Extensive Amplification of Human Regulatory T Cells Alters Their Functional Capacities and Targets Them to the Periphery

Gunter Rappl,¹ Annette Schmidt,² Cornelia Mauch,^{3,4} Andreas A. Hombach,¹ and Hinrich Abken^{1,4}

Abstract

Repetitive antigen encounters together with a strong CD28 co-stimulatory signal were recently identified as driving extensive amplification of human regulatory T (Treg) cells; however, the consequences of this on the functional capacities of Treg cells remain unknown. In this report, we reveal that T cell receptor (TCR)/CD28-triggered amplification *in vitro* converts CD4⁺CD25^{high}FoxP3⁺ Treg cells into a late memory phenotype associated with immunosenescence and loss of CD7. Accordingly, *ex vivo*-isolated CD7⁺ Treg cells have shortened telomeres and decreased telomerase expression compared to the majority of “mature” CD7⁺ Treg cells. Although they resist spontaneous apoptosis, amplified CD7⁺ Treg cells exhibit increased sensitivity to activation-induced cell death (AICD). Extensive amplification of Treg cells is, moreover, accompanied by an increased activation threshold, reduced suppressor capacities, and interleukin-10 (IL-10) secretion, but secretion of high amounts of IL-4. Concomitantly, amplified Treg cells express homing receptors targeting them to the periphery. This is confirmed *in vivo* by the extensive accumulation of CD7⁺ Treg cells with shortened telomeres in chronic inflammatory skin lesions, including atopic dermatitis and lichen ruber. Our data indicate that extensive amplification upon repetitive TCR/CD28 engagement alters the functional capacities of CD4⁺CD25^{high} Treg cells toward less suppressive cells, but potential mediators in sustaining inflammatory reactions through IL-4.

Introduction

REGULATORY (TREG) CELLS ARE KEY PLAYERS in the establishment and maintenance of peripheral tolerance.¹ Upon differentiation in the thymus, CD4⁺CD25^{high} Treg cells are exported in a naïve-like status into the circulation where they execute their suppressive functions, i.e., inhibition of extensive polyclonal T cell activation and control of self-reactive T cells that have escaped thymic negative selection. Alternatively, Treg cells can be generated in the periphery by conversion of nonregulatory naïve and memory T cells.² Treg cells enter terminal differentiation and proliferation upon long-lasting, repetitive stimulation, which was most recently identified to be delivered by T cell receptor (TCR) engagement plus a strong CD28 co-stimulatory signal.³ The functional capacities of Treg cells in an advanced proliferative stage, in terminal differentiation or in immunosenescence, however, remain to be elucidated.

On the other hand, several protocols for amplifying human Treg cells *in vitro* have been reported.^{4–6} Assuming that the functional capacities of Treg cells are adequately conserved upon extensive proliferation, *ex vivo*-amplified Treg cells are expected to be of therapeutic value upon adoptive transfer. This will particularly be the case when absence or dysfunction of Treg cells leads to immune-mediated pathologies, including autoimmunity, allergies, and graft rejection.^{6–8} Restoration of Treg cell numbers and/or function are expected to ameliorate disease, and there is great hope that *ex vivo*-amplified Treg cells upon adoptive transfer will execute their suppressor functions as do mature, freshly isolated Treg cells.⁹ Again, the consequences of extensive amplification on the functional capacities of Treg cells have so far not been explored.

One of the earliest markers in T cell ontogeny is CD7, a glycosaminoglycan, which is expressed on the majority of peripheral blood T cells, natural killer (NK) cells, and all thy-

¹Tumorigenetics, Department of Internal Medicine I, University of Cologne, Cologne, Germany.

²Institute for Circulation Research and Sports Medicine, German Sports University, Cologne, Germany.

³Tumorbiology, Department of Dermatology, University of Cologne, Cologne, Germany.

⁴Center for Molecular Medicine Cologne (CMMC), University of Cologne, Cologne, Germany.

mocytes. CD7, hence, has been widely used as a marker for postthymic T cells and for the diagnosis of malignancies in the early T cell development.¹⁰ Previously, we reported that lack of CD7 expression represents a stable phenotype of CD4⁺CD7⁻ T cell clones during long-term propagation for several months *in vitro*.¹¹ Because the majority of CD4⁺ T cells that lack CD7 expression exhibit the CD45RO⁺CD45RA⁻ memory phenotype, lack of CD7 expression may reflect a differentiation state associated with the late immune response.¹¹

Using loss of CD7 as a surrogate marker, we reveal here that prolonged TCR-triggered amplification of human Treg cells results in altered functional capacities, including reduced suppressor activities, entry into immunosenescence, and expression of homing receptors targeting them to the periphery. Accordingly, we found extensively amplified Treg cells accumulated in chronic inflammatory lesions in the peripheral organs *in vivo* where repetitive antigen encounter occurs. In addition to reduced suppressor capacities, it is interesting that proliferative advanced Treg cells secrete high amounts of IL-4, which converts them to potential mediators in sustaining chronic inflammatory reactions.

Materials and Methods

Human subjects

Healthy young blood donors were recruited for this study ($n = 30$; mean 30 ± 10 years), and buffy coats were obtained from the University of Cologne blood bank (Cologne, Germany). Subjects who were taking immunosuppressive drugs or who had a disease potentially affecting the immune system were excluded. All blood samples were taken with informed consent. The work was approved by the institutional review committee of the University of Cologne. The frequency of CD7⁻ T cells was the same in both sexes of the blood donors.

Antibodies, cytokines, and flow cytometric analyses

Fluorochrome-conjugated monoclonal antibodies were used according to the manufacturer's recommendations. OKT3 (ATCC CRL 8001) is a hybridoma cell line that produces the anti-CD3 monoclonal antibody (mAb) OKT3; 15E8 hybridoma cells¹² produce the anti-CD28 mAb 15E8. To detect surface and intracellular cytotoxic T lymphocyte antigen-4 (CTLA-4) expression, cells were stained with the phycoerythrin (PE)-conjugated anti-CTLA-4 mAb BNI3 (BD Biosciences, Heidelberg, Germany) as described.¹³ To analyze intracellular interleukin-2 (IL-2) or IL-4 expression upon stimulation, cells were activated, fixed, and permeabilized as described¹³ and stained with the PE-conjugated anti-IL-2 mAb MQ1-17H12 (Biolegend, Eching, Germany) or anti-IL-4 mAb 8D4-8 (Biolegend). To monitor killer cell lectin-like receptor G1 (KLRG-1) expression, cells were stained with Alexa-488-conjugated mouse anti-human KLRG-1 mAb clone 13A2 (kindly provided by H.P. Pircher, Institute of Immunology, Freiburg, Germany). For galectin-10 detection, cells were stained with the mouse anti-human galectin-10 mAb B-F42 (Diacclone, Cologne, Germany) as described.¹⁴ To monitor FoxP3 expression, cells were stained with the anti-human FoxP3 mAb 150D/E4 (kindly provided by Dr. A. Banham, Oxford, UK) as described.¹⁵ Alternatively, cells were

stained with the allophycocyanin (APC)-conjugated anti-FoxP3 mAb 3G3 (Miltenyi Biotec, Bergisch Gladbach, Germany). Cells were analyzed by flow cytometry using fluorescence-activated cell sorting (FACS; FACS Calibur or a FACS Canto flow cytometer, BD Biosciences). Viable lymphocytes were gated on the basis of forward/side scatter and negative 7-amino-actinomycin D (7-AAD) staining. Human recombinant (r) IL-2, IL-4, IL-10, IL-15, tumor growth factor- β 1 (TGF- β 1), and interferon- γ (IFN- γ) were purchased from R&D Systems (Wiesbaden, Germany).

Preparative isolation of CD4⁺ T cell subpopulations

Peripheral blood mononuclear cells (PBMCs) were isolated from healthy donors after informed consent. CD4⁺CD45RO⁺ T cells were obtained by negative depletion during magnetic cell sorting (MACS) procedures using the CD4⁺ memory T cell isolation kit (Miltenyi Biotec). Isolated memory T cells were separated into CD7⁺ and CD7⁻ subpopulations by an additional MACS separation step using the PE-conjugated anti-CD7 mAb CD7-6B7 (CALTAG, Hamburg, Germany) and anti-PE multisort beads (Miltenyi Biotec). After microbead removal using a "multisort-release reagent" (Miltenyi Biotec), CD7⁻ and CD7⁺ T cells were further separated according to their CD25 expression using anti-CD25 microbeads (Miltenyi Biotec).

