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Premature Aging of the Immune System in Children With Juvenile Idiopathic Arthritis

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Objective. **Juvenile idiopathic arthritis (JIA) is an autoimmune disease of the young. The pathogenesis is not completely understood. Premature aging, associated thymic involution, and compensatory autoproliferation could play important roles in the pathogenesis of autoimmunity. We undertook this study to determine whether patients with JIA demonstrate premature immunosenescence.**

Methods. **To test this hypothesis, we measured 3 indicators of aging: the percentages and total counts of peripheral blood naive T cells, the frequency of T cell receptor excision circles (TRECs) in naive T cells, and telomeric erosion and Ki-67 expression as estimates of the replicative history of homeostatic proliferation.**

Results. **JIA patients showed an accelerated loss of CD4+,CD45RA+,CD62L+ naive T cells with advancing age and a compensatory increase in the number of CD4+,CD45RO+ memory T cells. JIA patients demonstrated a significantly decreased frequency of TRECs in CD4+,CD45RA+ naive T cells compared with age-matched healthy donors ($P = 0.002$). TREC numbers correlated with age only in healthy donors ($P = 0.0001$). Telomeric erosion in CD4+,CD45RA+ naive T cells was increased in JIA patients ($P = 0.01$). The percentages of Ki-67–positive CD4+,CD45RA+ naive T cells were increased in JIA patients ($P = 0.001$) and correlated with disease duration ($P = 0.003$), which was also an independent factor contributing to telomeric erosion ($P = 0.04$).**

Conclusion. **Our findings suggest that age-inappropriate T cell senescence and disturbed T cell homeostasis may contribute to the development of JIA. In patients with JIA, dysfunction in the ability to reconstitute the T cell compartment should be considered when exploring new therapeutic strategies.**

Juvenile idiopathic arthritis (JIA), which currently is divided into 7 subtypes (1), is a heterogeneous inflammatory disorder of unknown etiology. Common to all forms of JIA is onset prior to age 16 years and duration of at least 6 weeks (2,3). Although several putative autoantigens have been implicated as candidate antigens in JIA, the "disease-triggering" antigen has not been identified (4). These findings support the concept that autoimmune diseases are not caused by a single autoantigen but rather result from a general dysfunction of peripheral immune system tolerance or homeostasis mechanisms.

Patients with T cell–mediated autoimmune diseases present immune system abnormalities that resemble the typical characteristics of immune dysfunction described in the elderly. Several lines of evidence suggest that T cells play an important role in the pathogenesis of adult rheumatoid arthritis (RA). Generally, the incidence of autoimmune diseases increases with advancing age. Thymic involution is supposed to be an important aspect of immunosenescence (5). In the process of thymic involution, the epithelial space (where T

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cell maturation and selection take place) is replaced by fatty tissue. This restructuring process causes a decline in the output of recent thymic emigrants. A space-filling autoproliferation mechanism, known as homeostatic proliferation, keeps peripheral T cell numbers at a constant level throughout life and becomes particularly important with advancing age (6). However, this mechanism induces replicative stress on peripheral T cells. Several research groups have observed T cell clonal expansions in the peripheral blood of RA patients (7–10) and have reported a population of T cells with signs of replicative stress in RA patients (8–12). Replicatively stressed cells have lost the expression of the major costimulatory molecule CD28, accompanied by phenotypic and functional changes (13).

Recent thymic emigrants have been identified by analyzing T cell receptor excision circles (TRECs), which are stable DNA episomes that form during T cell receptor rearrangement. Since TRECs are not replicated during mitosis, they are diluted out during cell divisions, which include not only priming of recent thymic emigrants to become memory T cells, but also homeostatic cell division of naive T cells. TREC numbers among CD4+ T cells decrease 50–100 times during aging (14–16). Absolute numbers of naive CD4+ T cells, as characterized by expression of CD45RA, decrease only by a factor of 2 or 3 with age (17).

Telomeres are TTAGGG-rich repeats located at the ends of chromosomes and have an important role in DNA replication and preservation of chromosome integrity (18). Telomere erosion has been considered a mitotic clock, with the telomere length approximately reflecting the life history of divisions of individual cells. Telomere lengths in naive T cells progressively decline with age (19). Therefore, consumption of telomeric DNA can be used to estimate the replicative history of cells in the T cell compartment. Studies in patients with RA (20) and in those with systemic lupus erythematosus (SLE) (21) have shown telomeric erosion in peripheral blood mononuclear cells (PBMCs) compared with controls.

Thus, premature aging, associated thymic involution, and compensatory autoproliferation could play important roles in the pathogenesis of autoimmunity. Moreover, premature aging of the immune system could be a risk factor for developing autoimmune disorders in genetically predisposed individuals in a susceptible environment (22). To determine whether pediatric patients with JIA have premature immunosenescence, we measured 3 indicators of aging: the percentages and total counts of peripheral blood naive T cells, the frequency of TRECs in naive T cells, and telomeric erosion and Ki-67 expression as estimates of the replicative history of homeostatic proliferation.

