Biological Evaluation of MILLIPLEX® MAP Human and Mouse Th17 Panels

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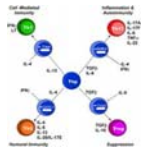
Introduction

Th17 cells are recently discovered T helper cells that play important roles in the establishment and maximization of the capabilities of the immune system. We recently developed MILLIPLEX® MAP Human and Mouse Th17 multiplex assays for simultaneous measurement of the cytokines that are secreted from Th17 cells and/or regulate Th17 cell differentiation and activation. In this study, we used the two Th17 panels to analyze the cytokine secretion in human and mouse PBMCs stimulated with lipopolysaccharide (LPS), concanavalin A (Con A) or Phytohemagglutinin (PHA). We also examined the effect of Adiponectin pretreatment on the LPS- or Con A-induced PBMC response. We examined cytokine levels in human plasma from sepsis, rheumatoid arthritis and lupus patients and examined *in vivo* mouse plasma cytokine response to LPS treatment. Our results demonstrated that LPS, Con A or PHA treatments induced significantly increased secretion of many cytokines including Th17 cell specific cytokines from human and mouse PBMCs (e.g. IL-6, TNF*α*, IL-17A, IL-17F and IL-22). The diseased human and the *in vivo* LPS-treated mice plasma samples also expressed increased levels of Th17 cell specific plasma cytokines. Adiponectin may regulate certain LPS-induced or Con A-induced cytokine secretion from PBMCs. In conclusion, the Th17 panels will be useful tools for Th17 cell-related cytokine profiling in various biological samples.

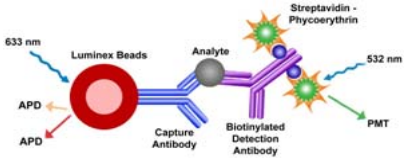


LPS, a component of Gram-negative bacterial cell walls, stimulates the innate immune response via TLR (Toll-like receptor) 4. Con A and PHA are lectins which bind to cell membrane carbohydrates and induce cell agglutination and T-cell mitosis and differentiation. Adiponectin, also called GBP-28, apM1, AdipoQ, and Acrp30, is a novel adipose tissue-specific protein that has structural homology to collagen VIII and X and Complement factor C1q, and circulates in human plasma at high levels. There has been a reported adiponectin attenuation of the TNFα response *in vivo* which indicates an anti-inflammatory role for the protein (Xu et al, 2003).

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 and resuspended in RPMI Media (Gibco) containing 10% FCS and 1% Penicillin/Stretomycin at 1 million cells per mL. The PBMCs were aliquoted at one million cells per well and incubated overnight at 37°C. The next day either LPS, Con A or PHA (Sigma) were added for final concentrations of 10, 20 or 20 μg/mL, respectively. For Adiponectin pretreatment studies, the Adiponectin (Sigma) was added to select wells to 10 ug/mL final concentration, 5 min prior to the addition of LPS, Con A or PHA. 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. Human disease plasma and *in vivo* LPS-challenged mouse plasma samples were obtained from Bioreclamation.

Immunoassay Protocol. 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 at 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 and further incubate at RT for 30 min. Lastly, wash beads two times, add 100 μL sheath fluid and read on Luminex instrumentation.

Results: Human Th17 Multiplex Panel

Table 1. Stimulation of Human PBMC

Human PBMCs were treated with LPS, Con A or PHA at 37°C for 48 hr and cell-free samples were collected and assayed as described. Approximate cytokine responses are indicated as (+) 40 to 100 pg/mL, (++) 101 to 1000 pg/mL or (+++) >1000 pg/mL. Unstimulated samples were 0-40 pg/mL for most analytes.

