Indications for a Disturbed Peripheral T-Cell Homeostasis in Juvenile Idiopathic Arthritis (JIA): Absent Expansion of CD28– T-Cells and No Decrease of Naive T-Cells in Cytomegalovirus-positive Patients with JIA

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ABSTRACT. ***Objective.*** To investigate the influence of latent cytomegalovirus (CMV) and Epstein-Barr virus (EBV) infections on CD28-expressing T-cell subpopulations and replicative senescence of naive T-cells as a marker for aging of the immune system in children with juvenile ideopathic arthritis (JIA).
Methods. T-cell subpopulations were analyzed from 24 patients with JIA and 61 healthy age-matched controls by fluorescence activated cell sorting. Relative telomere length (RTL) in CD4+CD28+CD45RA+ (naive) T-cells was measured by quantitative polymerase chain reaction.
Results. Although confirming known data of expansions of CD28– T-cells and tendency of decreasing naive T-cells in CMV-seropositive healthy individuals, our findings did not show a marked influence of latent EBV or CMV infection on CD28-expressing T-cells in patients with JIA. In contrast, CMV was an independent factor for loss of CD28, regardless of age, in healthy controls. Irrespective of serology results for CMV or EBV, patients with JIA showed signficantly decreased RTL compared to age-matched controls. Regression lines for RTL and age revealed decreased RTL with advancing age in CMV-positive and EBV-positive subjects. The evidence that findings for CMV-positive JIA patients did not resemble the findings of healthy CMV-positive controls, namely expansion of CD28– T-cells and decrease of naive T-cells, may support the theory of a disturbed peripheral T-cell homeostasis in JIA.
Conclusion. Diminished mechanisms of T-cell homeostasis and premature aging of the immune system may play a role in the pathogenesis of JIA. (First Release Feb 15 2008; J Rheumatol 2008;35:520–7)

Key Indexing Terms:
T-CELLS
CYTOMEGALOVIRUS
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EPSTEIN-BARR VIRUS

Juvenile idiopathic arthritis (JIA) is a heterogeneous inflammatory disorder, currently divided into different subtypes of which clinical classification is based on the number of involved joints and the presence of systemic symptoms and signs at disease onset[1]. All forms of JIA share onset before the age of 16 years and a chronicity of at least 6 weeks[2,3]. However, the etiology of the disease is still unknown.

Adult patients with rheumatoid arthritis (RA) showed significant changes in the peripheral T-cell repertoire resembling the findings of aged individuals and have been discussed under the aspect of increased incidence of autoimmune diseases with advancing age[4]. The most widely acknowledged phenotypic changes of the aged individual are the shift from CD45RA+RO– (naive) to CD45RA–CD45RO+ (memory) T-cells and the loss of the major costimulatory molecule CD28[5-8], which increases in frequency with age in the CD8+ T-cell population and to a lesser degree in CD4+ T-cells[9]. Loss of CD28 expression was suggested as a characteristic feature of immune dysfunction in the elderly and as a marker of replicative senescence for T-cells[10,11]. Patients with T-cell-mediated autoimmune dis-

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eases show immune system abnormalities that resemble the typical characteristics of immune dysfunction described in the elderly. Several research groups have observed T-cell clonal expansions in the peripheral blood of patients with RA[12-15] and reported a population of T-cells in RA patients with signs of replicative stress[14-19] that had lost CD28. The age-inappropriate high percentages of CD4+CD28– T-cells in RA, the proinflammatory capacities and resistance to apoptosis[18,20] of the CD28– subpopulation, and the early presence in disease and absent correlation with disease duration have suggested that CD4+CD28– T-cells are not only a marker for T-cell aging, but may be actively involved in the pathogenesis of autoimmune diseases[13,18,19,21,22]. One explanation for high frequencies of CD4+CD28– T-cells in autoimmune conditions is premature thymic involution with restoration of peripheral T-cell homeostasis by these cells[9,23], but viral infections can also cause massive proliferation of peripheral T-cells. Normally, most of these newly generated CD28– effector cells are deleted at the end of the response, thereby restoring total T-cell numbers to normal levels. However, defects in these deletion mechanisms can lead to several immune system abnormalities, including contracted T-cell receptor diversity and the accumulation of large numbers of clonally expanded T-cells. These T-cells can persist for many years and compromise immune competence. Several research groups have claimed that the CD4+CD28– T cells that emerge in the elderly are indeed cytomegalovirus (CMV)-specific cells[24,25]. Latent herpes virus infections, such as CMV or Epstein-Barr virus (EBV), have been shown to drive T-cell differentiation, to increase peripheral T-cell replication, and to trigger the formation of terminally differentiated CD28– T-cells[26-29]. Thus, CD4+CD28– T-cells emerge as a consequence of CMV infection, and were to an extent CMV-specific and produced interferon-γ only after stimulation with CMV antigen[24,30]. CMV seropositivity has been shown to be associated with the expansion of CD4+CD28– and CD8+CD28– T-cells in RA[31]. Evidence for increased synovial persistence of EBV and CMV has reinforced the notion of a contribution of these viruses in autoimmune arthritis and chronic inflammation[32-35].

