

Foxp3⁺ Cells Control Th2 Responses in a Murine Model of Atopic Dermatitis

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The role of Foxp3⁺ regulatory T (Treg) cells in atopic dermatitis (AD) is still unclear. In a murine AD model, the number of Foxp3⁺ cells increased in the allergen-exposed skin area and in the secondary lymphoid organs. Both Foxp3⁺ and Foxp3[−] IL-10⁺ T cells accumulated at the site of allergen exposure, and CD103⁺ effector/memory Foxp3⁺ Treg cells expanded gradually in the lymph nodes throughout the sensitization protocol. The depletion of Foxp3⁺ Treg cells led to significantly exacerbated skin inflammation, including increased recruitment of inflammatory cells and expression of T helper type 2 cytokines, as well as elevated serum IgE levels. The effect of depleting Treg cells during epicutaneous sensitization was mirrored off by a stronger inflammatory response also in the lungs following airway challenge. Thus, Treg cells have an important role in controlling AD-like inflammation and the transfer of allergic skin inflammation to the lungs.

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INTRODUCTION

Atopic dermatitis (AD) is a chronic inflammatory skin disease manifested by dry and itchy skin, skin lesions with increased numbers of T cells, eosinophils and mast cells, and elevated serum IgE levels in 80% of the patients. The life-time prevalence of AD has increased during the past decades, reaching 17% among school-aged children in some Western societies (Boguniewicz and Leung, 2010). Being the first step in the so-called atopic march, AD patients often develop food allergy, allergic rhinitis, or asthma later in life (Spergel, 2010).

Regulatory T (Treg) cells control immune homeostasis and balance immune responses during inflammation. Treg cells suppress immune responses by interacting with effector T

(Teff) cells or antigen-presenting cells (APC; Miyara and Sakaguchi, 2007; Vignali *et al.*, 2008), and their regulatory role in T helper type 2 (Th2) responses has been well established. Immune dysregulation, polyendocrinopathy, enteropathy, X-linked patients with a loss-of-function mutation in the Foxp3 gene suffer from food allergies, eczema, elevated IgE levels, and eosinophilia (Chatila *et al.*, 2000; Bennett *et al.*, 2001). In mice, the lack of Foxp3 leads to elevated serum IgE levels, eosinophilia, and increased production of Th2 cytokines (Lin *et al.*, 2005). Foxp3 controls signal transducer and activator of transcription (STAT6)-dependent Th2 polarization (Lin *et al.*, 2005) and interacts physically with GATA-3, most likely interfering with GATA3-dependent transcription of IL-4, IL-5, and IL-13 (Dardalhon *et al.*, 2008).

The role of Treg cells in AD is unclear. Treg cells can be divided into natural Foxp3⁺ Treg cells (nTreg) and either Foxp3⁺ or Foxp3[−] induced Treg cells (iTreg), including Foxp3[−] IL-10⁺ Tr1 and Foxp3[−] TGFβ⁺ Th3 cells (Belkaid, 2007; Vignali *et al.*, 2008). Some studies have observed increased numbers of peripheral blood Foxp3⁺ Treg cells in AD patients (Ou *et al.*, 2004; Reefer *et al.*, 2008; Ito *et al.*, 2009), whereas others have not (Szegedi *et al.*, 2009). Instead, in the latter study, the number of Tr1 cells was increased in the blood in AD patients. In the skin, in acute and chronic AD lesions, both the presence and absence of Foxp3⁺ cells have been reported, as well as the accumulation of Tr1 cells (Verhagen *et al.*, 2006; Schnopp *et al.*, 2007; Szegedi *et al.*, 2009). Thus, the role of Treg cells in AD needs to be further clarified.

To pin down the role of Foxp3⁺ Treg cells in AD, we analyzed Treg cells and studied the effect of depleting Foxp3⁺ Treg cells in the AD experimental mouse model. Our results show that Foxp3⁺ Treg cells are essential regulators of Th2-type inflammatory responses in the skin, and the effect of Treg cell depletion during skin sensitization transfers to the lung.

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Abbreviations: AD, atopic dermatitis; APC, antigen-presenting cell; BALF, bronchoalveolar lavage fluid; DC, dendritic cell; dLN, draining lymph node; DT, diphtheria toxin; EGFP, enhanced green fluorescent protein; iTreg, induced regulatory T cell; LN, lymph node; nTreg, natural regulatory T cell; OVA, ovalbumin; Teff, effector T; Treg, regulatory T

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RESULTS

Topical ovalbumin (OVA) sensitization increased cellular infiltration and the Th2 inflammatory response

Wild-type (WT) mice were sensitized as described in Materials and Methods and Figure 1a. Compared with phosphate-buffered saline (PBS)-treated mice, OVA-treated mice had significantly thickened skin with increased numbers of infiltrated eosinophils, T cells, and neutrophils (Figure 1b). The expression of IL-4, IL-13, and IL-10 mRNA (Figure 1c) and IL-10 protein (Supplementary Figure S1c online) in the skin was significantly increased in the OVA group, whereas IFN- γ remained low in both groups (Figure 1c). Serum levels of total and OVA-specific IgE were significantly elevated, whereas OVA-specific IgG2a remained low (Figure 1d).

Foxp3+ Treg cells accumulated in the OVA-treated skin

The published data concerning the engagement of Foxp3+ cells in AD has been controversial (Verhagen *et al.*, 2006; Schnopp *et al.*, 2007; Szegedi *et al.*, 2009). In this study, Foxp3+ cells clearly accumulated in the skin in the OVA-treated mice, but not in the PBS-treated controls (Figure 1e). Yet, the ratio of Foxp3+CD3+ to Foxp3-CD3+ cells

remained the same in the skin (Figure 1f) and in the draining LNs (Figure 1g) in both groups. Moreover, OVA treatment resulted in the accumulation of Foxp3- and Foxp3+IL-10+ -expressing cells in the skin (Supplementary Figure S1a online). Finally, the ratio of CD103-expressing effector/memory-type Treg cells increased steadily in the draining lymph nodes (dLNs) throughout the AD protocol, starting at 15% at the beginning of the protocol and reaching 25% at the end of the protocol. In the skin, most Foxp3+ Treg cells expressed CD103, with slightly higher percentages of CD103+ Tregs after OVA treatment (Figure 1h).

