

Dysregulation of innate immune receptors on neutrophils in chronic granulomatous disease

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Background: Chronic granulomatous disease (CGD) is the most common inherited disorder of neutrophil function, is caused by mutations in the reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, and results in recurrent bacterial infections.

Objective: We sought to investigate the expression and function of innate immune receptors on neutrophils in patients with CGD.

Methods: We quantified mRNA and protein expression of Toll-like receptors (TLRs), complement receptors, and chemokine receptors on neutrophils from 15 patients with CGD compared with that seen in healthy control subjects (n = 15) and control patients with bacterial pneumonia (n = 15). Phagocytosis, chemotaxis, and TLR function of isolated neutrophils were analyzed. The effect of NADPH oxidase inhibition on receptor expression and function was analyzed in control neutrophils.

Results: Neutrophils from patients with CGD had lower expression levels of TLR5, TLR9, CD11b, CD18, CD35, and CXCR1 compared with those from healthy control subjects, whereas similar or increased receptor expressions were found in patients without CGD but with bacterial pneumonia. Reduced TLR5 expression resulted in impaired neutrophil activation by bacterial flagella, reduced CD11b/CD18 expression was associated with impaired phagocytosis of *Staphylococcus aureus*, and reduced CXCR1 expression was associated with decreased chemotaxis. TLR5 and CD18 expression levels correlated with disease severity in patients with CGD. TLR5 and TLR9 expression were greater in patients with residual NADPH oxidase activity. Inhibition of the NADPH oxidase in control neutrophils *in vitro* decreased TLR5 and TLR9 expression and impaired TLR5 function.

Conclusion: These results provide the first evidence that innate immune receptors are dysregulated in patients with CGD.

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Key words: Chronic granulomatous disease, neutrophils, innate immune system, nicotinamide adenine dinucleotide phosphate oxidase, Toll-like receptors, complement receptors, Toll-like receptor 5, Toll-like receptor 9, CXCR1

Chronic granulomatous disease (CGD) is the most common inherited disorder of phagocytic function and is caused by defects in the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase that produces reactive oxygen species (ROS) during the respiratory burst. Phagocytes of patients with CGD are therefore unable to kill ingested microorganisms, which results in a concomitant increased susceptibility to bacterial and fungal infections.^{1,2} The NADPH oxidase is composed of the subunits gp91^{phox} and p22^{phox}, which form the membrane-bound cytochrome b558, and the 3 cytosolic components p47^{phox}, p67^{phox}, and p40^{phox}, which translocate to cytochrome b⁵⁵⁸ on activation. Patients with CGD typically have recurrent pyogenic infections starting from the first year of life and require prophylactic antibiotics and antimycotic treatment to prevent severe bacterial and fungal infections.¹⁻³ Despite recent advances in gene therapy of CGD,^{4,5} the disease can be lethal without sufficient causative treatment. Bone marrow transplantation might be curative and is recommended for patients with CGD with an HLA-identical sibling.⁶

Neutrophils are the most abundant immune cells in the human blood and represent the first line of antibacterial and antifungal host defense of the cellular innate immune system.⁷⁻⁹ The proper function of neutrophils is orchestrated by innate immune receptors, such as Toll-like receptors (TLRs) and complement receptors.^{10,11} On bacterial infection, neutrophils are recruited through the IL-8 receptors CXCR1 and CXCR2 to the site of inflammation,¹² recognize bacteria through TLRs,¹³ and phagocytose bacteria through complement and Fc receptors.¹⁴⁻¹⁷ Although several studies have analyzed cytokine profiles in CGD neutrophils,¹⁸⁻²⁰ the surface receptor expression and associated function of innate immune receptors on neutrophils from patients with CGD have not been defined thus far. Therefore we quantified mRNA expression and surface and intracellular protein expression levels of TLR1, TLR2, TLR4, TLR5, and TLR9 together with CD11b, CD18, and CD35 and CXCR1 and CXCR2 on neutrophils from patients with CGD in an infection-free interval compared with those from age-matched healthy control individuals. To further rule out effects related to bacterial infections, we included otherwise healthy age-matched patients with bacterial pneumonia. To examine whether receptor expression levels were associated with functional changes of neutrophils in CGD, we analyzed phagocytosis, chemotaxis, and TLR-mediated cell activation. To assess whether receptor changes are due to

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Abbreviations used

CGD: Chronic granulomatous disease
 DPI: Diphenyleneiodonium
 HBSS: Hanks' balanced salt solution
 NADPH: Nicotinamide adenine dinucleotide phosphate (reduced)
 PE: Phycoerythrin
 ROS: Reactive oxygen species
 TLR: Toll-like receptor

ROS-dependent mechanisms, we inhibited NADPH oxidase of control neutrophils *in vitro*.

METHODS**Patients**

Fifteen patients with CGD, 15 patients with bacterial pneumonia, and 15 age-matched healthy control subjects were selected for the study (Table I). The study was approved by the Institutional Review Board of the Children's Hospital Research Center, Ludwig-Maximilians-University, Munich, Germany, and by the Ethics Committee of the Medical Faculty, Ludwig-Maximilians-University, Munich, Germany. The study was performed according to the most recent version of the Declaration of Helsinki. Written informed consent was obtained from all patients and control subjects before the study. Details of the patients with CGD are shown in Table E1 in the Online Repository at www.jacionline.org.

Neutrophil isolation

Venous blood was drawn, and neutrophils were isolated immediately by means of density-gradient centrifugation over isotonic Percoll (Amersham Biosciences, Piscataway, NJ). After lysis of the erythrocytes, the neutrophils were harvested and washed twice with Hanks' balanced salt solution (HBSS). The purity of the neutrophil suspensions was 98% or greater, as assessed by means of microscopic examination of Pappenheim cytopsin preparations. Cell viability was assessed by means of trypan blue dye exclusion ($\geq 95\%$).

Flow cytometry

Freshly obtained neutrophils from peripheral blood were incubated with antibodies, washed 2 times, and analyzed by means of flow cytometry (FACSCalibur; Becton-Dickinson, Heidelberg, Germany). Gating of neutrophils was based on light-scatter properties. Ten thousand neutrophils per sample were analyzed. For intracellular protein detection, we performed intracellular flow cytometry using an IntraPrep intracellular staining kit (Beckman Coulter, Krefeld, Germany), according to the manufacturer's instructions. In brief, isolated neutrophils were fixed with formaldehyde (4%) and permeabilized with saponin before antibody staining. Fc blocking and isotype controls were used to exclude nonspecific staining. Results were reported as mean fluorescence intensity. Calculations were performed with Cell Quest analysis software (Becton-Dickinson).

Quantitative PCR

Expression levels were quantified in duplicate by means of real-time quantitative RT-PCR with the use of SYBR green and the iCycler iQ detection system (BioRad, Hercules, Calif). Cycle threshold values for genes of interest were normalized to β -actin and used to calculate the relative quantity of mRNA expression. Primers are listed in Table E2 in the Online Repository at www.jacionline.org.