Because some studies report problems with the regulatory nature of MACS-sorted Treg cells, e.g., that they are anergic, but not suppressive,¹⁶ we isolated by two alternative procedures: CD4⁺CD25^{high} T cells by FACS sorting. CD4⁺CD45RO⁺ cells were isolated from PBMCs by negative depletion and in a second step according to their CD25 expression by MACS techniques using the 'human regulatory T cell isolation kit' and anti-CD45RA microbeads (Miltenyi Biotec). Isolated CD25^{high} and CD25⁻ T cells were stained with the PE-Alexa700-conjugated anti-CD7 mAb CD7-6B7 (CALTAG) and separated into CD7⁺ and CD7⁻ subpopulations using a FACS Aria cell sorter (BD Biosciences).

Alternatively, CD4⁺ T cells with high CD25 expression (CD4⁺CD25^{high}) were isolated by MACS combined with FACS sorting, as reported previously.¹⁶ CD4⁺CD45RO⁺ T cells were obtained by negative depletion by MACS procedures using the CD4⁺ memory T cell isolation kit (Miltenyi Biotec). Isolated memory T cells were separated into CD7⁺ and CD7⁻ subpopulations by additional MACS separation using the fluorescein isothiocyanate (FITC)-conjugated anti-CD7 mAb 4H9 (BD Biosciences) and anti-FITC microbeads (Miltenyi Biotec). Half of the isolated CD7⁺ and CD7⁻ T cells were stained with the PE-conjugated anti-CD25 mAb M-A251 (BD Biosciences) and separated into CD25^{high} and CD25⁻ subpopulations using a FACS Aria cell sorter (BD Biosciences) or were incubated with 4 μ L of anti-CD25 beads per 10^7 cells for MACS isolation of CD25^{high} T cells.

The purity of MACS-sorted CD4⁺CD25⁺ and CD4⁺CD25⁻ T cells, as recorded by flow cytometry, was $98 \pm 1\%$ and $89 \pm 2\%$, respectively, and the purity of FACS-sorted cells was $>97\%$. A typical isolation procedure starting from 5×10^8 mononuclear cells from the peripheral blood resulted in 5×10^6 purified CD7⁺CD25⁺, 3×10^7 CD7⁺CD25⁻, 3×10^5 CD7⁻CD25⁺, and 8×10^6 CD7⁻CD25⁻ T cells. No significant difference in the yield of MACS- or FACS-sorted T cell subsets was found.

Cytokine detection

Cytokines in the culture medium were recorded by enzyme-linked immunosorbent assay (ELISA) using matched pairs of antibodies specific for IL-4 (clones 8D4-8 and MP4-25D2), IL-10 (clones JES3-9D7 and JES3-12G8), IFN- γ (clones NIB 42 and B133.5), and TGF- β 1 (clones A75-2 and A75-3) (all purchased from BD Biosciences). The detection limits of the assays were 15 pg/mL IL-4, 15 pg/mL IL-10, 15 pg/mL IFN- γ , and 10 pg/mL TGF- β 1, respectively.

Detection of apoptotic cells

Apoptotic cells were assessed by FACS analysis using the Annexin-V Apoptosis Detection Kit (BD Biosciences). Viable cells were detected as cells negative for annexin-V and 7-AAD staining.

Assay for suppressor activities

Analysis of suppressor activities of CD7⁺ Treg cells and the corresponding CD7⁺ Treg cells were performed as described recently.³ In brief, freshly isolated CD45RO⁺CD25⁺ T cells (1×10^7 cells/ml) were labeled with 5-carboxyfluorescein diacetate succinimidyl ester (CFSE) (1 μ M) (Molecular Probes, Göttingen, Germany) at room temperature for 3 min and washed three times in RPMI 1640 medium, 10% (vol/vol) fetal calf serum (FCS). CFSE-labeled CD45RO⁺CD25⁺ T cells (1×10^5 cells) were stimulated with the agonistic anti-CD3 mAb OKT3 (10 μ g/mL) plus the anti-CD28 mAb 15E8 (1 μ g/mL) in the presence of 1×10^5 CD45RO⁺CD25⁺ T cells or control cells, respectively. Cell proliferation was monitored by gating on viable cells and recording CFSE dilution after 6 days. In pilot experiments, CFSE-labeled cells in multiple rounds of proliferation were best detectable at day 6. The unstained cells were excluded by gating as described previously.¹⁷ Representative histograms are shown to demonstrate the validity of the assay. To calculate the percentage of cells that is inhibited in cell division, the following formula was used:

$$100 - [(\% \text{ CFSE-positive CD4}^+ \text{CD45RO}^+ \text{CD25}^- \text{ T cells in co-culture with CD4}^+ \text{CD45RO}^+ \text{CD25}^+ \text{ cells or CD4}^+ \text{CD45RO}^+ \text{CD25}^- \text{ cells}) / (\% \text{ CFSE-positive CD4}^+ \text{CD45RO}^+ \text{CD25}^- \text{ T cells only}) \times 100].$$

Proliferation assay

Cells (10^4 cells/100 μ L) were pulsed with bromodeoxyuridine (BrdU) for 6 hr and assayed for BrdU incorporation by ELISA using the Cell proliferation ELISA BrdU kit (Roche Diagnostics, Mannheim, Germany). BrdU incorporation directly correlates with cell proliferation.¹⁸

Telomere staining and analysis of telomerase expression

Telomere lengths were determined by fluorescence *in situ* hybridization (FISH) using the Telomere PNA Kit/FITC for flow cytometry (DAKO Cytomation, Hamburg, Germany) as described recently.¹⁹ Jurkat cells with a known telomere length of 10 ± 2 kb and 1301 CEM cells with telomere length of 30 ± 3 kb served as standards.^{20,21} Naive CD4⁺CD45RA⁺ T cells were isolated by MACS using the CD4⁺ Naive T Cell Isolation kit (Miltenyi Biotec). Cells were analyzed by flow

cytometry using a FACS Calibur (BD Biosciences) equipped with Cell Quest software. In biopsy sections, telomere lengths of individual cells were determined by quantitative fluorescence *in situ* hybridization (Q-FISH) using the Telomere PNA Kit/Cy3 for immunohistochemistry (DAKO Cytomation) as recently described.²² Multicolor immunofluorescence staining for CD4, CD7, and FoxP3 was performed as described. To record telomere-specific Cy3 fluorescence signals, regions of interest were gated around Cy3 spots and the mean fluorescence intensity of telomere spots (MFI/spot) was automatically quantified using the ImageJ software (U.S. National Institutes of Health). Telomerase expression was recorded by FACS analysis as recently described.^{19,23} Viable lymphocytes were gated based on forward/side scatter and negative 7-AAD staining.

Immunohistochemistry

Skin biopsies were snap-frozen in liquid nitrogen, processed for cryostat sections, and fixed with 4% (vol/vol) paraformaldehyde in phosphate-buffered saline (PBS) for 30 min at room temperature. Multicolor immunohistochemistry stainings were performed as described.²⁴ Sections were stained overnight in Tris-buffered saline (TBS), 0.8% (wt/vt) bovine serum albumin (BSA) with the mouse anti-CD4 mAb 4B12 (immunoglobulin G₁ [IgG₁]) (dilution 1:40) (Novocastra, Dossenheim, Germany), the mouse anti-CD7 mAb CBC.37 (IgG_{2b}) (dilution 1:50) (DAKO Cytomation), anti-CD25 mAb 4C9 (IgG_{2b}) (dilution 1:200) (Novocastra), polyclonal rabbit anti-human FoxP3 Ab ab10563 (dilution 1:800) (Abcam, Eching, Germany), and anti-CD127 mAb eBioRDR5 (IgG₁) (dilution 1:10) (eBiosciences, Frankfurt/Main, Germany), respectively. Slides were rinsed three times in TBS for 15 min. The following secondary antibodies were used: Cy3-conjugated goat anti-mouse IgG, Cy2-conjugated goat anti-mouse IgG2, Cy2-conjugated goat anti-mouse IgG, Cy5-conjugated goat anti-mouse IgG, and Cy5-conjugated goat anti-rabbit IgG (Jackson Immuno Research, West Grove, PA) in a 1:200 dilution for 1 hr at room temperature. Slides were rinsed three times in TBS for 15 min. For detection of multiple antigens, the slide was stained for the first antigen and blocked with TBS, 5% (wt/vol) BSA for 1 hr at room temperature before staining for the second antigen. Finally, slides were dehydrated, transferred into xylene, coverslipped with two drops of Entellan (ProSciTec, Thuringowa, Australia), and analyzed using the Zeiss Confocal Laser Scanning Microscope LSM 510 (Karl Zeiss, Oberkochen, Germany). Specificity of staining was assayed by incubation of the slides with the respective isotype-matched control antibodies.

