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The presumed hyporesponsive behaviour 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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Abstract

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 antigens, 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 (SF) 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 SF T cell hyporesponsiveness observed in bulk cultures can be attributed to spontaneous apoptosis ex vivo, which is associated with altered ratios of pro-apoptotic 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.

Introduction

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 interleukin IL-17 production, stimulation of B lymphocytes producing autoimmune Abs, and promotion of osteoclast differentiation1. Human genetic studies, experimental animal models of arthritis, and recent clinical experience have provided evidence consistent with a role for improper engagement of auto-reactive 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 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 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 antigens, 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 mis-folding of critical TCR signaling proteins, such as the CD3ζ chain[30;33] and LAT[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].
Here, 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 pro-apoptotic 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 out-patient clinics, with clinically active RA fulfilling the American College of Rheumatology (ACR) revised criteria for RA[38]. Patient characteristics are presented in Table I. At the time of sample collection eleven patients were receiving methotrexate (2.5-25 mg/week), four prednisolone (2.5-10 mg/day), one leflunomide (20 mg/day), five TNF-a antagonist therapy (adalimumab, 40 mg/2 weeks; etanercept, 25 mg twice a week or 50 mg/week; infliximab, 3 mg/kg intravenously [iv] every 8 weeks), 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

PB mononuclear cells (PBMCs) from healthy volunteers and PBMC and SF mononuclear cells (SFMCs) from RA patients were isolated by Ficoll-Isoplaque density gradient centrifugation (Nycomed, Pharma, Oslo, Norway). PB and SF T cells were purified from PBMC and SFMC using a negative isolation procedure (T Cell Negative Isolation Kit, Dynal Biotech, Oslo, Norway) 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 1x106/ml in IMDM medium supplemented with 10% FCS, L-glutamine, 25 mM HEPES, streptomycin (100 ng/ml) and penicillin (10U/ml) (all from Gibco, Invitrogen, Carlsbad, CA). T cells were stimulated with either 1 μg/ml anti-CD3 (clone 1XE) and 1 μg/ml anti-CD28 (clone 15E8) mAbs (both from Sanquin, Amsterdam, The Netherlands) or 1 ng/ml PMA and 1 μg/ml ionomycin (I) (both from Sigma-Aldrich, St. Louis, MO).

Measurement of T cell proliferation

T cell proliferation in bulk cultures was assessed by culturing T cells for 72 hours in a 96-well plate in the absence or presence of activating Abs. During the last 20 hours of culture, cells were pulsed with 1 mCi [^{3}H] thymidine (Amersham Biosciences, Piscataway, NJ). Cells were harvested and incorporated radioactivity measured using a 1450 Microbeta Plus Liquid Scintillation counter (Perkin Elmer, Waltham, MA). For single-cell analysis of T cell proliferation, PBTC and SFTC were resuspended at 5-10x106 cells/ml in PBS and labeled with 2.5 mM CFSE (Molecular Probes Europe BV, Leiden, The Netherlands) for 10 minutes at 37°C. Cells were washed and subsequently resuspended in complete culture medium. T cells (1x106/ml) were left unstimulated or stimulated for 72 hours at 37°C with anti-CD3 and anti-CD28 Abs. Proliferation was detected using a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA) 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 cells were calculated as previously described[39].

Detection of cytokine production

T cell culture supernatants were collected for cytokine analysis 24h (for IL-2) or 72h (TNF-α, IFN-γ) post-stimulation, and cytokine concentrations measured using a Bio-Plex Human 27-plex panel (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer's instructions. For single-cell analysis of T cell cytokine production, T cells were stimulated for six hours with anti-CD3/CD28 Abs or PMA/I, with 10 μg/ml Brefeldin A (Sigma-Aldrich) included for the last four hours 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-APC, IFN-γ-PE, or TNF-α-APC 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 APC- or FITC- labeled Annexin-V (IQ Products, Groningen, The Netherlands) for 30 minutes. Propidium iodide (PI; 5 mg/ml, Sigma-Aldrich) was added prior to analysis, and the percentage of viable cells quantified by flow cytometry.

