; DC, dendritic cell; DN, double-negative; DP, double-positive; FA, fluocinonide acetone; FMO, fluorescence minus one; GR, glucocorticoid receptor; ILC2, type 2 innate lymphoid cell; Oxa, oxazolone; qPCR, quantitative real-time PCR; REDD1, regulated in development and DNA damage response 1; *Redd1* KO, *Redd1*-deficient; ROS, reactive oxygen species; WT, wild-type.

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considered a T cell–dependent disease, keratinocytes, DCs, macrophages, mast cells, and neutrophils also play essential modifying roles in both the sensitization and elicitation phases (14).

Using *Redd1*-deficient (*Redd1* KO) mice and their isogenic wild-type (WT) counterparts, we assessed the role of REDD1 in murine contact dermatitis, a T cell–mediated disease model with features of ACD in human skin, induced by the topical application of the hapten oxalozone (Oxa) (14). Despite relatively normal indices of thymic T cell development and naive T cell residence in the draining lymph nodes of *Redd1* KO mice, these mice have profound deficits in early T cell activation after sensitization. REDD1 also selectively limits steady-state populations of many resident immune cell populations in the ear skin. We also show that *Redd1* mRNA is induced in WT ear skin within two hours of challenge and is required for normal inflammatory cell infiltration and the expression of numerous inflammatory genes upon Oxa challenge. These data demonstrate the widespread influence of REDD1 in controlling T cell and innate immune cell function in ACD.

Materials and Methods

Reagents

Oxa (4-ethoxymethylene-2-phenyl-2-oxazolin-5-1), fluocinolone acetone (FA), PMA, and ionomycin were purchased from Sigma-Aldrich (St. Louis, MO); brefeldin A was purchased from Thermo Fisher Scientific (Waltham, MA). Oxa was prepared as 1–2% solution in 100% ethanol immediately prior to use. FA was dissolved in acetone and kept at -20°C until use.

Ethics statement

All animal housing and treatments were performed in accordance with the animal protocols approved by the Northwestern University Animal Care and Use Committee, in strict adherence to the National Institutes of Health guidelines.

Animals

All experiments were performed on adult (7–8 wk old) female mice. *Redd1* KO mice were raised in-house (16). Isogenic F1 C57BL/6 $\times$ 129 mice were purchased from Taconic Farms (Germantown, NY) and acclimated in the same housing facility for at least 4 d before experiments.

Oxazolone sensitization and challenge

Animals were sensitized by a topical application of 100 μl of vehicle (ethanol) or 2% Oxa in ethanol to the shaved abdomen. Six days later, 10 μl of 1% Oxa was applied to both sides of right ear. Left ears were treated similarly with vehicle (see Fig. 1). For time course experiments, mice were challenged and sacrificed 0.5–24 h later.

Glucocorticoid FA effect on oxazolone challenge

WT and *Redd1* KO mice were sensitized with Oxa as described above. Six days later, 10 μl of FA solution in acetone or solvent control was applied to both sides of right ear 1 h prior to Oxa challenge treatment.

Morphometric analysis

Mice were sacrificed 48 h after Oxa challenge; ears were removed and fixed in 10% neutral-buffered formalin. H&E staining was done in formalin-fixed, paraffin-embedded samples. Ear thickness was determined from the top of the epidermal layer to the cartilage. At least 10 individual fields of view/slide in four individual ear samples (40 images per treatment group) were analyzed. H&E staining was performed at Northwestern Mouse Histology and Phenotyping Core.

Analysis of ear-swelling responses by measurement of ear weights

Mouse ears were challenged with 1% Oxa as described above. Animals were sacrificed 24, 48, and 72 h later, ears were harvested, and the weight of 5-mm ear punch, a readout of inflammation, was measured. Ears from mice that were sensitized but not challenged were used as controls.

Cell analysis by flow cytometry

Ears were harvested for flow cytometry 48 h after Oxa challenge. Axillary and inguinal lymph nodes were harvested 72 h after sensitization. Thymuses

were harvested from naive mice. Tissue was homogenized and treated with collagenase IV and DNase to prepare single-cell suspensions. Before staining with indicated Abs (listed in Supplemental Table I), samples were treated with Fc block (BioLegend, San Diego, CA) in 100 μl of FACS buffer for 30 min in the dark at 4°C . For intracellular staining, cells were stimulated with plate-bound CD3 Abs for 5 h, and 3 $\mu\text{g}/\text{ml}$ brefeldin A was added for the final 4 h. Cells were assayed for cytokine production using the Fixation & Permeabilization Kit (Thermo Fisher Scientific). Samples were processed on a BD FACSCanto II (BD Biosciences). Gating strategy is included in relevant figures as well as in Supplemental Fig. 1. Gates were determined based on fluorescence minus one (FMO) control staining samples. Abs used are shown in Supplemental Table I.

Real-time PCR and quantitative real-time PCR

Mouse ears were snap frozen in liquid N_2 and kept at -80°C . Frozen ears were ground in liquid N_2 using a mortar and pestle. RNA was extracted from ear tissues using E.Z.N.A. Total RNA Kit 1 (Omega Bio-tek, Norcross, GA). Random hexamers, Moloney murine leukemia virus reverse transcriptase (Thermo Fisher Scientific), and 1 μg of total RNA were used for reverse transcription. Gene-specific primers were designed using the National Center for Biotechnology Information Primer-BLAST tool (<http://www.ncbi.nlm.nih.gov/tools/primer-blast/>), and their sequences are presented in Supplemental Table II. Quantitative real-time PCR (qPCR) was performed with SsoAdvanced Universal SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA). Fold change was calculated as $2^{\Delta\Delta\text{Ct}}$, and the values were normalized to the housekeeping *Rpl27* transcript. All samples were tested in triplicate in two to three independent experiments.

Statistical analyses

Mean and SD were calculated using Microsoft Excel software. Statistical analyses of the data were performed by nonparametric unpaired two-tailed Student *t* test using GraphPad Prism software (version 7.03).

Results

Edema development in response to Oxa challenge is reduced in *Redd1* KO mice

To assess the effects of REDD1 on hypersensitive responses in the ear, *Redd1* KO and isogenic WT mice were sensitized with Oxa by topical application of 100 μl of 2% Oxa to shaved abdominal skin. Six days later, sensitized mice were treated with vehicle alone on the left ear or challenged with Oxa on the right ear (the experimental design is presented in Fig. 1A). We first evaluated the expression of *Redd1*, an immediate/early response gene (1), in mouse ears. As shown in Fig. 1B, Oxa treatment led to significant increases in *Redd1* expression 2 h postchallenge. The influence of REDD1 in ear inflammation induced by Oxa was assessed using morphometric analysis of ear thickness as a readout of edema/inflammation 48 h after Oxa challenge. Ear-swelling responses were observed in both WT and *Redd1* KO mice. However, the Oxa-induced increase in ear thickness was moderately, albeit significantly, reduced in *Redd1* KO mice (Fig. 1C, 1D).

