Quantitative Multiplex Analysis of Low-level Cytokine Expression

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Cytokines are immunomodulatory polypeptides that play key roles in both adaptive and innate immune responses. A generic term, 'cytokines' includes interleukins (acting as mediators among multiple immune cell types), chemokines (responsible for immune cell migration), lymphokines (produced by activated lymphocytes), adipokines (produced by adipocytes) 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.

The significant roles of cytokines in normal inflammatory responses and immune cell development and activation drive their involvement in a variety of disease types. Even low levels of the chronic inflammation mediated by cytokines 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 more detailed understanding of the immune system and its multi-faceted response to most antigens, especially those responses that make up the inflammatory process. In order to develop a more robust understanding of pro-inflammatory cytokine networks, it is critical to be able to quantitate multiple cytokines simultaneously.

This article describes development and use of a 21-plex assay for simultaneously detecting cytokines associated with Th1, Th2, and Th17 cells, based on the Luminex xMAP® technology and the MILLIPLEX® MAP Human High Sensitivity T Cell Panel (EMD Millipore Billerica, MA). This 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 cell markers. Although these standard panels measure a defined group of cytokines, each panel is customisable to enable the user to choose any number of analytes within the panel to meet specific research needs.

The article also presents data from collaboration with Barbara Nikolajczyk, PhD and Min Zhu, PhD of Boston University School of Medicine, who are studying the association between obesity and inflammation due to the overexpression of cytokines that support immune cell differentiation/activation.

Methods

For serum samples, 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 the 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 HbA1c<5.7%, Prediabetic Subjects (PD) with BMI 30-35 and HbA1A1c 5.7-6.5% 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 (500 mg 2x daily) as standard-of-care. The PBMCs were stimulated with plate-bound CD3 and soluble CD28 (2 µg/mL) at 1 x 106 cells/mL for 40 hours, as described previously [1].

Serum, plasma and PBMC supernatants were analysed using the MILLIPLEX® MAP kit, according to the kit protocol (HSTCMAG--28SK).

Results

The standard curves and standard concentrations for the 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)*.

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).

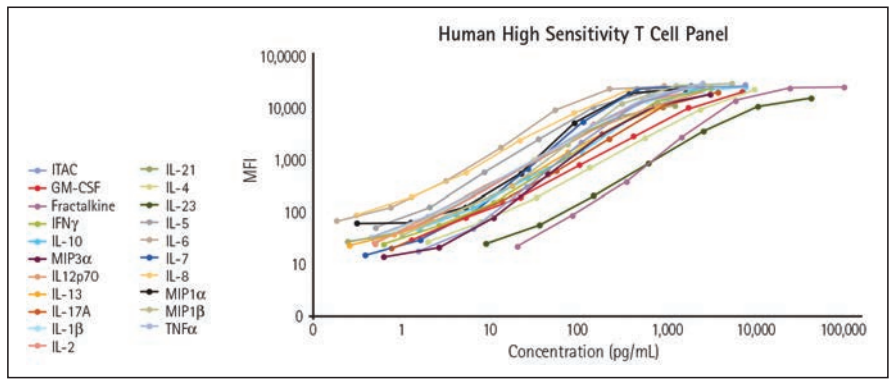

Figure 1. Standard curves of the MILLIPLEX® MAP Human High Sensitivity T Cell Panel.

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
1.46	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

Stability: Freeze/Thaw and Heat Stress

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*).

