Impaired number and function of lymphocytes in patients with common variable immunodeficiency: a consequence of activation-induced cell death

Alteração do número e da função de linfócitos em pacientes com imunodeficiência comum variável: uma consequência da morte celular induzida por alteração

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Abstract

Introduction: Common variable immunodeficiency (CVID) is an immunological disorder characterized by defective antibody production. **Objectives:** To study lymphocytes number, surface activation molecules, cell markers, lymphoproliferative response, cytokine production, and cell death. **Methods:** A study was led on thirty four patients with CVID selected from the Division of Clinical Immunology and Allergies of the Faculty of Medicine of São Paulo University (FMUSP), Brazil. Peripheral mononuclear blood cells (PBMC) of CVID patients and healthy individuals were evaluated in regard to the expression of cell surface markers, activation molecules, lymphoproliferative response, cytokine synthesis and apoptosis. **Results:** CVID patients showed decrease in T and B lymphocyte counts, CD25, CD69, CD40L, and CD70 expression, and low synthesis levels of IL-4 and IL-5. Furthermore, their lymphocytes were more susceptible to apoptosis following activation. **Conclusion:** The higher susceptibility to apoptosis following activation may also be responsible for the decrease in the expression of activation molecules and CD40L, in cytokine synthesis, and in levels of circulating T and B cells.

Key words: Apoptosis; Common variable immunodeficiency; human disease; antibodies.

Resumo

Introdução: A imunodeficiência comum variável (CVID) é uma enfermidade imune caracterizada pela produção deficiente de anticorpos. **Objetivo:** Avaliar o número de linfócitos, moléculas de ativação, resposta linfoproliferativa, produção de citocinas e morte celular. **Métodos:** Foram selecionados 34 pacientes com CVID na Divisão de Imunologia Clínica e Alergia da Faculdade de Medicina da Universidade de São Paulo (FMUSP), Brasil. Células mononucleares obtidas a partir de sangue periférico (PBMC) foram isoladas para avaliação de marcadores de superfície celular, moléculas de ativação, resposta linfoproliferativa, quantificação de citocinas e apoptose. **Resultados:** Os pacientes analisados apresentaram diminuição na contagem de linfócitos T e B, expressão de CD25, CD69, CD40L, CD70, e baixa produção de IL-4 e IL-5. Os linfócitos se apresentaram mais suscetíveis à apoptose pós-ativação. **Conclusão:** A maior susceptibilidade à apoptose pós-ativação pode ser responsável pela diminuição na expressão de moléculas de ativação e CD40L, síntese de citocinas e linfócitos T e B circulantes.

Descritores: Apoptose; Imunodeficiência comum variável; enfermidade humana; anticorpos.

Introduction

Common Variable Immunodeficiency (CVID) is one of the most frequent primary immunodeficiencies diagnosed in humans and is characterized by hypogammaglobulinemia and recurrent infections. Some patients may exhibit autoimmune illnesses, chronic bowel infections, granulomatous disease[1] or increased incidence of neoplasms, particularly lymphomas[2]. Patients generally have a normal or reduced number of T and B lymphocytes in peripheral blood. These lymphocytes may also have a reduced number of surface antigen receptors yet still be capable of recognizing an antigen on a first encounter. However, B cells from these patients become incapable of differentiating into plasma cells *in vivo*[3]. The resulting hypogammaglobulinemia maybe explains the recurrence of infections, which are associated with otitis, sinusitis and intestinal infections caused by *Giardia lamblia*, *Campylobacter jejuni* and or *Candida albicans*[4]. Frequent pulmonary infections may lead to bronchiectasis and chronic obstructive pulmonary disease[5]. More than 50% of the patients with CVID will also present defects in cellular immunity components[6]. Thus, part of the impairment of immunoglobulin synthesis may be due to inefficient help provided by T cells or to the presence of regulatory/suppressor cells[7]. The illness was also associated with the inversion of the CD4:CD8 ratio observed in the peripheral blood of some patients. Changes in the expression of CD40L and other activation molecules expressed on the surface of T cells have also been reported, associated with changes in cytokine synthesis[8,9].

In this study, we explored the number of T and B lymphocytes, the expression of surfaces molecule activation, cytokine synthesis, and the susceptibility of CD4 T lymphocytes to apoptosis in CVID patients.

Material and methods

Patients and controls

The study was based on thirty-four patients with positive diagnosis for CVID. They were selected by the Division of Clinical Immunology and Allergies of the Faculty of Medicine of the University of São Paulo (FMUSP), Brazil. The criteria for diagnosis were established by the World Health Organization[10], the Panamerican Group of Immunodeficiency (PAGID) and the European Society of Immunodeficiency (ESID)[11]. Clinical and laboratory criteria for the diagnosis of CVID were established by the Pan-American Group on Immunodeficiencies (PAGID), the European Society for Immunodeficiencies (ESID) and the Clinical Immunology Committee of the International Union of Immunological Societies (IUIS)[12]. All CVID patients received prophylactic treatment with intravenous immunoglobulin therapy (IVIG) at doses of 200-400 mg/kg every three to four weeks. None of the patients presented any opportunistic or metabolic disease or any sign of immunosuppressive therapy at the time of the study. The control group (n=33) comprised healthy individuals who showed no evidence of infectious disease, immunosuppressive treatment, or cancer. All procedures used in this study were approved and supervised by the Committee on Ethics in Research of the USP Faculty of Medicine, in accordance with the guidelines of the International Conference on Harmonization Good Clinical Practice and the Declaration of Helsinki, for the purposes of access to details of clinical records and the publication of patient data for research and informing the scientific community.

