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Altered Pattern of Naïve and Memory B cells and B1 Cells in Patients with Chronic Granulomatous Disease

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ABSTRACT

Chronic granulomatous disease (CGD) is a rare primary immunodeficiency disorder characterized by a greatly increased susceptibility to severe fungal and bacterial infections caused by defects in NADPH oxidase of phagocytic cells. We aimed to investigate immunophenotype alterations of naïve and memory B cells and B1a cells in peripheral whole blood from Iranian patients with CGD.

Flow cytometric analysis was performed on peripheral blood samples from 31 CGD patients and 23 healthy controls (HC) to study naïve (IgD+/CD27-), memory (CD27+) B and B1a (CD5+) cells. Soluble CD27 (sCD27) and immunoglobulins were also measured by ELISA and the nephelometric method, respectively.

We found significantly higher levels of naïve B cells and B1a cells but lower levels of memory B cells in CGD patients compared to HC. There was no significant difference in soluble CD27 (sCD27) alteration between CGD patients and HC.

Our findings suggested a role for NADPH oxidase in process of B cell differentiation and impairing conversion of naïve B cells to memory B cells and altered B1a cells in CGD patients. Increased susceptibility of CGD patients to opportunistic infections and autoimmune disorders could be partly explained by the altered phenotype of B lymphocytes in these patients.

Keywords: B-Lymphocytes; Chronic granulomatous disease; CD27; CD5; IgD; Naïve B cells; Memory B cells

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INTRODUCTION

Chronic granulomatous disease (CGD) is a disease of impaired phagocytosis, as neutrophils and monocytes from CGD patients fail to exhibit an

increase in oxygen metabolism during phagocytosis.¹ The disease is a rare genetic disorder (minimum estimated prevalence of 1/200,000 to 1/250,000) in the innate immune system characterized by severe and recurrent infections with mostly catalase-positive microorganisms. In these patients, phagocytes are not able to mount a respiratory burst to kill invading bacteria and fungi.² CGD is genetically caused by mutations in any of four proteins of the NADPH oxidase complex, including: gp91phox, p22phox (both subunits of the membrane cytochrome b558), p47phox, and p67phox (the cytosolic components of the enzyme complex).³ Besides, a fifth subunit (namely p40phox), was recently known which plays an important role in phagocytosis-induced superoxide production.⁴ Defect in gp91phox subunit results in X-linked (XL) and defect in any of the other subunits results in autosomal recessive (AR) type of the disease.

Interestingly, the presence of the phagocyte O₂⁻ generating NADPH oxidase in B cells is also well established. In these cells, the activity of NADPH oxidase is different from that in professional phagocytes; for instance, oxidants generated at low levels can act as signaling molecules.⁵ However, it has been shown that B cells also produce superoxide in response to various stimuli,⁶ but these B cells produce only a small amount of superoxide even when fully stimulated.⁷ The nature of components of the NADPH oxidase system and the mode of interaction appear to be the same in neutrophils and B lymphocytes. However, the rate of production of O₂⁻ by B lymphocytes is ten to twenty times lower than that of neutrophils which is probably revealed by the fact that cytochrome b558 is much less abundant in B cells compared to neutrophils.⁸ In contrast, B cells contain the same order of magnitude of the cytosolic factors of p67phox, p47phox and p40phox as those found in neutrophils.^{8,9} B lymphocytes use their slowly generated O₂⁻ and its direct product of dismutation H₂O₂ for other purposes like acting as second messenger in signal transduction pathways.⁸

Regarding studying the number of B lymphocytes, there is a universal marker for human memory B cells which can be used for distinguishing memory cells (CD27+) from naïve B cells (CD27-/IgD+).^{10,11} In clinical practice, membrane CD27 and its soluble form (sCD27) are used as lymphocyte subset and disease markers in the case of lymphoid malignancies, autoimmunity and transplant rejection.¹² The absence of memory B cells as a cause of the impaired

immunoglobulin production was demonstrated in vivo and in vitro in some of the immunodeficiency diseases such as common variable immunodeficiency (CVID) and X-linked hyper-IgM syndrome (XHIM).¹³

