View metadata, citation and similar papers at core.ac.uk

brought to you by CORE

provided by Elsevier - Publisher Connector

See related commentary on pg 2305

Composition of Innate Lymphoid Cell Subsets in the Human Skin: Enrichment of NCR$^+$ ILC3 in Lesional Skin and Blood of Psoriasis Patients

Marcel B.M. Teunissen[1,7], J. Marius Munneke[2,7], Jochem H. Bernink[3], Phyllis I. Spuls[1], Pieter C.M. Res[1], Anje te Velde[3], Stanley Cheuk[4], Marijke W.D. Brouwer[1], Stef P. Menting[1], Liv Eidsmo[4], Hergen Spits[5], Mette D. Hazenberg[2] and Jenny Mjösberg[6]

Innate lymphoid cells (ILCs) are increasingly appreciated as important regulators of tissue homeostasis and inflammation. However, their role in human skin remains obscure. We found that healthy peripheral blood CD117$^+$ ILC3, lacking the natural cytotoxicity receptor (NCR) NKp44 (NCR$^-$ ILC3), CD117$^-$NCR$^-$CRTH2$^-$ CD161$^+$ ILC1, and CRTH2$^+$ ILC2, express the skin-homing receptor cutaneous lymphocyte antigen (CLA). NCR$^+$ ILC3 were scarce in peripheral blood. Consistently, we identified in normal skin ILC2 and NCR$^-$ ILC3, a small proportion of CD161$^+$ ILC1, and hardly any NCR$^+$ ILC3, whereas NCR$^+$ ILC3 were present in cultured dermal explants. The skin ILC2 and NCR$^+$ ILC3 subsets produced IL-13 and IL-22, respectively, upon cytokine stimulation. Remarkably, dermal NCR$^-$ ILC3 converted to NCR$^+$ ILC3 upon culture in IL-1β plus IL-23, cytokines known to be involved in psoriatic inflammation. In line with this observation, significantly increased proportions of NCR$^+$ ILC3 were present in lesional skin and peripheral blood of psoriasis patients as compared with skin and blood of healthy individuals, respectively, whereas the proportions of ILC2 and CD161$^+$ ILC1 remained unchanged. NCR$^+$ ILC3 from skin and blood of psoriasis patients produced IL-22, which is regarded as a key driver of epidermal thickening, suggesting that NCR$^+$ ILC3 may participate in psoriasis pathology.

Journal of Investigative Dermatology (2014) **134,** 2351–2360; doi:10.1038/jid.2014.146; published online 24 April 2014

INTRODUCTION

Innate lymphoid cells (ILCs) is the collective term for a group of hematopoietic cells with a lymphoid morphology, all of which lack rearranged antigen-specific receptors such as those expressed by T and B cells (Spits and Di Santo, 2011; Spits and Cupedo, 2012). ILCs are defined as lineage-negative (Lin$^-$) cells with a lymphoid morphology that express the α-chain of the IL-7 receptor (CD127). ILCs are effector cells that perform their function via the release of cytokines and have been described to be involved in protective responses against microorganisms, in lymphoid tissue formation, in tissue remodeling after damage, and in the homeostasis of tissue stromal cells (Spits and Di Santo, 2011; Spits and Cupedo, 2012). The prototypic members of this family are the Natural killer (NK) cells that provide protection against viruses and tumors. Over the last decade, other members of the ILC family have been identified that are developmentally related to NK cells, as they all depend on the transcription factor Id2 and the common γ-chain of the IL-2 receptor (IL2Rγc). ILC subsets have been classified into three groups on the basis of transcription factor expression and cytokine production profile (Spits *et al.*, 2013). Group 3 ILCs (ILC3), which may express natural cytotoxicity receptors (NCR) including NKp44, rely on the transcription factor RORγt, respond to IL-1β and IL-23, and produce IL-17 and/or IL-22. These cells are involved in tissue remodeling and tissue protection in both mice and humans. ILC3 include the lymphoid tissue inducer (LTi) cells that are crucial for formation of lymph nodes during fetal development (Cupedo *et al.*, 2009). Another subset distinct from ILC3, ILC2, was recently described in the context of lung tissue protection and helminth parasite expulsion in the gut (Moro *et al.*, 2010; Neill *et al.*, 2010; Price *et al.*, 2010; Monticelli *et al.*, 2011). In humans, these cells express CRTH2 (Mjösberg *et al.*, 2011), lack NKp44, produce type 2 cytokines such as IL-5 and IL-13 (Mjösberg *et al.*, 2011, 2012), and are

[1]*Department of Dermatology, University of Amsterdam, Amsterdam, The Netherlands;* [2]*Department of Hematology, University of Amsterdam, Amsterdam, The Netherlands;* [3]*Tytgat Institute for Liver and Intestinal Research, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands;* [4]*Dermatology and Venereology Unit, Department of Medicine Solna, Karolinska Institutet, Solna, Sweden;* [5]*Department of Cell Biology and Histology, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands and* [6]*Center for Infectious Medicine, Department of Medicine Huddinge, Karolinska Institutet, Huddinge, Sweden*

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

Correspondence: Hergen Spits, Department of Cell Biology and Histology, Academic Medical Center, University of Amsterdam, 1105 AZ Amsterdam, The Netherlands. E-mail: hergen.spits@amc.uva.nl

Abbreviations: CLA, cutaneous lymphocyte antigen; ILC, innate lymphoid cell; Lin, lineage; NCR, natural cytotoxicity receptor

Received 4 June 2013; revised 13 February 2014; accepted 5 March 2014; accepted article preview online 21 March 2014; published online 24 April 2014

© 2014 The Society for Investigative Dermatology

GATA-3 dependent (Mjösberg *et al.*, 2012). More recently, we described a population of $CD117^-NKp44^-$ ILC that are distinct from NK cells and dedicated to the production of IFN-γ, and hence termed ILC1 (Bernink *et al.*, 2013). These cells express the transcription factor T-bet, respond to IL-12 plus IL-18 by production of IFN-γ, and are enriched in the inflamed ileum mucosa of patients with Crohn's disease (Bernink *et al.*, 2013).

As ILCs are crucial in mucosal defense, it is reasonable to assume that they are also involved in immune defense and tissue repair of the skin, which is one of the body's largest physical and immunological barriers. Indeed, a previous report demonstrated the presence of $NKp44^+$ ILCs in inflamed human skin (Cella *et al.*, 2009). More recently, $ROR\gamma t^+$ ILC3 have been observed in Aldara-induced skin inflammation (Pantelyushin *et al.*, 2012) that in some, but not all, aspects resembles psoriasis (Nestle *et al.*, 2009), one of the most common inflammatory disorders of the skin. Previous studies have ascribed IL-23, which induces IL-17 and IL-22 production from T-helper cells, as the essential cytokine for driving skin inflammation (Pantelyushin *et al.*, 2012). However, recent studies demonstrate that Aldara-induced psoriasiform skin inflammation occurs to the same extent in $Rag1/2^{-/-}$ mice lacking T and NK T cells (Hedrick *et al.*, 2009; Pantelyushin *et al.*, 2012), but not in $Rag2^{-/-}Il2r\gamma c^{-/-}$ mice that in addition lack ILC and conventional NK cells. These experimental studies suggest that skin-invading $ROR\gamma^+$ ILC3 can induce a pathology with some resemblance to psoriasis (Pantelyushin *et al.*, 2012).

Here we examined the ILC populations that are the most dominant in skin of healthy donors, $CD117^-CRTH2^-NCR^-$ $CD161^+$ ILC1, $CRTH2^+$ ILC2, and NCR^- ILC3. Few NCR^+ ILC3 were present in normal skin, whereas these NCR^+ ILC3 were present in cultured dermal explants. Skin ILC2 and NCR^+ ILC3 were functional as they responded to cytokine stimulation by secreting IL-13 and IL-22, respectively. NCR^- ILC3 cultured *in vitro* with IL-1β plus IL-23 converted to NCR^+ ILC3 cells. Strikingly increased proportions of NCR^+ ILC3 were present in human psoriatic skin lesions as compared with normal skin. The increase in NCR^+ ILC3 in the skin of psoriasis patients was mirrored by similar increases in peripheral blood NCR^+ ILC3 of psoriasis patients as compared with healthy individuals. Both blood and skin NCR^+ ILC3 produced IL-22, but very little IL-17. These data suggest that NCR^+ ILC3, by providing a source of IL-22, may participate in psoriasis pathology.

