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CLL-like monoclonal B cell lymphocytosis displays an increased inflammatory signature that is reduced in early stage chronic lymphocytic leukemia

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No potential conflicts of interest were disclosed.

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HIGHLIGHTS

- o An increased inflammatory signature exists in MBL, mainly mediated by monocytes
- o This inflammatory signature is reduced in early stage CLL, especially IGHV mutated
- o A similar increase of Th1 and cytotoxic CD4$^+$ T cells is detected in both entities
- o Tumor-supporting effects of monocytes are similarly observed in both entities

ABSTRACT

Several studies have demonstrated impaired immune cell functions in chronic lymphocytic leukemia (CLL) patients, contributing to tumor evasion and disease progression. However, in CLL-like monoclonal B cell lymphocytosis (MBL) studies are scarce. Herein, we characterized the immune environment in 62 individuals with clinical

MBL, 56 early stage CLL patients and 31 healthy controls. Gene expression arrays and qRT-PCR were performed on RNA from CD4^{+} peripheral blood cells; serum cytokines were measured by immunoassays; and HLA-DR expression on circulating monocytes as well as Th1, cytotoxic, exhausted and effector CD4^{+} T cells were characterized by flow cytometry. Besides, cell cultures of clonal B cells and CD14-enriched or depleted cell fractions were performed. Strikingly, MBL and early stage CLL differed in proinflammatory signatures. An increased inflammatory drive mainly orchestrated by monocytes was identified in MBL, which exhibited enhanced phagocytosis, pattern recognition receptors, IL8, HMGB1 and acute response signaling pathways, and increased proinflammatory cytokines (in particular IL8, IFN and TNF). This inflammatory signature was diminished in early stage CLL (reduced IL8 and IFN levels, IL8 signaling pathway and monocytic HLA-DR expression compared to MBL), especially in those patients with mutations in IGHV genes. Additionally, CD4^{+} T cells of MBL and early stage CLL showed a similar upregulation of Th1 and cytotoxic genes, and expanded CXCR3^{+} and perforin^{+} CD4^{+} T cells as well as PD1^{+} CD4^{+} T cells compared to controls. Cell culture assays disclosed tumor-supporting effects of monocytes similarly observed in MBL and early stage CLL. These novel findings reveal differences in the inflammatory environment between MBL and CLL, highlighting an active role for antigen stimulation in the very early stages of the disease, potentially related to malignant B cell transformation.

Introduction

Several alterations have been described in T cells of patients with chronic lymphocytic leukemia (CLL), presumably a consequence of chronic exposure to tumor cells [1-8]. Additionally, an elevated number of monocytes has been detected in peripheral blood (PB) of patients with the disease, displaying deregulated genes involved in phagocytosis and inflammation [9]. Serum cytokine levels have also been described to be altered in CLL [10], and increased levels of some cytokines such as CCL3 or IL8 have been associated with a worse outcome [11, 12].

CLL-like monoclonal B cell lymphocytosis (MBL) is defined as the presence of a clonal population of B lymphocytes in PB ($<5\times10^9$/l) having a phenotype consistent with CLL, without other clinical features of the disease. Its frequency increases with age, being present in >50% of people older than 90 years. Those cases with a clonal B cell count $>0.5\times10^9$/l are referred to as clinical MBL and are considered a premalignant condition. They show a progression rate to CLL requiring therapy of 1-2%/year. Indeed, virtually all cases of CLL are preceded by an MBL stage [13-17].

The role of immune interactions in MBL remains uncharacterized. While B cell expansions in patients with advanced CLL are accompanied by immune suppression that restrains antitumor immunity, prior investigations have shown that T cell function in MBL is only slightly deviated [1, 18]. Thus, although immunosuppressive features and T cell repertoire restrictions are already detectable in MBL and evolve toward a more suppressive profile across the disease stages [18-20], the functional immune cell fraction may play relevant roles in MBL dynamics and in clonal evolution leading to CLL. Understanding immune interactions in pre-leukemic individuals would also be relevant for deciphering molecular mechanisms underlying the natural history of the disease. In this study, we characterized PB CD4$^+$ cells (including CD4$^+$ T cells and monocytes) and serum samples of a large cohort of MBL and early stage CLL patients to elucidate more comprehensively these issues.