T cell development is not impaired in *Redd1* KO mice

As discussed, the development of ACD relies on efficient hapten sensitization of T cells (14). Although REDD1 is expressed in activated T cells (17), little is known regarding its role in T cell development and function. Thus, we first assessed the consequences of REDD1 deficiencies on early T cell differentiation by analyzing T cell populations in the thymus. As shown in Fig. 2A and 2B, proportions of CD4 and CD8 single-positive cells as well as CD4 CD8 double-positive (DP) and double-negative (DN) cells in the thymus are similar in both groups. The DN population, defined in part by distinct patterns of CD25 and CD44 expression, make up the earliest T cell precursors that ultimately give rise to mature CD4⁺ and CD8⁺ T cells. DN1 cells are considered pluripotent, and in addition to T cells, they can generate B cells, NK cells, NKT cells, DCs, and even myeloid cells. DN2 cell intrathymic differentiation potential is limited to T cells, type 2 innate lymphoid cells (ILC2s), and DCs,

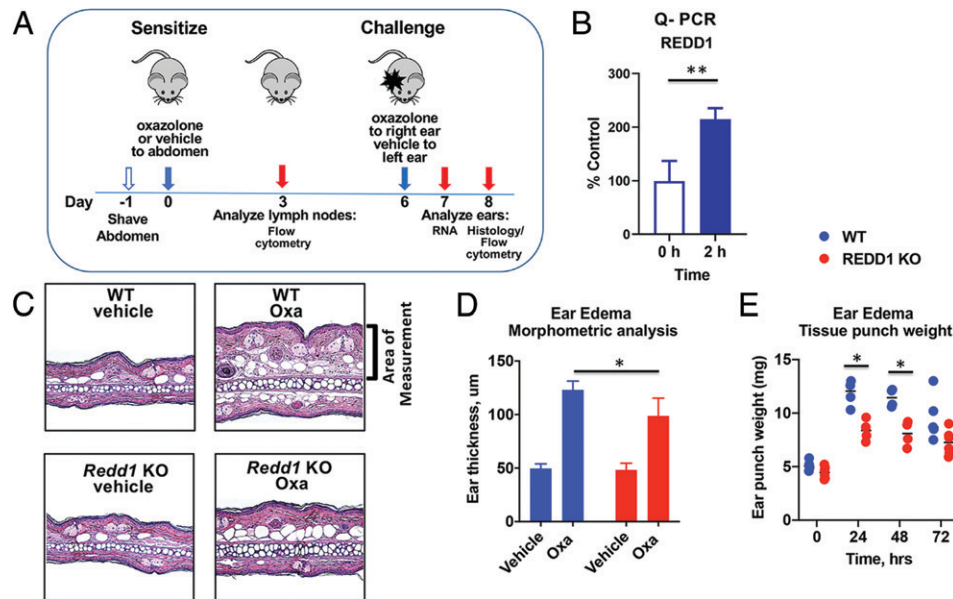


FIGURE 1. *Redd1* is induced in contact hypersensitivity responses and promotes edema. **(A)** Eight-week-old WT and *Redd1* KO female mice were sensitized by topical application of Oxa (100 μ l of 2% Oxa in ethanol) to shaved abdominal skin. Six days later, mice were challenged by Oxa application (10 μ l of 1% Oxa in ethanol) to both sides of the right ear. Left ears received ethanol vehicle alone. Immune indices in the draining lymph nodes were assessed at 3 d postsensitization and in the right and left ears at 48 h postchallenge. **(B)** qPCR analysis of *Redd1* expression in the ears of WT mice 2 h postchallenge. Data are expressed as percentage of vehicle control and presented as mean $\pm$ SD, $n = 3$ mice per group. $**p < 0.01$ by two-tailed unpaired *t* test, 0 versus 2 h after Oxa challenge. **(C)** Representative images of H&E staining of vehicle (control) and Oxa-challenged (Oxa) ear sections at 48 h after challenge. The bracket indicates the measured part of the ear. **(D)** Morphometric analysis of ear thickness, a measure of inflammation, was recorded for each section. Data are represented as mean $\pm$ SD of at least 10 measurements per ear, $n = 4$ individual samples per group. **(E)** Inflammation measured by increase in ear weight at 24, 48, and 72 h postchallenge relative to ears of sensitized only (0-h time point mice). Data represent individual ear measurements of four animals per group. $*p < 0.05$ by two-tailed unpaired *t* test, *Redd1* KO (red) versus WT (blue).

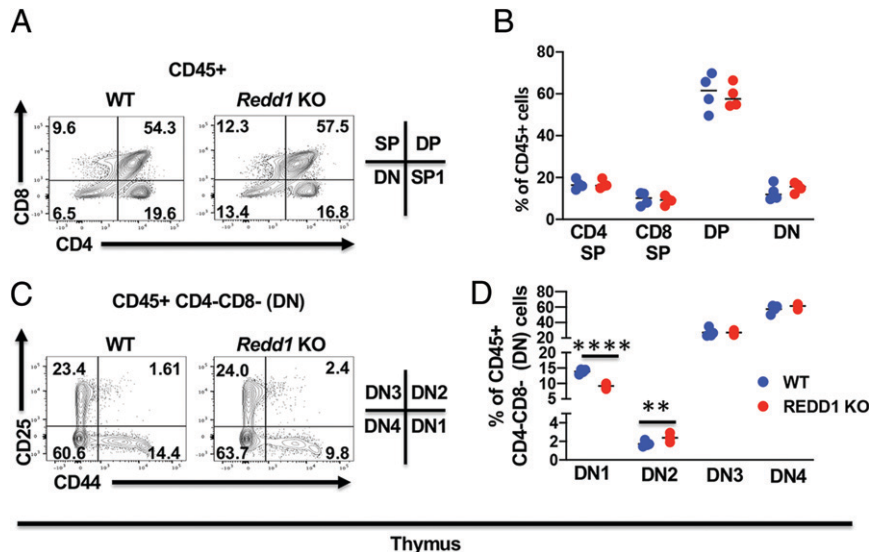
whereas DN3 and DN4 cells are fully committed T cell precursors (18–20). In *Redd1* KO mice, there is a significant reduction in thymic DN1 cells and a significant, albeit slight, increase in DN2 cells (Fig. 2C, 2D). However, DN3 and DN4 populations show no differences between groups, suggesting there are no major defects in thymic T cell development.

