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Cell apoptosis induced by hookworm antigens: a strategy of immunomodulation

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1. ABSTRACT

While several mechanisms of immunoregulation have been demonstrated for hookworm and other neglected tropical infections, the influence of apoptosis in the immunomodulation of hookworm infection is still poorly understood. In this study, we demonstrate the cytotoxic and pro-apoptotic activity of hookworm antigens in Jurkat T cells, mesenteric lymph nodes lymphocytes of healthy and hookworm-infected hamsters and during human natural infection. Our results showed that *in vitro* stimulation of Jurkat T cells with antigens induces a significant decrease of cell viability leading to a relevant increase of apoptotic cells. Similar results were also observed in experimental conditions, for both healthy and hookworm-infected hamsters` lymphocytes. Flow cytometric analysis demonstrated that hookworm-infected patients presented a significant increase of $CD4^+$, $CD8^+$, and $CD19^+$ lymphocytes in early and/or late apoptosis when compared with non-infected individuals. The downmodulation of TNF receptors, as well as the up-regulation of the pro-apoptotic genes belonging to the BCL-2 and P53 families, suggest that hookworm antigens induced apoptosis by an intrinsic mitochondrial pathway, acting as a sophisticated strategy to safeguard parasite long-term survival in their hosts.

2. INTRODUCTION

Human hookworm infection is a chronic parasitic disease caused by the blood-feeding nematodes *Ancylostoma* sp. and *Necator americanus*, that afflicts up to 740 million people worldwide (1). Besides its global importance, relatively little is known about the mechanisms by which hookworms manage to survive and indeed thrive, living in equilibrium with host immunity. Once exposed to the gastrointestinal extracellular niche, in confrontation with a potentially hostile environment, their persistent and chronic lifestyle is a persuasive evidence for their profound ability to modulate the hosts' immune response.

Several immunoepidemiological studies have established that hookworms can evade or downmodulate host´s immunity by myriad of excreted/secreted products (2, 3), which could be even applied for the treatment of human inflammatory diseases (4). Indeed, human hookworm infection has been classically related to a profound ablation of parasite specific T cell proliferative responses (''hyporesponsiveness'') (5). The mechanisms underlying T cell hyporesponsiveness during helminth infections have been associated with several factors such as regulatory cytokines (e.g., IL-10) (6, 7) secretion of IFN-γ by NK cells (8), modulation by regulatory T cells (9,10), impaired dendritic cell differentiation (5) and reduced expression of Toll-like receptors (11).

Cell apoptosis induced by parasites was also implicated as a mechanism of host immunomodulation potentiating parasite survival. In fact, the importance of apoptosis in the modulation of immune responses has already been very well established for protozoa infections. T and B lymphocytes or antigen presenting cells (APCs) apoptosis induced by *Leishmania donovani* (12), *Toxoplasma gondii* (13, 14), *Plasmodium ssp* (15, 16); *Trypanosoma cruzi* (17) and *Entamoeba histolytica* (18, 19) has been demonstrated as an efficient mechanism of host immune response evasion by these organisms (20). Moreover, T cells apoptosis has been associated with the control of pathology in a variety of helminth diseases, as described for both human and experimental schistosomiasis (21) and lymphatic filariasis (22). For hookworm infection, studies *in vitro*, showed that *Necator americanus* excreted/secreted products are able to induce apoptosis in a human immortalized line of T lymphocyte (Jurkat T cells) (23). However, the role of apoptosis into the immunomodulation of chronic *N. americanus*-infected patients and controlled experimental hookworm infection, as well as the apoptotic pathway induced by hookworm, have not been fully elucidated yet.

Thus, the aim of our study was to investigate the potential cytotoxic and pro-apoptotic effect of human hookworm crude extract (HEX) and excreted/secreted (ES) products in Jurkat T cells, mesenteric lymph nodes (MLN) lymphocytes of hookworm infected- and non-infected hamsters and T and B lymphocytes from healthy donors and *N. americanus*-infected patients. Furthermore, we investigated several apoptotic pathways, which might be induced by hookworm ES and HEX products, through the analysis of expression of multiple apoptotic genes. In the present study, we demonstrate the cytotoxic and pro-apoptotic activity of the hookworm antigens in *in vitro* and experimental conditions and during human natural infection. The downmodulation of TNF receptor family members (death receptors), as well as the up-regulation of the pro-apoptotic genes belonging to the BCL-2 and P53 families, observed by qPCR analysis, suggest that hookworm antigens induce apoptosis by an intrinsic mitochondrial pathway. Taken together, our results suggest that apoptosis induced by hookworms may contribute to downmodulate host's immunity and allow for protracted survival of this parasite in their hosts.

3. MATERIALS AND METHODS

3.1. Jurkat T cell culture

Human immortalized line of T lymphocytes (Jurkat cell, clone E6-1, ATCC, USA) was a kindly given by Dr. Gustavo Amarante-Mendes (São Paulo University, Brazil). This lineage was maintained in the logarithmic phase of growth in RPMI 1640 supplemented with 100 U/mL penicillin and 100ug/mL treptomycin (GIBCO BRL, Grand Island, NY) without fungizon, enriched with 2 mM of L-glutamine and 10% of fetal bovine serum. All cultures were maintained at 37°C in a humidified incubator with 5% CO_2. The media was changed twice weekly and regularly examined.

3.2. Parasites and experimental infection

Ancylostoma ceylanicum life cycle was maintained in Syrian golden outbred hamsters from Federal University of Minas Gerais. For all experiments, ten hamsters, male, 4-6 weeks-old, were infected with 100 third stage (L3) *A. ceylanicum* larvae by oral gavage. The parasite burden was evaluated by quantitative egg counts (McMaster technique), from 15 days post-infection up to 25 days p.i. Hamsters were then euthanized, and MLN were collected. Adult worms adhered to intestinal mucosa were recovered for hookworm antigen preparation. Other ten non-infected hamsters were used as controls. The maintenance and use of animals` samples were carried out in strict accordance with the recommendations from Brazilian College of Animal Experimentation (COBEA) guidelines. The protocol was approved by the Ethics Committee in Animal Experimentation (CETEA) of the Universidade Federal de Minas Gerais (Protocol# 66/08). All efforts were made to minimize suffering.

3.3. Isolation of mesenteric lymph nodes cells of hamsters

The mesenteric lymph nodes were harvested from non-infected and hookworm-infected hamsters with 25 days post-infection and transferred immediately to RPMI 1640 culture medium (Sigma, USA), supplemented with 10% heat-inactivated foetal calf serum and 1.6% L-glutamine, 3% antibioticantimycotic (Invitrogen, USA). In order to isolate the cells, the organs were mechanically macerated and the maceration product purified by a 70µm cell strainer (BD Falcon, USA). After that, the MLN cells were washed and cultured in a flat-bottomed 96-well plate

for cellular immune response evaluation to experimental hookworm infection.

3.4. Isolation and preparation of hookworm excretory–secretory (ES) products and adult crude extract (HEX)

For preparation of excretory–secretory (ES) products, worms were manually removed from the intestines of euthanized *Ancylostoma ceylanicum*-infected hamsters, washed in phosphate-buffered saline (PBS) several times, and then cultured overnight in RPMI 1640 containing 100 U/ml penicillin G sodium, 100 µg/ml streptomycin sulfate, and 0.25 µg/ml amphotericin B (all reagents from Sigma-Aldrich, St. Louis, MO) at 37°C with 5% CO_2 in a humidified incubator. The ES products were concentrated using centrifugal filter units with a 3 kDa-cutoff membrane (Centricon, Millipore, Bedford, MA) and were separated from debris by centrifugation (1400 rpm for 3 minutes). Supernatants were collected and stored at -80°C until further use.