RESULTS

Composition of human ILC subsets in normal human adult skin

Recently, we and others identified several ILC populations in humans that can be classified in three groups on the basis of the expression of c-Kit (CD117), NKp44, and CRTH2 (Spits *et al.*, 2013). As ILCs are involved in tissue immunity, remodeling, and epithelial barrier maintenance in the lung and gut (Sonnenberg and Artis, 2012; Spits and Cupedo, 2012; Walker *et al.*, 2013), we hypothesized that ILCs would also be involved in skin immunity and homeostasis. With this background, we set out to determine the composition of ILCs present in adult normal human skin. Freshly prepared, single-cell suspensions from dermis of healthy individuals contained a discrete population of $CD45^+$ cells lacking Lin markers for T cells (CD3, TCR), B cells (CD19), NK cells (CD94), myeloid and plasmacytoid dendritic cells (CD1a, CD11c, CD123, BDCA2), monocytes and macrophages (CD14), mast cells (FcεR1), and stem cells (CD34) (Figure 1a). These $CD45^+Lin^-$ cells with lymphoid morphology and high expression of CD127 represented $1.3 \pm 0.3\%$ of the $CD45^+$ dermal lymphocytes (Figure 1a and b). Approximately 25% ($24.2 \pm 9.9\%$) of these cells expressed CRTH2 (Figure 1a and b), a marker for human ILC2 (Mjösberg *et al.*, 2011). Interestingly, the IL-22-producing NCR^+ ILC3 population, which we previously described in tonsil and gut mucosa (Crellin *et al.*, 2010; Bernink *et al.*, 2013), could hardly be found in freshly prepared dermal-cell suspensions ($1.3 \pm 0.6\%$ of $CD45^+Lin^-CD127^+$ ILC, Figure 1a and b), which is similar to the very low proportions of NCR^+ ILC3 in peripheral blood (Figure 2). Importantly, the absence of NCR^+ ILC3 in these freshly prepared dermal-cell suspensions could not be explained by enzymatic cleavage of NKp44 as experiments showed that the enzymes used (dispase II and collagenase D) did not considerably affect expression of critical ILC markers such as CD127, CD117, CRTH2, or NKp44 (Supplementary Figure S1 online). Of note, we also observed a prominent population of $CD117^-CRTH2^-NKp44^-$ ILCs in both freshly isolated dermal-cell suspensions ($39.8 \pm 14.4\%$) and dermal crawl-out cultures ($24.8 \pm 11.0\%$) (Figure 1). Phenotypically, this population resembles the ILC1 that we recently described to be accumulated in Crohn's disease ileum (Bernink *et al.*, 2013). However, only a minority of this skin population expressed the pan-ILC marker CD161 (Figure 1), indicating that the ILC3-derived ILC1 that we identified (Bernink *et al.*, 2013) are very rare in the normal skin, comparable to our findings in non-inflamed intestine (Bernink *et al.*, 2013). Hence, this prevented us from performing functional studies of these cells.

Peripheral blood ILCs express the skin-homing marker CLA

A likely source of skin ILCs would be peripheral blood, in which, $CD117^-CRTH2^-NCR^-$ ILC1, $CRTH2^+$ ILC2, and NCR^- ILC3 are present (Mjösberg *et al.*, 2011; Bernink *et al.*, 2013). To test our hypothesis, we examined the expression of the skin-homing markers cutaneous lymphocyte antigen (CLA) and CCR10 on peripheral blood ILCs (Figure 2). Interestingly, both ILC2 and NCR^- ILC3 expressed the skin-homing marker CLA, whereas few cells expressed CCR10, which was restricted to the CLA^+ ILC population (Figure 2). Strikingly, the proportion of NCR^+ ILC3 was very low in peripheral blood from healthy donors (Figure 2), which correlates well with the very low percentages of NCR^+ ILC3 in freshly isolated normal skin (Figure 1).

Function of dermal ILC2

$CRTH2^+$ ILC2 were also present in so-called crawl-out dermal-cell suspensions (Figure 1b), representing cells that had migrated out of cultured dermal explants. Both freshly isolated and crawl-out $CRTH2^+$ ILC2 showed high expression

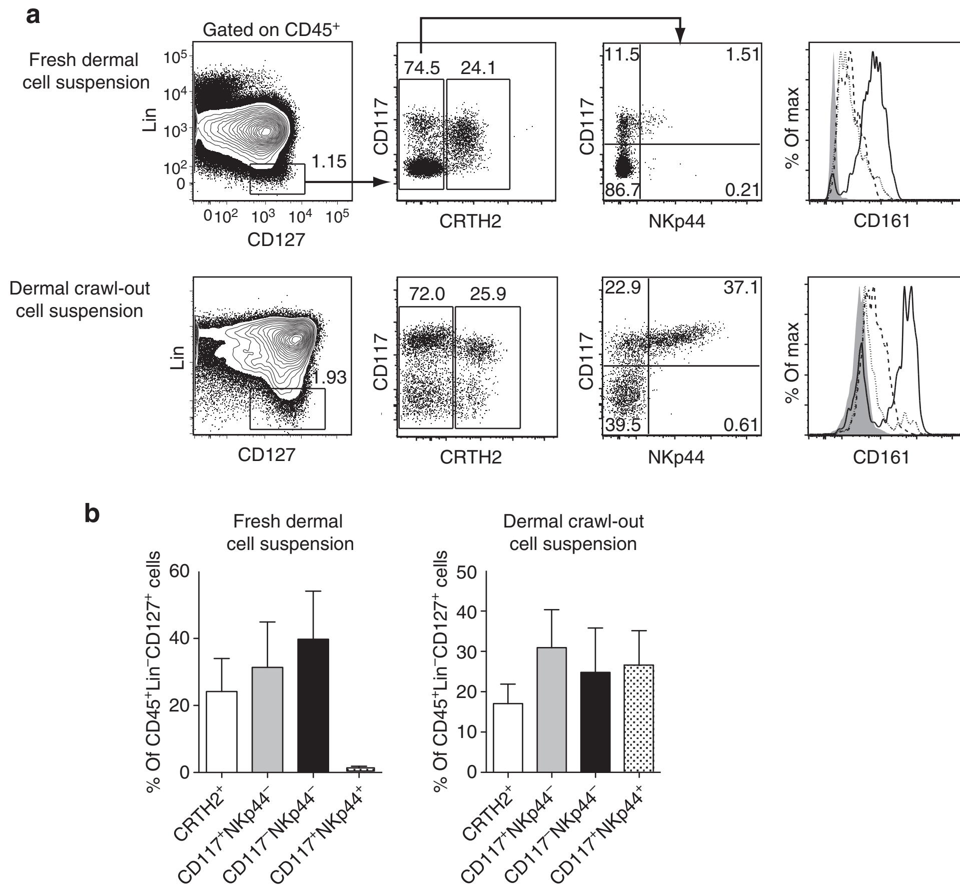


Figure 1. Innate lymphoid cells in normal human skin. (**a**) Flow cytometry analysis of Lin⁻CD127^hi ILC within the CD45$^+$ lymphocyte population in freshly prepared cell suspensions (upper panel) and crawl-out cells from dermal explant cultures (lower panel). Numbers in gates (outlined areas) and quadrants indicate percent cells in each throughout. Histograms depict CD161 expression by CRTH2$^+$ ILC2 (black line), CD117$^+$NKp44$^-$ cells (dashed line), CD117$^+$NKp44$^+$ ILC3 (dotted line), and CD117$^-$NKp44$^-$ cells (light gray shading). The lineage 'cocktail' included antibodies to CD1a, CD3, CD4, CD11c, CD14, CD19, CD34, CD123, TCRαβ, TCRγδ, BDCA2, FcεR1, and CD94. (**b**) ILC subpopulation distribution in normal human skin is dependent on the method used for sample preparation. Data are depicted as the mean percentage ± SD and are representative of 10 independent experiments with one donor each. ILC, innate lymphoid cell; Lin, lineage.

