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ROS-deficient monocytes have aberrant gene expression that correlates with inflammatory disorders of chronic granulomatous disease

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Abstract Chronic granulomatous disease is an immunodeficiency caused by an inability to produce reactive oxygen species. While the mechanism of hyper-sensitivity to infection is well understood in CGD, the basis for debilitating inflammatory disorders that arise in the absence of evident infection has not been fully explained. Herein it is demonstrated that resting and TLR-activated monocytes from individuals with CGD expressed significantly higher levels of inflammatory mediators than control cells; the expression in CGD cells resembled normal cells stimulated with lipopolysaccharide. The lack of acute illness, infection or circulating endotoxin in the blood of the CGD patients at the time of sampling was consistent with infection-free inflammation. The enhanced expression of inflammatory mediators correlated with elevated expression of NF-κB and was dependent on ERK1/2 signalling. The results are consistent with the hypothesis that ROS are anti-inflammatory mediators that control gene expression and potentially limit the development of sterile inflammatory disorders.

Abbreviations: AP-1, activator protein-1; CREB, cAMP response element-binding protein; CCL-, chemokine (C–C motif) ligand; CGD, chronic granulomatous disease; ERK-1/2, extracellular response kinase 1/2; GO, gene ontology; IFIT1, interferon-induced protein with tetratricopeptide repeats 1; IKK, I-κB-kinase; LAL, limulus amoebocyte lysate; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; Ncf1, neutrophil cytosolic factor 1; Nrf2, nuclear factor (erythroid-derived 2)-like 2; PGN, peptidoglycan; phox, phagocyte oxidase; qPCR, real-time quantitative PCR; ROS, reactive oxygen species; Sp3, specificity protein 3; TLR, Toll-like receptor.

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Introduction

Phagocytic oxidative killing is mediated in part by highly toxic reactive oxygen species (ROS) such as superoxide, hydrogen peroxide and hydroxyl radicals, which are generated by the NADPH-oxidase complex. In the late 1950s, a clinical syndrome was described in children as being characterized by recurrent life-threatening infections and widespread chronic granulomas [1]. It was later found that this condition, chronic granulomatous disease (CGD), results from mutations in one of the genes that encode components of the NADPH-oxidase complex. The result is a non-functional NADPH-oxidase complex and phagocytic cells that are defective in the production of ROS [reviewed in [2]]. Although the exact incidence of the disease is unknown, this is a rare disease, with approximately 1 case per 200–500 thousand individuals. The incidence varies with geographical location but there are no reports of racial or ethnic predilection. The most common form of the disease results from a mutation in the CYBB gene that encodes gp91^{phox}, the b subunit of cytochrome *b558*. As the CYBB gene is located on the X chromosome, this form of the disease is commonly referred to as X-linked CGD. An expected 50–70% of CGD cases result from a deficiency in gp91^{phox}, yielding an estimated 300–1000 cases in North America per year. The severity of the disease and mortality is highest in patients with this form of the disease.

In general, individuals with CGD are susceptible to a range of infections including those by *Aspergillus* species, *Staphylococcus aureus*, and *Burkholderia cepacia* complex bacteria. Patients with CGD also present with inflammatory conditions that occur in the absence of infections, including among others inflammatory bowel disease, inflammatory granulomatous obstruction of the bladder, esophagus and stomach, scarring acne, and 'sterile' pneumonitis [reviewed in [3]]. The inflammatory phenotypes are so compelling and similar to idiopathic inflammatory diseases that they often overshadow the underlying immunodeficiency [4]. These inflammatory disorders decrease the quality of life and shorten life expectancy in the absence of life-threatening infections. Even in patients with identical genetic defects, the disease is clinically heterogeneous thus the prognosis for individuals is difficult. It is important to recognize that death may be caused by an infection and/or the inflammatory disease. Indeed, two of the four patients investigated here died during the course of this study, one from an infection and the other from overwhelming and irreversible pulmonary inflammation in the absence of evident infection.

The inflammatory disorders remain a poorly understood aspect of the disease. It has been suggested that inflammatory disorders in CGD result from persistent, non-culturable microorganisms [5], although there is no compelling evidence for this possibility. Significantly, ROS, which are deficient in CGD, have functions besides oxidative killing, including roles in metabolism, cell death, apoptosis, induction of host defence genes, oxidative signalling and the regulation of inflammation [reviewed in [2,6]]. The absence or dysregulation of these functions might also account for the observed inflammatory disorders and explain how chronic inflammation in CGD patients can progress in the absence of infection. In particular, it has been suggested that ROS influence the activation of NF-κB [reviewed in [7]], a key factor responsible for the transcription of inflammatory genes [reviewed in [8]]; indeed the activity of NF-κB is known to be sensitive to cellular redox states.

Research in this field has tended to focus on neutrophils as these cells produce an abundance of ROS, particularly following cellular activation that can occur during phagocytosis of foreign microbes. We reported that PBMC from individuals with CGD demonstrate enhanced production of pro-inflammatory cytokines in response to TLR agonists [9], providing evidence that blood cells other than neutrophils are affected by this deficiency in ROS generation, and suggesting that these cells might contribute to the pathology of the inflammatory disorders associated with CGD. In this study, using microarray technology, bioinformatic interrogation and selective confirmation of gene expression by real-time PCR and ELISA, the profiles of gene expression in monocytes from individuals with CGD in the presence or absence of *in vitro* activation by the TLR agonist lipopolysaccharide (LPS) or peptidoglycan (PGN) were compared to similarly treated cells from normal individuals, indicating broad dysregulated expression of inflammatory genes in monocytes from four males with gp91^{phox}-deficient, X-linked CGD. In particular, the expression of NF-κB-regulated inflammatory genes was elevated in monocytes from the patients and this expression was highly co-dependent on the mitogen-activated protein kinase (MAPK) ERK1/2. Interestingly the most profound dysregulation of gene expression occurred in unstimulated CGD monocytes.

Materials and methods

Cell culture, PBMC isolation and PBMC stimulation

Four male patients, 18–32 years of age, with diagnosed X-linked CGD resulting from mutations in the gene for the gp91^{phox} component of the NADPH complex participated in this study. Previous work confirmed that PBMC from these individuals were devoid of ROS [9]. PBMC from healthy volunteers or the patients were prepared as previously described [10] in accordance with the University of British Columbia Clinical Research Ethics Board protocol C04-0193. PBMC (5×10^6 cells/ml) were stimulated for 4 h or 24 h at 37 °C in 5% CO_2 with 100 ng/ml repurified *Escherichia coli* 0111:B4 LPS or 10 μg/ml *Bacillus subtilis* PGN (Invivogen). The cationic human peptide LL-37 (LLGDFFRKSKEKIGKEFKRIVQRIKDFFR-NLVPRTES) was synthesized as described [10] and added to cells at 20 μg/ml alone or with LPS. Cells were pre-incubated for 30 min with inhibitors for ERK (10 μM PD98059; Cell Signalling Technologies), p38 (10 μM SB203580; Sigma), NF-κB (5 μM MG132; Calbiochem) or vehicle control, then activated with LPS for 1 h. The inhibitor concentrations and DMSO vehicle control were demonstrated to be non-toxic to PBMC. The gp91^{phox}P415H (X-CGD) and control PLB cell lines [11] were cultured under sterile conditions at 37 °C in 5% CO_2 in RPMI 1640 supplemented with 10% FCS, 2 mM L-glutamine, 1 mM sodium pyruvate, 100 U/ml penicillin and 100 μg/ml streptomycin (Fisher Scientific). Cells were seeded at 2×10^5 cells per well on 24-well plates and differentiated towards an adherent, myeloid phenotype by incubation for 15–18 h with 50 nM PMA (Sigma). Non-adherent cells were removed by washing and adherent cells

were cultured an additional 24 h (without PMA) before use in downstream assays.

Positive selection of CD14$^+$ peripheral blood monocytes

Following culture of PBMC, monocytes were positively selected using anti-CD14 conjugated magnetic beads (M450; Dynal; Invitrogen) following previously described protocols [12,13] which removed greater than 90% of CD14$^+$ cells from the PBMC population, as determined by flow cytometry analysis. Monocytes were stored in RNA Stabilization Reagent (Qiagen) at 4 °C for up to 24 h.

Detection of cytokines

Following culture of cells, the tissue culture supernatants were centrifuged and stored at −80 °C until analysis for cytokines using, for PBMC supernatants, a cytokine 5-Plex kit (Biosource International Inc) and Luminex 100™ StarStation software (Applied Cytometry Systems) as described previously [10], or for PLB and X-CGD cell line supernatants, standard human IL-8 and TNFα ELISA (R&D Systems).

