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INVESTIGATIVE REPORT

T-cell Receptor Excision Circles (TREC) in CD4+ and CD8+ T-cell Subpopulations in Atopic Dermatitis and Psoriasis Show Major Differences in the Emission of Recent Thymic Emigrants

Helle L. JUST[1], Mette DELEURAN[1], Christian VESTERGAARD[1], Bent DELEURAN[2] and Kristian THESTRUP-PEDERSEN[1]
[1]Department of Dermatology, Aarhus University Hospital, Aarhus, and [2]Department of Rheumatology, Institute of Medical Microbiology and Immunology, Aarhus University, Aarhus, Denmark

We used T-cell receptor excision circles (TREC) to evaluate thymic function in adult patients with atopic dermatitis and psoriasis. We observed that men, but not women, with atopic dermatitis had a significantly faster decline in TREC content with increasing age compared with healthy men. In contrast, both men and women with psoriasis had significantly reduced TREC levels, which were, on average, only 30% of that of healthy persons. In atopic dermatitis the levels of TREC declined with increasing levels of IgE, disease intensity and extent of eczema. Furthermore, patients with atopic dermatitis showed signs of altered thymus function, as they had a significantly greater variation in TREC content measured over time than healthy controls, especially within the CD8+ T-cell subpopulation. Because both atopic dermatitis and psoriasis patients have an increased number of T-cells, this indicates that atopic dermatitis patients can have compensatory emissions of thymic emigrants, whereas psoriatic patients do not, thus supporting different thymic function in these two diseases. ***Key words: T cells; thymus; atopic dermatitis; psoriasis.***

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Kristian Thestrup-Pedersen, Nygade 4, 1, DK-4800 Nykobing F, Denmark. E-mail: ktp56@hotmail.com

Atopic dermatitis (AD) is a disorder with accumulation of activated T cells in the skin and, in some patients, increased IgE production leading to type I allergies to environmental allergens (1). We have demonstrated a significant increase in thymic volume in infants and children with active AD, an increased number of lymphocytes in the skin, an increased telomerase activity and shortened telomere length in T cells both in blood and skin, and an increased number of double-positive CD4+CD8+ T cells in peripheral blood (2–5). These results suggest that patients with AD have a dysregulation of thymic T-cell selection and emission (6) and increased T-cell turn-over in the peripheral immune system.

Apart from their role in AD, T cells play a major role in the pathogenesis of psoriasis (7). The predominance of CD8+ T cells in the epidermis is a striking feature of psoriasis, while CD4+ T cells are predominant in the upper dermis (8).

The thymus is most active early in life and plays a central role for a continued, controlled emission of T cells, referred to as recent thymic emigrants (RTEs). In order to evaluate thymic function in patients with AD and psoriasis and because of a lack of phenotypic markers for RTEs, we measured T-cell receptor excision circles (TREC) among CD4+ and CD8+ T-cell subsets in blood.

When the T-cell receptor is formed a signal joint TREC is produced during the rearrangement of the T-cell receptor α gene segment in about two-thirds of $\alpha\beta$ T-cells. The δ gene is located within the T-cell receptor α gene locus. For $\alpha\beta$ T cells, the gene rearrangement is initiated by the deletion of the δ gene from the α gene locus. Deletion of the δ gene usually occurs in both alleles; therefore a maximum of 2 copies of TREC may exist in $\alpha\beta$ T cells immediately after completion of a corresponding rearrangement event (9, 10). Deletion of the δ locus from the α gene occurs during the double-positive (CD4+CD8+) stage before the positive and negative selection events.

The TREC DNA is localized in the cytoplasm of the T-cell and is not replicated during cell division. Therefore, the number of TREC is halved for each cellular doubling. If a T-cell contains TREC it is likely to be an RTE or a non-dividing cell.

