Atopic dermatitis induces the expansion of thymus-derived regulatory T cells exhibiting a Th2-like phenotype in mice

Verena Moosbrugger-Martinz a, *, Christoph H. Tripp a, Björn E. Clausen b, Matthias Schmuth a, Sandrine Dubraca, *

a Department of Dermatology, Venereology and Allergology, Medical University of Innsbruck, Innsbruck, Austria
b Institute for Molecular Medicine, University Medical Center of the Johannes Gutenberg-University Mainz, Mainz, Germany

Received: October 5, 2015; Accepted: January 7, 2016

Abstract

Atopic dermatitis (AD) is a widespread inflammatory skin disease with an early onset, characterized by pruritus, eczematous lesions and skin dryness. This chronic relapsing disease is believed to be primarily a result of a defective epidermal barrier function associated with genetic susceptibility, immune hyper-responsiveness of the skin and environmental factors. Although the important role of abnormal immune reactivity in the pathogenesis of AD is widely accepted, the role of regulatory T cells (Tregs) remains elusive. We found that the Treg population is expanded in a mouse model of AD, i.e. mice topically treated with vitamin D3 (VitD). Moreover, mice with AD-like symptoms exhibit increased inducible T-cell costimulator (ICOS)-, cytotoxic T-lymphocyte antigen-4 (CTLA-4)- and Glycoprotein-A repetitions predominant receptor (GARP)-expressing Tregs in skin-draining lymph nodes. Importantly, the differentiation of Tregs into thymus-derived Tregs is favoured in our mouse model of AD. Emigrated skin-derived dendritic cells are required for Treg induction and Langerhans cells are responsible for the biased expansion of thymus-derived Tregs. Intriguingly, thymus-derived Tregs isolated from mice with AD-like symptoms exhibit a Th2 cytokine profile. Thus, AD might favour the expansion of pathogenic Tregs able to produce Th2 cytokines and to promote the disease instead of alleviating symptoms.

Keywords: atopic dermatitis ● regulatory T cells ● thymic stromal lymphopoietin ● vitamin D3

Introduction

Atopic dermatitis (AD) is one of the most common inflammatory skin conditions, predominantly affecting infants and children. It is characterized by pruritus, eczematous lesions and skin dryness. Atopic dermatitis is a complex chronic relapsing inflammatory skin disorder involving immune hyper-responsiveness of the skin, epidermal barrier abnormalities, genetic susceptibility and environmental factors [1]. In its acute phase, AD is characterized by an abnormal production of thymic stromal lymphopoietin (TSLP), an alarmin secreted by keratinocytes and leading to expansion of TH2 cells via activation of Langerhans cells (LCs) [2–4]. Moreover, interleukin (IL)-17 has been shown to be present in acute AD [5]. A TH1/TH22 predominant immune response, dermal infiltration with inflammatory dendritic epidermal cells (IDECs), macrophages and eosinophils as well as bacterial superinfection are classical features of the chronic phase of AD [2].

Regulatory T cells (Tregs) play a critical role in the maintenance of peripheral tolerance and in the control of allergic responses. Despite a growing interest in the role of Tregs in the pathogenesis of AD, their precise role remains unclear. In both humans and mice, loss-of-function mutations in the FoxP3 gene lead to a multiorgan inflammatory response including skin inflammation resembling AD associated with elevated serum IgE levels, eosinophilia, allergic airway inflammation, food allergies and other autoimmune symptoms [6, 7]. These data suggest that lack of functional Tregs is sufficient to recapitulate important immunologic features of AD. Accordingly, lower circulating Tregs at birth and lower Treg numbers in cord blood predict a higher risk for the development of AD in the first year of life [8]. Furthermore, analysis of TH17 tissue from atopic children revealed significantly delayed maturation of TH2 Tregs as compared to age-matched, non-atopic controls [9]. However, studies in adults seem to dismiss this hypothesis. Some authors reported equal levels of circulating Tregs [10–12], whereas others found increased circulating Tregs, directly correlating with AD disease severity in patients with persisting AD in adulthood as compared to healthy controls [13–16]. Furthermore, there are con-
flicting reports about the presence of Tregns in inflammatory infiltrates of AD skin [10, 17, 18], and controversy exists about the immune suppressive capacity of Tregns in AD. Tregns from AD patients were shown to exhibit normal suppressive activity at baseline [11, 15] or after allergen-stimulation [19], but Tregns exhibiting reduced suppressive function have also been identified in patients with AD [13]. Intriguingly, stimulation with staphylococcal enterotoxin B leads to a Th2-dominated cytokine profile in circulating CCR6+ Tregns of AD patients [12, 14] and there is emerging evidence that Tregns can convert to Th2 cells, thereby contributing to AD instead of dampening the immune response [20].

Because the Treg population remains poorly characterized in AD, here we studied the phenotype and the dynamics of thymus-derived versus peripherally derived Tregns. Dendritic cells (DCs) are professional antigen-presenting cells and key players in regulating immunity and tolerance, including the instruction of Tregns. In light of the association of LCs with AD, we also investigated the role of skin-derived DCs in activating these cells.

