Ccr6 Is Dispensable for the Development of Skin Lesions Induced by Imiquimod despite its Effect on Epidermal Homing of IL-22–Producing Cells

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Expression of the chemokine receptor Ccr6 is shared by most IL-22–producing cells, and Ccr6-deficient mice showed decreased IL-22 production and skin inflammation upon IL-23 intradermal injections. To determine whether this observation might be extended to another psoriasis model, we applied imiquimod on Ccr6-deficient mice. Although epidermal IL-22 production was decreased because of a deficient recruitment of γδ T cells in these mice, they were not protected against psoriatic lesions. When primary epidermis or dermis tissue culture cells from nontreated mice were stimulated ex vivo with IL-1α/IL-2/IL-23, we observed that Ccr6 is crucial for *Il22* expression from epidermal but not dermal cultures. Taking advantage of Ccr6-LacZ–knock-in mice, we showed that Ccr6 is necessary for the homing of Ccr6-positive cells, probably a γδ T-cell subset, which represents the main potential IL-22 source in the epidermis. Similar results were observed in *Rag1*$^{-/-}$ epidermis and dermis primary cultures, in which a subset of innate lymphoid cells expressing Ccr6 represents the main potential source of IL-22. Taken together, our data show that Ccr6 is not required for the development of skin lesions induced by imiquimod despite its effect on epidermal homing of IL-22–producing cells.

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INTRODUCTION

Psoriasis is one of the most common immune-mediated chronic inflammatory skin disorders that affect approximately 2% of the general population. Several murine studies have highlighted a detrimental role for T helper (Th) 17/IL-23 pathway in the pathogenesis of psoriasis. In human psoriatic patients, elevated levels of Th17 cells and associated cytokines, including IL-23, IL-17, and IL-22, were also observed. The clinical efficacy of anti-IL-23p19 and anti-IL-17A monoclonal antibodies has been shown, highlighting the implication of Th17 response in psoriatic patients as well (Campa et al., 2016). However, these biological therapies are sometimes combined with significant adverse events. In this particular context, IL-22 and proteins associated with IL-22–producing cells might be other relevant targets in this disease.

Skin IL-22 production is induced by various stimuli including toll-like receptor ligands and inflammatory cytokines and plays an important role in bacterial and fungal infections as well as wound healing (Chan et al., 2015; Kagami et al., 2010; McGee et al., 2013; Sasaki et al., 2011). IL-22 acts on keratinocytes, promotes epithelial regeneration, and induces production of neutrophil chemotactic factors and antimicrobial peptides, thereby playing a key role in skin homeostasis (Boniface et al., 2005; Sa et al., 2007; Wolk et al., 2006). However, if these effects are not controlled, they can lead to chronic inflammatory processes such as psoriasis, in which the detrimental effect of IL-22 has been shown. IL-22–transgenic mice die soon after birth and exhibit acanthosis and hyperkeratosis, two typical features of psoriasis (Wolk et al., 2009). In the absence of IL-22, mice display less acanthosis in models of psoriasis induced by IL-23 or by imiquimod (Aldara; 3M Pharmaceuticals, St Paul, MN) (Tortola et al., 2012; Van Belle et al., 2012; Zheng et al., 2007). In these models, the main source of IL-22 is γδ T lymphocytes (Cochez et al., 2016; Pantelyushin et al., 2012), and more specifically the population of γδ^{low} T cells (Vγ2^{+} T cells), which are normally present in the dermis and migrate to the epidermis upon inflammation (Pantelyushin et al., 2012). By contrast, the γδ^{high} T cells (Vγ3^{+} T cells), also called dendritic epidermal T cells represent the main γδ T-cell population in the murine epidermis but do not produce IL-22 (Kadow et al., 2011). In Rag$^{-/-}$ mice lacking both T cells and B cells, IL-22 is still produced by Ccr6^{+}Thy1^{+} innate lymphoid cells (group 3 ILCs) (Cochez et al., 2016; Van Belle et al., 2012).

A common feature of the cell populations that are potential producers of IL-22 in the skin is the expression of the chemokine receptor Ccr6. This receptor is also found on dendritic cells, Th17 cells, and some B cells and regulatory T cells (Baba et al., 1997; Kleinewietfeld et al., 2005).

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Abbreviations: ILC, innate lymphoid cell; Th, T helper; WT, wild type

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Ccr6-deficient (*Ccr6*$^{-/-}$) display some abnormalities in the gut, showing underdeveloped Peyer's patches with a lack of myeloid dendritic cells (Cook et al., 2000; Varona et al., 2001). Similar defects in homing of regulatory T cells and Th17 cells in lung, mesenteric lymph nodes, and Peyer's patches are also described (Yamazaki et al., 2008). Moreover, Ccr6 also plays a role in the recruitment of activated T cells (Paradis et al., 2008) and regulatory T cells (Barth et al., 2012) in skin hypersensitivity models. *Ccr6*$^{-/-}$ mice have decreased ear swelling in a delayed-type hypersensitivity model, meaning that Ccr6 plays a proinflammatory role in this model (Varona et al., 2001). In addition, upon IL-23 intradermal injections, *Ccr6*$^{-/-}$ mice completely failed to develop psoriatic-like lesions (Hedrick et al., 2009). Compared with wild-type (WT) mice, *Ccr6*$^{-/-}$ mice displayed weaker *Il22* expression concomitant with a lack of γδ T-cell recruitment in the epidermis in response to IL-23 injections (Mabuchi et al., 2013).

IL-22, as well as Ccr6 and its ligand Ccl20, are strongly expressed in lesional psoriatic skin and in peripheral blood from psoriatic patients (Boniface et al., 2007; Homey et al., 2000), suggesting that these proteins could be involved in psoriasis. Here, we analyzed the requirement of Ccr6 for the development of skin inflammation and the local production of IL-22 after imiquimod treatment. In addition, we studied its effect in the homing of potential IL-22 producer cells in noninflammatory conditions.

RESULTS

In the imiquimod-induced psoriasis mouse model, Ccr6 is not required for skin inflammation but is involved in IL-22 production in the epidermis

Daily application of imiquimod on the ears of *Ccr6*$^{-/-}$ mice induced a similar swelling as in WT mice (Figure 1a, and see Supplementary Figure S1a online), as well as a similar acanthosis and gene expression of psoriasis-associated markers *K14*, *Cxcl3*, and *Ngp* (Figure 1b–f, and see Supplementary Figure S1b). When imiquimod was applied to the back skin of mice, similar results were obtained regarding the acanthosis (see Supplementary Figure S1c). These data indicate that *Ccr6*$^{-/-}$ mice were not protected from the psoriasis-like lesions induced in the imiquimod model. In addition, the *Il22* gene was expressed at the same level in WT and *Ccr6*$^{-/-}$ mice in the total ear (Figure 1g). By contrast, when we separated the dermis and epidermis, *Il22* expression was significantly and specifically lower in the epidermis from *Ccr6*$^{-/-}$ mice, showing that Ccr6 is at least partially required for IL-22 production at this level (Figure 1h and i). Analysis of infiltrating cells in the epidermis of these mice showed similar numbers of γδ^{high} T cells but a lower recruitment of γδ^{low} T cells in *Ccr6*$^{-/-}$ mice (see Supplementary Figure S1d). As a consequence, instead of γδ^{low} T cells, αβ T cells represent the main residual source of IL-22 in imiquimod-treated *Ccr6*$^{-/-}$ epidermis (see Supplementary Figure S1e).

