Functional Analysis of Recombinant Calreticulin Fragment 39–272: Implications for Immunobiological Activities of Calreticulin in Health and Disease

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*J Immunol* 2010; 185:4561-4569; Prepublished online 20 September 2010; doi: 10.4049/jimmunol.1000536

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Calreticulin (CRT) is a ubiquitous and major Ca\(^{2+}\)-binding resident protein, primarily resident in the endoplasmic reticulum (ER) of eukaryotic cells, and it plays a central role in intracellular Ca\(^{2+}\) homeostasis (1, 2). The full-length CRT is of 46 kDa with three domains followed by a four-amino acid ER retention sequence KDEL at the C terminus (1, 2). The lectin-like globular N domain (amino acid residues 18–197) of CRT is followed by a proline-rich P domain (residues 198–308) and a Ca\(^{2+}\)-binding C domain (1, 3–5). Similar to the heat shock proteins (HSPs), CRT is also a molecular chaperone in the ER, capable of facilitating peptide loading and correct folding of MHC class I molecules (2, 5, 6). Although CRT mainly localizes in intracellular compartments, it can also appear at the surface of various types of cells and plays roles in cell–cell interaction, cell–matrix interaction, and cell spreading (6–8). CRT has been found on the membrane surface of almost all types of immune cells, including monocytes/macrophages, neutrophils, T cells, and dendritic cells, exhibiting important functions in cell activation and clearance of apoptotic and tumor cells (9–12). Exposure of CRT on the membrane surface of tumor cells and apoptotic cells substantially increases their immunogenicity, rendering the cells recognized and attacked by phagocytosis more efficiently (13–16). However, molecular mechanisms for this phenomenon are not fully understood. We reason that the immunobiological functions of CRT are most likely attributable to its N rather than C domain, as the former is a lectin and capable of relatively high-affinity binding with various glycosylated protein molecules (3–5). In this study, we have expressed a murine CRT fragment (amino acid residues 39–272, including N domain and partial P domain) with a His\(_6\) tag (34 aa residues) in Escherichia coli and analyzed in detail its stimulatory activities for B lymphocytes and macrophages. We also provide evidence for the presence of soluble CRT in the sera of patients with autoimmune diseases.

### Materials and Methods

**Serum samples from patients and healthy subjects**

Peripheral blood was collected from patients with rheumatoid arthritis (RA) (n = 60; 23–80 y of age; mean, 50.2 y of age; 48 females) or systemic lupus erythematosus (SLE) (n = 32; 18–68 y of age; mean, 37 y of age; 27 females) attending the Department of Rheumatology, Peking University Third Hospital (Beijing, China) between 2008 and 2009. RA patients were diagnosed according to the criteria of the American College of Rheumatology (17). SLE patients fulfilled the American Rheumatism Association criteria for diagnosis of SLE (18). All RA and SLE patients donated blood samples while their disease was in an active phase. The blood samples were processed within 18 h of collection and the sera were stored at \(-80^\circ\)C until use. This study was reviewed and approved by the Ethics Committee of Peking University Health Science Center. Serum samples from healthy volunteers between 20 and 50 y of age (n = 48; mean age, 30.7 y; 28 females) were included as controls.
**Mice and their immunization**

Female BALB/c, C57BL/6, and nude mice between the age of 6 and 12 wk were purchased from the Chinese Academy of Military Medical Science (Beijing, China). C3H/HeN and C3H/HeJ mice were purchased from Vital River Laboratory Animal Technology (Beijing, China). All animals were maintained under specific pathogen-free conditions in the Department of Immunology, Peking University, and animal usage was conducted according to protocols approved by the Peking University Institutional Animal Care and Use Committee. Mice were immunized s.c. at the base of the tail with 100 µl of protein in total 50 µl of PBS. When booster immunization was needed, 50 µg of protein in 200 µl of PBS was injected i.p. Serum samples were collected by tail bleeding, aliquoted, and kept at −20°C until further use.

**Synthetic peptides and recombinant proteins**

A series of 15-mer overlapping peptides, covering amino acid residues 39–272 of murine CRT, were synthesized by Hanyu Biotech (Shenzhen, China). All synthetic peptides, supplied as lyophilized powder, were high-pressure liquid chromatography purified with purities >98%. The resultant peptides were dissolved in PBS at 2 mg/ml, filter sterilized, aliquoted, and stored at −80°C until use.