PBMC isolation and culture

Peripheral mononuclear blood cells (PBMCs) of CVID patients and healthy individuals were isolated from heparinized venous

blood by discontinuous gradient centrifugation with Isolymph (Gallard-Schlesinger Industries, Inc, Norway) and resuspended in DMEM (Sigma Chemical Co.) culture medium supplemented with gentamicin (40 µg/ml) and 5% pooled AB human serum (Human serum type AB-Biocell Laboratories, INC., Rancho Dominguez, C.A., USA) (complete culture medium), as described in the literature[13]. PBMCs ($5.0x10^5$/well) were cultivated in microculture flat-bottom plates (Costar, Cambridge, MA) at 37 °C and 5% CO_2, in the presence of an appropriate stimulus according to each experiment.

Cell surface markers analysis

To evaluate the expression of cell surface markers on PBMCs *ex vivo* of CVID patients and healthy individuals, the following monoclonal antibodies (mAb) were used: fluorescein isothiocyanate (FITC)-conjugated murine anti-CD4 and anti-CD19 and human anti-CD95; phycoerythrin (PE)-conjugated anti-CD8 and CD95L and CyChrome-conjugated anti-CD3 (Becton-Dickinson, Mountain View, CA). Saturating concentrations of fluorochrome-conjugated murine anti-human mAb were added to the resuspended pellet of $1.0x10^6$ cells for 30-minute incubation at 4 °C, followed by two washes in phosphate-buffered saline (PBS), pH 7.4, containing 0.5% AB positive human serum. The cells marked with their respective fluorochrome-conjugated mABs were analyzed on a FACS-Scan (Becton-Dickinson, Sunnyvale, CA) flow cytometer.

Kinetic study of CD25, CD69, CD70, and CD40L

PBMCs ($5.0x10^5$/well) were cultivated in microculture flat-bottom plates (Costar, Cambridge, MA) at 37 °C and 5% CO_2, in the presence of complete culture medium with phytohemagglutinin (PHA) (10 µg/ml), during 24, 48, 72, and 96 hours to evaluate the expression of CD25, CD69, and CD70. The expression of

CD40L was evaluated by culture of PBMCs only during the 72-hour period using a protocol described in the literature[14].

To evaluate the expression of activation molecules on the surface of PBMCs of CVID patients and healthy individuals, the mAb used in this study were fluorescein isothiocyanate (FITC)-conjugated anti-human CD25, CD40L, or CD69; phycoerythrin (PE)-conjugated anti-CD4, CD70, and CyChrome-conjugated anti-CD3 (Becton-Dickinson, Mountain View, CA). Saturating concentrations of the fluorochrome-conjugated murine anti-human mAb were added to a resuspended pellet of $0.5-1.0x10^6$ cells for 30-minutes incubation at 4 °C, followed by two washes with phosphate-buffered saline (PBS), pH 7.4, containing 0.5% AB positive human serum. The cells were analyzed on a FACS-Scan (Becton-Dickinson, Sunnyvale, CA) flow cytometer.

Lymphoproliferative response

Single-cell suspensions were prepared in DMEM from PBMCs of CVID patients and healthy controls. PBMC viability was verified by trypan blue exclusion of dead cells. Cells were resuspended in DMEM-C and were distributed at a concentration of $1.0x10^5$ cells/well in a 200 µl volume of complete culture medium, in flat-bottom, 96-well microtiter plates (Becton-Dickinson, Lincoln Park, NJ). Cells were stimulated by PHA (10 µg/ml) or Con-A (5 µg/ml), and all cell combinations were set up in triplicate. Plates were incubated for 48 hours at 37 °C and 10% CO_2 and pulsed for the final 18 hours with 1 µCi of methyl-[3H] thymidine ([3H] TdR). Radioactively-labeled material was automatically harvested and counted in a β–plate scanner (Pharmacia, France). The response was expressed by calculating the proliferation index from [3H] TdR incorporated in cultures divided by the values obtained for the PBMCs without [3H] TdR incorporation. The threshold value for positivity was set up at 20.

Cytokine synthesis

Cytokines were quantified by enzyme-linked immunosorbent assay (ELISA). The antibody-matched pair and the respective standard were purchased from Pharmingen (San Diego, CA) and used following the manufacturer's recommendations. The detection limit of the different cytokines was 7.8 pg/ml for IL-2, 15 ng/ml for IL-4, 31.2 pg/ml for IL-5, and 0.005 ng/ml for IFN-γ. To evaluate IL-2, IL-4, IL-5, and IFN-γ, PBMCs were cultured with PHA (10μg/ml). Prior kinetic studies indicated that IL-2 secretion predominantly occurs during the first 12 hours of culture; and IL-4, IL-5, and INF-γ during the initial 48 hours.

Evaluation of apoptosis

Apoptosis was assayed by the binding of annexin V protein to phosphatidylserine residues, a phospolipid of the inner side of the membrane that moves to the outer side at the initial stage of the apoptotic process, using a previously described protocol[15] modified by Vermes et al.[16]. PBMCs from controls and CVID patients were cultured in triplicate during 72 hour with PHA (10 μg/ml) and gently harvested from the wells at the indicated times, washed twice with PBS containing 0.5% AB positive human serum, and then resuspended in PBS at 1.0x10^6 cells/ml. Levels of cell suspension were stained with fluorochrome-conjugated murine mAb CyChrome-conjugated human anti-CD3 and PE-conjugated human anti-CD4 (Becton-Dickinson, Mountain View, CA), incubated for 30 min at 4 °C, and washed twice in PBS containing 0.5% AB positive human serum. The PBMCs were then washed twice with cold PBS, and resuspended in binding buffer (10 mM Hepes/NaOH, pH 7.4, 140 mM NaCl, and 2.5 mM CaCl$_2$) at a concentration of 1,0x10^6 cells/ml.

