Application Note

Quantitative multiplex analysis of low-level cytokine expression: MILLIPLEX® MAP Human High Sensitivity T Cell Panel

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Introduction

Cytokines are immunomodulatory polypeptides that play key roles in both adaptive and innate immune responses. A generic term, "cytokines" includes interleukins (acting as mediators between T cells), chemokines (responsible for T-cell migration), lymphokines (produced by activated Th cells), and myokines (produced by muscle cells). As regulators of the immune system, cytokines act at the recognition, activation, and/or effector phases of an immune response, modulating the development and functional activities of the subtypes of T cells, B cells and myeloid cells.

Their significant role in normal inflammatory response and immune cell development and activation drives their involvement in a variety of disease types. Even low levels of chronic inflammation are involved in many clinical and subclinical disease states. According to the CDC (Centers for Disease Control and Prevention, FASTSTATS - Leading Causes of Death. cdc.gov. 2011), low-level chronic inflammation contributes to at least seven of the 10 leading causes of mortality in the US, including cardiovascular disease, stroke, Alzheimer's disease, diabetes and cancer.

Consequently, research involving cytokines 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 inflammatory process. This deeper understanding is facilitated by the ability to study multiple cytokines simultaneously.

Based on the Luminex xMAP® technology, our MILLIPLEX® MAP Human High Sensitivity T Cell Panel (Cat. No. HSTCMAG-28SK) is a 21-plex multiplexed assay kit for simultaneously detecting cytokines significant to Th1, Th2, and Th17 cells. The customizable panel enables the user to choose any number of analytes within the panel to meet specific research needs. In addition, the panel is available in a premixed-bead format as either a 21-plex or a 13-plex kit, with the latter including only the Th1 and Th2 markers.

This application note summarizes the development, analytical validation studies and subsequent test results generated by the research team during the kit development process. Further experiments are also regularly conducted after the panel has been transferred to manufacturing, where it undergoes rigorous testing by both our Technical Transfer and Quality Assurance/ Quality Control teams.

We also present data from collaboration with Drs. Barbara Nikolajczyk and Min Zhu of Boston University School of Medicine studying the association between obesity and inflammation due to the overexpression of cytokines that support immune cell differentiation/ activation.

Methods

For serum samples, the blood was allowed to clot for 30 minutes before centrifugation for 10 minutes at 1000 x g. The serum was removed and either assayed immediately or stored at -20 °C. Plasma samples, with EDTA anticoagulant, were centrifuged at 1000 x g within 30 minutes of blood collection. Plasma was removed and assayed immediately or stored at -20 °C. Frozen samples were thawed completely, vortexed, and centrifuged prior to use, to remove particulates. Neat samples were added directly into the assay plate. Sepsis samples were obtained from Discovery Life Sciences, Los Osos, CA.

For Drs. Nikolajczyk and Zhu's obesity study, human peripheral blood mononuclear cells (PBMCs) were isolated from four groups of subjects (n=8 per group) defined as Healthy Subjects (Lean) with BMI<25, Non-Diabetic Obese Subjects (ND) with BMI 30-35 and A1c<5.6, Prediabetic Subjects (PD) with BMI 30-35 and A1c 5.6-6.5 who had never taken metformin and Prediabetic with Metformin Subjects (PD+Met) with BMI 30-35 and A1c 5.6-6.5 who were treated with metformin as standard-of-care. The PBMCs were stimulated with plate-bound CD3 and soluble CD28 (2 µg/mL) at 1 x 10^6 cells/mL for 40 hours. Culture supernatant (25 µL) was analyzed using the multiplexed assay panel.

