Resolving human circulating innate lymphoid cell heterogeneity using advanced single cell multiomic analysis

Application of BD Rhapsody™ Single-Cell Analysis System and BD FACSymphony™ high parameter cell analyzer for cell signature discovery and deep immunophenotyping

Features

- Simultaneous analysis of protein and gene expression at the single cell level
- Identification of signatures defining distinct subsets of human circulating innate lymphoid cells (ILCs)
- Single cell multiomic screening provides biological information critical for the development of high parameter flow cytometry panels

Innate lymphoid cells (ILCs) constitute a recently identified family of lymphoid cells that do not express rearranged antigen receptors and have key functions in inflammation, infection, cancer, metabolic disorders and allergies. They represent a heterogeneous population including classically defined natural killer (NK) cells as well as more recently described non-cytotoxic ILCs. Three major groups of non-cytotoxic ILCs have been characterized in mucosal and non-mucosal tissues based on distinct patterns of cytokine production and transcription factor expression. However, recent publications using single cell RNA sequencing (scRNA-Seq) or high-parameter mass cytometry showed the existence of more than just three groups of ILCs; hence better understanding of ILC

heterogeneity is needed to determine their roles in disease pathogenesis and to determine whether targeting of ILCs will help to prevent or treat these diseases. The emergence of single cell multiomic (scM) technologies provides an opportunity to further resolve ILC heterogeneity by enabling integration of phenotypic and transcriptional data from the same cell.

This data sheet provides an example of a complete workflow solution for deep characterization of ILCs (Figure 1). ILCs were enriched by sorting on a fluorescence-activated cell sorter prior to single cell multiomic analysis. Computational data analysis was used to identify proteins and genes that are differentially expressed by distinct subsets of ILCs. Information about relative antigen density and co-expression was then used to guide the design of a high-parameter flow cytometry panel to assess the distribution of ILC1 subsets across different donors.

