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Reference Values for CD4+ and CD8+ T Lymphocytes with Naïve or Memory Phenotype and Their Association with Mortality in the Elderly

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Key Words
Naïve T cells · Memory T cells · Immunosenescence · Mortality

Abstract
Background: Well-established reference values which take into account the influence of age on immune cell phenotype, and the impact of naïve or memory T cells on mortality have not been well defined in the elderly. ***Objective:*** The aim of this study was to evaluate the reference values for the peripheral number of total and naïve or memory CD4 and CD8 T cells in a healthy population in Italy, and to analyze whether the immune phenotype was associated with an increased risk of death among older adults. ***Methods:*** The number of total or naïve and memory CD4+ or CD8+ T cells was evaluated in the peripheral blood of 288 healthy people ranging in age from 20 to 107 years. Furthermore, to correlate peripheral immune phenotype with mortality rate after a 3-years follow-up, a retrospective analysis was performed on the results from those individuals aged >65 years at the time of the enrolment in the study. ***Results:*** The absolute number of total and naïve T cells was progressively reduced with increasing age in both the CD4+ and CD8+ T cell populations. The decrease was particularly evident for cells with naïve phenotype, since CD4-naïve and CD8-naïve T cells respectively showed a 4- and a 2- to 3-fold reduction in 70- to >90-year-old subjects in comparison with young adults. The number of CD4 memory T cells significantly increased with age. No significant age-related change was observed in the number of CD8+ memory T cells. Of the 194 subjects included in the study of association of immune phenotype with mortality, 121 were alive and 73 deceased during the 3-year follow-up. The impact of immune parameters on survival demonstrated that only the absolute number of CD8 memory T cells, after adjustment for age, correlated with increased mortality (OR 1.007, 95% CI 1.002–1.012, p = 0.01). The correlation was significant in female but not in male subjects. ***Conclusion:*** We provide reference values for total and naïve or memory CD4 and CD8 T cell populations, and demonstrate that the absolute number of CD8 memory T cells, after adjustment for age, correlates with increased mortality.

Introduction

Immune senescence has been associated with a numerical change in T cell number [1, 2] and in T cell repertoire, mainly represented by an expansion in memory and a reduction in naïve T lymphocytes [3–5]. Naïve T cells, i.e., lymphocytes which have never encountered their specific antigen, are essential for the induction of primary immune responses against new tumor antigens, and for the efficient generation of Th1-cell immunity which promotes cytotoxic T lymphocyte (CTL)-mediated responses. As opposed to naïve T cells, memory T lymphocytes accumulate in elderly individuals as a result of continuous antigen-induced turnover of populations that are specific for frequently encountered antigens [6].

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Analysis of T cell phenotype represents a useful aid in the diagnosis and management of a wide range of medical conditions, such as immunodeficiency, transplantation, cancer, and autoimmunity [7]. Nevertheless, the lack of well-established reference values which take into account the influence of age on immune cell phenotype, make the interpretation of the immunological data difficult and may lead to incorrect intervention. In this context, despite the observations which have demonstrated the age-related shift in the representation of naïve and memory phenotypes, the few studies performed until now compared a group of old subjects to a group of young donors without providing reference values for these lymphocyte populations in the different decades of human life.

Many studies performed in human subjects and in experimental animals have suggested a correlation between immune function and age-related risk of morbidity and mortality, although the contribution of the immune system to survival has not been extensively elucidated. In fact, few and controversial information has been derived from previous studies examining the relationship between immune status and survival. Lower lymphocyte total count was associated with an increased risk of mortality in two [8, 9] but not in another recent study [10]. Similarly, the association of high CD8+ and low CD4+, or a low CD4/CD8 ratio, with increased mortality was reported in some studies [1–10], but was not confirmed in a more recent report [11]. Other evidence has been reported on the association of higher mortality with decreased lymphocyte proliferation to mitogens [12, 13]. The relationship between naïve or memory CD4 or CD8 T cell subsets and mortality has not been characterized until now apart from a recent study examining a population of women with a mean age of 77 years in which no significant correlation was found [11].

In this study we report the age-related reference values for peripheral blood total or naïve and memory CD4+ or CD8+ T cells applicable to the healthy population in Italy and other regions with similar demographic characteristics. Furthermore, we analyzed whether the immune phenotype present in subjects at the time of the initial blood sample collection was associated with an increased risk of death among older adults.

