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Psoriasiform dermatitis is driven by IL-36–mediated DC-keratinocyte crosstalk

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Psoriasis is a chronic inflammatory disorder of the skin affecting approximately 2% of the world's population. Accumulating evidence has revealed that the IL-23/IL-17/IL-22 pathway is key for development of skin immunopathology. However, the role of keratinocytes and their crosstalk with immune cells at the onset of disease remains poorly understood. Here, we show that IL-36R–deficient (_Il36r_$^{-/-}$) mice were protected from imiquimod-induced expansion of dermal IL-17–producing γδ T cells and psoriasiform dermatitis. Furthermore, IL-36R antagonist-deficient (_Il36rn_$^{-/-}$) mice showed exacerbated pathology. TLR7 ligation on DCs induced IL-36–mediated crosstalk with keratinocytes and dermal mesenchymal cells that was crucial for control of the pathological IL-23/IL-17/IL-22 axis and disease development. Notably, mice lacking IL-23, IL-17, or IL-22 were less well protected from disease compared with _Il36r_$^{-/-}$ mice, indicating an additional distinct activity of IL-36 beyond induction of the pathological IL-23 axis. Moreover, while the absence of IL-1R1 prevented neutrophil infiltration, it did not protect from acanthosis and hyperkeratosis, demonstrating that neutrophils are dispensable for disease manifestation. These results highlight a central and unique IL-1–independent role for IL-36 in control of the IL-23/IL-17/IL-22 pathway and development of psoriasiform dermatitis.

Introduction

Psoriasis is the most common chronic inflammatory skin disease, which is characterized by epidermal hyperplasia (acanthosis) due to hyperproliferation and impaired differentiation of keratinocytes, scaling, and erythematous plaque formation, eventually resulting in loss of the protective skin barrier (1, 2). Another defining histological feature is an inflammatory infiltrate, consisting of neutrophils and mononuclear cells in the dermis and epidermis. However, little is known about the molecular mechanisms controlling the initial stages of psoriasis. According to the current understanding, a cross talk between skin-resident DCs and T cells, infiltrating macrophages and neutrophils, and keratinocytes through inflammatory mediators is thought to be responsible for the aberrant keratinocyte proliferation and differentiation and disease development. The precipitating event is the activation of DCs through TLR ligation by microbes or other environmental factors and the release of IL-12 and IL-23 that drive polarization into effector T cells secreting TNF-α, IFN-γ, IL-17, and/or IL-22. These cytokines cooperatively stimulate keratinocytes to produce a variety of growth factors and inflammatory mediators, ultimately fuelling the vicious cycle of cutaneous pathology (3).

Despite the inherent differences between human and mouse skin, substantial insights into the underlying mechanisms of skin inflammation have been gained using xenotransplantation of human psoriatic skin to immunodeficient mice (4, 5) or several mouse models of psoriasis-like disorders. The latter include T cell transfer into CB17 SCID/SCID mice (6); transgenic overexpression of several cytokines, such as IL-12p40 (7), IL-1α (8), and TGF-β (9), specifically in keratinocytes; dermal injection of IL-23 (10); and treatment with the TLR7 agonist imiquimod (IMQ) (11), which is clinically relevant as it is applied topically as a drug against skin cancer and known to induce psoriasis in susceptible patients (12, 13). Together, these models revealed that the cytokines IL-23, IL-17, and IL-22 (also termed the IL-23/IL-17/IL-22 axis) are key mediators of psoriasiform disease (6, 7, 10, 11, 14–16), with IL-23 secreted by some sort of DC which is responsible for development of pathological IL-17A– and IL-22–producing T cells. Notably, besides Th17 cells, IL-17 and IL-22 can be produced by γδ T cells (17, 18) and NKT cells (19), which all require IL-1 together with IL-23 for expansion (20–22). An important role of IL-23 and IL-17 in human psoriasis has become evident from genome-wide studies and the efficacy of biologics targeting these cytokines (23–26). Despite the well-accepted relevance of the IL-23/IL-17/IL-22 axis, no study has directly compared the individual knockout mice side by side in a psoriasis model to understand their relative contribution and relevance for disease development.

IL-36α, IL-36β, and IL-36γ (originally named IL-1F6, IL-1F8, and IL-1F9, respectively) are novel members of the IL-1 family of cytokines. Each binds specifically to the IL-36 receptor (IL-36R; also named IL-1Rrp2 or IL-1RL2), which leads to the recruitment of IL-1RAcP and activation of NF-κB. An IL-36 receptor antagonist (IL-36RN), also termed IL-1F5, inhibits signaling by binding to IL-36R (27, 28). Accumulating evidence implicates IL-36 cytokines in the development of psoriasis. Increased levels of IL-36 cytokines and IL-36R were observed in lesions of human psoriatic skin and confirmed in mouse models of psoriasis-like diseases (28–30). IL-36α overexpression in keratinocytes of K14-IL-36α transgenic mice was reported to result in a transient inflammatory skin disorder at birth that waned after 2 to 3 weeks of age (31) but rendered mice highly susceptible to 12-O-tetrade-
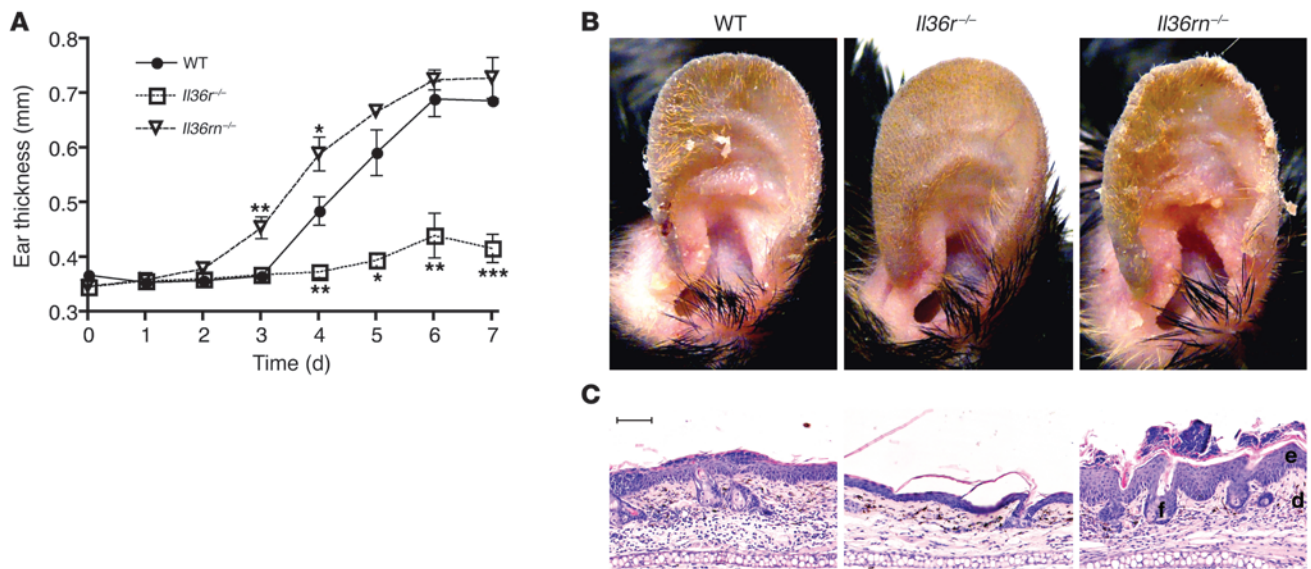
Figure 1
IL-36R and IL-36R antagonist play critical roles in the development of IMQ-induced psoriasiform dermatitis. Ears of both *Il36r*^{−/−} and *Il36rn*^{−/−} mice and WT mice (*n* ≥ 4 per group) were topically treated with an IMQ-containing cream (Aldara, MEDA Pharma) for 7 consecutive days. (**A**) Ear swelling was measured daily before treatment. Values show averages ± SEM. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. (**B**) At day 7, photographs were taken from whole ears or (**C**) H&E-stained ear sections representing the thickest epidermal region of each ear from individual mice representative of indicated groups. Epidermis (e), dermis (d), and hair follicle (f) are indicated. Data are representative of 4 experiments. Scale bar: 100 μm.

canoylphorbol-13-acetate and induced skin pathology reminiscent of human psoriasis (32). Moreover, removal of the *IL36RN* gene in K14-IL-36α transgenic mice induced chronic and aggravated skin abnormalities, and mutations in *IL36RN* were recently described in patients with pustular psoriasis (33, 34). IL-36 expression in keratinocytes was enhanced by IL-1α, TNF-α, and IL-17 and downregulated by neutralizing IL-22 (29). IL-17 induced IL-36 agonists more potently in human psoriasis-derived keratinocytes than in healthy keratinocytes, while *Il36rn* expression remained unaffected (35). Furthermore, IL-36 was able to induce its own expression as well as expression of various proinflammatory cytokines and augmented IL-17–mediated production of antibacterial peptides (29, 36).

