Evaluation of lymphocyte levels in a random sample of 218 elderly individuals from São Paulo city

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Background: *Age-associated changes in the immune system cause decreased protection after vaccination and increased rates of infections and tumor development.*

Methods: *Lymphocyte percentages were compared by gender and age to establish differences between subtypes. Three mL blood samples were obtained from 218 randomly selected individuals (60-101 years old) who live in São Paulo city. Blood was lysed with Tris phosphate buffer and stained for 30 minutes with monoclonal antibodies (CD3PerCP, CD4FITC, CD8Pe, CD19Pe) for analysis by flow cytometry. Statistical analysis was by ANOVA.*

Results: *The percentage of $CD4^+$ T cells (p-value = 0.005) and the CD4/CD8 ratio (p-value = 0.010) were lower in men, whereas the percentage of $CD8^+$ T cells was lower (p-value = 0.002) in women; the percentage of B cells ($CD19^+$) was similar between groups. Individuals grouped by gender and age range and compared showed a drop in $CD4^+$ cells in 75 to 79-year-old men (female: 46.1% ± 8.1% and male: 38.8% ± 10.5%; p-value = 0.023). Also, the 80 to 84-year-old group of men had a higher percentage of $CD8^+$ (female: 20.8% ± 8.2%, and male: 27.2% ± 8.2%; p-value = 0.032). Low percentages of B cells were detected in men in the 75 to 79-year-old (p-value = 0.003), 85 to 89-year-old (p-value = 0.020) and older than 90 year old (p-value = 0.002) age ranges.*

Conclusion: *Elderly men present with more changes in lymphocyte subsets compared to elderly women. These findings could demonstrate impairment in the immune response since the lower $CD4^+$ in men would provide less help to B cells (also lower in men) in terms of antibody production. In addition, the increase in $CD8^+$ cells in this group could represent chronic inflammation observed during the aging process.*

Keywords: *Aging; Sex distribution; Immune System; Lymphocytes; Flow Cytometry*

Introduction

The rates of morbidity and mortality due to infectious diseases are high in elderly individuals. This population is more susceptible to severe infections, presents a slower recovery from infections, and the response to vaccination is not effective in all individuals. It is believed that changes in the immune system occurring in individuals after the age of 60 (immunosenescence) provide adequate conditions for susceptibility to infectious diseases, autoimmunity and the development of cancer. As an example, influenza vaccine is protective in 40-60% of over 65-year-old patients[1] while in younger individuals this percentage is higher.[2-5]

Immunosenescence affects all cells from the immune system to some extent. In addition, the contribution of this system to longevity and healthy aging remains unknown.[6-8]

Subclinical pathogenic infections, which are very common in the elderly, cause persistent inflammation and thus contribute to tumor development, heart attacks and strokes.[9] It has already been demonstrated that in elderly individuals, persistent infections by herpes, cytomegalovirus (CMV) or parasites induce higher serum levels of pro-inflammatory factors (eg. IL-1, IL-6, tumor necrosis factor-alpha) leading to the aforementioned adverse clinical statuses.[10-13] In addition, older CMV seropositive adults present up to 25% of the total $CD8^+$ T cells pool specific for CMV immunodominant epitopes;[14] the expansion of CMV-specific $CD8^+$ T cells alters the capacity of the immune system to respond to other pathogens. These cells are able to secrete pro-inflammatory cytokines and contribute to an ongoing inflammatory process.[14]

Thymus involution, memory T cell accumulation, a decreased repertoire of naïve T cells and a diminished B cell population are changes that occur to the immune system

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during aging; this can contribute to a deficient response in elderly individuals. Predicting responsiveness to vaccination, infectious diseases and tumor development using biological markers that distinguish between healthy and immuno-senescent states is desirable as this might lead to more adequate therapies for the elderly population.

In order to determine possible changes in the subtypes of circulating lymphocytes in the elderly, these cells were evaluated in a random sample of 218 male and female individuals aged 60 to 101 years old from São Paulo city in Brazil. The percentages of $CD4^+$ and $CD8^+$ T cells, the $CD4^+$/$TCD8^+$ T cell ratio and the percentage of B cells ($CD19^+$) were evaluated.

Methods

A random sample of 218 individuals from São Paulo city who agreed to participate in this project was investigated. This population (men and women) were aged from 60 to 101 years old. From 3 mL of blood collected in EDTA from each individual, 100 µL were used to determine each cell phenotype. Blood (100 µL) was lysed with Tris buffered solution for 10 minutes and centrifuged at 377 g for 5 minutes. Cells were washed in PBS twice and centrifuged at 377 g for 5 minutes. Cells were incubated with monoclonal antibodies (CD3PerCP, CD4FITC, CD8Pe - tritest, CD19Pe; BD Biosciences, San Jose, California) to determine the percentages of T and B lymphocytes using flow cytometry in a FACSCalibur cell counter (BD Biosciences).

