Inflammatory Cues Direct Skin-Resident Type 1 Innate Lymphoid Cells to Adopt a Psoriasis-Promoting Identity

Beatrix D.G. Evers[1,6], Miriam Hils[1,6], Christoph Heuser[2], Inga M. Hölge[1], Désirée Argiriu[1], Yuliya Skabytska[1], Susanne Kaesler[1], Christian Posch[1,3,4], Percy A. Knolle[5] and Tilo Biedermann[1]

Innate lymphoid cells (ILCs) are gatekeepers in barrier organs, where they maintain tissue integrity and contribute to host defense as well as tissue repair. Inappropriate activation of ILCs, however, can lead to immunopathology with detrimental results. In this study, we focused on type 1 ILCs (ILC1s), which under inflammatory conditions constitute a poorly defined population with ambiguous functions. To delineate the properties of ILC1s in skin pathology, we used the well-established mouse model of imiquimod-induced psoriasis. Although ILC1s represented a minority among cutaneous lymphocytes in vehicle-treated controls, they rapidly expanded during early psoriasis and ultimately increased by >20-fold. This rapid increase was verified using two additional psoriasis models. Inflammatory ILC1s from imiquimod-treated skin were defined as CD44^{+}, CXCR6^{+}, and CD11b^{+} and substantially contributed to TNF-α and GM-CSF production, rendering them a potential candidate to shape the inflammatory infiltrate. In accordance with the psoriasis-specific microenvironment, skin ILC1s upregulated the IL-23 receptor whereas expression of the IL-12Rβ2 subunit was diminished. As a consequence, neutralization of IL-12 only had a minor impact, whereas blocking IL-23 reduced both ILC1 abundance and disease severity. Together, our findings identify skin ILC1s as a likely player in early psoriasis and a prospective target for therapeutic approaches.

JID Innovations (2023);**3:**100204 doi:10.1016/j.xjidi.2023.100204

INTRODUCTION

Psoriasis is one of the most common, chronic inflammatory skin disorders characterized by erythematous plaques and scaling (Nestle et al., 2009). Pathophysiologically, psoriasis is driven by innate immune cells, which fuel cutaneous pathology through T-cell activation and induction of keratinocyte proliferation (Zaba et al., 2009). Some of the key mediators known to promote disease progression are the Th1/Th17 cytokines TNF-α, GM-CSF, and IL-17A (Austin et al., 1999; Cai et al., 2011; Lowes et al., 2008; Scholz et al., 2017). Following yet unknown stimuli, TNF-α together with IL-17 creates a self-amplifying inflammatory response, which leads to epidermal cell proliferation and potentiates IL-17−induced transcription of several proinflammatory genes (e.g. TNF, IL-1β, and IL-6) (Chiricozzi et al., 2011; Wang et al., 2013).

Considerable insight into the mechanisms underlying psoriasis development has been gained using the mouse model of imiquimod-induced psoriasiform dermatitis. Repetitive application of the TLR7 agonist imiquimod induces γδ T−cell activity by dermal dendritic cells, which greatly mimics the processes observed in the chronic phase of human disease (Cai et al., 2011; Van der Fits et al., 2009). Despite these advances, little is known about the mechanisms and immune cells controlling the initial stages of psoriasis.

Innate lymphoid cells (ILCs) have recently emerged as an essential component in skin surveillance and defense against pathogenic assaults (Kansler and Li, 2019; Yang et al., 2017). On the basis of developmental requirements, phenotypic markers and the production of signature cytokines, ILCs can be divided into three main groups. Group 1 ILCs are T-bet dependent and are composed of NK cells and ILC1s, which protect against intracellular pathogens by IFN-γ production. ILC2s depend on the transcription factor GATA3 and control immunity to helminths as well as tissue repair by secretion of IL-5, IL-9, and IL-13. Finally, ILC3s are RORγt dependent and promote antimicrobial responses through IL-17 and IL-22 production (Artis and Spits, 2015).

Although a functioning interplay of ILCs with other immune cells promotes tissue integrity, inappropriate activation can lead to chronic inflammatory disorders and autoimmunity (Ebbo et al., 2017). ILC2s, for example, have been shown

[1]*Department of Dermatology and Allergology, TUM School of Medicine, Technical University of Munich (TUM), Munich, Germany;* [2]*Leibniz Institute for Immunotherapy, Department of Functional Immune Cell Modulation, Regensburg, Germany;* [3]*Department of Dermatology, Vienna Healthcare Group, Vienna, Austria;* [4]*Sigmund Freud University Vienna, Faculty of Medicine, Vienna, Austria; and* [5]*Institute of Molecular Immunology and Experimental Oncology, Technical University of Munich (TUM), Munich, Germany*

[6]*These authors contributed equally to this work.*

Correspondence: Tilo Biedermann, Department of Dermatology and Allergology, TUM School of Medicine, Technical University of Munich (TUM), Biedersteiner Str. 29, München 80802, Germany. E-mail: Tilo.Biedermann@tum.de

Abbreviations: DTHR, delayed type hypersensitivity reaction; ILC, innate lymphoid cell; TNCB, 2,4,6-trinitrochlorobenzene

Received 13 June 2022; revised 2 February 2023; accepted 27 February 2023; accepted manuscript published online 25 April 2023; corrected proof published online 19 June 2023

Cite this article as: JID Innovations 2023;3:100204

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to increase airway inflammation in a mouse model of eosinophilic asthma−like disease (Morita et al., 2015). Moreover, GM-CSF produced by intestinal ILC3s recruits and maintains inflammatory monocytes and thereby orchestrates acute inflammation (Pearson et al., 2016). Compared with ILC2s and ILC3s, the phenotypic properties of ILC1s remain less well-defined, which explains why their contribution to inflammatory disorders is currently under investigated (Jiao et al., 2016).

Here, we examined skin ILC1s in the mouse model of imiquimod-induced psoriasiform dermatitis and observed massive expansion in the early phase of inflammation. Early accumulation of ILC1s was confirmed using two additional psoriasis models, 2,4,6-trinitrochlorobenzene (TNCB)−induced delayed type hypersensitivity reaction (DTHR) and IL-23−induced psoriasis. Inflammatory ILC1s, defined by the expression of CD44, CXCR6, and CD11b, significantly contributed to the production of disease-promoting cytokines, such as TNF-α and GM-CSF. Moreover, this population was able to quickly adapt to the disease-specific microenvironment by increasing sensitivity toward the Th17-polarizing cytokine IL-23. In line, application of neutralizing IL-23 antibody reduced ILC1 abundance and disease severity, whereas neutralization of IL-12 only had a minor impact on both.

Together, our findings uncover dynamic adaptations of ILC1s in response to imiquimod-induced inflammatory stress, strongly indicating that this population significantly contributes to early disease pathology.

