Int. J. Morphol.,
39(3):789-796, 2021.

Reduced Naïve T Cell Numbers Correlate with Increased Low-Grade Systemic Inflammation During Ageing and Can be Modulated by Physical Activity

Un Número Reducido de Células T Vírgenes se Correlaciona con un Aumento de la Inflamación Sistémica de Bajo Grado Durante el Envejecimiento y puede ser Modulado por la Actividad Física

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MERELLANO-NAVARRO, E.; OLATE-BRIONES, A.; NORAMBUENA-MARDONES, L.; ROJAS-RAMOS, V.; PLATA-LUNA, M. A.; FAÚNDEZ-ACUÑA, J. Y.; MORAGA, J. C.; ESCOBEDO, N. & HERRADA, A. A. Reduced naïve T cell numbers correlate with increased low-grade systemic inflammation during ageing and can be modulated by physical activity. *Int. J. Morphol., 39(3)*:789-796, 2021.

SUMMARY: Age-associated decline of immune system, termed immunosenescence, is characterized by low-grade systemic inflammation, known as inflammaging, together with T-cell functional dysregulation. Although affecting all individuals, different environmental as well genetic factors impinge on the individual´s susceptibility or resilience to immunosenescence. Physical activity has been shown to improve autonomy and functionality in older adults. However, if physical activity affects immunosenescence or inflammaging remains unknown. The purpose of this study was to analyze immunosenescence and inflammaging in elderly individuals by measuring peripheral naïve T cells and interleukin (IL) -6 from peripheral blood and evaluate the impact of physical activity on T cell dysregulation and inflammaging. Thirty (30) elderly volunteers (10 males and 20 females), and 7 young controls (2 males ad 7 females), were recruited for this study. A methodology questionnaire was used to evaluate different parameters such as physical activity, and peripheral naïve $CD4^+$ and $CD8^+$ T cells and serum IL-6 were measured by FACS and ELISA respectively. Our results shown that naïve T cells decline, and IL-6 levels increase as older people age. Interestingly, we observed strong negative correlation between naïve T cells numbers and IL-6 levels in older adults, suggesting a direct link between reduced naïve T cell pool and increased inflammaging. Continuous physical activity during youth did not affect immunosenescence and inflammaging in elderly, but physical activity during elderly increase naïve T cell numbers and reduce inflammaging in older subjects. Our results showed reduced number of naïve T cells and increased levels of IL-6 as elder people get older. Moreover, the strong negative correlation between these parameters suggest that naïve T cells can have a direct suppressive activity over innate immune components. Furthermore, physical activity during elderly can reduce immunosenescence and inflammaging in older subjects.

KEY WORDS: Immune system; Naïve T cells; Immunosenescence; Inflammaging; Elderly; Physical activity.

INTRODUCTION

It is well known that ageing is responsible for diverse physiological dysfunctions in different organs and systems. One of the physiological systems showing dramatic changes by ageing is the immune system (Franceschi *et al*., 2017). This age-associated decline in immunity, known as immunosenescence, involves changes in both innate and adaptive immune components (Nikolich-Zugich, 2018). Regarding adaptive immune response, although affecting both humoral and cellular responses, changing in the T cell compartment is more evident and better documented. This is due mainly because of its role in orchestrate cellular responses and modulating of the immune system (Salam *et al*., 2013). Thus, diminished numbers of naïve T cells, primarily due to thymic involution, that leads to the shrinking of the TCR repertoire, together with increased numbers of memory T cells are mayor hallmarks of T cells immunosenescence (Lazuardi

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This work was supported by the following grants: Proyecto Enlace-Autónoma N° UA-EN2018-20 and FONDECYT de inicio N° 11190253 (A.H), FONDECYT de inicio N° 11160592 and FONDECYT Regular N° 1201562 (N.E).

et al., 2005). Along with the reduction in the adaptive immune arm, increased innate immune response leads to a chronic, low-grade inflammation, characterized by increased production of proinflammatory cytokines such as IL-6 and Tumor necrosis factor (TNF)-α, termed "inflammaging", another common factor during ageing (Franceschi & Campisi, 2014). A link between immunosenescence and inflammaging has been proposed by observation showing that memory $CD8^+$ T cells during ageing can acquire a senescent phenotype that could promote inflammaging (Akbar *et al*., 2016). Although this and other studies have described several factors capable of triggering inflammaging, it is less clear if negative signals or cells capable of modulating immune responses are compromised during ageing.

Physical activity (PA), described as any bodily movement resulting in energy expenditure, has been shown to improve the overall health, reducing the risk of suffering different disorders such as cardiovascular disease, diabetes and cancer among others (Hojman *et al*., 2018). In advanced ages, PA reduces the risk of contracting the most relevant non-communicable chronic disorders. Although older adults are less active, with 40 to 80 % of older people not meeting PA guidelines of at least 150 minutes of PA per week, PA in older individuals have been shown to provide beneficial effects on muscular strength, aerobic capacity, cognitive function and overall reducing the risk for chronic diseases (Sáez de Asteasu *et al*., 2017). However, how PA influences immunosenescence and inflammaging has received less attention. One recent study evaluating older people maintaining a high level of PA through cycling, showed increased numbers of circulating naïve T cells, and reduced numbers of memory T cells and IL-6 levels compared to healthy older controls, but PA did not show any effect over the frequency of senescent $CD8^+$ T cells (Duggal *et al*., 2018). However, a different study shows that aerobic fitness reduces senescent T cells (Spielmann *et al*., 2011). The differences between studies could be explained because different ages between groups, different physical status between participants, differential analysis used, the type of PA developed, among other variables. Moreover, since functional dependency, autonomy and physical conditions are different between older adults, if inflammaging or immunosenescence are distinct between groups remains to be evaluated.

Here, we described increased immunosenescence, characterized by the reduction of circulating naïve T cell numbers, and inflammaging, with increased levels of IL-6, as age increases. We observed strong negative correlation between reduced naïve T cells and increased inflammaging in the older population. Older people that realize PA have reduced inflammaging compared to sedentary older controls. Finally, older people with reduced autonomy showed enhanced immunosenescence and inflammaging compared to the autonomous older adults, but this can be attributed to the increase of age in this group.

MATERIAL AND METHOD

The study was performed in the city of Talca (Chile), through a single cross-sectional measurement. The sample was selected for convenience visiting day clubs, senior clubs, associations and senior residencies in different locations in the city. A final sample of 30 older participants and 7 young adults was obtained. Subjects suffering autoimmune diseases, infections in the last month, or body mass index (BMI) >40 kg/m^2 were excluded. The study was approved by the bioethics committee of the Universidad Autónoma de Chile (Ethical Application number 142-18). Subjects agreed to the study terms signing an informed consent document.

The application of the questionnaire and blood extraction were carried out on the same day in the health centers or senior residences. The total application time of the test was approximately 45 minutes. All measures were made by technical team who were previously trained.

Measurement instruments

Socio-demographic and lifestyle characteristics: A socio-demographic and lifestyle questionnaire, which included marital status, educational attainment and physical activity performed per week, was conducted on each individual.

Mean Arterial Pressure: Arterial pressure was assessed in two consecutive occasions via automated wrist sphygmomanometer, validated for its use in research studies (Omron, model HEM-6111). The results from this test were used in the criteria of exclusion "uncontrolled arterial hypertension".

Physical Functionality: Physical functionality was evaluated via Composite Physical Function scale (CPF), developed and validated by Rikli & Jones (1998), and adapted for its use in Chile (Merellano-Navarro *et al*., 2015). For sample characterization, CPF score was categorized as high functionality (score 24), moderate functionality (score between 14-23) and low functionality (score 13 or less).

Health-related quality of life: The Spanish version of the EQ-5D-5L was used to evaluate the HRQoL. The EQ-5D-5L value set based on Uruguayan population preferences was used to calculate this instrument since it is the only one developed for a South American country.

Physical Activity Level: International Physical Activity Questionnaire (IPAQ) short version: Questionnaire that measures the level of physical activity performed in the last seven days. It is a widely used and validated questionnaire in all populations, including older adults (Tomioka *et al*., 2011).

Flow cytometry analysis: Blood samples were received in heparinized tubes and immediately processed for flow cytometry analysis as described (Dagur & McCoy Jr., 2015). Briefly, samples were centrifuged, red blood cells were lysed using red blood cell lysis buffer, samples were washed, cells were counted and resuspended in PBS containing 2 % (w/v) BSA, with the following antibodies; anti-CD4 (clone SK3), anti-CD8a (clone RPA-T8), anti-CD3 (clone SK7), anti-CD25 (clone BC96), anti-CD45RA (clone HI100) and anti-CD45RO (clone UCHL1), all from Biolegend (San Diego, CA, USA). Samples were analyzed by flow cytometry using a FACS Calibur instrument and BD FACSDiva software (version 6.1.1; BD Biosciences, San Jose, CA, USA). $CD4^+$ naïve T cells were defined as $CD3^+CD4^+CD25^-CD45RA^{high}CD45RO^{low}$. $CD8^+$ naïve T cells were defined as $CD3^+CD8^+CD45RA^{high}CD45RO^{low}$.

ELISA analysis: Serum samples were obtained and stored at -80°C until use. IL-6 was measured using the ELISA MAX™ Deluxe Set Human IL-6 (San Diego, CA, USA) according to the manufacturer instructions. Samples were measured in an AutobioPHOmo microplate reader in a blinded manner as previously described (Herrada *et al*., 2020). Fourteen older volunteer samples and all young controls samples were below detection limits.

Statistical analysis. Statistical analysis was performed using GraphPad Prism 6 (GraphPad Software, San Diego, CA) software. Results are expressed as mean ± SD. Data were tested for normal distribution. Two-tailed Student's t test was used for comparison between the 2 groups. Pearson correlation test was used to find association between parameters. A p value < 0.05 was considered statistically significant (*p < 0.05; **p < 0.01; ***p < 0.001; ****p<0.0001).

RESULTS

Reduced circulating naïve T cells in older adults. In order to evaluate the number of circulating naïve T cells and serum IL-6, 30 older volunteers ranging from 65 to 94 years of age (average 77± 9 years) and 7 young volunteers ranging from 20 to 43 years of age (average 29±9 years), were recruited for this study. Table I lists the characteristics of older participants. 26 % of the participants were obese or overweight and 20 % of the older volunteers were categorized as active; 50 % of participants had low functionality. Health-related quality of life (HRQoL) measured by the EQ-5D-5L questionnaire, showed no significant differences between women versus men.

Table I. Characteristics of the study sample, by sex.

		All participants	Males	Female
		(n=30)	(n=10)	(n=20)
Age (years)		77.10 ± 9.083	78.20 ± 7.68	76.55 ± 9.84
Age Categories. n (%)				
	60-69	8 (26.7)	1 (10.0)	7 (35.0)
	70-74	5 (16.7)	2 (20.0)	3 (15.0)
	75-79	5 (16.7)	3 (30.0)	2 (10.0)
	80 +	12 (40)	4 (40.0)	8 (40.0)
BMI				
	Low weight	7(23.3)	1(10.0)	6(30.0)
	Normoweight	15 (50.0)	7 (70.0)	8(40.0)
	Overweight	5 (16.7)	2 (20.0)	3(15.0)
	Obese	3 (10.0)	0 (0)	3(15.0)
Physical Activity level by IPAQ				
	Low	19(63.3)	6(60.0)	13(65.0)
	Moderate	5(16.7)	0(0.0)	5(25.0)
	Higth	6(20.0)	4(40.0)	2(10.0)
CPF, n(%)				
	High functionality	7(23.3)	3(30.0)	4(20.0.)
	Moderate functionality	8(26.7)	0(0.0)	8(40.0)
	Low functionality	15(50.0)	7(70.0)	8(40.0)
EuroQol		0.7039 ± 0.33	0.699 ± 0.237	0.706 ± 0.373

Abbreviations: BMI: body mass index; CPF: composite physical function; IPAQ: International Physical Activity Questionnaire. Data are presented as mean ± Standard Deviation.