**Materials and methods**

**Animals**

Mice of inbred Balb/c and C57BL/6 strains were purchased from Charles River Laboratories (Sulzfeld, Germany). Mice expressing a diphtheria toxin receptor (DTR) under the control of the Langerin (CD207) gene were bred on a C57BL/6 background as described earlier [21]. All mice were used at 2–4 months of age and animal experiments were carried out according to governmental guidelines.

**Mouse treatments**

1α,25-dihydroxyvitamin D3 (1 nmol/ear) was dissolved in ethanol. Vehicle (ethanol) or vitamin D3 (VitD) were topically applied once daily onto inner and outer surfaces of mouse ears (10 μl/ear side) over a time period of 10 days (4 days treatment, 3 days no treatment, 3 days treatment) as described earlier [22]. Diphtheria toxin in PBS or PBS alone was injected intraperitoneally into Langerin-DTR mice on day 0 as described earlier [22]. Diphtheria toxin receptor (DTR) under the control of the Langerin (CD207) gene were bred on a C57BL/6 background as described earlier [21]. All mice were used at 2–4 months of age and animal experiments were carried out according to governmental guidelines.

**Animals**

**Materials and methods**

**Animals**

Mice of inbred Balb/c and C57BL/6 strains were purchased from Charles River Laboratories (Sulzfeld, Germany). Mice expressing a diphtheria toxin receptor (DTR) under the control of the Langerin (CD207) gene were bred on a C57BL/6 background as described earlier [21]. All mice were used at 2–4 months of age and animal experiments were carried out according to governmental guidelines.

**Mouse treatments**

1α,25-dihydroxyvitamin D3 (1 nmol/ear) was dissolved in ethanol. Vehicle (ethanol) or vitamin D3 (VitD) were topically applied once daily onto inner and outer surfaces of mouse ears (10 μl/ear side) over a time period of 10 days (4 days treatment, 3 days no treatment, 3 days treatment) as described earlier [22]. Diphtheria toxin in PBS or PBS alone was injected intraperitoneally into Langerin-DTR mice on day 0 as described earlier [22]. Diphtheria toxin receptor (DTR) under the control of the Langerin (CD207) gene were bred on a C57BL/6 background as described earlier [21]. All mice were used at 2–4 months of age and animal experiments were carried out according to governmental guidelines.

**Antibodies and reagents**

Directly labelled primary monoclonal antibodies (mAb) specific for mouse CD4, CD25, inducible T-cell costimulator (ICOS), CD11c, CCR7, IL-10 and MHCII were purchased from BD Biosciences (San Diego, CA, USA), and for detection of mouse CCR7, PD-L1, ICOS L, GITR L, CD11c, Glycoprotein-A repetitive predominant receptor (GARP) and IL-13 from eBioscience (San Diego, CA, USA). Directly labelled mAb for detection of cytotoxic T-lymphocyte antigen (CTLA)-4 was purchased from Biolegend (San Diego, CA, USA). For intracellular staining with anti-mouse mAb against FoxP3 (eBioscience) and anti-mouse mAb against Helios (Biolegend) cells were permeabilized and stained according to the manufacturer’s instructions. Cell viability was assessed by LIVE/DEAD Fixable Dead Cell Stain Kit (Invitrogen, Carlsbad, CA, USA) or Fixable Viability Dye (eBioscience). Biotinylated mAb against mouse CD103 and CD25 were purchased from BD-Biosciences, streptavidin PerCP Cy5.5 from Biolegend, and streptavidin APC from BD Biosciences. Directly labelled mAb against mouse Langerin (clone 929F.3) was purchased from Dendritics (Lyon, France) and used after permeabilization with Cytofix/Cytoperm kit (BD Biosciences), according to the manufacturer’s instruction. Purified mAb against IDO was purchased from Biolegend and detected with directly conjugated goat anti-rat immunoglobulin from BD Biosciences. 1α,25-dihydroxyvitamin D3 and DT were purchased from Sigma-Aldrich (St Louis, MO, USA).

**Analysis of DCs and lymphocytes in skin-draining lymph nodes of mice**

Auricular skin-draining lymph nodes (sdLNs) were collected from mice treated with VitD or vehicle on their ears and digested with collagenase D (Roche Diagnostics, Indianapolis, IN, USA) and DNase (Roche Diagnostics) for 25 min. at 37°C. Resulting single cell suspensions were counted in the haemocytometer, stained with mAb and analysed using flow cytometry as previously described [4]. Absolute cell numbers per auricular draining lymph node (LN) were calculated on the basis of flow cytometry analysis and haemacytometer cell counts. Mouse Tregns were identified by expression of CD4, CD25 and FoxP3, and distinction between induced and natural Tregns was made on the basis of Helios staining. CD11c+ CCR7+ cells in sdLNs were considered as emigrated DCs. Expression of CD103 was used to discriminate epidermal LCs (CD11c+ CCR7+ CD103− Langerin+) from Langerin+ dermal DCs (CD11c+ CCR7+ CD103+ Langerin+). CD11c+ CCR7+ CD103+− Langerin− cells were considered as Langerin dermal DCs and CD11c+ CCR7+ CD103+− Langerin− cells as ‘other DC’.