RESULTS

ILC1s rapidly accumulate in psoriasiform dermatitis

To investigate the function of ILC1s in psoriasis, the widely studied mouse model of imiquimod-induced psoriasiform dermatitis was used. First, we analyzed publicly available gene expression data from skin of vehicle- and imiquimod-treated mice (GSE60804) (Bai et al., 2015). To define transcripts suited best to identify ILC1s, recently published datasets from several studies focusing on ILCs were taken as a basis (Filipovic et al., 2018; Robinette et al., 2015; Sojka et al., 2014). We derived a signature of murine ILC1s that included *Klrb1c* (NK1.1), *Itga1* (CD49a), *Cxcr6* (CXCR6), *Cd44* (CD44), *Tbx21* (T-bet), and *Ifng* (IFN-γ). Indeed, the selected genes were more strongly expressed in lesional skin of imiquimod-treated mice (Figure 1a), pointing toward a potential role of ILC1s in this disease model. To further delineate the contribution of skin ILC1s to psoriasis formation, we enumerated group 1 ILCs by flow cytometry at different time points after psoriasis induction. Our initial analysis compared two different disease stages, the first before the onset of γδ T/Th17 cell−mediated inflammation (day 2−4) and the second when γδ T/Th17 cell−mediated inflammation was well established (day 7) (Pantelyushin et al., 2012; Tortola et al., 2012). As a marker for dermal inflammation and corresponding edema, ear swelling was quantified throughout the course of the experiment (Figure 1b). We examined $CD45^+CD3^-NK1.1^+$ group 1 ILCs according to the expression of the integrins α1 (CD49a) and α2 (CD49b), markers widely used to distinguish ILC1s from conventional NK cells, respectively (Figure 1c) (Peng et al., 2013; Sojka et al., 2014). Although ILC1s represented a minority among cutaneous lymphocytes under homeostatic conditions, they accumulated substantially within the first 4 days of imiquimod treatment (Figure 1d). Intriguingly, skin ILC1s showed markedly greater accumulation both in frequency and total numbers than conventional NK cells and outnumbered NK cells by >20 to 1 on day four. At day seven, ILC1 abundance was 50% lower than at the peak but still strongly elevated (Figure 1e). We confirmed the rapid increase of skin ILC1s in imiquimod-treated mice by fluorescence microscopy (Figure 1f). Notably, on day four of imiquimod treatment, when ILC1 abundance was highest with 20%, ILC3s and γδ T cells/Th17 cells comprised only ∼1 % and 4% of the inflammatory infiltrate, respectively (Figures 1g and h). To investigate, whether expansion of ILC1s in the skin was confined to this organ only or was a systemic phenomenon also found in the periphery, skin-draining lymph nodes were analyzed for ILC1 infiltration (Wang et al., 2018). Similar to the skin, ILC1s greatly accumulated in skin-draining lymph nodes in a time dependent manner, reflecting circulatory involvement (Figures 1i and j).

Taken together, these results demonstrate that ILC1s rapidly accumulate in imiquimod-treated skin and skin-draining lymph nodes before γδ T cells, ILC3s, and Th17 cells prevail in the inflammatory infiltrate, suggesting that they might play an important role in the early stages of disease.

ILC1s from imiquimod-treated skin are characterized by the expression of CD44, CXCR6, and CD11b

ILC1s can be found in a variety of tissues, including the liver, salivary gland, uterus, and small intestinal mucosa (Bernink et al., 2013, 2015; Cortez et al., 2017; Filipovic et al., 2018; Sojka et al., 2014). In the past, the hepatic subset was often considered the prototypic member of this population and a lot of markers used to characterize ILC1s are based on findings in the liver (Robinette et al., 2015; Turchinovich et al., 2018). In recent years, however, it became more apparent that organ-specific subsets often exhibit distinct phenotypic characteristics, complicating the definition of bona fide ILC1s (Fuchs, 2016; Jiao et al., 2016). Moreover, phenotypic markers defining the cutaneous subset have not been thoroughly investigated yet.

To obtain a more comprehensive picture of cutaneous group 1 ILCs, we performed a phenotypic analysis using flow cytometry. Notably, because of the paucity of cells retrieved from vehicle-treated skin and promiscuous results associated with it, we focused our analysis primarily on inflammatory conditions. First, we examined intracellular expression of the key transcription factors T-bet and Eomesodermin. As previously reported (Cortez and Colonna, 2016; Daussy et al., 2014; Gao et al., 2017; Robinette et al., 2015) skin ILC1s were T-bet$^+$ and Eomesodermin$^-$, whereas NK cells stained positive for both markers (Figures 2a and b). RORγt, a transcription factor important for ILC3 development, could not be detected in any of the subsets analyzed (Figures 2a and b). In correspondence with their hepatic counterparts, skin ILC1s were CXCR6$^+$ and CD44$^+$, clearly separating them from NK cells (Figures 2a and b). Other markers known to be important for chemotaxis and tissue residency, such as CXCR3 and CD103, were not expressed by either of the two subsets

