Aging-associated subpopulations of human CD8+ T-lymphocytes identified by their CD28 and CD57 phenotypes

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ABSTRACT

Background: During organismal aging, human T-cells shift towards less functional phenotypes, often called senescent cells. As these cells have not been well characterized, we aimed to relate surface markers of human T-cell senescence with characteristics of in vitro cellular aging and to further characterize these cells.

Methods: We identified, by flow cytometry, subpopulations of CD8+ T-cells based on CD57 and CD28 expression, and tested them for some markers of cellular senescence, apoptosis, differentiation and homing.

Results: Elderly persons presented significantly higher proportions not only of CD28−CD57+, but also of CD28+CD57+ cells. CD28+CD57+ cells had the highest expression of p16, p21, Bcl-2, CD95, CD45RO, CCR5 and PD-1, thereby arguing in favor of a senescent phenotype.

Conclusion: Among CD8+ T-lymphocytes, CD28+CD57+ cells represent a subset with some senescent features that are distinct from the CD28−CD57+ cells.

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1. Introduction

Reports on senescence of human cells have highly relied on in vitro observations, which might, however, not be true reflections of in vivo characteristics (Rodier and Campisi, 2011). As a consequence, the contribution of senescent cells to the human aging process has remained controversial. On the other hand, organismal aging invariably leads to a decrease in functionality. In the immune system, this results in immunosenescence, a complex process that includes a shift towards less functional T-cells that are often designated as senescent. Cell surface markers, mainly absence of CD28 or expression of CD57, have been used to identify T lymphocytes as senescent in vitro (Brenchley et al., 2003; Elfros, 1997).

Working with CD3+ T-cells, we have found the combined expression of CD28 and CD57 useful in distinguishing senescent subpopulations. In elderly compared with young persons, we have observed not only a higher prevalence of CD28−CD57+ cells, as expected, but also of a small subpopulation that is CD28+CD57+ (Onyema et al., 2012). The attention on CD28+CD57+ cells was first drawn in 2003 by Brenchley et al., who observed in six healthy individuals that 8% of memory CD8+ T-cells consisted of the CD28+CD57+ phenotype. In their study the expression of CD57 on T-cells was related to shorter telomeres and loss of proliferation capacity, possibly linking its presence to senescence (Brenchley et al., 2003). CD28+CD57+ T-cells differ from the more prevalent CD28−CD57+ cells, which are known to correspond to a highly differentiated subpopulation with effector function (Onyema et al., 2012). While CD28−CD57+ cells have been related to persistent viral infections and an immune risk profile (IRP) (Olsson et al., 2000; Wikby et al., 2002), we have found that CMV seropositivity was not related to the prevalence of CD28+CD57+ cells (Onyema et al., 2012). Based on these observations, CD28−CD57+ cells might be a different phenotype of senescent T-cells. However, their nature remains to be further elaborated.

Here, focusing on CD8+ T lymphocytes, we looked at various characteristics related to cellular senescence, apoptosis, T-cell differentiation and homing. We observed that the CD28+CD57+...
subpopulation increased with aging, presented some features of a senescent cell phenotype, and has differentiation characteristics of memory T-cells destined for tissue migration.

2. Material and methods

2.1. Participants

One group of young (N=11) and two groups of elderly participants (N=11+11) were recruited. First, to study the protein expression pattern of senescence and apoptosis modulators in CD8+ subpopulations of T-cells we recruited 11 young (median age 24±2.3 years; 5 females and 6 males) and 11 elderly (E1) (median age 84±18.5 years; 5 females and 6 males) Caucasians from the Belgian population. These 22 participants were the same as in our previous study; earlier studies have shown that the sample size we used was sufficient for the study (Mendez-Lagares et al., 2012; Njemini et al., 2008; Onyema et al., 2012; Pera et al., 2014; Zanni et al., 2003). All participants were living independently in the community, without major health or functional problems. They were included when they were nonsmokers, showed no signs of cognitive dysfunction, had no record of chronic inflammatory disorders, were free of any infectious illness for at least 6 weeks before sampling, and abstained from strenuous physical activity and exercise for at least 24 h before testing.

