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CD8⁺ tissue-resident memory T cells recruit neutrophils that are essential for flare-ups in contact dermatitis

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Short title: Neutrophils are essential for flare-ups in contact dermatitis

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Abstract

Background Allergic contact dermatitis (ACD) is classically described as a delayed-type hypersensitivity reaction. However, patients often experience flare-ups characterized by itching erythema, edema, and often vesicles occurring within hours after re-exposure of previously sensitized skin to the specific contact allergen. Recent studies have indicated that skin-resident memory T (T_{RM}) cells play a central role in ACD. However, the pathogenic role of T_{RM} cells in allergen-induced flare-ups is not known.

Methods By the use of various mouse models and cell depletion protocols, we investigated the role of epidermal T_{RM} cells in flare-up reactions to the experimental contact allergen 1-fluoro-2,4-dinitrobenzene (DNFB). The inflammatory response was measured by changes in ear-thickness and the cellular composition in epidermis was determined by flow cytometry and confocal microscopy. Finally, adoptive transfer and inhibitors were used to determine the role of T_{RM} cells, neutrophils and CXCL1/CXCL2 in the response.

Results We show that $CD8^+ T_{RM}$ cells initiate massive infiltration of neutrophils in the epidermis within 12 hours after re-exposure to the contact allergen. Depletion of neutrophils before re-exposure to the allergen abrogated the flare-up reactions. Furthermore, we demonstrate that $CD8^+ T_{RM}$ cells mediate neutrophil recruitment by inducing CXCL1 and CXCL2 production in the skin, and that blockage of the C-X-C chemokine receptor type 1 and 2 inhibits flare-up reactions and neutrophil infiltration.

Conclusion As the first, we show that epidermal $CD8^+ T_{RM}$ cells cause ACD flare-ups by rapid recruitment of neutrophils to the epidermis.

Key words

Allergic contact dermatitis, epidermal-resident T cells, neutrophils, CXCL1, CXCL2

Introduction

Allergic contact dermatitis (ACD) is a common T cell-mediated skin disease affecting about 10% of the adult population (1). ACD is induced by exposure of the skin to low-molecular weight chemicals called haptens or contact allergens that have the ability to react with proteins in the skin (2). Hapten-modified proteins are picked up by Langerhans and dendritic cells that subsequently migrate to the skin-draining lymph nodes where they activate specific T cells that recognize the hapten-modified self-protein in the context of major histocompatibility complex molecules. ACD is characterized by rapid flare-ups/exacerbations with intensely itching erythema, edema, and often vesicles occurring within hours after re-exposure to the specific contact allergen. Interestingly, these rapid flare-ups only develop at skin sites previously exposed to the contact allergen (3). Recent studies have indicated that skin-resident memory T (T_{RM}) cells play a central role in the flare-up reactions in ACD (4–7). Recently, Gaide et al. elegantly demonstrated that T_{RM} cells in the skin mediate rapid, intense flare-up reactions whereas circulating memory T cells mediate delayed, attenuated reactions in an experimental model of ACD (4). These observations were confirmed and extended in both mouse and man by Schmidt et al. who found that sensitization to a specific contact allergen induces a strong, long-lasting local memory that is mediated by allergen-specific IL-17A- and IFN γ -producing CD8 $^{+}$ T_{RM} cells in the epidermis (5). In line with these studies, Gamradt et al. demonstrated that long-lived CD8 $^{+}$ T_{RM} cells accumulated in the epidermis of skin exposed to contact allergens and that these CD8 $^{+}$ T_{RM} cells mediated the flare-up reactions following allergen challenge (6), and Gadsbøll et al. demonstrated that the intensity of the ACD flare-up reactions correlated with the number of CD8 $^{+}$ epidermal T_{RM} cells (7).

In healthy individuals, T_{RM} cells are crucial mediators of local infection control (8,9). Thus, recognition of pathogens by T_{RM} cells leads to rapid cytokine production that augment the ability of neighboring cells to resist and combat infection, and activates the endothelium in local blood vessels to recruit neutrophils and other leucocytes to the site of infection (10–15).

Although it has been shown that allergen-specific epidermal T_{RM} cells produce IL-17A and IFN γ within 4 hours after challenge with contact allergens (5), the pathogenic mechanisms induced by T_{RM} cells that lead to ACD flare-ups are not known. Here we show that CD8 $^{+}$ T_{RM} cells initiate infiltration of high numbers of neutrophils in the epidermis within 12 hours after re-exposure to contact allergen. Depletion of neutrophils before re-exposure to the contact allergen abrogated the

rapid flare-up reaction showing that neutrophils are essential for flare-up reactions. We demonstrate that CD8⁺ T_{RM} cells mediate neutrophil recruitment by inducing CXCL1 and CXCL2 chemokine production in the skin, and in accordance with the pathogenic role of the neutrophils, we found that blockage of the C-X-C chemokine receptor type 1 and 2 (CXCR1 and CXCR2) inhibited the flare-up reactions and neutrophil infiltration in parallel. In conclusion, this study shows that epidermal CD8⁺ T_{RM} cells cause ACD flare-ups by recruiting neutrophils to the epidermis.

Methods

Mice

6-8 weeks old C57Bl/6J mice were purchased from Janvier Labs (Le Genest-Saint-Isle, France) and used in all experiment of this study. All mice were housed in a specific pathogen-free animal facility at the Department of Experimental Medicine, University of Copenhagen in accordance with the national animal protection guidelines (license number 2018-15- 0201-01409).

Sensitization and challenge of CHS

Female mice were sensitized on three consecutive days (day 0-2) by epicutaneous painting with 0.15% 1-Fluoro-2,4-dinitrobenzene (DNFB) (Sigma-Aldrich, St. Louis, MO) diluted in a 1:4 solution of olive oil:acetone (OOA). Mice were painted either with 25 µl on the dorsum of both ears or with 50 µl on a similar sized area (2 cm²) on the shaved abdomen. Control groups were painted with OOA during sensitization. Mice were challenged after a minimum of 21 days by applying 25 µl DNFB on the dorsum of both ears. Ear thickness was measured using an electronic digital micrometer (Mitutoyo Corporation, Model: PK-1012CPX) and values presented were normalized to mean ear thickness within each group measured at 0 hours. Depending on the intended ex vivo analysis, mice were euthanized and the ears, spleen and the submandibular and cervical draining lymph nodes were isolated for further analyses. All experiments were repeated on at least two separate occasions and data were pooled from both experiments resulting in a minimum of eight mice per group (n = 8).

***In vivo* cellular depletion and receptor blocking and adoptive transfer**

Please see online supporting information's.

***Ex vivo* cellular phenotyping by flow cytometry (FC)**

Please see online supporting information's.

***Ex vivo* ImageStream analysis of epidermal neutrophils**

Please see online supporting information's.

***Ex vivo* fluorescent microscopy**

Please see online supporting information's.