of CD161 and a variable expression of CD117 (Figure 1), concurrent with the phenotype of ILC2 that we previously identified in human peripheral blood and nasal polyps (Mjösberg *et al.*, 2011, 2012). Freshly prepared and crawl-out flow cytometry–sorted dermal CRTH2$^+$ ILC2 responded to IL-33 plus thymic stromal lymphopoietin (TSLP) stimulation with secretion of IL-13 (Figure 3a), confirming their similarity to the previously described human ILC2 (Mjösberg *et al.*, 2011, 2012). In addition, ILC2 cell lines could be established, which maintained their expression of CRTH2, CD127, and CD117 (Figure 3b) and lacked markers for T and NK cells (CD3 and CD94, data not shown). Furthermore, these cells expressed CD25 (Figure 3b), a previously described marker for human ILC2 (Monticelli *et al.*, 2011). Upon stimulation with phorbol 12-myristate 13-acetate (PMA) plus ionomycin, these skin ILC2 cell lines produced IL-13 (Figure 3b). Furthermore, ILC2 cell lines responded to TSLP with increased production of IL-13 and IL-5, especially in the combination with IL-25, which together exerted a synergistic effect on inducing IL-13 production (Figure 3c and d). Noteworthy, addition of IL-33 to this mix did not further enhance IL-13 production, and TSLP plus IL-33 did not synergistically enhance IL-13 production as compared with TSLP or IL-33 alone (Figure 3c and d). Hence, in contrast to previously published data on ILC2 in polyps and blood where IL-33 and TSLP synergistically enhance type 2 cytokine production (Mjösberg *et al.*, 2012), ILC2 in the skin are mainly regulated by TSLP and IL-25 and to a lesser extent by IL-33. In contrast to blood and polyp ILC2 (Mjösberg *et al.*, 2012), skin ILC2 cell lines did not produce significant levels of IL-4, IL-9, or GM-CSF (data not shown). Of note, a minority of the cultured ILC2 co-expressed IFN-γ and IL-22, and to a lesser extent IL-17 (Figure 3b), in accordance with our previous studies of blood and polyp ILC2 (Mjösberg *et al.*, 2011, 2012). However, this was only seen with PMA/ionomycin stimulation but not with ILC2-specific stimulations such as IL-25, IL-33, and TSLP (data not shown). Hence, ILC2 seem to have the capacity to produce some IL-22 and IFN-γ; however, the physiological triggers and importance of this production remains unknown.

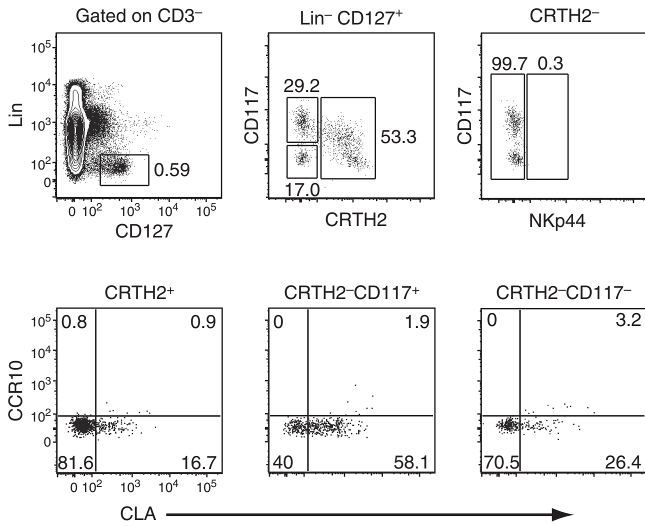


Figure 2. Peripheral blood ILCs express skin-homing marker cutaneous lymphocyte antigen (CLA). Gating for Lin⁻CD127^{hi} ILC subsets in peripheral blood of a healthy donor. Flow cytometry analysis of the expression of CLA and CCR10 by peripheral blood CRTH2$^+$, CRTH2⁻CD117$^+$, and CRTH2⁻CD117⁻ ILC. Data are from one experiment and representative of nine independent experiments with one donor each. ILC, innate lymphoid cell; Lin, lineage.

Capacity of skin NCR$^-$ ILC3 to convert into NCR$^+$ ILC3

Despite the very low numbers of NCR$^+$ ILC3 in cell suspensions prepared from freshly isolated skin, we consistently found a prominent CRTH2$^-$ ILC population (26.5 ± 8.6%) expressing CD161 and NKp44 (NCR$^+$ ILC3) (Figure 1a and b) in the crawl-out CD45$^+$Lin$^-$ ILCs from dermal explant cultures. Previously, we have documented that, in the tonsil, NKp44 identifies a population of ILC3 that responds to IL-1β plus IL-23 with production of IL-22 (Crellin *et al.*, 2010; Bernink *et al.*, 2013). In agreement with this, isolated crawl-out NCR$^+$ ILC3 from dermal explant cultures expressed RORγt (Figure 3e) and secreted IL-22 when they were exposed to IL-1β plus IL-23 for 4 days (Figure 3f), suggesting that NCR$^+$ ILC3 provide a source of IL-22 in skin.

We recently showed that NCR$^-$ ILC3 isolated from human tonsils and fetal intestine differentiate to NCR$^+$ ILC3 under the influence of IL-1β plus IL-23 (Bernink *et al.*, 2013). Hence, we reasoned that the NCR$^+$ ILC3 population found in crawl-out cultures might have arisen during the 3-day culture of the explant, deriving from a skin-resident NCR$^-$ ILC3 population. Indeed, such a NCR$^-$ ILC3 population, expressing CD161, could be found in both freshly prepared dermal-cell suspensions and crawl-outs derived from normal skin (Figure 1). When cultured in IL-1β plus IL-23, these cells readily converted into NCR$^+$ ILC3 (Figure 4a). The majority of these cells expressed CD161 and CD56 (Figure 4b). Of note, NCR$^-$ ILC3 did not give rise to CRTH2$^+$ ILC2 upon culture with either IL-1β plus IL-23 or TSLP plus IL-33 (Figure 4a).

In summary, in addition to differentiated ILC2, normal skin harbors a NCR$^-$ ILC3 population, which can convert into NCR$^+$ ILC3 with the capacity to produce IL-22.

NCR$^+$ ILC3 accumulate in skin lesions and peripheral blood of patients with psoriasis

Although the contribution of T cells to the pathogenesis of psoriasis is well established (Nestle *et al.*, 2009; Res *et al.*, 2010), it is becoming increasingly clear that the innate immune system also participates in the initiation and/or maintenance of psoriatic inflammation (Bos *et al.*, 2005). We set out to examine the presence and frequency of ILCs in freshly prepared lesional skin of psoriasis patients as compared with normal skin of healthy individuals. To this end, we collected blood, as well as biopsies from active lesions of psoriasis patients to be compared with normal skin and blood from healthy individuals. Strikingly, flow cytometric phenotypical analysis of the ILC populations revealed that psoriatic skin contained significantly higher proportions of total Lin$^-$CD127$^+$ ILCs (Figure 5b). In addition, significantly elevated frequencies of NCR$^+$ ILC3 were seen in both skin and blood from psoriasis patients as compared with normal skin and blood from healthy individuals (Figure 5a–d). Noteworthy, the frequency of NCR$^+$ ILC3 in the skin correlated with psoriasis severity (psoriasis area and severity index scores; $P = 0.042$; Figure 5e).

By contrast, the frequencies of ILC2 were similar in normal and psoriatic skin and blood (Figure 5a–d). Furthermore, we did not find any evidence for alterations in the frequency of CD161$^+$ ILC1 in psoriatic as compared with healthy skin (Figure 5b).

