Defective B-cell proliferation and maintenance of long-term memory in patients with chronic granulomatous disease

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Background: Chronic granulomatous disease (CGD) is a primary immune deficiency characterized by a defect in reactive oxygen species production. Although the effect of CGD mainly reflects on the phagocytic compartment, B-cell responses are also impaired in patients with CGD.

Objective: We sought to investigate how defective gp91phox expression in patients with CGD and CGD carriers might affect the B-cell compartment and maintenance of long-term memory.

Methods: We studied the B-cell compartment of patients with CGD in terms of phenotype and ability to produce reactive oxygen species and proliferate on stimuli differently directed to the B-cell receptor and Toll-like receptor 9. We further studied their capacity to maintain long-term memory by measuring cellular and serologic responses to measles.

Results: We show that the memory B-cell compartment is impaired among patients with CGD, as indicated by reduced total (CD19+CD27+) and resting (CD19+CD27+CD21+) memory B cells in parallel to increased naive (CD19+CD27−IgD+) B-cell frequencies. Data on CGD carriers reveal that such alterations are related to gp91phox expression. Moreover, proliferative capabilities of B cells on selective in vitro stimulation of B-cell receptor or Toll-like receptor 9 pathways were reduced in patients with CGD compared with those seen in age-matched healthy control subjects. Significantly lower plasma specific antibody levels and antibody-secreting cell numbers were also observed, indicating a poor ability to maintain long-term memory in these patients.

Conclusion: Altogether, our data suggest that patients with CGD present a defective B-cell compartment in terms of frequencies of memory B cells, response to in vitro stimulation, and maintenance of long-term antigen-specific memory.

Key words: Chronic granulomatous disease, B cell, proliferation, long-term memory, measles, memory B-cell compartment, reactive oxygen species deficiency

Chronic granulomatous disease (CGD) is a primary immune deficiency caused by defects in the phagocyte nicotinamide adenine dinucleotide phosphate (NADPH) oxidase. The phagocyte oxidase generates superoxide and other reactive oxygen species (ROS) by transferring electrons from NADPH to molecular oxygen and consists of the catalytic subunit gp91phox, which is structurally stabilized by p22phox protein, and the regulatory subunits p47phox, p40phox, p67phox, and Ras-related C3 botulinum toxin substrate (RAC).1,2 As a consequence, phagocytes of patients affected by CGD are unable to kill ingested microorganisms, resulting in augmented susceptibility to recurrent life-threatening pyogenic infections.3-6

Although CGD is primarily recognized as an oxidative deficiency of the phagocytic compartment, key cellular pathways, including lymphocyte function, were also shown to link to ROS production.7,8 Furthermore, patients affected by CGD have been described to present lower frequencies of circulating memory B cells,9,10 with an intact humoral immunologic memory.10 This was shown primarily in patients receiving immunosuppressive therapies.10

Recent mouse studies showed a direct relation between the B-cell stimulation and the production of ROS. Lower activity of the NADPH oxidase could impair B-cell receptor (BCR) signal strength, reducing activation and proliferation of B cells in response to surface immunoglobulin cross-linking.11,13 However, the role of ROS production in sustaining human B-cell function and long-term maintenance of memory B-cell responses remains poorly understood.

Here we performed an extensive phenotypic and functional characterization of B cells in the peripheral blood of patients with CGD who were not undergoing immunosuppressive therapies. Monitoring of B-cell proliferation on direct triggering of BCR pathways, Toll-like receptor (TLR) 9 pathways, or both revealed a partial impairment of B-cell function in patients with CGD. Subsequent analyses of vaccine-induced antibody responses against measles indicated defective long-term maintenance in terms of both serum antibody levels and numbers of circulating antibody-secreting cells (ASCs).
METHODS