Subjects and Methods

After written informed consent, human peripheral blood was obtained from 288 subjects (209 females and 79 males) ranging in age from 20 to 107 years. All study procedures and forms were approved by the local ethics committee. The health status of all people recruited for this study was assessed according to a protocol which included: (1) the assessment of current and past health status by means of a specific questionnaire; (2) the presence of diseases known to affect hematopoiesis (cancer, chronic infections, collagen vascular disease, and rheumatoid disease), and (3) the measurement of basic laboratory parameters. All donors were in good clinical condition at the time of enrolment in the study, and subjects in poor health or in therapy with drugs interfering with the immune system were excluded.

Peripheral blood mononuclear cells (PBMCs) were fractionated on Lympholyte H (Cederlane, Canada) and separated by density gradient centrifugation at 800 *g* for 20 min. Cells from the interface of the gradients where washed twice with Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline (PBS; Gibco/Life Technologies, Grand Island, N.Y., USA), and then counted and suspended in PBS.

Study Design for Analysis of Mortality

In the present study the blood drawing was conducted on subjects between 2002 and 2004. Retrospective analyses were performed on the results from those individuals aged >65 years at the time of enrolment, who were alive or had died 3 years after the initial blood sample collection. A total of 194 subjects was included in the study for analysis of the association of immune phenotype and mortality. Of these, 121 subjects (78 females, 43 males) were alive and 73 subjects (59 females, 14 males) had died during the 3-year follow-up. Causes of death included: cardiovascular diseases (n = 32); cancer (n = 12); pulmonary diseases (n = 4); cachexia (n = 16); neurodegenerative diseases (n = 2), and others (n = 7).

Monoclonal Antibodies and Flow Cytometric Analysis

PBMCs isolated by density gradient centrifugation were labelled with monoclonal antibodies anti-CD4 (PerCp) or anti-CD8 (PerCp) combined with anti-CD95 (PE) and anti-CD62L (FITC; all from Becton Dickinson, San Jose, Calif., USA) for 15 min at room temperature. At the end of the incubation, cells were washed in PBS, suspended in PBS, and immediately analyzed with a Coulter Epics XL flow cytometer equipped with an argon laser emitting at 488 nm. Ten thousand events were acquired. Analysis was done using Expo Cytometry Software by Applied Cytometry System. The absolute number of CD4+ and CD8+ naïve and memory T cells was calculated by the total number of lymphocytes per microliter of peripheral blood. The absolute number of naïve and memory CD4+ or CD8+ T cells in each subject was calculated as follows: (percentage of each cell population among total lymphocytes) × (total cell count)/100.

Statistical Analysis

Data were analyzed for statistical significance using parametric or nonparametric tests on the basis of the distribution of the data. Comparison of variables among groups were made by analysis of variance (ANOVA on ranks), and the Pearson correlation was used to correlate the data with the donor age. Significance was set at a 5% level ($p < 0.05$). To identify the independent predictors of death, multivariate analyses were performed by logistic regression. In order to assess the risk associated with the immune phenotype, the logistic model estimated the odds ratios adjusting for age as a potential confounder. Statistical analyses were performed using Sigma Stat software version 1.03 (Jandel Scientific, Germany) and Systat 10 (SPSS Science Marketing Department, Chicago, Ill., USA).

