In Situ Mapping of Innate Lymphoid Cells in Human Skin: Evidence for Remarkable Differences between Normal and Inflamed Skin

Marie-Charlotte Brüggen1,2,6, Wolfgang M. Bauer1,6, Bärbel Reininger1, Eduard Clim3, Catalin Captarencu4, Georg E. Steiner5, Patrick M. Brunner1, Barbara Meier2, Lars E. French2 and Georg Stingl1

Although innate lymphoid cells (ILCs) have recently been identified also in skin, their role in this organ remains poorly understood. In this study, we aimed at developing a technique to assess ILCs in situ and to determine their topographical distribution in human skin. We collected lesional skin biopsies from patients with atopic dermatitis and psoriasis (both n = 13) and normal human skin from healthy controls. After establishing immunofluorescence ILC in situ stainings, we developed an analysis approach (gating combined with manual validation) to reliably identify ILCs. Topographical mapping was obtained by automated calculations of the distances between ILCs and different cellular/structural elements of the skin. Whereas normal human skin harbored a very scarce ILC population (mostly ILC1s and AHR+ILC3s), atopic dermatitis and psoriasis skin was infiltrated by clearly visible ILC subsets. We observed atopic dermatitis skin to contain not only ILC2s but also a prominent AHR+ILC3 population. Conversely, we encountered almost equal proportions of ILC1s and RORC+ILC3s in psoriasis skin. Distance calculations revealed ILCs to reside near the epidermis and in close proximity to T lymphocytes. ILC mapping in situ will provide valuable information about their likely communication partners in normal and diseased skin and forms the basis for the appropriate mechanistic studies.


INTRODUCTION

The skin is a barrier organ directly exposed to the outside world. As such, it often faces environmental challenges ranging from immunogenic haptens to invading microbes. Protection from such danger signals is provided by a highly complex cutaneous immune network consisting of both innate and adaptive components (Modlin et al., 2012). Among the latter, a population of skin-resident T cells has been recognized as an important player (Clark, 2015). A novel subset of innate immune cells has more recently entered the stage of immune defense (Kim, 2015), namely innate lymphoid cells (ILCs). These leukocytes require IL-7 for their development and, thus, uniformly express the IL-7 receptor alpha chain CD127. They do not express any lineage (Lin) markers of other leukocyte populations and lack a recombinant antigen recognition receptor (Spits and Cupedo, 2012). What they do share with T-cell subsets, however, are striking similarities regarding their transcription factor (TF) and, hence, cytokine profiles. Based on the latter, three groups of noncytotoxic human ILCs can be defined (De Obaldia and Bhandoola, 2015; Eberl et al., 2015). Group 1 ILCs depend on the TF TBET, and generate IFN-γ and tumor necrosis factor-α, but no cytotoxic molecules. Group 2 ILCs express high levels of the GATA3 and produce T helper type 2 (Th2) cytokines (IL-4, IL-5, and IL-13). Group 3 ILCs constitute a group of cells displaying heterogeneous expression of the TFs’ nuclear receptor gamma (RORC), the AHR, and TBET (Serafini et al., 2015). Functionally, ILC3s produce variable amounts of IFN-γ, IL-22, and IL-17 (Eberl and Litman, 2004). This apparent heterogeneity of ILC3s, rather than being indicative of their multifocal origin, seems to be a sign of their plasticity imposed by different types of stimuli in their environment.

All ILCs have in common the capacity of immediately responding to different, often epithelial cell-derived mediators, mostly by cytokine production (Sonnenberg and Artis,
This innate response is not only biologically important by itself, but can also initiate and orchestrate adaptive immune responses (Eberl et al., 2015; Sonnenberg and Artis, 2015). Dependent on the type of ILC subset involved, the complex functional interplay of ILCs with other leukocyte populations may either result in inflammation or in the downregulation of inflammatory events. In addition, ILCs play an important role in tissue repair and, as described for a special subset of ILC3s, in the histogenesis of secondary lymphoid tissues (Eberl and Littman, 2004; Yoshida et al., 1999).

It is not entirely clear whether and, if so, how ILCs are embedded in the cutaneous immune network. Most data currently available on the occurrence of ILCs in skin are derived from the murine system. These point to the existence of a small ILC population in normal skin and suggest an involvement of ILC2s (Imai et al., 2013; Kim et al., 2013; Roediger et al., 2013; Salimi et al., 2013) and ILC3s (Pantelyushin et al., 2012) in mouse models of atopic dermatitis (AD) and psoriasis (Pso), respectively. Our knowledge about cutaneous ILCs in humans is currently limited to a few studies conducted on single cell suspensions isolated from skin (Dyring-Andersen et al., 2014; Salimi et al., 2013; Teunissen et al., 2014; Villanova et al., 2014).

