European Journal of Immunology

AhR modulates the IL-22-producing cell proliferation/recruitment in imiquimod-induced psoriasis mouse model

Perrine M. Cochez[1,2], Camille Michiels[1,2], Emilie Hendrickx[1,2], Astrid B. Van Belle[1,2], Muriel M. Lemaire[1,2], Nicolas Dauguet[2], Guy Warnier[1], Magali de Heusch[1,2], Dieudonnée Togbe[3,4], Bernhard Ryffel[3,4], Pierre G. Coulie[2], Jean-Christophe Renauld[1,2] and Laure Dumoutier*[1,2]*

[1] Ludwig Institute for Cancer Research, Brussels Branch, Belgium
[2] de Duve Institute, Université catholique de Louvain, Brussels, Belgium
[3] INEM-UMR7355, Molecular Immunology, University of Orleans, Orleans, France
[4] CNRS, UMR7355 and Artimmune SAS, Orleans, France

IL-22 has a detrimental role in skin inflammatory processes, for example in psoriasis. As transcription factor, AhR controls the IL-22 production by several cell types (i.e. Th17 cells). Here, we analyzed the role of Ahr in IL-22 production by immune cells in the inflamed skin, using an imiquimod-induced psoriasis mouse model. Our results indicate that IL-22 is expressed in the ear of imiquimod-treated *Ahr*$^{-/-}$ mice but less than in wild-type mice. We then studied the role of AhR on three cell populations known to produce IL-22 in the skin: γδ T cells, Th17 cells, and ILC3, and a novel IL-22-producing cell type identified in this setting: CD4$^-$CD8$^-$TCRβ$^+$ T cells. We showed that AhR is required for IL-22 production by Th17, but not by the three other cell types, in the imiquimod-treated ears. Moreover, AhR has a role in the recruitment of γδ T cells, ILC3, and CD4$^-$CD8$^-$TCRβ$^+$ T cells into the inflamed skin or in their local proliferation. Taken together, AhR has a direct role in IL-22 production by Th17 cells in the mouse ear skin, but not by γδ T cells, CD4$^-$CD8$^-$TCRβ$^+$ T cells and ILCs.

Keywords: AhR · IL-22 · Imiquimod · Innate lymphoid cells (ILCs) · Skin · T cells

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Introduction

Interleukin-22 (IL-22) is a key cytokine for the homeostasis of mucosa and barrier organs including lung, gut, vagina, and skin. Epithelial cells and keratinocytes express the receptor for IL-22. IL-22 itself is produced by T cells, mostly Th17 cells and γδ T cells, in an inflammatory environment. It is also produced by innate lymphoid cells 3 (ILC3), previously named Lti-like or NK-like cells, in response to stimuli from the microbiota [1]. Beside anti-bacterial, anti-fungal, and anti-viral activities [2–4], IL-22 exerts pro- or anti-inflammatory effects depending on the site of the inflammation. It plays a protective role in models of colitis and hepatitis

Correspondence: Dr. Laure Dumoutier
e-mail: laure.dumoutier@uclouvain.be

*Shared senior co-authorship

[5, 6] whereas it has a detrimental role in arthritis and in skin inflammation [7, 8].

The regulation of IL-22 production by T cells has been studied mainly in Th17 cells and shown to depend on IL-23 and transcription factors RORγt and AhR (aryl hydrocarbon receptor) [9, 10]. AhR is pivotal for IL-22 production by different cell types. It is required for IL-22 production by Th17 cells : *Ahr*$^{-/-}$ mice mount Th17 responses but with T cells that fail to produce IL-22 [9, 11]. AhR is crucial for the presence in the gut of ILC3, the main source of IL-22 in this organ [12, 13]. There is a higher expression of pro-apoptotic genes in ILC3 from *Ahr*$^{-/-}$ mice, suggesting an AhR involvement in ILC3 survival. Moreover Notch, which is activated by AhR, is required for ILC3 to be present in the gut. Finally, in γδ T cells, the role of AhR has been studied on cells elicited by heat-killed mycobacteria in the peritoneal cavity [14] and on Vγ6^{+} T cells, a lung-specific population, after chronic exposition to *Bacillus subtilis* [15]. In both models, IL-22 production was reduced in *Ahr*$^{-/-}$ mice.

AhR binds environmental toxins such as 2,3,7,8-tetrachlorodibenzo-p-dioxin, dietary components, and endogenous ligands [16]. The latter includes photo-oxidized compounds derived from the irradiation of L-tryptophan by visible or UV light, such as FICZ (6-formylindolo(3,2-ß)carbazole), which is produced in the human skin [17]. These photometabolites could induce cutaneous AhR activation in lupus erythematosus patients and this activation correlates with skin inflammation [18]. AhR expression is also important for several skin-resident cell types: it is required for the proliferation of Vγ3^{+} T cells or DETCs (Dendritic Epidermal T cells) [19] and for the maturation of Langerhans cells [20].

Psoriasis is a prevalent immune-mediated inflammatory skin disease. Th17 cell responses participate in psoriasis' pathogenesis, as shown by the clinical efficacy of anti-IL-17A monoclonal antibodies [21]. The other T-cell-derived cytokine that is highly expressed in lesions of human psoriasis is IL-22 [22]. IL-22-transgenic mice die soon after birth and exhibit two typical features of psoriasis: acanthosis and hyperkeratosis [23]. IL-22 plays an important role in the murine model of psoriasis that is induced by imiquimod (Aldara). In the original setting of this model, psoriasis-like lesions appear on a large area of the shaved back skin following daily topical application of imiquimod over 7 days. More recently, a second setting was used by several investigators including ourselves, in which imiquimod is applied on both sides of the ears [24]. In these two modalities of the model, *Il22-/-* mice display less acanthosis than wild-type (WT) mice, and less expression in the affected skin of *K14*, a marker of undifferentiated keratinocytes, and of chemokines such as *Ccl3* and *Cxcl3* ([8, 25] and our unpublished observations).

The main sources of IL-22 in the imiquimod model of psoriasis are γδ and αβ T lymphocytes [8, 26]. However, in the absence of T lymphocytes (*Rag2*$^{-/-}$ mice), IL-22 is still produced, from unknown source(s) [8]. We analyzed here the requirement of AhR for the development of skin inflammation and for the local production of IL-22 by various populations of T and non-T cells.

Results

IL-22 production is decreased in Ahr-deficient mice after imiquimod application on the ears

To investigate the role of AhR on IL-22 production in the imiquimod model of psoriasis, we applied Aldara®, a cream that contains imiquimod, on the ears. We chose this modality because the target skin surface is smaller than with the application on the shaved back skin. We hypothesized that this would result in less systemic inflammation and mimic human psoriasis more closely. *Ahr*$^{-/-}$ mice were partially protected against skin lesions, showing significantly less ear swelling than WT littermates (Fig. 1A), as well as less acanthosis (Supporting Information Fig. 1A). We observed similar results in another model, using repeated injections of IL-23 in the ears of *Ahr*$^{-/-}$ or littermate control mice (data not shown).

