Characterization of Innate Lymphoid Cells in Human Skin and Blood Demonstrates Increase of NKp44 + ILC3 in Psoriasis

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Innate lymphoid cells (ILCs) are increasingly appreciated as key regulators of tissue immunity. However, their role in human tissue homeostasis and disease remains to be fully elucidated. Here we characterize the ILCs in human skin from healthy individuals and from the inflammatory skin disease psoriasis. We show that a substantial proportion of IL-17A and IL-22 producing cells in the skin and blood of normal individuals and psoriasis patients are CD3-negative innate lymphocytes. Deep immunophenotyping of human ILC subsets showed a statistically significant increase in the frequency of circulating NKp44 + ILC3 in the blood of psoriasis patients compared with healthy individuals or atopic dermatitis patients. More than 50% of circulating NKp44 + ILC3 expressed cutaneous lymphocyte–associated antigen, indicating their potential for skin homing. Analysis of skin tissue revealed a significantly increased frequency of total ILCs in the skin compared with blood. Moreover, the frequency of NKp44 + ILC3 was significantly increased in non-lesional psoriatic skin compared with normal skin. A detailed time course of a psoriasis patient treated with anti–tumor necrosis factor showed a close association between therapeutic response, decrease in inflammatory skin lesions, and decrease of circulating NKp44 + ILC3. Overall, data from this initial observational study suggest a potential role for NKp44 + ILC3 in psoriasis pathogenesis.

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INTRODUCTION

The skin is the primary interface with the external environment and thus it is required to provide the first line of host defense against injury and infection (Kupper and Fuhlbrigge, 2004; Di Meglio *et al.*, 2011). Similar to lung and gut mucosal barriers, the skin is equipped with a diverse set of immune cells that are poised to react to different insults, yet retain the potential to cause autoimmunity.

Plaque-type psoriasis is a chronic inflammatory skin disease characterized by highly inflamed scaly lesions resulting from hyperproliferation of the epidermis and a prominent inflammatory infiltrate (Griffiths and Barker, 2007; Nestle *et al.*, 2009). The immunopathogenesis of psoriasis is based on a combination of genetic susceptibility and environmental risk factors triggering a pathogenic cross-talk between innate and adaptive immune cells (Lowes *et al.*, 2013). Recent advances in understanding the genetic basis of the disease suggest a significant involvement of the innate immune system. Indeed, 11 out of 36 psoriasis susceptibility loci identified in individuals of European ancestry encode plausible regulators of innate host defense (Tsoi *et al.*, 2012). The IL-23/IL-17/IL-22 axis bridges innate and adaptive immunity and is of critical importance in psoriasis (Diveu *et al.*, 2008; Di Cesare *et al.*, 2009). Although a contribution of conventional αβ T cells to IL-17 and IL-22 cytokine production is well-established (Hijnen *et al.*, 2013), the contribution of innate immune cell subsets is less well understood. Recent data in both mouse experimental models (Pantelyushin *et al.*, 2012) and in human immune-mediated pathologies, such as Crohn's disease (Geremia *et al.*, 2011), have established a potential role of innate lymphoid cells (ILCs) as key sources of IL-17 and IL-22 production in epithelial inflammatory disease.

ILCs are recombination-activating gene–independent cells and are identified by their lymphoid morphology as well as the absence of lineage markers for T/B cells and for other innate cells (Spits *et al.*, 2013). Three ILC groups have been described in both mouse and humans, related by their dependence on the transcriptional repressor inhibitor of

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Abbreviations: AA, lesional skin of AD patient; AD, atopic dermatitis; AN, non-lesional skin of AD patient; CLA, cutaneous lymphocyte–associated antigen; ILC, innate lymphoid cell; NN, normal skin; PASI, psoriasis area severity index; PN, non-lesional skin of psoriasis patient; PP, lesional skin of psoriasis patient; TNF, tumor necrosis factor