We investigated the correlation of CMV or EBV seropositivity to CD28+ and CD28– T-cell subpopulations and telomere length of CD4+ naive T-cells as a marker of peripheral replication in children with JIA.

MATERIALS AND METHODS

Study population. Peripheral blood mononuclear cells (PBMC) were obtained from 24 patients with JIA (14 patients with oligoarticular JIA, 9 with polyarticular JIA, and 1 with systemic JIA) who fulfilled the 1988 American College of Rheumatology criteria for JIA[1], and 61 healthy age-matched controls. Only healthy controls without signs of clinical infection, body temperature ≥ 38°C, or laboratory signs suspicious for infections [elevated C-reactive protein (CRP) or erythrocyte sedimentation rate (ESR), leukocytes above age range] were enrolled. None of the healthy controls had allergies, oncological or immunological disorders, or received immunosuppressive drugs. In patients with JIA, disease activity was evaluated by clinical examination and measurement of CRP and ESR. All patients had inactive disease at the evaluation. Mean age at diagnosis was 67 ± 53 (median 43, range 34–220) months. Ten patients had methotrexate therapy (dosage 10–20 mg/m^2 body surface/day), 2 cortisone (prednisone; dosage ≤ 0.2 mg/kg body weight), 17 received nonsteroidal antiinflammatory drugs (naproxen; dosage 10–15 mg/kg body weight in 2 doses), 2 had anti-tumor necrosis factor-α (TNF-α) therapy, and 5 patients had no medication at the time of evaluation. Eleven patients with JIA were positive for rheumatoid factor and 15 were positive for antinuclear antibodies. Mean CRP in patients with JIA was 0.4 ± 0.3 mg/dl (median 0.1, range 0.1 to 0.7). Clinical data were obtained by retrospective chart review. There were no subjects with double-positive serology for CMV and EBV in the JIA or control group. No subject showed serological or clinical signs of CMV or EBV reinfection or reactivation. CMV IgM and EBV IgM antibodies were negative in all cases. The study was performed according to the Declaration of Helsinki 2000 and approved by the local ethics committee, Medical University Innsbruck, Austria. All patients and controls gave their written informed consent.

CMV and EBV serology. Serum CMV antibodies (IgG and IgM) and serum EBV antibody (IgG and IgM) titers were determined by ELISA (Enzygnost, Dade Behring, Vienna, Austria) according to manufacturer's instructions.

Separation of T cell subsets. PBMC were isolated by using LymphoPrep™ (Axis Shield, Oslo, Norway) according to manufacturer's instructions. CD4+CD28+CD45RA+ T cells were separated by negative and positive selection by using a naive CD4+ T-cell kit and anti-human monoclonal CD28 antibodies (Miltenyi Biotec, Teterow, Germany), magnetic beads, and Auto MACS system with sterile columns (Miltenyi Biotec). Purity of separated CD4+CD28+CD45RA+ T-cells was checked using 4-color flow cytometry (FACS-Calibur flow cytometer; Becton Dickinson, Oxford, UK) and ranged from 97% to 99%.

