



HAL
open science

A novel point mutation in the gene promoter leading to a rare X minus chronic granulomatous disease variant – Impact on the microbicidal activity of neutrophils

Federica Defendi, Eva Decleva, Cécile Martel, Pietro Dri, Marie José Stasia

► **To cite this version:**

Federica Defendi, Eva Decleva, Cécile Martel, Pietro Dri, Marie José Stasia. A novel point mutation in the gene promoter leading to a rare X minus chronic granulomatous disease variant – Impact on the microbicidal activity of neutrophils. *Biochimica et Biophysica Acta - Molecular Basis of Disease*, 2009, 1792 (3), pp.201. 10.1016/j.bbadis.2009.01.005 . hal-00501601

HAL Id: hal-00501601

<https://hal.science/hal-00501601>

Submitted on 12 Jul 2010

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Accepted Manuscript

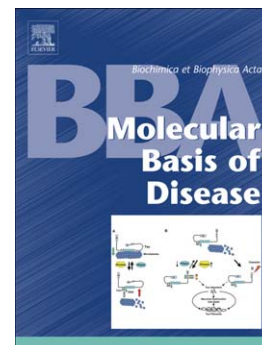
A novel point mutation in the *CYBB* gene promoter leading to a rare X minus chronic granulomatous disease variant – Impact on the microbicidal activity of neutrophils

Federica Defendi, Eva Decleva, Cécile Martel, Pietro Dri, Marie José Stasia

PII: S0925-4439(09)00006-4
DOI: doi:[10.1016/j.bbadis.2009.01.005](https://doi.org/10.1016/j.bbadis.2009.01.005)
Reference: BBADIS 62913

To appear in: *BBA - Molecular Basis of Disease*

Received date: 1 August 2008
Revised date: 7 January 2009
Accepted date: 7 January 2009



Please cite this article as: Federica Defendi, Eva Decleva, Cécile Martel, Pietro Dri, Marie José Stasia, A novel point mutation in the *CYBB* gene promoter leading to a rare X minus chronic granulomatous disease variant – Impact on the microbicidal activity of neutrophils, *BBA - Molecular Basis of Disease* (2009), doi:[10.1016/j.bbadis.2009.01.005](https://doi.org/10.1016/j.bbadis.2009.01.005)

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

A novel point mutation in the *CYBB* gene promoter leading to a rare X minus chronic granulomatous disease variant – Impact on the microbicidal activity of neutrophils

By ^{a,b}Federica Defendi, ^bEva Decleva, ^aCécile Martel, ^{b#}Pietro Dri and ^{a#}Marie José Stasia

From the ^aCentre Diagnostic et Recherche sur la Granulomatose Septique CGD, Laboratoire TIMC-IMAG UMR CNRS 5525, Université Joseph Fourier, Grenoble, France, the ^bDepartment of Life Sciences, Università di Trieste, Italy.

Supported by grants from the Université Joseph Fourier, Faculté de Médecine; the Région Rhône-Alpes, the Ministère de l'Éducation et de la Recherche, MENRT; the Direction Régionale de la Recherche Clinique ; the Primary Immunodeficiency Disease Consortium's National Institutes of Health to MJ Stasia, contract number N01-AI-30070; the Italian Telethon Foundation to P. Dri, Contract Grant Number GP07221.

Pietro Dri and Marie José Stasia contributed equally to this work

Key words: Chronic granulomatous disease, *CYBB* gene promoter, point mutation, cytochrome *b*₅₅₈, NADPH oxidase, microbicidal activity.

Name and address of corresponding author:

Name	Marie-José STASIA
Address	Centre Diagnostic et Recherche sur la Granulomatose Septique CGD Laboratoire TIMC-IMAG, UMR CNRS 5525, Université J. Fourier CHU Grenoble, BP 217 38043 Grenoble Cedex 9, France
Telephone	33 (0)4 76 76 54 83
Fax	33 (0)4 76 76 56 08
E-mail	mjstasia@chu-grenoble.fr

Abstract:

This article reports an atypical and extremely rare case of X-linked CGD in an Italian family characterized by a low expression of gp91 $phox$ (X91 $^-$ CGD). A novel point mutation in the *CYBB* gene's promoter (insertion of a T at position -54T to -56T) appeared to prevent the full expression of this gene in the patient's neutrophils and correlated with a residual oxidase activity in the whole cells population. The expression and functional activity of the oxidase in eosinophils appeared to be almost normal. Gel shift assays indicated that the mutation led to decreased interactions with DNA-binding proteins. The total O₂ $^-$ production in the patient's granulocytes (5–7% of normal) supported no microbicidal power after 45 min and 60 min of contact with *S. aureus* and *C. albicans*, respectively. Despite this residual oxidase activity, the patients suffered from severe and life-threatening infections. It was concluded that in these X91 $^-$ CGD neutrophils, the O₂ $^-$ production *per se* was not sufficient to protect the patient against severe infections.

1. Introduction

Chronic granulomatous disease (CGD) is a rare congenital immunodeficiency syndrome clinically characterized by severe recurrent bacterial and fungal infections, which are relatively resistant to treatment, by conventional means [1,2]. The majority of the patients are now diagnosed before their second birthday, although some may remain undiagnosed until later childhood or even adult life [3,4]. CGD is due to a defect in the NADPH oxidase system of phagocytes [2]. This enzymatic complex is composed of a membrane-bound flavocytochrome b_{558} , the redox center of the oxidase, and three cytosolic components: p47phox, p67phox and p40phox. Two small G proteins, rac2 and rap1A/B, are also involved in regulating NADPH oxidase activity. Cytochrome b_{558} , the terminal redox membranous component of the phagocyte NADPH oxidase, is a heterodimer composed of an α subunit, the small p22phox, and a β subunit, the heavy chain gp91phox or Nox2. Gp91phox is the flavin oxidase element capable of transferring electrons, while p22phox stabilizes the expression of this component [5]. The incidence of CGD is about 1 in 250,000 individuals. On the basis of the mode of inheritance, two forms of the disease are known: an autosomal form with mutations in *CYBA*, *NCF1* and *NCF2* genes encoding p22phox, p47phox and p67phox proteins, respectively, and an X-linked form with mutations in the *CYBB* gene encoding gp91phox, which accounts for more than 60% of CGD cases [5,7]. *CYBB* gene encompasses 13 exons spanning about 30 kb of human X chromosome genomic DNA [8,9]. In the so-called classical form of CGD which includes the majority of X-linked cases, cytochrome b_{558} is absent and there is no detectable oxidase activity (X91⁰). A number of cases with “variant” forms of the disease, called X91⁻ CGD have also been described, in which low levels of cytochrome b_{558} expression are accompanied by a proportionally decreased NADPH oxidase activity [10]. Mutations associated with this phenotype are usually located in the coding region (exons) of *CYBB* [11-16]. These variants are of interest because

they cause a structural disorganization leading either to an incomplete loss of protein or to a partial dysfunction, or both. Mutations in the upstream promoter region of *CYBB* leading to the $X91^-$ CGD phenotype also have been described. To our knowledge only four point mutations were reported in this region (C-52T, C-53T, T-55C and A-57C) [17-19]. These mutations are located between the “CCAAT” and the “TATA” boxes in a consensus binding site for the *ets* family of transcription factors of the *gp91phox* promoter responsible for *CYBB* transcription [17]. Despite the low expression of *gp91phox* protein and the residual NADPH oxidase activity in phagocytes, the clinical appearance of these specific $X91^-$ CGD patients varies considerably from mild forms [18] to severe clinical phenotypes with multiple life-threatening infections during their life [17,19]. This suggests that the levels of O_2^- production and other derived reactive oxygen species (ROS) by activated phagocytic cells are critical for effective protection against infections. A striking point is that in most of the $X91^-$ CGD cases characterized by a mutation in the *CYBB* promoter, the CGD diagnosis is made in adolescents (>10 years) or in adults.

We report a novel point mutation in the *CYBB* gene promoter (insertion of a T at position -54/-56) leading to a rare $X91^-$ CGD case in an Italian family with two members affected by CGD. The mutation caused a reduced expression of *gp91phox* at the mRNA and protein level associated with a decreased ability to generate O_2^- and H_2O_2 (~ 7% of control neutrophils). A decrease in the binding of nuclear factors to the mutated promoter region was found in electrophoretic mobility shift assays. However, the patient's eosinophils were NBT-positive, displayed DHR oxidation and were *gp91phox*-positive. Despite a weak respiratory burst activity, the patient's granulocytes did not kill either *S. aureus* or *C. albicans* even with long incubation times. The CGD diagnosis for both patients was made in adulthood. One of them died from a bilateral pneumonia while the other suffers from sporadic respiratory and skin infections that are kept under control by conventional antibiotic therapy. These

observations raise questions about the role and the levels of ROS needed for protection against bacterial and fungal infections.

2. Materials and Methods

2.1 Materials

Phorbol 12-myristate 13-acetate (PMA) and cytochrome c (type VI, from horse heart) were from Sigma Chemical Co. (St. Louis, MO). Percoll, reagents and molecular weight markers for SDS-PAGE were obtained from Amersham Pharmacia Biotech (Little Chalfont, Buckinghamshire, UK). Nitrocellulose sheets for Western blotting were purchased from Bio-Rad Laboratories. Monoclonal antibodies (MAbs) 449 and 48 were provided by D. Roos and A.J. Verhoeven. Mab 7D5 was provided by Clinisciences (Montrouge, France). Control IgG1 mAb from a mouse myeloma was purchased from Sigma Chemical Co. Alexa Fluor 647 rat anti-human CD294 (CRTH2) was purchased from BD Biosciences (San Jose, CA, USA).

2.2 Cases report of patients II-2 and III-5 of family C (Figure 1)

Patient II-2 was born in 1947. His mother died several years ago from undefined pathology while his father passed away recently. His son (III-1) is in good health and his daughters (III-2, III-3) are carriers of the disease. The patient sisters are both CGD carriers and one of them died recently. The brother died of pneumonia at the age of 14. Diagnosis of CGD was made in 1991 immediately after his nephew (patient III-5) was diagnosed as having CGD at the age of twenty during the terminal phase of the disease. Patient II-2 was relatively well until he was 5 years old. He subsequently had a history of skin abscesses, pulmonary infections with pleuritis treated with antibiotic therapy. The clinical history of patient II-2 is characterized by episodes of bronco-pneumonia, skin ulcero-necrotic lesions and an otitis

media with tympanic membrane perforation, which responded to antibiotic treatment. At present the patient is in a relatively good health and does not receive antibiotic prophylaxis.