The cell suspension was transferred to a 5 ml cytometer tube, and 5 μl of annexin V-FITC (BD Pharmingen, Los Angeles, CA) were added. Cells were gently vortexed and incubated in the dark for 15 minutes at room temperature, followed by the addition of 400 μl of binding buffer for analysis in a FACS-Scan (Becton-Dickinson, Sunnyvale, CA) flow cytometer. The results were expressed as a percentage of apoptotic cells per 10,000 counted cells.

Statistical analysis

Since percentages were not normally distributed, the Wilcoxon signed-rank paired test was used to compare the apoptosis score (%) in the different culture conditions among patients or controls; cell markers of PBMCs *ex vivo;* lymphoproliferative response; and cytokine production. The Mann-Whitney test and the Kruskal-Wallis test with Dunn's post-test were used additionally to compare nonpaired continuous data between two and three data sets, respectively. Differences were considered significant for *P*<0.05.

Results

Characterization of B-cell and T-cell subpopulations

In the CVID group, the majority of patients showed a statistically significant decrease in the total number of CD3, CD3CD4 T lymphocytes and CD19 B lymphocytes (Fig. 1A, 1B, 1D) when compared to the control group. Results of T and B cell phenotype demonstrate a statistical significant *P* values for comparisons between groups. No statistical difference was observed in the comparison of the number of T CD8 lymphocytes (CD3CD8) in both groups (Figure 1C). All results were presented as percentages with respect to the total number of PMBCs evaluated by FACS-Scan flow cytometry.

Figure 2 describes the percentage of PBMCs marked with CD95 and CD95L. In Figure 2A, the expression of the percentage of CD95 and CD95L on the surface of CVID PBMCs was higher when compared with that of groups of healthy individuals, showing a statistical difference. Figure 2B demonstrate a dot plot of PMBCs

of healthy controls with double marker CD95/CD95L in the upper right quadrant (3.66%), and Figure 2C a dot plot of PBMCs of CVID patients with double marker CD95/CD95L in upper right quadrant (6.90%).

Kinetic study of CD25, CD69, CD70 and CD40L

Expression of CD25 and CD69 were evaluated on PBMCs marked with murine mAB anti-CD3 and anti-CD4 and human anti-CD70 after activation with PHA during 24, 48, 72 and 96 hours. The kinetic study of theses molecules in these experimental times was necessary be-

cause their expression occurred in the first 24-48 hours, and decrease gradually after 72-96 hours. There were a lower percentage of positive CD25 (Figure 3A), CD69 (Figure 3B) and CD70 (Figure 3C) cells in the CVID group at all experimental times. These changes were statistical significant when compared with healthy patients. The gating strategy utilized for this study is represented in Figure 3.

Since CD40L is expressed by activated T CD4 lymphocytes and plays an essential role in the activation and in the immunoglobulin heavy chain isotype exchange of B cells, we next examined the expression of CD40L on the surface of T CD3CD4 lymphocytes after 72 hours of cul-
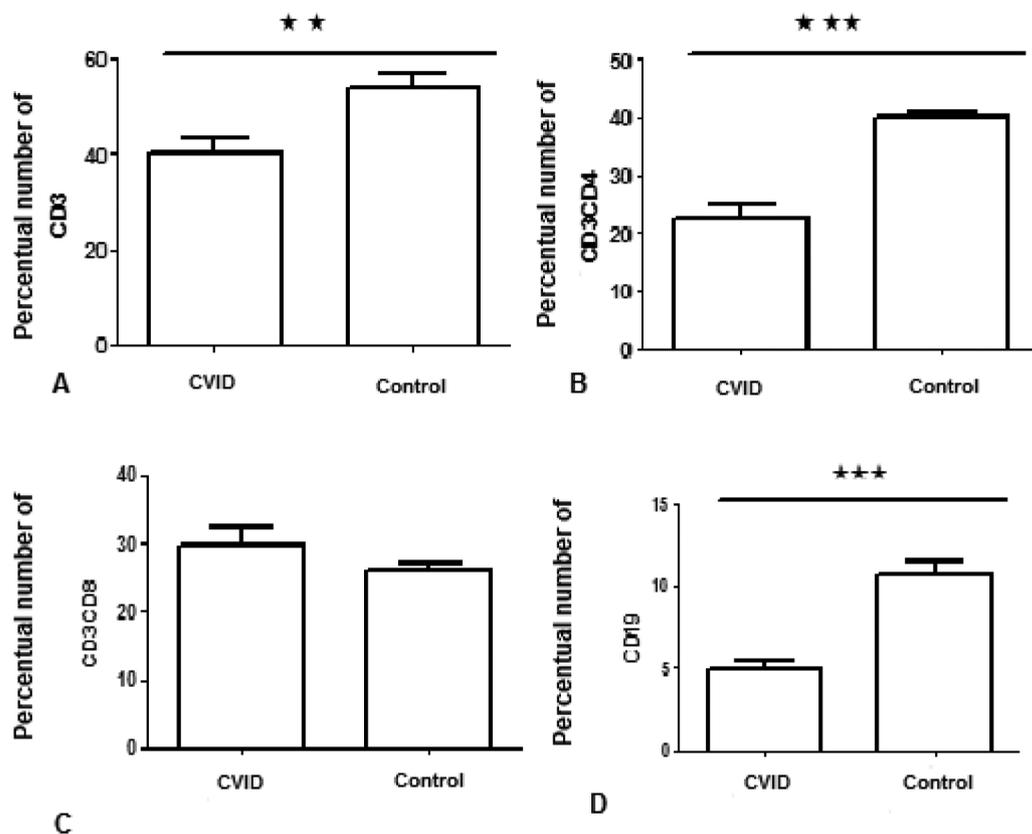

