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IL-7-Dependent Extrathymic Expansion of CD45RA$^+$ T Cells Enables Preservation of a Naive Repertoire[1]

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We have investigated the regulation of adult and cord blood CD45RA$^+$ T cell proliferation and apoptosis to identify factors that may control the naive T cell pool. Cord CD45RA$^+$ T cells were highly susceptible to spontaneous apoptosis as compared with CD45RA$^+$ T cells from adults. Apoptosis was prevented by the addition of IL-2, IL-4, IL-7, and IL-15 which signal via the γ-chain of the IL-2 receptor. IL-7 prevented the decrease in Bcl-2 and Bcl-x_L and induced cell cycling in up to 20% of cord T cells after 8 days, resulting in a threefold increase in cord T cell numbers. However, the expanded cells retained a CD45RA$^+$CD45RO$^-$ phenotype. Similar results were obtained with adult CD45RA$^+$ T cells. IL-7-expanded CD45RA$^+$RO$^-$ T cells expressed CD45RO after stimulation through the TCR. Investigations into the regulation of replicative senescence showed that after 12 days in culture with IL-7, cord blood CD45RA$^+$ T cell proliferation resulted in telomere shortening. Nevertheless, IL-7-expanded cord blood T cells still maintained longer telomeres than unstimulated adult T cells. IL-7 but not IL-2 could directly induce high telomerase activity which probably retarded the rate of telomere shortening in cord blood T cells. These results suggest that proliferation induced by IL-7 may be important for extrathymic expansion of neonatal CD45RA$^+$ T cells and may also contribute to the maintenance of the adult CD45RA$^+$ T cell pool. *The Journal of Immunology,* **1998, 161: 5909–5917.**

The immune system is regulated by homeostatic mechanisms which maintain the constant size of the lymphoid compartment concomitantly with the retention of selected Ag-experienced cells (1–3). An important component within this homeostatic network is the maintenance of the peripheral naive T cell repertoire. In newborns, essentially all cord blood T lymphocytes express the CD45RA isoform of the leukocyte common Ag, a marker associated with naive or unprimed T lymphocytes (4). Postnatal development is accompanied by an increase in size of the total lymphocyte compartment and appearance of primed cells that lose expression of the CD45RA isoform while acquiring expression of CD45RO (5). Since CD45RA$^+$ T cells are thought to be precursors of CD45RO$^+$ T lymphocytes (6, 7), it would be predicted that eventual deletion of CD45RA$^+$ T cell numbers as a result of repeated immune challenges would occur unless this subset is replenished. Thymic involution suggests that continuous output of CD45RA$^+$ T cells from the thymus may not be sufficient to maintain the size of the peripheral CD45RA$^+$ T cell pool (8).

Two possibilities for naive CD45RA$^+$ T cell maintenance throughout life have been suggested. In the first, reversion from CD45RO$^+$ to CD45RA$^+$ expression may occur in some cells (9). However, data from patients with viral infections suggest that the majority of the expanded CD45RO$^+$ population perish by apoptosis rather than revert to CD45RA$^+$ expression (10). A second possibility is that expansion of T cells within the CD45RA$^+$ T cell pool may occur in the presence of certain cytokines that induce proliferation without acquisition of CD45RO expression (11, 12).

Another homeostatic constraint on the T cell pool is telomere shortening as a result of proliferation (13–15). Telomeres are unique terminal chromosomal structures consisting of hexameric repeats ($(TTAGGG)_n$) and are involved in the maintenance of chromosomal integrity (16). Cell division leads to the loss of telomeric DNA; therefore telomere shortening acts as a mitotic clock, restricting the number of divisions that a cell can undergo (17, 18). Critically short telomeres signal growth arrest, a process known as replicative senescence (15). Previous studies have shown that CD45RO$^+$ T cells have shorter telomeres than CD45RA$^+$ T cells, suggesting that the former population has decreased replicative potential (19).

To investigate mechanisms that may regulate homeostasis of the naive T cell pool, we have studied both adult and cord blood CD45RA$^+$ T cells, the latter population being truly naive, not complicated by the presence of putative "back-converted" CD45RO$^+$ T cells (4, 5, 9). In adults, CD45RO$^+$ T cells are particularly prone to apoptosis because of their low expression of survival genes such as *bcl-2* but high expression of apoptosis-inducing molecules such as CD95 (Fas/Apo-1) (5, 7, 10, 20). Resting adult CD45RA$^+$ T cells are also susceptible to apoptosis but to a much lesser extent than CD45RO$^+$ T cells in either a resting or an activated state (21) (D. Pilling, A. N. Akbar, and M. Salmon, manuscript in preparation). Freshly isolated resting cord blood CD45RA$^+$ T cells, however, were highly susceptible to spontaneous apoptosis in culture, suggesting that neonatal and adult unprimed populations have some functional differences (22). Both cord and adult CD45RA$^+$ T cells can be induced to proliferate by IL-7, without conversion to a CD45RO$^+$RA$^-$ phenotype. Furthermore, we show for the first time that IL-7 alone can induce strong telomerase activity in CD45RA$^+$ T cells without the requirement for TCR stimulation. Although a decrease in the mean telomere

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Table I. *Abs used in this study*

Ab	Isotype	Code	Fluorochome	Source	Specificity
CD14	IgG1	Mo2		Immunotech	Monocytes
CD16	IgG	FcRγIII		Immunotech	NK cells neutrophils, macrophages
CD19	IgG1	RFB9		RFH[a]	B cells
CD45RA	IgG1	SN130	PE	RFH Conjugated by SBA	B cells, T cell subsets (naive T cells), monocytes, NK cells
CD45RO	IgG2a	UCHL1		UCH	T cell subsets (memory T cells), B cell subsets, monocytes, macrophages
CD3	IgG2a	UCHT1	FITC	RFH	Most peripheral T cells
CD4	IgG1	RFT4	PE	RFH Conjugated by SBA	Class II-restricted T cells
CD8	IgG1	RFT8	PE	RFH Conjugated by SBA	Class I-restricted T cells
CD95	IgG1	APO-1/FAS	FITC	Immunotech	Pro-apoptotic TNFR family
Bcl-2	IgG-1		FITC	Santa Cruz	Anti-apoptotic protein
Bcl-x_L	Polyclonal rabbit anti-human IgG		Second-layer anti-rabbit IgG-FITC	Santa Cruz	Anti-apoptotic protein

[a] RFH, Royal Free Hospital; SBA, Southern Biotechnology Associates, Birmingham, AL; UCH, kindly provided by Professor P. C. L. Beverley, University College and Middlesex School of Medicine, London, U.K.; Santa Cruz Biotechnology, Santa Cruz, CA; Immunotech, Birmingham, U.K.

length was observed after culture, telomerase up-regulation by IL-7 may enable bystander expansion of naive T cells by cytokines without significant telomere loss, thus preserving the replicative potential of the expanded cells.

