Integrin α_E(CD103) Is Involved in Regulatory T-Cell Function in Allergic Contact Hypersensitivity

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Murine contact hypersensitivity (CHS) is a dendritic cell (DC)-dependent T-cell-mediated inflammation with $CD8^+$ T cells as effectors and $CD4^+$ T cells as regulators (Treg cells) that models human allergic contact dermatitis. The integrin α_E(CD103) is expressed by some T-cell and DC subsets and has been implicated in epithelial lymphocyte localization, but its role in immune regulation remains enigmatic. We have identified a function for CD103 in the development of cutaneous allergic immune responses. CHS responses, but not irritant contact dermatitis, were significantly augmented in CD103-deficient mice in hapten-challenged skin. Phenotype and function of skin DCs during sensitization were normal, whereas adoptive transfer experiments revealed that the elevated CHS response in CD103-deficient mice is transferred by primed T cells and is independent of resident cells in recipient mice. While T-cell counts were elevated in challenged skin of CD103-deficient mice, the FoxP3 expression level of $CD4^+CD25^+$ Treg cells was significantly reduced, indicating impaired functionality. Indeed, Treg cells from CD103-deficient mice were not able to suppress CHS reactions during the elicitation phase. Further, CD103 on $FoxP3^+$ Treg cells was involved in Treg retention to inflamed skin. These findings indicate an unexpected dichotomous functional role for CD103 on Treg cells by modulating FoxP3 expression.

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INTRODUCTION

Contact hypersensitivity (CHS) in mice resembles human allergic contact dermatitis and, therefore, is a relevant model to study mechanisms underlying this common and burdensome condition (Becker, 2013; Mahler *et al.*, 2014). CHS is a dendritic cell (DC)-dependent T-cell-mediated immune response following epidermal sensitization and subsequent challenge with the relevant hapten (Grabbe and Schwarz, 1998; Nosbaum *et al.*, 2009). During sensitization, the skin is exposed to a specific hapten for the first time. Cutaneous DC take up the hapten, become activated through local production of pro-inflammatory mediators and migrate to draining lymph nodes (dLNs) where they prime hapten-specific T cells. Although for a long time, epidermal Langerhans cells (LCs) were considered to be the only DCs able to initiate CHS, more recent studies suggested that dermal DCs also contribute to its pathogenesis (Bennett *et al.*, 2007; Vocanson *et al.*, 2009; Honda *et al.*, 2013). The inflammatory elicitation (or challenge) phase after subsequent contact with the same hapten features an antigen-specific T-cell response along with profound infiltration of cells of the innate immune system. The clinical correlate is skin inflammation with edema, hyperemia, and epidermal damage, which peaks at 24–48 hours after re-exposure and progressively declines by different down-regulating mechanisms (Akiba *et al.*, 2002).

Both $CD4^+$ and $CD8^+$ T cells contribute to inflammatory responses. Hapten-specific $CD8^+$ cytotoxic T cells producing IFNγ and IL-17 are the primary effector cells in murine CHS to haptens such as oxazolone (OXA) or DNFB (Bour *et al.*, 1995; Kehren *et al.*, 1999; Akiba *et al.*, 2002). $CD4^+$ regulatory T (Treg) cells control the expansion of hapten-primed effector $CD8^+$ T cells and modulate the severity of CHS, presumably through production of IL-10 (Bour *et al.*, 1995; Gorbachev and Fairchild, 2004; Ring *et al.*, 2006; Ring *et al.*, 2011).

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Abbreviations: BM, bone marrow; BMDC, bone marrow–derived dendritic cell; CHS, contact hypersensitivity; DC, dendritic cell; dLN, draining lymph node; DNBS, 2,4-dinitrobenzene sulfonic acid hydrate; DNFB, 1-fluoro-2,4-dinitrobenzene; FoxP3, forkhead box P3; LC, Langerhans cell; OXA, oxazolone; 4-ethoxymethylene-2-phenyl-2-oxazolin-5-one; Treg, regulatory T cell; wt, wild-type

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However, the mechanisms underlying the fine-tuning of the "amplitudes" of such contact allergic reactions by Treg cells, the phenotype of Treg cells involved as well as Treg trafficking to and retention within the skin, are not yet fully understood.

The integrin α_E(CD103) is primarily expressed by epithelial lymphocytes of the intestine, lung, and skin, but also by subsets of mucosal mast cells, mucosal DCs, and dermal DCs (Kilshaw, 1999; Schön *et al.*, 2002; Pribila *et al.*, 2004). Although its function on DCs and mast cells remains elusive, it is thought that CD103 is not only involved in retention of lymphocytes to epithelial tissues but also in shaping and proper intraepithelial morphogenesis (Schön *et al.*, 1999; Schlickum *et al.*, 2008), as its only known ligand, E-cadherin, is expressed by epithelial cells such as keratinocytes and LCs (Cepek *et al.*, 1994; Karecla *et al.*, 1995; Kilshaw, 1999; Pauls *et al.*, 2001). Thus, the number of CD103-expressing lymphocytes is often increased in inflammatory skin diseases. Some studies suggested that CD103 expression on Treg cells but not on effector T cells is mandatory for their retention in inflammatory lesions and that dysregulation of CD103 leads to imbalanced local immunoregulation in the skin with augmented inflammatory responses (Lehmann *et al.*, 2002; Siegmund *et al.*, 2005; Suffia *et al.*, 2005). However, the points at which CD103 is required during this complex multistep immune response have not been clearly delineated.

We demonstrate that CHS developing in the absence of CD103 is more severe compared with wild-type (wt) mice. Moreover, while the number of T cells was increased, CD103 deficiency leads to a diminished FoxP3 expression on Treg cells suggesting a functional role. However, although CD103 is not mandatory for T-cell localization *per se*, it is crucial for Treg retention during CHS. Thus, it appears that CD103 deficiency augments inflammatory responses due to defective regulatory mechanisms by affecting both Treg function and retention.

RESULTS

Increased CHS reaction in CD103-deficient mice

CHS was induced using two different obligate haptens, namely OXA and 1-chloro-2,4-dinitrobenzene, in a large number of CD103-deficient mice and their wt counterparts (detailed in the legend to Figure 1a). The mice were sensitized on their shaved lower backs and challenged 5 days later on their right ears (the left ears served as internal vehicle controls). Although the overall dynamics of ear thickness as a surrogate parameter of cutaneous inflammation were similar in both genotypes, CD103-deficient mice developed a significantly stronger ear swelling response throughout the entire observation period. The difference in ear thickness between the strains became significant as early as 8–24 hours after the challenge, with a peak at 32–48 hours ($P<0.001$, $P<0.01$ or $P<0.05$, Figure 1a). Control mice, which were only challenged with either of the two haptens, did not mount an increased ear swelling, neither in wt, nor in CD103$^{-/-}$ mice (data not shown).