CGD patients are at risk for chronic inflammatory manifestations and autoimmune diseases, but it is not clear whether these disorders are secondary to altered superoxide production, or whether a defect in adaptive immune system, like defect in B lymphocytes, is involved.¹⁴ Moreover, we thought whether deficiency of NADPH oxidase components occurred in CGD patients can also affect their B cells. So, we aimed to study phenotype changes of the B cells in peripheral blood of patients with CGD using CD27+ B cells (as memory B cells marker) and IgD+/CD27- (as naïve B cells marker). We also investigated another subset of the B cells, defined by CD5 expression (known as B1a cells¹⁵) which might contribute to the CGD pathogenesis. Finally, we studied immunoglobulins (IgG, IgM and IgA) and sCD27 serum or plasma levels in this CGD population.

MATERIALS AND METHODS

Subjects

Thirty-one patients with CGD (CGD group) consuming cotrimoxazole and in some cases IFN- γ and/or itraconazole were included. All patients were diagnosed as CGD based on abnormal neutrophil oxidative burst assay (nitroblue tetrazolium (NBT) slide test and flow cytometry using dihydrorhodamine 123 (DHR) assay) at Immunology, Asthma and Allergy Research Institute; a main referral center for immunodeficiency disorder in Iran from December 2007 to June 2009. All of these patients were registered in IPIDR (Iranian Primary Immunodeficiency Registry).^{16,17} The CGD subtypes of the patients were also determined by western immunoblot detection of cytosolic and membrane NADPH oxidase components as described by Fattahi et al¹⁸. A healthy control group (HC), consisting 23 healthy; 10 males and 13 females, with the mean age of 13.2 \pm 12.2 years old (age range of 2-35 yrs), provided reference ranges for lymphocyte markers and soluble CD27 (sCD27) levels. We also obtained blood from two available XL-CGD carriers. An informed consent was obtained from all subjects or their parents.

Reagents

Goat anti-human IgD: RPE, Mouse anti-human

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CD27: FITC, Mouse anti-human CD5: FITC, Mouse anti-human CD19: RPE and lysing solution (IQ Product Company) were used in the experiments. All monoclonal antibodies (mAbs) were purchased from AbD Serotec, and used according to the manufacturer's recommendation.

sCD27 instant ELISA and immunoglobulin kits were purchased from the Binding Site company and the assays were performed according to the manufacturer's instructions.

Flow Cytometry

One milliliter of peripheral blood specimens were obtained from patients, and healthy controls. Anticoagulated (EDTA) samples were stained using the whole blood lysis method and analyzed on FACScan flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA) using CellQuest software. Control samples from healthy subjects were also analyzed simultaneously with the patients' samples. B cell subsets were identified by expression of CD19 in all experiments. To analyze subpopulation of the B cells, CD27, CD5 and IgD markers were used. At the acquisition and analysis steps, lymphocytes were selected in a gate by drawing a region around the cell population on the forward scatter (FCS)/side scatter (SSC) dot plot. Evaluation of expression of surface markers was assessed by looking at the relative number of positive cells. Isotype control antibodies were used to separate positive and negative cells on FITC and PE fluorescence channels.

Human sCD27 Instant ELISA

The Instant ELISA plate for sCD27 contained coating antibody and lyophilized detection antibody, streptavidin-HRP, and sample diluent. The plate wells also contained the ready-to-use standard curve containing serially diluted standard protein. So, an anti-CD27 mAb was coated to polystyrene microtiter wells to bind sCD27 present in serum and standard. After incubating 3 hours at room temperature and then washing, tetramethylbenzidine (TMB) substrate solution was added and was incubated for 10 minutes at room temperature. After adding stop solution, absorbance of patient and control samples and standards was measured at 450 nm in an ELISA reader. The concentration of sCD27 in serum was determined by interpolation with the standard curve, generated from seven sCD27 standards, ranging from 0.625 to 20 U/ml. A reference range was established from 16

healthy controls, analyzed concurrently with 29 patients' samples.

Immunoglobulin Measurement

Immunoglobulin serum levels of CGD patients were determined by nephelometric methods. The immunoglobulin levels (IgG, IgM and IgA) in the patients were compared with the normal ranges according to their classified age groups¹⁹. The age of the patients was divided into 7 groups as follows: 1-<2 years, 2-<3 years, 3-<6 years, 6-<9 years, 9-<12 years, 12-<16 years and 16 or older than 16 years.

