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Production of interferon-γ by natural killer cells and aging in chronic human schistosomiasis

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***Background*: Aging is associated with several alterations in the phenotype, repertoire and activation status of lymphocytes as well as in the cytokine profile produced by these cells. As a lifelong condition, chronic parasitic diseases such as human schistosomiasis overlaps with the aging process and no systematic study has yet addressed the changes in immune response during infection with *Schistosoma mansoni* in older individuals.**

***Aim*: Herein we study the influence of immunological alterations brought about by senescence in the course of schistosomiasis.**

***Materials and methods*: Individuals 10–95 years of age, from both sexes, from an endemic area for *S. mansoni* infection were matched by intensity of infection as measured by egg counts. We analyzed, as a parameter, cytokine expression by lymphocytes and natural killer cells after *in vitro* stimulation with soluble egg antigen and soluble worm antigen using flow cytometry.**

***Results*: We demonstrated that the frequency of $CD16^+$ interferon-γ (IFN-γ)$^+$ natural killer cells in negative individuals over the age of 70 years is significantly higher than in positive individuals after *in vitro* stimulation with *S. mansoni* antigen extracts. The frequency of these cells is increased in all individuals over the age of 50 years and only declines in positive individuals after 70 years of age. Analysis of either $CD4^?$ or $CD8^?$ cells after antigen stimulation show no significant increase in frequency of IFN-γ in negative or in positive individuals of this age group, suggesting that the effect on $CD16^+$ cells is not T-cell dependent.**

***Conclusion*: Since production of IFN-γ has been related to resistance to schistosome infection, our data suggest that age-associated changes in $CD16^+$ cells may play a role in controlling infection intensity in the elderly in *S. mansoni* endemic areas of Brazil.**

Key words: Interferon-γ, Aging, Natural killer, Schistosomiais

Introduction

The relationship between age and infection intensity in *Schistosoma mansoni* endemic areas is characterized by a steep rise in infection intensity during adolescence and a subsequent decline in infection intensity as the population ages. It is not yet clear whether this observed pattern is the result of changes in behavior or acquired immunity as the endemic population ages.[1,2] Recently, both Webster and coworkers[3] and our group[4,5] have reported that another rise in infection intensity occurs among the elderly, suggesting a loss of this partially acquired immunity by aged people. To date, there has been no systematic study of the immune response of individuals residing in *S. mansoni* endemic areas over the age of 60 years. This is despite the fact that senescence is associated with increased susceptibility to infection[6,7], along with numerous modifications in the immune system, including involution of the thymus, decline in the number of $CD3^+$ cells, a shift toward a greater proportion of $CD4^+$ T cells expressing a memory phenotype, loss of CD28 expression, and reduced potential to produce interleukin (IL)-2.[8,9] Although the number of B cells is reduced in old individuals,[10,11] the modest changes in the B-cell compartment may also be dependent on T cells.[12,13] Overall, the immune system of the elderly seems more activated, but less capable of responding to novel antigens.

Interestingly, innate immune responses are more resistant to change and natural killer (NK) cells are well preserved in healthy elderly subjects.[14] In fact, there is an age-related increase in $CD16^+CD57^-$ cells

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with high cytotoxicity capacity.[15] This increase in NK cells has been correlated with successful aging.[16]

The development of effector $CD4^+$ T helper cells is critical in determining the immunological outcome to helminth infection.[17,18] Previous studies have demonstrated that antigen-specific T cells in persons with chronic *S. mansoni* infection present poor proliferative responses to parasite antigens. Chronic infection is also characterized by a down-modulation of the inflammatory response, decrease in granuloma reaction, and cumulative fibrosis.[19] At this stage, high levels of IL-4, IL-5 and IL-10 are produced mostly by T cells.[20,21] The role of NK cells in schistosomiasis is not clear. In mice, treatment with anti-NK1.1 antibodies enhances hepatic fibrosis and reduces IL-12 production during the chronic phase, suggesting that liver $NK1.1^+$ cells are immunoregulatory cells at this stage.[22]

In this study, we observed a significant increase in $CD16^+$ IFN-γ^+ NK cells in individuals over the age of 50 years after *in vitro* stimulation with *S. mansoni* antigens (egg antigen and worm antigen). These cells increase in frequency as individuals age and are maintained at higher frequency in egg-negative subjects over 70 years old. However, in egg-positive individuals over 70 years of age, they decline. The frequency of IFN-γ^+ cells among antigen-stimulated $CD4^+$ and $CD8^+$ cells of egg-positive individuals of the same age group does not show any change when compared with egg-negative individuals or with other age groups. Our data suggest that IFN-γ^+ NK cells may play a role in the infection status of elderly individuals in endemic areas.