Next, as a follow-up to the analysis conducted at the protein level, a polymerase chain reaction (PCR) was performed on a second group of 11 elderly participants (E2) (5 females and 6 males; median age 78±4.9 years) to investigate the mRNA levels of the senescence and apoptosis modulators in the CD8+ subpopulations. The expression of receptors associated with T cell homing, activation and differentiation was also characterized in the E2 subpopulations. All participants had a normal proportion of lymphocytes in the peripheral blood (30.7±6.5%) and normal absolute lymphocyte counts (1805±418/mm3).

An approval for the study was received from the ethical committee of our institution and all participants provided written informed consent.

2.2. Blood cell preparation

Fresh peripheral venous blood samples, collected in EDTA tubes were incubated, within 6 h, in lysis buffer (NH4Cl—154.0 mM, EDTA—0.1 mM, and KHCO3—10.0 mM) to remove red blood cells and centrifuged at 2800 rpm for 4 min to obtain the peripheral blood leucocytes (PBL), which were used for analysis of the cell surface and intracellular markers. For cell sorting, peripheral blood mononuclear cells (PBMC) were isolated from fresh blood samples by density dependent centrifugation using lymphoprep™ gradient (Axis-Shield, Oslo, Norway) according to the manufacturer’s instruction.

2.3. Flow cytometry analysis

PBL from the subjects were stained with a panel of antibodies by direct immunofluorescence. The number of cells expressing various markers was quantified by five-color flow cytometry (Cytomics FC 500) (Beckman Coulter, Analis, Belgium). The following mouse anti-human monoclonal antibodies were used in appropriate combinations and concentrations: Alexa 488-anti-CD3 (BD Biosciences, Erembodegem, Belgium); FITC-anti-CD3 (BD Biosciences, Erembodegem, Belgium); FITC-anti-p53 (BD Biosciences, Erembodegem, Belgium); PE-cy5-anti-CD8+ (BD Biosciences, Erembodegem, Belgium); FITC-anti-p16 (Santa Cruz Biotech, Germany); ECD-anti-CD28 (Beckman Coulter); PE-cy7-anti-CD3 (Beckman Coulter); PE-anti-CD57 and the following FITC-conjugated antibodies: CD62L, CCR7, CCR5, CXCR2, CD45RO, and CD45RA (Biolegend, Lintec, Belgium). All antibodies were matched with isotype controls (Santa Cruz Biotech), except the ECD-conjugated isotype control that was obtained from Beckman Coulter. An extended panel of controls was used in order to exclude background fluorescence, fluorochrome interferences and dead cells. These included compensation controls based on data collected from single fluorochrome staining, fluorescence-minus-one controls that include all the fluorochromes used in a particular multicolor stain except the fluorochromes for which the threshold is to be determined, and dead cell exclusion control using 7-amino actinomycin-D (7-AAD) staining. Also, quality controls for the machine were performed daily by checking the detector voltage values for conformity with initial protocol and running daily verification of the dynamic range of the detectors using standardized quality control compensation beads.

For the identification of cell surface antigens about 5×10⁵ lymphocytes in 50 μl of PBS containing 1% BSA were incubated with 20 μl of appropriate combination of antibodies for 20 min at room temperature in the dark. After a washing step, cells were resuspended in 500 μl of FACScalibur® (BD Biosciences) and the samples were ready for flow cytometric analysis. For anti-CD57, anti-p15 and anti-p21 staining, the surface antigen labeling steps were followed by fixation and permeabilization steps with Fix and Perm® Cell Permeabilization kit (An Der Grub Bio Research, Austria) according to the manufacturer’s instruction. The antibodies were added during permeabilization. After the final incubation step, the cells were washed with PBS and were resuspended in 500 μl of FACScalibur® solution for flow cytometric analysis. For all samples, 100,000 PBL events were acquired for analysis. Firstly, the lymphocyte subpopulation was gated according to granularity and size in the forward scatter versus side scatter plot. To obtain a pure lymphocyte cluster, eliminate duplicates, and avoid contamination with monocytes, neutrophils, natural killer cells and cell debris, we gated on the CD3 fluorescence versus side scatter. Further, CD3+CD8+ lymphocytes were gated within the CD3+ cells on the CD3 versus CD8 dot plot. Flow cytometry dot plots and histograms were used to separate and identify different subpopulations of CD3+CD8+ lymphocytes based on their expression or non-expression of a combination of markers. Where necessary, the mean fluorescence intensity (MFI) of the markers in various subpopulations was also determined.

