

# Evidence for Less Marked Potential Signs of T-Cell Immunosenescence in Centenarian Offspring Than in the General Age-Matched Population

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People may reach the upper limits of the human life span at least partly because they have maintained more appropriate immune function, avoiding changes to immunity termed “immunosenescence.” Exceptionally long-lived people may be enriched for genes that contribute to their longevity, some of which may bear on immune function. Centenarian offspring would be expected to inherit some of these, which might be reflected in their resistance to immunosenescence, and contribute to their potential longevity. We have tested this hypothesis by comparing centenarian offspring with age-matched controls. We report differences in the numbers and proportions of both CD4<sup>+</sup> and CD8<sup>+</sup> early- and late-differentiated T cells, as well as potentially senescent CD8<sup>+</sup> T cells, suggesting that the adaptive T-cell arm of the immune system is more “youthful” in centenarian offspring than controls. This might reflect a superior ability to mount effective responses against newly encountered antigens and thus contribute to better protection against infection and to greater longevity.

**Key Words:** T cells—Immunosenescence—Aging—Longevity—Centenarian offspring.

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**I**NCREASING age is accompanied by a decreased ability of the immune system to protect against new antigenic challenges or to control chronic infections, and increased immune dysregulation also reflected in chronically elevated signs of systemic inflammation. These age-associated changes over the life span, loosely termed *immunosenescence* and *inflamm-aging*, are associated with a decline in the normal functioning of the different components of the immune system. Hence, there is a reduction in responsiveness as well as a functional deregulation of immune effector cells and an alteration of the cytokine and chemokine environment in the elderly population (1). T cells play a major role in defense against infection and in the secretion of pro- and anti-inflammatory factors, but in the elderly people, the balance of T-cell phenotypes in the peripheral blood is markedly different from that seen in most young people, in a manner most commonly interpreted as indicating impaired functionality in the former. This is thought to contribute to poorer vaccine responses, increasing incidence of infection, and reactivation of latent viruses in old people (2). Cross-sectional studies indicate that the most noticeable differences in young and old people lie in the frequencies and absolute numbers of naïve and different

differentiation stages of memory T cells. These data are generally interpreted as a dynamic process of decreasing frequency of naïve T cells and increasing frequency of memory T cells with age, due to conversion of naïve cells to memory cells after antigen contact, although in the absence of longitudinal studies, this assumption cannot be formally proven. However, limited longitudinal studies in the very elderly people have shown that an accumulation of late-stage differentiated CD8<sup>+</sup> T cells resulting in an inverted CD4:CD8 ratio and poor T-cell proliferative responses to mitogens formed the central plank of the “immune risk profile” (IRP), which did indeed predict mortality at 2-, 4-, and 6-year follow-up (3). It is of note that the number of naïve CD8<sup>+</sup> T cells was not part of the IRP. Intriguingly, cytomegalovirus (CMV) infection, however, was part of the IRP and has been postulated to be the main driver of the accumulation of late-stage differentiated CD8<sup>+</sup> T cells in the at-risk group. It is now clear that CMV itself influences T-cell phenotype distribution, independently of age, but becoming more marked in elderly populations with a higher percentage of CMV-infected individuals (4–6). In some studies, CMV infection is closely related to both a reduction of CD8<sup>+</sup> naïve T cells and increase of CD8<sup>+</sup> late-differentiated

effector memory (6), whereas in other studies, the loss of naïve cells was reported to be age dependent but not CMV dependent (7).

Because essentially no older individuals can escape from all age-related diseases, such as atherosclerosis, osteoporosis, sarcopenia, or insulin resistance, defining the elderly individuals as healthy is challenging. For this reason, the term “delayed aging” may better describe a group of people, such as centenarians, who have presumed genetic and functional advantages resulting in a lesser risk of developing major age-related diseases and, as a consequence, survive longer in a good condition (8). Studies of families with exceptionally long-lived participants have suggested that genetic background may contribute a very significant advantage in terms of longevity compared with the general population (9). Older individuals in such families also exhibit more “youthful” immune profiles in that their immune signatures are more similar to those of younger individuals than to age-matched (AM) controls. When infected with CMV, they appear resistant to the alterations in T-cell phenotype distribution caused by this virus in the general population (10). Centenarian offspring (CO) may be a special population of older individuals that, like their centenarian parent(s), could also have genetic and functional advantages that predispose them to healthy aging and longer survival (11–13). Data on B cells have shown that CO appear more similar to young donors than AM sporadic controls (14) and do not show the typical naïve–memory shift observed in the elderly people (15). Recently, we also demonstrated that the IgM memory B-cell pool present in CO is not reduced to the same extent as in their AM controls and remains more similar to that observed in young people (16). Here, we asked whether the T-cell compartment of CO is also resistant to the imputed age-associated changes observed in AM controls having no history of familial longevity. We report higher numbers and percentages of CD28<sup>+</sup>CD27<sup>+</sup>CD45RA<sup>+</sup>CD45RO<sup>-</sup> naïve cells within the CD4<sup>+</sup> as well as CD8<sup>+</sup> subsets in CO compared with the general elderly population, whereas there were

fewer CD28<sup>-</sup>CD27<sup>-</sup>CD45RA<sup>+</sup>CD45RO<sup>+</sup> late-differentiated CD8<sup>+</sup> T cells in CO. Moreover, CO had lower numbers and percentages of CD8<sup>+</sup>CD57<sup>+</sup> putatively senescent T cells compared with the general population of elderly people. These data on CO T-cell distribution imply that cellular immune capacity is likely to be better preserved in CO than AM random controls and could provide a survival advantage under certain conditions of environmental exposures to pathogens.