Statistical analyses

Data represent the mean $\pm$ standard deviation (SD) of at least five independent experiments. Statistical analysis was performed using the two-tailed Student test or the paired *t*-test; *p* < 0.05 was considered significant.

Results

Extensive amplification of Treg cells generates CD7⁺ Treg cells with reduced suppressor capacities

We asked whether the phenotype and functional capacities of mature Treg cells are retained after extensive ampli-

FIG. 1. *(Continues)*

fication *in vitro*. CD4⁺CD45RO⁺CD7⁺FoxP3⁺CD25⁺ Treg cells were isolated from the peripheral blood to high purity. Their functional capacities were recorded by a CFSE-based suppression assay, which monitors up to five division cycles of CFSE-labeled CD4⁺CD25⁻ responder cells within 6 days. Addition of Treg cells efficiently suppressed proliferation, whereas addition of CD7⁺CD25⁻ T cells did not (Fig. 1A). Treg cells were induced to proliferate by CD3 and CD28 stimulation in the presence of IL-2, giving rise to about 10 cell doublings within 14 days. Proliferation was monitored by CFSE dilution of labeled mature CD7⁺ Treg cells (Fig. 1B). We used CD7 as additional marker to identify mature T cells, because T cells in late proliferative senescence lose CD7 expression.¹⁹ After 14 days of stimulation, about 5×10^5 CD7⁻CD25⁺ T cells were generated from 5×10^6 CD7⁺CD25⁺ Treg cells. Proliferation is required to generate CD7⁻ Treg cells because blocking cell division by mitomycin C prevented generation of CD7⁻ cells from CD7⁺ Treg cells. Both, CD7⁺ and CD7⁻ Treg cells retained FoxP3 and CD25^{high} expression upon amplification (Fig. 1C), although FoxP3 expression was lower in CD7⁻ compared to CD7⁺ Treg cells (MFI 120.41 vs. 298.14). Amplified CD7⁻ Treg cells suppressed proliferation of allogeneic CD4⁺CD45RO⁺CD25⁻ T cells with significantly lower capacities than amplified CD7⁺ Treg cells (Fig. 1D). Suppressor activity is specific because CD4⁺CD25⁻ T cells did not inhibit proliferation of the allogeneic responder cells. Taken together, extensive amplification of peripheral blood CD7⁺ Treg cells *in vitro* generates CD7⁻ Treg cells with reduced suppressor capacities.

CD7⁻ Treg cells in the peripheral blood represent late memory Treg cells

Flow cytometric analyses of Treg cells in the peripheral blood revealed that the CD4⁺CD25^{high}-FoxP3⁺ T cell population harbored a subset of $9.5\% \pm 7.5\%$ cells, which in contrast to the majority of T cells, did not express CD7 (Fig. 2A). Both the CD7⁺ and CD7⁻ subsets of CD4⁺CD45RO⁺CD25^{high} T cells expressed FoxP3, although in significantly lower levels in CD7⁻ Treg cells compared to CD7⁺ Treg cells, i.e., MFI 110.46 vs. 252.81. CD4⁺CD45RO⁺CD25⁻ T cells as control did not express FoxP3.

CD7⁻ and CD7⁺ cells of the CD4⁺CD45RO⁺CD25^{high} Treg cell population were isolated to high purities from the peripheral blood by two alternative sorting procedures. First, CD4⁺CD45RO⁺ T cells were isolated by negative magnetic sorting and CD7⁺ and CD7⁻ T cells subsequently separated by positive enrichment of CD7⁺ cells. CD25^{high} T cells in the respective subsets were finally isolated by positive enrichment resulting in highly purified CD7⁺ and CD7⁻ subset cells of the CD4⁺CD45RO⁺CD25^{high} T cell population (Fig. 2B). FoxP3 was expressed in more than 95% of these cells. The corresponding CD45RO⁺CD25⁻ T cells lack FoxP3 expression, whereas some T cells with intermediate CD25 expression expressed FoxP3 as well, which is in agreement with recently published data.¹⁵ By an alternative cell isolation procedure, we first enriched CD4⁺CD45RO⁺CD25^{high} T cells by magnetic cell sorting and subsequently isolated the CD7⁺ and CD7⁻ subpopulations by FACS, or enriched CD4⁺CD45RO⁺CD7⁺ and CD4⁺CD45RO⁺CD7⁻ T cells by magnetic cell sorting, followed by isolation of the corresponding CD25^{high} and CD25⁻ subpopulations by flow cy-

tometric sorting. In this study, both MACS- and FACS-based separation procedures were used to isolate CD4⁺CD25^{high}-FoxP3⁺ T cells and their subpopulations, and both isolation procedures generated Treg cell subpopulations with similar functional capacities, i.e., proliferation, suppressor activity, cytokine secretion, and apoptosis sensitivity.

Both CD7⁻ and CD7⁺ subset cells of the *ex vivo*-isolated CD4⁺CD45RO⁺CD25^{high} Treg cell population suppressed proliferation of autologous CD4⁺CD45RO⁺CD25⁻ T cells (Fig. 3A). CD7⁻ Treg cells, however, exhibited significantly lower suppressor capacities toward the same target cells than the corresponding CD7⁺ Treg cells. Titration analyses revealed that a suppressor to responder cell ratio of 1:1 resulted in maximal suppressor activities of both CD7⁺ and CD7⁻ Treg cells (Fig. 3B). The same ratio is known for mature Treg cells.¹³ Suppressor activity is specific because CD7⁺ and CD7⁻ cells of the CD4⁺CD45RO⁺CD25⁻ T cell population did not inhibit proliferation of the autologous CD4⁺CD45RO⁺CD25⁻ T cells. We conclude that both CD7⁺ and CD7⁻ cells of the CD4⁺CD45RO⁺CD25^{high} T cell population *in vivo* are regulatory T cells, CD7⁻ subset cells, however, with lower suppressor capacities.

To prove the hypothesis that CD7⁻ Treg cells *in vivo* may represent cells in a more advanced differentiation stage compared to the majority of CD7⁺ Treg cells, we recorded a panel of surface markers related to the late memory phenotype (Fig. 3C). KLRG-1, which is associated with late memory T cells,²⁵ and CD57 were expressed at higher levels on CD7⁻ compared to CD7⁺ Treg cells, whereas CD27 and CD28 were expressed at lower levels. Galectin-10, recently described to be upregulated in CD4⁺CD25^{high} T cells,¹⁴ was expressed in higher numbers, although at lower levels, in CD7⁻ than in CD7⁺ Treg cells. Glucocorticoid-induced tumor necrosis factor receptor (GITR), which is associated with the modulation of regulatory T cell function,²⁶ was expressed in more CD7⁻ cells than in CD7⁺ Treg cells, although at identical expression levels in both subsets. CD62L was expressed at significantly lower frequencies and at lower levels in CD7⁻ cells compared to the corresponding CD7⁺ Treg cells. The IL-7R α chain was expressed in nearly equal numbers of CD7⁻ and CD7⁺ Treg cells, but at slightly higher levels in CD7⁺ compared to CD7⁻ Treg cells. Surface CTLA-4 expression was not detectable either on CD7⁻ or on CD7⁺ cells of the Treg cell population, whereas intracellular CTLA-4 was expressed in nearly equal numbers of cells and in similar levels. Upon extensive amplification *in vitro*, CD4⁺CD7⁻ Treg cells showed essentially the same phenotype as *ex vivo*-isolated CD4⁺CD7⁻ Treg cells (Fig. 3C). Particularly, CD4⁺CD7⁻ Treg cells expressed higher levels of KLRG-1 and lower levels of CD28, CD27, and CD62L compared to the corresponding CD7⁺ Treg cells. Taken together, reduced expression levels of CD27, CD28, and CD62L, and elevated levels of CD57 and KLRG-1 indicate CD7⁻ subset cells of CD4⁺CD45RO⁺CD25^{high} Treg cells as late memory Treg cells.

Therefore, we asked whether CD7⁻ Treg cells *in vivo* are at a more advanced proliferative stage, and therefore we monitored the relative telomere lengths of CD7⁺ and CD7⁻ cells of the CD4⁺CD45RO⁺CD25^{high} T cell population and of naïve T cells of the same individual by flow-FISH hybridization. Jurkat and 1301 CEM cells with known telomere lengths served as standards (Fig. 3D). As summarized in Fig.

