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Analysis of T and NK cell subsets in Sicilian population from young to supercentenarian: the role of age and gender

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Running title: Lymphocyte subsets from young people to supercentenarian

Key words: CMV, Gender, Supercentenarian, T Lymphocyte subsets

ABBREVIATIONS

CM, central memory

CMV, cytomegalovirus

EBV, Epstein-Barr virus

EDTA, ethylene diamine tetraacetic acid

EM, effector memory

FSC, forward scatter

NK, natural killer

PBMC, peripheral blood mononuclear cell

SSC, side scatter

TE, terminal effector

SUMMARY

Ageing dramatically affects number and function of both innate and adaptive arms of immune system, particularly T cell subsets, contributing to reduced vaccination efficacy, decreased resistance to infections and increased prevalence of cancer in the older people. In the present paper, we analysed the age-related changes in the absolute number of lymphocytes in 214 Sicilian subjects, and in the percentages of T and NK cells in a sub-cohort of donors. We compared these results with the immunophenotype of the oldest living Italian supercentenarian (111 years old). The results were also sorted by gender. The correlation between number/percentage of cells and age in all individuals and, separately, in males and females, was examined using a simple linear regression analysis. We did not record the increase in the rate of inversion of the CD4/CD8 *ratio* frequently reported as associated with ageing in literature. Our observation was the direct consequence of a flat average trend of CD4⁺ and CD8⁺ T cell percentages in ageing donors, even when gender differences were included. Our results also suggest that CD4⁺ and CD8⁺ subsets are not affected equally by age comparing females with males, and we speculated that gender may affect the response to CMV infection. The supercentenarian showed a unique immunophenotypic signature as regards the relative percentages of her T cell subsets, with CD4⁺ and CD8⁺ T cell percentages and CD4⁺ naïve T cell values in line with those recorded for the octogenarian subjects. This suggests that the supercentenarian has a naïve "younger" T cell profile comparable to that of a >80 year old female.

INTRODUCTION

The immune system undergoes a complex and progressive functional remodelling during the ageing process. Age-related alterations have been documented in both innate and acquired arms of the immune system, where some immune responses are diminished, leaving others unchanged or increased^{1,2}. One of the most commonly mentioned hallmarks of an aged immune system is a substantial decrease in naïve CD4⁺ and CD8⁺ T cells and their differentiation into memory lymphocyte subsets². This phenomenon is the result of events (thymic involution and life-long antigenic stimulation) that can occur independently, but also converge in the achievement of an "experienced" T cell profile, *i.e.*, low proliferating cells that are highly active in effector

cytokine production upon antigenic stimulation³. The involution of the thymus, the central organ of T-cell generation, starts at the time of puberty and is the main responsible for naïve T cell decline in the periphery and lymphoid organs during ageing^{2,4}. While CD4⁺ and CD8⁺ naïve T cell percentages generally decline with age, gender-related differences were demonstrated. In particular, females have shown higher frequencies in both CD4⁺ and CD8⁺ naïve T cells, probably due to a more elevated thymic function compared to males⁵.

Chronic stimulation by persistent viral infections, such as human cytomegalovirus (CMV) and, to some extent Epstein-Barr virus (EBV), could play a role in shaping age-related immune function, driving clonal expansion of specific T cells through repetitive stimulation. CMV is responsible for a persistent and latent viral infection that rarely causes obvious disease⁶. However, CMV seropositivity in older people has been included into an “immunological risk profile” predicting mortality⁷ (see also below). Phenotypically, CMV is thought to be responsible for increasing the presence of effector memory (EM) and terminally differentiated effector (TE) T cells with phenotypic and functional features of replicative senescence, especially in the cytotoxic compartment^{8,9}. Indeed, as CMV infection is mainly controlled by cytotoxic T cells, an accumulation of high numbers of CMV-specific senescent CD8⁺ T cells was observed with each cycle of viral reactivation⁴. A similar but slighter effect on clonal expansions of CD8⁺ T cells has been observed in response to EBV infection in the Swedish population⁷, although it does not seem to be confirmed in older people from West-Sicily¹⁰. In these subjects, the percentage of EBV-specific CD8⁺ cells was significantly lower than in young, showing decreased levels of CD27 and CD28, but no increase in CD45RA¹⁰.

Overall, the expansion of late-differentiated CD8⁺ T cells with ageing has been associated with an age-related increase in the rate of inversion of the CD4/CD8 *ratio* (*i.e.*, <1 vs. an expected *ratio* between 1-5 and 2.5 or more) in older people¹¹. This altered *ratio* is accordingly considered as a marker of frailty risk^{7,12,13}.

It becomes evident that the depicted alterations represent the direct consequence of lifelong antigen exposure or the reflection of the progressive adaptation and compensation of the individual to environmental *stimuli*. Overall, they are implicated in the increased frequency and severity of infections, cancer, and lowered responses to vaccination in older people, *i.e.*, the phenomenon called immunosenescence^{1,2,7}.

Innate immunity is also affected by age-related changes, as demonstrated by the increase in the overall absolute number and percentage of natural killer (NK) cells in the periphery in healthy

older people. This reflects a decrease in cytokine-producing CD56 high (CD56^{hi}) cells and the increased presence of cytotoxic CD56 low (CD56^{lo}) cells¹⁴⁻¹⁶.

Centenarians, individuals who have reached 100 years of age, are considered the best model of successful ageing as they avoid, delay or overcome age-related diseases, such as cancer, neurodegenerative and cardiovascular diseases¹⁷. Their relatively good state of health implies a surprisingly active immune system, which has been therefore extensively analysed worldwide¹⁸⁻²⁰. Becoming a centenarian was a rare phenomenon only a few decades ago. However, improvements in medical care and quality of life have led to a reduction in morbidity and mortality, with a consequent increase in life expectancy¹⁷.

According to the database from the Italian National Institute of Statistics, as of January 1, 2019, in Italy the number of centenarians (100+ years old) was 14,456 (84% females), semi-supercentenarians (105+ years old) were 1,112 (87% females) and supercentenarians (110+ years old) were 21, representing a doubling in these numbers compared to 2009²¹. At the time of revising this paper, according to the Italian supercentenarians database, the validated supercentenarians are 12 and the oldest living person in Italy is a woman 111 years old, born and living in Sicily²².

These demographic data show a significant gender difference in life expectancy, underlining the importance of a global analysis of an ageing-environment/gender interaction. Despite their biomedical relevance, gender differences seem to be still poorly considered and inadequately investigated in ageing studies²³.