IL-17 displays a similar expression profile as IL-22 (see Supplementary Figure S2a and b online), consistent with the fact that γδ^{low} T cells are an important source of this cytokine in this model (Pantelyushin et al., 2012). By contrast, IL-19, which is a keratinocyte-derived cytokine (Kunz et al., 2006), was expressed at the same level in total ear and epidermis of WT and *Ccr6*$^{-/-}$ mice (see Supplementary Figure S2c and d). Taken together, these results show that Ccr6 is not required for the inflammatory process in the imiquimod model, whereas this chemokine receptor is important for IL-22 production specifically in the epidermis because it induces γδ^{low} T cell recruitment.

Ccr6 deficiency is associated with reduced IL-22 production from epidermal but not dermal cells after primary ex vivo stimulation

When primary cells from the epidermis of untreated mice were cultured ex vivo and stimulated with various cytokines, *Il22* expression was mostly induced by IL-2, and maximal induction was obtained with a combination of IL-1α, IL-2, and IL-23 (see Supplementary Figure S2e). Using this combination of cytokines, we observed that *Il22* expression is lower in Ccr6-deficient epidermis cells both at the RNA (Figure 2a) and protein levels (Figure 2b). This effect was specific to the epidermis, because dermal cells from *Ccr6*$^{-/-}$ mice displayed the same *Il22* expression level as their WT counterparts (Figure 2c), in line with our observations in the imiquimod model. We also noticed that the production of IL-17 and IL-19, which can be induced by the same cytokine combination, was not affected by the absence of Ccr6 (see Supplementary Figure S2e and f). This observation indicates that Ccr6 is specifically required for either IL-22 production by epidermal cells or for their homing in noninflammatory epidermis.

Ccl20 is not required for ex vivo IL-22 production by epidermal cells

Although *Ccl20* gene expression was not up-regulated in epidermal cells by the IL-1α, IL-2, and IL-23 combination (Figure 2d), a similar basal expression level was detected at the RNA level in WT and *Ccr6*$^{-/-}$ mice at early time points. To test the hypothesis that this basal Ccl20 production might contribute to IL-22 induction in this model, we added either a blocking anti-Ccl20 monoclonal antibody or pertussis toxin, which inhibits signaling by G-protein coupled receptors such as Ccr6. As shown in Figure 2e and f, neither of these treatments affected *Il22* gene expression by epidermal tissue cells. These results suggest that Ccr6 does not have a direct effect via Ccl20 binding on *Il22* expression in epidermal tissue cells ex vivo.

Ccr6 is required for IL-22–producing cell homing in the epidermis

To define whether Ccr6 was required for epidermis homing of potential IL-22–producing cells, we first determined which epidermal cells produce IL-22 upon ex vivo stimulation. These primary cultures were composed of hematopoietic cells (CD45^{+} cells, approximately 20% of living cells) and keratinocytes (Figure 3a, left panel). We showed by magnetic sorting of cytokine-stimulated cells that IL-22 was produced by the CD45^{+} cell fraction (Figure 3b). Among CD45^{+} cells, two main populations were found (Figure 3a, central panel): CD11b^{+}γδ^{-} cells,

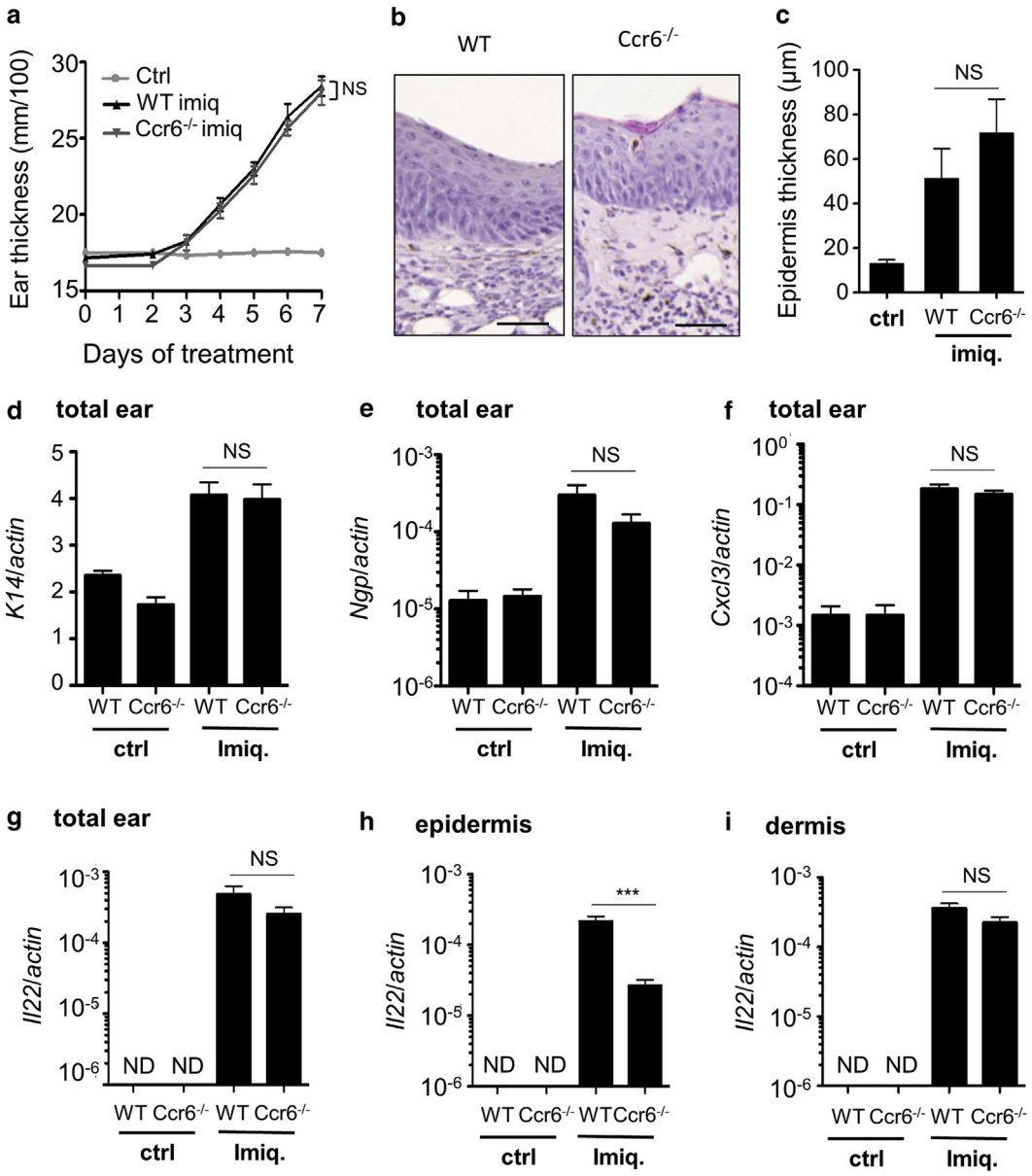

Figure 1. Ccr6 is dispensable for the development of skin lesions induced by imiquimod but is required for *Il22* expression in the epidermis. Imiquimod was applied daily on the ears of WT and *Ccr6*$^{-/-}$ mice, then quantitative real-time reverse transcriptase–PCR and histological analysis were performed. (**a**) Ear thickness was measured daily with a micrometer screw to evaluate acanthosis. n = six mice/group, representative of five experiments. (**b**) Hematoxylin and eosin staining of ear skin lesions. Original magnification = ×20. Scale bar = 50 µm. One representative picture is shown for each group. (**c**) Evaluation of acanthosis by measuring epidermis thickness on the ear skin sections. n = 6 mice/group, representative of two experiments. (**d**–**f**) *K14, Ngp,* and *Cxcl3* gene expression normalized against *β-actin.* (**g**–**i**) *Il22* gene expression in (**g**) total ear, (**h**) epidermis, and (**i**) dermis, normalized against ß-actin. n = 8 mice/group, pool of five experiments. Data are shown as mean ± standard error of the mean. In **a**, two-way analysis of variance with Bonferroni posttest was used; in **c**–**i** Mann-Whitney test was used. ***$P < 0.001$. ctrl, control; imiq., imiquimod; ND, not detected; NS, not significant; WT, wild type.

which include dendritic cells, and CD11b^{-}γδ^{+} cells. By sorting out these two populations, we observed that *Il22* was expressed by γδ T cells (Figure 3c).

As shown in Figure 3a (central panel), this population consists of γδ^{high} T cells (dendritic epidermal T cells) and a minor population of γδ^{low} T cells. Previous reports have shown that dendritic epidermal T cells do not produce IL-22 (Kadow et al., 2011), and the number of these cells was similar in the epidermis of WT and *Ccr6*$^{-/-}$ mice (Figure 3a, central and right panels, and 3d). By contrast, γδ^{low} T cells are well-known IL-22 producers (Cochez et al., 2016; Pantelyushin et al., 2012), but surprisingly, we observed only a marginal, non-statistically significant decrease in the number of these cells between WT and *Ccr6*$^{-/-}$ mice, both based on γδ TCR level of expression (Figure 3e) and on TCR-Vγ2 staining, which is specific for this population (Figure 3f). However, only 10% of γδ^{low} T cells expressed Ccr6 in normal epidermis (see Supplementary Figure S3a online), suggesting that only a small proportion of γδ^{low} T cells could be affected by the absence of Ccr6 and that this population would be the source of IL-22 in epidermis. We confirmed by cell sorting

