



Disruption of the Epidermal Barrier Induces Regulatory T Cells via IL-33 in Mice

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Disturbance of the epidermal barrier by UVR is associated with the release of antimicrobial peptides and inflammatory cytokines for the purpose of a danger response. On the other hand, UVR causes immunosuppression via regulatory T cells (Treg) that limit the inflammatory reaction. The concurrent induction of antimicrobial peptides and Treg by UVR may represent a counter-regulatory mechanism in response to barrier disruption, preventing microbial superinfection and sensitization to contact allergens, respectively, both of which cross impaired epidermis more easily. Thus, using a model of murine contact hypersensitivity we examined if disruption of the epidermal barrier only initiates similar counter-regulatory mechanisms via the generation of Treg. Sensitization through tape-stripped skin induced a weaker contact hypersensitivity response than in control mice. This was due to the induction of antigen-specific Treg, as demonstrated in adoptive transfer and depletion experiments utilizing DERE mice. Treg induction by tape stripping was linked to the expression of the alarmin IL-33, as blockade of IL-33 exacerbated contact hypersensitivity, whereas injection of IL-33 inhibited contact hypersensitivity and induced Treg. These results demonstrate that epidermal barrier disruption, in addition to danger signals, induces regulatory events that prevent exaggerated skin inflammation and that IL-33 appears to be critically involved in this process.

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INTRODUCTION

The majority of cutaneous inflammatory reactions, except deleterious or chronic ones, are subjected to physiological downregulatory processes that with time cause spontaneous resolution. In contrast to the pathways causing inflammation, these downregulatory processes are less well understood. However, further insight into these regulatory mechanisms may contribute to the development of novel anti-inflammatory strategies. There is also evidence for accompanying downregulatory processes during the early stages of inflammatory events. It has been shown that induction of contact hypersensitivity (CHS) not only results in the generation of T effector cells but also of regulatory T cells (Treg) (Glass et al., 1990; Maeda et al., 2008), although effector T cells ultimately come to dominate resulting in contact dermatitis. Nevertheless, Treg induced during sensitization are functional because their depletion results in a more pronounced CHS response, indicating an endogenous anti-inflammatory role for Treg (Xu et al., 1996).

Treg also prevent the initiation of cutaneous inflammatory responses, as demonstrated in UVR exposure. Physiological UVR doses that disturb the epidermal barrier (Jiang et al.,

2006) induce Treg when a contact allergen is applied to UVR-exposed skin (Schwarz, 2010). Immunosuppression induced by UVR was regarded as harmful because it was the general view that initiation of an immune response was beneficial for protection against cancer (Norval and Halliday, 2011; Ullrich and Byrne, 2012). However, contact allergens may penetrate more easily into skin that has a disrupted epidermal barrier (De Benedetto et al., 2012). Hence, the induction of Treg by UVR can also be regarded as a protective mechanism, preventing the induction of contact allergies (Schwarz, 2010).

A similar counter-regulatory protective mechanism after epidermal barrier disruption was described for antimicrobial peptides (AMPs). AMPs are induced on barrier disruption (Gläser et al., 2009a) or by UVR (Gläser et al., 2009b), giving rise to the speculation that AMPs play a role in protection against the invasion of microbes after barrier disruption. In addition to their antimicrobial activity, AMPs also possess the capacity to induce Treg (Navid et al., 2012). Given these properties, AMPs may therefore protect the host from microbial infection, while also constraining T-cell-driven reactions, thereby facilitating antimicrobial defense without collateral damage by the adaptive immune system.

On the basis of these observations, we have examined whether disruption of the epidermal barrier alone during murine CHS initiates similar counter-regulatory mechanisms via the induction of Treg as UVR and thereby reduces the risk of sensitization in the long term.

RESULTS

Epidermal barrier disruption suppresses CHS

Back-shaved C57BL/6J mice were tape stripped (Ahrens et al., 2011) and sensitized with the application of 2,4,6-trinitro-1-chlorobenzene (TNCB) to the back 24 hours later.

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Abbreviations: AMP, antimicrobial peptide; APC, antigen presenting cell; BMDC, bone marrow-derived dendritic cell; CHS, contact hypersensitivity; DT, diphtheria toxin; OXA, oxazolone; TEWL, transepidermal water loss; TNCB, 2,4,6-trinitro-1-chlorobenzene; Treg, regulatory T cell

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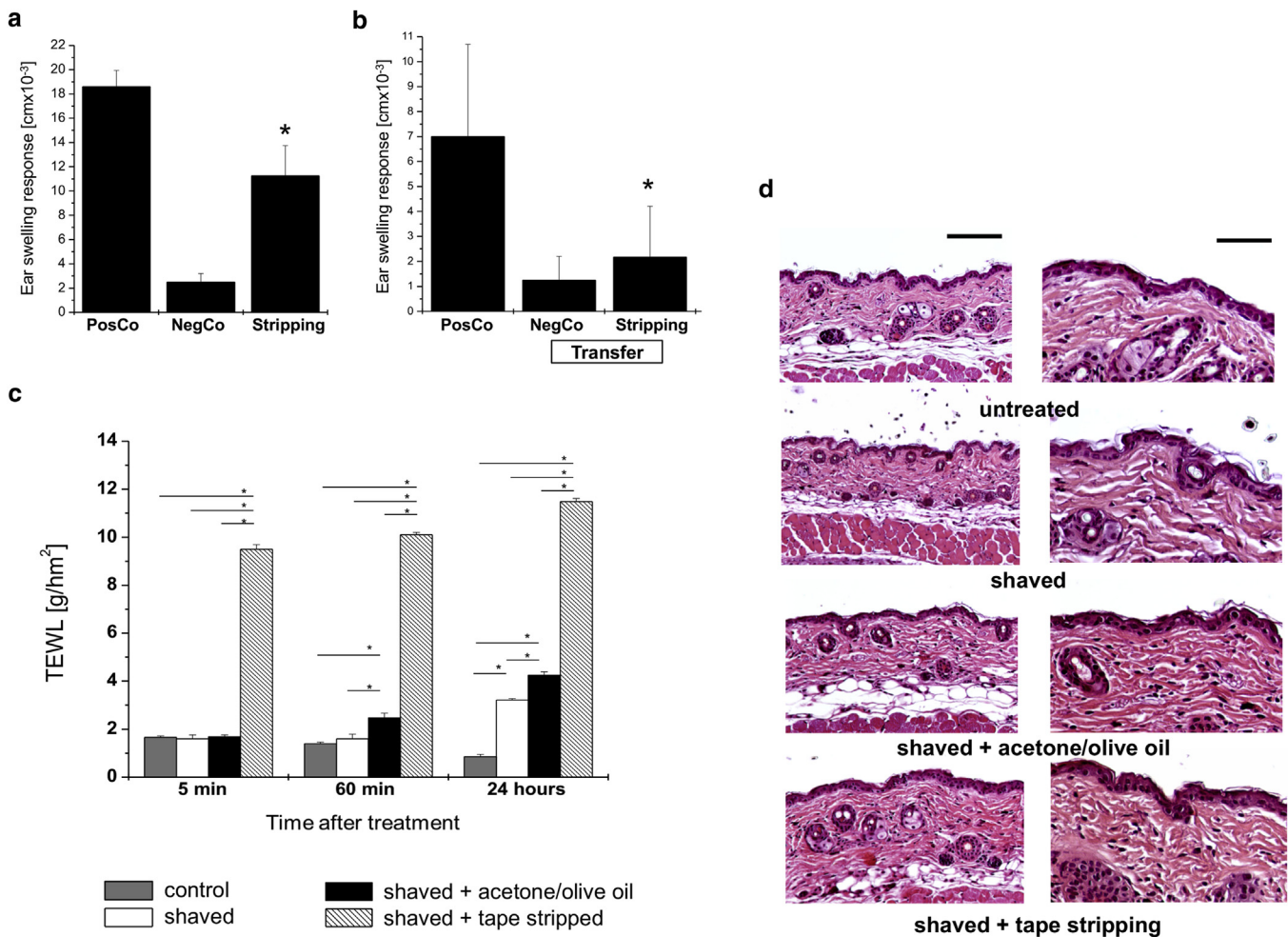