Here, we investigated age-related alterations in the absolute numbers of lymphocytes among 214 Sicilian subjects (22-111 age range) and in the percentages of circulating lymphocyte subsets of 41 Sicilian donors, between 25 and 111 years of age, focusing on T and NK cells. Blood cells from a subgroup of 27 healthy donors including the oldest living Italian supercentenarian were used for a more complete dissection of T cell subsets. Data were also analysed according to gender. To investigate whether the differences observed in T cell subpopulations are attributable to previous CMV and EBV infections, the same cohort was screened for CMV and EBV seropositivity.

MATERIALS AND METHODS

Study cohort

Participants were recruited between 2017 and 2020 within the project “Discovery of molecular and genetic/epigenetic signatures underlying resistance to age-related diseases and

comorbidities (DESIGN)", funded by the Italian Ministry of Education, University and Research. The Ethics Committee of Palermo University Hospital (Sicily, Italy) approved the study protocol (Nutrition and Longevity, No. 032017). The study was conducted in accordance with the Declaration of Helsinki and its amendments. All study participants (or their caregivers) gave their written informed consent prior to enrolment. A total of 214 healthy donors (females: 121; males: 93) were recruited. All study participants were Sicilians, selected on the basis of their health status and aged between 22 and 111 years. Exclusion criteria were immunodepression, stroke, cancer, or the use of immunomodulatory drugs within the previous six months. In addition, on July 13, 2020 Mrs. Maria Oliva, the oldest living person in Italy, according to the Italian supercentenarians database²², was recruited. To respect the privacy, all other donors were identified with an alphanumeric code. A database was created to handle the collected information. The participants underwent venipuncture in the morning, after a fasting period of 12 hours. The blood was collected in specific tubes containing ethylene diamine tetraacetic acid (EDTA) or no additives. Serum was separated by blood centrifugation of dry tubes and stored at -80°C before use. For more information about recruitment criteria, please see ref. n. 24.

Haematological parameters analysis

Whole blood was used for automated absolute leukocyte counts of 214 Sicilian subjects (22-111 age range), 121 females (mean age 68.82 years \pm 26.26 SD), and 93 males (mean age 63.62 years \pm 22.67 SD). Leukocyte, lymphocytes, neutrophil, and monocyte absolute numbers were counted at the Unit of Transfusion Medicine of University Hospital "Paolo Giaccone", Palermo, Italy.

Flow Cytometry analysis

Flow cytometry analysis was performed in a subgroup of 41 subjects (23 females and 18 males), aged 25-111 years. Peripheral blood was processed fresh to determine complete blood counts and to isolate peripheral blood mononuclear cells (PBMCs). PBMCs were isolated at Laboratory of Immunopathology and Immunosenescence of the Department of Biomedicine, Neurosciences and Advanced Diagnostics (BiND) of University of Palermo (Italy) from whole blood, using Ficoll-Paque (GE Healthcare, South Plainfield, NJ, USA) density gradient centrifugation according to the manufacturer instructions within 6 hours. PBMCs were frozen in 90% foetal bovine serum and 10% DMSO and stored at -80 °C. Part of the samples were sent on

dry ice to the King's College London, School of Cancer & Pharmaceutical Sciences, The Rayne Institute, where they were cryopreserved in liquid nitrogen tanks ($-180\text{ }^{\circ}\text{C}$) up to the day of analysis. The remaining sample were stored at $-70\text{ }^{\circ}\text{C}$ in the Laboratory of Immunopathology and Immunosenescence for a few days until their characterization at the Specialistic Oncology Laboratory Unit of ARNAS Civico Hospital of Palermo.

For cell thawing, the cryopreserved vials were first transferred to a $37\text{ }^{\circ}\text{C}$ water bath and washed with X-Vivo 15 (BioWhittaker, Walkersville, Maryland, USA). After centrifugation, the supernatant was discarded and PBMCs were resuspended in X-Vivo 15 for counting. Counts and viability were determined with a haemocytometer and trypan blue dye exclusion technique. With this method, dead cells appear blue and are distinguishable from viable cells. An average of $>90\%$ live cells were obtained for each count round.

For the analysis of T and NK cell subsets, PBMCs were thawed, washed, and counted. 1×10^6 PBMCs were first stained with Fixable Viability Dye eFluor™ 780 (eBioscience, San Diego, California, USA), following manufacturer instructions, and next incubated with various combinations of monoclonal antibodies (Supplementary Table 1). As a negative control, human unstained cells were used. Single stain controls were used for the automatic calculation of the compensation matrix. A minimum of 500,000 cells per sample were analysed in LSR Fortessa (BD Biosciences, San Jose, California, USA), for the samples processed at King's College London (UK), and in Navios EX (Beckman Coulter, Brea, California, USA), for the samples processed in Palermo (Italy).

Lymphocyte subsets were identified through forward scatter (FSC) and side scatter (SSC), and further checked in the SSC/CD45 dot plot. An exemplificative schematic representation of the applied gating strategy for T and NK cell analysis is displayed in Figure 1. After setting the first gate in the FSC/SSC dot plot in the lymphocyte region, events were gated in the CD3/SSC dot plot, as recommended by Rühle *et al.*²⁵. T cells were identified as CD3⁺ events. After exclusion of NKT (CD3⁺CD56⁺) cells, T cells were gated in the CD4/CD8 dot plot to define helper and cytotoxic subsets²⁵. CD4⁺ (helper) and CD8⁺ (cytotoxic) T cells were finally explored for CD197 and CD45RA expression in order to describe the fraction of naïve (CD197⁺CD45RA⁺), CM (CD197⁺CD45RA⁻), EM (CD197⁻CD45RA⁻), and TE cells (CD197⁻CD45RA⁺)²⁶. To complete T cell analysis, $\gamma\delta$ T cells were identified as CD3⁺ $\gamma\delta$ ⁺, without distinction between the various subtypes. NK cells were identified in the “non-T” pool of lymphocytes (CD3⁻, including NK and B cells) according to their positivity for CD56²⁵. After gating in the CD56/CD16 dot plot, NK

cells were classified in NK1 (CD3⁻CD56^{lo}CD16⁺) and NK2 (CD3⁻CD56^{hi}CD16⁻) as described elsewhere^{25,27}.

The analysed lymphocyte subsets are expressed as a fraction of the parental gated population, and reported as percentages in the graphics.

