

Multi-cohort study of the immune factors associated with *M. Tuberculosis* infection outcomes

Gaining a better understanding of the immune state of latent Mtb infection

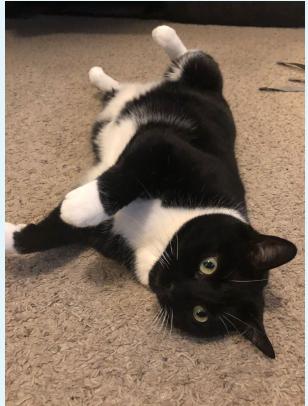
Published: 2018 Chowdhury et. al.

Alex Norman, Kewei Ye, Jordan Smiley, Lexi Wittstadt, Selena Halabi, Lola, Loki, Rebel, Rogue, Anna, Ophelia, Bagel, Ruby, Milo, Clegg



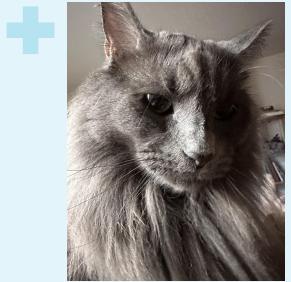


In the spirit of Polly Matzinger, our co-authors are...





Introduction



What is Tuberculosis?



Infection by *Mycobacterium tuberculosis* (*Mtb*)
Transmission through airborne bacteria



- Multiple different states of infection:
- Latent infection (LTBI) → ~¼ of the world's population
 - Active infection → 1 in 10 individuals, symptoms present, can spread disease



Worldwide, but higher prevalence in certain areas (sub-Saharan Africa and Asia)
10 million people infected every year, 1.5 million deaths (WHO)

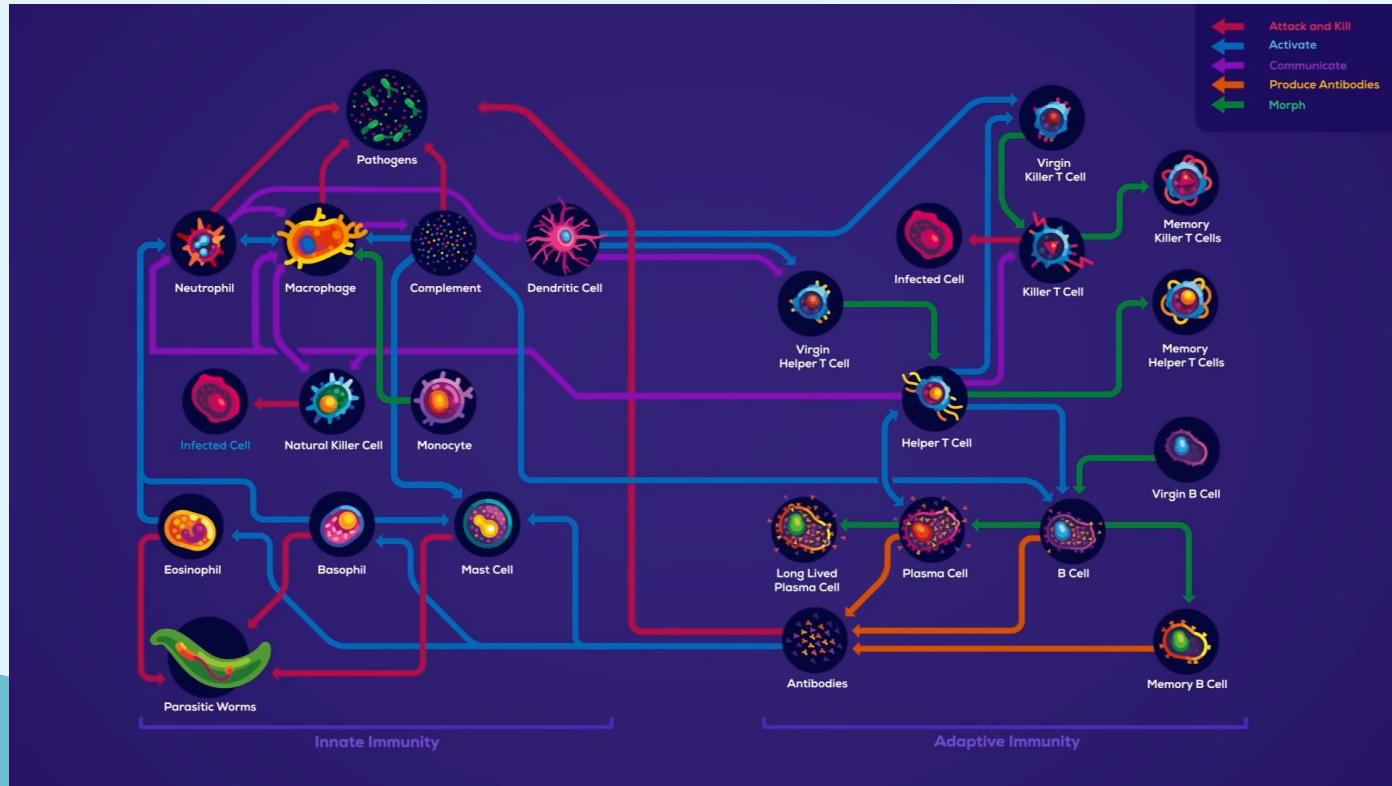


Symptoms:

- Fever/chills
- Chest pain
- Cough
- Fatigue
- Coughing up blood/mucus
- Weight loss



Important cell types to know



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NK cells (*natural killer cells*)

The diagram illustrates the innate immune system. A central purple circle labeled "Natural Killer Cell" contains a blue and yellow virus-like cell. Red arrows point from the NK cell towards various targets: "Pathogens" (a green cell), "Eosinophils" (a green cell with granules), "Mast Cell" (a small green cell), and "Parasitic Worms" (a green worm-like cell). Other cells shown include a "Dendritic Cell" (yellow starburst) and a "Neutrophil" (green cell). A legend at the top right indicates "Attack and Kill" with a red arrow pointing to the NK cell. The entire diagram is set against a light blue background with the text "Innate Immunity" at the bottom.