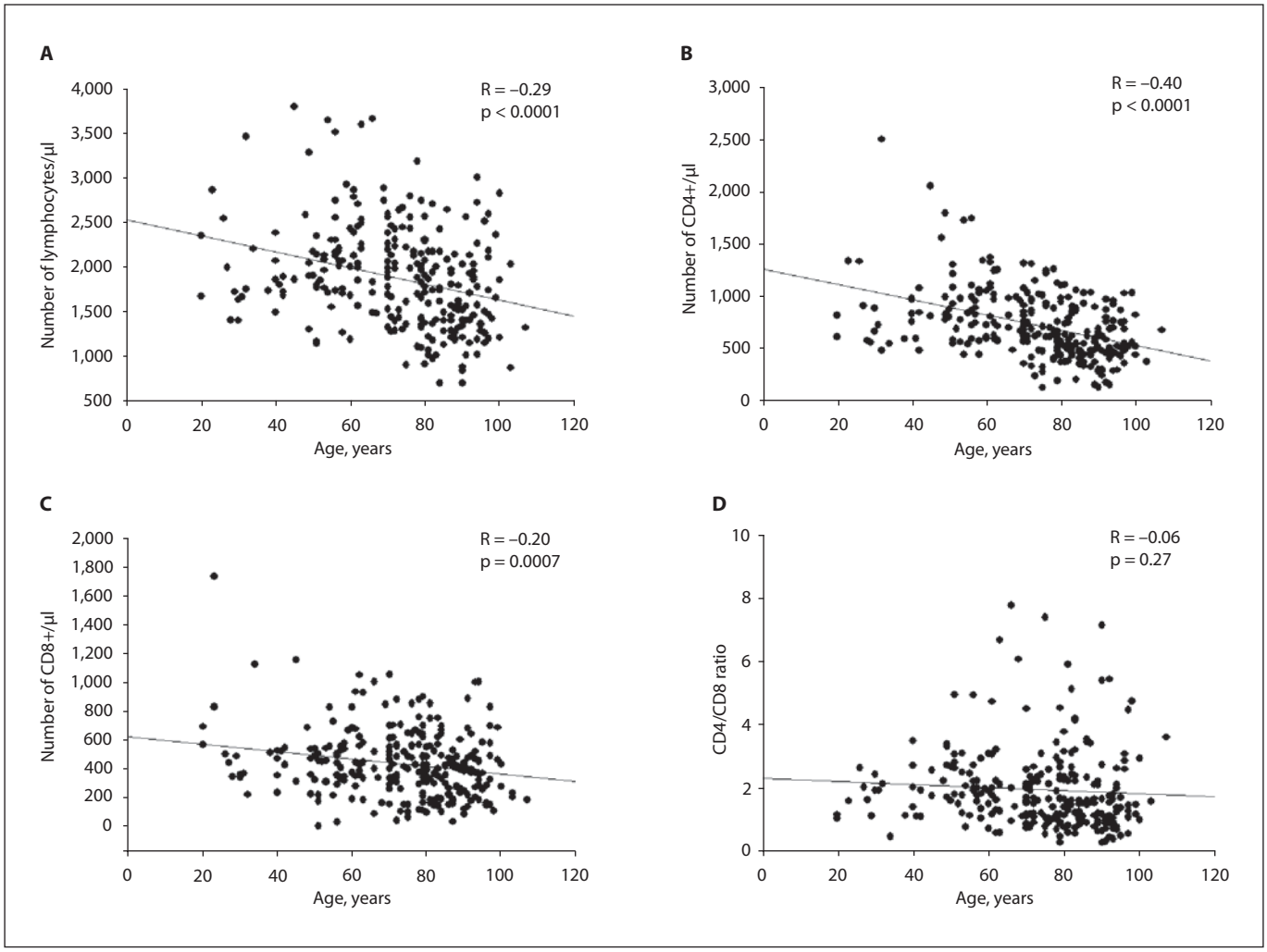

Fig. 1. Effect of age on the absolute number of lymphocytes and of CD4+ and CD8+ T cells. Data from 288 healthy donors, 20–107 years of age, are plotted as individual data points. R and p values were calculated by linear regression analysis. **A** Absolute number of lymphocytes in the peripheral blood. **B**, **C** Freshly isolated PBMCs from subjects of different ages were stained with mAbs anti-CD4 or anti-CD8 and analyzed by flow cytometry. **D** The ratio between the absolute number of circulating CD4+ cells and CD8+ cells is reported and plotted as a function of age.

Results

Ex vivo Analysis of Total or Naïve and Memory CD4+ or CD8+ T Cells

PBMCs from 288 healthy donors, 20–107 years of age, were analyzed for the absolute number of lymphocytes and for CD4+ or CD8+ T cells and plotted as individual data points. As shown in figure 1, the number of lymphocytes (fig. 1A; R = –0.29, p <0.0001) and of CD4+ (fig. 1B; R = –0.40, p <0.0001), or CD8+ (fig. 1C; R = –0.20, p = 0.0007) T cells significantly decreased with advancing age. The CD4/CD8 ratio did not show a significant relationship with age (fig. 1D; R = –0.06, p = 0.27).