FIG. 3. (Continued). CD7⁺ Treg cells exhibit the late memory phenotype with shortened telomeres. (A) CD7⁺ and CD7⁺ subset cells of CD4⁺CD45RO⁺CD25⁺ and CD4⁺CD45RO⁺CD25⁺ T cells were isolated as described in Fig. 2B. CD45RO⁺CD25⁺ T cells were labeled with CFSE (1 μ M) and stimulated (10⁶ cells/100 μ L) with anti-CD3 mAb OKT3 (10 μ g/mL) plus anti-CD28 mAb 15E8 (1 μ g/mL) for 6 days in the presence of CD7⁺CD4⁺CD45RO⁺CD25^{high} and CD7⁺CD4⁺CD45RO⁺CD25^{high} T cells (10⁵ cells each), respectively. Reduction in proliferation was recorded by flow cytometry as described. Only viable lymphocytes were gated for the assays. Data represent the means $\pm$ standard error of means (SEM) of five independent experiments. Representative histograms from one out of five independent experiments are shown. (B) CD7⁺ and CD7⁺ Treg cells were coincubated with CFSE-labeled CD4⁺CD45RO⁺CD25⁺ T cells in decreasing suppressor to responder cell ratios (1:1, 1:5, and 1:10). Proliferation of viable CFSE-labeled cells was monitored by flow cytometry. Data represent the means $\pm$ standard error of means (SEM) of five independent experiments. Statistical analyses (A and B) were made using a paired *t*-test. (C) Isolated CD4⁺CD45RO⁺CD25⁺ T cells were simultaneously stained with antibodies specific for CD4, CD25, CD7 plus one of the indicated markers or with isotype-matched control antibodies, respectively. In a parallel assay, the same Treg cells were amplified *in vitro* for 14 days (cf. Fig. 1) and stained as well. Only viable cells were analyzed by flow cytometry and gated on CD7⁺ and CD7⁺ cells, respectively. The assays were performed with T cells from five individuals, and results are given as the mean of positive cells (%) and the mean fluorescence intensity (MFI) $\pm$ SEM. Statistical analysis was performed using a paired *t*-test. **p* < 0.05. CD7⁺ cells compared with the corresponding CD7⁺ T cells. (D) CD4⁺CD45RO⁺CD25⁺ T cells and naïve T cells were isolated from the peripheral blood of the same individual and hybridized to the FITC-labeled, telomere-specific peptide nucleic acid (PNA) probe. Mock hybridizations served as controls. Cells were subsequently stained for CD7 using the CBC.37 antibody and viable cells were analyzed by flow cytometry (PNA probe, bold line; thin line, mock control). Telomere lengths of Jurkat (10 $\pm$ 2 kb) and 1301 CEM cells (30 $\pm$ 3 kb) served as length standards in the assay. One representative experiment out of five is shown. (E) Telomere lengths were calculated as described in Materials and Methods. (F) Telomerase expression in CD7⁺ and CD7⁺ cells of isolated CD4⁺CD45RO⁺CD25⁺ T cells was monitored by staining with the goat anti-human hTERT L-20 antibody and the FITC-conjugated anti-CD7 mAb 8H8.1. Only viable cells were analyzed by flow cytometry, respectively. All assays (D–F) were performed five times and mean values $\pm$ SEM are shown. Statistical analyses were made using a paired *t*-test.

FIG. 4. (Continues)

FIG. 4. (Continued).

FIG. 4. (Continued). CD7⁻ Treg cells exhibit altered functional capacities. (A) Isolated CD7⁻ and CD7⁺ cells of the CD4⁺CD45RO⁺CD25^{high} T cell population and the corresponding CD4⁺CD45RO⁺CD25⁻ T cells were cultured (10⁵ cells/100 μ L) for 2 days without stimuli (open columns) or in presence of the plate-bound agonistic anti-CD3 mAb OKT3 (10 μ g/mL) plus soluble anti-CD28 mAb 15E8 (1 μ g/mL) (closed columns). IFN- γ , IL-4, and IL-10 in the culture supernatants were recorded by ELISA at day 2. (B) To determine the threshold in activation, isolated CD7⁻ and CD7⁺ cells of the CD4⁺CD45RO⁺CD25^{high} T cell population were cultured (10⁵ cells/100 μ L) for 2 days in presence of increasing concentrations of plate-bound anti-CD3 mAb OKT3 (0–1 μ g/mL) plus soluble anti-CD28 mAb 15E8 (1–10 μ g/mL). IL-10 in the culture supernatant was monitored by ELISA at day 2. (C–E) To record spontaneous and activation induced apoptosis, isolated CD7⁻ and CD7⁺ cells of the CD4⁺CD45RO⁺CD25^{high} T cell population and the corresponding CD4⁺CD45RO⁺CD25⁻ T cells were cultured (10⁵ cells/100 μ L) for 4 days in medium without supplements (i) (w/o) or with IL-2 (ii) or IL-15 (iii) (10 ng/ μ L each), or in presence of the anti-CD3 mAb OKT3 (10 μ g/mL) plus anti-CD28 mAb 15E8 (1 μ g/mL) without (iv) or with IL-2 (v) or IL-15 (vi) (10 ng/mL each). Cells were simultaneously stained for CD7, CD25, annexin-V, and 7-AAD and analyzed by flow cytometry by gating on the CD7⁻CD25⁺, CD7⁺CD25⁺, and CD7⁺CD25⁻ subsets, respectively. Viable cells were assessed to be negative for both 7-AAD and annexin-V. (F) To monitor cell amplification, freshly isolated CD7⁻ and CD7⁺ T cells of the CD4⁺CD45RO⁺CD25^{high} population and the corresponding CD4⁺CD45RO⁺CD25⁻ T cells (10⁴ cells/100 μ L) were incubated for 6 days in medium without stimuli (open columns) or in presence of the anti-CD3 mAb OKT3 (10 μ g/mL) plus anti-CD28 mAb 15E8 (1 μ g/mL) (closed columns) and pulsed with BrdU. Cell proliferation and the number of proliferating cells was determined as described in Materials and Methods. The correlation figure (insert) validates the BrdU assay. Increasing numbers of CD45RO⁺CD25⁻ T cells were stimulated with the anti-CD3 mAb OKT3 (10 μ g/mL) plus the anti-CD28 mAb 15E8 (1 μ g/mL) and BrdU incorporation as well as cell numbers by Trypan Blue exclusion assay were determined. (G) MACS or FACS sorted CD7⁻ and CD7⁺ T cells of the CD4⁺CD45RO⁺CD25^{high} population and the corresponding CD4⁺CD45RO⁺CD25⁻ T cells (10⁴ cells/100 μ L) from the same donor were incubated for 6 days in medium without stimuli (i) (open columns) or in presence of the anti-CD3 mAb OKT3 (10 μ g/mL) plus anti-CD28 mAb 15E8 (1 μ g/mL) (closed columns) or in medium without stimuli (ii) (open columns) or on plates coated with the anti-CD3 mAb OKT3 (10 μ g/mL) plus plate-bound anti-CD28 mAb 15E8 (10 μ g/mL) (closed columns). To monitor cell amplification, cells were pulsed with BrdU. Data of all assays (Fig. 4) represent the means $\pm$ standard error of means (SEM) of five independent experiments. SEM of data in Fig. 4C–E, are below 3%. Statistical analysis was performed using a paired *t*-test. **p* < 0.05, CD7⁻ cells compared with the corresponding CD7⁺ T cells.

3E, the mean telomere length of CD7⁻ Treg cells in the peripheral blood was significantly shorter than that of the corresponding CD7⁺ Treg cells, indicating that CD7⁻ Treg cells are obviously in a more advanced proliferative stage than CD7⁺ Treg cells. This conclusion is in accordance to our observation that prolonged propagation *in vitro* generates CD7⁻ Treg cells. For comparison, naïve T cells had longer telomeres than CD4⁺CD25⁺ Treg cells, as previously described.²⁷ An increased number of CD7⁻ Treg cells expressed telomerase compared to CD7⁺ Treg cells, whereas the amount of telomerase per cell was significantly decreased in CD7⁻ Treg cells (Fig. 3F).

CD7⁻ Treg cells exhibit altered effector functions

We monitored the cytokines secreted by isolated CD7⁻ and CD7⁺ cells of the CD4⁺CD45RO⁺CD25⁺ Treg cell population upon CD3/CD28 engagement. As summarized in Fig. 4A, IL-10 secretion was induced in both CD7⁻ and CD7⁺ Treg cells upon stimulation; the levels of secreted IL-10, however, were significantly lower in CD7⁻ compared to CD7⁺ Treg cells. For comparison, IL-10 was detectable only in background levels in cultures of the corresponding CD4⁺CD45RO⁺CD25⁻ T cells. In contrast, IFN- γ secretion was very poorly induced in cells of both CD7⁺ and CD7⁻ Treg cells, but was induced efficiently in the corresponding cells of the CD4⁺CD45RO⁺CD25⁻ T cell population. Notably, IL-4 was highly expressed by CD7⁻ Treg cells upon CD3/CD28 stimulation compared to CD7⁺ Treg cells. Flow cytometric analyses confirmed that significantly higher numbers of CD7⁻ Treg cells ($14 \pm 2\%$) produce IL-4 compared to CD7⁺ Treg cells ($1 \pm 1\%$). No secreted TGF- β 1 was detected in cell culture supernatants of CD7⁺ and CD7⁻ Treg cells by ELISA nor did we detect the membrane-bound form of TGF- β 1 by flow cytometric analyses. For comparison, no IL-2 was detected in CD7⁺ or CD7⁻ Treg cells, but in the corresponding CD4⁺CD25^{low} T cells.

