Abnormal phenotypic distribution of regulatory and effector T cells in octogenarian and nonagenarian women

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SUMMARY

Introduction: aging is associated with several immunologic changes. Regulatory (Treg) and effector T cells are involved in the pathogenesis of infectious, neoplastic, and autoimmune diseases. Little is known about the effects of aging on the frequency and function of these T cell subpopulations.

Methods: peripheral blood mononuclear cells (PBMC) were obtained from 26 young (under 44 years old) and 18 elderly (above 80 years old) healthy women. T cell subpopulations were analyzed by flow cytometry.

Results: elderly individuals had lower frequency of several activated effector T cell phenotypes as compared with young individuals: CD3+CD4+CD25+ (3.82±1.93 versus 9.53±4.49; p<0.0001); CD3+CD4+CD25+CD127- (2.39±1.19 versus 7.26±3.84; p<0.0001); CD3+CD4+CD25+ (0.41±0.22 versus 1.86±0.85; p<0.0001); and CD3+CD4+CD25hiCD127- (0.06±0.038 versus 0.94±0.64; p<0.0001). Treg (CD3+CD4+CD25+Foxp3+) presented lower frequency in elderly individuals as compared to young adults (0.34±0.18 versus 0.76±0.48; p=0.0004) and its frequency was inversely correlated with age in the whole group (r=-0.439; p=0.013). The elderly group showed higher frequency of two undefined CD25hiFoxp3- phenotypes: CD3+CD4+CD25hiFoxp3- (15.05±7.34 versus 1.65±1.71; p<0.0001) and CD3+CD4+CD25hiCD127hiFoxp3- (13.0±5.52 versus 3.51±2.87; p<0.0001).

Conclusions: the altered proportion of different T cell subsets herein documented in healthy elderly women may be relevant to the understanding of the immunologic behavior and disease susceptibility patterns observed in geriatric patients.

Keywords: T-lymphocytes, aging, regulators T-lymphocytes.

INTRODUCTION

Aging is associated with decreased humoral immune response, shortened duration of protective immunity, decline in T cell diversity and thymus function, and decreased ability in T cell response to novel antigens. As a consequence, aging is associated with diminished immunity against microorganisms and malignant cells, as well as tolerance breakdown and increased frequency of autoimmune diseases. Naturally occurring regulatory T cells (Treg) are key regulators in the control of immune responses, with significant impact on autoimmunity, tumor immunity, infection, allergy and transplant tolerance. Among other characteristic features, these cells express the transcription factor Foxp3, high levels of membrane CD25, and low levels of membrane CD127. There have been some studies pointing to an increase in the proportion of CD25 regulatory T cells in the peripheral blood of aged BALB/c and C57BL/6 mice as well as in elderly people. Such imbalance might contribute to the decreased immune functional activity and
In face of the controversial literature on the frequency of Treg cells in aged people, and considering the evolving paradigm on Treg cell phenotypic characterization,\textsuperscript{28,29} we have studied the frequency of Treg cells based on the evaluation of a stringent phenotypic profile (CD3\textsuperscript{+} CD4\textsuperscript{+}CD25\textsuperscript{high}CD127\textsuperscript{med}Foxp3) in healthy volunteer women over a wide age range. With the understanding that the immune function is ruled by the equilibrium among several cellular phenotypes, we concurrently studied the frequency of activated effector T cells, a major T cell phenotype counteracted by Treg cells. We could not confirm previous reports of increased Treg cell frequency in elderly individuals. Instead, we observed a decline in the frequency of T cells, expressing markers related with cell activation and a higher proportion of two yet undefined Foxp3 CD25\textsuperscript{high} phenotypes in elderly women.

\section*{Methods}

\subsection*{Study population}

Eighteen octogenarian and nonagenarian women were recruited from the cohort of healthy elderly individuals at the geriatric division at Universidade Federal de São Paulo, with ages between 80 and 93 (85±64.6 years old). The young adult group (<44 years old) comprised 26 healthy women with age between 19 and 44 years (29.23±6.63 years old), recruited among workers at the Medical School at Universidade Federal de São Paulo. All enrolled individuals were not receiving immunosuppressive medication and had no chronic infection, malignancy or cognitive impairment. In addition, there was no previous or current evidence of allergy or autoimmune diseases. Several of the elderly individuals did receive medication for arterial hypertension, diabetes mellitus, hypercholesterolemia or age-related heart diseases. Informed consent was obtained from all participants and the study was approved by the institution’s ethics committee.

