Video Article

Single-cell Analysis of Immunophenotype and Cytokine Production in Peripheral Whole Blood via Mass Cytometry

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Abstract

Cytokines play a pivotal role in the pathogenesis of autoimmune diseases. Hence, the measurement of cytokine levels has been the focus of multiple studies in an attempt to understand the precise mechanisms that lead to the breakdown of self-tolerance and subsequent autoimmunity. Approaches thus far have been based on the study of one specific aspect of the immune system (a single or few cell types or cytokines), and do not offer a global assessment of complex autoimmune disease. While patient sera-based studies have afforded important insights into autoimmunity, they do not provide the specific cellular source of the dysregulated cytokines detected. A comprehensive single-cell approach to evaluate cytokine production in multiple immune cell subsets, within the context of "intrinsic" patient-specific plasma circulating factors, is described here. This approach enables monitoring of the patient-specific immune phenotype (surface markers) and function (cytokines), either in its native "intrinsic pathogenic" disease state, or in the presence of therapeutic agents (*in vivo* or *ex vivo*).

Video Link

The video component of this article can be found at https://www.jove.com/video/57780/

Introduction

Autoimmune diseases are a major cause of morbidity and mortality affecting 3-8% of the population. In the United States, autoimmune disorders are among the leading causes of death among young and middle-aged women (ages <65 years)[1,2]. Autoimmune disorders are characterized by heterogeneous clinical presentation and diverse underlying immunological processes. The spectrum of heterogeneity is well represented across different disorders, such as joint involvement in rheumatoid arthritis (RA) and neurological disease in multiple sclerosis (MS). However, this level of heterogeneity is also exemplified within a single disorder, such as systemic lupus erythematosus (SLE): some patients may present with renal pathology, while others suffer from hematologic or joint involvement[3].

The underlying immunopathogenesis in autoimmune disorders mirrors the clinical heterogeneity, involving auto- and hyper-activation of multiple innate and adaptive immune cell subsets, and concomitant dysregulated cytokine production. While cytokines play a pivotal role in the pathogenesis of autoimmune disease, understanding their specific role in the mechanism of disease has proven to be challenging. Cytokines are characterized by pleiotropy (one cytokine can have multiple effects on different cell types), redundancy (multiple cytokines can have the same effect), duality (one cytokine can have pro- or anti-inflammatory effects under different conditions), and plasticity (cytokines can be molded into a role different from its "original" one, depending on the environment)[4,5,6]. Consequently, population-level methods cannot distinguish heterogeneous cellular responses to the same "cytokine milieu". Similarly, study designs that focus on one specific aspect of the immune system (a single cell type or cytokine), do not offer a global assessment of all the elements involved in complex autoimmune disease. While patient sera-based studies have afforded important insights into autoimmunity, they do not provide the specific cellular source of the dysregulated cytokines detected.

Recently, we developed a single-cell proteomic approach to simultaneously assess multiple immune cell types, and detect their various cytokine perturbations in the milieu of patient specific "pathogenic" peripheral blood plasma circulating factors. The workflow described here is characterized by the use of intact peripheral whole blood samples, as opposed to isolated peripheral blood mononuclear cells (PBMCs). Peripheral whole blood represents the most physiologically relevant vehicle to study systemic immune-mediated disease, including 1) non-mononuclear blood cells often involved in autoimmune disease (*i.e.*, neutrophils, platelets), and 2) plasma circulating factors, such as nucleic acids, immune complexes, and cytokines, which have immune activating roles. To capture the "intrinsic pathogenic" dysregulated cytokine production, peripheral blood samples are processed immediately after the blood draw (T0, Time zero), and after 6 h of incubation at 37 °C (physiological body temperature) with a protein transport inhibitor in the absence of any exogenous stimulating condition (T6, Time 6 h), to detect

cytokine production (accumulation, T6-T0) that would reflect the "intrinsic" disease state (**Figure 1**). To study dysregulated processes that reflect over or under-activation of signaling pathways involved in immune responses relevant to the disease, peripheral blood samples are treated (6 h incubation at 37 °C with a protein transport inhibitor) with an exogenous stimulating condition that reflects disease pathogenesis, such as Toll-Like-Receptor (TLR) agonists in the case of SLE (T6 + R848, Time 6 h with 1 µg/mL R848), to detect cytokine production that would reflect a response to nucleic acids (comparing T0 vs. T6 vs. T6 + R848, **Figure 1**). To study immunomodulatory effects of available therapeutics *ex vivo*, as they pertain to the precise immune dysregulated processes for specific patients, peripheral blood samples are treated with a JAK inhibitor at the relevant therapeutic concentration (here, 5 uM ruxolitinib; T6 + 5R, Time 6 h with 5 uM ruxolitinib), to detect changes in "intrinsic" disease state in response to the drug (T0 vs. T6 vs. T6 + 5R, **Figure 1**). A JAK inhibitor was chosen for this study because JAK inhibitors have been shown to be successful in the treatment of autoimmune disorders such as RA.

To simultaneously evaluate the dysregulated processes described above in multiple immune cell subsets, peripheral blood samples from SLE patients and healthy controls were processed as described above and analyzed by mass cytometry. Mass cytometry, also known as Cytometry-Time-Of-Flight, offers single-cell analysis of over 40 parameters without issues of spectral overlap[7,8,9]. This technique utilizes rare earth metal isotopes in the form of soluble metal ions as tags bound to antibodies, instead of fluorophores[10]. Additional details regarding the mass cytometry technological platform (*i.e.,* tuning and calibration, sample acquisition) can be found in Leipold *et al.* and McCarthy *et al.*[11,12] The high-dimensionality of mass cytometry enables simultaneous measurement of multiple cytokines throughout innate and adaptive immune cell subsets with single-cell granularity (**Table of Materials**).