We described previously that IL-22 was a major factor mediating acanthosis in the imiquimod model. As shown in Fig. 1B, *Il22* gene expression in the imiquimod-treated ears was lower in *Ahr*$^{-/-}$ than in WT mice. Along the same line, the proportion of IL-22^{+} cells was lower in the ears of *Ahr*$^{-/-}$ mice (Fig. 1C). In contrast with these lower levels of skin inflammation and IL-22 production in *AhR*$^{-/-}$ mice, we observed no difference for the expression of gene *Il17* (Fig. 1D), and observed higher levels of expression of the genes encoding Tnf-α (Fig. 1D), Cxcl1, Cxcl2, and Cxcl5, implicated in neutrophil recruitment (Supporting Information Fig. 2).

We conclude that AhR aggravates ear skin inflammation induced by topical imiquimod, contributing to increased levels of IL-22 but decreased levels of other inflammatory mediators.

In contrast, Di Meglio et al. showed recently that AhR dampens the severity of imiquimod-induced skin lesions and IL-22 gene expression was higher in imiquimod-treated skin [27]. These results are different from ours but were obtained with topical imiquimod on the shaved back skin instead of the ears. We therefore tried to understand this discrepancy. At histological level, *Ahr*$^{-/-}$ mice displayed less acanthosis than WT animals after imiquimod on ears (Supporting Information Fig. 1A) but increased acanthosis after imiquimod on the back (Supporting Information Fig. 1D). Similar differences were observed for the epidermis thickness and numbers of epidermis layers (Supporting Information Fig. 1B,E). In addition, after topical imiquimod on the back skin, *Ahr*$^{-/-}$ mice had lost 20% of their weight by the end of the treatment (Supporting Information Fig. 1F) whereas after treatment on ears, *Ahr*$^{-/-}$ mice did not lose weight (Supporting Information Fig. 1C). We hypothesized that imiquimod treatment on the back skin could have systemic effects, which were exacerbated in *Ahr*$^{-/-}$ mice. In line with this hypothesis, serum level of IL-6 and liver expression of acute phase reactant genes *Saa1* and *Saa*3 increased significantly in *Ahr*$^{-/-}$ mice after topical imiquimod on the back but not on the ears (Supporting Information Fig. 1G,H). Finally, IL-22 gene expression was not modified in *AhR*$^{-/-}$ mice compared with WT mice when imiquimod was applied on back skin (Supporting Information Fig. 3A). These results suggest that topical imiquimod on the back skin induced a local and systemic

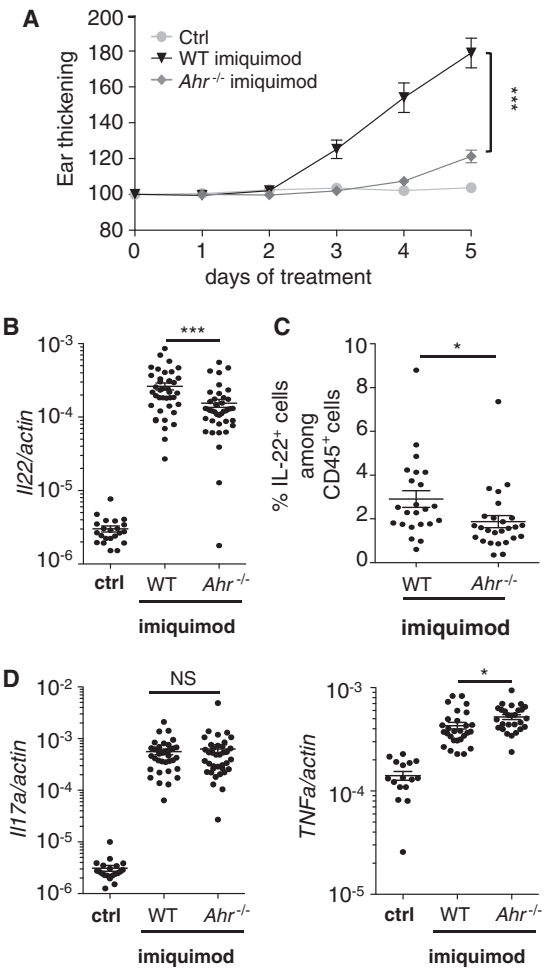

Figure 1. IL-22 production is decreased in *Ahr*-deficient versus WT mice after imiquimod application on ears. (A-D) *Ahr*$^{-/-}$ and WT C57BL/6 mice were treated daily with imiquimod on the ears for 5 days. (A) Ear thickness was measured daily with a micrometer screw to evaluate acanthosis. Data are shown as mean ± SEM and are pooled from five independent experiments ($n = 5$ mice/group in each experiment). (B and D) Ear epidermal cells were then analyzed by RT-qPCR. (B) *Il22* and (D) *Il17* and *Tnf* gene expression in ear normalized against ß-actin is shown. RNA was extracted from total ear. Data are shown as mean ± SEM and are pooled from six independent experiments ($n = 6$–7 mice/group in each experiment). (C) Flow cytometry analysis of IL-22$^+$ cells in the epidermis. Cells were gated on living CD45$^+$ cells and expression of IL-22 was analyzed (gating strategy for living CD45$^+$ cells is similar to Supporting Information Fig. 6E and for IL-22$^+$ cells to Fig. 2A). Data are shown as mean ± SEM and are pooled from five independent experiments ($n = 4$–5 mice/group in each experiment). Statistical analyses: A, Two-way ANOVA with Bonferroni post-test; B, Mann–Whitney test; C/D, unpaired *t*-test (NS = Not Significant, $^*p<0.05$ and $^{***}p<0.001$).

inflammation whereas treatment on the ears only induced local inflammation.

A new population of T cells producing IL-22 in imiquimod-treated ears

After imiquimod application on the ears, less CD45$^+$ cells produced IL-22 in *Ahr*$^{-/-}$ mice than in WT animals (Fig. 1C). We wished to know which IL-22-producing T cells were impacted by the absence of AhR. First, we used flow cytometry to phenotype the IL-22-producing T cells in the imiquimod-treated ears of WT mice. As shown in Fig. 2A, about 4% of the cells produced IL-22. They were equally distributed between γδ T cells and, surprisingly, CD4/CD8 double negative (DN) TCRβ T cells. This distribution was similar in dorsal skin (Supporting Information Fig. 3B). Taken together these data show that, in the imiquimod-treated epidermis of WT mice, IL-22 is mainly produced by T cells and the contribution of non-T cells is very small.

The production of IL-22 by γδ T cells in the imiquimod model is well established [8, 26]. Two populations of γδ T cells are present in the imiquimod-treated epidermis. The first consists of the dendritic epidermal T cells, which are resident cells with high levels of γδ TCR. These cells express mainly Vγ3 and do not produce IL-22 and are absent from *Ahr*$^{-/-}$ mice, as previously described (Supporting Information Fig. 4A) [19]. The second consists of cells that are recruited into the inflamed skin, carry lower levels of γδ TCR (γδ^{low}), and produce IL-22 [26].

The production of IL-22 by DN TCRß$^+$ T cells in the imiquimod model is a new observation. We could not find these cells in normal skin, indicating that they are recruited upon imiquimod treatment. We wondered whether they were TCRß$^+$ CD49b$^+$ or TCRß$^+$ NK1.1$^+$ NKT cells, which we found to be recruited in small numbers after imiquimod (Fig. 2B and C, central panels). We excluded the latter possibility, as the IL-22$^+$ DN TCRβ$^+$ cells were NK1.1$^-$ (Fig. 2B, left panel) and mostly CD49b$^-$ (Fig. 2C, left panel). By contrast, we show that these cells expressed Ccr6, Sca1, and Thy1 (Fig. 2D).

