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1 **Title:**

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3 **Dual function of Langerhans cells in skin TSLP-promoted Tfh cell**
4 **differentiation in mouse atopic dermatitis**

5

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16

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29 **Key words:** Atopic dermatitis; TSLP; Dendritic cells; Langerhans cells; Tfh; Th2; allergen
30 sensitization; mouse

31

32 ***Abbreviations used:***

33 AD: atopic dermatitis

34 BAL: Bronchoalveolar lavage

35 CT: Wild-type Control

36 DC: Dendritic cell

37 DEG: Differentially expressed genes

38 DT: Diphtheria toxin

39 DTR: Diphtheria toxin receptor

40 EDLN: Ear-draining lymph node

41 GC: Germinal center

42 H&E: Hematoxylin and eosin

43 IHC: Immunohistochemistry

44 Ig: Immunoglobulin

45 *i.n.: Intranasal*

46 *i.p.: Intraperitoneal*

47 LC: Langerhans cell

48 LMP: laser-assisted skin microporation

49 LN: Lymph node

50 NT: Non-treated

51 PAS: Periodic Acid Schiff
52 PCA: Principle component analysis
53 Tfh: T follicular helper
54 Th2: T helper type 2
55 ELISA: Enzyme-linked immunosorbent assay
56 TS: Tape stripping
57 TSLP: Thymic stromal lymphopoietin

58

59

60 **Key messages:**

61

- 62 • TSLP is critically involved in mounting Tfh/GC response in mouse AD driven by
63 MC903 or OVA-sensitization.
- 64 • LCs promote Tfh/GC response in MC903-induced AD.
- 65 • LCs suppress Tfh/GC response and Th2 skin inflammation in OVA sensitization-
66 induced AD.

67

68

69

70 **Abstract**

71

72 **Background:** Atopic dermatitis (AD) is one of the most common chronic inflammatory skin
73 diseases, usually occurring early in life, and often preceding other atopic diseases like asthma.
74 Th2 cell has been believed to play a crucial role in cellular and humoral response in AD, but
75 accumulating evidences have shown that T follicular helper (Tfh) cell, a critical player in
76 humoral immunity, is associated with disease severity and plays an important role in AD
77 pathogenesis.

78 **Objectives:** We aimed at investigating how Tfh cells are generated during the pathogenesis of
79 AD, particularly what is the role of keratinocyte-derived cytokine TSLP and Langerhans cells
80 (LCs).

81 **Methods:** We employed two experimental AD mouse models, triggered by the overproduction
82 of TSLP through topical application of MC903, or induced by epicutaneous allergen ovalbumin
83 (OVA) sensitization.

84 **Results:** We demonstrated that the development of Tfh cells and GC response were crucially
85 dependent on TSLP in MC903 model and OVA sensitization model. Moreover, we found that
86 LCs promoted Tfh cell differentiation and GC response in MC903 model, and the depletion of
87 Langerin⁺ DCs or selective depletion of LCs diminished the Tfh/GC response. By contrast, in
88 the model with OVA sensitization, LCs inhibited Tfh/GC response and suppressed Th2 skin
89 inflammation and the subsequent asthma. Transcriptomic analysis of Langerin⁺ and Langerin⁻
90 migratory DCs revealed that Langerin⁺ DCs became activated in MC903 model, whereas these
91 cells remained inactivated in OVA sensitization model.

92 **Conclusion:** Together, these studies revealed a dual functionality of LCs in TSLP-promoted
93 Tfh and Th2 cell differentiation in AD pathogenesis.

94

95 **Capsule Summary**

96

97 This study demonstrates that keratinocyte-derived cytokine TSLP plays a critical role in
98 promoting not only Th2 but also Tfh/GC response in the pathogenesis of atopic dermatitis,
99 which implicates a dual function of epidermal Langerhans cells.

100

101

102 **Introduction**

103

104 Atopic dermatitis (AD) is one of the most common chronic inflammatory skin diseases which
105 affects up to 20% of children and 3% of adults worldwide, with increasing prevalence in the
106 industrialized countries during the last 30 years ¹. AD is characterized by chronic cutaneous
107 inflammation, T helper type 2 (Th2) response and hyper immunoglobulin IgE. Patients
108 suffering from AD often present genetic risk factors in the form of mutations affecting the skin
109 barrier structure or the immune system ². Onset of AD usually occurs early in life and may lead
110 to allergen sensitization, which can trigger the progression from AD to other atopic diseases
111 such as asthma/allergic rhinitis, in a process called “atopic march” ^{3,4}.

112 It has been recognized that Th2 cell response is critically implicated in the pathogenesis of
113 AD. Previous studies from us and others using mouse models have established a central role of
114 the cytokine thymic stromal lymphopoietin (TSLP) expressed by epidermal keratinocytes in
115 promoting Th2 cell response and driving the pathogenesis of AD ⁵⁻⁸. In addition to Th2 cell
116 response, humoral immune response is another key feature of AD, with increased serum IgE
117 and IgG1 levels associated with AD, which contribute to AD pathology and the atopic march ⁹,
118 ¹⁰. For a long time, Th2 cell has been believed to play a crucial role both in cellular response
119 and humoral response, e.g. helping B cells to produce Igs. However, such knowledge has been
120 challenged with the identification of T follicular helper (Tfh) cell, which emerges to be a critical
121 player in humoral immunity and T cell memory ¹¹.

122 In lymphoid organs, Tfh cell differentiation process is believed to begin with an initial
123 dendritic cell (DC) priming of naive CD4⁺ T cells, which undergo a cell-fate decision with the
124 acquisition of master transcription factor Bcl6 expression and chemokine receptor CXCR5
125 expressed on cell surface to become early Tfh cells, of which CXCR5 promotes their migration

126 from T cell zone to the B cell follicles ^{12, 13}. The full differentiation and maintenance of Tfh
127 cells implicate the Tfh cell-B cell interaction, leading to GC Tfh cells which are phenotypically
128 defined by their high expression of CXCR5 and PD-1 ¹⁴. It has been shown that Tfh cells
129 coordinate generation of the GC, initiate help for antigen-specific B cells, and promote selection
130 of high-affinity B cells and differentiation into either memory B cells or long-lived plasma cells
131 ¹⁵. Recent studies have identified Tfh cells as an important source of IL-4, a master regulator in
132 type 2 immunity which was previously thought to be produced by Th2 cells, for providing
133 critical B-cell help by its anti-apoptotic and IgE and IgG1 class switch effects ¹⁶. In addition, it
134 was reported that Tfh cells produce IL-4 in a GATA3-independent manner ¹⁷, suggesting
135 distinct mechanisms employed by Tfh and Th2 cells in the regulation of IL-4.

136 Since their initial identification, the biological functions of Tfh cells and their mechanisms
137 of action in the onset and development of diseases have been studied in autoimmunity, infectious
138 diseases, immunodeficiencies and vaccination ¹⁸. Less is known on Tfh cells in the context of
139 AD and other atopic diseases, but more and more evidences have suggested that Tfh cells are
140 associated with disease severity and Tfh cells play an important role in the pathogenesis ¹⁹⁻²¹.
141 In human, alteration of circulating Tfh cells is correlated with severity of the disease in children
142 with AD ²², or with the comorbid association of allergic rhinitis with asthma ²³, and allergen-
143 specific T follicular helper cell counts are correlated with specific IgE levels and efficacy of
144 allergen immunotherapy ²⁴. In mice, it has been reported that Tfh cells are important for house
145 dust mite-induced asthma ²⁵ or peanut allergy ²⁶.

146 Despite of these accumulating evidences showing the importance of Tfh cells in atopic
147 diseases, how Tfh cells and humoral responses are generated and regulated in AD remained to
148 be investigated. In this study, by employing two experimental AD mouse models, one triggered
149 by the overexpression of TSLP in mouse skin through topical application of MC903 ^{6, 7, 27}, and
150 the other one induced by epicutaneous allergen ovalbumin (OVA) sensitization, we

151 demonstrated that skin TSLP plays a crucial role in driving/promoting Tfh cell differentiation
152 and GC response, in addition to its recognized role in promoting Th2 cell response. Moreover,
153 we investigated the role of skin DCs in mediating the Tfh cell differentiation. We uncovered a
154 dual functionality of epidermal langerhans cells (LCs) in TSLP-promoted Tfh/Th2 cell
155 differentiation in AD pathogenesis, and further explored the molecular insights by
156 transcriptomic analyses, thus shedding new light onto the long-standing controversy of LCs in
157 skin immunity.

158

159 **Methods**

160 Details on the methods used in this study are described in the Methods section in this article's
161 Online Repository, including Experimental mice; MC903 topical application; Epicutaneous
162 OVA sensitization and airway challenge; Depletion of Langerin⁺ DCs or LCs in mice; Cell
163 preparation for flow cytometry analyses; Surface staining for flow cytometry analyses; LN cell
164 culture and antigen stimulation; RNA sequencing; BAL cell analyses; ELISA; Histopathology;
165 IHC staining; RNA in situ hybridization and Statistics.

166

167 **Results**

168

169 **Topical MC903 treatment induces TSLP-dependent Tfh cell differentiation and GC** 170 **response**

171 We have previously reported that topical treatment with MC903, a low calcemic analog of
172 vitamin D3, induces the overproduction of TSLP (TSLP^{over}) and the pathogenesis of AD ⁶,
173 ⁷. To examine the Tfh cell differentiation and GC response in MC903-induced AD model,
174 Balb/c wildtype (WT) mouse ears were topically treated every other day from day (D) 0 to
175 D10 with MC903 and ear-draining lymph nodes (EDLN) were analyzed at D0, D7 and D11
176 (**Fig 1A**). Results showed that the frequency and number of CXCR5⁺ PD-1⁺ Tfh cells were
177 both increased in MC903-treated WT mice at D7 and further augmented at D11 (**Fig 1B**).
178 We next examined the expression of IL-4, a key signal provided by Tfh cells to sustain B
179 cell maturation, by taking use of *Il4/Il13* dual reporter 4C13R^{Tg/0} mice, in which AmCyan
180 and dsRed are expressed under the control of IL-4 and IL-13 regulatory elements,
181 respectively ²⁸. In agreement with a previous report ²⁹, CXCR5⁺ PD-1⁺ Tfh cells in EDLNs
182 express IL-4 (AmCyan) but not IL-13 (dsRed) (**Fig E1A**), and the IL-4 expression by Tfh
183 cells was augmented in MC903-treated 4C13R^{Tg/0} mice at both D7 and D11 (**Fig 1C**).

184 Together, MC903 treatment induces not only Tfh cell differentiation but also the production
185 of IL-4 by Tfh cells.

186 To examine whether the induction of Tfh cells in MC903 model is triggered by TSLP,
187 mice lacking TSLP (*Tslp*^{-/-})⁶ were subjected to MC903 treatment. Results showed that these
188 mice exhibited highly diminished Tfh cell frequency and number at D7 and D11, compared
189 to WT mice (**Fig 1B**). By breeding *Tslp*^{-/-} with 4C13R^{Tg/0} to generate *Tslp*^{-/-}/4C13R^{Tg/0} mice,
190 we showed that MC903-induced IL-4 expression in Tfh cells was abrogated in the absence
191 of TSLP (**Fig 1C**). In agreement with the recognized role of TSLP in Th2 cell differentiation,
192 we showed that the MC903-induced IL-4- or IL-13-expressing CXCR5⁻ CD4⁺ non-Tfh cells
193 (representing Th2 cells) were also abrogated in *Tslp*^{-/-} mice (**Fig E1B**). These results indicate
194 that the overproduction of TSLP triggers not only Th2 cell differentiation, but also Tfh cell
195 differentiation and IL-4 expression by these cells.

196 Next, we examined the GC response in MC903-treated Balb/c WT mice. The number
197 of GC B cells, identified as GL-7⁺ CD95⁺ B cells, exhibited an increase in MC903-treated
198 WT mice at D11, but not at D7 (**Fig 1D**). Such increase was abrogated in MC903-treated
199 *Tslp*^{-/-} mice (**Fig 1D**). This was confirmed by immunofluorescence (IF) staining for GCs (**Fig**
200 **E2A**). In addition, both IgG1⁺ and IgE⁺ B cells exhibited an increase in their numbers in
201 MC903-treated WT mice at D11, which was also abrogated in MC903-treated *Tslp*^{-/-} mice
202 (**Fig 1E**). Of note, we observed that most of the IgG1⁺ B cells were GL-7⁺ CD95⁺ (**Fig E1C**),
203 suggesting that these cells harbor a GC phenotype; however, this was not the case for IgE⁺
204 B cells (**Fig E1C**).

205 Taken together, these data indicate that the overproduction of TSLP triggers Tfh cell
206 differentiation and GC response.

207

208 **Depletion of Langerin⁺ DCs or LCs diminishes the TSLP^{over}-triggered Tfh/GC**
209 **response**

210 LCs reside in the epidermis as a dense network of immune system sentinels, in close
211 proximity to keratinocytes. We then asked whether LCs mediate the TSLP^{over}-triggered
212 Tfh/GC response. To this aim, we first employed Langerin-DTR knock-in mice (Lang^{DTR})
213 in which Langerin⁺ cells, including LCs and Langerin⁺ dermal DCs, express the human
214 diphtheria toxin receptor (DTR) and can thus be depleted upon injection of diphtheria toxin
215 (DT) ³⁰. Lang^{DTR} mice and their wildtype control littermates were intraperitoneally (*i.p.*)
216 injected with DT at D-2, D0 and every 4 days to maintain the depletion of Langerin⁺ cells
217 (named Lang^{DEP} and CT respectively), and were subjected to topical MC903 treatment (**Fig**
218 **2A**). Results showed that the TSLP^{over}-triggered Tfh cell differentiation was largely
219 diminished in Lang^{DEP} mice (**Fig 2B**). The expression of IL-4 (AmCyan) by Tfh cells was
220 also reduced in Lang^{DEP}/4C13R^{Tg⁰} mice (**Fig 2C**). Accordingly, GC B cell number was
221 lower and IgG1⁺ (however not in IgE⁺) B cell number was significantly decreased (**Fig 2D**).
222 Therefore, these results indicate that Langerin⁺ DCs play an important role in mediating the
223 TSLP^{over}-induced Tfh/GC response.

224 As LCs and Langerin⁺ cDC1s were both depleted in Lang^{DEP} mice, we next examined
225 whether LCs mediate Tfh cell differentiation by depleting selectively LCs using two
226 strategies: one took use of the differential recovery time between LCs and Langerin⁺ cDC1s
227 after DT-induced depletion as previously reported ³¹ (**Fig E3A-B**), and the other one
228 employed human Langerin-DTR (huLang^{DTR}) mice in which DT injection efficiently
229 depletes LCs but not Langerin⁺ cDC1s ³² (**Fig E3C-D**). In both cases, we showed that the
230 selective depletion of LCs led to a decrease in frequency and number of Tfh cells, suggesting
231 an important role for LCs in TSLP^{over}-triggered Tfh cell differentiation.

232

233 **Epicutaneous OVA sensitization induces a TSLP-dependent Tfh cell differentiation**
234 **and GC response**

235 We have previously reported that TSLP plays a crucial role for promoting skin sensitization
236 to allergens, using an experimental mouse protocol in which OVA sensitization through
237 tape-stripped (TS) skin leads to an allergic AD inflammation, accompanied by Th2 cell
238 response, and an increased production of OVA-specific IgG1 and IgE in sera ³³. Here, we
239 developed a novel experimental protocol, in which Precise Laser Epidermal System
240 (P.L.E.A.S.E.[®]) ³⁴ was used to disrupt skin barrier and to generate patterned micropores in
241 mouse skin. This protocol allowed us to deliver allergens to micropores at precise depths of
242 the epidermis, thereby achieving a higher efficiency and reproducibility of allergen
243 sensitization through the skin compared with experiments based on TS. We showed that
244 micropores at a depth of 30 μ m (30 μ m-LMP) on Balb/c WT mouse ears reached basal layer
245 of ear epidermis (**Fig 3A**). ELISA analyses indicated that the protein level of TSLP increased
246 at 48 hours after treatment (**Fig 3B**), in agreement with the previous studies showing that
247 barrier disruption induces TSLP production in mouse ³³ and human skin ³⁵. Notably, such
248 level of TSLP was comparable to our previously reported TSLP level in TS skin ³³, although
249 it was much lower compared to that of MC903-treated skin (**Fig 3B; see also Fig E13B**).
250 The administration of OVA did not further induce the TSLP level (**Fig 3B**). *In situ*
251 hybridization showed that TSLP RNA expression was restricted to epidermal keratinocytes
252 in LMP skin (**Fig 3C**).

253 As expected, OVA treatment on LMP ears (named “LMP/OVA”; **Fig 3D**) induced a
254 Th2-type skin inflammation in TSLP-dependent manner, showing that OVA sensitization-
255 induced infiltration of eosinophils and basophils (**Fig E4A-B**), Th2 cytokines (IL-4 and IL-
256 13) expression by T cells in the skin (**Fig E4C**) and by CXCR5⁻CD4⁺ cells in EDLNs (**Fig**
257 **E4D**), were all abolished in mice lacking TSLP. Examination of EDLNs revealed that both

258 frequency and number of Tfh cells were increased in LMP/OVA- compared to LMP/PBS-
259 treated WT mice, and such increase was largely diminished in *Tslp*^{-/-} mice (**Fig 3E**). Note
260 that LMP/PBS was not sufficient to induce Tfh cell differentiation (despite of the induction
261 of TSLP), but LMP plus OVA together promoted Tfh/GC response which was TSLP-
262 dependent (**Fig 3E**). Moreover, IL-4 production by Tfh cells was augmented in LMP/OVA-
263 treated *Tslp*^{+/+}/4C13R^{Tg/0} mice but not *Tslp*^{-/-}/4C13R^{Tg/0} mice (**Fig 3F**). GC B cell number
264 analyzed by flow cytometry (**Fig 3G**) and GC size analyzed by immunofluorescence (**Fig**
265 **E2B**) both showed an increase in LMP/OVA-treated WT mice, and this increase was
266 abrogated in the absence of TSLP. IgG1⁺ and IgE⁺ B cell numbers were also increased in
267 LMP/OVA-treated WT mice, and they were much lower in LMP/OVA-treated *Tslp*^{-/-} mice
268 (**Fig 3G**). Accordingly, serum levels of OVA-IgG1 and OVA-IgE were decreased in *Tslp*^{-/-}
269 mice compared to WT mice upon LMP/OVA treatment (**Fig 3H**). Together, these results
270 demonstrate that TSLP is crucially required for epicutaneous OVA sensitization-induced
271 Th2 and Tfh/GC responses.

272

273 **Depletion of Langerin⁺ DCs or LCs augments the Tfh/GC response induced by** 274 **epicutaneous OVA sensitization**

275 Based on the above data from MC903-induced AD, we had expected that Langerin⁺ DCs
276 would be crucially required for epicutaneous OVA-induced Tfh/GC response. To our
277 surprise, when subjected to 30μm-LMP/OVA sensitization (**Fig 4A**), Lang^{DEP} mice did not
278 exhibit a reduction in frequency and number of CXCR5⁺ PD-1⁺ Tfh cells, instead they tended
279 to be higher compared to CT mice (**Fig 4B**). More strikingly, IL-4 expression by Tfh cells
280 was higher in EDLN from LMP/OVA-treated Lang^{DEP}/4C13R^{Tg/0} mice (**Fig 4C**).
281 Accordingly, the GC B cell, IgG1⁺ and IgE⁺ B cell number were not reduced in LMP/OVA-
282 treated Lang^{DEP} mice (**Fig 4D**), and serum OVA-specific IgE and OVA-specific IgG1 were

283 higher or tended to be higher (**Fig 4E**). Thus, in contrast to our expectation, Langerin⁺ DCs
284 are not required for the Tfh/GC response in LMP/OVA-induced AD model; instead, they
285 appear to play a counteracting role.

286 Because LCs are located on the suprabasal layer of the epidermis, we suspected that
287 Langerin⁺ cells would be only required in Tfh cell differentiation when allergens are
288 encountered superficially on the skin. To test this possibility, LMP was performed at the
289 depth of 11 μ m, which disrupted only the cornified layer of the epidermis (**Fig 5A**). We
290 observed that the 11 μ m-LMP induced also the production of TSLP, even though its level
291 was lower compared to 30 μ m-LMP (**Fig 5B**). Treatment of wildtype control (CT) ears with
292 11 μ m-LMP/OVA induced significant increases (although milder than 30 μ m-LMP/OVA) in
293 Tfh cell frequency as well as GC B cell number, which were all abolished in *Tslp*^{-/-} mice
294 (**Fig 5C**), indicating that, despite of a low induction of TSLP, the Tfh/GC response promoted
295 by 11 μ m-LMP/OVA is still crucially dependent on TSLP. However, when Lang^{DEP} mice
296 were subjected to 11 μ m-LMP/OVA treatment, they exhibited a significant increase in the
297 frequency of Tfh cells, in IL-4 expression by Tfh cells, as well as in GC B cell, IgG1⁺ and
298 IgE⁺ B cell numbers in EDLNs (**Fig 5D-F**), accompanied by augmented serum levels of
299 OVA-IgG1 and OVA-IgE (**Fig 5G**). Similar results were also obtained with huLang^{DEP} mice
300 (**Fig 5H-I**), indicating that LCs significantly counteract the Tfh/GC response induced upon
301 the 11 μ m-LMP/OVA sensitization.

302 Furthermore, we sought to compare antigen-specific Tfh cells between CT and
303 huLang^{DEP} mice using an activation-induced marker assay³⁶. In this assay, the stimulation
304 of LN suspensions with specific antigen drives upregulation of CD154 (CD40L), CD25 and
305 OX40 on Tfh cells, providing a sensitive method for quantifying antigen-specific Tfh cells
306 in mice³⁶. We showed that *in vitro* stimulation with OVA drove the upregulation of CD154,
307 OX40 and CD25 in EDLN-derived Tfh cells from LMP/OVA-sensitized CT mice; and such

308 upregulation was significantly higher in Tfh cells from LMP/OVA-sensitized huLang^{DEP}
309 mice (**Fig 5J**), thus indicating a stronger OVA-specific Tfh cell differentiation in huLang^{DEP}
310 mice upon OVA sensitization.

311 Together, these data indicate that LCs suppress the TSLP-dependent Tfh/GC response
312 in epicutaneous OVA sensitization model.

313

314 **Langerin⁺ DCs or LCs limit epicutaneous OVA-induced Th2 skin inflammation and** 315 **the subsequent asthma**

316 Having observed the opposite role of Langerin⁺ DCs or LCs in Tfh/GC response in the two
317 mouse AD models, we further explored their involvement in the induction of Th2 cell
318 response. Upon MC903 treatment, Lang^{DEP}/4C13R^{Tg/0} mice exhibited a slight decrease in
319 IL-4 and a tendency of decrease in IL-13 production by CXCR5⁺CD4⁺ cells in EDLN (**Fig**
320 **E5A**), or by TCRβ⁺ cells in dermis (**Fig E5B**), which suggests a role, even though minor,
321 for Langerin⁺ DCs in the development of Th2 cell response. In contrast, upon 30μm-
322 LMP/OVA treatment, Lang^{DEP}/4C13R^{Tg/0} mice exhibited a higher Th2 cell response in both
323 skin (**Fig 6A**) and EDLN (**Fig E6**). This was in accordance with the observation that
324 LMP/OVA-sensitized Lang^{DEP} mice exhibited a stronger skin inflammation (**Fig 6B**),
325 accompanied with an increase in eosinophils and basophils (**Fig 6C**). Moreover, when
326 subjected to 11μm-LMP/OVA sensitization, both Lang^{DEP} and huLang^{DEP} mice exhibited an
327 enhanced AD-like skin inflammation compared to CT mice (**Fig E7**). Therefore, contrary to
328 their minor role in promoting Th2 cell response in MC903-AD, LCs suppress the Th2 cell
329 response in OVA-AD.

330 We further examined whether Langerin⁺ DCs limit the atopic march. Upon *intranasal*
331 (*i.n.*) OVA challenge following epicutaneous allergen sensitization (**Fig 6D**), the Lang^{DEP}
332 mice developed a much stronger asthmatic inflammation compared with CT mice, exhibiting

333 an increase in the number of eosinophils in bronchoalveolar lavage fluid (BAL) (**Fig 6E**),
334 and in RNA expression of Th2 cytokines IL-4, IL-5 and IL-13, as well as chemokine receptor
335 CCR3 (eosinophils) and MCPT8 (basophils) by BAL cells (**Fig 6F**). In addition, H&E
336 staining of lung sections of OVA-treated Lang^{DEP} mice revealed an increased peribronchial
337 and perivascular infiltration, and PAS staining showed an enhanced goblet cells hyperplasia
338 (**Fig 6G**). Similar results were obtained with huLang^{DEP} mice (**Fig E8 A-F**), indicating that
339 LCs counteract the asthma development following epicutaneous allergen sensitization. To
340 exclude the possibility that the enhanced asthmatic inflammation is due to any depletion of
341 lung DCs during the intranasal challenge, we subjected huLang^{DEP} mice to *i.p.* sensitization
342 with OVA/alum and *i.n.* OVA challenge, and observed that these mice developed similar
343 asthmatic inflammation as wildtype control mice (**Fig E8 G-H**). This suggests that the
344 limitation of asthma inflammation by LCs is indeed due to their role in suppressing the
345 epicutaneous allergen sensitization.

346 Taken together, these studies reveal opposite roles of LCs in two AD models: in MC903-
347 AD, LCs play an important role in priming Tfh/GC response; they participate but to a lesser
348 extend in promoting Th2 responses. In OVA-AD, LCs are neither required for Tfh/GC nor
349 Th2 responses, instead, they suppress OVA-induced Tfh/GC and Th2 responses as well as
350 the “atopic march”.

351

352 **Langerin⁺ migratory DCs from MC903-AD but not from OVA-AD mice present** 353 **profound transcriptomic changes**

354 We next conducted transcriptomic studies to explore molecular insights underlying the
355 opposite roles of Langerin⁺ DCs in Tfh and Th2 cell differentiation in MC903-AD and OVA-
356 AD, by taking use of Lang^{GFP} mouse line in which GFP reports the expression of Langerin
357 ³⁰. Lang^{GFP} mice were treated with MC903 (at D0, D2 and D4) or LMP/OVA (at D0 and

358 D3), and at D5, Langerin⁺ (GFP^{pos}) and Langerin⁻ (GFP^{neg}) migratory DCs (migDCs) were
359 sorted from EDLNs of non-treated (NT), MC903- or LMP/OVA-treated mice, and
360 proceeded to mRNA sequencing (**Fig E9A**). The time point at D5 was selected to compare
361 gene expression patterns of migDCs at the initiation stage of Tfh and Th2 cell differentiation.

362 Principle component analysis (PCA) for the RNAseq data revealed that the Pos_MC
363 (GFP^{pos} migDCs from MC903-treated Lang^{GFP} mice) was clearly separated from the Pos_NT
364 (GFP^{pos} migDCs from non-treated Lang^{GFP} mice); however, the Pos_OVA (GFP^{pos} migDCs
365 from LMP/OVA-treated Lang^{GFP} mice) was inseparable from the Pos_NT (**Fig 7A**).
366 Correspondingly, analyses of differentially expressed genes (DEGs) in Pos_MC vs Pos_NT
367 identified 756 upregulated and 559 downregulated genes (with a fold change >1.5 and
368 adjusted p<0.05; **Fig 7B**); in a sharp contrast, the comparison of Pos_OVA vs Pos_NT
369 revealed only 39 upregulated and 9 downregulated genes (**Fig 7B**). Therefore, in MC903-
370 AD, Langerin⁺ migDCs undergo profound transcriptomic changes, but in OVA-AD, they
371 present almost no, or very little, transcriptomic changes.

372 As to Langerin⁻ migDCs, PCA showed that Neg_MC (GFP^{neg} migDCs from MC903-
373 treated Lang^{GFP} mice), Neg_OVA (GFP^{neg} migDCs from LMP/OVA-treated Lang^{GFP} mice)
374 and Neg_NT (GFP^{neg} migDCs from non-treated Lang^{GFP} mice) were all clustered away from
375 each other (**Fig 7A**). Analyses of DEGs identified 710 upregulated and 698 downregulated
376 genes for Neg_MC vs Neg_NT; and 431 upregulated and 427 downregulated genes for
377 Neg_OVA vs Neg_NT (**Fig 7B**), suggesting that Langerin⁻ migDCs present major
378 transcriptomic changes in both MC903-AD and OVA-AD, with considerable numbers of
379 overlapped DEGs (249 upregulated and 215 downregulated).

