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Multiparameter grouping delineates heterogeneous populations of human IL-17 and/or IL-22 T-cell producers that share antigen specificities with other T-cell subsets

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The ontogenic relationship between pro-inflammatory populations of interleukin-17 (IL-17A)- and/or IL-22-producing T cells and other T-cell subsets is currently unclear in humans. To appreciate T helper cell-lineage commitment, we combined cytokine production profiles of in vitro expanded T-cell clones with T-cell receptor (TCR) clonotypic signatures. Moreover, ex vivo cytokine production profiles at the single-cell level were analyzed using an original approach based on the hierarchical cluster analysis of multi-parametric flow cytometry data. These combined approaches enabled the delineation of distinct functional T-cell subsets, including Th1, Th2, Tr1, Th17 cells and a highly poly-functional IL-22-producing T-cell population. Cluster analysis highlighted that the IL-22-producing T-cell population should be considered independently from the Th17 and Th1 subsets, although it was more closely related to the former. In parallel, we observed extensive TCRαβ sharing across all five subsets defined. The strategy described here allows the objective definition of cellular subsets and an unbiased insight into their similarities. Together, our results underscore the ontogenic plasticity of CD4$^+$ T-cell progenitors, which can adopt a differentiation profile irrespective of antigen specificity.

Keywords: IL-17A · IL-22 · Polyfunctionality · TCR repertoire · Th17


Supporting Information available online

Introduction

Effector CD4$^+$ T cells were originally subdivided into two T helper (Th) types, Th1 and Th2, characterized by their stable production of interferon-γ (IFN-γ) and IL-4/IL-5 respectively [1, 2]. The Th1/Th2 paradigm has been enriched by the discovery of CD4$^+$ Tregs, involved in the maintenance of self-tolerance and subdivided in turn into naturally occurring (nTregs) [3] and inducible (iTregs) Tregs [4]. The former express the FoxP3 transcription factor and their fate is determined in the thymus,

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while inducible Tregs acquire their regulatory properties in the periphery. This rather heterogeneous population includes both FoxP3$^+$ Tregs and IL-10-producing type 1 Tregs (Tr1) [5].

More recently, a pro-inflammatory IL-17-producing (Th17) subset involved in anti-microbial immunity and autoimmune inflammation [6, 7] has been described [8], characterized by the expression of IL-17A, CCR6 [9], CD161 [10] and the RORC transcription factor [9, 11]. IL-22-secretion was initially described as a typical Th17 cell feature [12], although results from several studies have suggested that IL-22-secreting cells should be considered distinct from Th17 cells. Indeed, T cells with skin homing potential producing IL-22, but not IL-17, have been described in healthy subjects [13–15], as well as in patients with atopic dermatitis [16]. Therefore, it is possible that IL-22 production could delineate a distinct subset and not merely a particular differentiation stage of Th17 cells. Nonetheless, the in vivo stability of CD4$^+$ T-cell subsets is debated [17], and it remains unknown as yet whether protective or pro-inflammatory T cells originate from common or distinct precursors [18].

IL-22 is a member of the IL-10 cytokine family, originally described as having pro-inflammatory activities in the liver, pancreas, intestine and skin [19]. IL-22 is mainly expressed by activated T cells, mast cells and NK cells and acts through a heterodimeric receptor containing the IL-10R2 and IL-22R1 chains. In contrast to the IL-10R, the IL-22R is not expressed on hematopoietic cells. Instead, IL-22 acts mainly on epithelial cells of the digestive and respiratory tracts, as well as on epidermal keratinocytes where it is involved in the induction of β-defensins and epithelium homeostasis [20, 21]. High IL-22 expression in skin lesions and serum levels of patients with active psoriasis suggests deleterious effects of this cytokine on tissue inflammation [22, 23]. Indeed, recent biologic therapies for psoriatic patients include anti-IL-23 treatment, a cytokine directly involved in the expansion of IL-17- and IL-22-secreting CD4$^+$ T cells [24, 25]. In contrast, although IL-22 transcripts are also elevated in inflamed lesions of patients with Crohn's disease [26], studies using mouse models of ulcerative colitis show that IL-22, produced by CD4$^+$ T cells and a subset of NK cells, had a protective effect [27]. Altogether, it is at present uncertain whether IL-22 exerts predominantly regulatory or pro-inflammatory effects.

The present study was undertaken in an attempt to clarify the phenotypic and functional plasticity of putative inflammation-inducing human CD4$^+$ T-cell subsets. Our goal was also to investigate the potential ontogenic relationships between these subsets, and other T-cell subsets, including induced Tregs. Our results argue for the existence of a highly polyfunctional IL-22-producing T-cell population, distinct from IL-17 "only"-producing T cells. Despite the pronounced functional differences, we found extensive TCRαβ sharing across all the effector and regulatory subsets defined. Our data therefore underscore the fact that one T-cell precursor is able to adopt multiple Th-subset profiles irrespective of antigen specificity.

Results

IL-22$^+$ cells are more polyfunctional than IL-17 "only"-secreting cells

To explore phenotypic and functional differences between IL-17A$^+$IL-22$^+$, IL-17A$^+$IL-22$^-$ and IL-17A$^-$IL-22$^+$ CD4$^+$ T cells, co-expression of IFN-γ, TNF-α, IL-2, CD161 and CCR6 was analyzed on circulating CD4$^+$ T cells using multiparametric flow cytometry (Fig. 1A and Supporting Information Fig. S1A). Circulating cytokine-secreting cells were present at similar proportions and absolute numbers in psoriasis patients and in controls (Supporting Information Fig. S1B). Also, the three combinations of IL-17A- and IL-22-secreting CD4$^+$ T cells were present with similar frequencies and absolute numbers in controls and psoriasis patients, although IL-17A$^+$IL-22$^+$ CD4$^+$ T cells were moderately, albeit non-significantly, increased in the latter (Fig. 1B).

