ARTHRITIS & RHEUMATISM
Vol. 65, No. 10, October 2013, pp 2567–2572
DOI 10.1002/art.38058

BRIEF REPORT

Deficient Thymic Output in Rheumatoid Arthritis Despite Abundance of Prethymic Progenitors

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Objective. **To determine the frequencies of common lymphoid progenitors (CLPs) and recent thymic emigrants (RTEs) in patients with rheumatoid arthritis (RA) and healthy control subjects.**

Methods. **Flow cytometry was performed to determine the frequencies of CLPs and RTEs in the peripheral blood of 101 control subjects and 51 patients with RA. Thirteen of these patients were also analyzed longitudinally for 6 months after initiation of treatment with a tumor necrosis factor (TNF) inhibitor.**

Results. **A significant correlation between the frequencies of CLPs and RTEs was observed in healthy control subjects. The frequencies of both CLPs and RTEs decreased with age and correlated inversely with absolute lymphocyte numbers in peripheral blood. In patients with RA, the frequencies of RTEs were significantly decreased compared with the frequencies in control subjects. Importantly, the frequencies of CLPs were significantly higher in patients with RA compared with control subjects. Therapeutic TNF blockade further increased the frequency of CLPs, thereby normalizing thymic output, as indicated by an increase in the number of RTEs.**

Conclusion. **Thymic insufficiency in RA is not attributable to an inadequate supply of progenitor cells to the thymus. Thus, insufficient numbers of RTEs could result from inadequate thymic T cell neogenesis, or alternatively, could be a consequence of high CD4+ T cell turnover, homeostatic proliferation, and subsequent dilution of the RTE population.**

The generation of T cell receptor excision circle (TREC)–positive recent thymic emigrants (RTEs) in humans declines progressively with increasing age. Homeostatic proliferation is possibly an extrathymic mechanism for the generation of new T cells, and lymphopenia and common γ-chain cytokines appear to be the main driving force (1). However, thymic generation of TREC-positive RTEs can be restimulated throughout adult life if an increased supply of T cells is required under conditions of lymphopenia.

Rheumatoid arthritis (RA) is associated with phenotypic alterations of T helper lymphocytes reminiscent of premature immunosenescence (2). In addition, RA is characterized by an age-inappropriate decrease in the number of CD4+ naive T cells and TREC-positive T cells (3), indicating decreased thymic output, diluting effects due to increased homeostatic maintenance proliferation, or both. Accelerated homeostatic proliferation of CD4+ T cells has also been observed in individuals who were thymectomized in early childhood, resulting in premature aging of T cells (4).

In theory, thymic output in RA could be insufficient due to a shortage of thymus-seeding precursor cells. In the human system, those precursors were initially characterized in bone marrow as lineage-negative (Lin−) CD34+CD10+ common lymphoid progenitors (CLPs) (5), and their phenotype was subsequently refined to Lin−CD34^{high}CD45RA+CD10+ (6). Six et al showed that CD34+CD10+CD24− progenitor cells are capable of migrating from the bone marrow and seeding the thymus (7). CLPs have recently been shown to have robust T cell potential regardless of CD7 expression, which appears to be a less important marker (8).

Supported by the BMBF (grant PtJ-Bio, 0315883) and the DFG (grant WA 2765/3-1).

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Submitted for publication January 21, 2013; accepted in revised form June 10, 2013.

Therefore, we decided to use CD10 expression as a marker defining the lymphoid commitment of human cells, in order to analyze the frequency of the best-characterized lymphoid-restricted progeny of hematopoietic stem cells (HSCs) (i.e., Lin−CD34+CD10+CD24− CLPs) in the peripheral blood of patients with RA and healthy control subjects. In order to simultaneously determine thymic output, we measured the frequency of CD4+CD31+CD45RA+ T cells, which represents a well-established surrogate marker for TREC-positive RTEs (9).

The results of the current study show a strong correlation between the frequencies of CLPs and RTEs in healthy control subjects. Compared with control subjects, patients with RA had a deficiency of RTEs despite a significantly increased number of thymic progenitors. Therapy with the tumor necrosis factor (TNF) inhibitor etanercept increased the frequency of thymic progenitors even further and almost normalized the deficient thymic output.