Material and methods

Studied subjects

Areas endemic for *S. mansoni* were identified from routine prevalence surveys undertaken by the Foundation for National Health. Seventy-three subjects who live in these areas (Virgem das Graças e Córrego do Onça—Minas Gerais, Brazil) were analyzed in a cross-sectional study. Extensive water contact studies performed in these endemic areas showed that exposure measures in total body minutes is not statistically different among age groups nor is it different among individuals with distinct levels of infection.[4] Participants were matched by gender and infection status in different groups (four age groups and two groups for infection status). Detailed information on the studied population is presented in Table 1. Negative individuals were those with no egg counts and only individuals with egg counts >100 eggs/g of feces were included in the positive group. Categories of infection are in accordance with the WHO/CDS/SIP (World Health Organization/Communicable Diseases/Schistosomiasis and Intestinal Parasitoses). None of the participants was affected by neoplastic or autoimmune diseases or was receiving chemotherapy that would impair immune function. All of the infected (positive) subjects had the intestinal form of schistosomiasis as determined by the absence of hepatic alterations on ultrasonography. Subjects classified as 'intestinal' were also defined as having eggs in their feces with no clinical symptoms other than occasional intestinal discomfort. Informed consent was obtained for all participants.

Test for infection status

Infection status was determined by the presence or absence of eggs on fecal samples. Individuals from the endemic area were asked for three consecutive days of fecal samples. Slide preparation of the stool samples occurred within 24 h of collection using the Kato–Katz thick smear technique, with two slides prepared from each day stool sample. Fecal egg counts for *S. mansoni* were measured by counting the number of eggs per slide and then determining the arithmetic mean of the eggs found in six slides (i.e. two from each of three day samples) as described by Katz and coworkers.[23]

Flow cytometry

Freshly collected blood was cultured in complete RPMI for 5 h with 25 μg/ml of either soluble egg antigen or soluble worm antigen preparation in the presence of 10 μg/ml of brefeldin. A short-term stimulation protocol was chosen to detect early events such as the activity of NK cells that do not

Table 1. Age and egg counts of individuals in the study sample from areas endemic for Schistosomiasis in Brazil

Age	No. of individuals						EPG (average ± SD)	
	Negative			Positive (>100 epg)			Negative	Positive >100 epg
	Total	F	M	Total	F	M		
10–24	12	6	6	6	4	2	0	291 ± 120
25–49	9	8	1	9	3	6	0	221 ± 107
50–69	19	12	7	5	1	4	0	285 ± 175
70–95	7	1	6	4	1	3	0	148 ± 42

F = females; M = males; EPG = eggs per gram of feces; SD = standard deviation.

require antigen presentation nor long-term stimulation for activation. Cells were then harvested, washed and stained with anti-CD16-FITC, anti-CD4-FITC or anti-CD8-FITC (Becton Dickinson, La Jolla, CA, USA) monoclonal antibodies. After lyses of red blood cells, fixation with 1% paraformaldehyde and permeabilization of the cells with saponin-containing buffer, they were stained with PE-labeled anti-IFN-γ antibody (Becton Dickinson). NK and NKT cells as well as subpopulations of T cells were identified by forward and side angle scatter on a FACScan flow cytometer (Becton Dickinson) and by the expression of CD16, CD4 or CD8. At least 30,000 events were analyzed using CellQuest software (Becton Dickinson).

Statistics

A one-way analysis of variance procedure was used to test the hypothesis that the means between age strata were different, with significance at $p < 0.05$. A Bonferonni post-hoc test was used for pair wise multiple comparisons to test the differences between each pair of means.

Results

In a cross-sectional study, we matched individuals by age in four strata that corresponded to the peak (10–24 years), plateau (25–49 years), decline (50–69 years), and then resurgence of infection intensity (70–95 years) in our endemic areas (Table 1). First, we observed that, in control cultures of cells from negative and positive individuals, there is a slight increase in the frequency of $CD16^+$ IFN-γ^+ cells in older individuals (Fig. 1A,B). However, after *in vitro* stimulation with parasite antigens (egg antigen and worm antigen), a significant and progressive rise in

