Extracellular Calreticulin Is Present in the Joints of Patients With Rheumatoid Arthritis and Inhibits FasL (CD95L)–Mediated Apoptosis of T Cells

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Objective. **The binding of FasL (CD95L) to its receptor, Fas (CD95), induces apoptosis. Studies have shown that in patients with rheumatoid arthritis (RA), T lymphocytes are resistant to FasL-induced apoptosis in vivo but are susceptible to FasL-induced apoptosis in vitro. Dysfunction in this mechanism may be an important contributor to the pathophysiology of RA. Thus, the present study was undertaken to determine which factors might inhibit FasL–Fas binding in vivo and those that would inhibit apoptosis of T lymphocytes in an in vitro model system.**

Methods. **Human Jurkat T cells rendered apoptotic by FasL exposure were analyzed by flow cytometry. Necrosis was determined according to measurement of lactate dehydrogenase release. Quantification of calreticulin in plasma and synovial fluid and of calreticulin–FasL binding was performed by enzyme-linked immunosorbent assay. Measurement of nitrite/nitrate in the plasma and synovial fluid was carried out by chemiluminescence assay.**

Results. **Extracellular calreticulin was present at a significantly higher concentration in the plasma (median 10.3 ng/ml, interquartile range [IQR] 14.8 ng/ml) and synovial fluid (median 10.3 ng/ml, IQR 12.0 ng/ml) of RA patients (each $P < 0.05$) compared with the plasma (median 3.1 ng/ml, IQR 1.3 ng/ml) and synovial fluid (median 2.9 ng/ml, IQR 0.9 ng/ml) of patients with psoriatic arthritis and the plasma of healthy control subjects (median 2.9 ng/ml, IQR 0.9 ng/ml). Calreticulin concentrations in the synovial fluid correlated with the tender and swollen joint counts and the activity scores on the 28-joint Disease Activity Score assessment. Calreticulin also bound directly to FasL. In vitro, calreticulin (2–16 ng/ml) inhibited FasL-induced apoptosis of Jurkat T cells.**

Conclusion. **Calreticulin was present at higher concentrations in the plasma and synovial fluid of RA patients. Calreticulin had the capacity to bind directly to FasL and to inhibit FasL-mediated apoptosis of Jurkat T cells, and thus might play a role in inhibiting apoptosis of inflammatory T cells in RA.**

Rheumatoid arthritis (RA) is characterized by the accumulation of leukocytes in the synovial compartments of the joints. T lymphocytes infiltrate the joint space and are known to be resistant to apoptosis (1). The ability of autoreactive T cells to undergo apoptosis is of great importance in the maintenance of tissue homeostasis in the joints. Synovial T cells appear to be highly differentiated and are susceptible to apoptosis in vitro, but appear to evade cell death in vivo. Studying the regulation of T cell apoptosis is important in understanding the mechanism of joint destruction observed in RA.

A number of studies have investigated the apoptotic regulatory systems of synovial T cells from the joints of RA patients (2,3), and results have suggested

that dysregulation of the survival protein Bcl-2 in either T cells or synovial cells may be implicated in T cell survival (4). Others have focused on interference of the apoptotic process mediated by the Fas antigen, which is expressed on 40–60% of CD3+ T cells in the synovium (5). There is a consensus that the Fas signaling pathway is involved in defective T cell (6) and synovial cell (7) apoptosis. The majority of studies have been performed on synovial cells, and these cells become resistant to apoptosis upon exposure to transforming growth factor β1 or may become resistant depending on differences in cell cycle components (8). Despite the Fas/FasL system being implicated in the pathogenesis of RA, there is surprisingly little information on factors present in the plasma or synovial fluid of patients with RA that might inhibit this important mechanism of apoptosis.

It has been shown that the intracellular chaperone calreticulin can be secreted from cells (9) and can bind directly to FasL in vitro (10). Further evidence of the presence of extracellular calreticulin comes from a number of studies in autoimmune diseases in which autoantibodies against calreticulin have been detected (11–13). Of note, calreticulin was recently identified as an autoantigen in patients with RA (14), although the plasma concentration of calreticulin itself has never been measured. We have previously shown that calreticulin can bind to a number of other immune-regulatory proteins, e.g., the first component of complement C1q, and such interaction impedes the ability of C1q to bind to immune complexes and activate the classical complement pathway (15,16). Furthermore, Ling et al (17) demonstrated that a shared epitope (consisting of a 5–amino acid sequence motif in position 70–74 of the hypervariable region of the HLA–DR β-chain, detected on lymphocytes present in the majority of RA patients) acts as a trigger for the production of the proinflammatory reactive oxygen species nitric oxide (NO˙), and that cell surface calreticulin plays a critical role in shared epitope signal transduction (18). In addition, in some cell systems, overexpression of calreticulin activates nitric oxide synthases, which thereby increases NO˙ production (19).

Thus, we investigated the ability of calreticulin to inhibit Fas/FasL-dependent T cell apoptosis and determined whether plasma/synovial fluid calreticulin concentrations are correlated with disease activity. Because T cells isolated from the synovial fluid of RA patients are predominantly resistant to FasL-mediated cell death, Jurkat T cells were chosen as a model cell. Jurkat cells share many phenotypic similarities with synovial T cells but are not resistant to FasL-induced cell death. We

wished to exploit this difference to determine whether components found in the synovial fluid of RA patients could induce T cell–like resistance to FasL-mediated apoptosis. Finally, we explored the possibility that extracellular calreticulin plays a role in the production and release of NO˙ from cells, since NO˙ has been implicated in the modulation of T cell apoptosis (20).

