Non-regulatory $CD8^+CD45RO^+CD25^+$ T lymphocytes may compensate for the loss of antigen-inexperienced $CD8^+CD45RA^+$ T cells in old age

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Abstract

The age-related decline in immune system functions is responsible for the increased prevalence of infectious diseases and the low efficacy of vaccination in elderly persons. In particular, the number of peripheral naive T cells declines throughout life and they exhibit severe functional defects in older age. However, we have recently identified a non-regulatory $CD8^+CD45RO^+CD25^+$ T cell subset that occurs in a subgroup of healthy elderly persons, who still exhibit an intact humoral immune response following influenza vaccination. We here demonstrate that $CD8^+CD45RO^+CD25^+$ T cells share phenotypic and functional characteristics with naive $CD8^+CD45RA^+CD28^+$ T cells from young persons, despite their expression of CD45RO. $CD8^+CD45RO^+CD25^+$ T cells also have long telomeres and upon antigenic challenge, they efficiently expand *in vitro* and differentiate into functional effector cells. The expanded population also maintains a diverse T cell receptor repertoire. In conclusion, $CD8^+CD45RO^+CD25^+$ T cells from elderly persons compensate for the loss of functional naïve T cells and may therefore be used as a marker of immunological competence in old age.

Keywords: aging; CD8 T cell; gene expression; immune system; naïve

Running title: Compensation for the loss of $CD8^+CD45RA^+CD28^+$ T cells in old age

Introduction

The immune system is subjected to a wide range of age-related alterations. As a consequence, infectious diseases are more frequent and severe in elderly persons and the efficacy of vaccinations is low (Grubeck-Loebenstein et al., 1998; Gavazzi and Krause, 2002). Due to the progressive involution of the thymus resulting in a reduced thymic output together with a life-long exposure to antigens, the number of naïve T cells dramatically declines with advancing age (Fagnoni et al., 2000). The small naïve T cell population in elderly persons also exhibits numerous functional defects, including a restricted T cell receptor (TCR) repertoire, shortened telomeres, a reduced interleukin (IL)-2 production and an impaired expansion and differentiation into effector cells (Haynes et al., 2003; Kohler et al., 2005; Pfister et al., 2006). Therefore, increasing age is associated with a decreasing ability to mediate effective immune responses to new antigens.

Another hallmark of immunological competence is the maintenance of functional memory cells. However, aging leads to the differentiation of memory cells into effector-memory cells, which have lost their proliferative capacity (Akbar and Fletcher, 2005). These highly differentiated effector-memory T cells are characterized by the lack of the co-stimulatory molecule CD28, display a highly restricted TCR repertoire and produce large amounts of the pro-inflammatory cytokine interferon (IFN)-γ yet no IL-2 (Almanzar et al., 2005). The accumulation of $CD28^-$ effector-memory T cells has been reported to trigger chronic inflammatory processes in elderly persons (Weyand et al., 2003; Raffeiner et al., 2005), to be associated with low efficacy of vaccination to induce antibody production in older age and to predict increased mortality (Saurwein-Teissl et al., 2002; Wikby et al., 2005). Chronic latent infections, such as with the cytomegalovirus accelerate the differentiation of naive and memory cells into $CD8^+CD28^-$ effector-memory T cells (Almanzar et al., 2005;

Weinberger et al., 2007). This further contributes to the decline and functional impairment of the naive and memory T cell pool in elderly persons.

We have recently described a novel, non-regulatory $CD8^+CD45RO^+CD25^+$ T cell population, which produces IL-2 and displays a highly diverse TCR repertoire (Herndler-Brandstetter et al., 2005). This population is of substantial size as it amounts to 23.7% ± 2.7% (mean ± SEM) of total $CD8^+$ T cells but occurs only in about 30% of healthy elderly persons. These persons are mostly CMV seronegative and still have a relatively intact immune system, as they can raise a protective humoral immune response following influenza vaccination (Schwaiger et al., 2003). We now demonstrate that $CD8^+CD45RO^+CD25^+$ T cells share phenotypic and functional characteristics with $CD8^+CD45RA^+CD28^+$ T cells from young persons and may therefore compensate for the loss of functional naïve T cells in old age.

