

Rapid allergen-induced interleukin-17 and interferon- γ secretion by skin-resident memory CD8⁺ T cells

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Summary

Background. Skin-resident memory T (T_{RM}) cells are associated with immunological memory in the skin. Whether immunological memory responses to allergens in the skin are solely localized to previously allergen-exposed sites or are present globally in the skin is not clear. Furthermore, the mechanisms whereby T_{RM} cells induce rapid recall responses need further investigation.

Objectives. To study whether contact allergens induce local and/or global memory, and to determine the mechanisms involved in memory responses in the skin.

Methods. To address these questions, we analysed responses to contact allergens in mice and humans sensitized to 2,4-dinitrofluorobenzene and nickel, respectively.

Results. Challenge responses in both mice and humans were dramatically increased at sites previously exposed to allergens as compared with previously unexposed sites. Importantly, the magnitude of the challenge response correlated with the epidermal accumulation of interleukin (IL)-17A-producing and interferon (IFN)- γ -producing T_{RM} cells. Moreover, IL-17A and IFN- γ enhanced allergen-induced IL-1 β production in keratinocytes.

Conclusions. We show that sensitization with contact allergens induces a strong, long-lasting local memory and a weaker, temporary global immunological memory response to the allergen that is mediated by IL-17A-producing and IFN- γ -producing CD8⁺ T_{RM} cells.

Key words: allergic contact dermatitis; IFN- γ ; IL-1 β ; IL-17A; local memory; tissue-resident T cells.

Skin-resident memory T (T_{RM}) cells play an important role during recall responses in the skin, but whether the generation of memory T cells results in localized or

widespread global immunological memory in the skin is still unclear (1–9). Following skin infection with vaccinia virus, specific T_{RM} cells were detected both locally at the area of infection and at distant sites (6). Likewise, it has been reported that T_{RM} cells are recruited both to allergen-exposed and to non-exposed skin (2). In contrast, it was shown that herpes simplex virus infections only lead to the generation of local memory at the site of infection (4). The existence of isolated, site-specific memory responses to antigens is supported by the local flare-up reactions that can occur at previously affected skin areas in patients with allergic contact dermatitis (ACD) following systemic re-exposure to the allergen (10,

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11). ACD is a T cell-mediated skin disease. It is induced following exposure of the skin to contact allergens, and it is a common disease affecting 27% of the European population (12). More than 30 years ago, Scheper et al. showed that local memory responses to specific allergens could develop in a guinea-pig model of ACD, and that this memory was most likely mediated by T cells (13). However, whether the local memory was attributable to resident or circulating memory T cells was not determined.

Interleukin (IL)-1 β is a central cytokine in ACD (14–16). Exposure of the skin to contact allergens leads to rapid induction of IL1 β expression (17). IL-1 β is produced by various cells in the skin, including keratinocytes, Langerhans cells, dendritic cells, and mast cells (18). Contact allergens can trigger the production of Toll-like receptors (TLRs), leading to IL-1 β production and inflammation (19, 20). Interestingly, T cell subsets that are capable of producing IL-17 and interferon (IFN)- γ have been associated with ACD (21–25). The importance of these cytokines in ACD is underscored by an impaired response to allergens in IL-17 and IFN- γ -knockout (KO) mice (22).

In the present study, we investigated the role of T_{RM} cells in the response to contact allergens. By using a mouse model of ACD, we found much stronger memory responses in skin areas that had previously been exposed to the allergen than in skin areas that had not previously been exposed to the allergen. The memory responses were most probably mediated by CD8⁺ T_{RM} cells via their rapid production of IL-17A and IFN- γ , as both cytokines enhanced allergen-induced IL-1 β production in keratinocytes. We show that similar mechanisms are involved in memory responses to nickel in humans. Thus, in patients with allergy to nickel, we found a rapid response to nickel after re-exposure to nickel only on the exact same areas of the skin that previously had been exposed to nickel. Furthermore, we found that the magnitude of the challenge responses to nickel in humans correlated with local recruitment of epidermal CD8⁺ T cells and the production of IL-17A and IL-1 β .

Materials and Methods

Study subjects and skin samples

Ten individuals with previous positive patch test reactions to nickel were enrolled after informed, written consent had been obtained in accordance with the Declaration of Helsinki principles for research involving human subjects. The study was approved by the ethics committee of Copenhagen (H-15004317) and the Danish Data Protection Agency. The individuals were patch tested twice by the use of 8-mm Finn Chambers® on Scanpor® tape with

pet. or nickel sulfate 5% in pet. (20 mg; Trolab®, Almirall Hermal, Reinbek, Germany). Twenty-four h after the second patch test, the reactions were scored according to the ESCD guideline (26). Skin biopsies (4 mm) were subsequently taken from all four skin sites.

Mice

C57BL/6 and B6(Cg)Rag2tm1.1Cgn (RAG2-KO) mice were purchased from Taconic (Ry, Denmark) and Jackson (San Diego, CA, USA), respectively. Mice were housed in specific pathogen-free facilities in accordance with national animal protection guidelines (license number: 2012-15-2934-00663). Four mice were used in each experimental group in at least two independent experiments.

