IL-23 induced in keratinocytes by endogenous TLR4 ligands polarizes dendritic cells to drive IL-22 responses to skin immunization

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Atopic dermatitis (AD) is a Th2-dominated inflammatory skin disease characterized by epidermal thickening. Serum levels of IL-22, a cytokine known to induce keratinocyte proliferation, are elevated in AD, and Th22 cells infiltrate AD skin lesions. We show that application of antigen to mouse skin subjected to tape stripping, a surrogate for scratching, induces an IL-22 response that drives epidermal hyperplasia and keratinocyte proliferation in a mouse model of skin inflammation that shares many features of AD. DC-derived IL-23 is known to act on $CD4^+$ T cells to induce IL-22 production. However, the mechanisms that drive IL-23 production by skin DCs in response to cutaneous sensitization are not well understood. We demonstrate that IL-23 released by keratinocytes in response to endogenous TLR4 ligands causes skin DCs, which selectively express IL-23R, to up-regulate their endogenous IL-23 production and drive an IL-22 response in naive $CD4^+$ T cells that mediates epidermal thickening. We also show that IL-23 is released in human skin after scratching and polarizes human skin DCs to drive an IL-22 response, supporting the utility of IL-23 and IL-22 blockade in AD.

INTRODUCTION

Atopic dermatitis (AD) is a pruritic inflammatory skin disease that affects >15% of children (Bieber, 2008). Acute AD skin lesions are characterized by epidermal and dermal thickening and by dermal infiltration of $CD4^+$ T cells and eosinophils, as well as predominant expression of the Th2 cytokines IL-4 and IL-13; expression of IL-22 is also significantly (Homey et al., 2006) elevated in AD skin lesions (Nograles et al., 2009; Khattri et al., 2014). A hallmark of AD is a dry itchy skin with a disrupted barrier function, which is caused in a large number of patients by filaggrin deficiency (Morar et al., 2007). Mechanical skin injury caused by scratching aggravates the defect in skin barrier function and leads to the release of cytokines that play key roles in driving the immune response to cutaneously encountered antigens (Homey et al., 2006). Epicutaneous (EC) sensitization with allergens plays an important role in the pathogenesis of AD (Kubo et al., 2012). Approximately 80% of patients with AD are sensitized to allergens, as indicated by elevated serum total IgE levels with specific IgE antibodies to environmental allergens (Leung, 2000). Importantly, application of allergens to the abraded uninvolved skin of patients with AD provokes an eczematous rash with characteristics of acute AD skin lesions (Mitchell et al., 1982).

IL-22 is a member of the IL-10 family of cytokines produced by adaptive Th17 and Th22 cells, innate lymphocytes that include γδ T cells and type 3 innate lymphoid cells (ILC3), and myeloid cells, including neutrophils (Xie et al.,

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Abbreviations used: AD, atopic dermatitis; AMP, antimicrobial peptides; CHX, cycloheximide; DLN, draining LN; DT, diphtheria toxin; EC, epicutaneous; GF, germ-free; HA, hyaluronic acid; H&E, hematoxylin and eosin; ILC, innate lymphoid cell; LC, Langerhans cell; SCM, skin-conditioned medium; SPF, specific pathogen–free.

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2000; Kreymborg et al., 2007; Duhen et al., 2009; Mielke et al., 2013; Zindl et al., 2013). The IL-22 receptor (IL-22R) is expressed on epithelial cells, including keratinocytes, but not immune cells (Wolk et al., 2004), indicating an important role for IL-22 signaling in mucosal barrier function. IL-22 signaling induces cell proliferation and expression of antiapoptotic genes (Radaeva et al., 2004; Zheng et al., 2008; Pickert et al., 2009), and thus exerts tissue repair activity and protects stem cells from injury (Radaeva et al., 2004; Dudakov et al., 2012; Hanash et al., 2012). IL-22 drives antimicrobial peptide (AMP) gene expression by epithelial cells in vitro, promotes complement-mediated resistance to intestinally absorbed pathogens, and exerts antiinflammatory activity in models of colitis (Aujla et al., 2008; Satoh-Takayama et al., 2008; Sonnenberg et al., 2012; Matsumoto et al., 2013; Hasegawa et al., 2014). Cutaneous injection of IL-22 in vivo causes keratinocyte proliferation and epidermal thickening (Zhang et al., 2012); in vitro application of IL-22 causes keratinocyte proliferation and the thickening of human epidermis reconstituted in a three-dimensional (3D) matrix (Boniface et al., 2005; Sa et al., 2007). *Il22* mRNA expression, and T cells that produce IL-22, but not IL-17, are significantly increased in the skin lesions of patients with AD (Guttman-Yassky et al., 2008; Nograles et al., 2009; Gittler et al., 2012). Furthermore, serum IL-22 levels are elevated in AD patients (Hayashida et al., 2011; Meephansan et al., 2011). However, the mechanisms of IL-22 production in AD and the exact role of IL-22 in the pathogenesis of the disease are not known.

IL-23 is an IL-12 family cytokine composed of a p19 subunit specific to IL-23 and a p40 subunit shared with IL-12 (Oppmann et al., 2000), and produced by epidermal Langerhans cells (LCs), DCs, macrophages, and keratinocytes (Andersson et al., 2004; Piskin et al., 2006; Chamilos et al., 2010). IL-23R is expressed on immune cells, including LCs, DCs, NK, NKT, γδ T cells, and Th17 cells (Rachitskaya et al., 2008; Awasthi et al., 2009; Tonel et al., 2010). IL-23 promotes Th17 polarization (McGeachy et al., 2009; Wu et al., 2013) and plays an essential role in the induction of *Il22* expression (Zheng et al., 2007; Graham et al., 2011). IL-23 expression is up-regulated in the skin of patients with AD, compared with healthy individuals (Guttman-Yassky et al., 2008).

We report that in addition to eliciting Th2-dominated skin inflammation, antigen application to mouse skin subjected to tape stripping, a surrogate for skin scratching, induces an IL-22 response that is necessary for epidermal thickening and keratinocyte proliferation. We describe for the first time a novel pathway in which endogenous TLR4 ligands released upon mechanical skin injury trigger keratinocyte production of IL-23, which targets the skin DCs to up-regulate their endogenous IL-23 production and drive IL-22 production by naive $CD4^+$ T cells. Similarly, IL-23 was released in human skin upon scratching and polarized human skin DCs to drive IL-22 production by T cells. Our findings suggest the utility of the ongoing trials of IL-23 and IL-22 blockade in AD.

RESULTS

EC sensitization elicits an antigen-specific IL-22 response important for epidermal thickening

We previously demonstrated that EC sensitization of mice by application of antigen to tape-stripped skin elicits a vigorous systemic Th2 response, and Th2-dominated skin inflammation with many of the characteristics of acute AD skin lesions, including dermal and epidermal thickening, infiltration with eosinophils, vigorous expression of *Il13*, modest expression of *Il17a*, and negligible expression of *Ifng* (Ma et al., 2002; He et al., 2007). We tested the hypothesis that cutaneous sensitization induces a systemic and local IL-22 response. EC sensitization with OVA, but not saline, resulted in a significant increase in serum IL-22 levels (Fig. 1 A). Cells from the draining LNs (DLNs) of mouse skin EC sensitized with OVA secreted IL-22 when stimulated with antigen in vitro (Fig. 1 B). FACS analysis of cells isolated from the skin revealed a significantly higher percentage of $CD3^+CD4^+$ T cells that express intracellular IL-22 in OVA-sensitized skin compared with saline-sensitized skin (Fig. 1 C). The percentage of $CD3^+CD4^+IL\text{-}22^+$ cells that coexpressed IL-17A was significantly higher in OVA-sensitized skin compared with saline-sensitized skin ($53 \pm 11.5\%$ versus $30 \pm 4.1\%$; $n = 4$ each; $P = 0.01$). These results demonstrate that EC sensitization with antigen results in a systemic IL-22 response and in local accumulation of IL-22-producing $CD4^+$ T cells.

Epidermal thickening and increased keratinocyte proliferation are characteristic features of AD (Bieber, 2008). Mouse skin that was EC sensitized with OVA exhibited a significant increase in epidermal thickening and keratinocyte proliferation, indicated by epidermal cell expression of the proliferation-associated antigen Ki67 (Fig. 1, D and E). Epidermal thickness and keratinocyte proliferation were not significantly increased in OVA-sensitized skin of $Il22^{-/-}$ mice (Fig. 1, D and E). Dermal thickening, eosinophil infiltration, and mRNA levels of *Il13* and *Il17a* were up-regulated comparably in OVA-sensitized skin of $Il22^{-/-}$ mice and WT controls (Fig. 1, F–H). EC sensitization did not up-regulate *Ifng* mRNA levels in the skin of WT BALB/c mice as previously reported (Jin et al., 2009), or $Il22^{-/-}$ mice on the same background (unpublished data). Expression of the AMP genes *Defb14*, *Slurp1*, *Lnc2*, *S100a9*, *Saa3*, and *Reg3g* was not significantly different in OVA-sensitized compared with saline-sensitized skin in WT or $Il22^{-/-}$ mice (unpublished data). Expression of *Defb14* and *Slurp1*, but not of the other AMP genes tested, was up-regulated in taped-stripped skin in WT mice, but to a significantly lesser extent in $Il22^{-/-}$ mice (17.1- and 1.5-fold versus 8.3- and 1.0-fold, respectively; $P < 0.05$ each). The level of IL-22 in EC-sensitized skin of WT mice may fall below the threshold for up-regulating the expression of AMP genes and/or is unable to overcome the inhibitory effect of Th2 cytokines on the up-regulation of these genes (Howell et al., 2006). There were no significant differences in the levels of IL-17A, IL-13, and IFN-γ secreted by OVA-stimulated cells from DLN, or in OVA-specific IgE

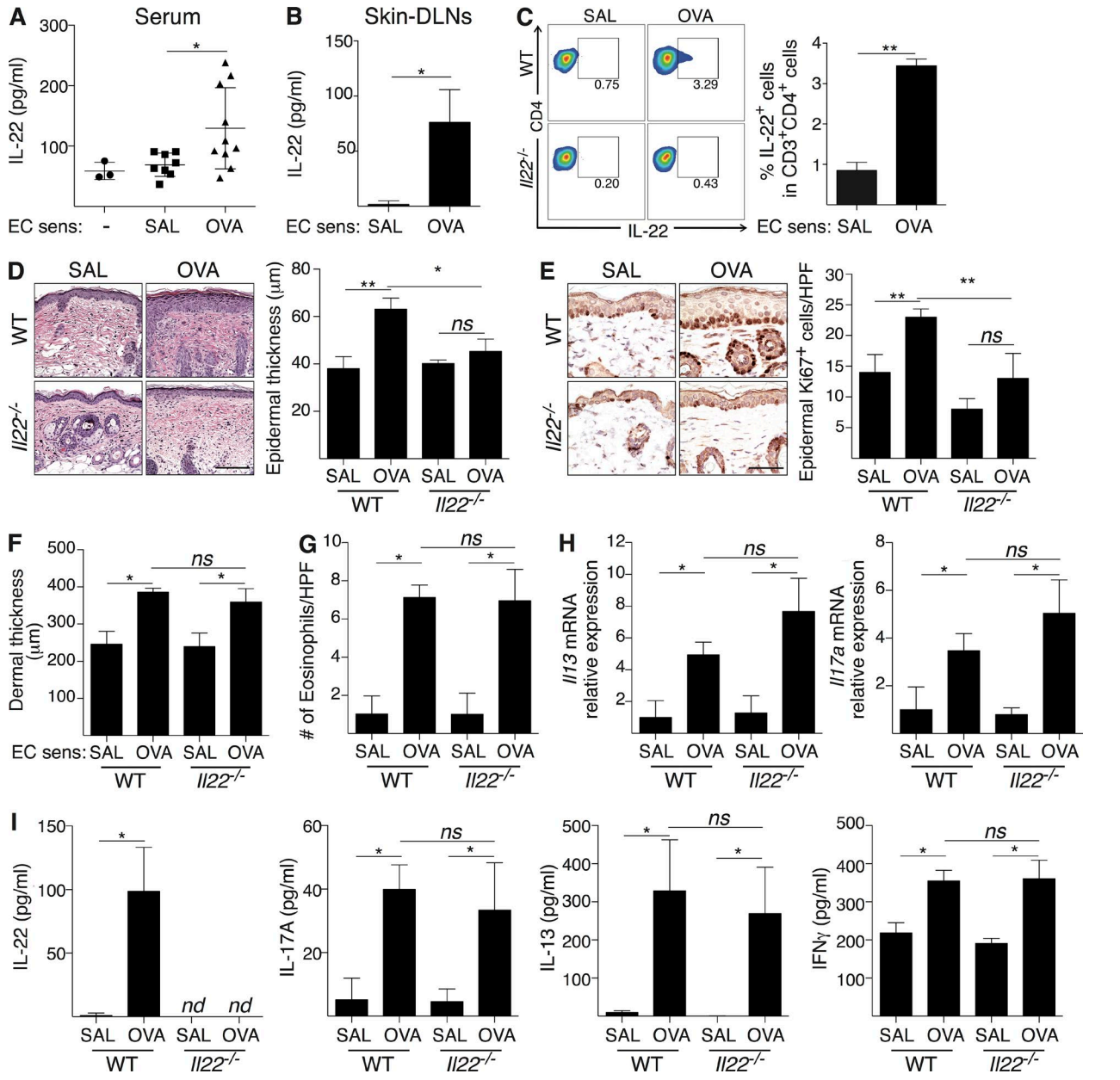

Figure 1. **EC sensitization elicits an IL-22 response important for epidermal thickening.** (A) Serum IL-22 levels in EC-sensitized BALB/c WT mice. (B) IL-22 secretion by skin DLN cells from EC-sensitized mice. (C) Representative FACS analysis and percentage of IL-22$^+$ cells among CD3$^+$CD4$^+$ T cells in the skin of EC-sensitized WT and *Il22*$^{-/-}$mice. (D and E) Representative H&E staining and quantitative analysis of epidermal thickness (D), and representative immunohistochemical staining and quantitative analysis of Ki67$^+$ cells in the epidermis (E) of skin from EC-sensitized *Il22*$^{-/-}$ mice and WT controls. (F–H) Dermal thickness (F), eosinophil infiltration (G), and cytokine expression (H) in EC-sensitized skin of *Il22*$^{-/-}$ mice and WT controls. Results in H expressed as fold increase relative to saline-sensitized skin of WT controls. (I) IL-22, IL-17A, IL-13, and IFN-γ secretion by skin DLN cells of EC-sensitized *Il22*$^{-/-}$ mice and WT controls. Results in A represent 3 unsensitized, 9 saline (SAL) sensitized, and 11 OVA-sensitized WT mice. Results in B and D–I represent five to six mice per group. Results in C represent three saline-sensitized and three OVA-sensitized WT mice. Horizontal lines and vertical bars in A and C, and columns and bars in B and D–I represent means and SEM. *, $P < 0.05$; **, $P < 0.01$; ns, not significant, two-tailed Student's *t* test.