Dramatic reduction of circulating naïve CD4⁺ and CD8⁺ T cells were detected in the older population when compared to younger controls (Fig. 1). No differences in the number of naïve T cells were found when older volunteers where categorized by sex or body mass index (data not shown). These results suggest that ageing is one of the main contributors in reducing circulating naïve T cell numbers in the older population.

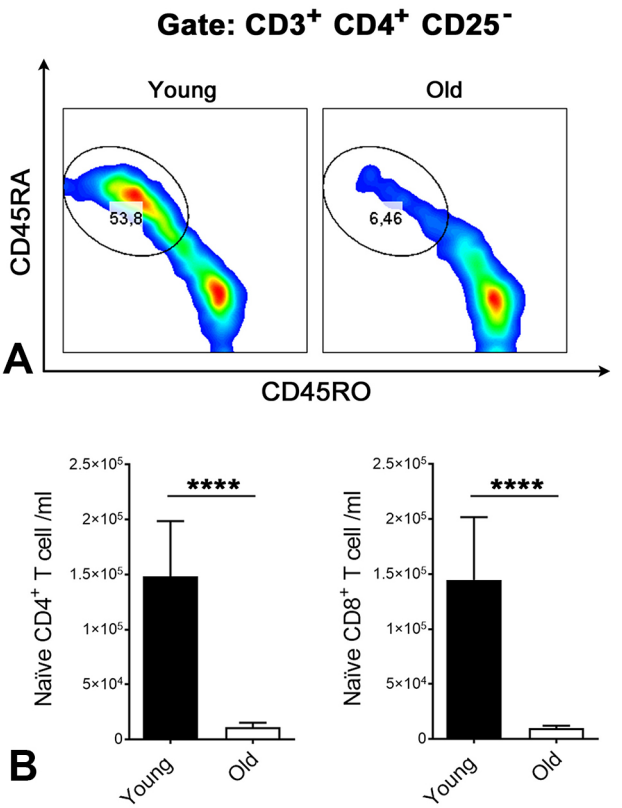


Fig. 1. Increased immunosenescence in older volunteers. A. Gating strategy used to define naïve CD4⁺ T cells and representative FACS profiles. B. Circulating numbers of naïve CD4⁺ (left) and naïve CD8⁺ (right) T cells, from younger and older volunteers. Error bars indicate SD. **** $p < 0.0001$ by two-tailed Student's t test.

Reduced immunosenescence and increased inflammaging as older adults get older. We next decided to analyze specifically the older population, evaluating the number of circulating naïve T cells and IL-6 levels from differently aged donors. Our results showed a negative correlation between naïve T cells and age, reducing both naïve CD4⁺ and CD8⁺ T cells as people get older (Fig. 2a). Moreover, inflammaging, measured by circulating levels of IL-6, positively correlated with age, suggesting that inflammaging also increased as older controls get older (Fig. 2b).

Reduced naïve T cell number correlates with increased inflammaging in the older population. Although inflammaging has been well described, the factors promoting inflammaging are still matter of debate. In fact, Regulatory T cells (Tregs), one of the main immune cells involved in dampening immune responses by promoting peripheral tolerance, seems to work even more efficiently during elderly (Garg *et al.*, 2014). Thus, it is possible that other cell(s) type(s) with potential suppressive capabilities could be affected during ageing. To explore this point further, we evaluated the association between circulating naïve T cell numbers and inflammaging, measured by serum IL-6 levels. We observed a strong negative correlation particularly between naïve CD4⁺ T cells and IL-6 levels (Figs. 3a and 3b), suggesting that naïve T cell could possess an unexpected suppressive activity over innate immune cell components.