EBV and CMV serology

Specific IgG antibodies to EBV nuclear antigen (EBNA) and CMV in serum of 41 subjects (23 females and 18 males), aged 25-111 years, were quantified by chemiluminescence immunoassay using the LIAISON[®] EBNA IgG kit (DiaSorin, Saluggia, Italy) and the LIAISON[®] CMV IgG II kit (DiaSorin, Saluggia, Italy) respectively, as recommended by the manufacturer. Measurements were performed by LIAISON XL (DiaSorin, Saluggia, Italy). The range upper limit was set at 180 U/mL for anti-CMV IgG and at 600 U/mL for anti-EBV IgG.

Statistical analysis

To analyse the percentages of T, NK, and NKT cells, flow cytometry data were analysed using FlowJo version 10.5.3 (Tree Star, Ashland, OR) and statistical analysis was performed with GraphPad Prism, version 8.1.2 (GraphPad Software, San Diego, California, USA).

Correlation between number/percent of cells and age in all individuals and in males and females were examined using a simple linear regression analysis. Figures were plotted as scatterplots with a linear regression line and 95% confidence bands. For each statistical analysis, only *p* values <0.05 were considered significant.

RESULTS

Analysis of haematological parameters

The number of leukocytes in peripheral blood of 214 subjects was analysed according to age and gender. Correlation analysis showed that neither age nor gender affected leukocyte, neutrophil, and monocyte counts (data not shown).

In contrast, there was a significant decline in lymphocyte counts (Figure 2) for males ($R^2=0.082$, $p=0.005$) but not for both genders ($R^2=0.016$, $p=ns$) or female subjects alone ($R^2=0.0005$, $p=ns$).

Analysis of T cell subsets

For the analysis of total CD4⁺ and CD8⁺ T cells, PBMCs were isolated from the blood of 41 healthy donors, 23 females (aged 25-111 years) and 18 males (aged 26-102 years), for the analysis of the percentages of T cells. PBMCs from 27 healthy donors (aged 25-111 years), divided by age and gender as represented in Table 1 were used for a more complete analysis of T cell subsets.

To investigate the effects of age and gender on human lymphocyte subsets, the percentages of CD4⁺ and CD8⁺ T cells in PBMC pool were determined by flow cytometric analysis using the gating strategy described in Figure 1. The percentage of CD4⁺ (Figure 3A) and CD8⁺ (Figure 3B) T cells remained almost constant with ageing, even when gender differences were included in the analysis. In contrast to the reported age-related increase in the rate of inversion of the CD4/CD8 *ratio*¹¹, a flat average trend of change in the CD4/CD8 *ratio* with age was recorded in all individuals merged together (Figure 4, $R^2=0.010$, $p=ns$) and analyzed according to gender (Figure 4, F: $R^2=0.007$, $p=ns$; M: $R^2=0.0001$, $p=ns$).

In order to investigate the impact of ageing upon T cell subsets, we examined age-related changes in the markers of differentiation within the CD4⁺ and CD8⁺ T cell subsets in PBMCs from a sub-cohort of 27 healthy donors, divided by age and gender as described in Table 1. Gating was performed on the CD4/CD8 dot plot, followed by CD4⁺ or CD8⁺ specific sub-gating according to CD45RA and CD197 (CCR7) expression as described above. An example of the applied gating strategy is displayed in Figure 5.

The percentages of CD4⁺ naïve cells significantly declined with age in the combined group of males and females (Figure 5A, $R^2=0.347$, $p=0.001$), but this was attributable to a significant decrease in CD4⁺CD197⁺CD45RA⁺ cells in female subjects ($R^2=0.662$, $p=0.0002$), while a constant trend was observed in the older males ($R^2=0.039$, $p=ns$). These results might suggest that older males were able to better maintain a naïve phenotype of CD4⁺ T cell subset during ageing. However, it should be taken into account that the young females in our cohort showed a higher percentage of naïve CD4⁺ T cells (ranging from 33% to 68%, mean 51.38 ± 11.42) than their male counterparts (ranging from 22% to 41%, mean 27.8 ± 7.45). Similarly, the significant increase in EM CD4⁺ T subset observed in ungrouped individuals ($R^2=0.345$, $p=0.001$) was mainly ascribable to the female group ($R^2=0.496$, $p=0.003$), while a significant decrease in CM ($R^2=0.163$, $p=0.033$) CD4⁺ T cells was recorded in males ($R^2=0.380$, $p=0.024$) but not in females. For TE CD4⁺ T cell subset, no significant ageing dependent effects were detected.

Consistent with other reports^{6,7,12,13}, in our sub-cohort the percentages of CD8⁺ naïve cells significantly declined with age ($R^2=0.769$, $p<0.0001$), being this piece of data confirmed in both females ($R^2=0.816$, $p<0.0001$) and males ($R^2=0.700$, $p=0.0013$). Data showed a concomitant significant increase in the percentage of both TE ($R^2=0.343$ $p=0.003$; F: $R^2=0.548$, $p=0.004$) and EM ($R^2=0.448$, $p=0.0004$; F: $R^2=0.438$, $p=0.014$; M: $R^2=0.482$, $p=0.018$), and a significant decrease in CM ($R^2=0.229$, $p=0.018$; F: $R^2=0.394$, $p=0.021$) CD8⁺ subsets (Figure 5B). Although naïve CD8⁺ cells as well as the less represented CM CD8⁺ subset declined dramatically with age, the concomitant increase in TE and EM CD8⁺ subsets contributed to the maintenance of an almost constant total CD8⁺ T cell percentage with age.

An overview of the differences among young donors, older people and long lived individuals (LLI; aged >90 years) in the proportions of T cell subsets is depicted in the pie charts in Figure 5A and B.

In order to evaluate the general effect of persistent viral infection on T cell subset distribution, we screened our sub-cohort of 27 subjects for CMV and EBV seropositivity. As shown in Table 1, all individuals were EBV-seropositive, while 16.6% of young donors and 100% of older subjects and LLI were CMV-seropositive. Thus, it would be plausible to hypothesize that the progressive accumulation of T cells with a TE and EM phenotype in older individuals of our cohort might be directly influenced by CMV infection, but not by EBV infection. However, a CMV-specific T analysis should be conducted to confirm this inference.

Analysis of $\gamma\delta$ T cells

We also analysed the $\gamma\delta$ T cell percentage from 25 healthy donors, 14 females (aged 31-102 years) and 11 males (aged 35-101 years), identified by their $\gamma\delta$ expression on CD3⁺ T cells. Correlation analysis in the combined group indicated that the percentage of $\gamma\delta$ T cells remained unchanged with age (Figure 6). However, when analysed separately, the male and female groups showed a reverse trend in the $\gamma\delta$ T cell percentages. Specifically, a lower trend in $\gamma\delta$ T cell percentages with age was observed in the female group, while in the males the fraction of these cells was increased.