**Detection of intracellular cytokines**

Isolated LN cells were cultured for 4 hrs with 1 μg/ml brefeldin A to block cytokine release; then stained and analysed by flow cytometry.

**Flow cytometry and immunohistochemistry**

Flow cytometry analysis was performed on a FACScalibur using CellQuest software (BD Immunocytometry Systems, San Jose, CA, USA) and results were analysed by FlowJo software (Tree Star, Ashland, OR, USA). Five- and six-colour stainings were carried out with a FACSCanto using FACSDiv software (BD Immunocytometry Systems). Epidermal sheets were separated from ear skin with 0.5 M ammonium thiocyanate (Merck, Westchester, PA, USA) as described previously [4], washed and stained with antimouse MHC-class II-FITC mAb for 1 hr at 37°C. Stainings were visualized by an Olympus BX60 epifluorescence microscope using a 40× objective. Fluorochrome- and isotype-matched immunoglobulins of irrelevant specificity served as negative controls.

**Statistical analysis**

Results are shown as mean ± SD, n represents the number of mice used per group. Data were analysed using a Student’s t-test for nor-
mally distributed values or a Mann–Whitney U-test, when values did not show a Gaussian distribution or when \( n < 5 \).

**Results**

**AD-like inflammation is associated with increased numbers of T\(_{\text{regs}}\)**

To study T\(_{\text{regs}}\) in AD, we topically treated mice with VitD to trigger high TSLP expression in the epidermis as observed in AD lesions [4, 22]. The inflammatory phenotype in these mice is similar to that observed in other TSLP-overexpressing mice and is characterized by an AD-like cutaneous inflammation containing Th2 CD4\(^+\) T cells expressing cutaneous homing receptors and by elevated serum IgE levels [4, 22–25]. Figure 1 depicts the kinetic of T\(_{\text{reg}}\) (CD4\(^+\)CD25\(^+\)FoxP3\(^+\)) and non-regulatory (CD4\(^+\)CD25\(^-\)/FoxP3\(^-\)) CD4\(^+\) cell expansion in sdLNs of mice upon treatments. Results show that VitD treatment significantly enhanced numbers of T\(_{\text{reg}}\) at all time-points when compared to vehicle treatment (Fig. 1A). In contrast, numbers of other CD4\(^+\) lymphocytes increased on days 3 and 5 but not on day 10 in sdLNs of VitD-treated mice when compared to controls (Fig. 1B). Maximal cell numbers were reached for both subsets on day 5 (Fig. 1A and B). Thus, although the expansion of T\(_{\text{reg}}\) was continuously promoted by VitD, the expansion of other CD4\(^+\) lymphocytes regressed between day 5 and day 10 (Fig. 1A and B). The variations in cell percentages (Fig. 1C and D) with decreased percentages of T\(_{\text{reg}}\) on day 3 and decreased percentages of other CD4\(^+\) lymphocytes on day 10 additionally suggest that the expansion of non-regulatory CD4\(^+\) lymphocytes precedes the expansion of T\(_{\text{reg}}\). Because AD-like symptoms in mice treated with VitD enhance over time, our data show that expansion of T\(_{\text{reg}}\) parallels symptom development in this AD model.

**T\(_{\text{reg}}\)s display an activated phenotype in AD-like inflammation**

To better characterize the CD4\(^+\)CD25\(^+\)FoxP3\(^+\) T\(_{\text{reg}}\) population in the VitD AD model, we measured the expression of various surface markers which are involved in T\(_{\text{reg}}\) function in mice with overt AD symptoms, i.e. on day 10 of treatment. Percentages of CD4\(^+\)CD25\(^+\)FoxP3\(^+\) T\(_{\text{reg}}\) expressing ICOS, CTLA-4 and GARP at their cell surface were increased in sdLNs of mice with AD when compared to controls (Fig. 2A and B). Hence, peripheral activated T\(_{\text{reg}}\)s are observed in AD-like inflammation.