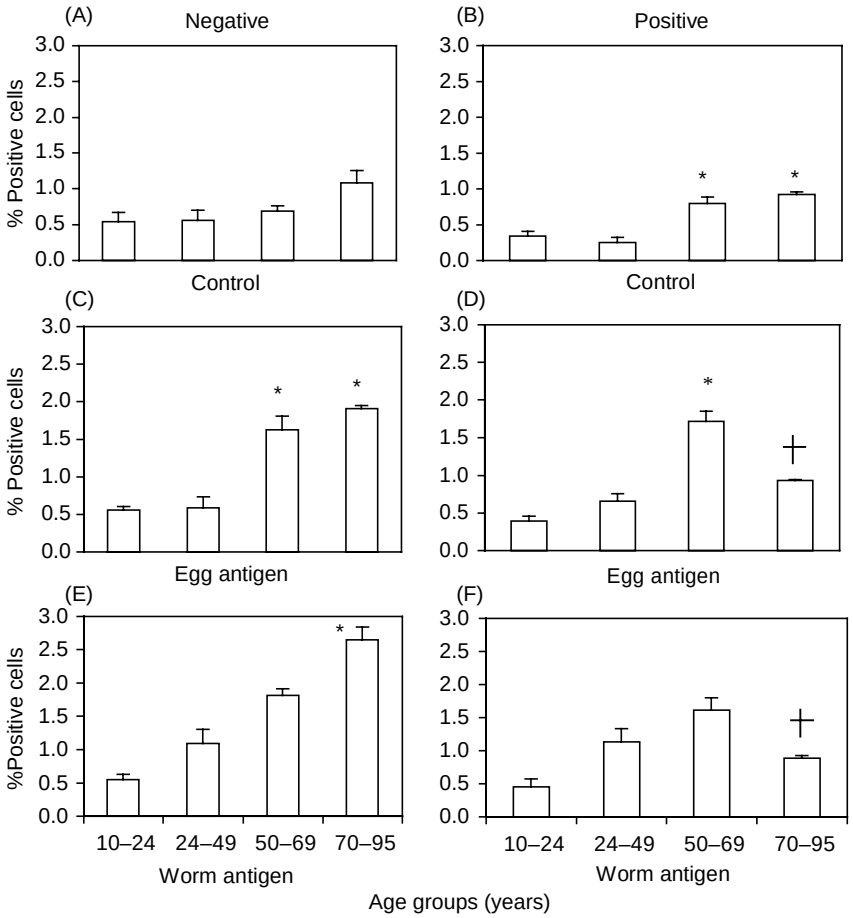

FIG. 1. Frequency of $CD16^+$ IFN-γ^+ cells according to age and infection status in individuals from areas endemic for schistosomiasis in Brazil. Seventy-three individuals from 10 to 95 years of age were analyzed in a cross-sectional study. Individuals who scored negative in the stool test for *S. mansoni* eggs were taken as negative subjects (A, C, E) and individuals who scored >100 eggs per gram of feces (epg) were taken as positive subjects (B, D, F). Freshly collected blood cells from both groups of individuals were cultured in complete RPMI for 5 h with either medium alone (control) (A, B), or 25 μg/ml of soluble egg antigen (egg antigen) (C, D) or 25 μg/ml of soluble worm antigen (worm antigen) (E, F). Cells were then stained for CD16 and for IFN-γ and analyzed by flow cytometry. Bars represent the average frequency of $CD16^+$ IFN-γ^+ cells±standard deviation of each age group. A one-way analysis of variance procedure was used to test differences among age groups, *significant differences. Pairs of means of negative and positive individuals at the same age group were tested by Bonferonni post-hoc test, +significant differences. $p < 0.05$.