Analysis of NK and NKT cells

NK and NKT cells from 40 healthy donors, 22 females (aged 25-101 years) and 18 males (aged 26-102 years) were analysed. NK cells (defined as CD3⁻, CD16^{negative to positive} and CD56⁺)

were divided into two subsets based on their CD56 and CD16 expression^{25,27}. The CD56^{lo}CD16⁺ subset (NK1, Figure 8A) is mainly responsible for natural cytotoxicity by releasing cytoplasmic granules containing perforin and granzymes B. By contrast, the CD56^{hi}CD16⁻ subset (Figure 8B) is described as secreting chemokines and cytokines.

An increase in the percentage of NK cells in healthy ageing has previously been reported, in association with a reduced fraction of CD56^{hi} NK cell subset and an expansion of the CD56^{lo} NK cells^{15,16}. Accordingly, NK cell percentages in the peripheral blood of the older people showed a significant increase depending on age (Figure 7, $R^2=0.124$, $p=0.025$), becoming not significant when genders were analysed separately.

Besides, when CD3⁺CD56⁺ cells were subdivided into CD56^{lo}CD16⁺ and CD56^{hi}CD16⁻ NK cells, there was a significant increase in cytotoxic NK cells in parallel with increasing age (Figure 8A, $R^2=0.184$, $p=0.006$). On the contrary, only a non-significant lower trend in the frequency of cytokine secreting NK cells in the older population was detected (Figure 8B, $R^2=0.008$, $p=ns$).

NKT cells, a cell type sharing some functional and phenotypic characteristics with NK cells²⁸, were determined by their CD3 and CD56 co-expression (Figure 9). Overall, a non-significant age-related increase was observed in the NKT cells ($R^2=0.023$ $p=ns$), mainly attributable to the female group.

Comparison with the T subset profile of a supercentenarian

The analyses of T cell subset included a cohort of 41 subjects with the oldest living Italian supercentenarian. In the graphs displaying gender merged analysis, the values for the supercentenarian are reported as a red dot. It is possible to note that in the CD4⁺ T cell percentage graph the red dot (Figure 3, 66.6%) seems to be close at the upper limit of the 95% confidence interval, while in the CD8⁺ T cell percentage graph the same point (Figure 3, 22.35%) is localized at the lower limit line. The resulting CD4/CD8 *ratio* was 3.03 (Figure 4), just located on the regression line, in contrast to the other dots that are widely scattered around the cohort regression line. The values of both CD4⁺ and CD8⁺ naïve T cells for the supercentenarian were lower than those observed in the young females (Figure 5 A,B). However, compared to the individuals aged 90-100 years, the CD4⁺ naïve T cell value for the supercentenarian (Figure 5A, 20.38%) is above the regression line, as in the case of values recorded for the octogenarian subjects. In contrast, the percentage of EM CD4⁺ T cells for the supercentenarian (Figure 5A, 65.44%) seems to be above

the line as the LLIs, differently from the octogenarian subjects whose values are clustered below. As regards the cytotoxic compartment, our Italian doyenne shows one of the highest values of EM CD8⁺ T cells (Figure 5B, 74.32%) but a percentage of CM CD8⁺ T cells (Figure 5B, 1.56%) comparable to their younger counterparts.

DISCUSSION

In the present study, we analysed the effect of ageing on the composition of immune system cell subsets, in correlation with gender and CMV and EBV serostatus, in a cohort of Sicilian donors, ranging from young individuals to a supercentenarian. We set our focus on T and NK cells. Our approach may be considered of some interest as it includes the immunophenotypic characterization of the supercentenarian immune system. Data about supercentenarian immune subsets are very rare in literature and, to the best of our knowledge, have only a transcriptomic focus²⁹.

In more than 15 years of research in the field, it is well documented that ageing is associated with a decrease in the absolute count of CD4⁺ T lymphocytes with higher numbers reported for females, expansion of effector/memory CD8⁺ T cells and contraction of both naïve CD4⁺ and CD8⁺ T cell compartment^{12,30-34}. These processes cannot be simply considered as the direct consequence of thymic involution since they are coupled with stimulation exerted by pathogens (especially by latent Herpes viruses) during the whole life of an individual^{2,7-9,32,34-38}. The most notable manifestation of the listed events is represented by the inverted CD4/CD8 *ratio*. The probability to detect an inverted CD4/CD8 ratio increases with age and is more prominent in males than in females^{11,32,33,39}. As stated in the Introduction, controversies still exist about this altered *ratio* since it is considered to be a marker of risk of frailty, although this association was not found in all populations studied³⁷.

The results of our study show a constant trend in the percentages of both CD4⁺ and CD8⁺ T cells with age. Consequently, we did not observe the described age-related increase in the rate of inversion of the CD4/CD8 *ratio*, whereas this value tends to increase in LLIs, and to decrease in the supercentenarian. As depicted by the linear regression graph, the total CD4⁺ and CD8⁺ T cell percentages in individuals aged 90-100 year old appear to exhibit different trends than those recorded in octogenarian subjects. Most 90-100 year old donors show lower CD4⁺ T cell percentages, in line with data for the supercentenarian, and higher CD8⁺ T cells percentages than octogenarian donors. It remains to be assessed if these fluctuations in CD4⁺ and CD8⁺ T cell

percentages are correlated with the probability of reaching a successful ageing. Another possible explanation may be represented by other concomitant causes determining the reduced detection of the CD4/CD8 *ratio* inversion in our pool of donors, like different lifestyle and environmental factors such as diet, gut microbiota, access to health care, exposure to pathogens, pollution as well as genetic background³⁷.

As mentioned above, an age-related shift from antigen-inexperienced naïve T lymphocytes to antigen-experienced memory and effector T cells has been extensively reported. These phenomena are due to both long-term repeated exposure to antigens together with reduced thymopoiesis^{2,5,12,35}. Our analyses demonstrated that age influences diversely lymphocyte subpopulations in the whole sampled population. Similar to previous reports³⁰⁻³², ageing was associated with a significant reduction in both naïve CD4⁺ and CD8⁺ T cells. Also, a significant reduction in CM CD4⁺ and CD8⁺ T cells was observed in our cohort, contradicting those reports recording an age-related increase in both T cell fractions^{31,40,41}. Finally, a significant increase in EM CD4⁺ and CD8⁺ T cells and in TE CD8⁺ T cells was observed, once again confirming previous reports documenting that the EM population increase with age^{30,40,42}.