To further characterize these DN TCRβ$^+$ cells present in the epidermis of WT mice treated for 5 days with imiquimod, we sorted the total population of CD4$^-$ CD8$^-$ TCRβ$^+$ cells as shown on Supporting Information Fig. 5 to analyze the expression of selected genes. As controls, we also sorted non-T cells (γδ$^-$ and TCRβ$^-$, representing 14.6% of CD45$^+$ cells), γδ$^+$ T cells (10.4%) and CD4$^+$ CD8$^-$ TCRβ$^+$ cells (1.3%). The DN TCRβ$^+$ cells represented 2.2% of the CD45$^+$ cells (Supporting Information Fig. 5). They expressed *Il22*, *Il17a*, *Ifng*, *Il23r,* and *Rorgt* genes. They did not express detectable level of *Il4* and *Foxp3* genes (Fig. 2E). In addition, we performed a microarray analysis on γδ$^+$, CD4$^-$ CD8$^-$ TCRβ$^+$ and CD4$^+$ CD8$^-$ TCRβ$^+$ cells. The pattern of gene expression for a selection of T-cell markers show that the gene profile of CD4$^-$ CD8$^-$ TCRβ$^+$ cells is different from the profiles of the two other populations (Supporting Information Fig. 6).

Impact of the absence of AhR on γδ^{low} and DN TCRβ$^+$ T cells producing IL-22 in imiquimod-treated ears

Next, we compared WT and *Ahr*$^{-/-}$ mice for two parameters: the numbers of γδ^{low} and DN TCRβ$^+$ in ears treated with imiquimod, and the proportions of these cells that produced IL-22. The numbers of γδ^{low} and DN TCRβ$^+$ cells, and their proportions among CD45$^+$ cells, were lower in *Ahr*$^{-/-}$ mice than in WT mice (Fig. 3A, D and Supporting Information Fig. 4). As very few if

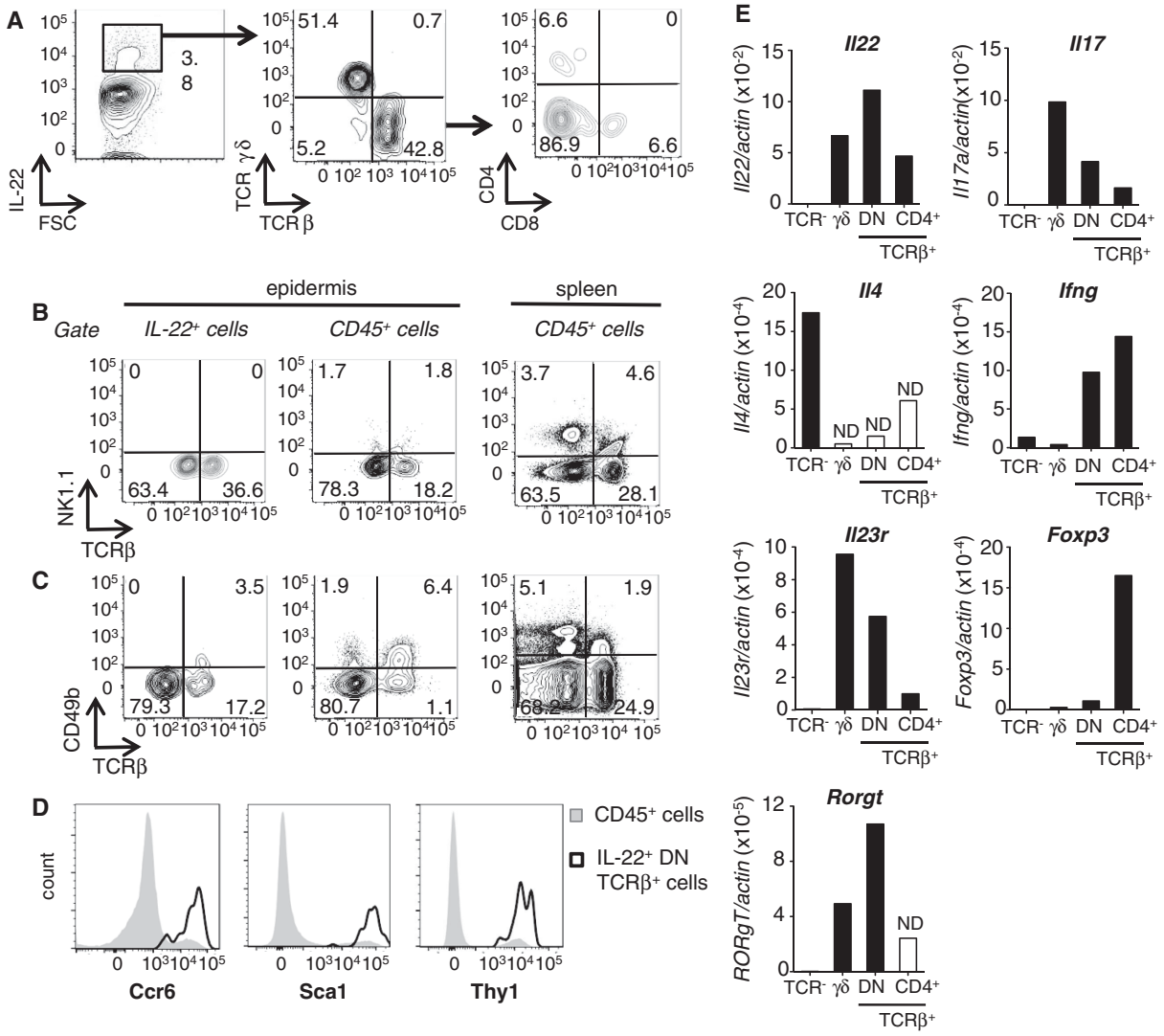

Figure 2. A new population of T cells producing IL-22 in imiquimod treated ears. (A–E) WT C57BL/6 mice were treated daily with imiquimod on the ears for 5 days. (A–D) Ear epidermal cells were analyzed by flow cytometry. (A) Characterization of IL-22$^+$ cells. Cells were gated on living CD45$^+$ cells. One representative graph is shown ($n = 12$). (B and C) NK1.1 (B) and CD49b (C) staining on IL-22$^+$ cells from the epidermis or CD45$^+$ from the spleen and from epidermis. One representative graph is shown for each staining ($n = 4$). (D) Characterization of surface markers, Ccr6, Sca1, and Thy1, on CD4$^-$ CD8$^-$ (DN) TCRβ$^+$ IL-22$^+$ cells among CD45$^+$ (gray filled) or IL-22$^+$ cells (bold line). One representative histogram is shown for each staining ($n = 4$). (E) Characterization of gene expression in CD4$^-$ CD8$^-$ (DN) TCRβ$^+$ cells by RT-qPCR. RNA was extracted from four sorted epidermis populations (among living CD45$^+$ cells): TCR$^-$ (TCRβ$^-$ and γδ$^-$), γδ^{low} (γδ), DN TCR β$^+$ and CD4$^+$ TCRβ$^+$ cells. Gene expression was normalized against ß-actin. Similar results were obtained in three different experiments (ND = not detected).

any of these cells were detected in normal skin, these differences resulted from a lower recruitment or proliferation.

