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4 T-cell phenotyping uncovers systemic features of atopic dermatitis and
5 psoriasis.

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53	
54	Capsule summary:
55	Deep-immunophenotyping of the circulatory T cell compartment using mass cytometry and
56	unsupervised clustering analysis, identifies phenotypic and functional differences in patients with
57	atopic dermatitis (AD) and psoriasis, highlighting the stronger systemic component associated
58	with AD.

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Key Words: mass cytometry, atopic dermatitis, psoriasis, unsupervised analysis, skin
inflammation.

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#### 63 Abbreviations:

- 64 ANOVA: Analysis of variance
- 65 AD: Atopic dermatitis
- 66 HD: Healthy donors
- 67 F: Functional
- 68 P: Phenotypic
- 69 DMSO: dimethyl sulfoxide
- 70 FOXP3: forkhead box P3
- 71 CCR: C C chemokine receptor 3

- 72 TNF: tumor necrosis factor
- 73 IFN: interferon
- 74 GM-CSF: granulocyte-macrophage colony-stimulating factor
- 75 MIP: macrophage inflammatory protein
- 76 MR-1: MHC-related protein 1
- 77 Th: T helper
- 78 CYTOF: Cytometry by time of flight
- 79 MAIT: mucosal-associated invariant T
- 80 tSNE: t-Distributed Stochastic Neighbor Embedding
- 81 PSO: psoriasis
- 82 RA: rheumatoid arthritis
- 83 T<sub>RM</sub>: Tissue resident memory T
- 84 T<sub>CM:</sub> central memory T
- 85 T<sub>EM:</sub> effector memory T
- 86  $T_{RcM:}$  recirculating memory T
- 87 T<sub>Reg:</sub> Regulatory T
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#### 92 **To the Editor**

Atopic dermatitis (AD) and psoriasis are T cell-mediated, chronic inflammatory skin conditions, 94 which are increasingly recognized as systemic rather than localized cutaneous diseases (1, 2). 95 Hence, a deeper understanding of the phenotypic and functional features of the circulatory T-cell 96 compartment may offer novel insights into their pathogenesis, generate opportunities for novel 97 targeted therapies, as well as, aid in the identification of potential biomarkers. We and others have 98 previously performed fluorescence-based immunophenotyping of circulatory T cells in psoriasis 99 and AD (1), and an earlier comparative study highlighted distinctive features of systemic activation 100 101 in T cells of AD patients (3). Here, we exploit the increased dimensionality of mass cytometry (cytometry by time of flight, CyTOF), which is able to detect over 50 different heavy metal 102 isotopes simultaneously, and the power of unsupervised analytical methods, to profile and compare 103 104 phenotypic and functional features of circulating CD4+ and CD8+ T cells of AD (n=15), psoriasis (n=19), and healthy donors (HD, n=9). 105

PBMCs were obtained from a total of 43 age-matched subjects (Fig. E1A), recruited into an observational clinical study approved by the London Bridge Research Ethics committee. Demographics and clinical characteristics of the subjects are shown in Table E1. A description of the methods is provided in the Methods section in this article's Online Repository at www.jacionline.org.

We ran a Phenotypic (P) panel, including 42 lineage, differentiation, activation, trafficking and homing markers, and a Functional (F) panel, including 27 phenotypic surface markers and 15 intracellular functional markers (e.g. cytokines, chemokines) (**Table E2**). Frequency of manually gated CD4+ and CD8+ T cells, as well as other major cell populations (**Fig. E1B**), did not significantly differ among AD, psoriasis and HD (**Fig. E1C**). Appling unsupervised clustering analysis to gated CD4+ and CD8+, we identified 144 cell clusters: 34 cell clusters in the CD4+ P 5

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and 31 in the CD8+ P dataset, as well as 51 in the CD4+ F and 29 in the CD8+ F dataset (**Fig. E1A, Fig. E2A**). Each dataset was visualized by applying a dimensionality reduction method, i.e. t-Distributed Stochastic Neighbor Embedding (tSNE) and coloring the cells according to the clusters they were assigned to. Clusters were curated and assigned to standard immune subsets, based on the mean expression of subset-defining markers, such as CD161 and V $\alpha$ 7.2 for mucosalassociated invariant T (MAIT) cells and CD103 for Recirculating memory T cells (T<sub>RcM</sub>) (**Fig. E2A, Fig. E3-6**).

We compared the average clusters' frequency within each of the four datasets and the means for each cytokine/chemokine in two-way comparisons applying False Discovery Rate (FDR) correction for multiple testing. Overall, we detected statistically significant differences (p-adj < 0.05) in 68 two-way comparisons (**Table E3**). Three cell populations were of particular interest: MAIT cells, T<sub>RcM</sub> CD8+ T cells, and CD49+ CD4+ Regulatory T (T<sub>Reg</sub>) cells.

Clusters CD8+P13 and CD8+ F14 displayed the hallmark features of MAIT cells, with high levels 129 of V $\alpha$ 7.2 and CD161 (Fig. 1A). Frequencies of MAITs were significantly reduced in psoriasis 130 patients versus HD and AD (Fig. 1B). This is in keeping with the reported reduction in circulating 131 MAITs in immune-mediated and autoimmune diseases such as RA, where they are 132 correspondingly enriched in the synovial fluid, possibly contributing to disease pathogenesis either 133 directly, or through recruitment of other cells (4). Thus, MAIT cells might preferentially migrate 134 from blood into the inflamed skin, and they have been indeed identified in lesional psoriatic skin 135 (5), although it is not known whether they are increased in inflamed *versus* healthy skin. 136

The vast majority of MAIT cells produced MIP1- $\beta$  and TNF (**Fig. 1C, E7A**), with AD producing significantly more than psoriasis (**Fig. 1D, E7B**), suggesting that they are more activated in allergic disease. As MAIT cells respond to MR-1-restricted vitamin B2 derivatives, and *Staphylococcus aureus* is equipped with the specific biosynthetic pathway for their production, their increased activation in AD might be linked to *S. aureus* infection.

