**Dissecting in vitro macrophage diversity through time: a computational analysis of RNA-seq data**

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**Abstract:**

**Introduction**: Macrophages are highly adaptable immune cells that play vital roles in host defence, tissue repair, and the regulation of inflammatory responses. Their remarkable plasticity enables them to respond to diverse microenvironmental signals and differentiate into distinct functional states. Unpolarised M0 macrophages, for example, can transition into either pro-inflammatory M1 macrophages or anti-inflammatory M2 macrophages, depending on the cytokines and stimuli they encounter. M1 macrophages are particularly noted for their response to interferon-gamma (IFN-γ) and lipopolysaccharide (LPS), which triggers the production of pro-inflammatory cytokines and nitric oxide, key factors in pathogen clearance and anti-tumour immunity (Ref). Understanding the diversity and functional specialisation of macrophage populations is crucial for developing targeted therapies for a range of diseases, including infections, cancer, and autoimmune disorders.

Despite this understanding, recent studies, such as that by Shalek et al. (2013), have highlighted the significant heterogeneity that can exist even within seemingly homogeneous populations of immune cells. This heterogeneity can have profound implications for the functional outcomes of immune responses. Consequently, it is essential to dissect the molecular underpinnings of this variability, particularly in macrophage populations transitioning from the M0 to M1 state under controlled in vitro conditions.

To explore the dynamic changes in macrophage populations over time and understand the activation-induced heterogeneity, this study employs both single-cell RNA sequencing (scRNA-seq) and bulk RNA sequencing technologies. These approaches allow for high-resolution analysis of macrophage heterogeneity, providing a deeper understanding of the molecular signatures and pathways that define the diverse functional states of these cells.

In order to enhance the resolution and accuracy of our analysis, we utilise two key gene lists identified in the study by Li et al. (2019): Polarisation Signature Genes (PSGs) and Activation-Induced Macrophage Differentiation Signature Genes (AMDSGs). PSGs are particularly effective in distinguishing between M1-like (pro-inflammatory) and M2-like (anti-inflammatory) macrophages, while AMDSGs are critical for mapping the progression from unpolarised to fully activated macrophage states. By incorporating these gene lists, we aim to accurately characterise the heterogeneity and dynamic transitions that occur during macrophage activation.

**Our Aim:** This study aims to understand activation-induced heterogeneity of macrophage populations in unbiased in vitro settings. To achieve this, we leverage single-cell RNA sequencing (scRNA-seq) and bulk RNA sequencing technologies to analyse the dynamic changes in macrophage populations over time.

**Methods:**

**Datasets Utilised**

This study analysed macrophage heterogeneity using three comprehensive single-cell RNA sequencing (scRNA-seq) datasets. The first dataset, sourced from (REF) provided data at two critical time points, 0 and 48 hours, allowing for the observation of early and late responses in macrophages. The second dataset, obtained from Nature Scientific Reports, included time points at 0, 6, and 24 hours, offering a broader temporal coverage to study intermediate stages of macrophage activation. The third dataset, from Nature, contained high-resolution data at 0, 2, 4, and 6 hours, essential for capturing rapid changes in gene expression and early cellular responses.

This study utilises three significant datasets to perform a comprehensive analysis of macrophage heterogeneity:

1. **Dataset 1**: The Journal of Clinical Investigation ([link](https://insight.jci.org/articles/view/126453#sd)) provides extensive scRNA-seq data on macrophages at time points 0,48 hours.
2. **Dataset 2**: From Nature Scientific Reports ([link](https://www.nature.com/articles/s41598-020-68766-w)), offering insights into the genetic expression profiles of various macrophage populations at 0,6,24 hours.
3. **Dataset 3**: From Nature ([link](https://www.nature.com/articles/s41586-018-0657-2)), which includes high-resolution scRNA-seq data crucial for in-depth analysis, scRNA-seq measured at 0,2,4,6 hours.

**Data Collection and Preparation**

The scRNA-seq datasets were collected from the aforementioned sources. The single-cell RNA-seq data from multiple time points and conditions were processed using the Seurat package. Data were filtered to retain high-quality cells, excluding those with high mitochondrial content or rRNA contamination. The datasets from different conditions and time points were merged and integrated to create a unified dataset, ensuring consistency and comparability across conditions. SCTransform normalisation was applied to mitigate technical variability, followed by PCA for dimensionality reduction. Clusters were identified using a graph-based clustering algorithm and visualised through UMAP and t-SNE projections. Pseudotime analysis with Slingshot provided insights into dynamic macrophage state transitions, tracing lineage paths across different time points.

**Single-Cell RNA Sequencing Analysis**

*Clustering Analysis*: Using R-coding, macrophages were grouped into distinct clusters based on their gene expression profiles, identifying various subpopulations within M0 and M1 macrophages.

*Pseudotime Analysis*: The macrophage subpopulations were arranged along a pseudotime trajectory to observe their dynamic changes and transitions over time.

*Functional Annotation*: The functional roles of each macrophage subpopulation were inferred by analysing active gene expressions, providing insights into their respective roles in immune responses.

**Validation**

*Flow Cytometry (FACS):* Flow cytometry validated the scRNA-seq analysis findings. This technique helped confirm the presence and functional characteristics of the identified macrophage subpopulations.

Results:

Discussion:

References: