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REVIEW

Genetics and immunopathology of chronic granulomatous disease

Marie José Stasia · Xing Jun Li

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Abstract Chronic granulomatous disease (CGD) is a primary immunodeficiency syndrome characterized by a greatly increased susceptibility to severe fungal and bacterial infections. CGD results from a failure of the reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase enzyme in the patient's phagocytes to produce superoxide. It is caused by mutations in any of four genes that encode the components of the NADPH oxidase. Investigation of CGD patients has identified the different subunits and the genes encoding them. Study of rare CGD variants has highlighted sequences involved in the structural stability of affected components or has provided valuable insights into their function in the oxidase activation mechanism. Functional and molecular CGD diagnosis tests are discussed in this review. Long-term antibiotic prophylaxis has been essential in fighting infections associated with CGD, but approaches based on hematopoietic stem cell transplantation and gene therapy offer great hope for the near future.

Keywords Chronic granulomatous disease · Primary immunodeficiency · NADPH oxidase · Rare variant · Bone marrow transplantation · Gene therapy

Abbreviation

CGD	chronic granulomatous disease
AR CGD	autosomal recessive chronic granulomatous disease
X CGD	X-linked chronic granulomatous disease
NADPH	reduced nicotinamide adenine dinucleotide phosphate
FAD	flavin adenine dinucleotide
INT	iodonitrotetrazolium
ROS	reactive oxygen species
PHOX	phagocytic oxidase
TPR	tetratricopeptide repeat
BMT	bone marrow transplantation

Introduction

Phagocytic leukocytes are essential cells of the innate immune system whose task is to rapidly respond to invading microbes. The responses of phagocytes to pathogens include phagocytosis, proteolytic destruction in the phagolysosomes, damage induced by superoxide, and reactive oxygen species (ROS) generated by membranous reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase. Primary phagocytic defects must be included in the differential diagnosis of life-threatening and recurrent infections and fever in children and occasionally

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M. J. Stasia (✉)
Centre Diagnostic et Recherche sur la Granulomatose Septique Chronique, Laboratoire TIMC/IMAG UMR CNRS 5525,
Université J. Fourier,
CHU 38043 Grenoble, France
e-mail: MJStasia@chu-grenoble.fr

X. J. Li
Department of Pediatrics (Hematology/Oncology),
Herman B Wells Center for Pediatric Research Indiana University School of Medicine,
1044 West Walnut Street,
Indianapolis, IN, 46202-5225, USA

in adults. The central importance of the phagocyte NADPH oxidase to innate host defense is illustrated in chronic granulomatous disease (CGD), a rare genetic disorder (estimated prevalence of 1/200,000 to 1/250,000) characterized by severe and recurrent infections with essentially catalase-positive microorganisms (which destroy their own hydrogen peroxide) due to the inability of phagocytes to mount a respiratory burst to kill invading bacteria and fungi.

History or what can we learn about NADPH oxidase through the discovery of CGD forms?

As long ago as 1933, Balbridge and Gerard [1] observed that when canine neutrophils engulf bacteria they consume large amounts of oxygen. This metabolic response was initially attributed to increased mitochondria energy until it was shown that this respiratory burst was resistant to mitochondria respiration inhibitors such as azide and cyanide [2]. Finally, it was found that under anaerobic conditions, while phagocytosis and degranulation occurred, phagocytes were unable to kill very common human bacterial pathogens [3]. Concomitantly in 1954, at an annual meeting of the Society for Pediatric Research, Janeway and colleagues [4] reported for the first time five cases of children with elevated serum gamma globulin levels who suffered from recurrent infections. At that time, the molecular basis of this disease had not been identified. Three years later, Landing and Shirley [5] described the same clinical features in two boys who presented infiltration of viscera with pigmented lipid histiocytes. In 1959, besides recurrent infections at epithelial surfaces such as skin, Bridges et al. [6] noted the presence of granuloma lesions in deep organs such as lung in four boys, describing "a fatal granulomatous disease." Since this familial disease occurred in boys, it was thought that it was an X-linked defect. Eight years later, Quie et al. [7] demonstrated for the first time that neutrophils purified from a male patient with this familial granulomatosis (now called CGD) were incapable of killing *Staphylococcus aureus* in vitro. At the same time, Holmes et al. [8] showed that neutrophils from CGD patients failed to exhibit an increase in oxygen metabolism during phagocytosis. Baehner and Nathan [9] were the first to use the nitroblue tetrazolium (NBT) reduction test to measure oxidase activity in leukocytes to diagnose CGD. Shortly before, Rossi and Zatti observed NADPH and NADH oxidation in leukocytes during phagocytosis and they proposed NADPH as a major substrate of the phagocytic oxidase [10, 11]. At nearly the same time, the presence of cytochrome b_{558} was discovered in the granules of neutrophils based on its absorption properties and on the fact that it could be reduced by NADH and NADPH under anaerobic conditions [12, 13]. Finally in 1978, 12 years after this discovery, Segal and Jones [14] identified this cytochrome as the NADPH oxidase in human neutrophils. They established the role of cytochrome b_{558} as the terminal electron-transporting element of the NADPH oxidase and reported that this element was missing in X-linked CGD neutrophils. The heterodimer nature of cytochrome b_{558}, which consists of two entities—the α or light chain or small subunit or p22*phox* and the β or heavy chain or large subunit or gp91*phox* (recently renamed NOX2)—was determined in 1987 [15, 16]. The discovery of the protein components of the NADPH oxidase complex was the direct consequence of studies in neutrophils collected from CGD patients. Indeed, the first European multicenter evaluation of incidence and relevance of CGD conducted in 1983 highlighted that in addition to an X-linked form of CGD, an autosomal recessive form of CGD existed with a normal amount of cytochrome b_{558} in neutrophils and affecting female patients [17]. One year later, Bromberg and Pick [18] demonstrated that, when using plasma membranes and cytosol purified from macrophages, NADPH oxidase activity could be induced in vitro in a cell-free system when stimulated by unsaturated fatty acids. Then, from 1985 to 1987, a variety of evidence confirmed that cytosolic factors were essential for NADPH oxidase activity and that defects in this activity caused autosomal recessive CGD [19–21]. The role of the last guanosine triphosphate (GTP)-dependent factor, rac1/2, essential for optimal NADPH oxidase activity, was discovered concomitantly by Abo et al. [22] and Knaus et al. [23] in neutrophils. The importance of the small G protein Rac2 was underlined when a severe immunodeficiency different from classical CGD was described in a 5-week-old male child and related to a dominant negative mutation in the RAC2 gene [24, 25].

NADPH oxidase and molecular genetics of CGD

NADPH oxidase is a multicomponent complex localized in the phagosomal and plasma membrane composed of a membranous component, flavocytochrome b_{558}, and cytosolic proteins p47*phox*, p67*phox*, and p40*phox* (*phox* for *ph*agocytic *ox*idase) and two small GTPase, Rac2 and Rap1A (Fig. 1). Cytochrome b_{558}, the redox center of the NADPH oxidase complex, is a heterodimer consisting of a large flavocytochrome NOX2 and a small protein p22*phox*. In unstimulated cells, the NADPH oxidase components are segregated into membrane and cytosolic locations. P40*phox*, p47*phox*, and p67*phox* are associated with a 1:1:1 stoichiometry in the cytosol. Rac2 is complexed in the cytoplasm with Rho-guanine nucleotide dissociation inhibitor. Upon activation, a series of protein–protein and

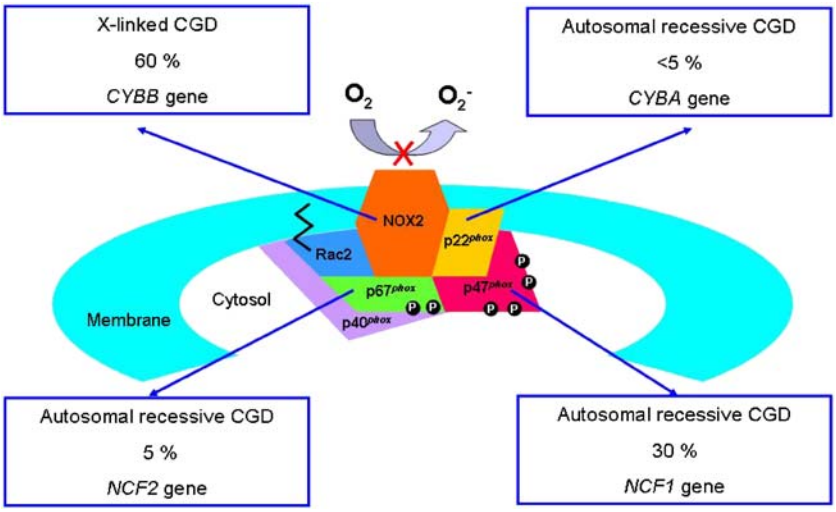

Fig. 1 Dysfunction of NADPH complex and chronic granulomatous disease. Hypothetical assembly of the NADPH oxidase components during activation. CGD is an inherited disorder resulting from failure of the NADPH oxidase activity in phagocytes. The most frequent form of the disease is the X-linked recessive defect in *CYBB* encoding NOX2. Three other forms of the disease are caused by autosomal recessive defects in *CYBA*, *NCF1*, and *NCF2* genes encoding p22*phox*, p47*phox*, and p67*phox* proteins, respectively

protein–lipid interactions occur. Both p47*phox* and p67*phox* are phosphorylated and translocate with p40*phox* to membrane-bound cytochrome b_{558}. Rac2 binds GTP and migrates to the membrane independently of the p67*phox*–p47*phox* complex. Then, in its complexed activated form, NADPH oxidase is able to transfer electrons from cytosolic NADPH to external molecular oxygen [26].

Recently, the research on nonphagocyte NADPH oxidase led to the discovery of two families of NOX homologs, *N*ADPH *ox*idase (NOX) and *du*al *ox*idase, expressed in several tissues and cells and involved in different pathological processes [27, 28]. All NOX family members contain a core structure consisting of six transmembrane domains (in which four heme-coordinating histidine residues are located) and a C-terminal cytosolic region (which contains heavily conserved binding sites for FAD and NADPH). This discovery has opened a huge field of research on NADPH oxidase not only of phagocytes but also involving many research domains related to the biomedical sciences ([28] and a review by W. Nauseef (2008) "NOX enzymes in immune cells: basic features". Seminars in Immunopathology, 30).

Genetically, CGD is a heterogeneous disease caused by mutations in any of four proteins of the NADPH oxidase complex, including NOX2 and p22*phox* (both subunits of the membrane cytochrome b_{558}), p47*phox*, and p67*phox* (the cytosolic components of this enzyme complex; Fig. 1, Table 1) [29]. All ethnic groups are equally affected. In 1986, the X-linked defective gene was the first gene involved in CGD cloned by reverse genetics from a cDNA library of differentiated human promyelocytic HL60 cells [30]. *CYBB* was also the first gene that allowed human disease to be identified according to its chromosomal location. A proximal location (Xp21.1) on chromosome X was suggested by linkage analysis using cloned, polymorphic DNA probes [31]. The X-linked recessive transmission type of CGD, characterized by mutations in the *CYBB* gene encoding NOX2, is the most frequent form of CGD (approximately 60% of cases; Table 1). Then Nunoi et al. [32] and Volpp et al. [33, 34] cloned and sequenced the cDNA encoding p47*phox* (the *NCF1* gene) and Leto et al. [35] cloned and sequenced the cDNA encoding p67*phox* (the *NCF2* gene) by screening a promyelocytic leukemia cDNA library. The genomic structure of the *NCF1* and *NCF2* genes was clarified in 1990 [36]. The structure and the chromosomal location of the *CYBA* gene encoding p22*phox*, the light chain of cytochrome b_{558}, were reported by Dinauer et al. [37]. The second most common form of CGD is autosomal recessive (ARCGD), accounting for approximately 30% of the cases. Most of the time, it is caused by the deletion of a GT from a GTGT tandem repeat at the first splice junction in the *NCF1* gene encoding p47*phox* (A47 CGD). In addition to these usual CGD types, mutations in the *CYBA* and *NCF2* genes encoding p22*phox* and p67*phox*, respectively, account for rare $A22^0$ CGD and $A67^0$ CGD, each accounting for less than 5% of cases (Table 1) [38].

X-linked CGD

The description of a patient who suffered from CGD and also from Duchenne muscular dystrophy and retinitis pigmentosa made it possible to localize the *CYBB* gene (OMIM number 306400) on the short arm of chromosome X [31]. The mutation responsible was a microdeletion in the Xp21 locus, affecting all the genes involved on these

Table 1 Characterization of NADPH oxidase complex components involved in CGD

		NOX2 or gp91*phox*	p22*phox*	p47*phox*	p67*phox*
Disease	Transmission	X-linked	Autosomal recessive	Autosomal recessive	Autosomal recessive
	Designation	X91 CGD	A22 CGD	A47 CGD	A67 CGD
	Subtype and frequency	$X91^0$ CGD (55%) $X91^-$ CGD (<5%) $X91^+$ CGD (<5%)	$A22^0$ CGD (5%) One case of $A22^+$ CGD	$A47^0$ CGD (30%)	$A67^0$ CGD (5%) One case of $A67^-$ CGD
Gene	OMIM	306400	233690	233700	233710
	Name	*CYBB*	*CYBA*	*NCF1*	*NCF2*
	Location	Xp21.1	16q24	7q11.23	1q25
	Size	30 kb	8.5 kb	15.2 kb	37 kb
	Number of exons	13	6	11	16
mRNA Protein	Size	4.7 kb	0.8 kb	1.4 kb	2.4 kb
	Synonyms	Heavy chain or β sub-unit	Light chain or α subunit		
	Number of amino acids	570	195	390	526
	Predicted molecular mass	65.0 kDa	20.9 kDa	44.6 kDa	60.9 kDa
	Molecular mass (SDS-PAGE)	76–92 kDa (smear)	22 kDa	47 kDa	67 kDa
	pI	9.26	10.1	6.12	9.58
	Tissue expression	Phagocytes B lymphocytes, neurones, cardiomyocytes, skeletal muscles, hepatocytes, endothelium	Ubiquitous	Phagocytes B lymphocytes, neurones, vascular cells, hepatocytes, endothelium	Phagocytes B lymphocytes, neurones, vascular cells, hepatocytes, endothelium
	Posttranslational modification	N-glycosylation			
	Cellular location in resting state	Plasma membrane Membrane of specific granules	Plasma membrane Membrane of specific granules	Cytoplasm	Cytoplasm
	Cellular location in activated state	Plasma and phagosome membranes	Plasma and phagosome membranes	Plasma and phagosome membranes	Plasma and phagosome membranes
	Phosphorylation after activation	?	Slight	Yes	Yes

diseases. European, American, and Japanese groups reported 106, 124, and 48 X-linked CGD mutations, respectively [39–41]. In addition, two databases gathered more than 200 mutations on the *CYBB* gene. The first one is the Human Gene Mutation Database (HGMD, http://www.hgmd.cf.ac.uk/ac/index.php) at the Institute of Medical Genetics in Cardiff (Wales) with 282 mutations reported to date [42]; the second is the Immunodeficiency database (IDbases, http://bioinf.uta.fi/CYBBbase/) of the Institute of Medical Technology–Bioinformatics in Tampere (Finland), reporting 244 mutations [43]. According to the HGMD database, single-nucleotide substitutions (missense or nonsense mutations including splicing or not) account for 58% of the defects; small deletions, insertions, and insertion–deletions account for 26% and large deletions and insertions for 14%. However, insertions in *CYBB* are less frequent than deletions, in accordance with findings in other genes [44]. The remaining cases are caused by mutations in the regulatory region of the *CYBB* promoter (1.5%) and complex rearrangements (0.5%). These results show that X-linked CGD is a very heterogeneous disease, caused by a large variety of mutations, except gene conversions. It should be emphasized that only three polymorphisms caused by missense mutations located in the encoding region of the *CYBB* gene have been reported [45].

X91^0 CGD

Most of the time, mutations in the *CYBB* gene lead to a lack of NOX2 expression because of the instability of the corresponding mRNA or protein. In these patients, NADPH oxidase activity is always totally abolished. This phenotype, called X91^0 CGD, is the most frequent (Table 1). It is usually caused by nonsense, missense, splice mutations, small deletions, and insertions, sometimes associated with frameshift and early termination of protein synthesis. In addition, large insertions, for example (part of) retrotransposons, or large deletions removing part of or the entire gene lead to X91^0 CGD [42, 43]. Generally, small deletions and insertions, often associated with frameshift in *CYBB*, lead to a sharp decrease in mRNA stability associated with the absence of NOX2 synthesis. This type of mutation is often caused by slipped mispairing during DNA replication at the replication fork, which often accounts for single base-pair deletions or insertions. In addition, deletions are often situated in very rich GC regions, previously demonstrated to be hot-spot consensus sequences for spontaneous small deletions in other genes [39, 46]. Mutations near or in the splice-junction sites in *CYBB* also cause CGD, with a mRNA processing defect (exon skipping) and a decrease in its stability. In accordance with the literature, mutations in *CYBB* are for the most part found in the donor (5′) splice site [39, 47]. Splice mutations have been estimated at 15–17% of all unique base substitutions in CGD, as in other human genetic diseases [39,40,47].

Nonsense mutations, which introduce a stop codon, affect the mRNA level to various degrees [39, 40]. When mRNA is stably transcribed, the corresponding truncated NOX2 protein is never immunodetected by Western blot analysis, suggesting either the absence of the specific epitope recognized by monoclonal antibodies, or, more probably, the instability of the mutated protein. The latter hypothesis can be confirmed by analysis of the difference absorption spectrum of cytochrome b_{558} from the membrane of the patient's neutrophils with the absence of the characteristic spectral bands at 426, 530, and 558 nm. This type of mutation accounts for approximately 50% of point mutations in the coding *CYBB* region [39,40,42,43]. Several mechanisms explaining single-nucleotide substitutions have been described [48]. One of the best-known mechanisms that can be applied to single-nucleotide substitution in *CYBB* is methylation-induced deamination of cytosine, leading to thymidine formation. This takes place in cytidine–phosphate–guanosine dinucleotides (5′ to 3′), also called CpG sequences, because they follow CG→TG and CG→CA changes [39,40,48]. In five X91^0 CGD patients, we demonstrated that nonsense mutations located specifically in exon 5 lead to the amplification of two reverse-transcription polymerase chain reaction (RT-PCR) products from the NOX2 mRNA, one corresponding to a cDNA fragment containing the mutated exon 5, the second missing that exon [49]. Parallel amplification of a housekeeping gene and control cDNA demonstrated that the PCR products did not appear to be artifacts. One possible explanation is that the introduction of a T into a purine-rich DNA sequence (splicing enhancers) within 30 bp of the nearest exon boundary is responsible for exon skipping [50]. This highlights that mutations found at the mRNA level must always be confirmed at the genomic level.

Approximately 50% of the point mutations in 13 exons of *CYBB* are missense mutations responsible for replacement of a single amino acid. Generally, these mutations do not affect mRNA stability but act on the level of NOX2 expression in phagocytic cells, leading to either X91^0, X91$^-$, or X91$^+$ CGD variants (Table 1). The superscripts minus or plus mean that NOX2 expression is diminished or normal, respectively. NADPH oxidase activity is always totally abolished in X91^0 and X91$^+$ CGD, while in X91$^-$ CGD neutrophils, this activity can be residual [51].

X91$^-$ CGD or what can we learn about cytochrome b_{558} synthesis?

Twenty-six mutations have been found in X91$^-$ CGD patients [45] (Table 2). These variants are of interest

Table 2 Mutations in *CYBB* gene causing X91^{-} CGD phenotypes

Mutation n°	cDNA nucleotide (or splice site) change	Mutation type	Amino acid change	Potential structural–functional domain	Functional analysis in neutrophils			
					NADPH oxidase (% of normal)	Cytochrome b 558 (% of normal)	Ratio activity–cyt b	Reference
1	A-57C	Promoter	NA	Regulation of NOX2 synthesis	Residual	Residual[a]	Normal	[52, 53]
2	T-55C	Promoter	NA	Regulation of NOX2 synthesis	1–5%	2–5%[a,b]	Normal	[51, 52]
3	C-53T	Promoter	NA	Regulation of NOX2 synthesis	1–5%	Residual[a]	Normal	[54, 55]
4	C-52T	Promoter	NA	Regulation of NOX2 synthesis	1–5%	Residual[a]	Normal	[54]
5	G66C	Missense	Trp18Cys[c]	I-transmembrane helix	NA	NA	NA	[58]
6	C170A	Missense	Ala53Asp[c]	II-transmembrane helix	NA	NA	NA	[39, 58]
7	C179T	Missense	Pro56Leu[c]	II-transmembrane helix	NA	NA	NA	[60]
8	G188T	Missense	Cys59Phe[c]	II-transmembrane helix	NA	NA	NA	[45]
9	C311T	Missense	His101Tyr[c]	Heme binding	0	10[a,b]	Abnormal	[61]
10	ex5AGgtaag→ex5AGgtaaa	Deletion	Traces of normal mRNA	?	2.4–8.0	5.0–17.0[b]	Normal	[68]
11	G478A	Missense	Ala156Thr[c]	II-extra loop	2.0–7.0	8[b]	Normal	[63,66,67]
12	A494G	Missense	Lys161Arg[c]	II-extra loop	NA	NA	NA	[66]
13	571–579 ATATTAATT deletion	Deletion	187–189 IleLeuIle del[c]	IV-transmembrane helix	NA	NA	NA	[45]
14	C590T	Missense	Ser193Phe[c]	II-intra loop (D-loop)	0.5–1.8	0.1–4.7[d]	Normal	[68]
15	G743C	Missense	Cys244Ser[c]	III-extra loop (near the glycosylation site)	3	39[b]	Abnormal	[63, 67]
16	G743A	Missense	Cys244Tyr[c]	III-extra loop (near the glycosylation site)	3.6	8.0–9.0[a]	Normal	[65]
17	G937A	Missense	Glu309Lys[c]	?	4	17[e]	Abnormal	[40]
18	AAG from base 955	Deletion	315 Lys[c]	?	12	20–30[a]	Normal	[65]
19	A985T	Missense	Ile325Phe[c]	?	4	5[e]	Normal	[40]
20	C1024A	Missense	His338Tyr[c]	The isoalloxazine ring of FAD-moiety-binding site	0.2–0.8	13–33[a,b,d]	Abnormal	[39,68,70]
21	C1028A	Missense	Pro339His[c]	The isoalloxazine ring of FAD-moiety-binding site	0	14–38[a,d]	Abnormal	[58,68,71]
22	G1178C	Missense	Gly389Ala[c]	?	13.0–19.0	21[b]	Normal	[65, 67]
23	3′ intron 9t(8nt)ag/g(8nt)ag	Splice site	Deletion exon 10 and traces of normal mRNA	?	0.2–8	0.1–2[d]	Normal	[68]
24	11-bp deletion: 2 bp of the end of exon 12 + 9 bp of the beginning of intron 12	Deletion	Frameshift at 524 and introduction of a stop codon at 534[c]	Lost of the nicotinamide-binding site (^{535}FLCGPE^{540})	0	Trace[a,b]	Normal	[49]
25	1,612–1,626 deletion	Deletion	AA 534–538 deletion[c]	Lost of the nicotinamide-binding site (^{535}FLCGPE^{540})	NA	NA	NA	[45]
26	Duplication of bases 1,672–1,702, stop codon	Deletion	Deletion of C term[c] 6 AA 565-570	?	2.1	16–24[e]	Abnormal	[65]