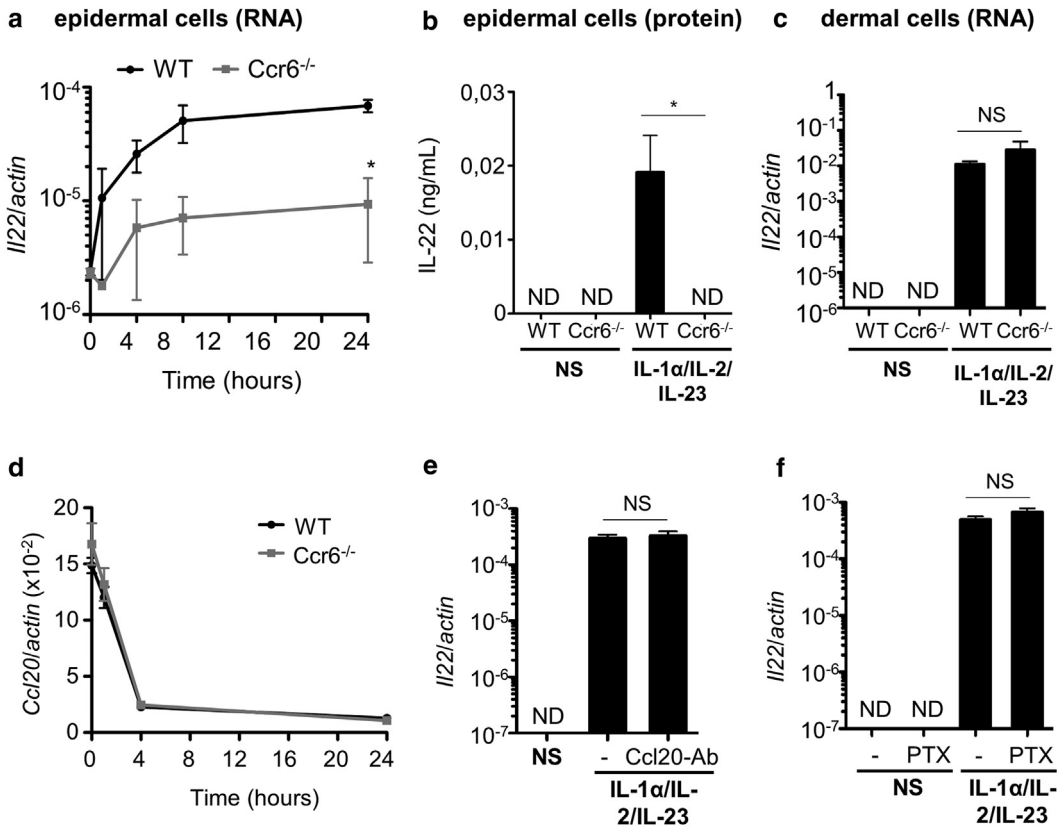

Figure 2. Ccr6 is required for *Il22* expression in the epidermal cell cultures but not via Ccl20 binding. Epidermal cells from WT and $Ccr6^{-/-}$ mice were stimulated ex vivo with IL-1α, IL-2, and IL-23; then quantitative real-time reverse transcriptase–PCR was performed. (**a**–**c**) Epidermal or dermal cells from WT and $Ccr6^{-/-}$ mice were stimulated ex vivo with IL-1α, IL-2, and IL-23, then quantitative real-time reverse transcriptase–PCR or ELISA was performed. (**a**) *Il22* gene expression kinetic in WT and $Ccr6^{-/-}$ epidermal cells, normalized against ß-actin (triplicates, representative of two experiments). (**b**) IL-22 production in supernatants of epidermal cells from WT and $Ccr6^{-/-}$ mice after 48 hours of stimulation. The ELISA detection threshold was 10 pg/ml, and in absence of detection, we considered that the samples contained 10 pg/ml to perform the statistical analysis. (**c**) *Il22* gene expression in dermal cells after 24 hours of stimulation, normalized against ß-actin (triplicates, representative of two experiments). (**d**) *Ccl20* gene expression kinetic in WT and $Ccr6^{-/-}$ epidermal cells, normalized against ß-actin (triplicates, representative of two experiments). (**e**) *Il22* gene expression in epidermal cells with or without blocking Ccl20 antibody, normalized against ß-actin (quadruplicates, representative of three experiments). (**f**) *Il22* gene expression in epidermal cells with or without pertussis toxin (PTX), normalized against ß-actin (quadruplicates, representative of two experiments). Data are shown as mean ± standard error of the mean. In **a** and **d**, two-way analysis of variance with Bonferroni posttest was used; in **b**, **c**, **e**, and **f** Mann-Whitney test was used. *$P < 0.05$. ND, not detected; NS, not significant; PTX, pertussis toxin; WT, wild type.

that *Il22* was mainly expressed by $Ccr6^{+}$ cells (Figure 3g, and see Supplementary Figure S3b). We then took advantage of the β-galactosidase expression in Ccr6-deficient mice to address the hypothesis that Ccr6 is required for epidermis homing. We observed that LacZ-positive cells were present in the dermis of $Ccr6^{-/-}$ mice at the same level as in $Ccr6^{+/-}$ mice (Figure 3h), indicating that a functional *Ccr6* gene is not required for the homing of this population in the dermis. In contrast, almost no LacZ-expressing cells were observed in the epidermis of $Ccr6^{-/-}$ mice compared with heterozygous littermate mice, indicating that their epidermis homing was impaired in the absence of Ccr6 (Figure 3i). Taken together, these results strongly suggest that the epidermis homing of a subset of Ccr6-positive $\gamma\delta^{low}$ T cells is impaired in the absence of Ccr6, resulting in lower IL-22 production.

Ccr6 is required for IL-22 production of epidermal but not dermal ILCs

We have shown previously that ILCs compensate for the absence of T cells by producing IL-22 in the imiquimod model (Cochez et al., 2016; Van Belle et al., 2012), and we took advantage of $Rag1^{-/-}Ccr6^{-/-}$ mice to study the effect of Ccr6 on this population. After ex vivo stimulation, we observed that *Il22* expression in the epidermis was lower in absence of Ccr6 both at the RNA (Figure 4a) and protein levels (Figure 4b), whereas it was not significantly different in the dermis (Figure 4c). We confirmed these observations by injecting the cytokine cocktail intradermally in the ear of mice. *Il22* was expressed in total ear of $Rag1^{-/-}Ccr6^{-/-}$ mice at the same level as in their $Rag1^{-/-}$ counterparts (Figure 4d) but not in the epidermis (Figure 4e).

Ccr6 is required for IL-22–producing ILC homing in the epidermis

In $Rag1^{-/-}$ mice, *Il22* was expressed by $CD45^{+}$ cells (Figure 5a, left panel, and 5b), which are mainly composed of $CD11b^{+}Thy1^{-}$ cells, including dendritic cells, and $CD11b^{-}Thy1^{+}$ cells, which correspond to ILCs in these mice (Figure 5a, central panel). By sorting out $CD11b^{+}$ and $CD11b^{-}$ populations after cytokine stimulation, we showed that *Il22* is expressed by $CD11b^{-}$ cell fraction, pointing to