Three CD8+ cell clusters identified in both Phenotypic and Functional dataset, i.e. i) CD8+ 142 P24/CD8+ F28, *ii*) CD8+ P15/CD8+ F18, and *iii*) CD8+ P27/CD8+ F29, were characterized by 143 the simultaneous expression of low levels of CCR7, and either skin-(CLA+, CCR4+, CCR10+) or 144 gut- (Intb7, CD49d as well as CCR9) homing markers (Fig. 1E). Features of these clusters broadly 145 map to effector memory T cells; both skin and gut-homing clusters expressed CD103, suggesting 146 147 that they previously resided in the tissue, as recently reported for CD4+ CD103+ cells by *Klicznik* et al (6). They may be en-route back from their target organ where they had acquired CD103 148 149 expression through exposure to TGF- $\beta$  of epithelial origin (6). Hence, we tentatively named them T<sub>RcM</sub>. T<sub>RcM</sub> were functionally characterized by widespread production of TNF and sizable 150 expression of IL-2, GM-CSF and IL-22 (Fig. 1F and E7C). Frequencies of all T<sub>RcM</sub> clusters were 151 significantly increased in AD as compared to HD and psoriasis (Fig. 1G and E7D), confirming 152 and expanding recent studies showing increased recirculation, frequency and activation of skin-153 154 homing T cells in AD (7). This is in keeping with the sequelae of non-cutaneous atopic manifestations known as atopic march. Of particular interest is the increase in gut-homing T<sub>RcM</sub>, 155 as AD often precedes the development of food allergy. 156

Skin- homing  $T_{RcM}$  (F28) were the major IL-22 producer within CD8+T cells (Fig. 1H), with AD patients producing significantly more IL-22 than HD (Fig. 1I). Interestingly, IL22 production in Skin- homing  $T_{RcM}$ , correlated with AD severity (Fig. 1J), highlighting the importance of IL-22 in the pathophysiology of AD, and in line with the beneficial effect of IL-22 blockade in AD clinical trials (8).

Lastly, three clusters within the CD4+ Phenotypic dataset displayed hallmarks of  $T_{Reg}$  cells: CD45RA+CD45RO- FOXP3+ CD25+ CD127- naïve  $T_{Reg}$  (P10), CD45RA-, CD45RO+, CD95+, FOXP3+ CD25+ CD127- CLA+ skin-homing memory  $T_{Regs}$  (P14), and CD45RA-, CD45RO+,

165 CD95+, FOXP3+ CD25+ CD127- CD49d+ memory  $T_{Reg}$  (P22) (**Fig. 2A**).

Frequencies of P22 memory T<sub>Reg</sub> were significantly reduced in AD versus psoriasis, with a 166 downward trend versus HD (Fig. 2B). Earlier studies have reported increased frequency of T<sub>Reg</sub> in 167 the blood of AD patients (9), although less phenotypic markers have been used, raising the question 168 of whether they were bona fide T<sub>Regs</sub> or a mixed population comprising activated-effector T cells 169 and T<sub>Reg.</sub> Discrepancies among studies could also be explained by their dynamic fluctuation 170 171 between the blood and skin compartment potentially driving disease relapses and remissions. This notion is supported by a previous report of Treg frequecny correlating with disease severity (3) 172 although we did not find this relationship in our data (data not shown). To validate our findings, 173 174 we combined clusters P10, P14 and P22 and performed a zoomed-in tSNE analysis (Fig. E7E). While CD45RA, CCR4 and CLA clearly separated  $T_{Regs}$  into three clusters (Fig. E7E), the 175 presence of additional cell subsets within cluster P14 was also evident, with dichotomous 176 expression of CLA and CD49d in P22 and P14(Fig. E7F). Thus, we applied manual gating to 177 further phenotype memory T<sub>Reg</sub> based on their expression of CLA, CD49d and CCR6. (Fig. 2C). 178 AD patients displayed an overall reduced frequency of non-skin homing CD49d+ T<sub>Reg</sub>, irrespective 179 of CCR6 expression, as compared to psoriasis, and with a downward trend versus HD, mirroring 180 the observed decrease in cluster P22. Moreover, CCR6+T<sub>Reg</sub> within skin-homing CLA+ cells were 181 also significantly reduced in AD versus HD, while CCR6-T<sub>Reg</sub> within skin-homing CLA+ cells 182 were significantly increased in psoriasis versus AD and HD (Fig. 2C). In contrast, non-T<sub>Reg</sub> 183 memory CLA+CCR6+ cells were significantly increased in AD as compared to HD and psoriasis. 184 Here, we have provided an exhaustive description of the circulatory T cell compartment, 185 186 highlighting phenotypic and functional differences in patients with AD and psoriasis. 187 Unsupervised analysis has overcome the bias of subjective gating by increasing resolution and capturing unexpected combinations of markers, e.g. identifying cell types that escape standard 188 classification strategies, e.g. T<sub>RcM</sub> expressing CD103. 189

190 Our data highlight the stronger systemic component associated with AD, as compared to psoriasis, and provide a resource to further investigate the phenotypic and functional features of the two 191 major inflammatory skin diseases. Future studies are needed to delineate the relationship of the 192 specific cell populations in blood and skin, explore their pathogenic potential, and ultimately assess 193 the utility of these findings for the identification of prognostic and/or predictive biomarkers, as 194 well as potential novel therapeutic targets. Our study proposes MAIT cells as of potential 195 pathogenic relevance for skin inflammation and calls for further studies aimed at assessing their 196 functional capacity in blood and skin of patients with inflammatory skin diseases. IL-22-197 producing skin-homing T<sub>RcM</sub> may be an attractive candidate for future studies to identify 198 biomarkers of response to IL-22 blockade in AD. Finally, this study highlights the utility of blood 199 and the use of profiling T cells by trafficking receptor expression patterns for discovery in the 200 context of tissue-specific immunological diseases. 201

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#### 277 Figure Legends

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# Figure 1: Mucosal-invariant T-cells (MAIT) and Recirculating memory CD8<sup>+</sup> T cells in skin disease

(A) Heatmap of mucosal-invariant T (MAIT) cells, within CD8+ Phenotypic and Functional

datasets. (**B**) Frequency of MAIT cells in atopic dermatitis (AD), healthy donor (HD) and psoriasis (PSO). (**C**) MIP-1 $\beta$ , TNF- $\alpha$ , IFN- $\gamma$ , GM-CSF, IL-2 and IL-17A production in MAIT cells, visualized in tSNE. (**D**) Cytokine production in MAIT cells, in AD, HD and PSO. (**E**) Heatmap of recirculating memory T cells (T<sub>RcM</sub>), within the CD8+ Phenotypic and Functional datasets. (**F**) TNF- $\alpha$ , GM-CSF, IL-2 and IL-22 production in T<sub>RcM</sub>, visualized in tSNE. (**G**) Frequency of T<sub>RcM</sub> in AD, HD and PSO. (**H**) Representative dot plots of AD. HD and PSO depicting expression of