Materials and Methods

Blood samples

Umbilical cord blood samples were obtained after uncomplicated births. Blood samples were collected into heparinized syringes and processed immediately. Heparinized venous peripheral blood samples obtained from healthy laboratory staff were used as adult control cells.

Cytokines used in the study

The cytokines used in this study were obtained from R&D Systems (Abingdon, U.K.) and used at pretitrated optimal concentrations of 5 ng/ml.

Lymphocyte subset purification

PBMC were separated using Ficoll-Hypaque (Nycomed, Oslo, Norway) density gradient centrifugation as previously described (10). CD45RA$^+$ T cells were separated by negative selection by immunomagnetic bead depletion. Cells were firstly incubated in a mixture of mAbs directed at the populations to be eliminated (CD16, CD14, CD19, and CD45RO; see Table I for details). Cord blood samples were preincubated with anti-glycophorin A (Dako, High Wycombe, U.K.) to eliminate nucleated RBC precursors. Cells were then incubated with Dynabeads (Dynal, Oslo, Norway) coated with affinity-purified goat anti-mouse Ig M450) at room temperature for 30 min. A magnetic particle concentrator allowed the recovery of unbound CD45RA$^+$ T cells. Two rounds of depletion were performed. Purified populations contained 85–95% CD3$^+$/CD45RA$^+$. In cord blood samples, contaminating CD3$^-$/RA$^-$ cells constituted <4% of gated populations, whereas the percentage of CD3$^-$/RA$^+$ ranged from 1 to 10%. CD45RA$^+$-enriched T cells were cultured, in the presence or absence of cytokines, in RPMI 1640 (Life Technologies, Paisley, U.K.) containing 10% FCS (M. B. Meldrum, Bourne End, U.K.), antibiotics (Life Technologies), and supplemented with L-glutamine (ICN Biomedicals, High Wycombe, U.K.).

Cell staining for flow cytometric analysis

FACScan (Becton Dickinson, Oxford, U.K.) analysis of T cell phenotype was performed as previously described (23) using different combinations of the Abs shown in Table I. Cells were incubated with Abs for 10 min and fixed in 2% paraformaldehyde in PBS before analysis. Analysis of the intracellular expression of Bcl-2 and Bcl-x_L required cell permeabilization with Permeafix (Ortho Diagnostic Systems, High Wycombe, U.K.). Data analysis was performed using LYSIS II Software.

Detection of apoptosis and cell cycle analysis

Apoptosis was detected as described previously (7, 10), first in cytocentrifuge preparations of cells by morphologic changes such as chromatin condensation, nuclear fragmentation, and decreased nuclear-cytoplasmic ratio after May-Grünwald-Giemsa staining. Secondly, the nucleus of cells in suspension was labeled with propidium iodide (PI[3], Sigma-Aldrich, Poole, U.K.) after permeabilization with 90% ethanol. PI binds to DNA, allowing the identification of apoptotic, resting, and proliferating cells by their variable DNA content. Apoptotic cells characteristically have lower DNA content and are smaller than resting viable cells which in turn have one-half the DNA content of proliferating cells.

Enumeration of the absolute numbers of lymphocytes

The number of viable cells surviving in culture, treated or untreated with the IL-2R common γ-chain cytokines, was assessed using the Cytoron Absolute Cytometer (Ortho). Samples were collected at different time points, fixed with 1% paraformaldehyde in PBS, and run through the Cytoron Absolute using preset gates for viable cells.

Activation of cytokine-expanded populations by anti-CD3 Ab

Anti-CD3-coated plates were prepared by overnight incubation of anti-CD3 (UCHT1, 10 ng/ml) at 37°C in coating buffer (Sigma-Aldrich). The plates were then washed twice with medium (RPMI 1640, containing 10% FCS), and cells were added at 10^6/ml. Cells cultured with or without anti-CD3 and in the presence of IL-2 R common γ-chain cytokines were harvested at 1 and 2 days after anti-CD3 stimulation. Response to anti-CD3 stimulation was evaluated by cell viability and PI staining to determine the relative degree of apoptosis and proliferation.

Assessment of the mean terminal restriction fragment (TRF) length

CD45RA$^+$-enriched cord blood T cells were analyzed before and after culture with IL-7. Cell samples (2×10^6 to 4×10^6 cells) were pelleted by centrifugation, transferred into Eppendorfs, and washed in PBS. The pellets were then snap-frozen in liquid nitrogen and stored at −70°C. Genomic DNA was extracted from frozen samples using the WIZARD genomic DNA purification kit (Promega, Southampton, U.K.) and digested for 6 h

[3] Abbreviations used in this paper: PI, propidium iodine; MFI, mean fluorescence intensity; TRAP, telomeric repeats amplification protocol; TRF, terminal restriction fragment.

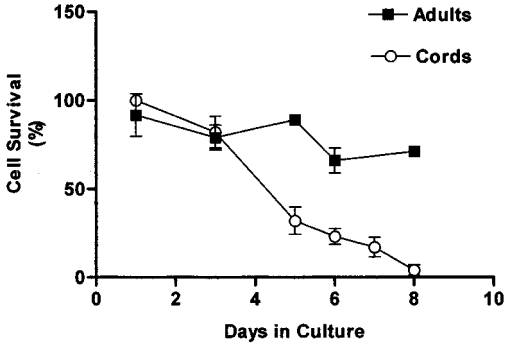

FIGURE 1. Cord vs adult CD45RA$^+$ T cell survival in vitro. Cord and adult T cells were enriched for CD45RA$^+$ subset using immunomagnetic bead depletion and cultured without exogenous stimuli under the same conditions. Cell recovery was determined using the Cytoron Absolute, and survival was calculated as a percentage of the initial input of cells. Mean survival values for 10 cord and 10 adult samples are shown, and the vertical bars represent the SEM

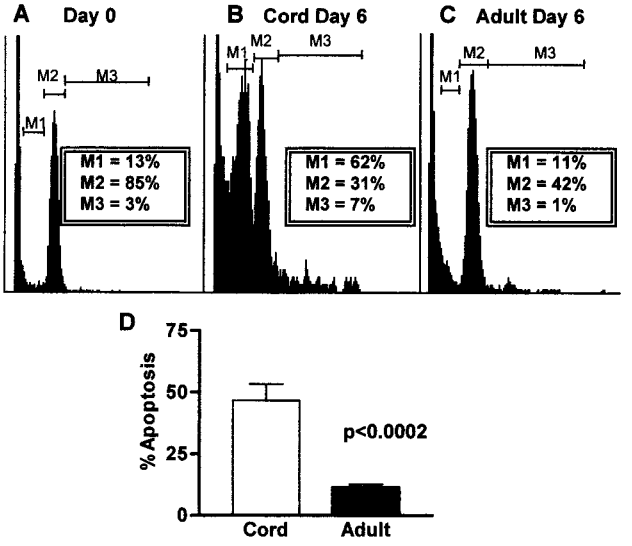

FIGURE 2. PI uptake by cord and adult T cells. *A*, *B*, and *C* are representative profiles obtained after PI staining and analysis on the FACScan. *A*, Freshly isolated cord blood CD45RA$^+$ T cells; *B*, cord CD45RA$^+$ T cells cultured for 6 days; *C*, Adult CD45RA$^+$ T cells cultured for 6 days without exogenous stimulus. M1 contains apoptotic cells, M2 resting viable lymphocytes, and M3 proliferating cells. *D* shows the mean percentage apoptosis in cord and adult cultures after 6 days in culture obtained from five cord and six adult samples. Vertical bars represent S.E.M. Student's *t* test revealed a statistically significant difference between the mean percentage apoptosis in the cord and adult samples; $p < 0.0002$.