\subsection*{Phenotypic evaluation of peripheral CD3 CD4 T lymphocytes}

The phenotype of peripheral CD3\textsuperscript{+}CD4\textsuperscript{+} T cells was analyzed according to the expression of CD25, CD127 and Foxp3 in 44 healthy women, divided according to two age strata: young and elderly women. Peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation in Ficoll Paque\textsuperscript{™} Plus (GE Healthcare Life Science, Pittsburgh, PA) and cryopreserved in 90\% fetal bovine serum (FBS) and 10\% dimethyl sulfoxide in liquid nitrogen. For labeling with antibodies, cells were quickly defrosted at 37\°C, centrifuged and re-suspended on fresh RPMI medium with 10\% FBS. Next cells were washed in phosphate buffer saline (PBS) and 0.5x10\textsuperscript{6} cells were incubated with FITC-labeled anti-CD127, APC-Cy3-labeled anti-CD3, PerCP-labeled anti-CD4, and PE-Cy7-labeled anti-CD25, according to the manufacturer’s instructions (Becton Dickinson, San Jose, CA). After 30 minutes at 4\°C cells, were washed with Macs\textsuperscript{®} buffer, fixed and permeabilized with Foxp3 fixation/permeabilization buffer (eBioscience, San Diego, CA) and, then, processed for Foxp3 staining using Foxp3 staining kit and APC-labeled anti-Foxp3 (eBioscience, San Diego, CA), according to the manufacturer’s instructions. For establishment of background staining and threshold for genuine expression of each phenotypic marker, we used the fluorescence minus one (FMO) method.\textsuperscript{30} Cells were analyzed in a FACSCanto\textsuperscript{®} flow cytometer (Becton Dickinson, San Jose, CA) and the obtained data were analyzed using the FlowJo software (Tree Star Inc, Ashland, OR). Three hundred thousand events were acquired for each sample. Parameters were expressed as mean and standard error (SE). Cell frequency was expressed as the percent frequency of each subpopulation relative to total CD3 cells.

\subsection*{Statistical analysis}

Comparison of quantitative parameters between two groups was performed using Student’s t test. Linear regression analysis (Spearman or Pearson correlation test, depending on the distribution pattern of the variable) was performed to determine the relationship between age in years and the frequency of different cell phenotypes. Differences were considered significant if p-values were less than 0.05.

\section*{Results}

The frequency of activated effector T cells was lower in elderly subjects as compared with young individuals, either when analyzing the CD3\textsuperscript{+}CD4\textsuperscript{+}CD25\textsuperscript{phenotype (3.82±1.93\% versus 9.53±4.49\%; p<0.0001), the CD3\textsuperscript{+} CD4\textsuperscript{+}CD25\textsuperscript{med}CD127\textsuperscript{phenotype (2.39±1.19\% versus 7.26±3.84\%; p<0.0001), the CD3\textsuperscript{+}CD4\textsuperscript{+}CD25\textsuperscript{high} phenotype (0.06±0.038\% versus 1.86±0.85\%; p<0.0001) and the CD3\textsuperscript{+}CD4\textsuperscript{+}CD25\textsuperscript{high}CD127\textsuperscript{phenotype (0.94±0.64\% versus 0.94±0.64\%; p<0.0001) (Figure 1 A-D and Table 1). In fact, there was an inverse correlation between the frequency of all these T lymphocyte activated phenotypes and
age (Table 1). Next, it was investigated whether the frequency of Treg cells, based on the expression of CD3, CD4, CD25, CD127, and Foxp3, was altered in elderly individuals (>80 years old) compared with young adults (<44 years old). In contrast to what we observed for effector T cells, there was an ambiguous representation of the several T cell phenotypes possibly associated with Treg function. No significant difference between elderly and young women was found in the frequency of phenotypes CD3<sup>-</sup>CD4<sup>+</sup>CD25<sup>-</sup>Foxp3 (1.51±0.95% versus 1.42±0.76%; p=0.74) and CD3<sup>+</sup>CD4<sup>-</sup>CD25<sup>-</sup>CD127<sup>-</sup>Foxp3 (1.02±0.75%; versus 1.26±0.60%; p=0.26) (Table 1). However, the CD3<sup>+</sup>CD4<sup>-</sup>CD25<sup>high</sup>Foxp3 and CD3<sup>-</sup>CD4<sup>+</sup>CD25<sup>high</sup>CD127<sup>+</sup>Foxp3 phenotypes, occurred at a decreased frequency in elderly individuals as compared to young people (0.47±0.16% versus 0.85±0.64%; p=0.014 and 0.34±0.18% versus 0.76±0.48%; p=0.004, respectively) (Figure 1 A-D and Table 1). In addition, the percentage of these two T cell phenotypes possibly associated with Treg function was found in the frequency of phenotypes CD3<sup>-</sup>CD4<sup>-</sup>CD25<sup>-</sup>Foxp3 and CD3<sup>+</sup>CD4<sup>-</sup>CD25<sup>high</sup>CD127<sup>+</sup>Foxp3 presented an inverse correlation with age among the 26 young and 5 elderly individuals (r=-0.355 p=0.049 and r=-0.439 p=0.013, respectively) (Table 1).