Quantification of chemokines in skin

Please see online supporting information's.

Statistics

Statistical comparisons were performed using GraphPad Prism version 7.0. Gaussian distributions were tested using Shapiro-Wilk's normality test and statistical significance were tested using One-way ANOVA, Two-way ANOVA repeated measures or Students unpaired t-tests as indicated in the figures. For conditions where Gaussian distribution was not found a non-parametric Mann-Whitney U test was performed. Multiple comparisons were adjusted post-hoc using Bonferroni's multiple comparisons test. Significance levels are illustrated in all figures as; Not significant (ns) = $p > 0.05$, * = $P \leq 0.05$, ** = $P \leq 0.01$, *** = $P \leq 0.001$, **** = $P \leq 0.0001$.

Results

Allergen-experienced skin harbors epidermal CD8⁺ T_{RM} cells and mounts intense and rapid flare-ups upon allergen re-exposure

The vast majority of previous studies on immune mechanisms involved in ACD have used the acute contact hypersensitivity (CHS) assay in mice. Importantly, in the acute CHS assay, sensitization and challenge with the contact allergen are separated by only five days, which is not enough time for adaptive immune memory to develop (4,16). Thus, the acute CHS assay does not truly reflect the conditions in human ACD where individuals repeatedly are exposed to the contact allergen resulting in the generation of memory T cells in the skin and the characteristic flare-ups. To study the role of T_{RM} cells versus circulating effector/memory T cells in ACD, we developed a modified CHS assay in which we sensitized mice on their ears or on the abdomen for three consecutive days with 2,4-dinitro-1-fluorobenzene (DNFB) or with the vehicle control (olive oil:acetone (OOA)). Twenty-four days after sensitization, we determined the presence of CD8⁺ T_{RM} cells in the epidermis isolated from ears of the mice by immunohistochemistry and flow cytometry. In accordance with previous studies, dendritic-shaped CD69⁺CD103⁺CD44⁺CD62L⁻ CD8⁺ T_{RM} cells were present in the epidermis of allergen-experienced skin from mice sensitized on the ears but not in allergen-naïve skin of mice sensitized on the abdomen (Fig. 1A, B and Fig. S1 for gating strategy) (5,6,8). Having established the model, we studied the nature of the challenge response in allergen-experienced skin versus allergen-naïve skin in sensitized mice as outlined in Fig 1C. Twenty-four days after sensitization on the ears or the abdomen, the mice were challenged on the ears and the inflammatory response and T cell infiltration were measured by changes in ear thickness and by flow cytometry, respectively, at zero to 96 hours after the challenge. In allergen-experienced skin that harbors epidermal CD8⁺ T_{RM} cells, we found an intense and rapid flare-up reaction peaking already 24 hours after allergen re-exposure (Fig. 1D). In contrast, we found that the ACD response in allergen-naïve skin in sensitized mice was less intense and furthermore delayed compared to the response in allergen-experienced skin. Thus, the ACD response in the allergen-naïve skin peaked at 72 hours in line with a classical delayed type IV allergic reaction and with the optimal time point for patch test readings in the clinical setting (17). We next determined the presence and recruitment of various T cell subsets to the epidermis during the challenge response in allergen-experienced and allergen-naïve skin. In accordance with the experiment described above, the epidermis of allergen-experienced skin contained CD8⁺ T_{RM} cells already at the time of challenge, whereas allergen-naïve skin did not (Fig. 1E, F and Fig. S1

for gating strategy). Interestingly, the number of CD8⁺ T_{RM} cells in the epidermis of allergen-experienced skin did not increase for the first 48 hours after challenge. At 96 hours, the number of CD8⁺ T_{RM} cells had increased in the allergen-experienced skin, and CD8⁺ T_{RM} cells were also found in allergen-naïve skin at this time point. CD8⁺ T effector memory (T_{EM}) cells infiltrated the epidermis of allergen-experienced and allergen-naïve skin with similar kinetics and were clearly detected 96 hours after the challenge (Fig. 1G). The number of epidermal CD8⁺ central memory (T_{CM}) cells were generally low or absent in all mice at all time points tested (Fig. 1H). Epidermal CD4⁺ T_{RM} cells were not generated during sensitization in our model (Fig. S2). However, an increase in CD4⁺ T_{RM} cells were seen 48 hours after the challenge (Fig. S2).

ACD flare-up reactions are dependent on neutrophils

As the flare-up reactions were not explained by recruitment of new T cells to the epidermis, we next investigated neutrophil recruitment following re-exposure to the contact allergen. Neutrophils are the major pathogen-fighting cells in the organism and central to this function is their ability to be recruited to sites of infection. Furthermore, neutrophils play key roles in acute inflammation leading to tissue injuries (18). Twenty-four days after sensitization on the abdomen or the ears, we challenged the mice on their ears and measured the neutrophil infiltration in the epidermis by flow cytometry at zero to 96 hours after the challenge. We found a clear difference in the number of infiltrating neutrophils between the epidermis of allergen-experienced and allergen-naïve skin (Fig. 2A-B, Fig S2G). Already 12 hours after re-exposure to the contact allergen, the number of epidermal neutrophils had strongly increased in allergen-experience skin, whereas neutrophils were not detected in allergen-naïve skin. At this early time point, the neutrophils actually outnumbered the CD8⁺ T_{RM} cells in the epidermis of allergen-experienced skin 6-8 times (Fig. 2B and Fig. 1F). After the swift increase, the number of neutrophils in the epidermis rapidly declined and nearly reached pre-challenge levels 96 hours after challenge of the allergen-experienced skin (Fig. 2A, B). Thus, a clear correlation was seen between neutrophil infiltration in the epidermis and the flare-up reaction in allergen-experienced skin after re-exposure to the allergen. In contrast to the rapid and intense neutrophil recruitment seen in allergen-experienced skin, only a minor increase in neutrophils was seen in allergen-naïve skin 48 hours after allergen challenge (Fig. 2A, B).

To determine whether the neutrophils were directly causative of the flare-up reactions seen in allergen-experienced skin, we sensitized mice on the ears for three consecutive days and injected

them i.v. at three days and just before the challenge with either neutrophil-depleting anti-Ly-6G monoclonal antibodies (mAb) or isotype control mAb as depicted in Fig 2C. In contrast to mice treated with the isotype control mAb, the flare-up reaction was severely inhibited in mice depleted for neutrophils (Fig 2D). Thus, the ACD response in allergen-experienced skin in mice depleted for neutrophils resembled the attenuated, delayed response seen in allergen-naïve skin in mice not depleted for neutrophils (Fig. 2D and Fig. 1D), showing that ACD flare-up reactions are dependent on neutrophils.