Results

$CD8^+CD45RO^+CD25^+$ T cells from elderly persons share phenotype and gene expression pattern with naïve $CD8^+CD45RA^+CD28^+$ T cells from young persons

It has recently been shown that $CD8^+CD45RA^+CD28^+$ T cells from elderly humans exhibit numerous defects, such as shortened telomeres, a restricted TCR repertoire and a decreased expression of the lymph node homing molecules CD62L and CCR7 (Pfister et al., 2006) (and unpublished observation). Thus, $CD8^+CD45RA^+CD28^+$ T cells can not be considered functionally naive in old age. However, we recently described a non-regulatory $CD8^+CD45RO^+CD25^+$ T cell subset which occurs in a subgroup of healthy elderly persons who have a good humoral immune response following vaccination (Herndler-Brandstetter et al., 2005). These cells have a highly diverse TCR repertoire and produce high amounts of the important T cell growth factor IL-2. We now hypothesized that $CD8^+CD45RO^+CD25^+$ T cells may compensate for the loss of antigen-inexperienced $CD8^+CD45RA^+CD28^+$ T cells in old age, despite their expression of the classical memory marker CD45RO. To prove this hypothesis, we first analyzed a panel of surface proteins known to be highly expressed on naïve $CD8^+CD45RA^+CD28^+$ T cells from young persons (Betz and Muller, 1998; Kaech et al., 2002). Both, $CD8^+CD45RO^+CD25^+$ T cells from elderly and $CD8^+CD45RA^+CD28^+$ T cells from young persons displayed a high expression of the lymph node homing molecule CD62L and the IL-6 receptor α chain (CD126), which were decreased after stimulation with anti-CD3 and IL-2, and are therefore expressed at low levels on memory cells (Figure 1). In contrast, the adhesion molecule CD11a, the chemokine receptor CCR5 and the natural killer cell molecule CD57 were up-regulated during stimulation and differentiation into memory and effector cells. $CD8^+CD45RO^+CD25^+$ T cells from elderly and $CD8^+CD45RA^+$ T cells from young persons exhibited a $CD28^+$ $CD11a^{dim}$ phenotype and did not express CCR5 and CD57.

To further specify the characteristics and lineage relationship of $CD8^+CD45RO^+CD25^+$ T cells from elderly persons, gene expression profile analysis was performed. Our results showed that $CD8^+CD45RO^+CD25^+$ T cells from elderly persons have a gene expression profile that resembles that of $CD8^+CD45RA^+CD28^+$ T cells from young persons but is distinct from $CD8^+CD45RO^+$ T cells from young and $CD8^+CD45RO^+CD25^-$ T cells from elderly persons. The T cell differentiation protein (MAL), transcription factor MYC and nitric oxide synthase interacting protein (NOSIP) were expressed at a similar high level in $CD8^+CD45RO^+CD25^+$ T cells from elderly and $CD8^+CD45RA^+CD28^+$ T cells from young persons (Figure 2). In contrast, the expression of granzyme B (GZMB) and natural killer cell group 7 sequence (NKG7), known to be highly expressed in antigen-experienced T cells (Kaech et al., 2002; Fann et al., 2005), was low in $CD8^+CD45RO^+CD25^+$ T cells from elderly and $CD8^+CD45RA^+CD28^+$ T cells from young persons.

$CD8^+CD45RO^+CD25^+$ T cells from elderly persons share functional properties with $CD8^+CD45RA^+CD28^+$ T cells from young persons

As naive T cells are by definition antigen-inexperienced, have thus not been exposed to their specific antigen and not undergone substantial propagation and differentiation, they still have long telomeres. Our results demonstrate that the telomere length of $CD8^+CD45RO^+CD25^+$ T cells from elderly persons (6.5 ± 0.4 kb) was similar to that of $CD8^+CD45RA^+CD28^+$ T cells from young persons (7.1 ± 0.1 kb). In contrast, $CD8^+CD45RO^+CD25^-$ T cells from elderly persons had significant shorter telomeres (5.1 ± 0.4 kb; Figure 3) compared to $CD8^+CD45RO^+CD25^+$ T cells from elderly and $CD8^+CD45RA^+CD28^+$ T cells from young persons.

