

Thymic output and functionality of the IL-7/IL-7 receptor system in centenarians: implications for the neolymphogenesis at the limit of human life

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Summary

During aging, the thymus undergoes a marked involution that is responsible for profound changes in the T-cell compartment. To investigate the capacity of the thymus to produce new cells at the limit of human lifespan, we analyzed some basic mechanisms responsible for the renewal and maintenance of peripheral T lymphocytes in 44 centenarians. Thymic functionality was analyzed by the quantification of cells presenting the T-cell receptor rearrangement excision circles (TREC). A new method based upon real-time PCR was used, and we found that most centenarians (84%) had undetectable levels of TREC+ cells. Six-color cytofluorimetric analysis revealed that centenarians had an extremely low number of naïve T cells; central memory and effector memory T cells were greatly increased, while terminally differentiated cells were as numerous as in young (aged 20–45) or middle-aged (aged 58–62) donors. Interleukin (IL)-7 and IL-7 receptor α -chain (CD127) levels were the same at all ages, as shown by ELISA, flow cytometry and real-time PCR. However, IL-7 plasma levels were higher in centenarian females than males. The presence of TREC+ cells and of very few naïve T lymphocytes suggests that in centenarians such cells could either derive from residues of thymic lymphopoietic

islets, or even represent long-living lymphocytes that have not yet encountered their antigen. IL-7 could be one of the components responsible, among others, for the higher probability of reaching extreme ages typical of females.
Key words: aging; IL-7; longevity; sjTREC; T cell; thymus.

Introduction

Several studies have demonstrated that immunological aging, defined as immunosenescence, is not characterized by a simple deterioration of the immune system but is part of a *continuum* of developmental processes with complex reorganizational events, compensatory mechanisms and qualitative alterations in function. Centenarians provide the best example of successful aging and their immune system is characterized by an extensive remodeling where some parameters increase, others decrease, while some remain unchanged (Cossarizza *et al.*, 1992; Franceschi *et al.*, 1995; Franceschi *et al.*, 1996; Haynes *et al.*, 2000; Pinti *et al.*, 2003).

Immunological aging is characterized by profound modifications of the thymus gland (Cossarizza *et al.*, 1996, 1997; Aspinall & Andrew, 2000; Haynes *et al.*, 2000; Taub & Longo, 2005). Indeed, shortly after birth the thymus undergoes a life-long process of involution whereby the organ is replaced by adipose tissue, accompanied by the loss of its main immune function (i.e. the production of T cells). Such an involution results in a reduction in the number of constituent thymocytes with age, a consequent shrinking of the thymus and finally a decline in the output of naïve T cells, typical of the gland. Far advanced age is thus characterized by a profound reduction and consequent exhaustion of naïve T-cell population and by a progressive accumulation of memory/effector T cells (Sansonetti *et al.*, 1997; Fagnoni *et al.*, 2000).

The modifications in peripheral T-cell populations reflect not only the involution of the thymus, but are also consequent upon the massive antigenic stimulation of a virgin immune system by numerous foreign antigens, that occurs principally between birth and puberty. Few data exist on the origin and renewal of virgin and memory T-cell compartments with age, especially at a time when the thymus has undergone a profound involution, as is the case in centenarians (Douek *et al.*, 1998; Poulin *et al.*, 1999; Douek & Koup, 2000). The direct evaluation of thymic output is now possible by the quantification of the so-called 'recent thymic emigrants' (RTE), the main cells that contribute to the naïve T-cell pool (that have the phenotype: CD45RA+, CD62L+, CCR7+, CD95–). RTE are characterized by the presence in the nucleus of circular episomal DNA molecules named 'T-cell receptor (TCR) rearrangement excision circles' (TREC) that are generated during the rearrangement of the TCR locus, and

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that can be measured by different assays, including real-time polymerase chain reaction (PCR) (Pinti *et al.*, 2003). Recent data show that TREC⁺ cells decline with age, and indeed donors older than 55 years have fourfold less cells than young individuals (Naylor *et al.*, 2005). No data are yet available on the production of TREC⁺ cells at the extreme limit of human lifespan.

The cytokine network undergoes a complex remodelling during aging, where an increase of proinflammatory molecules has been well documented, along with a decrease of a trophic agent such as interleukin (IL)-2 (Fagiolo *et al.*, 1993; Bagnara *et al.*, 2000; Franceschi *et al.*, 2000a,c; Gerli *et al.*, 2000; Franceschi *et al.*, 2001). Another cytokine, IL-7, has profound effects on the production of T lymphocytes and on the homeostasis of the T-cell pool (Fry & Mackall, 2001, 2005; Okamoto *et al.*, 2002; Bradley *et al.*, 2005), but scanty data exist on its production and utilization during aging. The activity of IL-7 is mediated by a receptor which is formed by a specific α -chain (IL-7R α , or CD127, expressed by most peripheral T cells), linked with the so called γ_c -chain, a molecule shared in common with receptors for other cytokines such as IL-2, IL-4, IL-9, IL-15, and IL-21. The formation of a heterodimer is crucial for signal transduction and therefore for the activity of this cytokine. Interestingly, the presence of high amounts of IL-7 receptor is associated with the permanent up-regulation of the antiapoptotic molecule bcl-2, which is accompanied by the down-regulation of CD95 (Ledru *et al.*, 1998; Guillemard *et al.*, 2001). By influencing intracellular levels of molecules such as bcl-2, bcl-x_L and caspase-3, IL-7 preserves T cells from apoptosis induced by glucocorticoids,

by cytokine withdrawal, and by radiation (Boise *et al.*, 1995; Hernandez-Caselles *et al.*, 1995; Seki *et al.*, 1995; Vella *et al.*, 1997; Hassan & Reen, 1998; Vella *et al.*, 1998). IL-7 can induce strong telomerase activity in CD45RA⁺ T cells without the requirement of TCR stimulation. It has been suggested that this cytokine-mediated expansion may contribute to the maintenance of a naïve T-cell repertoire in adults (Soares *et al.*, 1998).

In order to investigate thymic functionality and the IL-7/IL-7 receptor system during human longevity, we have analyzed the expression of TREC, IL-7 receptor and plasma levels of IL-7 in a group of 44 centenarians, who have been compared to young and middle-aged donors.

Results

Distribution of naïve, central and effector memory, and terminally differentiated lymphocytes in centenarians

Using flow cytometry, we measured the percentages of naïve, central and effector memory, and terminally differentiated cells in highly defined CD4⁺ and CD8⁺ T cells from 10 centenarians, 11 middle-aged and 12 young subjects. A typical example of the analysis performed on a centenarian's lymphocyte sample is shown in Fig. 1. To better identify CD4⁺ and CD8⁺ T lymphocytes, we first gated cells on the basis of their physical parameters (FSC and SSC), then on the basis of CD45 and CD3 expression. A gate was then set on CD4⁺ or CD8⁺ lymphocytes,

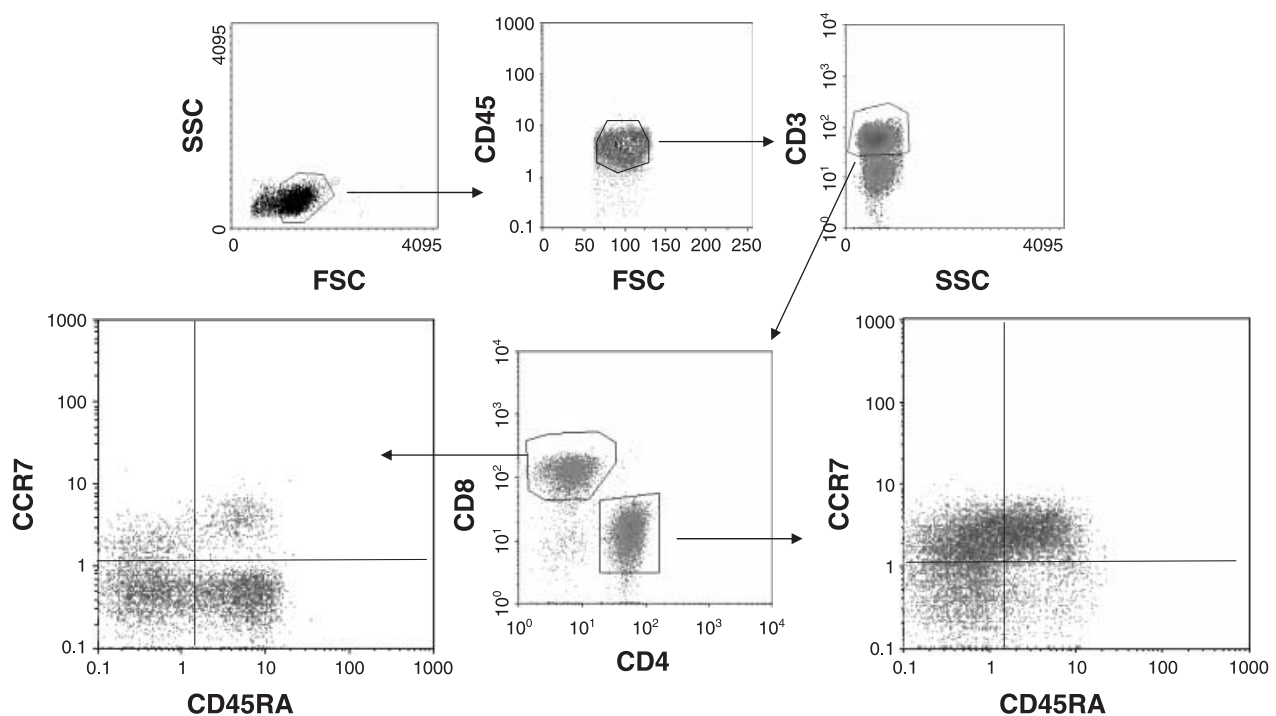
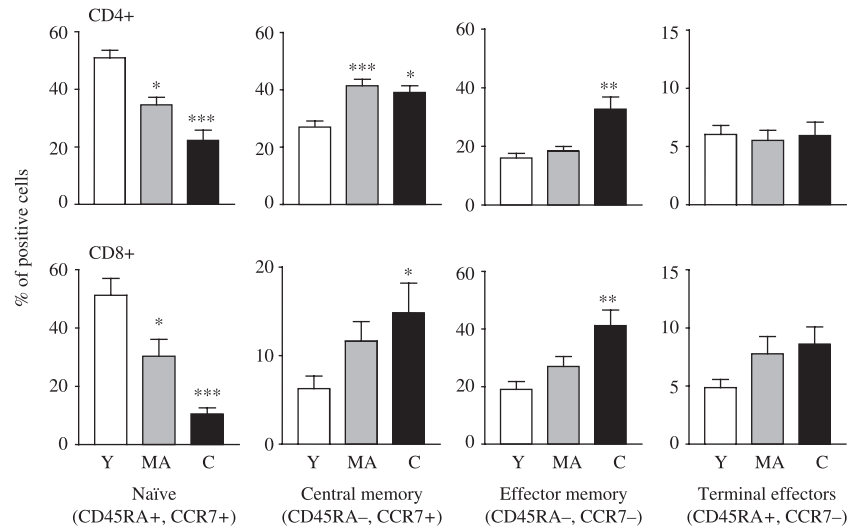