Current conventional clinical and laboratory parameters are often not sensitive or specific enough for detecting ongoing disease activity or the response to specific immunomodulators[13], reflecting the need to better delineate the underlying immune changes that drive flare-ups. Given the pervasiveness of cytokine dysregulation in autoimmune disease, a plethora of treatment approaches that use antibodies or small molecular inhibitors targeting cytokines or signaling proteins involved in the regulation of cytokine production have recently emerged. In its basic format, the peripheral blood analytical approach described here provides a platform to identify patient-specific dysregulated cell subsets and their abnormal cytokine production in autoimmune disease with systemic manifestations. This methodology allows for the personalization of therapeutic choices as specific dysregulated cytokines can be identified, and specific treatment options can be tested *ex vivo* to assess their ability to immunomodulate the patient specific disease process.

Protocol

All methods described here have been approved by the Colorado Multiple Institutional Review Board (COMIRB) of the University of Colorado. All described procedures below should be performed in a sterile tissue culture hood unless stated otherwise, with filtered pipette tips, and all reagents filtered.

1. Preparation of Reagents for Peripheral Whole Blood Processing

1. Prepare ruxolitinib stock aliquots (10–15 µL/vial for single use) at 10 mM by diluting the lyophilized reagent in DMSO as per the manufacturer's instructions (store at -80 °C). Keep DMSO concentrations in all assays including unstimulated controls below 0.2% (vol/vol).
2. Prepare R848 (resiquimod) stock aliquots (10–15 µL/vial for single use) at 1 µg/µL by diluting the lyophilized reagent in sterile water as the per manufacturer's instructions. Store at -80 °C.
3. Prepare lipopolysaccharide (LPS) stock aliquots (5 µL per vial for one time use only) at 1 µg/µL by diluting the lyophilized reagent in sterile water as per manufacturer instructions. Store at -80 °C.
4. Prepare the Cell Staining Media (CSM) using PBS, 0.5% BSA, and 0.02% NaN_3.
5. Acquire and keep ready the protein transport inhibitor (PTI) cocktail (**Table of Materials**).
6. Thaw and dilute the ruxolitinib stock vial (10 mM) 1:10 in sterile PBS (1 mM). Set aside at room temperature in a tissue culture hood.
7. Thaw and dilute the R848 stock vial (1 µg/µL) 1:10 in sterile PBS (0.1 µg/µL). Set aside at room temperature in a tissue culture hood.
8. Thaw and dilute the LPS stock vial (1 µg/µL) 1:100 in sterile PBS (0.01 µg/µL). Set aside at room temperature in a tissue culture hood.
9. Pre-warm sterile RPMI (no L-glutamine) in a standard 37 °C water bath (for at least 10 min).
10. Dilute the lyse/fix buffer by 1:5 in filtered ddH_2O (working concentration) on the benchtop (does not need to be sterile).
    Note: 1 mL of whole blood requires 20 mL of working concentration of lyse/fix buffer.
    1. Make lyse/fix buffer for 5 conditions of 1 mL of whole blood each (at least 100 mL). Aliquot 20 mL of the working concentration of lyse/fix buffer into 50 mL conical tubes per milliliter of whole blood (keep the lyse/fix solution at 37 °C until needed). Label the conditions on the conical tubes containing lyse/fix buffer as follows: T0 (Time zero), T6 + 5R (Time 6 h with 5 uM ruxolitinib), T6 + R848 (Time 6 h with 1 µg/mL R848), T6 + LPS (Time 6 h with 0.1 µg/mL LPS), T6 (Time 6 h).
11. Label round bottom sterile polystyrene tubes with caps and 1 mL microcentrifuge tubes with the same nomenclature as in step 1.10.

2. Stimulation and Processing of Peripheral Whole Blood (Figure 1)

1. Place the labeled round bottom polystyrene tubes from step 1.11 (T0, T6 + 5R, T6 + R848, T6 + LPS, T6) on a rack.
   Note: All the following steps are performed in this type of tube unless specified otherwise. Cap the tubes when transferring between the tissue culture hood and incubator to maintain sterility.
2. Add 1 mL of 37 °C RPMI (no L-glutamine) to each of the tubes (T0, T6 + 5R, T6 + R848, T6 + LPS, T6). Invert the blood collection tube several times, add 1 mL of whole blood to each tube, and pipette up and down several times to mix thoroughly with RPMI (dilution with RPMI prevents clumping of the blood during the 6 h incubation period).
3. Add 10 µL of the 1:10 ruxolitinib to T6 + 5R. Mix thoroughly with a P1000 pipette. Place the rack of tubes in the incubator at 37 °C and begin timing the incubation (T = 0).
4. Process the T0 sample as follows.