Statistical analysis

ANOVA was used to compare CD4, CD8, CD4/CD8 and CD19 cells in respect to two variables (age and gender). Differences were evaluated between the following age ranges: 60-64, 65-69, 70-74, 75-79, 80-84, 85-89 and more than 90 years old. Bartlett´s test was used for equal variances and significant differences between means were identified using Sidak's multiple comparisons.[15-17] The level of statistical significance was defined for a p-value < 0.05.

Results

On comparing lymphocyte phenotypes in respect to age and gender, the percentages of CD4 and CD8 T cells and the CD4/CD8 T cell ratio were statistically similar for age but not for gender (Figure 1). On the other hand, significant differences were detected only for age when percentages of B cells ($CD19^+$) were compared between age groups.

Possible differences between the percentages of cells of women and men in respect to age were then evaluated.

The percentage of $CD3^+CD4^+$ T cells in men was lower in all age groups compared to women (Table 1). However, a significant difference was only observed in the 75 to 79 age range (p-value = 0.023). An analysis of the percentage of

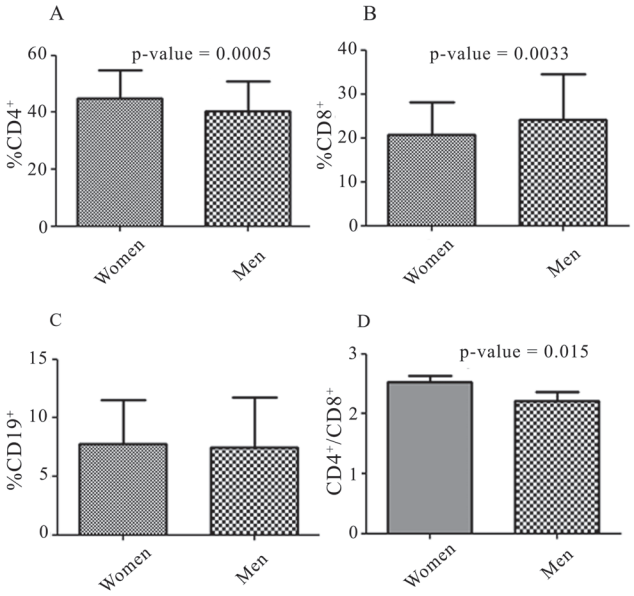

Figure 1 – Analysis of $CD3^+CD4^+$ T cells, $CD3^+CD8^+$ T cells, $CD19^+$ B cells and the $CD4^+/CD8^+$ ratio in the blood of 218 over 60-year-old individuals. Women presented with a higher percentage of $CD4^+$ T cells compared to men (p-value = 0.0005) whereas men presented with a higher percentage of $CD8^+$ T cells compared to women. The percentage of $CD19^+$ B cells was similar between women and men and the $CD4^+/CD8^+$ ratio was significantly higher in women.

Table 1. Percentage of $TCD3^+CD4^+$, $TCD3^+CD8^+$ and $TCD4^+/TCD8^+$ ratio determined in the peripheral blood of 60 to 101-year-old female and male individuals

	$CD3^+CD4^+$		$CD3^+CD8^+$		$CD4^+$/ $Cd8^+$ratio	
Age range (years)	Female %	Male %	Female %	Male %	Female	Male
60-64	45.6 ± 6.0 (n=9)	41.4 ± 15.3 (n=5)	24.6 ± 7.3 (n=9)	28.5 ± 11.4 (n=5)	2.1 ± 0.9 (n=9)	1.8 ± 1.1 (n=5)
65-69	43.9 ± 12.4 (n=19)	42.3 ± 11.0 (n=13)	18.8 ± 7.9 (n=19)	24.6 ± 9.8 (n=13)	2.8 ± 1.3 (n=19)	2.2 ± 1.4 (n=13)
70-74	44.7 ± 7.1 (n=16)	40.7 ± 7.8 (n=16)	22.3 ± 9.3 (n=16)	22.7 ± 11.4 (n=16)	2.5 ± 1.4 (n=16)	2.3 ± 1.2 (n=16)
75-79	46.1 ± 8.3 (n=21)	38.8 ± 10.5 (n=16)*	20.5 ± 5.2 (n=21)	23.6 ± 7.1 (n=16)	2.4 ± 0.8 (n=21)	1.9 ± 1.1 (n=16)
80-84	47.0 ± 12.5 (n=23)	40.5 ± 12.8 (n=13)	20.8 ± 8.2 (n=23)	27.2 ± 8.2 (n=13)#	2.6 ± 1.4 (n=23)	1.8 ± 1.3 (n=13)
85-90	41.6 ± 9.7 (n=18)	40.5 ± 10.5 (n=16)	19.6 ± 6.6 (n=18)	23.9 ± 12.2 (n=16)	2.4 ± 1.1 (n=18)	2.3 ± 1.4 (n=16)
90+	44.0 ± 9.4 (n=19)	38.7 ± 11.0 (n=14)	20.3 ± 8.1 (n=18)	24.7 ± 12.5 (n=14)	2.7 ± 1.6 (n=18)	1.9 ± 09 (n=14)