- in AD, HD and PSO. (H) Representative dot plots of AD, HD and PSO depicting expression of
- 287 IL-22 and GM-CSF in manually gated cells (red) within skin-homing  $T_{RcM}$  (bottom) compared to
- total CD8+ T cells (top). (I) IL-22 and GM-CSF production in skin-homing  $T_{RcM}$ , in AD, HD and
- 289 PSO. (J) Correlation between IL-22 production in skin =homing  $T_{RcM}$  and disease severity in AD
- 290 . Relative cell frequency (B, G) or cytokine expression (D, I) are shown, with circles representing
- 291 individual samples, and box and whiskers denoting minimum and maximum values.
- 292 \**p-adj*<0.05; \*\**p-adj*<0.01
- 293

#### Figure 2. Regulatory CD4+ T cells in skin disease.

(A) Heatmap of Regulatory T cells (T<sub>Regs</sub>), within the CD4+ Phenotypic dataset. (B) Frequency
 of T<sub>Regs</sub> in atopic dermatitis (AD), healthy donor (HD) and psoriasis (PSO) samples.
 Relative cell frequency is shown, with circles representing individual samples, and box
 and whiskers denoting minimum and maximum values. (C) Manual gating strategy to
 obtain Tregs subsets. Differences in cell frequencies were assessed by one -way ANOVA

300	followed by Dunn test for multiple correction, ****p-adj<0.0001, ***p-adj<0.001, **p-
301	<i>adj</i> <0.01, * <i>p-adj</i> <0.05.
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CD49d+ CCR6+FOXP3+ Treg С Cell frequency within CD4<sup>+</sup> ł 0.0 HD ..... PSO AD CD45R0 CD127 - multiple CCR6 CLA CD49d\* CCR6-FOXP3\* Treg Cell frequency within CD4\* 0.6and loand + 110ml + 110ml + 1 ..... ant furning a strong a strong and human CD45RA CD49d 1 CD25 Foxp3 -PSO CLA+ CCR6+FOXP3+Treg cell frequency within CD45RO'CLA\*CD25<sup>14</sup> (%) 0 0 (%) \* Ŧ CD127 CCR6 AD HD PSO 1 CLA\* CCR6 FOXP3\*Treg Cell frequency within CD45RO\* CLA\*CD25<sup>1</sup> (%) 0 0 (%) 0 0 0 0 0 0 . . . . . . . . . . CD25 Foxp3 ÷ 7 CLA+ CCR6+FOXP3 Cell frequency within CD45RO\*CLA\*CD25<sup>1</sup>) o 8 (%) 5 8 AD HD PSO ...... Ŧ AD HD PSO

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### Supplementary Material for Online Repository

# T-cell phenotyping uncovers systemic features of atopic dermatitis and psoriasis.

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#### **Online Methods**

#### **Study population**

Nine HD, 19 psoriasis and 15 AD patients, recruited at tertiary referral centers at Guy's and St Thomas' Hospital, Queen Elizabeth Hospital and West Middlesex University Hospital, were included in this study. Patients were not receiving any conventional or biological systemic therapy at the time of sampling. Healthy donors were nominally healthy at the time of sample collection and did not report any allergic or auto-immune disease. Full demographics can be found in **Table E1**. The study was conducted in accordance with the Declaration of Helsinki Principles, with written informed consent obtained from each volunteer and approved by the London Bridge Research Ethics committee (Reference 11/LO/1962). One additional healthy donor was recruited at SIgN, A\*STAR (IRB number: NUS-IRB-09-256) as internal control to be run with each batch.

#### **PBMCs isolation and cryopreservation**

PBMCs were isolated by blood centrifugation on a density gradient (Ficoll-Plaque Plus, VWR). Cells were viably frozen in RPMI 1640 (Life Technologies) supplemented with 11.25% HSA (Gemini Bio-Products)/10% DMSO (Sigma), stored in liquid nitrogen and shipped all together to SIgN A\*STAR in a shipper tank filled with liquid nitrogen. PBMCS from one additional healthy donor to be used as internal control (IC) were isolated at the SIgN, A\*STAR and cells were cryopreserved in 90% fetal bovine serum (FBS) (Life Technologies) plus 10% DMSO (MP Biomedicals).

#### **PBMCs stimulation and staining**

PBMCS were processed in 7 batches, each including the IC and samples from each BioDiP group (HD, AD and Pso). Cryopreserved cells were thawed, washed with pre-warmed RPMI 1640 supplemented with 1% FBS, 1X Penicillin/Streptomycin/L-glutamine, 1% 1M HEPES and 1X βmercaptoethanol (all Life Technologies), and rested overnight at 37°C. Next, cells were washed and split into two aliquots of up to  $3 \times 10^6$  cells each for staining with the Phenotypic Panel, including 42 surface and intracellular markers, and the Functional Panel, including 27 phenotypic markers and 14 cytokines (Table E2). For both panels, staining of surface markers (Surface I in Table E2) that are weakly expressed or are downregulated during polyclonal stimulation, was carried out in 96-well round bottom plates at 37°C for 30 minutes. Next, cells were either left untreated (Phenotypic Panel) or stimulated with 150 ng/ml phorbol-12-myristate-13-acetate (PMA) and 1 µM ionomycin (Sigma-Aldrich) in the presence of monensin and Brefeldin A (all eBioscience) for four hours for the detection of intracellular cytokines (Functional Panel). After incubation, cells were washed twice in cold PBS, incubated on ice with 100 µM cisplatin (Sigma-Aldrich) for 5 minutes for Live/Dead staining, washed with staining buffer and stained with fluorophore-tagged antibodies for 30 minutes on ice (Surface II, Table E2). Next, cells were washed twice in staining buffer and stained with metal isotope-labeled surface antibodies (Surface II, Table E2) for 30 minutes on ice.

