Analysis of Murine Th17 Cytokine Profiles Using Bio-Plex Pro Mouse Th17 Panel

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Bio-Plex Pro Multiplex Assays

Bulletin 6118

Introduction

T helper cells have classically been divided into two dominant effector subsets named Th1 and Th2 cells. Th1 cells activate macrophages and are highly effective in clearing intracellular pathogens. They are coupled to the sequential actions of IFN-γ and IL-12. Th2 cells, whose differentiation is driven by IL-4, are important for the production of immunoglobulin E and the clearance of extracellular organisms (Kimura and Kishimoto 2011). T helper cells producing IL-17 (Th17) are a new subset of T helper cells found to be associated with autoimmune diseases, such as rheumatoid arthritis, multiple sclerosis, psoriasis, and lupus (Di Cesare et al. 2009, Garrett-Sinha et al. 2008, Kebir et al. 2007, Shahrara et al. 2008, Sospedra and Martin 2008). In mice, Th17 differentiation and expansion is regulated by various cytokines, such as TGF-β, IL-6, IL-1β, TNF-α, and IL-23. The development of Th17 cells is negatively regulated by IFN-γ and IL-27 (Harrington et al. 2005, Stumhofer et al. 2006). The IL-17 family is composed of IL-17A, IL-17B, IL-17C, IL-17D, IL-17E (IL-25), and IL-17F (Kimura and Kishimoto 2011).

Using Luminex xMAP technology (Dale et al. 2008) and bulletin 5405, Bio-Rad has developed 32 mouse cytokine assays. These are configured into a mouse group I 23-plex and a mouse group II 9-plex (Table 1). To further expand our Bio-Plex Pro Mouse Cytokine Assay menu for Th17 studies, a mouse Th17 cytokine panel that includes ten new assays plus ICAM-1 was created (Table 1). These markers were selected because of their direct relevance to the mouse Th17 pathway. Including the six existing assays in mouse cytokine group I (green dots), a total of 17 mouse assays are available for the study of the mouse Th17 pathway (Table 1).

The validation studies described in this tech note cover the ten markers in the mouse Th17 panel. The assays were validated with mouse serum, plasma, and cell culture matrices. Validation criteria include assay working range lower and upper limit of quantification (LLOQ/ULOQ), sensitivity (limit of detection [LOD]), intra- and inter-assay precision, specificity and cross-reactivity, linearity of dilution, and parallelism to evaluate robustness in the key sample matrices mentioned above.

Table 1. Bio-Plex Pro Mouse Cytokine Assay menu.

		Mouse Group I	Mouse Group II	Mouse Th17
Mouse Assay	**1-Plex**	**23-Plex**	**9-Plex**	**10-Plex**
IL-1β	●	●		
IL-6	●	●		
IL-10	●	●		
IL-17A	●	●		
IFN-γ	●	●		
TNF-α	●	●		
IL-1α	●	●		
IL-2	●	●		
IL-3	●	●		
IL-4	●	●		
IL-5	●	●		
IL-9	●	●		
IL-12 (p40)	●	●		
IL-12 (p70)	●	●		
IL-13	●	●		
Eotaxin	●	●		
G-CSF	●	●		
GM-CSF	●	●		
KC	●	●		
MCP-1	●	●		
MIP-1α	●	●		
MIP-1β	●	●		
RANTES	●	●		
IL-17F	●			●
IL-21	●			●
IL-22	●			●
IL-23	●			●
IL-25	●			●
IL-27	●			●
IL-31	●			●
IL-33	●			●
CD40L	●			●
MIP-3α	●			●
ICAM-1	●			●
IL-15	●		●	
IL-18			●	
Basic FGF	●		●	
LIF	●		●	
M-CSF	●		●	
MIG	●		●	
MIP-2	●		●	
PDGF-BB	●		●	
VEGF	●		●	

(●) refers to the cytokines not related to Th17.

(●) refers to a subset of Th17 relevant mouse cytokines available as 6-plex and 10-plex panels.

(●) ICAM-1 is a mouse Th17 singleplex assay that cannot be multiplexed.