CD8⁺ T_{RM} cells are indispensable for ACD flare-ups and the rapid recruitment of neutrophils to the epidermis following re-exposure to allergen

As the ACD flare-up reactions and the rapid and intense recruitment of neutrophils were only seen after allergen re-exposure of allergen-experienced skin, and as allergen-experienced skin in contrast to allergen-naïve skin harbored CD8⁺ T_{RM} cells, we next investigated whether CD8⁺ T cells were directly required for neutrophil recruitment and flare-ups. We treated mice with anti-CD8 α mAb to deplete CD8⁺ T cells or with IgG isotype control mAb four times before and after sensitization of the mice on their ears as depicted in Fig. 3A. This treatment specifically depleted CD8⁺ T cells (Fig. S4). The mice were challenged on day 21 and the ear thickness was measured just before and 12 hours after the challenge. In contrast to control mice treated with the IgG isotype control mAb, the allergen-induced flare-up reaction was almost completely abrogated in mice treated with anti-CD8 α mAb (Fig 3B). To analyze the effect of the mAb treatment on the cellular composition in the epidermis, we prepared epidermal single cell suspensions 12 hours after the allergen challenge and determined the number of CD8⁺ T cells and neutrophils by flow cytometry. We found that treatment with anti-CD8 α mAb completely inhibited accumulation of CD8⁺ T cells in the epidermis (Fig. 3C) and profoundly reduced the recruitment of neutrophils to the epidermis (Fig. 3E, F).

To substantiate that the flare-up reactions were dependent on CD8⁺ T_{RM} cells, we next used a sex-match/sex-mismatch adoptive transfer model (11,19). As depicted in Fig 3F, we transferred lymphocytes from female or male donor mice sensitized with DNFB or OOA to naïve female recipient mice. The recipient mice were treated on the ears with the irritant sodium lauryl sulphate (SLS) for three days following the cell transfer to ensure recruitment of activated donor T cells into the epidermis of the recipient's ears in the absence of the allergen (Fig. S3A-C). In this sex-match/sex-mismatch model both circulating memory T cells and T_{RM} cells are present in recipient

mice that received cells from female donors, whereas only T_{RM} cells are left in recipients that received cells from male donors at the time of challenge (11,19). All the recipient mice were challenged with DNFB twenty-one days after the adoptive transfer. We found a similar increase in ear-thickness 12 hours after the challenge in mice that had received cells from female and male donors (Fig 3G). Taken together, these results indicated that CD8⁺ T_{RM} cells are indispensable for ACD flare-ups and the rapid recruitment of neutrophils to the epidermis following re-exposure of allergen-experienced skin to contact allergens.

CD8⁺ T_{RM} cells induce rapid release of CXCL1 and CXCL2 in the skin following re-exposure to allergen

CXCR1 and CXCR2 are the major chemokine receptors on neutrophils involved in neutrophil recruitment (20–27). The primary chemokines binding to CXCR1/2 in mice are CXCL1 (KC) and CXCL2 (MIP-2) (21,22,28). To determine whether CXCL1 and CXCL2 might be involved in the rapid recruitment of neutrophils to allergen-experienced skin following allergen challenge, we sensitized mice on the ears with either DNFB or OOA. Twenty-one days later, we challenged the mice with DNFB on the ears and measured the expression of CXCL1 and CXCL2 at 0, 3, 6 and 12 hours after the challenge. CXCL1 was up-regulated at 6 and 12 hours and CXCL2 was strongly up-regulated at 12 hours in allergen-experienced skin (Fig. 4A, B). Furthermore, we found that DNFB induced up-regulation of CXCL1 in allergen-naïve skin 6 hours after the challenge (Fig. 4A). By splitting the skin in epidermis and dermis, we found that whereas CXCL1 was mainly expressed in the dermis, CXCL2 was most intensely expressed in the epidermis of allergen-experienced skin (Fig. 4C, D). This was confirmed by immunohistochemical analyses, where we found strong expression of CXCL2 in the epidermis of allergen-experienced skin 12 hours after allergen challenge (Fig. 4E, F).

To determine the role of CD8⁺ T_{RM} cells in the production of CXCL1 and CXCL2, we treated mice with anti-CD8 α mAb to deplete CD8⁺ T cells or with IgG isotype control mAb four times before and after sensitization of the mice on their ears as depicted in Fig. 3A. The mice were challenged on day 21 and the concentration of CXCL1 and CXCL2 was measured in parallel with the number of CD8⁺ T_{RM} cells and neutrophils in the epidermis 12 hours after the challenge. We found that CXCL1 and CXCL2 expression and the number of neutrophils in the epidermis strongly correlated with the presence of epidermal CD8⁺ T_{RM} cells (Fig. 4G, H and Fig 3C, D).

CXCR1/2 antagonism inhibits ACD flare-up reactions and neutrophil recruitment

To further investigate whether CXCR1/2 were involved in the ACD flare-up reactions and the rapid recruitment of neutrophils to the epidermis after re-exposure to allergens of allergen-experienced skin, we sensitized mice with DNFB on the ears and treated them with the CXCR1/2 allosteric inhibitor reparixin or PBS just prior to and 8 hours after challenge as depicted in Fig. 5A. Ear thickness was measured 0, 6, 12, 24, 48, 72 and 96 hours after the challenge and recruitment of neutrophils to the epidermis was measured 12 hours after the challenge. We found that reparixin strongly inhibited both the flare-up reaction and the neutrophil recruitment normally seen in allergen-experienced skin (Fig 5B, C).

Discussion

In the present study, we demonstrate that ACD flare-up reactions in allergen-experienced skin are caused by neutrophils recruited to the site of allergen challenge by chemokines induced by local CD8⁺ T_{RM} cells.

Mouse models for ACD have been widely used to study immune responses to contact allergens. Up to now, the vast majority of these studies have used the acute CHS assay, where mice are sensitized on the abdomen or flank once or twice and then challenged on allergen-naïve skin on the ears only 5-7 days after the sensitization (16,29–34). In the acute CHS assay, the inflammatory response as measured as changes in ear thickness peaks 24 hours after the challenge (35,36). In contrast, in our model the challenge response seen in allergen-naïve skin peaked 72 hours after the challenge in accordance with a classical delayed type IV allergic reaction mediated by memory T cells, and in line with the optimal time for reading patch test responses in the clinic (17). Importantly, whereas our model allows time for the development of allergen-specific memory T cells, including epidermal CD8⁺ T_{RM} cells, the acute CHS assay where sensitization and challenge are separated by only 5-7 days does not allow for the development of memory T cells (4,16). Thus, as the acute CHS assay is analyzing boost-activation of effector T cells during the primary response and not memory responses, it does not reflect the underlying chronic conditions of ACD with allergen-specific memory T cells, where individuals repeatedly are exposed to contact allergens resulting in the characteristic flare-up reactions.