To assess whether the observed differences were specific for allergic immune reactions, we induced irritant contact dermatitis using Croton oil, which elicits acute inflammation without sensitization and, therefore, addresses innate immune responses in a T-cell-independent manner (Nosbaum *et al.*, 2009). The typical biphasic Croton oil-induced ear swelling response as a parameter for acute irritant inflammation was observed as early as 1 hour after exposure, with peak values after 6–8 hours (Figure 1b). Of note, there were no significant differences between CD103$^{-/-}$ and wt animals. In addition, cutaneous infiltration of mononuclear and polymorphonuclear leukocytes was similar in both genotypes (data not shown). Thus, deficiency of CD103 did not directly affect acute inflammatory processes.

When OXA-sensitized mice were evaluated histopathologically to assess the tissue distribution of various cell types involved in inflammatory processes, OXA-challenged skin showed a profound mixed inflammatory infiltrate including mononuclear, polymorphonuclear, and mast cells within the dermis, all of which were conspicuously more prominent in CD103-deficient mice (Figure 1c, four upper panels). Immunohistochemistry revealed massive dermal recruitment of CD11b$^+$ myeloid lineage cells (monocytes/macrophages) and Gr1$^+$ granulocytes, respectively, into inflamed skin (Figure 1c, four lower panels). Used as a second independent and quantitative method, flow cytometric analyses revealed markedly higher numbers of immigrating CD11b$^+$ monocytes/macrophages, CD11b$^+$/Gr1$^+$ neutrophils, and F4/80$^+$ macrophages upon OXA challenge in CD103$^{-/-}$ mice. Paralleling the macroscopic observations, these differences were most pronounced at 36 hours after OXA challenge (Figure 1d). Furthermore, a higher percentage of CD11b$^+$ and CD11b$^+$/Gr1$^+$, but not F4/80$^+$ cells cells within the infiltrate of CD103$^{-/-}$ mice became apparent. As these innate effector cells do not express CD103, they might not be the primary cell types mediating the observed differences. Therefore, we hypothesized that the stronger CHS-induced inflammation in CD103-deficient mice was due to altered function of cells expressing CD103, either DCs or T cells.

Normal DC function in CD103$^{-/-}$ mice during T-cell priming in the sensitization phase

Given that priming of hapten-specific T cells by DCs, particularly epidermal LCs, in dLNs during sensitization is mandatory in CHS (Grabbe *et al.*, 1995; Bennett *et al.*, 2007) and because the density of LCs influences CHS responses (Bennett *et al.*, 2007; Vocanson *et al.*, 2009), we examined DC migration, homing, maturation, and function in wt and CD103-deficient mice. On the basis of morphometric analyses of epidermal sheet preparations, we found that *in situ* counts and morphology of LCs showed no significant differences between CD103$^{-/-}$ and wt mice (Figure 2a), suggesting that CD103 was not required for the orthotopic localization and morphogenesis of LCs within the epidermis under steady-state conditions.

To elucidate the migration of skin DCs to the dLN, we followed DC migration after cutaneous painting with the fluorescent hapten, FITC, and analyzed FITC-loaded cells recovered from dLN by flow cytometry. All FITC-containing cells were MHCII/CD11c-positive, thus being DC migrated from the skin. Indeed, migration of DCs was restricted to dLN,