Figure 1: Expression of CD3, CD3CD4, CD3CD8 and CD19 on PBMC subsets. PBMCs were separated by centrifugation with Isolymph, marked with mAb conjugated with fluorochromes, and analyzed by FACS-Scan flow cytometry. Values of cells were expressed in percentages. The comparison between CVID patient's cells and those of control patients showed a statistically significantly decrease of T CD3, CD3CD4, CD19 lymphocytes (Mann-Whitney test, ** p<0.005; *** p<0.0001)
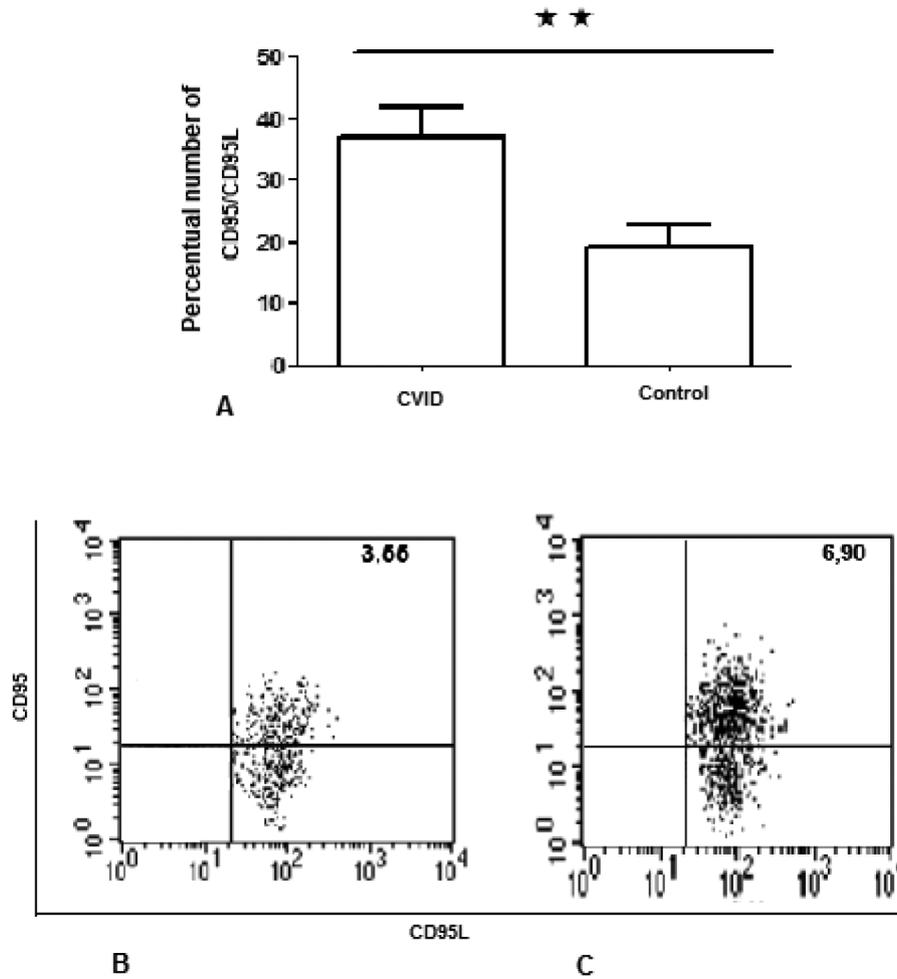
Figure 2: Expression of CD95 and CD95L on PBMC subsets. PBMCs were separated by centrifugation with Isolymph, marked with mAb conjugated with fluorochromes, and analyzed by FACS-Scan flow cytometry. Values were expressed in percentages. The comparison between CVID patients' cells and those of control patients demonstrated a statistically significant reduction of CD95 and CD95L (Mann-Whitney test, ** p<0.005) (2A). Dot plot representation of control patients (B) and CVID patients (C)

ture with PHA, because the expression of this molecule is observed only after this time. We found that CD40L expression was decreased in the CVID group (Figure 4D).

Lymphoproliferative response

Because cellular activation and proliferation is followed by apoptosis, we sought to measure the rate of proliferative responses in parallel cultures.

The response was expressed by calculating the proliferation index from [^{3}H] TdR incorporated in stimulated PBMCs, divided by the value obtained for non-stimulated PBMCs that incorporated [^{3}H] TdR. The stimulation of PBMCs with PHA or Con-A did not demonstrate a statistical difference between CVID patients and healthy controls (Figure 5), suggesting the maintenance of the proliferative response.

