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Quantitative and Functional Evaluation of Innate Immune Responses in Patients With Common Variable Immunodeficiency

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Abstract

Objectives: We evaluate the frequency and functional response of innate immune cells in peripheral blood (PB) from patients with common variable immunodeficiency (CVID) and healthy controls upon activation with agonists of the Toll-like receptors (TLR) *TLR2, TLR4*, and *TLR9*. In addition, several nonsynonymous single nucleotide polymorphisms (SNPs) within these TLR genes were examined.
Methods: Flow cytometry was used to perform immunophenotyping and evaluate the expression of cell surface markers. Levels of cytokines in the culture supernatants were evaluated using cytometric bead array technology. SNPs in the TLR genes were evaluated from genomic DNA using different sequencing techniques.
Results: Our results demonstrate that the frequency of CD1d-restricted TCR invariant natural killer T cells in PB was significantly reduced in the patients with CVID. A marked, though not significant, reduction in absolute numbers of plasmacytoid dendritic cells and natural killer cells was also observed in these patients. Interestingly, CD80 and CD86 expression on innate cells upon stimulation with TLR ligands was not altered in the patients although 3 of them exhibited low baseline levels of these surface molecules on monocytes compared to healthy controls. We also observed a significant increase in TNF-α levels in supernatants of PB mononuclear cells from CVID patients after stimulation with lipopolysaccharide. Finally, no association was found between the presence of nonsynonymous SNPs within the TLR genes and the clinical presentation of CVID.
Conclusions: Taken together, our study demonstrates than innate immune responses are disturbed in some CVID patients and prompts the evaluation of innate immunity genes as candidates to explain the CVID clinical phenotype.

Key words: Common variable immunodeficiency. Innate immunity. Lipoteichoic acid. Lipopolysaccharide. CpG oligodeoxynucleotides.

Resumen

Objetivos: Se evaluó la frecuencia y la respuesta funcional de las células de la inmunidad innata en la sangre periférica (SP) de pacientes con inmunodeficiencia común variable (IDCV) y en controles sanos tras la activación con agonistas de los genes de receptores tipo Toll (TLR) *TLR2, TLR4* y *TLR9*. Asimismo, se examinaron varios polimorfismos de un solo nucleótido (SNP) no sinónimos en estos genes de los TLR.
Métodos: Se empleó la citometría de flujo para determinar el inmunofenotipo y evaluar la expresión de los marcadores de la superficie celular. Se evaluaron los niveles de citocinas en los sobrenadantes de los cultivos mediante la técnica de citometría de flujo multiparamétrica (CBA). Se evaluaron los SNP en los genes de TLR a partir de ADN genómico con diferentes técnicas de secuenciación.
Resultados: Los resultados demuestran que la frecuencia de linfocitos T citolíticos naturales con TCR invariante restringidos por CD1d en la SP disminuyó significativamente en los pacientes con IDCV. Asimismo, se observó una reducción notable, aunque no significativa, de las cifras absolutas de células dendríticas plasmacitoides y linfocitos NK en estos pacientes. Cabe destacar que la expresión de CD80 y CD86 en las células innatas tras la estimulación con ligandos de TLR no se alteró en los pacientes, aunque 3 de ellos mostraron niveles iniciales bajos de estas moléculas de superficie en los monocitos en comparación con los controles sanos. Asimismo, se observó un aumento significativo de los niveles de TNF-α en los sobrenadantes de células mononucleares de SP en pacientes con IDCV tras la estimulación con lipopolisacáridos. Por último, no se observó ninguna relación entre la presencia de SNP no sinónimos en los genes de TRL y en la presentación clínica de IDCV.
Conclusiones: En conjunto, este estudio demuestra que las respuestas inmunitarias innatas están alteradas en algunos pacientes con IDCV y se requiere la evaluación de los genes inmunitarios innatos para explicar el fenotipo clínico de IDCV.

Palabras clave: Inmunodeficiencia común variable. Inmunidad innata. Ácido lipoteicoico. Lipopolisacárido. Oligodesoxinucleótidos CpG.

Introduction

Common variable immunodeficiency (CVID) is a primary immune disorder characterized by hypogammaglobulinemia and deficient production of specific antibodies against encapsulated bacteria. Most patients have recurrent sinopulmonary infections, although the first symptoms can be gastrointestinal inflammation or autoimmune manifestations [1]. Although several mutated genes have recently been described in association with the CVID phenotype [2-5], genetic causes remain elusive for the great majority of cases.

