Abnormal T cell differentiation persists in patients with rheumatoid arthritis in clinical remission and predicts relapse

C H Burgoyne,[1,2] S L Field,[1,2] A K Brown,[1,2] E M Hensor,[3] A English,[1,2] S L Bingham,[1,2] R Verburg,[4] U Fearon,[1,2] C A Lawson,[1,2] P J Hamlin,[5] L Straszynski,[2] D Veale,[1,2] P Conaghan,[1,2] M A Hull,[2,5] J M van Laar,[4] A Tennant,[3] P Emery,[1,2] J D Isaacs,[1,2] F Ponchel[1,2]

[1] Academic Unit of Musculoskeletal Disease, University of Leeds, Leeds, UK; [2] Leeds Institute of Molecular Medicine, University of Leeds, Leeds, UK; [3] Psychometric Laboratory, University of Leeds, Leeds, UK; [4] Rheumatology Department, Leiden University Medical Center, Leiden, The Netherlands; [5] Centre for Digestive Diseases, Leeds General Infirmary, Leeds, UK

Correspondence to: Dr Frederique Ponchel, Leeds Institute of Molecular Medicine, Clinical Sciences Building, St James's University Hospital, Leeds, LS9 7TF, UK; F.Ponchel@leeds.ac.uk

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ABSTRACT

Objectives: An abnormal CD4+ T cell subset related to inflammation exposure (inflammation-related cells, IRC) has been identified in rheumatoid arthritis (RA). Patients with inflammatory and non-inflammatory diseases were used to examine the relationship between inflammation and this T cell subset in vivo.

Methods: Blood was collected from healthy controls and patients with RA (active disease or in clinical remission), Crohn's disease and osteoarthritis. IRC and chemokine receptors were quantified by flow cytometry. Thymic activity and apoptotic factors were measured by real-time polymerase chain reaction. Circulating cytokines were measured by enzyme-linked immunosorbent assay. CXCR4 and SDF1 in synovial biopsies were measured using immunohistochemistry.

Results: IRC were identified in patients with RA ($p<0.0001$) and Crohn's disease ($p = 0.005$), but not in those with osteoarthritis. In RA in remission, IRC persisted ($p<0.001$). In remission, hyperproliferation of IRC was lost, chemokine receptor expression was significantly lowered ($p<0.007$), Bax expression dropped significantly ($p<0.001$) and was inversely correlated with IRC ($rho = -0.755$, $p = 0.03$). High IRC frequency in remission was associated with relapse within 18 months ($OR = 6.4$, $p<0.001$) and a regression model predicted 72% of relapse.

Conclusions: These results suggest a model in which, despite the lack of systemic inflammation, IRC persist in remission, indicating that IRC are an acquired feature of RA. They have, however, lost their hyper-responsiveness, acquired a potential for survival, and no longer express chemokine receptors. IRC persistence in remission confirms their important role in chronic inflammation as circulating precursors of pathogenic cells. This was further demonstrated by much higher incidence of relapse in patients with high IRC frequency in remission.

Rheumatoid arthritis (RA) is a chronic inflammatory disease of joints and extra-articular tissues, which results in severe disability and premature mortality.[1] The exact pathogenesis of RA remains uncertain; however, autoimmune processes are evidenced by major histocompatibility complex linkage,[2 3] autoantibody production and lymphocyte infiltration of the synovial tissue.[4] All of these features support the hypothesis of a T cell driven disease,[5–7] although this "T centric" paradigm has been challenged.[8 9]

We recently proposed a model of T cell differentiation and demonstrated divergences in relation to chronic inflammation in RA.[10] Isoforms of the tyrosine phosphatase CD45 coupled with lymph node homing receptor CD62L expression, were used to distinguish "naïve" from "memory" T cells. Using this model, we demonstrated the presence of atypical subsets of cells in RA, expressing aberrant combinations of naïve and memory markers.[10] One subset was characterised by the cell surface expression of $CD45RB^{high}$ CD45RA+ $CD45RO^{low}$ CD62L–. We named them inflammation-related cells (IRC), as the frequency of these cells in the periphery was directly correlated with systemic levels of inflammation measured by C-reactive protein (CRP). Retention of CD45RB and CD45RA expression by IRC, and their relatively high T cell receptor excision circle (TREC) content compared with naïve cells, suggested that they were relatively immature and we postulated that they resulted from inflammation-driven differentiation and proliferation of naïve cells.[10] They were also hyper-responsive to mitogen and antigen stimulation.

Cells with a similar phenotype have been described by other groups. Naïve CD4+ T cells treated with a combination of interleukin (IL)-2, IL-6 and tumour necrosis factor (TNF)-α or with IL-15 developed a phenotype similar to IRC, losing CD62L but retaining CD45RA and several other markers of activation.[11 12] Activation under similar conditions induces the expression of chemokine receptors such as CXCR4,[13] suggesting that such cells are destined to home to sites of inflammation. RA synovial T cells have been shown to share many features with cytokine-activated T cells, in particular with regard to their ability to induce macrophage production of TNF-α.[14]

Our current hypothesis is that, following an initiation phase, T cells in RA are essential to the perpetuation of the disease in a non-specific way. Chronic inflammation triggers cytokine T cell activation independently of antigen. These cytokine-activated T cells express chemokine receptors and home to inflamed tissue, where they perpetuate the disease ultimately leading to the destruction of cartilage and bone. In the current study, we used several cohorts of patients with RA, inflammatory and non-inflammatory disease controls and healthy individuals, to study the relationship between inflammation and T cell phenotype in vivo.