Contact sensitization to, and challenge with, allergens

Mice were sensitized for 3 consecutive days by topical administration on the dorsa of both ears with 25 μ l per ear of 0.15% 2,4-dinitrofluorobenzene (DNFB) (Sigma-Aldrich, Brøndby, Denmark) diluted in vehicle [olive oil/acetone (OOA), mixed 1:4]. For sensitization on the abdominal skin, mice were shaved 48 h prior to topical application of 50 μ l (Figs. 1 and 2) or 100 μ l (Fig. 4) of 0.15% DNFB for 3 consecutive days. In some experiments, mice were challenged by topical application of 25 μ l of 0.15% DNFB. The mice were killed by cervical dislocation.

Immunohistochemistry

Paraffin-embedded ears were cut into sections of 5 μ m, and stained with rabbit anti-mouse IL-1 β antibodies (H-153; Santa Cruz Biotechnology, Santa Cruz, CA, USA); this was followed by secondary staining with biotinylated goat anti-rabbit Ig antibodies (Vector Laboratories, Peterborough, UK), and counterstaining with Mayer's Hemalum. Sections of 3 μ m from human skin embedded in paraffin were incubated with mouse anti-CD8 (clone C8/144B; Nordic Biosite, Copenhagen, Denmark), anti-CD3 Optibody™ (clone BS103; 1:200; Nordic Biosite), or rabbit anti-IL-1 β (polyclonal; Proteintech, Manchester, UK).

For visualization of dinitrophenyl (DNP) conjugates, sections were treated with microwaves for 15 min in 10 mmol citrate buffer at pH 9, and this was followed by pre-incubation in 2% bovine serum albumin (BSA) for 10 min, and overnight incubation at 4°C with rabbit anti-DNP antibodies (D9656; Sigma-Aldrich). The samples were washed and incubated with goat anti-rabbit antibodies conjugated to Hilyte Fluor 488 (Novus Biologicals, Littleton, CO, USA).

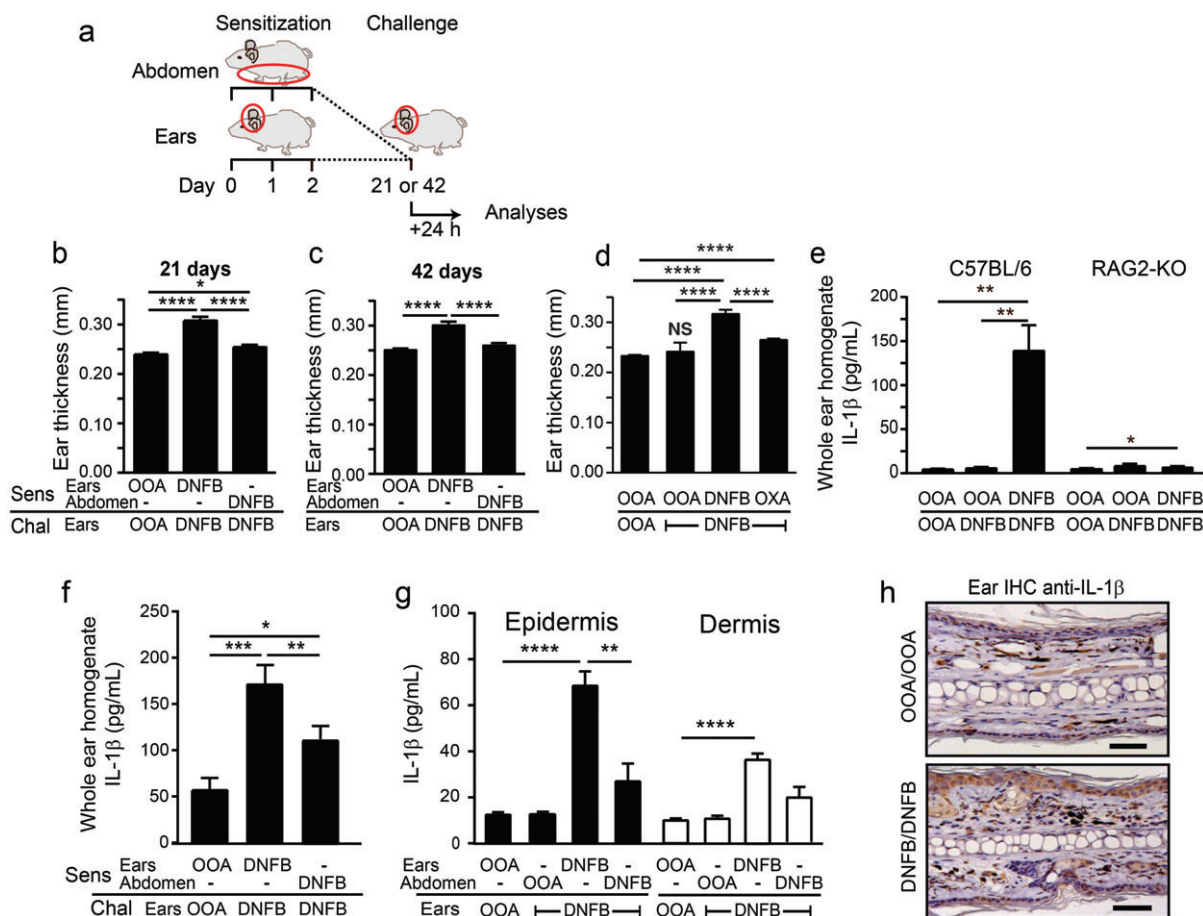


Fig. 1. A local immunological memory response to 2,4-dinitrofluorobenzene (DNFB) develops following exposure to DNFB. (a) C57BL/6 mice were sensitized and challenged. (b, c) Increase in ear thickness 3 weeks (b) and 6 weeks (c) after sensitization. (d) Increase in ear thickness in mice sensitized with either DNFB or oxazolone (OXA) following DNFB challenge. (e, f) Mice were sensitized with DNFB as described above, and challenged on the ears after 21 days. Interleukin (IL)-1 β levels were determined in ear homogenates by enzyme-linked immunosorbent assay. (g) The levels of IL-1 β in the epidermis and the dermis of challenged ears. (h) The distribution of IL-1 β determined by immunohistochemical (IHC) staining of ear sections. Scale bars: 100 μ m. The experiments were performed with 4 mice per group in two independent experiments. Error bars signify standard error of the mean. *t*-test: **** p < 0.0001; *** p < 0.0005; ** p < 0.001; * p < 0.05. KO, knockout; NS, not significant; OOA, olive oil/acetone.

