Early rheumatoid arthritis is associated with a deficit in the CD4⁺CD25^{high} regulatory T cell population in peripheral blood

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Objective. Our aim was to test the hypothesis that there is a deficit in the CD4⁺CD25^{high} regulatory T cell population in early rheumatoid arthritis (RA), either in size or functional activity.

Methods. Peripheral blood mononuclear cells were examined from subjects with early active RA who had received no previous disease-modifying therapy (n = 43), from individuals with self-limiting reactive arthritis (n = 14), from subjects with stable, well-controlled RA (n = 82) and from healthy controls (n = 72). The frequencies of CD4⁺CD25^{high} T-cells were quantified using flow cytometry, and function was assessed by the ability to suppress proliferation of CD4⁺CD25⁻ T-cells. Paired blood and synovial fluid was analysed from a small number of RA and reactive arthritis patients.

Results. There was a smaller proportion of CD4⁺CD25^{high} T-cells in the peripheral blood of early active RA patients (mean 4.25%) than in patients with reactive arthritis or in controls (mean 5.90 and 5.30%, respectively, P = 0.001 in each case). Frequencies in stable, well-controlled RA (mean 4.63%) were not significantly different from early active RA or controls. There were no differences in suppressor function between groups. Higher frequencies of CD4⁺CD25^{high} T-cells were found in synovial fluid than in blood in both RA and reactive arthritis.

Conclusions. These data demonstrate a smaller CD4⁺CD25^{high} regulatory T cell population in peripheral blood of individuals with early active RA prior to disease-modifying treatment. This may be a contributory factor in the susceptibility to RA and suggests novel approaches to therapy.

Key words: CD4⁺CD25^{high} T-cells, Regulatory T-cells, Early arthritis, Rheumatoid arthritis, Reactive arthritis.

Introduction

There is considerable evidence to support the involvement of T-lymphocytes in the pathogenesis of rheumatoid arthritis (RA), including the presence of both CD4⁺ and CD8⁺ T-cells in RA synovium [1–3]. Although the mechanisms that prevent autoimmunity remain poorly understood, substantial evidence supports the existence of a group of regulatory T-cells that contribute to peripheral tolerance via an active, dominant mechanism. For example, scid mice administered CD4⁺CD45RB^{high} T-cells from normal mice developed a severe wasting disease and colitis, which could be prevented by the simultaneous administration of CD4⁺CD45RB^{low} T-cells [4]. Furthermore, depletion of specific peripheral CD4⁺ T-cell subsets from healthy mice resulted in the development of organ-specific auto-immunity, which was preventable by reconstitution with distinct CD4⁺ T-cell populations, suggesting an active mechanism of self-tolerance [5].

Although a number of regulatory T-cell subsets have been described, those with the most potent regulatory function appear to reside within the naturally occurring CD25⁺ (IL-2 receptor α chain) subgroup of CD4⁺ T-cells [6–11], which constitute approximately 10% of CD4⁺ T-cells in healthy mice [6, 7]. These cells down-regulate the activation and proliferation of CD25⁻ T-cells, thereby suppressing harmful responses to self-antigens [6, 12]. They are anergic in vitro, and do not proliferate or secrete IL-2 in response to stimulation via the T-cell antigen receptor (TCR) [6, 13]. Other phenotypic surface markers have been identified including CD45RB^{low}, CD45RO⁺ and CD152⁺ (CTLA-4), although none of these are unique to the regulatory subset. For example, CD25 is transiently expressed upon activation of naïve CD4⁺ T-cells [14]. FOXP3, a transcriptional repressor, has been shown to be essential for both the development and function of regulatory T-cells [15].

There is substantial evidence for the presence of similar CD4⁺CD25⁺ regulatory T-cells in humans [13, 16–23]. However, only cells expressing the highest levels of CD25 (termed CD25^{high}) demonstrate potent regulatory function [16, 22, 24]. There are several reports describing the presence of CD4⁺CD25^{high} regulatory T-cells in RA [25–27], with conflicting conclusions, and also one report in juvenile idiopathic arthritis [28]. However, there are no data on the size of the circulating CD4⁺CD25^{high} T-cell population in individuals with early RA prior to the use of disease-modifying anti-rheumatic drug (DMARD) treatment or steroids. Given that these cells can be expanded in vitro without...
loss of regulatory function [18, 19, 29], there would be clear therapeutic implications if a deficit in their number and/or function were present. We therefore measured the size of the CD4⁺CD25<sup>high</sup> T-cell population in patients with recent onset RA, prior to therapy, and compared this with disease controls with self-limiting reactive arthritis or well-controlled RA, and to healthy controls. We postulated that abnormal T-cell regulation may underlie the persistent inflammation that is characteristic of RA.