MATERIALS AND METHODS

Patient cohorts

Ethical approval was obtained from Leeds Teaching Hospitals NHS Trust Ethics Committee and informed consent was obtained from each participant. A number of clinical groups were studied:

Rheumatoid arthritis cohorts

(1) Patients with active RA (n = 41, age range 20–73 years, CRP range 10–177 mg/l, 68% rheumatoid factor (RF) positive) comprised early, disease-modifying anti-rheumatic drug naïve (n = 22) and long-lasting, refractory RA (n = 19).

(2) RA in clinical remission (n = 42, age range 25–64 years, 57% RF positive). Consultant rheumatologists identified patients with RA whose disease was in remission. All patients satisfied the following criteria: (a) RA according to American College of Rheumatology (ACR) criteria; (b) over 18 years of age; (c) at least 12 months disease duration; (d) no disease flare within preceding 6 months; (e) stable therapy for 6 months; and (f) no clinical indication to change treatment. They had CRP below 11 mg/l for at least 6 months (median CRP 0, range 0–11 mg/l, with 78% of patients below detectable levels, the normal CRP range within our local population being 0–10 mg/l). Disease flare in this cohort was defined by sustained symptoms of tender or swollen joints, for two or more consecutive visits, involving more than one site and/or requiring a change in management (drug or dosage). Owing to the requirement for high cell numbers, all patients in remission were analysed for T cell differentiation, but only subsets of this cohort were used for the other experiments.

(3) Patients with long-lasting, severe RA (n = 12, age range 24–44 years, 75% RF positive) resistant to multiple conventional drugs and who received high-dose chemotherapy (HDC) followed by autologous haematological stem cell transplantation (ASCT).[15 16] Blood and synovial biopsy samples were available for analysis at baseline, during clinical remission (1 and 3 months) and at disease flare (9 or 12 months).

Disease controls

(1) Patients with osteoarthritis (OA; n = 12 age range 47–70 years) fulfilling ACR clinical criteria were recruited, had established disease with no elevation of CRP (CRP <10 mg/l).

(2) Patients with active Crohn's disease (CrD) (n = 13 age range 18–59 years) had established disease with moderate CRP (CRP range 5–45 mg/l).

Healthy controls

(1) Healthy controls were recruited from blood donors (n = 49, age range 24–72 years).

(2) Otherwise healthy individuals with mild cold-like symptoms, runny nose or sore throat, not requiring medication (n = 5). Serial samples were obtained during the first 5 days of symptoms, following resolution (10 days) and 2–3 weeks later.

T cell subset quantification and separation

Naïve, memory and IRC were identified in peripheral blood mononuclear cells based on their expression of CD45RB (bright or dull; Dako, Ely, Cambs, UK), CD45RA (+ or −, Serotec, Oxford, UK), CD45RO (−, dull or bright, Serotec) and CD62L (+ or −, Coulter, High Wycombe, Bucks, UK) as previously described.[10] Cells were further sorted, when required, using a FACS-Vantage (BD, Oxford, UK) under aseptic conditions.

T cell receptor excision circle measurements

TREC content was measured by real-time polymerase chain reaction as previously described.[17]

Proliferation assays

Proliferation assays were performed as described before,[10] using stimulation with phytohaemagglutin (1 and 10 μg/ml, Sigma), IL-2 (20 units/ml, Sigma), anti-CD3 antibody (OKT3, 1 μg/ml Ortho Biotech, Bridgewater, NJ, USA) with or without anti-CD28 antibody (12.5 μg/ml, YTH913 kindly provided by H Waldmann).

Serum cytokine measurements

TNF-α, IL-2, IL-6 and IL-7 levels in sera were measured by enzyme-linked immunosorbent assay (R&D Systems, Abingdon, Oxon, UK) according to the manufacturer's instructions. Minimum detection for the assay was 0.5 pg/ml for TNF-α, 7 pg/ml for IL-2, 0.7 pg/ml for IL-6 and 0.1 pg/ml for IL-7.

Chemokine receptor expression

PE-Cy5 conjugated antibodies (BD) were used to detect cell surface expression of the chemokine receptors CXCR3, CXCR4 or CCR5 in addition to CD45RB-FITC, CD45RA-PE and CD62L-ECD.

Bax, Bcl-2 and Bcl-xL expression

Peripheral blood mononuclear cells were lysed and RNA solubilised as described before and first-strand cDNA was synthesised using 400 U Superscript II reverse transcriptase (Invitrogen, Paisley, UK).[18] Real-time polymerase chain reaction was performed using an ABI Prism 7700 sequence detection system (Applied Biosystems, Warrington, Cheshire, UK) in the presence of SYBR-green. Primers were designed using Primer Express (Applied Biosystems). Transcription of the gene of interest was normalised to that of *GAPDH*. Primer sequences were:

GAPDH forward: 5′-AACAGGGACACCCACTCCTC-3′
GAPDH reverse: 5′-CATACCAGGAAATGAGCTTGACAA-3′
Bcl-2 forward: 5′-GTGGAGAGCGTCAACCGG-3′
Bcl-2 reverse: 5′-GGTTCAGGTACTCAGTCATCCACA-3′
Bax forward: 5′-GCCACTCCTCTGGGACCC-3′
Bax reverse: 5′-ACGCATTATAGACCACATCTGATGA-3′
Bcl-xL forward: 5′-ATACTTTTGTGGAACTCTATGGGAACA-3′
Bcl-xL reverse: 5′-CGTCAGGAACCAGCGGTT-3′.