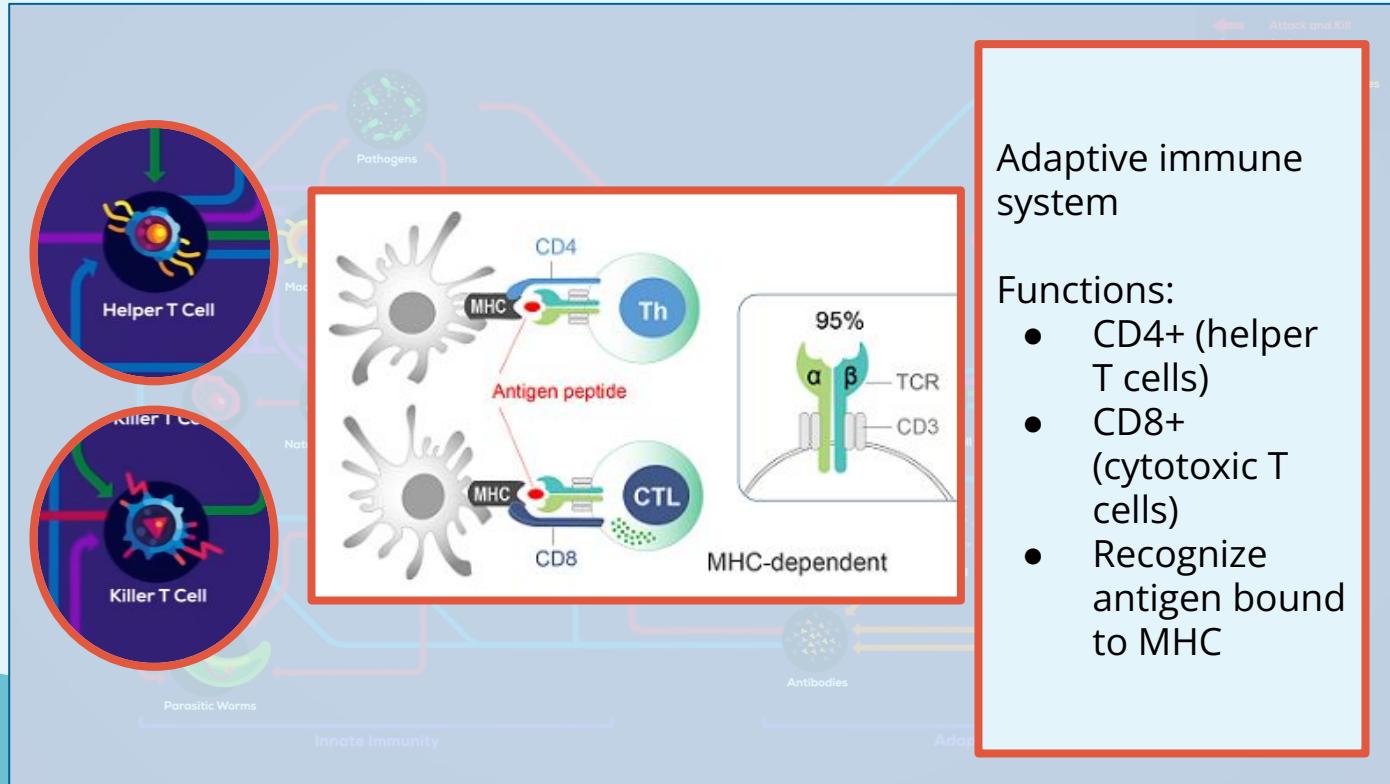
Innate immune system

Functions:

- Release cytokines (IFN- γ , TNF- α)
- Release cytotoxic granules (perforin, granzymes)



$\alpha\beta$ T cells



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Immune by Philipp Dettmer
Arigo Biolaboratories



$\gamma\delta$ T cells

The diagram illustrates the function of $\gamma\delta$ T cells in the innate immune system. On the left, a red-bordered box contains a schematic of a $\gamma\delta$ T cell receptor (1-5% of cells) with two orange Y-shaped chains (labeled γ and δ) binding to MHC molecules on a target cell. To the right, another $\gamma\delta$ T cell is shown attacking a target cell via MHC-independent receptors (MIC A/B and BTN2A/3A). The background shows a green worm-like parasite (Parasitic Worms) being attacked by other immune components like antibodies and complement. The overall title is "Innate Immunity".

Innate immune system

Functions:

- Commonly present in barrier tissues
- MHC-independent receptor recognition
- Release cytokines