Detection of senescence associated beta galactosidase (SA-Bgal) activity was based on the FlowReporter® lacZ Flow Cytometry kit in accordance with the manufacturer’s protocol (Life Technologies, Gent, Belgium).

2.4. Cell sorting

As it was not possible to sort sufficient number of all the subpopulations of interest from young participants for further analysis, CD8+ cells were only pre-concentrated from PBMC from the elderly participants by positive selection using anti-CD8-coated magnetic beads (MACS®, Miltenyi Biotec, Leiden, Netherlands) according to the manufacturer’s instruction. The purity of the pre-concentrated CD8+ cells was confirmed to be greater than 95% by flow cytometry. The cells were labeled with the relevant antibodies before sorting the CD3+ subpopulation into CD28+CD57−, CD28−CD57−, CD28−CD57− and CD28+CD57− fractions using a BD-FACSaria III (BD Biosciences). The gating procedure and antibodies used were similar to those used for flow cytometric analysis apart from the replacement of PE-cy5-anti-CD8 with APC-anti-CD8 of the same clone. Quality checks for the detectors were also conducted prior to each cell sorting with quality control beads and single-fluorochrome-conjugated antibody labeled cells. Dead cells were also excluded using 7-AAD. The purity and phenotype of the four subpopulations were also verified independently by flow cytometry (Cytomics FC 500).
2.5. RNA extraction, reverse transcription and polymerase chain reaction (PCR)

Total RNA was isolated from the different sorted CD8+ T-cell subpopulations using the Spin Protocol of the Total RNA Isolation System (Promega Benelux, Leiden, The Netherlands) according to the manufacturer’s instruction. The RNA samples were reverse transcribed to cDNA using the RevertAid H minus First Strand cDNA Synthesis Kit (Thermo Scientific, Germany) based on the manufacturer’s protocol. PCR was done on the cDNA samples using the KAPA2G™ Robust HotStart ReadyMix PCR Kit (Kapa Biosystems, Woburn, MA, USA) that enhances specificity of primer binding and sensitivity of sequence detection. The cycling was carried out in a GeneAmp PCR System 9600 (PerkinElmer, Waltham MA, USA). The cycle conditions were as instructed by Kapa Biosystems. Glycerolaldehyde 3-phosphate dehydrogenase (GAPDH) was used as the endogenous control. The following primer sets (Integrated DNA Technologies, Leuven, Belgium) were used:

p16  (i): Forward – CCAACGCACCCGAATAGTTACG  
Reverse – GCGCTGCCCCATCATCATG
(ii): Forward – AGCATGGAGGCTCTCGTGTA  
Reverse - CCATCATCATCACCTGGATCG
(iii): Forward – TTCGCTAAGTGCTCGGAGTT  
Reverse - AGCCCCCTCCTTCTTCTTCCT
p21: Forward – ATGAAATTCAACCCCCCTTCC  
Reverse – CCCTAGGCTGTGCTCACCCT
p53: Forward - CTCACCATCATCACACTGGA  
Reverse – TCATTAGCTCTCGGAACAT
CD95: Forward - TCAGTGAGGATGGGGAGAAG  
Reverse – CAGGCCCTCCAAGTCTCAG
Bcl-2: Forward - CAGCAGCTTCCCGCCGCTACGC  
Reverse – CCGCATGGGGCGCCGTACAGTTCC
GAPDH: Forward – GGAGTTCACTGGCGTCTTCC  
Reverse - GGTTCACACCACATGACGAAC

The ability of the primers to detect their respective genes was confirmed separately in 293T cells. PCR amplicons were analyzed following electrophoresis on a 1.5% agarose gel along with molecular size markers. Pictures were acquired using a CCD Camera connected to an image reader and a transilluminator (Vilber Lourmat, Cedex, France), and were analyzed using the Image-J software (NIH, Bethesda MD, USA).