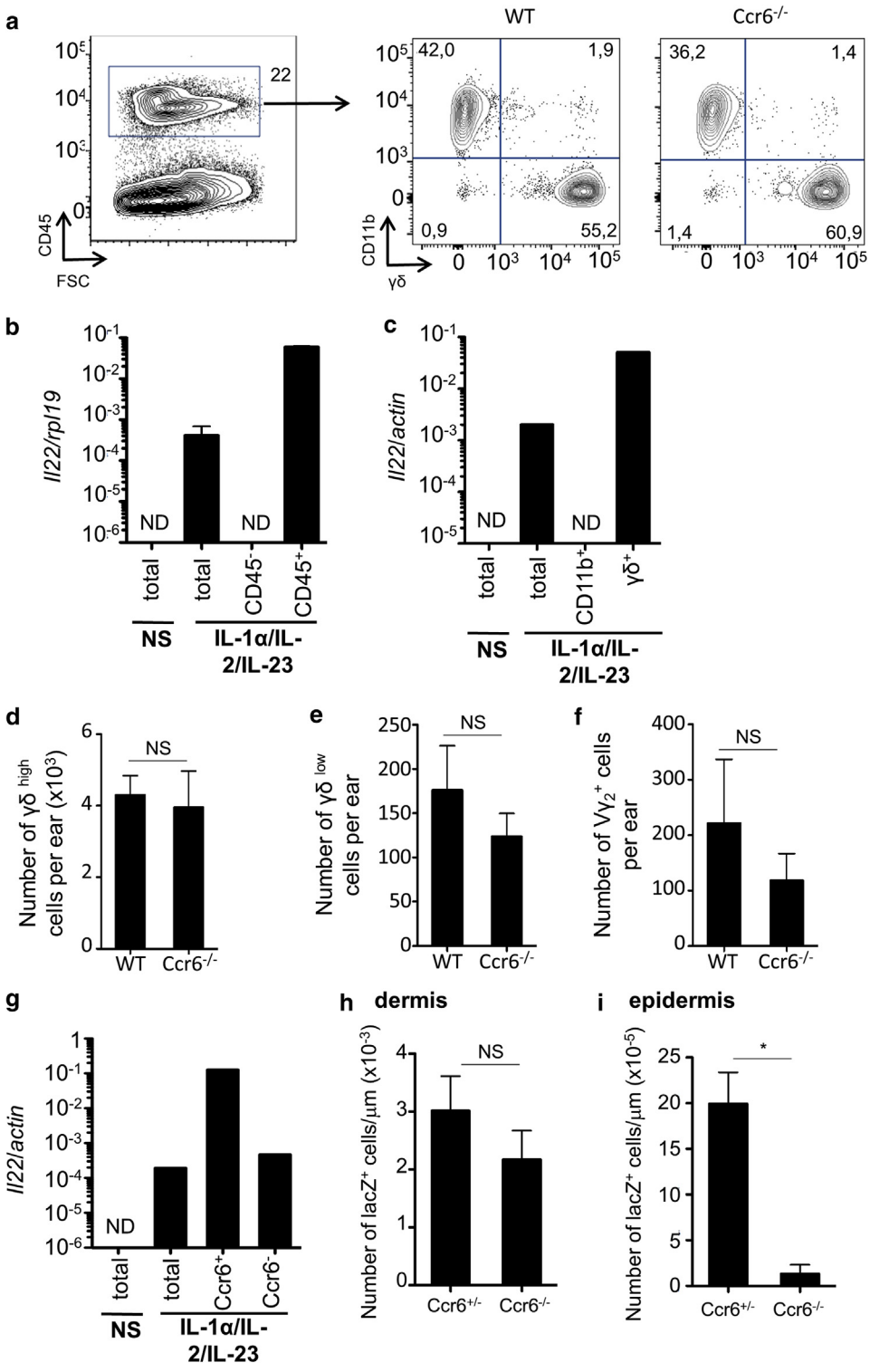

Figure 3. Ccr6 is required for epidermis homing of IL-22–producing γδ^{low} T cells. (**a**–**g**) Epidermal cells from WT (and *Ccr6*$^{-/-}$) mice were stimulated ex vivo with IL-1α, IL-2, and IL-23 (or not), then cell staining or cell sorting followed by quantitative real-time reverse transcriptase–PCR analysis were performed. (**a**) Staining of CD45-, γδ- and CD11b-positive cells among living epidermal cells (one representative graph). (**b**) *Il22* gene expression in WT epidermal cells normalized against *Rpl19* after magnetic sorting of CD45^{+} and CD45^{-} cells (triplicates, representative of three experiments). (**c**) *Il22* gene expression in WT epidermal cells normalized against ß-actin after FACS of CD11b^{+} and γδ^{+} cells (representative of three experiments). (**d**–**f**) Absolute numbers of (**d**) γδ^{high} and (**e**) γδ^{low} or (**f**) Vγ$_2$$^{+}$ T cells without stimulation among epidermal cells from WT and *Ccr6*$^{-/-}$ mice. n = 6 mice/group, representative of three experiments. (**g**) *Il22* gene expression in WT epidermal cells normalized against ß-actin after FACS of Ccr6^{+} and Ccr6^{-} cells (representative of three experiments). (**h, i**) Absolute numbers of LacZ^{+} cells after X-gal staining in (**h**) epidermis and (**i**) dermis of *Ccr6*$^{+/-}$ and *Ccr6*$^{-/-}$ littermates measured by histological analysis. n = 4 mice/group. Data are shown as mean ± standard error of the mean. In **d**–**h**, Mann-Whitney test was used. *P < 0.05. FSC, forward scatter; ND, not detected; NS, not significant; WT, wild type.

Thy1^{+} cells as the IL-22–producing cells (Figure 5c). In line with this result, Thy1^{+} cell depletion abolished the *Il22* expression (Figure 5d). The number of Thy1^{+} cells was similar in *Rag1*$^{-/-}$ and *Rag1*$^{-/-}$*Ccr6*$^{-/-}$ mice (Figure 5a, central and right panel, and 5e) but only 15% of Thy1^{+} cells expressed Ccr6 (data not shown). These observations suggest that the Ccr6-positive subset of ILCs is responsible for IL-22 production and requires Ccr6 for epidermal homing. This hypothesis was confirmed by analyzing LacZ expression in the skin, showing that the number of LacZ-positive cells was lower in the epidermis but not in the dermis of *Rag1*$^{-/-}$*Ccr6*$^{-/-}$ mice versus *Rag1*$^{-/-}$ mice (Figure 5f and g).

DISCUSSION

Taken together, our results indicate that, in noninflammatory skin, potential IL-22–producing cells, either γδ T cells

or ILCs, are present in both the dermis and epidermis and that Ccr6 is required for the homing of these cells in the epidermis but not in the dermis. Despite this effect on homing of epidermal IL-22–producing cells, Ccr6 deficiency does not affect global IL-22 production in total skin in the imiquimod mouse model. This observation explains why Ccr6 is not important for development of skin lesions in this model.

Several reports have shown that $\gamma\delta^{low}$ T cells represent the major source of IL-22 in murine skin. Both in the imiquimod model (Cochez et al., 2016) and upon IL-23 intradermal injections (Mabuchi et al., 2011), Ccr6 is required for the recruitment of these cells to the epidermis and the resulting *Il22* expression. Ccr6 deficiency did not appear to affect significantly the number of $\gamma\delta^{low}$ T cells in the epidermis of untreated mice, yet IL-22 production by these cells was lost in the absence of Ccr6. However, our experiments ruled out the possibility that Ccr6 could be involved directly in IL-22 production independently on its chemotactic activity. On one hand, *Il22* induction by ex vivo stimulation was not decreased by anti-Ccl20 blocking antibody nor by pertussis toxin, which blocks Ccr6 signaling to any other ligand, such as ß-defensin-2 (Niyonsaba et al., 2002). On the other hand, *Il22* expression is not increased in epidermis cells after Ccl20 ex vivo stimulation, alone or in combination with IL-1α/IL-2/IL-23 cocktail (data not shown).

In fact, only a minority of epidermis $\gamma\delta^{low}$ T cells express Ccr6, and our LacZ staining experiments on Ccr6-LacZ–knock-in mice strongly suggested that this receptor is indeed required for their homing, without affecting significantly the total number of $\gamma\delta^{low}$ T cells in the epidermis. A Ccr6-independent trafficking has been previously shown for dermis-to-epidermis trafficking of $CD4^+$ T cells, probably because other chemokine receptors are responsible for the homing of these cells to the epidermis (Tubo et al., 2011). Similarly, our data show that homing of Ccr6-positive $\gamma\delta^{low}$ T cells in the dermis does not require this receptor, suggesting that another receptor is involved in dermal homing, as previously shown for Ccr4 in blood-to-dermis trafficking of $CD4^+$ T cells (Tubo et al., 2011).

In $Rag1^{-/-}$ epidermis, the absence of γδ T cells is compensated for by an expansion and/or recruitment of ILCs, which are probably group 3 ILCs and provide an alternative source of IL-22 (Cochez et al., 2016; Van Belle et al., 2012). Our data showed that Ccr6 deficiency did not affect the absolute number of epidermal ILCs but decreased IL-22 production by this population. In fact, very similarly to the $\gamma\delta^{low}$ T cells, approximately 15% of epidermis ILCs express Ccr6 (data not shown), and our LacZ staining experiments show that this subpopulation depends on Ccr6 to move from dermis to epidermis.