## METHODS

### Participants

A total of 21 Sicilian CO have been identified and investigated (age range: 70.1±8.3; 10 men and 11 women), with at least one centenarian parent (>99 years). Fifteen AM controls (age range: 69.1±9.7; 7 men and 8 women), 10 old (O) participants (age range: 86.4±3.8; 5 men and 5 women), and 12 young participants (age range: 28.5±1.9; 7 men and 5 women) from Sicily were also included in the study. All participants were in good health at the moment of the recruitment, as revealed by blood tests (complete blood cell count, erythrocytes, C-reactive protein, liver function tests, iron, proteins). The controls were collected from the same population as the patient cohort. Characteristics of these individuals and their clinical history are summarized in Table 1.

The University Hospital Ethics Committee approved the study, and written informed consent was obtained from all participants according to Italian law.

All participants were tested for CMV serostatus by ELISA using CMV-IgG-ELISA PKS assays (Genesis Diagnostics, United Kingdom). All the elderly participants were positive for CMV antibody (CO, AM, and O), whereas none of the young participants were infected.

Whole blood was collected by venepuncture in vacutainer tubes containing ethylenediaminetetraacetic acid, at the same time of day for all participants. Peripheral blood

Table 1. Characteristics and Clinical History of Individuals Studied

	Young People	Centenarian Offspring	Age Matched	Old People
Participants	12	21	15	10
Gender	7 men, 5 women	10 men, 11 women	7 men, 8 women	5 men, 5 women
Age, mean (SD)	28.5 (1.9)	70.1 (8.3)	69.1 (9.7)	86.4 (3.8)
Prevalence of disease, <i>n</i> (%)				
Myocardial infarction	0 (0.0)	0 (0.0)	2 (13.3)	2 (20)
Stroke	0 (0.0)	1 (4.8)	2 (13.3)	1 (10)
Hypertension	0 (0.0)	3 (14.3)	5 (33.3)	2 (20)
Cancer	0 (0.0)	1 (4.8)	2 (13.3)	2 (20)
Rheumatoid arthritis	0 (0.0)	0 (0.0)	1 (6.7)	1 (10)
Level of education, <i>n</i> (%)				
Only primary education			2 (13.3)	10 (100)
Secondary education		19 (90.5)	11 (73.3)	
Higher education	12 (100)	2 (9.5)	2 (13.3)	

mononuclear cells were separated using a Ficoll/Hypaque gradient (Cedarlane Laboratories Limited, Ontario, Canada) and viably cryopreserved according to standard protocols.

### Flow Cytometry

To analyze T-lymphocyte subsets, peripheral blood mononuclear cells were adjusted to  $1 \times 10^6/\text{mL}$  and stained with antibodies. Direct immunofluorescence was performed with anti-CD3-AlexaFluor700, CD8-PerCP, CD28-PerCP-Cy5.5 (Becton Dickinson, Heidelberg, Germany), CD4-Qdot705 (Invitrogen, Karlsruhe, Germany), CD27-Qdot605 (Invitrogen), CD45RO-eFluor650 (eBioscience, San Diego, CA), CD45RA-PacificBlue (BioLegend, Biozol, Eching, Germany), and CD57-FITC (Immunotools, Friesoythe, Germany).

Cell viability was determined with RedVid (Invitrogen). All staining steps were performed in PFEA buffer (phosphate-buffered saline, 2% fetal calf serum, 2mM ethylenediaminetetraacetic acid, and 0.01% Na azide). Blocking of nonspecific binding sites was accomplished using human immunoglobulin GAMUNEX (Bayer, Leverkusen, Germany) or mouse serum (Caltag/Invitrogen, Karlsruhe, Germany). For each experiment, cells or mouse/rat  $\kappa$ -chain Comp Beads (Becton Dickinson) were stained with the corresponding fluorochrome-labeled antibodies and incubated for 20 minutes at 4°C in the dark. Human unstained cells were used as negative controls. After washing with PFEA, the cells or beads were resuspended and measured using an LSR-II flow cytometer and the acquisition software FACSDiva (Becton Dickinson). Data were analyzed using FlowJo software (Tree Star, Ashland, OR). For data analysis, dead cells (RedVid-positive) were excluded. CD3<sup>+</sup> living cells were gated within the side scatter/forward scatter (SSC/FSC) lymphocyte gate. Further analysis was performed using CD3<sup>+</sup>CD4<sup>+</sup> and CD3<sup>+</sup>CD8<sup>+</sup> gated populations.

### Statistics

All statistical analyses were performed with Graph-Pad Prism 4.0 using the Mann-Whitney nonparametric *U* test to compare two independent groups. Differences were considered significant with a *p* value  $\leq .05$ . Significant differences are indicated by \**p*  $\leq .05$ , \*\**p*  $\leq .01$ , \*\*\**p*  $\leq .001$ . Significant differences between young (Y) versus old (O) participants are not indicated because the purpose of this study is to compare CO with sporadic AM controls, not

with the young participants, where differences are clearly significant.

## RESULTS

### T-Cell Phenotype Distribution in CO, AM Controls, and Old and Young Participants

To determine the T-cell immune profile of CO compared with the general population, we first investigated the distribution of CD3<sup>+</sup>, CD4<sup>+</sup>, and CD8<sup>+</sup> T cells. Characteristics of individuals studied and their clinical history are summarized in Table 1. Young people tended to have a higher percentage of CD4<sup>+</sup> and fewer CD8<sup>+</sup> T lymphocytes than the elderly people and, as a consequence, their average CD4:CD8 ratio was greater (Table 2). There was a slight but nonsignificant increase in the mean percentage of CD4<sup>+</sup> T cells within the CD3<sup>+</sup> T-cell population in CO as a group compared with the AM controls. A similar slight trend is also visible for CD8<sup>+</sup> T cells, but in this case, CO had a lower percentage compared with AM controls. These slight shifts also affected the CD4:CD8 ratio. As a consequence, CO show a higher ratio compared with their AM controls (Table 2). The CD4:CD8 ratio cutoff for inclusion in the IRP is less than or equal to 1; the data presented here indicate that, as expected, no young controls fell into this group, whereas 3/10 old people (30%) had a CD4:CD8 ratio of less than or equal to 1 (Figure 1). In this respect, CO and AM controls differed little (*p* > .05), with 5/21 CO (23.8%) and 4/15 AM (26.6%) falling into the IRP group by this criterion (Figure 1).