Statistical Analysis

All data were analyzed using SPSS statistical software package version 13 (SPSS Inc, Chicago IL, USA). Comparisons between means were performed with Mann U Whitney test. Spearman correlation coefficient was used to assess relationship between two non-normally distributed continuous variables. A level of $p < 0.05$ was considered significant for all tests.

RESULTS

Patients' Characteristics

Thirty-one CGD patients including 14 females (45.2%) and 17 males (54.8%) were studied during a period of 1.5 years. The mean (\pm SD) age of the patients was 13.2 ± 8.7 years. Their age range was from 1.5 to 34 years old with the median age of 11 years. Based on the neutrophil function tests done in the patients' mothers, showing a mosaic population of oxidase-positive and oxidase negative neutrophils,¹⁸ two patients (6.5%) were recognized to have XL-CGD type. The rest of the patients (29 cases; 93.5%) had AR-CGD type. Western blot results also confirmed these results and revealed subtypes of the AR-CGD (Table 1). Among AR-CGDs, 16 cases (55.2%) had p47*phox* defect, 10 cases (34.5%) had p22*phox* defect and 3 cases (10.3%) had p67*phox* defect. Table 1 shows the characteristics of the patients in this study.

Flow Cytometric Analysis of B Cell Subpopulations

We found lower percentage of CD27+/CD19+ cells (memory B cells) in peripheral blood of the patients compared to HC group ($p < 0.0001$) (Figure 1A). In the CGD group, a median of 1.4% (range of 0-2.64%) of the B cells were positive for CD27,

Table 1. Subtypes of studied CGD patients determined by western immunoblotting

CGD Subtypes	Frequency (%)	Sex	Age (years)
AR-CGD	29 (93.5%)	12 M, 17 F	13.6± 8.8 13 (1.5-34)
p47 <i>phox</i>	16 (55.2%)	5 M, 11 F	16.7± 9.1 17.5 (4-34)
p67 <i>phox</i>	3 (10.3%)	1 M, 2 F	3.5± 2.3 3 (1.5-6)
p22 <i>phox</i>	10 (34.5%)	6 M, 4 F	11.6± 6.9 11.5 (3-22)
XL-CGD; gp91 <i>phox</i>	2 (6.5%)	2 M	7.9± 5.8 7.9 (3.8-12)

*Age data is presented as mean±SD and median (min-max).

Chronic granulomatous disease (CGD), X-linked (XL), Autosomal recessive (AR)