In accordance with recent studies, we found that CD8⁺ T_{RM} cells primarily localized in allergen-experienced skin and that ACD flare-up reactions correlated with the presence of CD8⁺ T_{RM} cells (4–7). The link between CD8⁺ T_{RM} cells and flare-up reactions seen in these studies likely reflects the flare-up reactions often observed in ACD patients after re-exposure of allergen-experienced skin to the contact allergen (3). We found that the rapid and intense flare-up reactions correlated with a massive recruitment of neutrophils to the epidermis of allergen-experienced skin following allergen re-exposure. In contrast, re-exposure to allergen did not result in T cell recruitment in neither allergen-experienced nor –naïve skin for the first 48 hours. Neither did re-exposure to allergen result in significant recruitment of neutrophils to allergen-naïve skin. Depletion of neutrophils abrogated the flare-up reactions and transformed the response in allergen-experienced

skin into a delayed type response normally seen in allergen-naïve skin, strongly indicating that neutrophils caused the flare-up reactions.

Previous studies using the acute CHS assay have indicated that neutrophils play a role during both the sensitization and challenge phase (16,29–33,37,38). One study found that sensitization by just one exposure of the skin to DNFB resulted in local inflammation and a moderate infiltration of neutrophils 24 hours after the exposure (39). Others found that neutrophils were required to activate dendritic cells during the sensitization phase (32) and that neutrophils were essential for recruitment of effector T cells to the site of allergen challenge during the elicitation phase (30). Thus, the challenge response seen in the acute CHS assay is likely elicited by local inflammation directly caused by the contact allergen. This inflammatory response results in recruitment of neutrophils that subsequently recruit effector T cells. Importantly, it should be stressed that allergen-specific memory T cells, and in particular local T_{RM} cells, are not found in the acute CHS assay in contrast to the conditions in patients with ACD and in our model. Diametrically opposed to the acute CHS assay, we demonstrate that $CD8^+$ T_{RM} cells recruit neutrophils to the site of allergen re-exposure. Thus, we found that $CD8^+$ T_{RM} cells rapidly induced production of CXCL1 in the dermis and in particular CXCL2 in the epidermis leading to massive recruitment of neutrophils to the epidermis. The important role of CXCL1 and CXCL2 in the recruitment of neutrophils and the pathogenesis of ACD was supported by the observation that treatment with a CXCR1/2 antagonist inhibited both neutrophil recruitment and the intensity of the allergic reaction following allergen re-exposure. The role of neutrophils in human ACD is less clear. The majority of studies investigating the cellular infiltration in the skin of humans with ACD have investigated punch biopsies obtained after exposure of previously allergen naïve skin sites to allergen (40–44). As we only observe the rapid, massive neutrophil recruitment in allergen-experienced skin harboring $CD8^+$ T_{RM} cells, we believe that neutrophils might play a role in flare-up reactions seen in human but that these cells are not found due to the predominant technique used up to now where previously allergen naïve skin sites has been investigated. However, this needs further investigation.

It has recently been shown that dermal $CD4^+$ T_{RM} cells play an important role for long-term local memory to contact allergens in BALB/c mice (38). In accordance with our study, a rapid infiltration of Gr-1⁺ cells (i.e. neutrophils) into previous sensitized skin sites was seen following

re-exposure with contact allergen. Furthermore, epidermal CD8⁺ T_{RM} cells outnumbered epidermal CD4⁺ T_{RM} cells more than 10 times (38). As we did not investigate the role of dermal-resident memory T cells, we cannot exclude that they play a role in our model as well. However, as we found that treatment with anti-CD8α mAb strongly inhibited production CXCL1 and CXCL2, recruitment of neutrophils and the flare-up response, we believe that CD8⁺ T_{RM} cells and not dermal CD4⁺ T_{RM} cells are responsible for these responses. Whether local immunological memory against allergens in human skin is solely maintained by CD4⁺ T_{RM} or CD8⁺ T_{RM} cells is still not known. However, as dermal CD4⁺ T_{RM} cells seem to survive for a prolonged period of time compared to epidermal CD8⁺ T_{RM} cells, these may be more important over time (6,38). Furthermore, the role of epidermal CD8⁺ T_{RM} cells in human ACD is still a matter of debate. A study investigating the retention of T cells in the skin following patch testing found very few CD8⁺ T cells in the skin 21 days after allergen-exposure (45). In contrast, we have previously shown that epidermal CD8⁺ T_{RM} cells are generated in the skin following patch testing with nickel of nickel allergic individuals (5). The reason for this discrepancy is not clear, but might be due to difference in allergen exposure in the two studies, as Moed et al. remove the patch test after 24 hours, whereas the patch test was first removed after 48 hours in our study (5,45). In support of this, we have recently shown that allergen-induced generation of CD8⁺ T_{RM} cells directly correlates with the allergen doses (7). Furthermore, the generation of CD8⁺ T_{RM} cells might also differ between allergens. Clearly, more human studies are needed to clarify this important issue.

In conclusion, this is the first study demonstrating that ACD flare-up reactions are caused by neutrophils recruited to the site of allergen exposure by chemokines induced by local CD8⁺ T_{RM} cells. The flare-up reactions were significantly reduced by CXCR1/2 antagonism suggesting CXC chemokines and receptors as future targets in treatment of ACD.

Author Contributions

Conceptualization of this study was led by ABF and CMB supported by JDJ, CG, NØ and AW. Formal analysis was led by ABF supported by VM. Funding acquisition was done by CMB and JDJ. Investigation was led by ABF supported by VM, AG, MHJ, and JFW. Methodology was led by ABF and CMB supported by CG. Project administration was done by ABF. Resources was obtain by CMB and CG. Supervision was done by CMB supported by JDJ. Validation was led by ABF supported by VM, AG, MHJ, and JFW. Visualization was done by ABF. Writing of the original draft was done by ABF, CMB and CG. Review and editing was done by all authors.

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Figure Legends

Figure 1. Allergen-experienced skin harbors epidermal CD8⁺ T_{RM} cells and mounts intense and rapid flare-ups upon allergen re-exposure

(A) CD8 α (red) and DAPI (blue) stained fluorescent microscopy images of non-challenged epidermal ear sheets isolated 24 days after sensitization with OOA or DNFB on the ears or abdomen. Scale bars; 100 μ m (20x images) and 20 μ m (63x image) (n = 2). (B) Representative dot plots of CD69 and CD103 expression in CD8 α ⁺TCR β ⁺CD44⁺CD62L⁻ epidermal cells isolated from non-challenged ears 24 days after sensitization with OOA or DNFB on the ears or abdomen (n = 8). (C) Experimental setup: Mice were exposed to DNFB or OOA at day 0-2 on the ears or abdomen. Mice were euthanized or challenged with DNFB on both ears on day 26. (D) Ear thickness was measured before challenge at 0 hours and 6, 12, 24, 48, 72 and 96 hours after challenge. Values are normalized to mean ear thickness at 0 hours (n = 8). (E-H) Mean number of live epidermal CD8⁺ T cell subsets in ear sheets isolated at 0, 12, 48 and 96 hours after the challenge (n = 8). (E) CD8⁺ T cells (CD8 α ⁺TCR β ⁺ cells); (F) CD8⁺ T_{RM} cells (CD103⁺CD69⁺CD44⁺CD8 α ⁺TCR β ⁺ cells); (G) CD8⁺ T_{EM} cells (CD44⁺CD8 α ⁺TCR β ⁺ and either CD103 or CD69 single positive or double negative cells); (H) CD8⁺ T_{CM} cells (CD44⁺CD62L⁺CD8 α ⁺TCR β ⁺). Statistical comparisons; Two-way ANOVA. Error bars; standard deviation (SD). Statistical significance levels; not significant (ns) = p > 0.05, * = P ≤ 0.05, ** = P ≤ 0.01, *** = P ≤ 0.001, **** = P ≤ 0.0001.