380

381 **Gene ontology analyses of DEGs in Langerin⁺ migDCs from MC903-treated mice**

382 Next, using the upregulated or downregulated DEGs identified in Pos_MC (vs Pos_NT) as
383 input, we performed cluster analyses of all the groups and generated heat map to visualize
384 trends of expression for genes across the different groups. Results are presented in **Fig E9B**
385 and **Fig E10A**. Further, we performed gene ontology (GO) analyses of the upregulated genes
386 in Pos_MC (**Fig 7C**), and examined whether these genes were also significantly upregulated
387 in Neg_MC (vs Neg_NT), and Neg_OVA (vs Neg_NT). We paid particular attention to the
388 upregulated genes shared in all the three groups (Pos_MC, Neg_MC and Neg_OVA),
389 standing here for “commonly upregulated” genes (highlighted in red in **Fig 7C**), as they
390 could be implicated in TSLP-promoted Tfh and/or Th2, a common feature shared by
391 MC903-AD and OVA-AD. Among them, we found genes related to: 1) “regulation of cell
392 migration”, many of which were reported to facilitate DC migration (*Mmp14*³⁷; *Stat5*³⁸;
393 *Nrp2*³⁹; *Sema7a*⁴⁰); 2) “T cell costimulation”: *Cd80* and *Cd86*⁴¹, *IL2ra*⁴², *Pdcd1lg2* (PD-
394 L2)⁴³, *Cd274* (PD-L1), *Gpr183* (EBI2)^{44,45}; 3) “cytokine signal”: *Il2ra*, *Tnfrsf11b* and *Ccl22*;
395 and 4) “transcription factors” such as *Ikzf4*, *Irf4*, *Stat4* and *Stat5a*.

396 We examined TSLP signaling pathway among the upregulated genes in Pos_MC. Using
397 the reported TSLP-regulated gene set⁴⁶, we identified *Cd84*, *Cd82*, *Ccl17*, *Ccl22* and *Tnfrsf11b*
398 (in the cluster with higher expression in Pos_MC than Neg_MC), as well as *Cish*, *Cd86*, *Cd80*,
399 *Cd274*, *Il2ra*, *Il6*, *ccr2*, *Tgfb1* (in the cluster with higher expression in Neg_MC than Pos_MC)
400 (**Fig 7D**). In addition, we identified *Irf4*, which has been recently shown to be downstream of
401 TSLP signaling in human migratory LCs⁴⁷. The upregulation of these TSLP-targeting genes
402 by Langerin⁺ migDCs suggests that these cells could be a direct responder to TSLP signaling,
403 although it remains to be demonstrated that TSLP signals through its receptor on LCs drive
404 their migration/activation. Besides these known TSLP downstream genes, more TSLP pathway
405 genes identified from those “commonly upregulated” genes can be envisaged.

406 Interestingly, we did not find *Tnfrsf4* (encoding OX40L) among the DEGs in Pos_MC,
407 despite that OX40L was reported to be TSLP-responsive gene and mediate TSLP-promoted
408 Th2⁴⁸ and Tfh⁴⁹ cell differentiation. Actually, OX40L expression by GFP^{pos} cells was barely
409 detected in Pos_NT, Pos_MC or Pos_OVA (**Fig 7E**). On the other hand, OX40L was expressed
410 in Neg_NT, and its expression was further upregulated in Neg_MC and Neg_OVA. Therefore,
411 it is unlikely that OX40L would be responsible for the Tfh-promoting function of Langerin⁺
412 DCs, while its precise function as a potential TSLP downstream factor in Langerin⁻ DCs
413 remains to be defined (**Fig 7E**).

414 Among the above-mentioned TSLP-regulated gene, IL-6 has been shown to be a critical
415 cytokine for Tfh cell differentiation^{50,51}. We thus tested whether IL-6 neutralization decreases
416 Tfh / GC response in MC903-AD. Results showed that IL-6 was not required for the initiation
417 of Tfh cell differentiation and the overall GC reaction (**Fig E11**), although it is possible that its
418 function in Tfh response is redundant with other signals as suggested by Eto et al⁵². Besides
419 IL-6, several other Tfh-promoting factors derived from DCs have been recently reported,
420 including IRF-4⁵³, IL-2Ra^{42,54} and EBI2 (*Gpr183*)^{44,45}, whose expression was all “commonly
421 upregulated” in Pos_MC, Neg_MC and Neg_OVA (**Fig 7D**). The role of these potential
422 candidates in TSLP-promoting Tfh cell differentiation remains to be examined.

423 Finally, among the downregulated genes (**Fig E10B**), less knowledge was available, but
424 we could see *Il12b* (IL-23/IL-12p40), whose expression in DCs was previously reported to be
425 suppressed by TSLP⁵⁵. Other commonly downregulated ones included genes related “T cell
426 costimulation” *Havcr2* (TIM3), *Lgals8* (Galectin 8); “Regulation of cell migration” *Adam15*
427 and *Ptk2* (negative regulators for cell migration) and “regulation of transcription” *Foxc2*, *Thrb*,
428 *Tcf7l2*, *Ehf* and *Lmo2*.

429

430 **Discussion**

431

432 In this study, we analyzed how Tfh cells were generated in two experimental AD mouse models,
433 triggered by the overproduction of TSLP by topical application of MC903, or induced by
434 epicutaneous OVA sensitization. We demonstrated a crucial role for TSLP in promoting Tfh
435 cells and GC response in MC903-AD as well as OVA-AD. Intriguingly, we revealed a dual
436 function of LCs in TSLP-promoted Tfh/Th2 cell differentiation: while they promoted Tfh cell
437 differentiation in MC903-AD, they inhibited Tfh/GC response and suppressed Th2 skin
438 inflammation and the atopic march in OVA-AD. This is schematically illustrated in **Fig 8**, and
439 is discussed below.

440

441 **1) TSLP: critical player for Th2 and Tfh cell response in AD**

442 It has been recognized that TSLP is overproduced in AD lesional skin ⁵⁶, however, its
443 expression varies from high to low, which could be related with the cause (e.g. genetic mutation
444 of *Spink5* which induces a high level of TSLP ⁵⁷ vs skin barrier impairment which induces a
445 low level of TSLP ³⁵), age (e.g. TSLP serum level in AD children is high at early stage and
446 decreases with age ⁵⁸), or the nature of disease (e.g. intrinsic or extrinsic AD). Our study
447 demonstrates that no matter in AD models associated with either high or low TSLP expression,
448 TSLP is crucial for promoting Tfh/GC response in AD. Recently, the link between TSLP and
449 Tfh cell differentiation was suggested by the study with human blood DC-T cell coculture
450 system ⁴⁹. Thus, the Tfh-promoting function of TSLP appears to be conserved between mouse
451 and human, which suggests that it is relevant and valuable to employ AD mouse models to
452 elucidate mechanisms underlying the TSLP (skin)-Tfh (draining LN) axis, particularly the
453 access of tissue and lymphoid organs is rather limited in human study.

454 Our data add new evidence that neutralization of TSLP or blocking TSLP downstream
455 pathway will be helpful for reducing Th2 and Tfh cell responses in AD. Notably, TSLP is
456 crucial for driving the downstream IL-4/IL-13 expression by Th2 cells, as well as IL-4
457 expression by Tfh cells. Indeed, blocking antibody against IL-4/-13R (Dupilumab), which may
458 actually target both Th2 and Tfh cell responses, has been shown to achieve significant
459 therapeutic effect on AD⁵⁹. Intriguingly, neutralization TSLP antibody Tezepelumab has been
460 demonstrated to significantly reduce annual asthma exacerbation rate in patients with
461 uncontrolled asthma⁶⁰. A recent study with Tezepelumab showed numeric improvements in
462 patients with moderate to severe AD, despite that there were certain limitations in that study
463 including patient selection, use of topical corticosteroids, duration of treatment and uncertain
464 inhibition of TSLP with the dose used⁶¹. Given the preclinical evidence for the role of TSLP
465 in AD, more clinical studies are required to evaluate TSLP as therapeutic target in AD.

466 It should be also noted that recent studies have recognized the importance of Tfh cells in
467 AD¹⁹⁻²¹, but the *in vivo* function of Tfh remains to be further delineated using AD mouse
468 models. This is challenged by the lack of appropriate tools to deplete Tfh cells. We are under
469 the way to generate mouse line in which DTR can be selectively expressed in Tfh cells, thus
470 allowing the DT-induced depletion of Tfh cells.

471

472 **2) LCs: function as migratory DCs to promote Tfh cell differentiation**

473 LCs represent one of the most studied but controversial DC subtypes. Our study shows that LCs
474 are importantly engaged in the initiation Tfh cell differentiation and GC response triggered by
475 TSLP^{over} in MC903-AD. This provides new evidence on the Tfh-promoting function of LCs in
476 AD, in addition to several studies reporting the requirement of LCs for humoral responses in
477 other contexts^{62,63,64}. In MC903-AD, we observed that LCs play a dominant role in Tfh cell
478 differentiation, although dermal langerin⁻ DCs may also contribute. On the other hand, langerin⁻

479 DCs (cDC2) appear to be the major player for the TSLP^{over}-induced Th2, while LCs have
480 somewhat but minor contribution. Nevertheless, to provide direct evidence for the contribution
481 of cDC2 in TSLP-driven Tfh and Th2 responses in AD, further studies could be performed
482 using DC-specific KO of IRF4 or Dock8 mice, which have impaired development and
483 migration of CD11b⁺ cDC2 ⁶⁵, or CD301b-DTR mice in which CD301b⁺ cDC2 can be
484 transiently depleted ⁶⁶.

485 There have been long debates on the migration, antigen uptake, and T cell differentiation
486 of LCs in different contexts; but transcriptomic study on migratory LCs in skin-draining LNs
487 under inflammatory pathological contexts was lacking. Our transcriptomic data are therefore of
488 value; however, one drawback is that migratory LCs and cDC1 were not separated in Langerin⁺
489 (GFP^{pos}) migDCs, thus the gene expression data still need to be cautiously interpreted
490 concerning LCs. Nevertheless, we have shown that Langerin⁺ migDCs in EDLN of MC903-
491 induced TSLP^{over} mice presented substantial transcriptional changes, suggesting that the
492 activation and migration of Langerin⁺ DC to the draining LNs underlie its function to prime
493 Tfh cell differentiation in MC903-AD. Indeed, when comparing numbers of GFP^{pos} and GFP^{neg}
494 migratory DCs in EDLN of MC903-treated Lang^{GFP} mice at D5, we observed that both were
495 increased (**Fig E12**), supporting that both Langerin⁺ DCs and Langerin⁻ DCs migrate to draining
496 LNs in MC903-AD.

497

498 **3) LCs: function as non-migratory cells in the skin to suppress Tfh/Th2 response?**

499 Our study revealed a suppressive role of LCs for epicutaneous OVA-induced Tfh and Th2 cell
500 differentiation. This is in contrast with two previous studies which reported a role of LCs in
501 provoking AD inflammation by using a tape stripping (TS) OVA sensitization model ^{67, 68}. To
502 examine whether the discrepancy is due to the different effects of LMP compared to TS, we
503 performed TS/OVA sensitization on mouse ears. Results showed that, similar to LMP/OVA,

504 TS/OVA-sensitized Lang^{DEP} mouse EDLNs exhibited increased frequency and number of Tfh
505 cells, increased IL-4 expression by Tfh cells, higher numbers of GC B cells, IgG1⁺ and IgE⁺ B
506 cells, with elevated OVA-IgG1 and OVA-IgE in sera (**Fig E13 A-G**). Moreover, when *i.n.*
507 challenged with OVA, Lang^{DEP} mice developed a stronger asthmatic inflammation (**Fig E13**
508 **H-I**). Therefore, the discrepancy with the previous reports ^{67, 68} is not explained by the
509 difference of LMP vs TS technique in epicutaneous OVA sensitization; rather, it could be due
510 to other factors remained yet to be determined, such as the allergen application method: topical
511 OVA vs long exposure (2-day) of OVA placed on patch-test tape; the difference of mouse
512 background: Balb/c vs C57Bl/6; or the site of allergen application: ear vs back.

513 Why are LCs not implicated in the promotion of Tfh/Th2 cell differentiation in EDLN in
514 this context? Transcriptomic analyses showed that in sharp contrast to MC903-AD, Langerin⁺
515 migDCs in OVA-AD presented almost the same transcriptomic program as in untreated mice,
516 suggesting an absence of migration/activation of these cells. Indeed, Langerin⁺ migDC numbers
517 in EDLNs from LMP/OVA-treated or TS/OVA-treated mice at D5 were nearly unchanged,
518 whereas Langerin⁻ migDC number was increased (**Fig E12**). This is in agreement with previous
519 studies showing that when skin was treated with fluorescence-conjugated OVA ⁶⁶, HDM ⁶⁹, or
520 Dextran ⁷⁰, antigen uptake and transport to draining LNs were mainly exerted by Langerin⁻ DCs.
521 Of note, it was recently shown that LCs can transfer antigen to cDC2 in the context of Langerin
522 mAb-mediated targeting ⁷¹. It will be interesting to see whether this occurs in AD models, and
523 whether efficiency of LC antigen transfer could be altered in the two models, as another possible
524 explication of different implication of LCs in Tfh cell differentiation.

525 Then how do LCs exert their anti-Tfh/Th2 role in OVA-AD? A recent study showed that
526 LCs played an immunosuppressive role when OVA was applied on the intact skin, in
527 accompany with the induction of IL-10 in LCs in skin-draining LNs ⁷². However, this does not
528 seem to be our case, because Langerin⁺ migDCs in EDLN did not exhibit any transcriptional

529 change of Treg-inducing signals including IL-10 and TGF β , or RALDH2. More likely, the anti-
530 Tfh/Th2 effect of LCs is related to their immune suppression function *in situ* in the skin, in
531 keeping with LC ontogeny not only as DCs but also as non-migratory macrophages^{73, 74}. It
532 should be further studied how LCs exert such functionality, for example, by limiting the
533 antigen-uptake by cDC2 in the skin, or by promoting local Tregs in OVA-sensitized skin^{75 76}.
534 Transcriptomic analysis of LCs isolated from the OVA-treated skin site may provide further
535 molecular insights.

536

537 **4) What signals switch the function of LCs?**

538 One intriguing question is what microenvironment cues and molecular signals switch the
539 function of LC between anti-Tfh/Th2 to pro-Tfh/Th2 in AD contexts. Notably, MC903-AD and
540 OVA-AD exhibit similar AD phenotype which is TSLP-dependent, but the quantity of TSLP
541 and the nature of antigen are different in these two models. In MC903-AD, MC903 induced a
542 high production of TSLP⁷ (**Fig 8**) which was sufficient to induce Tfh and Th2 cell
543 differentiation. As there was no administration of experimental allergen, the nature of antigen
544 implicated in T cell differentiation in MC903 model may involve endogenous antigens or
545 microbiota co-existing in the skin. On the other hand, in OVA-AD, the disruption of skin barrier
546 with LMP induced TSLP expression however to a much lower extent (**Fig 8**). It is possible that
547 LCs sense the quantity of TSLP. Indeed, as a danger signal, TSLP may convert the function of
548 LCs when its level is above certain threshold. *In vitro* studies have shown that TSLP triggers
549 DC migration⁷⁷, or promotes the survival, maturation and migration of human LCs, and
550 allogenic naïve CD4⁺ T cells cocultured with TSLP-conditioned LCs produced cytokines IL-4
551 and IL-13⁷⁸, but quantitative study on TSLP signaling has never been performed. It will be
552 interesting to explore whether and how quantitative TSLP signaling determines the role of LCs,
553 by conducting *in vivo* or *ex vivo* dose-dependent experiments. In addition, the nature and

554 quantity of antigens can be also involved in the functional switch of LCs. To unravel such
555 complexity, the emerging mathematic modeling^{79, 80} may eventually help to integrate multiple
556 parameters for a better understanding of functional switch of LCs.

557 It will be interesting to further explore in AD patients whether and how TSLP levels are
558 correlated with the states and function of LCs. A better understanding of what molecular switch
559 determines the function of LCs either as "pro-Tfh/Th2" or as "anti-Tfh/Th2", and of how LCs
560 exert such functions, will allow us to shape LCs to act in suppressing the skin inflammation,
561 limiting the allergen sensitization through AD skin, thus preventing the progression from AD
562 to asthma. On the other hand, the potential of LCs to induce Tfh cell differentiation and GC
563 response and the subsequent induction of antigen-specific antibodies has been of interest for
564 transcutaneous vaccination^{63, 81}. Therefore, the knowledge we obtain from this study should be
565 also insightful for LC-based skin vaccination, including the use of TSLP at an appropriate level
566 as an effective adjuvant for promoting Tfh cell differentiation and humoral response.

567

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819

820 **Figure Legends**

821

822 **FIG 1.** Overproduction of TSLP in the skin triggers Tfh differentiation and GC reponse in
823 MC903-induced AD mice. **A**, Experimental protocol. Mouse ears were topically treated with
824 MC903 or ethanol (EtOH; as vehicle control) every other day from day (D) 0 to D10 and
825 EDLNs were analyzed at D0, D7 and 11. **B**, Frequency and number of CXCR5⁺ PD-1⁺ Tfh cells
826 in EDLN from MC903-treated Balb/c wildtype (WT) and *Tslp*^{-/-} mice. **C**, Frequency of IL-4
827 (AmCyan)⁺ in Tfh cells and cell number of IL-4⁺ Tfh cells in EDLNs. **D-E**, Number of CD95⁺
828 GL-7⁺ GC B cells, IgG1⁺ B cells and IgE⁺ B cells in EDLNs. Values shown are means ± SEMs.
829 **B-D**, one-way ANOVA with Tukey's multiple comparison post-hoc test; **E**, unpaired t-test with
830 Welch's correction. Data are representative of 3 independent experiments with similar results.

831

832 **FIG 2.** Depletion of Langerin⁺ cells diminishes the MC903-induced Tfh/GC response. **A**,
833 Experimental protocol. Lang^{DTR} mice and wildtype littermate controls (CT) were *i.p.* injected
834 with DT at D-2 and D0 and then every 4 days. Mouse ears were topically treated with MC903
835 or EtOH every other day from D0 to D10 and EDLNs were analyzed at D11. **B**, Frequency and
836 number of Tfh cells. **C**, IL-4 (AmCyan) expression by Tfh cells. **D**, Total number of GC B cells,
837 IgG1⁺ and IgE⁺ B cells. Values shown are means ± SEMs; one-way ANOVA with Tukey's
838 multiple comparison post-hoc test. Data are representative of 3 independent experiments with
839 similar results.

840

841 **FIG 3.** OVA sensitization through laser-microporated (LMP) skin induces TSLP-dependent
842 Tfh/GC response. **A**, H&E staining of untreated or 30µm-LMP ears of Balb/c WT mice. The
843 red arrow points to a micropore with the disruption of the epidermis. Scale bar, 100 µm. **B**,
844 TSLP protein levels in ears of WT mice at 48h after the indicated treatment. **C**, RNAscope in

845 situ hybridization for TSLP in untreated or 30 μ m-LMP-ears at 48h after the microporation. The
846 black arrow points to one of the positive signals. Scale bar, 50 μ m. **D**, Experimental protocol
847 for OVA epicutaneous sensitization through LMP ears. OVA or PBS (vehicle) were topically
848 applied on LMP ears at D0, D4, D7 and D11 and EDLNs were analyzed at D13. **E-F**, Frequency
849 and cell number of Tfh cells (**E**) and IL-4 (AmCyan) producing Tfh cells (**F**) in EDLNs. **G**, GC
850 B cell, IgG1⁺ and IgE⁺ B cell numbers in EDLNs. **H**, Serum levels of OVA-IgG1 and OVA-
851 IgE. Values shown are mean \pm SEM; one-way ANOVA with Tukey's multiple comparison
852 post-hoc test. Data are representative of 3 independent experiments with similar results.

853

854 **FIG 4.** Depletion of Langerin⁺ cells does not reduce but rather tends to augment 30 μ m-
855 LMP/OVA-induced Tfh/GC response. **A**, Experimental protocol. Lang^{DTR} mice and wildtype
856 littermate controls (CT) were *i.p.* injected with DT at D-2, D0 and then every 4 days. Mouse
857 ears were treated by 30 μ m-LMP/OVA or 30 μ m-LMP/PBS at D0, D4, D7 and D11 and EDLNs
858 were analyzed at D13. **B**, Frequency and number of Tfh cells. **C**, IL-4 (AmCyan) expression
859 by Tfh cells. **D**, Number of GC B cells, IgG1⁺ and IgE⁺ B cells. **E**, Serum levels of OVA-
860 specific IgG1 and OVA-specific IgE in 30 μ m-LMP/OVA-sensitized Lang^{DEP} or CT mice. Data
861 are means \pm SEM; **B-D**, one-way ANOVA with Tukey's multiple comparison post-hoc test. **E**,
862 unpaired t-test with Welch's correction. Data are representative of 3 independent experiments
863 with similar results.

864

865 **FIG 5.** Depletion of Langerin⁺ cells or LCs enhances 11 μ m-LMP/OVA-induced TSLP-
866 dependent Tfh/GC response. **A**, H&E staining of untreated or 11 μ m-LMP ears of Balb/c WT
867 mice. The red arrow points to a micropore with the impairment of cornified layer. Scale bar,
868 100 μ m. **B**, TSLP protein levels in ears of WT mice. **C**, Comparison of Tfh cells and GC B
869 cells in EDLNs from WT or *Tslp*^{-/-} mice. **D-F**, Comparison of Tfh cells (**D**), IL-4 (AmCyan)

870 expression by Tfh cells (E) and number of GC B cells, IgG1⁺ B cells and IgE⁺ B cells (F) in
871 EDLNs from CT or Lang^{DEP} mice. G, Serum OVA-IgG1 and OVA-IgE levels. H, Experimental
872 protocol. I, Comparison of Tfh cells, GC B cells, IgG1⁺ and IgE⁺ B cells in CT and huLang^{DEP}
873 mice. J, Comparison of antigen-specific Tfh cells between LMP/OVA-sensitized CT and
874 huLang^{DEP} mice. EDLNs were *in vitro* stimulated with OVA or PBS (vehicle control), and
875 activation markers CD154, CD25 and OX40 expressed by EDLN-derived Tfh cells were
876 examined. Values shown are mean ± SEM; one-way ANOVA with Tukey's multiple
877 comparison post-hoc test. Data are representative of 2 independent experiments with similar
878 results.

879

880 **FIG 6.** Langerin⁺ cells counteract LMP/OVA sensitization-induced skin Th2 inflammation and
881 the subsequent asthmatic phenotype. **A**, IL-4 (AmCyan) and IL-13 (DsRed) expression in
882 TCRβ⁺ dermal cells. **B**, H&E staining of mouse ears. **C**, IHC staining of mouse ears with anti-
883 MBP (for eosinophils) or anti-MCPT8 (for basophils). Arrows point to positive signals. **D**,
884 Experimental protocol for OVA epicutaneous sensitization and airway challenge. Mice were
885 *i.p.* injected with DT at D-2, D0 and then every 4 days. Mice were either treated with OVA on
886 LMP ears at D0, D4, D7 and D11 or non-treated (NT). All mice were subjected to *i.n.*
887 instillation with OVA from D9 to D12, and analyzed at D13. **E**, Differential cell counting for
888 eosinophils (Eos), neutrophils (Neutro), lymphocytes (Lympho) and macrophages (Macro) in
889 BAL. **F**, RNA levels of indicated genes in BAL cells by RT-qPCR. **G**, Lung sections were
890 stained with H&E for histology or PAS for goblet cell hyperplasia analyses. B: bronchiole, V:
891 blood vessel. Scale bar, 100μm. Values shown are means ± SEM; one-way ANOVA with
892 Tukey's multiple comparison post-hoc test. Data are representative of 2 independent
893 experiments with similar results.

894

895 **FIG 7.** Transcriptomic analyses of migratory DCs in EDLNs of Lang^{GFP} mice upon MC903
896 treatment or epicutaneous OVA sensitization. Lang^{GFP} mice were treated with MC903 at D0,
897 D2 and D4 or 30µm-LMP/OVA on D0 and D3; EDLNs were collected at D5 for cell sorting
898 and RNAseq analyses. **A**, Left, percentage of variability explained by each Principal
899 Component. Right, principal component analyses showing PC1, PC2 and PC3. **B**, Venn
900 diagram showing the number of upregulated and downregulated genes (fold change > 1.5; p <
901 0.05; raw read > 200 in at least one sample of all groups), and the number of commonly
902 upregulated or downregulated genes between the comparisons, as indicated. Pos_NT, Pos_MC,
903 Pos_OVA: GFP^{Pos} migDCs from non-treated, MC903-treated or LMP/OVA-treated Lang^{GFP}
904 mice; Neg_NT, Neg_MC, Neg_OVA: GFP^{neg} migDCs from non-treated, MC903-treated or
905 LMP/OVA-treated Lang^{GFP} mice. **C**, Selected genes corresponding to gene ontology terms. *,
906 p<0.05; NS, non significant. **D**, Heatmaps of the reported TSLP pathway genes, which are
907 significantly upregulated in Pos_MC vs Pos_NT. **E**, Heatmap of Tnfsf4 (encoding OX40L)
908 from RNAseq data, and RT-qPCR analyses.

909

910 **FIG 8.** A schematic representation of the dual functions of LCs in regulating TSLP-dependent
911 Tfh cell and Th2 cell response, revealed by two experimental AD mouse models, triggered by
912 the overproduction of TSLP through topical application of MC903, or induced by epicutaneous
913 allergen ovalbumin (OVA) sensitization.

914

915

916 **Supplementary Figure Legends**

917

918 **FIG E1. (A)** CXCR5⁺ PD-1⁺ Tfh cells produce IL-4 (AmCyan) but not IL-13 (dsRed) in
919 EDLNs of MC903-treated 4C13R^{Tg0} mice at D11. 4C13R^{0/0} EDLNs were used as gating
920 control. **(B)** Frequency and number of CXCR5⁻ CD4⁺ (non-Tfh) cells producing IL-4
921 (AmCyan) or IL-13 (dsRed), representing Th2 cells, in EDLNs from Balb/c wildtype (WT) and
922 *Tslp*^{-/-} mice in the background of 4C13R^{Tg0}, treated with MC903 or ethanol, and analyzed at
923 D0, D7 and D11. **(C)** The majority of IgG1⁺ but not IgE⁺ B cells in EDLNs from MC903-
924 treated wildtype Balb/c mice are GL-7⁺ CD95⁺.

925

926 **FIG E2.** Germinal center staining. Wildtype (WT) and *Tslp*^{-/-} mice were treated with MC903
927 **(A)** or subjected to OVA-sensitization **(B)** as shown in FIG 1A and 4D respectively. EDLN
928 were collected and fixed overnight with 4% PFA at 4°C. After 2 times 30 minutes of wash in
929 PBS at room temperature (RT), samples were included in 4% low melting point agarose in PBS.
930 Vibratome sections of 100µm were blocked with 5% normal donkey serum (NDS), 0.1% Triton
931 X-100 in PBS and then stained overnight at 4°C with anti CD4-AlexaFluor 647 (RM4-5,
932 Biolegend, d=1/100; shown in blue), anti IgD-FITC (11-26c.2a, BD Biosciences, d=1/50;
933 shown in green) and biotinylated PNA (Vectorlabs, d=1/250; shown in red) diluted in 5% NDS,
934 0.1% Triton X-100 in PBS. Sections were subsequently incubated 1h at RT with Neutravidin-
935 Dylight550 (ref 84606, Thermofisher, d=1/200) diluted in PBS. After 2 washing of 30 minutes
936 with PBS at RT, sections were kept at 4°C in PBS containing Hoechst 33342 (Sigma Aldrich)
937 and images were acquired using Leica LSI confocal microscope. Measurements were
938 performed with ImageJ software. Data are means ± SEM; one-way ANOVA with Tukey's
939 multiple comparison post-hoc test.

940

941 **FIG E3.** Selective depletion of LCs leads to a diminished Tfh cell differentiation in MC903
942 model. **(A)** Experimental protocol. Lang^{DTR} mice and wildtype littermate controls were
943 *intraperitoneally (i.p.)* injected with diphtheria toxin (DT) at D-2 and D0. Mice were then
944 topically treated with MC903 or EtOH every other day from D13 to D19 and ear draining lymph
945 nodes (EDLN) were analyzed at D20. **(B)** Frequency and number of CXCR5⁺ PD-1⁺ Tfh cells
946 in Lang^{DEP} mice and CT at D20. **(C)** Experimental protocol. huLang^{DTR} mice and wildtype
947 littermate controls were intraperitoneally *i.p.* injected with DT at D-2 and D0. Mice were then
948 topically treated with MC903 or EtOH every other day from D0 to D10 and EDLN were
949 analyzed at D11. **(D)** Frequency and number of CXCR5⁺ PD-1⁺ Tfh cells in huLang^{DEP} mice
950 and CT at D11. Values shown are means ± SEMs; one-way ANOVA with Tukey's multiple
951 comparison post-hoc test. Data are representative of 2 independent experiments with similar
952 results.