Patient III-5, who is the nephew of patient II-2, was born in 1970 and died in 1990. He had no complaints and was healthy until the age of 19. In 1989 he developed severe multiple site infection of the skin with necrotic granuloma with involvement of the right latero cervical nodes and of the lung with pleural effusion sepsis that required hospitalization. *Serratia marcescens* was isolated from the blood and the lymph node. Lymph node and pleural lesions required surgical drainage. On that occasion, a marked defect in the respiratory burst of granulocytes was detected by a luminol-dependent chemiluminescence assay using opsonized zymosan as stimulating agent. Antibiotics including ceftazidime and netilmicin were administered and the patient recovered in five weeks. He was admitted again to hospital six months later, in March 1990, with fever, cough, dyspnoea and radiological evidence of a bilateral pneumonia. After a few days from admission the general conditions of the patient worsened and intensive care unit assistance was required. Two blood cultures were positive for *Staphylococcus sp.* (non aureus). *Pseudomonas aeruginosa* and *Candida albicans* were isolated from bronchial brushings. Antibiotic treatment, including ceftazimide, piperacilline, netilmicine and teicoplanine failed to control fever and pulmonary infection. After diagnosis was made, and before the molecular nature of the defect was known, it was hypothesized that the low activity of NADPH oxidase could be the result of enzyme instability. Accordingly, it was thought that "younger" cells could possess higher oxidase activity and kill microorganisms more efficiently. Therefore, GM-CSF was used with the aim of accelerating neutrophil release from the bone marrow and exploiting its known ability to prime neutrophils for enhanced response to agonists [20].

Human recombinant GM-CSF (kindly provided by Schering-Plough) was given, beginning with a dose of 5 µg/Kg/die escalating to a maximum of 10 µg/Kg/die and then modulating the

dose in order to maintain WBC count below $10 \times 10^9/l$. GM-CSF was given subcutaneously at 12 hrs intervals. Treatment with GM-CSF lasted for 22 days and led to a fast and sustained increase of the WBC and neutrophil count, without detectable effects on haemoglobin concentration and platelet count, and without side effects. However, in spite of the marked increase in the neutrophil count, there was no change in the percentage of NBT-positive cells and in the amount of superoxide anion produced after PMA stimulation (data not shown). After a slight and temporary clinical improvement, the patient's conditions worsened, with high fever, unconsciousness and increasing dyspnoea, which required mechanically assisted ventilation. Left pneumo-thorax developed and death occurred 38 days after admission. During the late course of the disease *Pseudomonas* and *Staphylococcus* were again isolated from bronchial brushings and from the blood, respectively.

2.3 Isolation of polymorphonuclear and mononuclear leucocytes

Human neutrophils were isolated from citrated blood samples of patients, their parents and healthy donors after their informed consent, by sequential dextran sedimentation and Ficoll-Hypaque differential density centrifugation, followed by erythrocyte lysis by hypotonic treatment, as described elsewhere [21]. The isolated neutrophils were resuspended in Ca^{2+} and Mg^{2+} free HEPES – buffered saline (HBS) solution containing 140 mM NaCl, 5 mM KCl, 5 mM Glucose, 5 mM HEPES, pH 7.4 and 0.2% bovine serum albumin (BSA). Immediately before use, cell suspensions were supplemented with 1 mM $CaCl_2$ and 1 mM $MgCl_2$. Isolated mononuclear cells were transformed with the B95-8 strain of Epstein Barr virus (EBV) and were grown in RPMI-1640 supplemented with 10% fetal calf serum, 100 U/ml penicillin, 50 μ g/ml streptomycin, at 37°C in a 5% CO_2 atmosphere [22].

2.4 Assay of superoxide anion production:

Superoxide anion was measured spectrophotometrically by the superoxide dismutase-inhibitable cytochrome c reduction at 550 nm, as previously described [19,23].

2.5 Assay of hydrogen peroxide production:

Hydrogen peroxide was measured by the dihydrorhodamine-1,2,3 (DHR) assay. In this assay, the nonfluorescent DHR is converted by hydrogen peroxide to the green fluorescent rhodamine 123 and analysed using flow cytometry [19]. Neutrophils were loaded with 2 μ M DHR 123 (Molecular Probes, Eugene, OR, USA) at room temperature for 30 minutes, in the dark. After loading and addition of 1 mM sodium azide, the samples were prewarmed at 37°C in a shaking water bath and after 10 minutes the cells were stimulated with 20 ng/ml phorbol-myristate acetate (PMA) for 15 minutes; the reaction was stopped by dilution with ice cold PBS. Samples were kept on ice and cell-associated fluorescence (FL-1) was measured by flow cytometry (FACScalibur™, BD Biosciences, San Jose, CA). In experiments where expression of the eosinophil marker CRTH2 was also measured, the samples were further incubated with 2.5 μ g/ml Alexa Fluor 647 rat anti-human CD294 (CRTH2) for 30 min and washed twice with cold HBS-BSA, before cytometry readings.

2.6 NBT test

Neutrophils were suspended at 0.2×10^6 /ml in HBS-BSA containing 1 mM EDTA to prevent cell aggregation and 1 mM NBT. The reaction was started by addition of 100 ng/ml PMA. After incubation for 15 min at 37°C cytopins were made, fixed with methanol and counterstained with a solution of 0.4% safranin in 25% ethanol. In some experiments the NADPH oxidase inhibitor diphenyleneiodonium (DPI) (f.c. 5 μ M) was included in the incubation mixture.

2.7 Cytochrome b_{558} spectroscopy

Reduced minus oxidized optical spectra of cytochrome b_{558} were recorded at room temperature on a DU 640 Beckman spectrophotometer, after addition of a few crystals of sodium dithionite, as previously reported [24]. A molar extinction coefficient $\epsilon_{426-410 \text{ nm}} = 200 \text{ mM}^{-1} \text{ cm}^{-1}$ for the Soret band was used for calculations.

2.8 Expression of gp91 $phox$ in neutrophils and eosinophils as measured by flow cytometry

Granulocytes (10^6 cells) were suspended in 0.1 ml HBS-BSA and incubated with 5 $\mu\text{g/ml}$ of mab 7D5 (anti-gp91 $phox$) [25] or control isotype-matched (IgG1) mab for 30 min on ice. The cells were washed twice and incubated with Alexa Fluor 488 goat-F(ab) $_2$ anti-mouse IgG1 (Invitrogen, Cergy Pontoise, France) for 30 min on ice. After two washes as above, the cells were incubated with 2.5 $\mu\text{g/ml}$ Alexa Fluor 647 rat anti-human CD294 (CRTH2) for 30 min on ice and washed [26, 27]. Cell-associated fluorescence (FL1 and FL4) was measured on 100,000 events .

2.9 Assay of microbicidal activity

The microbicidal activity was tested according to a recently described method [28]. Briefly, neutrophils (final concentration: $4 \times 10^6/\text{ml}$) were incubated at 37 °C with serum-opsonized *Staphylococcus aureus* (strain 502 A, ATCC 27217), *Escherichia coli* (ATCC 25922, a serum-resistant strain) or a clinical isolate of *Candida albicans* at a microorganism/neutrophil ratio of 3:1 for bacteria and 2:1 for the fungus, in HBS-BSA containing 1 mM CaCl_2 and MgCl_2 . At the indicated times of incubation aliquots were diluted 50 times in water brought to pH 11.0 with 1M NaOH. After 5 min at room temperature samples were vortexed, diluted in 0.9 % NaCl solution and plated on Petri dishes. After

overnight incubation, colony forming units (CFU) were counted and the percentage of killing was calculated with respect to the number of CFU at time 0 of incubation.

2.10 SDS-PAGE and immunoblotting

Neutrophil proteins were solubilised in 1% Triton X-100, separated by SDS-PAGE in 10% (w/v) acrylamide gel with a 5% (w/v) stacking gel [29], electrotransferred to nitrocellulose [30] and immunodetected using the monoclonal antibodies 449 and 48 directed against p22*phox* and gp91*phox*, respectively [31] and by polyclonal antibodies directed against p47*phox* and p67*phox* [32].

2.11 Electrophoretic mobility shift assays (EMSA)

Nuclear extracts. Nuclear extracts of undifferentiated PLB-985 cells were prepared with the NE-PER nuclear extraction reagents of the Pierce kit (Thermo Fisher Scientific Inc., Waltham, USA) according to the manufacturer's instructions and stored at -80°C . The protein concentration was determined with the BioRad protein assay kit (Biorad, Marnes-La-Coquette, France).

Oligonucleotides. Oligonucleotides used as double-stranded competitors and probes are as follows: WT *CYBB* [-68 to -30] bp (WT, 5'-ctatgcttcttccaatgaggaaatgaaaacagcag-3'); X91⁻ mutated *CYBB* (X91⁻ CGD, 5'-ctatgcttcttccaatgaggaaaatgaaaacagcag-3'). Oligonucleotide probes were labelled by biotin according to Biotin 3' End DNA Pierce Labelling Kit (Thermo Fisher Scientific Inc.), followed by annealing with the complementary strand oligonucleotides.

Electrophoretic mobility shift assays were performed in a 20- μl total volume with 10 μg of nuclear protein extract in 10 mM Tris, 50 mM KCl, 1 mM DTT (pH 7.5), 50 ng/ μL Poly (dI-

dC). Nuclear extracts were preincubated for 15 min on ice with or without a 20-, 50-, or 100-fold molar excess of unlabelled double-stranded oligonucleotide competitors followed by an additional 30 min on ice after the addition of 1 nM of biotin-labelled double-stranded oligonucleotides. Samples were subjected to a 5% polyacrylamide gel electrophoresis at 100 V for 2 h in a 0.5X Tris-Borate-EDTA buffer (pH 8.3) and transferred to a nylon membrane Hybond™ - N⁺ (Amersham, GE Healthcare, Munich, Germany). After transfer the membrane was immediately cross-linked for 45–60 s at 120 mJ/cm² using an UV-light cross-linker instrument equipped with 254-nm bulbs. The biotin-labelled DNA was detected by chemiluminescence using a luminol/enhancer solution and a stable peroxide solution (Pierce labelling kit). Membranes were exposed to an x-ray film for 15 min before developing.