Detection of DNP conjugates by western blot analysis

The ears were removed from the mice, snap-frozen in liquid nitrogen, and homogenized in lysis buffer [5 mM Tris base, 250 mM NaCl, 5 mM EDTA, 1% (vol/vol) Triton X-100, pH 7.4] with protease complete inhibitor cocktail (Roche, Hvidovre, Denmark) by use of the Precellys® technology (Bertin Technologies, Montigny-le Bretonneux, France) tissue homogenizer system at 4°C. Protein concentrations of the homogenates were determined with the Bradford assay. Samples were adjusted to 50 μ g per well, subjected to 10% sodium dodecylsulfate polyacrylamide gel electrophoresis (Bio-Rad, Copenhagen, Denmark) under reducing conditions, and blotted onto a poly(vinylidene

difluoride) membrane (Bio-Rad) by wet electrotransfer. Subsequent antigen probing was performed with rabbit anti-DNP-BSA antibodies (Sigma-Aldrich), followed by incubation with secondary horseradish peroxidase-conjugated swine anti-rabbit Ig antibodies (Dako, Glostrup, Denmark) and development with Western ECL substrate (GE Healthcare, Uppsala, Sweden).

Stimulation of PAM2.12 keratinocytes

The murine keratinocyte cell line PAM2.12 was maintained as previously described (14). Cells (1.5×10^5) were seeded in each well of a six-well plate in 2 ml of medium. After cells had been allowed to rest for 24 h, 10 ng/ μ l IFN- γ (Peprotech, Rocky Hill, NJ, USA), 10 ng/ μ l IL-17A

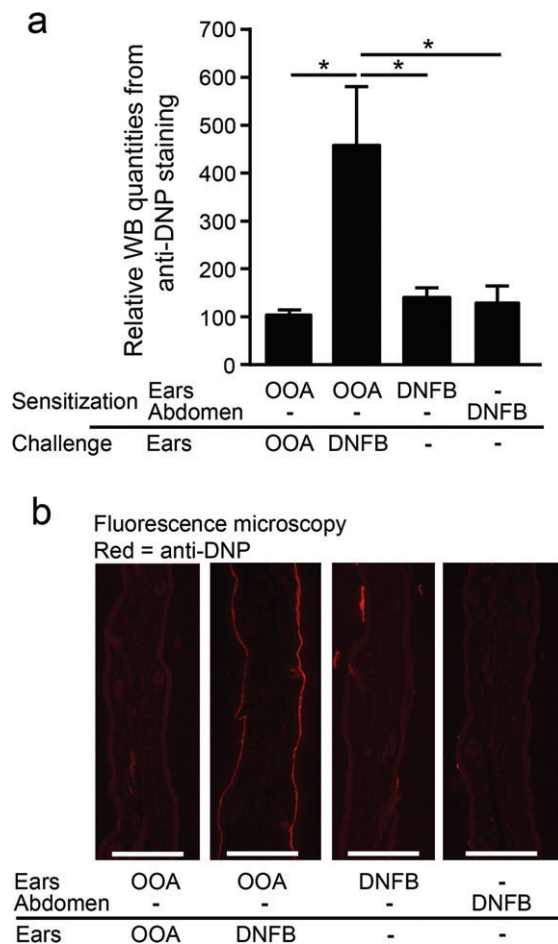


Fig. 2. The local memory response is not mediated by retention of 2,4-dinitrofluorobenzene (DNFB) in the skin. C57BL/6 mice were sensitized and challenged 21 days later with either DNFB or vehicle. (a) Quantification of the amounts of DNFB in ear homogenates determined by western blot (WB) analyses with anti-dinitrophenyl (DNP) antibodies to detect DNFB-modified proteins. (b) Immunohistochemical staining with anti-DNP antibodies of cross-sectioned ears. Scale bars: 200 μ m. The data shown are based on two independent experiments, each with 4 mice per group. Error bars signify standard error of the mean. *t*-test: **p* < 0.05. OOA, olive oil/acetone.

(R&D Systems, Minneapolis, MN, USA) and 0.25 μ g/ml 2,4-dinitrobenzenesulfonic acid hydrate (Sigma-Aldrich) were added as indicated to the cells. After incubation for 24 h, the cells were harvested and analysed.

IL-1 β measurements

Ear homogenates were prepared as previously described (14), and analysed for IL-1 β by enzyme-linked immunosorbent assay (eBioscience, San Diego, CA, USA).