953

954 **FIG E4.** TSLP is crucially required for 30µm-LMP/OVA-induced skin Th2 inflammation. **(A)**
955 Hematoxylin and eosin (HE) staining of mouse ears. **(B)** Immunohistochemistry staining of
956 mouse ears with anti-MBP antibody (for eosinophils) or anti-MCPT8 antibody (for basophils).
957 Arrow points to one of the positive cells. Scale bar, 100µm. **(C-D)** IL-4 (AmCyan) and IL-13
958 (dsRed) expression in TCRβ⁺ dermal cells **(C)** or CXCR5⁻ CD4⁺ (non-Tfh) cells **(D)**.

959

960 **FIG E5.** Depletion of Langerin⁺ cells slightly diminishes the MC903- induced Th2 cell
961 response. Comparison of IL-4 and IL-13 expression among CXCR5⁻CD4⁺ (non-Tfh) cells in the
962 EDLN **(A)**, or among TCRβ⁺ cells in the dermis **(B)** of EtOH- or MC903-treated control (CT)
963 or Lang^{DEP} mice, all in the background of 4C13R^{Tg/0}.

964

965 **FIG E6.** Depletion of Langerin⁺ cells increases the LMP/OVA-induced Th2 cell response in
966 EDLNs. Comparison of IL-4 and IL-13 expression among CXCR5⁺CD4⁺ (non-Tfh) cell in
967 EDLNs from LMP/OVA-treated control (CT) or Lang^{DEP} in the background of 4C13R^{Tg/0} mice.

968

969 **FIG E7.** 11 μ m-LMP/OVA-induced skin inflammation is enhanced in mice with the depletion
970 of Langerin⁺ DCs or LCs. Hematoxylin and eosin staining of ears from Lang^{DEP} (**A**, top) and
971 huLang^{DEP} (**B**, top) mice after 11 μ m-LMP/OVA sensitization. Immunohistochemistry for MBP
972 (eosinophils) and MCPT8 (basophils) of ears from Lang^{DEP} (**A**, bottom) and huLang^{DEP} (**B**,
973 bottom) mice after 11 μ m-LMP/OVA treatment. Scale bar, 100 μ m.

974

975 **FIG E8.** LCs counteract LMP/OVA sensitization-induced skin inflammation and the
976 subsequent asthmatic response. (**A**) Experimental protocol for OVA epicutaneous sensitization
977 and airway challenge. Mice were intraperitoneally injected with DT at D-2, D0. Mice were
978 either treated with OVA on LMP ears at D0, D4, D7 and D11 or ears were non treated (NT).
979 All mice were subjected to *intranasal* (*i.n.*) instillation with OVA from D9 to D12. Ears and
980 lungs were analyzed at D13. (**B**) H&E staining of mouse ears. Scale bar, 100 μ m. (**C**) IHC
981 staining of mouse 30 μ m-LMP/OVA ears with anti-MBP (for eosinophils) or anti-MCPT8 (for
982 basophils). (**D**) Differential counting of eosinophils (Eos), neutrophils (Neutro), lymphocytes
983 (Lympho) and macrophages (Macro) in BAL. (**E**) RNA levels of indicated genes in BAL cells
984 by RT-qPCR. (**F**) Lung sections were stained with H&E for histological analyses or PAS for
985 goblet cell hyperplasia analyses. B: bronchiole; V: blood vessel. Scale bar, 250 μ m. (**G**)
986 Experimental protocol for OVA *i.p.* sensitization and airway challenge. Mice were *i.p.* injected
987 with DT at D-2 and D0. Mice were *i.p.* sensitized with OVA/alum at D0 and D4, and subjected
988 to *i.n.* instillation with OVA from D9 to D12. Lungs were analyzed at D13. (**H**) Differential
989 cell counting in BAL. Data are means \pm SEM; unpaired two-tailed t-test.

990

991 **FIG E9.** Transcriptomic analyses of migratory DCs in EDLNs of Lang^{GFP} mice upon MC903
992 treatment or epicutaneous OVA sensitization. Lang^{GFP} mice were treated with MC903 at D0,
993 D2 and D4 or 30 μ m-LMP/OVA on D0 and D3; EDLNs were collected at D5 for cell sorting
994 and RNAseq analyses. **(A)** Gating strategy used to sort resident (res) and migratory (mig)
995 GFP^{pos} and GFP^{neg} DCs. **(B)** Heatmap generated with the input of upregulated genes identified
996 in Pos_MC compared with Pos_NT (FC > 1.5; p < 0.05; raw read > 200 in at least one sample
997 of the Pos groups), to visually assess the results of clustering on the data to observe trends of
998 expression for genes across all groups. Z score of the expression level is used to generate
999 heatmap. Pos_NT, Pos_MC, Pos_OVA: GFP^{pos} migDCs from non-treated, MC903-treated or
1000 LMP/OVA-treated Lang^{GFP} mice; Neg_NT, Neg_MC, Neg_OVA: GFP^{neg} migDCs from non-
1001 treated, MC903-treated or LMP/OVA-treated Lang^{GFP} mice.

1002 Two clusters C1 and C2 were revealed. The cluster C1 genes exhibited the expression trends:
1003 1) in non-treated groups, they had a lower expression in GFP^{pos} cells than in GFP^{neg} cells
1004 (Pos_NT < Neg_NT); 2) in MC903-treated groups, their expression in GFP^{pos} cells increased,
1005 reaching a similar or higher expression than non-treated GFP^{neg} cells (Pos_MC = or > Neg_NT),
1006 and their expression in GFP^{neg} cells was also increased (Neg_MC > Neg_NT), with a higher
1007 level than Pos_MC cells; 3) in OVA-treated groups, the expression of some genes was also
1008 increased in GFP^{neg} cells (Neg_OVA versus Neg_NT) (subcluters of C1: a, b and c) while
1009 others remained not changed. Together, expression features of the cluster C1 suggest that in the
1010 MC903-AD, Langerin⁺ migDCs acquire many gene expression of Langerin⁻ migDCs, and share
1011 the upregulation of these genes with Langerin⁻ migDCs; and moreover, the upregulation of
1012 some (although less) of these genes also occurs in Langerin⁻ migDCs (but not Langerin⁺
1013 migDCs) in OVA-AD.

1014 Different from the cluster C1, the cluster C2 genes were highly upregulated in Pos_MC; some
1015 of them were also upregulated in Neg_MC (but reaching a lower level) and very few of them
1016 were upregulated in Neg_OVA, suggesting that this cluster represents the upregulated genes
1017 rather specific for Langerin⁺ migDCs under MC903 treatment.

1018

1019 **FIG E10.** (A) Heatmap generated with the input of downregulated genes identified in Pos_MC
1020 compared with Pos_NT (FC > 1.5; p < 0.05; raw read > 200 in at least one sample of the Pos
1021 groups), to visually assess the results of clustering on the data to observe trends of expression
1022 for genes across all groups. Z score of the expression level was used to generate heatmap. (B)
1023 Selected genes corresponding to gene ontology terms for Cytokine activity, Regulation of
1024 transcription, Regulation of cell migration, or T cell costimulation. *, adjusted p<0.05; NS, non
1025 significant.

1026

1027 **FIG E11.** IL-6 neutralization does not significantly diminish Tfh cell differentiation and GC B
1028 cell numbers. (A) experimental scheme. 4C13R^{Tg/0} mice were i.p. injected with 200 mg anti-
1029 IL-6 neutralizing antibody (@IL-6; Clone MP5-20F3, BioXcell) every other day from D0 to
1030 D10, and mouse ears were topically treated with MC903 or EtOH every other day from D0 to
1031 D10. EDLNs were analyzed at D7 or D11. (B) Frequency and number of CXCR5⁺ PD-1⁺ Tfh
1032 cells. (C) IL-4 (AmCyan) expression by Tfh cells. (D) Frequency and number of CD95⁺ GL-7⁺
1033 GC B cells at D11. Data are means ± SEM, one-way ANOVA with Tukey's multiple
1034 comparison post-hoc test.

1035

1036 **FIG E12.** MC903 treatment leads to increased numbers of both langerin-GFP^{pos} and langerin-
1037 GFP^{neg} migratory DCs in EDLNs at D5. Lang^{GFP} mice were treated with MC903 at D0, D2 and
1038 D4, or 30µm-LMP/OVA at D0 and D3, or tape-stripping (TS)/OVA at D0 and D3, and EDLNs

1039 were collected at D5 for flow cytometry analyses. Absolute numbers of GFP-positive (GFP^{pos})
1040 and -negative (GFP^{neg}) migratory DCs in EDLN, compared with non-treated (NT), are shown.

1041

1042 **FIG E13.** Depletion of Langerin⁺ cells enhances the TS/OVA-induced Tfh/GC response and
1043 the subsequent asthmatic phenotype. **(A)** H&E staining of untreated or tape-stripped (TS)
1044 Balb/c wildtype mice. Arrow points to the absence of stratum corneum in TS-ear. Scale bar,
1045 100µm. **(B)** Dorsal side of ears of WT mice were tape-stripped 10 times and topical treated with
1046 200µg of OVA in 10µl PBS. TSLP protein levels in ears were measured by ELISA at 48h after
1047 treatment. **(C)** Experimental protocol. Lang^{DTR} mice and wildtype littermate controls (CT), in
1048 the background of 4C13R^{Tg/0}, were i.p. injected with DT at D-2, D0 and then every 4 days.
1049 OVA (200µg) were topically applied on TS-ears at D0, D4, D7 and D11. All mice were
1050 subjected to intranasal (i.n.) instillation with 50µg of OVA from D9 to D12 and analyzed at
1051 D13. **(D-F)** Frequency and number of Tfh cells **(D)**, IL-4 (AmCyan) expression by Tfh cells
1052 **(E)** and numbers of CD95⁺ GL-7⁺ GC B cells, IgG1⁺ and IgE⁺ B cells in EDLNs **(F)**. **(G)** Serum
1053 levels of OVA-IgG1 and OVA-IgE. **(H)** Differential cell counting for eosinophils (Eos),
1054 neutrophils (Neutro), lymphocytes (Lympho) and macrophages (Macro) in BAL. **(I)** H&E
1055 staining of lung sections. B: bronchiole; V: blood vessel. Scale bar, 250µm. Data are means ±
1056 SEM; unpaired two-tailed t-test.

1057

1 **Title:**

2

3 **Dual function of Langerhans cells in skin TSLP-promoted Tfh cell**
4 **differentiation in mouse atopic dermatitis**

5

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29 **Key words:** Atopic dermatitis; TSLP; Dendritic cells; Langerhans cells; Tfh; Th2; allergen
30 sensitization; mouse

31

32 ***Abbreviations used:***

33 AD: atopic dermatitis

34 BAL: Bronchoalveolar lavage

35 CT: Wild-type Control

36 DC: Dendritic cell

37 DEG: Differentially expressed genes

38 DT: Diphtheria toxin

39 DTR: Diphtheria toxin receptor

40 EDLN: Ear-draining lymph node

41 GC: Germinal center

42 H&E: Hematoxylin and eosin

43 IHC: Immunohistochemistry

44 Ig: Immunoglobulin

45 *i.n.: Intranasal*

46 *i.p.: Intraperitoneal*

47 LC: Langerhans cell

48 LMP: laser-assisted skin microporation

49 LN: Lymph node

50 NT: Non-treated

51 PAS: Periodic Acid Schiff
52 PCA: Principle component analysis
53 Tfh: T follicular helper
54 Th2: T helper type 2
55 ELISA: Enzyme-linked immunosorbent assay
56 TS: Tape stripping
57 TSLP: Thymic stromal lymphopoietin

58
59

60 **Key messages:**

61

- 62 • TSLP is critically involved in mounting Tfh/GC response in mouse AD driven by
63 MC903 or OVA-sensitization.
- 64 • LCs promote Tfh/GC response in MC903-induced AD.
- 65 • LCs suppress Tfh/GC response and Th2 skin inflammation in OVA sensitization-
66 induced AD.

67
68
69

70 **Abstract**

71

72 **Background:** Atopic dermatitis (AD) is one of the most common chronic inflammatory skin
73 diseases, usually occurring early in life, and often preceding other atopic diseases like asthma.
74 Th2 cell has been believed to play a crucial role in cellular and humoral response in AD, but
75 accumulating evidences have shown that T follicular helper (Tfh) cell, a critical player in
76 humoral immunity, is associated with disease severity and plays an important role in AD
77 pathogenesis.

78 **Objectives:** We aimed at investigating how Tfh cells are generated during the pathogenesis of
79 AD, particularly what is the role of keratinocyte-derived cytokine TSLP and Langerhans cells
80 (LCs).

81 **Methods:** We employed two experimental AD mouse models, triggered by the overproduction
82 of TSLP through topical application of MC903, or induced by epicutaneous allergen ovalbumin
83 (OVA) sensitization.

84 **Results:** We demonstrated that the development of Tfh cells and GC response were crucially
85 dependent on TSLP in MC903 model and OVA sensitization model. Moreover, we found that
86 LCs promoted Tfh cell differentiation and GC response in MC903 model, and the depletion of
87 Langerin⁺ DCs or selective depletion of LCs diminished the Tfh/GC response. By contrast, in
88 the model with OVA sensitization, LCs inhibited Tfh/GC response and suppressed Th2 skin
89 inflammation and the subsequent asthma. Transcriptomic analysis of Langerin⁺ and Langerin⁻
90 migratory DCs revealed that Langerin⁺ DCs became activated in MC903 model, whereas these
91 cells remained inactivated in OVA sensitization model.

92 **Conclusion:** Together, these studies revealed a dual functionality of LCs in TSLP-promoted
93 Tfh and Th2 cell differentiation in AD pathogenesis.

94

95 **Capsule Summary**

96

97 This study demonstrates that keratinocyte-derived cytokine TSLP plays a critical role in
98 promoting not only Th2 but also Tfh/GC response in the pathogenesis of atopic dermatitis,
99 which implicates a dual function of epidermal Langerhans cells.

100

101

102 **Introduction**

103

104 Atopic dermatitis (AD) is one of the most common chronic inflammatory skin diseases which
105 affects up to 20% of children and 3% of adults worldwide, with increasing prevalence in the
106 industrialized countries during the last 30 years ¹. AD is characterized by chronic cutaneous
107 inflammation, T helper type 2 (Th2) response and hyper immunoglobulin IgE. Patients
108 suffering from AD often present genetic risk factors in the form of mutations affecting the skin
109 barrier structure or the immune system ². Onset of AD usually occurs early in life and may lead
110 to allergen sensitization, which can trigger the progression from AD to other atopic diseases
111 such as asthma/allergic rhinitis, in a process called “atopic march” ^{3,4}.

112 It has been recognized that Th2 cell response is critically implicated in the pathogenesis of
113 AD. Previous studies from us and others using mouse models have established a central role of
114 the cytokine thymic stromal lymphopoietin (TSLP) expressed by epidermal keratinocytes in
115 promoting Th2 cell response and driving the pathogenesis of AD ⁵⁻⁸. In addition to Th2 cell
116 response, humoral immune response is another key feature of AD, with increased serum IgE
117 and IgG1 levels associated with AD, which contribute to AD pathology and the atopic march ⁹,
118 ¹⁰. For a long time, Th2 cell has been believed to play a crucial role both in cellular response
119 and humoral response, e.g. helping B cells to produce Igs. However, such knowledge has been
120 challenged with the identification of T follicular helper (Tfh) cell, which emerges to be a critical
121 player in humoral immunity and T cell memory ¹¹.

122 In lymphoid organs, Tfh cell differentiation process is believed to begin with an initial
123 dendritic cell (DC) priming of naive CD4⁺ T cells, which undergo a cell-fate decision with the
124 acquisition of master transcription factor Bcl6 expression and chemokine receptor CXCR5
125 expressed on cell surface to become early Tfh cells, of which CXCR5 promotes their migration

126 from T cell zone to the B cell follicles ^{12, 13}. The full differentiation and maintenance of Tfh
127 cells implicate the Tfh cell-B cell interaction, leading to GC Tfh cells which are phenotypically
128 defined by their high expression of CXCR5 and PD-1 ¹⁴. It has been shown that Tfh cells
129 coordinate generation of the GC, initiate help for antigen-specific B cells, and promote selection
130 of high-affinity B cells and differentiation into either memory B cells or long-lived plasma cells
131 ¹⁵. Recent studies have identified Tfh cells as an important source of IL-4, a master regulator in
132 type 2 immunity which was previously thought to be produced by Th2 cells, for providing
133 critical B-cell help by its anti-apoptotic and IgE and IgG1 class switch effects ¹⁶. In addition, it
134 was reported that Tfh cells produce IL-4 in a GATA3-independent manner ¹⁷, suggesting
135 distinct mechanisms employed by Tfh and Th2 cells in the regulation of IL-4.

136 Since their initial identification, the biological functions of Tfh cells and their mechanisms
137 of action in the onset and development of diseases have been studied in autoimmunity, infectious
138 diseases, immunodeficiencies and vaccination ¹⁸. Less is known on Tfh cells in the context of
139 AD and other atopic diseases, but more and more evidences have suggested that Tfh cells are
140 associated with disease severity and Tfh cells play an important role in the pathogenesis ¹⁹⁻²¹.
141 In human, alteration of circulating Tfh cells is correlated with severity of the disease in children
142 with AD ²², or with the comorbid association of allergic rhinitis with asthma ²³, and allergen-
143 specific T follicular helper cell counts are correlated with specific IgE levels and efficacy of
144 allergen immunotherapy ²⁴. In mice, it has been reported that Tfh cells are important for house
145 dust mite-induced asthma ²⁵ or peanut allergy ²⁶.

146 Despite of these accumulating evidences showing the importance of Tfh cells in atopic
147 diseases, how Tfh cells and humoral responses are generated and regulated in AD remained to
148 be investigated. In this study, by employing two experimental AD mouse models, one triggered
149 by the overexpression of TSLP in mouse skin through topical application of MC903 ^{6, 7, 27}, and
150 the other one induced by epicutaneous allergen ovalbumin (OVA) sensitization, we

151 demonstrated that skin TSLP plays a crucial role in driving/promoting Tfh cell differentiation
152 and GC response, in addition to its recognized role in promoting Th2 cell response. Moreover,
153 we investigated the role of skin DCs in mediating the Tfh cell differentiation. We uncovered a
154 dual functionality of epidermal langerhans cells (LCs) in TSLP-promoted Tfh/Th2 cell
155 differentiation in AD pathogenesis, and further explored the molecular insights by
156 transcriptomic analyses, thus shedding new light onto the long-standing controversy of LCs in
157 skin immunity.

158

159 **Methods**

160 Details on the methods used in this study are described in the Methods section in this article's
161 Online Repository, including Experimental mice; MC903 topical application; Epicutaneous
162 OVA sensitization and airway challenge; Depletion of Langerin⁺ DCs or LCs in mice; Cell
163 preparation for flow cytometry analyses; Surface staining for flow cytometry analyses; LN cell
164 culture and antigen stimulation; RNA sequencing; BAL cell analyses; ELISA; Histopathology;
165 IHC staining; RNA in situ hybridization and Statistics.

166

167 **Results**

168

169 **Topical MC903 treatment induces TSLP-dependent Tfh cell differentiation and GC** 170 **response**

171 We have previously reported that topical treatment with MC903, a low calcemic analog of
172 vitamin D3, induces the overproduction of TSLP (TSLP^{over}) and the pathogenesis of AD ⁶,
173 ⁷. To examine the Tfh cell differentiation and GC response in MC903-induced AD model,
174 Balb/c wildtype (WT) mouse ears were topically treated every other day from day (D) 0 to
175 D10 with MC903 and ear-draining lymph nodes (EDLN) were analyzed at D0, D7 and D11
176 (**Fig 1A**). Results showed that the frequency and number of CXCR5⁺ PD-1⁺ Tfh cells were
177 both increased in MC903-treated WT mice at D7 and further augmented at D11 (**Fig 1B**).
178 We next examined the expression of IL-4, a key signal provided by Tfh cells to sustain B
179 cell maturation, by taking use of *Il4/Il13* dual reporter 4C13R^{Tg/0} mice, in which AmCyan
180 and dsRed are expressed under the control of IL-4 and IL-13 regulatory elements,
181 respectively ²⁸. In agreement with a previous report ²⁹, CXCR5⁺ PD-1⁺ Tfh cells in EDLNs
182 express IL-4 (AmCyan) but not IL-13 (dsRed) (**Fig E1A**), and the IL-4 expression by Tfh
183 cells was augmented in MC903-treated 4C13R^{Tg/0} mice at both D7 and D11 (**Fig 1C**).

184 Together, MC903 treatment induces not only Tfh cell differentiation but also the production
185 of IL-4 by Tfh cells.

186 To examine whether the induction of Tfh cells in MC903 model is triggered by TSLP,
187 mice lacking TSLP (*Tslp*^{-/-})⁶ were subjected to MC903 treatment. Results showed that these
188 mice exhibited highly diminished Tfh cell frequency and number at D7 and D11, compared
189 to WT mice (**Fig 1B**). By breeding *Tslp*^{-/-} with 4C13R^{Tg/0} to generate *Tslp*^{-/-}/4C13R^{Tg/0} mice,
190 we showed that MC903-induced IL-4 expression in Tfh cells was abrogated in the absence
191 of TSLP (**Fig 1C**). In agreement with the recognized role of TSLP in Th2 cell differentiation,
192 we showed that the MC903-induced IL-4- or IL-13-expressing CXCR5⁻ CD4⁺ non-Tfh cells
193 (representing Th2 cells) were also abrogated in *Tslp*^{-/-} mice (**Fig E1B**). These results indicate
194 that the overproduction of TSLP triggers not only Th2 cell differentiation, but also Tfh cell
195 differentiation and IL-4 expression by these cells.

196 Next, we examined the GC response in MC903-treated Balb/c WT mice. The number
197 of GC B cells, identified as GL-7⁺ CD95⁺ B cells, exhibited an increase in MC903-treated
198 WT mice at D11, but not at D7 (**Fig 1D**). Such increase was abrogated in MC903-treated
199 *Tslp*^{-/-} mice (**Fig 1D**). This was confirmed by immunofluorescence (IF) staining for GCs (**Fig**
200 **E2A**). In addition, both IgG1⁺ and IgE⁺ B cells exhibited an increase in their numbers in
201 MC903-treated WT mice at D11, which was also abrogated in MC903-treated *Tslp*^{-/-} mice
202 (**Fig 1E**). Of note, we observed that most of the IgG1⁺ B cells were GL-7⁺ CD95⁺ (**Fig E1C**),
203 suggesting that these cells harbor a GC phenotype; however, this was not the case for IgE⁺
204 B cells (**Fig E1C**).

205 Taken together, these data indicate that the overproduction of TSLP triggers Tfh cell
206 differentiation and GC response.

207

208 **Depletion of Langerin⁺ DCs or LCs diminishes the TSLP^{over}-triggered Tfh/GC**
209 **response**

210 LCs reside in the epidermis as a dense network of immune system sentinels, in close
211 proximity to keratinocytes. We then asked whether LCs mediate the TSLP^{over}-triggered
212 Tfh/GC response. To this aim, we first employed Langerin-DTR knock-in mice (Lang^{DTR})
213 in which Langerin⁺ cells, including LCs and Langerin⁺ dermal DCs, express the human
214 diphtheria toxin receptor (DTR) and can thus be depleted upon injection of diphtheria toxin
215 (DT) ³⁰. Lang^{DTR} mice and their wildtype control littermates were intraperitoneally (*i.p.*)
216 injected with DT at D-2, D0 and every 4 days to maintain the depletion of Langerin⁺ cells
217 (named Lang^{DEP} and CT respectively), and were subjected to topical MC903 treatment (**Fig**
218 **2A**). Results showed that the TSLP^{over}-triggered Tfh cell differentiation was largely
219 diminished in Lang^{DEP} mice (**Fig 2B**). The expression of IL-4 (AmCyan) by Tfh cells was
220 also reduced in Lang^{DEP}/4C13R^{Tg⁰} mice (**Fig 2C**). Accordingly, GC B cell number was
221 lower and IgG1⁺ (however not in IgE⁺) B cell number was significantly decreased (**Fig 2D**).
222 Therefore, these results indicate that Langerin⁺ DCs play an important role in mediating the
223 TSLP^{over}-induced Tfh/GC response.

224 As LCs and Langerin⁺ cDC1s were both depleted in Lang^{DEP} mice, we next examined
225 whether LCs mediate Tfh cell differentiation by depleting selectively LCs using two
226 strategies: one took use of the differential recovery time between LCs and Langerin⁺ cDC1s
227 after DT-induced depletion as previously reported ³¹ (**Fig E3A-B**), and the other one
228 employed human Langerin-DTR (huLang^{DTR}) mice in which DT injection efficiently
229 depletes LCs but not Langerin⁺ cDC1s ³² (**Fig E3C-D**). In both cases, we showed that the
230 selective depletion of LCs led to a decrease in frequency and number of Tfh cells, suggesting
231 an important role for LCs in TSLP^{over}-triggered Tfh cell differentiation.

232

233 **Epicutaneous OVA sensitization induces a TSLP-dependent Tfh cell differentiation**
234 **and GC response**

235 We have previously reported that TSLP plays a crucial role for promoting skin sensitization
236 to allergens, using an experimental mouse protocol in which OVA sensitization through
237 tape-stripped (TS) skin leads to an allergic AD inflammation, accompanied by Th2 cell
238 response, and an increased production of OVA-specific IgG1 and IgE in sera ³³. Here, we
239 developed a novel experimental protocol, in which Precise Laser Epidermal System
240 (P.L.E.A.S.E.[®]) ³⁴ was used to disrupt skin barrier and to generate patterned micropores in
241 mouse skin. This protocol allowed us to deliver allergens to micropores at precise depths of
242 the epidermis, thereby achieving a higher efficiency and reproducibility of allergen
243 sensitization through the skin compared with experiments based on TS. We showed that
244 micropores at a depth of 30 μ m (30 μ m-LMP) on Balb/c WT mouse ears reached basal layer
245 of ear epidermis (**Fig 3A**). ELISA analyses indicated that the protein level of TSLP increased
246 at 48 hours after treatment (**Fig 3B**), in agreement with the previous studies showing that
247 barrier disruption induces TSLP production in mouse ³³ and human skin ³⁵. Notably, such
248 level of TSLP was comparable to our previously reported TSLP level in TS skin ³³, although
249 it was much lower compared to that of MC903-treated skin (**Fig 3B; see also Fig E13B**).
250 The administration of OVA did not further induce the TSLP level (**Fig 3B**). *In situ*
251 hybridization showed that TSLP RNA expression was restricted to epidermal keratinocytes
252 in LMP skin (**Fig 3C**).

253 As expected, OVA treatment on LMP ears (named “LMP/OVA”; **Fig 3D**) induced a
254 Th2-type skin inflammation in TSLP-dependent manner, showing that OVA sensitization-
255 induced infiltration of eosinophils and basophils (**Fig E4A-B**), Th2 cytokines (IL-4 and IL-
256 13) expression by T cells in the skin (**Fig E4C**) and by CXCR5⁻CD4⁺ cells in EDLNs (**Fig**
257 **E4D**), were all abolished in mice lacking TSLP. Examination of EDLNs revealed that both

258 frequency and number of Tfh cells were increased in LMP/OVA- compared to LMP/PBS-
259 treated WT mice, and such increase was largely diminished in *Tslp*^{-/-} mice (**Fig 3E**). Note
260 that LMP/PBS was not sufficient to induce Tfh cell differentiation (despite of the induction
261 of TSLP), but LMP plus OVA together promoted Tfh/GC response which was TSLP-
262 dependent (**Fig 3E**). Moreover, IL-4 production by Tfh cells was augmented in LMP/OVA-
263 treated *Tslp*^{+/+}/4C13R^{Tg/0} mice but not *Tslp*^{-/-}/4C13R^{Tg/0} mice (**Fig 3F**). GC B cell number
264 analyzed by flow cytometry (**Fig 3G**) and GC size analyzed by immunofluorescence (**Fig**
265 **E2B**) both showed an increase in LMP/OVA-treated WT mice, and this increase was
266 abrogated in the absence of TSLP. IgG1⁺ and IgE⁺ B cell numbers were also increased in
267 LMP/OVA-treated WT mice, and they were much lower in LMP/OVA-treated *Tslp*^{-/-} mice
268 (**Fig 3G**). Accordingly, serum levels of OVA-IgG1 and OVA-IgE were decreased in *Tslp*^{-/-}
269 mice compared to WT mice upon LMP/OVA treatment (**Fig 3H**). Together, these results
270 demonstrate that TSLP is crucially required for epicutaneous OVA sensitization-induced
271 Th2 and Tfh/GC responses.