Figure 1

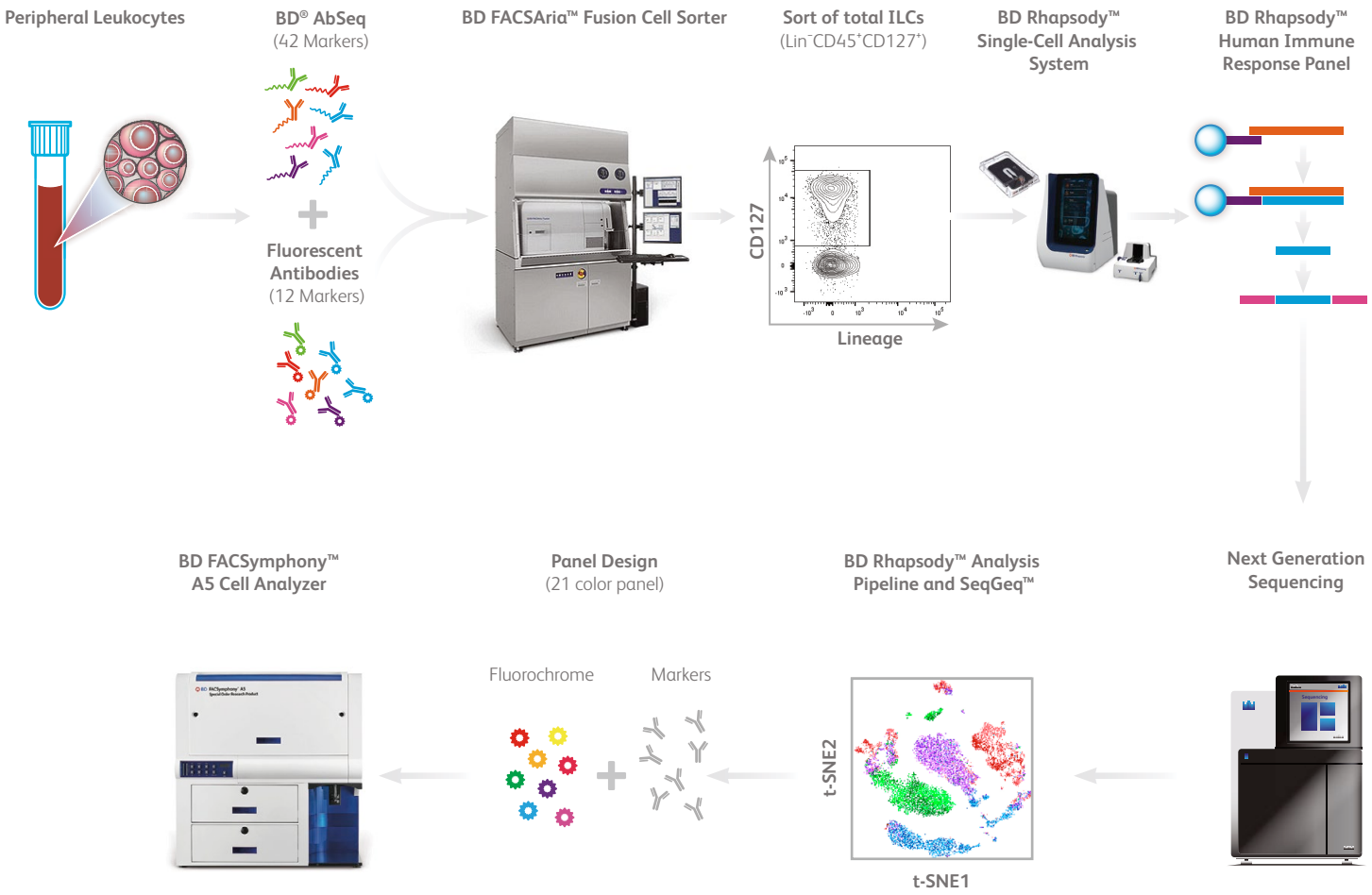

Figure 1. A complete workflow for high-parameter single cell ILC characterization using the BD Rhapsody Single-Cell Analysis System and the BD FACSymphony A5 Cell Analyzer

Peripheral blood leukocytes were isolated using HetaSep (STEMCELL Technologies) and labeled with a panel of 12 fluorochrome-conjugated antibodies for cell sorting (Table 1). Cells were also simultaneously stained with 42 oligonucleotide-conjugated AbSeq antibodies for downstream protein detection via next-generation sequencing. A BV510-labeled cocktail of CD19, CD14, TCRγδ, TCRαβ, CD123, CD1a, BDCA2 and FCεR1α antibodies was used as a dump channel to exclude lineage cells. To ensure clear resolution and exclusion of T and NK cells, CD3 and CD56 antibodies were additionally included in the sorting panel. Total ILCs were defined as Lineage-CD45$^+$CD127$^+$ cells and were sorted using a BD FACSAria Fusion cell sorter and loaded into the BD Rhapsody Single-Cell Analysis System for single cell capture. After sample retrieval, single cell BD® AbSeq and mRNA (BD Rhapsody Human Immune Response Panel) libraries were prepared for sequencing. Data was analyzed using BD Rhapsody Analysis Pipeline and SeqGeq™ software. Biological information obtained from the sequencing screening was used to guide the design of high parameter flow cytometry panels. The information derived from multiomic analysis was validated by analyzing peripheral blood ILCs from 4 different healthy donors using a 21-color flow cytometry panel on the BD FACSymphony A5 cell analyzer.

Table 1. List of fluorochrome-conjugated antibodies used for sorting of Lineage$^-$CD45$^+$CD3$^-$CD56$^-$CD127$^+$ total ILCs

Marker	Clone	Fluorochrome
CD19	SJ25-C1	BV510
CD14	MΦP9	
TCRγδ	11F2	
TCRαβ	IP26	
CD1a	HI149	
CD123	9F5	
BDCA2	V24-785	
FCεR1α	AER-37	
CD45	2D1	FITC
CD3	SK7	APC-H7
CD56	B159	PE-Cy7
CD127	HIL-7R-M21	BV421

Table 2. List of AbSeq oligonucleotide-conjugated antibodies used for single cell multiomic analysis of sorted total ILCs

Cells were co-stained with the markers CD3, CD56, CD14 and CD19 in fluorochrome- and oligonucleotide-conjugated formats in order to further exclude contaminant lineage cells during downstream multiomic analysis. The use of two distinct clones for CD56 was necessary in order to achieve resolution of both fluorochrome- and oligo-conjugated antibodies.

BD® AbSeq Panel			
Marker	Clone	Marker	Clone
CD2	RPA-2.10	CD90	5E10
CD3	SK7	CD94	HP-3D9
CD4	SK3	CD98	UM7F8
CD5	UCHT2	CD103	BER-ACT8
CD7	M-T701	CD117	YB5.B8
CD8	RPA-T8	CD152 (CTLA-4)	BNI3
CD11b	M1/70	CD161	DX12
CD11c	B-LY6	CD183 (CXCR3)	1C6/CXCR3
CD14	MΦP9	CD184 (CXCR4)	12G5
CD16	3G8	CD196 (CCR6)	11A9
CD19	SJ25C1	CD223 (LAG-3)	T47-530
CD25	2A3	CD226 (DNAM-1)	DX11
CD27	M-T271	CD274 (PD-L1)	MIH1
CD28	CD28.2	CD275 (B7-H2)	2D3/B7
CD34	581	CD278 (ICOS)	DX29
CD45RA	HI100	CD279 (PD-1)	EH12.1
CD49a	SR84	CD294 (CRTH2)	BM16
CD49d	9F10	CD314 (NKG2D)	1D11
CD56	NCAM16.2	CD335 (NKp46)	9E2/NKP46
CD62L	DREG-56	CD336 (NKp44)	p44-8
CD69	FN50	CD366 (TIM-3)	7D3