2.12 Preparation of RNA and DNA

Total RNA was isolated from either mononuclear cells or EBV-transformed B lymphocytes, using a modified single-step method [33, 34]. Genomic DNA was purified following classic procedures. Genomic DNA was also purified from the peripheral blood of patient II-2 and healthy donor according to the manufacturer's instructions (Wizard kit, Promega, France).

2.13 cDNA amplification and sequencing

First-strand cDNA was synthesized from 5 µg of total RNA from Epstein Barr virus immortalized B lymphocytes or native B lymphocytes or granulocytes by reverse transcriptase (RT) reaction according to the manufacturer's instructions (QBiogen, Illkirch, France). Total *gp91phox* cDNA was immediately amplified by PCR in three overlapping fragments [35]. Aliquots of 10 µl PCR products in bromophenol blue solution were run together with a scale of DNA ladders (markers VI, Roche Diagnostics, Indianapolis, IN, USA) on 1.5% (wt/vol)

agarose or on 5% acrylamide gels containing 1 µg/ml ethidium bromide. The bands were photographed under UV (Vilber Lourmat, Biolabo Scientific Instruments, Archamps, France). In some cases PCR products were purified from agarose gel with the Sephaglas kit (Amersham Pharmacia, Orsay, France). All RT-PCR products were sequenced by Cogenics (Meylan, France) using the Abi Prism automatic sequencer (Perkin Elmer, Foster City, CA, USA). For each patient, the complete encoding region of *CYBB* was sequenced.

2.14 Semi-quantitative PCR

Semi-quantitative PCR was performed to evaluate *CYBB* gene expression in mononuclear cells or granulocytes. Two microliters of cDNA (obtained as described before) was used in 100 µL of total reaction mixture containing 10 µL of 10X Qbiogen incubation mix supplemented with 0.2 mM Mg⁺⁺, 0.25 µM dNTP, specific primers 1 and 2* (see primer sequence in Table II), 0.25 µM β-actin primers (3 and 4*) and 25 mU/µL Taq DNA polymerase (QBiogene, Illkirch, France). The reaction mixture was thermally cycled in a Minicycler™ (MJResearch, St Bruno, Canada) once at 95°C for 5 min, 25 times at 95°C for 1 min, 57°C for 1 min, 72°C for 90 s and then once at 72°C for 7 min. Aliquots of 10-µl PCR products in bromophenol blue solution were run together with a scale of DNA ladders (marker XIV, Roche, Meylan, France) on 1.5% (wt/vol) agarose gels containing 1 µg/ml ethidium bromide and visualized on a UV transilluminator.

2.15 Genomic DNA amplification

Purified genomic DNA was amplified using a forward primer P1 CAGCAAGGCTATGAATGCTGTTC (position -427 in the *CYBB* promoter region and the backward one P2* GGAGAGCCCCTCATTACAG (position +45). PCR products were automatically sequenced by Cogenics (Meylan, France) using the Abi Prism automatic sequencer (Perkin Elmer, Courtaboeuf, France) with forward and backward primers [19].

2.16 Protein determination

Protein content was measured by using the Pierce^R method [36].

ACCEPTED MANUSCRIPT

3. RESULTS

Fig. 1 shows the pedigree of Family C studied in this paper. Patient II-2 was diagnosed with CGD immediately after his nephew (patient III-5) was diagnosed as having CGD at the age of 20 on the basis NBT test, PMA-induced superoxide production and cytochrome *b* content (Table 1). Patient II-2 was 43 at the time of diagnosis and his neutrophils in terms of respiratory burst activity (NBT and superoxide production) and cytochrome *b* content had characteristics similar to neutrophils of patient III-5 (Table 1). Production of superoxide anion by neutrophils from patients II-2 and III-5 after PMA stimulation was 7 and 5 % of control cells, respectively (Table 1). Neutrophils from family members II-3, II-4, III-2, III-3 and III-4 generated intermediate amounts of superoxide ranging from 34% to 60% of control (Table 1). These data are compatible with an X-linked mode of inheritance of the defect. This conclusion was confirmed by the results of the NBT test that showed that neutrophils from II-3, II-4, III-2, III-3 and III-4 were a mosaic in terms of NBT reduction, with two populations, one strongly positive, and the other negative while neutrophils from II-1 and III-1 were 100% NBT positive. In patients II-2 and III-5 a few cells were weakly NBT positive (Fig. 2 arrows and arrowheads). Such NBT positive cells were no longer apparent when DPI was included in the incubation mixture. Among the NBT positive cells, the more weakly positive (arrows) appeared to be neutrophils while the more strongly positive (arrowheads), based on the nuclear shape, the size and color of intracellular granules could be identified as eosinophils. The percentage of eosinophils as determined in Diff-Quick stained smears in patients II-2 and III-5 never exceeded 3% (data not shown).

To better establish if neutrophils from patient II-2 exhibited a NADPH oxidase activity, we measured the respiratory burst using a method much more sensitive than the NBT reduction test, i.e. the DHR oxidation assay. Fig. 3 shows that neutrophils from patient II-2 presented a homogeneous cell population with a peak response corresponding to about 5% of

control. Such a response was almost completely abolished by DPI. No response was observed with neutrophils from an X91⁰ CGD patient. In order to establish whether a population of NADPH oxidase-positive eosinophils, as indicated by the NBT reduction test, was detectable using the DHR assay in the granulocytes of patient II-2, we conducted an experiment in which eosinophils were identified with an anti-CRTH2 mAb and with acquisition of a number of events (100,000) larger than that used in Fig. 3 (10,000). Figure 4 shows that, indeed, a small percentage of DHR and CRTH2-positive cells (eosinophils) were present in the granulocyte preparation of patient II-2. To demonstrate the presence of cytochrome *b*₅₅₈ in the patients' neutrophils, reduced minus oxidized difference spectra of neutrophil homogenates and Western blot analysis were performed. As illustrated in Fig. 5A and Table 1, reduced-minus-oxidized spectra revealed a low but detectable amount of cytochrome *b*₅₅₈. The spectrum of X91⁰ CGD neutrophils did not present the peaks characteristic of cytochrome *b*₅₅₈. Cytochrome *b*₅₅₈ content in patients II-2 and III-5 was 14 and 10 % of normal neutrophils, respectively, while the neutrophils of a heterozygous member of family C (patient III-4) contained 44 % of control cytochrome *b*₅₅₈ (Table 1).

The presence of low amounts of cytochrome *b*₅₅₈ in neutrophils of these patients was confirmed by Western blot analysis using an identical amount of 1% Triton X100 soluble extract in all samples (50 µg). Fig. 5B shows that in comparison with normal cells, the two bands corresponding to gp91*phox* and p22*phox* were very faint. The specificity of the bands detected by the use of monoclonal antibodies directed against the two subunits of cytochrome *b*₅₅₈ was confirmed by the absence of gp91*phox* and p22*phox* in the extracts from neutrophils of an X91⁰ CGD patient. The cytosolic components of NADPH oxidase (p47*phox*, p67*phox*, p40*phox* and Rac2) detected by Western blot analysis were normal (data not shown). Since the spectroscopic and Western blot analyses do not distinguish eosinophils from neutrophils and since in the NBT slide test eosinophils appeared to stain more strongly than neutrophils

(Fig. 2), a flow cytometry experiment was devised that would distinguish *gp91phox* expression in neutrophils and eosinophils. Figure 6 shows the dot plot profiles of granulocytes from a normal subject (top panels) and from patient II-2 (bottom panels) incubated simultaneously with the anti-eosinophil mAb CRTH2 and with the anti-*gp91phox* 7D5 mAb antibodies (*gp91phox*) or an isotype-matched nonspecific mAb (control mAb). The percentage of eosinophils in the two cell preparations was 0.9 for the normal subject and 2.5 for patient II-2. The right panels show that both neutrophils (99.0%) and eosinophils (0.75%) from the normal subject (top panel) express *gp91phox*. In the case of patient II-2 (bottom panel), despite the low but measurable NADPH oxidase activity in his granulocytes, as shown previously (Figs. 2, 3 and 4), *gp91phox* was not detected in the CRTH2⁻ population (97%) and only eosinophils (1.9%) were *gp91phox*-positive.

RT-PCR and sequence analysis were performed to localize the mutation in the mRNA encoding *gp91phox* of patient II-2. mRNA was reverse-transcribed into three overlapping cDNA fragments with three pairs of oligonucleotide primers derived from the *gp91phox* cDNA and were sequenced as described in Materials and Methods. No mutations in the cDNA of *gp91phox* protein were found. A portion of the *gp91phox* promoter region in genomic DNA of *CYBB* was amplified by PCR [19]. Using sequence analysis, a thymidine insertion was found located between position -54T to -56T (Figure 7A). This mutation is 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 *gp91phox* promoter responsible for *CYBB* transcription (Fig. 7B). Genomic DNA from 50 control subjects was analyzed and no mutations were found.

Then electrophoretic mobility shift assays were performed to evaluate the impact of this new mutation of the *CYBB* promoter on the specific binding with regulatory transcription factors of a nuclear protein extract from PLB-985 cells. Figure 8 shows that a DNA/protein

complex (indicated by the arrow) is formed when a labelled wild-type double-strand oligonucleotide of the *CYBB* promoter region (-68 to -30) is used as a probe. The DNA/protein binding is specific as it is progressively inhibited by competition with a 20-, 50- and 100-fold molar excess of the unlabelled WT double-stranded oligonucleotide probe. On the contrary, adding an increasing amount of X91⁻ CGD unlabelled double-stranded oligonucleotide probe is ineffective.