PATIENTS AND METHODS

Patients and control subjects. The study group included 51 patients with definite RA according to the American College of Rheumatology/European League Against Rheumatism 2010 criteria for the classification of RA (10). The characteristics of the study populations are shown in Table 1. In 13 of the patients, treatment with etanercept was initiated because of a clinical requirement. Prior treatment with conventional disease-modifying antirheumatic drugs was continued, and the dynamics of the cell populations in these patients were analyzed longitudinally.

The control group included 101 subjects who were recruited from among healthy blood donors. Control subjects were matched with the RA cohort for both age and sex (median age 60 years [range 29–87 years], 32 men and 69 women). In addition, 30 younger control subjects (median age 29.5 years [range 18–43 years) were recruited in order to analyze the influence of age. All experiments with human materials were approved by the local ethics committee, and informed consent was obtained from all subjects.

Flow cytometric analysis. Peripheral blood mononuclear cells (PBMCs) were isolated from peripheral blood by Ficoll density-gradient centrifugation. To identify CLPs in peripheral blood, mononuclear cells were stained with allophycocyanin (APC)–conjugated anti-CD34 (AC136), phycoerythrin (PE)–conjugated lineage (Lin) antibody cocktail, PE–Cy7–conjugated anti-CD10 (HI10a), and fluorescein isothiocyanate–conjugated anti-CD24 (ML5). The Lin cocktail contained antibodies against CD2 (LT2), CD3 (BW264/56), CD4 (M-T466), CD14 (TüK4), CD15 (VIMC6), CD16 (3G8), CD19 (LT19), CD33 (AC104.3E3), CD56 (AF12-7H3), and glycophorin A (GA-R2). To identify RTEs in peripheral blood, mononuclear cells were stained with PerCP–Cy5.5–conjugated anti-CD4 (SK3), PE-conjugated anti-CD45RA (HI100), and APC-conjugated anti-CD31 (AC128). The antibodies were obtained from Miltenyi Biotech or BD Biosciences. Cells were analyzed on a FACSCalibur flow cytometer, using CellQuest software (BD Biosciences).

Lymphocyte count. The absolute lymphocyte count in whole blood was measured using an automated hematology analyzer (Cell-Dyn 3700; Abbott).

Statistical analysis. To assess statistical significance, Student's *t*-test or a Mann-Whitney rank sum test was used. Prior to all comparisons, a Kolmogorov-Smirnov test for normal distribution was performed.

RESULTS

Frequency of CLPs throughout life and correlation of CLP and RTE frequencies in peripheral blood. The healthy control subjects (n = 101) were selected based on an age distribution comparable with that in a typical RA cohort. After PBMCs were isolated, the frequency of RTEs was determined by flow cytometry as a percentage of total CD4+ T cells, using CD31 as a marker for RTEs. Lymphoid progenitors were quantified as Lin−CD34+CD10+ cells (Figure 1A), as described previously (7).

In healthy control subjects, the mean frequency of CLPs was 30 per 10^6 PBMCs, and the mean percentage of RTEs in all circulating CD4+ T cells was 46.7%.

Table 1. Characteristics of the rheumatoid arthritis patient cohorts*

	All patients (n = 51)	Etanercept-treated patients in the longitudinal study (n = 13)
Age, years	63.0 (28−84)	53.0 (28−65)
No. women/no. men	36/15	9/4
Disease duration, years	12.0 (1−36)	2 (1−13)
Age at disease onset, years	50.0 (18−77)	48.0 (25−62)
Rheumatoid factor positive, %	86.3	69.2
Anti-CCP positive, %	84.3	69.2
C-reactive protein, mg/dl	5.43 (0.3−113)	4.39 (0.5−16.3)
No. of tender joints (28 assessed)	2 (0−26)	3 (0−25)
No. of swollen joints (28 assessed)	5 (0−19)	4 (0−9)
Disease Activity Score in 28 joints	3.5 (1.2−6.3)	4.1 (1.9−6.0)
DMARD treatment, no. of patients		
Methotrexate	29	9
Leflunomide	2	1
Anti-TNF	4	0
Anti-TNF plus methotrexate	5	0
Anti–IL-6 plus methotrexate	1	0
Abatacept plus methotrexate	1	0
Quensyl plus methotrexate	1	1
Rituximab	3	0
None	5	2

* Except where indicated otherwise, values are the median (range). Anti-CCP = anti–cyclic citrullinated peptide; DMARD = disease-modifying antirheumatic drug; TNF = tumor necrosis factor; IL-6 = interleukin-6.