Age-related differences in T cell subset distribution may partly be explained by the presence of chronic viral infections. Being CMV more frequent in older individuals of our cohort, CMV seropositivity may have influenced the T cell subset specific expansion and survival with age^{30,40,42}. On the contrary, EBV infection seems to play no role, since all individuals in this study were EBV-seropositive. Indeed, accumulation of late-stage CD8⁺ T cells, some of which may indeed be senescent and contribute to age-related diseases⁴³, are predominantly observed in CMV-seropositive older people, whereas older people infected with other persistent Herpes viruses, as EBV, do not show similar effects seemingly limited to CMV³⁸. On the other hand, it has been claimed that a small proportion of CMV-infected individuals is able to counteract the pathological effects of the CMV-related accumulation of these cells, attaining longevity⁴⁴, as it seems to be happened in our LLIs including the supercentenarian.

Our data also demonstrated that $\gamma\delta$ T cells appeared to remain unchanged with increasing age. This is consistent with the demonstration by Argentati *et al.*⁴⁵, that the percentage of CD3⁺ $\gamma\delta$ T cells in the peripheral blood was heterogeneous in the different age groups, with mean values not significantly different among the young donors, older subjects, and centenarians.

NK cell subsets are known to be differentially affected by ageing with a gradual decrease in the more immature CD56⁺ NK cells, while the percentage and the absolute number of

CD56⁺CD16⁺ NK cells have been variably reported as maintained, increased, or decreased in the older subjects and consistently increased in the centenarians^{2,16,19,46,47}. Accordingly, our results showed a significant increase in cytotoxic NK cells with ageing, but no significant lower trend in the frequency of cytokine secreting NK cells in the older population. Finally, the number of circulating NK cells was slightly lower in females than in males.

NKT cells have been described to decrease in older people and increase in centenarians¹⁹. In our study, a slightly age-related increase was observed in the NKT cells, mainly attributable to the female group.

Besides these not-significant gender-related changes of NK and NKT cells, our data also suggest that the CD4⁺ and CD8⁺ T cell subsets are not affected by age in an equal manner between females and males. Indeed, the influence of gender was significant on various lymphocyte subpopulations. First, a significant age-related decline in the lymphocyte counts in males was identified. Males also showed significantly lower percentages of CM CD4⁺ T cells. The reduction in naïve and the increase in EM CD8⁺T cells were confirmed as significant in both genders, although the significance is higher in women, especially for the naïve compartment. This is in contrast with a previous report³², where both very old and middle-aged females showed a higher percentage of naïve CD8⁺ T cells than males. Females also exhibited a significant decrease in naïve CD4⁺ and CM CD8⁺ T cells.

Biological causes such as hormonal differences or the presence of two X chromosomes, in addition to other gender effects, could partially explain the age dependent differences in cell counts and percentages emerging comparing male and female donors in our study⁴⁸. We might also speculate that gender could impact the response to CMV infection. In fact, it is well known that females are more resistant to infections^{1,48}.

A recent epigenetics study has confirmed that PBMCs of females and males significantly differed after 65 years of age. This analysis revealed that older females have higher genomic activity for B and T cells, thus suggesting that the age-related decline in T cells is greater in men. Older males, instead, have higher activity for monocytes and inflammation⁴⁹.

Centenarians are considered a model of successful ageing^{17,50,51}. Therefore, supercentenarians, *i.e.* people who have reached 110 years of age, are a great model of successful ageing²⁹. Their characteristics of delayed onset of age-related diseases and compression of morbidity^{52,53} imply that their immune system remains functional²⁹. Here, we analysed the

immunological profile of the oldest living woman in Italy (111 years old), born and bred in Sicily, and compared her immunophenotype to that of Sicilian young, older and female LLIs donors.

From the graphs displaying values for naïve and EM CD4⁺ T cells, it is interesting to note that the octogenarians seem to have higher percentages of naïve CD4⁺ T cells than nonagenarians and centenarians and the supercentenarian seems to represent a meeting point between these two groups. Conversely, nonagenarians and centenarians show higher percentages of EM CD4⁺ T cells than octogenarians, but these values are similar to those reported for the supercentenarian. Our report is limited by the small number of analysed samples. However, it would be interesting to monitor these octogenarian subjects over time to see if with advancing age their immune profile in the context of CD4⁺ T cells changes like that of their older counterparts and, especially, that of the supercentenarian.

Recently, circulating immune cells of supercentenarians have been analysed at single-cell resolution. This permitted the identification of CD4⁺ T cells that have cytotoxic characteristics. This feature is truly unique to supercentenarians, as CD4⁺ T cells generally have helper functions, but no cytotoxic activity²⁹. This may represent an essential adaptation to achieve exceptional longevity by sustaining immune responses to infections and diseases.

Thus, an in-depth immunophenotypic and functional characterization of immune subsets of octogenarians followed over time could help to better understand if our experimental evidences, together with previously reported data, could be helpful in predicting the entity of changes affecting the immune system during ageing, and the probability of reaching an advanced age with a functional and adequately adapted defensive repertoire.

In conclusion, ageing dramatically affects both the relative presence and function of the different T cell subsets. These changes are likely to be among factors contributing to the reduced vaccination efficacy and decreased resistance to infections, as well as increased prevalence of cancer in the older population, *i.e.*, immunosenescence^{1,2,7}. However, our knowledge of immunosenescence is likely to be still very incomplete. More detailed data are needed, particularly on the immune phenotype of semi- and super-centenarians, to identify strategies that may counteract the effects of ageing on the immune system. A principal objective of such a kind of studies will be the identification of potential interventions that could reduce the incidence of and morbidity for age-associated diseases by better preservation and stimulation of functional immune competence in the course of chronological ageing.

Supercentenarians show a unique immunophenotypic signature as regards the relative percentages of their T cell subsets. Recent reports also confirm that their immune cells also show previously unknown functional properties²⁹. It would be reasonable to think that the key of successful ageing may be encountered in the supercentenarians' uncommon immune characteristics. An internationally coordinated effort would be highly recommendable in order to extend the characterization of supercentenarians' immune system beyond the geographical borders, and to create an immunophenotypic and genomic database to share the details at a worldwide level. Such a type of global commitment may be beneficial in terms of sharing these rare pieces of information and of speeding up the extrapolation of a possible predictive “successful-ageing-profile”.