1. Pipette the entire contents of the T0 tube into a labeled conical tube containing 20 mL of 37 °C working concentration lyse/fix buffer. To optimize cell recovery, rinse the tube with working concentration lyse/fix buffer. Mix by inverting the conical tube.
2. Incubate at 37 °C for 15 min to allow for lysis and fixation. Following fixation, perform all subsequent steps at the benchtop. Centrifuge cells at 500 x g for 5 min at room temperature.
3. Decant the supernatant. Resuspend the cells in 1 mL of ice cold PBS to break up the pellet, then fill the conical to a 15 mL volume with PBS.
4. Centrifuge the cells at 500 x g for 5 min at room temperature. Decant the supernatant. Repeat the PBS wash (steps 2.4.3–2.4.4) if pellets are red. Resuspend the cells in 1 mL of CSM to break up the pellet, then transfer to the labeled microcentrifuge tube (step 1.11) for antibody staining. Take a 10 µL sample to count cells using an automated cell counter or hemocytometer.
   Note: Since all cells are fixed at this point, it is not necessary to include a viability stain.
5. Centrifuge the cells at 500 x g for 5 min at room temperature. Aspirate the supernatant, leaving the pellet in ~60 µL of residual volume. Keep this pellet on ice until samples from other conditions have completed processing.

5. At T = 30 min, move the tube rack (step 2.3) to the tissue culture hood and perform the following.
   1. Add 10 µL of the 0.1 µg/µL R848 to T6 + R848. Mix thoroughly with a P1000 pipette.
   2. Add 10 µL of the 0.01 µg/µL LPS to T6 + LPS. Mix thoroughly with a P1000 pipette.
   3. Add 4 µL of protein transport inhibitor (stock at 500X) to T6, T6 + 5R, and T6 + LPS (but **NOT** to T6 + R848) and return the samples to the incubator until T = 6 h. Mix thoroughly with a P1000 pipette every 2 h.
      Note: R848 is an endoplasmic TLR agonist and therefore requires a temporal delay in the addition of the protein transport inhibitor to allow for access to its target receptor.

6. At T = 2 h, add 4 µL of protein transport inhibitor cocktail (stock at 500X) to the T6 + R848 tube. Mix all samples with a P1000 pipette and return the rack to the incubator until T = 6 h.
7. Mix the samples with a P1000 one more time at T = 4 h.
8. At T = 6 h, process T6, T6 + LPS, T6 + R848, and T6 + 5R blood sample tubes as described in step 2.4 for the T0 sample.
9. Store the cell pellets in residual CSM volume at -80 °C to process on a future date. Alternatively, proceed to barcoding and antibody staining without storing.

3. Barcode of Lysed/Fixed Blood Cells

1. Thaw the lysed/fixed cell samples from the -80 °C storage slowly on ice; a maximum of 20 samples can be labeled with unique barcodes and pooled using this system. Dilute the 10X barcoding perm buffer by 1:10 with PBS; make enough buffer for ~3 mL per sample.
2. Fill one non-sterile trough with the CSM and one with the 1X barcoding perm buffer. Add 1 mL of ice cold CSM to freshly thawed samples, mix thoroughly with a pipette, and transfer to the respective pre-labeled polypropylene cluster tubes.
3. Take a 10 µL sample to count cells using an automated cell counter or hemocytometer. Normalize the cell counts to 1.5–2 x 10^6 cells per sample in each cluster tube: remove and discard the volume of excess cells above 2 x 10^6 cells.
4. Centrifuge the cells at 600 x g for 5 min at room temperature. Resuspend the cells in 1 mL of 1X barcoding perm buffer with a multichannel pipette and centrifuge at 600 x g for 5 min at room temperature. Aspirate off the supernatant.
5. Line up the cluster tubes on a rack in the same order as indicated on the barcode key so the sample matches with its barcode. Add 800 µL 1X barcoding perm buffer by multichannel pipette to all samples in the cluster tubes without touching the cell pellet with the pipette tips (**no mixing**) to reduce cell loss. Set aside the rack with the cluster tubes.
6. Remove 20-plex Pd Barcoding Kit tube strips from -20 °C and thaw at room temperature. Add 100 µL of 1X barcoding perm buffer, mix thoroughly, and transfer 120 µL of the resuspended barcode mix into the corresponding cell samples in the cluster tubes.
7. Mix thoroughly by multichannel pipette so that there is no cross-contamination between individually barcoded samples. Incubate cluster tubes for 30 min at room temperature to allow the barcodes to label the cells.
8. Centrifuge at 600 x g for 5 min at room temperature. Aspirate the supernatant, then resuspend in 1 mL of CSM.
9. Centrifuge and resuspend in CSM again as in step 3.8.
   Note: Each sample is now labeled with a unique barcode, and samples are ready to be pooled.
10. Centrifuge at 600 x g for 5 min at room temperature and aspirate the supernatant. With a single pipette and using the same tip, transfer all cell pellets in ~70–80 µL residual volume to one polystyrene tube. Do **NOT** eject the pipette tip; set aside the single pipette with this tip.
11. With a multichannel pipette and new tips, add ~100 µL CSM to each original cluster tube to maximize cell recovery. With the single pipette with the tip that was set aside, transfer all cell pellets in ~100 µL residual volume to the same polystyrene tube.
12. Add CSM to top off the polystyrene tube (~3 mL). Count and record the cell number of the pooled barcode set. Centrifuge at 600 x g for 5 min at room temperature and aspirate the supernatant. Proceed to staining the barcoded samples on the same day.
    Note: It is normal to expect ~20–30% cell loss in the barcoding process.

4. Staining of Barcoded Lysed/Fixed Blood Cells and Preparation for Analysis on Mass Cytometry Instrument

Note: Each 1X titer of staining antibody (1 µL of antibody per 100 µL staining reaction), can usually stain 3–4 x 10^6 cells. Therefore, when all barcoded samples are pooled into one tube, the amount of antibody must be scaled up. If 20 barcoded samples amount to 30 x 10^6 cells, and each 1X titer can stain 3–4 x 10^6 cells, the barcoded sample only requires a 10X titer, as opposed to staining each sample individually, which would require a 20X amount of antibody (1X per individual tube). The concentration of the antibody to cell number should be carefully titrated for each individual antibody cocktail (not discussed here).