Treg cells from OVA-treated mice were less suppressive than Treg cells from PBS-treated mice

The IL-4-STAT6 pathway interferes with the generation of TGF- β -induced Foxp3+ Treg cells, as well as with the suppressive function of nTreg cells (Mantel *et al.*, 2007; Dardalhon *et al.*, 2008; Hadjur *et al.*, 2009). The OVA-treated mice underwent a strong Th2 shift, producing increased levels of Th2 cytokines in the OVA-exposed tissues (Figure 1c) and strongly elevated levels of IgE in the serum (Figure 1d). The Th2 shift was also evident *ex vivo* in

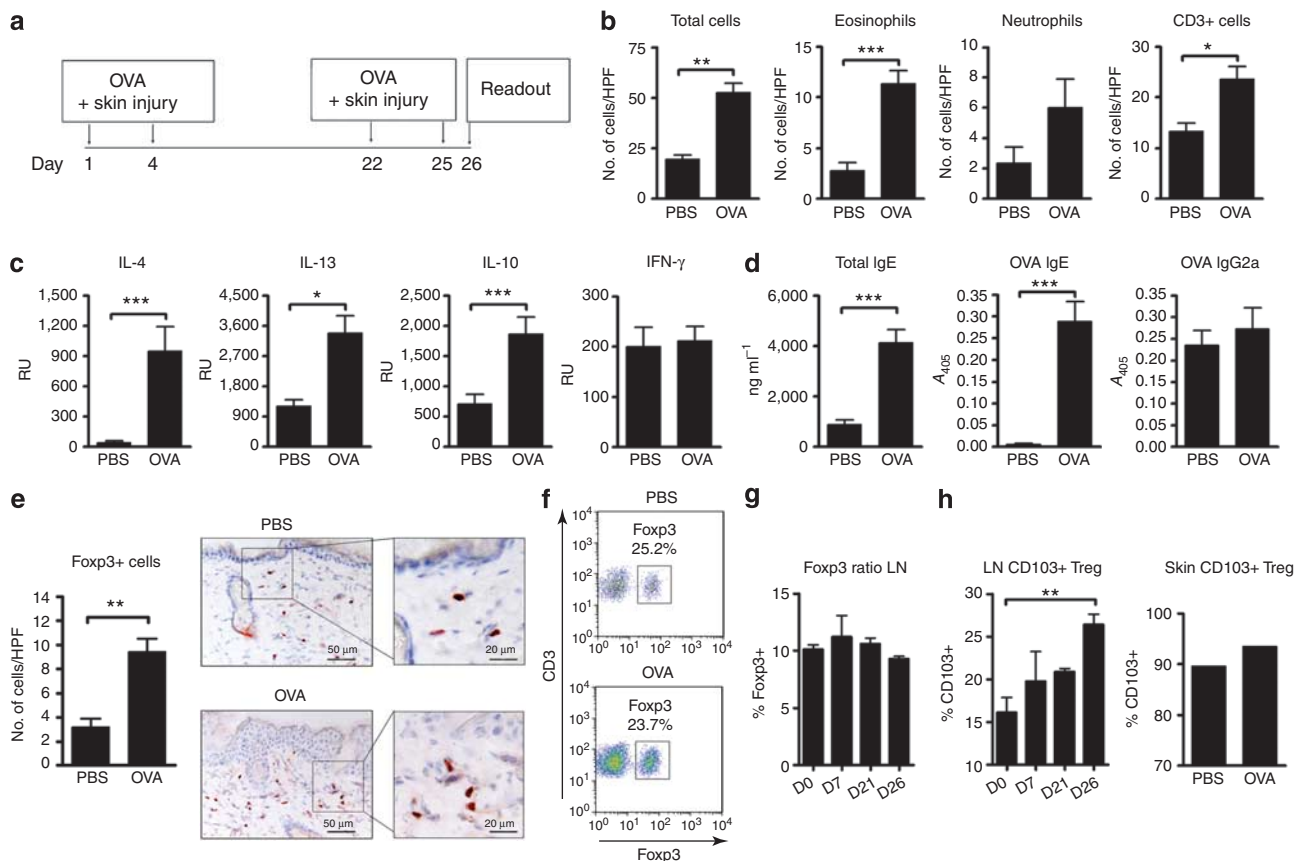


Figure 1. The AD model. (a) Topical ovalbumin (OVA) treatment led to (b) increased leukocyte recruitment and (c) elevated mRNA levels of IL-4, IL-13, and IL-10 in the skin, as well as (d) increased IgE serum levels in OVA-sensitized mice (OVA), compared with the control group phosphate-buffered saline (PBS). (e) Foxp3+ cell numbers in the skin increased in the OVA mice (bar = 50 μ m; in the insets bar = 20 μ m), (f) but the Foxp3+ : Foxp3- T-cell ratio remained similar in the PBS and OVA groups. (g) Foxp3+ / Foxp3- ratios in the dLNs were constant throughout the experiment (D0, 7, 21, 26 = analysis on days 0, 7, 21 and 26), (h) but the share of CD103+ Foxp3+ cells increased toward the end of the protocol. The percentage of CD103+ Foxp3+ cells increased slightly in the skin after OVA exposure, $n = 5-8$. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; bars represent mean $\pm$ SEM. dLN, draining lymph node; HPF, high-power field; RU, relative units; Treg, regulatory T cells.

α -CD3-stimulated LN cells (Figure 2a). To study the possible effect of the Th2 shift on the suppressive capacity of Foxp3 + Treg cells, sorted CD4 + Foxp3 – cells from the dLNs of OVA-treated mice were cocultured with CD4 + Foxp3 + T cells from either PBS- or OVA-treated mice. As shown in Figure 2b, Treg cells from the OVA-treated mice had reduced capacity to suppress the proliferation of the same responder T cells.

Diphtheria toxin (DT) treatment depleted most of the Foxp3 + cells

To assess the role of Treg cells in OVA-induced skin inflammation, mice were administered DT to transiently deplete Treg cells in DEREG mice (Figure 3a). The DT treatment resulted in significantly downregulated Foxp3 both at the mRNA and protein levels at the end of the AD protocol

(Figure 3b and c). Flow cytometric analyses of dLN cells further confirmed the greatly reduced expression of Foxp3 (Figure 3d).

Depletion of Foxp3 + cells significantly augmented the inflammatory response in OVA-sensitized mice

The AD model includes mild skin injury induced by tape stripping before applying OVA. The disruption of the skin barrier facilitates the penetration of the allergen through the epidermal layers, and is indispensable for the induction of OVA sensitization in the mice. The repeated tape stripping induced a modest skin inflammation in the WT PBS-treated irritation control mice compared with naive WT mice, including the recruitment of a small amount of CD3 + cells to the skin. Yet, these cells did not produce notable amounts of IL-4 or IL-13 (Figure 4a–c). Similar to naive WT mice, there

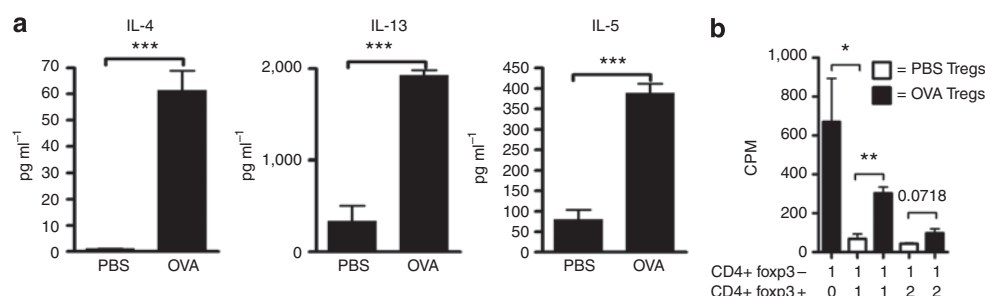


Figure 2. Th2 shift and Treg suppressive capacity in OVA sensitized mice. (a) dLNs from PBS- and OVA-treated mice were stimulated *ex vivo* with α -CD3, followed by measurement of Th2-type cytokines, $n=4$. (b) Treg cells from the OVA-treated mice had reduced capacity to suppress the proliferation of the responder T cells, $n=3$. * $P<0.05$, ** $P<0.01$, *** $P<0.001$; bars represent mean + SEM. CPM, counts per minute; dLN, draining lymph node; OVA, ovalbumin; PBS, phosphate-buffered saline; Th2, T helper type 2; Treg, regulatory T cells.