Decrease of IL-4 and IL-5 synthesis by PBMCs

Human IL-2, IL-4, IL-5 and IFN-γ production by PBMCs were examined in CVID patients and healthy controls after PHA stimulation. In this study, we found that IL-4 and IL-5 secretion is
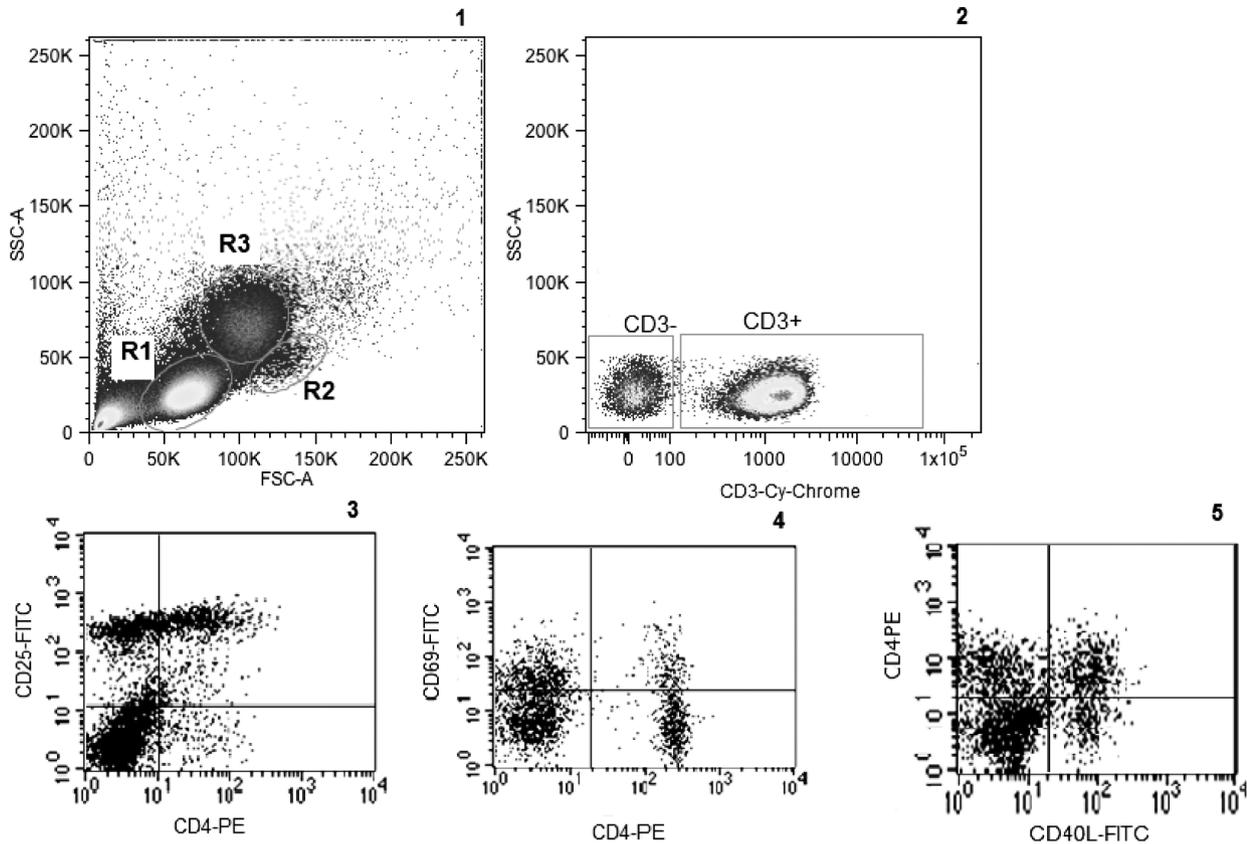
Figure 3: Strategy used for marking and triple analysis with FACS-Scan. In 1, a densit plot of PBMCs, where R1 represent the population of lymphocytes. In 2, a dot plot is created of R1, using SSC-A and CD3-Cy-Chrome parameters for visualizing T CD3⁺ cells. By gating CD3⁺ cells, new dot plots are created, with double marking for CD25-FITC and CD4-PE (3), CD69-FITC and CD4-PE, and CD40L-FITC and CD4-PE. The visualization of CD70 cells was made based on R1

lower in activated PBMCs of CVID patients when compared to healthy controls. The amounts of IL-2 and IFN-γ (Figure 6) were similar to healthy patients under the same conditions.

Apoptosis of T CD4 lymphocytes

Previous results of kinetics studies with CVID patients and healthy patients showed that the percentages of cells at early apoptosis stage (Annexin+/PI-) increased from day two to three in PHA-stimulated cultures, (data not show). In patients with CVID, PBMCs stimulated with PHA during 72 hours, and subsequently marked with Annexin-V showed statistically significant higher levels of apoptosis when compared with

healthy patients (Figure 7A). Figure 7B illustrate a dot plot of PBMC by health control marked with murine mAb anti-CD4 and annexin-V (upper right square, with 7.59% of positive cells for theses markers), and Figure 7C illustrate a dot plot of PBMCs by CVID patients marked with mAb anti-CD4 and annexin-V (upper right square, with 19.99% of positive cells to theses markers).

