Single cell multiomic analysis for the resolution of innate lymphoid cell heterogeneity

Background

Single cell multiomics approach for extensive immunophenotypical and transcriptional characterization of human circulating innate lymphoid cells (ILCs):

Workflow solution:

- Complete workflow solution including cell sorting, protein and mRNA detection, data analysis and high-parameter flow cytometry.
- The BD® Human Single-Cell Multiplexing Kit (SMK) allows sample pooling prior to cell sorting thus reducing and harmonizing sample manipulation.
- BD® AbSeq reagents can be used for purity check and contaminant exclusion prior to downstream analysis.

Single cell multiomic analysis:

- Unprecedented ability to simultaneously assess 42 proteins and 399 genes at the single cell level.
- Resolution of intra-donor ILC heterogeneity and identification of distinct subpopulations via combined protein and gene expression profiles mediated by unsupervised high-dimensional analysis.

Marker screening:

- Identify unique signatures defining distinct ILC subsets based on combined gene and protein expression analysis.
- Gain deep understanding of ILC biology (co-expression patterns and levels of expression of identified targets) to guide optimal flow cytometry panel design.
- Validate the observed signatures using high-parameter flow cytometry.

Innate lymphoid cells

- Innate immune cells play a role in inflammation, infection, cancer, metabolic disorders and allergies.
- Major ILC subsets have been characterized in several mucosal and non-mucosal tissues.
- ILCs are highly heterogeneous and broadly defined based on immunophenotype and function.
- Comprehensive phenotype of ILC subsets is still elusive and controversial.

Challenges of ILC characterization

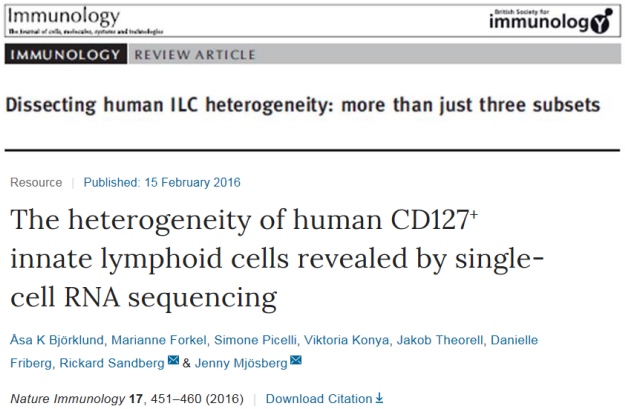
- ILCs are rare, highly heterogeneous and sensitive to cell manipulation.
- Conventionally, pre-enrichment followed by cell sorting is used to purify ILCs:
  - Extended cell processing has a detrimental impact on cell viability and/or protein and gene expression profiles.
  - Post-sort purity check is often not feasible due to the low number of cells, or would result in significant loss of precious cells.
- High-parameter flow cytometry (up to 35 parameters) and single cell RNA sequencing have been individually utilized to resolve ILC heterogeneity:
  - The identity of ILCs is still elusive and controversial.

Overcoming challenges of ILC characterization

We have optimized a workflow that minimizes and harmonizes cell processing for the simultaneous purification and downstream analysis of different donors:

- Co-staining cells with fluorochrome- and oligo-conjugated antibodies enables upstream and downstream lineage exclusion via cell sorting and AbSeq, respectively.
- Allows exclusion of potential sort contaminants and provides higher confidence in the data, especially when purity checks cannot be performed post-sort.
- The simultaneous analysis of 42 surface markers and 399 genes at the single cell level provides an unparalleled level of ILC characterization:
  - Identification of expression patterns that further refine the phenotype of ILC subsets.
  - The data can guide high-parameter flow cytometry panel design for validation and high-throughput analysis of ILCs from multiple donors.

The elusive and controversial identity of ILCs



Immunology

IMMUNOLOGY REVIEW ARTICLE

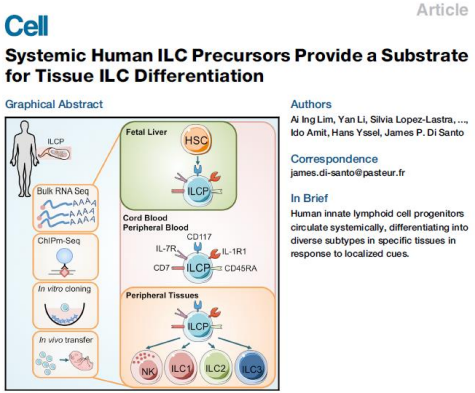
Dissecting human ILC heterogeneity: more than just three subsets

Resource | Published: 15 February 2016

The heterogeneity of human CD127+ innate lymphoid cells revealed by single-cell RNA sequencing

