Inflammation and repeated infections in CGD: two sides of a coin

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Abstract Chronic granulomatous disease (CGD) is an uncommon congenital immunodeficiency seen approximately in 1 of 250,000 individuals. It is caused by a profound defect in a burst of oxygen consumption that normally accompanies phagocytosis in all myeloid cells (neutrophils, eosinophils, monocytes, and macrophages). This “respiratory burst” involves the catalytic conversion of molecular oxygen to the oxygen free-radical superoxide, which in turn gives rise to hydrogen peroxide, hypochlorous acid, and hydroxyl radicals. These oxygen derivatives play a critical role in the killing of pathogenic bacteria and fungi. As a result of the failure to activate the respiratory burst in their phagocytes, the majority of CGD patients suffer from severe recurrent infections and rather unexplained prolonged inflammatory reactions that may result in granulomatous lesions. Both may cause severe organ dysfunction depending on the tissues involved. Preventive measures as well as rapid (invasive) diagnostic procedures are required to successfully treat CGD. Hematopoietic stem cell transplantation may be a serious option in some of the patients.

Keywords Infection · Inflammation · Granuloma · NADPH oxidase · IDO · Tryptophan · Kynurenine · IL-17 · Tregs

Background

Chronic granulomatous disease (CGD) is a rare genetic disorder caused by a defective NADPH oxidase enzyme complex [1, 2]. This complex enzyme is responsible for the burst of oxygen consumption (respiratory burst), which is associated with phagocytosis by professional phagocytes, i.e., neutrophils, eosinophils, monocytes, and macrophages. The enzyme reduces oxygen, giving rise to superoxide (O$_{2}^−$) and subsequent reactive oxygen species, which contribute to the killing of phagocytized microorganisms.

In the majority of CGD patients, the defective NADPH oxidase is responsible for an inadequate anti-microbial response, particularly to fungi, yeasts, and several bacterial species such as Staphylococcus species, Gram-negative bacteria such as Escherichia coli, nontyphoid Salmonella species, Klebsiella pneumoniae, and more rare species such as Burkholderia cepacia or actinomycosis species like Nocardia asteroides [2, 3].

The organs affected most are the lungs, skin, gastrointestinal tract, lymph nodes, and the liver. Pneumonia is a major complication that occurs, followed by lymphadenitis, infectious dermatitis, and the formation of abscesses. Although this points to underlying microbial infections, surprisingly, at most clinical episodes, no causative pathogen can be isolated.
The formation of granulomas is the other key feature in CGD, hence its name chronic granulomatous disease [1–3]. Granulomas are highly dynamic structures that, at least initially, center on the micro-organism or cells containing the intracellular micro-organism and at its border consist of a cellular barrier [4]. The formation of granuloma is particularly manifest in conditions with an inadequate antimicrobial defense, which may relate to the causative pathogen and/or to the condition of the host. In that respect, granuloma may be viewed as a means to contain persisting microorganisms and to seclude these from the host’s immune response. In CGD, the granulomas found are often sterile, indicating that the presence of granuloma does not appear to require the continued presence of microorganisms.

The granulomatous lesions can manifest themselves as obstruction in the gut, urinary tract, or pulmonary tree, but also result in a bleeding colitis with a clear failure to thrive. These two clinical manifestations, infection and inflammation, make the disease difficult to treat. Early diagnosis is beneficial to the patient because of prophylactic measures, but also when a disease episodes is at stake, early diagnostic procedures must be entertained to have a clue on what to do in clinical terms.

**Genetics**

The clinical presentation of CGD varies between patients, ranging from early to late in life and from mild to fatal [3]. At least some of this variation in presentation appears to be related to the underlying genetic defect. Mutations have been described for five subunits of the NADPH oxidase, i.e., gp91phox, p47phox, and much more seldom in p67 phox, p22phox, and p40 phox [2, 3, 5].

CYBB, the gene encoding the large gp91phox subunit of the transmembrane component cytochrome b558 of the NADPH oxidase complex, is localized on the X-chromosome. Genetic defects in CYBB are responsible for the majority of patients, most often affecting males. All other mutations are autosomal, which appear associated with a milder presentation as reflected by a higher median age at death and mean survival time and found among males and females equally [1, 5].

**Biochemistry**

A number of molecular processes involved in NADPH oxidase activation and regulation are known in some detail [1] and the current working model is schematically shown in Fig. 1. The catalytic core of NADPH oxidase consists of cytochrome b558, a heterodimeric membrane protein with subunits gp91phox and p22phox. The gp91phox subunit (also called Nox2) is a flavohemoprotein containing all the catalytically active prosthetic groups. The small subunit p22phox is a stabilizing protein for gp91phox and a docking site for cytosolic proteins that are indispensable for in vivo NADPH oxidase activation. The architecture of the NADPH oxidase (i.e., the separation between membrane-bound and cytosolic subunits) allows its activity to be tightly regulated.