REDD1 is required for optimal T cell activation and cytokine production in the lymph nodes of sensitized mice

Analyses of the lymph nodes of naive WT and *Redd1* KO mice revealed that CD4⁺ and CD8⁺ T cells are present in similar numbers (Fig. 3A, 3B), supporting the idea that REDD1 deficiencies do

not affect T cell development. However, sensitization, assessed at 3 d after Oxa treatment, results in a significant expansion of both CD4⁺ and CD8⁺ T cells in the draining lymph nodes of WT mice. Yet there was an actual reduction in total T cell numbers in KO mice (Fig. 3B), consistent with a previous in vitro study showing that REDD1 is required for optimal T cell proliferation and survival in response PHA or α CD3/CD28 Ab activation (17). Not surprisingly, T cell cytokine production also appears to be compromised in these mice. Not only are T cell numbers significantly lower in the lymph nodes of sensitized *Redd1* KO mice, but also a smaller proportion of CD4⁺, but not CD8⁺, T cells from *Redd1* KO lymph nodes expressed IFN- γ , IL-17, and IL-5 after in vitro reactivation

FIGURE 2. T cell development in the thymus is not impaired in *Redd1* KO mice. Single-cell suspensions were prepared from the thymuses of naive WT and *Redd1* KO mice. Cell populations were identified by flow cytometry after staining with indicated Abs. **(A)** Representative flow cytometry plots of thymic CD45⁺ CD4⁺ CD8⁺ cell populations. **(B)** Proportions of thymic CD4 and CD8 single-positive (SP) cells as well as CD4 CD8 DP and CD4 CD8 DN cells. **(C)** Representative flow cytometry plots of thymic CD45⁺ CD4⁺ CD8⁺ (DN) subset populations. **(D)** Proportions of thymic CD4 and CD8 DP (DN) cells from both groups of mice. Data shown are representative of one of two independent experiments with four mice per experimental group. $**p < 0.01$, $****p < 0.0001$ by two tailed unpaired *t* test.



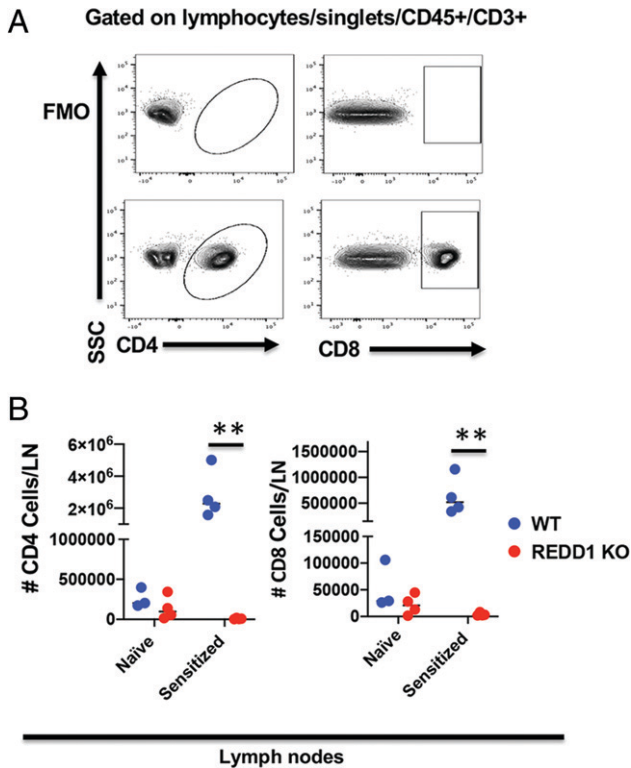


FIGURE 3. CD4⁺ and CD8⁺ T cell priming is impaired in lymph nodes of *Redd1* KO mice postsensitization. Single-cell suspensions were prepared from the ears of naive and WT and *Redd1* KO mice 48 h postsensitization. Cytokine expression was analyzed by flow cytometry after a 4-h in vitro restimulation with anti-CD3 Abs. **(A)** Flow cytometry gating strategy showing FMO controls. **(B)** A comparison of cell numbers in the lymph nodes of naive and sensitized mice. Data shown are representative of one of three experiments. ** $p < 0.01$ by two tailed unpaired t test.

with anti-CD3 Abs (Fig. 4). The mean fluorescence intensity of both CD4⁺ and CD8⁺ T cell cytokine responses was reduced as well. These results illustrate that REDD1 is essential for the early Ag-driven proliferation/expansion and cytokine responses of T cells in the lymph nodes postsensitization.

Redd1 KO mice display more CD45⁺ cells at steady state in ear skin but a reduced response to oxazolone challenge

To assess the effects of REDD1 on local T cell infiltration in response to Oxa challenge, we compared populations of total CD45⁺ cells, T cells, and ILC2s (Lineage[−], IL-7Rα⁺, and ST2⁺) in the ears of naive and sensitized/challenged mice using flow cytometry analysis (gating strategy for ILC2s is shown in Supplemental Fig. 1). Surprisingly, naive *Redd1* KO animals (non-sensitized and unchallenged) displayed increased proportions of CD45⁺ cells at steady state (Fig. 5A). CD45⁺ populations in WT ears made up ~1.78% of the total cells, whereas the mean CD45⁺ population in *Redd1* KO ears was 8.45%, approximately four times higher ($p < 0.0001$). This increased cellularity was reflected in significantly increased basal populations of CD4⁺ T cells, CD8⁺ T cells, and ILC2s in *Redd1* KO ears compared with those in WT ears.

We next compared these same populations in vehicle-treated left ears (sensitized only) and Oxa-treated right ears. To account for differences in baseline immune cell populations, data are expressed as the fold increase relative to naive mice from the same group. As shown in Fig. 5B, whereas both WT and KO mice exhibit significant CD45⁺ cell infiltration to the challenged ear relative to the

vehicle-only ear, the magnitude of infiltration is higher in WT mice (average 16.1-fold increase in WT ears versus 8.3 in *Redd1* KO ears; $p < 0.0001$). The difference in the infiltration of T cells in WT and *Redd1* KO mice was most striking. An average 12-fold increase in CD4⁺ T cells and an average 25-fold increase in CD8⁺ T cells were observed in the Oxa-challenged WT ears, whereas both the CD4⁺ and CD8⁺ T cell response was negligible in challenged *Redd1* KO animals. ($p < 0.01$). Thus, REDD1 plays a seminal role in facilitating T cell migration to the affected ear upon Oxa challenge.

