The Presumed Hyporesponsive Behavior of Rheumatoid Arthritis T Lymphocytes Can Be Attributed to Spontaneous Ex Vivo Apoptosis rather than Defects in T Cell Receptor Signaling


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The Presumed Hyporesponsive Behavior of Rheumatoid Arthritis T Lymphocytes Can Be Attributed to Spontaneous Ex Vivo Apoptosis rather than Defects in T Cell Receptor Signaling

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Genetic associations and the clinical success of compounds targeting TCR costimulatory proteins suggest an active role for TCR signaling in the initiation and perpetuation of rheumatoid arthritis (RA). Paradoxically, T cells isolated from affected joints in RA show impaired proliferative and cytokine responses following stimulation with mitogens and recall Ags attributed in part to chronic T cell exposure to oxidative stress and inflammatory cytokines. Therefore, it is uncertain how local autoreactive TCR signaling contributes to pathology in established RA. Using single-cell analysis, we show that in contrast to results obtained in bulk culture assays, T cells from the synovial fluid of RA patients proliferate and produce cytokines (IL-2, TNF-α, and IFN-γ) as efficiently, if not more so, than T cells isolated from healthy donors and RA patient peripheral blood following TCR/CD28 stimulation. RA synovial fluid T cell hyporesponsiveness observed in bulk cultures can be attributed to spontaneous apoptosis ex vivo, which is associated with altered ratios of proapoptotic Noxa and anti-apoptotic Mcl-1 expression. The absence of RA synovial T cell proliferation and cytokine production in situ, despite the capacity of these cells to support productive TCR signaling, suggests that T cells contribute to local pathology in established RA by TCR-independent mechanisms. The Journal of Immunology, 2009, 183: 621–630.

T lymphocytes are thought to contribute to synovitis and joint destruction in rheumatoid arthritis (RA) through multiple mechanisms. These include the pleiotropic activation of other synovial cells, such as macrophages and stromal fibroblast-like synoviocytes (FLS) via cell-cell contacts and IL-17 production, stimulation of B lymphocytes producing autoimmune Abs, and promotion of osteoclast differentiation (1). Human genetic studies, experimental animal models of arthritis, and recent clinical experience have provided evidence consistent with a role in improper engagement of autoreactive TCRs in the initiation and progression of disease in RA (1). However, our inability to detect significant T cell proliferation or cytokine production at sites of inflammation in RA, combined with observed defects in TCR-proximal signaling and TCR-dependent functional responses of RA synovial T cells, has raised questions regarding the role of TCR signaling in established RA and the mechanism of action of therapies targeting TCR costimulatory pathways (2–4).

The strongest evidence supporting an active role for TCR engagement in the initiation, if not the perpetuation, of disease in RA is data underlying the “shared epitope” hypothesis. Expression of specific HLA-DR1- and DR4-shared epitope alleles enhances the risk of the development of RA and contributes to disease severity (5–8). Additionally, polymorphisms in T cell gene products which influence the quality of TCR responses, such as PTPN22, PD-1, CTLA-4, TRAF1-C5, and CD40, have also been identified as candidate susceptibility genes in RA (1, 9–12). Finally, evidence consistent with an active role for TCR engagement in established arthritis is observed in clinical trials using soluble CTLA-4IG fusion protein (abatacept) to disrupt interaction of the TCR costimulatory protein CD28 with CD80/CD86 ligands expressed on synovial APCs and FLS. Initial clinical trials using abatacept to treat RA have demonstrated clear clinical benefits, even in patients refractory to therapy with biologicals that block TNF-α signaling (13–15).

T cells derived from both RA synovial tissue and synovial fluid (SF) display similar phenotypic and functional abnormalities. These T cells express surface markers characteristic of recent TCR stimulation, including CD44, CD45RO, CD69, HLA-DR, and VLA-4 (3, 16–20). These cells are primarily of a Th1 phenotype and are resistant to Th2 polarization ex vivo (21–24). Despite this, little direct evidence is available demonstrating that the TCR is functionally engaged in RA. Proliferation of synovial tissue T cells is not observed in situ and the

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2 Abbreviations used in this paper: RA, rheumatoid arthritis; SF, synovial fluid; FLS, fibroblast-like synoviocyte; PMA/I, PMA/ionomycin; HD, healthy donor; PB, peripheral blood; SFMC, SF mononuclear cell; PI, propidium iodide; RT-MLPA, reverse transcription multiplex ligation-dependent amplification.