PATIENTS AND METHODS

Study population. PBMCs were obtained from 22 patients with JIA (12 with oligoarticular JIA, 9 with polyarticular JIA, and 1 with systemic JIA) who fulfilled the 1998 International League of Associations for Rheumatology criteria for JIA (1,23) and from 37 healthy donors. Disease activity in JIA patients was evaluated by clinical examination as well as by measurement of the C-reactive protein (CRP) level and the erythrocyte sedimentation rate (ESR). All patients included in the study had an inactive disease state at the time of evaluation, defined by a normal CRP level and ESR and clinically stable disease for 3 months. Clinical data were obtained by retrospective chart review. None of the healthy donors had a personal or family history of inflammatory disease. Characteristics of the JIA patients and healthy donors are summarized in Table 1. The study was conducted in accordance with the Declaration of Helsinki (2000 version) and approved by the local ethics committee of the Medical University Innsbruck. All JIA patients and healthy donors gave their written informed consent to participate in this study.

Separation of T cell subsets. PBMCs were isolated by using LymphoPrep (Axis Shield, Oslo, Norway) according to the manufacturer's instructions. CD4+,CD45RA+ (naive) T cells were separated by negative selection using a naive CD4+ T cell isolation kit (Miltenyi Biotec, Teterow, Germany), magnetic beads, and an AutoMACS system with sterile columns (Miltenyi Biotec). The purity of separated CD4+, CD45RA+ T cells was checked using 4-color flow cytometry (FACSCalibur flow cytometer; Becton Dickinson, Oxford, UK) and ranged from 97% to 99%.

Quantification of T cell subsets. PBMCs were incubated for 20 minutes at room temperature in the dark with mouse monoclonal antibodies (mAb) specific for CD4, CD8, CD45RA, CD45RO, CD28, CD25, and CD62L labeled with fluorescein isothiocyanate, phycoerythrin, peridin chlorophyll protein, or allophycocyanin (all antibodies were purchased from BD PharMingen, San Jose, CA). After incubation, red blood cell lysis was performed with FACS Lysing Solution (BD PharMingen). Subsequently, cells were washed twice with phosphate buffered saline and fixed with 2% paraformaldehyde. All analyses were performed using a FACSCalibur flow cytometer utilizing CellQuest software (BD PharMingen). Results were expressed as the percentage of gated lymphocytes. According to phenotypic CD markers, CD4+, CD45RA+ and CD4+,CD45RA+,CD62L+ T cells were characterized as naive T cells, CD4+,CD45RO+ T cells were characterized as memory T cells, CD4+,CD25+,CD62L+ T cells were characterized as Treg cells, and CD4+,CD28− T cells were characterized as total effector T cells.

Quantification of TREC numbers. DNA was extracted from separated CD4+,CD45RA+ T cells using the QIAamp DNA Mini Kit (Qiagen, Chatsworth, CA). In order to remove contaminants that would interfere with polymerase chain reaction (PCR), DNA was purified by ethanol precipitation using 0.4*M* $LiCl_2$ and 2.5-fold the volume of 100% ethanol at

Table 1. Characteristics of the JIA patients and healthy donors*

Parameter	Healthy donors (n = 37)	JIA patients (n = 22)
Chronological age, median (range) months	162 (61–214)	156 (31–229)
No. of women/no. of men	22/15	15/7
Age at diagnosis, median (range) months	–	61 (12–195)
Disease duration, median (range) months	–	62 (5–160)
Medications, no. receiving		
MTX	–	10
Cortisone	–	4
NSAID	–	17
Anti-TNFα therapy	–	2
None	37	5
RF positive, no.	0	2
ANA positive, no.	0	15
CRP, median (range) mg/dl	0.09 (0.10–1.95)	0.14 (0.10–1.89)
Blood leukocytes, mean (range) $\times 10^9$/liter	6.7 (3.7–13.1)	6.8 (2.8–13.5)
Absolute neutrophils, mean (range) $\times 10^9$/liter	3.6 (1.1–8.9)	4.0 (1.5–9.9)
Absolute lymphocytes, mean (range) $\times 10^9$/liter	2.3 (1.3–4.1)	2.5 (0.9–11.1)
Blood erythrocytes, mean (range) $\times 10^{12}$/liter	4.8 (3.4–5.6)	4.7 (3.9–5.5)
Hemoglobin, mean (range) gm/liter	134.2 (103.0–154.0)	131.4 (94.0–149.0)
Blood platelets, mean (range) $\times 10^9$/liter	281.5 (157.0–445.0)	316.7 (251.0–500.0)

* JIA = juvenile idiopathic arthritis; MTX = methotrexate; NSAID = nonsteroidal antiinflammatory drug; anti-TNFα = anti–tumor necrosis factor α; RF = rheumatoid factor; ANA = antinuclear antibody; CRP = C-reactive protein.

−20°C for 30 minutes. After centrifugation, the pellet was washed twice with 70% ethanol to remove the remaining salts. The pellet was dissolved in nuclease-free water. Signal-joint TREC concentrations were determined by quantitative SYBR Green real-time PCR based on the coding TREC sequence, using an iCycler quantitative reverse transcriptase–PCR system (Bio-Rad, Hercules, CA). We designed primers to amplify a DNA fragment 82 bp across the remaining recombination sequence δrec/ψalpha (5′-CAC-ATC-CCT-TTC-AAC-CAT-GCT-3′ [forward] and 5′-GCC-AGC-TGC-AGG-GTT-TAG-G-3′ [reverse]). For quantification, we used the internal standard, as previously described (14). The PCR was run with 0.5 μg DNA, primers, and SYBR Green Supermix (Bio-Rad) in a final volume of 25 μl. Each experiment was performed in duplicate, and $\log_2$ dilutions of the internal standard were used to quantify the number of TRECs in each sample. To avoid bias arising from different numbers of naive T cells, the number of TRECs was calculated in relation to CD4+, CD45RA+ T cell numbers (24).