Inhibition of NADPH oxidase

We used diphenyleneiodonium chloride (DPI) as an irreversible *in vitro* inhibitor of the NADPH oxidase (K_i , 5.6 $\mu\text{mol/L}$). Peripheral blood isolated

TABLE I. Patient characteristics

	Patients with CGD	Patients with pneumonia	Control subjects
N	15	15	15
Age (y)	13 $\pm$ 12	13 $\pm$ 5	12 $\pm$ 6
Sex (M/F)	13/2	8/7	6/9
CRP (mg/dL)	0.4 $\pm$ 0.7	8 $\pm$ 6	0.2 $\pm$ 0.7
WBC (/ μL)	7,700 $\pm$ 3,900	17,200 $\pm$ 5,300	8,900 $\pm$ 3,000

Results are expressed as means $\pm$ SDs.

M, Male; F, female; CRP, C-reactive protein; WBC, white blood cell count.

neutrophils ($2 \times 10^6/\text{mL}$) from healthy control subjects were treated for 6 hours in RPMI with or without DPI (10 $\mu\text{mol/L}$). Afterward, neutrophils were washed, and surface receptor expressions, as well as receptor function, were analyzed as described below in detail.

Phagocytosis

Neutrophils were purified from peripheral blood of healthy control subjects and patients with CGD. *Staphylococcus aureus* bacteria were incubated with the fluorescent ligand Lucifer Yellow for 60 minutes at room temperature. After preopsonization with heat-inactivated serum (57°C for 60 minutes), the bacteria ($2 \times 10^7/\text{mL}$) were incubated at 37°C for 2 hours with peripheral blood-isolated neutrophils ($2 \times 10^6/\text{mL}$). Thereafter, the neutrophils were separated from the free bacteria by means of 3 centrifugations at 200g for 5 minutes. The Lucifer Yellow fluorescence of the isolated neutrophils was analyzed by flow cytometry.

Chemotaxis

Neutrophil chemotaxis was analyzed using a modified method of De Gendt et al.²¹ In brief, isolated neutrophils were labeled with 4 $\mu\text{g/mL}$ calcein acetomethyl ester for 45 minutes at 37°C before the start of the assay. After labeling, the cells were washed twice and resuspended in HBSS ($10^6/\text{mL}$). Calcein-labeled neutrophils (0.5×10^6 cells) were placed in the upper compartment, and IL-8 (72 amino acids; 10 nmol/L) was placed in the lower compartment of a Transwell filter system (3.0- μm pore size, 12-mm diameter; Costar, Cambridge, Mass). The Transwells were incubated for 60 minutes at 37°C. The percentage of cell migration was calculated by means of cell fluorescence in the lower versus the upper compartment.

Statistical analysis

Results are presented as means $\pm$ SD. Comparisons among all groups were performed with ANOVA, and comparisons between 2 patient groups were performed with the 2-sided t test.²² Correlation analysis was performed by calculating the 2-tailed Pearson correlation coefficient. A *P* value of less than .05 was considered significant. A correlation was assumed when the correlation coefficient was greater than 0.3. Statistical analysis was performed with Prism 4.0 (GraphPad Software, San Diego, Calif) and SPSS (SPSS, Inc, Chicago, Ill).

For a detailed description of patients, reagents, quantitative PCR, and *in vitro* assays, see the **Methods** section in the Online Repository at www.jacionline.org.

RESULTS**Impaired innate immune receptor expression on neutrophils from patients with CGD**

To examine the stability of receptor expressions, venous blood was taken from 3 patients with CGD at 3 infection-free time points within 1 year, which revealed a stable expression per individual and a robust difference in expression between individuals (see Fig E1 in the Online Repository at www.jacionline.org). Although TLR1, TLR2, and TLR4 expression levels on neutrophils did not differ, TLR5 and TLR9 expressions were lower in

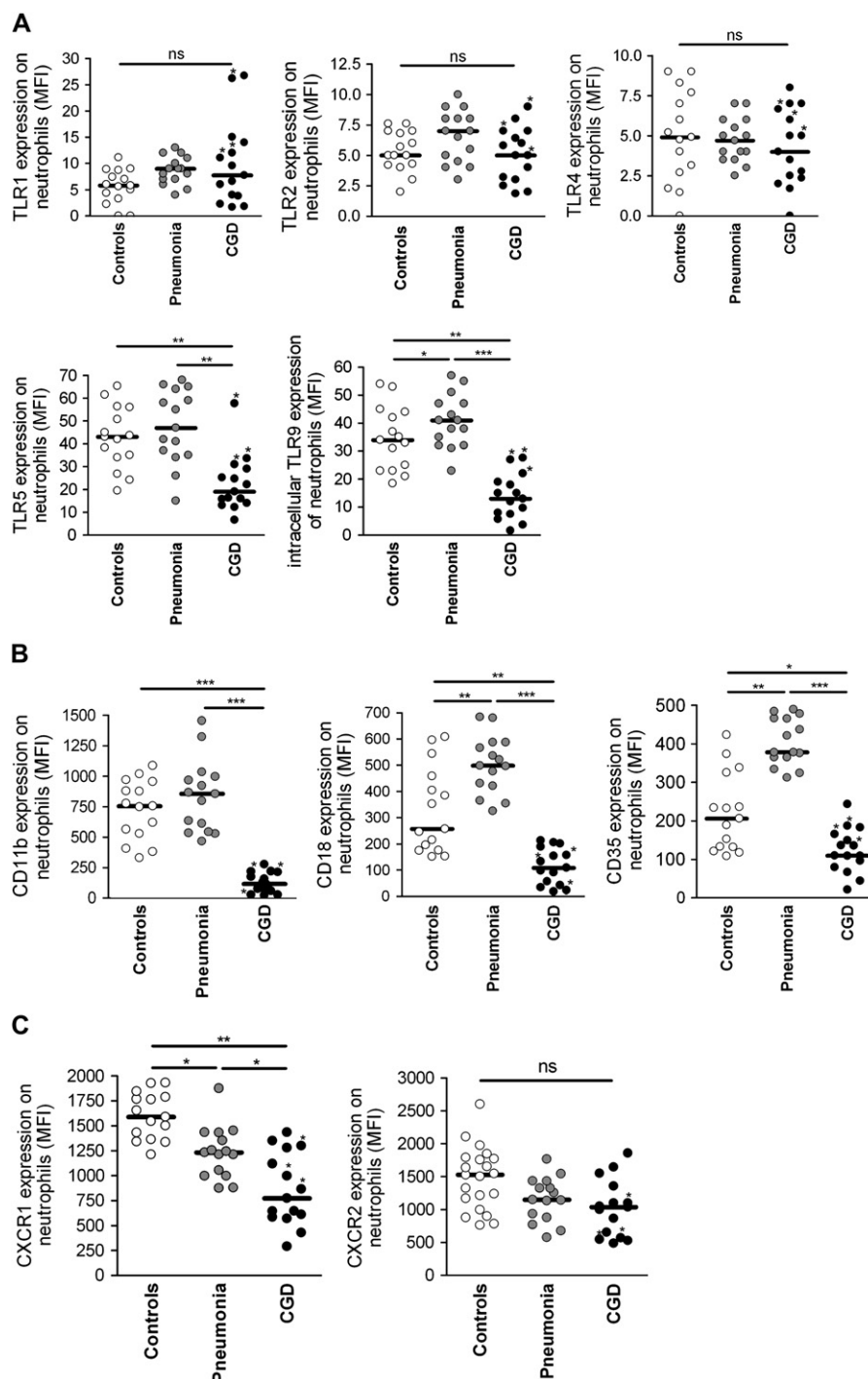


FIG 1. Receptor expression on neutrophils in patients with CGD, patients with pneumonia, and healthy control subjects. TLR (**A**), complement receptor (**B**), and IL-8 receptor (**C**) expression levels were quantified on neutrophils from patients with CGD ($n = 15$), patients with non-CGD pneumonia ($n = 15$), and healthy control subjects ($n = 15$) by means of flow cytometry. For TLR9 detection, neutrophils were permeabilized before antibody staining. Bars represent means. MFI, Mean fluorescence intensity (specific antibody staining minus isotype staining). * $P < .05$, ** $P < .01$, and *** $P < .001$ versus the respective patient group. Single asterisks indicate the 3 patients with CGD with residual NADPH oxidase activity.