DECLARATIONS

FUNDING

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ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The Ethics Committee of Palermo University Hospital (Sicily, Italy) approved the study protocol (Nutrition and Longevity, No. 032017), and written informed consent was obtained from all participant.

DATA AVAILABILITY STATEMENT

The datasets generated and/or analysed during the current study are not publicly available due to privacy reasons but are available in anonymized form from the authors on reasonable request.

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DISCLOSURE

The authors have no competing interests or conflicts of interest to declare.

AUTHORS CONTRIBUTIONS

MEL, FP performed statistical analysis of the data and drafted the manuscript. CC revised the manuscript and all authors contributed with the critical revising of the manuscript. MEL, MB, FG, NZ performed the cytometric analyses. FB, GG. performed the serologic analyses. MEL, CC, GC and FF conceived the study. CC was responsible with GA, AA, SA for patient recruitment and characterization. All authors checked statistical analysis of the data. All authors approved the final version of the manuscript

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Table 1. CMV and EBV serological status of the sub-cohort

Clinical features	Young (n = 12)	Older (n = 9)	LLI (n = 6)
Age (Years)			
Mean±SD	28.69±3.47	82.2±2.60	99.55±5.27
Range	25-34	79-88	93-111
Gender			
Female	8 (66.6%)	4 (44.4%)	3 (50%)
Male	4 (33.3%)	5 (55.5%)	3 (50%)
CMV serological status			
CMV+ [n, %]	2 (16.6%; 1 F, 1 M)	9 (100%; 4 F, 5 M)	6 (100%, 3 F, 3 M)
Anti-CMV IgG titer (U/mL)			
Range	103-160	44-177	160->180
EBV serological status			
EBV+ [n, %]	12 (100%; 8 F, 4 M)	9 (100%; 4 F, 5 M)	6 (100%, 3 F, 3 M)
Anti-EBV IgG titer (U/mL)			
Range	25.4->600	17.8 ->600	20.48->600

CMV, Cytomegalovirus; EBV, Epstein-Barr virus; F, females; LLIs, long-lived individuals; M, males; n, total number; SD, deviation standard; U/mL, units per millilitre.

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Legend to Figures

Figure 1. NK and T cell gating strategy. Gating strategies for the analysis of NK, CD4⁺ and CD8⁺ T cell subsets. The doublet exclusion on FCS-H vs FCS-A followed by SSC-H vs SSC-A is not shown. A representative donor is presented.

Figure 2. Correlations between lymphocyte counts and age. Linear regression analysis showing the relationship between lymphocyte count (10³/μL) and age in all individuals (N = 214) (black line), males (N = 93) (blue line) and females (N = 121) (pink line). Each point represents data from an individual healthy donor. The supercentenarian is shown in red in the graph on the left. The coefficient of determination and *p* values are shown on the graphs. R², R squared; ns, not significant; F, female; M, male.

Figure 3. Analysis of CD4 and CD8 T cells. Linear regression analysis showing the relationship between lymphocytes CD4⁺ % (A), CD8⁺ % (B) and age in all individuals (N = 41) (black line), males (N = 18) (blue line) and females (N = 23) (pink line). The coefficient of determination and *p* values are shown on the graphs. Each point represents data from an individual healthy donor. The supercentenarian is shown in red in the graphs on the left. R², R squared; ns, not significant; F, female; M, male.

Figure 4. Correlations between the CD4/CD8 ratio and age. CD4/CD8 ratio of 41 donors (M = 18; F = 23) was calculated by dividing the CD4 T cell percentage by CD8 T cell percentage. The supercentenarian is shown in red in the graph on the left. R², R squared; ns, not significant; F, female; M, male.

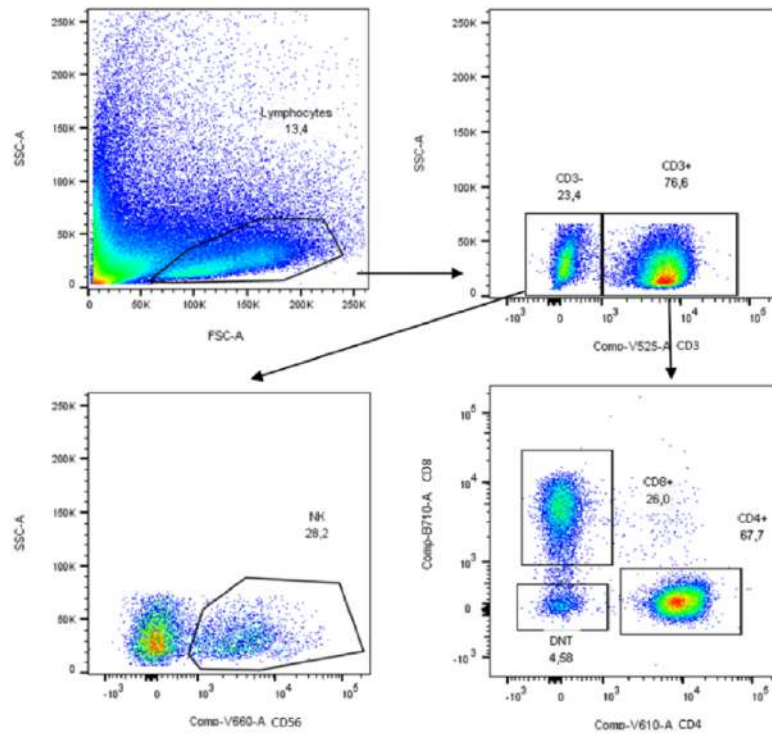
Figure 5. Analysis of CD4 and CD8 T cell subsets. Linear regression analysis shows the relationship between CD4⁺ (A), CD8⁺ (B) subsets and age in all individuals (N = 27) (black line), males (N = 12) (blue line) and females (F = 15) (pink line). The coefficient of determination and *p* values are shown on the graphs. Each point represents data from an individual healthy donor. The supercentenarian is shown in red in the graphs (A,B) on the left. R², R squared; ns, not significant; F, female; M, male. Right (A and B): representative FACS gating of (A) CD4⁺ and (B) CD8⁺ subsets. In the pie charts is depicted an overview of the differences among young donors, old people and LLI donors in the proportions of T cell subsets.

Figure 6. Analysis of γδT cells. Linear regression analysis shows the relationship between CD3⁺ γδ⁺ T cells and age in all individuals (N = 26) (black line), males (N = 12) (blue line) and females (N = 14) (pink line). The coefficient of determination and *p* values are shown on the graphs. Each point represents data from an individual healthy donor. R², R squared; ns, not significant; F, female; M, male.