at 37°C with restriction enzymes *Msp*I and *Rsa*I (both from Pharmacia Biotech, St. Albans, U.K.). The digests were then electrophoresed in a 0.7% agarose gel in Tris-acetate for 48 h at 20 V. The gel was then subjected to depurination, denaturation in an alkaline solution, and neutralization. The DNA was transferred onto a nitrocellulose membrane (Hybond N$^+$, Amersham Life Science, Amersham, U.K.) by Southern blotting and fixed by baking at 80°C. Before hybridization with the telomeric probe, the membranes were prehybridized with Rapid Hyb Buffer (Amersham). Hybridization with the telomeric specific probe $(TTAGGG)_3$, which had previously been labeled with [γ-^{32}P]ATP using polynucleotide kinase (Pharmacia), was performed at 42°C for 1 h. Autoradiographs were obtained by exposing the autoradiography film (Hyperfilm MP, Amersham) to the hybridized membrane at −70°C for the appropriate amount of time. TRF length was calculated by densitometry of autoradiographs within the linear range using Molecular Analyst software (Bio-Rad, Hemel Hempstead, U.K.). The mean TRF length was calculated as described (24).

Measurement of telomerase activity

A modified version of the telomeric repeat amplification protocol (TRAP) was used (Oncor, Gaithersburg, MD) as previously described (25). Extracts from varying cell numbers were used for telomeric elongation, using a [γ-^{33}P]ATP-end-labeled primer. These samples were used for PCR amplification (Perkin-Elmer Cetus, Norwalk, CT), using 25 to 28 cycles of 30 s at 94°C and 30 s at 59°C. The PCR products were run on a 12% polyacrylamide (Amersham) gel which was vacuum dried for exposure to autoradiography film (Hyperfilm MP, Amersham). Telomerase activity was calculated using the optical density of the telomeric repeat bands, divided by the strength of the internal PCR control band (which also served to indicate the absence of *Taq* inhibitors). Extracts from the immortalized 293 cell line provided the positive control. The negative controls were obtained by heat inactivation of the RNA template for each cell extract used. In addition, lysis buffer was used in place of cell extract in one reaction tube.

Results

Neonatal vs adult T cell survival

T lymphocytes from 10 umbilical cord blood samples and 10 normal adult individuals were prepared and cultured under the same conditions. Cord blood T cell populations contain <3% (range, 0.5–6%) CD45RO$^+$ T cells, whereas T cell populations from healthy adults contain 40% (range, 30–50%) of these cells (22). When CD45RA$^+$ T cells from adult and cord were isolated and cultured without added exogenous stimuli, similar survival rates for the first 72 h were observed in culture (Fig. 1). This was followed by a substantial decrease in the viability of cord blood T cells. By day 8, virtually all the cells in cord cultures were dead, whereas 75% of the cells in adult cultures were still viable. These data clearly show a deficiency in the ability of CD45RA$^+$ cord blood T cells to survive in culture compared with their adult counterparts.

Cell death occurring by apoptosis

The occurrence of apoptosis in cord blood CD45RA$^+$ T cells after 6 days in culture was demonstrated by increased PI incorporation as a sub-G_0 peak (Fig. 2*B*). The level of apoptosis in cultured cord T cell samples was greater than that observed in freshly isolated cord blood T cells (Fig. 2*A*) and adult CD45RA$^+$ T cells (Fig. 2*C*). The occurrence of apoptosis was also confirmed by the typical morphologic changes detected by May-Grünwald-Giemsa staining (data not shown). Fig. 2*D* represents the mean percentage apoptosis of cord and adult CD45RA$^+$ T cell populations cultured for 6 days showing a statistically significant increase in apoptosis in cord blood T cells ($p < 0.0002$).

Cord blood T cells can be rescued from apoptosis by IL-2R common γ-chain cytokines

In view of previous studies showing the ability of a group of cytokines sharing the γ-chain of the IL-2 receptor to prevent resting and activated T cell death (23, 26, 27), we investigated the effect of these cytokines (IL-2, IL-4, IL-7, and IL-15) on cord blood T cell apoptosis. Supplementing cord blood T cell cultures with these cytokines resulted in a significant increase in cell recovery at day 8 relative to the initial cell input (Fig. 3*A*). Both CD4$^+$ and CD8$^+$ T cells were expanded (data not shown). IL-7 produced the most striking results inducing a threefold increase in cell numbers compared with the initial input of cells. The greater effect of IL-7 compared with the other IL-2R common γ-chain-signaling cytokines was still observed when up to 10-fold higher concentrations of IL-2, IL-4, and IL-15 were used (data not shown). IL-6 was used as a control cytokine, and no rescue was achieved. The increase in cell recovery induced by IL-2 and especially IL-7 in both adult and cord blood T cells resulted in part from proliferation (Fig. 3*B*). Control adult and especially cord T cells cultured without added cytokines for 8 days showed a considerable decrease in viability (see