In order to study the impact of the defect of transcription factor binding on the mutated *CYBB* promoter region, the amount of gp91*phox* mRNA from patient II-2's mononuclear cells was estimated by semi-quantitative RT-PCR using β -actin, a housekeeping gene as an internal standard. The PCR was performed with 20, 25, and 30 cycles (data not shown). Finally, 25 cycles of PCR was chosen in order to be in the exponential phase of the PCR amplification and before reaching the plateau for both tested mRNA. As shown in Fig. 9, a 624-bp fragment of comparable intensity was obtained from the control, X91⁺ and X91⁰ CGD patients' gp91*phox* mRNA. In contrast, patient II-2's gp91*phox* mRNA amplification was very low while the signal of the housekeeping gene was similar in all the tested mRNA. We also did the same experiment with total ARN purified from the granulocyte population of patient II-2 and we obtained the same results (data not shown).

To determine the impact of the residual NADPH oxidase activity of neutrophils on microbicidal activity, in vitro killing assays were performed using *S. aureus*, *C. albicans* and *E. coli* as targets. Fig. 10 shows that the microbicidal activity of neutrophils from patient II-2 was superimposable to that of neutrophils from an X91⁰ CGD patient, i.e. no killing of *S. aureus* and *C. albicans*, normal killing of *E.coli*. No killing of both *S. aureus* and *C. albicans* could be observed even after prolonging to 90 min the time of incubation (data not shown). However the normal killing of *E. Coli* for both X91⁰ and X91⁻ CGD highlights that it was NADPH oxidase independent.

4. Discussion

The majority of genetic lesions found in X91⁻ CGD variants of chronic granulomatous disease are missense mutations or small deletions located in the encoding region of *CYBB*, whereas, up to now, only four point mutations have been described in the *CYBB* promoter region [17-19]. All these X91⁻ CGD variants are characterized by a low expression of cytochrome *b*₅₅₈ and display some NADPH oxidase activity.

We report here for the first time the occurrence of an insertion in the promoter of *CYBB* gene (T insertion between position -54 and -56) leading to a rare variant of X91⁻ CGD in an Italian family. On the basis of the amount of superoxide anion produced and the NBT test it was possible to demonstrate that neutrophils from the two members affected, II-2 and III-5, retained some oxidase activity (Table 1 and Figs. 1 and 2). Assay of hydrogen peroxide production by DHR 123 oxidation in neutrophils from patient II-2 showed a single peak of fluorescence, indicating that neutrophil population responded homogeneously to PMA with a respiratory burst. Such a response was only about 6 % of control (Fig. 3). The NADPH oxidase dependence of this response, as well as of the response observed in the NBT test was demonstrated by the complete sensitivity to inhibition by DPI (Fig 2 and Fig. 3). A homogeneous weakly responsive neutrophil population was also reported in other X91⁻ CGD patients but no identification of the specific *CYBB* mutation has been done for these subjects [37-38]. The eosinophils from patient II-2 strongly reduced NBT and oxidized DHR (Figs. 2, 3, and 4). In the neutrophil population from patient II-2 (which is RCTH2⁻), cytochrome *b*₅₅₈ could not be detected, probably because the amount of gp91*phox* expressed at the surface of each cell is low and is under the flow cytometry's sensitivity limit (Fig. 6). However, the low percentage of eosinophils in patient II-2 (about 2% of the granulocyte population) cannot explain the homogeneous weakly but DPI inhibited respiratory burst measured in the PMA-activated neutrophil population (Figs. 3 and 4).

A small population (5 % to 15 %) of granulocytes with completely normal respiratory burst activity has been previously found in X91⁻ CGD patients (T-55C mutation in the promoter region of *CYBB* [17]. In two other X91⁻ CGD cases caused by point mutations in the same *CYBB* promoter region (C-52T, C-53T), a small population of NBT-positive cells was described that turned out to be eosinophils [18]. The presence of oxidase positive eosinophils in these patients may be explained by the demonstration that gp91*phox* expression in eosinophils is regulated by specific transcription factors (GATA1 and GATA2) different from those operative in neutrophils monocytes and B-lymphocytic cells [39].

The new insertion mutation described here (Fig. 7) is located between the “CCAAT” and “TATA” box in a consensus binding sequence for the *ets* family of transcription factors of the gp91*phox* promoter site (5'-GAGGAAAT-3', lower strand, -57 to -50 bp), that are involved in eukaryotic gene transcription [40]. Mutations in this region are known to be related to the loss of association of DNA-binding proteins (Elf-1 and PU.1) and to prevent the full expression of the *CYBB* gene in neutrophils and monocytes [17, 41, 42]. Indeed, using EMSA we demonstrated a DNA-protein binding complex formation of nuclear extract from PLB-985 cells with the biotin-labelled wild-type double-strand oligonucleotide of the *CYBB* promoter region (-68 to -30). The specificity of the binding was demonstrated because an increasing amount of unlabelled WT oligonucleotide probe decreased the DNA-protein binding complex formation while the mutated X91⁻ oligonucleotide probe was ineffective (Fig. 8). The consequence of this transcription factor binding defect was a reduced gp91*phox* mRNA expression in mononuclear cells or granulocytes (Fig. 9) correlated with a diminished cytochrome *b*₅₅₈ expression in patient II-2's neutrophil population of (Fig. 5). The diminished expression of gp91*phox* in patients II-2 and patient III-5 correlates with the low respiratory burst of their neutrophils. The finding that the eosinophils of our patients were oxidase positive is in keeping with the above mentioned observation that expression of gp91*phox* in

eosinophils is regulated by transcription factors different from those active on neutrophils [39]. On the whole, the phenotypic profile of these cells is very similar to that of other X91⁻ CGD neutrophils with point mutations in the *CYBB* promoter (C-52T, C-53T, T-55C, A-57T) [17-19].

In order to understand the role of the residual NADPH oxidase activity in the microbicidal activity of X91⁻ CGD neutrophils, killing of Gram-positive (*S. aureus*) and Gram-negative (*E. coli*) bacteria and of a fungus (*C. albicans*) by patient II-2 neutrophils were assessed and compared with killing by X91⁰ CGD neutrophils (Fig. 10). As previously shown [28], neutrophils from the X91⁰ CGD patient were unable to kill both *S. aureus* and *C. albicans* and killed normally *E. coli*. The results obtained with patient II-2 neutrophils indicate that the residual NADPH oxidase activity of patient II-2 neutrophils (5-7 % of control) is not sufficient to guarantee any killing of *S. aureus* and *C. albicans* in vitro, even after long incubation times (90 min, data not shown). Bactericidal activity of X91⁻ CGD neutrophils was performed in only few studies [11,37]. The killing of *S. aureus* by X91⁻ CGD neutrophils in these studies was found only partially defective and comparable to that of X91⁰ CGD neutrophils. The discrepancy of these data with ours can be explained by the fact that techniques commonly used to assess bacterial killing were affected by a methodological flaw that undermined reliability of the results obtained [28]. In an additional study [18] a defective killing of *E. coli* by X91⁻ CGD neutrophils was demonstrated. In that study, killing of *E. coli* (strain ML35) was measured by following the kinetics of perforation of the bacterial cell envelope [43]. *E. coli* appear to be efficiently killed by non-oxidative mechanisms ([28,44] and data presented in this study). Therefore we have no ready explanation for the differences in killing between ref.18 and our results, considering that perforation assay of killing and the classical killing assay (CFU) were shown to give comparable results [43]. It is likely that the microbicidal defect demonstrated using the perforation technique does not reflect changes in

the number of viable bacteria detected as colony forming units or that the observed differences depend on the different strains of *E. coli* used. .

The strongly defective killing of *S. aureus* and *C. albicans* which are preferential pathogens found in CGD, in spite of the low O_2^- production may well explain the clinical conditions of patients II-2 and III-5, both of which suffered from recurrent and severe infections requiring hospitalization and patient III-5 died from pneumonia at the age of 20 when the diagnosis of CGD was made. It is the case for some previously described X91⁻ CGD patients with the same type of *CYBB* promoter's mutations [17, 45]. However the frequency and the severity of infections among the X91⁻ CGD variant patients characterized by point mutation in the same *CYBB* promoter region, vary considerably. Reasons for discrepancies between the infectious complications and the respiratory burst capabilities in such patients are unclear. However a mild clinical expression in these X91⁻ CGD variant patients can be explained by a high level of eosinophils (>10%) normally expressing NADPH oxidase in the blood [18]. However the compensation mechanism of eosinophil over-production is not known. Mothers who are carriers of the classical X91⁰ CGD are generally protected against severe infections even in cases of extreme lyonization, with < 15 % of neutrophils with a normal respiratory burst [46]. This indicates that even a low percentage of neutrophils with a normal respiratory burst is enough to protect against infections. In our study, neutrophils from patients II-2 and III-5 produced reactive oxygen species at a rate corresponding to 5-7 % of control. This response, however, is "diluted" among the whole cell population. Evidently, such a low level of oxidative metabolism, on a per cell basis, is not sufficient to allow microbial killing and to protect from infections. These observations raise questions about the levels of respiratory burst activity required for microbial killing and studies on this line are now underway using myeloid lines transfected with NOX mutants exhibiting various levels of oxidative activity.

Systematic characterization of gene mutations in CGD patients is essential for genetic counseling and prenatal diagnosis. It would be also of help in relating the clinical form of the disease to the type of gene affected and/or the type of mutation found. However, we believe that the number of X91- CGD variant cases with a well-documented clinical history and a defined genetic lesion described up-to-now is not sufficient to provide a clear and definitive correlation between mutation and severity of the clinical course.

Acknowledgments

The authors are grateful to Dr D. Roos and Dr A. J. Verhoeven for the generous gift of mAb449 and mAb48 antibodies raised against the two subunits of the cytochrome *b₅₅₈*. We extend our thanks to Dr. Marilena Granzotto for invaluable help in flow cytometry and Prof. B. Toussaint for helpful discussions concerning the EMSA experiments. Special thanks are extended to Lila Laval for her excellent secretarial work and to Linda Northrup for editing the manuscript.