1. Stain the cells using antibodies (**Table of Materials**) for surface staining and cytokine induction.

1. Make the surface staining cocktail by calculating the amount to be added and accounting for pipetting error (*i.e.*, if staining 20 barcoded samples of 2 x 10^6 cells in each sample, a 10X titer stain is required; for final volume calculations, compensate for pipetting error by making a 10.5X final staining solution).
2. Add 10X worth of surface staining volume to the barcoded cell pellet. To reduce cell loss, with the same tip, mix and measure up the volume of surface stains and cell pellet.
3. Based on the volume of cells and staining cocktail, add CSM to a final staining volume of 50 µL per number of cell samples (*i.e.*, if running a set of 20 samples, 50 µL X 20 = 1 mL). Incubate for 30 min at 4 °C. Agitate the sample every 15 min to promote even staining.
4. During the surface stain incubation, prepare the Perm/Wash buffer (**Table of Materials**) by diluting 1:10 with filtered ddH_2O. Prepare volumes of 2 mL for permeabilization, and 5 mL for washing. Keep at 4 °C or on ice.
5. Top off the surface staining tube with CSM, and centrifuge at 600 x g at 4 °C for 5 min. Aspirate the supernatant. Resuspend the barcoded sample in 2 mL of 4 °C, 1:10 dilution Perm/Wash buffer. Incubate at 4 °C for 20–30 min to fully permeabilize the cells.
6. Centrifuge at 600 x g at 4 °C for 5 min and aspirate the supernatant.
7. For intracellular staining, follow similar staining steps (as for surface staining). However, use the 4 °C, 1:10 dilution Perm/Wash buffer instead of the CSM to make the total staining volume so that the cells remain in a permeabilizing environment throughout the intracellular staining.
8. Add the intracellular antibody cocktail to the surface stained and permeabilized cell pellet (steps 4.1.2–4.1.17). Bring the total staining volume to 50 µL per number of cell samples. Incubate for 60 min at 4 °C. Agitate the sample every 15 min to ensure even staining.
9. During the intracellular staining, prepare the intercalator solution: 900 µL of filtered PBS + 100 µL of filtered 16% PFA (final concentration of 1.6% PFA) + 0.2 µL of 500 uM of intercalator dye (**Table of Materials**).
10. Top off the cell pellet + intracellular antibody cocktail with cold 1:10 Perm/Wash buffer.
11. Centrifuge at 600 x g at 4 °C for 5 min, and aspirate the supernatant. Top off the staining tube with CSM. Count and record the cell number. Centrifuge at 600 x g at 4 °C for 5 min, and aspirate the supernatant. Resuspend the cells in 1 mL of intercalator solution from step 4.9. Incubate for at least 20 min at room temperature for full intercalation, or overnight at 4 °C (samples in intercalator solution can stay at 4 °C for up to 1 week before running them on the mass cytometry instrument).

2. Prepare the cells for the mass cytometry instrument.
    1. Top off the tube with 3 mL of filtered ddH_2O, and centrifuge at 600 x g for 5 min at 4 °C. Resuspend the cells in 3 mL of filtered ddH_2O. Pass this suspension through a 100 µm filter to remove any debris or aggregates that could potentially clog the mass cytometry instrument.
    2. Count and record the cell number post-filtration. Centrifuge at 600 x g for 5 min at 4 °C

3. Prepare the calibration bead solution (**Table of Materials**) by diluting it 1:10 in filtered ddH_2O.
4. Resuspend the stained cells in the required volume of 1:10 diluted calibration bead solution to attain a cell concentration of ~1 x 10^6 cells/mL. Note: For a CyTOF1 at 45 µL/min, the recommended optimum is 5 x 10^5 cells/mL; for Helios at 30 µL/min, 7.5 x 10^5 cells/mL.
5. Proceed to run the sample on the mass cytometry instrument[9].

Representative Results

Figure 1 demonstrates the workflow for the stimulation and processing of the peripheral blood samples, including allocation of blood sample aliquots, timing of the addition of stimulation agents, protein transport inhibitor cocktail, and incubation times until the red blood cell (RBC) lysis and fixation. The choice of stimulating agents will depend on the signaling and cytokine pathways that are targeted for assessment. For example, in the protocol described here, TLR agonists are used to evaluate innate immune sensing mechanisms across multiple cell types. Representative extracellular (LPS, TLR4 agonist) and endoplasmic (R848, TLR7/8 agonist) agonists were chosen. Other stimulating agents include Phorbol 12-Myristate 13-Acetate (PMA) and Ionomycin (PMAIONO), which can act as T cell activators. Specific cytokines can also be used as stimulating agents, along with other specific B and T cell antigens.