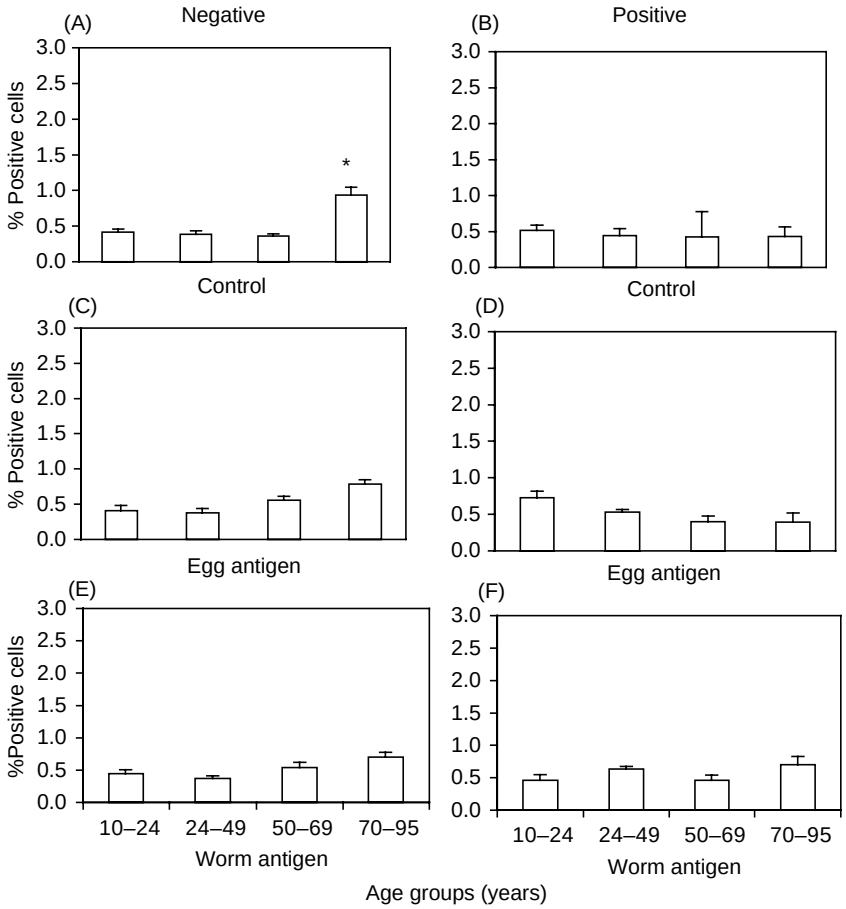

FIG. 2. Frequency of $CD4^+$ INF-γ^+ cells were analyzed according to age and infection status in individuals from areas endemic for schistosomiasis in Brazil. Seventy-three individuals from 10 to 95 years of age were analyzed in a cross-sectional study. Individuals who scored negative in the stool test for *S. mansoni* eggs were taken as negative subjects (A, C, E) and individuals who scored >100 eggs per gram of feces (epg) were taken as positive subjects (B, D, F). Freshly collected blood cells from both groups of individuals were cultured in complete RPMI for 5 h with either medium alone (control) (A, B), or 25 μg/ml of soluble egg antigen (egg antigen) (C, D) or 25 μg/ml of soluble worm antigen (worm antigen) (E, F). Cells were then stained for CD4 and for IFN-γ and analyzed by flow cytometry. Bars represent the average frequency of $CD4^+$ IFN-γ^+ cells±standard deviation of each age group. A one-way analysis of variance procedure was used to test differences among age groups, *significant differences. Pairs of means of negative and positive individuals at the same age group were tested by Bonferonni post-hoc test, +significant differences. $p < 0.05$.

$CD16^+$ IFN-γ^+ cells can be detected in individuals over 50 years old (Fig. 1C–F). Interestingly, only negative individuals over 70 years of age sustain this increase in the percentage of $CD16^+$ IFN-γ^+ cells (Fig. 1C,E). Among positive individuals over 70 years old, a significant decrease in the frequency IFN-γ^+ $CD16^+$ cells occurs after antigenic stimulation (Fig. 1D,F).

On the other hand, in control cultures of $CD4^+$ cells (Fig. 2A,B), the frequency of IFN-γ cells increases only in negative individuals over 70 years old, but after stimulation with either egg antigen (Fig. 2C,D) or worm antigen (Fig. 2E,F) this increase is no longer detectable.

In the $CD8^+$ cell population (Fig. 3), a higher frequency of IFN-γ cells occurred in the group of young individuals (10–24 years old). In negative individuals, these cells decline progressively as they age, and middle-aged individuals (24–49 years old) still maintain a high frequency of them. Conversely, in positive subjects, a drastic drop in the frequency of IFN-γ $CD8^+$ cells is detected in the 24-year-old to 49-year-old group and these cells stay at lower frequency in older individuals. Stimulation by either egg antigen (Fig. 3C,D) or worm antigen (Fig. 3E,F) yields no significant differences in the pattern of CD8 INF-γ cells between negative and positive individuals at any age strata.