The nomenclature of the mutations relates to the mRNA sequence NM_000397.
NA Data not available.
[a] Cytochrome b_{558} expression was determined by Western blot.
[b] Cytochrome b_{558} expression was determined by differential spectrum.
[c] These mutations are represented in NOX2 protein (Fig. 2).
[d] Cytochrome b_{558} expression was determined by flow cytometry with the 7D5 mAb.
[e] The method used to determine the amount of cytochrome b_{558} expressed was not detailed.

because they result from structural disorganization, leading to an incomplete loss of protein, partial dysfunction, or both.

Promoter of CYBB Four X91$^-$ CGD mutations (A-57C, T-55C, C-53T, and C-52T) are located in the promoter region of the *CYBB* gene (mutation nos. 1–4; Table 2) [51–55]. All these promoter mutations are located in a region between the "CCAAT" and the "TATA" boxes in a consensus binding site for the *ets* family of transcription factors of the NOX2 promoter site (5′-GAGGAAAT-3′, lower strand, −57 to −50 bp). These mutations strongly inhibit the binding of both Elf-1 and PU.1 (members of the *ets* family of transcription factors abundantly expressed in myeloid cells), suggesting that these mutations reduce NOX2 promoter activity, which results in a low level of NOX2 expression [52]. We found a homogeneous reduction of NOX2 expression and a measurable level of NADPH oxidase activity (3–9% of normal) in all the neutrophil populations from CGD patients containing a T-55C mutation. These patients suffered from severe clinical forms of CGD and presented multiple life-threatening infections in deep organs [51]. The amount of superoxide produced by their neutrophils is probably not sufficient to protect them against infections. However, the general idea is that X91$^-$ CGD patients are diagnosed later in life than X91^0 CGD patients and have a milder clinical course [29]. Other authors found a small homogeneous population of highly NBT-positive cells in some X91$^-$ CGD patients with a point mutation in the *CYBB* promoter (10–20% of the total phagocyte population) [52,53,55]. This positive clone was shown to be composed of eosinophils [54–56]. It has been demonstrated that NOX2 expression has a specific regulation in eosinophils by GATA transcription factors and not by Elf-1 and PU.1 [57]. Mild clinical forms of these specific X$^-$ CGD patients can be explained as a protection against infections caused by a high percentage of fully oxidase-competent eosinophils produced by an unknown compensatory mechanism [55].

Most of the X91$^-$ CGD patients had disease resulting from missense mutations that are equally distributed between the membranous NH2 terminal and the cytosolic COOH terminal part of NOX2 (Fig. 2, Table 2).

Transmembrane helices of NOX2 Trp18Cys and Ala53Asp, Pro56Leu, and Cys59Phe substitutions and the small deletion (187–189 IleLeuIle) are located in the transmembrane helices of NOX2 in the COOH terminus (Fig. 2) [39,45,58–60]. No precise data on the level of cytochrome b_{558} expression or NADPH oxidase activity are available (Table 2). However, these results suggest an important role of transmembrane helices in the stabilization of cytochrome b_{558} synthesis. One possibility is that these helices are regions interfacing with p22*phox* and that this subunit's interaction is necessary to stabilize both after their synthesis.

The His101Tyr mutation, located in one of the heme-binding sites in NOX2 (mutation no. 9, Table 2, Fig. 2), is a very interesting point mutation described by Tsuda et al. [61]. This mutation leads to the expression of 10% of the normal amount of NOX2 protein and inhibits the heme incorporation, as evidenced by the absence of a reduced-minus-oxidized differential spectrum of cytochrome b_{558}. In addition, no oxidase activity could be measured, probably because of the absence of electron transfer from FAD through the hemes. This study supported the previous prediction based on electron spin resonance studies on the heme environment of the neutrophil's cytochrome b_{558} [62]. Surprisingly, the His101Arg mutation leads to a classical X91^0 CGD phenotype [63]. This confirms that heme incorporation to NOX2 is required for the full expression of the large subunit of cytochrome b_{558}. [64].

Extracellular and intracellular loops The X$^-$ CGD mutations (Ala156 Thr and Lys161Arg, Cys244Ser, Cys244Tyr) are located in the second and third extracellular loops of NOX2 (mutation nos. 11, 12, 15, and 16, Table 2, Fig. 2), which contain the Asn residues as glycosylated sites [65–67]. They exhibit a slight but measurable oxidase activity (2–7% of normal control) related to the amount of cytochrome b_{558} expressed in the CGD patients' neutrophils, except for the Cys244Ser mutant (Table 2). In this latter case, cytochrome b_{558} measured by a differential spectrum was highly expressed compared to the NADPH oxidase activity [63, 67]. This was not found in the Cys244Tyr mutant [65]. Porter et al. [60] studied the biosynthesis of cytochrome b_{558} in Epstein-Barr-virus-immortalized B lymphocytes (EBV-LB) from the X91$^-$ CGD patients with Pro56leu and Cys244Ser mutations. For both, they found NOX2 as its 65-kDa high mannose precursor form in EBV-LB. These mutations probably disturbed the final maturation of NOX2. Finally, the Ser193Phe substitution (mutation no. 14; Table 2) located in the second intracellular loop of NOX2 (D-loop) leads to a proportional decrease in oxidase activity with the level of cytochrome b_{558} [68]. The D-loop is not only critical for the structural bearing of cytochrome b_{558} but is also essential for NADPH oxidase activity because of its involvement in electron transfer from FAD to oxygen, independently of cytosolic factor translocation [69]. Indeed, chimeric NOX2 proteins containing the D-loop of NOX1/3/4 support NADPH oxidase activity, suggesting that this region should play a similar role in NOX analog activation. In addition, for the first time, a "superoxidase" activity in both NOX2 mutants of the D-loop was demonstrated [69].

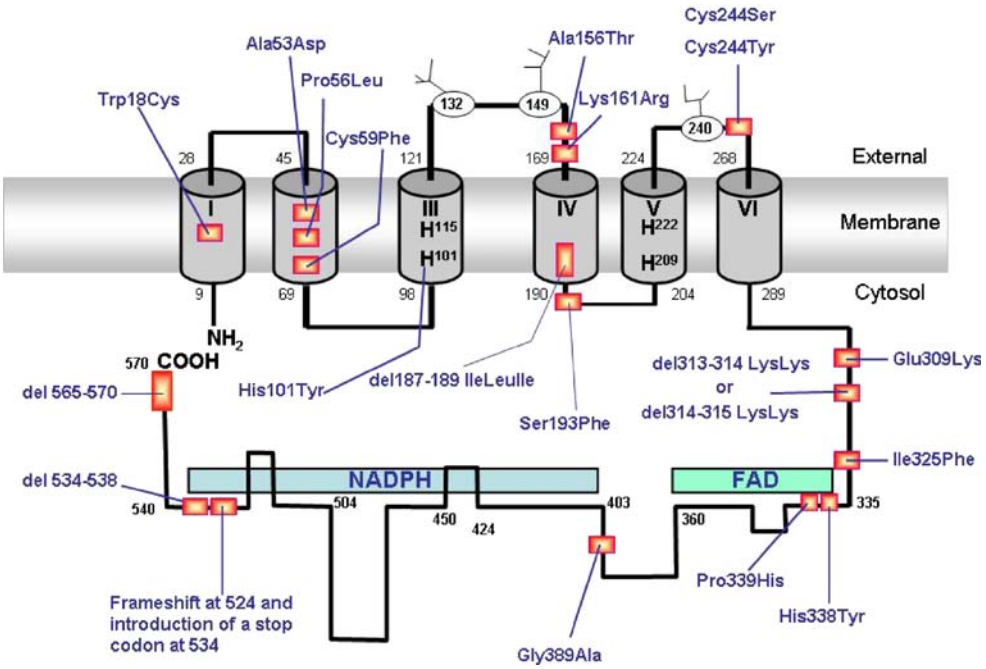

Fig. 2 X91⁻ CGD mutations in the potential structural and functional model of NOX2. Glycosylated asparagines are located in the external loops of NOX2 and numbered by their residue numbers. The four heme-binding histidines located in the third and fifth transmembrane domains are shown by single-letter code as H. The potential FAD- and NADPH-binding domains are illustrated by *full boxes*. Mutations causing X91⁻ CGD forms of CGD are equally distributed over the entire sequence of NOX2. Mutation nos. 1–4 (in the promoter of the *CYBB* gene), no. 10 (skipping of exon 5) and no. 23 (skipping of exon 10) in Table 2 are not represented

FAD-binding site Interestingly, two neighboring missense mutations (His338Tyr, Pro339His) are located in the putative binding site for the isoalloxazine ring of the FAD moiety (338HPFT motif; mutation nos. 20 and 21, Fig. 2), which is heavily conserved in the FNR family [39,58,68,70,71]. His338Tyr and Pro339His mutations inhibit nearly totally superoxide production in patients' neutrophils, while roughly one-third levels of the heme, in contrast to normal control, are conserved. The translocation of p47*phox* and p67*phox* occurred normally. However, the FAD content in patients' neutrophil membranes was as low as that of $X91^0$ CGD patients, suggesting complete deletion of FAD. These results indicate that His338 and Pro339 are critical residues for FAD incorporation into NOX2. This also suggests that incorporation of FAD into NOX2 is needed for the stability of NOX2 during synthesis, like the incorporation of hemes (mutation no. 9, Table 2).

NADPH-binding site Two X91⁻ CGD cases that originated from small deletions affect the nicotinamide of the NADPH-binding site (mutation nos. 24 and 25, Table 2) [45, 49]. In one of these patients, the introduction of a stop codon at position 534 leads to the total loss of this site. No oxidase activity could be detected, probably because of a defect in the NADPH binding. In addition, the cytochrome b_{558} expression was dramatically diminished [49]. Thus, it appears that the COOH terminus of NOX2 is essential for the stability of cytochrome b_{558} synthesis.