References

- [1] J.A Winkelstein, M.C. Marino, R.B. Jr Johnston, J. Boyle, J. Curnutte, J.I. Gallin, H.L. Malech, S.M. Holland, H. Ochs, P. Quie, R.H. Buckley, C.B. Foster, S.J. Chanock, H. Dickler, Chronic granulomatous disease. Report on a national registry of 368 patients. *Medicine (Baltimore)* 79 (2000) 155-169.
- [2] B.M. Segal, T.L. Leto, J.I. Gallin, H.L. Malech, S.M. Holland, Genetic, biochemical and clinical features of chronic granulomatous disease, *Medicine (Baltimore)* 79 (2000) 170-200.
- [3] B.L. Schapiro, P.E. Newburger, M.S. Klempner, M.C. Dinauer, Chronic granulomatous disease presenting in a 69-year-old man, *N. Engl. J. Med.* 325 (1991) 1786-1790.
- [4] P. Cathebras, C. Sauron, F. Morel, M.J. Stasia, An unusual case of sarcoidosis, *Lancet* 358 (2001) 294.
- [5] P.V. Vignais, The superoxide-generating NADPH oxidase: structural aspects and activation mechanism, *Cell. Mol. Life. Sci.* 59 (2002) 1428-1459.
- [6] D. Roos, M. De Boer, F. Kuribayashi, C. Meischl, R.S. Weening, A.W. Segal, A. Ahlin, K. Nemet, J.P. Hossle, E. Bertatowska-Matuskiewicz, H. Middleton-Price, Mutations in the X-linked and autosomal recessive forms of chronic granulomatous disease, *Blood* 87 (1996) 1663-1681.
- [7] J. Rae, P.E. Newburger, M.C. Dinauer, X-linked chronic granulomatous disease: mutations in the *CYBB* gene encoding the gp91 $phox$ component of respiratory burst oxidase, *Am. J. Hum. Genet.* 62 (1998) 1320-1331.
- [8] B. Royer-Pokora, L.M. Kundel, A.P. Monaco, S.C. Goff, P.E. Newburger, R.L. Baehner, F.S. Cole, J.T. Curnutte, S.H. Orkin, Cloning the gene for an inherited disorder-chronic granulomatous disease-on the basis of its chromosomal localization, *Nature* 322 (1986) 32-38.

- [9] R.L. Baehner, L.M. Kundel, A.P. Monaco, J.L. Haines, P.M. Conneally, C. Palmer, N. Heerema, S.H. Orkin, DNA linkage analysis of X chromosome-linked chronic granulomatous disease, *Proc. Natl. Acad. Sci. USA* 83 (1986) 3398-3401.
- [10] M.J. Stasia, X.J. Li, Genetics and immunopathology of chronic granulomatous disease, *Semin. Immunopathol.* 30 (2008) 209-35.
- [11] H.N. Bu-Ghanim, A.W. Segal, N.H. Keep, C.M. Casimir, Molecular analysis in three cases of X91 variant chronic granulomatous disease, *Blood* 86 (1995) 3575-3582.
- [12] M. Kaneda, H. Sakuraba, A. Ohtake, A. Nishida, C. Kiryu, A. Kakinuma, Missense mutations in the *gp91phox* gene encoding cytochrome *b₅₅₈* in patients with cytochrome *b* positive and negative X-linked chronic granulomatous disease, *Blood* 93 (1999) 2098-2104.
- [13] L.S. Yoshida, F. Saruta, K. Yoshikawa, K. Tatsuzawa, S. Tsunawaki, Mutation at histidine 338 of *gp91phox* depletes FAD and affects expression of cytochrome *b₅₅₈* of the human NADPH oxidase, *J. Biol. Chem.* 273 (1998) 27879-27886.
- [14] M. Tsuda, M. Kaneda, T. Sakiyama, I. Inana, M. Owada, C. Kiryu, T. Shiraishi, K. Kakinuma, A novel mutation at a probable heme-binding ligand in neutrophil cytochrome *b₅₅₈* in atypical X-linked chronic granulomatous disease, *Hum. Genet.* 103 (1998) 377-381.
- [15] J. Roesler, S. Heyden, M. Burdelski, H. Schafer, H.W. Kreth, R. Lehmann, D. Paul, J. Marzahn, M. Gahr, A. Rosen-Wolff, Uncommon missense and splice mutations and resulting biochemical phenotypes in German patients with X-linked chronic granulomatous disease, *Exp. Hematol.* 27 (1999) 505-511.
- [16] M.J. Stasia, P. Bordigoni, D. Floret, J.P. Brion, C. Bost-Bru, G. Michel, P. Gatel, D. Durant-Vital, M.A. Voelckel, X.J. Li, M. Guillot, E. Maquet, C. Martel, F. Morel, Characterization of six novel mutations in the *CYBB* gene leading to different sub-types of X-linked chronic granulomatous disease, *Hum. Genet.* 116 (2005) 72-82.
- [17] P.E. Newburger, D.G. Skalnik, P.J. Jopkins, E.A. Eklund, J.T. Curnutte, Mutations in the promoter region of the gene for *gp91phox* in X-linked chronic granulomatous disease with decreased expression, *J. Clin. Invest.* 94 (1994) 1205-1211.

- [18] R.S. Weening, M. De Boer, T.W. Kuijpers, V.M. Neeffjes, W.W. Hack, D. Roos, Point mutations in the promoter region of the *CYBB* gene leading to mild chronic granulomatous disease, *Clin. Exp. Immunol.* 122 (2000) 410-417.
- [19] M.J. Stasia, J.P. Brion, J. Boutonnat, F. Morel, Severe clinical forms of cytochrome *b*-negative chronic granulomatous disease (X91⁻) in three children with point mutation in the promoter region of the *CYBB* gene, *J. Infect. Dis.* 188 (2003) 1593-1604.
- [20] D.C. Dale, W.C. Liles, C. Llewellyn, T.H. Price, Effects of granulocyte-macrophage colony-stimulating factor (GM-CSF) on neutrophil kinetics and function in normal human volunteers. *Am. J. Hematol.* 57 (1998) 7-15.
- [21] P. Dri, R. Cramer, M.R. Soranzo, A. Comin, V. Miotti, P. Patriarca, New approaches to the detection of myeloperoxidase deficiency, *Blood* 60 (1982) 323-327.
- [22] L. Cohen-Tanugi, F. Morel, M.C. Pilloud-Dagher, J.M. Seigneurin, P. François, M. Bost, P.V. Vignais, Activation of O₂⁻ generating oxidase in heterologous cell-free system derived from Epstein-Barr-virus-transformed human B lymphocytes and bovine neutrophils, *Eur. J. Biochem.* 202 (1991) 649-655.
- [23] R. Menegazzi, R. Cramer, P. Patriarca, P. Scheurich, P. Dri, Evidence that tumor necrosis factor-induced activation of neutrophil respiratory burst on biological surfaces is mediated by the p55 TNF receptor, *Blood* 84 (1994) 287-293.
- [24] F. Morel, J. Doussièrè, M.J. Stasia, P.V. Vignais, The respiratory burst of bovine neutrophils. Role of *b* type cytochrome and coenzyme specificity, *Eur. J. Biochem.* 152 (1985) 669-679.
- [25] A. Yamauchi, L. Yu L, A.J. Pötgens, F. Kuribayashi, H. Nunoï, S. Kanegasaki, D. Roos, H.L. Malech, M.C. Dinauer, M. Nakamura. Location of the epitope for 7D5, a monoclonal antibody raised against human flavocytochrome *b*₅₅₈, to the extracellular peptide portion of primate gp91*phox*, *Microbiol. Immunol.* 45 (2001) 249-27.

- [26] K. Nagata, H. Hirai, K. Tanaka, K. Ogawa, T. Aso, K. Sugamura, M. Nakamura, S. Takano, CRTH2, an orphan receptor of T-helper-2-cells, is expressed on basophils and eosinophils and responds to mast cell-derived factor(s), *FEBS Lett.* 459(1999) 195-199.
- [27] H. Hirai, K. Tanaka, O. Yoshie, K. Ogawa, K. Kenmotsu, Y. Takamori, M. Ichimasa, K. Sugamura, M. Nakamura, S. Takano, K. Nagata, Prostaglandin D2 selectively induces chemotaxis in T helper type 2 cells, eosinophils, and basophils via seven-transmembrane receptor CRTH2, *J. Exp. Med.* 193 (2001) 255–261.
- [28] E. Decleva, R. Menegazzi, S. Busetto, P. Patriarca, P. Dri, Common methodology is inadequate for studies on the microbicidal activity of neutrophils, *J. Leukoc. Biol.* 79 (2006) 87-94.
- [29] U.K. Laemmli, Cleavage of structural proteins during the assembly of the head of bacteriophage T4, *Nature* 227 (1970) 680-685.
- [30] H. Towbin, T. Staehelin, J. Gordon, Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some application, *Proc. Natl. Acad. Sci. USA* 76 (1979) 4350-4354.
- [31] A.J. Verhoeven, G.J.M. Bolscher, L. Meerhof, R. van Zwieten, J. Keijer, R.S. Weening, D. Roos, Characterization of two monoclonal antibodies against cytochrome *b₅₅₈* of human neutrophils, *Blood* 73 (1989) 1686-1694.
- [32] S. Vergnaud, M.H. Paclet, J. El Benna, M.A. Pocidalo, F. Morel, Complementation of NADPH oxidase in p67-phox/p40-phox interaction, *Eur. J. Biochem.* 267 (2000) 1059-1067
- [33] P. Chomczynski, N. Sacchi, Single-step method of RNA isolation by acid guanidium thiocyanate-phenol-chloroform extraction, *Anal. Biochem.* 162 (1987) 156-159.
- [34] C. Trocmé, P. Gaudin, S. Berthier, C. Barro, P. Zaoui, F. Morel, Human B lymphocytes synthesize the 92-kDa gelatinase, matrix metalloproteinase-9, *J. Biol. Chem.* 273 (1998) 20677-20684.