DNA microarrays

RNA was isolated from monocytes with RNeasy Mini kit and analyzed using an Agilent 2100 Bioanalyzer (Agilent Technologies) as described previously [10]. Equal quantities of RNA from each of five healthy individuals were pooled per experimental condition. Samples from three of the CGD patients were subject to microarray analysis (against the control pool) on human 21K oligo-based gene arrays. Arrays were analyzed by ArrayPipe software version 1.6 [14] as previously described [10] and the data was deposited into ArrayExpress (ArrayExpress Accession number E-FPMI-9). Statistical predictions [15] yielded a false discovery rate of 20%, which was calculated experimentally to be 17% by the selective confirmation of more than 40 genes by qPCR. The lists of differentially expressed genes (a minimum 1.5-fold change in expression, $p<0.06$) obtained from ArrayPipe were compared to the total number of unique genes on the microarray (18730 genes, uploaded by Ensembl ID) using the Gene Ontology Tree Machine (GOTM, [16]) to identify GO categories with a significantly enriched ratio (ratio >1.0 and $p<0.01$) of differentially expressed genes. The enrichment ratio for each GO category is defined as the number of differentially expressed genes per category divided by the total number of genes from the microarray in the same category.

Quantitative real-time PCR (qPCR)

Differential gene expression was selectively validated using SuperScript® III Platinum® Two-Step qRT-PCR Kit with SYBR® Green [10], using 10 ng of total RNA as the starting material from each of the four patients and the pooled control. Primers were synthesized by Invitrogen and the sequences are available upon request.

Detection of endotoxin (LAL assay)

The LAL Chromogenic Endpoint Assay (Hycult Biotechnology) was used to determine a concentration of endotoxin (according to the best-fit-line of the standards) in the plasma from patients and control donors according to the manufacturer's instructions.

Production of ROS

ROS were detected using a luminol-enhanced CL system with a Mithras LB940 luminometer (Berthold) plate reader as previously described [9].

Table 1 Individuals in this study with X-linked chronic granulomatous disease

ID number	CGD0	CGD1	CGD37	CGD97
Clinical characteristics	Recurrent inflammatory bowel and esophageal obstruction.	Systemic infection with *Pseudallescheria boydii* during childhood. Since then healthy.	Inflammatory acne. Well until sudden death in 2005 at a location remote from Vancouver for which no cause was identified.	Progressive pulmonary inflammation. Multiple sterile open lung biopsies. Bone marrow transplantation in 2006. Died in 2007 awaiting a lung transplant.
Treatments and medications	TMP/SMX	IFNγ; TMP/SMX	IFNγ; TMP/SMX	TMP/SMX
Age at the time of first sampling	18	29	27	32
Date(s) of sampling	Sept 27, 2004 Jan 17, 2006	Sept 26, 2004 Dec 6, 2004 June 23, 2005 Oct 12, 2005	Oct 12, 2004	Sept 26, 2004
Health status over course of the study	Well during course of the study except for some episodes of bowel obstruction.	Well over course of the study.	Well until sudden unexplained death in the spring of 2005.	Pulmonary inflammation leading to death in May 2007.
Investigation	Microarray, RT-PCR, ELISA	Microarray, RT-PCR, ELISA	Microarray, RT-PCR, ELISA	RT-PCR, ELISA

TMP/SMX; trimethoprim/sulfamethoxazole, also known as co-trimoxazole.

Results

TLR signalling is dysregulated in monocytes from patients with CGD

We previously observed that CGD PBMC produce elevated protein levels of two pro-inflammatory cytokines, IL-6 and TNFα, compared to control cells in response to LPS and PGN [9]. As monocytes are a subset of PBMC that produce both ROS and substantial amounts of soluble inflammatory mediators, we predicted that this cell type may be responsible for the enhanced production of inflammatory cytokines by a mixed population of blood cells. Therefore we initially focused on gene expression patterns specifically in $CD14^+$ PBMC (monocytes) from CGD patients and healthy individuals using microarray technology.

To maximize the reliability of microarray analysis and minimize the impact of individual variability in gene expression, four individuals of the same gender and the same deficiency (in $gp91^{phox}$) were selected for the study and a control pool was constructed from 5 healthy individuals in the same general age bracket as the patients. A description of the persons with CGD can be found in Table 1. Control experiments indicated that the trends in gene expression in the control pool were consistent with those observed in other gene expression studies that implemented the same methods for *in vitro* cellular activation of primary cells and isolation of monocytes [13]. As anticipated, the expression of $gp91^{phox}$ was confirmed to be down-regulated in all patient samples.

Between 750 and 900 genes were identified as differentially expressed in CGD monocytes from individual patients compared with control monocytes under the various conditions. Of the dysregulated genes in CGD cells, approximately 59% of genes were up-regulated and 41% were down-regulated compared to controls. More than 60 real-time PCR experiments were performed to confirm the gene expression patterns reported from the microarray analysis and to determine absolute levels of expression. The gene expression (up- or down-regulation in monocytes from the various patients compared to the control) that was reported by microarray analysis had an 83% correlation with that determined by real-time PCR. While the magnitude of gene expression was never identical between individuals, the more accurate qPCR data demonstrated that, 90% of the time, the expression of individual genes was similar between the four different patients (i.e. less than 10% of the time the expression of a gene in one patient contrasted the expression in the 3 other patients).

The gene ontology (GO) tree machine (GOTM; [16]) was used to analyze the differentially expressed genes from the microarray analysis and determine the major biological processes and signalling pathways that were altered in CGD monocytes (Supplemental Tables 1 and 2). Previously, it was reported that ROS primarily alter the process of apoptosis in neutrophils [17–19]. Our analysis demonstrated that ROS deficiency in monocytes impacted on a much broader range of biological and cellular processes. Compared to normal, ROS-competent monocytes, there were significantly larger numbers of abnormally expressed genes in resting and TLR-activated CGD monocytes that were associated with the processes of cytokine/chemokine activity, the IKK/NF-κB signalling cascade, immune responses and cellular responses to stimuli. Consistent with dysregulation of these biological processes, a large number of the differentially expressed genes in resting and TLR-stimulated CGD monocytes were associated with the TLR4 signal transduction pathway as illustrated in Fig. 1 using Cytoscape clustering methodology [20]. Given that aberrant activation of TLR signalling pathways can promote disorders in both host defence and inflammation [21], both of which are clinical characteristics of CGD, the expression pattern of TLR-associated genes was investigated in more detail.

While, on the whole, differentially expressed genes in unstimulated CGD monocytes were up- or down-regulated with similar frequency, the majority (74%) of genes downstream of IKK and NF-κB in the TLR4 pathway had heightened expression in unstimulated CGD monocytes compared to control cells. Based on confirmatory qPCR experiments, the expression of 20/23 genes was elevated on average 13-fold higher in CGD cells than control cells in the absence of activation. Expression levels of the 23 genes are summarized

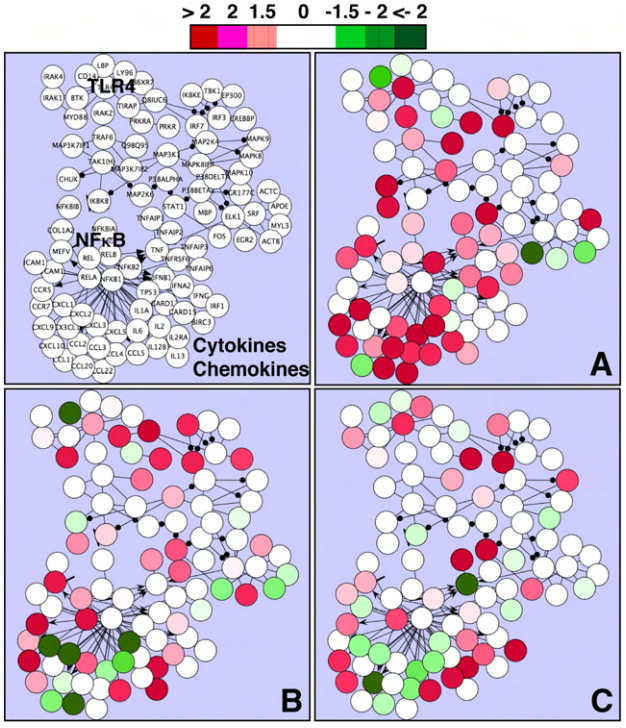

Figure 1 Differentially expressed genes in CGD monocytes map to the TLR signal transduction pathway. An interactive map of the TLR4 signal transduction pathway was integrated into Cytoscape [20], an open-source bioinformatics visualization software, as described previously [10]. Proteins and genes (nodes) are represented as circles while interactions are represented by lines between two interacting nodes. The microarray gene expression data was overlaid onto the nodes of this protein network. The node colour reflects the average gene expression in monocytes from patients with CGD relative to that in a control pool of monocytes after 4 h in the absence (A) or presence of LPS (B) or PGN (C). Green or red nodes respectively illustrate lower or higher expression of the gene in CGD monocytes relative to healthy controls. Gene names are indicated in the upper left panel.