Total cellular RNA was extracted from EL4 cells (American Type Culture Collection, Manassas, VA) using TRizol (Invitrogen, Carlsbad, CA) and then reverse transcribed using a SuperScript II reverse transcriptase kit according to the manufacturer’s instructions (Invitrogen). The resultant cDNA was employed as template for amplification of the gene encoding murine CRT fragment 39–272 by PCR using forward primer 5'-TCC GAA TCC AAA CAT AAG TCC GAT TTT TT-3' and reverse primer 5'-CGC GGA TCC AAA CAT AAG TCC GAT TTT TT-3'. The PCR product, verified by sequencing, was inserted into a pET28a expression vector (Novagen, Darmstadt, Germany), and the recombinant protein thus expressed contains a His-tag of 34 aa residues. The gene encoding the enhanced GFP (EGFP) was cloned from pEGFP-N1 plasmid (BD Clontech, Palo Alto, CA). A sequence overlapping extension PCR method was performed to prepare DNA encoding a fusion protein between CRT/39–272 and EGFP. The resulting plasmid, pCRT/EGFP, was digested with BglII and EcoRI, and the insert was subcloned into the expression vector pET28a. The Hiss-tagged recombinant proteins were expressed in *E. coli* BL21 (DE3) (Stratagene, La Jolla, CA) and purified using Ni-nitrilotriacetic acid resin (Novagen) following the manufacturer’s instructions. All proteins were desalted by being passed through PD10 columns (Pierce, Rockford, IL) in PBS. The protein concentration was determined using Coomassie protein assay reagent (Pierce). All recombinant proteins were used at >90% purity as judged by Coomassie blue-stained SDS-PAGE gels.

Recombinant mouse CD40L and recombinant fusion proteins between mouse CD14 and human IgG1 Fc fragment (rmCD14-Fc), mouse CD40 and human IgG1 Fc (rmCD40-Fc), mouse BAFFR and human IgG1 Fc (rmBAFFR-Fc) were purchased from R&D Systems (Minneapolis, MN).

**Western blotting**

The separated protein bands in SDS-PAGE gels were electroblotted onto polyvinylidene difluoride membranes at a constant current of 250 mA in transbuffer (50 mM Tris [pH 8.0] containing 0.192 M glycine and 20% methanol) using a Bio-Rad Trans-Blot Cell (Bio-Rad, Hercules, CA). The separated protein bands in SDS-PAGE gels were electroblotted onto nitrocellulose membranes in transbuffer (50 mM Tris [pH 8.0] containing 0.192 M glycine and 20% methanol) using a Bio-Rad Gel Doc 2000. (Applygen Technologies, Beijing, China). Data were recorded using the ECL detection system as recommended by the manufacturer (Pierce, Rockford, IL). The separated protein bands in SDS-PAGE gels were electroblotted onto nitrocellulose membranes in transbuffer (50 mM Tris [pH 8.0] containing 0.192 M glycine and 20% methanol) using a Bio-Rad Trans-Blot Cell (Bio-Rad, Hercules, CA). The separated protein bands in SDS-PAGE gels were electroblotted onto nitrocellulose membranes in transbuffer (50 mM Tris [pH 8.0] containing 0.192 M glycine and 20% methanol) using a Bio-Rad Gel Doc 2000.

**Cells and tissue culture**

All cells were cultured in complete RPMI 1640 supplemented with 10% (v/v) FBS (HyClone Laboratories, Logan, UT), 1% penicillin/streptomycin (100 U/ml), 1% L-glutamine (2 mM), and 2-ME (5 × 10⁻⁵ M). For preparation of human PBMCs, venous blood from healthy donors were 2-fold diluted using RPMI 1640 medium and overlaid on Ficoll lymphocyte separating solution (Sigma-Aldrich, St. Louis, MO) followed by centrifugation at 400 x g for 30 min at room temperature. The PBMCs were collected and washed twice with PBS, followed by resuspension in R10 medium.

For preparation of mouse peritoneal macrophages, mice were injected i.p. with 3% thioglycollate (1 ml/mouse) and the macrophages were retrieved from the peritoneum 3 d later using a syringe. For preparation of murine B and T cells, mouse spleens were gently disaggregated by pressing with the flat surface of a syringe plunger against a stainless steel sieve (200 mesh). RBCs were lysed by brief treatment with ACK lysis buffer. B cells were then purified by negative selection with a B cell isolation kit containing biotin-conjugated mAbs to CD43, CD4, and Ter-119 (Miltenyi Biotech, Bergisch Gladbach, Germany) following the manufacturer’s instructions. This procedure routinely yielded B cell preparations that were >95% positive for the B220 marker, as determined by FACS analysis using a FACSsort (BD Biosciences, San Jose, CA) and FCS Express software (De Novo Software, Los Angeles, CA). Naïve T cells were also purified by negative selection: single-cell suspensions of splenocytes were incubated for 2 h in a CO₂ incubator at 37°C in petri dishes, and the nonadherent cells were then depleted of B cells using CD19 microbeads (Miltenyi Biotech). The resultant cells were >90% positive for CD3 marker as determined by FACS analysis.