Regions with unknown functions A cytosolic region of NOX2, between the sixth transmembrane domain and the FAD-binding site, seems to be essential for preserving cytochrome b_{558} synthesis (Table 2, Fig. 2). Two point mutations, GLu309Lys and ILeu325Phe (mutations no. 17 and 19, Table 2), lead to a substantial decrease in oxidase activity (4% of control oxidase activity), while cytochrome b_{558} expression is 17% and 5% of normal, respectively. The deletion of one of the three Lys at position 313–315 (mutation no. 18) as well as the Gly389Ala substitution (mutation no. 22 located between the FAD and the NADPH-binding sites) seems to have a mild effect, because it preserved 12–19% of NADPH oxidase activity in the X⁻ CGD neutrophils, with 20–30% of cytochrome b_{558} expression compared to control cells [65, 67]. Moreover, for the Gly389Ala mutation, Porter et al. [60] found the mutated NOX2 in its mature form (91 kDa) in the corresponding EBV-LB, suggesting normal maturation steps in this mutant [65, 67]. The mutant characterized by the 565–570 amino acid deletion at the end of the NOX2 sequence (mutation no. 26) demonstrated the importance of this region in the stability of NOX2 and cytochrome b_{558} synthesis [65]. This mutant exhibits roughly 2% of normal oxidase activity but around 20% of cytochrome b_{558} expression. By probing the role of the COOH terminus of NOX2 using site-directed mutagenesis, Zhen et al. [72] found that the deletion 560–570 led to the absence of NADPH oxidase activity related to the absence of cytochrome b_{558} synthesis. Therefore, the last ten amino acids of the COOH terminal region of NOX2 can be considered an important region for the stability of cytochrome b_{558} synthesis and for bearing the NADPH oxidase activity. All these cytosolic regions can be potential p22*phox*-binding sites.

X91$^+$ CGD or what do we learn about NADPH oxidase complex activation?

Nineteen mutations have been reported to cause X91$^+$ CGD (Fig. 3) [49, 73]. Most of them are missense mutations, two are small deletions, and one is a deletion–insertion leading to a normal expression of mutated NOX2 proteins (Table 3). They are principally located in the COOH terminus cytosolic tail of NOX2, confirming that it is an important functional part of the protein but less involved in its structural stability. Some functional consequences of such rare mutations have been studied in only 12 patients (mutation nos. 3, 5–9, 11, 13–16, 19, Table 3).

Transmembrane helices of NOX2 Of the three mutations occurring in Arg54 located in the potential II-transmembrane domain of NOX2 near the propionate side chain of the first heme (Fig. 3), functional consequences have been studied in only one patient's neutrophils (mutation no. 3, Table 3) [74–76]. The Arg54Ser mutation affects the function of heme moiety of cytochrome b_{558}, as indicated by a subtle shift in the optical absorbance properties, a decreased midpoint of one heme from E_{m7}=−265 mV to E_{m7}=−300 mV, and the lack of electron transfer from the FAD moiety to heme. However, the membrane's translocation of p47*phox* and p67*phox* occurs normally in activated intact neutrophils from the CGD patient. These data imply that the electron transfer from FAD to oxygen requires both heme groups.

Unknown functional regions Only one double mutation (His303Asn–Pro304Arg) in NOX2 has been reported in the literature to cause X91$^+$ CGD (mutation no. 6, Table 3) [77]. The mutation is located in a site close to the putative FAD-binding site domain of NOX2 (Fig. 3). However, a normal level of FAD was found in the neutrophils' plasma membranes from this patient. Yet the p47*phox* and p67*phox* translocation to the plasma membrane was severely disrupted, suggesting that these residues were essential for the oxidase assembly. However, it is often difficult to get enough total blood to conduct functional analysis at the molecular level from the purified neutrophils of CGD patients. Cellular modeling of such human mutations is therefore necessary. An in vitro cellular model of X91^0 CGD has been developed by Dinauer's group [78]. The X-chromosome-linked CGD locus was disrupted by homologous recombination in the PLB-985 human myeloid cell line (KO PLB-985 cells). The PLB-985 cell line was previously obtained by Tucker et al. [79]. They demonstrated that NADPH oxidase activity can be totally restored by transferring the wild-type gp91*phox* cDNA in the KO PLB-985 cells and that, when transfected by the mutated gp91*phox* cDNA, these cells exactly mimic the phenotype of the original X91$^+$ CGD patient's neutrophils [78]. These KO PLB-985 cells were used to study the impact of the double missense mutation, His303Asp–Pro304Arg, and each single mutation on oxidase activity and assembly, to rule out a possible new polymorphism in the *CYBB* gene [80]. We found that even though the His303Asn mutation has a more severe inhibitory effect on NADPH oxidase activity and assembly than the Pro304Arg mutation, neither mutation can be considered a polymorphism. In addition, one X91$^+$ CGD mutant characterized by a Thr298–Thr302

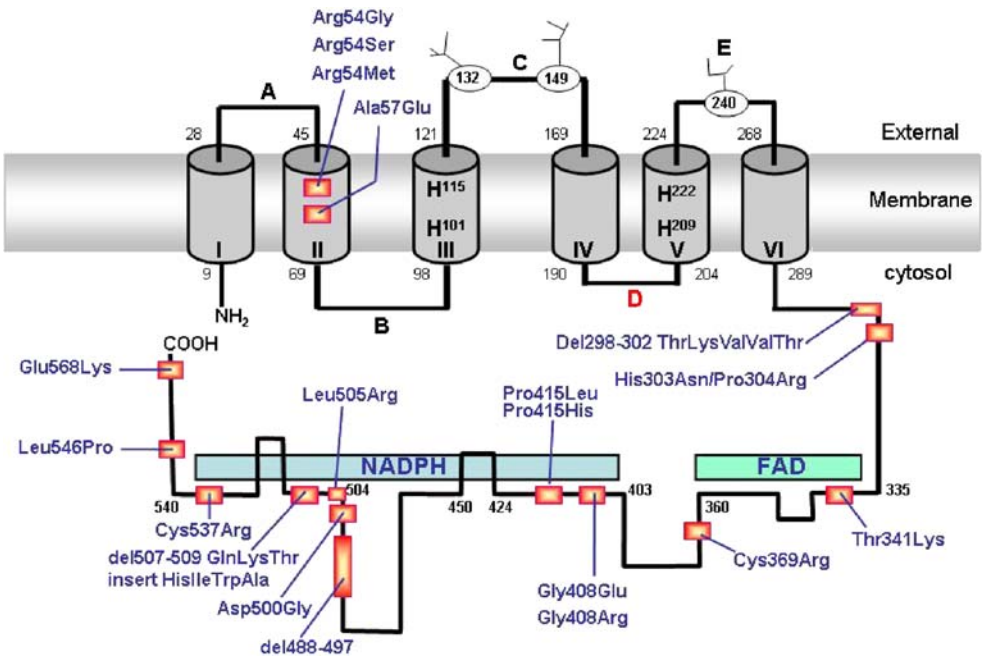

Fig. 3 X91$^+$ CGD mutations in the potential structural and functional model of NOX2. Glycosylated asparagines are located in the external loops of NOX2 and numbered by their residue numbers. The four heme-binding histidines located in the third and fifth transmembrane domains are shown by a single-letter code as H. The potential FAD- and NADPH-binding domains are illustrated by *full boxes*. Mutations causing X91$^+$ CGD forms of CGD are preferentially located in the COOH terminus cytosolic part of NOX2

Table 3 Mutations in *CYBB* gene causing X91^{+} CGD phenotypes

Mutation n°	cDNA nucleotide change	Mutation type	Amino acid change	Potential functional domain	Functional analysis				
					Membrane translocation of p47phox and p67phox	NADPH to FAD	FAD to hemes	Cellular model	Reference
1	A172G	Missense	Arg54Gly	II-transmembrane helix	ND	ND	ND		[58]
2	G173T	Missense	Arg54Met	II-transmembrane helix	ND	ND	ND		[76]
3	G174C	Missense	Arg54Ser	II-transmembrane helix	Normal	Normal	Defective		[74]
4	C182A174C	Missense	Ala57Glu	II-transmembrane helix	ND	ND	ND		[75]
5	902–916 deleted	Deletion	298–302 deletion	?	Defective for p67phox–p40phox	ND	ND		[81]
6	C919A–C923G	Missense	His303Asn/Pro304Arg	?	Defective	ND	ND	PLB-985 cells	[77, 80]
7	C1034A	Missense	Thr341Lys	The isoalloxazine ring of FAD-binding site (338HPFTLTSA)	Normal	ND	ND		[82]
8	T1117C	Missense	Cys369Arg	?	Defective	ND	ND		[82]
9	G1235A	Missense	Gly408Glu	Pyrophosphate of NADPH-binding site (^{405}MLVGAGIGVTPF^{416})	Defective	ND	ND		[82]
10	G1234C	Missense	Gly408Arg	Pyrophosphate of NADPH-binding site (405MLVGAGIGVTPF416)	ND	ND	ND		Unpublished
11	C1256A	Missense	Pro415His	Pyrophosphate of NADPH-binding site (^{405}MLVGAGIGVTPF^{416})	Normal	ND	ND	PLB-985 cells	[78,83,84]
12		Missense	Pro415Leu	Pyrophosphate of NADPH-binding site (^{405}MLVGAGIGVTPF^{416})	ND	ND	ND		Unpublished
13	3′ intron 11 ag/gg	Splice site	488–497 deletion	α-helix (484–504)	Normal	Defective	ND	PLB-985 cells	[90, 91]
14	A1511G	Missense	Asp500Gly	α-helix (484–504)	Defective	ND	ND	PLB-985 cells	[69, 89]
15	1,533–1,537 AAAGA deleted/CATCTGGG insert	Deletion–insert	507–509 GlnLysThr deletion/HisIleTrpAla insert	Adenine of NADPH-binding site (^{504}GLKQ^{507})	Normal	ND	ND		[87]
16	T1526G	Missense	Leu505Arg	Adenine of NADPH-binding site (^{504}GLKQ^{507})	Diminished	Diminished	ND	PLB-985 cells	[49, 86]
17	T1621C	Missense	Cys537Arg	Nicotinamide of NADPH-binding site (^{535}FLCGPE^{540})	ND	ND	ND		[40]
18	T1649C	Missense	Leu546Pro	?	ND	ND	ND		[68]
19	G1712A	Missense	Glu568Lys	?	Defective	ND	ND		[82]

Electron transfer from NADPH to FAD was assessed using the iodonitrotetrazolium reduction assay in a broken cell system (BCS) with purified plasma membranes from CGD patient neutrophils or transfected KO PLB-985 cells mimicking X^{+} CGD phenotypes. Electron transfer from NADPH to molecular oxygen was performed by SOD inhibitable cytochrome *c* reduction assay in a BCS. Electron transfer from FAD to hemes was deducted from both INT and cytochrome *c* reduction assay results. The nomenclature of the mutations relates to the mRNA sequence NM_000397. All the mutations shown in this table are represented in the NOX2 protein (Fig. 3).
ND Data not determined.

deletion (mutation no. 5, Table 3) located very close to the His303–Pro304 residues presented a normal p47*phox* translocation to the plasma membranes but a defective translocation for p67*phox* and p40*phox* [81]. This also confirms that p47*phox* translocation can occur independently of that of p67*phox* and p40*phox*. Then Leusen et al. [82] described a defective translocation of p47*phox* and p67*phox* to the plasma membranes in two $X91^+$ mutants Cys369Arg and Glu568Lys located near the ribityl of the FAD-binding site and in the COOH terminus tail of NOX2, respectively. The NADPH oxidase activity measured in a broken cell system in the presence of cytochrome *c* was defective in all these X^+ CGD mutants (mutation nos. 8 and 19, Table 3). These data provide strong evidence of an intimate relationship between the cytosolic domains of NOX2 involved in p47*phox* and/or p67*phox* binding and in electron transfer.

