

Attenuated TGF- β 1 responsiveness of dendritic cells and their precursors in atopic dermatitis

Wen-Ming Peng, Laura Maintz, Jean-Pierre Allam and Natalija Novak

Department of Dermatology and Allergy, University of Bonn, Bonn, Germany

The responsiveness of DCs and their precursors to transforming growth factor beta1 (TGF- β 1) affects the nature of differentiating DC subsets, which are essential for the severity of atopic dermatitis (AD). To evaluate TGF- β signaling in monocytes and monocyte-derived DCs of AD patients compared with that of controls, *in vitro* generated Langerhans cell (LC) like DCs, expression of TGF- β receptors, phospho-Smad2/3 and Smad7 were evaluated. Furthermore, TNF- α expression and synergistic effects of TNF- α upon TGF- β signaling and DC generation were evaluated. We found LC-like DC differentiation of monocytes from AD patients in response to TGF- β 1 was remarkably reduced and TGF- β 1 receptor expression was significantly lower compared with that of healthy controls. Attenuated TGF- β 1 responsiveness mirrored by lower phospho-Smad2/3 expression after TGF- β 1 stimulation and higher expression of inhibitory Smad7 was observed in monocytes from AD patients. During DC generation, mRNA expression of Smad7 was relatively higher in LC-like DCs of AD patients. Lower TNF- α expression of monocytes from AD patients might further contribute to attenuated TGF- β signaling in the disease since TNF- α had synergistic effects on TGF- β 1 signaling and LC generation through mediating the degradation of Smad7. Our results demonstrate alleviated TGF- β 1 signaling together with the amount of soluble co-factors might direct the nature of differentiating DCs.

Keywords: Atopic dermatitis · Dendritic cells · Monocytes · TNF- α · TGF- β

Introduction

Atopic dermatitis (AD) is one of the most common allergic inflammatory skin diseases affecting up to 20% of children and up to 3% of adults in the western society [1]. It has been shown that the interaction of inherited as well as environmental factors plays an important role in the pathogenesis of AD [1, 2].

Transforming growth factor beta1 (TGF- β 1) is a multifunctional cytokine and TGF- β 1 signaling is essential for the maintenance of immune tolerance [3]. Briefly, TGF- β 1 signaling is initiated by binding of TGF- β 1 to its heterodimeric transmembrane receptor complex, which is composed of type I (TGF β RI) and

type II (TGF β RII) receptors [4]. Binding of TGF- β 1 results in the phosphorylation and activation of TGF β RI, which then activates TGF β RII [4]. The activated TGF- β receptors induce phosphorylation of Smad2 and Smad3 [5, 6], which form a complex together with Smad4. The complex translocates into the nucleus and starts gene transcription and regulation [5–8]. The type III (TGF β RIII) receptor does not have signaling function but helps TGF- β to bind its receptors. Importantly, TGF- β 1 signaling is suppressed by the expression of inhibitory Smad molecules, namely Smad7, which mediates degradation of TGF β RI and suppresses the phosphorylation of Smad2/Smad3 [9, 10]. Several lines of evidence demonstrated that TGF- β 1 exerts strong suppressive functions on epidermal and mucosal inflammation [11, 12]. Animals with impaired TGF- β 1 signaling developed spontaneously lethal autoimmune inflammation [13, 14] and autoimmune diseases [15–17]. Moreover, evidence for modified TGF- β signaling in AD exists [18–20]. Furthermore, TGF- β 1 is the prerequisite for the generation of

Correspondence: Prof. Natalija Novak
e-mail: Natalija.Novak@ukb.uni-bonn.de

LCs both in vitro and in vivo [21–23] and keeps LCs in an immature state [24]. Accumulating data suggests that LCs represent a characteristic, resident subset of epidermal DCs with important tolerogenic functions [25–27]. Both Langerhans cell (LC) generation and TGF- β 1 signaling are important for the local immune response in AD [28]. Therefore, it was the purpose of this study to examine TGF- β 1 signaling in APCs in AD.

Results

Impaired LC-like DC differentiation of monocytes from AD patients

To analyze responsiveness of monocytes to TGF- β , the number of LCs differentiating from monocytes of patients with AD and healthy controls during TGF- β driven culture was investigated. CD1a⁺ DCs were analyzed on day 2, day 4, and day 6 of culture for the expression of Langerin (Fig. 1). Lower percentage of CD1a⁺Langerin⁺ DCs was detectable in AD patients on day 2, day 4, and day 6 of culture (Fig. 1A) compared with controls. Furthermore, mean value of expression of Langerin on CD1a⁺ DCs derived from monocytes from AD patients was also lower (Fig. 1B). These data provide evidence for lower TGF- β responsiveness of monocytes from patients with AD as compared with controls.