2.6. Statistical analysis

Statistics was done using IBM® SPSS® Statistics 20 (SPSS Inc., Illinois, USA). Data are expressed as median ± semi-interquartile range (SIQR). The Mann–Whitney U test was used for analyzing the differences between old and young participants. Differences in the expression of the various markers among CD28+CD57−, CD28+CD57+, CD28−CD57−, and CD28−CD57+ cells in each subject group were based on the Friedman’s test while the Wilcoxon Sign Rank test was used for post hoc analysis of the paired observations within the subjects. Differences were considered significant at two-sided p < 0.05. Significant values are shown in the figures.

3. Results

The gating procedure for the delineation of the different subpopulations is shown in Fig. 1A. Fig. 1B shows the proportion of CD8+ T-cells according to their combined expression of the surface markers CD28 and CD57 in young (N = 11) and E1 old (N = 11) participants. Cells expressing CD57 were more frequent in the older participants, irrespective of their CD28 status, while CD28− cells were more frequent only if they also expressed CD57. The frequency of the various CD8+ subpopulations in the second group of 11 elderly participants was very similar to that of the first one:

- p16: Forward – CCAACGCACCCGAATAGTTACG  
- Reverse – GCGCTGCCCCATCATCATG
- p21: Forward – ATGAAATTCAACCCCCCTTCC  
- Reverse – CCCTAGGCTGTGCTCACCCT
- p53: Forward - CTCACCATCATCACACTGGA  
- Reverse – TCATTAGCTCTCGGAACAT
- CD95: Forward - TCAGTGAGGATGGGGAGAAG  
- Reverse – CAGGCCCTCCAAGTCTCAG
- Bcl-2: Forward - CAGCAGCTTCCCGCCGCTACGC  
- Reverse – CCGCATGGGGCGCCGTACAGTTCC
- GAPDH: Forward – GGAGTTCACTGGCGTCTTCC  
- Reverse - GGTTCACACCACATGACGAAC

CD28+CD57+ 4.1 ± 1.6%, CD28−CD57+ 36.0 ± 11.4%, CD28−CD57− 18.4 ± 11.8%, and CD28−CD57− 40.4 ± 18.9%. To characterize the CD28+CD57+ cells, the expression of various senescence markers was examined in 11 young and E1 participants by flow cytometry. In our hands, it was impossible to demonstrate the presence of SA-B-gal and of p53 by flow cytometry with the available flow cytometry reagents.

When considering the 11 young and E1 old participants together, the expression of p16, given by MFI, was highest in CD28+CD57+ cells (comparison with the other three subpopulations: all p-values < 0.006). Separately, the young subjects presented similar significant differences; in E1 old subjects, the difference was almost significant compared to one of the subpopulations (Fig. 2A).
The MFI of p21, for both the young and E1 groups taken together, was also highest in CD28+CD57+ cells (comparison with the other three subpopulations: all p-values < 0.003). Separately, in young subjects the difference was significant with two subpopulations; in E1 old participants the difference was significant with one and almost significant with another subpopulation (Fig. 2B). For CD28−CD57− and CD28+CD57− subpopulations the MFI of p21 was significantly higher in E1 old participants than in young subjects.

To further characterize the CD28+CD57+ cells, the expression of apoptosis-related parameters, CD95 and Bcl-2, was examined in the young and E1 old participants by flow cytometry. The proportion of cells expressing CD95, for the young and E1 old participants taken together, was highest in CD28+CD57+ cells (comparison with the other three subpopulations: all p-values < 0.001). In both E1 old and young subjects, we observed significant differences in the proportion of cells expressing CD95 in the subpopulations in the order: CD28+CD57+ > CD28−CD57+ > CD28−CD57− > CD28+CD57− (Fig. 2C). The proportion of cells expressing CD95 was significantly higher in E1 old than in young subjects for all subpopulations except for CD28+CD57+ cells. For the young and E1 groups taken together, the
MFI of CD95 expressing cells was highest in the CD28+CD57+ cells (comparison with the other three subpopulations; all p-values < 0.032). In the young participants, the MFI of CD95 was significantly highest in the CD28+CD57+ cells; in the E1 old participants, this difference was significant with CD28−CD57+ and CD28−CD57− subpopulations (Fig. 2D). Cell populations that were CD28+ had a higher intensity of expression of CD95.