For the proportions of IL-22-producing cells, the results differed for γδ^{low} and DN TCRβ$^+$ cells. It was higher among the γδ^{low} cells from *Ahr*$^{-/-}$ mice than among those of WT mice (Fig. 3B and C, left panel). But it did not differ significantly among the DN TCRβ$^+$ cells of *Ahr*$^{-/-}$ and WT mice (Fig. 3E and F, left panel), as the lower numbers of IL-22$^+$ DN TCRβ$^+$ cells in *Ahr*$^{-/-}$ mice (Fig. 3F, right panel) paralleled those of the total TCRβ$^+$ DN cells (Fig. 3D). Noteworthy, the intensities of IL-22 staining were not lower in the γδ^{low} or DN TCRβ$^+$ cells from *Ahr*$^{-/-}$ mice than in those of WT mice (Fig. 3B and E), indicating similar levels of IL-22 production.

Taken together these results indicate that IL-22 production in the imiquimod-treated ears of *Ahr*$^{-/-}$ mice is lower than in WT animals because of a lower recruitment or proliferation of γδ^{low} T cells and DN TCRβ$^+$ cells, and not because of a reduced capacity of these cells to produce IL-22.

Impact of the absence of AhR on IL-22-producing Th17 cells in imiquimod-treated ears

Among CD4$^+$ T cells, *Ahr* is expressed only in cells committed to produce IL-17 or IL-22. In vitro and in the spinal cord, murine

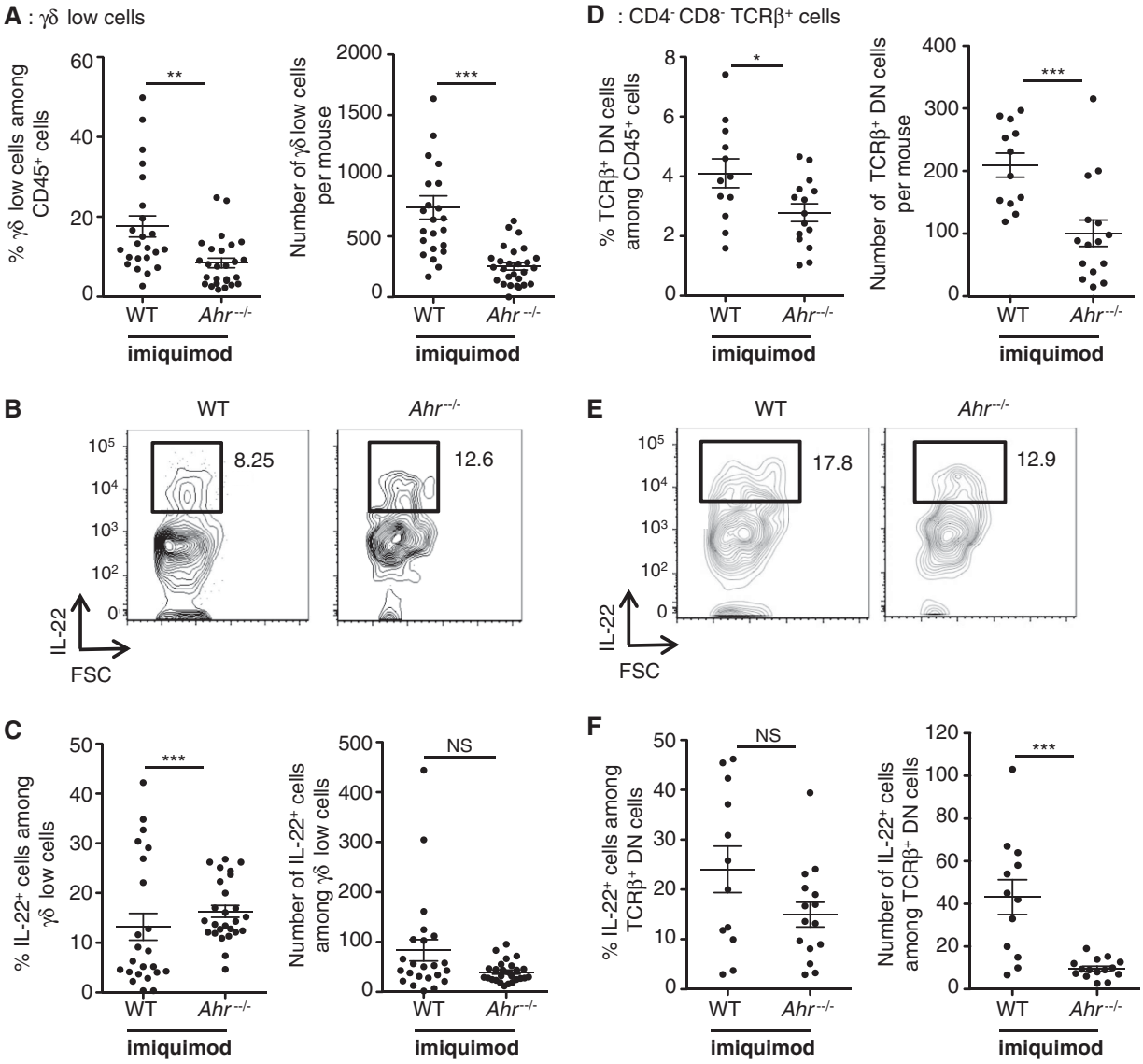

Figure 3. Impact of the absence of AhR on the $\gamma\delta^{low}$ and DN TCRβ^+ T cells that produce IL-22 in imiquimod-treated ears. (A–F) *Ahr*$^{-/-}$ and WT C57BL/6 mice were treated daily with imiquimod on the ears for 5 days. Ear epidermal cells were analyzed by flow cytometry. (A and D) Proportions and absolute numbers of $\gamma\delta^{low}$ T cells (A) and CD4$^-$ CD8$^-$ TCRβ^+ cells (D) among living CD45$^+$ cells. Data are shown as mean ± SEM and are pooled (A) from five independent experiments ($n = 5$ mice/group in each experiment) and (D) from three independent experiments ($n = 4$ mice/group in each experiment). (B and E) Contour plots of IL-22 production by $\gamma\delta^{low}$ T cells (B) and CD4$^-$ CD8$^-$ TCRβ^+ cells (E) among living CD45$^+$ cells. One representative plot is shown for each population. (C and F) Proportions of IL-22$^+$ cells among $\gamma\delta^{low}$ T cells (C) or CD4$^-$ CD8$^-$ TCRβ^+ cells (F). Cells were gated on living CD45$^+$ cells and then on TCRß$^-$ $\gamma\delta^{low}$ or CD4$^-$ CD8$^-$ TCRβ^+ cells, before assessing staining for IL-22. Data are shown as mean ± SEM and are pooled (C) from five independent experiments ($n = 4$–5 mice/group in each experiment) and (F) from three independent experiments ($n = 4$ mice/group in each experiment). Statistical analyses: Mann–Whitney test (NS = Not Significant, $^*p<0.05$, $^{**}p<0.01$, and $^{***}p<0.001$).