Preparation of single-cell suspensions from the epidermis and fluorescence-activated cell sorting analysis

Single-cell suspensions were prepared from the epidermis as previously described (14). The cells were stained with anti-CD69 (clone H1.2F3), anti-CD8a (clone 53-6.7), anti-T cell receptor (TCR) β -BV421 (clone H57), anti-TCR $\gamma\delta$ -BV711 (clone GL3), anti-IFN- γ (clone XMGI.2), anti-IL-17A (clone TC11-18H10), and fixable viability dye efluor 780 (all from BD Pharmingen, Albertslund, Denmark). For intracellular cytokine staining, cells were stimulated with phorbol 12-myristate 13-acetate (1.25 μ g/ml; Sigma-Aldrich), ionomycin (625 ng/ml; Sigma-Aldrich) and monensin (2.08 μ g/ml; Sigma-Aldrich) in complete RPMI-1640 for 4 h at 37°C.

Real-time quantitative polymerase chain reaction (qPCR)

mRNA was purified as previously described (27). qPCR was performed with 1 μ g of RNA by use of the Revert Aid First strand cDNA synthesis kit (Thermo Scientific, Copenhagen, Denmark). For qPCR, TaqMan[®] Universal Master Mix II with UNG (Applied Biosystems, Nærum, Denmark) was used according to the manufacturer's instructions, with the glyceraldehyde-3-phosphate dehydrogenase (mouse, Mm03302249_g1; human, Hs04420697_g1) IL-1 β (mouse, Mm00434228_m1; human, Hs01555410_m1), IFN- γ (Mm01168134_m1), IL-17A (mouse, Mm00439618_m1; human, Hs00174383_m1) and CD8 (Mm01182107_g1) Taqman probes (Life Technologies, Carlsbad, CA, USA).

Statistics

Statistical comparisons between experimental groups were performed with the two-tailed *t*-test for two samples. The levels of significance are indicated on the figures.

Results

Local and global immunological memory responses to DNFB following exposure to DNFB

To investigate whether exposure to contact allergens leads to the development of local or global immunological memory in the skin, we compared the magnitude of the challenge responses in ears from mice sensitized on either the ears or the abdomen (Fig. 1a). As compared with control mice treated with OOA, we found a significant increase in ear thickness after challenge with DNFB, both in mice sensitized on the ears and in mice sensitized on the abdomen (Fig. 1b). However, the increase in ear thickness was threefold higher in mice sensitized on the ears

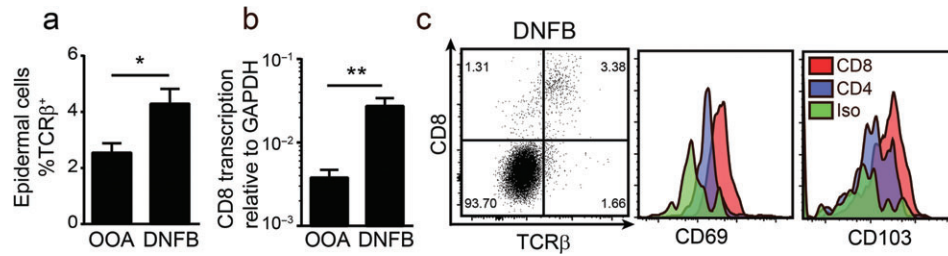


Fig. 3. Increased numbers of CD8⁺ skin-resident memory T cells in the epidermis following 2,4-dinitrofluorobenzene (DNFB) sensitization. C57BL/6 mice were sensitized on the ears with DNFB or olive oil/acetone (OOA) as a control. After 21 days, single-cell suspensions from the epidermis were analysed. (a) Cells were purified from the epidermis, and the proportion of T cell receptor (TCR)β⁺ cells was determined by flow cytometry. (b) CD8 mRNA expression relative to GAPDH expression as determined by qPCR. (c) Representative fluorescence-activated cell sorting plot of CD8 and TCRβ expression in epidermal single-cell suspensions, and histograms of CD69 and CD103 expression on TCRβ⁺ cells. Red histograms represent CD8⁺ TCRβ⁺ cells, and blue histograms represent CD4⁺ TCRβ⁺ cells. Error bars signify standard error of the mean. *t*-test: ***p* < 0.001; **p* < 0.05. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

than in mice sensitized on the abdomen. The local memory lasted for at least 6 weeks after sensitization, whereas global memory could not be detected at this point (Fig. 1c). Furthermore, the local memory seems to be dominantly hapten-specific, as only minor ear swelling was seen after challenge with DNFB in mice sensitized to oxazolone as compared with mice sensitized to DNFB (Fig. 1d). We could not detect DNFB in the skin 21 days after sensitization, and could thereby exclude the possibility that local retention of the allergen could explain our results (Fig. 2).

Next, we assessed the IL-1β levels induced in the skin by exposure to DNFB. We found a massive increase in the level of IL-1β following DNFB challenge on the ears in mice previously sensitized to DNFB on the ears as compared with control mice (Fig. 1e). Only a minor increase in the level of IL-1β was found in the ears of mice following a single exposure of the ears to DNFB (Fig. 1e). To determine whether memory responses to DNFB were dependent on the adaptive immune system, we determined IL-1β production in the ears following exposure and re-exposure to DNFB in RAG2-KO mice. In contrast to wild-type C57BL/6 mice, RAG2-KO mice showed only minute IL-1β production following DNFB challenge, indicating that the adaptive immune system is required for the strong memory response to DNFB in wild-type mice (Fig. 1e).

To determine whether the site of sensitization influenced the local production of IL-1β, we sensitized mice with DNFB on either the ears or the abdomen and challenged them on the ears (Fig. 1a). Correlating with the ear thickness, we found significantly higher production of IL-1β in the ears of mice that had been sensitized on the ears than in the ears of mice that had been sensitized on the abdomen (Fig. 1f). We found that the majority of the IL-1β was located in the epidermis, independently of whether mice were sensitized on the ears or the abdomen (Fig. 1g, h).