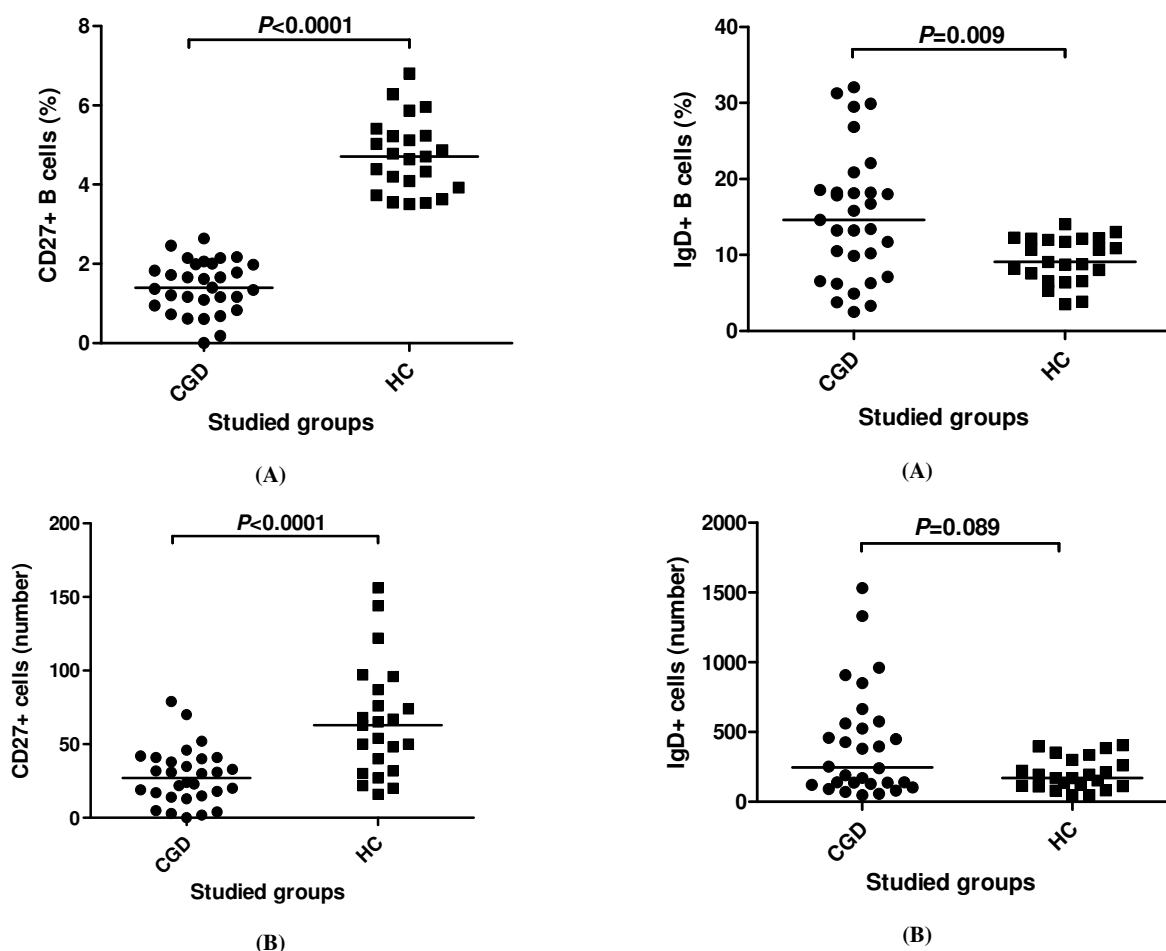


Figure 1. Percentage of CD27 expression (CD27+) on B cells in 31 CGD patients and 23 healthy control (HC) subjects; percentage levels (A), absolute counts(B)

Figure 2. Percentage of IgD expression (IgD+/CD27-) on B cells in 31 CGD patients and 23 healthy control (HC) subjects; percentage levels (A), absolute counts (B)

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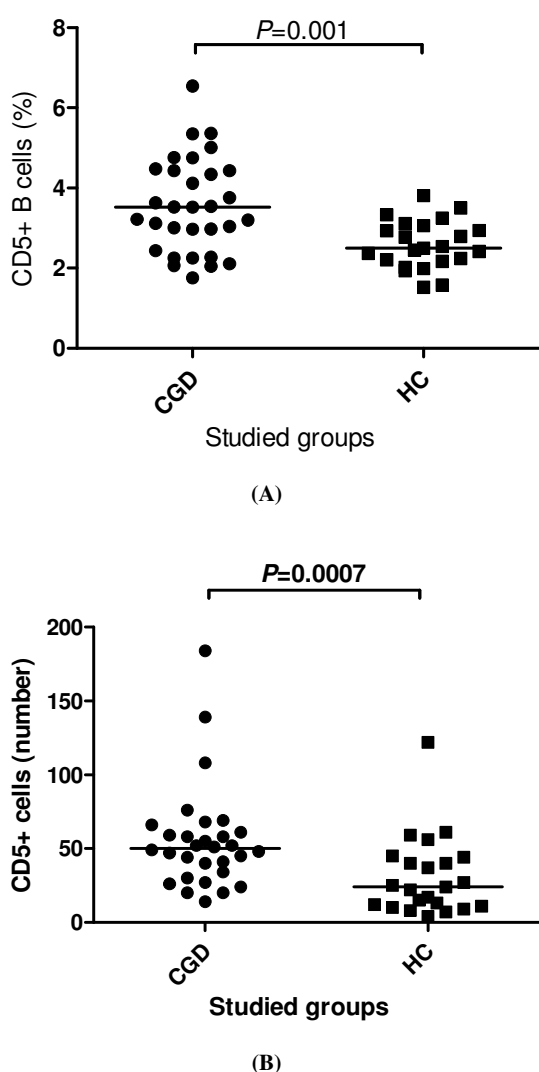


