1   *Mast cells and γδ T cells are largely dispensable for adaptive immune*

2   *responses after laser-mediated epicutaneous immunization*

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4   Isabella Joubert[1*], Daniel Kovacs [1,2*], Sandra Scheiblhofer[1], Petra Winter[1], Evgeniia Korotchenko[1],

5   Helen Strandt[1], Richard Weiss[1]

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7   [1] Department of Biosciences, University of Salzburg, Salzburg, Austria

8   [2] current address: Department of Hematology and Medical Oncology, Klinikum rechts der Isar and

9   TranslaTUM Cancer Center, Technische Universität München, München, Germany

10   [*] authors contributed equally

11

12   **Corresponding author:**

13   Assoc. Univ.-Prof. Dr. Richard Weiss

14   University of Salzburg, Department of Biosciences

15   Hellbrunnerstraße 34, 5020 Salzburg, Austria

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23   *Highlights*

24   • Laser microporation induces an inflammatory chemokine milieu at the site of immunization

25   •   γδ T cells and mast cells contribute to the steady-state or damage-induced cytokine milieu in

26   the skin

27   •   γδ T cells and mast cells are dispensable for adaptive immunity after laser-mediated

28   immunization

29

30 **Abstract**

31 **Background**

32 The skin resembles an attractive target for vaccination due to its accessibility and abundance of

33 resident immune cells. Cells like γδ T cells and mast cells (MCs) are part of the first line of defence

34 against exogenous threats. Despite being important mediators for eliciting TH2 immune responses

35 after epithelial stress, γδ T cell and MC function still remains to be completely understood. Here, we

36 aimed to characterize their roles in shaping adaptive immune responses after laser-mediated

37 epicutaneous immunization (EPI).

38 **Methods**

39 γδ T cell knock out, MC depleted, and wildtype control mice were immunized with mannan-

40 conjugated grass pollen allergen Phl p 5 (P5-MN) by laser-mediated EPI. After 2-3 immunizations,

41 cytokine expression, T helper polarization, and antigen-specific IgG1/IgE levels were analysed. The

42 local cytokine/chemokine milieu after laser microporation was determined.

43 **Results**

44 While the majority of inflammatory chemokines and cytokines induced by laser treatment was not

45 affected by the presence of γδ T cells or MCs, RANTES, was elevated in γδ T cell knock out mice, and

46 GROα and TSLP, were significantly decreased after MC depletion. However, absence of γδ T cells or

47 depletion of MC had no substantial effect on adaptive humoral or cellular immune responses after

48 laser-mediated EPI, except for slightly reduced IgG1 and effector T cell levels in MC depleted mice.

49 **Conclusions**

50 γδ T cells did not play a pivotal role in shaping the humoral and cellular adaptive immune response

51 after laser-mediated EPI, whereas MC depletion decreased numbers of effector T cells, indicating a

52 potential role of MCs in the activation and maturation of T cells after EPI.

1. Introduction

While vaccination via intramuscular (IM) or subcutaneous (SC) injection represents a straightforward and well-established method, in most cases, large doses of the respective vaccine are required to provide protective immunity. Moreover, due to the low number of resident antigen-presenting cells (APCs) in these target tissues, IM or SC inoculations require infiltrating leukocytes attracted by inflammatory signals at the site of vaccine administration [1]. To overcome the limitations of conventional injections, new methods have recently been developed with the focus on generating less invasive and more efficient delivery techniques for vaccines [2, 3].

Whereas some novel skin vaccination technologies rely on actual deposition of the vaccine within the skin, others aim at passive transport of the vaccine via barrier disrupted skin, e.g. by using laser microporation [3, 4]. Thereby, heat pulses are produced by highly focused thermal energy (e.g. infrared light) leading to thermal ablation at locally restricted skin areas [5] and thus generating skin micropores in a controlled manner by means of decomposition and vaporization of tissue. Laser microporation enables circumvention of the stratum corneum and promotes vaccine delivery to cutaneous APCs. In addition to enhanced antigen delivery, this technique provides an intrinsic adjuvant effect [6] and has shown promising results in prophylactic and therapeutic approaches against type I allergies and tumours in animal models [4, 7, 8].

As the barrier between host and environment, the skin is a highly immunocompetent organ harbouring diverse populations of resident immune cells including dendritic cells (DC), mast cells (MC), and γδ T cells, and thus represents an attractive target for vaccination [9]. While skin-resident APCs are continuously migrating to regional lymph nodes and the blood, sessile (epi)dermal cells are able to polarize immune responses by release of cytokines. An accumulating body of evidence suggests that vaccination via the skin enables dose sparing [10, 11], which is of special interest for treatment of immune-compromised and elderly patients as well as in cases of limited vaccine availability. The easily accessible upper layers of the skin furthermore allow for usage of smaller needles or even needle-free vaccination. This results in increased patient convenience and

80   compliance and reduces the risk of needle-stick injuries, thereby also offering advantages for

81   healthcare providers [12].

82   In the context of epicutaneous immunization (EPI), antigens are delivered to APCs mostly via passive

83   uptake. Based on the low efficiency of passive uptake, novel antigen formulations directly target

84   receptors on skin-resident DCs to promote antigen uptake. One approach is to actively target DCs by

85   engagement of their expressed C-type lectin receptors (CLRs) and thus significantly increase antigen

86   uptake [13]. Not only does targeting CLRs increase antigen uptake and enhance immunogenicity, but

87   also allows shaping of the subsequent immune responses [14]. CLRs comprise a large superfamily of

88   receptors, which recognize diverse carbohydrate moieties in a calcium-dependent or independent

89   manner through one or more conserved carbohydrate recognition domains (CRDs) or C-type lectin

90   domains (CTLDs). CLRs have evolved alongside pathogens and are often specific for distinct and

91   conserved carbohydrate motifs derived from viruses, bacteria, parasites or fungi [15]. A variety of

92   immune cells express CLRs such as dermal DCs (dDCs), Langerhans cells (LCs), macrophages,

93   neutrophils, and B lymphocytes. As pattern recognition receptors (PRRs), CLRs play various roles in

94   eliciting immune responses depending on the detected pathogen-associated molecular pattern

95   (PAMP). The potential of the carbohydrate mannan as an adjuvant capable of enhancing and shaping

96   the overall immune response has been demonstrated to rely on its capacity to engage CLRs [16, 17].