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Experimental Design

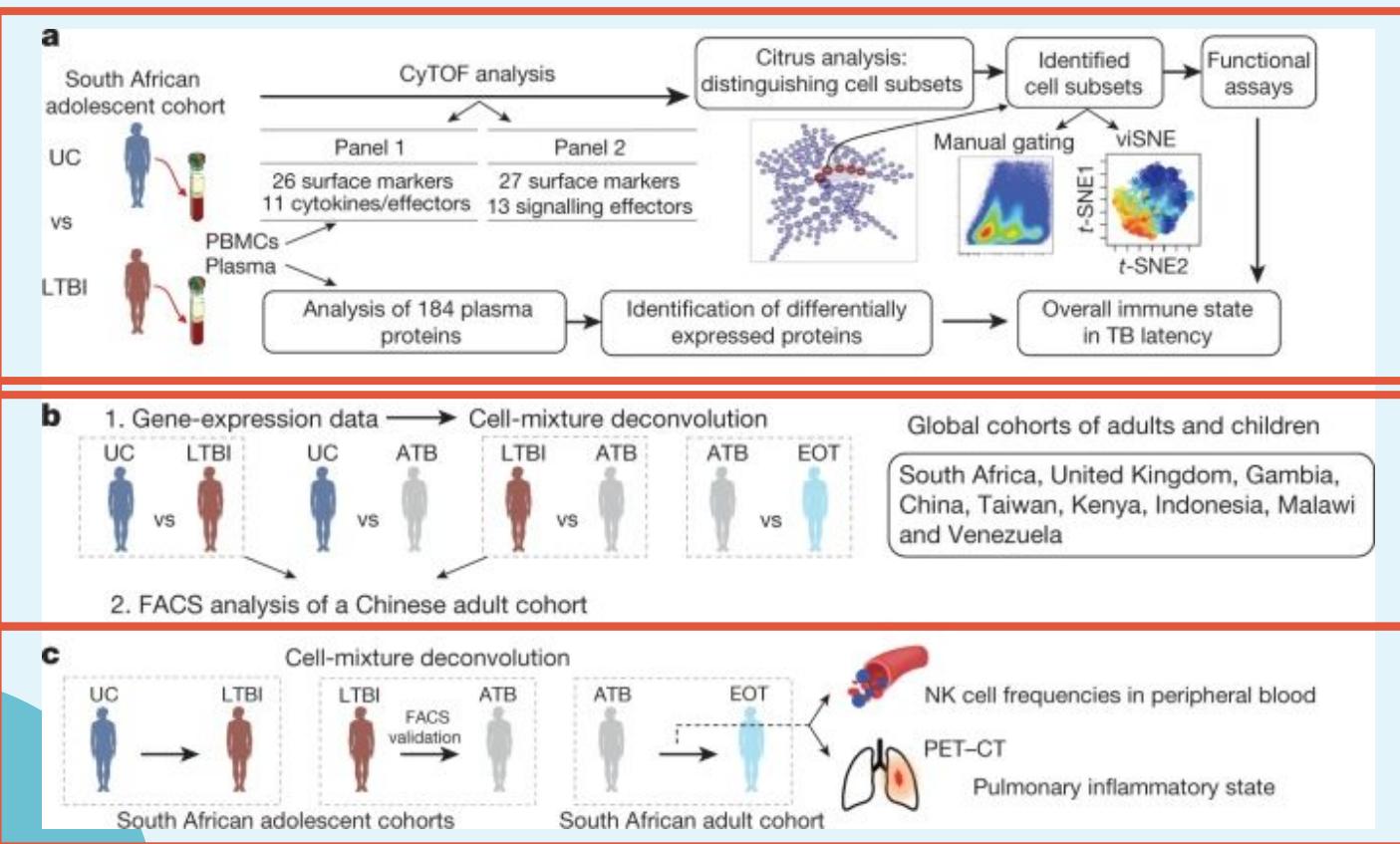


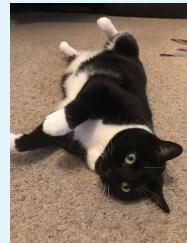
Figure 2

Figure 3

Figure 4



Methodology



Methodology: CyTOF

High-dimensional cytometry by time-of-flight (CyTOF): a proteomics technology that assesses the abundance of cell subsets, protein expression and activation of signalling pathways at the single-cell resolution

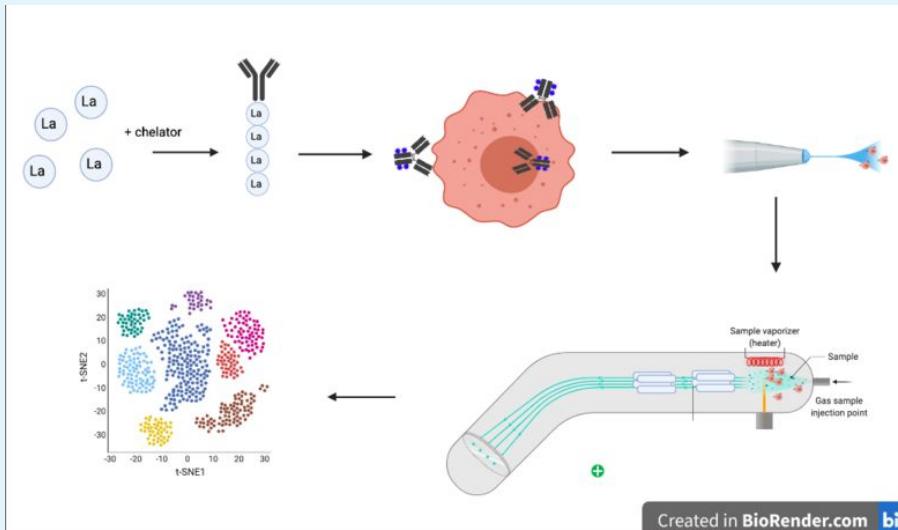
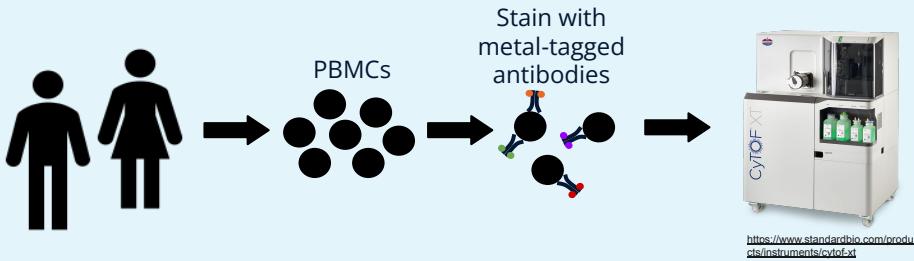
Used by authors to

- Broadly characterize the immune state
- Determine signalling capacity of immune cells in response to immune challenges

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The authors studied **peripheral blood mononuclear cells (PBMCs)** from uninfected and latently infected adolescents from South Africa

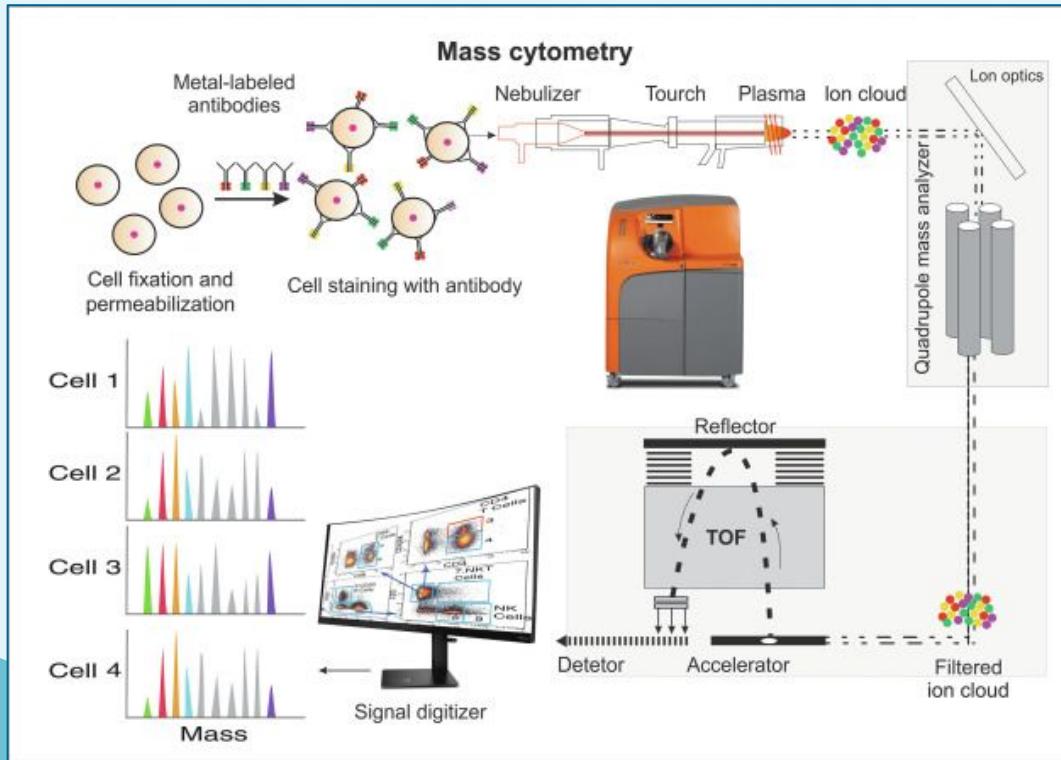


How does CyTOF work?



