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Inhibitory checkpoint receptors control CD8+ resident memory T cells to prevent skin allergy

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36 **Abbreviations**

37 ACD: Allergic contact dermatitis

38 Ag: Antigen

39 DC: Dendritic cells

40 DNFB: 2,4-dinitrofluorobenzene

41 DNP: Dinitrophenyl

42 dLN: Draining lymph node

43 DT: Diphtheria toxin

44 iDTR: Inducible diphtheria toxin receptor

45 IFN- γ : Interferon gamma

46 CHS: Contact hypersensitivity

47 ICR: Inhibitory checkpoint receptor

48 OXA: Oxazolone

49 Tcm: Central memory T cell

50 Tem: Effector memory T cell

51 Trm: Resident memory T cell

52

Abstract

Background: Tissue-resident memory T cells (Trm) are detrimental in numerous chronic inflammatory diseases, including allergic contact dermatitis (ACD).

Objectives: To analyze the contribution of Trm to the chronicity and severity of ACD and to define the local parameters regulating their development and functions.

Methods: We used an experimental model of ACD, i.e. contact hypersensitivity (CHS) to 2,4-dinitrofluorobenzene (DNFB) which is mediated by CD8+T cells.

Results: Our data show that early effector T cells accumulated in the skin during the acute CHS reaction and gave rise to epidermal CD8+ Trm expressing a specific set of immune checkpoint receptors (ICR), such as PD-1 and Tim-3. Those Trm remained in the epidermis for several weeks and mediated the eczema exacerbations, which developed upon allergen re-exposure without the contribution of circulating specific T cells. Furthermore, allergen-induced Trm reactivation was constrained, since treatment with ICR antagonists dramatically enhanced the magnitude and severity of eczema exacerbations. Finally, we show that the persistence of the allergen in the epidermis for long periods of time was responsible for both the development and maintenance of epidermal Trm as well as the sustained expression of ICR.

Conclusion: Although CD8+Trm cells are key for the pathophysiology of ACD, intrinsic mechanisms control their reactivation to prevent damaging immunopathology. Developing strategies targeting the reactivation of skin Trm *in situ* via their ICR should open new perspectives for the treatment of ACD.

Keywords: Resident memory T cells (Trm); eczemas; allergic contact dermatitis; inhibitory check point receptors; immunopathology.

Key Messages

- High numbers of hapten-specific CD8⁺ T_{rm} accumulate at the sites of healed eczema lesions in response to the long-term persistence of hapten moieties in the skin.

- CD8⁺ T_{rm} are key for the chronicity, severity and recurrence of the pathology.
- However, they express several inhibitory receptors, such as PD-1 or TIM-3, which keep them in check to preserve skin integrity and to avoid the development of severe immunopathology.

Capsule summary

The present study confirms the crucial role of epidermal CD8⁺ T_{rm} for the recurrences of allergic contact dermatitis and highlights the contribution of immune checkpoint receptors on CD8⁺ T_{rm} to prevent eczema flares upon allergen exposure.

Introduction

Resident memory T cells (Trm), which are classically identified by their stable expression of CD69 and CD103¹⁻⁴, constitute a recently described subset of memory T cells that do not re-circulate throughout the organism. They persist long-term within peripheral tissues and organs, where they organize an optimal front-line defense against invading pathogens or neoplastic cells^{1, 5, 6, 7, 8}.

Beyond their demonstrated active contribution to host protection, there is an emerging understanding that Trm also participate in several autoimmune/inflammatory diseases, in which they can instigate or augment the immunopathology^{9,10}. Trm have been described in different skin diseases such as psoriasis, vitiligo³, fixed drug eruption¹¹ or leukemic cutaneous T cell lymphoma^{12, 13}.

Allergic contact dermatitis (ACD) is a common chronic and relapsing T cell-mediated allergic skin disease. ACD expresses as eczema flares (also referred to as exacerbations), which consist in the worsening of the skin inflammation upon allergen exposure. Exacerbations usually develop on the same body sites, i.e. on previously affected but healed skin¹⁴. This observation has led to the concept of “local memory to allergens”, postulating that specific T lymphocytes (i) persist for several months in previous sites of eczema and, (ii) are important in flare-up reactivity and in chronic inflammation^{14, 15}. Recent works have shown that skin Trm are responsible for the development of these early and intense ACD flares¹⁶⁻¹⁸. However, little is known regarding the functional phenotype of Trm, their long term persistence, the antigen requirement for their development and survival, or the mechanisms which control their reactivation upon allergen exposure.

In this study, using the experimental model of contact hypersensitivity (CHS) to the chemical allergen 2,4-dinitrofluorobenzene (DNFB)¹⁹, we confirm the crucial role of epidermal CD8+ Trm in eczema exacerbations and we precisely define their phenotype and specificity. More importantly, we demonstrate that intrinsic cell mechanisms, i.e. the expression of inhibitory checkpoint receptors, prevent their uncontrolled reactivation to avoid eczema flares upon allergen exposure.

Methods:

A brief description of the Methods is provided below. See additional details in the Supplemental Methods section.

Animals and reagents

Mice, allergens (2,4-dinitrofluorobenzene [DNFB], 2,4-dinitrobenzenesulfonic acid [DNBS], or oxazolone) or mAbs used in this study are reported in the Supplementary Method section.

Contact hypersensitivity to DNFB and flare-up reactions

For most experiments, CHS was induced as previously described²⁰. Briefly, animals were sensitized epicutaneously on the shaved abdomen with 25µl of 0.5% DNFB on day -5, and were challenged on the left ear on day 0 with 2x5µl 0.13% DNFB while the right ear received the acetone/olive oil vehicle. Allergic animals were next retested at day 30, or months 3, 6 and 12, with different doses (0.01, 0.05 and/or 0.13%) of DNFB or with 1% OXA, both on healed (left ear) and/or naïve (right ear) skin, as indicated.

In some experiments, DNFB-allergic C57BL/6 or reconstituted CD3ε^{-/-} mice were depleted of circulating or of skin CD8⁺ T cells by anti-CD8 mAbs or diphtheria toxin (DT) injections, and treated with anti-PD-1 (RMP1-14, 200µg/ml), anti-TIM-3 (RMT3-23, 200µg/ml) or respective IgG2a isotype (2A3, 400 µg/ml) (all from BioXCell, West Lebanon, USA) at indicated time points.