Increased numbers of CD8⁺ T_{RM} cells in the epidermis following DNFB sensitization

Next, we investigated whether the site-specific challenge response could be ascribed to allergen-induced accumulation of CD8⁺ T_{RM} cells. To this end, we exposed mice to OOA or DNFB for 3 consecutive days on the ears, and 21 days after this we purified the cells from the epidermis and determined the distribution of TCRβ⁺ T cells. We found a significant increase in the number of TCRβ⁺ T cells in the epidermis of mice sensitized to DNFB as compared with control mice (Fig. 3a). In addition, we determined the expression level of CD8 in the epidermis by qPCR. We found a significant increase in the CD8 expression level in the epidermis from mice sensitized to DNFB (Fig. 3b). To characterize the CD8⁺ T cells found in the epidermis, we co-stained αβTCR⁺ CD8⁺ T cells for CD69 and CD103, which are known to be expressed by CD8⁺ T_{RM} cells (4, 28). We found that the majority of the αβTCR⁺ CD8⁺ T cells in the epidermis exposed to DNFB expressed both CD69 and CD103, indicating that these cells were CD8⁺ T_{RM} cells (Fig. 3c).

DNFB-induced CD8⁺ T_{RM} cells produce IL-17A and IFN-γ

It is known that IL-17A and IFN-γ augment allergen-induced production of IL-1β (22, 23). Therefore, we wanted to determine whether DNFB-induced CD8⁺ T_{RM} cells produce these cytokines, and thereby could be responsible for the strong memory response. Because relatively few cells can be purified from the epidermis of the ears, we sensitized mice on the abdomen for 3 consecutive days with DNFB or with OOA as a control. After 21 days, we purified cells from the abdominal epidermis and analysed them. As observed in the ears, the proportion of TCRβ⁺ cells was markedly increased in the epidermis from the abdomen after DNFB sensitization (Fig. 4a).

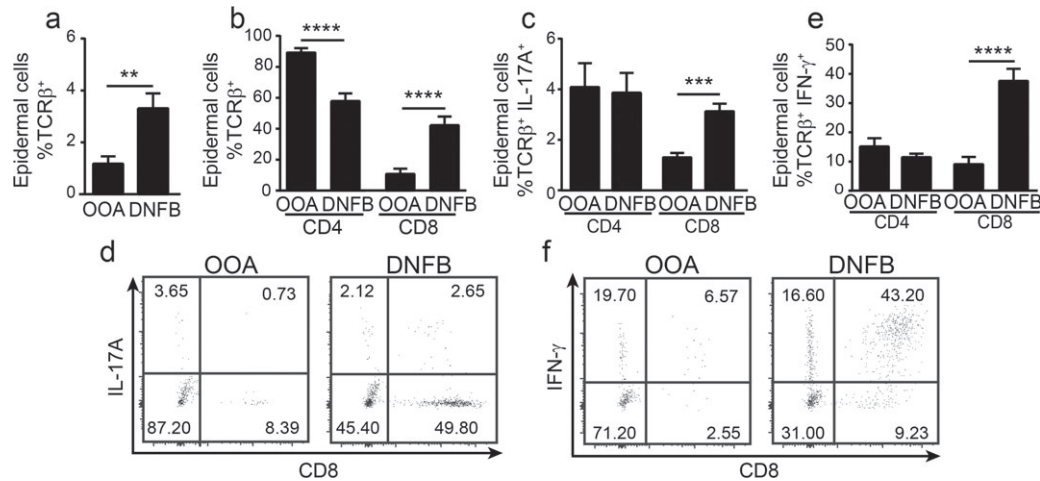


Fig. 4. 2,4-Dinitrofluorobenzene (DNFB)-induced CD8⁺ skin-resident memory T cells produce interleukin (IL)-17A and interferon (IFN)-γ. C57BL/6 mice were sensitized on the abdomen for 3 consecutive days. After 21 days, the cells were purified from the epidermis, and T cell receptor (TCR)β⁺ cells were analysed further by flow cytometry. (a) The frequency of TCRβ⁺ cells. (b) The distribution of CD4⁺ and CD8⁺ T cells (gate as CD8⁻ TCRβ⁺ and CD8⁺ TCRβ⁺ cells, respectively) in the TCRβ⁺ cell population. (c) The frequency of IL-17A-producing CD4⁺ and CD8⁺ TCRβ⁺ cells. (d) Representative dot plots of TCRβ⁺ cells stained for CD8 and IL-17A. (e) The frequency of IFN-γ-producing CD4⁺ and CD8⁺ TCRβ⁺ cells. (f) Representative dot plots of TCRβ⁺ cells stained for CD8 and IFN-γ. Error bars signify standard error of the mean. *t*-test: *****p* < 0.0001; ****p* < 0.0005; ***p* < 0.001. OOA, olive oil/acetone.

Likewise, CD8⁺ T cells represented the vast majority of the allergen-induced accumulation of TCRβ⁺ cells in the epidermis (Fig. 4a,b). Next, we determined the capacity of the epidermal T cells to produce IL-17A and IFN-γ. A twofold increase in the proportion of CD8⁺ IL-17A⁺ T cells was found in the epidermis exposed to DNFB as compared with the epidermis exposed to OOA (Fig. 4c,d). In parallel, we found a fourfold increase in the proportion of IFN-γ-producing CD8⁺ T cells in the epidermis exposed to DNFB as compared with the epidermis exposed to OOA (Fig. 4e,f). Interestingly, DNFB treatment did not change the distribution of either CD4⁺ IL-17A⁺ T cells or CD4⁺ IFN-γ⁺ T cells found in the epidermis (Fig. 4c–f).