Table 2 Gene expression by qPCR in monocytes from four CGD patients relative to expression in control cells

Gene name	CGD0	CGD1	CGD37	CGD97
TNF-α	2.5±0.0 [a]	3.3±0.8	3.6±0.2	2.0±0.1
IL-1β	4.1±0.3	4.3±0.2	6.6±0.3	3.1±0.3
IL-1F9	70.3±8.2	nd [b]	70.0±6.9	36.1±0.9
IL-1RN	22.7±0.3	10.2±0.5	8.9±0.5	22.6±0.8
IL-6	13.2±0.0	3.6±0.3	32.1±0.3	81.6±4.4
IL-10	2.9±0.4	2.1±0.1	10.0±0.0	1.8±0.3
IL-12β	4.6±0.0	nd	37.1±3.4	1.3±0.1
IFIT1	27.6±0.7	nd	37.8±2.8	11.5±3.9
CCL8	32.4±1.9	179.2±2.6	48.5±5.5	9.0±0.6
CXCL1	5.9±0.4	3.7±0.1	7.3±0.2	3.5±0.0
CXCL6	40.8±3.2	32.1±20.2	22.8±2.3	34.2±5.8
EBI3	1.9±0.1	0.6±0.5	4.6±0.2	1.2±0.1
CFLAR	2.0±0.5	4.6±0.0	2.3±0.0	1.1±1.0
MX2	18.2±1.2	nd	20.4±1.4	5.2±0.1
RIPK2	5.6±0.1	11.9±1.6	3.9±0.1	2.9±0.3
SOCS1	9.3±0.3	18.8±0.4	12.3±0.5	4.2±0.1
NF-κB2 (p100/p52)	1.6±0.3	0.9±0.4	2.6±0.1	0.9±0.1
RelB	1.1±0.1	0.9±0.0	1.0±0.0	0.8±0.0
c-Rel	0.9±0.0	0.9±0.1	1.4±0.0	0.7±0.0
NF-κB1 (p105/p50)	2.5±0.0	1.5±0.1	2.5±0.0	1.3±0.1
RelA (p65)	1.7±0.0	2.5±0.4	1.8±0.0	1.2±0.1
IκBζ	1.3±0.0	1.6±0.1	2.7±0.2	13.9±1.8
BATF	5.3±0.2	5.7±1.8	10.7±0.2	1.9±0.5

[a] Fold changes normalized to endogenous GAPDH in CGD cells then expressed relative to that in the pool of unstimulated control monocytes (fold change = 1.0) ± the standard deviation of 2 technical replicates.
[b] nd = not determined.

in Table 2 and examples of the RT-PCR results are shown in Figs. 2 (unstim) and 3A. Enhanced transcription of IL-6, IL-8 and TNF-α correlated with higher levels of protein in the TCS of unstimulated CGD monocytes (Fig. 3B), as determined by multiplex bead assays. The subset of up-regulated genes in resting CGD cells included a number of genes for classic pro-inflammatory cytokines and chemokines that are normally expressed only after cellular activation. The gene expression patterns of unstimulated patient cells had striking similarity to that normally observed in ROS-competent monocytic cells activated with LPS (Fig. 3C). In fact, most genes identified as differentially expressed (p-value ≤ 0.05 by Student's t-test) in unstimulated CGD monocytes (compared to unstimulated control cells) were expressed to a similar degree in normal monocytic cells stimulated for 4 h with LPS [10]. Supplemental Table 3 provides a list of the common up-regulated genes. Thus, unstimulated CGD monocytes had a transcriptional profile characteristic of TLR-activated cells and this hyper-inflammatory nature of the cells corresponded with the predisposition of individuals with CGD to exhibit sterile inflammatory disorders.

Following stimulation with the TLR agonists, LPS or PGN, for 4 h, 857 and 751 genes respectively, were differentially expressed in CGD monocytes compared to similarly stimulated control monocytes. More than half of these dysregulated genes (61% for LPS and 62% for PGN) were expressed at a higher level in CGD monocytes compared to control cells, on average 2-fold higher as determined by qPCR experiments. The expression levels of major inflammatory mediators, including IL-1β, IL-6, IL-8, IL-10, IL-12β, TNF-α, IFN-γ, IFIT1, and CCL8, were significantly up-regulated in CGD monocytes (examples shown in Fig. 2). Consistent with our previous findings in TLR-activated CGD PBMC [9] as well as a report that IL-8 was elevated in fMLF-induced CGD neutrophils [22], a corresponding increase in secreted IL-1β, IL-6, IL-8, and TNF-α proteins was confirmed by multiplex bead assay (Fig. 4 and data not shown).

These data demonstrate that the CGD mutation associated with the absence of ROS is also correlated with the enhanced transcription and translation of inflammatory mediators in peripheral blood monocytes, both unstimulated and stimulated with TLR agonists LPS or PGN. These studies expanded the range of pro-inflammatory cytokines confirmed to be up-regulated in CGD from the two described previously (TNFα and IL-6) to ten including IL-1β, IL1F9, IL1RN, IL-12β, IFIT1, CCL8, CXCL1, and CXCL6. The over expression of inflammatory genes was consistently observed in cells from all four patients compared to cells from healthy individuals. Further, these data indicated that ROS-deficient monocytes are likely responsible for the exaggerated levels

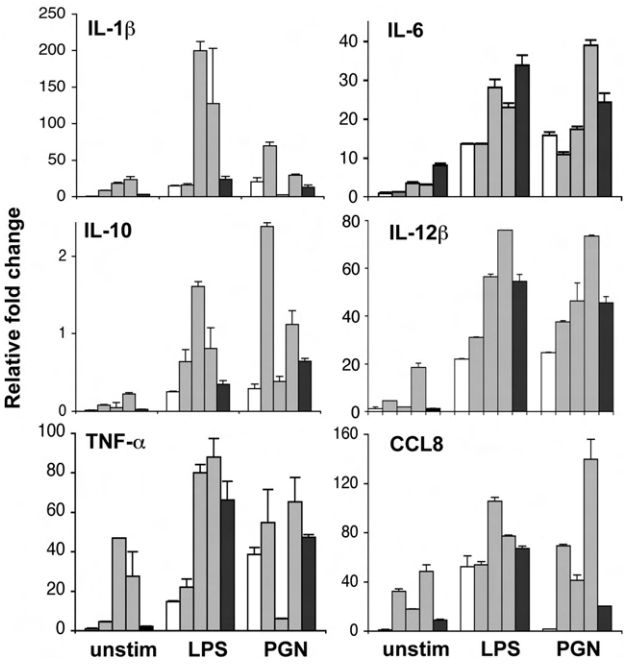

Figure 2 Inflammatory genes highly expressed in CGD monocytes following stimulation with TLR agonists. The relative expression (*Y*-axis) of pro-inflammatory cytokines and chemokines in monocytes from four CGD patients (grey bars) or a pool of five controls (white bars) following culture for 4 h (unstimulated) with LPS or PGN were measured by real-time PCR. The dark grey bars show gene expression in ROS-deficient monocytes that were not interrogated by microarray analysis, thus serving as an independent validation of the data. Fold changes were normalized to endogenous GAPDH then expressed relative to that in the pool of unstimulated control monocytes ± the standard deviation of 2 technical replicates.

