Biological Evaluation of the MILLIPLEX® MAP Mouse High Sensitivity T Cell Panel

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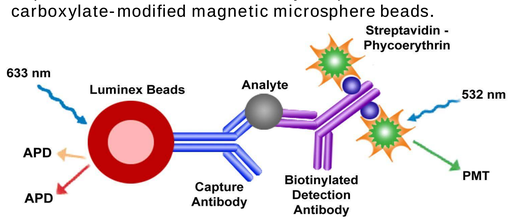
Introduction

Low levels of inflammation are involved in many clinical and sub-clinical disease states such as autoimmune diseases, metabolic diseases and cancer. Measuring picogram levels of cytokines is critical for understanding their pathogenesis. Model organisms, such as mice, offer unique challenges of sample availability and detection of low levels of cytokines. We recently developed a Mouse High Sensitivity T Cell Panel multiplex assay for simultaneous measurement of 18 mouse cytokines using Luminex® xMAP® technology. In this study, we analyzed 18 mouse cytokine levels in 25 µL of sample for both *in vitro*, with PMA-, PHA-, LPS-, Con-A, or calcium ionophore-challenged PBMC media samples, and *in vivo*, using LPS-challenged mice, obese mouse models and an aged-mouse model in serum samples. Typically, the response of mouse PBMCs to *in vitro* stimulants is difficult to study due to low levels of cytokine secretion. Using the MILLIPLEX® MAP Mouse High Sensitivity T Cell Panel, distinctly different stimulant-dependent cytokine responses were observed. The panel also detected low levels of serum cytokines *in vivo* using LPS-challenged mice (early onset of inflammation), obese mice (OB/OB and DB/DB) and an aged-mouse model (cytokine profiles of young vs. old mice).

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.

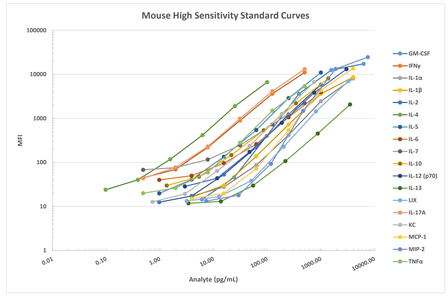


Immunoassay Protocol. The multiplex assay was performed in a 96-well plate. The detailed procedure is as follows:

- Wet the plate with 150 µL Wash Buffer for 10 min and decant.
- Add 50 µL standards or 25 µL samples and 25 µL Assay Buffer.
- Add 25 µL beads to all wells and incubate O/N at 4° C.
- Wash the beads three times then add 50 µL biotinylated detection Ab cocktail and incubate at RT for 1 hour.
- Add 50 µL Streptavidin-Phycoerythrin and further incubate at RT for 30 min.
- Wash beads three times, add 150 µL sheath fluid and read on Luminex® instrumentation.

Results

Analyte	Standard Curve Range (pg/mL)	Sensitivity (pg/mL)	Precision (%) Intra-assay	Precision (%) Inter-assay	Accuracy (%) In Matrix	Accuracy (%) In Samples	Linearity (%)
GM-CSF	6.1 - 25,000	5.33	6.5	4.6	102	66	119
IFNγ	0.49 - 2,000	0.15	3.9	3.3	97	68	130
IL-1α	3.91 - 16,000	1.54	5.4	4.7	94	64	155
IL-1ß	3.91 - 16,000	2.58	5.5	4.7	101	89	118
IL-2	0.98 - 4,000	0.80	4.8	6.0	89	105	98
IL-4	0.098 - 400	0.06	4.4	3.1	90	61	162
IL-5	0.98 - 4,000	0.53	6.1	2.9	85	82	84
IL-6	0.98 - 4,000	0.54	4.9	4.4	85	100	105
IL-7	0.488 - 2,000	0.98	7.6	7.0	95	113	75
IL-10	1.34 - 5,500	0.53	4.7	3.5	95	85	130
IL-12(p70)	2.93 - 12,000	1.29	3.6	3.9	91	89	111
IL-13	3.42 - 14,000	3.76	5.3	4.0	102	65	143
LIX	3.17 - 13,000	2.70	3.9	3.7	95	92	126
IL-17A	0.488 - 2,000	0.18	4.0	3.4	91	108	98
KC	0.732 - 3,000	0.45	5.0	7.3	82	78	140
MCP-1	3.91 - 16,000	3.00	3.5	3.4	94	122	99
MIP-2	7.32 - 30,000	9.06	5.3	4.6	99	29	181
TNFα	0.488 - 2,000	0.41	5.1	4.1	93	132	97