Fig. 1 Cytofluorimetric analysis of PBMC in a centenarian. In this representative example, a first gate was set on FSC vs. SSC to identify small lymphocytes and to eliminate cell debris. T cells were identified by gating on CD45⁺, CD3⁺ cells. The expression of CD45RA and CCR7 was then investigated in CD4⁺ and CD8⁺ cells.

Fig. 2 Naïve and memory T cells in centenarians, young and middle-aged donors. Data indicate the percentages (mean \pm SEM) of CD4+ and CD8+ naïve (CD45RA+CCR7+), central memory (CD45RA-CCR7+), effector memory (CD45RA-CCR7-) and terminal effector (CD45RA+CCR7-) T cells in young (Y), middle-aged (MA) and centenarian (C) subjects. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared to young healthy donors.



and the expression of CD45RA and CCR7 was analyzed on each main T-cell subset. Quadrants were drawn on the basis of adequate negative controls.

Figure 2 shows the results obtained in the three populations under investigation. It is noteworthy that while main changes occurred in the naïve, central memory and effector memory compartment, no significant age-related changes were present in the compartment of terminally differentiated cells either among CD4+ or CD8+ T cells.

Quantification of sjTREC+ cells

The evaluation of sjTREC levels was performed on peripheral blood mononuclear cells (PBMC) of 16 young subjects (13 females and 3 males), 13 middle-aged subjects (10 females, 3 males) and 25 centenarians (22 females and 3 males). All young subjects had detectable levels of TREC, which were undetectable in 3 out of 13 middle-aged donors and in 21 out of 25 centenarians ($P < 0.0001$ by chi-squared).

As far as the donors with detectable levels of TREC are concerned, we have observed an age-related significant decline of the number of sjTREC+ cells ($P = 0.012$ by Kruskal–Wallis test). Indeed, Fig. 3 indicates that young donors showed high levels of sjTREC+ cells (mean \pm SEM: $3.20\% \pm 0.48\%$) while middle-aged subjects had $2.13 \pm 0.47\%$ sjTREC+ cells, and centenarians $0.26 \pm 0.20\%$ sjTREC+ cells. The comparison between young and middle-aged groups revealed that there was no significant difference ($P > 0.05$ by Dunn's Multiple Comparison test). A statistical difference was observed comparing the groups of young and centenarian donors ($P < 0.01$); probably because of the low number of centenarians, no differences were present between these subjects and middle-aged donors.

IL-7 plasma concentration in males and females

We quantified IL-7 plasma levels in 29 young donors (15 females and 14 males), 31 middle-aged donors (16 females and 15

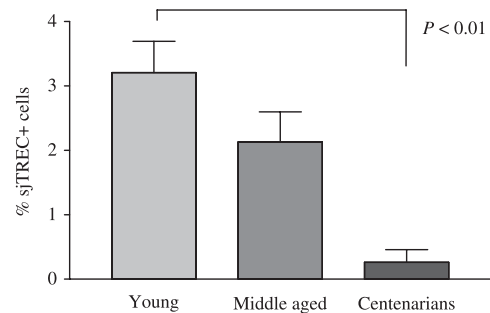


Fig. 3 Changes in TREC+ cells with age. The percentage of sj-TREC positive cells undergoes a dramatic age-dependent decline from young to middle-aged subjects and centenarians. Kruskal–Wallis test among the three groups revealed a $P < 0.0001$. No significant difference was observed between young and middle-aged donors. Note that only the values from individuals with detectable TREC levels (all young subjects, 10 out of 13 middle-aged donors, 4 out of 25 centenarians) are shown here.

males) and 44 centenarians (24 females and 20 males). In the three groups, IL-7 plasma concentrations were 5.68 ± 0.94 , 5.32 ± 0.77 , and 5.13 ± 0.67 pg mL⁻¹ (mean \pm SEM), respectively. Thus, if data from all subjects are considered together, plasma levels of IL-7 do not change with aging. We then analyzed plasma IL-7 levels in males and females separately (Fig. 4), and observed that IL-7 does not change significantly with age. However, the comparison between males and females in the three classes of age revealed a significant gender difference in centenarians, where values obtained in females were higher than those in males ($P = 0.044$). No correlation was found between plasma IL-7 levels and TREC+ cells.