PATIENTS AND METHODS

Antibodies and reagents. Mouse monoclonal antibodies (mAb) against human calreticulin were purchased from Cambridge Biosciences, while rabbit polyclonal antibodies against human calreticulin were developed as previously described (21). Horseradish peroxidase (HRP)–conjugated goat anti-mouse antibodies and HRP-conjugated goat anti-rabbit antibodies were obtained from Santa Cruz Biotechnology. A rabbit polyclonal anti-FasL antibody (ab15285) was obtained from Abcam. The enzyme-linked immunosorbent assay (ELISA) substrate 3,3′,5,5′-tetramethylbenzidine (TMB) was bought from Bio-Rad. Nunc Maxisorp 96-well plates and Amicon centrifugal concentrators were purchased from Fisher Scientific UK. Cayman Chemical cytotoxicity assays for the detection of lactate dehydrogenase (LDH) activity and soluble FasL were purchased from Enzo Life Sciences. Sulfuric acid was purchased from Sigma-Aldrich UK. Slide-a-lysers were purchased from Perbio Science UK. S-Monovette EDTA K_2 and lithium heparin tubes were purchased from Sarstedt. Native human calreticulin was purified from Jurkat cells using a method as previously described (22) or prepared in a recombinant form as previously reported (23).

Patients and samples. Plasma and synovial fluid samples were obtained from 32 patients with RA (28 female and 4 male; mean age 59.6 years, range 24–82 years) and from 7 patients with psoriatic arthritis (PsA) (mean age 33.3 years, range 19–54 years), and plasma samples were obtained from 30 patients with systemic lupus erythematosus (SLE) (28 female and 2 male; mean age 56.9 years, range 30–73 years) and from 27 healthy control subjects (21 female and 6 male; mean age 43.7 years, range 24–69 years). Peripheral blood samples were stored in S-Monovette EDTA tubes, and plasma was collected. All patients with RA fulfilled the American College of Rheumatology (ACR; formerly, the American Rheumatism Association) 1987 criteria for RA (24), all patients with SLE fulfilled the ACR 1982 criteria for SLE (25), and patients with PsA fulfilled the criteria of Moll and Wright (26). Local ethics approval was provided for all experiments, and informed consent was obtained from all study subjects (Multicenter Research Ethics Committee project number 06/Q2102/56 and Local Research Ethics Committee number 04/Q2102/87).

Cells. Jurkat T cells were grown in RPMI 1640 supplemented with 90 units/ml penicillin, 90 μg/ml streptomycin, 2 mM L-glutamine, and 10% volume/volume heat-inactivated fetal bovine serum at 37°C in an atmosphere of 5% CO_2. Jurkat cells were chosen for use in a model of CD95/CD95L-mediated apoptosis, since previous studies have shown that between 80% and 100% of synovial fluid T cells and Jurkat cells express CD95, compared with only 40% of peripheral blood lymphocytes (27,28). Synovial fluid T cells and Jurkat

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cells also have similar surface expression of integrins (29). In contrast, synovial T cells differ from Jurkat cells in their resistance to FasL-induced cell death (30).

Analysis of apoptotic genes in RA patients, SLE patients, and healthy subjects using quantitative reverse transcription–polymerase chain reaction (RT-PCR). The Fas apoptosis gene family, consisting of the genes encoding Fas, FasLG, FADD, CFLAR, CASP8AP2, and LRDD, was screened using quantitative RT-PCR TaqMan apoptotic tiled low density gene array (TLDA) (part no. 4378701; Applied Biosystems). Initially, RNA was isolated from 5×10^6 lymphocytes from all subjects using an RNeasy mini kit, and the RNA was then supplemented with 10 μl RNA stabilization reagent (Qiagen). Complementary DNA (cDNA) was reverse transcribed from 500 ng of total isolated RNA from 5 patients with RA, 7 patients with SLE, and 7 healthy controls, using the TaqMan Reverse Transcription kit (Applied Biosystems) in a total volume of 50 μl. Reaction mixtures for each sample contained 200 μl TaqMan Universal PCR Master Mix with 40 μl cDNA (corresponding to 400 ng RNA) in a total volume of 400 μl. The reaction mixture (4×100 μl) was then added to each loading port on the array, and 2 samples were analyzed per plate. Amplification conditions were as follows: 2 minutes at 50°C to activate uracil N-glycosylase, 10 minutes at 94.5°C (for activation), 40 cycles of denaturation at 97°C for 30 seconds, and annealing and extension at 59.7°C for 1 minute. Sample measurements were performed in duplicate on the same TLDA run. Data were analyzed using the comparative threshold cycle ($\Delta\Delta C_t$) method using relative quantification software (SDS2.2; Applied Biosystems), and gene expression in the samples was quantified relative to the levels of 3 housekeeping genes, *18S*, *ACTB* (for β-actin), and *GAPDH*. Quality control was achieved by comparison of the duplicate crossing points for each gene in each sample.

Measurement of calreticulin, nitrite/nitrate, and LDH in plasma and synovial fluid. The concentration of calreticulin in plasma and synovial fluid was quantified with the use of a sandwich ELISA. Briefly, 96-well Nunc Maxisorp plates were coated with 100 μl of rabbit polyclonal anticalreticulin IgG (2.1 mg/ml) at a 1:3,000 dilution in sodium carbonate buffer, pH 7.4, and the plate was left to incubate overnight at 4°C. Wells were then washed twice with phosphate buffered saline (PBS) containing 0.05% v/v Tween (PBST) and then twice with PBS to remove unbound polyclonal anticalreticulin. Residual binding sites were blocked with 5% weight/volume milk powder (Marvel) in PBST for 2 hours at 37°C. Wells were washed twice with PBST and twice with PBS. A standard curve of 100-μl aliquots of known concentrations of recombinant calreticulin diluted in 5% w/v milk/PBST was run on each plate. Plasma or synovial fluid samples were diluted 1:10 in milk/PBST, and triplicate 100-μl samples were added to the wells. The plates were incubated for 90 minutes at 37°C, and then washed as described above.