We report a deficit in the proportion of CD4⁺CD25<sup>high</sup> regulatory T-cells in the peripheral circulation of individuals with early active RA prior to disease-modifying treatment when compared with healthy controls, and contrast these findings with data from those with self-limiting reactive arthritis.

### Patients, materials and methods

**Patients**

Ethical approval for the project was obtained from the Leeds Local Research Ethics Committee, and all work was carried out in accordance with the Declaration of Helsinki. Blood samples were collected from individuals attending the Leeds Early Arthritis Clinic (early active RA and self-limiting reactive arthritis) following informed written consent. All patients with early active RA or with self-limiting reactive arthritis were DMARD-naïve, and had received no prior steroid therapy. Most patients had previously taken non-steroidal anti-inflammatory drugs. Patients with reactive arthritis were followed up to ensure that they subsequently resolved within 1 yr. Healthy control blood samples were similarly obtained from the Yorkshire Blood Transfusion Service. Controls were sex-matched to the early active RA group. Samples were also collected from patients attending a clinic for RA patients whose disease was stable and well-controlled, who possessed no clinically significant synovitis and were deemed to be in ‘remission’ by the assessing consultant rheumatologist. Most patients in this group were receiving DMARD therapy. Table 1 outlines the inclusion and exclusion criteria, demographics and clinical details of each group.

### Quantification of the CD4⁺CD25<sup>high</sup> subset

Peripheral blood was collected into tubes containing either heparin or acid citrate dextrose. Peripheral blood mononuclear cells (PBMCs) were separated by centrifugation over a sucrose density gradient, using Lymphoprep (Axis-Shield, Oslo, Norway). Cells were then labelled and analysed without prior freezing at all times. Antibodies used for flow cytometry analysis were anti-CD3-FITC (UCHT1, Serotec, Oxford, UK), anti-CD25-PE (CD25-3G10, Caltag, Burlingame, USA) and anti-CD4-Cy5 (MT310, DAKO, Glostrup, Denmark). Isotype-matched negative control antibodies (Beckman Coulter, Fullerton, USA) were also used. For quantification of the CD4⁺CD25<sup>high</sup> subset, cells were labelled with anti-CD3-FITC, anti-CD25-PE and anti-CD4-Cy5. Once labelled, cells were fixed using 1% formaldehyde buffer, and left for at least 24 h at 4°C prior to analysis. Analysis was performed using a Coulter EPICS XL-MCL flow cytometer and Coulter System II software Version 3.0. Forward and side scatter properties were used to exclude dead cells and debris from analysis. At least 30000 gated events were recorded from each sample. A typical CD25 expression profile is shown in Fig. 1A. There was no distinct boundary between CD25<sup>low</sup> and CD25<sup>high</sup> populations, and a number of factors were used to set a gate that discriminated between them. CD25<sup>high</sup> T-cells expressed slightly less CD3 and CD4 than CD25<sup>low</sup> and, additionally, very few CD4<sup>+</sup> cells expressed high levels of CD25, providing a ‘cellular negative’ population (Fig. 1B and C). Using these indicators, the sub-population of CD4⁺CD25<sup>high</sup> T-cells was quantified as a proportion of total CD4<sup>+</sup> T-cells, by an observer blinded to the patients’ or controls’ clinical details.

Paired peripheral blood and synovial fluid samples from RA and reactive arthritis patients were also analysed using the same techniques.