FAD-binding site The Thr341Lys mutation (mutation no. 7, Table 3) located in the putative FAD-binding domain (^{338}HPFT^{341} motif) of NOX2 leads to a normal cytosolic factor translocation to the membranes (Fig. 3) [82]. However, neither INT reductase activity, which reflects the electron transfer from NADPH to FAD, nor FAD content in the plasma membranes from the patient was measured.

NADPH-oxidase-binding site An interesting missense mutation Pro415His (mutation no. 11, Table 3) in NOX2 causing a case of $X91^+$ CGD was found in a conserved motif (G–X–G–X–X–P) involved in the binding of the pyrophosphate moiety of the NADPH-binding site (^{405}MLVGAGIGVTPF^{416}; Fig. 3) [83]. The translocation of p47*phox* and p67*phox* to the plasma membranes was not affected. The binding of the photoaffinity ligand 2-azido$^-$ $NADP^+$ was decreased in the neutrophil membranes from this patient, indicating that Pro415 is directly involved in the binding of NADPH [84]. Two other X^+ CGD mutations (mutation nos. 9 and 10, Table 3) are located in the same NADPH-binding site as mutation no. 11 (unpublished data, [81]). However, the Gly408Glu mutation leads to a defect in NADPH oxidase assembly (Table 3). The Gly408Arg and the Pro415Leu mutants from our lab are under investigation. Another missense mutation Leu505Arg, (mutation no. 16, Table 3) assumed to be involved in the adenine binding of the NADPH-binding site (^{504}GLKQ^{507}) according to sequence alignment with other ferredoxin reductases and close to the cytosolic α-helical loop (residues 484–504) [85], has been recently reported [49, 86]. This $X91^+$ CGD case was reproduced in the KO PLB-985 cells to investigate the functional and molecular consequence of this type of mutation [85]. We found that assembly and electron transfer from NADPH occurred partially in the Leu505Arg NOX2 mutant. Moreover, Leu505 seems not to be involved in the direct binding of NADPH. This finding calls into question the real position of the adenine of the NADPH-binding site in NOX2. Another $X91^+$ CGD mutation resulting from a deletion–insert mutation (^{507}Gln–Lys–Thr^{509} converted into His–Ile–Trp–Ala) near the assumed adenine of the NADPH-binding site [87] leads to a normal translocation of both p47*phox* and p67*phox* to the plasma membrane (mutation no. 15, Table 3). The last mutant found in this region is a Cys537Arg substitution in the Cys–Gly motif (^{535}FLCGPE^{540}) of the potential nicotinamide moiety of the NADPH-binding site (mutation no. 17, Table 3). However, the functional consequence of this mutation has not been elucidated [40].

Cytosolic α-helix loop In 1993, Taylor et al. emphasized that despite a high similitude of sequences between the FNR family and NOX2, the most remarkable difference was the addition of a large insertion of 20 residues, 484–504, forming a α-helical loop [85]. In their 3D model of the C-terminal tail of NOX2, which was built from the atomic structure of ferredoxin$^-$ $NADP^+$ reductase [88], the location of the large α-helical loop insert impaired accessibility to the nucleotide-binding site from the cytosol when the oxidase was in a resting state. They proposed that upon oxidase activation, NADPH access to the binding site could potentially be regulated by the interaction of this loop with oxidase cytosolic factors. Yet the introduction of this additional α-helical loop could question the positioning of the potential adenine of the NADPH-binding site ^{504}GLKQ^{507}. As noted above, Leu505 located at the end of this additional α-helical loop (mutation no. 16, Table 3) seems not to be directly involved in the binding of the adenine moiety of NADPH but is instead a residue located on the protein surface, which is probably important in the α-helical loop movements controlled by the p67*phox* interaction with NOX2 during the oxidase assembly, leading the NADPH access to its binding site [86]. A point mutation (Asp500Gly) located in the α-helical loop (mutation no. 14, Table 3) leads to a defective translocation of cytosolic proteins to the plasma membranes of defective neutrophils [89]. This suggests that Asp500 has a strategic position in NOX2, consistent with the model of Taylor et al. [85]. Using KO PLB-985 cells and site-directed mutagenesis of charged amino acids in the α-helical loop (residues 484–504), we highlighted that this region, and more precisely Asp484 and Asp500, are essential for proper assembly of the NADPH oxidase complex related to the electron transfer from NADPH to FAD during the time course of activation. The first functional analysis of an $X91^+$ CGD case in transfected KO PLB-985 cells was a splice-site mutation resulting in an in-frame deletion of 30 nucleotides encoding amino acids 488–497 of NOX2 [90,

91]. The 488–497 deletion of NOX2 located in the α-helical loop seems not to influence cytosolic factor translocation to the plasma membranes but alters the electron transfer from NADPH to FAD. Meanwhile, this deletion conserves the acidic amino acids Asp484 and Asp500, which seems to be essential in maintaining the oxidase activity and the complex assembly. In conclusion, the NADPH oxidase assembly process is intimately related to the electron transfer from NADPH to FAD, as proposed by the model developed by Taylor et al. [85].

Autosomal recessive CGD

Autosomal recessive CGD is caused by genetic defects in one of the three genes—*CYBA*, *NCF1*, and *NCF2*—encoding, respectively, p22*phox*, p47*phox*, and p67*phox* (Table 1). It is much less frequently found than the X CGD form and it affects female and male children alike. These three forms collectively account for approximately 30–40% of all CGD cases. Most of the mutations in the three genes are reported in the HGMD databases, http://www.hgmd.cf.ac.uk/ac/index.php and http://bioinf.uta.fi/ [42, 43]. European and Japanese groups reported mutations in *CYBA* [39, 41]; an American group published mutations in *CYBA*, *NCF1*, and *NCF2* [92], and recently Roos et al. [93] made an inventory of all the mutations found in *NCF1*. It should be noted that polymorphisms in the encoding regions of *CYBA*, *NCF1*, and *NCF2* are more frequent than in *CYBB* [92].

A47^0 CGD

The most frequent AR CGD form is caused by mutations in the *NCF1* gene (Table 1). This gene (OMIM number 233700) has been mapped to 7q11.23. It possesses 11 exons. The 5′ upstream region has been identified and no TATA and CAAT boxes were found, unlike the promoter region of *CYBB* [94]. In contrast with the large heterogeneity found in X CGD, a common mutation has been identified in approximately 95% of affected alleles analyzed worldwide. This mutation is a GT deletion (ΔGT) in a GTGT tandem repeat, corresponding to the first four bases of exon 2 [95]. Most patients have a homozygous GT deletion, which predicts a frameshift within a premature stop codon at amino acid 51, leading to a complete absence of p47*phox* protein from the patients' neutrophils (A47^0 CGD). Of approximately 100 patients investigated to date, only 12 patients were compound heterozygote for the ΔGT and one additional mutation; five patients were homozygote for a point mutation different from the ΔGT, and two patients had two different mutations on both alleles of *NCF1* but other than ΔGT [39,92–100]. The mutations other than the classical ΔGT at the beginning of exon 2 were small mutations (nonsense, missense mutations, and small deletion) and always led to A47^0 CGD. The reason that the ΔGT mutation predominates is that most normal individuals (>95%) have two *NCF1* pseudogenes (Φ*NCF1*) on each allele, which exhibit the ΔGT deletion, with more than 99% identity with the *NCF1* gene and they are physically close to the functional gene at 7q11.23. These Φ*NCF1* are the best-conserved unprocessed pseudogenes known [101]. They are located on each side of *NCF1*, one having the same orientation as *NCF1*, the other one having a reverse orientation [102]. Recent studies have demonstrated that the predominance of the ΔGT arises from recombination events between *NCF1* and its highly homologous pseudogene Φ*NCF1* [98,101,103,104]. Because of the presence of these Φ*NCF1* and the extreme homology between them and *NCF1*, it is hardly possible to detect carriers for A47^0 CGD by normal PCR and sequencing methods. In addition, the p47*phox* protein level and the NADPH oxidase activity in the phagocytes of carriers are indistinguishable from normal individuals. Reliable detection of other mutations in *NCF1* is also difficult. However, the pseudogenes are characterized by a single 30-bp block in intron 1, which is duplicated in the functional gene, and by a 20-bp duplication in intron 2, where *NCF1* has a single 20-bp stretch. A number of single nucleotides are also different between *NCF1* and Φ*NCF1s*. Therefore, gene and pseudogene-specific PCR, starting from cDNA or from genomic DNA, have been used [98,99,104,105]. A gene scan method based on the presence of the ΔGT to assess the ratio of *NCF1* genes to pseudogenes has also been developed [105, 106]. With the two technical approaches, they have succeeded in identifying mutations in *NCF1* other than the GT deletion in CGD patients who lack p47*phox* protein expression [93].

A22 CGD

Mutations in the *CYBA* gene encoding p22*phox* are extremely rare (frequency <5%) Table 1). The *CYBA* gene (OMIM number 233690) mapped to 16q24 has 6 exons. The promoter region of *CYBA* contains TATA and CCAC boxes and Sp1, γ-interferon, and nuclear factor κB sites [107]. The last update of *CYBA* mutations done in 2000 by Cross et al. [92] showed 26 different mutations [37,39,41,107–118]. Most of the mutations (15/28) are missense or nonsense (Table 4). Only one mutation is a large deletion (>10 kb) that removed all but the extreme 5′ coding region of the gene (mutation no. 1); three of them are real splice-site mutations caused by a base change in the GT 5′ donor sequence of the intron (mutation nos. 15, 20, 24, Table 4). All the small insertions or deletions led to a frameshift (mutation nos. 13, 14, 16, 23, 27, 28, Table 4)