Figure 2. ACD flare-up reactions are dependent on neutrophils

(A) Representative dot plots of Ly-6G expression in epidermal cells isolated at 0, 12, 48 and 96 hours after the challenge (n = 8). (B) Number of live epidermal (Ly-6G⁺) neutrophils in ear sheets isolated at 0, 12, 48 and 96 hours after challenge (n = 8). (C) Experimental setup: Mice were sensitized with DNFB at day 0-2 on both ears and injected with anti-Ly-6G mAbs or IgG isotype control at day 23 and day 26 just before the challenge. Mice were challenged with DNFB on both ears at day 26. (D) Ear thickness was measured before challenge at 0 hours and 6, 12, 24, 48, 72 and 96 hours after challenge. Values are normalized to mean ear thickness at 0 hours. Each dot/triangle represents the mean ear thickness of each group at each time point (n = 8). Statistical comparisons; Two-way ANOVA. Error bars; standard deviation (SD). Statistical significance levels; not significant (ns) = p > 0.05, * = P ≤ 0.05, ** = P ≤ 0.01, *** = P ≤ 0.001, **** = P ≤ 0.0001.

Figure 3. CD8⁺ T_{RM} cells are indispensable for ACD flare-ups and the rapid recruitment of neutrophils to the epidermis following re-exposure to allergen

(A) Experimental setup. Mice were sensitized with DNFB at day 0-2 on both ears and challenged with DNFB on both ears at day 21. Depletion of CD8⁺ cells were done by i.v. injection at day -1, 0, 3 and by one i.p. injection at day 8 with anti-CD8 α mAbs or IgG isotype control mAb. (B) Ear thickness 12 hours after challenge. Each dot represents ear thickness from one mouse normalized to the mean ear thickness measured before challenge at 0 hours (n = 16). (C-D) Flow cytometry data on living cells from epidermal ear sheets isolated 12 hours after challenge. Each dot represents the number of isolated cells from one mouse (n = 8). Number of (C) CD8⁺ T cells (CD8 α ⁺TCR β ⁺ cells), (D) neutrophils (Ly-6G⁺ cells). (E) Representative dot plots of Ly-6G expression in epidermal cells isolated 12 after challenge. (F) Experimental setup: Female and male donor mice were sensitized with DNFB or OOA at day 0-2 on both ears. Lymphocytes were isolated at day 5 and transferred i.v. into female recipient mice. Recipient mice were treated on both ears with SLS at day 5-7 and challenged with DNFB on both ears at day 26. (G) Ear thickness 12 hours after challenge. Each dot represents ear thickness from one mouse normalized to the mean ear thickness measured before challenge at 0 hours (n = 8). Donor treatment during sensitization and donor/recipient combinations are illustrated below the bars. Statistical comparisons; Students unpaired t-test (B-E) and one-way ANOVA (H). Error bars; standard deviation (SD). Statistical significance levels; not significant (ns) = p > 0.05, * = P $\leq$ 0.05, ** = P $\leq$ 0.01, *** = P $\leq$ 0.001, **** = P $\leq$ 0.0001.

Figure 4. CD8⁺ T_{RM} cells induce rapid release of CXCL1 and CXCL2 in the skin following re-exposure to allergen

(A-H) Mice were sensitized with DNFB at day 0-2 and challenged with DNFB on both ears at day 21. (A) CXCL1 and (B) CXCL2 chemokine concentrations from full skin samples obtained just before the challenge at 0 hours and at 3, 6 and 12 hours after the challenge. Each dot represents the chemokine concentration from one mouse (n = 8). (C) CXCL1 and (D) CXCL2 concentration in the dermis and epidermis obtained just before the challenge at 0 hours and 12 hours after challenge. Each dot represents the chemokine concentration from one mouse (n = 8). (E) Fluorescent microscopy images of cross-sections of ears from mice 0 and 12 hours (rows) after challenge with DNFB. The mice were previously sensitized on the ears with OOA or DNFB

(columns); CXCL2 (orange) and DAPI (blue). Scale bars; 100 μ m (n = 2). **(F)** Quantification of CXCL2 mean fluorescence intensity in epidermis (n = 8). **(G)** CXCL1 and **(H)** CXCL2 concentrations from full skin samples 12 hours after challenge of mice treated with anti-CD8 α mAbs or IgG isotype control mAb as depicted in Fig. 3a. Each dot represents the chemokine concentration from one mouse (n = 8). . Statistical comparisons; One-way ANOVA **(A-F)** and Students unpaired T-test **(G-H)**. Error bars; standard deviation (SD). Statistical significance levels; not significant (ns) = $p > 0.05$, * = $P \leq 0.05$, ** = $P \leq 0.01$, *** = $P \leq 0.001$, **** = $P \leq 0.0001$.

Figure 5. CXCR1/2 antagonism inhibits ACD flare-up reactions and neutrophil recruitment

(A) Experimental setup: Mice were sensitized with DNFB or OOA at day 0-2 and challenged with DNFB at day 21 on both ears. The mice were treated i.p. with reparixin or PBS 15 min before and 8 hours after the challenge. **(B)** Ear thickness was measured before challenge at 0 hours and 6, 12, 24, 48, 72 and 96 hours after challenge. Values are normalized to mean ear thickness at 0 hours. Each dot/triangle represent the mean ear thickness of each group at each time point (n = 8). **(C)** Number of neutrophils in epidermal ear sheets 12 hours after the challenge. Each dot represent ear thickness from one mouse normalized to the mean ear thickness measured before challenge at 0 hours (n = 8). Statistical comparisons; Two-way ANOVA **(B)** and one-way ANOVA **(C)**. Error bars; standard deviation (SD). Statistical significance levels; not significant (ns) = $p > 0.05$, * = $P \leq 0.05$, ** = $P \leq 0.01$, *** = $P \leq 0.001$, **** = $P \leq 0.0001$.