Ahr$^{-/-}$ CD4$^+$ T cells produce IL-17 but do not produce IL-22 [9]. It was not known whether AhR was required also for the production of IL-22 by CD4$^+$ T cells in the skin.

We evaluated that the number of IL-22-producing CD4$^+$ T cells in a mouse inflamed ear after imiquimod application is about 10 cells/ear, which is too low for a robust analysis (Fig. 2A, right panel). We resorted to the analysis of CD4$^+$ T cells in imiquimod-treated ears from irradiated *Rag2*$^{-/-}$ *IL-22*$^{-/-}$ mice reconstituted with bone marrow from WT or *Ahr*$^{-/-}$ mice. It has been shown that under these circumstances, skin immune cells are mostly Th17 cells [25]. We confirmed skin infiltration by Th17 cells (Fig. 4A, top panel). Some of these cells also produced IL-22 when the reconstituting cells were of WT origin. On the contrary, IL-22 was barely detected with bone marrow cells from *Ahr*$^{-/-}$ mice (Fig. 4A), leading to a sharp and reproducible decrease in the proportion of IL-22-producing cells among Th17 cells (Fig. 4B). Noteworthy, the proportion of IL-17-producing cells among CD4$^+$ T cells was unaffected (Fig. 4A).

These results are the first indication that AhR is required for the production of IL-22 by CD4$^+$ T lymphocytes in vivo in the skin.

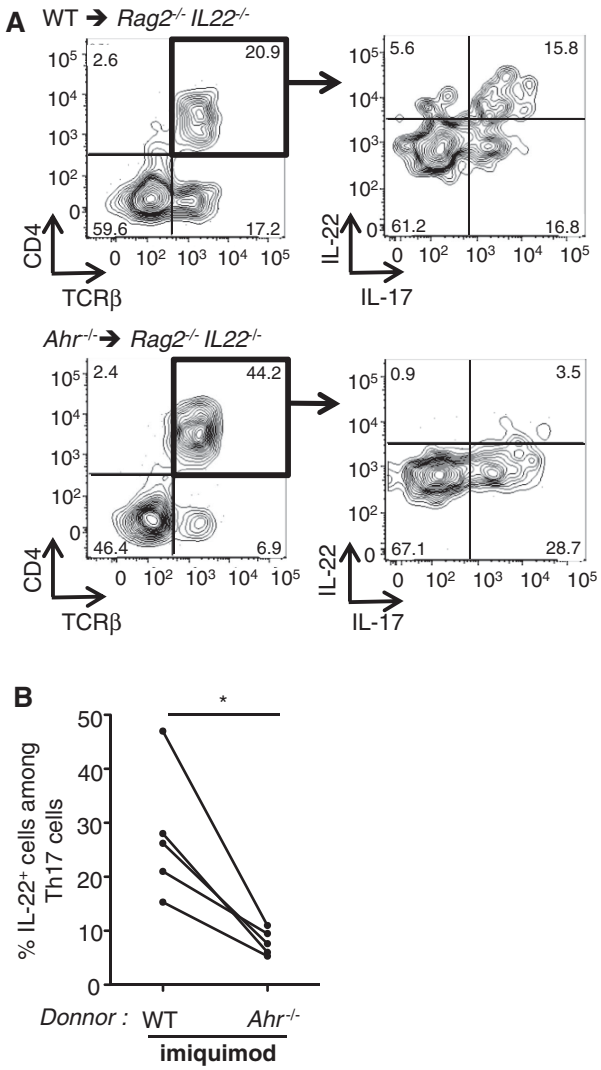

Figure 4. Impact of the absence of AhR on IL-22-producing Th17 cells in imiquimod-treated ears. (A–B) *Rag2*$^{-/-}$ *Il22*$^{-/-}$ mice reconstituted with *Ahr*$^{-/-}$ or WT C57BL/6 bone marrow cells were treated daily with imiquimod on the ears for 5 days. *Rag2*$^{-/-}$ *Il22*$^{-/-}$ mice were lethally irradiated and then reconstituted. After 4 weeks, imiquimod was applied. Ear epidermal cells were analyzed by flow cytometry. (A) Flow cytometry analysis of IL-22$^+$ and IL-17$^+$ cells among CD4$^+$ TCRß$^+$ in the epidermis. Cells were gated on living CD45$^+$ cells. One representative plot is shown for each group. (B) Proportions of IL22$^+$ cells among Th17 cells (TCRβ$^+$ CD4$^+$ IL-17$^+$). Cells were gated on living CD45$^+$ cells. Similar results were obtained in five different experiments, each experiment was represented with one line. Statistical analysis: paired *t*-test (*$p<0.05$).

Impact of the absence of AhR on IL-22-producing ILC3 in the imiquimod-treated ears of Rag2$^{-/-}$ mice

We showed previously that in the imiquimod-treated back skin of *Rag2*$^{-/-}$ mice, *Il22* transcription was quantitatively similar to that observed in the inflamed skin of WT animals [8]. We wished to verify this surprising result with imiquimod application on the ears, identify the non-T cells that produce IL-22 in this context, and examine the role of AhR.

As shown in Fig. 5A, *Il22* is transcribed at high levels in the imiquimod-treated ears of *Rag2*$^{-/-}$ mice, confirming our results obtained after imiquimod application on back skins. Flow cytometry experiments indicated that the IL-22-producing cells expressed CD45 (data not shown), making up ±4% of the CD45$^+$ cells (Fig. 5B), and expressed Sca1, Thy1, CD127, Ccr6, NKp46, and CD4 (Fig. 5C). This phenotype corresponds to that of a subgroup of ILC3 [28], characterized by the co-expression of CD4 and NKp46. ILC3 that produce IL-22 were recently shown to be present also in human psoriatic skin [29].

To analyze the role of AhR, we applied imiquimod on the ears of *Rag2*$^{-/-}$ *Ahr*$^{-/-}$ mice. These mice displayed less acanthosis (Fig. 5D), smaller numbers of epidermis epithelial layers (Fig. 5E), less ear swelling (Fig. 5F), and lower levels of *Il22* gene expression than *Rag2*$^{-/-}$ mice (Fig. 5A). We observed in the inflamed ears of *Rag2*$^{-/-}$ *Ahr*$^{-/-}$ vs *Rag2*$^{-/-}$ mice lower proportions of IL-22$^+$ cells among the CD45$^+$ cells (Fig. 5B). The intensities of IL-22 staining were similar in the two strains (data not shown). Moreover we observed lower proportions of Ccr6$^+$ cells among the CD45$^+$ cells and lower absolute numbers of Ccr6$^+$ cells in the inflamed ears of *Rag2*$^{-/-}$ *Ahr*$^{-/-}$ vs *Rag2*$^{-/-}$ mice (Fig. 5G). Altogether these data suggest that for ILC3 in this experimental setting, AhR is important for the recruitment or proliferation of the cells but not for *Il22* gene transcription.

Discussion

Our results indicate that in imiquimod-treated ears AhR contributes to acanthosis and is required for IL-22 production by Th17 but not by the other IL-22-producing cells: γδ T cells, CD4$^-$ CD8$^-$ TCRβ$^+$ T cells and ILC3. However for these three cell populations, AhR has a role in their recruitment or proliferation into the inflamed skin or in their local proliferation.