To demonstrate the experimental approach described in this protocol, **Figure 2**, **Figure 3**, and **Figure 4** show representative results obtained using LPS, R848, and PMAIONO as stimulating conditions. While the use of PMAIONO was not described in the protocol, data are shown in **Figure 3** and **Figure 4** to demonstrate that different (and selective) cell types and cytokines are activated/induced in response to TLR agonists (LPS and R848) versus a T cell activator (PMAIONO). Given that 26 surface markers (**Table of Materials**) have been used to demarcate multiple innate and adaptive immune cell subsets, various T, B, NK, monocyte, and dendritic cell subsets can be simultaneously studied within a single sample. Additionally, markers of immune cell activation are also included, such as CD86 or ICOS for B cells and PD1 for T cells. A representative limited gating strategy demonstrating the assessment of CD14hi monocytes is shown in **Figure 2**. However, further detailed and expanded gating of T, B, NK, monocyte, dendritic, and granulocytic cell subsets can be found in our previously published work in O'Gorman and Hsieh *et al.* and O'Gorman and Kong *et al.*[14,15] Additionally, unsupervised analysis methods including (and not limited to) SPADE[16], visNE[17], Citrus[18], Phenograph[19], and Xshift[20] can be used to evaluate mass cytometry data (not discussed here). Using CD14hi monocytes and CD4 T cells as representative innate and adaptive immune cell subsets, data demonstrating cytokine induction within these cell types are shown in **Figure 3**. A select number of cytokines was chosen to show that R848 selectively induces IL-12 (p40 subunit) and MCP1 in CD14hi monocytes while LPS does not (**Figure 3**). PMAIONO, a potent T cell activator does not induce cytokines in CD14hi monocytes (**Figure 3**) but does induce interferon gamma (IFNγ) and tumor necrosis factor alpha (TNFα) in CD4 T cells (**Figure 4**). Successful technique in peripheral blood sample stimulation and processing will demonstrate patterns of cytokine induction specific to stimulating conditions and immune subsets, as exemplified in **Figure 3** and **Figure 4**. For complete analysis of the 14 cytokines described here in the cell types captured within the 26 surface markers, please see O'Gorman and Hsieh *et al.*, and O'Gorman and Kong *et al.*[14,15]

To demonstrate how the experimental approach described in this protocol captures the "intrinsic" pathogenic state of a systemic autoimmune disease such as in SLE when compared to a healthy donor, **Figure 5** illustrates the cytokine induction (Mip1β and MCP1) in CD14hi monocytes found in the diseased peripheral blood sample only (part B), in the absence of any exogenous stimulation (T6 compared to T0). These cytokines are "modulated/decreased" by JAK inhibition therapy ruxolitinib (T6 + 5R compared to T6) in the diseased peripheral blood sample only (part B).

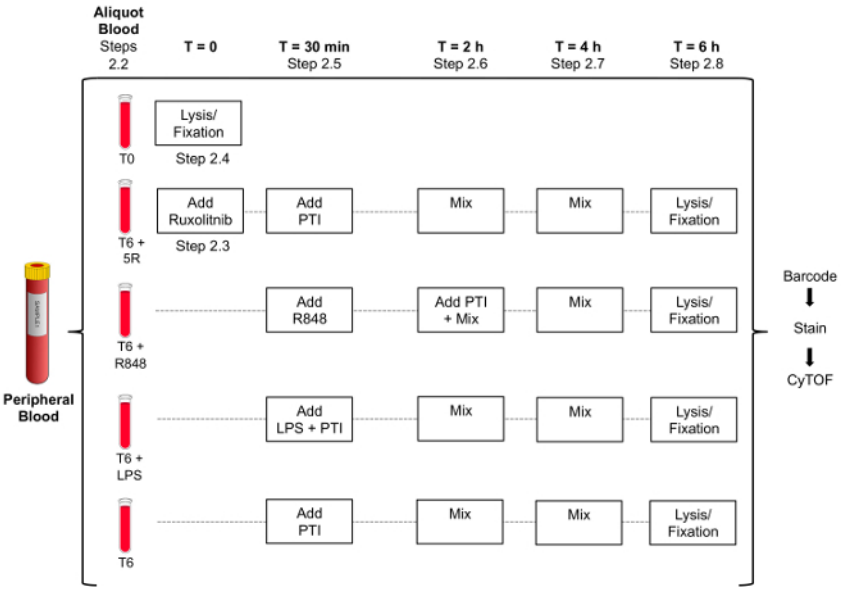

Figure 1. Experimental workflow for stimulation and processing of peripheral blood for mass cytometry analysis. Protocol for RBC lysis and fixation of disease peripheral blood sample immediately following collection (T0), or after 6 h incubation at 37 °C with a protein transport inhibitor (PTI) in the absence of any exogenous agent (T6), or with LPS (T6 + LPS), R848 (T6 + R848; protein transport inhibitor incubation for 4 h only), or ruxolitinib (T6 + 5R). Please click here to view a larger version of this figure.

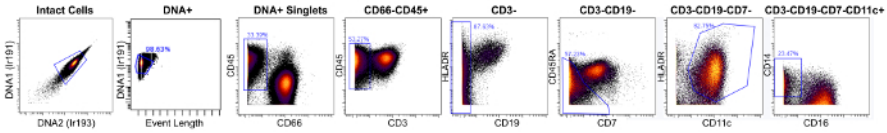

Figure 2. Representative gating strategy for the identification of CD14hi monocytes. Following fixation and RBC lysis, the individual lysed/fixed cell samples from a healthy donor were barcoded, labeled with 26 antibodies against surface markers, permeabilized and stained with 14 antibodies against cytokines (**Table of Materials**). The limited gating strategy representing the identification of CD14hi monocytes is shown. From left to right, each 2D plot represented is a population subset from the parent population that is gated (blue box) from the 2D plot immediately to the left. An extended gating strategy can be found in O'Gorman and Hsieh *et al.*, and O'Gorman and Kong *et al.*[14,15] Please click here to view a larger version of this figure.

