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CD25-Expressing CD8^+ T Cells Are Potent Memory Cells in Old Age^1

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We have recently described an IL-2/IL-4-producing CD8^+CD25^+ nonregulatory memory T cell population that occurs in a subgroup of healthy elderly persons who characteristically still have a good humoral response after vaccination. The present study addresses this specific T cell subset and investigates its origin, clonal composition, Ag specificity, and replicative history. We demonstrate that CD8^+CD25^+ memory T cells frequently exhibit a CD4^+CD8^− double-positive phenotype. The expression of the CD8 αβ molecule and the occurrence of signal-joint TCR rearrangement excision circles suggest a thymic origin of these cells. They also have longer telomeres than their CD8^+CD25^- memory counterparts, thus indicating a shorter replicative history. CD8^+CD25^+ memory T cells display a polyclonal TCR repertoire and respond to IL-2 as well as to a panel of different Ags, whereas the CD8^-CD25^- memory T cell population has a more restricted TCR diversity, responds to fewer Ags, and does not proliferate in response to stimulation with IL-2. Molecular tracking of specific clones with clonotypic primers reveals that the same clones occur in CD8^-CD25^+ and CD8^-CD25^- memory T cell populations, demonstrating a lineage relationship between CD25^- and CD25^- memory CD8^+ T cells. Our results suggest that CD25-expressing memory T cells represent an early stage in the differentiation of CD8^+ cells. Accumulation of these cells in elderly persons appears to be a prerequisite of intact immune responsiveness in the absence of naïve T cells in old age. The Journal of Immunology, 2005, 175: 1566–1574.

Infectious diseases are frequent and severe in elderly persons (1), and the efficacy of vaccinations is low (2). This is due to age-related changes within the immune system (3). The almost complete involution of the thymus in early adulthood (4) leads to a progressive and dramatic decrease in the numbers of naïve T cells (5). In contrast, CD8^-CD28^- effector T cell populations have been shown to accumulate with age (6). These cells are clonally restricted and produce large amounts of IFN-γ, but no IL-2 or IL-4 (7, 8). CD8^-CD28^- effector T cells have mainly been attributed detrimental effects in old age. Their accumulation has been reported to trigger chronic inflammatory processes in elderly persons (9, 10). High numbers of CD8^-CD28^- cells have also been shown to be associated with insufficient efficacy of vaccines to induce Ab production in old age (8, 11) and to predict higher mortality (12).

There is strong circumstantial evidence that CMV may be a dominant factor to drive CD8^- T cell differentiation and hereby induce premature immune senescence (13). CMV-specific CD8^- T cells have also been shown to occur as large expanded clones that may dominate the repertoire (14). In a recent publication (15), we demonstrated that aging as well as CMV infection lead to a decrease in the size of the naïve CD8^- T cell pool, but to an increase in the number of IFN-γ-producing CD8^-CD28^- effector T cells. The size of the CD8^- memory T cell population that produces IL-2 and IL-4 also increases with aging, but this increase is missing in CMV carriers. Lifelong latent CMV infection thus seems to diminish the size of the naïve and early memory T cell pool and to drive a Th1 polarization within the immune system. A recent publication demonstrates that the humoral immune response to influenza vaccination is reduced in CMV carriers (16). This finding indicates that lifelong CMV infection may restrict immunological diversity and thus compromise immunological memory in old age.

We have recently described a population of IL-2/IL-4-producing CD8^- T cells that display a central memory-like phenotype and constitutive CD25 expression, yet without regulatory function (17). This population characteristically occurs in a subgroup of healthy elderly persons still capable of raising a protective humoral immune response after influenza vaccination. CD8^-CD25^- T cells are virtually absent in elderly persons with CD8^-CD28^- effector cell accumulation and in young individuals, who characteristically have high numbers of naïve CD8^- T cells and low memory/effector counts (15).

We now demonstrate that nonregulatory CD8^-CD25^- T cells represent a memory T cell reservoir of great diversity in old age. This population may therefore be an important prerequisite for intact immune responses in elderly persons, in whom naïve T cells can no longer be regenerated.