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relatively low levels of IL-2 and IFN-γ which can be detected in RA synovial T cells are inconsistent with a contributory pathological role for TCR signaling in established RA (25–29). Ex vivo, RA synovial tissue and SF T cells are hyporesponsive to stimulation by pharmacological mitogens and recall Ags, both in terms of proliferation and cytokine secretion (2, 3, 25, 30–32). This may be a consequence of chronic T cell exposure to inflammatory cytokines and/or oxidative stress, which can lead to altered expression or misfolding of critical TCR signaling proteins, such as the CD3ζ chain (30, 33) and linker for activation of T cells (31, 32, 34).

The inability to provide evidence of direct TCR engagement in RA synovium, in combination with identified TCR-proximal signaling defects in RA synovial T lymphocytes in vitro, has led to the suggestion that these cells contribute to pathology through TCR-independent mechanisms (2, 3). Many properties of RA T cells, including surface expression of activation markers and cell-cell contact-dependent activation of macrophages and FLS, can be recapitulated by peripheral blood (PB) T cell exposure to IL-15 or a combination of inflammatory cytokines (19, 35, 36). Similar effects are observed in PB T cells chronically exposed to TNF-α (20, 30). Additionally, stimulation of PB T cells with a number of inflammatory cytokines, in combination with CD28 ligation, can reproduce oxidative stress observed in synovial T cells (37).

In this study, we report the unexpected finding that TCR signaling is functionally intact in freshly isolated RA SF T lymphocytes and fully capable of initiating cytokine production and T cell proliferation. Previously observed RA SF T cell hyporesponsiveness in bulk culture assays is due to spontaneous apoptosis of these cells ex vivo, associated with changes in the relative expression of the proapoptotic protein Noxa and anti-apoptotic Mcl-1. Our results suggest that the inability to detect evidence of TCR engagement in RA synovial tissue is unlikely a result of defects in TCR signaling, but rather, lack of TCR engagement.

### Materials and Methods

**Patients**

Paired PB and SF samples were obtained from patients attending our outpatient clinics, with clinically active RA fulfilling the American College of Rheumatology revised criteria for RA (38). Patient characteristics are presented in Table I. At the time of sample collection, 11 patients were receiving methotrexate (2.5–25 mg/wk), four prednisolone (2.5–50 mg/wk), one leflunomide (20 mg/day), five TNF-α antagonist therapy (adalimumab, 40 mg/2 wk; etanercept, 25 mg twice a week or 50 mg/wk; infliximab, 3 mg/kg i.v. every 8 wk), one had received rituximab treatment, and one patient was not receiving any medication at the time of arthrocentesis. All patients provided informed written consent and the study was approved by the Medical Ethics Committees of the Academic Medical Center (University of Amsterdam, Amsterdam, The Netherlands) and the Institute of Rheumatology (Warsaw, Poland).

### Cell isolation and culture

PBMCs from healthy volunteers and PBMCs and SF mononuclear cells (SFMCs) from RA patients were isolated by Ficoll-Isoaque density gradient centrifugation (Nycoderm; Pharma). PB and SF T cells were purified from PBMCs and SFMCs using a negative isolation procedure (T Cell Negative Isolation Kit; Dynal Biotech) in accordance with the manufacturer’s instructions. Purified T cells were >95% CD3+ as assessed by FACS analysis (see below). T cells were cultured at 1 × 10^6/ml in IMDM supplemented with 10% FCS, l-glutamine, 25 mM HEPES, 100 ng/ml streptomycin, and 10 U/ml penicillin (all from Life Technologies/Invitrogen). T cells were stimulated with either 1 μg/ml anti-CD3 (clone 1XE3) and 1 μg/ml anti-CD28 (clone 15E5) mAbs (both from Sanquin) or 1 ng/ml PMA and 1 μg/ml ionomycin (both from Sigma-Aldrich).