**LCs are the first DC subset to emigrate to sdLNs to potentially expand T\(_{\text{reg}}\)s in AD**

Earlier work has demonstrated that epidermal TSLP is overexpressed after topical treatment with the VitD analogue MC903 for 4 days [4]. As opposed to dermal DCs, LCs acquire an activated phenotype in the skin after MC903 treatment and increased migration to sdLNs [4]. These findings suggested that the activation of LCs may be associated with a biased Th2 response prior to the development of clinical
signs of AD [4]. On the other hand, activation and migration of LCs might also contribute to increased Treg expansion in early AD. We therefore analysed total numbers of CD4+ CD25+ FoxP3+ Tregs, LCs, Langerin+ and Langerin/C0 dermal DCs in sdLNs on days 0, 3 and 5 of topical VitD treatment. Numbers of CD4+ CD25+ FoxP3+ Tregs started to significantly increase in sdLNs as early as on day 3 (Fig. S1A), similar to emigrated LCs (Fig. S1B). Numbers of Langerin+ and Langerin/C0 dermal DCs and other DCs were not altered on day 3, but started to increase on day 5 (Fig. S1C–E), according to the literature [4, 26, 27]. Taken together, our results establish that, in the VitD AD model, LCs are the first skin DC subset to reach the sdLNs, which coincides with the beginning of Treg expansion. It was previously reported that VitD directly induces Tregs in vitro [28], challenging the requirement of skin-derived DCs in the development of Tregs after topical application of VitD. To address this issue, we removed the application sites (ears) 4 hrs after a single topical application of vehicle or VitD (3 nmol/ear) to prevent any skin DC migration [29]. As depicted in Figure S1, removal of the application sites prevented the increase of both Tregs (Fig. S1F) and DCs (Fig. S1G) in sdLNs of VitD-treated as compared to vehicle-treated mice. We verified that one-time application of 3 nmol VitD per ear elicits the same effects on the numbers of various DC subsets and Tregs in sdLNs than a daily treatment with 1 nmol VitD for 3 days (data not shown). Therefore, expansion of CD4+ CD25+ FoxP3+ Tregs is not because of a direct effect of VitD in this mouse model of early AD, but instead requires the migration of skin-derived DCs to sdLNs.

To further assess the tolerogenic function of skin DCs, we topically applied VitD to mice deficient for Langerin+ DCs, including LCs (Fig. S2). Numbers of CD4+ CD25+ FoxP3+ Tregs in sdLNs were increased on days 5 and 10 in VitD-treated mice depleted of Langerin+ DCs as compared to vehicle-treated controls (Fig. S3A and B). Therefore, the overall induction of Tregs might be initially dependent on LCs reaching firstly the sdLNs, whereas later on, other skin-derived DC subsets contribute to this expansion.

Thymus-derived Tregs are enhanced in AD

Helios, an Ikaros transcription factor preferentially expressed in human and mouse CD4+ FoxP3+ Tregs, was shown to allow discrimination between thymus-derived and peripherally induced Tregs [30, 31]. In VitD-induced AD-like inflammation, the percentages of thymus-derived (Helios+) Tregs were enhanced, whereas peripheral (Helios-) Tregs were reduced in sdLNs on day 10 of topical VitD treatment (Fig. 3A). Accordingly, thymus-derived Tregs displayed higher absolute numbers (Fig. 3B). Kinetic analysis revealed a predominant expansion of thymus-derived Tregs in sdLNs of mice as early as day 5 of topical VitD treatment (Fig. 4). Thus, our results indicate an early imbalance of the Treg compartment towards predominating thymus-derived Tregs in AD. Intriguingly, percentages of thymus-derived Tregs failed to increase after depletion of Langerin+ DCs in mouse skin at day 5 of VitD-treatment (Fig. 5). To identify the molecules providing the tolerogenic function to DCs, we screened for expression of various costimulatory and co-inhibitory molecules, without identifying significant changes in the expression of PD-L1, ICOS-L, GITR-L and IDO by VitD-exposed DCs (data not shown). Moreover, we detected only trace amounts of IL-10 production by skin-derived DCs in our experiments (data not shown). Thus, thymus-derived Tregs are increased in the VitD AD mouse model and Langerin+ DCs are required for the early expansion of thymus-derived Tregs in AD, via a still elusive mechanism.

Thymus-derived Tregs exhibit a Th2-like phenotype in AD

We first measured the percentages of overall Tregs producing IL-10 and IL-13 in the VitD model of AD. Tregs isolated from AD mice (day 10 of treatment) produced larger amounts of both IL-10 and IL-13 than Tregs isolated from healthy controls, regardless of the presence of Langerin+ DCs (Fig. S3C and D). In contrast, the production of IL-13 but not of IL-10 by Tregs was significantly increased earlier during the development of AD, i.e. 5 days after the start of VitD treatment (Fig. 6A and B). Depletion of Langerin+ DCs did not alter the produc-
tion of IL-13 (Fig. 6B), but increased the secretion of IL-10 by Tregs at early time-points (Fig. 6A). Moreover, the production of IL-13 was more strongly induced in Tregs than in CD4+ effector T cells in VitD-induced early and overt AD (Fig. S4). To further dissect the production of cytokines within the Treg compartment, we measured percentages of thymus- and peripherally derived Tregs producing IL-10 and IL-13. Thymus-derived Tregs were identified as the main source of IL-10 and IL-13 (Fig. 6C–E). Notably, we observed similar numbers of thymus-derived Tregs and effector T cells producing IL-13 in the VitD AD model (Fig. 6F). Moreover, both numbers of thymus-derived Tregs and effector T cells producing IL-13 were higher than numbers of peripherally derived Tregs. These findings emphasize the potential role of thymus-derived Th2-polarized Tregs in driving the pathogenic events leading to or sustaining AD and suggest that peripherally derived Tregs are small contributors to the overall Th2 cytokine production in VitD AD model (Fig. 6D and F). Furthermore, depletion of Langerin+ DCs did not alter the production of cytokines by thymus-derived Tregs in the VitD model of AD (Fig. 6C and D). In conclusion, we identified activated, IL-10-producing thymus-derived Tregs, concomitantly exhibiting a Th2-like phenotype in the VitD model of AD.