Material and Methods

Patients and samples

A total of 62 subjects with clinical CLL-like MBL and 56 untreated, early stage CLL patients (Binet A/Rai 0-I and $<20\times10^9$ clonal B cells/l) were evaluated. Patients were cared for at Hospital del Mar (n=50) and Hospital Vall d'Hebron (n=11) in Barcelona (Spain) and at Northwell Health (n=57) in New York (USA). Additionally, 31 age-matched healthy subjects were studied as controls. No case had evidence of infection at sampling. The main characteristics of the entire cohort are summarized in Table 1.

At the end of the study, all MBL cases remained asymptomatic and none progressed to CLL requiring therapy. The study was performed in accordance with National and International Guidelines (Professional Code of Conduct, Declaration of Helsinki) and approved by the Ethics Committee of Hospital del Mar, Barcelona (2011/4317/I). Fresh PB, cryopreserved PB mononuclear cells (PBMCs) and frozen serum samples were characterized by several methods (Supplementary Figure 1). Part of the samples were obtained from MARBiobanc (Barcelona). Data from Northwell Health were collected using REDCap [21].

Cell isolation and RNA extraction for gene expression analyses

Fresh PB was subjected to Ficoll density gradient centrifugation. Purified $CD4^+CD19^-$ (total $CD4^+$ cells, including $CD4^+$ T cells and monocytes), $CD4^+CD14^-$ ($CD4^+$ T cells) and $CD14^+$ cells (monocytes) were isolated from PBMCs based on positive selection methods employing immunomagnetic beads (CD19 Multisort Kit, CD19 MicroBeads, CD4 MicroBeads and CD14 MicroBeads) and autoMACS technology (Miltenyi Biotec, Bergisch Gladbach, Germany). A flow-chart describing all cell isolation procedures for gene expression analyses is shown in Supplementary Figure 2. To assess the purification process efficacy, 100000 cells of each sample were analyzed by flow cytometry using anti-CD3 (APC), anti-CD4 (PE), anti-CD14 (FITC) and/or anti-CD45 (PerCP-Cy5.5) antibodies (BD Biosciences, San Jose, CA, USA). Purity of 90% was achieved in all samples, except for two MBL subjects in which purities of total $CD4^+$ cells were 70%. Purified cells were stored at -80ºC in 1% BME RLT-plus buffer (QIAGEN, Venlo, The Netherlands). RNA was finally extracted following the RNeasy Plus Mini Kit protocol (QIAGEN).

Gene expression analysis by microarrays

RNA from total CD4$^+$ cells was employed for gene expression arrays in 9 healthy controls, 15 MBL and 14 CLL cases. All samples used for microarray experiments had an RNA integrity number (RIN) >7. Briefly, 30 ng of total RNA were retrotranscribed into cDNA and amplified (Ovation® Pico WTA System V2, NUGEN, Redwood City, CA, USA). The cDNA product was purified (MinElute Reaction Cleanup Kit, QIAGEN), yield and purity measured, and biotinylated fragments were added to the hybridization cocktail, finally hybridized on GeneChip Human Gene 2.0 ST array (Affymetrix, Santa Clara, CA, USA) following manufacturer's instructions. Data quality assessment was performed with the Expression Console (Affymetrix) and R (v3.1.1). A Robust Multi-array Average (RMA) algorithm, included in the aroma.affymetrix package, was used for data normalization [22]. To correct for batch effects, ComBat, implemented in the R package SVA, was employed [23]. Differential expression analysis was performed using *limma* R package [24]. *P*-values <0.05 together with a Fold Change |FC|>1.2 cut-off value were used to generate the list of differentially expressed genes. Gene annotations were obtained from the Affymetrix annotation file Human Gene 2.0 ST array (NetAffix version na34, hg19). UCSC database was employed (Feb. 2009 hg19, GRCh37) to complement Affymetrix annotations. Microarray data have been deposited in the Gene Expression Omnibus (GEO) database with series accession number GSE125791 [25]. A gene pathway analysis of the genetic signature of total CD4$^+$ cells was accomplished using Ingenuity Pathway Analysis (IPA, QIAGEN). Finally, to have an insight into the differential contribution of monocytes and T cells in gene expression, the BioGPS tool (http://biogps.org) was employed.