97   We have previously reported that EPI with allergen-mannan neoglycoconjuates by laser

98   microporation leads to more efficient antigen uptake by dDCs and LCs. In addition, we found that the

99   initiated immune response was skewed towards TH1/TH17, which is in accordance with the fungal

100   origin of mannan [17].

101   Nevertheless, the immune response mounted upon an antigen encounter is also highly dependent on

102   the microenvironment within the target tissue. Barrier disruption has been demonstrated to be

103   essential for enhancing skin permeation by altering the microarchitecture of the skin and increasing

104   the motility of embedded APCs. Furthermore, disrupting the stratum corneum has been shown to

105   provide a natural inherent adjuvant effect by inducing the secretion of pro-inflammatory cytokines,

106   chemokines and danger-associated molecular patterns (DAMPs) [18, 19]. More precisely, damaged

107   and necrotic keratinocytes release cytokines such as IL-25, IL-33 and TSLP ("alarmins"), which

108   activate other skin-resident cells and induce specific immune responses [20].

109   MCs and γδ T cells are distributed throughout the whole body in the mucosal, epithelial and

110   connective tissues. Hence, they are strategically positioned at the barrier between host and

111   environment, where exogenous material can enter. They both play important roles in innate

112   immunity and can be rapidly activated by stress signals and pathogens. The various protective

113   functions exerted by γδ T cells include anti-microbial defence, tumour surveillance, tissue

114   homeostasis, and wound repair [21, 22]. The term lymphoid stress-surveillance is commonly used to

115   describe the rapidly induced immune response initiated after stress-antigen engagement by the γδ T

116   cell receptor. Additionally, γδ T cells have been reported to directly sense epithelial dysregulation,

117   control tissue homeostasis by IL-13 production and promote IgE-dependent responses to protein

118   allergens in stressed tissue [23]. MCs are predominantly known for their essential role as mediators

119   of type I allergy and for their potential to secrete a variety of allergic mediators upon activation [24].

120   However, MCs cannot only be activated by antigen-dependent crosslinking of IgE:FcεRI, but also by a

121   variety of other stimuli, such as cytokines, neuropeptides and danger signals [25, 26]. Thus, MCs can -

122   depending on the context - elicit pro-inflammatory, anti-inflammatory as well as immunoregulatory

123   responses by releasing distinct patterns of mediators and cytokines [24], [25].

124   In addition to their protective functions and their role in allergic diseases, both γδ T cells as well as

125   MCs, are implicated in autoimmunity and malignancy, although the nature and extent of their

126   involvement in the etiopathology is debated [27, 28].

127   In the present study, we investigated the role of γδ T cells and MCs in shaping the adaptive immune

128   response following EPI using mannan-conjugated grass pollen allergen Phl p 5 after laser

129   microporation of skin.

130

131   2. **Material and Methods**

132   **Generation of allergen-mannan neoglycoconjugates**

133   Allergen-mannan neoglycoconjugates were generated by mild oxidation of the mannan moiety and

134   subsequent reductive amination with recombinant Phl p 5 (rPhlp 5) as previously described [17],

135   using a ratio of 5:1 (w/w) between carbohydrate and allergen. For more details, see supplementary

136   methods.

137

138   **Purification and characterization of Phl p 5-mannan neoglycoconjugates**

139   Phl p 5 mannan neoglycoconjugates (P5-MN) were subjected to size exclusion chromatography using

140   a 16/60 Sephacryl S-300 HR column (GE Healthcare) (Suppl. Fig. 1). Coupling efficiency of individual

141   glycoconjugate fractions was monitored by a 12.5% reducing SDS-PAGE using colloidal Coomassie

142   staining for protein visualization after which high molecular weight fractions were pooled (Suppl. Fig.

143   2). The hydrodynamic radius of this glycoconjugate pool was determined using dynamic light

144   scattering (DLS). Measurements were performed at a protein concentration of 1 mg/mL in PBS at

145   40% laser power for 20x5 seconds on a Viscotec DLS 802 dynamic light scattering instrument (Suppl.

146   Fig. 3).

147

148   **Animal experiments**

149   *Mice*

150   Tcrdt^{m1Mom} targeted mutant strain mice deficient in receptor expression in all adult lymphoid and

151   epithelial organs were purchased from the Jackson Laboratory. Mice were backcrossed for 14

152   generations to a BALB/c genetic background. For all experiments, homozygous T-cell receptor delta

153   chain (TCRd) knock-out mice and heterozygous littermates (controls) were used.

154   Transgenic Mas-TRECK (mast cell-specific enhancer-mediated toxin receptor-mediated conditional

155   cell knock out) mice have been previously described by Otsuka et al. and Sawaguchi et al. [29, 30].

156   MCs of this transgenic strain express the human diphtheria toxin receptor (hDTR) under the control

157   of an intronic enhancer (IE) element of the IL-4 gene. MCs in Mas-TRECK mice were conditionally

158   depleted by intraperitoneal injection of 250 ng Diphtheria toxin (DT) for five consecutive days prior

159   to the first immunization. Treatment with DT also leads to the complete temporary depletion of

160   basophils, since they express low levels of hDTR. C57BL/6 mice obtained from Charles River

161   Laboratories, Sulzfeld, Germany were used as controls. Prior to the experiments, all mice were

162   genotyped via PCR. To ensure the absence of MCs and γδ T cells at the time of immunization,

163   epidermal sheets and histological cryosections were prepared (Suppl. Fig. 4 and 5).

164

165   TCRd, Mas-TRECK and C57BL/6 mice were kept in the animal facility of the University of Salzburg and

166   were maintained according to the local animal care guidelines. All animal experiments were

167   approved by the Austrian Ministry of Science (permit number BMWFW-66.012/0014-

168   WF/V/3b/2017).

169

170   *Immunizations*

171   Immunizations were performed by laser microporation of the skin using the P.L.E.A.S.E© laser device

172   (Erbium-Yttrium aluminium garnet laser, Pantec biosolutions) followed by application of P5-MN in

173   solution to the laser-generated micropores as previously described [4]. Laser microporation was

174   performed with the following settings: 500 Hz, pulse duration of 50 μs, 5% pore density, 3 pulses per

175   pore and a total fluence of 8.4 J/cm^2. TCRd $^{-/-}$ and heterozygous control mice were immunized on

176   days 0, 14 and 28 with 1 μg P5-MN. MC-depleted Mas-TRECK and C57BL/6 control mice were

177   immunized on days 0 and 14 with 1 μg Phl p 5 mannan.