Regulation is crucial, since accidental or excessive production of ROS may lead to, for example, inflammatory tissue injury, carcinogenesis, and atherosclerosis. Several regulatory pathways exist in the onset and shutdown of NADPH oxidase activity, involving different intracellular signaling cascades. Phosphorylation of the cytosolic subunits p47phox, p67phox, and p40phox by protein kinases and the generation of lipid second messengers such as arachidonic acid, phosphatidic acid, and diacylglycerol by phospholipases are the two most important signaling mechanisms in this respect. Additionally, the small GTPase Rac2 also has a profound influence on the assembly of a functional NADPH oxidase in neutrophils, since a mutation in the GTP-binding site of Rac2 (as found in a patient) [6] results in a lack of NADPH oxidase activity. However, the exact role Rac2 plays in activation and regulation is still under debate [7].

**Pathophysiology of CGD**

The molecular defects in CGD are known, but how this leads to the resulting pathophysiology is not well understood. The key features in the pathophysiology of CGD are
the exaggerated inflammatory responses and the formation of granuloma. The exaggerated inflammatory responses in CGD may in part be explained by insufficient clearance of the causative microorganisms. Frequently though, inflamed sites are sterile, indicating that this microbial persistence may not be the only explanation. The first experimental data indicative of an exaggerated inflammatory response in CGD independent of an infectious agent came from a study in patients [8], clinical observations, and subsequently supported by experimental animal studies using gp91phox and p47phox knock-out mice [9, 10].

The hyperinflammatory reactivity of the host immune system was extended in in vitro studies showing that CGD phagocytes display exaggerated inflammatory mediator responses to various stimuli [11–13]. The absence of ROS in CGD phagocytes resulted in exaggerated TNF-α production [13] and inflammasome activity which—under certain conditions—may be held responsible for the IL-1β production in CGD [14–16].

**Granuloma formation and immune reactivity**

Recent studies have brought more insight into the structure of granuloma, even though the involvement of the various cell types in a granuloma may differ between tissues and among diseases.

Expression of the enzyme indoleamine 2,3-dioxygenase (IDO) at the border of granulomata is a common feature [17]. IDO, at the expense of superoxide, degrades the amino acid l-tryptophan to l-kynurenine, which is catabolized further (Fig. 2) [18].

L-tryptophan is an essential amino acid for most microbial pathogens, and thus its depletion halts bacterial growth and viral replication [19, 20]. In addition, low concentrations of l-tryptophan and enhanced concentrations of l-kynurenine and some of its metabolites halt cell-cycle progression and promote apoptosis of T lymphocytes, thereby diminishing the initiation of an immune response [21, 22]. Therefore, IDO expression in granulomata may halt bacterial growth and dampen T cell responses towards the causative pathogen.

Interferon-γ (IFN-γ) is the primary inducer of IDO expression. TNF-α released upon infection by dendritic cells and macrophages is able to induce the IFN-γ that in its turn can induce IDO activity in granuloma [17].

**Indoleamine 2,3-dioxygenase and CGD**

*Aspergillus* infection has recently been studied in a CGD mouse model, and activity of IDO was shown to be crucial for the survival of *Aspergillus* infection [23]. IDO converts l-tryptophan into l-kynurenine but requires superoxide as a cofactor for its activity. Secreted l-kynurenine subsequently acts as an anti-inflammatory agent, by mechanisms that are not completely understood but have been shown to induce cell death in pro-inflammatory γδ T cell subsets producing IL-17 [23]. Romani et al. thus concluded that hyperinflammation caused a lethal outcome for CGD mice upon challenge with *Aspergillus*, rather than a defective clearance itself, as was previously suggested in CGD patients with an overwhelming pulmonary aspergillosis [24]. This seems plausible in view of a large body of evidence showing that CGD patients often display exaggerated immune responses against immunologic challenges [13], and granulomas have been shown to develop in the absence of any detectable pathogen, even after wound sterilization or after injection of heat-inactivated pathogens [9].

Romani and coworkers underscore their conclusion by demonstrating that CGD mice, which all died upon *Aspergillus* challenge, survive this infection when treated with the IDO product l-kynurenine in combination with IFN-γ. In turn, wild-type mice, which normally survive this challenge, no longer overcome *Aspergillus* infection when treated with the IDO-inhibitor 1-MT [23]. Although the specificity of the challenge and the background of the CGD
mouse strain are subject for debate, the above-mentioned relevant findings have certainly opened a field of research at the boundary of innate and adaptive immunity and the complex interplay between the two defense systems.