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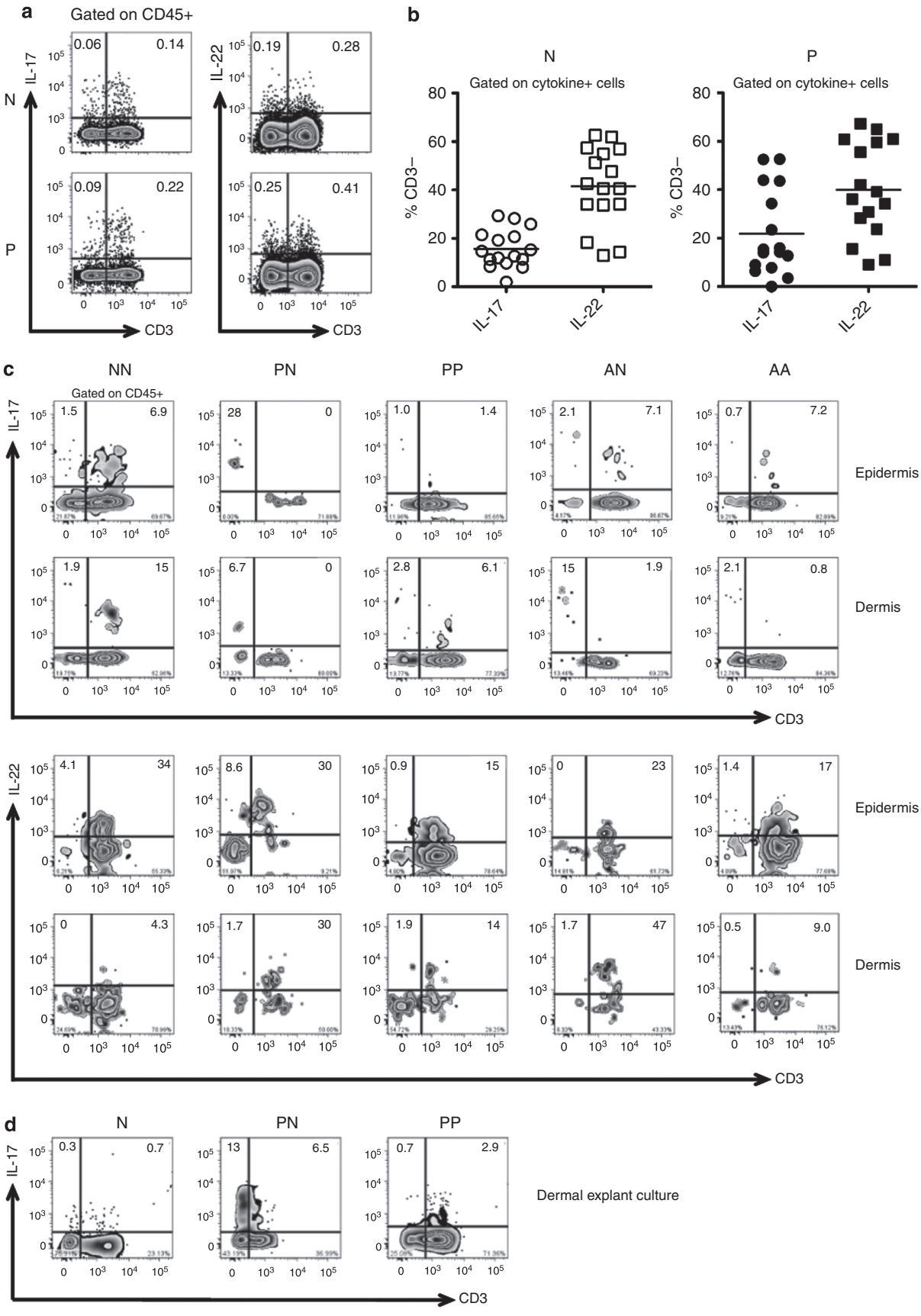

Figure 1. For caption please refer page 986.

DNA binding 2 (Id2) and on the IL-2Rγ chain (Yokota *et al.*, 1999). The ILC3 subset, which also includes lymphoid tissue inducer cells, is dependent on the transcription factor RORγT, as well as on the expression of the IL-7Rα chain. ILC3 can produce IL-17A and/or IL-22 upon stimulation (Takatori *et al.*, 2009). In humans, ILC3 can be subdivided on the basis of the expression of the natural cytotoxicity receptors NKp44, NKp46, and NKp30 (Cella *et al.*, 2009). NKp44+ ILC can produce IL-22 and are dependent on the aryl hydrocarbon receptor; conversely, human lymphoid tissue inducer cells and other NKp44− ILC3 produce IL-17A following stimulation (Hoorweg *et al.*, 2012). However, NKp44+ ILC3 isolated from the tonsil produce both IL-17 and IL-22, whereas NKp44− ILC3 show considerable plasticity, being able to develop either into NKp44+ ILC3 or into ILC1 cells producing IFNγ (Bernink *et al.*, 2013). ILC3 have been shown to produce IL-17 in the gut of inflammatory bowel disease patients (Bernink *et al.*, 2013), suggesting a potential role in immune-mediated diseases such as colitis and psoriasis.

Despite the growing interest about innate sources of proinflammatory cytokines, the ILC populations of human skin are ill-defined. Here, we explore their presence in psoriasis, as well as in the healthy skin and another inflammatory skin disease, atopic dermatitis (AD). We demonstrate that CD3-negative (CD3−) innate lymphocytes are major contributors to IL-17 and IL-22 production in both blood and skin of healthy individuals and psoriasis patients. We further show that the NKp44+ ILC3 subset of ILCs, previously demonstrated to produce IL-17 and IL-22 (Bernink *et al.*, 2013), is increased in frequency in both the peripheral blood and skin of psoriasis patients. Interestingly, in a psoriasis patient with a high baseline frequency of NKp44+ ILC3, the clinical response to anti–tumor necrosis factor (TNF) therapy was associated with a decrease of this population in the blood, implying that it may be either a useful biomarker or even a contributor to disease.

