Biological Evaluation of Human PBMC and Th17 cells using MILLIPLEX® MAP Cell Signaling and Cytokine Panels

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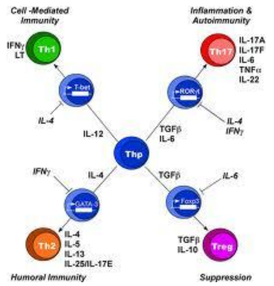
Introduction

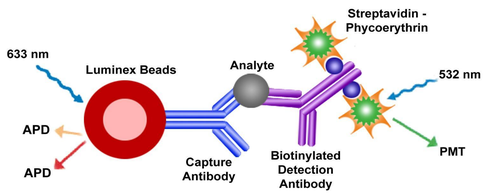
Peripheral Blood Mononuclear Cells (PBMC) are composed of all blood cells having a round nucleus. This includes lymphocytes, monocytes, and macrophages. The lymphocyte population is made up of T cells, B cells, and Natural Killer cells. Th17 cells are recently discovered T helper cells that play important roles in the establishment and maximization of the capabilities of the immune system. Th17 cells are derived from CD4+ lymphocytes which are prevalent in human PBMC samples. In this study, we used six Milliplex® Cell Signaling panels and the Human Th17 Circulating panel to compose a biochemical profile of PBMC before, during, and after a differentiation process. We also examined the effect of Fetal Bovine Serum treatment on undifferentiated and differentiated PBMC. Our differentiation process consisted of a 7-day treatment of four cytokines, IL-1β, IL-6, IL-23, and TGFβ, as demonstrated in *Zheng et al*. Our results from cell signaling panels demonstrated that several analytes showed increased constitutive expression and phosphorylation after differentiation and stimulation with Fetal Bovine Serum. Additionally, the human Th17 panel showed that cytokine treatments induced significantly increased secretion of many cytokines including Th17 cell specific cytokines from human PBMCs (e.g. IL-17F). In conclusion, the Th17 and cell signaling panels will be useful tools for Th17 cell-related cytokine and signaling profiling in PBMC and various biological samples.



Methods

Luminex® 200 system. This is a compact unit consisting of an analyzer, a computer, and software (Luminex Corporation, Austin, TX).

Microspheres. Magnetic microsphere beads were purchased from Luminex Corp. Each set of beads is distinguished by different ratios of two internal dyes yielding a unique fluorescent signature to each bead set. Capture antibodies were covalently coupled to the carboxylate-modified magnetic microsphere beads.



Sample Preparation. PBMCs (Bioreclamation) were thawed, washed, resuspended in RPMI Media (Gibco) 5 * 10^6 cells per mL, split into two aliquots and incubated overnight at 37°C. The next day, one aliquot was treated with four cytokines[1]: IL-1β, IL-6, IL-23, and TGFβ (R&D Systems) with final concentrations of 10 ng/mL, 50 ng/mL, 25 ng/mL, and 10 ng/mL respectively. Conditioned media was collected once per day for 7 days and centrifuged at 1100 RPM for 5 min. The cell-free supernatants were stored at -80°C prior to assaying in Multiplex Circulating format.

The cytokine-treated aliquot as well as the other aliquot were starved for 4 hours and treated with 10% Fetal Bovine Serum (Millipore). Media was collected at four time points: 0 (after starve, prior to treatment), 30 min, 2 hours, and 1 day. Media was centrifuged at 1100 rpm for 5 minutes. Supernatant was discarded and 200 µL of lysis buffer was added to cell pellets. Lysate samples were rocked for 15 min at 4°C and centrifuged at 12,000 rpm for 10 minutes. Lysate supernatant was collected, diluted to 1.4 mg/mL (protein concentration determined via BCA), and stored at -80°C prior to assaying in Multiplex Cell Signaling format.