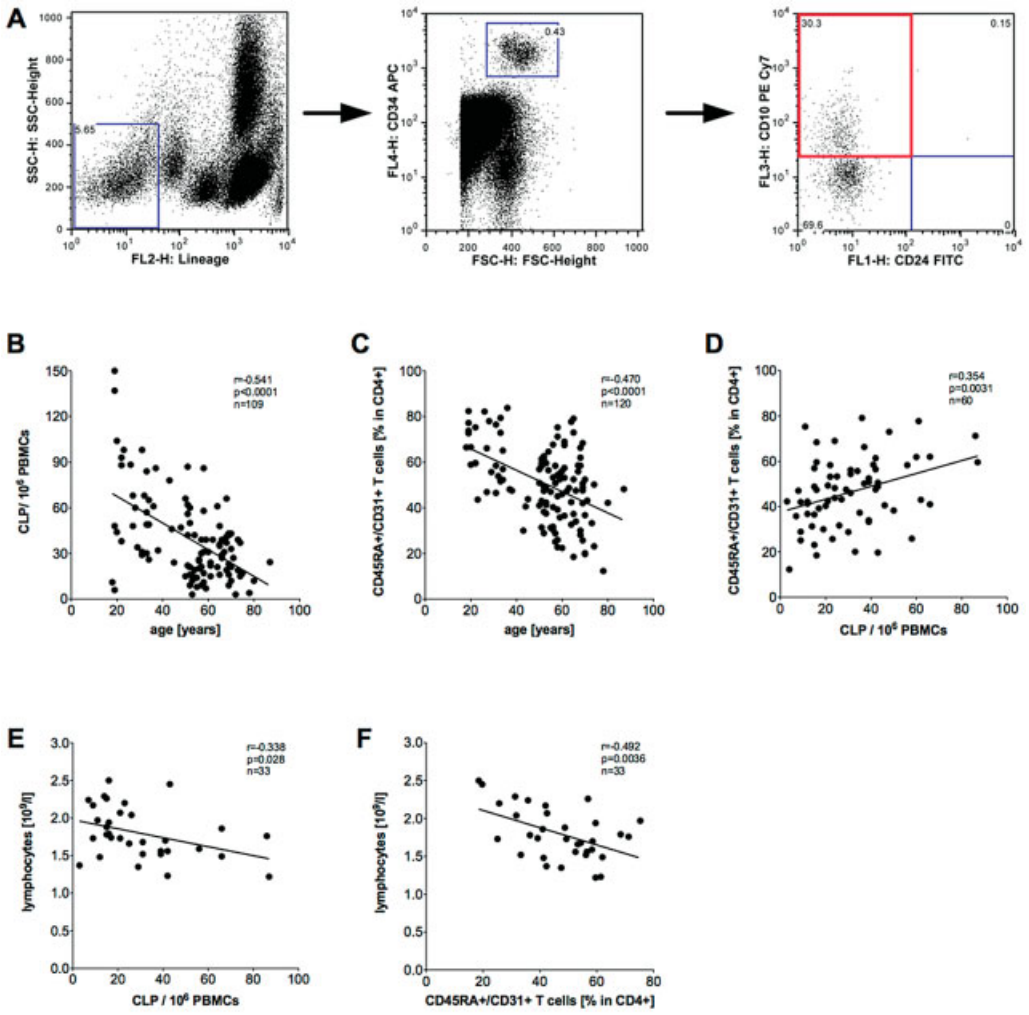

Figure 1. The frequency of common lymphoid progenitors (CLPs) declines with age and correlates with thymic output of recent thymic emigrants (RTEs) in healthy individuals. **A**, Flow cytometric analysis of Lin−CD34+CD10+CD24− progenitors in peripheral blood mononuclear cells (PBMCs). **B–F**, Correlations between the frequency of CLPs in peripheral blood and age (**B**), the frequency of RTEs and age (**C**), the frequencies of RTEs and CLPs (**D**), the lymphocyte count and the frequency of CLPs (**E**), and the lymphocyte count and the frequency of RTEs (**F**). APC = allophycocyanin; PE-Cy7 = phycoerythrin–Cy7; FITC = fluorescein isothiocyanate.

In order to more closely analyze the influence of age on the frequency of CLPs, a cohort of younger healthy control subjects (median age 29.5 years) was recruited. In the global analysis, a strong correlation between the frequency of CLPs and age was observed in both control cohorts (Figure 1B). In addition, the frequency of RTEs declined with age (Figure 1C), as previously reported (9). In the original age-matched control cohort, a highly significant positive correlation between the determined frequencies of CLPs and RTEs was observed (Figure 1D). In contrast, no significant correlation between the frequency of RTEs and that of Lin−CD34+ or CD34+ cells was detectable, while the frequency of CLPs correlated significantly with the frequency of CD34+ cells ($r = 0.3$, $P = 0.008$) (results not shown).

In order to estimate absolute numbers of CLPs in peripheral blood, absolute lymphocyte frequencies were determined from diagnostic complete blood cell (CBC) counts performed in 33 healthy donors at the time of CLP analysis and used as the denominator in the flow cytometric analysis. To calculate absolute cell numbers, lymphocyte numbers determined from the CBC count were equated to the number of cells present within the lymphocyte gate, and the determined percent frequen-

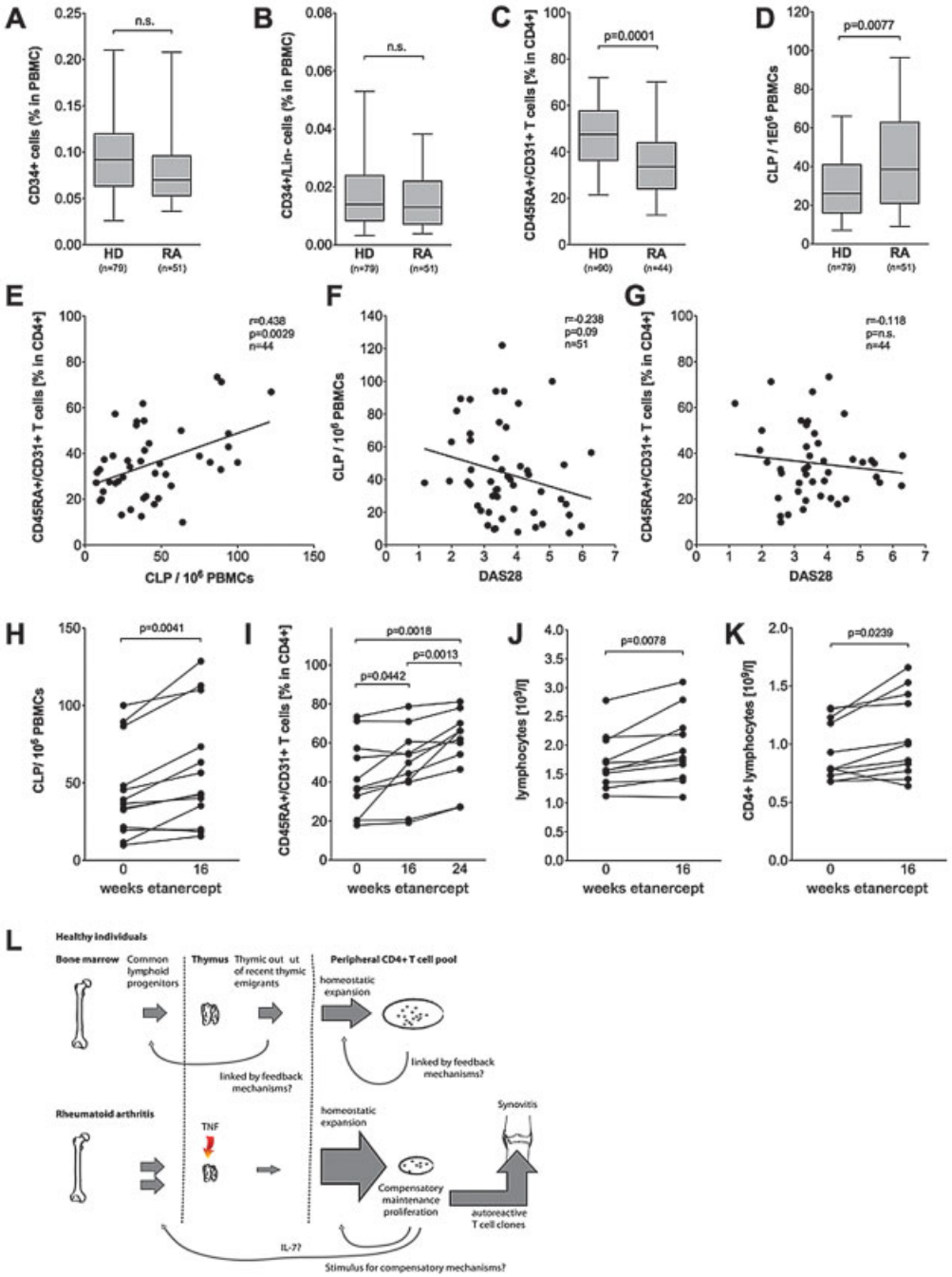

Figure 2. The frequency of CLPs is increased, the frequency of RTEs is decreased, and treatment with the tumor necrosis factor (TNF) inhibitor etanercept normalizes RTE frequencies. **A–D**, Frequencies of CD34+ hematopoietic stem cells (HSCs) (**A**), Lin−CD34+ cells (**B**), CD45+CD31+ RTEs (**C**), and Lin−CD34+CD10+CD24− CLPs (**D**) in the peripheral blood of healthy donors (HD) and patients with rheumatoid arthritis (RA). **E**, Correlation between the frequencies of CLPs and RTEs in the peripheral blood of patients with RA. **F**, Correlation between the frequency of CLPs and the Disease Activity Score in 28 joints (DAS28) in patients with RA. **G**, Correlation between the frequency of RTEs and the DAS28 in patients with RA. **H–K**, Frequency of Lin−CD34+CD10+CD24− CLPs (**H**), frequency of CD4+CD45RA+CD31+ RTEs (**I**), absolute numbers of lymphocytes (**J**), and absolute numbers of CD4+ lymphocytes (**K**) in the peripheral blood of patients with RA, at week 0, week 16, and/or week 24 of etanercept treatment. **L**, Schematic representation of different compartments of T cell generation in healthy individuals and patients with RA. Data in **A–D** are presented as box plots, where the boxes represent the 25th to 75th percentiles, the lines within the boxes represent the median, and the lines outside the boxes represent the 5th and 95th percentiles. NS = not significant (see Figure 1 for other definitions). Color figure can be viewed in the online issue, which is available at http://onlinelibrary.wiley.com/doi/10.1002/art.38058/abstract.