Group 2 innate lymphoid cells (ILC2s, nuocytes) are Lineage negative, ST2⁺, c-Kit⁺ (CD117), and IL-7Rα⁺ (CD127) immune cells that have been implicated in a 2,4,6-trinitrochlorobenzene-induced model of contact hypersensitivity (21) and Oxa-induced colitis (22). Skin-derived ILC2s express ST2, the IL-33R, and are enriched in skin lesions of atopic dermatitis patients (23). Although we observed ILC2s in the ears of naive animals of both genotypes, Oxa challenge did not significantly increase ILC2s in either WT or *Redd1* KO mice (Fig. 5A, 5B).

These patterns are also evident in nonlymphoid cells. Macrophages (CD11b⁺, Ly-6G[−], and CD11c), DCs (CD11c⁺ and Ly-6G[−]), and neutrophils (CD11b⁺ and Ly-6G⁺) exhibit significant increases in steady-state ear populations in *Redd1* KO mice relative to WT animals. Only mast cells (FcεR1⁺ and c-Kit⁺) show no differences between groups, whereas basophils are decreased in KO animals (Fig. 6A).

In WT mice, these same innate immune cell populations show apparent increases in the challenged ear in response to Oxa treatment, but only macrophages, neutrophils, basophils, and mast cells show significant fold increases over vehicle-treated ears (Fig. 6B). There is also a significant Oxa-induced infiltration of macrophages, neutrophils, and mast cells in *Redd1* KO mice. However, with the exception of mast cells, the fold increase in KO mice is diminished in all cell types compared with that in WT. For example, Oxa challenge resulted in an average 4.2-fold increase in ear macrophages in WT animals compared with only a 2.4-fold increase in *Redd1* KO mice ($p < 0.01$). Neutrophils are increased by an average of 12-fold in WT ears but only ~4.8-fold in *Redd1* KO ears ($p < 0.0001$).

Cytokine and chemokine expression are reduced in Redd1 KO mice after Oxa challenge

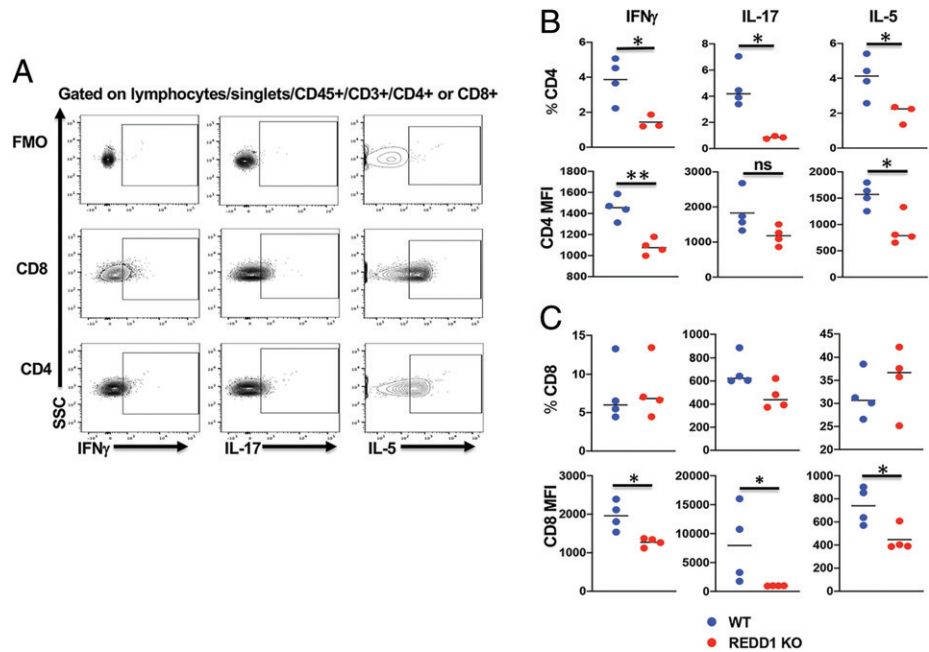
We next analyzed the expression of a variety of inflammatory genes in Oxa-challenged ears at 24 h postchallenge (Fig. 7). The induction of several cytokine genes, including *Il1b*, *Il6*, *Tnf*, *Ifng*, *Il33*, and *Il4*, is reduced in *Redd1* KO mice relative to WT mice, with *Il6*, *Tnf*, *Ifng*, and *Il33* exhibiting the most dramatic differences. The chemokine receptors *Cxcr3* and *Ccr4*, both of which regulate T cell migration, as well as *Cxcl10*, a chemoattractant for macrophages and DCs, also show impaired expression relative to WT mice.

In line with reduced *Il1b* expression, the inflammasome genes *Nlrp3* and *Casp1* are significantly reduced in expression, as is *Nox1*, which regulates membrane-associated reactive oxygen species (ROS) production. The expression of two genes associated with ILC2 effector function, thymic stromal lymphopoietin (*Tslp*) and amphiregulin (*Areg*), is lower in *Redd1* KO ears as well. *Il2* and *Il9* show a reduction in *Redd1* KO mice relative to WT values, but these differences do not reach statistical significance.

Glucocorticoids exhibit reduced anti-ACD activity in Redd1 KO mice

Topical glucocorticoids are widely used for the treatment of inflammatory skin diseases, including ACD (24). Thus, we asked whether *Redd1* status alters the anti-inflammatory effects of the glucocorticoid FA (class III potency glucocorticoid widely used in dermatology) in

FIGURE 4. Reduced T cell cytokine production in the draining lymph nodes of *Redd1* KO mice postsensitization. Cells were prepared as described in Fig. 3 and restimulated for 5 h with anti-CD3 Abs prior to staining with cytokine-specific Abs. **(A)** Flow cytometry gating strategy showing FMO control samples. **(B, C)** Relative cytokine production by CD4⁺ and CD8⁺ T cells depicted as the percentage of expressing cells (top row) and mean fluorescence intensity (MFI) (bottom row). Data are representative of two independent experiments. **p* < 0.05, ***p* < 0.01 by two-tailed unpaired *t* test.



the Oxa-ACD model. FA (0.025 and 0.25 μg/animal) was topically applied to the ear 1 h before Oxa challenge. Cellular infiltration was analyzed 48 h postchallenge and cytokine gene expression at 24 h. In WT mice, FA treatment reduced ear infiltration of total CD45⁺ cells by 2-fold (Fig. 8A) and drastically decreased induction of *Ifng*, *Tnf*, *IL1b*, *IL6*, *IL4*, and *IL33* (Fig. 8B). The anti-inflammatory effects of FA, especially at the higher dose, were significantly reduced in *Redd1* KO mice.