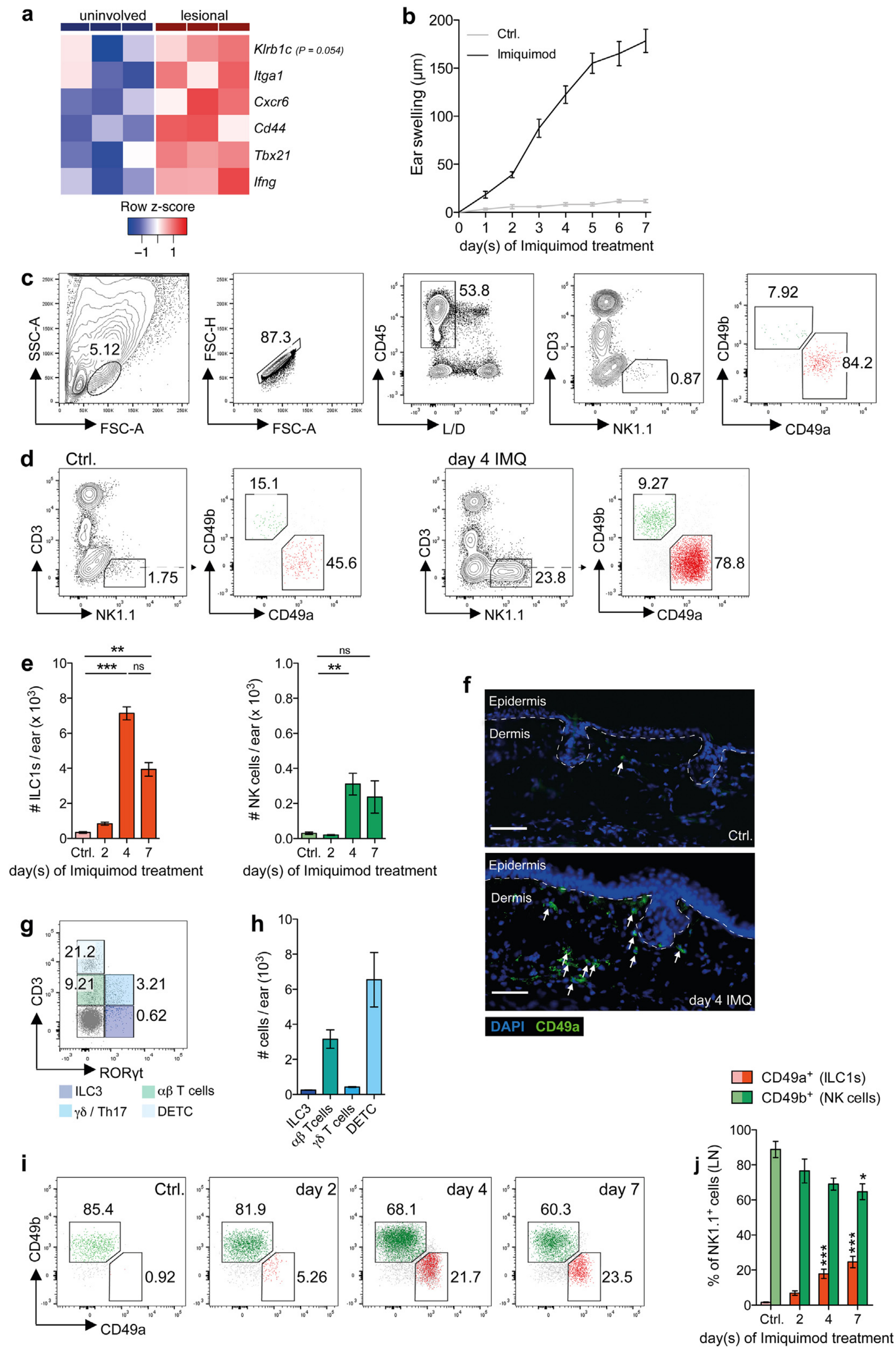

Figure 1. Skin ILC1s accumulate rapidly upon imiquimod treatment. (**a**) Differential expression of genes encoding markers of ILC1s in vehicle- and imiquimod-treated (lesional) skin. Selected genes and products: *Klrb1c*, NK1.1; *Itga1*, CD49a; *Cxcr6*, CXCR6; *Cd44*, CD44; *Tbx21*, T-bet; *Ifng*, IFN-γ. (**b**) Ear swelling. (**c**) Gating strategy. (**d**) Representative contour/dot plots from vehicle-treated ctrl and imiquimod-treated mice (day 4 IMQ). Imiquimod was applied for a total of four consecutive days. (**e**) Absolute numbers of skin ILC1s and NK cells during the course of imiquimod treatment. (**f**) Immunofluorescent images from vehicle-treated ctrl and imiquimod-treated mice (day 4 IMQ). Depicted are CD49a^{+} (green) and Dapi^{+} (blue) cells. Bar = 50 μm. Imiquimod was applied for a total of four consecutive days. (**g**) Gating strategy and (**h**) quantification of skin ILC3s defined as CD3^{-}RORγt^{+}, αβ T cells defined as CD3^{+}RORγt^{-}, γδ T/Th17 cells defined as CD3^{+}RORγt^{+}, and DETC defined as CD3^{high}RORγt^{-} on day four of imiquimod treatment. (**i**) Representative dot plots of ILC1s and NK cells from lymph nodes of vehicle-treated (Ctrl) and imiquimod-treated mice (day 2, 4, and 7). (**j**) Quantification of (i). Data shown are representative of one

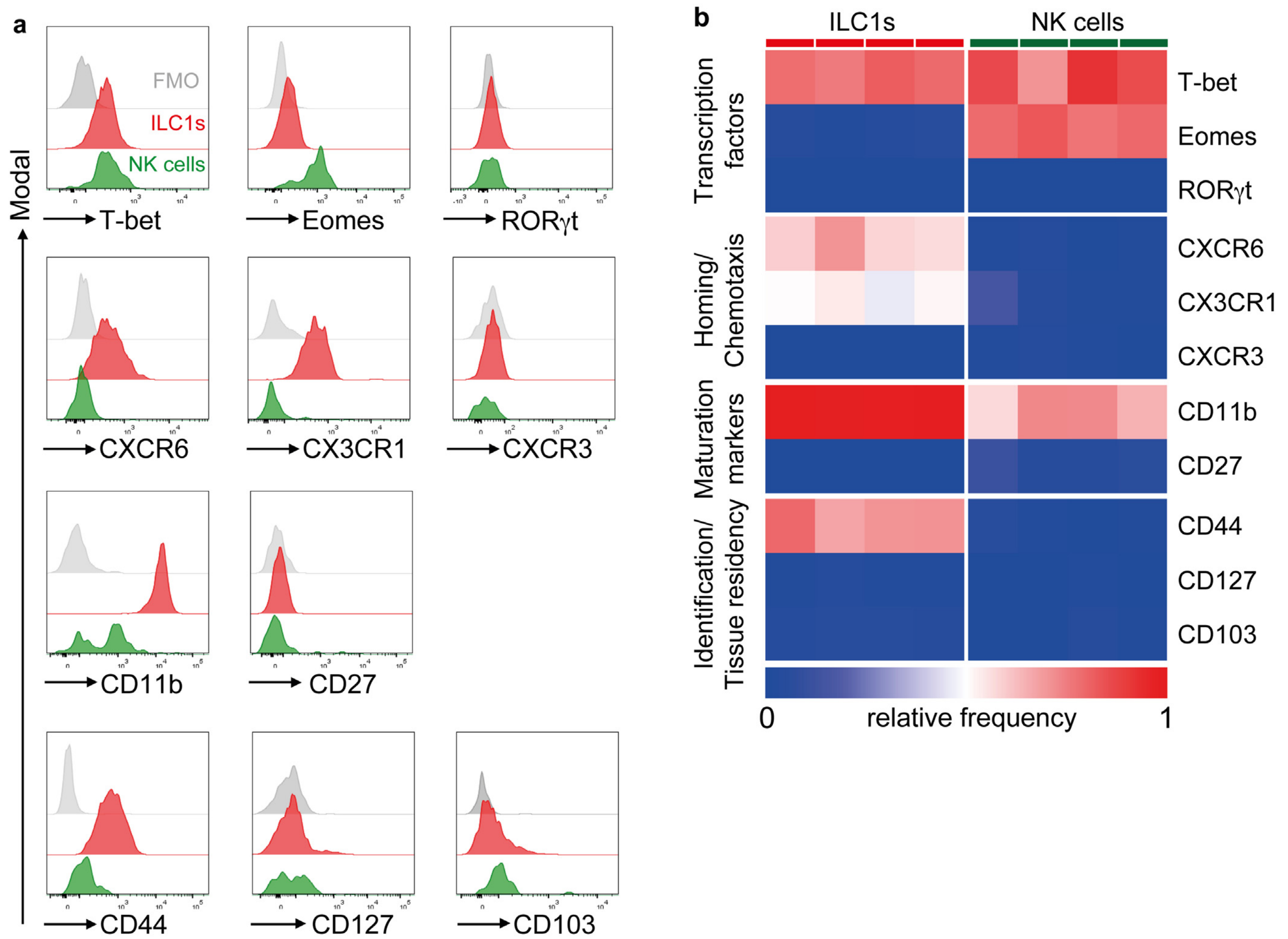

Figure 2. Characterization of skin ILC1s in imiquimod-induced psoriasiform dermatitis. (**a**) Representative histograms and (**b**) frequency of marker-positive cells among ILC1s and NK cells from mice treated with imiquimod for a total of four days. Data shown are representative of two independent experiments. Depicted are four animals per group. Eomes, eomesodermin; FMO, fluorescence minus one; ILC, innate lymphoid cell.

(Figures 2a and b). Furthermore, ILC1s from imiquimod-treated skin displayed a mature phenotype with low levels of CD27 and high levels of CD11b, complementing the inflammatory environment (Figures 2a and b). Next, we analyzed expression of the IL-7 receptor alpha chain (CD127), a marker commonly used to discriminate ILC1s from NK cells in the human setting. Interestingly, we noted that ILC1s from imiquimod-treated skin were predominantly CD127$^-$ (Figures 2a and b). Although differing from the human definition of bona fide ILC1s, our findings match those of other recent studies conducted in mice (Cortez et al., 2017; Doisne et al., 2015; Sojka et al., 2014).