The killer cell lectin-like receptor CD161 was recently reported to be preferentially expressed on Th17 precursor cells as well as on gut [10] and skin [28] homing Th17 cells, but the CD161 status of ex vivo IL-22-secretors is not known. CD161 expression (Supporting Information Fig. S2A) was found to be more pronounced on IL-17A-secreting CD4$^+$ T cells, as compared with cells producing IL-22 ($p = 0.0086$ and $p = 0.0102$ in healthy controls and psoriasis patients respectively) (Supporting Information Fig. S2B). Of note, CD161 expression is retained on IL-17A$^+$IL-22$^+$ cells (Supporting Information Fig. S2C).

We then investigated the expression of CCR6 on cytokine-secreting CD4$^+$ T cells. CCR6 is preferentially expressed on Th17 cells [9]; however, CCR6 expression was not studied on the combinatorial subsets of IL-17A- and IL-22-secreting CD4$^+$ T cells. Since CCR6 is extensively downmodulated by T-cell stimulation, we purified CCR6$^+$ and CCR6$^-$ lymphocytes (Supporting Information Fig. S2D) before stimulation and intracellular cytokine detection. We observed that CCR6 is indeed more frequently expressed on IL-17A-secreting CD4$^+$ T cells as compared with both IL-22- and IFN-γ-secreting CD4$^+$ T cells (Supporting Information Fig. S2E). Of note, CCR6 is expressed at similar levels on IL-17A$^+$IL-22$^-$ cells and IL-17A$^+$IL-22$^+$ cells, although a trend toward a modest decline in CCR6 expression is observed on the latter (Supporting Information Fig. S2F). Moreover, CCR6 expression was not associated with CD161 expression, since CCR6 levels are similar on CD161$^+$ and CD161$^-$ IL-17A- and/or IL-22-secreting CD4$^+$ T cells (Supporting Information Fig. S2G).

IL-22$^+$ CD4$^+$ T cells from both controls and psoriasis patients co-secrete TNF-α and IL-2 in larger proportions than IL-17A$^+$IL-22$^-$ CD4$^+$ T cells, irrespective of their IL-17A status (Fig. 1C), thus demonstrating that co-secretion of the latter cytokines is associated with IL-22 rather than with IL-17A secretion. Of note, IFN-γ and IL-17A/IL-22 secretion are almost mutually exclusive.

We conclude that IL-22-secreting CD4$^+$ T cells are more polyfunctional than IL-17A$^+$IL-22$^-$ cells, and that CD161 and CCR6 expression is a preferential feature of IL-17A-secreting CD4$^+$ T cells irrespective of their IL-22 status.