To assess the cytokine production from psoriasis-associated NCR$^+$ ILC3, we sorted and *in vitro* expanded these cells from the blood and dermal crawl-out cultures of psoriasis patients. Upon IL-1β/IL-23 plus PMA/ionomycin stimulation, blood and skin NCR$^+$ ILC3 produced IL-22, but few cells produced IL-17 (Figure 6a–e). A larger proportion of NCR$^+$ ILC3 in the skin as compared with the blood seemed to produce IL-22, both at resting and IL-1β/IL-23 plus PMA/ionomycin-stimulated conditions; however, due to the small number of observations, we were not able to assess this with statistical certainty. Importantly, we could also show that IL-22 was secreted from psoriasis blood–derived NCR$^+$ ILC3 (Figure 6f), but not NK cells or ILC2 (data not shown). IL-22 was secreted both spontaneously and in response to IL-1β plus IL-23 stimulation, which further increased the production of IL-22 (Figure 6f). No secreted IL-17 was detected from these NCR$^+$ ILC3.

Hence, our finding of increased numbers of NCR$^+$ ILC3 in lesional psoriatic skin and blood is likely to be clinically relevant, as these cells potently produce IL-22, a cytokine known to contribute to the epidermal thickening that is characteristic for this inflammatory skin disease (Zheng *et al.*, 2007).

DISCUSSION

In this study, we provide a detailed characterization of the Lin$^-$CD127$^+$ ILC phenotypes found in the normal skin of healthy humans. Furthermore, we show that all ILC populations found in the blood, including NCR$^-$ ILC3 and CRTH2$^+$ ILC2, have the capacity to migrate to and populate the skin, as part of these cells express CLA. Consistently, we identified in healthy human skin ILC2 and NCR$^-$ ILC3, but hardly any CD161$^+$ ILC1 or NCR$^+$ ILC3, whereas NCR$^+$ ILC3 were

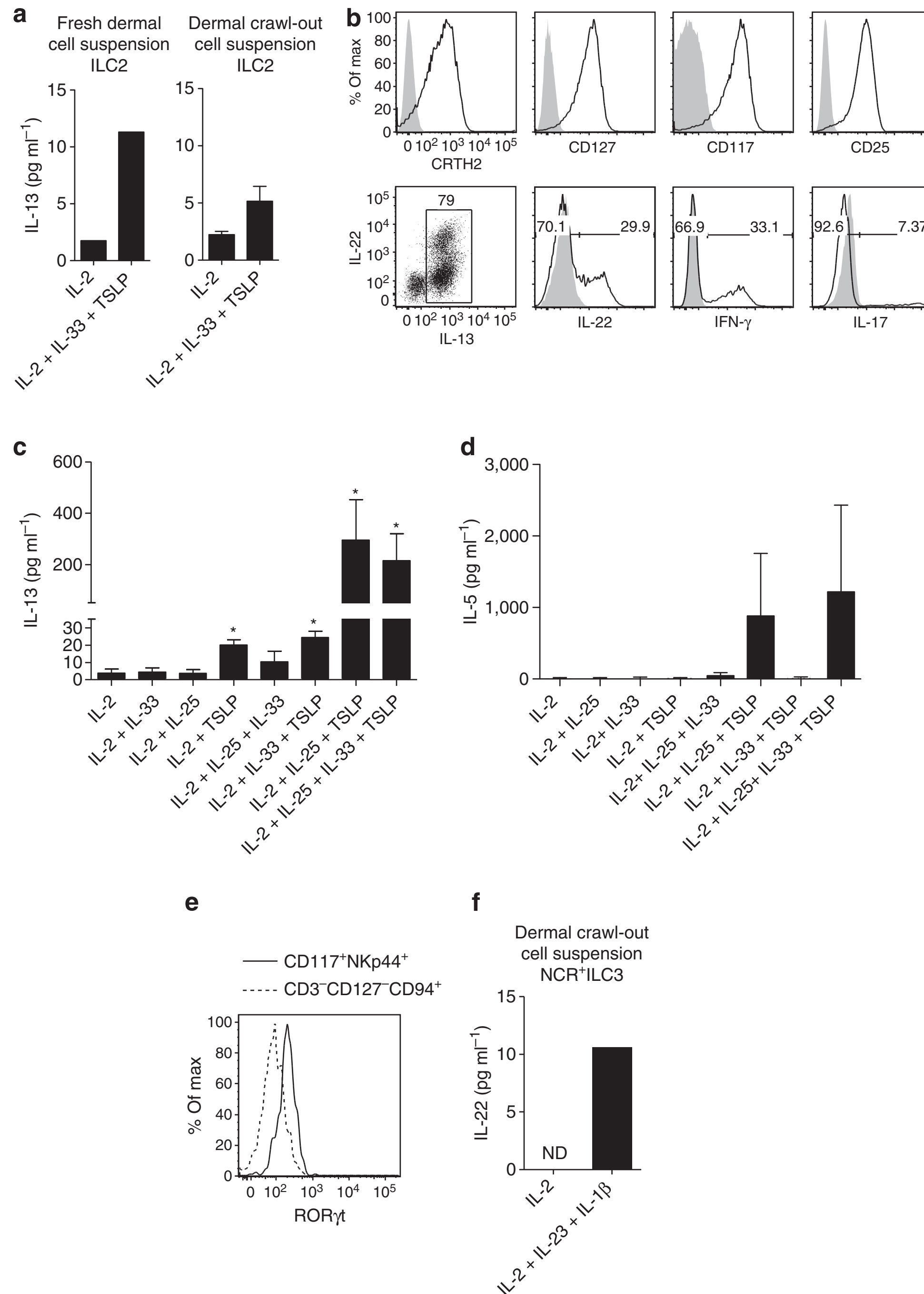


Figure 3. Cutaneous Lin−CD127hiCRTH2+ ILC2 produce IL-13 upon stimulation. (**a**) IL-13 secretion of freshly prepared and crawl-out dermal CRTH2+ ILC2 in response to IL-33 plus TSLP stimulation. (**b**) Flow cytometry analysis of cell lines generated from normal skin CRTH2+ ILC2 (upper panel) after stimulation with PMA and ionomycin, and staining for intracellular IL-13, IL-22, IFN-γ, or IL-17 (lower panel). (**c**, **d**) IL-13 or IL-5 secretion of expanded skin-derived ILC2 after stimulation with various combinations (horizontal axes) of IL-2 (1 U ml−1), IL-33 (50 ng ml−1), IL-25 (50 ng ml−1), and TSLP (50 ng ml−1). (**e**) RORγt expression is higher in dermal crawl-out CD117+NKp44+ (NCR+ ILC3) than in CD3−CD127−CD94+ (NK) cells. (**f**) IL-22 secretion from crawl-out Lin−CD127hiCD117+NKp44+ cells. Results were normalized to 2,000 cells (**a**, **f**) or 25,000 cells (**c**, **d**) per 200 μl in a 96-well plate. Data are representative of two experiments with one donor each (**b**) or from at least two experiments with one donor each (**a**, **c**, **d**–**f**; mean and SEM). ILC, innate lymphoid cell; Lin, lineage; NCR, natural cytotoxicity receptor; PMA, phorbol 12-myristate 13-acetate; TSLP, thymic stromal lymphopoietin.

present in cultured dermal explants. The ILC2 and NCR+ ILC3 subsets in healthy human skin were functional as they produced IL-13 and IL-22, respectively, upon cytokine stimulation. The scarcity of CD161+ ILC1 prevented us from performing functional studies of these cells. Extended characterization of the skin CRTH2+ ILC2 revealed that they could be expanded *in vitro* in high-dose IL-2 without loss of phenotype or function as they maintained CD127, CRTH2,

CD117, and CD25, lacked markers for T and NK cells (CD3 and CD94), and retained their capacity to produce IL-13 following *in vitro* culture. These data show that the skin ILC2 are a stable subset of cells that can be driven to proliferation by high-dose IL-2, a finding consistent with a recent paper describing mouse ILC2 (Roediger *et al.*, 2013). Interestingly, in contrast to blood and nasal polyp ILC2, which respond vigorously to TSLP plus IL-33 (Mjösberg *et al.*, 2012), TSLP plus IL-25 more potently triggered IL-13 production than the combination TSLP plus IL-33. Recently, Kim *et al.* (2013) published data from a mouse model of atopic dermatitis demonstrating that cutaneous allergic inflammation is critically dependent on TSLP-driven ILC2 responses. However, ILC2 inflammation in the mouse skin occurred independently of IL-25 and IL-33. In our study, we did not address the role for ILC2 in atopic dermatitis; however, our data on healthy human skin ILC2 clearly show that IL-25, and to a lesser extent also IL-33, enhances the capacity of TSLP to stimulate ILC2, findings that may have implications when considering these pathways as targets in the treatment of atopic dermatitis. The demonstration that CRTH2$^+$ ILC2 are present in normal human skin is in contrast with the data of Kim *et al.* (2013) who showed that, although ILC2, identified as Lin$^-$IL33R$^+$CD25$^+$ ILC, were present in healthy skin, they lacked expression of CRTH2. The reason for this discrepancy is unknown but may be attributed to the use of different cell surface–identification markers and flow cytometric gating strategies.