Analysis of CD127 expression at the cellular and molecular level

In seven donors per group, we could analyze the expression of CD127 on the surface of CD4+ and CD8+ T cells. Figure 5 shows a representative example of cytofluorimetric analysis. The

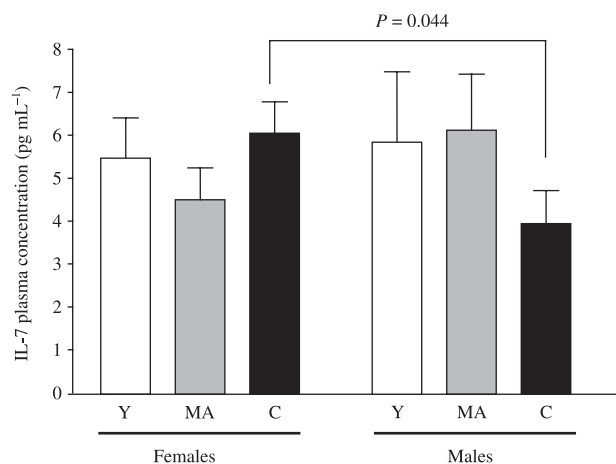


Fig. 4 Plasma interleukin-7 levels in the groups studied. Data refer to the plasma levels of IL-7 in young (Y), middle-aged (MA) and centenarians (C). We performed a gender-specific analysis by dividing the three populations in males and females, and observed a significant gender difference only in centenarians.

mean percentage of CD127+ cells in CD4+ was 87.77 ± 2.33 in young donors, 87.84 ± 1.72 in middle-aged donors and 88.80 ± 2.29 in centenarians. CD8+ cells expressing CD127 were 84.74 ± 3.95 , 85.63 ± 2.78 , and 86.67 ± 1.01 , respectively.

Thus, the percentage of CD127+ T lymphocytes does not change with age either in CD4+ or in CD8+ cells.

To confirm the data regarding protein expression with the quantification of mRNA that code for the α chain of the IL-7 receptor in these donors, we analyzed CD127 mRNA levels in resting PBMC (Fig. 6, upper panel) from 6 young and 6 middle-aged donors and 5 centenarians. Then, to evaluate whether mRNA production was also maintained in activated cells during aging, we treated lymphocytes with classical stimuli such as anti-CD3 mAbs or ionomycin plus TPA (Fig. 6, middle and lower panels, respectively). No differences between young and middle-aged donors, and centenarians were found in cells treated with anti-CD3 mAbs, that had a minimal effect on mRNA production. However, when a maximal stimulus for mRNA synthesis such as ionomycin plus TPA was applied, we found a statistically significant effect of age ($P = 0.0084$); young donors showed significantly higher values than middle-aged donors or centenarians ($P < 0.01$ and $P < 0.02$, respectively). No correlation was found between CD127 mRNA expression and TREC levels.

Discussion

The aim of this study was to investigate the behaviour of crucial parameters related to the homeostasis of T lymphocytes during human immunosenescence. By measuring TREC+ cells in