178

179   **Antigen-specific antibody measurements**

180   10-13 days after the last immunization, mice were sacrificed, and blood samples were drawn either

181   from the saphenous vein or the retrobulbar sinus. Phl p 5-specific IgG1 antibodies were detected by

182   luminometric ELISA. To assess cell-bound Phl p 5-specific IgE, a basophil activation test was

183   conducted as described [31, 32].

184

185

186   **Lymphocyte cultures, flow cytometry, and cytokine measurements**

187   Isolated lymphocytes from spleens and lymph nodes were cultured in a sterile 96-well tissue culture

188   plate (U-bottom; Greiner) at a density of $4x10^6$ cells/mL in the presence of 10 µg/mL rPhl p 5 in T cell

189   medium (RPMI-1640; 10% FCS, 25mM HEPES, 2mM L-Glu, 100 µg/mL streptomycin, 100 U/mL

190   penicillin) for five days. At day five, culture supernatants were harvested, and cytokine profiles were

191   assessed either by using ProcartaPlex multiplex cytokine panels (Thermo Fisher) or LegendPlex

192   Mouse Th cytokine panel (BioLegend), respectively. On the same day, restimulated cells were

193   analysed by flow cytometry. In brief, cells were washed with DPBS, blocked with an anti-CD16/32

194   containing hybridoma supernatant, and stained for the extracellular markers using anti-CD4 (APC-

195   Cy7-labelled; clone GK1.5, eBioscience; 1:400), anti-CD44 (BV650-labelled; clone IM7, BioLegend;

196   1:100), anti-CD62L (FITC-labelled; clone MEL-14, BioLegend; 1:200) and live/dead staining (fixable

197   viability dye eFluor 506; Thermo Fisher; 1:1000) for 30 min on 4°C. For intracellular staining, cell

198   fixation and permeabilization was performed using the FoxP3 Staining Buffer Sets (Tonbo

199   Biosciences) according to the manufacturer's instructions and 10% naïve mouse serum (in

200   permeabilization buffer) was used for additional blocking. After 30 min of incubation with

201   intracellular markers for FoxP3 (APC-labelled; clone FJK-16s, Invitrogen; 1:100), GATA3 (BV421-

202   labelled; clone 16E10 A23, BioLegend; 1:200), RORγT (PE-labelled; clone B2D, Thermo Fisher; 1:100)

203   and T-bet (PE-Cy7-labelled; clone 4B10, BioLegend; 1:100) at RT in the dark, cells were washed twice

204   with permeabilization buffer and dissolved in FACS buffer (PBS, 1% BSA, 2mM EDTA) for analysis

205   using a CytoFLEX S flow cytometer (Beckman Coulter).

206

207   **Laser-induced cytokine-milieu in murine skin**

208   Skin areas of $TCRd^{-/-}$, $TCRd^{-/+}$, DT-treated and untreated Mas-TRECK mice were excised 6 hours and 24

209   hours after laserporation and then homogenized in 20-fold volume of DPBS containing 1% of a

210   protease inhibitor cocktail (Sigma, P8340) using an Ultra-Turrax homogenizer (Ika, Staufen,

211   Germany). Samples were centrifuged for 10 min at 21.000 x g, and then the supernatants were 0.22

212   µm filtered and finally analysed for cytokine/chemokine release using the ProcartaPlex multiplex

213   assay (Thermo Fisher)

214

215   **Statistical analysis**

216   Unpaired or paired Student's t-tests were used for comparing two groups. Effect of genotype and

217   laser treatment on skin chemokines/cytokines was assessed by two-way ANOVA followed by Tukey's

218   or Holm-Sidak's post hoc test as indicated (Prism 6, GraphPad Software). Data are expressed as

219   means ± SEM. (P-value range is indicated: *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$). Flow cytometry

220   profiles and microscopy images are representative of repeated experiments.

221

222   ## 3. Results

223   *Contribution of Mast cells and γδ T cells to the cytokine/chemokine milieu at the site of laser*

224   *microporation*

225   Knowing that both γδ T cells and MCs are sentinels and damage sensors, we evaluated the

226   contribution of these cell types to the locally-induced cytokine and chemokine milieu after laser-

227   induced skin damage. To this end, the skin of TCRd$^{-/-}$, TCRd$^{-/+}$, DT-treated and untreated Mas-TRECK

228   mice was analysed for cytokine/chemokine secretion either 6 h or 24 h after laser microporation.

229   Non-laser microporated mice were included as controls. As expected, in wild-type mice, laser

230   microporation led to an increase of a broad panel of cytokines and chemokines. Both in TCRd$^{-/-}$, TCRd$^-$

231    $^{/+}$ and Mas-TRECK mice, cytokines GROα, MCP-1, TSLP, IL-6, TNF-α, and MCP-5 peaked 6 h after laser

232   microporation (Fig. 1, A and B), whereas the highest levels of MIP-1α, MIP-2, MIP-3α, RANTES, and

233   IP-10 were observed at the 24 h time point. Contrary, MIP-1α, MIP-2, MIP-3α, RANTES, and IP-10

234   peaked earlier (6 h) in Mas-TRECK mice (Fig. 1B). Tables 1 and 2 summarize the significant changes in

235   cytokine/chemokine concentrations in the skin following laser microporation both in TCRd$^{-/-}$, TCRd$^-$

236    $^{/+}$and Mas-TRECK mice. γδ T cells did not alter the locally-induced cytokine/chemokine milieu after

237   laser microporation, with the exception of RANTES, which was significantly higher in TCR$^{-/-}$ mice. Also,

238   steady-state IL-6 (NS) and MCP-5 (P<0.05) levels were decreased in absence of γδ T cells (Fig. 4A). In

239   accordance with that, we found that IL-6 and MCP-5 peak levels after laser microporation were

240   elevated in the presence of γδ T cells, confirming a significant contribution of γδ T cells to the

241   production/regulation of these chemokines (also see Table 1).

242   In Mas-TRECK mice, we observed a decrease in the secretion of cytokines such as GROα (P<0.05),

243   MIP-1α (NS), MIP-2α (NS), and TSLP (P<0.05) dependent on the presence of MCs after laser

244   microporation (Fig. 1B). In addition, we found a DT-mediated effect on the release of all assessed

245   cytokines/chemokines with the exception of IL-6 and RANTES (significant only for IP-10, see Table 2).