and IgG1 levels between EC-sensitized *Il22*$^{-/-}$ mice and WT controls (Fig. 1 I and not depicted). These results suggest that IL-22 is essential for keratinocyte proliferation and epidermal thickening in antigen-sensitized skin.

IL-22–producing CD4$^+$ T cells drive epidermal thickening and keratinocyte proliferation

IL-22 is produced mainly by TCRαβ CD4$^+$ Th cells and CD45$^+$CD3$^-$ ILC3s (Rutz et al., 2013). In contrast to the sig-

nificant increase in the percentage of $CD3^+CD4^+IL\text{-}22^+$ cells (Fig. 1 C), there was no significant change in the percentage of $CD45^+CD3^-Lin^-CD90^+IL\text{-}22^+$ ILCs in OVA-sensitized skin compared with saline-sensitized skin (Fig. 2 A). Furthermore, mean fluorescence intensity of IL-22 staining by these cells was not significantly changed (unpublished data). To ascertain whether IL-22–producing TCRαβ $CD4^+$ T cells are sufficient to drive epidermal thickening and keratinocyte proliferation in response to EC antigen challenge, we adoptively transferred OVA-specific $CD4^+$ T cells that had been polarized to IL-22 production in vitro into naive recipients, which were then subjected to cutaneous OVA challenge. As previously reported (Zheng et al., 2007; Yeste et al., 2014), naive $CD4^+CD62L^+$ T cells from TCR-OVA transgenic DO11.10 mice that were activated with anti-$CD3^+$anti-CD28 and IL-23 in the presence of neutralizing antibodies to IL-4, IFN-γ, and TGFβ expressed significantly more *Il22* mRNA compared with DO11.10 cells activated under neutral Th0 conditions (Fig. 2 B). These cells expressed Il17a, *Il13*, and *Ifng* mRNA levels that are comparable to those expressed by Th0 cells. They were designated as Th22 cells for simplicity. Application of OVA to tape-stripped skin resulted in a significant increase in epidermal thickness and keratinocyte proliferation in WT recipients of Th22-polarized DO11.10 cells, compared with WT recipients of Th0 DO11.10 cells (Fig. 2, C and D). There was no significant increase in epidermal thickness or keratinocyte proliferation in bovine serum albumin challenged skin of WT recipients of Th0 DO11.10 cells or Th22-polarized DO11.10 cells (unpublished data). The difference in the ability of Th0 and Th22 cells to cause epidermal thickening was not caused by a difference in skin homing, because the numbers of $CD4^+KJ1\text{-}26^+$ donor cells in OVA-sensitized skin were not significantly different in recipients of Th0 cells and Th22 cells (unpublished data). Epidermal thickening and keratinocyte proliferation at the site of OVA challenge were comparable in $Il22^{-/-}$ and WT recipients of Th22-polarized DO11.10 cells (Fig. 2, C and D), ruling out a role for recipient-derived IL-22. These results indicate that Th22-polarized $CD4^+$ T cells are sufficient to drive epidermal thickening and keratinocyte proliferation in response to EC sensitization.

IL-22 and IL-17A are both essential for the induction of epidermal hyperplasia after intradermal injection of rIL-23 in mouse skin (Rizzo et al., 2011). OVA-sensitized skin in $Il17a^{-/-}$ mice, like in $Il22^{-/-}$ mice, demonstrated no significant increase in epidermal thickness (Fig. 2 E). Because the adoptively transferred Th22 cells we used, as well as the recipients, express *Il17a*, we examined whether IL-17A contributed to the ability of Th22 cells to drive epidermal thickening. Treatment of WT recipients of Th22 cells with neutralizing antibody to IL-17A, but not control IgG, abolished epidermal thickening upon cutaneous challenge with OVA (Fig. 2 F). These observations indicate that IL-22 and IL-17A play nonredundant roles in driving epidermal thickening in our model.

Both nonhematopoietic and hematopoietic sources of IL-23 are required for the IL-22 response to EC sensitization

IL-23 acts directly on naive $CD4^+$ T cells to drive their differentiation into Th22 cells in vitro (Zheng et al., 2007; Yeste et al., 2014). Consistent with this observation, $Il23r^{-/-}$ mice EC sensitized with OVA failed to exhibit an increase in serum IL-22 levels (Fig. 3 A). Moreover, cells from the DLNs of these mice failed to secrete IL-22 after OVA restimulation in vitro (Fig. 3 B). However, they secreted IL-13 and IFN-γ in amounts comparable to cells from WT controls, but secreted significantly less IL-17A (Fig. 3 B), in agreement with the role of IL-23 in amplifying the Th17 response (Stritesky et al., 2008). There was no increase in epidermal thickness, or $Ki67^+$ epidermal cells in the OVA-sensitized skin in $Il23r^{-/-}$ mice (Fig. 3, C and D), consistent with the failure of these mice to mount an IL-22 response to EC sensitization. $Il12p35^{-/-}$ mice, which lack IL-12, mounted a normal IL-22 response, but as expected, failed to mount an IFN-γ response to EC sensitization (Fig. 3 E). These findings demonstrate that IL-23 is critical for the IL-22 response to EC sensitization that drives epidermal thickening.

Both radiosensitive hematopoietic cells and radioresistant nonhematopoietic cells, including keratinocytes, are potential sources of IL-23 (Piskin et al., 2006; Guttman-Yassky et al., 2008; Chamilos et al., 2010). To determine the contribution of IL-23 derived from nonhematopoietic cells versus hematopoietic cells in the IL-22 response to EC sensitization, we generated BM chimeras using CD45 congenic WT mice and $p40^{-/-}$ mice, which lack both IL-23 and IL-12, as a role for IL-12 in the IL-22 response to EC sensitization has been already excluded (Fig. 3 E). FACS analysis of the DCs in the skin DLNs of the BM chimeras using CD45.1- and CD45.2-specific mAbs revealed that >90% of the $CD11c^+$ DCs were donor derived (unpublished data). 8 wk after BM reconstitution, the chimeras were EC sensitized with OVA for 7 wk. Cells from the skin DLNs of OVA-sensitized WT→WT chimeras secreted significantly more IL-22, IL-17A, IL-13, and IFN-γ in response to OVA stimulation than cells from saline-sensitized controls (Fig. 4, A and B). In contrast, cells from the skin DLNs of OVA-sensitized WT→$p40^{-/-}$ and $p40^{-/-}$→WT chimeras, failed to secrete IL-22 in response to OVA stimulation, but produced comparable amounts of IL-13 and notably, IL-17A as control WT→WT chimeras (Fig. 4, A and B). The normal IL-17A secretion in these chimeras suggests that IL-23 from either hematopoietic or nonhematopoietic cell sources is sufficient to sustain a normal IL-17A response to EC sensitization. As expected, IFN-γ production was not increased in $p40^{-/-}$→WT chimeras whose DCs lack IL-12 (Fig. 4 B). OVA-sensitized skin in WT→WT chimeras exhibited a significant increase in epidermal thickness and $Ki67^+$ epidermal cells compared with control saline-sensitized skin (Fig. 4, C–F). In contrast, WT→$p40^{-/-}$ and $p40^{-/-}$→WT chimeras did not exhibit any significant increase in epidermal thickness or $Ki67^+$ epidermal cells in OVA-sensitized skin (Fig. 4, C–F), consistent with their failure to mount an IL-22

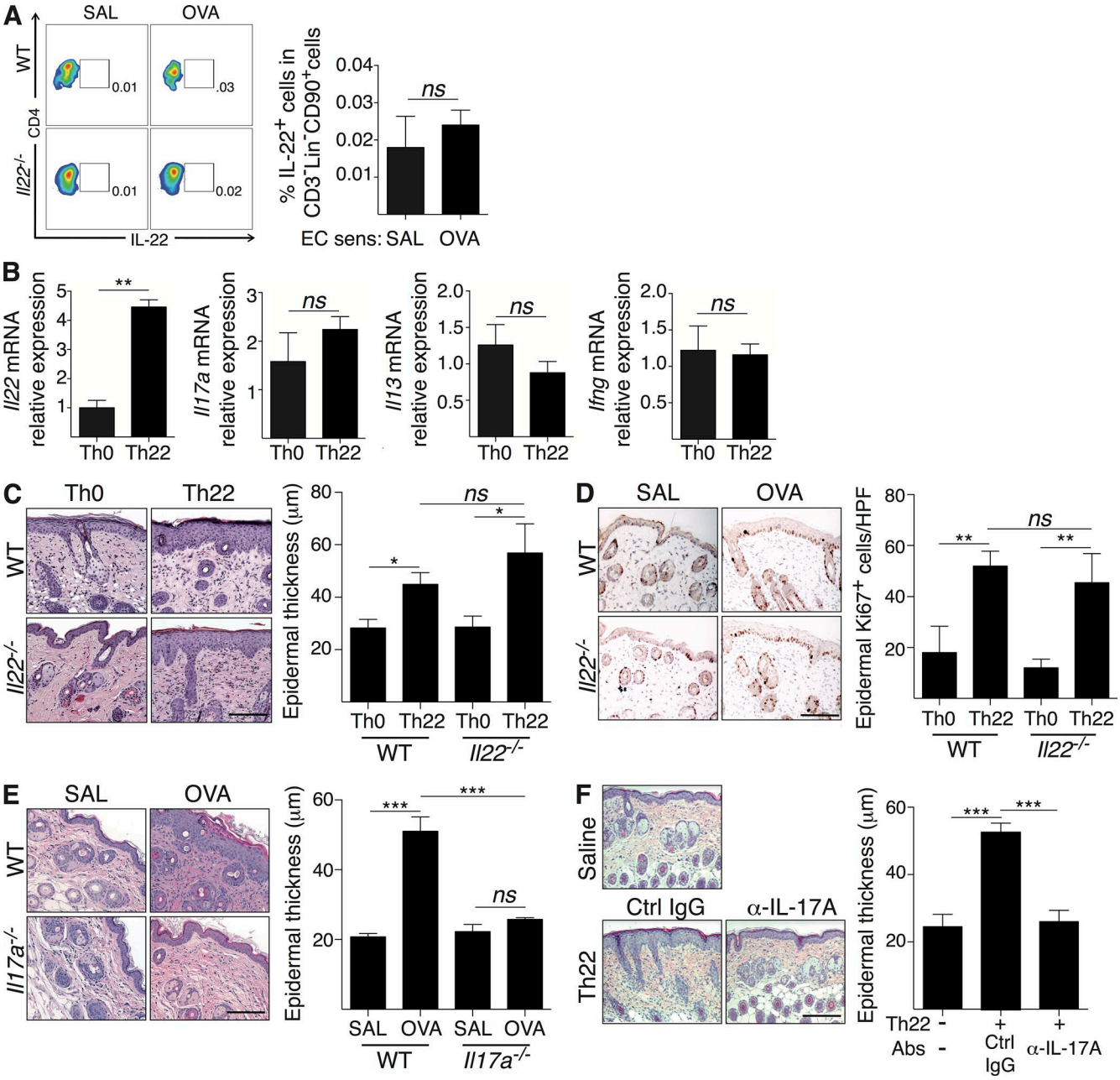

Figure 2. **Th22 cells are sufficient to cause epidermal thickening and keratinocyte proliferation.** (A) Representative FACS analysis and percentage of IL-22 producing CD45$^+$ Lin$^-$CD90$^+$ ILCs in the skin of EC-sensitized WT and *Il22*$^{-/-}$ mice. Lin$^-$ cells refer to gating out cells that express CD3, CD11b, CD11c, F4/80, B220, CD19, FcεR1α, CD49b, and GR1 using mAbs. (B) Cytokine mRNA expression by naive CD4$^+$CD62L^{hi} DO11.10 T cells stimulated in vitro for 5 d using anti-CD3 and anti-CD28 mAbs alone (Th0 condition) or with addition of rIL-23, and neutralizing mAbs to IL-4, IFN-γ, and TGF-β (Th22 condition). Results are expressed as fold increase relative to Th0 condition. (C and D) Representative H&E staining and quantitative analysis of epidermal thickness (C) and representative immunohistochemical staining and numbers of Ki67$^+$ cells (D) in OVA-challenged skin of naive WT and *Il22*$^{-/-}$ recipients of DO11.10 Th22 or Th0 cells. (E) Representative H&E staining and quantitative analysis of epidermal thickness in 7 wk EC-sensitized skin of *Il17a*$^{-/-}$ mice and WT controls. (F) Representative H&E staining and quantitative analysis of epidermal thickness in OVA-challenged skin of WT recipients of DO11.10 Th22 cells and anti–IL-17A neutralizing antibody or IgG isotype control. Saline recipients were used as controls. Results represent three mice/group in A, 4 independent experiments in B, and five to six mice per group in C–F. Horizontal lines and vertical bars in A, and columns and bars in B–F, represent means and SEM. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ns, not significant, two-tailed Student's *t* test.

