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Fas and FasL expression in leukocytes from Chronic Granulomatous Disease patients.

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Key words: CGD, Fas/FasL, T lymphocytes, neutrophils.

Abstract. Chronic Granulomatous Disease (CGD) is a primary immunodeficiency characterized by defects in superoxide (O_2-) production, which result from mutations in one of the four NADPH oxidase components, predisposing to bacterial and fungal infections. Besides the O_2-defect, it has been described that neutrophils from CGD patients are resistant to cell death, a phenomenon that has been connected to chronic inflammation and predisposition to autoimmune diseases. A diminished expression of Fas and its counterpart FasL, molecules known to play a major role in cell death, has been described in lymphocytes depleted of O_2-reactive oxygen species (ROS), suggesting an involvement of ROS in Fas/FasL expression. In this work, Fas and FasL expressions were analyzed in T cells and neutrophils from two CGD families, previously known to harbor two different molecular defects: absence of either p47-phox or p67-phox. We found that T lymphocytes from CGD patients express low levels of Fas and FasL, while a diminished FasL expression was observed on neutrophils from a CGD A470 patient. These defects may contribute to understand altered cell death in CGD patients.

Expresión de Fas y FasL en leucocitos de pacientes con Enfermedad Granulomatosa Crónica

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Palabras clave: EGC, Fas/FasL, linfocitos T, neutrófilos.

Resumen. La Enfermedad Granulomatosa Crónica (EGC) es una inmunodeficiencia primaria caracterizada por un defecto en la producción de superóxido (O2-), que se genera como consecuencia de mutaciones en uno de los cuatro componentes del complejo NADPH oxidasa y predispone a infecciones

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por bacterias y hongos. Además de los defectos en la producción de O_2-, se ha descrito que los neutrófilos de los pacientes con EGC exhiben una resistencia a la muerte celular, evento que se asocia con la inflamación crónica y predisposición a enfermedades autoinmunes. Se ha descrito que linfocitos en medios desprovistos de O_2-especies reactivas del oxigeno (ROS), muestran reducida expresión de Fas y FasL, moléculas que juegan un papel relevante en el control de la muerte celular, sugiriendo la participación de los ROS su regulación. En este trabajo analizamos la expresión de Fas y FasL en linfocitos T y neutrófilos en dos familias portadores de dos defectos genéticos diferentes asociados con EGC: ausencia de p47-phox o de p67-phox. Evidenciamos una baja expresión de Fas y FasL en los linfocitos T de los pacientes con EGC, pero solo los neutrófilos de los pacientes con defecto de p47-phox, fueron incapaces de expresar FasL. Estos defectos pudieran contribuir a entender la alteración de la muerte celular observada en los pacientes con EGC.

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INTRODUCTION

The hallmark of chronic granulomatous disease (CGD) is total absence or severely diminished levels of superoxide (O2-). The rare disease occurs in about 1 per 250.000 individuals. CGD is genetically and biochemically heterogeneous, and it is the result of absence or intensely diminished amount of any of four phox protein components, encoded by their respective genes, resulting in either X-chromosome-linked or autosomal recessive inheritance [1]. gp91-phox mutation is the unique X-chromosome-linked form of the disease and accounts for nearly two-thirds of all cases, whereas the autosomal recessive forms, account for the remaining 36% of cases. The most common autosomal recessive form results from a defect in p47-phox, whereas a rare form occurs because a deficiency of p67-phox or p22-phox [2]. Impaired recognition, activation of apoptotic pathway and clearance of apoptotic cells may contribute to CGD inflammation [3-5]. A delay in physiologic apoptosis has been shown in neutrophils from CGD patients [6, 7], and gene expression profiling studies support the notion that defective neutrophil apoptosis could play a role in chronic granulomatous lesions typical of the disease [8].