cies of CLPs were used to calculate the absolute numbers. The results confirmed the correlation between the frequencies of CLPs and RTEs. In addition, however, an inverse correlation between absolute lymphocyte numbers and the frequencies of both CLPs and RTEs was observed in healthy control subjects (Figures 1E and F).

Decreased frequency of RTEs and increased frequency of lymphoid progenitors in patients with RA. For the analysis of thymopoiesis in RA, 51 patients were matched with healthy control subjects for age and sex. The frequency of CD34+ HSCs and their Lin−CD34+ HSC progeny did not differ between patients with RA and healthy control subjects (Figures 2A and B). In agreement with reports in the literature, however, the frequencies of RTEs in patients with RA were significantly lower than those in healthy control subjects (Figure 2C). In contrast, the frequency of CLPs was significantly higher in patients with RA than in healthy control subjects (Figure 2D). The age of the patients was inversely correlated with both the frequency of CLPs ($r = -0.278$, $P = 0.05$) and the frequency of RTEs ($r = -0.467$, $P = 0.0014$). The analysis of disease duration prior to study enrollment and the current therapeutic regimen revealed no correlations (results not shown). Comparable with results in the healthy control group, a significant correlation between CLP and RTE frequencies was also observed in patients with RA (Figure 2E).

The results of CBC counts were available for 42 of the patients with RA and revealed mild but significant lymphopenia in patients compared with control subjects (median 1.74 gm/liter versus 1.52 gm/liter; $P = 0.0081$), which has been described previously (11). Calculation of absolute CLP numbers confirmed the significant difference between healthy control subjects and patients with RA described above (median 57.1 CLPs/ml blood versus 94.3 CLPs/ml blood; $P = 0.0124$).

Effect of therapeutic TNF blockade with etanercept on the frequency of thymic progenitors in RA. In the cross-sectional study of 51 patients with RA, no significant influence of disease activity on the frequency of CLPs (Figure 2F) or RTEs (Figure 2G) was detectable. A trend toward a decreased CLP frequency with increasing disease activity did not reach statistical significance.