MILLIPLEX® MAP Multiplexed Assay Protocol

The multiplex assays were performed in 96-well plates according to the product instructions supplied for the MILLIPLEX® MAP Human High Sensitivity T Cell Panel (Cat. No. HSTCMAG-28SK). The detailed procedure is as follows:

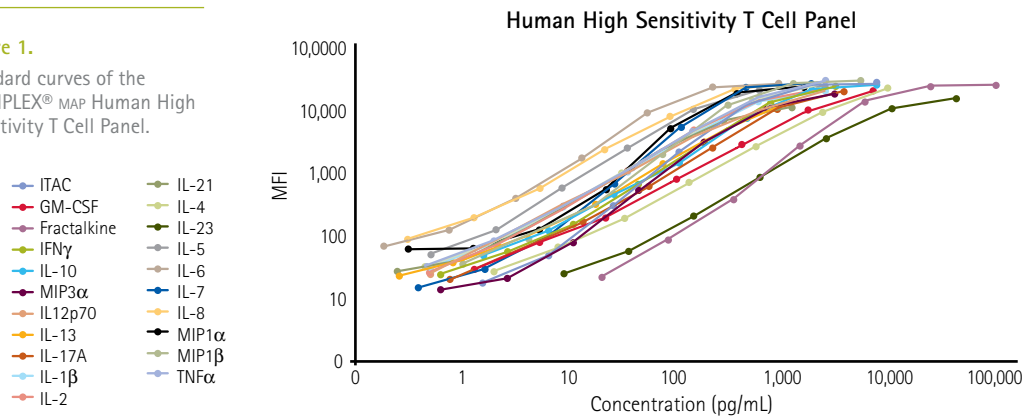
- Wet the plate with 150 µL assay buffer for 10 minutes and decant.
- Reconstitute standards and controls in serum matrix provided in the kit.
- Add 50 µL standards or controls to appropriate wells.
- Add 25 µL samples and 25 µL assay buffer to the sample wells.
- Add 25 µL beads to all wells and incubate overnight at 4 °C.
- Wash the beads three times, add 50 µL biotinylated detection antibody cocktail and incubate at room temperature (RT) for 1 hour.
- Add 50 µL streptavidin-phycoerythrin and further incubate at RT for 30 minutes.
- Wash beads three times, add 150 µL sheath fluid and read on a Luminex® instrument.

Results

The standard curves and standard concentrations for the MILLIPLEX® MAP Human High Sensitivity T Cell Panel are shown in Figure 1 and Table 1, respectively. The minimum detectable concentrations (Table 2) indicate the sensitivity for most assays to be less than 1 pg/mL. The standard curves show a broad linear range of detection for all the analytes in the panel (Figure 1).

Figure 1.

Standard curves of the MILLIPLEX® MAP Human High Sensitivity T Cell Panel.



ITAC	GM-CSF	Fractalkine	IFNγ	IL-10	MIP-3α	IL-12p70
1.46	1.22	18.31	0.61	1.46	0.61	0.49
5.86	4.88	73.24	2.44	5.86	2.44	1.95
23.4	19.53	292.97	9.77	23.4	9.77	7.81
93.8	78.1	1171.9	39.1	93.8	39.1	31.3
375	312.5	4687.5	156.2	375	156.25	125
1500	1250	18750	625	1500	625	500
6000	5000	75000	2500	6000	2500	2000

IL-13	IL-17A	IL-1β	IL-2	IL-21	IL-4	IL-23
0.24	0.73	0.49	0.49	0.24	1.83	7.93
0.98	2.93	1.95	1.95	0.98	7.32	31.74
3.91	11.72	7.81	7.81	3.91	29.30	126.95
15.6	46.9	31.3	31.3	15.6	117.2	507.8
62.5	187.5	125	125	62.5	468.75	2031.25
250	750	500	500	250	1875	8125
1000	3000	2000	2000	1000	7500	32500

IL-5	IL-6	IL-7	IL-8	MIP1α	MIP1β	TNFα
0.49	0.18	0.37	0.31	0.31	0.92	0.43
1.95	0.73	1.46	1.22	1.22	3.66	1.71
7.81	2.93	5.86	4.88	4.88	14.65	6.84
31.3	11.7	23.4	19.5	19.5	58.6	27.3
125	46.875	93.75	78.125	78.125	234.375	109.375
500	187.5	375	312.5	312.5	937.5	437.5
2000	750	1500	1250	1250	3750	1750

Table 1.