In this study, we sought to develop an in situ approach allowing to detect and quantify ILCs in the skin, and, importantly, to obtain a detailed topographic description of these cells in the cutaneous microenvironment. By doing so, we hoped to gain new insights into the role of ILCs in skin biology under physiologic (normal human skin [NHS]) and pathologic conditions (AD and Pso).

RESULTS

In situ identification of ILCs in human skin by a newly developed two-step validation algorithm

Our first aim was to establish multicolor immunofluorescence (IF) in situ stainings for ILC subsets in human skin. We used a microscope equipped with four filters capable of discriminating DAPI, green fluorescent protein, Cy3, and Cy5 fluorescence.

A major requirement for the identification of ILCs is to reliably exclude the expression of markers for any other leukocyte population (Eberl et al., 2015), especially T cells. We implemented this by including the relevant Lin markers (i.e., for natural killer cells [CD16, CD94], Th cells [CD3, CD4], B cells [CD20], Langerhans cells [CD1a], myeloid dendritic cells [CD11c], monocytes [CD14], stem/endothelial cells [CD34], neutrophils [CD15], plasmacytoid dendritic cells [CD123], and mast cells [CD203c]; Supplementary Figure S1a online) in a single fluorescence channel. Another channel was used for the identification of TFs that are known to be a prerequisite for the development and functional specialization of, on the one hand, different ILC subpopulations (TBET for ILC1, GATA3 for ILC2, RORC, and AHR for ILC3), and, on the other, Th-cell subtypes (Shih et al., 2014). TF stainings were established using differentiated, sorted Th-cell subsets (Supplementary Figure S1b and c). GATA3 expression in keratinocytes (Kaufman et al., 2003) served as an additional positive validation. We then followed either of two staining strategies to unequivocally identify ILCs in situ (Supplementary Table S1 online). In staining protocol A, we deliberately counterstained with CD3 for the precise exclusion of T cells and also for comparison of Th cell and ILC subtypes. In staining protocol B, we included CD3 in the lineage cocktail and used the third and remaining channel for the depiction of CD45 or of prototypic ILC markers (CD127, CD161, chemoattractant receptor-homologous molecule expressed on T helper type 2, CD117, NKP44, and NKP46).

When examining tissue sections of NHS stained in this way by conventional (i.e., eye based) epiluminescence microscopy, we were able to occasionally identify Lin<sup>−</sup> TF<sup>+</sup> cells classified as ILCs but could not grasp the complete picture. The paucity of ILCs and small field of views were the main limiting factors. To overcome this problem, we scanned and recorded the entire skin sections. We then used analysis software (TissueFAXS) to apply an automated algorithm on the scanned images measuring the mean IF intensities of the stained markers in cells (identified with DAPI). Software analysis allowed graphical depiction of the cells as events in dot plots, allowing for gating strategies similar to conventional FACS data assessment. Concerned that the slightest “contamination” of the ILC gate with falsely positive cells would bias results, we implemented a manual validation step in our software. This algorithm cropped out all cells automatically identified as ILCs on a single-cell basis (Figure 1a, Supplementary Figure S2 online) allowing for the manual reassessment of the different fluorescence channels and the cells’ morphologic shape. Each cell was manually confirmed or rejected by two observers (Figure 1a).

Using these measurements, we reliably identified a very small population of Lin<sup>−</sup> CD3<sup>−</sup> cells expressing one of the ILC-associated TFs (Figure 1a). Similar numbers of Lin<sup>−</sup> TF<sup>+</sup> cells were encountered when we used staining protocol B, in which we assessed the expression of phenotypic ILC markers in the Lin<sup>−</sup> CD3<sup>−</sup> TF<sup>+</sup> cell population (Figure 1b). We were thereby able to show that these cells are indeed ILCs, as evidenced by their positive expression of CD45, CD127, CD161, or CRTH2 in the case of GATA3<sup>+</sup> ILC2s (Figure 1b–h and Supplementary Figure S3a–c online).