Discussion

The goal of these studies was to assess the role of the stress-response gene REDD1 in the development of Oxa-induced ACD in

mice. We demonstrate that REDD1 exerts striking and widespread proinflammatory effects in this disease model. REDD1 has a profound effect on both ACD phases: 1) it supports the initial expansion of CD4⁺ and CD8⁺ cells and Th1/Th17/Th2 differentiation in draining lymph nodes (sensitization), and 2) it promotes the local edema response and infiltration of CD4⁺ and CD8⁺ T cells, macrophages, DCs, and neutrophils into the ear after challenge (elicitation). In agreement with the reduced numbers of infiltrating leukocytes in challenged ears of *Redd1* KO mice, the induction of many proinflammatory cytokines and chemokines was also significantly suppressed. The diminished early T cell response after sensitization in mice lacking REDD1 is the key deficit that sets the stage for the subsequent muted inflammatory responses observed in

FIGURE 5. *Redd1* KO mice display more T cells at steady state in ear skin but a reduced response to Oxa challenge. Single-cell suspensions were prepared from the ears of naive, vehicle-treated (sensitized only) and Oxa-challenged WT and *Redd1* KO mice 48 h postchallenge. Cell lineages were identified by flow cytometry. **(A)** Proportions of CD45⁺, CD4⁺, and CD8⁺ T (gated on CD45⁺/CD3⁺ cells) and ILC2s (CD45⁺, Lineage[−], IL-7Ra⁺, and ST2⁺) in the ears of untreated mice. **(B)** Innate immune cell increases in response to Oxa challenge. Data are presented as fold increase relative to ears of naive mice of corresponding genotype. Data are compiled from two to three independent experiments. **p* < 0.05, ***p* < 0.01, ****p* < 0.001, *****p* < 0.0001 by two-tailed unpaired *t* test.

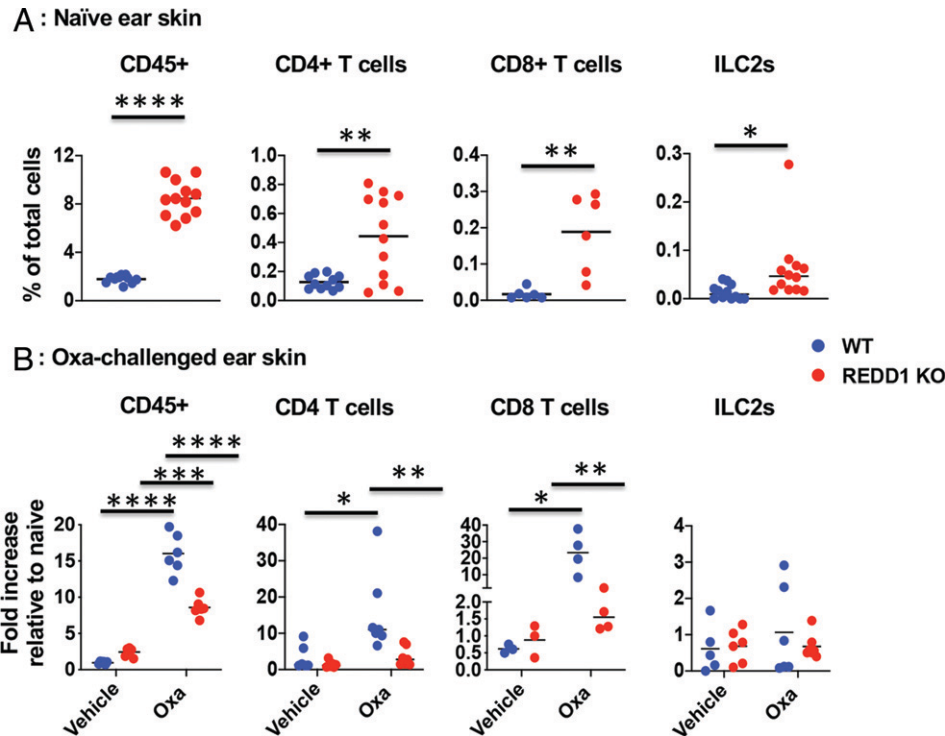
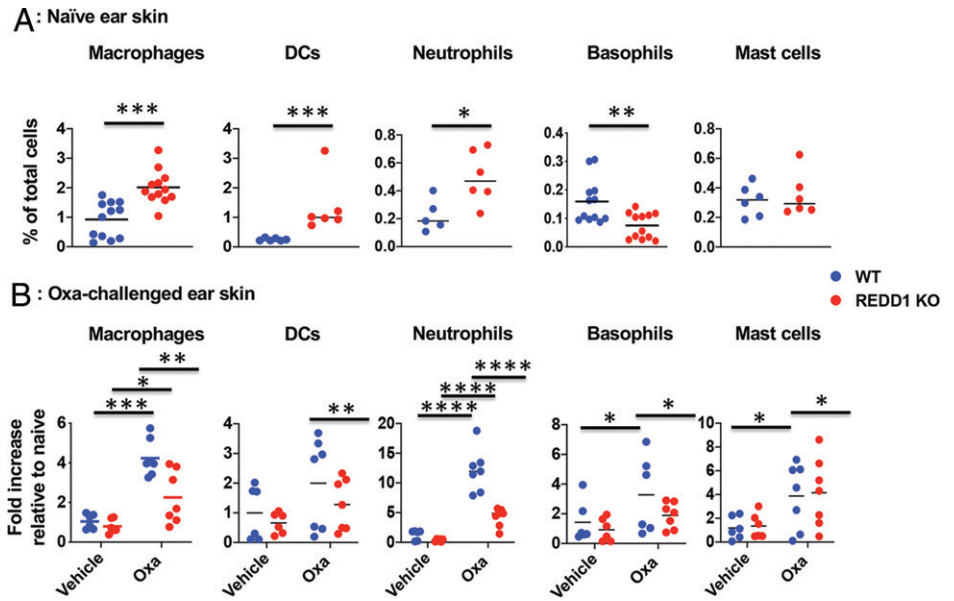