**Flow cytometric analysis**

Cells were collected, washed with 1% BSA (Sigma-Aldrich) in PBS, and the pellets (1 × 10⁶ cells/tube) were incubated for 30 min at 4°C with 20 µl of PE-conjugated anti-mouse B220 and also FITC-conjugated Abs against mouse CD80, CD86, CD69, mouse MHC class II (I²F), or FITC-conjugated isotype control Abs (BioLegend, San Diego, CA). After washing, the cells were subjected to analysis using a fluorescence-activated cell sorter (FACScan; BD Biosciences, Rutherford, NJ).

**Proliferation assays**

Proliferation assays of B and T cells were performed using 96-well tissue culture plates (Nunc. Roskilde, Denmark). Purified mouse B cells (4 × 10⁹ cells/well) or T cells (2 × 10⁷ cells/well) were stimulated, in triplicates, with rCRT/39–272, rEGFP, LPS, Con A or synthetic CRT peptides, or gamma ray-irradiated stimulator cells in 200 µl of R10 medium for 72 h in a 5% CO₂ incubator at 37°C. For the last 8 h of incubation, the cells were pulsed with 0.2 µCi/well of [³H]thymidine (Atom HighTech, Beijing, China) and the cultures were subsequently harvested on an automatic cell harvester (Tomtec, San Diego, CA) and the radioactivity was counted in a beta-counter (EG&G Wallac, Turku, Finland).

**Cytokine and Ig production assays**

Freshly prepared human PBMCs or BALB/c mouse peritoneal macrophages (1 × 10⁹ cells/well) were stimulated with rCRT/39–272 (30 µg/ml), LPS (3 µg/ml), or peptidoglycans (20 µg/ml; Sigma-Aldrich) in R10 medium in 96-well tissue culture plates for 48 h in a 5% CO₂ incubator at 37°C. The concentration of TNF-α in the culture supernatant was determined using ELISA kits (BioLegend) following the manufacturer’s instructions. Standard curves were established using mouse recombinant TNF-α, and the assay detection limit was 7.8 pg/ml.

Freshly prepared mouse B cells were stimulated with rCRT/39–272 in R10 medium in 24-well Costar plates for 6 d. The concentrations of mouse IgM, IgG1, and IgA were determined using a mouse Ig ELISA Quantitation Set (Bethyl Laboratories, Montgomery, TX) according to the manufacturer’s instructions. Standard curves were established using mouse IgM, IgG1, and IgA, and the assay detection limits were 15.6, 3.9, and 15.6 ng/ml, respectively.

**PCR for activation-induced cytidine deaminase, Iµ-Cα1, and Iλ1-Cα**

Expression of the activation-induced cytidine deaminase (AID) gene and the presence of looped-out DNA Iµ–Cα1 and Iλ1–Cα1 following class switching in B cells were detected using the methods described by previous investigators (19–21). Total cellular RNA was extracted from rCRT/39–272-stimulated B cells using TRizol (Invitrogen) and was reverse transcribed using SuperScript II RT (Invitrogen). This was used as a template for amplification of cDNA (349 bp) encoding an AID fragment in PCR by an initial denaturing step of 95°C for 5 min followed by 34 cycles of PCR (94°C for 30 s, 50°C for 30 s, 72°C for 30 s). As an internal control, cDNA (370 bp) encoding a β-actin fragment was amplified by an initial denaturing step of 95°C for 5 min followed by 23 cycles of PCR (94°C for 30 s, 55°C for 30 s, 72°C for 30 s). The Iµ–Cα1 DNA (408 bp) was amplified by an initial denaturing step of 95°C for 5 min followed by 34 cycles of PCR (94°C for 30 s, 45°C for 30 s, 72°C for 30 s).