Although defective adaptive immune responses, with impairment of T-cell and B-cell maturation and activation, are common features in most CVID patients [1], attention has been recently focused on defects of the innate immune system as a possible explanation for CVID heterogeneity. Altered dendritic cell (DC) distribution in peripheral blood (PB) as well as defective T-cell priming capacity of DCs after antigenic or allogeneic stimulation have been described in CVID [6-11]. These findings have been associated with decreased expression of major histocompatibility complex class II and costimulatory molecules and reduced production of interleukin (IL) 12 by DCs. On the other hand, increased production of IL-12 by monocytes has been also reported in CVID [12]. Some evidence has also demonstrated reduced levels of circulating natural killer (NK) cells [13]. More importantly, however, CVID plasmocytoid DCs (pDCs) and B cells exhibit impaired responses to CpG stimulation in vitro [14]. A more detailed understanding of the status of innate immune cells upon activation with different toll-like receptor (TLR) ligands is necessary to elucidate specific defects in innate immune responses in CVID patients that may explain the variability in clinical symptoms.

It is also possible that subtle changes in TLR genes alter amino acid sequences in CVID patients. An example would be nonsynonymous single nucleotide polymorphisms (SNPs), which would influence the cellular response to TLR-stimulation and thus confer altered susceptibility to infections or inflammatory diseases. Within the gene encoding TLR4, the cosegregating SNPs Asp299Gly and Thr399Ile, which affect the extracellular domain of the molecule conferring hyporesponse to lipopolysaccharide (LPS) stimulation in vitro and in vivo, positively correlate with several infectious diseases [15]. SNPs of the gene encoding TLR2 also have an impact on susceptibility to infections by modifying cell response to lipoteichoic acid (LTA) and other stimuli [16,17]. On the other hand, a number of rare nonsynonymous SNPs have also been reported within the coding region of TLR9, although the significance of this in pathogen defense is still uncertain [18].

In the present study, we found that PB mononuclear cells (PBMCs) from CVID patients were responsive to *TLR2*, *TLR4*, and *TLR9* agonists at the same level as those from healthy controls. Also, no association was found between the presence of nonsynonymous SNPs within these TLR genes and the phenotype observed in the patients. However, PBMCs from these patients did exhibit a significant increase in the production of tumor necrosis factor (TNF)-α upon stimulation with LPS in comparison with healthy controls. Moreover, CD1d-restricted TCR invariant natural killer T (iNKT) cells were significantly decreased in the PB of most of the CVID patients. More detailed studies should be performed to evaluate if other TLR-mediated events are altered in CVID. iNKT immune responses should also be investigated in more detail to ascertain if their function is also defective in CVID and to explore how this phenomenon affects iNKT:B cell crosstalk.

Materials and Methods

Study Population

Six patients (5 women and 1 man; age range, 22-52 years) with CVID as defined by the International Union of Immunological Societies [19] were enrolled in this study. Their clinical and immunological characteristics are shown in Table 1. All were under intravenous immunoglobulin (IVIG) replacement therapy. Their specific antibody deficiency was verified by the lack of protective levels of antibodies to rabies virus after vaccination. These patients had no CVID-associated transmembrane activator and calcium-modulator and cyclophilin-ligand interactor (TACI) mutations and 2 of them (patients #3 and #4, Table 1) were found to be homozygous for the recently described CD19 mutation [4]. Samples were collected immediately before IVIG infusion. The control group included 5 healthy volunteers (4 women and 1 man; age range, 20-46 years). All the individuals evaluated in this study were from the metropolitan area of Medellin in Colombia and gave their informed consent. The study obtained approval from the local ethics committee.

Antibodies and Reagents

Fluorochrome-labeled monoclonal antibodies (mAbs) against human molecules CD3, CD11c, CD14, CD16, CD19, CD56, CD69, CD80, CD86, CD123, HLA-DR, and the invariant CDR3 loop of human canonical Vα24Jα18 TCR α chain for the identification of iNKT cells (clone 6B11), lineage markers (Lin, a mixture of anti-CD3, CD14, CD16, CD19, CD20, and CD56) and the corresponding isotype control antibodies were purchased from Becton Dickinson (San Jose, California, USA). FITC-labeled anti-Vß11 and PE-labeled anti-Vα24 were obtained from Beckman Coulter Immunotech (Marseille, France). Fcγ receptor (FcγR)-blocking antibody was provided by Miltenyi Biotec (Bergisch Gladbach, Germany).

LTA purified from *Staphylococcus aureus* was obtained from InvivoGen (San Diego, California, USA), and LPS from *Escherichia coli* 0127:B8 was purchased from Sigma-Aldrich (Saint Louis, Missouri, USA). The A-Class CpG oligodeoxynucleotide (ODN) 2216 (sequence 5'-ggGGGA**CG**AT**CG**TCggggG-3', lower case, phosphorothioate linkage; upper case, phosphodiester linkage 3' of the base) with no detectable endotoxin levels was kindly provided by the Coley Pharmaceutical Group (Wellesley, Massachusetts, USA).

Quantification of Innate Immune Cells and Flow Cytometry

The absolute number of eosinophils, neutrophils,

lymphocytes, and monocytes was calculated on the basis of total and differential blood cell counts using Wright staining of blood smears and conventional light microscopy. The frequency and phenotype of myeloid DCs (mDCs) (Lin-/CD11c$^+$/HLA-DR$^+$), pDCs (defined as Lin-/CD123$^+$/HLA-DR$^+$), monocytes (CD14$^+$/CD3-), NK cells (CD3-/CD16$^+$/CD56$^+$), iNKT cells (6B11$^+$/CD3$^+$), and NK T cells (CD3$^+$/CD56$^+$) were determined by 3- or 4-color flow cytometry. Briefly, cells were incubated in the dark with the corresponding mAbs for 20 minutes at room temperature. Nonspecific mAb binding was controlled by blocking FcγR with 20 μL of blocking reagent per 1×10^7 cells (20 minutes at 4ºC). Erythrocytes were lysed by incubation of the cell suspensions with 1X FACS lysing solution (Becton Dickinson) following the manufacturer´s instructions. Finally, cells were fixed with 250 μL of 2% formaldehyde. Appropriate isotype-matched control antibodies were also included. Flow cytometry was performed using the Becton Dickinson FACSORT and analyzed using CellQuest software (Becton Dickinson).

Isolation and Culture of Mononuclear Cells

PBMCs were obtained from heparinized blood samples by density gradient centrifugation using lymphocyte separation medium (BioWhittaker, Walkersville, Maryland). Viability of PBMCs was determined by trypan blue exclusion. For cell culture, PBMCs (1×10^6/mL) were suspended in complete culture media (RPMI 1640 supplemented with 10% of heat-inactivated fetal bovine serum, 100 U/mL of penicillin, 100 μg/mL of streptomycin, and 2 mM of L-glutamine) and stimulated with/without 15 μg/mL of LTA, 2 μg/ml of LPS, or 4 μg/mL of A-Class CpG ODN 2216 for 24 hours at 37ºC in 5% CO_2. Cell surface markers were also analyzed by flow cytometry using specific mAbs as described before. Culture supernatants were collected and stored at –70ºC for measurement of cytokine concentration by flow cytometry or enzyme-linked immunosorbent assay (ELISA).

Measurement of Cytokines

Levels of IL-12p70, TNF-α, IL-10, IL-6, and IL-1ß in PBMC culture supernatants were determined by flow cytometry using the BD Cytometric Bead Array (CBA, Human Inflammatory Kit, BD Biosciences Pharmingen, San Diego, California, USA), following the manufacturer's instructions. The assay sensitivities were 1.9, 3.7, 3.3, 2.5, and 7.2 pg/mL, respectively. Interferon (IFN) α was measured using a commercial ELISA kit (PBL Biomedical Laboratories, Piscataway, New Jersey, USA) following the manufacturer's instructions (detection limit, 3.5 pg/mL).

Detection of Polymorphisms in TLR Genes

Genomic DNA was isolated from blood samples using the Puregene DNA Isolation Kit (Gentra Systems, Minneapolis, Minnesota, USA) following the manufacturer´s instructions. For *TLR2*, 2 SNPs located at positions 17023 (C/T, Arg677Trp) and 17252 (G/A, Arg753Gln) of the gene were studied. Specific primers flanking both SNPs in a single polymerase chain reaction (PCR) product (approximately 370 base pairs [bps]) were used. For the *TLR4* gene, 2 SNPs located at positions 8843 (A/G, Asp299Gly) and 9143 (C/T, Thr399Ile) were also analyzed. Specific primers flanking both SNPs in a single PCR product (approximately 509 bps) were used as well. Additionally, the *TLR9* gene (from position +474 to +3765) was sequenced using primer walking. We further confirmed these findings by amplifying and sequencing 3 different PCR products using specific primers targeting the regions +745 to +1290, +1291 to +1837, and +3324 to +3837 of the *TLR9* gene. Primer design and synthesis was carried out by Macrogen Corporation (Seoul, Korea). PCR product amplification and sequencing was also performed by Macrogen. Sequences were analyzed and edited using Chromas version 2.33 (http://en.bio-soft.net/dna/chromas.html). A homology search against the human genome database at the National Center for Biotechnology Information was performed using nucleotide Blast (Blast-n version 2.2). Multiple alignments to map SNPs were performed using the program ClustalW version 1.83 (http://www.ebi.ac.uk/clustalw/index.html). Some sequences were submitted to the GenBank with accession numbers GQ228032, GQ228034 for the *TLR2* gene sequences; GQ903701 for the *TLR4* gene sequence; and FJ946868, FJ946869, FJ946870, FJ946871, FJ946873, FJ946874, FJ946875, and FJ946876 for the *TLR9* gene sequences.