In order to dissect the influence of current disease activity and response to therapy on deficient thymic output and the increased availability of lymphoid progenitors in RA, the cell populations were quantified in a cohort of patients with RA who were observed longitudinally before and during 24 weeks of treatment with etanercept. In the prospective analysis, the frequencies of lymphoid progenitors in this patient cohort increased further during therapy (from a mean ± SD of 44.2 ± 8.3 CLPs/10^6 PBMCs at baseline to 58.4 ± 10.5 CLPs/10^6 PBMCs at week 16) (Figure 2H). More importantly, TNF blockade normalized the RTE deficiency by significantly increasing the frequency of RTEs in the peripheral circulation to almost normal levels (mean ± SD percent CD31+ cells in CD4+ cells 41.8 ± 5.9 at baseline, 48.6 ± 5.6 at week 16, and 58.1 ± 5.5 at week 24) (Figure 2I). The absolute lymphocyte count (Figure 2J) and the calculated absolute CD4+ T cell count (Figure 2K) also increased during anti-TNF therapy.

DISCUSSION

This study is the first to provide evidence that the frequency of Lin−CD34+CD10+CD24− CLPs in healthy individuals is quantitatively linked to thymic output, indicating that CD10+ HSC progeny are indeed a precursor population seeding the thymus and giving rise to T cells. In addition, the study showed that thymic output correlates inversely and closely with both the frequency of CLPs and absolute lymphocyte numbers, which strongly suggests that a tight feedback mechanism controls thymic output. In addition, the results showed that thymic output correlates inversely and closely with both the frequency of CLPs and absolute lymphocyte numbers, which strongly suggests that a tight feedback mechanism controls thymic output (Figure 2L).

In patients with RA, the frequency of RTEs was decreased despite significantly increased frequencies of prethymic T cell precursors, and absolute lymphocyte numbers in patients were reduced in comparison with those in control subjects. Studies of therapeutic T cell depletion with agents such as Campath 1H have shown that reconstitution of the peripheral T cell compartment is delayed in patients with RA (12). One possible explanation for our results is that intrathymic blockade of T cell development in RA leads to RTE deficiency, which is accompanied by a compensatory increase in the frequency of prethymic lymphoid progenitors. Alternatively, a higher demand for T cells in order to replenish the peripheral T cell pool could induce excessive homeostatic proliferation, which dilutes the concentration of TREC and the frequency of RTEs in the naive T cell pool. The most likely scenario is the coexistence of compromised thymic function and increased T cell proliferation.

Cytokines are another pivotal factor influencing T cell homeostasis (and possibly thymic function) in RA. Interleukin-7 (IL-7) is a cytokine known to exert a profound influence on physiologic T cell homeostasis in

healthy individuals, and this could provide a link between shrinkage of the peripheral T cell pool, the supply of CLPs from bone marrow, and thymic output. Published reports on the role of IL-7 in RA are conflicting, but serum levels of IL-7 have been shown to be significantly decreased in untreated patients with very early RA (13). We previously showed that successful anti-TNF treatment significantly increased serum levels of IL-7 in responding patients with RA while simultaneously normalizing at least part of the oligoclonal CD4 T cell compartment of these patients (14). Accordingly, IL-7 could be part of a compensatory feedback loop that is present in patients with RA and acts by increasing the availability of those precursors. A reduction in disease activity (i.e., the inflammatory burden and the TNF load) strengthens this feedback mechanism and leads to a surge of CLPs during treatment and to increased thymic output of RTEs.

In RA, TNF is another pivotal cytokine. TNF is physiologically expressed in the thymus (15) and promotes T cell development, but overexpression of TNF results in lymphopenia and thymic involution (16). Our group previously demonstrated that in patients with RA, surface-expressed, membrane-anchored TNF drives maintenance proliferation of T cells (11). Accordingly, the paucity of RTEs in RA might be the result of elevated TNF expression. The increased number of RTEs observed during successful anti-TNF therapy (to levels comparable with those in normal control subjects) suggests that TNF blockade alleviates the RTE deficiency in RA, which in turn indicates that the TNF load seen in RA contributes to this defect.

In conclusion, these findings show that the shortage of RTEs seen in RA is likely not attributable to a shortage of CLPs but rather is the result of thymic or postthymic perturbances.

AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Wagner 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 conception and design. Wagner, Baerwald, Rossol.
Acquisition of data. Wagner, Schatz, Baerwald, Rossol.
Analysis and interpretation of data. Wagner, Baerwald, Rossol.

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