New Flow-Based, Multiplex Assays Allow Simultaneous Quantification of 13 Soluble Factors Related to Macrophage/Microglia or B Cell Functions

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Abstract

Immune responses are mediated by innate immune cells (*e.g.,* macrophages) and subsequently, adaptive immune cells (*e.g.,* B cells). Macrophages secrete an array of cytokines to affect their functions in host defense, tissue repair and immunoregulation. Pro-inflammatory macrophages (M1) produce cytokines, such as IL-12 p70, TNF-α, IL-6, IL-1β, IL-12 p40, IL-23, IFN-γ, and IP-10, while anti-inflammatory and tissue repairing macrophages (M2) release a different set of factors, such as IL-4, IL-10, IL-6, Arginase, TARC and IL-1RA. B cells, in addition to antibody production, also secrete an array of cytokines that mediate Th1- and Th2-like immune responses. These cytokines are produced by regulatory B cells (*e.g.,* IL-10, TGF-β1) and B effector cells, namely Be1 (*e.g.,* TNF-α, TNF-β, IFN-γ, and IL-12 p70) and Be2 cells (*e.g.,* IL-2, IL-4, IL-6, TNF-α, and IL-13). Other cytokines associated with activation and survival of B cells such as APRIL, BAFF, and CD40L are also important targets in B cell related processes.

We have developed new assay panels targeting macrophage/microglia and B cells, using fluorescence–encoded beads that are suitable for use on general lab flow cytometers (for a complete list of panels available, please visit: **biolegend.com/legendplex**). Each panel allows simultaneous quantification of 13 related analytes. Each antibody pair was carefully optimized for assay specificity, sensitivity, accuracy and reproducibility. These panels have been validated by detecting expected changes in biological samples. These panels are of high quality, low cost and ease of use, providing an alternative multiplex solution to the biomedical research community.

Materials and Methods

1. Partial list of Instruments and settings are as shown.

Flow Cytometer	Reporter Channel	Reporter Channel Emission	Beads Classification Channel	Classification Channel Emission	Compensation needed?
BD FACS Calibur™ (single laser)	FL2	575 nm	FL3	670 nm	Yes
BD FACS Calibur™ (dual laser)	FL2	575 nm	FL4	660 nm	No
BD FACSCanto™, BD FACSCanto™ II	PE	575 nm	APC	660 nm	No
BD LSR, BD LSRII, BD LSRFortessa™	PE	575 nm	APC	660 nm	No
BD FACSAria™	PE	575 nm	APC	660 nm	No
Beckman Coulter CytoFLEX	PE	585 nm	APC	660 nm	No
BD Accuri ™ C6	FL2	585 nm	FL4	675 nm	No

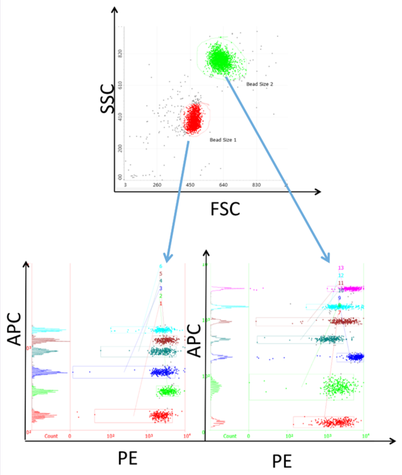
2. 96-well microtiter filter plates , V- or U-bottom plates, vacuum pump and filtration manifold.
3. Capture antibody immobilized beads, biotinylated detection antibody cocktail, streptavidin-phycoerythrin (PE) conjugate, assay buffer, and wash buffer.
4. Data analysis software and software dongle.
5. **Biological Sample Preparation:**

   Human monocytes from healthy donors were isolated using Ficoll-Paque™ (GE Healthcare) followed by positive selection with the MojoSort™ Human CD14⁺ Monocytes Isolation Kit (BioLegend, San Diego). Cells were then differentiated and treated with the appropriate stimulations as described in the figure legend.

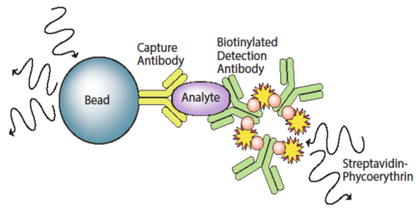
   Human B cells from healthy donors were isolated using Ficoll-Paque™ (GE Healthcare) followed by negative selection using the MojoSort™ Human B Cell (CD43⁻) Isolation Kit (BioLegend, San Diego). Cells were then treated with the appropriate stimulations as described in the figure legend.

   Cell culture supernatants were collected after 2 days.