Åsa K Björklund, Marianne Forkel, Simone Picelli, Viktoria Konya, Jakob Theorell, Danielle Friberg, Rickard Sandberg & Jenny Mjösberg

Nature Immunology **17**, 451–460 (2016) | Download Citation



Cell

Article

Systemic Human ILC Precursors Provide a Substrate for Tissue ILC Differentiation

Graphical Abstract

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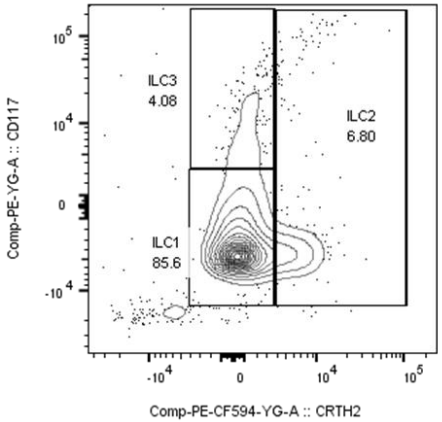
In Brief
Human innate lymphoid cell progenitors circulate systemically, differentiating into diverse subtypes in specific tissues in response to localized cues.

- A population of CD4+ T cell-like "contaminants" has been reported in circulating ILC1 (Simoni *et al. Immunity 2017; comments from Ziegler and Spits).*
- Circulating cells expressing the conventional ILC3 phenotype (Lin−CD127+CD117+CD294−) have been shown to be enriched in ILC precursors (ILCP), lacking expression of RORgt and IL-17/IL-22 output upon stimulation (Lim et al. *Cell 2019)*.
- Transcriptional heterogeneity has also been observed in tonsil ILCs via singe cell RNA sequencing.

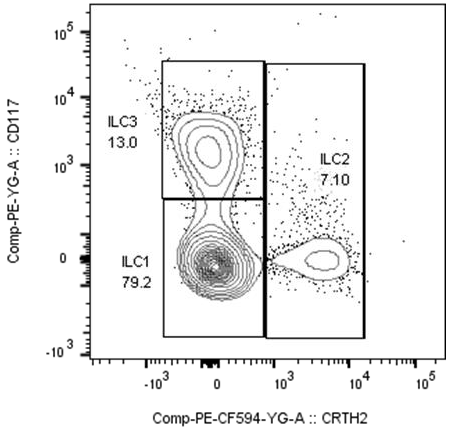
Workflow solution

Pre-sorting cell preparation

PBMC (CPT)



Whole Blood (HetaSep™)



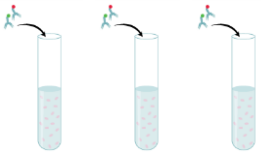
- Several magnetic enrichment methods consistently resulted in low ILC yield.
- This would require, in some cases, more than 100 mL of blood to obtain ~30,000 ILCs.
- Minimal cell manipulation enabled by HetaSep™-preserved ILCs and yielded enough ILCs from 15 mL of blood.
- How can cell sorting time be minimized when processing more than 1 donor?

Workflow

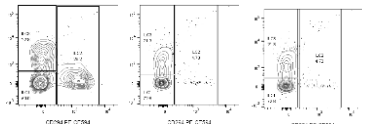
1. RBC Lysis (HetaSep™)

Donor 1 Donor 2 Donor 3

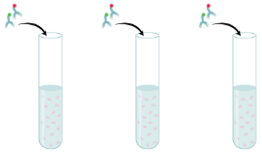
2. Stain a Sample Aliquot for Flow Cytometry Analysis



3. ILC Subset Analysis and Enumeration



4. Stain with SMK, Sort and AbSeq Panels

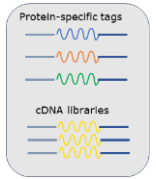


5. Pool Samples

6. Sort Lin-CD127⁺ Total ILCs

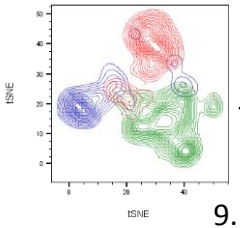
7. Single Cell Capture

8. Library Preparation



9. Sequencing

10. Data Analysis



11. High-parameter Flow Cytometry Validation

Panels

Step 2

Flow Cytometry Panel	
Marker	**Fluorochrome**
Lineage	BV510
CD45	FITC
CD3	APC-H7
CD56	PE-Cy™7
CD127	BV421
CD161	APC
CD117	PE
CD294	PE-C594