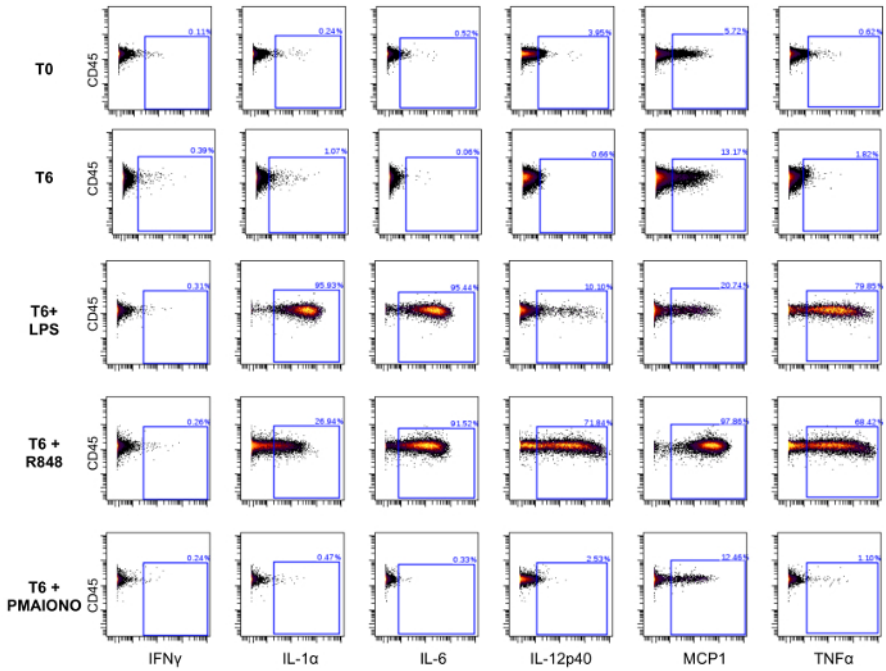

Figure 3. Example of cytokine induction in CD14hi monocytes following stimulation with LPS, R848, and PMAIONO. A representative mass cytometry analysis of peripheral blood samples from a healthy donor at time zero (T0), unstimulated (T6), and following stimulation with LPS (T6+LPS), R848 (T6+R848), and PMAIONO (T6+PMAIONO) is shown. An example of the cytokine induction in CD14hi monocytes is shown; selected cytokines with specificity to the stimulating agent used (TLR agonist vs. T cell activator) are depicted. Extended data for all cytokine induction across multiple immune cell subsets ascertained with this mass cytometry antibody panel can be found in O'Gorman and Hsieh *et al.*, and O'Gorman and Kong *et al.*[14,15] Please click here to view a larger version of this figure.

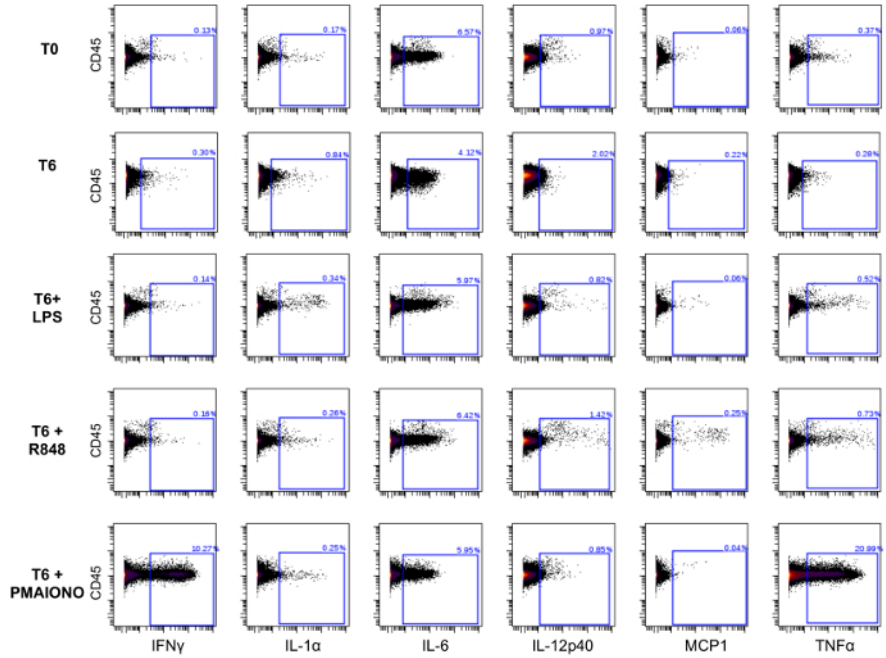

Figure 4. Example of cytokine induction in CD4 T cells following stimulation with LPS, R848, and PMAIONO. A representative mass cytometry analysis of peripheral blood samples from a healthy donor at time zero (T0), unstimulated (T6), and following stimulation with LPS (T6+LPS), R848 (T6+R848), and PMAIONO (T6+PMAIONO) is shown. Example of cytokine induction in CD4 T cells is shown; selected cytokines with specificity to the stimulating agent used (TLR agonist vs. T cell activator) are depicted. Extended data for all cytokine induction across multiple immune cell subsets ascertained with this mass cytometry antibody panel can be found in O'Gorman and Hsieh *et al.*, and O'Gorman and Kong *et al.*[14,15] Please click here to view a larger version of this figure.