Standard concentrations in the MILLIPLEX® MAP Human High Sensitivity T Cell Panel (pg/mL).

ITAC	GM-CSF	Fractalkine	IFNγ	IL-10	MIP-3α	IL-12p70
1.24	0.33	7.75	0.47	0.51	0.79	0.16

IL-13	IL-17A	IL-1β	IL-2	IL-21	IL-4	IL-23
0.24	0.31	0.14	0.18	0.14	1.07	3.06

IL-5	IL-6	IL-7	IL-8	MIP1α	MIP1β	TNFα
0.10	0.11	0.43	0.12	0.93	0.69	0.16

Table 2.

Minimum detectable concentrations in the MILLIPLEX® MAP Human High Sensitivity T Cell Panel (pg/mL).

Cross-reactivity

Potential analyte cross-reactivity within the assays was determined with a single standard cross-reactivity test. Each individual standard was tested in the presence of multiplexed beads and detection antibodies. All standards had less than five percent cross-reactivity with the other assays (data not shown).

Stability: Freeze/Thaw and Heat Stress

The effect of multiple freeze-thaw cycles or heat stress on serum sample analyte values was examined using the MILLIPLEX® MAP Human High Sensitivity T Cell Panel kit. No analyte in the panel was affected (10% less than control) by up to three freeze-thaw cycles of the samples (Figure 2). Only Fractalkine and MIP1β concentrations were affected (40-50% less than control) by temperature stress on the serum samples (24 hours RT or 2 hours at 37 °C, Figure 3).

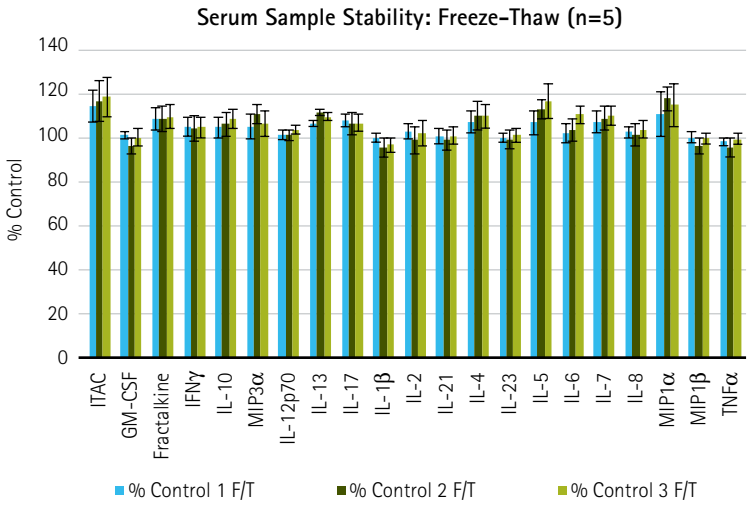


Figure 2.

Effect of freeze-thaw (F/T) cycles on the serum sample concentrations of analytes in the MILLIPLEX® MAP Human High Sensitivity T Cell Panel.

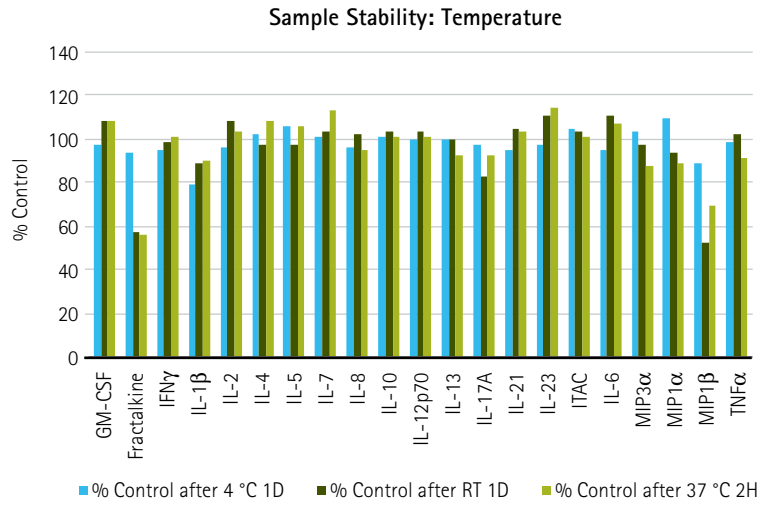


Figure 3.