Discussion

In this study we discovered higher numbers of overall Tregs with a specific expansion of thymus-derived Tregs in mice with AD. Furthermore, our results indicate that AD is associated with the expansion of thymus-derived Tregs exhibiting a Th2 phenotype and that LCs seem to be responsible for this biased Treg differentiation. However, other cells or factors from the microenvironment in sdLNs might also contribute to shaping the unusual cytokine profile of thymus-derived Tregs. Irrespectively, our data strongly suggest that Th2-like Tregs actively contribute to the development of AD [32].

Several groups reported increased Tregs in the peripheral blood [13–16] and skin lesions [10] of AD patients, whereas others did not [10–12, 18]. In support of the former, we here report increased numbers of CD4+ CD25+ FoxP3+ Tregs in sdLNs of mice at different disease stages of AD development (Fig. 1). The T-cell-specific costimulatory molecule ICOS is up-regulated after cell activation and binding to its ligand (ICOS-L). This step is essential for Treg survival, proliferation and memory rather than for their activation [33]. Indeed, reduced numbers of CD4+ FoxP3+ Tregs have been observed in ICOS knockout mice in the steady-state and upon immunization [34]. CTLA-4 is a CD28 homologue that is up-regulated after activation of effector T cells and Tregs. It is associated with Treg suppressive function, although this remains controversial [35]. GARP (or LRRC32), a Treg-specific activation marker, is part of the receptor for latency-associated peptide/latent transforming growth factor-β complex [36].

In the VitD mouse model of AD, percentages of Tregs expressing ICOS (Fig. 2A), CTLA-4 and GARP (Fig. 2B) and producing IL-10 (Fig. 6A and C, Fig. S3C) were increased, indicating an activated phenotype. However, expansion of activated Tregs in VitD AD-like inflammation is unable to counteract ongoing AD. A similar situation is highly probable in AD patients [10, 13–16].

Tregs can be divided into two subcategories, namely thymus-derived and peripherally induced Tregs. While Helios is expressed in all CD4+ CD8– FoxP3+ mouse thymocytes [30], neuropilin discriminates thymic Tregs from peripheral Tregs only in the steady-state [37]. Therefore, Helios is currently the most discriminative marker for thymus-derived Tregs [30]. We found that the ratio of thymus-derived (Helios+) Tregs over peripherally derived (Helios−) Tregs was increased in sdLNs during AD-like inflammation (Fig. 3), with enhanced expansion of thymus-derived (Helios+) Tregs starting early in the development of the disease (Fig. 4). Thymus-derived Tregs are involved in self-tolerance and were shown to be activated by microbes [32]. Moreover, they are important for the control of Th1 immune responses [38]. Thus, thymic Tregs might be less efficient at counteracting Th2-related diseases such as AD when compared to peripherally induced Tregs.

The Treg population is heterogeneous. Indeed, Tregs can acquire alternative effector or hybrid fates, associated with promotion rather than inhibition of inflammation under certain conditions [32]. Accordingly, increased production of IL-5 and IL-13 has been described in skin-homing Tregs of AD patients [12, 14]. High expression of GATA3, as observed in Tregs located at barrier sites such as the skin and gut...
might enable Tregs to produce Th2 cytokines. In our VitD AD model, we found increased percentages of IL-13-producing Tregs in sdLNs (Fig. 6B and D, Figs S3D and S4), similar to Th2 Tregs in the skin of AD patients [12, 14]. This might potentially confer a pro- rather than an anti-inflammatory phenotype to Tregs in AD.

When we analysed the cytokine production by Tregs more thoroughly, we found that thymus-derived Tregs were the main source of IL-10 and IL-13 in the VitD AD model (Fig. 6C-E). Therefore, we demonstrate here for the first time that expansion of thymus-derived Tregs exhibiting a Th2-like phenotype is promoted in AD. In fact, the numbers of thymic Tregs secreting IL-13 were similar to the numbers of effector T cells producing IL-13 (Fig. 6F) and the percentages of thymus-derived Tregs secreting IL-13 were significantly increased compared to effector T cells (Fig. 6D and E). This strongly suggests a pathogenic role of thymus-derived Th2-polarized Tregs in AD. Indeed, these Tregs might exert poor immunosuppressive properties despite their capacity to produce IL-10 and consequently contribute to the development of AD-like inflammation. It would be of particular interest to test this hypothesis by assessing the overall immunosuppressive capacity of these Th2 Tregs. Unfortunately, due to the nuclear...
localization of Helios, the lack of Helios-EGFP mice, and the missing specificity of neuropilin as a surface marker during inflammation, it is not possible to purify Th2 thymus-derived Tregs for further in vitro immunosuppressive assays.

