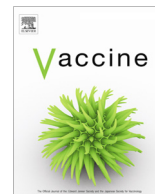




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Mast cells and $\gamma\delta$ T cells are largely dispensable for adaptive immune responses after laser-mediated epicutaneous immunization

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ABSTRACT

Background: The skin resembles an attractive target for vaccination due to its accessibility and abundance of resident immune cells. Cells like $\gamma\delta$ T cells and mast cells (MCs) are part of the first line of defence against exogenous threats. Despite being important mediators for eliciting TH2 immune responses after epithelial stress, $\gamma\delta$ T cell and MC functions still remain to be completely understood. Here, we aimed to characterize their roles in shaping adaptive immune responses after laser-mediated epicutaneous immunization (EPI).

Methods: $\gamma\delta$ T cell knock out, MC-depleted, and wildtype control mice were immunized with mannan-conjugated grass pollen allergen Phl p 5 (P5-MN) by laser-mediated EPI. After 2–3 immunizations, cytokine expression, T helper polarization, and antigen-specific IgG1/IgE levels were analysed. Furthermore, the local cytokine/chemokine milieu after laser microporation was determined.

Results: The majority of inflammatory chemokines and cytokines induced by laser treatment were not affected by the presence of $\gamma\delta$ T cells or MCs. However, RANTES was elevated in $\gamma\delta$ T cell knock out mice and GRO α , TSLP, and IL-33 were significantly decreased after MC depletion. The absence of $\gamma\delta$ T cells or depletion of MCs had no substantial effect on adaptive immune responses after laser-mediated EPI, except for slightly reduced IgG1 and effector T cell levels in MC-depleted mice.

Conclusions: $\gamma\delta$ T cells did not play a pivotal role in shaping the humoral and cellular adaptive immune response after laser-mediated EPI. MC depletion decreased the numbers of effector T cells, indicating a potential role of MCs in the activation and maturation of T cells after EPI.

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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]. This generates 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

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(MC), and $\gamma\delta$ 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 compliance and reduces the risk of needle-stick injuries, thereby also offering advantages for health-care providers [12].

In the context of epicutaneous immunization (EPI), antigens are delivered to APCs mostly via passive uptake. Due to the low efficiency of passive vaccine uptake, novel antigen formulations directly target receptors on skin-resident DCs to promote antigen uptake. One approach is to actively target DCs by engagement of their expressed C-type lectin receptors (CLRs) [13]. Not only does targeting CLRs increase antigen uptake and enhance immunogenicity, but also allows shaping of the subsequent immune responses [14]. CLRs comprise a large superfamily of receptors, which recognize diverse carbohydrate moieties in a calcium-dependent or independent manner through one or more conserved carbohydrate recognition domains (CRDs) or C-type lectin domains (CTLDs). CLRs have evolved alongside pathogens and are often specific for distinct and conserved carbohydrate motifs derived from viruses, bacteria, parasites or fungi [15]. A variety of immune cells express CLRs, such as dermal DCs (dDCs), Langerhans cells (LCs), macrophages, neutrophils, and B lymphocytes. As pattern recognition receptors (PRRs), CLRs play various roles in eliciting immune responses depending on the detected pathogen-associated molecular pattern (PAMP). The potential of the carbohydrate mannan as an adjuvant capable of enhancing and shaping the overall immune response has been demonstrated to rely on its capacity to engage CLRs [16,17]. We have previously reported that laser-mediated EPI with allergen-mannan neoglycoconjugates leads to more efficient antigen uptake by dDCs and LCs. In addition, we found that the initiated immune response was skewed towards TH1/TH17, which is in accordance with the fungal origin of mannan [17].

Nevertheless, the immune response mounted upon an antigen encounter is also highly dependent on the microenvironment within the target tissue. Barrier disruption has been demonstrated to be essential for enhancing skin permeation by altering the microarchitecture of the skin and increasing the motility of embedded APCs. Furthermore, disrupting the stratum corneum has been shown to provide a natural inherent adjuvant effect by inducing the secretion of pro-inflammatory cytokines, chemokines and danger-associated molecular patterns (DAMPs) [18,19]. More precisely, damaged and necrotic keratinocytes release cytokines such as IL-25, IL-33 and TSLP ("alarmins"), which activate other skin-resident cells and induce specific immune responses [20].

MCs and $\gamma\delta$ T cells are distributed throughout the whole body in the mucosal, epithelial and connective tissues. Hence, they are strategically positioned at the barrier between host and environment, where exogenous material can enter. They both play important roles in innate immunity and can be rapidly activated by stress signals and pathogens. The various protective functions exerted by $\gamma\delta$ T cells include anti-microbial defence, tumour surveillance, tissue homeostasis, and wound repair [21,22]. The term lymphoid stress-surveillance is commonly used to describe the rapidly induced immune responses initiated after stress-antigen engagement by the $\gamma\delta$ T cell receptor. Additionally, $\gamma\delta$ T cells have been reported to directly sense epithelial dysregulation, control tissue homeostasis by IL-13 production and promote

IgE-dependent responses to protein allergens in stressed tissue [23]. MCs are predominantly known for their essential role as mediators of type I allergy and for their potential to secrete a variety of allergic mediators upon activation [24]. However, MCs cannot only be activated by antigen-dependent crosslinking of IgE: Fc ϵ R1, but also by a variety of other stimuli, such as cytokines, neuropeptides and danger signals [25,26]. Thus, MCs can - depending on the context - elicit pro-inflammatory, anti-inflammatory as well as immunoregulatory responses by releasing distinct patterns of mediators and cytokines [24,25].

In addition to their protective functions and their role in allergic diseases, both $\gamma\delta$ T cells and MCs are implicated in autoimmunity and malignancy, although the nature and extent of their involvement in the etiopathology is debated [27,28].

In the present study, we investigated the role of $\gamma\delta$ T cells and MCs in shaping the adaptive immune response following laser-mediated EPI, using mannan-conjugated grass pollen allergen Phl p 5.

2. Material and methods

2.1. Generation of allergen-mannan neoglycoconjugates

Allergen-mannan neoglycoconjugates were generated by mild oxidation of the mannan moiety and subsequent reductive amination with recombinant Phl p 5 (rPhl p 5) as previously described [17], using a ratio of 5:1 (w/w) between carbohydrate and allergen. For more details, see supplementary methods.

2.2. Purification and characterization of Phl p 5 mannan neoglycoconjugates

Phl p 5 mannan neoglycoconjugates (P5-MN) were subjected to size exclusion chromatography using a 16/60 Sephacryl S-300 HR column (GE Healthcare) (Suppl. Fig. 2). Coupling efficiency of individual glycoconjugate fractions was monitored by a 12.5% reducing SDS-PAGE using colloidal Coomassie staining for protein visualization after which high molecular weight fractions were pooled (Suppl. Fig. 3). The hydrodynamic radius of this glycoconjugate pool was determined using dynamic light scattering (DLS). Measurements were performed at a protein concentration of 1 mg/mL in PBS at 40% laser power for 20x5 seconds on a Viscotec DLS 802 dynamic light scattering instrument (Suppl. Fig. 4).

2.3. Animal experiments

2.3.1. Mice

Tcrdt^{m1Mom}-targeted mutant strain mice deficient in receptor expression in all adult lymphoid and epithelial organs were purchased from the Jackson Laboratory. Mice were backcrossed for 14 generations to a BALB/c genetic background. For all experiments, homozygous T-cell receptor delta chain (TCRd) knock-out mice and heterozygous littermates (controls) were used.

Transgenic Mas-TRECK (mast cell-specific enhancer-mediated toxin receptor-mediated conditional cell knock out) mice have been previously described by Otsuka et al. and Sawaguchi et al. [29,30]. MCs of this transgenic strain express the human diphtheria toxin receptor (hDTR) under the control of an intronic enhancer (IE) element of the IL-4 gene. MCs in Mas-TRECK mice were conditionally depleted by intraperitoneal injection of 250 ng Diphtheria toxin (DT) for five consecutive days prior to the first immunization. Treatment with DT also leads to the complete temporary depletion of basophils, since they express low levels of hDTR. C57BL/6 mice obtained from Charles River Laboratories, Sulzfeld, Germany were used as controls. Prior to the experiments, all mice were genotyped

via PCR. To ensure the absence of MCs and $\gamma\delta$ T cells at the time of immunization, epidermal sheets and histological cryosections were prepared (Suppl. Fig. 5 and 6).

TCRd, Mas-TRECK and C57BL/6 mice were kept in the animal facility of the University of Salzburg and were maintained according to the local animal care guidelines. All animal experiments were approved by the Austrian Ministry of Science (permit number BMWFW-66.012/0014-WF/V/3b/2017).

2.3.2. Immunizations

Immunizations were performed by laser microporation of the skin using the P.L.E.A.S.E[®] laser device (Erbium-yttrium aluminium garnet laser, Pantec biosolutions) followed by application of P5-MN in solution to the laser-generated micropores as previously described [4]. Laser microporation was performed with the following settings: 500 Hz, pulse duration of 50 μ s, 5% pore density, 3 pulses per pore and a total fluence of 8.4 J/cm². TCRd^{-/-} and heterozygous control mice were immunized on days 0, 14 and 28 with 1 μ g P5-MN. MC-depleted Mas-TRECK and C57BL/6 control mice were immunized on days 0 and 14 with 1 μ g Phl p 5 mannan.

2.4. Antigen-specific antibody measurements

10–13 days after the last immunization, mice were sacrificed and blood samples were drawn either from the saphenous vein or the retrobulbar sinus. Phl p 5-specific IgG1, IgG2a (TCRd mice) or IgG2c (Mas-TRECK mice) antibodies were detected by luminometric ELISA. To assess cell-bound Phl p 5-specific IgE, a basophil activation test (BAT) was conducted as described [31,32].

2.5. Lymphocyte cultures, flow cytometry, and cytokine measurements

Isolated lymphocytes from spleens and lymph nodes were cultured in a sterile 96-well tissue culture plate (U-bottom; Greiner) at a density of 4×10^6 cells/mL in the presence of 10 μ g/mL rPhl p 5 in T cell medium (RPMI-1640; 10% FCS, 25 mM HEPES, 2 mM L-Glu, 100 μ g/mL streptomycin, 100 U/mL penicillin) for five days. At day five, culture supernatants were harvested, and cytokine profiles were assessed either by using a ProcartaPlex multiplex cytokine panel (IFN- γ , TNF- α , IL-1 β , IL-4, IL-5, IL-6, IL-10, IL-13, IL-17A, IL-23, VEGF-A, G-CSF, GRO α , Thermo Fisher) or LegendPlex Mouse Th cytokine panel (IFN- γ , TNF- α , IL-2, IL-4, IL-5, IL-6, IL-9, IL-10, IL-13, IL-17A, IL-17F, IL-21, IL-22, BioLegend), respectively. On the same day, restimulated cells were analysed by flow cytometry. In brief, cells were washed with DPBS, blocked with an anti-CD16/32 containing hybridoma supernatant, and stained for extracellular markers using anti-CD4 (APC-Cy7-labelled; clone GK1.5, eBioscience; 1:400), anti-CD44 (BV650-labelled; clone IM7, BioLegend; 1:100), anti-CD62L (FITC-labelled; clone MEL-14, BioLegend; 1:200) and live/dead staining (fixable viability dye eFluor 506; Thermo Fisher; 1:1000) for 30 min at 4 °C. For intracellular staining, cell fixation and permeabilization was performed using the FoxP3 Staining Buffer Sets (Tonbo Biosciences) according to the manufacturer's instructions and 10% naïve mouse serum (in permeabilization buffer) was used for additional blocking. After 30 min of incubation with intracellular markers for FoxP3 (APC-labelled; clone FJK-16s, Invitrogen; 1:100), GATA3 (BV421-labelled; clone 16E10 A23, BioLegend; 1:200), ROR γ T (PE-labelled; clone B2D, Thermo Fisher; 1:100) and T-bet (PE-Cy7-labelled; clone 4B10, BioLegend; 1:100) at RT in the dark, cells were washed twice with permeabilization buffer and dissolved in FACS buffer (PBS, 1% BSA, 2 mM EDTA) for analysis using a CytoFLEX S flow cytometer (Beckman Coulter).

2.6. Laser-induced cytokine-milieu in murine skin

Skin areas of TCRd^{-/-}, TCRd^{+/-}, DT-treated and untreated Mas-TRECK mice were excised 6 h and 24 h after laserporation and then homogenized in 20-fold volume of DPBS containing 1% of a protease inhibitor cocktail (Sigma, P8340) using an Ultra-Turrax homogenizer (Ika, Staufen, Germany). Samples were centrifuged for 10 min at 21.000g, the supernatants were 0.22 μ m filtered, and finally analysed for cytokine/chemokine release using a Procarta Multiplex assay (GRO α , IP-10, MCP-1, MIP-1 α , RANTES, TNF- α , IL-6, MIP-2, TSLP, MCP-3, Thermo Fisher) and a MILLIPLEX MAP Mouse Chemokine kit (MIP-3 α , MCP-5, Millipore). IL-1 α , IL-1 β , IL-18, IL-33 (all LegendPlex Multiplex assay), and IL-25 (sandwich ELISA, Thermo Fisher) were measured 20 h after laserporation.

2.7. Statistical analysis

Unpaired or paired Student's t-tests were used for comparing two groups. The effect of genotype and laser treatment on skin chemokines/cytokines was assessed by two-way ANOVA followed by Tukey's or Holm-Sidak's post hoc test as indicated (Prism 6, GraphPad Software). Data are expressed as means $\pm$ SEM. (P-value range is indicated: *P < 0.05, **P < 0.01 and ***P < 0.001). Flow cytometry profiles and microscopy images are representative of repeated experiments.