Discussion

Our results demonstrate a significant increase in apoptosis of CD4 cells of CVID patients upon polyclonal stimulus, associated
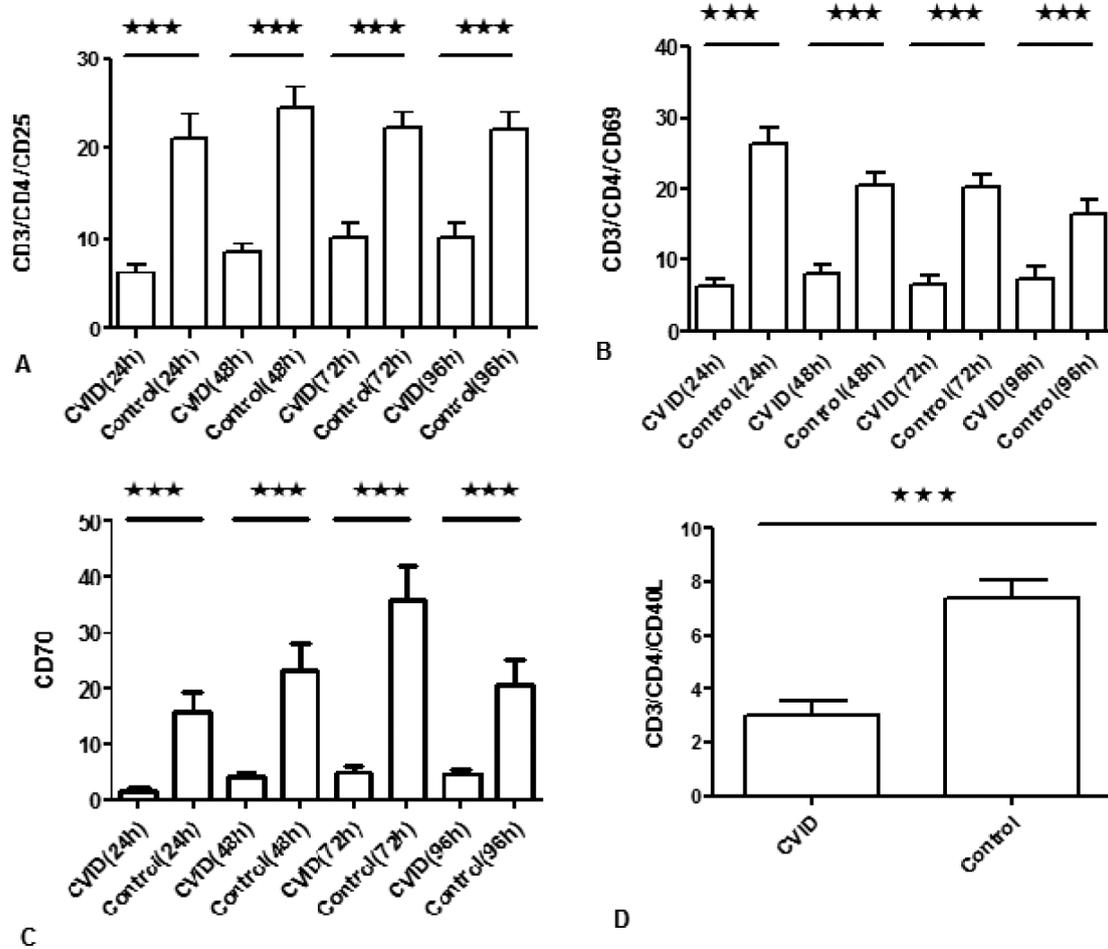
Figure 4: Kinetic study of CD25, CD69, CD70 and CD40L expression. The expression of CD25 (4A), CD69 (4B) and CD70 (4C), was statistically lower in the CVID group than in controls at all experimental times (Kruskal-Wallis test with Dunn's posttest, *** p<0.0001). Polyclonal stimulation significantly decreased the percentage of CD40L in CVID patient's CD3CD4 cells (4D) (Mann-Whitney test, *** p<0.0001). Values are expressed in percentages

with a decrease in expression of markers of cellular activation (CD25, CD40L, CD69, and CD70). Other effects observed were a decrease in cytokines IL-4 and IL-5 synthesis, T and B lymphopenia, and an increase in CD95 and CD95L expression in T CD4 cells. The potential physiologic relevance these results suggest a role for apoptosis on CD4 cells, and that the reduction in the number of these cells may play a critical role in the regulation of effective immune adaptive response and immunoglobulin production.

High levels of IL-2 were found in supernatants of CVID patient's PBMC culture, excluding cell death by negligence (associated with IL-2 deprivation)[17]. Signaling through CD95 and CD95L has been demonstrated to induce apoptosis of transformed cell lines and chronically activated T cell clones[18,19].

The increased susceptibility to apoptosis and enhanced CD95 expression on different subpopulations are considered factors that contribute to CVID pathogenesis[20]. Our results showed the increased CD95 and CD95L expression *ex vivo* on PBMCs of CVID patients, correlated with reduction in the number of T and B lymphocytes in peripheral blood, IL-2 synthesis, normal lymphoproliferative response, and higher levels of T lymphocyte apoptosis after polyclonal stimulation with PHA. The results described by our
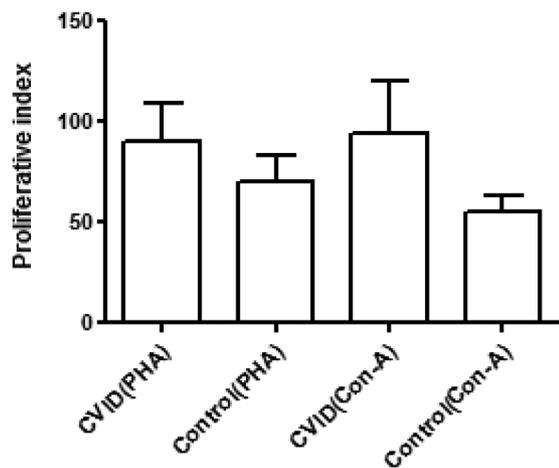
Figure 5: Lymphoproliferative response to PHA and Con-A. PBMCs were separated by centrifugation with Isolymph and cultivated in the presence of PHA or Con-A. The response was expressed by the proliferation index calculated from [^{3}H] TdR incorporated in cultures with non-pulsed PBMCs. Threshold value for positivity was set up at 20. Proliferative response to PHA and Con-A of PBMCs of both groups did not show a statistically significant difference