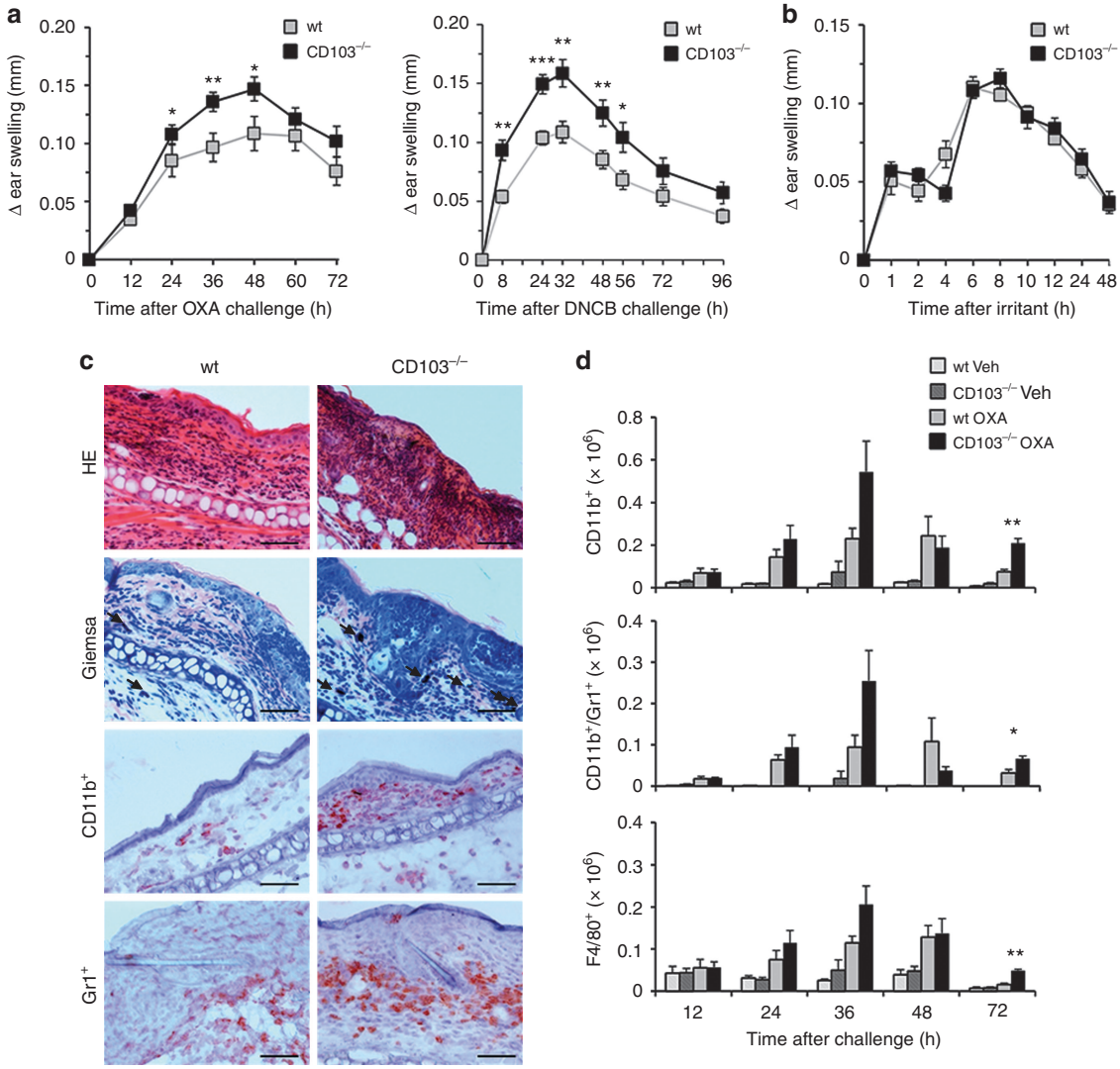

Figure 1. Increased allergic contact hypersensitivity (CHS), but normal irritant contact dermatitis in CD103$^{-/-}$ mice. (**a**) Normalized ear swelling of oxazolone (OXA)- or DNCB-treated wild-type (wt) and CD103$^{-/-}$ mice were measured as differences in ear thickness in at least four independent experiments ($n = 10$–42; mean ± SEM), respectively. (**b**) Ear swelling after Croton oil-induced irritant contact dermatitis are displayed ($n = 11$–12; mean ± SEM). (**c**) At 36 hours after challenge, representative tissue sections (out of three) were stained with hematoxylin and eosin (HE), Giemsa-solution, or antibodies against CD11b or Gr1. Examples of mast cells in the Giemsa-stained sections are indicated by arrows. Scale bars = 50 μm. (**d**) Ear cells of wt and CD103$^{-/-}$ mice were analyzed for CD11b$^+$, CD11b$^+$/Gr1$^+$, and F4/80$^+$ expression by flow cytometry. ($n = 3$–7; mean ± SEM). *$P < 0.05$; **$P < 0.01$ (unpaired Student's t test). DNCB, 1-chloro-2,4-dinitrobenzene.

as non-dLN and spleen did not harbor any FITC-positive cells, irrespective of the mouse strain used (data not shown). Approximately 2.5–3.0 × 10^5 antigen-loaded DCs migrated to each dLN in both CD103$^{-/-}$ and wt mice with similar dynamics for an observation period of up to 72 hours (Figure 2b). To assess DC phenotypes, classical DC activation markers including the adhesion molecule, CD62L (L-selectin), the chemokine receptor, CCR7, regulating homing and trafficking of DC from the skin to secondary lymphoid tissues, and the co-stimulatory molecules CD40, CD83, and CD86, respectively, were assessed by flow cytometry. Expression of these markers was similar within the FITC-positive populations of both strains (Figure 2c). Further analysis of FITC-loaded skin-derived DC in dLN of wt mice showed that the vast majority did not express CD103 (12.16% (SEM = 2.07%); data not shown) and are, therefore, presumably LCs, as these cells do not express CD103.

In functional activation experiments, bone marrow–derived DCs (BMDCs) from CD103$^{-/-}$ mice stimulated hapten-specific CD4$^+$ or CD8$^+$ T-cell proliferation with equal efficiency compared with BMDCs from wt mice (Supplementary Figure S1 online). This indicated that CD103 expression on DCs was not required for the stimulation of antigen-reactive T cells in CHS. BMDC preparations from both CD103$^{-/-}$ and wt mice contained >85% DCs as assessed by the expression of CD11c (data not shown), and their maturation phenotype was identical

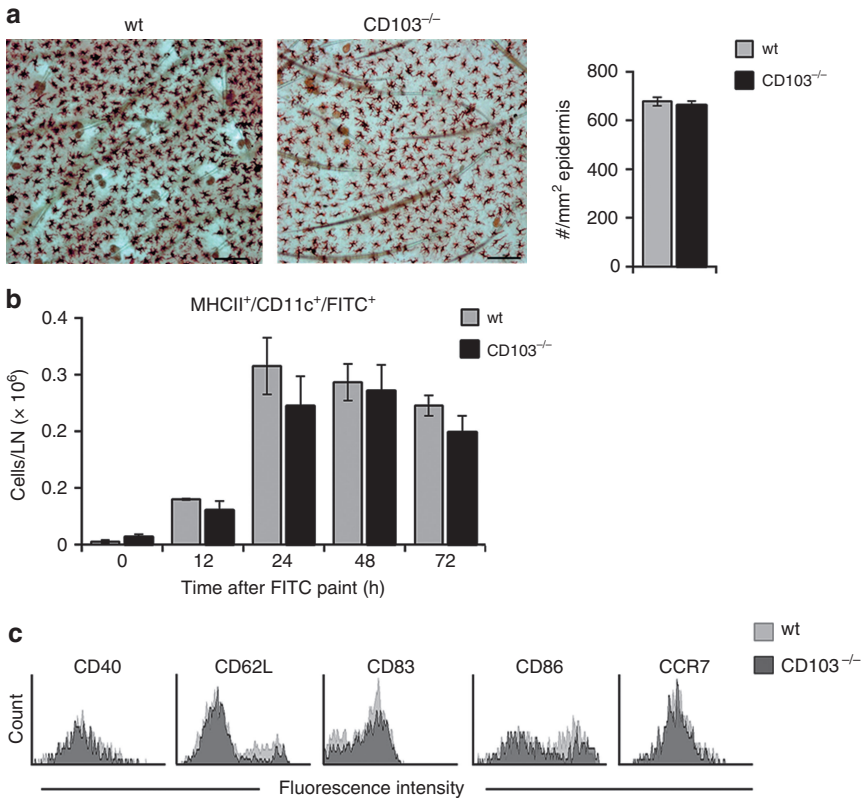

Figure 2. Skin dendritic cells of CD103$^{-/-}$ mice show normal phenotype and function. (**a**) Number of MHCII$^+$ Langerhans cells in epidermal sheets from naïve mice are displayed as number per mm^2 ($n = 6$; mean ± SEM). Representative preparations are shown (left). (**b** and **c**). After cutaneous FITC-paint, dLN cells were analyzed for (**b**) the presence of FITC-loaded MHCII$^+$CD11c$^+$ DC ($n = 3$–11; mean ± SEM) and (**c**) the expression of activation markers CD40, CD62L, CD83, CD86, or CCR7 within the MHCII$^+$CD11c$^+$FITC$^+$ populations. Representative overlay histograms of the expression of indicated surface markers are shown (one out of five independent experiments).

with respect to MHCII, CD86 and CD40 expression (Supplementary Figure S1 online).

Altered DC functions during the elicitation phase cannot be formally excluded. A recent study demonstrated that dermal dendritic cells form clusters with effector T cells during the elicitation phase leading to optimal antigen-specific activation and proliferation of T cells (Natsuaki *et al.*, 2014).

CD103 deficiency increases cutaneous T-cell infiltration in CHS

Both CD8$^+$ and CD4$^+$ T cells act as either effector or regulatory cells in CHS through the production of IFNγ and/or IL-4/IL-10 (Vocanson *et al.*, 2009; Honda *et al.*, 2013). Given that early CD8$^+$ T-cell infiltration induces skin inflammation in CHS (Bour *et al.*, 1995; Kehren *et al.*, 1999; Akiba *et al.*, 2002), we assessed T-cell infiltration into mouse ears 24 hours after OXA challenge, i.e., prior to the massive macrophage and granulocyte recruitment into the skin. While the total cell numbers of CD103$^{-/-}$ ears did not differ significantly from wt ears, the number of T cells (TCRαβ$^+$) was significantly increased in ears of CD103$^{-/-}$ mice by 62.4% ($P < 0.01$; Figure 3a). Similar differences were seen when CD8$^+$ and CD4$^+$ T-cell populations and even CD4$^+$CD25$^+$Treg cells were analyzed separately (Figure 3a). The relative composition of the T-cell infiltrate was not different between the two strains, indicating that T-cell populations increased equally (data not shown).