Cells for the Phenotypic Panel were then washed, fixed in Foxp3 Transcription Factor Fixation/Permeabilization buffer (eBioscience), washed in Permeabilization buffer (Biolegend) and stained with metal isotope-labeled antibodies against intracellular phenotypic markers (Intra Table E2, Phenotypic Panel), each step carried out on ice for 30 minutes. Next, cells were incubated with metal isotope-conjugated streptavidin for 10 minutes, washed and fixed in 2% paraformaldehyde (PFA; Electron Microscopy Sciences) at 4°C, overnight. Next day, cells were washed twice with 1X perm buffer, once with PBS, and incubated with cellular barcodes for 30 minutes (Surface III, **Table E2**). Next, cells were washed once with 1X perm buffer, incubated in staining buffer for 10 minutes on ice, and DNA was labelled with 250 nm iridium intercalator (Fluidigm Corporation) in PBS-2% PFA at room temperature for 20 minutes. Finally, cells were washed twice with staining buffer, re-suspended in 90% FBS + 10% DMSO in a 96-well round bottom plate and stored immediately at -80°C, prior to CyTOF acquisition.

Following Surface II staining, cells for the Functional Panel were washed twice with staining buffer, once in PBS and fixed in 2% PFA with at 4°C overnight. Next day, cells were washed twice with 1X perm buffer and stained with intracellular antibodies (Intra, **Table E2**, Functional Panel) on ice for 30 minutes. Next, cells were washed, labelled with cellular barcodes and DNA intercalator, cryopreserved and stored until CyTOF acquisition, as described for the Phenotypic Panel.

#### Cytometry by-time-of-flight (CYTOF)

Prior to CyTOF acquisition, cryopreserved stained cells from each batch were thawed, washed twice and re-suspended in staining buffer. Aliquots of cells from all samples within a batch were pooled and filtered through a 0.35  $\mu$ m strainer into a 5 ml polystyrene tube. Pooled, filtered cells were washed twice in water before final re-suspension in water at 0.5 x 10<sup>6</sup> cells/ml. EQ Four Element Calibration Beads (Fluidigm Corporation) were added to a concentration of 1% prior to acquisition. Cells acquisition was performed on a CyTOF2 mass cytometer (Fluidigm Corporation).

#### Data cleaning and manual gating

Raw data were manually cleaned and curated. Patient AD6 was excluded from the overall analysis for low cell viability. Samples run in batch 1 were excluded for unsupervised analysis of the Phenotypic Panel due to suboptimal staining (**Table E1**). Data were normalized with beads and files corresponding to the same batch were concatenated, using the Fluidigm CyTOF software. Obvious dead cells were removed to facilitate debarcoding. Manual debarcoding using Infinicyt software was performed to obtain the individual FCS files for each sample. Manual gating to identify CD4+ and CD8+ T populations for each sample was performed using Cytobank (**Fig. E1B**). From this step onward, each panel (Phenotypic and Functional) and each manually gated T cell compartment (CD4+ T, CD8+ T) was pre-processed, processed for clustering and analyzed independently, resulting in 4 different datasets, Functional CD4, Functional CD8, Phenotypic CD4 and Phenotypic CD8.

#### T cell datasets pre-processing, QC and clustering

For each dataset, 10,000 CD4+T or CD8+T cells were randomly selected from each sample (or the entire sample was used if it contained less than 10,000 cells) and merged into a single FCS file. Marker expression for all markers except Time and Event length, was asinh-transformed using a cofactor of 5. In order to minimize batch differences, a normalization step was applied to both Panels. For the Functional Panels, CD4+ and CD8+ T-cells were normalized dividing the expression of each marker (except Time, Event length and DNA) by the mean of that marker in the intact live cells from the corresponding batch. In the Phenotypic Panel, CD4 and CD8 T-cells were normalized dividing the expression of each marker in the intact live cells from the corresponding batch. Independent clustering for each of the 4 datasets was performed using the clustering algorithm PhenoGraph in its python implementation (1). The

specific marker combinations, and values of the parameter k, the number of nearest neighbor used to build the similarity graph, used for each dataset are listed in **Table E4**.

Quality of normalization and clustering was assessed by visualizing the frequency of each cluster in the IC and 9 HD. Minimal instability was observed among the IC across different batches, while variability among HD was much larger, as expected for true biological variation, which we confirmed by comparing the coefficient of variation of IC and HD (p<0.001 for all 4 datasets) (**Fig. E2B** and data not shown).

#### Dimensionality reduction, visualization and cluster interpretation

Dimensionality-reduction for visualization purposes was performed using the t-Distributed Stochastic Neighbor Embedding algorithm (2) as implemented in the R package *Rtsne*. Representative tSNE plots for each of the 4 datasets were built by randomly selecting 60.000 cells from each dataset. The perplexity parameter was set to 30. The median tSNE1 and tSNE2 coordinates of the cells belonging to each cluster are shown as black dots and labeled by their cluster ID. To enhance visualization, cells were colored according to the clusters they were assigned to. As expected, cells belonging to a specific, colour-coded cluster were found on adjacent coordinates in the tSNE plots (Fig E2A). Additional tSNE plots representative for specific clusters were created by randomly selecting 30.000 cells belonging to those clusters. The perplexity parameter was set to 30 and only cytokines were used to generate the tSNE plots.

Each cluster was manually analyzed and assigned to standard immune subsets, based on the mean expression of subset-defining markers, such as CD161 and V $\alpha$ 7.2 for mucosal-associated invariant T (MAIT) cells and CD103 for Recirculating memory T cells (T<sub>ReM</sub>) (**Fig. E3A**). To confirm that the markers used were reliable discriminators for the indicated populations, we combined the profile of selected clusters with the tSNE projection and colour-graded the expression of the most

defining markers for each cell population on the tSNE plot (Fig E3B). As expected, maximal expression of the defining markers co-localized with the tSNE coordinates of the respective cluster shown in Fig. E2A. Moreover, visual inspection of density dot plots further confirmed that identified cell cluster were real biological entities, when compared with a negative control population cluster CD8+ F20.

#### IgE Measurement

IgE were measured in the serum of AD patients using Human IgE Ready-Set-GO from Affymetrix according the manufacturer's instructions.