Dendritic cells are antigen-presenting cells regulating immunity and tolerance, respectively, by priming effector T cells and expanding Tregs [40]. In the VitD AD model, T$_\text{reg}$ expansion in sdLNs required the presence of skin-derived DCs (Fig. S1F and G). The time course of T$_\text{reg}$ induction revealed that, unexpectedly, LCs are the first cutaneous DC subset to reach sdLNs in our experimental setup (Fig. S1A–E). VitD-induced production of TSLP by keratinocytes might primarily trigger LCs, whereas dermal DCs migrate to sdLNs more quickly after

Fig. 6 Langerin$^+$ dendritic cells are dispensable for cytokine-producing phenotype of thymus-derived regulatory T cells in murine AD-like inflammation. Production of IL-10 (A) and IL-13 (B) by overall CD4$^+$ FoxP3$^+$ T$_\text{reg}$ in sdLNs from ETOH versus Vit D-treated Langerin-DTR mice, injected with PBS (+ Langerin$^+$ DC) or diphtheria toxin (– Langerin$^+$ DC), at day 5 of treatment. Percentages of effector T cells (E) and numbers of effector T cells, thymus- and peripherally derived T$_\text{reg}$ (F) producing IL-13 in sdLNs of ETOH or Vit D-treated mice, at day 5 of treatment. Data are representative of one to three independent experiments and were analysed with a Student’s $t$-test, $n=6–8$. n.s. not significant.
skin immunization with DNB [4, 41]. Previous results established that LCs promote Treg proliferation upon RANK signalling [42]. However, depletion of LCs and Langerin+ dermal DCs did not affect the size of the overall CD4+ CD25+ FoxP3+ Treg population in dlNs of VitD-induced AD mice (Fig. S3A and B) but specifically abolished the induction of thymus-derived Treg (Fig. 5). Moreover, depletion of LCs and Langerin+ dermal DCs did not affect the production of IL-13 by Tregs, regardless of Helios expression (Fig. 6B and D, Fig. S3D). Thymus-derived Treg are involved in antimicrobial responses [32] and LCs are a privileged DC subset sensing microbe-derived antigens in AD [43]. Thus, the expansion of thymus-derived Treg might be attributed to LCs, while their cytokine production might rather be determined by other cells or factors within the microenvironment of the dlNs. Langerhans cell-derived IL-10 can promote Treg expansion [44, 45], but in our experiments, IL-10 was not detectable in skin-derived DCs following VitD treatment (data not shown). Furthermore, expression of PD-L1, ICOS L, GITR L and IDO by skin-derived DCs might only have a supporting role in Treg expansion in the VitD AD mouse model (data not shown). Thus, the question how LCs or other DCs promote the expansion of Treg or otherwise impact on their phenotype remains unanswered.

In summary, our work represents the first study demonstrating a preferential expansion of activated CD4+ CD25+ FoxP3+ thymus-derived Treg exhibiting a Th2 phenotype in a mouse model of AD. Furthermore, differentiation of thymus-derived Treg seems to depend on LCs, while their cytokine profile might rather be determined by DC phenotype and their microenvironment. Hence, Treg in AD might contribute to the disease rather than playing their role of immunosuppressive cells and thus might represent potential new therapeutic targets.

Acknowledgements

S.D. had funds through (FWF-P28039-B13 and FWF-P21449-B13). We are grateful to Ernst R. Werner, Innsbruck Medical University, for valuable comments and to Martin Thurnher for providing access to the FACS Canto in his laboratory. We would like to thank Nikolaus Romani and Patrizia Stoitzen for critically reading of the manuscript. We appreciate the support of the European SKINBAD COST Action BM0903.

Conflicts of interest

Christoph H. Tripp was employed by the COMET Center ONCOTYROL, which is funded by the Austrian Federal Ministries BMVIT/BMWFJ (via FFG) and the Standortagentur Tirol. We further appreciate the participation of the TILAK hospital holding company, who serves as a partner in the Oncotyrol research program.

Supporting information

Additional Supporting Information may be found in the online version of this article:

Figure S1 (A–E) Numbers of CD4+ CD25+ FoxP3+ Tregs (A) and DC (B–E) in dlNs in mice treated with ETOH or Vit D.

Figure S2 Depletion of Langerin-expressing DCs in epidermis (A–D) and dlNs (E–H) from Langerin-DTR mice, topically treated with ETOH (A, B, E and F) or Vit D (C, D, G and H), after intraperitoneal injection of PBS (A, C, E and G) or DT (B, D, F and H) on day −2, day +2, day +6 and day +8.

Figure S3 (A and B) Numbers of Treg in dlNs from Langerin-DTR mice, injected with PBS (+ Langerin+ DC) or diphtheria toxoid (− Langerin+ DC), at day 5 (A) and day 10 (B) of treatment.

Figure S4 Percentages of IL-13-producing effector and total Treg in dlNs of ETOH or Vit D-treated mice at day 5 (A) and day 10 (B) of treatment.