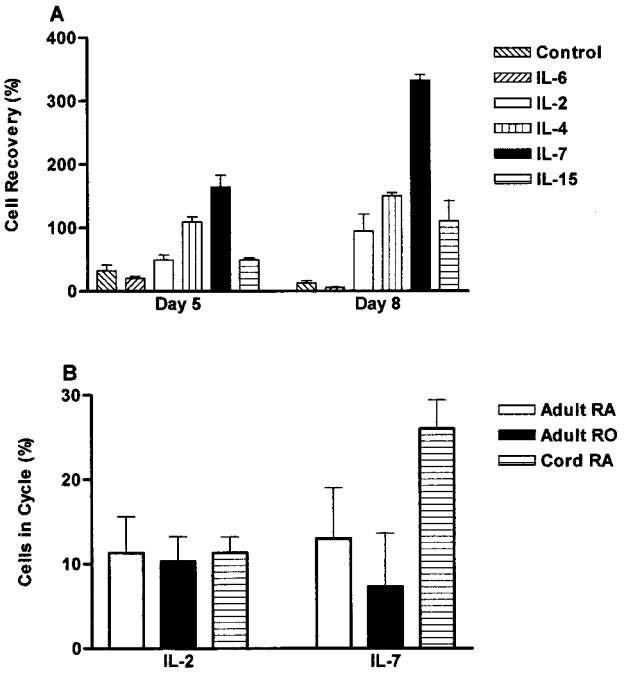

FIGURE 3. IL-2R common γ-chain cytokines promote cord blood T cell survival. Cord blood CD45RA$^+$ T cells were cultured with and without IL-2, IL-15, IL-7, IL-4, and IL-6 (5 ng/ml). Cell recovery was determined at several time points in culture using the Cytoron Absolute cytometer (*A*). The mean of five cord samples is shown. Vertical bars show the S.E.M. whereas survival is represented as a percentage of the initial input of cells. Cord and adult T cells, cultured for 8 days with IL-2 or IL-7, were stained with PI to determine the number of cells in cycle and analyzed on the FACScan. *B*, Mean and S.E.M. for three experiments are shown.

Fig. 1). In four cord and four adult CD45RA$^+$ T cell samples studied, only 4.4 ± 1.4% (mean ± S.E.M.) and 2.2 ± 1.5%, respectively, of residual control cells cultured without cytokines for 8 days were in cycle.

Phenotype of proliferating cord blood T cells

Adult and cord CD45RA$^+$RO$^-$T cells have been shown to convert to a "primed" phenotype (CD45RO$^+$RA$^-$) upon stimulation (4, 6, 22). We investigated whether proliferation induced by cytokines alone caused a similar switch. Cells cultured with or without IL-2 or IL-7 were harvested at several time points and analyzed for CD3, CD45RA, and CD45RO expression. The mean results from six cord blood samples are shown in Fig. 4*A*. The absolute numbers of CD3$^+$/RA$^+$ cells were always increased in cytokine-supplemented cultures as compared with control cultures. Interestingly, proliferation in these cells was not accompanied by a switch to CD45RO$^+$RA$^-$ (Fig. 4*D*). IL-7 produced the most pronounced effect, not only in terms of absolute numbers of viable cells recovered but also in the extent of CD45RA$^+$ T cell proliferation induced. Similarly, adult CD3$^+$CD45RA$^+$ T cells cultured in the presence of these cytokines did not switch to CD45RO$^+$ expression (data not shown).

To investigate whether expansion of CD45RA$^+$ cord T cells by IL-7 had a selective effect on certain T cell clones, a panel of 19 different TCR anti-Vβ Abs was tested on three cord blood samples before and after culture in IL-7. The staining profiles in CD4- and CD8-positive T cells showed that there was no selective expansion in any particular Vβ family in any of these samples (not shown).

Bcl-2 and Bcl-x_L expression in cord blood T cells

To determine whether the susceptibility of cord blood T cell to apoptosis was caused by changes in the constitutive expression of Bcl-2 or Bcl-x_L which prevent apoptosis (28, 29), freshly isolated cord and adult T cells were stained for these proteins and analyzed by flow cytometry. It was found that both proteins were expressed at the same concentrations in cord blood and adult peripheral blood T cells before culture (data not shown). Bcl-2 and Bcl-x_L levels were then measured after culture in the presence or absence of the survival-promoting cytokines (Fig. 5). After 7 days in culture without exogenous addition of cytokines, Bcl-2 and Bcl-x_L levels decreased, when compared with day 0 (Fig. 5, *A* and *B*). A comparison of the mean percentage decrease in Bcl-2 levels after culture

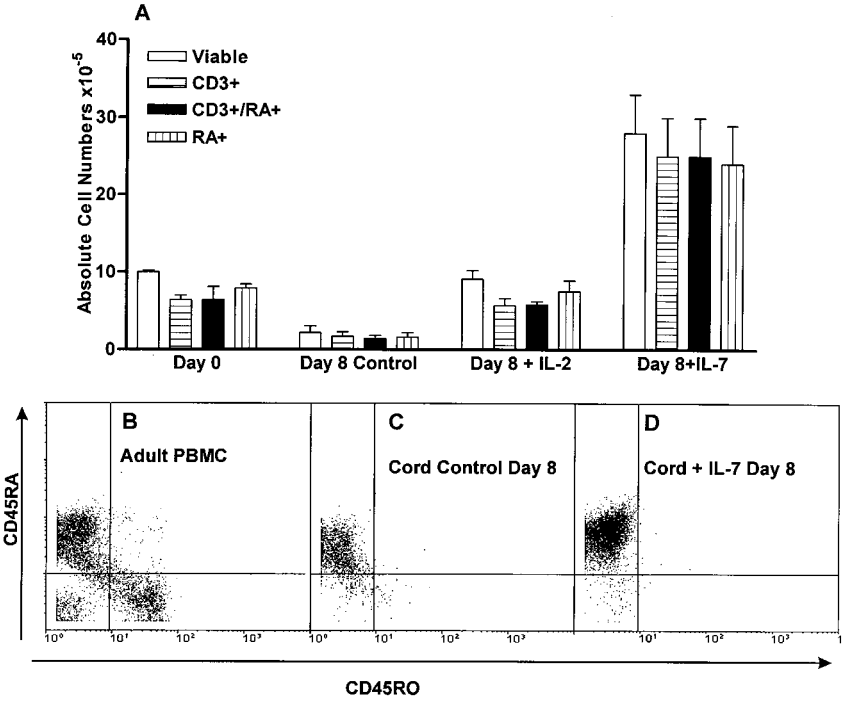

FIGURE 4. Effect of IL-2R common γ-chain cytokines on CD45RA/RO expression. Purified cord CD45RA$^+$ T cells were cultured in the presence or absence of IL-2 or IL-7 (*A*). Cells were harvested from culture at day 8, counted on the Cytoron absolute cytometer, and analyzed for CD3 and CD45RA expression. The mean result of six cord blood samples is shown. Vertical bars, SEM. Typical FACScan profiles of CD4RA/RO expression are shown for fresh adult PBMC (*B*) and cord blood T cells after 8 days in culture without (*C*) or with IL-7 (*D*).

