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Short communication

Low DNA damage in peripheral lymphocytes of healthy elderly individuals with inverted CD4:CD8 ratio

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ABSTRACT

The aim of this study was to evaluate the DNA damage in peripheral lymphocytes and the frequencies of CD8+ T cells expressing CD25, CD28 and CD45ro in aged individuals with inverted CD4:CD8 ratio. Blood samples of elderly individuals (aged > 65) with normal CD4:CD8 ratio (n = 8) and inverted CD4:CD8 ratio (n = 8) were collected to identify the expression of CD25+, CD28+ and CD45ro+ in CD8+ T cells. DNA damage index was evaluated by the alkaline comet assay which was performed in lymphocytes treated with different concentrations of methyl methanesulfonate (MMS) (control non-treatment, 2×10^{-5} M, 4×10^{-5} M) for 1, 2 or 24 h. Elderly individuals with inverted CD4:CD8 ratio presented low frequency of CD8+ CD28+. Moreover, low DNA damage was observed in lymphocytes of elderly with inverted CD4:CD8 ratio in different doses of MMS. Aged individuals with inverted CD4:CD8 ratio presented lower DNA damage events in peripheral lymphocytes, suggesting a resistance for cell death in T cells of individuals with immune risk profile.

1. Introduction

Aging is associated with higher reactive oxygen species (ROS), proinflammatory mediators and remodeling of T-cells phenotype in a condition named as immunosenescence [1]. During aging, several features such as progressive thymic involution, accumulation of senescent CD8+ T cells (CD28−) and latent virus reactivation induce an inversion in CD4:CD8 ratio [2]. Moreover, Ferguson et al. [3] described that the low lymphoproliferative response and the inverted CD4:CD8 ratio are positively associated with higher mortality in elderly individuals regardless of their health status. Collectively, these results indicate that inverted CD4:CD8 is an immune risk factor associated with several chronic conditions, but mainly with immunosenescence.

The accumulation of DNA damage during the life plays a pivotal role in the cellular and organism senescence and dysfunction [4]. In each cellular division the machinery involved in the replication of DNA can be a target of different agents (i.e. hormones, ROS, cytokines) that may cause DNA damage [4]. Thus, chronic DNA damage in lymphocytes is a marker of cellular senescence associated to the increased production of inflammatory mediators, contributing to inflammaging, a hall mark of immune senescence [5,6]. Thus, the damaged DNA structure leads to senescent and less functional T cell activity in aging [5].

Although several cell alterations have been associated to senescence, the relationship between DNA damage and the CD4:CD8 ratio in aged individuals is largely unknown. Here, the DNA damage induced by methylmethanesulfonate (MMS) and an immunophenotype profile (consisting of CD25, CD28, CD45ro) were evaluated in healthy elderly subjects with normal or inverted CD4:CD8 ratio.

2. Methods

Sixteen healthy non-institutionalized elderly subjects (8 men and 8 women, aged 65.68 ± 4.35 years old; body mass index 27.25 ± 2.50 kg/m^2) were randomly recruited by random home visits in districts of Gravatai (Brazil) which was performed by the research