We then analyzed freshly isolated PBMCs from young adulthood to the extreme limit of human life using 3-color fluorescence with CD62L and CD95 co-expression either with CD4+ or CD8+ T cells. The results in figure 2A and C indicate that the absolute number of CD62L+CD95– T cells, which has recently been considered the standard phenotype to define naïve T cells [3], progressively decreased with the increasing age of subjects both among CD4+ T cells (fig. 2A; R = –0.48, p <

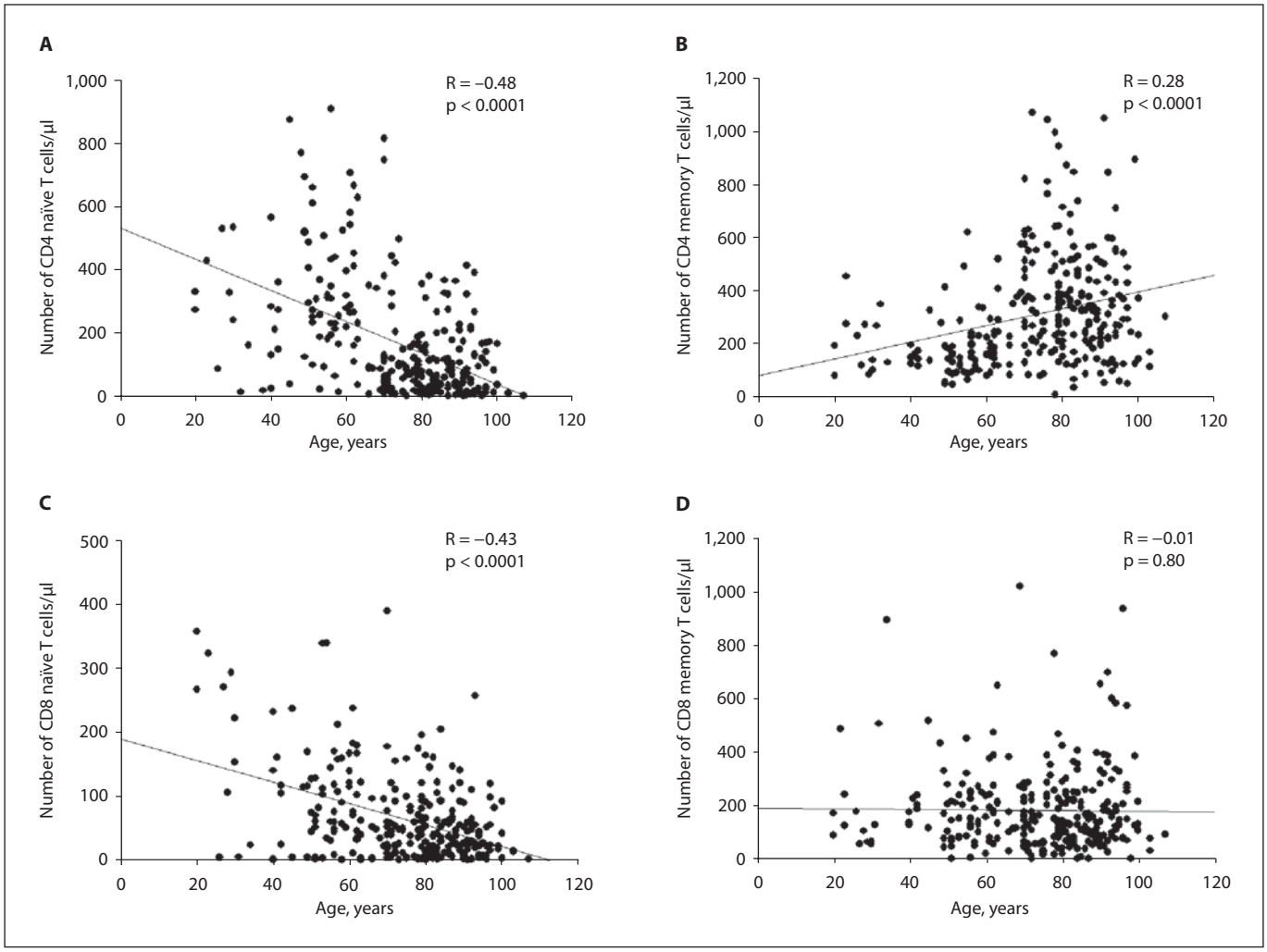

Fig. 2. Age-related changes in the absolute number of CD4+ and CD8+ naïve and memory T cells. Absolute number of naïve and memory T cells among CD4+ (**A**, **B**) and CD8+ (**C**, **D**) cells from 288 donors were plotted as individual data points. Cell staining was performed as reported in 'Subjects and Methods'. R and p values were calculated by linear regression analysis.