To analyze the time-dependent acquisition of effector function, expression of IFN-γ and GZMB in $CD8^+CD45RA^+CD28^+$ and $CD8^+CD45RO^+$ T cells from young as well as

$CD8^+CD45RO^+CD25^+$ and $CD8^+CD45RO^+CD25^-$ T cells from elderly persons after stimulation with anti-CD3/anti-CD28 was analyzed by quantitative RT-PCR. The results revealed lower gene expression of IFN-γ and GZMB before and after stimulation in $CD8^+CD45RA^+CD28^+$ T cells from young and $CD8^+CD45RO^+CD25^+$ T cells from elderly persons compared to their respective control populations (Figure 4).

$CD8^+CD45RO^+CD25^+$ T cells can efficiently be expanded *in vitro* and differentiate into functional effector cells

To address the question whether $CD8^+CD45RO^+CD25^+$ T cells have the potential to expand efficiently and retain an effector phenotype over a prolonged period of time, $CD8^+CD45RO^+CD25^+$ T cells from elderly persons were stimulated *in vitro* for 28 days with anti-CD3 and IL-2. Our results revealed a substantial expansion of $CD8^+CD45RO^+CD25^+$ T cells upon repeated stimulation (Figure 5A), similar to $CD8^+CD45RO^+CD25^-$ T cells (data not shown). During *in vitro* expansion, the down-regulation of the lymph node homing molecules CD62L and CCR7 as well as CD126, MAL and MYC combined with the up-regulation of the tissue homing marker CXCR3 indicated differentiation of a naive-like cell type into effector cells that are capable of homing to peripheral tissues after antigenic stimulation (Figure 5B). In addition, IFN-γ production increased (Figure 5C) and GZMB gene expression was up-regulated 4-fold after expansion (Figure 5D). Long-term stimulated $CD8^+CD45RO^+CD25^+$ T cells also had a high GZMA and TNF-α gene expression and did not express IL-10 (data not shown).

We also analyzed the effect of *in vitro* expansion on TCR repertoire diversity of $CD8^+CD45RO^+CD25^+$ T cells. In accordance with previous work we could show that the repertoire of the unstimulated population is highly diverse (Figure 6). After 28 days, only minor variations in the clonal composition of $CD8^+CD45RO^+CD25^+$ T cells were found.

During stimulation few individual clones changed in size, but virtually no clones were lost. Altogether, our results revealed that *in vitro* stimulation of $CD8^+CD45RO^+CD25^+$ T cells led to a rapid expansion and differentiation into functional effector cells and only to minor variations in the TCR repertoire.

Discussion

The age-related involution of the thymus and the life-long exposure to pathogens leads to a dramatic decrease in naive T cell numbers thus limiting the host's ability to cope with a variety of infectious diseases and to mount protective immune responses following vaccination (Barker and Mullooly, 1982; Saurwein-Teissl et al., 2002; Wolters et al., 2003). Recent results have additionally shown that aged $CD8^+$ T cells which display a $CD45RA^+CD28^+CD62L^+$ phenotype and should thus per definition be naive, exhibit severe defects such as shortened telomeres, a restricted TCR repertoire and decreased effector functions (Haynes et al., 2003; Kohler et al., 2005; Pfister et al., 2006). However, a subgroup of healthy elderly persons can still raise a protective humoral immune response after influenza vaccination (Saurwein-Teissl et al., 2002; Schwaiger et al., 2003). These elderly persons characteristically possess high numbers of $CD8^+CD45RO^+CD25^+$ T cells and low $CD8^+CD28^-$ effector T cell counts. We have already demonstrated that $CD8^+CD45RO^+CD25^+$ T cells are non-regulatory, have a highly diverse TCR repertoire and express the co-stimulatory molecules CD28 and CD27 as well as the lymph node homing markers CD62L and CCR7 (Schwaiger et al., 2003; Herndler-Brandstetter et al., 2005). We therefore hypothesized that $CD8^+CD45RO^+CD25^+$ T cells may be antigen-inexperienced, but have acquired their CD45RO phenotype during homeostatic proliferation and/or long-term residence in lymphoid and/or extra-lymphoid organs (Murali-Krishna and Ahmed, 2000; Mazo et al., 2005). Although only limited data from elderly humans are available, studies in mice have indicated that homeostatically proliferated naïve T cells acquire a memory-like phenotype due to the up-regulation of CD44, CD122 and Ly6C (Cho et al., 2000; Goldrath et al., 2000). To ascertain whether $CD8^+CD45RO^+CD25^+$ T cells from elderly persons may compensate for the loss of naïve $CD8^+CD45RA^+CD28^+$ T cells, we determined their phenotypic characteristics. FACS analysis revealed that the surface molecules CD62L,