RT-MLPA Procedure and Analysis

Total T cell mRNA was isolated using a GenElute RNA isolation kit (Sigma-Aldrich). Reverse transcription multiplex ligation-dependent probe amplification (RT-MLPA) of pro- and anti- apoptotic genes was performed as previously described[40]. Briefly, RNA was reverse-transcribed using a gene-specific probe mix (MRC Holland, Amsterdam, The Netherlands). 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-carboxy-fluorescein-labeld primer. PCR products were applied to an ABI 3100 capillary sequen-

cer (Applied Biosystems, Warrington, United Kingdom), and data processed with Genescan and Genotyper software (both from Applied Biosystems). Final analyses were conducted with Microsoft Excel spreadsheet software (Microsoft, Redman, WA). 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 mg/ml leupeptin, 1 mM PMSF, 0.1 mM TLCK, and 2 mg/ml trypsin inhibitor. Lysates were cleared by centrifugation at 13k rpm for 15 minutes, and protein expression analyzed by standard western blotting procedures as previously reported in detail[37]. Proteins were resolved by SDS-PAGE, transferred to PVDF membrane (Bio-Rad Laboratories), and blots probed with antibodies against Noxa (Imgenex, San Diego, CA), Bim (Chemicon, Temecula, CA), Bcl-2 (Alexis, San Diego, CA), Bcl-X_L (BD Transduction Laboratories, Lexington, KY), Mcl-1 (BD Biosciences Pharmingen, San Diego, CA), β-actin and ERK 1/2 (Santa Cruz Biotechnology, Santa Cruz, CA).

Statistical Analysis

Values between groups were compared using one-way ANOVA followed by Dunnett's post hoc test using RA SF T lymphocytes as the reference group. Variables that were not normally distributed were rank transformed prior to the analyses. Comparisons within groups were done using a t-test or Mann-Whitney test where appropriate. To correct for multiple testing, overall p-values were corrected using the False Discovery rate (FDR) and p-values < 0.05 were considered statistically significant.

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

Characteristic	Median (range)
Age (years)	52 (25-85)
Male:female	3:10
Disease duration (years)	9 (1-17)
Erythrocyte sedimentation rate (mm/hour)	57 (23-120)
C-reactive protein (mg/L)	64 (2-117)
Rheumatoid factor (kU/L)	102 (<1-438)
Anticitrullinated peptide Abs (kAU/L)	2438 (2-15951)

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 T lymphocytes were refractory to TCR/CD28-induced proliferation. Freshly isolated HD PB, and paired RA PB and SF T cells were stimulated for 72 hours in the presence of anti-TCR/CD28 Abs, and proliferation measured by incorporation of [^{3}H]-thymidine during the last 20 hours of culture (Figure 1). In the absence of TCR/CD28 stimulation, [^{3}H]-thymidine incorporation was barely detectable in all samples, and did not differ statistically between HD PB, RA PB, and RA SF T cell populations. In accordance with previous inde-

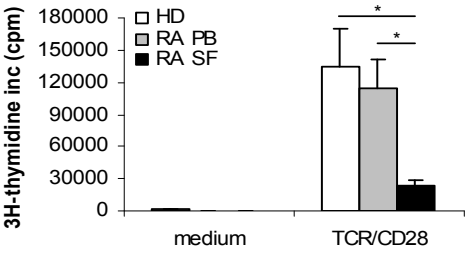

Figure 1. RA SF T lymphocyte proliferative responses are depressed in bulk cultures. *HD PB, RA PB, and RA SF T lymphocytes were cultured in the presence of anti-TCR/CD28 antibodies for 72 hours, pulsed for an additional 20 hours with [3H]-thymidine, and [3H]-thymidine incorporation quantified (n=3). Mean values of counts per minute (cpm) +/- SEM are indicated. * $p < 0.05$.*

pendent observations, RA SF T lymphocyte proliferation was significantly reduced following TCR/CD28 stimulation, compared to HD (82% reduction, $p < 0.05$) or RA PB T cells (79% reduction, $p < 0.05$).