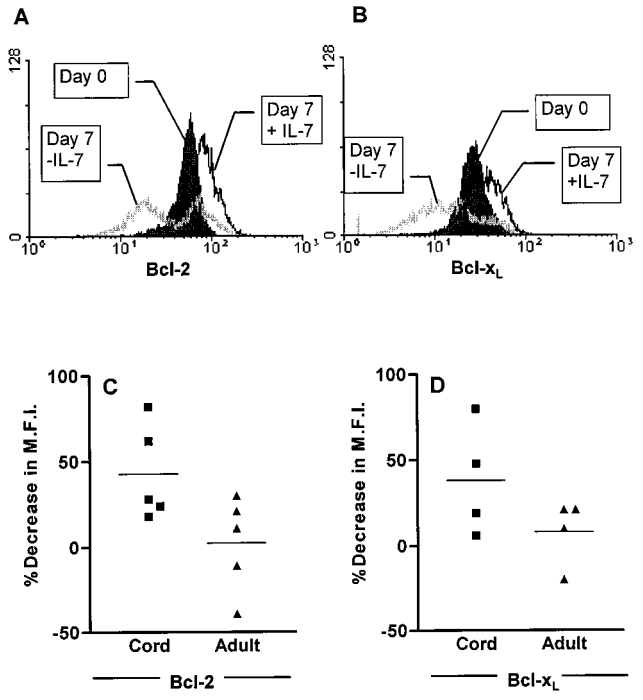

FIGURE 5. Bcl-2 and Bcl-x_L expression by cord blood CD5RA$^+$ T cells before and after culture in the presence or absence of IL-7. Freshly isolated cells and cells harvested from culture at day 7 were investigated for Bcl-2 (*A*) and Bcl-x_L (*B*) expression by flow cytometry (one representative experiment is shown). The percentage decrease in Bcl-2 expression (MFI) for five cord and five adult CD45RA$^+$ T cell samples after 7 days without added cytokines are shown in *C*. The percentage decrease in Bcl-x_L expression in CD45RA$^+$ T cells from four adults and four cord samples under similar conditions is shown in *D*. Horizontal bars indicate the mean percentage decrease in expression for the different groups.

in cord and adult CD45RA$^+$ T cells showed that the mean expression by cord T cells decreased by 42.9%, whereas that of adults decreased by 3% (Fig. 5*C*). The mean expression of Bcl-x_L was decreased by 35% in cultured CD45RA$^+$ cord T cells compared with the starting population, whereas in adults Bcl-x_L expression decreased by a mean of 11% without cytokines (Fig. 5*D*). Although both Bcl-2 and Bcl-x_L were consistently decreased in all the cord samples tested after culture, the magnitude of the decrease was variable. One reason for this was that the decrease in expression of these proteins occurred earlier in some cells than in others, which resulted in a bimodal expression of these molecules in cultured cord T cells (representative experiment shown in Fig. 5, *A* and *B*). Although these bimodal profiles convincingly demonstrate a decrease in expression of Bcl-2 and Bcl-x_L in a substantial proportion of cells, the net mean fluorescence intensity (MFI) of these bimodal peaks showed little change compared with the original population (Fig. 5*A*; MFI day 0 is 54 whereas MFI of cells cultured for 7 days without IL-7 is 50). In addition, the preferential death of cultured cord blood CD45RA$^+$ T cells with low expression of the apoptosis-regulating molecules underestimates the extent of the decrease, since the remaining viable cells have apparently higher concentrations of these molecules. Adult T cells, on the other hand, do not decrease the expression of these molecules to the same extent as cord T cells and are relatively resistant to apoptosis under similar conditions (see Fig. 1).

When cord blood T cells were cultured in the presence of different IL-2R common γ-chain cytokines, Bcl-2 and Bcl-x_L expression increased in every experiment performed (Fig. 6; representative staining profile for CD45RA$^+$ T cells cultured with or without IL-7 in Fig. 5, *A* and *B*). Although all the IL-2R common γ-chain cytokines increase Bcl-2 and Bcl-x_L expression, the greater effect of IL-7 on cell survival and recovery suggests that signaling components of the IL-7R, other than the γ-chain, also have a role in the effects of this cytokine.

Effects of anti-CD3 activation on the phenotype of cytokine-expanded T cells

We next investigated whether cytokine-expanded cord blood CD45RA$^+$ T cells could be induced to express CD45RO after TCR ligation. Following stimulation with immobilized anti-CD3 for 48 h in the absence of APCs and costimulatory signals, cord blood CD45RA$^+$ RO$^-$ T cells that had been expanded by cytokines for 6 days acquired CD45RO$^+$ expression (Fig. 7, *E–H*). Although the majority of cells at 48 h showed dual expression of both CD45RA and CD45RO (Fig. 7), at later time points (5 days), the majority of cells were CD45RA$^-$,CD45R0$^+$, confirming our previous results (6, 7, 22). Freshly isolated cord blood CD45RA$^+$ T cells that were not cultured in cytokines could also convert to CD45RO expression after anti-CD3 stimulation as demonstrated previously (22). In addition, the small number of CD45RA$^+$ T cells remaining after 7 days of culture in the absence of cytokines could also convert to CD45RO expression upon stimulation with anti-CD3 Ab (data not shown).

CD95 is a cell surface molecule expressed by T cells upon activation. Triggering by its ligand (CD95L) induces a cascade of intracellular events culminating in apoptosis (30–32). Although cord blood CD45RA$^+$ T cells express lower concentrations of

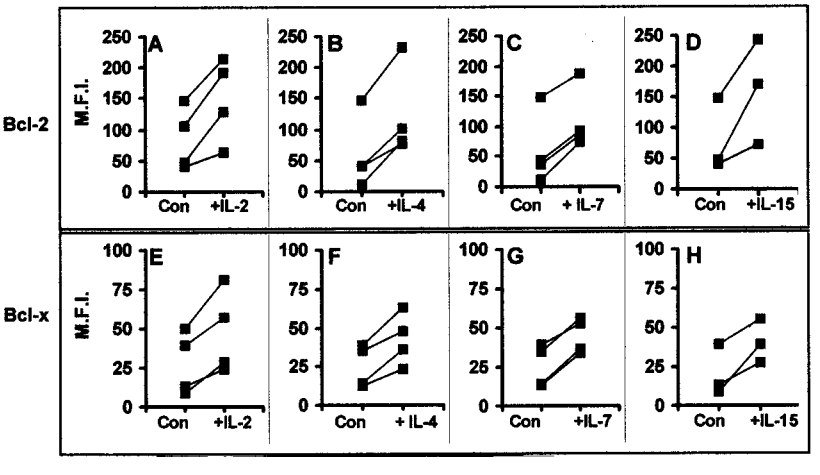

FIGURE 6. Increase in Bcl-2 (*A–D*) and Bcl-x_L (*E–H*) expression of cord blood CD45RA$^+$ T cells in the presence of IL-2R γ- chain cytokines after 7 days of culture as compared with control cultures (Con) without cytokines. Each pair of points represents the MFI of an individual cord CD45RA$^+$ T cell sample in the presence or absence of the cytokine indicated. IL-6 used as a control did not prevent apoptosis, and similar cell numbers to the control cultures were left after 7 days of culture. These residual IL-6-cultured cells did not show enhanced Bcl-2 or Bcl-x_L expression.