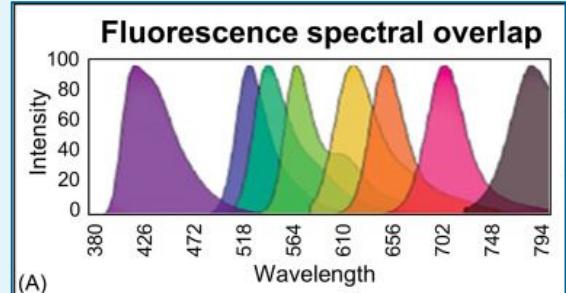


How does CyTOF work?



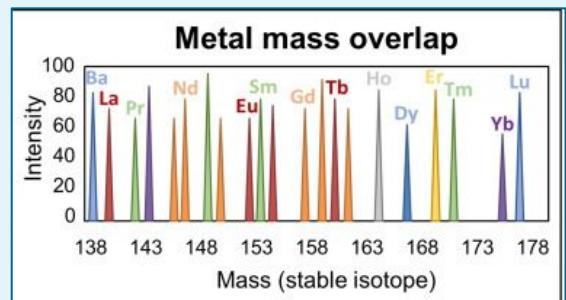
Minakshi et al., *Single-Cell Omics* Volume 1 Ch. 14, 2019

Flow Cytometry



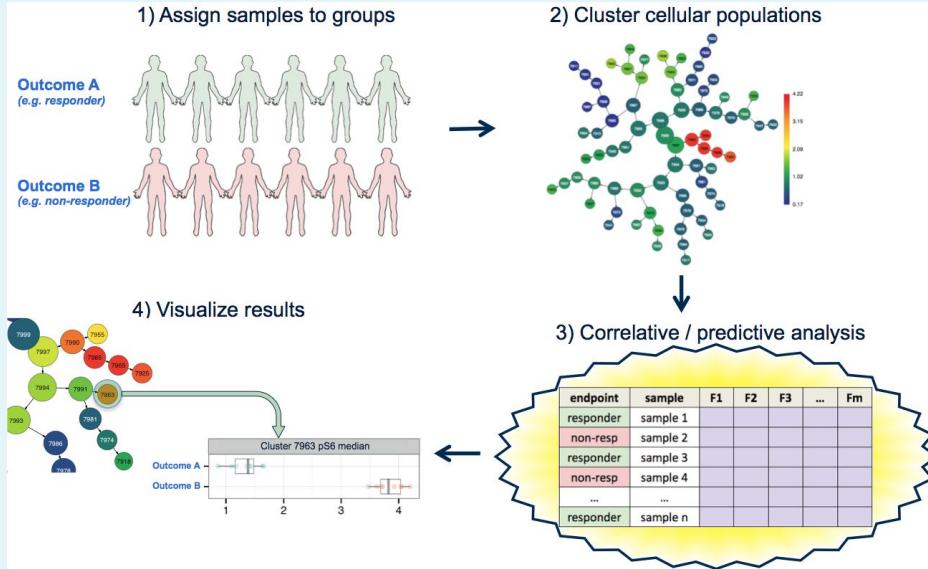
Blair et al., *Platelets (Fourth Edition)*, Ch. 35, 2019

↓
CyTOF



Blair et al., *Platelets (Fourth Edition)*, Ch. 35, 2019

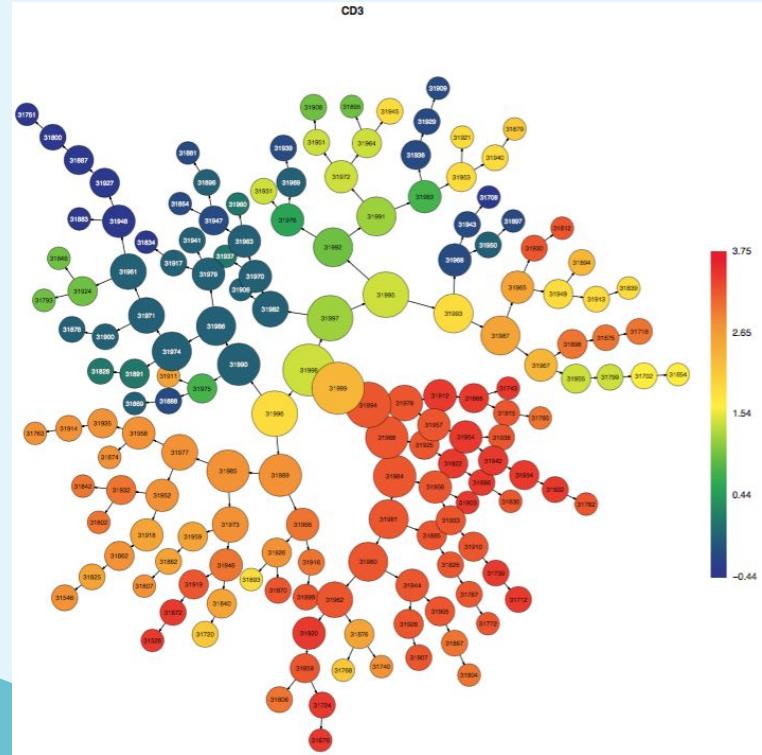
CITRUS (Cluster Identification, Characterization and Regression)



An algorithm that leverages unsupervised clustering and supervised predictive and correlative statistical association models to call out significant feature differences between experimental groups.

<https://support.cytobank.org/hc/en-us/articles/226940667-Overview-of-CITRUS>.