Table 4 Mutations in the *CYBA* gene causing A22 CGD

Mutation n°	cDNA nucleotide change	Mutation type	Amino acid change	CGD type	References
1	Large deletion >10 kb	Deletion	ND	$A22^0$	[37]
2	5′ intron 1(−4) agtg deleted	Deletion	Insertion 79 bp of intron 1 at the beginning of exon 2	$A22^0$	[41, 199]
3	Exon 2–exon 3	Deletion	ND	$A22^0$	[114]
4	C7T	Nonsense	Gln3stop	$A22^0$	[41, 115]
5	G26A	Nonsense	Trp9stop	$A22^0$	[114]
6	G27A	Nonsense	Trp9stop	$A22^0$	[41]
7	G70A	Missense	Gly24Arg[a]	$A22^0$	[114]
8	G71A	Missense	Gly24Arg[a]	$A22^0$	[41]
9	G74T	Missense	Gly25Val[a]	$A22^0$	[114]
10	G107A	Nonsense	Trp36stop	$A22^0$	[114]
11	T155C	Missense	Leu52Pro[a]	$A22^0$	[114]
12	A158T	Missense	Glu53Val[a]	$A22^0$	[112]
13	C between 162C and 166C	Insertion	Frameshift	$A22^0$	[114]
14	G between 166G and 172G	Insertion	Frameshift	$A22^0$	[110, 112]
15	5′ intron 3 gt→tt	Splice site	Exon 3 deleted	$A22^0$	[114]
16	244C	Deletion	Frameshift	$A22^0$	[37, 114]
17	C268T	Missense	Arg90Trp[a]	$A22^0$	[114]
18	G269A	Missense	Arg90Gln[a]	$A22^0$	[37]
19	A281G	Missense	His94Arg[a]	$A22^0$	[110]
20	5′ intron 4 gt→at	Splice site	Exon 4 deleted	$A22^0$	[110]
21	C354A	Missense	Ser118Arg[a]	$A22^0$	[37, 114]
22	36-bp deletion between 5′ intron 4 and 3′ exon 5	Deletion	179-bp insertion of 3′ intron 4 + 21-bp deletion of 5′ exon 5	$A22^0$	[116]
23	267–273 deletion from exon 5	Deletion	Frameshift	$A22^0$	[117, 118]
24	5′ intron 5 gt→ct	Splice site	Exon 5 deleted	$A22^0$	[113]
25	C371T	Missense	Ala124Val[a]	$A22^0$	[41, 92]
26	C467A	Missense	Pro156Gln[a]	**$A22^+$**	[109, 111]
27	472–484 deletion from exon 6	Deletion	Frameshift	$A22^0$	[45]
28	34-bp deletion from exon 6	Deletion	Frameshift	$A22^0$	[41]

Numbering from the ATG. The only case of $A22^+$ CGD is in bold.
[a] These mutations are represented in p22*phox* protein (Fig. 4A).

except in mutation no. 3 where the entire exon 2 and exon 3 were deleted from the genomic DNA. Yet the genetic reason for this exon's skipping was not determined [114]. It seems that since 2000 no more than two new mutations have been described in *CYBA* [116–118]. The first one is a 36-bp deletion in the intron 4–exon 5 junction leading to abnormal intronic sequence incorporation in the p22*phox* mRNA by the activation of a cryptic site (mutation no. 22, Table 4). The second one is a 7-bp deletion in exon 5 leading to a frameshift and a premature stop codon at position 188 [117, 118]. Missense mutations leading to $A22^0$ CGD are principally located in the potential transmembrane passages of p22*phox* (Fig. 4A). This highlights the amino acids and/or the sequences involved in the structural stability of p22*phox*. Perhaps some of these regions are possible interaction sites with NOX2. Finally, the only missense mutation, Pro156Gln (mutation n° 26, Table 4), leading to the unique $A22^+$CGD, is located in the potential cytosolic C-terminal tail of p22*phox* [109, 111]. Pro156 is in a potential Src homology (SH3) binding domain of p22*phox* [120]. The proline 156 to glutamine substitution inhibits the ex vivo and in vitro p47*phox* and p67*phox* translocation from the cytosol to the plasma membranes. Most likely, binding of p47*phox* is disturbed because p47*phox* is thought to interact first with cytochrome b_{558} (and p22*phox* more precisely) [119, 120]. In addition, p67*phox* failed to translocate to the membrane-bound cytochrome b_{558} in the p47*phox*-deficient CGD, but the translocation of p47*phox* is not impaired in p67*phox*-deficient CGD [121].

A67 CGD

The last and extremely rare autosomal recessive form of CGD is the form caused by mutations in the *NCF2* gene encoding p67*phox*, accounting for approximately 5% of CGD cases (Table 5). The *NCF2* gene (OMIM number 233710) mapped to 1q25 possesses 16 exons (Table 1)

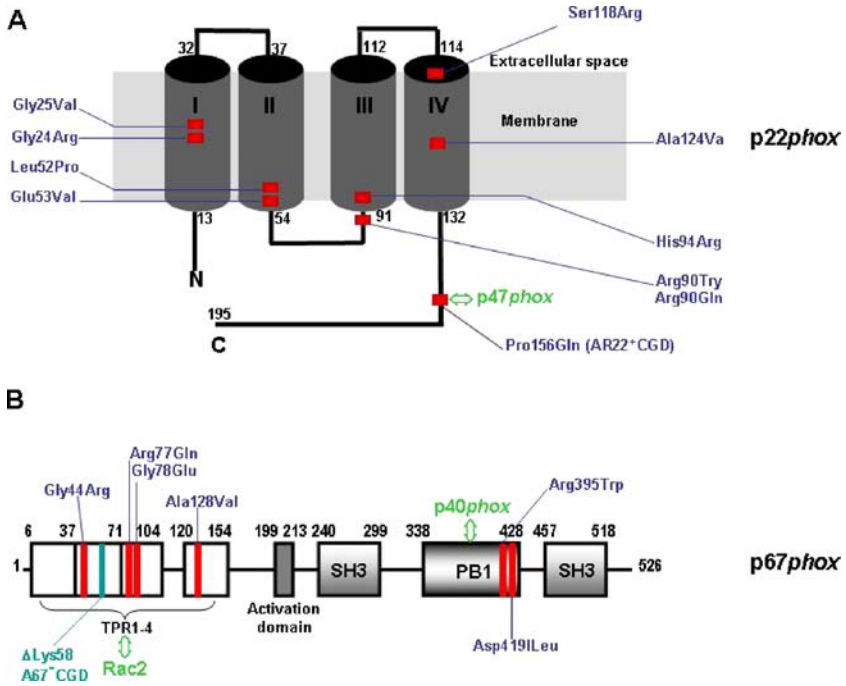

Fig. 4 Location of point mutations leading to $A22^{0/+}$ CGD and $A67^{0/-}$ CGD in the potential structural models of p22*phox* and p67*phox* proteins. **A** Missense mutations located in the first to the forth membrane-spanning domains (numbered with Greek numbers) lead to $A22^{0}$ CGD. The only missense mutation causing an $A22^{+}$ CGD form is located in the cytosolic COOH terminus tail of p22*phox* in a polyproline-rich domain involved in the binding of p47*phox* protein. **B** The majority of missense mutations leading to $A67^{0}$ CGD are located in the tetratricopeptide repeat domains of p67*phox*, which are involved in Rac2 binding during NADPH oxidase activation. An exceptional mutation is the Lys58 deletion in one allele of the *NCF2* gene causing a single $A67^{-}$ CGD case characterized by a defect in Rac2 binding during NADPH oxidase activation. Two missense mutations are located in the PB1 domain involved in the p40*phox* interaction at the resting and activated states of NADPH oxidase. However, the A67 CGD subtypes caused by these two point mutations were not documented

[122–124]. Its promoter region has been well defined. It contains PU.1, IRF1, and ICSBP transcriptional activation sequences, like the *CYBB* gene [122]. Because of the homologous *cis*-element in the *CYBB* and *NCF2* genes, they are regulated by common transcription factors [123, 124]. The last update of *NCF2* mutations revealed 18 different mutations [92,118,125–133]. Most A67 CGD patients had no expression of the p67*phox* protein with normal levels of mRNA [134]. However, an $A67^{0}$ CGD mutation (a T-to-C transition in the conservative 5′ splice site of intron 3) resulted in a deletion in mRNA from 174 to 258 bp, leading to a dramatic reduction in mRNA expression (mutation no. 8, Table 5). This mutation generated a premature TGA stop codon at position 60, resulting in the absence of p67*phox* in the patient containing this T–C transition [126]. Deletion and insertion account for eight cases out of 21, including only two insertions (mutation nos. 13 and 19; Table 5). Ten missense and nonsense mutations were described as were three splice-site mutations caused by a point mutation in the 5′ part of the intron in the donor site (mutation nos. 8, 11, and 17). All the missense mutations led to $A67^{0}$ CGD and were located in the tetratricopeptide repeat (TPR) domains of p67*phox* (Fig. 4B). This demonstrated that the TPR domains are structurally important for p67*phox* protein stability. Only one patient ($A67^{-}$ CGD) expressing half-normal amounts of one amino-acid-deleted (Lys58) p67*phox* has been reported [129]. The patient was a compound heterozygote for a triplet nucleotide deletion in the *NCF2* gene, predicting an in-frame deletion of Lys 58 (mutation no. 4, Table 5) and a larger 11- to 13-kb deletion in the other allele (mutation no. 1). The Lys58 deletion led to the mutated p67*phox* synthesis but disturbed its interaction with Rac. In contrast to normal neutrophils, in which p47*phox* and p67*phox* translocate to the plasma membrane upon cell activation, the patient's cells did not show this translocation, indicating that an interaction between p67*phox* and Rac is essential for the translocation of these cytosolic proteins. Yet this is not in agreement with what was observed in an $A67^{0}$ CGD patient in whom the translocation of p47*phox* occurred normally [121]. Moreover, this CGD patient is the only case caused by defective binding of Rac with p67*phox*. Later, using different experimental approaches, several teams demonstrated the direct binding between these two proteins as a key step in the assembly of the active NADPH oxidase

Table 5 Mutations in the *NCF2* gene causing A67 CGD

Mutation n°	cDNA nucleotide change	Mutation type	Amino acid change	CGD type	Reference
1	11- to 13-kb deletion AAGAAGGAC	Deletion	ND	**A67^{-}**[a]	[128, 129]
2	55–63 deletion	Deletion	19–21 LysLysAsp	A67^{0}	[132, 133]
3	G130C	Missense	Gly44Arg[b]	A67^{0}	[92]
4	170–172 or 171–173 or 172–174 deletion	Deletion	58Lys[b]	**A67^{-}**[a]	[128, 129]
5	C196T	Nonsense	Arg66stop	A67^{0}	[133]
6	G230A	Missense	Arg77Gln[b]	A67^{0}	[133]
7	G233A	Missense	Gly78Glu[b]	A67^{0}	[125]
8	5′ intron 3 GT→GC	Splice site	Deletion of exon 3	A67^{0}	[126]
9	C298T	Nonsense	Gln100stop	A67^{0}	[133]
10	C304T	Nonsense	Arg102stop	A67^{0}	[132]
11	5′ intron 4 GT→AT	Splice site	Del of Ex 3 and 4 or ex 4 or 5 nucleotides of 3′ exon 4	A67^{0}	[132, 133]
12	C383T	Missense	Ala128Val[b]	A67^{0}	[133]
13	AG after 397A (or 399G)	Insertion	Frameshift	A67^{0}	[127]
14	A479T and A481G	Dle missense	AspLys160-161 ValGlu	A67^{0}	[131]
15	728A	Deletion	Frameshift	A67^{0}	[132]
16	835–836 AC	Deletion	Frameshift	A67^{0}	[133]
17	5′ intron 9 GT→AT	Splice site	Del of exons 8 and 9	A67^{0}	[130]
18	1,169–1,173 CTAAG	Deletion	Frameshift	A67^{0}	[118, 132]
19	Duplication of 1.1 kb including ex 9 and ex 10	Insertion	Low amount of abnormal mRNA	A67^{0}	[138]
20	C1250T	Missense	Arg395Trp[b]	A67[c]	[43]
21	A1256T	Missense	Asp419Ile[b]	A67[c]	[117]

Numbering from the ATG. The only case of A67^{-} is in bold.
[a] This patient suffers from rare A67^{-} CGD and is heterozygous for both mutations (mutation nos. 1 and 4).
[b] These mutations are represented in p67*phox* protein (Fig. 4B).
[c] The A67 CGD subtype or variant was not determined.

complex [135–137]. Three new mutations (mutation nos. 19 to 21, Table 5) were recently described, resulting in 21 different mutations found in the *NCF2* gene. The first one (mutation no. 19) involves exon 9 and exon 10 and is the result of tandem duplication of approximately 1.1 kb caused by the juxtaposition of intron 8 to intron 10 [138]. In this case, the mRNA is dramatically reduced, but using RT-PCR the authors found two abnormal bands, one containing the duplication of exon 9 and 10, the other one revealing the presence of a second exon 10 between exons 8 and 10. The two most recently described mutations, Arg395Trp and Asp419Ile (mutations n°20 and 21, Table 5), are located in the C-terminal tail of p67*phox*, where only polymorphisms have been found until now [43, 117]. Unfortunately, the authors did not determine the subtypes of these mutations (0, −, or +). An interesting point to underline is that Borgato et al. and our group [118, 138] observed that the absence of p67*phox* protein expression leads to the absence or the reduction of p40*phox* expression. This confirms that p67*phox* and p40*phox* protein stability are related [139, 140]. However, in the 21 cases reported, p40*phox* expression was rarely documented.