Decreased expression of TGF- β 1 receptors on monocytes from patients with AD

TGF- β 1 binds to TGF β RI and TGF β RII to initiate the transduction of TGF- β signaling. Therefore, we analyzed the expression of TGF β RI and TGF β RII on freshly isolated human monocytes from AD patients and control individuals by flow cytometry. The extracellular expression of TGF β RI protein was significantly lower on monocytes from patients with AD ($p = 0.008$), the expression of TGF β RII was also significantly lower on monocytes from patients with AD ($p = 0.030$) (Fig. 2A). The lower expression of TGF β RI on monocytes of patients with AD was further confirmed on mRNA level by real-time PCR assays ($p = 0.041$) (Fig. 2B). Interestingly, TGF β RII mRNA expression correlated negatively with total IgE serum levels of AD patients ($r = 0.761$; $p = 0.017$). These results suggest that disease severity mirrored by higher total serum IgE level might influence TGF β RII expression. Lower expression of TGF β RI and II might result in lower TGF- β binding capacity of monocytes from patients with AD.

Increased Smad7 expression and attenuated monocyte responsiveness in AD upon TGF- β 1 stimulation

In the next series of experiments, we evaluated constitutive and TGF- β -induced expression of activating and inhibitory Smad molecules by immunoblotting in unstimulated monocytes of AD

patients and healthy controls. We detected significantly higher constitutive expression of suppressive Smad7 in unstimulated monocytes of AD patients as compared with monocytes from healthy controls ($p = 0.001$) (Fig. 3A and C). Furthermore, we investigated TGF- β -mediated induction of phospho-Smad expression in monocytes from AD patients and control individuals. Upon TGF- β stimulation, expression of phospho-Smad2/3 was lower in monocytes from patients with AD as compared with those from healthy controls (Fig. 3B and D). These data provide evidence for modified baseline and TGF- β -induced Smad signaling in monocytes of patients with AD.

Higher relative Smad7 mRNA expression in MoDCs of patients with AD

Next we analyzed relative Smad3 and Smad7 mRNA expression of MoDCs of patients with AD and healthy controls under TGF- β driven in vitro culture in a time kinetic. While relative Smad3 mRNA expression was downregulated from day 2 of culture in both MoDCs of AD patients as well as healthy controls, relative Smad7 mRNA expression was upregulated in patients with AD as well as in controls (Fig. 4). Relative upregulation of Smad7 mRNA expression as compared with baseline Smad7 mRNA expression was higher in MoDCs of patients with AD during whole culture, with a peak on day 2 of culture ($p = 0.026$). Relative Smad3 mRNA expression correlated negatively with total serum IgE levels of AD patients ($r = 0.682$; $p = 0.043$), indicating that Smad3 expression might be the lower, the higher total serum IgE levels in AD patients are.

TNF- α synergizes with TGF- β 1 on TGF- β signaling

To investigate the association of TNF- α signaling with TGF- β signaling, we stimulated human monocytes with 10 ng/mL TGF- β , 50 ng/mL TNF- α , 10 ng/mL TGF- β together with 50 ng/mL TNF- α , respectively, or left them unstimulated. We could show that TNF- α enhanced TGF- β 1 signaling by selectively increasing the expression of activating phospho-Smad2/3, while MAPK phospho-p44/42 expression level remained unaffected (Fig. 5A). Immune blotting results demonstrated that TNF- α mediated downregulation of the expression of Smad7 during DC generation in vitro (Fig. 5B). Because cytokines exert functions in an autocrine/paracrine mode, as a next step, we evaluated baseline TNF- α expression of monocytes from patients with AD as compared with those from healthy controls. Relative TNF- α mRNA expression of monocytes from patients with AD, analyzed with the help of real-time PCR assays was significantly lower than TNF- α mRNA expression of monocytes from healthy controls ($p = 0.001$) (Fig. 5C). Moreover, we added TNF- α to the DC culture to evaluate whether exogenous TNF- α could rescue attenuated LC generation in response to TGF- β in patients with AD. Induction of Langerin-expressing DCs was significantly lower in

