Single-Cell, 42-Plex Cytokine Analysis
- from Immune Defense to Immuno Pathogenesis

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The failed HIV Merck vaccine study: a step back or a launching point for future vaccine development?

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Abstract
The world of human immunodeficiency virus (HIV) vaccines has suffered a baffling setback. The first trial of a vaccine designed to elicit strong cellular immunity has shown no protection against infection. More alarmingly, the vaccine appeared to increase the rate of HIV infection in individuals with prior immunity against the adenovirus vector used in the vaccine. A new study in this issue suggests that a different vaccine approach—using a DNA prime/poxvirus boost strategy—induces polyfunctional immune responses to an HIV immunogen. The disappointing results of the recent vaccine trial suggest that a more thorough assessment of vaccine-induced immune responses is urgently needed, and that more emphasis should be placed on primate models before efficacy trials are undertaken.

The Merck candidate vaccine showed good HIV-specific immunogenicity in Phase I and II studies (see http://www.hvttn.org/science/1107.html for the recently released STEP trial results) as measured mostly by a single parameter: the IFN-γ ELISPOT assay. The polyfunctional T helper (Th)1 cells that make higher levels of cytokines on an individual cell basis (e.g., as assessed by mean fluorescence intensity for IFN-γ staining) have been associated with protection in vaccine trials against other microbes as well as in HIV-infected elite controllers (31, 32). Both adenovirus-based vaccines and the DNA/NYVAC vaccine trigger T cells that produce at least three different cytokines in response to the HIV immunogen. The DNA/NYVAC vaccine elicited a greater frequency of CD8+ and Th1 cells in a statistically significant way, compared to the vaccine D3,
Dynamic Functional Heterogeneity

Effector Function: 
- TNFα or IL2
- TNFα + IL2 + IFNγ
- IFNγ + (IL2 or TNFα)

Naïve CD4+ T cell → Central Memory T Cells (CCR7+) → Polyfunctional T cells → Effector Memory T Cells (CCR7-) → Terminally differentiated T cells → Apoptosis

Mario Roederer et al., Nature Review Immunology, 8, 247 (2008).

What the T cell immunobiology tells us: a single parameter ELISpot in most cases detects the terminally differentiated cells, which will undergo apoptosis soon and provide little protective response. This explains in part why the Merck’s trial failed.

What is the desired T cell function analysis tool? ALL functions (40+) on the SAME cell!
a) Cross sectional view

- Glass slide
- Single cell
- PDMS
- Microchambers
- Antibody array

b) Single-cell suspension

- Part (i) - Antibody barcode array slide is super-imposed
- Part (ii) - PDMS micro-chamber array for single-cell capture

- Incubate for 12-24h, capture secreted cytokines by barcodes
- Remove the barcode slide & complete the sandwich assay

- Positions of microchambers

- 3 colors x 15 bars = 45-plex per single cell

- Read out all the spots that lit up in a microchamber
Ultra-high Density Antibody Array

20 microchannels for creating 20-plex 1D microarray
Ultra-high Density Antibody Array
Single Cell Intracellular Protein Assay

Single Cell Western Blotting
(Herr Lab at UC Berkeley)

Single Cell Proteomic Barcode Chip
(Heath Lab at Caltech)
From Academic Prototype to Commercial Product

Conflict of Interest Disclosure:
Rong Fan is co-founder of IsoPlexis Inc, a company that develops single-cell multi-protein assays for monitoring cancer immunotherapy.
Highly multiplexed profiling of single-cell effector functions reveals deep functional heterogeneity in response to pathogenic ligands

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Human Macrophage Response to LPS
Single-Cell Data Quantification
PCA of High Dimensional Single Cell Data

*MIF regulates innate immune responses through modulation of Toll-like receptor 4, Nature 414, 920–924 (2001)*
Clustering of High-Dimensional Single-Cell Data
Measure Cytokine Secretion from The Same Single Cells Before and After Stimulation

The puzzle is solved – our results suggest that MIF potentiates the activation of macrophages in response to LPS and the subsequent production of TLR-4 signaling-associated pro-inflammatory cytokines.
A phenotypically identical cell population still has intrinsic dynamic macrostructure, which is essential for the collective response and the population-level function.
Polyfunctional cells – the small subset of cells with “superpowers”

Protective Cellular Immunity (innate & adaptive)
- High quality and more potent immune protection or defense.

Superheroes

Human Diseases (autoimmune, cancer, etc.)
- Who are they??