Supplementary Figure S1

Flow cytometry gating strategy for identification of neutrophils and T cell subsets in single cell suspensions from ear epidermis

Each gating plot shows a representative example obtained from DNFB-challenged mice sensitized 26 days earlier with **(A)** OOA or **(B)** DNFB. To illustrate all gated populations in both **(A)** and **(B)** plots SSC-A/FSC-A, FSC-W/FSC-A, FSC-W/Viablity Dye and FSC-W/Ly-6G show epidermal cells 12 hours after challenge and plots TCR β /CD8 α , CD62L/CD44, CDCD69/CD103 show epidermal cells 96 hours after challenge. As illustrated with red arrows FSC-W/Ly-6G and TCR β /CD8 α plots are gated on live single cells. CD62L/CD44 plots are gated on TCR β ⁺CD8 α ⁺ cells, and CD69/CD103 plots are gated on TCR β ⁺CD8 α ⁺CD44⁺CD62L⁻ cells. Identification of each subset are indicated by transparent color of the target quadrant: Neutrophils (brown), CD8⁺ T cells (blue), CD8⁺ T_{CM} cells (yellow), CD8⁺ T_{EM} (red) and CD8⁺ T_{RM} (green). The percentage of

cells within each gate or quadrant is shown. Similar gating strategy was used for CD4⁺ cells subsets.

Supplementary Figure S2

T cell kinetics in the epidermis, submandibular and cervical draining lymph nodes upon allergen challenge.

(A-F) Mice were exposed to DNFB or OOA at day 0-2 on the ears or abdomen. Mice were euthanized or challenged with DNFB on both ears on day 26. Each dot represent the number of isolated cells from one mouse (n = 8). (A-D) Mean number of live epidermal CD4⁺ T cell subsets in ear sheets isolated at 0, 12, 48 and 96 hours after the challenge. (A) CD4⁺ T cells (CD4⁺TCRβ⁺ cells); (B) CD4⁺ T_{RM} cells (CD103⁺CD69⁺CD44⁺CD4⁺TCRβ⁺ cells); (C) CD4⁺ T_{EM} cells (CD44⁺CD4⁺TCRβ⁺ and either CD103 or CD69 single positive or double negative cells); (D) CD4⁺ T_{CM} cells (CD44⁺CD62L⁺CD8α⁺TCRβ⁺). (E-F) Mean number of live CD8⁺ and CD4⁺ T cell subsets in the submandibular and cervical draining lymph nodes (dLN) isolated at 0, 12, 48 and 96 hours after the challenge. (E) CD8⁺ T cells (CD8⁺TCRβ⁺ cells); (F) CD4⁺ T cells (CD4⁺TCRβ⁺ cells). (G) Images of epidermal (Ly-6G⁺) neutrophils from a DNFB sensitized mouse 12 hours after DNFB challenge. Images were captured using an Amnis ImageStream^X Mk II Imaging flow cytometer showing a bright field (BF), a Ly-6G⁺ staining (pink) and an overlay image of each cell. Statistical comparisons; Two-way ANOVA. Error bars; standard deviation (SD). Statistical significance levels; not significant (ns) = p > 0.05, * = P ≤ 0.05, ** = P ≤ 0.01, *** = P ≤ 0.001, **** = P ≤ 0.0001

Supplementary Figure S3

SLS treatment results in the generation of DNFB-induced CD8 T_{RM} cells in the epidermis in an adoptive transfer model.

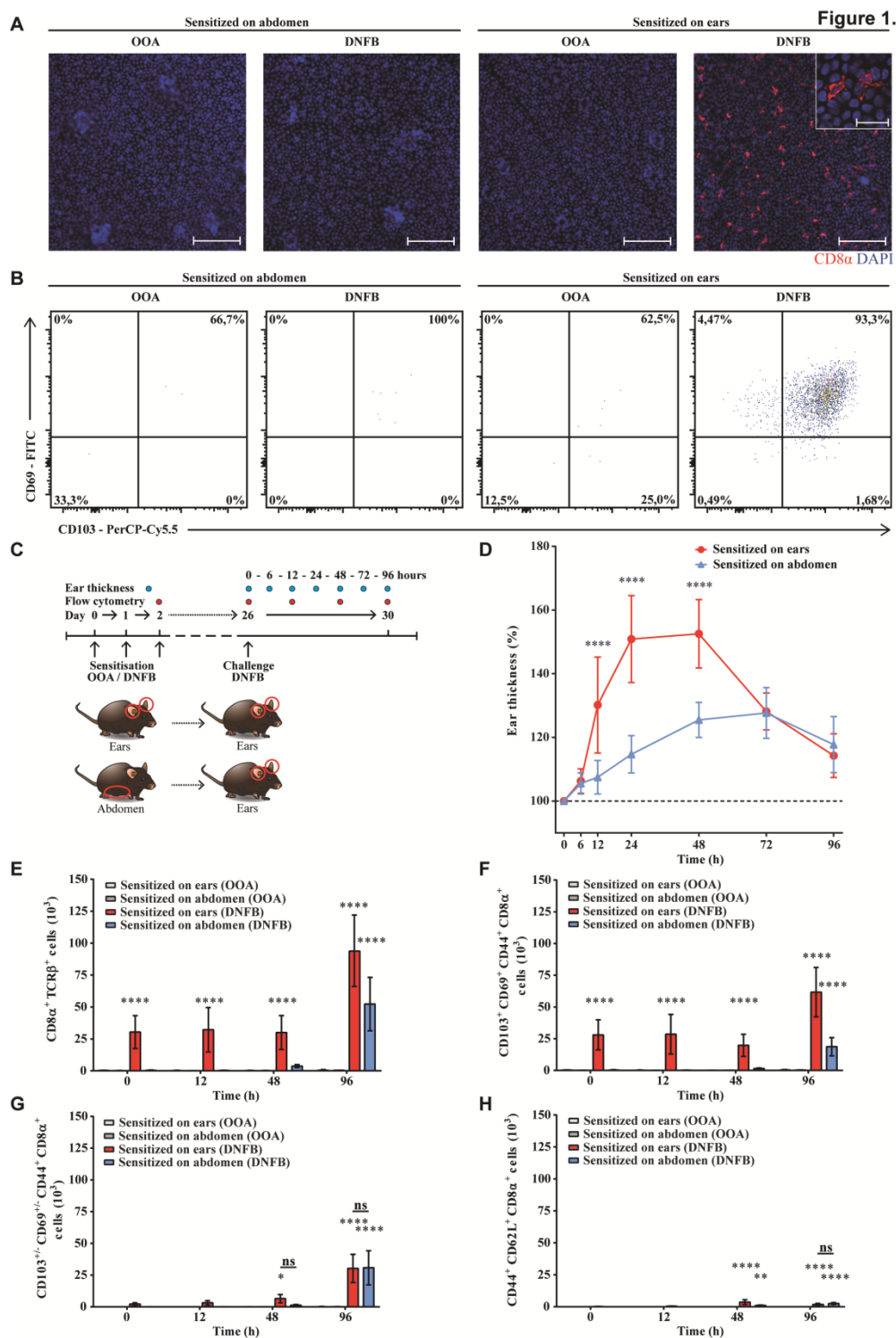
Female donor mice were sensitized with DNFB or OOA at day 0-2 on both ears. Lymphocytes were isolated at day 5 and transferred i.v. into female recipient mice. Mice were treated on both ears with SLS at day 5-7. Epidermal cells was isolated from both ears on day 21. (A-C) Show epidermal cells isolated from mice only treated with SLS (no transfer) or from recipient mice following transfer of lymphocytes from OOA or DNFB donors. (A) Number of CD8α⁺TCRβ⁺ T cells. (B) CD8⁺ T_{RM} cells (CD103⁺CD69⁺CD44⁺CD8α⁺TCRβ⁺ cells). (C) Representative dot plots of CD8α and TCRβ expression in live epidermal cells isolated from one mouse from each group.