### Costimulatory Molecules on CD4<sup>+</sup> and CD8<sup>+</sup> T Cells

Next, the expression of the costimulatory molecules CD27 and CD28 on CD4<sup>+</sup> and CD8<sup>+</sup> cells was analyzed. These two markers are useful to identify the stage of differentiation of T cells because they are expressed in the early stage and not in the latest stage of differentiation with CD28 downregulated before CD27 on CD8<sup>+</sup> T cells and vice versa in CD4<sup>+</sup> cells (17,18). We therefore compared T-cell differentiation status between the four groups analyzed. The percentage of early differentiated CD4<sup>+</sup>CD27<sup>+</sup> cells (Figure 2A) in young people is significantly higher than in any of the elderly groups (CO, AM, O). Nonetheless, within the elderly population, CO have on average a significantly greater percentage of CD4<sup>+</sup>CD27<sup>+</sup> cells than their AM controls (or the old group). The same result was observed

Table 2. Lymphocyte Subpopulations in Young People, Centenarian Offspring (CO), Age-Matched (AM) Controls, and Old People

Lymphocyte Subpopulations	Young People (age 28.5 ± 1.9), mean ± SD	CO (age 70.1 ± 8.3), mean ± SD	AM (age 69.1 ± 9.7), mean ± SD	Old People (age 86.4 ± 3.8), mean ± SD
CD3 <sup>+</sup>	73.4 ± 16.7	71.5 ± 8.7	72.2 ± 7.2	73.9 ± 8.1
CD4 <sup>+</sup>	58.2 ± 11.2	55.5 ± 18.9	47.1 ± 14.3	48.8 ± 15.6
CD8 <sup>+</sup>	23.3 ± 6.6	24.2 ± 9.9	30.3 ± 11.6	31.6 ± 8.4
CD4:CD8 ratio	2.6 ± 0.9	2.4 ± 1.9	1.9 ± 1.6	1.8 ± 1

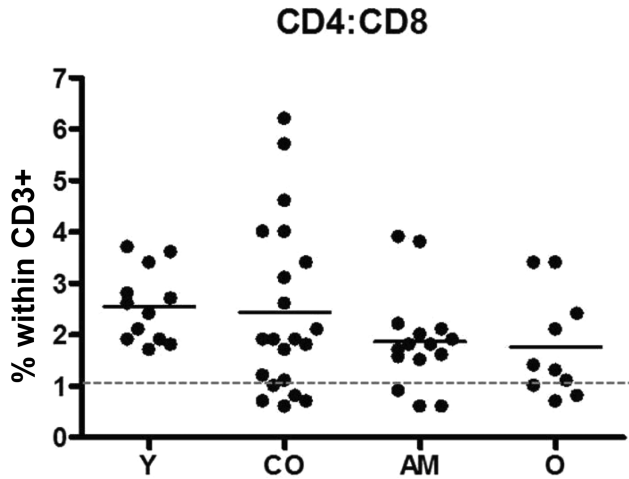


Figure 1. CD4:CD8 ratio in young (Y) controls, centenarian offspring (CO), age-matched (AM) controls, and old (O) people. Peripheral blood mononuclear cells were stained with CD3, CD4, and CD8 antibodies. The CD4:CD8 ratio was calculated within CD3<sup>+</sup> cells. Bars represent medians. Significant differences evaluated by Mann–Whitney nonparametric *U* testing are indicated by \**p* ≤ .05, \*\**p* ≤ .01, \*\*\**p* ≤ .001. The horizontal dotted line indicates a CD4:CD8 ratio of ≤1.

analyzing the absolute cell number (data not shown). For CD4<sup>+</sup>CD28<sup>+</sup> cells, we observed a similar pattern, with a higher expression of CD28 in young people compared with the older group; CO again had more CD4<sup>+</sup>CD28<sup>+</sup> cells than their AM controls, although in this case, the difference did not reach significance (*p* = .06, data not shown).

Within the CD8 subset, again, significantly higher percentages of CD27<sup>+</sup> and CD28<sup>+</sup> cells were observed in young people compared with any of the elderly groups (CO, AM, O). Also here, there was a significant difference between CO and AM controls for the expression of CD27 as percentages (Figure 2B) and absolute numbers (data not shown). A trend toward a higher percentage of CD28<sup>+</sup> cells was observed (*p* = .1, data not shown).