Likewise, analyses of skin dLN during the elicitation phase revealed a significantly more pronounced inflammation in CD103$^{-/-}$ mice, as evinced by higher total leukocyte counts as well as elevated numbers of TCRαβ$^+$, CD8$^+$, CD4$^+$, and CD4$^+$CD25$^+$ T cells ($P < 0.01$ or $P < 0.001$ in all cases; Figure 3b). Therefore, the "clinical" increase of CHS responses in CD103-deficient mice was associated with increased T lymphocyte infiltration/recruitment to sites of cutaneous inflammation as well as into the dLN.

CD103 deficiency–dependent increase of CHS is mediated by T cells in adoptive transfer experiments

To directly examine the role of primed T cells for the CHS response, naïve wt or CD103$^{-/-}$ mice were injected intravenously with dLN cells from OXA-sensitized wt or CD103$^{-/-}$ donor mice. Compositions of cell suspension were comparable between strains with 25.86 ± 0.93% and 29.48 ± 0.62% total TCRαβ$^+$ cells including 62.6 ± 0.65%/63.86 ± 1.12% CD4$^+$ T cells and 31.4 ± 0.68%/30.63 ± 1.2% CD8$^+$ T cells in wt and CD103$^{-/-}$ mice, respectively (data not shown). When these adoptively transferred mice were challenged, primed dLN cells from CD103$^{-/-}$ mice induced a

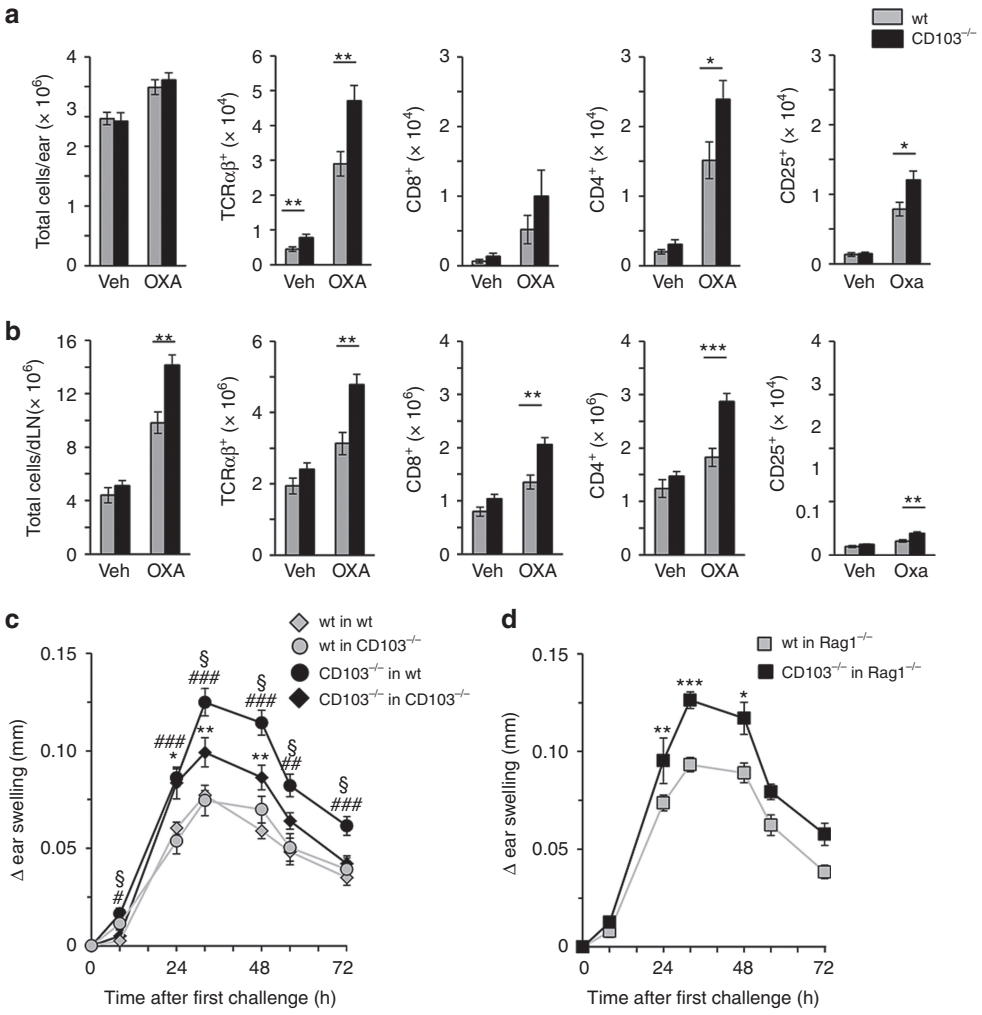

Figure 3. Increased contact hypersensitivity (CHS) reaction in CD103$^{-/-}$ mice is T-cell dependent. (**a** and **b**) Cellular infiltrations in ears (**a**) and draining lymph nodes (dLN) (**b**) were analyzed flow cytometrically 24 hours after challenge. Total cell number and the number of TCRαβ, CD4, and CD8 lymphocytes were calculated ($n=12$; mean ±SEM). (**c** and **d**) Naïve wild-type (wt) or CD103$^{-/-}$ mice (C) or Rag1$^{-/-}$ mice (**d**) were adoptively transferred with LN cells (2×10^7) isolated from oxazolone (OXA)-sensitized wt or CD103$^{-/-}$ donor mice and subsequently challenged with OXA 24 and 32 hours after transfer. Ear swelling is displayed as change in ear thicknesses ($n=9$–11; mean ±SEM). $^{*,\#,\S}P<0.05$; $^{**,\#\#,\S\S}P<0.01$; $^{***,\#\#\#,\S\S\S}P<0.001$ (unpaired Student's *t* test, *comparing transfer of CD103-deficient cells into CD103-deficient recipients versus wt cells into wt recipients, $^{\#}$comparing transfer of CD103-deficient cells into wt recipients versus wt cells into wt recipients, §comparing transfer of CD103-deficient cells into CD103-deficient recipients versus CD103-deficient cells into wt recipients).

significantly more pronounced ear swelling response compared with transfer of dLN cells from wt mice, irrespective of the recipient genotype (increase by 28.3–67.8% after 32 hours; $P<0.01$ or $P<0.001$ in all cases; Figure 3c). Thus, T cells were fully primed, homed to the skin, and elicited a CHS response, which was significantly enhanced when CD103 was lacking, supporting the hypothesis that increased CHS responses in CD103$^{-/-}$ mice were mediated by altered T-cell functions.