Data analysis was initially performed by manually gating the three major groups of circulating ILCs conventionally defined as CD117$^-$ CD294$^-$ ILC1, CD117$^{+/-}$CD294$^+$ ILC2 and CD117$^+$CD294$^-$ ILC progenitors/ILC3 (ILCP/ILC3) (Figure 2A). We then performed a differential expression analysis between the three ILC groups using single cell AbSeq and mRNA data and identified unique protein and mRNA signatures for each group (Fig 2B). We also observed expected expression patterns for key transcription factors defining ILC groups. For example, the gene encoding T-bet (*TBX21*) was exclusively expressed in a subset of ILC1 cells, whereas *GATA3* was expressed at highest levels in ILC2 cells. As recently reported, *RORC*, encoding RORγδ, was partially expressed by circulating ILC progenitors displaying an ILC3 phenotype (CD117$^+$CD294$^-$ ILCP/ ILC 3 cells).

The single cell heatmap also revealed heterogeneity within each ILC group as most of the represented markers were not uniformly expressed by all the cells. These data therefore suggest that additional subsets exist within each group of ILCs. For example, CD4 was exclusively expressed by the majority, but not all of ILC1 cells. This observation is in agreement with previous reports and was further confirmed by bi-variate plot analysis clearly showing discrete subsets of CD4$^+$ and CD8$^+$ cells within ILC1 but not ILC2 or ILCP/ILC3 cells (Figure 3A). To further investigate the heterogeneity of ILC1 cells, we then performed differential gene and protein expression analysis between the three subsets of CD4$^+$CD8$^-$, CD4$^-$CD8$^+$ and CD4$^-$CD8$^-$ ILC1 cells. Figure 3B shows a single cell heatmap displaying representative protein and gene signatures associated with each subset and illustrated the high heterogeneity and complexity of the ILC1 subset. These data demonstrate the power of a single cell multiomic approach to screen a high number of proteins and genes and discover critical markers required to define and discriminate unique cell subsets within highly heterogeneous samples.

Figure 2A

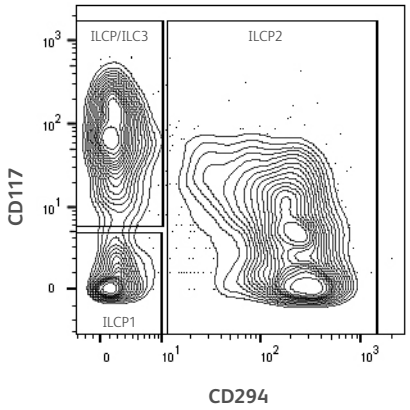

Figure 2B

Figure 2. Single cell multiomic analysis reveals heterogeneity of total ILCs

A. Gating strategy for analysis of ILC groups. Cells were gated based on conventional phenotypes, ILC1 ($CD117^{-}CD294^{-}$), ILC2 ($CD117^{+/-}CD294^{+}$), ILC progenitor cells ILCP/ILC3 ($CD117^{+}CD294^{-}$). **B.** Single cell heat map displaying representative proteins (blue) and genes (red) differentially expressed between the three main ILC groups.