Figure 3. Percentage of CD5 expression on B cells in 31 CGD patients and 23 healthy control (HC) subjects; percentage levels (A), absolute counts (B)

compared to a median of 4.71% (range of 3.51-6.8%) in the HC group ($p<0.0001$). The same direction was found when we compared the absolute count of the CD27 positive cells between the group of the patients and HC (Figure 1B). Two XL-CGD carriers showed almost the same levels of CD27+/CD19+ cells (1.56% and 2.12%) as XL-CGD patients.

Moreover, the percentage of IgD+/CD27- naïve B cells in peripheral blood B lymphocytes was significantly higher in the patients compared to the HC group (Figure 2A and 2B; $p=0.009$). There was a median of 14.6% (range of 2.5-32.06%) IgD+/CD27- cells in the patients compared to a median of 9.1%

(range of 3.54-14.04%) in the HC group. The levels of IgD+/CD27- lymphocytes in the patients was significantly correlated with their age; patients with older age had lower percentage of IgD+/CD27- lymphocytes (Spearman's $\rho=-0.40$, $p=0.02$) as well as lower number of IgD+/CD27- lymphocytes (Spearman's $\rho=-0.63$, $p<0.0001$). The percentage of naïve B cells was 20.5 and 8.5 in the two XL-CGD carriers.

In addition to the higher levels of naïve B cells, a significant higher level of CD5/CD19 positivity was also observed in the patients ($p=0.001$) (Figure 1C). A median of 3.52% (range from 1.76 to 6.54%) of B lymphocytes in the patients was CD5 positive, compared to a median of 2.5% (ranged from 1.52 to 3.81%) in the HC group ($p=0.001$) as shown in figure 3A while figure 3B compares absolute counts of the CD5 positive cells between the two groups. Along with the percentage results, there was a significantly higher level of absolute count of the CD5 positive cells in the patients compared to the HC ($p<0.0001$). The levels of CD5 positive lymphocytes in the patients was significantly correlated with their age; patients with older age had higher percentage of CD5 positive lymphocytes (Spearman's $\rho=0.55$, $p=0.001$). However, this age dependency was not found when absolute count of the CD5 positive cells was considered for analyzing this correlation. The percentage of CD5+ cells was 3.87 and 3.21 in the two XL-CGD carriers.

We did not find any significant difference between subtypes of CGD patients regarding any levels of B lymphocyte phenotypes (data not shown).

Serum Levels of sCD27 and Immunoglobulins

We assayed sCD27 concentration in serum of 29 CGD patients and 16 HC subjects. The patients did not show any significant difference in the sCD27 levels compared to HC ($p=0.094$; mean \pm sd of 76.79 ± 24.59 U/ml in CGD patients versus 64.37 ± 20.72 U/ml in HC).

The level of CD27+ expression on B cells in the patients was negatively correlated with the sCD27 levels (Spearman's $\rho=-0.45$ and $p=0.015$) (Figure 4) which can indicate lower CD27+ levels is because of their conversion to sCD27 secretion in serum. Moreover, to determine increase of immunoglobulin levels along with sCD27, we measured IgA, IgG and IgM levels and compared their levels with sCD27 concentration of patients' serum. As serum

Table 2. The mean of immunoglobulin levels in studied CGD patients compared to the normal range classified by age groups