Interestingly, transfer of dLN cells from CD103$^{-/-}$ donor mice into wt recipient mice induced a significantly higher ear swelling response than transfer of these cells into CD103$^{-/-}$ recipient mice (increase by 126% after 32 hours; $P<0.05$; Figure 3c). We cannot explain this discrepancy by now, but it is conceivable that other cells are involved in the regulation of the skin response (Natsuaki *et al.*, 2014).

To further elucidate the role of hapten-specific lymphocytes and to exclude intrinsic T (and B)-cell biases, we adoptively transferred dLN cells from previously sensitized animals into immunodeficient Rag1$^{-/-}$ mice devoid of functional T and B cells. Again, adoptive transfer of CD103$^{-/-}$ dLN cells correlated with a significantly stronger ear swelling response compared with the transfer of wt cells (increase by 35.5% after 32 hours; $P<0.001$; Figure 3d). These results indicated that elevated CHS responses in CD103$^{-/-}$ mice were primarily, if not exclusively, transferred by primed T cells and (largely) independent of resident cells in recipient mice.

When dLNs from OXA-sensitized mice were adoptively transferred into Rag1-deficient mice and the recipients were challenged with DNFB, the minuscule ear swelling responses in mice reconstituted with either wt or CD103-deficient dLN

cells were identical to those of mice without transfer ($n=3$; mean ± SEM), thus indicating antigen specificity.

Ex vivo hapten-specific T-cell responses are augmented in CD103$^{-/-}$ mice

To further dissect the impact of CD103 on CD4$^+$ and CD8$^+$ T-cell function, we assessed the ability to recall a hapten-specific T-cell response, i.e., the proliferative response of *in vivo* primed T cells upon re-stimulation with haptenized BMDC *in vitro*. Interestingly, a higher portion of CD8$^+$ T cells, but not CD4$^+$ T cells, proliferated in CD103$^{-/-}$ mice compared with wt mice (increase by 25%; $P<0.01$; Figure 4a) indicating a role of CD103 in T-cell proliferation. It is conceivable that proliferation of CD8$^+$ T cells is either

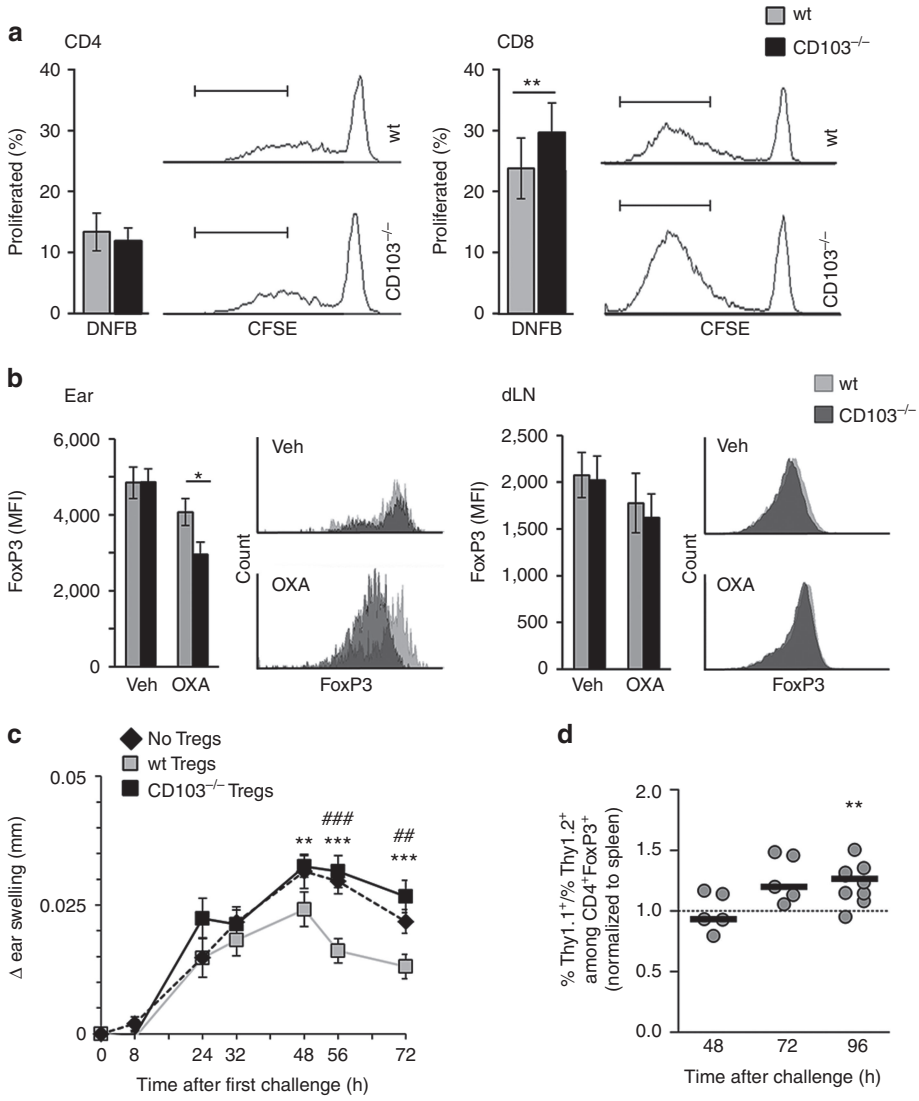

Figure 4. Altered FoxP3$^+$ Treg cells in CD103$^{-/-}$ mice during contact hypersensitivity (CHS). (**a**) CFSE-labeled draining lymph node (dLN) cells (1×10^5) from DNFB-sensitized wild-type (wt) or CD103$^{-/-}$ mice were co-cultured with DNBS-loaded wt-BMDC at a T cell:DC ratio of 10:1. Proliferation of CD4$^+$ and CD8$^+$ T cells, respectively, was analyzed after 72 hours as percentage of proliferated cells by flow cytometry ($n=3$; mean ± SEM). Representative CFSE profiles of CD4$^+$ or CD8$^+$ T cells are shown with bar indicating proliferating cells (one out of three independent experiments). $^{**}P<0.01$ (unpaired Student's *t* test). (**b**) Cellular infiltrations in ears and dLN were analyzed 24 hours after challenge as described in Figure 3, and TCRαβ$^+$CD4$^+$CD25$^+$ cells were analyzed for FoxP3 expression. Data are expressed as mean fluorescence intensitiy (MFI) and one representative profile of FoxP3 expression is shown (wt in light gray; CD103$^{-/-}$ in dark gray) ($n=9$–12; mean ± SEM). $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$ (unpaired Student's *t* test). (**c**) Naïve Rag1$^{-/-}$ mice were adoptively transferred with Treg cells (5×10^5) isolated from naïve wt or CD103$^{-/-}$ donor mice together with 2×10^7 dLN cells from OXA-sensitized wt mice. Reconstituted mice were subsequently challenged with OXA 24 and 32 hours after transfer. Ear swelling is displayed as differences in ear thickness ($n=12$; mean ± SEM). $^{**,\#\#}P<0.01$; $^{***,\#\#\#}P<0.001$ (unpaired Student's *t* test, *comparing the recipients of CD103$^{-/-}$ Treg cells with recipients of wt Treg cells, # comparing the recipients of wt Treg cells with the group "no Tregs"). (**d**) Lethally irradiated Balb/c mice reconstituted with a 1:1 mixture of bone marrow (BM) from wt (Thy1.1$^+$) and CD103$^{-/-}$ (Thy1.2$^+$) donors were sensitized and challenged with DNFB. At 48, 72, and 96 hours after the challenge, cells were isolated from spleens and ears and analyzed by flow cytometry. The graph depicts the ratios of wt (Thy1.1$^+$) and CD103$^{-/-}$ (Thy1.2$^+$) among CD4$^+$FoxP3$^+$ cells normalized to the ratios in the spleen ($n=5$ or 10; median; the dotted line indicates a ratio of 1). $^{**}P<0.01$ (Wilcoxon signed-rank test compared with a hypothetical value of 1). BMDC, bone marrow–derived dendritic cell; CFSE, carboxyfluorescein diacetate succinimidyl ester; DNBS, 2,4-dinitrobenzenesulfonic acid hydrate.