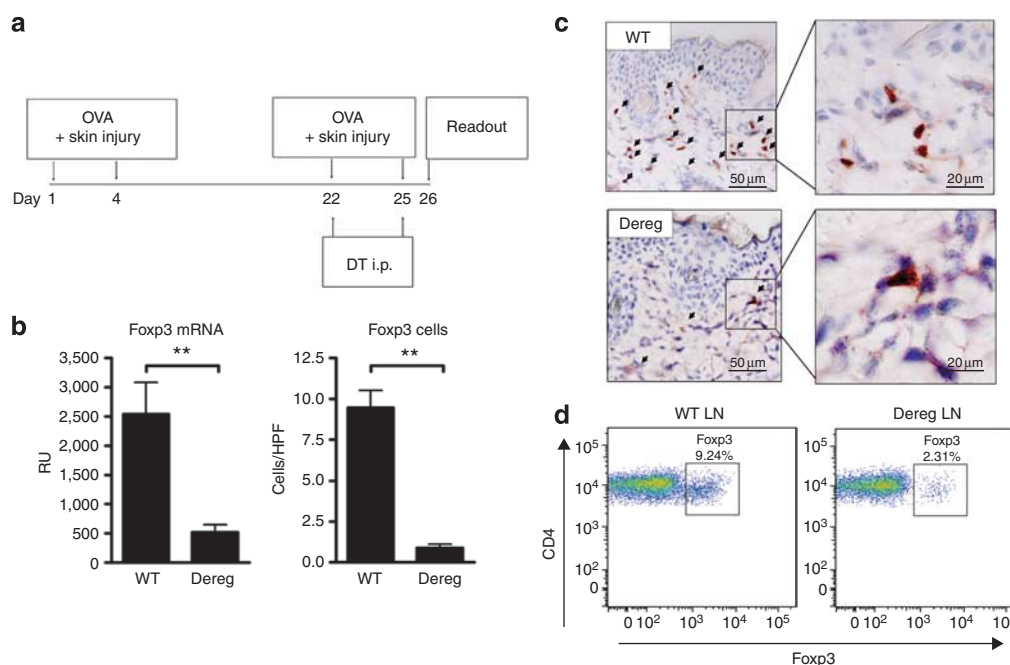


Figure 3. Depletion of Foxp3 + cells in DEREG mice. (a) Depletion protocol. (b) Foxp3 mRNA expression in the skin after DT treatment, and the number of Foxp3 + cells in the skin, based on immunohistochemical (IHC) staining, $n=5-8$. (c) IHC staining of Foxp3 in the skin (bar = 50 μ m; in the insets bar = 20 μ m) and (d) flow cytometric analysis of Foxp3 + cells in the dLN. ** $P<0.01$, bars represent mean + SEM. DT, diphtheria toxin; HPF, high-power field; LN, lymph node; OVA, ovalbumin; RU, relative units; WT, wild type.

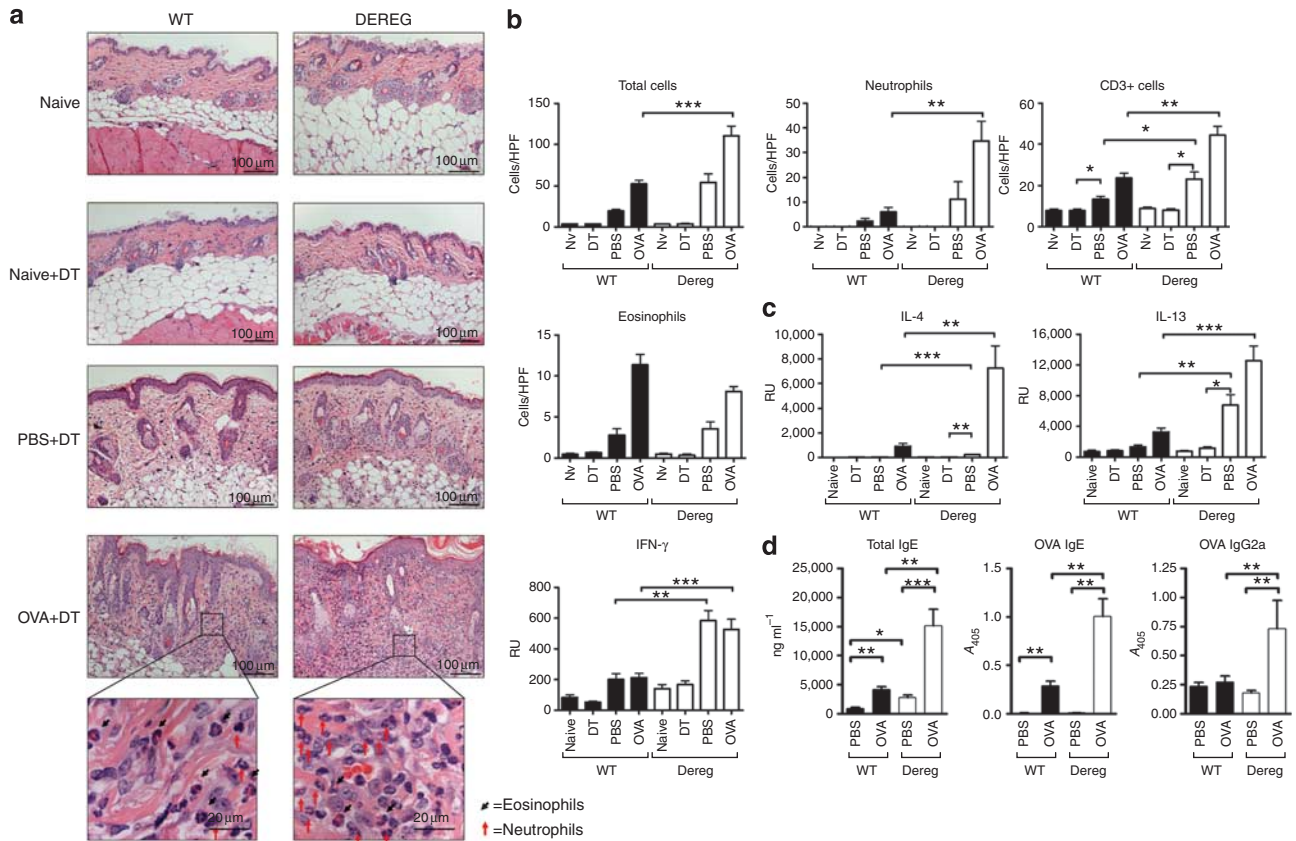


Figure 4. The effect of Treg cell depletion on AD-like skin inflammation. (a) Hematoxylin-eosin (H&E) staining of skin samples from wild-type (WT) mice (left panels) and DERE mice (right panels). Bar = 100 µm, in the insets bar = 20 µm. (b) Inflammatory cell counts, (c) inflammatory cytokine mRNA in the skin, and (d) OVA-specific IgE and IgG2a serum levels were elevated in the Foxp3⁺-depleted, OVA-treated mice, $n = 5-8$. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; bars represent mean + SEM.

were no signs of skin inflammation in the DT-treated naive DERE mice (Figure 4a). However, in the DT-treated DERE PBS-treated irritation controls, there was a remarkable influx of CD3⁺ T cells and expression of some inflammatory mediators at the site of tape stripping (Figure 4a-c). Tape stripping combined with OVA exposure resulted in significantly increased recruitment of inflammatory cells to the skin, increased local expression of Th2-type cytokines, and elevated levels of total and OVA-specific IgE in the serum in WT mice (Figure 4). In the OVA DERE mice, these parameters were further increased compared with WT mice (Figure 4, Supplementary Figures S1 and S2 online). In the dLNs, the depletion of Treg cells led to significantly elevated numbers of OVA-specific IL-13-producing T cells, but marginal numbers of OVA-specific IFN-γ-expressing T cells (Supplementary Figure S3 online).