NHS harbors a sparse dermal ILC population

In a first set of experiments, we searched for ILCs residing in epidermal, upper dermal, and hypodermal areas of NHS. This analysis revealed a very sparse ILC population in the upper dermis (Figure 2a), whereas hypodermal areas as well as the epidermis were essentially devoid of CD45<sup>−</sup> Lin<sup>−</sup> cells expressing ILC TFs (data not shown). Approximately half of upper dermal ILCs belonged to the group 1 subset (TBET<sup>+</sup>). AHR-expressing ILC3s accounted for the second most prominent subgroup in NHS followed by RORC<sup>+</sup> ILC3s, whereas hardly any GATA3<sup>+</sup> ILC2s were identified. With the exception of ILC2, this composition of ILC subpopulations corresponds to what we and others observed using FACS analysis of dermal cell suspensions (Supplementary Figure S4 online).

Next, we sought to assess the spatial arrangement of ILCs within the skin, especially their topographical distribution with regard to the epidermis and to vascular structures. Therefore, we developed and applied an algorithm calculating the distance of each validated ILC to the epidermis as well as to the nearest blood vessel. These measurements (Figure 2b)
revealed all ILCs to reside in a close (<30 μm) distance to the epidermis. In contrast, ILCs did not occupy the perivascular areas of the dermis (Figure 2c).

**Diverse ILC subsets infiltrate cutaneous AD versus Pso lesions**

We next asked which changes in number and composition the cutaneous ILC population would undergo in AD and Pso, both inflammatory skin diseases driven by distinct T-cell subsets (Nogales et al., 2009). When compared with NHS, we found considerably increased numbers of ILCs (Figures 2d–g and 3a) in both AD and Pso skin lesions (both n = 13). In AD, this numerical increment mainly affected GATA3⁺ ILC2s and AHR⁺ ILC3s (Figures 2g and 3a). AHR⁺ ILC3s even represented the most prominent ILC subset in AD. In Pso skin, by contrast, we observed an expansion of...
both ILC1s and ILC3s (Figures 2d–f and 3a), but hardly of ILC2s.

When determining the topographical relationship between ILCs and the skin’s structural components, in AD and Pso lesions, we found an almost random distribution of these cells within the upper dermis with no signs of perivascular clustering (Figure 3b and c). In fact, none of the subsets was located in a close distance (Figure 3f) from blood vessels but rather far (≥80 μm) or very far (≥150 μm) away from them (Figure 3d). As far as their topographic relationship to the epidermis is concerned, ILC subsets in AD and Pso were found to reside at an intermediate distance (50–60 μm; Figure 3e and f, Supplementary Figure S5 online) from the basal cell layer. With the exception of RORC⁺ILC3s, this distribution was similar to that observed in NHS.
ILCs are in intimate contact with T cells

On the basis of the observation that murine ILCs engage in a biologically relevant functional interaction with T lymphocytes (Halim et al., 2014), we studied the localization of human skin ILCs vis-à-vis their T-cell counterparts.

Strikingly, our analyses revealed a close proximity (maximal distance: 30 μm) between ILCs and T cells in both AD and Pso (Figure 4a and b, Supplementary Figure S6a–c online). Even in NHS, ILCs were found not further than 70–80 μm away from T cells (Figure 4b). A particularly intimate relationship (<10 μm) was observed between ILC1s and T cells in Pso lesions.

We next investigated the contribution of T cells to the closest cellular environment of ILCs. We developed an analysis algorithm for this purpose, in which the whole cellular content of the upper dermal area was screened to calculate the percentage of T cells in a defined radius around validated ILCs (Figure 4c). Direct cell-cell contact...
corresponded to an analysis radius of 3 μm. The “inner circle” of the ILC microenvironment was arbitrarily defined with a 9 μm radius (Figure 4d). The percentage of T cells directly adjacent to all ILCs ranged between 3% and 30%, with the notable exception of AHR⁺ILC3s of AD skin, which were surrounded by a dense mantle of T cells (Figure 4e, Supplementary Figure S6d–f). We ensured that our results were not biased by overlapping cells; the analysis of a 3 μm and a 5 μm (corresponding to our section thickness) radius yielded essentially the same results (data not shown). The proportion of T cells within the radius of 9 μm ranged between 15% and 50% and did not differ greatly between the various ILC subsets (Figure 4f).