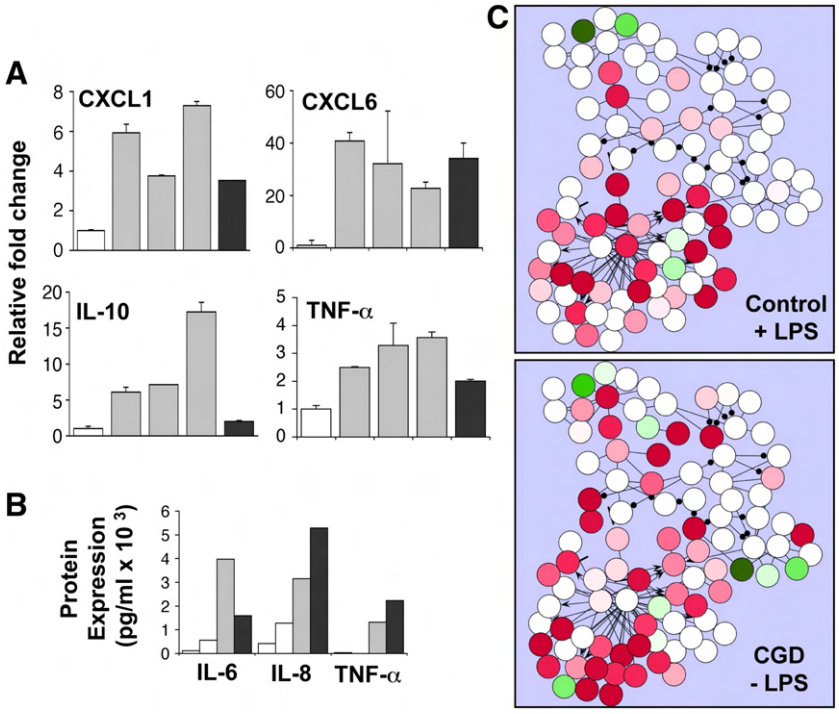

Figure 3 Unstimulated CGD monocytes have profoundly heightened expression of inflammatory genes and transcriptional signatures similar to TLR-activated control (ROS-competent) monocytes. (A) The relative expression (*Y*-axis) of chemokines (CXCL1, CXCL6) and inflammatory cytokines (TNF-α, IL-10) in unstimulated monocytes from four patients with CGD (grey bars) or unstimulated monocytes from healthy controls (white bars) were measured by real-time PCR following 4 h culture *in vitro*. Fold changes were normalized to endogenous GAPDH then expressed relative to that in unstimulated control monocytes ± the standard deviation of 2 technical replicates. (B) Mutiplex bead assays were used to measure pro-inflammatory cytokines (pg/ml × 10^3 ± standard deviation; *Y*-axis) in the tissue culture supernatants of PBMC from 2 healthy donors (white bars) and 2 CGD patients (grey bars) after 4 h culture *in vitro* in the absence of cellular activation. The dark grey bars show gene expression (in A) and protein secretion (in B) in ROS-deficient monocytes that were not interrogated by microarray analysis. (C) Microarray gene expression data was overlayed on the TLR4 pathway illustrating gene expression in THP-1 monocytic cells in response to stimulation with LPS for 4 h (upper panel) [10]. The lower panel shows a similar pattern of gene expression in (4 h) unstimulated CGD monocytes relative to that in unstimulated controls. The legend for node colours and gene names are as in Figure 1.

of cytokines observed in the tissue culture supernatants (TCS) following the stimulation of a mixed population of leukocytes with TLR agonists. Thus monocytes are an important cell type contributing to the inflammatory phenotype of CGD patients.

Lack of up-regulation of anti-inflammatory mediators

The expression of inflammatory mediators other than the classic inflammatory cytokines and chemokines discussed

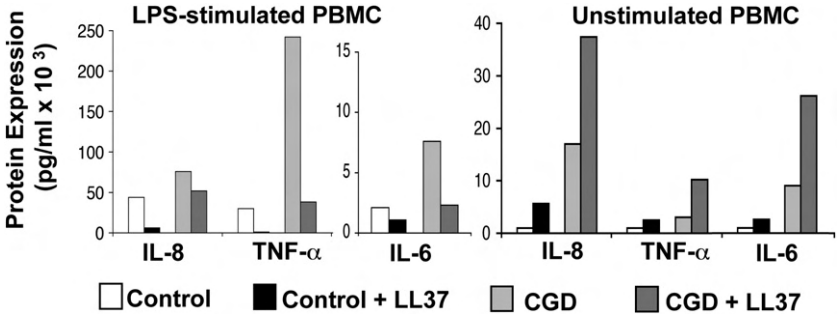

Figure 4 CGD monocytes have intrinsic inflammatory transcriptional activity. The expression of pro-inflammatory cytokines IL-6, IL-8 and TNF-α (*Y*-axis) after 24 h culture of PBMC from healthy individuals (white bars) or one CGD patient (light grey bars) in the absence (right panel) or presence of LPS (left panel) and the absence or presence of the anti-inflammatory peptide LL-37 (black bars for control and dark grey bars for CGD) as determined by multiplex bead assays. Shown is a representative result of the experiment that was performed three times with PBMC from the different control donors and the same CGD patient.

Table 3 Gene expression by microarray analysis of anti-inflammatory genes in CGD monocytes relative to gene expression in control cells

Gene name	Gene description	Unstimulated		+LPS		+PGN	
		4 h	24 h	4 h	24 h	4 h	24 h
TGFB2	TGF-beta 2 has suppressive effects on interleukin-2 dependent T-cell growth.	−1.1[a]	−1.1	−1.4	−1.0	1.2	−1.5
TGFBR2	Type I/type II TGF-beta receptors.	−1.7	1.0	1.2	−1.5	1.1	−1.5
IL-13	Inhibits inflammatory cytokine production. May be critical in regulating inflammatory and immune responses.	1.3	−1.5	1.4	−1.2	1.1	1.1
IL-18BP	Binds to IL-18 and inhibits its activity. Functions as an inhibitor of the early T_H1 cytokine response.	1.5	−1.2	1.1	1.2	1.5	−1.3
IL-1F5	Is a highly and a specific antagonist of IL-1F9.	−1.6	1.0	1.0	−1.1	1.1	1.0
NF-κBIA	Inhibits NF-κB by complexing with and trapping it in the cytoplasm. May be involved in regulation of transcriptional responses to NF-κB, including cell adhesion, immune and pro-inflammatory responses, apoptosis, differentiation and growth.	1.1	1.3	−1.1	1.0	1.0	−1.0
NF-κBIB	Inhibits NF-κB by complexing with and trapping it in the cytoplasm. The unphosphorylated form resynthesized after cell stimulation is able to bind NF-κB allowing its transport to the nucleus and protecting it to further IKBα-dependent inactivation.	1.1	−1.0	1.1	1.1	1.0	−1.0
NF-κBIE	Inhibits NF-κB by complexing with and trapping it in the cytoplasm.	1.1	1.4	1.2	1.0	1.0	−1.0
NF-κBIL1	Inhibits NF-κB by sequestering it in the cytoplasm or also inhibits NF-κB-DNA binding.	−1.1	−1.1	−1.1	1.0	1.0	1.0

[a] The average ratio of gene expression in monocytes from 4 patients with CGD relative to expression in control cells (fold change = 1.0).

above were also elevated in CGD monocytes. For example, the gene for IL-1 family member 9 (IL-1F9), a ligand of the IL-1 receptor related protein 2 (IL-1RL2/IL-1R-rp2) and a stimulant of NF-κB activity, was up-regulated in unstimulated (2.9 fold) and LPS-stimulated (1.8 fold) CGD monocytes compared to the expression of this gene in control unstimulated or LPS-stimulated monocytes. While IL-1F9 gene expression was enhanced, IL-IF5, a specific antagonist of IL-1F9, was not (Table 3). Likewise in monocytes, genes that encode other anti-inflammatory mediators, such as the cytokines TGFβ2, IL-13, IL-18-binding protein (IL-18BP) and the TGFβ receptor (TGFBR2) did not demonstrate elevated expression in CGD monocytes compared to controls (fold change was <1.5; Table 3). These data indicate that the defect in CGD monocytes caused a specific (pro-inflammatory) cellular dysfunction, rather than a general enhancement of gene transcription and was consistent with the hypothesis that the CGD cells are intrinsically pro-inflammatory.