Adult worm crude extract (HEX) was prepared by direct maceration of parasites using a tissue grinder and a cell disruptor (Sonifier Cell Disruptor, Branson Sonic Power Co., Danbury, CT, USA). The procedure was repeated five times, with 1-min intervals between disruptions. All of the antigen preparations used in this experiment were passed through a 0.22-µm low-protein binding syringe filter (Millipore), and the resulting protein concentration was determined using a BCA protein assay kit (Pierce). Both hookworm antigens were tested negative for endotoxin content by the Limulus lysate assay (sensitivity of 0.06 U/ml; Cambrex, USA), and stored in aliquots at -80 °C until needed.

3.5. Study population

The study was conducted in endemic areas for *N. americanus* in the Northeast Minas Gerais State, Brazil. Thirteen volunteers were recruited over the course of two months (Table 1). These volunteers reside in areas of moderate *N. americanus* transmission and presented with low to moderate (up to 642 epg) intensity of *Necator* infection. Individuals were selected on the basis of not having any other helminth infection (mono-infected) and presenting no other medical condition. The presence of *Necator* infection was determined by formalin–ether sedimentation and, if positive, two more stool samples were analyzed by the Kato–Katz fecal thick-smear technique and parasite load was expressed as eggs per gram of feces (epg) (24). Seventeen hookworm-naive individuals were enrolled as healthy non-infected individuals from Belo Horizonte, Minas Gerais State, Brazil, where no transmission occurs. None of these individuals had a history of *Necator* infection and all presented with egg-negative stool and no specific antibodies to *Necator* crude antigen extracts. Furthermore, the nutritional status of non-infected volunteers (controls) was similar to those presented by hookworm-infected individuals as determined by anthropometric measurements. The nutritional status of adults was determined using the absolute body mass index and classified as eutrophic (18.5–24.9 kg/m2), underweight (<18.5 kg/m2) or overweight (≥25 kg/m2) (25).

Approximately 24 mL of blood from *N. americanus*-infected patients and healthy donors was collected in heparinized tubes and PBMCs separated by Ficoll-Hypaque reagents (Sigma, USA) for further use in studies of proliferative cellular response. Haematological parameters of these individuals were evaluated by an automated haematology instrument (Coulter, USA), using whole blood collected in 5 mL tubes containing EDTA. For the use of human`s samples, this study was approved by the Ethical Committee on Research of Universidade Federal de Minas Gerais (COEP) (Protocol #ETIC0449.0.203.000-09). Written consent was obtained from all individuals prior to enrollment in this study.

3.6. Cell viability and cell proliferation assay

MTT assay was carried out to assess the cell viability in Jurkat T cells and MLN cells from non-infected and hookworm-infected hamsters, as previously described (26). The proliferative response of PBMCs to hookworm antigens was also determined after stimulation with different concentrations of ES and HEX antigens. Briefly, aliquots of 180 µL cell suspension in RPMI 1640 medium supplemented with 10% heat-inactivated foetal calf serum or 5% human sera, 1.6% L-glutamine (Sigma, USA), 3% antibiotic-antimycotic (Invitrogen, USA), were cultured in a flat-bottomed 96-well plate, with 20 µL of ES and HEX antigens at 3.1, 6.2, 12.5, 25, 50 and 100 µg/mL, for 48 hours in an atmosphere of 5% CO_2 at 37°C. This step was followed by the addition of 20 µL of MTT solution (5 mg/mL) and incubation of the plates for 4 hours. After incubation the supernatant was carefully removed from the wells, followed by the addition of 100 µL DMSO with thorough mixing. Later, the mean optical density at 570 nm was determined on an ELISA reader (Molecular Devices, USA). Cell viability was expressed as the percentage of control absorbance obtained in untreated cells after subtracting the absorbance from appropriate blanks. Proliferation inhibition index of non-stimulated cells was used as reference value (0% of inhibition) and percentages of ES- and HEX-stimulated cells were calculated from this value.

3.7. DNA labeling and flow cytometry analysis

DNA fragmentation as a measurement of the level of apoptosis was quantified by cell cycle analysis of total DNA content as described by Nicoletti et al. (27) with slight modifications. A total of 2 x 10^5 cells were resuspended in 0.3 mL of hypotonic fluorochrome solution (HFS) (50 µg/mL propidium iodide and 0.1% Triton X-100 in 0.1% sodium citrate) 48-h after stimulation with ES and HEX antigens at 100 µg/mL. After incubating for 4 hours with HFS at 4°C in the dark, the fluorescence of individual nuclei was measured using a FACS flow cytometer (Becton–Dickinson, Mountain View, CA). All experiments were performed in triplicates and repeated three times.

3.8. Annexin V and Propidium iodide cell staining

The whole blood of *Necator*-infected patients and healthy donors was collected in vacutainer tubes containing heparin (Becton Dickinson, USA) which were diluted 1:10 proportion in culture medium supplemented with 5% of AB Rh-positive heat inactivated normal human serum and 1.6%

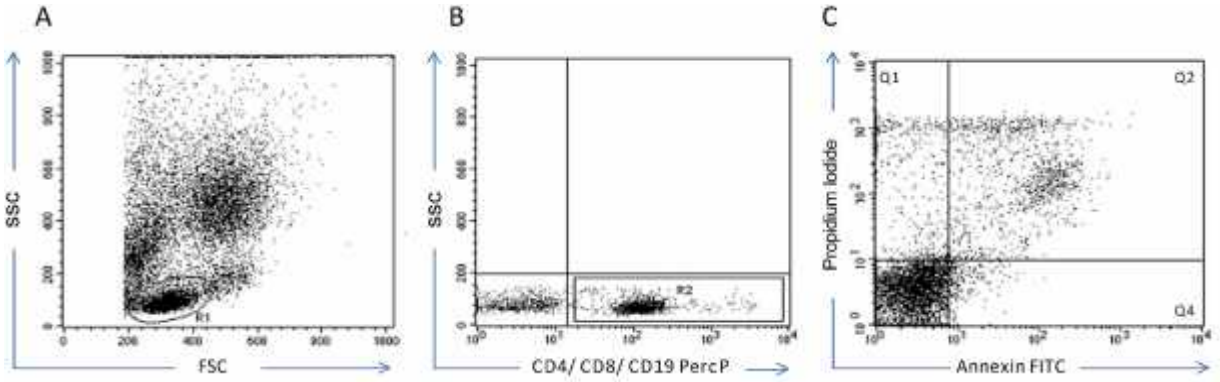

Figure 1. Flow cytometric analysis of peripheral blood lymphocytes subpopulation stained with annexin V/FITC and Propidium Iodide. (A) Cellular profile of lymphocyte population in side scatter (SSC) x forward scatter (FSC) distribution. (B) Cellular profile of lymphocytes, selected by the gate R1, positive for CD4, CD8 and CD19 PercP surface markers. (C) Apoptotic profile of $CD4^+$, $CD8^+$ and $CD19^+$ lymphocytes, selected in the gate R2, stained with Annexin V/FITC and Propidium iodide (PI). Viable cells in Q3 (Annexin V^- and PI^-), early cell apoptosis in Q4 (Annexin V^+ and PI^-), late cell apoptosis in Q2 (Annexin V^+ and PI^+) and death cells in Q1 (Annexin V^- and PI^+).