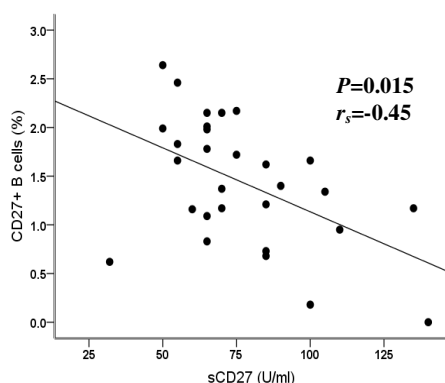
Age (years)	No.	IgG		IgA		IgM	
		Mean	Normal Range	Mean	Normal Range	Mean	Normal Range
1-≤2	1	11.02	3.49-11.36	3.11	0.13-1.02	0.72	0.4-2.29
2-≤3	0	-----	4.82-12	-----	0.22-1.18	-----	0.54-2.09
3-≤6	8	13.48	5.53-13.07	2.3	0.33-1.8	0.55	0.56-2.18
6-≤9	2	14.95	6.46-14.51	3.4	0.28-2.22	0.51	0.55-2.32
9-≤12	4	17.28	6.13-15.12	2.13	0.57-2.56	1.17	0.7-2.84
12-≤16	4	16.17	6.67-14.64	3.43	0.77-2.19	0.55	0.49-2.61
≥16	12	15.49	6.58-18.37	3.92	0.71-3.6	0.85	0.4-2.63

* The normal range in the age groups has been cited from: Lentner C. ed. Geigy Scientific Tables, Vol 1, 8th ed. Basle, Switzerland. Ciba-Geigy Ltd, Table 36, p154.¹⁹

immunoglobulin concentrations are age-dependent, thus we classified the patients to the age groups (Table 2).

We found that 12 of 31 CGD patients had above normal levels of IgG concentration, 13 of 31 CGD patients had IgA concentration above normal levels and all of the patients had IgM concentration in the normal levels. The mean of each immunoglobulin in the CGD patients compared to the normal range are shown in table 2 by the age groups. As it is shown in the table, all of the age groups in the CGD patients had approximately normal mean of the IgM levels whereas the mean levels of IgA was higher than the normal range in almost all age groups of the patients (9-12 years had an exception). Likewise, most age groups (except 1-2 years and adults) had above normal range of the mean levels of IgG.

There was no significant correlation between the levels of sCD27 and any of the immunoglobulins (IgG, IgA or IgM).

**Figure 4.** Correlation between CD27 expression and sCD27 serum concentrations in CGD patients

DISCUSSION

In the present study, we investigated subpopulations of B cells in peripheral whole blood from 31 CGD patients and 23 HC with approximate equal sex ratio (female/male ratio of 1.2 in patients and 1.3 in HC group) and similar age range.

We immunophenotypically investigated memory B cells by using CD27 as a memory marker of B cells and naïve B cells by using IgD. We found an alteration of peripheral B cells: a significantly lower level of memory B cells and higher levels of naïve B cells in CGD patients compared to HC. These findings suggest that deficiency of NADPH oxidase components can affect the differentiation of naïve B cells to memory B cells and especially influence the expression of CD27 on memory B cells. Indeed, there might be a deficiency in conversion of naïve B cells to memory B cells in CGD patients. Regarding the responsible mechanism, it has been demonstrated that the NADPH oxidase components (mostly p47*phox*) in B cells may be involved in a system other than the superoxide-producing NADPH oxidase and play a role in the signaling system and activation of some transcription factors in B cells.⁷ In accordance with our findings, Bleesing et al showed profound reduction in the contribution of CD27+ B cells to the peripheral B cell compartment in CGD patients and showed its correlation with the defective NADPH oxidase system.²⁰ Reduced CD27+ B cells has been also shown in patients with other immunodeficiency disorders like CVID^{13,21} and XHIM.²² In these diseases, crucial signaling molecules inducing terminal differentiation into memory B cells was suggested to be causative

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factor.¹³ In CGD, one possible causative factor is alteration of CD40/CD40L (CD154) which affects the production of memory B cells in germinal centers in CGD patients.¹⁴ The signaling through the CD40/CD40L system, a crucial element in the generation of memory B cells, may be adversely affected in patients with CGD. The reduced CD154 expression in T lymphocytes as well as CD40 expression on neutrophils has been shown in CGD patients.¹⁴ There is a connection between O₂⁻ generated by NADPH oxidase and CD40L induction.¹⁴ Deficiency in productions of NADPH oxidase in CGD patients might therefore indirectly affect the production of memory B cells, impair differentiation of naïve B cells to memory B cells and cause reduced memory B cells.