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Flow cytometry
Immunofluorescence surface staining was performed by adding a panel of conjugated mAbs to freshly prepared PBMCs. The Abs used were CD3 (FITC or PE), CD4 (PE-Cy7 or allophycocyanin), CD8 (PerCP), CD45RO (PE or allophycocyanin; clone 2A3), CD28 (PE or allophycocyanin), CD44 (FITC), CD45RO (FITC, PE, or allophycocyanin), CD62L (allophycocyanin, CCR7 (PE), HLA-DR (PE), CD94 (FITC), CD95 (PE), NKB1 (FITC), CD158αβ (FITC), and CD158bβ (FITC; all from BD Pharmingen) and chemoattractant receptor of Th2 cells (cRT2α; PE, Miltenyi Biotec). After staining, cells were fixed in 2% formaldehyde, and fluorescence was measured with a FACSCalibur flow cytometer (BD Pharmingen).

Cell purification
Preparation of PBMCs was performed by density gradient centrifugation (Ficoll-Hypaque; Amersham Biosciences). CD8+ CD45RO+ CD25+ T cells were enriched from PBMCs in a series of separations using magnetic beads as described previously (17). Briefly, PBMCs were first depleted of naïve and NK cells by the application of CD45RA and CD56 microbeads (Miltenyi Biotec) and a LD depletion column (Miltenyi Biotec). Subsequently, CD8+ T cells were positively selected using CD8 Multisort microbeads (Miltenyi Biotec). After removal of the CD8 Multisort microbeads, the CD8+ T cells were stained with an allophycocyanin-conjugated mAb recognizing CD25 (BD Pharmingen). Cells were washed twice with PBS supplemented with 0.5% BSA and 2 mM EDTA, pH 7.2, and CD25+ cells were obtained by positive selection using anti-allophycocyanin microbeads (Miltenyi Biotec) and applying a LS column (Miltenyi Biotec). The purity of the obtained population was >90%. The CD25+ fraction was depleted of residual CD25+ cells in an additional LD column (Miltenyi Biotec), resulting in a purity of >95%. We also performed cell sorting of PBMCs by staining with mAbs for CD3 (FITC), CD3 (PE), CD4 (PE-Cy7), and CD25 (allophycocyanin) using a FACSDiva software (BD Biosciences). The purity of the CD25+ and CD25− CD45RO+ populations assessed by FACS was >93%.

[3H]Thymidine incorporation
All cells were cultured in RPMI 1640 (Invitrogen Life Technologies) supplemented with 10% FCS (Sigma-Aldrich) and 1% penicillin-streptomycin (Invitrogen Life Technologies). Isolated CD8+ CD45RO+ CD25+ or CD8+ CD45RO− CD25− T cells were cultured together with autologous irradiated (30 Gy) PBMCs as APCs (105 cells/well) at a density of 105 cells/well in 96-well plates and were stimulated with anti-CD3 mAb (OKT3; 30 ng/ml; Orthoclone; Transplant), OKT3 and rIL-2 (20 ng/ml; Novartis), or rIL-2 alone. For MLR cultures, irradiated (30 Gy) PBMCs as APCs, anti-CD3 mAb (OKT3; 30 ng/ml; Orthoclone; Transplant), OKT3, and 1 μCi [3H]thymidine (ICN Pharmaceuticals) were added for control purpose. PCR amplification was performed using a CD25 forward primer (5′-CGGCAAAGAGTGGGACAAAGA-3′; MWG) and primers (MWG) specific for each of the human Vβ families assessed by FACS Vantage (BD Biosciences). The purity of the CD25+ and CD25− CD45RO+ populations assessed by FACS was >93%.

CFSE labeling
The fluorescent dye CFSE (Molecular Probes) was used to determine proliferation and differentiation. Cells were suspended in PBS at a concentration of 1 × 106/ml. An equal volume of 1 μM CFSE in PBS was added, and cells were incubated in the dark at room temperature for 10 min. Unbound or deacetylated CFSE was quenched by adding 5% FCS, followed by two washing steps with culture medium. CD8+ CD45RO+ CD25+ or CD8+ CD45RO− CD25− T cells were cultured together with autologous, irradiated (30 Gy) PBMCs as APCs (105 cells/well) at a density of 105 cells/well in 24-well plates and were stimulated with PHA (1 μg/ml; Sigma-Aldrich) for 4 days. CFSE-labeled cells were costained with PE-, PerCP-, and allophycocyanin-conjugated mAbs and analyzed on a FACSCalibur flow cytometer.