CD11a, CCR5 and CD57 were expressed at similar levels in $CD8^+CD45RO^+CD25^+$ and $CD8^+CD45RA^+CD28^+$ T cells. Noteworthy, $CD8^+CD45RO^+CD25^+$ and $CD8^+CD45RA^+CD28^+$ T cells expressed the interleukin 6 receptor α chain (CD126), which is known to be highly expressed in naïve T cells (Willinger et al., 2005) and is down-regulated following antigenic stimulation (Betz and Muller, 1998). We also compared the gene expression profile of $CD8^+CD45RO^+CD25^+$ T cells from elderly persons with that of $CD8^+CD45RO^+CD25^-$ T cells from elderly persons and $CD8^+CD28^+$ and $CD8^+CD45RO^+$ T cells from young persons. The T cell differentiation protein MAL and the transcription factor MYC, both known to be highly expressed in naïve $CD8^+$ T cells (Holmes et al., 2005; Fann et al., 2006a), had an equally high expression in $CD8^+CD45RO^+CD25^+$ from elderly and $CD8^+CD45RA^+CD28^+$ T cells from young persons. In contrast, GZMB and NKG7 were highly expressed in $CD8^+CD45RO^+$ T cells (Kaech et al., 2002), but had a comparable low expression in $CD8^+CD45RO^+CD25^+$ and $CD8^+CD45RA^+CD28^+$ T cells.

The telomere length of $CD8^+CD45RO^+CD25^+$ T cells from elderly persons and $CD8^+CD45RA^+CD28^+$ T cells from young persons were similar, indicating a similar replicative history. Though, $CD8^+CD45RO^+CD25^+$ T cells had much longer telomeres (6.5 kb) compared to $CD8^+CD45RO^+CD25^-$ (5.1 kb) as well as $CD8^+CD45RA^+CD28^+$ T cells (4.5 kb) from elderly persons (Herndler-Brandstetter et al., 2005; Pfister et al., 2006). The differences in telomere length between $CD8^+CD45RO^+CD25^+$ and $CD8^+CD45RO^+CD25^-$ T cells from elderly persons was however not due to recent activation of $CD25^+$ cells, as demonstrated previously (Herndler-Brandstetter et al., 2005).

Memory T cells show a rapid and robust acquisition of effector function following antigenic stimulation, which is a key feature distinguishing them from naïve T cells (Veiga-Fernandes et al., 2000; Fann et al., 2006a). We therefore analyzed the gene expression kinetics of IFN-γ and GZMB after TCR-mediated stimulation. Our results revealed that both,

$CD8^+CD45RO^+CD25^+$ T cells from elderly and $CD8^+CD45RA^+CD28^+$ T cells from young persons show decreased IFN-γ and GZMB expression before and after stimulation compared to the corresponding memory $CD8^+$ T cell population. The results obtained by gene expression kinetic analysis further support our hypothesis that $CD8^+CD45RO^+CD25^+$ T cells from elderly persons may resemble naïve-like cells that compensate for the loss of $CD8^+CD45RA^+CD28^+$ T cells in elderly persons.

In mice it has been reported that aged naïve T cells exhibit numerous functional defects, such as a decreased production of IL-2 and a decreased generation of functional effector cells (Haynes et al., 2000). We therefore addressed the question, whether $CD8^+CD45RO^+CD25^+$ T cells might, despite their similarity to young naïve cells, be impaired in their capacity to expand and maintain effector functions over a longer time period. Our results demonstrate that $CD8^+CD45RO^+CD25^+$ T cells efficiently expand during long-term *in vitro* culture, as has been shown for $CD8^+CD45RA^+CD28^+$ from young persons (Wallace et al., 2006), strongly up-regulate IFN-γ and GZMB expression and maintain a high IL-2 production. They may also be able to migrate to peripheral tissues, as indicated by their expression of CXCR3.