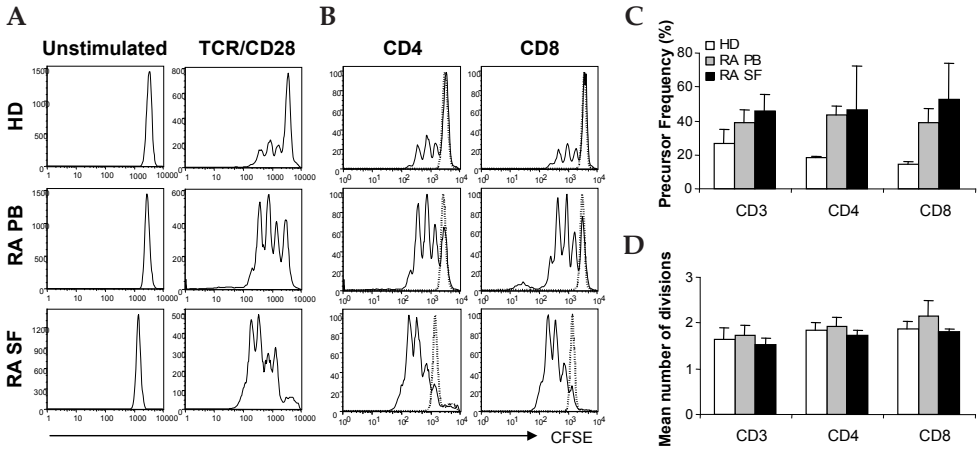

Figure 2. Single cell analysis fails to reveal defects in RA SF T lymphocyte proliferative responses. *T cell proliferative responses were assessed by FACS analysis of T cell CFSE dilution following stimulation with anti-TCR/CD28 antibodies. A, Representative histograms of CFSE labeled T cells cultured in the absence (left panels) or presence (right panels) of activating anti-TCR/CD28 for 72 hours (n=5). B, Representative histograms of CFSE dilution in CD4$^+$ (n=2) and CD8$^+$ (n=2) T lymphocytes in the absence (broken line) and presence (solid line) of anti-TCR/CD28 stimulation. C, Precursor frequency and D, mean number of HD PB (white bars), RA PB (gray bars) and RA SF (black bars) T cell divisions calculated after anti-TCR/CD28 stimulation. Mean values +/-SEM are indicated.*

We next attempted to confirm RA SF T cell proliferative hyporesponsiveness at the single-cell level, and 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 three days prior to 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 to HD PB T lymphocytes (Figure 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 (Figure 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 to HD or RA PB T cells (Figure 2C). Rather, the precursor frequency of RA SF T cells (45%) was increased compared to 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 (Figure 2C). No differences were observed between 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 (Figure 2D). 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 [^{3}H]-thymidine incorporation experiments (Figure 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 SF T cell

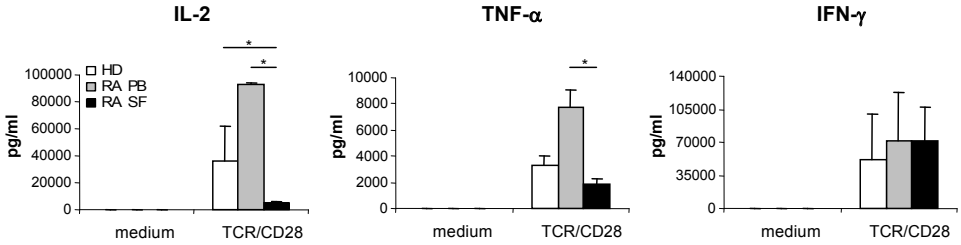

Figure 3. IL-2 and TNF-α production is decreased in cell culture supernatants of TCR/CD28-stimulated RA SF T lymphocytes. *HD PB (white bars), RA PB (gray bars) and RA SF T lymphocytes (black bars) were left unstimulated or stimulated with anti-TCR/CD28 antibodies, and medium collected for cytokine analysis by ELISA. Cells were stimulated for 24 hours to detect IL-2 production (left panel, n=3) and for 72 hours to detect TNF-α (middle panel, n=3) and IFN-γ (right panel, n=3) production. Values represent the mean and SEM of cytokine concentrations (pg/ml). * $p < 0.05$.*