Immunohistochemistry

Cryostat serial sections of synovial tissue (4 μm) were cut and dried overnight at 37°C. Sections were fixed in 4% paraformaldehyde for 10 min. Endogenous peroxidase was quenched by treatment with 3% H_2O_2 for 5 min, followed by pretreatment with 3% normal serum for 20 min. Sections were incubated at room temperature for 1 h with specific monoclonal antibodies to SDF1 and CXCR4 (R&D Systems). An irrelevant isotype-matched monoclonal antibody acted as a negative control. A three-stage immunoperoxidase labelling technique incorporating avidin–biotin-immunoperoxidase complex was used (kit from Vector Laboratories, Burlingame, CA, USA). Slides were counterstained with haematoxylin and mounted. Coded slides were analysed using a semi-quantitative scoring method (0–4), which has been previously validated[19] (0 = no staining, 1 = 1–25%, 2 = 25–50%, 3 = 50–75%, 4 = >75% staining).

Statistical analysis

Linear variables were not normally distributed among the different groups of patients or controls, so non-parametric tests were used throughout. Medians and ranges were used to describe data distributions. Spearman's rank correlation coefficient was used to correlate two variables. Mann–Whitney U test was used to compare groups, and the Kruskal–Wallis test was used for comparisons between more than two independent samples. p-Values were adjusted for significance according to number of multiple comparisons performed. Binary logistic regression was then used to identify dependency between clinical parameters and laboratory outcomes. For this analysis continuous variables were dichotomised as below and above median. Both demographic and clinical variables were entered individually into a logistic regression model to obtain their unadjusted odds ratios. Those that were deemed at least borderline significant ($p \leqslant 0.1$) were then entered into a logistic regression in a step-wise fashion using SPSS version 11. The variables analysed were: (1) demographics: sex, age; (2) clinical: disease duration, remission duration, age at disease onset, satisfying ACR remission criteria,[20 21] presence of RF, family history, past and current drug history, RA complications (nodules); (3) laboratory: frequency of naïve and IRC CD4+ T cells, expression of apoptotic factors, levels of circulating cytokines, expression of chemokine receptors on IRC.

RESULTS

Peripheral CD4+ T cell differentiation pattern in relation to inflammation

To establish whether factors other than inflammation could generate IRC, we compared patients with OA and CrD and otherwise healthy individuals with a mild infection not requiring medication. In health, very few IRC were observed in a representative individual (fig 1). IRC were abundant in active RA as previously described.[10] In clinical remission, IRC remained present. At the cohort level, IRC frequency was high in active RA compared with healthy controls (table 1, $p<0.001$) and remained high in clinical remission ($p<0.001$). In patients with OA the differentiation pattern resembled healthy controls (fig 1), but in CrD it was closer to active RA. IRC frequency was significantly higher in patients with CrD compared with an age-matched control ($p<0.005$). In contrast, the IRC frequency in OA was not different from that of an age-matched, control cohort. During the first 5 days of a mild infection, the frequency of IRC was increased by fivefold compared with a control group ($n = 10$, age-matched, data not shown). Following resolution of symptoms after 10 days, IRC were reduced in the circulation (less than twofold) and their frequency returned to normal within 2–3 weeks.

In 12 patients with RA who achieved clinical remission following HDC and ASCT, we analysed IRC at 1 and 3 months post-ASCT and in seven patients who had a relapse at 1 year post-treatment. HDC reduced the number of circulating IRC (fig 2A, $p<0.001$); however, between 1 and 3 months IRC numbers had significantly increased ($p<0.001$), but the total number of other CD4+ T cell subsets (naïve, effector and memory cells) had not.[22] The frequency of IRC at relapse was high and comparable with pretreatment (on average 25% and 27% respectively). A direct relationship between IRC frequency and CRP was previously observed in patients with active RA,[10] and this was also reflected in this group of 12 patients pretreatment (fig 2B, black circles, $rho = 0.682$, $p = 0.01$). A major reduction in CRP was observed during the remission period between 1 and 3 months,[15 16] and IRC frequency was not related to CRP at these times (grey and white squares). A trend toward CRP and IRC frequency being correlated at relapse was observed (white circles, $rho = 0.474$, $p = 0.133$, $n = 7$).

Proliferation and differentiation from naïve cells

We showed previously that IRC differentiate and proliferate from naïve cells under the drive of inflammation, resulting in TREC dilution.[10] In patients with RA in remission, in whom we had previously detected thymic activity,[22] the TREC content of naïve CD4+ T cells was high and within the normal range, demonstrating an absence of TREC dilution through proliferation (table 1). These results suggested that there is no proliferation and differentiation of naïve cells into IRC during remission. The mechanism by which IRC may differentiate in remission is, therefore, different from that in active disease.