Study subjects

Ten patients with CGD, 13 age-matched healthy control subjects (HCs), and 4 CGD carriers (mothers of 4 of the enrolled patients) were enrolled at the University Department of Pediatrics, Unit of Immune and Infectious Diseases, Children’s Hospital Bambino Gesù, Rome, Italy. Participating patients and their family members provided written consent for evaluation and follow-up. All experiments were reviewed and approved by the appropriate institutional review board. Patients were considered to have the X-recessive form of the disease, as previously described.1,13 The CGD group consisted of 9 patients with the X-linked form and 1 with the autosomal recessive form of CGD. Patients’ characteristics are listed in Table I. All patients with CGD were clinically stable, and only 1 patient was receiving immunosuppressive therapy (azathioprine) because of a concomitant inflammatory bowel disease. Nonetheless, no evidence for differences in terms of both cellular and humoral immunity was found compared with the other patients with CGD. According to the national routine vaccination protocol, all patients and HCs received measles vaccination (Priorix; GlaxoSmithKline, Research Triangle Park, NC) between the 2 groups (Fig 1, A). In contrast, numbers of naive B cells (CD19+ IgD+CD27-) were comparable between the 2 groups (Fig 1, C). In addition, higher frequencies of switched memory B cells (CD19+ IgD-CD27+) were greater in patients with CGD compared with those in HCs in terms of percentages (P = .049; Fig 1, B) and total counts (P = .022; Fig 1, D). Among mature B-cell subsets, patients with CGD showed differences in percentages of resting memory B cells (CD19+CD10-CD21+CD27+) when compared with HCs (P = .0053). Other B-cell subsets, such as tissue-like (CD19+CD10+CD21-CD27+), activated memory (CD19+CD10-CD21+CD27+), and immature transitional (CD19+CD24+CD38+CD10+) cells, were comparable between the 2 groups (Fig 1, E). In addition, higher frequencies of
double-negative (CD19−CD27− IgD−) cells were observed among patients with CGD compared with HCs, although a statistical difference was not reached (P = .081; Fig 1, B).

To rule out whether these observations were related to lymphopenia, we re-evaluated the above data, taking into account absolute white blood cell counts (Table 1). Normal white blood cell counts and a nonsignificant increase in total B-cell counts were observed in patients with CGD compared with HCs (Fig 1, D). Interestingly, although the number of naive B cells was increased among patients with CGD (P = .022), total memory (P = .0059) and resting memory (P = .043) B-cell counts were consistently lower in patients with CGD compared with those in HCs. In conclusion, our data indicate that patients with CGD present a selective paucity in resting memory B-cell counts, together with an accumulation of naive B cells.

**B-cell proliferation is impaired on single BCR or TLR9 stimulation among patients with CGD**

To investigate B-cell functional responses in patients with CGD, we next sought to assess the ability of B cells from patients with CGD to proliferate on different *in vitro* stimulations engaging the BCR or TLR9 pathways. Total PBMCs from patients with CGD and HCs were labeled with carboxyfluorescein succinimidyl ester and cultured for 5 days in the presence of different stimuli, and B-cell proliferation was subsequently analyzed by means of flow cytometry (Fig 2, A). PWM is widely used as a B-cell stimulatory reagent to elicit B-cell expansion with concomitant differentiation of B cells into ASCs.

B cells from patients with CGD showed a significantly lower proliferation after PWM stimulation (P = .009) compared with those from HCs (Fig 2, B). The B-cell mitogenic effect of PWM was recently shown to result from synergistic activity of the pokeweed lectin and microbial TLR2/9 ligands present in the PWM preparations. To investigate whether reduced B-cell proliferation in patients with CGD after PWM stimulation is related to perturbations in BCR or TLR9 pathways, we conducted single or combined stimulations using anti-Ig for BCR cross-linking and CpG for TLR9 stimulations. B cells from patients with CGD showed a significantly lower proliferation after single stimulation with either anti-Ig (P = .019) or CpG (P = .017) compared with HCs.