Tetramer staining
To determine the expansion potential of CD8+ CD45RO+ CD25+ and CD8+ CD45RO− CD25− T cells upon stimulation with specific Ags, cells from HLA-A2-positive donors were seeded at a concentration of 2.5 × 105 cells/well together with the same number of autologous, irradiated (30 Gy) PBMCs as APCs, antigenic peptides (2 μg/ml), and rIL-2 (20 ng/ml). All subjects had positive EBV, but negative anti-HBc and anti-HBs, Ab serology. Only one person had a positive CMV Ab serology. Primers used were FLP5SDFPSV (hepatitis B virus [HBV] core), CLGGLTMV (EBV/MP; Priommine), GILGFVFL (influenza virus matrix FLU M1,8–66; Fundacion Instituto de Immunologia de Colombia), and NLVP-MVAT (human CMV pp65; Bachem). After 1 wk of culture, the cells were harvested, and the frequencies of Ag-specific cells in the two subpopulations were determined by tetramer staining (Pirimmine). Propidium iodide (1 μg/ml; BD Pharmingen) was added directly before analysis to exclude dead cells.

RNA isolation and TCR CDR3 spectratyping
RNA isolation and first-strand cDNA synthesis were performed as previously described (8). Briefly, total RNA was extracted from purified CD8+ CD45RO+ CD25+ and CD8+ CD45RO− CD25− T cells using Tri-Reagent (Sigma-Aldrich). Glycogen (Roche) was added as a carrier for RNA isolation at a concentration of 2 μg/ml. cDNA synthesis was performed applying the reverse primer (5′-ACATGGCGAAGTGAGCGGAGC-3′) and primers (MWG) specific for each of the human Vβ families and a specific primer for the C region of the β-chain (labeled with the fluorescent dye marker 6-FAM) as described previously (18). After an initial incubation at 95°C for 15 min, optimal cycling conditions were 95°C for 1 min, 58°C for 1 min, and 72°C for 1 min for 34 cycles, followed by a final extension period at 72°C for 20 min. For analysis of CD25 mRNA expression, additional RNA from PBMCs, stimulated with PHA for 48 h, was used for control purpose. PCR amplification was performed using a CD25 forward primer (5′-GAG AAA GAC CTC CGC TTC ACC-3′) and reverse primer (5′-CGA GTG GCT AGT GTT TTC TG-3′; MWG) (19) and 31 cycles. CDR3 spectratyping was performed with some modifications as previously described (20, 21). An aliquot of the PCR product was diluted in 16 μl of deionized formamide and 1.2 fmol of internal lane standard GeneScan-350 TAMRA (Applied Biosystems) with the fluorescent dye marker 6-FAM as described previously (20, 21). Analysis of the PCR products was performed using a capillary electrophoresis system (Gene Scan-350 TAMRA) in a capillary (36 cm) and a separation buffer consisting of 89% formamide, 10 mM Tris, 2.5 mM EDTA, and 0.5% Genescan-350 TAMRA (Applied Biosystems). The fluorescent products were analyzed using a software program (Applied Biosystems) with the Local Southern method for fragment size estimation. In the case of a normal distribution of individual clones within the Vβ family, a Gaussian profile was depicted. The appearance of a dominant peak suggested the presence of an oligoclonal or clonal T cell population. The occurrence of dominant clonal expansion within the different Vβ families was quantified using a diversity score between 1 and 3 (20): 1 was assigned to a Gaussian distribution, 2 corresponded to a pattern with one to three peaks above the Gaussian background, and 3 corresponded to one predominant peak above the Gaussian distribution.

Sequenceing and molecular tracking of TCR Vβ clones
cDNA of purified CD8+ CD45RO+ CD25− T cells was used to amplify the Vβ3 and Vβ16 regions including a single predominant peak. For ease of identification into a sequencing vector, the Vβ3 and Vβ16 primers were synthesized with EcoRI restriction sites at their 5′ terminus. Hence, the purified PCR products were digested with EcoRI before subcloning into pBS-SK−. Sequence analyses were performed by MWG, and adequate primers were identified.
detecting the predominant clone within TCR Vβ3 (5'-CAG TTG TGG GGG GGC CAC-3') and Vβ16 (5'-CAG CAG CCA AGA GGG AGA ACA GTA C-3') were chosen using the ClustalW program ((www.ebi.ac.uk/ clustalw/)) (22, 23). To determine whether these predominant clones also occur in CD25+ T cells, RNA and cDNA preparations of purified CD8+CD45RO+CD25+ were performed. A first round of PCR using the same conditions as those described above was performed before amplification using the two clonotype-specific primers. The amplification products were analyzed by 2.5% agarose gel electrophoresis.