In the imiquimod-treated epidermis, IL-22 is produced not only by $\gamma\delta^{low}$ T cells but also by a population of $CD4^-CD8^-$(DN)$TCR\beta^+$ cells (Cochez et al., 2016). In contrast to the $\gamma\delta^{low}$ T cells, the number of $TCR\beta^+$ cells recruited to the epidermis upon imiquimod treatment is similar in WT and $Ccr6^{-/-}$ mice (data not shown). This suggests that these cells also express other chemokine receptors, which might compensate for the absence of Ccr6. Because *Il22* expression is decreased but not abolished in epidermis from $Ccr6^{-/-}$ mice, this residual *Il22* production might result from this population.

Ccr6 has been proposed as a potential target in psoriasis based on promising observations in IL-23–treated mice (Hedrick et al., 2010). In this model, which consists of intradermal injections of IL-23 every other day for 16 days, $Ccr6^{-/-}$ mice were totally protected, and ear thickness, acanthosis, and cell infiltrate were totally abolished in these mice (Hedrick et al., 2009). Decreased *Il22* expression was proposed as the mechanism underlying this observation, because IL-22 injections induced similar lesions in WT and $Ccr6^{-/-}$ mice (Hedrick et al., 2009). Our observations in the imiquimod model ascribe a more restricted effect to Ccr6, mainly in epidermis trafficking of IL-22–producing cells. This discrepancy might result from the difference in the experimental models, because imiquimod application leads to a global and massive cytokine production such as IL-23, IL-36, IL-1, TNF-α, IL-17, and IL-22 (Tortola et al., 2012; Van Belle et al., 2012; van der Fits et al., 2009), sometimes even inducing systemic effects (Cochez et al., 2016; Grine et al., 2016). This multifactorial cytokine production probably has broader effects than IL-23 only, maybe by inducing higher and more diverse immune cell recruitment.

Translating observations from mouse psoriasis models to human patients remains a challenging issue. In human skin, γδ T cells are less abundant, both in epidermis and in dermis, and their role remains to be discovered, whereas they are considered key players in mouse models. However, mouse studies were instrumental in showing the role of Th17/IL-23 pathways and paved the way for anti-IL-23p19, anti-IL-17, and anti-IL-17R therapies that successfully reverse clinical features of psoriasis in 80–90% of patients (Campa et al., 2016). In this context, further experiments, including additional preclinical models for skin inflammation and psoriasis-like disease, will be needed to better characterize the pathologic conditions in which Ccr6 targeting may have optimal clinical efficiency.

MATERIALS AND METHODS

Mice and treatments

All mice used in this study were bred in our animal facility under specific pathogen-free conditions. $Ccr6^{-/-}$ C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME) (stock number 005793). $Rag1^{-/-}$ C57BL/6 mice were acquired from S. Goriely (Université libre de Bruxelles, Brussels, Belgium) (Mombaerts et al., 1992). $Rag1^{-/-}Ccr6^{-/-}$ mice were obtained by crossing $Rag1^{-/-}$ C57BL/6 mice with $Ccr6^{-/-}$ C57BL/6 mice. Heterozygous breeding pairs were generated, and littermates were used for the experiments. All animal experiments were performed in compliance with national and institutional guidelines and were approved by the local ethical committee. For cytokine cocktail injection, 8-week-old mice received 500 ng IL-1α (Biolegend, San Diego, CA), 250 ng IL-23 (eBioscience, Waltham, MA) and 15,000 U IL-2 (Novartis, Basel, Switzerland) by intradermal injection in the ear, under anesthesia. Ears were harvested 9 hours after cytokine injection. For the imiquimod model, 8-week-old mice were shaved