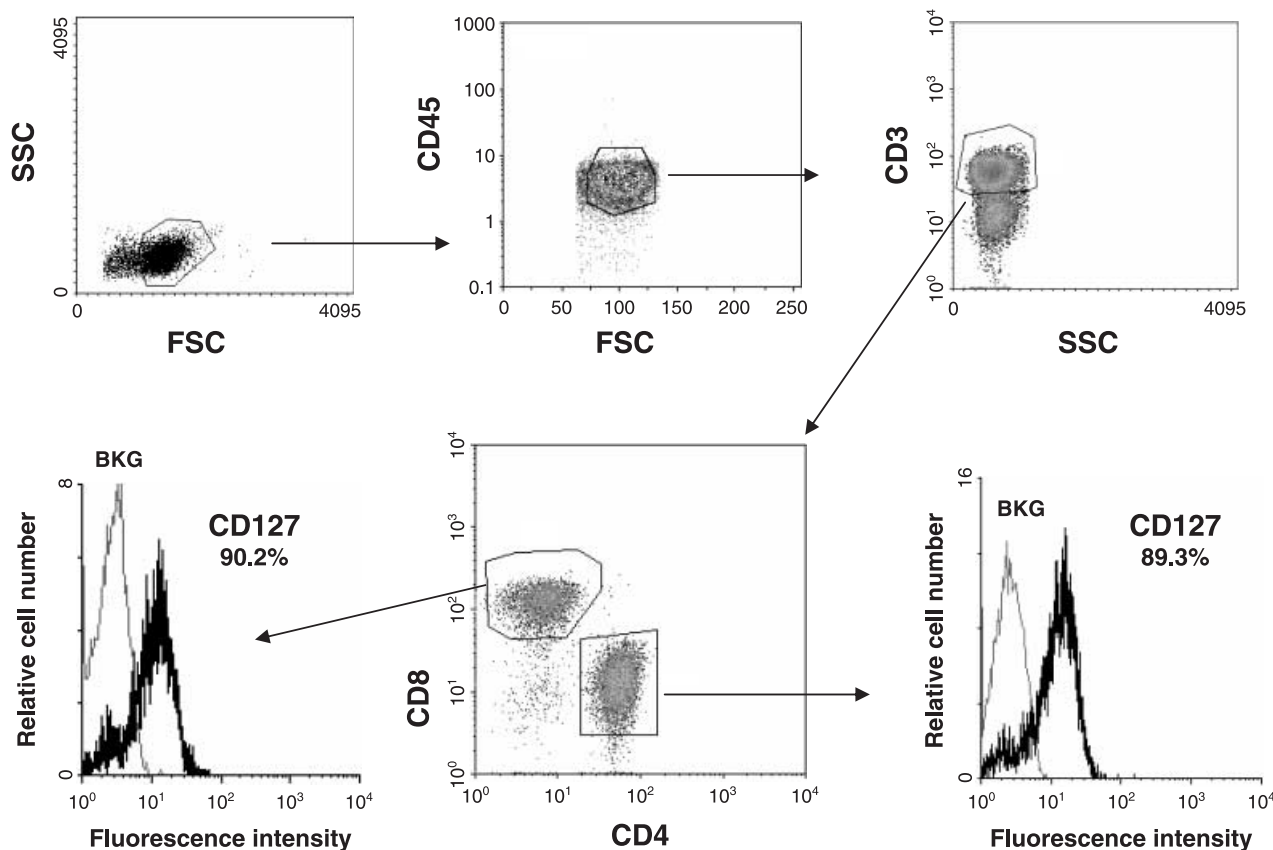


Fig. 5 Cytofluorimetric analysis of CD127 expression in cells from a centenarian. Representative example of the cytometric analysis of CD127 expression on CD4+ and CD8+ T cells from a centenarian. First, CD45+ cells were gated, then T cells were identified by gating on CD3+ cells. Another gate was subsequently done either on CD4+ or CD8+ T cells. The percentage of CD127+ cells was calculated taking into account the adequate fluorescence background (BKG).

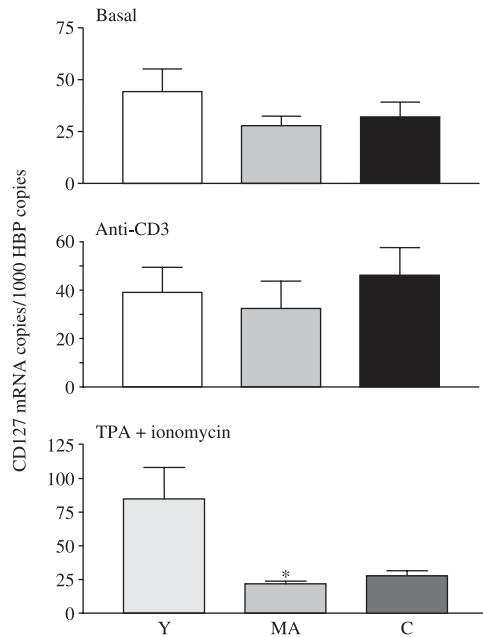


Fig. 6 Expression of CD127 mRNA in cells from the studied groups. Data indicate the amount of copies of CD127 mRNA per 1000 copies of HBP mRNA in PBMC from young (Y), middle-aged (MA) and centenarians (C) without any stimulation (basal, upper panel) and after stimulation with anti-CD3 mAb (middle panel) and TPA plus ionomycin (lower panel). A significant difference was observed only with the last treatment, in middle-aged vs. young donors (* $P < 0.01$).

centenarians, we have analyzed the capacity of the thymus to produce new lymphocytes at the limit of human lifespan, and then we have investigated the functionality of the IL-7/IL-7 receptor system.

Previous studies have shown that an age-related reduction in virgin cells exists among CD4+ and CD8+ T lymphocytes, and this is also the case with centenarians (Fagnoni *et al.*, 2000). However, these studies, could not fully analyze the different populations of naïve and memory cells. Here we show that substantial changes occur with age in the compartment of naïve, central and effector memory lymphocytes, but not in the compartment of terminally differentiated cells, i.e. those that re-express CD45RA on the cell surface but do not expose CCR7 or other homing receptors such as CD62L (Champagne *et al.*, 2001). Interestingly, most of these cells express markers of cell death/activation such as CD95 (Lugli *et al.*, manuscript in preparation). It is thus likely that these cells represent a part of the previously reported population of CD45RA+ (virgin) cells, mainly present within the subset of CD8+ T cells during human aging (Cossarizza *et al.*, 1996). Terminally differentiated cells are able to produce cytokines such as IL-2, perforin and TNF- α that are crucial for several functions, including triggering of proliferative events or the control of infectious agents. This could indicate that the immune system has to maintain a fixed percentage of cells capable of performing the final step of the immune response until the extreme limit of human life.