Results

Allergen-specific CD8⁺ Trm persist for months within the epidermis and dermis of healed CHS lesions

In this study, we used DNFB allergic mice, which were sensitized (day-5) on the abdomen and challenged 5 days later (day 0) on the left ear with DNFB, leading to a CHS reaction peaking at day 2 and resolving by day 15 (**Fig.1A**). DNFB allergic mice were then analyzed at different time points after the resolution of CHS (day 30, months 2, 6 and 12) for the presence of Trm, as well as for their functional properties. In this model, the left ear, which received DNFB at day 0 and developed a CHS response at day 2 is called the “healed skin”, whereas the right ear which received the vehicle and did not develop CHS is called the “naive skin”. At day 30 and thereafter, naïve and healed skin look identical without any clinical or histological sign of inflammation.

To confirm that Trm persist at the sites of CHS skin inflammation^{16, 17, 18}, healed and naive skin of DNFB-allergic mice were first collected at day 30 and processed for the presence of CD8⁺ and/or CD4⁺ T cell subsets by FACS and immunofluorescence microscopy. While the epidermis and the dermis of naive skin were nearly exclusively seeded with CD4⁺ T cells, increased numbers of CD8⁺ T cells were recorded in healed CHS lesions (**Fig.1B and Fig.S1**). Approximately 70% of epidermal and dermal CD8⁺ T cells co-expressed the two canonical Trm markers CD103 and CD69²¹, emphasizing the residency properties of these cells (hereafter referred to as Trm) (**Fig.1C**).

Time course experiments next demonstrated that CD8⁺Trm progressively accumulated in the epidermis during the resolution of acute CHS inflammation, with a gradual increase between day 2 and day 15 post DNFB challenge (**Fig.S2A**). Trm numbers then stabilized between days 15 and 30, along with complete resolution of the skin inflammation, before they underwent a progressive and profound attrition up to 12 months post challenge (**Fig.1D**). Of note, depletion of circulating, but not of skin-resident CD8⁺ T cells by anti-CD8⁺ mAb treatment at day 2 post challenge, did not change the numbers of epidermal Trm recorded in the skin (**Fig.S2B&C**), indicating that skin Trm mainly derive from *in situ* proliferation of early effector cells present at the peak of the CHS response (**Fig.S2D**).

To determine the proportion of allergen-specific Trm among CD8⁺ Trm

present in the healed lesion of DNFB-allergic mice, skin CD8⁺ and CD4⁺ T cell subsets were subsequently collected at 30 days, FACS sorted and re-activated *ex vivo* with allergen-pulsed bone marrow-derived dendritic cells (BMDCs). The following day, the number of spot forming cells (SFCs) producing IFN- γ , IL-17a or IL-4 was determined by enzyme-linked immunospot (ELISpot) assay. While approx. 1% of epidermal and 0.2% of dermal CD8⁺ T cells of healed CHS lesions were antigen-specific and produced IFN- γ (**Fig.1E**), IL-17a- or IL-4- were not detected (**data not shown**). Comparatively, the frequency of DNFB-specific cells among total CD8⁺ T cells from dLNs, which comprise a mix of effector (Tem) and central (Tcm) memory T cells and dLN Trm, was <0.1%.

Time course experiments revealed a progressive and significant reduction in the numbers of DNFB-specific cells during the months following DNFB challenge, with an approx. 10 fold decrease at month 12, when compared to day 30 (**Fig.1F**).

Thus, these data show that healed CHS lesions are enriched for allergen-specific CD8⁺ Trm which persist in the skin for months.

CD8⁺ Trm mediate DNFB-induced flare-up reactions

We next examined the contribution of CD8⁺ Trm to the allergen-induced eczema flares. When DNFB-allergic animals were re-challenged on the healed skin at day 30 with a standard dose of 0.13%, they developed potent flare-up reactions, with a more severe magnitude than the response induced on naive skin and the background response induced in non-allergic animals (**Fig.2A, left part**). The flare-up CHS reactions were antigen-specific, since application of the irrelevant strong allergen oxazolone (OXA) on the healed or naive skin of DNFB-allergic animals triggered only background responses of similar magnitude to the one induced in non-allergic animals (**Fig.2A, right part**).

To demonstrate the contribution of DNFB-specific CD8⁺ Trm to the exaggerated reaction recorded in healed lesions upon allergen-retest, we developed an original inducible diphtheria toxin receptor (iDTR)-based approach, using DNFB-allergic CD3 ϵ ^{-/-} lymphopenic recipient mice, reconstituted prior to the induction of DNFB-CHS with iDTR-CD8⁺ and WT-CD4⁺ T cells collected by FACS sorting from iDTR/CD4^{cre} and C57BL/6 donor mice, respectively. Flare-up reactions upon retest were completely abrogated in recipient mice injected with diphtheria toxin (DT) (one

day before re-challenge), while untreated or anti-CD8 mAb treated control mice developed a full flare-up response (**Fig.2B**). Conversely to anti-CD8b mAb treatment which only depletes circulating CD8+ T cells, DT injection depleted all the circulating and skin CD8+ T cells, while sparing non-CD8+ T cells in these recipients (**Fig.S3**).

Our results thus show that antigen-specific CD8+ Trm are of prime importance for the development of recurrent flare-up reactions recorded on healed lesions of DNFB-allergic mice.

CD8+ Trm are hypo-responsive to low DNFB doses

We next examined flare-up reactions induced in allergic animals by low DNFB doses, i.e. below the standard dose of 0.13%. As expected, repeated applications of low DNFB doses on healed skin induced an intense flare-up reaction. It required 3 consecutive administrations of DNFB 0.05% (on days 0, 2, and 6) to generate flares of similar magnitude to the one provoked by a single optimal dose (**Fig.3A**). By contrast, 6 consecutive administrations (on days 0, 2, 6, 8, 13, and 15) of DNFB 0.01% failed to induce a flare-up reaction. On naïve skin, 6 consecutive administrations of DNFB 0.05% (on days 0, 2, 6, 8, 13 and 15) did not induce significant flares (**data not shown**). These data indicate that, although specific Trm are seeded in the skin at significant density, they are actively restrained to prevent excessive immunoreactivity in response to DNFB application below a certain threshold (**Fig.3A**).

Epidermal CD8+ Trm express a restricted set of inhibitory check point receptors

In this respect, we investigated the expression of different inhibitory checkpoint receptors (ICRs) by skin CD8+ Trm²². We noticed that these cells expressed high levels of PD-1²³, TIM-3²⁴ or 2B4²⁵ (**Fig.3B**), as well as CD5²⁶, but not TIGIT²⁷, LAG-3, CTLA-4²⁸ (**Fig.S4**), compared to CD8+ Tem and Tcm or naïve CD8+ T cells isolated from the dLNs of DNFB-allergic mice. CD160 and BTLA²⁹ were also not observed on skin CD8+ Trm, while VISTA³⁰ was detected at a low level ((**Fig.S4**). Further analysis showed that approx. 55% of CD8+ Trm expressed PD-1 (**Fig.3C, top**). In both the PD-1+ and PD-1- subpopulations, a majority of the cells co-expressed TIM-3 and 2B4 [**Fig.3C, bottom**].