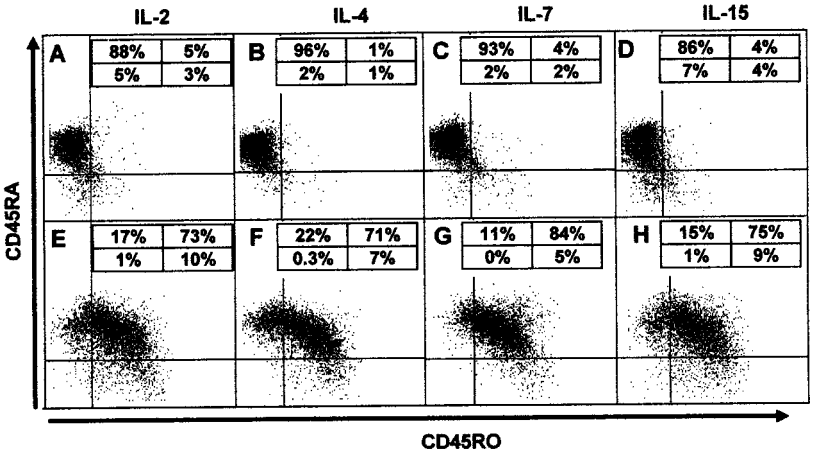

FIGURE 7. CD45RA$^+$ to CD45RO$^+$ conversion by cord blood T cells after anti-CD3 stimulation. After a 6-day period of culture in medium supplemented with the different cytokines indicated, cord blood T cells were stimulated with immobilized anti-CD3. After 48 h, CD45RA and CD45RO expression was analyzed in CD3-stimulated (*E–H*) and control non-CD3-stimulated (*A–D*) cultures. The numbers represent the percentage of cells found in each quadrant.

CD95 than unstimulated adult CD45RA$^+$ T cells, the IL-2R common γ-chain cytokines induced striking increases in CD95 expression in both CD4$^+$ and CD8$^+$ T cells (data not shown). It was possible, therefore, that the major outcome of anti-CD3 stimulation was death rather than proliferation of cytokine-expanded CD45RA$^+$ T cells and that the cells that convert to CD45RO were only a minor residual subset. To clarify this, we examined the extent to which apoptosis and proliferation occurred after activation with anti-CD3 (Fig. 8). After 24 h, there was an increase in apoptosis but proliferating cells were also present. After 2 days (Fig. 8) and 5 days (not shown), proliferating cells predominated, and cell numbers increased, indicating that cytokine-expanded CD45RA$^+$ T cells could be induced to proliferate by TCR ligation.

Telomere length and telomerase activity and in cord blood T cells

Telomere length has been used to compare the replicative history as residual replicative capacity of T cell subpopulations, although the modulating effects of telomerase, which is able to add back telomeric sequences, must also be considered. We therefore looked for telomere shortening after IL-7-induced proliferation. CD45RA$^+$ cord blood T cells were cultured with anti-CD3 in the presence or absence of IL-2 or IL-7. Samples were collected for both telomere length (Fig. 9) and telomerase activity analysis (Fig. 10).

Telomere length was analyzed in four different cord blood samples before and after 12 days in IL-7-supplemented medium (2 representative cord samples shown in Fig. 9). We found that the extensive proliferation induced by IL-7 caused the mean TRF to decrease by only 0.5 kb (Fig. 9). Nevertheless, IL-7-cultured cord blood T cells still maintained longer telomeres than control freshly isolated adult CD45RA$^+$ T cells.

We then investigated telomerase activity in these samples. Freshly isolated cord blood CD45RA$^+$ T cells had very low telomerase activity (Fig. 10) which was maintained in cells cultured for 1 day with either IL-2 or IL-7 but increased in anti-CD3-stimulated cells. Telomerase activity was substantially higher after 4 days in culture in both anti-CD3-treated cultures and cells cultured with IL-7 alone. Cells cultured with IL-2 showed only a small up-regulation of telomerase. Fig. 10 shows one representative experiment of four performed to date. Under all experimental conditions, telomerase activity returned to basal levels at later time points (e.g., day 11), even when cytokines were replenished.

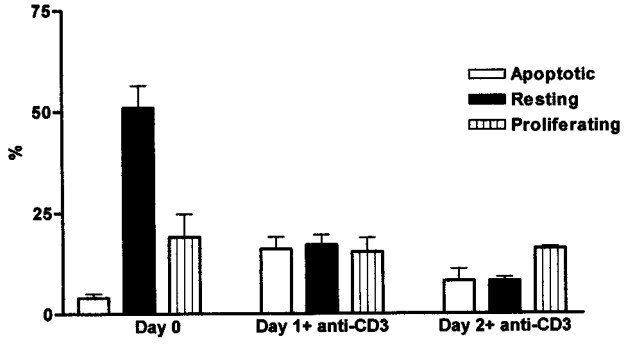

FIGURE 8. Effect of anti-CD3 on proliferation and apoptosis of IL-7-expanded cord blood T cells. After a 6-day period of culture with IL-7, cord blood CD45RA$^+$ T cells were transferred to anti-CD3-coated plates. PI incorporation was analyzed before and after 1 and 2 days of anti-CD3 stimulation. The results represent the mean and S.E.M. of five separate experiments.

Discussion

The study of cord blood T cell function is becoming increasingly important because of its potential use in bone marrow reconstitution with low incidence of graft-vs-host disease (33). In addition, the identification of factors that maintain a naive (CD45RA$^+$) T cell repertoire may be important for the understanding of why depletion of this compartment occurs in patients with HIV infection (34). For these reasons, we have investigated the differential regulation of apoptosis and proliferation in cord blood and adult CD45RA$^+$ T cells to identify factors that may regulate the peripheral naive T cell repertoire.

Neonatal CD45RA$^+$ T cells were more susceptible to spontaneous apoptosis than adult CD45RA$^+$ T cells. This was associated with the inability of the former population to maintain the expression of anti-apoptotic molecules such as Bcl-2 and Bcl-x_L. Since neonatal CD45RA$^+$ T cells are susceptible to apoptosis, factors that prevent death must be present in vivo to enable expansion of the immune system in newborns (4). We have shown that the IL-2R γ-chain-signaling cytokines (in particular IL-7) are a group of factors that not only prevent apoptosis but also induce proliferation of cord blood CD45RA$^+$ T cells. One possible reason for the preferential action of IL-7 in neonatal T cells is the constitutive expression of the IL-7 receptor on cord compared with adult CD45RA$^+$ T cells (35, 36). These data support and extend previous reports showing that postthymic peripheral T cells in neonates require the presence of additional factors to attain full maturity (22) by revealing that the presence of anti-apoptotic factors is also required. Previous studies support the hypothesis that IL-7 and other IL-2R common γ-chain-signaling cytokines may have a central involvement in the maintenance of homeostasis of T cells at