PA at youth did not affect immunosenescence or inflammaging during elderly. It has been previously demonstrated that PA has profound effect over both innate and adaptive immune system in healthy adults (Koelwyn *et al.*, 2015). However, how PA at youth modulates immune system during aging has not been yet evaluated. We decided to assess this point, by categorizing our older volunteers according the development of chronic PA during youth by using the widely used and validated International Physical Activity Questionnaire (IPAQ) and evaluating immunosenescence and inflammaging (Hagstromer *et al.*,

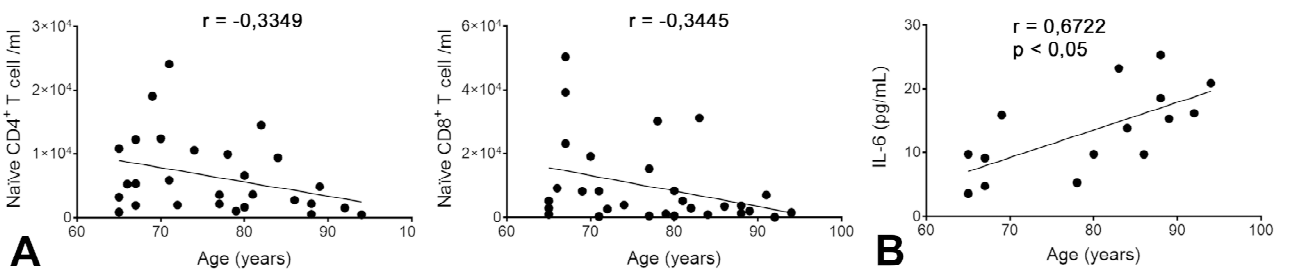


Fig. 2. Effect of ageing over immunosenescence and inflammaging in the older population. A. Correlation between circulating naïve CD4⁺ (left) and CD8⁺ (right) T cells versus age in the older population. B. Correlation between serum IL-6 levels versus age in the older population. Pearson correlation coefficient ranges are shown for each graph.

2006). We did not observe any significant differences between number of circulating naïve T cells or serum IL-6 levels between both, Active and Sedentary groups (Fig. 4), suggesting that the development of PA during youth has no significant effect over the immune system during aging.

PA during elderly increased naïve T cell numbers and reduced inflammaging. We next asked if PA during aging in the elderly has any effect over immunosenescence and inflammaging, by categorizing the older adults in Active (high PA) or Sedentary (low PA) by using IPAQ. Our results indicated that Active older adults showed increased circulating naïve T cells and lower serum IL-6 levels compared to Sedentary older volunteers (Fig. 5). We did not find significant difference between the two groups with respect to age, sex or body mass (not shown). These results suggest that PA during elderly could modulate immune system, reducing immunosenescence and inflammaging in older adults.

Finally, we categorized our older volunteers according to their functionality by using the Composite Physical Function scale (CPF) (Rikli & Jones). Although we did not observe differences between number of circulating naïve $CD4^+$ or $CD8^+$ T cells between both groups (not shown), we found increased IL-6 levels in the low functionality group (see in Additional file 1: Fig. S1a). However, this observation could in part be explained by the increased average age of this group compared to the high functionality group (Fig. S1b).

Collectively, these findings indicate that immunosenescence and inflammaging dramatically increase during aging, and there is a strong negative correlation between $CD4^+$ naïve T cell reduction and higher serum IL-6, suggesting a possible suppressive role of this population over innate immune cell components. Moreover, PA during aging improves the immune system, slowing naïve T cells decrease and reduced serum IL-6 levels.

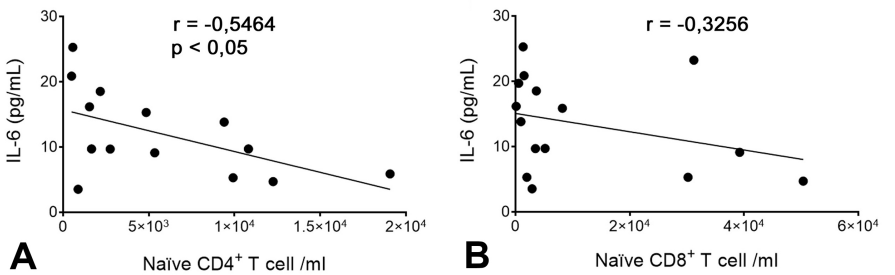


Fig. 3. Strong negative correlation between circulating naïve $CD4^+$ T cell numbers and IL-6 levels in older volunteers. A. Correlation between circulating naïve $CD4^+$ T cells versus serum IL-6 levels in the older population. B. Correlation between naïve $CD8^+$ T cells versus serum IL-6 levels in the older population. Pearson correlation coefficient ranges are shown for each graph.