Figure 3A

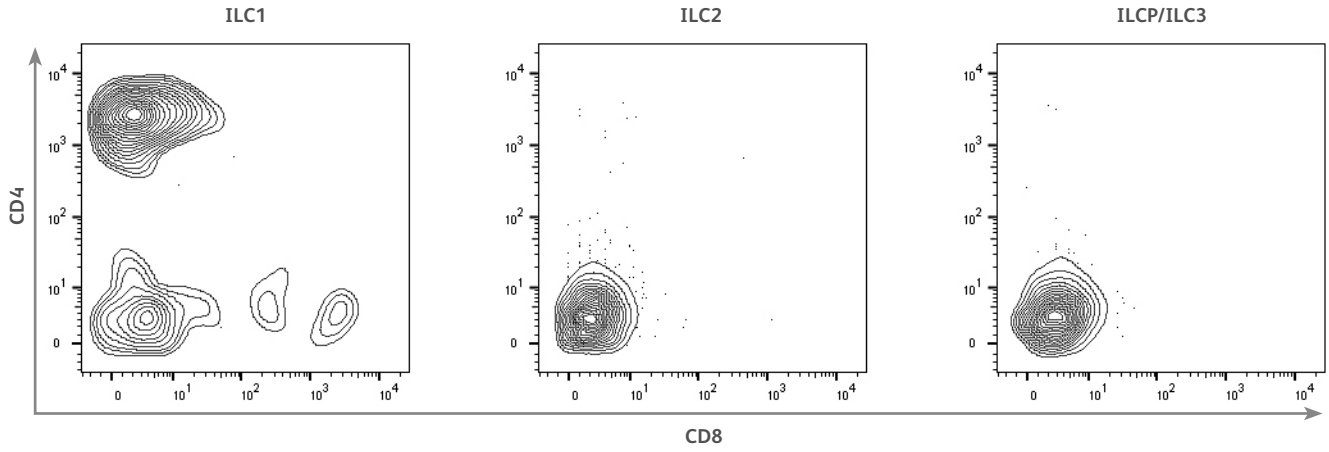

Figure 3B

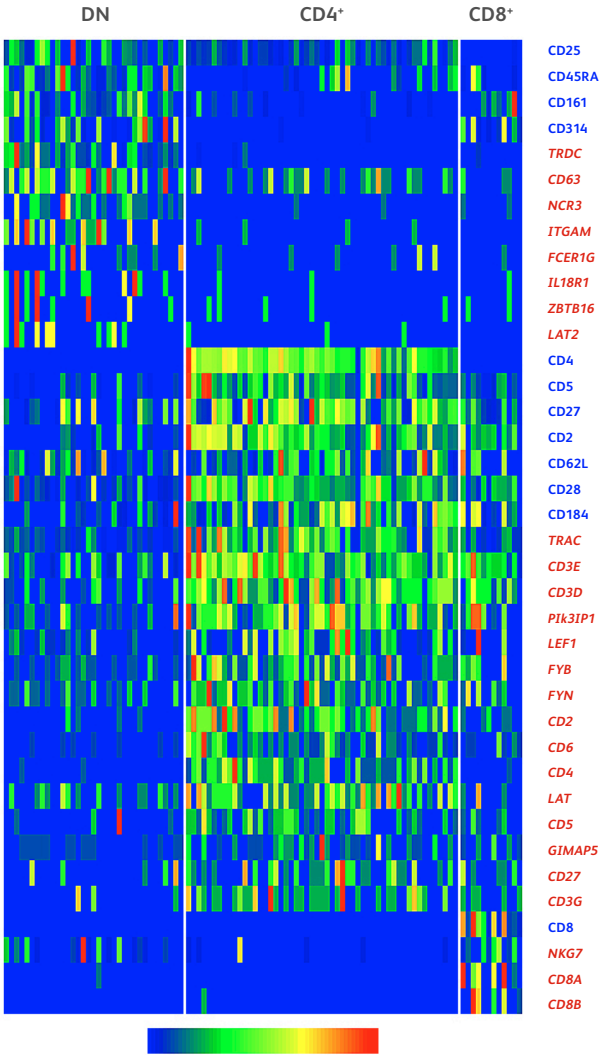

Figure 3. Single cell multiomic analysis reveals heterogeneity of ILC1 cells

A. Contour plots showing CD4 and CD8 expression for three groups of ILCs, demonstrating CD4 and CD8 expression exclusively in ILC1 cells. **B.** Single cell heat map displaying representative proteins (blue) and genes (red) differentially expressed between double negative (DN), $CD4^{+}CD8^{-}$ ($CD4^{+}$) and $CD4^{-}CD8^{+}$ ($CD8^{+}$) ILC1 subsets.

Next, we demonstrate how the information obtained by single cell multiomic screening can be leveraged to design high parameter flow cytometry panels for routine use, for example, to deeply characterize ILCs across a large number of donors or tissues. Of the 42 AbSeq markers tested, our analysis identified 13 surface markers differentially expressed between the three subsets of ILC1. We also identified one differentially expressed gene (*CD63*) coding for the surface protein CD63 (Fig. 3B). We therefore designed a 21-color flow cytometry panel that included lineage markers, markers required for the gating of the three main ILC groups, the 13 differentially expressed markers identified by AbSeq screening, and the surface marker CD63 based on the differential expression of its corresponding gene. The design of this panel was guided and facilitated by the information on relative antigen density and co-expression patterns that were obtained from the single cell multiomic screening (Table 3).