Apoptosis of inflammatory cells represents a physiological mechanism to prevent secondary uncontrolled necrosis and hyperinflammation, followed by tissue damage [9]. Apoptotic cells externalize phosphatidylserine (PS), which is recognized through PS-receptors. This interaction enables the uptake of apoptotic cells by phagocytes (for instance, macrophages), a process termed "efferocytosis" [10]. Both apoptosis and efferocytosis of apoptotic cells by macrophages have been reported to be impaired in CGD patients [11]. This may lead to unbalanced necrosis with release of intracellular proteases/oxidants and an increased risk of developing autoimmune disease in CGD patients [12].

Fas, also known as CD95 or APO-1, is a member of the TNF/NGF receptor superfamily. The Fas gene encodes a 45-kDa type 1 transmembrane protein that is constitutively expressed on the surface of a broad range of cells or tissues, including neutrophils and lymphocytes among others lineages [13]. Fas is involved in a signal transduction pathway that results in pro-

grammed cell death from Fas positive cells [14], and its natural ligand is FasL. FasL also known as CD95L, is a member of the TNF/NGF superfamily, a 40-kDa type II transmembrane protein that is induced in neutrophils [15], T lymphocytes, and B cells, after stimulation [13]. It has been shown that FasL expression [16] and apoptosis Fas/Fasl-induced is extremely delayed in absence of ROS [7]. Therefore, altered expression or induction of Fas/FasL could be expected in CGD patients. In this study we found that CGD patients have low expression of CD95 and CD95L in resting and stimulated T lymphocytes. Additionally, a diminished expression of CD95L was observed in stimulated neutrophils from a $A47^0$ CGD patient. These results may contribute to understand resistance to cell death in CGD patients.

MATERIALS AND METHODS

CGD patients and families

Blood samples were obtained from three CGD patients (2 with $A67^0$ and 1 with $A47^0$), seven heterozygous family members and five control individuals. The experimental protocol was approved by the ethics committee of University of Los Andes and a written informed consent was obtained from all subjects. The first patient is a 24-year-old female who was diagnosed with CGD at age 11 years during a functional and genetic screening of the entire family because of a previous case of CGD in her brother, who died from a pulmonary infection due to Mycobacterium tuberculosis. She is currently asymptomatic. The second and third patients are brothers, males aged 26 and 14 years diagnosed with the disease at ages 14 years and 15 months, respectively. The second patient had an episode of pulmonary nocardiosis 8 years before this study, when two of his brothers died in an intensive care unit because of the same infection. No history of infectious diseases was reported for the third patient . None of the CGD patients participating in this study had any signs of acute illness or infection. Anti- nuclear antibody (ANA) and Rheumatoid factor (RF), were negative and C-reactive protein (CRP) values for all patients were normal.

Reagents and antibodies

Goat anti-human-p47-phox, rabbit anti-human-p22-phox, goat anti-human-p67-phox, rabbit anti-human-gp91-phox, HRP-conjugated anti-goat and HRP-conjugated anti-rabbit antibodies were purchased from Santa Cruz Biotechnology, CA, USA. Phenylmethylsulfonyl fluoride (PMSF). Bis-acrylamide, acrylamide, 2β-mercaptoethanol, Triton X-100 and Amonium Persulfate (APS), were purchased from BIO-RAD CA, USA. RPMI-1640 with L-Glutamine (0.3g/L), PHA and HEPES were purchased from GIBCO, BRL®, Gaithersburg MD, USA. Ficoll-Hypaque™ (Lymphoprep) was purchased from NYCOMED Pharma, AS, Oslo, Norway. Luminol was purchased from Pierce, Rockford, IL, USA. CD3 PerCp-conjugated antibody, CD95 FITC-conjugated, CD95L Biotin-conjugated and Streptavidin phycoerythrin (PE)-conjugated were purchased from Becton Dickinson Co. (San Jose, CA, USA). Dihydrorhodamine-123 (DHR-123) was purchased from Molecular Probes (Eugene, OR, USA). Phorbol-12-myristato-13-acetato (PMA) was purchased from Sigma (St Louis, MO).