Cytokine driven proliferation

We investigated the presence of cytokines able to induce the proliferation of T cells (IL-2, IL-6 and TNF-α[11 12]) (table 1). IL-6 was below detectable levels in remission and health, and high in active disease. IL-2 and TNF-α were detected in patients in remission; however, their presence was not correlated with IRC frequency. Notably, some of the highest IRC frequencies were detected in patients with no detectable IL-2 or TNF-α. We used a regression analysis to predict whether one cytokine could replace the other or act in combination. The model did not identify any significant association, confirming our data on TREC content.

Hyper-responsiveness

IRC were isolated by cell sorting from eight patients from whom we had obtained samples at times when disease was active and in remission (fig 3). As shown previously,[10] IRC from samples taken during active disease (grey bars) responded to mitogen (phytohaemagglutin) and full T cell receptor stimulation (anti-CD3/CD28).[10] In contrast, both responses were lost in remission (black bars). Cells were equally unresponsive to IL-2 and partial T cell receptor stimulation (anti-CD3).

Cell trafficking

We measured the expression of CXCR3, CXCR4 and CCR5 on CD4+ T cells with an IRC phenotype (table 1). We did not observe cells with double positivity for any chemokine receptor and CD62L, confirming that lymph node and tissue homing were mutually exclusive on IRC. The proportion of chemokine receptor positive cells with an IRC phenotype was significantly reduced for all three receptors in remission compared with active disease ($p<0.007$ for all).

We next investigated whether cells expressing CXCR4 could be detected in synovial tissue under conditions of remission. We also examined the levels of expression of SDF1, one of the chemokines responsible for attracting T cells to sites of inflammation. We obtained synovial tissue before HDC ($n = 2$), 3 months after ASCT ($n = 3$), and at relapse ($n = 1$).[19 23] Strong CXCR4 expression was detected on small isolated cells in both inflamed tissues (fig 4A), but not in the tissue obtained when disease was in remission. SDF1 expression was detected in all samples. SDF1 scores were, however, reduced in remission compared with baseline and relapse (fig 4B). The visual analogue score (VAS) for degree of synovitis was recorded at the time of tissue sampling. A significant correlation between SDF1 expression and VAS score was observed ($rho = 0.784$, $p = 0.05$).

