European Journal of Immunology

Enhanced inflammatory responses of chronic granulomatous disease leukocytes involve ROS-independent activation of NF-κB

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Reactive oxygen species (ROS) generated by the cellular NADPH-oxidase are crucial for phagocytic killing of ingested microbes and have been implicated as signaling molecules in various processes. For example, ROS are thought to be involved in activation of the transcription factor NF-κB, central for mediating production of proinflammatory cytokines in response to inflammatory stimuli. Several studies have demonstrated that inhibitors of the NADPH-oxidase interfere with NF-κB activation and production of proinflammatory cytokines. Curiously, patients with chronic granulomatous disease (CGD), an immunodeficiency characterized by an inability to produce ROS, are not only predisposed to severe infections, but also frequently develop various inflammatory complications indicative of exaggerated inflammatory responses. Here, we show that human CGD leukocytes display a hyperinflammatory phenotype with increased production of proinflammatory cytokines in response to stimulation with Toll-like receptor agonists. The hyperinflammatory phenotype was also evident in mononuclear cells from CGD mice ($gp91^{phox-/-}$), but not in control cells in the presence of NADPH-oxidase inhibitor diphenyleneiodonium, probably reflecting NADPH-oxidase-independent effects of the inhibitor. Furthermore, we show that the major steps involved in NF-κB activation were intact in human CGD cells. These data indicate that ROS were nonessential for activation of NF-κB and their production may even attenuate inflammation.

Received 29/8/06
Revised 6/12/06
Accepted 24/1/07

[DOI 10.1002/eji.200636651]

Key words: Immunodeficiencies · Inflammation · NF-κB pathway

Introduction

Chronic granulomatous disease (CGD) is a very unusual, primary immunodeficiency affecting approximately 1/250 000 births; the affected individuals are hypersusceptible to a narrow range of bacterial and fungal pathogens, most notably *Staphylococcus aureus*, *Burkholderia cepacia* complex and various *Aspergillus* species [1]. The underlying defect is caused by one of several mutations in genes that encode different

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Abbreviations: **CBA:** cytometric bead array · **CL:** chemiluminescence · **CGD:** chronic granulomatous disease · **DPI:** diphenyleneiodonium · **EthD-1:** ethidium homodimer · **p65i:** p65 inhibitor peptide · **PGN:** peptidoglycan

subunits of the NADPH-oxidase, which is responsible for production of ROS that are necessary for full antimicrobial activity of phagocytes. In addition to being hypersusceptible to infection, CGD patients are also predisposed to a number of different inflammatory complications. It has been argued that these complications are secondary to failure to completely eradicate various infections, but in fact many of the inflammatory symptoms occur in the absence of any obvious infectious antecedent and the inflammatory sites are often sterile [2–4]. Two murine CGD models exist, each of which is characterized by an inability to eradicate infecting microbes as well as by exuberant inflammatory responses upon experimental challenge [5–7]. These observations in humans and mice lacking a functional NADPH-oxidase suggest that ROS play a critical role in inflammatory regulation. Furthermore, ROS have been implicated as signaling molecules of importance in a variety of cell signaling systems. Given the fact that ROS are, per definition, highly reactive and can alter a number of key organic structures (DNA, proteins, lipids, *etc*), their involvement in signaling appears to be logical, although the details are obscure.

The best-characterized proinflammatory signaling pathway resulting in transcription of inflammatory genes, involves activation of the transcription factor NF-κB. This pathway is typically activated through ligation of genome-encoded receptors such as the TLR that recognize conserved microbial signatures [8]. The NF-κB family is composed of five different members/subunits - p50, p52, p65, c-Rel and RelB [9]. In resting cells, NF-κB heterodimers are present in the cytoplasm, where they are bound to the inhibitory protein I-κBα. Upon activation, I-κBα is phosphorylated and eventually degraded by ubiquitination, releasing the NF-κB subunits that become available for nuclear translocation. Once within the nucleus, NF-κB binds to specific NF-κB consensus sequences in the genome and initiates gene transcription [10]. Activation of NF-κB is generally regarded as a proinflammatory event and this transcription factor is considered to be subject to redox regulation [11]. Recent reports imply that NADPH-oxidase-derived ROS are directly involved in the activation of NF-κB [12–15]. These published reports, suggesting that lack of ROS production leads to decreased NF-κB activation and subsequently to decreased inflammatory responses, are difficult to reconcile in the context of the gross inflammatory phenotypes displayed by both CGD patients [3, 4] and mice [5, 6].

We investigated the role of NADPH-oxidase-derived ROS in the context of NF-κB-driven cytokine production by using PBMC from CGD patients with mutations in $gp91^{phox}$ (X-linked CGD). We found that CGD cells exhibited a hyperinflammatory phenotype and produced significantly more IL-6 and TNF-α than control cells upon stimulation with a variety of substances, including the bacterial-derived TLR agonists LPS and peptidoglycan (PGN). We also show that NF-κB signaling in response to LPS was intact in human CGD cells, indicating that ROS derived from the NADPH-oxidase were nonessential for NF-κB activation. Instead, the lack of a functional NADPH-oxidase conferred exaggerated production of proinflammatory cytokines, which implies a role for ROS in dampening inflammation.