To assess the phenotype of the remaining/regenerated subsets of Foxp3⁺ and Foxp3⁻ regulatory T cells after the depletion of Foxp3⁺ T cells in the DERE mice, LN cells were stained for PD-1 and Helios. PD-1 is expressed both by nTreg and iTreg cells, but at a higher level by iTreg cells, and is essential for the conversion of naive T cells into iTreg cells (Haribhai *et al.*, 2011). Helios, in turn, is a marker for nTreg cells (Thornton *et al.*, 2010). In the DERE mice, the frequency of PD-1⁺ Foxp3⁺ and PD-1⁺ Foxp3⁻ cells was significantly

higher, and the percentage of Helios⁺ Foxp3⁺ T cells slightly lower (Supplementary Figure S4 online) than in the WT mice.

T cells and dendritic cells proliferated and were activated in the absence of Foxp3⁺ cells

Treg cells control inflammation by suppressing Teff cells directly or via dendritic cells (DCs; Tang and Bluestone, 2008). The depletion of Foxp3⁺ cells led to significantly increased numbers and proliferation rates of CD3⁺CD4⁺ and CD3⁺CD8⁺ T cells, as well as CD11c⁺CD3⁻ DCs in the dLNs of OVA-treated mice (Figure 5a and b). Moreover, a larger part of the T-cell and DC populations expressed activation marker CD69 (Figure 5c) and costimulatory molecules CD80 and CD86 (Figure 5d), respectively. CD80 and CD86 were also elevated at the mRNA level in the skin in the DERE mice (Figure 5e). Finally, there was a clear accumulation of CD11b^{hi}CD11c⁺ DCs (Hammad *et al.*, 2009) and skin-derived EpCam⁺ DCs in the dLN in the Treg-depleted mice (Figure 5f and Supplementary Figure S5 online).

Depletion of Treg cells during epicutaneous sensitization modulated the lung inflammation after airway challenge

More than 50% of AD patients develop food allergy, allergic rhinitis, or asthma by 3 years of age (Boguniewicz and Leung, 2010), suggesting a connection between skin sensitization

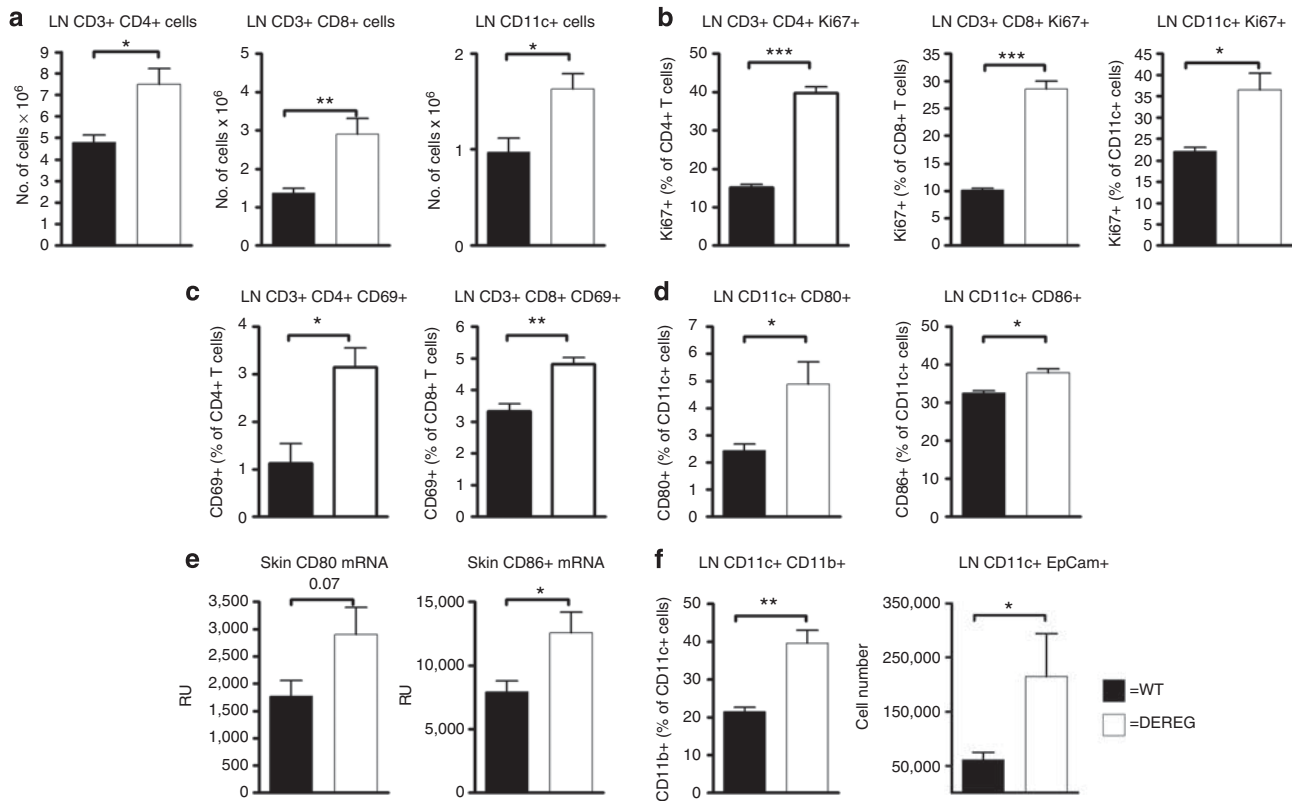


Figure 5. T-cell and DC function after Treg-cell depletion. (a) Numbers of CD3⁺CD4⁺, CD3⁺CD8⁺, and CD3⁺CD11c⁺ cells in the dLN in OVA-treated WT and DEREK mice. (b) T-cell and DC proliferation based on Ki67 expression. (c) T-cell activation and (d) the expression of costimulatory molecules CD80 and CD86 on dendritic cells (DCs). (e) CD80 and CD86 mRNA expression in the skin of DEREK mice. (f) The amount of CD11b⁺ DCs and EpCam⁺ DCs in the LNs, $n = 4-8$. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; bars represent mean $\pm$ SEM.

and subsequent allergy. The skin–lung association has also been demonstrated in a mouse model (Lehto *et al.*, 2005). To examine the effects of Treg cell depletion during skin sensitization on subsequent lung inflammation, mice were subjected to airway challenge 5 days after the end of the AD protocol, and Treg cells were depleted during the second sensitization week (Figure 6a). The lack of Treg cells during skin sensitization led to exacerbated lung inflammation after intranasal OVA, including increased cellular infiltration (Figure 6b), upregulated IL-4 expression (Figure 6c), and increased numbers of eosinophils and CD4⁺ T cells in the bronchoalveolar lavage fluid (BALF; Figure 6d). Yet, the production of mucus and expression of IL-13 were not affected by the lack of Treg cells (Figure 6c and data not shown). By the time of readout, the Foxp3⁺ to Foxp3[−] T-cell balance had been restored in the lymph nodes (LN), but in the BALF Foxp3⁺ cells were outnumbered by Teff (Figure 6e).