Villains
JAK–STAT Pathway Activation in Malignant and Nonmalignant Cells Contributes to MPN Pathogenesis and Therapeutic Response

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**Myelofibrosis**

Myelofibrosis is a severe bone marrow disorder induced by mutation or damage of one or several types of hematopoietic stem/progenitor cells. It results in extensive scarring (fibrosis) in bone marrow. It may also lead to severe systemic symptoms including anemia, weakness, fatigue, and often, an enlarged spleen (splenomegaly), which makes the patient really suffer.
**JAK2 Mutation**

Normal  

JAK2V617F mutation  

MPLW515L mutation

Activation of genes important in proliferation and survival  
& inflammatory cytokines

Elevated Levels of Circulating Cytokines in Patients in MPN

Tefferi et al. Journal of Clinical Oncology (2011)
Verstovsek et al. NEJM (2010)
**Question we ask:** how hematopoietic cells in bone marrow produce inflammatory cytokines to drive MPN pathogenesis?

**The end goal:** more precise monitoring of disease and identifying pathogenic cell subpopulations for therapeutic targeting.
PCA reveals a substantially increased degree of cellular heterogeneity in single-cell cytokine functions in MF.

Mapping cytokines to single cells indicates the presence of two wings – myeloid and lymphoid lineages.

Observed aberrant cytokine secretion pattern and “skewed” hierarchical structure of hematopoiesis in MF.

Increased Frequency of Polyfunctional Hematopoietic Cells in MF

- Significantly greater fraction of cells secreting at least one cytokine in MF
- Majority of BM cells from MigR1 produced fewer than 2 cytokines
- Greater fractions of ‘polyfunctional’ cells that secrete 2 or more cytokines simultaneously from MF mice
Stat3 was selectively deleted in MPLW515L-mutant BM cells.

- Mutant-specific Stat3 deletion did not reduce cytokine production or disease severity.
- STAT3 signaling activated in both mutant and normal hematopoietic cells for MPN pathogenesis.

Mutation-specific STAT3 Deletion Alone Did Not Markedly Reduce Disease Severity
Single-Cell Cytokine Profile: Lineage + BM Cells

(a) LIN+GFP+ MPN

(b) LIN+GFP- MPN

(c) LIN+MigR1
Single-Cell Cytokine Profile: Lineage - BM Cells

(d) LIN-GFP+ MPN

(e) LIN-GFP- MPN

(f) LIN-MigR1
Cytokine Production in Myelofibrosis Originates in Malignant and Nonmalignant Hematopoietic Cells
Treatment of Chronic Myelogenous Leukemia by Blocking Cytokine Alterations Found in Normal Stem and Progenitor Cells

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Convert and Conquer: The Strategy of Chronic Myelogenous Leukemic Cells

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http://dx.doi.org/10.1016/j.ccell.2015.04.012
Towards Human MF Study

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<tr>
<td>PMF 5</td>
<td>Human MF</td>
<td>Circulating CD34+</td>
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Single-cell Cytokine Analysis of Bone Marrow Cells From PMF Patients with JAK2 Mutation

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<tr>
<th>Cytokine</th>
<th>Healthy subject (HC4) Single-cells, n=655</th>
<th>JAK2V617F PMF patient (PMF4) Single-cells, n=1672</th>
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<tbody>
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<td>GMCSF</td>
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Perspective: Polyfunctional Strength Index as a Potential Biomarker

**Multifunctional 3 cytokines**

- VEGF
- MIP-1β
- IL-12
- IL-10
- IL-8
- IL-6
- IL-1β

**Legend**

- * indicates p-value<0.05
- ** indicates p-value<0.01
- *** indicates p-value<0.001

**nd** indicates no statistical significance

**Graph**

- **Primary Myelofibrosis**
- **Healthy Control**

**Image**

Microscopy images showing tissue sections.
Conclusions

- We successfully developed a microchip technology for single-cell, 42-plex cytokine profiling – highest multiplexing recorded to date for single-cell protein secretion assay.

- Phenotypically identical immune cells show a dynamic population macrostructure associated with varying levels of activation states.

- Polyfunctional subpopulation dominates the protective immune response, and once dysregulated becomes the main driver of pathogenesis.

- The cytokine functions of both mutant and “normal” bone marrow cells are substantially skewed and both contribute to MF pathogenesis.

- Single-cell cytokine profile is distinct between MF patients and control, and potentially a biomarker for predicting disease progression.

- Superheroes do exist (at least in biology)! However, their superpower may turn them into villains.
- How to know and how to control? Our device offers the solution.
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Go Bears!