**ELISAs**

Different ELISA systems were developed in this study for detection of CRT-binding Abs or ligands (CRT-based ELISA), soluble CRT (sandwich ELISA), or CRT-binding Abs or ligands (CRT-based ELISA).
ELISA), and CRT-Abs/CRT complexes (immune complex [IC] ELISA). For CRT-based ELISA, ELISA plates were coated at 4°C overnight with rCRT/39–272 (2 μg/ml) in carbonate buffer (pH 9.6) and subsequently incubated with blocking solution (1% BSA in PBS) for 2 h at 37°C. The wells were washed five times with PBS containing 0.05% Tween 20 (PBS-T) and then 100 μl of diluted human sera, or recombinant fusion proteins containing human IgG1 Fc fragment (R&D Systems) in PBS were added in triplicates and incubated for 2 h at 37°C. After five washes with PBS-T, the plates were incubated with HRP-labeled goat anti-human IgM or IgG Abs (SouthernBiotech) for 1 h at 37°C. The reaction was developed with 100 μl of O-phenylenediamine (OPD) (Sigma-Aldrich) for 5 min and stopped with 100 μl of 2 M H₂SO₄. OD was measured at 492 nm in an ELISA spectrophotometer (Titertek Multiscan Plus Mk II; ICN Flow Laboratories, Irvine, U.K.). For sandwich ELISA for detection of soluble CRT, mAbs against a linear epitope (mAb153.24) and a conformational epitope (mAb153.8) within the sequence of CRT/39–275 were provided by Dr. Boquan Jin (Forth Military Medical University, Xian, China). The former Ab, purified from hybridoma ascites using saturated ammonium sulfate precipitation, was used as capture Ab, while mAb153.8 was labeled with HRP using a peroxidase labeling kit (Dojindo Molecular Technologies, Kumamoto, Japan) and the resultant HRP-conjugated product (HRP-mAb153.8) was employed as detection Ab in the assay. Purified rCRT/39–272 was used as reference for establishing standard curves; detection sensitivity of this ELISA system was 0.6 ng/ml. All serum samples were 1/10 diluted before assay. For IC ELISA, essentially a sandwich ELISA was used with mAb153.24 and HRP-conjugated goat anti-human IgG as capture and detection Abs, respectively, which are capable of detecting ICs between CRT and anti-CRT Ab in human sera. For glycan-based ELISA for screening carbohydrate ligands of CRT, ELISA plates were coated at 4°C overnight with individual glycans, including carrageenan, algicin acids, hyaluronic acids, chondroitin sulfate, heparin, laminarin, α-methylmannoside (α-MM), α-methylglucoside (α-MG), dextran, and LPS (Sigma-Aldrich), at 50 μg/ml in carbonate buffer (pH 9.6) followed by washes and blocking steps. rCRT/39–272 (2 μg/ml in PBS) was then added to the glycan-coated wells and incubated for 2 h at room temperature. HRP-mAb153.8 was added after further washes, which was followed by OPD development and OD₄₉₂ measurement.

Comparison of relative affinities of EGFP-specific Abs

An EGFP-based ELISA (using rEGFP as coating Ag at 2 μg/ml) was also developed for detection of EGFP-specific Abs. The NaSCN method, as described by Pullen et al. (22), was employed for comparing the relative affinities of EGFP-specific IgG Abs in sera from mice before or after a booster immunization with rEGFP or rCRT-EGFP. Briefly, serum samples (diluted in PBS, 100 μl/well) were added, in triplicates, to the wells of ELISA plates precoated with rEGFP. The chaotropic agent NaSCN (50 μl/well at different concentrations in PBS) was then added and incubated for 30 min at 37°C followed by washes with PBS-T. Subsequent steps were performed as described for the ELISAs.

Statistical analysis

All experiments were repeated at least three times, and the results are expressed as mean ± SD. Statistical analysis was performed using the independent samples t test or two-sided, paired t test between groups using the SPSS 14.0 program (SPSS, Chicago, IL). Differences were considered statistically significant at p < 0.05.

FIGURE 1. Biochemical characterization of rCRT/39–272. Structural characteristics of full-length CRT and the recombinant product rCRT/39–272 are illustrated (A). In the N domain of CRT, two cysteine residues at positions 137 and 163 form an intramolecular disulfide bond (-S-S-) while residues Y109, K111, Y128, and D317 are involved in oligosaccharide binding (oblong diamond symbol). rCRT/39–272 covers the N domain (aa 39–197) and partial P domain (aa 198–272) with a His-tag of 34 aa residues at the N terminus. Affinity-purified rCRT/39–272 (lane 1), rEGFP (lane 2), and rCRT-EGFP (lane 3) were run in two identical SDS-PAGE 12% gels, one of which was stained with Coomassie blue (B). Protein molecular mass markers (M) were loaded in the left-hand side lane. Protein bands in the unstained gel were transferred onto polyvinylidene difluoride membrane for Western blotting with rabbit anti-human CRT Abs (1/1000 diluted) and HRP-conjugated goat anti-rabbit IgG for detection (C). The results were visualized by ECL detection system and recorded using Bio-Rad Gel Doc 2000. The rCRT/39–272 protein was further analyzed using nondenaturing native SDS-PAGE 8% gel in the presence or absence of 2-ME (D). Lanes 1 and 3 contain CRT/39–272, and lanes 2 and 4 contain an unrelated recombinant 20-kDa protein, Hrs-1, as control. Protein bands in the gels were visualized by Coomassie blue staining. The ability of rCRT/39–272 to bind carbohydrate structures was analyzed using glycian-based ELISAs (E). Wells in the polyvinyl plates were coated with carbohydrate preparations, including carrageenan, algicin acids, hyaluronic acids, chondroitin sulfate, heparin, laminarin, α-MM, α-MG, dextran, and LPS, and then sequentially treated, in triplicates, with rCRT/39–272 (2 μg/ml), HRP-mAb153.8, followed by development with OPD. Wells not coated with any carbohydrate Ags (None) were included as control. The results are expressed as OD₄₉₂ with SD. *p < 0.05. Results are representative of three independent experiments.
Results