Step 4

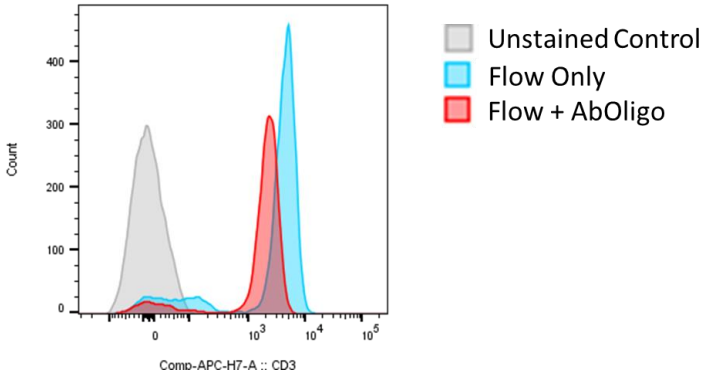
42-plex AbSeq Panel		
CD103	CD90	CD98
CD161	CD62L	CD294
CD11b	CD16	LAG3
CD69	CD184	B7-H1
CD278	CD117	B7-H2
CD25	CD314	TIM3
CD183	CD335	PD-1
CD4	CD226	CTLA-4
CD196	CD94	CD49d
CD7	CD57	CD336
CD11c	CD28	CD45RA
CD8	CD34	CD27
CD3*	CD2	CD19
CD14	CD5	CD56*

Cell Sorting Panel	
Marker	**Fluorochrome**
Lineage	BV510
CD45	FITC
CD3*	APC-H7
CD56*	PE-Cy™7
CD127	BV421

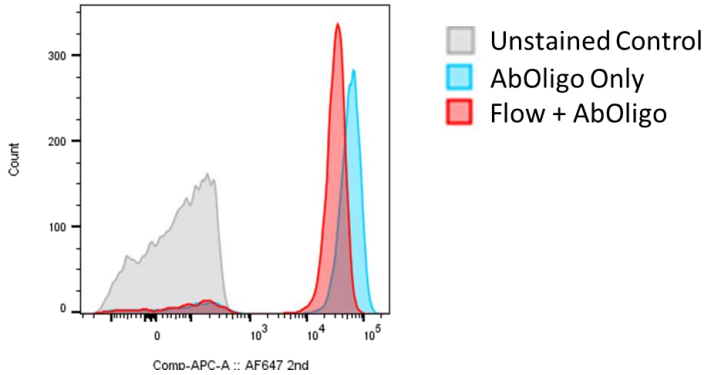
*Cells were co-stained with fluorochrome- and oligo-conjugated CD3 and CD56 antibodies.

Flow cytometry analysis of CD3 reagents

CD3 APC-H7



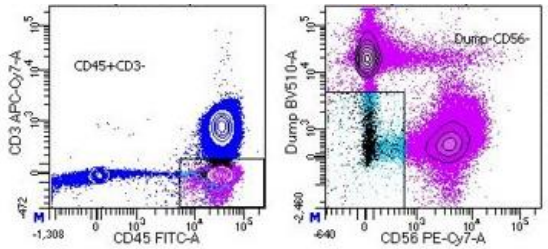
CD3 AbSeq + AF647 Probe



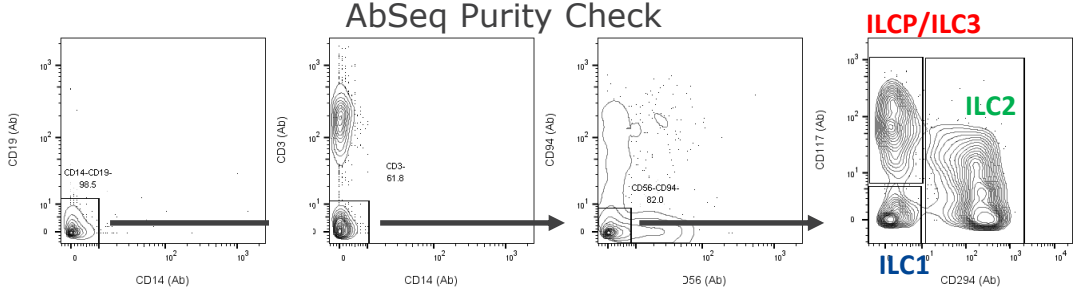
- Here, we validate co-staining a same target with both fluorochrome- and oligo-conjugated antibodies.
- The resolution of CD3 APC-H7 is minimally impacted by co-staining with CD3 AbSeq (1:1), determined by flow cytometry analysis.
- Similarly, the resolution of CD3 AbSeq (detected by AF647 probe) is minimally reduced in the presence of CD3 APC-H7, determined by flow cytometry analysis.
- The data shows that co-staining CD3 APC-H7 and CD3 AbSeq is feasible and enables resolution of both formats for upstream and downstream exclusion of T-cell contaminants.