A.

246
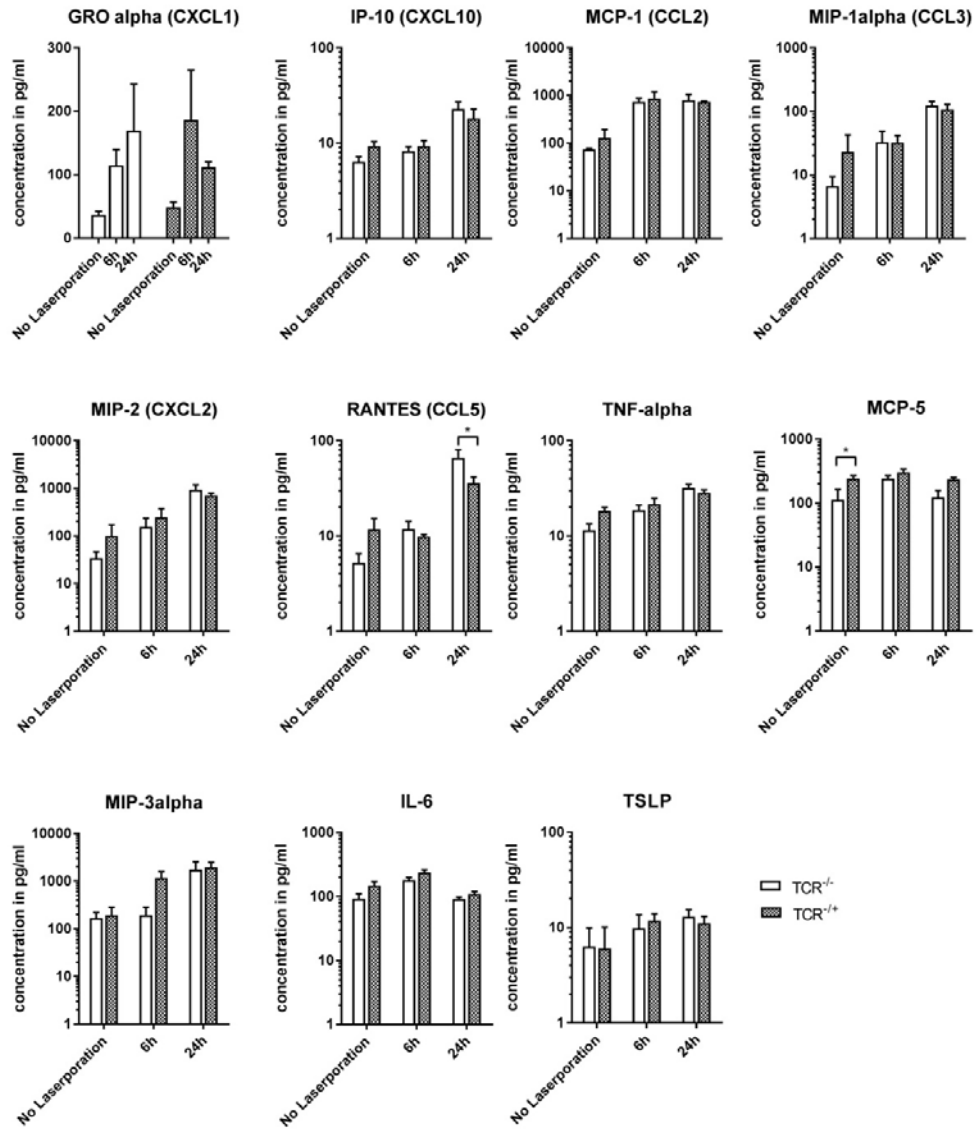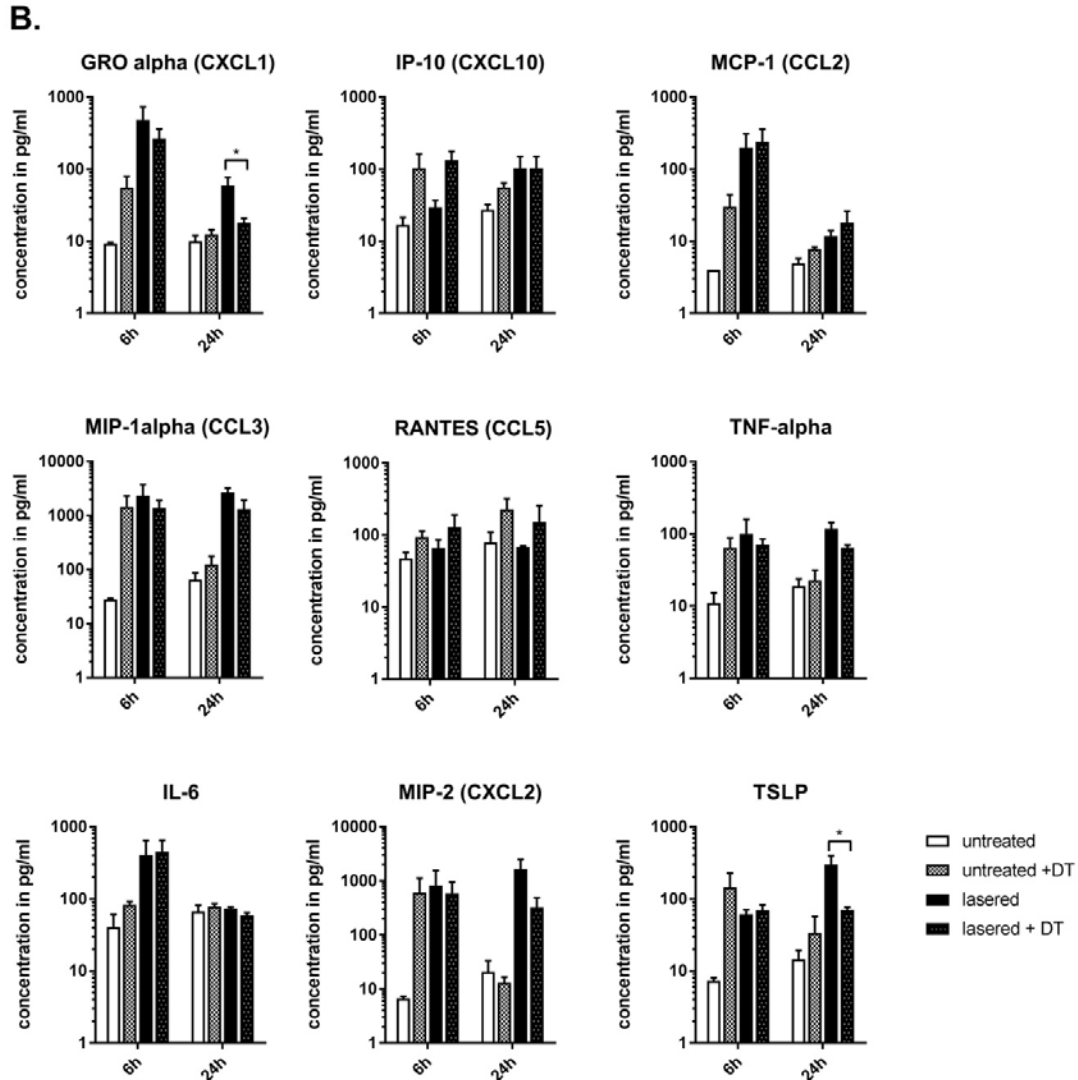
Figure 1: Cytokine/chemokine milieu at the site of laser microporation. Cytokine/chemokine concentrations measured in supernatants of homogenized skin from TCRd (A) or MasTRECK (B) mice 6 h and 24 h after laser microporation using cytokine multiplex assay. Non-laser microporated mice (untreated) were used as controls. Data (n=4) is depicted as cytokine/chemokine concentration in pg/mL. Statistical significance between TCRd$^{-/-}$ and TCRd$^{+/-}$ mice or MC depleted (+DT) vs non-depleted Mas-TRECK mice was assessed by two-way ANOVA followed by Holm-Sidak's multiple comparison post hoc test. * P<0.05.