Statistical comparisons; One-way ANOVA (A-B). Error bars; standard deviation (SD). Statistical significance levels; not significant (ns) = $p > 0.05$, * = $P \leq 0.05$, ** = $P \leq 0.01$, *** = $P \leq 0.001$, **** = $P \leq 0.0001$.

Supplementary Figure S4

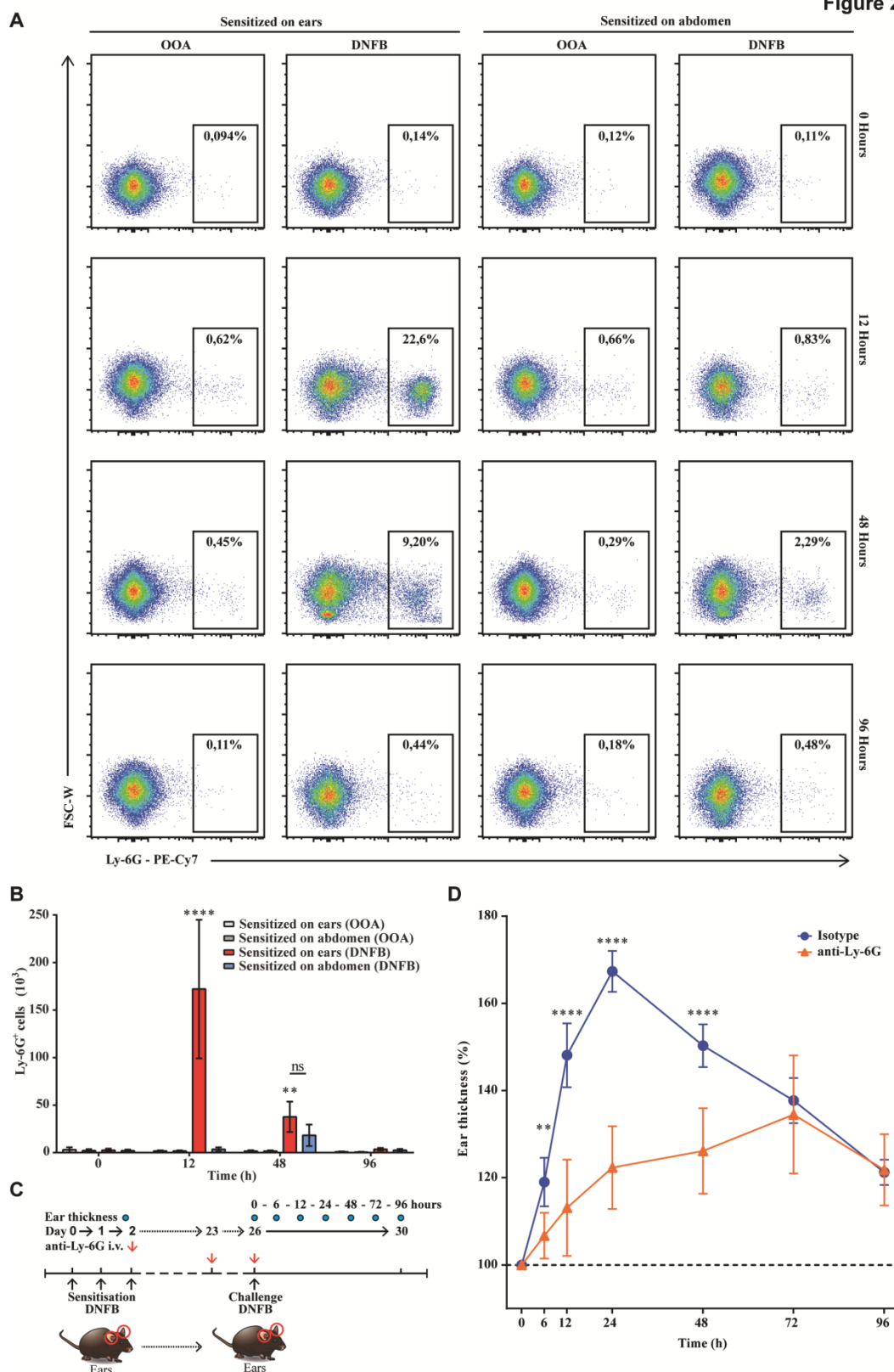
I.v depletion of CD8⁺ T cells with anti-CD8 α monoclonal antibodies do not show off target depletion of other immune cells—.