Skin ILC1s significantly contribute to TNF-α and GM-CSF production in psoriasiform dermatitis feeding the psoriasis-specific TNF-α/IL-23/IL-17-axis

Because local and systemic expansion of ILC1s in psoriasiform dermatitis was conspicuous, we were keen to find out whether this population contributes to the TNF-α/IL-23/IL-17-axis central for the pathophysiology of psoriasis. To this end, 6×10^4 CD3$^-$NK1.1$^+$CD49a$^+$ cells from imiquimod-treated skin were sorted and cultured in the presence of phorbol 12-myristate 13-acetate and ionomycin. Supernatants were used to screen for multiple pro- and anti-inflammatory cytokines. Intriguingly, we observed that ILC1s from imiquimod-treated skin were able to produce a broad range of different proinflammatory mediators. Besides IFN-γ and TNF-α as well-described ILC1-secreted cytokines, substantial amounts of GM-CSF, IL-1α, and IL-6 were detected in culture supernatants (Figure 3a). To further delineate, which of these cytokines might be relevant for psoriasis progression, we analyzed the dataset GSE60804 for differential gene expression (Bai et al., 2015). Although *Ifng, Tnf, Il17a,* and *Csf2* were substantially upregulated in lesional skin, *Il1a* and *Il6* were unchanged (Figure 3b). Moreover, the significance of IL-1α and IL-6 as well as IFN-γ in the pathogenesis of psoriasis remains controversial (Blauvelt, 2017; González-López et al., 2008; Harden et al., 2015; Ogata et al., 2012). Therefore, we

experiment with three animals per group (a) or are representative of two independent experiments (b-j). Depicted are six (e and j) or four (h) animals per group. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. Ctrl, control; FSC, forward scatter; ILC, innate lymphoid cell; ns, not significant; SSC, side scatter.

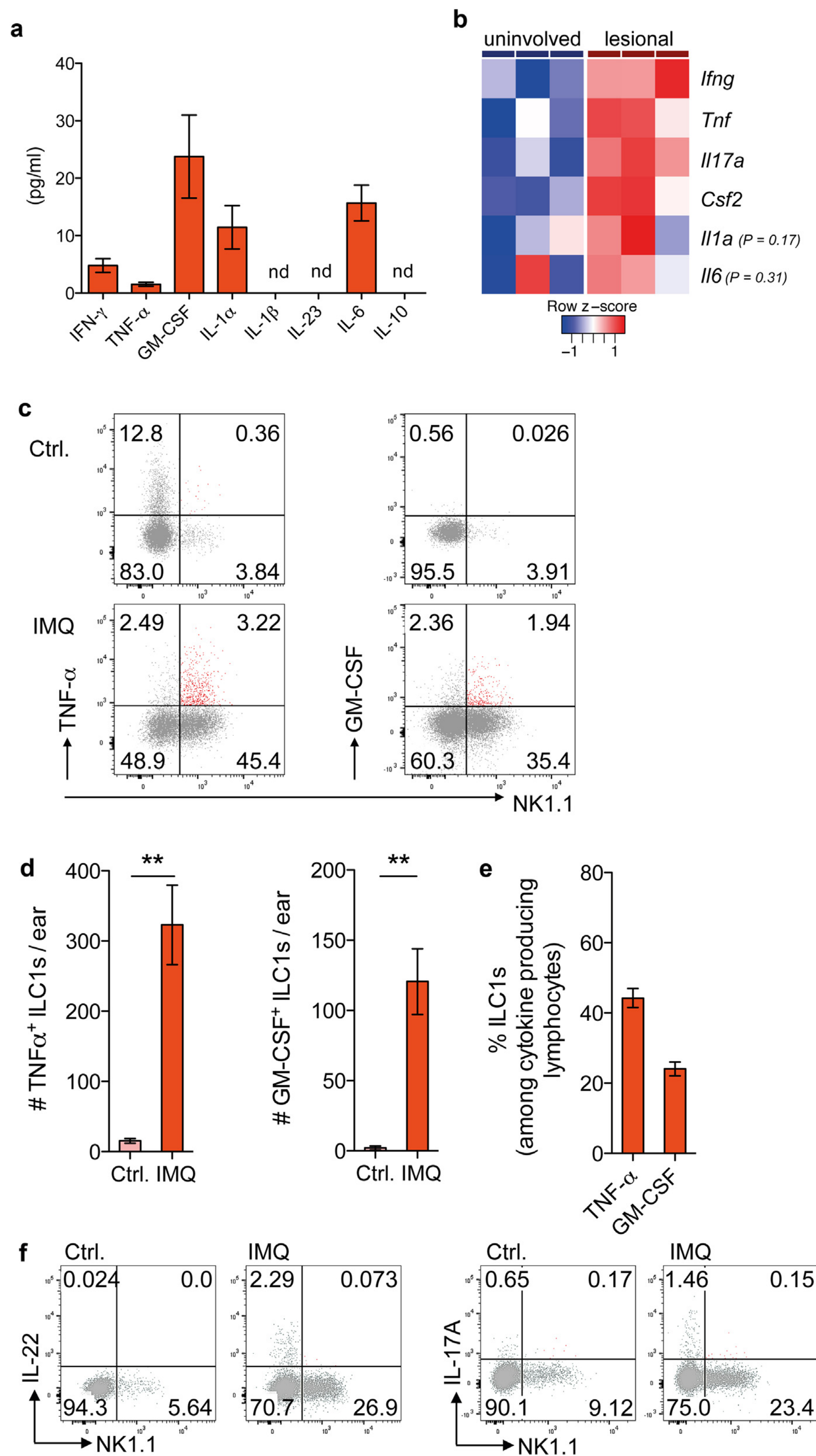

Figure 3. Skin ILC1s from imiquimod-treated skin acquire a disease promoting identity. (**a**) Supernatants of 6 × 10^4 sorted CD3⁻NK1.1⁺CD49a⁺ ILC1s from IMQ (day 4) were analyzed for cytokine production. (**b**) Differential expression of genes encoding proinflammatory cytokines in vehicle- (uninvolved) and imiquimod-treated (lesional) skin, curated manually (data derived from the website of the Gene Expression Omnibus: https://www.ncbi.nlm.nih.gov/geo; GSE60804). Selected genes and products: *Ifng*, IFN-γ; *Tnf*, TNF-α; *Il17a*, IL-17A; *Csf2*, GM-CSF; *Il1a*, IL-1α; *Il6*, IL-6. (**c**) Representative dot plots and (**d**) absolute quantification of the cytokines TNF-α and GM-CSF produced by ILC1s from Ctrl and IMQ. Imiquimod was applied for a total of four consecutive days. (**e**) Quantification of ILC1s among TNF-α and GM-CSF producing lymphocytes. (**f**) Representative dot plots of the cytokines IL-22 and IL-17A produced by ILC1s from Ctrl and IMQ. Imiquimod was applied for a total of four consecutive days. No valid signal was detected. (a) Data shown are representative of two independent experiments with eight animals, pooled to perform triplet analyses of 6 × 10^4 sorted CD3⁻NK1.1⁺CD49a⁺ ILC1s. Data shown are representative of one experiment with three animals per group (b) or are representative of two independent experiments. Depicted are four animals per group (d and e). $^{*}P < 0.05$, $^{**}P < 0.01$. Ctrl, vehicle; ILC, innate lymphoid cell; IMQ, imiquimod-treated skin; nd, not detectable.

excluded these cytokines from further analysis and focused on the contribution of ILC1s to total TNF-α and GM-CSF using flow cytometry. ILC1s from psoriasiform skin secreted substantial amounts of TNF-α and GM-CSF in contrast to cells from vehicle-treated skin (Figures 3c and d). In fact, at their peak, ILC1s constituted an ample source of TNF-α and GM-CSF, accounting for up to 40% of cytokine-producing lymphocytes (Figure 3e). IL-17A and IL-22, two cytokines known to drive psoriasis development (Van Belle et al., 2012), could not be detected (Figure 3f).

Taken together, these data show, that upon imiquimod-induced inflammation, a sizable proportion of skin ILC1s acquire a disease-promoting phenotype defined by secretion of TNF-α and GM-CSF and therefore have the ability to indirectly fuel IL-23/IL-17 signaling (Chiricozzi et al., 2011; Na et al., 2016; Powell et al., 2012).

ILC1s are abundant in psoriasiform skin despite application of NK1.1-depleting antibody

As a proof-of-concept experiment and to exclude a potential "bystander" function, we anticipated depleting ILC1s using NK1.1-depleting antibody. To this end, 200 μg of NK1.1 antibody was injected intraperitoneally every other day, starting one day before imiquimod application. However, after 4 days of imiquimod treatment, we did not observe reduced ear swelling and inflammation (Figure 4a). Flow cytometric analysis revealed that we were not able to significantly diminish skin ILC1s in psoriasiform dermatitis (Figure 4b) however, systemic depletion was successful (Figure 4c). This phenomenon can likely be ascribed to the tissue-resident properties of ILC1s and their role as local sentinels (Gasteiger et al., 2015).

Skin ILC1s dynamically adapt to the inflammatory microenvironment

Because depletion of skin ILC1s was not successful and further models allowing for targeted depletion of this population are lacking, we opted for an alternative approach. Consequently, we sought to confirm the expansion of ILC1s in the early phase of psoriasis, using two additional models.

First, we tested the model of TNCB-induced DTHR (Guenova et al., 2015). Although psoriasis and DTHR are different diseases, they share clinical as well immunological similarities, including Th1 and Th17 involvement (Bai et al., 2016; Pavel et al., 2023; Donglang et al., 2021). As in imiquimod-treated skin, application of TNCB led to a significant increase in ear swelling (Figure 5a) and ILC1 abundance (Figure 5b). Interestingly, we observed that, besides ILC1s, NK cells greatly accumulated in TNCB-treated skin, accounting for ∼40% of infiltrating NK1.1^{+} lymphocytes (Figure 5b). This phenomenon has been described in other models of contact hypersensitivity and reflects a strong NK cell–mediated inflammatory arm, synergizing with the Th1/Th17 axis in DTHR (van den Boorn et al., 2016).

Second, we applied recombinant IL-23 intradermally for a total of four consecutive days. In line with our hypothesis, there was marked edema (Figure 5c) as well as significant accumulation of skin ILC1s (Figure 5d). NK cells were rather scarce compared to ILC1s, corroborating our results from the imiquimod model (Figure 5d). ILC1 accumulation, however, was minor compared to imiquimod, which led to the question of how ILC1s are orchestrated at the site of inflammation. Although several older studies showed that ILC1s are readily activated through IL-12, recent data demonstrate that nuanced functional adaptation of ILCs to the inflammatory microenvironment is important for disease development (Bernink et al., 2015; Bielecki et al., 2021; Colonna, 2018). Therefore, we examined ILC1s from vehicle-treated controls and imiquimod-treated mice for expression of appropriate cytokine receptors. Interestingly, we noted that ILC1s from psoriasiform skin downregulated the IL-12Rβ2 subunit but increased expression of the IL-23 receptor, complementing the psoriasis-specific cytokine milieu (Figure 5e). Thus, ILC1s may require inflammatory cues to gain sensitivity to IL-23.

To test the functional relevance of this observation, we examined the functional impact of IL-12 or IL-23 neutralization on inflammation and in particular, on skin ILC1 abundance. Therefore, we used neutralizing anti-IL12p75 or IL-23p19 antibodies while applying imiquimod for a total of four consecutive days. Although IL-12p75 blockade tended to reduce disease severity, anti-IL-23p19 antibody–treated animals showed significantly decreased ear swelling and ILC1 numbers compared to PBS-treated controls (Figures 5f and g). These results indicate that, in line with inflammation and skin thickness, ILC1 abundance resolved upon administration of IL-23p19 antibody.

Although we were not able to give direct proof, our data suggest that either directly or indirectly, ILC1s are regulated by the inflammatory microenvironment and promote disease initiation.

DISCUSSION

Because of their potential impact in health and disease, ILCs have recently been studied in various settings. Initially, NK cells were thought to be the only member of group 1 ILCs. This paradigm, however, radically changed when a noncirculating subset that developed separately from NK cells was described (Diefenbach et al., 2014; Klose et al., 2014). On the basis of their preferential localization within tissues, these cells were first designated as tissue-resident NK cells and are now largely referred to as ILC1s (Peng et al., 2013; Sojka et al., 2014). Knowledge on the characteristics of ILC1s mainly originates from analyses of steady state, whereas their presence and function in chronic inflammatory diseases remains poorly defined.

To address this notion, we focused on skin ILC1s in the murine model of imiquimod-induced psoriasiform dermatitis. Surprisingly, we noted substantial infiltration of this population at early time points after disease induction with a peak at day four. At this peak, skin ILC1s outnumbered the key players in the pathogenesis of psoriasis, ILC3s and γδ T cells/Th17 cells, by >15:1 and 7:1, respectively. When inflammation was fully established on day 7, ILC1 numbers declined, potentially opening a niche for other infiltrating cell populations (Cai et al., 2011; Bielecki et al., 2021). Moreover, we observed that under homeostatic conditions lymph node–derived ILC1s were almost absent, whereas in imiquimod-treated mice ILC1s accounted for up to 23% of lymph node–derived NK1.1^{+} group 1 ILCs. These data not only strengthen the concept of ILC1-mediated early inflammation, but also hint toward systemic involvement in psoriasis, which has been increasingly appreciated over the past decade (Grozdev et al., 2014). Moreover, they help to address the following question: Do ILC1s contribute to psoriasis induction or are they just a bystander population? We cannot give a fully satisfying answer to this, because depletion of skin ILC1s using NK1.1-depleting antibody was ineffective. Moreover, appropriate mouse models specifically targeting skin ILC1s are so far lacking. However, it seems rather unlikely that this population just "passes-by," considering that they account for up to 20% of all infiltrating