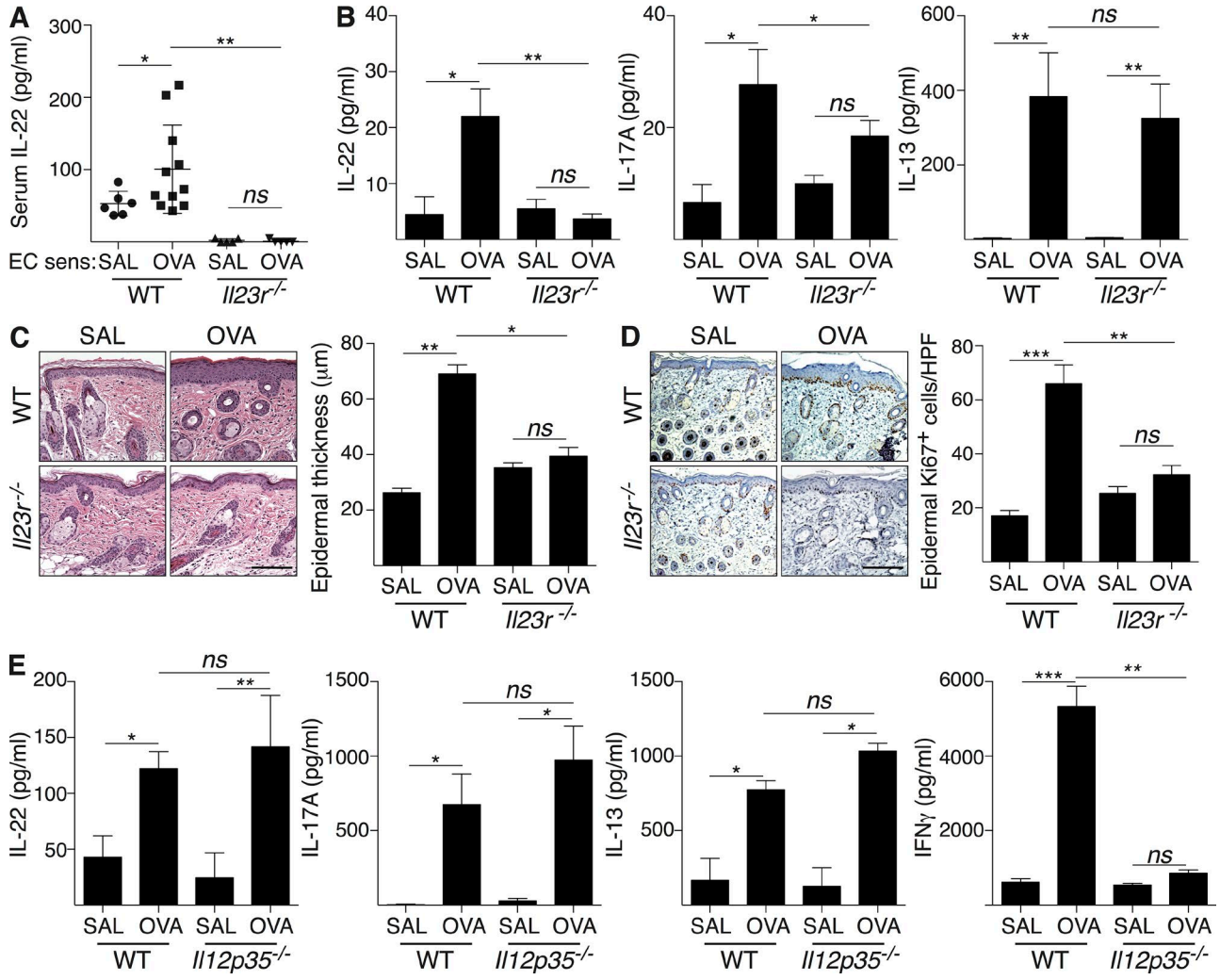

Figure 3. **IL-23 is essential for the IL-22 response to EC sensitization and for epidermal thickening and keratinocyte proliferation.** (A and B) Serum IL-22 levels (A) and cytokine secretion OVA-stimulated skin DLN cells (B) in EC-sensitized *Il23r*$^{-/-}$ mice and WT controls. (C and D) Representative H&E staining and epidermal thickness (C) and representative immunohistochemical staining and numbers of Ki67^{+} cells in the epidermis (D) of skin from EC-sensitized *Il23r*$^{-/-}$ mice and WT controls. Bars: (C and D) 100 µm. (E) Cytokine secretion by skin DLN cells from EC-sensitized *Il12p35*$^{-/-}$ mice and WT controls. Results in A represent 6 and 11 WT mice EC sensitized with saline and OVA, respectively, and 5 *Il23r*$^{-/-}$ mice EC sensitized with saline and OVA, respectively. Results in B–E represent five to six mice per group. Horizontal lines and vertical bars in A represent means and SEM, respectively. Columns and bars in B–E represent mean and SEM. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ns, not significant, two-tailed Student's *t* test.

response to EC sensitization. These results indicate that IL-23 derived from both radioresistant nonhematopoietic cells and radiosensitive hematopoietic cells is important for driving the IL-22 response to EC sensitization.

Tape stripping of the skin induces IL-23 expression in keratinocytes

We examined whether tape stripping up-regulates *Il23* expression in the skin. PCR analysis revealed that *Il23p19* mRNA expression was significantly up-regulated in mouse skin 6 h after tape stripping, but returned to baseline after 24 h (Fig. 5 A). Furthermore, explants of mouse skin obtained immediately after tape stripping and cultured for 24 h released IL-23 into their supernatants (Fig. 5 B). This release was dependent on de novo protein synthesis, as it was blocked by the addition of the protein synthesis inhibitor, cycloheximide (CHX).

Potential sources of IL-23 in the skin include keratinocytes and LCs in the epidermis and DCs in the dermis. We examined *Il23p19* mRNA levels in epidermis and dermis isolated from skin 6 h after tape stripping. *Il23p19* mRNA was induced strongly in the epidermis, but only modestly in the dermis (Fig. 5 C). The purity of the dermal preparations was confirmed by their virtual lack of mRNA expression of the keratinocyte-specific gene *Krt5*. Immunohistochemical staining demonstrated that 6 h after tape stripping IL-23 pro-

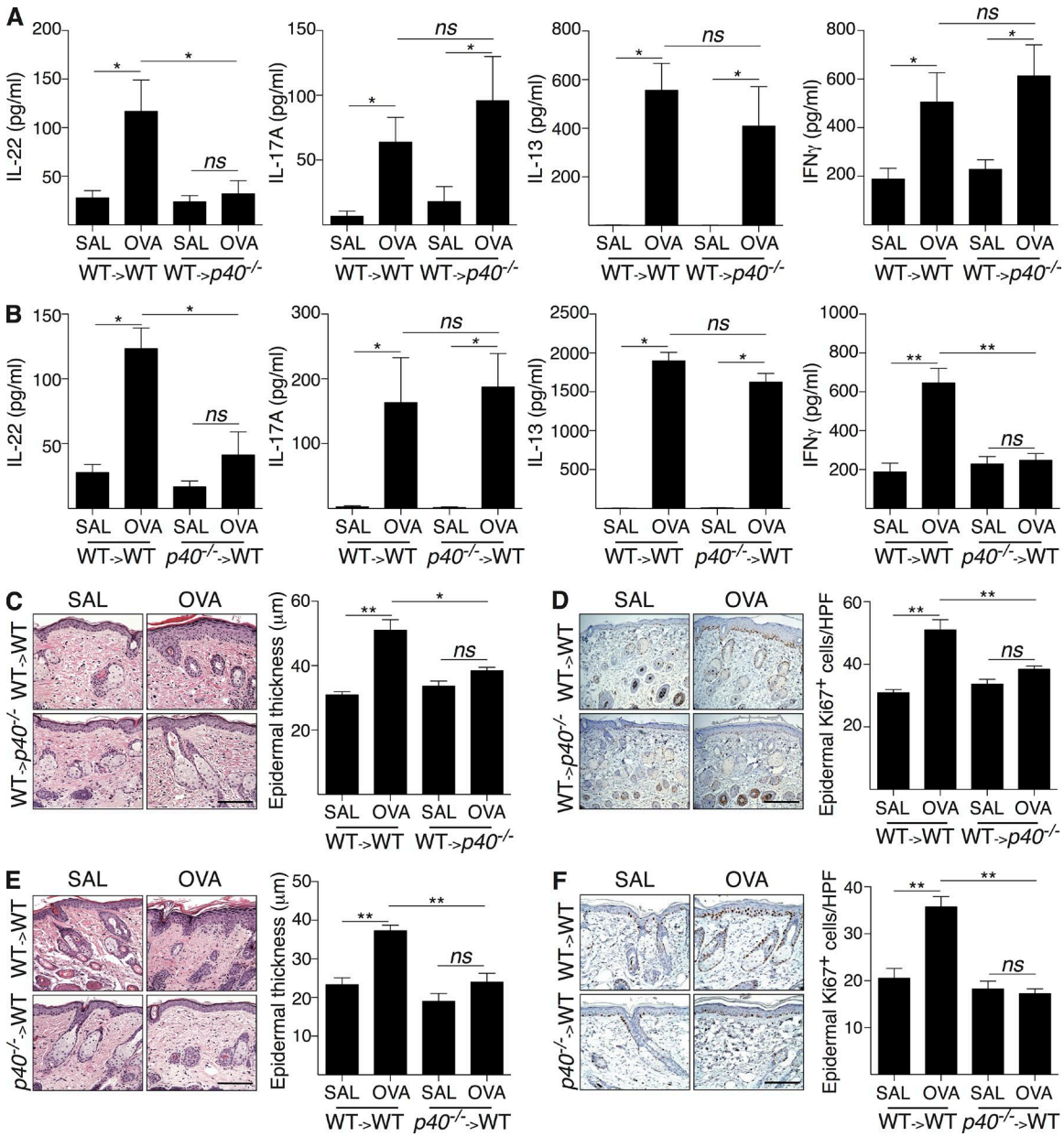

Figure 4. **IL-23 derived from both nonhematopoietic and hematopoietic cells is important for the IL-22 response to EC sensitization.** (A and B) Cytokine secretion by OVA-stimulated skin DLN cells from WT→*p40*$^{-/-}$, *p40*$^{-/-}$→WT, and WT→WT control chimeras. (A and B) Representative H&E staining of the skin and epidermal thickness (C and E) and representative immunohistochemical staining of the skin and numbers of Ki67^{+} cells in the epidermis (D and F) in EC-sensitized WT→*p40*$^{-/-}$, *p40*$^{-/-}$→WT, and WT→WT control chimeras. Bars: (C–F) 100 µm. Results in A–F represent five to six mice per group. Columns and bars in A–F represent mean and SEM. *, $P < 0.05$; **, $P < 0.01$; ns, not significant, two-tailed Student's *t* test.

tein expression was up-regulated primarily throughout the epidermis (Fig. 5 D). Treatment of *Langerin-eGFP-DTR* mice with diphtheria toxin (DT) resulted in the effective depletion of Langerin^{+} LCs in the epidermis, but had no effect on the up-regulation of *Il23p19* mRNA expression in the skin after tape stripping (Fig. 5 E). These results indicate that tape stripping of the skin up-regulates IL-23 expression primarily in epidermal keratinocytes.