References

1. Elias PM, Schmuth M. Abnormal skin barrier in the etiopathogenesis of atopic dermatitis. Curr Opin Allergy Clin Immunol. 2009; 9: 437–46.
2. Leung DY, Guttman-Yassky E. Deciphering the complexities of atopic dermatitis: shifting paradigms in treatment approaches. J Allergy Clin Immunol. 2014; 134: 769–79.
3. Ebner S, Nguyen VA, Forstner M, et al. Thymic stromal lymphopoietin converts human epidermal Langerhans cells into antigen-presenting cells that induce proinflammatory T cells. J Allergy Clin Immunol 2007; 119: 982–90.
4. Elentner A, Finke D, Schmuth M, et al. Langerhans cells are critical in the development of atopic dermatitis-like inflammation and symptoms in mice. J Cell Mol Med. 2009; 13: 2658–72.
5. Eyerich K, Pennino D, Scarpioni C, et al. IL-17 in atopic eczema: linking allergen-specific adaptive and microbial-triggered innate immune response. J Allergy Clin Immunol. 2009; 123: 59–66 e4.
6. Lin W, Truong N, Grossman WJ, et al. Allergic dysregulation and hyperimmunoglobulinemia E in Foxp3 mutant mice. J Allergy Clin Immunol. 2009; 116: 1106–15.
7. d’Hennezel E, Bin Dhuban K, Torgerson T, et al. The immunogenetics of immune dysregulation, polyendocrinopathy, enteropathy, X linked (IPEX) syndrome. J Med Genet. 2012; 49: 291–302.
8. Hinz D, Bauer M, Roder S, et al. Cord blood Tregs with stable FOXP3 expression are influenced by prenatal environment and associated with atopic dermatitis at the age of one year. Allergy. 2012; 67: 380–9.
9. Tulic MK, Andrews D, Crook ML, et al. Changes in thymic regulatory T-cell maturation from birth to puberty: differences in atopic children. J Allergy Clin Immunol. 2012; 129: 199–206 e1–4.
10. Szegedi A, Barath S, Nagy G, et al. Regulatory T cells in atopic dermatitis: epidermal
dendritic cell clusters may contribute to their local expansion. Br J Dermatol. 2009; 160: 984–93.

11. Brandt C, Pavlovic V, Radbruch A, et al. Low-dose cyclosporine A therapy increases the regulatory T cell population in patients with atopic dermatitis. Allergy. 2009; 64: 1588–96.

12. Lin YT, Wang CT, Chao PS, et al. Skin-homing CD4+ Foxp3+ T cells exert Th2-like function after staphylococcal superantigen stimulation in atopic dermatitis patients. Clin Exp Allergy. 2011; 41: 516–25.

13. Ou LS, Goleva E, Hall C, et al. Regulatory T cells in atopic dermatitis and subversion of their activity by superantigens. J Allergy Clin Immunol. 2004; 113: 756–63.

14. Reeter AJ, Satinover SM, Solga MD, et al. Analysis of CD25hiCD4+ "regulatory" T-cell subsets in atopic dermatitis reveals a novel T(H)2-like population. J Allergy Clin Immunol. 2008; 121: 415–22 e3.

15. Ito Y, Adachi Y, Makino T, et al. Expansion of FOXP3-positive CD4+ CD25+ T cells associated with disease activity in atopic dermatitis. Ann Allergy Asthma Immunol. 2009; 103: 160–6.

16. Hijnis D, Haeck I, van Kraats AA, et al. Cyclosporin A reduces CD4(-)CD25(+) regulatory T-cell numbers in patients with atopic dermatitis. J Allergy Clin Immunol. 2009; 124: 856–68.

17. Schnopp C, Rad R, Weidinger A, et al. FoxP3-positive regulatory T cells are present in the skin of generalized atopic eczema patients and are not particularly affected by medium-dose UVA1 therapy. Photodermatol Photoimmunol Photomed. 2007; 23: 81–5.

18. Verhagen J, Akdis M, Traidl-Hoffmann C, et al. Absence of T-regulatory cell expression and function in atopic dermatitis skin. J Allergy Clin Immunol. 2006; 117: 176–83.

19. Vukmanovic-Stejic M, McQuaid A, Birch KE, et al. Relative impact of CD4+ CD25+ regulatory T cells and tacrolimus on inhibition of T-cell proliferation in patients with atopic dermatitis. Br J Dermatol. 2005; 153: 759–7.

20. Agrawal R, Wniewierski JA, Woodfolk JA. The role of regulatory T cells in atopic dermatitis. Curr Probl Dermatol. 2011; 41: 112–24.

21. Bennett CL, van Rijn E, Jung S, et al. Inducible ablation of mouse Langerhans cells diminishes but fails to abrogate contact hypersensitivity. J Cell Biol. 2005; 169: 569–76.

22. Li M, Hener P, Zhang Z, et al. Topical vitamin D3 and low-calcemic analogs induce thymic stromal lymphopoietin in mouse keratinocytes and trigger an atopic dermatitис Proc Natl Acad Sci USA. 2006; 103: 11736–41.

23. Li M, Messaddeq N, Teletin M, et al. Retinoid X receptor ablation in adult mouse keratinocytes generates an atopic dermatitis triggered by thymic stromal lymphopoietin. Proc Natl Acad Sci USA. 2005; 102: 14795–800.