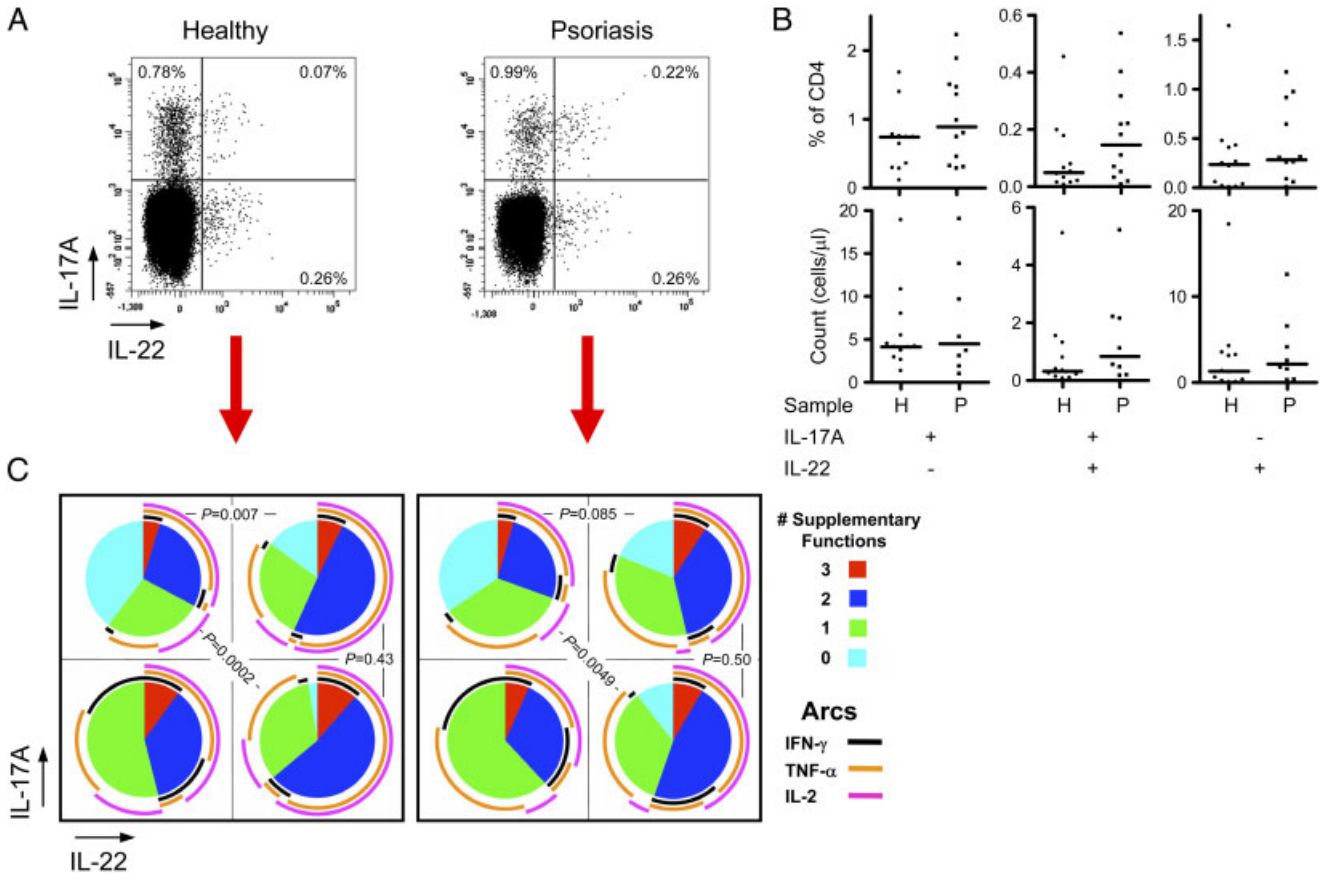

Figure 1. Characterization of IL-17A- and/or IL-22-secreting $CD4^+$ T cells in healthy controls and psoriasis patients. (A) Representative flow cytometric analysis of IL-17A and IL-22 intra-cellular expression by peripheral $CD4^+$ T cells following PMA/ionomycin-stimulation. (B) Frequencies (upper panel) and absolute cell count (bottom panel) of $IL\text{-}17A^+IL\text{-}22^-$, $IL\text{-}17A^+IL\text{-}22^+$ and $IL\text{-}17A^-IL\text{-}22^+$ cytokine-secreting $CD4^+$ T cells in healthy controls (H, $n = 12$) and psoriasis patients (P, $n = 12$). (C) Pie chart representation in quadrants corresponding to subsets defined in (A) gated on cytokine-secreting $CD4^+$ T cells. The pies represent the capacity of each IL-17A/IL-22 combinatorial subset to secrete none (0) or any (1, 2 or 3) of the three additional cytokines IFN-γ, TNF-α and IL-2, e.g. the red pie slice indicates the proportion of cells producing three cytokines (IFN-γ, TNF-α and IL-2), in addition to IL-17A and/or IL-22 as defined by the four quadrants. The arcs of the pies indicate which and how much of the three cytokines are involved in the polyfunctionality. Horizontal lines indicate median frequencies, and *p*-values were calculated using the pie statistic tool integrated in the Spice software and the Wilcoxon signed rank test.

Objective T-cell subset definition based on hierarchical cluster analysis

Unlike other Th subsets, the putative Th22 subset has as yet no unique transcription factor assigned to it and has been characterized only by its capacity to produce IL-22 in the absence of IL-17. Therefore, we applied multiparametric flow cytometry analysis to objectively determine whether this IL-22-secreting population represents an individualized subset.

We used fluorescence intensity values extracted from ex vivo flow cytometry data files (Figs. 1 and 2A). This enabled us to evaluate the IFN-γ, IL-17A and IL-22 cytokine secretion patterns of thousands of single-cell events and to order these patterns according to a distance tree obtained through hierarchical cluster analysis (Fig. 2B). In the dendrogram plot obtained, the largest distance change occurs between the third and fourth junctions, which corresponds to four major parallel branches leading to four individual clusters. Cells mainly secreting IFN-γ, IL-17A or IL-22 are grouped into three separate clusters, coined Th1, Th17 or Th22 respectively, whereas cells secreting none or only low levels of these three cytokines were grouped into a fourth cluster (Fig. 2C). Using three parameters, a maximum of eight (2^3) possible clusters could have been expected. The obtention of only four separate clusters is therefore likely due to the fact that only certain cytokine combinations exist frequently enough at the single-cell level to be detected as a T-cell cluster. Hierarchical cluster delineation results were validated using non-hierarchical cluster analysis (kappa inter-classification comparison agreement value $\kappa = 0.98$). We conclude that this type of analysis can be used to objectively delineate T-cell clusters sharing identical features.

Closely related, yet distinct Th17 and Th22 cell subsets

We then attempted to determine, using this approach, whether IL-22-secreting cells are more similar to the Th1 or Th17