Biochemical properties of rCRT/39–272

The amino acid sequences of human and mouse CRT fragment 39–272 have 96% homology (23). The structural characteristics of rCRT/39–272 and its relative localization in the full-length CRT molecule are illustrated in Fig. 1A. Affinity-purified rCRT/39–272 was >90% pure as judged by Coomassie blue-stained SDS-PAGE gels and was specifically recognized by rabbit anti-human CRT Abs in Western blotting assays (Fig. 1B, 1C). Similar to full-length CRT (24), rCRT/39–272 was highly soluble and formed homodimers and oligomers in solution as evidenced by non-denaturing and non-reducing SDS-PAGE gel electrophoresis (Fig. 1D). Recombinant EGFP and a fusion protein between CRT/39–272 and EGFP (rCRT-EGFP) with histidine tags were also expressed in E. coli and were included in this study.

The lectin-like N domain of CRT is known to be able to bind oligosaccharide Glc3Man5GlcNAc2 (3, 4). In our carbohydrate-based ELISAs, rCRT/39–272 was able to selectively bind carrageenan, algic acid, and hyaluronic acids, but not chondroitin sulfate, heparin, laminarin (β1,3-glucan with β1,6- and β1,4-linked branches), α-MM, α-MG, dextran (α1,6-glucan), and LPS (Fig. 1E).

Immunostimulatory effect of rCRT/39–272 in vitro

As illustrated in Fig. 2A and 2B, rCRT/39–272 was able to induce vigorous proliferation of freshly isolated BALB/c mouse splenic B, but not T, cells. Additionally, freshly prepared mouse peritoneal macrophages and human PBMCs responded to rCRT/39–272 stimulation in vitro by production of cytokines, such as TNF-α (Fig. 2E, 2F). LPS contamination of the rCRT/39–272 preparation was minimal, as polymyxin B (an efficient LPS inhibitor) did not show substantial inhibitory effect on the B cell activation ability of rCRT/39–272 (Fig. 2C). Additionally, rEGFP (expressed and purified using the same system) did not show any B cell stimulatory activity in parallel experiments. The immunostimulating effect of rCRT/39–272 was apparently conformation-dependent, because none of the series of overlapping 15-mer synthetic peptides covering its amino acid sequence was able to activate murine B cells in proliferation assays (Fig. 2D).

Phenotypic and functional maturation in B cells following rCRT/39–272 stimulation was also observed. Treatment with rCRT/39–272 led to upregulation of CD69 and MHC class II, but not CD80, in murine B cells (Fig. 3A–C). BALB/c mouse B cells that had been treated with rCRT/39–272, but not with rEGFP, were substantially more effective in inducing proliferative responses of allogeneic T cells from C57BL/6 mice in vitro (Fig. 3D).

Potent immunogenicity and adjuvanticity of rCRT/39–272

CRT is a widely expressed conserved household protein in mammalian cells and should theoretically be tolerated by the immune system as a typical self-Ag. However rCRT/39–272 was extraordinarily immunogenic in mice, as high-titer CRT-specific IgM and IgG Abs were produced in both BALB/c and nude mice following immunization with rCRT/39–272 in the absence of adjuvant (Fig. 4A, 4E). In contrast, rEGFP was able to elicit relatively low-titer Ab responses in BALB/c but not in nude mice in parallel experiments (Fig. 4B, 4F). The CRT fragment also exhibited potent adjuvanticity, as its linking to EGFP enabled the fusion protein rCRT-EGFP to induce EGFP-specific IgG as well IgM responses in nude mice.

FIGURE 2. Immunostimulatory effect of rCRT/39–272 to B cells and macrophages. Freshly purified BALB/c mouse T (A) and B (B) cells (4 × 10⁶ cells/well) were stimulated with Con A (10 μg/ml), rCRT/39–272 (30 μg/ml), LPS (3 μg/ml), or rEGFP (30 μg/ml) in triplicate wells in 96-well plates for 72 h. Mouse splenic B cells were also stimulated with rCRT/39–272 (30 μg/ml) or LPS (3 μg/ml) in the presence or absence of polymyxin B at 10 μg/ml (C). For assessment of conformation dependence of rCRT/39–272, a series of twenty-five 15-mer synthetic peptides (P1–P25) covering its amino acid sequence were employed (10 μg/ml final concentration) to stimulate BALB/c mouse splenic B cells in a proliferation assay, with rCRT/39–272 (30 μg/ml), LPS (3 μg/ml), and Con A (10 μg/ml) as controls (D). In all proliferation assays, the cells were pulsed with [³H]Tdr during the last 8 h of culture, and incorporated radioactivity was subsequently counted using a beta-counter. The results are expressed as mean [³H]Tdr incorporation (cpm) ± SD. In cytokine production assays, freshly prepared BALB/c mouse peritoneal macrophages and human PBMCs (1 × 10⁶ cells/well) were stimulated with rCRT/39–272 (10 μg/ml) and LPS (3 μg/ml) in 96-well plates for 48 h. The culture supernatant was then quantitatively assayed, in triplicate wells, for the presence of TNF-α using an ELISA kit, and the results are expressed as mean concentration (pg/ml) of TNF-α ± SD (E, F). *p < 0.05; **p < 0.01. TdR, thymidine deoxyribose.
mice (Fig. 4G). rCRT-EGFP was also much more efficient than rEGFP in eliciting humoral responses in BALB/c mice (Fig. 4C). Affinity maturation of EGFP-specific IgG Abs in BALB/c mice, but not in nude mice, after a booster rCRT-EGFP immunization was observed using the NaSCN displacement method (22) (Fig. 4D, 4H).