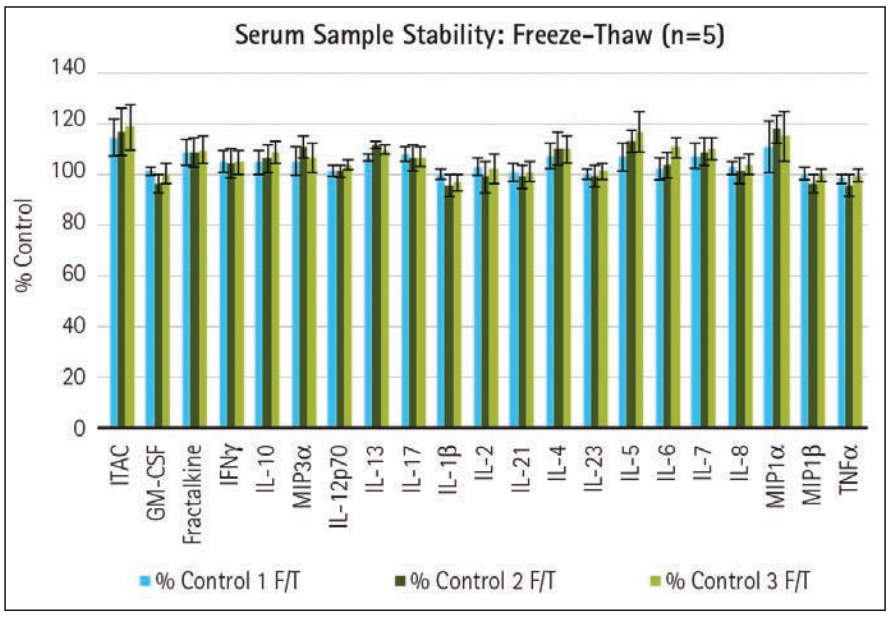

Figure 2. Effect of freeze-thaw (F/T) cycles on the serum sample concentrations of analytes.

Table 2. Minimum detectable concentrations.

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

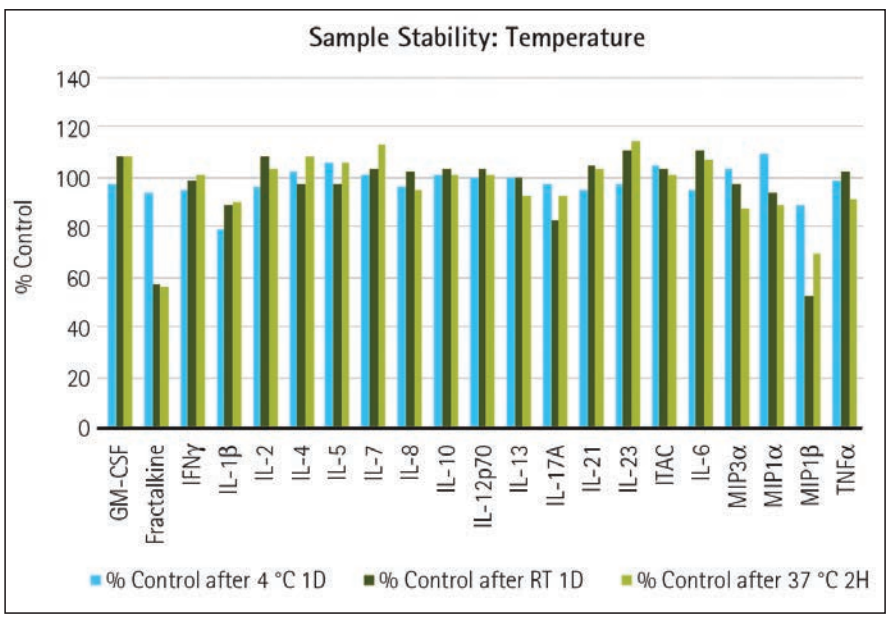

Figure 3. Effect of temperature stress on the serum sample concentrations of analytes.

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).

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

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).

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 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*).

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 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 other kit (*Table 4*).

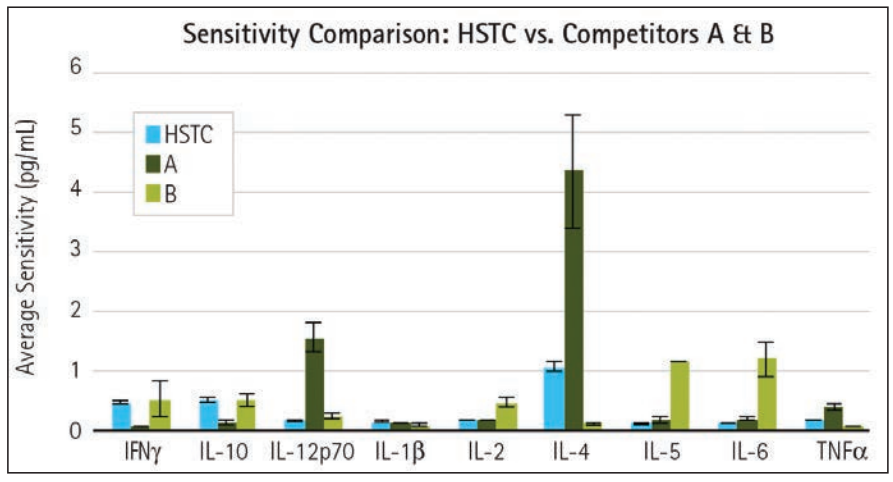

Figure 4. Comparing sensitivity of the MILLIPLEX® MAP Human High Sensitivity T Cell Panel vs. non-EMD Millipore High Sensitivity Multiplexed Assay Kits.