The milder epidermal acanthosis that we observed in *Ahr*$^{-/-}$ vs WT mice after imiquimod treatment on ears was associated with lower levels of *Il22* but similar or higher levels of the other acanthosis-inducing inflammatory cytokines such as *Il1*, *Il17*, *Il19*, *Il23,* and *Il36* (Fig. 1D and data not shown). In as much as acanthosis is cytokine-driven, our results suggest that when imiquimod is applied to the ears, IL-22 is responsible for most of the epidermis thickening, although the decrease of IL-22 expression in *AhR*$^{-/-}$ skin is only partial and other unidentified factors might also be involved.

Neutrophil influxes were higher in the epidermis of *Ahr*$^{-/-}$ mice, in line with higher levels of chemokines associated with neutrophil recruitment (Supporting Information Fig. 2). It suggests that AhR has an anti-inflammatory role in the skin, even though it contributes to acanthosis and IL-22 production. Di Meglio et al. also described an anti-inflammatory effect of AhR in the imiquimod-treated back skin of *Ahr*$^{-/-}$ mice, in which they observed neutrophilia [27].

Imiquimod application on ears or back of *Ahr*$^{-/-}$ mice does not have identical consequences on the treated skin. After 5 days,

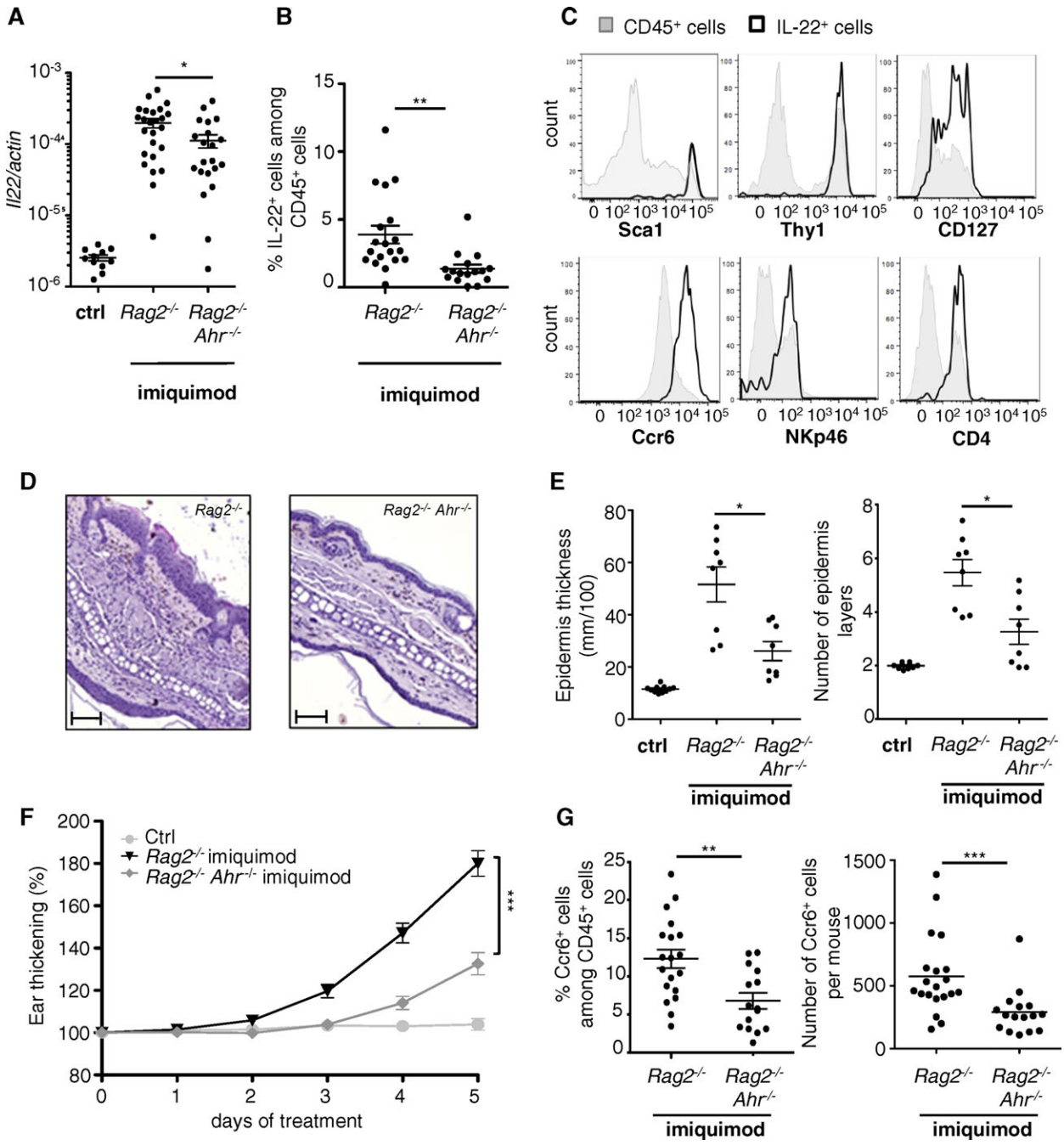

Figure 5. Impact of the absence of AhR on IL-22-producing ILC3 in imiquimod-treated ears of *Rag2*$^{-/-}$ mice. (A–G) *Ahr*$^{-/-}$ *Rag2*$^{-/-}$ and *Rag2*$^{-/-}$ mice were treated daily with imiquimod on the ears for 5 days. *Ahr*$^{-/-}$ *Rag2*$^{-/-}$ and *Rag2*$^{-/-}$ mice are littermate mice generated by crossing *Rag2*$^{-/-}$ BALB/c mice with *Ahr*$^{-/-}$ B6. (A) *Il22* gene expression in ear epidermal cells was analyzed by RT-qPCR and normalized against ß-actin. RNA was extracted from total ear. Data are shown as mean ± SEM and are pooled from four independent experiments ($n = 5$ mice/group in each experiment). (B) Flow cytometry analysis of IL-22$^+$ cells in the epidermis. Cells were gated on living CD45$^+$ cells and expression of IL-22 was analyzed (Gating strategy for living CD45$^+$ cells similar is to Supporting Information Fig. 6E and for IL-22$^+$ cells to Fig. 2A). Data are shown as mean ± SEM and are pooled from four independent experiments ($n = 5$ mice/group in each experiment). (C) Characterization of ear IL-22$^+$ cells in *Rag2*$^{-/-}$ mice by flow cytometry with staining of different markers on CD45$^+$ (gray filled) or IL-22$^+$ cells (bold line). One representative histogram is shown for each staining ($n = 3$). (D) H&E staining of skin lesions (original magnification ×20, scale bar = 100 μm). One representative picture is shown for each group. (E) Evaluation of acanthosis by measuring epidermis thickness and counting the number of epidermis layers in the skin sections. Data are shown as mean ± SEM and are pooled from two independent experiments ($n = 4$ mice/group in each experiment). (F) Ear thickness was measured daily with a micrometer screw to evaluate acanthosis. Data are shown as mean ± SEM and are pooled from four independent experiments ($n = 5$ mice/group in each experiment). (G) Proportions and absolute numbers of Ccr6$^+$ cells in the epidermis among living CD45$^+$ cells (Gating strategy for living CD45$^+$ cells is similar to Supporting Information Fig. 6E). Data are shown as mean ± SEM and are pooled from three independent experiments ($n = 5$–6 mice/group in each experiment) Statistical analyses: (A) Two-way ANOVA with Bonferroni post-test; (C) Mann–Whitney test; (D) unpaired *t*-test; (E/G) Mann–Whitney test (*$p<0.05$, **$p<0.01$, and ***$p<0.001$).