L-glutamine (Sigma, USA), 3% antibiotic-antimycotic (Invitrogen, USA) in 24 wells plate. After that, we evaluated the apoptotic profile of $CD4^+$, $CD8^+$ and $CD19^+$ lymphocyte population from both groups by Annexin V/FITC and propidium iodide (PI) cell staining (BD Biosciences, USA) in two different moments; initially, without any antigenic stimulation and finally, after 48 hours of ES and HEX antigens stimulation (5ug/mL and 50ug/mL). Briefly, the erythrocytes were lysed with ammonium chloride (150mM) and washed twice in PBS. Cultured cells were then stained with monoclonal antibodies conjugated with PerCP specific for CD4, CD8 and CD19 (All from Becton Dickinson, USA) cell surface markers during 30 minutes in the dark at room temperature and later stained with Annexin V/FITC and PI. Phenotypic analyses were performed by flow cytometry with a FACScan flow cytometer (BD Biosciences, USA). Data were collected on 1 x 10^5 lymphocytes (gated by forward and side scatter) and analyzed using Cell Quest Pro™ software (BD Biosciences, USA) (Figure 1).

3.9. Confocal microscopy

Jurkat T cells (2 x 10^5 cells) were stimulated for 48 hrs with adult *A. ceylanicum* ES products and crude extract HEX, and then double-stained with Hoechst 33342/Propidium Iodide for viability assays. Briefly, cells were labeled with Hoechst 33342 at 1 µg/ml. The labeling consisted of 30 min incubation at 37°C in the dark. During the last 15 minutes, Propidium iodide (Sigma-Aldrich; St. Louis, MO, USA) was added to the cells at a concentration of 1 µg/ml. After labeling, dyes were removed by washing the cells twice with PBS. Cells were then resuspended in culture medium and observed using a Zeiss LSM 510 Meta Confocal Microscope equipped with a 63x oil-immersion objective (CEMEL, ICB-UFMG). Image analysis was carried out with Zeiss LSM Image Browser software.

3.10. Apoptotic pathways triggered by hookworm antigens

In order to determine the apoptotic pathways triggered by hookworm, Jurkat cells were incubated with placebo (CT group), ES products (ES group) and crude extract (HEX group), at a final concentration of 100 µg/mL. After 48hs of incubation at 37°C and 5% CO_2 air, cells were recovered and washed with PBS. Subsequently, cells were submitted to a total RNA extraction protocol using NucleoSpin® RNA II kit (Macherey-Nagel, Germany). The total RNA was quantified according to standard procedures using spectrophotometer and evaluated in agarose gel to confirm its integrity (Sambrook et al, 1989). The cDNAs were performed with Superscript II (Invitrogen, USA) using 120ng of total RNA, according to manufacturer's instructions. Afterwards a RT-PCR using previously established constitutive human primers (28) has been performed in order to confirm cDNA synthesis.

The apoptotic transcripts have been evaluated using a Human Apoptosis RT^2 Profiler™ PCR Array kit (SABiosciences, USA) in a qPCR machine (7500, Applied Biosystem, USA), according to manufacturer's instructions. Eighty four transcripts involved with programmed cell death were evaluated. The qPCR data were analyzed by PCR Array Data Analysis Web Portal (SABiosciences, USA) and the results were expressed using the method of $2^{-\Delta\Delta Ct}$ (29).

3.11. Statistical analysis

The one-sample Kolmogorov-Smirnov test was used to determine whether variability followed a normal distribution pattern. One-way ANOVA test followed by Bonferroni's Multiple Comparison Test was used to evaluate the statistical differences into cell viability and proliferation assay and to compare the groups of hookworm antigens-stimulated cells with non-stimulated cells. Unpaired t test was used to determine the differences of parametric variables between *Necator*-infected individuals and healthy donors. The maximum residual test (Grubb´s test) was used to detect possible outliers. All statistics were carried out using Prism 5.0 for Windows (GraphPad Software Inc., USA).

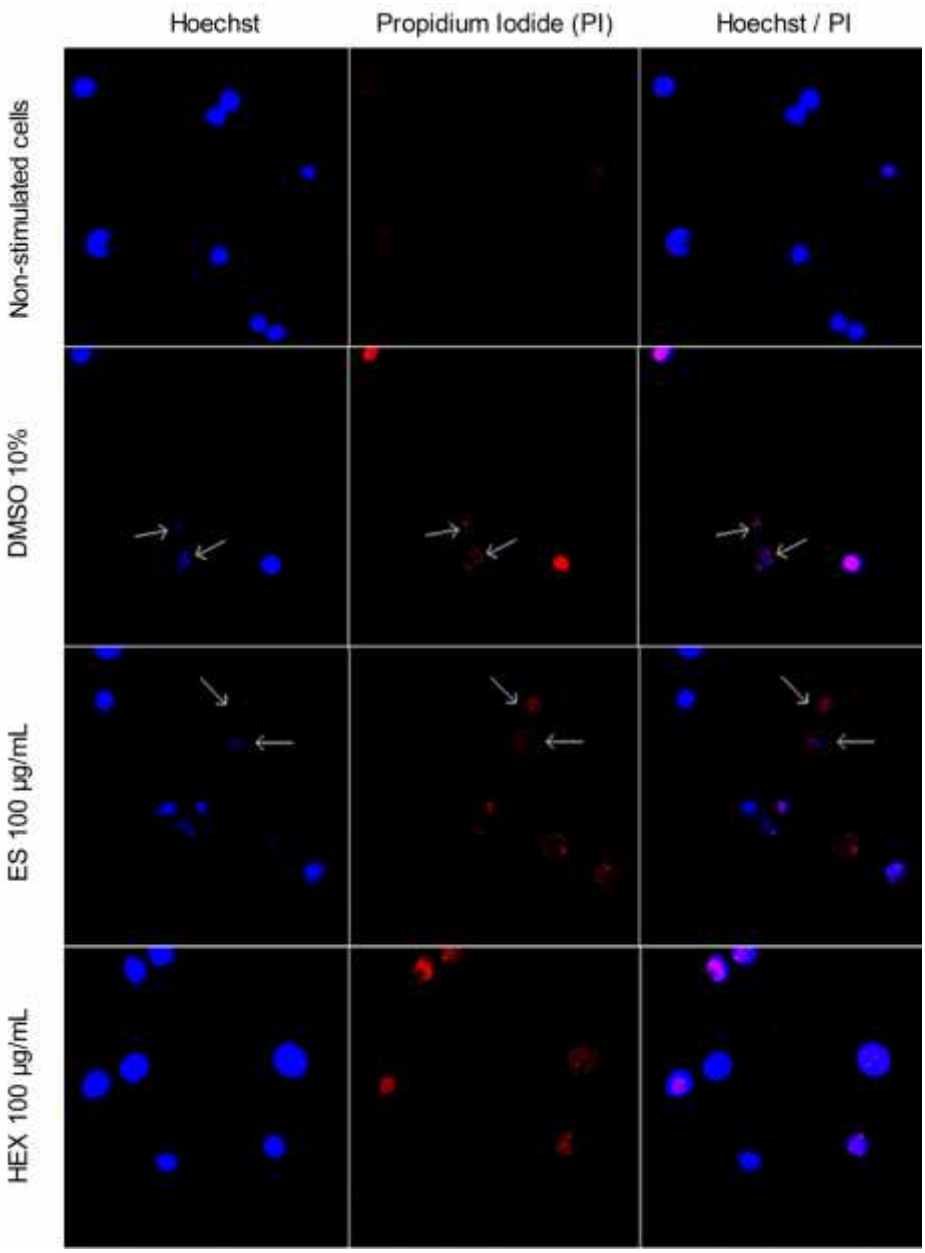

Figure 2. Jurkat T cells undergo apoptosis after hookworm antigens, *in vitro*, stimulation. Confocal microscopy images of Jurkat T cells stained with hoechst (blue), which evaluates nuclear condensation, and propidium iodide (red), which evaluates cellular death, after *in vitro* stimulation for 48 hours with DMSO 10% (positive control), hookworm ES products and HEX extract. Qualitative analysis performed in comparison to non-stimulated cells.