Exogenous IL-23 polarizes migratory skin DCs that capture antigen to drive IL-22 production by naive CD4^{+} T cells

Because IL-23 expression in nonhematopoietic cells was required for the IL-22 response to EC sensitization, we tested the hypothesis that exogenous IL-23, such as IL-23 released by keratinocytes in tape-stripped skin, polarizes skin-derived DCs to drive the differentiation of naive CD4^{+} T cells into IL-22–producing cells. qPCR analysis showed that *Il23r*

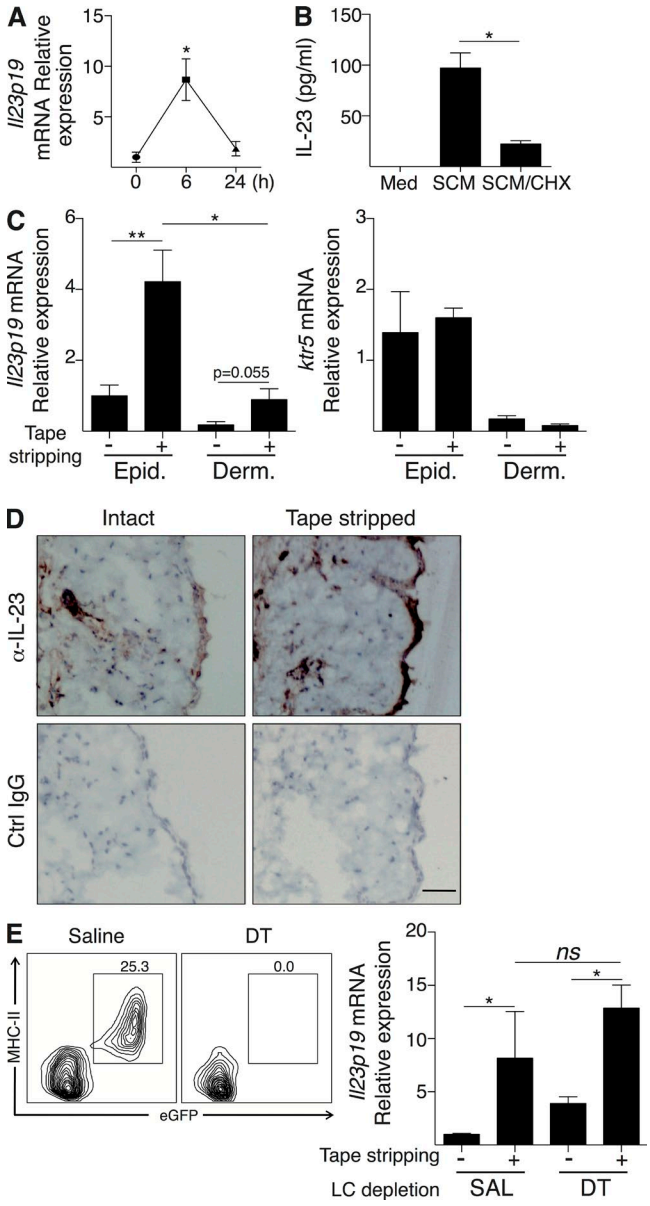

Figure 5. **Tape stripping induces IL-23 expression in keratinocytes of epidermis.** (A) *Il23p19* mRNA expression in ear skin of BALB/c mice at 0, 6, and 24 h after tape stripping. Results are expressed as fold increase relative to nontape-stripped skin. (B) IL-23 release by explants of skin minced with scissors and cultured for 24 h with or without 10 µg/ml CHX. (C) Expression of *Il23p19* mRNA (left) and *Keratin 5* mRNA (right) in dermis and epidermis isolated from ear skin 6 h after tape stripping. Results are expressed as fold increase relative to epidermis of nontape-stripped skin. (D) Representative immunohistochemical staining for IL-23 in the skin. Staining with an isotype-matched irrelevant antibody (Ctrl IgG) was used as a negative control. Bars, 100 µm. (E) Representative FACS analysis of $CD11c^+Langerin^+$ cells in ears from *Langerin-eGFP-DTR* mice treated by *i.v.* administration of DT or saline 24 h before sacrifice and qPCR analysis of *Il23p19* mRNA expression 6 h after tape stripping the ear skin of *Langerin-eGFP-DTR* mice that were administered DT or saline 24 h before tape stripping. Results are expressed as fold increase relative to nontape-stripped skin of saline-treated *Langerin-eGFP-DTR* mice. Results represent at least 5 mice for each group in A–C and E and 3 mice in D. Columns and bars represent mean and SEM. *, $P < 0.05$; **, $P < 0.01$; ns, not significant, two-tailed Student's *t* test.

mRNA levels are considerably higher in DCs isolated from the skin and skin DLN, than in DCs isolated from the spleen of unmanipulated WT mice (Fig. 6 A). Furthermore, FACS analysis detected IL-23R expression on a subset of $CD11c^+$ DCs in skin and skin DLN, but on very few splenic $CD11c^+$ DCs in unmanipulated WT mice (Fig. 6 B). To examine whether IL-23 polarizes DCs to drive an IL-22 response in $CD4^+$ T cells, DCs were treated with rIL-23 for 24 h, extensively washed, and then co-cultured with naive $CD4^+$ OT-II T cells in the presence of $OVA_{323-339}$ peptide. Pretreatment with IL-23 caused DCs from the skin and skin DLN, but not spleen, to drive significantly more IL-22 production by naive $CD4^+$ T cells (Fig. 6 C). As expected, IL-17A production in cultures containing IL-23 pretreated DCs from skin DLN was also increased, but IL-13 and IFN-γ production were unaffected (Fig. 6 D). IL-23 pretreatment of DCs from the skin DLN of *Il23r*$^{-/-}$ mice failed to augment their ability to drive IL-22 production by co-cultured OT-II T cells stimulated with $OVA_{323-339}$ peptide (unpublished data), demonstrating that the polarizing effect of rIL-23 on DCs is specific. Exogenous IL-23 caused DCs from the skin DLN to up-regulate *Il23p19* mRNA expression and to secrete IL-23 (Fig. 6, E and F). The ability of IL-23 primed DCs to increase IL-22 production by co-cultured naive $CD4^+$ OT-II T cells was significantly reduced by the addition of neutralizing antibody to IL-23 to the co-cultures (Fig. 6 G). More importantly, IL-23–primed DCs from *Il23p19*$^{-/-}$ mice failed to induce IL-22 production by naive $CD4^+$ OT-II T cells (Fig. 6 H). These findings indicate that exogenous IL-23 polarizes DCs to drive an IL-22 response by up-regulating endogenous IL-23 expression.

IL-23 in mechanically injured skin polarizes migratory skin DCs to drive an IL-22 response by inducing endogenous IL-23 expression

Pretreatment of DCs from skin DLN of WT mice with skin-conditioned medium (SCM) from explants of tape-stripped skin from WT mice caused DCs to drive IL-22 and IL-17A production by naive $CD4^+$ OT-II T cells in the presence of $OVA_{323-339}$ peptide (Fig. 7 A). SCM-treated DCs from skin DLN of *Il23r*$^{-/-}$ mice caused significantly less IL-22 and IL-17A production by T cells compared with SCM-treated WT DCs (Fig. 7 A). These results demonstrate that IL-23 is released in tape-stripped skin in amounts sufficient to polarize skin DCs to drive the differentiation of IL-22–producing T cells.

To ascertain the role of keratinocyte-derived IL-23 in polarizing skin DCs to drive IL-22 production, we generated conditioned medium from explants of epidermal and nonepidermal layers derived from tape-stripped skin of WT and *Il23p19*$^{-/-}$ mice. Conditioned medium from the epidermal

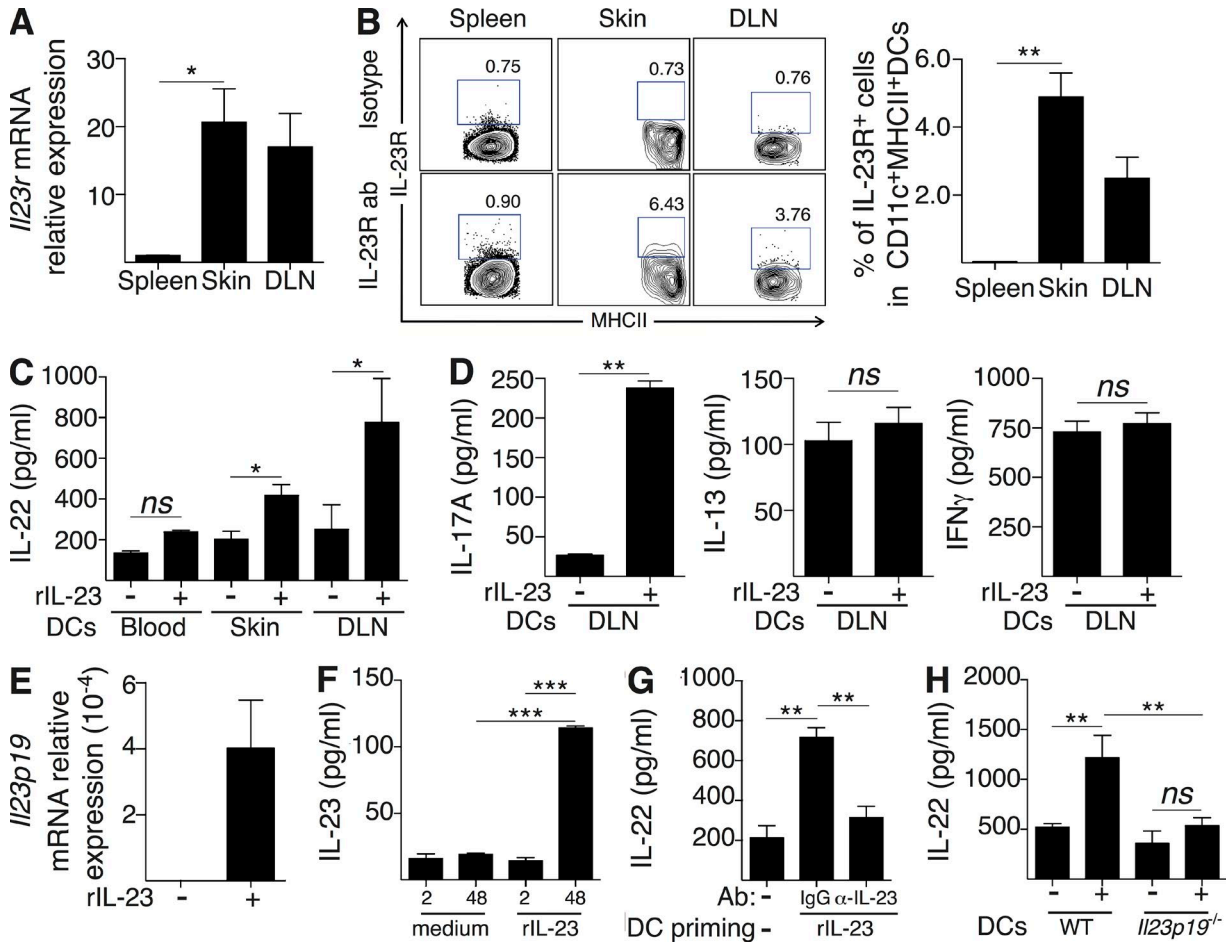

Figure 6. **IL-23 polarizes DCs from skin DLN to drive IL-22 production by naive CD4⁺ T cells by inducing them to express endogenous IL-23 expression.** (A) qPCR analysis of Il23r mRNA expression by CD11c⁺ DCs from spleen, skin and skin DLN. Results as expressed relative to splenic DCs. (B) Representative FACS analysis of IL-23R expression by CD11c⁺MHCII$^{+/high}$ DCs and percentage of IL-23R⁺CD11c⁺MHCII$^{+/high}$ DCs in spleen, skin, and skin DLN from WT mice. (C) IL-22 secretion by CD4⁺ OT-II cells cultured with DCs isolated from spleen, skin, and skin DLN of WT mice pretreated with rIL-23 or medium for 24 h. (D) IL-17A, IL-13, and IFN-γ secretion by CD4⁺ OT-II T cells cultured with rIL-23 primed DCs isolated from the skin DLN of WT mice. (E) Expression of *Il23p19* mRNA by skin DLN DCs from WT mice cultured for 3 h with rIL-23. (F) IL-23 secretion by DCs from skin DLN of WT mice after preincubation with rIL23 for 16 h, followed by extensive washing and culture for the indicated times. (G) Effect of IL-23 neutralizing antibody or its isotype control on the ability of IL-23 primed and washed DCs isolated from skin DLN to drive IL-22 production by naive CD4⁺ OTII T cells. (H) IL-22 production by naive CD4⁺ OTII T cells stimulated with $OVA_{323-339}$ peptide and IL-23 primed DCs derived from the skin DLN of WT or *Il23p19*$^{-/-}$ mice. Results in A–H represent DCs obtained from four to six mice for each group. Columns and bars represent mean and SEM. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ns, not significant, two-tailed Student's *t* test.

layer of tape-stripped skin of WT, but not *Il23p19*$^{-/-}$, mice caused DCs from skin DLN to drive IL-22 production by naive CD4⁺ OT-II T cells in the presence of $OVA_{323-339}$ peptide (Fig. 7 B). IL-17A production was significantly less in cultures containing DCs treated with epidermal layer conditioned medium from *Il23p19*$^{-/-}$ mice compared with WT controls (Fig. 7 B). Treatment of DCs with conditioned medium from explants of nonepidermal layers of tape-stripped skin had no significant effect on IL-22 or IL-17A production (unpublished data). Because keratinocytes appear to be the major, if not sole, source of IL-23 in tape-stripped skin (Fig. 5 E), these results suggest that keratinocytes are the major, if not exclusive, source of IL-23 that polarizes DCs in mechanically injured skin to drive IL-22 production by naive CD4⁺ T cells.