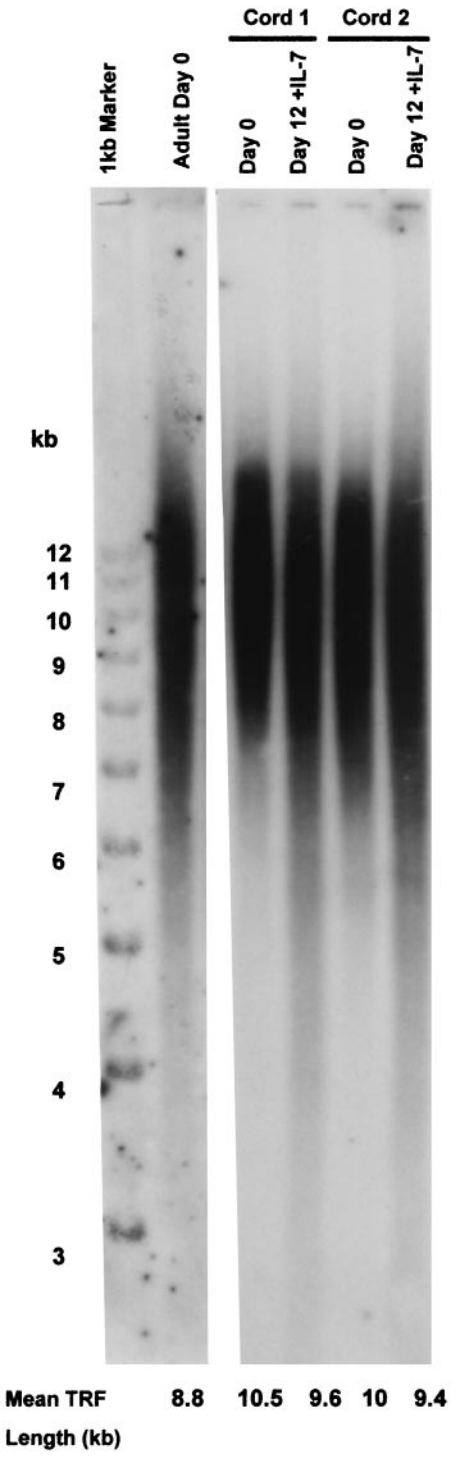

FIGURE 9. Mean terminal restriction length analysis on cord blood T cells. Autoradiography of TRF analysis on one representative adult T cell sample and two of four cord blood T cell samples analyzed. Cord blood samples were collected at day 0 and after 12 days in the presence of IL-7. All the tracks shown were obtained from samples processed at the same time and are on the same gel. The mean TRF length is indicated on the bottom of each track. Similar results were obtained on two additional cord samples tested. The m.w.s (kilobases) of the bands from the 1-kb DNA ladder are included.

both early and late stages of differentiation (37–40). These studies also suggest that IL-7 exerts its action on cord and adult T cell survival in part via a Bcl-2-dependent mechanism, which supports our current and previous observations (23, 37, 38). Our data, reported here, suggest that IL-2, IL-4, and IL-15, which also signal via the γ-chain of the IL-2R, up-regulate Bcl-2 and Bcl-x_L in cord $CD45RA^+$ T cells. IL-7, however, consistently has a greater effect on survival of these T cells. This suggests that Bcl-2 and Bcl-x_L up-regulation is not the only way by which IL-7 exerts its effect, other signals perhaps mediated by the IL-7R α-chain may also contribute to the proliferation/survival of cord $CD45RA^+$ T cells (38). In addition to its role in maintaining homeostasis, IL-7 has also been shown to be essential for the functional development of neonatal T cells and induces the initial expression of IL-4 by activated neonatal $CD4^+$ T cells (35).

The non-Ag-specific expansion of $CD45RA^+$ T cells may also be induced by other cytokines apart from IL-7. It has been reported that a combination of IL-2, TNF-α, and IL-6 can stimulate $CD45RA^+$ RO^- T cell proliferation without a switch to $CD45RO^+RA^-$ (11, 12). In both these and our studies, the cytokine-expanded cells can be stimulated through the TCR to switch to CD45RO expression (11, 12). It is of interest that cytokine-expanded $CD45RA^+$ T cells express increased levels of CD95 and other adhesion molecules (12) (N. Borthwick and A. Akbar, unpublished observations). Previous studies demonstrated that a proportion of freshly isolated $CD45RA^+$ T cells in adults express increased levels of adhesion molecules (41, 42). It has been suggested that these $CD45RA^+$ T cells were revertant $CD45RO^+$ T cells (41, 42). In contrast, our results together with those of Unutmaz et al. (12) suggest the possibility that these cells may be cytokine-expanded $CD45RA^+$ T cells. We have also shown that IL-4 alone could induce the proliferation of cord $CD45RA^+$ T cells in agreement with previous reports (43) and have extended these observations by showing that that conversion to $CD45RO^+$ did not occur. Other cytokines that induce bystander T cell proliferation (44) should now also be investigated for their effect on the $CD45RA^+$ T cell pool.

Cell proliferation is generally associated with telomere shortening, which results in replicative senescence (13–16). Telomeric repeats are synthesized by telomerase, a riboprotein that uses its RNA component as a template (45, 46). Telomerase is constitutively expressed on germline and tumor cells (15). Although most somatic cells do not express telomerase, the low activity seen in resting T cells is increased upon activation in vitro (14, 47–51) and in vivo[4]. Up-regulation of telomerase activity in activated T lymphocytes may retard the loss of telomeres and the development of senescence (13–16). We therefore investigated whether cytokine-driven expansion of $CD45RA^+$ T cells induced changes in telomerase activity and telomere length. We found low telomerase activity in ex vivo naive T cells derived from cord blood, confirming previous observations on peripheral blood T cells (48, 52, 53). Surprisingly, we found that upon culture with IL-7, cord blood T cells strongly up-regulated telomerase activity to the same extent as anti-CD3-stimulated cells. After an initial burst of strong telomerase activity which lasted for 4–6 days, it declined dramatically. In agreement with previous reports, we found no significant telomerase up-regulation upon culture with IL-2 alone. Cord blood T cells cultured with IL-7 were found to have decreased telomere length after a 12-day period, suggesting that under these in vitro conditions, telomerase activity may not sufficient to completely abrogate the progress toward replicative senescence. Nevertheless, the mean TRF length was still longer in IL-7-expanded $CD45RA^+$ T cells than in adult $CD45RA^+$ T cells. It would be predicted that

[4] Maini, M. K., M. V. D. Soares, C. F. Zilch, A. N. Akbar, and P. C. L. Beverley. Telomerase up-regulation in an acute viral infection: a mechanism to maintain the replicative capacity of $CD8^+$ T cells undergoing clonal expansion. *Submitted for publication.*