Immunoassay Protocols. For supernatant samples: The multiplex assay was performed in a 96-well plate. The detailed procedure is as follows: wet the plate with 150 µL assay buffer for 10 min and decant. Add 25 µL standards or samples, 25 µL beads, 25 µL assay buffer in sample wells or 25 µL matrix in standard wells and incubate O/N at 4°C. Wash the beads two times then add 25 µL biotinylated detection Ab cocktail and incubate at RT for 1 hour. Add 25 µL Streptavidin-Phycoerythrin (SAPE) and further incubate at RT for 30 min. Lastly, wash beads two times, add 100 µL sheath fluid and read on Luminex instrumentation.

For lysate Samples: The multiplex assay was performed in a 96-well plate. The detailed procedure is as follows: wet the plate with 50 µL assay buffer for 10 min and decant. Add 25 µL beads, 25 µL assay buffer in blank wells, and 25 µL lysate at 0.8 mg/mL to sample or control wells and incubate O/N at 4°C. Wash the beads two times then add 25 µL biotinylated detection Ab cocktail and incubate at RT for 1 hour. Wash the beads one time then add 25 µL SAPE and further incubate at RT for 15 min. Do not remove SAPE and add 25 µL amplification buffer. Remove SAPE/amplification buffer and resuspend beads in 100 µL assay buffer. Read on Luminex instrumentation.

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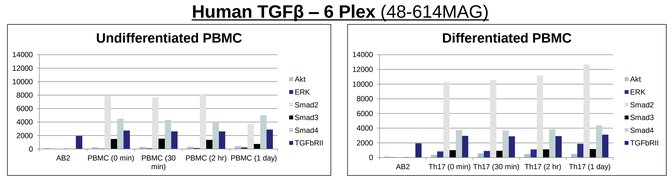
Results: Cell Signaling Multiplex Panels

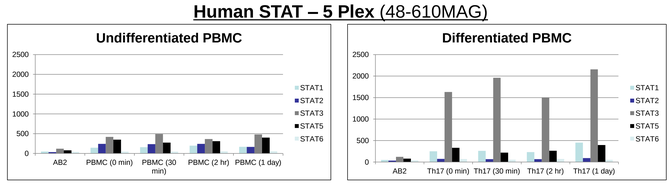
Graphs 1-12. Stimulation of Human PBMCs with FCS

Human PBMCs were treated with 10% Fetal Bovine Serum (FBS), before and after differentiation. Differentiation consisted of a 7-day treatment of four cytokines as described by *Zheng et al*[1].

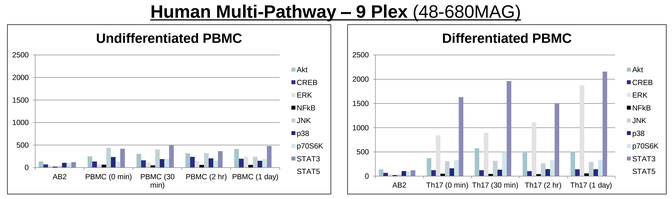
Human TGFβ – 6 Plex (48-614MAG)



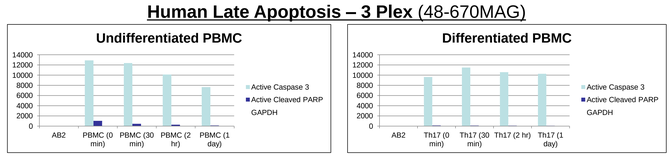
Human STAT – 5 Plex (48-610MAG)



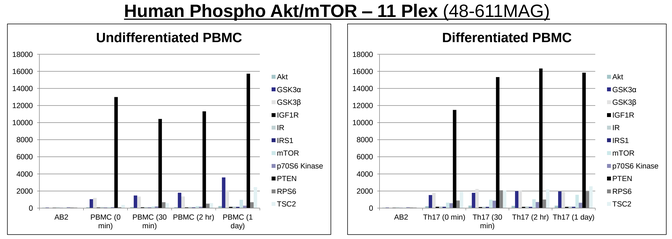
Human Multi-Pathway – 9 Plex (48-680MAG)