4. RESULTS

4.1. Hookworm excretory-secretory (ES) products induce apoptosis in Jurkat T cells

The cytotoxic and pro-apoptotic effect of hookworm antigens were first assessed in Jurkat T cells. Cell apoptosis induced by hookworm products was detected by confocal microscopy, and further quantified by MTT assay and flow cytometry. As shown in Figure 2, Jurkat T cells incubated with ES products were driven to death [propidium iodide (PI) staining] and exhibited condensed fragmented nuclei (Hoechst staining), compared to control cells, which had normal intact chromatin and negative staining for PI. Additionally, the cytotoxicity results revealed that *in vitro* stimulation with ES products (at 50 and 100µg/mL) statistically reduced ($p = 0.0074$) the cell viability of Jurkat T cells to 73.3% and 62.3% respectively (Figure 3A), with a characteristic dose-response pattern ($p = 0.0004$; $r = -0.9910$). No differences were seen after hookworm HEX stimulation ($p > 0.05$ for all) (Figure 3B). Interestingly, DNA fragmentation analysis by flow cytometry demonstrated that hookworm ES

Table 1. Demographic, parasitological and hematological parameters of *Necator americanus*-infected patients and egg-negative individuals (Mean and range)

Parameters	*Necator*-infected patients (n=13)	Non-infected donors (n=17)
Age (years)	33.07 (20 - 59)	37.47 (21 – 55)
Intensity of infection[1]	110.61 (4 - 648)	0
Hemoglobin (g/dL)	14.43 (11.8 – 16.4)	14.24 (12.9 – 16.5)
Whole blood count (cells/mm^3)	7200 (2800 – 10900)	7635 (5300 – 10700)
Eosinophil (cells/mm^3)	405.98 (88.5 – 1711.3)[2]	203.75 (108.0 – 549.0)
Monocytes (cells/ mm^3)	491.73 (182.9 – 708.5)[2]	212.59 (70.0 – 432.0)
Lymphocytes (cells/mm^3)	2101.94 (1055.6 – 3368.9[2]	2582.88 (1378 - 4278)
CD4$^+$ Lymphocytes (cells/mm^3)	956.63 (535.7 – 1456.5)[2]	1736.8 (833.5– 3146.5)
CD8^{high} Lymphocytes (cells/mm^3)	434.83 (114.0 – 969.2)	744.6 (293.0 – 1183.7)
CD19$^+$ Lymphocytes (cells/mm^3)	287.60 (127.7 – 636.7)	581.4 (292.3 – 1112.7)

[1]Number of eggs per gram of feces; [2]Statistically different from non-infected donors (P<0.05).

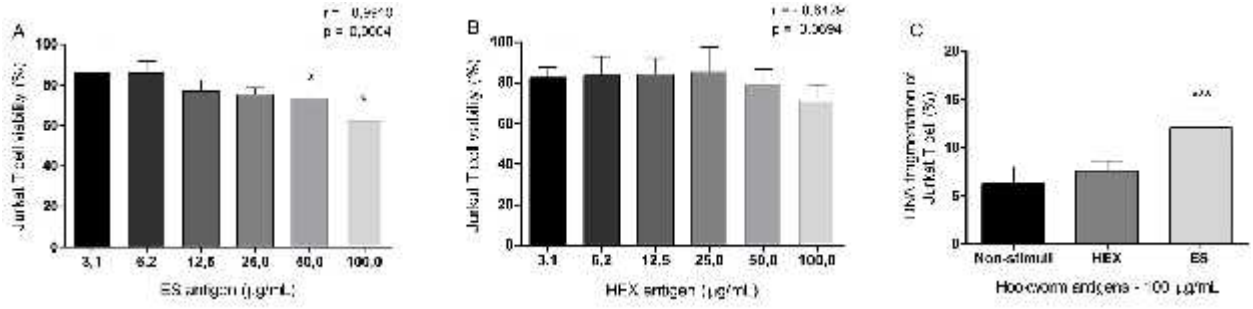

Figure 3. The apoptotic profile of Jurkat T cells. Jurkat T cells were stimulated, *in vitro*, for 48 hours, with *A. ceylanicum* excretory-secretory (ES) products and adult crude extract (HEX). Induction of cell death and DNA fragmentation by hookworms extracts was analyzed by MTT assay (A and B) and flow cytometry respectively (C). Data are presented as mean ± SD from triplicates of three separate experiments. Statistical differences with non-stimulated cells are indicated in each graph with the significant P values: *p<0.05 and ***p< 0.001. The p and r values represent the statistical correlation analysis.

products stimulation statistically increases (p < 0.0001) the percentage of Jurkat T cells with fragmented DNA (apoptotic cells) when compared with non-stimulated cells (12.08% vs 6.28%) (Figure 3C). For these assays, etoposide (14.75µM) was used as a positive control of apoptosis, resulting in a cell viability reduction of 81% and a DNA fragmentation induction of 40.9% (data not shown).

4.2. Cell apoptosis induced by hookworm antigens is also observed in a controlled experimental primary infection in hamsters

Once observed a significant pro-apoptotic activity of hookworm ES products in Jurkat T cells, we further analyzed the induction of apoptosis during a controlled experimental hookworm primary infection by evaluating the apoptotic profile of MLN lymphocytes of non-infected and hookworm-infected hamsters. Analysis of cytotoxicity in infected hamsters revealed that hookworm ES products at 50 and 100µg/mL induce a statistical dose-dependent decrease (p < 0.0001; r= -0.9153) of cell viability to 80.01 and 73.01% respectively (Figure 4A). Additionally, flow cytometry analysis showed a relevant increase in the percentual of MLN lymphocytes with fragmented DNA after ES products stimulation (p= 0.0073), when compared to control group (CT = 1.93%; HEX = 10.1%; ES = 25.35%) (Figure 4E).