Telomere length analysis. Determination of relative telomere length was performed by calculating the ratio of a quantitative PCR product from the same sample using specific primers for telomeres and a single copy gene, as described previously (25,26). Quantitative PCR is the method of choice for determining telomere length in small extractable quantities of DNA, as was the case in our study.

Ki-67 staining. Naive T cells in the cell cycle were identified by expression of the Ki-67 nuclear antigen. Determination of Ki-67 expression on CD4+,CD45RA+ T cells was performed by cytospin preparation (Cytospin 4; Shandon, Waltham, MA) of 5×10^3 CD4+,CD45RA+ T cells. After fixation with 4% paraformaldehyde, cells were stained first with mouse anti-human Ki-67 antibody (MIB-1 clone; Dako, Glostrup, Denmark) and second with peroxidase-conjugated goat anti-mouse mAb (Dako). The second antibody was developed with 3,3′-diaminobenzidine tetrahydrochloride (iVIEW DAB Detection; Ventana Medical Systems, Tucson, AZ). Immunohistochemistry was performed using a fully automated Nexes system (Ventana Medical Systems). For microscopic orientation, counterstaining was performed with Hematoxylin Counterstain and Bluing Reagent (Ventana Medical Systems). Stained cells were examined by 2 independent investigators (MP and AB) by light microscopy (Eclipse 800; Nikon, Tokyo, Japan). Percentages of Ki-67–positive cells were calculated per total CD4+,CD45RA+ T cell count on each cytospin preparation.

Statistical analysis. The Mann-Whitney U test for independent variables was used to compare healthy donors with JIA patients. Spearman's rank correlation coefficient was used to correlate percentages and numbers of T cell subsets, the relative telomere length, percentages of Ki-67–positive CD4+,CD45RA+ T cells, and the TREC numbers in

Table 2. Total counts of T cell subsets, numbers of TRECs, and relative telomere length in JIA patients and healthy donors*

	Healthy donors		JIA patients		
	Mean ± SD	Median (range)	Mean ± SD	Median (range)	*P*†
T cell subset					
CD3+	1.97 ± 0.39	1.90 (1.20–2.80)	1.70 ± 0.56	1.50 (1.10–3.0)	0.01
CD3+,CD4+	1.12 ± 0.26	1.08 (0.78–1.84)	0.88 ± 0.38	0.73 (0.33–1.83)	0.001
CD4+,HLA−DR+	0.11 ± 0.07	0.09 (0.03–0.36)	0.08 ± 0.03	0.08 (0.02–0.2)	0.04
CD4+,CD25+,CD62L+	0.24 ± 0.13	0.20 (0.11–0.77)	0.16 ± 0.11	0.15 (0.0004–0.52)	0.002
CD4+,CD28+	1.07 ± 0.26	1.04 (0.63–1.78)	0.81 ± 0.40	0.69 (0.02–1.73)	0.001
CD4+,CD28−	0.01 ± 0.01	0.01 (0.001–0.07)	0.01 ± 0.01	0.01 (0.001–0.05)	0.05
CD4+,CD45RA+	0.53 ± 0.23	0.55 (0.03–1.06)	0.47 ± 0.34	0.36 (0.006–1.44)	0.08
CD4+,CD45RA+,CD62L+	0.65 ± 0.22	0.66 (0.17–1.24)	0.55 ± 0.34	0.45 (0.01–1.39)	0.04
CD4+,CD45RO+	0.30 ± 0.11	0.31 (0.03–0.57)	0.42 ± 0.19	0.41 (0.003–0.92)	0.002
TRECs	325.1 ± 231.5	244.1 (114.8–1,370.2)	251.5 ± 198.8	175.4 (87.4–688.5)	0.002
Relative telomere length	2.0 ± 1.1	2.0 (0.03–4.3)	1.1 ± 0.6	1.0 (0.01–3.0)	0.01

* Values for T cell subsets are the number of cells ($\times 10^3/\mu$l). Values for T cell receptor excision circles (TRECs) are the number of TRECs per 10^3 CD4+,CD45RA+ T cells. Values for relative telomere lengths of CD4+,CD45RA+ T cells are a ratio of polymerase chain reaction products of telomeres and a single copy gene used as a standard (25,26). JIA = juvenile idiopathic arthritis.
† By Mann-Whitney U test.

CD4+,CD45RA+ T cells with chronological age, age at diagnosis, and disease duration (SPSS version 15.0; SPSS, Chicago, IL). Stepwise multiple linear regression including age, sex, disease duration, and current therapy (steroids, methotrexate) was used to determine relationships between clinical parameters and laboratory values. *P* values less than 0.05 were considered significant.