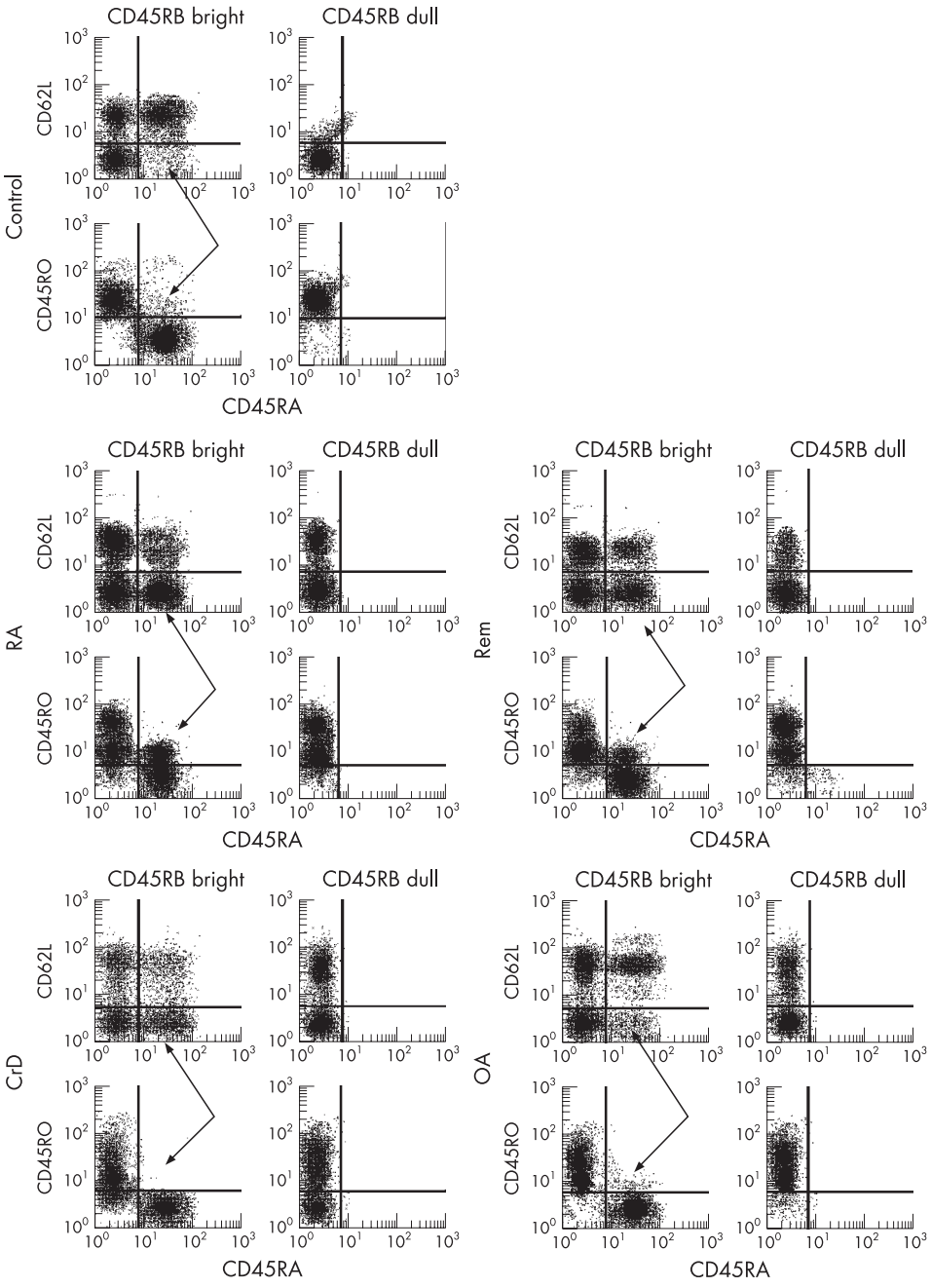

Figure 1 T cell differentiation remains abnormal in patients with rheumatoid arthritis (RA) in clinical remission. Representative flow cytometry plots for a healthy control (age 48), and a patient with active RA (age 45), osteoarthritis (OA, age 60) and Crohn disease (CrD) (age 45), and a patient with RA in remission (rem, age 42). CD4+ T cells were isolated by negative selection using magnetic beads. Cells were labelled with antibodies against CD45RB, CD45RA, CD45RO and CD62L. Cell populations were first gated according to CD45RB expression. Plots represent two-colour analysis of each gated CD45RB bright or dull population. Inflammation-related cells (IRC) are $CD45RB^{bright}$ CD45RA+ CD62L− $CD45RO^{dull}$ (arrows). IRC were present in the healthy control at a very low frequency. The differentiation pattern in the patient with OA resembled the control. IRC were abundant in the patients with active RA and CrD, as well as in the patient with RA in clinical remission.

Survival drive

We examined the expression of the pro- and anti-apoptotic factors Bax, Bcl-2 and Bcl-xL (table 1). Owing to the limited amount of blood available from each participant, we used total peripheral blood mononuclear cells for this analysis. There was no difference in expression of the anti-apoptotic factors, Bcl-2 and Bcl-xL, between health, remission and active disease. The expression of the pro-apoptotic factor Bax was significantly reduced in remission compared with active disease ($p<0.001$) or controls ($p<0.001$). In addition, there was an inverse relationship between the level of Bax and the frequency of IRC in remission (rho = −0.755, p = 0.030). These data suggest that a mechanism lowering the expression of Bax may be involved in the persistence of IRC in remission.

Cytokine rescue is another known mechanism by which T cells can survive. Having found that there was no effect of IL-2 on IRC frequency, we measured IL-7 (table 1), another known T cell survival factor. Patients with active RA had significantly less IL-7 than controls, as previously reported.[22] Patients in remission displayed variable IL-7,[22] but there was no relationship between serum IL-7 levels and IRC frequency in remission.

Prediction of disease flare

Nearly half of the patients in remission (20 of 41) experienced a disease flare within 18 months of disease cessation. Frequency of IRC was significantly higher in patients subsequently relapsing (p = 0.001, OR = 6.4). We therefore analysed factors that could predict disease flare. We first analysed parameters individually (table 2). RF positivity (p = 0.041, OR = 3.9) and, possibly, having nodules (p = 0.121, OR = 3.4) were significant in predicting relapse. Binary logistic regression analysis revealed that IRC frequency (p = 0.010, OR = 7.6) and presence of RF

Table 1 IRC frequency, chemokine receptor expression, circulating cytokines and expression of apoptotic factors (median (range))

IRC frequency (% of CD4+ T cells)	**Disease group**	**Control group,* n**	
Active RA (n = 41)	11.3 (0.7–45)	1.3 (0.1–6.5), n = 30	
Remission (n = 41)	9.3 (0.9–32)	1.3 (0.1–6.5), n = 30	
OA (n = 12)	11.3 (6.4–30.6)	6.4 (1.2–34.4), n = 20	
CrD (n = 13)	9.3 (2.2–14.4)	1.0 (0.9–3.2), n = 20	
TREC content†	**CD4+ T cells**	**Naïve CD4**	
Controls (n = 33)	5.7 (1.5–11.0)‡	8.0 (2.3–15.0)	
Active RA (n = 35)	0.93 (0.05–4.65)	3.35 (0.13–8.96)	
Remission (n = 18)	5.15 (1.70–17.80)‡	6.4 (1.06–20.11)	
Chemokine receptors§	**CXCR3**	**CXCR4**	**CCR5**
Active RA (n = 10)	31.9 (16.4–54)	27.8 (2.4–47)	17.3 (0–66)
Remission (n = 12)	10.7 (2.2–23.5)	4.4 (0–23)	1.0 (0–4.9)
Circulating cytokines¶¶	**IL-2¶**	**IL-6¶**	**TNF-α¶**
Controls (n = 10)	bd (bd-8.8)**	bd	bd
Active RA (n = 10)	bd	13.44 (2.55–16.8)	21 (bd-86)††
Remission (n = 20)	bd (bd-40.6)‡‡	bd	1.85 (bd-19.4)§§
Circulating cytokines¶¶	**IL-7**		
Controls (n = 10)	13.85 (9.23–22.30)		
Active RA (n = 10)	7.0 (2.95–9.83)		
Remission (n = 20)	9.02 (2.47–22.85)		
Apoptotic factors***	**Bax**	**Bcl-2**	**Bcl-xL**
Controls (n = 10)	0.041 (0.02–0.18)	0.053 (0.014–0.32)	0.21 (0.11–0.59)
Active RA (n = 10)	0.045 (0.012–0.19)	0.026 (0.008–0.060)	0.168 (0.07–0.35)
Remission (n = 20)	0.023 (0.007–0.068)	0.034 (0.005–0.18)	0.106 (0.007–0.42)