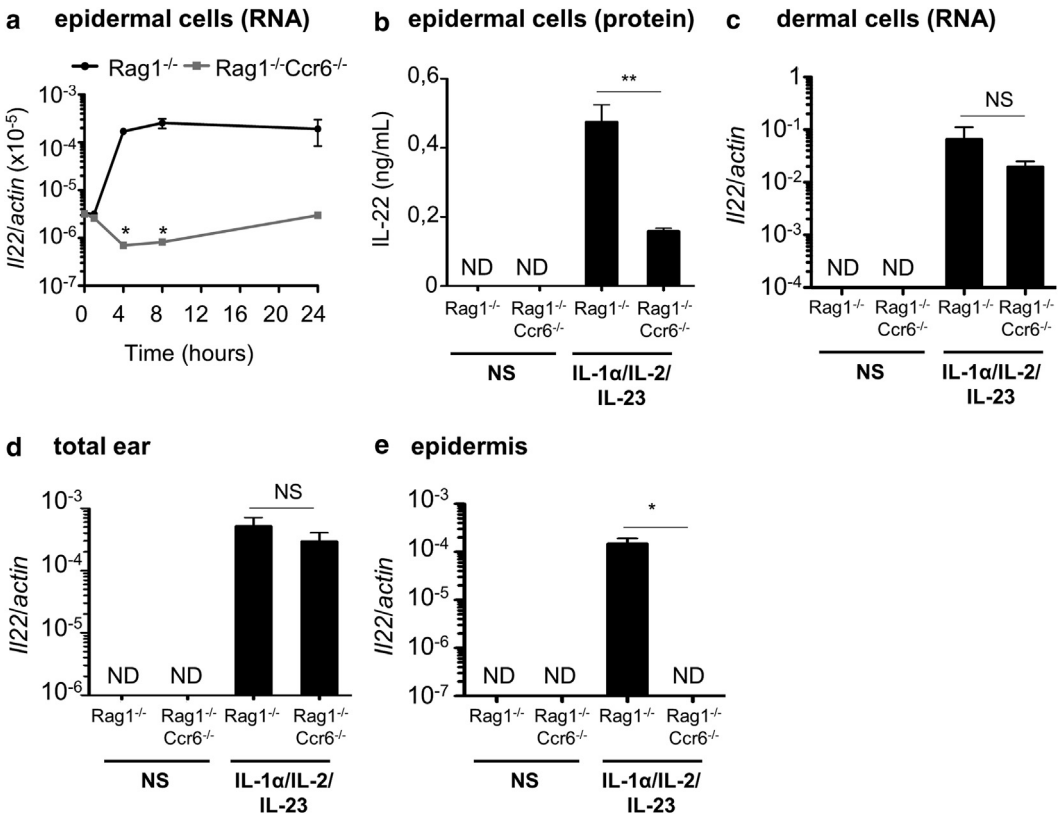

Figure 4. Ccr6 is required for *Il22* expression in the epidermis but not in the dermis of *Rag1*$^{-/-}$ mice. (**a**–**c**) Epidermal or dermal cells from *Rag1*$^{-/-}$ and *Rag1*$^{-/-}$*Ccr6*$^{-/-}$ mice were stimulated ex vivo with IL-1α, IL-2, and IL-23, then quantitative real-time reverse transcriptase–PCR or ELISA were performed. (**a**) *Il22* gene expression kinetic in *Rag1*$^{-/-}$ and *Rag1*$^{-/-}$*Ccr6*$^{-/-}$ epidermal cells, normalized against ß-actin (triplicates, representative of three experiments). (**b**) IL-22 production in supernatants of epidermal cells from *Rag1*$^{-/-}$ and *Rag1*$^{-/-}$*Ccr6*$^{-/-}$ mice after 72 hours of stimulation. (**c**) *Il22* gene expression in dermal cells after 24 hours of stimulation, normalized against ß-actin (triplicates, representative of two experiments). (**d**–**e**) *Il22* gene expression in (**d**) total ear and (**e**) epidermis normalized against ß-actin after intradermal injection of IL-1α, IL-2, and IL-23 in *Rag1*$^{-/-}$ and *Rag1*$^{-/-}$*Ccr6*$^{-/-}$ mice. Ears are collected after 9 hours and then quantitative real-time reverse transcriptase–PCR was performed (triplicates, representative of three experiments). The quantitative real-time reverse transcriptase–PCR detection threshold was 10 copies and, in the absence of detection, we considered that the samples contained 10 copies to perform the statistical analysis. Data are shown as mean ± standard error of the mean. In **a**, two-way analysis of variance with Bonferroni posttest was used; in **b**–**e**, Mann-Whitney test was used. *$P < 0.05$, **$P < 0.01$. ND, not detected; NS, not significant.

behind the ears and received five daily topical applications of imiquimod (0.8 mg per ear) from a commercially available cream (Aldara 5%; 3M Pharmaceuticals). Ear thickness was measured every day with a micrometer screw (Mitutoyo, Aurora, IL). For histology, tissue blocks of ear skin biopsy samples were prepared using routine methods. Consecutive frozen sections were stained following the protocol of the LacZ Tissue Staining Kit (Invivogen, San Diego, CA). Stained slides were digitized using a Mirax Midi scanner (Carl Zeiss MicroImaging, Oberkochen, Germany) equipped with a Zeiss Plan-Apochromat 20 Numerical Aperture 0.80 objective lens (Carl Zeiss) and a Hitachi HV-F22 acquisition camera (Hitachi, Tokyo, Japan), providing an object pixel size of 0.23 µm. Image files were analyzed with the Mirax Viewer software (Carl Zeiss). Two independent evaluators analyzed the staining.

Single-cell suspension and FACS staining

Ears were collected and incubated overnight at 4 °C in Dispase II at 2.5 U/ml (Roche, Basel, Switzerland) in phosphate buffered saline before separation of epidermis and dermis with forceps. Epidermal cells were further isolated by digestion with DNAse I (Sigma) at 0.5 mg/ml for 20 minutes at room temperature (Salgado et al., 1999) and dermal cells, by digestion with collagenase D (Roche) at 2.5 mg/ml and DNAse I (Sigma) at 0.1 mg/ml for 45 minutes at 37 °C. After mechanical disruption, cells were filtered on 40-µm nylon filters to obtain a single-cell suspension. Cells were then pre-incubated with 10 µg/ml of purified rat anti-mouse CD16-CD32 mAb (Fc Block; BD Pharmingen, San Diego, CA) before incubation with a viability marker (LIVE/DEAD Fixable Near-IR Dead Cell Stain Kit, Life Technologies, Carlsbad, CA) and 2 µg/ml of antibodies for extracellular staining for 1 hour at 4 °C. Cells were analyzed with a FACS Fortessa (BD Biosciences). Gating included forward and side scatter, a viability marker, and CD45 expression. Data were analyzed with FlowJo software (Tree Star, Ashland, OR). For FACS sorting experiments, we used a FACSAria III (BD Biosciences). A 85-µm nozzle was used with a sheath pressure of 45 pounds per square inch, a drop drive frequency 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), PE-labeled anti-TCRγδ (GL3), PercP-labeled anti-Thy1.2 (30-H12), FITC-labeled anti-CD11b (M1/70), and PE-labeled anti-Ccr6 (29-2L17).

Real-time quantitative PCR and cytokine measurement

For the total ear, RNA was directly extracted from a piece of ear. For the ex vivo experiment, cells from epidermis or dermis were