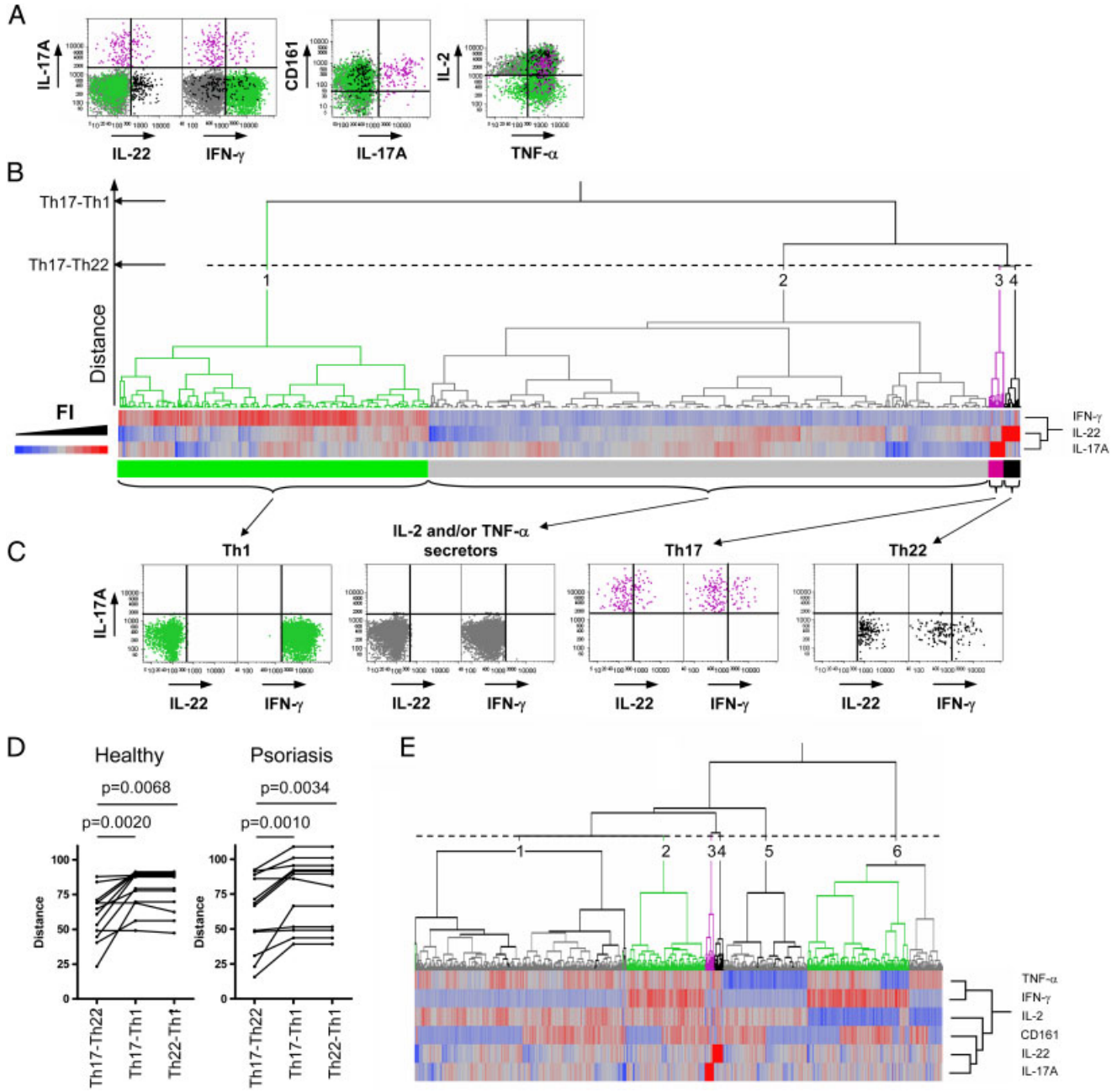

Figure 2. Closely related IL-17A- and IL-22-secreting subsets objectively defined in circulating blood using hierarchical cluster analysis. (A) Projection of cluster analysis results on originating raw flow cytometry analysis of peripheral $CD4^+$ T cells from a healthy individual, following PMA/ionomycin-stimulation. Cytokine-secreting cells are color-coded according to their cluster of origin, as defined just below. (B) Representative distance tree ordering individual $CD4^+$ T cells ($n = 10\,600$) into four main clusters (labeled 1–4 in the tree) according to three parameters: intracellular IFN-γ, IL-17A and IL-22 levels. Fluorescence intensities (FI) are indicated using a color scale (low and high cytokine levels are blue and red, respectively). Cluster 1 regroups IFN-γ main producers (labeled Th1 – green), cluster 3: IL-17A main producers (Th17 – magenta), cluster 4: IL-22 main producers (Th22 – black). Cells not significantly secreting any of these three cytokines are in cluster 2 (gray). (C) Cytokine secretion patterns of cells belonging to the clusters defined above. (D) Th17-Th22, Th17-Th1 and Th22-Th1 distances were measured as shown in (B) in healthy donors ($n = 12$) and psoriasis patients ($n = 12$). *p*-Values were calculated using the Wilcoxon signed rank test. (E) Representative distance tree ordering the same $CD4^+$ T-cell sample as above into six main clusters according to six parameters (IFN-γ, IL-17A, IL-22, IL-2, TNF-α and cell surface CD161). The color code for clusters was determined above using three parameters. The dotted line in the tree indicates at which branching level optimal clusters were defined.

subset. As shown in Fig. 2B, the branching point at which IFN-γ-secreting cells are parted from IL-17A- and/or IL-22-secreting cells is more distant from the extremity of the tree, as compared with the branching at which the latter are split into two subsets. As the magnitude of the distance for a given branch point separating two given clusters is directly correlated

with their degree of phenotypical differences, Th22 cells appear more closely related to Th17 than to Th1 cells, in PBMCs from the healthy individual taken as an example (Fig. 2B).

To confirm this observation, cluster analysis was repeated using PBMCs from a series of healthy ($n = 12$) and psoriasis ($n = 12$) individuals. The results from this analysis confirmed that, in both groups, the distance of the branching point segregating the Th17 and Th22 subsets is significantly shorter than the distance segregating Th1 and any of the latter two subsets (Fig. 2D). Additional parameters (IL-2, TNF-α and CD161) were introduced in order to test their influence on the analysis. As shown in Fig. 2E, the global clustering pattern was conserved when six parameters were used, except for Th1 cells, which were grouped into two distinct clusters according to their capacity to secrete IL-2 or not. Altogether, six major clusters were defined using six parameters. This result further confirms the restricted number of dominant T-cell subsets sharing identical features, since here sixty-four (2^6) different clusters could theoretically have been delineated. According to this analysis, IFN-γ^+IL-2^+ cells would phenotypically be more related to IL-17A- and IL-22-secreting cells, than IFN-γ^+IL-2^- producers. Of note, the IL-17A and IL-22 parameters were found to cluster together and, importantly, away from IFN-γ. The same pattern was repeatedly observed in 20 out of 24 individuals analyzed (data not shown). Thus, Th17 and Th22 subsets are distinguishable and defined as separate entities, even when a more complex analysis is performed.

T-cell clones with distinct cytokine patterns

As shown above, IL-17A- and IL-22-secreting cells are relatively scarce in periphery, even in psoriasis patients (Fig. 1 and Supporting Information Fig. S1). To determine whether these cells are more abundant in inflamed tissue lesions, infiltrating T cells were expanded in vitro from both healthy skin and psoriasis lesions of the same patients ($n = 3$) and their cytokine production profiles analyzed by multiparametric flow cytometry (Supporting Information Fig. S3A). The proportion of IFN-γ-secreting Th1 cells did not differ between T cells originating from healthy skin or psoriatic skin lesions. In contrast, IL-17A- and IL-22-secreting cells were more abundantly derived from lesional skin (Supporting Information Fig. S3B). This observation led us to use such lesions as a source of T cells to generate $CD4^+$ T-cell clones with various Th profiles, including Th17 and Th22 cells. Hierarchical cluster analysis performed on the cytokine pattern of skin-infiltrating T-cell clones obtained from two psoriasis patients yielded distance trees that highlighted their organization into five dominant groups, each characterized by a typical cytokine secretion profile (Fig. 3A and Supporting Information Fig. S4A). The number of clusters obtained was validated using the non-hierarchical cluster analysis (data not shown) with an excellent inter-classification comparison index (kappa agreement value $\kappa = 0.89$ and 0.70 respectively). The inter-cluster differences were confirmed through the computation of the mean relative cytokine productions in each proposed cluster, followed by inter-cluster comparisons (Fig. 3B and Supporting Information Fig. S4B). This analysis confirmed that IFN-γ was most increased in the first cluster, as compared with other clusters ($p < 0.0001$ for both patients), IL-10 in the second cluster ($p < 0.0001$), IL-4 ($p = 0.001$ and $p = 0.0065$, 1st and 2nd patient respectively) and IL-5 ($p < 0.0001$) in the third, IL-17 in the fourth ($p < 0.0001$) and IL-22 in the fifth ($p < 0.0001$) (Fig. 3B and Supporting Information Fig. S4B). The clusters were therefore named Th1, Tr1, Th2, Th17 and Th22 respectively.