MILLIPLEX® MAP Mouse High Sensitivity T Cell Panel Detects Low Levels of Analytes in Typically Undetectable Samples (n=16 normal mice*)

Analyte	MHSTC (%)	Comp. A (%)	Analyte	MHSTC (%)	Comp. A (%)
GM-CSF	13		IL-10	100	
IFNγ	94	100	IL-12 (p70)	63	
IL-1α	100		IL-13	100	
IL-1β	56		LIX	100	
IL-2	93	62	IL-17A	100	
IL-4	100	79	KC	100	
IL-5	88		MCP-1	100	
IL-6	75	100	MIP-2	88	
IL-7	88		TNFα	100	100

*Normal Mice: 4 CD-1, 4 Balb/C, 4 C57Bl/6, 4 Swiss-Webster

MILLIPLEX® MAP Mouse High Sensitivity T Cell Panel Detects Low Levels of Analytes in Mouse PBMC Culture Media

PBMC Media (pg/mL)*	GM-CSF	IFNγ	IL-1α	IL-1β	IL-2	IL-4	IL-5	IL-6	IL-7
Unstimulated Cells	13.8	2.61	30.1	0	0	0.23	0	53	5.89
(+) PMA	10.3	2.12	26.0	4.35	0	0.18	1.04	71	4.05
(+) PHA	10.3	2.33	23.9	0	0	0.23	0	59	5.43
(+) Con A	11.3	3.74	29.6	0	0	0.21	0	56	5.84
(+) LPS	12.3	3.39	33.2	14.35	0	0.24	1.15	728	2.75
(+) A21387	10.3	2.22	17.8	0	0	0.17	0	51	2.62
(+) All of the above	54.9	4.77	40.2	6.13	1.8	0.30	4.05	544	4.24

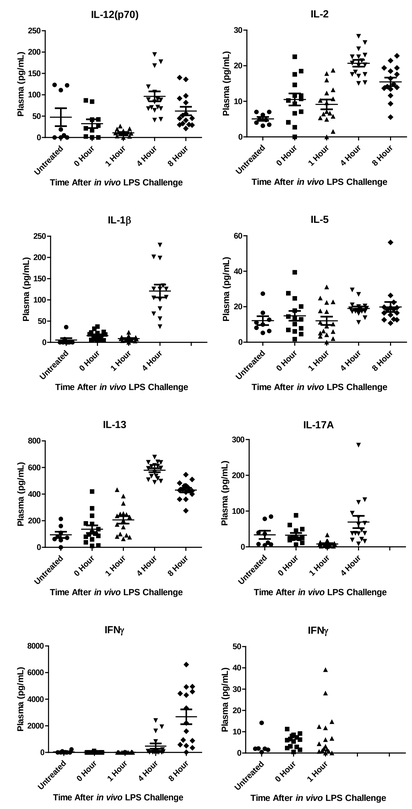
PBMC Media (pg/mL)*	IL-10	IL-12 (p70)	IL-13	LIX	IL-17A	KC	MCP-1	MIP-2	TNFα
Unstimulated Cells	6.7	0	8.2	109	0.94	156	33	491	8.6
(+) PMA	24.2	0	10.4	134	1.35	216	110	803	12.2
(+) PHA	7.2	0	13.3	99	7.02	154	27	463	8.9
(+) Con A	8.9	0	8.2	104	23.99	137	65	405	8.4
(+) LPS	40.6	0	14.1	515	1.1	3937	97	12053	204.9
(+) A21387	4.9	0	4.3	97	0.46	154	21	462	8.4
(+) All of the above	119.4	0	18.0	656	25.08	4266	951	19470	318.4

