An Alternative Pathway of Imiquimod-Induced Psoriasis-Like Skin Inflammation in the Absence of Interleukin-17 Receptor A Signaling

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Topical application of imiquimod (IMQ) on the skin of mice induces inflammation with common features found in psoriatic skin. Recently, it was postulated that IL-17 has an important role both in psoriasis and in the IMQ model. To further investigate the impact of IL-17RA signaling in psoriasis, we generated IL-17 receptor A (IL-17RA)–deficient mice (IL-17RA^{del}) and challenged these mice with IMQ. Interestingly, the disease was only partially reduced and delayed but not abolished when compared with controls. In the absence of IL-17RA, we found persisting signs of inflammation such as neutrophil and macrophage infiltration within the skin. Surprisingly, already in the naive state, the skin of IL-17RA^{del} mice contained significantly elevated numbers of Th17- and IL-17-producing γδ T cells, assuming that IL-17RA signaling regulates the population size of Th17 and γδ T cells. Upon IMQ treatment of IL-17RA^{del} mice, these cells secreted elevated amounts of tumor necrosis factor-α, IL-6, and IL-22, accompanied by increased levels of the chemokine CXCL2, suggesting an alternative pathway of neutrophil and macrophage skin infiltration. Hence, our findings have major implications in the potential long-term treatment of psoriasis by IL-17-targeting drugs.

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INTRODUCTION

Psoriasis is one of the most common chronic inflammatory diseases of the skin, affecting 2–3% of the population (Wagner *et al.*, 2010). Up to now there is no cure known for this disease, which is associated with extensive psychological and physical burden. This disease evolves over time in a complex interplay of heterogeneous genetic and environmental factors (Di Cesare *et al.*, 2009). Psoriasis is not only a skin disorder but may also include inflammation of the joints in psoriasis arthritis (Winchester *et al.*, 2008) and comorbidities such as metabolic syndrome with increased insulin resistance, increased cardiovascular risk (Vena *et al.*, 2010; Mehta *et al.*, 2011), and an increased risk of mortality (Gelfand *et al.*, 2007).

Several risk factors and pathways are known to enable the development of psoriasis. Genetic factors seem to have a role when interacting with environmental factors such as drugs, stress, or streptococcal infection (Griffiths and Barker, 2007). Further, endogenous antimicrobial peptides (β-defensins and cathelicidins) are overexpressed in psoriasis skin (Ong *et al.*, 2002; Griffiths and Barker, 2007). In complex with extracellular self-DNA, these peptides seem to be able to evoke autoimmunity by triggering plasmacytoid dendritic cells (DCs) of the skin (Gilliet *et al.*, 2008). In a mechanism not yet fully understood, the IL-23/IL-17 axis is critically involved in the development of human psoriasis (Blauvelt, 2008). Th17 cells are found in lesions from psoriatic patients (Harper *et al.*, 2009) and may be the key factors in psoriasis (Di Cesare *et al.*, 2009). In addition, clinical signs of psoriasis can be induced in mouse skin by intradermal injection of the IL-17-supporting cytokine IL-23 (Chan *et al.*, 2006).

Apart from genetic approaches (Gudjonsson *et al.*, 2007), xenograft transplantation (Conrad and Nestle, 2006; Wagner *et al.*, 2010) or the IL-23-induced dermatitis model (Mabuchi *et al.*, 2011), imiquimod (IMQ)-induced psoriasis in mice, represents a model that mimics the skin disease particularly well (van der Fits *et al.*, 2009; Cai *et al.*, 2011). IMQ is a toll-like receptor 7/8 ligand (Gilliet *et al.*, 2004) and a potent immune activator that is used for treatment of actinic

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Abbreviations: IMQ, imiquimod; PASI, psoriasis area and severity index; WT, wild-type

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keratosis and superficial basal cell carcinomas (Geisse *et al.*, 2002; Szeimies *et al.*, 2004), but as a side effect it can also provoke psoriasis-like skin flares in predisposed humans (Wu *et al.*, 2004). Daily topical application of IMQ on the skin of mice leads to a psoriasis-like dermatitis with many hallmarks of human psoriasis, such as the formation of microabscesses, skin thickening, hyperkeratosis, acanthosis, scaling, and erythema (Greaves and Weinstein, 1995; Griffiths and Barker, 2007; van der Fits *et al.*, 2009). This model was shown to be critically dependent on the IL-23/RORγt/IL-17 axis (van der Fits *et al.*, 2009; Cai *et al.*, 2011; Pantelyushin *et al.*, 2012) in mice of BALB/c or C57BL/6 background. IMQ application results in an increased dermal proliferation and infiltration of neutrophils, mononuclear cells, $CD4^+$ T cells (especially Th17 cells), $CD11c^+$ DCs, plasmacytoid DCs, and modified differentiation of keratinocytes. In addition to Th17 cells, γδ T cells producing IL-17A were recently shown to have a pivotal role in the development of psoriasis (Mabuchi *et al.*, 2011; Pantelyushin *et al.*, 2012). In the murine skin, γδ T cells are present in the epidermis (Hayday and Tigelaar, 2003), where they have a dendritic morphology, and therefore have been named dendritic epidermal T cells (Havran and Jameson, 2010). In addition, they exist in the dermis of mice as well—that is the place where they are located in the human skin (Ebert *et al.*, 2006). Cai *et al.* (2011) recently showed that it is mainly the innate dermal γδ T cells that are the major IL-17A–producing cells in the skin after IL-23 injection. The frequency of IL-17A-producing dermal γδ T cells in humans suffering from psoriasis was increased in comparison with healthy controls (Cai *et al.*, 2011). In addition, it was shown that dermal γδ T cells were obligatory for the IL-23-initiated disease, whereas both γδ and αβ T cells did have important roles in the IMQ model (Cai *et al.*, 2011; Mabuchi *et al.*, 2011).

It was previously shown (van der Fits *et al.*, 2009) that IL-17RA-deficient mice develop reduced erythema, scaling, and skin thickening under IMQ treatment. Furthermore, the same group also found that the number of infiltrating $GR1^+$ neutrophils in the dermis was reduced in comparison with the control group. Here, we describe a mouse strain in which the IL-17RA can be deleted in a Cre-dependent manner. These mice were crossed so that the IL-17RA was inactivated in the germ line, thus resulting effectively in IL-17RA knockout mice, herein termed $IL\text{-}17RA^{del}$ mice. Naive $IL\text{-}17RA^{del}$ mice contained massively elevated levels of Th17- and IL-17A-producing dermal γδ T cells in the skin. Increased numbers of Th17 and γδ T cells in lymph nodes and spleen were present, indicating that signaling via IL-17RA regulates the size of these populations in the skin and in other organs. We found that in these mice the IMQ-induced disease was clinically reduced, but signs of inflammation such as infiltration of neutrophils and macrophages persisted in the skin. Importantly, we found significantly elevated levels of IL-6, IL-22, and CXCL2 in the skin of IMQ-treated $IL\text{-}17RA^{del}$ mice compared with controls, thereby revealing an alternative pathway for psoriasis development in the absence of IL-17RA signaling.