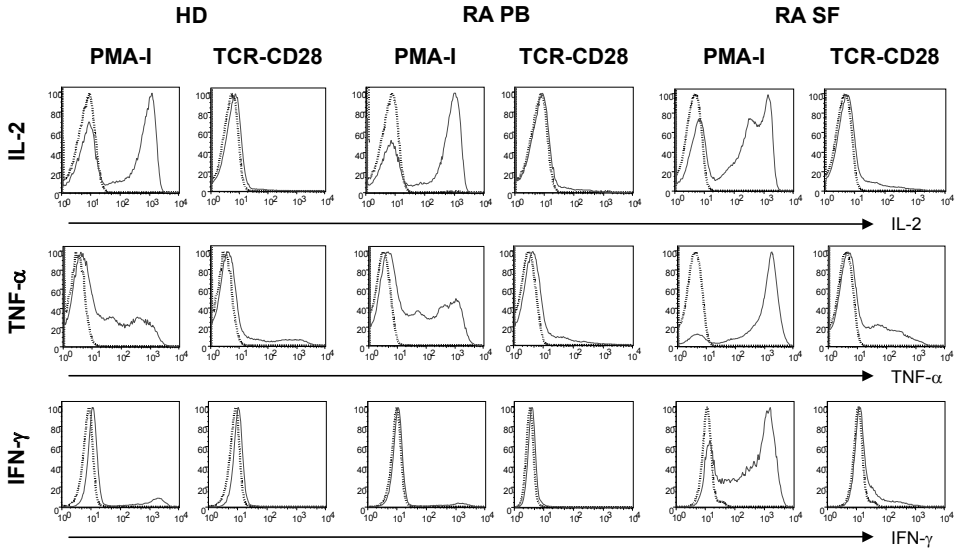

Figure 4. Single cell analysis reveals robust TCR-dependent cytokine production by RA SF T lymphocytes. *Representative histograms of IL-2 (top row), TNF-α (middle row) and IFN-γ production (bottom row) in HD, RA PB and RA SF T lymphocytes, following T cell stimulation in the absence (dashed black lines) or presence (red solid lines) of PMA/I or anti-TCR/CD28 antibodies. Cells were stimulated for 6 hours and Brefeldin A included for the last 4 hours of culture. Cells were fixed, permeabilized, stained with anti -IL-2, -TNF-α , and -IFN-γ antibodies, and cytokine-producing cells detected by FACS analysis.*

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 to autologous RA PB T cells ($p < 0.001$) (Figure 3). Additionally, TNF-α production in RA SF T cells was decreased compared to 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-γ.

We next analyzed TCR/CD28-induced cytokine production by intracellular staining and flow cytometry (Figures 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-α- and IFN-γ- producing T lymphocytes was elevated in RA SF (Figure 5A). Unexpectedly, following TCR/CD28 stimulation, RA SF T cell IL-2 production was significantly increased when compared to TCR/CD28-stimulated RA PB ($p < 0.005$) or HD T cells (p

< 0.005) (Figure 5B). Also TNF-α and especially IFN-γ production was significantly increased in SF T cells compared to HD or RA PB T cells. The frequency of IFN-γ 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 $CD4^+$ (Figure 5C) and $CD8^+$ cells (Figure 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