Analysis of signal-joint TCR rearrangement excision circles (sjTRECs)

DNA isolation of purified CD8+CD45RO+CD25+ and CD8+CD45RO+CD25− T cells was performed using the Puregene DNA Purification Kit (Genta Systems) as previously described (24). The sjTRECs of CD8+CD45RO+CD25+ and CD8+CD45RO+CD25− T cells were amplified and directly quantified using Light Cycler (Roche) using known starting numbers of standard sjTREC molecules. Then real-time PCR was performed using Quatitect SYBR Green (Qiagen) as previously described (25). Briefly, 100 ng of DNA were added to a mix of 1× Quatitect SYBR Green PCR Master Mix, 200 ng/ml BSA, 1.5 mM MgCl2, 0.5 mM dNTPs, 1× SYBR Green (Qiagen) as previously described (25). Briefly, 100 ng of DNA were added to a mix of 1× Quatitect SYBR Green PCR Master Mix, 200 ng/ml BSA, 1.5 mM MgCl2, 0.5 mM dNTPs, and SYBR Green (Qiagen) as previously described (25).

Telomere length analysis by flow cytometric fluorescence in situ hybridization (flow FISH)

The telomere lengths of CD8+CD45RO+CD25+ and CD8+CD45RO+CD25− T cells were determined using flow FISH with a fluorescent-labeled peptide nucleic acid (PNA) telomere probe. The lymphoblastic leukemia T cell line 1301, which has unusually long telomeres and is tetraploid (26), was used as a standard for each telomere length measurement (27). The lymphocytes were fixed and permeabilized using the standard procedure with the Cytotox/Cytoperm kit (BD Biosciences). Three milliliters of the Cy5-labeled PNA telomere probe (Applied Biosystems) or an equivalent quantity of correspondingly labeled control Ab (BD Pharmingen) was added to hybridization buffer. The cells were incubated in a hybridization buffer containing 70% formamide at 82°C for 10 min, then snap-cooled on ice and incubated at room temperature for 90 min. After three rounds of washing in a posthybridization buffer and three rounds in PBS, the fluorescence of the Cy5-labeled PNA telomere probe was analyzed for the two T cell subsets on a FACSCalibur flow cytometer. Together with the fluorescence of the lymphoblastic leukemia cell line 1301, the individual telomere length was calculated.

Statistical analysis

An independent-samples t test was performed to compare CD8+CD45RO+CD25+ and CD8+CD45RO+CD25− T cells using SPSS software for Windows version 11.5. A value of p < 0.05 was considered statistically significant.

Results

CD8+CD25+ T cells have an early memory surface phenotype and can coexpress CD4

As a first step we analyzed CD25 mRNA expression in CD8+CD45RO+CD25+ and CD8+CD45RO+CD25− T cells after cell sorting, as described in Materials and Methods (Fig. IA). CD8+CD25+ T cells displayed a constitutive expression of CD25 mRNA, whereas CD8−CD25− cells failed to do so (Fig. IB). CD25 mRNA analysis was performed using PCR and CD25-specific primers (15). Surface phenotype analysis of CD25+ and CD25− T cells by four-color flow cytometric analysis additionally revealed that both subpopulations were Ag-experienced T cells, because they expressed CD45RO, CD44high, and CD95 (Fig. 1D). CD25+ T cells also expressed the lymph node-homing markers CD62L and CCR7, thus displaying a central-memory-like phenotype, whereas CD25− T cells were CCR7− and had mostly a low expression of CD62L. In contrast to CD25− cells, a major fraction of CD25+ cells expressed CRTH2, indicating a Tc2 phenotype. A substantial proportion of CD8+CD25+ cells also coexpressed the CD4 molecule. These cells truly expressed a CD4+CD8− double-positive phenotype, because there were no contaminating CD4+CD8− T cells (Fig. 1C). Because CD25 and CD4 surface molecules are thought to be increased only on activated CD8+ T cells, we determined additional activation markers. However, CD8+CD25+ T cells did not express the early activation marker CD69, the chronic activation marker HLA-DR, or the late differentiation marker CD57 (Fig. 1D). They were also negative for receptors such as CD56, CD16, CD94, NKB1, CD158a/h, and CD158b/j and were thus not NK T cells.