By measuring TREC, we observed that patients with psoriasis have significantly low TREC contents compared with normal controls. In contrast, patients with AD at the age of 20 years have normal TREC levels, but have a faster decline in their TREC level over time. This was especially seen in men in their CD8+ T-cell subpopulation compared with normal healthy volunteers. We observed that patients with AD over time could produce high TREC levels, indicating periodic high emission of RTE. This was most pronounced in the CD8+ T-cell population.

METHODS

Patients and healthy controls

Patients with AD or psoriasis were recruited from the Department of Dermatology, Aarhus University Hospital. Among 35 adult patients with AD, 10 had mild eczema, 6 had moderate

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eczema and 19 had severe to very severe eczema. All 24 patients with psoriasis had active, severe psoriasis. Healthy controls were recruited among blood donors. There were no significant differences between patients and controls regarding gender and age (Table I). In addition, a group of 7 patients with AD was observed over a period of 6 months. Each month the patients were clinically scored with SCORAD and blood samples were obtained. To investigate the variance in TREC content in healthy individuals, a group of 6 healthy persons were observed over a period of 4 months. Informed consent was obtained from each participant and the research protocol was approved by the ethics committee of Aarhus County (journal number: 2001.0161).

T-cell subset separation

Blood samples (30 ml) were collected into heparin-coated tubes and peripheral blood mononuclear cells (PBMCs) were recovered using Lymphoprep (Nycomed, Oslo, Norway) gradient centrifugation. CD4+ and CD8+ T cells were subsequently isolated from PBMC by positive immunoselection using Dynal CD4+ beads or CD8+ beads according to the manufacturer's guidelines (Dynal, Oslo, Norway). Briefly, total PBMCs were resuspended in phosphate-buffered saline (PBS) containing 2% foetal calf serum (FCS) at a concentration of 5×10^6 cells/ml. CD4+ and CD8+ isolation beads, previously washed twice in PBS containing 2% FCS, were added at a ratio of 4–10 beads per positive cell and were incubated at 4ºC for 30 min under constant rotation. After incubation, beads were washed 4 times in PBS containing 2% FCS on the Dynal magnet, after which the beads with the positive selected cells were resuspended in 300 µl cell lysis solution for DNA extraction. Flowcytometric tests showed cell purity of approximately 95% in the positively selected subpopulations.

Real-time PCR quantification of TREC

DNA was purified from CD4+ and CD8+ T cells using the Purescript kit according to manufacturer's guidelines (Gentra Systems, Inc. Minneapolis, MA, USA). After DNA extraction samples were stored at –80ºC until TREC analyses were performed. To detect TREC a real-time quantitative PCR (RQ-PCR) method was used. The PCR was performed in a 25 µl reaction containing 1× Platinum® Quantitative PCR Supermix-UDG (Invitrogen, Carlsbad, CA, USA), 4mM $MgCl_2$, 900 nM forward and reverse primers (300 nM for β-2-microglobulin), 200 nM probe and 5 µl isolated DNA. PCR conditions were 50ºC for 2 min, followed by 95ºC for 10 min, after which 45 cycles of amplification were carried out (95ºC for 15 s, 60ºC for 1 min). 250 ng DNA was amplified and each sample was analysed in triplicate using Rotor-Gene 2000 or Rotor-Gene 3000 from Corbett Research (New South Wales, Australia). To normalize for the input DNA, the constant gene segment of the TCRA gene Cα constant region and β2m were amplified in every DNA sample tested and expressed as TREC per 150,000 cells. The sequences of the TREC primers and probes were: forward primer: 5′-TCC CTT TCA ACC ATG CTG ACA-3′, reverse primer: 5′-TGC AAC-TCG TGA GAA CGG -3′, and probe: FAM-5′-CAC GGT GAT GCA TAG GCA CCT GC-3′-TAMRA.