Altogether, these data suggest that Th1, Th2, Tr1, Th17 and Th22 orientation can be objectively distinguished by cluster analysis of cytokine production profiles. The Th22 subset should therefore clearly be distinguished from the previously recognized Th17 subset.

Extensive TCRαβ sharing between functionally distinct $CD4^+$ T-cell subsets

We then used TCRα and TCRβ clonotypic analysis to assess whether the commitment of these functionally distinct subsets of $CD4^+$ T cells would be antigen-driven or TCR-independent. Surprisingly, only 45 different clonotypes were used by the 66 T-cell clones derived from the skin biopsy of a psoriasis patient. Eight different clonotypes were extensively shared between subsets and represented 39% of the T-cell infiltrate (Fig. 4). One clone was shared by four different subsets. TCR sharing between the Th17 and Th22 subset, with only one clone shared, was not more extensive than that between other subsets. TCR sharing between functionally distinct T-cell clones was confirmed in a skin biopsy from a second psoriasis patient. In this case, TCR sharing was less extensive, but clones overlapping between Th17 and Th22 as well as Th17 and Th2 were nonetheless identified among the 59 skin-derived T-cell clones analyzed (Supporting Information Fig. S4C). These results demonstrate that none of the five Th cell types use a strictly dedicated TCR repertoire.

Discussion

The original definition of Th lymphocyte subsets [1] was based on the analysis of mouse T-cell clones retaining stable cytokine patterns under various stimulation regimens and over extended time periods, whereas subsequent Th definitions have frequently been coined using bulk populations that have not always been corroborated at the single-cell level [29]. In the present study, we combined multiparametric analysis of cytokine production profiles and TCR clonotypic signatures to study the functional diversity of circulating T cells and skin-infiltrating effector T cells at the clonal level. In doing so, we found that T cells bearing canonical Th17 signatures, such as IL-17A, IL-22, CCR6 and CD161 expression, can in fact be assigned to phenotypically and functionally heterogeneous subsets.

Through direct ex vivo analysis of circulating T cells from healthy controls we confirmed that the cell surface marker