### Proliferation and suppression assays

For proliferation and suppression assays, CD4<sup>+</sup> T-cells were negatively selected from PBMCs, using magnetic depletion with MACS (LS – Miltenyi Biotech, Bisley, UK) columns and CD4<sup>+</sup> T-cell enrichment cocktail (StemCell Technologies, Vancouver,
Canada). Negatively selected cells were then labelled with anti-CD25-PE and anti-CD4-Cy5 and separated into CD4⁺CD25⁻, CD4⁺CD25low and CD4⁺CD25high fractions using a FACS Vantage cell sorter, with gates set as shown in Fig. 1D. Fractions were resuspended in RPMI 1640 medium (Invitrogen, Paisley, UK) containing 10% human AB serum (Sigma-Aldrich, Poole, UK), penicillin 100 IU/ml and streptomycin 10 μg/ml (Sigma-Aldrich) and 2 mM l-glutamine (Invitrogen). Purities of each of the sorted cell fractions were above 90% (and usually above 95%).

Subsequent assays were performed in 96-well round bottomed plates (Sigma) with 20 μl total volume/well. Each well contained 10⁴ autologous antigen presenting cells (APCs) (irradiated [60Gy] CD4⁻ cells, derived during CD4⁺ T-cell isolation). For later assays, due to technical problems with the irradiator, APCs were prepared by pre-treatment with mitomycin C (Sigma-Aldrich). Proliferation of sorted cell subsets (CD4⁺CD25⁻, CD4⁺CD25low or CD4⁺CD25high, 10⁴/well) was compared using phytohaemagglutinin (PHA) (Sigma-Aldrich) 1 μg/ml or soluble anti-CD3 (OKT3, Janssen-Cilag, High Wycombe, UK) at 1 μg/ml as mitogens. The ability of CD4⁺CD25low and CD4⁺CD25high subsets to inhibit proliferation of CD4⁺CD25⁻ cells was assessed in co-culture experiments in which 10⁴ CD4⁺CD25low or CD4⁺CD25high cells were mixed with 10⁴ CD4⁺CD25⁻ cells prior to the addition of mitogens. Cells were incubated at 37°C with 5% CO₂ for 4 days for PHA-stimulated cells, or for 6 days for anti-CD3-stimulated cells. After this time, 1μCi [5³H]-thymidine (Amersham Biosciences, Little Chalfont, UK) in 5 μl medium was added, and cells incubated for a further 16 h. Cells were subsequently harvested on to glass fibre filters using a β plate harvester (EG&G Wallac Harvester 96-Mach IIIM) and [³H]-thymidine incorporation into DNA measured by liquid scintillation counting (Beta Plate Scint – Perkin-Elmer, UK).

For samples from early active RA and reactive arthritis patients, PBMCs were stored at −80°C for up to 4 days prior to sorting and culture, for practical reasons. However, an experiment performed comparing the suppressive properties of regulatory T-cells before and after freeze-thawing did not reveal any loss of suppressive capacity after storage (data not shown).

Statistics

A one-sample Kolmogorov-Smirnov test was used to determine that data relating to CD4⁺CD25high T-cells from the control population followed a normal distribution. A one-way analysis of variance (ANOVA) was performed to demonstrate the presence of differences between groups as a whole, and then an independent samples t-test was used to perform comparisons between means of specific clinical groups. Correlations between age and proportion of CD4⁺CD25high cells within the control group were sought initially using Pearson's correlation coefficient. Linear regression was performed to seek any effect from age, sex, disease duration and C-reactive protein (CRP). A Bonferroni correction was performed when multiple comparisons were made.
The Mann–Whitney U test was used to compare suppression between groups. All analyses were performed using SPSS 11.0 for Windows.

Results

Determinants of the CD4^+CD25^{high} T-cell proportion in healthy controls

Within the control group, the proportion of CD4^+CD25^{high} T-cells was similar in males and females. There was a very weak negative correlation between proportion of CD4^+CD25^{high} cells and age ($r = -0.335$, $P = 0.004$). This finding was confirmed using regression analysis ($P = 0.010$) as discussed below, and an appropriate correction was made in the subsequent analysis.