* In the 75 to 79-year-old age range there was a significant difference for $CD3^+CD4^+$ T cells between women and men (p-value = 0.023)

In the 80 to 84-year-old age range there was a significant difference for $CD3^+CD8^+$ T cells between women and men (p-value = 0.032)

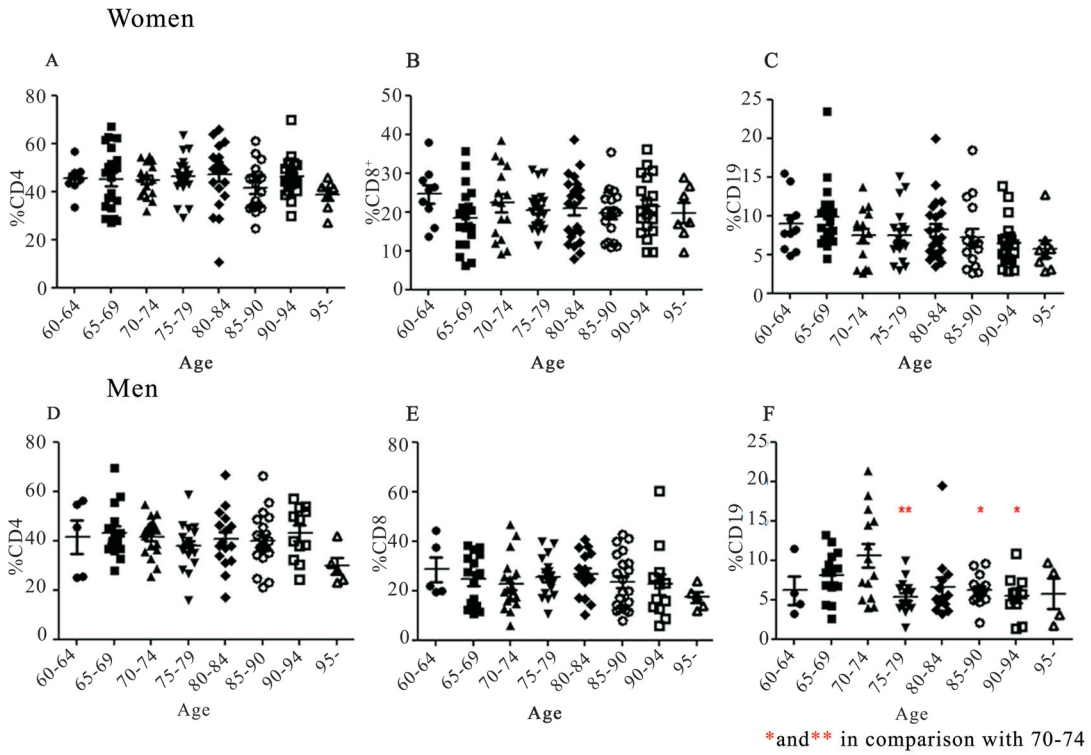

Figure 2 – Percentages of $CD3^+CD4^+$ T cells, $CD3^+CD8^+$ T cells and $CD19^+$ B cells of women (A, B and C) and men (D, E and F). No significant differences were seen for CD4 (A), CD8 (B) and CD19 (C) cell populations in women of different ages. Men presented with differences in CD19 cell populations (F) in the 70-74 age range compared with the 75-79 age range (p-value = 0.003), 85-89 age range (p-value 0.02) and the 90-94 age range (p-value 0.03). No significant differences were observed for the CD4 (D) and CD8 (E) cell populations in men

$CD3^+CD8^+$ T cell lymphocytes showed that this cell population was higher for men in all age groups with a significant difference for the 80-84 age range (p-value = 0.032). There was no significant difference in the CD4/CD8 T cell ratio on comparing women and men with similar ages.