DCs that capture antigen in the skin migrate to skin DLNs where they activate antigen-specific T cells (Warger et al., 2007). DCs isolated from DLNs 24 h after application of OVA to the tape-stripped skin of C57BL/6 mice induced IL-22, as well as IL-17A, IL-13, and IFN-γ secretion by co-cultured naive CD4⁺ OT-II T cells in the absence of the addition of any exogenous $OVA_{323-339}$ peptide (Fig. 7 C). DCs isolated from the skin DLN of tape-stripped OVA-exposed skin of *Il23r*$^{-/-}$ mice induced significantly less IL-22 production

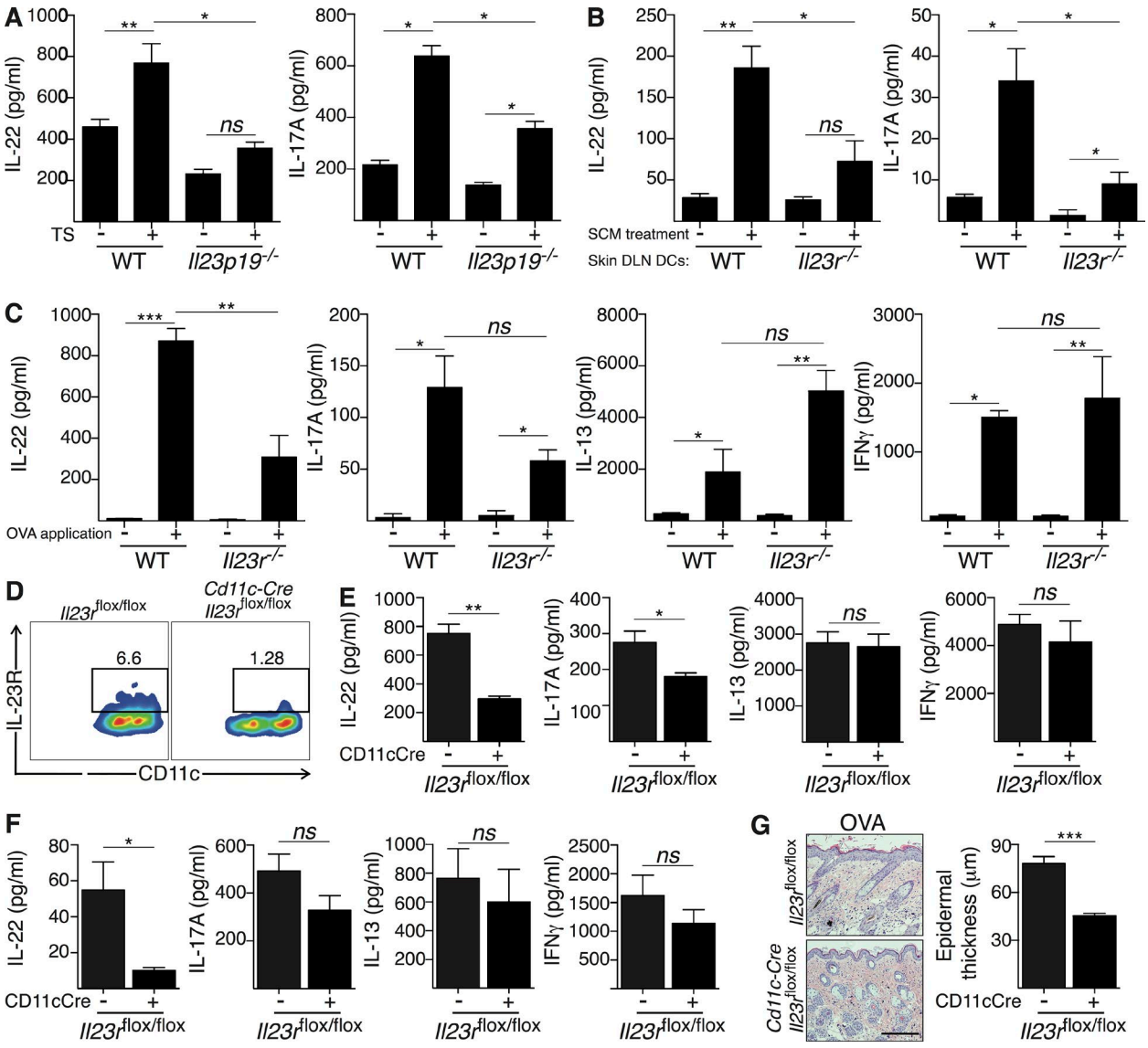

Figure 7. **IL-23 in mechanically injured skin polarizes migratory skin DCs to drive an IL-22 response by inducing endogenous IL-23 expression.** (A) Cytokine secretion of IL-22 and IL-17A by CD4$^+$ OT-II T cells cultured with DCs isolated from skin DLN of WT or *Il23r*$^{-/-}$ mice and pretreated for 24 h with skin explant–conditioned medium (SCM) harvested from cultured explants of tape-stripped (+) or with medium (–) as control. (B) Cytokine secretion of IL-22 and IL-17A by CD4$^+$ OT-II T cells cultured with DCs isolated from skin DLN of WT mice and pretreated for 24 h with conditioned medium harvested from cultured epidermal layer explants of tape-stripped (+) or unmanipulated (–) skin from WT and *Il23p19*$^{-/-}$ mice. (C) Cytokine secretion by CD4$^+$ OT-II T cells cultured with DCs isolated from the skin DLN of WT or Il23r$^{-/-}$ mice 24 h after tape striping and OVA application. No exogenous OVA were added to the cultures. (D) FACS analysis of IL-23R expression by DCs from the skin of *Cd11c-Cre*$^{Tg/0}$*Il23r*$^{flox/flox}$ mice and *Il23r*$^{flox/flox}$ controls. (E) Cytokine secretion by CD4$^+$ OT-II T cells cultured with DCs isolated from the skin DLN of *Cd11c-Cre*$^{Tg/0}$*Il23r*$^{flox/flox}$ mice and *Il23r*$^{flox/flox}$ controls 24 h after tape striping and OVA application. (F) Cytokine secretion by OVA-stimulated skin DLN cells from *Cd11c-Cre*$^{Tg/0}$*Il23r*$^{flox/flox}$ mice and *Il23r*$^{flox/flox}$ controls that were EC sensitized with OVA for 7 wk. (G) Representative H&E staining and epidermal thickness of skin in OVA EC-sensitized *Cd11c-Cre*$^{Tg/0}$*Il23r*$^{flox/flox}$ mice and *Il23r*$^{flox/flox}$ controls. Bar, 100 μm. Results in A-G represent four to six mice for each group. Columns and bars represent mean and SEM. *, P < 0.05; **, P < 0.01; ***, P < 0.001; ns, not significant, two-tailed Student's *t* test.

by naive CD4$^+$ OT-II T cells compared with DCs from WT controls (Fig. 7 C); IL-17A production was decreased as expected, but IL-13 and IFN-γ production was not significantly different (Fig. 7 C). To ascertain whether IL-23 acts directly on DCs to polarize them to drive IL-22 production in CD4$^+$ T cells, we made use of *Cd11c-Cre*$^{Tg/0}$*Il23r*$^{flox/flox}$ mice. DCs in skin DLN of these mice expressed negligible levels of IL-23R (Fig. 7 D). DCs isolated from the DLN of tape-stripped skin of OVA-exposed skin of *Cd11c-Cre*$^{Tg/0}$*Il23r*$^{flox/flox}$ mice induced significantly less IL-22 production by naive CD4$^+$

OT-II T cells compared with DCs from *Il23r*$^{flox/flox}$ control littermates (Fig. 7 E). IL-17A production was, as expected, modestly decreased, but IL-13 and IFN-γ production was unaffected (Fig. 7 E). We also examined the role of IL-23R expression by DCs in the systemic IL-22 response to EC sensitization with antigen. In contrast to *Il23r*$^{flox/flox}$ controls, cells from the skin DLNs of *Cd11c-Cre*$^{Tg/0}$*Il23r*$^{flox/flox}$ mice EC sensitized with OVA failed to increase IL-22 secretion in response to OVA stimulation in vitro (Fig. 7 F); IL-17A, IL-13, and IFN-γ secretion were not significantly different from control littermates (Fig. 7 F). Furthermore, in contrast to OVA-sensitized skin of *Il23r*$^{flox/flox}$ controls, OVA-sensitized skin of *Cd11c-Cre*$^{Tg/0}$*Il23r*$^{flox/flox}$ mice failed to exhibit a significant increase in epidermal thickening (Fig. 7 G). Collectively, these results demonstrate that keratinocyte-derived IL-23 polarizes migratory skin DCs to drive an IL-22 response by directly engaging their IL-23R, inducing them to produce endogenous IL-23.

Endogenous TLR4 ligands drive the induction of IL-23 expression in tape-stripped skin

Tape stripping breaches the epidermal barrier formed by dead corneocytes, allowing ligands derived from the skin microbial flora to access live skin cells. Induction of *Il23p19* mRNA was comparable in the tape-stripped skin from germ-free (GF) mice and WT controls reared under specific pathogen–free (SPF) conditions (Fig. 8 A), suggesting that the microbiota is dispensable for the induction of *Il23p19* mRNA expression in tape-stripped skin. Tape stripping triggers the release of endogenous damage-associated molecular pattern molecules, which include TLR ligands, and IL-1 family cytokines (Gregorio et al., 2010; Guiducci et al., 2010). IL-1 family cytokines and TLR ligands signal via the adaptor molecule MyD88, except for TLR3, which signals via TRIF. Tape stripping completely failed to induce *Il23p19* mRNA expression in the skin of *Myd88*$^{-/-}$ mice (Fig. 8 B). In contrast, it induced comparable *Il23p19* mRNA expression in the skin of *Trif*$^{-/-}$ mice and WT controls (Fig. 8 B). To determine the potential role of IL-1 family cytokines in *Il23p19* expression by tape-stripped skin we examined mice deficient in IL-1R1, IL-18R, or the IL-33 receptor ST2, encoded by *Il1r1*, *Il18r*, and *Il1rl1*, respectively. Tape stripping induced comparable levels of *Il23p19* mRNA expression in the skin of *Il1r1*$^{-/-}$, *Il18r*$^{-/-}$, and *Il1rl1*$^{-/-}$ mice and of *Il1r1*$^{-/-}$ mice treated with neutralizing antibodies to IL-18 and ST2, compared with WT controls (Fig. 8 C), suggesting that TLR ligands are sufficient for mediating IL-23 induction in mechanically injured skin.

Tape stripping induced normal *Il23p19* mRNA expression in the skin of *Tlr2*$^{-/-}$ and *Tlr5*$^{-/-}$ mice and *Unc93b*$^{-/-}$ mice, which fail to signal via TLR3, TLR7, and TLR9 (Fig. 8 D). In contrast, it induced significantly less *Il23p19* mRNA expression in the skin of TLR4 mutant C3HeJ mice than WT controls (Fig. 8 D). Tape stripping of mouse skin has been shown to cause the release of the endogenous TLR4 ligand, low molecular weight hyaluronic acid (low MW HA; (Tammi et al., 2005). Tape stripping up-regulated the expression of genes encoding the endogenous TLR4 ligands, S100a8 and S100a9 (Fig. 8 E). Intradermal (i.d.) injection of purified low MW HA and rS100a8, but not PBS vehicle, induced *Il23p19* mRNA expression in the skin of WT mice, but not TLR4 mutant mice (Fig. 8 F). Moreover, stimulation with low MW HA up-regulated *Il23p19* mRNA expression in an NF-κB– and ERK-dependent, but p38- and JNK–independent manner in the mouse keratinocyte cell line Pam212, as determined using selective pharmacologic inhibitors (Fig. 8 G). The aforementioned results indicate that endogenous TLR4 ligands are important drivers of *Il23p19* expression in the mechanically injured skin.

TLR4 is essential for the IL-22 response to EC sensitization

We next examined the role of TLR4-dependent IL-23 induction in the in vivo polarization of DCs after application of antigen to tape-stripped skin. DCs from DLNs of OVA-exposed tape-stripped skin of *Tlr4*$^{-/-}$ mice induced significantly less IL-22 secretion by naive CD4^{+} OT-II T cells than the corresponding DCs from WT controls (Fig. 9 A); IL-17A production was diminished, but the difference did not reach statistical difference. IL-13 and IFN-γ production were comparable to controls, suggesting that lack of TLR4 did not alter the capacity of skin DCs to capture antigen or migrate to DLNs. CD11c^{+}MHCII^{high}DQ-OVA^{+} migratory DCs isolated from the DLN of tape-stripped and DQ-OVA–painted skin of *Tlr4 mut.* mice exhibited significantly reduced *Il23* mRNA levels (Fig. 9 B), but their expression of the Th17 polarizing cytokines *Il6*, *Il1b*, and *Tnfa* was comparable to controls.

