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

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

3

4

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27 **Running title:** CD8+ Trm in skin allergy.

28

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30 AMS are employed by Pierre Fabre. All other authors are public employees from
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32

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35

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

53 **Abstract**

54

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

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

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

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

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

77

78

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

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85

86 **Key Messages**

87

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

91 • CD8+ T_{rm} are key for the chronicity, severity and recurrence of the pathology.

92 • However, they express several inhibitory receptors, such as PD-1 or TIM-3,
93 which keep them in check to preserve skin integrity and to avoid the development of
94 severe immunopathology.

95 **Capsule summary**

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

100

101 **Introduction**

102

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

108 Beyond their demonstrated active contribution to host protection, there is an
109 emerging understanding that Trm also participate in several
110 autoimmune/inflammatory diseases, in which they can instigate or augment the
111 immunopathology^{9,10}. Trm have been described in different skin diseases such as
112 psoriasis, vitiligo³, fixed drug eruption¹¹ or leukemic cutaneous T cell lymphoma^{12,}
113¹³.

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

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

132 **Methods:**

133

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

136

137 **Animals and reagents**

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

141

142 **Contact hypersensitivity to DNFB and flare-up reactions**

143 For most experiments, CHS was induced as previously described²⁰. Briefly, animals
144 were sensitized epicutaneously on the shaved abdomen with 25µl of 0.5% DNFB on
145 day -5, and were challenged on the left ear on day 0 with 2x5µl 0.13% DNFB while
146 the right ear received the acetone/olive oil vehicle. Allergic animals were next
147 retested at day 30, or months 3, 6 and 12, with different doses (0.01, 0.05 and/or
148 0.13%) of DNFB or with 1% OXA, both on healed (left ear) and/or naïve (right ear)
149 skin, as indicated.

150 In some experiments, DNFB-allergic C57BL/6 or reconstituted CD3ε^{-/-} mice were
151 depleted of circulating or of skin CD8⁺ T cells by anti-CD8 mAbs or diphtheria toxin
152 (DT) injections, and treated with anti-PD-1 (RMP1-14, 200µg/ml), anti-TIM-3 (RMT3-
153 23, 200µg/ml) or respective IgG2a isotype (2A3, 400 µg/ml) (all from BioXCell, West
154 Lebanon, USA) at indicated time points.

155 **Results**

156

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

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

169 To confirm that Trm persist at the sites of CHS skin inflammation ^{16, 17, 18},
170 healed and naive skin of DNFB-allergic mice were first collected at day 30 and
171 processed for the presence of CD8⁺ and/or CD4⁺ T cell subsets by FACS and
172 immunofluorescence microscopy. While the epidermis and the dermis of naive skin
173 were nearly exclusively seeded with CD4⁺ T cells, increased numbers of CD8⁺ T
174 cells were recorded in healed CHS lesions (**Fig.1B and Fig.S1**). Approximately 70%
175 of epidermal and dermal CD8⁺ T cells co-expressed the two canonical Trm markers
176 CD103 and CD69 ²¹, emphasizing the residency properties of these cells (hereafter
177 referred to as Trm) (**Fig.1C**).

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

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

189 present in the healed lesion of DNFB-allergic mice, skin CD8⁺ and CD4⁺ T cell
190 subsets were subsequently collected at 30 days, FACS sorted and re-activated ex-
191 vivo with allergen-pulsed bone marrow-derived dendritic cells (BMDCs). The
192 following day, the number of spot forming cells (SFCs) producing IFN- γ , IL-17a or IL-
193 4 was determined by enzyme-linked immunospot (ELISpot) assay. While approx. 1%
194 of epidermal and 0.2% of dermal CD8⁺ T cells of healed CHS lesions were antigen-
195 specific and produced IFN- γ (**Fig.1E**), IL-17a- or IL-4- were not detected (**data not**
196 **shown**). Comparatively, the frequency of DNFB-specific cells among total CD8⁺ T
197 cells from dLNs, which comprise a mix of effector (Tem) and central (Tcm) memory T
198 cells and dLN Trm, was <0.1%.

