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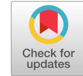
Impaired X-CGD T cell compartment is gp91phox-NADPH oxidase independent



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ABSTRACT

Chronic granulomatous disease (CGD) is a phagocytic disorder characterized by a defective production of reactive oxygen species (ROSs). Although infections and granuloma formation are the most common manifestations in CGD patients, a significant number of patients experienced autoimmunity and inflammatory diseases suggesting that adaptive immune abnormalities might be involved.
Here we investigated T-cell compartment and showed that CGD patients had a skewed TCRV-beta distribution in CD8+ T cells, particularly in older patients, and a reduced proliferative responses toward mitogens compared to healthy donors (HD). Afterwards we studied the role of gp91phox protein in causing these alterations and demonstrated that human T cells do not express gp91phox and TCR-stimulated ROS generation is gp91phox-NADPH oxidase independent. Finally, we proved that the NADPH oxidase is not active in the T cell compartment even when forcing gp91phox expression transducing T cells from X-CGD and HD with a SIN lentiviral vector (LVV) encoding the gp91phox cDNA.

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1. Introduction

Chronic granulomatous disease (CGD) is a rare phagocytic disorder caused by defects in any one of genes encoding the NADPH oxidase subunits. CGD is genetically heterogeneous and is characterized by an autosomal recessive (AR-CGD; p22phox, p47phox, p67phox) or X-linked (X-CGD; gp91phox) inheritance. The X-linked CGD is the most frequent form (65% of cases described) and clinically it is more severe than AR-CGD [1].

The oxidase complex plays a key role in both antimicrobial host defence and inflammation, through the production of reactive oxidative species (ROS) essential for the killing of bacteria, fungi and parasites [1]. Although granulocytes in CGD patients are able to engulf the pathogens, failure to produce ROS prevents the clearance of the microorganisms. As a consequence, CGD patients are susceptible to recurrent life-threatening pyogenic infections, particularly those caused by catalase-positive bacteria and fungi. Moreover, CGD often have poor wound healing and chronic inflammation leading to granuloma formation [2]. Given the improved treatment of infections and survival in CGD patients, nowadays the increased susceptibility to inflammatory and autoimmune diseases has become an increasing relevant issue in patients. At the moment, Systemic Lupus- and Discoid Lupus-Erythematosus, thrombocytopenic purpora, arthritis and inflammatory bowel disease have been often described in CGD patients, suggesting a protective role of ROS in autoimmune disease [3–5]. The evidence that, in addition to phagocytes, other cells of the innate and adaptive immune system such as B lymphocytes and dendritic cells (DC) are able to produce moderate levels of oxygen radicals, highlights the key role that ROS have in regulating immune system interaction and function [6,7]. Particularly we showed in CGD patients that the lower frequency of memory B-cells and higher frequency of naïve B-cells, with their reduced ability to proliferate and differentiate upon *in vitro* BCR-stimulation, are dependent on the defect in BCR-derived ROS production by NADPH oxidase complex [8]. On the other hand, the presence of functional NADPH oxidase complex in T lymphocytes remains

Abbreviations: CGD, Chronic Granulomatous Disease; NADPH, Nicotinammide adenina dinucleotide fosfato; TCR, T-cell reception; ROS, Reactive oxygen species; PBMC, Peripheral blood mononuclear cells; SIN-LVV, Self-inactivating lentiviral vector.

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controversial. Although T cells contain low levels of ROS that occur upon T-cell receptor (TCR) stimulation, it is unclear if oxygen species are actually derived from respiratory burst enzymes or from mitochondria and how they affect T cell functionality [9–11]. Interestingly, T cell lymphopenia and reduced proliferation to mitogens in CGD patients [12], suggest a link between ROS production and the susceptibility to infections. However, how a molecular defect in CGD could influence T-cell function and consequently play a role in the pathogenesis of the disease remains poorly understood.

Here, we performed a phenotypic and functional characterization of T-cell compartment in CGD patients and investigated the role of gp91phox-NADPH oxidase in the production of ROS in T cells. We analysed lymphocyte proliferation upon TCR-stimulation and TCR-Vβ repertoire and found a reduced proliferative capacity in X-CGD together with age-related skewed distribution of TCR repertoire in CD8+ T cells. Moreover, we demonstrated that after TCR stimulation ROS were produced regardless of the gp91phox-NADPH oxidase expression. Furthermore, transducing with a SIN lentiviral vector (LVV) encoding the gp91phox protein [13] T cells collected from X-CGD and HD subjects, we proved that forced gp91phox expression does not induce NADPH oxidase activity.

2. Material and methods

2.1. Patients and informed consent

The study enrolled nine male CGD patients (10 gp91phox-CGD and 1 p22phox-CGD) during follow up procedures. All patients analysed did not express the protein (gp91° or p22°). Blood samples were taken from HD and CGD after obtaining informed consent following standard ethical procedures with approval of the Children's Hospital Bambino Gesù Ethical Committee. Both HD and CGD were free of infection at follow-up and patients were off corticosteroid and immunosuppressive therapy at the time of the blood sampling or previously. Clinical and molecular data of CGD patients are summarized in Table 1.

2.2. T-cell proliferation assay

Peripheral blood mononuclear cells (PBMCs) from 11 CGD patients and 8 age-related HDs were obtained by centrifugation using Ficoll-Paque (Euroclone) and stimulated with PHA 5 μg/ml (Sigma Chemical, Milan, Italy) or OKT3 3 μl/ml (stock supernatant obtained from OKT3 cell line, ATCC-Sigma, MI, Italy) for 72 h. PBMC from the same sample were cultured for 3 and 6 days without any stimulus as negative control. T cells proliferation was investigated as described [14] and results were indicated as stimulation index (SI), defined as the ratio between the average counts of stimulated and unstimulated PBMC. Lymphoproliferation towards mitogens, was considered positive if PHA > 35.000 cpm or OKT3 > 25.000 cpm.

2.3. TCR spectratyping

PBMC from 11 patients and 8 age related HD, were fractionated into $CD4^+$ and $CD8^+$ subsets using anti-CD4+ or anti-CD8+ monoclonal antibody-coupled magnetic beads (Dynal AS, OSLO, Norway) following manufacturer's instructions. The purity of the two subsets was >95%. Total RNA was extracted by TRIzol® (Gibco-BRL/Life Technologies) in accordance with the manufacturer's instructions. T cell receptor CD4 and CD8 V beta repertoire were investigated essentially as described [15]. Particularly, we defined collected data as polyclonal (p), polyclonal altered (pa) and skewed/perturbed (sk) patterns, respectively with a Gaussian “bell shaped distribution”, a non-Gaussian-like distribution and with a skewed profiles consisted of 1–4 peaks or a multipeak pattern with one solitary peak >50% of the total area or one or more deleted peaks.

2.4. Flow-cytometry studies

Cells were stained with antibodies against: anti-human CD3-PERCP (BD), anti-human $gp91^{phox}$-FITC (anti-flavocytochrome B558, clone 7D5) (MBL, Medical and Biological Laboratories Co., Japan), CD45RA-APCH7 (BD), CD4-APC (BD), CD8-APC (BD). Intracellular staining was performed using cytofix-cytoperm (BD) in accordance with the manufacturer's instructions. FACS Canto II (Becton-Dickinson, USA) and Flowjo (Tree Star, Inc.) were used to collect and analyze the data obtained.

2.5. Detection of ROS in T cells

T cells were isolated from PBMC using PanT Cell Isolation Kit (Miltenyi Biotec) and stimulated with OKT3 (10 μl/ml), αCD28 (10 μg/ml) (BD) or phorbol 12-myristate 13-acetate (PMA) (Sigma) for 48 h. To investigate ROS production cells were further incubated for 10 min at 37 °C in water bath in the dark with 0.5 μM 2′,7′-dichlorodihydrofluorescein diacetate (DCFDA; Invitrogen) or with dihydrorhodamine (DHR) 123 DHR (Diidrorhodamine). Oxidative reactions were stopped and fixed with paraformaldehyde in phosphate buffer saline. ROS emission was measured at 530/30 using 488 nm laser excitation on a FACSCanto (BD) with DIVA software. Data were subsequently analyzed with the FlowJo software (Tree Star, Inc.) and reported as Stimulation Index (SI) calculated as ratio between mean of fluorescent intensity (MFI) of stimulated cells and MFI of un-stimulated cells.

2.6. Transduction

PBMCs from X-CGD patients and HD were cultured in presence of OKT3 (10 μl/ml)/αCD28 (10 μg/ml;BD)/IL2 (100 UI/ml, SIGMA) for 2 days and then washed, and transduced with LV PGK.gp91 at MOI 50 in presence of IL2 (100 UI/ml) and Polybrene (4 μg/ml) (SIGMA). PGK.

Table 1
Clinical and molecular data of CGD patients enrolled for the study.