DISCUSSION

Although the importance and function of Treg cells in the field of allergy have been under intensive investigation, the role of Treg cells in AD is still not clear. We explored Treg cells in a murine model of AD, where an allergic inflammatory response develops in the skin after recurrent OVA applications through injured skin. The inflammatory response in the model closely resembles the hallmarks of AD,

including increased infiltration of lymphocytes and eosinophils, production of Th2 cytokines, and elevation of serum IgE levels. The number of Foxp3⁺ cells increased both in the dLNs and in the skin in the OVA-treated mice compared with the PBS-treated mice, but the Treg/Teff ratio was tightly maintained throughout the exposure protocol. OVA treatment also significantly increased the numbers of both Foxp3⁺ and Foxp3[−] IL-10-producing T cells, indicating the engagement of Tr1 cells. Furthermore, the phenotype of Foxp3⁺ Treg cells changed over time, with a gradually increasing share of CD103⁺ Treg cells toward the end of the sensitization protocol. CD103 is a surface marker for memory/effector Treg cells, and an important molecule for the retention of Treg cells at the site of inflammation (Huehn *et al.*, 2004; Siegmund *et al.*, 2005; Suffia *et al.*, 2005). Expectedly, the great majority of skin-infiltrated Treg cells was CD103⁺.

Several lines of evidence indicate that the function of Foxp3⁺ Treg cells is altered in patients with allergies. For example, Th2 responses are less effectively inhibited in atopic individuals (Bellinghausen *et al.*, 2003; Grindebacke *et al.*, 2004; Ling *et al.*, 2004), and key constituents of Th2-type signaling pathways, including IL-4, STAT6, and GATA3, interfere with the generation of Treg cells and the suppressive capacity of established Treg cells (Mantel *et al.*, 2007; Dardalhon *et al.*, 2008; Hadjuri *et al.*, 2009). We show that

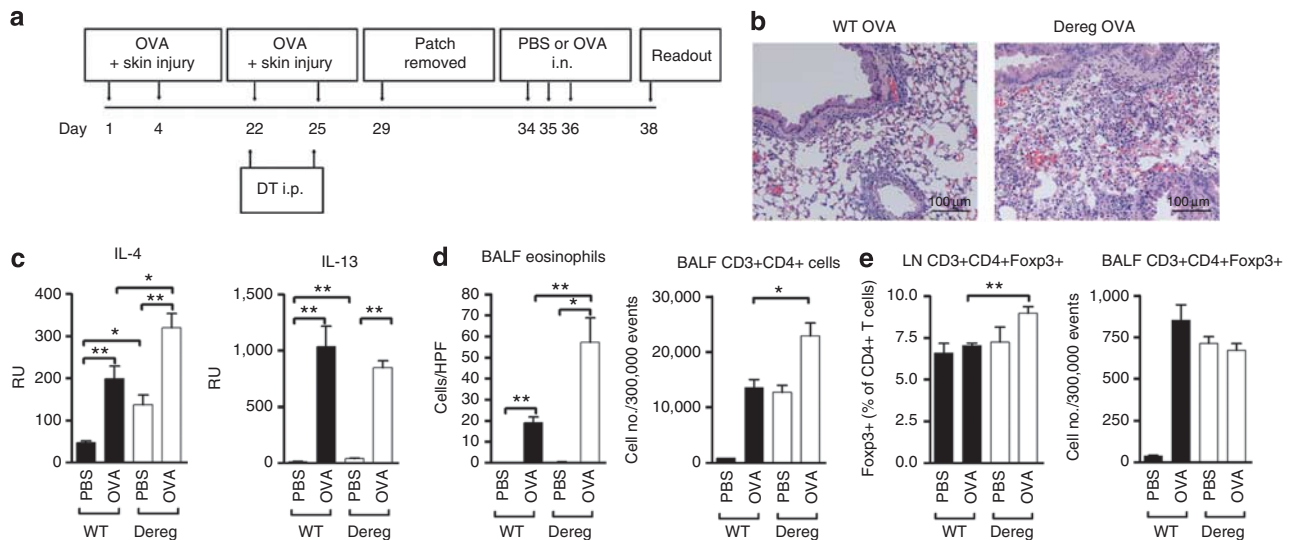


Figure 6. The effect of depleting Foxp3⁺ cells during epicutaneous sensitization on subsequent lung inflammation. (a) The exposure protocol and (b) hematoxylin-eosin (H&E) staining of lung tissue. Bar = 100 μm. (c) The expression of IL-4 and IL-13 mRNA in the lung ($n = 5-7$) and (d) the number of eosinophils and CD4⁺ T cells in the BALF increased in DERE mice. (e) The ratio of Foxp3⁺ cells in the LNs and Foxp3⁺ cell numbers in the BALF, $n = 2-3$. * $P < 0.05$, ** $P < 0.01$; bars represent mean + SEM. BALF, bronchoalveolar lavage fluid; HPF, high-power field; LN, lymph node; OVA, ovalbumin; PBS, phosphate-buffered saline; RU, relative units; WT, wild type.

the strong Th2 shift that occurs in the Balb/c mice upon OVA sensitization is associated with impaired suppressive capacity of Foxp3⁺ Treg cells. AD is associated with a Th2-polarizing genetic background (Brown and McLean, 2009), and one might speculate that the disease may develop upon systemic Th2 polarization with consequences for Treg functions.

Nevertheless, we show that also potentially defective Foxp3⁺ Treg cells have a crucial role in controlling AD-like skin inflammation. Depleting naive DERE mice of their Treg cell compartment had no effect on the skin, proving that the exaggerated skin inflammation in the DERE mice is likely not a consequence of the sole deletion of Treg cells. Tape stripping alone induced modest skin inflammation in the PBS-treated irritation WT controls, and, not surprisingly, a much stronger response in the DT-treated DERE mice. The purpose of the tape stripping is to mimic the impaired skin barrier function that occurs in AD patients (Marenholz *et al.*, 2006; Palmer *et al.*, 2006; Kim *et al.*, 2008) and to facilitate the penetration of the allergen through the skin. Tape stripping combined with OVA exposure further exacerbated the inflammatory response, resulting in significantly increased cellular infiltration, activation of Th2 cells, and highly elevated production of OVA-specific IgE. All these inflammatory parameters increased substantially after the depletion of Treg cells in the OVA-treated DERE mice. Together, the results show that Foxp3⁺ Treg cells are essential in controlling both skin injury-induced inflammation and allergen-specific immune responses, both of which are important components of AD-associated pathology.