Time course analysis revealed a rather constant PD-1 expression on CD8+ Trm from days 2 to 15 post DNFB challenge (approx. 80%), associated with a progressive decline in the following months (approx. 20% at month 12). By comparison, the percentage of TIM3+ cells remained stable throughout the protocol (approx. 80%), while the percentage of 2B4+ cells increased dramatically from around 10% at day 4 to almost 80% from day 30 to month 12 (**Fig.3D**).

Of note, the number of DNFB-specific IFN- γ -producing T cells among CD8+PD1+ Trm was two times higher compared to their PD1- counterparts, as revealed by ELISpot assay at day 30 post DNFB challenge (**Fig.S5**).

Blocking ICR signaling leads to severe CHS responses upon challenge with low DNFB doses

We then tested for the functional relevance of ICRs expressed on skin Trm, and their ability to prevent eczema exacerbation. Blocking ICR *in vitro* using anti-PD-1 and anti-TIM-3 mAbs increased the reactivity of purified epidermal CD8+ Trm and their capacity to produce IFN- γ (**Fig.4A**) and Granzyme-B (**data not shown**) upon *ex vivo* re-activation with allergen-pulsed BMDCs. Of note, the impact of blocked TIM-3 signaling was more modest than for PD-1, with an increase of approx. 40% and 160% regarding the number of IFN- γ -producing cells, respectively. More importantly, *in vivo* blockade of these two ICRs triggered severe flare-up CHS reactions, when DNFB-allergic animals (depleted of all circulating CD8+ T cells by anti-CD8 mAb treatment just before re-challenge) were repeatedly re-exposed to low DNFB doses (0.05%), which induced only a very mild inflammation in untreated animals (**Fig.4B**). Of note, the acute depletion of skin CD8+ Trm by DT injection using our iDTR-based model, abrogated the development of the CHS reaction induced by anti-PD-1 and anti-TIM-3 blockade (**Fig.S6**).

Taken together, our results show that PD-1 and, to a lesser extent, TIM-3, control the reactivation of epidermal CD8+ Trm and prevent the development of CHS responses when individuals are re-exposed to low allergen doses.

Allergen persists for several weeks within the epidermis

To better understand why CD8+ Trm are restrained in the skin and maintain ICR expression for weeks, we first investigated how long DNFB, which is

administrated epicutaneously, persists inside the tissue. Indeed, sustained expression of ICRs has previously been associated with recent or chronic stimulation of T cells by their cognate Ag²².

Using standard immunofluorescence staining of DNP-moieties generated at different time points after DNFB painting and light sheet fluorescence microscopy (LSFM), we observed that around 40% of the epidermal compartment was stained by anti-DNP mAbs at day 2 post challenge. Interestingly, approx. 10% was still stained at days 8 and 15 and about 5% remained labeled at day 30 post challenge, with a pronounced accumulation of CD8+ T cells (detected by using anti-CD8b mAb staining) around DNP-stained areas (**Fig.5** and **movies S1-S6**).

Our results thus revealed an underestimated persistence of chemical allergen-moieties in the skin, for at least one month.

Allergen persistence amplifies the number of CD8+ Trm and modulates their ICR phenotype

To determine whether the long-term persistence of the allergen impacted the ICR expression on skin Trm, we compared cells generated via the application of the cognate Ag (DNFB) or via a prime and pull strategy³¹. The latter allows recruiting Ag-specific T cells into the skin in an antigen-independent manner, via the application of an inflammatory but structurally irrelevant molecule. Here, we used the strong contact allergen OXA. In addition, to track specific CD8+ Trm, we developed a new CHS model based on the use of a MHC class I-restricted ovalbumin peptide bearing a dinitrophenyl (DNP)-moiety (DNP-peptide)³², which displays similar features to its DNFB counterpart, but in which specific CD8+ T cells can be detected using fluorescently labeled dextramers loaded with the cognate DNP-peptide (**Fig.S7**).

Using the prime and pull strategy, the absence of cognate antigen in the skin should lead to (i) a significant decrease of CD8+ Trm numbers, as previously reported³³⁻³⁵, (ii) a modulation of their ICR phenotype due to the lack of TCR stimulation. Hence, while we observed that similar numbers of specific CD8+ T cells were recruited into the epidermis 2 days after the challenge with DNP-peptide or OXA, only $\frac{1}{4}$ of the number of epidermal specific CD8+ Trm induced by cognate Ag challenge was detected 40 days after OXA challenge (**Fig.6A**). Moreover, significantly less TIM-3 but not PD-1 expression was detected on dextramer+ CD8+

T cells at day 2 post OXA versus DNP challenge, indicated by a drop in the GMFI from approx. 3000 to 2200 (**Fig.6B-C**). At day 40 post challenge, the expression of both PD-1 and TIM-3 was decreased (**Fig.6B-C**). Conversely, we did not observe any difference in the expression of 2B4 between the two recruitment strategies (**Fig.6B-C**).

Therefore, long term persistence of the allergen in the skin not only amplifies the number of specific CD8⁺ T_{rm} that populate the epidermis, but also partially modulates their phenotype.

Discussion

Tissue-resident memory T cells are considered detrimental in numerous human inflammatory diseases, including skin allergies. In the present study, we capitalized on a reference mouse model of ACD to study the local parameters regulating Trm development and functions during pathological processes. We report that, in allergic animals, the sites of previously inflamed skin are seeded with a high frequency of specific CD8⁺ Trm, in both the epidermis and the dermis. These cells are instrumental for the recurrence and severity of eczema, since they are sufficient to evoke intense flare-up reactions upon allergen challenge, without help from their circulating counterparts. However, albeit they are highly pathogenic, specific CD8⁺ Trm display certain levels of hypo-responsiveness. They express a specific pattern of inhibitory receptors such as PD-1 and TIM-3 (but also high levels of 2B4 and CD5), which attenuate their reactivity and prevent excessive responses when individuals are re-exposed to the allergen. Finally, a major observation was the underestimated long-term persistence of the allergen inside the epidermis, which drives the expansion of early CD8⁺ T cell effectors and their differentiation into Trm, but which also modulates their ICR expression to keep them in check. Our results therefore identify intrinsic factors that control pathogenic Trm to preserve skin integrity and to avoid the development of chronic and severe immunopathology.