patients with CGD compared with those in healthy control subjects (Fig 1, A). Surface expressions of the complement receptors CD11b, CD18, and CD35 were also decreased on neutrophils from patients with CGD compared with those seen in healthy control subjects (Fig 1, B). These receptor alterations in patients with

CGD were probably not due to frequent bacterial infections because neutrophils from age-matched patients without CGD but with bacterial pneumonia had similar (TLR5 and CD11b) or even increased (TLR9, CD18, and CD35) expression levels compared with those seen in healthy control subjects. The surface

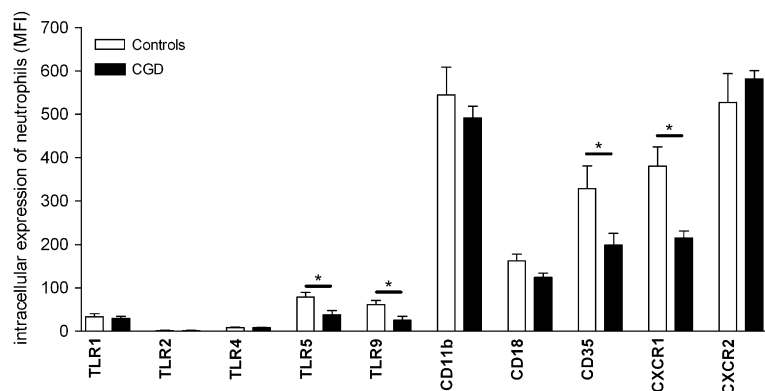


FIG 2. Intracellular flow cytometry. Intracellular protein expression was quantified by means of flow cytometry, as described in the Methods section. Neutrophils were fixed and permeabilized before antibody staining. Bars represent means $\pm$ SDs. MFI, Mean fluorescence intensity (specific antibody staining minus isotype staining). * $P < .05$, patients with CGD versus healthy control subjects.

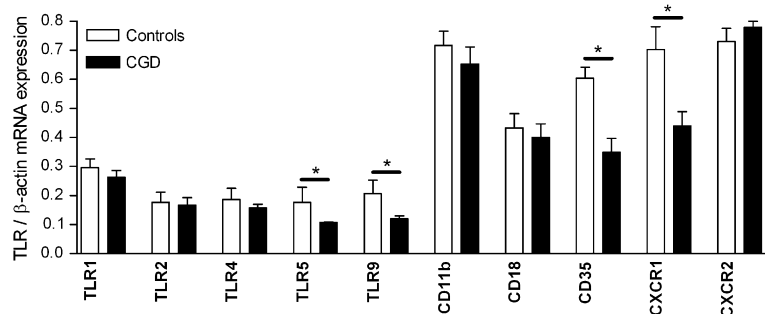


FIG 3. mRNA expression. Neutrophils were isolated from freshly drawn peripheral blood. RNA was extracted, and mRNA levels were quantified by means of quantitative RT-PCR, as described in the Methods section. mRNA expression is shown normalized to β -actin expression. Bars represent means $\pm$ SDs. * $P < .05$, patients with CGD versus healthy control subjects.

expression of CXCR1 was reduced on neutrophils from patients with CGD compared with that seen in both healthy control subjects and patients with pneumonia, whereas CXCR2 expression did not differ among the groups (Fig 1, C). Children with pneumonia had slightly reduced CXCR1 expression levels compared with those seen in healthy control children. Stratification for inheritance revealed that patients with autosomal-recessive CGD had reduced CD11b and CD18 expression levels compared with those seen in patients with CGD with X-chromosomal inheritance, whereas the other receptors studied showed no difference (see Fig E2 in the Online Repository at www.jacionline.org). Among the patients with autosomal-recessive CGD, the single patient bearing a mutation in the p22^{phox} subunit had higher CD11b and CD18 receptor levels than the other p47^{phox} patients.

Intracellular receptor expression and mRNA levels

To further investigate whether reduced surface receptor expressions on CGD neutrophils might be the result of surface receptor internalization or reduced receptor protein synthesis, we performed intracellular flow cytometry and quantified mRNA expression levels of the respective receptors. TLR5, TLR9, CD35, and CXCR1 intracellular protein levels in patients with CGD were decreased, whereas the CD11b and CD18 intracellular receptor pools did not differ from those of control subjects (Fig 2). Similarly, patients with CGD expressed lower mRNA levels of

TLR5, TLR9, CD35, and CXCR1 compared with those seen in healthy control neutrophils, whereas CD11b and CD18 mRNA expression did not differ (Fig 3). We tested whether mobilization of intracellular CD11b/CD18 receptor pools to the cell surface²³ might be impaired on CGD neutrophils. Therefore we incubated neutrophils from patients with CGD and healthy control subjects with TLR ligands and analyzed the resulting CD11b/CD18 surface expression. The TLR2 ligand Pam3CSK4 induced significantly less CD11b/CD18 surface expression on neutrophils from patients with CGD compared with that seen on neutrophils from healthy control subjects (see Fig E3 in the Online Repository at www.jacionline.org).

Functional relevance of impaired receptor expression on CGD neutrophils

To examine whether decreased receptor expression on CGD neutrophils might be associated with functional neutrophil abnormalities, we analyzed phagocytosis, chemotaxis, and TLR function by neutrophils from patients with CGD compared with those by neutrophils from healthy control subjects. Neutrophils from patients with CGD had a decreased capacity to phagocytose *S aureus* bacteria after preopsonization (Fig 4, A). Neutrophil chemotaxis toward IL-8 was reduced in patients with CGD compared with that in seen healthy control subjects (Fig 4, B). Because TLR5 surface expression was strongly decreased in patients

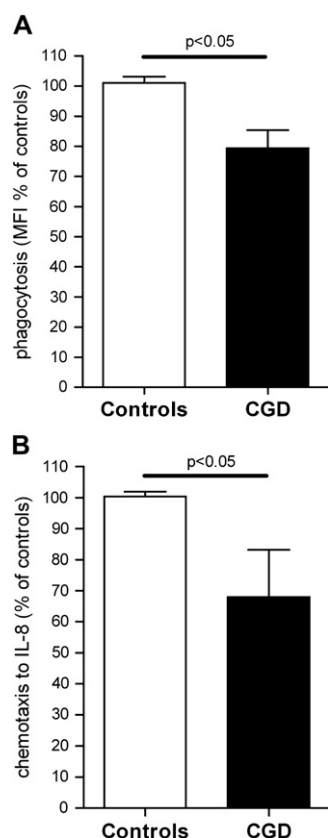


FIG 4. Functional relevance. Bars represent means $\pm$ SDs. Data are representative for 5 independent experiments. **A**, Phagocytosis. *S aureus* bacteria were incubated with the fluorescent ligand Lucifer Yellow for 60 minutes at room temperature. After preopsonization, the bacteria (2×10^7 /mL) were incubated at 37°C for 2 hours with neutrophils (2×10^6 /mL). After the incubation period, the neutrophils were separated from the free bacteria by means of 3 centrifugations at 200g for 5 minutes. The Lucifer Yellow fluorescence of the isolated neutrophils was analyzed by means of flow cytometry. Phagocytosis by neutrophils from patients with CGD is shown as the percentage related to phagocytosis by neutrophils isolated from peripheral blood of healthy control subjects (= 100%). **B**, Chemotaxis. Isolated neutrophils were labeled with 4 μ g/mL calcein acetomethylester and were placed in the upper compartment of a Transwell filter system (3.0- μ m pore size, 12-mm diameter), with IL-8 (10 nmol/L) in the lower compartment. The Transwells were incubated for 60 minutes at 37°C. Chemotaxis by neutrophils from patients with CGD is shown as the percentage related to chemotaxis of neutrophils isolated from peripheral blood of healthy control subjects (= 100%).