ROS-deficient cells are inherently pro-inflammatory

None of the patients had acute illness or infection at the time that blood was donated for the study. Furthermore, an LAL assay performed on the plasma from the patients' blood excluded the possibility that the observed hyper-inflammatory phenotype of the CGD cells was due to circulating endotoxin *in vivo*. There was no significant difference in the average level of endotoxin in plasma from six healthy individuals (including plasma from 4/5 individuals whose monocytes were part of the control group for the microarray and qPCR analyses) and the four individuals with CGD (3.6 ± 0.7 and 3.9 ± 1.7 pg/ml endotoxin in control and CGD plasma respectively). To further address the possibility that TLR agonists, including LPS, were pre-activating the PBMC *in vivo*, cells were activated *ex vivo* in the presence or absence of LPS and a human host defence peptide, LL37. This peptide potently blocks gene transcription induced by certain bacterial signature molecules including Gram positive lipoteichoic acid, Gram negative LPS and bacterial CpG oligonucleotides through TLRs 2, 4, and 9, respectively but not endogenous pro-inflammatory mediators [10]. In the absence of TLR agonists, the peptide modestly promotes the expression of certain pro-inflammatory chemokines [10]. As anticipated, the peptide inhibited >90% of IL-6, IL-8 and TNF-α production by LPS-activated PBMC from both CGD patients and healthy donors (Fig. 4). If PBMC from the CGD patients had been previously activated by bacterial ligands *in vivo*, the peptide alone would be anticipated to have suppressed cytokine induction from resting CGD cells compared to the cells from healthy individuals. In contrast, the peptide moderately promoted expression of cytokines by both unstimulated PBMC from CGD patients and, to a lesser extent, healthy donors (Fig. 4). To demonstrate conclusively that a deficiency in ROS production is sufficient to promote the expression of inflammatory mediators, we evaluated the

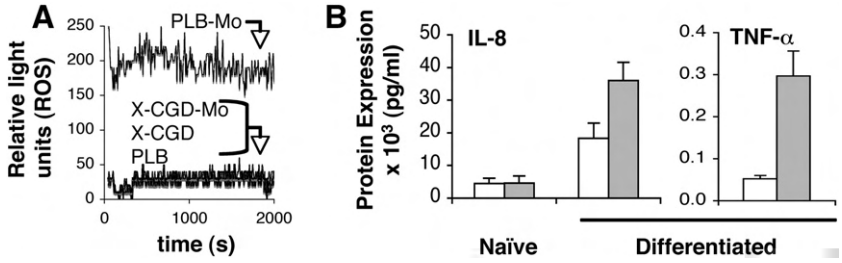

Figure 5 ROS-deficient gp91phoxP415H PLB-X-CGD cell line produced more IL-8 and TNFα than control, ROS-competent PLB cells. (A) The production of radicals was detected in control PLB cells differentiated towards a myeloid phenotype (PLB-Mo) but not in naive PLB cells (PLB), naive or differentiated gp91^{phox}P415H cells (X-CGD and X-CGD-Mo). Radicals were measured over time (*X*-axis) and radical production by control primary neutrophils in this experiment (not shown) was ~1000 relative light units. (B) Spontaneous production of IL-8 (left graph) and TNFα (right graph) was detected (pg/ml × 10^3 ± standard deviation, *Y*-axis) in the tissue culture supernatant from naive or differentiated control PLB cells (white bars) and X-CGD cells (grey bars) over a 24-hour period (see Materials and methods for details).

expression of IL-8 and TNFα in a control, ROS-competent PLB cell line and a mutant, gp91^{phox}-deficient cell line (X-CGD) [11]. The cell lines were cultured in the naive state as well differentiated towards a myeloid phenotype (see Materials and methods for details) in order to promote radical production in the control cells (Fig. 5A). No radicals were detected in naive cells (control or X-CGD) or differentiated X-CGD cells. Differentiated, unstimulated gp91^{phox}-deficient cells, like primary monocytes from CGD patients, produced elevated levels of the inflammatory mediators IL-8 and TNFα (2.0±0.4, n=4 and 5.3±3.2, n=4 respectively) compared to control, differentiated (ROS-producing) cells (Fig. 5B). IL-8, but not TNFα, was detected in naive cell culture supernatants (again, without stimulation) and in this case, the levels of IL-8 were equivalent between control and X-CGD cells. This is consistent with the observed inability of the naive PLB cells to produce radicals. It is noteworthy that the cells were cultured in the presence of antibiotics and the enhanced secretion of TNFα and IL-8 could be observed in the absence of cellular activation, a phenomenon also observed in the CGD cells. Together with the results that significant levels of endotoxin were not detected in the plasma of patients and that a peptide known to block TLR-induced inflammatory cytokine production had no negative impact on cytokine production by the unstimulated CGD cells, it is proposed that the robust production of inflammatory proteins by CGD cells did not result from *in vivo* activation by circulating endotoxin or TLR-2 -4 or -9 agonists.

ROS-deficient cells have heightened expression of NF-κB subunits

Our findings (Figs. 1–5) expand on the findings of previous reports in demonstrating the heightened expression of a broad range of NF-κB-regulated cytokines in the absence of ROS [9,23–25]. These observations contrast with several reports that indicate that production of ROS is obligatory for NF-κB activity [23,26–29]. We previously demonstrated [9] that inhibitors of the activation of NF-κB were able to block the TLR-induced production of pro-inflammatory cytokines in both CGD and control monocytes (confirmed here for secretion of TNF-α and

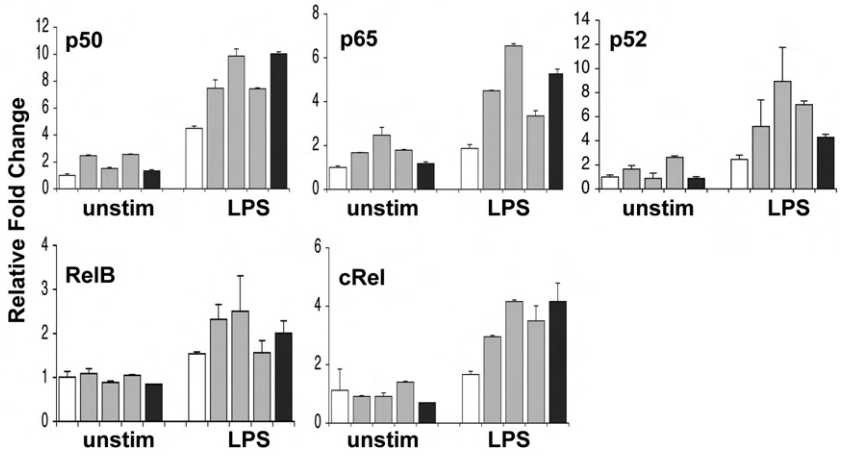

Figure 6 Induction of NF-κB subunit expression in CGD monocytes. The relative expression (Y-axis) of NF-κB subunits (NF-κB1 (p50), NF-κB2 (p52), RelA (p65), RelB and c-Rel) in 4 h unstimulated or LPS-stimulated monocytes from healthy individuals (white bars) or from each of the four individuals with CGD (grey bars) were determined by real-time PCR. The dark grey bars show gene expression in ROS-deficient monocytes that were not interrogated by microarray analysis. Fold changes were normalized to endogenous GAPDH and expressed relative to that in unstimulated control monocytes±the standard deviation of 2 technical replicates.

IL-6 by both CGD and control cells (Fig. 7). In these studies there were no observed differences between CGD and control cells with respect to the abundance of the p50 subunit of NF-κB, phosphorylation and degradation of IkBα or the kinetics, over the first hour, of p50 translocation into the nucleus following activation with LPS. Thus early activation of NF-κB itself was apparently not altered in CGD cells. We examined here if prolonged signalling by NF-κB or altered signalling via other arms of the TLR signalling pathway might be responsible for the enhanced expression of inflammatory mediators in CGD monocytes.

To replenish cytoplasmic pools of NFκB, the depletion of cytoplasmic NFκB is accompanied by new transcription of NFκB subunits [8]. Thus a higher level of gene expression of NFκB subunits is consistent with more rapid turnover. The p50 and p65 subunits form the transcriptionally active NF-κB heterodimer most often implicated in the expression of inflammatory genes. Consistent with the elevated expression of inflammatory genes in CGD monocytes, the genes for NF-κB subunits NF-κB1 (p50) and RelA (p65) were expressed at significantly higher levels in unstimulated CGD monocytes compared to control monocytes, as determined by qPCR (Fig. 6). Likewise, NF-κB1 (p50) and RelA (p65) as well as NF-κB subunits NF-κB2 (p52), RelB and c-Rel were induced to a higher level in LPS-stimulated CGD monocytes compared to control cells (Fig. 6). Therefore, it seems likely that the heightened expression of NF-κB subunits could sustain signalling in unstimulated and activated CGD monocytes, particularly since several negative regulators of NF-κB (NF-κBIA, NF-κBIB, NF-κBIE, NF-κBIL1) were not up-regulated in CGD monocytes (Table 3).

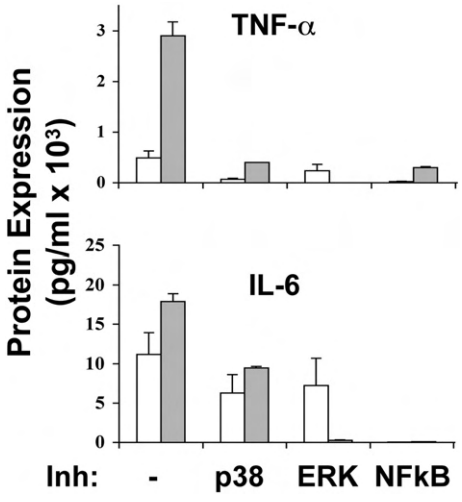

Figure 7 Induction of inflammatory cytokines in CGD monocytes is dependent on ERK. Mutiplex bead assays were used to measure pro-inflammatory cytokines (pg/ml × 103 ± standard deviation, Y-axis) in the tissue culture supernatants of PBMC from 3 healthy donors (white bars; standard deviation calculated from 3 biological replicates) and 1 CGD patient (grey bars; standard deviation calculated from 3 technical replicates) after 1 h culture *in vitro* in the presence of LPS alone or with chemical inhibitors of p38, ERKor NF-κB (X-axis).