research suggest that stimulated T lymphocytes die due to classical activation induced cell death (AICD). Iglesias et al. showed increased spontaneous apoptosis and CD95 expression on the CD4 and CD45RA (activation marker) subsets from lymphopenic CVID patients. The CVID patients presented a profound reduction in absolute counts, mainly affecting the T CD4 CD45RA subpopulation and the authors suggest that apoptosis could be one of the mechanisms implicated in the lymphopenia[21].

This phenomenon is a consequence of persistent activation of lymphocytes with disseminated antigens presented by antigen-presenting cells (APC) in CVID patients with acute, chronic, or recurrent infections in the form of pneumonia, sinusitis, and otitis. Activation of T cells stimulates synthesis and expression of CD95 on the surface of these cells, and persistent activation stimulates *de novo* synthesis of CD95L. The interaction of these two molecules results in

death of persistently activated T cells. Activated T cells have been implicated in the apoptosis by interaction of CD95 and CD95L, a process called AICD, a homeostasis mechanism that limits the clonal expansion of autoreactive T cells and regulates peripheral tolerance. The increased susceptibility to apoptosis and enhanced CD95 expression on different subpopulations have been suggested as events that could contribute to several diseases, e.g. systemic lupus erythematosus[22], HIV infection[23] and polymicrobial sepsis[24].

We extended the studies evaluating the expression of T cell surface molecules relevant in activation (CD25, CD69) and T-B interaction (CD40L, CD70). The results showed a decrease in CD25, CD69 and CD70 at all experimental times, associated with decreased expression of CD40L on activated T cells from CVID patients. CD69 expression during the early stages of cellular activation plays an important role in conferring cognate interaction between cells of the immune system. The activation, multiplication, and synthesis of immunoglobulins by B lymphocytes dependent on T lymphocytes requires the synthesis of cytokines by T CD4 lymphocytes and physical contact between activated T lymphocytes (CD40L) and B lymphocytes (CD40)[25]. CD40/CD40L interaction is essential to activation, clonal expansion, immunoglobulin class switching, and V gene hypermutation on B cells[26]. CD70/CD27 interaction plays a minor role in B cell proliferation, but it's very necessary for the formation of immunoglobulin-producing cells[27]. Our results were obtained by polyclonal stimuli during four days, and we observed lower expression of different cell markers in T CD4 lymphocytes. Our findings are supported by the results presented by Lin et al.[28], who suggest that the preactivated status *in vivo* of PBMCs in children as a consequence of recurring infections can lead to an increase in apoptosis, augmented by the administration of Il-2 and Il-15.

Other studies focused T cells from CVID patients, showed abnormalities of mitogen-induced lymphokine gene expression by decreased expression of IL-2, IL-4, IL-5 and
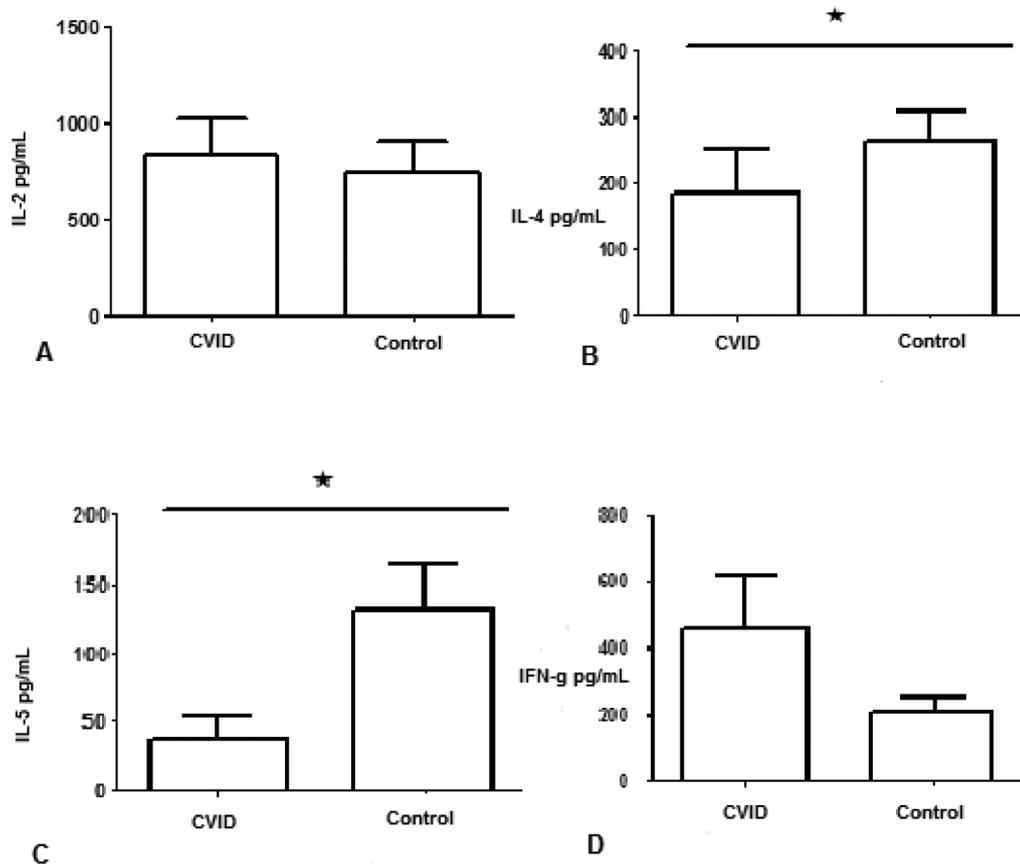
Figure 6: Cytokines levels secreted by PBMCs. The levels of IL-4 and IL-5 produced by PBMCs of CVID patients were significantly lower than those in the healthy control group (Mann-Whitney test, ∗ $p<0.05$), whereas the levels of production of IL-2 and IFN-γ did not show a statistically significant difference