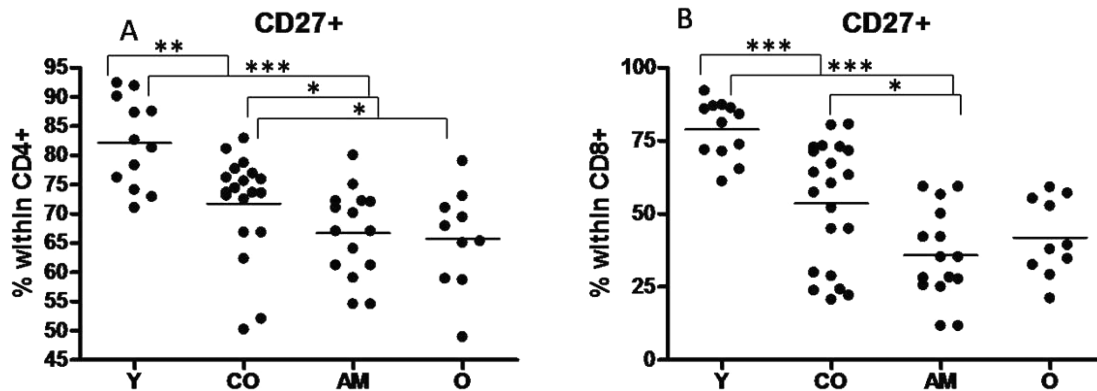


Figure 2. Costimulatory molecule CD27 expression in young (Y) controls, centenarian offspring (CO), age-matched (AM) controls, and old (O) people. Peripheral blood mononuclear cells of 12 young controls (age range: 28.5 ± 1.9; 7 men and 5 women), 21 CO (age range: 70.1 ± 8.3; 10 men and 11 women), 15 AM controls (age range: 69.1 ± 9; 7 men and 8 women), and 10 old (O) participants (age range: 86.4 ± 3.8; 5 men and 5 women) were stained for CD3, CD4, CD8, and CD27. The percentages of CD27<sup>+</sup> (early differentiated) lymphocytes are shown for CD4<sup>+</sup> (A) and CD8<sup>+</sup> T cells (B). Bars represent medians. Significant differences evaluated by Mann–Whitney nonparametric *U* testing are indicated by \**p* ≤ .05, \*\**p* ≤ .01, \*\*\**p* ≤ .001.

### Naïve and Memory T Cells Within the CD4<sup>+</sup> and the CD8<sup>+</sup> Populations

Naïve and memory T cells can be identified by analyzing the expression of two isoforms of the protein phosphatase CD45 (CD45RA and CD45RO), with naïve cells more likely to express CD45RA and memory cells CD45RO. Although assessing the expression of CD45RA and CD45RO provides some information on T-cell differentiation status (Figures 3B and 4B), they are not by themselves sufficient to distinguish between naïve and memory cells as CD45RA is also reexpressed by late-differentiated memory cells (19). For this reason, we have analyzed the expression of CD45RA and CD45RO together with CD27 and CD28 to identify T-cell phenotypes in detail. As previously reported, we considered the phenotype CD27<sup>+</sup>CD28<sup>+</sup>CD45RA<sup>+</sup>CD45RO<sup>-</sup> as the naïve T cells and CD27<sup>-</sup>CD28<sup>-</sup>CD45RA<sup>+</sup>CD45RO<sup>+</sup> as the late-differentiated cells (20).

We confirmed here that naïve CD4<sup>+</sup> (CD27<sup>+</sup>CD28<sup>+</sup>CD45RA<sup>+</sup>CD45RO<sup>-</sup>) cells are more frequent in young people than in any of the groups of elderly people (Figure 3A). The percentage of naïve cells in AM controls (and old participants) is again significantly lower than in CO (*p* = .005, Figure 3A and B). The same result was observed analyzing the absolute cell counts of naïve CD4<sup>+</sup> (*p* = .002, Figure 3C). Differences were more noticeable when we analyzed naïve and late memory CD8<sup>+</sup> T cells in detail. There were fewer naïve CD8<sup>+</sup> T cells in all elderly groups (Figure 4A and B), but CO again had a significantly higher proportion of naïve CD8<sup>+</sup>CD27<sup>+</sup>CD28<sup>+</sup>CD45RA<sup>+</sup>CD45RO<sup>-</sup> cells than their AM controls (*p* = .005). Also in this case, the absolute cell number of naïve CD8<sup>+</sup> cells is significantly higher in CO compared with AM (*p* = .002, Figure 4C). Reciprocally, a higher percentage of late-differentiated CD4<sup>+</sup> cells (CD27<sup>-</sup>CD28<sup>-</sup>CD45RA<sup>+</sup>CD45RO<sup>+</sup>) in the older groups is less evident in CO although this difference did not reach significance, neither as percentage (*p* = .1, Figure 5A) or absolute number of cells (*p* = .1, Figure 5B).