Role of p38 and ERK1/2 MAP kinases in enhanced inflammation of CGD monocytes

MAP kinases are also part of the TLR signalling cascade that, either independently or through the activation of transcription factors such as AP-1, Elk, Ets-1 and CREB, influence the expression of NF-κB-regulated genes. Thus, inhibitors of the MAPK ERK1/2 and p38 were used to determine the contribution of these signalling cascades to the enhanced expression of TLR-inducible genes in CGD monocytes. An inhibitor of ERK1/2 significantly reduced inflammatory cytokines produced by CGD cells (98 ± 1% and 100 ± 0% reduction of IL-6 and TNF-α production respectively) yet only had a partial effect on IL-6 and TNF-α production by control PBMC (43 ± 28% and 54 ± 28% respectively) (Fig. 7). An inhibitor of p38 MAPK demonstrated a milder inhibition with similarly reduced expression of these inflammatory cytokines in tissue culture supernatants from CGD (37 ± 5% and 83 ± 0% reduction in IL-6 and TNF-α respectively) and control (48 ± 12% and 84 ± 7% reduction in IL-6 and TNF-α respectively) cells (Fig. 7). These results indicate that the heightened expression of TLR-induced, NF-κB-dependent genes by CGD cells had an enhanced dependence on ERK1/2 signalling after exposure to TLR agonists and may indicate that under normal conditions, ROS suppress ERK1/2 signalling to control the transcription of pro-inflammatory mediators.

Discussion

Phagocytes, primarily neutrophils, have been extensively studied in the pathology of CGD since their ability to kill and clear bacteria and fungi early in an infection is in large part dependent on the production of ROS. Relatively few studies have evaluated the contribution of mononuclear cells to CGD pathology and the development of inflammatory disorders associated with this condition. Using microarrays to gain insight into the inflammatory conditions associated with CGD, and the contribution of monocytes to the disease, we obtained evidence that was consistent with the hypothesis that primary monocytes are an important element in CGD-associated hyper-inflammatory conditions, in that pro-inflammatory genes in these cells are consistently up-regulated at the level of transcription. Indeed resting monocytes from CGD patients had profound alterations in gene expression and a transcriptional profile highly similar to TLR-activated inflammatory monocytes from non-CGD individuals.

As CGD is a rare disease, the findings from this study were limited to an analysis of four individuals with diagnosed X-linked CGD resulting from a mutation in gp91^{phox}. The simplest, most direct evaluation of gene expression in three of the patients was rendered possible through the use of a reference control pool that behaved similarly to control cells from individual volunteers employed in other studies by our group [10,13]. Cells from one patient that was not included in the microarray analysis served as an independent validation of the microarray data in qPCR and ELISA. We reiterate that the array data is only a true reflection of gene expression in the three patients studied and contains approximately 17% false positive results. We suggest that more biologically significant information with an expectedly lower false discover rate than that associated with the

microarray can be found in Supplemental Table 1, highlighting ROS-dependent biological and cellular processes rather than individual genes. While tempting, the small sample size and use of a pooled control cautions us about extrapolation of the microarray results to other individuals that suffer from CGD.

Infection can induce pro-inflammatory responses, which upon induction are often transient in the absence of other immune processes. Thus we were faced with three prospective possibilities. First it was possible that previous suggestions concerning infections by uncharacterized and presumably unculturable organisms [5] might be responsible for the observed hyper-inflammatory state. As shown in Fig. 5, a gp91^{phox}-deficient cell line cultured *in vitro* in the presence of antibiotics and absence of cellular stimulation produced higher levels of TNF-α and IL-8 protein than ROS-competent control cells. This is probably the most compelling, supporting evidence that ROS deficiency is sufficient to mediate the over expression of inflammatory genes in a sterile environment. In conjunction with an absence of clinical symptoms of illness in our CGD patients, comparable low levels of circulating endotoxin in patients and controls and the inability of LL-37 anti-inflammatory peptide to knock down the cytokine response in resting CGD monocytes this strongly argued against the possibility that bacterial or fungal agonists of TLR receptors or other pro-inflammatory stimuli, endogenous or exogenous, were responsible for the elevated expression of inflammatory mediators in blood monocytes from CGD patients.

It is difficult to conclusively determine if the over expression of inflammatory molecules in ROS-deficient cells drives the development of inflammatory diseases or if repeated infections that are slow to resolve in CGD individuals are the catalyst for the inflammatory disease. A second possibility then, is that inflammation is re-triggered from time to time by infections but an imbalance, due to a deficient ability to generate ROS, blocks the control of inflammatory responses such that they fail to return to baseline after initial stimulation. Defective regulation of the expression of inflammatory genes appears sufficient to drive inflammatory disorders; administration of sterile *Aspergillus fumigatus* hyphae to the lungs of CGD mice induced the exaggerated production of KC (equivalent to Gro-α in humans), IL-1β, and TNF-α [30], and it has been correspondingly shown that elevated levels of chemokines contribute to granuloma formation [31], a characteristic inflammatory symptom of many CGD patients. Our data also tends to favour this explanation, namely that ROS are required to properly control inflammatory responses resulting in the typical spiking responses of pro-inflammatory cytokines that are associated with acute inflammation, peaking at 4–6 h and declining thereafter. Consistent with this we saw a modest decline in several anti-inflammatory molecules in CGD monocytes.

The third possibility is that the oxidant–antioxidant imbalance caused by the CGD mutation leads to continuous stimulation of inflammatory responses. The oxidant–antioxidant balance is influential to immune cell function [reviewed in 32]. Since most transcription factors contain DNA-binding Zn-finger domains and Zn-finger coordination is a redox-sensitive cellular event, DNA-binding and transcription are likely to change with cellular redox states. Indeed, AP-1, NF-κB, CREB, Sp3, and Nrf2 are known to be redox-sensitive transcription factors [reviewed in 33]. The sensitivity may result from alterations in Zn-finger coordination but could also result from changes in protein oxidation or activation of upstream, redox-sensitive phospho-tyrosine kinases [reviewed in 6,7,32-35]. Studies aimed to address the role(s) of ROS in cellular processes have, more often than not, utilized chemical antioxidants and inhibitors of the NADPH-oxidase to create ROS-deficient cells. This is a logical approach given the scarcity of individuals with CGD. In such studies, artificial ROS deficiency causes a cellular inflammatory phenotype that is opposite to the phenotype of cells from individuals with CGD [9]. The contradictory results regarding the role(s) of ROS in cellular processes may be due to the use of such chemical inhibitors that have inherently pleiotropic effects on cells, or of cells from different species, lineages and states of activation [reviewed in 7,34,35].

Our *in vitro* evidence for an anti-inflammatory role of ROS and not the contradictory reports that suggest a pro-inflammatory role, is consistent with several *in vivo* studies. For example, mice with allelic polymorphisms in the Ncf1 gene (p47^{phox}) and a lower capacity to produce ROS are more susceptible to develop severe arthritis [36,37] and experimental autoimmune encephalomyelitis [38]. The gp91^{phox}-deficient mice are susceptible to severe, T-cell-dependent (collagen-induced) arthritis [39] and persistent, severe, sterile hyper-inflammation and tissue necrosis [40]. It also intriguing that the maternal carriers of CGD patients have a higher incidence of discoid lupus, an autoimmune disease of the rheumatic family [41]. This leads us to suggest that ROS are imperative molecules that control inflammation during microbial challenge and prevent the development of inflammatory disorders (seen in CGD).