Exclusion of sort contaminants via BD AbSeq

Sort Gating Strategy

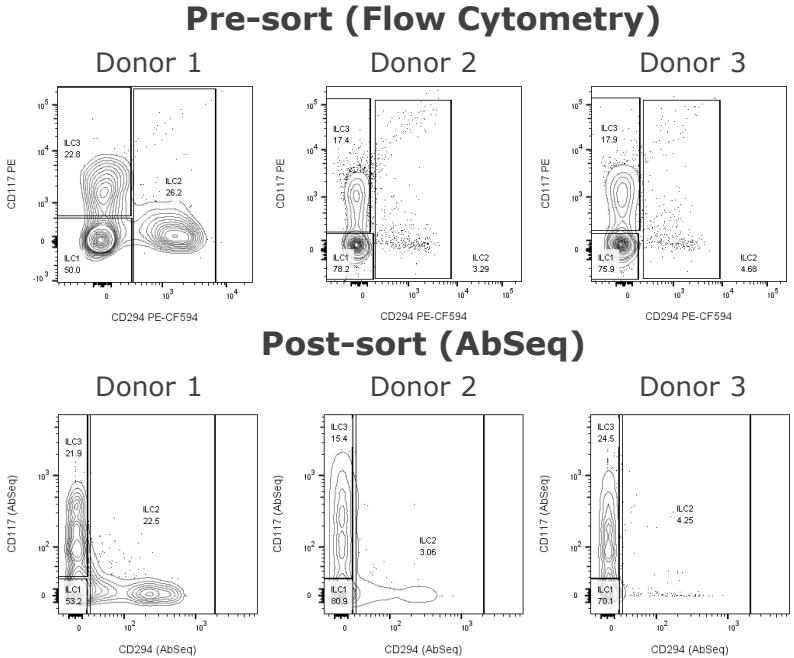


AbSeq Purity Check



- A purity check can be performed with flow cytometry, however, no further action can be taken.
- Including the lineage marker in the AbSeq panel makes it possible to exclude contaminants in downstream analysis. This is important, considering that even a 5% CD3 contamination would impact the confidence in the data.
- The AbSeq purity check can also be used when post-sort flow cytometry purity checks cannot be performed due to the low yield of target cells.