Sequences of the Cα primers and probes were: forward primer: 5′-CCT GAT CCT CTT GTC CCA CAG-3′, reverse primer: 5′-GGA TTT AGA GTC TCT CAG CTG GTA CA-3′, and probe: FAM-5′-ATC CAG AAC CCT GAC CCT GCC G-3′-TAMRA. Sequence of the β2m primers and probes were: forward primer: 5′-TAA AAC TTA ATG TCT TCC TTT TTT TTC TC-3′, reverse primer: 5′-AAA CAT TTT CTC AAG GTC AAA AAC TTA-3′, and probe: FAM-5′-CCT CCA TGA TGC TGC TTA CAT GTC TC-3′-TAMRA. These three primer combinations resulted in amplicons of 129, 70 and 75 bp, respectively.

To produce a TREC standard, DNA from a pool of PBMCs from five healthy donors was amplified for 30 cycles using standard PCR conditions. The PCR product was visualized with ethidium bromide on 2% agarose gel. The amplified fragment was purified from the gel using Wizard® PCR Preps DNA Purification System (Promega, Madison, WI, USA). The agarose isolated product, corresponding to TREC, was then cloned into pGEM®-T Vector (Promega). DNA sequence analysis was performed on the isolated clone to verify that no sequence mutation was introduced during the PCR-based cloning strategy. The sequence of the primers used for generating the TREC standard was: forward primer: 5′-CCA CAT CCC TTTCAACCA-3′, reverse primer: 5′-GTG TTT TCG GCC GTG A-3′ (amplicon of 237 bp).

IgE values

Total IgE concentrations were measured by a two-site sandwich immunoassay using the ADVIA Centauer Total IgE assay (Bayer Diagnostic, Tarrytown, NY, USA).

Accuracy of TREC measurements

We pooled DNA from 4 patients with AD, split the sample into ten different tubes and performed TREC analysis weekly for 10 weeks. The results showed a mean value of TREC on 2.493 with a standard deviation (SD) of 284 (11%), thus giving a good reproducibility. As the tests of patient DNA were run in large batches to avoid inter-experiment variation, the observed differences must relate to biological changes. T-cell lines were established from patients with AD as described previously (5). After 9–12 cell doublings we took T lymphocytes and measured TREC. We never observed TREC in T cells from cell lines likely due to their strong proliferation and thus reduced TREC ($n = 6$, results not shown).

Statistical analysis

The TREC decline with increasing age was investigated by linear regression and the difference between patients and controls was tested by independent *t*-test of the slopes, as the TREC concentrations tested with normal plots were shown to fulfil the criteria for normal distribution. The TREC concentrations are expressed in mean ± SD. Kruskal-Wallis one-way analysis of variance was used to test for differences between gender, whereas correlation between TREC concentrations and disease activity was tested by Spearman's rho analysis. A Mann-Whitney *U*-test was used to test if AD patient's variance in TREC content over time was different from the variance in TREC content in healthy controls. In all cases $p < 0.05$ was considered significant.

Table I. *Characteristics of patients and healthy controls*

Characteristics	Atopic dermatitis	Psoriasis	Controls
Cross-sectional study	($n = 46$)	($n = 24$)	($n = 41$)
Men % (n)	37% (17)	54% (13)	54% (22)
Women % (n)	63% (29)	46% (11)	46% (19)
Age, years (range)			
Men	36 (20–51)	44 (28–58)	36 (19–51)
Women	31 (18–57)	37 (23–55)	28 (20–48)
Time-course study	($n = 7$)		($n = 6$)
Men % (n)	20% (1)		33% (2)
Women % (n)	80% (6)		67% (4)
Age, years (range)	23 (21–33)		31 (25–37)

RESULTS

Absolute count of lymphocytes and TREC

We have chosen to describe our data as TREC per 150,000 T cells. To confirm that the TREC content in a given pool of peripheral T cells was not dependent on the total amount of T cells present, we compared the total number of lymphocytes in blood with the TREC values and did not observe any correlation between the total lymphocyte number and TREC (data not shown).