- [35] M.J. Stasia, B. Lardy, A. Maturana, P. Rousseau, C. Martel, P. Bordigoni, N. Demaurex, F. Morel, Molecular and functional characterization of a new X-linked chronic granulomatous disease variant (X91⁺) case with a double missense mutation in the gp91 phox -cytosolic C-terminal tail, *Biochem. Biophys. Acta.* 1586 (2002) 316-330.
- [36] P.K. Smith, Krohn, G.T. Hermanson, A.K. Mallia, F.H. Gartner, M.D. Provenzano, E.K. Fujimoto, N.M. Goeke, B.J. Olson, D.C. Klenk, Measurement of protein using bicinchoninic acid, *Anal. Biochem.* 150 (1985) 76-85.
- [37] D. Roos, M. de Boer, N. Borregard, O.W. Bjerrum, N.H. Valerius, R.A. Seger, T. Muhlebach, B.H. Belohradsky, R.S. Weening, Chronic granulomatous disease with partial deficiency of cytochrome b_{558} and incomplete respiratory burst: variants of the X-linked, cytochrome b_{558} -negative form of the disease, *J. Leukoc. Biol.* 51 (1992) 164-171.
- [38] R.A. Seger, L. Tiefenauer, T. Matsunaga, A. Wildfeuer, P.E. Newburger, Chronic granulomatous disease due to granulocytes with abnormal NADPH oxidase activity and deficient cytochrome-b, *Blood* 61(1983) 423-428.
- [39] D. Yang, S. Suzuki, L.J. Hao, Y. Fujii, A. Yamauchi, M. Yamamoto, M. Nakamura, A. L. Kumatori, Eosinophil-specific regulation of gp91 phox gene expression by transcription factors GATA-1 and GATA-2, *J. Biol. Chem.* 275 (2000) 9425-9492.
- [40] E.A. Eklund, W. Luo, D.G. Skalnik, Characterization of three promoter elements and cognate DNA binding protein(s) necessary for IFN-gamma induction of gp91 phox transcription, *J. Immunol.* 157 (1996) 2418-2429.
- [41] K.S. Voo, D.G. Skalnik, Elf-1 and PU.1 induce expression of gp91 phox via a promoter element mutated in a subset of chronic granulomatous disease patients, *Blood* 93 (1999) 3512-3520.
- [42] S. Suzuki, A. Kumatori, I.A. Haagen, Y. Fujii, M.A. Sadat, H. Li Jun, Y. Tsuji, D. Roos, M. Nakamura, PU.1 as an essential activator for the expression of gp91 phox gene in human

peripheral neutrophils, monocytes, and B lymphocytes, Proc. Natl. Acad. Sci. U.S.A. 95 (1998) 6085-6090.

[43]M.N. Hamers, A.A. Bot, R.S. Weening, H.J. Sips, D. Roos, Kinetics and mechanism of the bactericidal action of human neutrophils against *Escherichia coli*, Blood 64 (1984) 635-641.

[44]B.K. Rada, M. Geiszt, K. Kaldi, C. Timar, E. Ligeti, Dual role of phagocytic NADPH oxidase in bacterial killing, Blood 104 (2004) 2947-2953.

(45)R.C. Woodman, P.E. Newburger, P. Anklesaria, R.W. Erickson, J. Rae, M.S. Cohen, J.T. Curnutte, A new X-linked variant of chronic granulomatous disease characterized by the existence of a normal clone of respiratory burst-competent phagocytic cells, Blood 85 (1995) 231-241.

[46] M. Anderson-Cohen, S.M. Holland, D.B. Kuhn, T.A. Fleisher, L. Ding, S. Brenner, H.L. Malech, J. Roesler, Severe phenotype of chronic granulomatous disease presenting in a female with a de novo mutation in *gp91phox* and a non familial, extremely skewed X chromosome inactivation, Clin. Immunol. 109 (2003) 308–317.

Table 1

Superoxide anion (O_2^-) production, NBT reduction, and cytochrome b_{558} content of neutrophils from family C members and the members of a family with a CGD patient affected by the classical X91⁰ form of the disease. For experimental details see Materials and Methods. In parenthesis number of experiments.

Subjects	O_2^- production (nmol/20 min/10 ⁶ cells)	NBT reduction (% NBT-positive neutrophils)	Cyt. <i>b</i> content pmol/mg cell protein
Family C			
II-1	226.0	100 strong	NT
II-2	13.2	100 weak to negative	20.0
II-3	68.1	52 strong 48 weak to negative	NT
II-4	83.3	53 strong 47 weak to negative	NT
III-1	217.3	100 strong	NT
III-2	118.9	64 strong 36 weak to negative	NT
III-3	91.3	53 strong 47 weak to negative	NT
III-4	19.8	56 strong 44 weak to negative	72.5
III-5	8.9± 1.8(3)	100 weak to negative	14.8±4.3 (3)
Healthy donor	197.9±7.1 (3)	100 strong	166.0
Family of patient with X91⁰ CGD			
Father	181.9	100 strong	100.7
Mother	99.2	61 strong 39 negative	62.5
Son	<0.5	100 negative	<5

Table II

Sequences of oligonucleotide primers used for the semi-quantitative RT-PCR amplification of *gp91phox* cDNA.

Gene	Primers	Sequence	Position
β -actin	1	CCTCTGCCACCATGGGGA ACT	294
	2*	GACTTCAAAGTAAGACCTCCGGATG	1131
<i>gp91pho</i>	3	ATCTGGCACCACACCTTCTACAATGAGCTGCG	2
	4*	CGTCATACTCCTGCTTGCTGATCCACATCTGC	624

* Anti-sense strand.

Figure legends

Fig 1. Pedigree of family C. Individuals II-3, II-4, III-2, III-3, III-4 were proven to be carriers for X-linked CGD on the basis of NBT reduction and superoxide production (Table 1). In contrast, individuals II-1 and III-1 were normal in terms of NBT reduction and superoxide production (Table 1). (□), normal male; (○), normal female; (●), carrier female; (■), affected male; (†), deceased; NT, not tested.

Fig 2. NBT test in neutrophils from the patient II-2. Purified neutrophils from patient II-2 or a healthy donor were pre-incubated or not with 5 μ M DPI and stimulated with 20 ng/ml PMA in presence of 1 mM NBT for 15 min at 37 °C. Arrows indicate neutrophils weakly NBT positive; arrowheads indicate eosinophils more strongly NBT positive.

Fig 3. Flow cytometric histograms of hydrogen peroxide generation from X CGD patients. Dihydrorhodamine-1,2,3 (DHR)-loaded neutrophils (10^6 cells/ml) from a healthy donor, patient II-2 and a X91⁰ CGD patient were incubated for 15 min at 37°C without (resting) or with 20 ng/ml PMA or PMA in the presence of 5 μ M DPI, as described in Materials and Methods, and analyzed by flow cytometry.

Fig 4. Flow cytometric dot plots of hydrogen peroxide generation from neutrophils and eosinophils in granulocyte preparations from a healthy subject (top panels) and patient II-2 (bottom panels) at rest (left panels) or after stimulation with PMA (right panels). Dihydrorhodamine- 1,2,3 (DHR)-loaded granulocytes (10^6 cells/ml) were incubated for 15 min at 37°C without or with 20 ng/ml PMA. After dilution with ice-cold PBS the cells were incubated with Alexa Fluor 647 rat anti-human CD294 (CRTH2) for 30 min and washed, before cytometry readings, as described in Materials and Methods.

Fig. 5. Reduced minus oxidized spectra and Western blot analysis of cytochrome b_{558} of neutrophils from the X CGD patients. The experiments were performed on 1% Triton X100 soluble extract of neutrophils. **(A)** Reduced minus oxidized difference spectra of cytochrome b_{558} from neutrophils of a healthy donor, patient II-2 and an X91⁰ CGD patient. **(B)** Immunodetection of the α (p22 $phox$) and β (gp91 $phox$) subunits of cytochrome b_{558} neutrophils of patient II-2, an X91⁰ CGD patient and a healthy donor. All samples were subjected to SDS-PAGE and blotted onto a nitrocellulose sheet. Cytochrome b_{558} α and β subunits were revealed with monoclonal antibodies 449 (α subunit) and 48 (β subunit), as described in Materials and Methods. The results of one experiment representative of the two performed, with similar results, are reported.

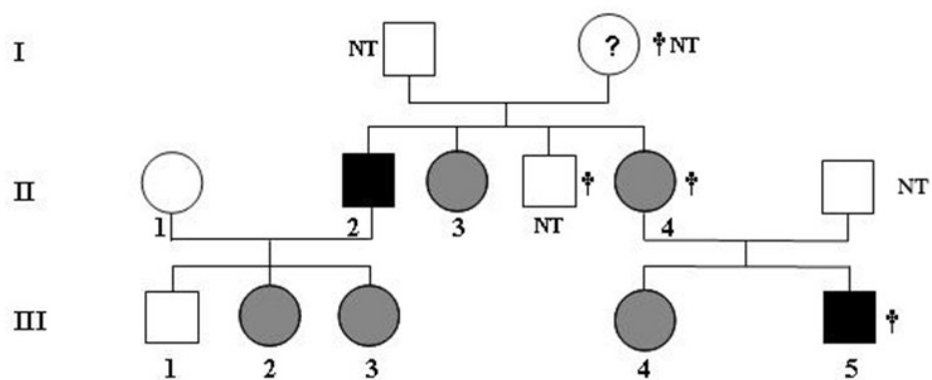
Fig. 6. Surface expression of gp91 $phox$ on neutrophils and eosinophils. Granulocyte preparations from a healthy subject (top panels) and from patient II-2 (bottom panels) were stained with the anti gp91 $phox$ mAb 7D5 and the eosinophil specific anti-CRTH2 mAb (right panels), or a control mAb and CRTH2 (left panels). For experimental details, see Materials and Methods.

Fig 7. Analysis of mutations in the *CYBB* gene from patient II-2. PCR products were gel-purified and automatically sequenced by Cogenics, Meylan, France, with forward and backward primers P1 and P2*, as described in Materials and Methods. **(A)** Thymidine insertion detected in the *CYBB* promoter region of patient II-2, compared to a healthy donor sequence. **(B)** Schematic representation of part of the *CYBB* promoter region, with localization of the “CCAAT” and “TATA” boxes, the ATG starting codon and the point mutation described in A.