CGD diagnostic tests

The common and usual diagnostic characteristic of CGD is the absence of respiratory burst in stimulated phagocytes. In some rare variants such as X91^{-} CGD subtypes, as previously described, there is a small amount of normal NADPH oxidase activity, i.e., roughly 1–20% (Table 2). The other phagocyte functions, including chemotaxis, adhesion, phagocytosis, and degranulation of intracytoplasmic granule populations, are normal. The simplest, the most robust, and the least expensive screening test is the reduction of the NBT. This is easily performed by exposing neutrophils to NBT together with a soluble stimulus such as phorbol myristate acetate, formyl peptide,]or with a particulate stimulus such as opsonized latex beads, bacteria, or zymosan (to test the ability of cells to phagocytize at the same time). Then the yellow water-soluble NBT dye is reduced to dark blue insoluble formazan in the activated cells. This test is valid with total blood or purified white cells or purified neutrophils, even if the blood sample is transported and preserved (<48 h). However, a control blood sample carried and preserved in the same manner as the patient's sample and a control fresh

blood sample are needed to ascertain the results. More sensitive techniques such as luminol or lucigenine chemiluminescence exist, where fewer than 10^5 cells can be taken for one test, to measure hydrogen peroxide or superoxide, respectively. The major disadvantage is the expression of the results in arbitrary units (relative luminescence unit [RLU]) and the calculation method (maximum RLU or peak or the sum of total RLUs during a total measurement time that varies depending on the stimuli used). Then fresh purified neutrophils must be used for reproducible results. The flow cytometry method (fluorescent-activated cell sorting [FACS]) is also available using fluorescent probes (dihydrorhodamine-1,2,3 [DHR] or 2′7′-dichlorofluorescein diacetate) to measure intracellular ROS production, but the equipment is sophisticated and expensive and the blood sample must be freshly drawn. The NBT test, chemiluminescence measurement, and the FACS can evaluate intracellular ROS production after soluble or particulate stimuli activation. Fluorescence measurement following resorufine oxidation by ROS is a highly sensitive method able to quantify only extracellular hydrogen peroxide production. A less sensitive but more specific measurement of NADPH oxidase activity is superoxide-inhibitable cytochrome *c* reduction using a classical dual-beam spectrophotometer. However, it assesses only external O_2^- production. The reference method is still the measurement of oxygen consumption of stimulated phagocytes in the presence of cyanide, with an oxygen-sensitive electrode. However, it is necessary to use at least two to five million purified neutrophils per test. The NBT test and the FACS analysis have the advantage of being able to detect the carrier state in female relatives of X-linked CGD patients, who often show a mixed population of NBT-positive or oxidized DHR-positive populations and negative cells in a nearly identical proportion. Sometimes inactivation of the X chromosome in these carriers has given different proportions of positive and negative cells depending on what X chromosome is preferentially inactivated, providing from normal to pathological results. With the other methods used in cases of X91 CGD carriers (kinetics of cytochrome *c* reduction, chemiluminescence, or fluorescence), only a reduction in NADPH oxidase activity is observed and it is not as easy to use as the NBT test and the FACS methods to evaluate carrier status. Carriers of ARCGD are never detected by the measurement of NADPH oxidase activity in their neutrophils. Their detection is best achieved by genetic analysis. However, first the gene involved in the disease has to be determined. The missing protein of the NADPH oxidase components (except for rare CGD variants where all the oxidase proteins are expressed) has to be determined using Western blot in the CGD patient's neutrophils with specific antibodies. Flavocytochrome b_{558} can also be evaluated in a Triton-X100-soluble extract (to avoid myeloperoxidase contamination for the spectral analysis) from the CGD patient's neutrophils, by a reduced-minus-oxidized difference spectrum. If the cytochrome b_{558} signal is missing, the subunit involved remains to be determined because each subunit stabilizes the other. Meanwhile, NOX2 is clearly involved if there is evidence of X-linked transmission, either in the familial history or by the detection of female carriers in relatives. A history of consanguinity in the parents and/or a female CGD patient can evoke an AR inheritance. Definitive evidence of molecular lesions used for genetic counseling and in prenatal diagnosis is achieved by sequencing the appropriate gene. Since the location of genetic mutations is never known (except for the majority of $A47^0$ CGD cases), RT-PCR from the corresponding mRNA and sequencing of the resulting PCR product are the easiest first steps in determining the genetic lesion in the majority of cases. This cannot be applied for large deletions or insertions or other mutations that cause unstable mRNA. Single-strand conformation polymorphism or simple restriction fragment length polymorphism can also be informative in defined cases. However, the genetic defect must always be confirmed at the genomic level.

Clinical features of CGD

CGD is characterized by an unusual predisposition to infection with bacteria and fungi, resulting in severe recurrent bacterial and fungal infections and granuloma formation [29]. The severe recurrent bacterial and fungal infections normally are difficult to treat using conventional means. CGD patients usually present a clinical syndrome in the first few years of life with cervical or inguinal lymphadenitis, liver abscesses, osteomyelitis, pneumonia, or skin infections [38]. Rarely is CGD diagnosed later in adulthood [141, 142]. Based on a study of a registry including 368 CGD patients in the US, the common complications in CGD are pneumonia (79%), suppurative adenitis (53%), subcutaneous abscess (42%), liver abscess (27%), osteomyelitis (25%), and sepsis (18%) [38]. A recent study of 60 CGD patients in Italy demonstrated that pneumonia (47%) and lymphadenitis (45%) are the most common infections, followed by dermatitis (26%), subcutaneous abscess (20%), liver abscess (16%), and osteomyelitis (16%) [142]. The microorganisms responsible for the majority of infections in CGD are *S. aureus*, Gram-negative enteric bacilli (including *Serratia marcescens*, *Salmonella* species, and *Burkholderia cepacia*) and *Aspergillus* species. Catalase-negative bacteria are rarely involved in CGD infection because of microbe-generated H_2O_2 in the phagosomes of CGD cells [38, 142]. The other hallmark of CGD is the development of chronic inflammatory granuloma, characterized by obstruction in hollow organs.

Half of CGD patients had gastric outlet obstruction, 10% urinary tract obstruction, and 17% colitis–enteritis [38]. In a review of 140 CGD patients, gastrointestinal involvement was reported to be a common and recurring problem in CGD [143]. Current and early prophylaxis with antibiotics and antifungals allows most patients to survive into adulthood. Adolescent and adult CGD is increasingly characterized by inflammatory complications, such as granulomatous lung and inflammatory bowel disease, requiring immunosuppressive therapy [144] (see also M. Schappi and K.H. Krause's review in this NOX issue). A variety of disorders (e.g., lupus syndrome) have been reported in CGD patients without infectious etiology [38]. The registry data showed that pneumonia and/or sepsis due to *Aspergillus* and *Burkholderia* are the most common causes of death in CGD patients [38].

X-linked CGD patients, accounting for 65% of CGD cases, have been reported to have more severe clinical complications and higher mortality rates than those with $A47^0$ CGD. X91 CGD patients have a higher mortality rate (5%) than A47 CGD patients (2%) per year [38]. In general, female carriers of $X91^0$ CGD, with 10% normal granulocytes, are asymptomatic; in rare cases, female carriers with the same or a higher proportion of normal circulating neutrophils may have a clinical manifestation of a host defense defect [29].

Clinical management of CGD

Antimicrobial prophylaxis

Long-term antimicrobial prophylaxis is the mainstay of treatment for CGD patients. Adequate prophylaxis of bacterial infections with trimethoprim-sulfamethoxazole (or dicloxacillin in CGD patients who are allergic to sulfa) is indicated for the management of patients with CGD [29,145,146]. Trimethoprim-sulfamethoxazole prophylaxis decreases the incidence of nonfungal infections without increasing the incidence of fungal infections [145, 147]. An open-label study of long-term itraconazole in Europe showed excellent tolerance and a reduced rate of *Aspergillus* infections compared to historical controls [148]. A double-blinded, randomized, placebo-controlled study at the National Institute of Health (NIH) demonstrated that prophylaxis of itraconazole prevented both serious and superficial fungal infections in CGD patients, with excellent tolerance [149]. The minimal side effects of rash, increased liver function values, and headache were observed in this study; these minor toxic effects were resolved on discontinuation of the drug [149]. The mortality rate for CGD patients is believed to be approximately 2–5% per year [38]. A follow-up study of 21 British children with CGD diagnosed since 1990 revealed that all 21 patients were thriving and developmentally normal under the treatment of prophylactic co-trimoxazole and itraconazole at the time of CGD diagnosis, suggesting that the prognosis of CGD could be improved with the development of antimicrobial prophylaxis [150]. Ketoconazole has been reported to be ineffective in reducing fungal infections in CGD patients [147].

Interferon-γ

Long-term prophylactic trimethoprim-sulfamethoxazole has greatly reduced the infection rate in CGD patients. Interferon-γ, an immunomodulatory cytokine, has further decreased the rate of infection among CGD patients [143, 151]. Interferon-γ has been shown to increase NADPH oxidase activity in some rare variants of $X91^-$ CGD with neutrophils and monocytes characterized by a very low but detectable oxidase activity. However, interferon-γ did not significantly increase phagocyte superoxide production from classical $X91^0$ and AR CGD [152, 153]. A multicenter study of 128 CGD patients showed that interferon-γ reduced the serious infections in CGD patients with a subcutaneous injection three times weekly [151]. In this study, the benefit of interferon-γ prophylaxis in CGD patients was observed in both X-linked and autosomal recessive forms of CGD. Interferon-γ therapy was well tolerated without serious toxicity. A previous 12-month, randomized, double-blind, placebo-controlled trial in Europe showed that interferon-γ used as an infection prophylaxis is safe and justified [154]. Long-term interferon-γ therapy for 76 CGD patients has been reported to be effective and well tolerated in a 9-year open-label study in the US [143]. The prolonged use of interferon-γ appears safe and shows persistent reduction in the rate of serious infection and mortality. No enhancement of proinflammatory complications, such as granuloma formation, was observed. Interferon-γ is also effective in reducing infections in a CGD mouse model [155]. Interferon-γ is believed to enhance the oxidant-independent antimicrobial pathways. However, the molecular mechanisms associated with host defense improvement induced by interferon-γ in CGD patients are unknown. Interferon-γ is now recommended as lifelong therapy for infection prophylaxis in CGD patients [147], but the cost of long-term prophylactic interferon-γ is high and it needs to be injected intramuscularly, making the compliance to this treatment rather poor [156].