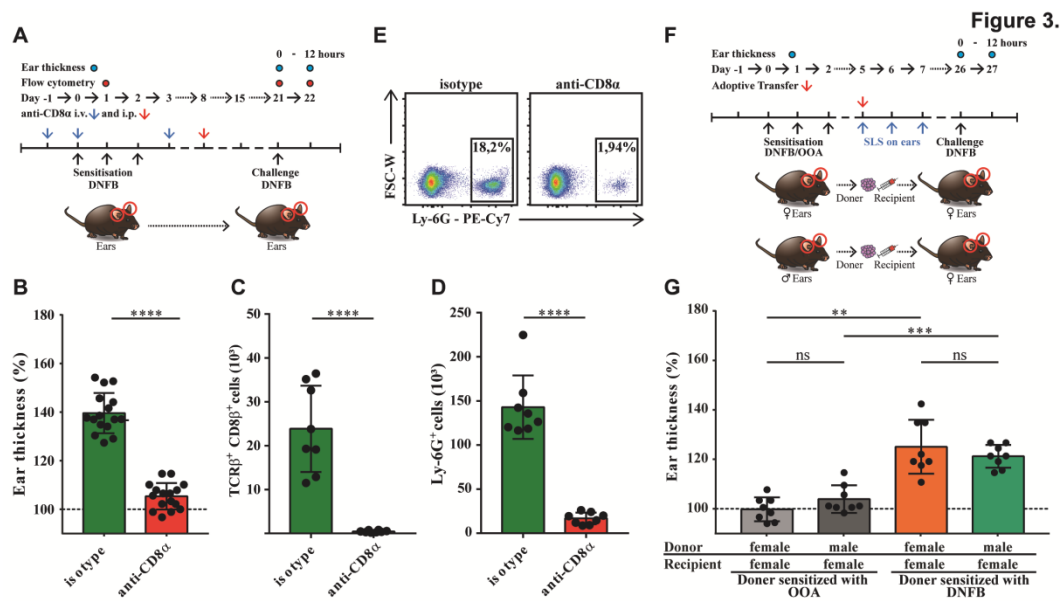
Depletion of CD8⁺ cells were done by i.v. injection at day 0 and day 3 with anti-CD8 α mAbs or IgG isotype control mAb and spleen were isolated at day 4. (A-F) Graphs show the number of cells obtained by flow cytometry on living cells from spleen. Each dot represent the number of isolated cells from one mouse (n = 8). Representative dot plots from one mouse treated with or IgG isotype control mAb and one mouse treated with anti-CD8 α mAbs. Each dot represent a single cell. (A) CD8 β ⁺TCR β ⁺ T cells. (B) CD4⁺TCR β ⁺ T cells. (C) CD11b⁺ cells. (D) NK1.1⁺ cells. (E) TCR γ δ ⁺ T cells. (F) CD11c⁺ cells. Statistical comparisons; Students unpaired T-test (A-F). Error bars; standard deviation (SD). Statistical significance levels; not significant (ns) = $p > 0.05$, * = $P \leq 0.05$, ** = $P \leq 0.01$, *** = $P \leq 0.001$, **** = $P \leq 0.0001$.



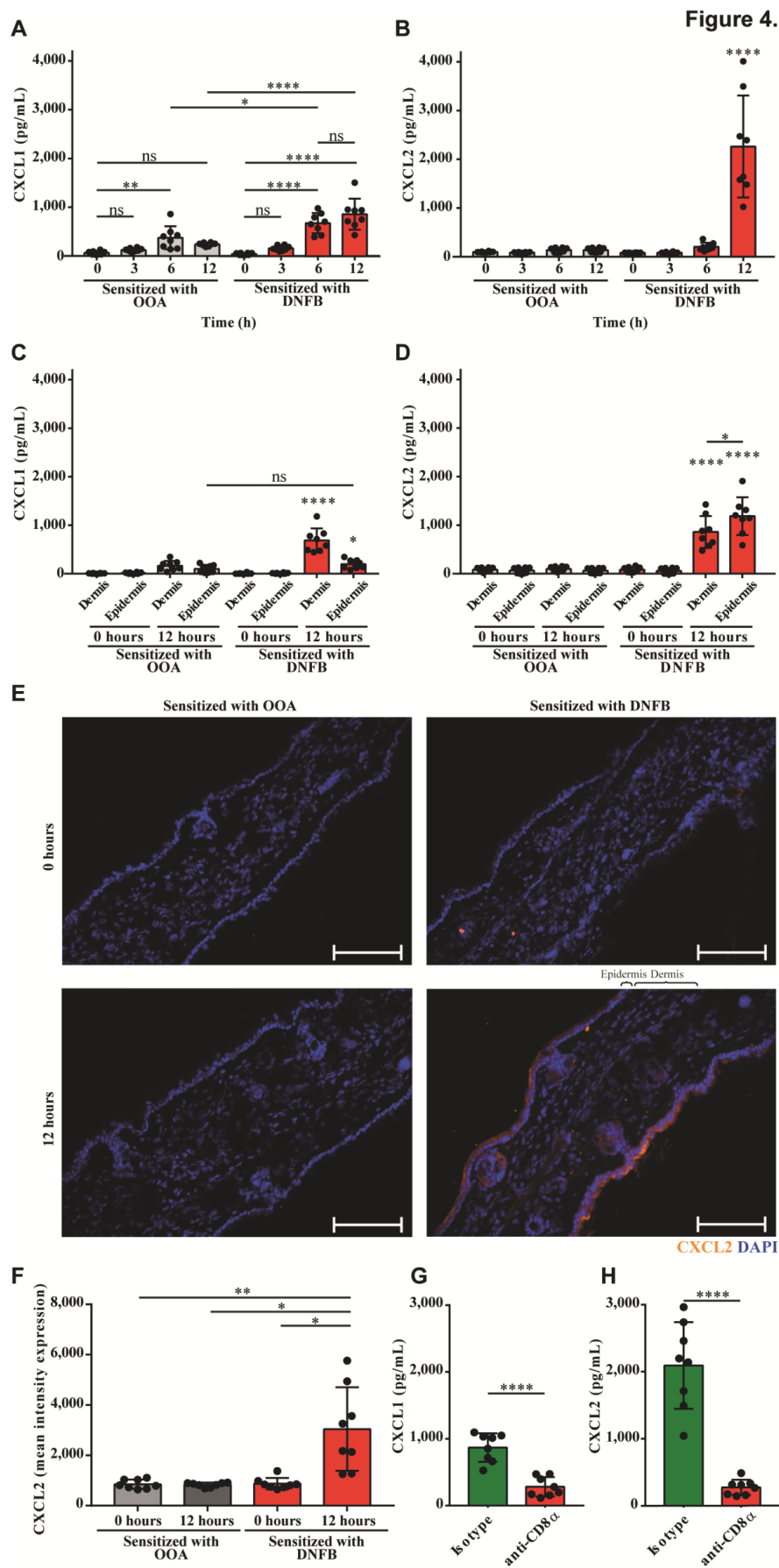
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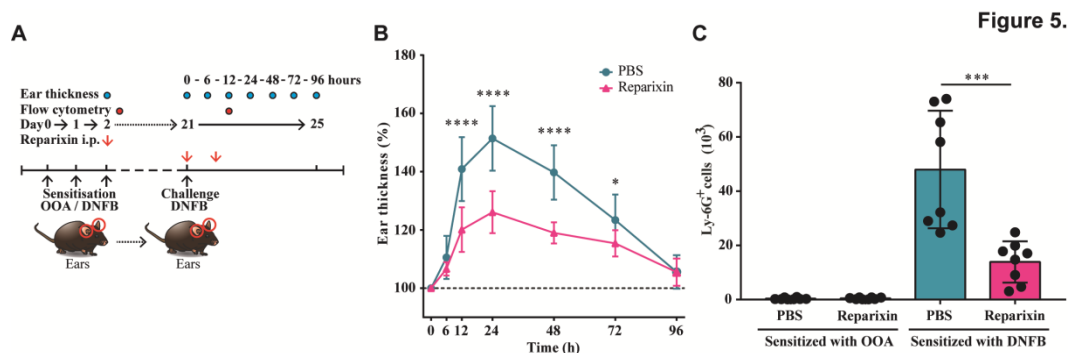
Figure 2.





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