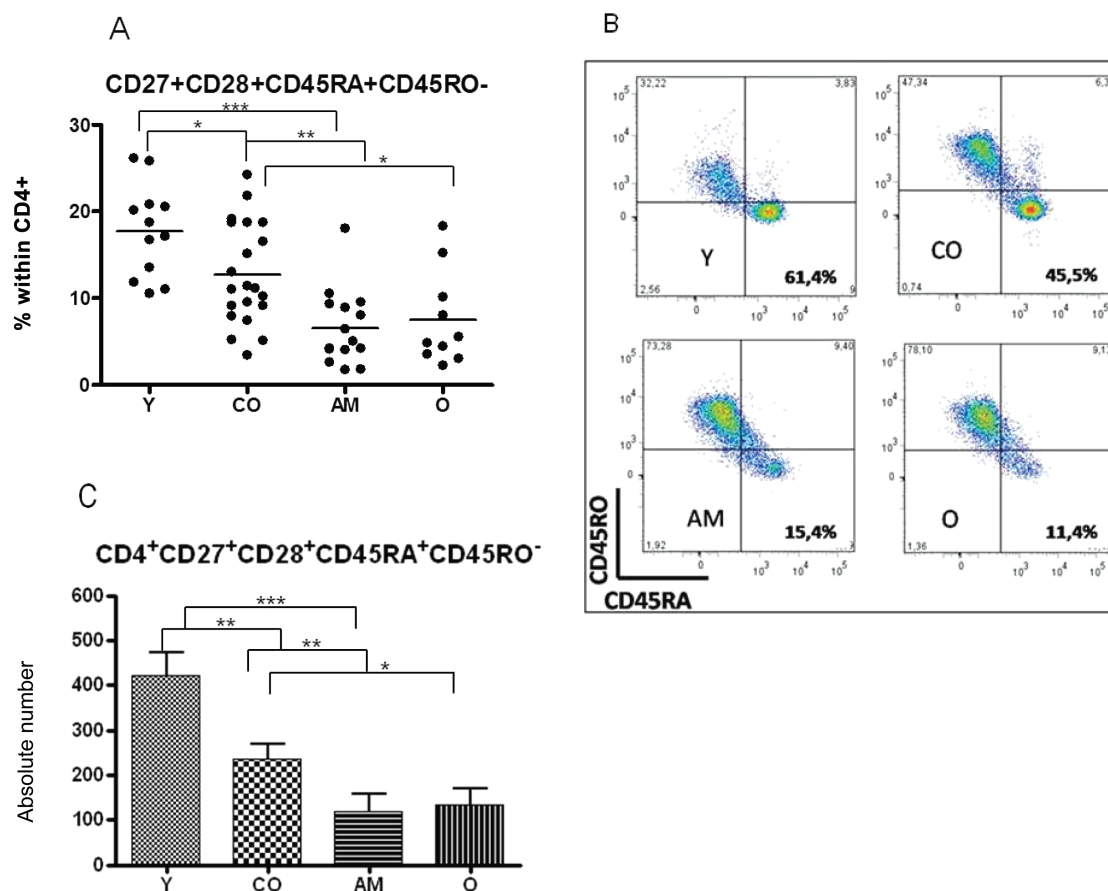


Figure 3. CD4<sup>+</sup> naïve cells. Peripheral blood mononuclear cells from young (Y) controls, centenarian offspring (CO), age-matched (AM) controls, and old (O) people as in Figure 2 were stained with CD3, CD4, CD28, CD27, CD45RA, and CD45RO to identify naïve cells. Percentages (A) and absolute cell counts (C) of CD28<sup>+</sup>CD27<sup>+</sup>CD45RA<sup>+</sup>CD45RO<sup>-</sup> naïve cells of 12 young controls, 21 CO, 15 AM controls, and 10 old participants. Bars represent medians. (B) Representative dot plots of naïve CD4<sup>+</sup> T-cell distribution in a young control (upper left), in a CO (upper right), in an AM (lower left), and in an old participant (lower right). Significant differences are indicated by \* $p \leq .05$ , \*\* $p \leq .01$ , \*\*\* $p \leq .001$ .

Along the same lines, the percentage of late-differentiated (CD8<sup>+</sup>CD27<sup>-</sup>CD28<sup>-</sup>CD45RA<sup>+</sup>CD45RO<sup>+</sup>) cells was significantly lower in CO compared with both AM controls and old people both as percentage ( $p = .003$ , Figure 5C) and absolute number of cells ( $p = .002$ , Figure 5D).

#### Potentially Senescent CD4<sup>+</sup> and CD8<sup>+</sup> T Cells

Previous studies have suggested that lifelong antigenic exposure may lead to increased frequencies of end stage-differentiated CD8<sup>+</sup> T cells that have often been designated “senescent” in the literature. These cells are characterized *inter alia* by the expression of the inhibitory receptor CD57 (21). Therefore, we analyzed the percentage of CD57<sup>+</sup> cells, finding that the older groups had a significantly higher percentage of CD57<sup>+</sup> cells than young people. Although for CD4<sup>+</sup> cells, there were no differences between CO and AM controls or older people ( $p > .05$ , data not shown), percentages of CD8<sup>+</sup>CD57<sup>+</sup> cells were significantly lower in CO than in AM controls and older people ( $p = .0005$ , Figure 6), suggesting that fewer “senescent” T cells are accumulating

in CO. The same trend was present when we analyzed absolute cell numbers.

#### DISCUSSION

Compromised and dysregulated immunity is commonly assumed to be a cause of the increased susceptibility and sensitivity to infectious disease and poor response to vaccination in old people. The immune system, which is constantly challenged by external and internal agents, undergoes dramatic changes with age. These cumulative changes include those resulting from decreased thymic output following puberty, alterations in lymphocyte population dynamics in late life, and reduced intracellular signaling within those cells (22,23). Alterations in T-cell immunity imputed to occur dynamically with aging are reflected in altered numbers and proportions of T-cell phenotypes. One of the consequences of the developmentally programmed involution of the thymus in humans is the rapidly declining output of naïve T lymphocytes after puberty. In aged humans, after the age of 70, naïve CD4<sup>+</sup> T cells have declined to such an extent that