Gene expression analysis by quantitative RT-PCR (qRT-PCR)

To confirm the gene expression levels detected by microarrays in total CD4$^+$ cells, RNA from the same samples (excluding the two cases with purities <90%) and from an additional independent cohort consisting of 8 MBL and 5 CLL subjects (purity 90%, 49 cases in total) was subjected to qRT-PCR validation. To elucidate the different contributions of CD4$^+$ T cells and monocytes in gene expression, RNA from additional

CD14-depleted CD4$^+$ cells (CD4$^+$ T cells) and CD14$^+$ cells (monocytes) was subjected to the same qRT-PCR validation panel (in 4 healthy and 8 MBL; and 3 healthy and 9 MBL individuals, respectively). This panel consisted of 13 selected genes (*CCL2, CLU, CXCL5, DEFA1, FGFBP2, GZMH, ITGAM, NEXN, NKG7, PPBP, RAB31, TUBB1* and *VSTM1*; assays detailed in Supplementary Table 1) significantly upregulated by gene expression arrays and related to inflammation, Th1 cells and cytotoxic pathways. *GUSB* and *GAPDH* were employed as housekeeping genes. A total of 400 ng of RNA were analyzed in this customized gene panel, using Taqman Low Density Arrays (TLDA, Life Technologies, Foster City, CA) according to manufacturer's instructions. For qRT-PCR data analysis, outlier replicates, defined by a SD >0.5 and a qRT-PCR threshold cycle (Ct) >40, were excluded. The Ct measures, defined as the mean of the expression of a studied gene minus the mean of the expression of the selected housekeeping genes, were compared.

Flow cytometry analysis

Cryopreserved PBMCs from 8 controls, 11 MBL and 10 CLL subjects were assessed. PBMCs were washed and labeled with the following antibodies: anti-CD45 (PerCP-Cy5.5), anti-CD14 (FITC), anti-CD3 (V450), anti-CD4 (PE), anti-CD4 (APC), anti-HLA-DR (PE-Cy7), anti-CXCR3 (PerCP-Cy5.5), anti-perforin (AF488), anti-granzyme B (FITC), anti-PD1 (PE) and anti-CD27 (PE). FVS510 was employed to discriminate viable from non-viable cells, and the Fixation/Permeabilization Solution Kit to stain intracellular molecules. Isotype controls (PE, PerCP-Cy5.5, PE-Cy7 and APC) and fluorescence-minus-one controls were used to correctly assess antigen expression. All reagents were purchased from BD Biosciences. Data acquisition was performed in a BD FACSCanto II cytometer and analyzed using the FACSDiva software (BD Biosciences). The gating strategy to define monocytes and CD4$^+$ T cells and evaluate the expression of the antigens of interest is detailed in Figure 1.