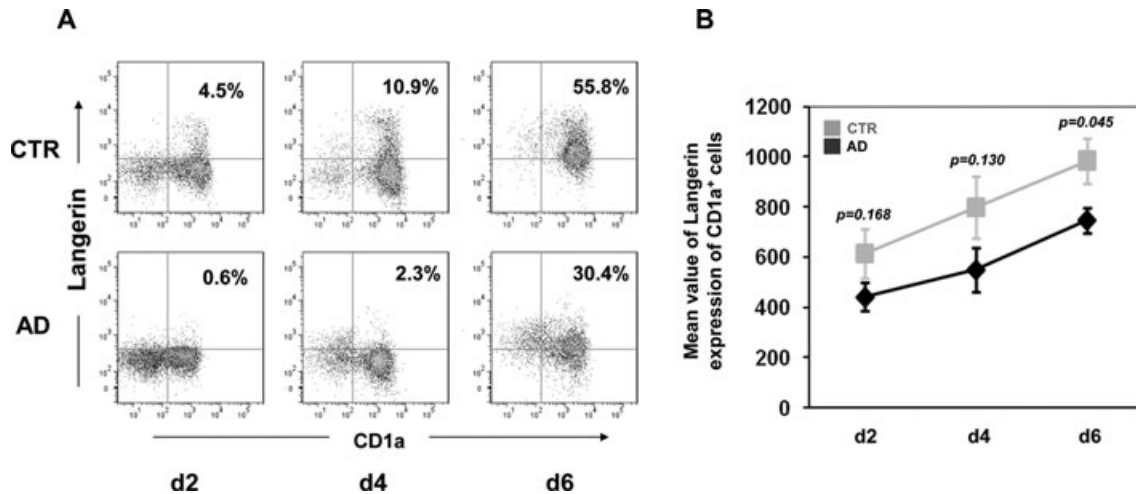


Figure 1. Lower generation of Langerin⁺CD1a⁺ DCs from monocytes of patient with AD. (A) The percentages of CD1a⁺Langerin⁺ DCs on day 2, day 4, and day 6 of culture are shown. One representative experiment of six performed is shown. (B) Mean value of Langerin expressed by CD1a⁺ DCs derived from monocytes of healthy controls (gray line) or patients with AD (black line) on day 2, day 4, and day 6 is shown as relative expression. Data are shown as mean $\pm$ SEM of samples pooled from six independent experiments. Statistical significance was determined by t-test.

monocyte-derived DCs of patients with AD as compared with controls. Therefore, we conclude that supply of TNF- α is not capable to rescue lower TGF- β responsiveness of monocytes and monocyte-derived DCs of patients with AD (Fig. 5D).

Discussion

In this study, we demonstrated that monocytes from patients with AD display lower responsiveness to TGF- β in vitro, which