CD8⁺Trm are major drivers of the chronicity and severity of eczema.

In this study, allergic skin responses were triggered by the epicutaneous application of DNFB, a chemical allergen/hapten endowed with strong sensitizing properties. We observed that the majority of local memory cells which populated the epidermis and dermis of previously affected skin were CD8⁺ Trm. We failed to detect allergen-specific CD4⁺ T cells producing IFN- γ , IL-4, and IL-17.

We demonstrated that epidermal CD8⁺ Trm are sufficient to prompt severe eczema exacerbations, which in consequence categorize them as prominent drivers of the chronicity and severity of eczema. The concept of local skin memory, to explain the strong and accelerated allergen-induced inflammatory reaction at the site of previously affected skin, was introduced more than three decades ago by Scheper et al.¹⁵. So far, major but only indirect demonstrations have suggested that skin Trm, and notably CD8⁺Trm, are responsible for the development of these early and

intense flares¹⁴⁻¹⁷. The most solid evidence came from the study by Gaide et al., which reported that flare-up reactions are not prevented when allergic animals are injected with drug compounds that block the egress of memory T cells from the lymphoid organs¹⁶. In the present study, the sharp elimination of iDTR+ CD8+ Trm from the skin and the subsequent abrogation of allergen-induced flares firmly demonstrated the contribution of Trm to the allergen-induced exacerbations, excluding the potential involvement of other tissue resident cells such as dermal ILCs or liver NK memory cells³⁶⁻³⁸. Besides, the fact that the application of the irrelevant allergen OXA gave similar inflammation on healed and naive skin in DNFB-allergic mice, enabled us to exclude the possibility that the intense flares recorded at the sites of healed lesions were due to bystander activation of non-allergen-specific Trm, solely as the results of the inflammatory environment and cytokine signaling on CD8+ Trm³⁹⁻⁴¹.

The long-term persistence of chemical allergen-moieties in the skin promotes the formation of CD8+ Trm.

The mechanisms that drive the formation and fate of skin Trm are complex and so far only partially understood. Here, we observed that the accumulation of epidermal CD8+ Trm correlated with the long-term persistence of the allergen in the skin (at least > than 30 days), with a progressive decline from day 0 to day 30. Of note, the allergen persistence at day 30 was mainly observed in keratinocytes from hair follicles. Since epidermal renewal needs only 8-10 days in mice, this observation strongly suggests that the allergen is stocked in long-lived slow-cycling epidermal stem cells, which represent a small fraction of interfollicular epidermis and hair follicles⁴²⁻⁴⁴. Hence, one could speculate that, mimicking viral immunity controlling viruses integrated in our genomes⁴⁵, allergen-specific CD8+Trm are placed here to survey long-lived skin cells that continuously present allergen-epitopes. It will be crucial to determine exactly how long an allergen can persist in the skin, and whether its presence is functional and prompts Trm activation and/or survival, avoiding progressive attrition of specific Trm pools from the epidermis (as shown in **Fig.1F**). Here, confirming previous studies, we demonstrated that while a significant number of allergen-specific Trm can be recruited to the skin upon prime and pull strategy, this number is dramatically amplified when cells recognize their cognate Ag in the skin^{33,}

⁴⁶.

CD8⁺ Trm are placed at the skin interface, but they are kept in check.

The most striking finding of our study was certainly the demonstration that, despite their key role in the recurrence of the pathology, allergen-specific CD8⁺ Trm are tightly regulated, and express ICRs, which prevent them from unnecessary strong reactivation upon allergen exposure. Several investigators have previously reported the expression of different inhibitory molecules by CD8⁺ Trm, not only in cancer ^{47,48}, but also in viral infections ⁴⁹⁻⁵², autoimmune uveoretinitis ⁵³ or at homeostasis ^{54, 55}. Besides, recent comparison of the gene-expression pattern of lung Trm versus circulating Tem in humans demonstrated that Trm expressed a restrained genetic program with high expression of CTLA4, BTLA, LAG3, SPRY1, A2AR, PD-1, TWIST1, BACH2 genes ⁵⁵. Here, we reported that allergen-induced Trm expressed at their surface different levels of a restricted set of ICRs (PD-1, TIM-3, 2B4, but also a high level of CD5, a low level of VISTA, but not of TIGIT, BTLA, LAG-3, CD160 or CTLA-4), corroborating the hypothesis that the reactivation of Trm must be tightly controlled to ensure sufficient but limited reactivity. Interestingly, a previous study demonstrated that the pattern of ICR coexpression and the number of receptors simultaneously expressed by the same CD8⁺ Trm affect the magnitude of the restraint on the Trm ²². In this study, we observed that the pattern of ICR coexpression varies over time, with progressive increase of 2B4, compared to PD-1 or TIM-3, during the first month post DNFB challenge, and late decline of PD-1, but not of TIM-3 or 2B4 expression, after 6-12 months. Our results then question about the environmental signals that participate in modulating the level of ICR expression and thereby the functions of skin Trm. It was recently suggested that the acquisition of a specific inhibitory program could be associated to the affinity/avidity of the initial TCR-pMHC interactions between Trm precursors and antigen presenting cells ⁵¹. Here, we showed that the persisting allergen in the skin not only amplified the number of CD8⁺ Trm populating the epidermis, but also maintained PD-1 and TIM-3 expression, which is reminiscent of a recent work describing the presence of clonally expanded Trm-like PD1⁺CD8⁺ cells in the synovial fluid of patients suffering from chronic juvenile idiopathic arthritis ⁵⁶. However, beyond the antigen persistence, it will be interesting to determine whether hair follicle-derived IL-15 or IL-7 cytokines ⁵⁷, or TGF- β ⁵⁸, which were reported to be instrumental for Trm differentiation, are also susceptible to modulate the ICR program on skin Trm; the more so since we have

431 observed that these three cytokines are synthesized during the resolution of the
432 DNFB inflammation (**data not shown**). Finally, it will also be important to determine
433 the respective role of epidermal Langerhans cells, dendritic epidermal T cells
434 (DETCs), T regulatory cells or innate lymphoid cells... in the control of Trm
435 reactivation via ICR acquisition and/or ligation.

436 In conclusion, our results provide important information for considering the
437 unique nature of allergen-induced CD8⁺ Trm and the mechanisms that preserve their
438 unwanted reactivation and the subsequent development of chronic or severe skin
439 allergy. The development of strategies targeting the reactivation of skin Trm *in situ* via
440 their ICRs should open new avenues to treat ACD.