There are a number of experimental findings that also contrast with those reported by Romani’s group. First of all, Murray et al. [25] showed that IFN-γ-exposed monocytes and monocyte-derived macrophages from a CGD patient degraded tryptophan just like cells from a healthy individual. This suggests that superoxide from the NADPH oxidase is not the essential cofactor for IDO activity. In fact, there are several studies that suggest that IDO may acquire its reducing equivalents from prosthetic groups such as heme and flavin [26, 27], unrelated to the NADPH oxidase enzyme.

Another major aspect of the study by Romani et al. is that L-kynurenine is not accumulating in CGD mice and L-kynurenine suppletion restores the defective down-regulation of the inflammatory γδ T cells. IDO is the rate-limiting enzyme in the kynurenine cascade, meaning that the IDO-generated L-kynurenine is usually very quickly catabolized. Thus, an alternative explanation for the findings by Romani et al. could be that L-kynurenine is more rapidly degraded in CGD patients than in healthy individuals. If so, the lack of L-kynurenine cannot be taken as a measure of defective IDO activity. In a CGD cohort study, these issues were addressed most recently by the group of Malech, who demonstrated that IFN-γ induces normal levels of L-kynurenine in cultures of monocytes, dendritic cells, and PMN from gp91phox and p47phox-deficient human CGD patients. L-kynurenine accumulation was dose- and time-dependent, as was that of the downstream metabolite 3-OH anthranilic acid. Furthermore, urinary and serum levels of L-kynurenine and a variety of other tryptophan metabolites were elevated rather than suppressed in CGD donors [28].

As explicitly stated, the data in CGD patients only demonstrated the lack of abnormal IDO activity in blood cells, but little is known about tissue activity or localization. In preliminary immunohistochemical studies, a marked expression of IDO was noticed at the rim of granuloma in lung tissue from CGD patients (Fig. 3). Infection was excluded in these pathological patient-derived tissue samples. Neutrophils were found predominantly in the center of the granuloma, T cells both in the center and outside of the granuloma, whereas macrophages particularly localized at the border of the granuloma. These macrophages expressed most if not all of the IDO protein detected in these tissue samples. In particular, the neutrophils showed virtually no IDO protein expression, in contrast to the findings in the mouse model as reported by Romani et al. [23].

It was noted that the IDO expression in CGD tissue is much more abundant than that in granulomatous tissue from patients with sarcoidosis and tuberculosis (Lutter, van der Loos, unpublished). Since IDO activity induces apoptosis, apoptotic cells in the vicinity of IDO expression in CGD tissue can be taken as an indicator of IDO activity. Indeed, marked numbers of apoptotic (active caspase-3
positive) cells were found, predominantly in the center and border of the granulomata and consisting of neutrophils, T cells, and macrophages. These findings—taken together with the findings on L-kynurenine and IDO activity in CGD patients recently published by Malech et al. [28]—suggest that the general activity may be normal, while locally induced IDO activity may still be present in the affected tissues. It is unclear whether this markedly increased yet localized IDO activity in CGD patients directly contributes to the clinical manifestations of hyperinflammation in CGD. In a previous study by one of us, it was shown that IDO activity may lead to the increased production of inflammatory mediators by airway epithelial cells, which will lead to an exaggerated inflammatory reaction [29]. In an experimental pneumonia model, a similarly IDO-mediated enhanced production of both TNF-α and IL-10 was found [30]. IL-10 is known to inhibit neutrophil function and thus contribute to the increased bacterial load observed in this lung model. Together, this could indicate that an enhanced IDO activity in granulomata may enhance the recruitment of inflammatory cells and at the same time prevent their functioning, which may lead to a prolongation of the inflammatory response.

**T lymphocytes in inflammation and neutrophilia**

Discoveries made over the past two decades have markedly changed the way we assume immunological processes are being regulated. Not the least of these was the identification and cloning of several new cytokines, including IL-17 [31]. These discoveries provided the impetus to explore outside the Th1-Th2 cell paradigm in search of answers to explain the effector T cell responses that occur independently of known Th1 and Th2 cell-signaling pathways. It was clear that a strong T cell-dependent response can occur even in the absence of the Th1 cell-promoting factors IFN-γ, signal transducer and activator of transcription-1 (STAT1) and STAT4 or the Th2 cell promoting factors IL-4, STAT6 and GATA-binding protein-3 (GATA3). Through the study of the role of IL-23 in autoimmunity, it was discovered that an alternative T cell subset can promote chronic inflammation and tissue damage [32]. The Th17 cell was identified as a cell, in which IL-6-STAT3 activation of the transcriptional regulator retinoic acid receptor-related orphan receptor-γt (RORγt) controls the lineage fate of IL-17, IL-21, and IL-22-producing T cells (known as Th17 cells), that are highly responsive to IL-1-receptor-I (IL-1R1) and IL-23R signaling [32].