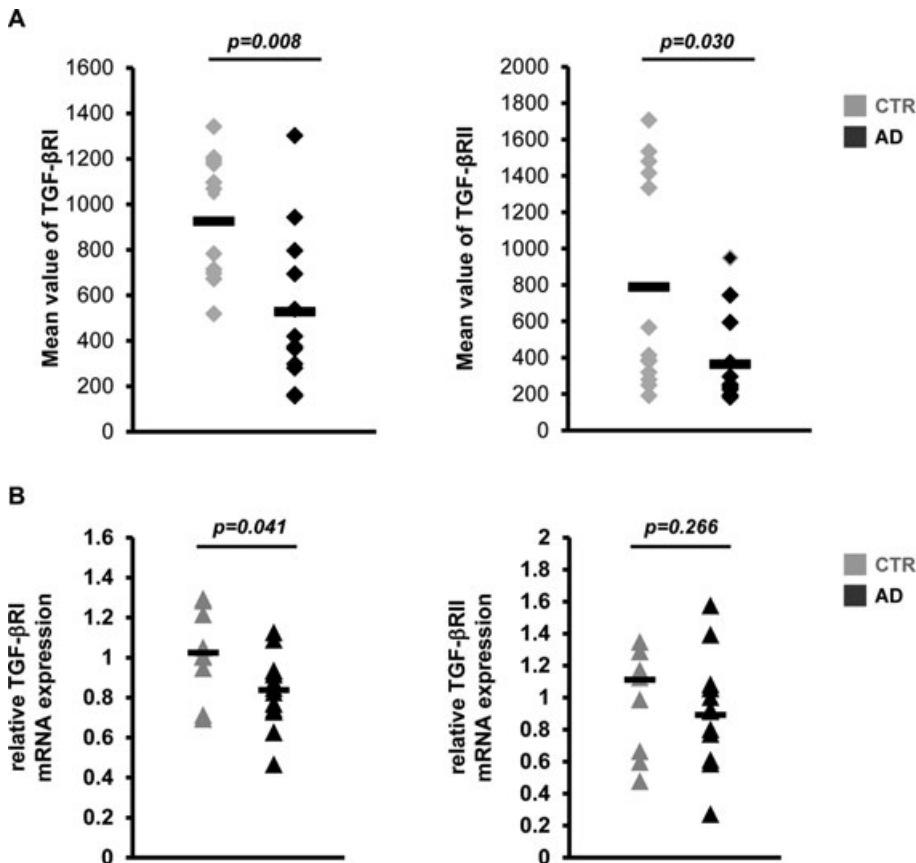


Figure 2. Decreased expression of TGF β RI and TGF β RII on monocytes of patients with AD. (A) Surface expression of TGF β RI (left) and TGF β RII (right) of freshly isolated human monocytes was evaluated by flow cytometry. Mean value of TGF β RI and TGF β RII on monocytes from patients with AD (black symbols, $n = 13$) and healthy controls (CTR, gray symbols, $n = 12$) is shown. (B) Relative mRNA expression of TGF β RI and TGF β RII of monocytes of patients with AD (AD, black symbols, $n = 9$) and healthy controls (CTR, gray symbols, $n = 14$) is shown. Bars represent the means. Data shown are from pooled data from the number of samples mentioned above. Statistical significance was determined by Mann-Whitney U test.

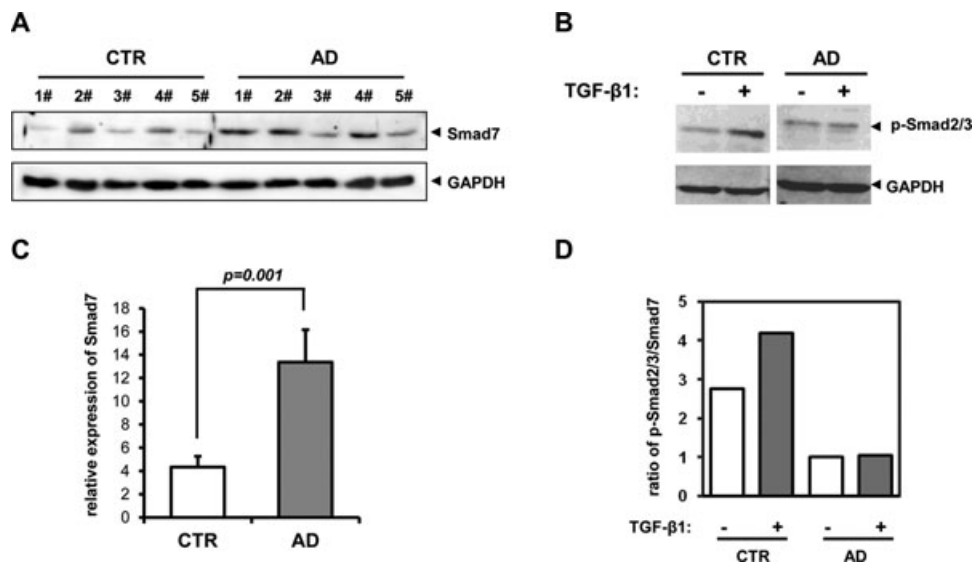


Figure 3. Increased Smad7 expression and lower TGF- β responsiveness of monocytes from patients with AD. (A) Immunoblots on Smad7 expression of freshly isolated monocytes from patients with AD and control individuals are shown. GAPDH was used as loading control. (B) Immunoblotting results of phospho-Smad2/3 expression of monocytes from patients with AD and control individuals stimulated with 2 ng/mL TGF- β 1 for 1 h are shown. Data shown are from one experiment representative of three performed. (C) Densitometric analysis of Smad7 expression of freshly isolated monocytes from patients with AD and control individuals is shown as mean $\pm$ SEM, results from $n = 13$ of CTR and $n = 16$ of patients with AD. Statistical significance was determined by Mann–Whitney U test. (D) Densitometric analysis of phospho-Smad2/3 levels of monocytes with and without TGF- β 1 stimulation is depicted, data from one experiment representative of three performed are shown.

results most likely from a lower TGF β RI and TGF β RII expression on their cellular surface and higher TGF- β signaling inhibiting Smad7 expression. Moreover, TNF- α mRNA expression was lower in monocytes of the peripheral blood of patients with AD, which is of critical importance in view of the synergistic effects of TNF- α on TGF- β signaling in terms of propagating Langerin expression of DCs and enhancing phospho-Smad2/3 expression and suppressing Smad7 expression of monocytes in response to TGF- β stimulation. We show, for the first time, evidence for attenuated TGF- β signaling in DC precursor cells in patients with AD. This attenuated signaling might diminish the protective and protolerogenic effects of TGF- β on human monocytes on one hand and impact on the amount of differentiating LCs in the skin on the other, and contribute thereby to the breakdown of the homeostasis and tolerance in the skin in AD.