IL-36 is thought to act mainly in an autocrine fashion on skin-resident cells like keratinocytes and fibroblasts, which express high amounts of IL-36 and IL-36R. However, lower levels of IL-36R are also expressed on bone marrow–derived DCs (BMDCs) and CD4+ T cells. Consistently, when treated with superactive forms of IL-36, DCs upregulated CD80, CD86, and MHC class II and produced proinflammatory cytokines like IL-12, IL-1β, TNF-α, and IL-23 (37).

Clearly, several feedback loops exist in the regulatory circuits of psoriasis. But the individual contributions of the different cytokines and their relative impact on skin pathology are not well understood. In this work, we show that IL-36 cytokines are master regulators of the IL-23/IL-17/IL-22 pathway and development of cutaneous pathology in response to IMQ.

Results

IL-36R is essential for the development of IMQ-induced psoriasis. We investigated the development of IMQ-induced psoriasiform dermatitis in IL-36R–deficient (*Il36r*^{−/−}) mice by topical treatment of ear skin with an IMQ-containing cream (Aldara). As shown

in Figure 1, *Il36r*^{−/−} mice were protected from the pathological manifestations of psoriasis, including ear swelling, acanthosis, erythema, skin flaking, and hyperkeratosis. To assess the role of IL-36RN in this disease model, we generated IL-36R antagonist-deficient (*Il36rn*^{−/−}) mice (Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI63451DS1) and treated them with Aldara. Consistent with the results above, mice lacking IL-36RN showed increased severity of key disease features (Figure 1).

IL-36 regulates the recruitment of inflammatory cells and the expansion of IL-17A–producing γδ T cells in the skin. To further characterize the cellular and molecular pathways affected by IL-36, we characterized and quantitated the inflammatory ear infiltrate by flow cytometry. The lesions of WT mice showed a remarkable infiltration of CD45+ leucocytes, consisting mainly of neutrophils and macrophages, which was absent in *Il36r*^{−/−} mice and 3-fold enhanced in *Il36rn*^{−/−} mice compared with that in WT mice (Figure 2, A–D). γδ T cells showed a much more pronounced expansion than αβ T cells and accounted for the majority of T cells in the affected ear during the course of disease in WT mice (Figure 2, E and F). The expanded γδ T cells were mainly composed of dermal γδ TCR^{lo} cells, but not γδ TCR[hi] dendritic epidermal T cells, and the former were by far the most prominent IL-17A producers (Figure 2I). Notably, γδ T cells and consequently IL-17A–producing cells failed to expand in *Il36r*^{−/−} mice (Figure 2, E, G, and H), while αβ T cells remained virtually unaffected by the loss of IL-36R (Figure 2F). Characterization of the few IL-17A producers in *Il36r*^{−/−} mice showed that they were also mainly composed of γδ T cells (Figure 2J), indicating that absence of IL-36R signaling affected primarily the expansion of γδ T cells and to a lesser extent their propensity to produce IL-17A (Figure 2K). *Il36rn*^{−/−} mice showed no differences in expansion of γδ T cells and IL-17A–producing cells. The changes observed in the skin infiltrates were partially
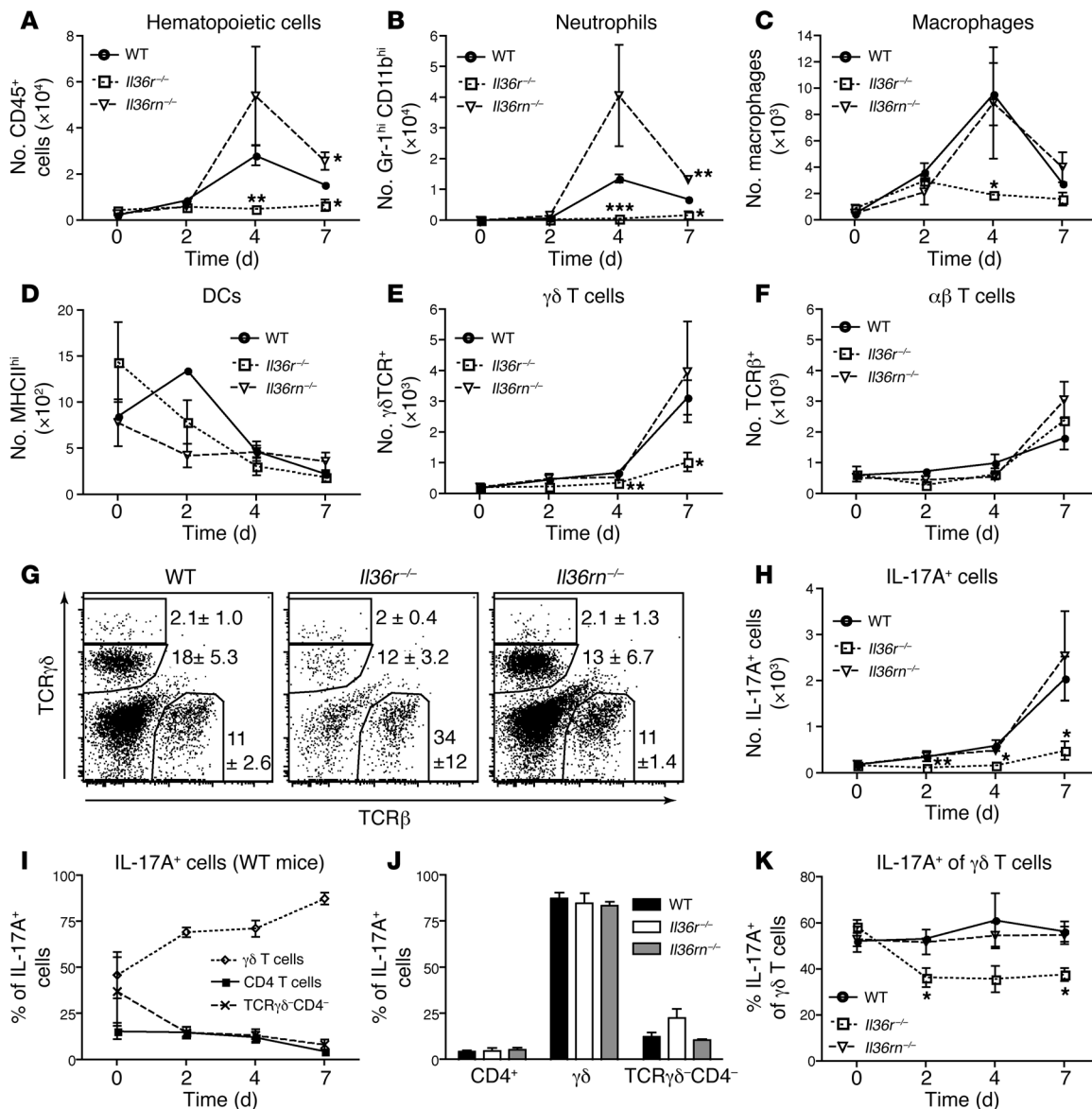
Figure 2
IL-36 regulates the recruitment of inflammatory cells and the expansion of IL-17A–producing γδ T cells in the skin. Mice were treated as described in the legend to Figure 1. At days indicated, mice (*n* = 3/group) were sacrificed and immune cells in the ear were characterized by flow cytometry. Graphs show total numbers (± SEM) of (**A**) CD45+ hematopoietic cells, (**B**) Gr-1hiCD11b+ neutrophils, (**C**) CD11b+MHCII–/lo macrophages, (**D**) MHCIIhi DCs, (**E**) γδ T cells, and (**F**) αβ T cells per ear. (**G**) Dot plots show expression of TCRγδ and TCRαβ on CD45+ cells. The TCRγδhi population represents dendritic epidermal T cells. Values represent average percentages ± SD of the gated populations from groups of mice. (**H**) Total numbers of IL-17A–producing cells in the ears at indicated days. (**I**) Characterization of cell types producing IL-17A in WT mice throughout the course of IMQ treatment. (**J**) Percentages of CD4+ T cells, γδ T cells, and CD4–γδ– cells among IL-17A producers in ears of indicated groups of mice at day 7 after treatment. (**K**) Percentages of IL-17A producers among γδ T cells in ears of indicated groups of mice. Data are representative of 4 independent experiments. (**H–K**) Values show averages ± SEM. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

mirrored in the draining lymph nodes (dLNs). During the course of disease, we observed a reduction in cellularity and frequencies of neutrophils and macrophages in *Il36r*–/– mice, albeit this decrease was not as strong as that in the ears (Supplemental Figure 2, A–C). Similar to the skin, γδ but not αβ T cells expanded vigorously and were the main producers of IL-17A (Supplemental Figure 2, D–G). However, IL-17A–producing γδ T cells were only slightly reduced in dLNs of *Il36r*–/– mice (Supplemental Figure 2, D and F). These results demonstrate that IL-17 is primarily derived from dermal γδ

T cells, in agreement with recent reports (38, 39), and that IL-36R is indispensable for expansion of pathogenic IL-17–producing γδ T cells and development of skin disease.