Interestingly, we demonstrated a very similar apoptotic profile for MLN cells of non-infected hamsters, when also stimulated with hookworm antigens. Notably, a significant dose-dependent decrease of cell viability was induced by ES products (p<0.0001; r=-0.8267) and HEX antigen (p=0.0127; r=-0.8571) (Figure 4C and D). On the other hand, a statistical increase of lymphocytes with fragmented DNA after ES products stimulation (p< 0.0001) was also observed in non-infected hamsters when compared with their non-stimulated cells. Conversely, no differences were seen after HEX stimulation (p>0.05) (CT= 2.67%; HEX = 2.71%; ES = 9.59%) (Figure 4E). Noteworthy, hookworm-infected hamsters presented significantly higher levels of apoptotic lymphocytes than non-infected hamsters, after ES (25.35% vs 9.59%) and HEX stimulation (10.15% vs 2.71%) (Figure 4F). In this way, the high immune cell activation levels observed during hookworm infection may increase the susceptibility of lymphocytes to the pro-apoptotic effect of hookworm antigens.

4.3. Induction of apoptosis by hookworm antigens is confirmed in peripheral lymphocyte population of chronic *N. americanus*-infected patients

After demonstrating the potential pro-apoptotic activity of hookworm antigens in experimental models, we evaluated the apoptotic cell profile of *N. americanus*-infected patients, all residents in endemic regions of Minas Gerais State, Brazil. Initially we characterized the whole blood cell profile of chronic hookworm-infected patients (Table 1), and further evaluated the proliferative capacity of their PBMC in face of hookworm antigenic stimulation. Finally, we demonstrated, by flow cytometry, the apoptotic profile of *N. americanus* infected-patients lymphocyte population after staining these cells with the apoptotic biomarkers, Annexin-V/FITC and propidium iodide.

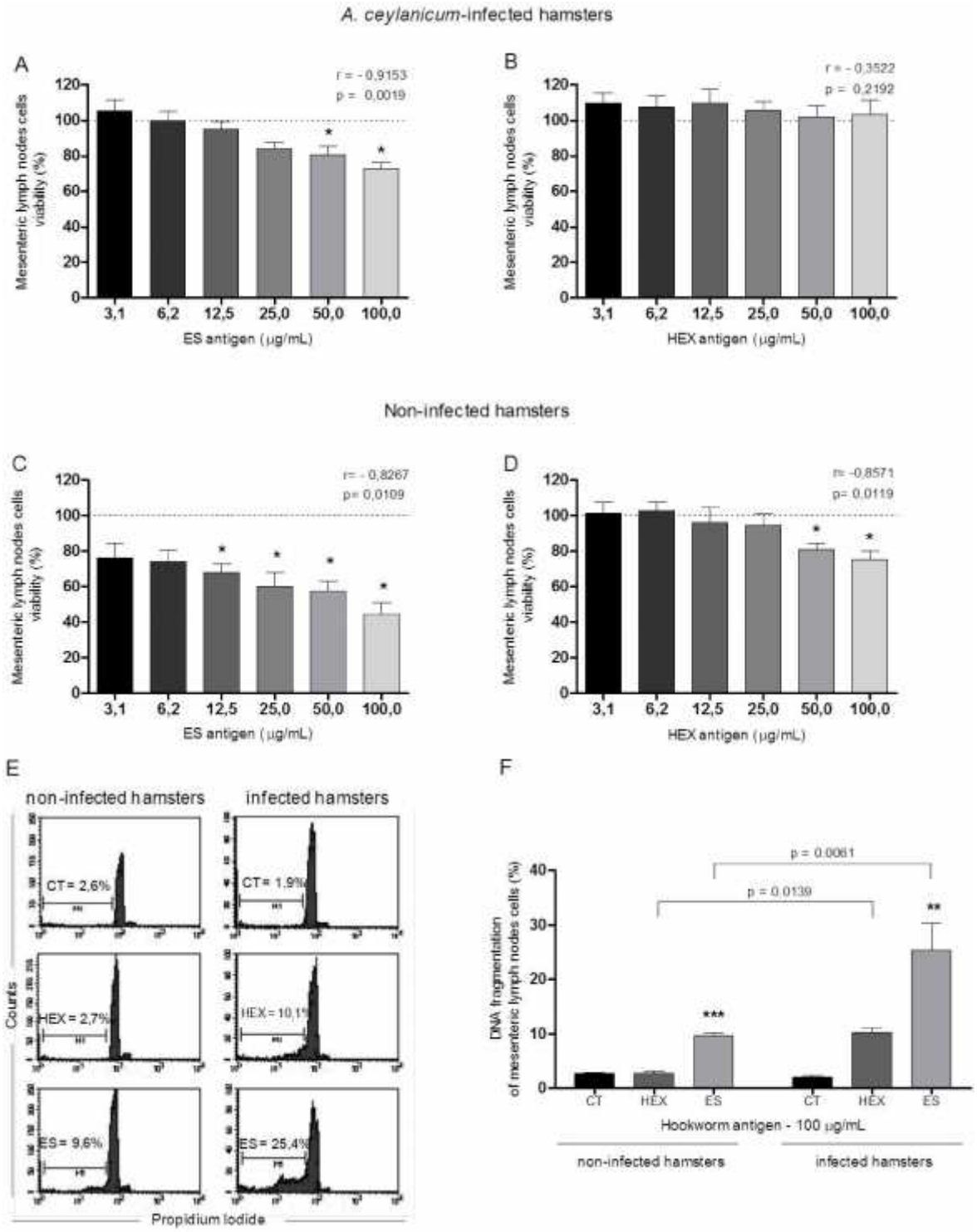

Figure 4. The apoptotic profile of hamsters MLN cells. MLN cells from non-infected (7) and hookworm-infected hamsters (5), were stimulated, *in vitro*, for 48 hours, with *A. ceylanicum* excretory-secretory (ES) products and adult crude extract (HEX). Induction of cell death and DNA fragmentation by hookworm antigens was analyzed by MTT assay (A,B,C,D) and flow cytometry (E,F) respectively. Data are presented as mean ± SD from triplicates of three separate experiments. Statistical differences with non-stimulated cells are indicated in each graph with the significant P values: *p<0.05, **p<0.01 and ***p< 0.001. The p and r values represent the statistical correlation analysis.

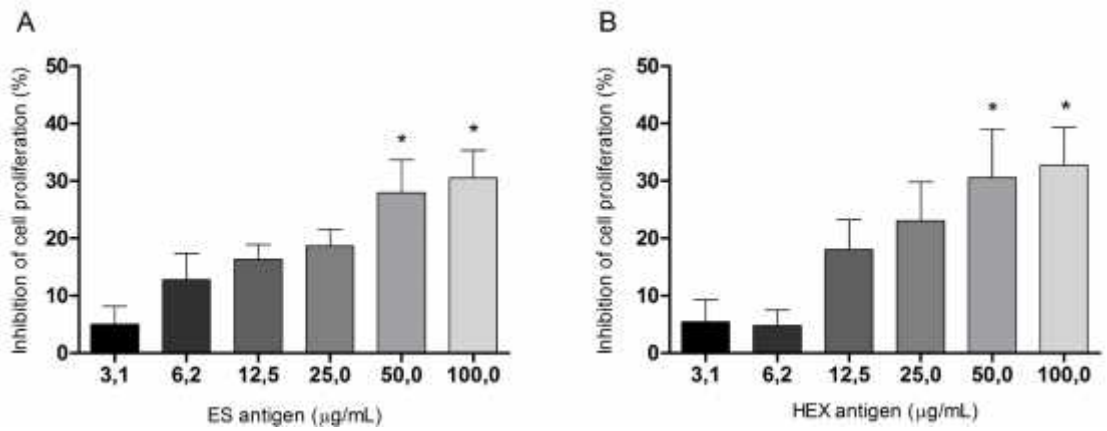

Figure 5. Hookworm antigens inhibit human peripheral cell proliferation. PBMCs proliferation inhibition from *N. americanus*-infected patients (7), induced by different concentration of *Ancylostoma ceylanicum* excretory-secretory (ES) products (A) and adult crude extract (HEX) (B). Data are presented as mean ± SD from seven hookworm-infected patients. Statistical differences with non-stimulated cells are indicated by an asterisk ($p<0.05$).