RESULTS AND DISCUSSION

Identification of a population of IL-17 and IL-22 producing CD3− immune cells in the blood and skin of psoriatic patients

We recently embarked on a systematic analysis of peripheral immune cells in patients with inflammatory skin disease. During our analysis of Th17 cells in the blood of psoriasis patients, we observed that there was a consistent population of CD3− innate lymphocytes capable of producing IL-17A. Similarly, when we assessed IL-22 production, we could readily identify CD3− cells, which were making IL-22 (Figure 1a and b). In the peripheral blood, these cells account for about 20% of IL-17 (normal, mean: 15.65%±1.95; psoriasis, mean: 21.83±4.40%) and about 40% of IL-22 (normal, mean: 41.49%±4.04; psoriasis, mean: 39.95%±4.91) producing cells. To assess whether these cytokine-producing CD3− cells are also present in the skin, we isolated cells from the skin of normal healthy individuals (N) or patients with either psoriasis (P) or AD (A). Distinct populations of CD3+ and CD3− cells could be clearly identified in the skin and CD3 discrimination was not affected by enzymatic tissue digestion; fluorescence minus one control and isotype controls were used to define the CD3 gate and exclude nonspecific binding (Supplementary Figure S1 online). In agreement with our peripheral blood data, CD3− IL17+ cells could be clearly identified in the dermis and epidermis of healthy normal skin (NN), lesional (PP), and non-lesional (PN) skin of psoriatic, as well as in lesional (AA) and non-lesional (AN) skin of AD patients (Figure 1c and Supplementary Figure S2 online). CD3− IL-22+ cells could be identified in epidermal samples from healthy volunteers, psoriasis and AD patients; they could not be identified in normal dermis (NN), but could be found in both non-lesional (PN and AN) and lesional samples (PP and AA) from patients with skin disease (Figure 1 and Supplementary Figure S2 online). The frequency of these cells varied considerably between individuals (Supplementary Figure S2 online) and as a result no statistically significant differences were observed; however, non-lesional psoriatic epidermis seemed to harbor the highest median frequency of CD3− IL-17+ and CD3− IL-22+ cells (15.9% and 3.8% of CD45+ cells, respectively). In addition, we performed dermal explant cultures of NN ($n=2$), PP ($n=5$), and PN ($n=2$) skin, allowing cells to migrate out of the tissue over 4–5 days. Migratory immune cells confirmed the presence of CD3− cells capable of producing IL-17 (Figure1d), as well as IL-22 (data not shown).

Our data point to the existence of an IL-17 and IL-22 producing CD3− immune cell subset in the blood and skin of healthy individuals and patients with inflammatory skin disease.

Extensive immunophenotyping of psoriasis peripheral blood cells reveals an enrichment in NKp44+ ILC3

To further dissect immune cells subsets in healthy individuals and patients with psoriasis or AD, we performed extensive immunophenotyping of peripheral blood mononuclear cells (PBMCs). No apparent differences were found in the populations of T, B, natural killer, and myeloid cells between healthy individuals and patients with inflammatory skin disease (Figure 2a). On the basis of the recent demonstration of ILCs as important contributors to IL-17 and IL-22 production in models of inflammatory skin disease (Pantelyushin *et al.*, 2012) and gut inflammation (Geremia *et al.*, 2011), we developed a panel of antibodies (Supplementary Table 4

Figure 1. Identification of CD3-negative (CD3−) lymphocytes in blood and skin that produce IL-17 and IL-22. Peripheral blood mononuclear cells (PBMCs) or skin cells from psoriasis patients (P), healthy individuals (N), or atopic dermatitis (AD) patients were stimulated with phorbol 12-myristate 13-acetate/ionomycin and stained for surface markers and cytokines for flow cytometric analysis. (**a**) Representative plots showing IL-17A and IL-22 production in PBMCs from N ($n=16$) and P ($n=16$). (**b**) Frequencies of CD3− cells within IL-17A+ or IL-22+ cells in PBMCs. (**c**) Representative dot plots for CD3− cells producing IL-17 and IL-22 from the epidermis and dermis of normal (NN, $n=4$ (epidermis), 5 (dermis)), psoriasis non-lesional (PN, $n=4$ (epidermis), 3 (dermis)), psoriasis lesional (PP, $n=5$ (epidermis and dermis)), AD non-lesional (AN, $n=5$ (epidermis), 2 (dermis)), and AD lesional (AA, $n=8$ (epidermis), 7 (dermis)) skin. (**d**) Representative plots of dermal skin explants from NN ($n=2$), PN ($n=2$), and PP ($n=5$) skin.