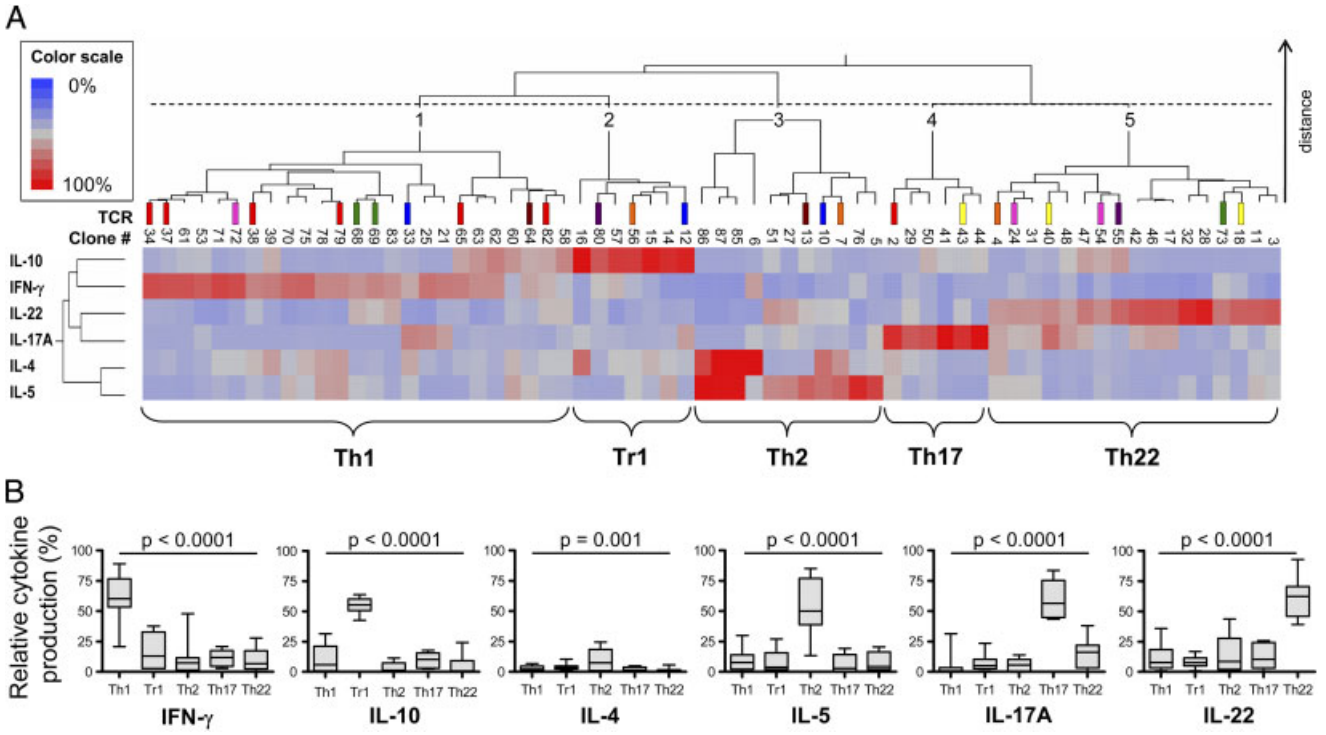

Figure 3. Cytokine secretion profile of skin-infiltrating T cells in a psoriasis patient. (A) Sixty-six T-cell clones were obtained by limiting dilution culture of skin-infiltrating T cells. Indicated cytokines were measured in individual culture supernatants. The color scale representation reflects the molar proportion contributed by a given cytokine among all other measured cytokines. Hierarchical cluster analysis was performed as in Fig. 2 Clonotypic signatures were obtained through TCRα and TCRβ sequencing. Shared TCRαβ clonotypes are color-coded and indicated. This representation highlights that, for instance, clones ♯10, ♯12 and ♯33 are assigned to distinct subgroups according to their cytokine profile, but nevertheless derive from the same precursor (identical "blue" TCRαβ clonotype). Also, identical "yellow" clonotype is expressed by IL-17A$^-$IL-22$^+$ IL-17A$^+$IL-22$^-$ and IL-17A$^+$IL-22$^+$ cells. (B) Comparison of relative cytokine production among each identified subset of T-cell clones. Horizontal lines within boxes indicate median values, lower and upper box sides respectively indicate lower (25th) and upper quartiles (75th) and whiskers indicate lowest and highest values. The dotted line in the tree indicates at which branching level optimal clusters were defined. *p*-values are calculated with a Kruskal-Wallis test. See Supporting Information Fig. 4 for an independent experiment on a second patient.

CD161, which was recently shown to be expressed on Th17 precursor cells, is also expressed on a significant proportion of mature IL-17A-producing CD4$^+$ T cells [10]. Ramirez et al. studied CD161 expression on in vitro generated IL-17- and IL-22-secreting CD4$^+$ T cells and observed expression confined to IL-17-secreting CD4$^+$ T cells [30]. In line with this in vitro study, we observed that ex vivo CD161 expression is significantly higher on IL-17A-secreting CD4$^+$ T cells, either co-expressing IL-22 or not, as compared with both IL-17A$^-$IL-22$^+$ and IL-17A$^-$IL-22$^-$ CD4$^+$ T cells. CD161 expression is therefore more strongly associated with IL-17A-secretion than with IL-22-secretion. CCR6 expression is another typical feature of the Th17 subset [9]. We therefore investigated CCR6 expression on IL-17A-secreting CD4$^+$ T cells in relation with IL-22 expression. We found that CCR6 was expressed on IL-17A-secreting CD4$^+$ T cells independently of IL-22 co-secretion. Moreover, the observation that CCR6 and CD161 surface expression on IL-17A-secreting CD4$^+$ T cells are not associated indicates that the two homing receptors can act independently and possibly target different tissues or organs.

We furthermore observed that IL-22-secreting CD4$^+$ T cells secrete IL-2 and TNF-α more frequently than IL-17A$^+$IL-22$^-$ CD4$^+$ T cells, thus demonstrating that a high degree of polyfunctionality is a feature associated with IL-22-, but not with IL-17A-secretion. Finally, we observed that IFN-γ and IL-17A/IL-22 secretion are virtually mutually exclusive at the single-cell level. This most likely reflects the fact that, like in mice [31], IFN-γ is also a negative regulator of IL-17A-secretion in humans. Volpe et al. previously showed a strong correlation between IL-22 and IFN-γ production in supernatants from in vitro differentiated polyclonal T-cell cultures [32]. However, while certain polarizing conditions can indeed drive bulk CD4$^+$ populations to produce both IL-22 and IFN-γ, it is unclear whether both cytokines are produced by the same cell. In summary, we conclude from our results that IL-17A$^-$IL-22$^+$ cells show elevated polyfunctionality, IL-17A$^+$IL-22$^-$ cells express CCR6 and CD161, and IL-17$^+$ IL-22$^+$ cells share both features.

We found that circulating cytokine-secreting T cells are phenotypically and functionally similar in psoriasis patients and healthy controls. Only TNF-α-secretion by IL-22-producing T cells was diminished in psoriasis patients, as compared with those of healthy controls. As expected, psoriasis skin lesions appear enriched in IL-17A- and IL-22-secreting CD4$^+$ T cells [33]. We therefore used these lesions as a source for T-cell clones of various Th cell profiles, expecting a significant proportion of IL-17A and/or IL-22-producing T cells that are otherwise found at very low frequencies in peripheral blood. We postulated that

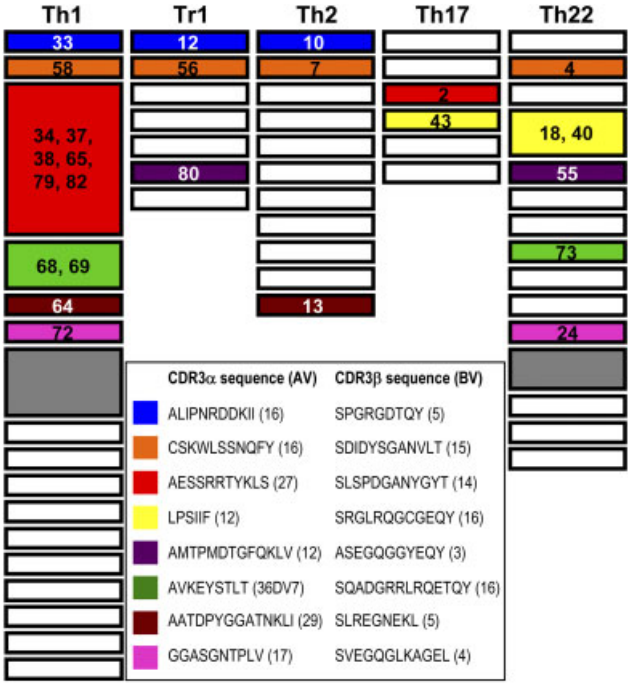

Figure 4. Extensive TCR sharing between functionally distinct skin-infiltrating T-cell subsets. TCRα and TCRβ CDR3 sequences along with AV (TCRα variable region) and BV (TCRβ variable region) usage corresponding to T-cell clone clusters defined in Fig. 3. Clonotypes shared between subsets are color coded. White boxes harbor clonotypes not shared with other subsets. Gray sequences are repeated but only within the same subset. Box size is proportional to the number of identical sequences. Reference numbers for shared T-cell clones are indicated inside the boxes. CDR3α and CDR3β sequences for clonotypes shared between subsets are indicated with their corresponding color code.