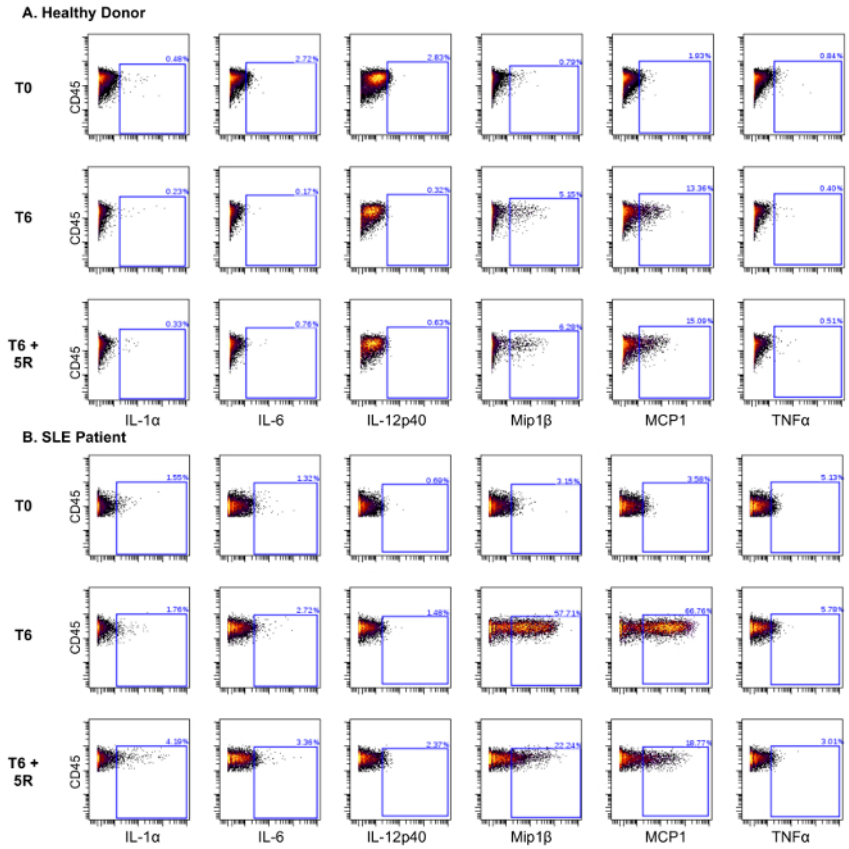

Figure 5. Example of cytokine induction in CD14hi monocytes from SLE disease patient. A representative mass cytometry analysis of peripheral blood samples from healthy donor (**A**) and SLE disease patient (**B**) are shown. Examples of the cytokine induction in CD14hi monocytes; selected cytokines with specificity to the SLE disease "intrinsic" pathogenic process (T6) (*i.e.*, induction of MIP1β and MCP1 only in SLE patient), and the effect of immunomodulator ruxolitinib (T6 + 5R) are depicted. Extended data for all cytokine induction across multiple immune cell subsets ascertained with this mass cytometry antibody panel can be found in O'Gorman and Kong *et al.*[15] Please click here to view a larger version of this figure.

Discussion

Here we describe a novel, single-cell, proteomic approach to simultaneously assess multiple immune cell types and detect their various cytokine perturbations in the milieu of patient specific "pathogenic" peripheral blood plasma circulating factors. This method employs peripheral whole blood as the analytical vehicle, and mass cytometry as the tool for the evaluation of immune cellular phenotypic and functional abnormalities. The method is readily applicable to human and mice studies[21], and is compatible with other immune functional analysis, such as detecting signaling abnormalities[14].

The user should be cognizant of a number of variables that can affect the success of this procedure. Based on our experience, blood samples should be processed within 2 h of collection to avoid baseline induction of intracellular cytokines (data not shown). Blood samples should remain at room temperature (25 °C) while awaiting processing. Blood samples can be collected in either sodium heparin or EDTA tubes, without interference with the described assay. Live and dead cell discrimination can be evaluated by mass cytometry using cisplatin[22]. However, such evaluation is omitted in the protocol described above assuming blood is processed immediately (or within 2 h) after collection. Additionally, even if samples were delayed in processing, the lack of cryopreservation allows for the omission of live/dead discrimination. We would, however, recommend the use of cisplatin for viability discrimination for any study in which cell death could affect the outcome of the downstream analysis.

In addition, we have found that the careful choice and titration of the stimulating agent is essential to evaluating targeted immune pathways/cytokine induction. First, the choice of the stimulating agent should be based on 1) the immune pathways intended to engage/evaluate, and 2) targeted functional readouts (whether signaling proteins or cytokine production). For example, if the goal were to evaluate T cell activation and different T helper cell (Th) subsets and their respective cytokine induction, PMAIONO or a combination of anti-CD3/anti-CD28 would be an appropriate stimulating condition. However, if the goal were to evaluate monocyte subset activation, a TLR agonist (or a combination of them) may be best. Second, the concentration for each of these stimulating agents needs to be optimized to elicit cell activation and cytokine induction, without severely altering the cell surface markers. Use of too little stimulating agent will not lead to the activation of desired cell types and no cytokine induction will be observed, while use of too much will lead to excessive cell death, and changes in the immune cell surface markers such that populations identified in the unstimulated condition will not be present in the stimulated condition. Third, the kinetics of the stimulation is also an important variable. While 6 h has been previously published as an optimal stimulation timeframe to capture maximal induction of pro-inflammatory cytokines in response to the TLR stimulation[14,23], a different stimulation timepoint may be needed for other agents. Similarly, the

use of any therapeutic drug *in vitro* with the peripheral blood samples should also be titrated in the same manner as the stimulating agent, while paying close attention to concentration and timepoint.