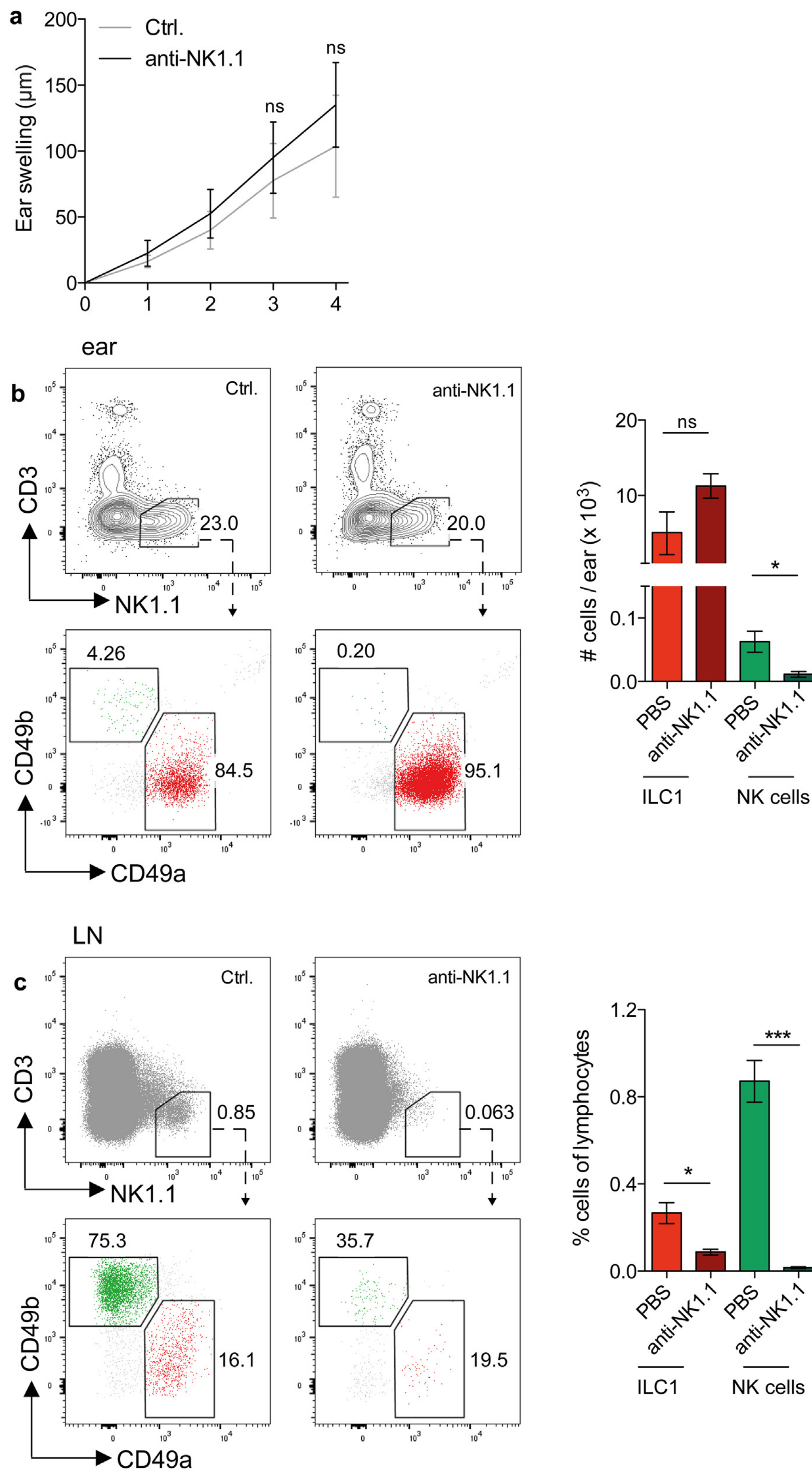

Figure 4. Skin ILC1s are abundant despite application of NK1.1-depleting antibody. (**a**) Ear swelling of PBS- or anti-NK1.1 antibody–treated animals after four consecutive days of Imiquimod application. PBS or anti-NK1.1 antibody was injected every other day starting one day before imiquimod treatment. (**b**) Representative contour/dot plots and quantification of skin ILC1s and NK cells on day four of imiquimod treatment. (**c**) Representative dot plots and quantification of ILC1s and NK cells from skin-draining LNs. Data shown are representative of two independent experiments. Depicted are four animals per group. *$P < 0.05$, ***$P < 0.001$. ILC, innate lymphoid cell; LN, lymph nodes; ns, not significant.

lymphocytes during the first days of psoriasiform dermatitis. Another factor arguing against a bystander role of skin ILC1s is the potential to quickly adapt to the psoriasis-specific microenvironment by downregulation of the IL-12Rβ2 subunit and upregulation of the IL-23 receptor. In accordance with this phenotypic shift, ILC1s were significantly reduced in mice that were treated with IL-23–neutralizing antibody.

To confirm our results obtained in imiquimod-induced psoriasiform dermatitis, we included two additional murine models in our analyses: TNCB-induced DTHR and IL-23–induced