with CGD, we analyzed whether the loss of TLR5 expression on CGD neutrophils resulted in impaired TLR5-mediated neutrophil activation. The recognition of flagellin by neutrophils, measured based on intracellular calcium release, was decreased in neutrophils from patients with CGD compared with that seen in neutrophils from healthy control subjects (see Fig E4 in the Online Repository at www.jacionline.org). Blocking of TLR5 abolished the effect of flagellin on intracellular calcium levels, indicating signaling through TLR5. Treatment of neutrophils with isotype antibodies had no effect.

NADPH oxidase inhibition

To further investigate whether the decreased expression and impaired function of innate immune receptors was related to the genetic defect in patients with CGD, we inhibited NADPH

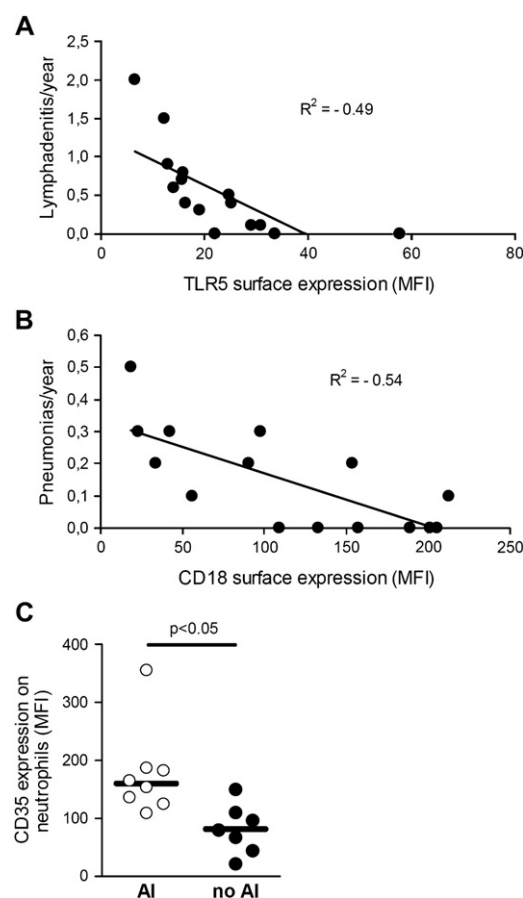


FIG 5. Clinical correlations. The correlation of TLR5 (**A**) and CD18 (**B**) expression levels on neutrophils and the frequency of lymphadenitis (Fig 5, A) and pneumonias (Fig 5, B) per year are shown in patients with CGD. **C** shows CD35 expression in patients with CGD with ($n = 8$) or without ($n = 7$) autoimmune (AI) symptoms. MFI, Mean fluorescence intensity. Correlation analysis was performed by calculating the 2-tailed Pearson correlation coefficient.

oxidase activity in neutrophils from healthy control subjects *in vitro*. NADPH oxidase inhibition resulted in a decrease in TLR5 and TLR9 expression (see Fig E5, A, in the Online Repository at www.jacionline.org), whereas the other receptor expressions analyzed did not change significantly (see Fig E5, B). Consistent with loss of TLR5 expression, ROS inhibition impaired the TLR5-dependent response of neutrophils toward flagellin (see Fig E5, C). These results are in accordance with our observation that the 3 individuals with CGD with a residual NADPH oxidase activity had the highest TLR5 and TLR9 expression levels among all patients with CGD (Fig 1, asterisks, and see Fig E6 in the Online Repository at www.jacionline.org).

Association with clinical parameters

To evaluate whether the observed alterations in innate immune receptors might be of relevance for the clinical course of patients with CGD, we performed statistical correlation analysis with CGD disease severity-associated parameters. TLR5 surface expression levels correlated inversely with the frequency of lymphadenitis (Fig 5, A), whereas CD18 surface expression levels correlated inversely with the frequency of pneumonias in these patients (Fig 5, B). When patients with CGD were stratified

according to the occurrence of autoimmune-associated symptoms, patients with autoimmune symptoms yielded higher CD35 surface expression levels on neutrophils (Fig 5, C).

DISCUSSION

The present study provides the first evidence that both the expression and function of innate immune receptors on neutrophils are impaired in patients with CGD. Decreased expressions of TLR5, TLR9, complement receptors, and CXCR1 resulted in an impairment of the associated neutrophil functions of pathogen recognition, phagocytosis, and chemotaxis. These receptor dysregulations were not due to frequent bacterial infections in patients with CGD because (1) age-matched patients without CGD but with bacterial pneumonia did not show these receptor alterations and (2) the receptor downregulations could be linked, at least in part, to the genetic defect in CGD. The correlation of TLR5 and CD18 with disease severity suggests a clinical relevance for these receptors in patients with CGD.

Given the high number of surface receptors expressed on neutrophils and the different stimuli and mechanisms involved in receptor downregulation,^{8,11,24} the question arises whether the receptor changes observed in this study have any relevance in patients with CGD. We studied key receptors involved in pathogen recognition (TLRs), phagocytosis (complement receptors), and chemotaxis (CXCR1/2) by neutrophils and found that distinct receptors in patients with CGD are dysregulated compared with those in age-matched healthy control subjects and patients with bacterial infection. Several lines of evidence support a pathophysiologic and clinical relevance of these findings: (1) longitudinal analysis of receptor expression revealed that the observed changes were not immunologic snapshots because of the actual patient condition but revealed a stable intraindividual and interindividual receptor expression; (2) the receptor downregulations observed in patients with CGD were in marked contrast to the upregulations found in patients with non-CGD pneumonias; (3) receptor alterations were associated with functional impairment of CGD neutrophils; (4) TLR5 and TLR9 expressions were linked to NADPH oxidase activity, as evidenced by the observation that patients with CGD with a residual oxidase activity had higher expression levels than patients without any enzyme activity and by the effect of NADPH oxidase inhibition on receptor expression and function *in vitro*; and (5) TLR5 and CD18 expression levels on neutrophils correlated with CGD disease severity parameters.