Patients	Gender	Age (y)	DNA change	Protein expression	NADPH function	Severe infections	Noninfectious complications
Pt1	Male	18	del Xp11.4	Gp91°	No residual activity	Lung abscess, lymphadenitis, pyodermitis (*Serratia*)	IBD
Pt2	Male	11	c.1357T > A	Gp91°	No residual activity		IBD
Pt3	Male	30	c.1006G > T	Gp91°	No residual activity	Sepsis (*Staphylococcus*), Osteomyelitis (*C. albicans*)	IBD
Pt4	Male	3	c.83G > A	Gp91°	No residual activity	Liver abscess, meningitis (*Mycobacterium tuberculosis*)	
Pt5	Male	13	c.1076G > C	Gp91°	No residual activity	Lung aspergillosis, suppurative lymphadenitis	
Pt6	Male	10	del Xp11.4	Gp91°	No residual activity	Lung aspergillosis, lung abscess	CE
Pt7*	Male	2	c.252G > A	Gp91°	No residual activity		
Pt8	Male	5	c.1287delT/c.1290delC	Gp91°	No residual activity	Lung aspergillosis	Granulomatous Cistitis
Pt9	Male	6	c.295-301del GTGCCCG	p22°	No residual activity	BCG infection, liver abscess, lymphadenitis	
Pt10	Male	12	c.338-2A > C/p.Cys85ArgFsX15	Gp91°	No residual activity	Osteomyelitis, pyodermitis, lung pneumoniae	IBD
Pt11*	Male	42	c.252G > A	Gp91°	No residual activity	Lung pneumoniae, liver abscess, pyodermitis	

IBD (inflammatory bowel disease); CE (celiac disease).
* Pt7 is the nephew of Pt11.

Table 2
Phenotypic investigations of CD4+ and CD8+ T cell subsets.

Patients	Age(y)	T cells memory/naive phenotype** (gated on CD45 + CD3+ cells)			
		%CD4/RA+	%CD4/RO+	%CD8/RA+	%CD8/RO+
Pt1	*18*	37	44	73	15
Pt2	*11*	70	30	78	22
Pt3	*30*	35	65	49	51
Pt4	*3*	nd	nd	nd	nd
Pt5	*13*	nd	nd	nd	nd
Pt6	*10*	64	36	55	44
Pt7	*1*	88	8	92	8
Pt8	*5*	61	39	60	40
Pt9	*6*	43	57	40	60
Pt10	*12*	59	41	56	44
Pt11	*42*	27	73	49	51
HD	**Normal range for age from *Shearer WT et al., JACI 2003*				
	2–6 y	71	16	86	9
	6–12 y	59	21	80	12
	>***12 y***	53	28	79	13

gp91 LVV designed, production and efficiency of transduction on human myeloid and CD34+ cells were previously described [13]. After 7 days of culture, cells were investigated for the presence of gp91phox protein and ROS-generation capacity using DHR and DCFDA substrates. Cells were cultured for 14 days after transduction in order to eliminate non-integrated vector forms and vector copy number per genome (VCN) were quantified by quantitative Real-Time PCR (Q-PCR) as described [13].

2.7. Statistical analysis

Collected data were processed using Prism 5 (GraphPad Software, San Diego, CA). The mean values of repeated immunological parameters were calculated. The Shapiro-Wilcoxon test was used to evaluate the Gaussian distribution of overall immunological parameters. Statistical comparisons between patients and HD were calculated with non-parametric analyses (one-way ANOVA) when no Gaussian distribution was found and exact p values were obtained, otherwise t-students' test and column statistics was used. A significant threshold of $p < 0.05$ was used to assess the statistical differences in patients versus healthy control values.

3. Results

3.1. Lymphocytes from CGD patients have reduced proliferative capacity

Immunological investigations revealed that all CGD patients showed a normal total B- and T-lymphocytes number, but reduced peripheral memory B-cell compartment. An imbalance toward memory phenotype, investigated by the differential expression of CD45 isoforms, for both CD4+ and CD8+ T-cell was observed (Table 2).

In order to investigate the proliferation capacity of PBMCs after mitogen stimulation, cells isolated from CGD patients and HDs were stimulated with PHA or OKT3. The patient's lymphocytes showed a statistically significant reduction of mitogen responsiveness to PHA (SI HD 82.41 ± 10.4 *vs* SI CGD 43.54 ± 9.3 $p = 0.027$) and OKT3 (SI HD 93.86 ± 17.2 *vs* SI CGD 30.08 ± 7.19, $p = 0.027$) respect to age-matched healthy donors (Fig. 1).

3.2. Skewed distribution of CD8-TCR repertoire in CGD patients seems to be influenced by age

Molecular analysis of T-cell receptor (TCR) repertoire of CGD patients and HDs was studied by measuring the CDR3 heterogeneity length of variable beta regions by spectratyping. Sorted CD4 and CD8 T cells were investigated for 24 TCR-V beta different families.

Results showed that CGD patients presented a normal polyclonal profile in the CD4+ T cells, but a skewed distribution in CD8+ T cells with respect to HD ($p < 0.001$) (Fig. 2B). In order to check if abnormalities in CD8+ T cell populations were age-dependent, the results were reanalysed considering two different sub-groups of patients: pediatric (nPP = 6; age < 12) and non-pediatric (nNPP = 5; age > 12). The analysis showed that the distribution profile in CD8+ T cells is significantly ($p < 0.05$) skewed in NPP CGD with respect to HD (Fig. 2C), with no evidence for preferential skewing of particular V beta subfamily.