### Measurement of T cell proliferation

T cell proliferation in bulk cultures was assessed by culturing T cells for 72 h in a 96-well plate in the absence or presence of activating Abs. During the last 20 h of culture, cells were pulsed with 1 μCi of[^H]thymidine (Amersham Biosciences). Cells were harvested and incorporated radioactivity was measured using a 1450 Microbeta Plus Liquid Scintillation Counter (PerkinElmer). For single-cell analysis of T cell proliferation, PB and SF T cells were resuspended at 5–10 × 10^6 cells/ml in PBS and labeled with 2.5 μM CFSE (Molecular Probes Europe) for 10 min at 37°C. Cells were washed and subsequently resuspended in complete culture medium. T cells (1 × 10^6/ml) were left unstimulated or stimulated for 72 h at 37°C with anti-CD3 and anti-CD28 Abs. Proliferation was detected using a FACSCalibur flow cytometer (BD Biosciences) and CellQuest Pro software (BD Biosciences). The precursor frequency (percentage of cells in the initial population that underwent one or more divisions) and the mean number of divisions per proliferating cell were calculated as previously described (39).

### Detection of cytokine production

T cell culture supernatants were collected for cytokine analysis 24 h (for IL-2) or 72 h (TNF-α, IFN-γ) after stimulation, and cytokine concentrations were measured using a Bio-Plex Human 27-plex panel (Bio-Rad) according to the manufacturer’s instructions. For single-cell analysis of T cell cytokine production, T cells were stimulated for 6 h with anti-CD3/CD28 Abs or PMA/ionomycin (PMA/L) with 10 μg/ml brefeldin A (Sigma-Aldrich) included for the last 4 h of stimulation. Cells were fixed with 4% (w/v) paraformaldehyde/PBS and permeabilized with 0.5% (w/v) BSA/PBS containing 0.1% (w/v) saponin (Sigma-Aldrich). Cells were then incubated with conjugated anti-IL-2-allophycocyanin, anti-IFN-γ-PE, or TNF-α-allophycocyanin Abs (all from BD Biosciences). The percentage of positively stained cells and the mean fluorescent intensity of staining were measured by flow cytometry as above.

### Apoptosis detection

Cells were washed in ice-cold HEPES buffer and incubated with allophycocyanin- or FITC-labeled annexin V (IQ Products) for 30 min. Propidium iodide (PI, 5 μg/ml; Sigma-Aldrich) was added before analysis and the percentage of viable cells were quantified by flow cytometry.

### Reverse transcription multiplex ligation-dependent probe amplification (RT-MLPA) procedure and analysis

Total T cell mRNA was isolated using a GenElute RNA isolation kit (Sigma-Aldrich). RT-MLPA of pro- and antiapoptotic genes was performed as previously described (40). Briefly, RNA was reverse-transcribed using a gene-specific probe mix (MRC Holland). The obtained cDNA was annealed to MLPA probes and covalently linked with Ligase-65 (MRC Holland). Ligation products were amplified and fluorescently labeled by PCR using one unlabeled and one 6-carboxyfluorescein-labeled primer. PCR products were applied to an Applied Biosystems 3100 capillary sequencer and data were processed with Genescan and Genotyper software (both from Applied Biosystems). Final analyses were conducted with Microsoft Excel spreadsheet software. The sum of all peak data was set at 100% to normalize for fluctuations in total signal between samples. Individual peaks for each gene product were then calculated relative to the total value.