441 **Author contributions**

442 PG, LL, VM, VL and PR carried out the experiments and analysed the data.

443 AN, SG, DR, AMS participated in the interpretation of the data.

444 JFN and MV designed the experiments and supervised this study.

445 PG, JFN and MV wrote the manuscript.

446 All authors had final approval of the submitted and published version.

447

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454

Figure legends

Figure 1: Allergen-specific CD8⁺ Trm persist long-term in the epidermis and dermis of healed CHS lesions

A. Protocol: CHS response to DNFB to track the persistence and function of DNFB-specific Trm.

B. Quantification of skin T cell subsets. Histograms depict mean cell numbers \pm SEM of CD8⁺ and CD4⁺ T cell subsets present in the epidermis or dermis of healed and naive skin, at day 30 post challenge, as determined by FACS analysis.

C. Canonical Trm marker expression. FACS plots and histograms depict mean percentages \pm SEM of CD8b⁺CD103⁺CD69⁺ T cells among CD45⁺ cells present in the epidermis and dermis of healed skin, or in dLNs, at day 30.

D. Long-term persistence of skin Trm. The mean numbers \pm SEM of CD8⁺CD103⁺CD69⁺ Trm was also assessed at months 3, 6 and 12.

E&F. DNFB-specific T cell responses. Mean number of DNFB-specific CD8⁺ or CD4⁺ T cells (\pm SEM) determined by IFN- γ -ELISpot at day 30 (**E**). The persistence in the epidermis of IFN- γ -producing DNFB-specific cells was also analyzed at months 3, 6 and 12 (**F**).

All results are representative of two or three independent experiments with 4-10 mice per group.

n.s.=not significant, *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001. One-way ANOVA or Mann-Whitney U test.

Figure 2: Skin CD8⁺ Trm mediate site- and Ag-specific flare-up reactions in DNFB-allergic mice.

A. CHS response of DNFB-allergic mice to DNFB or OXA. DNFB-allergic C57BL/6 mice were re-challenged with DNFB or oxazolone (OXA) on healed skin lesions or naive skin at day 30. As control, DNFB and OXA were applied on the ears of naïve mice. The following day, CHS reaction was determined by measuring the mean ear oedema (μ m) \pm SEM.

B. Depletion of skin CD8⁺ Trm abrogates CHS response to DNFB. CHS to DNFB was induced in CD3 ϵ ^{-/-} mice reconstituted with iDTR-CD8⁺ and WT-CD4⁺ T cells. At day 30 and 60 and 1 day after DT or anti-CD8 mAb i.p. injections, mice were re-

challenged with DNFB. The following day, CHS reaction was determined by measuring the mean ear oedema (μm) \pm SEM.

All results are representative of two or three independent experiments with 4-5 mice per group.

n.s.=not significant, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. One-way ANOVA.

Figure 3: Epidermal CD8⁺ Trm, but not circulating memory CD8⁺T cells, express several inhibitory checkpoint receptors (ICR).

A. CHS response to repeated applications of low DNFB doses. DNFB-allergic C57BL/6 mice were re-challenged (arrows) with low DNFB doses (0.05% at days 0, 2, and 6; or 0.01% at days 0, 2, 6, 8, 13, and 15) or with a single standard DNFB dose (0.13% at day 0). CHS reaction was determined by measuring the mean ear oedema (μm) \pm SEM.

B. ICR expression by epidermal or dLN CD8⁺ memory T cell subsets. FACS plots depict representative PD-1, TIM-3 or 2B4 expression by epidermal CD8⁺ Trm and dLN CD8⁺ Tem, Tcm or naïve cells, at day 30 post DNFB challenge.

C. ICR co-expression by CD8⁺ Trm. Mean percentages \pm SD of TIM-3 and 2B4 co-expression on gated epidermal PD-1⁺ and PD-1⁻ CD8⁺ Trm subsets, at day 30. Dendritic epidermal T cells (DETCs) are shown as control.

D. ICR expression by epidermal CD8⁺ T cells during the course of CHS response to DNFB and after its resolution. Mean percentages \pm SD of PD-1⁺, TIM-3⁺ and 2B4⁺ cells among epidermal CD8⁺ Trm from day 2 to month 12.

All results are representative of two or three independent experiments with 4-5 mice per group or time point.

* $P < 0.05$. One-way ANOVA.

Figure 4: Blocking ICR signaling increases the reactivity of CD8⁺ T cells *in vitro* and exacerbates the flare-up CHS reactions *in vivo*.

A. DNFB-specific T cell responses in the presence of ICR blocking mAbs. CD8⁺ T cells from healed epidermis were co-cultured overnight with haptenized (DNBS; 0.4mM) or non-haptenized (NH) BMDCs in the presence of anti-PD-1 and/or anti-TIM-3 mAb. IgG2a was added to control co-cultures. The following day, the number of specific T cells was determined by IFN- γ ELISpot. Results are expressed as the mean number of SFC/ 10^6 CD8⁺ T cells \pm SEM.

B ICR antagonists increase CHS responses to low DNFB doses. DNFB-allergic mice were re-challenged with low DNFB doses (0.05%, at days 30, 32, 34), while ICR blockade was maintained by i.p. injection of anti-PD-1 and anti-TIM-3 mAbs at days 26, 28, 30 and 32. As control, DNFB-allergic mice were injected with IgG2a mAbs. Circulating CD8⁺ T cells were depleted by i.p. injection of anti-CD8 mAbs at day 27. CHS reaction was determined by measuring the mean ear oedema (μm) \pm SEM.

All results are representative of three independent experiments with 5-15 mice per group.

n.s.=not significant, *P<0.05, **P<0.01. Mann-Whitney U test.

Figure 5: Allergen persistence in the skin after the resolution of CHS to DNFB.

Illustrative and quantitative analysis of DNP moieties present in the skin during the course of CHS to DNFB.

A. Threshold segmentation images. Epidermal sheets collected before and 2, 8, 15 or 30 days after DNFB challenge of DNFB-sensitized animals were explored for the presence of DNP moieties (green), CD8b⁺ T cells (red), and DETCs (yellow) by standard immunofluorescence staining. Representative photographs acquired on a Lightsheet Z.1 microscope after threshold segmentation using Bitplane Imaris are shown. Control isotype staining was performed on epidermal sheets collected at day 8. White arrows highlight the pronounced accumulation of CD8⁺ T cells around DNP-stained areas.

B. Quantification. The percentage of epidermal area stained by anti-DNP mAbs was calculated throughout the CHS response to DNFB by determining the total epidermal area (μm^3) analyzed by DAPI nuclear staining and the DNP-stained area (μm^3). For each individual mouse 3-4 pictures were analyzed.