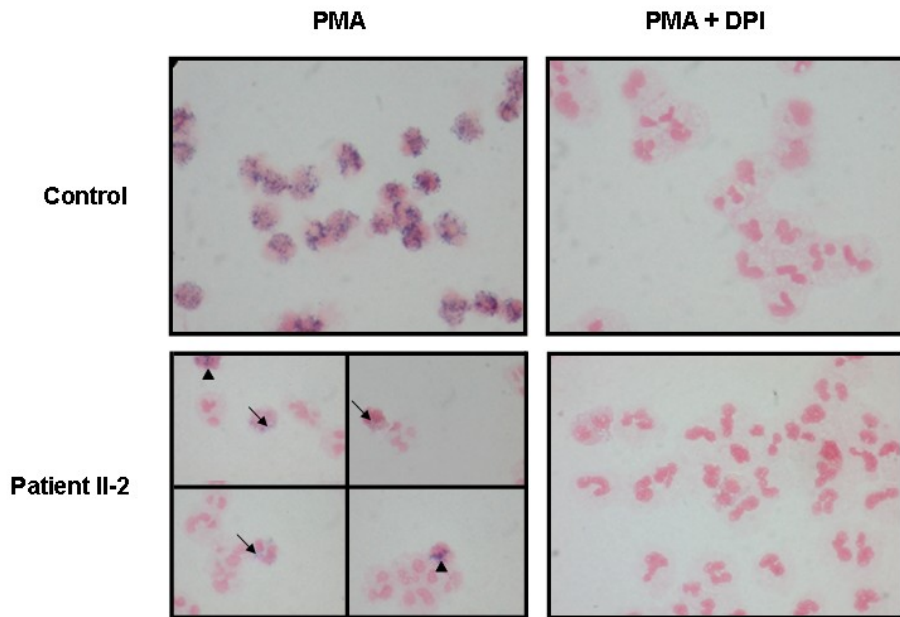
Fig. 8. Electrophoretic mobility shift assay. Gel shift analysis was performed as described in Materials and Methods using the -68- to -30-bp region of the wild-type (WT) *CYBB* promoter as biotin labelled probe. The WT-labelled probe was incubated with 10 µg of nuclear extract from undifferentiated PLB 985 cells after preincubation with 20-, 50-, or 100-fold molar excess of unlabelled double-stranded WT or X91⁻ CGD competitor oligonucleotide as indicated. The data are representative of three separate experiments.

Fig. 9. Semi-quantitative RT-PCR amplification of gp91*phox* mRNA from patient II-2. The gp91*phox* mRNA from patient II-2, X91⁺ and X91⁰ CGD patients and from a healthy subject were amplified by RT-PCR using primers 1 and 2* (see Table II for sequences and positions). The housekeeping gene of β-actin was used as internal control. M, DNA size markers. The data are representative of three separate experiments.

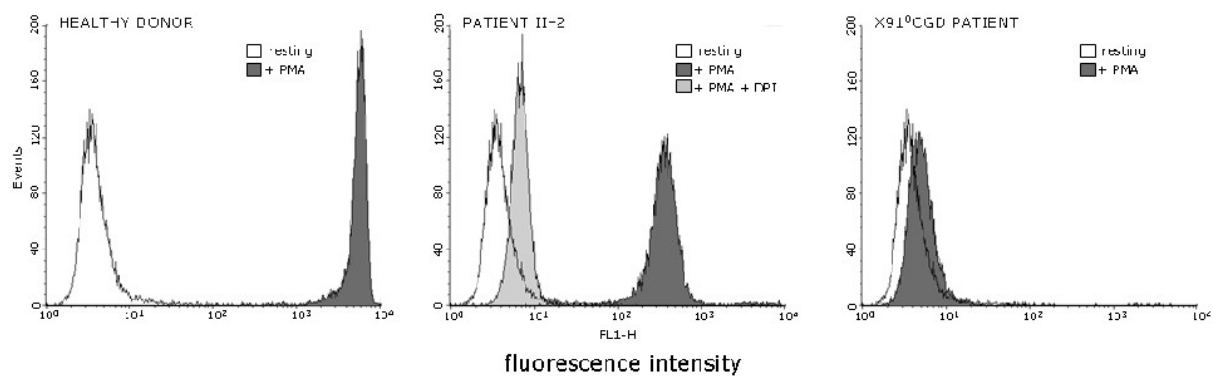
Fig 10. Microbicidal activity of neutrophils from X CGD patients. Neutrophils from a control subject, patient II-2 and a patient with X91⁰ CGD were incubated with opsonized *S. aureus* at MOI 3-5:1, *C. albicans* at MOI 1-2:1 and *E.coli* at MOI 3-5:1. Data are expressed as percent killing after 45 min incubation for *S. aureus* and *E. coli*, and after 60 min incubation for *C. albicans*. The results are means of 3-4 experiments for patient II-2 and 2-3 experiments for the X91⁰ CGD patient. For experimental details, see Materials and Methods.



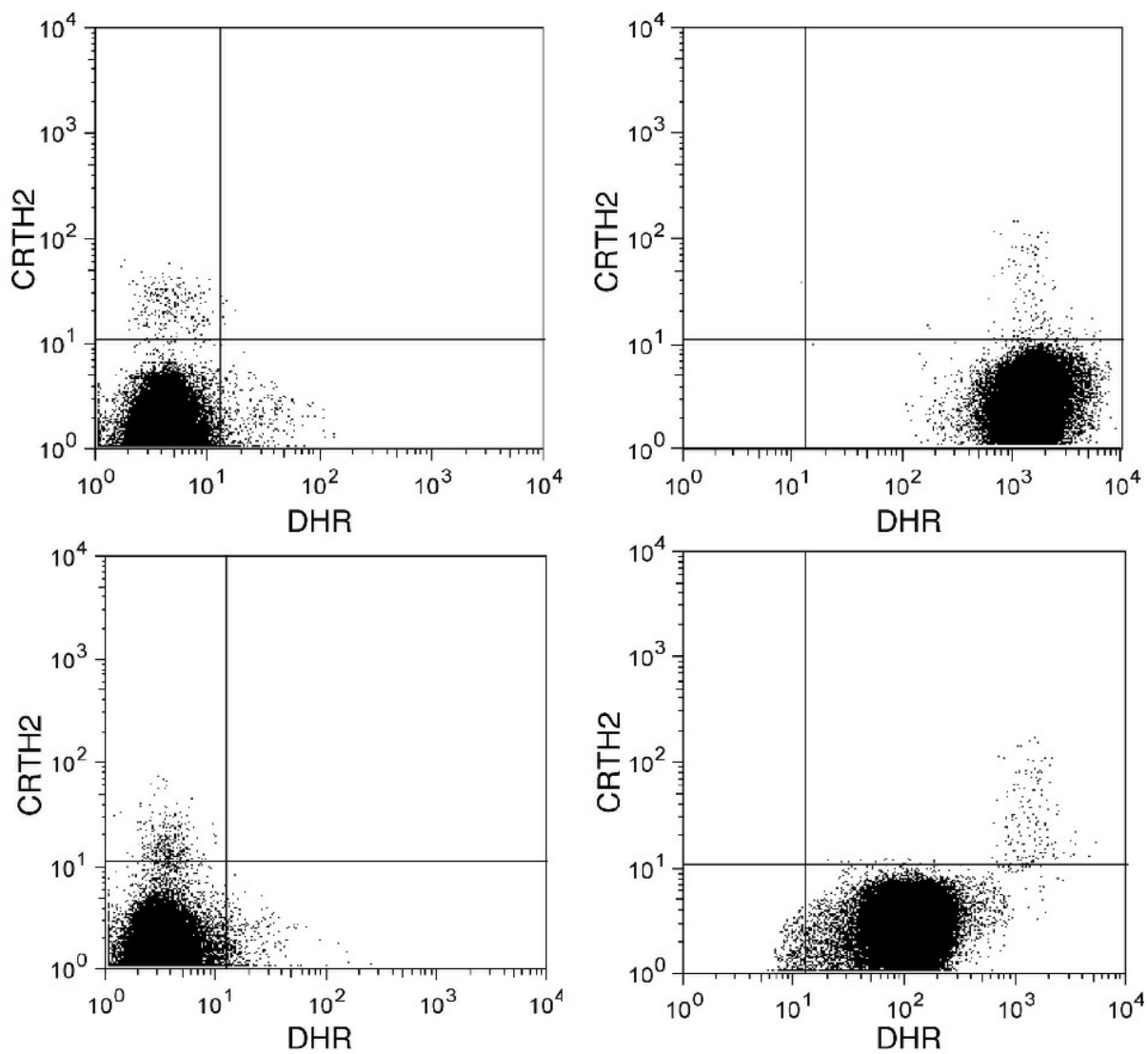
ACCEPTED



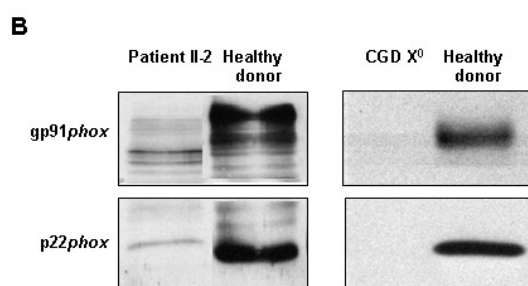
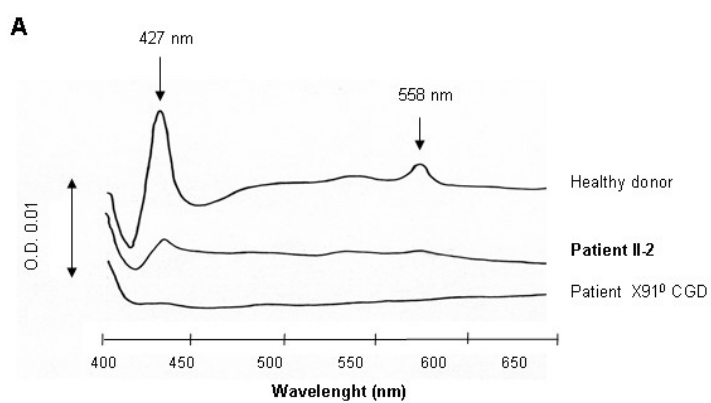
ACCEPTED