DT treatment resulted in extensively reduced numbers of Foxp3⁺ Treg cells in the DERE mice. Nevertheless, the expression of IL-10 was significantly elevated in the DERE mice, indicating Foxp3⁺ Treg cell-independent IL-10 production in the skin, which is in line with previous AD

studies where the general view is that Foxp3⁺ Treg cells are not the main IL-10 producers (Caproni *et al.*, 2006; Schlotter *et al.*, 2011). Macrophages, DCs, and neutrophils are all significant producers of IL-10 (Wills-Karp *et al.*, 2001), as well as keratinocytes and other skin cells (Vocanson *et al.*, 2009), and the contribution of Foxp3⁺ Tr1 cells cannot be excluded (Hawrylowicz and O'Garra, 2005). A larger share of Foxp3⁺ T cells in DERE mice expressed PD-1 compared with WT, which may indicate the presence of elevated numbers of Foxp3⁺ iTreg cells in DERE mice after Foxp3 depletion (Haribhai *et al.*, 2011).

Treg cells suppress inflammatory responses through several mechanisms, such as the production of suppressive cytokines, inhibition of Teff cell proliferation, or modulation of DC maturation and function (Vignali *et al.*, 2008). The temporary deletion of Treg cells resulted in a significantly larger share of proliferating T cells, as well as activated T cells in the dLNs. Thus, T-cell expansion and activation are important targets of Treg cell-mediated regulation. Furthermore, AD skin lesions are characterized by the infiltration of CD11b^{hi} inflammatory dendritic epidermal cells (Wollenberg *et al.*, 1999; Schuller *et al.*, 2001). These cells express costimulatory molecules CD80 and CD86, which are further upregulated in AD (Schuller *et al.*, 2001). Temporary deletion of Treg cells during OVA challenge resulted in a significantly increased share of CD11b^{high}, as well as skin-derived EpCam⁺ DCs in the LNs and elevated expression of CD80 and CD86 on the DCs. Clearly, the absence of Treg cells modulated both DC activation and differentiation.

Sensitization to an allergen via the skin can lead to the development of lung inflammation, if the same allergen is subsequently encountered in the airways (Lehto *et al.*, 2005). The depletion of Treg cells during cutaneous sensitization to OVA resulted in a stronger inflammatory response in the

lungs after OVA airway challenge. Although Treg/Teff ratios in the LNs had been restored, Teff cells outnumbered Treg cells locally in the lungs, indicating divergent regulation of the allergen-specific response.

To conclude, we show that Foxp3⁺ Treg cells are essential suppressors of AD-like skin inflammation, and that the presence of Treg cells at the time of epicutaneous protein exposure is important for the regulation of subsequent airway inflammation.

MATERIALS AND METHODS

Mice and experimental models

DEREG (depletion of regulatory T cells) mice (Lahl *et al.*, 2007) in C57/Black background were backcrossed to Balb/c mice (Scanbur, Sollentuna, Sweden) at least five times. These mice harbor genes for enhanced green fluorescent protein (EGFP) and DT receptor under the control of Foxp3 promoter. Mice were genotyped and grouped into DEREG mice and WT mice. For suppression assay and Treg kinetics studies, C.Cg-Foxp3^{tm2Tch}/J mice (The Jackson Laboratory, Bar Harbor, ME) that express EGFP under the control of Foxp3 promoter were used. All the procedures concerning animals were accepted by Social and Health Services of State Provincial Office of Southern Finland.

In the AD model, OVA sensitization was carried out as previously described with a few modifications (Spergel *et al.*, 1998). Briefly, 10- to 14-week-old female mice were anesthetized and their backs were shaved. Skin injury was induced by repeated tape stripping, after which 100 µg of OVA in 100 µl of PBS, or PBS only, was applied on a sterile patch and attached to the tape-stripped area with an adhesive tape. Treatment was repeated once during the 1-week sensitization period after which a 2-week resting period was kept before a second, similar kind of sensitization week. The mice were killed and samples collected 24 hours after the last treatment. DT (Calbiochem, La Jolla, CA) was administered intraperitoneally during the second sensitization week simultaneously with the patch treatment: 1 µg during the first and 0.5 µg during the second treatment. DT was administered similarly in naive mice. In the asthma model, the sensitization was carried out as in the AD model. Four days after the last patch treatment, patches were removed, and 5 days thereafter mice were given 50 µg of OVA in 50 µl of PBS (OVA group) or 50 µl PBS (PBS group) intranasally on three consecutive days and samples were collected 48 hours after last treatment.

Histology

Skin or lung tissue was fixed in 10% formalin and embedded in paraffin. Multiple 4-µm-thick sections were stained with hematoxylin-eosin or periodic acid Schiff.

Immunohistochemistry

For immunohistochemical staining, samples obtained from exposed skin were immersed in Tissue-Tek oxacalcitriol compound (Sakura Finetek, Alphen aan den Rijn, Netherlands) and frozen on dry ice. Frozen sections (4 µm) were fixed with cold acetone and immunoperoxidase staining was used to detect CD3 (BD Biosciences, NJ), Foxp3 (eBiosciences, San Diego, CA), or IL-10 (Abcam, Cambridge, UK). Biotin-conjugated secondary antibody anti-rat IgG (H+L) was purchased from Vector Laboratories (Servion, Switzerland).

RNA isolation and real-time quantitative PCR

Skin from the exposed area or lung tissue was homogenized in Trisure (Bioline, London, UK). RNA was extracted according to the manufacturer's instructions and used as a template for cDNA synthesis. Real-time quantitative PCR analysis was performed with 7500 Fast Real Time PCR system (Applied Biosystems, Carlsbad, CA) using commercial primers and probes. Ribosomal 18S was used as an endogenous control. All primers and probes were purchased from Applied Biosystems.

Serum IgE antibodies

Total IgE levels in the serum were measured as previously described (Savinko *et al.*, 2005). For measurements of OVA-specific IgE and IgG2a, the plate was coated with rat anti-mouse IgE/IgG2a mAb (BD Biosciences, NJ). Diluted sera were allowed to bind overnight and bound IgE/IgG2a was detected with biotinylated OVA, Streptavidin-HRP (BD Biosciences, NJ), and peroxidase substrate reagents (Kirkegaard & Perry Laboratories, Gaithersburg, MD). Absorbance at 405 nm was read with an automated ELISA reader (Titertek Multiscan, Eflab, Turku, Finland).

Cytokine ELISAs

LN cell suspensions were stimulated with α-CD3, and cytokine production was measured at 48 hours with commercial ELISA kits. OptEIASet Mouse IL-5 (BD Biosciences, San Jose, CA) was used for detection of IL-5, Mouse IL-4 ELISA Ready-SET-Go! (eBioscience, San Diego, CA) for IL-4, and Quantikine M MURINE mouse IL-13 (R & D Systems, Minneapolis, MN) for IL-13.