272

273 **Depletion of Langerin⁺ DCs or LCs augments the Tfh/GC response induced by** 274 **epicutaneous OVA sensitization**

275 Based on the above data from MC903-induced AD, we had expected that Langerin⁺ DCs
276 would be crucially required for epicutaneous OVA-induced Tfh/GC response. To our
277 surprise, when subjected to 30μm-LMP/OVA sensitization (**Fig 4A**), Lang^{DEP} mice did not
278 exhibit a reduction in frequency and number of CXCR5⁺ PD-1⁺ Tfh cells, instead they tended
279 to be higher compared to CT mice (**Fig 4B**). More strikingly, IL-4 expression by Tfh cells
280 was higher in EDLN from LMP/OVA-treated Lang^{DEP}/4C13R^{Tg/0} mice (**Fig 4C**).
281 Accordingly, the GC B cell, IgG1⁺ and IgE⁺ B cell number were not reduced in LMP/OVA-
282 treated Lang^{DEP} mice (**Fig 4D**), and serum OVA-specific IgE and OVA-specific IgG1 were

283 higher or tended to be higher (**Fig 4E**). Thus, in contrast to our expectation, Langerin⁺ DCs
284 are not required for the Tfh/GC response in LMP/OVA-induced AD model; instead, they
285 appear to play a counteracting role.

286 Because LCs are located on the suprabasal layer of the epidermis, we suspected that
287 Langerin⁺ cells would be only required in Tfh cell differentiation when allergens are
288 encountered superficially on the skin. To test this possibility, LMP was performed at the
289 depth of 11 μ m, which disrupted only the cornified layer of the epidermis (**Fig 5A**). We
290 observed that the 11 μ m-LMP induced also the production of TSLP, even though its level
291 was lower compared to 30 μ m-LMP (**Fig 5B**). Treatment of wildtype control (CT) ears with
292 11 μ m-LMP/OVA induced significant increases (although milder than 30 μ m-LMP/OVA) in
293 Tfh cell frequency as well as GC B cell number, which were all abolished in *Tslp*^{-/-} mice
294 (**Fig 5C**), indicating that, despite of a low induction of TSLP, the Tfh/GC response promoted
295 by 11 μ m-LMP/OVA is still crucially dependent on TSLP. However, when Lang^{DEP} mice
296 were subjected to 11 μ m-LMP/OVA treatment, they exhibited a significant increase in the
297 frequency of Tfh cells, in IL-4 expression by Tfh cells, as well as in GC B cell, IgG1⁺ and
298 IgE⁺ B cell numbers in EDLNs (**Fig 5D-F**), accompanied by augmented serum levels of
299 OVA-IgG1 and OVA-IgE (**Fig 5G**). Similar results were also obtained with huLang^{DEP} mice
300 (**Fig 5H-I**), indicating that LCs significantly counteract the Tfh/GC response induced upon
301 the 11 μ m-LMP/OVA sensitization.

302 Furthermore, we sought to compare antigen-specific Tfh cells between CT and
303 huLang^{DEP} mice using an activation-induced marker assay³⁶. In this assay, the stimulation
304 of LN suspensions with specific antigen drives upregulation of CD154 (CD40L), CD25 and
305 OX40 on Tfh cells, providing a sensitive method for quantifying antigen-specific Tfh cells
306 in mice³⁶. We showed that *in vitro* stimulation with OVA drove the upregulation of CD154,
307 OX40 and CD25 in EDLN-derived Tfh cells from LMP/OVA-sensitized CT mice; and such

308 upregulation was significantly higher in Tfh cells from LMP/OVA-sensitized huLang^{DEP}
309 mice (**Fig 5J**), thus indicating a stronger OVA-specific Tfh cell differentiation in huLang^{DEP}
310 mice upon OVA sensitization.

311 Together, these data indicate that LCs suppress the TSLP-dependent Tfh/GC response
312 in epicutaneous OVA sensitization model.

313

314 **Langerin⁺ DCs or LCs limit epicutaneous OVA-induced Th2 skin inflammation and** 315 **the subsequent asthma**

316 Having observed the opposite role of Langerin⁺ DCs or LCs in Tfh/GC response in the two
317 mouse AD models, we further explored their involvement in the induction of Th2 cell
318 response. Upon MC903 treatment, Lang^{DEP}/4C13R^{Tg/0} mice exhibited a slight decrease in
319 IL-4 and a tendency of decrease in IL-13 production by CXCR5⁺CD4⁺ cells in EDLN (**Fig**
320 **E5A**), or by TCRβ⁺ cells in dermis (**Fig E5B**), which suggests a role, even though minor,
321 for Langerin⁺ DCs in the development of Th2 cell response. In contrast, upon 30μm-
322 LMP/OVA treatment, Lang^{DEP}/4C13R^{Tg/0} mice exhibited a higher Th2 cell response in both
323 skin (**Fig 6A**) and EDLN (**Fig E6**). This was in accordance with the observation that
324 LMP/OVA-sensitized Lang^{DEP} mice exhibited a stronger skin inflammation (**Fig 6B**),
325 accompanied with an increase in eosinophils and basophils (**Fig 6C**). Moreover, when
326 subjected to 11μm-LMP/OVA sensitization, both Lang^{DEP} and huLang^{DEP} mice exhibited an
327 enhanced AD-like skin inflammation compared to CT mice (**Fig E7**). Therefore, contrary to
328 their minor role in promoting Th2 cell response in MC903-AD, LCs suppress the Th2 cell
329 response in OVA-AD.

330 We further examined whether Langerin⁺ DCs limit the atopic march. Upon *intranasal*
331 (*i.n.*) OVA challenge following epicutaneous allergen sensitization (**Fig 6D**), the Lang^{DEP}
332 mice developed a much stronger asthmatic inflammation compared with CT mice, exhibiting

333 an increase in the number of eosinophils in bronchoalveolar lavage fluid (BAL) (**Fig 6E**),
334 and in RNA expression of Th2 cytokines IL-4, IL-5 and IL-13, as well as chemokine receptor
335 CCR3 (eosinophils) and MCPT8 (basophils) by BAL cells (**Fig 6F**). In addition, H&E
336 staining of lung sections of OVA-treated Lang^{DEP} mice revealed an increased peribronchial
337 and perivascular infiltration, and PAS staining showed an enhanced goblet cells hyperplasia
338 (**Fig 6G**). Similar results were obtained with huLang^{DEP} mice (**Fig E8 A-F**), indicating that
339 LCs counteract the asthma development following epicutaneous allergen sensitization. To
340 exclude the possibility that the enhanced asthmatic inflammation is due to any depletion of
341 lung DCs during the intranasal challenge, we subjected huLang^{DEP} mice to *i.p.* sensitization
342 with OVA/alum and *i.n.* OVA challenge, and observed that these mice developed similar
343 asthmatic inflammation as wildtype control mice (**Fig E8 G-H**). This suggests that the
344 limitation of asthma inflammation by LCs is indeed due to their role in suppressing the
345 epicutaneous allergen sensitization.

346 Taken together, these studies reveal opposite roles of LCs in two AD models: in MC903-
347 AD, LCs play an important role in priming Tfh/GC response; they participate but to a lesser
348 extent in promoting Th2 responses. In OVA-AD, LCs are neither required for Tfh/GC nor
349 Th2 responses, instead, they suppress OVA-induced Tfh/GC and Th2 responses as well as
350 the “atopic march”.

351

352 **Langerin⁺ migratory DCs from MC903-AD but not from OVA-AD mice present** 353 **profound transcriptomic changes**

354 We next conducted transcriptomic studies to explore molecular insights underlying the
355 opposite roles of Langerin⁺ DCs in Tfh and Th2 cell differentiation in MC903-AD and OVA-
356 AD, by taking use of Lang^{GFP} mouse line in which GFP reports the expression of Langerin
357 ³⁰. Lang^{GFP} mice were treated with MC903 (at D0, D2 and D4) or LMP/OVA (at D0 and

358 D3), and at D5, Langerin⁺ (GFP^{pos}) and Langerin⁻ (GFP^{neg}) migratory DCs (migDCs) were
359 sorted from EDLNs of non-treated (NT), MC903- or LMP/OVA-treated mice, and
360 proceeded to mRNA sequencing (**Fig E9A**). The time point at D5 was selected to compare
361 gene expression patterns of migDCs at the initiation stage of Tfh and Th2 cell differentiation.

362 Principle component analysis (PCA) for the RNAseq data revealed that the Pos_MC
363 (GFP^{pos} migDCs from MC903-treated Lang^{GFP} mice) was clearly separated from the Pos_NT
364 (GFP^{pos} migDCs from non-treated Lang^{GFP} mice); however, the Pos_OVA (GFP^{pos} migDCs
365 from LMP/OVA-treated Lang^{GFP} mice) was inseparable from the Pos_NT (**Fig 7A**).
366 Correspondingly, analyses of differentially expressed genes (DEGs) in Pos_MC vs Pos_NT
367 identified 756 upregulated and 559 downregulated genes (with a fold change >1.5 and
368 adjusted p<0.05; **Fig 7B**); in a sharp contrast, the comparison of Pos_OVA vs Pos_NT
369 revealed only 39 upregulated and 9 downregulated genes (**Fig 7B**). Therefore, in MC903-
370 AD, Langerin⁺ migDCs undergo profound transcriptomic changes, but in OVA-AD, they
371 present almost no, or very little, transcriptomic changes.

372 As to Langerin⁻ migDCs, PCA showed that Neg_MC (GFP^{neg} migDCs from MC903-
373 treated Lang^{GFP} mice), Neg_OVA (GFP^{neg} migDCs from LMP/OVA-treated Lang^{GFP} mice)
374 and Neg_NT (GFP^{neg} migDCs from non-treated Lang^{GFP} mice) were all clustered away from
375 each other (**Fig 7A**). Analyses of DEGs identified 710 upregulated and 698 downregulated
376 genes for Neg_MC vs Neg_NT; and 431 upregulated and 427 downregulated genes for
377 Neg_OVA vs Neg_NT (**Fig 7B**), suggesting that Langerin⁻ migDCs present major
378 transcriptomic changes in both MC903-AD and OVA-AD, with considerable numbers of
379 overlapped DEGs (249 upregulated and 215 downregulated).

380

381 **Gene ontology analyses of DEGs in Langerin⁺ migDCs from MC903-treated mice**

382 Next, using the upregulated or downregulated DEGs identified in Pos_MC (vs Pos_NT) as
383 input, we performed cluster analyses of all the groups and generated heat map to visualize
384 trends of expression for genes across the different groups. Results are presented in **Fig E9B**
385 and **Fig E10A**. Further, we performed gene ontology (GO) analyses of the upregulated genes
386 in Pos_MC (**Fig 7C**), and examined whether these genes were also significantly upregulated
387 in Neg_MC (vs Neg_NT), and Neg_OVA (vs Neg_NT). We paid particular attention to the
388 upregulated genes shared in all the three groups (Pos_MC, Neg_MC and Neg_OVA),
389 standing here for “commonly upregulated” genes (highlighted in red in **Fig 7C**), as they
390 could be implicated in TSLP-promoted Tfh and/or Th2, a common feature shared by
391 MC903-AD and OVA-AD. Among them, we found genes related to: 1) “regulation of cell
392 migration”, many of which were reported to facilitate DC migration (*Mmp14*³⁷; *Stat5*³⁸;
393 *Nrp2*³⁹; *Sema7a*⁴⁰); 2) “T cell costimulation”: *Cd80* and *Cd86*⁴¹, *IL2ra*⁴², *Pdcd1lg2* (PD-
394 L2)⁴³, *Cd274* (PD-L1), *Gpr183* (EBI2)^{44,45}; 3) “cytokine signal”: *Il2ra*, *Tnfrsf11b* and *Ccl22*;
395 and 4) “transcription factors” such as *Ikzf4*, *Irf4*, *Stat4* and *Stat5a*.

396 We examined TSLP signaling pathway among the upregulated genes in Pos_MC. Using
397 the reported TSLP-regulated gene set⁴⁶, we identified *Cd84*, *Cd82*, *Ccl17*, *Ccl22* and *Tnfrsf11b*
398 (in the cluster with higher expression in Pos_MC than Neg_MC), as well as *Cish*, *Cd86*, *Cd80*,
399 *Cd274*, *Il2ra*, *Il6*, *ccr2*, *Tgfb1* (in the cluster with higher expression in Neg_MC than Pos_MC)
400 (**Fig 7D**). In addition, we identified *Irf4*, which has been recently shown to be downstream of
401 TSLP signaling in human migratory LCs⁴⁷. The upregulation of these TSLP-targeting genes
402 by Langerin⁺ migDCs suggests that these cells could be a direct responder to TSLP signaling,
403 although it remains to be demonstrated that TSLP signals through its receptor on LCs drive
404 their migration/activation. Besides these known TSLP downstream genes, more TSLP pathway
405 genes identified from those “commonly upregulated” genes can be envisaged.

406 Interestingly, we did not find *Tnfsf4* (encoding OX40L) among the DEGs in Pos_MC,
407 despite that OX40L was reported to be TSLP-responsive gene and mediate TSLP-promoted
408 Th2⁴⁸ and Tfh⁴⁹ cell differentiation. Actually, OX40L expression by GFP^{pos} cells was barely
409 detected in Pos_NT, Pos_MC or Pos_OVA (**Fig 7E**). On the other hand, OX40L was expressed
410 in Neg_NT, and its expression was further upregulated in Neg_MC and Neg_OVA. Therefore,
411 it is unlikely that OX40L would be responsible for the Tfh-promoting function of Langerin⁺
412 DCs, while its precise function as a potential TSLP downstream factor in Langerin⁻ DCs
413 remains to be defined (**Fig 7E**).

414 Among the above-mentioned TSLP-regulated gene, IL-6 has been shown to be a critical
415 cytokine for Tfh cell differentiation^{50,51}. We thus tested whether IL-6 neutralization decreases
416 Tfh / GC response in MC903-AD. Results showed that IL-6 was not required for the initiation
417 of Tfh cell differentiation and the overall GC reaction (**Fig E11**), although it is possible that its
418 function in Tfh response is redundant with other signals as suggested by Eto et al⁵². Besides
419 IL-6, several other Tfh-promoting factors derived from DCs have been recently reported,
420 including IRF-4⁵³, IL-2Ra^{42,54} and EBI2 (*Gpr183*)^{44,45}, whose expression was all “commonly
421 upregulated” in Pos_MC, Neg_MC and Neg_OVA (**Fig 7D**). The role of these potential
422 candidates in TSLP-promoting Tfh cell differentiation remains to be examined.

423 Finally, among the downregulated genes (**Fig E10B**), less knowledge was available, but
424 we could see *Il12b* (IL-23/IL-12p40), whose expression in DCs was previously reported to be
425 suppressed by TSLP⁵⁵. Other commonly downregulated ones included genes related “T cell
426 costimulation” *Havcr2* (TIM3), *Lgals8* (Galectin 8); “Regulation of cell migration” *Adam15*
427 and *Ptk2* (negative regulators for cell migration) and “regulation of transcription” *Foxc2*, *Thrb*,
428 *Tcf7l2*, *Ehf* and *Lmo2*.

429

430 **Discussion**

431

432 In this study, we analyzed how Tfh cells were generated in two experimental AD mouse models,
433 triggered by the overproduction of TSLP by topical application of MC903, or induced by
434 epicutaneous OVA sensitization. We demonstrated a crucial role for TSLP in promoting Tfh
435 cells and GC response in MC903-AD as well as OVA-AD. Intriguingly, we revealed a dual
436 function of LCs in TSLP-promoted Tfh/Th2 cell differentiation: while they promoted Tfh cell
437 differentiation in MC903-AD, they inhibited Tfh/GC response and suppressed Th2 skin
438 inflammation and the atopic march in OVA-AD. This is schematically illustrated in **Fig 8**, and
439 is discussed below.

440

441 **1) TSLP: critical player for Th2 and Tfh cell response in AD**

442 It has been recognized that TSLP is overproduced in AD lesional skin ⁵⁶, however, its
443 expression varies from high to low, which could be related with the cause (e.g. genetic mutation
444 of *Spink5* which induces a high level of TSLP ⁵⁷ vs skin barrier impairment which induces a
445 low level of TSLP ³⁵), age (e.g. TSLP serum level in AD children is high at early stage and
446 decreases with age ⁵⁸), or the nature of disease (e.g. intrinsic or extrinsic AD). Our study
447 demonstrates that no matter in AD models associated with either high or low TSLP expression,
448 TSLP is crucial for promoting Tfh/GC response in AD. Recently, the link between TSLP and
449 Tfh cell differentiation was suggested by the study with human blood DC-T cell coculture
450 system ⁴⁹. Thus, the Tfh-promoting function of TSLP appears to be conserved between mouse
451 and human, which suggests that it is relevant and valuable to employ AD mouse models to
452 elucidate mechanisms underlying the TSLP (skin)-Tfh (draining LN) axis, particularly the
453 access of tissue and lymphoid organs is rather limited in human study.

454 Our data add new evidence that neutralization of TSLP or blocking TSLP downstream
455 pathway will be helpful for reducing Th2 and Tfh cell responses in AD. Notably, TSLP is
456 crucial for driving the downstream IL-4/IL-13 expression by Th2 cells, as well as IL-4
457 expression by Tfh cells. Indeed, blocking antibody against IL-4/-13R (Dupilumab), which may
458 actually target both Th2 and Tfh cell responses, has been shown to achieve significant
459 therapeutic effect on AD⁵⁹. Intriguingly, neutralization TSLP antibody Tezepelumab has been
460 demonstrated to significantly reduce annual asthma exacerbation rate in patients with
461 uncontrolled asthma⁶⁰. A recent study with Tezepelumab showed numeric improvements in
462 patients with moderate to severe AD, despite that there were certain limitations in that study
463 including patient selection, use of topical corticosteroids, duration of treatment and uncertain
464 inhibition of TSLP with the dose used⁶¹. Given the preclinical evidence for the role of TSLP
465 in AD, more clinical studies are required to evaluate TSLP as therapeutic target in AD.

466 It should be also noted that recent studies have recognized the importance of Tfh cells in
467 AD¹⁹⁻²¹, but the *in vivo* function of Tfh remains to be further delineated using AD mouse
468 models. This is challenged by the lack of appropriate tools to deplete Tfh cells. We are under
469 the way to generate mouse line in which DTR can be selectively expressed in Tfh cells, thus
470 allowing the DT-induced depletion of Tfh cells.

471

472 **2) LCs: function as migratory DCs to promote Tfh cell differentiation**

473 LCs represent one of the most studied but controversial DC subtypes. Our study shows that LCs
474 are importantly engaged in the initiation Tfh cell differentiation and GC response triggered by
475 TSLP^{over} in MC903-AD. This provides new evidence on the Tfh-promoting function of LCs in
476 AD, in addition to several studies reporting the requirement of LCs for humoral responses in
477 other contexts^{62,63,64}. In MC903-AD, we observed that LCs play a dominant role in Tfh cell
478 differentiation, although dermal langerin⁻ DCs may also contribute. On the other hand, langerin⁻

479 DCs (cDC2) appear to be the major player for the TSLP^{over}-induced Th2, while LCs have
480 somewhat but minor contribution. Nevertheless, to provide direct evidence for the contribution
481 of cDC2 in TSLP-driven Tfh and Th2 responses in AD, further studies could be performed
482 using DC-specific KO of IRF4 or Dock8 mice, which have impaired development and
483 migration of CD11b⁺ cDC2 ⁶⁵, or CD301b-DTR mice in which CD301b⁺ cDC2 can be
484 transiently depleted ⁶⁶.

485 There have been long debates on the migration, antigen uptake, and T cell differentiation
486 of LCs in different contexts; but transcriptomic study on migratory LCs in skin-draining LNs
487 under inflammatory pathological contexts was lacking. Our transcriptomic data are therefore of
488 value; however, one drawback is that migratory LCs and cDC1 were not separated in Langerin⁺
489 (GFP^{pos}) migDCs, thus the gene expression data still need to be cautiously interpreted
490 concerning LCs. Nevertheless, we have shown that Langerin⁺ migDCs in EDLN of MC903-
491 induced TSLP^{over} mice presented substantial transcriptional changes, suggesting that the
492 activation and migration of Langerin⁺ DC to the draining LNs underlie its function to prime
493 Tfh cell differentiation in MC903-AD. Indeed, when comparing numbers of GFP^{pos} and GFP^{neg}
494 migratory DCs in EDLN of MC903-treated Lang^{GFP} mice at D5, we observed that both were
495 increased (**Fig E12**), supporting that both Langerin⁺ DCs and Langerin⁻ DCs migrate to draining
496 LNs in MC903-AD.

497

498 **3) LCs: function as non-migratory cells in the skin to suppress Tfh/Th2 response?**

499 Our study revealed a suppressive role of LCs for epicutaneous OVA-induced Tfh and Th2 cell
500 differentiation. This is in contrast with two previous studies which reported a role of LCs in
501 provoking AD inflammation by using a tape stripping (TS) OVA sensitization model ^{67, 68}. To
502 examine whether the discrepancy is due to the different effects of LMP compared to TS, we
503 performed TS/OVA sensitization on mouse ears. Results showed that, similar to LMP/OVA,

504 TS/OVA-sensitized Lang^{DEP} mouse EDLNs exhibited increased frequency and number of Tfh
505 cells, increased IL-4 expression by Tfh cells, higher numbers of GC B cells, IgG1⁺ and IgE⁺ B
506 cells, with elevated OVA-IgG1 and OVA-IgE in sera (**Fig E13 A-G**). Moreover, when *i.n.*
507 challenged with OVA, Lang^{DEP} mice developed a stronger asthmatic inflammation (**Fig E13**
508 **H-I**). Therefore, the discrepancy with the previous reports ^{67, 68} is not explained by the
509 difference of LMP vs TS technique in epicutaneous OVA sensitization; rather, it could be due
510 to other factors remained yet to be determined, such as the allergen application method: topical
511 OVA vs long exposure (2-day) of OVA placed on patch-test tape; the difference of mouse
512 background: Balb/c vs C57Bl/6; or the site of allergen application: ear vs back.

513 Why are LCs not implicated in the promotion of Tfh/Th2 cell differentiation in EDLN in
514 this context? Transcriptomic analyses showed that in sharp contrast to MC903-AD, Langerin⁺
515 migDCs in OVA-AD presented almost the same transcriptomic program as in untreated mice,
516 suggesting an absence of migration/activation of these cells. Indeed, Langerin⁺ migDC numbers
517 in EDLNs from LMP/OVA-treated or TS/OVA-treated mice at D5 were nearly unchanged,
518 whereas Langerin⁻ migDC number was increased (**Fig E12**). This is in agreement with previous
519 studies showing that when skin was treated with fluorescence-conjugated OVA ⁶⁶, HDM ⁶⁹, or
520 Dextran ⁷⁰, antigen uptake and transport to draining LNs were mainly exerted by Langerin⁻ DCs.
521 Of note, it was recently shown that LCs can transfer antigen to cDC2 in the context of Langerin
522 mAb-mediated targeting ⁷¹. It will be interesting to see whether this occurs in AD models, and
523 whether efficiency of LC antigen transfer could be altered in the two models, as another possible
524 explication of different implication of LCs in Tfh cell differentiation.

525 Then how do LCs exert their anti-Tfh/Th2 role in OVA-AD? A recent study showed that
526 LCs played an immunosuppressive role when OVA was applied on the intact skin, in
527 accompany with the induction of IL-10 in LCs in skin-draining LNs ⁷². However, this does not
528 seem to be our case, because Langerin⁺ migDCs in EDLN did not exhibit any transcriptional

529 change of Treg-inducing signals including IL-10 and TGF β , or RALDH2. More likely, the anti-
530 Tfh/Th2 effect of LCs is related to their immune suppression function *in situ* in the skin, in
531 keeping with LC ontogeny not only as DCs but also as non-migratory macrophages^{73, 74}. It
532 should be further studied how LCs exert such functionality, for example, by limiting the
533 antigen-uptake by cDC2 in the skin, or by promoting local Tregs in OVA-sensitized skin^{75 76}.
534 Transcriptomic analysis of LCs isolated from the OVA-treated skin site may provide further
535 molecular insights.

536

537 **4) What signals switch the function of LCs?**

538 One intriguing question is what microenvironment cues and molecular signals switch the
539 function of LC between anti-Tfh/Th2 to pro-Tfh/Th2 in AD contexts. Notably, MC903-AD and
540 OVA-AD exhibit similar AD phenotype which is TSLP-dependent, but the quantity of TSLP
541 and the nature of antigen are different in these two models. In MC903-AD, MC903 induced a
542 high production of TSLP⁷ (**Fig 8**) which was sufficient to induce Tfh and Th2 cell
543 differentiation. As there was no administration of experimental allergen, the nature of antigen
544 implicated in T cell differentiation in MC903 model may involve endogenous antigens or
545 microbiota co-existing in the skin. On the other hand, in OVA-AD, the disruption of skin barrier
546 with LMP induced TSLP expression however to a much lower extent (**Fig 8**). It is possible that
547 LCs sense the quantity of TSLP. Indeed, as a danger signal, TSLP may convert the function of
548 LCs when its level is above certain threshold. *In vitro* studies have shown that TSLP triggers
549 DC migration⁷⁷, or promotes the survival, maturation and migration of human LCs, and
550 allogenic naïve CD4⁺ T cells cocultured with TSLP-conditioned LCs produced cytokines IL-4
551 and IL-13⁷⁸, but quantitative study on TSLP signaling has never been performed. It will be
552 interesting to explore whether and how quantitative TSLP signaling determines the role of LCs,
553 by conducting *in vivo* or *ex vivo* dose-dependent experiments. In addition, the nature and

554 quantity of antigens can be also involved in the functional switch of LCs. To unravel such
555 complexity, the emerging mathematic modeling ^{79, 80} may eventually help to integrate multiple
556 parameters for a better understanding of functional switch of LCs.

557 It will be interesting to further explore in AD patients whether and how TSLP levels are
558 correlated with the states and function of LCs. A better understanding of what molecular switch
559 determines the function of LCs either as "pro-Tfh/Th2" or as "anti-Tfh/Th2", and of how LCs
560 exert such functions, will allow us to shape LCs to act in suppressing the skin inflammation,
561 limiting the allergen sensitization through AD skin, thus preventing the progression from AD
562 to asthma. On the other hand, the potential of LCs to induce Tfh cell differentiation and GC
563 response and the subsequent induction of antigen-specific antibodies has been of interest for
564 transcutaneous vaccination ^{63, 81}. Therefore, the knowledge we obtain from this study should be
565 also insightful for LC-based skin vaccination, including the use of TSLP at an appropriate level
566 as an effective adjuvant for promoting Tfh cell differentiation and humoral response.

567

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593

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819

820 **Figure Legends**

821

822 **FIG 1.** Overproduction of TSLP in the skin triggers Tfh differentiation and GC reponse in
823 MC903-induced AD mice. **A**, Experimental protocol. Mouse ears were topically treated with
824 MC903 or ethanol (EtOH; as vehicle control) every other day from day (D) 0 to D10 and
825 EDLNs were analyzed at D0, D7 and 11. **B**, Frequency and number of CXCR5⁺ PD-1⁺ Tfh cells
826 in EDLN from MC903-treated Balb/c wildtype (WT) and *Tslp*^{-/-} mice. **C**, Frequency of IL-4
827 (AmCyan)⁺ in Tfh cells and cell number of IL-4⁺ Tfh cells in EDLNs. **D-E**, Number of CD95⁺
828 GL-7⁺ GC B cells, IgG1⁺ B cells and IgE⁺ B cells in EDLNs. Values shown are means ± SEMs.
829 **B-D**, one-way ANOVA with Tukey's multiple comparison post-hoc test; **E**, unpaired t-test with
830 Welch's correction. Data are representative of 3 independent experiments with similar results.

831

832 **FIG 2.** Depletion of Langerin⁺ cells diminishes the MC903-induced Tfh/GC response. **A**,
833 Experimental protocol. Lang^{DTR} mice and wildtype littermate controls (CT) were *i.p.* injected
834 with DT at D-2 and D0 and then every 4 days. Mouse ears were topically treated with MC903
835 or EtOH every other day from D0 to D10 and EDLNs were analyzed at D11. **B**, Frequency and
836 number of Tfh cells. **C**, IL-4 (AmCyan) expression by Tfh cells. **D**, Total number of GC B cells,
837 IgG1⁺ and IgE⁺ B cells. Values shown are means ± SEMs; one-way ANOVA with Tukey's
838 multiple comparison post-hoc test. Data are representative of 3 independent experiments with
839 similar results.