Differences in mean TREC values in patients with AD and psoriasis

Overall, women have a higher TREC content than men in all three groups ($p < 0.05$), with the highest levels observed in normal healthy volunteers followed by AD and psoriasis (Fig. 1). TREC levels were always higher in the CD8+ T-cell subpopulation compared with CD4+ T cells. By extending this analysis to AD and psoriasis we observed that gender also seems to influence thymus production of the CD4+ and the CD8+ T-cell subpopulation during disease (Fig. 1). Men suffering from AD have a significantly lower mean TREC level in their CD8+ T cells, but not in their CD4+ T cells in comparison with healthy men. In women TREC did not differ between AD and healthy females, neither in the CD4+ nor the CD8+ T-cell subpopulation. This is in sharp contrast to what is seen in psoriasis, where both men and women had, on average, 70% reduced TREC levels of both CD4+ and CD8+ T cells compared with healthy controls (Fig. 1).

Patients with AD and psoriasis have age-related changes in the level of TREC

It is well known that thymic output declines with increasing age. However, little is known about whether this decline is influenced by gender or varies in different T-cell subpopulations. We sorted peripheral blood CD4+ and CD8+ T cells from males and females with AD, psoriasis and normal healthy volunteers. We used the linear correlations between increasing age and individual TREC values as an expression of decline in thymus function (Fig. 2). We confirm that normal healthy volunteers have a decline in their thymus function with increasing age.

Both AD males and females have the same thymic output around the age of 20 years, but as AD males become older their ability to produce both CD4+ and CD8+ T-cells declines faster than expected (CD4+: $p = 0.02$, CD8+: $p < 0.01$), whereas this was not observed in women with AD.

Both male and female psoriatic patients had a significantly lower level of TREC compared with healthy volunteers. At the age of 20 years their TREC levels are equivalent to what is seen in 35-year-old normal healthy volunteers. However, the decline in TREC with increasing age is similar to what is seen in healthy controls (Fig. 2).