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Methods

The mouse cytokine assays were designed according to a capture sandwich immunoassay format (Figure 1). The capture antibody–coupled beads were first incubated with antigen standards or samples followed by incubation with biotinylated detection antibodies. After washing away the unbound biotinylated antibodies, the beads were incubated with a reporter streptavidin-phycoerythrin (SA-PE) conjugate. Following removal of excess SA-PE, the beads were passed through the Bio-Plex Array Reader, which measures the fluorescence of the bead and of the bound SA-PE. Incubations were performed at room temperature for durations shown in Table 2. All washes were performed using a Bio-Plex Pro Wash Station. Data acquisition was performed using Bio-Plex Manager Software 6.2 at low photomultiplier tube setting.

Table 2. Bio-Plex Pro Mouse Cytokine Assay incubation and detection guidelines.

	Incubation Time, min		
	Samples + Beads	Detection Antibody	SA-PE
All panels	30	30	10

Results

Assay Performance and Quality

Performance characteristics of the Th17 assays were examined according to the following parameters: assay sensitivity, precision, accuracy, assay working range, cross-reactivity, matrix effects, and validation with biological samples. Assay sensitivity, defined as LOD, was evaluated by adding two standard deviations to the mean median fluorescence intensity (MFI) of 11 zero standard replicates. Of the 11 assays investigated, three (IL-22, IL-27, and MIP-3α) were detected in serum at ≤1 pg/ml, four (IL-17F, IL-21, IL-25, and CD40L) at ≤10 pg/ml, and four (IL-23, IL-31, IL-33, and ICAM-1) at ≤40 pg/ml (Table 3). The results in cell culture media showed comparable performance, with the exception of IL-21 and IL-23, which recorded greater than 10 pg/ml difference and higher and lower LOD, respectively.

Table 3. Limit of detection (pg/ml) in serum and cell culture media.

Matrix	IL-17F	IL-21	IL-22	IL-23	IL-25	IL-27	IL-31	IL-33	CD40L	MIP-3α	ICAM-1*
Serum	8	7	0.5	32	3	1	36	22	4	1	14
Cell culture media	3	41	1	20	4	3	38	28	4	1	16

* Values derived from singleplex format.