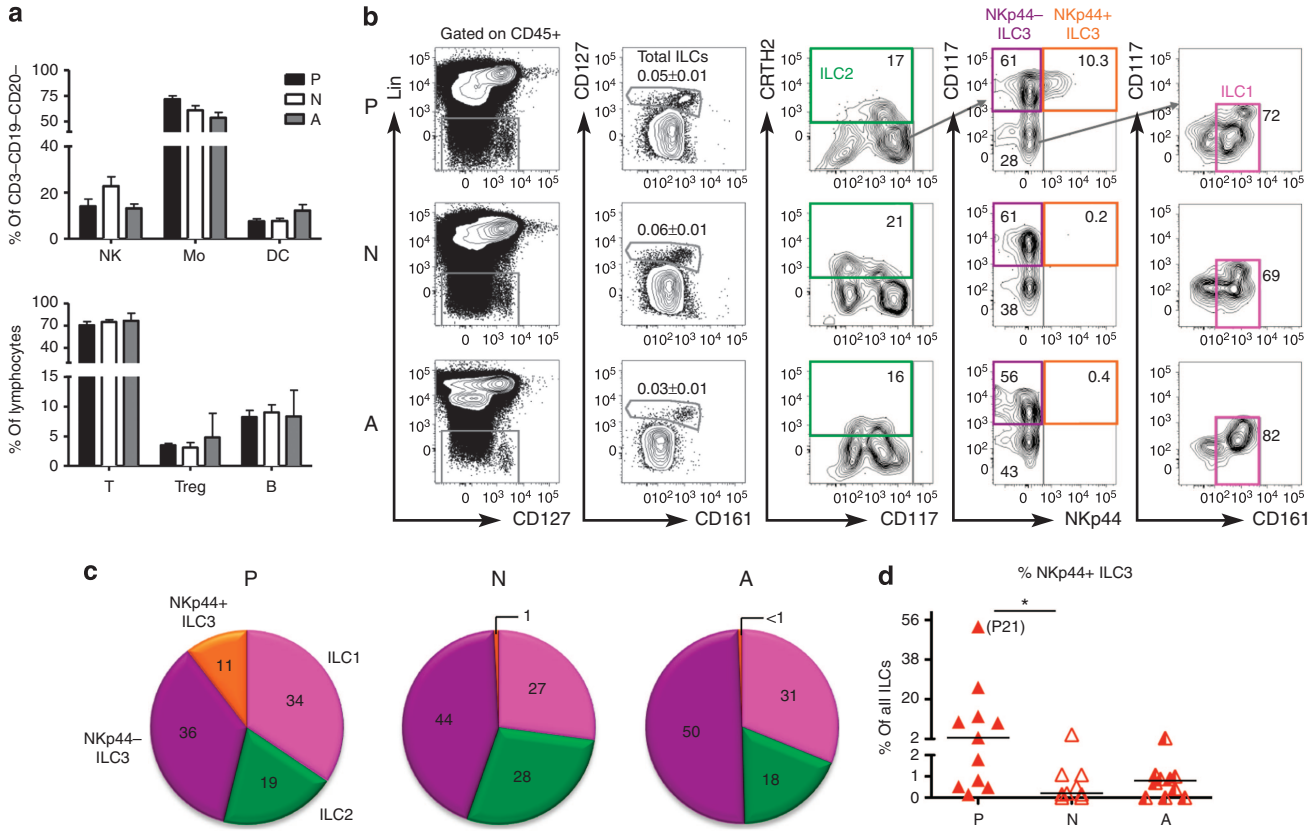

Figure 2. Deep immunophenotyping of peripheral blood mononuclear cells (PBMCs) shows increased frequency of NKp44+ innate lymphoid cell 3 (ILC3) in the blood of psoriasis patients. (**a**) Mean frequencies ± SEM of myeloid (top) and lymphocytic (bottom) cell populations within PBMCs of psoriasis (P) (black, $n=9$), healthy individuals (N) (white, $n=7$), and AD patients (A) (gray, $n=10$). (**b**) Analysis of total ILCs defined as Lin−CD127+ within live CD45+ lymphocytes in P ($n=11$), N ($n=9$), and A ($n=10$) (mean % ± SEM). ILC subpopulations were defined as shown in representative plots: ILC1 (pink), ILC2 (green), NKp44+ ILC3 (orange), and NKp44− ILC3 (purple). (**c**) Pie charts showing average frequencies of ILC subpopulations in P, N, and A. (**d**) Median frequencies of NKp44+ ILC3; each filled (P), empty (N), or shaded triangle (A) represents an individual donor. Kruskal–Wallis, followed by Dunn's multiple comparison test was performed, *$P<0.05$. DC, dendritic cell; Mo, monocyte; NK, natural killer cell; Treg, regulatory T cell.

online) allowing for the differentiation between the major human ILC subsets (Spits *et al.*, 2013). Analysis of the total ILC population defined as lymphoid/CD45+/Lin−/CD127+ in PBMCs of healthy individuals showed a mean frequency of 0.06 ± 0.01% ($n=9$) in agreement with previously reported data (Bernink *et al.*, 2013). The total ILC frequency was similar in psoriasis patients ($n=11$, mean 0.05 ± 0.01%) compared with healthy controls, but was somewhat decreased in AD patients ($n=10$, mean 0.03 ± 0.01%, $P<0.05$ vs. normal) (Figure 2b). The distinct populations of ILCs were defined as shown in Figure 2b: ILC2 (CRTH2+, green frame), ILC3 (CRTH2− CD117+) and either NKp44− (purple frame) or NKp44+ (orange frame), and ILC1 (CRTH2−/CD117−/CD161+, pink frame). Figure 2c shows the distribution of the different subsets, as mean frequency, in normal, psoriasis, and AD patients. In healthy individuals, the NKp44− ILC3 population was the most frequent, accounting for more than 40% of all ILCs (median: 45%, (36–54%)). The frequency of ILC1 and ILC2 varied somewhat among healthy individuals (ILC1 median: 24% (19–42%) and ILC2 median: 25% (19–45%)) (Figure 2c). Noticeably, the NKp44+ population of ILC3 was negligible in healthy individuals ranging from undetectable to 1% in eight out of nine individuals and 4% in one additional individual. No significant differences were observed in the distribution of ILC subsets in AD patients compared with healthy volunteers (Figure 2c). However, in psoriasis patients there was a statistically significant ($P<0.05$) increase in the median frequency of NKp44+ ILC3 cells from 0.2% (0–3.9%) in healthy individuals to 2.6% (0.1–53%) in patients (Figure 2d). The frequency of NKp44+ ILC3 in psoriasis patients appeared to be bimodally distributed, with one group of patients having low frequencies similar to those seen in healthy individuals (6 of 11) and a second group in which the NKp44+ ILC3 population ranged from 9.2 to 53% of ILC (5 of 11). Although the relatively small number of patients makes it difficult to perform proper stratification, no association was found between the bimodal frequency of NKp44+ ILC3 and either psoriasis patient clinical or demographic features (age, sex, psoriasis area severity index (PASI), age of disease onset, presence of comorbidities), or the frequency of other peripheral cell populations (cutaneous lymphocyte–associated antigen-positive (CLA+) ILCs, CD3, CD4 and CD8 T cells, T regulatory cells, T-helper type 17, natural killer cell, monocytes, and dendritic cell). Further