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

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

204

205 ***CD8⁺ Trm mediate DNFB-induced flare-up reactions***

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

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

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

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

229

230 ***CD8+ Trm are hypo-responsive to low DNFB doses***

231 We next examined flare-up reactions induced in allergic animals by low DNFB
232 doses, i.e. below the standard dose of 0.13%. As expected, repeated applications of
233 low DNFB doses on healed skin induced an intense flare-up reaction. It required 3
234 consecutive administrations of DNFB 0.05% (on days 0, 2, and 6) to generate flares
235 of similar magnitude to the one provoked by a single optimal dose (**Fig.3A**). By
236 contrast, 6 consecutive administrations (on days 0, 2, 6, 8, 13, and 15) of DNFB
237 0.01% failed to induce a flare-up reaction. On naive skin, 6 consecutive
238 administrations of DNFB 0.05% (on days 0, 2, 6, 8, 13 and 15) did not induce
239 significant flares (**data not shown**). These data indicate that, although specific Trm
240 are seeded in the skin at significant density, they are actively restrained to prevent
241 excessive immunoreactivity in response to DNFB application below a certain
242 threshold (**Fig.3A**).

243

244 ***Epidermal CD8+ Trm express a restricted set of inhibitory check point*** 245 ***receptors***

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

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

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

264

265 ***Blocking ICR signaling leads to severe CHS responses upon challenge with*** 266 ***low DNFB doses***

267 We then tested for the functional relevance of ICRs expressed on skin Trm,
268 and their ability to prevent eczema exacerbation. Blocking ICR *in vitro* using anti-PD-
269 1 and anti-TIM-3 mAbs increased the reactivity of purified epidermal CD8+ Trm and
270 their capacity to produce IFN- γ (**Fig.4A**) and Granzyme-B (**data not shown**) upon ex
271 vivo re-activation with allergen-pulsed BMDCs. Of note, the impact of blocked TIM-3
272 signaling was more modest than for PD-1, with an increase of approx. 40% and
273 160% regarding the number of IFN- γ -producing cells, respectively. More importantly,
274 *in vivo* blockade of these two ICRs triggered severe flare-up CHS reactions, when
275 DNFB-allergic animals (depleted of all circulating CD8+ T cells by anti-CD8 mAb
276 treatment just before re-challenge) were repeatedly re-exposed to low DNFB doses
277 (0.05%), which induced only a very mild inflammation in untreated animals (**Fig.4B**).
278 Of note, the acute depletion of skin CD8+ Trm by DT injection using our iDTR-based
279 model, abrogated the development of the CHS reaction induced by anti-PD-1 and
280 anti-TIM-3 blockade (**Fig.S6**).

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

284

285 ***Allergen persists for several weeks within the epidermis***

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

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

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

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

300

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

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

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

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

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

329 **Discussion**

330

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

349

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

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

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

363 intense flares ¹⁴⁻¹⁷. The most solid evidence came from the study by Gaide et al.,
364 which reported that flare-up reactions are not prevented when allergic animals are
365 injected with drug compounds that block the egress of memory T cells from the
366 lymphoid organs ¹⁶. In the present study, the sharp elimination of iDTR+ CD8+ Trm
367 from the skin and the subsequent abrogation of allergen-induced flares firmly
368 demonstrated the contribution of Trm to the allergen-induced exacerbations,
369 excluding the potential involvement of other tissue resident cells such as dermal ILCs
370 or liver NK memory cells ³⁶⁻³⁸. Besides, the fact that the application of the irrelevant
371 allergen OXA gave similar inflammation on healed and naive skin in DNFB-allergic
372 mice, enabled us to exclude the possibility that the intense flares recorded at the
373 sites of healed lesions were due to bystander activation of non-allergen-specific Trm,
374 solely as the results of the inflammatory environment and cytokine signaling on CD8+
375 Trm ³⁹⁻⁴¹.