Single-cell preparations and flow cytometry

Single-cell suspensions from the inflamed skin were prepared by cutting the skin sample into small pieces, passing through a 70-µm cell strainer, washing with PBS, and filtering again through a 40-µm cell strainer (BD Biosciences). LN cells were isolated by crushing the nodes and filtering through a 100-µm strainer. BALF cells were used as such. Antibodies PE (phycoerythrin)-Cy7-conjugated anti-CD3 (BD Biosciences), PerCP-Cy5-conjugated anti-CD4, Alexa Fluor700-conjugated anti-CD8, APC-conjugated CD103, PE-conjugated PD-1, PerCP-Cy5.5-conjugated CD11b, APC-Alexa750-conjugated CD11c, PE-conjugated EpCam, PE-conjugated CD86, and APC-conjugated CD80 (eBiosciences) were used for surface stainings. For intracellular stainings, cells were fixed and permeabilized with a commercial Foxp3 staining kit (eBiosciences) according to the manufacturer's instructions, and the cells were stained with PE- or Alexa Fluor700-conjugated Foxp3, APC-conjugated CD69 (eBiosciences), AlexaFluor 647-conjugated Helios (Biolegend, San Diego, CA), or PE-conjugated Ki-67 (BD Biosciences). For intracellular cytokine detection, cells were stimulated with OVA (50 µg ml⁻¹) or Phorbol myristate acetate (20 ng ml⁻¹) and Ionomycin (1 µg ml⁻¹) in the presence of Brefeldin A (10 µg ml⁻¹) for 4 hours, fixed and permeabilized with Fix and Perm kit from Caltag (CA), and stained with PE-conjugated anti-IL-13, APC-conjugated IFN-γ, or IL-10 (eBiosciences, CA). Samples were run with FACS Cantoll (BD Biosciences) and results analyzed with the FlowJo software (Tree Star, Ashland, OR).

Suppression assay

CD4⁺ cells were isolated from LN cell suspensions with the Robosep CD4⁺ cell-positive selection kit (StemCell Technologies,

Grenoble, France), and sorted into eGFP+ (~Foxp3+) and eGFP- (~Foxp3-) fractions. CD4+ Foxp3- cells from OVA-treated mice were seeded at 3×10^4 cells per well in complete RPMI-1640 medium (Invitrogen Life Technologies, Carlsbad, CA), and stimulated with α -CD3 ($0.75 \mu\text{g ml}^{-1}$) and α -CD28 ($2.5 \mu\text{g ml}^{-1}$) in the presence of CD4+ Foxp3+ cells from PBS- or OVA-treated mice at responder suppressor cell ratios as indicated. The cells were cultured in 37°C/5% CO₂ for 72 hours and pulsed with 1 μCi [³H]thymidine per well (Amersham Biosciences Europe, Freiburg, Germany) during the last 18 hours. Incorporated radioactivity was determined using a liquid scintillation counter (Trilux 1450 Microbeta, Wallac, Turku, Finland). Results are expressed as mean counts per minute of triplicate wells.

Statistical analyses

Statistical analyses were performed with *t*-test and nonparametric Mann-Whitney *U*-test using the GraphPad Prism software (GraphPad Software, LaJolla, CA).

CONFLICT OF INTEREST

The authors state no conflict of interest.

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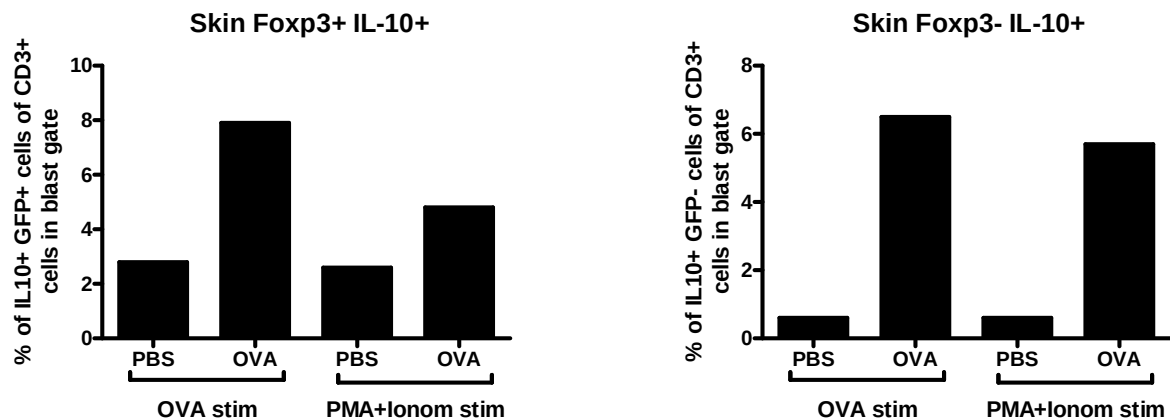
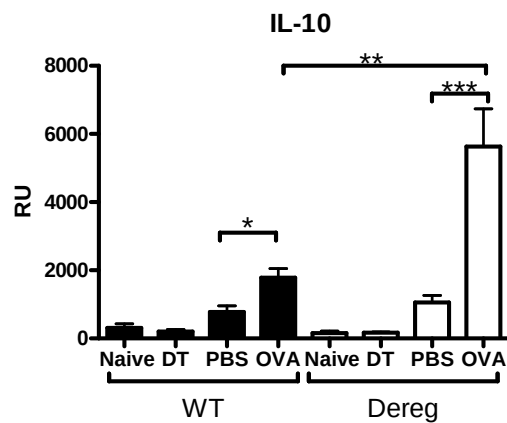
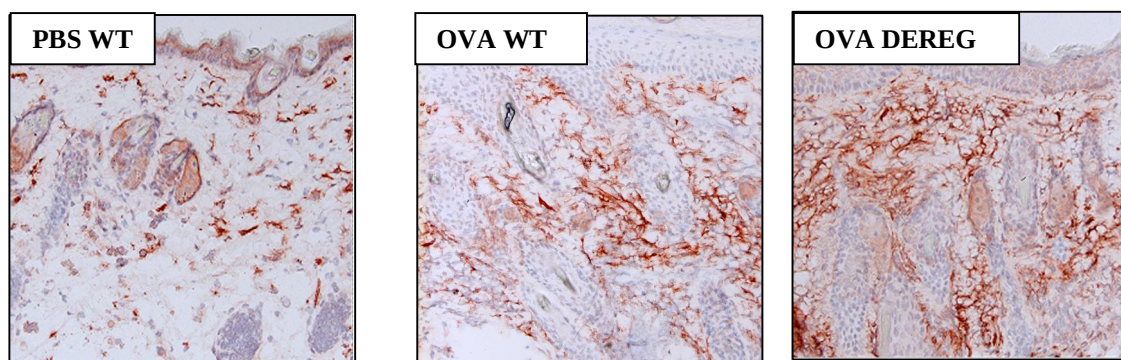
SUPPLEMENTARY MATERIAL