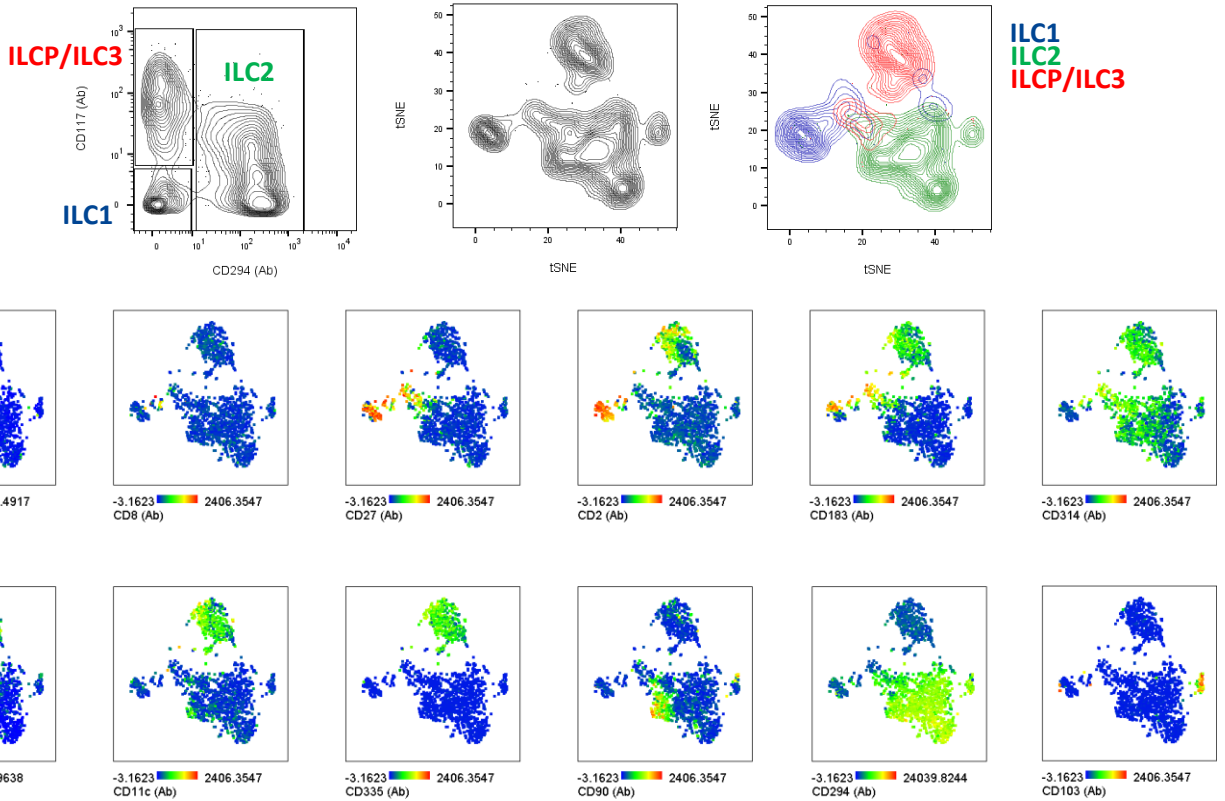
Pre- and post-sort ILC subset analysis



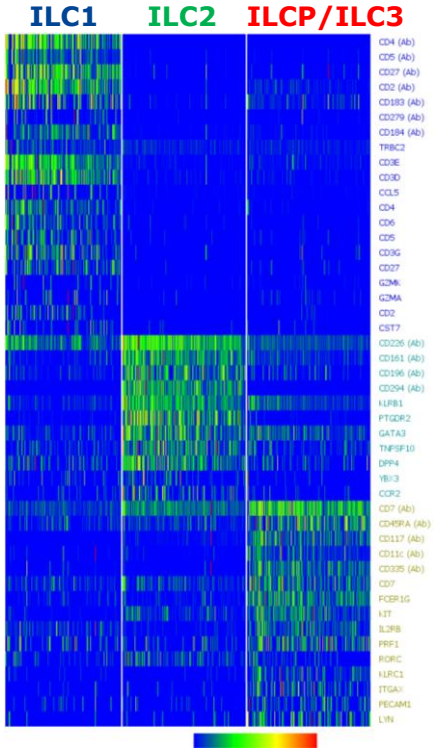
- Comparable resolution and quantitation of major ILC subsets were seen using BD AbSeq assays and flow cytometry.
- The BD® Human Single-Cell Multiplexing Kit makes it possible to combine samples from three donors for sorting. Results for each donor can be parsed after deconvolution of the sequencing result.