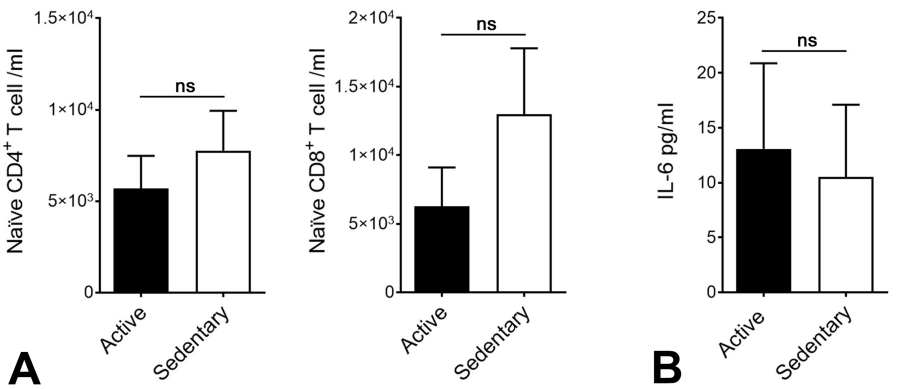


Fig. 4. Ageing immune system is not affected by the realization of PA during youth. Older volunteers were categorized according to the development of chronic PA during youth and circulating naïve T cells and serum IL-6 levels were analyzed. A. Circulating numbers of naïve $CD4^+$ (left) and naïve $CD8^+$ (right) T cells, from older adults who declared to have performed intense PA during youth (Active) versus older controls (Sedentary) are shown. B. Serum IL-6 levels from both groups are shown. Error bars indicate SD. ns: not significant

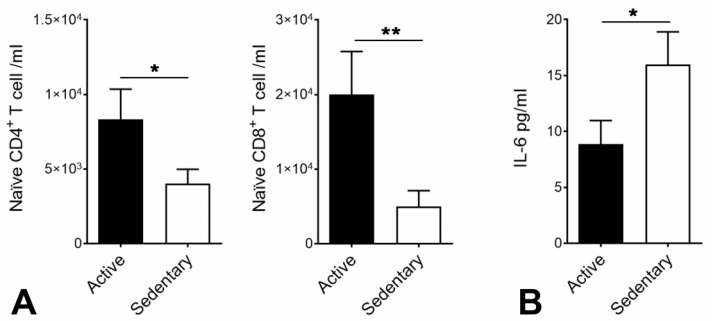
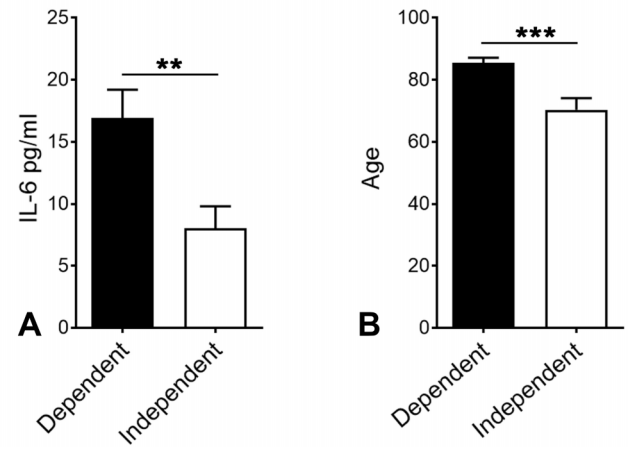


Fig. 5. PA during elderly slow down immunosenescence and inflammaging. Older volunteers were categorized according to the development of PA during elderly and circulating naïve T cells and serum IL-6 levels were analyzed. A. Circulating numbers of naïve $CD4^+$ (left) and naïve $CD8^+$ (right) T cells, from Active older adults versus Sedentary older controls are shown. B. Serum IL-6 levels from both groups are shown. Error bars indicate SD. * $p < 0.05$; **$p<0.01$ by two-tailed Student's t test.