All results are representative of two independent experiments with 3 mice per time point.

Figure 6: Accumulation of epidermal Trm and maintenance of ICR expression is antigen-dependant

Naïve C57BL/6 mice were immunized i.p. with DNP-peptide pulsed-BMDCs and challenged 5 days later on the left ear with the DNP-peptide in DMSO or with an irrelevant hapten, oxazolone (OXA).

556 The number of DNP-peptide specific CD8⁺ T cells recruited in the epidermis via the
557 Ag-specific or the non Ag-specific approach (“prime and pull strategy”) was
558 measured by FACS at days 2 and 40, using fluorescently labeled dextramers loaded
559 with the respective DNP-peptide (**A**). Results are depicted as mean values
560 (percentage) ±SEM.

561 The expression levels of PD-1, TIM-3, and 2B4 by DNP-specific CD8⁺ T_{RM} after Ag-
562 specific (DNP-peptide) or non Ag-specific (Oxazolone) recruitment was assessed.
563 Representative histograms (day 40) (**B**) and mean values of respective geometric
564 mean fluorescence intensity (GMFI, days 2 and 40) ±SEM (**C**) are shown.

565 All the results are representative of three independent experiments with 4-5 mice per
566 group.

567 n.s.=not significant, *P<0.05, **P<0.01. One-way ANOVA.

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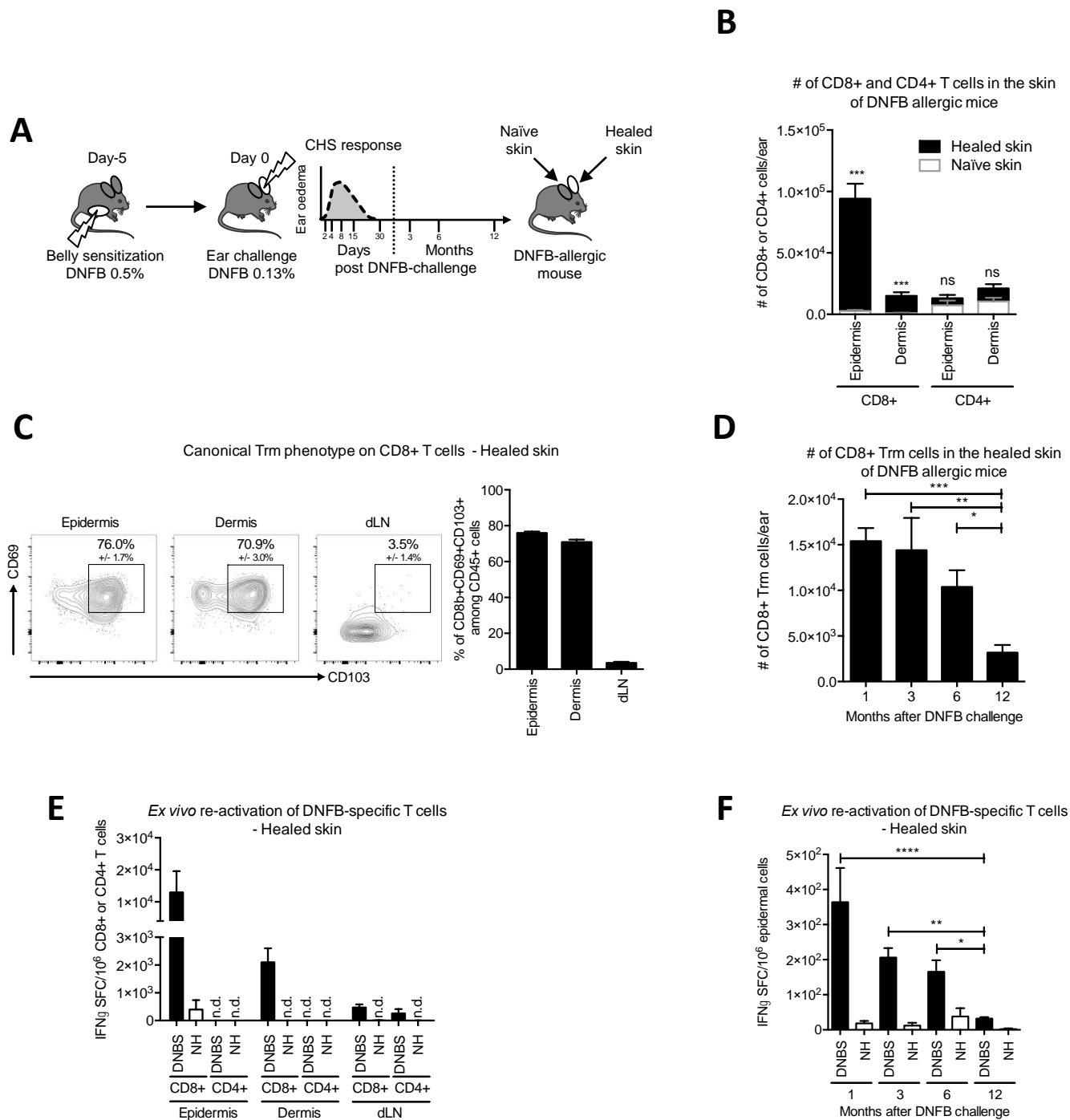
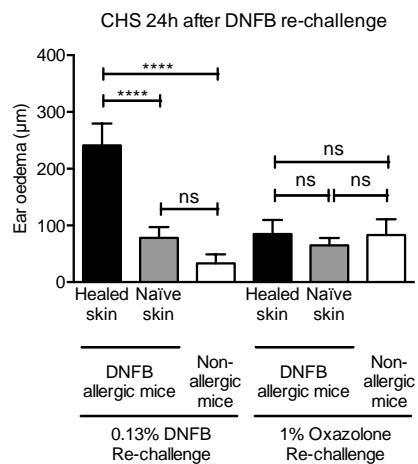
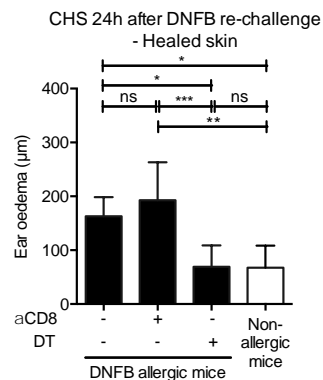