To monitor the activation threshold of CD7⁺ and CD7⁻ Treg cells, we incubated the respective Treg cells in a cross-titration setting with increasing amounts of the agonistic anti-CD3 and anti-CD28 antibodies and recorded IL-10 secretion. As summarized in Fig. 4B, the amount of stimuli required to induce the same amount of secreted IL-10 was approximately 10-fold higher in CD7⁻ than in CD7⁺ Treg cells, indicating that CD7⁻ Treg cells exhibit an increased activation threshold compared to CD7⁺ Treg cells.

Without antigenic stimulation, the majority of CD4⁺CD25^{high} T cells rapidly enter apoptosis *in vitro*.²⁸ Here we assessed the frequency of apoptotic cells of freshly isolated CD7⁻ and CD7⁺ Treg cells upon CD3/CD28 stimulation *in vitro*. As summarized in Fig. 4C, CD7⁻ Treg cells are more resistant to spontaneous apoptosis *in vitro* than CD7⁺ Treg cells. Upon CD3/CD28 stimulation, however, CD7⁻ Treg cells rapidly enter apoptosis, whereas CD7⁺ Treg cells are rescued from apoptosis. Addition of IL-2 poorly, but of IL-15 more effectively prevented apoptosis of CD7⁻ Treg cells compared to incubation without cytokines (Fig. 4D,E). CD7⁺ Treg cells, in contrast, were rescued by IL-2 from apoptosis, whereas IL-15 had no significant effect. CD7⁻ Treg cells obviously require IL-15 and CD7⁺ Treg cells IL-2 for survival.

Both CD7⁻ and CD7⁺ Treg cells exhibited no significant proliferative capacities in presence of agonistic anti-CD3 plus anti-CD28 antibodies (Fig. 4F,G), but proliferation of both Treg cell subsets was induced by immobilized stimuli as recorded by BrdU-incorporation (Fig. 4G). For comparison, the corresponding cells of the CD4⁺CD45RO⁺CD25⁻ T cell population are induced to proliferate under both conditions. Essentially the same results were obtained with MACS- and FACS-sorted cells from the same donor (Fig. 4G). Treg cells of both CD7⁻ and CD7⁺ subtypes obviously require strong TCR/CD3 and CD28 engagement to initiate their amplification. CD7⁻ Treg cells, however, exhibited significantly lower proliferation capacities under identical conditions than the corresponding CD7⁺ Treg cells.

TABLE 1. EXPRESSION OF HOMING RECEPTORS ON *EX VIVO*-ISOLATED AND *IN VITRO*-EXPANDED CD7⁻ AND CD7⁺ CELLS OF CD4⁺CD25^{high} T CELLS

	<i>Ex vivo-isolated</i> CD4 ⁺ CD25 ^{high} T cells				<i>In vitro-expanded</i> CD4 ⁺ CD25 ^{high} T cells			
	CD7 ⁻		CD7 ⁺		CD7 ⁻		CD7 ⁺	
	[%]	MFI	[%]	MFI	[%]	MFI	[%]	MFI
Integrin α 4 β 1	92 $\pm$ 1	236 $\pm$ 20	69 $\pm$ 2	177 $\pm$ 12	95 $\pm$ 2	240 $\pm$ 18	66 $\pm$ 3	172 $\pm$ 11
Integrin α 4 β 7	29 $\pm$ 3	232 $\pm$ 9	31 $\pm$ 2	209 $\pm$ 10	28 $\pm$ 2	229 $\pm$ 8	33 $\pm$ 3	206 $\pm$ 11
CD103	<1	N.D.	6 $\pm$ 1	98 $\pm$ 5	<1	N.D.	8 $\pm$ 2	100 $\pm$ 6
CCR4	97 $\pm$ 2	195 $\pm$ 8	77 $\pm$ 3	168 $\pm$ 9	98 $\pm$ 1	191 $\pm$ 7	74 $\pm$ 3	161 $\pm$ 8
CCR7	15 $\pm$ 1	160 $\pm$ 13	41 $\pm$ 2	201 $\pm$ 18	12 $\pm$ 2	155 $\pm$ 12	45 $\pm$ 2	210 $\pm$ 19
CCR10	20 $\pm$ 1	181 $\pm$ 2	4 $\pm$ 2	65 $\pm$ 3	22 $\pm$ 2	188 $\pm$ 3	6 $\pm$ 3	68 $\pm$ 4
CLA	43 $\pm$ 4	222 $\pm$ 13	17 $\pm$ 5	187 $\pm$ 15	41 $\pm$ 2	234 $\pm$ 15	15 $\pm$ 2	195 $\pm$ 11
CD31	3 $\pm$ 3	16 $\pm$ 4	20 $\pm$ 5	23 $\pm$ 8	5 $\pm$ 2	18 $\pm$ 6	22 $\pm$ 3	26 $\pm$ 6

CD4⁺CD25^{high} T cells were isolated from the peripheral blood of 5 healthy donors or from *in vitro* simulation assays at day 14 (cf. Fig. 1). Cells were stained with antibodies specific for CD7 and for the indicated markers. Isotype-matched antibodies served as controls. Cells were analyzed by flow cytometry and gated on CD7⁺ and CD7⁻ cells, respectively. Data are given as the mean of positive cells [%] and the mean fluorescence intensity (MFI) $\pm$ SEM of five independent experiments.

Note: MFI, mean fluorescence intensity; N.D., not determined.

FIG. 5. CD7⁻ Treg cells accumulate in inflammatory lesions in the periphery. **(A)** Serial sections of biopsies from reactive lymph node, psoriasis, atopic dermatitis, and lichen ruber were stained simultaneously for: (i) CD4, CD7, FoxP3; (ii) CD4, CD7, CD25; (iii) CD4, CD25, FoxP3; and (iv) CD4, CD127, FoxP3, respectively. Sections were analyzed by laser-scan microscopy as described in Materials and Methods. Specificity of cell staining was assayed by incubation of the slides with the respective isotype-matched control antibodies. The numbers of CD7⁺ cells (open bars) and CD7⁻ cells (closed bars) with CD4⁺FoxP3⁺ and with CD4⁺CD25⁺ phenotypes, respectively, were determined. **(B)** For quantitative recording of FoxP3-specific Cy5 fluorescence signals, regions of interest were gated on Cy5 spots and the mean fluorescence intensity (FU, fluorescence unit) per spot (MFI/spot) was determined using the ImageJ software (U.S. National Institutes of Health). A minimum of 100 cells of each cell population in each biopsy section was recorded. Data represent the mean $\pm$ SEM. * $p < 0.05$, CD7⁻ cells compared with the corresponding CD7⁺ T cells. **(C and D)** Laser scan micrographs of immunofluorescence analyses of a lichen ruber and reactive lymph node biopsy are shown. (Circles) CD7⁻CD4⁺ T cells; (squares) CD7⁺CD4⁺ T cells. Note that nearly all FoxP3⁺ cells coexpress CD4 and CD25, but have downregulated CD127 (IL-7R α) expression. Magnification, 630 $\times$. (HE) overview of the biopsy section upon hematoxylin eosin stain. **(E)** Serial biopsy sections of the psoriasis lesion were hybridized with a telomere-specific PNA probe, followed by staining for CD4 and CD7 and for FoxP3 and CD7, respectively. (Circles) CD7⁻; (squares) CD7⁺ T cells. Magnification, 630 $\times$. For quantitative recording of telomere-specific Cy3 fluorescence signals, regions of interest were gated on Cy3 spots of the CD7⁻FoxP3⁺ and CD7⁺FoxP3⁺ cells, respectively, and the mean fluorescence intensity per spot (MFI/spot) was determined using the ImageJ software (U.S. National Institutes of Health). MFI per telomere spot directly correlates with the mean telomeric restriction fragment length as recorded by Southern blot hybridization.²² A minimum of 100 cells of each cell population in each biopsy section was recorded. Data represent the mean $\pm$ SEM. Statistical analyses (Fig. 5) were performed using a paired *t*-test test. (FU) fluorescence unit. (Continues $\longrightarrow$)

FIG. 5. (Continued).