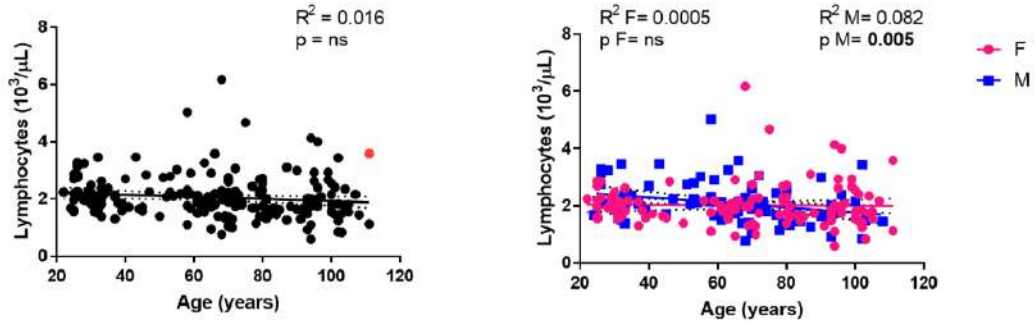
Figure 7. Analysis of NK cells. Linear regression analysis showing the relationship between CD3⁻CD56⁺ NK cells and age in all individuals (N = 40) (black line), males (N = 18) (blue line) and females (N = 22) (pink line). The coefficient of determination and *p* values are shown on the graphs. Each point represents data from an individual healthy donor. R², R squared; ns, not significant; F, female; M, male.

Figure 8. Analysis of NK subsets. Linear regression analysis showing the relationship between cytotoxic (A), secreting cytokines (B) NK cell subsets and age in all individuals (N = 40) (black line), males (N = 18) (blue line) and females (N = 22) (pink line). The coefficient of determination and *p* values are shown on the graphs. Each point represents data from an individual healthy donor. Center: representative FACS gating of CD56^{lo}CD16⁺ and CD56^{hi}CD16⁻ NK cell subsets; R², R squared; ns, not significant; F, female; M, male.

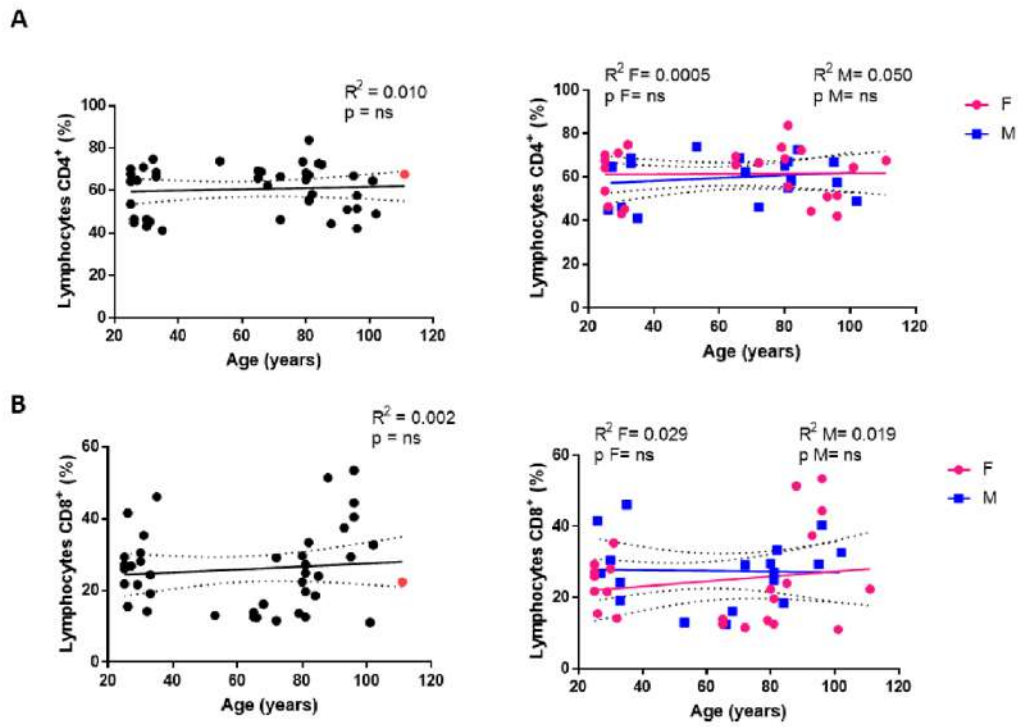
Figure 9. Analysis of NKT cells. Linear regression analysis showing the relationship between CD3⁺CD56⁺ NKT cells and age in all individuals (N = 40) (black line), males (N = 18) (blue line) and females (N = 22) (pink line). The coefficient of determination and *p* values are shown on the graphs. Each point represents data from an individual healthy donor. Right: an example of the gating strategy for NKT cells. R², R squared; ns, not significant; F, female; M, male.