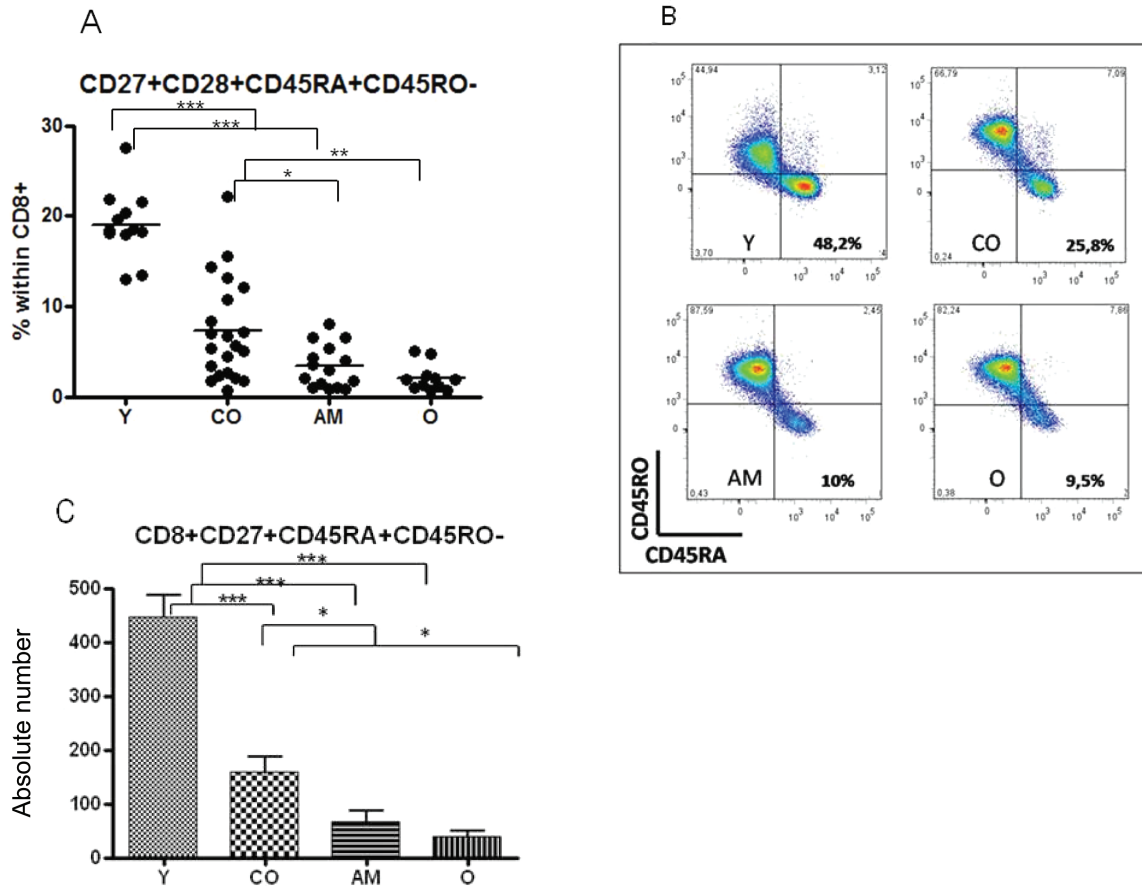


Figure 4. CD8 naïve cells. Peripheral blood mononuclear cells from young (Y) controls, centenarian offspring (CO), age-matched (AM) controls, and old (O) people as in Figure 2 were stained with CD3, CD8, CD28, CD27, CD45RA, and CD45RO to identify naïve cells. Percentages (A) and absolute cell count (C) of CD28<sup>+</sup>CD27<sup>+</sup>CD45RA<sup>+</sup>CD45RO<sup>-</sup> naïve cells of 12 young controls, 21 CO, 15 AM controls, and 10 old participants. Bars represent medians. (B) Representative dot plots of naïve CD8<sup>+</sup> T-cell distribution in a young control (upper left), in a CO (upper right), in an AM (lower left), and in an old participant (lower right). Significant differences are indicated by \* $p \leq .05$ , \*\* $p \leq .01$ , \*\*\* $p \leq .001$ .

their capacity to help B cells to provide efficient humoral responses may be compromised (24–26). As a result of thymic involution, the peripheral T-cell repertoire is not replenished, correlating with the impaired immune response that characterizes old individuals (27). Thus, the elderly people have low percentages of naïve T cells that display numerous functional defects and are associated with mortality, primarily from infectious diseases. The mirror image of this situation is an increased representation of late-differentiated memory T cells that occurs as a consequence of a lifetime's exposure to microbial and other agents.

Similar changes in the CD8<sup>+</sup> subpopulation take place even earlier in life than in CD4<sup>+</sup> T cells (28,29). In particular, in the CD8 compartment, late-differentiated CD45RA<sup>+</sup>CD28<sup>-</sup> T cells accumulate with age and their increased frequencies inversely correlate with vaccine responses (30–32). Thus, it is clear that T cells become significantly less able to engage in effective immune responses and to become functional memory cells as we age, as also seen in mice (33). Many of these CD8<sup>+</sup> T cells with a late-differentiated phenotype are likely to be CMV specific and their accumulation is

much less marked in older CMV-seronegative individuals (6,8–10). Indeed, it was demonstrated that repeated exposure to antigens directly affects the T-cell arm and pathogens and thus directly contribute to immunosenescence (29,34). Chronic CMV infection has an enormous impact on all the changes observed in elderly people. It seems that not only chronological age alone but also persistent CMV infection “accelerates” what is commonly interpreted as immune aging, materially influencing the distribution of T-cell phenotypes in the peripheral blood of old individuals (1,10,35–37). In general, CD4<sup>+</sup> as well as CD8<sup>+</sup> T cells are affected by chronic CMV response, although the magnitude of the effect is greater for CD8<sup>+</sup> T cells (38,39), and other herpes viruses probably contribute little (40).

Recently, we have demonstrated that CMV-seropositive individuals from families enriched for longevity have higher percentages of naïve T cells and lower percentages of CD45RA-reexpressing and late-differentiated effector memory T cells than the general AM population. These people also have lower levels of the proinflammatory marker C-reactive protein compared with AM CMV-seropositive