rCRT/39–272 induces Ig class switching by murine B lymphocytes in vitro

The fact that rCRT/39–272 was able to induce production of specific IgG Abs in nude mice indicates its ability in triggering Ig class switching in B cells in the absence of T cell help. To confirm
this, freshly isolated splenocytes of nude mice (~85% B cells, almost no detectable T cells as judged by flow cytometric analysis) were stimulated for 6 d with rCRT/39–272, or rEGFP as specificity control, in the presence or absence of rmlL-4. The culture supernatant was then assayed for total Abs of IgM, IgA, and IgG classes. As illustrated in Fig. 5A and 5B, rCRT/39–272 alone was sufficient in inducing IgM and IgA production by murine B cells; it was also able to produce the induction of IgG1 by murine B cells in the presence of rIL-4 (Fig. 5C). In support of this, expression of AID in rCRT-stimulated B cells 2 d after rCRT/39–272 stimulation was detected by using semiquantitative RT-PCR (Fig. 5D). The successful rearrangement of Ig gene was further confirmed by the detection of the recombinated DNA I_μ-C_μ1 and also looped-out circular DNA I_λ1-C_λ in these cells.

**CRT functions through the CD14/TLR4 pathway**

In many ways the immunobiological activity of rCRT/39–272 resembles that of LPS, which activates B cells and macrophages mainly through the CD14/TLR4 pathway (25). Interestingly, purified splenic B cells and peritoneal macrophages from C3H/HeJ mice (expressing nonfunctional mutant TLR4) were much less responsive to rCRT/39–272 stimulation compared with C3H/HeN mice (expressing wide-type TLR4) (Fig. 6A, 6B). CD14 is known to be an adaptor molecule for TLR4 for recognition of LPS, while CD40 and BAFFR are involved in transducing Ig class-switching signals in B cells (26, 27). ELISA experiments were carried out to analyze specific interaction between CRT and these molecules. As illustrated in Fig. 6C, rCRT/39–272 was able to bind recombinant soluble protein rmCD14-Fc (a fusion protein between murine CD14 and human IgG1 Fc fragment), but not rmCD40-Fc or rmBAFFR-Fc, in a highly specific manner. In parallel experiments, rmCD14-Fc and rmCD40-Fc were able to specifically bind LPS and CD40 ligands (CD40L), respectively (Fig. 6D, 6E).

**Detection of soluble CRT in patient sera**

Given that CRT can appear on the membrane surface of various types of cells (6–11), it could potentially also be cleaved and released into the extracellular environment and exist in soluble forms in vivo. By using a sandwich ELISA system developed in our laboratory, we were able to detect soluble CRT in the sera from 14 out of 60 patients (23.3%) with RA and 7 out of 32 patients (21.9%) with SLE, but not in any of the 48 serum samples from healthy subjects (Fig. 7A), revealing a strong association between the prevalence of serum CRT and these autoimmune diseases. Interestingly, the average level of serum CRT in RA patients was ~5-fold higher than that of SLE patients (24.31 ± 8.97 versus 4.70 ± 1.89 ng/ml; p < 0.05). It has previously been reported that CRT is associated with increased anti-CRT Ab titers (28, 29). However, by using the rCRT/392–272-based ELISA, we detected CRT-specific IgG Abs in sera from not only the patient groups but also from control subjects, and average levels of such Abs in the three groups were not significantly different (Fig. 7B). The presence of both CRT and CRT-Abs in sera would undoubtedly lead to formation of anti-CRT–Ab/CRT complexes, which are identifiable using our IC ELISA system. As shown in Fig. 7C, levels of such immune complexes in CRT-positive patient sera (n = 21) were significantly higher than those of CRT-negative patient sera (n = 64) (p < 0.001).