Figure 1. Flow cytometry gating strategy.
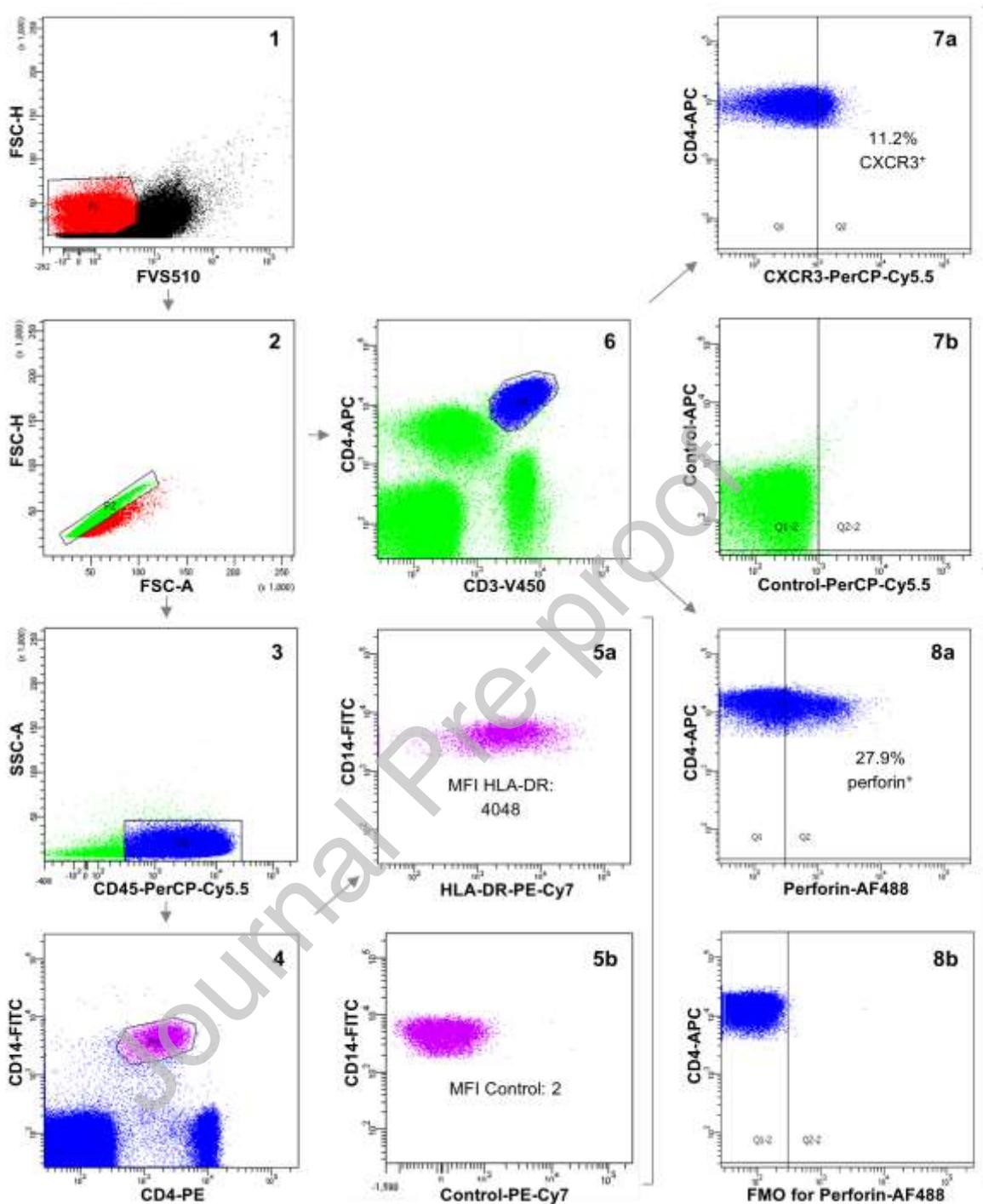
FVS510 was used to select viable cells (1). After gating for singlets (2), CD45$^+$CD14$^+$CD4$^+$ cells (monocytes) were selected (3 and 4) and linear mean fluorescence intensity (MFI) values of HLA-DR were registered (5a). The absence of fluorescence for PE-Cy7 on monocytes was confirmed employing isotype control (5b). On the other hand, CD3$^+$CD4$^+$ cells (CD4$^+$ T cells) were

gated (6). The percentage of CD4$^+$ T cells that were positive for CXCR3 (7a) and PD1 or negative for CD27 was assessed employing isotype controls (7b), whereas the percentage of CD4$^+$ T cells that were positive for perforin (8a) and granzyme B was evaluated using fluorescence-minus-one (FMO) controls (8b). In the plots, fluorescence intensity values are transformed into logarithmic scale.

Multiplex cytokine analysis

In 24 healthy subjects, 40 MBL and 44 CLL cases, serum levels of the following 20 cytokines were quantified: IL1 , IL2, IL4, IL5, IL6, IL8, IL10, IL12, IL15, IL17A, IFN , IFN , TNF , GM-CSF, CCL3, CCL4, CCL19, CXCL10 and CXCL11 using the U-PLEX Platform (Meso Scale Discovery, Rockville, MD, USA) and CXCL9 using Human CXCL9/MIG Quantikine ELISA Kit (R&D Systems, Minneapolis, MN, USA). All determinations were performed in duplicate and concentrations were reported in picograms per milliliter. To study the potential cytokine interactions, their respective coding genes were obtained and genomic data was analyzed using IPA.