257 **Table 1:** Laser-related effects on the cytokine/chemokine milieu 6 h or 24 h in TCRd mice after laser
258 microporation. Data was analysed by two-way ANOVA (time 0h, 6h, 24h vs. genotype) and Tukey's
259 multiple comparisons test (0h vs. 6h and 0h vs. 24h). * P<0.05, ** P<0.01, *** P<0.001, ****
260 P<0.0001, NS = not significant.

Cytokine/Chemokine	6h laser effect	24h laser effect	genotype effect
GROα	NS	NS	NS
IL-6	***	NS	*
IP-10 (CXCL10)	NS	**	NS
MCP-1 (CCL2)	NS	**	NS
MIP-1α (CCL3)	NS	****	NS
MIP-2 (CXCL2)	NS	****	NS
RANTES (CCL5)	NS	****	NS
MIP-3α	NS	**	NS
TNF-α	NS	***	NS
TSLP	NS	NS	NS
MCP-5	*	NS	*

261
262 **Table 2:** Laser- and DT-related effects on different skin cytokines/chemokines 6 h or 24 h after laser
263 microporation in MasTRECK mice were analysed by two-way ANOVA (DT treatment vs. laser
264 treatment). * P<0.05, ** P<0.01, NS = not significant.

| Cytokine/Chemokine	6h laser effect	6h DT effect	24h laser effect	24h DT effect
GROα	*	NS	*	NS
IL-6	*	NS	NS	NS
IP-10 (CXCL10)	NS	*	NS	NS
MCP-1 (CCL2)	*	NS	NS	NS
MIP-1α (CCL3)	NS	NS	**	NS
MIP-2 (CXCL2)	NS	NS	*	NS
RANTES (CCL5)	NS	NS	NS	NS
TNF-α	NS	NS	**	NS
TSLP	NS	NS	*	NS

265

266 *Mast cells and γδ T cells play no essential role in induction of humoral Th2 responses after EPI*

267 To investigate the role of γδ T cells and MCs in the systemic immune response after EPI with P5-MN,

268 specific IgG1 and cell-bound IgE levels were measured by ELISA and BAT, respectively. Elevated IgG1

269 serum levels and cell-bound Phl p 5-specific IgE were found in all groups after EPI, suggesting an

270 ongoing humoral immune response. In general, TCRd mice produced higher levels of IgG1 and IgE

271 compared to Mas-TRECK mice (C57BL/6) due to their BALB/c genetic background. Unlike TCRd$^{-/-}$,

272 TCRd$^{-/+}$ mice showed no reduction of antigen-specific IgG1 production after EPI with P5-MN (Fig. 2A).

273 However, we found a significant decrease in serum IgG1 levels in MC-depleted Mas-TRECK mice

274 compared to C57BL/6 WT mice, thereby indicating at least a partial role of MCs in the TH2-

275   dependent switch to IgG1. Despite the differences in IgG1 levels in MC-depleted mice, in our

276   experimental setting, we observed no significant differences in cell-bound antigen-specific IgE.

277   Furthermore, cell-bound antigen-specific IgE levels were comparable between TCRd$^{-/-}$ and TCRd$^{-/+}$

278   mice (Fig 2B). Thus, both γδ T cells as well as MCs play no major role in the induction of humoral TH2

279   responses after two (MCs) or three (γδ T cells) epicutaneous immunizations with P5-MN.