Supplementary information. Additional File 1: Figure S1. Reduced functionality increased inflammaging and immunosenescence, but it can be explained by the differences in age between groups. Older volunteers were categorized according to their functional status and serum IL-6 levels were analyzed. A. Serum IL-6 levels from both groups is shown. B. Age average between both groups. Error bars indicate SD. ** $p < 0.01$; ***$p<0.001$ by two-tailed Student's t test.

DISCUSSION

Inflammaging and immunosenescence are two key events occurring during aging in the elderly that affect immune system. Although these processes have been generally accepted to occur independently, the relationship between immunosenescence and inflammaging has begun to be elucidated (Fulop *et al*., 2018). Here, by evaluating young and older volunteers from the Maule Region, Chile, we provide evidence showing that reducing numbers of naïve T cells, especially $CD4^+$ T cells negatively correlates with increasing levels of serum IL-6. Our results indicate that these processes could be directly connected, suggesting that performing PA during aging has a beneficial effect on the immune system.

Diminished naïve T cells activation and proliferation in aged mice and humans have been previously demonstrated (Goronzy *et al*., 2015). The main determinant of these changes seems to be explained in part by the age-related regression of the thymus (Lazuardi *et al*.). In fact, patients thymectomized during early childhood showed premature immunosenescence with dramatic reduction of naïve T cells and accumulation of oligoclonal memory T cells (Sauce *et al*., 2009). Our results showed a strong negative correlation between circulating naïve $CD4^+$ T cell and serum IL-6 levels, suggesting a suppressive capability of this population over innate immune cells. Moreover, we observed increased levels of $CD25^+$ Tregs in the aged volunteers (not shown), similar to what has been previously published (Garg *et al*.). Interestingly, human immunodeficiency virus infection (HIV), is characterized by $CD4^+$ T cell depletion and also by elevated IL-6 levels, mimicking some aspects observed during ageing (Breen *et al*., 1990). A negative correlation between peripheral $CD4^+$ T cell numbers but not Tregs and IL-6 levels has been observed in HIV patients, closely resembling what we observed in our study regarding older adults (González-Hernández *et al*., 2012). Although the possible suppressive activity of naïve $CD4^+$ T cells could have implications beyond ageing, further studies are necessary to confirm these findings.

PA during lifetime reduces the risk cancer, cardiovascular disease, and other inflammatory disorders. How PA during youth could affects the immune system while aging remains largely unknown. Here, by using a validated socio-demographic and lifestyle characteristics questionnaire, we evaluated this question and found no differences regarding immunosenescence or inflammaging parameters between older adults that performed ongoing PA during youth versus the control group. Since we did not categorize the type of PA developed during youth, our results could in part be explained by different routines of PA (type of exercise, dose, among others), that could affect in different ways the immunosenescence parameters

in individuals (Turner, 2016). Further studies specifically focusing on the different types of exercise, dose, time per week among other parameters are necessary to determine the contribution of specific PA during youth to immunosenescence and inflammaging during aging.

We have also evaluated the effect of PA in the elderly. Our results showed increased number of circulating naïve T cells and reduced IL-6 levels in active older adults (i.e., accumulating 150 minutes of moderate intensity activity on a weekly basis), compared to non-active older controls. These results are in line with a recent study showing that active older cyclist people have increased number of naïve T cells and reduced levels of IL-6 compared to sedentary older controls (Duggal *et al*.). Although in that study they include adults below 60 years old and they did not take into account the differences of age between both groups, our results showed that age is the main factor in triggering immunosenescence and inflammaging in the older population and also highlights the importance of PA during aging to slow these processes.