FIGURE 6. *Redd1* KO mice display more innate immune cells in ear skin at steady state but a reduced response to Oxa challenge. Single-cell suspensions were prepared from the ears of naive, vehicle-treated (sensitized only), and Oxa-challenged WT and *Redd1* KO mice 48 h postchallenge. Cell lineages were identified by flow cytometry. **(A)** Proportions of innate immune cells in the ears of untreated mice. Macrophages: Ly-6G⁺/CD11b⁺; mast cells: c-Kit⁺/FcεR1⁺; DCs: Ly-6G⁺/CD11c⁺/CD11b⁺; neutrophils: Ly-6G⁺/CD11b⁺; basophils: c-Kit⁺/FcεR1⁺; ILC2s: Lineage⁺/IL-7Rα⁺/ST2⁺. Data represent the compilation of two to three independent experiments. **p* < 0.05, ***p* < 0.01, ****p* < 0.001, by two-tailed unpaired *t* test. **(B)** Cell increases in response to Oxa challenge. Data are presented as fold increase relative to ears of naive mice of corresponding genotype. Data are compiled from two to three independent experiments. **p* < 0.05, ***p* < 0.01, ****p* < 0.001, *****p* < 0.0001 by two-tailed unpaired *t* test.



challenged ears. Lower numbers of activated T cells and a reduced capacity to produce cytokines results in fewer T cells available to migrate to the inflamed tissues to become reactivated. This, in turn, leads to less recruitment of other immune cells. However, there is

also a clear migratory defect in REDD1-deficient T cells, as shown by the reduced infiltration relative to WT animals. The diminished expression of *Cxcr3* and *Ccr4* in *Redd1* KO mice, which regulate tissue migration of Th1 and Th2 cells, respectively, supports this

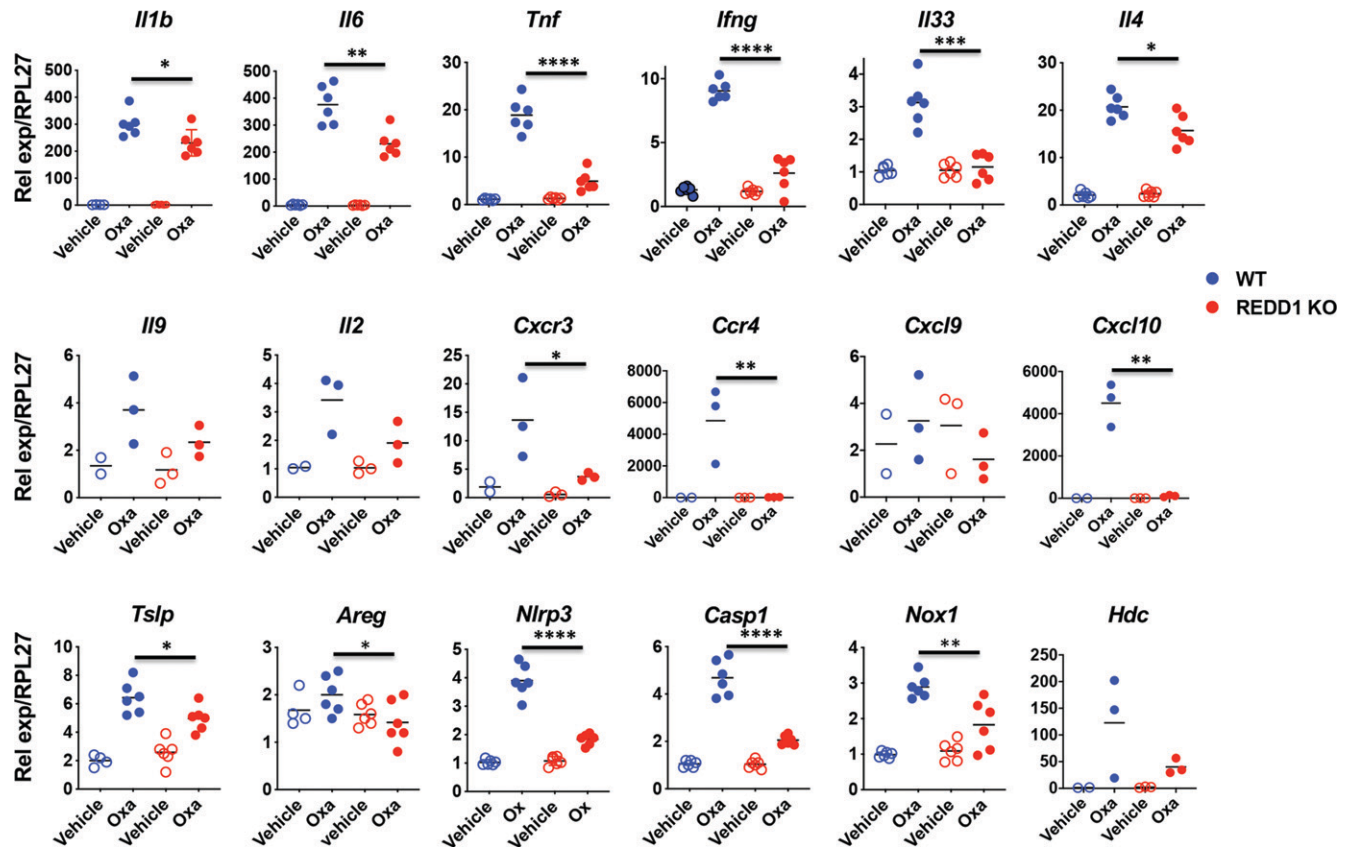


FIGURE 7. Reduced inflammatory gene expression in Oxa-challenged *Redd1* KO mice. Total RNA was isolated from mouse ears 24 h after Oxa challenge, and gene expression was assessed by qPCR analyses. Results were normalized to the expression of housekeeping gene *Rpl27*. *Hprt1* was also used to normalize a subset of data with similar results. Data (*n* = 3–6 mice per group) are presented as mean ± SD. Data shown are representative of two independent experiments with three to six mice per experimental group. **p* < 0.05, ***p* < 0.01, ****p* < 0.001, *****p* < 0.0001 by two-tailed unpaired *t* test. Open circles denote vehicle-treated ears and closed circles represent Oxa-treated ears.