FIG. 5. (Continued).

CD7⁻ Treg cells express homing receptors directing them into peripheral organs

We asked whether the striking differences in the functional capacities of CD7⁺ and CD7⁻ Treg cells are reflected by different tissue distributions. Therefore, we monitored the expression of a panel of surface markers associated with homing and migration (Table 1). CCR7 was expressed in less numbers and at lower levels on CD7⁻ compared to the CD7⁺ Treg cells. C-C chemokine receptor type 7 (CCR7)-deficient T cells are reported to be prone to persist in the skin by their inability to emigrate into lymph and back to blood.²⁹ In line with that, cutaneous lymphocyte antigen (CLA), associated with skin homing,³⁰ was expressed in higher frequencies and at higher levels in CD7⁻ compared to CD7⁺ Treg cells. CD31, a marker expressed by naïve Treg cells,³¹ was expressed on slightly higher numbers of cells and in higher levels by CD7⁺ compared to CD7⁻ Treg cells. Integrins $\alpha 4\beta 1$ and $\alpha 4\beta 7$ are receptors for T cell migration into inflamed tissues and intestinal mucosal sites, respectively.^{32,33} Integrin $\alpha 4\beta 1$ was more predominantly expressed on CD7⁻ than on CD7⁺ Treg cells, whereas integrin $\alpha 4\beta 7$ was nearly equally expressed in both Treg cell subsets. The chemokine receptor CCR4, associated with trafficking of specialized cutaneous memory T cells to the skin,³⁰ was expressed on nearly all CD7⁻ Treg cells, whereas 77% of CD7⁺ Treg cells express CCR4. In line with that, the chemokine receptor CCR10, associated with skin-homing of T cells,³⁴ was expressed on a significantly higher percentage and on higher levels of CD7⁻ compared to CD7⁺ Treg cells. CD103, associated with intestinal memory cells,³⁵ was not detectable on CD7⁻ Treg cells, whereas some CD7⁺ Treg cells expressed CD103. We found the same pattern of homing receptors on *ex vivo*-isolated and on *in*

vitro-generated CD7⁻ Treg cells. Taken together, CD7⁻ Treg cells display a set of homing receptors indicating that these cells home to the skin, migrate into inflamed tissues, and persist in the periphery.

CD7⁻ Treg cells accumulate in chronic inflammatory skin lesions

We monitored lymphocyte infiltrates of chronic inflammatory skin lesions including lichen ruber, atopic dermatitis, and psoriasis for the presence of CD7⁻ and CD7⁺ Treg cells (Fig. 5). Recording of CD4⁺CD25⁺FoxP3⁺ Treg cell subsets *in situ* by immunofluorescence staining revealed that CD7⁻ Treg cells accumulate in these chronic inflammatory skin lesions compared to their distribution in the peripheral blood. In lichen ruber and atopic dermatitis, CD7⁻ Treg cells constituted 90% and 75%, respectively, of infiltrating Treg cells, whereas these cells represent less than 10% of Treg cells in the peripheral blood. In the reactive lymph node, for comparison, CD7⁻ Treg cells were not significantly increased compared to their distribution in the peripheral blood. Nearly all FoxP3⁺ T cells coexpress CD4 and CD25, but have downregulated CD127 (IL-7R α) expression. Quantitative recording of FoxP3 expression *in situ* confirmed that CD7⁻ Treg cells expressed FoxP3 at lower levels per cell compared to CD7⁺ T cells within the same lesion, which is in accordance with the expression pattern of the corresponding Treg cells from the peripheral blood (Fig. 5B). Although these entities of chronic inflammatory pathologies were analyzed exemplarily, the survey clearly sustains our conclusion that CD7⁻ Treg cells exhibit a marked tropism to the periphery compared to their distribution in the peripheral blood.

To monitor whether CD7⁻ Treg cells in the inflammatory lesion have a longer replicative history in comparison to the corresponding CD7⁺ Treg cells of the same lesion, we recorded the telomere lengths *in situ* by Q-FISH, combined with multicolor immunofluorescence analyses, to identify the respective cells. The assay was validated by analysis of telomere lengths of normal human diploid fibroblasts in cryostat sections of healthy skin, as recently described.³⁶ Analyses of infiltrating cells in a psoriasis lesion is shown in Fig. 5E. Quantitative recording of MFIs of telomere spots revealed that CD4⁺FoxP3⁺CD7⁻ T cells had shorter telomeres than the corresponding CD7⁺ T cells in the same lesion (Fig. 5E). This sustains our conclusion that CD7⁻ Treg cells in chronic inflammatory lesions are in a more advanced replicative stage of T cell development than the corresponding CD7⁺ Treg cells.

Discussion

Recently, repetitive antigen encounter together with a strong CD28 signal were reported to stimulate the *ex vivo* amplification of Treg cells,³ the consequences of which, however, remained unknown. Here we have revealed that extensive TCR/CD28-triggered proliferation of human Treg cells results in altered functional capacities, including reduced suppressor activity, secretion of IL-4, display of markers associated with late memory and immunosenescence, and expression of homing receptors targeting these cells to the periphery. One additional hallmark of extensively am-

FIG. 6. Proposed model of late Treg cell differentiation. Treg cells constitute a separate branch of the T cell lineage and undergo positive selection in the thymus, which is mediated by high-affinity interactions of the T cell receptor and yet undefined additional signals. After entering into circulation and peripheral presentation of antigen, naïve Treg cells differentiate into memory-like Treg cells. Chronic antigen stimulation, however, drives late memory differentiation of Treg cells with altered functional capacities and homing properties.

plified T cells is the loss of CD7 expression, which is based on the following observations: (1) CD7⁺ T cells are generated *in vitro* from highly purified CD7⁺ Treg cells by extensive amplification. Accordingly, generation of CD7⁺ T cells requires multiple cell divisions because blocking CD7⁺ Treg cell proliferation prevented CD7⁺ T cell generation. (2) CD7⁺ Treg cells isolated from peripheral blood have significantly shorter telomeres than CD7⁺ Treg cells of the same donor. Shorter telomeres were also found for tissue-infiltrating Treg cells in inflammatory lesions, as revealed by *in situ* monitoring of telomere lengths. Chronic inflammatory lesions may provide strong TCR and CD28 co-stimulatory signals, both required to induce proliferation of Treg cells. On the other hand, CD7⁺ Treg cells express less Ki67 compared to CD7⁺ Treg cells in the same lesion (unpublished data), implying that, upon extensive amplification, CD7⁺ Treg cells may have reached the Hayflick limit of proliferative capacities. CD7⁺ T cells generated *in vitro* from CD7⁺ Treg cells still exhibit functional attributes of regulatory T cells because they express FoxP3 and execute suppressive activities toward allogeneic CD4⁺CD45RO⁺CD25⁺ T cells as do the *ex vivo* isolated CD7⁺ Treg cells. The suppressive capacity of CD7⁺ Treg cells, however, is lower than that of CD7⁺ Treg

cells, a result that was found consistently for both *in vitro*-generated and *ex vivo*-isolated CD7⁺ Treg cells. Reduced suppressor capacities of CD7⁺ Treg cells are unlikely to be due to contamination with effector T cells, because after 2 weeks of amplification more than 98% of the cells exhibit the FoxP3⁺CD25^{high} phenotype. In contrast to a recent report³⁷ that 40% of cells after 2 weeks of proliferation lack FoxP3 expression, we used an extraordinarily strong TCR plus CD28 signal, provided by immobilized agonistic antibodies, in the presence of IL-2 to induce proliferation of Treg cells.³

CD7⁺ Treg cells with CD4⁺CD45RO⁺CD25^{high} phenotype fulfill the criteria of cells in late memory development, which is summarized in Fig. 6. Both, homeostatic proliferation and repeated antigen exposure lead to late memory differentiation, which is characterized by loss of CD45RA and CCR7, by strong CD45RO and weak CD62L expression, and by shortened telomere lengths.³⁸ The model moreover postulates that CD7⁺ Treg cells represent a late memory differentiation stage of Treg cells, but not a separate lineage of T cells. Accordingly, we did not detect CD7⁺ Treg cells with shorter telomere lengths than CD7⁺ Treg cells. Moreover, CD7⁺ Treg cells differ from Tr1 cells as follows: lower levels of IL-10 are secreted, no TGF-β1 is secreted, but high levels of IL-4 are secreted, whereas Tr1 cells are characterized by high levels of IL-10 and TGF-β1 and moderate amounts of IFN-γ and IL-5 upon TCR stimulation.³⁹

After extensive amplification, the suppressor capacities of CD7⁺ Treg cells are substantially reduced. This holds for both *ex vivo*-isolated and *in vitro*-generated CD7⁺ Treg cells. Reduced suppressor capacities of CD7⁺ Treg cells are associated with reduced FoxP3, CD27, and CD31 expression. Reduction in suppressor capacities upon extensive *ex vivo* amplification of Treg cells is likely to have dramatic consequences for their efficacy in the adoptive immunotherapy of various autoaggressive diseases, including diabetes and transplant rejection. Consequently, therapeutic protocols should prefer short-term *ex vivo* amplification of Treg cells and/or depletion of CD7⁺ cells from the Treg cell transplant.

