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

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Introduction

T helper cells have been classically 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, the differentiation of which is driven by IL-4, are important for the production of immunoglobulin E and the clearance of extracellular organisms (Kimura and Kishimoto 2010). IL-17 producing T helper cells (Th17) are a new subset of T helper cells recently found to be associated with autoimmunity 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 2010) .

Using Luminex xMAP technology (Dale et al. 2008) and bulletin 5405, Bio-Rad has previously developed 32 mouse cytokine assays, which are configured into a mouse group I 23-plex and a mouse group II 9-plex, respectively (Table 1). To further expand our Bio-Plex Pro mouse cytokine assay menu for Th17 studies, a mouse cytokine group III that includes 11 new assays 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 (Th17 panel A, Table 1, 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 11 markers in group III. The assays were validated with mouse serum, plasma, and cell culture matrices. Validation criteria include assay working range (LLOQ/ULOQ), sensitivity (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 Group III
Mouse Assays	**1-Plex**	**23-Plex**	**9-Plex**	**8+3-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-23p19	●			●
IL-31	●			●
IL-33	●			●
CD40L	●			●
MIP-3α	●			●
ICAM-1	●			●
IL-25	●			●
IL-27p28	●			●
IL-15	●		●	
IL-18			●	
Basic FGF	●		●	
LIF	●		●	
M-CSF	●		●	
MIG	●		●	
MIP-2	●		●	
PDGF-BB	●		●	
VEGF	●		●	

(●) refer to a subset of Th17 relevant mouse group I (6-plex) and group III (8-plex) assays designated as mouse Th17 panel A and B respectively.
(●) designate group III singleplex assays that cannot be multiplexed with either the mouse group I 6-plex or the mouse group III 8-plex panel due to cross reactivity or differences in sample dilution factors. Mouse group I and III assays cannot be multiplexed with each other for the same reasons. IL-25 and IL-27p28 can be assayed together in a 2-plex format.

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 conjugate (SA-PE). 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 6.0 at low PMT setting.

Table 2. Bio-Plex Pro mouse cytokine assay incubation and detection guidelines.

	Incubation time, min		
	Samples + bead	Detection Ab	SA-PE
Group I and II	30	30	10
Group III*	60	30	10

* Includes 8-plex (Th17 panel B) and singleplex assays

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 limit of detection (LOD), was evaluated by adding two standard deviations to the mean median fluorescence intensity (MFI) of ten zero standard replicates. Of the 11 assays investigated, seven (IL-17F, IL-22, IL-23p19, IL-33, CD40L, MIP-3α, and ICAM-1) were detected at ≤1 pg/ml, three (IL-21, IL-31, IL-25) at ≤10 pg/ml, and one (IL-27p28) at ≤30 pg/ml (Table 3). The results in RPMI cell culture media showed comparable performance, with the exception of IL-25 and IL-27p28, which recorded higher and lower LOD respectively.

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

Matrix	IL-17F	IL-21	IL-22	IL-23p19	IL-31	IL-33	CD40L	MIP-3α	IL-25*	IL-27p28*	ICAM-1**
Serum	0.1	2.8	0.1	0.7	5.2	0.2	0.4	0.3	9.0	29.2	0.8
RPMI	0.3	0.2	0.3	1.0	5.0	0.8	0.8	0.1	55.8	13	0.5

* Mean LOD derived from five multiplex assays in a 2-plex format.
** Mean LOD derived using a singleplex format in a serum matrix and multiplex format in RPMI.