The decrease in the number of naïve T cells was accompanied by a dramatic reduction in the functionality of the thymus, as

revealed by the quantification of TREC+ cells. In most centenarians we could not detect any TREC molecules; since the methodology we used has an estimated sensitivity of about 1 cell in 20 000 (since in each PCR tube the DNA equivalent of 20 000 cells is amplified), it cannot be excluded that the frequency of TREC+ cells was lower than this value. In any case, a detectable number of TREC+ lymphocytes that can be defined as 'recent thymic emigrants' were indeed present in few of them. It is well known that the thymus loses its capacity to generate T cells after puberty, and that the involution is clearly maximal at extreme advanced ages (Pawelec *et al.*, 2002; Franceschi & Bonafe, 2003). It is generally thought that, when the thymus progressively undergoes the profound age-related involution, the output of naïve T cells is not sufficient to maintain the size of the peripheral naïve T-cell pool. Thus (yet) unknown mechanisms, including the capacity of forming T cells in sites other than the thymus, have been hypothesized (Abo, 2001).

Our data show that minimal amounts of sjTREC+ cells can be present in centenarians. This observation has two possible, not mutually exclusive, interpretations. The first is that a minimal functional capacity of the thymus still exists, and is likely responsible for the output of naïve T lymphocytes. Previous studies have shown that TREC+ cells were detected in elderly people (up to 78 years), indicating the presence of a continuous thymic output of TREC+ naïve T cells even in aged subjects, who were, however, 20–25 years younger than those we analyzed (Douek *et al.*, 1998). These naïve T cells could derive from residues of thymic lymphopoietic islets, and indeed it has been demonstrated that the relative number of TREC+ cells present within thymocytes isolated from healthy donors is constant with increasing age despite the age-related involution of thymic tissue and the consequent loss in the total number of thymocytes. Thus, diminished thymic output would lower the number of naïve T cells produced, but does not change the amount of TREC+ cells within RTE (Jamieson *et al.*, 1999). It should be emphasized that the age-related decrease in the amount of TREC+ cells can be due not only to a decreased thymic function, but also to an expansion of T cells in the periphery (Hazenbergh *et al.*, 2001, 2003). Indeed, it is well known that with age the number of activated peripheral T cells increases, along with the presence of expanded T-cell clones (Sanson *et al.*, 1993; Wack *et al.*, 1998). The sum of T-cell activation and proliferation can thus contribute, at least in part, to the decrease in TREC that we have found in centenarians.

A second possible explanation is that TREC+ cells have an extremely long lifespan. In a group of patients thymectomized 3–39 years prior to analysis, TREC+ cells were detectable, even if their number was one order of magnitude lower than healthy controls (Douek *et al.*, 1998). Clearly, such cells were not recently produced by the thymus, but were likely those produced when the thymus was still present. As a consequence, the detection of TRECs in adults or aged individuals cannot be taken as an absolute measure of thymic activity, but is the sum of a residual thymic function and the presence of life-long naïve T cells, that could mask for many years the absence of a thymic output.

IL-7 is a crucial molecule for the immune system. Not only does it have a trophic action on precursor T cells, but it can also act as a survival factor for mature T lymphocytes, and further on the survival of naïve T cells (Fry *et al.*, 2001). There is evidence that IL-7 has a nonredundant role in supporting not only survival but also homeostatic expansion of naïve CD4+ and CD8+ T cells *in vivo* (Rathmell *et al.*, 2001). IL-7 is also a potent modulator of thymus-independent T-cell regeneration and extrathymic maturation (Laky *et al.*, 1998, 2000). It is thus logical that the production of this cytokine should be kept constant throughout life, and indeed we observed that IL-7 plasma levels did not change significantly among young or adult donors and centenarians. However, we found that, in centenarians, females had higher IL-7 levels than males. In general, among centenarians, women outnumber men all over the world. In Italy, a gradient exists from north to south, and the ratio between women and men decreases from about 7 : 1 in northern Italy to 3 : 1 in southern Italy (Passarino *et al.*, 2002). In some areas, such as the province of Nuoro in the island of Sardinia, this ratio is as low as 1 : 1 (Deiana *et al.*, 1999). The reasons for such a gradient are at present unknown, and differences related to differential mortality in women and men, as well as those related to social, genetic and anthropological factors, may play a role (Franceschi *et al.*, 2000b). For these reasons we have compared females and males, and found a higher IL-7 plasma level in women.

To further investigate the IL-7/IL-7 receptor system, we have analyzed the expression of CD127 at the cellular and molecular level, and found that CD127 was expressed at a constant rate on resting CD4+ and CD8+ surface throughout life. This was confirmed by the quantitative analysis of mRNA expression, which remained stable even after the stimulation of TCR with anti-CD3 mAbs. Treating resting lymphocytes with ionomycin and TPA resulted in a significant decrease in the production of CD127 mRNA both in middle-aged donors and in centenarians. This could indicate that the capacity to respond to a maximal stimulus with the massive production of CD127 mRNA is lost in middle-aged individuals, long before reaching the end of their life.