affected directly or the higher turnover is due to impaired regulatory mechanisms, a hypothesis that was tested in the following experiments.

Augmented CHS responses in CD103-deficient mice are associated with alterations of FoxP3$^+$ Treg cells

Treg cells are a critical CD4$^+$ T-cell population controlling immune homeostasis and regulating inflammation (Sakaguchi *et al.*, 2008; Rudensky, 2011). Many studies have described their capability to suppress CHS responses by limiting the expansion of hapten-primed effector CD8$^+$ T cells (Dubois *et al.*, 2003; Gorbachev and Fairchild, 2004; Ring *et al.*, 2006; Ring *et al.*, 2011; Honda *et al.*, 2013). In consequence, enhanced and prolonged inflammation might result from diminution of Treg activity, which can, in principle, be caused by defective function, traffic, and/or retention.

Besides high expression of the IL-2 receptor α-chain (CD25), the best Treg marker is the transcriptional regulator forkhead-box P3 (FoxP3), which is restricted to the Treg lineage and is thought to be a master regulator for Treg maturation and function (Fontenot *et al.*, 2005). When we assessed FoxP3 expression within the cutaneous CD4$^+$CD25$^+$ T-cell population 24 hours after challenge, its expression in this population was higher by 37.7% in wt compared with CD103$^{-/-}$ mice ($P<0.05$; Figure 4b). Because the intensity of FoxP3 expression is linked to the functionality of Treg cells (Rudensky, 2011), these data indicate a higher suppressive capacity of wt Treg cells.

Treg cells can, in principle, suppress immune reactions either at peripheral effector sites or during the expansion and differentiation of T cells in the dLN (Dubois *et al.*, 2003; Vocanson *et al.*, 2009). However, the FoxP3 expression levels within the dLN were not significantly different in our two genotypes (Figure 4b). Thus, we conclude, that CD103 was involved in *in situ* Treg function within the skin, but not at the level of dLN.

Consistent with this notion, CD103 is thought to facilitate adhesion, retention, and morphogenesis of lymphocytes within epithelial tissues (Kilshaw, 1999; Schön *et al.*, 1999; Schlickum *et al.*, 2008), including inflamed skin, but not peripheral lymph nodes (Lehmann *et al.*, 2002; Siegmund *et al.*, 2005; Suffia *et al.*, 2005). Indeed, in wt mice, we determined that CD103 was poorly expressed on Treg cells within lymphoid tissues, but highly expressed within the skin (20% in dLN vs. 80% in the skin of total Treg cells; data not shown), which is consistent with previous reports (Lehmann *et al.*, 2002; Anz *et al.*, 2011).

Treg cells from CD103-deficient mice fail to suppress CHS effector reactions

To further determine the role of Treg cells for the suppression of CHS effector reactions, we adoptively transferred naïve Treg cells into reconstituted Rag1$^{-/-}$ recipient mice. As expected, transfer of Treg cells from wt mice strongly suppressed the ear swelling in Rag1$^{-/-}$ recipient mice after challenge with OXA (Figure 4c). In contrast, ear swelling of Rag1$^{-/-}$ recipient mice reconstituted with Treg cells from CD103$^{-/-}$ mice was comparable with that of Rag1$^{-/-}$ mice without any Treg cells (Figure 4c). These data demonstrate that CD4$^+$CD25$^+$ Treg cells lacking CD103 are unable to regulate cutaneous inflammatory reactions.

CD103 supports the retention of Treg cells in CHS

Besides the involvement of CD103 in regulatory functions of Treg cells, its expression has been implicated in T-cell recruitment and/or retention during inflammation, e.g., in *Leishmania*-mediated skin inflammation (Suffia *et al.*, 2005). We further investigated the role of CD103 expression on Treg cells for their localization to inflamed skin in a slightly modified CHS model using repetitive sensitization with the strong hapten DNFB. For this purpose, mixed bone marrow (BM) chimeric mice were generated by reconstituting lethally irradiated mice with BM from wt and CD103$^{-/-}$ donor mice at a 1:1 ratio. The proportion of wt (Thy1.1$^+$) and CD103$^{-/-}$ (Thy1.2$^+$) Treg cells detected within the inflamed ears upon hapten challenge was then monitored over time by flow cytometry. Of note, 96 hours after the challenge, wt Treg cells were detected at significantly higher numbers compared with their CD103$^{-/-}$ counterparts (increase by 25%; $P<0.01$; Figure 4c).

Taken together, we conclude that the higher number of infiltrating TCRαβ T cells in the skin of CD103$^{-/-}$ mice is a consequence of insufficient termination or quenching of the immune response due to both altered Treg function and retention.

DISCUSSION

The function of the α_E(CD103) integrin for immune responses is still enigmatic in many aspects. We have now provided experimental evidence that this receptor is important for the regulation of allergic CHS in mice. By using two different haptens, namely OXA and 1-chloro-2,4-dinitrobenzene, we could show that this dysregulation is independent of the model used, T helper type 1 or T helper type 2, respectively.