IFN-γ[29,30], and our results demonstrated impaired production of IL-4 and IL-5 by PBMCs, but not IL-2 or IFN-γ. Since IL-2 is essential to AICD, this reinforces our hypothesis that T lymphocytes of CVID patients presented an increase in apoptosis, which leads to a decrease in expression of activation molecules(CD25, CD69), interaction between T and B (CD40L e CD70), and cytokine synthesis (IL-4, IL-5), with inadequate signaling for B lymphocytes to produce immunoglobulins.

Conclusion

Our data support that the CVID patients in our study showed evidence of T and B lympho-

phenia, a decrease in the expression of activation molecules (CD25, CD69, CD40L and CD70) on the surface of PBMCs after *in vitro* polyclonal activation, and decreased synthesis of IL-4 and IL-5 in comparison with normal individuals. The same patients showed higher expression of CD95 and CD95L on peripheral blood mononuclear cells (PBMCs) and increased susceptibility to apoptosis following activation, which may also be responsible for the decrease in the expression of activation markers, in Th2 cytokine synthesis, and in the decreased number of T and B circulating cells in peripheral blood. These phenomena, taken together, promote the development and persistence of hypogammaglobulinemia.
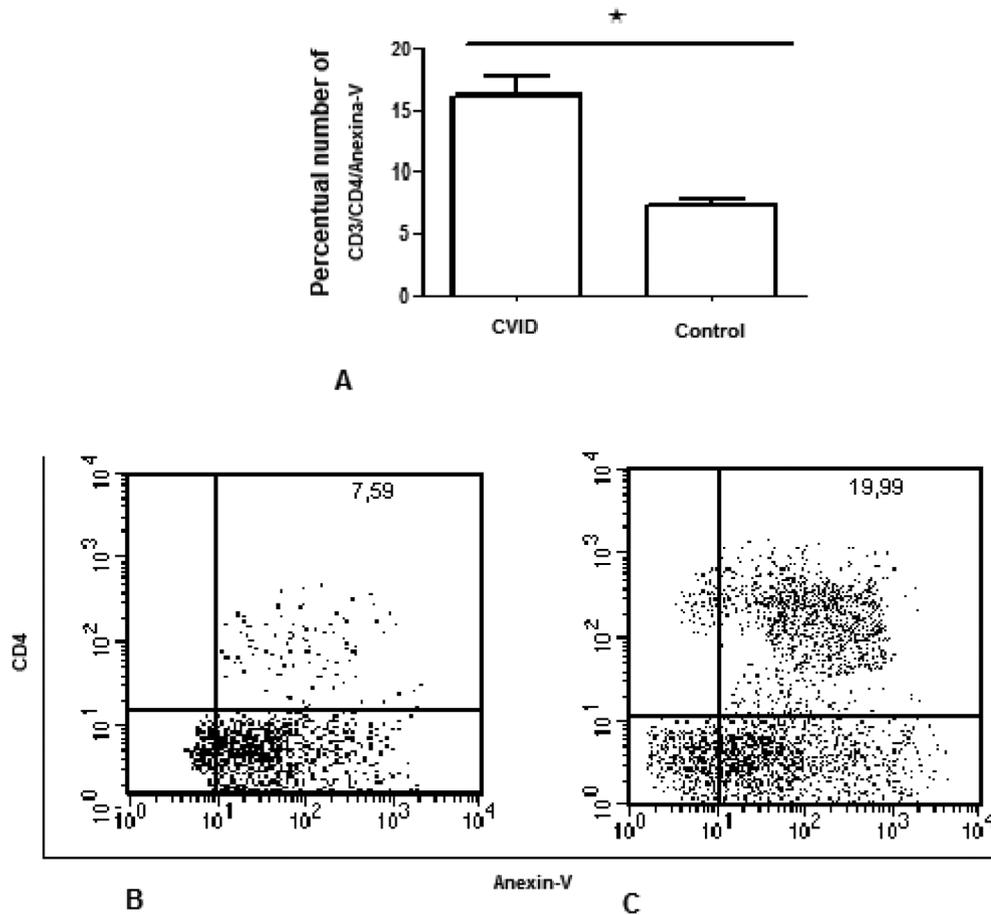
Figure 7: Apoptosis evaluation. PBMCs were stimulated with PHA during 72 hours and subsequently marked with mAb anti-CD3, anti-CD4 and Annexin-V. There was an increased apoptosis rate of T CD3CD4 lymphocytes from patients with CVID compared to healthy patients (Mann-Whitney test, ∗ p<0.05) (7A). Dot plot representation of CD4/Annexin-V cells (7.59%; B) of a healthy individual and of a CVID patient (19.99%; 7C)

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