Cytokine Response	LPS	Con A	PHA
IL-1β	+++	++	++
IL-2	+	+++	+++
IL-4		++	+
IL-5		++	+
IL-6	+++	+++	+++
IL-9		++	++
IL-10	++	++	++
IL-13		+++	++
IL-17A		++	++
IL-17F		+++	++
IL-21		+	+
IL-23	+	+	
IL-27	++	+	++
IL-31		++	++
IFNγ		+++	+++
GM-CSF	++	+++	+++
MIP-3α	++	++	++
TNFα	+++	+++	+++
TNFβ		++	++

Table 2. Stimulation of Human PBMC: Representative Study

Human PBMCs were treated with LPS or Con A alone or after 5 min pretreatment with Adiponectin then incubated for 48 hr at 37°C and cell-free samples were collected and assayed as described. Values are pg/mL.

pg/mL			IL-17F	GM-CSF	IFNg	IL-10	MIP-3a	IL-12p70	IL-13	IL-15	IL-17A	IL-22	IL-9	IL-1b	IL-33	IL-2	IL-21	IL-4	IL-23	IL-5	IL-6	IL-17E	IL-27	IL-31	TNFa	TNFb	IL-28A
Sample	sample	A (ug/mL)	19	20	25	27	28	33	35	37	39	43	45	46	47	48	52	53	54	55	57	66	72	73	75	76	77
Unstimulated	1	0	34	33	10	10	15	27	6	5	7	135	10	2	3	7	10	36	194	3	101	0	103	29	17	16	35
0 hr	2	0	12	32	6	1	7	4	16	0	0	29	0	4	0	4	3	17	123	3	76	0	0	0	19	0	0
0 hr	3	0	19	37	8	2	10	7	23	4	0	38	0	2	0	5	6	25	150	4	80	0	0	23	22	0	0
48 hr	1	0	61	37	76	11	24	42	107	8	6	187	10	5	11	33	23	47	366	5	106	0	201	37	29	19	53
48 hr	2	0	13	65	7	2	6	3	12	6	0	0	0	0	0	6	0	16	150	4	68	0	0	22	22	0	0
48 hr	3	0	13	0	10	2	6	3	16	0	0	30	0	0	0	7	0	16	176	0	75	0	0	0	24	0	0
Adiponectin Alone	1	0.2	40	49	48	12	18	26	81	5	0	157	10	4	6	31	13	40	298	3	88	0	137	27	24	0	34
48 hr	2	0.2	16	58	8	3	8	5	11	3	0	34	0	2	2	6	5	15	132	0	75	0	0	30	22	15	0
	3	0.2	18	0	24	2	10	7	24	3	0	51	7	2	3	10	6	16	132	3	74	0	44	33	26	0	0
	1	0.4	16	0	16	10	9	8	26	3	0	81	6	3	0	34	4	17	0	3	74	0	51	0	16	0	0
	2	0.4	12	0	7	2	6	3	10	0	0	0	0	0	0	5	0	13	132	0	69	0	0	24	23	0	0
	3	0.4	15	53	10	2	7	3	16	3	0	16	0	2	0	9	0	0	114	3	80	0	37	0	32	0	0
	1	10	29	61	38	12	17	13	53	7	0	92	5	3	4	36	8	22	132	0	121	0	92	30	34	0	0
	2	10	14	73	10	3	12	4	13	3	0	37	5	0	0	7	3	16	123	0	107	0	34	21	36	0	0
	3	10	17	48	23	4	15	5	25	5	0	48	0	0	3	6	3	15	141	0	117	0	55	27	40	0	0
LPS alone	1	0	48	0	80	887	122	22	53	11	7	121	13	47	3	47	21	35	323	5	3471	0	264	42	1388	18	32
48 hr	2	0	46	897	54	902	297	8	17	7	8	39	11	185	5	17	21	28	786	5	>10000	0	270	31	2484	16	24
	3	0	65	511	95	937	237	12	40	9	8	68	10	169	7	20	23	48	786	5	>10000	0	328	42	2389	20	28
LPS and Adiponectin	1	0.2	61	0	67	881	248	18	78	8	8	121	12	44	8	50	28	41	434	5	3481	0	313	33	1709	23	30
48 hr	2	0.2	113	704	80	917	611	14	27	10	10	53	14	150	3	17	22	31	1115	3	>10000	0	345	38	3131	19	27
	3	0.2	61	745	54	838	221	11	32	11	6	56	11	201	5	11	27	34	894	6	>10000	0	317	47	2367	17	37
	1	0.4	34	114	47	811	97	13	37	8	7	87	13	44	0	31	18	24	246	4	3160	0	217	31	1120	0	24
	2	0.4	53	503	45	790	232	7	21	10	0	41	8	120	0	12	22	24	636	3	>10000	0	240	36	1942	15	24
	3	0.4	88	424	65	822	247	13	41	11	7	66	12	202	3	14	22	35	1017	4	>10000	0	294	42	2377	21	38
	1	10	48	63	101	873	360	13	46	14	11	115	14	62	7	65	24	29	315	3	>10000	0	246	29	2078	0	32
	2	10	101	877	85	869	657	14	28	8	10	66	15	133	4	20	25	30	1075	4	>10000	0	306	44	3032	15	31
	3	10	112	775	119	950	802	18	31	10	12	61	12	180	7	20	28	34	1619	4	>10000	0	386	35	3417	18	32
Con A alone	1	0	1581	6628	>40000	941	552	36	5091	20	263	184	371	148	14	3160	58	1315	753	988	708	0	472	140	>10000	1717	50
48 hr	2	0	1378	3306	8400	130	133	17	2165	10	97	66	68	501	8	2689	47	984	661	631	472	0	134	76	4513	529	46
	3	0	781	1636	10032	136	78	18	1815	11	41	54	38	35	6	621	37	865	349	706	290	0	160	58	4329	409	37
Con A and Adiponectin	1	0.2	1040	2475	10008	102	161	20	2220	12	78	72	48	154	7	2220	50	1212	409	712	317	0	129	55	4740	487	38
48 hr	2	0.2	1017	2303	11167	126	156	25	2561	15	45	64	49	86	7	1961	51	1414	527	1008	306	0	183	60	>10000	520	37
	3	0.2	702	1551	10249	93	403	26	2099	12	57	72	45	81	9	4332	109	1319	443	420	391	0	181	61	>10000	404	38
	1	2.4	1186	2363	11910	121	187	29	2463	13	64	65	47	109	7	2085	62	1279	485	920	458	0	183	62	>10000	518	37
	2	10	2549	7726	>40000	909	749	30	4960	20	354	182	353	336	12	4744	76	1184	778	939	1700	0	483	154	>10000	1914	63
	3	10	1472	3111	10438	99	214	20	2083	12	75	65	48	199	8	1998	47	1035	460	813	1010	0	175	65	>10000	476	35
	1	10	821	2325	9997	110	159	26	2536	9	47	44	49	85	7	1835	49	1176	485	954	304	0	204	52	>10000	593	32