Patient demographics and clinical details

A summary of the demographics of each patient group is shown in Table 1. There was a small difference in the mean ages of these groups (mean age 52 yr in early active RA and 44 in healthy controls). An appropriate adjustment was made in the subsequent regression analysis. As expected, the mean age of the self-limiting reactive arthritis group was lower than other groups [29]. All groups were well-matched for sex. The mean disease duration in the early active RA group was 6.6 months and 1.7 med in RA in the self-limiting reactive arthritis group. The early active RA group had a mean CRP of 36.5 mg/l, whereas the reactive arthritis group had a mean of 35.4 mg/l and the stable, well-controlled RA group 5.5 mg/l. The majority of the early active RA group was rheumatoid factor (RF)-positive (74.4%) compared with none of those with self-limiting reactive arthritis. Of those with stable, well-controlled RA, 46.3% were RF-positive at the time of assessment although, 64% had a positive RF during the course of their disease. Most of those with stable, well-controlled RA were on DMARD therapy (92.7%). None of those recruited to the early active RA or stable well-controlled RA, 46.3% were RF-positive at the time of assessment although, 64% had a positive RF during the course of their disease.

Most patients in the reactive arthritis group had mono- or oligoarticular disease several weeks after either a gastrointestinal or steroid therapy. The remainder had a symmetrical polyarticular disease following a respiratory tract infection.

Comparison of proportion of CD4^+CD25^{high} T-cells between patient groups

The proportion of CD4^+CD25^{high} T-cells in each group was determined as a percentage of total CD4^+ T-cells (Fig. 2). An initial one-way ANOVA comparing proportions of CD4^+ T-cells that were CD25^{high} between all clinical groups was highly significant ($P = 0.001$). Having performed Bonferroni correction for multiple comparisons, the critical $P$ value for the independent $t$-test was 0.01. There was a significantly smaller proportion of CD4^+ T-cells that were CD25^{high} in patients within the early active RA group (mean 4.25%) when compared with controls (mean 5.30%, $P = 0.001$) as shown in Fig. 2. There was also a significant difference between the early active RA group and those with self-limiting reactive arthritis (mean 5.90%, $P = 0.001$). Amongst individuals who had well-controlled stable RA, intermediate values were observed (mean 4.64%), which were not significantly different from the early active RA group ($P = 0.209$), but showed a trend to being lower than controls ($P = 0.024$). There was no significant difference between controls and those with self-limiting reactive arthritis ($P = 0.262$). The $t$-tests, therefore, highlighted significant differences in the proportion of CD4^+CD25^{high} cells between controls and early active RA patients, and between self-limiting reactive arthritis and early active RA patients.

In the regression model, age had a small (Beta $= -0.193$) but significant ($P = 0.010$) effect. There was no correlation between the proportion of CD4^+CD25^{high} T-cells and CRP or disease duration. In the regression analysis, correcting for age did not change the conclusions reached from the $t$-tests alone ($P$ values for early active RA compared with controls $= 0.010$ and for early active RA compared with reactive arthritis $P = 0.041$, with no other significant differences between groups).

Paired peripheral blood and synovial fluid analysis of CD4^+CD25^{high} regulatory T-cells

None of the patients presenting with early active RA had sufficiently swollen joints to warrant aspiration of synovial fluid on clinical grounds alone. Therefore, five paired blood and synovial fluid samples were obtained from RA patients with more established disease (four of whom were taking DMARDs and one of whom was taking low dose prednisolone), and

![Fig. 2. Percentage of CD4^+ T-cells expressing high levels of CD25 in each clinical group. Asterisks (***) represent significant differences ($P < 0.05$) between the mean value of the early active RA group as compared with controls, and between the early active RA group and reactive arthritis group. Corresponding unadjusted $P$-values were: early active RA and controls, $P = 0.001$; reactive arthritis and controls, $P = 0.262$; stable well-controlled RA and controls, $P = 0.024$; early active RA and reactive arthritis, $P = 0.001$; and early active RA and stable well-controlled RA, $P = 0.209$.](image)
three paired samples were obtained from reactive arthritis patients (all of whom were DMARDs and steroid-naïve). In four out of five of the RA paired samples, the proportion of CD4⁺CD25<sup>high</sup> regulatory T-cells was higher in synovial fluid than in peripheral blood (Fig. 3). However, in all of the reactive arthritis samples this was also the case.