Single cell multiomic analysis

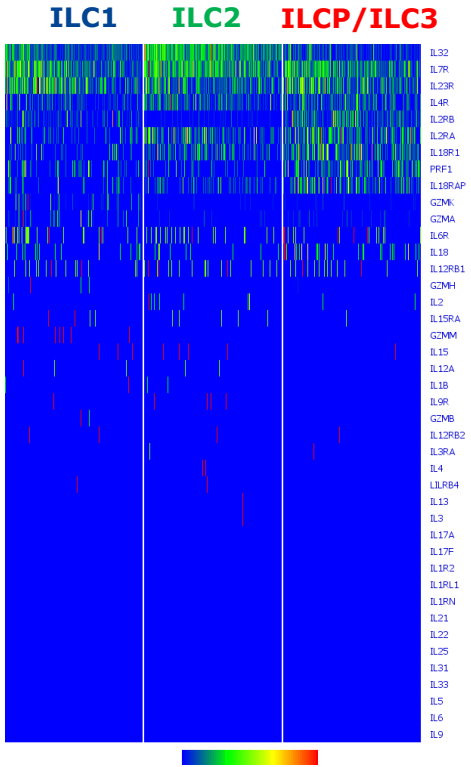
Heterogeneity of major ILC subsets

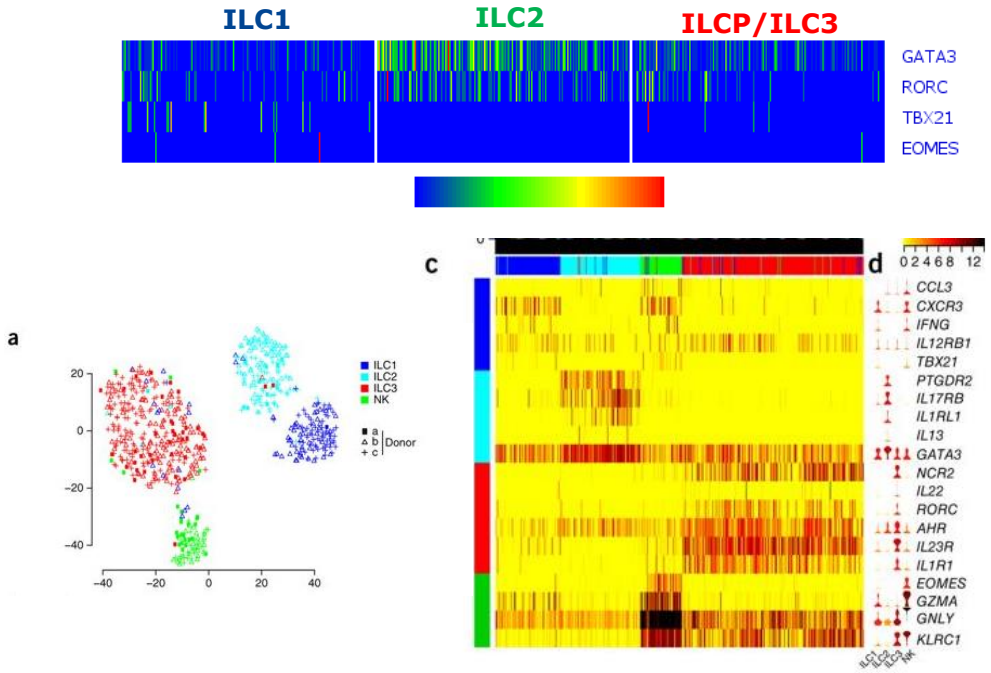


Differential protein and gene expression at the single cell level



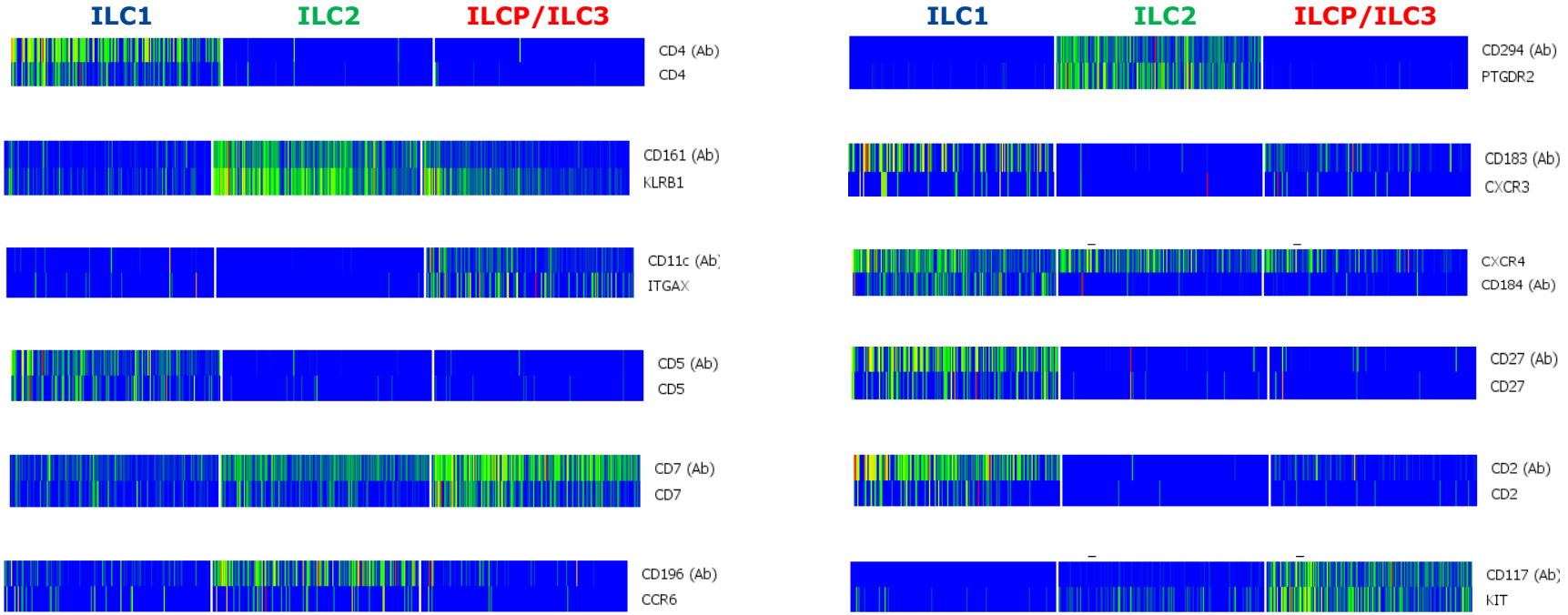
Cytokine, effector molecule and transcription factor expression analysis