Quantification of T-cell subsets. PBMC were incubated with monoclonal mouse anti-human antibodies specific for CD4, CD8, CD45RA, and CD28 labeled with fluorescein isothiocyanate (FITC), phycoerythrin (PE), PerCP, or antigen-presenting cells (APC) (all antibodies purchased from BD Pharmingen, San Jose, CA, USA) for 20 min at room temperature in the dark. After incubation, red blood cell lysis was performed with FACS-lysing solution (BD Pharmingen) for 15 min at room temperature. Subsequently, cells were washed twice with phosphate buffered saline and fixed with 2% paraformaldehyde. All analyses were performed using a FACS-Calibur flow cytometer utilizing CellQuest software (BD Pharmingen). Results were expressed as percentage of gated lymphocytes.

Telomere length analysis. Studies in patients with RA[36] and systemic lupus erythematodes[37] have shown telomeric erosion in PBMC from patients compared with healthy controls. Telomeres are highly preserved TTA GGG-rich repeats located at the ends of chromosomes and have an important role in DNA replication and preservation of chromosome integrity[38]. 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 peripheral T-cells progressively decline with age[39] and can serve as a marker for replicatively stressed cells. Telomere lengths in CD4+CD28+CD45RA+ (naive) T-cells served as a marker for cellular senescence. DNA was extracted from separated CD4+CD28+CD45RA+ T-cells using a QIAamp DNA Mini Kit (Qiagen, Chatsworth, CA, USA). To remove residues that would interfere with polymerase chain reaction (PCR), DNA was purified by precipitation as follows: DNA was precipitated with 1 in 10 of the total volume of 4 M $LiCl_2$ and 2.5-fold the volume of 100% ethanol at –20°C for 30 min. Determination of relative telomere length (RTL) was performed by calculating the ratio of a quantitative PCR reaction product from the same sample using specific primers for telomeres and a single copy gene as described[40,41]. Quantitative PCR is the method of choice for determining telomere length in small extractable quantities of DNA as in our case.

Statistical analysis. Nonparametric tests were used throughout. The Mann-Whitney U-test for independent variables was used to compare controls with JIA patients (SPSS, Version 12.0, Chicago, IL, USA). Correlation between RTL and age was calculated by Spearman's rank correlation coefficient. Multiple regression analysis (subjects enrolled in the analysis: JIA patients: n = 20; healthy controls: n = 61) was used to investigate dependency of measures (T-cell subpopulations and RTL) from age, methotrexate use, and CMV and EBV seropositivity. Cortisone or anti-TNF-α therapy was used in only 2 patients of our cohort. Their data were therefore not included in the multiple regression model.

RESULTS

All subjects showed normal CD4+ and CD8+ T-cell levels according to age[42] and there was no significant difference between patients with JIA and controls (Table 1). Initially, we compared the proportion of peripheral CD4+ and CD8+ T-cells expressing CD45RA and the costimulatory surface marker CD28 in JIA patients serologically positive for CMV (Figure 1) and negative controls. CMV-positive controls differed significantly from CMV-negative controls in percentages of CD28+ (CD4+: $p < 0.01$; CD8+: $p < 0.05$) (Figure 1A, 1B). As CMV infection is known not only to increase CD28– effector T-cells, but also leads to a decrease in the size of the naive CD8+ T-cell pool[29], CD45RA+ was measured to evaluate naive CD28+ T-cells. However, naive T-cells were not significantly influenced by CMV in patients or controls (Figure 1C, 1D). EBV seropositivity did not significantly influence the CD28+ or CD28– T-cell pool (Figure 2).

However, RTL in naive T-cells is known to decrease with advancing age[39]. Telomere erosion may be enhanced by oligoclonal expansion of CD28+ T-cells after chronic viral antigen stimulation[43]. Therefore, RTL in CD4+CD28+CD45RA+ T-cells of CMV- (Figure 3A, 3B) or EBV-positive individuals (Figure 4A, 4B) was evaluated. Irrespective of serology results for CMV or EBV, patients with JIA showed significant decreased RTL compared to age-matched controls. Regression lines for RTL and age revealed decreased RTL with advancing age of individuals in CMV-positive subjects (Figure 3B; patients with JIA: $R = –0.714$, $p = 0.11$; controls: $R = –0.467$, $p = 0.11$) and in EBV-positive subjects (Figure 4B; patients with JIA: $R = –0.597$, $p = 0.053$; controls: $R = –0.417$, $p = 0.048$), although this was significant only in EBV-positive controls.