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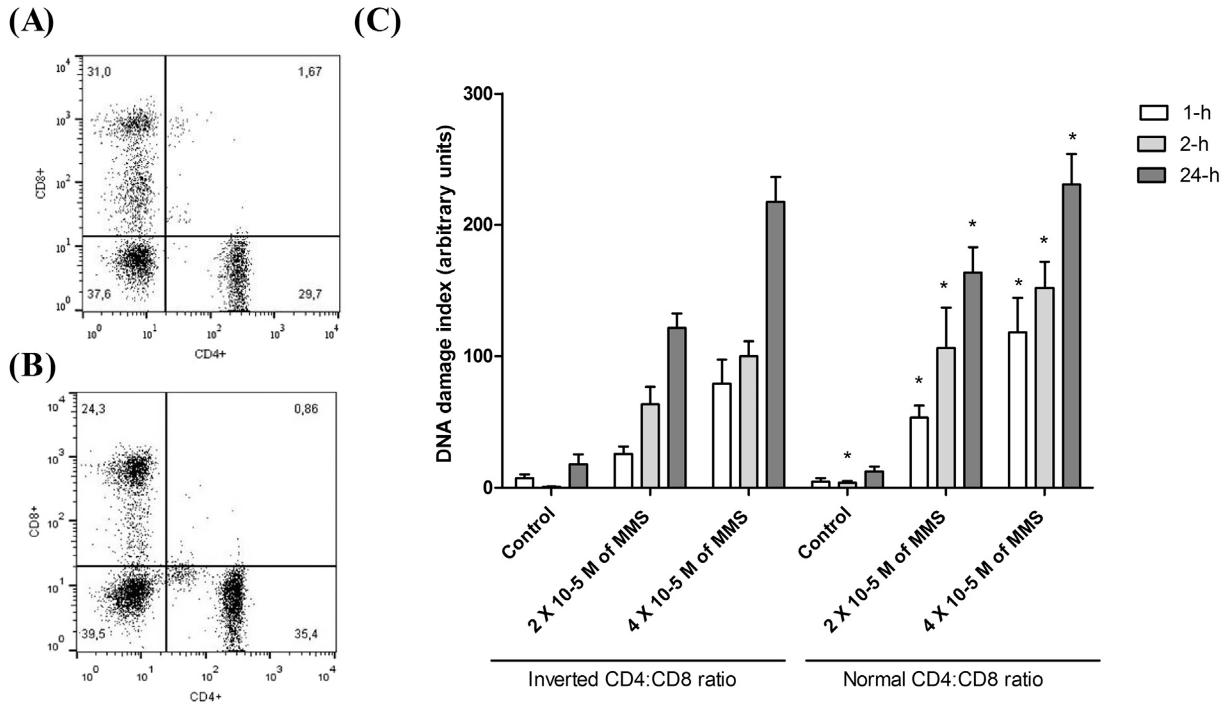

Fig. 1. Flow cytometry dot plot analyses of peripheral frequencies of CD4+ and CD8+ T cells in elderly with inverted (Fig. 1A) and normal (Fig. 1B) CD4:CD8 ratio. The Fig. 1C shows the DNA damage index of blood samples from elderly with inverted CD4:CD8 ratio and normal CD4:CD8 ratio incubated with control untreated, 2×10^{-5} M of MMS and 4×10^{-5} M of MMS during 1 h, 2 h and 24 h.

* Denotes statistical difference from elderly with inverted CD4:CD8 ratio at the same time ($p \leq 0.05$).

group with the assistance of teams of the Family Health System Health Strategy. All participants were nonsmokers, with cognitive ability, not taking medication or supplements affecting the immune system and free from any infectious illness for 6-weeks prior to testing. Patients with serology positive for HIV, cardiovascular diseases, metabolic disorders, acute infections, neoplasia and autoimmune diseases were excluded. All subjects signed informed consent and this study protocol, and the Ethics Committee from the University approved this study. Individuals (n = 8) with a CD4:CD8 < 1 were designed in the group inverted CD4:CD8 ratio.

Patients arrived to laboratory at 8:00–9:00 a.m. in fasting state to blood collection. 10 mL of venous blood from the antecubital vein were collected into heparinized tubes by a capacitated professional. Immunophenotyping was carried out using a direct immunofluorescence technique. Monoclonal antibodies specific for CD3, CD4, CD8, CD25, CD28, CD45ro, conjugated with specific fluorochromes (PE, FITc) as well as the appropriate isotype controls, were purchased from Becton Dickinson (San Jose, CA, USA). Flow cytometry was performed using a FACSCalibur instrument and CellQuest software (Becton Dickinson, San Jose, CA, USA). Lymphocyte population was gated on the basis of forward scatter (FSC-H) versus side scatter (SSC-H) and appropriated control antibodies as CD14 + CD45+. A minimum of 20.000 cells per gate were obtained with each sample. Data are reported as the peripheral frequency of CD3+ CD4+, CD3+ CD8+, CD8+ CD25+, CD8+ CD28+, and CD8+ CD45ro+ on lymphocyte gate.