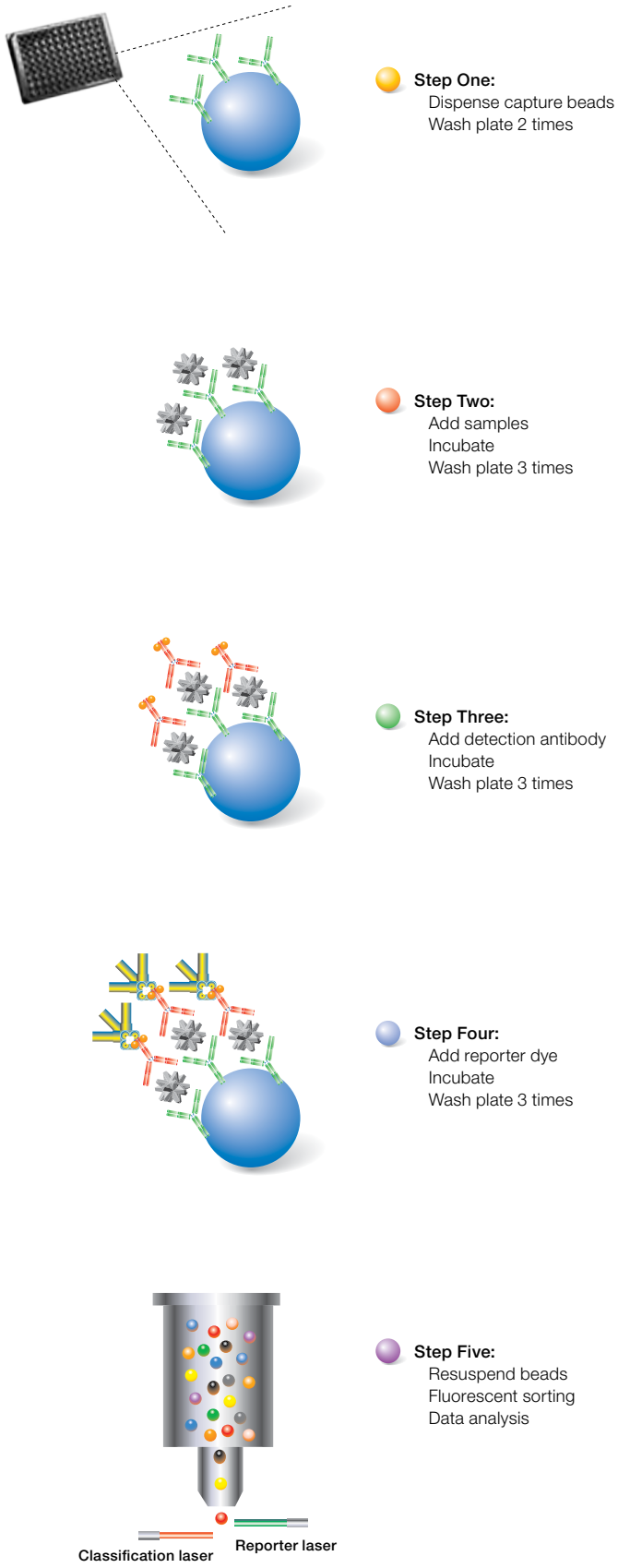

Fig. 1. Schematic representation of a sandwich-based Bio-Plex assay workflow.

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 four replicate wells. 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 three independent assays, in which a mean inter-assay %CV of ≤15% was achieved. The precision in RPMI was comparable or better (data not shown).

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

Targets	Intra-assay Precision		Inter-assay Precision
	Multiplex	Singleplex	Mean of 2-Level Spike
IL-17F	5.0	4.7	5
IL-21	3.5	5.8	15
IL-22	4.3	6.1	5
IL-23p19	4.6	7.5	6
IL-31	6.3	5.7	6
IL-33	4.7	4.0	12
CD40L	3.6	4.0	14
MIP-3α	6.2	5.6	13
IL-25*	9.0	4.8	12
IL-27p28*	8.8	6.5	6
ICAM-1**	—	4.0	8

* Values derived from a 2-plex format.
** Values derived from singleplex format.

Assay working range is defined as the range between the lower limit of quantification (LLOQ) and the upper limit of quantification (ULOQ) in which an assay is both precise and accurate. The ranges of these assays were determined for both serum and RPMI 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 RPMI matrices.

Matrix	Serum		RPMI	
Working Range	LLOQ	ULOQ	LLOQ	ULOQ
IL-17F	0.7	6,362	0.7	11,567
IL-21	5.5	8,207	5.5	5,660
IL-22	0.5	8,433	0.5	8,433
IL-23p19	3.4	55,764	13.6	55,764
IL-31	2.5	41,313	10.1	41,313
IL-33	0.5	7,343	1.8	7,343
CD40L	3.6	11,245	0.7	11,245
MIP-3α	0.7	1,660	3.7	3,794
IL-25*	168.0	98,266	383.9	98,266
IL-27p28*	63.4	117,521	28.7	117,521
ICAM-1**	2.9	23,723	1.5	23,723

* Values derived from a 2-plex format in both matrices.
** Values derived from singleplex format in a serum matrix.