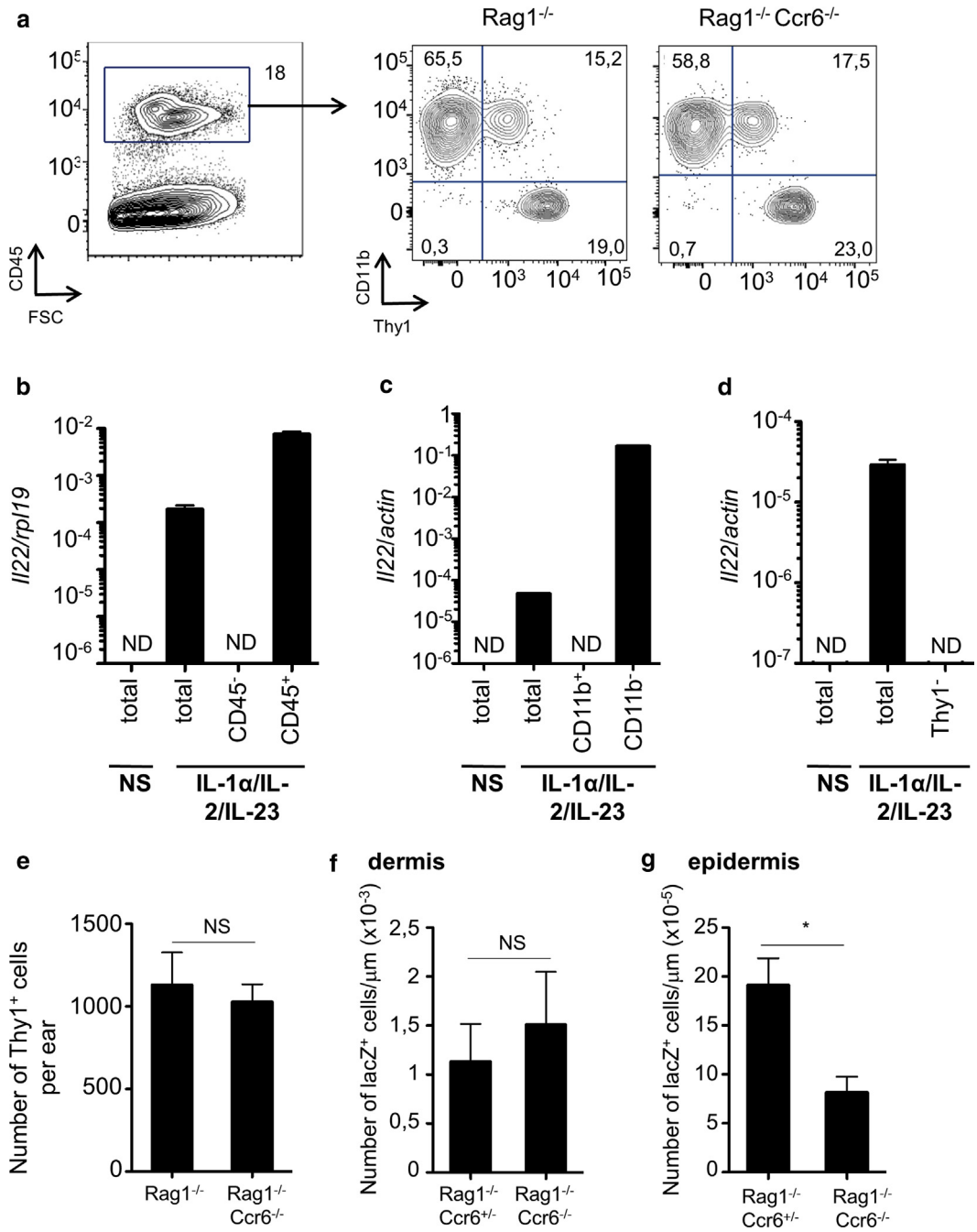

Figure 5. Ccr6 is required for epidermis homing of IL-22-producing ILCs. (**a**–**e**) Epidermal cells from *Rag1*$^{-/-}$ and *Rag1*$^{-/-}$*Ccr6*$^{-/-}$ mice were stimulated ex vivo with IL-1α, IL-2, and IL-23 (or not), then cell staining or cell sorting, followed by quantitative real-time reverse transcriptase–PCR, were performed. (**a**) Staining of CD45-, Thy1-, and CD11b-positive cells among living epidermal cells (one representative graph). (**b**) *Il22* gene expression in *Rag1*$^{-/-}$ epidermal cells normalized against *Rpl19* after magnetic sorting of CD45^{+} and CD45^{-} cells (triplicates, representative of three experiments). (**c**) *Il22* gene expression in *Rag1*$^{-/-}$ epidermal cells normalized against ß-actin after FACS of CD11b^{+} and CD11b^{-} cells. (**d**) *Il22* gene expression in *Rag1*$^{-/-}$ epidermal cells normalized against ß-actin after magnetic depletion of Thy1^{+} cells (triplicates, representative of four experiments). (**e**) Absolute numbers of Thy1^{+} cells without stimulation among epidermal cells from *Rag1*$^{-/-}$ and *Rag1*$^{-/-}$*Ccr6*$^{-/-}$ mice. n = 4 mice/group, representative of two experiments. (**f, g**) absolute numbers of lacZ^{+} cells after X-gal staining in (**f**) epidermis and (**g**) dermis of *Rag1*$^{-/-}$*Ccr6*$^{+/-}$ and *Rag1*$^{-/-}$*Ccr6*$^{-/-}$ littermates measured by histological analysis. n = 3 mice/group. Data are shown as mean ± standard error of the mean. In **e**, Mann-Whitney test was used. *$P < 0.05$. FSC, forward scatter; ILC, innate lymphoid cell; ND, not detected; NS, not significant.

incubated with IL-1α (10 ng/ml, Biolegend), IL-23 (10 ng/ml, eBioscience), and IL-2 (400 U/ml, Novartis) at 37 °C. Pertussis toxin was used at 100 ng/ml (VWR, Radnor, PA) and anti-Ccl20 (clone MMCCL20.1.4) blocking antibody at 20 µg/ml (a kind gift from Muriel Lemaire from our laboratory). For cell depletion, epidermal cell suspension was depleted of Thy1^{+} cells using anti-CD90.2 magnetic MicroBeads (Miltenyi Biotec, North Rhine-Westphalia, Germany). Total RNA was isolated from cells, ears, epidermis, or dermis with the TriPure isolation reagent (Roche). Reverse transcription was performed on 1 µg of total RNA with oligo(dT) primer (Eurogentec, Liège, Belgium) and Moloney murine leukemia virus reverse transcriptase (Invitrogen).

Quantitative PCR amplifications were performed on cDNA obtained from 25 ng of total RNA, using primer sets and TaqMan probes corresponding to murine *β-actin*, *Il17a*, *Il19*, *Il22*, and *Ngp* with quantitative PCR Mastermix TaqMan Takyon (Eurogentec). The sequences of primers and probe were as follows: *Il19*, 5′-GAGCGATGTCAGGTGCACAGAC-3′, 5′-CTTAAGGG CAGCAGATGAGACCTC-3′, and probe, 5′-CTGCAGTCAG GAAGCCACCAATG-3′ and Ngp, 5′-GACTGCGACTTCCTGGAG GATG-3′, 5′-GTATCCTCTCGACTGCAATCCCTG-3′, and probe, 5′-CAGTCAACCTCCCTGACCTTG-3′. For *Rpl19*, *K14*, and *Cxcl3*, quantitative PCR was done using MasterMix Takyon for SYBR Green (Eurogentec). The sequences of primers were as follows: *Rpl19*, 5′-GAAGGTCAAAGGGAATGTGTTCA-3′ and 5′-CCTTGT CTGCCTTCAGCTTGT-3′ and *K14*, 5′-GAGGACGCCCACCTTT CATCTTCC-3′, 5′-CCATCGTGCACATCCATGACCTTG-3′. For *β-actin*, *Il17a*, *Il22*, and *Cxcl3*, the sequences have been described (Van Belle et al., 2012). Samples were first heated for 10 minutes at 95 °C before amplification as follows: 40 cycles of two-step PCR program at 95 °C for 10 seconds and at 60 °C for 1 minute. For SYBR Green quantitative PCR, 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. Gene expression was normalized against *β-actin* except for the comparison between immune cells and keratinocytes, for which we used *Rpl19*. IL-22 secretion was measured in cell culture supernatants after 24 or 48 hours. ELISA specific for murine IL-22 was performed using mouse Abs generated in our laboratory (MH22B2 and rabbit polyclonal anti-mIL-22). IL-22 concentrations were calculated by means of a standard curve generated via the use of calibrated standards.

Statistics

Results are presented as the mean ± standard error of the mean. Statistical significance between treated groups of WT (or $Rag1^{-/-}$) and $Ccr6^{-/-}$ (or $Rag1^{-/-}Ccr6^{-/-}$) mice was assessed with one-tailed unpaired Student *t* test in parametric conditions, Mann-Whitney test in nonparametric conditions, and two-way analysis of variance with Bonferroni posttest for the ear thickening curves, using the Prism software (GraphPad Software, La Jolla, CA).

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at www.jidonline.org, and at http://dx.doi.org/10.1016/j.jid.2016.12.023.

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