Conversely, comparable B-cell responses between patients with CGD and HCs were observed when exposed to combinations of CpG plus anti-Ig or PWM plus anti-Ig (Fig 2, B). To investigate whether there was lower proliferation of B cells in CGD patients with stimuli singularly directed to BCR or TLR9, we further monitored cell proliferation of memory (CD19−CD27− IgD−) and naive (CD19−CD27+ IgD−CD10+) B cells (see Fig E1 in this article’s Online Repository at www.jacionline.org). Partial impairments were observed in both memory and naive B-cell proliferation on single stimuli, which were compensated when these stimuli were given together, as shown for total B cells. Collectively, these data suggest that synergistic engagement of BCR and TLR9 signaling pathways might be required to compensate for intrinsic defects in CGD-derived B cells.

**ROS after PMA stimulation in B cells of patients with CGD**

Small and localized amounts of ROS have been reported to modulate lymphocyte activation and proliferation. To investigate whether the above-described alteration in B-cell proliferation from patients with CGD is related to NADPH oxidase activity, we used a flow cytometric assay to specifically measure ROS levels in B cells from patients with CGD and HCs after B-cell activation. Our results show that the modulation of ROS activity, intended as a fold increase of ROS after PMA stimulation, is significantly lower among patients with CGD compared with that seen in HCs (P = .006, Fig 3). To exclude that measured ROS in B cells originated through diffusion from bystander cells among total PBMCs, we repeated the above experiments with purified B cells isolated by means of negative selection. Comparable results were observed between sorted B cells and CD19− B cells gated out of total PBMCs (see Fig E2, A, in this article’s Online Repository at www.jacionline.org), thus confirming our observation that B cells from patients with CGD carry intrinsic defects of NADPH oxidase.

**Impaired proliferation is associated with lower frequencies of measles-specific ASCs**

Measles-specific antibodies were reported to be maintained through life and to correlate with the level of total memory B cells in the periphery. For this reason, this antigen is used as a model to dissect long-term memory maintenance and analyzed in this study. *In vivo* exposure to cognate antigens (in the form of reinfection or booster vaccination) results in the activation of memory B cells and their subsequent proliferation and differentiation into antibody-secreting daughter cells. To assess whether the impaired B-cell proliferation could affect the capacity of memory B cells to differentiate into ASCs, we measured the *in vitro* differentiation of total PBMCs after stimulation with PWM and CpG alone or in combination (Fig 4, A).

Results showed that measles-specific ASC frequencies are lower in patients with CGD compared with those seen in HCs. These data were not affected by differences in total IgG-secreting cells between patients with CGD and HCs. Moreover, this result was confirmed on single stimulation (PWM; P = .033 and CpG; P = .018), whereas combined stimulation (PWM+CpG) only partially recovered specific memory B cells in these patients (Fig 4, B). Overall, these data confirm the results of the proliferation experiments, suggesting that B cells from patients with CGD need simultaneous triggering of BCR and TLR9 to differentiate into ASCs.

Although total IgG was not different between patients with CGD and HCs, revealing a good control of the inflammatory phase of the disease, patients with CGD had lower frequencies of serum antibodies against measles (P = .0234; Fig 4, C). In addition, 4 of 10 patients had antibody levels of less than protective levels (Fig 4, C), according to standard correlates of seroprotection. Further analyses demonstrated that among patients with CGD, there is an age-related waning of measles serologic immunity (r = −0.7, P = .022; Fig 4, D).
<table>
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<th>Participant no.</th>
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<th>Mutation</th>
<th>Protein change</th>
<th>CGD type</th>
<th>Sex</th>
<th>Age</th>
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<th>RBC (10^6/μL)</th>
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<td>12.8</td>
<td>4.75</td>
<td>6.13</td>
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Mean (SD) 6.33 — — — — 19.2 (13.6) 12.71 (0.55) 5.03 (0.18) 6.8 (0.51) 4.31 (0.42)

AZA, Azathioprine; CRP, C-reactive protein; del, deletion; dup, duplication; E, eosinophils; ESR, erythrocyte sedimentation rate; F, female; Hb, hemoglobin; ITCZ, itraconazole; L, lymphocytes; M, male; N, neutrophils; ND, not determined; ns, nonsense mutation; PLT, platelets; RBC, red blood cells; ssm, splice site mutation; TMP-SMX, trimethoprim/sulfamethoxazole; WBC, white blood cell count.