In this protocol, we use fresh peripheral whole blood samples, as intracellular cytokine induction (and detection) is more robust and effective when compared to cryopreserved samples. We describe in the protocol here the use of whole blood as opposed to PBMCs, as the presence of plasma circulating factors contributes to the "intrinsic" nature of the immune abnormalities related to the disease process. These plasma-circulating factors may interfere with stimulating conditions added to the peripheral whole blood, such as the addition of anti-IgM and anti-IgG to engage the B cell receptor, as they are bound by plasma circulating antibodies and red blood cells. Therefore, careful choice of the stimulating conditions when using peripheral whole blood is crucial for successful experiment read outs. Using peripheral whole blood has its advantages (*in vivo* platform) and disadvantages (interfering plasma circulating factors). However, PBMCs offer a different set of "pros" and "cons." When fresh PBMCs (as opposed to whole blood) undergo the same process/protocol and with stimulation using different TLR agents, we observed the same "pattern" of cytokine induction, albeit to a slightly lesser magnitude[14,23]. Following this protocol, the use of frozen PBMCs leads to a less robust cytokine induction, and therefore we would recommend against directly comparing results from samples processed after cryopreservation with those processed fresh.

Given the caveat of fresh versus frozen samples, and the preference for the use of fresh samples for optimal data quality, the protocol described here is suitable for prospective human studies where patient samples are collected over a timeframe of months to years. Peripheral blood samples can be processed as described, and once they reach the RBC lysis and fixation stage, samples can be stored in -80 °C and stained in batches later. This technical approach, while advantageous for most human studies, also poses the challenge of using antibodies that can bind to the target epitope after fixation. The **Table of Materials** lists the clones for the respective antibodies that have been tested and demonstrate specific staining post fixation. Barcoding of multiple samples (n = 20) offers the advantages of 1) decreased technical variability, as multiple samples are stained together in one tube, therefore making comparisons of samples across conditions fairly consistent and reliable; 2) reduction of total antibody use (see protocol section 4.1); and 3) the barcoding scheme of "6 different palladium isotopes/choose 3" leads to the exclusion of doublets (cells positive for more than 4 palladium isotopes). These advantages are discussed in detail in Zunder and Fick *et al.*[24] Additionally, the barcoding process requires that cells be fixed, as they need to be mildly permeabilized for the intercalation of the barcoding reagent (palladium isotopes)[24]. Therefore, live staining cannot be performed following this protocol. It should be noted that live cell barcoding (as opposed to fixed cell barcoding described here) is a possibility[25,26], but in the protocol here, cells are fixed so they can be stored and batch processed together.

Batch sample processing, while advantageous from a labor and time efficiency standpoint, also has its own challenges. With batch processing there is a need for a carefully curated normalization process. This normalization process can be addressed at different steps of the mass cytometry workflow. First, the concentration of antibody to cell number should be kept consistent across batches. We have found that an effective way to achieve this consistent concentration is by 1) careful titration of the antibody concentration to cell number, and 2) normalization of the cell number of each of the samples that are barcoded together (*i.e.*,1 million cells per sample, for all 20 barcoded samples). Second, to account for instrument signal intensity variability (different days of tuning, calibration, and signal decay over run time), samples should be resuspended and analyzed on the mass cytometer with an appropriate normalization bead solution (**Table of Materials**), followed by file normalization after data acquisition[27] (but prior to debarcoding). Individual samples are debarcoded after the entire barcoded file has been normalized using the tools described in Zunder and Finck *et al.*[24] Third, to account for manual antibody staining technical variability, we recommend the inclusion of an "anchor" sample with each barcode set that is analyzed for a single study, *i.e.*, a single sample that is from one donor (human studies) and has been processed in the same way as the other study samples. This sample should be generated once in large enough quantity to be distributed into each barcode set analyzed for the study. These anchor samples from each barcode will be used to generate a coefficient of variance to correct for signal variability across the study samples for that barcode (*i.e.*, anchor sample from barcode 8 will be used to correct for variability in barcode 8).

Another important optimization step is the assembly of the mass cytometry antibody panel, which will assess relevant immune cell subsets and cytokines. Some of the key variables in the antibody panel assembly include the choice of clones (addressed above), antibody titer for minimal background signal, choice of antibody targets and isotope channels based on antigen abundance and channel sensitivity, "compensation" of signal spillover based on isotope impurity and oxidation, and individual antibody lot variability. These optimization steps are addressed elsewhere[28] and not within the scope of the protocol described here.

Using this single-cell proteomic approach to studying immune abnormalities in peripheral blood samples, it is possible to identify abnormal phenotypes of immune cell subsets, whether it is population frequency differences or changes in the expression of specific cell surface markers (such as markers of cell activation). Additionally, it is also possible to identify functional abnormalities within the immune cell subsets, such as under or overproduction of cytokines. Lastly, the process of comparing the sample immediately fixed (T0) versus fixed after 6 h of incubation without an exogenous agent (T6) demonstrates "intrinsic" pathogenic processes that lead to immune cell type and cytokine abnormalities (T6-T0). This approach could be tailored to the study of a variety of systemic immune-mediated processes, and the engagement of multiple immune cell subsets and pathways depending on the stimulation agent.

Disclosures

The authors have nothing to disclose.

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