376

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

379 The mechanisms that drive the formation and fate of skin Trm are complex
380 and so far only partially understood. Here, we observed that the accumulation of
381 epidermal CD8+ Trm correlated with the long-term persistence of the allergen in the
382 skin (at least > than 30 days), with a progressive decline from day 0 to day 30. Of
383 note, the allergen persistence at day 30 was mainly observed in keratinocytes from
384 hair follicles. Since epidermal renewal needs only 8-10 days in mice, this observation
385 strongly suggests that the allergen is stocked in long-lived slow-cycling epidermal
386 stem cells, which represent a small fraction of interfollicular epidermis and hair
387 follicles ⁴²⁻⁴⁴. Hence, one could speculate that, mimicking viral immunity controlling
388 viruses integrated in our genomes ⁴⁵, allergen-specific CD8+Trm are placed here to
389 survey long-lived skin cells that continuously present allergen-epitopes. It will be
390 crucial to determine exactly how long an allergen can persist in the skin, and whether
391 its presence is functional and prompts Trm activation and/or survival, avoiding
392 progressive attrition of specific Trm pools from the epidermis (as shown in **Fig.1F**).
393 Here, confirming previous studies, we demonstrated that while a significant number
394 of allergen-specific Trm can be recruited to the skin upon prime and pull strategy, this
395 number is dramatically amplified when cells recognize their cognate Ag in the skin ³³.
396 ⁴⁶.

397

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

399 The most striking finding of our study was certainly the demonstration that,
400 despite their key role in the recurrence of the pathology, allergen-specific CD8+ Trm
401 are tightly regulated, and express ICRs, which prevent them from unnecessary
402 strong reactivation upon allergen exposure. Several investigators have previously
403 reported the expression of different inhibitory molecules by CD8+ Trm, not only in
404 cancer ^{47,48}, but also in viral infections ⁴⁹⁻⁵², autoimmune uveoretinitis ⁵³ or at
405 homeostasis ^{54,55}. Besides, recent comparison of the gene-expression pattern of lung
406 Trm versus circulating Tem in humans demonstrated that Trm expressed a
407 restrained genetic program with high expression of CTLA4, BTLA, LAG3, SPRY1,
408 A2AR, PD-1, TWIST1, BACH2 genes ⁵⁵. Here, we reported that allergen-induced
409 Trm expressed at their surface different levels of a restricted set of ICRs (PD-1, TIM-
410 3, 2B4, but also a high level of CD5, a low level of VISTA, but not of TIGIT, BTLA,
411 LAG-3, CD160 or CTLA-4), corroborating the hypothesis that the reactivation of Trm
412 must be tightly controlled to ensure sufficient but limited reactivity. Interestingly, a
413 previous study demonstrated that the pattern of ICR coexpression and the number of
414 receptors simultaneously expressed by the same CD8+ Trm affect the magnitude of
415 the restraint on the Trm ²². In this study, we observed that the pattern of ICR co-
416 expression varies over time, with progressive increase of 2B4, compared to PD-1 or
417 and TIM-3, during the first month post DNFB challenge, and late decline of PD-1, but
418 not of TIM-3 or 2B4 expression, after 6-12 months. Our results then question about
419 the environmental signals that participate in modulating the level of ICR expression
420 and thereby the functions of skin Trm. It was recently suggested that the acquisition
421 of a specific inhibitory program could be associated to the affinity/avidity of the initial
422 TCR-pMHC interactions between Trm precursors and antigen presenting cells ⁵¹.
423 Here, we showed that the persisting allergen in the skin not only amplified the
424 number of CD8+ Trm populating the epidermis, but also maintained PD-1 and TIM-3
425 expression, which is reminiscent of a recent work describing the presence of clonally
426 expanded Trm-like PD1+CD8+ cells in the synovial fluid of patients suffering from
427 chronic juvenile idiopathic arthritis ⁵⁶. However, beyond the antigen persistence, it will
428 be interesting to determine whether hair follicle-derived IL-15 or IL-7 cytokines ⁵⁷, or
429 TGF- β ⁵⁸, which were reported to be instrumental for Trm differentiation, are also
430 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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452 assistance with flow cytometry and microcospy.

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454

455 **Figure legends**

456

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

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

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

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

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

469 **E&F. DNFB-specific T cell responses.** Mean number of DNFB-specific CD8⁺ or
470 CD4⁺ T cells (\pm SEM) determined by IFN- γ -ELISpot at day 30 (**E**). The persistence in
471 the epidermis of IFN- γ -producing DNFB-specific cells was also analyzed at months 3,
472 6 and 12 (**F**).

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

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

477

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

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

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

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

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

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

493

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

496 **A. CHS response to repeated applications of low DNFB doses.** DNFB-allergic
497 C57BL/6 mice were re-challenged (arrows) with low DNFB doses (0.05% at days 0, 2,
498 and 6; or 0.01% at days 0, 2, 6, 8, 13, and 15) or with a single standard DNFB dose
499 (0.13% at day 0). CHS reaction was determined by measuring the mean ear oedema
500 (μm) \pm SEM.

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

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

507 **D. ICR expression by epidermal CD8+ T cells during the course of CHS**
508 **response to DNFB and after its resolution.** Mean percentages \pm SD of PD-1+, TIM-
509 3+ and 2B4+ cells among epidermal CD8+ Trm from day 2 to month 12.

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

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

513

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

516 **A. DNFB-specific T cell responses in the presence of ICR blocking mAbs.** CD8+
517 T cells from healed epidermis were co-cultured overnight with haptenized (DNBS;
518 0.4mM) or non-haptenized (NH) BMDCs in the presence of anti-PD-1 and/or anti-
519 TIM-3 mAb. IgG2a was added to control co-cultures. The following day, the number
520 of specific T cells was determined by IFN- γ ELISpot. Results are expressed as the
521 mean number of SFC/ 10^6 CD8+ T cells \pm SEM.

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

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

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

532

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

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

536 **A. Threshold segmentation images.** Epidermal sheets collected before and 2, 8,
537 15 or 30 days after DNFB challenge of DNFB-sensitized animals were explored for
538 the presence of DNP moieties (green), CD8b⁺ T cells (red), and DETCs (yellow) by
539 standard immunofluorescence staining. Representative photographs acquired on a
540 Lightsheet Z.1 microscope after threshold segmentation using Bitplane Imaris are
541 shown. Control isotype staining was performed on epidermal sheets collected at day
542 8. White arrows highlight the pronounced accumulation of CD8⁺ T cells around DNP-
543 stained areas.

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

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

550

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

553 Naïve C57BL/6 mice were immunized i.p. with DNP-peptide pulsed-BMDCs and
554 challenged 5 days later on the left ear with the DNP-peptide in DMSO or with an
555 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) \pm 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) \pm 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.