Table 4. Comparing percent sample detection of the MILLIPLEX® MAP Human High Sensitivity T Cell Panel vs. non-EMD Millipore kit.

		% 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

The panel was further validated in a study done in collaboration with Dr Nikolajczyk and Dr Zhu. 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.

Table 5. Characteristics of study subject

		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

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 function, with a statistically significant decrease in levels of the anti-inflammatory cytokine, IL-10. These results are consistent with our demonstration that T cell stimulation (αCD3/αCD28) induces TNFα production by CD14+ myeloid cells [2].

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. This IL-10 boosting action of metformin is consistent with the demonstration that metformin decreases function of the inflammasome in insulin resistant individuals [3].

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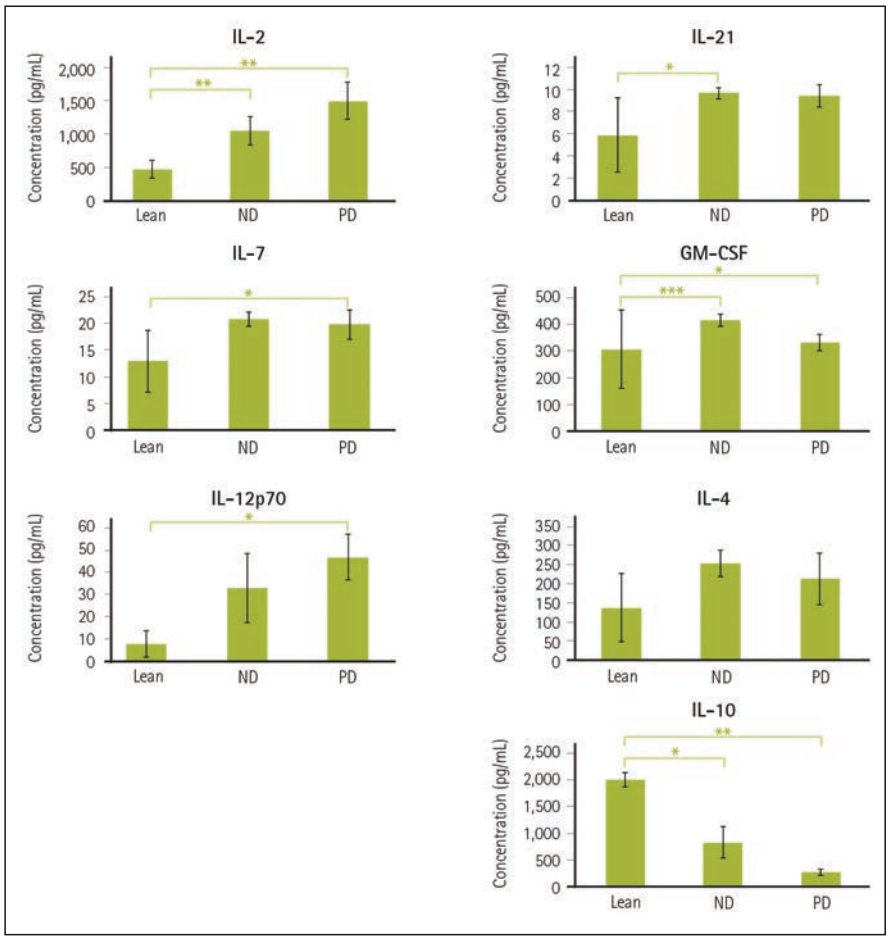