840

841 **FIG 3.** OVA sensitization through laser-microporated (LMP) skin induces TSLP-dependent
842 Tfh/GC response. **A**, H&E staining of untreated or 30µm-LMP ears of Balb/c WT mice. The
843 red arrow points to a micropore with the disruption of the epidermis. Scale bar, 100 µm. **B**,
844 TSLP protein levels in ears of WT mice at 48h after the indicated treatment. **C**, RNAscope in

845 situ hybridization for TSLP in untreated or 30 μ m-LMP-ears at 48h after the microporation. The
846 black arrow points to one of the positive signals. Scale bar, 50 μ m. **D**, Experimental protocol
847 for OVA epicutaneous sensitization through LMP ears. OVA or PBS (vehicle) were topically
848 applied on LMP ears at D0, D4, D7 and D11 and EDLNs were analyzed at D13. **E-F**, Frequency
849 and cell number of Tfh cells (**E**) and IL-4 (AmCyan) producing Tfh cells (**F**) in EDLNs. **G**, GC
850 B cell, IgG1⁺ and IgE⁺ B cell numbers in EDLNs. **H**, Serum levels of OVA-IgG1 and OVA-
851 IgE. Values shown are mean \pm SEM; one-way ANOVA with Tukey's multiple comparison
852 post-hoc test. Data are representative of 3 independent experiments with similar results.

853

854 **FIG 4.** Depletion of Langerin⁺ cells does not reduce but rather tends to augment 30 μ m-
855 LMP/OVA-induced Tfh/GC response. **A**, Experimental protocol. Lang^{DTR} mice and wildtype
856 littermate controls (CT) were *i.p.* injected with DT at D-2, D0 and then every 4 days. Mouse
857 ears were treated by 30 μ m-LMP/OVA or 30 μ m-LMP/PBS at D0, D4, D7 and D11 and EDLNs
858 were analyzed at D13. **B**, Frequency and number of Tfh cells. **C**, IL-4 (AmCyan) expression
859 by Tfh cells. **D**, Number of GC B cells, IgG1⁺ and IgE⁺ B cells. **E**, Serum levels of OVA-
860 specific IgG1 and OVA-specific IgE in 30 μ m-LMP/OVA-sensitized Lang^{DEP} or CT mice. Data
861 are means \pm SEM; **B-D**, one-way ANOVA with Tukey's multiple comparison post-hoc test. **E**,
862 unpaired t-test with Welch's correction. Data are representative of 3 independent experiments
863 with similar results.

864

865 **FIG 5.** Depletion of Langerin⁺ cells or LCs enhances 11 μ m-LMP/OVA-induced TSLP-
866 dependent Tfh/GC response. **A**, H&E staining of untreated or 11 μ m-LMP ears of Balb/c WT
867 mice. The red arrow points to a micropore with the impairment of cornified layer. Scale bar,
868 100 μ m. **B**, TSLP protein levels in ears of WT mice. **C**, Comparison of Tfh cells and GC B
869 cells in EDLNs from WT or *Tslp*^{-/-} mice. **D-F**, Comparison of Tfh cells (**D**), IL-4 (AmCyan)

870 expression by Tfh cells (**E**) and number of GC B cells, IgG1⁺ B cells and IgE⁺ B cells (**F**) in
871 EDLNs from CT or Lang^{DEP} mice. **G**, Serum OVA-IgG1 and OVA-IgE levels. **H**, Experimental
872 protocol. **I**, Comparison of Tfh cells, GC B cells, IgG1⁺ and IgE⁺ B cells in CT and huLang^{DEP}
873 mice. **J**, Comparison of antigen-specific Tfh cells between LMP/OVA-sensitized CT and
874 huLang^{DEP} mice. EDLNs were *in vitro* stimulated with OVA or PBS (vehicle control), and
875 activation markers CD154, CD25 and OX40 expressed by EDLN-derived Tfh cells were
876 examined. Values shown are mean ± SEM; one-way ANOVA with Tukey's multiple
877 comparison post-hoc test. Data are representative of 2 independent experiments with similar
878 results.

879

880 **FIG 6.** Langerin⁺ cells counteract LMP/OVA sensitization-induced skin Th2 inflammation and
881 the subsequent asthmatic phenotype. **A**, IL-4 (AmCyan) and IL-13 (DsRed) expression in
882 TCRβ⁺ dermal cells. **B**, H&E staining of mouse ears. **C**, IHC staining of mouse ears with anti-
883 MBP (for eosinophils) or anti-MCPT8 (for basophils). Arrows point to positive signals. **D**,
884 Experimental protocol for OVA epicutaneous sensitization and airway challenge. Mice were
885 *i.p.* injected with DT at D-2, D0 and then every 4 days. Mice were either treated with OVA on
886 LMP ears at D0, D4, D7 and D11 or non-treated (NT). All mice were subjected to *i.n.*
887 instillation with OVA from D9 to D12, and analyzed at D13. **E**, Differential cell counting for
888 eosinophils (Eos), neutrophils (Neutro), lymphocytes (Lympho) and macrophages (Macro) in
889 BAL. **F**, RNA levels of indicated genes in BAL cells by RT-qPCR. **G**, Lung sections were
890 stained with H&E for histology or PAS for goblet cell hyperplasia analyses. B: bronchiole, V:
891 blood vessel. Scale bar, 100μm. Values shown are means ± SEM; one-way ANOVA with
892 Tukey's multiple comparison post-hoc test. Data are representative of 2 independent
893 experiments with similar results.

894

895 **FIG 7.** Transcriptomic analyses of migratory DCs in EDLNs of Lang^{GFP} mice upon MC903
896 treatment or epicutaneous OVA sensitization. Lang^{GFP} mice were treated with MC903 at D0,
897 D2 and D4 or 30 μ m-LMP/OVA on D0 and D3; EDLNs were collected at D5 for cell sorting
898 and RNAseq analyses. **A**, Left, percentage of variability explained by each Principal
899 Component. Right, principal component analyses showing PC1, PC2 and PC3. **B**, Venn
900 diagram showing the number of upregulated and downregulated genes (fold change > 1.5; p <
901 0.05; raw read > 200 in at least one sample of all groups), and the number of commonly
902 upregulated or downregulated genes between the comparisons, as indicated. Pos_NT, Pos_MC,
903 Pos_OVA: GFP^{Pos} migDCs from non-treated, MC903-treated or LMP/OVA-treated Lang^{GFP}
904 mice; Neg_NT, Neg_MC, Neg_OVA: GFP^{neg} migDCs from non-treated, MC903-treated or
905 LMP/OVA-treated Lang^{GFP} mice. **C**, Selected genes corresponding to gene ontology terms. *,
906 p<0.05; NS, non significant. **D**, Heatmaps of the reported TSLP pathway genes, which are
907 significantly upregulated in Pos_MC vs Pos_NT. **E**, Heatmap of Tnfsf4 (encoding OX40L)
908 from RNAseq data, and RT-qPCR analyses.

909

910 **FIG 8.** A schematic representation of the dual functions of LCs in regulating TSLP-dependent
911 Tfh cell and Th2 cell response, revealed by two experimental AD mouse models, triggered by
912 the overproduction of TSLP through topical application of MC903, or induced by epicutaneous
913 allergen ovalbumin (OVA) sensitization.

914

915

916 **Supplementary Figure Legends**

917

918 **FIG E1. (A)** CXCR5⁺ PD-1⁺ Tfh cells produce IL-4 (AmCyan) but not IL-13 (dsRed) in
919 EDLNs of MC903-treated 4C13R^{Tg0} mice at D11. 4C13R^{0/0} EDLNs were used as gating
920 control. **(B)** Frequency and number of CXCR5⁻ CD4⁺ (non-Tfh) cells producing IL-4
921 (AmCyan) or IL-13 (dsRed), representing Th2 cells, in EDLNs from Balb/c wildtype (WT) and
922 *Tslp*^{-/-} mice in the background of 4C13R^{Tg0}, treated with MC903 or ethanol, and analyzed at
923 D0, D7 and D11. **(C)** The majority of IgG1⁺ but not IgE⁺ B cells in EDLNs from MC903-
924 treated wildtype Balb/c mice are GL-7⁺ CD95⁺.

925

926 **FIG E2.** Germinal center staining. Wildtype (WT) and *Tslp*^{-/-} mice were treated with MC903
927 **(A)** or subjected to OVA-sensitization **(B)** as shown in FIG 1A and 4D respectively. EDLN
928 were collected and fixed overnight with 4% PFA at 4°C. After 2 times 30 minutes of wash in
929 PBS at room temperature (RT), samples were included in 4% low melting point agarose in PBS.
930 Vibratome sections of 100µm were blocked with 5% normal donkey serum (NDS), 0.1% Triton
931 X-100 in PBS and then stained overnight at 4°C with anti CD4-AlexaFluor 647 (RM4-5,
932 Biolegend, d=1/100; shown in blue), anti IgD-FITC (11-26c.2a, BD Biosciences, d=1/50;
933 shown in green) and biotinylated PNA (Vectorlabs, d=1/250; shown in red) diluted in 5% NDS,
934 0.1% Triton X-100 in PBS. Sections were subsequently incubated 1h at RT with Neutravidin-
935 Dylight550 (ref 84606, Thermofisher, d=1/200) diluted in PBS. After 2 washing of 30 minutes
936 with PBS at RT, sections were kept at 4°C in PBS containing Hoechst 33342 (Sigma Aldrich)
937 and images were acquired using Leica LSI confocal microscope. Measurements were
938 performed with ImageJ software. Data are means ± SEM; one-way ANOVA with Tukey's
939 multiple comparison post-hoc test.

940

941 **FIG E3.** Selective depletion of LCs leads to a diminished Tfh cell differentiation in MC903
942 model. **(A)** Experimental protocol. Lang^{DTR} mice and wildtype littermate controls were
943 *intraperitoneally (i.p.)* injected with diphtheria toxin (DT) at D-2 and D0. Mice were then
944 topically treated with MC903 or EtOH every other day from D13 to D19 and ear draining lymph
945 nodes (EDLN) were analyzed at D20. **(B)** Frequency and number of CXCR5⁺ PD-1⁺ Tfh cells
946 in Lang^{DEP} mice and CT at D20. **(C)** Experimental protocol. huLang^{DTR} mice and wildtype
947 littermate controls were intraperitoneally *i.p.* injected with DT at D-2 and D0. Mice were then
948 topically treated with MC903 or EtOH every other day from D0 to D10 and EDLN were
949 analyzed at D11. **(D)** Frequency and number of CXCR5⁺ PD-1⁺ Tfh cells in huLang^{DEP} mice
950 and CT at D11. Values shown are means ± SEMs; one-way ANOVA with Tukey's multiple
951 comparison post-hoc test. Data are representative of 2 independent experiments with similar
952 results.

953

954 **FIG E4.** TSLP is crucially required for 30µm-LMP/OVA-induced skin Th2 inflammation. **(A)**
955 Hematoxylin and eosin (HE) staining of mouse ears. **(B)** Immunohistochemistry staining of
956 mouse ears with anti-MBP antibody (for eosinophils) or anti-MCPT8 antibody (for basophils).
957 Arrow points to one of the positive cells. Scale bar, 100µm. **(C-D)** IL-4 (AmCyan) and IL-13
958 (dsRed) expression in TCRβ⁺ dermal cells **(C)** or CXCR5⁻ CD4⁺ (non-Tfh) cells **(D)**.

959

960 **FIG E5.** Depletion of Langerin⁺ cells slightly diminishes the MC903- induced Th2 cell
961 response. Comparison of IL-4 and IL-13 expression among CXCR5⁻CD4⁺ (non-Tfh) cells in the
962 EDLN **(A)**, or among TCRβ⁺ cells in the dermis **(B)** of EtOH- or MC903-treated control (CT)
963 or Lang^{DEP} mice, all in the background of 4C13R^{Tg/0}.

964

965 **FIG E6.** Depletion of Langerin⁺ cells increases the LMP/OVA-induced Th2 cell response in
966 EDLNs. Comparison of IL-4 and IL-13 expression among CXCR5⁺CD4⁺ (non-Tfh) cell in
967 EDLNs from LMP/OVA-treated control (CT) or Lang^{DEP} in the background of 4C13R^{Tg/0} mice.
968

969 **FIG E7.** 11 μ m-LMP/OVA-induced skin inflammation is enhanced in mice with the depletion
970 of Langerin⁺ DCs or LCs. Hematoxylin and eosin staining of ears from Lang^{DEP} (**A**, top) and
971 huLang^{DEP} (**B**, top) mice after 11 μ m-LMP/OVA sensitization. Immunohistochemistry for MBP
972 (eosinophils) and MCPT8 (basophils) of ears from Lang^{DEP} (**A**, bottom) and huLang^{DEP} (**B**,
973 bottom) mice after 11 μ m-LMP/OVA treatment. Scale bar, 100 μ m.
974

975 **FIG E8.** LCs counteract LMP/OVA sensitization-induced skin inflammation and the
976 subsequent asthmatic response. (**A**) Experimental protocol for OVA epicutaneous sensitization
977 and airway challenge. Mice were intraperitoneally injected with DT at D-2, D0. Mice were
978 either treated with OVA on LMP ears at D0, D4, D7 and D11 or ears were non treated (NT).
979 All mice were subjected to *intranasal* (*i.n.*) instillation with OVA from D9 to D12. Ears and
980 lungs were analyzed at D13. (**B**) H&E staining of mouse ears. Scale bar, 100 μ m. (**C**) IHC
981 staining of mouse 30 μ m-LMP/OVA ears with anti-MBP (for eosinophils) or anti-MCPT8 (for
982 basophils). (**D**) Differential counting of eosinophils (Eos), neutrophils (Neutro), lymphocytes
983 (Lympho) and macrophages (Macro) in BAL. (**E**) RNA levels of indicated genes in BAL cells
984 by RT-qPCR. (**F**) Lung sections were stained with H&E for histological analyses or PAS for
985 goblet cell hyperplasia analyses. B: bronchiole; V: blood vessel. Scale bar, 250 μ m. (**G**)
986 Experimental protocol for OVA *i.p.* sensitization and airway challenge. Mice were *i.p.* injected
987 with DT at D-2 and D0. Mice were *i.p.* sensitized with OVA/alum at D0 and D4, and subjected
988 to *i.n.* instillation with OVA from D9 to D12. Lungs were analyzed at D13. (**H**) Differential
989 cell counting in BAL. Data are means \pm SEM; unpaired two-tailed t-test.

990

991 **FIG E9.** Transcriptomic analyses of migratory DCs in EDLNs of Lang^{GFP} mice upon MC903
992 treatment or epicutaneous OVA sensitization. Lang^{GFP} mice were treated with MC903 at D0,
993 D2 and D4 or 30 μ m-LMP/OVA on D0 and D3; EDLNs were collected at D5 for cell sorting
994 and RNAseq analyses. **(A)** Gating strategy used to sort resident (res) and migratory (mig)
995 GFP^{pos} and GFP^{neg} DCs. **(B)** Heatmap generated with the input of upregulated genes identified
996 in Pos_MC compared with Pos_NT (FC > 1.5; p < 0.05; raw read > 200 in at least one sample
997 of the Pos groups), to visually assess the results of clustering on the data to observe trends of
998 expression for genes across all groups. Z score of the expression level is used to generate
999 heatmap. Pos_NT, Pos_MC, Pos_OVA: GFP^{pos} migDCs from non-treated, MC903-treated or
1000 LMP/OVA-treated Lang^{GFP} mice; Neg_NT, Neg_MC, Neg_OVA: GFP^{neg} migDCs from non-
1001 treated, MC903-treated or LMP/OVA-treated Lang^{GFP} mice.

1002 Two clusters C1 and C2 were revealed. The cluster C1 genes exhibited the expression trends:
1003 1) in non-treated groups, they had a lower expression in GFP^{pos} cells than in GFP^{neg} cells
1004 (Pos_NT < Neg_NT); 2) in MC903-treated groups, their expression in GFP^{pos} cells increased,
1005 reaching a similar or higher expression than non-treated GFP^{neg} cells (Pos_MC = or > Neg_NT),
1006 and their expression in GFP^{neg} cells was also increased (Neg_MC > Neg_NT), with a higher
1007 level than Pos_MC cells; 3) in OVA-treated groups, the expression of some genes was also
1008 increased in GFP^{neg} cells (Neg_OVA versus Neg_NT) (subcluters of C1: a, b and c) while
1009 others remained not changed. Together, expression features of the cluster C1 suggest that in the
1010 MC903-AD, Langerin⁺ migDCs acquire many gene expression of Langerin⁻ migDCs, and share
1011 the upregulation of these genes with Langerin⁻ migDCs; and moreover, the upregulation of
1012 some (although less) of these genes also occurs in Langerin⁻ migDCs (but not Langerin⁺
1013 migDCs) in OVA-AD.

1014 Different from the cluster C1, the cluster C2 genes were highly upregulated in Pos_MC; some
1015 of them were also upregulated in Neg_MC (but reaching a lower level) and very few of them
1016 were upregulated in Neg_OVA, suggesting that this cluster represents the upregulated genes
1017 rather specific for Langerin⁺ migDCs under MC903 treatment.

1018

1019 **FIG E10.** (A) Heatmap generated with the input of downregulated genes identified in Pos_MC
1020 compared with Pos_NT (FC > 1.5; p < 0.05; raw read > 200 in at least one sample of the Pos
1021 groups), to visually assess the results of clustering on the data to observe trends of expression
1022 for genes across all groups. Z score of the expression level was used to generate heatmap. (B)
1023 Selected genes corresponding to gene ontology terms for Cytokine activity, Regulation of
1024 transcription, Regulation of cell migration, or T cell costimulation. *, adjusted p<0.05; NS, non
1025 significant.

1026

1027 **FIG E11.** IL-6 neutralization does not significantly diminish Tfh cell differentiation and GC B
1028 cell numbers. (A) experimental scheme. 4C13R^{Tg/0} mice were i.p. injected with 200 mg anti-
1029 IL-6 neutralizing antibody (@IL-6; Clone MP5-20F3, BioXcell) every other day from D0 to
1030 D10, and mouse ears were topically treated with MC903 or EtOH every other day from D0 to
1031 D10. EDLNs were analyzed at D7 or D11. (B) Frequency and number of CXCR5⁺ PD-1⁺ Tfh
1032 cells. (C) IL-4 (AmCyan) expression by Tfh cells. (D) Frequency and number of CD95⁺ GL-7⁺
1033 GC B cells at D11. Data are means ± SEM, one-way ANOVA with Tukey's multiple
1034 comparison post-hoc test.

1035

1036 **FIG E12.** MC903 treatment leads to increased numbers of both langerin-GFP^{pos} and langerin-
1037 GFP^{neg} migratory DCs in EDLNs at D5. Lang^{GFP} mice were treated with MC903 at D0, D2 and
1038 D4, or 30µm-LMP/OVA at D0 and D3, or tape-stripping (TS)/OVA at D0 and D3, and EDLNs

1039 were collected at D5 for flow cytometry analyses. Absolute numbers of GFP-positive (GFP^{pos})
1040 and -negative (GFP^{neg}) migratory DCs in EDLN, compared with non-treated (NT), are shown.
1041

1042 **FIG E13.** Depletion of Langerin⁺ cells enhances the TS/OVA-induced Tfh/GC response and
1043 the subsequent asthmatic phenotype. **(A)** H&E staining of untreated or tape-stripped (TS)
1044 Balb/c wildtype mice. Arrow points to the absence of stratum corneum in TS-ear. Scale bar,
1045 100µm. **(B)** Dorsal side of ears of WT mice were tape-stripped 10 times and topical treated with
1046 200µg of OVA in 10µl PBS. TSLP protein levels in ears were measured by ELISA at 48h after
1047 treatment. **(C)** Experimental protocol. Lang^{DTR} mice and wildtype littermate controls (CT), in
1048 the background of 4C13R^{Tg/0}, were i.p. injected with DT at D-2, D0 and then every 4 days.
1049 OVA (200µg) were topically applied on TS-ears at D0, D4, D7 and D11. All mice were
1050 subjected to intranasal (i.n.) instillation with 50µg of OVA from D9 to D12 and analyzed at
1051 D13. **(D-F)** Frequency and number of Tfh cells **(D)**, IL-4 (AmCyan) expression by Tfh cells
1052 **(E)** and numbers of CD95⁺ GL-7⁺ GC B cells, IgG1⁺ and IgE⁺ B cells in EDLNs **(F)**. **(G)** Serum
1053 levels of OVA-IgG1 and OVA-IgE. **(H)** Differential cell counting for eosinophils (Eos),
1054 neutrophils (Neutro), lymphocytes (Lympho) and macrophages (Macro) in BAL. **(I)** H&E
1055 staining of lung sections. B: bronchiole; V: blood vessel. Scale bar, 250µm. Data are means ±
1056 SEM; unpaired two-tailed t-test.

1057

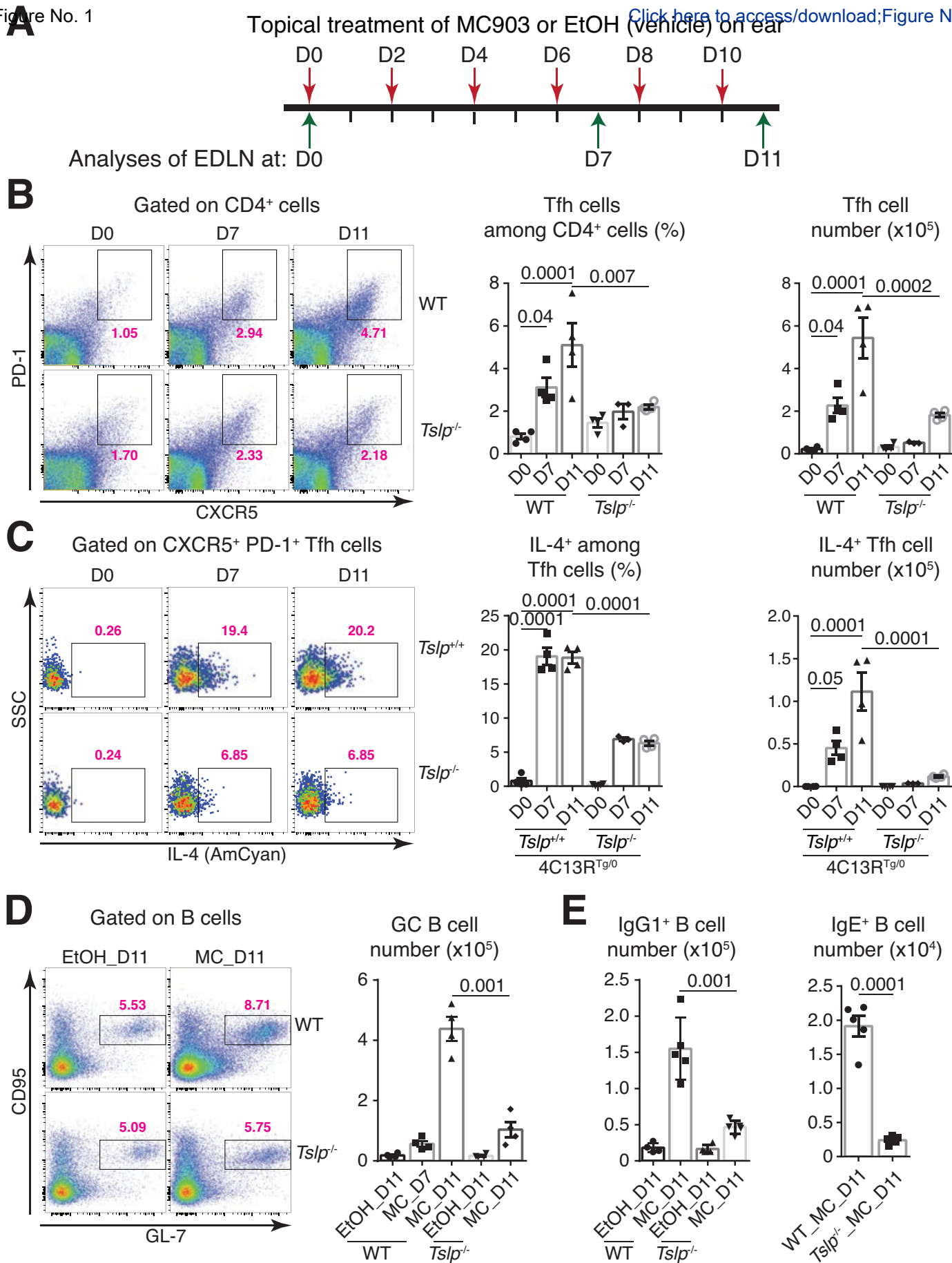


FIG 1

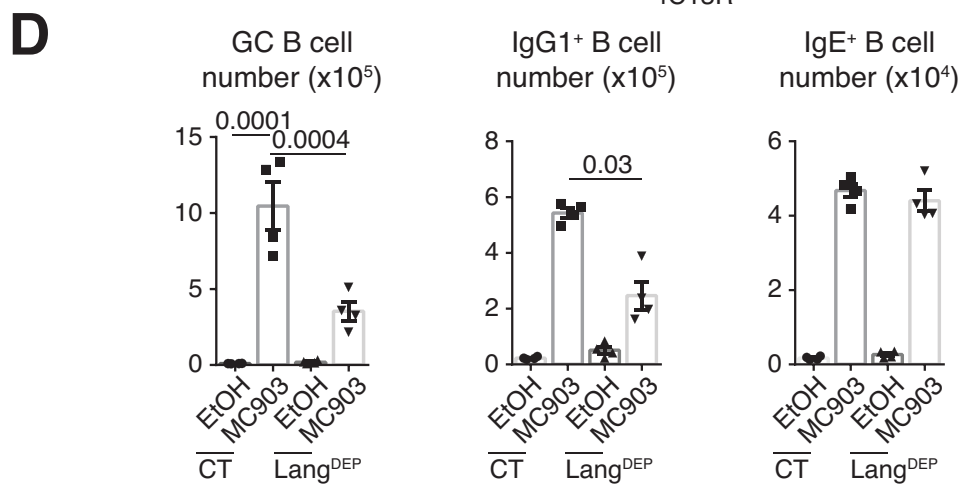
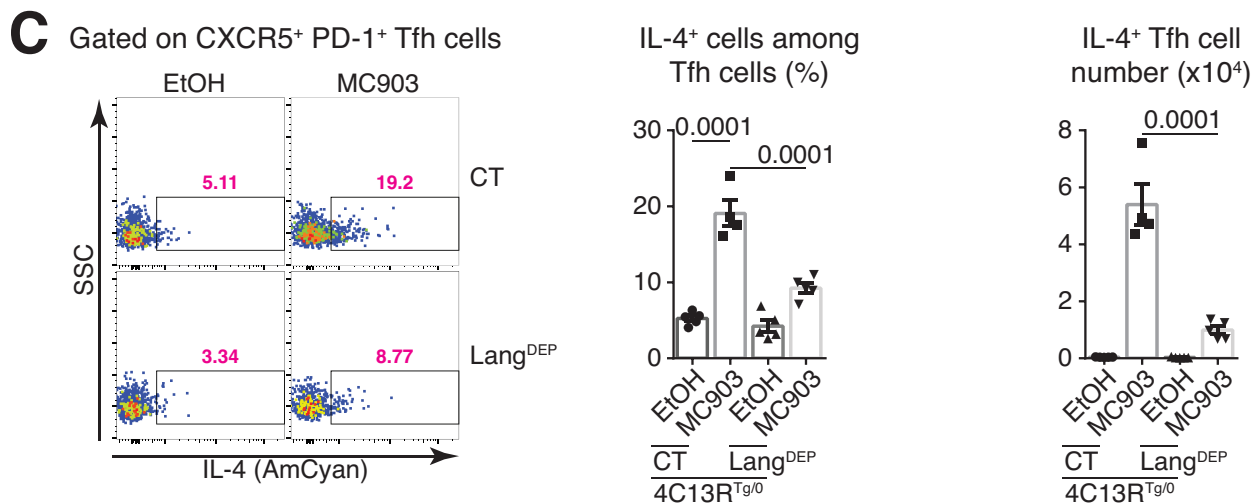
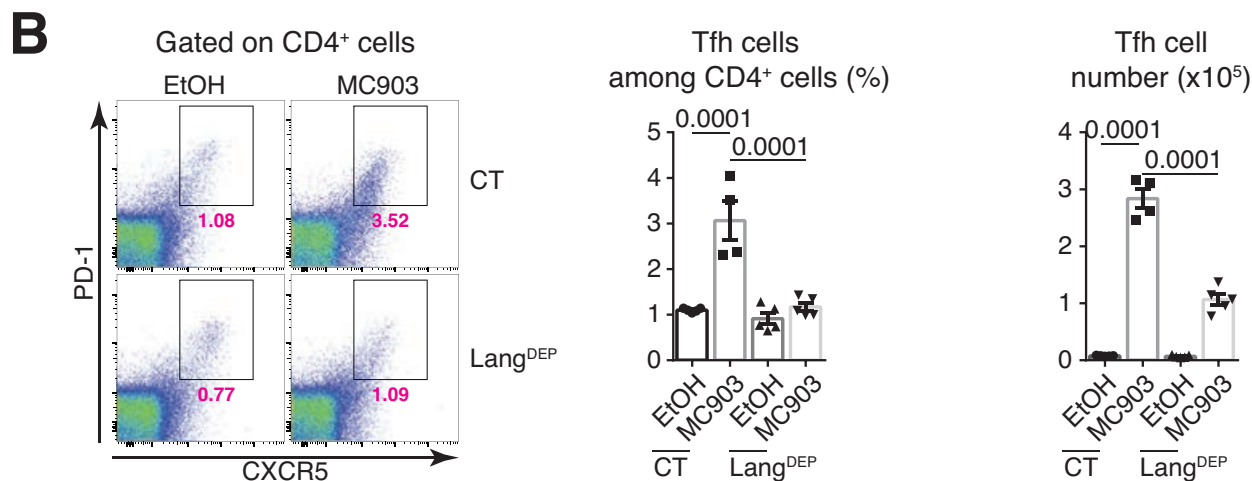
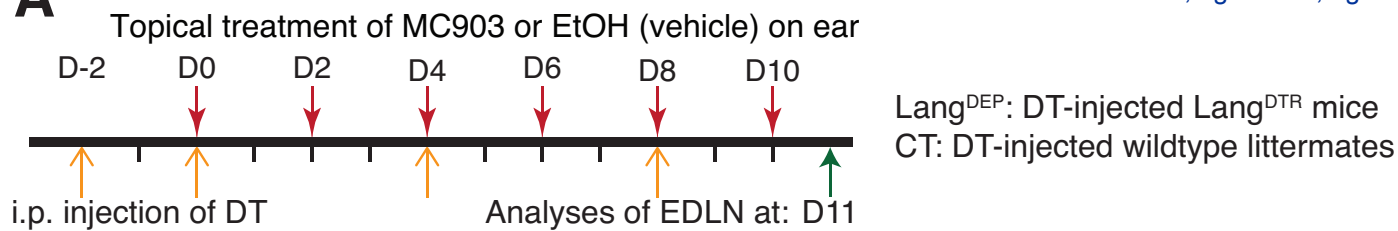