Tumor cell survival analysis

Cryopreserved PBMCs from 6 MBL subjects and 5 CLL patients, all of them with mutated IGHV genes, were employed. Purified tumor B cells were isolated based on negative selection methods employing immunomagnetic beads (B-CLL Cell Isolation Kit) and autoMACS technology (Miltenyi Biotec). To assess the purification process efficacy, 100000 cells of each sample were analyzed by flow cytometry using anti-CD33 (FITC), anti-CD4 (PE), anti-CD20 (PerCP-Cy5.5) and anti-CD5 (APC) antibodies (BD Biosciences). Purity of 90% of tumor B cells was achieved for all samples (median: 98.2%, range: 90.1-99.2). Additionally, CD14-enriched cell fractions and CD14-depleted PBMCs were

obtained using CD14 MicroBeads (Miltenyi Biotec). The same flow cytometry panel was employed to evaluate the percentage of monocytes in the CD14-enriched cell fraction (median: 85.9%, range: 51.3-95.3), total PBMCs and CD14-depleted PBMCs (median: 4.7 vs. 0.9% respectively, *P*-value=0.003). A total of 1×10^5 tumor cells were cultured alone or in the presence of 6×10^5 cells of the CD14-enriched fraction, besides cell cultures of 1×10^6 total PBMCs and CD14-depleted PBMCs. Cells were resuspended in 200 µl of RPMI 1640 medium supplemented with 1% Fetal Bovine Serum (FBS), L-glutamine (4 mM), penicillin (100 U/ml) and streptomycin (100 µg/ml) in MW96 assay plates (Corning Inc., Corning, NY). Cells were then centrifuged at 200 x g for 4 minutes and cultured for 24 hours at 37ºC and 5% CO_2. After that period, cells were washed and labeled with anti-CD33 (FITC), anti-CD4 (PE), anti-CD20 (PerCP-Cy5.5), anti-CD5 (APC) and FVS510, which allowed the identification of two subpopulations (viable and non-viable cells) within the tumor cell clone in all included patients, as shown in Supplementary Figure 3. Data acquisition was performed in a BD FACSCanto II cytometer and analyzed using FlowJo (FlowJo LLC, Ashland, OR). The effect of monocytes on tumor viability was assessed as the difference between the viability of tumor cells cultured with the CD14-enriched cell fraction minus the viability of tumor cells cultured alone.

Statistical analysis

The t-test and Mann-Whitney U test were used to estimate statistical significance of the differences identified between independent groups, whereas the results of the tumor cell survival analysis comparing different conditions within the same subject were evaluated using the Wilcoxon test. Linear regression analysis and Spearman correlations were calculated to evaluate the relationship between gene expression

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Author contributions

G.B., A.P. and B.E. designed the whole research study, analyzed all data and wrote the manuscript. G.B. and A.P. processed the samples and performed the experiments. B.S., G.F., F.P. and N.C. participated in the design and interpretation of serum studies. N.C. performed major contributions in the discussion of the results and manuscript writing. L.N. and M.A. analyzed raw gene expression data. X.C. and A.F. helped to the design and analysis of flow cytometry experiments. E.P. contributed to perform microarray and qRT-PCR assays. P.Y.C. contributed to perform cytokine immunoassays. Y.K. helped with data management. M.R.R. contributed to samples processing. E.G., E.A., K.R.R., P.A. and F.B. provided patient data and samples. A.C. helped to perform cell culture assays. All authors read the last version of the manuscript.
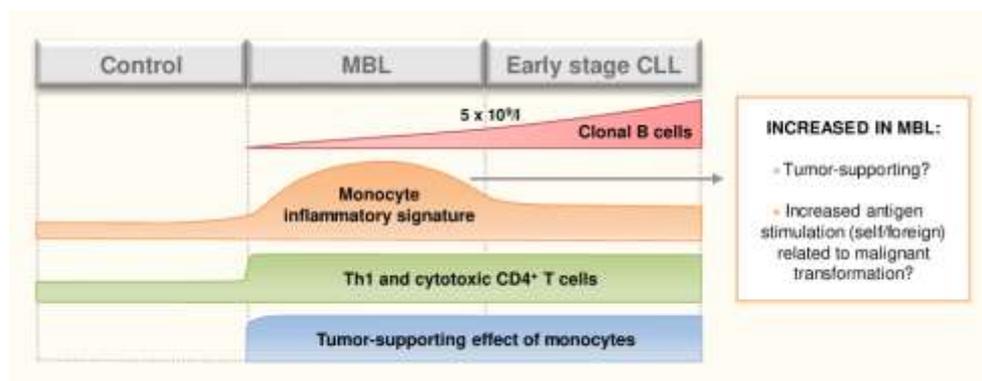
Blanco et al, GRAPHICAL ABSTRACT

Table 1. Characteristics of the study cohort at the time of analysis and last follow-up.