Statistical Analysis

Results are presented as means (SD). Statistical comparisons between pairs of groups were performed using the Mann-Whitney U test, with a confidence level of 95%. Statistical comparisons between 3 or more groups were performed using the nonparametric Kruskal-Wallis test, with a confidence level of 95%. We also applied the Shapiro Wilk test to evaluate normality (Statistica version 5) and Bartlett's test to evaluate equality of variances (GraphPad Prism 4.0 Software). A P value of <.05 was considered significant.

Results

Numbers of Innate Immune Cells in Peripheral Blood From CVID Patients

In order to quantify the innate immune cells in PB from CVID patients and healthy controls, percentages of the different cell types were determined by either light microscopy or flow cytometry, and absolute numbers were calculated using total white cells counts. No differences were observed in the absolute number of neutrophils, eosinophils, monocytes, mDCs, or NK T cells in PB from CVID patients and healthy controls (Table 2). Patients #1 and #2 (Table 1) exhibited a marked decrease in the absolute number of pDCs in PB in comparison to age- and sex-matched healthy controls (pDCs cell numbers, 1.84 and 1.18, respectively, compared to a mean of 9.2 [4.7] for healthy controls). Based on normal PB cell numbers reported by Commans-Bitter et al [20] and those of our healthy controls, we also observed reduced NK cells in patients #1 and #2 (NK cell numbers, 8.80 and 30.47, respectively, compared to a mean of 189.90 [80.57] for healthy controls). These patients had CVID status MB0 and Ib as

Table 1. Clinical and Immunological Parameters of Patients With Common Variable Immunodeficiency (CVID) Included in This Study

Patient	Age, y	Sex	Age at the First Symptom, y	Infections	Other Symptoms	IgG[a]	IgA[a]	IgM[a]	Lymphoproliferation			CVID Status[b]
									PHA	Cand.	TT	
1	43	F	30	URI, pneumonias, DAD, DCD, *Helicobacter pylori,* APD, conjunctivitis	Parotid gland swelling, artralgias	0	6.7	12.3	N	N	N	Ib MB0
2	38	F	23	URI, pneumonias, DAD, conjunctivitis, HBV infection, ocular	Gastric adenocarcinoma	216	22.6	29	N	D	D	Ib MB0
3	40	M	7	URI, pneumonias, *Helicobacter pylori*, APD, conjunctivitis	Seborrheic dermatitis	148	18	47	N	N	N	II MB1
4	52	F	5	URI, pneumonias, DAD, conjunctivitis, erysipelas, neck abscesses	None	262	18.9	63.6	N	D	D	II MB0
5	35	F	18	URI, pneumonias, DAD	None	43.8	6.6	23.4	N	N	N	II MB2
6	22	F	10	URI, pneumonias, DAD	None	0	2.1	5.7	N	N	N	II MB1

Abbreviations: APD, acid-peptic disease; Cand, Candidin; D, decreased compared to values in healthy individuals; DAD, diarrheic acute disease; DCD, diarrheic chronic disease; F, female; HBV, hepatitis B virus; Ig, immunoglobulin; M, male; N, normal; TT, tetanus toxoid; URI, upper respiratory infection.
[a]At diagnosis (mg/dL, all patients presented levels 2 or more confidence intervals below the normal ranges for age).
[b]As proposed by Warnatz et al [21] or Piqueras et al [22].

defined by Warnatz et al [21] and Piqueras et al [22], respectively. Interestingly, iNKT cells were significantly reduced in PB in 5 of our 6 CVID patients compared to controls (Table 2); the exception was patient #6 (Table 1). However, the decrease in the absolute numbers of iNKT cells was not associated with other clinical or immunological parameters analyzed in this study or with CVID status as defined by Warnatz et al or Piqueras et al.

Modulation of Costimulatory Molecules on the Surface of Innate Immune Cells From CVID Patients and Healthy Controls After Stimulation With TLR Agonists

Although previous reports have shown that CVID patients exhibit defective expression of costimulatory molecules on the surface of monocyte-derived DCs after stimulation with LPS [6,7,11], no studies have been conducted to evaluate the expression of these molecules in other innate immune cells after stimulation with TLR agonists. For this reason, we evaluated the surface expression of the costimulatory molecules CD80 and CD86 in mDCs, pDCs, and monocytes from PBMCs after stimulation with LTA, LPS, and CpG in CVID patients and controls. All these stimuli induced an increase in the expression levels of CD80 and CD86 on the surface of mDCs and pDCs from both patients and controls. As monocytes were analyzed, LPS and CpG but not LTA induced an upregulation of these molecules in comparison to unstimulated cells in both groups (data not shown). No significant differences between patients and controls were observed for CD80 or CD86 expression levels in mDCs, pDCs, or monocytes after stimulation with the TLR agonists. Interestingly, monocytes in CVID patients #2, #4, and #6 (Table 1) exhibited low baseline levels of CD80 and CD86 in comparison to healthy controls (Figure 1, CD80 mean fluorescence intensities [MFI] of 6.99, 14.75, and 11.08, respectively, compared to a mean of 108.2 [16.3] for monocytes from controls. The corresponding MFI for CD86 was 127.59, 256.08, and 145.21, respectively, compared to a mean of 443 [40.23] for monocytes from controls). Nonetheless, these cells were responsive to stimulation with the TLR agonists used in this study at the same levels as those from healthy controls