The alkaline comet assay was performed as described by Singh et al. [7] and Tice et al. [8]. In brief, blood samples for each subject were treated with MMS (M4016/Sigma, St. Louis, MO, USA) at different doses: (a) control - not treated with MMS, (b) 2×10^{-5} M and (c) 4×10^{-5} M. The treated blood samples were incubated for 1, 2 or 24 h at 37 °C. Lymphocytes were embedded in 95 μl of 0.75% low melting point agarose. After solidification, the slides were placed in lysis buffer (2.5 M NaCl, 100 mM EDTA and 10 mM Tris, pH 10.0–10.5, with freshly added 1% Triton X-100 and 10% dimethyl sulfoxide [DMSO]) for a minimum of 1 h and a maximum of two weeks. Subsequently, the slides were incubated in freshly-made alkaline buffer (300 mM NaOH and 1 mM EDTA, pH > 13) for 20 min. The DNA was electrophoresed for 15 min at 25 V (0.90 V/cm) and 300 mA, and then the buffer was neutralized with 0.4 M Tris (pH 7.5). Finally, the DNA was stained with ethidium bromide (2 μg/ml) (Sigma). The slides were coded for blind analysis. Using a fluorescence microscope equipped with BP546/12 nm excitation filter and a of 590 nm barrier filter, images of 100 randomly selected cells (50 cells from each of two replicate slides) from each person were analyzed. Cells were rated visually into five classes according to tail size, from no tails (0), to maximally long tails (4), resulting in a single DNA damage score for each subject, and consequently for each group studied. Thus, the damage index (DI) of the group can range from 0 (all cells with no tails, 100 cells × 0) to 400 (all cells with maximally long tails, 100 cells × 4) [8].

Kolmogorov-Smirnov test was used to evaluate the normality of distribution of each group for all variables, and values are displayed as mean ± standard deviation (SD). Analysis of variance (ANOVA) of three-way repeated measurements was adopted taking concentrations of MMS, time of incubation and group as factors. Multiple comparisons of mean differences among groups were checked with Bonferroni's post hoc test. Differences in immunophenotyping between groups were evaluated by unpaired Student *t*-test. Statistical evaluations were performed using a computer statistic package (SPPS 20.0, USA). A $p < 0.05$ was considered statistically significant.

3. Results

Considering that low CD4:CD8 ratio is a marker of negative prognostic in aging [3], we pooled the data from aged individuals according to CD4:CD8 ratio (normal vs. inversed). Both groups were homogeneous regarding age (normal CD4:CD8 = 65.3 ± 3.29 years; inverted CD4:CD8 ratio = 66.93 ± 2.91 years), BMI (normal CD4:CD8 ratio = 28.15 ± 3.91 kg/m^2; inverted CD4:CD8 ratio = 27.63 ± 4.21 kg/m^2), gender (normal CD4:CD8 ratio = 5 females; inverted

Table 1
Peripheral frequency (%) of CD25, CD28 and CD45ro on CD8+ T cells between elderly with inverted and normal CD4:CD8 ratio.

	Elderly with inverted CD4:CD8 ratio (n = 8)	Elderly with normal CD4/CD8 ratio (n = 8)
CD25	3.31 ± 0.59	3.24 ± 0.57
CD28	40.32 ± 2.43[a]	56.03 ± 3.23
CD45RO	47.87 ± 5.18	49.48 ± 5.89

Data presented as mean ± standard deviation.
[a] Denotes significant difference between groups ($p \le 0.05$).

CD4:CD8 ratio = 6 females), education, ethnicity (normal CD4:CD8 ratio = 4 caucasians; inverted CD4:CD8 ratio = 4 caucasians), smoking and drinking habits.

Lower frequencies of CD4+ T cells (28.68 ± 1.43 vs. 44.30 ± 2.39%; $p < 0.001$) and higher proportions of CD8+ T cells (50.01 ± 3.77 vs. 29.19 ± 2.05%; $p < 0.001$) were found in elderly with inverted CD4:CD8 ratio compared to normal CD4:CD8 ratio. The Fig. 1A shows a dot plot of one elderly subject with inverted CD4:CD8 ratio (0.95) and the dot plot of Fig. 1B shows a dot plot of elderly individual with normal CD4:CD8 ratio (1.45). The Fig. 1C shows the DNA damage index on lymphocytes of aged individuals with inverted or normal CD4:CD8 ratio exposed to different concentrations of MMS at different times. Elderly subjects with inverted CD4:CD8 had low DNA damage index after 2-h of incubation in control untreated model ($p = 0.026$). Moreover, the MMS treatment with 2×10^{-5} M and 4×10^{-5} M showed low DNA damage index after 1 h ($p = 0.023$ and $p = 0.037$, respectively), 2 h ($p = 0.02$ and $p = 0.04$, respectively), and 24 h ($p = 0.026$ and $p = 0.047$, respectively) in lymphocytes from elderly with inverted CD4:CD8 ratio than those with normal CD4:CD8 ratio.