Peripheral blood mononuclear cells (PBMC) isolation and stimulation

PBMC were isolated from heparinised blood by density gradient sedimentation over Lymphoprep. Freshly isolated PBMC (98% viable by trypan blue exclusion) were resuspended in RPMI 1640 supplemented with 10% heat-inactivated FCS, 100 units/mL of penicillin, and 100 mg/mL of strep-

tomycin and were stimulated with 20 μg/mL of PHA at 37°C during 48 hours.

Polymorphonuclears (PMNs) isolation and stimulation

PMN were purified from citrated (3.8%) blood, mixed with 6% dextran solution (mol Wt 500.000) and, incubated at room temperature for 30 min. After red blood cell depletion, leukocyte-enriched supernatant was collected and layered on Ficoll-Hypaque (2:1 ratio) (1077, Sigma, St. Louis, Mo). Then, a density gradient centrifugation was performed for each sample at 400 g for 30 min at 18°C. PMN were obtained from the bottom. Red blood cells contained in PMN pellet were hypotonic lysed using cold distilled water. This procedure consistently resulted in a highly purified (98%) and viable (95%) polymorphonuclear cell population, visualized with acridine orange [18] (1 mg/mL) diluted at 1:25 ratio and trypan blue exclusion stain, respectively. Freshly isolated neutrophils were resuspended in RPMI 1640 supplemented with 10% heat-inactivated FCS, 100 units/mL of penicillin, and 100 mg/mL of streptomycin and were stimulated with 100 ng/mL of PMA at 37°C during 60 min.

Superoxide production

Flow cytometry analysis of neutrophil respiratory burst activity was measured using a modification of a previously published method [19]. Briefly, freshly isolated neutrophils (1×10^6 cells/mL) were preloaded with DHR-123 (1 μmol/L) at 37°C for 15 min. Afterwards, cells were incubated with 100 ng/mL PMA. Cells were analyzed on a FACScan flow cytometer (Becton Dickinson, San Jose, CA). A total of 10,000 events were collected from each sample.

Protein immunoblotting

Resting PMN (1×10^6 cells/100 μL) were lysed in buffer A containing 50mM TrisCl, pH 8, 1% Triton X-100, 150 mM NaCl, 1 mM EDTA, 1mM PMSF, 1 μg/mL leupeptin/aprotinin, 1mM sodium orthovanadate, incubated on ice for 15 min; and lysates were clarified by centrifugation at 14,000 X g for 10 min at 4°C. Supernatants were mixed in sample buffer heated in a boiling water bath for 3 min, separated by electrophoresis on 10% SDS polyacrylamide gels, transferred to poly(vinylidene difluoride) membranes (Millipore), and probed with different antibodies: anti-p22-phox, anti-gp91-phox, anti-p47-phox, anti-p67-phox. The antibody-labeled protein bands were detected by autoradiography after enhanced chemiluminescense (Super Signal, Pierce, Rockford, IL).

Detection of CD95 in PMN and T lymphocytes

To analyze CD95 expression before and after stimulation, cells were washed twice with cold PBS, PMN were stained with a monoclonal anti-CD95 FITC-conjugated antibody and PBMC were double stained with anti-CD95 FITC-conjugated antibody and anti-CD3 PerCp-conjugated antibody, during 30 min at 4°C, washed twice with cold PBS-EDTA and fixed in a PBS solution containing 1% paraformaldehyde, during 10 min at room temperature. After fixation, cells were analyzed by flow cytometry.

Detection of CD95 ligand (CD95L) in PMN and T lymphocytes

CD95L cell surface expression was assessed by flow cytometry. PMN and PBMC were primary stained with a monoclonal anti-FasL biotin-conjugated antibody, in a solution containing 1% BSA, during 30 min at 4°C. Cells were washed twice with cold PBS-EDTA. PMN were incubated with streptavidin-PE and PBMC were double labeled with streptavidin-PE and anti-CD3-PerCp during 30 min at 4°C, washed twice with PBS-EDTA and fixed with 1% paraform-

aldehyde in PBS. Cells were analyzed by flow cytometry.