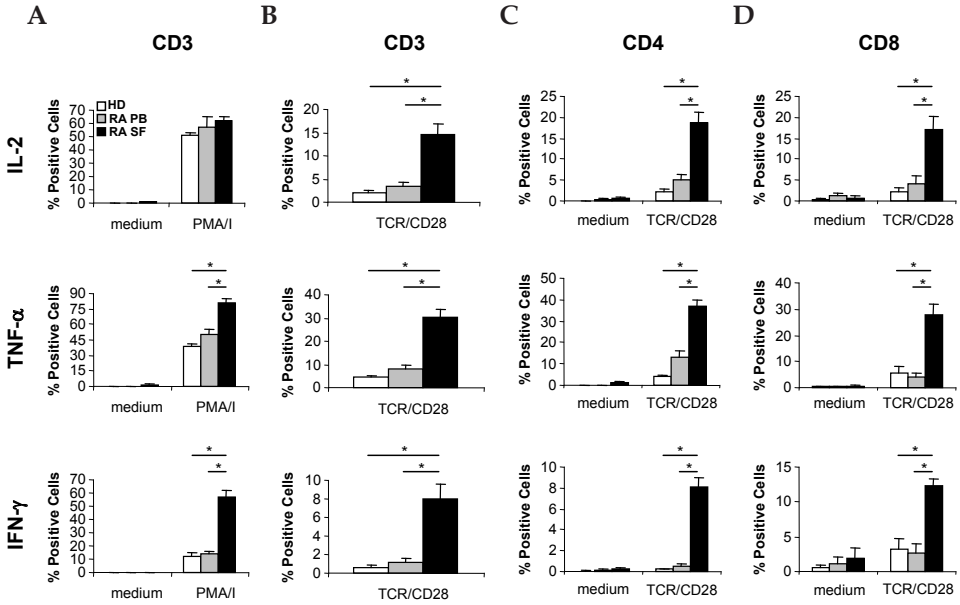

Figure 5. TCR-dependent cytokine production is enhanced in RA SF T lymphocytes. *Cytokine production in HD PB (white bars), RA PB (gray bars) and RA SF T lymphocytes (black bars) in the absence or presence of PMA/I or TCR/CD28 stimulation was assessed by intracellular staining for IL-2 (upper panels), TNF-α (middle panels), and IFN-γ (lower panels) and FACS analysis. A, T cells were left unstimulated or stimulated with PMA/I for 6 hours, and Brefeldin A included for the last 4 hours of culture. Cells were fixed, permeabilized, stained with anti–IL-2, -TNF-α, and -IFN-γ antibodies, and cytokine-producing cells detected by FACS analysis. B. Cytokine production in T lymphocytes stimulated with anti-TCR/CD28. Data was obtained in the same experiments presented in A and presented in separate graphs to facilitate visualization of differences in T cell cytokine responses. C, Cytokine production in $CD4^+$ and D, $CD8^+$ T cell subsets. Values represent the mean and SEM of the percentage of positive cells in $CD3^+$ (n=6), $CD4^+$ (n=4), and $CD8^+$ (n=3) populations from independent experiments. * $p < 0.05$.*

in cytokine production observed between ELISA analysis of bulk cultures (Figure 3) and single cell analysis were not attributable to patient heterogeneity or drug treatment, as 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.

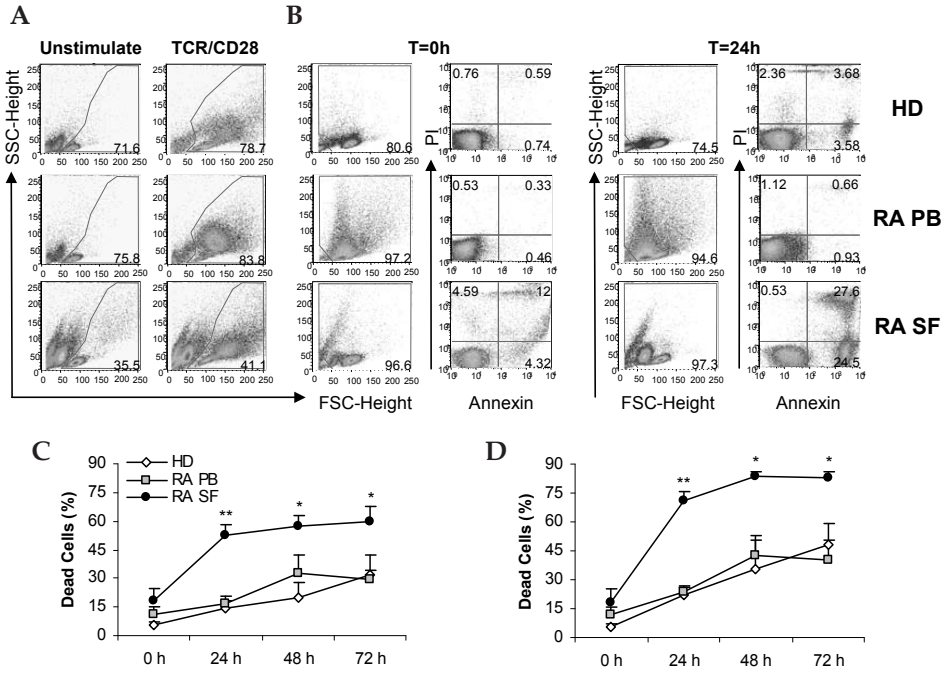

Figure 6. RA SF T lymphocytes undergo spontaneous apoptosis ex vivo. *A, Representative forward scatter (FSC)/side scatter (SSC) dot plots of HD PB (upper panels), RA PB (middle panels), and RA SF T lymphocytes (lower panels) after 72 hours in culture in the absence (unstimulated) or presence of activating anti-TCR/CD28 antibodies. Numbers indicate the percentage of viable gated cells. B, Representative FSC/SSC dot plots and stainings of HD PB (upper panels), RA PB (middle panels), and RA SF T lymphocytes (lower panels) with annexin V and propidium iodide (PI) immediately after isolation (T=0) and after 24 hours (T=24h) in culture. C and D, The percentage of apoptotic HD PB (open diamonds), RA PB (gray squares), and RA SF T lymphocytes (black circles) as detected by annexin V/PI staining and FACS analysis after 0, 24, 48, and 72 hours in culture in the absence (C) or presence (D) of activating anti-TCR/CD28 antibodies. Values represent the mean percentage of apoptotic cells and SEM of 9 independent experiments. *$p < 0.05$, **$p < 0.001$.*