Results

CGD leukocytes produce increased amounts of proinflammatory cytokines after stimulation

We stimulated PBMC from CGD patients and healthy controls with a battery of well-characterized proinflammatory substances, including ultra-purified *Escherichia coli* LPS, and measured the release of cytokines IL-6 and TNF-α after 20 h of culture. IL-6 production from CGD cells was significantly increased compared to control cells, for all the stimulators used except IL-1β (Fig. 1A); IL-6 production in response to this stimulus was very low in general compared to the responses to, *e.g*. LPS or peptidoglycan (PGN). Control cells produced only minute amounts of IL-6 in response to IL-1β and CGD cells, again, displayed an enhanced response. The hyperresponsiveness of CGD cells was also apparent in terms of TNF-α production (Fig. 1B), indicating that the lack of ROS did not inhibit the production of proinflammatory cytokines, but instead led to exaggerated proinflammatory responses.

Several previous reports have argued that ROS are needed for proper NF-κB-driven cytokine production. Most of these studies have employed various antioxidants or NADPH-oxidase inhibitors to abrogate ROS; in the presence of these agents NF-κB activation was blocked [16–18] and cytokine production diminished [12, 19, 20]. In the presence of diphenyleneiodonium (DPI; a NADPH-oxidase inhibitor), a marked decrease in TNF-α production after stimulation was recorded (Fig. 1C). This was consistent with previously published data, but not with the hyperresponsive phenotype of CGD cells, indicating that this inhibitor did not replicate the conditions of CGD cells, at least with respect to cytokine production.

Enhanced proinflammatory cytokine responses of murine CGD spleen cells

As opposed to inbred strains of mice, the human population has substantial genetic variability, such that relatively high inter-individual variations can be antici-

pated. To ascertain whether the absence of a functional NADPH-oxidase was the reason behind the hyperinflammatory phenotype seen for human CGD cells, we performed experiments using mononuclear spleen cells from gp91$^{phox-/-}$ mice and age- and gender-matched WT mice of isogenic background (CB57BL/6J). As was the case for the human CGD cells, the murine CGD cells responded to stimulation with significantly higher production of proinflammatory cytokines than WT cells (Fig. 2A and B). In general, LPS and PGN were very poor stimuli for cytokine production from murine cells, but IL-6 production from CGD cells was still enhanced compared to WT cells in response to these stimuli (data not shown). Interestingly, a particularly potent inflammatory stimulus for murine CGD cells was formalin-killed *S. aureus* (Fig. 2A and B), a bacterial pathogen to which CGD patients are especially susceptible. As the mice used in these experiments were genetically identical, except for the gp91^{phox} gene, the data strongly suggested that the hyperinflammatory phenotype was indeed due to the absence of a functional NADPH-oxidase. We also monitored cellular survival in the presence or absence of stimulation and found no significant differences between CGD and WT cells (Fig. 2C), indicating that the hyperinflammatory phenotype of CGD cells was not due to increased cell survival in culture.

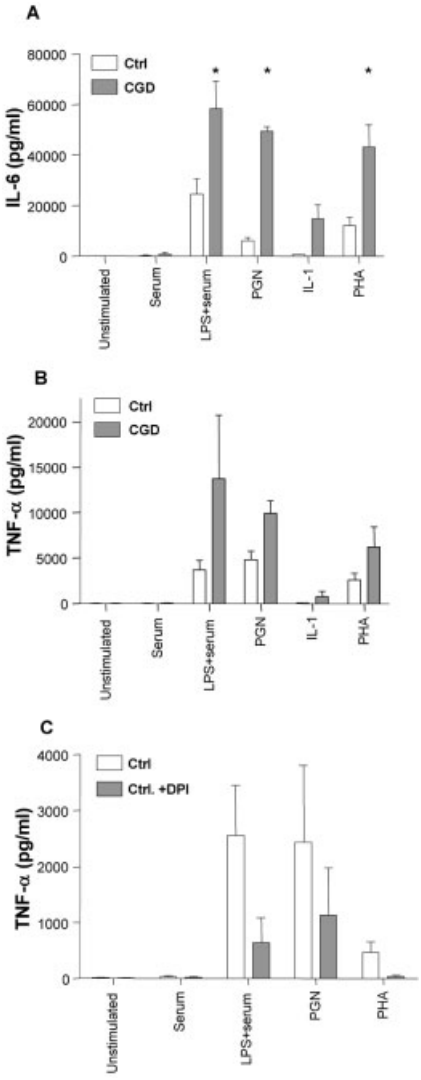

Figure 1. Hyperinflammatory phenotype of CGD cells. PBMC from healthy controls (open bars) and CGD patients (filled bars) were cultured in the presence of medium (unstimulated), human AB serum (1%), LPS (100 ng/mL in the presence of 1% serum), PGN (10 μg/mL), IL-1β (250 ng/mL) or PHA (10 μg/mL) for 20 h after which the cell-free supernatants were analyzed by ELISA. CGD cells responded to stimulation by producing more IL-6 (A) and TNF-α (B). In a separate series of experiments, control PBMC were preincubated in the presence (filled bars) or absence (open bars) of DPI (10^{-5} M) for 45 min before stimulation as above. After 20 h of incubation, cell-free supernatants were analyzed for TNF-α content by ELISA; DPI inhibited cytokine production (C). Shown are the mean + SD of three-to-four independent experiments. Asterisks denote statistically significant differences between control and CGD cells ($p < 0.05$).