3.3. Human T cells do not express gp91phox and TCR-stimulated ROS generation is gp91phox-NADPH oxidase independent

To determine if the defect in T cell compartment of CGD patients was linked to NADPH oxidase deficiency, T cells collected from X-CGD patients (gp91°) and HDs were studied for the presence of gp91phox on cell surface and intracellular compartment, and for the involvement of an NADPH oxidase in TCR-stimulated reactive oxygen species (ROS) generation.

As expected, the protein is expressed on of neutrophils, monocytes and B-cells' cell surface (100%) from HD, but it was absent in the subsets from X-CGD patients. Interestingly, the expression of gp91phox protein resulted undetectable on the cell surface of T cells from both patients ($n = 2$ with missense mutation, and $n = 2$ with whole gene deletion) and HDs ($n = 6$) (Fig. 3A).

Since previous studies on gp91phox expression in T cells were performed by intracellular staining [16] we analysed gp91phox using this method. The intracellular staining performed on CD3 cells revealed that percentage and mean fluorescence intensity (MFI) of gp91phox was similar between HD (99%; MFI = 7558) and both X-CGD with whole CYBB gene deletion (98%; MFI = 4813) and with missense

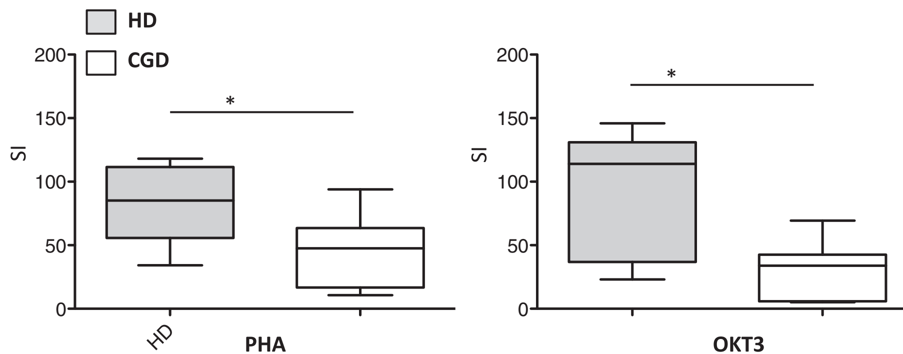

Fig. 1. Lymphocytes from CGD patients have reduced proliferative capacity. Box plot graph showing the distribution of proliferation after PHA or OKT3 stimulation, in age-matched HD ($n = 171$, grey boxes) and X-CGD patients ($n = 11$, white boxes). The Box represents the interquartile range of the sample, with a horizontal bar at the median value and the whiskers defining the highest and lowest values. Statistical analysis was performed using one-way ANOVA test; $^{***}p < 0.05$.