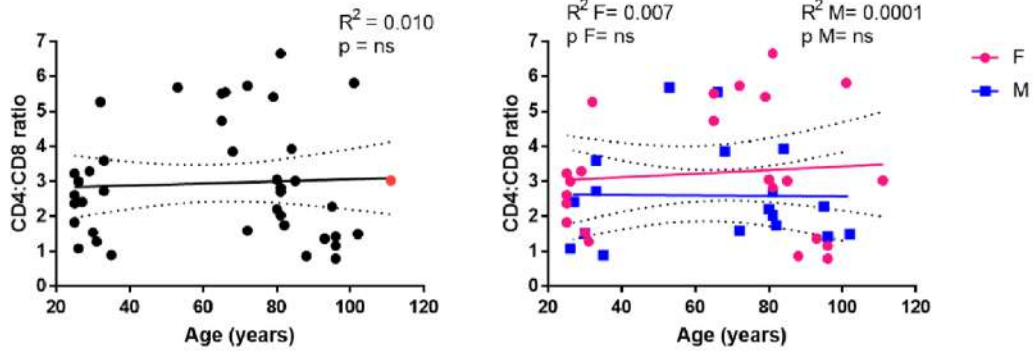
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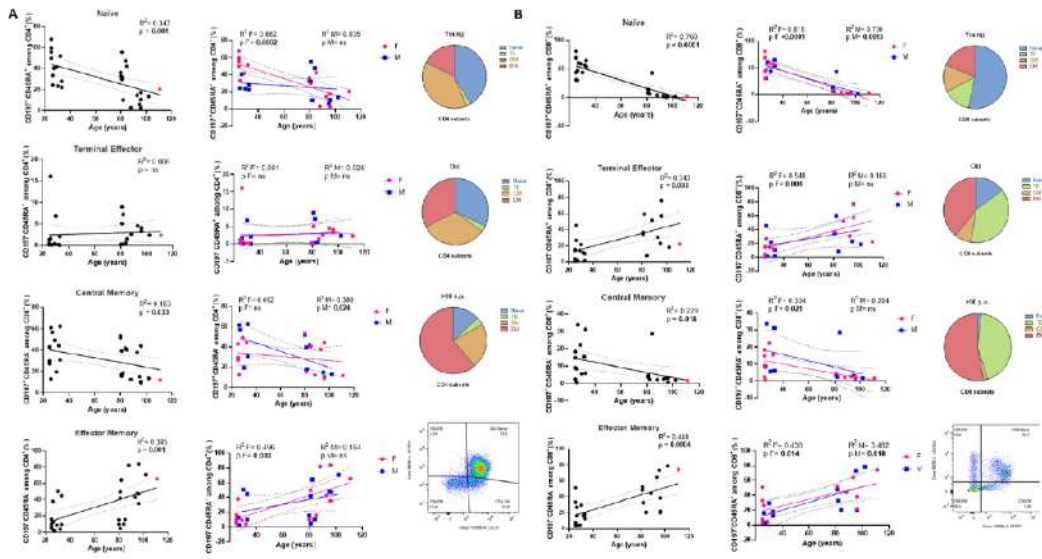
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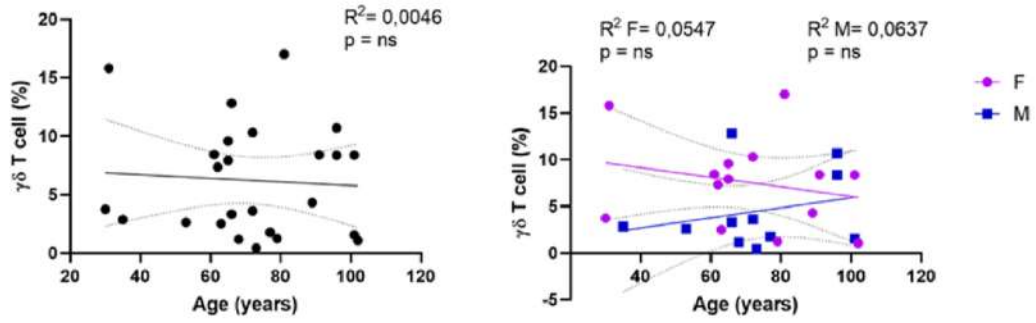
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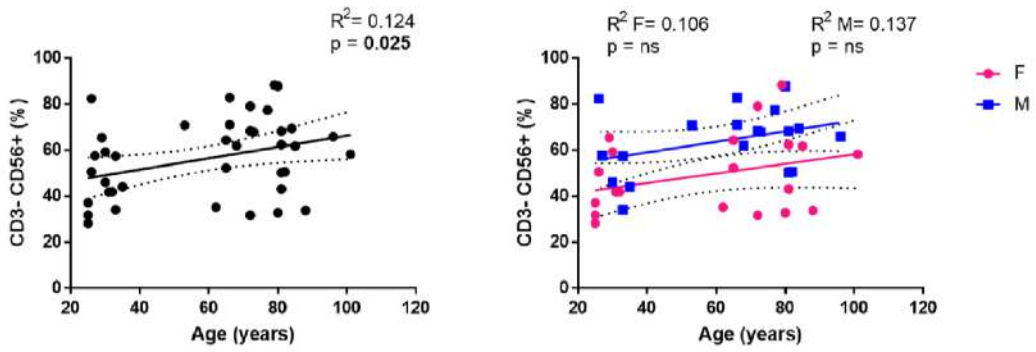
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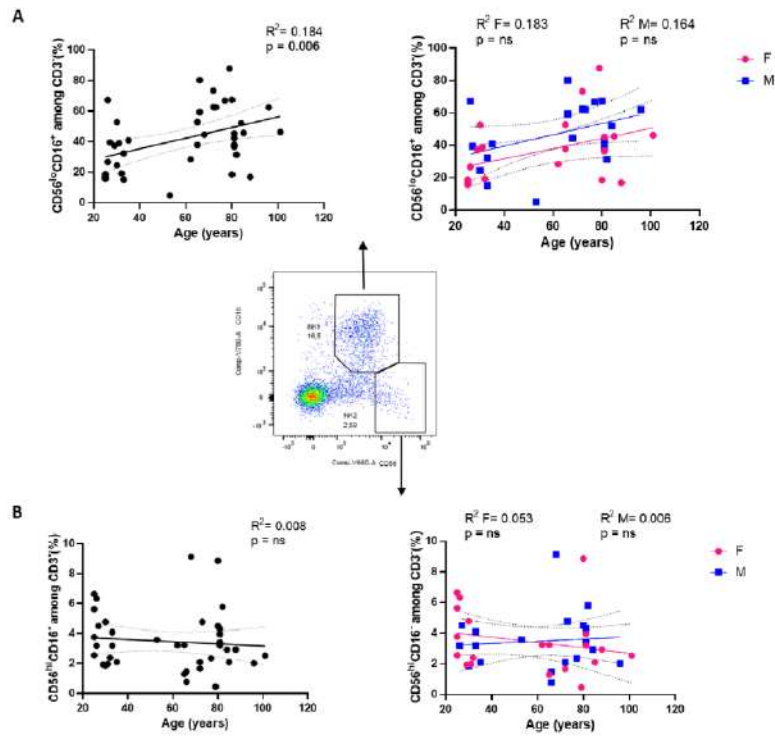
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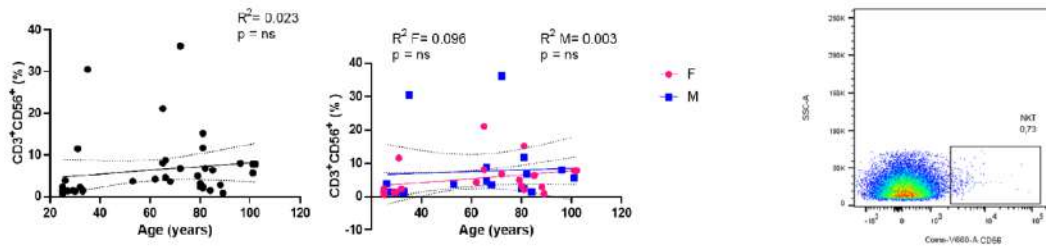
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