Stem cell transplantation

Although lifelong prophylaxis with antimicrobial drugs and interferon-γ reduce the incidence of infection in CGD patients, the overall annual mortality is still high (2–5%) [146,157,158]. Because CGD results from a defect in

myeloid lineage cells, stem cell transplantation is a potentially curative option for CGD patients when an HLA-matched donor is available [29,146,147, and 159]. The first reported bone marrow transplantation (BMT) in a 3.5-year-old boy with CGD failed after 2 months because of tissue rejection [160]. A survey of the European experience (1985–2000) showed that the overall success rate of unmodified hemopoietic allograft combined with myeloablative conditioning for those with an HLA-identical donor is 81%, with an overall mortality of 15% [158, 161]. A review of cases revealed that 20 of 24 CGD patients were alive and were disease free 1–7 years after transplantation; most patients were conditioned with busulfan and cyclophosphamide [162].

However, graft-versus-host disease (GVHD) and inflammatory flare-ups at infectious sites are the major risks associated with BMT [158, 161]. In order to reduce the risk of GVHD and minimize the toxicity induced by myeloablative conditioning, Malech and colleagues [157] developed a CGD treatment with nonmyeloablative conditioning and a T-cell-depletion hematopoietic stem cell allograft. In this study, the proportion of circulating donor neutrophils in eight of ten CGD patients was 33–100%, and preexisting granulomatous lesions resolved in patients with successful transplantation. Unfortunately, three of four adult CGD patients developed acute GVHD and three of ten patients died. Recently, Sastry et al. [163] reported successful allogeneic bone marrow transplantation with reduced intensity conditioning for a case of X91 CGD complicated by severe invasive aspergillosis, indicating reduced intensity conditioning should be considered as an alternative to standard myeloablative conditioning for CGD.

Although the successful cases of CGD stem cell transplantation are promising, the morbidity and mortality (~10%) associated with BMT discourages physicians from recommending and using this therapeutic approach [29, 147]. The time of transplantation is considered a critical factor. Ideally, the infections should be under control before BMT [29, 158]. The transplantation appears to be most successful if performed in infancy or early childhood [147]. Seger and colleagues [158] proposed that CGD patients with an HLA-matched sibling and recurrent invasive infections and/or inflammatory, steroid-dependent disease should be considered as prime candidates for stem cell transplantation.

Gene therapy

CGD is caused by mutations in any of four genes encoding subunits of the NADPH oxidase complex. In general, X-linked CGD female carriers with at least 10–20% normal neutrophils do not have clinical syndrome, suggesting that clinical benefit from gene therapy might occur in partially functionally corrected granulocytes [146]. For CGD patients without an HLA-matched donor, gene therapy becomes an attractive and promising option. Gene therapy for CGD has been applied in cellular level studies, murine CGD models, and clinical trials [29,146,161]. NADPH oxidase activity could be restored by gene transfer into EBV-transformed B cells and primary monocytes from patients with X-linked or autosomal recessive forms of CGD in vitro [29,146,164–167]. Peripheral blood progenitors and bone marrow $CD34^+$ from CGD patients were used as a target for genetic correction of A47 CGD, X91 CGD, and A67 CGD, and reconstitution of respiratory burst activity was observed [168–171]. High-level reconstitution of respiratory burst activity in the human X CGD PLB-985 cell line has been reported by Dr. Dinauer's group [172]. The development of $gp91^{-/-}$ and $p47phox^{-/-}$ mouse models made clinically relevant evaluation of CGD gene therapy possible [161]. Basically, the hematopoietic stem cells that were transduced with retroviral vectors encoding gp91*phox* or p47*phox* were transplanted into gp91*phox*- or p47*phox*-deficient mice, respectively, with lethal or sublethal irradiation [173–178]. These studies showed that NADPH oxidase activity was restored in neutrophils and the resistance to challenge with bacteria and fungi was increased, suggesting that the correction of CGD could be achieved by gene transfer into hematopoietic stem cells.

Adenovirus has been used to express p47*phox* and gp91*phox* in monocytes from CGD patients, achieving successful correction of NADPH oxidase activity [167, 179]. Functional reconstitution of the NADPH oxidase was observed in adeno-associated virus-2 (AAV) vector-mediated gene therapy for p47*phox* and gp91*phox* [180, 181]. However, human elongation factor-1alpha silenced a high percentage of clones with integrated rAAV [181]. The majority of vectors used in CGD gene therapy are retroviruses, including murine stem cell virus and the Moloney murine leukemia virus-based retroviral vector (MFGS) [171–175]. Promising correction of NADPH oxidase activity was achieved in $gp91^{-/-}$ and $p47phox^{-/-}$ knockout mouse models and $CD34^+$ cells from an $A67^0$ CGD patient [171–175]. However, retroviral vectors only infect dividing cells and carry the risk of insertional mutagenesis [182–184]. For transduction efficiency and safety reasons, lentiviral vectors have been tried in $X91^0$ CGD gene therapy. Significant correction of NADPH oxidase activity was observed in the third generation self-inactivating lentivector-mediated gp91*phox* gene therapy [185]. The transduction efficiency in Epstein-Barr-virus-transformed B cells from $X91^0$ CGD or $A47^0$ CGD patients has been enhanced by selection for human multidrug-resistant gene expression [186, 187]. The results showing that the concentrated RD114-pseudotyped MFGS-gp91*phox* vector achieved high levels of functional

correction of X CGD suggest that higher titer of virus may increase the transduction efficiency for CGD gene therapy [188].

Although the correction of CGD on animal models is promising, clinical trials of CGD gene therapy have not yet been successful. Clinical trials of five $A47^0$ CGD and five $X91^0$ CGD patients by Harry Malech at the NIH, including the first clinical trial, showed that peak levels of 0.004–0.13% oxidase-corrected peripheral granulocytes and were observed at 3–6 weeks, with the effect lasting several months after infusion [38,189–192]. Autologous mobilized $CD34^+$ peripheral blood stem cells were transduced with a retrovirus vector encoding gp91*phox* or p47*phox*. The third clinical trial of two $X91^0$ CGD patients showed that superoxide production was detected in both patients in 0.1% peripheral blood neutrophils and persisted for 9 months [193]. No bone marrow conditioning, which is believed to have a proliferative advantage over nontransduced cells, was used in any of these clinical trails. Cyclophosphamide has been used as a mild myelosuppressive conditioning regimen in an $X91^0$ CGD patient [194]. However, gene-marked granulocytes from peripheral blood amounted to 1% shortly after reinfusion and decreased to almost undetectable levels 3 months later. The same group developed a partially myeloablative dose of busulfan combined with a new protocol in two adult $X91^0$ CGD patients. This encouraging study showed that gene-marked peripheral blood granulocytes ranged from 12% to 31% during the first 4–5 months after treatment, and similar amounts of functionally corrected granulocytes were found in both patients, which contributed to the eradication of refractory bacterial and fungal infections from which patients had suffered for many years [161,195,196]. The number of gp91*phox*-transduced cells increased up to 40–60% of total peripheral blood granulocytes 10 months after transplantation and remained at the same level for the next 12–14 months without abnormal myeloid proliferation. One patient died of severe bacterial sepsis following colon perforation 27 months after gene therapy. The cause of death is still under investigation [161, 197].

In conclusion, CGD is a promising candidate for gene therapy. New strategies including vector design, in vivo selection of transduced hematopoietic stem cells, development of myeloid-specific promoters to restrict the transgene specifically expressed in myeloid compartment, and bone marrow conditioning will contribute to the successful gene correction of CGD.

Final comments

Since the first description of "a fatal granulomatous disease" in the 1960s, a great deal has been learned about the molecular origin of CGD. The NADPH oxidase failure of phagocytes has been well understood and its activation mechanism has been partially elucidated. However, the molecular processes of the termination of its transient activity are less thoroughly understood [198]. Studies on patients with CGD have provided important information on the genetics of the enzyme and have highlighted the existence of membranous and cytosolic components of the NADPH oxidase complex. Then the identification of potent natural tools for $X91^-$ and $X91^+$ CGD variants, characterized by diminished or normal mutated NOX2 expression, respectively, has helped to map sequences that are essential for the stability of NOX2 (and cytochrome b_{558}) or involved in the activation mechanism of the NADPH oxidase complex. Although direct functional studies on neutrophils from CGD patients have provided clear progress in the current knowledge of the activation process of the NADPH oxidase (e.g., the study of $A22^+$ CGD and the $A67^-$ CGD), functional analysis of the protein defect at the molecular level requires a large amount of biological material. The cellular modeling approach of such human mutations by directed site mutagenesis and stable transfection in the KO PLB-985 cell line is an interesting alternative and an efficient approach to probing the role of new regions of NOX2.

Working on an international level to corroborate research findings has contributed to furthering and disseminating the knowledge of rare diseases, resulting in improved inherited immunodeficiency syndrome detection and in the management of CGD patients, who are now protected against severe and life-threatening infections earlier than before. It should be noted that alongside the sophisticated methods used to measure the failure of NADPH oxidase activity in phagocytes, a simple stain test (the NBT reduction) is needed to establish the biological diagnosis. Significant progress in CGD molecular genetics can now offer genetic counseling and prenatal diagnosis of all CGD forms. One of the major advances in this field is the understanding of the relationship between the *NCF1* gene and its pseudogenes, which elucidate the occurrence of the most frequent autosomal recessive mutations in CGD (a GT deletion at the beginning of exon 2). Recently, new technical approaches have succeeded in evaluating the GT signature in potential carriers and identifying new mutations that are different from the GT deletion.

Long-term antibiotic and antifungal prophylaxis and interferon-γ is still recommended for CGD patients. Aggressive antibiotic and high-dose interferon-γ treatment has been advocated during severe infectious episodes. Granulocyte transfusion is another option for CGD patients with life-threatening infections and in infections refractory to antibiotics and surgical treatment. The most important advances in recent years have been the development of

autologous hematopoietic stem cell gene therapy and allogeneic transplant approaches. Bone marrow transplantation is a potentially curative treatment for CGD patients with an HLA-matched sibling, especially in their infancy or early childhood. However, this treatment has a slight risk of transplantation-related morbidity and mortality, especially in patients with other severe debilitating illnesses. CGD is a promising candidate for the development of gene therapy targeted at hematopoietic stem cells. It was recently demonstrated that this approach was feasible and may significantly ameliorate acute and chronic infections for CGD patients. Hopefully, the improvements in the safety and efficacy of gene therapy could help achieve a clinically significant correction of CGD.

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