Recently, CD4⁺CD25^{high} Treg cells with diminished FoxP3 or CD27 expression were reported to exhibit reduced suppressor capacities,^{40–42} whereas CD31⁺ Treg cells are more potent suppressor cells.³¹ Accordingly, in healthy elderly subjects, CD7⁺ Treg cells correspondingly accumulate in the peripheral blood with aging (E.K. Meuer, unpublished observations), in patients with vigorous Th2 responses and in diseases with a long history of antigen stimulation, such as multiple sclerosis, type I diabetes, and psoriasis, Treg cells are reported to be associated with reduced suppressor activities.^{43,44} Repeated antigen stimulation and inflammatory conditions over a long time may result in the conversion from CD7⁺ to late memory CD7⁺ Treg cells, which, due to their reduced suppressor capacities, contribute to a functional deficiency of the Treg cell compartment.

Interestingly, highly amplified Treg cells, exhibit a number of unique properties that are different from the majority of mature Treg cells, the most prominent of which is the expression of skin homing receptors. Amplified Treg cells lack CCR7, but express CLA, integrin α4β1, CCR4, and CCR10 capable of directing the cells to the periphery. Loss of CCR7 results in the inability of CD4⁺ T cells to emigrate from the

skin back to the lymphoid organs.²⁹ We found CD7⁺ Treg cells accumulated at high levels in inflammatory lesions in the periphery where they represent up to 90% of infiltrating Treg cells. In inflamed central organs like reactive lymph nodes, in contrast, these cells are not amplified compared to the peripheral blood. *In situ* analysis confirmed that CD4⁺FoxP3⁺CD7⁺ T cells in chronic inflammatory skin lesions have shorter telomeres than the corresponding CD7⁺ T cells within the same lesion.

These data sustain the concept that proliferation of Treg cells under chronic inflammatory conditions results in the accumulation of CD7⁺ Treg cells, which, due to lack of CCR7 expression, are unable to migrate back into the lymph system, thereby leading to their accumulation in the lesion. IL-15 highly expressed in chronic inflammatory lesions⁴⁵ prevents apoptosis of CD7⁺ Treg cells, thereby sustaining their accumulation in the lesion. IL-15 will, moreover, contribute to reduce the overall suppressor capacities of Treg cells as recently shown.⁴² Although highly amplified in numbers, CD7⁺ Treg cells are likely not to be capable to limit the immune reaction due to their reduced suppressor capacities. Quite the reverse, IL-4 secreted by CD7⁺ Treg cells sustains inflammatory reactions by recruitment of mast cells, which, for their part, are involved in the pathogenesis of a number of immunological skin diseases.^{46–48} In this context, extensive Treg cell amplification upon strong TCR/CD28 engagement is likely to sustain the inflammatory reaction due to driving mature Treg cells into the late memory CD7⁺ Treg cell compartment with reduced suppressor capacities and proinflammatory IL-4 secretion. Accordingly, Treg cells with IL-4 and IL-10 expression were recently found in the peripheral blood of patients with atopic dermatitis.⁴⁹ Taken together, our data indicate that extensive amplification of Treg cells under chronic stimulatory conditions alters their functional properties dramatically and targets the cells to the periphery.

Acknowledgments

We would like to thank Anika Voss, Petra Hofmann, Birgit Hops, and Frank Steiger for technical support. We are gratefully to Dr. A. Banham (Nuffield Department of Clinical Laboratory Sciences, University of Oxford, Oxford, UK), Dr. H. P. Pircher (Institute of Immunology, Freiburg, Germany), and Dr. H. Derradji (Laboratory of Radiobiology, Belgian Nuclear Research Centre, SCK CEN, Mol, Belgium) for supplying us with antibodies and cell lines. Our study was supported by the Deutsche Forschungsgemeinschaft, Bonn, Cluster of Excellence "Cellular Stress Responses in Aging-Associated Diseases" (CECAD) and by the Koeln Fortune Program of the Faculty of Medicine, University of Cologne.

References

- Levings MK, Allan S, d'Hennezel E, Piccirillo CA. Functional dynamics of naturally occurring regulatory T cells in health and autoimmunity. *Adv Immunol* 2006;92:119–155.
- Walker MR, Carson BD, Nepom GT, Ziegler SF, Buckner JH. *De novo* generation of antigen-specific CD4⁺CD25⁺ regulatory T cells from human CD4⁺CD25⁺ cells. *Proc Natl Acad Sci USA* 2005;102:4103–4108.
- Hombach AA, Kofler D, Hombach A, Rappl G, Abken H. Effective proliferation of human regulatory T cells requires a strong costimulatory CD28 signal that cannot be substituted by IL-2. *J Immunol* 2007;179:7924–7931.
- Hoffmann P, Eder R, Kunz-Schughart LA, Andreesen R, Edinger M. Large-scale *in vitro* expansion of polyclonal human CD4⁺CD25^{high} regulatory T cells. *Blood* 2004;104:895–903.
- Gregori S, Bacchetta R, Passerini L, Levings MK, Roncarolo MG. Isolation, expansion, and characterization of human natural and adaptive regulatory T cells. *Methods Mol Biol* 2007;380:83–105.
- Boschiero L, Nacchia F, Fior F, Cordiano C, Tridente G, Bellisola G. Specific alloantigen self-control by regulatory T cells in organ transplantation: a review. *Transplant Proc* 2007;39:2013–2017.
- Kang SM, Tang Q, Bluestone JA. CD4⁺CD25⁺ regulatory T cells in transplantation: progress, challenges and prospects. *Am J Transplant* 2007;7:1457–1463.
- Roncarolo MG, Battaglia M. Regulatory T-cell immunotherapy for tolerance to self antigens and alloantigens in humans. *Nat Rev Immunol* 2007;7:585–598.
- Bluestone JA, Thomson AW, Shevach EM, Weiner HL. What does the future hold for cell-based tolerogenic therapy? *Nat Rev Immunol* 2007;7:650–654.
- Stillwell R, Bierer BE. T cell signal transduction and the role of CD7 in costimulation. *Immunol Res* 2001;24:31–52.
- Reinhold U, Abken H. CD4⁺CD7⁺ T cells: a separate subpopulation of memory T cells? *J Clin Immunol* 1997;17:265–271.
- Van Lier RA, Brouwer M, Aarden LA. Signals involved in T cell activation. T cell proliferation induced through the synergistic action of anti-CD28 and anti-CD2 monoclonal antibodies. *Eur J Immunol* 1988;18:167–172.
- Dieckmann D, Plottner H, Berchtold S, Berger T, Schuler G. *Ex vivo* isolation and characterization of CD4⁺CD25⁺ T cells with regulatory properties from human blood. *J Exp Med* 2001;193:1303–1310.
- Kubach J, Lutter P, Bopp T, Stoll S, Becker C, Huter E, Richter C, Weingarten P, Warger T, Knop J, Müllner S, Wijdenes J, Schild H, Schmitt E, Jonuleit H. Human CD4⁺CD25⁺ regulatory T cells: proteome analysis identifies galectin-10 as a novel marker essential for their anergy and suppressive function. *Blood* 2007;110:1550–1558.
- Roncador G, Brown PJ, Maestre L, Hue S, Martinez-Torrecuadrada JL, Ling KL, Pratap S, Toms C, Fox BC, Cerundolo V, Powrie F, Banham AH. Analysis of FoxP3 protein expression in human CD4⁺CD25⁺ regulatory T cells at the single-cell level. *Eur J Immunol* 2005;35:1681–1691.
- Baecher-Allan C, Wolf E, Hafler DA. Functional analysis of highly defined, FACS-isolated populations of human regulatory CD4⁺CD25⁺ T cells. *Clin Immunol* 2005;115:10–18.
- Game DS, Hernandez-Fuentes MP, Lechler RI. Everolimus and Basiliximab permit suppression by human CD4⁺CD25⁺ cells *in vitro*. *Am J Transplant* 2005;5:454–464.
- Huong PL, Kolk AH, Eggelte TA, Verstijnen CP, Gilis H, Hendriks JT. Measurement of antigen specific lymphocyte proliferation using 5-bromo-deoxyuridine incorporation. An easy and low cost alternative to radioactive thymidine incorporation. *J Immunol Methods* 1991;140:243–248.
- Rappl G, Schrama D, Hombach A, Meuer EK, Schmidt A, Becker JC, Abken H. CD7⁺ T cells are late memory cells generated from CD7⁺ T cells. *Rejuvenation Res* 2008;11:543–556.
- Kapoor V, Telford WG. Telomere length measurement by fluorescence *in situ* hybridization and flow cytometry. *Methods Mol Biol* 2004;263:385–398.