24. Chappaz S, Flueck L, Farr AG, et al. Increased TSLP availability restores T- and B-cell compartments in adult IL-7 deficient mice. Blood. 2007; 110: 3862–70.

25. Yoo J, Omori M, Gyarmati D, et al. Spontaneous atopic dermatitis in mice expressing an inducible thymic stromal lymphopoietin transgene specifically in the skin. J Exp Med. 2005; 202: 541–9.

26. Nakajima S, Igyarto BZ, Honda T, et al. Langerhans cells are critical in epidermatitis sensitization with protein antigen via thymic stromal lymphopoietin receptor signaling. J Allergy Clin Immunol. 2012; 129: 1048–56 e6.

27. Soumelis V, Reche PA, Kanzier H, et al. Human epithelial cells trigger dendritic cell mediated allergic inflammation by producing TSLP. Nat Immunol. 2002; 3: 673–80.

28. Baek F, Korf H, Oberberg L, et al. The vitamin D analog, TXS27, promotes a human CD4+ CD25(high)CD127(low) regulatory T cell profile and induces a migratory signature specific for homing to sites of inflammation. J Immunol. 2011; 186: 132–42.

29. Flacher V, Tripp CH, Haid B, et al. Skin langerin(+) dendritic cells transport intradermally injected anti-DEC-205 antibodies but are not essential for subsequent cytotoxic CD8(+) T cell responses. J Immunol. 2012; 188: 2146–55.

30. Thornton AM, Korty PE, Tran DO, et al. Expression of Helios, an Ikaros transcription factor family member, differentiates thymic-derived from peripherally induced Foxp3(+) regulatory T cells. Proc Natl Acad Sci USA. 2010; 108: 3433–41.

31. Dhamne C, Chung Y, Alosi AM, et al. Peripheral and thymic foxp3(+) regulatory T cells in search of origin, distinction, and function. Front Immunol. 2013; 4: 253.

32. Sawant DV, Vignali DA. Once a Treg, always a Treg? Immunity Rev. 2014; 259: 173–91.

33. Simpson TR, Quezada SA, Allison JP. Regulation of CD4 T cell activation and effector function by inducible costimulator (ICOS). Curr Opin Immunol. 2010; 22: 326–32.

34. Busse M, Krech M, Meyer-Bahlburg A, et al. ICOS mediates the generation and function of CD4+ CD25+ Foxp3+ regulatory T cells conveying respiratory tolerance. J Immunol. 2012; 189: 1795–82.

35. Bour-Jordan H, Bluethone JA. Regulating the regulators: costimulatory signals control the homeostasis and function of regulatory T cells. Immunol Rev. 2009; 229: 41–66.

36. Zhou AX, Kozhaya L, Fujii H, et al. GARP–TGF-beta complexes negatively regulate regulatory T cell development and maintenance of peripheral CD4+ T cells in vivo. J Immunol. 2013; 190: 5057–64.

37. Weiss JM, Bilate AM, Gobert M, et al. Neuropilin 1 is expressed on thymus-derived natural regulatory T cells, but not mucosa-generated induced Foxp3+ T reg cells. J Exp Med. 2012; 209: 1723–42, S1.

38. Dhaianaut M, Coquereille C, Uzureau S, et al. Thymus-derived regulatory T cells restrain pro-inflammatory Th1 responses by downregulating CD70 on dendritic cells. EMBO J. 2015; 34: 1336–48.

39. Wohlfert EA, Grainger JR, Bouladoux N, et al. GATA3 controls Foxp3(+) regulatory T cell fate during inflammation in mice. J Clin Invest. 2011; 121: 4503–15.

40. Schlitzer A, McGovern N, Ginhoux F. Dendritic cells and monocyte-derived cells: two complementary and integrated functional systems. Semin Cell Dev Biol. 2015; 41: 9–22.

41. Kissenpfennig A, Henri S, Dubois B, et al. Dynamics and function of Langerhans cells in vivo: dermal dendritic cells colonize lymph node areas distinct from slower migrating Langerhans cells. Immunity. 2005; 22: 643–54.

42. Loser K, Meling A, Losser S, et al. Epidermal RANKL controls regulatory T-cell numbers via activation of dendritic cells. Nat Med. 2006; 12: 1372–9.

43. Yoshiida K, Kubo A, Fujita H, et al. Distinct behavior of human Langerhans cells and inflammatory dendritic epidermal cells at tight junctions in patients with atopic dermatitis. J Allergy Clin Immunol. 2014; 134: 856–64.

44. Yoshihi R, Kabashima K, Sugita K, et al. IL-10-producing Langerhans cells and regulatory T cells are responsible for depressed contact hypersensitivity in grafted skin. J Invest Dermatol. 2009; 129: 705–13.

45. Igyarto BZ, Jenison MC, Dudda JC, et al. Langerhans cells suppress contact hypersensitivity responses via cognate CD4 interaction and Langerhans cell-derived IL-10. J Immunol. 2009; 183: 5085–93.