FIG 2

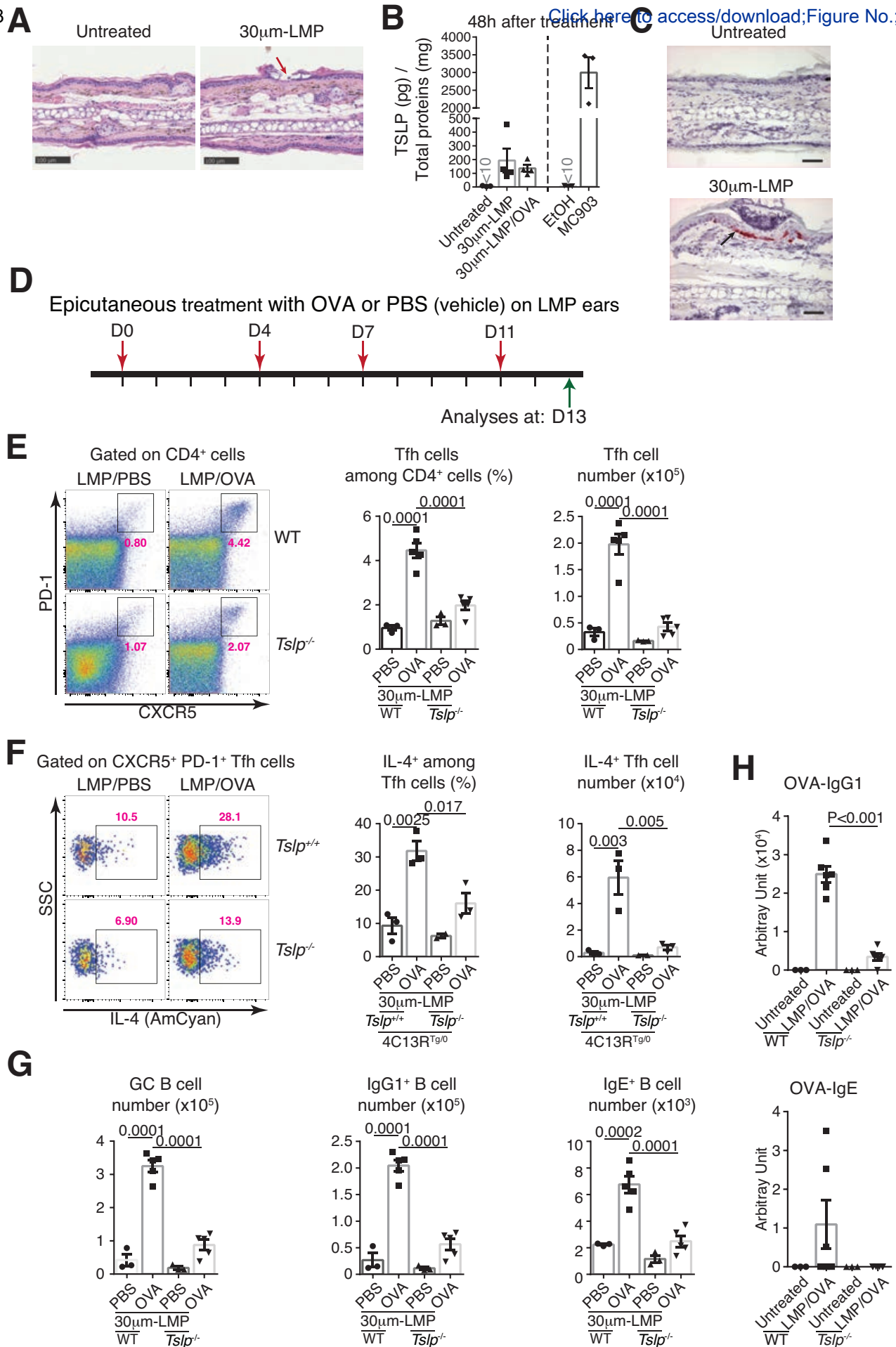
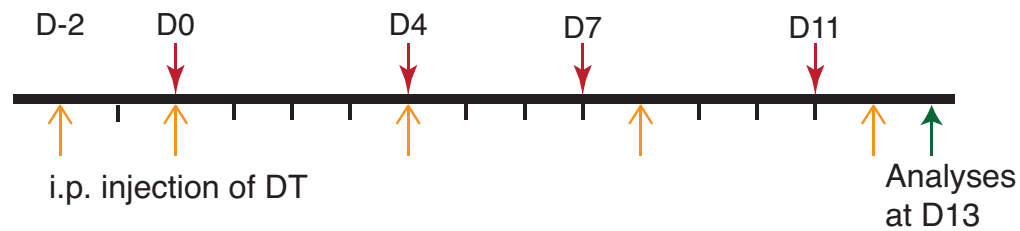
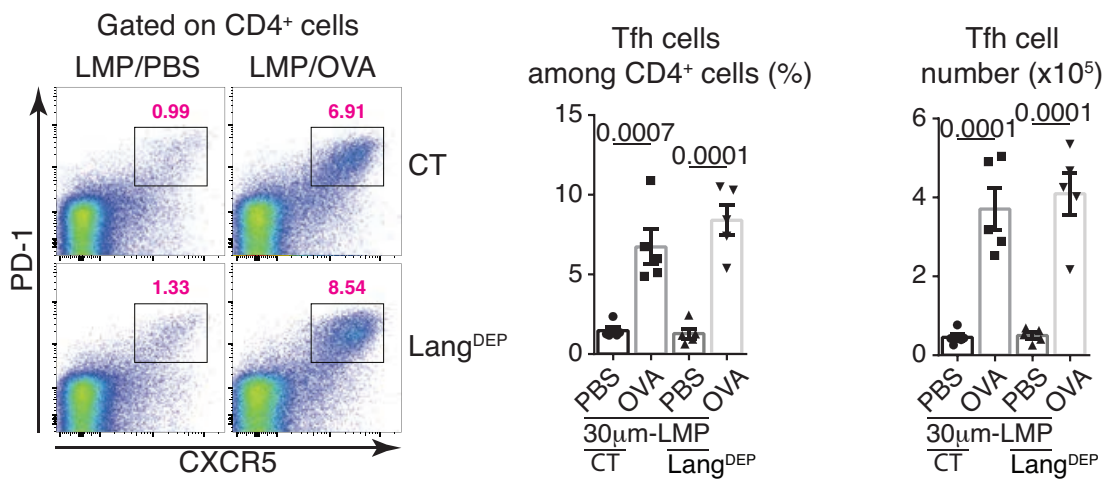
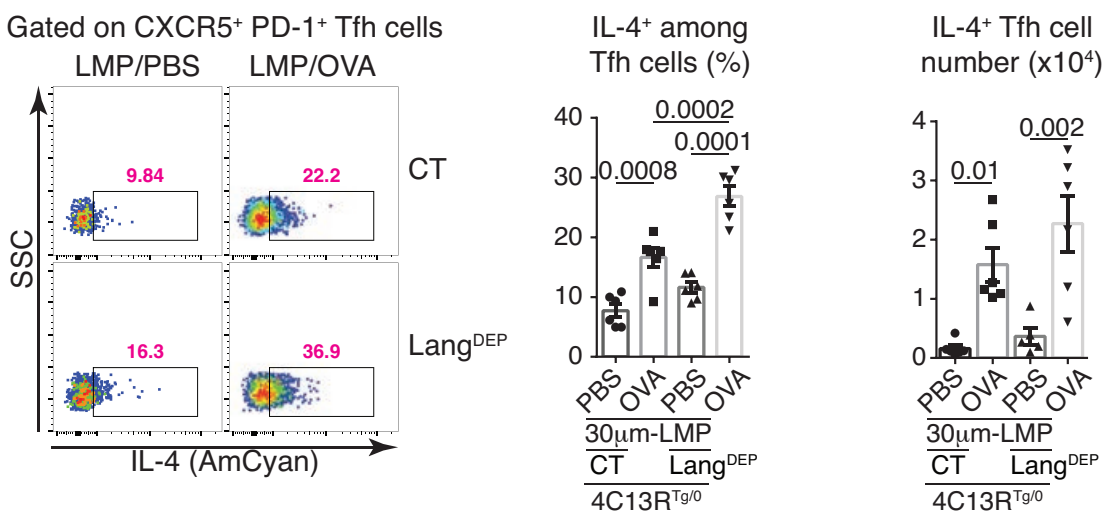
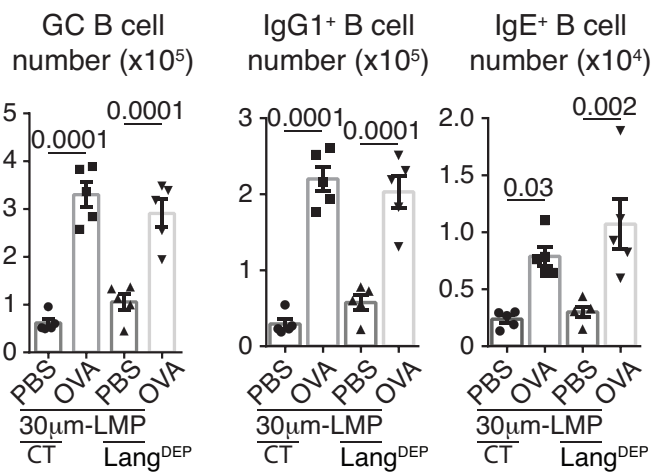
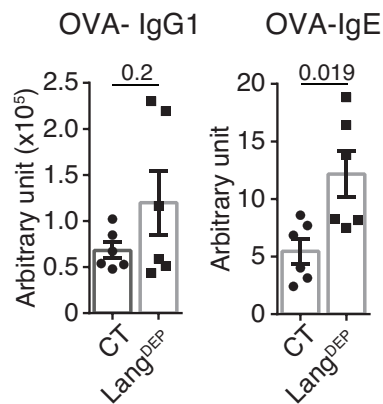


FIG 3

AEpicutaneous treatment with OVA or PBS (vehicle) on 30 μ m LMP ears**B****C****D****E****FIG 4**

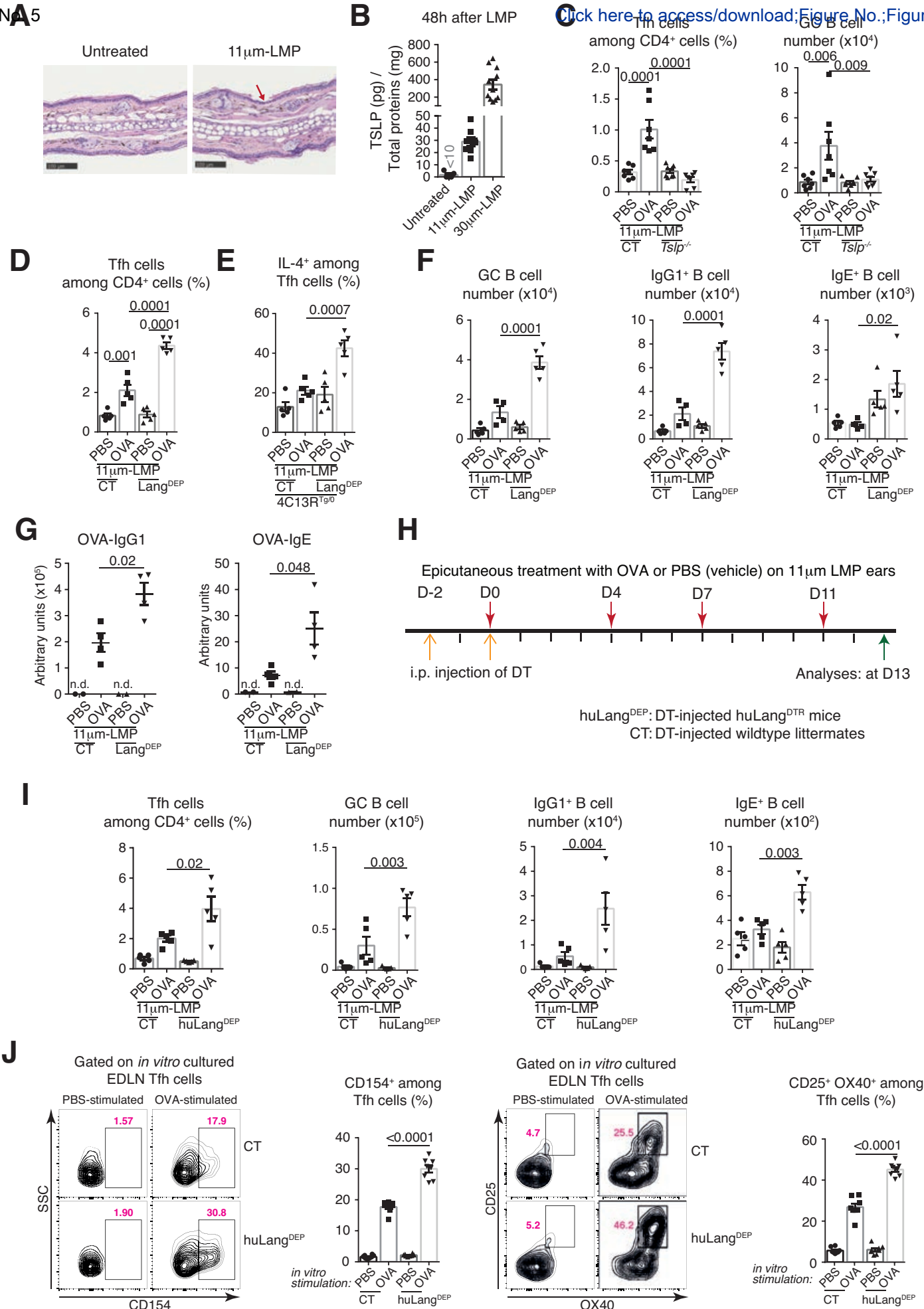


FIG 5

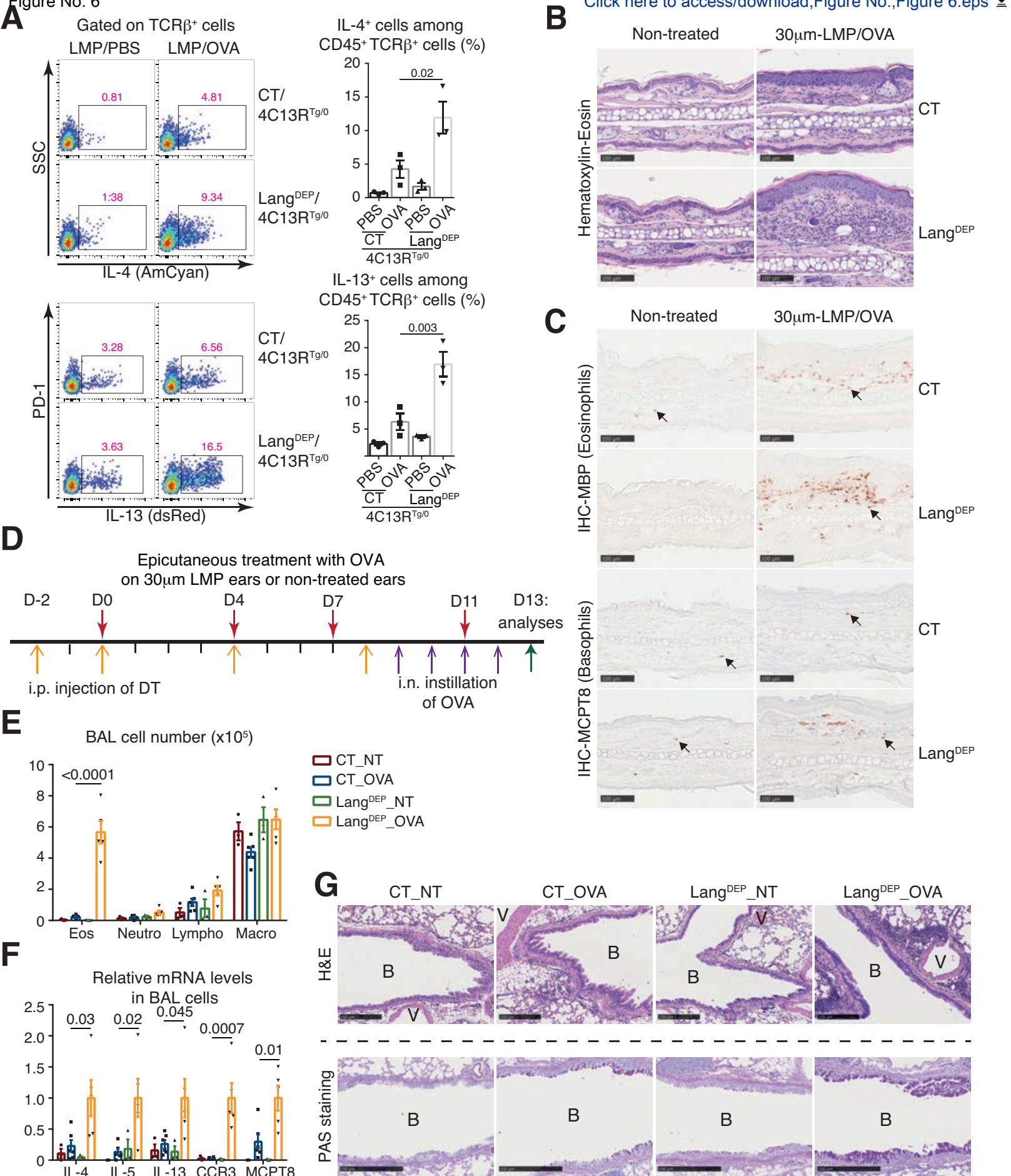


FIG 6

Figure No. 7

Click here to access/download/figure/NonFig7.eps

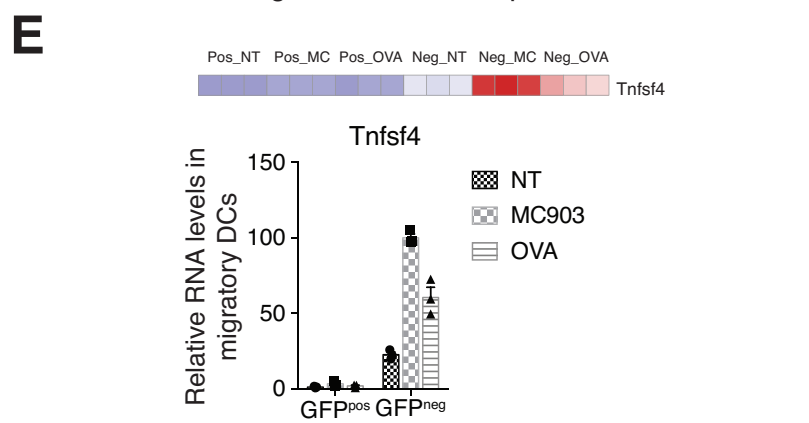
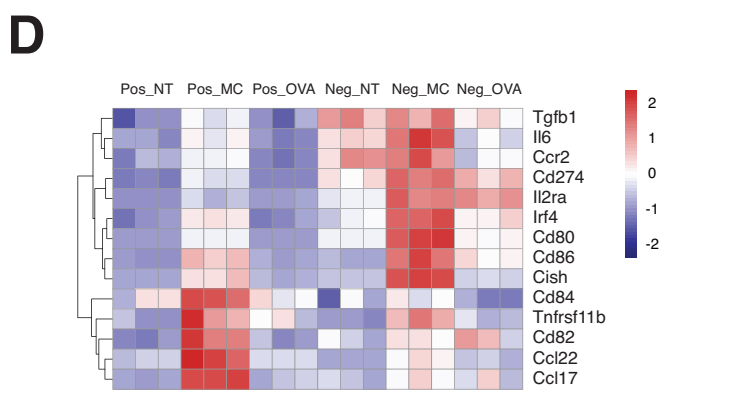
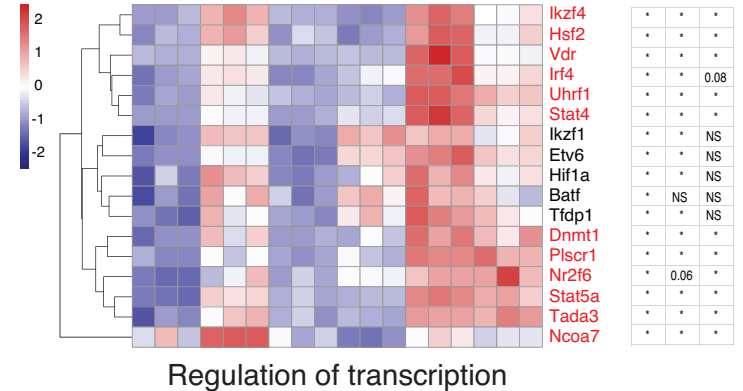
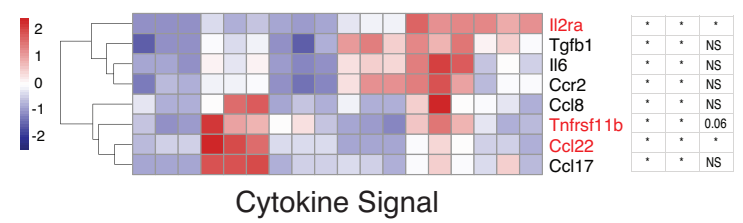
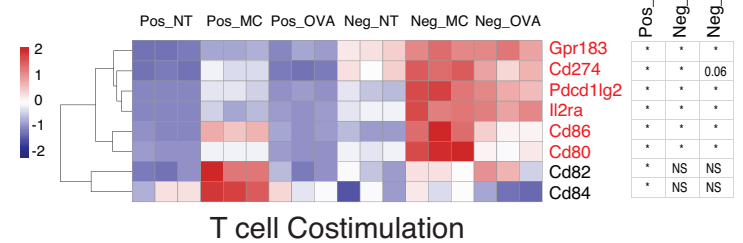
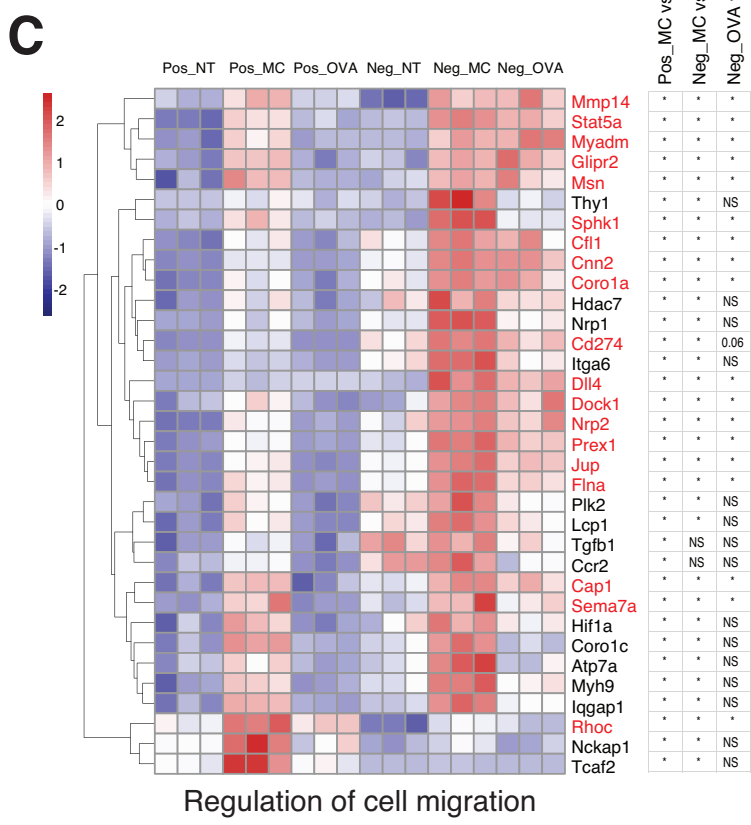
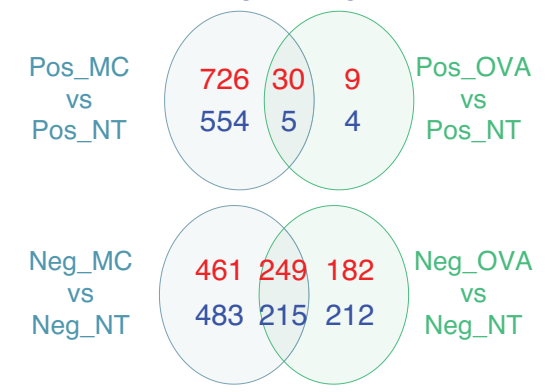
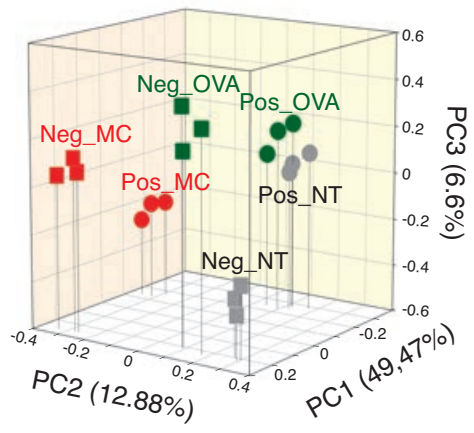
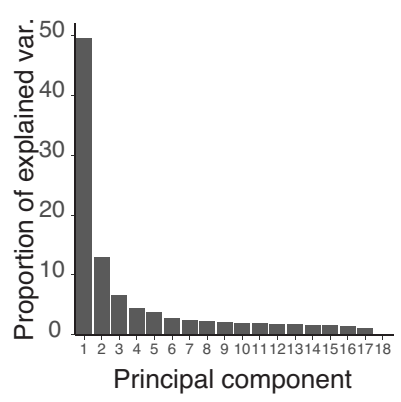