21. Hultdin M, Grönlund E, Norrback K, Eriksson-Lindström E, Just T, Roos G. Telomere analysis by fluorescence *in situ* hybridization and flow cytometry. *Nucleic Acids Res* 1998;26:3651–3656.
22. Ferlicot S, Youssef N, Feneux D, Delhommeau F, Paradis V, Bedossa P. Measurement of telomere length on tissue sections using quantitative fluorescence *in situ* hybridization (Q-FISH). *J Pathol* 2003;200:661–666.
23. Ali AS, Chopra R, Robertson J, Testa NG. Detection of hTERT protein by flow cytometry. *Leukemia* 2000;14:2176–2181.
24. Schmidt A, Ladage D, Steingen C, Brixius K, Schinköthe T, Klinz FJ, Schwinger RH, Mehlhorn U, Bloch W. Mesenchymal stem cells transigrate over the endothelial barrier. *Eur J Cell Biol* 2006;85:1179–1188.
25. Voehringer D, Koschella M, Pircher H. Lack of proliferative capacity of human effector and memory T cells expressing killer cell lectin like receptor G1 (KLRG-1). *Blood* 2002;100:3698–3702.
26. Mc Hugh RS, Whitters MJ, Piccirillo CA, Young DA, Shevach EM, Collins M, Byrne MC. CD4⁺CD25⁺ immunoregulatory T cells: gene expression analysis reveals a functional role for the glucocorticoid-induced TNF receptor. *Immunity* 2002;16:311–323.
27. Valmori D, Merlo A, Souleimanian NE, Hesdorffer CS, Ayyoub M. A peripheral circulating compartment of natural naive CD4⁺ Tregs. *J Clin Invest* 2005;115:1953–1962.
28. Taams LS, Smith J, Rustin MH, Salmon M, Poulter LW, Akbar AN. Human anergic/suppressive CD4⁺CD25⁺ T cells: a highly differentiated and apoptosis-prone population. *Eur J Immunol* 2001;31:1122–1131.
29. Debes GF, Arnold CN, Young AJ, Krautwald S, Lipp M, Hay JB, Butcher EC. Chemokine receptor CCR7 required for T lymphocyte exit from peripheral tissues. *Nat Immunol* 2005;6:889–894.
30. Kupper TS, Fuhlbrigge RC. Immune surveillance in the skin: mechanisms and clinical consequences. *Nat Rev Immunol* 2004;4:211–222.
31. Haas J, Fritzsche B, Trübswetter P, Korporal M, Milkova L, Fritz B, Vobis D, Krammer PH, Suri-Payer E, Wildemann B. Prevalence of newly generated naive regulatory T cells (Treg) is critical for Treg suppressive function and determines Treg dysfunction in multiple sclerosis. *J Immunol* 2007;179:1322–1330.
32. Stassen M, Fondel S, Bopp T, Richter C, Müller C, Kubach J, Becker C, Knop J, Enk AH, Schmitt S, Schmitt E, Jonuleit H. Human CD25⁺ regulatory T cells: two subsets defined by the integrins alpha 4 beta 7 or alpha 4 beta 1 confer distinct suppressive properties upon CD4⁺ T helper cells. *Eur J Immunol* 2004;34:1303–1311.
33. Berlin C, Berg EL, Briskin MJ, Andrew DP, Kilshaw PJ, Holzmann B, Weissman IL, Hamann A, Butcher EC. Alpha 4 beta 7 integrin mediates lymphocyte binding to the mucosal vascular addressin MAdCAM-1. *Cell* 1993;74:185–195.
34. Homey B, Alenius H, Müller A, Soto H, Bowman EP, Yuan W, Mc Evoy L, Lauerma AI, Assmann T, Bünnemann E, Lehto M, Wolff H, Yen D, Marxhausen H, To W, Sedgwick J, Ruzicka T, Lehmann P, Zlotnik A. CCL27-CCR10 interactions regulate T cell-mediated skin inflammation. *Nat Med* 2002;8:157–165.
35. Johansson-Lindbom B, Agace WW. Generation of gut-homing T cells and their localization to the small intestinal mucosa. *Immunol Rev* 2007;215:226–242.
36. O'Sullivan JN, Finley JC, Risques RA, Shen WT, Gollahon KA, Moskovitz AH, Gryaznov S, Harley CB, Rabinovitch PS. Telomere length assessment in tissue sections by quantitative FISH: image analysis algorithms. *Cytometry Part A* 2004;58A:120–131.
37. Crellin NK, Garcia RV, Levings MK. Flow cytometry-based methods for studying signaling in human CD4⁺CD25⁺FoxP3⁺ T regulatory cells. *J Immunol Methods* 2007;324:92–104.
38. DeJaco C, Duftner C, Schirmer M. Are regulatory T cells linked with aging? *Exp Gerontol* 2006;41:339–345.
39. Groux H, O'Garra A, Bigler M, Rouleau M, Antonenko S, de Vries JE, Roncarolo MG. A CD4⁺ T-cell subset inhibits antigen-specific T cell responses and prevents colitis. *Nature* 1997;389:737–742.
40. Venken K, Hellings N, Thewissen M, Somers V, Hensen K, Rummens JL, Medaer R, Hupperts R, Stinissen P. Compromised CD4⁺CD25^{high} regulatory T-cell function in patients with relapsing-remitting multiple sclerosis is correlated with a reduced frequency of FoxP3-positive cells and reduced FoxP3 expression at the single-cell level. *Immunology* 2008;123:79–89.
41. Valencia X, Yarboro C, Illei G, Lipsky PE. Deficient CD4⁺CD25^{high} T regulatory cell function in patients with active systemic lupus erythematosus. *J Immunol* 2007;178:2579–2588.
42. Ruprecht CR, Gattorno M, Ferlito F, Gregorio A, Martini A, Lanzavecchia A, Sallusto F. Coexpression of CD25 and CD27 identifies FoxP3⁺ regulatory T cells in inflamed synovia. *J Exp Med* 2005;201:1793–1803.
43. Tsaknaris L, Spencer L, Culbertson N, Hicks K, LaTocha D, Chou YK, Whitham RH, Bakke A, Jones RE, Offner H, Bourdette DN, Vandenbark AA. Functional assay for human CD4⁺CD25⁺ Treg cells reveals an age-dependent loss of suppressive activity. *J Neurosci Res* 2003;74:296–308.
44. Taams LS, Palmer DB, Akbar AN, Robinson DS, Brown Z, Hawrylowicz CM. Regulatory T cells in human disease and their potential for therapeutic manipulation. *Immunology* 2006;118:1–9.
45. Budagian V, Bulanov E, Paus R, Bulfone-Paus S. IL-15/IL-15 receptor biology: a guided tour through an expanding universe. *Cytokine Growth Factor Rev* 2006;17:259–280.
46. Navi D, Saegusa J, Liu FT. Mast cells and immunological skin diseases. *Clin Rev Allergy Immunol* 2007;33:144–155.
47. Fischer M, Harvima IT, Carvalho RF, Möller C, Naukkarinen A, Enblad G, Nilsson G. Mast cell CD30 ligand is upregulated in cutaneous inflammation and mediates degranulation-independent chemokine secretion. *J Clin Invest* 2006;116:2748–2756.
48. Zalewska A, Brzezinska-Blaszczyk E, Omulecki A, Pietrzak A. Functional studies of skin mast cells in lichen planus. *Arch Dermatol Res* 1997;289:261–264.
49. Bellinghausen I, Klostermann B, Knop J, Saloga J. Human CD4⁺CD25⁺ T cells derived from the majority of atopic donors are able to suppress Th1 and Th2 cytokine production. *J Allergy Clin Immunol* 2003;111:862–868.

Address for correspondence and reprints:
 Univ.-Prof. Dr. Hinrich Abken
 Center for Molecular Medicine Cologne
 University of Cologne
 Kerpener Str. 62, D-50924
 Köln, Germany

E-mail: hinrich.abken@uk-koeln.de

Received: March 17, 2008

Accepted: July 31, 2008