Figure 1. Disruption of epidermal barrier suppresses the CHS response. (a) Mice were tape stripped on the back (Stripping) and sensitized 24 hours later with TNCB. Five days thereafter, ears were challenged with TNCB and ear swelling was measured 24 hours later. CHS was determined as the thickness of the hapten-challenged ear compared with the thickness of the vehicle-treated ear and expressed in $\text{cm} \times 10^{-3}$ (mean + standard deviation). Bar graph data show one from three independent experiments; $n = 6$ per group; $P_{\text{ANOVA}} < 1 \times 10^{-5}$, $*P_{\text{Student}}$ Stripping versus PosCo < 0.002 . (b) Lymph node cells and splenocytes were obtained from mice (a, Stripping) and injected intravenously into naïve mice. Twenty-four hours later, recipients were sensitized with TNCB. Five days later, ears were challenged and ear swelling was measured after 24 hours. $P_{\text{ANOVA}} < 1 \times 10^{-6}$, $*P_{\text{Student}}$ Stripping Transfer versus PosCo < 0.05 . (c) TEWL was measured at the back of mice after various treatments. (d) Biopsies taken from back skin were stained with hematoxylin and eosin and histologically analyzed. Scale bar left panel = 50 μm , right panel = 100 μm . ANOVA, analysis of variance; CHS, contact hypersensitivity; NegCo, negative control; PosCo, positive control; TEWL, transepidermal water loss; TNCB, 2,4,6-trinitro-1-chlorobenzene.

Five days after sensitization, ears were challenged with TNCB and ear swelling measured 24 hours thereafter. Stripping suppressed the CHS response, when compared with positive controls (Figure 1a). To analyze whether stripping induced Treg, lymph node cells and splenocytes obtained from tape-stripped donors (Figure 1a) were injected into naïve animals (Figure 1b). Twenty-four hours later, mice were sensitized with TNCB. The recipients of cells from tape-stripped animals were suppressed in their CHS response, suggesting that Treg had developed in the stripped donors.

To confirm that barrier disruption was achieved by tape stripping, transepidermal water loss (TEWL) was monitored. Twenty stripping repeats caused an increased TEWL in comparison to untreated skin (Figure 1c). Shaving alone resulted in a minor increase of TEWL. The application of acetone/olive oil on the shaved back marginally increased the TEWL. Thus, neither shaving nor application of acetone/olive oil caused a major disturbance of the epidermal barrier.

To exclude that tape stripping was causing severe skin damage that may affect our results, biopsies were obtained from tape-stripped and shaved skin and analyzed histopathologically. In these samples, no signs of cell death, spongiosis, ulceration, subcutaneous bleeding, or inflammation were detected (Figure 1d). This result implies that tape stripping only disturbed the epidermal barrier without causing severe trauma-induced damage.

Tape stripping induces antigen-specific Foxp3⁺ Treg

To determine if Treg generated during epidermal damage were acting in an antigen-specific manner, lymph node cells and splenocytes were obtained from tape-stripped and TNCB-sensitized mice and injected intravenously into naïve recipients, which were subsequently sensitized against either TNCB or the unrelated hapten oxazolone (Oxa). Five days later, the recipients were challenged with TNCB or Oxa, respectively. Cells isolated from tape-stripped and