FIG 1. The B-cell phenotype of patients with CGD is characterized by lower memory and higher naive cell counts. Representative gates (A) and comparisons of B-cell percentages (B and C) and absolute counts (D) are shown. Horizontal bars represent medians. P values were determined with the nonparametric Mann-Whitney test. CD19^+ cells established the B-cell population, and expression of IgD, CD27, CD21, CD24, CD38, and CD10 was used to define total naive (CD27^-IgD^-), total memory (CD27^-IgD^+), double-negative (DN; CD27^-IgD^-), resting memory (Rem), tissue-like (TL), activated memory (AM), naive (Na), and immature transitional (Tr) cells. FSC, Forward scatter; SSC, side scatter.
not found in HCs (Fig 4, E). All patients with CGD who presented with a lower response after in vitro stimulation for ELISpot assay and serologically unprotected to measles presented common phenotypic characteristics compared with other patients with CGD (Fig 5). In fact, ELISpot assay nonresponders presented lower levels of total (P = .022) and resting memory (P = .0356) B cells compared with ELISpot assay responders. ELISpot assay responders showed lower naive B-cell counts (P = .0044) compared with ELISpot assay nonresponders with CGD (Fig 5). No correlation with age was found between responders and nonresponders. Together, these results identify a relation between impaired maintenance of memory response and phenotype in patients with CGD.

### TABLE I. Continued

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<th>L (10^3/μL [%])</th>
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<th>Ferritin (ng/mL)</th>
<th>IgA (mg/dL)</th>
<th>IgG (mg/dL)</th>
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**FIG 2.** B-cell proliferation of patients with CGD is reduced if a single stimulus directed to BCR or TLR9 is given. Representative gate of carboxyfluorescein succinimidyl ester (CFSE)–diluted B cells (A) and comparisons of proliferating B-cell percentages between patients with CGD (n = 10) and HCs (n = 13; B) are shown in the scatter dot plots. Horizontal bars, Medians. Statistics have been defined with the nonparametric Mann-Whitney test, and only significant P values (P < .05) have been reported in the figure. SSC, Side scatter.
To investigate whether B-cell defects could be found in a competitive setting, we investigated CGD carriers. We observed that mothers with CGD express different levels of gp91phox-positive B cells (Fig 6, B) and CD14<sup>+</sup> cells (data not shown).

We further show a partial B-cell impairment among CGD carriers compared with that seen in HCs. In fact, higher levels of total naive and lower total memory levels are found among CGD carriers. In addition, resting memory cell counts are significantly lower compared with those in HCs ($P = .028$; Fig 6, D). Similar data are shown in the proliferation experiments, where CGD carriers show a lower proliferation rate on single

**FIG 3.** ROS activity is hampered in B cells affected by CGD. Representative gate (A) and comparisons (B) between patients with CGD and HCs in terms of modulation of ROS with and without PMA stimulation on gated CD19<sup>+</sup> cells are shown. Modulation of ROS is defined as the ratio of the geometric mean of M2–2′,7′-dichlorodihydrofluorescein diacetate (DCFDA) before and after PMA stimulation. Means and SEMs are shown in the graph. Statistics have been defined with the nonparametric Mann-Whitney test. SSC, Side scatter.

**FIG 4.** In vitro and in vivo responses to measles in patients with CGD are lower compared with those seen in HCs. Representative ELISpot assay analysis (A) and comparisons (B) between patients with CGD and HCs are shown in the graph. Open triangles, Average of the IgG spots; horizontal bars, medians. Specific antibodies for measles and total IgG (C) and correlation (Spearman test) with age in patients with CGD (D) and HCs (E) are shown. KLH, Keyhole limpet hemocyanin.
stimuli. In line with this, mothers with CGD with higher naive B cells in parallel to lower proliferation rates on single stimulations singularly directed to TLR9 or BCR is compensated if the congenital defective production of ROS could impair B-cell lymphocyte activation and proliferation after specific stimuli. In patients with CGD, this altered production of ROS renders the patient’s phagocytes unable to kill ingested microorganisms, resulting in increased susceptibility to bacterial and fungal infections. However, several pieces of evidence indicate the importance of ROS, although at different concentrations, to act as secondary messengers in lymphocyte activation.8,39,40 Furthermore, it has been shown that these functional defects seem inversely related to the selective advantage in B cells. These results fit with recent data showing that female carriers, who are usually considered unaffected, display clinical manifestations that often underlie an autoimmune pathogenesis.38