Immune tolerance of skin to common environmental components is essential for the physiologic functions of normal skin,

while cutaneous hypereactivity to environmental components is a distinct feature of AD [1]. As a key regulatory cytokine, TGF β 1 inhibits activation and maturation of DCs [23, 29], suppresses the activation of LC-like cells after allergen stimulation [30]. In addition, tolerogenic LC-like DCs can be generated by incubation of myeloid precursors with TGF- β 1 [23, 24], which promotes Langerin expression in a dose-dependent manner [31]. In epidermal skin lesions of AD patients, two important myeloid DC subpopulations, namely LCs and inflammatory dendritic epidermal cells (IDECs) can be detected. It is assumed that LCs that are also presented in nonlesional AD skin and skin of healthy individuals, primarily maintain a state of tolerance in the skin [26, 32]. In contrast, activated IDECs that are only present in significant numbers in lesional skin of patients with AD might contribute profoundly to the local inflammatory environment in AD skin by the secretion of proinflammatory cytokines and chemokines [33]. The relation of LCs to IDECs in the epidermis might therefore direct the course

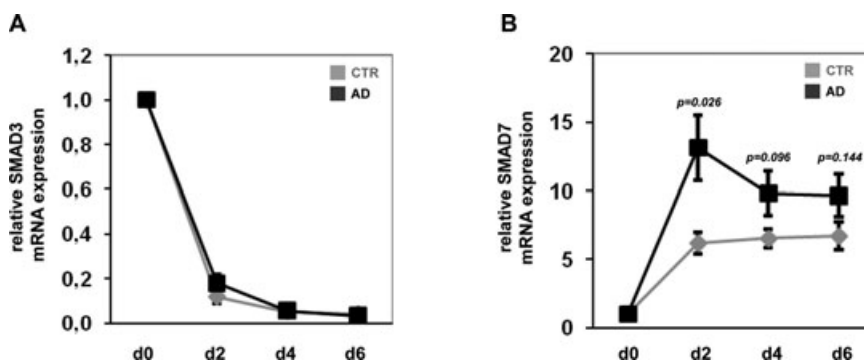


Figure 4. Smad7 mRNA expression is modulated during DC differentiation. (A) Smad3 and (B) Smad7 mRNA expression on day 2, day 4, and day 6 of culture are pooled from seven independent experiments performed. Statistical significance was determined by Mann–Whitney U test.