ROS production

To test if the stimuli used were able to induce ROS production in WT cells, we employed a sensitive luminol-enhanced chemiluminescence system (CL) [21]. Spleen cells from WT mice produced measurable levels of ROS when equilibrated (in the absence of stimulation) at 37°C; these levels were clearly enhanced upon stimulation with CpG (Fig. 2D). Cells from CGD mice on the other hand produced no ROS at all and the light emission (reflecting superoxide production) from these cells was indistinguishable from light emission in the absence of cells (not shown). These data indicated that WT cells produced ROS in response to CpG and confirmed the need for gp91^{phox} for ROS production. We also corroborated these findings with human PmBC, comparing cells from one healthy control donor with cells from one CGD patient. In a similar manner, the control cells produced clearly detectable levels of ROS, whereas the CGD cells were completely devoid of ROS production regardless of stimulation (not shown).

Cytoplasmic phosphorylation and degradation of I-kBα

Next, we investigated how the NF-κB signaling pathway was executed in the absence of ROS by subjecting PBMC from CGD patients or healthy controls to LPS stimulation. After stimulation, cells were harvested at different time-points and cytoplasmic and nuclear extracts were prepared. Cytoplasmic extracts from stimulated PBMC were immunoblotted using a monoclonal antibody directed against the phosphorylated form of I-κBα;

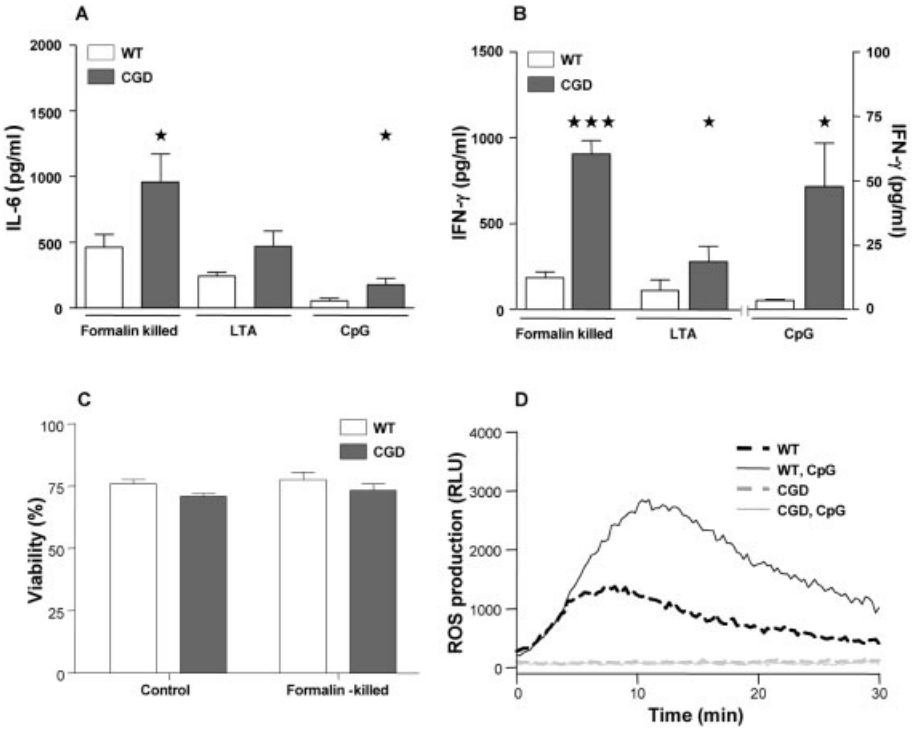

Figure 2. Hyperinflammatory phenotype and the absence of ROS production of murine gp91$^{phox-/-}$spleen mononuclear cells. Mononuclear cells from WT (open bars) or CGD (filled bars) mice were cultured in the presence of formalin-killed *S. aureus* (MOI 1:25), lipoteichoic acid (2 μg/mL) or CpG-containing oligodeoxynucleotides (3.3 μM) for 24 h after which cell-free supernatants were analyzed for IL-6 (A) or IFN-γ (B) using CBA. (C) Viability of WT (open bars) and CGD (filled bars) cells in the presence or absence (control) of stimulation with formalin-killed *S. aureus* for 24 h was analyzed by flow cytometry and the cell-impermeable nuclear dye EthD-1 that only permeates dead cells. (D) Spleen cells from WT (black lines) or CGD (gray lines) were stimulated with buffer (background; dotted lines) or CpG (3.3 μM; solid lines) and the generation of ROS was followed by luminol-enhanced CL. Shown are representative experiments (including cells from four mice of each type) that were repeated independently at least four times (A and B), mean + SD ($n = 4$; C), and a representative experiment repeated four times (D). Asterisks denote statistically significant differences between control and CGD cells ($^{*}p < 0.05$; $^{***}p < 0.0005$).