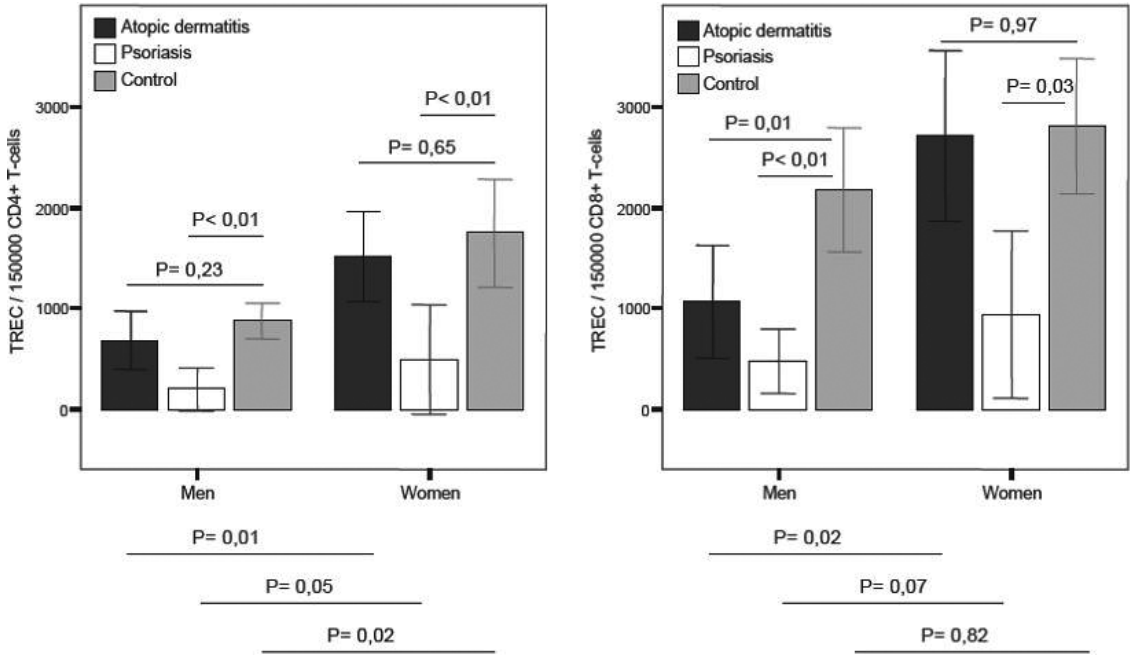

Fig. 1. Comparison of T-cell receptor excision circles (TREC) per 150,000 CD4+ or CD8+ T cells between healthy males and females, patients with psoriasis and patients with atopic dermatitis. The bars shows mean value of TREC and the error bars represent 95% confidence interval of mean. *p*-levels between the individual columns are shown.