Haematological analysis showed that chronic mono-infected patients present a relevant reduction on the absolute number of lymphocytes ($p=0.0499$), associated with a decrease in $CD4^+$, $CD8^{high}$ and $CD19^+$ cells (Table 1). Cell proliferation assay demonstrated that antigenic stimulation during 48 hours induces a dose-dependent inhibition of PBMC proliferation in *N. americanus* infected-patients. Hookworm ES products, at 50 and 100 µg/mL, significantly inhibited the cellular proliferation by 27.91% and 30.48% respectively, while HEX antigen, similarly, reduced the proliferative response in 30.56% and 32.69%, at the same concentrations (Figure 5).

When the apoptotic profile of lymphocyte population without antigenic stimulation was evaluated, we observed that *N. americanus*-infected patients presented a significant increase ($p=0.0052$) in the percentual of $CD4^+$ T lymphocytes in early apoptosis (Annexin V^+), in comparison to healthy non-infected individuals (Figure 6A). Additionally, we observed a statistical increase in the percentual of $CD8^+$ T (Figure 6B) and $CD19^+$ B cells (Figure 6C) in early ($p=0.0269$ and $p<0.0001$ respectively) and late apoptosis (Annexin V^+/ PI^+) ($p=0.0248$ and $p=0.0065$ respectively) (Figure 6E and 6F). These findings suggest that chronic *Necator americanus*-infected patients present an important alteration of lymphocyte homeostasis.

Interestingly, this apoptotic profile was further confirmed by *in vitro* antigenic stimulation of lymphocytes from *N. americanus*-infected patients. ES stimulation induced an increase of $CD4^+$ T cells in early ($p=0.0366$) and late ($p=0.0274$) apoptosis (Figure 7A and D). Considering $CD8^+$ T cells, ES products stimulation reflected in the augmentation of early apoptosis ($p=0.0104$) (Figure 7B). Finally, the stimulation of $CD19^+$ B cells with ES and HEX antigens induced an increase ($p= 0.0343$) of early apoptotic cells (Figure 7C), followed by a HEX-dependent increase ($p=0.0004$) of late apoptosis (Figure 7F).

4.4. Hookworm antigens might induce apoptosis by an intrinsic mitochondrial pathway

Once demonstrated the induction of apoptosis in lymphocytic lineage of human and experimental models by hookworm antigens, the next step was to identify the possible apoptotic pathway activated by these antigens through the analysis of pro- and anti-apoptotic genes expression after *in vitro* stimulation with ES and HEX antigens in human Jurkat T cells.

In this study, twenty four out of the eighty-four apoptosis-related genes initially evaluated were up or downmodulated by ES or HEX stimulation. Initially, these genes were grouped and categorized according to their respective families and function (Figure 8). An increase on the expression of anti-apoptotic genes, BAG-3 and BCL-2L10, members of BCL-2 family, was observed after HEX stimulation. Conversely, HEX antigen also up regulated the expression of the pro-apoptotic genes BIK (Bcl-2 interacting killer), BAD (Bcl‑2 antagonist of cell death) and BNIP3, in comparison to non-stimulated cells (Figure 8A). Interestingly, these genes are members of the BH3‑only subfamily, which can antagonize the anti-apoptotic effect of BCL-2 and drive the cells to apoptosis. On the other hand, ES products downmodulated the expression of the following pro-apoptotic genes from caspase family: CASP1 (Caspase 1), CASP14 (Caspase 14), CASP5 (Caspase 5) and CARD8 (Caspase recruitment domain family, member 8). Curiously, HEX extract induced a downregulation of CASP2 (Caspase 2), but also an upregulation of CASP1 and CASP5, when compared with non-stimulated cells (Figure 8B). The analysis of TNF receptor family, known as death receptors of extrinsic pathway, showed that ES and HEX antigens downmodulate the expression of several pro-apoptotic genes: CD40, FADD (GIG3/MORT1), LTBR (TNFRSF3), TNFR10B (CD262/DR5), TNFR11B (OCIF/OPG), TNFR1A (CD120a/FPF), TNFR25 (APO-3/DDR3). In opposition, HEX up regulated the CD27 gene expression (Figure 8C).

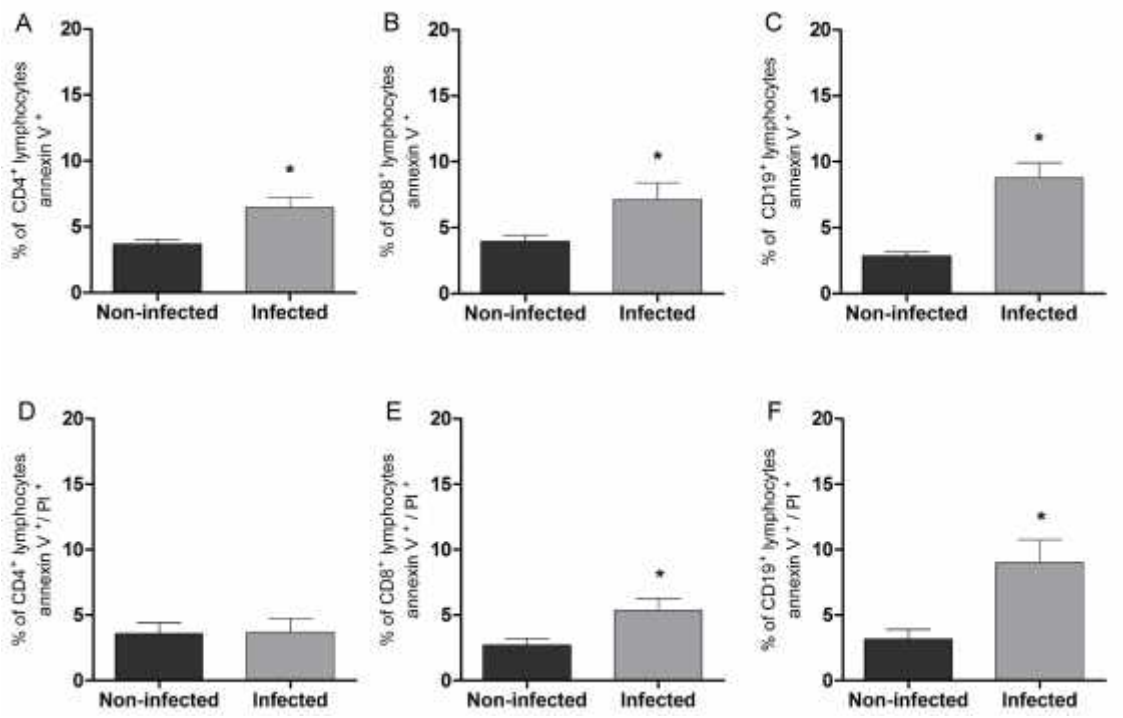

Figure 6. The apoptotic profile of hookworm-infected patients. *Ex vivo* analysis of the percentage of peripheral lymphocytes subpopulations, $CD4^+$, $CD8^+$ and $CD19^+$, from non-infected donors (17) and hookworm-infected patients (13), stained with Annexin V-FITC (A,B,C), and Annexin V-FITC and Propidium Iodide (D,E,F), indicating early apoptosis and late apoptosis respectively. Data are presented as mean ± SD and statistical differences between the groups are indicated by an asterisk ($p<0.05$).