Table 3. Human Disease Samples

Human disease plasma samples were obtained from Bioreclamation and assayed in the Human Th17 Panel as described. Values are the sample range in pg/mL for 16 sepsis, 4 rheumatoid arthritis (RA) and 4 lupus samples.

Cytokine Response	Sepsis (n=16)	RA (n=4)	Lupus (n=4)
IL-1β	0-129	0-143	0-30
IL-2	0-160	0-514	0-108
IL-4	0-735	0-3	ND
IL-5	0-88	0-5	ND
IL-6	31-703	0-373	0-11
IL-9	0-185	0-143	ND
IL-10	0-1603	0-2	0-8
IL-12p70	0-126	0-185	0-23
IL-13	12-300	0-11	0-1
IL-15	9-80	0-89	0-13
IL-17A	0-111	0-1	ND
IL-17E/IL-25	0-12479	0-18634	0-951
IL-17F	15-1038	0-1237	0-79
IL-21	22-254	0-34	0-3
IL-22	37-2042	0-729	ND
IL-23	0-13183	0-12472	0-1685
IL-27	2333-17179	505-8777	429-804
IL-28A	200-2974	0-1123	1-313
IL-31	0-4614	0-3013	0-17
IL-33	0-1202	0-320	0-10
IFNγ	0-445	0-30	ND
GM-CSF	61-3262	0-1493	0-320
MIP-3α	48-1207	0-127	7-48
TNFα	20-4355	4-72	2-16
TNFβ	0-386	0-474	0-13

Results: Mouse Th17 Multiplex Panel

Table 4. Stimulation of Mouse PBMC

Mouse PBMCs were treated with LPS, Con A or PHA at 37°C for 48 hr, then cell-free samples were collected and assayed as described. Approximate cytokine responses are indicated as (+) 1 to 20 pg/mL, (++) 21 to 500 pg/mL or (+++) >500 pg/mL. Unstimulated samples were not detectable for most samples except IL-6 at approximately 30 pg/mL.