Except for TLR3, TLR1 through TLR9 have been described to be expressed by neutrophils.²⁵ The latter study further demonstrated that stimulation of neutrophils with specific TLR ligands resulted in an increased phagocytosis, respiratory burst, and IL-8 production by neutrophils *in vitro*, suggesting that TLR signaling plays a crucial role in priming of neutrophil functions in human diseases.²⁵ Here we show that TLR5 and TLR9 are expressed at lower levels by neutrophils in patients with CGD, whereas other TLRs involved in bacterial host defense are expressed to a similar extent in patients with CGD and in healthy control subjects or patients with bacterial pneumonia. TLR5 is the receptor for flagellin, the major constituent of bacterial flagella, which provides mobility and represents a universal virulence factor for both gram-positive and gram-negative bacteria.²⁶ Accordingly, an emerging body of evidence suggests that recognition of flagella through TLR5 might represent one of the key mechanisms by which bacteria are sensed by the innate

immune system.²⁷ Given these previous findings, our observation that TLR5 expression is decreased and TLR5-mediated neutrophil activation is blunted in patients with CGD suggests that the sensing of flagellin and the TLR5-mediated priming of neutrophils might be impaired in patients with CGD. Our finding that inhibition of NADPH oxidase, mimicking the CGD neutrophil phenotype *in vitro*, resulted in a decreased TLR5 expression and an impaired TLR5-mediated neutrophil activation points toward an intracellular association between ROS and TLR5 expression. The correlation of TLR5 with the frequency of lymphadenitis in CGD finally suggests a clinically relevant role for TLR5 dysregulation in the course of CGD.

TLR9 is expressed intracellularly in the membrane of endosomes, such as phagolysosomes.²⁸ Recently, Alvarez et al²⁹ demonstrated that TLR9-mediated activation of neutrophils results in upregulation of complement receptors and an increased IL-8 production, indicating that TLR9 signaling activates neutrophil functions. Jozsef et al³⁰ found that CpGs delay apoptosis and induce IL-8 production by neutrophils through peroxynitrite signaling.³¹ Based on these reports, we speculate that the loss of complement receptors might be linked to dysregulation of TLR9 expression in these patients. The decrease of TLR9 expression after inhibition of the NADPH oxidase further suggests a pathophysiologic link between ROS signaling and TLR9 expression in CGD neutrophils.

Gaither et al³² found a reduced expression of CD35 on the neutrophil surface and in cell extracts from patients with CGD and hyper-IgE syndrome compared with that seen in healthy control subjects and patients with recurrent infections. Since then, to the best of our knowledge, no study has been published on complement or other innate immune receptors on neutrophils in patients with CGD. In line with the study by Gaither et al,³² we found reduced CD35 expression levels on neutrophils from patients with CGD. Furthermore, CD11b and CD18 expression levels on neutrophils from patients with CGD were also reduced compared with those seen in healthy control subjects and matched patients with non-CGD bacterial pneumonia. Interestingly, CD11b surface expression was reduced even to a larger extent than CD35 receptors. However, because Gaither et al³² used different methods than we did, in particular rosette formation, binding of radiolabeled anti-CR1, and an immunoradiometric assay for neutrophil cell extracts, these results can hardly be compared on a quantitative level with our findings.

IL-8 recruits and activates neutrophils through the G protein-coupled chemokine receptors CXCR1 and CXCR2.¹² Whereas multiple CXC chemokines bind to CXCR2, IL-8 is the only high-affinity ligand for CXCR1. Our finding that CXCR1, but not CXCR2, expression was reduced on neutrophils in patients with CGD raises the question of which factors induce the low CXCR1 expression on neutrophils from patients with CGD. Activation of NADPH oxidase was found to negatively regulate IL-8 mRNA expression in neutrophils, and the absence of NADPH oxidase in neutrophils from patients with CGD resulted in an increase of IL-8 production.¹⁹ Despite the fact that we did not quantify circulating IL-8 levels in our patients with CGD, the previously reported overproduction of IL-8 by neutrophils from patients with CGD might represent a possible underlying cause for the downregulation of CXCR1 receptors found on these neutrophils.

Our results obtained from functional assays suggest that neutrophils in patients with CGD are not only restricted in terms of NADPH oxidase-mediated intracellular oxidative killing but

display further phenotypical and functional abnormalities related to innate immune receptors. We found that reduced complement receptor expression by neutrophils from patients with CGD was associated with impaired bacterial opsonophagocytosis. Although further indications exist that neutrophils from patients with CGD display impaired phagocytosis,^{33,34} Hasui et al³⁵ analyzed phagocytosis of CGD neutrophils and found 2 distinct CGD patient subgroups, one with a normal and one with an increased phagocytic capacity. We speculate that the discrepancy between these studies might be due to the experimental procedure or patient selection. Nevertheless, the marked loss of surface complement receptors on neutrophils from patients with CGD is in accordance with the results of our phagocytosis assays and supports the presence of an impaired complement-dependent opsonophagocytosis in CGD. Our patients with CGD were free of any infection in the 2 weeks before the study, rendering an influence of recent or actual bacterial infections unlikely. The heterodimer CD11b/CD18 (Mac-1) acts as an adhesion molecule and facilitates leukocyte migration to sites of inflammation.³⁶ Based on the fact that our patients with pneumonia had remarkably high CD11b and CD18 expression levels on neutrophils whereas patients with CGD had decreased CD18 expression and expression levels in CGD correlated inversely with the occurrence of pneumonia, the decreased CD18 surface expression on CGD neutrophils might impair neutrophil migration into peripheral inflammatory compartments, such as the lung.

Because CD11b has been described to be mobilized from intracellular pools on activation²³ and CD11b and CD18 mRNA and intracellular protein expression did not differ between patients with CGD and healthy subjects, we hypothesized that impaired receptor mobilization rather than receptor synthesis might cause the low CD11b and CD18 surface expression on neutrophils from patients with CGD. Indeed, we found a deficiency of neutrophils from patients with CGD to upregulate CD11b and CD18 on stimulation with the TLR2 ligand Pam3CSK4. These findings might suggest that neutrophils from patients with CGD exhibit an intrinsic impairment to mobilize complement receptors to the cell surface that, in turn, might result in impaired opsonophagocytosis, as we observed in patients with CGD *ex vivo*.

In addition to their impaired host defense, patients with CGD frequently have autoimmune phenomena, such as granuloma formation, suggesting an uncontrolled inflammatory response in these patients.³⁷ Previous studies found an upregulation of several genes involved in inflammation in neutrophils from patients with CGD by using microarray analysis,²⁰ as well as an enhanced production of proinflammatory cytokines on stimulation with TLR2/4 ligands, which was independent of NADPH oxidase function.¹⁸ Our studies demonstrate that among all TLRs analyzed, only TLR5 and TLR9 expression were decreased in patients with CGD, which was reproduced by NADPH oxidase inhibition *in vitro*. Based on these findings, we speculate that certain TLR pathways, in particular TLR5 and TLR9, are impaired in patients with CGD through a mechanism that is causally linked to the deficient ROS production in these cells.

In summary, our study provides the first evidence that innate immune receptors on neutrophils in patients with CGD are phenotypically and functionally impaired. The correlation of TLR5 and CD18 with disease severity suggests a clinical relevance of these receptors in patients with CGD. The dysregulation of innate immune receptors on neutrophils in patients with CGD allows further insights into neutrophil function and should

stimulate novel diagnostic and therapeutic approaches for patients with CGD.

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Clinical implications: Innate immune receptors can provide a novel diagnostic and therapeutic target in patients with chronic granulomatous disease.