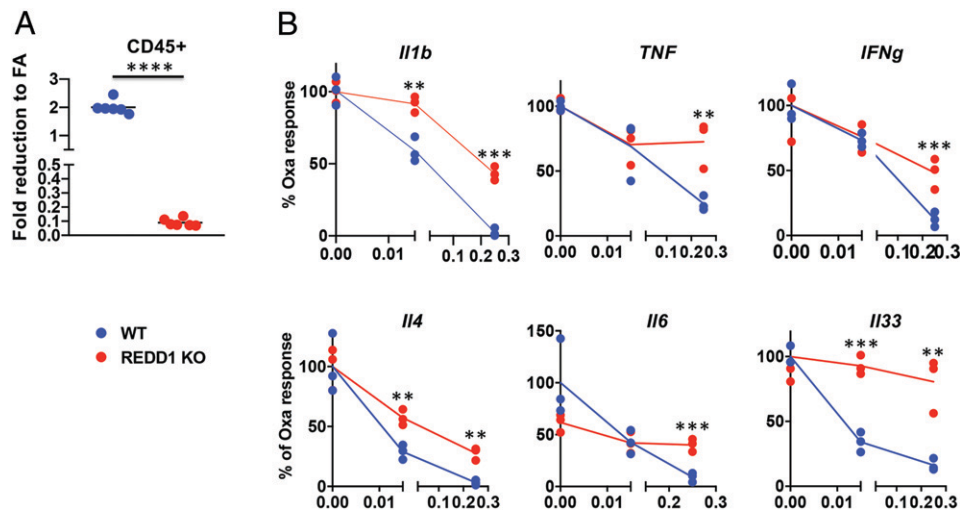


FIGURE 8. *Redd1* KO mice show reduced sensitivity to the anti-inflammatory effects of glucocorticoid FA. Eight-week-old female WT and *Redd1* KO mice were sensitized by topical application of Oxa (100 μ l of 2% Oxa in ethanol), as depicted in Fig. 1. Six days later, right ears were pretreated with solvent control (acetone) or either 25 or 250 ng of FA and, 1 h later, challenged by application of 1% Oxa (10 μ l). (A) Single-cell suspensions were prepared from Oxa-challenged right ears (pretreated with solvent or 250 ng of FA) of WT and *Redd1* KO mice 48 h after the challenge. Cells were analyzed by flow cytometry using dye-conjugated, CD45-specific Abs, as described in *Materials and Methods*. Data are representative of two experiments with three to four mice per group. (B) Total RNA was isolated from right ears 24 h after Oxa challenge. qPCR analyses were performed using mouse-specific primers. Data are presented as percentage of solvent control-challenged ears (no FA) of parent control group, which was set at 100%. These data represent the results of one experiment with three mice per group at each of three FA concentrations. ** p < 0.01, *** p < 0.001, **** p < 0.0001 by two-tailed unpaired t test.

idea. It was previously shown that REDD1 is inducibly expressed in T cells in vitro (17), so it is likely that it is acting intrinsically to regulate activation, including expression of tissue homing molecules, in this disease setting. Yet this protein is also expressed in other cells, and the possibility exists that APCs in the secondary lymphoid organs are impaired in T cell-activating ability in KO mice. Studies are ongoing to distinguish between these scenarios.

There is a notably higher proportion of resident immune cells in the ear skin of naive mice in *Redd1* KO mice. The overall lack of response to challenge suggests that these cells are less responsive to activating signals as well and that REDD1 impacts the functionality of innate immune cells and perhaps regulates their homeostatic maintenance and migratory capacity.

Because REDD1 is best known as an mTOR inhibitor, we predicted that REDD1 deletion would relieve mTOR inhibition and promote inflammation, leading to increased T cell activation and immune cell trafficking. Indeed, there is evidence that mTOR activation, rather than inhibition, is implicated in T cell-mediated inflammation. Ag receptor-mediated activation of resting T cells involves mTOR-dependent protein synthesis and an increase in glycolysis necessary for rapid T cell proliferation and cytokine-driven differentiation and leading to variable effector functions and chemokine-induced migration to the site of inflammation. CD4⁺ T cells lacking mTOR fail to differentiate into Th1, Th2, or Th17 effector cells and, instead, default to the Foxp3⁺ regulatory T cell differentiation pathway (25). Blockade of mTOR signaling in Ag-activated CD8⁺ T cells leads to the development of memory CD8⁺ T cells instead of effector cytotoxic T cells (25). mTOR also regulates T cell trafficking, promoting T cell egress from lymph nodes (26). The suppressed T cell response to Oxa treatment in *Redd1* KO mice we observe may reflect the differences of the controlled conditions of in vitro studies compared with the more complicated in vivo disease settings in which multiple cell types and signaling pathways are functioning. More likely, some proinflammatory effects of REDD1 do not require mTOR, an idea supported by several studies. For example, *Redd1* KO mice exhibit decreased inflammation in an endotoxin shock model that is mTOR independent (13). Based on

decreased ROS production and Nox-1 and Gpx3 expression in these mice, it was proposed that REDD1 acts via the induction of oxidative stress. REDD1 is also known to activate the proinflammatory NF- κ B pathway via an atypical mechanism mediated by physical interaction with its inhibitor I κ B α (9, 12). Finally, the REDD1-HIF-1 α feedback loop that mediates adaptive responses to oxidative stress, important for some inflammatory processes, also does not involve mTOR (5). Our observation of reduced *Nox1* gene expression in *Redd1* KO mice supports a role for oxidative stress in promoting ACD. Reduced expression of inflammasome-related genes, which are activated by ROS, including *Nlrp3*, *Casp1*, and *Il1b*, is also consistent with this idea.

The actions of glucocorticoids are mediated by the glucocorticoid receptor (GR), a well-studied transcription factor (24). REDD1 is a known GR target gene, and as we reported previously, REDD1, via a feed-forward loop, controls GR signaling and overall transcriptional response to glucocorticoids, especially gene activation important for gluconeogenesis and atrophic adverse effects of glucocorticoids (16). At the same time, we found that neither induction of inflammation nor glucocorticoid anti-inflammatory responses were affected in *Redd1* KO animals treated with the skin irritant croton oil (16). Unexpectedly, in Oxa-induced ACD, FA was less effective at inhibiting inflammation in *Redd1* KO mice at lower doses (25 and 250 ng/animal), as measured by the reduced cellular infiltration to the ear and suppression of proinflammatory cytokines (Fig. 8). There are major differences in the cellular mechanisms initiating inflammation in these two models. The major active component of croton oil is PMA (27), which directly induces keratinocytes to produce PGs and IL-1 β (12, 28) to initiate the inflammatory response (12, 29, 30). In the ACD model, induction of inflammation is dependent on hapten-peptide complexes activating T cells, which triggers proliferation and cytokine production, resulting in inflammation (14). Using Illumina OligoArray, we previously found that REDD1 status did not significantly interfere with downregulation of proliferative and proinflammatory genes in epidermal keratinocytes in mouse skin treated with FA (16). However, the changes

in glucocorticoid transcriptome in *Redd1* KO T cells remain to be investigated.

In summary, our data provide the first evidence, to our knowledge, that REDD1 is necessary for the full induction of inflammation associated with allergic contact hypersensitivity and affects both the sensitization and elicitation phase of the disease as well as the response to glucocorticoids. Although REDD1 is an inhibitor of mTOR, other mechanisms, including cell-specific mechanisms, may be involved in the proinflammatory role of this factor. The potent actions of REDD1 in inflammatory disease make it an attractive target for therapeutics and provide strong rationale for further mechanistic studies.