IL-36R on radioresistant skin-resident cells is crucial for psoriasis. IL-36R is predominantly expressed in epithelial tissues, including the skin (36), but it has also been detected in DCs and T cells (37). To distinguish the importance of IL-36R on nonhematopoietic skin-resident cells compared with that on radiosensitive hematopoietic cells in disease development, we generated recipro-
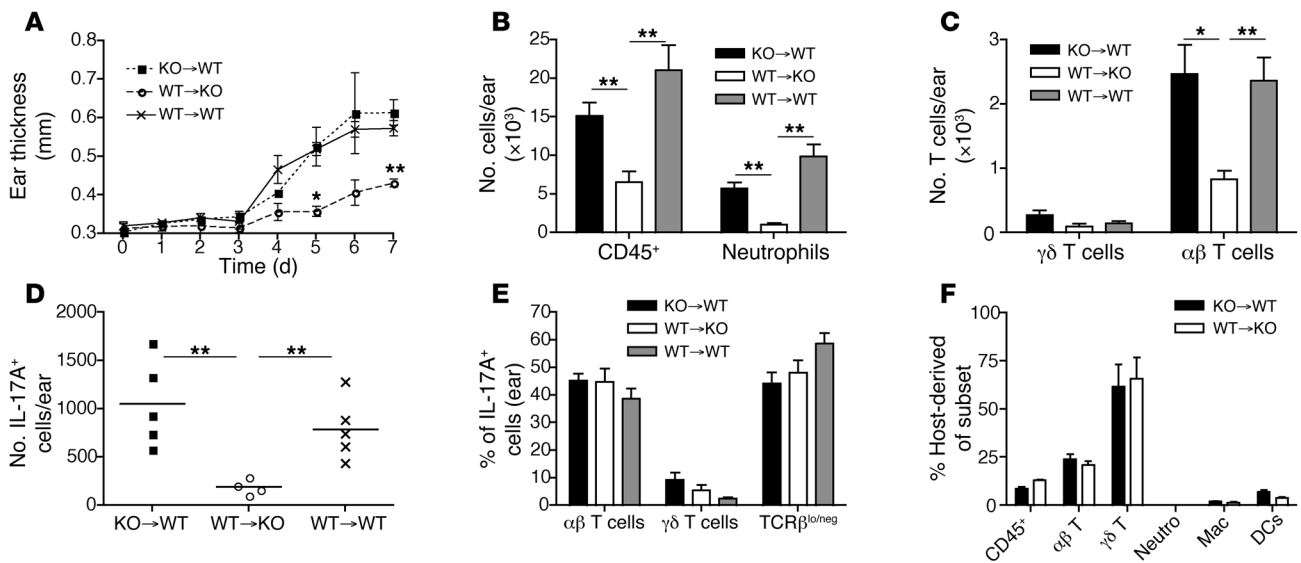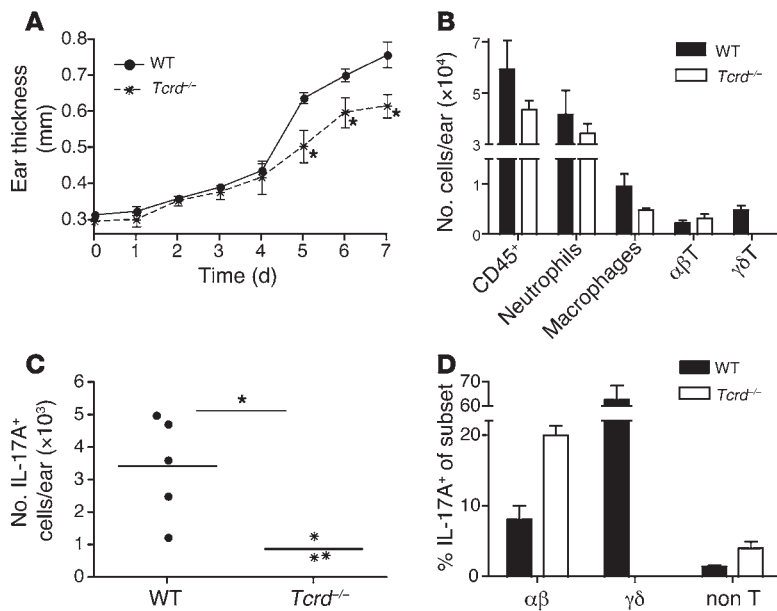
Figure 3
IL-36R on radioresistant resident cells is crucial for the expression of IL-17A in the skin and development of IMQ-induced skin inflammation. Reconstituted bone marrow chimeras, *Il36r*−/− CD45.2 BM → WT CD45.1 mice (KO→WT), WT CD45.1 BM → *Il36r*−/− CD45.2 mice (WT→KO), and WT CD45.1 BM → WT CD45.1 mice (WT→WT), were treated with Aldara cream, as described in the legend to Figure 1. (**A**) Ear thickness was monitored daily. (**B**–**F**) At day 7, cell populations in the ear were characterized by flow cytometry. Total numbers of (**B**) CD45+ hematopoietic cells and neutrophils, (**C**) γδ and αβ T cells, and (**D**) IL-17A–producing cells in ears of indicated chimeras are shown. (**E**) Characterization of IL-17A+ cells. (**F**) Percentage of host-derived remaining hematopoietic cell populations that survived the irradiation and were not replaced. (**A**–**C**, **E**, and **F**) Values indicate averages ± SEM of groups. (**D**) Symbols represent individual mice, and horizontal lines indicate averages of groups. *$P < 0.05$; **$P < 0.01$.

cal bone marrow chimeras. Lethally irradiated C57BL/6 mice were reconstituted with *Il36r*−/− bone marrow (CD45.2+ KO → CD45.1+ WT) and vice versa (CD45.1+ WT → CD45.2+ KO). CD45.1+ WT → CD45.1+ WT chimeras served as controls. We found that the expression of IL-36R on radioresistant skin-resident cells, but not on hematopoietic bone marrow–derived cells, was essential for pathology upon IMQ treatment (Figure 3A). Accordingly, the swelling and the cutaneous inflammatory response, in particular neutrophil recruitment, were impaired in the absence of IL-36R on skin-resident cells, while mice lacking IL-36R on radiosensitive leukocytes showed swelling and an inflammatory response analogous to that of WT → WT mice (Figure 3, A and B). Similarly, recruitment of neutrophils into dLNs was affected in the absence of IL-36R on nonhematopoietic skin-resident cells (data not shown). Detailed analysis of T cell populations in the ears showed an up to 90% drop in numbers of dermal γδ T cells in all 3 groups of chimeras when compared with those in nonirradiated mice (Figure 2, E and G, and Figure 3C). This indicates that in nonirradiated WT mice a fetal-derived radiosensitive γδ T cell subset expanded in response to IMQ and is responsible for the IL-17 production. Interestingly, the αβ T cell expansion observed in the irradiated chimeras was dependent on IL-36R expression by skin-resident cells (Figure 3C), while it was independent of IL-36R in nonirradiated mice (Figure 2F). In addition, IL-17A production in the skin lesions of the WT → WT chimeras was found in TCRβhi and TCRβlo αβ T cells, which were reduced in WT → KO chimeras but not in KO → WT chimeras (Figure 3, D and E). Staining of CD45.1 (WT) and CD45.2 (KO) cells enabled us to verify that most (>90%) of the CD45+ cells in ear skin of the chimeras originated from donor bone marrows (Figure 3F). Interestingly, the few γδ T cells present in the skin of chimeras were derived

primarily from the host, but they played a minor role, as their numbers were limited relative to IL-17A–producing αβ T cells. Together, these results demonstrate that the expression of IL-36R by skin-resident cells, such as keratinocytes and fibroblasts, is pivotal for the cutaneous pathology.

IL-17+ αβ T cells can partially mediate psoriasiform disease in the absence of IL-17+ γδ T cells. Previous reports suggested that IL-23 promotes the expansion of pathogenic IL-17–producing γδ T cells (38, 39). Surprisingly, we found that in *Tcrd*−/− mice ear swelling was only moderately inhibited and that the inflammatory infiltrate was insignificantly reduced (Figure 4, A and B), despite a substantial decrease in the number of IL-17A–producing cells (Figure 4C). Notably, αβ T cells and a minor population of non-T cells accounted for the remaining IL-17A production in *Tcrd*−/− mice (Figure 4D), corroborating data in WT → WT chimeras, which lacked dermal γδ T cells. Taken together, our results suggest that IL-17–producing αβ T cells (Th17 cells) can partially compensate for the absence of dermal γδ T cells as mediators of cutaneous pathology.