### SDS-PAGE and Western blotting

T cells were washed with ice-cold PBS and lysed in buffer containing 1% CHAPS, 20 mM Tris-HCl (pH 7.4), 135 mM NaCl, 1.5 mM MgCl2, 10% glycerol, 1 mM EGTA, 2 mM Na3VO4, 10 mM NaF, 2 μg/ml leupeptin, 1 mM PMSF, 0.1 mM tosyl lysine chloromethyl ketone, and 2 μg/ml trypsin

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**Table I. Clinical features of RA patients (n = 13) included in the study**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Median (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>52 (25–85)</td>
</tr>
<tr>
<td>Male:female</td>
<td>3:10</td>
</tr>
<tr>
<td>Disease duration (years)</td>
<td>9 (1–17)</td>
</tr>
<tr>
<td>Erythrocyte sedimentation rate (mm/h)</td>
<td>57 (23–120)</td>
</tr>
<tr>
<td>C-reactive protein (mg/L)</td>
<td>64 (2–117)</td>
</tr>
<tr>
<td>Rheumatoid factor (kU/L)</td>
<td>102 (&lt;1–438)</td>
</tr>
<tr>
<td>Anticitrullinated peptide Abs (kAU/L)</td>
<td>2438 (2–15951)</td>
</tr>
</tbody>
</table>

a Median and range are given for each characteristic.
T lymphocytes were refractory to TCR/CD28-induced proliferation. Freshly isolated healthy donor (HD) PB and paired RA PB and SF T cells were stimulated for 72 h in the presence of anti-TCR/CD28 Abs, and proliferation was measured by incorporation of \(^{3}H\)thymidine during the last 20 h of culture (Fig. 1). In the absence of TCR/CD28 stimulation, \(^{3}H\)thymidine incorporation was barely detectable in all samples and did not differ statistically among HD PB, RA PB, and RA SF T cell populations. In accordance with previous independent observations, RA SF T lymphocyte proliferation was significantly reduced following TCR/CD28 stimulation, compared with HD (82% reduction, \(p < 0.05\)) or RA PB T cells (79% reduction, \(p < 0.05\)).

We next attempted to confirm RA SF T cell proliferative hyperresponsiveness at the single-cell level and to determine whether residual proliferative responses might be limited to specific T cell subpopulations. To accomplish this, HD PB, RA PB, and RA SF T cells were labeled with CFSE and cultured in the absence or presence of anti-TCR/CD28 Abs for 3 days before analysis by flow cytometry. Surprisingly, in contrast to results from bulk T cell analysis, CFSE dilution in total RA SF CD3\(^+\) T lymphocytes was not reduced compared with HD PB T lymphocytes (Fig. 2A). Indeed, proliferation was most robustly observed in RA SF T lymphocytes, followed by RA PB T lymphocytes. This relative enhanced TCR/CD28-induced proliferation of RA SF T cells was observed in both CD4\(^+\) and CD8\(^+\) T cell subsets (Fig. 2B). After calculating the percentage of T cells in the initial populations that underwent one or more cell divisions, we found that the precursor frequency of RA SF T cells was not depressed compared with HD PB or RA PB T cells (Fig. 2C). Rather, the precursor frequency of RA SF T cells (45%) was increased compared with HD T cells (27%), although this difference did not reach statistical significance (\(p = 0.152\)). Again, this trend was observed in both CD4\(^+\) and CD8\(^+\) T cell subsets (Fig. 2C). No differences were observed among HD PB, RA PB, and RA SF T cells in terms of the mean number of cell divisions achieved following TCR/CD28 stimulation, either in total CD3\(^+\) T lymphocytes or in CD4\(^+\) and CD8\(^+\) T cell subsets.

**Results**

**RA SF TCR is competent to support T cell proliferative responses**

In initial analyses of RA SF T cell responses to TCR ligation, we first attempted to confirm previously published reports that RA SF
Differences in RA SF T cell proliferation observed between bulk cultures and single-cell analyses were unlikely due to patient heterogeneity or drug treatment, as RA SF T cells from two of the three patients studied in [3H]thymidine incorporation experiments (Fig. 1) were assessed in parallel by CFSE dilution. Thus, in contrast to observations made in analyses of bulk T cell populations, single-cell analysis of RA SF T cell responses to TCR/CD28 stimulation reveals that these cells can proliferate as well, if not better, than their HD PB or RA PB counterparts. This may be due to an increased frequency of precursors competent to initiate proliferation.