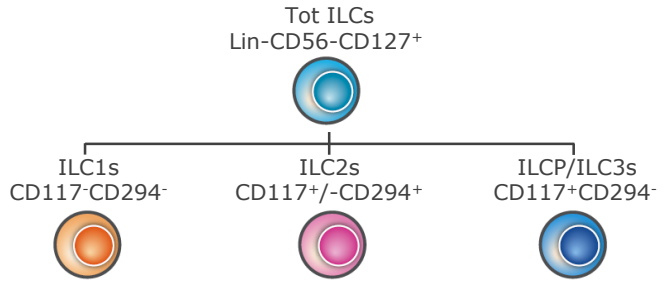


Tonsil ILCs (*Bjorklund, Nat. Immunology 2016*)

Correlation between gene and protein expression



Extensive immunophenotypic and transcriptional analysis of ILC major subsets



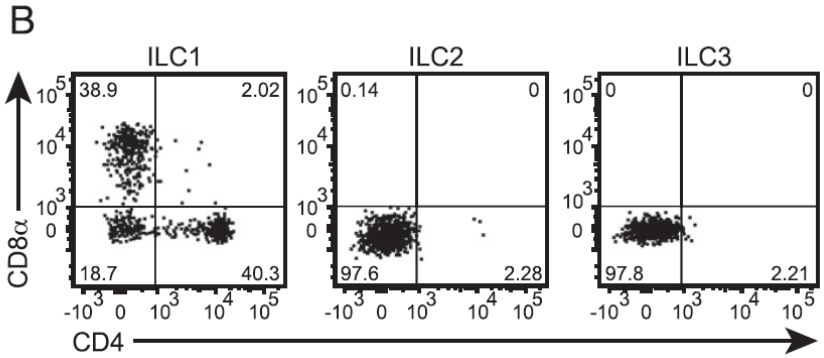
CD4	+	-	-
CD5	++	-	-
CD27	++	-	-
CD2	++	-	++
CD183	+	-	-
CD184	+	-	-
CD6	+	-	-
CD294	-	+	-
CD90	-	+	-
CD161	+	++	+
CD196	+	++	+
CD192	+	++	+
CD117	-	-	+
CD11c	-	-	+
CD335	-	-	+
CD122	+	-	+
CD94	+/-	-	-

ILC1 heterogeneity

The Journal of Immunology

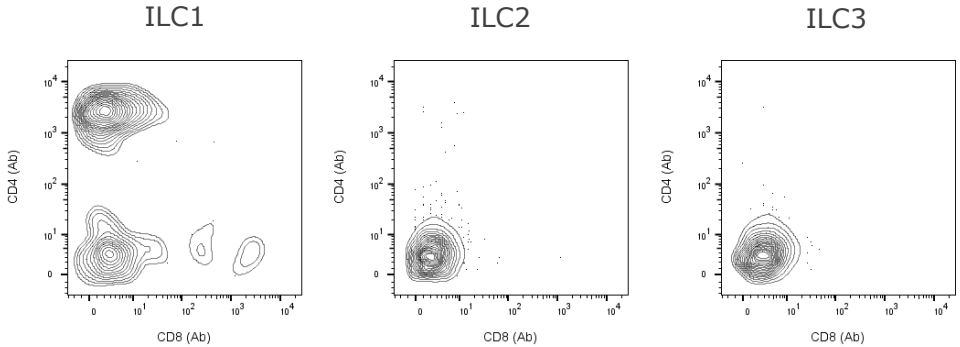
CD4$^+$ Group 1 Innate Lymphoid Cells (ILC) Form a Functionally Distinct ILC Subset That Is Increased in Systemic Sclerosis

Florence Roan, Thomas A. Stoklasek, Elizabeth Whalen, Jerry A. Molitor, Jeffrey A. Bluestone, Jane H. Buckner and Steven F. Ziegler

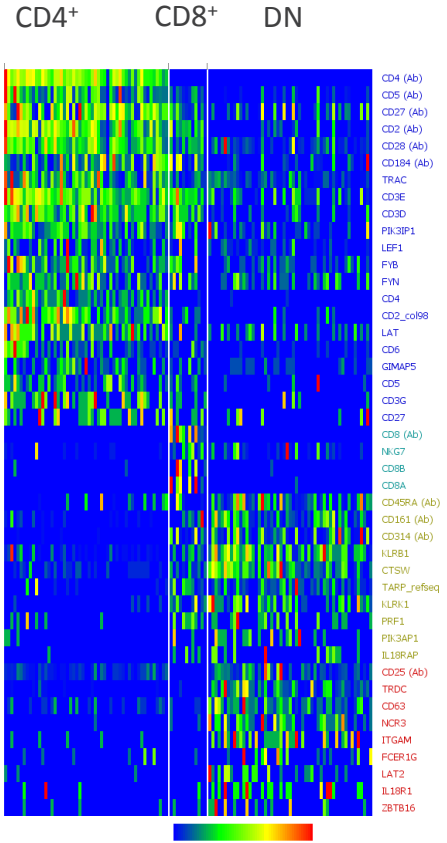


- This study confirmed the existence of a subset of circulating ILC1 cells expressing T-cell markers CD4, CD8, CD5, intracellular CD3e but not surface CD3e and TCR.
- These cells might represent more than just a T-cell contaminant and have a role in disease and homeostasis.