Bcl-2 expression (both as percentage of expressing cells and as MFI) was highest among CD28+CD57+ cells; the differences were significant as well for the young and E1 participants taken together (comparison with the other three subpopulations; all p-values < 0.001), as for both age groups separately. Of note is the significantly higher intensity of Bcl-2 in CD28+CD57+ cells of young than in E1 old subjects (p < 0.005). (Fig. 2E and F).

To complement the flow cytometry data, the expression of p16, p21, p53, CD95 and Bcl-2 at the mRNA level in the four subpopulations was examined by PCR in freshly sorted primary cells from a second group of 11 (E2) elderly subjects (Fig. 3). For
these experiments we focused on elderly persons. The low frequency of CD28+CD57+ cells among the young subjects makes the sorting of these cells practically impossible from the young subjects. Also, as we have earlier analyzed for differences between the young and E1 elderly group, we aimed at further characterization of the cells among E2 elderly subjects, especially the CD28+CD57+ cells that looked similar in the young and E1 elderly groups. However, this might not exclude completely the presence of some differences among CD28+CD57+ cells in some yet to be identified markers between the young and elderly groups. Among the E2 elderly participants, the expression of p21 and p95 was significantly highest in CD28+CD57+ cells (p < 0.05), with the values in the following order CD28+CD57+ > CD28–CD57+ > CD28–CD57– > CD28+CD57–. No significant difference was observed in the expression of Bcl-2 and p53 among the E2 old participants. In all the subpopulations, p16 mRNA was undetectable. Subsequently, as a control, we tested three different primers, including one that previously detected p16 in a study on primary T-cells (Liu et al., 2009). All the primers allowed us to demonstrate p16 expression in 293T cells, a human cell line that expresses high levels of p16 (Wang et al., 2008) (data not shown).

Still among the E2 elderly subjects, the CD28+CD57+ subpopulation surface markers were further examined and compared with those of the other subpopulations. The homing receptors CD62L and CCR7 were most frequently expressed by the CD28+ subpopulations and were highest among CD28+CD57– cells (Fig. 4A and B). The expression of CD45RA was significantly more frequent (p ≤ 0.007) among CD28– cells and was highest among CD28–CD57+ cells (Fig. 4C). CD45RO expression was significantly more frequent (p < 0.002) among CD28+ cells and was highest among CD28+CD57+ cells (Fig. 4D). Fig. 4E shows that the proportion of CCR5+ cells was significantly (p < 0.005) highest in CD28+CD57+ cells compared with the other subpopulations. CXCR2 expression was significantly highest (p < 0.01) among CD28–CD57+ cells compared with the other subpopulations that expressed it at a much lower proportion (Fig. 4F). PD-1 was mostly expressed (p ≤ 0.003) on CD28+CD57+ cells (Fig. 4G). A summary of the levels of expression of the different markers among the E2 elderly participants is shown in Table 1.

Fig. 3. Expression of p21, p53, CD95, and Bcl-2 mRNA in CD8+ T-cell subpopulations in a group of 11 elderly (E2) subjects. Semi-quantitative representation of the mRNA levels of (A) p21, (B) p53, (C) CD95, and (D) Bcl-2 in the subpopulations. Columns represent median values and error bars SIOR.

4. Discussion

When considering the cell surface markers CD28 and CD57, we observed in CD8+ T-cells from old (E1) compared to young subjects not only significantly higher proportions of the CD28–CD57+ cell population (34.5% versus 6.0%), as expected, but also of a small population that was characterized as CD28+CD57+ (3.0% versus 0.8%). Both subpopulations were CD57+, emphasizing the importance of CD57 expression in T-lymphocyte aging, independent of the CD28 phenotype.