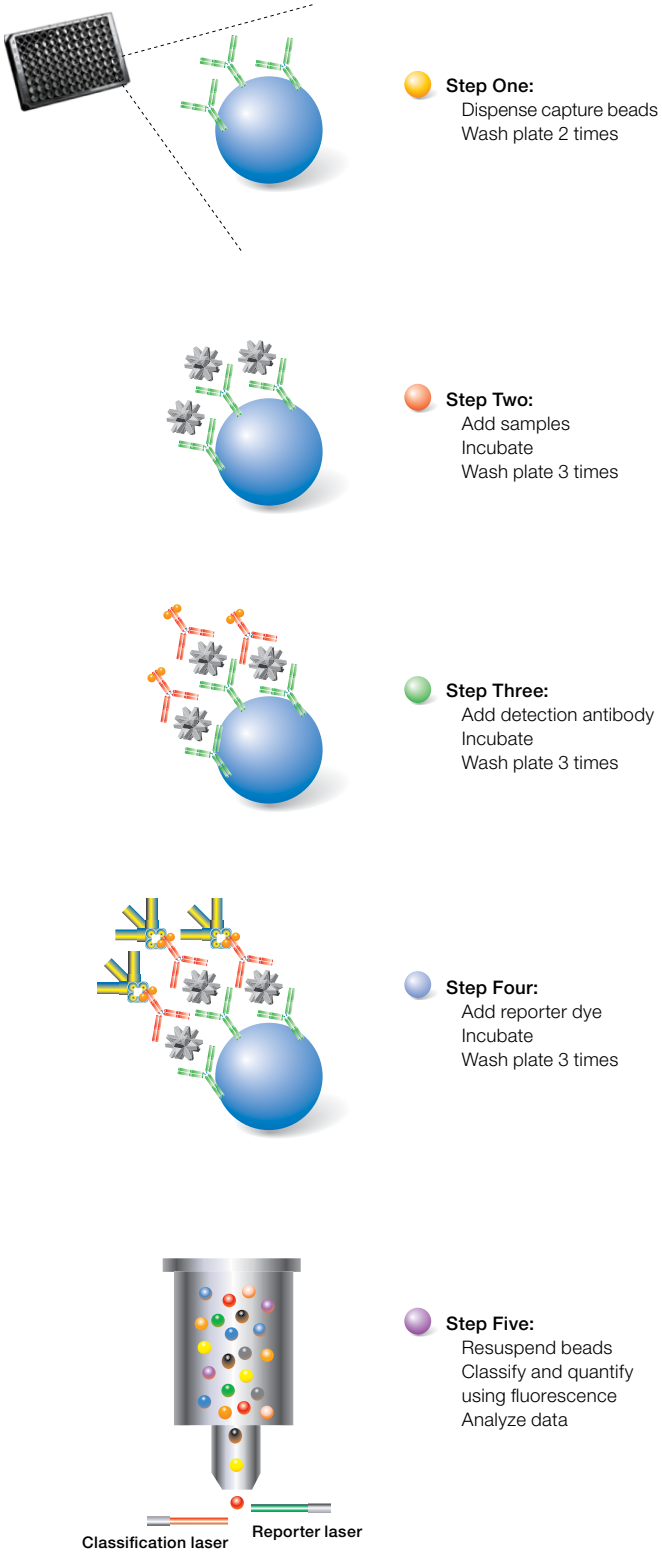

Fig. 1. Schematic representation of a sandwich-based Bio-Plex Pro Assay workflow.

Evidence of reproducibility was reported in both intra- and inter-assay precision in serum matrix (Table 4). Intra-assay precision was calculated as the coefficient of variation (%CV) among fluorescence values of within-plate replicate wells. The precision values reflect a mean of eight standard points from three plates and two operators. Overall, these assays demonstrated a mean intra-assay %CV of ≤10%. Inter-assay precision was calculated as the mean %CV of the observed concentrations of two levels of spike controls. The precision reflects two independent assays, in which a mean inter-assay %CV of ≤10% was achieved. The precision in cell culture media was comparable or better (data not shown).

Table 4. Precision profile — intra- and inter-assay %CV.

Target	Standard		Control
	Intra-Assay %CV 10-Plex	Intra-Assay %CV Singleplex	Inter-Assay %CV
IL-17F	5.9	3.9	1.7
IL-21	4.4	9.1	3.2
IL-22	4.7	3.9	1.4
IL-23	4.5	4.1	1.7
IL-25	2.8	7.8	1.4
IL-27	2.7	3.9	0.4
IL-31	3.0	4.8	2.0
IL-33	3.9	7.4	2.7
CD40L	2.8	4.4	1.9
MIP-3α	2.7	4.5	2.9
ICAM-1*	–	3.0	5.0

* Values derived from singleplex format.

Assay working range is defined as the range between LLOQ and ULOQ in which an assay is both precise and accurate. The ranges of these assays were determined for both serum and cell culture media (Table 5). Assay working ranges were also investigated in the singleplex format using the same serum matrix. The results are generally comparable to the values obtained using the multiplex format (data not shown). The consistency of these ranges is often dictated by the overall precision in preparing the assay reagents.

Table 5. Multiplex assay working ranges in serum and cell culture media matrices.

	Serum		Cell Culture Media	
Target	LLOQ	ULOQ	LLOQ	ULOQ
IL-17F	25	86,159	6	99,280
IL-21	30	31,793	56	56,917
IL-22	1	21,071	1	20,770
IL-23*	14	273,553	14	272,946
IL-25	5	71,463	7	74,008
IL-27	4	60,530	4	60,297
IL-31*	85	–	67	–
IL-33	43	170,779	39	159,751
CD40L	8	136,300	10	106,461
MIP-3α	1	5,499	2	6,486
ICAM-1*	81	90,319	20	82,750

* Values derived from singleplex format.