IL-36 mediates cutaneous pathology mainly but not only by induction of the IL-23/IL-17/IL-22 pathway. In the light of the above results, we reassessed the roles of the IL-23/IL-17/IL-22 pathway by comparing the respective knockouts with each other and with *Il36r*−/− mice in the IMQ model. Expectedly, both *Il23a*−/− and *Il17a*−/− mice showed impaired disease development, although the *Il23a*−/− mice were better protected. Skin flaking and erythema were visibly reduced in *Il17a*−/− mice and almost undetectable in *Il23a*−/− mice (data not shown). Accordingly, *Il17a*−/− and, in particular, *Il23a*−/− mice showed reduced skin swelling compared with that of WT mice (Figure 5A). Cellular analysis revealed that neutrophilia in the ears was only partially dependent on IL-17A

Figure 4
IL-17A production and skin disease can be partially mediated by αβ T cells in γδ T cell–deficient mice. Mouse ears were treated with Aldara as described above. (**A**) Ear thickness was monitored daily. Values show averages ± SEM ($n \geq 3$). (**B–D**) At day 7, cell populations in the ear were characterized by flow cytometry. (**B**) Total numbers of indicated populations and (**C**) IL-17A–producing cells per ear are shown. Symbols represent individual mice, and horizontal lines indicate averages of groups. Values indicate averages ± SEM. (**D**) Percentages of αβ T cells, γδ T cells, and non–T cells among IL-17A–producing cells in ears. *$P < 0.05$.

expression, while it was completely abrogated in the absence of IL-23 (Figure 5B). IL-23 deficiency almost completely prevented the expansion of γδ T cells, including the IL-17A producers (Figure 5, C and D). The few remaining γδ T cells showed a reduced propensity to produce IL-17A (Figure 5E). In addition, IL-17A–producing αβ T cells were reduced in the skin and dLNs of $Il23a^{-/-}$ mice (Figure 5, E and F). We reasoned that the remaining neutrophil influx and skin swelling observed in $Il17a^{-/-}$ mice was due to the presence of other pathogenic effector cytokines such as IL-22. Indeed, ear thickness and skin neutrophilia were similarly reduced in $Il22^{-/-}$ and $Il17a^{-/-}$ mice (Figure 5, G and H), suggesting similar effector activities elicited by the 2 cytokines. The number of IL-17A–producing cells was slightly increased in $Il22^{-/-}$ mice compared with that in WT mice, possibly indicating some compensatory mechanisms (Figure 5I). Regardless, skin pathology was more severe in $Il17a^{-/-}$ and $Il22^{-/-}$ mice than in $Il36r^{-/-}$ mice. To assess the combined effects of IL-17 and IL-22, we inhibited IL-22 in $Il17a^{-/-}$ mice using neutralizing antibodies (Figure 5, J–L). In this case, most effector functions and pathological manifestations of the disease were gone. Skin swelling was similarly reduced in $Il22^{-/-}$ mice and WT mice treated with anti–IL-22 mAb, confirming efficient neutralization of IL-22 in the latter (data not shown). Nevertheless, in comparison with $Il36r^{-/-}$ mice, residual ear swelling could be observed (Figure 5J). Together, these experiments suggest that IL-36 drives skin lesion development mainly by induction of the IL-23/IL-17/IL-22 axis, but it can promote skin thickening to a minor extent in the absence of these cytokines.

IL-36 promotes IL-17A production in the skin and IMQ-induced psoriasis independently of IL-1R signaling. Considering that IL-23–mediated expansion of IL-17A–producing γδ T cells has been reported to depend on IL-1 (38), and IL-36α overexpression in the skin promotes expression of inflammatory mediators including IL-1α (31), we reasoned that IL-1 and IL-23 might be downstream of IL-36 and cooperate in IMQ-driven psoriasis. However, IL-1R1 deficiency did not protect mice from IMQ-induced psoriasiform dermatitis. Ear swelling, acanthosis, and skin flaking were comparable in $Il1r1^{-/-}$

and WT mice (Figure 6, A and B). Furthermore, IL-17A–producing T cells expanded normally in ears of $Il1r1^{-/-}$ mice (Figure 6, D–F). Nevertheless, recruitment of neutrophils was considerably reduced, although not to the level of that in $Il36r^{-/-}$ mice (Figure 6C). Thus, IL-1R signaling is important for neutrophil influx but redundant for the development of IL-17A–producing γδ T cells and the pathology of IMQ-induced psoriasiform dermatitis.

IL-36 promotes expression of itself along with chemokines and keratinocyte mitogens in dermal mesenchymal cells and keratinocytes. Since IL-36R was primarily required on skin-resident cells but not on hematopoietic cells for disease development (Figure 3), we wanted to analyze the direct effects of IL-36 and IMQ on nonhematopoietic skin-resident cells. Therefore, we sorted CD45⁻ cells from either epidermis or dermis. The majority of epidermal CD45⁻ cells are keratinocytes, whereas dermal CD45⁻ cells consist predominantly of mesenchymal cells (fibroblasts, adipocytes, and vascular cells). These cell populations were cultured with IMQ and superactive forms of IL-36β and IL-36γ (40). IL-36β potently induced expression of IL-36 cytokines, the neutrophil- and T cell–attracting chemokines CXCL1 and CCL20, and the keratinocyte mitogens G-CSF and TGF-α (Figure 7A). Similar results were obtained using IL-36γ for stimulation (data not shown). In contrast, IMQ showed no such activity (Figure 7A), consistent with the absence of TLR7 expression on CD45⁻ dermal mesenchymal cells and keratinocytes (Figure 7B).

DCs are crucial for the induction of IMQ-driven psoriasis. Considering the abundance of TLR7 expression on DCs and its absence on CD45⁻ keratinocytes and dermal mesenchymal cells (Figure 7B), we speculated that DCs are the main sensors of IMQ and producers of IL-23. To assess the role of DCs in the IMQ psoriasis model, we injected diphtheria toxin into CD11c-DTR mice, which effectively depleted all CD11c⁺ DCs until analysis of mice at day 4 after IMQ treatment (Figure 8B). Absence of DCs protected mice from ear swelling, neutrophil infiltration, T cell expansion, and development of IL-17–producing cells (Figure 8, A and C–E). Notably, stimulation of BMDCs with IMQ or IL-36β induced expression of $Il23a$ as well as $Il36a$ and $Il36g$ (Figure 8F).
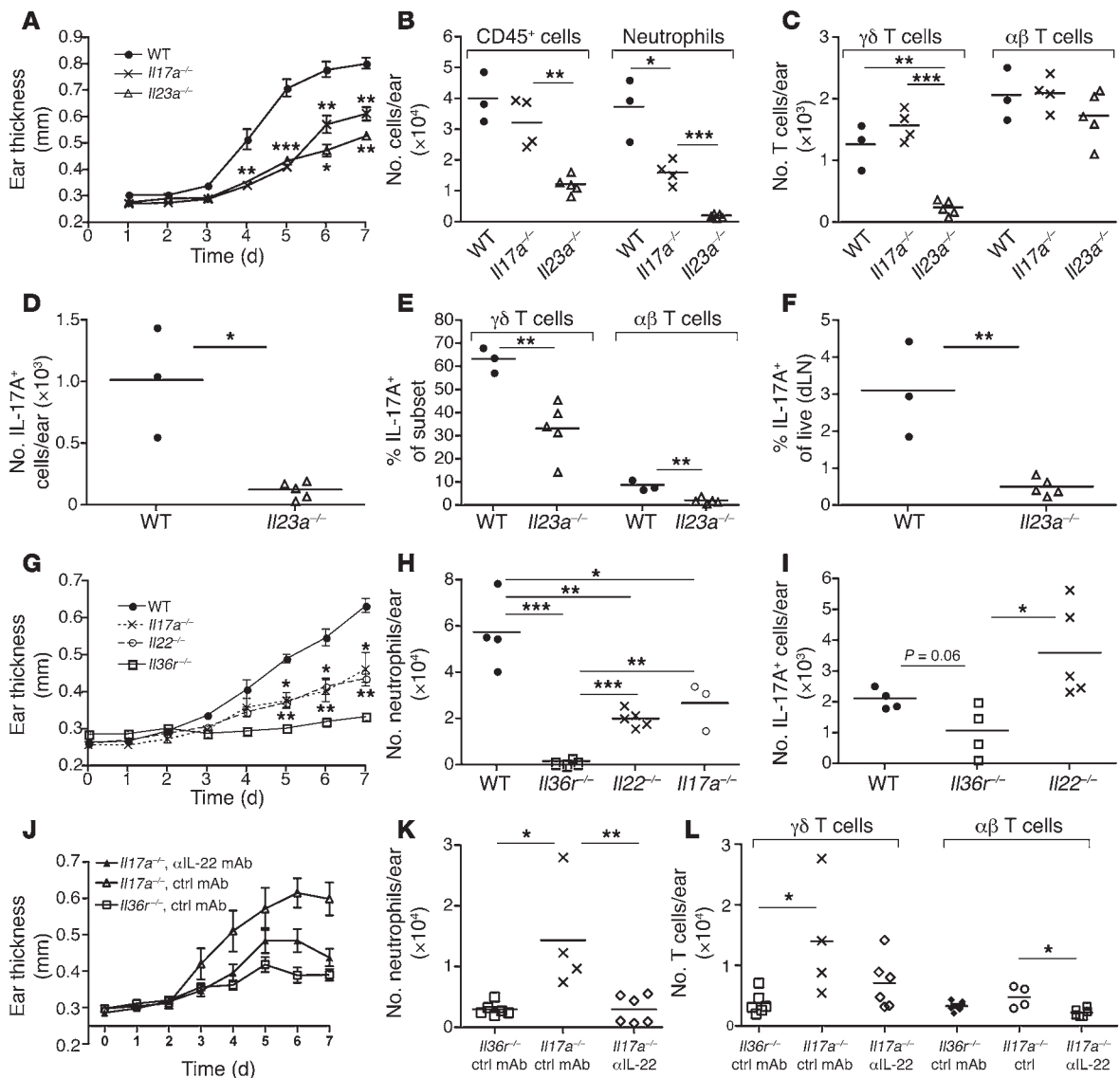
Figure 5