Among patients with CGD, the defective proliferation and differentiation into ASCs in response to stimuli that are solely directed to TLR9 (CpG) or BCR (anti-Ig) is compensated if these stimuli are given together. Accordingly, restoration of the proliferative ability on combined stimulation with both BCR and TLR could be due to activation of naive B cells, in which the synergistic activation of both pathways is required.28,29

In view of this, it is possible that dysfunctional NADPH oxidase activity on naive B cells in vivo might directly affect the capability of these cells to differentiate into memory cells. In fact, patients with CGD have been reported to have lower total memory B-cell counts (CD19+CD27+ and resting memory B-cell counts (CD19+CD10−CD21+CD27+).10 In the present study we confirmed these findings.

Further investigations were performed on measles-specific immunity to study the effects of CGD on the maintenance of long-term serologic memory. To date, measles-specific antibodies are known to be long term and directly correlated with levels of total memory B cells in the periphery.50 Results pointed out significantly lower serum antibody levels against measles and impaired differentiation of measles-specific memory B cells to ASCs among patients with CGD compared with HCs. Interestingly, an age-related reduction in measles-specific antibody levels was found among patients with CGD. This further points out the impaired recall/maintenance of antigen-specific memory B cells in patients with CGD, which, in the case of measles, should confer serologic memory for the entire lifespan in healthy subjects.50

A similar scenario occurs in other diseases characterized by chronic immune activation,16,30,31 in which the capacity to confer durable protection after vaccination is reduced and related to low memory B-cell frequencies.2,33

Thus a possible explanation for the loss of long-term memory in patients with CGD can be an ineffective polyclonal activation of memory B cells, which usually maintain serologic memory.28,34,35 However, we also found numbers of CD27/IgD double-negative B cells, which were previously described as increased in elderly healthy subjects.36 HIV-infected children, and patients undergoing kidney transplantation,37 to be higher among patients with CGD compared with those seen in HCs. This might support the hypothesis that B cells from patients with CGD could also be unresponsive to stimuli because of premature aging. In HIV-infected children and patients undergoing kidney transplantation, this is due to B-cell chronic activation.37

This suggest that B cells from patients with CGD might be subjected to a similar chronic activation status as a result of the inflammatory milieu.

To further investigate the B-cell defect in the context of a competitive setting, we studied B cells from mothers with CGD. CGD carriers showed a partial impairment in terms of phenotype and proliferation on single stimuli. Interestingly, our data show that these functional defects seem inversely related to the selective advantage in B cells. These results fit with recent data showing that female carriers, who are usually considered unaffected, display clinical manifestations that often underlie an autoimmune pathogenesis.38

On the other hand, our investigation has focused on understanding whether the congenital defective production of ROS could impair B-cell lymphocyte activation and proliferation after specific stimuli. In patients with CGD, this altered production of ROS renders the patient’s phagocytes unable to kill ingested microorganisms, resulting in increased susceptibility to bacterial and fungal infections. However, several pieces of evidence indicate the importance of ROS, although at different concentrations, to act as secondary messengers in lymphocyte activation.8,39,40

DISCUSSION

In the present study we focused on the analysis of B-cell phenotype and function in patients affected by CGD, with the aim of assessing whether the deficit in NADPH oxidase characterizing these patients could directly affect B-cell functions in a setting free of immunosuppressive drugs. This might be particularly important because inhomogeneous effects of immunosuppressive drugs on different total B-cell subtypes and antigen-specific memory B cells can occur. First, we confirmed that patients affected by CGD present lower frequencies of memory B cells9,10 and higher frequencies of naive B cells. In addition, we show that both naive and memory B cells from these patients present a lower ability to proliferate and differentiate into ASCs in vitro on stimulation singularly directed to TLR9 or BCR. Such impairment is related to a suboptimal capacity of these patients to maintain long-term memory responses, as revealed by the dissection of measles-specific antibody responses.