3. Results

3.1. Contribution of mast cells and $\gamma\delta$ T cells to the cytokine/chemokine milieu at the site of laser microporation

Knowing that both $\gamma\delta$ T cells and MCs are sentinels and damage sensors, we evaluated the contribution of these cell types to the locally-induced cytokine and chemokine milieu after laser-induced skin damage. To this end, the skin of TCRd^{-/-}, TCRd^{+/-}, DT-treated and untreated Mas-TRECK mice was analysed for cytokine/chemokine secretion either 6 h or 24 h after laser microporation. Non-laser microporated mice were included as controls. As expected, in wild-type (WT) mice, laser microporation led to an increase of a broad panel of cytokines and chemokines. Both in TCRd^{-/-}, TCRd^{+/-} and Mas-TRECK mice, cytokines GRO α , MCP-1, TSLP, IL-6, TNF- α , and MCP-5 peaked 6 h after laser microporation (Fig. 1, A and B), whereas the highest levels of MIP-1 α , MIP-2, MIP-3 α , RANTES, and IP-10 were observed at the 24 h time point. Contrary, MIP-1 α , MIP-2, MIP-3 α , RANTES, and IP-10 peaked earlier (6 h) in Mas-TRECK mice (Fig. 1B). Tables 1 and 2 summarize the significant changes in cytokine/chemokine concentrations in the skin following laser microporation both in TCRd^{-/-}, TCRd^{+/-} and Mas-TRECK mice. $\gamma\delta$ T cells did not alter the locally-induced cytokine/chemokine milieu after laser microporation, with the exception of RANTES, which was significantly higher in TCR^{-/-} mice. Also, steady-state IL-6 (NS) and MCP-5 (P < 0.05) levels were decreased in absence of $\gamma\delta$ T cells (Fig. 1A). In accordance with that, we found that IL-6 and MCP-5 peak levels after laser microporation were elevated in the presence of $\gamma\delta$ T cells, confirming a significant contribution of $\gamma\delta$ T cells to the production/regulation of these chemokines (also see Table 1).

In Mas-TRECK mice, we observed a decrease in the secretion of cytokines such as GRO α (P < 0.05), MIP-1 α (NS), MIP-2 (NS), TNF- α (NS), and TSLP (P < 0.05) dependent on the presence of MCs after laser microporation (Fig. 1B). In addition, we found a DT-mediated effect on the release of all assessed cytokines/chemokines with the exception of IL-6 and RANTES (significant only for IP-10, see Table 2).

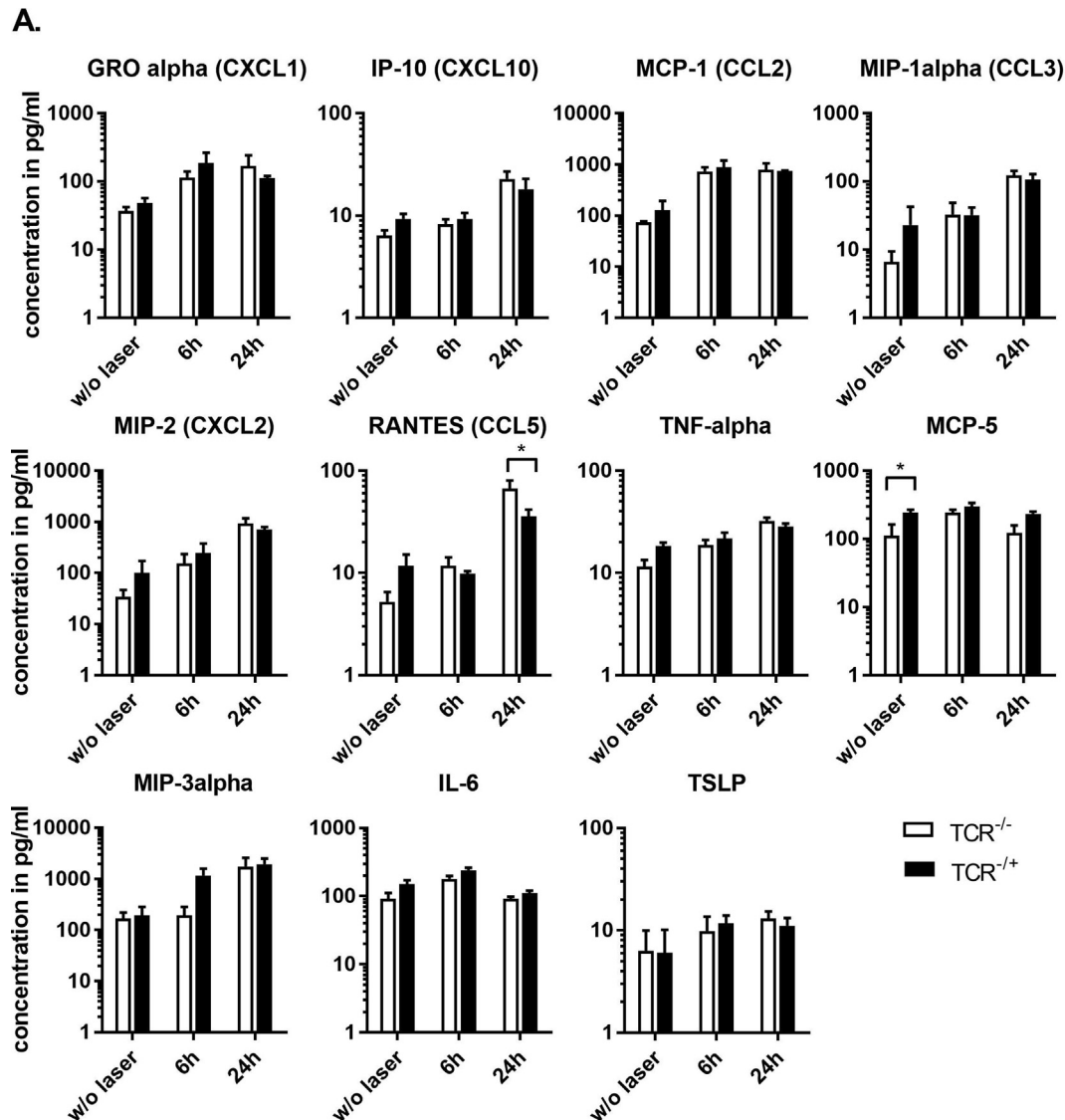


Fig. 1. Cytokine/chemokine milieu at the site of laser microporation. Cytokine/chemokine concentrations measured in supernatants of homogenized skin from TCRd^{-/-} (A) or MasTRECK (B) mice 6 h and 24 h after laser microporation using cytokine multiplex assay. Non-laser microporated mice (untreated) 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.

Skin barrier disruption can lead to production of cytokines of the IL-1 family, such as IL-1 β , and IL-18 in their active form, and of “alarmins” including IL-1 α , IL-25, and IL-33. These cytokines are constitutively expressed in epithelial cells such as keratinocytes and thus can be readily released upon cell injury or death [33]. Due to the disruption of epithelial cells in our homogenized skin samples, we would not expect a difference between samples of laser treated and untreated skin, or a major contribution of MCs or $\gamma\delta$ T cells. However, since MCs have been shown to secrete substantial amounts of IL-33 upon activation via IgE cross-linking within 20 h [34], we also investigated the secretion of IL-1 α , IL-1 β , IL-18, IL-25, and IL-33 20 h after laser microporation in Mas-TRECK and TCRd mice. As shown in suppl. Fig. 7, depletion of MCs indeed had a dramatic effect on the IL-33 levels in the skin, whereas absence of $\gamma\delta$ T cells had no significant impact. Surprisingly, IL-33 was significantly induced by laserporation in both mouse strains, indicating *de novo* synthesis of IL-33 in addition to release of pre-stored IL-33. While IL-1 β was induced by laserporation in TCRd^{-/-} and TCRd^{+/-} mice, it was below the detection limit in Mas-TRECK mice. As expected, high levels of IL-1 α were

found in all skin homogenates, although levels were slightly lower in the laserporated groups. This suggests a release of IL-1 α by the laserporation process but no induction of *de novo* synthesis. IL-25 was found in similar levels in the skin, independent of genotype or laser treatment.

3.2. Mast cells and $\gamma\delta$ T cells play no essential role in induction of humoral TH2 responses after EPI

To investigate the role of $\gamma\delta$ T cells and MCs in the systemic immune response after EPI with P5-MN, specific IgG1 and cell-bound IgE levels were measured by ELISA and BAT, respectively. Sera were diluted 1:1000 for IgG1 and 1:100 for IgG2a/2c measurements to be in the linear range of the ELISA assay. Titration confirmed that the sera were not in a plateau at these dilutions (Suppl. Fig. 8). Elevated IgG1 serum levels and cell-bound Phl p 5-specific IgE were found in all groups after EPI, suggesting an ongoing humoral immune response. In general, TCRd mice produced higher levels of IgG1 and IgE compared to Mas-TRECK mice (C57BL/6) due to their BALB/c genetic background. Unlike

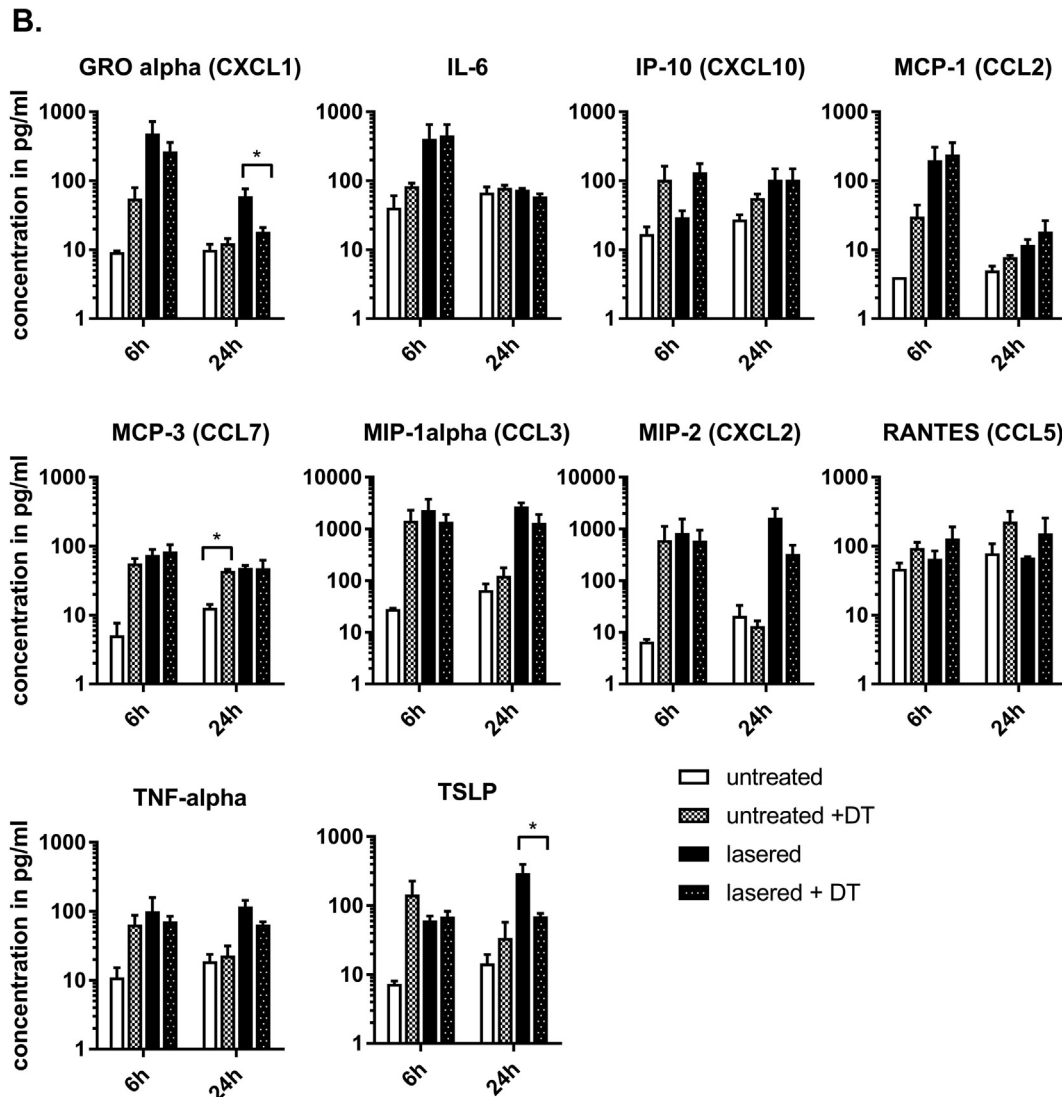


Fig. 1 (continued)

Table 1

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

Cytokine/Chemokine	6 h laser effect	24 h laser effect	genotype effect
GRO α	NS	NS	NS
IL-6	***	NS	*
IP-10 (CXCL10)	NS	**	NS
MCP-1 (CCL2)	**	**	NS
MCP-3 (CCL7)	NS	NS	NS
MCP-5 (CCL12)	*	NS	*
MIP-1 α (CCL3)	NS	****	NS
MIP-2 (CXCL2)	NS	****	NS
MIP-3 α	NS	**	NS
RANTES (CCL5)	NS	****	NS
TNF- α	NS	***	NS
TSLP	NS	NS	NS

TCRd^{-/-} mice, TCRd^{+/-} mice showed no reduction of antigen-specific IgG1 production after EPI with P5-MN (Fig. 2A). However, we found a significant decrease in serum IgG1 levels in MC-depleted Mas-TRECK mice compared to C57BL/6 WT mice, indicating at least a partial role of MCs in the TH2-dependent

switch to IgG1. Despite the differences in IgG1 levels in MC-depleted mice, in our experimental setting, we observed no significant differences in antigen-specific IgG2c as well as cell-bound antigen-specific IgE. Furthermore, antigen-specific IgG2a and cell-bound antigen-specific IgE levels were comparable between TCRd^{-/-} and TCRd^{+/-} mice (Fig. 2B and C). Thus, both $\gamma\delta$ T cells and MCs play no major role in the induction of humoral TH2 responses after two (MCs) or three ($\gamma\delta$ T cells) epicutaneous immunizations with P5-MN.