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METHODS

Patients

Fifteen patients with CGD, 15 patients with bacterial pneumonia, and 15 age-matched healthy control subjects were selected for the study (Table I). The study was approved by the Institutional Review Board of the Children's Hospital Research Center, Ludwig-Maximilians-University, Munich, Germany, and by the Ethics Committee of the Medical Faculty, Ludwig-Maximilians University, Munich, Germany. The study was performed according to the most recent version of the Declaration of Helsinki. Written informed consent was obtained from all patients and control subjects before the study. The CGD group included patients with CGD with a mean age of 13 ± 12 (SD) years. Diagnostic criteria for patients with CGD were a pathologic Nitroblue tetrazolium test, dihydrorhodamine 123 test, or both and confirmation with genetic analysis. A further inclusion criterion was the absence of current infection for 2 weeks before the study. Residual O_2^- production was detected by using a cytochrome c reduction assay. The patients with CGD were treated with itraconazole (mean, 5 mg/kg/d) and cotrimoxazole (mean, 5 mg/kg/d). Details of the patients with CGD are shown in Table E1. The pneumonia group included subjects with community-acquired pneumonia with a mean age of 13 ± 5 (SD) years. Pneumonia was diagnosed based on infiltrates in chest radiographs, laboratory findings (C-reactive protein, white blood cell count, and erythrocyte sedimentation rate), and clinical signs of pneumonia (cough, dyspnea, tachypnea, and fever). The healthy control group included 15 children without infections for 2 weeks before the study with a mean age of 12 ± 6 (SD) years.

Reagents

Recombinant IL-8 (72 amino acids) was from PeproTech (Rocky Hill, NJ). Percoll ($\delta = 1.078$ g/mL) was from Amersham Biosciences (Piscataway, NJ). HBSS and DPI were from Sigma-Aldrich (St Louis, Mo). Recombinant flagellin (*Salmonella typhimurium*) and nonmethylated CpG oligonucleotides type A (5'-ggGGGACGATCGTCggggg-3') were from Invivogen (San Diego, Calif). The following monoclonal anti-human antibodies were from eBioscience (San Diego, Calif): mouse IgG1 anti-TLR1-phycoerythrin (PE), mouse IgG2a anti-TLR2-fluorescein isothiocyanate, mouse IgG1 anti-TLR4-PE, and rat IgG2a anti-TLR9-PE. Mouse IgG2a anti-TLR5-PE was from Imgenex (San Diego, Calif). The following monoclonal anti-human antibodies were from BD PharMingen (San Diego, Calif): CD35-PE mouse IgG1, mouse IgG1-PE, and mouse IgG2a-PE. The following monoclonal anti-human antibodies were from R&D Systems (Minneapolis, Minn): CD11b-PE mouse IgG2a, CD18-PE mouse IgG1, anti-CXCR1-PE mouse IgG2b, and anti-CXCR1-PE mouse IgG1. Functional blocking mouse anti-human TLR5 and isotype mouse IgG antibodies were from Santa Cruz Biotechnologies (Santa Cruz, Calif) and were used as described previously.^{E1} *S aureus* (no. 10390) was from the American Type Culture Collection. All

reagents, buffers, and media were free of LPS (<0.01 ng/mL), as assessed by means of Limulus assay (Sigma-Aldrich, St Louis, Mo).

Quantitative PCR

Peripheral blood-isolated neutrophils were mixed with Trizol LS Reagent (Invitrogen Life Technologies, Carlsbad, Calif) and stored at -20°C until mRNA extraction. Total RNA was isolated according to the manufacturer's instructions (Invitrogen), treated with DNase, and immediately reverse transcribed by using random hexamer primers and Superscript II RT (Invitrogen). Contamination with genomic DNA was controlled by means of cDNA synthesis reaction without reverse transcriptase. Expression levels were quantified in duplicate by means of real-time quantitative RT-PCR with the use of SYBR green and the iCycler iQ detection system (BioRad, Hercules, Calif). Cycle threshold values for genes of interest were normalized to β -actin and used to calculate the relative quantity of mRNA expression. β -actin was selected as the normalizing gene because it was stable under the *in vitro* conditions tested. Primers are listed in Table E2.

In vitro stimulation

The effect of the TLR ligands LPS (100 ng/mL; TLR4), Pam3CSK4 (1 μg /mL; TLR1/2), peptidoglycan (1 μg /mL; TLR2/NOD2), CpG-DNA (100 μg /mL; TLR9), flagellin (1 μg /mL; from *S typhimurium*; TLR5), and zymosan (50 μg /mL; TLR2/6) was assessed on CD11b and CD18 surface expression of neutrophils. Therefore isolated neutrophils (5×10^6) were stimulated in RPMI 1640 medium for 1 hour with TLR ligands at 37°C and 5% CO_2 . After the incubation period, neutrophils were washed twice with ice-cold PBS, incubated with CD11b or CD18 antibodies for 40 minutes, washed 2 times with PBS, and analyzed by means of flow cytometry, as described above. Where indicated, neutrophils were treated with anti-TLR5 (20 μg /mL) or anti-IgG isotype (20 μg /mL) antibodies before exposure to flagellin. After the incubation period, neutrophils were washed twice with ice-cold PBS, and free cytoplasmic calcium was analyzed as described in the Methods section.

Free calcium concentration

Changes in the cytosolic free calcium concentration in neutrophils were measured with Fura 2 (Invitrogen), as described previously.^{E2}

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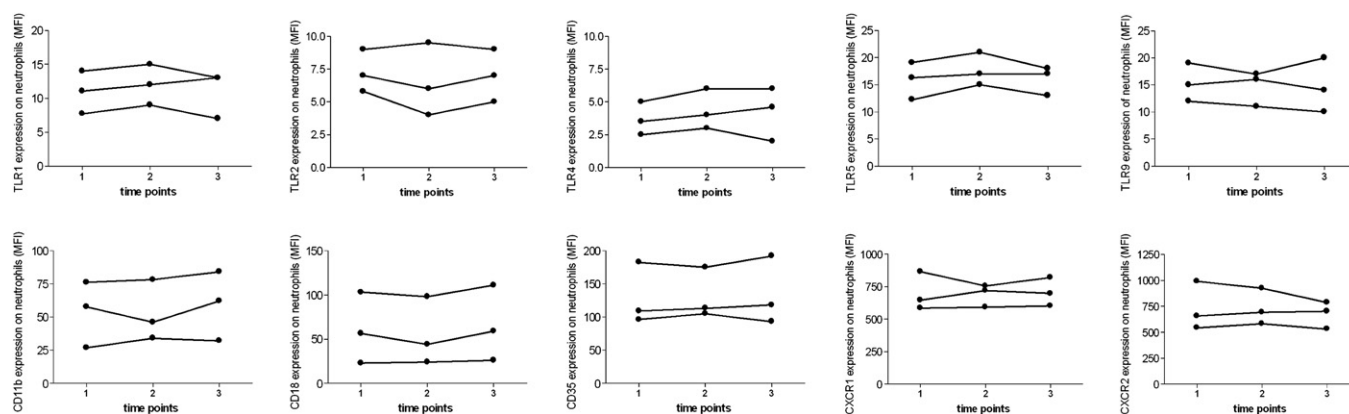


FIG E1. Receptor stability. Receptor expression levels analyzed were determined by means of flow cytometry by using peripheral blood-isolated neutrophils obtained from 3 representative patients with CGD at 3 different time points within 1 year (mean interval, 3 ± 2 months [mean $\pm$ SDs]) to assess receptor expression stability. Each dot represents a single patient with CGD. MFI, Mean fluorescence intensity (specific antibody staining minus isotype staining).