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

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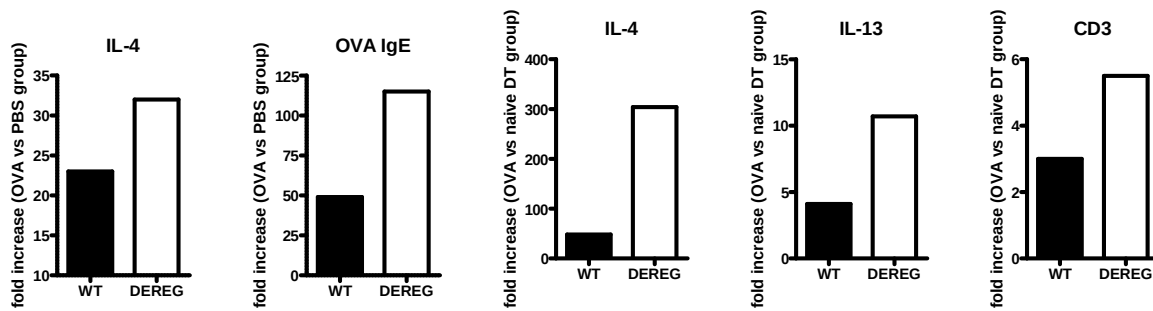
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a**b****c**

Supplementary figure 1

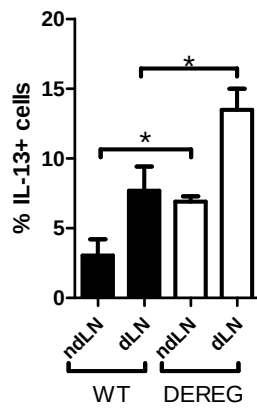
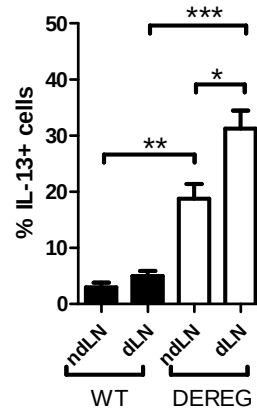
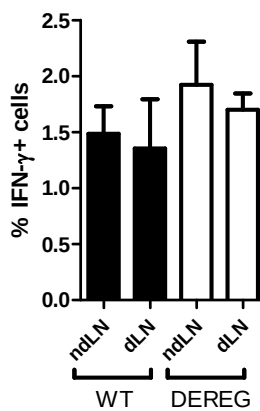
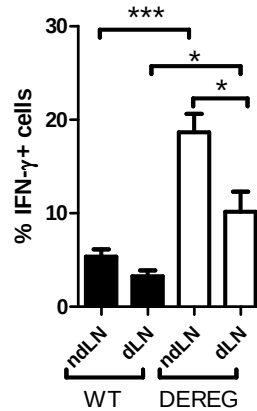
a) Flow cytometric analysis of the skin revealed increased percentages of both Fxp3+ and Fxp3- IL-10+ cells in OVA sensitized mice compared with PBS control. **b)** When Fxp3+ cells were depleted, the expression level of IL-10 was remarkably upregulated in the skin of DEREK OVA mice compared with WT both at mRNA and **c)** protein level.

a**b**



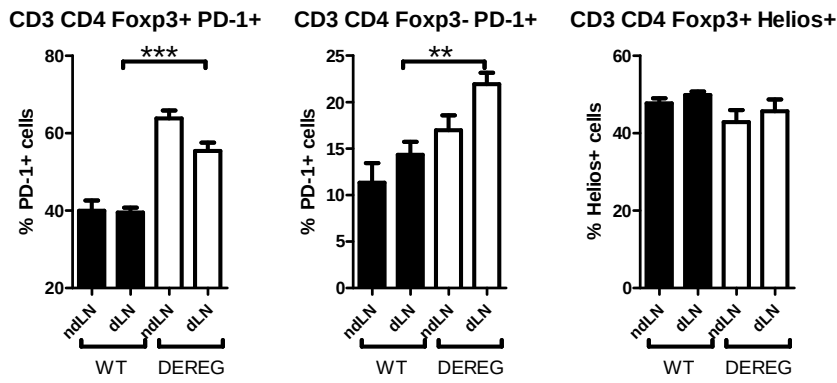
Supplementary figure 2

a) The mRNA expression of IL-4 increases approximately 20 fold in WT OVA vs. WT PBS group, whereas the increase in DERE OVA vs. DERE PBS group is over 30 fold. Similarly, OVA-specific IgE levels increase more drastically in DERE mice compared with WT mice. **b)** In addition, if expression levels or increases in CD3⁺ cell numbers are compared between OVA treated mice and DT treated naive mice, the fold increase is even further enhanced in DERE mice compared with WT mice.

CD3 IL-13+ (OVA stimulation)**CD3 IL-13+ (PMA+Ionom stimulation)****CD3 IFN-γ+ (OVA stimulation)****CD3 IFN-γ+ (PMA+Ionom stimulation)**

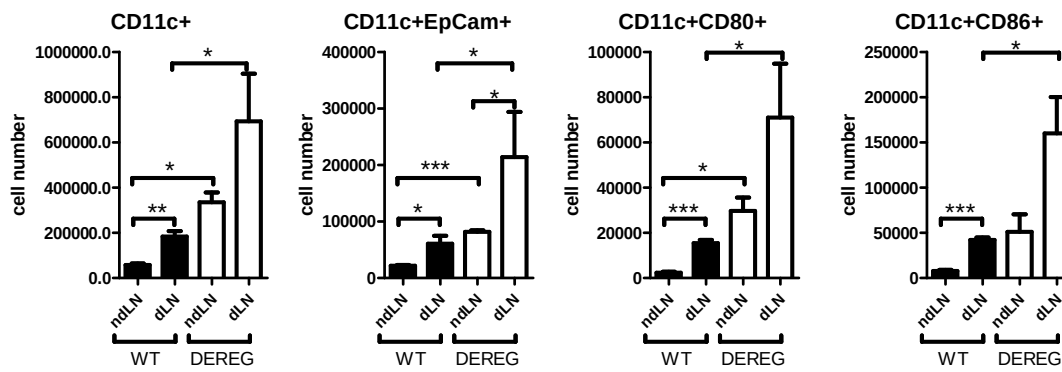
Supplementary figure 3

In OVA stimulated LN cell suspensions the share of IL-13 producing cells was higher in the draining LNs (dLN) compared with non-draining LNs (ndLN), and at a significantly higher level in DERE mice compared with WT mice. In the PMA+ ionomycin stimulated cells the difference between WT and DERE mice was even more pronounced. The share of IFN- γ producing Th1 cells was at a low level in the OVA-stimulated cell cultures, and similar between WT and DERE mice. In the PMA+ ionomycin stimulated cell cultures, however, the share of Th1 cells was significantly higher in the DERE mice. The results indicate a Th2 polarized cytokine milieu in the dLNs, while non-draining LNs were Th1 polarized, and antigen-specific Th2 polarized cells were much more frequent in DERE mice. * $P < 0.05$ ** $P < 0.01$ *** $P < 0.001$, bars represent mean + SEM



Supplementary figure 4

a) The percentages of PD-1⁺ Foxp3⁺ and Foxp3⁻ T cells were increased in the non-draining (ndLN) and draining (dLN) LNs of DERE mice compared with WT. **b)** Helios expression was slightly lower in DERE mice. **P < 0.01 ***P < 0.001, bars represent mean + SEM



Supplementary figure 5

The number of DCs and EpCam+, CD80+ or CD86+ DCs was elevated in the draining LNs (dLNs) compared with non-draining LNs (ndLNs) and significantly more in DERE mice compared with WT mice. *P<0.05 **P<0.01 ***P< 0.001, bars represent mean + SEM