3.3. TH2 cytokine production following EPI is elevated in the absence of mast cells

Having found that both $\gamma\delta$ T cells and MCs had little effect on humoral TH2 responses, we were prompted to investigate whether these cells influence TH2 cytokine release. To address this question, splenocytes from immunized mice and naïve controls were restimulated with Phl p 5 *in vitro* for five days and cytokine secretion was measured by ProcartaPlex/LegendPlex multiplex analysis. Whereas splenocytes from naïve mice displayed only low baseline secretion of cytokines upon Phl p 5 exposure (<10 pg/mL for TNF- α , GRO α , IL-5, and IL-13; all others < 1 pg/mL), splenocytes from immunized mice produced significant amounts of

Table 2

Laser- and DT-related effects on the skin cytokine/chemokine milieu 6 h or 24 h after laser microporation in MasTRECK mice. Data was analysed by two-way ANOVA POST HOC? (DT treatment vs. laser treatment). * $P < 0.05$, ** $P < 0.01$, NS = not significant, n.d. = not done.

Cytokine/Chemokine	6 h laser effect	6 h DT effect	24 h laser effect	24 h DT effect
GRO α	*	NS	*	NS
IL-6	*	NS	NS	NS
IP-10 (CXCL10)	NS	*	NS	NS
MCP-1 (CCL2)	*	NS	NS	NS
MCP-3 (CCL7)	**	NS	*	NS
MCP-5 (CCL12)	n.d.	n.d.	n.d.	n.d.
MIP-1 α (CCL3)	NS	NS	**	NS
MIP-2 (CXCL2)	NS	NS	*	NS
MIP-3 α	n.d.	n.d.	n.d.	n.d.
RANTES (CCL5)	NS	NS	NS	NS
TNF- α	NS	NS	**	NS
TSLP	NS	NS	*	NS

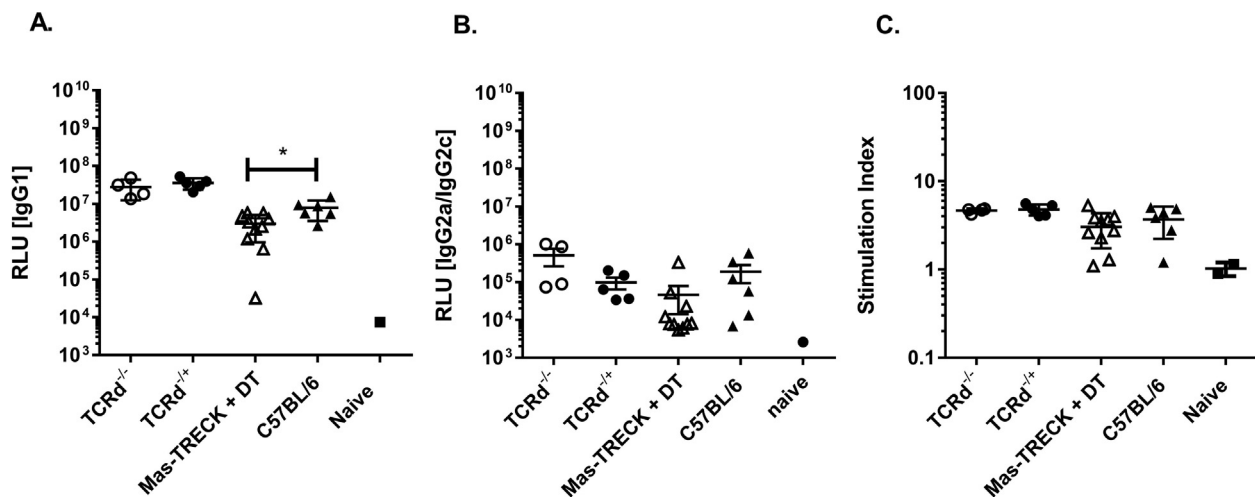


Fig. 2. Antigen-specific IgG1, IgG2a/2c, and IgE antibody titers after laser-mediated EPI with P5-MN. A luminometric ELISA was performed with 1:1000 (IgG1) or 1:100 (IgG2a/2c) diluted serum samples and Phl p 5-specific IgG1 (A.) and IgG2a (TCRd) or IgG2c (Mas-TRECK/C57BL/6) (B.) levels were measured in terms of RLU (relative light units). 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 (C.). 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. * $P < 0.05$.

pro-inflammatory, TH1, TH2, and TH17 cytokines. As expected, splenocytes from Mas-TRECK mice secreted higher levels of the TH1 cytokine IFN- γ and lower levels of TH2 cytokines IL-4, IL-5, and IL-13 compared to TCRd mice due to their genetic background. Again, we observed no significant changes in the secretion of cytokines between TCRd^{-/-} and TCRd^{+/-} mice in our experiments (Fig. 3A). We also found no major differences between MC-depleted mice and C57BL/6 WT mice, except for slightly higher concentrations of IL-4 and IL-10 (Fig. 3B). Additionally, we detected increased levels of the TH2 cytokines IL-4, IL-5 and IL-10 in *ex vivo* restimulated skin draining lymph node (SDLN) cells in MC-depleted Mas-TRECK mice (Suppl. Fig. 9). Other innate cytokines/chemokines that were included in the panel are shown in Suppl. Fig. 10. Taken together, these findings suggest that in our experimental setting, the induction of TH2 cytokines was elevated in the absence of MCs, which is in contrast to the observed reduction of IgG1. The secretion of pro-inflammatory, TH1 and TH17 cytokines, however, was found to be independent from the presence of MCs. $\gamma\delta$ T cells had no effect on the TH cell profile induced by EPI.

3.4. Presence of mast cells contributes to T cell activation and maturation but not to T helper polarization

Based on the differences we detected in cytokine secretion profiles of restimulated splenocytes and SDLN cells in MC-depleted

mice, we asked whether $\gamma\delta$ T cells or MCs influence T cell activation or polarization.

Intra- and extracellular staining of SDLN cells from P5-MN immunized mice revealed no differences in the percentages of naïve, central memory, and effector CD4⁺ T cells between TCRd^{-/-} and TCRd^{+/-} groups, both *ex vivo* (Fig. 4A) as well as after 5d of *in vitro* stimulation with Phl p 5 (Fig. 4B). We furthermore found no differences in the expression of TH transcription factors T-bet, GATA3, ROR γ T and FoxP3 between the groups. While the percentage of naïve CD4⁺ T cells was significantly higher in MC-depleted mice, the amount of effector T cells was lower, implying a role of MCs in T cell activation and maturation. We found that the expression of key TH transcription factors was independent of MC depletion (Fig. 4C). Thus, we could not confirm an influence of MCs on TH2 polarization on the transcription factor level.

3.5. Mast cells and $\gamma\delta$ T cells play no essential role in the adaptive immune response against non-glycosylated antigen

Initially, we wanted to investigate the role of MCs and $\gamma\delta$ T cells in laser-mediated EPI using mannan neoglycoconjugates, as this vaccine type was demonstrated to be especially potent when applied to laser-microporated skin [17]. As we found no impact of MCs and $\gamma\delta$ T cells on the adaptive immune response after EPI using P5-MN, we asked whether we would see similar results using

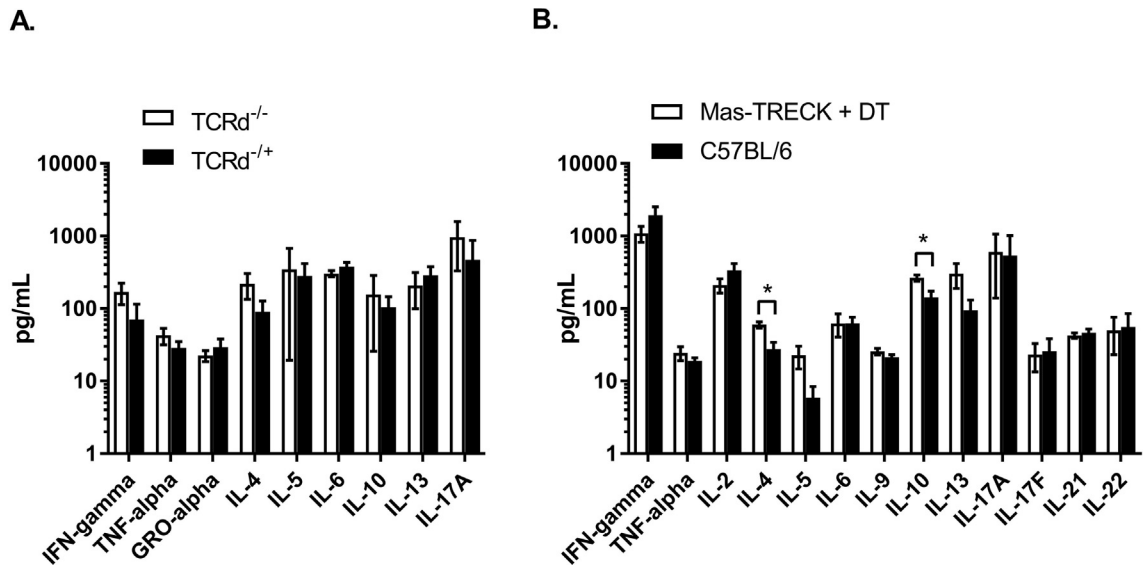


Fig. 3. Cytokine profiles of cultured splenocytes from P5-MN-immunized mice. Cytokine production in cell culture supernatants of restimulated splenocytes was measured using ProcartaPlex (TCRd mice) or LegendPlex (Mas-TRECK mice) multiplex analysis. (A.) TCRd^{-/-} (n = 4), TCRd^{-/+} (n = 5), and (B.) DT-treated Mas-TRECK (n = 10) and C57BL/6 (n = 6) mice. *P < 0.05.

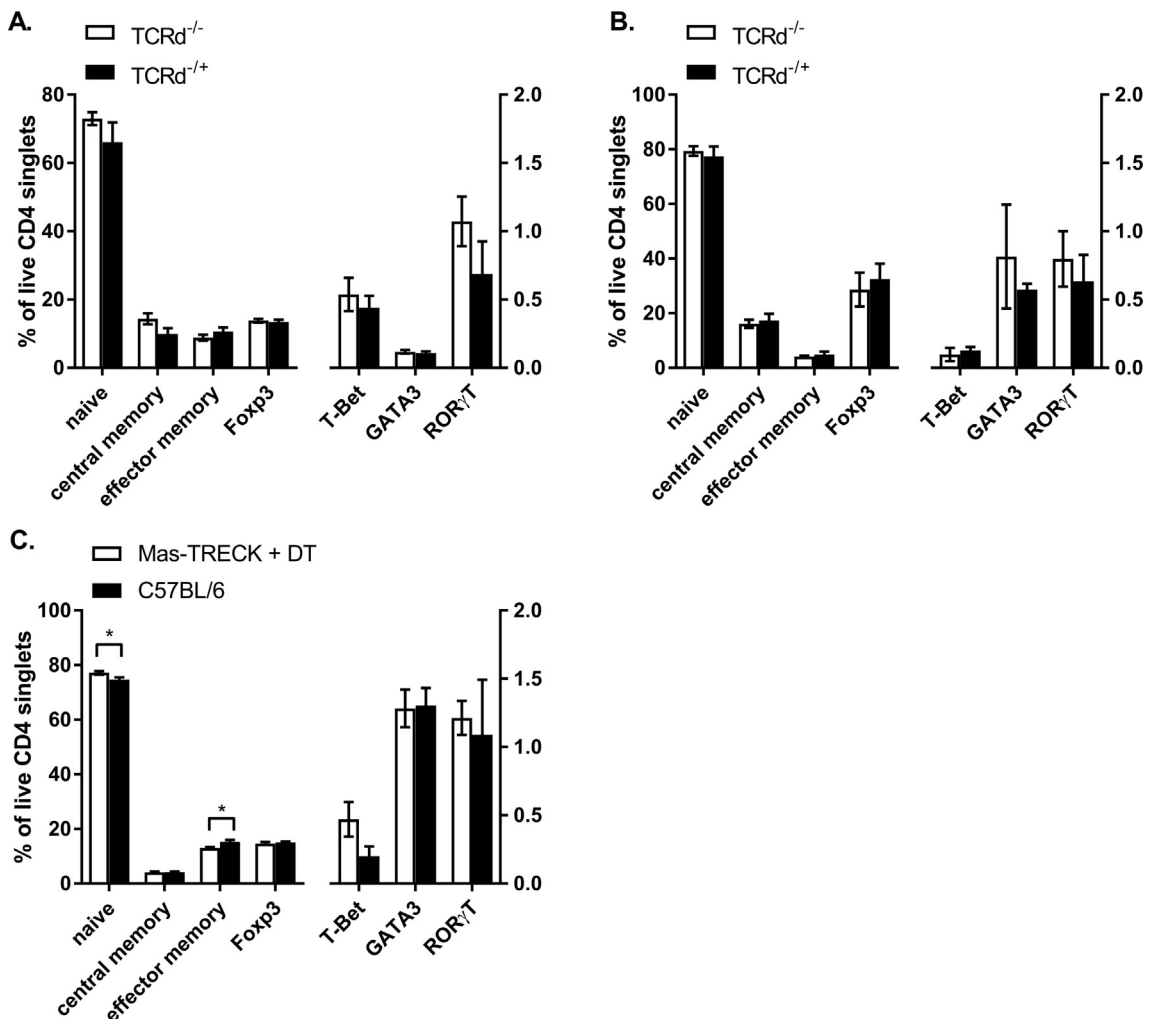


Fig. 4. T helper polarization in SDLN cells of P5-MN immunized mice. Extracellular markers CD4, CD62L and CD44 were used to determine the distribution of naive, central memory, and effector CD4⁺ T cells. Viable and singlet CD4⁺ T cell subset distribution (left Y-axis) and the percentage of FoxP3, GATA3, RORγT and T-bet-producing CD4⁺ T cells (right Y-axis) were determined using flow cytometry. (A.) TCRd^{-/-} (n = 4), TCRd^{-/+} (n = 5) *ex vivo* or (B.) after 5 days *in vitro* stimulation with Phl p 5. (C.) DT-treated Mas-TRECK (n = 10) and C57BL/6 (n = 6) *ex vivo*. *P < 0.05.

a non-glycosylated antigen. Using recombinant Phl p 5 we were able to confirm that also in the absence of glycoconjugation, both cell types had no significant impact on antibody responses (suppl. Fig. 11), cytokine responses (suppl. Fig. 12), and cellular composition of SDLNs (suppl. Fig. 13).