Effect of temperature stress on the serum sample concentrations of analytes in the MILLIPLEX® MAP Human High Sensitivity T Cell Panel.

Precision

Intra-assay precision (%CV) was determined from eight duplicates of the standard controls (Table 3A) and inter-assay precision (%CV) was determined from twelve independent replicates of the standard controls (Table 3B).

3A.

ITAC	GM-CSF	Fractalkine	IFNγ	IL-10	MIP-3α	IL-12p70
3.4	1.7	1.6	4.1	3.5	2.6	5.7
IL-13	**IL-17A**	**IL-1β**	**IL-2**	**IL-21**	**IL-4**	**IL-23**
3.3	3.0	3.1	2.4	2.7	2.4	2.1
IL-5	**IL-6**	**IL-7**	**IL-8**	**MIP1α**	**MIP1β**	**TNFα**
2.7	4.0	2.4	2.5	2.1	1.4	2.9

3B.

ITAC	GM-CSF	Fractalkine	IFNγ	IL-10	MIP-3α	IL-12p70
13.5	12.7	12.4	19.2	15.4	16.0	13.4
IL-13	**IL-17A**	**IL-1β**	**IL-2**	**IL-21**	**IL-4**	**IL-23**
16.2	15.9	12.8	14.1	12.3	13.9	15.4
IL-5	**IL-6**	**IL-7**	**IL-8**	**MIP1α**	**MIP1β**	**TNFα**
15.8	17.2	14.7	13.6	12.3	12.5	13.9

Table 3.

Intra-assay precision (%CV) of the MILLIPLEX® MAP Human High Sensitivity T Cell Panel (3A). Inter-assay precision (%CV) of the MILLIPLEX® MAP Human High Sensitivity T Cell Panel (3B).

Recovery

Assay accuracy was determined as the percentage of the observed concentration of known amount of standard spiked into serum matrix. The percent recoveries were between 96% and 106% for all assays (data not shown).

Sensitivity Comparison

The sensitivity of the MILLIPLEX® MAP Human High Sensitivity T Cell Panel was compared to the sensitivity of two high sensitivity multiplexed Luminex® assay kits from different suppliers. The MILLIPLEX® MAP assay was found to have, overall, higher sensitivity than the competitor kits (Figure 4).

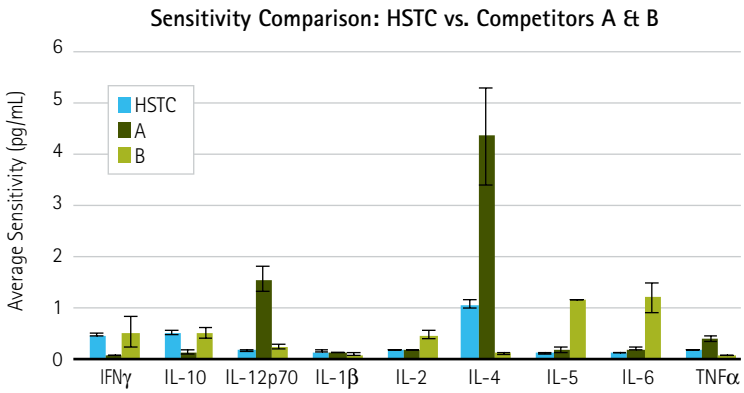


Figure 4.

Comparing sensitivity of the MILLIPLEX® MAP Human High Sensitivity T Cell Panel vs. non-Merck Millipore High Sensitivity Multiplexed Assay Kits.