Citrus Example



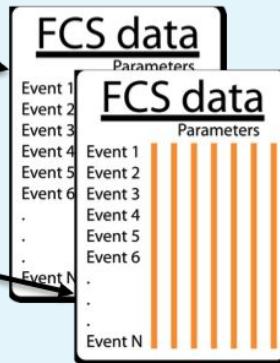
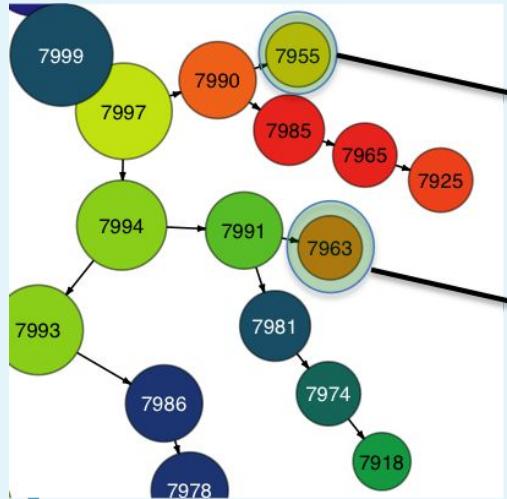
Cluster Characterization Median & Abundance



<https://support.cytobank.org/hc/en-us/articles/227268328-Analysis-and-Intepretation-of-CITRUS-Results>



Steps of Citrus Analysis



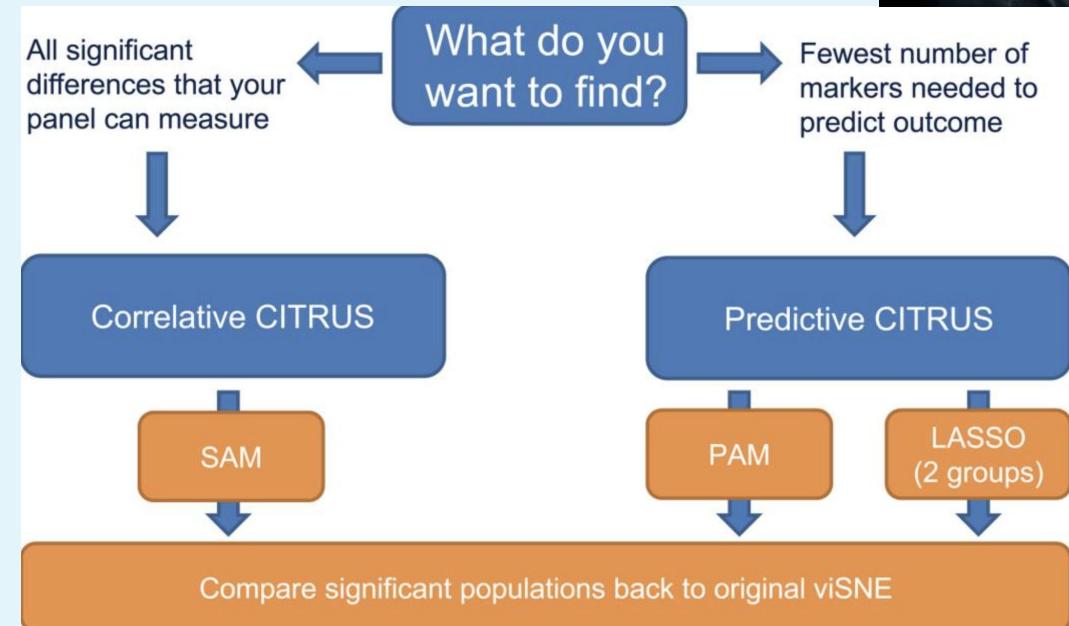
<https://support.cytobank.org/hc/en-us/articles/228224108-Exporting-Clusters-from-a-CITRUS-Analysis-as-New-FCS-Files>

1. FCS files of normalized, 'live cell/no beads' samples were randomly sampled for n single-cell events.
2. Collected single-cell events were pooled and iteratively hierarchically clustered based on similarity of expression of subsets of the measured channels.
3. The pooled dataset was split back into its constitutive samples, and the relative abundance of cells in each cluster was computed, as well as the median expression of each functional marker in each cluster.
4. Used the SAM algorithm in Citrus, which assesses FDR by permutations.

SAM algorithm - Significant Analysis of Microarrays

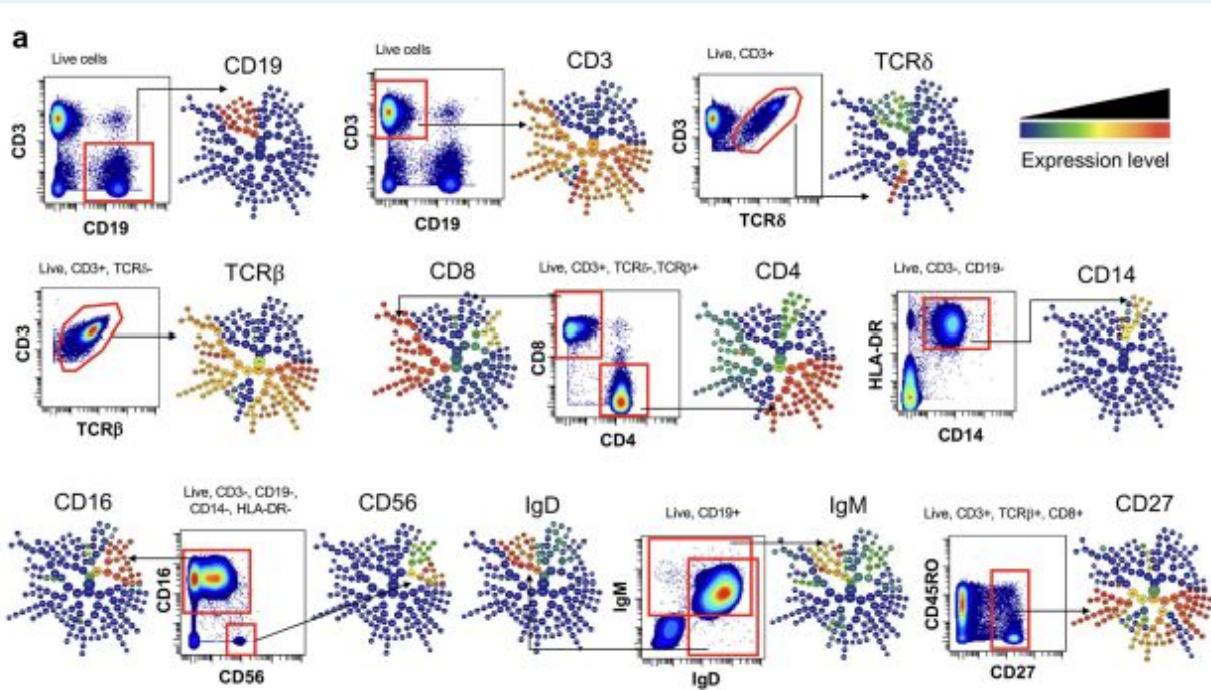


- Used for **large-scale gene or protein expression** data like those collected with microarrays.
- Applied a **t-test** at the **individual gene or protein level**
 - determines whether the expression pattern for that gene or protein is significant.