We also found higher levels of CD5 positive cells (B1a cells) in CGD patients compared to HC group. The secretion of autoantibodies in some autoimmune diseases has identified B-1a cells (CD5+) as potential contributors to the development of these kinds of diseases, such as lupus and discoid lupus. The increased B1a cells in Sjogren's syndrome and rheumatoid arthritis has been also reported.²³ B1a cells are the major source of natural antibodies, which are serum poly-reactive and weakly autoreactive. Several mechanisms have been suggested to explain the possible role of B-1a cells in autoimmune pathogenesis, such as production of pathogenic autoantibodies, presentation of self-antigens to autoreactive T cells, and/or their ability to secrete cytokines such as IL-10.^{15,24} We found higher levels of CD5+ B cells (B1a cells) in CGD patients which is in accordance with previous reports indicating increased frequency of discoid and systemic forms of lupus in CGD patients and the X-linked carriers.^{15,20} The number of XL-CGD carriers in our study was not enough to compare their CD5+ cells levels with HC statistically but still its levels in two studied carriers was high and almost at the same levels as that found in CGD patients. Elevated levels of CD5+ B cells in CGD patients may explain the increased frequency of autoimmune diseases in these patients and may connect this finding to deficiency of NADPH oxidase in the B cells of CGD patients.

Regarding the changes in the expression of the B cell markers by the age of our patients, we only found decreased percentage as well as absolute number of IgD+/CD27- (naïve B-cells), but not other studied

markers. This finding is in line with the previous study showing decreased percentage of naïve B cells with age.²⁵

We did not find significantly increased levels of sCD27 in CGD patients compared to HC group suggesting decreased CD27 expression is not resulted from a specific lack of CD27 expression on B cells. Regarding immunoglobulins, we did not find any significant alteration in IgM levels in CGD patients (its levels was in the normal range). Besides, some patients had slightly higher levels of IgG and IgA than the normal range suggesting likely presence of systemic and mucosal infections in these patients, consistent with increased levels of IgG and IgA, respectively. Along with these findings, Carnide et al reported hypergammaglobulinemia as a common occurrence in 18 CGD patients²⁶. This finding in CGD patients may contribute in the defense against opportunistic infections and persistence of inflammation. However, we can not speculate that CGD and other immunodeficiency disorders like XHIM and CVID share a similar pathogenic mechanism. Besides, serum IgG, IgA, and IgM levels were found to be essentially within normal limits in the CGD patients studied by Bleesing et al.²⁰

The mature B cell differentiation and immunoglobulin production are regulated by at least two of the major cell to cell signaling pathways. As expressed above, CD40/CD154 interactions act in an early phase of B cell activation and induce the expansion of a memory B cell pool¹⁴. It was also demonstrated that memory B cells are differentiated into plasma cells by the activated helper T cells via CD27/CD70 in the presence of several cytokines such as IL-10 and IL-2.^{27,28} These findings strongly suggest that memory B cells are necessary to produce large amounts of immunoglobulins by differentiating into plasma cells through the contact with CD70.²² Serum sCD27 levels may therefore be important in diseases characterized by abnormalities in B-cell differentiation or activation. It has been shown that the levels of serum sCD27 in primary Sjogren's syndrome patients increase and correlate with the elevated serum IgG concentrations. This suggests that the plasma cells or B cells undergoing plasma cell differentiation may be responsible for the production of both sCD27 and IgG. Consequently, elevation of serum sCD27 and hypergammaglobulinemia could be because of an increased plasma cell differentiation (as the main

source of sCD27).²⁹ We found negative correlation between expression of CD27 and sCD27 secretion in the CGD patients and hypergammaglobulinemia in some of the patients. These could result from differentiation of memory B cells to plasma cells in these patients. However, we did not find significantly increased sCD27 levels parallel to elevated immunoglobulin levels (IgG, IgA). Future investigations with larger group of the patients may clarify hypergammaglobulinemia with unknown causes and deficiency of NADPH oxidase in adaptive immune system in CGD patients.

However, our findings suggest a role for NADPH oxidase enzyme in the process of B cell differentiation. It can phenotypically affect subsets of B cells and impair conversion of naïve B cells to memory B cells in CGD patients. This can play an important role in these patients to be more susceptible to recurrent infections. Additionally increased CD5+ B cells in these patients can be an explanation for higher frequency of autoimmune disorders in CGD patients.

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