The meaning of the gender-related difference in IL-7 plasma levels is not clear since there is no difference in surface expression of CD127. A better preservation of the IL-7/IL-7R system in females can be hypothesized that can reflect a higher efficacy in maintaining T-cell homeostasis. In any case, the data relating to the IL-7/IL-7R system suggest that the ability of peripheral lymphocytes to respond to IL-7 is well preserved during aging. Further studies are needed to clarify whether IL-7 contributes to our understanding of the female gender-related higher probability of reaching extreme ages.

Experimental procedures

Patients and cells

We studied a total of 44 centenarians with a mean age of 100 years (range: 98–105), 20 males and 24 females. All these

subjects were in relatively good condition without relevant acute or chronic disease affecting the immune system, and gave informed consent for the studies here described.

As controls, we have studied two groups of healthy subjects of different ages: 31 middle-aged subjects with a mean age of 60 years (range: 58–62, 16 males and 15 females), and 29 young donors with a mean age of 22 years (range: 20–25, 14 males and 15 females). All subjects were enrolled and selected according to the criteria described in the SENIEUR protocol (Ligthart *et al.*, 1990; Guerreschi *et al.*, 2003).

Peripheral blood mononuclear cells (PBMC) were separated from freshly collected blood according to standard procedures.

Cytofluorimetric analysis

To quantify the main populations of virgin and memory cells (i.e. naïve, central memory, effector memory, terminally differentiated) (Sallusto *et al.*, 1999), we used a cytometric approach with fluorochrome-labelled monoclonal antibodies (mAbs), according to standard methods (Cossarizza *et al.*, 2004). Frozen PBMC (1×10^6) from young ($n = 12$), middle-aged ($n = 11$) healthy donors and centenarians ($n = 10$) were thawed, washed twice with cold Hanks' balanced salt solution, resuspended in 100 μ L PBS and stained with the following mAbs: anti-CD45 (conjugated with APC), anti-CD3 (APC-Cy7), anti-CD4 (PE-Cy5), anti-CD8 (PE), anti-CD45RA (FITC) and anti-CCR7 (PE-Cy7); mAbs were from Serotec (Oxford, UK), R&D Systems (Minneapolis, MN, USA) and BD Biosciences (San José, CA, USA). For the analysis of the expression of CD127, we used PE-conjugated anti-CD127 mAbs (R & D Systems), along with anti-CD4 (PE-Cy5), anti-CD45 (APC), anti-CD8 (FITC) and anti-CD3 (APC-Cy7) mAbs. Negative controls were prepared by incubating cells with fluorochrome-labelled isotype-matched mAbs directed against irrelevant antigens, according to standard procedures.

Cells were incubated for 30 min at 4 °C, then washed with PBS, resuspended in PBS containing 1% formaldehyde and analyzed by flow cytometry (Pinti *et al.*, 2004). At least 50 000 events inside the lymphocyte gate were acquired for each sample. Cells were analyzed using a Cyflow ML (Partec GmbH, Münster, Germany), equipped with a blue solid state laser (λ 488 nm, 200 mW, kept at 50 mW), a red diode laser (λ 635 nm, 25 mW), a UV Mercury lamp HBO (100 long life, 100 W, not used in this study), a green solid state laser (λ 532 nm, 100 mW, not used in this study), and a CCD camera. Data were acquired and analyzed by Flomax 3.0 (Partec) and WinMDI 2.8 (by Dr J. Trotter, Scripps University, La Jolla, CA, USA) under Windows, and FlowJo 6.3 (Treestar Inc., Ashland, OR, USA) under MacOSx.

Quantification of IL-7 plasma levels

Non-heparinized plasma specimens (collected in EDTA) were obtained from the subjects and stored at -80 °C until use. Aliquots were thawed and analyzed at least in duplicate by using a commercially available, ultrasensitive immunoassay that detects both free IL-7 and IL-7 bound to carrier proteins or

soluble IL-7 receptors (R&D System), following the manufacturer's instructions.

Detection of sjTREC+ cells

Quantification of sjTREC content was measured by an original method that has been recently developed. This assay was performed by two parallel real-time PCRs that quantify sjTREC or nuclear DNA (nDNA) using the same construct, known amounts of which were amplified as reference molecules. Briefly, DNA was extracted from PBMC according to standard methods (Pinti *et al.*, 2002). In the first reaction, we quantified sjTREC and the primers used were: sjDir (5'-CACATCCCTTTCAACCATGCT-3') and sjRev (5'-GCC AGC TGC AGG GTTAGG-3'). The TaqMan probe used for sjTREC (5'-FAM- ACACCTCTGGTTTTGTAAAG GTGCCACT-BlackHole Quencher1-3') was included in the reaction mixture throughout PCR as a real-time detector for the amplified product. We prepared a mix consisting of IQ Supermix 2X (Bio-Rad, Hercules, CA, USA), 500 nM of primers and 200 nM of sjTREC probe. One cycle of denaturation (95 °C for 6 min) was performed, followed by 45 cycles of amplification (94 °C for 30 s, 58 °C for 60 s). PCR was carried out in an iCycler Thermal cycler (Bio-Rad), and all samples were analyzed in triplicate.