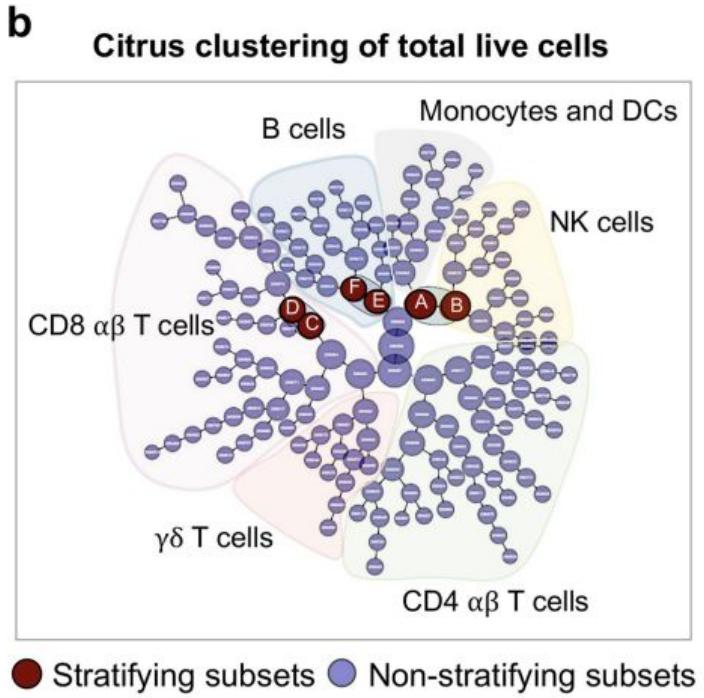
Polikowsky et al. *Mass Cytometry: Methods and Protocols* (2019): 309-332.

Citrus - in this paper



Used for comparison between cell subset abundance and functional marker expression in PBMCs from uninfected controls and subjects with LTBI.

Citrus - in this paper



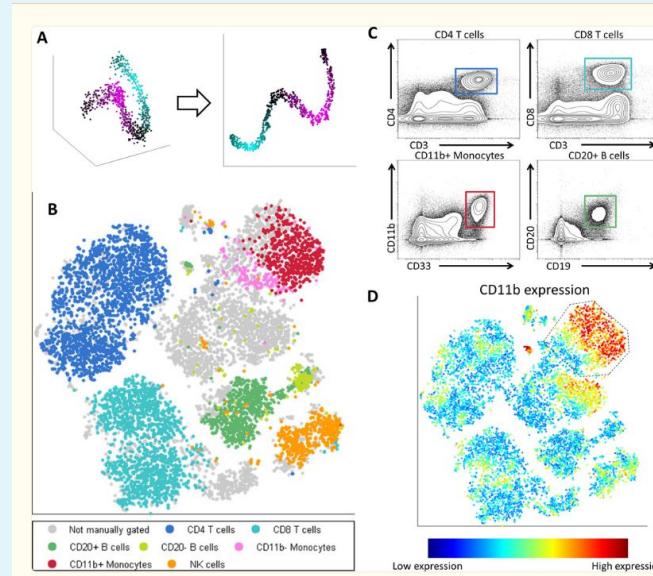
c

Stratifying subsets	Phenotype	Cell populations
A	CD16+, CD3-, CD19-, HLA-DR+/-, CD86+/-, CD56+/-, CD33+/-, CD27-	Total CD16+ cells
B	CD16+, CD56+/-, CD8low/-, CD3-, CD19-, CD14-, HLA-DR-	Total NK cells
C	CD3+, TCR $\alpha\beta$ +, CD8+, CD69-, CD45RO+/-, CD38-/low, CD27-	CD38-/low CD27- CD8+ $\alpha\beta$ T cells
D	CD3+ TCR $\alpha\beta$ + CD8+ CD69- CD45RO+/-, CD38-, CD27-	CD38-CD27-CD8+ $\alpha\beta$ T cells
E	CD19+, CD20+, HLA-DR+	Total B cells
F	CD19+, CD20+, HLA-DR+, IgD+, IgM+	Total Naïve B cells

viSNE (Visualization of High-Dimensional Single-Cell Data)



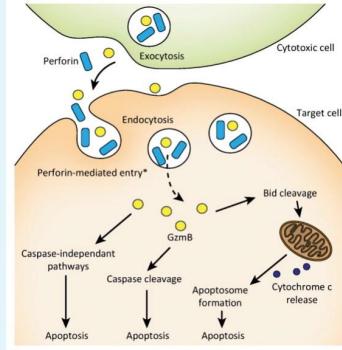
- Reduces high parameter biological data down to two dimensions
- Single cell resolution is still maintained
- Allows researchers to identify phenotypically distinguishable cell subsets and gate single cell events
- Visualization can be limited



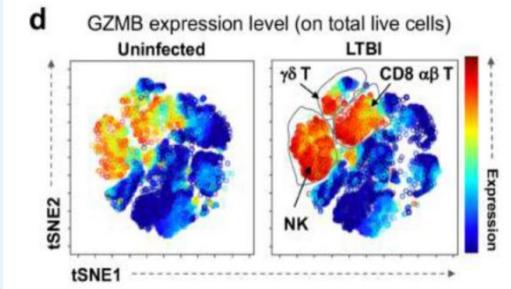
From Amir el-AD et al.
Nat Biotechnol 2013

viSNE Analysis Findings

- Granzyme B (GZMB) expression level in 14 uninfected patients and 14 LTBI patients analyzed
- GZMB released by lymphocytes and NK cells to kill infected and tumor cells
- Significantly more expression found in all cell subsets for LTBI patients over uninfected patients



Hiebert et al., *Trends in Molecular Medicine*, 2012





Cell Mixture Deconvolution

Computational technique

- Measures proportions of different cell types in a mixed sample
- Bulk gene expression data to identify individual cell types
- Percentage of the total live cells