Calreticulin binding was detected using an mAb against calreticulin (Cambridge Biosciences) in a dilution of 1:2,000 in milk/PBST, followed by incubation for 2 hours at 37°C. The wells were washed as described above, and a secondary HRP-conjugated goat anti-mouse IgG (Santa Cruz Biotechnology) was added to 100-μl aliquots, diluted 1:2,000 in milk/PBST, and incubated for 1 hour at 37°C. The wells were washed as described above, and 100 μl TMB/well was added.

The reaction was developed for 30 minutes at room temperature in the dark and was then terminated by adding 50 μl of 2N H_2SO_4 to each well. The plates were assessed by ELISA on a BMG Labtech FLUROstar plate reader at 450 nm.

Nitrite and nitrate measurements were performed in the plasma from healthy control volunteers and patients with SLE and in the plasma and synovial fluid samples from patients with RA. Before the samples were analyzed for nitrite and nitrate concentrations, they were deproteinized using zinc sulfate precipitation (31). This was performed by adding 200 μl ultrapure water and 300 μl 0.3M NaOH to 100 μl plasma or synovial fluid samples, followed by incubation at room temperature for 5 minutes. Zinc sulfate solution (300 μl, 5% w/v) was added to the mixture, followed by incubation for 10 minutes at room temperature. Each sample was centrifuged at 10,000g for 15 minutes, and the supernatant was analyzed for nitrite and nitrate using a Sievers nitric oxide analyzer (Sievers NOA 280; Analytix) according to the method described previously (32). Briefly, samples were refluxed in either 0.1M vanadium III chloride and 1M HCl at 95°C (for nitrate analysis) or in 0.3M sodium iodide and glacial acetic acid at room temperature (for nitrite analysis). These conditions reduce both nitrate and nitrite to NO$^{.}$. The NO$^{.}$ reacts with ozone, causing light emission, which is detected and measured by chemiluminescence assay. To determine the concentrations of nitrite and nitrate in the samples, standard curves were created using known concentrations of $NaNO_2$ or $NaNO_3$. Throughout the sample measurements, known standards were periodically injected to determine the efficiency of the reduction reaction. Samples were analyzed in triplicate.

LDH is a marker enzyme for cell membrane integrity, since it is present in the cytosol of cells and is released during cell lysis and necrosis. A Cayman Chemical LDH assay was used to measure the activity of LDH present in the plasma and synovial fluid samples. The assay was performed according to the manufacturer's protocol.

Generation of apoptotic cells. Jurkat T cells were incubated in 96-well plates with FasL for 4 hours in a humidified atmosphere of 5% CO_2 at 37°C. A concentration of FasL of 0.78 nM and an incubation period of 4 hours were chosen because these conditions have been shown, in preliminary work for this study, to induce ~30% apoptosis. After incubation, the cells were assessed for apoptosis using annexin V and 7-aminoactinomycin D (7-AAD) staining and a Beckman Coulter Quanta SC flow cytometer and Cell Lab Quanta software. Early apoptotic cells stained positive with annexin V alone and late apoptotic cells stained positive with annexin V and 7-AAD, while necrotic cells only stained positive with 7-AAD.

Analysis of FasL binding to calreticulin. Aliquots (100 μl) of native calreticulin (10 μg/ml) in NaCO$_3$ buffer (pH 9.6) were added to each individual well of a 96-well Nunc Maxisorp plate. The plate was incubated overnight at 4°C and then washed with PBST. Unoccupied absorption sites were blocked with 3% w/v bovine serum albumin (BSA) in PBST for 1 hour at 37°C. The plates were then washed as described above. Aliquots (100 μl) of either 0, 1, or 2 μg/ml FasL diluted in 3% w/v BSA in PBST were added to triplicate wells and incubated for 1 hour at 37°C. The plates were again washed as described above.

FasL binding to calreticulin was detected by adding to

each well 100 μl of rabbit polyclonal anti-FasL antibody (1 μg/ml diluted in 3% w/v BSA in PBST; Abcam) and incubating the plates for 1 hour at 37°C. The wells were washed as described before, and 100-μl aliquots of HRP-conjugated goat anti-rabbit antibody (Santa Cruz Biotechnology) at a 1:2,000 dilution in 3% BSA in PBST were added to each well. The plates were then incubated at 37°C for 1 hour. The plates were washed in PBST, and 100 μl TMB/well was added and developed for 30 minutes at room temperature in the dark. Each reaction was terminated by adding 50 μl of 2N H$_2$SO$_4$, and the plates were read on an ELISA plate reader at 450 nm.

Assessment of the ability of calreticulin to inhibit FasL-mediated apoptosis. To determine whether the binding of calreticulin to FasL inhibited FasL-induced apoptosis, calreticulin and FasL were preincubated together for 1 hour at room temperature, after which the coincubated proteins were added to cultures with Jurkat cells. The effect of calreticulin and FasL on early and late-stage apoptosis was assessed by staining with fluorescein isothiocyanate–annexin V and 7-AAD (15 minutes at 4°C). Inhibition of apoptosis was performed in 96-well plates into which varying concentrations of calreticulin (0, 1, 2, 4, 8, and 16 μg/ml) were added alone or in combination with 0.78 nM FasL, followed by incubation for 1 hour at room temperature. Jurkat cells (1 $\times$ 10^6 cells/ml) were then added to each well to a final volume of 250 μl. The Jurkat cells were then incubated for a further 4 hours in a humidified atmosphere with 5% v/v CO$_2$ at 37°C.

After incubation, the cells were removed from the 96-well plates and assessed for apoptosis by flow cytometry. To assess the ability of calreticulin to specifically inhibit FasL-mediated apoptosis, inhibition of calreticulin binding to FasL by the anticalreticulin antibody (0–25 nM) was conducted by coincubating calreticulin with anticalreticulin for 1 hour at room temperature, followed by incubation with 0.78 nM FasL for 1 hour at room temperature. The proteins were then added to cultures with Jurkat cells (1 $\times$ 10^6 cells/ml), followed by incubation for 4 hours in the same manner as outlined above.