while neutrophilia is present in both cases, acanthosis and IL-22 production are more pronounced in the back than in the ears ([27] and our results). In addition, we observed serum acute phase reactants (IL-6, *Saa*) and weight loss but only after application on the back. Thus, in *Ahr*$^{-/-}$ mice, imiquimod applied on the back skin leads to systemic inflammation, which in turn might be necessary but not sufficient for acanthosis and IL-22 production in the inflamed skin. Several factors might explain why systemic inflammation follows imiquimod application on the back skin but not on the ears: differences in the areas of treated skin, in the amount of imiquimod applied per unit area, or in attributes of the skin. We estimate that we treated 3.4 cm^2 of skin on the ears versus 10.4 cm^2 on the back. We applied approximately 1.6 mg of imiquimod on the two ears versus 3.1 mg on the back, resulting in applications of 0.5 and 0.3 mg/cm^2 for ears and back, respectively. Thus the level of systemic inflammation increases with the areas of treated skin (3-fold difference between ears and back) and not with the amounts of applied imiquimod per unit area. We feel that other and possibly more important differences might be intrinsic to the treated skins such as the quality of the vasculature, the density of TLR7$^+$ cells, the kinetics of their migration into the blood stream or a difference in the microbial communities in different skin areas. It would be interesting to treat a smaller area of the back skin of *Ahr*$^{-/-}$ mice with a smaller amount of imiquimod to distinguish between skin intrinsic or extrinsic factors. Di Meglio et al. did almost that, applying 1.5 mg of imiquimod on back skin areas that appear on a figure to amount to 4 cm^2 [27]. We suspect that in that setting acanthosis and local IL-22 production, which they observed, were accompanied by systemic inflammation. If this proves to be the case, location matters more than quantity for imiquimod to induce systemic inflammation.

In the imiquimod-treated ears IL-22 is mainly produced by three different types of cells: γδ T cells, ILC and CD4$^-$ CD8$^-$ (DN) TCRβ$^+$ cells. To the best of our knowledge the latter cells have not been described to produce IL-22. These T cells are CD4$^-$ CD8$^-$ TCRβ$^+$ RORγT$^+$ IL-23R$^+$ IL-17$^+$ and IL-22$^+$. We were not able to confirm the expression of a TCR-α chain due to the lack of a good tool to address this question. These cells are very similar to a population of entheseal resident T cells that express CD3$^+$ CD4$^-$ CD8$^-$ RORγT$^+$ IL-23R$^+$ IL-17$^+$ and IL-22$^+$ [30]. In addition, the skin DN TCRβ$^+$ cells are phenotypically similar to T cells described in two models of bacterial infection, and whose IL-22 production is likely but has not been shown [31, 32]. In the inflamed ear skin of *Ahr*$^{-/-}$ mice we found less DN TCRβ$^+$ cells than in the inflamed ear skin of WT mice, but the production of IL-22 by these cells was normal. The effect of AhR could be direct because DN TCRβ$^+$ cells seem to express low level of AhR RNA (Supporting Information Fig. 6) but it could also be indirect and depend on other cells, as keratinocytes. Indeed, keratinocytes express *Ahr* and may be involved, as their expression of constitutively activated AhR was shown to lead to acanthosis and expression of chemokines such as CCL20 [33]. Which immune cells are recruited in that context is not known.

The second population of cells that produce IL-22 in imiquimod-treated ears is γδ^{low} T cells. Our results show that AhR supports the recruitment or proliferation of these cells into the inflamed skin but has no influence on their capacity to produce IL-22. At odds with our results, two studies reported that AhR was required for IL-22 production by γδ T cells. First, IL-22 production by γδ T cells in lungs infected with *Bacillus subtilis* was lower in *Ahr*$^{d/d}$ than in WT mice [15]. *Ahr*^{d} has a reduced affinity for ligands [34]. In that study it was the number of IL-22-producing cells that was reduced, with no obvious difference in the amount of IL-22 produced per cell. Second, IL-22 production by γδ T cells in the peritoneal cavity of mice injected with heat-killed *M.tuberculosis* was lower in *Ahr*$^{-/-}$ than in WT mice [14]. Here the recruitment of γδ T cells was unchanged. Thus the role of AhR on IL-22 production by γδ T cells is contextual.

ILC3 are the third population of cells that produce IL-22 in the imiquimod-treated ears. We studied these cells in *Rag2*$^{-/-}$ mice, in which ILCs are the main source of IL-22 after the administration of imiquimod [8]. Here we show that in the inflamed ears of these mice the absence of AhR leads to lower levels of IL-22 and less infiltration by ILC3. These results are reminiscent of observations made in the gut, where AhR was required for the presence of ILC3 [35]. Two mechanisms have been proposed: c-Kit and Notch. *Kit* 's promoter is activated by AhR and c-Kit regulates the development of a subpopulation of ILC3 [36]. Notch is involved in the AhR-dependent presence of NKp46$^+$ ILC3 in the small intestine lamina propria [13]. As the ILC3 studied here do also express NKp46, it is possible that Notch is involved in the differentiation or recruitment of ILC3 in the skin. Noteworthy, ILC3 were recently shown to be enriched in human psoriatic skin as compared with normal skin, with an important contribution to IL-22 production [29].

Altogether our results indicate that AhR plays a role in the recruitment or the proliferation of all three populations of cells that produce IL-22 in imiquimod-treated ears. Surprisingly, AhR is not necessary for IL-22 production by these cells. It contrasts with the requirement of AhR for IL-22 production by Th17 cells, which we confirmed here for skin Th17 cells in a bone marrow transfer model. The reason why AhR is required for IL-22 production specifically by Th17 needs further studies but might reflect interaction or competition with other transcription factors. For instance, c-Maf, which is induced by TGF-β, binds to the IL-22 promoter and blocks its expression. In this condition, AhR activation is required to overcome the suppression by TGF-ß and c-Maf [37]. This mechanism could be specific to Th17 cells because TGF-β is involved in their differentiation. Whatever the molecular mechanism by which AhR modulates IL-22 expression or recruitment of IL-22 producing cells, as IL-22 is deleterious in inflammatory skin diseases such as psoriasis, inhibiting AhR could ameliorate skin lesions. However, AhR has also a protective role by decreasing cutaneous neutrophilia. It is only the in vivo testing of candidate AhR inhibitors that will determine the clinical applicability of such drugs for skin inflammatory disorders.