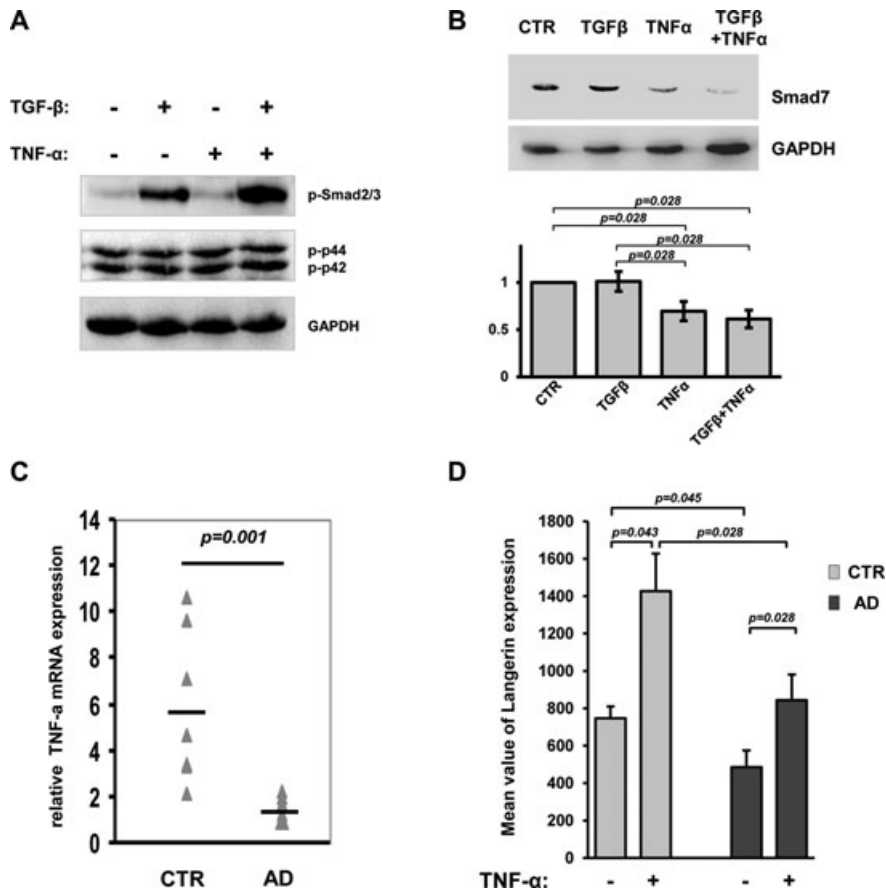


Figure 5. TNF- α synergizes with TGF- β 1 on TGF- β 1 signaling. (A) Human monocytes were stimulated with either 10 ng/mL TGF- β 1, 50 ng/mL TNF- α , or 10 ng/mL TGF- β 1 together with 50 ng/mL TNF- α for 1 h at 37°C. Unstimulated cells were used as control. After stimulation, cells were collected and subjected to immunoblot for expression of phospho-p44/42 MAPK and phospho-Smad2/3. TNF- α further increases TGF- β -induced phospho-Smad2/3 expression. One representative experiment of three performed is shown. (B) LC-like DCs were generated with or without TNF- α as described in Materials and methods. Immune blot (top) and densitometric analysis (bottom) of Smad7 expression on day 6 is shown as mean $\pm$ SEM, samples pooled from six independent experiments. (C) Relative TNF- α mRNA expression of monocytes from patients with AD ($n = 7$) and healthy individuals ($n = 7$) is shown. (D) Human monocytes from patients with AD ($n = 5$) and healthy controls ($n = 5$) were cultured with or without TNF- α as described in Materials and methods. After 6 days of culture, the mean value of Langerin expressed by CD11a $^{+}$ DCs was determined by flow cytometry. Data are shown as mean $\pm$ SEM of the indicated number of samples. Statistical significance was determined by Mann-Whitney U test for independent samples and Wilcoxon test for related samples.

of AD with a higher relative number of LCs in nonlesional skin and skin successfully treated and a relatively higher number of IDECs at the onset of inflammation [31, 34]. We have demonstrated that TGF- β 1 in combination with tacrolimus enhanced generation of LC-like DCs from precursor cells, moving putatively the balance of differentiating DCs to tolerogenic LCs [31]. Here, our results demonstrated that human monocytes from patients with AD have an impaired TGF- β signaling, which lessens the tolerogenic function on skin DCs by decreasing the generation of LCs.

Generally, cross-talking of signaling pathways induced by coexisting cytokines in cellular microenvironment determines the outcome of cell differentiation and responsiveness. As a key regulatory cytokine, TGF- β signaling is also under regulation of other signaling pathways. It has been shown that growth factors such as epidermal growth factor and insulin-like growth factor activate the Ras/MAPK pathway and induce MAPK-mediated phosphorylation of Smad1/2, which counteracts phosphorylation of TGF- β receptor mediated phosphorylation of Smad molecules [35]. Furthermore, activation of protein kinase C by tyrosine kinase receptors and G protein coupled receptors also phosphorylates TGF- β receptor-activated Smads and prevents binding of Smad3/4 complex to genetic DNA [36]. Moreover, TGF- β inhibits both TNF- α -conducted NF- κ B activation by upregulating the expression of I κ B- α [37] and IFN- γ induced STAT1-dependent activation of epithelial cells, but not macrophages [38]. As a co-factor,

TNF- α exerts synergistic effects with TGF- β in terms of enhancing human LC generation [39]. However, the mechanism was largely unknown. Our results demonstrate that TNF- α synergizes with TGF- β signaling by enhancing phosphorylation of Smad2/3 on one hand and suppressing expression of Smad7 in human monocytes on the other hand. Lower TNF- α expression observed in monocytes of AD patients presented here is in line with previous data showing that PBMCs of patients with AD express reduced levels of TNF- α mRNA [40]. Since TNF- α exerts its functions in a paracrine and autocrine manner, lower TNF- α baseline expression might further attenuate TGF- β signaling of monocytes from patients with AD. However, TNF- α added to the culture medium did not rescue lower TGF- β responsiveness of monocytes and monocyte-derived DCs of patients with AD.

In our study, monocytes from patients with AD showed phenotypes with low TGF- β receptor expression, low phosphorylation of Smad2/3 toward TGF- β stimulation, and overexpression of Smad7. Since Smad7 suppresses TGF- β signaling through preventing phosphorylation of smad2/3 and mediating degradation of TGF- β receptor [9, 10], the observation places Smad7 in a remarkable position of AD. The expression of Smad7 is upregulated by stimulation of multiple signals, such as activation of STAT1 pathway by IFN- γ or IL-7 and activation of NF- κ B pathway by TNF- α [41–43]. Furthermore, overexpression of Smad7 has been reported in several inflammatory diseases, such as inflammatory

bowel disease [44], multiple sclerosis [45] and *Helicobacter pylori*-associated gastritis [46]. Therefore, it is possible that overexpression of Smad7 in the monocytes of patients with AD associates with inflammation state of the disease. However, overexpression of Smad7 may also result from post-transcriptional modification mechanisms, such as ubiquitin ligases Smurf1 and Smurf2-mediated translocation of smad7 from nucleus to plasma [47, 48], Arkadia and JAB1 (Jun activation domain-binding protein 1) mediated Smad7 ubiquitination and degradation, and TGF- β signaling coactivator p300-mediated acetylation, which protects Smad7 from ubiquitination and degradation [49]. The factors that induce overexpression of Smad7 in monocytes from patients with AD still need to be clarified.