ILC and Th-cell patterns in AD and Pso are similar but not identical

The close vicinity between ILCs and T cells prompted us to ask whether the TF-based composition of ILCs mirrored the one of their Th cell counterparts in AD and Pso. The
analysis of IF stainings for Th-cell subsets (Figure 5a) revealed GATA3+ Th2 cells to clearly predominate the CD3+CD4+ GATA3+ T-cell infiltrate in AD (see also Figure 5b). AHR+, putative Th22 cells were the second most prominent subset in AD, followed by TBET+ Th1 and RORC+ Th17 cells. Pso lesions, on the other hand (Figure 5a, c, and d), contained considerable numbers of Th1 as well as Th17 cells.

A comparison of the relative ILC and Th-cell subset composition in NHS, AD, and Pso skin (Figure 5c) showed no absolute correlation between TF-defined ILC and Th-cell lineages. In AD, Th2 cells and AHR+ ILC3s predominated within the Th-cell and ILC infiltrate, respectively. In Pso, we found a RORC+/AHR+ ILC3 predominance among ILCs juxtaposed to a Th17 pattern among CD4+ T lymphocytes. TBET expression was prevailing in both ILCs and T cells of NHS. A sizable proportion of AHR-positive cells were only found in the ILC, but not the Th-cell fraction of NHS.

Next, we were interested in whether T cells in close proximity (<9 μm and <3 μm) to ILCs belong to the respective “mirror” subsets. Overall, this was surprisingly not the case (Figure 5e and f, Supplementary Figure S6). Only 3–5% of the cells surrounding ILCs belonged to their respective adaptive T-cell counterparts. Exceptions to this were Th2 cells/ILC2s in AD; in both a 3 μm and 9 μm radius, we found them to make up for 10–20% of all neighboring cells.
DISCUSSION

Although previous human studies have used flow cytometric approaches to identify ILCs in peripheral organs, our aim was to provide an in situ characterization of ILCs in skin under homeostatic (NHS) and inflammatory conditions (AD and PsO). We established an IF staining panel for ILCs and developed a computed analysis algorithm allowing enumeration and topographic assessment of ILCs on a single-cell basis. Our analysis of ILC subpopulations was based on the fact that the expression of certain TFs is a prerequisite for the differentiation of ILCs into distinct lineages (Eberl et al., 2015). The same TFs are responsible for the polarization of Th-cell subsets (Shih et al., 2014), allowing us to subsequently compare these two lymphocyte populations in situ. A limiting factor in choosing this approach is the known plasticity of ILCs and in part overlapping TF expression. GATA3, for example, is not only expressed by ILC2 and Th2 cells but is also required early during ILC lineage specification and ILC3 homeostasis (De Obaldia and Bhandoola, 2015; Yagi et al., 2014). RORC<sup>+</sup>/AHR<sup>+</sup>ILC3s can differentiate into TBET<sup>+</sup>ILC1s under special conditions (Bermink et al., 2013). In addition, some controversy as to the assignment of ILC3 to a distinct lineage still exists (Hughes et al., 2014). These caveats should be kept in mind when snap-shot analyzing ILC subsets either by FACS or IF.

Our investigations revealed a very sparse ILC population in NHS consisting almost exclusively of TBET<sup>+</sup>ILC1s and AHR<sup>+</sup>ILC3s. In contrast, inflamed skin harbored much larger numbers of ILCs. AD was found to mainly host AHR<sup>+</sup>ILC3s and GATA3<sup>+</sup>ILC2s. In PsO lesions, TBET<sup>+</sup>ILC1s and ILC3s predominated, whereas GATA3<sup>+</sup>ILC2s were almost absent. Topographically, ILCs were mainly clustered beneath the dermoepidermal junction and exhibited a very close spatial relationship to T lymphocytes.

Some of our data on NHS, that is, the presence of ILC3s and ILC1s, correspond to those obtained by Teunissen et al. and Villanova et al. in flow cytometric studies. In contrast, these authors as well as our own group (Supplementary Figure S4a) identified approximately 10% of all ILCs in dermal cell suspensions as ILC2s by FACS analysis, but we hardly found any GATA3<sup>+</sup>ILC2s in tissue sections by IF. Kim et al. (2013), in contrast, were not able to demonstrate CRTH2 expression on ILC2s in NHS or patients with AD by FACS. The reasons for this discrepancy are not entirely understood and may include: (i) differences in ILC density in different body locations, as it has been reported for T cells (Foster et al., 1990); (ii) phenotypic alterations during isolation and purification procedures; (iii) differences in the sensitivity of the staining and detection method. The high threshold of detection applied in our study together with lower mean fluorescence intensities measured by IF in comparison to FACS and the paucity of ILCs in NHS may have led to a slight underestimation of the actual number of ILCs in NHS.