The adaptive Th17 cell pathway was inadequate to explain the early IL-17-mediated immune responses that have crucial roles during stress responses and host defense. The IL-17-mediated immune pathway is induced within hours following epithelial cell injury or activation of pattern-recognition receptors (PRRs), which is not enough time for the development of Th17 cells. IL-17 produced within 4–8 h after microbial infection was shown to enhance neutrophil chemotaxis by promoting IL-6, G-CSF, and CXC-chemokine ligand-8 (CXCL8; also known as IL-8) production and to trigger rapid, nonspecific immunity to infectious agents [33].

A hallmark of innate immunity is its rapid response to pathogens. Innate IL-17-producing cells can induce epithelial cell secretion of granulopoietic factors such as G-CSF, which recruit large numbers of neutrophils crucial for effective and rapid control of bacterial and fungal pathogens. IL-17 also synergizes with other cytokines, such as IL-1, IL-6, and TNF-α that promote activation of tissue infiltrating neutrophils to effectively eliminate extracellular pathogens, such as *Klebsiella pneumoniae*, *Staphylococcus aureus*, and *Candida albicans*. Several innate cell subsets could cooperate to promote this immediate IL-17-mediated response. In the lung, skin, liver, and gut, the γδ T cell subset has been implicated as a primary source of early IL-17 production in several in vivo models of infection. Initial studies looked at extracellular pathogens that target the lungs, such as *K. pneumoniae*, and showed that IL-23 was a main stimulator of innate IL-17 production [34].

It is important to note that innate IL-17 populations not only interact with pathogens during infection but also interact with the commensal flora to maintain mucosal homeostasis. IL-17 also has an important role in maintaining mucosal barrier integrity both during homeostasis and pathogenic insults. One way IL-17 can preserve tissue integrity is by enhancing synthesis of tight junction proteins, such as claudin. These tight-junction proteins form interconnecting ultrastructures between epithelial cells to keep out gut luminal contents and commensal organisms. Another mechanism for the protective effects of IL-17 is the induction of antimicrobial agents such as β-defensins, regenerating (ReG) proteins, S100 proteins, lipocalins, and lactoferrins. These microbicidal agents are predominantly produced by epithelial cells, as well as neutrophils and macrophages that constitutively express IL-17R. It is notable that IL-17 often works cooperatively with IL-22. In the airways, IL-17 and IL-22 have been shown to work together to promote the secretion of human β-defensin-2, β-defensin-3 and calgranulin by bronchial epithelial cells, essential for the killing of bacterial and fungal pathogens [35], a defect that may explain the recurrent skin and lung infections in the autosomal-dominant Hyper-IgE syndrome, as caused by mutations in STAT3.

Also in the gut mucosa, IL-17 and IL-22 may have synergistic effects on the induction of antimicrobial proteins such as ReG3; by gastrointestinal epithelial cells...
known as Paneth cells, which have an important role in the control of pathogens and limits dissemination of commensal bacteria that could penetrate a disrupted epithelial barrier. These innate and adaptive memory immune cells can be assumed to act in concert to produce a basal level of IL-17 and IL-22, which then maintain a constitutive level of antimicrobial proteins [36].

IL-17-mediated activity during infections in CGD

IL-17 augmented inflammation, but, paradoxically, impaired neutrophil antifungal host defense in mice [37]. As mentioned earlier, Romani et al. demonstrated that IDO-mediated l-tryptophan metabolism along the kynurenine pathway is defective in CGD mice. As a consequence, unrestrained γδ T cell and αβ Th17 expansion, defective regulatory Treg activity, and acute inflammatory lung injury leading to mortality occurred in CGD mice when challenged with intratracheal Aspergillus fumigatus [23]. Although complete protection from invasive fungal disease and reversal of the hyperinflammatory phenotype were achieved by recombinant IFN-γ and administration of a natural l-kyurenine distal to the IDO blockade was suggested, beneficial effects in this infection model were also induced by IL-17 neutralization or depletion of γδ T cells (which produce IL-17) in CGD mice [23, 38]. Similar to Aspergillus challenge, intratracheal zymosan (a sterile fungal cell wall constituent that ligates Toll-like receptor 2 and dectin-1), caused dramatically augmented lung inflammation and IL-17 production and diminished Treg responses in CGD compared to wild-type mice, emphasizing the intrinsic regulatory role of the NADPH oxidase on inflammation [39]. George-Chandy et al. recently showed that ptp91/phox-negative dendritic cells (DCs) enhance induction of both Th17 and Th1 responses in vitro compared to wild-type DCs [40]. T cell development was dependent exclusively on the NADPH oxidase status of the responding T cells. To date, we did not detect a defect in Th17 development in CGD patients, making alternative explanations well possible.