Table 3. List of fluorochrome-conjugated antibodies used for deep characterization of ILC1 cells via high-parameter flow cytometry

Marker	Clone	Fluorochrome or Dye	ILC1 subsets		
			DN	CD4$^+$	CD8$^+$
Viability	N/A	7-AAD	-	-	-
CD19	SJ25-C1	BB700	-	-	-
CD14	MϕP9				
TCRγδ	11F2				
TCRαβ	IP26				
CD1a	HI149				
CD123	9F5				
BDCA2	V24-785				
FCeR1α	AER-37				
CD3	UCHT1	BUV661	-	-	-
CD56	NCAM16.2	BUV563	-	-	-
CD94	HP-3D9	BV786	-	-	-
CD117	YB5.B8	BV711	-	-	-
CD294	BM16	PE-CF594	-	-	-
CD127	HIL-7R-M21	BV421	+	+	+
CD4	SK3	APC-H7	-	+	-
CD8	RPA-T8	BUV496	-	-	+
CD5	UCHT2	BUV805	+/-	++	+
CD27	M-T271	APC	+	+	+
CD2	RPA-2.10	BV605	-	++	++
CD184	12G5	PE	+/-	+	+/-
CD62L	SK11	BB515	+	+	+
CD45RA	HI100	BUV395	+	+/-	+
CD25	2A3	BV480	+	+/-	-
CD28	CD28.2	BV750	+/-	++	+
CD161	DX12	BUV737	+	-	+
CD63	H5C6	PE-Cy7	+	+	-
CD196	11A9	APC-R700	+/-	+/-	+/-
CD314	1D11	BV650	+	-	+/-

To confirm equivalent protein measurement via AbSeq and flow cytometry methods, cells from the same donor as used for the single cell multiomic study were used for ILC1 characterization by flow cytometry. After exclusion of lineage and dead cells (dump channel), CD127$^+$CD117$^-$CD294$^-$ ILC1 subset was identified and divided into three groups based on CD4 and CD8 expression (Figure 4A). The expression of each target marker was then assessed within each ILC1 subset (Figure 4B). The protein expression patterns across these three ILC1 subsets were overall consistent with the AbSeq results (Figure 4C) confirming the correlation between AbSeq and flow cytometry. For example, using either method, expression of CD314 (NKG2D) was specifically observed in subsets of DN cells, in the majority of CD8$^+$ cells, and not in the CD4$^+$ cells.