The role of ILCs in NHS escapes us at the present time. Intriguingly, certain ILC3s are involved in fetal development of secondary lymphoid tissues (Yoshida et al., 1999). One could speculate that ILCs, in addition to forming a first line of antimicrobial defense (Klose et al., 2014), contribute to skin histogenesis/organogenesis. This raises questions about the time point at which ILCs start populating this organ and whether in adults ILCs are permanently residing/renewing in skin or transiting from the blood. The previously described expression of cutaneous lymphocyte-associated antigen on circulating ILCs suggests their entry from blood (Teunissen et al., 2014; Villanova et al., 2014). Further studies are needed to investigate the time of entry of ILCs into the skin and their possible role in antimicrobial defense or as promoters of tissue remodeling/repair (Dudakov et al., 2015).

As opposed to the rare occurrence of ILCs in NHS, we found AD lesions to be prominently populated by AHR<sup>+</sup>ILC3s and GATA3<sup>+</sup>ILC2s. Their adaptive Th-cell counterparts consisted mostly of Th2, and, to a lesser extent, of Th22 cells.

Concerning ILC2s, our results are in keeping with observations by other investigators demonstrating this leukocyte subset in cell suspensions isolated from human AD lesions and murine AD-like skin (Bonefeld and Geisler, 2016). Concerning the latter, mostly keratinocyte-derived cytokines (e.g., thymic stromal lymphopoietin) activate ILC2s. Their main effector function in AD seems to be the release of cytokines further accentuating epidermal barrier breaching (Salimi et al., 2013). Particularly striking regarding the potential role of ILCs in AD was our observation that among T cells directly adjacent to ILC2s, the majority belonged to the Th2 subset. This may indicate that ILC2s modulate the Th2 response in AD. Studies in murine allergic asthma models support a Th2-promoting role of ILC2s (Halim et al., 2014, 2016).

An unexpected finding of our investigations was the identification of substantial amounts of AHR<sup>+</sup>ILC3s in AD skin. The TF AHR is known to be not only an important modulator of IL-22 production of ILC3s but is also necessary for their development and maintenance (Cella and Colonna, 2015; Hughes et al., 2014; Mjosberg et al., 2012). In analogy to the role of Th22 cells in AD (Czarnowicki et al., 2015; Nograles et al., 2009), IL-22 producing ILC3 could be coreponsible not only for inflammation but also for lichenification of the skin, a hallmark of AD (Zheng et al., 2007).

The question whether AHR<sup>+</sup>ILC3s and ILC2s act in concert with their Th-cell counterparts and thus, to which extent they contribute to the pathogenic process in a given situation, remains to be elucidated. Because cytokine release by ILCs occurs in an antigen-independent fashion, one may hypothesize that ILCs play an important role especially in the intrinsic form of AD, which, as opposed to the extrinsic one, is not associated with allergen sensitization and/or elevated IgE levels (Weidinger and Novak, 2016).

When compared with NHS, PsO skin sections were found to harbor substantially increased numbers of ILC1s as well as of ILC3s. ILC2s, in contrast, were only rarely encountered. Again, all ILCs showed a preferential topographic distribution near the epidermis. Strikingly, all ILC subsets were found to reside close to T lymphocytes, which, as reported by others, consisted mainly of Th1 and Th17 cells (Kryczek et al., 2008; Lowes et al., 2008).

As far as the ILC3 subset is concerned, our in situ data are complementary to FACS studies published previously (Dyring-Andersen et al., 2014; Teunissen et al., 2014; Villanova et al., 2014) pointing to the existence of an ILC3
population in PsO skin. In contrast to findings by others (Teunissen et al., 2014), we observed an abundance of TBET⁺ILC1s in cutaneous PsO lesions. Although we cannot exclude the possibility that these cells directly originate from the common ILC precursor, we should not forget that ILC3s can downregulate RORC and, reciprocally, increase the expression of TBET. Concurrent with these changes, the cells acquire the capacity to produce IFN-γ and tumor necrosis factor-α at the expense of IL-17 and IL-22 secretion (Serafini et al., 2015). It is currently unknown whether TBET⁺ILC1s play a disease-promoting role in PsO. A possible argument against this possibility is the recently published finding that neutralization of IL-17A is even more efficient in the treatment of moderate-to-severe plaque PsO than the blockade of IL-12/IL-23p40 (Thaci et al., 2015). Whether TBET⁺ILC1s may have perhaps a dampening effect on their neighboring Th17 populations remains to be determined.