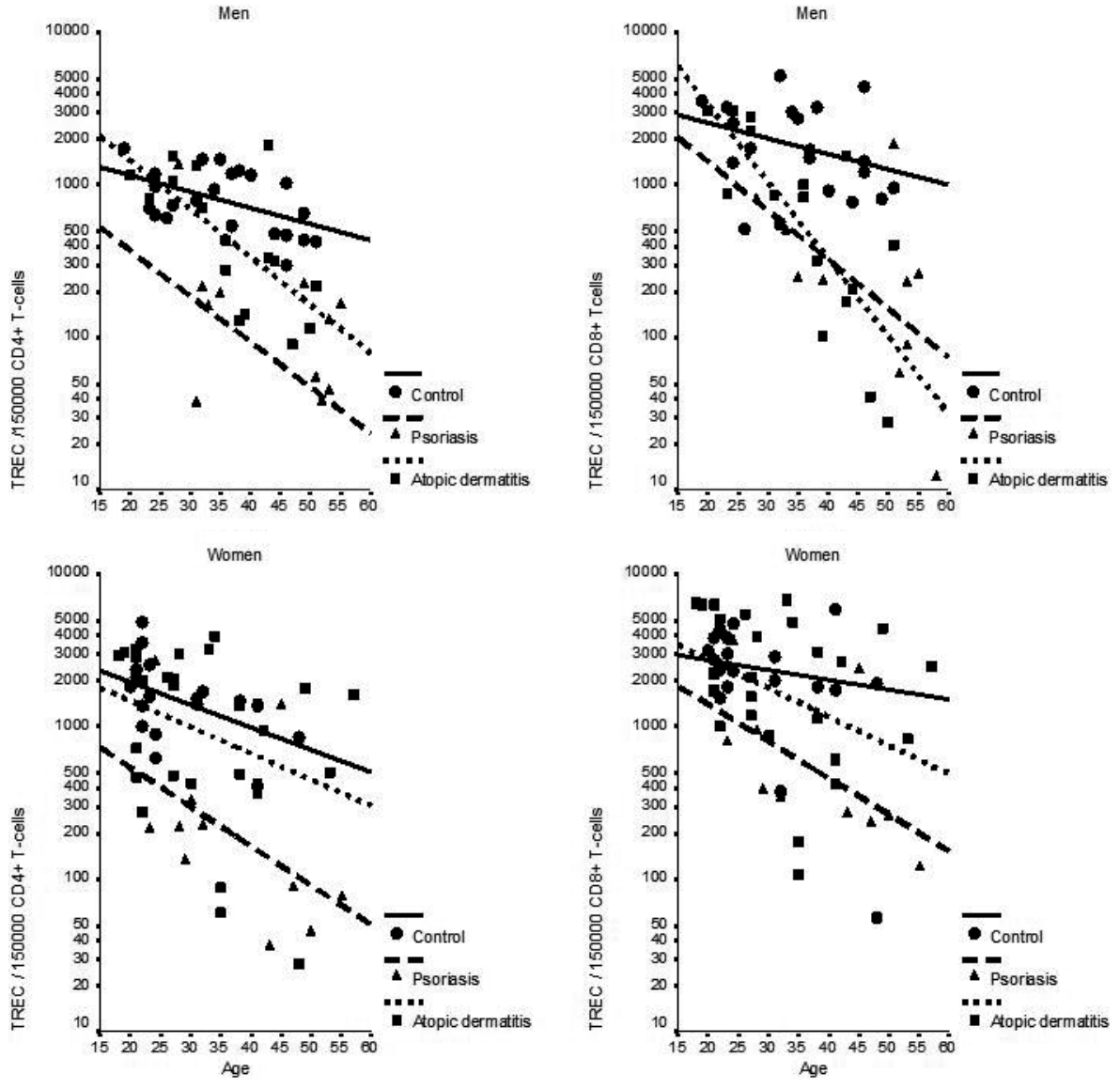

Fig. 2. The number of T-cell receptor excision circles (TREC) per 150,000 CD4+ or CD8+ T cells in healthy males and females (–), patients with psoriasis (--) and patients with atopic dermatitis (•••). Best fit linear regression lines shows the correlation between age and TREC values.

TREC and IgE values in patients with atopic dermatitis

The majority of adult patients with AD have high IgE. Total IgE values were obtained for 32 of the AD patients. Nine patients (28%) had normal IgE values (< 150 kU/l), 3 (9%) had a total IgE value between 150 and 500 kU/l, and 20 (63%) had a total IgE value over 500 kU/l. There was a significant reduction in the TREC content of both CD4+ and CD8+ T cells with increasing IgE (correlation coefficient (CC) = –0.26, $p = 0.039$ and CC = –0.29, $p = 0.020$, respectively) (Fig. 3).

TREC and disease activity in patients with atopic dermatitis

We correlated individual levels of TREC with different measures of disease activity in AD. In the cross-sectional study we did not observe a correlation between TREC values and the degree of eczema (data not shown). AD patients, who were observed over time, were clinically scored by SCORAD. When correlating the TREC concentration in both CD4+ and CD8+ T cells to total SCORAD, only the CD8+ subpopulation reached the level of significance (data not shown). This is not surprising, as SCORAD consists of three individual components: area of involved skin and thereby the T-cell infiltration, intensity which also reflects the T-cell activation and subjective symptoms, itching and sleep loss. Examining the individual components of SCORAD, TREC in both the CD4+ and CD8+ T-cell subpopulation was reversely correlated both with extent (CD4+ r = –0.34, $p = 0.008$ and CD8+ r = –0.40, $p = 0.003$) and intensity (CD4+ r = –0.27, $p = 0.045$ and