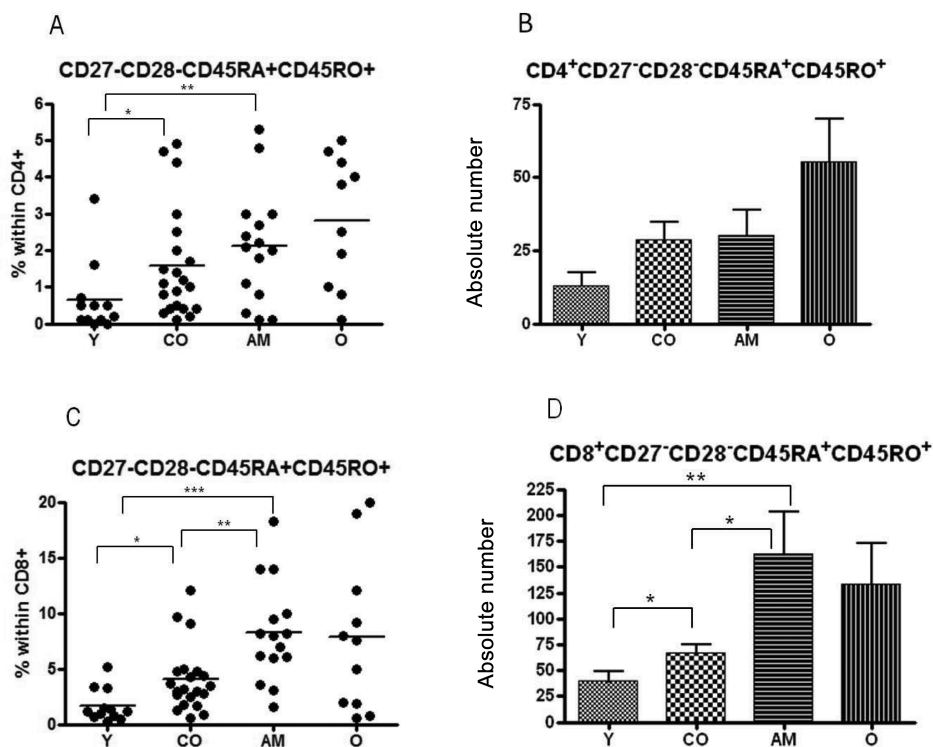


Figure 5. CD4<sup>+</sup> and CD8<sup>+</sup> late-differentiated cells. Peripheral blood monuclear cells from young (Y) controls, centenarian offspring (CO), age-matched (AM) controls, and old (O) people as in Figure 2. The percentages and absolute cell count of late differentiated CD28<sup>-</sup>CD27<sup>-</sup>CD45RA<sup>+</sup>CD45RO<sup>+</sup> within CD4<sup>+</sup> cells (A and B) and within CD8<sup>+</sup> cells (C and D) are shown. Significant differences between the four groups analyzed evaluated by Mann–Whitney nonparametric *U* testing are indicated by \**p* ≤ .05, \*\**p* ≤ .01, \*\*\**p* ≤ .001.

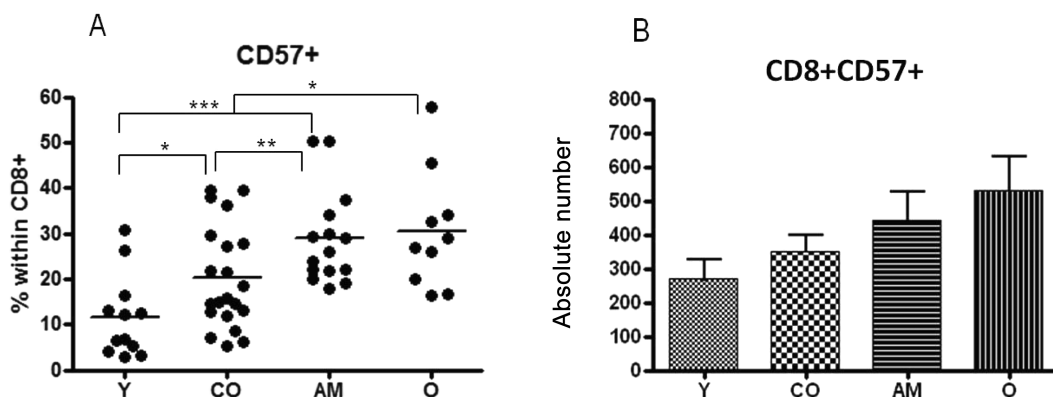


Figure 6. CD8<sup>+</sup>CD57<sup>+</sup> “senescent” cells. Peripheral blood monuclear cells from young (Y) controls, centenarian offspring (CO), age-matched (AM) controls, and old (O) people as in Figure 2 were stained with CD3, CD8, and CD57. Percentages (A) and absolute number (B) of CD8<sup>+</sup>CD57<sup>+</sup> cells in 12 young controls, 21 CO, 15 AM controls, and 10 old people are shown. Bars represent medians. Differences between the four groups analyzed evaluated by Mann–Whitney nonparametric *U* testing are indicated by \**p* ≤ .05, \*\**p* ≤ .01, \*\*\**p* ≤ .001.

controls, suggesting a lower proinflammatory status despite CMV infection (less “inflamm-aging”). The analysis of immune signatures of offspring genetically enriched for longevity has revealed marked differences compared with the general elderly population that might better result in protection not only from infectious disease but also from cardiovascular morbidity and other inflammatory diseases

such as diabetes (10). Such family studies facilitate the identification of genes that are also expected to affect on immune function in old age.

In the present study, we performed a phenotypic analysis of the T-cell arm of adaptive immunity in a group of Sicilian CO, a special population of elderly people presumed to be genetically advantaged for longevity, and who may share

some of the same characteristics as members of long-lived families. Here, we have analyzed very rare people with at least one centenarian parent (100–107 years of age) and compared these results with those of a previous article on familial longevity (10) in which the individuals studied ranged from 40 to 70 years of age with at least one parent having at least one sibling that lived to be more than 90. Thus, the present study specifically sought phenotypic similarities in cohorts derived in a very different manner (offspring of sporadic vs familial longevity). To emphasize the likely genetic contribution, rather than the environmental, we selected the present cohort from a southern European population, which will have experienced very different exposures (food, weather conditions, culture, pathogens, etc.). Also given the fact that these populations are likely to be genetically different, we sought to identify prevalent-shared immune signatures despite all these possible confounding differences in the population. The results indicate some striking similarities between the two different cohorts, emphasizing the potential biological relevance to longevity of these findings under quite different conditions.