studies are required to confirm or rule out a genetic influence on the cell frequency of this population.

Successful treatment of psoriasis with anti-TNF antibody is associated with a decrease of circulating NKp44+ ILC3: a case study

We next asked whether NKp44+ ILC3 frequencies change during treatment of psoriasis with cytokine-targeted therapy. TNF synergizes with IL-23 to drive IL-17A production by ILC (Powell *et al.*, 2012). We had the opportunity to closely follow the clinical progression of patient P21 who had the highest frequency of circulatory NKp44+ ILC3 during treatment with an anti-TNF mAb (adalimumab). P21 was off any systemic treatment at the time the initial blood sample was taken and was then commenced on adalimumab. The PASI, which measures disease severity, was 21.2, indicating substantial disease. Subsequent blood samples were taken at 1, 4, and 12 weeks after commencing treatment. There were no gross changes in the frequencies of B cells, natural killer cells, myeloid cells, or T cells during adalimumab therapy (Figure 3a). In contrast, there was a marked shift in the frequencies of ILC3 populations in the blood with a 75% reduction in the NKp44+ ILC3 population and a corresponding increase in the NKp44− ILC3 population (Figure 3b). The shift in the absolute number of NKp44+ ILC3 was closely associated with a reduction in disease severity and extent measured by PASI, which decreased from 21.2 to 13.6 over time during treatment with adalimumab (Figure 3c).

Although only a single case, this result is potentially instructive, suggesting that frequency of circulating NKp44+ ILC3 might reflect disease severity and/or response to treatment with anti-TNF therapy. In addition, it highlights a potential role for TNF in the differentiation of human NKp44+ ILC3, which could be the subject of further investigation beyond the scope of this initial descriptive study.

ILC3 preferentially express the skin-homing CLA

To assess if there is a population of ILCs with skin-homing potential, we analyzed the expression of the skin-homing marker CLA on ILC populations and T cells in the blood. Interestingly, ILCs of healthy individuals expressed CLA at high frequency (mean 33 ± 4%), significantly higher than on CD8 T cells (mean 11 ± 5%, $P<0.01$) and higher than on CD4 T cells (mean 18 ± 5%), although this did not reach statistical significance (Figure 4a). A significant increase in the frequency of CLA+ ILCs was also seen in the blood of psoriasis and AD patients where the frequency of CLA expression was 2- to 4-fold higher on ILCs than on either CD4 or CD8 T cells (Figure 4a). In healthy individuals, the expression of CLA on NKp44− ILC3 was particularly high (mean 48 ± 15%), and significantly increased when compared with ILC2 ($P<0.05$; mean 18 ± 12%). Similarly, the expression of CLA was also high on NKp44− ILC3 in patients with inflammatory skin disease (psoriasis, mean 56 ± 22%; AD, mean 56 ± 16%) (Figure 4b).

NKp44+ ILC3 were only present at high enough frequency in psoriasis patients to assess CLA expression; interestingly, these cells expressed similarly high levels of CLA to

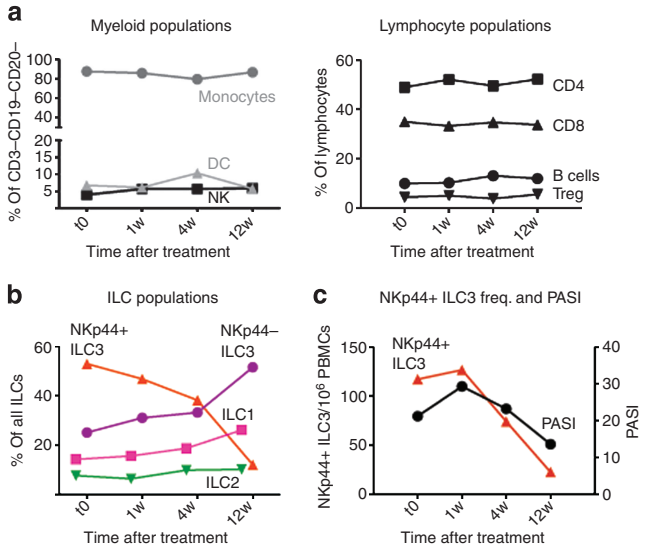

Figure 3. Reduction in NKp44+ innate lymphoid cell 3 (ILC3) population during successful treatment of psoriasis with anti–tumor necrosis factor (TNF) therapy. Case study of a psoriasis patient before and at indicated intervals after commencing therapy with the anti-TNF mAb adalimumab. Time-course analysis showing (**a**) the frequency of myeloid cell populations within CD45+/CD3−/CD19−/CD20− cells (top left); the frequency of B and T cell populations within CD45+ lymphocytes (top right); (**b**) the frequency of ILC1, ILC2, NKp44− ILC3, and NKp44+ ILC3 cells within total ILCs; and (**c**) the absolute count of NKp44+ ILC3 within 10^6 peripheral blood mononuclear cells (PBMCs), (left *y*-axis) plotted against disease severity expressed as psoriasis area severity index (PASI) (right *y*-axis). DC, dendritic cell; NK, natural killer cell; Treg, regulatory T cell; w, weeks.