**Suppressive capacity of CD4⁺CD25<sup>high</sup> regulatory T-cells**

Functional assays using healthy control cells (n = 8) confirmed that the CD4⁺CD25<sup>high</sup> subset contained cells that were both anergic and suppressive. CD4⁺CD25<sup>high</sup> cells alone proliferated poorly in response to both soluble anti-CD3 and PHA (Fig. 4),
There were no significant differences seen between groups in T-cells in rheumatic diseases without the confounding influence of steroids or other drug therapies. We have shown these cells to be both anergic and suppressive in control individuals. We found no difference in the suppressive capacity of CD4^+CD25^{high} T-cells from RA, reactive arthritis or healthy controls.

In patients with stable well-controlled RA, the proportion of CD4^+CD25^{high} T-cells was intermediate between that seen in controls and in untreated early active RA, with no significant difference from either group. Many of the well-controlled RA patients were on DMARDs, however, and it is not possible to exclude an effect of these drugs on peripheral lymphocyte subsets. Furthermore, a significant proportion of these patients may flare if DMARDs were stopped, which suggests that their disease is suppressed by drugs rather than modulated by an immune regulatory response [31].

One implication of these findings is that, in the context of a polygenic disease, a relatively small deviation from normal in a mechanism regulating peripheral immune tolerance may be sufficient to convert a self-limiting inflammatory event into a persistent auto-immune disease. The recent discovery that a polymorphism in the gene for CTLA-4 is linked to a variety of auto-immune states has similar implications [32]. In this regard, it is notable that CTLA-4 is a marker for, and may play a role in the function of, the CD4^+CD25^{high} regulatory T-cell subset [33]. It is intriguing that we found a small but significant fall in the number of regulatory T-cells with age. Although this finding requires replication, auto-immune diseases increase with age, which could reflect corresponding changes in the size of the regulatory T-cell pool.

An alternative explanation for our data is that regulatory cells are recruited to sites of inflammation in an attempt to suppress disease, resulting in a relative reduction in the peripheral blood population. Functional regulatory CD4^+CD25^{high} T-cells have previously been isolated from RA synovial fluid [34]. However, we found that there were increased proportions of CD4^+CD25^{high} T-cells in the joints of both RA and reactive arthritis patients, and, therefore, this alone is not a sufficient explanation for the lower frequency of regulatory T-cells seen in the early active RA group compared with the self-limiting reactive arthritis group in the peripheral circulation. Although there may be some migration of cells, there appears to be an intrinsic difference between individuals who develop RA and those who develop self-limiting reactive arthritis. It is possible that the CD4^+CD25^{high} T-cell population expands within the synovial compartment, rather than (or in addition to) the cells purely homing from the blood to joints.

Synovial fluid analysis is complicated by the presence of abundant activated T-cells within the joints that have a similar phenotype to regulatory T-cells. Currently there is no unique surface marker that identifies the latter population of cells. We took extreme care in identifying this subset, however, based not only on high levels of CD25 expression but also on subtle variations in the intensity of the CD3 and CD4 cell surface markers. Analysis of all flow cytometry results was also performed without the observer’s knowledge of the clinical or control group, to avoid potential bias. FOXP3 is a more specific marker for regulatory T-cells, although there is evidence to suggest that it can also be induced in CD4^+CD25^- T-cells and, therefore, is not necessarily unique to regulatory T-cells in humans (unlike the situation in mice) [35, 36]. This is not a cell surface molecule, however, and current detection methods largely rely upon transcriptional analysis of cell populations. We analysed FOXP3 expression using real-time RT–PCR in a subset of individuals with early RA and in healthy controls. High levels of FOXP3 expression were detected in peripheral blood CD4^+CD25^{high} T-cells from both RA and controls, relative to CD4^+CD25^- and CD4^+CD25^{low} T-cells (data not shown).

It is also important to stress that a number of other types of regulatory lymphocyte have also been described, particularly in animal models [37]. These include different CD4^+ T-cell subsets but also cells of the innate immune system such as NKT-cells and...
yT-cells. Similar to our current work, NKT-cells are deficient in diabetes-prone NOD mice and may also be reduced in number in humans with diabetes [38]. There is also evidence that regulatory T-cells can be induced, with a similar phenotype to the naturally occurring CD4\(^+\)CD25\(^{high}\) population in terms of surface markers [23, 39].