Flow cytometry analysis of surface molecules

Three-color flow cytometry was performed by FACSort (Beckton & Dickinson, San José, CA), following the manufacturer's instructions. Gating criteria used to identify cell populations was based on forward and side scatter parameters for PMN, and forward and CD3 fluorescence parameters for T lymphocytes. Evaluation of expression was assessed by looking at the percentage of positive cells to each used markers. Isotype control antibodies were used to separate positive and negative cells on FITC and PE channels. A minimum of 10.000 events were collected for each analysis.

Statistical analysis

The data represents means ± SD. Statistical analysis was performed by Student's *t* test. A two-tailed P value of <0.05 was considered significant

RESULTS

Detection of p47-phox and p67-phox in CGD patients

Two unrelated families with CGD were diagnosed ten years ago by superoxide production, and DNA sequencing [20, 21]. Western blotting was performed to demonstrate the complete absence of p47phox or p67phox protein components (Fig. 1). Absence of p47-phox (Fig. 1a) and p67-phox (Fig.1b) was observed. ROS production was analyzed by flow cytometry, and a remarkable reduced superoxide production was also observed (Figs. 1c and 1 d).

T lymphocytes from CGD patients show low expression of Fas

To determinate Fas expression in T lymphocytes and neutrophils from CGD patients, CD95 was measured in resting and

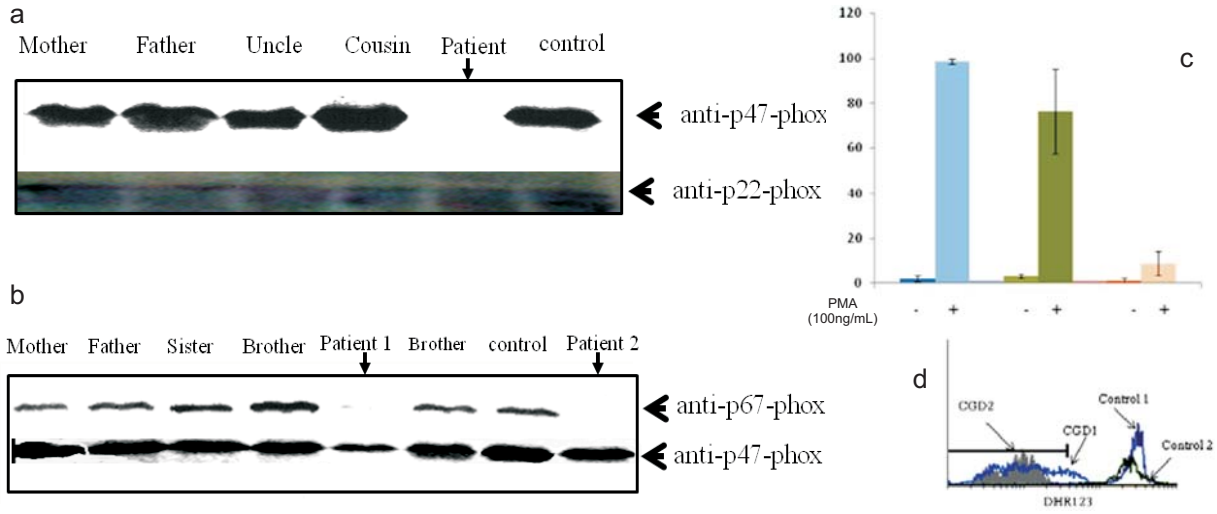

Fig. 1. Western blotting of NADPH-oxidase component. 1a. p47-phox detection by western blot. Neutrophils (1×10^6 cels/100 μL) were subject to 10% SDS-PAGE. Western blotting was performed by using goat anti-human p47phox. 1b. p67-phox detection by western blot. Neutrophils (1×10^6 cels/100 μL) were subject to 10% SDS-PAGE. Western blotting was performed by using goat anti- human p67phox. 1c. Shows ROS production in neutrophils from controls, family members and CGD patients. 1d. A representative histograms showing ROS production after stimulation in 2 CGD patients and 2 controls individuals.