Discussion

Usually the few available studies on aging and schistosome infection have neglected people over 60 years old despite the increasing number of the oldest olds in endemic areas for the disease. Reports on young and middle-aged individuals as well as in mice show that IFN-γ production by T cells is important in the protective immune response during schistosome infection.[24–28] Our data suggest that the

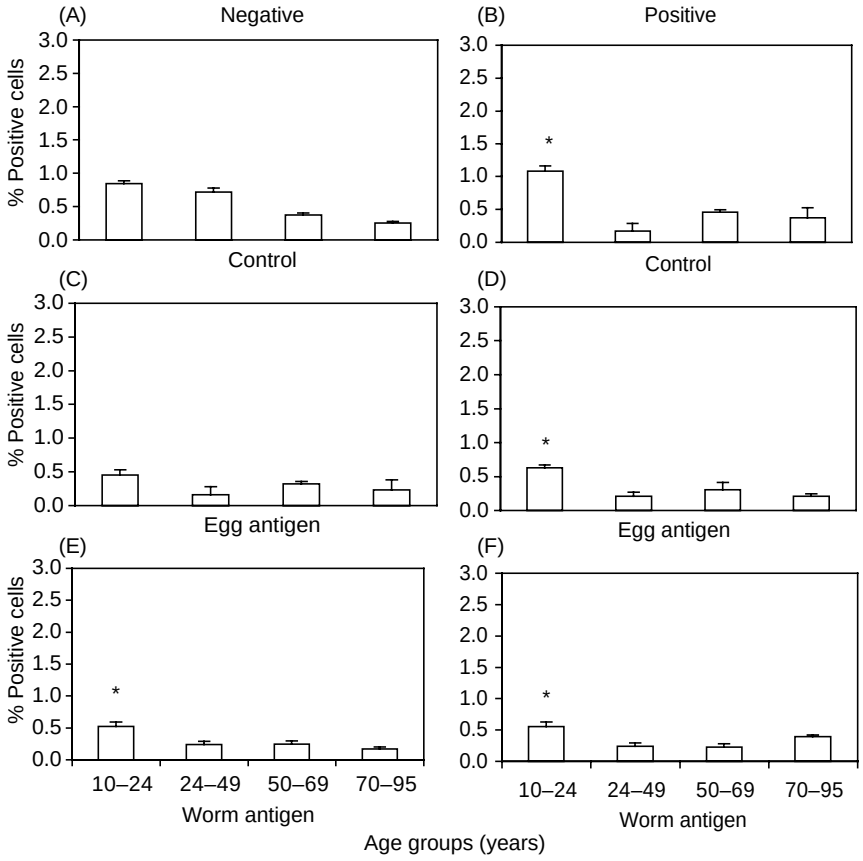

FIG. 3. Frequency of $CD8^+$ INF-γ^+ cells were analyzed according to age and infection status in individuals from areas endemic for Schistosomiasis in Brazil. Seventy-one individuals from 10 to 95 years of age were analyzed in a cross-sectional study. Individuals who scored negative in the stool test for *S. mansoni* eggs were taken as negative subjects (A, C, E) and individuals who scored >100 eggs per gram of feces (epg) were taken as positive subjects (B, D, F). Freshly collected blood cells from both groups of individuals were cultured in complete RPMI for 5 h with either medium alone (control) (A, B), or 25 μg/ml of soluble egg antigen (egg antigen) (C, D) or 25 μg/ml of soluble worm antigen (worm antigen) (E, F). Cells were then stained for CD8 and for IFN-γ and analyzed by flow cytometry. Bars represent the average frequency of $CD8^+$ IFN-γ^+ cells±standard deviation of each age group. A one-way analysis of variance procedure was used to test differences among age groups, *significant differences. Pairs of means of negative and positive individuals at the same age group were tested by Bonferonni post-hoc test, +significant differences. $p < 0.05$.

cytokine profile of NK cells is related to infection status in the elderly. Usually partial immunity to infection is acquired by adults between 25 and 49 years, with a further decline in infection seen in the following decades.[29] Several immunological factors seem to be involved in the acquisition of this partial immunity, with increased INF-γ production by T cells considered a crucial part of the process.[25,27,29] In the current study, we observed that there is an increase in the frequency of $CD16^+$ IFN-γ^+ cells in individuals over 50 years who live in endemic areas for *S. mansoni*. However, in subjects over 70 years old, negative individuals have significantly higher numbers of $CD16^+$ IFN-γ^+ cells than positive individuals after *in vitro* stimulation of their peripheral blood cells with both egg antigen (Fig. 1B,C) and worm antigen (Fig. 1E,F). This suggests that negative individuals are those who sustain a high frequency of IFN-γ^+ NK cells as they age. Interestingly, in individuals of the same group, the frequency of $CD4^+$ and $CD8^+$ cells from either negative or positive individuals that produce IFN-γ are not increased upon *in vitro* stimulation for 5 h with the same antigens, suggesting that the increase in IFN-γ expression by NK cells at this time point is T-cell independent (Figs. 2 and 3). It is likely that 5 h is too short a period of time for antigen presentation and stimulation of T cells in culture. On the other hand, NK cells may interact directly with proteins present in parasite extracts being stimulated earlier than T cells. Mechanisms of innate immunity are usually more promptly responsive to antigen stimuli that acquired immune responses.[30] The nature of this interaction between NK cells and parasite antigens is currently under investigation. Of note, the frequency of $CD4^+$ IFN-γ^+ cells is slightly increased in control cultures of negative but not of positive individuals over 70 years old (Fig. 2A,B). These differences are not observed after *in vitro* stimulation suggesting that $CD4^+$ IFN-γ^+ cells may also be related to infection status in the elderly but their involvement does not correlate with $CD16^+$ IFN-γ cells.