RA SF T cells display increased cytokine production following TCR stimulation

Given the discordance of our results with previous observations regarding RA SF T cell proliferative responses to TCR/CD28 stimulation, we next assessed RA SF T cell cytokine responses. In agreement with previous reports, following TCR/CD28 stimulation, IL-2 production in supernatants of bulk RA SF T cell cultures was severely impaired compared with autologous RA PB T cells \((p < 0.001;\) Fig. 3). Additionally, TNF-\(\alpha\) production in RA SF T cells was decreased compared with RA PB T cells \((p < 0.005)\). A similar trend was observed when comparing RA SF T cells with HD PB T cells. In contrast, HD PB, RA PB, and RA SF T cells all produced similar levels of IFN-\(\gamma\).

We next analyzed TCR/CD28-induced cytokine production by intracellular staining and flow cytometry (Figs. 4 and 5). In line with previous reports (22, 24), pharmacological stimulation of HD PB, RA PB, and RA SF T cells with PMA/I resulted in IL-2 production in a similar frequency of cells, while the frequency of TNF-\(\alpha\)- and IFN-\(\gamma\)-producing T lymphocytes was elevated in RA SF (Fig. 5A). Unexpectedly, following TCR/CD28 stimulation, RA SF T cell IL-2 production was significantly increased when compared with TCR/CD28-stimulated RA PB \((p < 0.005)\) or HD T cells \((p < 0.005;\) Fig. 5B). Also, TNF-\(\alpha\) and especially IFN-\(\gamma\) production was significantly increased in SF T cells compared with HD or RA PB T cells. The frequency of IFN-\(\gamma\)-producing T cells after TCR/CD28 stimulation was as high as 13 times that of HD T cells \((p < 0.005)\). The same pattern of cytokine production observed in the CD3\(^+\) T cell population could be observed in both CD4\(^+\) (Fig. 5C) and CD8\(^+\) cells (Fig. 5D). The mean fluorescent intensity of cytokine staining in RA SF T cells was similar to or higher than that observed in HD PB and RA PB T cells (data not shown), indicating that decreased cytokine production observed in RA SF T cell bulk cultures was not due to inefficient cytokine production by responding cells. Additionally, differences in cytokine production observed between ELISA analysis of bulk cultures (Fig. 3) and single-cell analysis were not attributable to patient heterogeneity or drug treatment, since all three of the patients assessed by ELISA were studied in parallel single-cell analyses.
Together, these results demonstrated that at the single-cell level, the frequency of TCR-responsive lymphocytes is elevated in RA SF.

RA SF T cell hyporesponsiveness in bulk cultures is secondary to spontaneous ex vivo apoptosis

One possible explanation for the observed discrepancy between RA SF T cell proliferative and cytokine responses in bulk cultures and single-cell analyses could be changes in RA SF T cell viability, as these cells have been reported to quickly undergo apoptosis ex vivo (41, 42). Assays using [3H]thymidine incorporation and tissue culture supernatant ELISA analyses cannot accurately account for apoptosis which may occur during extended cell culture. Initial examination of live cell gating of HD PB, RA PB, and RA SF T lymphocytes 72 h after isolation and CFSE labeling suggested a significant loss of viability of RA SF T cells under these culture conditions (Fig. 6A). We confirmed this by performing annexin V/PI stainings on HD PB, RA PB, and RA SF T cells. Apoptosis was measured immediately after T cell isolation and after 24 h (Fig. 6B). Immediately following T cell isolation, similar percentages of apoptotic cells were observed in all samples. However, after 24 h in culture, RA SF T cells displayed almost four times higher levels of apoptosis compared with HD T cells (p < 0.001) and RA PB T cells (p < 0.001). RA SF T cells continued to undergo apoptosis at a higher rate than the other T cell populations after 48 and 72 h in culture (Fig. 6C) and remained elevated compared with HD (p < 0.001) and RA PB (p < 0.001) controls in the presence of TCR/CD28 stimulation (Fig. 6D).