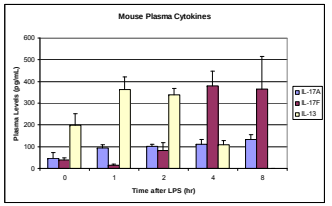
Cytokine Response	LPS	Con A	PHA
IL-1β	+		
IL-2		+	+
IL-4		+	
IL-5	+		
IL-6	+++	++	++
IL-10	+		
IL-17A		++	+
IL-17F		++	+
IL-22		+	
MIP-3α	+		
TNFα	++		

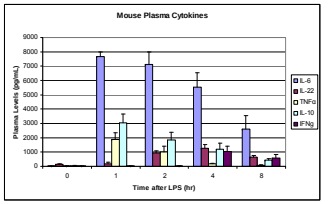
Table 5 . *in vivo* LPS Effect on Mouse Plasma Cytokine Levels

8-12 week old CD-1 Mice were injected with 1 mg/kg E.Coli 055:B5 LPS (Sigma-Aldrich) suspension in saline, IP, disodium EDTA plasma was collected at various time points and assayed as described. N=8, pg/mL (SEM)

hr	IL-1β	IL-6	IL-10	IL-12p70	IL-13	IL-15
0	3 (3)	31 (22)	53 (16)	44 (17)	198 (55)	57 (5)
1	11 (3)	7648 (352)	3053 (591)	106 (38)	363 (58)	75 (17)
2	102 (46)	7129 (871)	1855 (516)	80 (12)	339 (30)	48 (5)
4	60 (13)	5542 (1017)	1192 (429)	77 (10)	108 (20)	44 (6)
6	20 (6)	2616 (921)	442 (93)	114 (29)	0 (0)	124 (48)
hr	IL-17A	IL-17E	IL-17F	IL-22	IL-23	IL-27
0	45 (26)	3914 (1456)	40 (8)	158 (34)	460 (120)	1383 (494)
1	94 (14)	6049 (694)	15 (6)	190 (96)	962 (190)	2093 (535)
2	102 (11)	6392 (476)	82 (37)	923 (163)	680 (77)	2183 (433)
4	113 (21)	5044 (617)	379 (69)	1278 (250)	612 (70)	1961 (384)
6	132 (22)	3590 (510)	365 (151)	661 (84)	538 (48)	2805 (992)
hr	IL-28B	CD40L	GM-CSF	IFNg	MIP-3a	TNFa
0	137 (15)	157 (24)	45 (20)	20 (12)	96 (19)	20 (6)
1	171 (38)	167 (48)	70 (12)	27 (10)	157 (73)	1871 (489)
2	239 (59)	131 (8)	188 (52)	38 (9)	3307 (758)	1017 (376)
4	199 (17)	110 (5)	57 (13)	1052 (370)	8360 (5979)	164 (42)
6	228 (45)	201 (26)	39 (12)	592 (246)	352 (61)	70 (20)

Figures 1 & 2 . *in vivo* LPS-effect on Mouse Plasma Cytokine levels. Data from Table 3





Summary

- LPS, Con A or PHA treatment induced secretion of cytokines from Human or Mouse PBMC including Th17 cell specific cytokines.
- Human sepsis, RA and lupus plasma samples contain significant levels of many cytokines including Th17 cell specific cytokines.
- Mouse *in vivo* LPS-stimulated plasma samples contain significant levels of many cytokines including Th17 cell specific cytokines.
- Adiponectin may regulate cytokine secretion from PBMC.
- MILLIPLEX Th17 panels are powerful tools for cytokine profiling in biological samples.

References:

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2. Xu A, et al, The fat-derived hormone adiponectin alleviates alcoholic and nonalcoholic liver diseases in mice, J Clin Invest 112:91-100 (2003).

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