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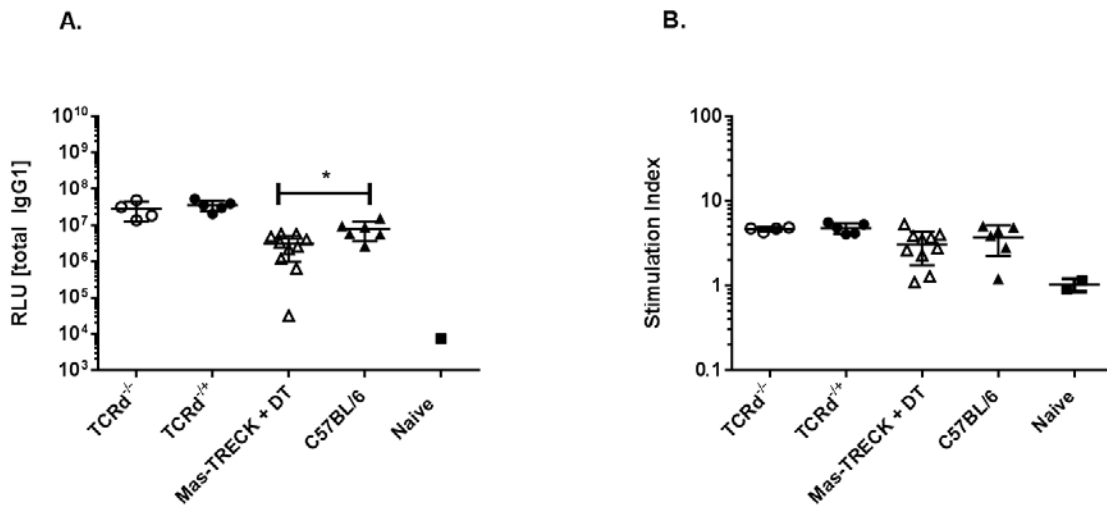
Figure 2. Antigen-specific IgG1 and IgE antibody titers after laser-mediated EPI with P5-MN. A) A luminometric ELISA was performed with 1:1000 diluted serum samples and Phl p 5-specific IgG1 levels were measured in terms of RLU (relative light units) **B)** The basophil activation due to IgE crosslinking after restimulation with 10 ng/mL Phl p 5 was measured in terms of CD200R upregulation via flow cytometry. Mean fluorescence intensity (MFI) of CD200R was normalized to untreated samples. TCRd$^{-/-}$ (n=4), TCRd$^{-/+}$ (n=6), DT-treated Mas-TRECK (n=10), C57BL/6 (n=6) mice and naïve (n=1-2) mice. *P<0.05

290

291   *TH2 cytokine production following EPI is elevated in the absence of mast cells*

292   Having found that both γδ T cells and MCs had little effect on humoral TH2 responses, we were

293   prompted to investigate whether these cells influence TH2 cytokine release.   To address this

294   question, splenocytes from immunized mice and naïve controls were restimulated with Phl p 5 *in*

295   *vitro* for five days and cytokine secretion was measured by ProcartpaPlex/LegendPlex multiplex

296   analysis. Whereas splenocytes from naïve mice displayed only low baseline secretion of cytokines

297   upon Phl p 5 exposure (<10pg/mL for TNF-α, GROα, IL-5, and IL-13; all others <1pg/mL), splenocytes

298   from immunized mice produced significant amounts of pro-inflammatory, TH1, TH2, and TH17

299   cytokines. As expected, splenocytes from MasTRECK mice secreted higher levels of the TH1 cytokine

300   IFN-γ and lower levels of TH2 cytokines IL-4, IL-5, and IL-13 compared to TCRd mice due to their

301   genetic background. Again, we observed no significant changes in the secretion of cytokines between

302   TCRd$^{-/-}$ and TCRd$^{-/+}$ mice in our experiments (Fig. 3A). We also found no major differences between

303   MC-depleted mice and C57BL/6 control mice except for slightly higher concentrations of IL-4 and IL-

304   10 (Fig. 3B). Additionally, we detected increased levels of the TH2 cytokines IL-4, IL-5 and IL-10 in *ex*

305   *vivo* restimulated cells from draining lymph nodes from MC-depleted Mas-TRECK mice (Suppl. Fig. 6).

306   Taken together, these findings suggest that in our experimental setting, the induction of TH2

307   cytokines was elevated in the absence of MCs, which is in contrast to the observed reduction of IgG1.

308   The secretion of pro-inflammatory, TH1 and TH17 cytokines, however, was found to be independent

309   from the presence of MCs. γδ T cells had no effect on the TH cell profile induced by EPI.
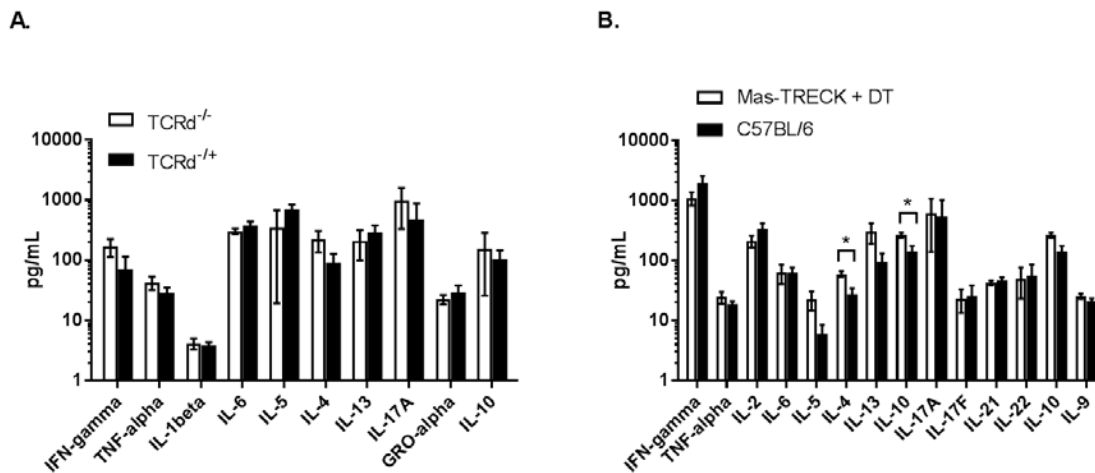
310
311   **Figure 3: Cytokine profiles of cultured splenocytes from immunized mice.** Cytokine production in
312   cell culture supernatants of restimulated splenocytes was measured using ProcartpaPlex (A) or
313   LegendPlex (B) multiplex analysis. **A.)** TCRd$^{-/-}$ (n=4), TCRd$^{-/+}$ (n=5), and **B.)** DT-treated Mas-TRECK
314   (n=10) and C57BL/6 (n=6) mice. *P<0.05
315

316   *Presence of mast cells contributes to T cell activation and maturation but not to T helper polarization*

317   Based on the differences we detected in cytokine secretion profiles of restimulated splenocytes and

318   SDLN cells at least in MC-depleted mice, we asked whether γδ T cells or MCs influence T cell

319   activation or polarization.