stimulated cells. We found that both unstimulated and stimulated neutrophils from CGD patients, show similar percentage of Fas molecules, as compared with controls and family group (Figs. 2a and 2b). However, a significantly diminished expression of CD95 was observed on resting and stimulated T cells from CGD patients, regardless of their defects (2 $A67^0$ and or 1 $A47^0$) (Figs. 3a and 3b).

CGD patients are unable to upregulate FasL in T cells, following stimulation

FasL was expressed at low concentrations in unstimulated cells (neutrophils and T cells) from all studied individuals, without significant differences (Fig. 4a). A PMA induced expression of FasL was observed in PMN from family members, control group and $A67^0$ patients, but no changes in this molecule were observed in a $A47^0$ CGD patient (Figs. 4a and 4b). T lymphocytes from CGD patients show low expression of CD95L following stimulation, regardless of their genetic defect (Fig. 5).

DISCUSSION

This study shows that CGD patients analyzed in this families showed a defective PHA-dependent induction of Fas and FasL in lymphocytes and FasL in neutrophils. It has been previously described that neutrophils from CGD patients are reported to be resistant to apoptosis [7] expressing diminished or delayed phosphatidylserine on cell surface [12], an important requirement to identify and engulf apoptotic cells [22]. These defects have been associated with hyperinflammation and autoimmune diseases observed during CGD [23]. Components of the NADPH oxidase have been