RESULTS

The $IL\text{-}17RA^{del}$ mice

Th17 cells are key factors in psoriasis as demonstrated by clinical and experimental data (Di Cesare *et al.*, 2009; Waisman, 2012). IL-17A is the hallmark cytokine of Th17 cells and is produced by γδ T cells in addition (Jensen *et al.*, 2008). In addition to IL-17A, Th17 cells were shown to express IL-17F, IL-21, IL-22, and IL-6 (Waisman, 2011). IL-17A and IL-17F are structurally and genetically very similar, and can both bind to the heterodimeric IL-17RA/IL-17RC receptor (Iwakura *et al.*, 2011; Zepp *et al.*, 2011). To delete IL-17RA, we flanked exons 4–7 with loxP sites (see Figure 1a and Supplementary Fig S1 online). The neomycin resistance cassette, necessary during the generation process of the mouse strain, was removed by Flippase Recognition Target (FRT) sites using Flippase recombinase. The resulting mouse is thereafter named $IL\text{-}17RA^{Fl}$ mouse. Crossing this mouse strain to the deleter Cre mouse (Schwenk *et al.*, 1995) resulted in a germ line–transmitted complete deletion of the *IL-17RA* gene, herein named as the $IL\text{-}17RA^{del}$ strain (Figure 1a and Supplementary Fig S1 online). Reverse transcriptase–PCR was applied to RNA of spleens of wild-type (WT), $IL\text{-}17RA^{Fl}$, and $IL\text{-}17RA^{del}$ mice with primers that bind to exon 4 and 8. In $IL\text{-}17RA^{del}$ mice, no PCR product could be detected, whereas the $IL\text{-}17RA^{Fl}$ mice expressed the IL-17RA receptor in a manner similar to WT control mice (Figure 1b).

Psoriasis-like disease in the absence of IL-17RA signaling

Recently, it was reported by van der Fits *et al.* (van der Fits *et al.*, 2009) that application of IMQ-containing Aldara cream results in a psoriasis-like disease that is dependent on the IL-17/IL-23 cytokine axis. To test whether $IL\text{-}17RA^{del}$ mice are resistant to this skin inflammatory disease, we applied IMQ containing cream once daily on the shaved back skin and on the ears of the mice for 4–9 consecutive days, as described by van der Fits *et al.*, 2009. Mice were used at 7 and 8 weeks of age at the start of treatment. $IL\text{-}17RA^{del}$ mice showed a significantly reduced erythema on day 4 (Figure 1c), but surprisingly other scoring parameters such as skin thickness or scaling were only mildly reduced in $IL\text{-}17RA^{del}$ mice compared with WT C57BL/6 control mice (Figure 1d). These findings are summed as the cumulative psoriasis area and severity index (PASI) score (combining the three parameters), which was significantly reduced only up to day 4 of disease (Figure 1d) in $IL\text{-}17RA^{del}$ mice compared with controls. Application of sham cream (containing all ingredients as the IMQ cream except for the active component) did not lead to any sign of skin inflammation in both WT and $IL\text{-}17RA^{del}$ mice.

To further quantify the epidermal hyperplasia, we performed blinded measurements of the epidermal thickness on the basis of hematoxylin and eosin–stained slices from mice treated with IMQ for five consecutive days. In both IMQ-treated groups, WT and $IL\text{-}17RA^{del}$ mice, the epidermal thickness was significantly higher than in sham-treated WT mice (Figure 1e). In line with the skin thickness measurements shown in Figure 1d, the difference between WT and $IL\text{-}17RA^{del}$ mice after treatment was minor (WT mice had