The diversity of the TCR repertoire is of utmost importance to raise effective immune responses (Nikolich-Zugich et al., 2004), but is severely restricted in elderly individuals, in particular in the $CD8^+$ T cell pool. This may be due to the decreased output of new naïve T cells as well as continuous antigenic challenge, caused during infections with viruses such as the cytomegalovirus or the human immunodeficiency virus (Khan et al., 2002; Pawelec et al., 2004; Fletcher et al., 2005; Naylor et al., 2005). $CD8^+CD45RO^+CD25^+$ T cells have a diverse TCR repertoire, which is maintained even after repeated *in vitro* challenge with anti-CD3 and IL-2. This indicates that the broad majority of antigen specificities within the $CD8^+CD45RO^+CD25^+$ T cell population possess an intact proliferative capacity. Therefore,

$CD8^+CD45RO^+CD25^+$ T cells appear to fulfill tasks carried out by naïve $CD8^+CD45RA^+CD28^+$ T cells in young adults.

However, about 70% of the elderly population does not possess substantial numbers of this cell type (Schwaiger et al., 2003). For these persons it may be beneficial to multiply the few remaining $CD8^+CD45RO^+CD25^+$ T cells by *in vitro* expansion. The resulting population could then be stored and re-transfused in the case of immune deficiency such as after chemo- or radiation therapy. Furthermore, our results greatly suggest that high numbers of $CD8^+CD45RO^+CD25^+$ T cells represent a good bona fide biomarker of immunological competence in older age.

Materials and Methods

Volunteers

Peripheral blood samples were obtained from healthy young (20-35 years) and elderly (>65 years) volunteers. Only elderly persons were chosen for the study known to have more than 15% $CD45RO^+CD25^+$ cells within their $CD8^+$ T cell population and a good humoral immune response following influenza vaccination, determined by an increase in antibody titers to a post-vaccination level of greater than 40 for all three influenza strains (Herndler-Brandstetter et al., 2005). All subjects were CMV seronegative, except two persons who had low CMV antibody titers. Before bleeding, a health check was performed for each participant. All participants had given their informed written consent and blood collection was approved by the Ethics Committee of Innsbruck Medical University.

T cell purification

Peripheral blood mononuclear cells (PBMC) were seperated by Ficoll-Hypaque® density gradient centrifugation (Amersham Biosciences, Uppsala, Sweden). $CD8^+CD45RO^+CD25^+$ and $CD8^+CD45RO^+CD25^-$ T cells from elderly persons were enriched from PBMC using magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany) as described previously (Herndler-Brandstetter et al., 2005). For the isolation of $CD8^+CD45RA^+CD28^+$ (naïve) and $CD8^+CD45RO^+$ (memory) T cells from young persons, $CD8^+$ T cells were positively selected using CD8 MultiSort microbeads (Miltenyi Biotec). After enzymatic removal of the CD8 MultiSort microbeads, $CD8^+CD45RO^+$ T cells were isolated with the aid of CD45RO microbeads. The $CD8^+CD45RO^-$ fraction was then stained with an allophycocyanin-conjugated mAb recognizing CD28 (BD Pharmingen). $CD8^+CD45RA^+CD28^+$ T cells were obtained by positive selection using anti-allophycocyanin microbeads and a LS column (Miltenyi Biotec). The purity of the isolated $CD8^+$ T cell subpopulations was >93%.

Flow cytometry

Immunofluorescence surface staining of freshly prepared PBMC was performed using the following conjugated mAbs: CCR5 (PE), CCR7 (PE), CD3 (FITC or PE), CD4 (FITC or APC), CD8 (PerCP), CD11a (PE), CD25 (PE or APC; clone 2A3), CD27 (PE), CD28 (PE or APC), CD45RO (FITC, PE or APC), CD57 (FITC), CD62L (APC), CD126 (PE), CXCR3 (FITC), HLA-DR (PE) (BD Pharmingen, San Jose, USA) and chemoattractant receptor of Th2 cells (CRTH2; PE) (Miltenyi Biotec, Bergisch Gladbach, Germany). Fluorescence was monitored with a FACSCalibur® flow cytometer (BD Pharmingen). The production of cytokines before and after long-term culture of $CD8^+CD45RO^+CD25^+$ T cells was assessed by stimulating the cells for 4 hours with phorbol myristate acetate (PMA; 30 ng/ml) and ionomycin (500 ng/ml) in the presence of brefeldin A (10 µg/ml). Cells were permeabilized using the Cytofix/Cytoperm kit (BD Pharmingen) and intracellular staining of IFN-γ, IL-2 and IL-4 (BD Pharmingen) was performed.