RESULTS

Reduction of naive T cell numbers with advancing age in JIA patients. Initially, we compared the total counts (Table 2) and the percentages (Table 3) of peripheral CD4+ T cells expressing naive and memory surface markers in JIA patients and healthy donors. Traditionally, isoforms of CD45, along with expression of the lymph node homing receptor CD62L, have been used to distinguish naive from memory T cells. Therefore, these markers were used to examine the T cell maturity in our patients and healthy donors. JIA patients showed lower total counts of CD4+ T cells ($P = 0.001$) and CD4+,CD45RA+,CD62L+ T cells ($P = 0.04$) compared with healthy donors (Table 2). Although there was high interindividual variation, JIA patients demonstrated an accelerated decrease in the number of CD4+ naive T cells with advancing age (Figures 1A and B and Table 4). In contrast to healthy donors, at the time of evaluation, patients with JIA showed a correlation between a decrease in the number of naive T cells ($P = 0.006$) and a compensatory increase in the number of CD45RO+ T cells ($P = 0.02$) with chronological age (Figures 1A and C). Studies on human aging have identi-

Table 3. Percentages of T cell subsets and Ki-67–positive CD4+,CD45RA+ T cells in JIA patients and healthy donors*

	Healthy donors		JIA patients		
	Mean ± SD	Median (range)	Mean ± SD	Median (range)	*P*†
T cell subset					
CD3+	67.4 ± 7.2	66.2 (55.9–82.5)	65.8 ± 7.8	65.1 (42.3–80.5)	0.66
CD4+	37.9 ± 6.9	38.8 (25.8–49.7)	39.8 ± 7.6	40.6 (19.5–51.0)	0.28
HLA–DR+	5.7 ± 2.8	4.8 (1.8–15.7)	4.9 ± 1.8	5.1 (1.3–8.8)	0.68
CD25+,CD62L+	22.1 ± 12.2	18.9 (10.8–78.1)	20.0 ± 18.0	19.3 (0.1–74.4)	0.29
CD28+	95.1 ± 3.6	96.3 (79.9–98.9)	91.4 ± 19.8	95.2 (63.2–98.7)	0.85
CD28−	1.4 ± 1.4	1.2 (0.1–8.4)	1.1 ± 1.1	0.7 (0.1–5.5)	0.33
CD45RA+	47.4 ± 15.5	50.7 (3.2–69.4)	49.5 ± 17.7	47.7 (1.2–79.2)	0.90
CD45RA+,CD62L+	61.9 ± 13.9	61.3 (27.5–88.2)	64.9 ± 9.2	63.4 (53.6–81.3)	0.30
CD45RO+	39.6 ± 18.2	36.1 (0.1–75.9)	36.9 ± 13.3	41.3 (5.8–53.1)	0.73
Ki-67–positive T cells	0.2 ± 0.2	0.09 (0.01–1.7)	0.8 ± 0.7	0.5 (0.02–0.2)	0.001

* Values for CD3+ and CD4+ T cells are the percentage of total lymphocytes. Values for Ki-67–positive T cells are the percentage of CD4+, CD45RA+ T cells. Values for all other T cell subsets are the percentage of CD4+ T cells. JIA = juvenile idiopathic arthritis.
† By Mann-Whitney U test.