phosphorylation occurred as early as 20 min after LPS stimulation, peaked at 40 min and then declined (Fig. 3) as anticipated given that phosphorylation marks I-κBα for ubiquitination and proteasomal degradation [22]. CGD cells tended to display weak phosphorylation of I-κBα even in the absence of stimulation, possibly indicative of enhanced resting levels of NF-κB activity, but in terms of magnitude- and kinetics of phosphorylation, CGD cells responded in a similar manner as the control cells. Both normal and CGD cells degraded cytosolic I-κBα upon LPS stimulation with a slightly more robust degradation displayed by CGD cells (Fig. 3). These data indicated that NADPH-oxidase-derived ROS were not needed for phosphorylation and degradation of I-κBα in human PBMC. Degradation of I-κBα and subsequent activation of NF-κB appeared to occur *via* the normal ubiquitination proteasome pathway since production of both IL-6 and TNF-α from control and CGD cells was completely abrogated in the presence of proteasome inhibitor MG-132 (not shown).

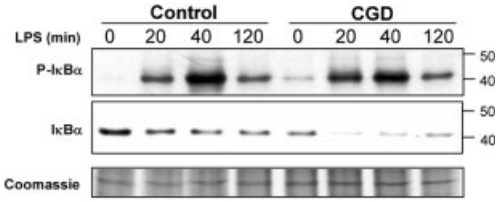

Figure 3. Phosphorylation and degradation of cytoplasmic I-κBα in response to LPS. PBMC from healthy controls and CGD patients were equilibrated at 37°C for 30 min, stimulated with LPS (100 ng/mL in the presence of 1% serum) for indicated periods of time and cytoplasmic and nuclear fractions were prepared. The cytoplasmic fractions (14 μg/sample) were immunoblotted with antibody directed against the phosphorylated form of I-κBα (upper panel), followed by blotting with antibody against total I-κBα (middle panel) after stripping of the membranes. CGD cells displayed slightly elevated resting levels of I-κBα phosphorylation and responded to stimulation by a more pronounced degradation of this protein. Equal loading was ascertained by Coomassie Blue staining of the membranes (lower panel). Representative blots from four different experiments are shown.

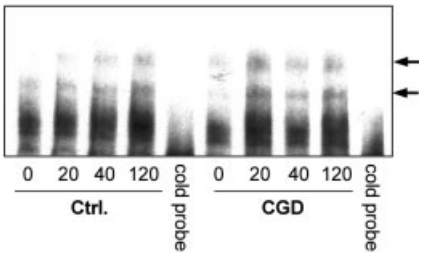

Figure 4. Induction of NF-κB DNA binding by LPS. Nuclear extracts (5 μg) from PBMC stimulated with LPS for the indicated times were subjected to EMSA using a biotin-labeled NF-κB consensus sequence oligonucleotide as a probe. CGD extracts exhibited slightly higher NF-κB-binding activity than control extracts in the absence of stimulation and the activity was markedly increased upon stimulation. Addition of excess unlabeled NF-κB probe (cold probe) was used as a competitor (added to the LPS samples treated for 40 min), to determine the specificity of DNA binding. NF-κB-specific complexes, which were out competed by excess cold probe, are indicated by arrows; one representative experiment of four independent experiments is shown.

Nuclear translocation of NF-κB-binding proteins

After nuclear translocation, NF-κB subunits bind promoter elements of various proinflammatory genes that contain the NF-κB consensus sequence [10]. Using EMSA, we assayed nuclear extracts from human PBMC for NF-κB binding activity, both before and after LPS stimulation. In control cells, NF-κB-binding activity was induced 20 min after LPS stimulation, and the binding continued to increase for up to 120 min (Fig. 4). In CGD cells, some NF-κB-binding activity was evident even in the absence of stimulation. This activity was, however, markedly increased upon LPS stimulation in a manner similar to that seen for control cells (Fig. 4). Specificity of the NF-κB binding was demonstrated by performing the reaction in the presence of excess unlabeled NF-κB probe (cold probe), which completely abolished the signals, from both control and CGD cell nuclear extracts.