0.0001) and CD8+ T cells (fig. 2C; R = -0.43, $p < 0.0001$). As shown in figure 2B and D, the analysis of the CD62L–CD95+ phenotype, which has been demonstrated to define memory T cells, revealed that CD62L–CD95+ T cells increased significantly and progressively with age among CD4+ T cells (fig. 2B; R = 0.28, $p < 0.0001$) but not CD8+ T cells (fig. 2D; R = 0.01, p = 0.80).

Reference Values for Total or Naïve and Memory CD4+ or CD8+ T Cells during Aging

In order to establish the reference values for total and naïve or memory CD4 or CD8 T cells during aging, data were grouped into 6 classes of age and expressed as mean ± SD with double-sided confidence intervals, as shown in table 1. No significant change in the absolute number of CD4+ T cells was observed in subjects ranging from 20 to 69 years of age. Afterwards, the absolute number of CD4+ lymphocytes significantly decreased in 70- to 79-year-old donors, and were further reduced in 80- to 89- and >90-year-old subjects ($p < 0.05$ vs. 20- to 45- and 46- to 59-year-old subjects; table 1). A similar kinetics was observed in CD8+ T cell number, with a significant decrease in subjects aged >80 years ($p < 0.05$ vs. 20- to 45-year-old subjects). The numbers of both CD4+ and CD8+ naïve T cells dropped in subjects aged >69 years ($p < 0.05$ vs. <69-year-old subjects). On the contrary, the

Table 1. Reference values of naïve and memory CD4 or CD8 T cells during aging

Donor age years	Number of subjects	Female/ male ratio	Gender	Lymphocyte population					
				CD4[1]	CD8[2]	CD4 naïve[3]	CD4 memory[4]	CD8 naïve[5]	CD8 memory
20–45	28	22/6	All	912 ± 484[a]	564 ± 342[a]	473 ± 539[a]	190 ± 94[a]	160 ± 115[c]	216 ± 196
			Female	949 ± 516	461 ± 193	450 ± 527	176 ± 80	136 ± 106	198 ± 144
			Male	768 ± 340	763 ± 483	557 ± 639	238 ± 133	213 ± 154	281 ± 347
46–59	38	30/8	All	934 ± 345[a]	442 ± 153	384 ± 272[a]	183 ± 123[a]	99 ± 78[a]	173 ± 105
			Female	969 ± 354	424 ± 159	412 ± 290	190 ± 129	94 ± 73	161 ± 110
			Male	799 ± 293	513 ± 155	265 ± 138	154 ± 100	100 ± 97	208 ± 84
60–69	32	19/13	All	868 ± 259[c]	519 ± 270[c]	298 ± 200[a]	234 ± 127[a]	80 ± 64[a]	233 ± 209
			Female	942 ± 286	458 ± 207	357 ± 206	204 ± 117	86 ± 69	201 ± 160
			Male	780 ± 201	594 ± 315	204 ± 155	272 ± 133	71 ± 60	274 ± 263
70–79	65	41/24	All	709 ± 287[d]	449 ± 213	126 ± 156[b]	406 ± 239[b]	57 ± 62[d]	154 ± 124
			Female	696 ± 265	455 ± 230	109 ± 119	424 ± 224	56 ± 52	159 ± 107
			Male	730 ± 324	462 ± 202	152 ± 201	376 ± 263	62 ± 75	138 ± 151
80–89	69	53/16	All	610 ± 237[b]	376 ± 182[b]	97 ± 104[b]	366 ± 183[b]	50 ± 47[d]	156 ± 100
			Female	618 ± 242	361 ± 163	105 ± 106	361 ± 180	52 ± 48	149 ± 101
			Male	600 ± 212	426 ± 235	69 ± 95	384 ± 197	48 ± 46	170 ± 100
>90	56	48/9	All	558 ± 226[b]	390 ± 203[b]	87 ± 98[b]	329 ± 211[b]	36 ± 41[b]	226 ± 188
			Female	570 ± 226	399 ± 206	94 ± 105	329 ± 222	35 ± 43	228 ± 169
			Male	562 ± 310	341 ± 192	66 ± 48	296 ± 157	35 ± 36	211 ± 279