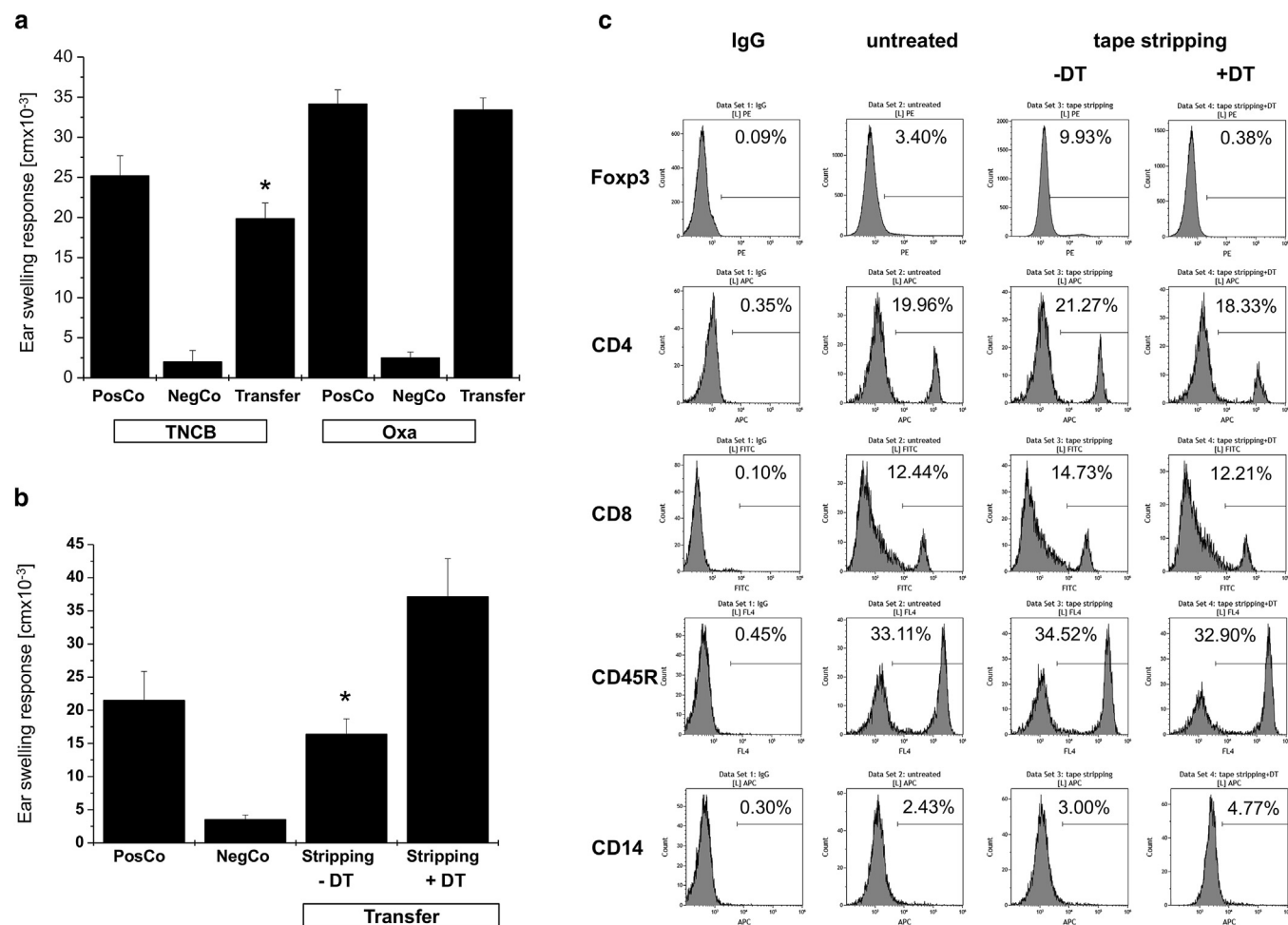


Figure 2. Tape stripping induces antigen-specific Foxp3⁺ Treg. (a) Lymph node cells and splenocytes were obtained from tape-stripped and TNCB-sensitized mice (Figure 1a, Stripping) and transferred intravenously into naïve animals (Transfer) 24 hours before sensitization with TNCB or oxazolone (Oxa). After 5 days, ears were challenged with the respective hapten and ear swelling was measured after 24 hours. $P_{ANOVA} < 1 \times 10^{-8}$, $*P_{Student}$ TNCB Transfer versus TNCB PosCo < 0.0005 . Negative controls were challenged only. $P_{ANOVA} < 1 \times 10^{-9}$, $*P_{Student}$ Stripping-DT versus PosCo < 0.01 . (b) DERE mice were tape stripped on the back and sensitized 24 hours later with TNCB. Forty-eight hours after sensitization one group of tape-stripped animals received DT on three consecutive days. Five days after sensitization, splenocytes and lymph node cells were obtained from DERE mice and injected intravenously into naïve wild-type mice. Recipients were sensitized against TNCB 24 hours after injection. Five days later, challenge with TNCB was performed. Positive controls comprised mice that were sensitized and challenged. (c) Lymph node cells and splenocytes from DERE mice (b) and untreated animals were stained with the respective antibodies and subjected to analysis by flow cytometry. ANOVA, analysis of variance; APC, antigen presenting cell; DT, diphtheria toxin; NegCo, negative control; PE, phycoerythrin; PosCo, positive control; TNCB, 2,4,6-trinitro-1-chlorobenzene.

TNCB-sensitized donors reduced the recipient CHS response against TNCB but not Oxa (Figure 2a).

Foxp3 is a key marker for Treg (Ramsdell and Ziegler, 2014) and is also expressed by UVR-induced Treg (Schwarz et al., 2011). To assess if tape stripping induced Foxp3⁺ Treg, we used DERE mice. These transgenic animals express a diphtheria toxin (DT) receptor-enhanced green fluorescent protein as a fusion protein controlled by Foxp3 promoter/enhancer elements (Lahl et al., 2007). Thus, the injection of DT depletes Foxp3-expressing cells in these mice. The injection of DT into wild-type mice did not have any impact on the CHS response (data not shown). DERE mice were tape stripped on the back and sensitized 24 hours later with TNCB. Forty-eight hours after sensitization, one group of tape-stripped animals received DT on three consecutive days. Five days after sensitization, splenocytes and lymph node cells were obtained from DERE mice and injected

intravenously into naïve wild-type mice. Recipients were sensitized against TNCB 24 hours later and challenged after 5 days. Cells obtained from tape-stripped mice suppressed CHS in the recipients (Figure 2b). In contrast, sensitization was not reduced on the transfer of cells obtained from DERE mice treated with DT, indicating that tape-strip-induced Treg express Foxp3.

Although Foxp3 is considered a specific Treg marker, not all subtypes of Treg express Foxp3 and other non-Treg cells can occasionally express Foxp3 (Devaud et al., 2014). To determine that injection of DT into DERE mice depleted predominantly Treg but no other immune cells, lymph node cells and splenocytes were isolated from tape-stripped DERE mice with and without DT treatment as well as from untreated animals and analyzed by flow cytometry. Cells were stained with antibodies against Foxp3, CD4, CD8, CD45R, and CD14 before analysis (Figure 2c). On tape

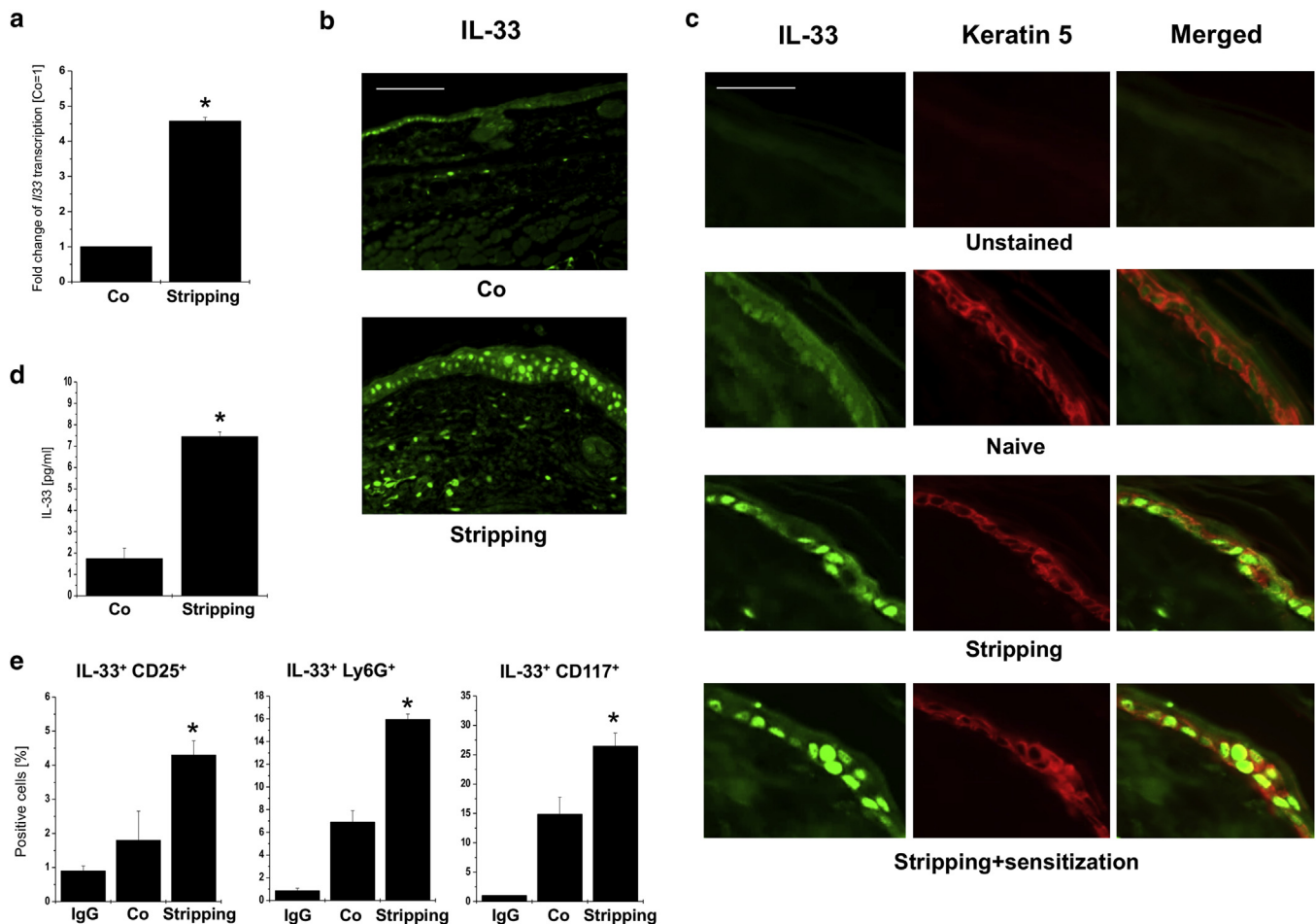


Figure 3. IL-33 is induced by barrier disruption and predominantly expressed by keratinocytes. (a) Mice were tape stripped on the back and sensitized 24 hours later with TNCB. After 24 hours, back skin was obtained. Naïve mice served as control. RNA was isolated and transcription of *Il33* was measured by quantitative RT-PCR. Relative transcription was normalized to the transcription of *Actb* and *Hprt*. * $P_{\text{Student}} < 0.05$. (b) Paraffin sections of back skin from sensitized and tape-stripped mice and untreated animals were stained for the expression of IL-33. Scale bar = 100 μm . (c) Costaining was performed with an anti-keratin 5-antibody. Scale bar = 50 μm . (d) Ears were tape stripped or left untreated and split into dorsal and ventral sides. After 48 hours, supernatants were taken and subjected to ELISA. * $P_{\text{Student}} < 0.05$. (e) Ears were tape stripped or left untreated. After 24 hours, ears were dissected, single-cell suspensions were obtained, stained simultaneously with an antibody against IL-33 and the T-cell marker CD25, the mast cell marker CD117 (c-Kit) or Ly6G, a marker for neutrophils, and subjected to analysis by flow cytometry. $P_{\text{ANOVA}} < 0.05$, * P_{Student} Stripping versus Co < 0.05 . ANOVA, analysis of variance; TNCB, 2,4,6-trinitro-1-chlorobenzene.

stripping, Foxp3-positive cells were upregulated compared with untreated mice. The injection of DT abrogated this effect completely. After DT injection, the number of Foxp3-positive cells was reduced to a lower number than that seen in untreated mice, implying that almost all Foxp3-expressing cells were depleted. In contrast, no major changes were observed in the number of cells expressing CD4, CD8, CD45R, or CD14, either on tape stripping or on injection of DT.