Statistical analysis. Data were checked for normality. Data that were not normally distributed were analyzed using the Mann-Whitney U test and Wilcoxon's signed rank test for statistical comparison of unpaired and paired samples, respectively. Statistical correlation was assessed using Spearman's rank correlation coefficients. A probability level set at P values less than 0.05 was chosen as the threshold for statistically significant differences between groups, as determined by the Mann-Whitney U test, Wilcoxon's signed rank test, and Spearman's rank correlation analysis. The statistical significance of differential gene expression determined on TLDA in samples from normal, RA, and SLE subjects was determined by Mann-Whitney U test using pairwise comparisons of individual $\Delta\Delta C_t$ measurements (RA patients compared with controls and SLE patients compared with controls) for each sample group. The threshold for significance was set at P values less than or equal to 0.05.

RESULTS

Expression of Fas signaling genes. To ascertain whether the decreased expression of Fas and associated apoptosis family genes might be responsible for the lack
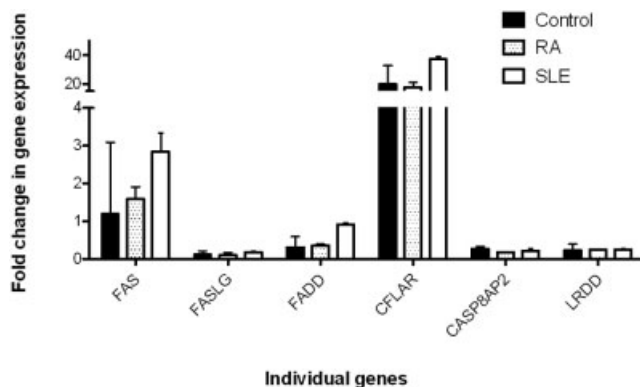

Figure 1. The expression of Fas signaling genes in peripheral blood lymphocytes from patients with rheumatoid arthritis (RA) (n = 5), patients with systemic lupus erythematosus (SLE) (n = 7), and healthy control subjects (n = 7), as determined by quantitative reverse transcription–polymerase chain reaction. Results are presented as the mean and SD fold change relative to 3 housekeeping genes.

of Fas-induced apoptosis that has been observed in RA patients, quantitative RT-PCR was performed. The expression levels of Fas, FasLG, FADD, CFLAR, CASP8AP2, and LRDD were not significantly different in lymphocytes from patients with RA compared with those from patients with SLE and healthy controls (Figure 1).

Concentrations of calreticulin and LDH in plasma and synovial fluid samples from patients. The median concentrations of calreticulin in the plasma and synovial fluid of patients with RA (median 10.3 ng/ml, interquartile range [IQR] 14.8 ng/ml and median 10.3 ng/ml, IQR 12.0 ng/ml, respectively) were significantly higher (P < 0.05) compared with the median calreticulin concentrations in the plasma of healthy controls (median 2.9 ng/ml, IQR 0.9 ng/ml), plasma of patients with SLE (median 2.8 ng/ml, IQR 2.6 ng/ml), and plasma and synovial fluid of patients with PsA (median 3.1 ng/ml, IQR 1.3 ng/ml and median 2.9 ng/ml, IQR 0.9 ng/ml, respectively) (Figure 2).

Measurements of LDH activity in the samples were performed in order to ascertain whether the calreticulin detected in the RA patients' plasma and synovial fluid was released as a result of necrosis of the cells. There was a significant increase in LDH activity in the plasma and synovial fluid of RA patients (median 0.12 arbitrary units [AU]/minute, IQR 0.08 AU/minute and median 0.18 AU/minute, IQR 0.12 AU/minute, respectively) compared with that in the plasma of healthy controls (median 0.09 AU/minute, IQR 0.06 AU/minute), plasma of patients with SLE (median 0.11
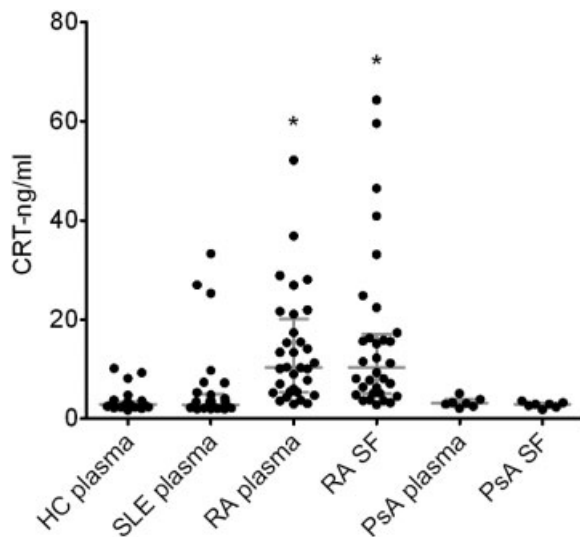
Figure 2. Concentrations of calreticulin (CRT) in autoimmune diseases. A sandwich enzyme-linked immunosorbent assay was used to measure the concentration of calreticulin in plasma from healthy control (HC) subjects (n = 27), plasma from patients with systemic lupus erythematosus (SLE) (n = 30), and matched plasma and synovial fluid (SF) samples from patients with rheumatoid arthritis (RA) (n = 32) and patients with psoriatic arthritis (PsA) (n = 7). Bars show the median and interquartile range. $*$ = $P < 0.05$ versus all other groups.

analyses were performed to assess correlations between the concentrations of calreticulin in RA patients' plasma and synovial fluid and the swollen and tender joint counts and Disease Activity Score in 28 joints (DAS28) (33) (Figure 4). A significant correlation was observed between calreticulin concentrations in the synovial fluid and the tender joint counts (r_s = 0.373, P = 0.04), swollen joint counts (r_s = 0.370, P = 0.04), and DAS28 (r_s = 0.440, P = 0.02). The correlation between calreticulin concentrations and the DAS28 in RA patients was also significant in the plasma (r_s = 0.439, P = 0.02), but the correlations between plasma calreticulin concentrations and the tender joint and swollen joint counts did not reach statistical significance (r_s = 0.342, P = 0.06 and r_s = 0.344, P = 0.06, respectively). A Bonferroni correction for multiple comparisons was not performed, because this study was focused only on the relationship of joint inflammation and mobility with calreticulin levels, and the mathematical correction is not recommended for a limited number of related comparisons (34).