Data are expressed as the mean ± SD of the number of cells per microliter in the peripheral blood (95% CI).
[1] $p < 0.05$: b vs. a; d vs. a; b vs. c.
[2] $p < 0.05$: b vs. a; b vs. c.
[3] $p < 0.05$: b vs. a.
[4] $p < 0.05$: b vs. a.
[5] $p < 0.05$: b vs. a; b vs. c; d vs. c.

number of CD4+ memory cells greatly increased after the age of 69 years ($p < 0.05$ vs. <69-year-old subjects). The number of CD8+ memory T cells did not show significant age-related changes. As reported in table 1, when total or naïve and memory CD4 or CD8 T cells were analyzed separately in female and male subjects, no statistically significant gender difference was found.

Immune Parameters as Predictors of Death

Retrospective analyses were performed on the results from those individuals aged >65 years at the time of enrolment in the study who were alive (n = 121, 78 females, 43 males, F/M ratio = 1.81) and those who had died (n = 73, 59 females, 14 males, F/M ratio = 4.21) 3 years after the initial blood sample collection. The impact of immune parameters on survival was then evaluated in a multivariate logistic regression analysis. As shown in table 2, the absolute number of CD8 memory T cells, after adjustment for age, correlated with increased mortality (OR 1.007, 95% CI 1.002–1.012, p = 0.01). The other immune parameters examined did not correlate with the mortality rate (table 2). When female and male subjects were analyzed separately, the absolute number of CD8 memory T cells correlated with increased mortality in female but not in male subjects (OR 1.010, 95% CI 1.004–1.017, p = 0.002 for females; OR 1.000, 95% CI 0.989–1.011, p = 0.982 for males; table 2).

Discussion

Age-related changes in the immune system, which are generally referred to as immunosenescence, are well documented and concern primarily the adaptive immune responses [14–17]. These changes are mainly represented by alterations in T cell phenotype and functions [18]. Nev-

Table 2. Multivariate analysis assessing predictors of mortality

Variables	Gender	Odds ratio	95% CI	p value
Age	All	0.781	0.727–0.838	0.000
	Female	0.771	0.705–0.843	0.000
	Male	0.749	0.634–0.885	0.001
Lymphocytes	All	0.999	0.998–1.000	0.223
	Female	0.999	0.998–1.000	0.138
	Male	1.000	0.998–1.002	0.761
CD4[1]	All	1.001	0.997–1.004	0.723
	Female	1.000	0.997–1.004	0.776
	Male	1.006	0.995–1.018	0.275
CD8[1]	All	0.997	0.993–1.001	0.158
	Female	0.996	0.991–1.001	0.091
	Male	0.999	0.988–1.009	0.785
CD4/CD8 ratio	All	0.967	0.537–1.742	0.911
	Female	0.929	0.491–1.757	0.821
	Male	0.687	0.074–6.375	0.741
CD4 naïve[1]	All	0.998	0.993–1.003	0.346
	Female	0.998	0.993–1.003	0.369
	Male	0.995	0.978–1.012	0.558
CD4 memory[1]	All	1.002	0.999–1.004	0.303
	Female	1.002	0.999–1.005	0.251
	Male	0.996	0.987–1.005	0.391
CD8 naïve[1]	All	0.999	0.991–1.008	0.891
	Female	1.001	0.992–1.011	0.771
	Male	0.986	0.947–1.028	0.512
CD8 memory[1]	All	1.007	1.002–1.012	0.008
	Female	1.010	1.004–1.017	0.002
	Male	1.000	0.989–1.011	0.982

CI = Confidence interval.

[1] Lymphocytes, CD4 and CD8 T cells and their naïve and memory subsets have been considered as absolute number.

ertheless, even though the age-associated kinetics of the peripheral representation of total and naïve or memory CD4 or CD8 T cells has been described, no detailed data have been reported to date on the reference levels of these lymphocyte populations in aging populations.