The same approach was used to quantify nDNA, which was required to obtain the percentage of sjTREC positive cells. In this case, primers GenDir (5'-GGCTCTGTGAGGGATATAAAGACA-3') and GenRev (5'-CAAACCACCCGAGCAACTAATCT-3'), designed on FasL gene sequence, present in two copies in the human genome (obviously, one in each chromosome), were used. The TaqMan probe Genprobe (5'-TexasRed-CTGTCCGTTTCCT-GCCGGTGC-BlackHole Quencher2-3') was included in the reaction mixture throughout PCR as a real-time detector for the amplified product. We prepared a mix consisting of IQ Supermix 2X (Bio-Rad), 600 nM of GenDir, 400 nM of GenRev and 300 nM of Genprobe. One cycle of denaturation (95 °C for 6 min) was performed, followed by 45 cycles of amplification (94 °C for 30 s, 60 °C for 60 s). The primers were synthesized by Operon (Cologne, Germany).

The regions used as templates for the two amplifications (that is, those of sjTREC and nDNA) were cloned tail to tail in a vector (pGEM-11Z, from Promega, Madison, WI, USA), to have a ratio of 1 : 1 of the molecules used as reference. Then, serial known dilutions of this vector, amplified in triplicate, were included in each PCR run to generate a standard curve from which the relative copy number of either sjTREC or nDNA present in the unknown samples was determined. Then, the percentage of sjTREC positive cells was simply obtained from the ratio between the relative values of sjTREC and nDNA (obtained vs. the same vector), multiplied by 2 (as two copies of the nuclear gene are obviously present in a cell) and by 100.

Considering that in each PCR determination we used an amount of DNA that was equivalent to a maximum of 20 000 cells, the assay we used had the theoretical capacity to reveal a percentage of TREC+ cells as low as 0.005%.

In vitro PBMC stimulation for the modulation of CD127 mRNA production

For the analysis of CD127 mRNA expression, freshly collected PBMC were stimulated with anti-CD3 monoclonal antibody (mAb) (clone OKT3, IgG, 20 ng mL⁻¹; American Type Culture Collection, Manassas, VA, USA), or with 3 ng mL⁻¹ 12-O-tetradecanoylphorbol-13-acetate (TPA) plus 2 µM ionomycin (both from Sigma Inc., St. Louis, MI, USA), for 4 h at 37 °C, in RPMI 1640 medium containing 10% fetal calf serum, 2 mM L-glutamine, 100 IU mL⁻¹ penicillin, 100 µg mL⁻¹ streptomycin (complete medium), then collected, extensively washed and processed. Cell pellets were used for the extraction of RNA and quantification of CD127 mRNA.

Quantification of CD127 expression

Total RNA was extracted from PBMC using RNeasy Minikit from Qiagen (Alameda, CA, USA), following instructions supplied by the manufacturer. One microgram of RNA was reverse transcribed using Random hexamers, with RevertAid First strand cDNA Synthesis Kit by Fermentas (Vilnius, Lithuania). The total volume of reverse transcription reaction was 25 µL; 1/25 of cDNA was used for each real-time PCR amplification that was a multiplexing reaction. Each reaction amplifies at the same time IL-7α mRNA and housekeeping gene L13/HBP mRNA and was in triplicate. Expression of the messengers was calculated as relative expression referred to 1000 copies of HBP mRNA, based upon differences in the threshold cycle of mRNA for IL-7Rα and threshold cycle of HBP mRNA, as described (Pinti *et al.*, 2003).

Selected primers were: IL-7Rα Dir (5'-GAAACAAATGGACG-CATGTGAA-3'), IL-7Rα Rev (5'-TCAGGGATGGATCGAACTTTA-3'), IL-7RαProbe (5'-FAM-CAGAGAAAGCTCCAACCGGCAGC-BlackHole Quencher2-3'), HBPDdir (5'-ACCGGTAGTGGATCTT-GGCTTT-3'), HBP Rev (5'-GCTGGAAGTACCAGGCAGTGA-3'), and HBPprobe (5'-TexasRed-TCTTCTCTTCTCTCCAGGGTGGCT-BlackHole Quencher2-3'). The primers were synthesized by Operon (Cologne, Germany). A portion of each cDNA was used for quantitative PCR in a 50 µL of PCR mix consisting of PCR buffer 1× (Promega), MgCl₂ 3 mM, dNTPs 400 nM, 2 U Taq polymerase, 200 nM of each primer for HBP amplification, HBP probe 300 nM, 400 nM of each primer for IL-7Rα amplification and IL-7Rα probe 200 nM.

Statistical analysis

Statistical analysis was performed by Kruskal–Wallis test, Dunn's Multiple Comparison test and chi-squared using Prism 3.03 under Windows XP. A *P*-value < 0.05 was considered significant.

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