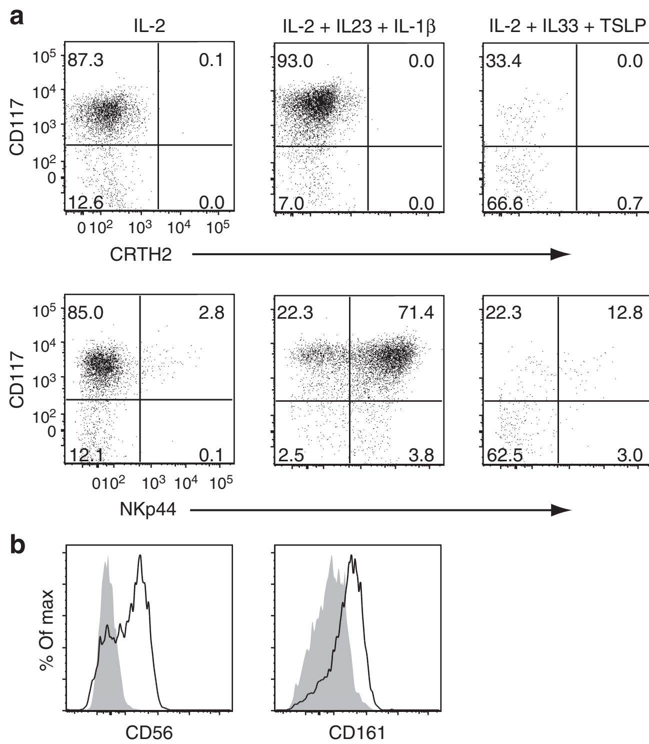
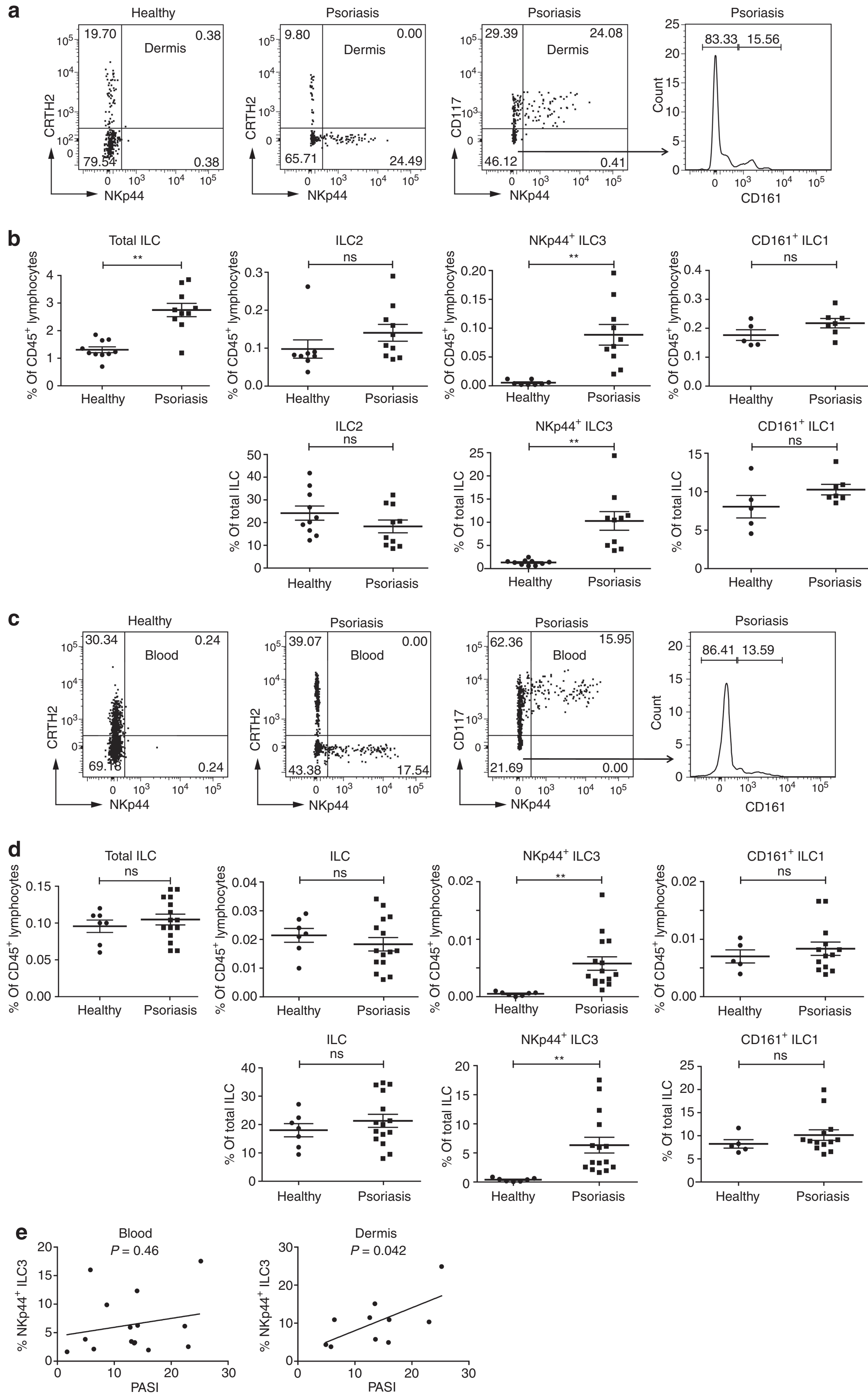


Figure 4. Normal skin harbors a NCR$^-$ ILC3 population, which can be converted to NCR$^+$ ILC3. (**a**) Differentiation of highly purified normal skin CD117$^+$NKp44$^-$ ILCs into NKp44$^+$ ILC3 after stimulation for 10 days with feeder cells and either 100 U ml^{-1} IL-2 alone (two dot plots left side) or a combination of IL-2, IL-23 plus IL-1β (50 ng ml^{-1}) or IL-2, IL-33 plus TSLP. (**b**) Flow cytometry analysis of the expression of CD56 and CD161 by *in vitro* differentiated NKp44$^+$ ILC3 (black line) and NKp44$^-$ ILC3 (light gray shading). Data are representative of two experiments with one donor each (**a**, **b**). ILC, innate lymphoid cell; NCR, natural cytotoxicity receptor; TSLP, thymic stromal lymphopoietin.