Nuclear translocation of NF-κB p50

The NF-κB species most heavily implicated in proinflammatory responses is the p50/p65 heterodimer. The EMSA showed specific binding of NF-κB to consensus sequences, but did not distinguish between the various NF-κB subunits. To ascertain whether p50 was translocated into the nucleus upon LPS stimulation, we performed immunoblotting of nuclear extracts using an antibody directed to this subunit. Minor background levels of p50 were detected in the nuclei of resting cells, but the signal was robustly increased after LPS stimulation (Fig. 5A). We also subjected nuclear extracts to an NF-κB p50 ELISA in order to quantify p50 translocation and binding activity. Accumulation of

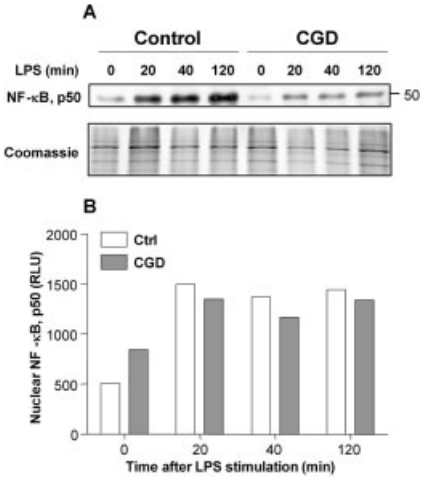

Figure 5. Nuclear translocation of NF-κB p50 subunit. PBMC from healthy controls or CGD patients were stimulated with LPS for the indicated times and nuclear extracts (9 μg) were subjected to immunoblotting (A) with an antibody against the NF-κB p50 subunit (upper panel). The kinetics of NF-κB p50 translocation was similar for control and CGD cells. Equal loading was ascertained by Coomassie Blue staining of membranes after stripping (lower panel); one representative blot from four independent experiments is shown. Nuclear extracts from healthy controls (open bars) or CGD patients (filled bars) were subjected to ELISA to quantify the levels of NF-κB p50 present in the nucleus at the indicated time-points after LPS stimulation. Similar amounts of NF-κB p50 were present in nuclear extracts from control and CGD cells (B). The graph depicts mean values from two independent experiments.

nuclear p50 occurred in a very similar fashion in both control and CGD cells (Fig. 5B), indicating that both translocation and DNA binding of p50 was unimpeded in the absence of ROS.

Translocation of p65 is needed for the production of proinflammatory cytokines

Only three out of five NF-κB members, *i.e.* p65, c-Rel and RelB, contain the transactivation domains needed to form transcriptionally active DNA complexes [23]. Thus, p50 needs to partner with a transactivation domain-containing component in order to activate gene transcription. We employed a cell-permeable p65 inhibitor peptide (p65i), which has been shown to inhibit specifically NF-κB activation [24]. In the presence of p65i, LPS-induced production of both IL-6 (Fig. 6A) and TNF-α (Fig. 6B) was markedly decreased in control and CGD cells. At the concentration used (100 μM), the control peptide only modestly decreased cytokine production, indicating that the inhibitory action of p65i was not due to cytotoxic/unspecific effects and that regardless of the presence of ROS, LPS-induced production of IL-6 and TNF-α required phosphorylation and translocation of NF-κB p65.

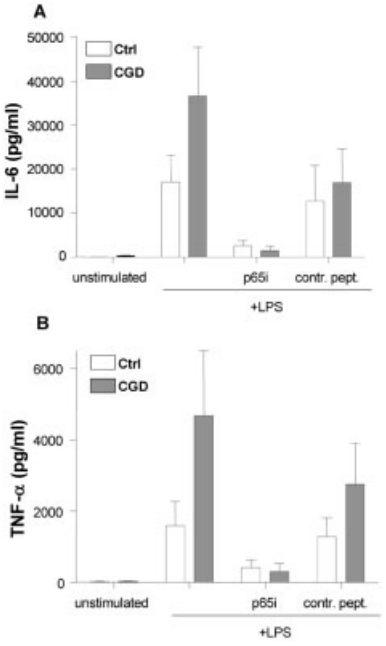

Figure 6. Involvement of NF-κB p65 in the hyperinflammatory responses. PBMC from healthy controls (open bars) and CGD patients (filled bars) were preincubated for 1 h in the presence of medium, p65i (100 μM) with a decoy phosphorylation site corresponding to Ser276 on NF-κB p65, or the control peptide (100 μM) that lacks the Ser276 site. The cells were then stimulated with LPS (100 ng/mL in the presence of 1% serum) for 24 h in culture and the cell-free supernatants were analyzed using the CBA for IL-6 (A) and TNF-α (B). In both control and CGD cells, p65i abolished cytokine production in response to LPS, whereas the control peptide only slightly decreased the responses. The graphs show mean + SEM of three independent experiments performed in triplicate.