Cell culture

Short-term stimulation

$CD8^+CD45RA^+CD28^+$ and $CD8^+CD45RO^+$ T cells were isolated from young persons, and $CD8^+CD45RO^+CD25^+$ and $CD8^+CD45RO^+CD25^-$ T cells were isolated from elderly persons. The T cell subsets were stimulated for 20 or 70 hours with Dynabeads® CD3/CD28 T cell expander (Invitrogen Dynal, Oslo, Norway) using one bead per T cell as recommended by the manufacturer. After washing with phosphate-buffered saline (PBS), total RNA was isolated as described below.

Long-term stimulation

Purified $CD8^+CD45RO^+CD25^+$ T cells were cultured together with irradiated (30 Gy) autologous PBMC as antigen presenting cells (10^6 cells/well) at a density of 10^6 cells/well in 24-well plates and stimulated with OKT3 (30 ng/ml, Orthoclone, Transplant, Vienna, Austria) and IL-2 (20 ng/ml, Novartis, Basel, Switzerland). Thereafter, cells were recultivated on days 7, 14 and 21 by adding new culture medium (10% FCS/RPMI 1640), OKT3, IL-2 and irradiated autologous PBMC. On days 4, 11, 18 and 25 IL-2 was added as well as additional fresh culture medium. The growth status of the cells was monitored twice a week. Cells were stained with trypan blue and counted after one, two, three and four weeks of culture. Before and after four weeks of stimulation, RNA was isolated and phenotype and intracellular cytokine production was analyzed.

cDNA array and data analysis

Total RNA was extracted from purified T cell subsets using Tri Reagent (Sigma-Aldrich). Glycogen (Roche, Basel, Switzerland) was added as a carrier for RNA precipitation at a concentration of 1 μg/ml. The yield and quality of the RNA was determined by spectrophotometric and agarose gel analysis. Radiolabeled cDNA probe was prepared using oligo(dT)-primers and [α-^{33}P]dCTP. Nylon filter cDNA arrays (JHU/NIH MGC1 human 9k filter representing approximately 6424 unique genes (Nadon et al., 2005), a kind gift from Kevin Becker, Genome Center of the National Institute on Aging, Baltimore, USA) were hybridized for 48 hours at 65°C (5x Denhardt's solution, 5x SSC, 1% SDS), washed with 0.2x SSC, 0.1% SDS at 68°C and exposed to Fuji Imaging Plates for 24 - 48 hours. The plates were scanned by means of a Fuji BAS-1800-II reader and the images were processed using AIDA Image Analyzer 3.51 software (Raytest). The primary intensity values were normalized and compared between $CD8^+$ T cell subsets. For hierarchical cluster analysis MultiExperiment Viewer (MeV) v4.0b software was chosen (Saeed et al., 2003) and complete

linkage clustering was performed applying Euclidean distance. Genes were considered differentially regulated between $CD8^+$ T cell subsets with a fold difference greater than two and $p<0.05$ for Pavlidis template matching (PTM). Markers of naive vs. antigen-experienced T cells from young persons were chosen on the basis of array data and the above mentioned selection criteria. The obtained gene list corresponded well with results from literature (Willinger et al., 2005; Fann et al., 2006b). Only genes with a high expression in naive and memory cells were used for quantitative RT-PCR (n=19). The Panther (Protein Analysis Through Evolutionary Relationships) Classification System was then used as a resource to classify genes according to their respective function (Mi et al., 2005).