Beads Classification



Assay Principle



Assay Protocol

25 µL Matrix or Assay Buffer
25 µL Standard or samples
25 µL beads

Shaking for 2h, RT
Vacuum and wash twice

25 µL Detection Antibody

Shaking for 1h, RT
No vacuum, no wash

25 µL Streptavidin-Phycoerythrin

Shaking for 30 min, RT
Vacuum and wash twice

Read on a flow cytometer

Representative Standard Curves

Human B Cell Panel

Human Macrophage / Microglia Panel

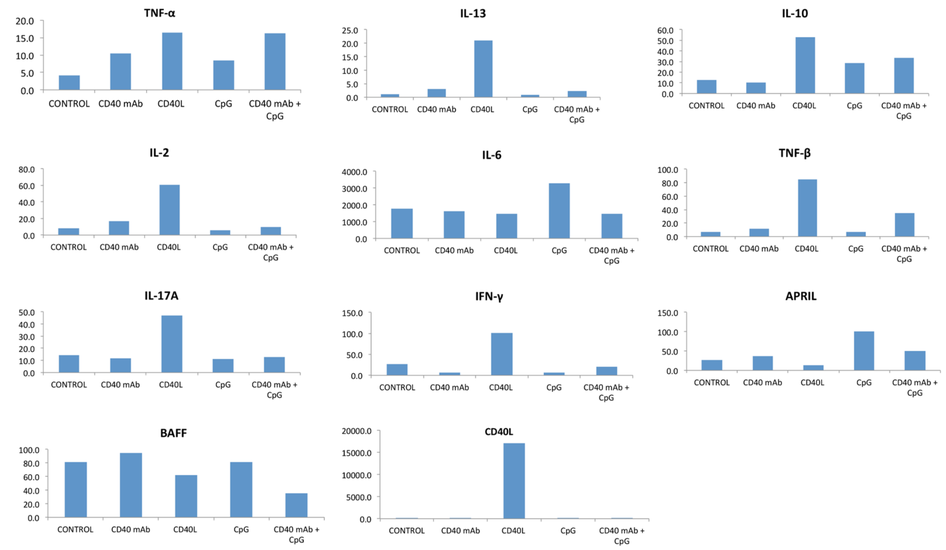
Panel Design and Sensitivities

B cell Panel	B Effector 1 subpanel	B Effector 2 subpanel	B cell Activator subpanel	Sensitivity (pg/mL)
TNF-α	TNF-α	TNF-α		1.2
IL-13		IL-13		1.2
IL-4		IL-4		1.1
IL-10				1.3
IL-6		IL-6		1.2
IL-2		IL-2		3.3
TNF-β	TNF-β			4.0
IFN-γ	IFN-γ			1.2
IL-17A				2.1
IL-12 p70	IL-12 p70			1.4
APRIL			APRIL	13.7
BAFF			BAFF	10.4
CD40L			CD40L	7.0

Macrophage/ Microglia Panel	M1 subpanel	M2 subpanel	Sensitivity (pg/mL)
IL-12 p70	IL-12 p70		1.3
TNF-α	TNF-α		1.3
IL-6	IL-6	IL-6	0.9
IL-4		IL-4	0.8
IL-10		IL-10	0.9
IL-1β	IL-1β		0.9
Arginase		Arginase	64.4
CCL17		CCL17	1.4
IL-1RA		IL-1RA	72
IL-12 p40	IL-12 p40		4.7
IL-23	IL-23		1.3
IFN-γ	IFN-γ		1.6
CXCL10	CXCL10		1.9

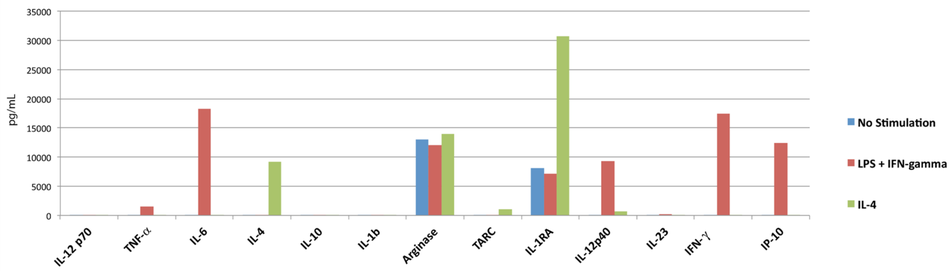
Biological Validation

Total B cells isolated from PBMC were stimulated and supernatants analyzed with B cell Panel



Human B cells (1 x 10^6 cells/mL) were isolated and cultured under various conditions (activating CD40 mAb, 5 µg/mL; CD40L, 1 µg/mL; CpG, 5 µg/mL). Supernatants were collected after 48 hours and assayed with the Human B Cell Panel. The results (all in pg/mL) are shown. Note: no detectable levels of IL-4 and IL-12 p70 were found under these conditions. The high concentration of CD40L detected in CD40L treated samples is due to the added CD40L recombinant protein.

M1/M2 Macrophages polarized under different conditions (M1 vs. M2) display different cytokine profiles as measured using the Macrophage/Microglia Panel



Monocytes were isolated and differentiated for 5 days using GM-CSF (25 ng/mL). Differentiated monocytes were polarized under either M1 or M2 conditions for 48 hr with LPS (100 ng/mL) + IFN-γ (25 ng/mL) or IL-4 (20 ng/mL). The adherent cells were washed, rested for 2 days in fresh media and re-stimulated with the same agents for another 48 hr. The cell culture supernatant samples were collected and tested using the Human Macrophage/Microglia Panel.

Conclusions

We have developed bead-based multiplex assays for simultaneous quantification of B cell and macrophage/microglia related cytokines.

These assay panels offer user friendly software and high performance, low cost, easy-to-use kits that don't require specialized instruments.

The utility of these multiplex assays was validated by using relevant biological samples, including serum, plasma, cell culture supernatants, offering useful tools for biomedical research.