Multiple regression analysis ($R = 0.482$) demonstrated that the most important factor influencing RTL in CD4+CD28+CD45RA+ T-cells was age (standardized coefficient = –0.451, $p = 0.078$), whereas CMV (standardized coefficient = 0.205, $p = 0.403$), EBV (standardized coefficient = 0.052, $p = 0.86$), or methotrexate use (standardized coefficient = –0.162, $p = 0.562$) did not show any influence on this measure. Similar results were obtained by multiple regression analysis ($R = 0.414$) in controls, in whom age was also the most important measure (standardized coefficient = –0.406, $p = 0.052$), whereas CMV (standardized

Table 1. Characteristics of patients with JIA and healthy controls.

	Patients with JIA	Control Group
CMV-negative	n = 16	n = 17
Age, mo	131 ± 55 (140; 42–210)	147 ± 36 (154; 60–213)
Total CD4+ T-cell counts	0.6 ± 0.1 (0.6; 0.4–0.8)	0.5 ± 0.2 (0.4; 0.1–0.8)
% of CD3+ T-cells	39.1 ± 9.3 (40.2; 27.1–55.2)	36.6 ± 6.3 (34.5; 22.5–50.1)
Total CD8+ T-cell counts	0.3 ± 0.2 (0.3; 0.1–0.6)	0.3 ± 0.2 (0.3; 0.1–0.6)
% of CD3+ T-cells	22.1 ± 4.3 (22.8; 16.2–33.1)	25.5 ± 6.7 (22.8; 15.1–35.7)
CMV-positive	n = 6	n = 16
Age, mo	137 ± 42 (146; 72–190)	145 ± 41 (152; 60–218)
Total CD4+ T-cell counts	0.5 ± 0.3 (0.6; 0.1–0.9)	0.4 ± 0.2 (0.4; 0.1–0.8)
% of CD3+ T-cells	41.2 ± 4.2 (42.3; 31.1–52.0)	37.8 ± 7.5 (39.8; 25.4–48.2)
Total CD8+ T-cell counts	0.3 ± 0.2 (0.4; 0.1–0.8)	0.3 ± 0.2 (0.2; 0.1–0.7)
% of CD3+ T-cells	22.8 ± 3.3 (22.4; 16.7–27.8)	24.9 ± 6.2 (24.3; 14.3–34.1)
EBV-negative	n = 11	n = 5
Age, mo	120 ± 49 (128; 42–188)	118 ± 35 (122; 56–166)
Total CD4+ T-cell counts	0.7 ± 0.4 (0.6; 0.2–1.2)	0.5 ± 0.2 (0.5; 0.2–0.9)
% of CD3+ T-cells	41.9 ± 9.8 (43.4; 26.8–55.9)	39.9 ± 6.4 (40.9: 31.2–52.0)
Total CD8+ T-cell counts	0.3 ± 0.1 (0.3; 0.1–0.5)	0.3 ± 0.2 (0.3; 0.1–0.7)
% of CD3+ T-cells	21.1 ± 4.5 (20.9: 13.0–27.9)	27.1 ± 6.3 (24.6; 18.4–35.2)
EBV-positive	n = 11	n = 28
Age, mo	140 ± 54 (156; 34–220)	151 ± 37 (159; 64–229)
Total CD4+ T-cell counts	0.6 ± 0.4 (0.6; 0.1–1.3)	0.4 ± 0.2 (0.4; 0.1–0.8)
% of CD3+ T-cells	37.6 ± 6.6 (35.1; 27.4–47.1)	36.6 ± 6.9 (38.3; 25.9–46.1)
Total CD8+ T-cell counts	0.3 ± 0.2 (0.3; 0.1–0.7)	0.3 ± 0.2 (0.2; 0.1–0.6)
% of CD3+ T-cells	23.3 ± 3.5 (23.9; 18.1–29.4)	24.9 ± 6.4 (23.2; 15.2–34.2)

Values are mean ± SD (median; range). JIA: juvenile idiopathic arthritis; CMV: cytomegalovirus; EBV: Epstein-Barr virus.