Sample Detection

Initial sample testing compared the percent sample detection between the MILLIPLEX® MAP Human High Sensitivity T Cell Panel and a high sensitivity multiplex Luminex® assay kit from a different supplier. Both normal serum samples (n=9) and serum samples from sepsis patients (n=16) were tested. The MILLIPLEX® MAP Human High Sensitivity T Cell Panel detected analytes at a similar or greater frequency than did the non-Merck Millipore kit (Table 4).

		% Samples Detected									
Sample	**Kit**	**GM-CSF**	**IFNγ**	**IL-1β**	**IL-2**	**IL-4**	**IL-5**	**IL-6**	**IL-8**	**IL-10**	**IL-12p70**	**TNFα**
Sepsis (n=16)	MILLIPLEX® MAP	88	94	56	88	88	69	100	100	94	94	100
	Competitor	100	6	100	50	88	50	94	100	75	25	100
Normal (n=9)	MILLIPLEX® MAP	100	100	11	89	67	56	100	100	89	78	100
	Competitor	89	0	33	22	78	22	33	89	22	0	100

Table 4.

Comparing percent sample detection of the MILLIPLEX® MAP Human High Sensitivity T Cell Panel vs. non-Merck Millipore kit.

The MILLIPLEX® MAP Human High Sensitivity T Cell Panel was further validated in a study done in collaboration with Drs. Barbara Nikolajczyk and Min Zhu of Boston University School of Medicine. To investigate immune cell function in obese and prediabetic subjects, four groups of subjects (n=8 per group, as described in Methods section and in Table 5.) were recruited following informed consent. The study design was cross-sectional.

		Lean Median (range)	ND Median (range)	PD Median (range)	PD+Met Median (range)
	Age (years)	33 (24-59)	40 (30-58)	39 (35-59)	52 (30-58)
	A1c (%)	N/A	5.2 (4.9-5.4)	5.9 (5.6-6.2)	6.0 (5.7-6.2)
	BMI (kg/m²)	<25	32.3 (30-35)	33.3 (32-35)	32.4 (30-34)
	Glucose	N/A	91 (73-106)	90 (81-100)	97 (71-126)
		N (8 total)	N (8 total)	N (8 total)	N (8 total)
Sex	Females	2	6	7	7
Sex	Males	6	2	1	1
Race	White/non-Hispanic	8	4	1	3
Race	African-American	0	3	6	3
Race	Hispanic	0	1	1	2
Race	Asian	0	0	0	0

Table 5.

Characteristics of study subjects.

T cells from subjects in the Lean, ND, PD and PD+Met groups were stimulated in the context of PBMCs with plate-bound CD3 and soluble CD28 (Figures 5 and 6). Figure 5 shows cytokine secretion patterns indicative of Th1, B Cell and myeloid immune cell development, with a statistically significant decrease in levels of the anti-inflammatory cytokine, IL-10.