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 both in serum and RPMI. Overall, the standard recovery is comparable in both matrices, with most targets recovering within 80–120%.

Table 6. Standard Recovery in Serum Matrix

Targets	Sample							
	S1	S2	S3	S4	S5	S6	S7	S8
IL-17F	105	99	101	100	99	102	99	101
IL-21	**	80	98	105	96	103	98	101
IL-22	117	96	101	100	100	99	102	99
IL-23p19	101	98	101	101	98	102	100	99
IL-31	100	99	102	99	99	101	105	89
IL-33	101	98	103	100	98	99	110	89
CD40L	100	100	100	99	102	100	96	107
MIP-3a	80	123	94	103	99	100	100	98
IL-25*	100	103	96	97	105	**	**	**
IL-27p28*	100	99	102	100	98	101	**	**

* Values derived from a two-plex format.
** Recovery values obtained outside the assay working range.

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 Th17 relevant markers on panels A and B. 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 were 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.

Targets	R^2	
	Serum	Plasma
IL-17F	0.996	0.958
IL-21	1.000	0.959
IL-22	0.999	0.993
IL-23p19	1.000	1.000
IL-31	0.999	1.000
IL-33	1.000	1.000
CD40L	1.000	1.000
MIP-3α	1.000	1.000
IL-25*	0.988	0.999
IL-27p28*	1.000	1.000
ICAM-1**	0.9991	0.9997

* Values derived from a 2-plex format.
** Values derived from a singleplex format.

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 prepared in the serum-based standard diluent.

The percentage difference is summarized in Table 8. In the serum, the level of difference ranges between 0.5 and 18.6%. In plasma, the difference ranges between 0.1 and 37.3%. This demonstrates assay parallelism between the standard reference matrix and the mouse serum and plasma tested.

Analysis of Mouse Serum and Plasma Samples

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

Targets		Slope Difference, %	
		Mouse Serum vs Standard Diluent	Mouse Plasma vs Standard Diluent
8-plex	IL-17F	3.7	1.7
	IL-21	14.4	18.9
	IL-22	0.9	3.0
	IL-23p19	1.7	6.6
	IL-31	6.7	3.5
	IL-33	0.7	1.2
	CD40L	1.6	0.1
	MIP-3α	12.6	37.0
2-plex	IL-25	2.2	10.7
	IL-27p28	5.2	7.0
Singleplex	IL-17F	1.6	15.2
	IL-21	18.6	24.0
	IL-22	0.5	10.9
	IL-23p19	3.3	2.2
	IL-31	0.8	3.2
	IL-33	2.1	1.0
	CD40L	11.4	11.0
	MIP-3α	12.6	37.3
	IL-25	3.1	13.1
	IL-27p28	2.6	4.4
	ICAM-1	14.5	16.6

The mouse Th17 assays were further validated by collecting sample measurements in various biological samples such as mouse serum, plasma, broncheoalveolar lavage fluid, and cell culture samples. Table 9 provides an overview of the percentage of samples measured within the assay working ranges.

In a study comparing normal and LPS-stimulated mouse serum, the levels of most Th17 cytokines were significantly elevated after a single-dose LPS challenge (Figure 2).

Table 9. Percentage of biological samples tested falling within mouse Th17 working assay ranges.

Targets	Percent of Samples Measured within Assay Range*
IL-17F	100
IL-21	59
IL-22	92
IL-23p19	99
IL-31	59
IL-33	67
CD40L	70
MIP-3α	99
IL-25**	32
IL-27p28**	82
ICAM-1***	100

* N=188

** Values derived from a 2-plex format.

***Values derived from singleplex format.

Conclusions

The newly configured Bio-Plex Pro mouse Th17 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 ELISA. Similar to all Bio-Plex cytokine assay panels, the mouse Th17 assay panel is also compatible to other Luminex readers, such as FLEXMAP3D and MAGPIX.