$Il36r^{-/-}$ mice are better protected than mice lacking IL-23, IL-17A, or IL-22. (**A**, **G**, and **J**) Ears of indicated groups of mice were treated with Aldara, and ear thickness was analyzed as described above. Values show averages ± SEM. Cell populations in the ear were characterized by flow cytometry at day 7. Total numbers of (**B**, **H**, and **K**) CD45+ hematopoietic cells and neutrophils, (**C** and **L**) γδ and αβ T cells, and (**D** and **I**) IL-17A–producing cells in ears of indicated mice. (**E**) Percentage of IL-17A producers among γδ and αβ T cells in ear lesions. (**F**) Percentage of IL-17A–producing cells gated on lymphocytes in dLNs. (**J–L**) $Il17a^{-/-}$ mice treated with neutralizing anti–IL-22 or control (ctrl) mAb compared with $Il36r^{-/-}$ mice treated with control mAb. (**B–F**, **H**, **I**, **K**, and **L**) Symbols represent individual mice, and horizontal lines indicate averages of groups. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

Discussion

Current evidence implicating IL-36 cytokines in the development of psoriasis is mainly based on transgenic mice that develop dermatitis due to forced overexpression of IL-36α in the epidermis (31, 32) and the recent identification of IL-36R antagonist loss-of-function mutations in patients with a rare and very severe form of psoriasis termed generalized pustular psoriasis (33, 34). To our knowledge, our study demonstrates for the first time that IL-36R signaling is absolutely crucial for control of the pathogenic IL-23/IL-17/IL-22 axis and development of psoriasiform dermatitis in response to environmental cues triggering TLR7 on skin DCs (i.e., using topical application of IMQ). IL-36R knockouts

were (completely) protected from IMQ-induced skin pathology observed in WT mice, including hyperkeratosis, acanthosis, neutrophil recruitment, and expansion of IL-17–producing T cells. Moreover, mice lacking IL-36R antagonist showed exacerbated disease. Together, these data suggest that the balance of endogenous IL-36R ligands and IL-36R antagonist plays a pivotal role in IMQ-induced skin disease.

IMQ treatment of WT mice resulted in vigorous expansion of IL-17–producing dermal γδ T cells that are mainly responsible for the psoriasiform disease, as suggested recently (38). However, we also found that αβ T cells can contribute substantially to cutaneous pathology and inflammation when γδ T cells are
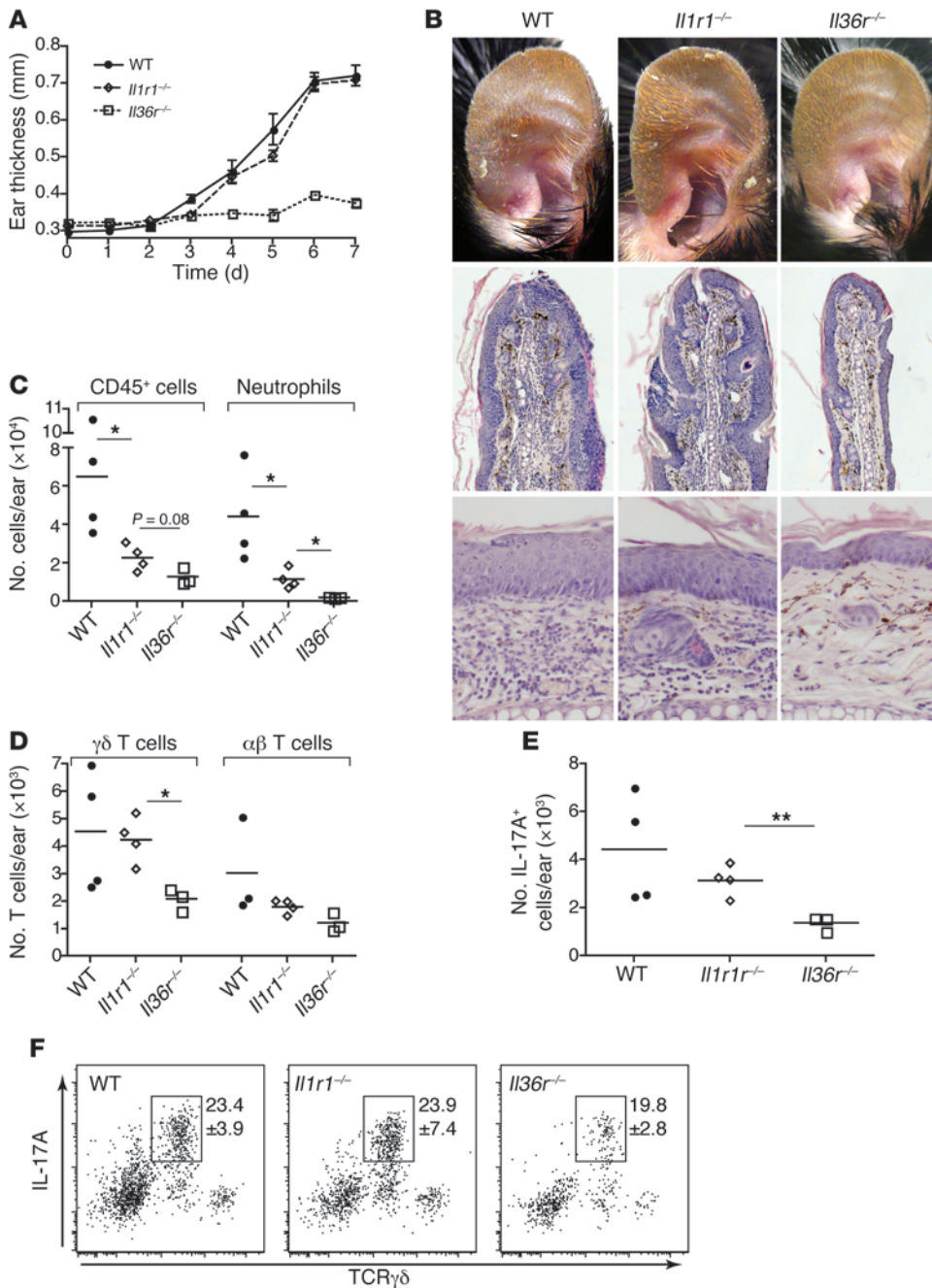
Figure 6
IL-36 promotes IL-17A expression in the skin and IMQ-induced psoriasis independent of IL-1R1 signaling. *Il1r1*$^{-/-}$, *Il36r*$^{-/-}$, and WT mice were treated with Aldara, as described in the legend to Figure 1. (**A**) Ear thickness was monitored daily. Values show averages ± SEM (*n* ≥ 3). (**B**) At day 7, photographs were taken from whole ears (top row) and H&E-stained ear sections, representing the ear tip (middle row) and the thickest epidermal region (bottom row) from individual mice representative of indicated groups. Scale bar: 100 μm. On day 7, cells prepared from ears were analyzed by flow cytometry. The graphs show the total numbers of (**C**) infiltrating CD45$^+$ hematopoietic cells and neutrophils, (**D**) αβ and γδ T cells, and (**E**) IL-17A–producing cells per ear. (**C**–**E**) Symbols represent individual mice, and horizontal lines indicate averages of groups. (**F**) Dot plots showing IL-17A–producing cells and γδ T cells. Values indicate frequencies of IL-17$^+$γδ$^+$ T cells in adjacent gated fields. *P < 0.05; **P < 0.01.

absent. TCRδ-deficient mice showed only a moderate reduction in ear swelling and no differences in neutrophilia, probably due to a 2-fold increase in frequencies of Th17 cells compared with WT mice. Interestingly, in concordance with a recent report showing that dermal γδ T cells are radiosensitive and not reconstituted after transfer of adult bone marrow upon irradiation (41), we found that Th17 cells but not γδ T cells expanded in the skin upon IMQ treatment of WT → WT chimeras. Thus, Th17 cells can compensate, at least in part, for the absence of γδ T cells in mice, indicating functional plasticity. This is interesting in the context of human skin in which αβ T cells are far more prominent than γδ T cells, although presence of the latter has also been described in psoriatic lesions (38). Regardless, our

data demonstrate that IL-36R signaling was essential for the expansion of both IL-17$^+$ γδ T cells and IL-17$^+$ αβ T cells (in the absence of γδ T cells).