Although CD103 was expressed by subsets of both T lymphocytes and DCs, the latter potentially possessing regulatory functions (Annacker *et al.*, 2005; Coombes *et al.*, 2007; Honda *et al.*, 2013), we could not detect altered DC functions during the pathogenesis of CHS. The skin harbors different subsets of DCs, which seem not only to have different but also redundant roles during CHS (Edelson *et al.*, 2010; Noordegraaf *et al.*, 2010; Romani *et al.*, 2010). Instead, the lack of CD103 on Treg cells was associated with the enhanced cutaneous inflammation during CHS in CD103-deficient mice compared with their wt counterparts. Of note, the function of CD103 appeared to be dichotomous: On the one hand, CD103 was associated with higher expression of FoxP3 in Treg cells, and thus it is likely that Treg cell function is altered. On the other hand, it contributed to the retention of Treg cells at sites of inflammation. CD103 is relevant for tissue-specific localization not only of CD8$^+$ epidermal T cells (Pauls *et al.*, 2001) and CD8$^+$ resident memory T cells (Gebhardt and Mackay, 2012), but also of CD4$^+$CD25$^+$ Treg cells (Suffia *et al.*, 2005). Our findings extend previous studies showing that CD103 expression on CD4$^+$CD25$^+$ Treg cells, but not on CD4$^+$CD25$^-$ effector T cells, was necessary for

their retention at the site of *Leishmania major* infection. As a consequence, CD103$^+$ Treg cells interfered with the clearance of this infection (Suffia *et al.*, 2005). How and to what extent CD103-expressing CD4$^+$ and/or CD8$^+$ effector/resident-memory T cells are involved in the pathogenesis of CHS is not clear (Gebhardt and Mackay, 2012).

By contrast, in CD103-expressing tumor-infiltrating FoxP3$^+$ Treg cells, CD103 was not involved in homing or retention of FoxP3$^+$ cells within the tumor tissue, but rather in mediating their suppressive function (Anz *et al.*, 2011). Indeed, CD103 expression on tumor-infiltrating FoxP3$^+$ Treg cells increased the potency of these cells (Lehmann *et al.*, 2002; Anz *et al.*, 2011), suggesting that Treg cells from CD103$^{-/-}$ mice might be ineffective regarding their suppressive capacity. This supports previous studies suggesting that CD103 is a marker for a subset of Treg cells with high suppressive capacity (Lehmann *et al.*, 2002; Banz *et al.*, 2003).

As of now, the exact mechanisms of how Treg cell functions are controlled are not fully understood. CD103 expression on Treg cells may have different implications when studying different inflammatory processes, such as colitis, tumor growth, or skin infection. Thus, the concept of a tissue-specific regulation is a reasonable hypothesis, although modifying factors have not been identified yet. In addition, the mechanistic link between CD103 and FoxP3 expression remains unknown. Although the molecular interplay between CD103 expression versus CD103 ablation and FoxP3 regulation is not known, it has become clear that the level of FoxP3 expression is directly linked to the suppressive capacity of Treg cells (Rudensky, 2011), and instability of FoxP3 expression seems to be linked to the flexible adaptation of regulatory functions (Feuerer *et al.*, 2009). Thus, an impaired suppressive capacity of Treg cells or even a conversion into potential effector T cells due to phenotypic alterations and T-cell plasticity might be responsible, at least in part, for the enhanced CHS responses in CD103$^{-/-}$ mice. The regulation of FoxP3 expression in CD103$^+$ and CD103$^-$ Treg cells and their suppressvie capacity is, therefore, an interesting open question.

Moreover, expression of both CD103$^-$ and FoxP3 is induced by transforming growth factor (TGF)-β.[14] Indeed, TGFβ induces CD103 expression on T cells and even the generation of CD103-expressing FoxP3$^+$ Treg cells from naïve T cells *in vitro* (Pauls *et al.*, 2001; Rao *et al.*, 2005). Thus, increased levels of TGFβ, which are present in the local microenvironment during inflammatory processes, might contribute to the Treg-mediated control of inflammation by a feed-forward-loop, as much as CD103 expression mediates increased Treg retention which leads to the upregulation of FoxP3 due to the presence of TGFβ. This seems to be likely for CD103$^+$ tumor-infiltrating Treg cells (Anz *et al.*, 2011) or Treg cells during gastrointestinal parasite infections (Reynolds and Maizels, 2012). It is conceivable that this is true for Treg cells during skin inflammation as well, where CD103-mediated retention exposes Tregs to TGFβ within the epithelial microenvironment for a longer period of time, thus enhancing their FoxP3 expression. To further address the question of T-cell plasticity, it is likely that previously non-regulatory T cells are converted to CD103$^+$ Treg cells in the presence of TGFβ.

It is reasonable to assume that unraveling the molecular crosstalk between CD103 and FoxP3 will provide a handle on targeting regulatory mechanisms in inflammation. It is also conceivable that CD103 is involved in transcriptional regulation of other surface molecules, chemokines, or cytokines, which are crucial for traffic and function of Treg cells, thus acting indirectly. In any case, our study of CHS now provides experimental evidence that CD103 is involved in the "fine-tuning" of T-cell–mediated skin inflammation through influencing retention and FoxP3 expression of Treg cells.

MATERIALS AND METHODS

Mice

C57BL/6J wt, Rag1$^{-/-}$ (The Jackson Laboratory, Bar Harbor, ME) and α_E(CD103)-deficient (CD103$^{-/-}$) mice (Schön *et al.*, 1999) backcrossed on C57BL/6J genetic background were bred at the animal care facility of the University Medical Center Göttingen. For chimeric mice experiments, Balb/c (Bundesinstitut für Risikobewertung, BfR, Berlin, Germany), congenic Thy1.1xBalb/c and CD103$^{-/-}$ mice on a Balb/c background were bred at the animal facility of the German Rheumatism Research Center (DRFZ, Berlin, Germany). Mice were housed under specific pathogen-free conditions with food and water *ad libitum*. Age-matched mice (8–12 weeks) were used in all experiments. All animal experiments were performed in accordance with the institutional, state, and federal guidelines and were approved by local institutional animal care advisory committees and the respective permit authorities.

Allergic CHS

Mice shaved on their lower back were sensitized once topically with 100 µl of 3% OXA (w/v) (4-ethoxymethylene-2-phenyl-2-oxazolin-5-one; Sigma Aldrich, Munich, Germany) or 1% 1-chloro-2,4-dinitrobenzene (Sigma Aldrich). On d5, mice were challenged with 20 µl of 1% OXA or 1% 1-chloro-2,4-dinitrobenzene on the right ear and 20 µl of vehicle (ethanol) on the left ear. Ear thicknesses were measured using a digital caliper (Kroeplin, Schlüchtern, Germany), and the increase in ear swelling was determined as the normalized difference of ear thicknesses between the OXA-treated versus the vehicle-treated side of the same mouse.

For experiments using chimeric mice, mice were sensitized with 20 µl of 0.5% DNFB (Sigma Aldrich) in acetone/oil (4:1; v/v) on d0 and d2. On d4, mice were challenged with 10 µl/ear of 0.2% DNFB. Single-cell suspensions of ears and spleen were prepared and assessed by flow cytometric analyses.

Irritant CHS

Acute irritant inflammatory reactions were induced by paining 20 µl of 2.5% Croton oil (Sigma Aldrich) in acetone (v/v) or the vehicle (acetone) to the right ear. Increase in ear swelling was measured as described above.