4. Discussion

Laser microporation represents a novel method for skin-based vaccination, exploiting the abundance of APCs in superficial skin layers. The microporation process itself has been shown to create a local inflammatory milieu thereby providing an adjuvant effect. This could potentially obviate the need for additional adjuvants in vaccine formulations. By targeting receptors on DCs with antigen coupled to glycans such as mannan, a more efficient uptake of the antigen can be facilitated. Furthermore, we were able to demonstrate a synergistic effect between microporation and directly addressing APCs, which could not be observed following intradermal injection of the vaccine [17]. Whereas the role of various skin-resident dendritic cell types and Langerhans cells in eliciting immune responses following laser-facilitated skin immunization has been studied in detail [35–37], it remains to be elucidated whether innate immune cells contribute to these reactions. In the current study, we therefore investigated the impact of $\gamma\delta$ T cells and MCs for production of antigen-specific antibodies as well as local and systemic chemokine and cytokine secretion following laser-mediated EPI. As antigen we chose the grass-pollen allergen Phl p 5 coupled to mannan, which is a promising candidate for allergen-specific immunotherapy via the skin as it combines high immunogenicity with low allergenicity [17].

Skin-resident dendritic epidermal T cells (DETCs) in mice as well as their human counterparts exclusively express $\gamma\delta$ T cell receptors and are able to modulate immune responses initiated in the epithelium. Both have been implicated in maintenance of the immunologic integrity of the skin, acting as sensors of cellular dysregulation by recognizing microbial moieties or ligands expressed by tumor cells [23,38]. It has been previously shown that DETCs are essential for strong TH2-associated atopic responses upon antigen encounter in the context of cutaneous epithelial stress. In the current study, we investigated the role of these cells in immune reactions following antigen delivery via barrier disrupted skin.

Our study shows that the role of $\gamma\delta$ T cells in shaping the local cytokine/chemokine milieu following laser microporation is marginal and consequently, they have no effect on eliciting adaptive immune responses after laser-mediated EPI.

Upon activation, DETCs have been reported to be potent producers of pro-inflammatory cytokines (IL-2, IL-3, GM-CSF), TH1 effector cytokines (IFN- γ , TNF- α), TH2 effector cytokines (IL-4, IL-5, IL-10, IL-13) and TH17 effector cytokines (IL-17) [39,40]. Additionally, activated $\gamma\delta$ T cells are known to secrete the chemokines lymphotactin (XCL1), RANTES (CCL5) [22] and the macrophage-homing chemokines MIP-1 α (CCL3) and MIP-1 β (CCL4) [41]. However, we found that the cytokine and chemokine expression in the skin after laser microporation is largely independent of $\gamma\delta$ T cell presence (Fig. 1A, Table 1, and suppl. Fig. 7). Surprisingly, the production of both IL-6 and MCP-5 (CCL12) was reduced in untreated mice lacking $\gamma\delta$ T cells, suggesting that $\gamma\delta$ T cells might play a role in steady-state production of these chemokines (Fig. 1A).

In our experimental setting, the induction of antigen-specific IgG1 and IgE (Fig. 1.) as well as the secretion of TH2 cytokines of *in vitro* restimulated splenocytes (Fig. 3A) did not differ between TCRd^{-/-} and TCRd^{+/-} mice.

In contrast, Strid et al. have demonstrated a crucial role for $\gamma\delta$ T cells in producing antigen-specific IgG1 and IgE after allergen

application to stressed epithelia [23]. However, in this study a different method for barrier disruption, i.e. tape stripping, was used. By repeatedly pressing an adhesive film to the surface of the skin and then abruptly removing it, only the uppermost skin layers, the stratum corneum, gets abraded. Unlike laser microporation, this technique has to be rated as much less invasive. Moreover, the tape-stripped skin area is inevitably larger compared to the constrained area of 10 mm² treated with the laser, on which the (epi)dermal tissue between the laser micropores is left intact [16]. In the above-mentioned work, communication between epithelial cells and DETCs via the activating receptor NKG2D was crucial for the induction of TH2-biased responses. One could speculate that repeated tape-stripping induces a more pronounced upregulation of the NKG2D ligand Rae-1 on keratinocytes compared to laser microporation, thereby leading to enhanced activation of DETCs. Finally, besides the diverging technique used for barrier disruption, Strid et al. applied not only a different allergen, i.e. ovalbumin, but also a higher dosage (100 μ g vs. 1 μ g) compared to our approach, a fact that could also contribute to the discrepant outcomes.

Taken together, our data indicate that systemic immune responses following laser-mediated EPI are not significantly modulated or regulated by $\gamma\delta$ T cells. However, we propose that $\gamma\delta$ T cells are rather responsible for shaping the local inflammatory milieu under steady-state conditions.

MCs act as potent producers of various pro-inflammatory and TH2 cytokines [42,43] and have been attributed to play a role in DC stimulation and migration. Furthermore, MCs can both directly and indirectly regulate T cell recruitment, activation, proliferation, and cytokine secretion (reviewed in [44]). Therefore, we hypothesized that MC and basophil depletion would impair TH2 polarization and consequently promote TH1 responses.

However, the cytokine and chemokine milieu in the skin after laser microporation was largely unaffected by the depletion of MCs with the exception of TSLP, GRO- α , and IL-33 (Fig. 1, Table 2, and suppl. Fig. 7), which were significantly downregulated in MC-depleted mice. Similarly, a decreased induction of MIP-1 α and MIP-2 was detected in the absence of MCs (not significant). Interestingly, we observed that DT treatment by itself caused the induction of most skin chemokines. We have shown that MCs constitute a major depot of the alarmins TSLP and IL-33, but not IL-1- α or IL-25. We thus reasoned that the DT-induced depletion of MCs leads to the release of immune mediators and alarmins from necrotic MCs and causes the activation of other skin-resident innate cells.

Nevertheless, depletion of MCs had only slight effects on the adaptive T cell response after laser-mediated EPI. Consistent with our stated hypothesis, MC-depleted mice show significantly decreased Phl p 5-specific serum IgG1 titers compared to WT control mice after EPI (Fig. 2A). However, there were no changes in antigen-specific IgG2c levels (Fig. 2B). Thus, we suggest that MCs play a potential role in the TH2-dependent class switch to IgG1. In contrast to those findings, antigen-specific cell-bound IgE levels were not affected by the presence of MCs (Fig. 2B). We found no differences in the secretion of pro-inflammatory cytokines by antigen-restimulated lymphocytes between MC-depleted and WT control mice (Fig. 3B). Unexpectedly, we observed an increased production of IL-4 and IL-10 by antigen-restimulated splenocytes in MC-depleted mice, contrasting our finding of reduced serum IgG1 (Fig. 2A). As basophils, - which are known to secrete large amounts of IL-4 upon IL-33-stimulation - are depleted alongside MCs in our model, this is even more surprising. Thus, our results are contrary to data proposing either a role of MCs in promoting TH2 immunity in response to skin barrier disruption [45] or in promoting TH1/TH17 polarization [46]. Since cytokine expression was measured 12 days after the last immunization, we speculate that MCs and basophils only influence the early cytokine expression,

but are rather dispensable for shaping the adaptive immune response after two or more epicutaneous immunizations using laser microporation.

FACS analysis revealed that MC-depleted mice show a significantly higher percentage of naïve T cells and a concomitant decrease of effector T cells among isolated lymphocytes compared to WT control mice (Fig. 4). This confirms a role of MCs in T cell activation/maturation and is consistent with data indicating a role of MCs in the pre-priming of DCs via ICAM/LFA-1, which can enhance subsequent T cell priming [29,47]. In line with our cytokine data, we observed no difference in transcription factor expression between MC-depleted mice and WT control mice, suggesting that MCs are dispensable for T helper cell polarization via transcription factor expression. Collectively, we propose that MCs are mostly dispensable for humoral TH2-immune responses in laser-mediated EPI, but may play a role in T cell activation/maturation. Finally, we conclude that MCs mainly contribute to the laser-induced cytokine/chemokine milieu by either secretion or regulation of TSLP and GRO- α .

6. Conclusion

In summary, we did not observe a major role of $\gamma\delta$ T cells or MCs in shaping the immune response after laser-mediated EPI with Phl p 5 mannan. However, they can contribute to the steady-state or damage-induced cytokine milieu in the skin, respectively.

7. Limitations of the study

C57BL/6 mice have the same genetic background as Mas-TRECK mice and were also born and held in the same animal facility. While they should be a suitable control group with respect to certain factors like their specific microbiome, they did not undergo DT treatment and are therefore not an optimal control. Due to their low expression of the human DTR, DT treatment also results in transient basophil depletion. Furthermore, we have shown that DT-treatment can alter the chemokine milieu in the skin. Thus, immunological outcomes in the subsequent experiments cannot be considered solely a MC-dependent effect, but rather have to be considered MC- or basophil-dependent or DT-induced. This is of special interest regarding TH2 cytokine expression, since basophils are known to be producers of IL-4 and hence able to promote TH2 polarization. To overcome these limitations and exclusively elucidate the role of MCs in EPI in further studies, novel constitutive or inducible MC-deficient models like Mcpt5-Cre; R-DTA or Mcpt5-Cre; iDTR might be employed.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.vaccine.2019.11.051>.

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Supplementary Data

1.1 Methods

Generation of allergen-mannan glycoconjugates

Allergen-mannan neoglycoconjugates were generated by mild oxidation of mannan and subsequent reductive amination. For this purpose, 50mg of mannan (α -1,6-glycosidic-linked mannan from *S. cerevisiae*, Sigma) was dissolved in 1 mL of NaIO₄ solution (11.91 mg/mL) and incubated at RT for 1 h under constant stirring in the dark, thereby resulting in an oxidation degree of 20%. For removal of excess NaIO₄, a NAP-5 column (prepacked with sephadex G-25, GE Healthcare) was used according to the manufacturer's instructions. For coupling of oxidized mannan to Phl p 5, mannan was mixed with recombinant Phl p 5 (LPS content <0.3 pg/ μ g) to reach a ratio (w/w) of 5:1 between mannan and the allergen. 12.5% of freshly prepared NABH₃CN (sodium cyanoborohydride; 20 mg/mL in dH₂O) was added and the reaction was incubated at RT under constant stirring in the dark overnight. To reduce any remaining aldehyde groups, 10% of NaBH₄ (5 mg/mL in 50 mM borate buffer, pH 9.5) was added to the reaction, followed by a 6 h incubation at 4°C under constant stirring in the dark.

Analysis and characterization of neoglycoconjugates

The newly generated Phl p 5 mannan glycoconjugates were subjected to size exclusion chromatography using a 16/60 Sephacryl S-300 HR column (fractionation range: 10.000-1.500.000 Da, GE Healthcare) with an ÄKTA chromatographic system (GE Healthcare). The coupling efficiency of the neoglycoconjugate fractions was monitored by applying the fractions to a 12.5% reducing SDS-PAGE using colloidal Coomassie staining for protein visualization and fractions containing high molecular weight conjugates were pooled. The hydrodynamic radius of this glycoconjugate pool was measured using dynamic light scattering. Measurements were performed at a protein concentration of 1 mg/mL in DPBS at 40% laser power for 20x5 s on a dynamic light scattering instrument (DLS 802, Viscotek).