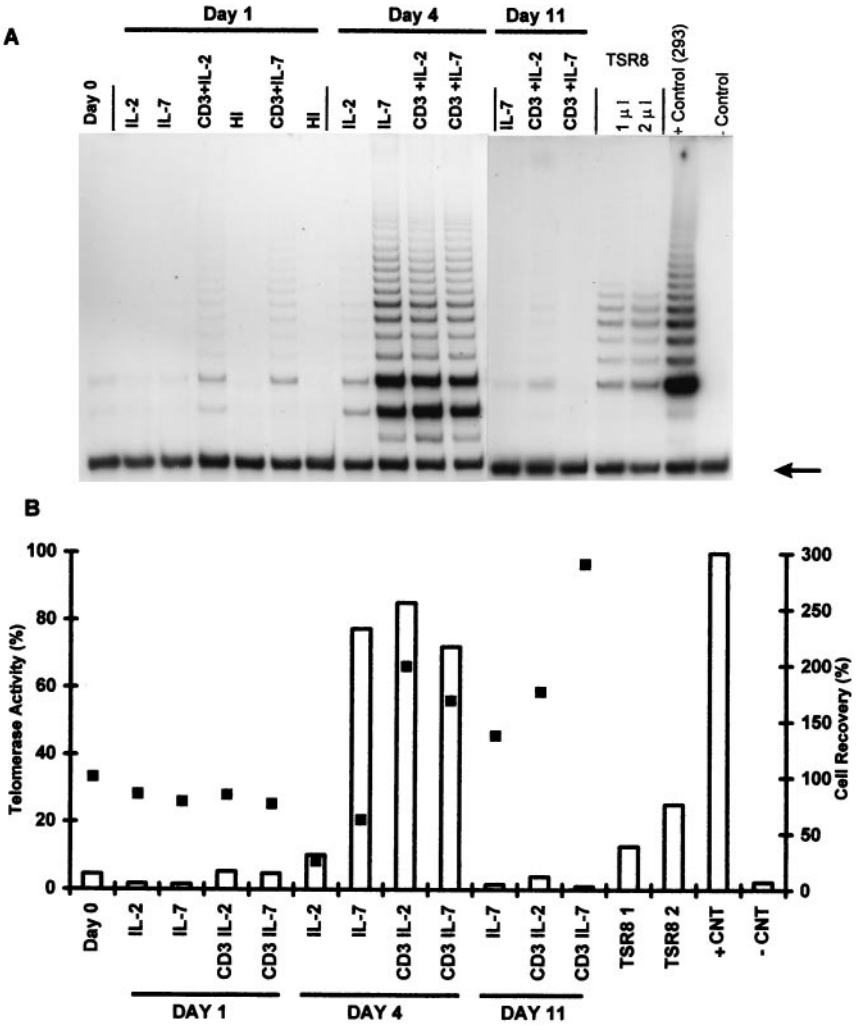

FIGURE 10. Telomerase activity in cord blood T cells. Telomerase activity was measured in CD45RA$^+$-enriched cord blood T cell samples, supplemented with IL-2 or IL-7 and in the presence or absence of anti-CD3. Cultured cells were harvested at days 0, 1, 4, and 11. Activity was measured on extracts from 5×10^5 cells using the TRAP assay described earlier (see *Materials and Methods*). Autoradiography of TRAP assay acrylamide gel showing a representative experiment from four performed. *A*. Cell extract heat inactivated at 85°C for 10 min before PCR reaction to inactivate the RNA component of telomerase (*HI*). The arrow shows the internal PCR control 36-bp band. The control template (oligo containing the same sequence as TS primer plus 8 telomeric repeats) *TSR8* was used in two dilutions (1 and 2 μl of oligo added, TSR8 1 and 2). The positive control (*+CNT*) is the 293 transformed cell line which was used for calculating the relative telomerase activity as a percentage of the activity obtained for that transformed line. For the negative control (*−CNT*) the cell lysis buffer replaced extract. Relative telomerase activity and cell recovery analysis on cord blood T cells (*B*). Telomerase activity was calculated as a percentage of the activity obtained by the positive control cell line. Cell recovery was calculated as a percentage of the initial input of cells and is indicated for each condition by ■.

excessive expansion by CD45RA$^+$ T cells would eventually result in senescence and growth arrest. Since IL-7-expanded T cells acquire resistance to apoptosis, this may be an important mechanism, which protects against uncontrolled cytokine-mediated expansion of the naïve/unprimed T cell pool. These results are compatible with observations that in aged subjects, CD45RA$^+$ T cells have shorter telomeres than similar cells from young individuals, the former cells having progressed further toward senescence than the latter (19). Although this may be the result of reversion of CD45RO$^+$ T cells with shorter telomeres to CD45RA$^+$ expression, our current data suggest that bystander activation by cytokines, which aged cells have experienced more frequently, may also contribute to these observations. Collectively, these results suggest that IL-7 contributes to the maintenance of the naive T cell pool by slowing down rather than completely abrogating the development of senescence of expanded CD45RA$^+$ T cells.

The factors that regulate the size of the CD45RA$^+$ T cell pool in adults are not well defined, but clearly, mechanisms that enable the replenishment of CD45RA$^+$ T cells throughout life are required to prevent the eventual loss of this subset through recruitment, by activation, into the CD45RO$^+$ pool followed by removal through apoptosis or replicative senescence (14, 54). Apart from reversion of cells from CD45RO to CD45RA (9), we now suggest that expansion of CD45RA$^+$ T cells by IL-7 may be a mechanism for extrathymic expansion of the developing T cell system in neonates in a non-Ag-specific manner and may also contribute to the maintenance of the CD45RA$^+$ T cell pool in adults.

An important question is whether there is sufficient IL-7 production in vivo to enable CD45RA$^+$ T cell stimulation to occur. It has been shown that IL-7 is found in adult human intestinal epithelial cells and is intimately involved in the regulation of mucosal lymphocyte proliferation (36). Also, the recently reported development of chronic colitis in IL-7-transgenic mice indicates that chronic inflammation at this site may be mediated by excessive colonic epithelial cell-derived IL-7 (55). We have also recently demonstrated that there is high level of IL-7 expression by epithelial cells in neonatal gut (L. Poulter, D. Howie, T. T. MacDonald, and A. Akbar, unpublished observations) and that considerable levels of T cell proliferation can be observed in this tissue (56). These data are compatible with the idea that IL-7-driven proliferation may take place in certain anatomic sites in both adults and neonates.

In summary, we have provided evidence that CD45RA$^+$ T cell proliferation induced by cytokines such as IL-7 which induce proliferation without a switch to CD45RO expression. This cytokine-mediated expansion may have an important role in extrathymic expansion of neonatal cells during growth and may also contribute to the maintenance of a naive T cell repertoire in adults. Further investigations into the mechanisms that regulate the homeostasis of the CD45RA$^+$ T cell pool are important to rationalize ways in which the naive T cell compartment may be reconstituted in patients who are immunodeficient for various reasons.

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