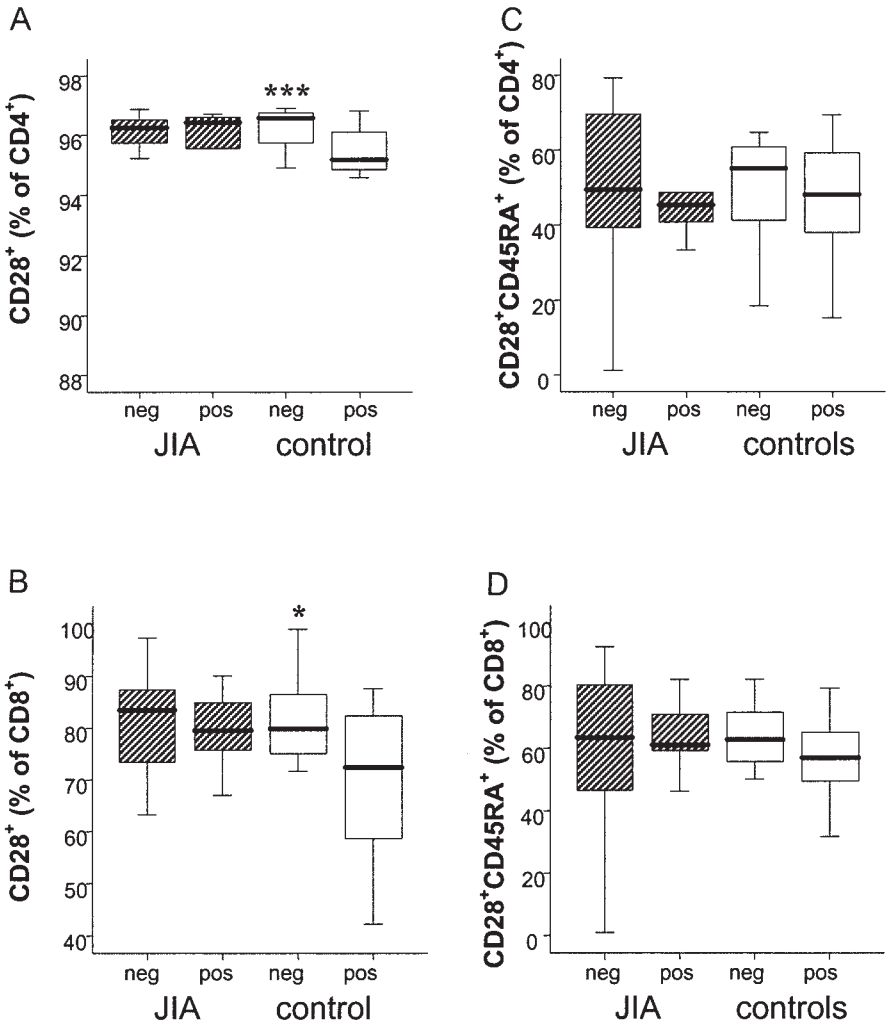

Figure 1. Influence of CMV on T-cell subpopulations. Box plots represent percentages of T-cell subpopulations of CMV-negative (n = 16) or CMV-positive patients with JIA (n = 6) and age-matched controls (CMV-negative: n = 17; positive: n = 16). Differences (Mann-Whitney U-test) between CMV-negative and CMV-positive patients with JIA or controls: *p < 0.05; ***p < 0.01.

coefficient = –0.053, p = 0.783) or EBV (standardized coefficient = –0.012, p = 0.953) showed no influence on RTL.

For the T-cell subpopulations, CMV or EBV seropositivity was not an independent factor in patients with JIA, whereas in contrast, CMV seropositivity was an independent factor driving loss of CD28 in the healthy controls (p = 0.015 for CD4+CD28+ and CD4+CD28– T-cells; p = 0.029 for CD8+CD28+ T-cells and p = 0.02 for CD8+CD28– T-cells; Table 2). Chronological age or EBV infection did not significantly influence the proportions of T-cell subpopulations (Table 2).