Assay accuracy (also defined as recovery) was calculated as the percentage of the observed concentration value of a target antigen relative to the expected value. This parameter was evaluated using standard points and spiked controls in both multiplex and singleplex configurations, and in both serum and cell culture media. Overall, the standard recovery is comparable in both matrices, with most targets recovering 80–120% (Table 6).

Table 6. Percentage standard recovery.

Target	S1	S2	S3	S4	S5	S6	S7	S8
IL-17F	76	126	95	99	104	99	98	101
IL-21	–	104	98	101	98	103	96	–
IL-22	80	109	99	99	102	99	100	100
IL-23	101	98	102	99	100	100	100	98
IL-25	–	100	100	100	101	98	103	99
IL-27	96	107	95	101	104	97	100	101
IL-31	99	102	97	104	97	99	114	71
IL-33	105	99	99	101	99	100	99	–
CD40L	–	104	99	102	97	99	110	88
MIP-3α	–	89	116	95	99	103	98	101
ICAM-1	–	103	98	102	96	106	95	–

Assay specificity was examined by performing single-antigen and single-detection cross-reactivity studies. The single-antigen study evaluates the specificity of a capture antibody. This was conducted by testing an individual antigen in the presence of multiplexed capture beads and detection antibody. The single-detection study evaluates the specificity of the detection antibody. This was conducted by testing the individual detection antibody in the presence of multiplexed antigens and capture beads. In this study cross-reactivity was performed specifically on the relevant Th17 markers in the 6-plex and 10-plex panels. Data analysis was weighted on the second highest standard concentration point. The results showed that the degree of cross-reactivity within each panel is well below 1% (data not shown).

Linearity of dilution was assessed by spiking known quantities of recombinant antigens into native serum and plasma matrices. A series of seven serially diluted spiked samples was prepared for each matrix. ICAM-1 was evaluated by using mouse serum or plasma with high endogenous levels. The observed and expected sample concentrations within assay working range were plotted for each analyte in the multiplex. The correlation coefficient (R^2) value generated by linear regression analysis reflects the linearity of dilution for that assay (Table 7).

Table 7. Dilution linearity in serum and plasma matrices.

	R^2	
Target	Serum	Plasma
IL-17F	1.00	1.00
IL-21	1.00	1.00
IL-22	0.99	1.00
IL-23	1.00	1.00
IL-25	1.00	1.00
IL-27	1.03	1.00
IL-31	1.01	1.00
IL-33	1.00	1.00
CD40L	0.99	0.99
MIP-3α	0.99	0.99

Note: ICAM-1 cannot be spiked at high concentration.

To support the assumption that the antibody-binding characteristics are similar between the standard reference matrix and the diluted serum or plasma sample matrices, assay parallelism was investigated by comparing the slope of the spike concentration response curve in mouse serum or plasma with that of the serum-based standard diluent.

The percentage difference is summarized in Table 8. In the serum, the level of difference ranges between 0.3 and 26%. In plasma, the difference ranges between 0.3 and 31.7%. This demonstrates assay parallelism between the standard reference matrix and the mouse serum and plasma tested.

Table 8. Assay parallelism in mouse serum and plasma matrices.

	Slope Difference, %	
Target	Mouse Serum vs. Standard Diluent	Mouse Plasma vs. Standard Diluent
IL-17F	26.0	31.7
IL-21	–4.7	–4.0
IL-22	0.3	–2.5
IL-23	–9.3	–3.3
IL-25	4.0	2.5
IL-27	–2.2	0.3
IL-31	–0.6	–3.2
IL-33	–0.3	–4.0
CD40L	–0.6	–5.9
MIP-3α	5.1	–5.4

Analysis of Mouse Serum and Plasma Samples

The mouse Th17 assays were further validated by collecting sample measurements in various biological samples, such as mouse plasma and cell culture samples.