Together, TGF- β response depends on the activation of 'positive' signaling pathways such as stimulation of TGF- β 1 in the microenvironment and 'negative' signaling pathways such as expression of suppressive molecules including Smad7. Therefore, treatments either increasing local TGF- β concentration or decreasing threshold of TGF- β 1 stimulation might represent promising therapeutic options to enhance immune regulatory functions of TGF- β in inflammatory skin diseases such as AD. Because of strong side effects and potential carcinogenicity of TGF- β 1 [50], down-regulating Smad7 expression to restore TGF- β 1 responses and negatively regulate pro-inflammatory pathways would represent a good alternative strategy. In line with this concept, it has been shown that phosphorylation of Smad3 is restored and NF- κ B activation is inhibited by endogenous TGF- β 1 after Smad7 is down-regulated with antisense oligonucleotides [51]. Furthermore, a very recent study demonstrated an effect of anti-Smad7 treatment in inflammatory bowel disease [52]. Our study suggests TGF- β signaling molecules might be a potential therapeutic target for the treatment of AD.

Materials and methods

Reagents

Monoclonal mouse anti-human TGF β RI (Clone: MM0015–8G33) antibody was from Abcam (Cambridge, UK). Monoclonal mouse anti-human TGF β RII (Clone: 25508) and TGF β RIII (Clone: 31349) antibodies were from R&D Systems (Wiesbaden-Nordenstadt, Germany). Phycoerythrin (PE) labeled CD207/Langerin (clone: DCGM4) was from Beckman Coulter, PE-labeled T6RD1 mAb from Coultertronics (Krefeld, Germany) was directed against CD1a. FITC- or Phycoerythrin-cyanine (PE-Cy5)-labeled mAb against CD1a (clone: HI149) and PE-labeled IgG2b mAb against CD14 (clone: MfP9) were obtained from BD Biosciences (Heidelberg, Germany). FITC-conjugated goat anti-mouse (GaM/FITC) antibody was from Jackson Laboratories (West Grove, PA, USA). Normal mouse serum for blocking purposes was obtained from Dianova (Hamburg, Germany). Rabbit mAb Phospho-p44/42 mitogen-activated protein kinase (MAPK) (clone: 20G11) was from New England Biolabs (Frankfurt, Germany). Rab-

bit polyclonal antibody against Smad7 and mouse monoclonal anti-GAPDH antibodies were from Santa Cruz Biotechnology (Heidelberg, Germany). Rabbit polyclonal anti-phospho-Smad2/3 was from Millipore (Schwalbach, Germany). All other reagents were obtained from Sigma Aldrich (Taufkirchen, Germany) unless otherwise indicated.

Isolation of monocytes from the peripheral blood

The study was approved by the ethics committee, University of Bonn. Patients with AD ($n = 38$; 17 male, 21 female, mean age 35.8 ± 2.6 , age range 15–76, mean IgE level 4618.1 ± 1237.2 kU/L) and healthy controls ($n = 28$, 12 male, 16 female, mean age 34.7 ± 2.1 , age range 21–56, mean IgE level 59.7 ± 19.1 kU/L) from the Department of Dermatology and Allergy, University of Bonn, Germany, were included in the study after giving their informed consent. AD was diagnosed on the basis of a skin examination by experienced dermatologists using the criteria of Hanifin and Rajka [53]. Patients under systemic treatment were excluded from the study. The severity of eczema was assessed by using the Severity Scoring of Atopic Dermatitis system (SCORAD) [54]. Monocytes were isolated from peripheral blood with a modified density gradient protocol using Nycoprep gradient centrifugation (Axis-Shield PoC AS, Oslo, Norway) as described previously [31]. Cells were cultured in very low endotoxin 1640 medium (Biochrom, Berlin, Germany) with 1% antibiotics/antimycotics and 10% inactivated FCS.