Toluidine blue staining (mast cell depletion)

To confirm the absence of mast cells at the time of immunization, small samples of ear tissue from Mas-TRECK mice were excised 18 days after the last treatment with diphtheria toxin (DT). Therefore, mice were put under light isoflurane (2.5% v/v) anaesthesia and lidocaine cream was applied. Tissue samples were embedded in O.C.T compound (Tissue-Tek, Sakura) and frozen in the gaseous phase of a liquid nitrogen chamber. 5 µm cryo-sections were generated using a cryostat microtome (Leica CM1950) and mounted on poly-L-lysine coated glass slides (Thermo Scientific). Slides were washed in PBS-T (PBS with 0.05% Tween®-20) before they were stained by dipping the slides in a 50 mL Greiner tube of toluidine blue working solution (2.8 g toluidine blue in a solution of 4 g urea dissolved in 120 mL dH₂O and 280 mL of 70% isopropanol) for ten times. Slides were washed with PBS-T before they were destained by dipping into 50 mL of 70% isopropanol with 0.5% HCl. After destaining, sections were immediately analysed under a light microscope (Hun, Wilovert 30).

Flow cytometry (basophil depletion)

After 250 ng diphtheria toxin (DT) was intraperitoneally injected for five consecutive days, blood samples were drawn from the saphenous vein to confirm depletion of basophils in Mas-TRECK mice. Blood samples were diluted (1:10) with 1.5 mg/mL Li-heparin in PBS to avoid coagulation. An equal volume of staining mix was added containing anti-mouse CD3 (APC-labelled; clone 145-2C11, BioLegend), anti-mouse CD45-B220 (PerCp-Cy5-labelled; clone RA3-6B2, BioLegend), and anti-mouse IgE (FITC-labelled; clone RTK2071, BioLegend) diluted 1:50 in FACS buffer (1% BSA, 2 mM EDTA in PBS). Samples were incubated for 30 min at 4°C in the dark. After incubation, blood samples were washed with 400 µL FACS buffer before erythrocyte lysis was performed by addition of 300 µL 1xRBC lysis buffer (eBioscience). After 5 min of incubation at RT, cells were washed with 600 µL of FACS buffer. The supernatant was discarded and the cell pellet was dissolved in 100 µL FACS buffer. The samples were then analysed on a FACS Canto II (BD Biosciences) by gating for basophils (anti-IgE⁺, anti-CD45-B220⁻, and anti-CD3⁻).

Epidermal sheets (monitoring of $\gamma\delta$ T cells)

To determine the presence of $\gamma\delta$ T cells in the epidermal layer of the skin, epidermal sheets were generated from ear tissue after mice were sacrificed by cervical dislocation. Therefore, 3.8% NH_4SCN (ammonium thiocyanate in dH_2O ; Carl Roth GmbH) was warmed to 37°C before a 500 μL drop was placed into a petri dish (35 mm). The dorsal and ventral side of the ear were separated and incubated on the drop of 3.8% NH_4SCN with the inner side facing down for 20 min. The dorsal and ventral side of the ear were washed on a drop of PBS in a 24-well plate (Greiner) before the epidermis was separated from the dermis. The separated epidermis was fixed by floating on acetone for 1-2 min at RT, before it was washed again by floating on a drop of PBS. The epidermal sheet was then placed on a drop of staining mix containing anti-mouse CD3 (AlexaFluor647-labelled; clone 172A, BioLegend) diluted 1:200 in PBS and incubated for 30 min at 37°C . Nuclei were stained by putting the epidermal sheet on a drop of PBS containing Hoechst 33342 staining dye (dilution 1:1000; Thermo Fisher) for 3-5 min at RT. The epidermal sheets were washed twice with PBS and once with dH_2O before they were mounted on glass slides using Roti Mount Aqua (Carl Roth GmbH). To detect $\gamma\delta$ T cells, the slides were placed under a fluorescence microscope (Olympus IX70) with an appropriate filter.

Generation of skin micropores and immunization

One day prior to laserporation, an area of roughly 3 cm^2 at the lower back of mice was shaved using an electric clipper, followed by treatment with depilatory cream (Veet sensitive). The depilatory cream was applied for 30 s under light anaesthesia (2.5% v/v isoflurane) before it was removed with a wet sponge. The next day, mice received ketamine/xylazine anaesthesia (80 mg ketamine and 7.5 mg xylazine per kg body weight) by intraperitoneal injection. Mice underwent laser microporation and the allergen suspension (1 μg of Phl p 5 mannan in 20 μL DPBS) was applied onto the laser-induced micropores using a pipette tip. After all the liquid was taken up, the treated area was covered with an adhesive tape (OPSITE Flexifix, Smith & Nephew).

IgG subclass ELISA

A luminometric ELISA was performed to determine the production of Phl p 5-specific serum IgG1 after immunization with Phl p 5 mannan glycoconjugates. Therefore, a 96-well plate (flat white chimney; Greiner) was coated with 1 µg/mL recombinant Phl p 5 diluted in PBS (50 µL per well) overnight at 4°C. After incubation at RT for 1 h, the coating solution was discarded. 200 µL of blocking buffer (2% skim milk, blotting grade, and 0.1% Tween®-20 in PBS) was added to each well and incubated 1 h at RT. The plate was washed with PBS-T (PBS with 0.1% Tween-20) using an automated plate washer (Tecan 96PW Microplate Washer). Meanwhile, serum dilutions (1:1000 and 1:10000) were prepared in blocking buffer. 50 µL of each serum dilution was added to the wells and incubated for 1 h at RT. After another washing step, 50 µL of the secondary antibody (goat anti-mouse IgG1-HRP, Bio-Rad; goat anti-mouse IgG2c-HRP, Bio-Rad; rabbit anti-mouse IgG2a-HRP, antibodies-online.com) was added as a 1:1000 dilution in blocking buffer. The plate was incubated for 1 h at RT. After a final washing step, 50 µL of an ELISA BM chemiluminescence substrate (Roche) was added and after 2-3 min the luminescence was measured in relative light units (RLU) using a plate reader (Tecan infinite 200Pro; integration time of 1000 ms and attenuation set to automatic).

Basophil activation test

For the measurement of cell-bound allergen-specific IgE levels, a basophil activation test was performed. Briefly, 30 µL of heparinized blood sample was mixed with either 30 µL RPMI (stimulation control), 30 µL RPMI with 20 ng/mL rPhl p 5 (final concentration 10 ng/mL) or 30 µL RPMI with 200 ng/mL rPhl p 5 (final concentration 100 ng/mL) in a 96-well plate (V-bottom plate; Greiner) and incubated at 37°C and 5% CO₂ for 2 h. 80 µL of ice-cold FACS buffer (1% BSA, 2mM EDTA in PBS) was added to each well and the plate was spun down at 260 g for 5 min at 4°C. The supernatants were aspirated and pellets were resuspended in 30 µL staining mix containing anti-mouse IgE (FITC-labelled; clone RME-1, BioLegend), anti-mouse CD4 (PerCp/Cy5.5-labelled; clone GK1-5, BioLegend), anti-mouse CD200R (APC-labelled; clone OX110, eBioscience), and anti-mouse CD19 (PE/Cy7-labelled; clone eBio1D3, BD Biosciences), all diluted 1:200 in FACS buffer and incubated on ice for 25

min. Then 80 μ L of FACS buffer was added and the plate was spun down for 5 min at 260 g at 4°C. The supernatants were aspirated and the pellet was resuspended in 100 μ L 1xRBC lysis buffer (eBioscience). After 5 min of incubation at RT, the plate was spun down, supernatants were discarded and 100 μ L FACS buffer was added. After a final washing step, the pellets were dissolved in 50 μ L FACS buffer and the median fluorescence intensity of CD200R was analysed on a FACS Canto II flow cytometer (BD Biosciences, Franklin Lakes, New Jersey) by gating on non-T/non-B/IgE^{high} cells.

Gating strategy for the basophil activation test:

FSC/SSC were used to gate for the cell population and the FSC height and width were used to determine singlet cells. Antibodies specific for the extracellular markers CD4 and CD19 were used to exclude T cells and B cells, respectively. Therefore, to gate on the basophil population IgE⁺, CD4⁻ and CD19⁻ cells were considered. Activation status of the basophil population following the treatment was determined by analysis of the median fluorescence intensity of CD200R.

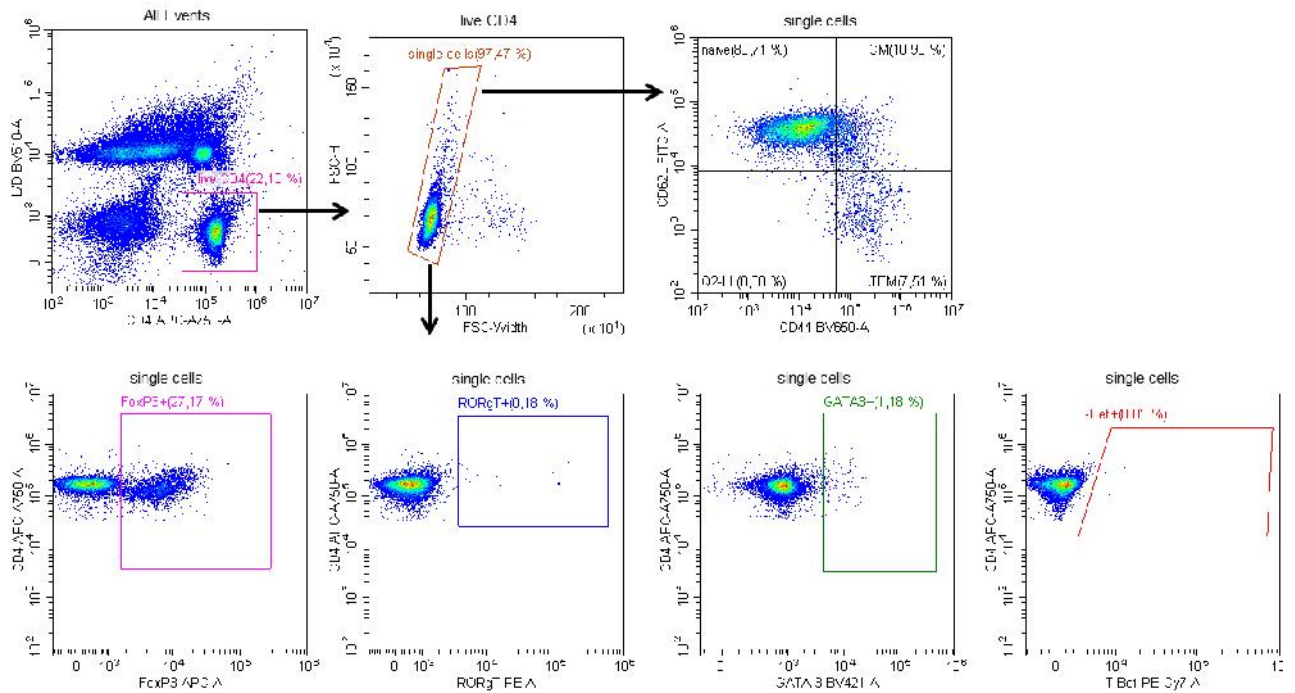
T helper cell polarization

After mice were sacrificed, spleens and skin-draining lymph nodes (inguinal, axillary, and brachial) were prepared. Lymph nodes and spleens were homogenized and after sedimentation of debris, the monodisperse supernatants of LN samples were transferred into 15 mL tubes with DPBS. Red blood cell lysis was performed with spleen samples using Ammonium-Chloride-Potassium lysis buffer (ACK buffer; 0.15 M NH₄Cl, 10 mM KHCO₃, 0.1 mM Na₂EDTA, pH 7.2). All samples were spun down at 260 g for 5 min. The supernatants were discarded and the pellets were washed with 5 mL DPBS. Pellets were dissolved in 1 mL (spleen) or 200 μ L (lymph nodes) T cell medium (RPMI-1640; 10% FCS, 25 mM HEPES, 2 mM L-Glu, 100 μ g/mL streptomycin, 100 U/mL penicillin). Cells were diluted to a concentration of 4x10⁶ cells/mL and 75 μ L of each cell suspension was seeded in a sterile 96-well tissue culture plate (U-bottom; Greiner). 75 μ L of Phl p 5 (10 μ g/ml) was added and the plate was incubated at 37°C and 5% CO₂. After 5 days of incubation, culture supernatants were removed and the remaining restimulated lymphocytes were washed twice with 100 μ L of DPBS before 20 μ L of

supernatant from anti-CD16/32 antibody (clone 2.4G2) producing hybridoma (ATCC number HB-197) was added to the cells to block FcγR and incubated for 5min at 4°C in the dark. 20 µL of extracellular staining mix were added to each well containing a live/dead staining (fixable viability dye eFluor 506; Thermo Fisher; 1:1000), anti-mouse CD62L (FITC-labelled; clone MEL-14, BioLegend; 1:200), anti-mouse CD44 (BV650-labelled; clone IM7, BioLegend; 1:100), and anti-mouse CD4 (APC-Cy7-labelled; clone GK1.5, eBioscience; 1:400) in DPBS. After 30 min incubation at 4°C in the dark, 100 µL of ice-cold FACS buffer was added to each well. The cells were spun down and the supernatant was discarded. The cells were then resuspended in 150 µL ice-cold Fix/Perm buffer (FoxP3 staining buffer kit; eBioscience). The cells were incubated for 60 min at 4°C in the dark before they were spun down and the supernatant was discarded. Two washing steps with 100 µL Perm buffer (FoxP3 staining buffer kit; eBioscience) were performed before the cell pellets were dissolved in 20 µL of 10% naïve mouse serum in Perm buffer for additional blocking. After 5 min of incubation at RT, 20 µL of the intracellular staining mix was added containing anti-mouse FoxP3 (APC-labelled; clone FJK-16s, Invitrogen; 1:100), anti-mouse T-bet (PE-Cy7-labelled; clone 4B10, BioLegend; 1:100), anti-human/mouse GATA3 (BV421-labelled; clone 16E10 A23, BioLegend; 1:200), and anti-mouse RORγT (PE-labelled; clone B2D, Invitrogen; 1:100). Cells were incubated with mouse serum and intracellular staining mix at RT in the dark for 30 min followed by two final washing steps with 100 µL Perm buffer were performed. Cells were resuspended in 80 µL FACS buffer (1% BSA, 2 mM EDTA in PBS) and analysed using a CytoFLEX S flow cytometer (plate mode; Beckman Coulter).