No statistical differences were found in CD25+ and CD45ro+ expression in CD8+ T cells between groups ($p > 0.05$). However, elderly with inverted CD4:CD8 ratio presented diminished CD28+ expression in CD8+ T cells ($p = 0.011$) (Table 1).

4. Discussion

Previous data highlighted the role of DNA damage in lymphocytes and the development of several aging-associated diseases [6]. To the best of our knowledge, this is the first study to identify the lower rates of DNA damage in lymphocytes exposed to different concentrations of MMS and low frequency of CD28+ expression on CD8+ T-cells in elderly individuals with inverted CD4:CD8 ratio.

Aging impacts several co-stimulatory molecules related to the functional activity of T cells. In our study, participants with inverted CD4:CD8 ratio presented also lower frequencies of CD28+ expression in CD8+ cells. The loss of CD28 expression in T cells correlates with a reduction in the CD4:CD8 ratio in aging individuals [9] and affects mainly the CD8+ subset of T cells [10]. Moreover, the aging “immune risk profile” has been associated with higher terminally differentiated effector CD8+ T cells, higher concentrations of systemic tumor necrosis factor alpha and inflammatory cytokines, and loss of CD28+ on cell surface of T cells [1,2].

Endogenous and exogenous agents, such as ROS and inflammatory cytokines, can cause DNA damage that is involved in the aging process. Accumulation of DNA damage can lead to replication errors causing point mutations or chromosomal rearrangements. These, in turn, may be responsible for cell aging, cancer and neurodegenerative diseases [5]. Different approaches, using a variety of cell types, indicate an age-related increase in DNA damage. In the comet assay, although total leukocytes are used, the majority of these cells (80–85%) corresponds to lymphocytes [4,8].

In the present work, two different MMS concentrations were tested to assess DNA damage in blood cells of aged individuals. Interestingly, elderly individuals with inverted CD4:CD8 ratio had lower DNA damage index in all concentrations at 1-h, 2-h and 24-h of incubation. Higher DNA damage is induced due to ROS generation, lack of histones and a less efficient DNA repair mechanisms [11]. In this way, Muller and coworkers [12] showed that elderly individuals with inverted CD4:CD8 ratio presented higher levels of ferric reducing ability of plasma and an antioxidant marker, and lower levels of lipid peroxidation than those with normal CD4:CD8 ratio. Indeed, T cells that have undergone excessive rounds of cell division have short telomeres, are more resistant to apoptosis, and undergo proliferative arrest (senescence) [13,14]. Taken together, these data indicate that aged individuals with inverted CD4:CD8 ratio may accumulate senescent T cells that are more resistant to cell death due to alterations in DNA repair mechanism. Future studies should be conducted to evaluate aspects of DNA breaks and repair mechanisms with other methods of DNA damage, such as ultraviolet irradiation, in elderly individuals presenting immune risk profile.

Our study have some limitations, mainly the lack of cellular senescence (i.e. CD27, CD57, KLRG1) and an exhaustion marker (PD-1) in both CD4+ and CD8+ T cells. However, we highlight that the loss of CD28 in CD8+ T cells is a classic marker of “immune risk profile” associated with pivotal clinical outcomes. Moreover, future studies should be conducted for evaluating the role of latent virus infection, inflammaging, telomerase activities and the presence of latent virus infection, such as cytomegalovirus, in DNA damage of lymphocytes in healthy aging. Finally, the small sample size of our study reduces the generalization of the results. However, no past studies evaluated the DNA damage in peripheral lymphocytes of elderly with inverted CD4:CD8 ratio.