CD8+CD25+ cells are not gut-derived and have a shorter replicative history than their CD8+CD25− counterparts

To exclude that the two memory CD8+ populations were gut-derived, we analyzed the expression of CD8αβ and the occurrence of sjTRECs in sorted CD8+CD45RO+CD25+ and CD8+CD45RO−CD25− T cells. Double staining of CD8+CD25+ and CD8+CD25− T cells with mAbs against the α and β CD8 receptor chains revealed that all cells within each subpopulation were αβ double-positive (Fig. 2A). None of the cells had a CD8αα phenotype, which would have
thymus derived (data not shown). The sjTREC concentrations varied from person to person, but were generally relatively low. The number of sjTRECs in CD25+ T cells was, on the average, still 3.5 times higher than that in CD25− cells, suggesting a shorter replicative history of this specific T cell subset. This concept was also supported by the telomere length analysis of sorted CD8+CD45RO−CD25− and CD8+CD45RO+CD25+ T cells using flow FISH technology. In all subjects studied, CD25+ T cells had longer telomeres than CD25− T cells (Fig. 2B).

Upon PHA stimulation, CD8+CD25+ T cells proliferate and partially down-regulate CD62L, but retain their HLA-DR−CD4+ phenotype

Having established that CD8+CD25+ T cells were thymus derived and had a shorter replicative history than their CD25− counterparts, we next aimed at determining their differentiation and proliferation profile upon stimulation. Cell proliferation tracking of purified CD8+CD45RO−CD25− and CD8+CD45RO+CD25+ T cells was performed using the fluorescent dye CFSE, which penetrates cell membranes and couples to proteins resulting in stable, long-term intracellular retention. CFSE segregates equally between daughter cells upon cell division, resulting in a 2-fold decrement in cellular fluorescence intensity. Upon stimulation with PHA, both subsets divided up to five times and retained their CD45RO (data not shown) and CD28 (Fig. 3) phenotypes. Sixty-five percent of the CD25+ T cells down-regulated their initially high CD62L expression, but did not become HLA-DR as 13% of the CD25− T cells did. CD8 single-positive CD25+ as well as CD25− T cells did not up-regulate the CD4 molecule upon stimulation, whereas CD8+CD4+CD25+ T cells maintained their CD4+CD8+ double-positive phenotype and proliferated as well as CD8 single-positive cells.

FIGURE 2. CD8+CD25+ T cells are not gut-derived and have longer telomeres than CD8+CD25− T cells. CD8+CD45RO−CD25+ and CD8+CD45RO+CD25+ T cells were isolated from persons with >15% CD25-expressing CD8+ T cells, who characteristically have a good humoral immune response after influenza vaccination. A, Double staining was performed using mAbs against the α and β CD8 receptor chains. All cells within each subpopulation were αβ double positive. One of eight experiments for CD8+CD45RO−CD25+ cells is shown. B, Telomere length was determined for the sorted subpopulations using a fluorescent peptide nucleic acid probe and flow FISH as described in Materials and Methods. The resulting fluorescent signal (gray curves) was plotted on a logarithmic scale against the signal from the leukemia T cell line 1301 (empty curves). Telomere length was calculated against the known telomere length of this cell line. One of three experiments performed is shown.

FIGURE 3. Proliferation and differentiation of CFSE-labeled CD8+CD25+ and CD8+CD25− T cells. Purified CD8+CD45RO−CD25+ (A and C) and CD8+CD45RO+CD25+ T cells (B and D) from elderly persons with >15% CD25-expressing CD8+ T cells, who characteristically have a good humoral immune response after influenza vaccination, were labeled using the fluorescent dye CFSE and analyzed before (A and B) and after 4 days of stimulation with PHA (C and D) with the aid of a FACSCalibur. Cells were costained using mAbs against CD4, CD28, CD62L, and HLA-DR. Numbers in the graphs indicate the percentage for the respective quadrant. One of three experiments is shown.
CD8⁺CD25⁺ T cells proliferate better upon stimulation with IL-2 or alloantigen than their CD25⁻ counterparts

We also investigated the proliferative capacity of purified CD8⁺CD45RO⁺CD25⁺ and CD8⁺CD45RO⁺CD25⁻ cells upon stimulation with IL-2, alloantigen, OKT3, or OKT3 in combination with IL-2 as described in Materials and Methods. After 1 wk of culture, [³H]thymidine incorporation was measured. The results are expressed as the mean cpm ± SD from three independent experiments. *p < 0.05, CD25⁺ vs CD25⁻ cells. B, On the last day of culture, a photograph of CD25⁺ and CD25⁻ cells was taken to demonstrate the different growth characteristics after stimulation with OKT3 and IL-2.