Interestingly, there was a strikingly higher frequency of two CD25<sup>+</sup> phenotypes in elderly people as compared with the young group (Figure 2A-B, Table 1). The relative frequency of the CD3<sup>-</sup>CD4<sup>-</sup>CD25<sup>high</sup>Foxp3 phenotype was 15.05±7.34% in the elderly group versus 3.51±2.87% in the young group (Figure 2C-D, Table 1). The relative frequency of the CD3<sup>-</sup>CD4<sup>-</sup>CD25<sup>high</sup>CD127<sup>-</sup>Foxp3 phenotype was 13.0±5.52% in the elderly group against 3.51±2.87% in the young group (Figure 2A-B, Table 1). The relative frequency of the CD3<sup>-</sup>CD4<sup>-</sup>CD25<sup>high</sup>CD127<sup>-</sup>Foxp3 versus CD3<sup>-</sup>CD4<sup>-</sup>CD25<sup>high</sup>CD127<sup>-</sup>Foxp3 was significantly lower frequency in elderly as compared to young individuals. These findings suggest a possible decrease in the production of Treg cells in elderly individuals. Studies from 2005 to 2008 have found an increased frequency of circulating Treg cells in elderly people.13-15,17-21,31

The apparent discrepancy of the present findings and earlier studies in the literature may be related to the combination of phenotypic markers used for definition of Treg cells. In fact, these results are more comparable with more

### Table 1: Relative frequency of different CD3 CD4 T cell phenotypes in elderly and young healthy women.

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<th>T lymphocyte phenotype</th>
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<th>Mean and standard deviation*</th>
<th>Correlation with age</th>
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* Relative frequency expressed as percentage of CD3 cells.
recent studies that used phenotypic markers similar to these and showed no increase in Treg cell frequency in elderly individuals.\textsuperscript{4,26} Other possible factors contributing to discrepancy in the literature include peculiarities in subject selection and methodological aspects (e.g., frozen versus fresh cells, cell handling protocols, and flow cytometry parameters). In particular, it should be noted that the present study analyzed only females, and it has been shown previously that sexual hormones may influence the frequency of Treg cells.\textsuperscript{32} The actual impact of the modest magnitude of decrease in Treg cell frequency observed in this series (average 0.69% to 0.29%) on tolerance maintenance remains to be determined. It should be noticed that such a decrease was observed in successful octogenarians and nonagenarians, and, therefore, should be regarded as a tendency along the normal aging process. However, this may lead to predisposition for surpassing the threshold necessary for immune disequilibrium in some individuals, favoring the appearance of autoantibodies and autoimmune diseases in susceptible individuals. Although B and T lymphocyte response to antigenic stimulus has been shown to decline in aged in-
individuals, paradoxically there is an increase in the frequency of autoantibodies and organ-specific autoimmune diseases in elderly people. This apparent incongruity might be reconciled by the finding of a reduced frequency and function of Treg cells. Therefore, further studies are granted to investigate whether the slight reduction in Treg frequency observed in this study is functionally relevant to tolerance maintenance in elderly individuals.