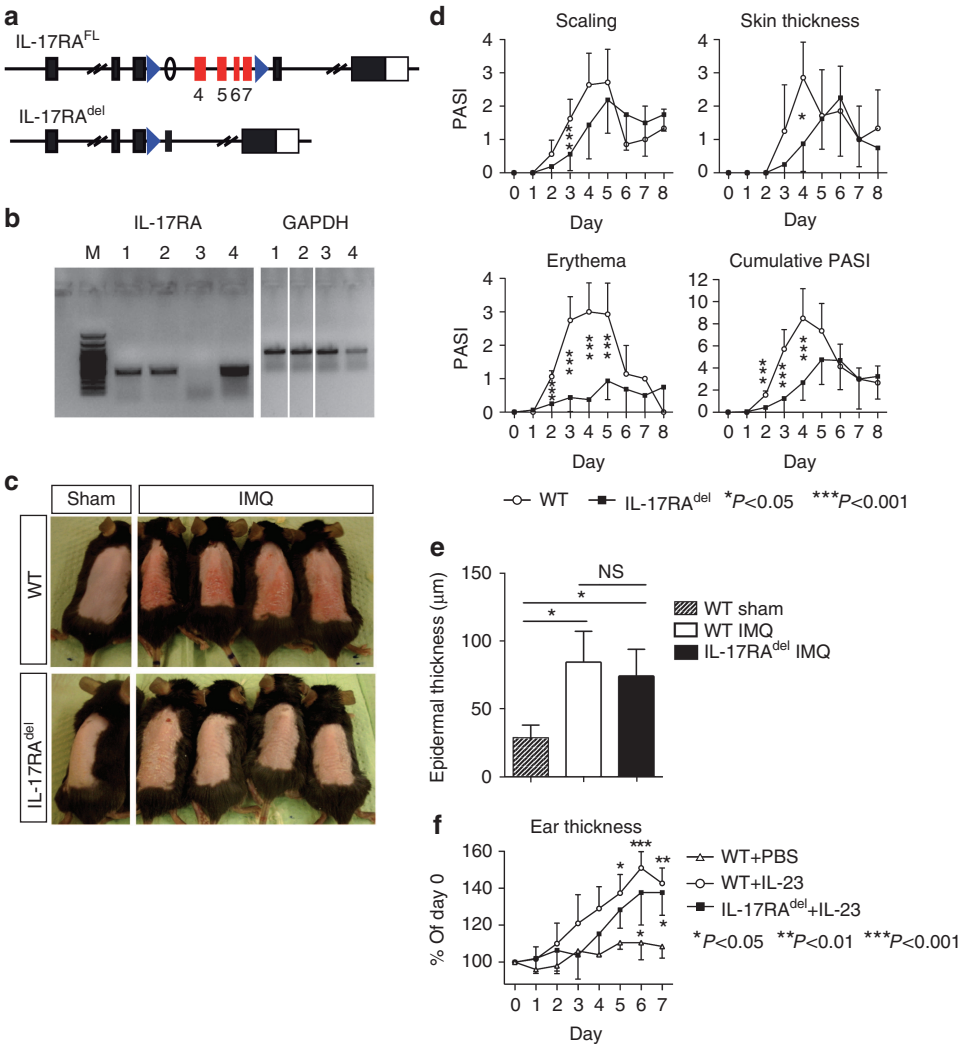

Figure 1. IL-17RA^{del} mice show a milder form of imiquimod (IMQ)-induced psoriasis in comparison with control mice. (**a**) Construction of the IL-17RA^{del} mouse strain: exons 4–7 of the mouse *IL-17RA* gene are flanked by LoxP sites, indicated by blue triangles (IL-17RA^{Fl}). After crossing this mouse strain with a Deleter Cre mouse, exons 4–7 (red boxes) are deleted. Exons are indicated as boxes. The small oval indicates the Flippase Recognition Target site, which flanked the neomycin resistance cassette during the generation process of this mouse line and is removed in the IL-17RA^{Fl} and the IL-17RA^{del} mice. (**b**) IL-17RA^{del} mice express no wild-type (WT) IL-17RA messenger RNA (mRNA) in reverse transcriptase (RT)–PCR: IL-17RA^{Fl} mice show normal levels of IL-17RA mRNA (lanes 1 and 2) compared with WT (lane 4). In contrast, IL-17RA^{del} mice (lane 3) do not express IL-17RA mRNA. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as internal control. (**c**) Phenotypical presentation of mouse back skin of 8-week-old WT and IL-17RA^{del} mice that were treated with IMQ on the shaved and depilated back skin and on the right ear over 7 days. The photo was taken on day 4. Mouse one was the internal sham-treated control in each group, which shows no disease development. (**d**) Daily scoring of scaling, skin thickness, and erythema of IL-17RA^{del} and control mice under IMQ treatment over 8 days. The cumulative psoriasis area and severity index (PASI) includes scaling, skin thickness, and erythema of IMQ-treated animals. Scale from 0 to 4. Shown is the mean ± standard deviation (SD; $n = 4$ per experiment, representing three independent experiments with comparable results; the statistical analysis of significance is described in the methods section). (**e**) Microscopical quantification of epidermal hyperplasia after 5 days of treatment ($n = 3$, blinded measurements were performed at three different regions and do not include the stratum corneum to avoid measurement falsification due to fixation artifacts). (**f**) Ears of WT and IL-17RA^{del} mice were injected intradermally for 8 days with 20 µl phosphate-buffered saline (PBS), alone or containing 500 ng IL-23. Ear thickness was measured daily and shows percentagewise increase of the starting values ($n = 3$, significance is shown in comparison with the PBS-treated WT control).

2.9 times and IL-17RA^{del} mice 2.6 times thicker epidermis than sham-treated WT mice). The hematoxylin and eosin staining show the appearance of hyperkeratosis, acanthosis, and microabscesses in IMQ-treated WT and IL-17RA^{del} mice (Supplementary Figure S2 online).

To validate the results of our IMQ-induced model and to test whether a more defined model of disease pathogenesis would be similarly independent of IL-17RA signaling, we used the IL-23 injection model as described before (Chan *et al.*, 2006). After intradermal IL-23 injection for seven consecutive days in the ears of WT and IL-17RA^{del} mice, we detected a significant increase in the ear thickness in mice of both groups compared with the phosphate-buffered saline-treated mice (Figure 1f). In line with the IMQ model,

inflammation in the IL-23 injection model was delayed by 1 day only in IL-17RA-deficient mice compared with control mice without showing a significant difference in ear thickness on the individual days.

Cellular infiltrates in the IMQ model

Next, we analyzed cellular infiltrates in the skin of IMQ-treated ears after 8 days of treatment. When analyzed on day 9 of IMQ treatment, we detected an increased frequency of $CD11b^+$ cells in both mouse groups in comparison with the sham-treated mice (Figure 2a–c). Interestingly, we found significantly more $CD11b^+$ cells in the ears of the IL-$17RA^{del}$ mice in comparison with IMQ-treated WT animals (Figure 2a–c). These infiltrates were composed mainly of macrophages but also of inflammatory monocytes and $GR1^{hi}$ neutrophils (Figure 2a, right). Despite the higher number of $CD11b^+$ cells in IMQ-treated IL-$17RA^{del}$ mice compared with controls, the composition of myeloid cellular infiltrates was not significantly altered (Figure 2a, right) compared with IMQ-treated WT mice (Figure 2b and c). As plasmacytoid DCs are implicated in disease development following IMQ treatment, we stained and quantified plasmacytoid DCs of these animals on day 5 of treatment and found similar numbers of plasmacytoid DCs in WT and IL-$17RA^{del}$ mice (Supplementary Figure S3 online). We further histologically analyzed the skin for the infiltration of neutrophils (Figure 2d) and macrophages (Figure 2e). As seen in these figures, the skin of WT and IL-$17RA^{del}$ mice treated with the sham cream contained considerable numbers of these innate cell populations, reflecting the effect of the nonspecific irritation of the sham treatment. Once treated with IMQ-containing cream, the levels of both neutrophils and macrophages were drastically increased in both mouse groups. These data clearly show that at this time point of disease the lack of IL-17RA signaling does not mitigate infiltration of innate myeloid cells, which points to alternative mechanisms of disease in these mice.

Deletion of IL-17RA results in increased number of IL-17A-producing cells in the skin of IMQ-treated mice

Our findings demonstrate that mice lacking signaling via the IL-17RA are susceptible to IMQ-induced psoriasis-like disease, which is further manifested by accumulation of innate immune cells in the skin. Although IL-17 signaling is impeded in these mice, IL-17-producing cells may be involved in this inflammatory process by the secretion of other inflammatory cytokines. Indeed, we found that the skin of sham-treated IL-$17RA^{del}$ mice contained high numbers of $CD4^+$ T cells (Figure 3b), of which about 50% also expressed IL-17A as seen by intracellular staining (Figure 3a). In contrast, sham-treated WT mice were, as expected, devoid of Th17 cells in the skin, and only after treatment with IMQ showed a small but significant infiltration of these cells. Upon treatment with IMQ, the skin of IL-$17RA^{del}$ mice retained a level of about 40% Th17 cells of the total CD4 T cells, but it is noteworthy that the total number of CD4 cells was highly increased compared with WT mice (Figure 3b and c). We also analyzed these mice for systemic effects of the IMQ treatment on cytokine-producing CD4 and γδ-T cells. In agreement with previous reports describing a higher level of IL-17A in the serum and the lung and higher differentiation of Th17 cells *in vitro* in IL-17RA KO animals (Ye *et al.*, 2001; Nagata *et al.*, 2008; Smith *et al.*, 2008), we found highly increased levels of IL-17A-expressing Th17 and γδ T cells also in our sham-treated IL-$17RA^{del}$ animals. Upon IMQ treatment, only Th17 cells in the lymph nodes further increased in these animals (Supplementary Figure S4 online) as compared with control mice.