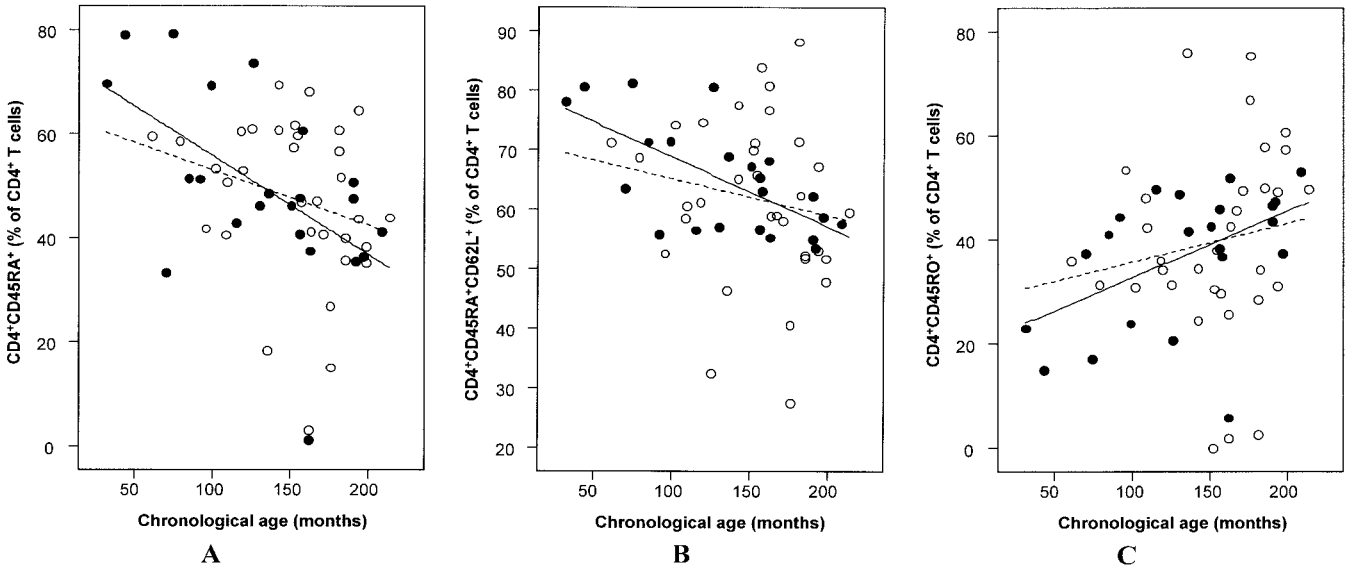

Figure 1. Correlations of CD4+,CD45RA+, CD4+,CD45RA+,CD62L+, and CD4+,CD45RO+ T cells with chronological age. Dot-blots represent the percentages of CD4+,CD45RA+ T cells (for patients with juvenile idiopathic arthritis [JIA], r = –0.564, $P = 0.006$; for healthy donors, r = −0.316, $P = 0.07$) (**A**), CD4+,CD45RA+,CD62L+ T cells (for JIA patients, r = −0.605, $P = 0.003$; for healthy donors, r = 0.260, $P = 0.14$) (**B**), and CD4+,CD45RO+ T cells (for JIA patients, r = 0.490, $P = 0.02$; for healthy donors, r = 0.258, $P = 0.15$) (**C**). Open circles and dashed line represent healthy donors; solid circles and solid line represent JIA patients.

fied the loss of CD28 expression on both CD4+ and CD8+ T cells as a biologic indicator of aging in the immune system (27). However, in both JIA patients and healthy donors, the frequencies of CD4+,CD28− T cells did not increase significantly with advancing age (Table 4).

Reduction of TREC numbers in CD4+, CD45RA+ T cells from JIA patients. Thymic activity and output of recent thymic emigrants in patients with JIA were assessed by measuring the numbers of TRECs in CD4+,CD45RA+ T cells in the peripheral blood of

Table 4. Correlations of total counts/percentages of T cell subsets, numbers of TRECs, relative telomere length, and percentages of Ki-67–positive CD4+,CD45RA+ T cells in JIA patients and healthy donors with chronological age, age at diagnosis, and disease duration*

	Healthy donors, chronological age		JIA patients					
			Chronological age		Age at diagnosis		Disease duration	
	r_s	P	r_s	P	r_s	P	r_s	P
T cell subset								
CD3+	−0.705/0.117	0.0001/0.51	−0.877/0.159	0.001/0.48	−0.334/0.150	0.13/0.51	−0.536/0.159	0.01/0.48
CD3+,CD4+	−0.254/0.416	0.14/0.01	−0.765/0.167	0.001/0.46	−0.335/0.002	0.13/0.99	−0.419/0.036	0.05/0.88
CD4+,HLA−DR+	−0.308/−0.105	0.07/0.55	−0.524/0.202	0.01/0.37	−0.322/−0.045	0.14/0.84	−0.183/0.258	0.42/0.25
CD4+,CD25+,CD62L+	0.244/0.374	0.17/0.03	−0.792/0.172	0.0001/0.44	−0.359/0.219	0.10/0.33	−0.416/−0.182	0.05/0.42
CD4+,CD28+	−0.253/0.236	0.14/0.17	−0.805/−0.163	0.0001/0.47	−0.281/0.077	0.21/0.73	−0.515/−0.037	0.01/0.87
CD4+,CD28−	0.239/0.107	0.17/0.54	−0.035/0.117	0.87/0.60	0.172/0.059	0.44/0.74	−0.206/−0.006	0.36/0.97
CD4+,CD45RA+	−0.284/−0.316	0.11/0.07	−0.759/−0.564	0.0001/0.006	−0.364/−0.390	0.10/0.07	−0.370/0.001	0.09/0.99
CD4+,CD45RA+,CD62L+	−0.228/0.260	0.20/0.14	−0.792/−0.605	0.0001/0.003	−0.359/−0.292	0.10/0.19	−0.416/−0.150	0.05/0.51
CD4+,CD45RO+	0.086/0.258	0.63/0.15	−0.186/0.490	0.41/0.02	−0.056/0.185	0.80/0.41	−0.145/0.256	0.52/0.25
TRECs	−0.818	0.0001	0.154	0.49	−0.063	0.78	0.023	0.92
Relative telomere length	−0.430	0.02	−0.424	0.06	−0.217	0.36	−0.197	0.41
Ki-67–positive CD4+, CD45RA+ T cells	−0.662	0.0001	0.057	0.80	−0.344	0.12	0.608	0.003

* Spearman's rank correlation coefficients are shown for total counts/percentages of T cell subsets, numbers of TRECs per 10^3 CD4+,CD45RA+ T cells, relative telomere lengths of CD4+,CD45RA+ T cells, and percentages of Ki-67–positive CD4+,CD45RA+ T cells. See Table 2 for definitions.