Importantly, we identified IL-1β plus IL-23-responsive IL-22-producing NCR$^+$ ILC3 in cell suspensions obtained from dermal explant cultures derived from the skin of healthy individuals. These cells were scarce in freshly isolated skin-cell suspensions. This led us to hypothesize that these cells were derived from NCR$^-$ ILC3 that were present in both freshly isolated dermal-cell suspensions as well as in dermal crawl-out cultures. Indeed, in accordance with what we previously observed in fetal intestine and tonsil (Bernink *et al.*, 2013), dermal NCR$^-$ ILC3 cultured with IL-1β plus IL-23 converted to NCR$^+$ ILC3. This observation is intriguing in the context of skin inflammation, as IL-1β and IL-23 are upregulated in psoriatic lesions (Debets *et al.*, 1997; Lee *et al.*, 2004; Piskin *et al.*, 2006) and implicated in pathogenesis of the disease (Zheng *et al.*, 2007). Indeed, we found an accumulation of IL-22-producing NCR$^+$ ILC3 in freshly isolated lesional skin and blood of psoriasis patients. Paralleling our findings, it was recently reported that the number of ILC3 is increased in 12-day-old crawl-out cultures of psoriasis skin (Dyring-Andersen *et al.*, 2014). This study did not, however, assess the frequency of different ILC populations in freshly isolated skin biopsies or blood of psoriasis patients, which is likely to yield a more relevant view of the *in vivo* ILC status of these patients. Furthermore, during the review process of our manuscript, another study demonstrated an accumulation of NCR$^+$ ILC3 in psoriasis (Villanova *et al.*, 2014). This paper suggested NCR$^+$ ILC3 as sources of both IL-22 and IL-17 in psoriasis. However, Villanova *et al.* (2014) did not demonstrate cytokine production from pure ILC populations in the skin. Instead, they examined CD45$^+$CD3$^-$ cells, which, although enriched for

▶

Figure 5. Accumulation of NCR$^+$ ILC3 in lesional skin and peripheral blood of psoriasis patients. (**a**) CD45$^+$Lin$^-$CD127$^+$ lymphocytes from healthy or psoriatic lesional skin, representing the total ILC population, were subdivided into ILC2 and ILC3 on the basis of the mutually exclusive expression of CRTH2 and NKp44. CD161$^+$ ILCs that were negative for CRTH2, NKp44, and CD117 were regarded as ILC1. (**b**) Summary of the percentages of ILC subsets in human skin. In the top row, ILC subsets are depicted as a percentage of CD45$^+$ lymphocytes, while in the bottom row ILC subsets are depicted as a percentage of total ILC. (**c**) Peripheral blood of healthy donors contains ILC2 and ILC1, but is devoid of NCR$^+$ ILC3, whereas blood of psoriasis patients contains a clear population of NCR$^+$ ILC3. (**d**) A summary of the ILC subsets in blood depicted as percentage of CD45$^+$ lymphocytes in the top row and depicted as a percentage of total ILCs in the bottom row. (**e**) Correlation analysis of dermal NCR$^+$ ILC3 frequency and psoriasis area and severity index (PASI) score. Sample numbers may vary between groups. Horizontal lines indicate mean values ± SD. $^{**}P<0.001$. (**e**) Correlation analysis of blood and dermal NCR$^+$ ILC3 frequency and PASI score. ILC, innate lymphoid cell; Lin, lineage; NCR, natural cytotoxicity receptor.


a
Healthy
Dermis
Psoriasis
CRTH2
NKp44
CD117
Count
CD161
b
Total ILC
ILC2
NKp44+ ILC3
CD161+ ILC1
% Of CD45+ lymphocytes
% Of total ILC
Healthy
Psoriasis
ns
c
Blood
d
ILC
e
P = 0.46
P = 0.042
% NKp44+ ILC3
PASI

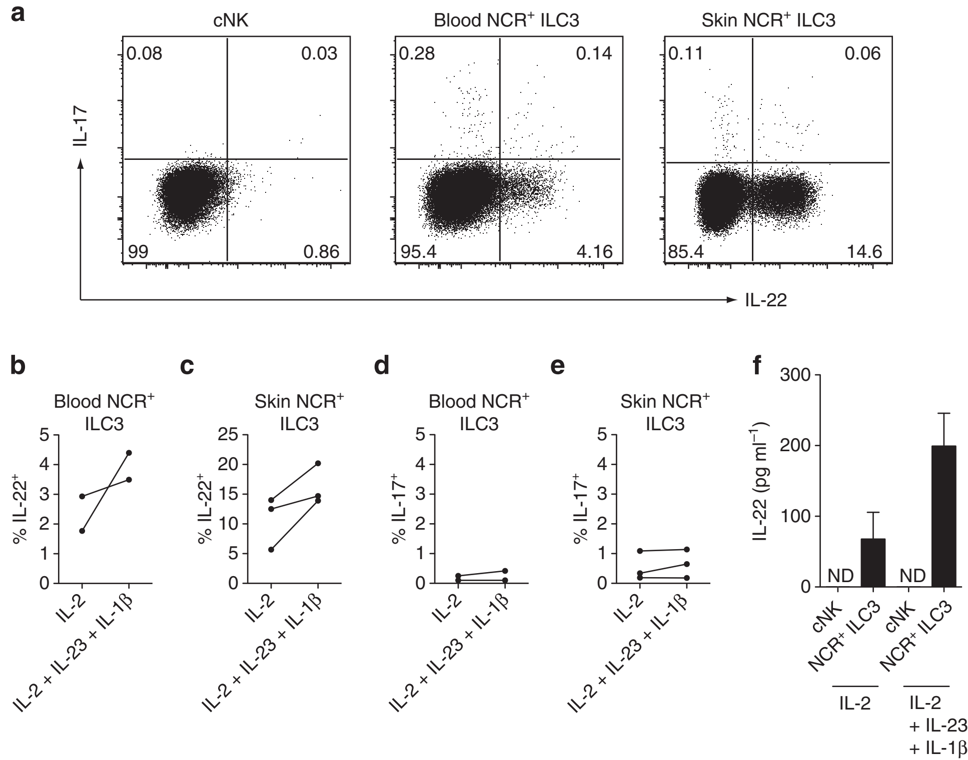


Figure 6. NCR$^+$ ILC3 from lesional skin and peripheral blood of psoriasis patients produce IL-22. (**a**) Flow cytometric cytokine analysis of cell lines generated from conventional NK cells (cNK) and NCR$^+$ ILC3 from peripheral blood and skin-biopsy crawl-out cells from psoriasis patients. Cells were cultured with IL-2 (10 U ml^{-1}), IL-23 plus IL-1β (both 50 ng ml^{-1}) for 3 days, stimulated with PMA/ionomycin for 6 hours, and then stained for intracellular IL-22 and IL-17. (**b–e**) Percentage of IL-22$^+$ (**b**, **c**) and IL-17$^+$ (**d**, **e**) blood- and skin-derived NCR$^+$ILC3 after stimulation with PMA/ionomycin after 3 days of pre-incubation with either 10 U ml^{-1} IL-2 alone or in combination with IL-23 plus IL-1β (50 ng ml^{-1}). (**f**) IL-22 secretion of expanded blood-derived conventional NK cells and NCR$^+$ ILC3 after stimulation with either 10 U ml^{-1} IL-2 alone or in combination with IL-23 plus IL-1β (50 ng ml^{-1}). Results were normalized to 8,000 cells per 200 μl in a 96-well plate (**f**; mean and SEM). Data are representative of two (blood) or three (skin) psoriasis patients. Each patient sample was examined in independent experiments. ILC, innate lymphoid cell; NCR, natural cytotoxicity receptor; PMA, phorbol 12-myristate 13-acetate.

ILC, are not pure, and their results need to be interpreted with caution. In a set of preliminary experiments, we confirmed that CD45$^+$CD3$^-$ cells isolated from the skin produce IL-17 following stimulation with PMA/ionomycin (Supplementary Figure 2). This observation, together with our demonstration of very low frequencies of IL-17$^+$NCR$^+$ ILC3, suggests that IL-17 is produced by cells distinct from NCR$^+$ ILC3 within the CD45$^+$CD3$^-$ population. Villanova *et al.* (2014) did not assess which cytokines might drive the IL-17 or IL-22 production from NCR$^+$ ILC3. Hence, our study further extends these observations, as we demonstrated that the IL-22 production, and to a lesser extent the IL-17 production, was stimulated in purified blood and skin-derived NCR$^+$ ILC3 by the presence of IL-1β plus IL-23. In addition, we show that culture of NCR$^-$ ILC3 in IL-1β plus IL-23 increases the frequency of NCR$^+$ ILC3 *in vitro*, suggesting that these cytokines not only stimulate cytokine production from NCR$^+$ ILC3 but may also contribute to the accumulation of NCR$^+$ ILC3 in psoriasis.