DISCUSSION

We investigated the influence of latent CMV and EBV infections on the peripheral T-cell pool and replicative senescence of naive T-cells as a marker for aging of the immune system in children with JIA. While confirming known data[26-29] of expansions of CD28– T-cells and tendency of decreasing naive T-cells[29] in CMV-seropositive healthy individuals, our findings did not show a marked influence of latent EBV or CMV infection on CD28-expressing T-cells in patients with JIA. The finding that patients with JIA displayed normal percentages of CD28+ and CD28– T-cells compared to age-matched controls was in contrast to findings in adult patients with arthritis, but is in accord with data from other studies, in which clonal expansions in the peripheral blood CD4+ T-cells of children with JIA were also not observed[3]. A recent study showed an association between CMV and CD4+CD28– T-cells, which may represent a diverse cell subset containing both autoreactive and virus-specific T cells, and in adult patients with autoimmune disorders[44]. However, the subsequent loss of

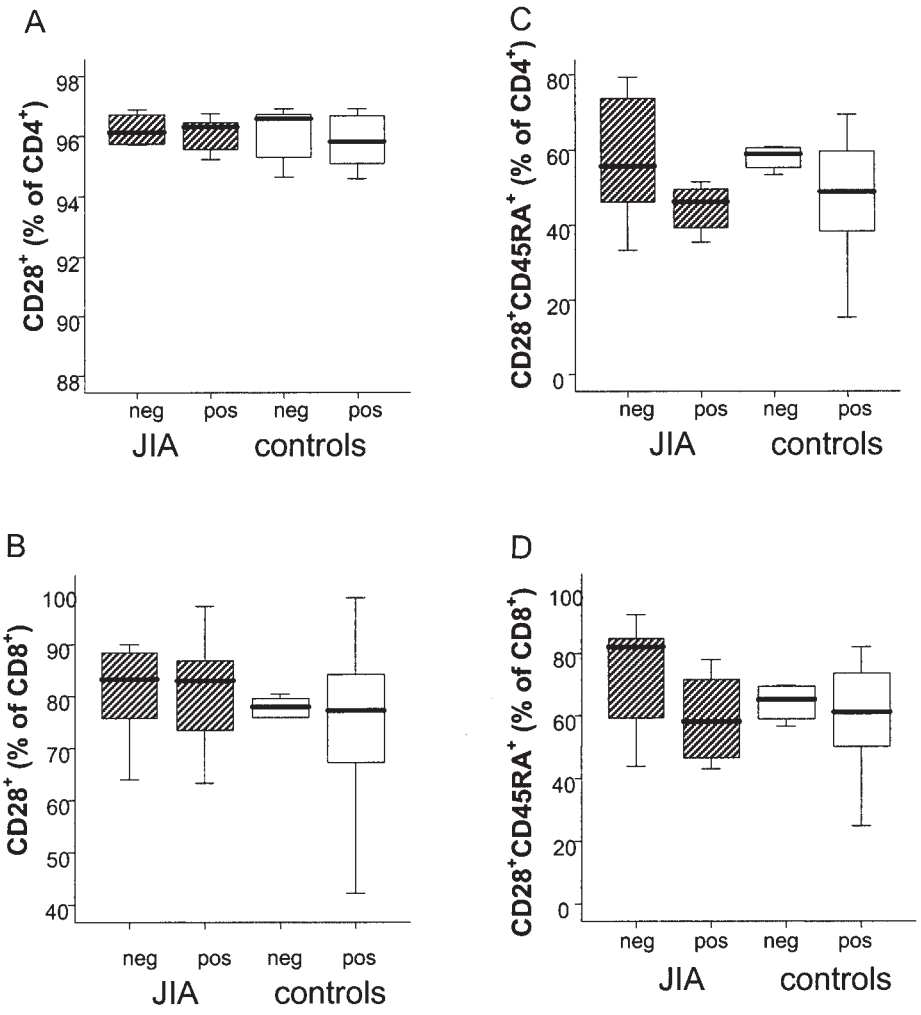

Figure 2. Influence of EBV on T-cell subpopulations. Box plots represent percentages of T-cell subpopulations of EBV-negative (n = 11) or EBV-positive patients with JIA (n = 11) and age-matched controls (EBV-negative: n = 5; positive: n = 28). Differences (Mann-Whitney U-test) between EBV-negative and EBV-positive patients with JIA or controls.