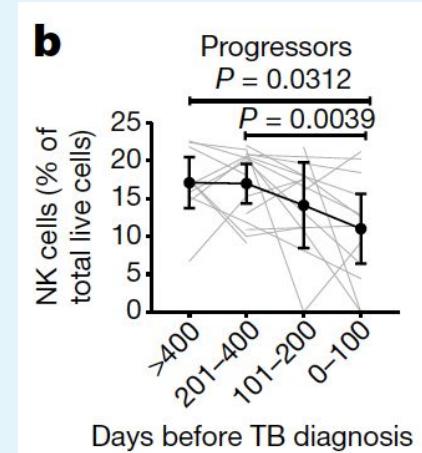
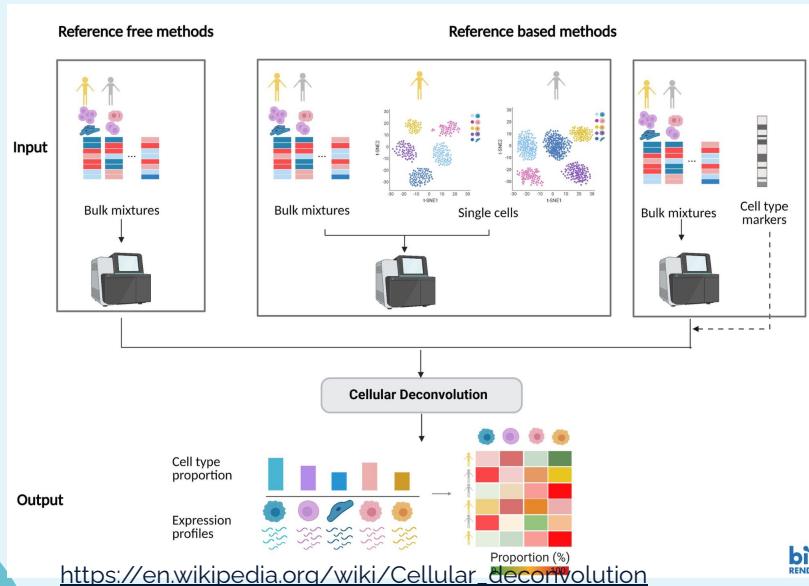


Fig. 4b Statistical Significance of NK cell levels

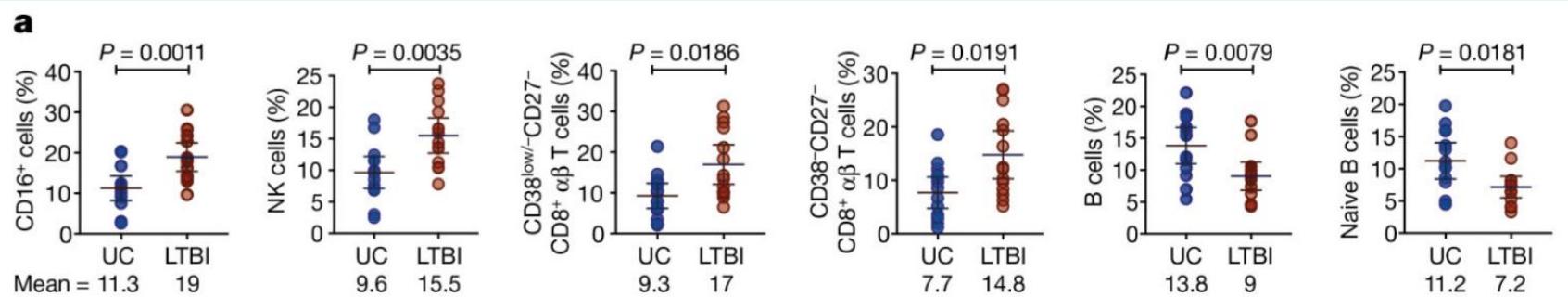
Result

S



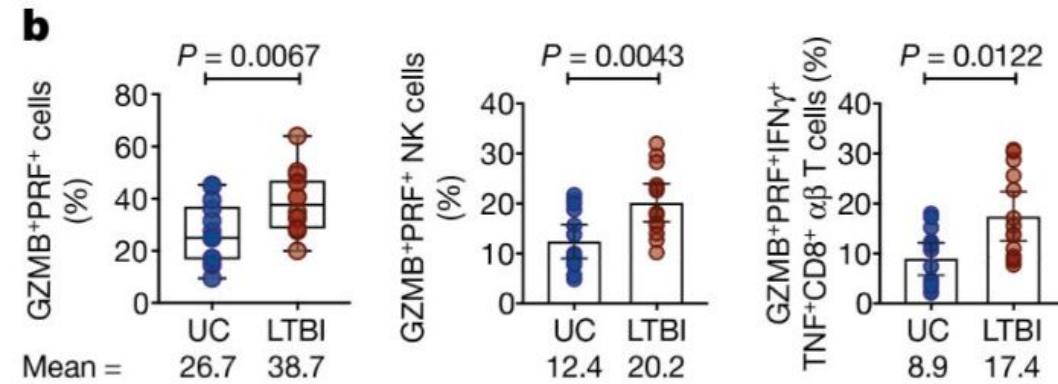
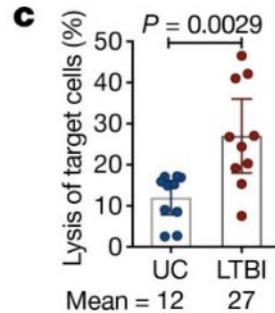
Analysis of cell subsets defined by surface protein expression

Significant differences in the percentage of immune effector cell subsets between samples from uninfected controls and individuals with LTBI were identified.



Enhanced cytotoxic potential

Granzyme B (GZMB)-and perforin (PRF)-expressing cells were **significantly higher** in individuals with LTBI.

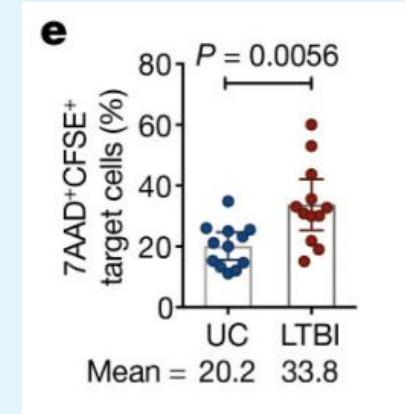
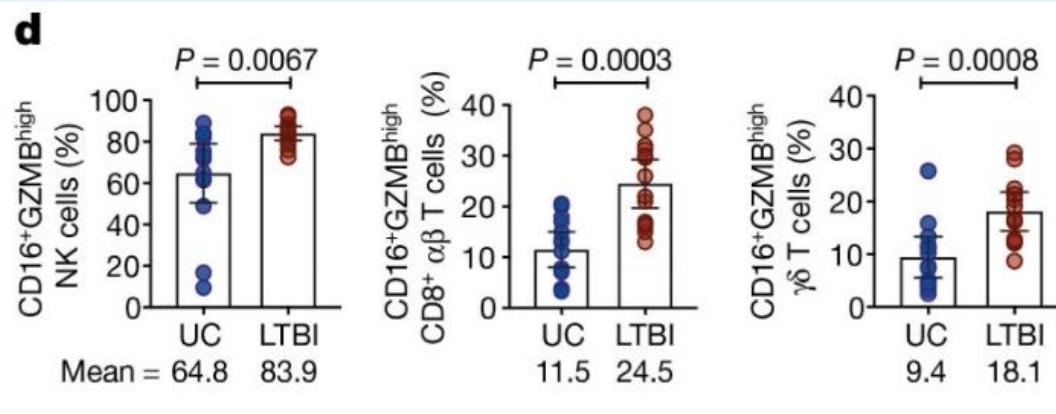