We have recently demonstrated that offspring of a centenarian parent also have a “younger” B-cell as well as T-cell profile that could help them resist infection (14,16). Here, we have analyzed the T-cell immune profile of CO (age range:  $70.1 \pm 8.3$ ) compared with equally elderly people without a familial history of longevity (age range:  $69.1 \pm 9.7$ ), and also with unrelated very elderly individuals (age range:  $86.4 \pm 3.8$ ). All were evaluated for CMV status and found to be uniformly positive. Thus, any differences seen between CO and other elderly people cannot be solely due to CMV infection, whereas differences between any of the elderly groups and the young groups, who were all CMV-negative, are mostly likely caused by CMV.

The distribution of the CD4<sup>+</sup> and CD8<sup>+</sup> subsets within CD3<sup>+</sup> T cells was analyzed in terms of the CD4:CD8 ratio because this is one of the immunological parameters included in the IRP in the Swedish OCTO/NONA longitudinal studies (41–43). As there are few studies of the IRP in different populations, it remains of interest to explore its presence and relevance in non-Swedish cohorts. We found that CO had a higher CD4:CD8 ratio compared with their AM controls, again appearing more similar to the CMV-negative young people in this respect, despite being CMV-positive. As previously demonstrated for the B-cell arm of the immune system (14,15), the T-cell arm is probably better preserved in CO than in sporadic AM controls. This could be a consequence of an increased thymic output, due to slower involution or, alternatively, to less primarily CMV-driven peripheral post thymic expansion that causes a significant contraction of the peripheral T-cell receptor repertoire in most elderly people (44). This remains to be investigated, for example, by assessing T cell receptor (TREC) levels.

Analysis of CD27 and CD28 expression, which defines an early stage of T-cell differentiation, confirmed higher

percentages in young people compared with the elderly people. Comparison of the four groups revealed that the percentages of CD4<sup>+</sup>CD27<sup>+</sup> cell subsets were significantly higher in the presumed genetically advantaged CO group compared with their AM controls, with CO again looking more similar to the young groups. For CD28 expression on CD4<sup>+</sup> and CD8<sup>+</sup> cells, the differences between CO and controls remained trends but did not reach statistical significance. Next, we evaluated naïve T cells within the CD4<sup>+</sup> and CD8<sup>+</sup> populations. Our recent detailed classification defined them as CD27<sup>+</sup>CD28<sup>+</sup>CD45RA<sup>+</sup>CD45RO<sup>-</sup> (20). As expected, CD8<sup>+</sup> naïve T cells were more frequent in young donors than in the general elderly population, but here we also saw fewer CD4<sup>+</sup> naïve cells, not always seen in every published study or in our own previous studies on other populations. This may be due to population effects and the relatively small numbers of donors tested here, or to true population differences in Sicilians. Using these phenotyping panels, we had previously observed fewer CD4<sup>+</sup> naïve cells in Western populations only under pathological conditions (notably, Alzheimer’s disease) (20,45). However, we have observed a more pronounced effect on CD4<sup>+</sup> naïve cells in a non-European population where people are considered old at a much earlier chronological age than in the West and might well be expected to have a higher pathogen load (46). The patterns observed in the Sicilian elderly population seem to fall midway between those of the commonly studied northern European and U.S. populations, and the rural Pakistani population studied by Alam and colleagues.

The presence of fewer naïve cells is commonly mirrored by accumulations of memory cells, which have a more restricted repertoire for recognizing pathogens to which the individual was previously exposed. Consistent with the higher level of naïve T cells in CO, percentages of late-differentiated CD8<sup>+</sup> memory T cells (CD27<sup>-</sup>CD28<sup>-</sup>CD45RA<sup>+</sup>CD45RO<sup>+</sup>) were significantly lower in CO than in the general elderly population and were in fact similar to those in the young group. Although the differences between young (CMV<sup>-</sup>) and elderly (CMV<sup>+</sup>) groups can mostly be attributed to the expansion of CMV-specific clones, the lowest percentage of memory T cells observed in CO (who were also all CMV<sup>+</sup>) may be due to more efficient maintenance of immune surveillance against this potentially dangerous pathogen. Consistent with this, the analysis of putatively senescent CD57-expressing cells within CD8<sup>+</sup> T lymphocytes, that previous studies have reported as increased in elderly people when compared with young people (21), also indicated that CO showed a phenotype more similar to the CMV-negative young people than the CMV-positive AM controls and very old people. In this respect, CO do appear very similar to the offspring of long-lived parents in familial longevity studies (10). Seeking shared genetic parameters in these two disparate populations might therefore assist in



identifying the nature of the genes possibly contributing to extended human longevity.

Taken together, data reported here suggest that CO do not show the typical naïve/memory trend observed in the random elderly people, despite all being CMV-positive. The adaptive immune system (both B- and T-cell arms) appears “better conserved” in the offspring of centenarians (ie, more like that seen in younger CMV-negative people), and less susceptible to the major effects that CMV infection has on the general population. This may contribute to their ability to resist typical age-related diseases and imbue them with an increased probability to reach the extreme limits of human life as their centenarian parent did. The similarity between these results and those from the Leiden Longevity Study of familial longevity suggests the presence of longevity-promoting genes shared between quite different populations conveying resistance to the effects of CMV infection on immune signatures commonly believed to be mark deleterious immune function.

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M.P. and G.P. designed the experiments; M.P., S.B., and D.G. performed experiments; M.B. and A.M. contributed to sample collection; G.C.-R., C.C., and G.P. provided material necessary for performing experiments; M.P., S.B., and G.P. wrote the manuscript; all authors have seen and approved the final draft of the manuscript.

#### CONFLICT OF INTEREST

The authors declare no competing financial interests.

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