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 [^{3}H]-thymidine incorporation and tissue culture supernatant ELISA analyses can not 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 hours after isolation and CFSE labeling suggested a significant loss of viability of RA SF T cells under these culture conditions (Figure 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 hours (Figure 6B). Immediately following T cell isolation, similar percentages of apoptotic cells were observed in all samples. However, after 24 hours in culture, RA SF T cells displayed almost four times higher levels of apoptosis as compared to HD T cells ($p < 0.001$) and to 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 hours in culture (Figure 6C), and remained elevated compared to HD ($p < 0.001$) and RA PB ($p< 0.001$) controls in the presence of TCR/CD28 stimulation (Figure 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 (Figure 7A). However, mRNA expression of the pro-apoptotic BH3-only family member Noxa was increased approximately two-fold in RA SF T lymphocytes compared to HD ($p < 0.05$) and RA PB ($p < 0.05$) T cell populations (Figure 7B). The expression of Bim, NIP3 and Puma, three other pro-apoptotic Bcl-2 family members known to regulate T cell apoptosis was equivalent between RA SF and other T cells (Figure 7A). Among

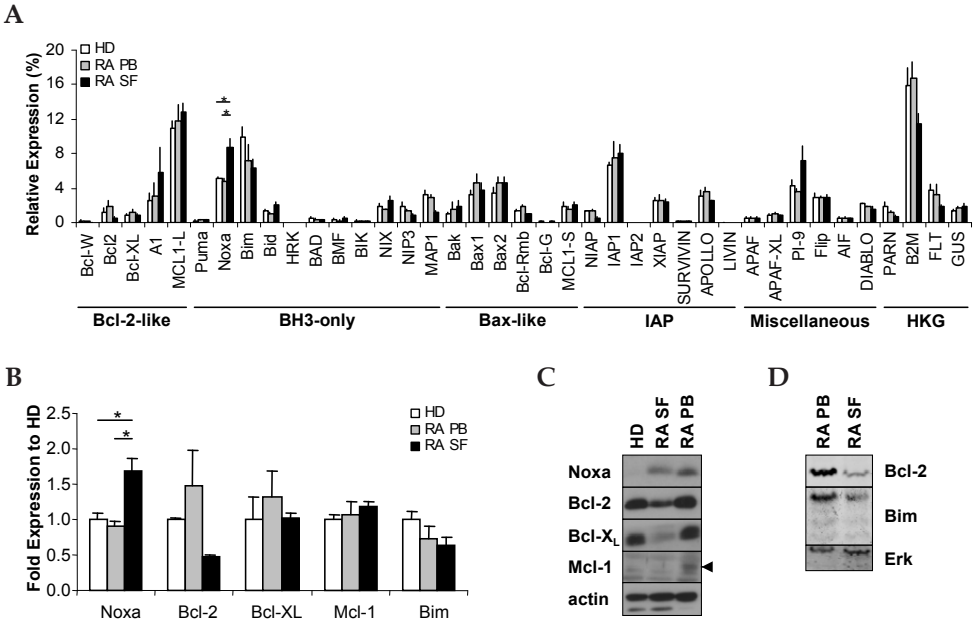

Figure 7. Expression of pro- and anti-apoptotic proteins is altered in RA SF T lymphocytes. *A, MLPA analysis of gene expression in mRNA from HD PB (n=4), RA PB (n=3) and SF T lymphocytes (n=3). The results were calculated and expressed as percentage of the total signal (relative expression) of all genes examined. Mean values and SEM are indicated. * $p < 0.05$. B, Expression of selected genes presented as the mean and SEM relative to HD PB T lymphocytes. Relative expression of genes in HD PB T cells has been normalized to a value of 1. C, Western blotting of whole cell lysates of freshly isolated HD PB, RA SF and RA PB T lymphocytes with antibodies against Noxa, Bcl-2, Bcl-X_L, Mcl-1 and actin. The arrow indicates Mcl-1 expression. One of three representative independent experiments is shown. D, Western blotting of RA PB and RA SF T cell lysates as in C, using antibodies against Bcl-2, Bim and ERK.*