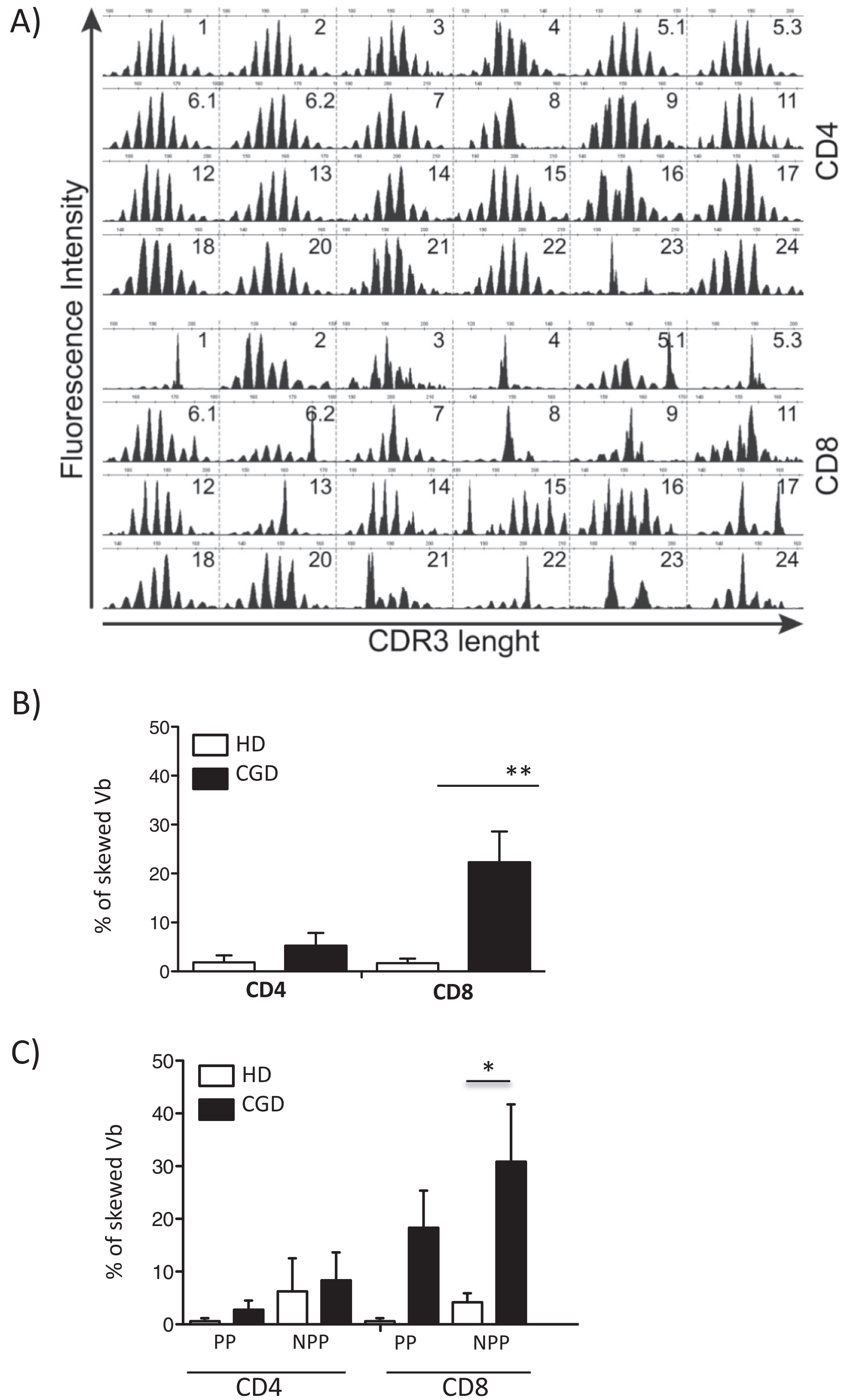

Fig. 2. Skewed distribution of CD8-TCR repertoire in CGD patients seems to be influence by age. A) Spectratyping representative peak distribution in subsets of CD4 and CD8 cells. B) The mean ± SEM of skewed Vb in CD4 and CD8 subsets from HD ($n = 11$ black box) and CGD patients ($n = 11$ white box) were showed. Horizontal line indicates statistical analysis performed by Student's *t*-test: $^{*}p < 0.001$. C) Obtained results represented considering paediatric (PP $n = 6$; age < 12) and non-pediatric (NPP $n = 5$; age > 12) patients. *p*-Values: $^{*}p < 0.05$ were determined by the Student's *t*-test.