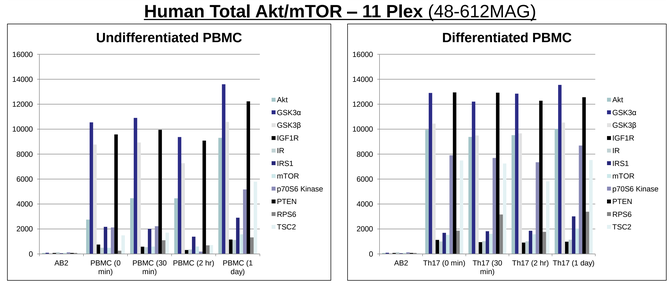
Human Late Apoptosis – 3 Plex (48-670MAG)



Human Phospho Akt/mTOR – 11 Plex (48-611MAG)

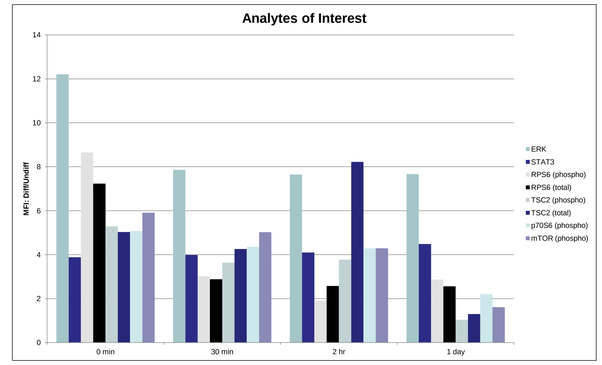


Human Total Akt/mTOR – 11 Plex (48-612MAG)



Graph 13. Analytes of Interest

Certain analytes showed a dramatic increase in expression after differentiation of human PBMCs.



Results: Human Th17 Multiplex Panel

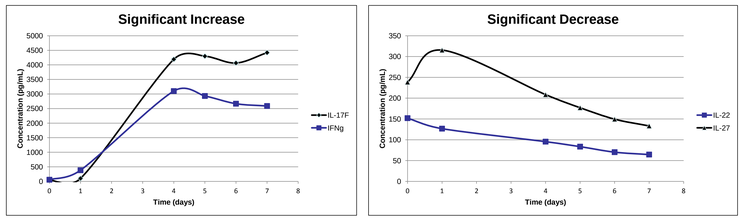
Table 1. Differentiation of Human PBMC

Human PBMCs were treated with TGF-β, IL-6, IL-23 and IL-1β at 37°C for 7 days. Cell-free samples were collected and assayed as described. Approximate cytokine responses are indicated as (+) 20 to 100 pg/mL, (++) 100 to 500 pg/mL, (+++) 500 to 2000 pg/mL, or (++++) >2000 pg/mL.

Cytokine Response	Day 0	Day 1	Day 4	Day 7
IFNg	+	++	++++	++++
IL-2		+	+	
IL-4	+	+		
IL-5			+	+
IL-9			++	++
IL-10		++	++	+
IL-17A			++	++
IL-17F	+	+	++++	++++
IL-22	++	++	+	+
IL-28A	+	+	+	
MIP-3α	+	++	++	++
TNFα	+	+	+	+

Graphs 14-15. Differentiation of Human PBMC

Certain cytokines showed dramatic increases or decreases in concentration throughout the differentiation process.



Summary

- Treatment of human PBMC with four cytokines induced significant cell differentiation.
- MILLIPLEX® Cell Signaling kits provide powerful tools in evaluating subtle changes in PBMC signaling.
- Differentiated PBMC samples contain significant levels of many cytokines including Th17 cell specific cytokines.
- MILLIPLEX® Th17 panels provide powerful tools for cytokine profiling in biological samples.

References:

1. Zheng et al, Induction of Th17 Differentiation by Corneal Epithelial-Derived Cytokines, J Cell Physiol. 222(1):95-102 (2010).

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