To study the production of cytokines related to Th17 cells, we injected mice with lipopolysaccharide (LPS), a toxin derived from the outer membrane of gram-negative bacteria, and measured cytokine concentration using the Bio-Plex Pro Mouse Th17 Cytokine 10-Plex Panel (Zambrano-Zaragoza et al. 2014, Copeland et al. 2005). Briefly, increasing concentrations of LPS solutions were prepared in phosphate buffered saline (PBS) and animals were dosed with 0.2 ml of LPS or PBS by single intraperitoneal injection. Plasma K2 ethylenediaminetetraacetic acid (EDTA) was collected 2 hr after injection and was diluted 4-fold for testing with the Bio-Plex Pro Mouse Th17 10-Plex Panel and 100-fold for testing with the ICAM-1 singleplex assay. These data clearly demonstrate the dose response increase in IL-17F, IL-22, IL-23, IL-27, MIP-3α, and ICAM-1 to LPS and the utility of these Bio-Plex Pro Assays in studying Th17 cytokines related to immunoresponse to pathogens (Figure 2). Of note is the more than 1,000-fold increase in IL-22 and IL-27.

To investigate the utility of Bio-Plex Pro Panels in detecting Th17 cytokines in cell culture growth media, EL4B5, a CD40L-expressing mouse thymoma cell line, and RAW 264.7, a commonly used model of mouse macrophages, were activated (Werner-Favre et al. 1994, Raschke et al. 1978). Growth medium was collected from EL4B5 cultures treated for 48 hr with three conditions, no compounds or combinations of two or four compounds known to stimulate T cells (Table 9), and from RAW 264.7 treated with no LPS and 10 µg/ml LPS for 16 and 48 hr (Table 10). For ease of use, the Bio-Plex Pro Mouse Th17 6-Plex, Th17 10-Plex, and ICAM-1 were blended to a 17-plex panel, and 50 µl of growth medium was tested undiluted. We observed the strongest production of IL-17A, IL-17F, IL-21, IL-22, IL-10, and IFN-γ after 48 hr treatment of condition 3 with IL-6, LPS, phorbol 12-myristate 13-acetate (PMA), and ionomycin (Figure 3). Treatment of RAW 264.7 with 10 µg/ml LPS for 48 hr led to the strongest production of IL-23, IL-25, IL-27, IL-31, IL-33, ICAM-1, IL-1β, IL-6, and TNF-α. CD40L (Figure 3) and MIP-3α (Figure 4) were generated consistently regardless of stimuli.