#### **Statistical Analysis**

Frequencies per sample were computed for all clusters in each of the four datasets. Variability within IC and HD was evaluated by calculating coefficient of variation (CV) for each cluster within both groups and performing Mann-Whitney U test comparison for each dataset. Mean normalized expression values for each cytokine per sample in each cluster were also computed for the Functional Panel dataset. Statistical significance for differences in frequencies and mean normalised expression across groups was computed using a linear mixed effects model based on the following formula (in the coding language R):

 $expression/frequency \sim study\_group * visit\_number + age + sex + ethnicity + batch +$ 

(1|subject\_ID)

That is, where the subject ID represents the random effect variable, and the other variables (including the interaction term between study\_group and visit\_number) are fixed effects. Models were fitted using the R package lmer and contrasts of interest were extracted using the

function glht from the R package multcomp. This method was chosen to account for the fact that

multiple samples were coming from the same donor and as there were missing samples for some of the time points (where a paired test would have required to drop out many samples). p-values were corrected for the number of clusters (frequency) or cluster-cytokine pairs (mean normalised expression) tested using the Benjamini-Hochberg procedure. For manually gated populations differences were assessed by one-way ANOVA followed by Tukey's test for multiple correction. Correlation between IL-22 production and disease severity in AD patients, measured by SASSAD, was assessed by two-tailed Pearson correlation.Tests with a corrected p-value lower than 0.05 were considered significant

### **Supplementary References**

E1. Levine JH, Simonds EF, Bendall SC, Davis KL, Amir el AD, Tadmor MD, et al. Data-Driven Phenotypic Dissection of AML Reveals Progenitor-like Cells that Correlate with Prognosis. Cell. 2015;162(1):184-97.
E2. van der Maaten L, Hinton G. Visualizing Data using t-SNE. J Mach Learn Res. 2008;9:2579-605.

### **Supplementary Figure Legends**

#### Figure E1: T cell profiling in inflammatory skin disease

(A) Experimental workflow for the analysis of CD4+ and CD8+ T cells of atopic dermatitis (AD), healthy donor (HD) and psoriasis (PSO) patients by mass-cytometry. PBMCs were stained for phenotypic markers (Phenotypic Panel) or stimulated with PMA/Ionomycin and stained for cytokine production (Functional panel). Four datasets were obtained by pooling manually gated CD4+ or CD8+ cells from each experiment and performing unsupervised clustering analysis. Differential analysis of frequencies and cytokine expression levels was then performed. (B) Gating strategy used to manually identify major cell populations within PBMCs samples. Representative plots shown are from the Phenotypic panel. (C) Pie charts showing mean percentages of main lymphocyte populations within CD45+ cells identified in the Phenotypic panel in AD, HD and PSO.

#### Figure E2: Evaluation of clusters frequency within healthy donors and internal control

(A) tSNE plots of colour-coded T cell clusters within CD4+ and CD8+ T cells, identified in the Phenotypic and Functional panels. Labelled black dots represent the centroid (based on median) of the corresponding cluster. (B) Evaluation of cluster frequency within healthy donor and Internal control. Bar-plots of relative cluster frequency of 9 healthy donors (HD) and one internal control (IC) run each time, within CD4+ Phenotypic, CD4+ Functional, CD8+ Phenotypic, and CD8+Functional datasets.

#### Figure E3: Cell clusters identified in the Functional CD8 dataset

(A) Marker analysis and attribution of the 29 clusters identified within the CD4+ Phenotypic dataset to standard cell populations. Normalized expression of all cells assigned to each cluster is represented by colors in the heatmap, and non-normalized raw values are shown as histograms.
(B) MAIT and Recirculating Memory T cells identified within the CD8+ Functional dataset are visualized on tSNE plot coloured using normalized values of selected markers (left), and as dotplots in black (right). Cluster F20, shown in blue, is the negative control.

#### Figure E4: Cell clusters identified in the CD4+ Functional dataset

Marker analysis and attribution of the 51 clusters identified within the CD4+ Functional dataset to standard cell populations. Normalized expression of all cells assigned to each cluster is represented by colours in the heatmap, and non-normalized raw values are shown as histograms.

#### Figure E5: Cell clusters identified in the CD4+ Phenotypic dataset

Marker analysis and attribution of the 34 clusters identified within the CD4+ Phenotypic dataset to standard cell populations. Normalized expression of all cells assigned to each cluster is represented by colours in the heatmap, and non-normalized raw values are shown as histograms.

#### Figure E6: Cell clusters identified in the CD8+ Phenotypic dataset

Marker analysis and attribution of the 31 clusters identified within the CD8+ Phenotypic dataset to standard cell populations. Normalized expression of all cells assigned to each cluster is represented by colours in the heatmap, and non-normalized raw values are shown as histograms.

# Figure E7. MAIT, Recirculating memory CD8+ T and T regulatory CD4+ T cells in inflammatory skin disease

A) tSNE visualization of cytokine expression in Functional CD8+ cluster F14 using pooled cells from AD, HD and psoriasis. (B) Cytokine production in cluster F14, in AD, HD and psoriasis. (C) tSNE visualization of cytokine and PD-1 expression in the Functional CD8+ cluster F28 using pooled cells from AD, psoriasis and HD. (D) Box plot showing frequency of TRcM clusters F18, F29 and F29 in the Functional CD8+ dataset. (E) TRegs in clusters P10, P14 and P22 in the CD4+ Phenotypic dataset were merged and coloured by cluster, or according to the raw expression of the indicated markers (F). \*  $p \le 0.05$ .





В



С







25

٥

IC B2 IC B3 ICB4 IC B5 IC B6 IC 87

IC B1

%

F13 F30 F47

-

F14 F31 F48 F15 F32 F49 F16 F33 F50

F17 F34 F51

F11 F26

F12 F27

F13 F28

F14 F29

F15

HD2 HD4 HD5 HD6 HD8 HD8 HD8

25

HD1

В

25

0

IC B3 IC 84 ICB5 ICB6 IC B7

IC B1 IC B2

25

0

HD1





raw expression values

CD8+F14: MAIT Cells

CD8+ F18/18/29: Recirculating Memory T Cells



В

mean relative expression across all samples



0 1 2 3 4 5

.