Materials and methods

Mice and treatments

All mice used in this study were bred in our animal facility under specific pathogen-free conditions. *Ahr*$^{-/-}$ C57BL/6 mice [38] were provided by D. Togbe (CNRS UMR7355. Orléans, France). *Rag2*$^{-/-}$ BALB/c mice were purchased from Taconic (Ejby, Denmark). *Rag2*$^{-/-}$ *Il22*-deficient mice were generated by crossing *Rag2*$^{-/-}$ with *Il22*-deficient BALB/c mice [39]. *Rag2*$^{-/-}$ *Ahr*$^{-/-}$ mice were generated by crossing *Rag2*$^{-/-}$ BALB/c mice with *Ahr*$^{-/-}$ B6 mice and littermate were used for the experiments. For adoptive transfer experiments, bone marrow cells obtained by flushing femurs of WT or *Ahr*$^{-/-}$ mice were depleted of T cells using anti-CD5 magnetic MicroBeads (Milteny Biotec) and injected i.v. (10^7 cells) into irradiated *Rag2*$^{-/-}$ *Il22*$^{-/-}$ mice. After 4 weeks, mice were treated with imiquimod for 4 or 5 days. All animal experiments were performed in compliance with national and institutional guidelines and were approved by the local ethical committee.

Eight-week-old mice were shaved behind the ears or on the back and received five daily topical applications of imiquimod (0.8 mg per ear or 3.125 mg on the back) from a commercially available cream (Aldara 5%; 3M Pharmaceuticals). Ear thickness was measured every day with a micrometer screw (Mitutoyo) and mice were weighed daily. Paraffin tissue blocks of ear or dorsal skin biopsies were prepared using routine methods. Consecutive sections were stained with H&E and scanned with Mirax (Zeiss). Two evaluators analyzed the staining.

Single-cell suspension and FACS staining

Ears were dissected and incubated overnight at 4°C in dispase II at 1U/ml (Roche). After separation of epidermis and dermis with forceps, epidermal cells were isolated by digestion with DNAse I (Roche) at 0.5 mg/ml during 20 min at room temperature [40]. After a mechanical disruption, cells were filtered on 40-mm nylon filters to obtain a single-cell suspension and incubated for 3 h at 37°C in GolgiStop solution (BD Biosciences). Cells were then preincubated with 10 μg/mL of purified rat anti-mouse CD16-CD32 mAb (Fc Block; BD Pharmingen) before incubation with a viability marker (LIVE/DEAD® Fixable Near-IR Dead Cell Stain Kit, Life) and 2 μg/mL of antibodies to extracellular staining for 1 h at 4°C. Then, for intracellular cytokine staining, cells were fixed, permeabilized, using the Cytofix/Cytoperm Plus Kit (BD Biosciences) and finally stained with MH22B2 (anti–IL-22) or MM17F3 (anti-IL-17A) Abs, produced in our laboratory [41]. The specificity of the IL-22 staining was verified in IL-22$^{-/-}$ mice. Cells were analyzed with an FACS Fortessa (BD Biosciences). Gating included forward and side scatter, a viability marker and CD45 expression. Data were analyzed with the FlowJo software (Tree Star). For FACS sorting experiments, we used a FACSAria III (BD Biosciences). A 85 μm nozzle was used with a sheath pressure of 45 psi, a drop drive frenquency of 65 kHz, and a four-way purity mode (0-32-0). Sample injection chamber and collection tubes were maintained at 4°C. RNA was extracted after the sorting. The following antibodies from Biolegend were used: PercP or AlexaFluor700-labeled anti-CD45 (30-F11), AlexaFluor700-labeled anti-Ly6G/C (Gr1), PE-labeled anti-Ccr3 (J073E5), PE-Cy7-labeled anti-CD4 (GK1.5), AlexaFluor700-labeled CD8a (53-6.7), FITC-labeled anti-TCRβ (H57-597), PE-labeled anti-TCRγδ (GL3), PE-labeled anti-CD127 (SB/199), AlexaFluor488-labeled anti-CD25 (PC61), PE-labeled anti-Ccr6 (29-2L17), PercP-labeled anti-Thy1.2 (30-H12), PE-labeled anti-CD49b (Dx5), PE-labeled anti-NK1.1 (PK136), and FITC-labeled anti-NKp46 (29A1.4).

RT-qPCR

Total RNA was isolated from ears with the TriPure isolation reagent (Roche). Reverse transcription was performed on 1 μg of total RNA with oligo(dT) primer (Eurogentec) and Moloney murine leukemia virus reverse transcriptase (Invitrogen, Carlsbad, CA, USA). Quantitative PCR (qPCR) amplifications were performed on cDNA obtained from 25 ng of total RNA, using primer sets and TaqMan probes corresponding to murine β-*actin*, *Il4*, *Il17a*, *Il22*, *Ifng*, *Rorgt,* and *Tnfa* with qPCR Mastermix TaqMan (Eurogentec). For *Foxp3*, *Il23r*, *Saa1,* and *Saa3*, qPCR was done using MasterMix for SYBR Green (Eurogentec). The sequences of primers and probe were provided in Supporting Information Table 1. Samples were first heated for 10 min at 95°C, before amplification as follows: 40 cycles of two-step PCR program at 95°C for 10 s and 60°C for 1 min. For SYBR Green qPCR, melting point analysis was carried out by heating the amplicon from 60 to 95°C. A standard curve with known concentrations of a cloned cDNA fragment was used for each gene.

Microarray

Whole transcripts were amplified from 100 ng total RNA, converted into double-strand cDNA, and then into labeled cRNA with the GeneChip® 3′ IVT PLUS Reagent Kit (Affymetrix). Fifteen microgram DNA was fragmented and hybridized on Genechip® Mouse Genome 430 2.0 array (Affymetrix). Microarray data are available in the ArrayExpress database (http://www.ebi.ac.uk/arrayexpress) under accession number E-MTAB-4453.

Statistics

Results are presented as the mean ± SEM. Statistical significance between groups of WT (or *Rag2*$^{-/-}$) and *Ahr*$^{-/-}$ (or *Rag2*$^{-/-}$ *Ahr*$^{-/-}$) mice was assessed with one-tailed unpaired Student's *t*-test in parametric conditions, Mann–Whitney test in nonparametric conditions and two-way ANOVA with Bonferroni post-test for the ear thickening curves, using the Prism software (GraphPad software).

Acknowledgements: We are very grateful to Sabrina El Bachiri for expert statistical assistance. This work was supported by the Belgian Programme on Interuniversity Poles of Attraction initiated by the Belgian State, Prime Minister's Office, Science Policy Programming, and by the Actions de Recherche Concertées of the Communauté Française de Belgique. L.D. is a Research Associate and A.B.V.B. is Research Fellow with the Fonds National de la Recherche Scientifique, Belgium. P.M.C. was supported by the L'Oréal-Unesco grant and is Research Fellow with the Fonds National de la Recherche Scientifique, Belgium.

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

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Abbreviations: **AhR**: Aryl hydrocarbon receptor · **ILC3**: Innate Lymphoid cells group · **3/ DN TCRß**: Double Negative TCRß

Full correspondence: Dr. Laure Dumoutier, Ludwig Institute for Cancer Research, Avenue Hippocrate, 74, B-1200 Brussels, Belgium
Fax: +32-2-764-74-30
e-mail: laure.dumoutier@uclouvain.be

Received: 15/9/2015
Revised: 11/2/2016
Accepted: 14/3/2016
Accepted article online: 22/3/2016