RA SF T cells have altered expression levels of Noxa, Bcl-2, and Bcl-xL

To investigate in more detail the mechanisms which might be responsible for the increased susceptibility of RA SF T cells to apoptosis ex vivo, we quantified the relative expression of gene products known to be direct regulators of apoptosis. To accomplish this, total mRNA from freshly isolated HD PB, RA PB, and RA SF T lymphocytes was subjected to a RT-MLPA assay, allowing simultaneous quantification of expression of 34 important regulators of apoptosis. Expression profiles of HD PB, RA PB, and RA SF T cells were remarkably similar (Fig. 7A). However, mRNA expression of the proapoptotic BH3-only family member Noxa was increased 2-fold in RA SF T lymphocytes compared with HD (p < 0.05) and RA PB (p < 0.05) T cell populations (Fig. 7B). The expression of Bim, NIP3, and Puma, three other proapoptotic Bcl-2 family members known to regulate T cell apoptosis was equivalent between RA SF and other T cells (Fig. 7A). Among antiapoptotic gene products associated with T cell survival, we saw no differences in the expression levels of Bcl-xL or the Noxa-binding partner Mcl-1 (Fig. 7B). An ~50% reduction in Bcl-2 expression was observed, but did not reach statistical significance.
As many Bcl-2 family members are also subjected to post-translational modifications affecting protein stability, we examined protein expression of pro- and antiapoptotic proteins in whole cell lysates (Fig. 7, C and D). Consistent with mRNA data, Noxa protein expression was elevated in RA SF T cells compared with HD PB T cells. Surprisingly, protein expression of Noxa was even higher in RA PB T cells. Mcl-1, which antagonizes Noxa-induced apoptosis, was only detectable in RA PB T cells, but not HD PB or RA SF T cells. Although previous analysis of RA SF T cells by intracellular FACS staining identified elevated Bcl-xL expression as a proposed compensatory prosurvival mechanism in RA SF T cells (41), we found that Bcl-xL was hardly detectable by Western blotting (Fig. 7C). Antiapoptotic Bcl-2 protein expression in RA SF T cells was severely depressed (Fig. 7, C and D), but this occurred in parallel with decreased expression of the proapoptotic Bcl-2-binding partner Bim (Fig. 7D).

The ratio of Noxa and Mcl-1 expression in RA SF T cells ex vivo favors apoptosis

Our collective data suggested a link between the susceptibility of RA SF T cells to apoptosis and the relative expression levels of Noxa vs Mcl-1. However, although the relative balance of expression of these proteins has been previously shown to regulate T cell apoptosis under various conditions, including environmental stress, cytokine withdrawal, and Ag stimulation (43, 44), we noted no differences in apoptotic rates of HD PB, RA PB, and RA SF T cells immediately after isolation. Therefore, we performed a comparative analysis of HD PB, RA PB, and RA SF T cell gene expression immediately following isolation and after 24 h in culture. Ex vivo culture of HD PB T cells led to a significant down-regulation of Noxa mRNA expression compared with RA SF T cells (p < 0.05; Fig. 8A). A trend toward down-regulation of Mcl-1 expression was observed in RA SF T cells, while Bcl-xL expression remained comparable in each T cell population after 24 h in culture. Ex vivo culture of HD PB T cells led to a significant down-regulation of Noxa mRNA expression compared with RA SF T cells (p < 0.05; Fig. 8A). A trend toward down-regulation of Mcl-1 expression was observed in RA SF T cells, while Bcl-xL expression remained comparable in each T cell population after 24 h in culture. Ex vivo culture of HD PB T cells led to a significant down-regulation of Noxa mRNA expression compared with RA SF T cells (p < 0.05; Fig. 8A). A trend toward down-regulation of Mcl-1 expression was observed in RA SF T cells, while Bcl-xL expression remained comparable in each T cell population after 24 h in culture.
culture, little differences were observed in the relative expression of Bim. However, significant down-regulation of Bcl-2 expression was observed in HD PB ($p < 0.05$) and RA PB T cells ($p < 0.05$) and a similar trend was observed in RA SF T cells. Ratios of Bim mRNA expression relative to Bcl-2 increased by a similar degree in HD PB, RA PB, and RA SF T cells (Fig. 8B, right panel). Thus, within the context of general increases in Bim:Bcl-2 ratios in T cells during culture, the failure of RA SF T cells to down-regulate Noxa expression relative to Mcl-1 may promote selective apoptosis of this T cell population ex vivo.