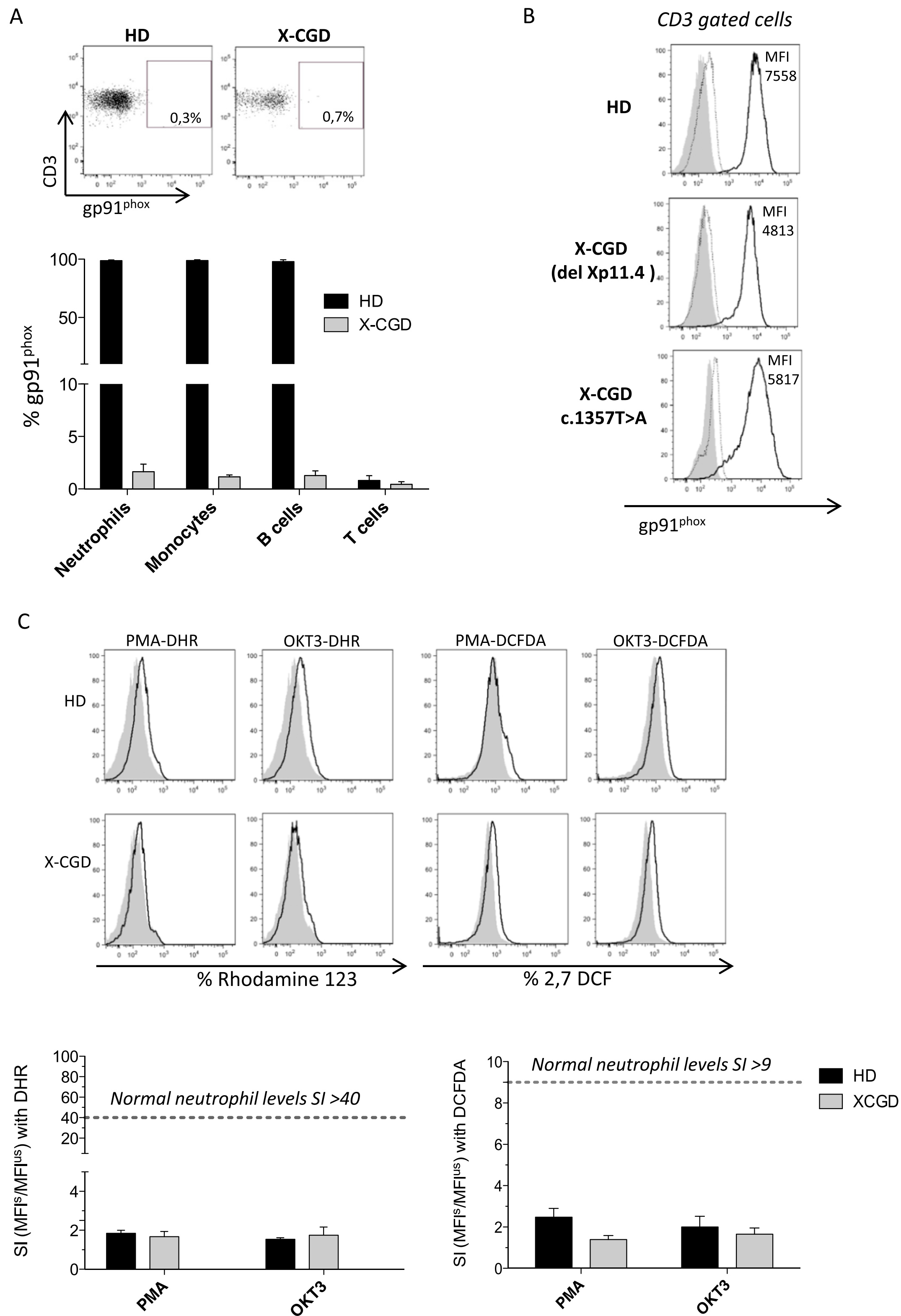

Fig. 3. Human T cells do not express gp91phox and TCR-stimulated ROS generation is gp91phox-NADPH oxidase independent. A) Gp91phox expression on different peripheral cellular subsets. Representative FACS-plots showing gp91phox expression on CD3 cellular surface (upper panel) and bar graph representing the protein expression ± SEM on neutrophils, monocytes and B-cells from HDs ($n = 6$) and CGD patients ($n = 2$ with missense mutation, and $n = 2$ with whole gene deletion) (lower panel). B) Representative overlay histograms of intracellular gp91phox expression into sorted CD3 cells from HD, X-CGD with whole CYBB gene deletion (del Xp11.4) and X-CGD with missense mutation (c.1357T > A). Black line indicates gp91phox antibody, dotted line indicates isotype control and solid grey indicates unmarked control. C) Representative histograms of percentage of rhodamine 123 or 2,7 DCF produced by CD3 cells after PMA or OKT3/CD28 stimulation (upper panel). Bar histograms showing the ROS production evaluated considering the stimulation index (SI) = $MFI_{\text{stimulated cells}}/MFI_{\text{unstimulated cells}}$ ± SEM. Normal level evaluated on neutrophils were also showed (lower panels).