CD28– T-cells may manifest in autoimmune conditions in later life, as environmental factors such as chronic viral infections causing oligoclonal expansions in the mature T-cell subset may have a greater influence in older individuals than in children. The evidence that CMV-positive patients with JIA did not resemble the findings of healthy CMV-positive controls may support the theory of a disturbed peripheral T-cell homeostasis in JIA. Although limited by small numbers in the regression model, CMV was an independent factor for loss of CD28 in healthy controls regardless of age. This difference of patients with JIA compared to controls may also reflect the abnormal behavior of the peripheral T-cell pool in response to environmental factors, such as viral infections, in patients with JIA. However, interpretation of our data is limited by the heterogeneity of the disease itself[45] and the fact that it was not possible to determine the age at primary CMV or EBV infection in our study groups. Possibly, a relatively short observation time between infection and evaluation may bias the results.

Naive T-cells have been shown to be decreased by CMV infection[29] in the elderly. In our cohort, telomeric loss was not accelerated in the naive T-cell pool of the CMV-positive groups. However, patients with JIA were found to have significantly lower RTL in CD4+ naive T-cells compared to age-matched healthy controls. Thus, our study supports the hypothesis that diminished mechanisms of T-cell homeostasis[46] and premature aging of the immune system may play a role in the pathogenesis of JIA. The concept of premature T-cell senescence was also supported by studies that analyzed telomere lengths in the T-cell compartment of patients with RA[36]. There, most notably, telomeric erosion also affects naive T-cells. Telomeric shortening occurred in RA patients with very early disease and remains unaffected by progressive disease, suggesting that it is not an epiphenomenon of

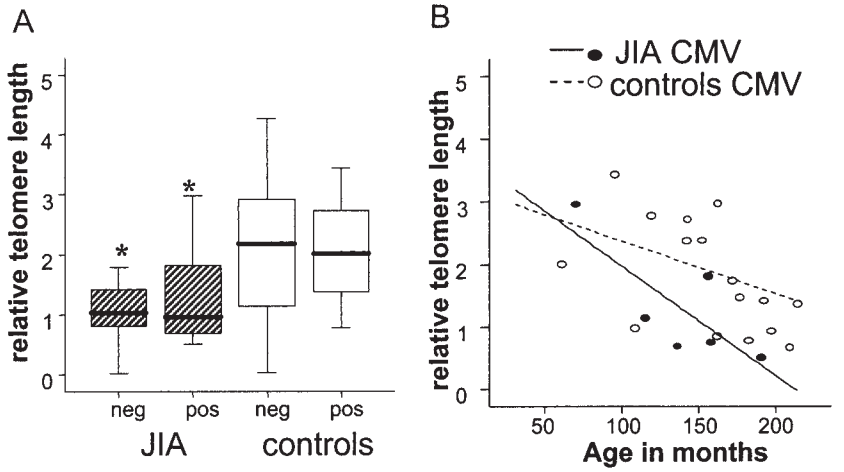

Figure 3. Influence of CMV on relative telomere length (RTL) in CD4+CD28+CD45RA+ T-cells. A. Box plots represent RTL of CMV-negative (n = 16) or CMV-positive patients with JIA (n = 6) and age-matched controls (CMV-negative: n = 17; positive: n = 16). B. Regression line shows correlation between RTL and age of subjects. Differences (Mann-Whitney U-test) between patients with JIA and controls (CMV-negative or CMV-positive group): *p < 0.05.