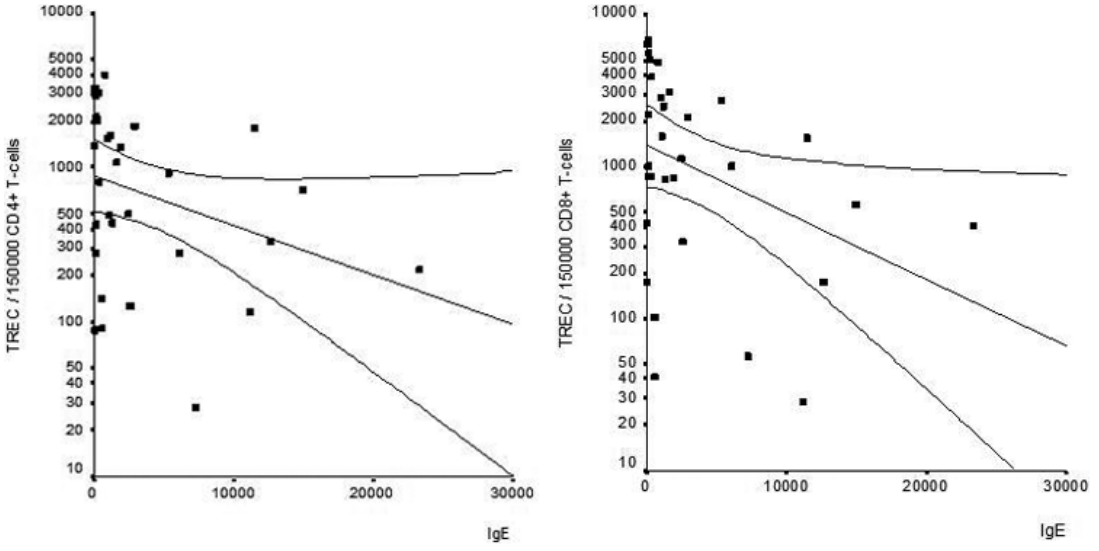

Fig. 3. Correlations between IgE values and T-cell receptor excision circles (TREC) values from patients with atopic dermatitis. Linear regression was used to test the correlation between TREC values in CD4+ and CD8+ T cells, respectively, and IgE values. The lines represent the linear regression line and the 95% confidence interval mean regression prediction lines.

CD8+ r = –0.35, $p = 0.010$), whereas the more subjective symptoms of itching and sleep loss did not show any correlation.

Variation in TREC levels over time

A time course study was conducted in order to evaluate whether thymus has a fluctuant function. As mentioned above, thymus function decreases with age. As our purpose was to examine whether patients with AD have changes in their thymic activity, we only included patients under the age of 40 years.

Normal healthy volunteers show very little change in their TREC values over time, supporting that their thymus is working at a constant level. This is in contrast with AD, where we observed a large variation in the amount of TREC in T cells over a time period of 6 months (Fig. 4). This variation was observed in both the CD4+ and CD8+ T-cell subpopulation. Compared with healthy controls we found it to be significant ($p = 0.022$ and $p = 0.008$ for CD4+ and CD8+ T cells, respectively). The variation of TREC in CD8+ T cells was significantly larger than among CD4+ T cells. The fluctuation in TREC, with highs and lows, supports a connection between thymus function and AD disease and shows that patients with AD can increase their thymic output.

DISCUSSION

In this study we report that adults suffering from psoriasis have significantly reduced TREC levels in peripheral blood T cells, whereas patients with AD have normal or only slightly reduced levels. Because TREC reflects the T-cell output from the thymus, these observations also reflect different thymus metabolism in these two diseases.

We observed a clear gender difference as females have significantly higher TREC levels than men, both in healthy controls and in patients. Furthermore, are the TREC levels in CD8+ T cells always above the levels in CD4+ T cells. Finally, TREC levels are significantly reduced with age. Our findings regarding gender differences and age-related decreases of TREC have been observed by other authors (11–15). This difference seems to be connected to the balance between androgen and oestrogen, as patients with prostate cancer that have sex steroid ablation show a rise in their TREC level (16)

Our observations in psoriasis are new, with TREC levels reduced to only 30%, compared with levels seen in healthy persons of the same age and sex. A similar picture has been observed in rheumatoid arthritis, HIV infection, systemic lupus erythematosus, cutaneous T-cell lymphoma, hepatitis C infection, head and neck cancer patients, and in children undergoing partial thymectomy (17–26). In all cases this has been explained to be caused by an increased turn-over of the peripheral T cells in these diseases, not compensated by an increased thymic output. We, therefore, find it interesting that AD differs from the diseases mentioned above. Although, TREC levels are normal in women with AD, TREC levels in men with AD, in CD8+ T cells are clearly reduced related to age. Why women with AD keep their thymus function, in contrast to men, is unclear. The reason for this could be that boys develop AD earlier in life than girls (27), and as childhood is a crucial time for development of the immune system, this would indicate that the CD8 subpopulation is worn down in males.