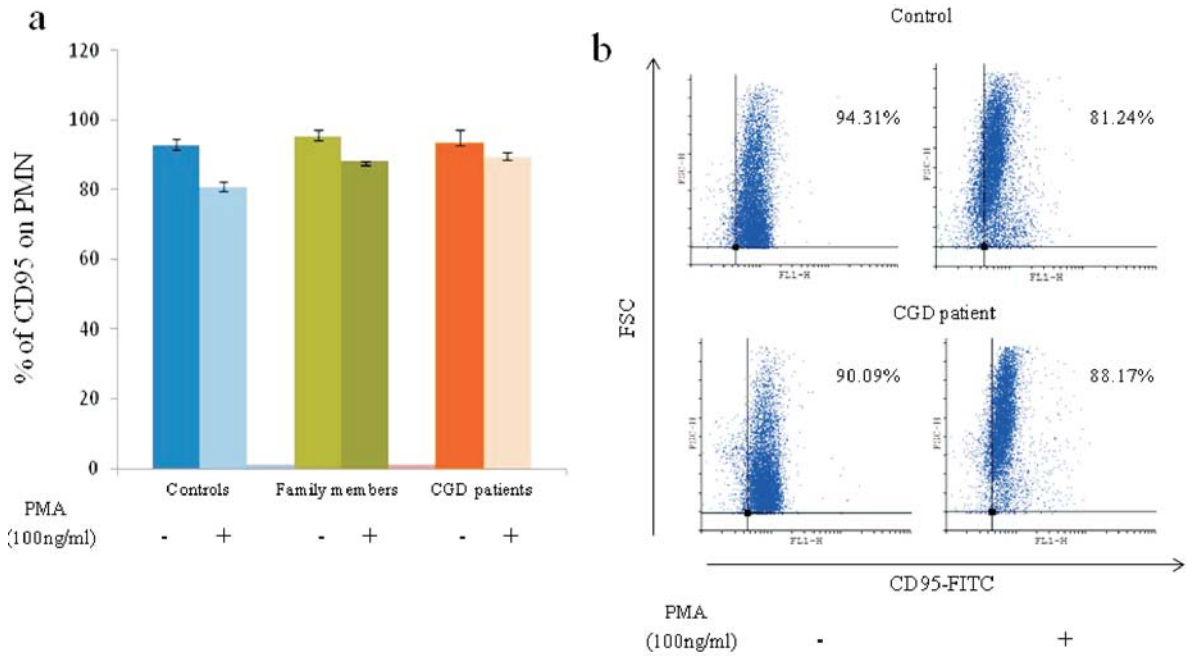

Fig. 2. Expression of CD95 on neutrophils in a group of control subjects, family members and CGD patients (a) Shows the level of expression of CD95 in neutrophils from the different groups, with and without stimulation, expressed of percentage of expression and, represented in standard deviation (SD). Left panel represents controls, middle panel family member and right panel CGD patients. (b) It is a representative dot plot showing the expression of CD95 in neutrophils from control (top) and CGD patient (bottom).

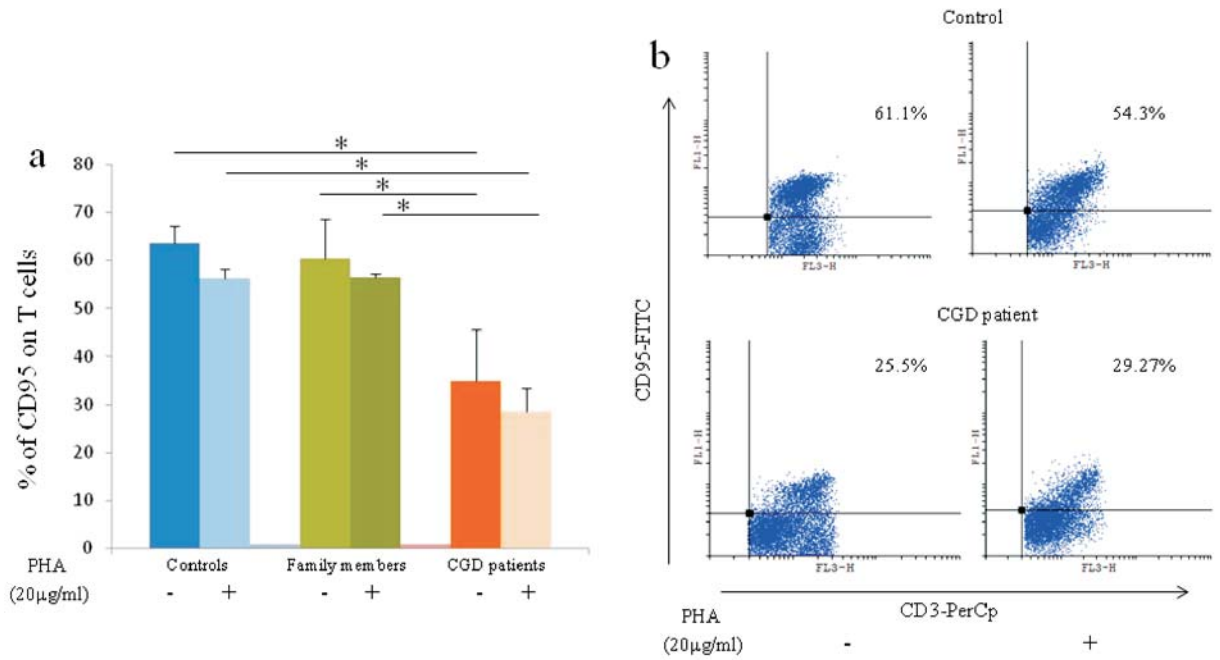

Fig. 3. Expression of CD95 on T lymphocytes in a group of control subjects, family members and CGD patients (a) Shows the level of expression of CD95 in T lymphocytes from the different groups, with and without stimulation, expressed of percentage of expression and, represented in standard deviation (SD). Left panel represents controls, middle panel family member and right panel CGD patients, (*$p < 0.01$). (b) It is a representative dot plot showing the expression of CD95 in T lymphocytes from control (top) and CGD patient (bottom).