In summary, we have developed an algorithm-based in situ analysis technique enabling us to reliably quantify and, importantly, topographically characterize ILCs in human skin. As opposed to flow cytometric studies conducted so far, our approach additionally allows us to accurately describe the structural and cellular environment of ILCs on a single-cell basis. Deeper insights into the nature of cutaneous ILC subpopulations and their surroundings in human skin will constitute a solid basis for future analyses of the functional relationship between ILCs and other constituents of the cutaneous immunological microcosmos.

MATERIALS AND METHODS

Study design, skin sampling, and processing

We collected lesional biopsies from patients with PsO (n = 13) and AD (n = 13) treated at Division of Immunology, Allergy and Infectious Diseases, Dermatology Department of Vienna’s Medical University, or at the Dermatology Department of the University Hospital Zurich between 2009 and 2015. NHS (n = 10) was obtained as tissue discarded from abdominoplasties. Patient characteristics are displayed in Supplementary Table S2 online. Details of skin sampling and processing are given in the Supplementary Material and Methods online.

The study was approved by the Ethics Committees of the Medical University of Vienna (EK700/2009) and of the University of Zurich (EK 647/2006) and conducted according to the Declaration of Helsinki. All study subjects participated voluntarily and gave written informed consent.

IF stainings, section scanning

A complete list of antibodies used in this study is shown in Supplementary Table S3 online. Details of the performed multicolor IF stainings and the scanning of sections are given in the Supplementary Material and Methods online.

Images displayed in the figures were taken with Zeiss LD Plan-Neofluar objectives (primary objective ×20/0.4, ocular objective ×10) using a PC pixelfly camera (Carl Zeiss Inc, Jena, Germany) and exported from the StrataQuest software as TIFF images. Some images were processed in Adobe Photoshop CS6 (Adobe Systems, San Jose, CA).

Automated analysis of IF stainings, validation step

The computed analysis of skin sections was performed using StrataQuest V64 (TissueGnostics GmbH, Vienna, Austria). A detailed description of the created algorithms and analysis layers is given in the Supplementary Material and Methods and Supplementary Figure S2. Briefly, a first set of analysis layers was applied to automatically differentiate between epidermal, upper dermal, and hypodermal areas and detected regions manually validated. ILCs were gated based on the IF mean intensity of the respective channels; cutoffs were adapted to isotype controls.

A specific tool was developed to manually validate/reject all events (ILCs) on a single cell basis in the gates of interest regarding their staining as well as the cell shape. The validation step was performed by two independent observers.

Topographic analyses

Two additional analysis layers were created in the program to explore the topographic distribution of manually validated cells. In the first topography layer, the shortest distance of each ILC nucleus in the dermal area to (i) the epidermis and to (ii) other cells of interest was calculated (precision: 0.31 μm). The second topography layer calculated the distance between validated ILCs and the closest blood vessel. Blood vessel detection was based on their morphological shape (i.e., endothelial-shaped cells surrounding a lumen) and the mean intensity of green fluorescent protein expression (CD34 included in the lineage cocktail) followed by manual corrections.

Proximity analysis

This algorithm layer was created to more precisely assess the cellular environment of validated ILCs by screening the complete cellular content of the upper dermis with regard to its distance to every validated ILC. The percentage of T cells in a defined radius (3 μm and 9 μm) of validated ILCs was calculated.

Statistical analysis

All statistical analyses were performed using GraphPad Prism software (Graphpad, San Diego, CA). For comparison of means between more than two groups (NHS, AD, and PsO), one-way analysis of variance with the Tukey post-test was used for all experiments. A P value <0.05 was considered statistically significant.

CONFLICT OF INTEREST

The authors state no conflict of interest.

ACKNOWLEDGMENTS

We thank Robin Ristl from the Department of Statistics of the Medical University of Vienna for his statistical advice. This work was supported, in part, by the Austrian Science Fund (DK W1248-B13) and the Medical University of Vienna.

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at www.jidonline.org, and at http://dx.doi.org/10.1016/j.jid.2016.07.017.

REFERENCES

Czarnowicki T, Gonzalez J, Shemer A, Malajian D, Xu H, Zheng X, et al. Severe atopic dermatitis is characterized by selective expansion of circulating TH2/TC2 and TH22/TC22, but not TH17/TC17, cells within the