Finally, we observed an up-regulation of P73 (tumor protein p73) and GADD45 (growth arrest and DNA-damage-inducible) after ES stimulation, while HEX extract downmodulated P53BP2 (tumor protein p53 binding protein, 2) and PAF-1 (apoptotic peptidase activating factor 1) genes, all from intrinsic pro-apoptotic p53 family (Figure 8D).

5. DISCUSSION

Apoptosis is a tightly regulated multi-step pathway that is responsible for cell death not only during development, but also in adult multicellular organisms, in which it partially controls cell numbers and tissue homeostasis (30, 31). Unlike necrosis, the net result of apoptosis is the safe removal of unwanted cells without release of toxic intracellular substances or the local incitement of inflammation (32). In addition, apoptosis has been established as a crucial modulator of host-parasite interaction (33). Recent immunological studies have shown that helminths induce cell apoptosis as an efficient strategy to evade the host immune response, mainly through the creation of a site of 'immune privilege' around themselves (22, 23), allowing for long term parasite survival in the host lumen (2). In this context, the present study sheds lights on the potential contribution of apoptosis as a mechanism of immunomodulation during human and experimental hookworm infection.

Herein, we have demonstrated the cytotoxic and pro-apoptotic effect of *A. ceylanicum* antigens in human and experimental cells lines. Initially, Jurkat T cells viability assay revealed a dose-dependent cytotoxic activity of hookworm ES products, which was also reported by Chow et al. (23). In our study, we have shown that ES and HEX stimulation decrease MLN cells viability in both hookworm-infected and non-infected hamsters, process that can be explained by hookworm antigens-induced apoptosis. Our results suggest that the apoptotic death observed after ES and HEX stimulation could reflect in cell proliferative suppression during hookworm infection, likely resulting in the hyporesponsiveness of MLN cells already described in experimental hookworm infection (34-36). Interestingly, previous studies have demonstrated that lymphocytes from MLN of hookworm infected-hamsters present an impaired lymphoproliferative response to specific adult antigens in late patency (34). Although it is difficult to distinguish whether an inability to proliferate or increased levels of apoptosis is the primary defect, it is important to consider programmed cell death induction as an efficient strategy of immunomodulation in helminth infections. We hypothesized that the profound ablation of hookworm antigens specific cell proliferative responses could be at

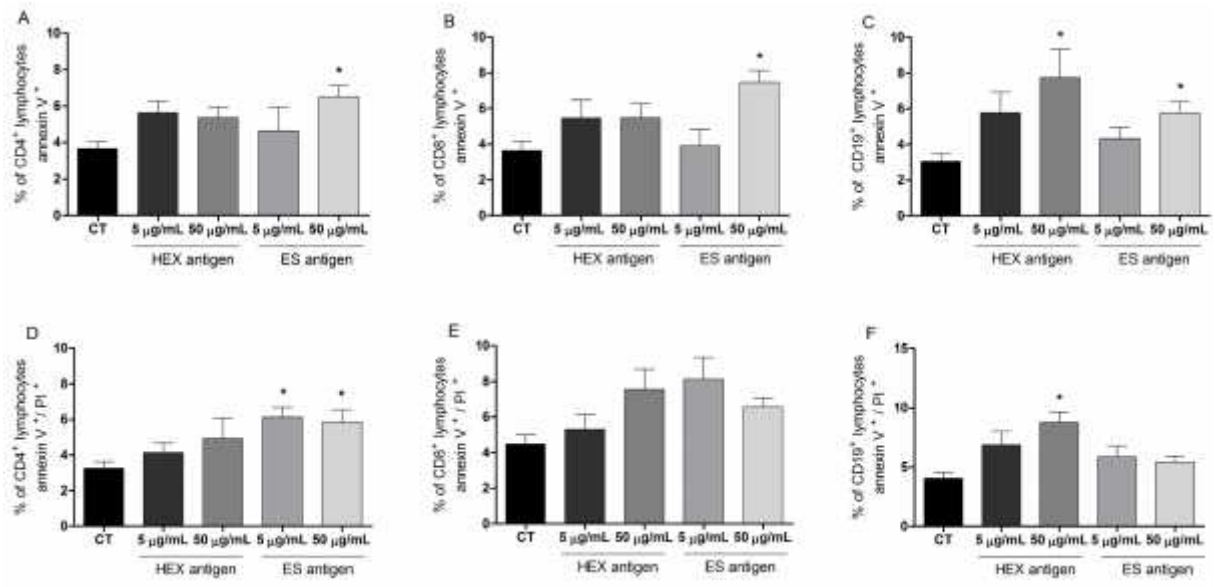

Figure 7. The apoptotic profile of hookworm-infected patients, after hookworm antigens in vitro stimulation. Percentage of peripheral lymphocytes subpopulation, $CD4^+$, $CD8^+$ and $CD19^+$, of hookworm-infected patients (10), stained with Annexin V-FITC (A,B,C), and Annexin V-FITC and Propidium Iodide (D,E,F), after 48 hours of stimulation, *in vitro*, with *Ancylostoma ceylanicum* excretory-secretory (ES) products and adult crude extract (HEX). Data are presented as mean ± SD and statistical differences with non-stimulated cells (CT) are indicated by an asterisk ($p<0.05$).

least partially related to hookworm-induced apoptosis during hookworm infection. Indeed, Jenson et al. (22) showed that *Brugia pahangi* microfilariae can induce lymphocyte apoptosis, a phenomenon that contribute to parasite persistence during human filariasis.

In our study, we have also shown that hookworm-infected hamsters present higher levels of apoptosis after exposure to hookworm antigens than non-infected hamsters. The differences between the propensity of MLN cells from infected and non-infected hamsters to undergo apoptosis may be related to the highly activated immunological status observed during hookworm infection (37) or signals received upon re-exposure to hookworm antigens. On the other hand, hookworm-naive lymphocytes were also driven to apoptosis after antigen specific stimulation. These results suggest that directing cells to apoptosis is not simply related to the host immune response against the parasite, but specifically mediated by hookworm excreted/secreted products. Indeed, several immunomodulatory macromolecules from adult hookworms ES products have already been characterized, including a tissue inhibitor of metalloproteinase (TIMP) (38), Natural Killer cells binding ligand (39), metalloproteases that inactivates eotaxin (40), neutrophil inhibitory factor (NIF) (41), macrophage migration inhibitory factor (42), anti-oxidants and anti-inflammatory proteins (2), and now an apparent cell apoptosis-inductor factor.