IL-33 is induced on barrier disruption

IL-33 is a novel IL-1-like cytokine that is constitutively expressed by epithelial cells at barrier sites. IL-33 functions as an endogenous danger signal (alarmin), and in response to tissue damage, it is released into the extracellular space and consequently drives T helper 2 responses by signaling via ST2 (Molofsky et al., 2015). Conversely, IL-33 has also been reported to exhibit the capacity to promote Treg function in the intestine (Duan et al., 2012; Schiering et al., 2014). Thus,

we were interested in examining the effect of tape stripping on IL-33 expression.

RNA was isolated from back skin of naïve and before sensitization tape-stripped mice. Quantitative RT-PCR analysis revealed an upregulation in the transcription of *Il-33* 24 hours after stripping (Figure 3a). To identify the cellular source of IL-33, immunohistochemistry using an anti-mouse IL-33 antibody was performed. Increased expression of IL-33 was primarily found in the epidermis after barrier disruption (Figure 3b), confirming previous observations (Morita et al., 2012). Given the extent of epidermal IL-33 staining, it was likely that keratinocytes were the main source of the alarmin. Keratinocytes were confirmed as the primary source of IL-33 by double staining with anti-IL-33 and anti-keratin 5 antibodies (Figure 3c). Although keratin 5 assembled into high-affinity filament complexes, IL-33 was expressed predominantly in the nucleus, where it functions as a nuclear factor in addition to its role as a soluble cytokine (Moussion et al., 2008).

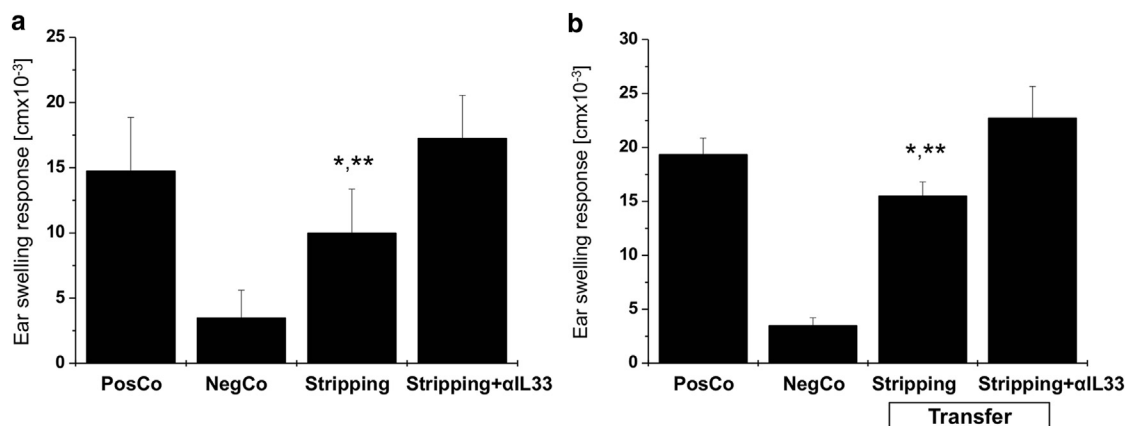


Figure 4. Tape-stripping-induced suppression of CHS is mediated by IL-33. (a) Mice were tape stripped on the shaved back 24 hours before sensitization with TNCB. One group was injected intraperitoneally on two consecutive days with 5 μ g of a neutralizing anti-IL-33 antibody 24 hours before tape stripping. Five days after sensitization, ears were challenged and ear swelling was measured after 24 hours. $P_{ANOVA} < 0.005$, $*P_{Student}$ Stripping versus PosCo < 0.05 ; $**P_{Student}$ Stripping versus Stripping+ α IL-33 < 0.05 . (b) Lymph node cells and splenocytes were obtained from mice (a, Stripping and Stripping+ α IL-33) and injected intravenously into naïve animals (5×10^7 per mouse). Twenty-four hours after transfer, recipients were sensitized with TNCB. Five days later, ears were challenged and ear swelling was measured after 24 hours. $P_{ANOVA} < 1 \times 10^{-5}$, $*P_{Student}$ Stripping versus PosCo < 0.05 ; $**P_{Student}$ Stripping versus Stripping+ α IL-33 < 0.05 . ANOVA, analysis of variance; CHS, contact hypersensitivity; NegCo, negative control; PosCo, positive control; TNCB, 2,4,6-trinitro-1-chlorobenzene.

Ears from tape-stripped and untreated mice were divided into dorsal and ventral sides and placed into culture medium. After 48 hours, supernatants were collected and IL-33 levels determined by ELISA. Supernatants from tape-stripped ears contained significantly more IL-33 than from control tissue (Figure 3d). The upregulation of IL-33 was independent of sensitization as it could be induced by tape stripping only.

Although keratinocytes appeared to be the major source of IL-33 on tape stripping, IL-33 was also expressed by cells within the dermis. For further characterization, single-cell suspensions from untreated and tape-stripped ears were stained with an anti-IL-33 antibody and subjected to flow cytometry analysis. Double-staining with an anti-CD25, anti-Ly6G, and anti-CD117 antibody revealed that CD25⁺ T cells, neutrophils, and mast cells also expressed IL-33 (Figure 3e).

Tape-stripping-induced suppression of CHS is mediated by IL-33

To clarify whether IL-33 is involved in the tape-stripping-induced suppression of CHS, one group of mice was injected intraperitoneally with a neutralizing IL-33 antibody 24 hours before stripping. Twenty-four hours after stripping, mice were sensitized. As expected, mice that were tape stripped only displayed a reduced CHS response (Figure 4a). In contrast, mice given anti-IL-33 had ear swelling comparable to positive controls. This result indicates that IL-33 is important for the tape-stripping-induced CHS suppression.

Lymph node cells and splenocytes obtained from donors (Figure 4a) were injected into naïve animals that were then sensitized (Figure 4b). The recipients of cells obtained from tape-stripped animals were suppressed, whereas CHS reactions in the recipients of cells from anti-IL-33-treated and tape-stripped animals were unaffected, indicating that a lack of IL-33 signaling subsequently impaired Treg development.

IL-33 induces Treg

To further assess if IL-33 alone could recreate the effect of tape stripping, we injected one group of mice

intraperitoneally with IL-33. Another group was tape stripped but did not receive IL-33. One day after tape stripping or IL-33 injection, mice were sensitized and 5 days later challenged. Tape-stripped mice were suppressed in their CHS response (Figure 5a). A similar suppressive effect was observed in mice that were treated with IL-33 instead of stripping.