in vitro expanded clones were likely to reflect the functional and phenotypic diversity of T cells infiltrating the lesion. It is of note that the culture conditions used in the present study support a functionally stable clonal growth over time [34] and does not favor the outgrowth of a particular Th lymphocyte population, as shown by the wide diversity of cytokine secretion profiles obtained. Therefore, although these data are in part derived from the study of in vitro-expanded cells, they are nevertheless likely to reflect functional sub-divisions existing in the un-manipulated T-cell infiltrate.

Hierarchical cluster analysis was used here for the first time for the objective delineation of distinct phenotypes of $CD4^+$ T cells at the single-cell level. Cluster analysis refers to a family of multivariate techniques designed to delineate subgroups sharing similar characteristics within a studied population. This approach was previously used to analyze correlations between cytokines produced in bulk T-cell cultures under various conditions [35], but was not applied to subset definition, nor to ex vivo single-cell analysis. We used canonical cytokine signatures, IFN-γ, IL-4, IL-5, IL-10, IL-17A and IL-22 in order to segregate T-cell clones in Th1, Th2, Tr1, Th17 and Th22 cells respectively. Ubiquitously produced cytokines were not included in the analysis. In particular, TNF-α was not selected, as production of this cytokine is not restricted to the Th1 subset [14]. The cytokines used for cluster analysis were selected on the basis of their recognized contribution to characterize both previously defined and potential $CD4^+$ T-cell subset profiles. In the future, other parameters may be introduced in order to possibly identify other functionally meaningful subsets. To increase the power of the analysis, it is also possible to rely on fluorescence intensity values extracted from ex vivo flow cytometry data files (Fig. 2). The latter approach is, we believe, an important way to make inroads into analysis of complex cellular populations. Indeed, this strategy allows the objective definition of cellular subsets and unbiased insight into their similarities since an unlimited number of single cells can be processed, with minimal cellular manipulations.

The analysis of ex vivo stimulated T cells and in vitro cultured T-cell clones enabled us to distinguish objectively between classic Th1, Th2 and Tr1 subsets and to underscore the existence of a distinct IL-22-producing T-cell subset with a high degree of polyfunctionality, as compared with IL-17A-producing cells. Thus, pro-inflammatory T cells cannot be considered as a single entity represented by IL-17 and IL-22 co-producing T cells. According to the clustering algorithm used here, IL-22-secreting T cells were nevertheless found more closely related to IL-17A-secreting T cells than to the other subsets. However, TCR sharing was not more extensive between IL-17A- and IL-22-secreting $CD4^+$ T cells than between the other subsets studied here, as each defined subset was found to share TCR clonotypes with several other subsets. Similar conclusions have been drawn from the analysis of the $CD8^+$ T-cell compartment. Following the transfer of single antigen-specific naïve $CD8^+$ T cells in recipient mice, it was shown that different types of effector cells, as well as long-living memory T cells, each with a wide range of diversity, could develop out of a single naïve precursor cell [36]. More recent fate-mapping studies show that mouse Th17 cells are intrinsically unstable, and can transform into Th1 and Th22 type cells in vitro [37] and in vivo [38, 39]. Our study supports the notion that reprogramming of established Th-type cells may occur in a clinical setting. Additional longitudinal studies on unmanipulated samples are required in order to determine whether Th-type programming of the same clonal lineage corresponds to early or late events. Interestingly, we here observed that the extent of TCR overlap varied between two individuals analyzed. Again, longitudinal studies might help to understand whether these differences are related to lesional evolution.

Altogether, these data indicate that naive precursor T cells can adopt a differentiation profile irrespective of antigen specificity. These results also support the existence of a distinct IL-22-producing T-cell subset distinguishable from Th17 cells by low CD161 expression and a high degree of polyfunctionality. It is presently unclear whether the latter phenotype corresponds to a higher degree of differentiation, as well as whether the distinctions between IL-17- and IL-22-producing T cells are stable over time. Such putative transitions should be monitored longitudinally at the single-cell level, in order to prove that a given highly differentiated T-cell can modify its programme, resulting in the expression of a totally different sets of cytokines.

Materials and methods

Polyfunctional analysis of human samples

Psoriasis vulgaris patients ($n = 12$) receiving no or only moderate immunosuppressive treatments were age- and sex-matched with healthy controls ($n = 12$) (Table 1). Skin and blood samples were obtained following acquisition of patients' informed consent. The study protocol was reviewed and approved by the local ethics committees of Pitié-Salpêtrière Hospital, Paris and C.H.U. de Montpellier.