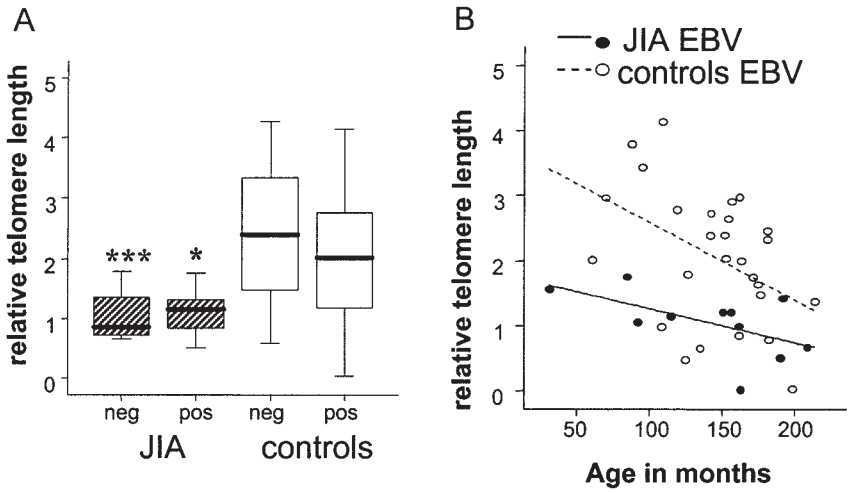

Figure 4. Influence of EBV on relative telomere length (RTL) in CD4+CD28+CD45RA+ T-cells. A. Box plots represent RTL of EBV-negative (n = 11) or EBV-positive patients with JIA (n = 11) and age-matched controls (EBV-negative: n = 5; positive: n = 28). B. Regression line shows correlation between RTL and age of subjects. Differences (Mann-Whitney U-test) between patients with JIA and controls (EBV-negative or EBV-positive group): *p < 0.05; ***p < 0.01.

Table 2. Multivariate regression analysis for CD28+ T-cells and age, CMV, and EBV seropositivity in healthy controls.

Measures	CD4+CD28+ Standardized coefficient R = 0.482	p	CD4+CD28– Standardized coefficient R = 0.482	p	CD8+CD28+ Standardized coefficient R = 0.413	p	CD8+CD28– Standardized coefficient R = 0.421	p
Chronological age	–0.246	0.175	0.246	0.175	0.084	0.657	–0.065	0.718
CMV	–0.433	0.015	0.433	0.015	–0.399	0.029	0.416	0.02
EBV	–0.019	0.916	0.019	0.916	–0.007	0.970	–0.006	0.914

CMV: cytomegalovirus; EBV: Epstein-Barr virus.

the disease process itself[9]. In contrast to the concept of replicative senescence caused by latent virus infections, CMV or EBV did not accelerate telomeric loss in patients with JIA. Most notably, age was the main factor driving telomeric erosion in patients with JIA and controls. Probably, enhanced telomeric erosion in CD4+ naive T-cells in patients with JIA compared to age-matched controls can be attributed to factors driving replicative senescence other than CMV or EBV infections. However, the role of CMV or EBV infections on local inflammation sites in patients with JIA[47-49] and also influence of these viruses on systemic afflictions of the peripheral T-cell pool and proinflammatory cytokines in elderly persons with autoimmune conditions[32-34,50,51] may not be neglected. Herpes viruses have been reported to play a role in RA and monitoring of the viral DNA load has been suggested, although alternative explanations are possible for a high viral load: defects in cellular immunity in patients with RA may result in a relatively high viral load[52-54] and/or patients with RA may be more prone to infection and/or reactivation[55]. However, our patients with JIA may be too young to show changes found in adult arthritis or loss of T-cell receptor diversity[56]. Changes in the RTL in the naive T-cell pool may precede alterations of clonal expansions of highly specialized effector T-cells in later life.

The decline in immunocompetence with age is accompanied by a steadily increasing incidence rate of autoimmune diseases[9]. Although the incidence of autoimmune diseases increases with advancing age, immunosenescence alone is clearly not sufficient to induce autoimmunity. It is speculation whether the loss of telomeres has functional consequences that may explain the increased susceptibility of patients with JIA to develop infections, as seen in patients with RA[57]. These findings may predict that the ability of patients with JIA to react to novel antigens, such as during infection, may be compromised. However, our findings illustrate that autoimmunity is a complex, multifactorial disorder. Premature aging could be a risk factor for developing autoimmunity in genetically prone individuals in a susceptible environment. It remains to be investigated if lifelong chronic antigenic stress, which is commonly caused by infection with persistent activating herpes viruses, causes the accumulation of anergic, apoptosis-resistant CD28– T-cells in later life of patients with JIA and whether these cells are CMV-specific. It also remains to be proven if changes such as decreased RTL or absent alterations of T-cell subset proportions under viral stress are in accord with an emerging "immunological risk profile" with loss of immune competence in the elderly, who bear the risk of higher infection rates and autoimmune diseases[58-62].

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