CrD, Crohn disease; IL, interleukin; IRC, inflammation-related cells; OA, osteoarthritis; RA, rheumatoid arthritis; TREC, T cell receptor excision circle.
*Disease and control group were aged matched for this analysis. †Measured by real-time polymerase chain reaction as previously reported[17] and ‡inversely correlated with age.[23] §Percentage of cells with an IRC phenotype expressing the receptor. ¶Cytokines below detectable level are reported as bd. **Detected in two of 10 controls. ††Detected in eight of 10 patients with active RA. ‡‡Detected in nine of 20 patients with RA in remission. §§Detected in 13 of 20 patients with RA in remission. ¶¶Measured by enzyme-linked immunosorbent assay (pg/ml of serum). ***Measured by real-time polymerase chain reaction and normalised to *GAPDH*.

(p = 0.046, OR = 4.8) each significantly contributed to the model. There was no significant further interaction between these factors. The main effects model was a reasonably good one, predicting 76% of relapse and 66% of non-relapse (72% correct, $p<0.05$), and demonstrated good fit to the data (Hosmer and Lemeshow test p = 0.989).

DISCUSSION

We previously reported that abnormal differentiation of T cells in active RA was associated with inflammation, as measured by CRP.[10] We have confirmed these findings in another inflammatory disease, CrD, and longitudinally in RA, and also shown a similar transient phenomenon in healthy individuals with a mild infection. Despite a lack of systemic inflammation and meeting additional clinical criteria for remission, we observed the persistence of IRC in the circulation of asymptomatic patients with RA. To explain this persistence, we showed that, the mechanism by which IRC are generated under inflammatory conditions is different from the mechanism by which they persist when inflammation is well controlled. We propose that, IRC in remission are the product of T cell release from synovial tissue, where they have acquired an increased resistance to apoptosis. Taken together, these data suggest that the T cell differentiation abnormalities observed in active disease are a feature of RA, further amplified by inflammation but ''suspended'' in remission.

The presence of IRC in remission was unexpected with regard to our previous work[10] and our current longitudinal data demonstrating a direct relationship between IRC frequency and CRP in active disease. The frequency of IRC was not associated with any other parameters. The persistence of IRC in remission, therefore, suggested different mechanisms for their emergence (as described before[10]) and their persistence in remission. There are several possible explanations for these observations. Frequency of an individual subset of cells (IRC among CD4+ T cells) depends on entry, exit, self-renewal or cell death. Entry may depend on differentiation from another subset into IRC or, alternatively, entry into the circulation from a tissue compartment. Exit would imply that IRC are either differentiating into another circulating subset or exiting the circulation to enter another tissue compartment. Self-renewal could be triggered by different stimuli, the most likely being cytokines. Cell death could be blocked either by cytokine rescue or other mechanisms specific to the disease. Subclinical inflammation has been reported in the joint of 96% of patients in clinical remission using advanced imaging analysis.[24] Markers of systemic inflammation are below or within the normal range in these patients, but could nevertheless be influential in our observations. We therefore analysed these parameters in more detail, comparing active disease and remission.

Chemokines and their receptors are important factors determining the distribution of T cells within tissue. The