NKp44− ILC3 and significantly more than ILC2 (psoriasis, mean % CLA+: NKp44+ ILC3 54 ± 18% vs. ILC2 14 ± 4%, $P<0.001$). These results imply that both circulating NKp44− and NKp44+ ILC3 express skin-homing markers and are poised to home to the skin.

NKp44+ ILC3 are increased in PN and PP skin of psoriasis patients

To confirm their role in skin inflammation, we isolated and characterized ILCs in skin biopsies from psoriasis patients and skin obtained from healthy individuals after surgical procedures. We did not have the opportunity to investigate the skin of AD patients, where clearly ILC2 are increased in the lesional skin (Kim *et al.*, 2013). In preliminary experiments, we found that optimal cell yield/viability was obtained by collagenase digestion of whole skin. Gating strategy and representative plots of the different ILC subsets in healthy and psoriasis skin are shown in Figure 4c. ILC populations were significantly enriched in the skin compared with blood (PP skin vs. P blood, $P<0.01$; NN skin vs. N blood, $P<0.001$), with a median frequency of 0.6% (0.4–4.4%) of CD45+ lymphocytes in normal skin ($n=6$) and 1.4% (1.0–2.3%) in lesional psoriasis skin ($n=5$) (Figure 4d), which represents over 10- and 30-fold more, respectively, than the frequencies observed in blood (Figure 2b).

ILC populations in normal skin ($n=6$) were variable between individuals (ILC1 median: 19% (9–32%); ILC2