*Figure 5. Cytokine production by isolated and stimulated PBMCs from healthy (Lean), non-diabetic obese (ND) and prediabetic (PD) subjects. Isolated PBMCs were stimulated with plate-bound CD3 and soluble CD28 (2 µg/mL) at 1 x 106 cells/mL for 40 hours. Culture supernatant (25 µL) was analysed using the MILLIPLEX® MAP High Sensitivity T Cell Kit. (A1-3) T/Th1-associated IL-2, IL-7 and IL-12p70; (B) B cell-associated IL-21; (C) myeloid- and Th1-associated GM-CSF; (D) Th2-associated IL-4; or (E) anti-inflammatory IL-10. N=8 per group; *, P<0.05; **, P<0.01; ***, P<0.001, analysed by one-way ANOVA.*

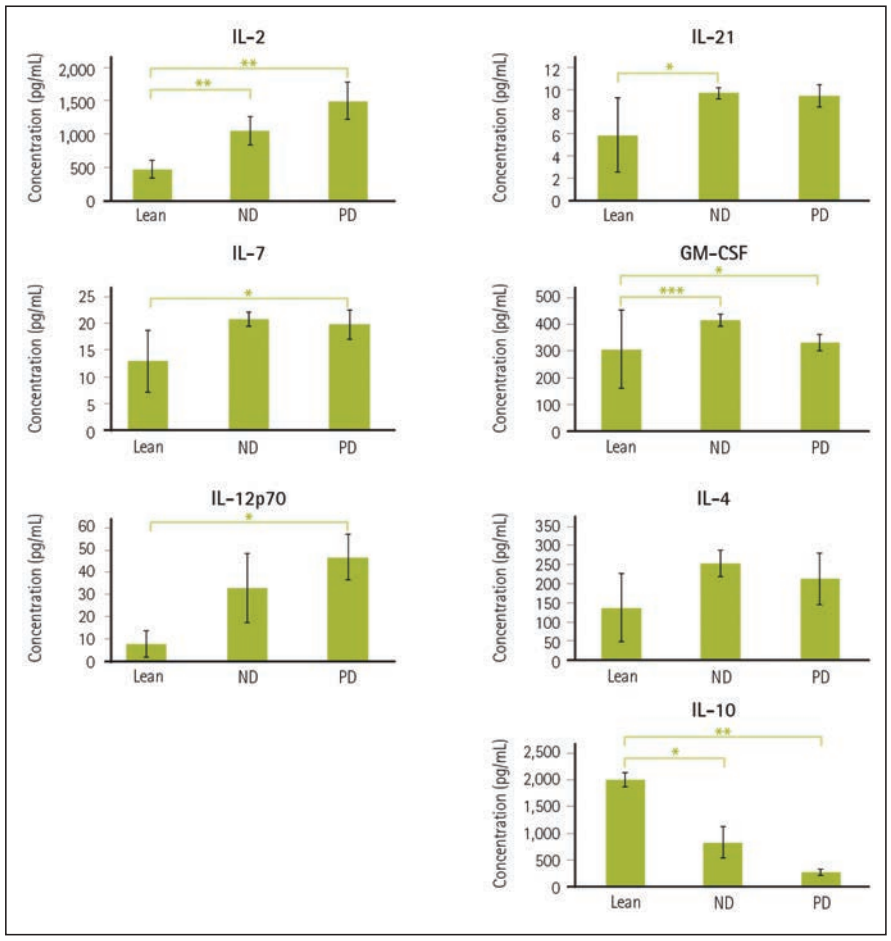

*Figure 6. In vivo usage of 'anti-inflammatory' T2D drug metformin has limited impact on the cytokine profile in PBMCs from prediabetic (PD) and prediabetic on metformin therapy (PD+Met) subjects. PBMCs from prediabetic subjects who do (PD + Met) or do not (PD) take metformin were stimulated and analysed as above. PBMCs from all prediabetic subjects secrete similar amounts of see changes in the following from the previous figure (A) T/Th1-associated 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, analysed by 2 tailed 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 both antigens and physiological perturbation, especially those responses that make up the immune cell-mediated inflammatory process.

The 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.

References

1. Jagannathan-Bogdan M, McDonnell ME, Shin H, Rehman Q, Hasturk H, Apovian CM, Nikolajczyk BS. Elevated proinflammatory cytokine production by a skewed T cell compartment requires monocytes and promotes inflammation in type 2 diabetes. J Immunol. 2011 Jan 15;186(2):1162-72.

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