Quantitative RT-PCR

cDNA was synthesized from total RNA using a Reverse Transcription System (Promega, Madison, USA). Quantitative RT-PCR experiments were performed using the LightCycler® 480 System (Roche Diagnostics, Basel, Switzerland), SYBR Green I Master (Roche Diagnostics) and β-Actin as housekeeping gene for relative quantification of candidate genes. Sequence-specific oligonucleotide primers were designed to amplify cDNA fragments using Primer3 software (Rozen and Skaletsky, 2000) and were synthesized by MWG Biotech, Ebersberg, Germany (Table 1). RT-PCR was performed in a total volume of 22 µl and primers were used at a final concentration of 1.4 pmol/µl. The RT-PCR amplification protocol was as follows: initial incubation at 95°C for 8 min followed by 50 amplification cycles (95°C for 15 sec, 57°C for 8 sec and 72°C for 15 sec). PCR products were subsequently loaded onto a 1% agarose gel containing ethidium bromide and visualized by UV illumination. Normalized threshold cycle (CT) values ($\Delta CT = CT_{gene} - CT_{ACTB}$) were used to calculate the fold differences (fold difference = $2^{-\Delta\Delta CT}$) (Livak and Schmittgen, 2001). Relative expression

values were obtained using the following formula: Relative expression value = Log_{10} ($2^{-\Delta\Delta CT}$) + 5.

Telomere length analysis by flow FISH

The telomere length of $CD8^+CD45RA^+CD28^+$ T cells from young persons and $CD8^+CD45RO^+CD25^+$ and $CD8^+CD45RO^+CD25^-$ T cells from elderly persons was determined using flow cytometric fluorescence in situ hybridization (flow FISH) as described previously (Herndler-Brandstetter et al., 2005). The fluorescence of the Cy5-labeled PNA telomere probe was analyzed for the three T cell subsets on a FACSCalibur® flow cytometer. Based on the fluorescence intensity of the lymphoblastic leukemia cell line 1301, the relative telomere length was calculated (Fehrer et al., 2006).

TCR CDR3 spectratyping

cDNA of unstimulated and long-term stimulated $CD8^+CD45RO^+CD25^+$ T cells was synthesized as described above. TCR Vβ transcripts were amplified by PCR using a HotStarTaq Master Mix Kit (Qiagen, Hilden, Germany) and primers (MWG, Ebersberg, Germany) specific for each of the human Vβ families and a specific primer for the constant region of the β-chain (labeled with the fluorescent dye 6-FAM) as described previously (Herndler-Brandstetter et al., 2005).

Statistical analysis

Statistical analysis was performed using a two-tailed Student's *t* test. Differences were considered significant when the *P* value was less than 0.05.

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Table 1 List of primers used for quantitative RT-PCR analysis

Gene symbol	Gene name	Primer sequence (5' – 3')
ACTB	Actin, beta	Fwd: TCC TTC CTG GGC ATG GAG T Rvs: TCT CCT TCT GCA TCC TGT CG
CD126	Interleukin 6 receptor, alpha	Fwd: TCCTCTGCATTGCCATTGT Rvs: GTGGGGAGATGAGAGGAACA
GZMB	Granzyme B	Fwd: GACCCAGCAGTTTATCCCTGT Rvs: CTGGGCCTTGTTGCTAGGTA
IFNG	Interferon gamma	Fwd: TGG AGA CCA TCA AGG AAG ACA Rvs: GCG ACA GTT CAG CCA TCA CT
MAL	Mal, T cell differentiation protein	Fwd: CTGGGTGATGTTCGTGTCTG Rvs: GACTGAGGCGCTGAGGTAAA
MYC	V-myc myelocytomatosis viral oncogene homolog (avian)	Fwd: GGATTCTCTGCTCTCCTCGAC Rvs: CCTGCCTCTTTTCCACAGAA
NKG7	Natural killer cell group 7 sequence	Fwd: GCC TGA TGT TCT GCC TGA TT Rvs: GAA CAG CCA TAA TGC TGA AGG
NOSIP	Nitric oxide synthase interacting protein	Fwd: CTTGCCACGATCCTGTTGT Rvs: TGAAGCTCCTTCTGCTCCTC