γδ T cells have been described in the skin of untreated mice (Hayday and Tigelaar, 2003) and were recently implicated in IMQ-induced psoriasis-like disease in mice and in human psoriasis patients (Cai *et al.*, 2011; Pantelyushin *et al.*, 2012). We were therefore interested to analyze whether these cells can also be found in the skin of the IL-$17RA^{del}$ mice. In mice, dermal γδ T cells can be easily distinguished from the dendritic epidermal T cells by the expression level of the γδ T-cell receptor, with low expression marking the dermal γδ T cells. As can be seen in Figure 3a, we found by intracellular staining a clear population of IL-17A-producing γδ T cells in WT mice (Figure 3a, right). In contrast to the Th17 cells, the proportion of IL-$17A^+$ γδ T cells increased much more in WT mice after IMQ treatment, as reported previously (Cai *et al.*, 2011). Similar to the Th17 cells, we also found a high proportion of dermal γδ T cells in the skin of the sham-treated IL-$17RA^{del}$ mice, which only slightly changed after treatment (Figure 3a). The total number of the IL-$17A^+$ γδ T cells were similar after IMQ treatment in WT and IL-$17RA^{del}$ mice (Figure 3c). We further analyzed IL-17A expression on day 9 of IMQ treatment by immunohistochemistry. As expected by the flow cytometry data, sham-treated IL-$17RA^{del}$ mice showed high levels of IL-17A production (Figure 3d), partially coassociated with increased numbers of CD4 T cells. Upon IMQ treatment, IL-17A levels in the skin further increased in IL-$17RA^{del}$ mice, similar to CD4 levels (Figure 3d), but at this late time point barely no IL-17A staining was detectable by immunofluorescence in IMQ-treated WT mice. As IL-17A-positive cells only partially overlapped with $CD4^+$ T cells, γδ T cells were probably responsible for the residual increase in IL-17A in the skin under IMQ treatment.

IMQ-treated IL-$17RA^{del}$ mice exhibit high levels of IL-6 and IL-22 in the skin

As signaling of both IL-17A and IL-17F via IL-17RA is interrupted in IL-$17RA^{del}$ mice, we wondered whether other cytokine-mediated signaling pathways might account for the disease seen in these animals after IMQ application. We therefore analyzed the messenger RNA expression of several proinflammatory cytokines and chemokines in the skin of IMQ-treated mice by reverse transcriptase–PCR. As expected by the flow cytometry data, IL-17A messenger RNA expression was greatly enhanced in the skin of IL-$17RA^{del}$ mice compared with control animals (Figure 4). Further, the expression of the proinflammatory cytokines IL-22 and IL-6, as well as the chemokine CXCL-2, was strongly upregulated in IMQ-treated IL-$17RA^{del}$ mice compared with IMQ-treated

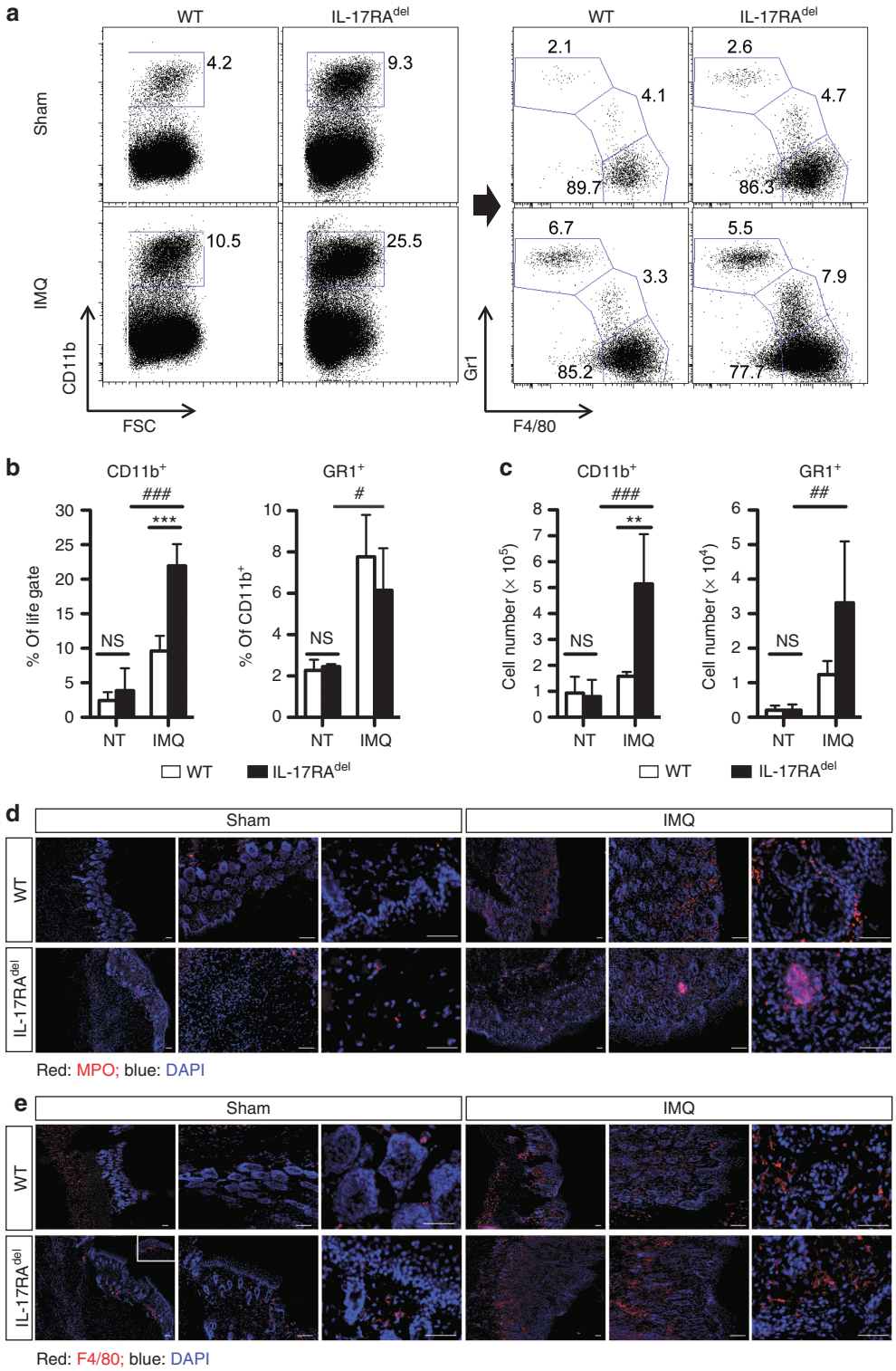

Figure 2. Invasion of neutrophilic granulocytes and macrophages to the skin of imiquimod (IMQ)-treated mice. IL-17RA^{del} and control mice in comparison with sham-treated controls after 8 days of treatment. (**a**) Flow cytometric analysis of the ear skin using the indicated antibodies. Right: CD11b^{+} gated cells showing GR1^{+} granulocytes and F4/80^{+} monocytes/macrophages. Panels are representative for three independent experiments including 3–4 mice each with comparable results. (**b**) CD11b^{+} cells invading the ears of IMQ- and sham-treated wild-type (WT) and IL-17RA^{del} mice shown as percentage of acquired cells of the live gate (analyzed with one-way analysis of variance (ANOVA)). (**c**) Total number of CD11b^{+} cells and GR1^{+} granulocytes in the ears of IMQ- and sham-treated WT and IL-17RA^{del} mice (analyzed with one-way ANOVA). (**d**, **e**) Immunofluorescence of back skin crysosections of IL-17RA^{del} and control mice after 9 days of sham or IMQ treatment: (**d**) Red = myeloperoxidase (MPO), blue = 4′,6-diamidino-2-phenylindole (DAPI). (**e**) Red = F4/80, blue = DAPI. Different magnifications are presented. Bar = 100 μm; in highest original magnification bar = 50 μm.