As mentioned above, there is overwhelming evidence for a pivotal role of the IL-23/IL-17/IL-22 axis in development of disease, with IL-23 driving development of pathological IL-17– and IL-22–producing T cells in the skin. Our analysis of mice lacking IL-23, IL-17, or IL-22 confirmed a pathological effector function of these cytokines but also revealed interesting differences, especially in comparison with *Il36r*$^{-/-}$ mice. Neutrophil infiltration and ear swelling were comparably reduced (~60%), but not abrogated, in *Il22*$^{-/-}$ and *Il17a*$^{-/-}$ mice, suggesting that both cytokines exert important but partially redundant effector functions in the
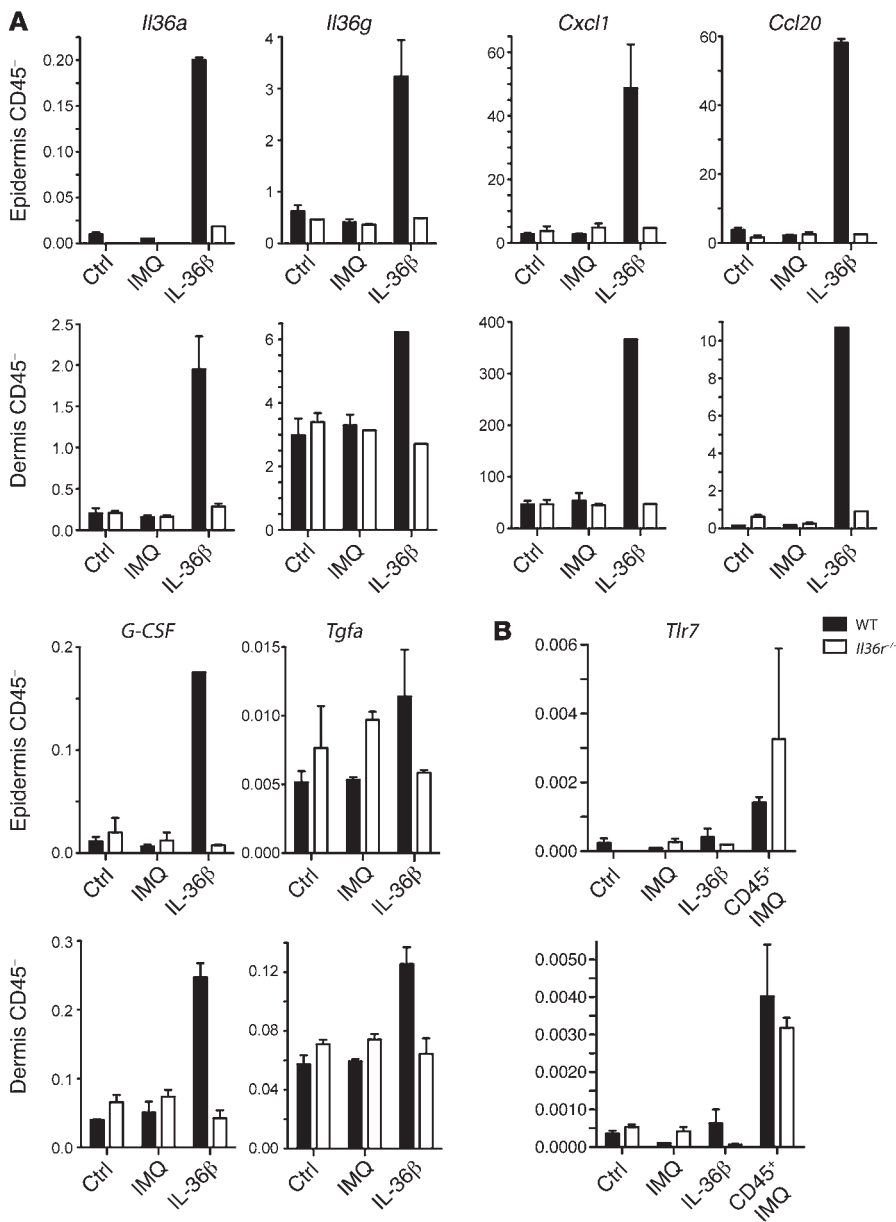
Figure 7
Autocrine IL-36 production by skin-resident cells promotes expression of chemokines and growth factors involved in disease manifestation. Ear dermis and epidermis were separated. CD45+ hematopoietic cells and CD45− dermal and epidermal cells, consisting almost exclusively of dermal mesenchymal cells and epidermal keratinocytes, respectively, were purified (>99%) by cell sorting. CD45− dermal and epidermal cells were cultured for 8 hours in the absence (Ctrl) or presence of IMQ or recombinant IL-36β. (**A**) Expression of indicated genes was measured by real-time PCR. (**B**) *Tlr7* expression in CD45+ and CD45− cells from dermis and epidermis stimulated as indicated. Values show mean ± SEM normalized to *G6PD*.

development of IMQ-induced lesions. Indeed, IL-17 and IL-22 have been shown to cooperate additively in the upregulation of antimicrobial peptides, including β-defensin-2 and S100A9 in keratinocytes (42). Consistently, neutralization of IL-22 in *Il17a*$^{-/-}$ mice inhibited skin swelling and neutrophil recruitment more than the absence of either cytokine (Figure 5, J–L). In line with previous reports demonstrating a critical role of IL-23 in psoriasis mouse models (7, 10, 11, 14, 15) and in human psoriasis (23–26), IMQ-treated *Il23a*$^{-/-}$ mice showed a striking reduction in skin swelling, concomitant with the absence of neutrophils and IL-17+ γδ T cells (Figure 5, B–E) and *Il22* mRNA levels (43). Nevertheless, absence of IL-23 or both IL-17 and IL-22 resulted in a measurable, increased skin swelling compared with *Il36r*$^{-/-}$ mice (Figure 5). These results suggest that IL-36 contributes to a small but not negligible degree to lesion development beyond induction of IL-23/IL-17/IL-22 axis in the IMQ psoriasis model. Notably, 12-O-tetradecanoylphorbol-13-acetate treatment of K14-IL-36α

transgenic mice induced psoriasiform dermatitis also on a lymphocyte-deficient *rag2*$^{-/-}$ background, indicating that T cells and their pathological mediators, IL-17 and IL-22, are completely dispensable in this transgenic mouse model (32).