Discussion

CGD is a very rare disorder caused by an inability of the affected individuals' leukocytes to produce ROS. This inability is due to genetic defects in genes encoding the NADPH-oxidase; as a result, CGD patients frequently face problematic infections with a narrow range of fungal and bacterial pathogens [1]. Regardless of the exact mechanism behind ROS-mediated microbial killing, the lack of ROS production from CGD phagocytes confers defective phagocytic killing [25], resulting in an increased susceptibility to infection. In addition to predisposition to life-threatening infection, CGD patients suffer from an array of inflammatory complications, most prominently granuloma formation in hollow viscera, inflammatory bowel disease, progressive sterile pulmonary inflammatory disease, and lupus-like syndromes in patients and their obligate heterozygous mothers [4]. To date, a convincing explanation for the exaggerated inflammatory responses seen in CGD patients is lacking, and the notion that the inflammatory state stems from sub-clinical infections (due to the decreased antimicrobial action) cannot explain, for example, why granulomas are typically sterile [3, 4]. Functionally, CGD cells have been shown to display prolonged intracellular calcium transients upon stimulation, leading to exaggerated cellular responses [26]. ROS have also been implicated in numerous processes of potential importance for the inflammatory conditions displayed by CGD patients: neutrophil cell death (both apoptosis [27] and necrosis [28]), secondary clearance of apoptotic cells [29, 30], and functional down-regulation of immunocompetent lymphocytes [31]. In addition, rats with decreased capacity to produce ROS display increased susceptibility to autoimmune arthritis [32]. Very recent data showing that ROS may dampen the reactivity of neighboring T cells suggests an intriguing mechanism whereby ROS production could quench autoimmune responses by decreasing the number of reduced thiol groups on T cell surfaces [33]. Future studies will hopefully determine how these ideas relate to the hyperinflammatory phenotype of human CGD.

Several inflammatory pathways converge on the transcription factor NF-κB, *e.g.* those triggered by recognition of conserved microbial signatures by genome-encoded TLR [34]. As ROS are able to react with, and alter, most types of biomolecules, they have been implicated in a variety of cell-signaling processes including NF-κB activation [11]. The role of ROS in NF-κB activation has been studied using a variety of techniques, *e.g.* a murine model for CGD [15], or cells in the presence of various antioxidants/NADPH-oxidase inhibitors [12, 13, 19, 35]. These reports suggest that a lack of ROS production leads to diminished NF-κB activation and thus decreased inflammatory responses, *i.e.* a fundamentally different scenario from that seen in CGD patients. Here, we show that leukocytes from CGD patients are hyperresponsive to inflammatory stimulation and produce more proinflammatory cytokines than cells with a functional NADPH-oxidase. We also show that DPI, an inhibitor of the NADPH-oxidase, failed to confer the hyperinflammatory phenotype of CGD cells and is thus a poor tool for studying the role of ROS in inflammatory signaling. This is in line with data describing ROS-independent effects of antioxidants and NADPH-oxidase inhibitors [36, 37] and points to the importance of interpreting data generated with such substances with great caution. Because CGD is such a rare condition and CGD patients are not readily available, many investigators employ CGD mice to study the role of NADPH-oxidase derived ROS. A critical role for ROS in the activation of NF-κB was recently proposed, whereby $p47^{phox\ -/-}$ CGD mice were unable to activate NF-κB to the same extent as mice with a functional NADPH-oxidase upon intratracheal infection with *Pseudomonas aeruginosa* [15]. Furthermore, the

CGD mice had markedly lower levels of TNF-α in their lungs after infection and were impaired in bacterial clearance. Our contrasting data, showing dramatically enhanced production of proinflammatory cytokines from murine CGD mononuclear spleen cells, were generated using standardized *in vitro* conditions whereas the previous study employed a complex *in vivo* model of infection, making direct comparisons difficult. It should also be noted that the gp91$^{phox-/-}$ murine model used here differ from the p47^{phox}-deficient mouse employed by Sadikot *et al*. [15]. These different mouse strains represent the two most common genetic deficiencies among human CGD patients (gp91^{phox} being the X-linked form of CGD and p47^{phox} representing an autosomal recessive form), and differences in the severity of symptoms between these patient groups have been reported [1]. It should be noted that the murine spleen cell preparations used in our study were not identical to the human PBMC preparations in terms of cellular composition. Murine spleen cell preparations represent a well-established mixed leukocyte system and in both the murine and human systems, lymphocytes were the dominating cell type; no significant differences in cell composition were found between WT/control and CGD preparations. Our data also show that cells from the gp91^{phox}-deficient mice were completely devoid of ROS production, regardless of stimulation, whereas WT cells produced ROS both spontaneously and in response to stimulation.

Relatively few studies have been published on the inflammatory responses of human CGD cells, probably due to the scarcity of CGD patients. The available reports invariably describe increased inflammatory reactions in CGD cells/patients [3, 38–40], and a recent microarray study described up-regulation of various proinflammatory genes in unstimulated CGD cells compared to controls [41]. Thus, the data presented in our work are consistent with these published studies and suggest that ROS exhibit a dampening effect on inflammatory reactions, which fits very well with clinical data on exuberant inflammatory responses in CGD patients [4, 42, 43]. The level at which this dampening occurs and the mechanistic details behind the phenomenon remains to be elucidated.

The hyperinflammatory phenotype of CGD leukocytes was evident regardless of the nature of the stimulus, indicating general dysregulation rather than an abnormality in a specific activation pathway and/or a specific cell type. A plausible explanation to the increased cytokine production displayed by CGD cells could be that these cells survived better than WT cells in culture. In contrast, CGD cells were slightly less viable than WT cells after 24 h in culture, although the difference was not statistically significant. These data clearly indicated that the exaggerated cytokine production displayed by CGD cells *in vitro* was not due to increased survival in culture. Although we could not detect any profound differences in the abilities of CGD cells to execute the various steps involved in NF-κB activation, other ROS-dependent cell signaling systems might be involved in the hyperresponsiveness of CGD cells. One such system is the pathway that involves AP-1, a dimeric transcription factor that is implicated in inflammatory signaling and has recently been connected to inflammatory complications such as psoriasis and arthritis [44]. Evidence suggests that AP-1 is redox-sensitive [45] and can be activated by ROS [46]. Whether AP-1 activation is somehow defective or abnormal in CGD cells has, to our knowledge, never been investigated.