We also examined the role of TLR4 in the IL-22 response to EC sensitization. In contrast to WT controls, serum IL-22 levels failed to rise in TLR4 mutant mice after EC sensitization with OVA for 7 wk (Fig. 9 C). Cells from the skin DLNs of OVA-sensitized TLR4 mutant mice failed to increase IL-22 secretion in response to OVA stimulation (Fig. 9 D); IL-17A and IFN-γ secretion were not significantly different from WT controls, but IL-13 secretion was decreased, consistent with a previous report that TLR4 signaling is involved in IL-13 production (Eisenbarth et al., 2002). OVA-sensitized skin TLR4 mutant mice failed to exhibit a significant increase in epidermal thickening, or in Ki67^{+} epidermal cells (Fig. 9, E and F). Collectively, these results suggest that endogenous TLR4 ligands released in the skin after mechanical injury induce local IL-23 expression, and play an important role in driving the IL-22 response to cutaneous sensitization.

IL-23 polarizes human skin DCs to drive an IL-22 response

We investigated whether the mechanism for IL-22 induction after EC sensitization we have elucidated in mice is applicable to humans. To this end, we tested whether scratching induces IL-23 release in human skin, and evaluated the effect of IL-23 on the polarization of human skin DCs in vitro. Significantly more IL-23 was released by explants of scratched

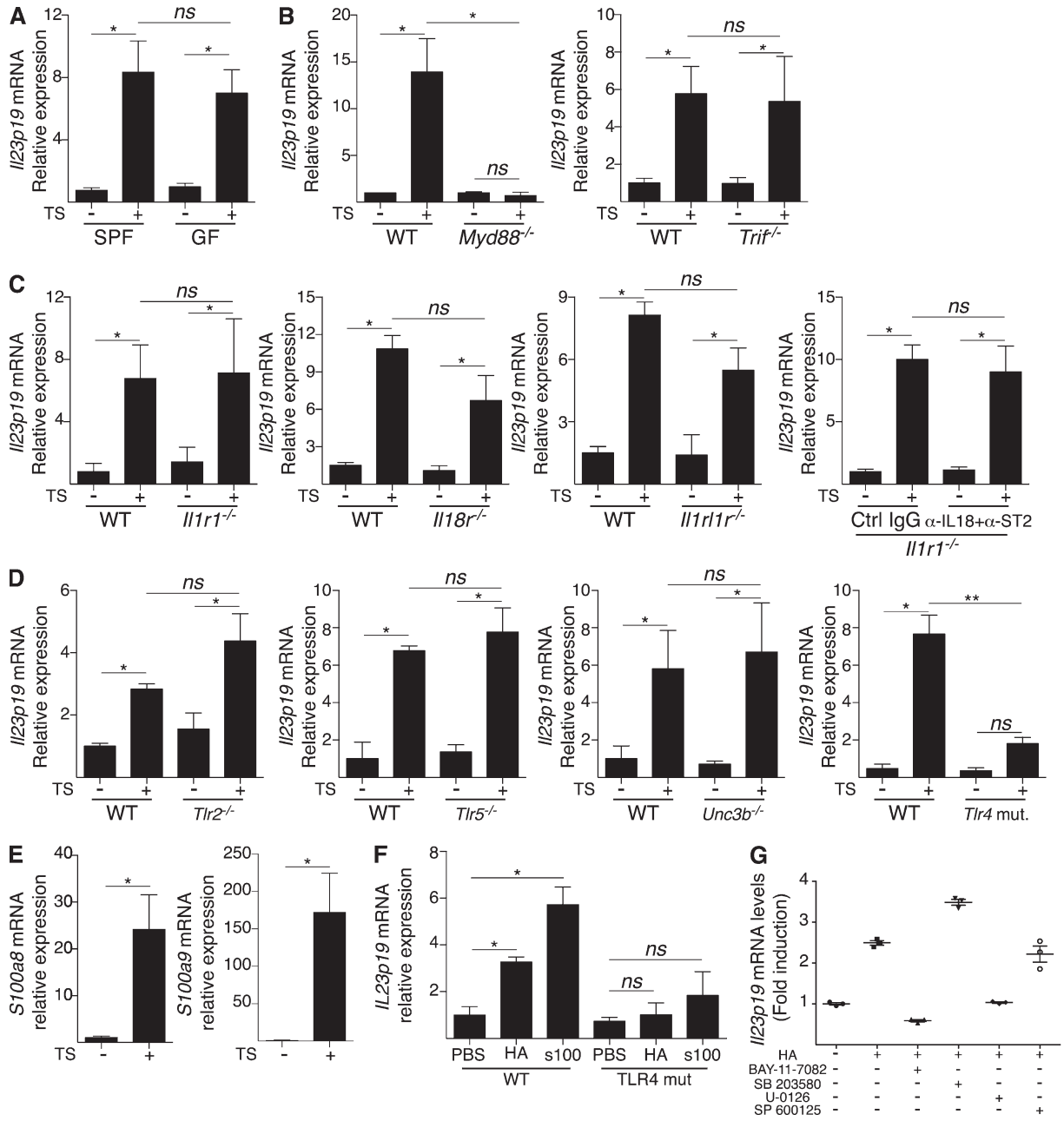

Figure 8. **TLR4 is essential for the induction of IL-23 expression in mouse skin by tape stripping and for the IL-22 response to EC immunization.** (A–D) *Il23p19* mRNA expression 6 h after tape stripping of the ear skin of WT mice housed under GF versus specific pathogen–free (SPF) conditions (A); *Myd88*$^{-/-}$ and *Trif*$^{-/-}$ mice and WT controls (B); *Il1r1*$^{-/-}$, *Il18r*$^{-/-}$, *Il1rl1*$^{-/-}$, and *Il1r1*$^{-/-}$ mice treated with anti-ST2 and anti–IL-18–neutralizing mAbs or control IgG (C); *Tlr2*$^{-/-}$, *Tlr5*$^{-/-}$, *Unc93b*$^{-/-}$, and TLR4 mutant (mut.), C3HeJ mice, and WT controls (D). (E) S100a8 and S100a9 mRNA expression 6 h after tape stripping of ear skin. (F) Il23p19 mRNA expression 3 h after i.d. injection of low MW HA or rS100a8 in the back skin of TLR4 mutant mice and WT controls. (G) Il23p19 mRNA expression in Pam212 cells stimulated for 6 h with low MW HA with or without preincubation with the indicated inhibitors of NFKβ (BAY-11-7082), p38 (SB 203580), ERK (U-0126), or JNK (SP600125). Results in A–F are derived from at least five mice for each group. Results in G represent four independent experiments. Columns and bars in A–F and horizontal and vertical bars in G represent mean and SEM. *, $P < 0.05$; **, $P < 0.01$; ns, not significant, two-tailed Student's *t* test.

normal skin than explants of unmanipulated skin from the same individual (Fig. 10 A). A fraction of epidermal LCs (5%) and dermal DCs (~12%) in normal human skin express IL-23R on their surface as indicated by FACS analysis using anti–huIL-23R–specific mAb (Tonel et al., 2010). Using the same mAb, we found that a negligible fraction (<0.9%) of

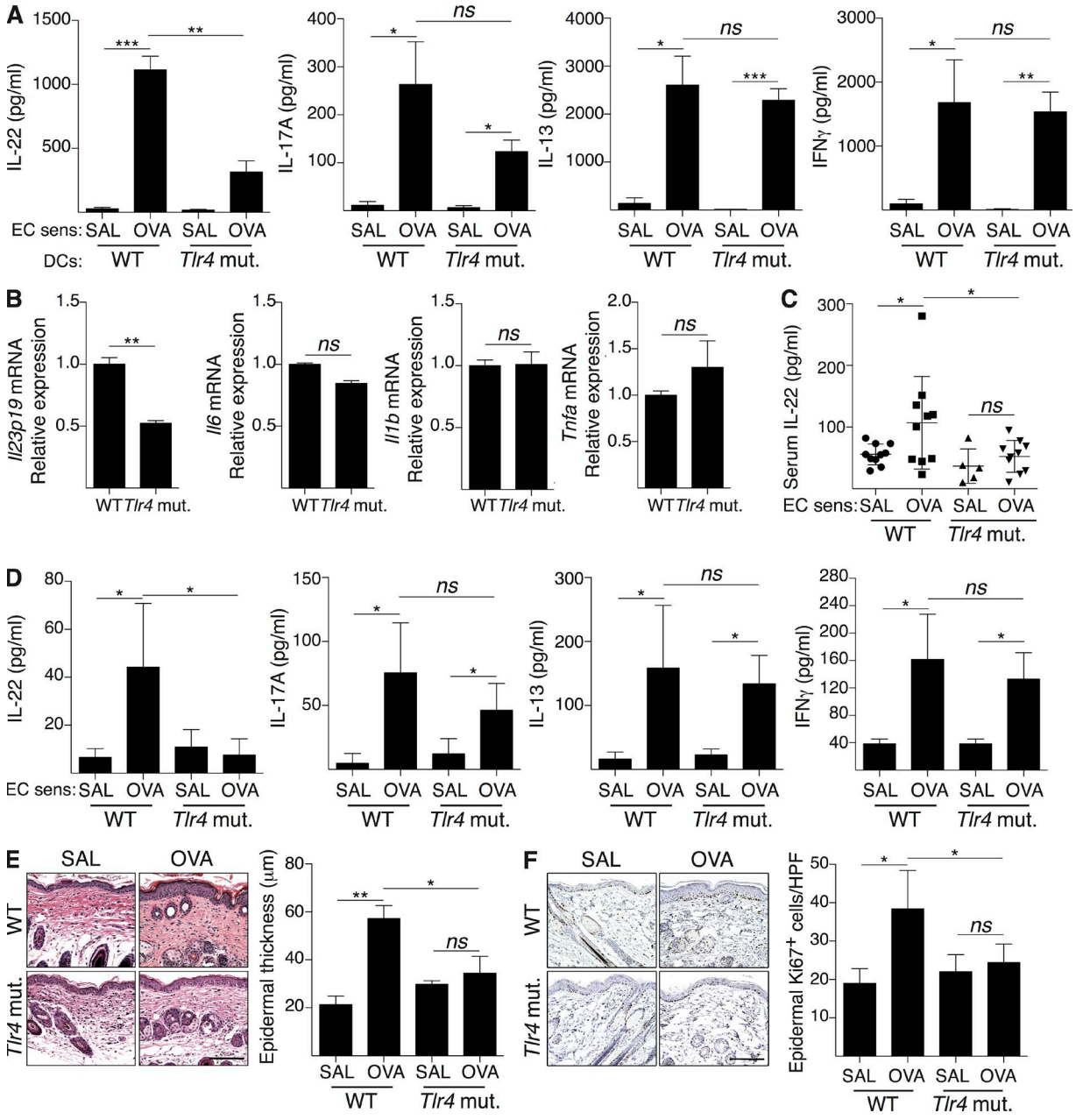

Figure 9. **TLR4 is essential for the IL-22 response to EC sensitization.** (A) Cytokine secretion by naive CD4$^+$ OTII cells cultured with DCs isolated from the skin DLN of Tlr4$^{-/-}$ mice or WT controls 24 h after tape stripping and OVA application. No exogenous OVA were added to the cultures. (B) qPCR analysis of cytokine mRNA levels in DQ-OVA$^+$CD11c$^+$MHCII^{high} migratory skin DCs from TLR4 mut. mice and WT controls sorted from skin DLNs 24 h after DQ-OVA application to tape-stripped skin. (C) Serum IL-22 levels in 7 wk EC-sensitized TLR4 mut. mice and WT controls. (D) Cytokine secretion by OVA-stimulated skin DLN cells from TLR4 mut. mice and WT controls EC sensitized for 7 wk. (E and F) Representative H&E staining and epidermal thickness of skin (E), and representative immunohistochemical staining and numbers of Ki67$^+$ cells in the epidermis (F) in EC-sensitized TLR4 mut. mice and WT controls. Bar, 100 µm. Results in A, B, and D–F are derived from at least five mice for each group. Results in C represent 10 and 5 saline-sensitized WT and TLR4 mut. mice, respectively, and 9 and 9 OVA-sensitized WT and TLR4 mut. mice, respectively. Columns and bars in A and C–E, and horizontal and vertical bars in B, represent mean and SEM. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ns, not significant, two-tailed Student's *t* test.

peripheral blood CD11c$^+$ DCs express surface IL-23R; however, a fraction of dermal DCs (~25%) and epidermal LCs (~15%) express IL-23R (Fig. 10 B). Priming with exogenous IL-23 significantly increased the ability of human skin LCs, but not blood DCs, to drive IL-22 production by naive CD4$^+$ T cells stimulated with anti-CD3$^+$ anti-CD28–coated beads (Fig. 10 C). IL-23 priming of human skin LCs also significantly increased their ability to drive IL-17A production, but