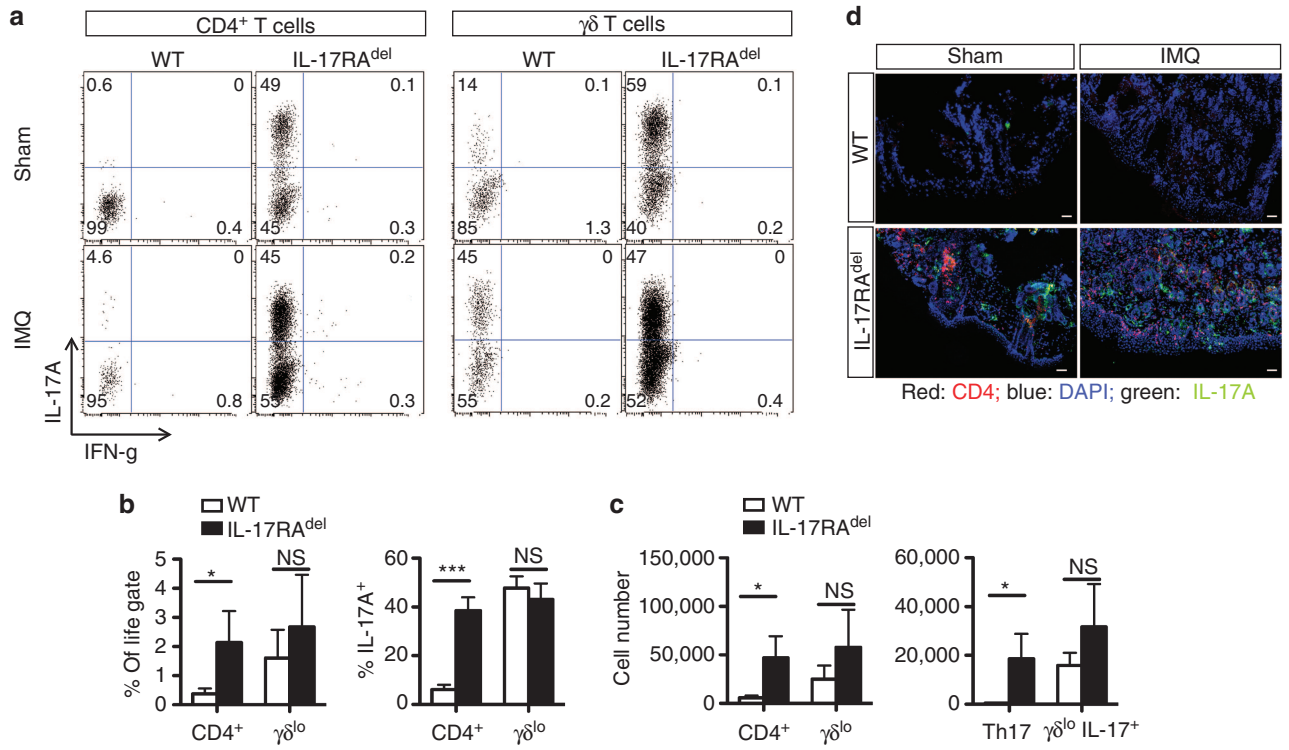

Figure 3. Invasion of Th17– and IL-17A–producing γδT cells in the back skin upon imiquimod (IMQ) treatment of IL-17RA^{del} and control mice in comparison with the sham-treated controls after day 7. (**a**) Flow cytometric analysis of cells isolated from the skin and stained with the indicated antibodies. Cells were pregated on B220^{-}, CD3^{+}, and on CD4^{+} cells (for Th17) or on γδ-TCR^{lo} for dermal γδ T cells. Panels are representative for three independent experiments each including 3–4 mice with comparable results. (**b**) Percentages of CD4^{+} and γδ-TCR^{lo} cells invading the skin of IMQ-treated WT and IL-17RA^{del} mice (left) and percentage of IL-17A^{+} CD4^{+} T cells (Th17) and IL-17A–producing γδT cells invading the skin of the indicated groups (right). (**c**) Total number of CD4^{+} and γδ-TCR^{lo} cells invading the skin of IMQ-treated WT- and IL-17RA^{del} mice (left) and Th17– and IL-17A–producing dermal γδ T cells (right). (**d**) Immunofluorescence of skin crysosections of IL-17RA^{del} and control mice with sham and IMQtreatment: red = CD4, green = IL-17A, blue = 4′,6-diamidino-2-phenylindole (DAPI). Bar = 100 μm.

WT mice. IL-6, IL-22, and CXCL-2 were shown to be associated with skin inflammation and neutrophil recruitment to sites of inflammation (Luttikhuizen *et al.*, 2007; Di Cesare *et al.*, 2009). In addition, also tumor necrosis factor-α was similarly high in IL-17RA^{del} mice as in WT IMQ-treated animals. In contrast, we found a significantly lower expression of IL-23p19, CCR6, and GM-CSF in the skins of IMQ-treated IL-17RA^{del} mice compared with IMQ-treated WT mice (Figure 4). The lower GM-CSF is in line with the lack of a functional receptor for IL-17A in these mice, as this cytokine is secreted in response to IL-17 signaling (McGeachy, 2011).

Sources of IL-6 and IL-22 in skin of IMQ-treated animals

We aimed to determine the cellular source of IL-6 in the skin of these mice. By intracellular staining, we found significantly more skin-infiltrating dermal (γδ-TCR^{lo}) and epidermal (γδ-TCR^{high}) γδ T cells (Figure 5a and b). We also found an increase in IL-6-producing CD4 T cells in IMQ-treated IL-17RA^{del} mice when compared with WT mice (Figure 5b) or with sham-treated IL-17RA^{del} mice. We further stained skin-infiltrating cells for IL-22 in conjunction with IL-17A and found that IL-22 was expressed by dermal γδ T cells (Figure 6a and b) and by CD4^{+} T cells (Figure 6c). Notably, the proportion of dermal γδ T cells and CD4 T cells that produce IL-22 in IMQ-treated IL-17RA^{del} mice is significantly higher compared with IMQ-treated control animals (Figure 6d). These findings suggest that, although IL-17 cytokines produced by αβ and γδ T cells are unable to signal via IL-17RA, the expanded cells in IL-17RA^{del} that express these cytokines may contribute to the pathogenic process in the skin by producing other proinflammatory cytokines, such as IL-6 and IL-22.

DISCUSSION

Our results demonstrate that a complete deletion of the IL-17 receptor A chain (IL-17RA^{del} mice) results in delayed and milder IMQ-induced psoriasis—but nevertheless the disease develops also without IL-17 signaling via IL-17RA. Most likely, the disease develops via elevated amounts of cytokines such as IL-6, IL-22, and the chemokine CXCL2, secreted by IL-17-producing αβ and γδ T cells.