10 11 12 13 14 15

density







## Table E1 Demographics and clinical data

Patient	Ethnicity	Age	Sex	Disease onset	Disease phenotype	Disease	Comorbidities	Recurrent	serum IgE	Notes
						Seventy		meetions	(10/1)	
AD1	Mixed	18	М	not known	Generalised;flexural pattern	Generalised;flexural SASSAD 30 Asthma, Allergic S.aureus; HSV pattern Rhinocongintivitis (eczema herpeticum)		23207 ± 2030	Excluded from unsupervised analysis (Phenotypic Panel)	
AD2	Black	25	м	Childhood	Follicular/papular phenotype; extensor pattern	Follicular/papular not known Asthma, Allergic not known phenotype; extensor pattern Rhinocongintivitis		not known	855 ± 56	Excluded from unsupervised analysis (Phenotypic Panel)
AD3	White	26	М	Childhood	Generalised;flexural pattern	neralised;flexural SASSAD 17 Asthma, Allergic S.aureus; HSV (eczema herpeticum)		1664 ± 27	Excluded from unsupervised analysis (Phenotypic Panel)	
AD4	White	50	М	Childhood	Generalised;flexural pattern	ineralised;flexural not known Asthma, Allergic not known ttern Rhinocongintivitis, Food allergies		83 ± 3		
AD5	White	32	М	Childhood	Generalised;flexural pattern	not known	None	not known	2722 ± 50	
AD6	White	59	F	Childhood	Generalised; flexural prominence	SASSAD 31	Asthma, Allergic Rhinocongintivitis	not known	36292 ± 155	Excluded from overall analysis for low cell viability
AD7	Black	57	F	Childhood	Generalised;flexural pattern; hand and foot eczema	ralised;flexural SASSAD19 None S.aureus (abscesses) na		1205 ± 15		
AD8	White	47	М	Childhood	Generalised;flexural pattern; hand and foot eczema	SASSAD 24	Asthma, Allergic Rhinocongintivitis	Extensive molluscum contagiosum	5243 ± 175	
AD9	White	49	М	Childhood	Generalised;flexural pattern	SASSAD 27	Asthma, Allergic Rhinocongintivitis	S.aureus; HSV (eczema herpeticum)	1118 ± 58	
AD10	White	35	М	not known	Generalised;flexural pattern	not known	Asthma, food allergies	HSV (eczema herpeticum)	5452 ± 55	
AD11	White	60	М	Adult	Generalised;flexural pattern; hand eczema	SASSAD17	Metals allergy	not known	243 ±4	
AD12	Black	40	М	Childhood	Generalised;flexural pattern	SASSAD 20	Asthma, Allergic Rhinocongintivitis	not known	1318 ± 36	
AD13	Asian	30	М	not known	Generalised;flexural pattern; hand eczema	SASSAD 15	Allergic Rhinocongintivitis	S.aureus	757 ± 5	
AD14	Asian	65	М	not known	Generalised;flexural pattern	SASSAD21	Allergic Rhinocongintivitis	S.aureus; HSV (eczema herpeticum)	6516 ± 172	
AD15	Asian	36	М	not known	Generalised;flexural pattern; hand eczema	SASSAD 15	None	S.aureus	17654 ±724	
PSO1	White	27	M	Teenager	Plaque-psoriasis	PASI 10.5	Psoriatic arthritis	not applicable	not applicable	Excluded from unsupervised analysis (Phenotypic Panel)
PSO2	White	48	м	Adult	Plaque-psoriasis	PASI 21.2	none	not applicable	not applicable	
PSO3	White	40	М	Childhood	Plaque-psoriasis	PASI 10.2	none	not applicable	not applicable	
PSO4	White	37	F	Childhood	Plaque-psoriasis	PASI 23.3	none	not applicable	not applicable	
PSO5	White	37	м	Adult	Plaque-psoriasis	PASI 12.8	none	not applicable	not applicable	
PSO6	White	50	М	Adult	Plaque-psoriasis	PASI 33.4	liver disease	not applicable	not applicable	
PSO7	White	53	F	Teenager	Plaque-psoriasis	PASI 14.1	none	not applicable	not applicable	
PSO8	White	58	F	Childhood	Plaque-psoriasis	PASI 9.9	none	not applicable	not applicable	Excluded from unsupervised analysis (Phenotypic Panel)

PSO9	White	47	м	Teenager	Plaque-psoriasis	PASI 17.3	none	not applicable	not applicable	
PSO10	White	50	м	Adult	Plague-psoriasis	PASI 15.3	Hyperthension,	not applicable	not	
							hypercolerestemia		applicable	
PSO11	Asian	19	м	Childhood	Plaque-psoriasis	PASI 22.7	none	not applicable	not applicable	Excluded from the analysis of Phenotypic markers
PSO12	Asian	37	м	Childhood	Plaque-psoriasis	PASI 17.9	none	not applicable	not applicable	
PSO13	White	48	М	Adult	Plaque-psoriasis	PASI 12.6	none	not applicable	not applicable	
PSO14	White	45	М	Teenager	Plaque-psoriasis	PASI 25	none	not applicable	not applicable	
PSO15	Asian	28	М	Teenager	Plaque-psoriasis	PASI 35.5	none	not applicable	not applicable	
PSO16	White	36	F	Adult	Plaque-psoriasis	PASI 17.6		not applicable	not applicable	
PSO17	White	61	F	Adult	Plaque-psoriasis	PASI 13.5	asthma	not applicable	not applicable	
PSO18	White	33	F	Adult	Plaque-psoriasis	PASI 13.7	asthma	not applicable	not applicable	
PSO19	White	35	F	Adult	Plaque-psoriasis	PASI 26.7	none	not applicable	not applicable	
HD1	White	50	м	not applicable	not applicable	not applicable	not applicable	not applicable	not applicable	Excluded from the unsuprvised analysis of Phenotypic markers
HD2	White	53	м	NA	NA	NA	NA	NA	NA	Excluded from unsupervised analysis (Phenotypic Panel)
HD3	White	32	м	NA	NA	NA	NA	NA	NA	
HD4	White	28	F	NA	NA	NA	NA	NA	NA	
HD5	White	53	F	NA	NA	NA	NA	NA	NA	
HD6	White	48	М	NA	NA	NA	NA	NA	NA	
HD7	White	31	м	NA	NA	NA	NA	NA	NA	
HD8	White	24	м	NA	NA	NA	NA	NA	NA	
HD9	White	32	м	NA	NA	NA	NA	NA	NA	