568

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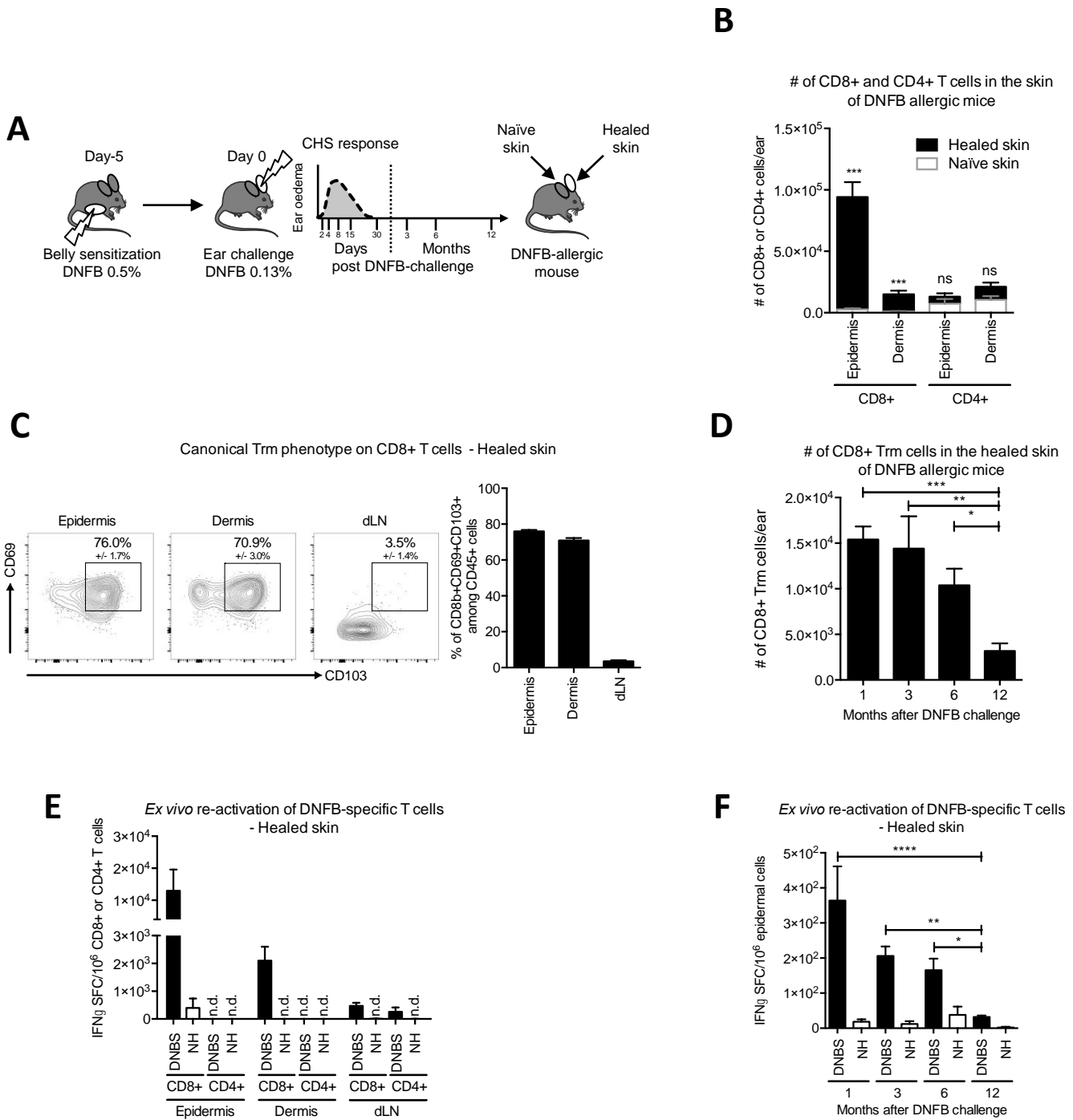
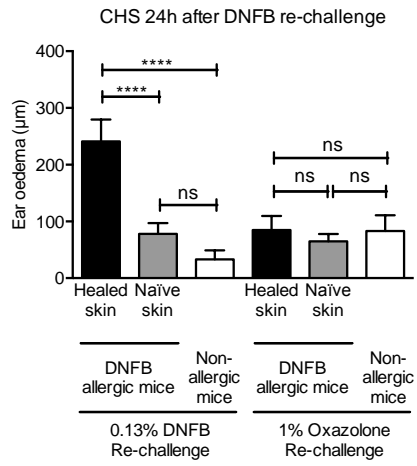
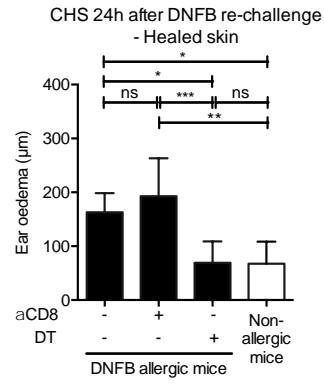