identified in phagocytic and non-phagocytic cells [24], in non-phagocytic cells, such as T lymphocytes, expression of NADPH-oxidase component play a role in signal transduction pathways, immune response polarization and apoptosis [25], hence CGD patients are significantly less efficient to induce regulatory response and Tregs development [26] probably associated to TGF-β defect [27], but show elevated IL-17 response and T helper 17 polarization [28]. It has been also described that NADPH oxidase p47 phox-deficient mice develop inflammation and cell proliferation in secondary lymphoid organs, without any evidence of infection, whatsoever, associated with hyperplasia of B cells and T lymphocyte accumulations [29], suggesting a failure in homeostatic mechanisms. Apoptosis play an important in keeping the immune response in balance and it is essential in T cells negative selection and maturation [30, 31]. Within this context, although our study included a limited amount of patients, yet previously studied and published for the genetic defects, our results may contribute to understand further the homeostasis deregulation in cell death apoptosis described in CGD patients.

Fas/FasL have been involved in homeostasis of immune response during activation-induced cell death (AICD) [32], critical to ensure protective immunity and avoiding autoimmunity by deleting autoreactive T cells of [33]. Recent reports suggest that Fas and FasL expression is somehow regulated by ROS production [34]. ROS are quickly generated after TCR acti-

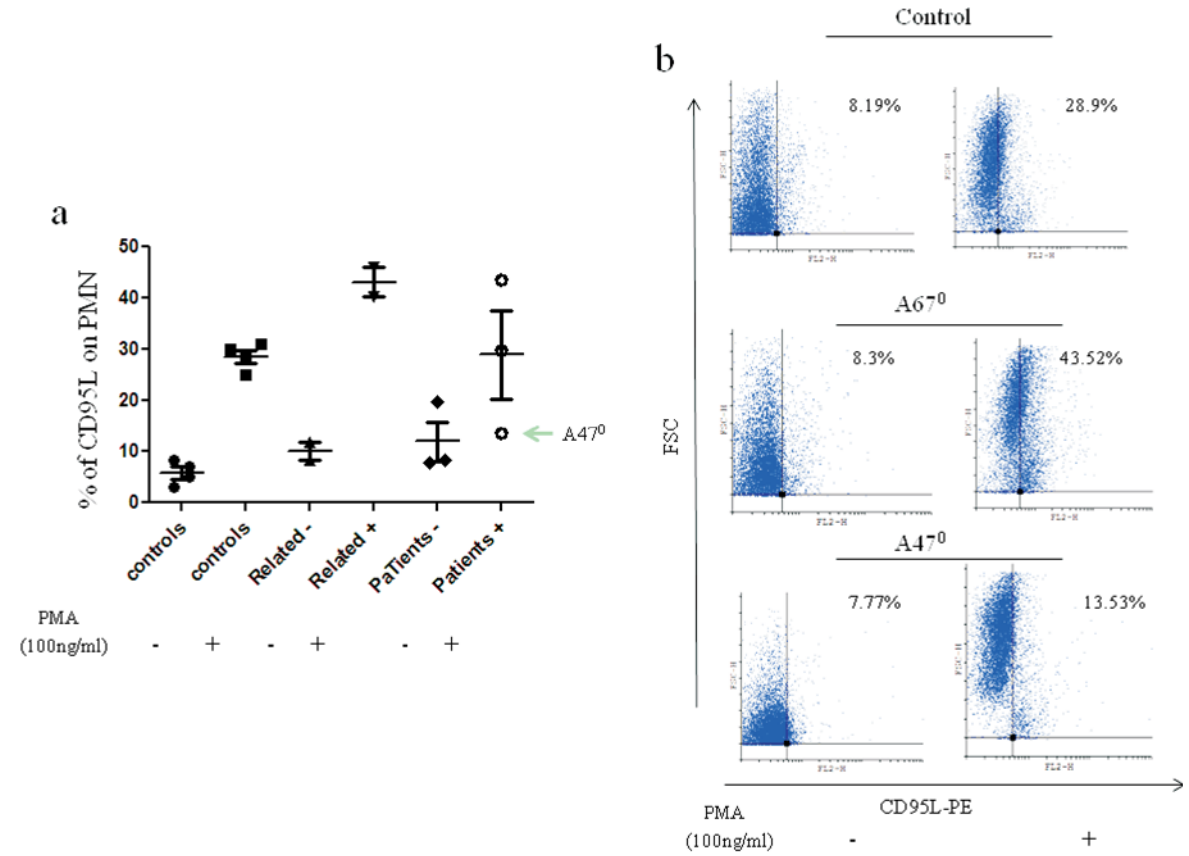

Fig. 4. Expression of CD95L on neutrophils in a group of control subjects, family members and CGD patients. (a) Shows the level of expression of CD95L in neutrophils from the different groups, with and without stimulation, expressed of percentage of expression and, represented in standard deviation (SD). Left panel represents controls, middle panel family member and right panel CGD patients. (b) It is a representative dot plot showing the expression of CD95L in neutrophils from control (top), A67⁰ CGD patient (middle) and A47⁰ CGD patient (bottom).