CD8⁺CD25⁺ T cells recognize a panel of different Ags. Isolated CD8⁺CD45RO⁺CD25⁺ and CD8⁺CD45RO⁺CD25⁻ cells from HLA-A2-positive elderly persons with >15% CD25-expressing CD8⁺ T cells who characteristically have a good humoral immune response after influenza vaccination were stimulated with immunodominant peptides derived from HBV, influenza virus, EBV, or CMV (2 μg/ml) in the presence of IL-2 (20 ng/ml). Irradiated autologous PBMCs were used as APCs. After 1 wk, the cells were stained for CD8, and the frequency of Ag-specific cells was analyzed using tetramer staining. A, The graph shows a representative FACS profile. Numbers in the graphs indicate the percentages of Ag-specific cells after culture. B, CD25⁺ and CD25⁻ T cell responses to antigenic peptide stimulation in different subjects. Three experiments were performed for each Ag. Results from subjects who did not have a response to the specific Ag were not included in the graphs.
CD8+CD25+ T cells recognize a panel of different Ags

To obtain additional information about the Ag reactivity of CD8+CD25+ T cells, we compared the propagation of cells of different Ag specificities in CD8+CD45RO+CD25+ and CD8+CD45RO+CD25− T cells by stimulating the two subpopulations with a panel of antigenic peptides (Fig. 5). The HBV core protein-derived peptide FLPSDFFPESP induced a weak response in CD25+ and no response in CD25− cells. Stimulation of CD25+ cells with the influenza virus matrix protein M1-derived peptide FLU M158–66 led to the propagation of a relatively large number of Ag-specific cells. In contrast, only a few M158–66 peptide-specific cells were found when the CD25+ population was stimulated with the M158–66 peptide. Stimulation with immunodominant peptides from EBV (LMP2) and CMV (pp65) led to the propagation of different Ag specificities in CD8+ T cells, we compared the propagation of cells of respective Ag specificities in both subpopulations.

CD8+CD25+ T cells have a highly diverse TCR repertoire

To analyze the clonal composition of the CD8+CD45RO+CD25+ and CD8+CD45RO−CD25− T cell subsets, we used immuno-scope technology. TCR CDR3 spectratyping demonstrated that the CD25+ population was mostly polyclonal, whereas the CD25− subset had a more restricted clonality (Fig. 6A). Peaks of a certain size, which were dominant in the CD25− population, could frequently also be identified as slightly enlarged in the CD25+ fraction. In the CD25+ population, 83 ± 5% of the amplified Vβ families corresponded to a diversity score of 1, 2, or 3 was assessed in CD25+ B of three identical experiments. In the CD25− population, 83% CD25+ expressing CD8+ T cells, who characteristically have a good humoral immune response after influenza vaccination, were studied. Eight randomly chosen Vβ families are shown. The graph represents one of three identical experiments. B, Deviations from the Gaussian profile were classified using a diversity score (of 1–3). The number of Vβ families corresponding to a diversity score of 1, 2, or 3 was assessed in CD25+ and CD25− cells in three different individuals. In each person the number of clones of one diversity type was expressed as a percentage of all 24 Vβ families (100%). The bars represent mean values of the percentage of families with a certain diversity score in three subjects. CD25+ and CD25− T cells are compared.

FIGURE 6. CD8+CD25+ T cells have a highly diverse repertoire. A, The clonal composition of the 24 Vβ families was analyzed by CDR3 spectratyping in isolated CD8+CD45RO+CD25+ and CD8+CD45RO−CD25− T cells. Only persons with >15% CD25+expressing CD8+ T cells, who characteristically have a good humoral immune response after influenza vaccination, were studied. Eight randomly chosen Vβ families are shown. The graph represents one of three identical experiments. B, Deviations from the Gaussian profile were classified using a diversity score (of 1–3). The number of Vβ families corresponding to a diversity score of 1, 2, or 3 was assessed in CD25+ and CD25− cells in three different individuals. In each person the number of clones of one diversity type was expressed as a percentage of all 24 Vβ families (100%). The bars represent mean values of the percentage of families with a certain diversity score in three subjects. CD25+ and CD25− T cells are compared.