To further investigate the interplay between IL-1β, IFN-γ and IL-17A during the local memory response to contact allergens, we monitored the time-course of *IL1β*, *IFNγ* and *IL17A* expression in the epidermis following exposure to DNFB in mice that were or were not sensitized to DNFB. Both *IL1β* and *IFNγ* expression were detectable in the epidermis of non-sensitized and sensitized skin prior to the challenge with DNFB (0 h), with the highest expression levels being found in sensitized skin. In contrast, *IL17A* expression was not detectable at any time-point in non-sensitized mice (Fig. 5a–c). An increase in *IL1β* expression was found 4 h after challenge in both non-sensitized and sensitized skin (Fig. 5a). In sensitized skin, *IL1β* expression further increased after 24 h, whereas a plateau was reached after 4 h in non-sensitized epidermis (Fig. 5a). Interestingly, whereas the expression

of *IFNγ* did not change in non-sensitized epidermis upon DNFB challenge, a 50-fold increase in *IFNγ* expression was found 4 h after DNFB challenge in sensitized epidermis (Fig. 5b). Furthermore, whereas *IL17A* expression could not be detected in non-sensitized epidermis upon DNFB exposure, *IL17A* expression could be detected in sensitized epidermis 4 h after DNFB exposure, and this level was stable for at least 24 h after the challenge (Fig. 5c).

Finally, we determined whether IFN-γ and/or IL-17A had an effect on DNBS-induced *IL1β* expression in keratinocytes. We stimulated cultures of PAM2.12 cells with IFN-γ, IL-17A, or DNBS, or combinations of these, for 24 h, and then determined *IL1β* expression. Whereas IFN-γ treatment did not induce *IL1β* expression (Fig. 5d), IL-17A induced a twofold increase in *IL1β* expression (Fig. 5d). As expected from our previous results, DNBS induced increased expression of *IL1β* (Fig. 5d) (14). Addition of IFN-γ, IL-17A or, especially, the combination of the two during DNBS stimulation increased *IL1β* expression as compared with the *IL1β* expression induced by DNBS stimulation alone (Fig. 5d).

Patients with nickel allergy have distinctly localized memory responses to nickel at previously exposed sites

Next, we wanted to determine whether the local memory response to DNFB in mice translated to similar conditions in humans. To investigate this, we recruited 10 patients

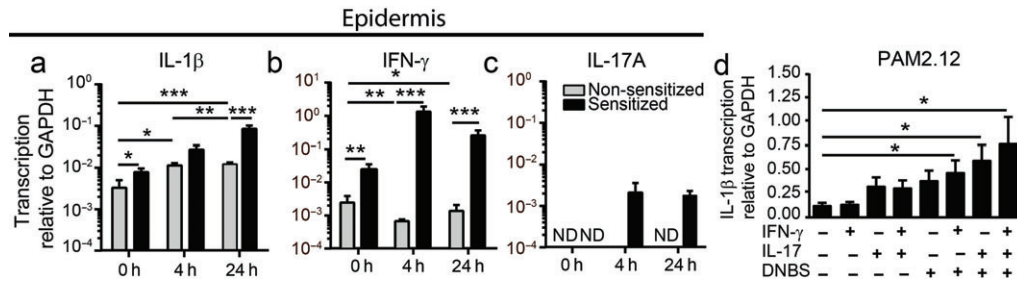


Fig. 5. Interleukin (IL)-17A and interferon (IFN)- γ levels increase rapidly during memory responses. Mice were either left untreated or sensitized with 2,4-dinitrofluorobenzene (DNFB) on the ears for 3 consecutive days. After 21 days, the epidermis from groups of non-sensitized (grey bars) or sensitized (black bars) mice were analysed before challenge (0 h) or 4 or 24 h after challenge with DNFB for expression of (a) *IL1 β* , (b) *IFN γ* and (c) *IL17A* by real-time quantitative polymerase chain reaction (qPCR). (d) PAM2.12 cells were treated with 0.25 μ g/ml 2,4-dinitrobenzenesulfonic acid hydrate (DNBS), 10 ng/ml IFN- γ or 10 ng/ml IL-17A, either alone or at the indicated combinations. After 24 h, the induction of *IL1 β* expression was assessed by qPCR. Error bars signify standard error of the mean. *t*-test: ****p* < 0.0005; ***p* < 0.001; **p* < 0.05. ND; not detectable.

with allergy to nickel, and patch tested them with either nickel or pet. as a control at areas previously unexposed to nickel, as shown in Fig. 6a. After 21 days, the patients were challenged on exactly the same skin areas with a second patch test, as indicated in Fig. 5a, and after 24 h the reactions were scored. Visible reactions were only found on skin areas that had been exposed to nickel both in the first and the second patch tests (Fig. 6b and Table 1). Next, we examined the expression level of *IL1 β* in the different test areas. Interestingly, we found increased *IL1 β* expression in all areas that had been exposed to nickel during either the first test, the second test, or both tests (Fig. 6c). However, the expression level of *IL1 β* was highest in skin that had been exposed to nickel during both the first and the second patch tests (Fig. 6c). As in the ACD mouse model, we found that IL-1 β in human ACD primarily was located in the epidermis (Fig. 6d). Interestingly, IL-17A was found in four of seven skin areas that had been exposed to nickel during both the first and the second patch tests, but in only one of 14 skin areas that had been exposed to nickel only once (Table 1 and Fig. 6c). The presence of IL-17A correlated with the clinical score and the levels of IL-1 β . Finally, we found that CD8⁺ T cells were recruited to the interphase between the epidermis and the dermis after exposure to nickel during the first patch test (Fig. 6d).