Figure 4A

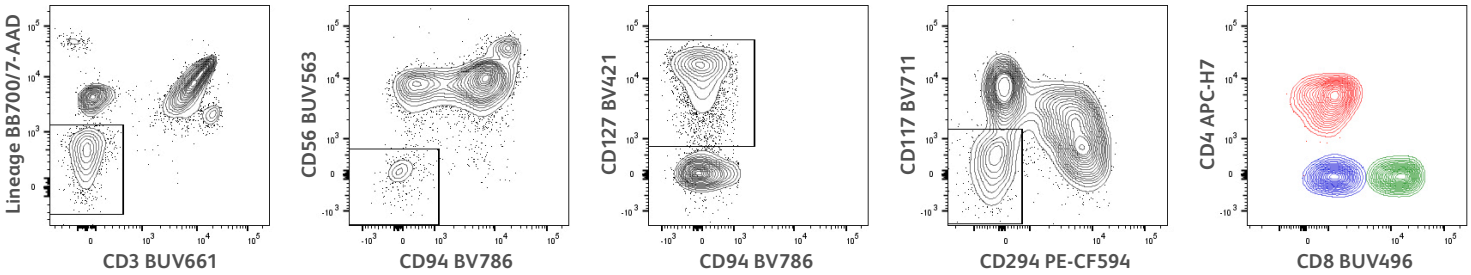

Figure 4B

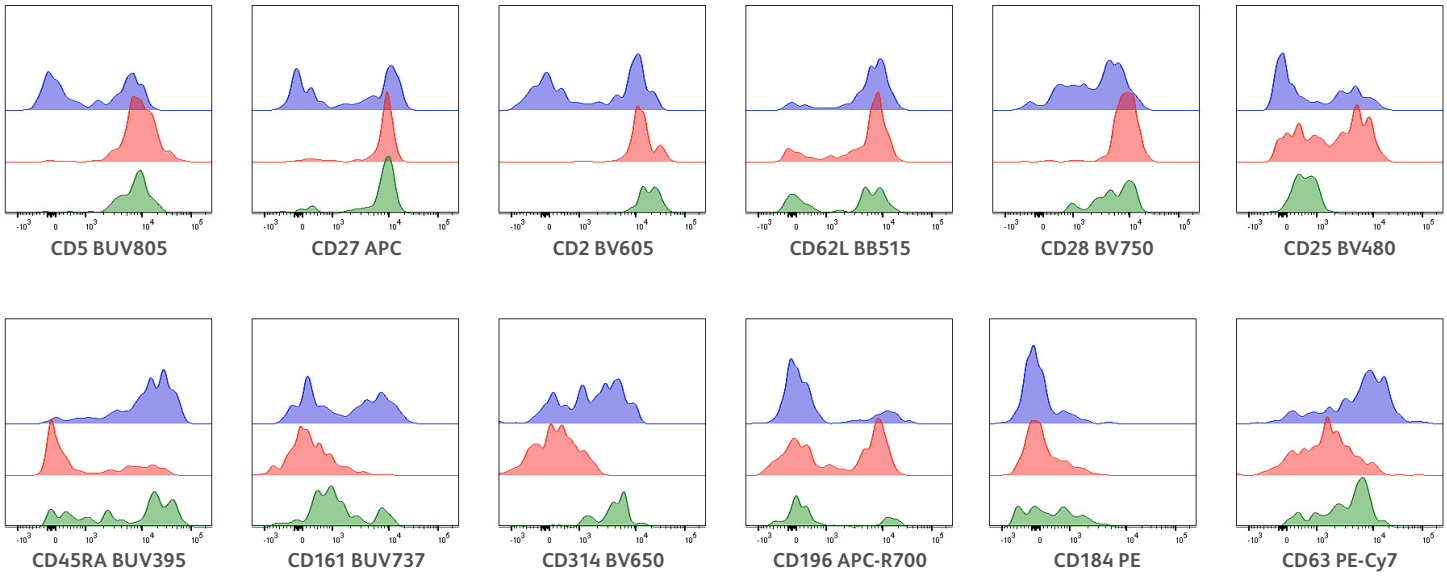

Figure 4C

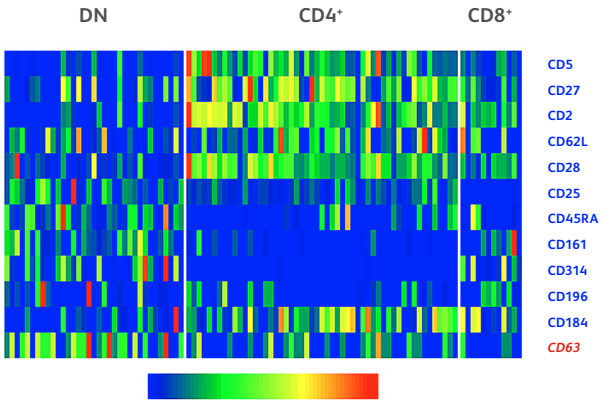

Figure 4. Assessment of markers on the surface of ILC1 cells using a 21-color flow cytometry panel

Fresh PBMCs from the same healthy donor used for the single cell multiomic study were stained with the fluorochrome-conjugated antibodies listed in Table 3. Samples were acquired on the BD FACSymphony A5 Cell Analyzer. **A.** The gating strategy to define ILC1 cells is shown. Lineage$^-$CD3$^-$CD56$^-$CD127$^+$CD117$^-$CD294$^-$ ILC1 cells were further gated into three subsets: CD4$^-$CD8$^-$ (blue), CD4$^+$CD8$^-$ (red) and CD4$^-$CD8$^+$ (green). **B.** Protein expression patterns across the three ILC1 subsets. **C.** Summary of the AbSeq and scRNA-Seq data described in Figure 3 showing overall consistent protein (blue) expression patterns, as compared to high-parameter flow cytometry analysis. Relatively similar patter expression for the gene CD63 (red) was also observed.

Having demonstrated the quality and validity of the high parameter panel, this assay was then used to investigate the distribution of ILC1 subsets across 4 healthy donors. We used unsupervised computational analysis to eliminate bias introduced by manual gating and to facilitate the analysis of high dimensional data. First, we gated on ILC1 cells and performed dimensionality reduction resulting in the generation of a t-Distributed Stochastic Neighbor (t-SNE) plot for all 4 concatenated donors. Next, we used PhenoGraph algorithm for the identification of distinct ILC1 sub-clusters. To facilitate visualization, the 13 clusters identified by PhenoGraph were overlayed onto the t-SNE map (Figure 5A). Subsequently, the same t-SNE map was generated for each individual donor to visualize differences in cluster distribution. Cluster 3 (orange) is evident only in donor 2 while cluster 4 (green) is present mainly in donors 2 and 4. Figure 5B shows the quantitation of the frequency of cells belonging to each cluster across the 4 donors.