Figure legends

Figure 1. Phenotypic characteristics of unstimulated $CD8^+$ T cell subsets from young and elderly persons. (A) The expression of the surface molecules CD62L, CD11a, CD126, CCR5 and CD57 on $CD8^+CD45RO^+CD25^+$ T cells from elderly persons and $CD8^+CD45RA^+CD28^+$ (naïve) and $CD8^+CD45RO^+$ (memory) T cells from young persons was determined by flow cytometry. Numbers indicate the percentage of positive cells within the respective T cell subset. One representative example of three independent experiments is shown. (B) Bars represent the mean percentage ± SEM of CD62L, CD11a, CD126, CCR5 and CD57 expression on $CD8^+CD45RO^+CD25^+$ T cells from elderly persons (black) and $CD8^+CD45RA^+CD28^+$ (grey) and $CD8^+CD45RO^+$ T cells from young persons (white) of at least three independent experiments per group. $P < 0.01$, *, $CD8^+CD45RO^+CD25^+$ vs. $CD8^+CD45RO^+$; +, $CD8^+CD45RO^+CD25^+$ vs. $CD8^+CD45RA^+CD28^+$; #, $CD8^+CD45RA^+CD28^+$ vs. $CD8^+CD45RO^+$.

Figure 2. Gene expression profile of unstimulated $CD8^+$ T cell subsets from young and elderly persons. The gene expression of $CD8^+CD45RO^+CD25^+$ T cells from elderly persons (black squares), and $CD8^+CD45RA^+CD28^+$ (grey squares) and $CD8^+CD45RO^+$ T cells from young persons (open squares) was analyzed by quantitative RT-PCR. Bars represent the mean gene expression level ($\log_{10}$) ± SEM (three subjects per group).

Figure 3. Telomere length of unstimulated $CD8^+$ T cell subsets from young and elderly persons. Bars represent the mean fluorescent signal ± SEM of $CD8^+CD45RO^+CD25^+$ and $CD8^+CD45RO^+CD25^-$ T cells from elderly persons, and $CD8^+CD45RA^+CD28^+$ T cells from young persons. * $P < 0.05$; three subjects per group.

Figure 4. Time-dependent acquisition of effector functions by $CD8^+$ T cell subsets from young and elderly persons. The relative gene expression level (log_{10}) of IFN-γ and granzyme B (GZMB) before as well as 20 and 70 hours after stimulation with anti-CD3/anti-CD28 of $CD8^+CD45RA^+CD28^+$ (naive; grey) and $CD8^+CD45RO^+$ T cells (memory; black) from young persons as well as $CD8^+CD45RO^+CD25^+$ (grey) and $CD8^+CD45RO^+CD25^-$ T cells (black) from elderly persons was analyzed by quantitative RT-PCR. The figure shows one of two independent experiments.

Figure 5. $CD8^+CD45RO^+CD25^+$ T cells from elderly persons can be efficiently expanded *in vitro* and retain an effector phenotype. (A) *In vitro* expansion profile of $CD8^+CD45RO^+CD25^+$ T cells from elderly persons after repeated stimulation with anti-CD3 and IL-2. Mean values ± SEM from five independent experiments are shown. (B) Phenotypic profile before (black) and after (white) repeated stimulation with anti-CD3 and IL-2 of $CD8^+CD45RO^+CD25^+$ T cells. Bars represent mean values ± SEM from five independent experiments. * $P < 0.05$; before vs. after long-term stimulation. (C) Cytokine production profile of $CD8^+CD45RO^+CD25^+$ T cells before and after long-term stimulation with anti-CD3 and IL-2. The percentage of cytokine-producing cells was determined after 4 hours stimulation with PMA and ionomycin in the presence of brefeldin A. The bars represent mean values ± SEM of three independent experiments. * $P < 0.05$; before vs. after long-term stimulation. (D) Relative expression intensity (log_{10}) of candidate genes before (first point) and after 28 days of stimulation with anti-CD3 and IL-2 (second point) of $CD8^+CD45RO^+CD25^+$ T cells. The relative expression intensities represent mean values from three independent experiments.

Figure 6. T cell receptor repertoire of $CD8^+CD45RO^+CD25^+$ T cells before and after *in vitro* expansion. The clonal composition of 24 Vβ families was analyzed by CDR3 spectratyping of $CD8^+CD45RO^+CD25^+$ T cells from elderly persons before and after 28 days of *in vitro* expansion using anti-CD3 and IL-2. Five randomly chosen TCR Vβ families from one of three independent experiments are shown before and 28 days after *in vitro* stimulation. The arrows indicate minor changes in the size of individual clones after long-term *in vitro* stimulation.