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The Rheumatoid Arthritis Shared Epitope Triggers Innate Immune Signaling via Cell Surface Calreticulin

Song Ling, Xiujun Pi, and Joseph Holoshitz

The shared epitope (SE), carried by the vast majority of rheumatoid arthritis patients, is a 5-aa sequence motif in the third allelic hypervariable region of the HLA-DRβ chain. We have recently demonstrated that the SE acts as an allele-specific ligand that triggers NO-mediated pro-oxidative signaling in opposite cells. The identity of the cell surface molecule that interacts with the SE is unknown. Using affinity chromatography purification, cell-binding assays, surface plasmon resonance, and time-resolved fluorescence resonance energy transfer techniques, we have identified cell surface calreticulin (CRT) as the SE-binding molecule. SE-triggered signaling could be blocked by anti-CRT Abs or Abs against CD91 and by CRT-specific antisense or small-interfering RNA oligonucleotides. Embryonic fibroblasts from \textit{crt}^{-/-} or CD91-deficient mice failed to transduce SE-triggered signals. Exogenously added soluble CRT attached to the cell surface and restored SE-triggered signaling responsiveness in \textit{crt}^{-/-} cells. These data indicate that cell surface CRT, a known innate immunity receptor, which has been previously proposed as a culprit in autoimmunity, plays a critical role in SE-triggered signal transduction. The \textit{Journal of Immunology}, 2007, 179: 6359–6367.

Genetic associations with particular class I or class II MHC alleles have been noticed in many autoimmune diseases. Based on the known role of MHC molecules in thymic selection and Ag presentation, it has been postulated that the mechanism underlying these associations involves T cell repertoire selection and/or presentation of self-Ags. Evidence to support this paradigm exists in some diseases, but in other cases, the basis for MHC-disease association remains unclear. For example, over 90% of rheumatoid arthritis (RA) patients carry \textit{HLA-DRB1} alleles, which encode a 5-aa sequence motif, known as the shared epitope (SE), in position 70–74 in the β1 domain of the HLA-DR molecule (1). The mechanistic basis of SE-RA association is presently unknown.

Class II MHC and classical class I MHC molecules are adaptive immune system glycoproteins, which specialize in Ag presentation to T cells carrying the CD4 or CD8 surface markers, respectively. In addition to their ability to present antigenic peptides, class I MHC molecules can trigger innate immunity signals through a pair of ligands located, respectively, in the α1 and α2 domains of the class I molecule (2). The α2 domain of the class I MHC superfamily is of particular interest, because it contains ligands, which interact with a diverse repertoire of inhibitory or activating innate immunity receptors, such as killer Ig-like receptors (KIR), the NKG2/C/CD94 heterodimeric receptor, and the NKG2D receptor (2–4). Moreover, this domain has been found to contain ligands for nonimmune system receptors, such as transferrin receptor (5) or the V2R pheromone receptor (6). Thus, the class I MHC α2 domain appears to have preserved throughout evolution an ability to interact with a wide range of receptors mediating diverse biological functions.

It is noteworthy that x-ray crystallography studies have demonstrated that despite their functional divergence through evolution, the β1 domain of the class II HLA-DR molecule and the α2 domain of the class I HLA molecule have a remarkably similar tridimensional structure (7). It is therefore intriguing that the SE, located in the HLA-DRβ1 domain, is capable of triggering innate signaling in opposite cells (8, 9), reminiscent of innate immune signaling triggered by class I α2 domain ligands (reviewed in Ref. 10).

In previous studies, we have found that the SE acts as a ligand that activates NO-mediated pro-oxidative signaling in opposite cells (8, 9). For example, cells carrying SE-positive \textit{HLA-DRB1} alleles displayed increased constitutive NO production, which resulted in higher levels of reactive oxygen species and higher susceptibility to oxidative DNA damage. The signaling aberration could be transferred to SE-negative cells by cDNA transfection, indicating that SE-associated signaling activity is not due to linkage disequilibrium with another gene. Importantly, recombinant proteins, HLA-DR tetramers, and short synthetic peptides containing the SE motif could all mimic the signaling effect of the native positive DR molecule.

To better understand the mechanisms involved in SE-triggered signaling, in this study, we have undertaken to identify the SE-binding receptor. Using peptide affinity chromatography, N-terminal amino acid sequencing, surface plasmon resonance (SPR), time-resolved fluorescence resonance energy transfer (TR-FRET), and cell-binding assays, we have determined that cell surface calreticulin (CRT) is a SE-binding molecule. The involvement of CRT in SE-triggered signaling was confirmed by demonstrating an inhibitory effect with anti-CRT Abs and with CRT-targeted antisense or small-interfering RNA
Materials and Methods

Reagents and cells
cCRT and its truncated N-terminal domain were purified as previously described (11). Recombinant human heat shock protein (HSP) 60 was donated by Dr. H. Gaston (University of Cambridge, Cambridge, U.K.). All recombinant proteins were used at over 90% purity. Rabbit anti-CRT polyclonal Ab PA3–900 was purchased from Affinity Bio Reagents. The mouse recombinant proteins were used at over 90% purity. Rabbit anti-CRT or anti-HSP60 as above.