320   Analysis of intra- and extracellular staining of Phl p 5-restimulated SDLN cells revealed no differences

321   in the percentages of naïve, central memory, and effector CD4$^+$ T cells or the amount of transcription

322   factor expression between TCRd$^{-/-}$ and TCRd$^{-/+}$ groups (Fig. 4). While the percentage of naïve CD4$^+$ T

323   cells was significantly higher in MC-depleted mice, the amount of effector T cells was lower, thereby

324   implying a role of MCs in T cell activation and maturation. In particular, we found that expression of

325   key TH transcription factors T-bet, GATA3, RORγt and FoxP3 was independent of MC depletion. Thus,

326   we could not confirm an influence of MCs on TH2 polarization on the transcription factor level.
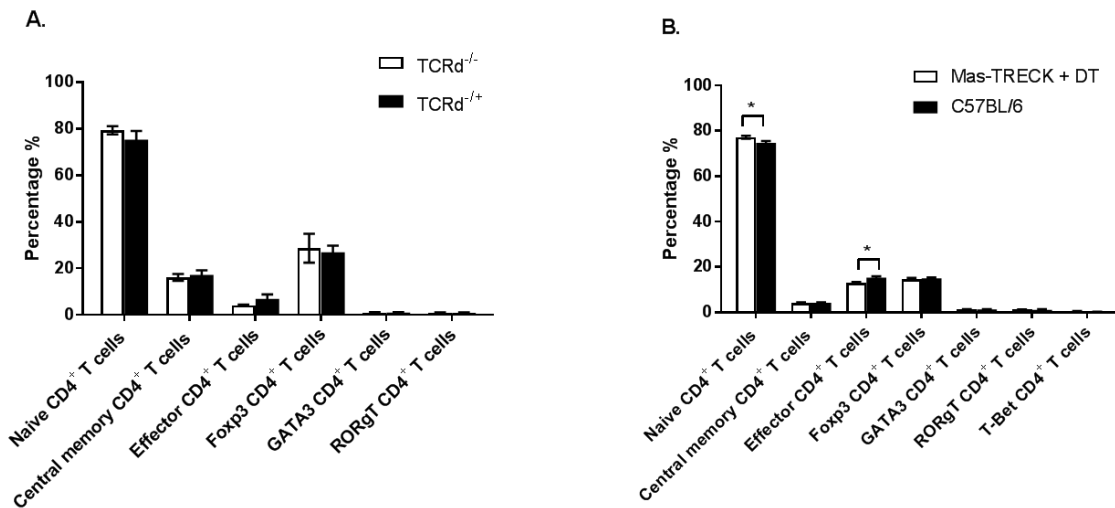
327
328   **Figure 4: T helper polarization in Phl p 5-restimulated SDLN cells**. Extracellular markers CD4, CD62L
329   and CD44 were used to determine the distribution of naïve, central memory, and effector CD4$^+$ T
330   cells. Viable and singlet CD4$^+$ T cell subset distribution and the percentage of FoxP3, GATA3, RORγT
331   and T-bet-producing CD4$^+$ T cells was determined using flow cytometry. **A.)** TCRd$^{-/-}$ (n=4), TCRd$^{-/+}$
332   (n=5), **B.)** DT-treated Mas-TRECK (n=10) and C57BL/6 (n=6). *P<0.05

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338 **4. Discussion**

339 Laser microporation represents a novel method for skin-based vaccination exploiting the richness of

340 superficial skin layers in antigen presenting cells. Furthermore, the microporation process itself has

341 been shown to locally create an inflammatory milieu, thereby providing an adjuvant effect, which

342 potentially obviates the need for adjuvants in vaccine formulations. By targeting receptors on

343 dendritic cells with antigen coupled to glycans such as mannan, we have also demonstrated that a

344 synergistic effect between microporation and directly addressing antigen presenting cells can be

345 achieved, which could not be observed following intradermal injection [17]. Whereas the role of

346 various skin-resident dendritic cell types and Langerhans cells in eliciting immune responses

347 following laser-facilitated skin immunization has been studied in detail [33-35], it remains to be

348 elucidated whether innate immune cells contribute to these reactions. In the current study we

349 therefore investigated the impact of γδ T cells and MCs for production of specific antibodies as well

350 as local and systemic chemokine and cytokine secretion following laser-mediated EPI. As antigen we

351 chose the grass-pollen allergen Phl p 5 coupled to mannan, which is a promising candidate for

352 allergen-specific immunotherapy via the skin as it combines high immunogenicity with low

353 allergenicity [17].

354 Skin-resident dendritic epidermal T cells (DETCs) in mice as well as their human counterparts

355 exclusively express γδ T cell receptors and are able to modulate immune responses initiated in the

356 epithelium. Both have been implicated in maintenance of the immunologic integrity of the skin

357 acting as sensors of cellular dysregulation by recognizing microbial moieties or ligands expressed by

358 tumor cells [23, 36]. As it has been previously shown that DETCs are essential for strong TH2-

359 associated atopic responses upon antigen encounter in context with cutaneous epithelial stress, it

360 was obvious to investigate the role of these cells in immune reactions following antigen delivery via

361 barrier disrupted skin.

362 Our current study shows that the role of γδ T cells in shaping the local cytokine/chemokine milieu

363 following laser microporation is marginal and they consequently have no effect on eliciting adaptive

364 immune responses after laser-mediated EPI.

365   Upon activation, DETCs have been reported to be potent producers of pro-inflammatory cytokines

366   such as IL-2, IL-3, GM-CSF, IFN-γ and TNF-α and they can also produce TH1 effector cytokines (IFN-γ,

367   TNF-α), TH2 effector cytokines (IL-4, IL-5, IL-10, IL-13) and TH17 effector cytokines (IL-17) [37, 38].

368   Additionally, activated γδ T cells are known to secrete the chemokines lymphotactin (XCL1) and

369   RANTES (CCL5) [22] and also produce the macrophage-homing chemokines MIP-1α (CCL3) and MIP-

370   1β (CCL4) [39]. However, we found that the cytokine and chemokine expression in the skin after laser

371   microporation is largely independent of γδ T cell presence (Fig. 1A and Table 1). A surprising finding

372   was that the production of both IL-6 and MCP-5 (CCL12) was reduced in untreated mice lacking γδ T

373   cells, thereby suggesting them to play a role in steady-state production of these chemokines (Fig.

374   1A).

375

376   In our experimental setting, the induction of antigen-specific IgG1 and IgE (Fig 1.) and the secretion

377   of TH2 cytokines such as IL-4, IL-5, IL-10 and IL-13 of *in vitro* restimulated splenocytes (Fig. 3A) do

378   not differ between TCRd$^{-/-}$ and TCRd$^{-/+}$ mice and therefore do not depend on the presence of γδ T

379   cells.