Figure 1

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

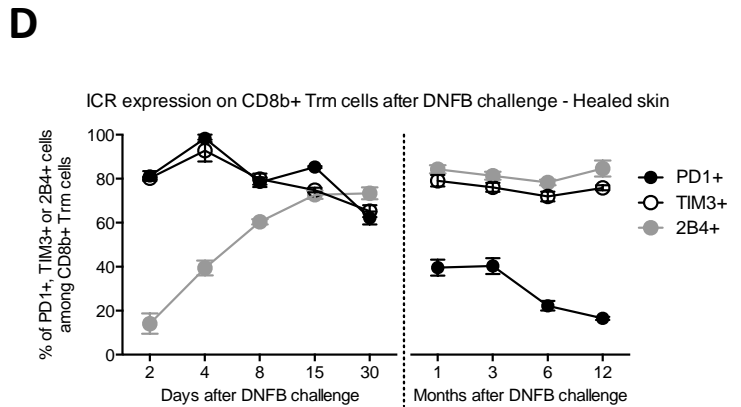
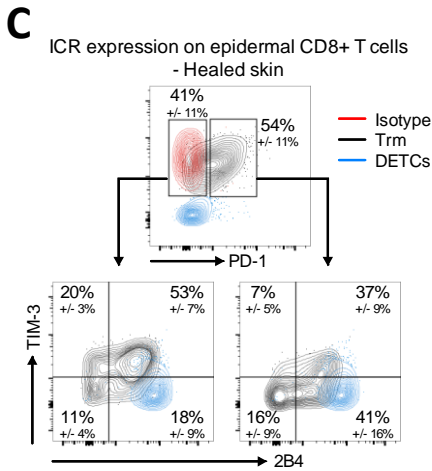
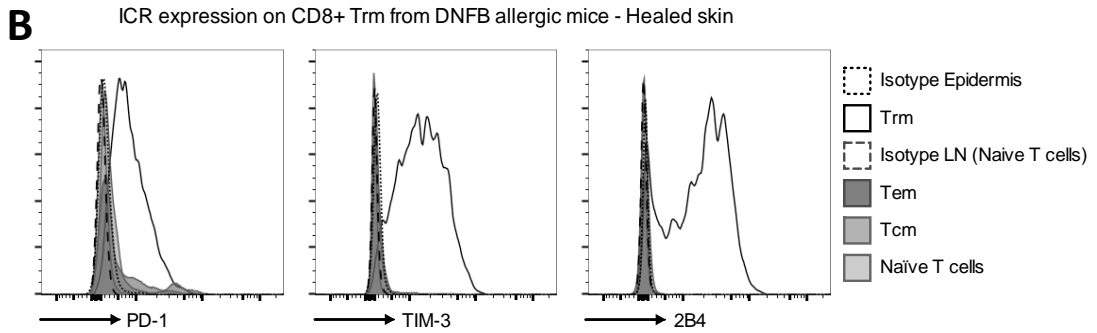
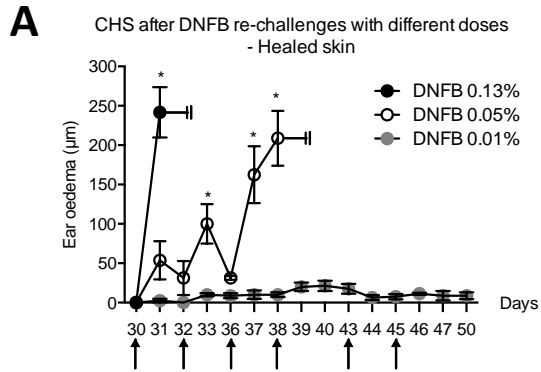