All together, our findings suggest that the elevated levels of IL-1β and IL-23 found in the skin of psoriasis patients support conversion of NCR$^-$ ILC3 into NCR$^+$ ILC3, the latter of which constitutes a source of IL-22 in the skin, contributing to the epidermal thickening that is characteristic for this inflammatory skin disease (Nakajima, 2012). Interestingly, elevated numbers of NCR$^+$ ILC3 were also present in peripheral blood of psoriatic patients. This raises the possibility that NCR3$^+$ ILC3 are not only generated in the skin but also elsewhere and then migrate to the skin. Of note, we did not find any evidence for altered frequencies of CD161$^+$ ILC1 in lesional skin of psoriasis patients. As these cells are primarily induced by IL-12 (Bernink *et al.*, 2013), this suggests that the effects of IL-23 dominate those of IL-12 in psoriatic skin lesions. It will be highly interesting to see whether the numbers of NCR$^+$ ILC3 in the peripheral blood and skin are modulated by therapy targeting IL-23 in psoriasis.

IL-22 is not only produced by NCR$^+$ ILC3 but also by T cells in psoriasis. As T cells are more abundant, it is tempting to assume that they represent the major source of IL-22. However, the relatively lower frequency of NCR$^+$ ILC3 as compared with T cells does not diminish the importance of NCR$^+$ ILC3. A good example of this comes from studies of ILC3 in the gut, where these cells are also rare. Mice lacking IL-22-producing ILC3 show strikingly increased susceptibility

to the pathogen *Citrobacter rodentium* (Satoh-Takayama *et al.*, 2008). Furthermore, ILC3 and T-helper cells may have complementary functions. Again, using the *C. rodentium* infection model, it was shown that IL-22 was induced from ILC3 and Th22 cells in sequential waves, each crucial for host defense (Basu *et al.*, 2012). Hence, although numerically inferior, NCR$^+$ ILC3 have a nonredundant function in the gut, which may very well also be the case in the skin.

In conclusion, we demonstrate that the ILC compartment in normal human skin comprises functional ILC2 as well as NCR$^-$ ILC3, the latter of which can be converted to NCR$^+$ ILC3. In psoriatic skin lesions, however, these NCR$^+$ ILC3 are present in increased proportions, and as these NCR$^+$ ILC3 provide a source of IL-22, they may, together with T cells, participate in the pathogenesis of psoriasis.

MATERIALS AND METHODS

Skin and blood collection

The study protocols were reviewed and approved by the Medical Ethical Committee of the Academic Medical Center, Amsterdam, following the Declaration of Helsinki protocols. All material was collected after patient's written informed consent. Normal adult skin was obtained as residual tissue after plastic surgery of the breast or abdomen. Patients with active psoriasis vulgaris who visited the Department of Dermatology at the Academic Medical Center in Amsterdam donated 4 mm punch biopsies from an active lesion. All patients refrained from any systemic therapy or phototherapy for at least 4 weeks before participation and topical treatments were not allowed 14 days before baseline. The mean psoriasis area and severity index of the patients was 10.7 ± 6.9. Some of the skin donors (healthy and psoriatic) also donated peripheral blood that was sampled via venipuncture.

Preparation of dermal and epidermal skin suspensions

Thin sheets of 0.3 mm thickness were shaved from residual normal skin by an electrodermatome and were treated overnight at 4 °C with 0.3% (wt/vol) dispase II (Roche, Almere, The Netherlands) in PBS to enable the separation of dermis and epidermis. To get fresh cell suspensions, the dermis was fragmented by scissors and incubated in IMDM with 0.5% (wt/vol) collagenase D (Roche), 50 U ml^{-1} DNAse I (Sigma-Aldrich, St Louis, MO), and 0.5% (vol/vol) FCS for 90 minutes at 37 °C. Alternatively, to allow spontaneous migration of cells from the tissue, the dermis was cut into small pieces of 2–4 cm squares and was cultured in IMDM with 10% FCS for 3 days. Skin biopsies from lesional skin of psoriasis patients were treated subsequently with dispase and collagenase as described above.

Flow cytometry analysis and sorting

The following antibodies to human proteins were used: FITC-conjugated anti-CD1a (HI149), anti-CD3 (OKT3), anti-CD4 (RPA-T4), anti-CD11c (3.9), anti-CD14 (HCD14), anti-CD16 (3G8), anti-CD19 (HIB19), anti-CD34 (581), anti-CD94 (DX22), anti-CD123 (6H6), anti-FcεR1α (AER-37), phycoerythrin (PE)-conjugated anti-CCR10 (6588-5) anti-CD94 (HP-3D9), anti-CD161 (HP-3G10), anti-NKp44 (P44-8), peridinin chlorophyll protein–cyanine 5.5 (PerCP-Cy5.5)-conjugated anti-CD117 (104D2), Alexa Fluor 647–conjugated anti-NKp44 (P44-8), Pacific Blue-conjugated anti-CLA (HECA-452), allophycocyanin-indotricarbocyanine (APC-Cy7)-conjugated anti-CD25 (BC96), Brilliant Violet 421–conjugated anti-CD161 (HP-3G10), Alexa Fluor 700–conjugated anti-CD3 (UCHT1), anti-CD56 (HCD56; all from BioLegend, San Diego, CA); FITC-conjugated anti-TCRαβ (IP26), anti-TCRγδ (B1), PE-conjugated anti-CD16 (3G8), Alexa Fluor 647–conjugated anti-CRTH2 (CD294; BM16), APC-Cy7-conjugated anti-CD45 (2D1), V500-conjugated anti-CD3 (UCHT1), and isotype-matched control antibodies conjugated to Alexa Fluor 700 (MOPC-21), PE, APC, or PE-Cy7 (X40; all from BD Biosciences, Mountain View, CA); R Phycoerythrin-Cyanine 5.5 (PE-Cy5.5)-conjugated anti-CD117 (104D2D1), R Phycoerythrin-Cyanine 7 (PE-Cy7)-conjugated anti-CD127 (R34.34; both from Beckman Coulter, Woerden, The Netherlands), and FITC-conjugated anti-BDCA2 (CD303; AC144; Milenyi Biotec, Leiden, The Netherlands). Intracellular RORγt staining was carried out using PE-conjugated anti-human RORγt (clone AFKJS-9; eBioscience, San Diego, CA) and the Foxp3 permeablilization/fixation kit according to the manufacturer's instructions.

Generation of ILC lines

Purified Lin$^-$CD127$^+$CRTH2$^+$, Lin$^-$CD127$^+$CRTH2$^-$CD117$^+$NKp44$^+$, and Lin$^-$CD127$^+$CRTH2$^-$CD117$^-$NKp44$^-$ ILC populations were stimulated with 1 μg ml^{-1} phytohemagglutinin (Oxoid, Hampshire, UK), 25 Gy–irradiated allogeneic peripheral blood mononuclear cells, 50 Gy–irradiated Epstein–Barr virus–transformed JY human B cells, and 100 U ml^{-1} IL-2 (Novartis, Arnhem, The Netherlands) in Yssel's medium (prepared in-house (Yssel *et al.*, 1984)) supplemented with 1% (vol/vol) human AB serum. Culture medium containing IL-2 was refreshed two times per week and cells were re-sorted before further analysis.