The IMQ-induced psoriasis-like mouse model recapitulates many hallmark features of human psoriasis, including clinically hyperkeratosis, erythema, and scaling, and immunologically neutrophil microabscesses and infiltration of γδ T cells and Th17 cells to the skin (van der Fits *et al.*, 2009). This model is used to induce a fast, reproducible, and efficient

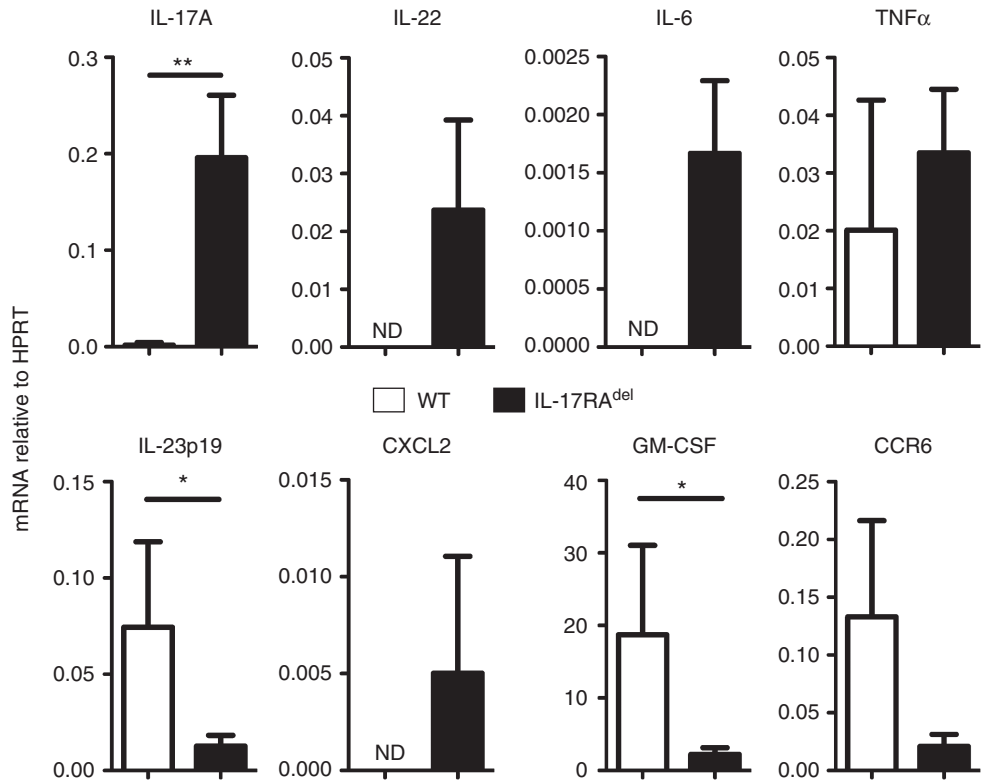

Figure 4. Expression of cytokines in the back skin of imiquimod (IMQ)-treated IL-17RA^{del} control mice after 7 days of treatment. Quantitative real-time reverse transcriptase (RT)–PCR analysis of the indicated genes in the skin of IMQ-treated IL-17RA^{del} mice in comparison with IMQ-treated control mice. Expression level shown relative to the housekeeping gene *HPRT* ($n = 4$ from two independent experiments with comparable results).

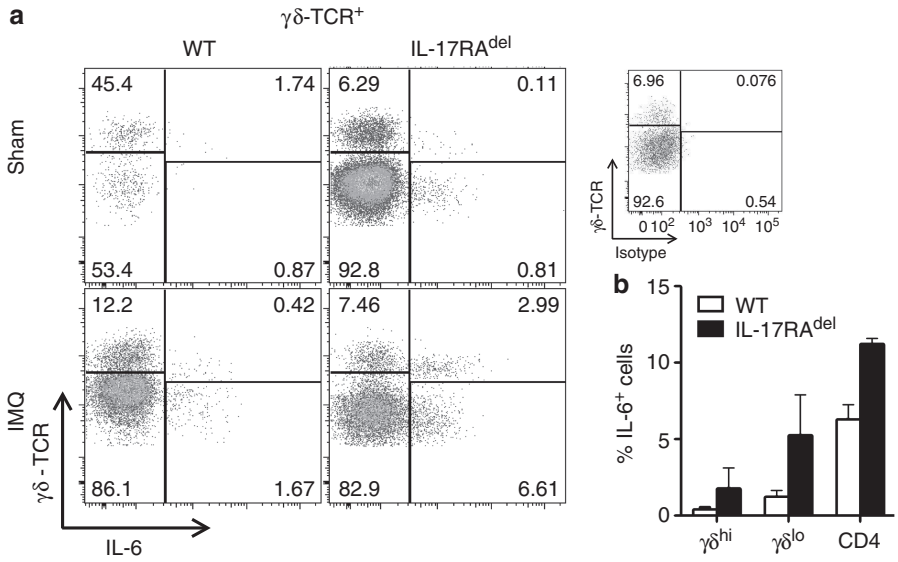

Figure 5. Elevated IL-6 production of T cells in the skin of imiquimod (IMQ)-treated IL-17RA^{del} and wild-type (WT) mice. (**a**) Flow cytometric analysis of IL-6–producing dermal γδ-TCR^{lo} and epidermal γδ-TCR^{hi} cells in the skin of IMQ- or sham-treated IL-17RA^{del} and WT mice. (**b**) IL-6–producing T cells. All cells are pregated on CD11b^{-} and CD3^{+}.

psoriasis-like pathology, which is based on the IL-23/IL-17 axis (Shear *et al.*, 2008). van der Fits *et al.* (2009) showed that the IMQ-induced skin inflammation in mice with a complete deletion of the IL-17RA receptor is profoundly suppressed compared with WT control mice. Here we show that the PASI score of IL-17RA^{del} mice is significantly reduced in comparison with the control group when treated with IMQ, but that there is no reduction of the IMQ-induced skin thickening and from day 4 onward there is no difference in scaling between the two groups.