Schistosomiasis is a disease that is marked by an acute inflammatory phase dominated by Th1 cytokine responses followed by a chronic stage where inflammatory Th1 response cohabits with immunomodulatory processes that include production of regulatory cytokines such as IL-10.[19,20] The downsizing of inflammatory reaction against schistosome eggs in the liver and in the intestine with a concomitant switch in the cytokine pattern to a Th2/T-regulatory type of T-cell responses are important in limiting tissue damage.[31] Chronic stages of infection are already present in young and middle-aged individuals in endemic areas since they acquire infection very early in their lives. These regulatory circuits of the chronic phase seem to co-exist in middle-aged individuals with other immunological mechanisms that are able to provide protection to re-infection. Most of these mechanisms dependent on T cells.[25,27,29]

In our study, an increased frequency of $CD8^+$ IFN-γ cells was observed in all individuals of the young group (10–24 years old). After that, a drastic decline in the expression of this cytokine is detected in positive but not in negative individuals over 24 years of age regardless of their infection status and antigen stimulation *in vitro*, indicating that $CD8^+$ cells may be related to infection status in young and middle-aged subjects. It is possible that other Th1 cytokines, such as tumor necrosis factor-α (TNF-α), or humoral immune responses, such as specific IgE production, also act as important players in the acquired immunity to *S. mansoni* in young and middle-aged negative individuals. Some authors have already suggested a protective role for specific IgE in experimental models of infection with *S. mansoni*[32,33] and in human schistosomiasis.[34] Others have found no correlation between specific IgE and resistance to infection.[35,36] Production of TNF-α by T cells have been implicated in protection against re-infection by *S. mansoni* in humans,[27] although some authors have shown that TNF-α may be also associated with increased risk of periportal fibrosis and aggravation of disease.[27]

As individuals age, major immunological events such as T-cell activation and cytokine production are progressively altered. It is possible that the decline in immunological activities in the elderly will combine with chronically sustained suppressive mechanisms to prevent a protective immune response to *S. mansoni* antigens in some individuals. Indeed, the proportion of IFN-γ^+ $CD4^+$ T cells is decreased in positive individuals over 70 years old in the endemic areas we studied (Fig. 2A,B). At the same time, the immunomodulatory mechanisms must be fully active in these individuals since they are infected but bearing an intestinal form of the disease. In this scenario, healthy aging in endemic areas may imply alternative mechanisms of protective immune responses against re-infection that coexist with immunoregulation. Our data suggest that there is an association between infection status and frequency of antigen-stimulated INF-γ^+ NK cells among individuals over 70 years of age and that this phenomenon is T-cell independent. It is already reported that innate immunity is well preserved in the healthy elderly and that NK cells may represent a replacement, in older individuals, for the function exerted by T cells. Systematic research on immunosenescence in humans have shown that NK and NKT cells may be considered a marker of successful aging, and centenarians show an increased number of these cells *ex vivo*.[14,37] In our study, CD16 was used as a marker for NK cells, and since they were not tested for CD3 expression it is possible that $CD16^+$ cells producing IFN-γ include populations of NK and NKT cells. This is the first study to report on the immunological status of the elderly infected with *S. mansoni*. Longitudinal studies may reveal in more detail the important role for NK cells among old individuals residing in endemic areas. Due to the progressive increase in the aged population in endemic areas, these studies are crucial for the design and implementation of disease control strategies.

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Informed consent — The study was approved by the National Ethics Committee of Brazil and the Internal Review Board of the Centro de Pesquisas Rene Rachou.

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