FIGURE 7. The same clones that dominate the CD8+CD25+ T cell repertoire also occur in the CD8+CD25+ T cell population. A, Having analyzed the clonal composition of CD8+CD45RO+CD25− and CD8+CD45RO−CD25− T cells by CDR3 spectratyping (Fig. 6), we performed molecular tracking of dominant clones. We therefore chose two Vβ regions, displaying a dominant clone within the CD25− population, and performed sequence analysis to ascertain the identities of these dominant clones and to create adequate primers. Arrows indicate clones with the same CDR3 length, which occur in the CD25+ and CD25− populations. B, Clone Vβ3− and clone Vβ16−specific primers were then used to amplify the cDNA of CD25+ and CD25− T cells. PCR products were analyzed by 2.5% agarose gel electrophoresis.
families had a Gaussian or close to Gaussian CDR3 size distribution pattern (diversity score, 1), 14 ± 6% had minor deviations, and 3 ± 2% had pronounced deviations from the Gaussian profile (Fig. 6B). In contrast, a Gaussian profile was found in only 24 ± 8% of Vβ families in the CD25+ population, whereas 42 ± 3% displayed minor deviations from the Gaussian profile (diversity score, 2), and 34 ± 10% were dominated by a single dominant peak (diversity score, 3).

The same clones that dominate the CD8+ CD25− T cell repertoire also occur in the CD8+ CD25+ T cell population

Using immunoscope technology, peaks with the same CDR3 length were found in both CD8+ CD25+ and CD8+ CD25− cells (indicated by arrows in Fig. 7A), but within some Vβ families one such clone dominated only the CD8+ CD25+ population, whereas the CD8+ CD25+ population displayed a polyclonal repertoire. We therefore wanted to ascertain the identity of these dominant clones by extracting the RNA of isolated CD8+ CD45RO+ CD25− T cells from a donor previously shown to exhibit such dominant clones within the Vβ3 and Vβ16 regions (Fig. 7A). Sequencing of these two TCR Vβ regions including the dominant clones was performed as described in Materials and Methods. Our results revealed that each of the two single peaks analyzed contained only one T cell clone. Using clonotype-specific primers, these clones were also detected in CD8+ CD25+ T cells (Fig. 7B). The results of molecular tracking of dominant clones demonstrate a lineage relationship between CD25− and CD25+ memory CD8+ T cells. In view of the fact that CD25+ T cells have a larger TCR repertoire and a shorter replicative history, we propose a differentiation pathway from CD25− to CD25+ memory CD8+ T cells as they prolife-rate.

Discussion

We recently reported that IL-4-producing CD8+ T cells with a CD25+ memory phenotype accumulate in a subgroup of healthy elderly persons who still have an intact humoral immune response after influenza vaccination (17). These apparently beneficial CD8+ CD25+ T cells are rare or even absent in elderly persons with latent CMV infection, who characteristically have high percentages of CD8+ CD28− effector cells (15, 29–31). CD8+ CD25+ T cells are also rare in young individuals who still have high numbers of naive T cells (15, 17). This indicates that shrinkage of the naive T cell population could increase the available space and facilitate nonspecific memory T cell turnover (29), resulting in a larger CD8+ CD25− memory T cell population in elderly adults (15, 17). However, a change in the composition of the CD8+ T cell pool toward cells of a higher differentiation stage in the elderly (14, 32, 33), as observed in patients with latent CMV infection, negatively correlates with the occurrence of CD8+ CD25+ cells (15). CD8+ CD28− T cells that expand in response to persistent antigenic challenge thus seem to have a survival advantage over CD25+ memory T cells (34, 35), indicating loss of diversity and loss of intact memory in the presence of chronic antigenic stimulation.

The analysis of CD8+ CD25+ and CD8+ CD25− memory T cells in the present study provides novel insights into TCR repertoire diversity, replicative history, and lineage relationship of CD8+ T cells in old age. CD8+ CD25− T cells display a central memory-like phenotype, as shown by their expression of CD45RO, CD28, and the lymph node-homing markers CD62L and CCR7. These cells display a constitutive expression of the IL-2R α-chain (CD25) and CD25 mRNA. They have not been recently activated, because they do not express CD69 and HLA-DR. Apart from regulatory T cells, CD25 expression has been detected on human CD4+ CRTH2+ cells (36), CD25 has also been reported to be expressed at higher levels on Th2 than on Th1 cells upon stimulation (37). Moreover, IL-2, apart from IL-4, plays a central role in Th2 differentiation and has been shown to stabilize the accessibility of the Il4 gene (38). Thus, constitutive expression of the high affinity IL-2R on memory Tc2 cells may have a functional assignment in the maintenance of type 2 cells and seems not to be exclusively expressed on regulatory T cells. Furthermore, CD8+ CD25+ T cells grow well after stimulation with IL-2 only. This may allow them to survive and divide in a bystander manner in vivo, even in the absence of their specific Ag. IL-7 and IL-15 could play additional roles in this process (34) as could IL-4 and IL-9 (39). Turnover in response to nonspecific stimuli allows CD8+ CD25− cells to persist for extended periods and ensures the maintenance of a diverse memory T cell pool in old age. Due to their IL-4 production, CD8+ CD25+ cells can prevent loss of the CD28 molecule (40), assist memory cell generation (41), induce MHC class II up-regulation on Ag-presenting B cells, and promote Ab isotype switching to IgG1 and IgE (42).