ILC1 heterogeneity



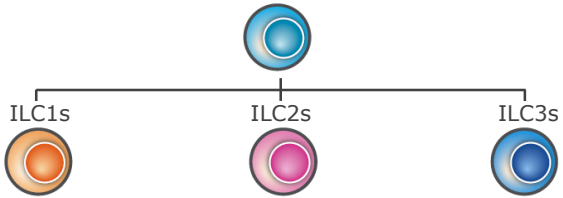
- In agreement with previous reports, $CD4^+$ and $CD8^+$ cells are observed within $CD3^-$ ILC1, but not ILC2 and ILC3.
- Differential gene and protein expression analysis was performed to identify signatures defining each ILC1 subset.



Flow cytometry validation

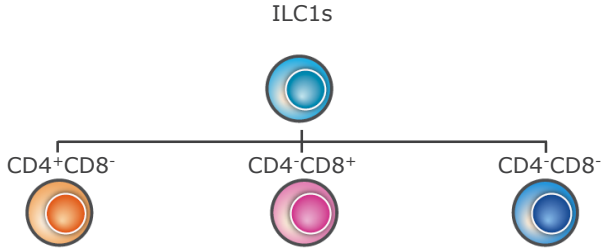
High-parameter flow cytometry panel for extensive characterization of major ILC subsets

Lin-CD127+ ILCs



Lineage-viability	-	-	-	PerCP-Cy5.5/BB700/7AAD
CD3	-	-	-	BUV661
CD56	-	-	-	BUV563
CD94	-	-	-	BV711
CD127	+	+	+	BV421
CD4	+	-	-	BUV496
CD5	+	-	-	APC-Cy7
CD27	+	-	-	APC
CD2	+	-	+	BV605
CD183	+	-	-	BUV395
CD6	+	-	-	BUV805
CD294	-	+	-	BV750
CD90	-	+	-	PE-CF594
CD161	+	+	+	BUV737
CD196	+	+	+	APCR700
CD103	-	+	-	BV786
CD117	-	-	+	PE
CD11c	-	-	+	PE-Cy7
CD335	-	-	+	BV650
CD122	+	-	+	BB515
CD31	-	-	+	BV480

High-parameter flow cytometry panel for deep characterization of ILC1s



Lineage-viability	-	-	-	PerCP-Cy5.5/BB700/7AAD
CD3	-	-	-	BUV661
CD56	-	-	-	BUV563
CD94	+/-	+/-	+/-	BV786
CD117	-	-	-	BV711
CD294	-	-	-	BV750
CD127	+	+	+	BV421
CD4	+	-	-	APC-H7
CD8	-	+	-	BUV496
CD5	+	+	-	BUV805
CD27	+	-	+	APC
CD2	+	+	-	BV605
CD184	+	-	-	PE
CD62L	+/-	+/-	+/-	BB515
CD45RA	+	-	+	BUV395
CD25	-	-	+	BV480
CD28	+	+	+/-	PE-CF594
CD161	-	+	+	BUV737
CD63	-	-	+	PE-Cy7
CD196	+/-	+	+/-	APC-R700
CD314	-	+/-	+	BV650

Conclusions

- Optimized complete workflow solution for the sorting, single cell multiomic analysis and high-throughput high-parameter flow cytometry analysis of rare human circulating ILCs.
- Unprecedented level of ILC characterization based on simultaneous analysis of 42 proteins and 399 genes at the single cell level:
  - Assessment of correlation between gene and protein expression.
  - Identification of immunophenotypic and transcriptional signatures enabling resolution of ILC subsets.
- Our observations are consistent with the literatures reporting ILC immunophenotypic and transcriptional heterogeneity and highlight the value of simultaneously assessing protein and gene expression at the single cell level for a more refined ILC characterization.
- The biological information gathered using the BD Rhapsody™ Single-Cell Analysis system can be used to guide the design of high-parameter flow cytometry panels and enable high-throughput analysis on larger cohorts of donors.
- Functional assays performed using putative ILC subsets would ultimately be required to define cell identity and role.

Thank you!

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