Figure 5A

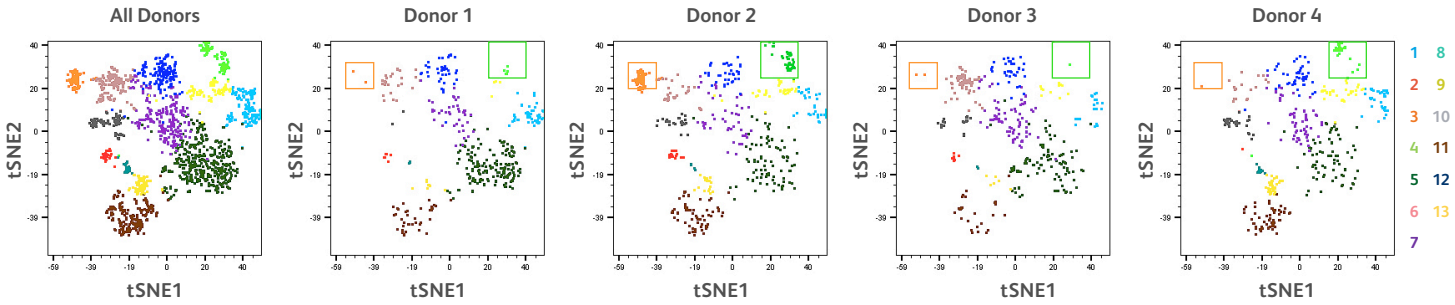

Figure 5B

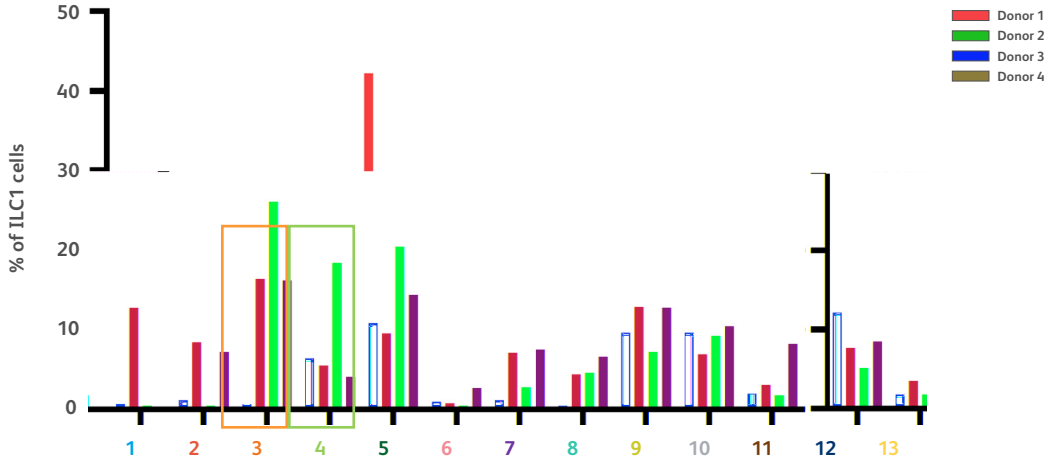

Figure 5. High-dimensional analysis of subpopulations of ILC1 cells across different donors

Fresh leukocytes from four healthy donors were stained with the fluorochrome-conjugated antibodies listed in Table 3. **A.** Data from all four donors were combined and analyzed by FlowJo PhenoGraph plugin, then shown for each individual donor. **B.** The distribution of each cluster within the ILC1 subset was quantified across all donors.

We then used the Cluster Explorer FlowJo plug in to characterize the immunophenotype of the cells in each cluster. This tool provides information on the levels of expression of selected markers in cells belonging to the chosen cluster. For example, the cells in cluster 3 were defined by high expression of CD4, CD62L and CD25, and the lack of expression of CD45RA and CD27. A manual gating strategy was developed accordingly to validate the signature and confirm the presence of this $CD4^{+}CD62L^{+}CD45RA^{-}CD25^{+}CD27^{-}$ ILC1 subset only in donor 2 (Figure6).