A significant difference was found for the percentage of B cells (CD19) only for age. No statistical differences were found on comparing women of different age ranges. On the other hand, for men there was a statistical difference on comparing the 70 to 74-year-old age range with the 75 to 79-year-old (p-value = 0.003), 85 to 89-year-old (p-value = 0.020) and more than 90-year-old (p-value = 0.002) age ranges (Figure 2).

Discussion

Changes in the immune system occurring during aging have been associated with increases in morbidity and mortality.[18] A Swedish study (Immune Longitudinal Study) established high levels of CD8 and low levels of CD4 and CD19 cells as predictive for mortality within 2 years.[19] Using a random sample of elderly individuals from São Paulo city as the study group the same predictive parameters cited above were evaluated to see the effects of gender and age on the percentages of peripheral lymphocytes.

The percentage of $CD3^+CD4^+$ T cells was higher in women than in men when all individuals were compared without taking age into account. Moreover, the percentage of $CD3^+CD8^+$ T cells was higher in men than in women.

$CD3^+CD4^+$ T lymphocytes are essential to provide help for the proliferation (through the production of IL-2) and differentiation of other cells (i.e. IL-4 and IL-5 inducing B cell production of antibodies). Therefore, the low percentage of $CD3^+CD4^+$ T lymphocytes observed in men suggests that they are more likely to present impaired immune responses against pathogens and after vaccination.

Cytotoxic T lymphocytes increase in the elderly due to chronic infections (i.e. CMV) causing the accumulation of oligoclonal $CD8^+$ T cells which interfere in immune responses either to vaccinations or infections.[20,21] In this study men had a higher percentage of $CD3^+CD8^+$ T cells compared to women. On comparing ages this percentage was significantly higher in 80 to 84-year olds. De Fanis et al.[22] evaluated whether changes in the percentage of immune system cells could be related to frailty in 72 to 94-year old patients. Patients who were classified as frail had a significantly higher percentage of $CD8^+$ T cells and a lower percentage of $CD4^+$ T cells than the non-frail group. The results of this study suggest than elderly men present not only an impaired immune response but could also be frailer than women.

In this study there was a trend toward a higher CD4/CD8 ratio in women of all age groups. Hussein et al.[23] showed that young healthy individuals presented a mean CD4/CD8 ratio of 1.8 ± 0.8 which was slightly higher in over 50-year-old individuals with osteoarthritis (2.2 ± 0.4). Wikby et al.[24] observed similar CD4/CD8 ratios in healthy young men and women (1.55 for men and 1.77 for women). These authors showed that the CD4/CD8 ratio increases significantly during aging whereas in men this ratio remained relatively unchanged.

A Swedish study comparing age, matched 20 to 92-year-old male and female individuals and showed that women present a higher percentage of $CD3^+CD4^+$ cells than men during the entire adult lifespan. Moreover, the CD4/CD8 ratio was higher, albeit without statistical significance, in 20 to 59-year-old women whereas from 60 to 92 years old this ratio was significantly higher in women. The authors believe that there are specific gender differences in the processes that produce mature $CD4^+$ and $CD8^+$ cells in the thymus and that this could, at least in part, explain the higher longevity observed in women.[24]

It has been shown that in over 60-year-old individuals, B lymphocytes ($CD19^+$) are lower not only in numbers[25] but also in function. The capacity of these cells to produce antigen-specific antibodies after vaccination is less due to reduced expansion and differentiation. However the immune response generated before the immunosenescence process is maintained, as was showed by Yu et al.[26] during an evaluation of individuals who survived an influenza epidemic in 1918. The results of this study add new information to published data as the male population presented a trend toward a lower percentage of B cells than the female population. In the male group a more dramatic decrease in the B cell percentage was observed from the age of 75 years old. This result was different for women who presented a more constant percentage of B cells in all age ranges. This result suggests that men could be more susceptible to infections or produce lower antibody levels after vaccination.

In the elderly population the production of antibodies may also be impaired by changes in T helper cells which should send stimuli to B cells. In agreement this study, found that in men the percentage of $CD3^+CD4^+$ T cells was lower than in women. Therefore, the decreased percentage of B cells observed for men could be due to the decreased percentage (and maybe also function) of T cells found in male individuals.

Conclusion

This study showed that in a random sample of elderly from São Paulo, men suffered greater changes in lymphocyte subsets compared to women. These findings may represent impairment of the immune system as the lower percentage of $CD4^+$ cells in men would provide less help to B cells (also fewer in men) to produce antibodies. In addition, the increase in the percentage of $CD8^+$ T cells in this group may represent chronic inflammation observed during the aging process. This profile observed in men reflects the parameters established in the Immune Longitudinal Study which are predictive of 2-year mortality rates. More studies are needed to confirm whether cell functions are also impaired differently according to gender and age in order to provide the best therapy for aging people.

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