At the protein level, the highest expression of the senescence markers p16 and p21 was encountered in the CD28+CD57+ subpopulation, suggesting that these cells could correspond to the concept of in vitro senescence. While the higher expression of p21 was confirmed at the mRNA level, we detected no p16 mRNA in any of the subpopulations. Others also have reported difficulties in identifying p16 mRNA in primary, unstimulated T-lymphocytes, but not after stimulation and culturing (Erickson et al., 1998; Ye et al., 2012). Our demonstration of the presence of p16 mRNA in a human cell line known to express high level of p16, reduces the chances of a technical problem (Wang et al., 2008). Nevertheless, the possibility of a technical cause might be linked to other...
shortcomings, including the low detection sensitivity of the semi-quantitative gel-based PCR technique (Mitchell and Iadarola, 2010), which we adopted for the analysis of mRNA level, against the more sensitive quantitative technique, especially the Taqman protocol that proved successful elsewhere for the detection p16 mRNA in human PBMC (Jiu et al., 2009).

The highest proportion of Bcl-2 expressing cells and the highest MFI were found in CD28+CD57+ cells. This strong Bcl-2 expression could contribute to promoting senescence by inhibiting apoptosis, even under enhanced expression of pro-apoptotic factors in cells as previously reported (Rincheval et al., 2002). Although reports indicate that CD95 mediated apoptosis is associated with the down regulation of Bcl-2 (Petrovas et al., 2009), we observed that CD95, a surface receptor triggered by members of the TNF family that modulates apoptotic and survival signals (Peter et al., 2007), was also most frequently and in the highest concentration, expressed on CD28+CD57+ cells. Reports have shown that the expression of CD95 increases during T-cell differentiation, with enhanced presence in more differentiated memory phenotypes (Aggarwal and Gupta, 1998; Fagnoni et al., 2000). Therefore, our observation would portend the CD57+ subpopulations as the most differentiated cells by our subpopulation classification. However, among cells expressing CD95, the highest intensity (MFI) of CD95 was observed in CD28+CD57+ and CD28+CD57− cells. This might indicate a possible role of CD95 in the long term survival of these cells as CD95 signaling can promote pro-survival mechanisms (Krammer, 2000; Peter et al., 2007; Vallejo et al., 2000), including its role in promoting the survival and proliferation of cancer cells as well as regeneration of liver tissue (Chen et al., 2010). In this scenario, the role of CD95 would complement rather than antagonize Bcl-2, which might have played out among CD28+CD57+ cells.

Fig. 4. Flow cytometry analysis of T cell homing, activation and differentiation markers expression in CD8+ T-cell subpopulations in a group of 11 elderly subjects. Proportion of cells expressing (A) CD62L, (B) CCR7, (C) CD45RA, (D) CD45RO, (E) CCR5, (F) CCR2, (G) PD1. Columns represent median values and error bars SIQR.
Table 1  
Expression of the different markers among the subpopulations in the elderly. (–) implies not expressed while (+) indicates a degree of expression and ranges from (+) as lowest to (++++) as highest.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>CD28+CD57+</th>
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CD95 signaling has been shown to up-regulate p21 (Hingorani et al., 2000). The parallel trend in CD95 and p21 expression in CD28+CD57+ cells could also point to a single modulator. p53, a major promoter of cellular senescence, is known as a positive modulator of p21 and CD95 expression (Herbig, Jolbing, Chen, & Sedivy, 2004; Ruiz-Ruiz, Robledo, Cano, Redondo, & Lopez-Rivas, 2003). Its regulation is reported to occur mainly at the protein level (Jenkins, Durell, Mazur, & Appella, 2012). However, using flow cytometry, we were not able to detect p53 protein, or phosphorylated-p53 [pS392-Ser37] that is necessary for DNA damage response activation (Dohoney et al., 2004) with the available fluorochrome conjugated antibodies. The observed similarity in p53 mRNA among the four subpopulations is in accordance with other reports portraying similar levels of p53 expression between senescent and non-senescent cells (Jackson and Pereira-Smith, 2006).