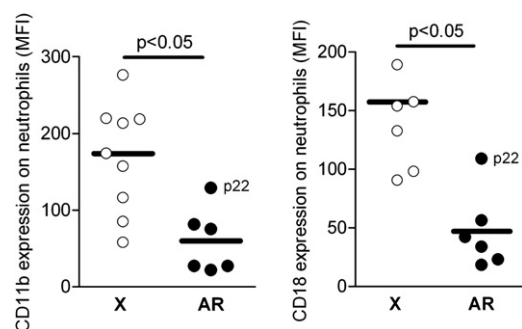


FIG E2. CGD inheritance and complement receptor expression. CD11b and CD18 surface expression levels are shown in patients with CGD with X-chromosomal (X; n = 9) or autosomal-recessive (AR; n = 6) inheritance. Bars represent means $\pm$ SDs. *MFI*, Mean fluorescence intensity (specific antibody staining minus isotype staining); *p22*, a single autosomal-recessive patient with CGD bearing a mutation in the *p22^{phox}* subunit.

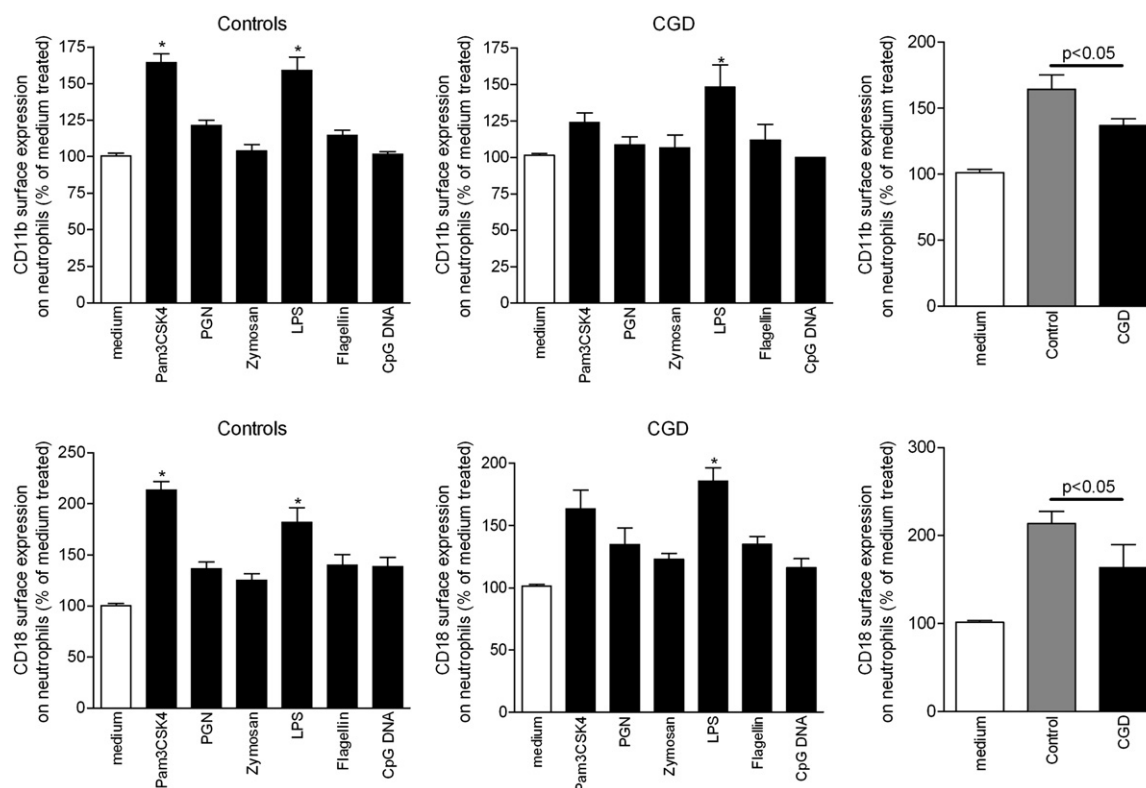


FIG E3. CD11b/CD18 upregulation. Neutrophils obtained from patients with CGD or healthy control subjects were incubated in RPMI medium at 37°C and 5% CO₂ for 1 hour with LPS (100 ng/mL), Pam3CSK4 (1 μg/mL), peptidoglycan (1 μg/mL), CpG-DNA (100 μg/mL), flagellin (1 μg/mL), or zymosan (50 μg/mL). After the incubation period, CD11b (A) or CD18 (B) surface expression was quantified by means of flow cytometry. Bars represent means ± SDs of 3 independent experiments each. *MFI*, Mean fluorescence intensity (specific antibody staining minus isotype staining). * $P < .05$ versus medium-treated neutrophils. The right panels show CD11b=CD18 expression after medium or Pam3CSK4 stimulation.

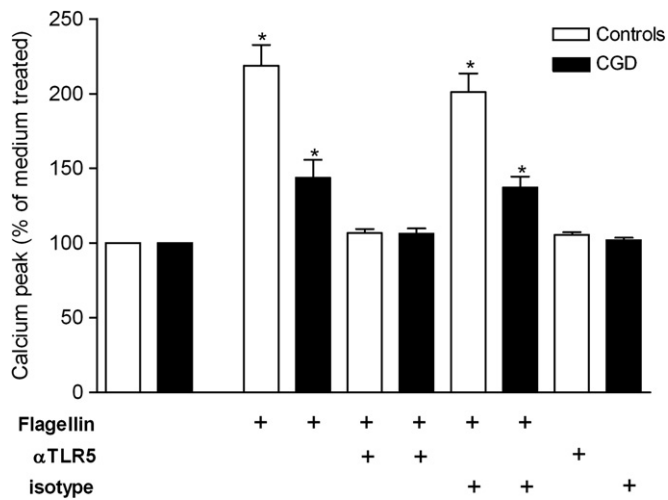


FIG E4. TLR5 signaling. Neutrophils isolated from peripheral blood of healthy control subjects or patients with CGD were incubated in RPMI medium at 37°C and 5% CO₂ for 1 hour with flagellin (1 μg/mL). TLR5 signaling was inhibited by pretreatment of neutrophils for 30 minutes with anti-TLR5 (αTLR5; 20 μg/mL). As a control, isotype antibodies (20 μg/mL) were used instead of anti-TLR5 antibodies. After the incubation, intracellular calcium levels were measured as described in the Methods section. Bars represent means ± SD of 5 independent experiments. Intracellular calcium levels are presented as a percentage related to medium-treated neutrophils (= 100%). **P* < .05 versus medium-treated neutrophils.