*Cytokine levels in media of mouse PBMC after 24 hours with various agents

PBMCs (Bioreclamation) were thawed, washed and resuspended in RPMI Media (Gibco) containing 10% FCS and 1% Penicillin/Streptomycin at 10^6 cells/mL. The PBMCs were aliquoted at 10^6 cells/well and incubated overnight at 37° C. The next day either PMA, A23187, LPS, Con A, PHA (Sigma) or all, were added for final concentrations of 0.025, 0.01, 1, 5, or 20 µg/mL, respectively. Conditioned media was collected at various time points, centrifuged at 15,000 RPM for 10 min, and the cell-free supernatants were stored at -80° C prior to assaying in multiplex format.

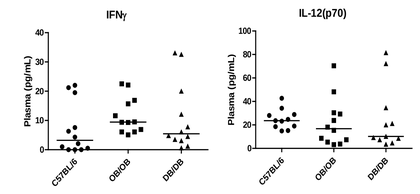
Results

In vivo LPS-challenge Plasma Cytokine Levels



The *in vivo* LPS-challenged mouse plasma samples were obtained from Bioreclamation. CD-1 mice were injected IP at 1 mg/kg with a 1 mg/mL suspension of *E. coli* 055:B5 LPS (Sigma) in saline. At various time points approximately 0.5 mL of plasma (sodium EDTA) was collected from each mouse and stored at -80° C prior to assaying in multiplex format.

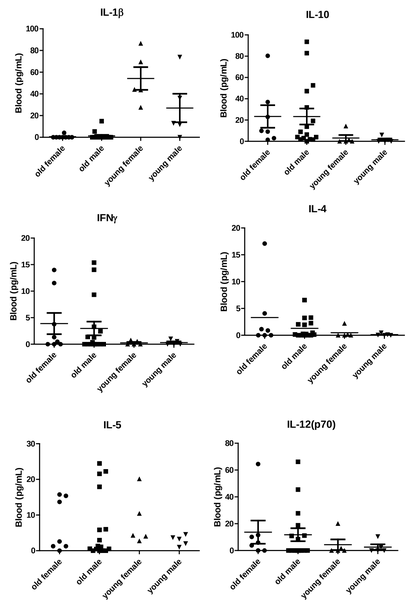
Diabetic Mouse Model K/O Samples



OB/OB obese mice cannot produce the hormone leptin which inhibits hunger. In DB/DB obese mice, the leptin receptor is inactive. OB/OB, DB/DB and C57Bl/6 control mice were from BioreclamationIVT, Westbury, NY.

Results

Aging Mouse Model Samples



C57Bl/6 Mice, young: 4 months; old: 20 to 22 months.

Summary

Low levels of chronic inflammation are involved in many clinical and subclinical disease states. Consequently, research investigating low levels of cytokine expression plays a significant role in achieving a deeper understanding of the immune system and its multi-faceted response to most antigens, especially those responses that make up the immune cell-mediated inflammatory process. Merck's MILLIPLEX® MAP Mouse High Sensitivity T Cell Magnetic Bead Panel provides researchers with an analytically validated "must-have" assay, not only to study low-level cytokine expression, but also to quantify multiple cytokine secretion levels simultaneously and in a biologically relevant context. The MILLIPLEX® MAP Mouse High Sensitivity T Cell Panel features 18 configurable mouse cytokine assays and requires only 25 µL of each sample. Representative data shown here exemplifies the value of this kit for the study of relevant cytokine biomarkers both *in vitro* for stimulated PBMC and for *in vivo* studies, examining the early stages of inflammation for LPS-challenge models and for use in metabolic-disease and aging animal models. The MILLIPLEX® MAP Mouse High Sensitivity T Cell Magnetic Bead Panel is a powerful tool for cytokine profiling in biological samples.

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