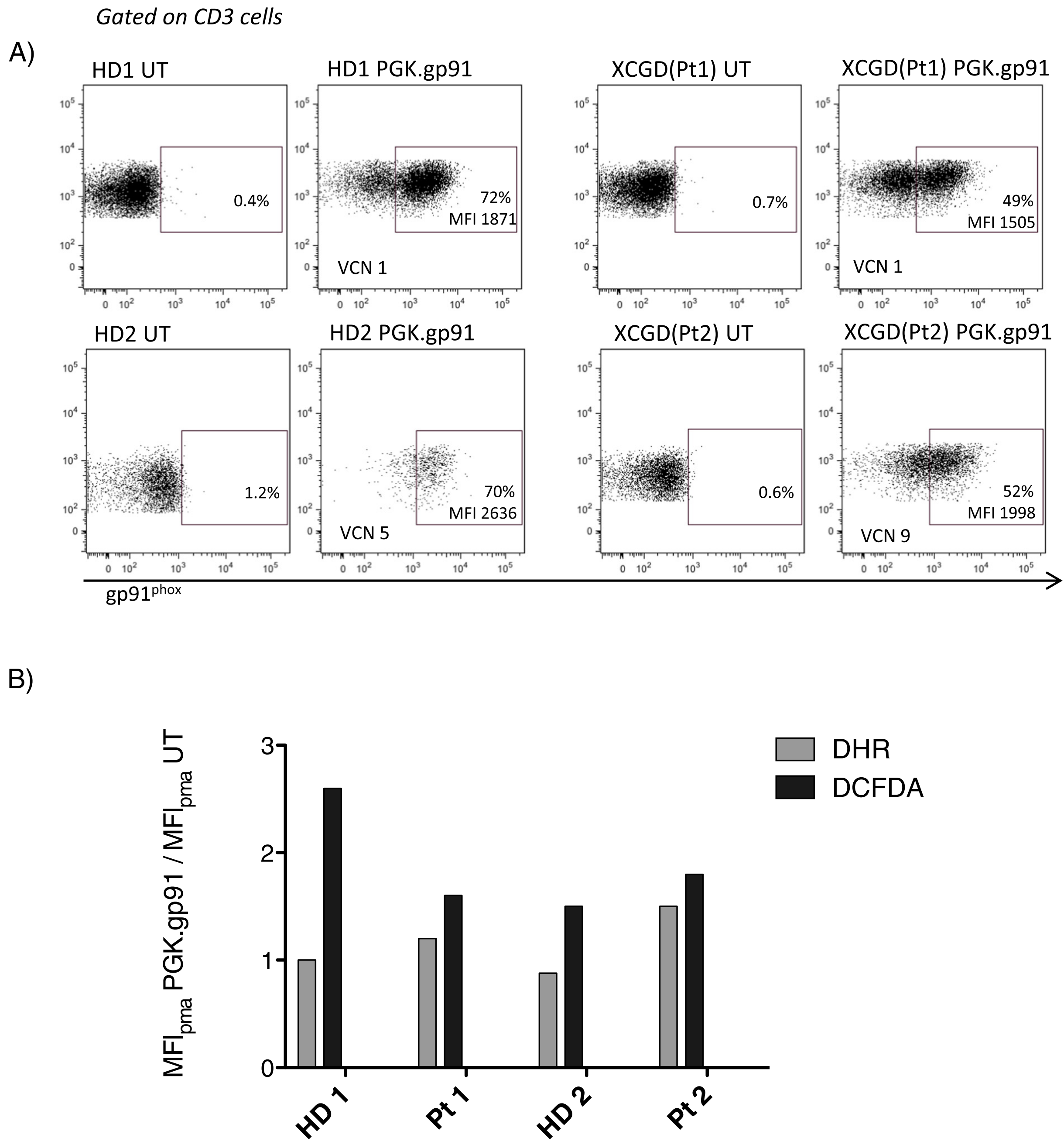

Fig. 4. Forced gp91^{phox} expression in X-CGD and HD T cells do not induce gp91phox-NADPH oxidase activity. A) Plots showing the gp91phox transgene expression on CD3 gated cells from X-CGD patients and HD after transduction with PGK.gp91 LV. Percentage, MFI and vector copy number (VCN) were showed. B) The degree of ROS production in transduced cells was considered as ratio between MFI of PMA stimulated PGK.gp91 transduced cells and MFI of PMA un-transduced cells.

mutation (97%; MFI = 5817), suggesting that the fluorescence is due to background staining of the gp91phox antibody (7D5) (Fig. 3B).

Next, we investigated the production of oxidants from CD3 cells after nonspecific stimulation phorbol-12-myristate-13-acetate (PMA)-mediated and specific OKT3/CD28 TCR-stimulation. We performed the experiments using the dihidrorhodamine 123 (DHR123) and cell-permanent 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA) probes, which in presence of ROS are converted respectively to the highly fluorescent rhodamine 123 (directly via H_2O_2) and 2′, 7′ dichlorofluorescein (DCF) (initially via esterase and then via H_2O_2) [17].

Results showed that both X-CGD and HD cells after prolonged culture with stimuli induced a minimal oxidation of both probes (Fig. 3C upper panel). In particular, the difference between the stimulation index (SI) evaluated in HD ($n = 7$) and X-CGD ($n = 7$) is not statistically significant for both probes DHR (SI PMA: 1.8 ± 0.2 vs 1.7 ± 0.5 and SI OKT3: 1.5 ± 0.2 vs 1.8 ± 0.8) and DCFDA (SI PMA: 3 ± 0.7 vs 1.4 ± 0.2 and SI OKT3: 2.5 ± 0.7 vs 1.7 ± 0.3), despite a tendency to a slight increase of the oxidation detected by the first probe in HD rather than in X-CGD (Fig. 3C lower panels). These results suggested that ROS generated after TCR-stimulation (SI < 3) were independent from NADPH oxidase activity.

3.4. Forced gp91phox expression in X-CGD and HD T cells do not induce gp91phox-NADPH oxidase activity

To investigate if the forced gp91phox expression in T cells was able to induce the NADPH oxidase activity, T cells from X-CGD patients and HDs were cultured in presence of OKT3/CD28/IL2 and transduced (Multiplicity of Infection-MOI 50) with a LV expressing human codon optimized gp91phox cDNA under the control of a ubiquitous cellular promoter (PGK.gp91).

After 7 days of culture the gp91phox transgene protein was presented on all transduced cells (Fig. 4A) with variable expression levels in both X-CGD (49–52%; VCN = 1–9) and HD (72–70%; VCN = 1–5). We tested the functionality of the oxidase activity and found that by DHR assay both HD and patients did not have the activity of NADPH oxidase ($0,88 < MFI_{pma}$ PGK.gp91/MFI_{pma} UT < 1.5). However by DCFDA assay we found a slight increase of the oxidase activity ($1.5 < MFI_{pma}$ PGK.gp91/MFI_{pma} UT < 2.6) in both HD and patients probably caused by the influence of cytochrome c levels that could give a signal without any change in cellular peroxide levels. These results suggested that the NADPH oxidase complex is not functional in T cells.