Our data clearly show that CGD cells are competent to activate the NF-κB pathway in the absence of ROS and present a general hyperinflammatory phenotype in response to multiple different stimuli, *e.g*. TLR stimulation. Although at present we can only speculate on the underlying reason for the hyperinflammatory phenotype of CGD cells, it seems obvious that the lack of ROS as microbicidal effectors is only part of the explanation for CGD pathology.

Materials and methods

Patients and isolation of PBMC

Peripheral blood was obtained from healthy volunteers and four male gp91^{phox}-deficient patients (X-linked CGD). None of the patients was infected at the time of the experiments and blood from one patient was analyzed in detail; white blood cell count, red blood cell count, as well as differential counts for granulocytes, lymphocytes, monocytes, eosinophils and basophils were all within normal range (not shown). All of the donors provided informed consent and the study was conducted according to UBC Clinical Research Ethics protocol C04–0193.

PBMC were isolated by mixing peripheral blood at a ratio of 1:1 with RPMI 1640 supplemented with 10% FCS, 2 mM glutamine and 1 nM sodium pyruvate (R10 media; all from Invitrogen, Burlington, Ontario, Canada) in an endotoxin-free bottle. This solution was then overlayed on Ficoll-Paque Plus (Amersham Pharmacia Biotech, Baie D' Urfe', Quebec, Canada) and centrifuged (1500 rpm) at room temperature. The resulting PBMC layer, consisting mainly of lymphocytes (T cells, B cells and NK cells) and monocytes, was washed twice in PBS and the cells resuspended in R10 media. In some instances, the PBMC preparations were further characterized by flow cytometry and cellular compositions were always within the normal range as reported [47, 48].

Cytokine assays

PBMC in R10 medium at 2×10^6/mL were equilibrated in a 96-well plate and incubated at 37°C in 5% CO_2 for 30 min before starting the experiments. Stimulation was achieved by the addition of the following: R10 (unstimulated); 100 ng/mL ultra-pure *E. coli* LPS (Invivogen, San Diego, CA) in the presence of 1% human AB serum (Sigma Chemical, St. Louis, MO) as a source of LPS-binding protein; 10 μg/mL PGN (Invivogen); 250 ng/mL IL-1β (Research Diagnostics, Flanders, NJ) or 10 μg/mL PHA (Sigma). After 20 h of culture, supernatants were collected and stored at –80°C until evaluated by ELISA (BD Biosciences, Mississauga, Ontario, Canada) according to the manufacturer's instructions. When DPI (Sigma) was used, the cells were preincubated with this agent (10^{-5} M) for 45 min before stimulation. When MG-132 (Calbiochem, San Diego, CA) was used, the cells were pretreated with this proteasome inhibitor (5 μM) for 30 min before stimulation.

In certain experiments, PBMC were preincubated with 100 μM of p65i, 100 μM control peptide [NF-κB p65 (Ser276) Inhibitory Peptide set; Imgenex], or medium 60 min before stimulation. After 24 h, the supernatants were collected and frozen at –80°C. These samples were analyzed using the Cytometric Bead Array (CBA) Human Inflammation Kit (BD Biosciences) as outlined in the manufacturer's instructions. Following data acquisition on a BD FACSCalibur (BD Biosciences) flow cytometer, the concentration of each cytokine in the samples and standards was determined using the BD CBA software.

Stimulation and preparation of cellular extracts

PBMC in R10 media at 10^7/mL were equilibrated at 37°C in 5% CO_2 for 30 min before starting the experiments after which 100 ng/mL LPS in the presence of 1% human AB serum as a source of LPS-binding protein, was added. At the indicated time points, the samples were placed on ice, washed in ice-cold PBS and cytoplasmic and nuclear extracts were obtained using the NE-PER kit (Pierce, Rockford, IL) in the presence of Complete mini, EDTA-free protease inhibitor cocktail (Roche Diagnostics, Basel, Switzerland) at 4°C. Protein content was determined using the BCA Protein Determination Kit (Pierce), and the samples were aliquoted and stored at –80°C.

Western blotting

Equal amounts of cytoplasmic or nuclear extracts were separated on 12% SDS polyacrylamide gels, transferred to PVDF membranes, and immunoblotted. Primary antibodies against phospho-I-κBα, I-κBα, and NF-κB p50, and secondary HRP-linked antibodies were obtained from Cell Signaling Technology (Beverly, MA). Visualization was conducted using CL peroxidase substrate (Sigma) and blots were routinely treated with stripping buffer (Pierce) before reprobing. Anti-phospho-antibodies were always used on un-stripped membranes. After blotting was finished, the membranes were stripped again and Coomassie Blue stained to ascertain equal loading.