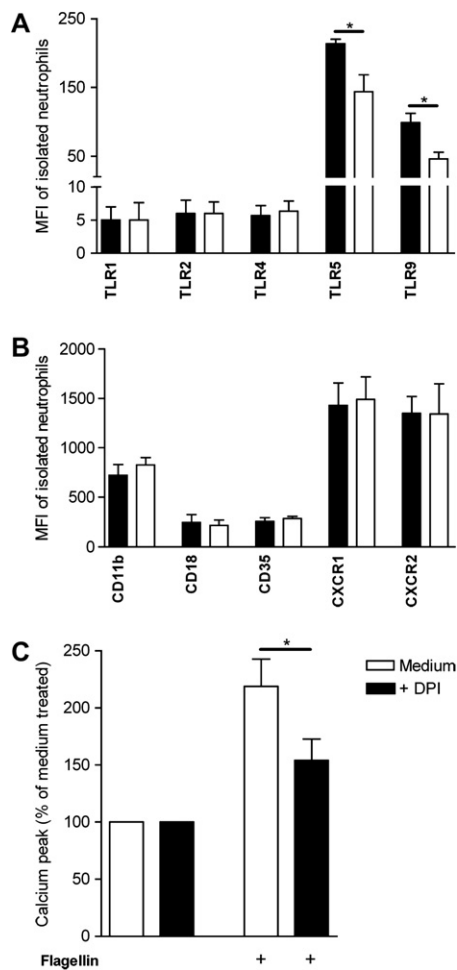


FIG E5. Inhibition of NADPH oxidase. Peripheral blood-isolated neutrophils were treated for 6 hours in RPMI with or without DPI (10 μ mol/L). Afterward, neutrophils were washed, and TLR (A) or other innate immune receptor expressions (B), as well as TLR5 receptor function (C), were analyzed as described elsewhere in detail. * $P < .05$, DPI versus medium-treated neutrophils.

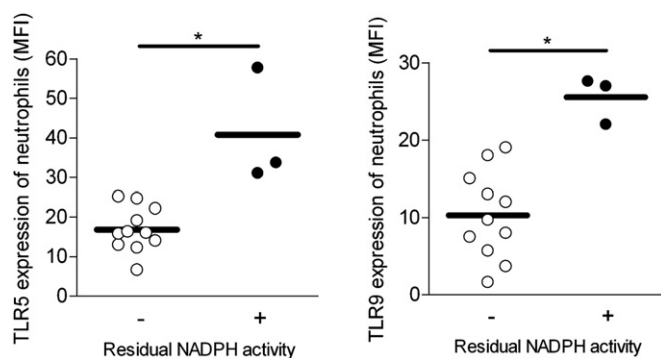


FIG E6. Residual NADPH oxidase. The *left panel* shows TLR5 expression levels and the *right panel* shows TLR9 expression levels of neutrophils in patients with CGD with (n = 3) or without (n = 12) residual NADPH oxidase activity. *MFI*, Mean fluorescence intensity (specific antibody staining minus isotype staining). * $P < .05$.

TABLE E1. Detailed characteristics of patients with CGD

Patient no.	Age (y)	Sex	Inheritance	Mutation	Nucleotide change	Amino acid or mRNA change	CRP (mg/dL)	WBC (g/L)	NBT test (% positive)	DHR test	Prophylactic medication
1	1	M	gp91, X-chromosomal	Nonsense	1140 G → A	380 Trp → stop	3.6	17.7	0% PMA, 0% <i>Candida</i> species	Negative	Itraconazole, cotrimoxazole
2	18	M	gp91, X-chromosomal	Missense	1028 C → A	339 Pro → His	0.5	8.3	0% PMA, 0% <i>Candida</i> species	Negative	Itraconazole, cotrimoxazole
3	5	M	p47, autosomal	Deletion	GTGT deletion Exon 2	Frameshift	0.03	7.4	4% PMA, 15% <i>Candida</i> species	Positive	Itraconazole, cotrimoxazole
4	9	M	p47, autosomal	Deletion	GTGT deletion Exon 2	Frameshift	0.05	7.5	6% PMA, 5% <i>Candida</i> species	Positive	Itraconazole, cotrimoxazole
5	11	M	gp91, X-chromosomal	Missense	578 C → T	193 Ser → Phe	0.1	5.3	0% PMA, 0% <i>Candida</i> species	Negative	Itraconazole, cotrimoxazole
6	9	M	p47, autosomal	Deletion	GTGT deletion Exon 2	Frameshift	0.15	6.1	0% PMA, 0% <i>Candida</i> species	Negative	Itraconazole, cotrimoxazole
7	12	M	gp91, X-chromosomal	Deletion	80-83 TCTG	Frameshift	0.32	4.7	0% PMA, 0% <i>Candida</i> species	Negative	Itraconazole, cotrimoxazole
8	16	M	gp91, X-chromosomal	Missense	1106 T → C	Frameshift	0.49	4.1	0% PMA, 0% <i>Candida</i> species	Negative	Itraconazole, cotrimoxazole
9	31	M	gp91, X-chromosomal	Insertion	A after A742	Frameshift	0.1	3.7	0% PMA, 0% <i>Candida</i> species	Negative	Itraconazole, cotrimoxazole
10	11	M	gp91, X-chromosomal	Deletion	1497A deletion	Frameshift	0.41	7.8	0% PMA, 0% <i>Candida</i> species	Negative	Itraconazole, cotrimoxazole
11	54	M	p47, autosomal	Deletion	GTGT deletion Exon 2	Frameshift	0.1	5.8	10% PMA, 7% <i>Candida</i> species	Positive	None
12	8	F	p22, autosomal	Deletion	AGTG deletion	Activated cryptic donor splice site in intron 1	0.2	6.7	0% PMA, 0% <i>Candida</i> species	Positive	Itraconazole, cotrimoxazole
13	4	M	gp91, X-chromosomal	Nonsense	469 G → T	157 Arg → stop	0.92	15.5	0% PMA, 0% <i>Candida</i> species	Negative	Itraconazole, cotrimoxazole
14	4	M	gp91, X-chromosomal	Nonsense	469 G → T	157 Arg → stop	0.12	10.7	0% PMA, 0% <i>Candida</i> species	Negative	Itraconazole, cotrimoxazole
15	12	F	p47, autosomal	Deletion	GTGG deletion Exon 2	Frameshift	0.1	4	0% PMA, 0% <i>Candida</i> species	Negative	Itraconazole, cotrimoxazole

Results are expressed as means ± SDs.

CRP, C-reactive protein; WBC, white blood cell count; PMA, phorbol 12-myristate 13-acetate; NBT, Nitroblue tetrazolium; DHR, dihydrorhodamine 123; M, male; F, female.

TABLE E2. Primers

Gene	Forward	Reverse
β-actin	CTCCGTGGCCTTAGCTGTG	TTTGGAGTACGCTGGATAGCCT
TLR1	CTGGTATCTCAGGATGGTGTGC	TTGGAGTTCTTCTAAGGGTATGTTCC
TLR2	GGCCAGCAAATTACCTGTGTG	AGGCGGACATCCTGAACCT
TLR4	CTGCAATGGATCAAGGACCA	TTATCTGAAGGTGTTGCACATTCC
TLR5	TCGAGCCCCCTACAAGGGAA	CACTGAGACTCTGCTATACAAGCTA
TLR9	TGGTGTTGAAGGACAGTTCTCTC	CACTCGGAGGTTTCCCAGC
CD11b	GCTGTITAACATCACCAACGG	TCATGTCTGAATCCACTCTGG
CD35	TGGCATGGTGCATGTGATCA	TCAGGGCCTGGCACTTCACA
CD18	AGCGACCTCAGGGAGTACCAT	GTCGTGGTGGCACTCTTGAAA
CXCR1	GAG CCC CGA ATC TGA CAT T	AGCAGACACTGCAACACA C
CXCR2	ACA GCT ACT TGG GAG GCT GA	TGCAGTGGTCACACCATT TT