Reports by Moreland et al. and Harfouche et al. have demonstrated hyper-phosphorylation of the MAPK p38, respectively, in ROS-deficient neutrophils and human endothelial cells [42,43], although the inhibitor experiments reported here indicate only a partial dependency of the CGD inflammatory phenotype on p38. A more robust and significant contribution to the heightened production of inflammatory cytokines in ROS-deficient monocytes came from the ERK1/2 MAPK signalling pathway. MAP kinases such as ERK1/2 and p38 are activated, in part, by redox-sensitive phosphorylation on tyrosine residues. Over active ERK1/2 signalling would presumably enhance activity of the transcription factors such as AP-1 and Elk-1 and promote the expression of genes that are dependent and/or co-dependent on these transcription factors and NF-κB. While the initial LPS-induced translocation of the p50/p65 NF-κB heterodimer to the nucleus occurred with similar kinetics in CGD and control monocytes [9], it was observed here that the genes encoding p50, p65 and other subunits of NF-κB, but not NF-κB inhibitory molecules, were elevated in CGD monocytes (Fig. 6, Table 3). This imbalance in positive and negative regulators could allow prolonged NF-κB signalling in CGD cells without a compensatory suppression. Thus we conclude that a loss of ROS-dependent restraint on ERK1/2 signalling pathways and/or levels of NF-κB is sufficient to drive the over expression of genes in CGD cells. Consequently, in addition to the already established contributions of ROS in host defences via oxidative killing of microbes and stimulation of neutrophil apoptosis, we hypothesize that ROS contribute to host defences by controlling the expression of

inflammatory genes. A detailed analysis of redox-sensitive events in MAPK and NF-κB pathways under homeostatic conditions and upon immune challenge will be the focus of future investigations.

An imbalance in cytokines and chemokines, as seen in CGD, may alter cell-to-cell communication and cellular differentiation responses and cause damage to host tissues that would probably result in defective immune defences and prolong the inflammatory response after microbial challenge. Reports prior to this one demonstrated that phagocytosis or fMLF stimulation of CGD neutrophils [18,22] or LPS-activation of monocyte-derived CGD dendritic cells [25] led to enhanced cytokine production (compared to control cells).

The severity of inflammatory disease among our four patients varied considerably however, this variation was not reflected by grossly different patterns of gene expression. In fact, the pattern of gene expression was consistent in all four patients for 90% of the genes evaluated by qPCR. This argues that the genes we identified are implicitly associated with the disease yet also suggests that other genetic modifiers, such as single nucleotide polymorphisms (SNP), may be important in the progression and severity of inflammatory conditions in CGD. As reported by CB Foster et al. [44], seven genes, namely myeloperoxidase, mannose-binding lectin, $F_c\gamma$ receptors IIa, IIIa, IIIb, TNFα and IL-1 receptor antagonist were identified in 129 CGD patients to have polymorphisms that are predicted to influence the host inflammatory response. The authors suggest that while polymorphisms in these genes may have little or no effect on the general population, they may have significant effects on persons with CGD or other immune-compromised individuals. Intriguingly, five genes containing SNPs suggested by Foster to be associated with CGD inflammation, were annotated on our array and three ($F_c\gamma$ receptor IIIa, TNFα and IL-1 receptor antagonist) were differentially expressed in the CGD patients compared to the controls.

In this study, 2 of the 4 studied patients with CGD were receiving IFN-γ treatment. We failed to observe any obvious alterations in gene expression profiles that differentiated these patients from the two who were not receiving IFN-γ. This is consistent with a report by Kobayashi et al. that gene expression in neutrophils was very similar in CGD patients receiving a diverse range of medications including prednisone, anti-virals/fungals/bacterials or IFN-γ [18]. While there is a definite prophylactic benefit of IFN-γ treatment of CGD in humans and mice, years of *in vitro* and *in vivo* studies have failed to show consistent effects of IFN-γ that would explain its effectiveness [45,46]. Furthermore, while IFN-γ is effective in preventing infection, it appears to have no effect on the inflammatory disorders of CGD [46]. Collectively the studies strongly suggest that IFN-γ, and possibly other medical treatments, may not alter gene transcription but are more likely to exert their positive effect(s) via alterations to cellular post-transcriptional/translational events.

These data are thus consistent with the hypothesis that ROS are critical in maintaining cellular inflammatory homeostasis by dampening the expression of inflammatory genes, which is influenced by altered signalling through MAP kinases. Furthermore, our results suggest that the robust hyper-inflammatory state of unstimulated CGD monocytes plays a significant role in the pathology of inflammatory disorders associated with CGD. These studies provide a platform for the identification of ROS-regulated genes (and therefore potential insight into the mechanism of action of ROS), genes that are essential for the development of chronic inflammatory disorders, and points of intervention for development of novel anti-inflammatory agents for treatment of CGD and other inflammatory disorders.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.clim.2008.06.005.

References

[1] R.A. Bridges, H. Berendes, R.A. Good, A fatal granulomatous disease of childhood, Am. J. Dis. Child 97 (1959) 387–408.
[2] J. Bylund, D. Goldblatt, D.P. Speert, Chronic granulomatous disease: from genetic defect to clinical presentation. Adv. Exp. Med. Biol. 568 (2005) 67–87.
[3] B.H. Segal, T.L. Leto, J.I. Gallin, H.L. Malech, S.M. Holland, Genetic biochemical, and clinical features of chronic granulomatous disease, Medicine 79 (2000) 170–200.
[4] J.S. Huang, D. Noack, J. Rae, B.A. Ellis, R. Newbury, A.L. Pong, J.E. Lavine, J.T. Curnutte, J. Bastian, Chronic granulomatous disease caused by a deficiency in p47(phox) mimicking Crohn's disease, Clin. Gastroenterol. Hepatol 2 (2004) 690–695.
[5] D.E. Greenberg, L. Ding, A.M. Zelazny, F. Stock, A. Wong, V.L. Anderson, G. Miller, D.E. Kleiner, A.R. Tenorio, L. Brinster, D.W. Dorward, P.R. Murray, S.M. Holland, A novel bacterium associated with lymphadenitis in a patient with chronic granulomatous disease, PLoS Pathog 2 (2006) e28.
[6] H. Kimura, T. Sawada, S. Oshima, K. Kozawa, T. Ishioka, M. Kato, Toxicity and roles of reactive oxygen species, Curr. Drug Targets Inflamm Allergy 4 (2005) 489–495.
[7] G. Gloire, S. Legrand-Poels, J. Piette, NF-kB activation by reactive oxygen species, Fifteen years later, Biochem. Pharmacol. 72 (2006) 1493–1505.
[8] M.S. Hayden, S. Ghosh, Signalling to NF-kB, Genes Dev. 18 (2004) 2195–2224.
[9] J. Bylund, K.L. MacDonald, K.L. Brown, P. Mydel, V.L. Collin, R.E. Hancock, D.P. Speert, Enhanced inflammatory responses of chronic granulomatous disease leukocytes involve ROS-