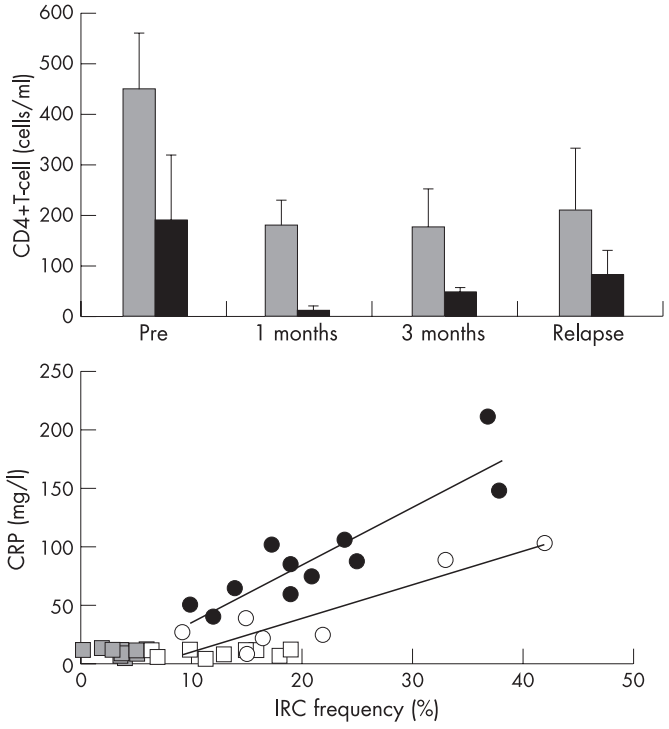

Figure 2 Inflammation-related cell (IRC) frequency correlates with C-reactive protein (CRP) in active disease but not in remission. (A) Following high-dose chemotherapy and autologous haematological stem cell transplantation, the number of IRC (black bars) post-treatment was greatly reduced (compare pretreatment with 1 month, $p<0.001$). During remission, IRC numbers increased (compare 1 and 3 months remission, $p<0.001$), but total cells numbers (grey bars) did not increase significantly. At time of relapse (1 year) IRC frequency reached pretreatment levels in terms of ratio to total numbers. (B) In the same patients, a relationship between CRP and IRC frequency was observed pretreatment (black circles rho = 0.682, p = 0.014), but was lost at 1 month (grey squares). During the remission period (white squares) IRC numbers increased; however, no relationship with CRP was observed. At relapse (white circles, seven of 12 patients) the CRP-IRC frequency relationship was apparent, but not significant (rho = 0.465, p = 0.133).

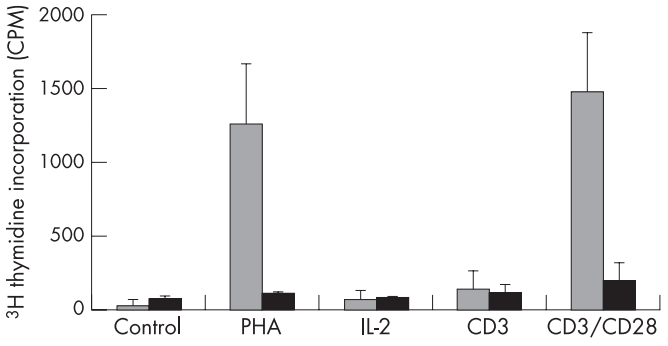

Figure 3 Inflammation drives the hyper-responsiveness of immature atypical cells. CD4+ T cells were separated and labelled as described in fig 1 and further sorted under aseptic conditions according to their inflammation-related cell phenotype. Proliferation in response to mitogen (phytohaemagglutin (PHA) 10 μg/ml), interleukin (IL)-2 (20 units/ml) and T cell receptor stimulation (anti-CD3 1 μg/ml and anti-CD28 5 μg/ml) was measured using a ^{3}H-thymidine incorporation assay ((CPM) after 5 days in culture (20 000 cells/well)). Samples collected during active disease are represented by grey bars and samples collected during remission by black bars. Hyper-responsiveness to PHA and full T cell receptor stimulation of inflammation-related cells in active disease was not observed in remission.

chemokine/receptor pair SDF-1/CXCR4 has been shown to be important for the accumulation of T cells in synovial tissue in RA.[25] Our results show that tissue homing is not sustained on IRC in remission (table 1). As IRC are also not differentiating de novo in remission, this suggests that the factors inducing expression of chemokine receptors on their surface are now missing. In support of this hypothesis, we showed previously that CD4+ T cell infiltration, particularly of CD4+ T cells with a CD45RA+ phenotype, was significantly reduced in biopsies from patients responding to HDC.[19] In contrast, CD8+ T cells, B cells, macrophages and natural killer cells remained in the tissue. We also showed that SDF-1 expression in the synovium is reduced in remission, but comparable with baseline at relapse. We further demonstrated that small cells expressing CXCR4 are detected in synovial tissue in active disease, but not in remission. Our data therefore suggest that IRC are destined to home to tissues in active disease, but not in remission when they recirculate.

The proliferation and hyper-responsiveness of IRC in active disease were not present in the absence of systemic inflammation. Similar cells were observed transiently in otherwise healthy individuals with a mild infection. This suggested that, either a defect in the regulation of cell death at the end of an infectious/inflammatory episode, or the presence of an unidentified survival/growth factor may participate in the pathogenesis of RA, by maintaining cells that should otherwise have died. IL-2, IL-7 and other γc cytokines are known to induce T cell survival by upregulating the Bcl-2 and Bcl-xL anti-apoptotic factors,[26] but our results do not support such a mechanism. Cell–cell contact-dependent interaction with stromal cells results in an environmental blockade of apoptotic cell death in synovial T cells in RA.[27–29] Survival through the downregulation of Bax expression may be a novel mechanism by which (stromal) factors in the synovium reduce T cell susceptibility to apoptosis.

Altogether our results point to a lack of effect of inflammation on the features of IRC in remission that are associated with these cells in active disease. The DAS28 score and CRP level, which are measures of disease activity, were not associated with IRC frequency. A need to reach a threshold of inflammation, in order to activate the "bad side" of IRC (hyper-responsiveness, chemokine receptor expression), could explain our results. The non-fulfilment of this requirement would reflect the absence of systemic inflammation. On the other hand, our data could also be explained by a subclinical inflammatory drive to the differentiation of IRC. The higher frequency of IRC in patients in remission destined for relapse could reflect subclinical inflammation, which has been identified in this cohort using highly sensitive imaging techniques.[24] Therefore, the current work extended our imaging findings by providing a circulating biomarker predictive of relapse.