anti-apoptotic gene products associated with T cell survival, we saw no differences in the expression levels of Bcl-X_L or the Noxa-binding partner Mcl-1 (Figure 7B). An approximately 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 anti- apoptotic proteins in whole cell lysates (Figures 7C and 7D). Consistent with mRNA data, Noxa protein expression was elevated in RA SF T cells compared to 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-X_L expression as a proposed compensatory pro-survival mechanism in RA SF T cells[41], we found that Bcl-X_L was hardly detectable by western blotting (Figure 7C). Anti-apoptotic Bcl-2 protein expression in RA SF T cells was severely depressed (Figures 7C and 7D), but this occurred in parallel with decreased expression of the pro-apoptotic Bcl-2 binding partner Bim (Figure 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 versus Mcl-1. However, while 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 antigen stimulation[43;44], we noted no differences in apoptotic rates of HD PB, RA PB and RA SF T cells immediately post-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 hours in culture. Ex vivo culture of HD PB T cells led to a significant down-regulation of Noxa mRNA expression as compared to RA SF T cells ($p < 0.05$) (Figure 8A). A trend towards down-regulation of Mcl-1 expression was observed in RA SF T cells, while Bcl-X_L expression remained comparable in each T cell population after 24 hours (Figure 8A). Paired analysis of the ratio of Noxa expression relative to Mcl-1 in each sample immediately after isolation and after 24 hours culture demonstrated that apoptosis in RA SF T cells was associated with an inability of RA SF T cells to down-regulate the Noxa/Mcl-1 ratio (Figure 8B, left panel). Relative expression levels of Bim to Bcl-2, especially in the context of Fas signaling, have recently been demonstrated to regulate T cell survival and prevent autoimmunity in mice[45-48], so we therefore assessed potential changes in HD PB, RA PB and RA SF T cell expression of Bim and Bcl-2 over time (Figure 8A). After 24 hours in 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 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 (Figure 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

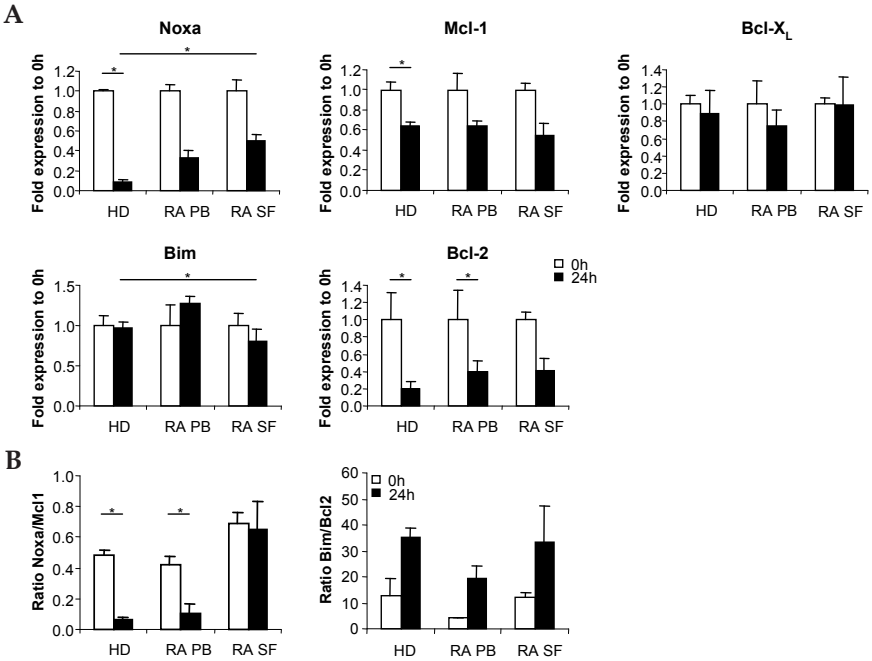

Figure 8. RA SF T lymphocytes fail to downregulate expression of Noxa relative to Mcl-1 following isolation. *A, Expression of selected genes from HD PB, RA PB, and RA SF T lymphocytes immediately after isolation (0h, white bars) and after 24 hours (24h, black bars) in culture. Values are normalized to 1 for each T cell source at 0h. B, The ratio of relative expression of Noxa/Mcl-1 (left panel) and Bim/Bcl-2 (right panel) in HD PB, RA PB and RA SF T lymphocytes immediately after isolation (0h, white bars) or after 24 hours in culture (24h, black bars). Shown are the means and SEM of 3 independent experiments.*

T cells to down-regulate Noxa expression relative to Mcl-1 may promote selective apoptosis of this T cell population ex vivo.