NF-κB p50 ELISA

Equivalent amounts of nuclear extracts from control or CGD PBMC were analyzed for NF-κB subunit p50 content by StressXpress NF-κB p50 ELISA Kit (Stressgen Bioreagents, Victoria, BC, Canada) according to manufacturer's instructions. Luminescence was detected with SpectraFluor Plus Multifunction Microplate Reader (Tecan Systems, CA).

EMSA

Nuclear extracts (5 μg), from resting and LPS-stimulated cells, were mixed with buffer (10 mM Tris pH 7.5, 100 mM KCl, 5 mM $MgCl_2$, 1 mM DTT, 0.75% glycerol, and 2 mM EDTA), 1.8 μg of poly(dI-dC), in the presence or absence of 50 μg/mL unlabeled NF-κB probe and incubated for 20 min at room temperature. Biotin-labeled NF-κB probe (0.25 μg/mL; probe set from Panomics, Redwood City, CA) was added to all samples, which were incubated for 20 min at room temperature before addition of loading buffer and separation on 7% native polyacrylamide gels in a running buffer of 0.5 x TBE buffer (50 mM Tris pH 8.0, 45 mM boric acid, 0.5 mM EDTA). The reactions were then transferred to nylon membranes, UV-cross-linked and visualized using the LightShift Chemiluminescent EMSA Kit (Pierce) according to the manufacturer's instructions.

Mouse strains

For stimulation experiments, 8-week-old female WT (CB57BL/6J) and NADPH-oxidase-deficient ($Cybb^{-/-}$) mice, which lack the fragment of chromosome X that encodes $gp91^{phox}$ (X-linked CGD) were purchased from B&K Universal AB (Sollentuna, Sweden) and maintained in the animal facility of the Department of Rheumatology and Inflammation Research, Göteborg University. The experiments were performed with the approval of the Ethical Committee of Göteborg University.

In vitro stimulation of murine spleen mononuclear cells

Spleens were removed from mice and placed in sterile PBS on ice. Using a cell strainer (BD Falcon 70-μm nylon), the cells were filtered into 10 mL of sterile, cold PBS. The suspension was centrifuged (250 × *g*, 5 min, 4°C) and the pellet resuspended in 10 mL of cold, sterile ammonium chloride pH 6.8 to lyse residual erythrocytes. The cells were then washed three times with the sterile PBS solution and counted. This procedure repeatedly generated preparations consisting mainly of lymphocytes (85%) and monocytes/macrophages (10%) as assessed by differential cell counting and flow cytometric analysis of size and granularity (not shown). No significant differences in cell composition were noted between spleen cells from WT and CGD mice. Cells were seeded onto 24-well plates (2×10^6/well) in 1 mL of Iscove's modified Dulbecco's medium (Sigma) and incubated with formalin-killed *S. aureus* (LS-1 strain; MOI 1:25), 2 μg/mL *S. aureus* lipoteichoic acid (Invivogen) or with 3.3 μM of a completely phosphorothioated CpG motif, containing oligodeoxynucleotide (5′-TCCATGACGTTCCTGCT-3′), which was synthesized

by Scandinavian Gene Synthesis AB (Köping, Sweden). After 24 h of incubation, the supernatants were harvested and frozen at –70° for future analysis. Cells were analyzed for viability by flow cytometry using the fluorescent nuclear dye ethidium homodimer (EthD-1; 1 μM; Molecular probes) that is impermeable to viable cells. The BD CBA Mouse Inflammation Kit (BD Biosciences) was used to measure the levels of IL-6 and IFN-γ in the supernatants according to manufacturer's instructions. Analysis was performed on a FACSCalibur using the BD CellQuest and BD CBA softwares (BD Biosciences).

Production of ROS

A luminol-enhanced CL system was used with a Mithras LB940 (Berthold technologies; Bad Wildbad, Germany) plate reader and disposable 96-well plates containing 220-μL reaction mixtures. Each well contained 2×10^5 spleen cells, HRP (4 U/mL) and luminol (2×10^{-5} M) in Krebs-Ringer phosphate buffer (KRG, pH 7.3) containing glucose (10 mM), Ca^{2+} (1 mM), and Mg^{2+} (1.5 mM). The cells were transferred to 37°C, stimulated with CpG (3.3 μM), light emission recorded continuously and data are expressed as relative light units.

Acknowledgements: We thank Rebecca C. Ma and Malgorzata Kubica for outstanding technical assistance and all participating patients and their families for partaking in this study. This study was funded by grants from the Swedish Society for Medical Research, the King Gustav V:s 80-year Foundation, the Frederik & Ingrid Thuring Foundation and the Swedish Medical Research Council (JB); the Canadian Cystic Fibrosis Foundation, British Columbia Lung Association, the David Chow Memorial Fund and the Canadian Institutes for Health Research (DPS); Genome Canada, Genome BC and Genome Prairie (RH). RH is a Canada Research Chair, KB received a postdoctoral fellowship from the CIHR Translational Research in Infectious Diseases program and KM received a studentship from CCFF.

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