Concentrations of nitrite and nitrate in plasma and synovial fluid samples from patients. Measurements of nitrite and nitrate were performed on the patients' samples as an indirect assessment of the gen-

AU/minute, IQR 0.06 AU/minute), and plasma and synovial fluid of patients with PsA (median 0.11 AU/minute, IQR 0.09 AU/minute and median 0.12 AU/minute, IQR 0.06 AU/minute, respectively) (Figure 3). Moreover, a statistically significant increase in the median LDH activity in the plasma as compared with the synovial fluid of patients with RA was also observed. These data indicate that in the RA patients' plasma, cell necrosis was occurring, and even more necrosis was occurring in the synovial fluid of RA patients.

Analyses of correlations between the calreticulin concentrations and LDH activity in RA patients' plasma and synovial fluid were performed to assess whether calreticulin was being released from necrotic cells. There was no significant correlation ($P > 0.05$) between the calreticulin concentrations and LDH levels in the RA patients' plasma or synovial fluid (results not shown). These data indicate that calreticulin was not predominantly released from the cells into the RA patients' plasma or synovial fluid by necrosis.

Correlation of plasma and synovial fluid calreticulin levels with RA disease activity. Calreticulin concentrations in RA plasma and synovial fluid were correlated with disease activity. Spearman's rank correlation
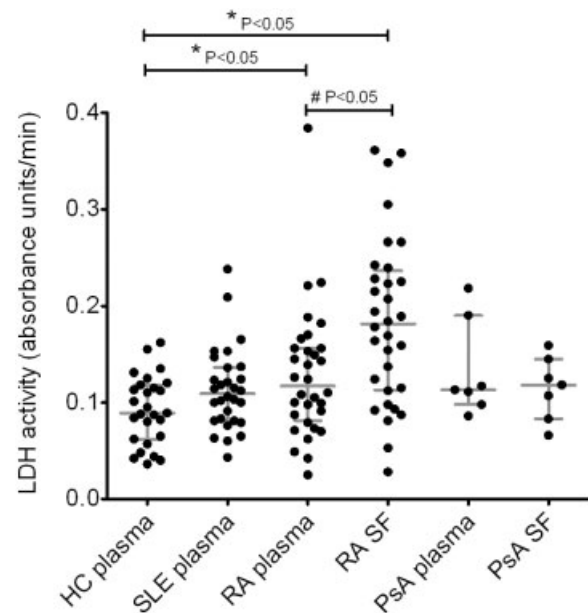