FIG 7

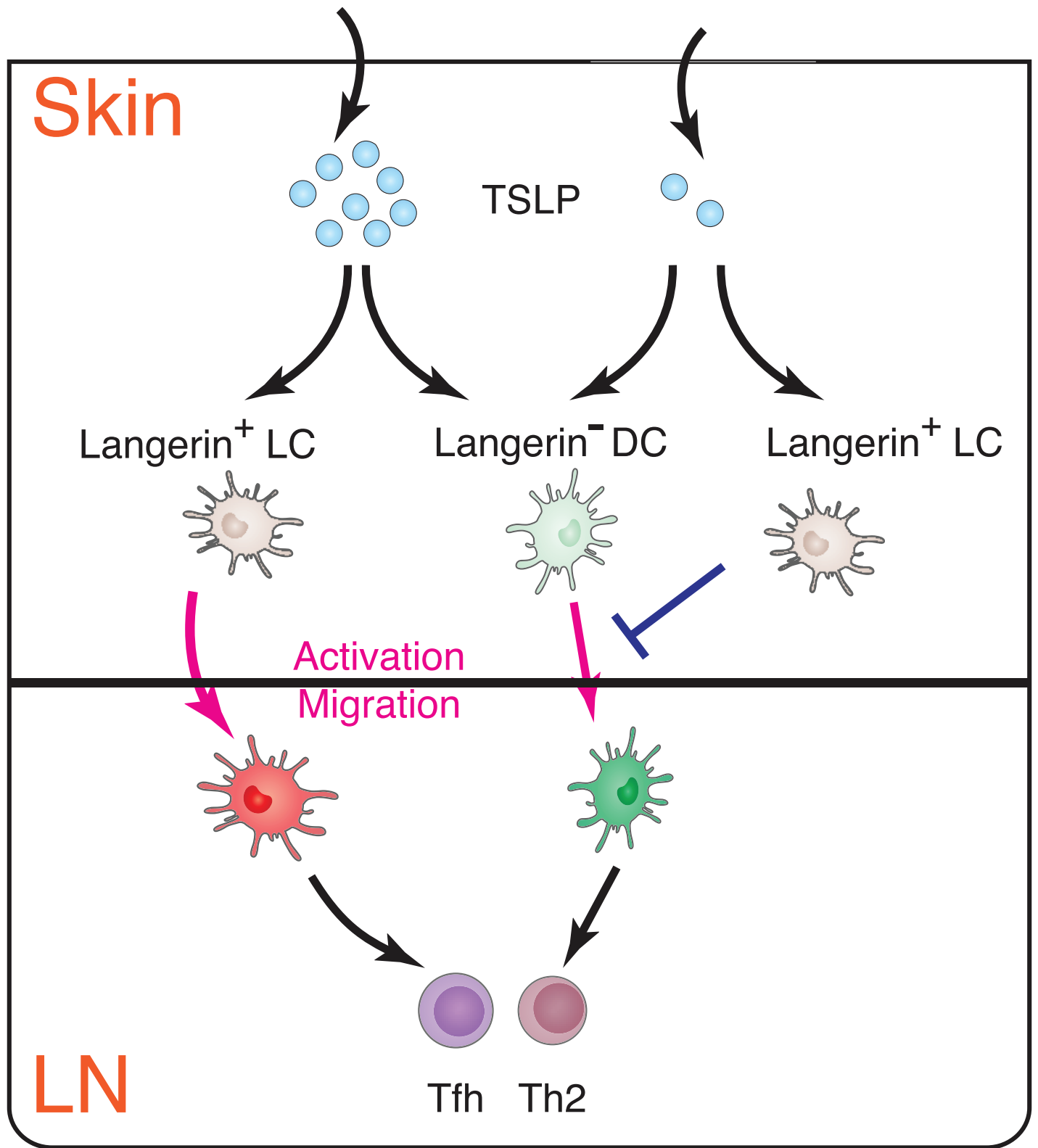


FIG 8

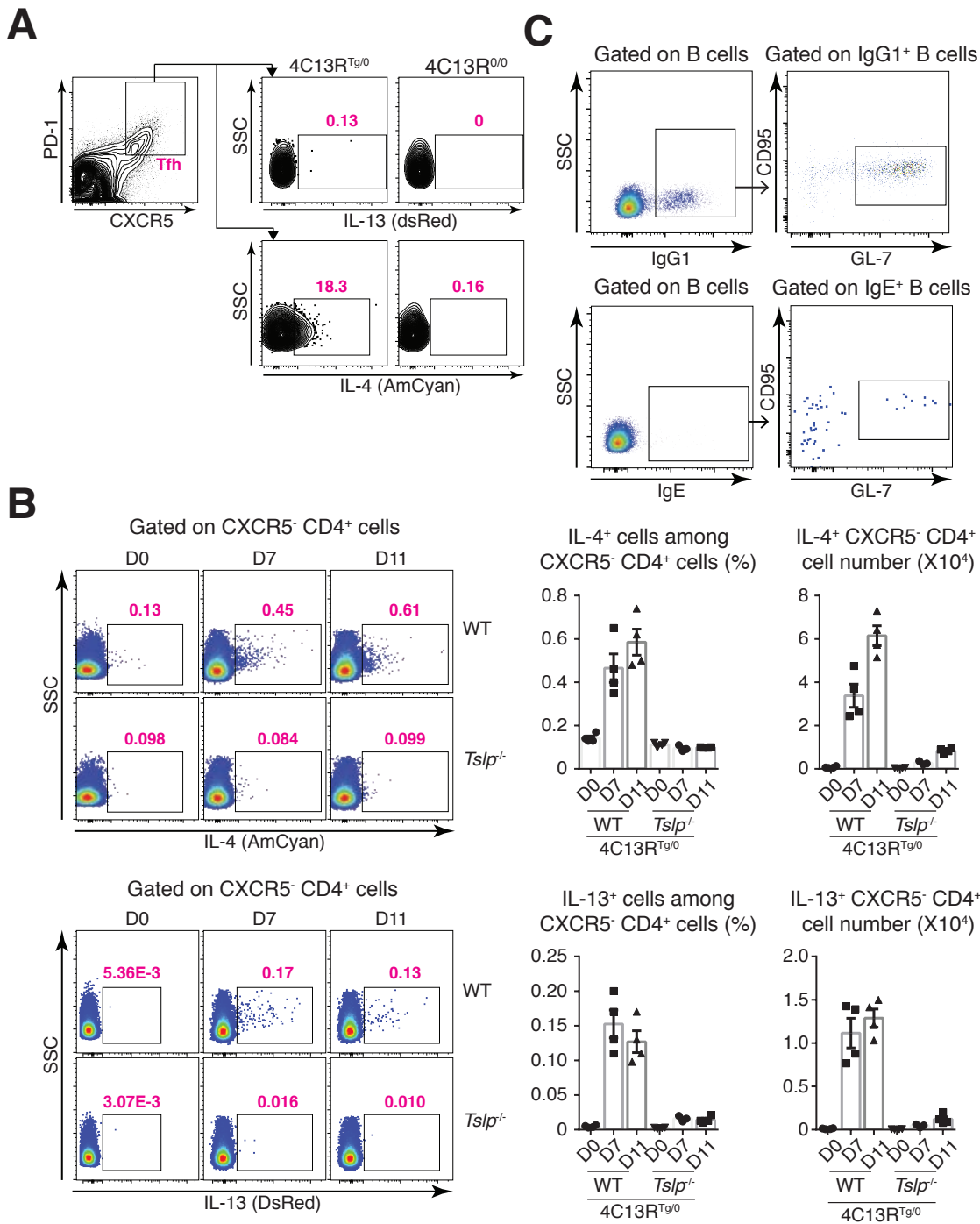


FIG E1.

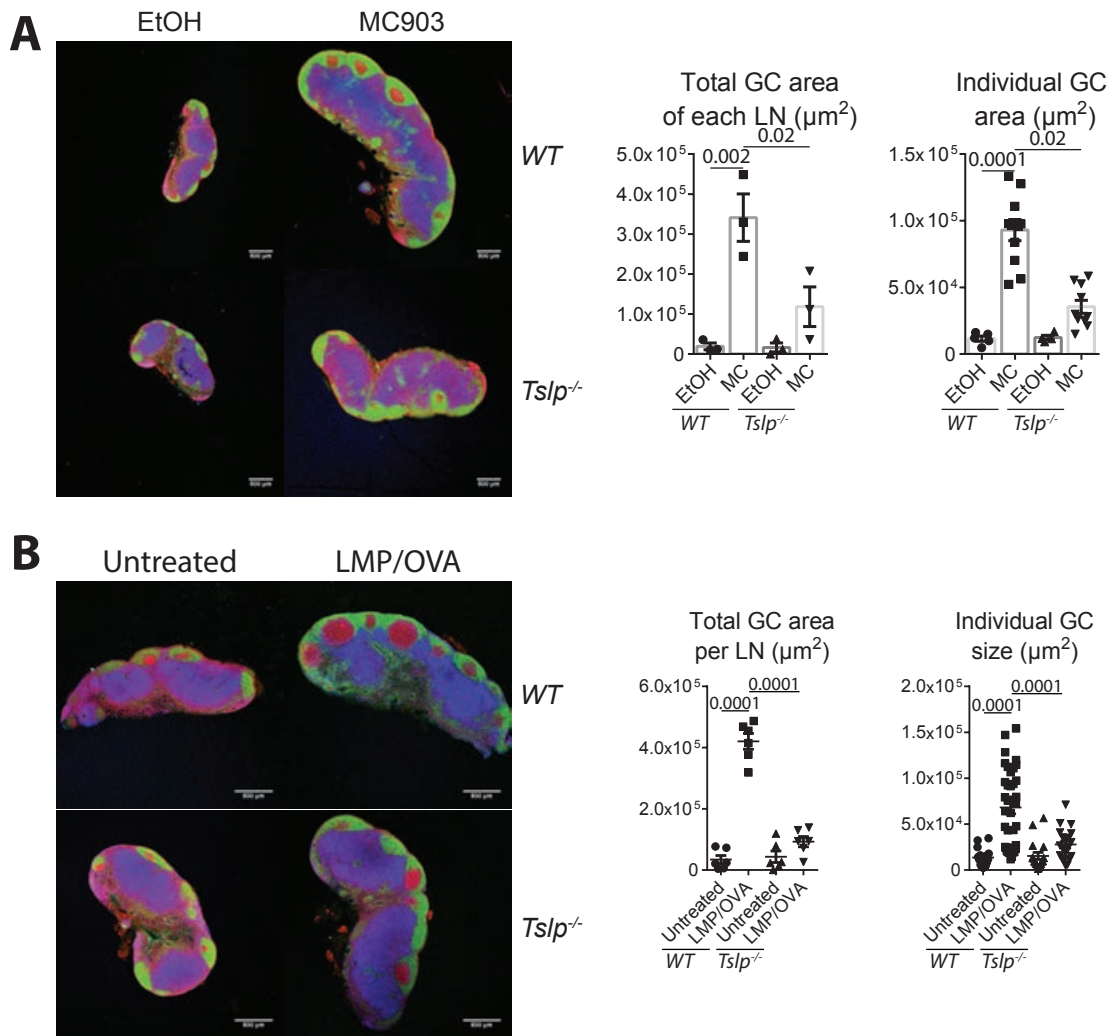


FIG E2.

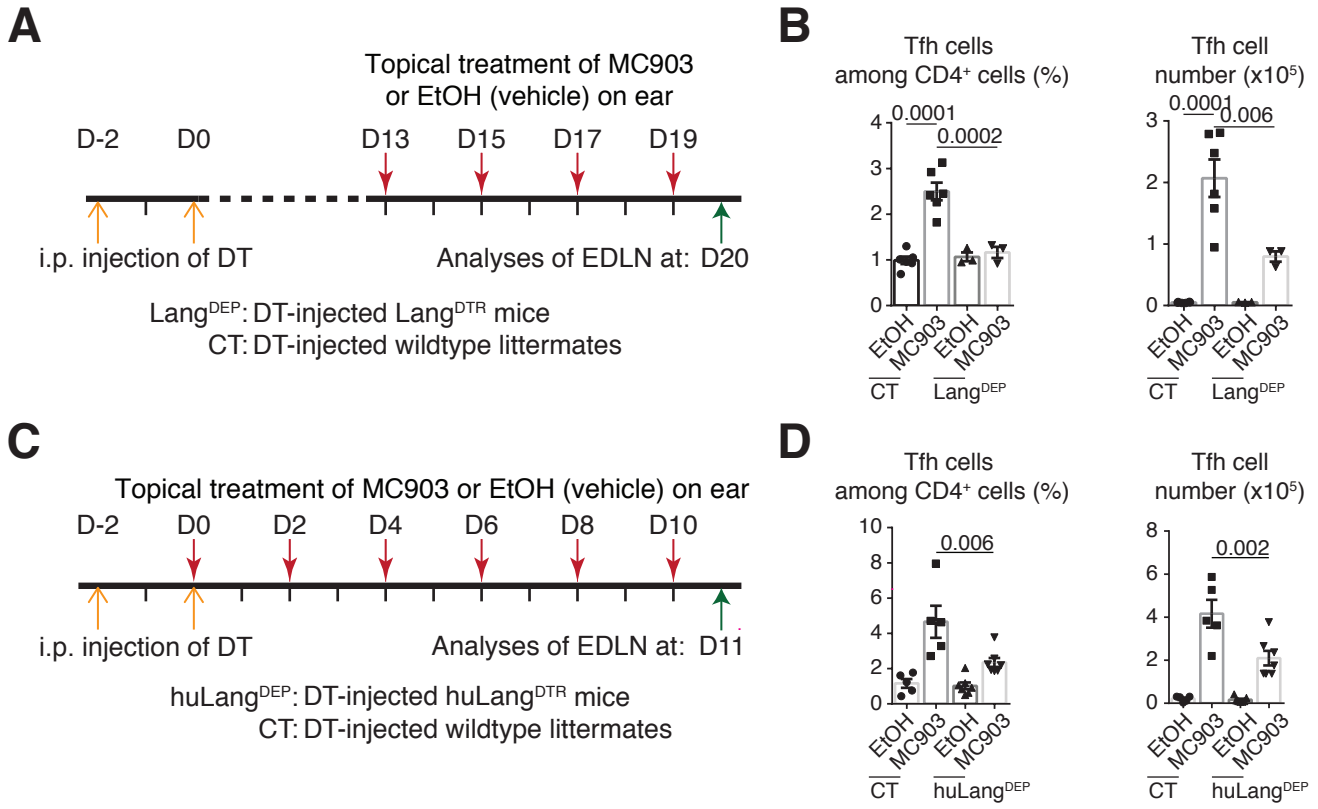


FIG E3.

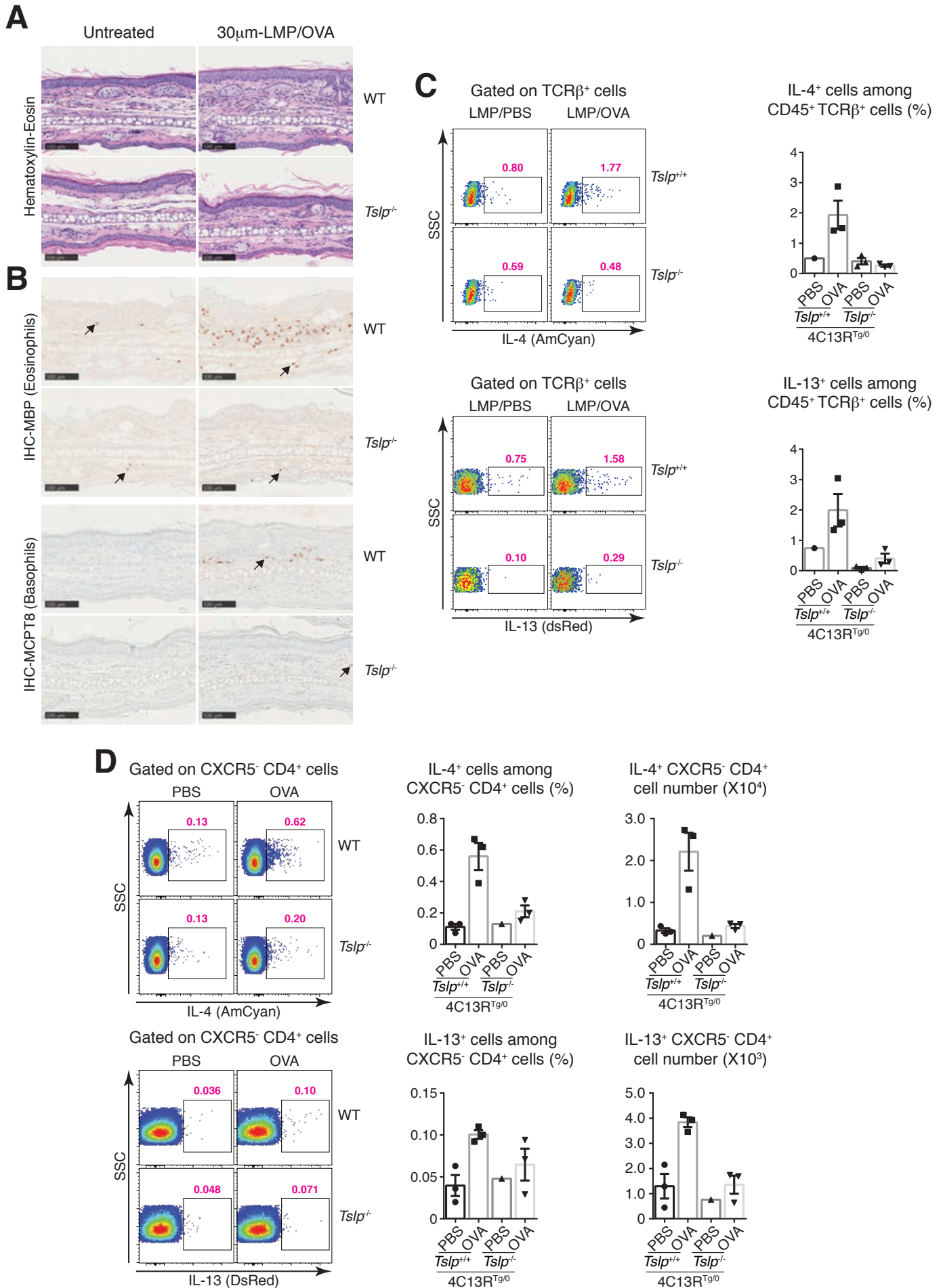


FIG E4.

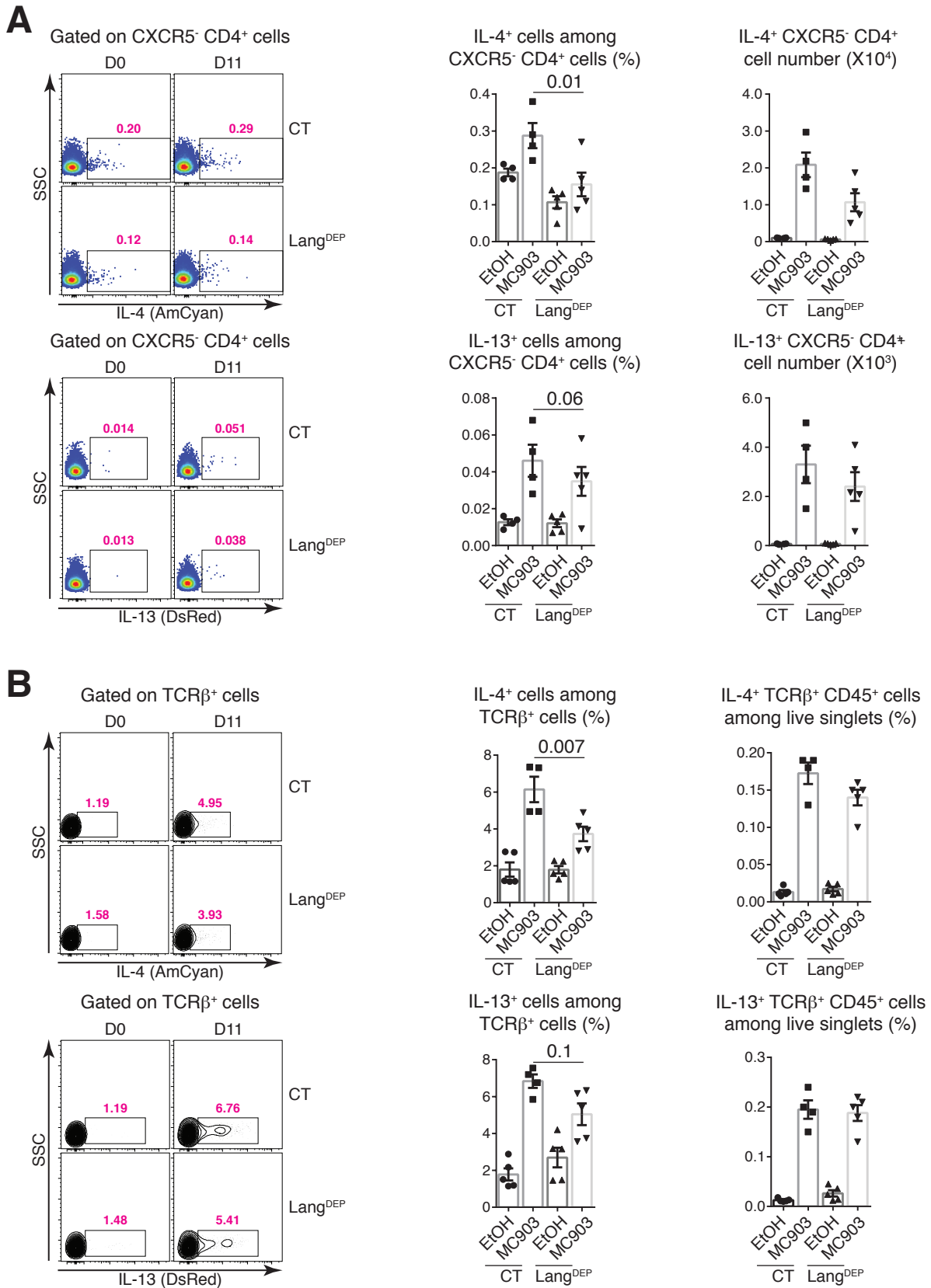


FIG E5.

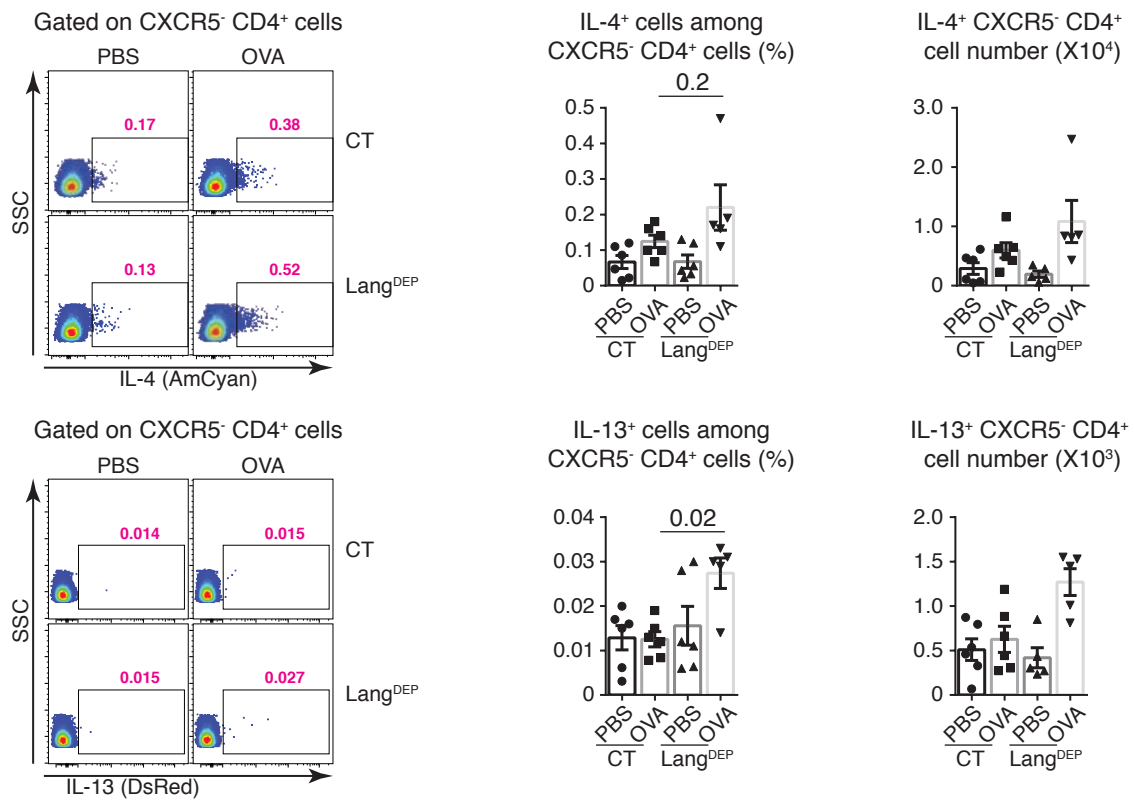
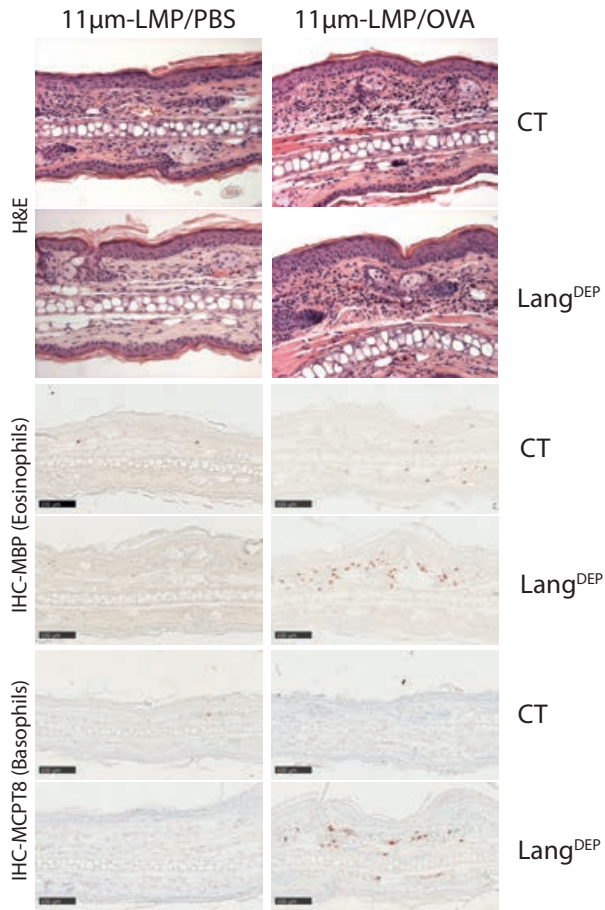
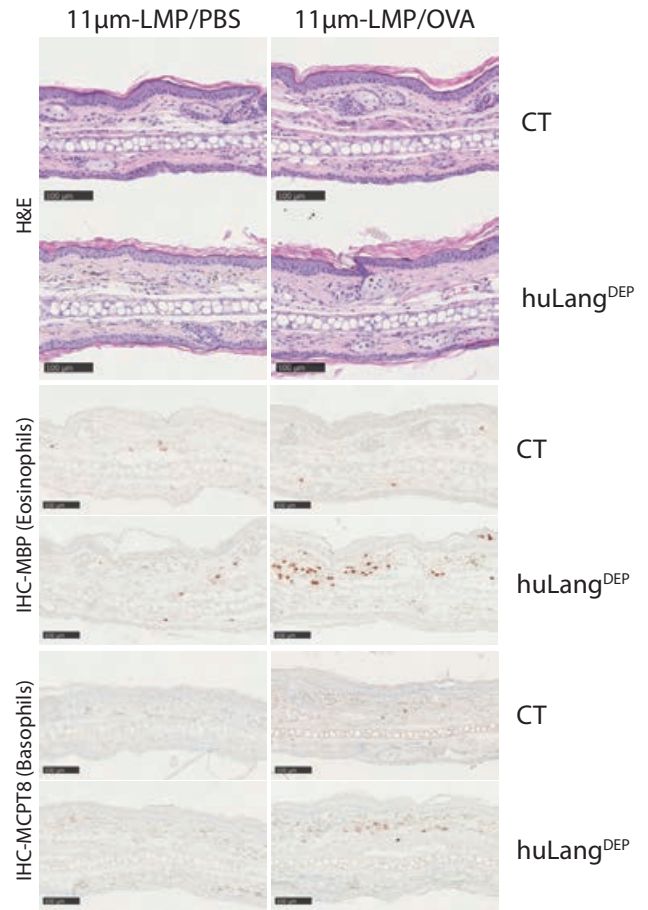


FIG E6.