To further characterize the CD28+CD57+ cell population, we analyzed the expression of homing and differentiation markers. As expected, CD62L and CCR7, identifying cells that home to the secondary lymphoid organs, were most expressed by CD28+CD57+ cells; CD28–CD57+ cells showed a weak expression, while that of CD28–CD57+ was intermediate. (Sallusto, Lenig, Forster, Lipp, & Lanzavecchia, 1999). The CD28+CD57+ subpopulation had a greater proportion of cells expressing the memory and effector marker, CD45RO (Sallusto, Geginat, & Lanzavecchia, 2004), which has also been associated with T-cells undergoing senescence in vitro (Effros, 1998). Together with the high expression of CCR5, it characterizes CD28+CD57+ cells as effector or highly differentiated memory cells for non-lymphatic tissue migration (Fukada, Sobao, Tomiyama, Oka, & Takiguchi, 2002). It has been demonstrated that subpopulations of CD8+ T-lymphocytes enriched with senescent cells, resulting mostly from the exhausted and terminally differentiated cells, increases importantly during strenuous physical exercise (Simpson, Florida-James et al., 2007; Simpson, Cosgrove et al., 2008). It therefore seems likely that at rest, senescent lymphocytes are located mainly outside the blood circulation. The CD28+CD57+ cells appear to behave similarly and their presumed presence in the extra-lymphatic tissues might contribute to their low concentration in the circulation. Although all the subpopulations showed PD-1 expression, a T-cell differentiation marker that is highly expressed on exhausted CD8+ T-cells (Day et al., 2006), the highest level was found among CD28+CD57+ cells, in line with the assumption that exhausted T-cells have a higher tendency of undergoing senescence. On the contrary, the highest proportion of CXCR2 expression was observed among the CD28–CD57+ subpopulation (1/4 of the cells). As CXCR2 is a chemokine receptor associated with senescence reinforcement (Acosta et al., 2008), this might hint at the existence of a subpopulation with more pronounced senescent attributes among CD28–CD57+ cells. Such a subpopulation might explain some inconsistencies that have raised doubt on the senescent nature of CD28–CD57+ lymphocytes, including their remaining capacity to proliferate and undergo reversible differentiation (Chong et al., 2008; Geginat et al., 2001; Schwendemann, Choi, Schirrmacher, & Beckhove, 2005).

From the foregoing, a pertinent differentiation relationship appears to exist among the different subpopulations of CD8+ T-cells. It is known that CD28–CD57− cells arise from CD28+CD57− cells (Borthwick, Lowdell, Salman, & Akbar, 2000; Labalette et al., 1999), with CD28−CD57+ cells differentiating from CD28−CD57− cells, while CD28+CD57+ cells might emerge directly from CD28+CD57− cells, possibly after a series of cell division cycles (Scheuring, Sabzevari, & Theofilopoulos, 2002). In Fig. 5, a pathway of CD8+ T-cells towards senescence is proposed: CD28+CD57+ cells arise from CD28+CD57− cells, while CD8−CD57+ cells that show lesser tendency to proliferate and greater effector functions compared with CD28−CD57− cells (Brenchley et al., 2003; Ibegbu et al., 2005) arise from CD28−CD57− cells.

Our study presents limitations. As we set out to study cells as they are in vivo, we excluded subculturing and in vitro experiments. Multiple statistical testing was done, implicating that type I errors cannot be excluded when p-values were near the limit of significance. Also, the very low concentration of the CD28+CD57+ cells restricted our techniques; we did not succeed in having reliable Western blot analysis for some of the parameters and could not perform an extensive determination of the senescence markers. Although the flow cytometry protocol has been adjudged as highly sensitive for the detection of SA-B-gal, an often cited senescence marker, it was not possible to demonstrate its presence in our non-cultured cell model (Noppe et al., 2009). The observation of SA-B-gal activity has been mainly restricted to cells undergoing senescence after serial cultivation in culture (Dimri et al., 1995; Kurz, Decary, Hong, & Erusalimsky, 2000), where it has been related to the increase in lysosomal enzyme activity that results from increased lysosomal mass associated with exponential cell growth in culture (Kurz et al., 2000).

We conclude that CD28+CD57+ cells present several features related to a senescent phenotype. However, a more extensive analysis of senescence markers will be needed to fully characterize these cells and establish their senescence identity.

Conflict of interest

None of the authors has a conflict of interest to report.
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