Table 2. Quantitative Evaluation of Innate Immune Cells in Peripheral Blood From Patients With Common Variable Immunodeficiency (CVID) and Healthy Controls[a]

Cell Types	No. of Cells/μL		*P* Value[b]
	CVID Patients (n=6)	Healthy Controls (n=5)	
Total leukocytes	6440 (1995) (4140-9350)	7908 (2291) (5720-11150)	.2321
Neutrophils	4315 (1013) (3219-5610)	5343 (1981) (3718-8140)	.3312
Eosinophils	92 (148) (0-374)	148 (112) (0-246)	.1751
Monocytes	394 (207) (200-691)	315 (102) (194-401)	.3961
mDCs	9.2 (5.5) (2.6-16.9)	12.1 (3.3) (7.6-15.8)	.3312
pDCs	4.9 (3.2) (1.1-8.7)	9.2 (4.7) (3.1-14.1)	.0628
NK cells	94 (77) (8.8-225)	189.9 (80.5) (100-298)	.0628
iNKT cells	0.6 (0.9) (0-2.5)	4.4 (5.6) (1-14.5)	.0152*
NK T cells	61 (53) (6.5-125)	65 (23) (31-97)	.9372

Abbreviations: iNKT, invariant natural killer T cells; DCs, dendritic cells; mDCs, myeloid dendritic cells; NK, natural killer; pDCs, plasmacytoid dendritic cells.
[a]Each value represents the mean (SD); ranges are shown on the second line.
[b]Significance was set at *P*<.05.

(Figure 1B).

TLR Agonist-Mediated Upregulation of CD69 on NK Cell Surface in CVID Patients

Taking into account that 2 of our CVID patients had reduced NK cell numbers in PB and considering that NK cells are known to express all known TLR mRNA (TLR1-10) and to become activated after stimulation with TLR ligands [23], we evaluated the expression of CD69 on NK cells (CD3-/CD56$^+$/CD16$^+$) after the exposure of PBMCs from CVID patients and controls to LTA, LPS, and CpG ODN. Compared to unstimulated cells, the expression of CD69 on NK cells was upregulated after the stimulation of PBMCs with LPS and CpG ODN but not with LTA in either CVID individuals or controls (data not shown). On the other hand, no significant differences in the percentages or MFI of CD69$^+$ NK cells from patients and controls were observed after stimulation of PBMCs with the different TLR agonists (Figure 2). Patient #6 showed high percentages of CD69$^+$ cells in unstimulated and LPS-stimulated NK cells in PBMCs. Patient #5 exhibited high CD69 MFI in NK cells upon stimulation with CpG-ODN. Interestingly, patients #1 and #2, who exhibited reduced NK cell numbers in PB, were fully responsive to LPS and CpG-ODN compared to healthy controls, indicating that NK cells in PB from CVID patients are fully capable of becoming activated after TLR4 and TLR9 engagement.