TREC measurements reflect not only the thymic function, but are influenced by the peripheral T-cell

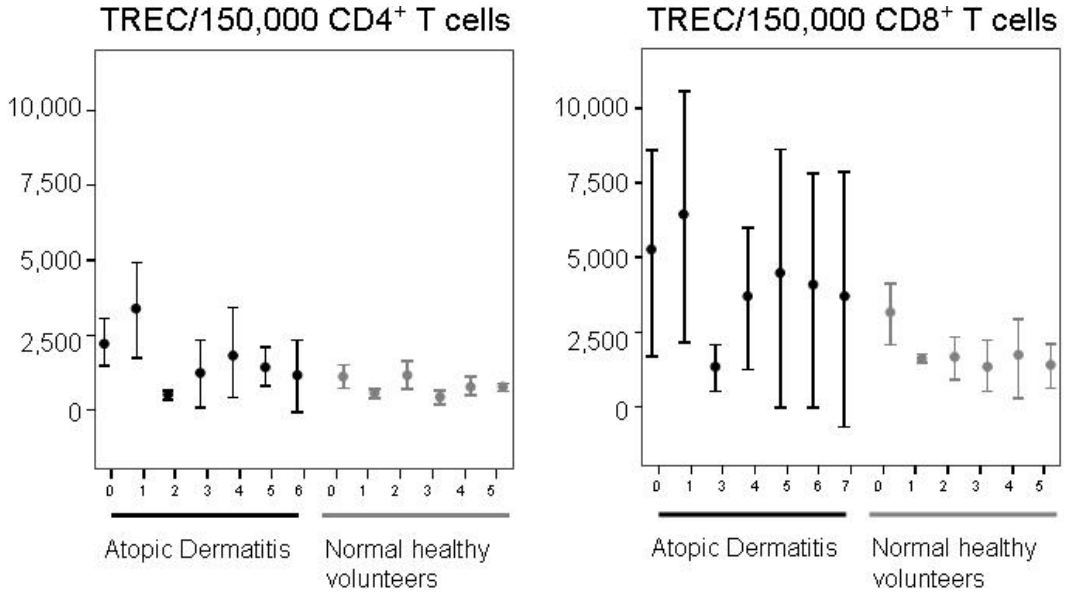

Fig. 4. T-cell receptor excision circles (TREC) values over time for the individual patients with AD and healthy volunteers. Each patient and healthy volunteer is presented with their mean TREC concentration and error bars representing 2 standard deviations to illustrate the variation in TREC values over a time period.

metabolism. Bearing this in mind, it is still the best way to measure RTE (28). In contrast to the stable levels of TREC in healthy controls, patients with AD below the age of 40 years have significant larger variations in their TREC levels, especially in the CD8+ T-cell population. This indicates that AD patients have bursts in their emission of RTE especially in the CD8+ population, presumably reflecting the degree of inflammation in these patients. As TREC correlates inversely to disease activity and IgE, fluctuations in RTE take place in connection with disease flares, supporting our hypothesis that the thymus plays an important role in the pathogenesis of AD. This is further supported by previous studies, which show that the number of T-cells in the skin, of even normal looking skin of patients with AD, are significantly increased (4).

Although one has to be careful in comparing disease activity between different diseases, we find it interesting that AD differs from psoriasis, and many other chronic inflammatory diseases regarding thymus metabolism. TREC has been reported to be decreased in HIV, rheumatoid arthritis, systemic lupus erythematosus, cutaneous T-cell lymphoma, hepatitis C, head and neck cancer (18–26). We believe that our observations of both AD and psoriasis are new and, together with our previous observations on increased numbers of lymphocytes in the skin (4) and shortened telomeres in both patients with AD and psoriasis (3), indicate that there is an increased turn-over of T cells in the peripheral immune system of both diseases. Whereas telomere length and telomerase activity had an equal pattern in patients with AD and psoriasis, the TREC measurements show an almost opposite finding. Because TRECs reflects thymic output, patients with AD must therefore have a much larger thymic activity (28).

Our results indicate that patients with AD are capable of compensatory mechanisms leading to increased thymic emission, especially in the CD8+ T subpopulation, in contrast to patients with psoriasis. Therefore, further studies of RTEs are needed in order to dissect the trafficking of RTEs in chronic inflammatory diseases.

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