Measurement of cytokine production

Purified fresh and expanded ILCs were cultured for 3–4 days at 2,000 cells per well in a round-bottomed 96-well plate in Yssel's medium supplemented with 1% (vol/vol) human serum in the presence or absence of 10 U ml^{-1} IL-2 and stimulated with one of the following cytokines or combinations thereof: 50 ng ml^{-1} TSLP, 50 ng ml^{-1} IL-25, or 50 ng ml^{-1} IL-33 (all from R&D systems, Abingdon, UK) for Lin$^-$CD127$^+$CRTH2$^+$ ILCs; 50 ng ml^{-1} IL-23 and 50 ng ml^{-1} IL-1β (both from R&D systems) for Lin$^-$CD127$^+$CRTH2$^-$CD117$^+$NKp44$^+$ ILCs and 50 ng ml^{-1} IL-12 and 50 ng ml^{-1} IL-18 (both from R&D systems) for Lin$^-$CD127$^+$CRTH2$^-$CD117$^-$NKp44$^-$ ILCs. IL-13, IL-17, IL-22, and IFN-γ were measured in culture supernatants by enzyme-linked immunosorbent assays from R&D systems or Sanquin (Amsterdam, The Netherlands). Alternatively, multiple cytokine detection (including IL-4, IL-9, and GM-CSF) in the supernatants was performed using the MILLIPLEX MAP Human Cytokine/Chemokine Panel (Merck Millipore, Billerica, MA) and the Bio-Rad Bioplex-200 analysis instrument (Bio-Rad, Hercules, CA), all according to the manufacturer's instructions. The Bioplex Manager 4.1 software was used for data analysis. To detect intracellular cytokines, ILCs were stimulated for 6 hours with 10 ng ml^{-1} PMA (Sigma) and 500 nM ionomycin (Merck) in the presence of GolgiPlug (BD Biosciences) for the final 4 hours of culture. In some experiments, IL-23 and IL-1β (50 ng ml^{-1}) was added for the whole culture period. Cytofix/Cytoperm kit (BD Biosciences) was used for permeabilization during subsequent staining and washing steps. We used the following antibodies: APC-conjugated anti-IL-13 (JES10-5A2) and APC-

conjugated anti-IL-17 (BL168) from BioLegend; PE-conjugated anti-IL-22 (142928) from R&D systems and PE-conjugated anti-IFN-γ (B27) from BD Biosciences. Data were acquired with LSR-Fortessa equipment (BD Biosciences) and analyzed with FlowJo software (TreeStar, Ashland, OR).

Statistical analysis

Statistical significance was determined with Student's *t*-test and Pearson correlation analysis using SPSS software.

CONFLICT OF INTEREST

The authors state no conflict of interest.

ACKNOWLEDGMENTS

We thank B Hooibrink and Faas van Dooren, AMC, Amsterdam, for valuable help with flow cytometric sorting and Luminex analysis, respectively.

SUPPLEMENTARY MATERIAL

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

REFERENCES

Basu R, O'Quinn DB, Silberger DJ *et al.* (2012) Th22 cells are an important source of IL-22 for host protection against enteropathogenic bacteria. *Immunity* 37:1061–75

Bernink JH, Mjösberg JM, Peters CP *et al.* (2013) Human type 1 innate lymphoid cells accumulate in inflamed mucosal tissues. *Nat Immunol* 14:221–9

Bos JD, de Rie MA, Teunissen MB *et al.* (2005) Psoriasis: dysregulation of innate immunity. *Br J Dermatol* 152:1098–107

Cella M, Fuchs A, Vermi W *et al.* (2009) A human natural killer cell subset provides an innate source of IL-22 for mucosal immunity. *Nature* 457:722–5

Crellin NK, Trifari S, Kaplan CD *et al.* (2010) Human NKp44+IL-22+ cells and LTi-like cells constitute a stable RORC+ lineage distinct from conventional natural killer cells. *J Exp Med* 207:281–90

Cupedo T, Crellin NK, Papazian N *et al.* (2009) Human fetal lymphoid tissue-inducer cells are interleukin 17-producing precursors to RORC+ CD127+ natural killer-like cells. *Nat Immunol* 10:66–74

Debets R, Hegmans JP, Croughs P *et al.* (1997) The IL-1 system in psoriatic skin: IL-1 antagonist sphere of influence in lesional psoriatic epidermis. *J Immunol* 158:2955–63

Dyring-Andersen B, Geisler C, Aggerbeck C *et al.* (2014) Increased number and frequency of group 3 innate lymphoid cells in non-lesional psoriatic skin. *Br J Dermatol* 170:609–16

Hedrick MN, Lonsdorf AS, Shirakawa AK *et al.* (2009) CCR6 is required for IL-23-induced psoriasis-like inflammation in mice. *J Clin Invest* 119: 2317–29

Kim BS, Siracusa MC, Saenz SA *et al.* (2013) TSLP elicits IL-33-independent innate lymphoid cell responses to promote skin inflammation. *Sci Transl Med* 5:170ra16

Lee E, Trepicchio WL, Oestreicher JL *et al.* (2004) Increased expression of interleukin 23 p19 and p40 in lesional skin of patients with psoriasis vulgaris. *J Exp Med* 199:125–30

Mjösberg J, Bernink J, Golebski K *et al.* (2012) The transcription factor GATA3 is essential for the function of human type 2 innate lymphoid cells. *Immunity* 37:649–59

Mjösberg JM, Trifari S, Crellin NK *et al.* (2011) Human IL-25- and IL-33-responsive type 2 innate lymphoid cells are defined by expression of CRTH2 and CD161. *Nat Immunol* 12:1055–62

Monticelli LA, Sonnenberg GF, Abt MC *et al.* (2011) Innate lymphoid cells promote lung-tissue homeostasis after infection with influenza virus. *Nat Immunol* 12:1045–54

Moro K, Yamada T, Tanabe M *et al.* (2010) Innate production of T(H)2 cytokines by adipose tissue-associated c-Kit(+)Sca-1(+) lymphoid cells. *Nature* 463:540–4

Nakajima K (2012) Critical role of the interleukin-23/T-helper 17 cell axis in the pathogenesis of psoriasis. *J Dermatol* 39:219–24

Neill DR, Wong SH, Bellosi A *et al.* (2010) Nuocytes represent a new innate effector leukocyte that mediates type-2 immunity. *Nature* 464:1367–70

Nestle FO, Kaplan DH, Barker J (2009) Psoriasis. *N Engl J Med* 361:496–509

Pantelyushin S, Haak S, Ingold B *et al.* (2012) Rorgammat+ innate lymphocytes and gammadelta T cells initiate psoriasiform plaque formation in mice. *J Clin Invest* 122:2252–6

Piskin G, Sylva-Steenland RM, Bos JD *et al.* (2006) *In vitro* and *in situ* expression of IL-23 by keratinocytes in healthy skin and psoriasis lesions: enhanced expression in psoriatic skin. *J Immunol* 176:1908–15

Price AE, Liang HE, Sullivan BM *et al.* (2010) Systemically dispersed innate IL-13-expressing cells in type 2 immunity. *Proc Natl Acad Sci USA* 107:11489–94

Res PC, Piskin G, de Boer OJ *et al.* (2010) Overrepresentation of IL-17A and IL-22 producing CD8 T cells in lesional skin suggests their involvement in the pathogenesis of psoriasis. *PLoS One* 5:e14108

Roediger B, Kyle R, Yip KH *et al.* (2013) Cutaneous immunosurveillance and regulation of inflammation by group 2 innate lymphoid cells. *Nat Immunol* 14:564–73

Satoh-Takayama N, Vosshenrich CA, Lesjean-Pottier S *et al.* (2008) Microbial flora drives interleukin 22 production in intestinal NKp46+ cells that provide innate mucosal immune defense. *Immunity* 29:958–70

Sonnenberg GF, Artis D (2012) Innate lymphoid cell interactions with microbiota: implications for intestinal health and disease. *Immunity* 37:601–10

Spits H, Artis D, Colonna M *et al.* (2013) Innate lymphoid cells - a proposal for uniform nomenclature. *Nat Rev Immunol* 13:145–9

Spits H, Cupedo T (2012) Innate lymphoid cells: emerging insights in development, lineage relationships, and function. *Annu Rev Immunol* 30:647–75

Spits H, Di Santo JP (2011) The expanding family of innate lymphoid cells: regulators and effectors of immunity and tissue remodeling. *Nat Immunol* 12:21–7

Villanova F, Flutter B, Tosi I *et al.* (2014) Characterization of innate lymphoid cells in human skin and blood demonstrates increase of NKp44+ ILC3 in psoriasis. *J Invest Dermatol* 134:984–91

Walker JA, Barlow JL, McKenzie AN (2013) Innate lymphoid cells—how did we miss them? *Nat Rev Immunol* 13:75–87

Yssel H, De Vries JE, Koken M *et al.* (1984) Serum-free medium for generation and propagation of functional human cytotoxic and helper T cell clones. *J Immunol Methods* 72:219–27

Zheng Y, Danilenko DM, Valdez P *et al.* (2007) Interleukin-22, a T(H)17 cytokine, mediates IL-23-induced dermal inflammation and acanthosis. *Nature* 445:648–51