The characterization of the immune response in experimental infection represents an important tool to elucidate the basis of immunomodulation mechanisms in the human hookworm infection. In order to investigate whether the cell apoptosis observed in the experimental models contributes to the immunomodulation of human hookworm infection, we assessed the apoptotic profile of peripheral lymphocytes from chronic *N. americanus* infected-patients. Our results revealed that hookworm infected-individuals of endemic regions present a relevant reduction on the absolute number of lymphocytes and subpopulations. Similarly, Onyemelukwe and Mousa (43) and Geiger et al. (37) also demonstrated that human hookworm infection is characterized by a reduction of T and B lymphocytes respectively. Moreover, we observed that ES and HEX antigens can considerably inhibit the proliferative capacity of PBMCs from infected-individuals, supporting several studies that associate chronic human hookworm infection with T cell hyporesponsiveness (5, 43-47). Additionally, we observed that chronic *Necator*-infected patients naturally present a persistent alteration of lymphocyte homeostasis, characterized by an increase of $CD4^+$, $CD8^+$ and $CD19^+$ cells in apoptosis process. Interestingly, a similar apoptotic profile was observed after *in vitro* ES and HEX stimulation, which increased the percentage of these cells in early and late apoptosis, in comparison to non-stimulated cells. These findings suggest that hookworm antigens would constantly drive lymphocytes to apoptosis *in vivo*, contributing to the downmodulation of immune response *in situ* and for hookworm survival in the host intestinal lumen. It is well known that both cellular and humoral responses are elicited by human hookworm infection, which primarily aims to provide protection against worm colonization (47). According to Dondji et al. (36) the experimental depletion of $CD4^+$ T cells is associated with a threefold increase in intestinal hookworm burden, suggesting that hookworm-associated depletion of $CD4^+$ lymphocyte populations

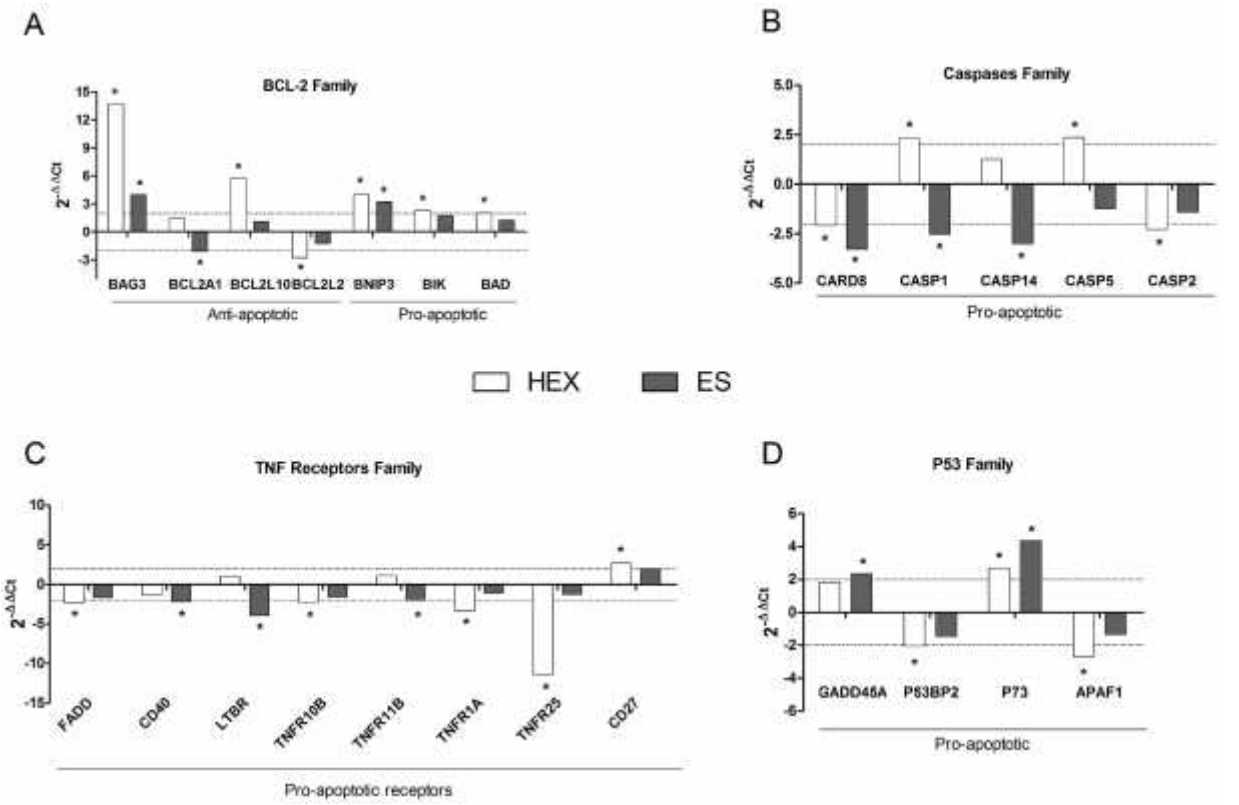

Figure 8. Apoptotic pathways induced by hookworm antigens. Analysis of genes expression of BCL-2 (A), Caspases (B), TNF receptors (C) and P53 (D) families, from Jurkat T cells, after stimulation with adult *Ancylostoma ceylanicum* excreted/secreted products (ES) and crude extract (HEX). The fold-change values ($2^{-\Delta\Delta Ct}$) were represented by the bars in comparison with non-stimulated cells.

represents a specific strategy for parasite survival. Furthermore, Th2 and B cells play a pivotal role in the generation of protective immunity in vaccines targeting hookworm larvae (44).

Finally, we identified possible apoptotic pathways triggered by hookworms ES and HEX antigens to modulate host immune response. Despite not having found one specific apoptotic pathway, the combined analysis of apoptotic genes expression allowed us to select different families of genes up or down-regulated by these antigens. Initially, we observed a downmodulation of TNF receptor family members, CD40, FADD, TNFR10B (CD262/DR5), TNFR11B (OCIF/OPG), TNFR1A (CD120a/FPF), TNFR25 (APO-3/DDR3) which are essentials for apoptotic extrinsic pathway activation. Moreover, our results showed an up-regulation of BNIP3, BIK and BAD, as well as, TP73 e GADD45A, which are pro-apoptotic genes associated with the BCL-2 and P53 families, respectively. Taken together, these findings suggest that hookworm antigens induce apoptosis by an intrinsic mitochondrial pathway. This hypothesis is also supported by Chow *et al.* (23), who demonstrated that cell apoptosis induced by *Necator americanus* ES products does not involve FasL and FasR molecules, important mediators of apoptotic extrinsic pathway.

Although hookworm antigens induced a downmodulation of Caspase family genes, the expression of the initiators (8 and 9) and executioner caspases (3, 6 and 7) were not altered after hookworm antigen exposure. In fact, several caspases may induce apoptosis but there is no evidence they play crucial role in apoptosis as described for caspases 3, 8, and 9 (48). Therefore, additional studies are still necessary to elucidate whether hookworms are inducing cell death by intrinsic mitochondrial caspase-dependent or -independent apoptotic pathway.

In conclusion, we demonstrated the potential cytotoxic and pro-apoptotic activity of the hookworm antigens against T and B lymphocytes, which may contribute to the downmodulation of immune response and persistence of human hookworm infection. New experiments using molecular inhibitors to block specific apoptotic pathways should be performed in order to describe in detail the mechanisms of apoptosis induced by hookworms.

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Abbreviations: ES: excretory/secretory; HEX: hookworm crude extract; MLN: mesenteric lymph nodes; PI: propidium iodide; BCL-2: B-cell lymphoma 2

Key Words: Apoptosis, Hookworm, Immune response, Immunomodulation, Hookworm infection, Hookworm antigens

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