	Control (n=31)	MBL (n=62)	CLL Binet A (n=56)
Age	64 (42-88)	69 (45-89)	64 (37-89)
Absolute lymphocyte count (x10^9 cells/l)	1.7 (0.4-3.2)	5.0 (0.9-10.1)	13.2 (7.9-24.2)
Clonal B cell count (x10^9 cells/l)	NA*	2.1 (0.5-4.8)	10.2 (5.8-19.4)
Rai stage			
0	NA	NA	48 (85.7%)
I			8 (14.3%)
Mutated IGHV	NA	48/54 (88.9%)	32/54 (59.3%)
Treated patients	NA	0	11 (19.6%)
Deaths	NA	3 (4.8%)	6 (10.7%)
Follow-up from recruitment (months)	NA	48 (0-117)	67 (0-143)

Values are given as median (range) or number (%). NA: not applicable.

*Polyclonal B cell count: 0.07x10^9 cells/l (0.04-0.21).

Table 2. Serum cytokine levels of healthy controls, MBL subjects and CLL patients.

Cytokine	Control (n=24)		MBL (n=40)		CLL (n=44)		*P*-value		
	Median	Range	Median	Range	Median	Range	MBL vs. Control	CLL vs. Control	CLL vs. MBL
, /	0.26	0.07-5.85	0.35	0.07-124.27	0.47	0.07-114.13	0.475	0.061	0.219
IL2	1.49	0.44-8.07	1.49	0.44-11.80	1.66	0.44-16.49	0.633	0.273	0.483
IL4	0.21	0.06-0.79	0.21	0.06-40.67	0.08	0.06-1.34	0.875	0.270	0.210
IL5	1.44	0.45-4.97	2.01	0.52-4.42	1.51	0.45-4.19	**0.004**	0.558	**0.005**
IL6	2.04	1.16-151.09	3.62	1.16-1431.63	2.80	0.34-1689.05	**0.021**	0.080	0.495
IL8	22.05	6.15-5216.03	238.01	7.21-6810.22	20.73	4.00-8807.27	**0.005**	0.980	**0.019**
IL10	0.74	0.39-1.66	0.93	0.39-3.74	0.88	0.39-5.03	**0.017**	0.179	0.451
IL12	1.07	0.60-3.57	1.07	0.60-18.42	1.07	0.60-3.08	0.299	0.358	0.824
IL15	3.21	1.03-7.65	2.92	0.51-7.09	2.88	0.51-7.92	0.787	0.787	0.865
IL17A	2.51	0.51-13.14	2.52	0.51-21.39	2.52	0.51-16.69	0.199	0.196	0.895
,) 1 .	1.23	0.45-6.58	1.98	0.45-7.95	2.20	0.45-14.91	0.286	0.306	0.913
,) 1	11.24	3.30-42.08	16.69	1.44-151.14	13.24	3.30-69.33	**0.014**	0.577	**0.026**

All	7 1).	2.25	0.50-29.74	4.99	1.30-69.19	3.30	0.70-28.21	**0.015**	0.140	0.149
	GM-CSF	0.23	0.09-1.05	0.42	0.09-1.51	0.24	0.09-0.88	0.151	0.305	0.590
	CCL3	22.67	17.80-2925.30	46.00	19.05-2913.37	39.13	4.61-6427.70	**0.001**	0.070	0.395
	CCL4	114.53	56.66-1753.44	176.57	76.92-2358.09	151.61	29.75-2915.75	**0.012**	0.114	0.275
	CCL19	109.36	52.26-449.53	131.95	3.17-871.66	108.35	4.53-1561.50	0.071	0.908	0.078
	CXCL9	70.98	15.70-1044.45	165.53	15.70-1166.00	151.78	15.70-1982.50	**0.003**	**0.001**	0.964
	CXCL10	188.10	102.88-985.02	325.21	10.73-1904.78	230.79	5.25-735.04	**0.016**	0.581	**0.029**
	CXCL11	32.37	13.15-122.40	47.05	10.60-213.10	53.69	8.09-315.39	**0.010**	**0.038**	0.844

determinations were performed in duplicate. Cytokine values are reported in picograms per milliliter (pg/ml). Significant P-values (<0.05) are highlighted in bold.