Lymph node cells and splenocytes isolated from donors (Figure 5a) were injected into naïve animals (Figure 5b). Twenty-four hours later mice were sensitized with TNCB. The recipients of cells from tape-stripped donors or from IL-33-injected mice were suppressed in their ear-swelling response, indicating Treg development in both donors.

To prove that IL-33 induced Foxp3⁺ Treg, DREG mice were treated by either tape stripping or intraperitoneal injection with IL-33 and sensitized 24 hours later. Forty-eight hours thereafter half of each treatment group received DT on three consecutive days. Five days after sensitization, splenocytes and lymph node cells were obtained from all four treatment groups and injected intravenously into naïve wild-type mice. Recipients were sensitized 24 hours after injection and challenged 5 days later. Cells isolated from IL-33-injected mice suppressed CHS in the recipients in a similar manner as cells obtained from tape-stripped animals (Figure 5c). Sensitization was not reduced by the transfer of cells obtained from either IL-33-injected or tape-stripped DREG mice that had been treated with DT, indicating that IL-33 induces Foxp3⁺ Treg.

Antigen-presenting cells are modulated by IL-33

To elucidate whether IL-33 affects antigen-presenting cells (APC), bone marrow-derived dendritic cells (BMDC) were cultured in the presence of IL-33, hapten-coupled, and injected subcutaneously into naïve mice. After 5 days, an ear challenge was performed. The recipients of IL-33-stimulated BMDC were significantly suppressed in their CHS response, compared with mice receiving untreated hapten-coupled BMDC (Figure 6a).

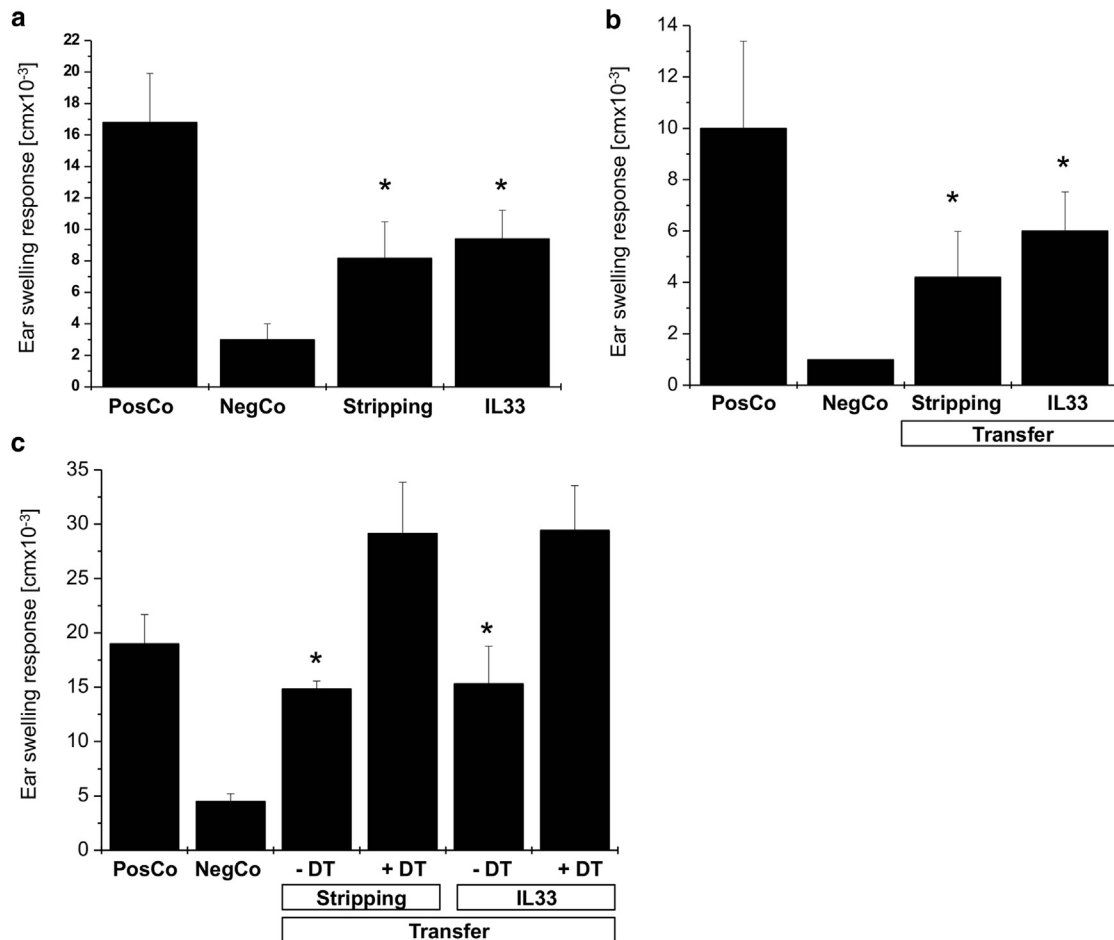


Figure 5. IL-33 suppresses CHS via the induction of Treg. (a) Mice were tape stripped on the back skin 24 hours before sensitization with TNCB. One additional group was injected intraperitoneally with 2 μ g IL-33 instead of stripping. Five days after sensitization, ears were challenged and ear swelling was measured after 24 hours. $P_{ANOVA} < 5 \times 10^{-5}$, $*P_{Student}$ Stripping versus positive control (PosCo) < 0.002 ; IL-33 versus PosCo < 0.002 . (b) Lymph node cells and splenocytes were obtained from mice (a, Stripping; IL-33) and injected intravenously into naïve animals (5×10^7 per mouse). Twenty-four hours after transfer, recipients were sensitized with TNCB. Five days later, ears were challenged and ear swelling was measured after 24 hours. $P_{ANOVA} < 0.001$, $*P_{Student}$ Stripping versus PosCo < 0.01 ; IL-33 versus PosCo < 0.05 . (c) DERE mice were tape stripped on the back or injected intraperitoneally with 2 μ g IL-33 and sensitized 24 hours later with TNCB. Forty-eight hours after sensitization, one group of tape stripped and IL-33 injected animals received 1 μ g DT on three consecutive days. Five days after sensitization, splenocytes and lymph node cells were obtained from DERE mice and injected intravenously into naïve wild-type animals (5×10^7 per mouse). Recipients were sensitized against TNCB 24 hours after injection. Five days later, challenge with TNCB was performed. Positive controls comprised mice that were sensitized and challenged; negative controls were challenged only. $P_{ANOVA} < 1 \times 10^{-10}$, $*P_{Student}$ Stripping versus PosCo < 0.05 ; IL-33 versus PosCo < 0.05 . ANOVA, analysis of variance; CHS, contact hypersensitivity; DT, diphtheria toxin; NegCo, negative control; TNCB, 2,4,6-trinitro-1-chlorobenzene.

Splenocytes and lymph node cells were obtained from both groups and injected intravenously into naïve wild-type mice. Recipients were sensitized 24 hours later and challenged 5 days thereafter. Cells isolated from mice given IL-33-treated BMDC were subsequently able to suppress CHS in recipient animals compared with cells from mice that had received untreated BMDC (Figure 6b), indicating that IL-33 can act on APC to influence Treg development.

Expression of costimulatory molecules determines the activation status of DC (Jung and Choi, 2013). B7-H3 exhibits inhibitory function for antigen presentation, whereas major histocompatibility complex class II and B7-2 are required for the induction of immune responses. An alteration of the expression of costimulatory molecules by IL-33 on APC was addressed by flow cytometry. IL-33 reduced the expression of B7-2 and major histocompatibility complex class II, whereas

B7-H3 was upregulated (Figure 6c), implying that IL-33 shifts APC from a stimulatory to a regulatory phenotype.