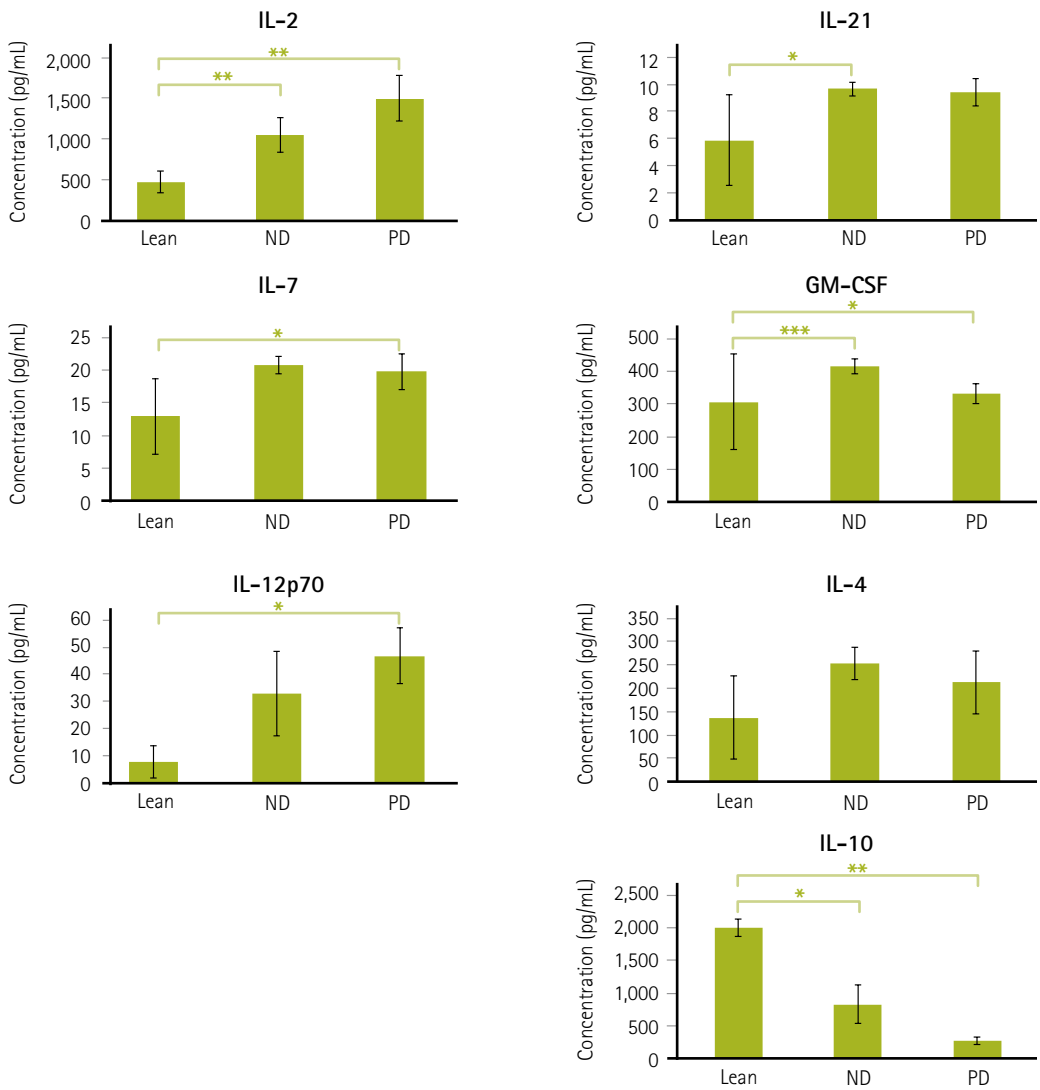


Figure 5.

Cytokine production by isolated and stimulated PBMCs from healthy (Lean), non-diabetic obese (ND) and prediabetic (PD) subjects. Isolated PBMCs were stimulated with plate-bound CD3 and soluble CD28 (2 µg/mL) at 1 x 10^6 cells/mL for 40 hours. Culture supernatant (25 µL) was analyzed using the MILLIPLEX® MAP High Sensitivity T Cell Kit. (A1-3) T/Th1-supportive IL-2, IL-7 and IL-12p70; (B) B cell-supportive IL-21; (C) myeloid-supportive GM-CSF; (D) Th2-supportive IL-4; or (E) anti-inflammatory IL-10. N=8 per group; *, P<0.05; **, P<0.01; ***, P<0.001, analyzed by one-way ANOVA.

While Figure 6 does not show a significant decrease in pro-inflammatory cytokine levels in prediabetic subjects taking metformin, there is a statistically significant increase in the anti-inflammatory cytokine, IL-10 (Figure 6).