Pepitide affinity chromatography

Three-milliliter affinity matrices were packed into Bio-Rad PolyPrep columns. Columns were washed with 16 ml each of the following wash buffers: 0.1 M NaHCO3, 0.5 M NaCl (pH 8.0), then 0.5 M CH3COONa (pH 4.0) and, finally, with PBS (Ca2+ and Mg2+ free) at pH 7.5. M1 cell suspensions were washed with PBS and resuspended in 750 μl of lysis buffer. Cells were passed 12 times through a 26-G needle using a 3-ml syringe. Cell lysates were kept on ice for 20 min and subsequently microcentrifuged at 15,000 rpm for 30 min in 4°C. Supernatants were loaded, 0.7 ml on each column. Columns were incubated overnight in 4°C with gentle rotation. At the end of incubation, columns were washed with 32 ml of pre-elution buffer in 4°C and transferred to room temperature to equilibrate. Bound proteins were eluted successively with 20 mM glycine buffer as follows: 10 ml of glycine at pH 4.0, then, 6 ml of at pH 3.0, and finally 6 ml at pH 2.0. Protein concentration was determined by the Pierce Coomassie Plus assay at wavelength 595. The eluates were concentrated with Centricon 10 filters. Fifteen microliters per lane were loaded onto NuPage 4–12% gradient MOPS gels, using a standard protocol recommended by the manufacturer (Invitrogen Life Technologies). Proteins were transferred to polyvinylidene difluoride membranes, stained with Coomassie and processed for sequencing by the University of Michigan Protein Structure Facility, or directly stained with the Bio-Rad Silver Stain Plus kit, following the manufacturer’s instructions.

Table I  N-terminal amino acid sequencing

<table>
<thead>
<tr>
<th>Band No.</th>
<th>Molecular Mass (kDa)</th>
<th>Sequencea</th>
<th>Homology (Swissprot Accession Number)</th>
<th>Codon No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>18</td>
<td>PEPAKSAAP</td>
<td>Human histone H2B.1 (Q93080)</td>
<td>2–11</td>
</tr>
<tr>
<td>2</td>
<td>22</td>
<td>DEKKKGQKVT</td>
<td>Human cyclophilin B precursor (P23284)</td>
<td>26–35</td>
</tr>
<tr>
<td>3</td>
<td>27</td>
<td>LHTDGDKAFV</td>
<td>Human Pre-mRNA splicing factor SF2, P32 subunit/GC1q-R protein (Q07021)</td>
<td>74–83</td>
</tr>
<tr>
<td>4</td>
<td>29</td>
<td>KGKDPNKFR</td>
<td>Human HMG-2 (P26583)</td>
<td>2–11</td>
</tr>
<tr>
<td>5</td>
<td>31</td>
<td>KGDPKKPRG</td>
<td>Human HMG-1 (P09426)</td>
<td>3–11</td>
</tr>
<tr>
<td>6</td>
<td>38</td>
<td>FVKVXXKNAYFKYb</td>
<td>Human 60S ribosomal protein L5 (P46777)</td>
<td>3–16</td>
</tr>
<tr>
<td>7</td>
<td>39</td>
<td>FVQVQVNP</td>
<td>Human GAPDH (P04406)</td>
<td>2–11</td>
</tr>
<tr>
<td>8</td>
<td>42</td>
<td>PVDQVLPEQF</td>
<td>Human fructose-bisphosphate aldolase A (P040155)</td>
<td>2–11</td>
</tr>
<tr>
<td>9</td>
<td>50</td>
<td>EPAVYFKEQF</td>
<td>Human calcitulin precursor (P27797)</td>
<td>18–27</td>
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<tr>
<td>10</td>
<td>53</td>
<td>DAEEDDIHLV</td>
<td>Human protein disulfide isomerase precursor (P07237)</td>
<td>18–27</td>
</tr>
<tr>
<td>11</td>
<td>58</td>
<td>AKDVKFGADA</td>
<td>Human 60-kDa heat shock protein precursor, HSP60 (P10809)</td>
<td>27–36</td>
</tr>
</tbody>
</table>

a  In single letter form.

b  “X”, Unsuccessful determination of amino acid identity.
Biotinylation of cell surface proteins

M1 cells were incubated with 0.5 mg/ml EZ-Link Sulfo-NHS-LC-Biotin (Pierce) for 10 min at 37°C. The reaction was terminated by adding Tris-HCl (pH 7.5) at a final concentration of 50 mM. Cells were then washed in PBS, centrifuged, and solubilized in PBS containing 2% Nonidet P-40, followed by a brief sonication. Biotinylated proteins were purified on ImmunoPure-immobilized monomeric avidin columns (Pierce) using the protocol provided by the manufacturer. The bound biotinylated proteins were eluted from the column with 5 mM d-biotin in PBS containing 1% Nonidet P-40. The eluted proteins were concentrated on Centricon YM-3 columns (Millipore) before being subjected to peptide affinity chromatography.