DISCUSSION

Intact epidermal barrier is crucial for maintaining cutaneous homeostasis. This notion has been illustrated by a variety of dermatoses with barrier breakdown and by the pathogenic relevance of an impaired barrier due to filaggrin mutations in atopic dermatitis (Irvine et al., 2011; Weidinger and Novak, 2016). Removing the corneal layer by stripping with adhesive tapes is one of the best and most frequently used models for epidermal barrier (dys)function. Tape stripping is associated with an increased TEWL, alteration of differentiation markers and keratins (Ekanayake-Mudiyanse et al., 1998), and release of cytokines (Hu et al., 2017). Tape stripping is considered a nondestructive method used to

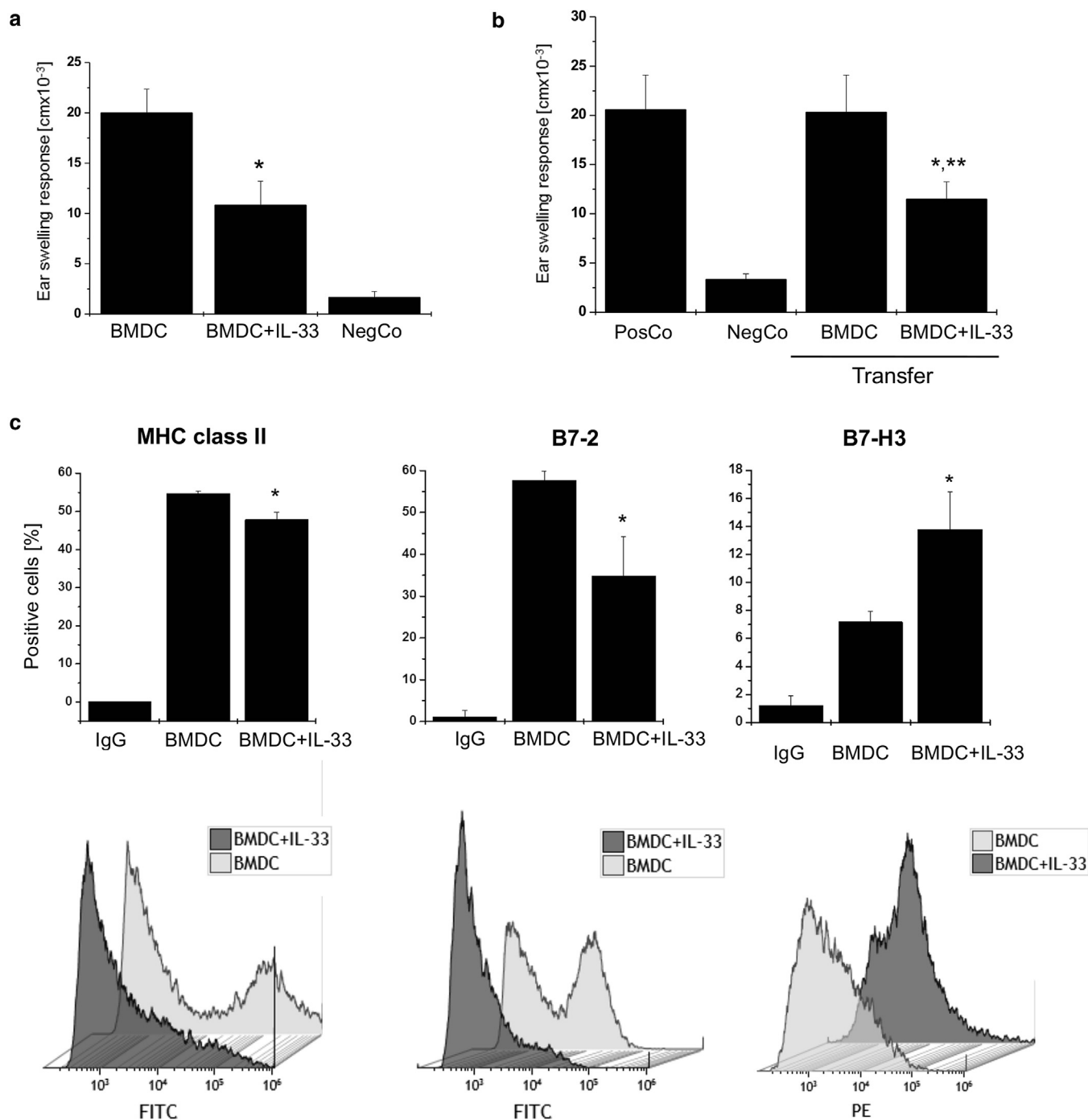


Figure 6. Antigen presenting cells are modulated by IL-33. (a) BMDC were incubated with or without 10 ng/ml IL-33. After 8 days, adult BMDC were harvested, pulsed with trinitrochlorobenzenesulfonic acid (1 mM for 30 minutes), and washed and injected subcutaneously into naïve C57BL/6J mice. After 5 days, ear challenge against TNCB was performed, and 24 hours later ear swelling was measured. Negative control (NegCo) mice were only challenged. $P_{ANOVA} < 0.001$, $*P_{Student} < 0.005$ versus BMDC. (b) Lymph node cells and splenocytes obtained from mice (a) were injected intravenously (5×10^7 per mouse) into naïve mice (Transfer). Twenty-four hours later, recipients were sensitized against TNCB, and 5 days later ears were challenged. $P_{ANOVA} < 0.001$, $*P_{Student} < 0.001$ versus positive control (PosCo); $**P_{Student} < 0.001$ versus BMDC. (c) BMDC cultured as described in (a) were analyzed by flow cytometry for the expression of MHC class II, B7-2, and B7-H3. $P_{ANOVA} < 0.001$, $*P_{Student} < 0.05$ versus BMDC. Histograms show representative data. ANOVA, analysis of variance; BMDC, bone marrow-derived dendritic cell; MHC, major histocompatibility complex; PE, phycoerythrin; TNCB, 2,4,6-trinitro-1-chlorobenzene.

disturb the epidermal barrier without inducing epidermal trauma. Accordingly, we observed only an increased TEWL on tape stripping but not cell death, bleeding, inflammation, or ulceration. Shaving or application of acetone/olive oil did

not compromise the epidermal barrier because TEWL was only minimally enhanced by these procedures.

The effects of stripping on sensitization are controversial, although one would expect stronger sensitization due to easier

penetration of allergens. Accordingly, enhanced CHS responses to dinitrofluorobenzene and picryl-chloride have been observed (Nishijima et al., 1997). In contrast, Oka et al. (1985) reported reduced CHS responses to dinitrochlorobenzene on tape stripping. Impaired sensitization was initially explained by the finding that Langerhans cells are depleted by tape stripping (Streilein et al., 1982). Previously, Langerhans cells were regarded as the crucial APC population in the skin and that their depletion was associated with the loss of sensitization (Toews et al., 1980). More recently, Langerhans cells have been shown to be dispensable for sensitization (Noordegraaf et al., 2010; Stoecklinger et al., 2007) and that the main APC population for sensitization is dermal dendritic cells that are presumably not directly affected by tape stripping.

These discrepancies may be resolved by the observation that during the generation of an immune response both immunostimulatory and downregulatory pathways are activated. The balance between these two pathways is crucial for the effective resolution of immune reactions. The concurrent induction of T effector cells and Treg was first described by Glass et al. (1990) and confirmed by the observation that CHS is more pronounced in mice when CD4⁺ T cells (which includes Treg) are depleted (Xu et al., 1996). This also explains enhanced CHS in DERE mice on depletion of Treg (Figures 2b and 6c) (Lehtimäki et al., 2012; Schwarz et al., 2017).