Inflammatory and regulatory T cells

Tregs and IL-17-producing T cells mediate opposing responses. IL-17 stimulates production of G-CSF, GM-CSF, TNF-α, and chemokines that regulate myelopoiesis and neutrophil recruitment to inflammatory sites. The IL-23/IL-17 axis (IL-23 expands Th17 cells) mediates several experimental autoimmune disorders, including colitis, collagen-induced arthritis, and experimental autoimmune encephalomyelitis, and is a promising target for drug development. T cells producing IL-17, which is known to drive chronic inflammation, are held in check by regulatory T cells. Although a potential role of IL-17-producing cells will very well be possible, this mechanism requires further confirmation in human CGD patients. On the other hand, Montagnoli et al. [38] previously showed that naturally occurring Tregs are recruited early in the inflammatory response to Aspergillus in wild-type mice, and are capable of suppressing inflammation.

Apart from IDO activity and localization of Th17 reactivity, a regulatory defect in the Treg-mediated control of the adaptive immune system may be present in CGD. The role of Tregs in the exaggerated innate immune reactivity loop in CGD was suggested by previous studies in mice and rats with mutant Ncf1 alleles (encoding p47phox) associated with defective ROS production. Due to a less functional NADPH oxidase complex, these animals are more susceptible to develop autoimmune diseases as compared to littermates with normal ROS production [41, 42]. This is in line with the observation that patients with CGD who are unable to produce ROS have a higher incidence of autoimmune diseases such as granulomatous colitis or Crohn’s-like disease (CD), rheumatoid arthritis (RA), or systemic lupus erythematosus (SLE) [43, 44].

In the rat model for CGD, the T cells were responsible for mediating the higher susceptibility to pristane-induced arthritis [41, 45]. However, T cells themselves do not express p47phox and do not produce ROS via NADPH oxidase [46]. Instead, the antigen-presenting cells were involved in aberrant adaptive immune reactivity under these conditions. Macrophages are far more efficient in producing ROS as compared to B cells and dendritic cells (DC) [47]. A transgenic mouse was developed that expressed functional p47phox in macrophages only, on a p47phox-mutated background. These mice were equally protected against collagen-induced arthritis as wild-type littermates, indicating that macrophage-derived ROS are sufficient to prevent the induction of unwanted T cell reactivity [47].

Regulatory T cells (Tregs) can suppress activation and proliferation of effector T cells and thereby diminish most immune responses. Autoimmunity can therefore be the result of a defective Treg system [48] and successful treatment of autoimmune disease with Tregs has been reported in mouse models [49, 50]. Reasoning along these lines, Treg induction via ROS can be mediated via macrophages, as was reported before [51]. In collaboration, Keldeman et al. indeed observed that such a mechanism of ROS-dependent Treg induction seems to be relevant to CGD. Macrophage-derived ROS could induce Tregs under normal conditions. In contrast, macrophages from CGD patients were significantly less efficient in inducing Tregs (Keldeman et al. PNAS 2011). Thus, macrophages...
producing ROS help induce Tregs for proper immune regulation, a regulatory loop that seems to be impaired in CGD. Previously it has been found that T cell suppression by Tregs induced by anti-inflammatory macrophages is dependent on membrane-bound TGF-β [51]. A role for ROS to activate TGF-β was suggested before [52, 53], and this may subsequently determine the induction of Tregs.

In sum, when macrophages prevent T cell reactivity by producing ROS, the activation signal needs to overcome a certain threshold level for T cell activation and this fail-safe mechanism may thus prevent unwanted inflammation and autoimmunity.

Conclusions

To date, the abundant presence of results in experimental mouse models as well as in human CGD patients indicate that the NADPH oxidase complex is able to restrain inflammation by modulating redox-sensitive innate immune pathways. When challenged with either intratracheal zymosan or LPS, NADPH oxidase-deficient (CGD p47 phox pathways. When challenged with either intratracheal inflammation by modulating redox-sensitive innate immune that the NADPH oxidase complex is able to restrain mouse models as well as in human CGD patients indicate

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