5. Conclusion

In conclusion, this study is the first report to demonstrate that aged individuals with inverted CD4:CD8 ratio had low DNA damage index and CD28+ expression than those with normal CD4:CD8 ratio. These features suggest a tolerance for apoptosis and cell death of T cells from individuals with immune risk profile.

Conflict of interest

The authors declare that they have no conflict of interest.

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References

[1] A. Bektas, S.H. Schurman, R. Sen, L. Ferrucci, Human T cell immunosenescence and inflammation in aging, J. Leukoc. Biol. 102 (2017) 977–988.
[2] G. Pawelec, Y. Barnett, R. Forsey, D. Frasca, A. Globerson, J. McLeod, C. Caruso, C. Franceschi, T. Fülöp, S. Gupta, E. Mariani, E. Mocchegiani, R. Solana, T cells and aging, Front. Biosci. 7 (2002) (1056-1018).
[3] F.G. Ferguson, A. Wikby, P. Maxson, J. Olsson, B. Jahansson, Immune parameters in a longitudinal study of a very old population of Swedish people: a comparison between survivors and nonsurvivors, J. Gerontol. A Biol. Sci. Med. Sci. 50 (1995) 378–382.
[4] Y. Li, J.J. Goronzy, C.M. Weyand, DNA damage, metabolism and aging in proinflammatory T cells: rheumatoid arthritis as a model system, Exp. Gerontol. 105 (2018) 118–127.
[5] D.R. Turner, A.A. Morley, R.S. Seshadri, J.R. Sorell, Age-related variations in human lymphocyte DNA, Mech. Ageing Dev. 17 (1981) 305–309.
[6] S. Scarpaci, D. Frasca, P. Barattini, L. Guidi, G. Doria, DNA damage recognition and repair capacities in human naïve and memory T cells from peripheral blood of

young and elderly subjects, Mech. Ageing Dev. 124 (2003) 517–524.
[7] N.P. Singh, M.T. McCoy, R.R. Tice, E.L. Schneider, A simple technique for quantification of low levels of DNA damage in individual cells, Exp. Cell Res. 175 (1988) 184–191.
[8] R.R. Tice, E. Agurell, D. Anderson, B. Burlinson, A. Hartmann, H. Kobayashi, Y. Miyamae, E. Rojas, J.C. Ryu, Y.F. Sasaki, Single cell gel/comet assay: guidelines for in vitro and in vivo genetic toxicology testing, Environ. Mol. Mutagen. 35 (2000) 206–221.
[9] G. Pfister, D. Weiskopf, L. Lazuardi, R.D. Kovaiou, D.P. Cioca, M. Keller, B. Lorbeg, W. Parson, B. Grubeck-Loebenstein, Naive T cells in the elderly: are they still there? Ann. N. Y. Acad. Sci. 1067 (2006) 152–157.
[10] B. Luz Correa, A.P. Ornaghi, G. Cerutti Muller, P. Engroff, R. Pestana Lopes, I. Gomes da Silva Filho, J.A. Bosch, C. Bonorino, M.E. Bauer, The inverted CD4:CD8 ratio is associated with cytomegalovirus, poor cognitive and functional states in older adults, Neuroimmunomodulation 21 (2014) 206–212.
[11] O.A. Ross, P. Hyland, M.D. Curran, B.P. McIlhatton, A. Wikby, B. Johansson, A. Tompa, G. Pawelec, C.R. Barnett, D. Middleton, Y.A. Barnett, Mitochondrial DNA damage in lymphocytes: a role in immunosenescence? Exp. Gerontol. 37 (2002) 329–340.
[12] G.C. Muller, M.G.V. Gottlieb, B.L. Correa, I. Gomes Filho, R.N. Moresco, M.E. Bauer, The inverted CD4:CD8 ratio is associated with gender-related changes in oxidative stress during aging, Cell. Immunol. 296 (2015) 149–154.
[13] P.J. Rochette, D.E. Brash, Progressive apoptosis resistance prior to senescence and control by the anti-apoptotic protein BCL-xL, Mech. Ageing Dev. 129 (2008) 207–214.
[14] C. Spaulding, W. Guo, R.B. Effros, Resistance to apoptosis in human CD8+ T cells that reach replicative senescence after multiple rounds of antigen-specific proliferation, Exp. Gerontol. 34 (1999) 633Y44.