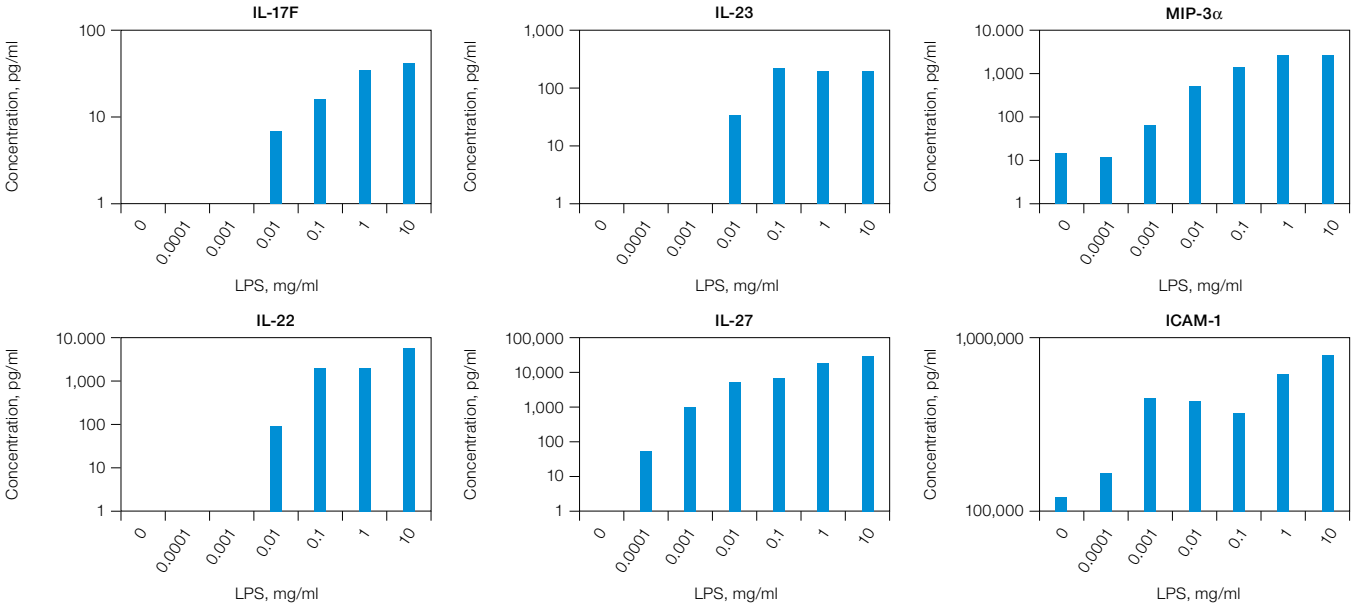

Fig. 2. Plasma from normal mice and mice treated with increasing concentrations of LPS.

Table 9. Three different treatment conditions of the EL4B5 cell line.

	48 hr Treatment, µg/ml		
Compound	**1**	**2**	**3**
IL-6	0	0.05	0.05
LPS	0	0	1
PMA	0	0	1
Ionomycin	0	0	1

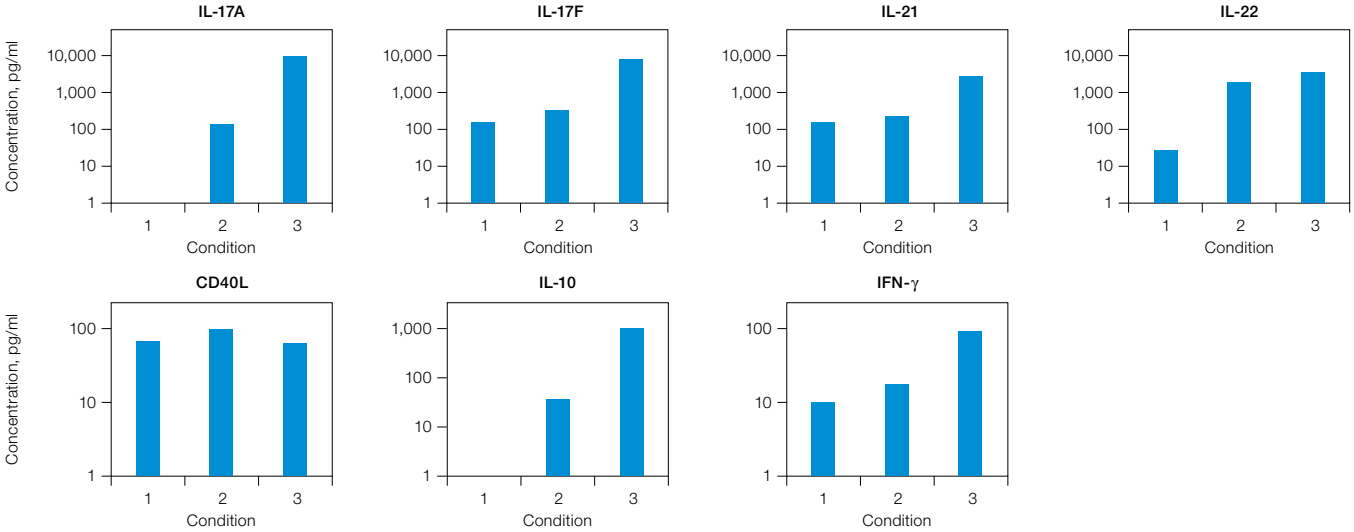

Fig 3. EL4B5 cell line Th17 cytokine expression after 48 hr of no treatment and treatment with different combinations of IL-6, LPS, PMA, and ionomycin.

Table 10. Treatment of the RAW 264.7 cell line with 0 and 10 µg/ml LPS at different time points.