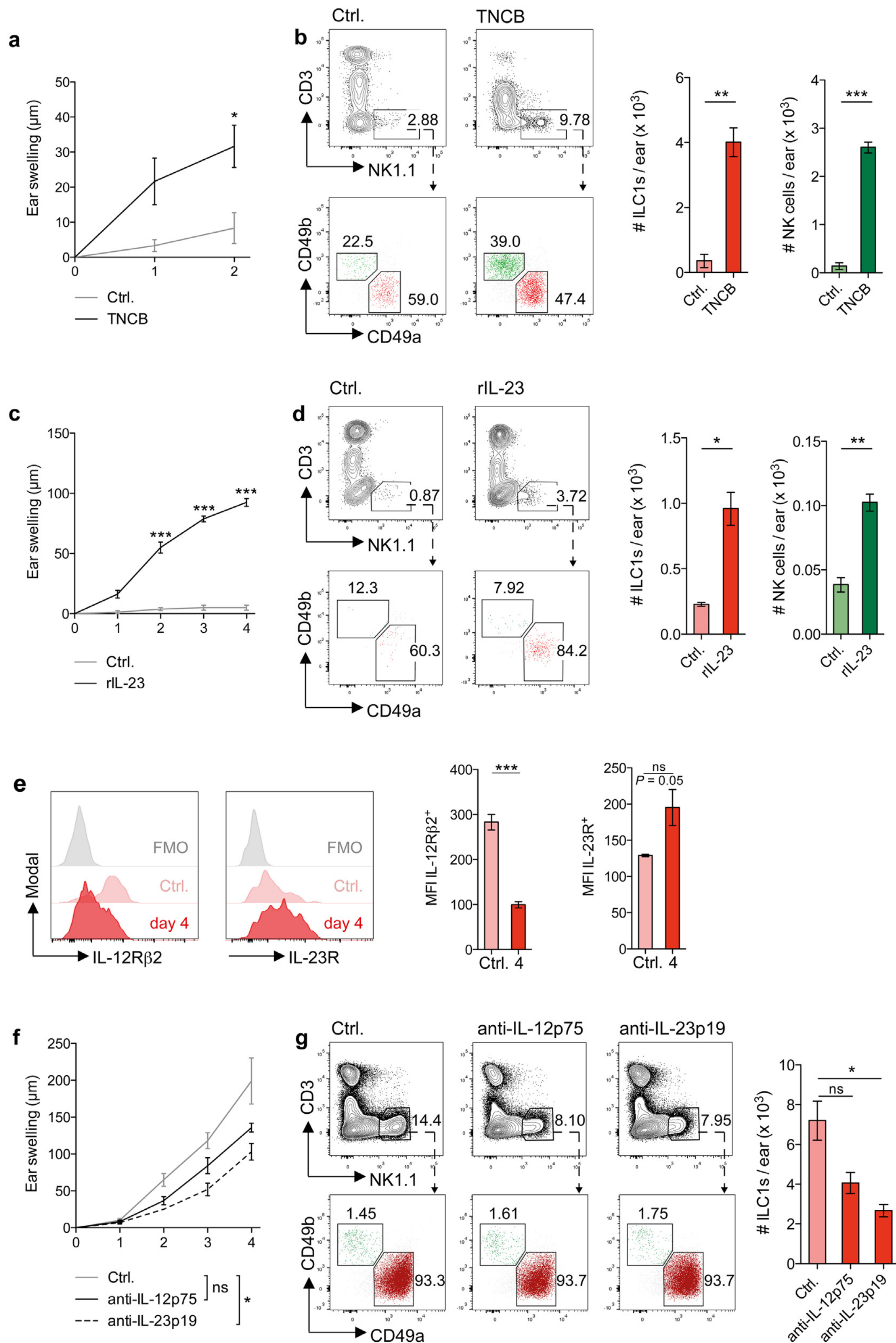

Figure 5. Skin ILC1s dynamically adapt to the inflammatory environment. (**a**) Ear swelling of acetone/olive oil-treated Ctrl and TNCB mice. (**b**) Representative contour/dot plots and quantification of ILC1s and NK cells from PBS (Ctrl) and TNCB skin. (**c**) Ear swelling of PBS-treated control mice (Ctrl) and rIL-23 mice. (**d**) Representative contour/dot plots and quantification of ILC1s and NK cells from PBS and rIL-23 skin (day 4 of PBS or rIL-23 treatment). (**e**) Representative histograms and quantification of IL-12Rβ2 and IL-23R expression by skin ILC1s from Ctrl or Imiquimod-treated mice (day 4). (**f**) Ear swelling of Imiquimod and PBS (Ctrl), anti-IL-12p75- or anti-IL-23p19 mice. PBS, anti-IL-12p75 or anti-IL-23p19 antibodies were injected every other day starting one day prior to Imiquimod treatment. (**g**) Representative contour/dot plots and quantification of Imiquimod and PBS, anti-IL-12p75, or anti-IL-23p19 skin (day 4 of Imiquimod treatment). Data are representative of two independent experiments. Depicted are three (a and b) or four (c-g) animals per group. *$P < 0.05$,

psoriasis. Both models confirmed significant expansion of ILC1s at early time points after disease induction, however to a lesser extent than in the imiquimod model. In TNCB-induced DTHR this might be because of an additional, strong NK cell response known to synergize with the Th1/Th17 axis (van den Boorn et al., 2016), which could not be observed in imiquimod-induced psoriasiform dermatitis. In IL-23–induced psoriasis, on the other hand, the intricate interplay between various disease-initiating cytokines, which might be required to endow ILC1s with sensitivity to IL-23 to subsequently promote disease progression, was skewed only toward IL-23 (Bromley et al., 2013; Kabashima and Nomura, 2017).

To further characterize skin ILC1s under inflammatory conditions, we performed a thorough flow cytometric analysis. One defining distinction between NK cells and ILC1s is the expression of the integrins α1 (CD49a) and α2 (CD49b) (Peng et al., 2013; Sojka et al., 2014) as well as the transcription factors T-bet and Eomesodermin (Daussy et al., 2014). Although ILC1s are $CD49a^{+}$ and T-bet–dependent, NK cells are $CD49b^{+}$ and require both transcription factors for development (Simonetta et al., 2016). However, there are exceptions to this rule, because thymic as well as salivary ILC1s share a number of features with NK cells, including expression of CD49b and Eomesodermin (Cortez and Colonna, 2016; Gabrielli et al., 2017; Vosshenrich et al., 2006). Similar to previous reports, we observed that skin ILC1s expressed CD49a, CXCR6, and CD44, markers proposed to be important for homing and tissue residency (Jiao et al., 2016). Another defining marker often used to distinguish ILC1s from NK cells in humans is the IL-7 receptor alpha chain (CD127). Several reports on murine hepatic as well as uterine ILC1s, however, revealed poor expression of CD127 and suggested that this marker is not necessarily definitive in all settings (Cortez et al., 2017; Filipovic et al., 2018; Fuchs, 2016; Sojka et al., 2014; Wang et al., 2018). Moreover, mice deficient in IL-7 or the IL-7 receptor have normal ILC1 numbers, indicating that this cytokine is dispensable for their development (Daussy et al., 2014; Fuchs, 2016; Klose et al., 2014; Satoh-Takayama et al., 2010). This incomplete clarity on how to properly define ILC1s in different organs and/or under inflammatory conditions might also explain discrepancies observed in human studies. Recently, three articles on the distribution of ILCs in patients with psoriasis were published. Two of these studies included CD127 in their flow cytometric analysis to define ILC1s in the skin and peripheral blood (Teunissen et al., 2014; Villanova et al., 2014). The third study mainly focused on the expression of transcription factors (Brüggen et al., 2016). Strikingly, although the first two studies mainly described an increase in ILC3 abundance (Teunissen et al., 2014; Villanova et al., 2014), the third study also found a substantial rise in ILC1 numbers, rendering this subset as one of the most abundant ILC populations in lesional skin of psoriasis patients (Brüggen et al., 2016). In conclusion, further refinement of phenotypic characteristics in the human skin will be required to more precisely investigate the contribution of ILC1s to inflammation. Our phenotypic analysis might help to guide this process in the future.

When examining the potential function of ILC1s in the course of psoriasis induction, we found this subset to be an ample source of multiple proinflammatory mediators driving disease. These included TNF-α, GM-CSF, IL-1α, and IL-6. Although IL-1α and IL-6 have been discussed controversially in the context of psoriasis, TNF-α and GM-CSF are able to activate $CD4^{+}$ T cells and induce differentiation of macrophages toward a proinflammatory phenotype, respectively (McGeachy, 2011; Mylonas, 2018; Na et al., 2016). Neutralization of GM-CSF in murine psoriasis has also been shown to reduce expression of TNF-α and to ameliorate imiquimod-induced psoriasiform dermatitis (Scholz et al., 2017). TNF-α together with IL-17A, on the other hand, is able to increase the production of IL-23 and other proinflammatory cytokines, such as IL-1β and IL-6 in keratinocytes (Chiricozzi et al., 2011; Ehst et al., 2021). Furthermore, TNF-α has been described to induce IL-17 producing cells in a synergistic fashion together with IL-23 (Powell et al., 2012). This feed-forward loop initiated by antigen-presenting cells and TNF-α–producing ILC1s might also be relevant in early psoriasis, directing ILC3s, γδ T cells, and Th17 cells to the site of inflammation.

Together, our data support the concept of early ILC1-mediated inflammation as a “supporting act” until IL-17–producing ILC3s, γδ T cells, and Th17 cells take over. Our data show that skin ILC1s are able to quickly adapt to the disease-specific environment and to exhibit a proinflammatory identity that can drive early pathology. These insights could help to better understand the dynamics of ILC1 responses under inflammatory conditions and might contribute to improve management of chronic inflammatory diseases.

MATERIALS AND METHODS

Mice

C57BL/6N mice were bred at the Technical University of Munich (Klinikum rechts der Isar) under specific pathogen-free conditions. Mice were used at 8–12 weeks of age. Littermates of the same sex were randomly assigned to vehicle or imiquimod groups in all experiments. All animal experiments were performed in accordance with national and institutional guidelines for animal care and were approved by the Governmental Review Board Oberbayern (Regierung von Oberbayern).

Vehicle and imiquimod treatment

Ears of C57BL/6N mice were treated with 15 mg/ear of 5% imiquimod cream (Aldara, Meda Pharma, Solna, Sweden) for a total of 2–7 days or 15 mg/ear of white petrolatum (Bombastus-Werke AG, Freital, Germany), referred to as vehicle controls, for a total of 4–7 days.

For cytokine neutralization, mice were injected intraperitoneally every second day starting one day before imiquimod treatment. Mice received 200 μg of anti-IL-23p19 (G23-8, BioXcell) or IL-12p75 (R2-9A5, BioXcell) antibodies. Control mice received PBS.