Figure 1

A**B****Figure 2**

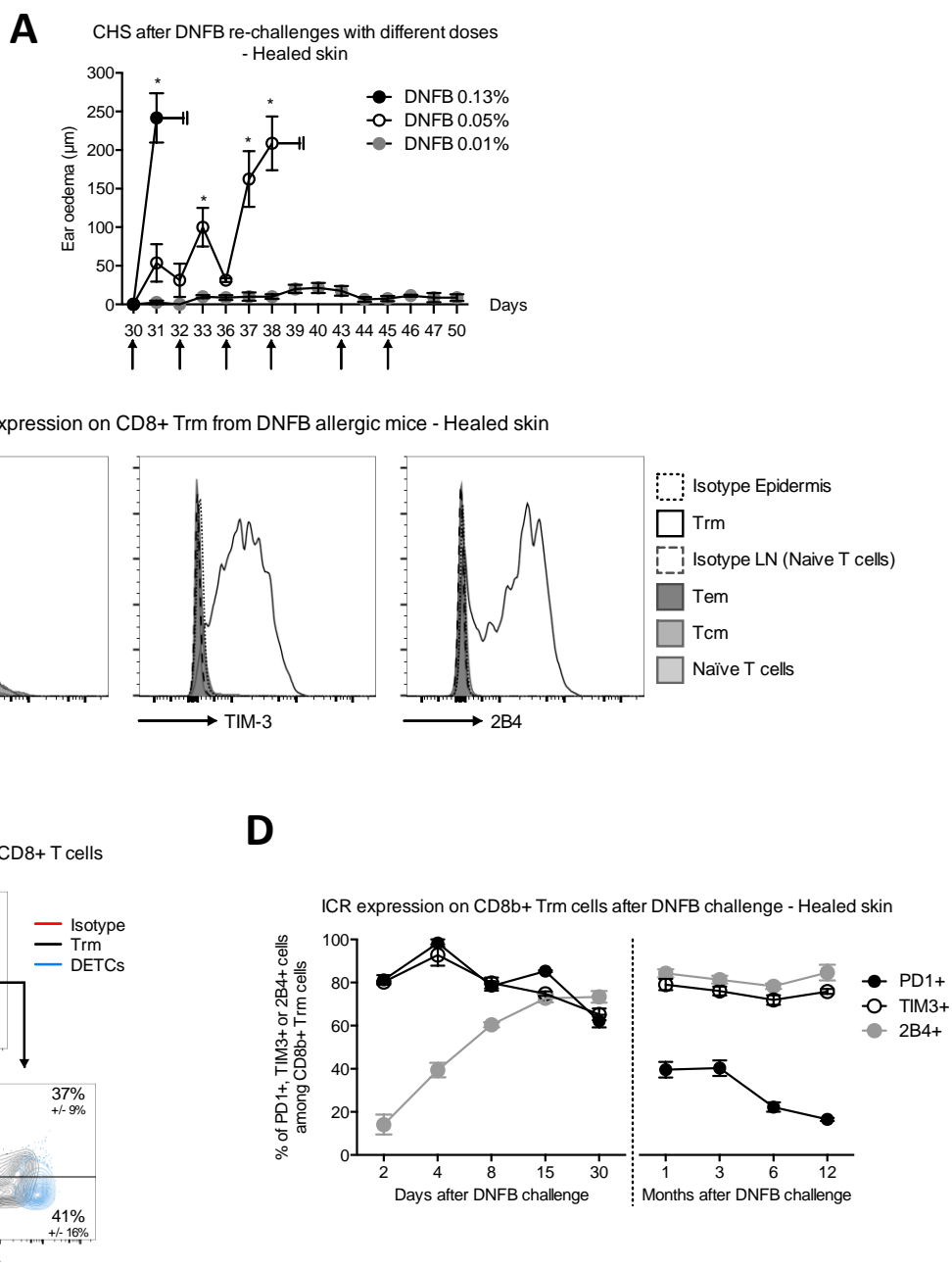


Figure 3

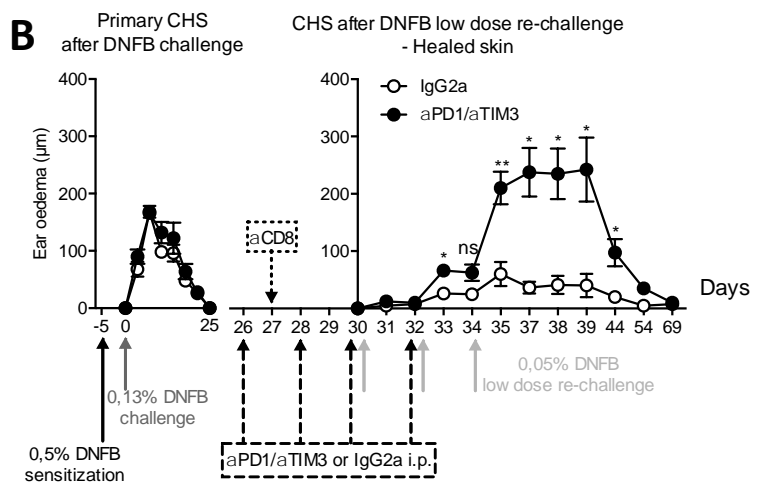
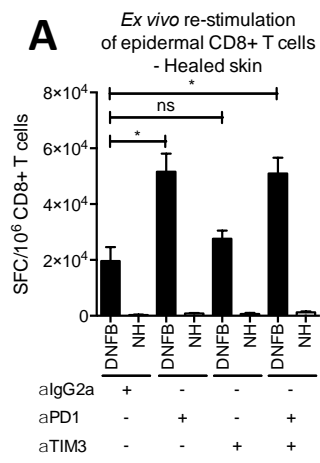
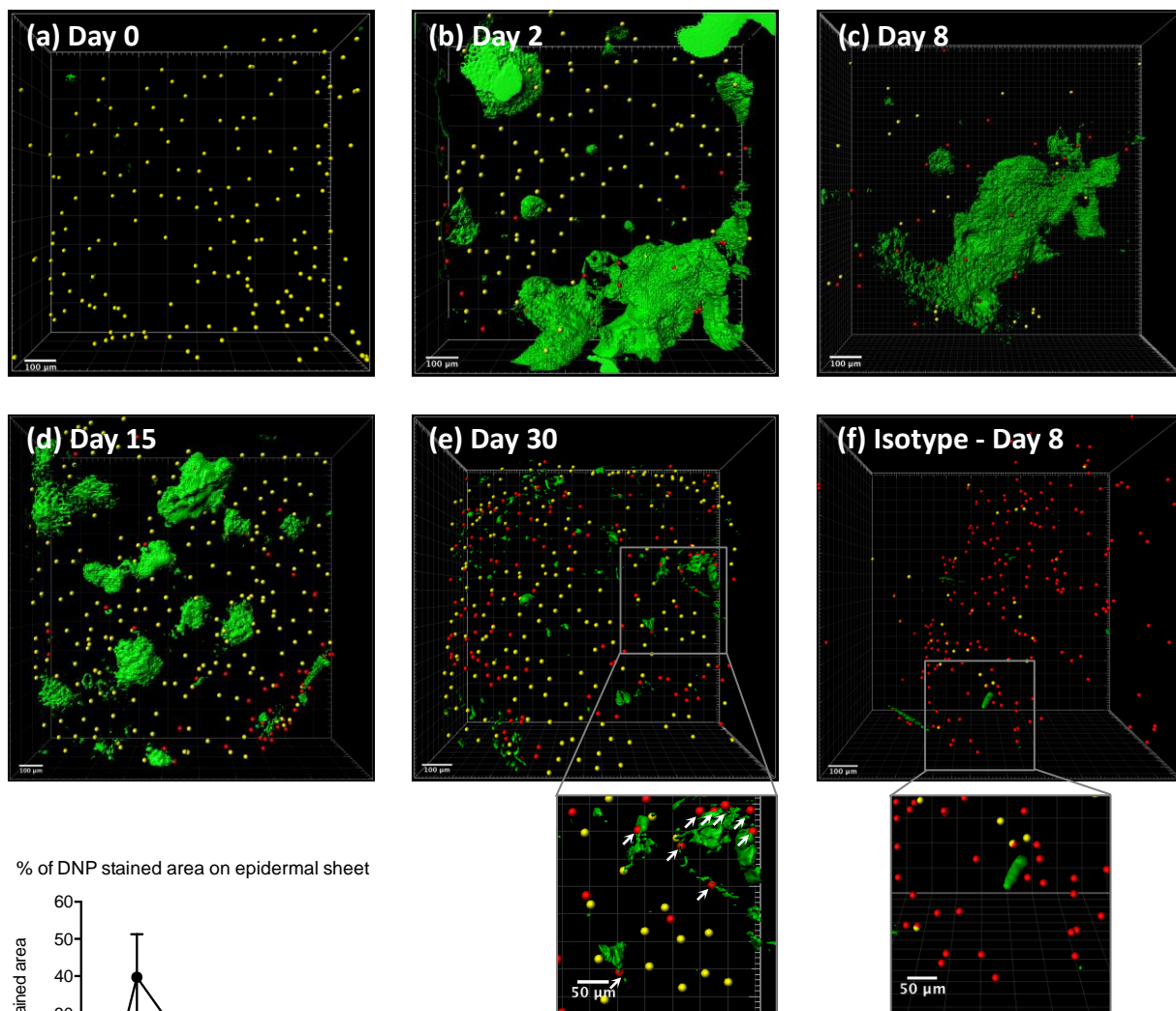


Figure 4

A**B**

% of DNP stained area on epidermal sheet

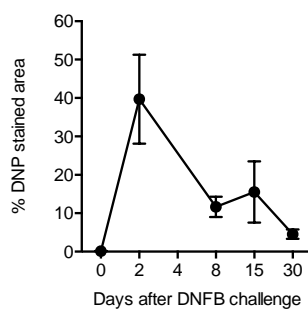
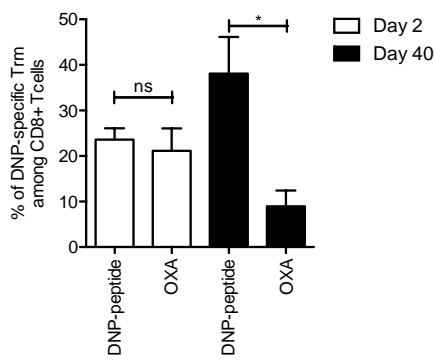