Figure 3

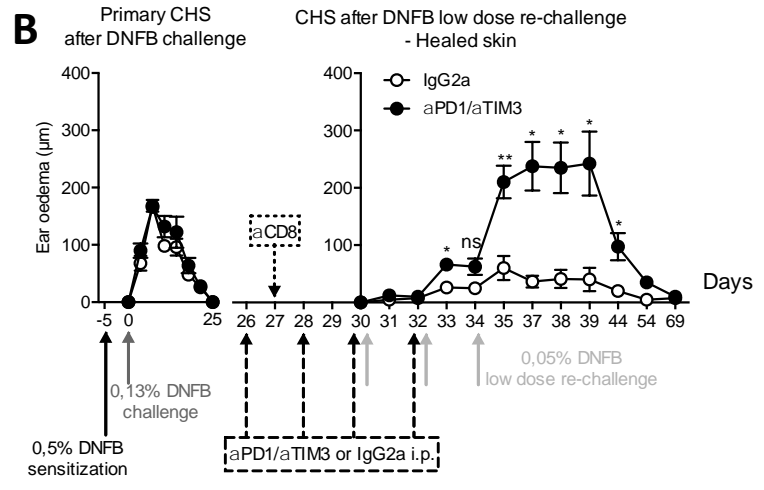
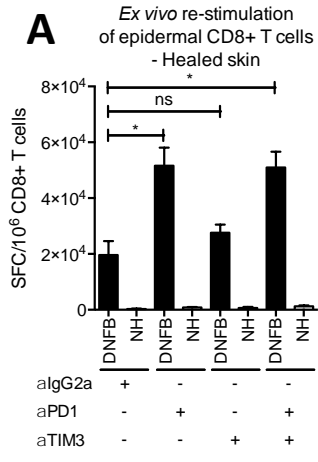
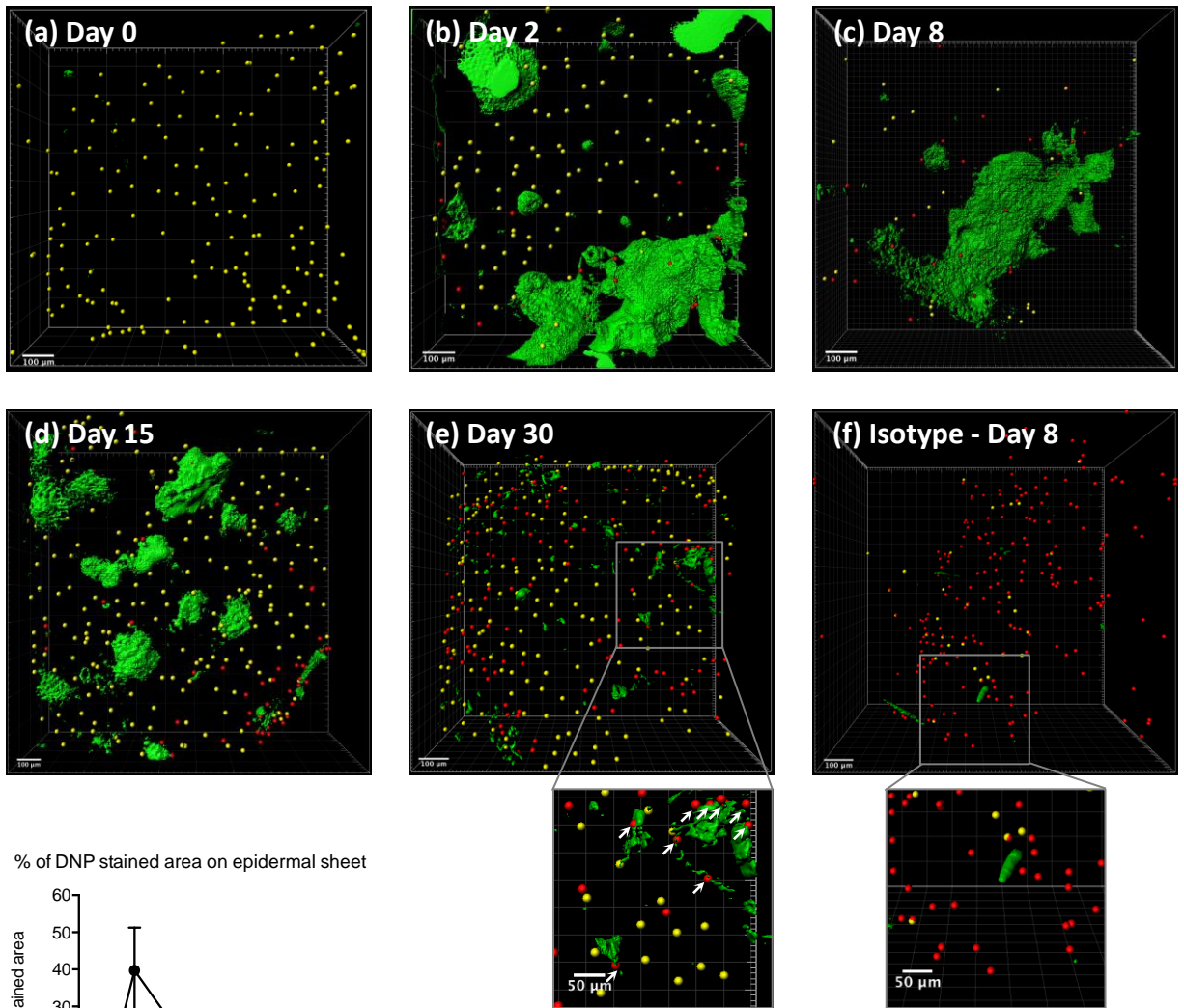