While not implicating T cells as primary pathogenic players, our data are consistent with such a model. We showed previously that IRC develop in the periphery from naïve T cells, non-specifically under the drive of inflammation.[10] We showed in this study that IRC in active disease express chemokine receptors, which may drive them to relocate to the inflamed synovium. The movement of IRC in and out of the synovium would, however, be biased towards exit in remission, releasing IRC into the circulation. T cell survival factors expressed in the synovium may alter the local balance of pro- and anti-apoptotic factors.[25 28 31] IRC, which have been primed

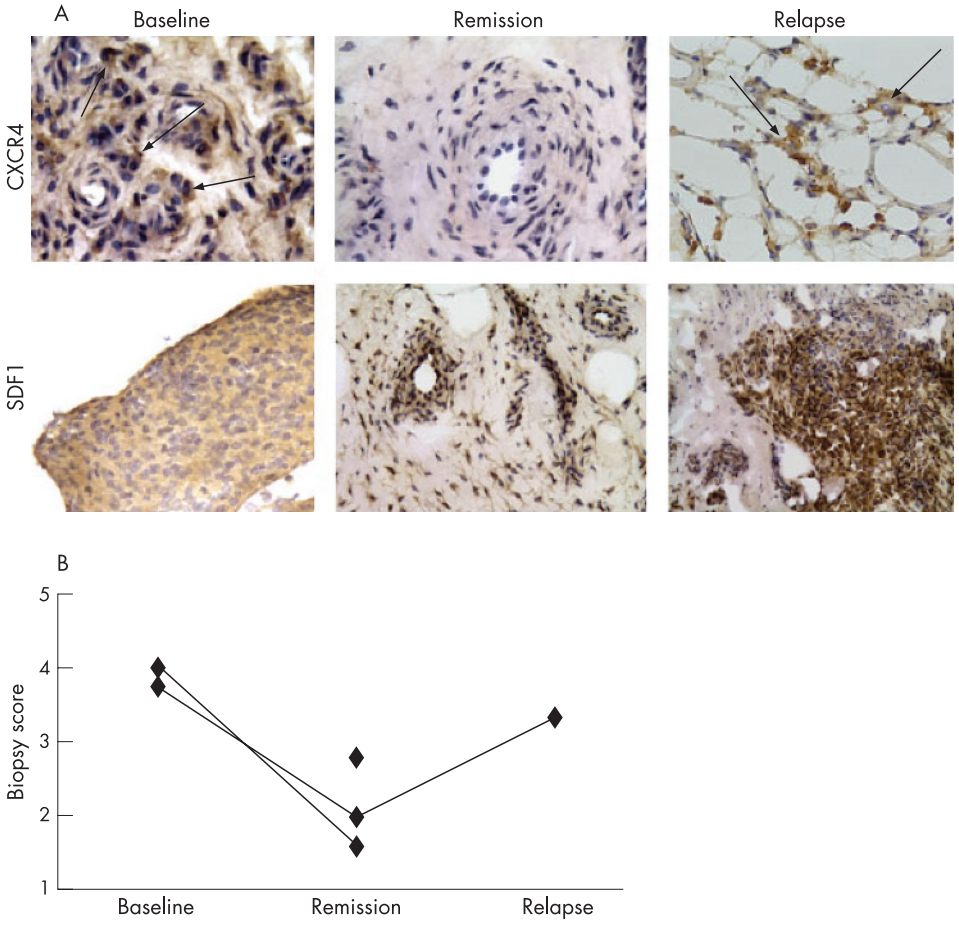

Figure 4 Immunohistochemistry analysis of synovial tissue in active disease and in remission. Biopsies were taken at baseline (n = 2), 3 months in the remission period induced by high-dose chemotherapy (n = 3) and at time of relapse (n = 1, 12 months). Staining for SDF1 and CXCR4 expression was performed according to standard procedures. (A) Magnification 400× for CXCR4 and 200× for SDF1. Arrows indicate CXCR4-positive cells. (B) Slides were analysed using a semi-quantitative scoring method (0 = no staining, 1 = 1–25%, 2 = 25–50%, 3 = 50–75%, 4 = >75% staining). SDF-1 data are presented as an average score for the entire biopsy surface. Lines indicate consecutive biopsies from the same patient.

towards survival in the synovium, can therefore persist in the periphery, until a new inflammatory episode triggers them to initiate relapse. Thus, these cells are a potential tool for monitoring a patient's response to treatment. In addition, treatments that reduce joint inflammation (such as TNF blockade) allowing the release of potentially pathogenic cells (such as IRC) into the circulation, followed by treatments that would kill these cells specifically, may provide new therapeutic avenues to be explored.

Table 2 Analysis of factors predicting relapse

	OR	p-Value
Individual parameter		
ACR criteria	0.538	0.435
Age	0.760	0.780
Age at onset	0.844	0.813
Sex	1.090	0.907
Disease duration	1.180	0.802
Remission duration	0.850	0.631
RF	3.900	0.041
NSAID	0.455	0.261
Current methotrexate	1.015	0.481
Current sulfasalazine	0.933	0.483
Total DMARD	0.677	0.794
Nodules	3.40	0.121
IRC	6.4	0.009
Regression analysis		
IRC	7.576	0.010
RF	4.844	0.046
Combining effect analysis		
IRC×RF	1	0.558

ACR, American College of Rheumatology; DMARD, disease-modifying anti-rheumatic drug; IRC, inflammation-related cells; NSAID, non-steroidal anti-inflammatory drug; RF, rheumatoid factor.

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