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Disclosures

The authors have no financial conflicts of interest.

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SUPPLEMENTARY MATERIAL

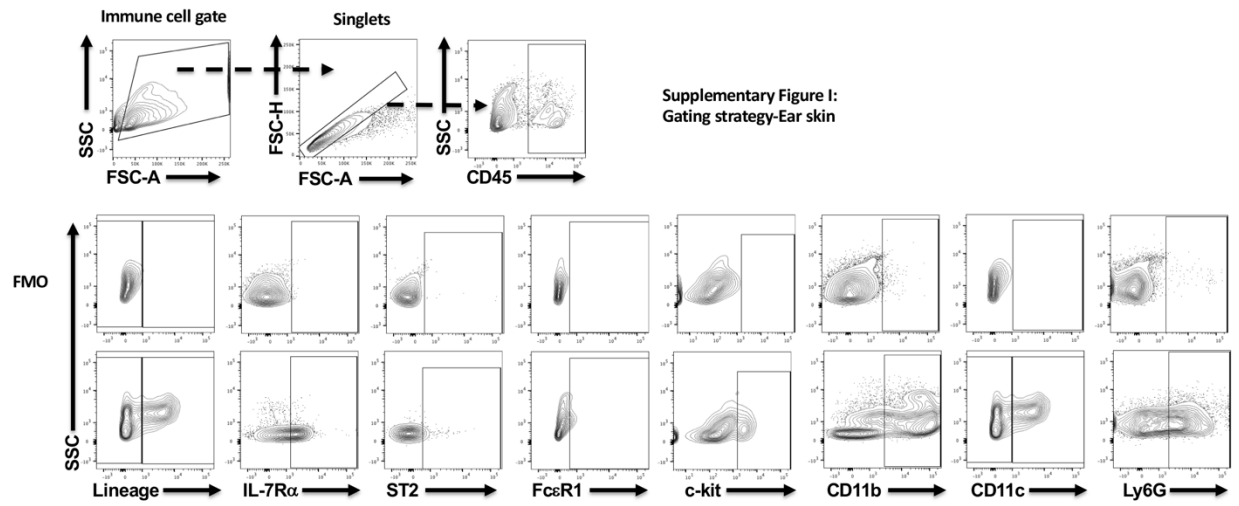
Supplementary Table I. Antibodies used to identify inflammatory cell populations.

Antibody	Clone	Source
CD45	30-F11	BD Biosciences
CD3	145-2C11	Biolegend
CD4	Gk 1.5	Biolegend
CD8	53-6.7	Biolegend
CD11b	M1/70	Biolegend
CD11c	N418	Biolegend
CD19	6D5	Biolegend
c-kit (CD117)	2B8	Biolegend
F4/80	BM8	Biolegend
FcεR1α	MAR-1	Biolegend
IFNγ	XMG1.2	Biolegend
IL4	11B11	eBioscience
IL5	TRFK%	Biolegend
IL-7Rα	A7R34	Biolegend
IL13	eBio13A	eBioscience
IL17	TC11-18H10.1	Biolegend
SiglecF	E50-2440	BD Biosciences
Ly6G/Ly6C	RB6-8C5	Biolegend
ST2	DIH9	Biolegend
CD25	PC61	Biolegend
CD44	IM7	Biolegend
Lineage cocktail Ter119, Gr-1, B220, CD3, and CD11b	Part 78022	Biolegend

Lineage cocktail (part 78022, Biolegend) for innate lymphoid cells includes antibodies against Ter-119, Gr-1, B220, CD3, and CD11b.

Supplementary Table II. Primers used for qRT-PCR analysis

Gene	Primer seq, sense/antisense 5'-3'	Gene	Primer seq, sense/antisense 5'-3'
Areg	AGGCGAATCGCTTTCTGGGG CCCCTGTGGAGAGTTCACTGC	IL9	TCATGACTTCCGTGACGCTTT GTTTTCTTCCTCAGAGCCCTGTT
Casp1	ACACGTCTTGCCCTCATTATCTG C C CACTCCTTGTTTCTCTCCACG	IL13	GCAGCATGGTATGGAGTGTG TGGCGAAACAGTTGCTTTGT
Ccr4	TCATGACTTCCGTGACGCTTT GTTTTCTTCCTCAGAGCCCTGTT	IL17	TCTCCACCGCAATGAAGACC CACACCCACCAGCATCTTCT
Cxcl9	TCTTTTCCTCTTGGGCATCATCTT TTTCCCCCTCTTTTGCTTTTCTT	Ifng	GCTCTGAGACAATGAACGCT AAAGAGATAATCTGGCTCTGC
Cxcl10	ATCCCGAGCCAACCTTC CTTAGATTCCGGATTGAGACAT	IL33	TGGACCTCGCACTAAAATCATTG CGAAAGGAAGAGATGGCCTTAA
Cxcr3	CCTGCGCAGCCCAAGTCCTAAC ACGCCTGGCCCTCTGTGCTATTT	Nlrp3	AGAAGAGACCACGGCAGAAG CCTTGGACCAGGTTTCAGT
Hdc	GAGTACGCTGACTCCTTCACCT CAGAGTTGGCATGTCGGAGGTA	Nox1	TCCATTTCTTCCTGGAGTGGCAT GGCATTGGTGAGTGCTGTTGTTCA
IL1b	CAGGCAGGCAGTATCACTCA AGGTGCTCATGTCCTCATCC	Rpl27	GCCCTGGTGGCTGGAATTGACC TTGCGCTTCAAAGCTGGGTCCC
IL2	CCTGAAACTCCCCAGGATGC TCAAATCCAGAACATGCCGC	Hprt1	GTTGGATACAGGCCAGACTTTGTT GAGGGTAGGCTGGCCTATAGGCT
IL4	TCGGCATTGTTGAACGAGGTC GAAAAGCCCCGAAAGAGTCTC	Tnf	CCCACGTCGTAGCAAACC GCAGCCTTGTCCTTGAA
IL5	TCACCGAGCTCTGTTGACAA CCACACTTCTTTTTTGCG	Tslp	GACCTGACTGGAGATTTGAAAGGG GACAAGCTGGCTTGCTCTCACA
IL6	AACCCAAGGGCATTTCATC CACCGCATCTATCACCACAG		



Supplementary Figure I: Gating strategy of ear skin. Single cell suspensions of ear skin were stained with the indicated fluorescently labeled antibodies. Gating is based on FMO (fluorescence minus one) control cell samples.