**Discussion**

In the present study, we have successfully characterized the immunobiological functions of CRT fragment 39–272, providing important insights into our understanding of the immune-potentiating effects of CRT reported by previous investigators (6–16). This recombinant soluble fragment of CRT is able to not only drive murine B cell activation (vigoruous proliferation, IgM production, and secretion of cytokines) and phenotypical maturation but also triggers Ig class switching by B cells with or without T cell help. It is also capable of inducing proinflammatory cytokine (e.g., TNF-α) production by mouse macrophages and human PBMCs in vitro. DimORIZATION and oligomerization of full-length CRT has previously been reported (24). It is possible that oligomerization of soluble CRT is of crucial importance for its ability to cross-link membrane surface receptors leading to cell activation. Although several membrane

**FIGURE 5.** Ig class switching by murine B cells under stimulation of rCRT/39–272 in vitro. Freshly isolated splenocytes (4 × 10^6 cells/well) from nude mice were treated with rEGFP (30 μg/ml), rCRT/39–272 (30 μg/ml), or LPS (3 μg/ml) in the presence or absence of rmlL-4 (20 ng/ml) for up to 6 d in 24-well Costar plates. Concentrations of total IgM (A), IgA (B), and IgG1 (C) were determined using mouse Ig ELISA quantitation kits, and the results are expressed as mean concentration (ng/ml) ± SD. *p < 0.05. Cells collected on day 2 of the culture were used for extraction of total cellular RNA and DNA for detection of mRNA encoding AID and also looped-out DNAs I_μ1–C_μ and I_λ1–C_λ using semiquantitative PCRs. The resultant PCR products were run in agarose gels in the following loading order: lane 1, medium alone; lane 2, rCRT/39–272; lane 3, rCRT/39–272 plus IL-4; lane 4, rEGFP; lane 5, rEGFP plus IL-4 (D).
surface molecules, including CD59 (30), CD91 (31, 32), and scavenger receptors (33, 34), have been proposed as functional CRT receptors by previous investigators, our data illustrate that CRT exerts its stimulatory effect on B cells and macrophages mainly through the CD14/TLR4 pathway. Note, however, that rCRT/39–272 at a higher concentration (30–100 μg/ml) was also able to moderately upregulate the expression of the activation markers CD86 and CD69 in mutant TLR4-expressing B cells from C3H/HeJ mice (data not shown), indicating the possible involvement of signaling pathways other than CD14/TLR4 in mediating the stimulatory effect of CRT on B cells and macrophages.

The adjuvanticity of rCRT/39–272 in T cell-deficient nude mice is attributable to its ability to effectively activate B cells and trigger their Ig class switching without CD4+ T cell help (Fig. 5B, 5C). The fusion protein rCRT-EGFP was also much more effective than rEGFP in inducing humoral responses against EGFP in BALB/c mice, and the EGFP-specific IgG Abs showed clear affinity maturation after a booster immunization (Fig. 4D). Additionally, chemical conjugation with rCRT/39–272 enabled β-glucan polysaccharides to induce specific IgG Abs in BALB/c mice (C. Hong, unpublished observation). These results suggest that CRT could also enhance Ab production by B cells through T cell-dependent pathways. Indeed, CRT-specific CD4+ T cell lines and clones were relatively easily established, after several rounds of feeding with rCRT/39–272-pulsed syngenic feeder cells, from draining lymph node cells of mice that had been s.c. immunized with rCRT/39–272 (data not shown).

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FIGURE 6. rCRT/39–272 activates murine B cells and macrophages through the CD14/TLR4 pathway. Freshly purified splenic B cells (4 × 10^5 cells/well) from C3H/HeN and C3H/HeJ mice were stimulated, in triplicates, with rCRT/39–272 at various concentrations or with LPS (3 μg/ml) in 96-well plates for 72 h. During the last 8 h of culture, the cells were pulsed with [3H]Tdr, and the results are expressed as [3H]Tdr incorporation (cpm) ± SD (A). Freshly prepared peritoneal macrophages (1 × 10^6 cells/well) from C3H/HeN and C3H/HeJ mice were also stimulated with rCRT/39–272 (0.3–10 μg/ml), LPS (3 μg/ml), or peptidoglycan (20 μg/ml) in 96-well plates for 48 h. Concentration of TNF-α in the culture supernatant was determined using an ELISA kit, and the results are expressed as mean ± SD (B). Modified CRT-based ELISA was performed for identification of CRT-specific ligands (C), in which plate-bound CRT/39–272 was allowed to react with recombinit soluble fusion protein mCD14-hIgG1-Fc (rmCD14), mCD40-hIgG1-Fc (rmCD40), or mBAFFR-hIgG1-Fc (rmBAFFR) in triplicate wells for 1 h, followed by HRP-conjugated goat anti-human IgG with OPD as substrate. The results are expressed as mean OD492 ± SD. *p < 0.05; **p < 0.01. As additional specificity controls, LPS-based (D) and CD40L-based (E) ELISAs were also carried out in similar fashion. Results are representatives of three independent experiments. PGN, peptidoglycan.