Migration, homing, and maturation assay *in vivo*

For migration assays, mice were painted once with 200 µl of 1% FITC Isomer I (Merck Millipore, Schwalbach, Germany) in acetone/DBP

(1:1; v/v). Cells from the inguinal dLN were isolated and processed further for flow cytometric analyses.

Adoptive transfer experiments

On d5 after sensitization, dLNs were prepared and single cell suspensions of 2×10^7 sensitized dLN cells in 150 µl phosphate-buffered saline were transferred *via* retro-orbital injection into anesthetized recipient mice. For adoptive transfer of Treg cells, CD4$^+$CD25$^+$ cells were enriched from naïve mice using the "Regulaotry T cell Isolation Kit" (Miltenyi, Bergisch Gladbach, Germany) and 5×10^5 Treg cells were injected together with dLN per recipient mouse. After 24 and 32 hours, the mouse ears were challenged with 1% OXA to induce CHS.

Generation of chimeric mice

Recipient Balb/c mice received Enrofloxacin (Bayer, Leverkusen, Germany) in sterile drinking water *ad libitum* for 1 week. Subsequently, mice were lethally irradiated (10.5 Gy) using a gamma irradiation device (Rad Source Technologies, Suwanee, GA). One day later, BM cells from donor mice were depleted from mature CD90$^+$lymphocytes (e.g., Thy1.1$^+$ in wt (Balb/cxThy1.1) and Thy1.2$^+$ in CD103$^{-/-}$ donors) by magnetic depletion using an AutoMACSPro separator (Miltenyi) according to the manufacturer's instructions. CD90-depleted BM from wt and CD103$^{-/-}$ donors was then combined at a 1:1 ratio and 5×10^6 cells were injected intravenous into irradiated recipient mice. Mice continuously received the antibiotic regimen with Enrofloxacin for another 4 weeks. Five weeks later, chimerism was certified in peripheral blood and successfully engrafted mice were subjected to experimental procedures after 5–6 weeks of reconstitution.

Generation of BMDCs

BMDCs were generated as described previously (Lutz *et al.*, 1999; Braun *et al.*, 2010). Briefly, BM cells from femur and tibiae were cultured (2×10^6) in the presence of 10% (v/v) murine GM-CSF-containing supernatant (Zal *et al.*, 1994) for 8 days. Subsequently, non-adherent cells were loaded with 0.5 mM DNBS (2,4-dinitrobenzenesulfonic acid hydrate; Sigma Aldrich) for 24 hours and assessed for expression of the indicated cell surface markers and used in hapten-specific T-cell proliferation assays.

Ex vivo hapten-specific T-cell proliferation

For *ex vivo* proliferation assays, mice were sensitized with 100 µl 0.3% DNFB in acetone/oil (4:1; v/v) and on day 5 dLN cells were isolated. Carboxyfluorescein diacetate succinimidyl ester-stained (1×10^5; Sigma Aldrich) cells were co-cultured with DNBS-stimulated BMDCs at the indicated for 72 hours. Cell division was analyzed by measuring carboxyfluorescein diacetate succinimidyl ester on CD4$^+$ and CD8$^+$ T cells using flow cytometry.

Histology and immunohistochemistry

For morphological analyses, mouse ears were either deep frozen in Tissue-Tek O.C.T. Compound (Sakura Finetek, Leiden, Netherlands) or fixed in 4% paraformaldehyde solution (v/v) and subsequently embedded in paraffin. For immunohistochemical analyses of epidermal sheets, ear halves were incubated in 50 mM EDTA/phosphate-buffered saline (v/v) and the epidermis was peeled off the connective tissue.

Hematoxylin and eosin-staining was performed using paraffin-embedded sections of 3 µm. For Giemsa-staining, samples were incubated with Giemsa-solution (Sigma Aldrich) and differentiated with 0.1% acetic acid (v/v). During immunohistochemistry, 5 µm sections of frozen samples were fixed with methanol, followed by inactivation of endogenous peroxidase with 0.3% H_2O_2, blocking of endogenous biotin with Biotin-Blocking System (DAKO, Hamburg, Germany) and unspecific binding with 5% rabbit serum. The samples were incubated with the respective primary antibody (CD11b, M1/70, Immunotools, Friesoythe, Germany; Gr1, RB6–8C5, BD, Heidelberg, Germany) followed by incubation with the relevant horseradish peroxidase-labeled secondary antibody (Vector, Burlingame, CA). After incubation with streptavidin-peroxidase (Vector) and AEC$^+$-Solution (DAKO), samples were finally counterstained with hematoxylin (DAKO). Microscopic analyses were performed using an Axioskop2 (Zeiss, Göttingen, Germany) microscope and the Axiovision software Rel 4.7 (Zeiss).

Flow cytometry (fluorescence-activated cell sorting analysis)

To prepare single cell solution, ears were digested in Liberase solution (Roche Diagnostics, Mannheim, Germany), homogenized and filtrated. Lymph nodes were collected, homogenized, and filtrated on ice. After blocking Fc receptors with anti-mouse CD16/32 mAb (2.4G2; BD), cells were subsequently stained with indicated mAb: Gr1 (RB6–8C5; Immunotools), CD11b (M1/70; BD), F4/80 (CI:A3-1; Serotec, Puchheim, Germany), TCRαβ (H57–597; BioLegend, San Diego, CA or Immunotools), CD4 (RM4–5; BD), CD4 (H129.19; BioLegend), CD8 (53-6.7; BioLegend or Miltenyi), CD8 (YTS169.4; Immunotools), CD25 (PC61; BioLegend), CD103 (2E7; BioLegend), CD11c (N418; BioLegend), MHC class II (M5/114.15.2; BioLegend), CD40 (2/23; BD), CD86 (GL1; BD), CD62L (MEL-14; Immunotools), CCR7 (4B12; BioLegend), CD83 (Michel-19; BioLegend), CD90.1 (Thy1.1; HIS51; BD), CD90.2 (Thy1.2; 53-2.1; BD). Intracellular staining with anti-FoxP3 (MF-14; BioLegend) was performed using the FoxP3 Buffer Kit (BD) or anti-FoxP3 (FJK-16s; eBioscience, San Diego, CA) with Mouse Regulatory T Cell Stainig Kit (eBioscience). Viability was determined using 7-aminoactinomycin D solution (BD) or Zombie-NIR (BioLegend). The stained cells were detected in the BD FACSCantoII (BD) and data were analyzed by the FACSDiva software (BD). Data from chimeric mouse experiments were assessed with LSRII operated with FACSDiva software (BD) and analyzed by FlowJo-software (TreeStar, Ashland, OR).

Statistics

Statistical analyses were performed using the unpaired, two-tailed Student's *t*-test to compare groups of independent samples. For statistical analyses of experiments using chimeric mice, the "Wilcoxon signed-rank" test was performed in comparison to a hypothetical value of 1. *P*-values < 0.05 were considered statistically significant.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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