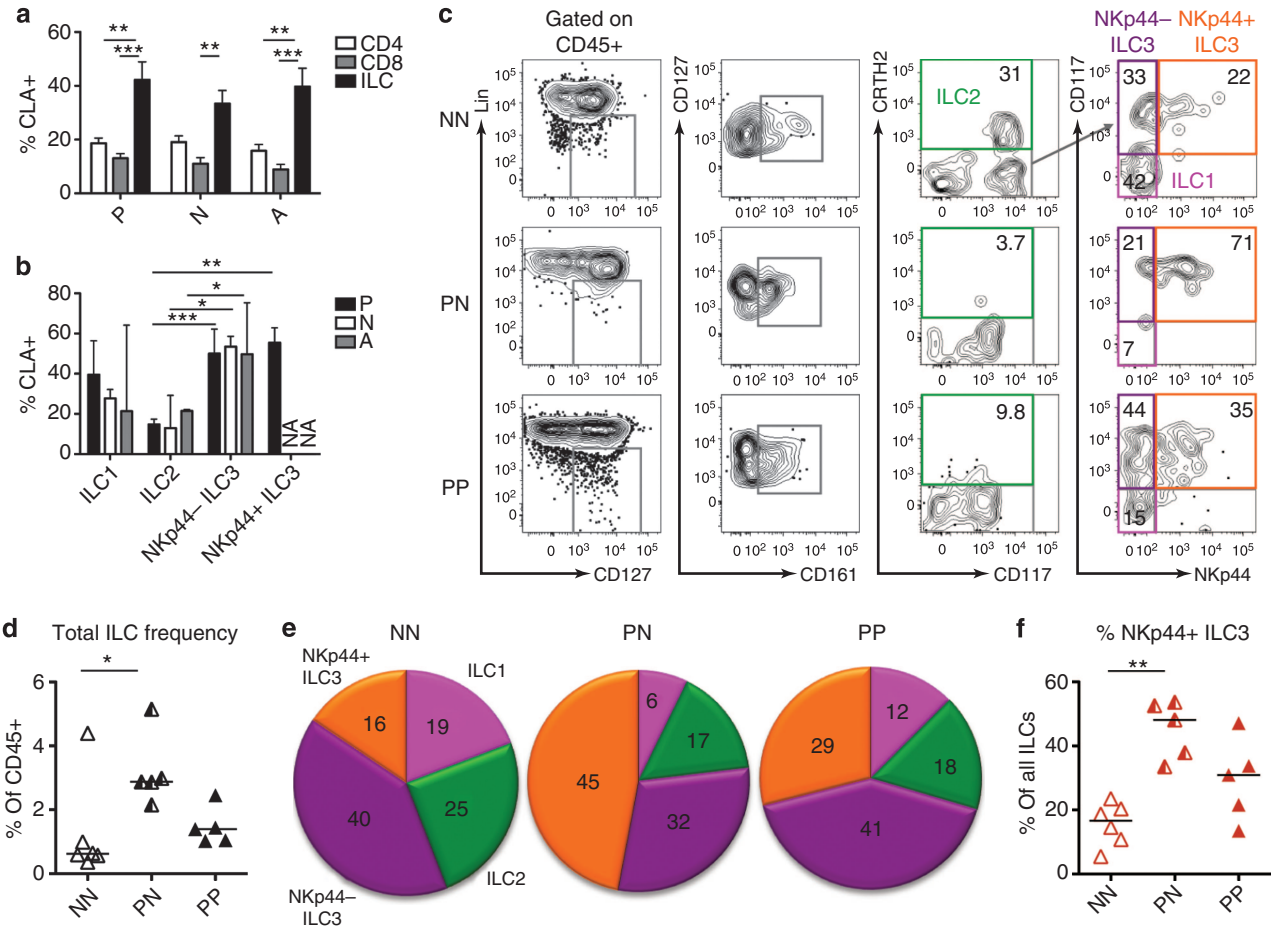

Figure 4. Circulating NKp44+ innate lymphoid cell 3 (ILC3) express skin-homing CLA and are enriched in the skin of psoriasis patients. (**a**–**b**) Peripheral blood mononuclear cells (PBMCs) and (**c**–**f**) collagenase-digested skin cells were immunophenotyped. (**a**) Frequency (mean ± SEM) of CLA+ cells within CD4s, CD8s, and ILC from psoriasis (P, $n=9$), healthy individuals (N, $n=6$), and atopic dermatitis (AD) patients (A, $n=7$). (**b**) Frequency (median ± interquartile range) of CLA+ cells within ILC subsets. (**c**) Plots representative of NKp44+ ILC3 show gating of ILC populations in normal (NN, $n=6$), psoriasis non-lesional (PN, $n=5$), and psoriasis lesional (PP, $n=5$) skin. (**d**) Total ILC frequency, (**e**) ILC subset distribution, and (**f**) frequency of NKp44$^+$ ILC3 in NN, PN, and PP skin. (**d**, **f**) Each open (NN), shaded (PN), and filled (PP) triangle represents an individual donor. (**a**) One-way analysis of variance with Bonferroni post-test or (**b**, **d**, **f**) Kruskal–Wallis with Dunn's multiple comparison test. *$P<0.05$, **$P<0.01$, and ***$P<0.001$.

median: 16.3% (4–69%); ILC3 NKp44− median: 46% (9–65%); ILC3 NKp44+ median: 13% (5–24%)). Figure 4e shows distribution of the different subsets, as the mean frequency, in normal, non-lesional, and lesional psoriasis skin. Interestingly, the overall frequency of ILCs in PN skin was significantly increased compared with healthy skin (median 2.9% (2.2–5.1%), PN vs. NN, $P<0.05$) (Figure 4d). Moreover, the distribution of ILC populations was highly skewed, with a significant enrichment of the NKp44+ ILC3 to a median frequency of 48% (34–54%) (PN vs. NN, $P<0.05$) (Figure 4f). This population also appeared to be enriched in psoriasis lesions in comparison with normal skin, although this did not reach statistical significance (median 31% (13–47%)) (Figure 4f). However, because of the overall enrichment of ILCs in lesional (PP) compared with PN skin (PP median: 2,100 (500–3,600) ILC per cm^2 skin. PN median: 800 (100–1,400) ILC per cm^2 skin), there was an increased overall density of NKp44+ ILC3 in each lesional (PP) sample when compared with the matched non-lesional sample (PN) ($n=5$, PP median 440 (250–1,200) cells per cm^2 skin, PP median 260 (60–770) cells per cm^2 skin, PN vs. PP, $P<0.05$).

The enrichment of ILCs in the skin suggests that these cells are important players in tissue immunity. The increase in frequency of ILCs and particularly the NKp44+ ILC3 subset in non-inflamed skin of psoriasis patients is particularly interesting, as it indicates that these cells are not bystanders recruited to the skin following T cell activation. NKp44+ ILC3 have previously been shown to produce IL-17 and IL-22 (Bernink *et al.*, 2013), which is consistent with the trend observed of increased CD3− cytokine-producing cells in PN samples (Figure 1c and Supplementary Figure S2 online). The enrichment in PN samples suggests that these cells may be strategically placed and poised to start the psoriatic disease process if appropriate stimuli occur. As such, they could represent the innate counterparts to the tissue-resident memory T cells of adaptive immunity, which are known to reside in the skin to act as the first line of defense in the tissue (Boyman *et al.*, 2007; Clark, 2010) and provide long-term peripheral

immunity (Gebhardt *et al.*, 2009; Jiang *et al.*, 2012). Activation of resident memory T cells is necessary and sufficient for the development of psoriatic lesions from human non-lesional skin transplanted onto an AGR129-immunodeficient mouse (Boyman *et al.*, 2004), and skin-resident ILCs could contribute by providing the necessary activation signals. In this sense, ILCs would have a role in initiating the psoriasis plaque, which is then sustained by proliferating T cells, which are present at high frequency in lesional skin. Further studies, for instance using the xenotransplant model (Boyman *et al.*, 2004), are required to elucidate ILC function in initiating the psoriasis plaque.

Our findings are complementary but distinct to our previous description of the role of Vγ9Vδ2 γδ T cells in psoriasis, which are recruited to the psoriasis skin under inflammatory conditions (Laggner *et al.*, 2011). Our observation that Vγ9Vδ2 γδ T cells represent only a minority of IL-17 production in non-lesional skin is now explained by the discovery of NKp44+ ILC3 as potentially major innate contributors of IL-17 and IL-22 production in conditions of skin homeostasis and inflammatory pathology.