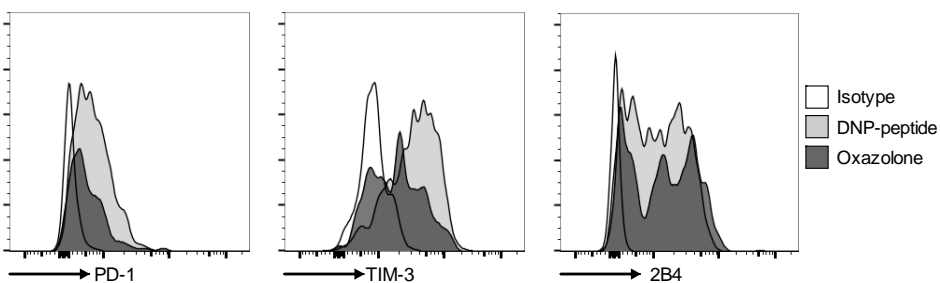


Figure 5

A % of epidermal DNP-specific CD8+Trm in DNP-peptide allergic mice



B ICR expression on epidermal DNP-specific CD8+ Trm - Healed skin



C

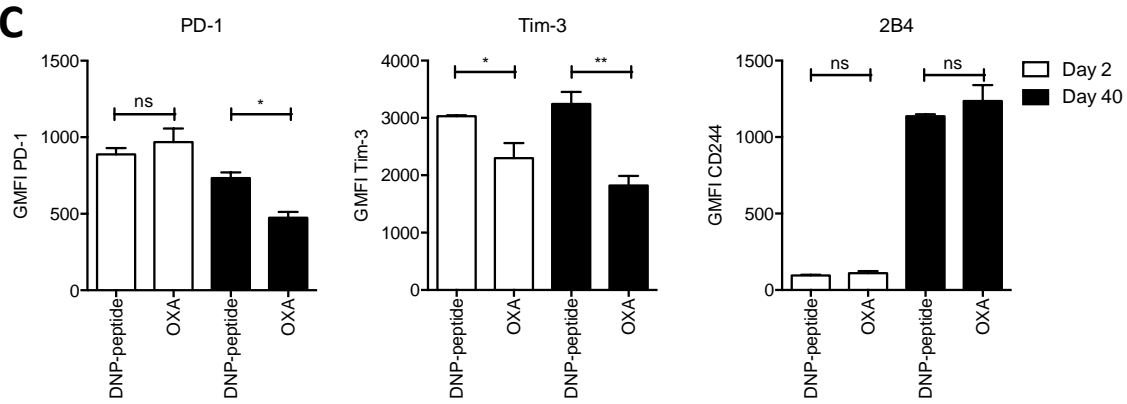


Figure 6



Inhibitory checkpoint receptors control CD8+ resident memory T cells to prevent skin allergy