Discussion

In this study, we show that exposure to contact allergens leads to a strong site-specific and a weaker global immunological memory response to the allergen in the skin. Thus, we found increased ear swelling and levels of IL-1 β in the ears of mice that were both sensitized and challenged on the ears as compared with mice

that were sensitized on the abdomen and challenged on the ears. We show that allergen-induced local memory is associated with the accumulation in the epidermis of CD8⁺ T_{RM} cells that are able to produce IL-17A and IFN- γ . Furthermore, we found that IFN- γ and, especially, IL-17A enhanced allergen-induced IL-1 β production in keratinocytes. Finally, we show the existence of a distinct local memory response to nickel in the skin of patients with allergy to nickel. The inflammatory memory response in the patients correlated with accumulation of CD8⁺ T cells in the interphase between the dermis and epidermis, and with the presence of increased levels of IL-1 β and IL-17A in the affected skin.

In accordance with a recent study by Gaide et al., we found that T_{RM} cells accumulate in the skin following DNFB sensitization (2). Studies using DNFB, virus or *Leishmania major* infections have shown that T_{RM} cells are not only recruited locally, but also become globally seeded in the skin (2, 6, 29, 30). However, even though T_{RM} cells were seeded globally in the skin, the challenge responses were more pronounced in skin areas previously exposed to the specific antigen (2, 4, 6). In contrast, another study found that, following infections with herpes simplex virus, only localized seeding of T_{RM} cells in the infected skin took place (4). We found that sensitization to DNFB induced a strong and long-lasting local memory response and a weaker, temporary global memory response to DNFB in the skin. In contrast to our findings, Gaide et al. found that the global skin memory response to DNFB was long-lasting (2). Several conditions may explain these differences. First, Gaide et al. determined the response by investigating the presence of various T cell clones, whereas we determined the inflammatory response (2). Furthermore, Gaide et al. used a higher dose of DNFB and a smaller area of exposure than we

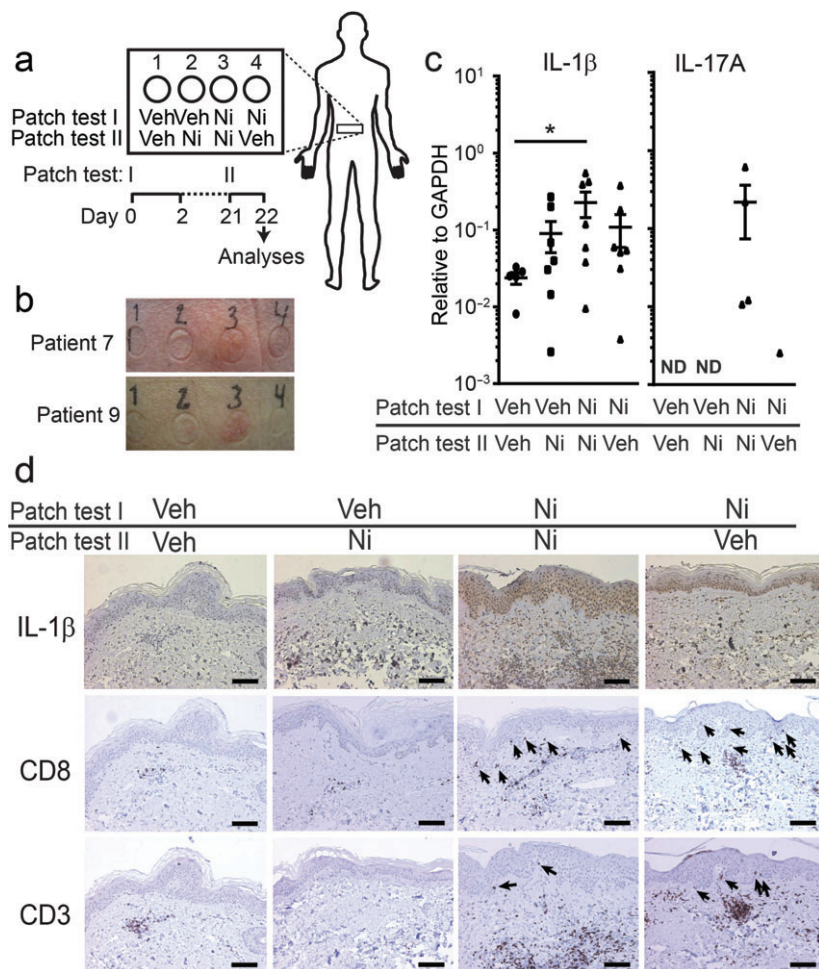


Fig. 6. Patients with nickel allergy have distinctly localized memory responses to nickel at previously exposed sites. Ten individuals with allergy to nickel were patch tested twice with either 5% NiSO₄ in pet. (Ni) or pet. (Veh) at four precisely defined skin areas with a 21-day interval. Twenty-four hours after application of the second patch test, the local immune responses were analysed. (a) Schematic model for the experimental setup. (b) Photograph of the test area from 2 representative individuals 24 h after application of patch test II. (c) Expression levels of IL1 β and IL17A in the punch biopsies from 7 individuals. (d) Histological staining for interleukin (IL)-1 β , CD8 and CD3 was performed on biopsies from 3 individuals. Arrows indicate cells positive for CD8 and CD3 staining. Representative histological stains are shown. *t*-test: **p* < 0.05. GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ND.