Discussion

Here, 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 activation and proliferation remains intact in the SF of RA patients. This finding stands in sharp contrast to currently held notions regarding the cellular and

molecular mechanisms underlying our inability to detect T cell cytokine production and proliferation in RA SF and synovial tissue. Initial studies characterizing the responsiveness of RA SF and synovial tissue T cells to TCR triggering, recall antigens, and pharmacological mitogens presented clear evidence of depressed IL-2 production and proliferation in these lymphocytes (reviewed in[2;3]). However, the accuracy and reliability of thymidine incorporation measurements, as well as cytokine determination in supernatants, are critically dependent upon the comparison of equivalent cell numbers. Our data suggest that previous reports of RA SF TCR signaling defects, at least in terms of proliferation and cytokine production, reflect limitations of the experimental systems used in these studies in accounting for apoptosis, and not the functional capacity of RA SF T cells. Although previous studies have demonstrated that T cells derived from RA synovial tissue, like those derived from SF, are hyporesponsive to TCR and mitogenic triggering, further studies will be needed to reassess the functional capacity of RA synovial TCR signaling in situ.

Productive TCR engagement of autoreactive T lymphocytes should readily lead to detectable local T cell cytokine generation and proliferation in RA synovial tissue. However, in established RA, no significant synovial tissue T cell cytokine production or proliferation has been observed in situ[27;28;49], and no significant spontaneous production of IL-2, IFN-γ or TNF-α has been observed in freshly isolated RA SF T cells (this manuscript and[22-24;49]). Spontaneous IL-17 production has been observed in RA synovial tissue T cells[50;51], although the presence of spontaneous IL-17 production in RA SF T cells is controversial[49;52]. Similarly contradictory reports exist regarding whether IL-17 levels are elevated in RA SF compared to disease controls[29;36;51;52]. The lack of obvious T cell cytokine production and proliferation in established RA has led to the idea that TCR signaling is repressed in the presence of chronic inflammatory mediators, and that at this stage of disease, synovial T cells contribute to pathology by TCR-independent mechanisms[2;3]. A number of TCR-independent in vitro model systems have been developed, each of which recapitulates at least some phenotypic characteristics of RA synovial T cells. In both human PB T cells and in murine T cell hybridomas, chronic TNFa or cytokine cocktail exposure results in decreased CD3ζ protein expression and concomitant diminished proliferative responses to CD3/CD28 stimulation, yet promotes T cell activation of monocytes by cell-cell contact[19;30;33]. Additionally, coincubation of RA SF T cells with IL-15 can induce IL-17 production[36], while a subset of RA synovial tissue and SF T cells respond to cytokine cocktails by producing IFN-γ[53]. Studies in model systems may thus benefit most from functional readouts distinct from TCR-dependent cytokine production and proliferative responses.

The balance between survival and apoptosis in T lymphocytes, under homeostatic or inflammatory conditions, is tightly regulated by expression and post-translational modification of Bcl-2 family proteins. Bcl-2 family proteins known to regulate T cell survival include pro-apoptotic Bax, Bim, NIP3, Noxa, and Puma, and anti-apoptotic Bcl-2, Bcl-X_L, and Mcl-1[54]. Specific association of Bim to its anti-apoptotic binding partners, Bcl-2 and Mcl-1, and Noxa binding to Mcl-1, has been demonstrated[55]. Therefore, shifts in expression levels of these interacting Bcl-2 members are expected to affect the threshold for T cell apoptosis. Although no significant apoptosis of RA synovial T lymphocytes is observed in situ or immediately following isolation from tissue or SF (data presented here and[41;42]), previous studies indicated 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 anti-apoptotic 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-X_L protein expression[41;42;56]. 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-X_L expression, compared to 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-X_L protein expression in RA SF T cells was hardly detectable, in contrast with previous reports. Although the reason for this discrepancy is unknown, Bcl-X_L expression in RA SF T cells was previously assessed by intracellular FACS staining without independent verification by western blotting. mRNA expression of the pro-apoptotic 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 pro-apoptotic 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 as compared to 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 rea-

son 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]. While 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 CLL, increases in the ratio of Noxa/Mcl-1 when Bcl-2 expression is limited also drive cellular apoptosis[57].

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 RA4. 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 if 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 antigen presenting cells and FLS[58], direct targeting of RA synovial and SF T cells, which abundantly express CD80/86[59;60], 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 immune-mediated inflammatory diseases to which this compound might be applied therapeutically.

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