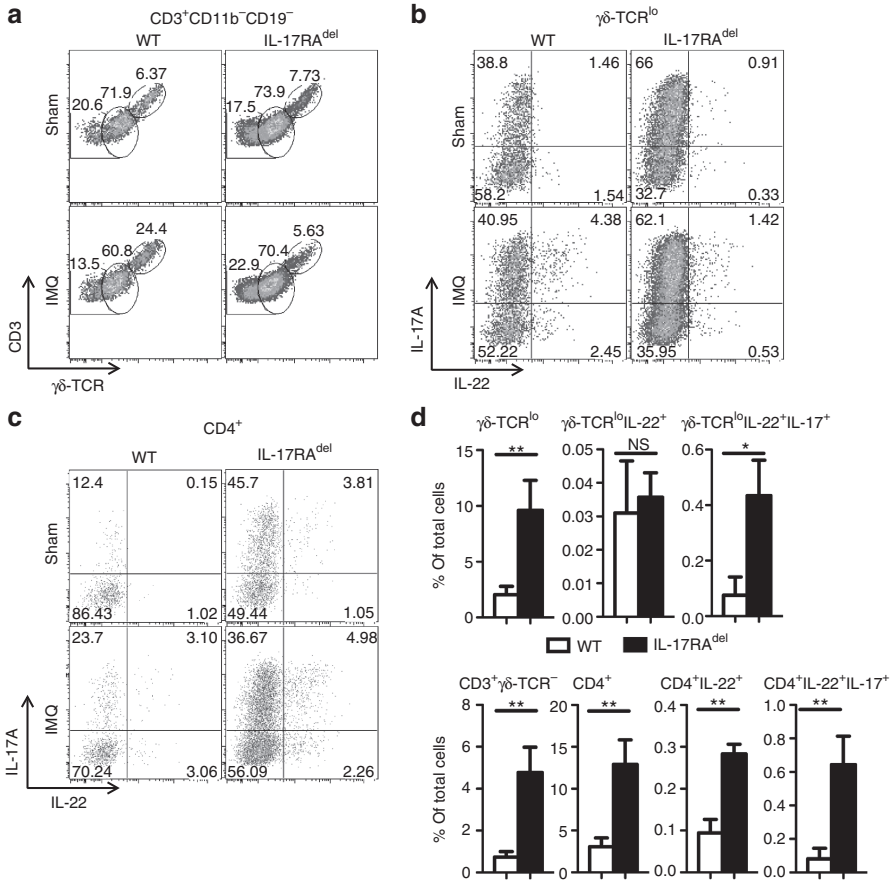

Figure 6. IL-22 production of T cells in the ear skin of imiquimod (IMQ)-treated IL-17RA^{del} and wild-type (WT) mice. (**a**) CD3$^+$ T cells in the skin: cells were pregated on CD3$^+$, CD11b$^-$, and CD19$^-$. (**b**, **c**) Flow cytometric analysis of IL-22–producing dermal γδ-TCR^{lo} and CD4$^+$ T cells in the skin of IMQ- or sham-treated IL-17RA^{del} and WT mice. (**d**) Statistical analysis of IL-22–producing γδ-TCR^{lo} and CD4$^+$ T cells shown as percentage of total cells. Bars represent $n=3$ per group; Student's *t*-test was used for statistical analysis.

It was shown that in IL-17RA KO mice both mobilization of peripheral neutrophils and their influx to infected organs is significantly impaired and delayed (Huang *et al.*, 2004). However, this does not mean that the neutrophil invasion or activation is completely abolished in fungal or bacterial infection—only that it is weakened and milder. In addition, the level of macrophage inflammatory protein in the serum of *Toxoplasma gondii*–infected IL-17RA KO mice was lower compared with controls but was not abolished (Kelly *et al.*, 2005). The latter is in agreement with our findings showing a mild development of IMQ-induced psoriasis in the IL-17RA^{del} mice. Nevertheless, also under conditions of IL-17RA deletion, activated neutrophils and macrophages are attracted to the site of inflammation.

Although IL-17C can also attract neutrophils when injected into the skin (Ramirez-Carrozzi *et al.*, 2011), this cytokine cannot be attributed to neutrophil attraction in IL-17RA^{del} mice, as IL-17C signaling, similar to that of IL-17A, F, and E, depends on IL-17RA (Chang *et al.*, 2011; Iwakura *et al.*, 2011; Song *et al.*, 2011). In the absence of IL-17A/F/E/C signaling, other cytokines secreted or induced by the elevated levels of IL-17A-producing γδ T cells and Th17 cells may take over. IL-6 is known to promote neutrophil induction and activation both *in vivo* and *in vitro* (Romani *et al.*, 1996). This proinflammatory cytokine was detected more than 20 years ago in human psoriatic lesions, and it was suggested that IL-6 enhances keratinocyte proliferation (Grossman *et al.*, 1989; Kishimoto *et al.*, 1992). Moreover, IL-6 is an important factor to switch differentiation of monocytes from DCs to macrophages (Chomarat *et al.*, 2000) and contributes to neutrophil recruitment (Hurst *et al.*, 2001). The increased level of IL-6 in the skin of IMQ-treated IL-17RA^{del} mice compared with treated WT skin supports a possible role of IL-6 in disease development in the absence of IL-17RA signaling.

The pivotal role of γδ T cells in human psoriasis has been stressed recently (Cai *et al.*, 2011). Cai *et al.* showed that, upon IL-23 stimulation, dermal γδ T cells produce IL-17A, which leads to disease progression in mice. In psoriasis patients, the numbers of γδ T cells are also elevated in the affected skin and produce large amounts of IL-17A. γδ T cells not only promote inflammation via IL-17 but are also known to produce IL-22 (Mabuchi *et al.*, 2011; Ness-Schwickerath and Morita, 2011). IL-22 signals to keratinocytes causing their activation and hyperproliferation. Activated keratinocytes produce downstream proinflammatory cytokines, chemokines, and also antimicrobial peptides, which are able to recruit immune cells to the inflammed skin, where they are in turn activated (Zheng *et al.*, 2007; Nograles *et al.*, 2008).

It was previously shown that IL-17F binding to IL-17RA regulates the production of IL-17A *in vivo* and that CD4 T cells from IL-17RA KO mice produce higher amounts of IL-17A *in vivo* (Nagata *et al.*, 2008). It was also shown that IL-17A or F treatment inhibits the expression of IL-17A (Smith *et al.*, 2008). In line with this, we not only found increased systemic levels of IL-17A in the IL-17RA-deficient mice but also increased numbers of IL-17A-producing $CD4^+$ and γδ T cells in the skin, LN, and spleen of these mice, even when naive. The reason for this increase of IL-17A-producing T cells is not yet clarified and is a subject of further investigation in our laboratory. Despite the initially elevated numbers of IL-17-competent cells, there is no skin inflammation in untreated IL-17RA^{del} mice, and only when we apply IMQ psoriasis develops in these animals. This suggests that IMQ indirectly activates the preexisting Th17 and γδ T cells, which secrete, in addition to IL-17, other cytokines such as IL-6 and IL-22 that initiate psoriasis independent of IL-17.

In conditions of IL-17RA deficiency, the effect of the Th2-associated IL-17E (IL-25) is also weakened: IL-17E is usually known as ''protective'' cytokine ameliorating diabetes (Emamaullee *et al.*, 2009) and suppressing experimental autoimmune encephalomyelitis (Kleinschek *et al.*, 2007). Weakening the IL-17E effect may result in the activation of a proinflammatory response, which could also add to inflammation induced by IL-6 and IL-22 produced by the elevated levels of IL-17-producing cells in the skin of IL-17RA^{del} mice.