Taken together, we establish a potentially important role for NKp44+ ILC3 in the pathogenesis of psoriasis paving the way for the investigation of such cells as therapeutic targets or biomarkers in the management of psoriasis.

MATERIALS AND METHODS

Subjects

Thirty-six healthy individuals (18 men and 18 women; mean age 44 years) were included in the study. Thirty-four psoriatic (29 men and 5 women; mean age 42 years) and 10 AD (5 men and 5 women; mean age 37 years) patients were recruited at St John's Institute of Dermatology (Guy's and St Thomas' Hospital), West Middlesex University Hospital, and Queen Elizabeth Hospital, after examination by expert clinicians. Unless otherwise stated in Supplementary Table 2 online, psoriasis and AD patients were off biologic and systemic immunosuppressive therapy for at least 2 weeks. Full demographics of participants are provided in Supplementary Tables 1–3 online. Our study was conducted in accordance with the Declaration of Helsinki Principles, written informed consent was obtained from each participant, and approved by the institutional review board of Guy's and St Thomas' Hospital (Guy's Research Ethics Committee, Reference 06/Q0704/18) and London Bridge (London Bridge Research Ethics Committee, Reference 11/LO/1962).

Sample collection

Skin biopsies (4–6 mm punch biopsy) were obtained from psoriasis and AD patients from both lesional and non-lesional skin, and healthy discarded skin was obtained from plastic surgery. One psoriatic patient (P21; Supplementary Table 2 online and Figure 3) was followed during the course of therapy with adalimumab (human mAb anti-TNF), with blood collected before and after 1, 4, and 12 weeks of therapy.

PBMC isolation and storage

PBMCs were isolated from the blood by density centrifugation over Lymphocyte Separation Medium LSM 1077 (PAA Laboratories, Pasching, Austria) and frozen in RPMI 1640 (Life Technologies, Carlsbad, CA) containing 11.25% human serum albumin (Gemini Bio-Products, West Sacramento, CA) + 10% DMSO (Sigma, St Louis, MO) and stored in liquid nitrogen.

Skin sample processing

Skin immune cells were obtained either by spontaneous migration out of the tissue (dermal explants) or by enzymatic isolation.

Dermal explants: Epidermis and dermis were separated using dispase (5 mg ml^{-1}; StemCell, Grenoble, France) treatment at 4 °C overnight. The dermis was then cut into small pieces and cultured in complete RPMI (RPMI containing 1% penicillin/streptomycin and 10% fetal calf serum; all from Life Technologies) for 3–5 days at 37 °C/5% CO_2. Non-plastic adherent cells that had migrated out of the dermis 3–5 days after culture were harvested for flow cytometric analysis.

Enzymatic isolation: Epidermis and dermis were separated using EDTA treatment (15 mM; Life Technologies) for 1 hour at 37 °C and then incubated in collagenase (0.8 mg ml^{-1}; Worthington, Lakewood, NJ) at 4 °C overnight. For whole skin fresh cell suspensions, the skin was incubated in collagenase overnight at 37 °C.

Multiparameter flow cytometry

PBMCs were thawed and either stimulated for intracellular cytokine production on a custom-made stimulation lyoplate (BD Biosciences, San Jose, CA) containing phorbol 12-myristate 13-acetate, ionomycin, and golgi inhibitors (monensin and brefeldin A), at 37 °C/5% CO_2 for 5 hours and stained on a custom-made staining lyoplate (BD Biosciences) as described previously (Villanova *et al.*, 2013) or stained with liquid antibody cocktails for surface markers.

Skin cells were either stimulated with phorbol 12-myristate 13-acetate (Sigma; 50–100 ng ml^{-1}), ionomycin 1 μg ml^{-1} (Calbiochem, Darmstadt, Germany), monensin (BD Bioscience; 3 μM), and/or brefeldin A (BD Bioscience; 5 μM) at 37 °C/5% CO_2 for 5 hours and stained with liquid cocktails for intracellular cytokine production, or stained with liquid antibody cocktail for surface marker analysis.

Lyophilized antibody present on lyoplates and liquid antibodies used are listed in Supplementary Table 4 online.

Intracellular staining was performed using the BD Human Foxp3 Buffer kit (BD Bioscience) according to the manufacturer's instructions. Dead cells were excluded from the analysis by staining with Live Dead Aqua/Yellow (Life Technologies).

Samples were acquired on a SORP Fortessa (BD Bioscience), and data analyzed using the DIVA software (BD Bioscience) or FlowJo software (Treestar, Ashland, OR).

Statistical analysis

Indicated populations were assessed for normal Gaussian distribution with D'Agostino and Pearson omnibus normality test and then analyzed by one-way analysis of variance, followed by Bonferroni post test or Kruskal–Wallis, followed by Dunn's multiple comparison test, as appropriate, using Prism version 5.0 (GraphPad Software, La Jolla, CA). Paired lesional and non-lesional skin samples were compared using Wilcoxon's signed-rank test. Unless stated otherwise, mean values are given ± SEM and median values with (range). Values of $P<0.05$ were considered significant.

CONFLICT OF INTEREST

FN has been a consultant for companies producing anti-TNF reagents for treatment of patients with psoriasis.

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SUPPLEMENTARY MATERIAL

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

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