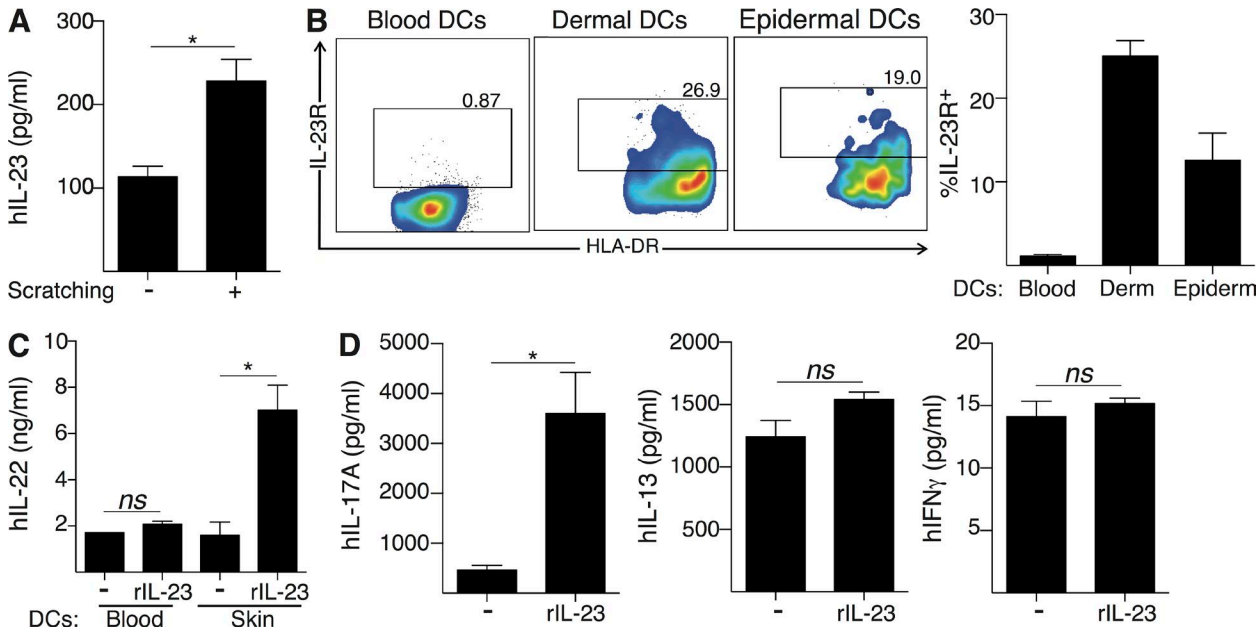

Figure 10. **IL-23 is released in human skin by scratching and polarizes human skin DCs to drive an IL-22 response.** (A) IL-23 release by explants of unmanipulated human skin and skin obtained 6 h after scratching from the same individual. (B) FACS analysis of IL-23R expression by adult human blood DCs, and foreskin dermal DCs and epidermal LCs. (C) Secretion of IL-22, by allogeneic human naive $CD4^+$ cells stimulated with anti-CD3– and anti-CD28–coated beads in the presence of rIL-23 pretreated and washed LCs from the skin or the blood of normal subjects. (D) Secretion of IL-13, IFN-γ, and IL-17A by allogeneic human naive $CD4^+$ cells stimulated with anti-CD3– + anti-CD28–coated beads in the presence of rIL-23 primed LCs from human skin. Results in A, C, and D represent three independent experiments. Results in B represent two independent experiments each, including two to three subjects. Columns and bars represent mean and SEM. *, $P < 0.05$; ns, not significant, two-tailed Student's *t* test.

had no detectable effect on their ability to drive IL-13 and IFN-γ production (Fig. 10 D).

DISCUSSION

We have demonstrated step-by-step the mechanism leading to epidermal thickening in a mouse model of AD elicited by application of antigen to skin mechanically injured by tape stripping. Endogenous TLR4 ligands released by mechanical skin injury cause keratinocytes to release IL-23, which drives migratory skin DCs to produce endogenous IL-23 and polarize naive antigen-specific T cells in skin DLNs. This polarization mount an IL-22 response to cutaneously introduced antigen that causes keratinocyte proliferation and epidermal thickening, hallmark features of AD.

EC sensitization by application of OVA antigen to tape-stripped mouse skin resulted in elevated serum IL-22 levels, IL-22 secretion by skin DLN cells in response to stimulation with OVA, and accumulation of $CD3^+CD4^+IL\text{-}22^+$ T cells in OVA-sensitized skin. Elevated serum IL-22 levels and increased numbers of $CD4^+IL\text{-}22^+$ T cells in the skin are features of AD (Nograles et al., 2009). Thus, our findings further extend the similarity between our EC sensitization model of allergic skin inflammation and AD.

We demonstrated that IL-22 is essential for epidermal thickening and keratinocyte proliferation in OVA-sensitized mouse skin, as both were absent in *Il22*$^{-/-}$ mice. In complementary experiments, we showed that keratinocyte proliferation and epidermal thickening were similarly induced in OVA-challenged skin of naive recipients of Th22-polarized $CD4^+$ cells. We also provided evidence that IL-22 and IL-17A play nonredundant roles in the epidermal thickening of OVA-sensitized skin. Together with our previous demonstration that TCRαβ cells are critical for skin inflammation and epidermal thickening in our model (Woodward et al., 2001), our results indicate that IL-22 produced by $CD4^+$ T cells is necessary for keratinocyte proliferation and epidermal thickening in EC-sensitized skin.

The IL-22 response to EC immunization was strictly dependent on IL-23. *Il23r*$^{-/-}$ mice EC sensitized with OVA failed to exhibit a rise in serum IL-22, cells from their skin DLN failed to secrete IL-22 in response to OVA stimulation, and there was no increase in epidermal thickness or keratinocyte proliferation in their OVA-sensitized skin. These findings are consistent with the central role IL-23 plays in driving the in vitro differentiation of naive $CD4^+$ T cells into IL-22–producing cells (Zheng et al., 2007). By using BM chimeric mice, we obtained evidence that IL-23 from nonhematopoietic and hematopoietic cell sources is required for the IL-22 response to EC immunization. Cells from the DLN of OVA-sensitized skin in both WT→*Il12p40*$^{-/-}$ and *Il12p40*$^{-/-}$→WT chimeras failed to secrete IL-22 in response to OVA stimulation. Consistent with their absent IL-22 response, the chimeras

exhibited no significant increase in epidermal thickness or keratinocyte proliferation in OVA-sensitized skin. Residual IL-23 in the chimeras may have been sufficient to normally amplify IL-17A production, explaining their intact IL-17A response to EC sensitization.

Tape stripping strongly up-regulated the expression of *Il23* mRNA and IL-23 in the epidermis. Keratinocytes were the major source of *Il23* mRNA, as there was no detectable contribution by epidermal LCs. There was a modest up-regulation of *Il23* mRNA in the dermis that could reflect the contribution of skin DCs. A key finding in our study is that endogenous TLR4 ligands are the major drivers of *Il23* up-regulation in tape-stripped skin. This was indicated by the observation that *Il23* mRNA expression in the skin after tape stripping was intact in GF mice, but severely impaired in TLR4- and MyD88-deficient mice. TLR4 ligands released after tissue injury include S100a8, S100a9, low MW HA, HMGN1, fibrinogen, oxidized-low density lipoproteins, several heat shock proteins, and β2-defensin, a product of epithelial cells, including keratinocytes (Yu et al., 2010). We demonstrated that tape stripping up-regulates *S100a8* and *S100a9* mRNA expression in mouse skin, and that i.d. injection of rS100a8 and low MW HA induced cutaneous *Il23* mRNA expression. Keratinocytes express functional TLR4 and secrete proinflammatory cytokines in response to TLR4 ligation (Song et al., 2002). We specifically demonstrated that the endogenous TLR4 ligand low MW HA induced *Il23p29* expression in the keratinocyte cell line Pam212, suggesting that endogenous TLR4 ligands induce IL-23 expression in keratinocytes after mechanical skin injury. The role of TLR4 in eliciting an IL-22 response to cutaneously introduced antigen was established by demonstrating that DCs derived from the DLN of OVA-exposed tape-stripped skin of TLR4-deficient mice were significantly impaired in their ability to express *Il23* mRNA and to drive IL-22 production by naive $CD4^+$ T cells. Furthermore, OVA-sensitized TLR4-deficient mice did not show a significant increase in serum IL-22, and their DLN cells were severely impaired in their ability to secrete IL-22 in response to OVA stimulation. The capacity of migratory of skin DCs from TLR4-deficient mice to support IL-17A production and the IL-17A response of these mice to EC sensitization, were decreased, albeit not significantly, compared with WT controls. These results are consistent with the observation that skin migratory DCs from OVA-sensitized skin of TLR4-deficient mice expressed normal mRNA levels of *Il6*, *Il1b*, and *Tnfa*, which are essential for Th17 polarization, but decreased levels of *Il23*, which amplifies IL-17A production by Th17 cells, but is essential for IL-22 production. Consistent with their impaired IL-22 response, TLR4-deficient mice demonstrated no significant epidermal thickening of OVA-sensitized skin. Thus, by inducing IL-23 expression in the skin, endogenous TLR4 ligands released after mechanical skin injury are essential for polarizing skin DCs to drive an IL-22 response to cutaneously introduced antigen.

We observed that the IL-23R is expressed on a subpopulation of DCs in the skin and skin DLN, but is not detectable on splenic DCs. IL-23 up-regulates the expression of its receptor (Ghoreschi et al., 2010); thus, constitutive expression of IL-23 in the skin may drive IL-23R expression by skin DCs. We further observed that rIL-23, as well as IL-23 released by explants from total and epidermal layer, but not nonepidermal layer, of tape-stripped mouse skin, polarized DCs from skin DLN in vitro to drive IL-22 production by naive $CD4^+$ T cells. Exogenous IL-23 was shown to induce endogenous expression of *Il23* mRNA expression and IL-23 secretion by DCs from skin DLN; this was critical for their ability to induce an IL-22 response by naive $CD4^+$ T cells in vitro. We used mice with selective deficiency in IL-23R expression in DCs to demonstrate that IL-23 directly acts on the IL-23R of skin DCs that capture antigen and migrate to DLN to up-regulate their endogenous IL-23 expression, causing them to drive IL-22 production by naive $CD4^+$ T cells. Overall, the data strongly suggest that IL-23 released by keratinocytes after mechanical injury primes $IL\text{-}23R^+$ skin-derived migratory DCs to express endogenous IL-23, which polarizes them to drive an IL-22 response to cutaneously introduced antigen. The priming action of keratinocyte-derived IL-23 on skin DCs to induce an IL-22 response parallels the priming action of keratinocyte-derived TSLP and IL-33 on DCs to induce a Th2 response (Soumelis et al., 2002; Leyva-Castillo et al., 2013; Tordesillas et al., 2014), indicating that keratinocyte release of cytokines is a primary early event in the adaptive immune response to cutaneous sensitization. Nociceptive sensory neurons are important for driving IL-23 production by skin DCs in a psoriasis model of skin inflammation elicited by application of the TLR7 ligand imiquimod, suggesting that signals from nonhematopoietic cells other than keratinocytes may also contribute to IL-23 production in skin DCs (Riol-Blanco et al., 2014).

Our findings support a paradigm shift in understanding the mechanisms that drive the IL-22 response to cutaneous sensitization in AD. We have identified a novel pathway in which IL-23 released by keratinocytes after mechanical skin injury targets skin DCs, up-regulating their endogenous IL-23 production and polarizing them to drive an IL-22 response in naive T cells, which in turn mediates epidermal thickening. The relevance of this pathway to the pathophysiology of AD in humans is supported by the observation that human keratinocytes express biologically active IL-23 (Piskin et al., 2006), and by our finding that scratching induces IL-23 expression in human skin, and that exogenous IL-23 polarizes human LCs to drive IL-22 production by naive $CD4^+$ T cells. Moreover, AD skin lesions demonstrate elevated levels of the endogenous TLR4 ligands IL-23 and IL-22 (Guttman-Yassky et al., 2008; Nograles et al., 2009; Gittler et al., 2012), and serum IL-22 levels are elevated in AD (Hayashida et al., 2011; Kanda and Watanabe, 2012). Our findings suggest that IL-23 and IL-22 blockade, which are currently being clinically tested, may prove useful in patients with AD.

MATERIALS AND METHODS

Mice

C57BL/6J WT, BALB/c WT, TLR4 mutant (C3HeJ), *Tlr4*$^{-/-}$, *Il18r*$^{-/-}$, *Il12p40*$^{-/-}$, *Tlr2*$^{-/-}$, *Tlr5*$^{-/-}$, *Il1r1*$^{-/-}$, *Il12p35*$^{-/-}$, *Langerin-eGFP-DTR*, *Cd11c-Cre*, and B6.SJL-Ptprc^{a}Pep3^{b}/BoyJ (OT-II) transgenic mice were purchased from the Jackson Laboratory. C3HeB/FeJ mice were purchased from Taconic. DO11.10 TCR transgenic mice were purchased from Charles River Laboratory. *Myd88*$^{-/-}$, *Trif*$^{-/-}$ (Hise et al., 2007), *Il22*$^{-/-}$ (Zheng et al., 2007), *Il23r*$^{-/-}$ (Chan et al., 2006), *Unc93b1*$^{-/-}$ (Kim et al., 2008; provided by H. Ploegh, Whitehead Institute for Biomedical Research, Cambridge, MA), *Il23p19*$^{-/-}$ (Edgerton et al., 2009; provided by M. Oukka, Center of Immunity and Immunotherapies, Seattle Children's Research Institute, Seattle, WA), and *Il1rl1*$^{-/-}$ mice (Barlow et al., 2013) were previously described. All mice were kept in a pathogen-free environment and fed an OVA-free diet. GF mice were bred at the gnotobiotic Research Animal Facility at The University of Chicago. All procedures performed on the mice were in accordance with the Animal Care and Use Committee of the Children's Hospital Boston.

