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 gp91^{phox} 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 gp91^{phox} 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 measles-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.
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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 gp91^{phox}, which is structurally stabilized by p22^{phox} protein, and the regulatory subunits p47^{phox}, p40^{phox}, p67^{phox}, 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).

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Abbreviations used
Anti-Ig: $F(ab')_2$ fragment goat anti-human IgA+IgG+IgM
ASC: Antibody-secreting cell
BCR: B-cell receptor
CGD: Chronic granulomatous disease
HC: Healthy control subject
NADPH: Nicotinamide adenine dinucleotide phosphate
PMA: Phorbol 12-myristate 13-acetate
PWM: Pokeweed mitogen
ROS: Reactive oxygen species
TLR: Toll-like receptor

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.[14] 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 12 and 15 month of age and at 5 years of age.

Flow cytometric analyses

PBMCs were obtained by using density gradient centrifugation with Ficoll-Hypaque EDTA (Amersham Pharmacia Biotech, Little Chalfont, United Kingdom). Control samples from healthy volunteers were analyzed concurrently with experimental samples. PBMCs were stained, as previously described.[15] In all experiments B-cell subsets were identified based on $CD19^+$ expression. Other antibodies included were CD21, CD27, IgD, CD38, CD10, CD3, CD24, and CD27, all from BD (Franklin Lakes, NJ). All antibodies were previously titrated. Viable lymphocytes were identified with Live/Dead violet stain (Invitrogen, Carlsbad, Calif). Samples were analyzed on an LSRFortessa (BD) with Diva software. Data analysis was performed with FlowJo software (Tree Star, Ashland, Ore).

Proliferation assays

Total PBMCs were labeled for 7 minutes at 37°C with 0.25 μmol/L carboxyfluorescein succinimidyl ester (Invitrogen), according to the manufacturer's protocol. Cells were resuspended at a concentration of 2.5×10^5 cells per well and cultured at 37°C in the presence of 2.5 μg/mL $F(ab')_2$ Fragment Goat Anti-Human IgA+IgG+IgM (anti-Ig; Jackson ImmunoResearch, West Grove, Pa), 10 μg/mL pokeweed mitogen (PWM; Sigma, St Louis, Mo), and 5 μg/mL CpG-DNA ODN 2006 (CpG; Hycult Biotech, Plymouth Meeting, Pa) mixed in different combinations. After 5 days of culture, cells were resuspended; labeled for CD19 or CD19, CD27, CD10, IgD, and Live/Dead Violet stain; and analyzed by using flow cytometry on an LSRFortessa (BD) with Diva 6.x software. Cells per well (5×10^4) were acquired by using flow cytometry. An average of 68% recovery was found among the samples, with no significant differences between the different conditions. Data analysis was performed with FlowJo software (Tree Star).

ELISpot assay

PBMCs were stimulated with 10 μg/mL PWM and 5 μg/mL CpG for 5 days. ELISpot 96-well filtration plates (MSIPS4510; Millipore, Temecula, Calif) were precoated with either 0.5 μg of anti-IgG (109-006-088; Novakemi, Handen, Sweden) or 2 μg per well of measles antigen and subsequently loaded with 3×10^5 cells per well. Plates were then processed, as previously described,[16] and analyzed with an ELISpot reader. The frequency of memory B cells was calculated as related to total PBMCs. The ability of memory B cells to differentiate into ASCs in response to BCR stimulation, TLR9 stimulation, or both was evaluated, as previously described.[15]

Detection of ROS in B cells

Total human PBMCs or purified B cells were resuspended at a concentration of 2.5×10^5 cells per well and incubated for 30 minutes at 37°C in the presence or absence of phorbol 12-myristate 13-acetate (PMA; 1 μmol/L). Cells were further incubated for 45 minutes at 37°C in the dark with 0.5 μmol/L 2′,7′-dichlorodihydrofluorescein diacetate (Invitrogen) and labeled with anti-CD19 (BD). ROS emission was measured with a FACSCanto (BD). Purified B cells were obtained by means of negative selection, according to the manufacturer's protocol (MACS System; Miltenyi Biotec, Bergisch Gladbach, Germany). After isolation, the purity of $CD19^+$ cells was greater than 95%.

Quantification of measles-specific plasma antibodies

Plasma antibody titers against measles were measured with the Enzygnost Anti-measles Virus/IgG ELISA kit (Dade Behring, Deerfield, Ill), according to the manufacturer's instructions. The protective threshold was set at 0.12 IU/mL for measles.[17]

$gp91^{phox}$ Expression on B cells of CGD carriers

Total PBMCs of 4 patients, their mothers, and HCs were labeled with anti-human $gp91^{phox}$ (Medical and Biological Laboratories, Nagoya, Japan), CD19-allophycocyanin and CD14-phycoerythrin (BD) were added, and the cells were incubated for 20 minutes at 4°C, washed, and analyzed on FACSCanto II (BD).

Statistics

The Mann-Whitney and Wilcoxon matched-pairs signed-rank tests were used to compare median values between patients and HCs by using GraphPad Prism software (GraphPad Software, La Jolla, Calif).

RESULTS

Characterization of B-cell subsets in patients with CGD

Whether the deficit in NADPH oxidase characterizing patients with CGD can alter the development and function of B cells remains not fully explored. Comparison of patients with CGD with sex- and age-matched HCs revealed significantly lower percentages of switched memory B cells ($CD19^+CD27^+IgD^-$) in patients with CGD ($P = .041$; Fig 1, *B*). In contrast, numbers of naive B cells ($CD19^+CD27^-IgD^+$) 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, *C*). In addition, higher frequencies of