The concurrent induction of T effector cells and Treg may explain our observation that the CHS response is mitigated when sensitization takes place in tape-stripped skin. We are not arguing that the mice after stripping are not sensitized, but that their response is mitigated by the concurrent induction of Treg. These Treg express Foxp3 and may belong to the CD4⁺CD25⁺ subtype. They act antigen-specifically and appear similar to Treg induced by UVR that also disturbs the epidermal barrier (Jiang et al., 2006). However, we have not yet characterized the tape-strip-induced Treg in such detail as to prove whether they are identical to UVR-induced Treg.

Our results add to the concept that both stimulatory and downregulatory events are induced during CHS and that the final outcome is dependent on the dominance of one of the pathways. Previously, the downregulation of an immune response was regarded as dangerous because the view dominated that any immune reaction is beneficial. This concept, however, does not apply to allergic contact dermatitis. Hence, concurrent induction of downregulatory events can be regarded as beneficial, as demonstrated for UVR, which through its immunosuppressive properties may protect individuals from developing allergic contact dermatitis and autoimmune diseases (Schwarz, 2010). A similar compensatory mechanism has been described for AMPs that are induced by UVR (Gläser et al., 2009b). Thus, the upregulation of AMPs by UVR may help to combat microbial infections that can easily penetrate into skin with a barrier compromised by UVR. It is unclear whether the importance of the downregulatory component can be extrapolated to any immune reaction; however, we think that studying these mechanisms may help to better understand the various final outcomes of immune/inflammatory responses.

We identified IL-33 as a crucial mediator in the generation of Treg induced by tape stripping. Stripping is known to stimulate the release of several cytokines, including IL-1

family members (Hänel et al., 2013). The IL-1 family comprises IL-1 α , IL-1 β , IL-18, and IL-33 (Hänel et al., 2013; Savinko et al., 2012). We confirmed the upregulation of these cytokines on tape stripping (data not shown). IL-33 was of interest to us as it was initially described as a danger molecule (alarmin) alerting the immune system on trauma or infection but has been recognized to attenuate the development of chronic intestinal inflammation (Groß et al., 2012) and to promote Treg function in the intestine (Schiering et al., 2014). Our results led us to hypothesize that IL-33 is crucial for the downregulation of CHS by tape stripping because stripping induced the expression of IL-33 and importantly the inhibitory stripping effect could be blocked by anti-IL-33 antibody treatment. Conversely, exogenous IL-33 inhibited the induction of CHS and induced Treg.

IL-33 appears to induce Treg by shifting APC into a regulatory phenotype. This is functionally relevant because IL-33-treated BMDC on subcutaneous injection did not induce sensitization but Treg. In this context, it should be mentioned that IL-33 has already been reported to induce the development of tolerogenic CD103⁺ DC that promote Treg development via expression of indoleamine 2,3-dioxygenase (Duan et al., 2012).

It may appear surprising that IL-33, whose primary function as an alarmin is to activate inflammation and the immune system, can exert immunosuppressive features as well. This pleiotropic behavior may be in line with the plasticity of the immune system whose reactions do not appear to be a one-way road but can switch their outcome during the course of an immune response. The same appears to apply for epidermal barrier disruption that on the one hand causes the release of cytokines and AMPs as a danger response, but on the other hand turns down exaggerated immune responses via the induction of Treg, as demonstrated in this study.

MATERIALS AND METHODS

Mice

Seven- to eight-week-old female C57BL/6J mice were purchased from Charles River Laboratories (Sulzfeld, Germany). DERE mice were provided by T. Sparwasser (Lahl et al., 2007). To deplete Foxp3-positive cells, 1 μ g DT (Merck, Darmstadt, Germany) was injected intraperitoneally. Mice were bred and housed under specific pathogen-free conditions in compliance with relevant laws and institutional guidelines. Experiments were approved by the institutional review board.

Chemicals and antibodies

The following chemicals were used: TNCB, trinitrochlorobenzenesulfonic acid, 4-ethoxymethylene-2-phenyl-2-oxazolin-5 (Oxazolone; all from Sigma-Aldrich, Darmstadt, Germany), recombinant mouse IL-33 (carrier-free, BioLegend, Zum Grundtal, Germany), anti-IL-33 antibody (R&D, Wiesbaden-Nordenstadt, Germany). IL-33 levels in supernatants were determined by ELISA (BioLegend).

For immunofluorescence: biotin-labeled anti-mouse IL-33 antibody, Alexa 488-conjugated streptavidin, Alexa 594-conjugated anti-rabbit antibody (all from BioLegend), anti-keratin-5 antibody (Covance, Richmond, CA).

For flow cytometry (FC500; Beckman-Coulter, Krefeld, Germany): biotin-labeled anti-mouse IL-33 antibody; Alexa 488-conjugated

streptavidin; allophycocyanin-conjugated anti-CD25, anti-CD117, anti-CD45R, anti-Ly6G antibody; biotin-conjugated anti-CD14, Alexa 594-conjugated anti-rabbit antibody and allophycocyanin-conjugated streptavidin (all from BioLegend); allophycocyanin-conjugated anti-CD4, anti-Foxp3, fluorescein-conjugated anti-CD8, anti-B7-2, anti-major histocompatibility complex class II antibody (all from Miltenyi, Bergisch Gladbach, Germany); phycoerythrin-conjugated B7-H3 antibody (eBioscience, Santa Clara, CA).

Contact hypersensitivity

Mice were sensitized by painting 50 μ l TNCB (1.0%) or Oxa (2.0%) in acetone:olive oil, 4:1 on the shaved back. After 5 days, a challenge was performed with 20 μ l 0.5% TNCB or 1% Oxa on the left ear. Twenty-four hours later, ear swelling was determined as the swelling of the hapten-treated ear compared with vehicle-treated ear and expressed in cm^{-3} . Positive controls were sensitized and challenged, negative challenged only.

Barrier disruption

Shaved back skin was stripped with adhesive cellophane tape (20 times). TEWL was measured using an evaporimeter (Tewameter 300; Courage-Khazaka Electronic, Cologne, Germany).

Bone marrow-derived dendritic cells

BMDC were generated by culture of bone marrow cells obtained from tibias (Schwarz and Schwarz, 2010). BMDC were labeled with 1 mM trinitrochlorobenzenesulfonic acid for 30 minutes at 37°C. After washing, 7×10^5 cells were injected subcutaneously into naive mice.

Adoptive transfer

Single-cell suspensions were obtained by passing lymph nodes and spleens through a 100- μ m cell strainer. Cells (5×10^7) were injected intravenously into naïve mice that were sensitized 24 hours thereafter.

Quantitative real-time PCR

RNA was extracted (peqGOLD TriFast, VWR, Darmstadt, Germany), treated with DNaseI, and converted to complementary DNA by RT. Quantitative detection of *Il33* was performed with the StepOnePlus system (Applied Biosystems, Life Technologies, Darmstadt, Germany) using a SYBR Green Master Mix (Life Technologies). Primer sequences are available on request. Relative gene expression was normalized to *Actb* and *Hprt* according to the delta cycle threshold method.

Statistical analysis

Statistical analysis was performed using Student's *t*-test. For multiple comparisons, we carried out one-way analysis of variance in order to assess whether at least two groups significantly differ. For pairwise post hoc analysis, we applied Bonferroni correction. Values of $P < 0.05$ were considered statistically significant. Unless otherwise stated, data show one representative of three independently performed experiments with at least six mice per group.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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REFERENCES

Ahrens K, Schunck M, Podda GF, Meingassner J, Stuetz A, Schroder JM, et al. Mechanical and metabolic injury to the skin barrier leads to increased expression of murine beta-defensin-1, -3, and -14. *J Invest Dermatol* 2011;131:443–52.

De Benedetto A, Kubo A, Beck LA. Skin barrier disruption: a requirement for allergen sensitization? *J Invest Dermatol* 2012;132:949–63.