Figure 3. Activity of lactate dehydrogenase (LDH) in autoimmune diseases. Extracellular LDH activity was assessed by colorimetric assay in plasma from healthy control subjects (n = 27), plasma from patients with SLE (n = 30), and matched plasma and synovial fluid samples from patients with RA (n = 32) and patients with PsA (n = 7). Bars show the median and interquartile range. See Figure 2 for other definitions.
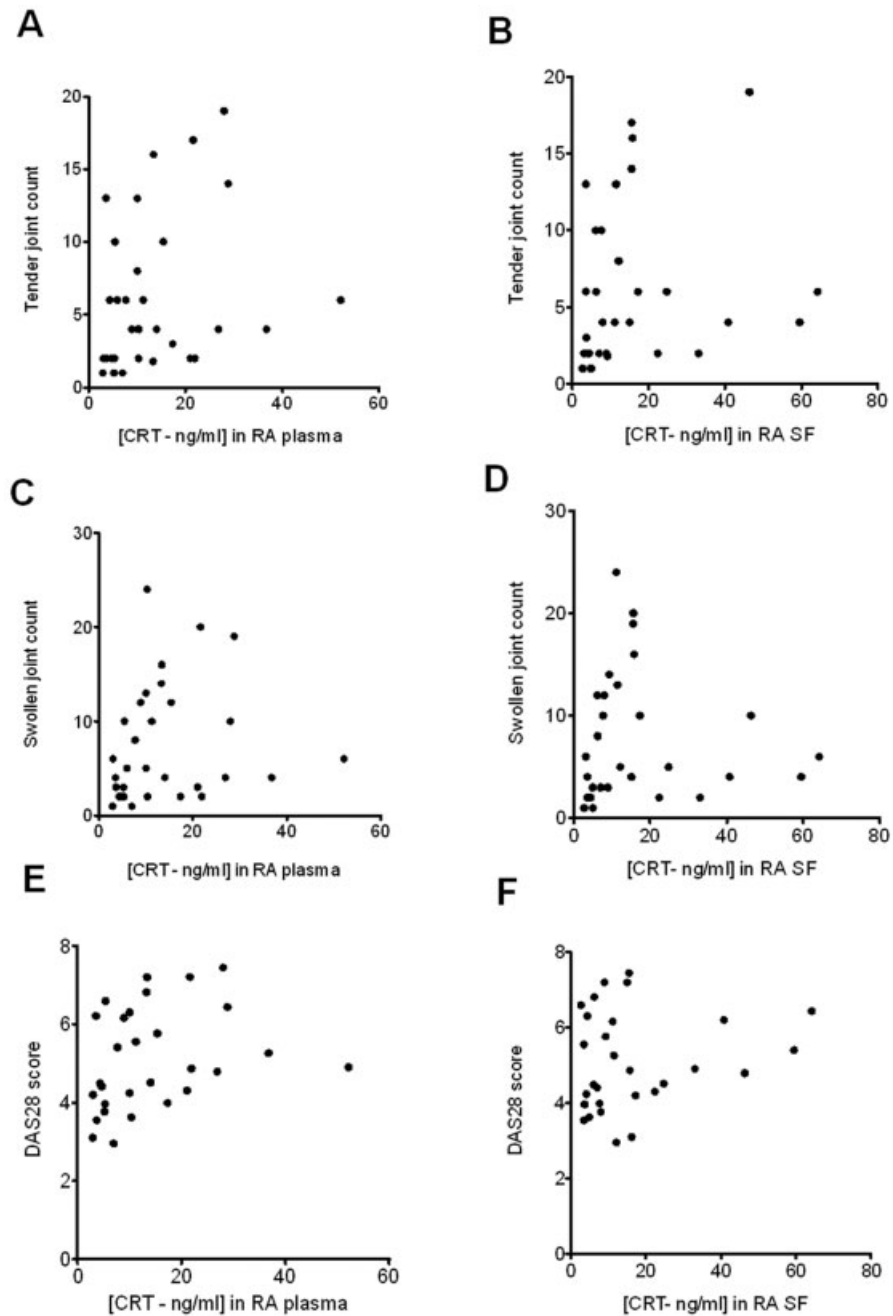
Figure 4. Correlations between calreticulin concentrations in the plasma (**A, C,** and **E**) and synovial fluid (**B, D,** and **F**) of patients with RA and the level of disease activity. The disease activity was measured by assessing the tender joint count (**A** and **B**), the swollen joint count (**C** and **D**), and the 28-joint Disease Activity Score (DAS28) (**E** and **F**). Correlations with the calreticulin concentration, as determined by Spearman's rank correlation coefficient analyses, were as follows: for plasma calreticulin and tender joint count, $r_s = 0.342$, $P = 0.059$ (**A**); for synovial fluid calreticulin and tender joint count, $r_s = 0.373$, $P = 0.039$ (**B**); for plasma calreticulin and swollen joint count, $r_s = 0.344$, $P = 0.058$ (**C**); for synovial fluid calreticulin and swollen joint count, $r_s = 0.370$, $P = 0.040$ (**D**); for plasma calreticulin and DAS28, $r_s = 0.439$, $P = 0.017$ (**E**); and for synovial fluid calreticulin and DAS28, $r_s = 0.440$, $P = 0.017$ (**F**). See Figure 2 for other definitions.
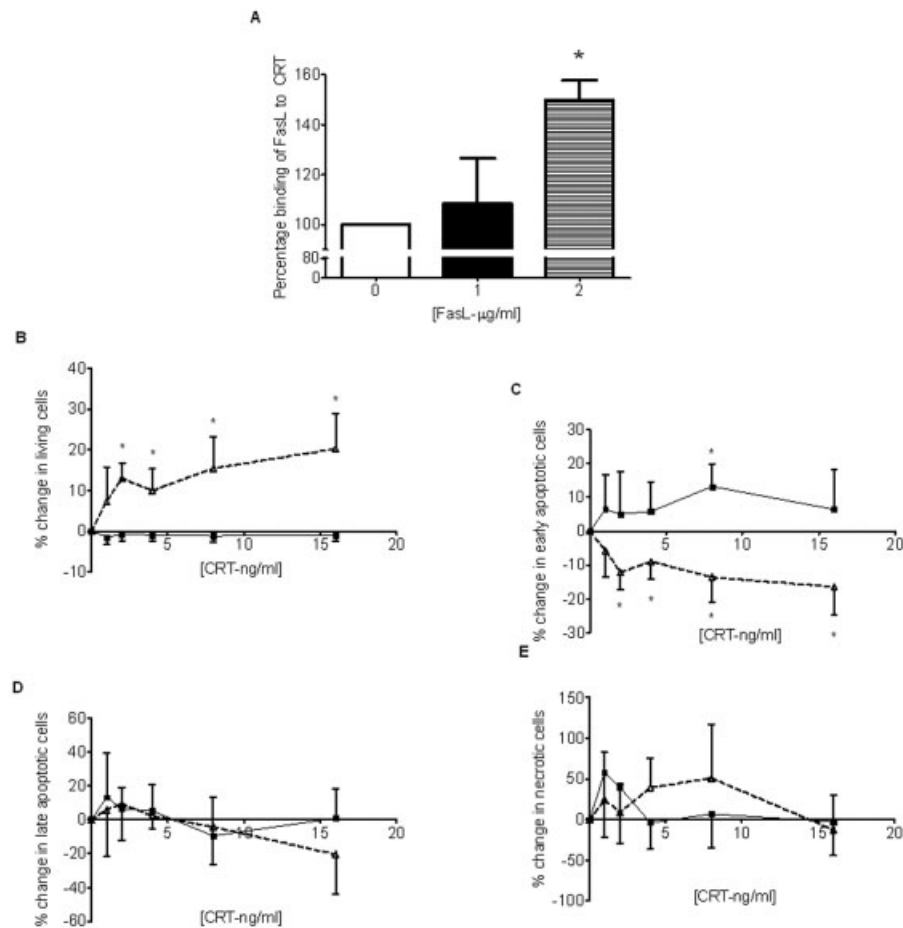
Figure 5. Demonstration of calreticulin (CRT) binding to FasL, and inhibition of FasL-induced apoptosis by calreticulin in Jurkat T cells. **A,** Calreticulin (10 μg/ml) was immobilized on microtiter plates and incubated with FasL (0, 1, and 2 μg/ml). FasL binding to calreticulin was detected using an anti-FasL antibody followed by a horseradish peroxidase–conjugated goat anti-rabbit antibody (binding details are described in Patients and Methods). Bars show the mean and SD. ∗ = $P < 0.05$ versus no FasL. **B–E,** The percentage of living (**B**), early apoptotic (**C**), late apoptotic (**D**), and necrotic (**E**) Jurkat T cells in the presence of increasing concentrations of calreticulin (0–16 ng/ml), with or without FasL, was detected by flow cytometry. Where added, the concentration of FasL was 0.78 nM. Bars show the mean and SD normalized data from 4 individual experiments performed in triplicate. In cases where no error bars are visible, the SD is smaller than the data point. The baseline level of living cells after treatment with 0.78 nM FasL was $37.9 \pm 16.2\%$. ∗ = $P < 0.05$ versus no calreticulin. Triangles represent FasL and calreticulin treatment; squares represent calreticulin treatment alone.

eration of NO˙. There was a statistically significant increase in the concentration of nitrite in RA plasma (median 0.99 μM, IQR 0.72) and RA synovial fluid (median 1.25 μM, IQR 0.89) as compared with that in the plasma from healthy controls (median 0.74 μM, IQR 0.17). There was no significant difference in the concentrations of nitrate present in the plasma of RA patients compared with the plasma of healthy controls.