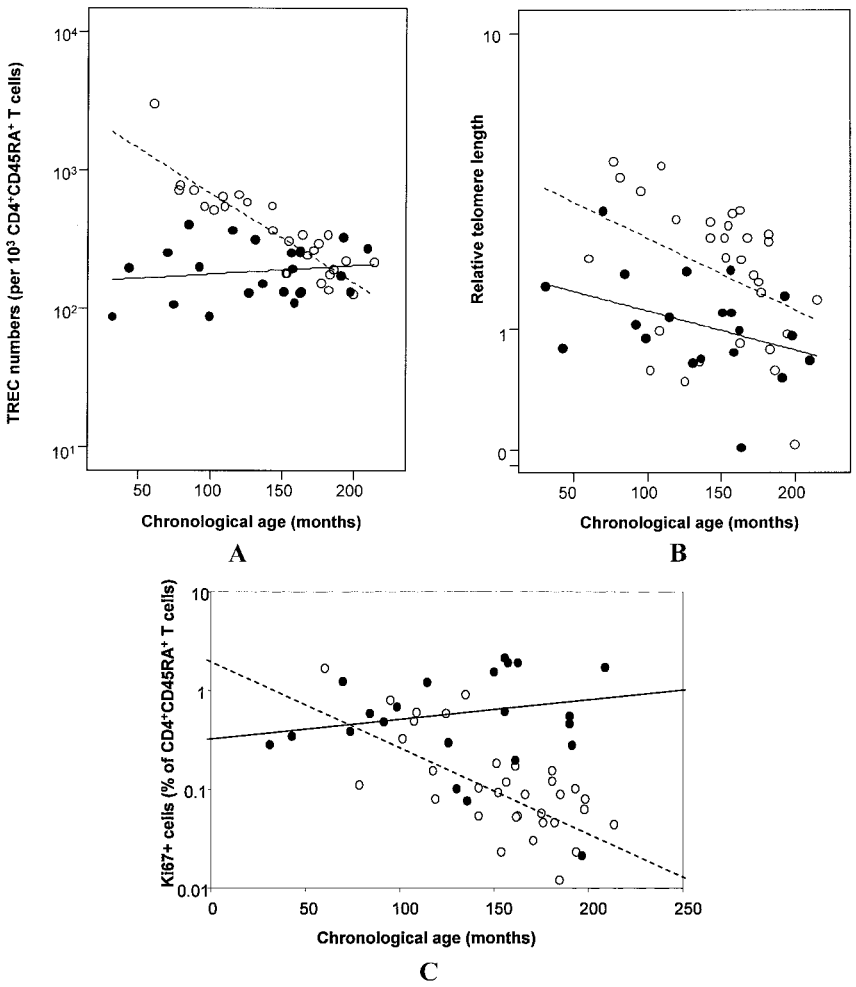

Figure 2. Correlations of T cell receptor excision circle (TREC) numbers, relative telomere length, and percentages of Ki-67–positive cells with chronological age. Dot-blots represent TREC numbers (for patients with juvenile idiopathic arthritis [JIA], r = 0.154, $P = 0.49$; for healthy donors, r = −0.818, $P = 0.0001$) (**A**), relative telomere length of CD4+,CD45RA+ T cells (for JIA patients, r = −0.424, $P = 0.06$; for healthy donors, r = −0.430, $P = 0.02$) (**B**), and percentages of Ki-67–positive cells (for JIA patients, r = 0.057, $P = 0.8$; for healthy donors, r = −0.662, $P = 0.0001$) (**C**). Open circles and dashed line represent healthy donors; solid circles and solid line represent JIA patients.

JIA patients and healthy donors. In healthy donors, there was a significant inverse correlation between age and the number of TRECs in the peripheral blood CD4+,CD45RA+ T cell pool ($P = 0.0001$), reflecting the physiologic decrease in thymic function with age (Figure 2A and Table 4). In JIA patients, TREC numbers did not correlate with age (Figure 2A and Table 4), and JIA patients had fewer TRECs per 10^3 CD4+, CD45RA+ T cells than did age-matched healthy donors ($P = 0.002$) (Table 2).

Telomere shortening and Ki-67 expression in CD4+,CD45RA+ T cells in JIA patients. TREC concentrations are influenced by 2 parameters: the output of newly generated thymic T cells and the dilution of TREC+ T cells through replication of peripheral T cells. The reduction in the number of TRECs in JIA patients could indicate already-impaired thymic T cell production at an early age, which would induce compensatory growth of mature T cells. An age-inappropriate decline in the number of TRECs could also result from a primary dysregulation of peripheral T cell expansion (20). In either case, the CD4+ T cells of JIA patients should have undergone increased replication. To provide an estimate of the replicative history, the relative telomere length of peripheral CD4+,CD45RA+ T cells was examined (Figure 2B). In healthy donors and JIA patients, the relative telomere length declined progressively with age ($P = 0.02$ and $P = 0.06$, respectively)

Table 5. Multiple regression analysis of demographic parameters and therapy in JIA patients*

Parameter	CD4+, CD45RA+ T cells		CD4+, CD45RO+ T cells		CD4+, CD45RA+, CD62L+ T cells		TRECs per 10^3 CD4+, CD45RA+ T cells		Relative telomere length		Ki-67–positive CD4+, CD45RA+ T cells	
	r	*P*	r	*P*	r	*P*	r	*P*	r	*P*	r	*P*
Chronological age	−0.705	0.01	0.543	0.04	−0.766	0.004	0.021	0.95	−0.038	0.87	0.094	0.65
Disease duration	−0.059	0.83	−0.053	0.85	0.101	0.69	0.012	0.97	−0.548	0.04	0.570	0.01
Methotrexate treatment	−0.009	0.97	0.353	0.13	−0.244	0.24	−0.106	0.71	−0.277	0.21	0.328	0.09
Steroid treatment	−0.354	0.17	0.057	0.82	−0.113	0.62	−0.077	0.81	−0.098	0.71	0.200	0.34
Sex	−0.006	0.98	0.252	0.28	−0.119	0.56	0.012	0.97	−0.376	0.10	0.22	0.30