Discussion

In this study, we demonstrate that at the single-cell level, RA SF T lymphocytes produce IL-2 and proliferate as well as, if not more so, than HD and RA PB T cells. Our studies provide evidence that the intrinsic capacity of T lymphocytes to undergo TCR-dependent
that RA synovial T cells display a phenotype favoring rapid apoptosis. Using intracellular FACS staining, it was observed that synovial T cells expressed low levels of antiapoptotic Bcl-2 (41). Under these circumstances, it was proposed that synovial T cell apoptosis in situ was actively suppressed by cell-cell contacts, signaling of IL-2 and IL-15 via the IL-2 receptor common γ-chain, and/or CD28 costimulation, each of which could enhance Bcl-xL protein expression (41, 42, 57). In our analysis of mRNA expression in RA SF T cells, we did observe a modest decrease in Bcl-2 expression, but no differences in Bcl-xL expression, compared with HD and RA PB T lymphocytes. Consistent with mRNA data, Bcl-2 protein expression was selectively down-regulated in RA SF T cells. Surprisingly, Bcl-xL protein expression in RA SF T cells was hardly detectable, in contrast with previous reports. Although the reason for this discrepancy is unknown, Bcl-xL expression in RA SF T cells was previously assessed by intracellular FACS staining without independent verification by Western blotting. mRNA expression of the proapoptotic Bcl-2-binding partner Bim was similar in freshly isolated HD PB, RA PB, and RA SF T cells, but at the protein level was depressed in RA SF T cells.

During T cell culture ex vivo, we observed a dramatic increase in the ratio of Bim mRNA to that of Bcl-2, although this occurred to a similar degree in HD PB, RA PB, and RA SF T cells and could not be clearly linked with the selective induction of apoptosis in RA SF T cells. However, we did note a significant increase in expression of proapoptotic Noxa, which is up-regulated following TCR triggering or IL-7/IL-15 stimulation and determines the apoptosis susceptibility of T cells exposed to environmental stress (43). Also in line with mRNA data, we readily detected elevated Noxa protein expression in RA SF T cells compared with HD PB T cells. However, Noxa protein was even more elevated in RA PB T lymphocytes, although apoptosis of these cells ex vivo was no greater than observed in HD PB T lymphocytes. The reason for this inconsistency may lie in the observation that RA PB T cells, unlike their SF counterparts, also express elevated levels of Mcl-1, protecting them from Noxa-mediated apoptosis. In murine T cells, Mcl-1 plays a general role in protecting T cells against apoptosis during development, activation, and differentiation (44). Although HD and RA PB T cells quickly down-regulated transcription of Noxa ex vivo, this process was delayed in RA SF T cells, leading to a persistently increased Noxa:Mcl-1 ratio. This specific failure to decrease Noxa:Mcl-1 ratios in RA SF T cells, in combination with general increases of Bim:Bcl-2 ratios in T cells during culture, might push RA SF T cells into apoptosis. In chronic lymphocytic leukemia, increases in the ratio of Noxa:Mcl-1 when Bcl-2 expression is limited also drive cellular apoptosis (58).

Although numerous studies noting the clinical efficacy of abatacept in RA are now available, there are no reports describing the direct effects of this compound on T cell function in RA (4). At least implicitly, clinical benefits are usually interpreted in terms of the ability of abatacept to block requisite costimulatory signaling of CD28 during the TCR-dependent activation of autoreactive T cells. Given that TCR signaling is intact in SF T cells, it will be of interest to determine whether abatacept exerts its effects through the inhibition of rare TCR-dependent activation events, currently below our detection threshold, or suppresses inflammation in RA by alternative mechanisms, such as reverse signaling to CD80/86 expressing APCs and FLS (59), direct targeting of RA synovial and SF T cells, which abundantly express CD80/86 (60, 61), or effects on peripheral mononuclear cell populations. Continued efforts to understand the molecular mechanisms by which abatacept achieves clinical efficacy in RA may identify additional immunemediated inflammatory diseases to which this compound might be applied therapeutically.
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