$^{**}P < 0.01$, $^{***}P < 0.001$. Anti-IL-12p75, anti-IL-12p75 treated; anti-IL-23p19, anti-IL-23p19 treated; ctrl, control; ILC, innate lymphoid cell; IL-12Rβ2, IL-12 receptor beta subunit 2; ns, not significant; rIL-23; rIL-23 treated; TNCB, TNCB-treated.

For depletion of skin ILC1s, mice were injected intraperitoneally every second day starting one day before imiquimod treatment. Mice received 200 μg of NK1.1 (PK136, BioXcell) antibody.

TNCB-induced DTHR

C57BL/6N mice were sensitized on their shaved abdomens with 2% TNCB (Sigma-Aldrich, St. Louis, MO) in acetone/olive oil (4:1). After 7 days, ears were challenged with 1% TNCB in acetone/olive oil (4:1). Analysis was performed on day 2 after challenge. Control mice received acetone/olive oil (4:1).

Intradermal IL-23 injection

Mouse ears were injected intradermally with 15 μl PBS or 500 ng rmIL-23 in PBS (BioLegend, San Diego, California) daily for four days.

Cell isolation from tissues and flow cytometry

Freshly isolated ear tissue was diced and subsequently digested with 0.25 mg/ml Liberase TL (Roche) at 37 °C for 135 minutes. Epithelial fragments were removed by filtration (EASYstrainer 70 μm mesh, Greiner Bio-one, Kremsmünster, Austria). Lymph nodes were passed over a 40 μm cell strainer (EASYstrainer, Greiner Bio-one) and washed with PBS. Single-cell suspensions were stained with Live/Dead Fixable Aqua dead cell stain kit (Invitrogen, Waltham, MA) for 20 minutes to exclude dead cells and treated with Fc-blocking anti-mouse CD16/32 antibodies (TruStain FcX; BioLegend). Cells were subsequently stained with fluorochrome-conjugated antibodies against the following surface antigens: CD45, CD3, NK1.1, CD49a, CD49b, CD44, CCR6, CD27, CD11b, CX3CR1, CXCR3, CD127, CD103, IL-23R (all BioLegend), and IL-12Rβ2 (R&D Systems, Minneapolis, MN).

Restimulation of cells for intracellular cytokine staining

Cells isolated from ear tissue were stimulated for 1.5 hours with the cell activation cocktail containing phorbol 12-myristate 13-acetate /ionomycin and brefeldin A (BioLegend). Stimulated cells were stained with the Live/Dead Fixable Aqua dead cell stain kit (Invitrogen), stained for surface markers, fixed with the FoxP3 Fixation/Permeabilization kit (Invitrogen) in accordance with the manufacturer's instructions and stained with antibodies against IFN-γ, TNF-α, GM-CSF, IL-17A, and IL-22 (all BioLegend).

Fluorescence-activated cell sorting and in vitro culture of ILCs

ILC1s were sorted by flow cytometry (BD FACSAria Fusion; Becton Dickinson, Franklin Lakes, NJ) from imiquimod-treated skin based on the absence of CD3 but on the expression of NK1.1 and CD49a. Purity was determined by reanalysis of a fraction of sorted cell samples and exceeded 95%. In 90 μl RPMI 1640 supplemented with 10% fetal calf serum, penicillin, streptomycin, HEPES, glutamine, nonessential amino acids and 50 mM β-mercaptoethanol (complete RPMI), 6×10^4 ILC1s were cultured for 20 hours. For the last 4 hours, 50 ng/ml phorbol 12-myristate 13-acetate and 2.5 μg/ml ionomycin were added (total volume 100 μl). Cytokine expression in the cell-free supernatant was assessed using a bead-based cytokine detection assay (LEGENDPlex; BioLegend) according to the manufacturer's instructions.

Immunofluorescence images

Ear tissue was embedded in TissueTek optimal cutting temperature freezing medium (Sakura Finetek, Rijn, the Netherlands), frozen in liquid nitrogen and stored at -80 °C until further processing. 20 μm sections were fixed in ice-cold acetone for 10 minutes. Slides were rinsed in PBS twice and blocked with blocking buffer (Dako) for 30 minutes in a humid chamber at room temperature. The primary antibody (purified hamster anti-rat/mouse CD49a; Becton Dickinson) was kept overnight at 4 °C. The secondary antibody (goat anti-hamster IgG AlexaFluor488; BioLegend) was added after two washing steps for 1 hour at room temperature. Sections were counterstained with DAPI, mounted in aqueous mounting media (Abcam, Cambridge, United Kingdom) and analyzed on a Leica DM 4000B (Leica-Microsystems, Wetzlar, Germany) microscope.

Analysis of gene expression data

Publicly available datasets were downloaded from the Gene Expression Omnibus (GEO, https://www.ncbi.nlm.nih.gov/geo). Normalized data were log2-transformed and manually curated. Differential expression of genes was analyzed as follows: one per row; change >1-fold (log2 value) and false-discovery rate ≤0.05. All data were processed within R using the following packages: Biobase, GEOquery, limma and gplots. Data from the gene expression dataset GSE60804 were used in this paper.

Statistical analyses

Statistical analyses were performed using GraphPad Prism software. Statistical significance for two groups was determined using an unpaired two-tailed Student *t*-test. Statistical analyses for three or more groups were performed by ANOVA with Tukey multiple comparison test. Results are expressed as mean ± SEM. P values <0.05 were considered significant.

Data availability statement

The dataset analyzed in this study (GSE60804) was downloaded from the Gene Expression Omnibus (GEO, https://www.ncbi.nlm.nih.gov/geo).

ORCIDs

Beatrix D.G. Evers: http://orcid.org/0000-0002-3422-8121
Miriam Hils: http://orcid.org/0000-0003-4268-0342
Christoph Heuser: http://orcid.org/0000-0003-1410-7573
Inga M. Hölge: http://orcid.org/0000-0002-3689-6457
Désirée Argiriu: http://orcid.org/0000-0001-9787-9346
Yuliya Skabytska: http://orcid.org/0000-0002-8731-2917
Susanne Kaesler: http://orcid.org/0000-0002-4029-7895
Christian Posch: http://orcid.org/0000-0003-0296-3567
Percy A. Knolle: http://orcid.org/0000-0003-2983-0414
Tilo Biedermann: http://orcid.org/0000-0002-5352-5105

CONFLICT OF INTEREST

The authors state no conflict of interest.

ACKNOWLEDGMENTS

This research was funded by Else-Kröner-Fresenius-Forschungskolleg (BDGE). This work was also supported in parts by grants from Deutsche Forschungsgemeinschaft (DFG, German Research Foundation) to TB, CRC 1335 P17, CRC 1371 P06; GRK2668/1 (2022) - Projectnumber: 435874434/ A2, bilateral funding from Luxembourg National Research Fund (FNR) project C17/BM/11656090 – DFG DACH-Lead-Agency BI696/12-1.

AUTHOR CONTRIBUTIONS

Conceptualization: BDGE, TB; Data curation: BDGE, MH; Formal Analysis: BDGE, MH; Funding Acquisition: BDGE, TB; Investigation: BDGE, MH, DA; Methodology: BDGE, MH, CH, IMH, YS, SK, CP, PAK, TB; Project Administration and Resources: TB; Supervision: TB; Validation: MH, SK; Visualization and Writing-Original Draft: BDGE, MH, TB; Writing-Review and Editing: BDGE, MH, CH, SK, TB

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