FIGURE 7. Detection of soluble CRT, anti-CRT Abs, and CRT/Ab complexes in patient sera. Serum samples (1/10 diluted) from patients with RA (n = 60) or SLE (n = 32) and from healthy human subjects (n = 48) were individually assayed for the presence of soluble CRT using sandwich ELISAs, and the results are expressed as CRT concentrations (ng/ml) (A). Average levels of the serum CRT in patient groups, shown by horizontal lines, were compared with those of healthy subjects. ELISAs based on rCRT/39–272 and rEGFP (for specificity control) were carried out for detection of CRT-specific IgG Abs in the same groups of sera (1/200 diluted) with HRP-conjugated goat anti-human IgG as detection Abs. The results are shown as absorbance readings at OD492 (B). For detection of CRT/Ab complexes, patient sera (1/200 diluted), divided into CRT-positive (n = 21) and CRT-negative (n = 64) groups, were individually assayed using anti-CRT mAb-based ELISAs with HRP-conjugated goat anti-human IgG as detection Abs (D). *p < 0.05; **p < 0.01; ***p < 0.001.
CRT is a glycosylated protein, and prokaryotically expressed CRT (e.g., rCRT/39–272) may functionally differ from that of mammalian cells due to different protein glycosylation mechanisms. To address this question, we constructed a eukaryotic expression vector encoding secreted form CRT/39–272 with a hemagglutinin tag. The resultant recombinant protein, expressed by 293 cells and purified using hemagglutinin-Ab-agarose, exhibited similar stimulatory activities compared with rCRT/39–272 in terms of both stimulating B cell proliferation and inducing TNF-α secretion by macrophages in vitro (data not shown). Another concern with regard to the interpretation of our data was whether the C domain of CRT could modify the biological activity of the N domain. To exclude this possibility, we expressed a “full-length” CRT, rCRT/18–412 (including N, P, and C domains, without the leader and KDEL sequences), in E. coli and compared it with rCRT/39–272 for the ability to activate B cells and macrophages in vitro, with no apparent differences having been observed (data not shown).

CRT and HSPs share many similar immunobiological activities (35, 36), although they have no apparent structural homologies. Both CRT and HSPs are ER chaperones capable of assisting peptide loading and correct folding of MHC molecules (2, 6, 35, 36). HSPs on the membrane surface of various types of cells (e.g., activated lymphoid cells and tumor cells) exert strong immunobiological functions (37–42). Similar to CRT, HSP60, HSP70, HSP90, and gp96 are able to directly activate immune cells in vitro (35, 36). Many HSPs (e.g., HSP60, HSP70, HSP70L1, and HSP96) have been shown to use the TLRs as signaling pathways (43–45), and the stimulatory signals mediated by HSP60 and HSP70 have been shown to be dependent on CD14 (46, 47). CRT shares a common receptor, CD91, with gp96, HSP90, and HSP70 (29). Importantly, however, HSPs have so far not been shown to be able to trigger Ig class switching by B cells either in vivo or in vitro. The finding of soluble CRT in the sera of RA and SLE patients (Fig. 7) is of great interest. Note that soluble CRT was also observed in synovial fluid from 3 out of 11 RA patients (data not shown). Given that rCRT/39–272 exhibits potent immunostimulatory effects in vitro (Fig. 2), it is reasonable to suggest that soluble CRT may play important roles in the pathogenesis of autoimmune disorders, such as RA and SLE. The origin of soluble CRT in patient sera and synovial fluid is unclear although, similar to HSPs, it may be released from necrotic cells (48). Previous investigators have reported elevated levels of CRT-specific autoantibodies in SLE patients (28, 29). In our study, however, similar levels of serum Abs against rCRT/39–272 were found in patients with RA or SLE and also in healthy subjects (Fig. 7B). The lack of the C domain in our recombinant CRT fragment is a possible explanation, but further investigation is needed to fully clarify these discrepancies. Another factor that needs to be considered is the fact that soluble CRT in the sera may interfere with quantitation of CRT-Abs in ELISA experiments.

Taken together, our results indicate that CRT is a potent immunostimulatory molecule for B cells and macrophages via the TLR4/CD14 pathway. It also exhibits strong adjuvanticity, capable of driving Ig class switching in B cells with or without T cell help. The presence of soluble CRT in sera is strongly associated with RA and SLE and may therefore be considered a biomarker for such diseases. The role for CRT in the pathogenesis of autoimmune disorders merits further investigation.

Acknowledgments
We thank Prof. Boqun Jin (Forth Military Medical University, Xi’an, China) for kindly preparing the mAbs against CRT.

Disclosures
The authors have no financial conflicts of interest.

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