CD4\(^+\)CD25\(^{high}\) T-cells have recently been shown to be reduced in number in patients with active lupus [40], and impaired in their suppressor function (but not in number) in multiple sclerosis [41, 42] and in RA patients prior to receiving anti-TNF\(^\alpha\) therapy [26]. Data from children with juvenile idiopathic arthritis show that the benign, self-remitting form of the disease is associated with a higher frequency of CD4\(^+\)CD25\(^{high}\) T-cells in blood than the form with a more persistent course [28]. Parallels can potentially be drawn between this data and ours, in that our adult inflammatory arthritis group with a self-remitting course (reactive arthritis) had higher CD4\(^+\)CD25\(^{high}\) T-cell numbers than adults with persistent inflammatory arthritis (RA). A number of other groups have examined the frequency of regulatory T-cells in the peripheral blood of individuals with RA, with varying conclusions. There are several possible explanations for the discrepancy between these published results and the data here. Cao et al. [34] detected no difference between the frequency of total CD4\(^+\)CD25\(^{+}\) T-cells (i.e. including CD4\(^+\) T-cells expressing low levels of CD25) in RA and controls, looking at individuals with long-standing disease on DMARDs. The following year, the same group examined the CD4\(^+\)CD25\(^{high}\) population of T-cells and noted a reduced frequency in the RA patients (in accordance with our data) [25]. van Amelsfort et al. [27] detected an increase in total CD4\(^+\)CD25\(^{+}\) T-cells in those with RA compared with controls, studying patients with long-standing disease on a range of DMARDs. Ehrenstein et al. [26] did not find a difference in CD4\(^+\)CD25\(^{high}\) T-cell frequency between individuals with active RA on DMARDs (who subsequently received biological therapy and presumably many of whom had established disease) and healthy controls. Liu et al. [43] showed no difference between total CD4\(^+\)CD25\(^{+}\) T-cell frequencies in RA and controls. Finally, Möttönen et al. [44] detected no difference in the total CD4\(^+\)CD25\(^{+}\) T-cell frequency in blood between patients with established RA on DMARDs or steroids and healthy controls.

There are important differences between all of these studies and the data presented here. Firstly, RA patients in the above studies have had disease of several years’ duration and, are therefore, in the phase of established inflammation. Secondly, no previous work has examined the frequency of regulatory T-cells without the influence of DMARDs. Some of the previous studies have included patients on steroid therapy, which is known to affect CD25 expression and lymphocyte function [45]. Of greater importance is the fact that many of these studies have documented the total CD4\(^+\)CD25\(^{+}\) T-cell frequency, which includes CD4\(^+\)CD25\(^{low}\) T-cells that are likely to be recently activated. In this respect, there are major differences in what is actually being quantified between studies, making comparisons of results difficult. Finally, it may also be of note that a range of different anti-CD25 monoclonal antibodies has been used for flow cytometry analysis. This should not have a major influence on conclusions from comparisons between healthy control and disease groups, although one might expect differences in the range of frequencies reported by researchers using different antibodies.

A number of groups have now shown that it is possible to both isolate regulatory lymphocytes and expand them \textit{ex vivo}, without any loss of suppressive activity [18, 19, 29, 46, 47]. In the context of the current study, this suggests novel regimens for treating auto-immune disease. At present, severe auto-immunity is being treated in some centres by high dose cytotoxic therapy with stem cell rescue (autologous stem cell transplantation). It should be possible to supplement these and similar approaches by the infusion of expanded autologous regulatory cells if a deficiency in their number or function were confirmed in auto-immune disease.

In summary, we have demonstrated a reduction in the CD4\(^+\)CD25\(^{high}\) regulatory T-cell subset in the peripheral blood of patients with early active RA prior to therapy. These data have important implications in the context of the control of inflammation and development of autoimmunity. It is now important to replicate these findings in early RA and to seek a similar phenomenon in other auto-immune diseases. Our data also suggest novel paradigms for the treatment of auto-immune diseases.

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<td>Lower regulatory T-cell numbers may pre-dispose to persistent auto-immune disease such as RA.</td>
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<td>A unique surface marker for regulatory T-cells would facilitate their study in human disease.</td>
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Regulatory T-cells in early RA