Figure 6

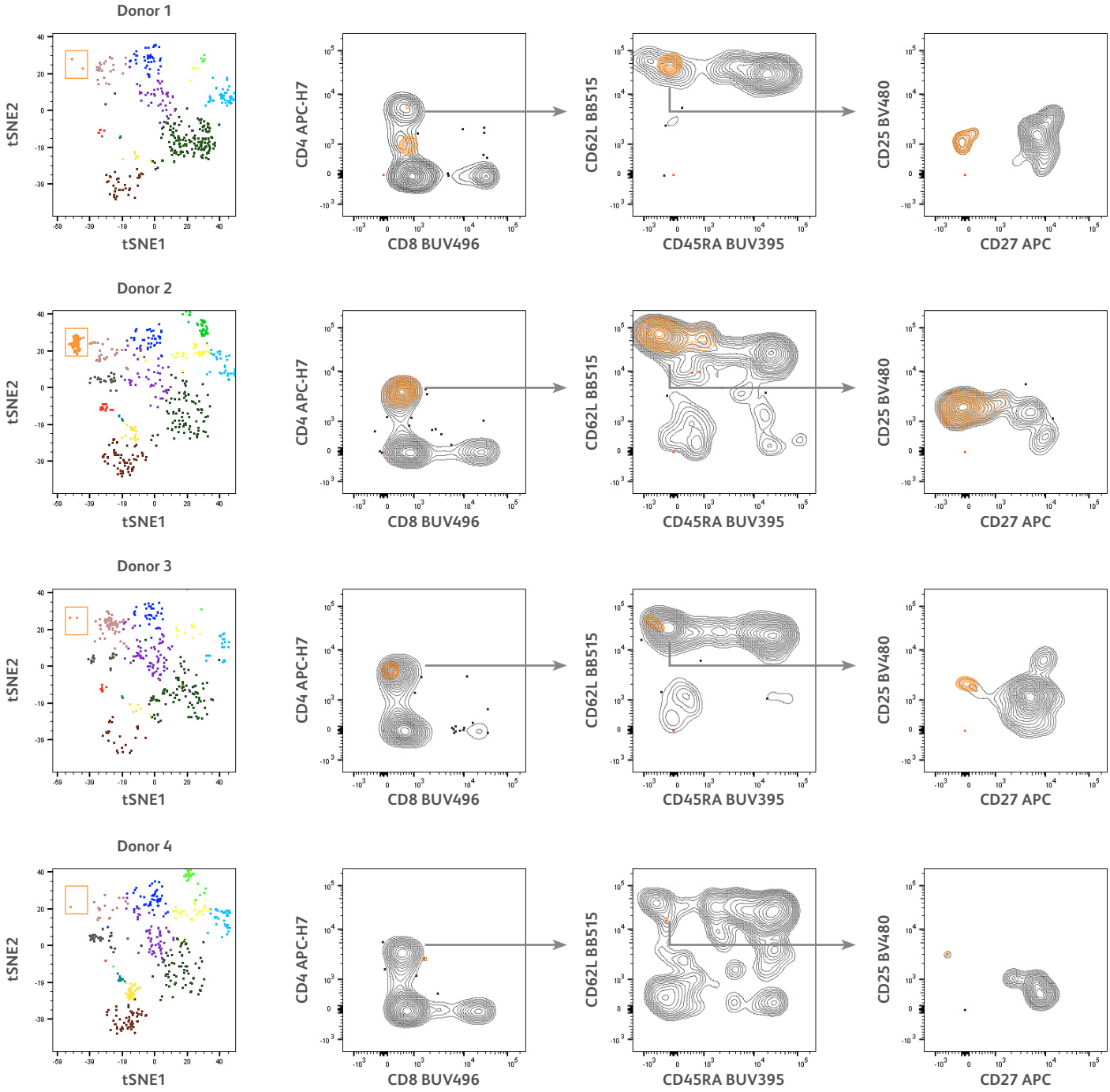

Figure 6. Confirmation of ILC1 sub-cluster phenotype

The phenotype of cluster 3 was validated using conventional manual gating strategy.

In conclusion, we have demonstrated a complete workflow for deep characterization of circulating human ILCs. Cell sorting enabled purification of total ILCs for simultaneous downstream analyses of the expression of 42 proteins and 399 genes. Single cell multiomic analysis revealed complex immunophenotypic and molecular signatures refining the identity of the three main groups of ILCs. The analysis also revealed heterogeneity within ILC1 cells leading to the discovery of markers defining distinct ILC1 cell populations. The biological information provided by the single cell multiomic screening was instrumental for the design of a high parameter flow cytometry panel that could be used to deeply characterize ILC1 cells from multiple donors. Unsupervised computational analysis, mediated by plugins available in FlowJo software, led to the observation of differential distribution of ILC1 cell populations across different donors. It also enabled the identification of a specific cluster that was present in only one of the donors tested. The advent of high parameter cell sorters will enable purification of cells and facilitate the downstream workflows demonstrated here to ultimately define the identity and function of putative new subsets of ILCs.

Product information

Systems and software
Description
BD Rhapsody™ Single-Cell Analysis System
BD FACSAria™ Fusion Cell Sorter
BD FACSymphony™ A5 Cell Analyzer
FlowJo™
SeqGeq™

Reagents
Description
BD Rhapsody™ Human Immune Response Panel
BD® AbSeq Antibody-oligonucleotide Conjugates
BD Fluorescent Antibodies
BD Rhapsody™ Targeted mRNA and AbSeq Reagent Kit

Class 1 Laser Product.

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