380   In contrast, Strid et al. have demonstrated a crucial role for γδ T cells in producing antigen-specific

381   IgG1 and IgE after allergen application to stressed epithelia [23]. However, in this study a different

382   method for barrier disruption, i.e. tape stripping, was used. By repeatedly pressing an adhesive film

383   to the surface of the skin and then abruptly removing it, only the uppermost skin layers, the stratum

384   corneum, gets abrased. Unlike laser microporation, this technique has to be rated as much less

385   invasive. Also the tape-stripped skin area is inevitably larger compared to the constrained area of 10

386   mm^2 treated with the laser, on which the (epi)dermal tissue between the micropores is left intact

387   [16]. In the above mentioned work, communication between epithelial cells and DETCs via the

388   activating receptor NKG2D was crucial for induction of TH2-biased responses. One could speculate

389   that repeated tape-stripping induces a more pronounced upregulation of the NKG2D ligand Rae-1 on

390   keratinocytes than laser microporation, thereby leading to enhanced activation of DETCs. Finally,

391   besides the diverging technique used for barrier disruption, Strid et al. applied not only a different

392   allergen, i.e. ovalbumin, but also a higher dosage (100 μg vs. 1 μg) compared to our approach, a fact

393   that could also contribute to the discrepant outcomes.

394   Taken together, our data indicate that systemic immune responses following laser-mediated EPI are

395   not significantly modulated or regulated by γδ T cells, however; we propose that γδ T cells are rather

396   responsible for shaping the local inflammatory milieu under steady state conditions.

397

398   MCs act as potent producers of various pro-inflammatory and TH2 cytokines [40, 41] and have been

399   attributed to play a role in DC stimulation and migration. Furthermore, MCs can both directly or

400   indirectly regulate T cell recruitment, activation, proliferation, and cytokine secretion (reviewed in

401   [42]). Therefore, we hypothesized that MC and basophil depletion would impair TH2 polarization and

402   consequently promote TH1 responses.

403   However, the cytokine and chemokine milieu in the skin after laser microporation was largely

404   unaffected by the depletion of MCs with the exception of TSLP and GROα, which were significantly

405   downregulated in MC-depleted mice. Similarly, a decreased induction of MIP-1α, and MIP-2α was

406   detected in the absence of MCs (not significant). Interestingly, we observed that DT treatment by

407   itself caused the induction of most skin chemokines. We thus reasoned that the DT-induced

408   depletion of MCs leads to the release of immune mediators and alarmins from necrotic MCs and

409   causes the activation of other skin-resident innate cells.

410   Nevertheless, depletion of MCs had only slight effects on the adaptive T cell response after EPI.

411   Consistent with our stated hypothesis, MC-depleted mice show significantly decreased Phl p 5-

412   specific serum IgG1 titers compared to C57BL/6 control mice after EPI (Fig. 2A). Thus, it should be

413   considered that MC play a potential role in the TH2-dependent class switch to IgG1. In contrast to

414   those findings, antigen-specific cell-bound IgE levels were not affected by the presence of MCs (Fig.

415   2B). However, we found no difference in the secretion of pro-inflammatory cytokines by antigen-

416   restimulated lymphocytes between MC-depleted and WT C57BL/6 control mice (Fig. 3B).

417   Unexpectedly, we observed an increased production of IL-4 and IL-10 by antigen-restimulated

418   splenocytes in MC-depleted mice contrasting our finding of reduced serum IgG1 (Fig. 2A). As

419 basophils, which are known to secrete large amounts of IL-4 after IL-33-stimulation, are depleted

420 alongside MCs in our model, this is even more surprising. Thus, our results are contrary to data

421 proposing either a role of MCs in promoting TH2 immunity in response to skin barrier disruption [43]

422 or in promoting TH1/TH17 polarization [44]. Since cytokine expression was measured 12 days after

423 the second immunization has been performed, we speculate that MCs and basophils only influence

424 the early cytokine expression, but are rather dispensable in shaping the adaptive immune response

425 after two or more epicutaneous immunizations using laser microporation.

426 FACS analysis revealed that MC-depleted mice show a significantly higher percentage of naïve T cells

427 among isolated lymphocytes, compared to WT control mice (Fig. 4) and a concomitant decrease of

428 effector T cells, confirming a role of MCs in T cell activation/maturation. This is consistent with data

429 indicating a role for MCs in priming of DCs via ICAM/LFA-1 that can in turn enhance T cell priming [29,

430 45]. In line with our cytokine data, we observed no difference in transcription factor expression

431 between MC-depleted mice and WT mice, thereby suggesting that MCs are dispensable for T helper

432 cell polarization via transcription factor expression. Collectively, we propose that MCs are mostly

433 dispensable for humoral TH2-immune responses in laser-mediated EPI, but may play a role in T cell

434 activation/maturation. Finally, we concluded that MCs contribute to the laser-induced cytokine

435 profile by either secretion or regulation of TSLP and GROα.

436

437   6. **Conclusion**

438 In summary, we did not observe a major role of γδ T cells or MCs in shaping the immune response

439 after laser-mediated EPI with Phl p 5 mannan. However, they can contribute to the steady-state or

440 damage-induced cytokine milieu in the skin, respectively.

441

442   7. **Limitations of the study**

443 C57BL/6 mice have the same genetic background as Mas-TRECK mice and were furthermore born

444 and held in the same animal facility. While they should be a suitable control group in respect of

445 certain factors like their specific microbiome, they did not undergo DT treatment and are therefore

446   not an optimal control. Due to their low expression of the human DTR, DT treatment also results in

447   transient basophil depletion. Furthermore, we have shown that DT-treatment can alter the

448   chemokine milieu in the skin. Thus, immunological outcomes in the subsequent experiments cannot

449   be considered solely a MC-dependent effect, but rather have to be considered MC- or basophil-

450   dependent or DT-induced. This is of special interest regarding TH2 cytokine expression, since

451   basophils are known to be producers of IL-4 and hence able to promote TH2 polarization. To

452   overcome these limitations and exclusively elucidate the role of MCs in EPI in further studies novel

453   constitutive or inducible MC-deficient models like Mcpt5-Cre, R-DTA, Mcpt5-Cre or iDTR might be

454   employed.

455

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459

460   **9. Conflict of interest**

461   The authors have no conflict of interest.

462

463   **10. References**

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