Peripheral blood mononuclear cells (PBMCs) were purified by centrifugation over ficoll (PAA, France) and incubated in RPMI-1640 medium, supplemented with 1 μg/mL PMA and 1 μg/mL ionomycin (Sigma-Aldrich, France) at 37°C. After 1 h of stimulation, cytokine secretion was blocked following the addition of 2.5 μg/mL monensin and 5 μg/mL brefeldin A (Sigma-Aldrich). After 16 h of culture, cells were collected, washed and incubated with directly conjugated anti-CD3-Cascade Yellow (DAKOCytomation, Glostrup, Denmark), anti-CD4-APC/Cy7, anti-CD161-PECy5 (BD Biosciences, San Jose, CA, USA) and anti-CD8-Alexa405 (Caltag, Burlingame, CA, USA). Cells were washed and permeabilized with Cytofix/Cytoperm™ (BD Biosciences) and incubated with pre-titrated anti-IL-2-FITC, anti-TNF-α-PECy7, anti-IFN-γ-Alexa700, (BD Biosciences), anti-IL-17A-PE (Clone 64CAP17) and anti-IL-22-Alexa647 (Clone 22URTI), (eBiosciences, San Diego, CA, USA) for 20 min at room temperature. Finally, 10^6 cell events were analyzed on a BD LSRII apparatus using FACSDiva (BD Biosciences) and FlowJo (Tree-Star) softwares. Unstimulated cells for each sample, treated under the same experimental conditions served as negative controls, and background values were subtracted from the analysis of the stimulated samples. Polyfunctional statistical analysis was performed using Pestle Ver. 1.6.2 and Spice Ver. 4.2.3 software (Mario Roederer, ImmunoTechnology Section, VRC/NIAID/NIH) [40].

Table 1. Clinical information for patient cohort and healthy controls

	Psoriasis patients ($n = 12$)	Healthy controls ($n = 12$)
Male sex (%)	9/12 (75%)	8/12 (67%)
Median inclusion age, years (±SD)	51.6 (15)	50.8 (19)
Median time since diagnosis, years (±SD)	17 (15)	N/A
Disease severity	3 benign 4 moderate 5 severe	
Median inflamed skin coverage (%) (±SD)	27% (23)	N/A
Systemic immunosuppresive treatment*	1/12	N/A
Anti-TNF-α therapy	None	N/A

SD, standard deviation; N/A, not applicable. *Cyclosporine 75 mg twice a day.

$CD4^+$ T-cell clones

Punch skin biopsies were cultivated in 1 mL of Yssel's culture medium [41] supplemented with 1% human AB^+ serum and 10 ng/mL rIL-2 (R&D Systems, Abingdon, UK) in the presence of anti-CD3 and anti-CD28-coated beads (Dynal Biotech). After 10–14 days, T-cells were cloned by limiting dilution and cultured in the presence of rIL-2 (10 ng/mL), irradiated (45 Gy) allogeneic PBMCs, irradiated (60 Gy) EBV-LCL JY and 2 μg/mL PHA (Murex, Beckenham, UK), as described [42]. After another 10–14 days, T-cell clones were stimulated with anti-human CD3 and CD28 monoclonal antibodies for 48 h. Culture supernatants and cell pellets were collected for ELISA analysis of cytokine secretion and TCRα and TCRβ variable region sequencing.

Cytokine ELISA of cell culture supernatants

Levels of IL-4, IL-5, IL-10, IL-17A, IL-22 and IFN-γ in cell culture supernatants were determined by cytokine-specific ELISA, as previously described [43]. None of the six cytokines monitored were detected in cell culture supernatants from non-stimulated T-cell clones.

TCR clonotypic analysis

Total RNA was extracted using RNAeasy Mini Kit (Qiagen), according to the manufacturer's recommendations. Complementary DNA (cDNA) was synthesized using reverse-transcription (RT) core kit (Eurogentec, Seraing, Belgium) with random hexamer primers. Amplification reactions were performed using an α or β common-region (AC or BC) specific primer and a TCRα or TCRβ variable-region (AV or BV) specific primer as previously described [44, 45]. In brief, 1 μL of RT product was brought to a final reaction volume of 30 μL containing 15 mM Tris-HCl, 1.5 mM $MgCl_2$, 50 mM KCl, pH 8.0, 20 pM of each dNTP, 1.5 U blocked Taq DNA polymerase (AmpliTaq Gold, Applied Biosystems, Foster City, CA, USA), 0.4 mM of AC- or BC-primer and 0.4 mM of AV- or BV-specific primer. After an initial denaturation step of 10 min at 94°C, the reactions were subjected to 40 cycles of PCR (94°C for 30 s, 58°C for 40 s, 72°C for 50 s), followed by a final extension step of 5 min at 72°C. Runoff products were purified using Sephadex gel and filter plates (Multiscreen, Millipore, Billerica, MA, USA) before they were sequenced using fluorescent chain-terminating inhibitors (BigDye Terminator v1.1 kit) and an automated capillary sequencer (ABI Prism 3700 DNA Analyzer, Applied Biosystems). CDR3α and CDR3β definitions as well as AV and BV nomenclature are according to IMGT (http://imgt.cines.fr).

Hierarchical cluster analysis

Cytokines were selected for cluster analysis on the basis of their recognized contribution to characterize both known and potential $CD4^+$ T-cell subsets. Cytokine secretion levels of PMA and calcium ionophore-activated $CD4^+$ T cells were determined ex vivo at the single-cell level using a BD LSRII apparatus. Fluorescence intensity values were directly extracted from the corresponding Flow Cytometry Standard (FCS) files using Flow Cytometry Standard Extract Utility (Earl F. Glynn, Stowers Institute for Medical Research, KS, USA) and analyzed using Ward's method (see below). T-cell clone clustering was based on cytokine ELISA measurements in culture supernatants. In that case, the molar concentration of each cytokine measured was expressed as the percentage of the six measured cytokines produced by a given T-cell clone and normalized in order to express results independently of their measurement scale.

Agglomerative hierarchical cluster analysis according to Ward's [46] was performed using the JMP7 software (SAS Software, NC, USA). The optimal number of clusters was identified according to the largest distance change between successive junctions of the dendrogram plot. Validity and reproducibility of the classification obtained with hierarchical cluster analysis was assessed using non-hierarchical k-means cluster analysis, in which the optimal number of clusters identified through hierarchical cluster analysis was pre-specified. Reproducibility of the classifications obtained with both hierarchical and non-hierarchical clustering was assessed by determination of the kappa value.

Statistical analysis

Differences between groups and clusters were tested using Mann-Whitney *U*-test (unpaired), Wilcoxon signed rank test (paired) and Kruskal-Wallis test. All tests were two-sided and a p-value <0.05 was considered statistically significant.

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