Time Point, hr	LPS Concentration, µg/ml
0	0
16	10
48	10

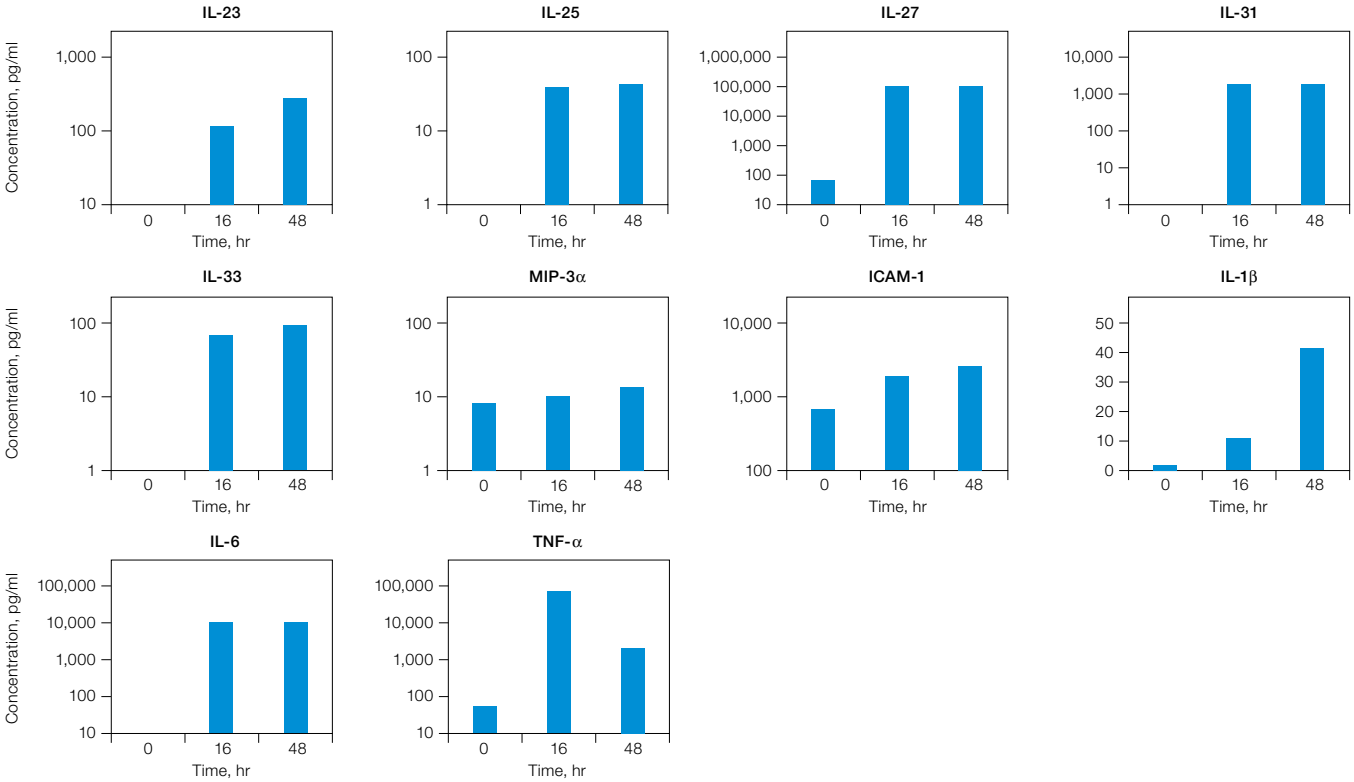

Fig. 4. Evaluating Th17 cytokine expression in time course treatment of cell line RAW 264.7 with 0 (0 hr) and 10 µg/ml (16 and 48 hr) of LPS.

Conclusions

The newly configured Bio-Plex Pro Mouse Th17 Cytokine Magnetic Bead–Based Multiplex Assays are designed to meet the demands of the academic and pharmaceutical research communities. By measuring multiple analytes simultaneously, the mouse Th17 panel helps reduce time, cost, and sample volume compared to more traditional systems such as enzyme-linked immunosorbent assay (ELISA). Similar to all Bio-Plex Cytokine Assay Panels, the Mouse Th17 Assay Panel is also compatible with Luminex Readers, such as FLEXMAP 3D and MAGPIX.

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