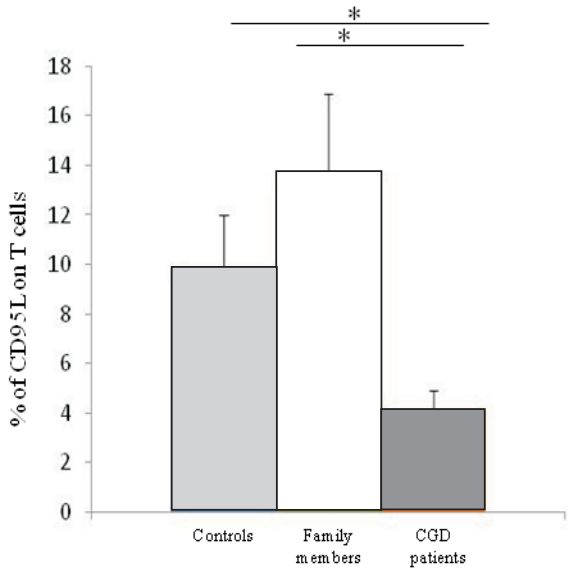

Fig. 5. Expression of CD95L on T lymphocytes in a group of control subjects, family members and CGD patients. (a) Shows the differences (stimulation-unstimulation) of expression of CD95L in T lymphocytes from the different groups, with and without stimulation, expressed of percentage of expression and, represented in standard deviation (SD). Left panel represents controls, middle panel family member and right panel CGD patients, (*$p < 0.01$).

vation, followed by p38 MAPK and JNK activation pathways which contribute to increase FasL expression during AICD [35, 36]. In this work we observed that T lymphocytes from CGD patients express constitutive low levels of Fas and did not increase FasL expression following proper stimulation. On the contrary, neutrophils from CGD patients were capable of upregulating FasL expression following stimulation, except for neutophils from a A470 patient who did not respond accordingly. Consistent with our study, previous analysis showed that expression of Fas on monocytes from CGD patients, was not altered [37]. Fas is a key molecule to trigger apoptosis in sensitive cells [38, 39], but membrane-bound CD95L is essential to induce cytotoxic activity in CD95 positive cells [40].

ROS may contribute to apoptosis by the up-regulation of FasL expression [41] and is a major mechanism responsible for apoptosis of monocytes after phagocytosis of *S. aureus* involves CD95-CD95L interactions [42]. Therefore, low expression of CD95 and CD95L on T cells from CGD patients could delay physiologic cell death and homeostasis, contributing with autoinflammatory manifestations in CGD patients. Further studies are required, with a larger number of patients allowed to extrapolate these results and therefore understand the role played by ROS and NADPH-oxidase components, in CD95/CD95L expression and their involvement in CGD inmunopathogenesis.

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