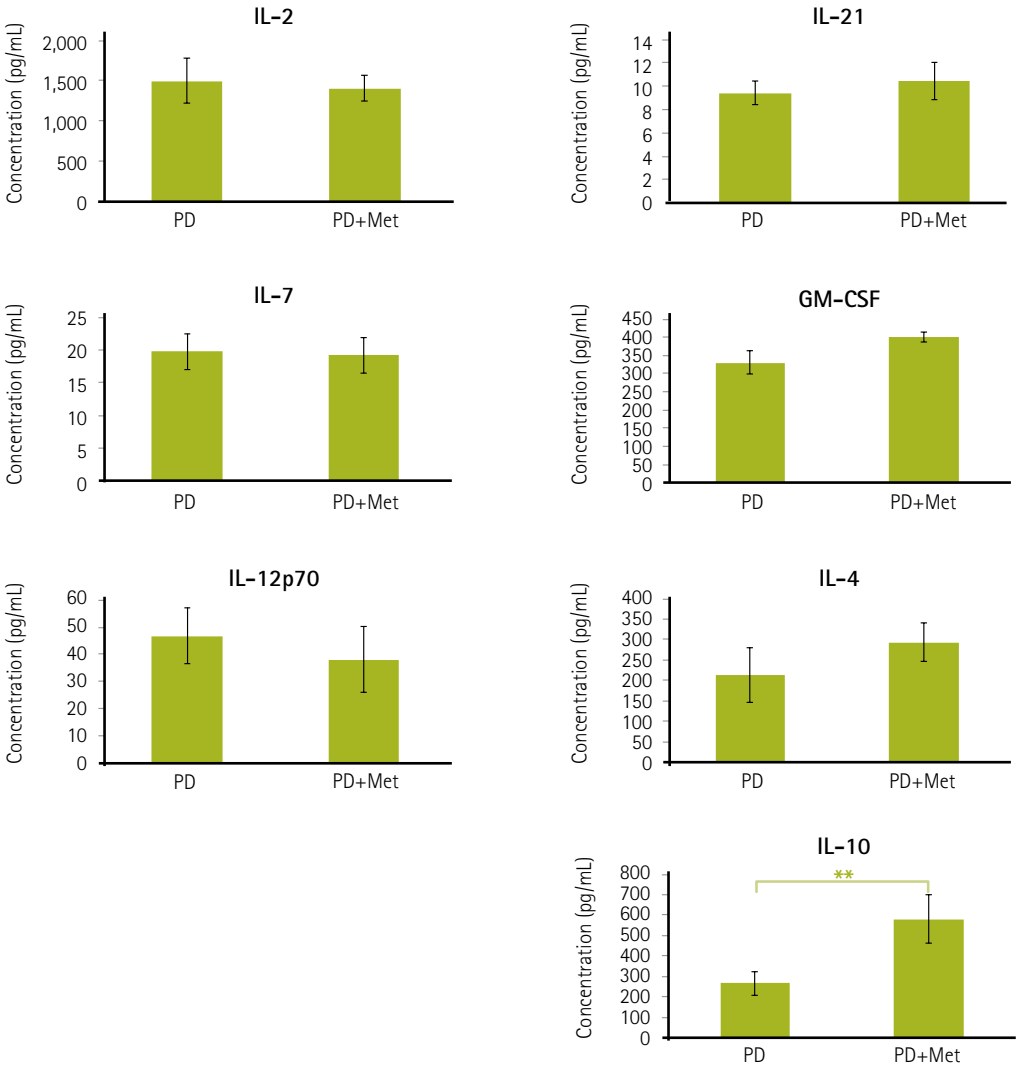


Figure 6.

In vivo usage of "anti-inflammatory" T2D drug metformin has limited impact on the cytokine profile in PBMCs from prediabetic (PD) and prediabetic on metformin therapy (PD+Met) subjects. PBMCs from prediabetic subjects who do (PD + Met) or do not (PD) take metformin were stimulated and analyzed as above. PBMCs from all prediabetic subjects secrete similar amounts of (A) T/Th1-supportive IL-2, IL-7 and IL-12p70, (B) B cell-supportive IL-21, (C) myeloid-supportive GM-CSF, and (D) Th2-supportive IL-4. (E) Increased amounts of anti-inflammatory cytokine IL-10 were produced in PD+Met compared to PD samples. N=8 per group; **, P<0.01, analyzed by unpaired t-test.

Conclusions

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 Millipore's MILLIPLEX® MAP Human 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.

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