How is the immune system status in functional versus dependent older adults is still unknown. By using the CPF scale, we observed increased inflammaging and immunosenescence in the dependent group compared to the functional adults. However, our observations can in part, be explained by the different mean of age between both groups. Thus, and although interesting, more research focusing in functional versus dependent older people of similar age is necessary to validate our observation.

Some limitations of our work should be considered. First, the number of participants was relatively low, and the differences in the age between the older volunteers was significant, although the sample size allowed statistical comparisons that showed significant changes. Second, we evaluated PA in older adults by using validated questionnaires but did not directly assay PA in our volunteers. However, we consider that this study provides enough evidence to correlate naïve T cell number reduction and inflammaging, to explore possible suppressive capabilities of naïve T cells over innate immune components. Furthermore, to evaluate different PA interventions in older people and their differential impact over immunosenescence and inflammaging.

In summary, our observations indicate that immunosenescence and inflammaging dramatically increase as people get older. There is a strong negative correlation between circulating naïve $CD4^+$ T cells and serum IL-6 levels in older adults, suggesting that naïve T cells can have a direct suppressive activity over innate immune components. Furthermore, PA in the elderly may reduce immunosenescence and inflammaging in older subjects.

ACKNOWLEDGEMENTS

We are grateful to the Casa de Reposo Cade Ltda., especially Pabla Gutierrez for helping us to recruit older volunteers and to Amparo Gonzalez for technical assistance.

FUNDING. This work was supported by the following grants: Proyecto Enlace-Autónoma N° UA-EN2018-20 and FONDECYT de inicio N° 11190253 (A.H), FONDECYT de inicio N° 11160592 and Fondecyt Regular N° 1201562 (N.E).

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RESUMEN: El deterioro del sistema inmunológico asociado con la edad, denominado inmunosenescencia, se caracteriza por una inflamación sistémica de bajo grado, conocida como inflamaging, junto con una desregulación funcional de las células T. Aunque afectan a todos los individuos, diferentes factores ambientales y genéticos inciden en la susceptibilidad o resiliencia del individuo a la inmunosenescencia. Estudios anteriores han demostrado que la actividad física mejora la autonomía y la funcionalidad en los adultos mayores, aunque como la actividad física impacta a la inmunosenescencia e inflammaging es aún desconocido. El propósito de este estudio fue analizar la inmunosenescencia e inflammaging en personas de edad avanzada, midiendo las células T vírgenes y la interleucina (IL)-6 de sangre periférica, junto con evaluar el impacto de la actividad física sobre la inflamación basal y la inmunosenescencia. Treinta voluntarios ancianos (10 hombres y 20 mujeres) y 7 controles jóvenes (2 hombres y 5 mujeres) fueron incluidos en este estudio. Para medir actividad física, autonomía y dependencia se utilizó un cuestionario de metodología, junto con evaluar el número de células T $CD4^+$ y $CD8^+$ periféricas vírgenes e IL-6 sérica mediante FACS y ELISA, respectivamente. Nuestros resultados muestran que las células T vírgenes disminuyen y los niveles de IL-6 aumentan a medida que las personas mayores envejecen. Curiosamente, observamos una fuerte correlación negativa entre el número de células T vírgenes y los niveles de IL-6 en adultos mayores, lo que sugiere un vínculo directo entre la reducción de la reserva de células T vírgenes y el aumento de la inflamación. La actividad física durante la juventud no afectó la inmunosenescencia ni la inflamación en los ancianos, pero la actividad física durante la vejez aumenta el número de células T vírgenes y reduce la inflamación en los adultos mayores. Estos

resultados sugieren que inmunosenescencia e inflammaging parecen estar directamente conectados, además de concluir que el desarrollo de actividad física durante la vejez reduce la inmunosenescencia y la inflamación basal en adultos mayores.

PALABRAS CLAVE: Sistema inmunológico; Células T virgenes; Inmunosenescencia; Inflamación; Anciano; Actividad física.

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Received : 08-01-2021
Accepted : 01-03-2021