Gating strategy:

First, live CD4 cells were gated using fixable viability dye eFluor 506 and anti-CD4. Subsequently, FSC height and width were used to determine single cells. Antibodies specific for the extracellular markers CD62L (lymph node homing) and CD44 were used to differentiate between naïve, central memory (TCM), and effector memory (TEM) CD4⁺ live lymphocytes. Regulatory, TH1, TH2, and TH17 cells were gated based on their expression of intracellular transcription factors FoxP3, T-Bet, GATA-3, and RORγT, respectively.



Supplementary Figure 1. Gating of T cell subpopulations. Live CD4 T cells were gated and doublets were excluded. Single cells were separated into naïve (CD62L+CD44-), central memory (TCM, CD62L+CD44+), and effector memory (TEM, CD62L-CD44+) T cells. Regulatory T cells, TH17, TH2, and TH1 cells were gated based on their expression of FoxP3, RORγT, GATA3, and Tbet, respectively.

Cytokine production

After isolated lymphocytes from spleen and lymph nodes were restimulated *in vitro* with 10 µg/mL Phl p 5 for five days, supernatants were transferred into a new 96-well plate (flat bottom; Greiner). For quantification of the cytokines TNF-α, IFN-γ, IL-4, IL-5, IL-6, IL-10, IL-13, IL-17, and MCP-1 produced by *ex vivo* restimulated lymphocytes from immunized TCRd mice, ProcartaPlex multiplex cytokine panels (Invitrogen) were used according to the manufacturer's protocol. For quantification of cytokines TNF-α, IFN-γ, IL-2, IL-4, IL-5, IL-6, IL-10, IL-13, IL-17A, IL-17F, IL-21, IL-22 produced by *ex vivo* restimulated lymphocytes from immunized mast cell-depleted Mas-TRECK mice and C57BL/6 mice a LegendPlex Mouse TH cytokine panel (BioLegend) was performed according to the manufacturer's protocol.

183

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

185 Mice were shaved and depilated one day prior to laser treatment as described for immunizations.
186 Two non-overlapping areas of 1 cm² on the back were microporated using the P.L.E.A.S.E laser device
187 (laser settings: 8.3 J/cm², 50 µs pulse duration, 500 Hz frequency, 5% pore density, 3 pulses). 6 h and
188 24 h after laser microporation the mice were sacrificed by cervical dislocation. Control groups that
189 were not microporated were both shaved and depilated after they had been sacrificed. The lasered
190 skin area (non-microporated skin in the case of the control group) was excised and transferred into
191 12 mL tubes. The weight of the skin tissue was determined and a 20x amount of DPBS (+1% protease
192 inhibitor cocktail; Sigma, P8340) was added. The skin was homogenized on ice using an IKA® ULTRA-
193 TURRAX® tissue homogenizer (speed of 18000/min; 6x10 s, 10 s breaks in between). The
194 homogenized skin samples were transferred into 1.5mL Eppendorf tubes and centrifuged at >20.000
195 g for 15 min. The supernatants were filtered through a 0.22 µm SpinX column (Corning) before the
196 concentrations of MIP1α, RANTES, GROα, MIP2α, MCP1, MCP5, MIP3α, IP-10, IL-6, TNF-α, and TSLP
197 (for TCRd mice), and TSLP, IL-6, TNF-α, MCP1, MIP1α, RANTES, MCP3, IP-10, GROα, and MIP2α (for
198 Mas-TRECK mice) were analysed using ProcartaPlex multiplex assay (eBioscience) as described
199 before.

200

201 **1.2 Results**

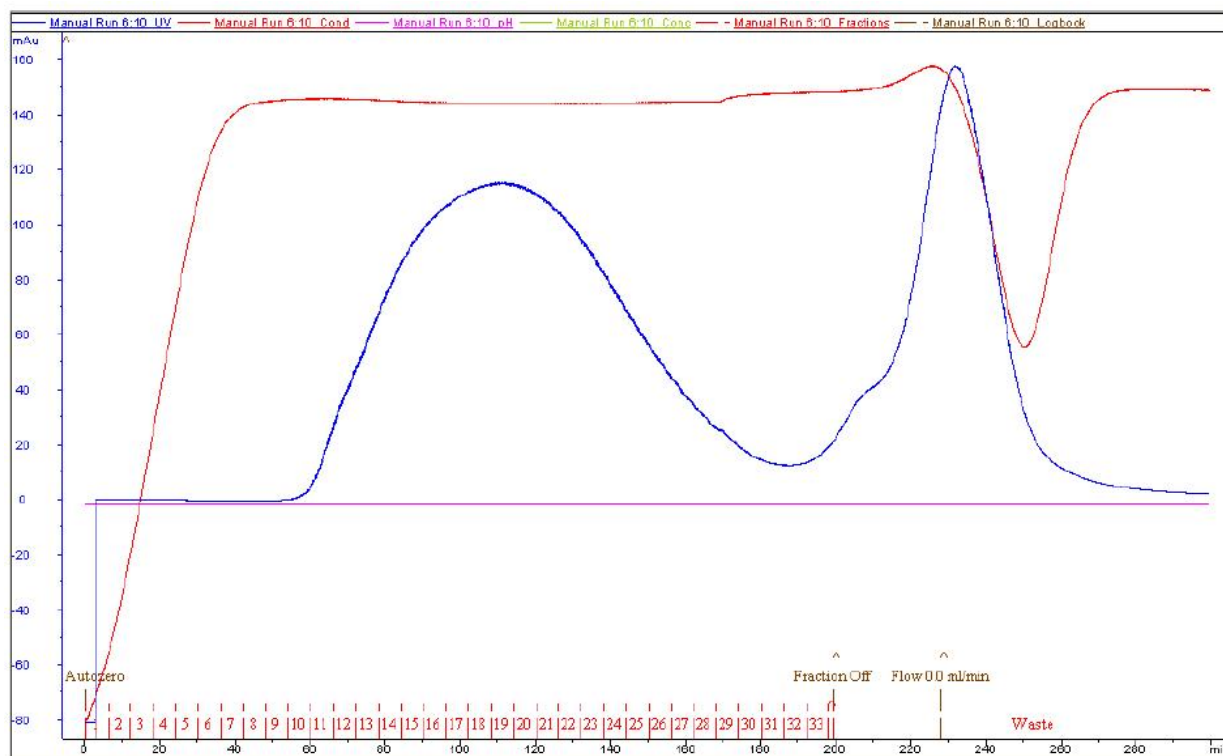
202

203 ***Purification and characterization of Phl p 5 mannan neoglycoconjugates***

204 *Size exclusion chromatography*

205 Size exclusion chromatography (SEC) was performed to separate the Phl p 5 mannan
206 neoglycoconjugates by size. Therefore, a HiPrep 16/60 Sephacryl S-300 HR column was used on an
207 ÄKTA prime chromatography system. 2 mL of the freshly generated Phl p 5 mannan
208 neoglycoconjugates were loaded onto the sample loop with a constant flow rate of 0.5 mL/min.
209 Elution was performed with PBS and the eluate was collected in 3 mL fractions starting at minute 0 of

the SEC until after the first peak ended at minute 200. Due to the larger size, mannan conjugated Phl p 5 is eluted first as its size prevents retention in the pores of the adsorbent. Therefore, the first visible peak in the chromatogram (Suppl. Fig. 1) represents the eluted neoglycoconjugates (starting



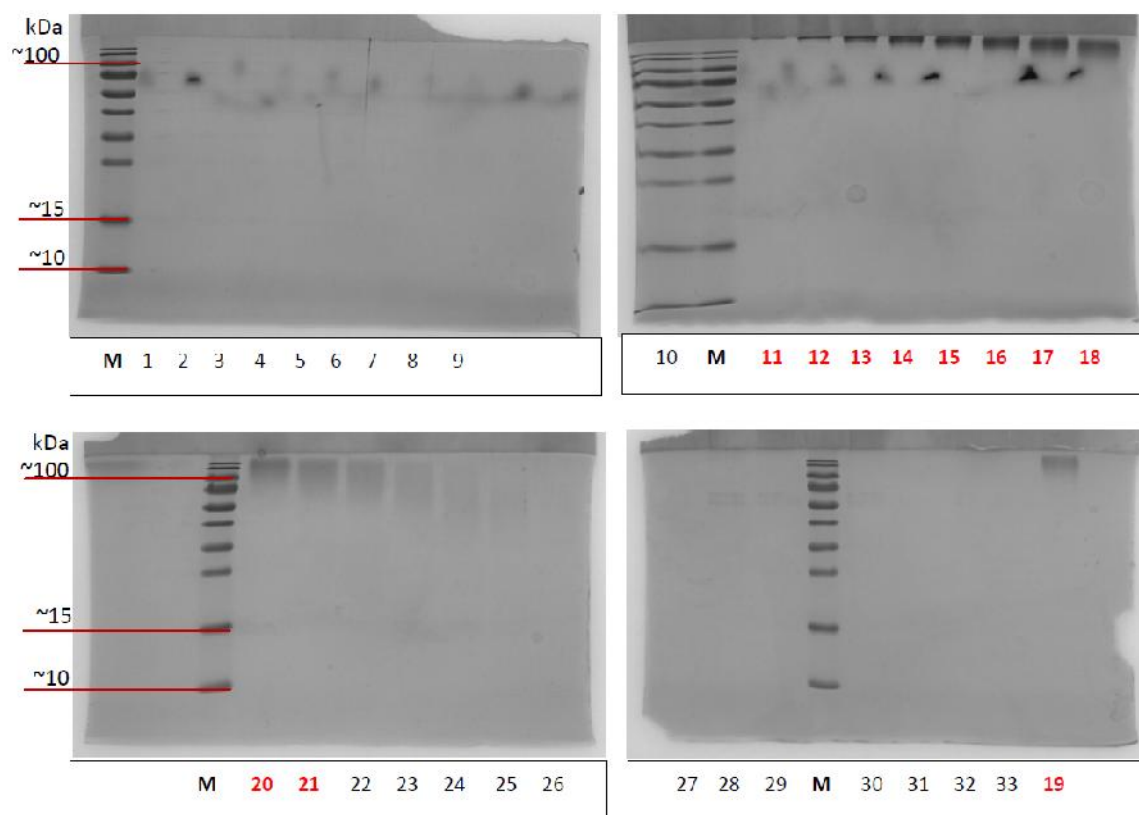
at minute 60 of SEC), whereas the second peak (starting at minute 200 of SEC) resembles unconjugated mannan that also absorbs at 280 nm.

Supplementary Figure 2. Size exclusion chromatogram of P5-MN neoglycoconjugates. P5-MN conjugates were separated by size using a HiPrep 16/60 Sephacryl S-300 HR column on an ÄKTA prime chromatography system. The blue and the red curve represent changes in the absorbance at 280 nm and in conductivity/absorbance, respectively.

Reductive amination is an effective way to generate allergen-neoglycoconjugates

After collecting the different fractions, we wanted to investigate whether the separation by SEC was efficient and resulted in the enrichment of high molecular weight Phl p 5 mannan neoglycoconjugates. To evaluate molecular weight (MW) and also coupling efficiency of the individual fractions after SEC, we performed an SDS-PAGE on a reducing 12.75% gel and visualized proteins by staining with colloidal Coomassie. Suppl. Fig. 2 clearly depicts that the MW of the individually obtained fractions (11-21) was approximately higher than 100 kDa, i.e., more than 3-fold

227 larger compared to unconjugated Phl p 5 (MW = 30kDa). In addition, our gel images revealed no
 228 detectable unconjugated Phl p 5 in all analysed fractions (1-33). Thus, SDS-PAGE verified the effective



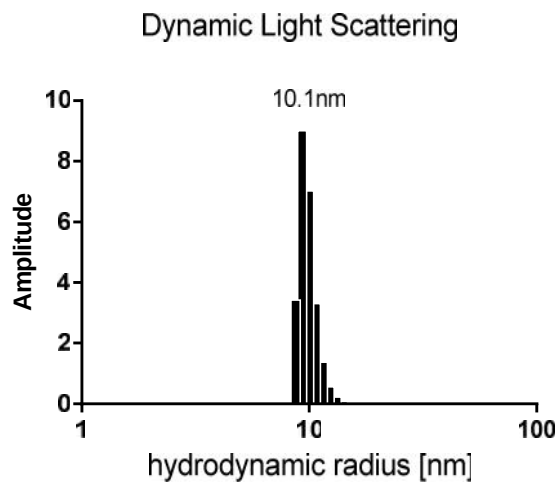
229 and controlled generation of high molecular weight Phl p 5 mannan neoglycoconjugates with a high
 230 coupling efficiency.