IL-36 has also been shown to upregulate IL-1α expression (31, 37), which can support keratinocyte survival and proliferation through the induction of FGF7 and GM-CSF in fibroblasts (44). Moreover, IL-1 together with IL-23 is essential for expansion of IL-17–producing γδ T cells (22, 38). This led us to hypothesize that IL-36 mediates IMQ psoriasis through the induction of IL-1. Indeed, IL-1 has also been believed to play a pivotal role in cutaneous pathology in mice and humans. IL-1Ra deficiency (45–47) or transgenic overexpression of IL-1α in keratinocytes (8) results in severe cutaneous inflammation similar to transgenic overexpression of IL-36α. However, our data revealed that epidermal hyperplasia and skin flaking was unaffected in IMQ-treated *Il1r1*$^{-/-}$ mice, while neutrophil infiltration was inhibited. These
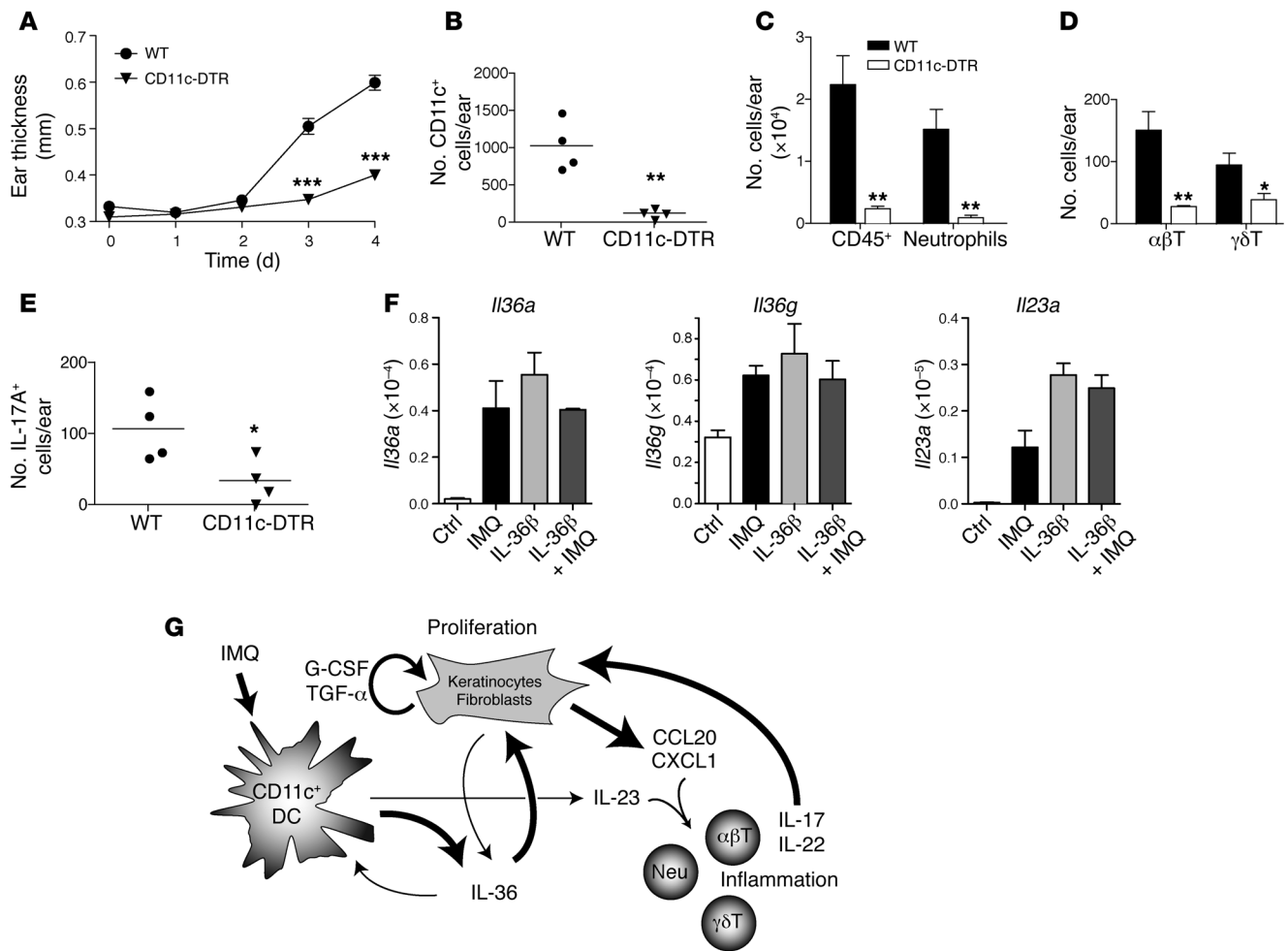
Figure 8
DCs produce IL-36 upon IMQ treatment and are pivotal for IMQ-induced cutaneous pathology. CD11c-DTR transgenic and WT control mice were injected with diphtheria toxin 6 hours prior to daily treatment with Aldara. (**A**) Ear thickness was monitored daily. Values show averages ± SEM ($n \geq 4$). (**B–E**) On day 4, cells prepared from ears were analyzed by flow cytometry. Numbers of (**B**) CD11c+ DCs, (**C**) CD45+ cells and neutrophils, (**D**) αβ and γδ T cells, and (**E**) IL-17–producing cells per ear are shown. Symbols represent individual mice, and horizontal lines indicate averages of groups. (**F**) *Il36a*, *Il36g*, and *Il23a* mRNA expression in BMDCs cultured for 6 hours in the absence (Ctrl) or presence of IMQ, recombinant IL-36β, or IMQ and IL-36β, measured by real-time PCR. Values show mean ± SEM normalized to *HPRT*. (**G**) Scheme illustrating development of psoriasiform dermatitis driven by DC-keratinocyte crosstalk via autocrine and paracrine IL-36 loops. Neu, neutrophil. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

findings uncouple neutrophil recruitment and tissue damage; the former being mediated by IL-1 and the latter by IL-36 in an IL-1–independent manner.

Thus, comparing pathological manifestations in mice lacking IL-23, IL-17, IL-22, IL-36R, or IL-1R following IMQ treatment allowed us to rank the importance of these cytokines and their contribution for IMQ-induced psoriasiform dermatitis as follows: IL-36R ≥ IL-23 > IL-22, IL-17 >> IL-1R.

So where and how do IL-36 cytokines act in the process of lesion development following IMQ encounter? Results from bone marrow chimeras revealed that IL-36R signaling on skin-resident cells, including keratinocytes and fibroblasts, but not on hematopoietic cells was essential for cutaneous pathology, indicating that IL-36 acted locally in the skin and was not essential for activation of innate or adaptive immune cells, including DCs and T cells. Yet DCs were essential to kick off the pathologic cytokine network, as they responded to IMQ with production of IL-36 and IL-23

(Figure 8F), while keratinocytes and dermal mesenchymal cells did not respond to IMQ due to the absence of TLR7 (Figure 7B and ref. 48). Stimulation of BMDCs with IL-36 agonists triggered IL-23 and IL-36 cytokine expression (Figure 8F), possibly together with other proinflammatory factors (37), comparably to TLR7 ligation by IMQ. Consequently, depletion of all CD11c+cells, including conventional and plasmacytoid DCs as well as Langerhans cells, prevented disease induction. While depletion of langerin+ DCs did not change IMQ-induced skin pathology, suggesting that Langerhans cells are not critically involved in this model (B.E. Clausen, personal communication), plasmacytoid DCs known to express high levels of TLR7 remain to be tested (49). Our data suggest that TLR7 engagement on conventional and/or plasmacytoid DCs triggers IL-36 production that acts in cis (autocrine) to promote the DC proinflammatory response and in trans (paracrine) to upregulate IL-36 cytokine production by skin-resident cells, including keratinocytes and fibroblasts. However, it is

the IL-36R signaling on skin-resident cells that is crucial for development of psoriasiform lesions, as demonstrated by the results of the IL-36R/WT crisscross bone marrow chimeras. Indeed, culture of epidermal keratinocytes and dermal mesenchymal cells with IL-36 agonists potently induced IL-36 cytokine expression (Figure 7A) concomitant with the upregulation of chemokines that promote recruitment of neutrophils and dermal CCR6+ γδ T cells (50). In addition, IL-36 triggered expression of keratinocyte mitogens by epidermal and dermal cells.

Our results suggest that autocrine IL-36 production by epidermal and dermal skin-resident cells may account for the remaining skin swelling that we have observed in the absence of IL-23, IL-17, and IL-22. However, dysregulated keratinocyte growth and differentiation, together with the production of antimicrobial peptides, is certainly amplified by IL-17A and IL-22 produced by the expanded dermal γδ T cells (29). Although human keratinocytes were reported to produce IL-23 (51), IL-12p40 expression was undetectable in CD45− cells sorted from epidermis and dermis and stimulated with IMQ, IL-36β, or IL-36γ (data not shown), indicating that keratinocytes and dermal mesenchymal cells do not produce IL-23 under these conditions and that DCs are solely responsible for IL-23 production.

Taken together, we have established a central IL-36–mediated crosstalk between DCs and skin-tissue cells, such as keratinocytes and fibroblasts. Our results suggest the mechanism illustrated in Figure 8G. TLR7 engagement by IMQ triggers IL-23 and IL-36 cytokine production in DCs. While DC-derived IL-36 may augment the DC inflammatory response in cis (autocrine), it is mainly required for activation of keratinocytes and dermal mesenchymal cells in trans (paracrine). When activated, they produce IL-36 in an autocrine fashion along with chemokines that attract neutrophils (i.e., CXCL1) and dermal CCR6+ γδ T cells (i.e., CCL20) and growth factors that promote keratinocyte hyperproliferation, such as G-CSF and TGF-α.

Due to increased understanding of the molecular mechanisms of immune pathology, current therapies use biologics targeting endogenous mediators of inflammation, such as TNF-α, IL-23, or IL-17, that have shown efficacy in clinical trials (1, 2, 52). However, inhibition of these cytokines, which are critical for certain types of immune responses, may render individuals susceptible to infection, underlining the need for tissue-specific targets. Our data, together with recent reports of patients with pustular psoriasis having mutations in the *IL36RN* gene (33, 34), suggest that blocking IL-36R signaling might be a promising approach for the specific treatment of psoriasis.