Analyses of correlations between the concentra-tions of calreticulin and those of nitrite or nitrate present in the plasma and synovial fluid of RA patients were performed using Spearman's rank correlation co-efficient analyses (at a level of significance of $P < 0.05$), but no significant correlations were found (results not shown).

Inhibition of FasL-induced early apoptosis by binding of calreticulin to FasL. Calreticulin binding to FasL has been observed previously (10), and this was confirmed by our results on ELISA (Figure 5A). The
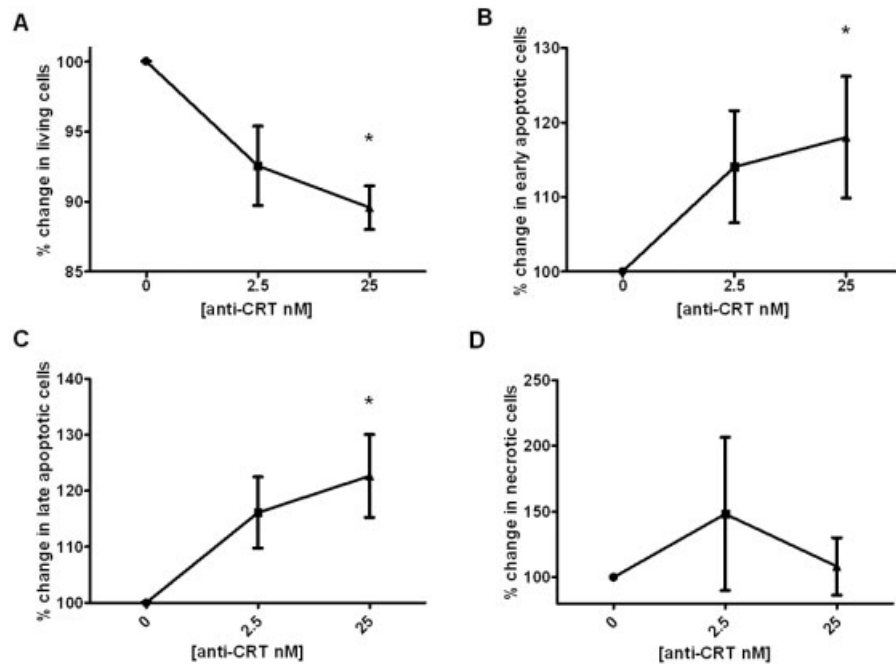
Figure 6. Anticalreticulin (anti-CRT) blocking of calreticulin inhibition of FasL-mediated apoptosis. Jurkat cells were treated with 0.78 nM FasL in the presence of calreticulin (16 ng/ml; 0.34 nM final concentration), which resulted in ~48% of cells surviving (results normalized to 100%). Calreticulin (16 ng/ml; 0.34 nM final concentration) was coincubated with a range of anti-CRT IgG concentrations (0–25 nM) and then incubated with 0.78 nM FasL, before addition to the Jurkat T cells (1×10^6). A significant reduction in living cells was observed following incubation with FasL, calreticulin, and 25 nM anti-CRT (**A**). Simultaneously, an increase in early apoptosis (**B**) and late apoptosis (**C**) of Jurkat cells occurred in the presence of 25 nM anti-CRT and 16 ng/ml (0.34 nM) calreticulin. Necrotic cell death was unaffected (**D**). Bars show the mean ± SD results from 3 individual experiments performed in triplicate. ∗ = $P < 0.05$ versus cultures with 0.78 nM FasL and 16 ng/ml calreticulin, by Mann-Whitney U test.

normalized results showed that calreticulin bound to FasL in a dose-dependent manner.

Having confirmed that FasL binds to calreticulin, we investigated whether calreticulin could inhibit FasL-induced apoptosis. The addition of 1 ng/ml (0.02 nM) calreticulin along with 0.78 nM FasL had no significant effect on the level of living Jurkat T cells as compared with FasL treatment alone (Figure 5B). However, coincubation of calreticulin at a concentration of 2, 4, 8, or 16 ng/ml (0.04, 0.08, 0.16, or 0.34 nM, respectively) with 0.78 nM FasL significantly inhibited FasL-mediated apoptosis of Jurkat cells as compared with FasL treatment alone. The addition of calreticulin alone (1–16 ng/ml; 0.02–0.34 nM) had no significant effect on Jurkat cell death (Figure 5B). Furthermore, the addition of calreticulin (2, 4, 8, or 16 ng/ml; 0.04–0.34 nM) with 0.78 nM FasL significantly decreased the number of early apoptotic cells by between 10% and 20% as compared with

FasL treatment alone (Figure 5C). The treatment of Jurkat T cells with FasL and a range of concentrations of calreticulin (1–16 ng/ml; 0.02–0.34 nM) or calreticulin alone (1–16 ng/ml) had no significant effect on the percentage of late apoptotic cells (Figure 5D) or necrotic cells (Figure 5E). These data indicate that preincubation of calreticulin with FasL is able to significantly inhibit the early apoptosis induced by FasL alone.