## Table E2 Staining panels

Phenotypic I	Panel			Functional F	Panel		
Metal	Antibody / Target	Company (clone)	Staining step	Metal	Antibody / Target	Company (clone)	Staining step
103	Cellular Barcode	Synthesized in house	Surface III	103	Cellular Barcode	Synthesized in house	Surface III
104	Cellular Barcode	Synthesized in house	Surface III	104	Cellular Barcode	Synthesized in house	Surface III
105	Cellular Barcode	Synthesized in house	Surface III	105	Cellular Barcode	Synthesized in house	Surface III
106	Cellular Barcode	Synthesized in house	Surface III	106	Cellular Barcode	Synthesized in house	Surface III
108	Cellular Barcode	Synthesized in house	Surface III	108	Cellular Barcode	Synthesized in house	Surface III
110	Cellular Barcode	Synthesized in house	Surface III	110	Cellular Barcode	Synthesized in house	Surface III
113	Cellular Barcode	Synthesized in house	Surface III	113	Cellular Barcode	Synthesized in house	Surface III
89	CD45	Fluidigm (HI30)	Surface II	89	CD45	Fluidigm (HI30)	Surface II
112/114	Qdot800-CD14	Invitrogen (TuK4)	Surface II	112/114	Qdot800-CD14	Invitrogen (TuK4)	Surface II
115	CD57	Biolegend (HCD57)	Surface II	115	CD57	Biolegend (HCD57)	Surface II
139	TCRγδ PE (Primary)	Invitrogen (5A6.E9)	Surface II	139	TCRγδ PE (Primary)	Invitrogen (5A6.E9)	Surface II
	(Secondary)	Biolegend (PE001)			(Secondary)	Biolegend (PE001)	
140	CD3	Biolegend (UCHT1)	Surface II	140	CD3	Biolegend (UCHT1)	Surface II
141	HLA-DR	Biolegend (L243)	Surface II	146	CD8α	Biolegend (SK1)	Surface II
142	CLA	Biolegend (HECA-452)	Surface II	147	CD45RA	Biolegend (HI100)	Surface I
143	CD38	Biolegend (HIT2)	Surface II	148	CLA	Biolegend (HECA-452)	Surface II
144	Granzyme B	Abcam (CLB- GB11)	Surface II	149	CD4	Biolegend (SK3)	Surface I
145	CD62L	BD Biosciences (DREG-56)	Surface II	150	CD103	eBioscience (B- Ly7)	Surface I
146	CD8α	Biolegend (SK1)	Surface II	152	CD25	Biolegend (M- A251)	Surface I
147	CD45RO	Biolegend (UCHL1)	Surface II	153	CD107a	BD Biosciences (H4A3)	Surface II
148	νδ2	Biolegend (B6)	Surface II	154	CD40L	eBioscience (24-31)	Surface II
149	CD4	Biolegend (SK3)	Surface II	155	CTLA-4	BD Biosciences (BNI3)	Surface II
150	CD103	eBioscience (B- Ly7)	Surface II	156	νδ2	Biolegend (B6)	Surface II
151	CCR4	R&D Systems (205410)	Surface II	158	CD56	BD Biosciences (NCAM16.2)	Surface II
152	Ki-67	BD Biosciences (B56)	Intra	159	Integrin β7	Biolegend (FIB504)	Surface I
153	CD49a	Biolegend (TS2/7)	Surface II	161	CCR9	Biolegend (L053E8)	Surface I

154	CCR10	R&D Systems (314305)	Surface II	163	CD161	Biolegend (HP- 3G10)	Surface I
	Foxp3 Biotin (Primary)	eBioscience (PCH101)		164	CD19	Biolegend (HIB19)	Surface II
155	Streptavidin (Secondary)	Synthesized in house	Intra	165	Vα7.2	Biolegend (3C10)	Surface I
156	CCR7	R&D Systems (150503)	Surface I	166	CXCR5	BD Biosciences (RF8B2)	Surface I
157	CD27	eBioscience (LG.7F9)	Surface I	168	CCR2	Biolegend (K036C2)	Surface I
158	CD56	BD Biosciences (NCAM16.2)	Surface II	171	CCR6	Biolegend (G034E3)	Surface I
159	Integrin β7	Biolegend (FIB504)	Surface I	173	CCR4	R&D Systems (205410)	Surface I
160	PD-1	eBioscience (eBioJ105)	Intra	175	CCR5	Abcam (HEK/1/85a)	Surface I
	Vδ1 FITC (Primary)	Miltenyi Biotec (REA173)		209	CD16	Fluidigm (3G8)	Surface II
161	anti-FITC (Secondary)	Biolegend (FIT- 22)	Surface II	141	IFN-y	eBioscience (4S.B3)	Intra
162	CD95	Biolegend (DX2)	Surface II	142	TNF-α	eBioscience (MAb11)	Intra
163	CXCR3	R&D Systems (49801)	Surface II	143	IL-8	Biolegend (E8N1)	Intra
164	CCR9	Biolegend (L053E8)	Surface I	144	Granzyme B	Abcam (CLB- GB11)	Intra
165	Vα7.2	Biolegend (3C10)	Surface I	145	IL-17F	eBioscience (SHLR17)	Intra
166	CXCR5	BD Biosciences (RF8B2)	Surface II	151	IL-2	eBioscience (MQ1-17H12)	Intra
167	CD49d	Biolegend (9F10)	Surface II	157	MIP-1β	BD Biosciences (D21-1351)	Intra
168	CCR2	Biolegend (K036C2)	Surface I	160	PD-1	eBioscience (eBioJ105)	Intra
169	CD25	Biolegend (M- A251)	Surface II	162	IL-5	Biolegend (JES1-39D10)	Intra
170	CD161	Biolegend (HP- 3G10)	Surface I	167	IL-9	BD Biosciences (MH9A4)	Intra
171	CCR6	Biolegend (G034E3)	Surface II	169	IL-4	Biolegend (MP4-25D2)	Intra
172	CD45RA	Biolegend (HI100)	Surface II	170	IL-10	eBioscience (JES3-9D7)	Intra
173	CD19	Biolegend (HIB19)	Surface II	172	GM-CSF	Biolegend (BVD2-21C11)	Intra
174	CX3CR1	Biolegend (K0124E1)	Surface II	174	IL-22	Biolegend (Poly5161)	Intra
175	CCR5	Abcam (HEK/1/85a)	Surface I	176	IL-17A	Biolegend (BL168)	Intra
176	CD127	Biolegend (A019D5)	Surface II	191/193	DNA	Fluidigm	DNA
209	CD16	Fluidigm (3G8)	Surface II	195	CisPlatin	Sigma-Aldrich	Live/dead
191/193	DNA	Fluidigm	DNA				
195	CisPlatin	Sigma-Aldrich	Live/dead				