4. Discussions and conclusion

Phagocytes, primarily neutrophils, have been extensively studied in the pathogenesis of CGD because of their ability to kill and clear bacteria and fungi during the infection through the production of ROS by the NADPH oxidase complex. In the last years various studies showed that several non-phagocytic leukocytes, such as B-lymphocytes, dendritic and NK cells, are capable of producing moderate amount of ROS, suggesting that ROS are not only harmful and mediators of oxidative stress, but also have immune regulatory functions, especially when produced in lower amounts [1,18].

In this regard we have recently demonstrated that CGD patients have lower memory and higher naïve B-cells counts related to impaired long-term memory in CGD, and that impaired B-cells are dependent on defective ROS-production by BCR [8]. The ability of T lymphocytes to generate ROS upon TCR stimulation has been also investigated by Belikov AV et al. [19] that showed how T lymphocytes exhibit a very small burst production reactive oxygen products in response to mitogen stimulus, and that a portion of this burst is missing in mouse and human CGD T lymphocytes.

In contrast other groups were unable to detect any ROS production by T lymphocytes following TCR stimulation [12]. It is likely that ROS influence T-lymphocytes signalling, however it is not clear whether T-lymphocytes produce ROS themselves or whether ROS in particular H_2O_2 diffuse from APCs to T-lymphocytes [20]. Interestingly, slight abnormalities in T-lymphocytes have been reported in CGD patients, including diminished T-cell number, a tendency to reduced proliferative responses and diminished expression of CD40L on T cells, suggesting a possible role of ROS in T cells [21]. In order to better define the role of T cells in CGD patients we performed a careful phenotypic and functional characterization of T-cell and we evaluated the role of gp91phox-NADPH oxidase in the production of ROS by T cells.

Our data showed that CGD T lymphocytes have significantly reduced proliferative response toward mitogens. Furthermore, we demonstrated significantly higher skewing of TCR-V beta in pheripheral CD8+ T cells among CGD patients compared to healthy controls, with no evidence for preferential skewing of particular V beta subfamily. Particularly, the frequency of skewed TCR-Vβ subfamilies in older patients was significantly higher than that observed in younger patients and HD. Because none of the patients had clinical and laboratory evidence of acute infection at the time of sample collection, we conclude that these alterations reflect a stable state of the TCR repertoire, consolidated over time, in CGD patients.

The clinical relevance of a contracted T-cell repertoire and reduced T cell proliferation in CGD is unclear; T cell compartment has been investigated in some studies. Although infections and granuloma formation remain the most common manifestations in patients with CGD, there is a significant subset of CGD patients who experienced a broad variety of autoimmune and inflammatory disease suggesting the possibility of concomitant adaptive immune abnormalities [22].

Whether the decreased and skewed TCR Vβ diversity might be involved in the pathogenesis of immunodeficiency and autoimmunity or might be the consequence of chronic antigen exposure remains to be clarified in patients with CGD as well as underlying molecular mechanisms.

In order to define if impaired TCR-expression and function were dependent on defective gp91phox expression, we investigated the involvement of NADPH-oxidase in the generation of ROS in TCR-stimulated T cells from CGD patients and HD. Initially we assessed that human T cells didn't express gp91phox protein on the cellular surface and the production of ROS is gp91phox-NADPH oxidase independent. Finally, forcing the expression of gp91phox protein transducing T cells from X-CGD patients and HD with LV.PGK.gp91, we showed that gp91phox-NADPH oxidase complex did not generate ROS, probably for the absence of other NADPH oxidase subunits [23].

In summary our results indicate that X-CGD patients a skewed CDR3 size distribution and reduced proliferative response toward mitogens. Most importantly, we unambiguously demonstrate that T cell abnormalities are not due to the absence of gp91phox protein expression and function into the NADPH-oxidase complex. Indeed, in T cells, ROS production is not mediated by gp91phox in physiological and *in vitro* conditions. Our results add to the knowledge of T-cell defects in CGD and highlight the necessity to extend the study of different cellular immune components in CGD patients in order to understand the complex pathogenic mechanism underlying the relations between innate and adaptive immunity.

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Author contributions

MC designed and performed experiments, analysed the data, performed statistical analyses and wrote the paper; FC and GDM performed laboratory analysis and contributed to the data collection and revision of the manuscript; BG produced the LVV; AC, NC, DAP, PR provided patient's samples and clinical information, contributed to data discussion and drafting of the paper; AA supervised the project and revised the paper; AF supervised the project, designed the research, wrote and revised the paper. All authors critically revised and approved the final version of the manuscript.

Conflict of interest

All authors declare no conflict of interest.

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