NK cells from individuals with LTBI showed significantly **higher target cell lysis** than those from uninfected controls

Pronounced ADCC

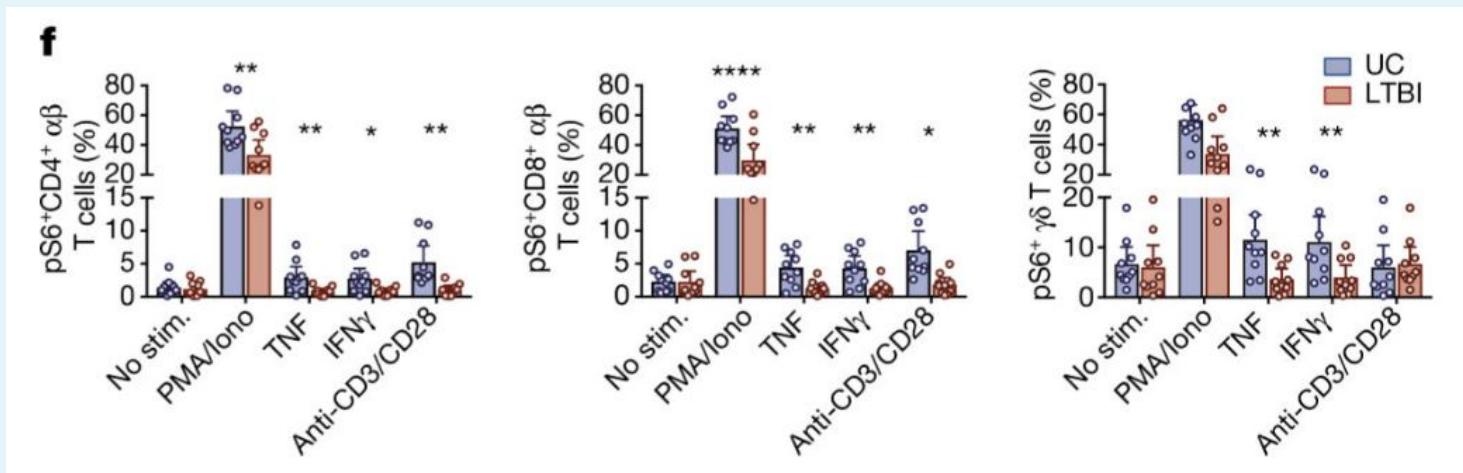
2d. Higher percentages of CD16⁺GZMB^{high} cells within the compartments of the NK cells, CD8+ $\alpha\beta$ T cells and $\gamma\delta$ T cells in PBMCs from individuals with LTB

2e. BMCs from individuals with LTBI also mounted significantly higher antibody-dependent cell-mediated cytotoxicity (ADCC) responses than those from uninfected controls



Signaling Capacity of Immune Cells

Fig. 2f. In individuals with LTBI, all T cell subsets exhibited diminished responsiveness through the S6 signalling pathway, irrespective of the stimulation condition, with the exception of $\gamma\delta$ T cells after stimulation with both anti-CD3 and anti-CD28, as compared to cells from uninfected controls





Discussion

CyTOF



Advantages

- Applications to other illnesses (example HIV)
- Shows a “**big picture**” of disease progression
 - **Simultaneous analysis** of cell processes
 - Observe **concurrent cell behavior** (signalling, proliferation, etc) under given conditions
 - Greater number of targets than possible with traditional flow cytometry (which has spectral overlap)
 - Spectral flow cytometry can address this!

Disadvantages

- Cell loss, requires high number of samples, **no cell recovery**
- **Challenging** to measure **small molecule metabolites**
- Reduced sensitivity of metal masses signal with respect to fluorescence signals, making the **analysis of low signals difficult**
- **Slower** than flow cytometry
 - **Complexity** in data analysis





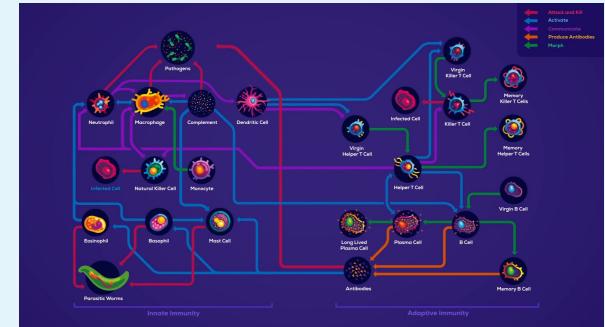
Summary of the Paper

CyTOF and cell deconvolution

- Changes in the levels of **NK cells** and **inflammatory state**
 - predict **progression** on Mtb and **response to treatment**
- Better understanding of the **pathophysiology** of latent TB
- Identify *multiple* factors that influence **patient outcomes**
- Very useful for patients with autoimmune diseases (such as HIV/AIDS)
 - TB is most fatal in this population
 - NK cells show progressive impairment

CyTOF + CITRUS + viSNE

- **Machine learning combination** to understanding the complexity of biological systems



Kurzgesagt,
Immune by Philipp
Dettmer

References

- 1) Amir el-AD, Davis KL, Tadmor MD, Simonds EF, Levine JH, Bendall SC, Shenfeld DK, Krishnaswamy S, Nolan GP, Pe'er D. viSNE enables visualization of high dimensional single-cell data and reveals phenotypic heterogeneity of leukemia. *Nat Biotechnol.* 2013 Jun;31(6):545-52. doi: 10.1038/nbt.2594. Epub 2013 May 19. PMID: 23685480; PMCID: PMC4076922.
- 2) Matthew H. Spitzer, Garry P. Nolan, *Mass Cytometry: Single Cells, Many Features*, Cell, Volume 165, Issue 4, 2016, Pages 780-791, ISSN 0092-8674,
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- 4) Polikowsky, Hannah G., and Katherine A. Drake. "Supervised machine learning with CITRUS for single cell biomarker discovery." *Mass Cytometry: Methods and Protocols* (2019): 309-332.
- 5) Bruggner, Robert V., Bernd Bodenmiller, David L. Dill, Robert J. Tibshirani, and Garry P. Nolan. "Automated identification of stratifying signatures in cellular subpopulations." *Proceedings of the National Academy of Sciences* 111, no. 26 (2014): E2770-E2777.
- 6) RayBiotech. "SAM: Statistical Analysis of Microarrays." RayBiotech Learning Center.
<https://www.raybiotech.com/learning-center/sam/#:~:text=SAM%20is%20a%20method%20used,value%20cut%2Doff%20of%200.01>.



Thanks!

Do you have any questions?



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