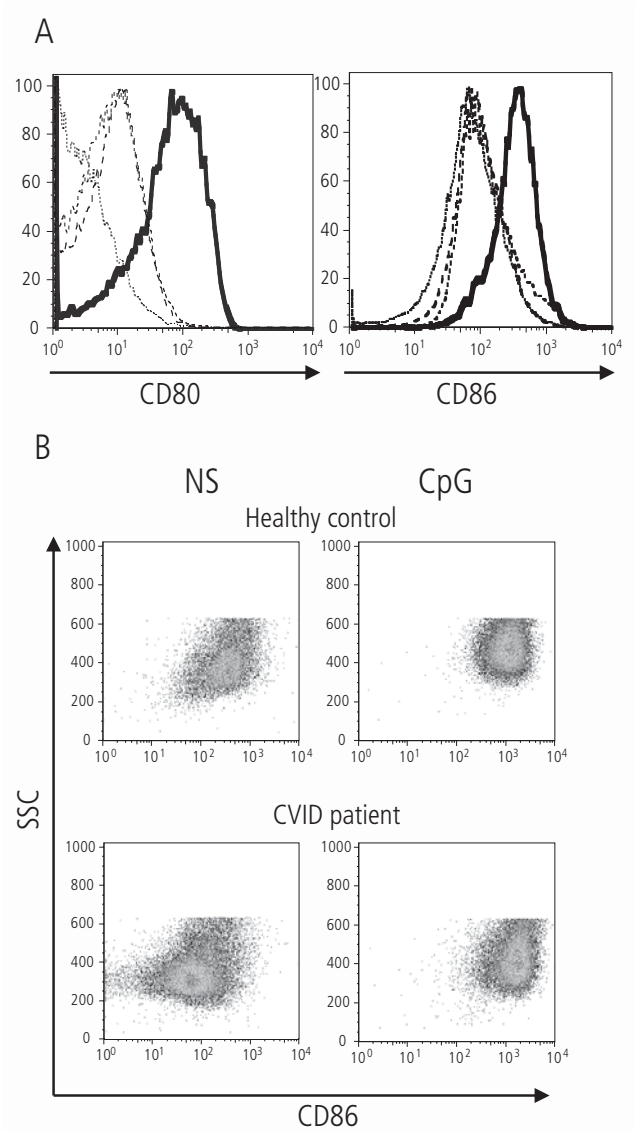

Figure 1. CD80 and CD86 expression on monocytes from patients with common variable immunodeficiency (CVID) and healthy controls. Peripheral blood mononuclear cell (PBMC) monocytes from CVID patients and healthy controls were evaluated for expression of CD80 and CD86 at the baseline levels or after stimulation with the different Toll-like receptor agonists using flow cytometry. A, Histograms representing the baseline median fluorescence intensity of these markers in monocytes from 3 CVID patients (dotted lines, patient #2; dashed lines, patient #4, and long dashed lines, patient #6) and 1 healthy control (no dashed line).B, Dot plots from the CD86 surface expression of PBMC monocytes obtained from 1 healthy control and 1 CVID patient after stimulation with and without CpG ODN. NS indicates nonstimulated cells; SSC, side scatter.

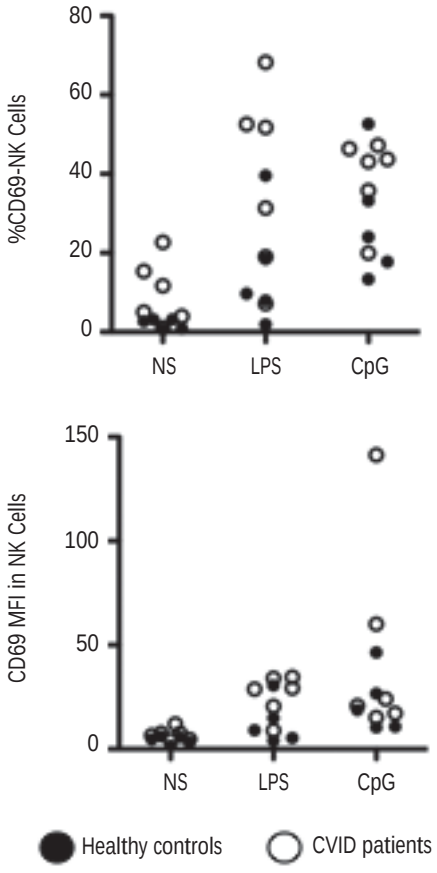

Figure 2. Modulation of C69 expression by Toll-like receptor agonists on natural killer cells from patients with common variable immunodeficiency (CVID) and healthy controls. Peripheral blood mononuclear cells from CVID patients (white circles) and controls (black circles) were stimulated 24 hours with and without LPS or CpG ODN and the expression of CD69 on natural killer (NK) cells was evaluated by flow cytometry. Results are shown as the percentage of CD69 positive cells (A) and CD69 median fluorescence intensity (MFI) (B). NS indicates nonstimulated cells.

Secretion of Cytokines by TLR Agonist-Stimulated PBMCs From CVID Patients

Next, we evaluated the production of cytokines in PBMCs from CVID patients and controls after stimulation with the different TLR ligands. As shown in Figure 3, stimulation with LTA and LPS induced the secretion of IL-12p70, TNF-α, IL-10, IL-6, and IL-1ß in PBMCs from both CVID patients and healthy controls. The stimulation of PBMCs with CpG-ODN also induced the secretion of these cytokines albeit to a much lesser extent than LTA or LPS (data not shown). However, only CpG-ODN was able to considerably increase the levels of IFN-α in PBMCs from both groups (Figure 3). When levels of the different cytokines in PBMC culture supernatants from patients and controls were compared, we observed significantly greater production of TNF-α in PBMCs from all the CVID patients upon stimulation with LPS, a finding that could be due to the constant microbial stimulation of innate immune cells observed in these patients.