Figure 4

A**B**

% of DNP stained area on epidermal sheet

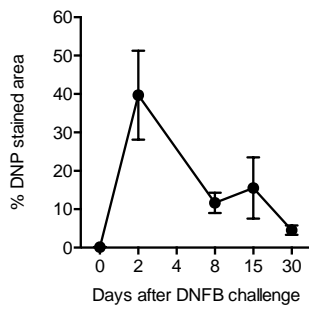
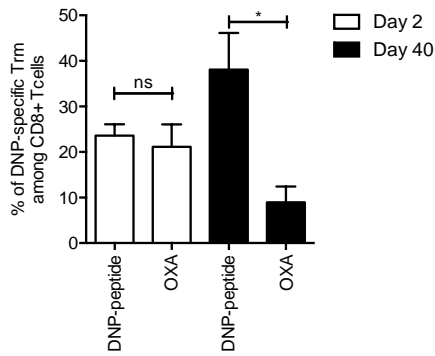


Figure 5

A

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

**B**

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

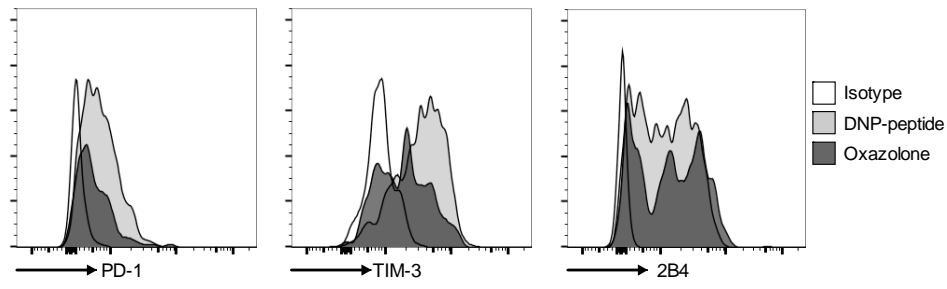
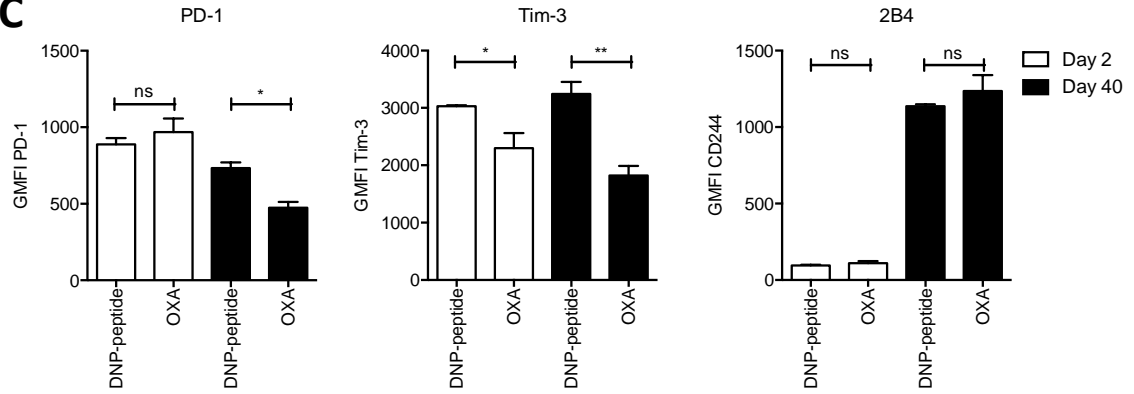
**C**

Figure 6



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