## Table E3 Class Comparisons

Variable	Two-way	Dataset	Cluster	adj p	Estimate	Interpretation
	comparisons		(-Cytokine)	value		
Cell	AD vs HD	CD8+	P15	0.01918	5.4545	TRcM ↑ in AD
Frequency		Phenotypic	P27	0.04711	0.3009	TRcM 个 in AD
			P24	0.04711	1.2636	TRcM 个 in AD
		CD8+	F18	0.03012	3.7208	TRcM 个 in AD
		Functional	F28	0.04756	1.3014	TRcM 个 in AD
			F29	0.04756	0.2971	TRcM 个 in AD
	Pso vs AD	CD4+ Phenotypic	P22	0.02238	0.597	CD49d+ Tregs ↓ in AD
		CD8+	P15	0.00688	-4.8345	TRcM 个 in AD
		Phenotypic	P27	0.00688	-0.2957	TRcM 个 in AD
			P13	0.00688	-5.3525	MAITs $\downarrow$ in Pso
			P24	0.00688	-1.2771	TRcM 个 in AD
			P8	0.03115	3.2531	
		CD8+	F18	0.02473	-3.4072	TRcM 个 in AD
		Functional	F28	0.02473	-1.3535	TRcM 个 in AD
			F29	0.04425	-0.2861	TRcM 个 in AD
	Pso vs HD	CD8+	P13	0.02057	-5.159	MAITs $\downarrow$ in Pso
		Phenotypic	P18	0.01999	1.3257	
			Р3	0.02057	-11.2144	
		CD8+	F6	0.01198	-2.6657	
		Functional	F1	0.01198	-3.0584	
Cytokine expression	AD vs HD	CD8+ Functional	F28-IL-22	0.0346	4.6189	↑ IL-22 Skin homing TRcM in AD
	Pso vs AD	CD4+ Functional	F36-IL-2	8.96E- 07	-1.6209	
			F17-IL-2	0.00076	-1.4239	
			F15-IL-5	0.00076	-0.1376	
			F20-IL-2	0.0016	-0.9294	
			F6-IL-2	0.00189	-0.8553	
			F1-IL-2	0.00189	-0.7572	
			F17-IL-4	0.00331	-1.0255	
			F10-IL-2	0.00413	-0.7834	
			F15-GM-CSF	0.00413	-2.87	
			F35-IL-2	0.00413	-0.8284	
			F28-IL-2	0.00413	-2.1091	
			F15-IL-2	0.00446	-1.5149	
			F3-	0.00489	-0.274	
			Granzyme_B			
			F7-IL-2	0.00526	-0.7901	

		F34-	0.00551	-0.4253	
		Granzyme_B			
		F26-TNFα	0.00555	-0.4811	
		F17-IL-10	0.00583	-1.0828	
		F3-IL-17F	0.00634	-0.3753	
		F26-IL-2	0.00847	-0.9284	CD4+ T cells
					more activated
			0.00050	2 1 1 4 4	in AD
		F26-GIVI-CSF	0.00859	-2.1144	
		F15-IL-4	0.00905	-1.4349	
		F26- Granzyme_B	0.00958	-0.2734	
		F3-TNF	0.01183	-0.5216	
		F48-GM-CSF	0.01221	-5.5909	
		F11-IL-2	0.01292	-1.1134	
		F19-IL-2	0.01292	-1.5154	
		F34-IL-22	0.01356	-1.2253	
		F40-IL-2	0.01523	-1.4544	
		F28-GM-CSF	0.01745	-3.1101	
		F32-IL-2	0.01745	-0.7572	
		F17-TNF	0.02153	-0.4009	
		F3-IL-2	0.02316	-1.4063	
		F1-IL-17F	0.02316	-0.0657	
		F17-GM-CSF	0.02341	-0.6456	
		F26-IL-22	0.03003	-0.9507	
		F21-IL-2	0.03239	-1.3264	
		F36-IL-5	0.04464	-0.0782	
	CD8+	F14-TNFα	0.01791	-0.8907	MAITs more
	Functional	F14-MIP1β	0.03995	-1.1912	activated in AD
Pso vs HD	CD4+	F15-IL-4	0.01818	-1.6613	CD4+ T cells not
	Functional	F47-IFNγ	0.0293	-2.218	over-activated
		F47-MIP-1β	0.0293	-0.7495	in PSO
		F40-IFNγ	0.0293	-0.4006	
		F28-IL-4	0.0293	-1.849	
		F31-IFNγ	0.0293	-0.6983	
		F44-MIP-1β	0.04397	-0.6579	
		F44-GM-CSF	0.04397	-1.113	

Clustering markers										
Functional	CD4 dataset	Functional	CD8 dataset	Phenotypic	CD4 dataset	Phenotypic (	Phenotypic CD8 dataset			
147	CD45RA	147	CD45RA	172	CD45RA	172	CD45RA			
148	CLA	148	CLA	142	CLA	142	CLA			
150	CD103	150	CD103	150	CD103 (αε Integrin)	150	CD103 (αε Integrin)			
152	CD25	155	CTLA-4	169	CD25	159	Integrinβ7			
155	CTLA-4	159	Integrinβ7	159	Integrinβ7	170	CD161			
156	Vd2	163	CD161	170	CD161	165	Va7.2			
159	Integrinβ7	165	Va7.2	165	Va7.2	166	CXCR5			
163	CD161	166	CXCR5	166	CXCR5	168	CCR2			
165	Va7.2	168	CCR2	168	CCR2	171	CCR6			
166	CXCR5	171	CCR6	171	CCR6	151	CCR4			
168	CCR2	173	CCR4	151	CCR4	175	CCR5			
171	CCR6	175	CCR5	175	CCR5	145	CD62L			
173	CCR4	154	CD40L	145	CD62L	141	HLA-DR			
175	CCR5			141	HLA-DR	147	CD45RO			
				147	CD45RO	144	Granzyme B			
K value				144	Granzyme B	152	Ki67			
K=10		K=100		152	Ki67	153	CD49a (α1 Integrin)			
				153	CD49a (α1 Integrin)	154	CCR10			
				154	CCR10	156	CCR7			
				155	Foxp3	157	CD27			
				156	CCR7	160	PD-1			
				157	CD27	162	CD95			
				160	PD-1	163	CXCR3			
				162	CD95	164	CCR9			
				163	CXCR3	167	CD49d (α4 Integrin)			
				164	CCR9	174	CX3CR1			
				167	CD49d (α4 Integrin)	176	CD127			
				174	CX3CR1					
				176	CD127					
				K value						
				K=10		K=25				

## Table E4 Markers used for clustering