Interestingly, there was a strikingly higher frequency of two yet undefined CD4+CD25- phenotypes (CD3+CD4+CD25-Foxp3 and CD3+CD4+CD25-CD127-Foxp3) in elderly as compared with young women (Figure 2A-B, Table 1). Accordingly, there was a significant correlation between the frequency of these two T cell phenotypes and age (r=0.71 and 0.74, respectively, p < 0.0001) (Figure 2C-D). This has not been reported previously in elderly individuals, possibly due to a lack of focus on these particular T cell subsets. However, in previous studies the authors and others have shown these cells to be increased in patients with active systemic lupus erythematosus. Bonelli et al. have shown that CD3+CD4+CD25-Foxp3 cells are able to suppress effector T cell proliferation, but not IFN-γ production in vitro. In view of this, we hypothesize that these cells may represent intermediate or incomplete phenotypes towards Treg or effector T cells. It is possible that the increased frequency of these incomplete phenotypes in elderly people would be a consequence of counteracting mechanisms in a senescent immune system.

In the present study, the authors demonstrated some interesting peculiarities in the frequency of effector and regulatory T CD4 cells in the peripheral blood of healthy octogenarians and nonagenarians. These were characterized by a decrease in the frequency of activated effector T cell phenotypes and of the T cell phenotype, most strictly associated with Treg function (CD3+CD4+CD25-Foxp3). Interestingly, there was a strikingly increased frequency of two still undefined CD4+CD25+Foxp3 and CD3+CD4+CD127-Foxp3. The knowledge obtained about diverse T cell subsets in elderly people should be of practical relevance to the search for means of increasing the efficacy of vaccination, boosting immunity in cancer, and avoiding tolerance breakdown,
Distribuição fenotípica anormal das células T reguladoras em mulheres octogenárias e nonagenárias

Introdução: o envelhecimento está associado a diversas alterações imunológicas. Células T reguladoras e efetoras estão envolvidas na patogênese de enfermidades infecciosas, neoplásicas e autoimunes. Pouco se sabe acerca dos efeitos da idade sobre a frequência e a função dessas populações celulares.

Métodos: células mononucleares do sangue periférico foram obtidas de participantes saudáveis (26 de idade inferior a 44 anos e 18 acima de 80 anos). As subpopulações celulares foram analisadas por citometria de fluxo.

Resultados: o grupo constituído por idosas apresentou menor frequência de vários fenótipos de células T efetoras ativadas em comparação com jovens: CD3⁺CD4⁺CD25⁺ (3,82±1,93 versus 9,53±4,49, p<0,0001); CD3⁺CD4⁺CD25⁺CD127⁺ (2,39±1,19 versus 7,26±3,84, p<0,0001); CD3⁺CD4⁺CD25⁺FoxP3⁺ (0,41±0,22 versus 1,86±0,85, p<0,0001); CD3⁺CD4⁺CD25⁺FoxP3⁺ (0,06±0,038 versus 0,94±0,64, p<0,0001). As células T reguladoras CD3⁺CD4⁺CD25⁺FoxP3⁺ apresentaram menor frequência em indivíduos idosos em comparação com adultos jovens (0,34±0,18 versus 0,76±0,48, p=0,0004) e sua frequência foi inversamente correlacionada com a idade em todos o grupo (r=-0,439; p=0,013). O grupo de idosas apresentou maior frequência de dois fenótipos indefinidos (CD25⁺FoxP3⁺), células CD3⁺CD4⁺CD25⁺FoxP3⁺ (15,05±7,34 versus 1,65±1,71, p<0,0001) e células CD3⁺CD4⁺CD25⁺FoxP3⁺ (13,01±5,52 versus 3,51±2,87, p<0,0001).

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Palavras-chave: linfócitos T, envelhecimento, linfócitos T reguladores.

RESUMO

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