231 **Supplementary Figure 3. SDS-PAGE of P5-MN neoglycoconjugate fractions.** Fractions 1-33 were
 232 analysed on a 12.75% gel. PageRuler™ Prestained Protein Ladder, 10 to 180 kDa was used as marker
 233 for identification of uncoupled Phl p 5. Fractions marked in red were pooled for further analysis.
 234

235 ***Phl p 5 mannan neoglycoconjugates are heterogeneous in size***

236 To determine the hydrodynamic radius and characterize size distribution profile of the Phl p 5
 237 mannan glycoconjugates, we performed a dynamic light scattering (DLS) analysis of the pooled
 238 fractions 11-13 in PBS. Pooled fractions were repeatedly measured at a protein concentration of 1
 239 mg/mL using the solvent settings for PBS. As expected, the mean hydrodynamic radius of the
 240 neoglycoconjugate pool estimates to 10.1 nm (Suppl. Fig. 3) and therefore is 3-fold higher than the
 241 mean hydrodynamic radius of unconjugated Phl p 5 ($R_H = 3$ nm, not shown). Furthermore, the

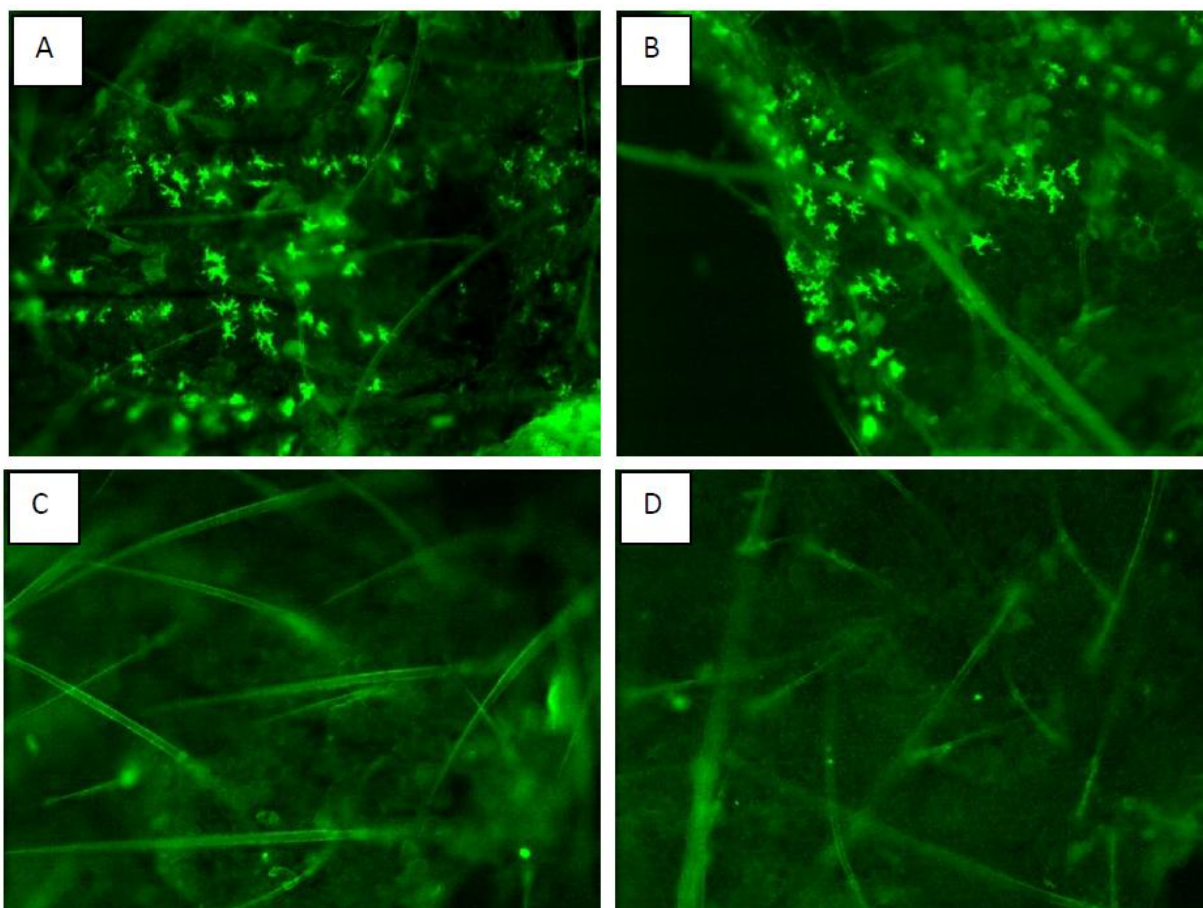
obtained size distribution demonstrates heterogeneity, suggesting the presence of bigger aggregates in the solution.



Supplementary Figure 4. Measurement of the hydrodynamic radius and size distribution of pooled fractions 11-13 by DLS.

Visual verification of $\gamma\delta$ cells and mast cells in skin samples

Epidermal sheets were prepared from the ears from sacrificed mice at the end of the immunization experiments to verify the presence and absence of $\gamma\delta$ T cells in TCRd^{+/+} and TCRd^{-/-} mice, respectively. Suppl. Fig. 4 shows representative examples of epidermal sheets from TCRd^{+/+} (A, B) and TCRd^{-/-} (C, D) mice. Consistent with their genotype, epidermal sheets from TCRd^{-/-} showed no detectable $\gamma\delta$ T cells, whereas in TCRd^{+/+} $\gamma\delta$ T cells with a dendritic morphology were observed. Thus, we were able to validate TCRd^{-/-} mice as a suitable model to study effects depending on the presence of $\gamma\delta$ T cells.

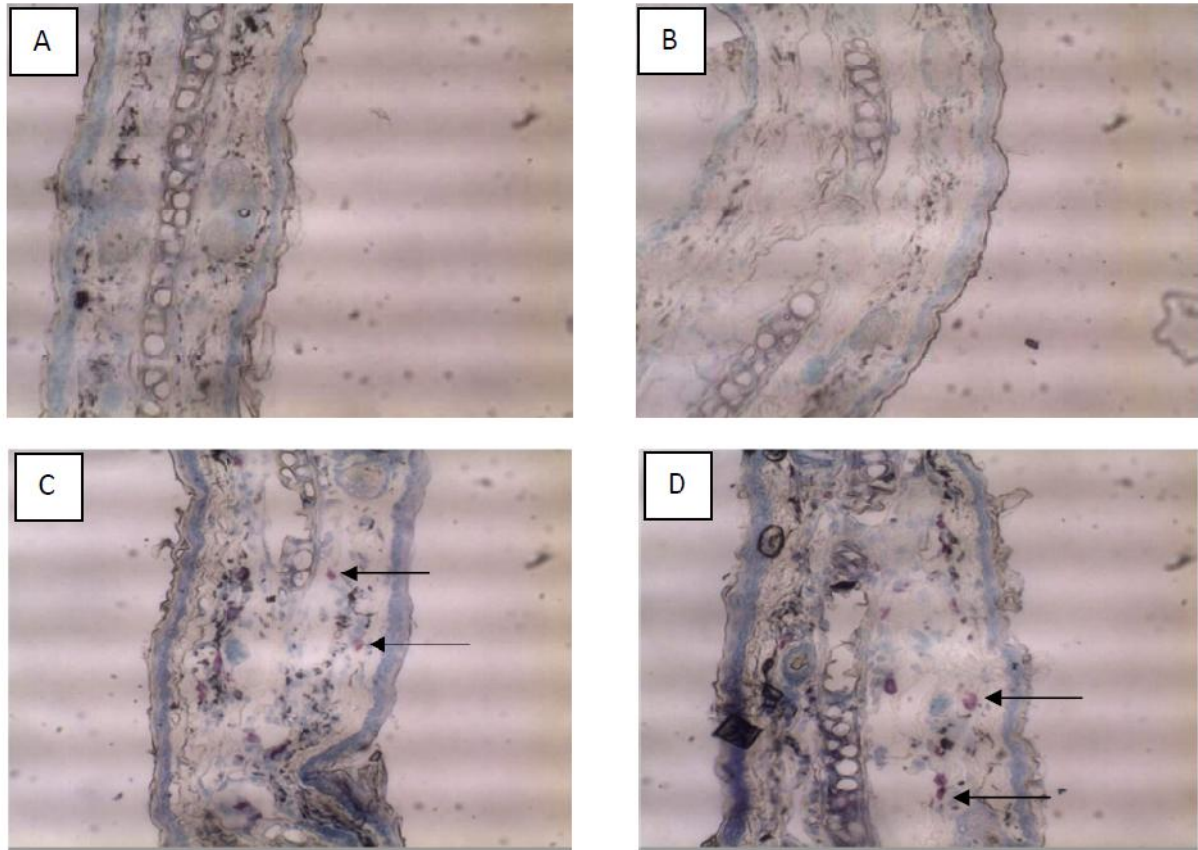


Supplementary Figure 5. Fluorescence microscopy pictures of epidermal sheets. Epidermal sheets (ES) from TCRd^{-/+} mice (A and B) or TCRd^{-/-} mice (C and D) were stained with anti-CD3 antibody (Alexa Fluor 647) to visualize γδ T cells in the epidermis.

To demonstrate that MCs were depleted at the time of immunization, small samples of ear tissue were prepared after the first immunization (day 18) followed by a histological analysis. It has been reported that MCs and basophils are depleted for at least 12 days after cessation of DT treatment [28], and we verified that MCs were still depleted on day 18 after the last DT application. Cryo-sections of both MC-depleted Mas-TRECK mice and C57BL/6 control mice were prepared and metachromatically stained with toluidine blue to visualize connective-tissue MCs in the skin. We only observed MCs in C57BL/6 control mice (Suppl. Fig. 5; C and D) as identified by their red-purple color. In contrast, DT-treated Mas-TRECK cryo-sections (Suppl. Fig. 5; A and B) displayed a complete absence of MCs. Surprisingly, MC were still completely absent from the connective-tissue in the skin

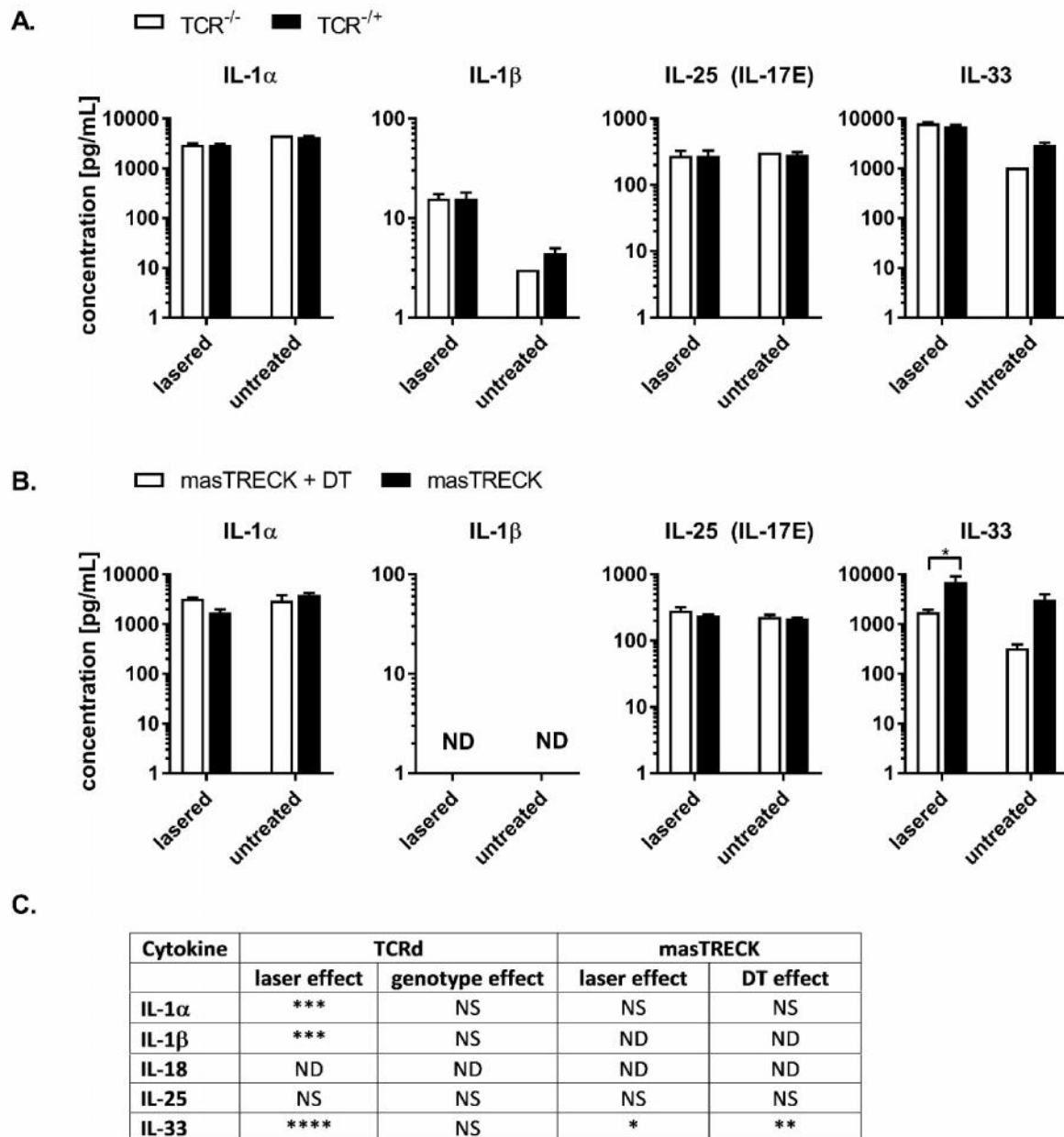
271 at day 18. Taken together, we were able to confirm the conditional, temporal and specific depletion
272 of MCs in skin of Mas-TRECK mice by application of DT.