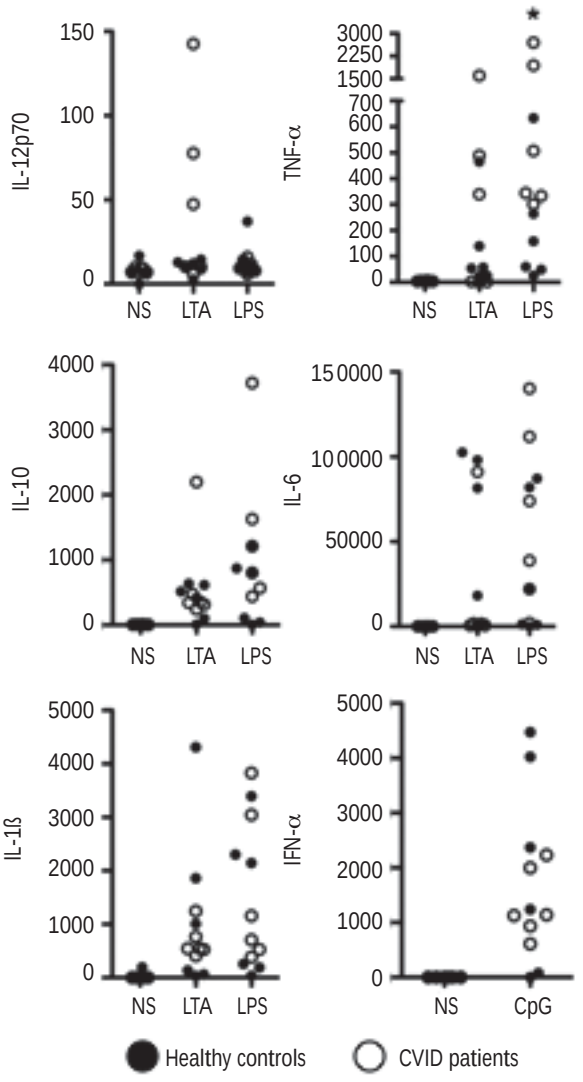

Figure 3. Secretion of cytokines by Toll-like receptor (TLR) agonist-stimulated Peripheral blood mononuclear cells (PBMCs) from patients with common variable immunodeficiency (CVID) and healthy controls. PBMCs from CVID patients (white circles) and controls (black circles) were stimulated with different TLR ligands as described in the Materials and Methods section. Subsequently, cytokine levels were evaluated in the supernatants by flow cytometry (IL-12p70, TNF-α, IL-10, IL-6, IL-1ß) or enzyme-linked immunosorbent assay (IFN-α). Results are presented as concentration in micrograms/mL. *P<.05. Only statistical differences between cells from patients and controls when the same stimulus is evaluated are shown. IFN indicates interferon; IL, interleukin; NS, nonstimulated cells; TNF, tumor necrosis factor.

Analysis of Polymorphisms in TLR2, TLR4, and TLR9 Genes

We also investigated the presence of several previously reported SNPs in the *TLR2, TLR4*, and *TLR9* genes in patients with CVID and healthy controls. Within the TLR2

Table 3. Frequency of Single Nucleotide Polymorphisms (SNPs) Within the *TLR2*, *TLR4,* and *TLR9* Genes in Patients with Common Variable Immunodeficiency (CVID) and Healthy Controls

Gene	*TLR2*						*TLR4*						*TLR9a*					
SNP	C17023T			G17252A			A8719G			C9019T			G1174A			G2848A		
Genotypes	C	CT	TT	GG	GA	AA	AA	AG	GG	CC	CT	TT	GG	GA	AA	GG	GA	AA
CVID	6/6	–	–	6/6	–	–	6/6	–	–	6/6	–	–	3/6	2/6	1/6	1/6	2/6	3/6
Controls	5/5	–	–	4/5	1/5	–	5/5	–	–	5/5	–	–	1/5	4/5	–	1/5	1/5	3/5

[a]SNP offsets were calculated by taking the A of the *TLR9* start codon as position 1.

gene, we evaluated the frequency of 2 nonsynonymous SNPs, Arg677Trp (C17023T) and Arg753Gln (G17252A). As shown in Table 3, we did not observe the presence of the Arg677Trp SNP in any of the individuals studied. A heterozygous individual for Arg753Gln was found in the healthy control group. In agreement with previously reported evidence [16], we found that PBMCs from this individual did not exhibit impaired response to stimulation with LTA (data not shown). Regarding the *TLR4* gene, 2 nonsynonymous SNPs, Asp299Gly (A8719G) and Thr399Ile (C9019T), were studied as well. These 2 SNPs were not present in our group of CVID patients or healthy controls (Table 3). In the last part of this study, an extensive region of the *TLR9* gene was sequenced from genomic DNA from CVID patients and controls by primer walking. Seven SNPs have been reported within this gene, 2 of which (G1174A and G2848A) have been found at high frequencies in the Hispanic population [18]. Our work showed that these 2 SNPs were also highly frequent in our study groups. Of the individuals analyzed, 1 (9%) was homozygous and 6 (54%) were heterozygous for the G1174A, and 6 were homozygous (54%) and 3 (27%) were heterozygous for G2848A. However, the presence of these 2 SNPs in homozygous or heterozygous individuals does not correlate with PBMC response to stimulation with CpG-ODN (data not shown).