Generation of LC-like DCs

To generate LC-like DCs in vitro, monocytes were cultured for up to 6 days in very low endotoxin 1640 medium (Biochrom, Berlin, Germany) with 500 U/mL GM-CSF (PeproTech, London, UK), 500 U/mL IL-4 (Miltenyi Biotec, Bergisch Gladbach, Germany) and 2 ng/mL natural platelet-derived TGF- β 1 (R&D systems, Wiesbaden, Germany) to yield LC-like DCs. To avoid any antagonistic effect of IL-4 on TGF- β 1, IL-4 was added only on day 0 to cell culture. In addition, cells were fed every second day with half of the cytokine dosage, and fresh medium was exchanged on day 4.

In some experiments, 50 ng/mL TNF- α (R&D systems) was added together with other cytokines on day 0, day 2, and day 4 to the culture. After 6 days of culture, percentage of CD1a⁺Langerin⁺ LC-like DCs was evaluated by flow cytometry.

Flow cytometric analysis

Flow cytometric staining was performed as described before [31]. Finally, the cells were acquired on an FACS-Canto (Becton Dickinson GmbH, Heidelberg, Germany) as described in detail elsewhere and analyzed by FACSDiva (Becton Dickinson) and FlowJo (TreeStar, Ashland, OR, USA) software.

RNA isolation, reverse transcription, and quantitative real-time PCR

Total RNA was isolated by the NucleoSpin® RNA II or XS kit (Machery-Nagel GmbH & Co. KG, Dueren, Germany) including digestion of genomic DNA and was subjected to cDNA synthesis with TaqMan RT reagents with random hexamers according to the manufacturer's instructions (Applied Biosystems, Darmstadt, Germany). The prepared cDNA was amplified with the help of TaqMan® Gene Expression Master Mix (Applied Biosystems) and Applied Biosystems pre-designed TaqMan® Gene Expression Assays according to the recommendations of the manufacturer on an ABI Prism 7300 Sequence Detection System (Applied Biosystems). The following primers including probes were used: TNF- α (Hs00174128.m1), TGF β RI (Hs00610319.m1), TGF β RII (Hs00559661.m1), Smad3 (Hs00969210.m1), Smad7 (Hs00998193.m1), and 18s rRNA endogenous control (4310893E) (Applied Biosystems). All assays were performed according to the manufacturer's instructions. Relative quantification and calculation of the range of confidence was performed using the comparative $\Delta\Delta$ CT method [55]. All analyses were conducted in duplicates.

Immunoblotting

Monocytes ($\sim 4 \times 10^6$ cells/sample) from either AD patients or healthy individuals were collected and washed three times with cold PBS. After that, cells were lysed by scraping into ice-cold RIPA buffer (150 mM NaCl, 1.0% IGEPALP® CA-630, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0) with 1 mM PMSF, 5 μ g/mL aprotinin, 5 μ g/mL leupeptin, and 1% phosphatase inhibitor cocktail (Santa Cruz Biotechnology). After 15 min of extraction at 4°C with rocking, cell lysates were collected by centrifugation at $15\,000 \times g$ for 10 min. After determination of protein concentration, lysates containing equal amounts of protein were subjected to 12% SDS-PAGE. Protein was then electrotransferred to PVDF membranes (Millipore, Billerica, MA). Blots were blocked with 5% nonfat milk in Tris-buffered saline (20 mM Tris, pH 7.5, and 0.15 M NaCl) containing 0.1% Tween-20 for 1 h at room temperature and incubated with 1:1000 diluted primary antibodies described elsewhere overnight at 4°C, followed by HRP-conjugated secondary antibodies (1:2000) (Santa Cruz Biotechnology). Proteins were detected by enhanced chemiluminescence Western blot detection system (ECL, Amersham Biosciences, Freiburg, Germany). Molecular weights and variations in phosphorylation were determined by Fujifilm LAS 3000 image analysis (Fujifilm Europe GmbH, Düsseldorf, Germany).

Statistical analysis

Statistical analysis was performed with SPSS 17.0 for Windows (SPSS, Chicago, III). Quantitative values were compared between the different groups by using the *t*-test and Mann–Whitney U test

for independent samples and Wilcoxon test for related samples. For *t*-test, normal distribution of data was examined by Shapiro–Wilk test. Correlation analysis was performed by Pearson test. Results are given as mean $\pm$ SEM, respectively. *P* values were two-sided and subject to a significance level of 5%.

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Conflict of interest: The authors have declared no financial or commercial conflict of interest.

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Abbreviations: AD: atopic dermatitis · IDEC: inflammatory dendritic epidermal cell · LC: Langerhans cell · TGF- β 1: Transforming growth factor beta1

Full correspondence: Prof. Natalija Novak, Department of Dermatology and Allergy, University of Bonn, Sigmund-Freud-Str. 25, 53105 Bonn, Germany
Fax: +49-228-287-14333
e-mail: Natalija.Novak@ukb.uni-bonn.de

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