273

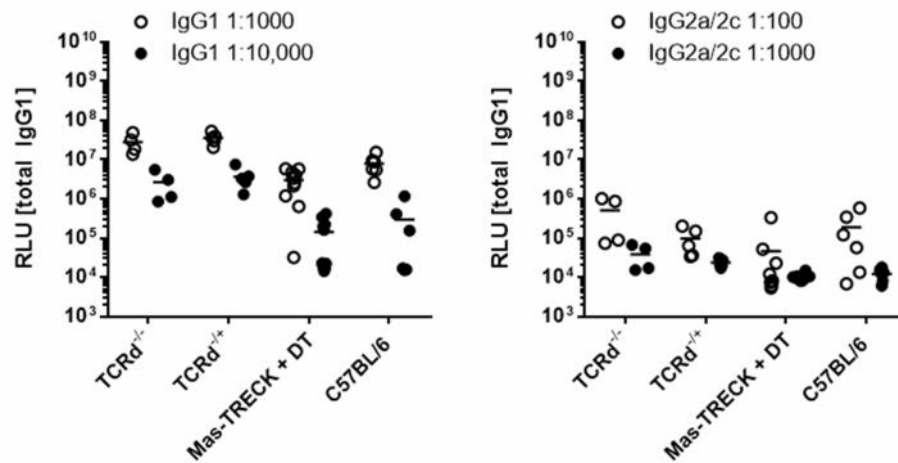


274

275 **Supplementary Figure 6. Microscopic pictures of cryo-sections from ear skin.** To visualize
276 connective-tissue MCs in the skin of Mas-TRECK mice (A and B) and C57BL/6 controls (C and D), cryo-
277 sections from ear skin were prepared on day 18 after the final DT treatment and metachromatically
278 stained with toluidine blue.



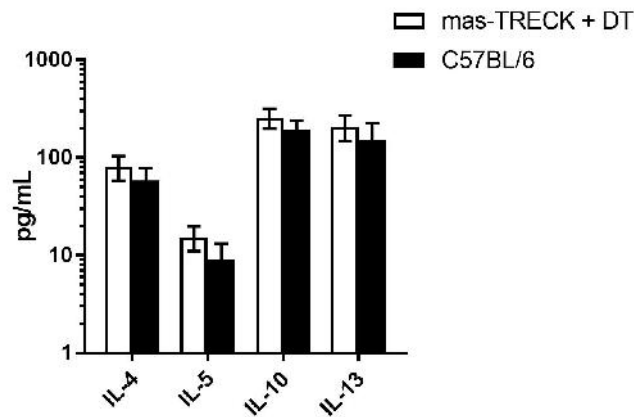
Supplementary Figure 7. IL-1 family cytokines and alarmins in homogenized skin samples. Cytokine concentrations measured in supernatants of homogenized skin from TCRd (A) or MasTRECK (B) mice 20h after laser microporation using cytokine multiplex assay (IL-1α, IL-1β, IL-18, IL-33) or sandwich ELISA (IL-25). Non-laser microporated mice (untreated) were used as controls. Data (n=4, except for TCR^{-/-} control where n=1) is depicted as cytokine concentration in pg/mL. (C) Statistical significance between TCR^{-/-} and TCR^{+/-} 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, ** P<0.01, *** P<0.001, **** P<0.0001, NS = not significant, ND = not detectable



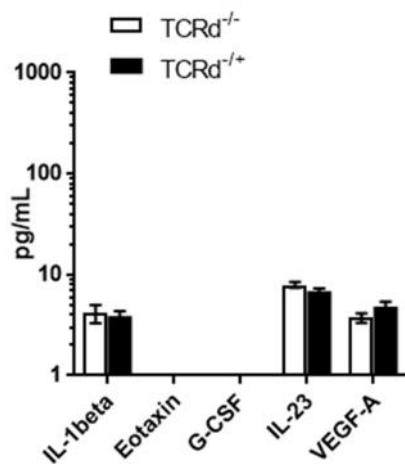
Supplementary Figure 8. Titration of sera from mice immunized with P5-MN. A luminometric ELISA was performed with 1:1000 and 1:10,000 (IgG1) or 1:100 and 1:1000 (IgG2a/2c) diluted serum samples and Phl p 5-specific IgG1 (**A**) and IgG2a (TCRd) or IgG2c (Mas-TRECK/C57BL/6) (**B**) levels were measured in terms of RLU (relative light units). TCRd^{-/-} (n=4), TCRd^{+/-} (n=6), DT-treated Mas-TRECK (n=10), C57BL/6 (n=6) mice.

Antigen-restimulated lymphocytes secrete elevated levels of TH2 cytokines in the absence of MCs

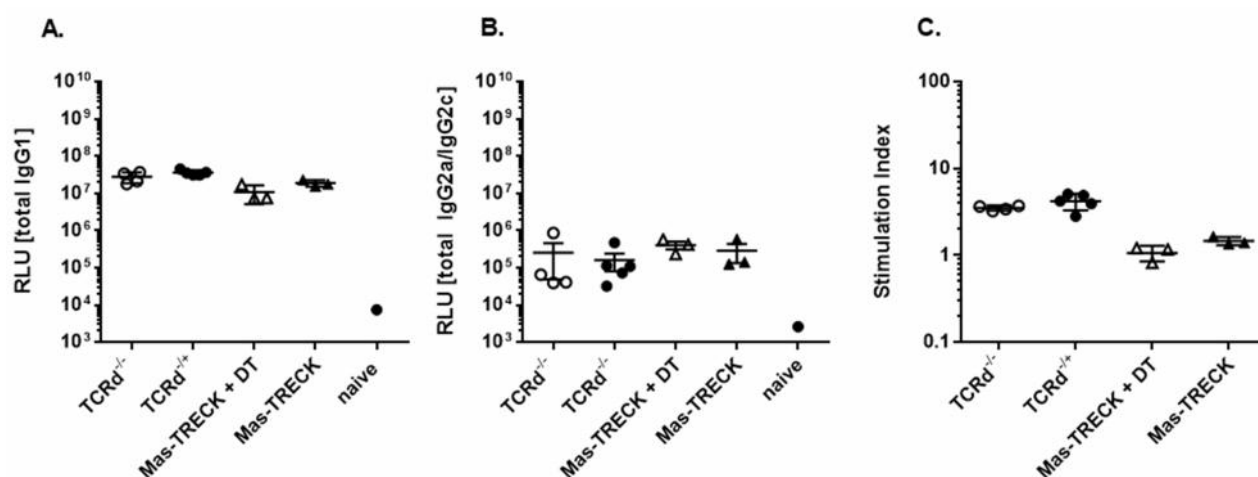
Isolated lymphocytes from skin draining lymph nodes from immunized Mas-TRECK and C57BL/6 mice were restimulated with 10 µg/mL recombinant Phl p 5 *in vitro* for five days and cytokine secretion in the culture supernatants was measured by LegendPlex multiplex analysis. We observed that lymphocytes isolated from MC-depleted mice show a consistent, however non-significant, trend of higher TH2 cytokine secretion compared to C57BL/6 control mice (Suppl. Fig. 6). This data points towards a role of MCs in polarization T helper type 2 cells.



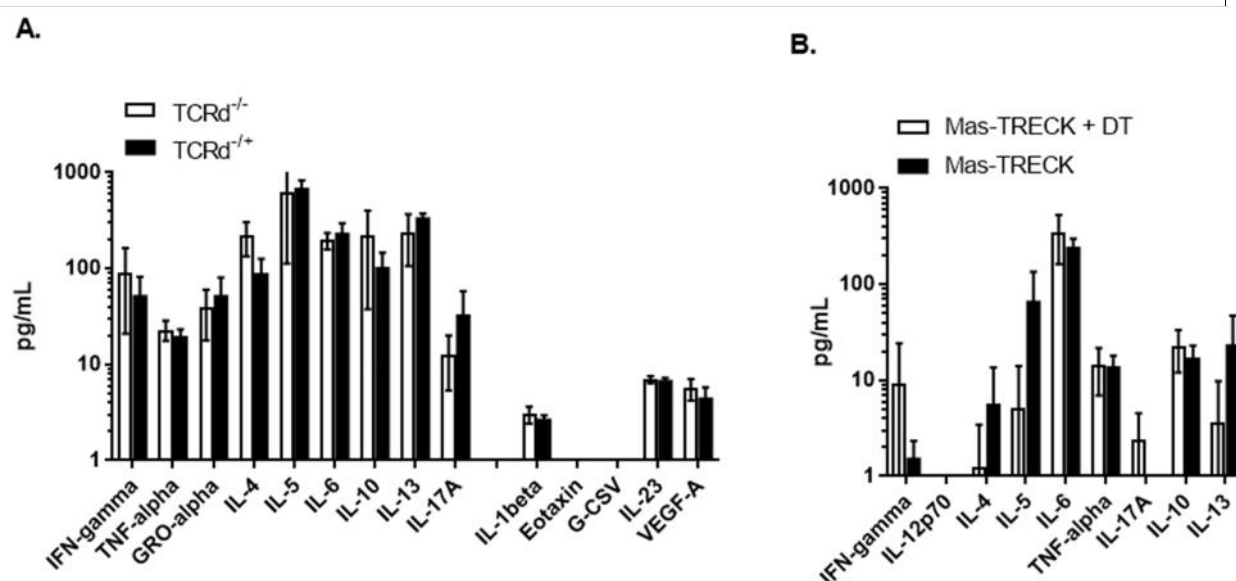
Supplementary Figure 9. Impact of MC depletion on TH2 cytokine secretion by antigen-restimulated lymphocytes. Skin-draining LN cells were isolated after EPI with P5-MN and cultured for 5 days in the presence of Phl p 5. Datasets for DT-treated Mas-TRECK (n=10) and C57BL/6 (n=6) mouse groups were analysed using a one-way ANOVA and Tukey's post hoc test with a single pooled variance and a significance level of 95% using GraphPad Prism 6 software. Data is shown as means $\pm$ SEM.



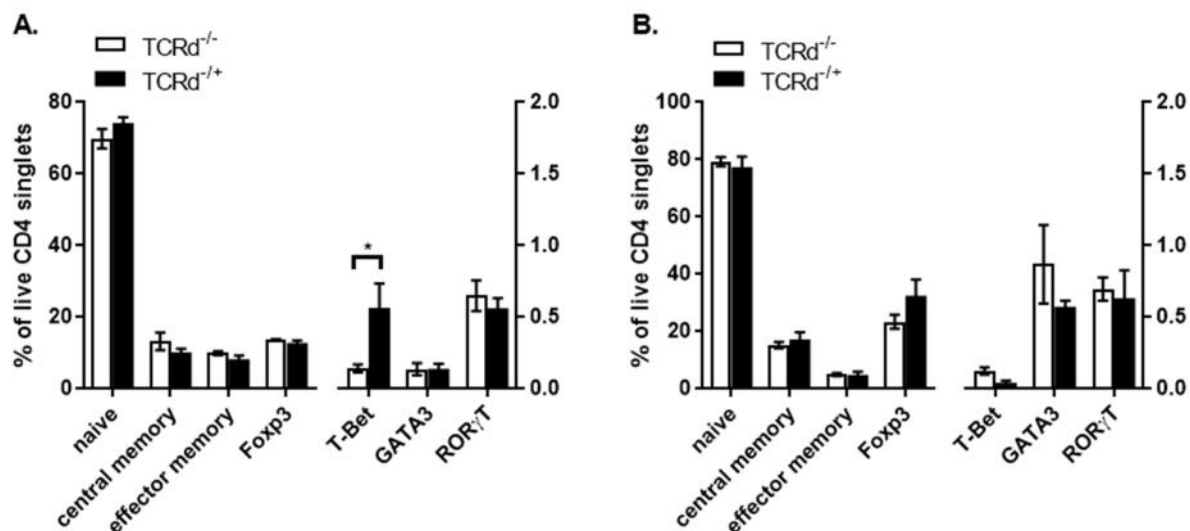
Supplementary Figure 10. Innate cytokine profiles of cultured splenocytes from P5-MN immunized mice. Cytokine production in cell culture supernatants of restimulated splenocytes was measured using ProcartaPlex multiplex analysis. TCRd^{-/-} (n=4), TCRd^{+/-} (n=5).



Supplementary Figure 11. Antigen-specific IgG1, IgG2a/2c, and IgE antibody titers after laser-mediated EPI with Phl p 5. A luminometric ELISA was performed with 1:1000 diluted serum samples and Phl p 5-specific IgG1 (A) and IgG2a (TCRd) or IgG2c (Mas-TRECK/C57BL/6) (B) levels were measured in terms of RLU (relative light units) (C) 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=5), DT-treated (+DT) or untreated Mas-TRECK (n=3), naïve (n=1) mice.



Supplementary Figure 12. Cytokine profiles of cultured splenocytes from Phl p 5-immunized mice. Cytokine production in cell culture supernatants of restimulated splenocytes was measured using ProcartaPlex (A) or LegendPlex (B) multiplex analysis. A.) TCRd^{-/-} (n=4), TCRd^{+/-} (n=5), and B.) DT-treated (+DT) or untreated Mas-TRECK (n=3) mice.



Supplementary Figure 13. T helper polarization in SDLN cells of Phl p 5-immunized mice. Extracellular markers CD4, CD62L and CD44 were used to determine the distribution of naïve, central memory, and effector CD4⁺ T cells. Viable and singlet CD4⁺ T cell subset distribution (left Y-axis) and the percentage of FoxP3, GATA3, RORγT and T-bet-producing CD4⁺ T cells (right Y-axis) were determined using flow cytometry. **A.)** TCRd^{-/-} (n=4), TCRd^{+/+} (n=5) *ex vivo* or **(B)** after 5 days *in vitro* stimulation with Phl p 5. *P<0.05