Il23r$^{flox/flox}$ mice

Conditional *Il23r*$^{flox/flox}$ mice have been generated using 129SV ES cells in collaboration with genOway (Lyon, France). The proximal loxP site was inserted together with a neomycin selection cassette flanked by FRT sites within intron 3 of the *Il23r* gene, the distal loxP site was introduced within intron 4. The resultant mouse line was bred to a FLP deleter-line, which ubiquitously expresses Flp-recombinase to excise the neomycin selection cassette. In the final *Il23r*$^{flox/flox}$ line, the 1.1-kb region harboring exon 4 was flanked by two loxP sites. After backcrossing onto a C57BL/6 background for 10 generations, mice were bred to *Cd11c-Cre*^{Tg} mice to achieve the DC-specific knockout. Conditional deletion of the allele using Cre-recombinase results in deletion of the exon 4 encoding for part of the first fibronectin domain, resulting in frameshift in the open reading frame.

Flow cytometry

Cells were preincubated with FcγR-specific blocking mAb (2.4G2; eBioscience) and washed before staining with the following mAbs: PE-anti-CD45.1 (A20) and FITC-anti-CD45.2 (104) from Biolegend; APC-anti-CD45 (2D1), PerCP-eFluor-780-anti-MHC-II (AF6-120.1), APC-eFluor-780-anti-CD11C (N418), PE-Cy7-anti-CD4 (GK1.5), eFluor-450-anti-CD3 (17A2), APCeFluor780-anti-CD90 (53–2.1), and PE-anti-IL-22 (1H8PWSR) from eBioscience; and APC anti-IL-23R (753317) from R&D Systems. For ILC staining, biotin-anti-CD11b (M1/70), biotin-anti-CD11c (N418), biotin-anti-F4-80 (BM8), biotin-anti-B220 (RA3-6B2), biotin-anti-CD19 (1D3), biotin-anti-FcεR1α (MAR-1), biotin-anti-CD49b (DX5), and biotin-anti-Gr1 (RB6-8C5; all from eBioscience), followed by Streptavidin-BV605 (BD), were used as linega gate. ILCs were defined as CD45^{+}CD3^{-}Lin^{-} cells.

Intracellular staining of IL-22 was performed according to the manufacturer's instructions (eBioscience). Human blood DCs were stained using anti–human mAbs to HLA-DR (eBioscience), CD11c (eBioscience), and IL-23R (R&D Systems). Live cells were pregated by Fixable Viability Dye eFluor-506 (eBioscience). Cells were analyzed on LSR-Fortessa (BD), and the data were analyzed with FlowJo software.

Preparation of epidermal and dermal sheets of mice

Ears from mice were split into dorsal and ventral halves, cartilage and s.c. tissue were removed, and skin was floated on 3.8% ammonium thiocyanate (Sigma-Aldrich) in 100 mM sodium phosphate/100 mM potassium phosphate for 20 min at 37°C.

EC sensitization

6–8-wk-old-female mice were epicutaneously sensitized for 7 wk, as described previously (He et al., 2007). In brief, EC sensitization consists of three 1-wk cycles of tape stripping. For each cycle, 6–8-wk-old female mice were anesthetized, and then their back skin was shaved and tape-stripped with a film dressing (TegadermTM; 3M). 2 wk rest intervals were observed between the cycles. Epicutaneous sensitization consisted of applying a 1 cm^2 gauze containing 100 µg OVA (Sigma-Aldrich) to the skin after each tape stripping and securing it with a film dressing. Analyses were done at day 49.

Adoptive transfer of in vitro–polarized DO11.10 CD4^{+} T cells and skin challenge

Naive CD4^{+} T cells from spleens of DO11.10 TCR transgenic mice were magnetically sorted using naive CD4^{+} T cell isolation kit, mouse (Miltenyi Biotec). Next, these cells were cultured in plate-bound antibodies against CD3 (145-2C11; 2 µg/ml; eBioscience) and soluble anti-CD28 (PV-1; 2 µg/ml; BioLegend), in the presence of IL-23 (40 ng/ml; R&D Systems), anti–IL-4 (11B11; 10 µg/ml; BioLegend), anti–IFN-γ (XMG1.2; 10 µg/ml; BioLegend), and anti–TGF-β1,2,3 (1D11; 10 µg/ml; R&D Systems) neutralizing antibodies to generate Th22 cells for 5 d. After extensive washings, 5 × 10^6 polarized T cells were adoptively transferred i.v. to naive WT or *Il22*$^{-/-}$ recipients, which were challenged the same day by EC sensitization with OVA (100 µg/100 µl PBS). For in vivo IL-17A neutralization, anti–IL-17A (17F3; 500 µg/ml; BioXCell) neutralizing antibodies or Control IgG1 (MOPC-21; 500 µg/ml; BioXCell) was i.p. injected 1 h before and 48 h after OVA challenge. 7 d later, the challenged skins were examined by histology and immunohistochemistry.

Tape stripping of mouse skin and quantitative RT-PCR

Ears or shaved back skin of anesthetized mice were tape stripped six and eight times, respectively. For blockade of endogenous IL-18 and ST2, anti–IL-18 (93-10C; 100 µg/mouse; R&D Systems), and anti-ST2 (DJ8; 100 µg/mouse; MD Bioproducts) antibodies or control rat IgG1 antibody were i.p. injected

1 h before tape stripping. 6 h after tape stripping, total skin RNA was extracted in the lysis buffer solution provided in the RNAqueous extraction kit (Ambion). Reverse transcription was performed with an iScript cDNA synthesis kit (Bio-Rad Laboratories). PCR reactions were run on an ABI Prism 7300 (Applied Biosystems) sequence detection system platform. TaqMan primers and probes were obtained from Life technologies. The housekeeping gene β_2-microglobulin was used as a control. Relative mRNA expression was quantified using the $2^{-\Delta\Delta Ct}$ method, as previously described (He et al., 2007).

In vivo depletion of Langerin$^+$ cells

Langerin-eGFP-DTR mice were injected i.p. with DT (1 μg in 200 μl saline; List Biological Laboratories). 24 h after DT injection, epidermis and dermis were separated by treatment of dispase II (1 mg/ml; Gibco) overnight at 4°C, and LC depletion in the epidermis was measured by FACS analysis.

Ear excision and PTX treatment

Ears were excised 5 h after tape stripping and OVA immunization. PTX (0.5 μg in 50 μl saline; Sigma-Aldrich) was injected i.d. for 4 consecutive days before tape stripping and immunization with OVA on the same skin area.

Intradermal injection of HA and rS100a8

Low endotoxin, select-HA (25 μg in 50 μl PBS, <0.1 EU/mg endotoxin; Sigma-Aldrich), rS100a8 (1 μg in 50 μl PBS; Vandal et al., 2003), or PBS as a control was i.d. injected into shaved back skin of WT or TLR4 mutant mice. 3 h after injection, skin samples were harvested for quantitative RT-PCR analysis.

Histology and immunohistochemistry

Skin specimens were fixed in 10% buffered formalin and embedded in paraffin. Multiple 4-μm sections of skin were stained with hematoxylin and eosin (H&E) by Histo-Scientific Research Laboratories. Fixed sections were stained with rabbit anti–IL-23p19 antibody (3795; ProSci incorporated) or rabbit anti-Ki67 antibody (NB110-89719; Novus Biologicals).

Cell culture and in vitro cytokine expression

Single-cell suspensions of skin DLN (axillary and inguinal LN) were cultured in complete RPMI 1640 (JRH Biosciences) supplemented by 10% FCS, 1 mmol/liter sodium pyruvate, 2 mmol/liter L-glutamine, 0.05 mmol/liter 2-mercaptoethanol, 100 U/ml penicillin, and 1 mg/ml streptomycin at 4–8 × 10^6/ml in 24-well plates for 4–5 d in the presence of OVA (100 μg/ml). After 96 h of culture, cytokine levels (IL-17A, IL-22, IL-13, and IFN-γ) of cell-free supernatants were determined by ELISA according to the manufacturer's instructions (eBioscience). Tape-stripped skin explants (1 cm^2) were chopped and cultured in complete RPMI. The supernatant (or skin explant conditioned medium; SCM) was harvested after 18 h IL-23 was measured in the SCM by means of ELISA kit according to the manufacturer's instructions (sensitivity, 8 pg/ml; eBioscience). Pam212 cells, mouse keratinocyte cell line, were preincubated with inhibitors for NFKβ (BAY-11-7082), MAPK (SB 203580), ERK (U-0126), or JNK (SP600125), and then activated with low endotoxin, select-HA (10 μg/ml, <0.1 EU/mg endotoxin; Sigma-Aldrich) or PBS as control for 6h. Total RNA was extracted in the lysis buffer solution provided in the RNAqueous extraction kit (Ambion). Reverse transcription was performed with an iScript cDNA synthesis kit (Bio-Rad Laboratories). PCR reactions were run on an ABI Prism 7300 (Applied Biosystems) sequence detection system platform. TaqMan primers and probes were obtained from Life Technologies. The housekeeping gene β_2-microglobulin was used as a control. Relative mRNA expression was quantified using the $2^{-\Delta\Delta Ct}$ method, as previously described (He et al., 2007).

Serum IL-22 levels

Mouse serum IL-22 was determined using quantikine ELISA kit (sensitivity, 8.2 pg/ml; R&D Systems).

Isolation and functional analysis of mouse skin and skin DLN DCs

CD11c$^+$ DCs of mouse skin and skin DLN (axillary and inguinal) were purified as described previously (Oyoshi et al., 2010). DCs were primed with SCM or recombinant IL-23 (20 ng/ml; R&D Systems) for 24 h. After extensive washing, DCs were co-cultured with naive CD4$^+$CD62L$^+$ T cells isolated by using naive CD4 T cell isolation kit II (Miltenyi Biotec) in the presence of $OVA_{323–339}$ peptide (4 μM; AnaSpec Inc.) for 4–5 d. In some experiments, neutralizing antibody goat anti–mouse IL-23 (He et al., 2007) was used at 10 μg/ml. For in vivo DC priming experiment, OVA (1 mg in 100 μl saline) or saline was epicutaneously applied to shaved, tape-stripped skin of WT, Tlr4$^{-/-}$, and *Il23r*$^{-/-}$ mice. 24 h after sensitization, DCs were isolated from axillary and inguinal LNs and naive CD4$^+$ T cells were purified from spleen of OT-II mice. DLN CD11c$^+$ cells (10^5) were co-cultured in duplicates with OT-II CD4$^+$ T cells (10^5) without addition of exogenous OVA protein for 5 d. Cell-free supernatant was used to detect mouse IL-22, IL-17, IL-13, and IFN-γ with ELISA Ready SET Go kits (eBioscience) with sensitivity of 8, 4, 4, and 15 pg/ml, respectively.

Generation of BM chimeras

8-wk-old recipient CD45.2$^+$ WT and *Il12p40*$^{-/-}$ mice were lethally irradiated (1,200 rad delivered in two doses of 600 rad each at 3-h intervals), and injected i.v. with 5 × 10^6 BM cells obtained from congenic CD45.1$^+$ WT mice and vice versa. Chimerism was assessed by measuring the percentages of donor cells in the chimeric mice blood 8 wk after BM reconstitution.

IL-23 release after scratching of normal human skin

After obtaining informed consent, the inner side of the forearm of healthy nonallergic adult subjects was scratched 30 times with a #11 sterile blade with care not to draw any

blood. 6 h later, a 4-mm punch biopsy was obtained from the scratched site and another one from a skin site on the contralateral forearm. The skin sample was chopped with scissors and cultured in complete RPMI medium for 18 h and IL-23 protein released in the supernatants was measured by ELISA (eBioscience).

Isolation and functional analysis of human skin DCs

Normal human skin samples were obtained as the discarded products of dermatologic surgery. Subcutaneous fat was removed, and remaining tissue was washed with PBS. The dermal layer was scratched as described above and incubated with Dispase II (1 mg/ml; Gibco) overnight at 37°C. Epidermis and dermis were separated with forceps. Epidermal sheets were chopped with scissors and incubated with Liberase (0.3 mg/ml; Roche) at 37°C for 1.5 h LCs were isolated from epidermal cell suspension by using a CD1a isolation kit (Miltenyi Biotec). Blood DCs were isolated by using Blood Dendritic Cell Isolation kit II (Miltenyi Biotec). DCs were primed with recombinant IL-23 (100 ng/ml; R&D Systems) for 24 h. After extensive washing, DCs were co-cultured with allogeneic naive CD4 T cells (Miltenyi Biotec) in the presence of anti-CD3/CD28 beads (Miltenyi Biotec) for 5 d. Cell-free supernatants were used to detect IL-22, IL-17A, IL-13, and IFN-γ by ELISA kit (eBioscience).

Statistical analysis

Statistical significance was determined by the two-tailed Student's *t* test. $P < 0.05$ was considered statistically significant.

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