Phenotypic characterization revealed that up to 20% of the CD8+ CD25+ T cell population coexpressed the CD4 molecule. The origin, function, and role of circulating CD4+ CD8− T cells are still a matter of debate. However, the expression of the CD8αβ molecule suggests that CD4+ CD8− double-positive T cells were not derived from the gut, but were most likely thymus derived. Performing cell proliferation tracking using the fluorescent dye CFSE, we demonstrated that purified memory CD8+ T cells do not acquire CD4 coexpression upon stimulation with PHA (Fig. 3) or OKT3 and IL-2 (unpublished observation), but maintain their double-positive phenotype within the CD8+ CD25+ population. It is therefore likely that peripheral double-positive cells represent a distinct memory T cell population that increases with age (43–45). Due to the coexpression of CD4, double-positive cells can enhance the interaction with APCs by serving as an adhesion and a co-stimulatory molecule, interacting with MHC class II (46).

We also demonstrate that the TCR repertoire of CD8+ CD25+ memory cells is highly diverse, whereas the CD8+ CD25− subset has a more restricted clonality. This is in agreement with previous results showing that CD8+ central memory cells have a greater TCR diversity than CD8+ effector memory cells (47). Together with telomere length analysis, our results demonstrate a shorter replicative history for CD8+ CD25+ T cells. This has also been shown with respect to Ag specificity, because CD8+ CD25+ T cells contain greater numbers of different specificities, which proliferate rapidly upon Ag encounter (Fig. 5). Our results suggest that continuous differentiation of CD8+ T cells throughout life leads to the loss of certain Ag specificities, but to the unproportional accumulation of others, for instance for persistent Ags, and appears to be associated with the inability to raise a sufficient cellular immune response to pathogens such as influenza (11, 17). Moreover, it seems likely that the relatively oligoclonal CD8+ CD45RO+ CD25− cell population contains precursors of the relatively nonresponsive effector cells described by many groups (7, 48). Studies presently being performed in our laboratory are aimed at elucidating this possibility.

We also performed molecular tracking of clones, a technique used for the identification of putatively pathogenetic T cell clones in aplastic anemia (23) or rheumatoid arthritis (49) and to monitor the persistence and localization of adoptively transferred T cells in tumor immunotherapy (50). We used this method to ascertain that clones dominating certain TCR Vβ families within the CD8+ CD25+ population also occur in the CD8+ CD25− subset. The data shown in Fig. 7 are in accord with our results achieved by CDR3 spectratyping and telomere length analysis and...
enabled us to propose a lineage relationship from CD25+ to CD25- memory CD8+ T cells.

New vaccination approaches aimed at supporting the long-term survival of immune-competent CD8+CD25+ memory T cells should be considered, because they would have multiple beneficial effects for the elderly population. Booster immunization approaches for elderly persons could, for instance, take advantage of recent progress in DNA vaccine research. By inducing both humoral and cellular immune responses, cytokine DNA, especially IL-2, but also IL-7 or IL-15, could be used as an adjuvant specifically targeting CD8+CD25+ memory T cells and thus augmenting immunity even in subjects with a low CD8+CD25- T cell frequency.

In conclusion, our results suggest that CD25-expressing CD8+ T cells represent a memory T cell reservoir of great diversity in old age. We propose that CD8+CD25+ cells represent an early stage in the differentiation of CD8+ T cells and demonstrate a lineage relationship from CD25+ to CD25- memory CD8+ T cells. Loss of the physiologically occurring CD8+CD25+ memory population due to the accumulation of highly differentiated CD8+CD28- effector cells should, however, be prevented by immunotherapeutic measures, because CD25-expressing memory CD8+ T cells appear to be a prerequisite for intact immune responsiveness in the absence of naive T cells in old age.

Disclosures

The authors have no financial conflict of interest.

References

susceptible than Th1 cells to the suppressive activity of CD25+ regulatory thymocytes because of their responsiveness to different cytokines. Blood 103: 3117–3121.