In addition to the classical therapeutic options that are already in use for psoriasis, more selective anti-inflammatory treatments such as anti-tumor necrosis factor-α (Gudjonsson and Elder, 2008), anti-IL-6R (Tocilizumab), and anti-IL-12/IL-23 (Ustekinumab) are already licensed. In addition, a monoclonal antibody neutralizing IL-17A (Secukinumab) is currently tested in clinical trials (Kurzeja *et al.*, 2011). In light of our findings, one might consider to monitor long-term anti-IL-17A-treated patients for the development of increased numbers of IL-17-producing γδ T cells and Th17 cells.

In conclusion, we here demonstrate that IMQ-induced skin inflammation appears in the absence of IL-17RA signaling. Hence, besides the classical IL-23/IL-17 axis of neutrophil recruitment to the skin, other efficient pathways of neutrophil attraction and inflammation do exist, which are activated in the absence of IL-17RA signaling. This compensatory process should be taken into consideration when developing anti-psoriatic drugs targeting the IL-17 signaling pathway.

MATERIALS AND METHODS

Mice

All animal experiments were conducted in accordance with the guidelines of the central animal facility institution of the University of Mainz.

Detection of IL-17RA

Total RNA from spleens of WT, IL-17RA^{Fl}, and IL17RA^{del} mice was extracted using the RNeasy Mini Kit (Qiagen, Hilden, Germany). One microgram of total RNA was reverse transcribed using the SuperScript II reverse transcriptase (Invitrogen Life Technologies, Paisley, UK) according to the manufacturer's protocol using oligo $(DT)_{12–18}$ primer. Complementary DNA was amplified with the following primers specific for IL-17A receptor A (IL-17RA): 5′-GCAGCTGAACACCAATGAGC-3′ and 5′-GCAGCACCAGTGAA ACTTGC-3′. Glyceraldehyde-3-phosphate dehydrogenase was used as a reference gene (5′-ACCACAGTCCATGCCATCAC-3′ and 5′-TAC AGCAACAGGGTGGTGGA-3′).

IMQ-induced psoriasis

Eight-week-old female mice (IL-17RA^{del} and controls) were shaved and depilated on their back. One group was treated with Aldara cream (Meda AB, Solna, Sweden) containing 5% IMQ and the other with sham cream (Heib *et al.*, 2007) on one ear (5 mg) and on their back (55 mg) over 4–9 days once daily.

Intradermal IL-23 injections

We administered intradermal injection of 20 μl of phosphate-buffered saline containing 500 ng of recombinant mouse IL-23 (eBioscience, San Diego, CA) into the ears of anesthetized mice using a 30-gauge needle every day for eight consecutive days. Before and after injection, ear thickness was measured daily. Values of day 0 were taken as the starting value. Ear measurements were taken at the center of the ears using a dial thickness gage (Mitutoyo, Kawasaki, Japan). After 8 days, the mice were killed and tissue was collected.

PASI score for mice

The human PASI score describes the degree of erythema, scaling of skin, skin thickness, and affected area to characterize the severity of psoriasis in patients. We used a modified PASI score for the IMQ-induced psoriasis in mice consisting of the parameters skin thickness, scaling, and erythema. The thickness of back skin was measured in duplicates to triplicates using a thickness gage. A cumulative PASI score or the individual score concerning one parameter (skin thickness, scaling, erythema) is shown.

Flow cytometry

Skin was cleaned of fat tissue and incubated in a Liberase (Roche, Basel, Switzerland) and DNAse solution (Sigma-Aldrich, St Louis, MO) for 1.5 hours (back skin) or 1 hour (ears) before finally shredding with gentleMacs Dissociator (Miltenyi Biotec, Bergisch Gladbach, Germany). Lymph nodes and spleen were mechanically dissociated to yield a single-cell solution and were treated with ammonium chloride–potassium buffer for red blood cell lysis. Cells were treated with Fc-block (eBioscience). Surface staining was performed with CD11b ef450 (eBioscience), CD11b PE-Cy7 (eBioscience), CD11c allophycocyanin (APC) (BD, San Diego, CA), PDCA-1 Bio (Miltenyi Biotec), Streptavidin V500 (BD), Ly6c (BD), GR-1 APC (eBioscience), F4/80 PE (eBioscience), B220 ef780 (eBioscience), B220 PerCp (BD), CD3 PerCP (BD), CD4 V500 (BD), and γδ T-cell receptor FITC (BD). For intracellular cytokine staining, the cells were activated for 4 hours in phorbol 12-myristate 13-acetate (50 ng ml^{-1}) and Ionomycin (750 ng ml^{-1}) in the presence of Brefeldin A (1 mg ml^{-1}). Thereafter, cells were surface-stained, washed, and fixed in 2% paraformaldehyde in phosphate-buffered saline for 10 minutes on ice. Cells were then permeabilized with saponine buffer (0.1% saponine, 1% BSA, and 0.02% NaN3). Intracellular staining was performed with IL-17A PE (eBioscience), IFN-γ

APC (BD), IL-22 PE (BioLegend, San Diego, CA), and IL-6 PE (eBioscience) in saponine buffer. As intracellular isotype control, IgG1,κ PE (eBioscience) and IgG2a,κ (eBioscience) were used. Samples were acquired using a FACSCanto II flow cytometer (BD) and analyzed with FlowJo (TreeStar, San Carlos, CA).

Real-time reverse transcriptase–PCR

Total RNA was isolated from the skin using the RNeasy Mini Kit (Qiagen) after controlled crushing with Tissue Lyzer (Qiagen) and Proteinase K (Qiagen) incubation. Complementary DNA was prepared using the first-strand synthesis kit from Invitrogen Life Technologies. One microgram of complementary DNA was used for a quantitative real-time reaction using the QuantiTect SYBR Green reaction mixture (Qiagen) on white 96-well plates (Roche) with primer mixes from Qiagen as described on their homepage (https://www1.qiagen.com/GeneGlobe/Default.aspx). Real-time PCR was performed on a Roche Lightcycler 480II. The relative expression levels of the respective samples to HPRT are calculated with the delta-delta Ct method (Roche software) (Livak and Schmittgen, 2001).

Histology

Immunofluorescence of 10-μm cryosections was performed using the fluorescence microscope Olympus IX81 (Olympus, Tokyo, Japan) and the TSA Cy3 and TSA Fluorescein system (PerkinElmer, Waltham, MA) as recommended by the company. The following primary antibodies were used: F4/80 (BD), myeloperoxidase (Abcam, Cambridge, MA), CD4 (BD), and IL-17A (Santa Cruz Biotechnology, Santa Cruz, CA). The slides were incubated for 30 minutes at room temperature with the biotinylated secondary antibody (Dianova, Hamburg, Germany). Nuclei were counterstained with Hoechst 33342 (Invitrogen).

Statistical analysis

Data were analyzed for statistical significance using either the two-tailed unpaired Student's *t*-test or where indicated using the one-way analysis of variance test. Values of $P<0.001$, $P<0.01$, and $P<0.05$ were marked by three, two, and one asterisks (or rhomb), respectively. Columns in figures represent means ± standard deviation.

CONFLICT OF INTEREST

The authors state no conflict of interest. The manuscript contains a part of the medical thesis of K. El Malki.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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