independent activation of NF-kB. Eur. J. Immunol. 37 (2007) 1087–1096.
[10] N. Mookherjee, K.L. Brown, D.M.E. Bowdish, S. Doria, R. Falsafi, K. Hokamp, F. Roche, R. Mu, G.H. Doho, J. Pistolic, J.-P. Powers, J. Bryan, F.S.L. Brinkman, R.E.W. Hancock, Modulation of the Toll-like receptor-mediated inflammatory response by the endogenous human host defence peptide LL-37, J. Immunol. 176 (2006) 2455–2464.
[11] L. Zhen, A.A. King, Y. Xiao, S.J. Chanock, S.H. Orkin, M.C. Dinauer, Gene targeting of X chromosome-linked chronic granulomatous disease locus in a human myeloid leukemia cell line and rescue by expression of recombinant gp91phox. Proc. Natl. Acad. Sci. U. S. A. 90 (1993) 9832–9836.
[12] K.L. Brown, A. Maiti, P. Johnson, Role of sulfation in CD44-mediated hyaluronan binding induced by inflammatory mediators in human $CD14^{+}$ peripheral blood monocytes, J. Immunol. 167 (2001) 5367–5374.
[13] M.G. Scott, E. Dullaghan, N. Mookherjee, N. Glavas, M. Waldbrook, A. Thompson, A. Wang, K. Lee, S. Doria, P. Hamill, J.J. Yu, Y. Li, O. Donini, M.M. Guarna, B.B. Finlay, J.R. North, R.E. Hancock, An anti-infective peptide that selectively modulates the innate immune response. Nat. Biotechnol. 25 (2007) 465–472.
[14] K. Hokamp, F.M. Roche, M. Acab, M.E. Rousseau, B. Kuo, D. Goode, D. Aeschliman, J. Bryan, L.A. Babiuk, R.E.W. Hancock, F.S. Brinkman, ArrayPipe: a flexible processing pipeline for microarray data, Nucl. Acids Res. 32 (2004) W457–W459.
[15] J.D. Storey, A direct approach to false discovery rates, J. Roy. Stat. Soc. Series B 64 (2002) 479–498.
[16] B. Zhang, D. Schmoyer, K. Kirov, J. Snoddy, GOTree Machine (GOTM): a web-based platform for interpreting sets of interesting genes using gene ontology hierarchies, BMC Bioinformatics 5 (2004) 16.
[17] J.R. Brown, D. Goldblatt, J. Buddle, L. Morton, A.J. Thrasher, Diminished production of anti-inflammatory mediators during neutrophil apoptosis and macrophage phagocytosis in chronic granulomatous disease (CGD), J. Leukoc. Biol. 73 (2003) 591–599.
[18] S.D. Kobayashi, J.M. Voyich, K.R. Braughton, A.R. Whitney, W.M. Nauseef, H.L. Malech, F.R. DeLeo, Gene expression profiling provides insight into the pathophysiology of chronic granulomatous disease, J. Immunol. 172 (2004) 636–643.
[19] J. Bylund, P.A. Campsall, R.C. Ma, B.A. Conway, D.P. Speert, Burkholderia cenocepacia induces neutrophil necrosis in chronic granulomatous disease, J. Immunol. 174 (2005) 3562–3569.
[20] P. Shannon, A. Markiel, Q. Ozier, N.S. Baliga, J.T. Wang, D. Ramage, N. Amin, B. Schwikowski, T. Ideker, Cytoscape: a software environment for integrated models of biomolecular interaction networks, Genome Res. 13 (2003) 2498–2504.
[21] P. Cristofaro, S.M. Opal, Role of Toll-like receptors in infection and immunity: clinical implications, Drugs 66 (2006) 15–29.
[22] J.A. Lekstrom-Himes, D.B. Kuhns, W.G. Alvord, J.I. Gallin, Inhibition of human neutrophil IL-8 production by hydrogen peroxide and dysregulation in chronic granulomatous disease. J. Immunol. 174 (2005) 411–417.
[23] M.S. Byun, K.I. Jeon, J.W. Choi, J.Y. Shim, D.M. Jue, Dual effect of oxidative stress on NF-kappaB activation in HeLa cells, Exp. Mol. Med. 34 (2002) 332–339.
[24] M. Hayakawa, H. Miyashita, I. Sakamoto, M. Kitagawa, H. Tanaka, H. Yasuda, M. Karin, K. Kikugawa, Evidence that reactive oxygen species do not mediate NF-kappaB activation, EMBO J. 22 (2003) 3356–3366.
[25] M. Vulcano, S. Dusi, D. Lissandrini, R. Badolato, P. Mazzi, E. Riboldi, E. Borroni, A. Calleri, M. Donini, A. Plebani, L. Notarangelo, T. Musso, S. Sozzani, Toll receptor-mediated regulation of NADPH oxidase in human dendritic cells, J. Immunol. 173 (2004) 5749–5756.
[26] R. Schreck, P. Rieber, P.A. Baeuerle, Reactive oxygen intermediates as apparently widely used messengers in the activation of the NF-kappa B transcription factor and HIV-1, EMBO J. 10 (1991) 2247–2258.
[27] S.H. Korn, E.F. Wouters, N. Vos, Y.M. Janssen-Heininger, Cytokine-induced activation of nuclear factor-kappa B is inhibited by hydrogen peroxide through oxidative inactivation of IkappaB kinase. J. Biol. Chem. 276 (2001) 35693–35700.
[28] K. Asehnoune, D. Strassheim, S. Mitra, J.Y. Kim, E. Abraham, Involvement of reactive oxygen species in Toll-like receptor 4-dependent activation of NF-kB1, J. Immunol. 172 (2004) 2522–2529.
[29] Q. Li, J.F. Engelhardt, Interleukin-1b induction of NFkB is partially regulated by H_2O_2-mediated-activation of NFkB-inducing kinase kinase, J. Biol. Chem. 281 (2006) 1495–1505.
[30] D.E. Morgenstern, M.A. Gifford, L.L. Li, C.M. Doerschuk, M.C. Dinauer, Absence of respiratory burst in X-linked chronic granulomatous disease mice leads to abnormalities in both host defence and inflammatory response to *Aspergillus fumigatus*. J. Exp. Med. 185 (1997) 207–218.
[31] X. Gao, T.J. Standiford, A. Rahman, M. Newstead, S.M. Holland, M.C. Dinauer, Q. Liu, A.B. Malik, Role of NADPH oxidase in the mechanism of lung neutrophil sequestration and microvessel injury induced by Gram-negative sepsis: studies in $p47phox^{-/-}$ and $gp91phox^{-/-}$ mice. J. Immunol. 168 (2002) 3974–3982.
[32] J.A. Knight, Free radicals, antioxidants, and the immune system. Ann. Clin. Lab. Sci. 30 (2000) 145–158.
[33] H. Liu, R. Colavitti, I.I. Rovira, T. Finkel, Redox-dependent transcriptional regulation, Circ. Res 97 (2005) 967–974.
[34] A. Bowie, L.A. O'Neill, Oxidative stress and nuclear factor-kappaB activation: a reassessment of the evidence in the light of recent discoveries.Biochem, Pharmacol. 59 (2000) 13–23.
[35] F.C. Fang, Antimicrobial reactive oxygen and nitrogen species: concepts and controversies, Nat. Rev. Microbiol. 2 (2004) 820–832.
[36] K.A. Gelderman, M. Hultqvist, J. Holmberg, P. Olofsson, R. Holmdahl, T cell surface redox levels determine T cell reactivity and arthritis susceptibility, PNAS 103 (2006) 12831–12836.
[37] K.A. Gelderman, M. Hultqvist, A. Pizzolla, M. Zhao, K.S. Nandakumar, R. Mattsson, R. Holmdahl, Macrophages suppress T cell responses and arthritis development in mice by producing reactive oxygen species, J. Clin. Invest. 117 (2007) 3020–3028.
[38] M. Hultqvist, P. Olofsson, J. Holmberg, B.T. Bäckström, J. Tordsson, R. Holmdahl, Enhanced autoimmunity, arthritis, and encephalomyelitis in mice with a reduced oxidative burst due to a mutation in the Ncf1 gene, PNAS 101 (2004) 12646–12651.
[39] A. George-Chandy, I. Nordström, E. Nygren, I.M. Jonsson, J. Postigo, L.V. Collins, K. Eriksson, Th17 development and autoimmune arthritis in the absence of reactive oxygen species. Eur. J. Immunol. 38 (2008) 1118–1126.
[40] M.G. Schäppi, C. Deffert, L. Fiette, G. Gavazzi, F.R. Herrmann, D.C. Belli, K.-H. Krause, Branched fungal b-glucan causes hyperinflammation and necrosis in phagocyte NADPH oxidase-deficient mice, J. Pathol. 214 (2008) 434–444.
[41] J.A. Winkelstein, M.C. Marino, R.B. Johnston Jr., J. Boyle, J. Curnutte, J.I. Gallin, H.L. Malech, S.M. Holland, H. Ochs, P. Quie, R.H. Buckley, C.B. Foster, S.J. Chanock, H. Dickler, Chronic granulomatous disease. Report on a national registry of 386 patients, Medicine 79 (2000) 155–169.
[42] J.G. Moreland, A.P. Davis, J.J. Matsuda, J.S. Hook, G. Bailey, W.M. Nauseef, F.S. Lamb, Endotoxin priming of neutrophils requires NADPH oxidase generated oxidants and is regulated by the anion transporter ClC-3, J. Biol. Chem. (Oct 1 2007) (ahead of print).
[43] R. Harfouche, N.A. Malak, R.P. Brandes, A. Karsan, K. Irani, S.N. Hussain, Roles of reactive oxygen species in angiopoietin-1/tie-2 receptor signalling, FASEB J. 19 (2005) 1728–1730.

[44] C.B. Foster, T. Lehrnbecher, F. Mol, S.M. Steinberg, D.J. Venzon, T.J. Walsh, D. Noack, J. Rae, J.A. Winkelstein, J.T. Curnutte, S.J. Chanock, Host defence molecule polymorphisms influence the risk for immune-mediated complications in chronic granulomatous disease. J. Clin. Invest. 102 (1998) 2146–2155.
[45] S.H. Jackson, G.F. Miller, B.H. Segal, M. Mardiney, J.B. Domachowske, J.I. Gallin, S.M. Holland, IFN-gamma is effective in reducing infections in the mouse model of chronic granulomatous disease (CGD). J. Interferon Cytokine Res. 21 (2001) 567–573.
[46] B.E. Marciano, R. Wesley, E.S. De Carlo, V.L. Anderson, L.A. Barnhart, D. Darnell, H.L. Malech, J.I. Gallin, S.M. Holland, Long-term interferon-γ therapy for patients with chronic granulomatous disease, Clin. Infect. Dis. 39 (2004) 692–699.