After demonstrating that binding of calreticulin to FasL could inhibit FasL-mediated apoptosis of Jurkat cells, we determined whether a specific antibody against calreticulin could block the calreticulin-mediated inhibition of FasL-induced apoptosis. As shown in Figures 6A–D, there was a concentration-dependent blocking of calreticulin inhibition of FasL-mediated apoptosis by anticalreticulin. This inhibition became statistically significant when 25 nM anticalreticulin was preincubated with 16 ng/ml (0.34 nM) calreticulin.

DISCUSSION

RA is an autoimmune disease that is characterized by the presence of autoreactive T cells in the peripheral blood and synovial joints (35). Synovial T cells orchestrate a wide array of clinical sequelae, including chronic inflammation, cartilage damage, and bone erosion (36). It has been hypothesized that dysfunctional or resistant apoptosis of T cells could be integral to the pathogenesis of RA, promoting the progressive destruction of joint and cartilage structures (1,3,7). The trigger for T cell resistance is unclear, but an impaired Fas signaling pathway has been suggested to be involved in defective T cell apoptosis (6).

Calreticulin is known to bind to a number of immunologically important proteins, such as C1q, and collectins (15). It recently has been demonstrated that calreticulin binds to apoptotic regulatory proteins such as TRAIL and FasL (10). In the present study, we confirmed the findings of Duus and coworkers (10) that demonstrate that calreticulin binds to FasL. In view of the evidence of an apoptosis-resistant population of T cells in the synovial fluid of RA patients and the fact that FasL binding to Fas on the surface of cells is an important mechanism of noninflammatory cell death, we sought to determine whether the calreticulin–FasL interaction impeded FasL-mediated apoptosis of T cells. We demonstrated that coincubation of FasL with varying concentrations of calreticulin (0–16 ng/ml) reduced Jurkat T cell death by up to 20% compared with the level of cell death with FasL alone. The significant reductions in FasL-mediated apoptosis occurred with calreticulin concentrations well within the range of values that were observed in the plasma and synovial fluid of RA patients (3–40 ng/ml).

The mechanism of calreticulin release or secretion from cells is uncertain. Release may be via the secretory pathway (37) or by an unconventional mechanism (38). Our own findings suggest that calreticulin release is regulated by a NO˙-dependent mechanism (39). Previously, we demonstrated that calreticulin is released from activated neutrophils by the process of necrosis (15). In view of this, we measured the LDH concentrations in our patient groups and compared the values with extracellular calreticulin concentrations. We observed a significant increase in the median LDH activity in the plasma of RA patients compared with that in the plasma of healthy controls (Figure 3); this could possibly be attributed to the increase in liver toxicity transiently observed in RA patients who were receiving treatment with methotrexate (40). A further significant increase in median LDH activity was observed in the synovial fluid samples from RA patients as compared with matched plasma samples from the same patients, suggesting that the increased LDH activity was being produced locally in the synovial joint. However, there was no significant correlation between LDH activity and calreticulin concentrations in the RA samples in which calreticulin levels were significantly increased as compared with those in the healthy and disease control groups (results not shown). This indicates that calreticulin levels in the synovial fluid of RA patients are likely to be released through non-necrotic mechanisms.

Our observation of a correlation between calreticulin production and disease activity provides further evidence for a possible role of calreticulin in RA. A proposed ligand for calreticulin on the surface of lymphocytes in the vast majority of RA patients is the shared epitope (18). It has been hypothesized that calreticulin binds to this 5–amino acid sequence motif in the third allelic hypervariable region of the HLA–DR β-chain. This binding leads to increased NO˙ production and promotes immune dysregulation (41).

In view of these observations, we measured the concentrations of the stable end products of NO˙ production, namely, nitrate and nitrite, in both the plasma and synovial fluid samples from disease and control groups. A significant increase in the level of nitrite in the plasma and synovial fluid of RA patients compared with that from patients with PsA was found in this study. Earlier studies also demonstrated increased concentrations of nitrite and nitrate in the plasma and synovial fluid of RA patients (42–45). There was no association between the concentrations of calreticulin and those of nitrite or nitrate in the plasma or synovial fluid of RA patients (results not shown). A possible reason for this lack of association is that membrane-bound and intracellular calreticulin is involved in the increased production of NO˙. In the present study, we only measured the levels of extracellular calreticulin present in the RA patients' plasma and synovial fluid. A second possible reason for the lack of correlation between calreticulin concentrations and nitrite and nitrate concentrations is that NO˙ is a reactive molecule that can form a number of metabolites other than nitrite and nitrate. Therefore, by limiting the measurements to nitrite and nitrate, we were not measuring the total NO˙ production within the RA patients' plasma or synovial fluid (46).

The present results suggest an additional role for extracellular calreticulin in apoptosis. Previous studies have determined that extracellular calreticulin–C1q interactions mediated the recognition and clearance of

apoptotic blebs via the C1q/calreticulin/CD91 apoptotic pathway (47), which is impaired in some autoimmune diseases (48). Thus, extracellular calreticulin may have a number of roles to play during apoptosis, by inhibiting FasL-mediated apoptosis, but may aid in the clearance of apoptotic debris once cells have undergone apoptosis.

In conclusion, calreticulin was shown to bind to FasL and inhibit Jurkat T cell death. In other autoimmune diseases, anticalreticulin autoantibodies are prevalent. This is not the case in RA. This raises the possibility that autoantibodies against calreticulin may neutralize the ability of calreticulin to inhibit FasL/Fas-mediated cell death. In addition, other mechanisms of inhibition of FasL-mediated cell death are also known to occur (for review, see ref. 49), involving overexpression of antiapoptotic molecules. For example, overactivation of survival signals (e.g., sphingosine 1-phosphate) can inhibit JNK expression and suppress activation of G protein–coupled proapoptotic executioner caspases (50). Infiltrating T cells, within the synovial lining layer, express the antiapoptotic protein Bcl-2, the levels of which are correlated with T cell resistance to Fas apoptosis (51). Overall, this suggests that multiple pathways contribute to the resistance of lymphocyte apoptotic death during the pathogenesis of joint destruction in patients with RA.

AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Eggleton had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.
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