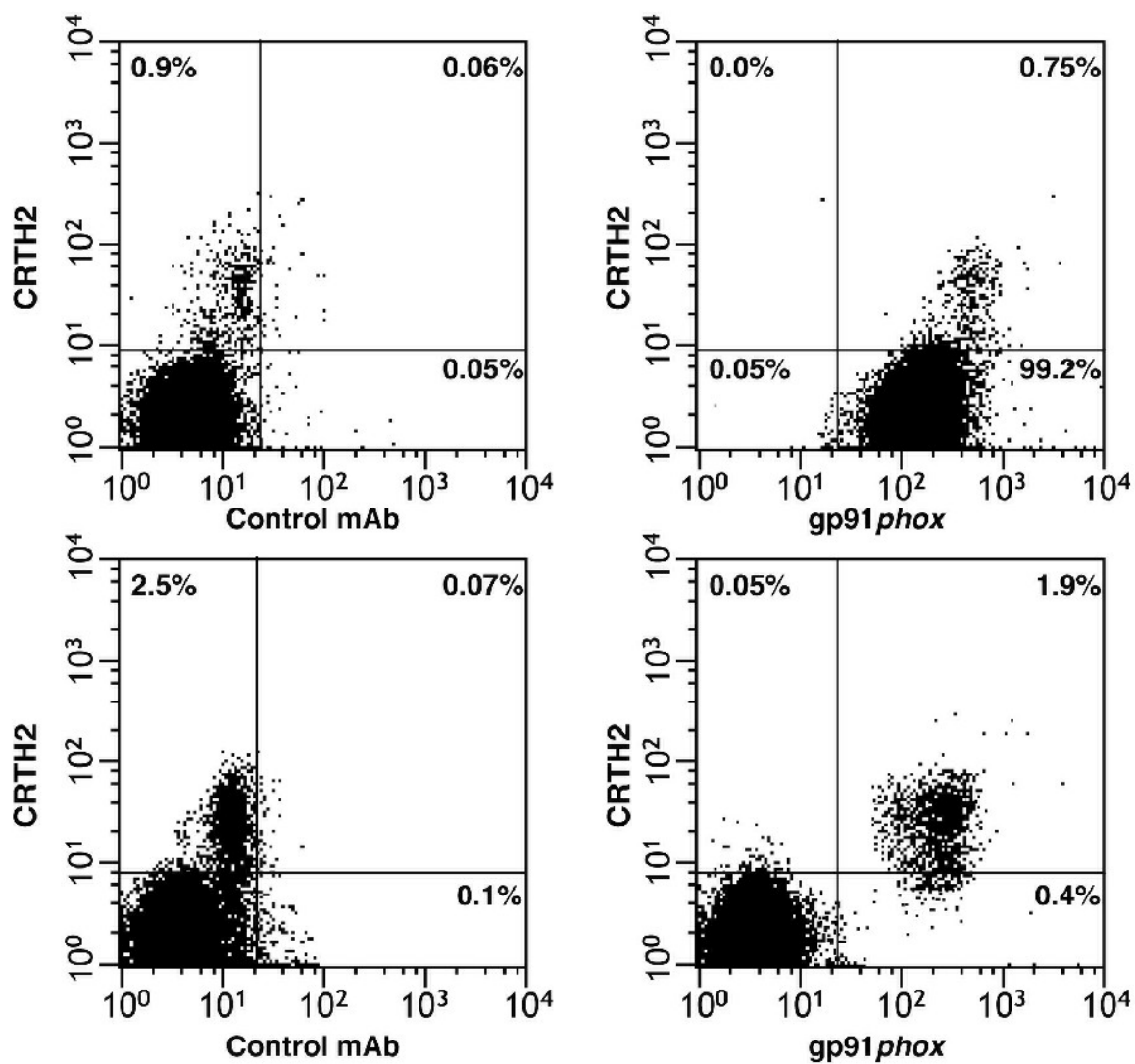
ACCEPTED



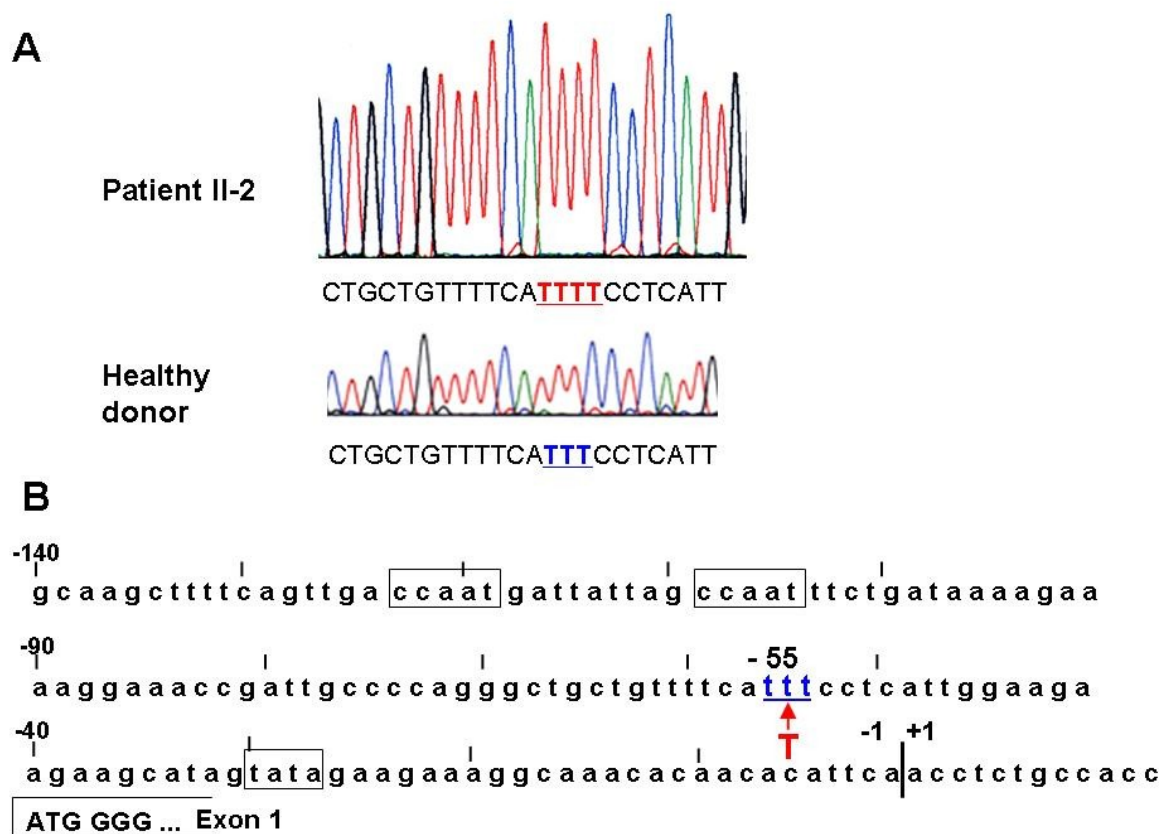
ACC

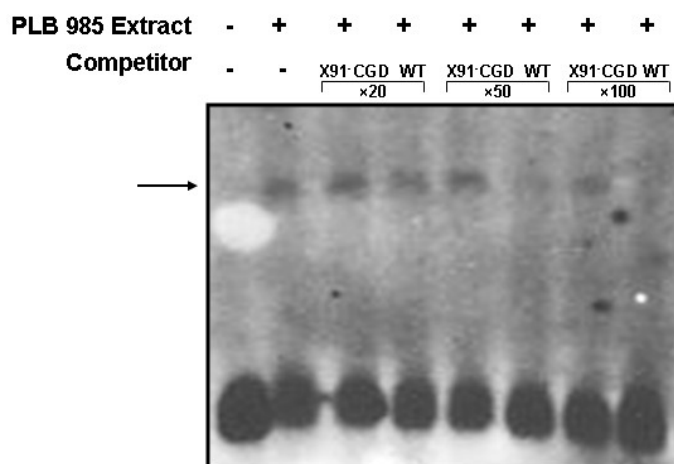


ACCEPTED

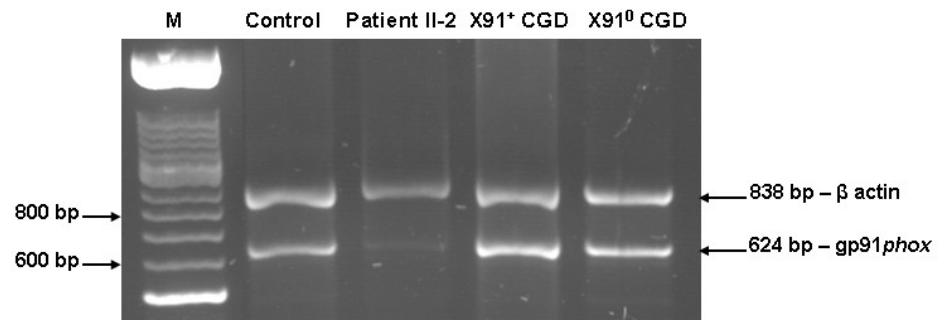


AC

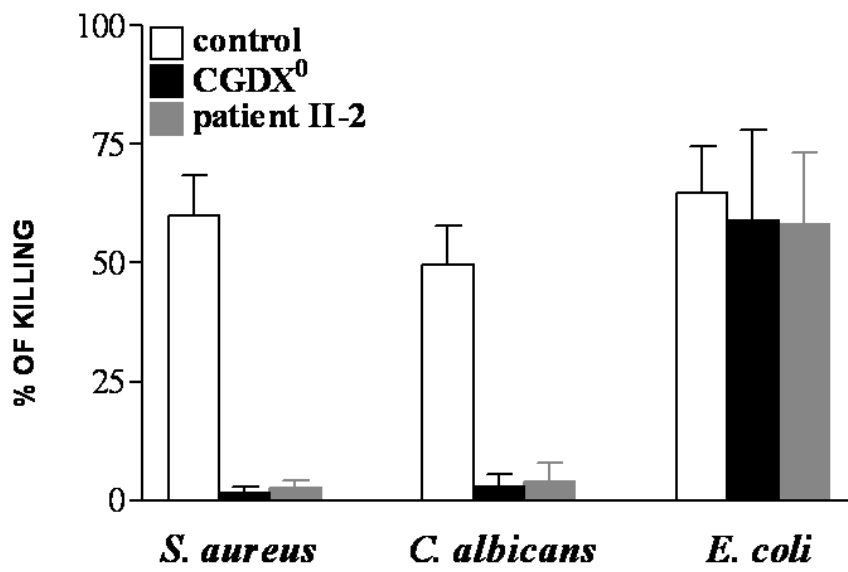




ACCEPTED



ACCEPTED



ACCEPTED