A**B****FIG E7.**

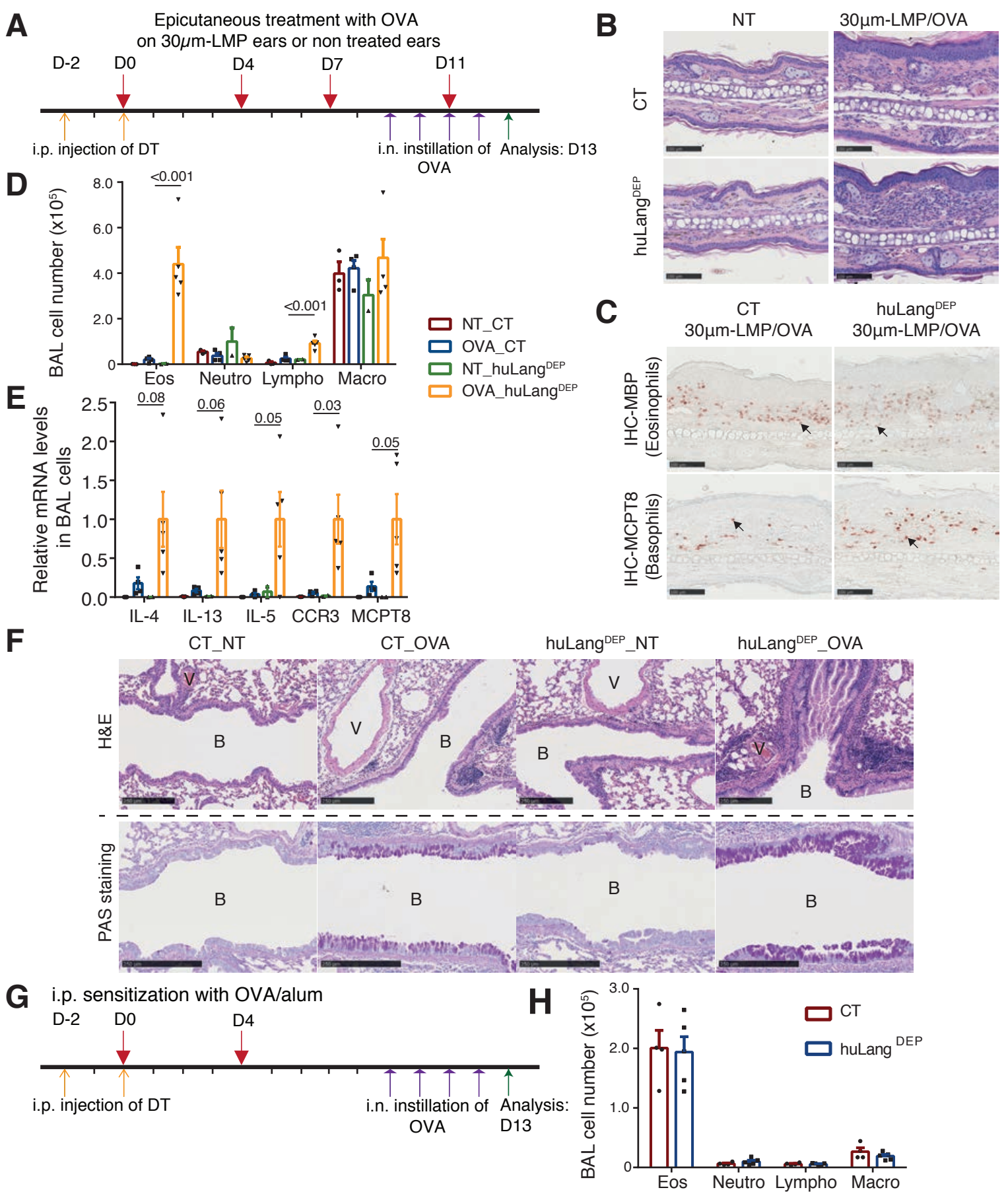
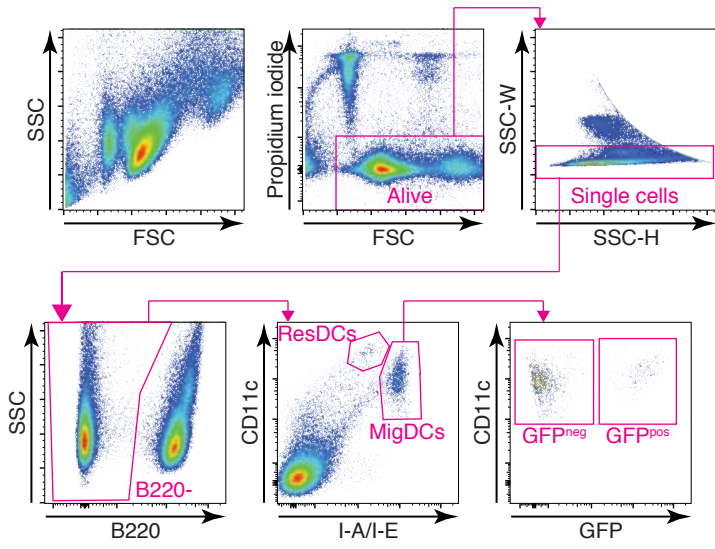
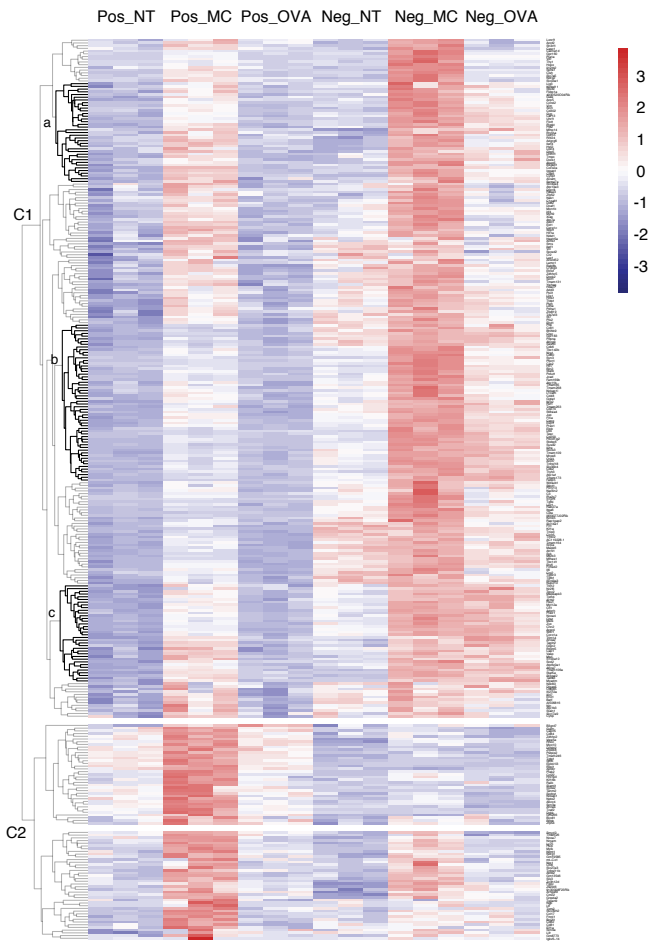


FIG E8.

A**B****FIG E9.**

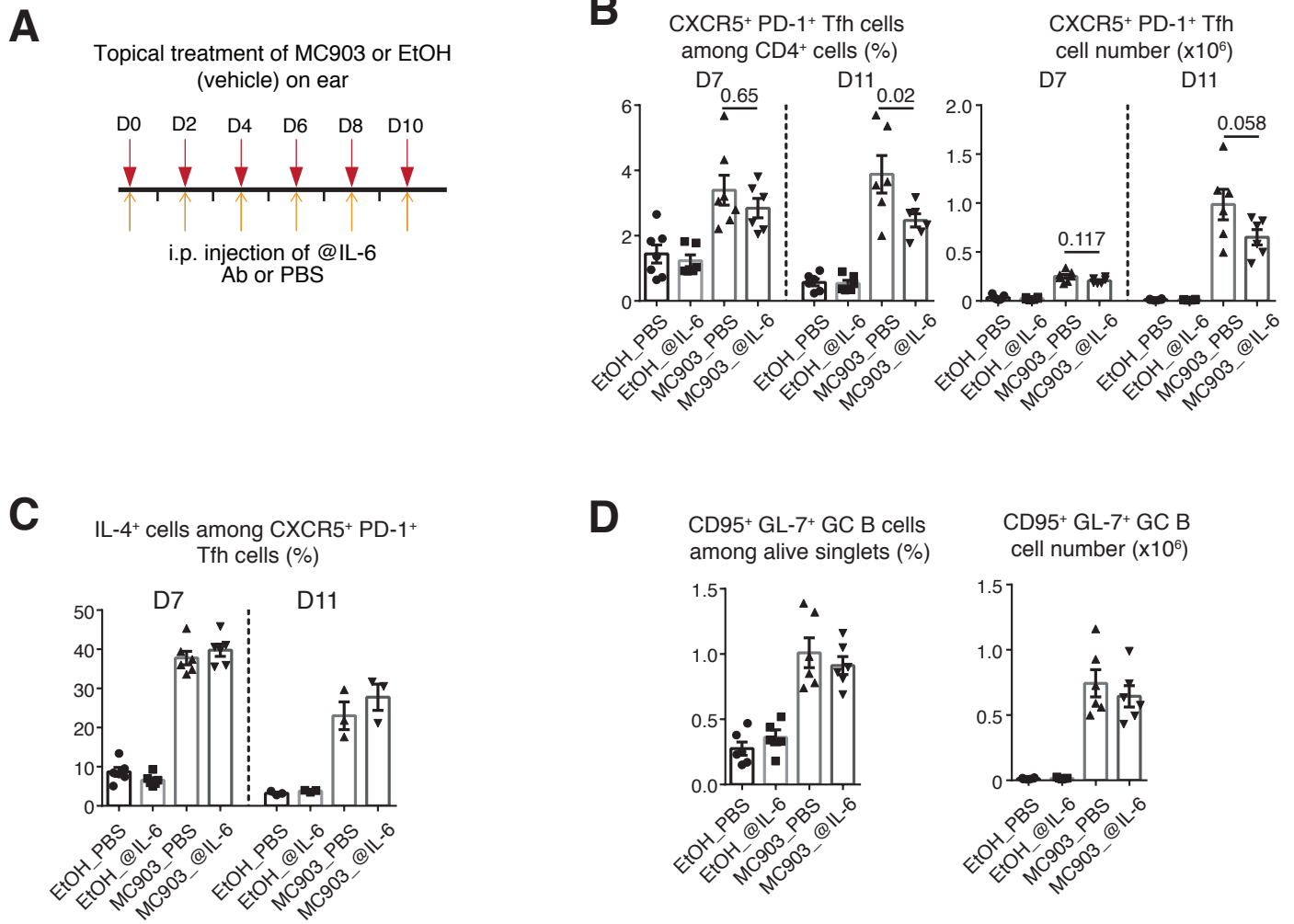


FIG E11.

Migratory DC cell number in EDLNs at D5 ($\times 10^5$)

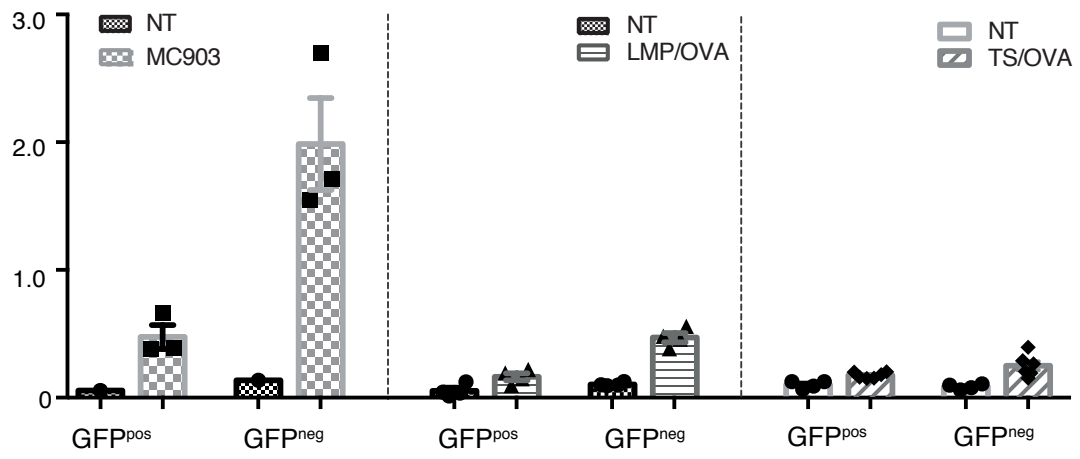


FIG E12.

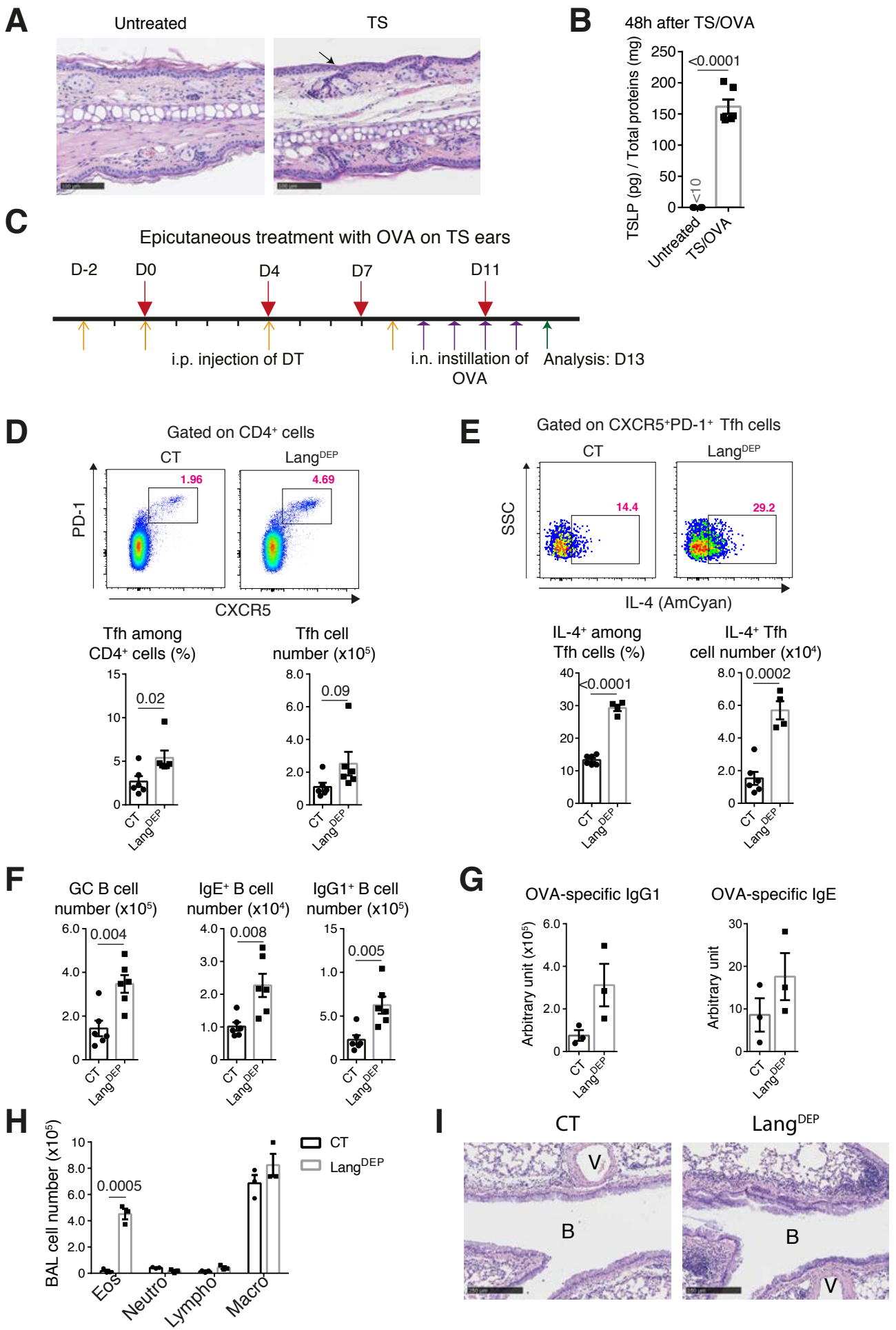


FIG E13.

Methods

Experimental mice. Balb/c mice were purchased from Charles River Laboratory. *Tslp*^{-/-1}, 4C13R^{Tg/0} ², Langerin^{DTR} ³ and huLangerin^{DTR} ⁴ were as described and were all backcrossed to >99.9 % Balb/c genetic background. Lang^{GFP} reporter mice ³ were in C57BL/6J background. Breeding and maintenance were performed under institutional guidelines, and all of the animal experiments were approved by the animal care and ethics committee of animal experimentation of the IGBMC.

MC903 topical application. MC903 (Calcipotriol, Sigma) was dissolved in 100% ethanol and topically applied on mouse ears (2 nmol in 25 μ l per ear) as previously described ⁵.

Epicutaneous OVA sensitization and airway challenge. Laser-assisted skin microporation (LMP) was performed using P.L.E.A.S.E.[®] research system (Pantec Biosolutions) on the dorsal side of mouse ears. For the depth of 30 μ m (30 μ m_LMP): 2 pulses per pore, with fluence of 7.5 J/cm², pulse length of 75 μ s, RepRate of 500 Hz and power of 1.0 W; for the depth of 11 μ m (11 μ m_LMP): 1 pulse per pore with fluence of 1,8 J/cm², pulse length of 50 μ s, RepRate of 500 Hz and power of 0.7 W. In all cases, the pore array size was set 14 mm and the pore density was set 15%. To induce epicutaneous OVA sensitization, 10 μ l of sterile PBS solution containing 200 μ g of OVA (Sigma-aldrich) were applied immediately on LMP ear skin at the time points indicated in experimental schemes in the Figures. In case of airway challenge, 25 μ L of saline solution containing 50 μ g of OVA was intranasally instilled.

Depletion of Langerin⁺ DCs or LCs in mice. Lang^{DTR} or huLang^{DTR} mice were intraperitoneally injected with diphtheria toxin (DT; Sigma-Aldrich) (1 μ g per 25 g body weight) at the time points indicated in the experimental schemes in the Figures. The DT-injected wild-type littermate mice were used as controls.

Cell preparation for flow cytometry analyses. For cell preparation from ear-draining lymph nodes (EDLN) for Tfh/GC staining, EDLNs were dissociated with piston, passed through a 70 μ m strainer

(Falcon) and resuspended in PBS containing 0.5% BSA and 2mM EDTA. Cells were then centrifuged and resuspended in FACS buffer (PBS containing 1% FCS and 2mM EDTA), counted and used for FACS staining. In case of preparation of EDLN cells for DC staining, EDLNs were cut in small pieces and incubated 30 minutes at 37°C in 2mg/mL collagenase D (Roche), 0.25mg/mL DNase I (Sigma) and 2.5% foetal calf serum (Thermofisher) in PBS prior passing through the strainer.

For preparation of dermal cells, ears were split into ventral and dorsal halves and floated 1h at 37°C on a PBS solution containing 4mg/ml Dispase (Gibco). Dermis was subsequently separated from epidermis and incubated 1h at 37°C with 1mg/ml collagenase D, 0.25mg/ml DNase I and 2.5% of foetal calf serum in PBS. Cells were passed through a 70µm cell strainer and resuspended in PBS containing 0.5% BSA and 2mM EDTA. Cells were then centrifuged and resuspended in FACS buffer, counted and used for FACS staining.

Surface staining for flow cytometry analyses. Two million cells were used for antibody staining. Cells were first incubated with anti-CD16/CD32 (clone 93, eBioscience) to block unspecific binding, followed by surface staining with the following antibody panels : CD11c biotin (clone HL3), IgE biotin (clone R35-72), CD95 PE-Cy7 (clone Jo2), CD19 FITC (clone 1D3), CXCR5 biotin (clone 2G8), CD4 Alexa Fluor 700 (clone RM-5), CD4 BV421 (clone GK1.5), streptavidin BV605 were from BD Biosciences; CD8a PerCP-Cy5.5 (clone 53-6.7), B220 APC (clone RA3-6B2), GL-7 PE (clone GL-7), I-A/I-E PE (clone M5/114.15.2) and streptavidin APC were from eBioscience. PD-1 PE-Cy7 (clone RMP1-30), IgG1 PerCP-Cy5.5 (clone RMG1-1) were from Biolegend. Viability staining was performed by adding propidium iodide to a final concentration of 4 µg/mL prior to cell passing with the cytometer. Stained cells were analysed on a Fortessa or LSRII flow cytometer (BD Biosciences). Results were analysed using FlowJo (Treestar).

LN cell culture and antigen stimulation. To identify OVA-specific Tfh cells by activation-induced marker assay ⁶, one million of freshly isolated EDLN single cell suspensions were cultured in 96-well U-bottom plate in 200µl of medium (RPMI 1640 supplemented with 10% FCS, HEPES, 0.05mM 2-

mercaptoethanol, 100U/ml penicillin, 100U/ml streptomycin), stimulated with 500µg/ml of OVA or PBS (vehicle) for 18h. Anti-CD154 BV650 antibody (clone MR1, BD Biosciences) was added to all culture conditions. After the culture, cells were incubated with anti-CD16/CD32 (Clone 93, eBioscience) to block unspecific binding, and stained with viability dye 506 (eBioscience) and antibody panels: B220 FITC (clone RA3-6B2, Biolegend), CD4 BV421 (clone GK1.5, BD Biosciences), CXCR5 biotin (Clone 2G8, BD Biosciences), Streptavidin PE (eBioscience), PD-1 PE-Cy7 (clone RMP1-30, Biolegend), OX40 APC (clone OX86, eBioscience) and CD25 PerCP-Cy5.5 (clone PC61, BD Biosciences).

RNA sequencing. Migratory DCs from EDLNs were FACS-sorted with ARIA II (BD) (see Fig 8A for sorting strategies). RNA was extracted using RNeasy Micro Kit (Qiagen). RNA-seq was performed in IGBMC high-throughput mRNA sequencing facility. Full length cDNAs were generated from 1ng of total RNA using Clontech SMART-Seq v4 Ultra Low Input RNA kit for Sequencing (Takara Bio Europe, Saint Germain en Laye, France) according to manufacturer's instructions with 12 cycles of PCR for cDNA amplification by Seq-Amp polymerase. Six hundred pg of pre-amplified cDNA were then used as input for Tn5 transposon tagmentation by the Nextera XT DNA Library Preparation Kit (96 samples) (Illumina, San Diego, CA) followed by 12 cycles of library amplification. Following purification with Agencourt AMPure XP beads (Beckman-Coulter, Villepinte, France), the size and concentration of libraries were assessed by capillary electrophoresis. Libraries were sequenced as 50bp single-end reads on an Illumina HiSeq 4000 sequencer.

Reads were preprocessed in order to remove adapter, polyA and low-quality sequences (Phred quality score below 20). After this preprocessing, reads shorter than 40 bases were discarded for further analysis. These preprocessing steps were performed using cutadapt version 1.10. Reads were mapped onto the mm10 assembly of mouse genome using STAR version 2.5.3a. Read counts have been normalized across samples with the median-of-ratios method proposed by Anders and Huber ⁷, to make these counts comparable between samples. Comparisons of interest were performed using the method proposed by Love et al. ⁸ and implemented in the DESeq2 Bioconductor library version 1.16.1. P-values were adjusted for multiple testing using the Benjamini and Hochberg method. Gene expression

quantification was performed from uniquely aligned reads using htseq-count version 0.6.1p1, with annotations from Ensembl version 96 and "union" mode. The RNA-Seq data have been deposited in the NCBI's Gene Expression Omnibus (GEO) and are accessible as GSE149039.

Bronchoalveolar lavage (BAL) cell analyses. BAL was taken in anaesthetized mice by instilling and withdrawing 0.5 ml of saline solution (0.9% NaCl, 2.6mM EDTA) in the trachea. After six times lavages, BAL fluid was centrifuged, and BAL cells were counted using a Neubauer hemocytometer. 5×10^4 BAL cells were cytopspined and stained with Hemacolor kit (Merck) to identify macrophages, lymphocytes, neutrophils and eosinophils. After differential counting to obtain their frequencies, the number of each cell type was calculated according to the total BAL cell number and the frequency. For RT-qPCR analyses, RNA was extracted from BAL cells using NucleoSpin RNA XS kit (Macherey-Nagel), reverse transcribed by using random oligonucleotide hexamers and SuperScript IV Reverse Transcriptase (Invitrogen) and amplified by means of quantitative PCR with LightCycler 480 SYBR Green kit (Roche), according to the manufacturer's instructions. Relative RNA levels were calculated with hypoxanthine phosphoribosyl- transferase (HPRT) as an internal control. For analyses of each set of gene expression, an arbitrary unit of 1 was given to the samples with the highest level, and the remaining samples were plotted relative to this value. Sequences of qPCR primers are: Hprt (TGGATACAGGCCAGACTTTG ; GATTCAACTTGCCTCATCTTA; 161 bp); Il4 (GGCATTTTGAACGAGGTCAC; AAATATGCGAAGCACCTTGG; 132 bp); Il5 (AGCACAGTGGTGAAAGAGACCTT; TCCAATGCATAGCTGGTGATTT; 117 bp); Il13 (GGAGCTGAGCAACATCACACA; GGTCCCTGTAGATGGCATTGCA; 142 bp); Ccr3 (TAAAGGACTTAGCAAAATTCACCA; TGACCCCAGCTCTTTGATTC; 150 bp); Mcpt8 (GTGGGAAATCCCAGTGAGAA; TCCGAATCCAAGGCATAAAG; 160 bp).

Enzyme-linked immunosorbent assay (ELISA). To measure TSLP levels by ELISA, mouse skin was chopped and homogenized with a Mixer Mill MM301 (Retsch, Dusseldorf, Germany) in lysis buffer (25 mmol/L Tris pH 7.8, 2 mmol/L EDTA, 1 mmol/L dithiothreitol, 10% glycerol, and 1% Triton X-100)

supplemented with protease inhibitor cocktail (Roche). Protein concentrations of skin extract were quantified by using the Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, Calif), and TSLP levels in skin extracts were determined with the DuoSet ELISA Development Kits (R&D Systems, Minneapolis, Minn).

To measure OVA-specific IgG1 and IgE in sera, microtiter plates were coated with OVA and then blocked with BSA. Serum samples were incubated in the coated plates overnight at 4°C followed by incubation with a biotinylated rat anti-mouse IgE (BD Biosciences; clone R35-118) or IgG1 (BD Biosciences; clone A85-1). Extravidin horseradish peroxidase (Sigma) and TMB (tetramethylbenzidine) Substrate Reagent Set (BD Biosciences) were used for detection. Levels of OVA-specific IgG1 and OVA-specific IgE were calculated relevant to a pre-prepared serum pool from OVA-sensitized and challenged mice and expressed as arbitrary units.

Histopathology. Mouse ears and lungs were fixed in 4% paraformaldehyde overnight at 4°C and embedded in paraffin. 5µm sections were stained with hematoxylin & eosin (H&E). For periodic Acid Schiff (PAS) staining, slides were incubated with 0.5% aqueous periodic acid (Alfa Aesar), washed with water and incubated 15 minutes in Schiff's reagent (Merck). Slides were counterstained with hematoxylin and differentiated with acid alcohol.

Immunohistochemistry (IHC). For IHC staining of major basic protein (MBP) and mast cell protease 8 (MCPT8), 5µm paraffin sections were treated with 0.6% H₂O₂ to block endogenous peroxidase activity before antigen retrieval with either Pepsin (for IHC of MBP; Life technologies) or citric buffer (10 mmol/L citric acid, pH 6; for IHC of MCPT8). Slides were then blocked with normal rabbit serum (Vector Laboratories) and incubated overnight with primary antibody (Rat anti-mouse MBP antibody (Mayo Clinic, Rochester); Rat anti-mouse TUG8 (Biolegend)). Slides were then incubated with biotinylated rabbit anti-rat IgG (dilution: 1/300) and treated with AB complex (Vector Laboratories). Staining was finally visualized with AEC high-sensitivity substrate chromogen solution (Dako) and counterstained with hematoxylin.

RNA in situ hybridization. Mouse ears were fixed in formalin and embedded in paraffin. RNA in situ hybridization was performed on freshly 5µm sections using RNAscope® 2.5 HD Reagent Kit-RED (Advanced Cell Diagnostics, Hayward, CA, USA) according to the manufacturer's instructions. Probe Mm-Tslp was used for detection of TSLP (Cat 432741).

Statistics. Data were analyzed using GraphPad Prism 6. Comparison of two samples was performed either by Student's two-tailed unpaired t-test with Welch's correction or the Mann–Whitney rank sum nonparametric test depending on results from the Kolmogorov–Smirnov test for normality. Comparison of more than two samples was performed by ordinary one-way ANOVA followed by Tukey's post-hoc test.

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