Devaud C, Darcy PK, Kershaw MH. Foxp3 expression in T regulatory cells and other cell lineages. *Cancer Immunol Immunother* 2014;63:869–76.

Duan L, Chen J, Zhang H, Yang H, Zhu P, Xiong A, et al. Interleukin-33 ameliorates experimental colitis through promoting Th2/Foxp3(+) regulatory T-cell responses in mice. *Mol Med* 2012;18:753–61.

Ekanayake-Mudiyansele S, Aschauer H, Schmook FP, Jensen JM, Meingassner JG, Proksch E. Expression of epidermal keratins and the cornified envelope protein involucrin is influenced by permeability barrier disruption. *J Invest Dermatol* 1998;111:517–23.

Gläser R, Meyer-Hoffert U, Harder J, Cordes J, Wittersheim M, Kobliakova J, et al. The antimicrobial protein psoriasin (S100A7) is upregulated in atopic dermatitis and after experimental skin barrier disruption. *J Invest Dermatol* 2009a;129:641–9.

Gläser R, Navid F, Schuller W, Jantschitsch C, Harder J, Schroder JM, et al. UV-B radiation induces the expression of antimicrobial peptides in human keratinocytes in vitro and in vivo. *J Allergy Clin Immunol* 2009b;123:1117–23.

Glass MJ, Bergstresser PR, Tigelaar RE, Streilein JW. UVB radiation and DNFB skin painting induce suppressor cells universally in mice. *J Invest Dermatol* 1990;94:273–8.

Groß P, Doser K, Falk W, Obermeier F, Hofmann C. IL-33 attenuates development and perpetuation of chronic intestinal inflammation. *Inflamm Bowel Dis* 2012;18:1900–9.

Hänel KH, Cornelissen C, Lüscher B, Baron JM. Cytokines and the skin barrier. *Int J Mol Sci* 2013;14:6720–45.

Hu L, Mauro TM, Dang E, Man G, Zhang J, Lee D, et al. Epidermal dysfunction leads to an age-associated increase in levels of serum inflammatory cytokines. *J Invest Dermatol* 2017;137:1277–85.

Irvine AD, McLean WH, Leung DY. Filaggrin mutations associated with skin and allergic diseases. *N Engl J Med* 2011;365:1315–27.

Jiang SJ, Chen JY, Lu ZF, Yao J, Che DF, Zhou XJ. Biophysical and morphological changes in the stratum corneum lipids induced by UVB irradiation. *J Dermatol Sci* 2006;44:29–36.

Jung K, Choi I. Emerging co-signaling networks in T cell immune regulation. *Immune Netw* 2013;13:184–93.

Lahl K, Lodenkemper C, Drouin C, Freyer J, Arnason J, Eberl G, et al. Selective depletion of Foxp3+ regulatory T cells induces a scurfy-like disease. *J Exp Med* 2007;204:57–63.

Lehtimäki S, Savinko T, Lahl K, Sparwasser T, Wolff H, Lauerma A, et al. The temporal and spatial dynamics of Foxp3+ Treg cell-mediated suppression during contact hypersensitivity responses in a murine model. *J Invest Dermatol* 2012;132:2744–51.

Maeda A, Beissert S, Schwarz T, Schwarz A. Phenotypic and functional characterization of ultraviolet radiation-induced regulatory T cells. *J Immunol* 2008;180:3065–71.

Molofsky AB, Savage A, Locksley RM. Interleukin-33 in tissue homeostasis, injury and inflammation. *Immunity* 2015;42:1005–19.

Morita H, Arae K, Ohno T, Kajiwara N, Oboki K, Matsuda A, et al. ST2 requires Th2-, but not Th17-, type airway inflammation in epicutaneously antigen-sensitized mice. *Allergol Int* 2012;61:265–73.

Moussion C, Ortega N, Girard J-P. The IL-1-like cytokine IL-33 is constitutively expressed in the nucleus of endothelial cells and epithelial cells in vivo: a novel 'alarmin'? *PLoS One* 2008;3:e3527.

Navid F, Boniotto M, Walker C, Ahrens K, Proksch E, Sparwasser T, et al. Induction of regulatory T cells by a murine beta-defensin. *J Immunol* 2012;188:735–43.

Nishijima T, Tokura Y, Imokawa G, Seo N, Furukawa F, Takigawa M. Altered permeability and disordered cutaneous immunoregulatory function in mice with acute barrier disruption. *J Invest Dermatol* 1997;109:175–82.

Noordegraaf M, Flacher V, Stoitzner P, Clausen BE. Functional redundancy of Langerhans cells and Langerin(+) dermal dendritic cells in contact hypersensitivity. *J Invest Dermatol* 2010;130:2752–9.

Norval M, Halliday GM. The consequences of UV-induced immunosuppression for human health. *Photochem Photobiol* 2011;87:965–77.

Oka D, Nakagawa S, Ueki H. The suppressive effect of tape-stripping treatment of guinea-pig skin on the induction of contact sensitivity by

- intra-dermal injection of haptenated epidermal cells. *Arch Dermatol Res* 1985;278:102–6.
- Ramsdell F, Ziegler SF. FOXP3 and scurfy: how it all began. *Nat Rev Immunol* 2014;14:343–9.
- Savinko T, Matikainen S, Saarialho-Kere U, Lehto M, Wang G, Lehtimäki S, et al. IL-33 and ST2 in atopic dermatitis: expression profiles and modulation by triggering factors. *J Invest Dermatol* 2012;132:1392–400.
- Schiering C, Krausgruber T, Chomka A, Frohlich A, Adelman K, Wohlfert EA, et al. The alarmin IL-33 promotes regulatory T-cell function in the intestine. *Nature* 2014;513:564–8.
- Schwarz A, Navid F, Sparwasser T, Clausen BE, Schwarz T. In vivo reprogramming of UV radiation-induced regulatory T-cell migration to inhibit the elicitation of contact hypersensitivity. *J Allergy Clin Immunol* 2011;128:826–33.
- Schwarz A, Bruhs A, Schwarz T. The short-chain fatty acid sodium butyrate functions as a regulator of the skin immune system. *J Invest Dermatol* 2017;137:855–64.
- Schwarz A, Schwarz T. UVR-induced regulatory T cells switch antigen-presenting cells from a stimulatory to a regulatory phenotype. *J Invest Dermatol* 2010;130:1914–21.
- Schwarz T. The dark and the sunny sides of UVR-induced immunosuppression: photoimmunology revisited. *J Invest Dermatol* 2010;130:49–54.
- Stoecklinger A, Grieshuber I, Scheiblhofer S, Weiss R, Ritter U, Kissenpfennig A, et al. Epidermal langerhans cells are dispensable for humoral and cell-mediated immunity elicited by gene gun immunization. *J Immunol* 2007;179:886–93.
- Streilein JW, Lonsberry LW, Bergstresser PR. Depletion of epidermal langerhans cells and Ia immunogenicity from tape-stripped mouse skin. *J Exp Med* 1982;155:863–71.
- Toews GB, Bergstresser PR, Streilein JW. Epidermal Langerhans cell density determines whether contact hypersensitivity or unresponsiveness follows skin painting with DNFB. *J Immunol* 1980;124:445–53.
- Ullrich SE, Byrne SN. The immunologic revolution: photoimmunology. *J Invest Dermatol* 2012;132:896–905.
- Weidinger S, Novak N. Atopic dermatitis. *Lancet* 2016;387:1109–22.
- Xu H, Dilulio NA, Fairchild RL. T cell populations primed by hapten sensitization in contact sensitivity are distinguished by polarized patterns of cytokine production: interferon gamma-producing (Tc1) effector CD8+ T cells and interleukin (Il) 4/Il-10-producing (Th2) negative regulatory CD4+ T cells. *J Exp Med* 1996;183:1001–12.