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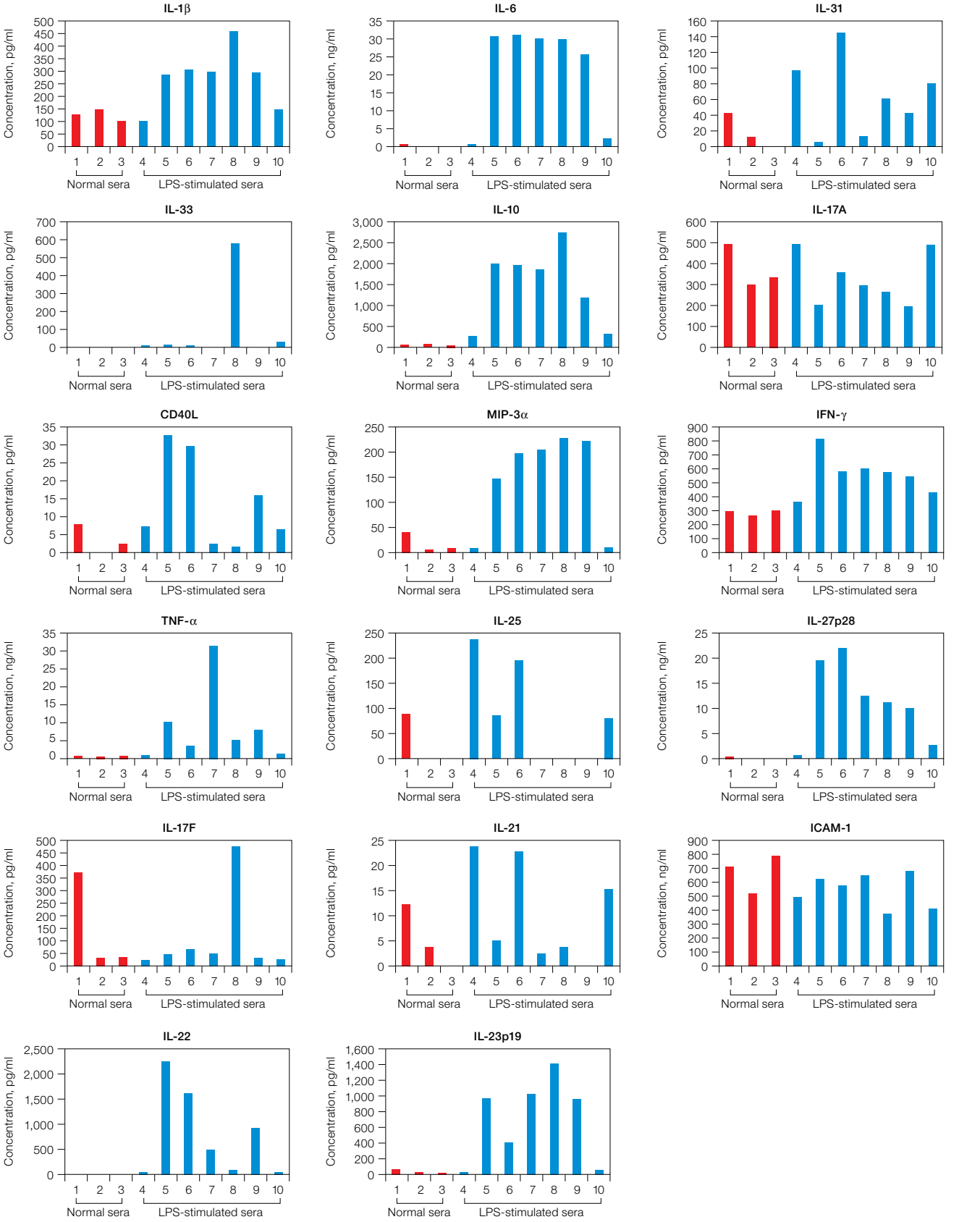

Fig. 2. Elevation of Th17 markers in sera of LPS-challenged mice (strain C57BL/6).

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