Discussion

In order to elucidate the contribution of innate immunity to the physiopathology or clinical heterogeneity of CVID, in the present study we evaluated the frequency and functional response of innate immune cells in CVID patients, using a model of PBMC activation with TLR agonists. Importantly, our study demonstrated reduced levels of iNKT cells in PB from 80% of the patients analyzed. Although the mechanism underlying low iNKT cell numbers in CVID remains unclear, it is important to point out that these cells have been linked to the development of appropriate humoral immune responses through the secretion of cytokines and the provision of cognate help to B cells for isotype switching and antibody secretion [24]. Thus, a defect in iNKT cells would induce defective B-cell function and antibody production as observed in CVID. This hypothesis is supported by recent findings from Fulcher et al [25], who found a marked decrease in the proportion of iNKT cells in CVID patients and particularly in those with low or absent isotype-switched memory B cells, suggesting that defective iNKT cell numbers may result in hypogammaglobulinemia and contribute to antibody failure in CVID. However, our data are in conflict with the findings of Fulcher et al since in our case the CVID status of patients with low iNKT cell levels in PB was characterized by absent, low, or even normal switched memory B cells. Considering that many previous reports have demonstrated altered iNKT cell numbers or function in patients with diseases characterized by autoreactive tissue damage [26], another possible explanation for our results is that decreased iNKT cell numbers in CVID are the manifestation of a regulatory loop to control cell inflammation. Although the significant increase found in the production of TNF-α in the supernatants of PBMCs from our CVID patients following stimulation with LPS support the hyperinflammatory state of our patients, more studies are necessary to evaluate which mechanisms induce a decrease in iNKT cell numbers in CVID. Moreover, considering previous findings by our group that have shown that pDCs initiate iNKT cell/mDC crosstalk [27], detailed research should be done to ascertain if impaired pDC function in CVID might influence levels of iNKT cells.

Interestingly, we observed a significant decrease in the absolute numbers of NK cells in the PB of CVID patients #1 and #2 in comparison with controls, although these cells were fully capable of becoming activated after TLR4 and TLR9 ligation. IVIG treatment does not seem to be responsible for this phenomenon since all of the patients were receiving replacement therapy and only 2 exhibited decreased NK cell levels in PB. These results are in agreement with those of Aspalter et al [13], who reported NK cell concentrations in PB below the normal range in 60% of CVID patients. However, no correlation was observed between IgG serum levels after IVIG infusion and NK cell numbers in those patients. The authors also showed that high-dose IVIG did not affect PB NK cell levels in patients with eczema or vasculitis. Therefore, we still have no explanation for the decrease in absolute numbers of NK cells in some CVID patients. Although these patients had the same CVID status (Ib/MB0 as defined by Warnatz et al [21] and Piqueras et al [22]), and 1 of them had a gastric adenocarcinoma, more studies are necessary to elucidate the clinical outcome of NK cell defects in CVID.

Our own data also show that baseline expression of CD80 and CD86 is altered in monocytes from some CVID patients although these cells can respond normally to stimulation with TLR agonists by increasing the surface expression of these

costimulatory molecules at the same levels as monocytes from healthy controls do. We still do not know if this is a transient phenomenon since previous reports have described a transient but reproducible CD80 expression defect in stimulated B cells from CVID patients with recurrent episodes of cyclic neutropenia, skin vasculitis, and infections [28].

Taken together, the results of the present study demonstrate that the frequency of innate immune cells such as pDCs, NK cells, and iNKT cells in PB is altered in CVID. We still do not know if intrinsic defects in the activation of iNKT cells are responsible for the quantitative differences observed in the other innate cells in PB from CVID patients or how these innate pathways influence B-cell function or antibody production. These findings encourage us to analyze in detail the functional activity of iNKT cells in CVID patients and the pathways involved in iNKT-B cell crosstalk. On the other hand, we have also demonstrated that the presence of nonsynonymous SNPs within the TLR2, TLR4 and TLR9 genes are not associated with clinical variability in CVID patients. More studies are also necessary to elucidate the mechanisms responsible for chronic inflammation and autoimmune manifestations in this syndrome.

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