* r indicates the regression coefficient for each variable, by analysis of variance. Standard regression coefficients for the calculated model were as follows: for CD4+,CD45RA+ T cells, r = 0.623; for CD4+,CD45RO+ T cells, r = 0.632; for CD4+,CD45RA+,CD62L+ T cells, r = 0.714; for TRECs per 10^3 CD4+,CD45RA+ T cells, r = 0.256; for relative telomere length, r = 0.737; for Ki-67–positive CD4+,CD45RA+ T cells, r = 0.744. See Table 2 for definitions.

(Table 4). The distributions of relative telomere length in healthy donors and JIA patients were different, with most of the JIA patients showing a shorter relative telomere length than age-matched healthy donors (P = 0.01) (Figure 2B and Table 2).

Proliferation rates of CD4+,CD45RA+ T cells were estimated by measuring the fraction of Ki-67–expressing cells. JIA patients had a significantly higher proportion of CD4+,CD45RA+ T cells in the cell cycle than did healthy donors (P = 0.001) (Table 3). However, in contrast to healthy donors, the percentages of Ki-67–positive CD4+,CD45RA+ T cells in JIA patients did not correlate with chronological age (P = 0.8) but instead correlated with disease duration (P = 0.003) (P = 0.0001 for the correlation with chronological age in healthy donors) (Figure 2C and Table 4), which was an independent factor for the proportion of Ki-67–positive CD4+,CD45RA+ T cells (Table 5).

T cell subsets, TREC numbers, relative telomere length or Ki-67 expression, and clinical variables. In JIA patients, there was no correlation of any of the parameters (TREC numbers, the relative telomere length, and Ki-67 expression of naive T cells) with each other. In healthy donors, there was a significant correlation between TREC numbers and the relative telomere length (r = 0.463, P = 0.01) of naive T cells. Multiple regression analysis identified disease duration as the clinical variable correlating with relative telomere length and Ki-67 expression in the JIA cohort (P = 0.04 and P = 0.01, respectively) (Table 5). Current therapy and sex did not influence the numbers of T cell subsets, TREC numbers, relative telomere length, or percentages of Ki-67–expressing CD4+ naive T cells. Classification of JIA, antinuclear antibody positivity, or rheumatoid factor (RF) positivity did not influence the numbers of T cell subsets, TREC numbers, relative telomere length, or percentages of Ki-67–expressing CD4+ naive T cells in the multiple regression model, possibly causing a Type II error (data not shown). The most important factor for accelerated decrease in the number of naive T cells was chronological age (P = 0.01) (Table 5).

DISCUSSION

This study examined TREC content, telomere erosion, and the percentage of naive T cells as indicators of immunosenescence in JIA patients compared with age-matched controls. We found that JIA patients showed an accelerated loss of CD4+, CD45RA+, CD62L+ naive T cells with advancing age. An accelerated decrease in the number of naive T cells in JIA patients may reflect loss of the ability of the thymus to produce recent thymic emigrants or the loss of compensatory proliferation of naive T cells and restoration of the naive peripheral T cell pool. Additionally, inappropriate trafficking of antigen-inexperienced T cells, with bypass of lymph nodes caused by the loss of CD62L expression, may, under certain circumstances, promote anergy induction through nonprofessional antigen presentation (9). The peripheral lymphocyte homeostasis appears to be reconstituted by the expansion of memory T cells (28–30), as observed in the elderly, when the thymus fails to generate new naive T cells after thymic involution. However, abnormalities in T cell homeostasis were evident very early in disease, with no progression over the course of the disease, suggesting a primary defect in T cell generation and/or maturation in the pathogenesis of JIA.

JIA patients demonstrated significantly de-

creased TREC numbers in CD4+,CD45RA+ naive T cells at an early age compared with age-matched controls, whereas a correlation of TREC contents with age was present only in healthy donors. The frequencies of TREC-positive cells are known to decline sharply between age 15 years and age 20 years, corresponding to the time of thymic involution (14,31). Thus, the age-inappropriate low TREC numbers observed in JIA patients may be due to premature thymic involution, which may also be accelerated in these patients. Age-inappropriate degeneration of thymic epithelium, which is required to support the differentiation of precursor cells into mature T cells (32), is a possible mechanism for premature thymic involution. However, several biologic parameters could complicate the interpretation of TREC data (33). TREC numbers can be diluted by peripheral T cell proliferation. Chronic T cell stimulation induces increased T cell turnover in JIA patients, as evidenced by age-inappropriate short T cell telomere lengths in JIA patients. Decreased thymic output will induce compensatory autoproliferation, a process that contributes to TREC dilution. Age-inappropriate thymic involution and increased peripheral T cell turnover may each contribute to the lower TREC numbers observed in JIA patients.