This study provides reference values for the absolute number of total and naïve or memory CD4+ and CD8+ T cells with advancing age, reporting levels of these lymphocyte subsets for each decade of life. Furthermore, the study investigated the impact of immune parameters on survival demonstrating that the absolute number of CD8 memory T cells, after adjustment for age, correlates with increased mortality.

We show that the absolute number of both CD4 and CD8 T cells progressively decreases with advancing age, mainly because of a great age-related reduction in the absolute number of naïve T cells. On the contrary, the absolute number of memory CD4 but not CD8 T cells significantly increases with age in elderly subjects. The mean values of these lymphocyte populations are significantly different in aged subjects from those reported in young-adult age, particularly in people older than 70 years: CD4-naïve and CD8-naïve T cells showed a 4- and a 2- to 3-fold reduction, respectively, in 70- to >90-year-old subjects in comparison with young-adult ages, whereas CD4 memory T cells were 1.5- to 2-fold increased.

Earlier observations have demonstrated an age-related shift in the representation of naïve and memory phenotypes with a decrease in naïve T cells and an accumulation of memory lymphocytes. However, in the studies performed to date a cumulative group of old subjects was compared to a group of young donors, without providing reference values for these lymphocyte populations for the different decades of human life. Xu et al. [19] found that the number of memory cells exceeded the number of naïve cells in donors aged >70 years, whereas the converse was true for young donors. Similarly, Stulnig et al. [20] found a shift from naïve to memory phenotype in elderly subjects aged 65–74 years in comparison to young donors aged 20–32 years. More recently, Bisset et al. [21] reported reference values for naïve and memory CD4 or CD8 T cells in 70 healthy subjects. Considering 2 groups of subjects with mean ages of <50 or >50 years, they found a significant age-related decrease in both naïve CD4 and CD8 T cells, and an increase in memory CD4 T lymphocytes. The range in age of each group in this study included about two decades of life and the older donors were aged 71 years. As opposed to the studies reported above, in our study we provide for the first time reference values for subjects ranging from 20 to 107 years grouped into 6 clusters of increasing age. From our data clearly emerges that the number of naïve CD4 or CD8 T cells significantly decreased in subjects aged >69 years.

Several studies have examined the relationship between immune status and survival. Murasko et al. [18] reported that poor responses to three T cell mitogens were associated with increased mortality during aging. In 2 other studies, anergic individuals were found to have a higher mortality rate as compared with non-anergic subjects [13, 22]. A combination of high CD8+ and low CD4+ percentages and poor lymphocyte proliferation responses was associated with higher mortality in studies of older adults in Sweden [1, 11, 23]. In a case-control

study conducted in 122 women aged 65 and over, there was no significant correlation between cases and controls in any of the T cell subsets studied which included CD4+ and CD8+ T cells and subsets of these cells defined by expression or non-expression of CD28, CD45RA (naïve T cells), and CD45RO (memory T cells) [11]. In our study we found a significant relationship between the CD8 memory T cell number and diminished survival. The correlation was statistically significant in female but not in male subjects. This evidence might seem contradictive since this cell population is relevant for the protection against infectious and cancerous diseases. However, it is well known that highly differentiated T cells accumulate in elderly subjects as a result of continuous antigen-induced turnover of populations that are specific for frequently encountered antigens [7]. It has been suggested that the expansion of functionally suboptimal specific CD8 T cells might smother other memory T cell populations through competition for space or growth factors [24, 25]. This may be the case with cytomegalovirus (CMV), which has been found to induce accelerated and extreme T cell differentiation and has been associated with decreased survival of elderly subjects [26]. In this context, CMV seropositivity has been included in a cluster of immune parameters, defined as immune risk phenotype, that have been shown recently to predict the early mortality of elderly humans [27]. In our study we did not examine the CMV seropositivity of the population studied and we can only speculate that the association of memory CD8 T cell number with mortality is linked to a potential expansion of nonfunctional specific CD8 T cells.

In conclusion, we provide reference values for total and naïve or memory CD4 and CD8 T cell populations in the different decades of human life, and we demonstrate that the absolute number of CD8 memory T cells, after adjustment for age, correlates with increased mortality. When female and male subjects were analyzed separately, the absolute number of CD8 memory T cells correlated with increased mortality in female but not in male subjects.

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