Methods

Mice and antibodies. Il36r−/− (31), *Il36rn−/−* (generated as described below), *Il1r1−/−* (53), *Il17a−/−* (54) (provided by Y. Iwakura, The Institute of Medical Science, University of Tokyo, Tokyo, Japan), *Il23a−/−* (55) (provided by R. Kastelein, Schering-Plough Biopharma, Palo Alto, California, USA), and *Il22−/−* mice (56) were bred and maintained under specific pathogen–free conditions in our animal facility (BioSupport). All knockout mice were backcrossed to C57BL/6 >7 generations. C57BL/6 mice were purchased from Charles River Inc. For experiments, age- and sex-matched mice (at 7 to 12 weeks of age) were used. The following antibodies from BioLegend were used: FITC-labeled anti-CD45.1 and anti–IL-17A; PerCP/Cy5.5-labeled anti-CD11b; PerCP-labeled streptavidin; PE/Cy7-labeled anti-CD45.2; and APC-labeled anti-CD11c, anti-CD45.1, and anti–IFN-γ. The following antibodies and reagents were purchased from eBioscience: FITC-labeled anti-

CD4 and anti–Ly-6G; PE-labeled anti-TCRβ, anti-CD4, and anti-MHCII; APC-labeled anti–GR-1; biotin-labeled anti-TCRγδ; and the viability dye eFluor780. Recombinant IL-36β (aa 31–183) was purchased from R&D Systems (catalog no. 7060-ML). R837 (IMQ) was purchased from Tocris.

Generation of knockout mice. Il36rn−/− mice were generated using a targeting vector designed to remove part of exon 1 and exon 2 by homologous recombination. 129S5/SvEv embryonic stem cells were transfected with the targeting vector by electroporation. Targeted embryonic stem clones were identified by Southern blot analysis and microinjected into C57BL/6 blastocysts. PCR was used for routine genotyping of the mice. The following primers were used: WT exon 1, 5′-TGGAGCTCATGAT-GGTTCTG-3′; WT intron 1, 5′-AGGATCCTGCTCAGTTCTTCC-3′; KO 5′ arm, 5′-AAGGGCAGATCTGATGGATGC-3′; Neo, 5′-ATGACTGGGCA-CAACAGACA-3′. *Il36rn*-targeted mice were backcrossed at least 7 times on C57BL/6 background before performing the experiments.

Treatment of mice. For induction of psoriasiform dermatitis, mouse ears were treated as described previously (11) for 7 consecutive days with Aldara Crème containing 5% Imiquimodum (provided by MEDA Pharma GmbH). For neutralization of IL-22, mice were treated with 200 μg anti–IL-22 (AM 22.1) or control anti-human IL-9R mAb (AH9R4) 4 hours prior application of Aldara at days 0, 3, and 5.

Generation of bone marrow chimeras. Bone marrow cells were obtained from flushing femurs and tibias of donor WT CD45.1 and *Il36r−/−* CD45.2 mice Lethally irradiated recipient mice (WT CD45.1 and *Il36r−/−* CD45.2 mice) were then reconstituted by intravenous injection of bone marrow cells. Recipient mice were treated with antibiotics (Borgal 24%, Veterinaria AG) for 5 weeks. Treatment with Aldara was performed 8 weeks after reconstitution.

Histopathology of ear skin. At day 7, mice were euthanized, and ears were removed and fixed in 95% ethanol/1% aceton for subsequent embedding in paraffin. Tissue sections were deparaffinized and stained with H&E for histological analysis.

Cell preparation. Single-cell suspensions from auricular lymph nodes were prepared by digestion with 1 mg/ml collagenase type IV (Sigma-Aldrich) and subsequent pressing of the organ through 40-μm pore size strainers (BD Biosciences). To obtain a single-cell suspension from ears, ear halves were separated and cut into small pieces before collagenase digestion. Following digestion, the tissue was mechanically disrupted and pressed through 40-μm pore size strainers. For preparation of epidermal and dermal skin-resident cells, ear halves were separated and incubated in 0.1% Trypsin IMDM for 50 minutes at 37°C. Dermis and epidermis were separated, cut into small pieces, and digested in 0.1% Trypsin IMDM plus 0.1 mg/ml DNase for 30 minutes at 37°C. Dermis was further digested with collagenase for 30 minutes at 37°C. Single-cell suspension was made, and cells were washed once with IMDM 10% FCS, stained, and sorted on the FACSAria (BD Biosciences). CD45− epidermal cells were plated out in defined keratinocyte medium (Gibco) (containing 10−10 M cholera toxin), and CD45− dermal mesenchymal cells were plated out in pure IMDM at 10^5 cells per well in a 96-well plate. After overnight starvation, cells were stimulated for 8 hours as indicated in Figure 7.

Immunofluorescence, surface staining, and analysis. Cells were initially stained with the viability dye eFLuor780 in PBS. Successively, cells were incubated with Fc receptor blocking mAb (clone 2.4G2) in PBS 2% FCS, after which staining with relevant fluorescently labeled antibodies was performed. For subsequent flow cytometric analysis using a FACSCanto II (BD Biosciences), cells were washed after a 15-minute incubation time and resuspended in PBS 2% FCS. FACS data was then analyzed using FlowJo (Tree Star Inc.).

In vitro restimulation and intracellular staining. To analyze cytokine production, single-cell suspensions were incubated for 2 hours at 37°C with PMA (10−7 M; Sigma-Aldrich), monensin (2 mg/ml; Sigma-Aldrich), and ionomycin

(1 mg/ml; Sigma-Aldrich). After performing surface stainings as described above, cells were fixed with 4% formalin and permeabilized with PBS 2% FCS supplemented with 0.5% saponin. Intracellular stainings with fluorescently labeled antibodies were performed for 30 minutes in PBS 2% FCS with 0.2% saponin. For flow cytometric analysis, cells were washed and resuspended in PBS 2% FCS.

DC culture and stimulation. BMDCs were generated from bone marrow cells cultured for 10 days in complete RPMI medium supplemented with GM-CSF. At day 10, cells were stimulated with IMQ (R837) at 5 μg/ml and/or recombinant IL-36β (10 ng/ml). After 6 hours, supernatant was removed, and the cells were lysed in TRI Reagent and processed for RT-PCR as described below.

Quantitative RT-PCR. Total RNA was isolated from ears using TRI Reagent (Molecular Research Center Inc.) and reverse transcribed with GoScript (Promega). Quantitative real-time RT-PCR was performed with Brilliant SYBR Green (AGILENT-Stratagene) on an iCycler (Bio-Rad Laboratories). Expression was normalized to *G6PD* or *HPRT* as indicated in figure legends. The following primers were used: IL-36α (forward) 5′-TGCCCACTCATTCTGACCCA-3′ and (reverse) 5′-GTGCCACAGAG-CAATGTGTC-3′; IL-36γ (forward) 5′-ATGGACACCCTACTTTGCTG-3′ and (reverse) 5′-TGTCCGGGTGTGGTAAAACA-3′; IL-23p19 (forward) 5′-CACCTCCCTACTAGGACTCAGC-3′ and (reverse) 5′-CTGCCACTGCT-GACTAGAAC-3′; G-CSF (forward) 5′-TGCACTATGGTCAGGACGAG-3′ and (reverse) 5′-GGGGTGACACAGCTTGTAGG-3′; TGF-α (forward) 5′-CTGAGTGACTCACCCGTGGC-3′ and (reverse) 5′-TCTGCATGCT-CACAGCGAAC-3′; TLR7 (forward) 5′-CCACCAGACCTCTTGATTCC-3′ and (reverse) 5′-CCATCGAAACCCAAAGACTC-3′; CXCL-1 (forward) 5′-GCCTATCGCCAATGAGCTG-3′ and (reverse) 5′-ATTCTTGAGTGT-GGCTATGA-3′; CCL20 (forward) 5′-AATCTGTGTGCGCTGATCCA-3′ and (reverse) 5′-CCTTGGGCTGTGTCCAATTC-3′; GAPDH (forward) 5′-AAATTCAACGGCACAGTCAAG-3′ and (reverse) 5′-TGGTGGTGAA-GACACCAGTAG-3′; and G6PD (forward) 5′-CTACAGGTTCAGATGAT-GTC-3′ and (reverse) 5′-CAGCTTCTCCTTCTCCATTG-3′.

Statistics. Two-tailed paired and unpaired *t* tests were performed using Prism 4.0 (GraphPad software). A *P* value of less than 0.05 was considered significant. Data presented show values ± SEM, if not indicated otherwise in figure legends.

Study approval. All animal experiments were approved by the veterinary authorities of the Canton of Zurich (Kantonales Veterinäramt Zürich).

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