did, and these parameters are known to be critical for sensitization (2, 31, 32). In addition, Gaide et al. boosted the response, which most likely affected the generation of long-lasting global memory (2). This is in accordance with studies showing that allergen-induced inflammatory responses correlate with the allergen dose (33), and that the level of the inflammatory response plays an important role in memory development (34, 35). Our results from the patients with allergy to nickel strongly supported the existence of very distinct local memory responses to allergens. Although these patients had been exposed to nickel many times at other skin sites, they did not show memory responses at previously unexposed skin areas, but developed a strong memory response exactly at the site that had been previously exposed to nickel.

In addition to T_{RM} cells, peripheral memory T cells are also generated following exposure of the skin to contact allergens (2). An allergic individual can be tested for allergic responses by patch testing various areas of the skin.

The response following patch testing is normally evaluated 72 h after application of the test. In this study, we evaluated the response 24 h after the second nickel test. We find it likely that the response seen in the skin 24 h after exposure to the allergen is primarily mediated by T_{RM} cells. In contrast, the response seen 72 h after exposure most probably results from the combined effects of T_{RM} cells and peripheral memory T cells. However, this notion needs further investigation.

CD8⁺ T_{RM} cells are known to produce IFN- γ rapidly in response to antigen challenge, thereby putting the local tissue in an alerted state via the induction of proinflammatory cytokines and chemokines (1, 36). We found that, although the expression of IL1 β increased following exposure to DNFB in both non-sensitized and sensitized skin, the increase was 10-fold higher in sensitized skin than in non-sensitized skin. This is in good agreement with the ability of contact allergens to induce IL1 β expression via two pathways, namely by an innate pathway involving TLRs, and by a pathway-dependent on effector

Table 1. Correlation between clinical score, IL-17A and IL-1 β .

Patient no.	Age (years)	Sex	Clinical score				Ni/Ni	
			Patch test II				Transcription relative to GAPDH	
			Veh/Veh	Veh/Ni	Ni/Ni	Ni/Veh	IL-17A	IL-1 β
1	58	Female	–	–	–	–	0.669	0.392
2	69	Female	–	–	–	–	Not detectable	0.038
3	43	Female	–	–	++	–	0.235	0.551
4	45	Female	–	–	–	–	Not detectable	0.010
5	55	Female	–	–	++	–	0.011	0.119
6	67	Male	–	–	–	–	Not detectable	0.060
7	56	Female	–	–	+++	–	0.012	0.426
8	50	Female	–	–	+	–	Used for IHC	Used for IHC
9	75	Female	–	–	+	–	Used for IHC	Used for IHC
10	56	Female	–	–	–	–	Used for IHC	Used for IHC

GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IHC, immunohistochemistry; IL, interleukin; Veh, vehicle.

cytokines produced by T cells (22, 37). In contrast to *IL1 β* expression, increased expression of *IFN γ* and induction of *IL17* after DNFB exposure were only found in sensitized skin, indicating that these cytokines are produced by memory cells recruited to the skin as a consequence of the sensitization. As we found an increased percentage of CD8⁺ T_{RM} cells producing IL-17A and IFN- γ in the epidermis 21 days after sensitization, we think it likely that these cells are the cells mediating the increased expression levels of *IL17A* and *IFN γ* found in sensitized skin following challenge with DNFB. Although only a minority of T_{RM} cells produce IL-17A as compared with IFN- γ , these might still play an important role in enhancing allergen-induced IL-1 β production, as IL-17A seems to be a potent enhancer of DNBS-induced IL-1 β production. This is also supported by the observation that blocking IL-17A signalling has a stronger effect on allergen-induced IL-1 β production than blocking IFN- γ signalling (22, 23). The correlation that we found between the expression levels of *IL1 β* and *IL17* in the skin of the patients with allergy to nickel also supports the importance of IL-17A-producing T_{RM} cells in memory responses to allergens. However, in 1 of the patients (patient 1), we found high expression levels of *IL1 β* and *IL17* but no clinical symptoms. The reason for this is

unknown, but might be related to the fact that the patient was treated with prednisone for other medical reasons in between the two nickel tests.

In their response to skin infections, T_{RM} cells most probably play an important and beneficial role (1, 4, 6). It has been shown that virus-specific T_{RM} cells in the skin can trigger a state of pathogen alert, increasing the response to unrelated pathogens following co-exposure to the specific and unrelated pathogen (1). In contrast to the beneficial role during infections, one might expect that the same mechanism increases the risk of developing ACD to contact allergens when skin is simultaneously exposed to a mixture of contact allergens. For example, fine fragrances often consist of 10–100 or even more potential contact allergens (38), and perfume allergy could thus be an example of 'ACD spreading'. In relation to this, it is surprising that as many as 4.1% of the population have contact allergy to one or more fragrance allergens, as these are generally classified as weak to moderate contact allergens (39). We suggest that, once allergen-specific T_{RM} cells are present in the skin, they will increase the risk of the development of additional allergies, as they readily induce a strong inflammatory response when new allergens are presented together with the allergen that originally induced the ACD.

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