These data are in contrast to a previous report24 in mice with g91phox deficiency, suggesting that ROS production is inversely related to BCR proliferation. On the other hand, our results support previous observations that ROS activity can enhance protein tyrosine kinase by inhibiting protein tyrosine phosphatase activity after BCR stimulation.25,27

FIG 5. Patients with CGD not producing measles-specific ASCs have lower memory and higher naive B cells. Among patients with CGD, 2 groups were created according to numbers of ASCs for measles. Responders and nonresponders have been selected according to the number of ASCs/106 cells greater than or less than the mean plus SD. The nonparametric Mann-Whitney test was used to analyze data. Box plots show means and SEMs. Na, Naive cells; Rem, resting memory cells; Tr, immature transitional cells.
that H$_2$O$_2$ plays a critical role in the activation of the antigen receptor on lymphocytes because this signaling cascade requires not only activation of kinases but, more importantly, inhibition of phosphatases that are critically regulated by H$_2$O$_2$. This evidence has been confirmed on B cells from mutant mice for HVCN1 or Ncf1, thus emulating the alteration of NADPH oxidase activity of patients with CGD. Such an experimental model showed altered B-cell function and proliferative ability. Our results show that ROS production in B cells after PMA stimulation is lower among patients with CGD compared with HCs. Thus we present evidence for an intrinsic decrease in total NADPH activity in B cells; a caveat in our findings is the limitation of available methods to directly link ROS production to B-cell signaling rather than to proliferation.

In conclusion, we report that B cells from patients with CGD show an altered proliferation and differentiation into specific ASCs on single stimuli in parallel to a reduced capacity to maintain long-term serologic memory. In addition, patients with CGD with lower values of specific ASCs showed common B-cell phenotypic characteristics, such as low memory and high naive B-cell percentages. Hence this evidence could guide clinicians in performing more specific B-cell assays in patients presenting with such a B-cell phenotype and in evaluating these patients as eligible for a personalized immunization schedule.

It is still unclear why specific stimuli, singularly directed to the BCR or TLR9 in patients with CGD, elicit lower proliferation compared with HCs and why partial recovery is seen on combined stimulation with TLR9 and BCR. Future efforts are needed to find out whether this is due to a higher percentage of naive B cells in patients with CGD compared with HCs or whether this is due to a direct role of ROS production deficiency because of the NADPH oxidase defect, which in turn affect the specific single stimuli of BCR and TLR9 expression. A deeper knowledge of how NADPH oxidase affects B-cell function could shed light on the mechanisms underlying long-term memory maintenance. This evidence might play a key role in the development of new vaccination adjuvants and the design of new survey models and revaccination programs to maintain a
long-term protective memory in patients affected by immune deficiencies.

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Key messages

- Patients with CGD present an altered cellular distribution in the B-cell compartment.
- Combined stimuli to BCR and TLR9 in patients affected by CGD are required to elicit an efficient B-cell proliferation and ASC differentiation.
- There are lower memory and higher naive B-cell counts related to impaired long-term memory in patients with CGD.

REFERENCES

FIG E1. Proliferation of memory and naive B cells. Representative gates (A) and graphs of gated memory (B) and naive (C) B cells are shown. The Mann-Whitney test was used to analyze data. Means and SDs are shown in the graphs. CFSE, Carboxyfluorescein isothiocyanate; SSC, side scatter.
FIG E2. ROS production in gated and sorted B cells and myelocytic cells. A, Median fluorescence intensity of gated and sorted B cells are shown in unstimulated cells and after PMA stimuli. B, Modulation of ROS in myelocytic cells and B cells of patients with CGD and HCs. *DCFDA, 2',7'-Dichlorodihydrofluorescein diacetate.