These findings are consistent with the results of other studies that have shown decreased TREC numbers in RA patients (9,20). Those studies showed that the frequency of TREC-positive T cells was significantly lower in RA patients compared with that in age-matched controls. This reduced frequency in the number of TREC-positive T cells was seen as evidence of a diminution of thymic production at an earlier age. Individuals susceptible to RA would enter adulthood with less thymic activity, while the subsequent age-dependent decline in thymic output would not differ between patients and controls (20). A similar reduction in the number of TREC-positive T cells in patients with RA was described in a previous study, which favors the hypothesis that the reduced thymic output is still reversible in adult patients (9). That study also demonstrated that reduced thymic output was associated with phenotypic changes in the compartment of naive T cells, clearly demonstrating downstream events of reduced thymic output. The data can also be explained by a history of increased turnover in the naive compartment. TRECs are not replicated during proliferation, and therefore TREC concentrations are influenced as much by peripheral turnover as by the influx of new TREC-positive T cells (33–35).

In JIA patients, one possible explanation for decreased TREC numbers and telomere shortening in naive T cells is that increased homeostatic proliferation compensates for defective thymic output, and that accelerated telomeric erosion is a consequence of compensatory cell renewal (36). In a study of patients with RF-positive RA in whom the disease developed between age 20 years and age 30 years, telomeres showed evidence of age-inappropriate shortening in naive and memory T cells (20). These observations correlate with our results, indicating telomere loss already at an early stage of the disease.

Homeostatic T cell proliferation already contributes to T cell generation in the early months of life (37,38). Formation of the naive T cell repertoire is largely concluded during early adolescence. Although JIA patients already showed fewer TRECs and shortened telomeres in naive T cells compared with age-matched healthy donors at an early age, the difference was less evident in adolescence. It is possible that maintenance of homeostasis in the T cell pool is overtaken by compensatory replication of mature T cells (39) in adolescence, and therefore alterations of the naive T cell pool become less evident by this age. In JIA patients, the variability of T cell subset numbers, relative telomere length, Ki-67–positive naive T cells, and TREC numbers may reflect the different disease activity and classification of various clinical pictures and manifestations of JIA. However, in contrast to the situation in healthy donors, TREC numbers, relative telomere length, and Ki-67 expression did not correlate with each other in JIA patients, and each parameter did not correlate with chronological age. We interpreted these conflicting results as a sign of disturbed T cell homeostasis in JIA patients.

However, the influence of disease duration on relative telomere length and Ki-67 expression in naive T cells may reflect a history of increased homeostatic proliferation of naive T cells, as seen in RA (40). The finding that telomere shortening also affects naive T cells is indicative of replicative stress occurring early in T cell ontogeny in patients with JIA. One possible explanation for this is that the influx of newly generated T cells from the thymus is decreased in individuals at risk of JIA, and that this reduced regenerative capacity increases homeostatic proliferation of the peripheral T cell compartment. Thus, telomere shortening in JIA patients could be due to chronic activation and/or proliferation occurring in autoimmune conditions. This concept is analogous to alterations of the immune system in senescence presenting as immune exhaustion, telomere shortening, a decrease in TREC-containing T

cell subpopulations, a rise in the number of abnormal T cell subsets, and a higher incidence of autoimmune disease (41).

The most widely acknowledged phenotypic change is the loss of CD28, which happens more frequently in the CD8+ T cell population with age; this also occurs in CD4+ T cells, although to a lesser degree (42). Loss of CD28 expression was suggested as a marker of replicative senescence for T cells (43). A clonal expansion of CD8+,CD28– T cells was found in elderly individuals (44–46) and in patients with RA (8,47,48). CD8+,CD28– T cells preserve telomerase activity in SLE patients (21). In contrast to adult arthritis, according to our data, clonal expansions in the peripheral blood CD4+ T cells of children with JIA were not observed (3). We were unable to detect a subsequent loss of CD28 on CD4+ T cells with advancing age in JIA patients. However, these mechanisms may manifest in autoimmune conditions in later life, since environmental factors such as chronic viral infections causing oligoclonal expansions in the mature T cell subset may have more influence in older individuals.

Taken together, these findings indicate that the immune system of JIA patients is considerably older than their chronological age, as also seen in RA patients (35). The immune system of JIA patients shows phenotypic and functional signs of premature immunosenescence. The age-inappropriate senescence of T cells may contribute to the autoimmune manifestations of JIA. Reduced thymic output induces compensatory autoproliferation of T cells, and this process can lead to premature T cell senescence. These findings, already observed in young children with JIA, illustrate that premature aging of the immune system and disturbed T cell homeostasis with an impact on the naive T cell pool could be a contributing factor to the immunologic abnormalities associated with autoimmunity and aging (49). Finally, the dysfunction in reconstituting the T cell compartment should be considered when exploring new therapeutic strategies for patients with JIA.

AUTHOR CONTRIBUTIONS

Dr. Prelog had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study design. Prelog, Strasak, J. Brunner.

Acquisition of data. Prelog, Schwarzenbrunner, Sailer-Höck, Kern, Klein-Franke, Ausserlechner, Koppelstaetter, A. Brunner, Duftner, Dejaco.

Analysis and interpretation of data. Prelog, Schwarzenbrunner, Sailer-Höck, Kern, Klein-Franke, Ausserlechner, Koppelstaetter, A. Brunner, Duftner, Dejaco, Strasak, Zimmerhackl.

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Statistical analysis. Prelog, Schwarzenbrunner, Strasak.

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