Oligonucleotide transfection

CRT sense and antisense phosphorothioate oligonucleotides were synthesized by Operon Technologies: antisense, 5’-GGATAGCAGCATGGCGGCCG-3’; sense, 5’-CGGCCCGCCATGCTGCTATCC-3’.

M1 cells were cultured in 6-well plates to 60–70% confluence and treated with 0.2 µM oligonucleotide, using lipofectin as a carrier.
were cultured in medium for 48–96 h before experiments were performed. For siRNA experiments, a crt-targeted 21-nt (5′-AAGAGAAGATAAGCCCTGAT-3′) was designed using the Qiagen siRNA software. A scrambled 21-nt siRNA was used for control (Qiagen). Briefly, 107 L cells were transfected with an Amaxa nucleofector apparatus. After transfection, cells were cultured for 48–96 h before analyzing gene-silencing effects. Levels of mRNA were measured by quantitative real-time RT-PCR using TaqMan probes. CRT transcription levels were normalized to the housekeeping control gene hydroxymethylbilane synthase (HMBS).

Surface plasmon resonance
A Biacore2000 Biosensor System (Pharmacia/LKB Biotechnology) was used to assay the interaction of soluble synthetic peptides with CRT. A SPR assay is based on a biosensor chip with a dextran-coated gold surface that is coated with a covalently immobilized protein. Binding of an injected ligand (the “analyte”) to the immobilized protein results in changes of the SPR that are directly proportional to the amount of bound ligand. Results are read in real time as resonance units (RU).

Before use, biosensor chips CM5 (Biacore) were preconditioned in water at 100 μl/min by applying two consecutive 20-μl pulses of 50 mM NaOH, followed by 10 mM HCl, and finally 0.1% SDS. CM5 surface was activated by a 7-min injection of 200 mM 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride in 50 mM N-hydroxysuccinimide. Purified CRT or its truncated N domain was immobilized by standard primary amines coupling in 25°C in HBS-EP buffer (10 mM HEPES (pH 7.4), 150 mM NaCl, 1 mM EDTA, and 0.005% surfactant P-20), at a flow rate of 10 μl/min. The protein was injected manually at a rate of 100 μl/min until approaching 5500 RU. The remaining activated groups were blocked by ethanolamine (1 M).

Binding assays were performed at 25°C in a binding buffer (10 mM HEPES (pH 7.4), 50 mM KCl, 0.5 mM CaCl2, 100 μM ZnCl2, and 0.005% surfactant P-20) at a flow rate of 10 μl/min. Peptides were in the analyte at different concentrations, ranging from 0 to 500 μM. Each experiment was repeated at least three times. The binding data were analyzed using the BIAevaluation version 3.0.1 program (Biacore).

TR-FRET
Biotin-labeled peptides were synthesized by BioWorld. All experiments were performed in an assay buffer of 25 mM HEPES (pH 7.7), 20 mM KCl, 0.5 mM CaCl2, and 0.1% BSA. Experiments were conducted in ProxiPlate 384-well microtiter plates (PerkinElmer) in 25-μl volumes. Five microtiter plates of monoclonal anti-GST Ab labeled with europium cryptate in assay buffer was added to a mixture of 10 μl of 100 nM GST-CRT fusion protein and 5 μl of biotin-labeled peptide at different concentrations. After 1 h of incubation, 5 μl of 150 mM Cy5-labeled streptavidin was added and the plates were incubated overnight at 4°C. Energy transfer was measured by exciting at 340 nm and monitoring the emission for 50 μl at 670 nm, using a Fusion εHT Universal Microplate Analyzer (PerkinElmer Life Sciences) configured for time-resolved fluorescence after a 400 μs delay.

Cell-binding assays
L cell transfectants were incubated overnight in a 96-well plate (6 × 10⁴ cells/well). M1 cells were labeled with 5-chloromethylfluorescein diacetate (Molecular Probes/Invitrogen Life Technologies). Labeled cells were incubated with or without anti-CRT Ab at 1/100 dilution for 45 min. M1 cells were added at 2 × 10⁶well to the L cell monolayers and allowed to bind to the L cells during a 15-min culture at 37°C. Wells were then washed three times with PBS to remove nonbinding cells and the remaining cells were fixed with 10% formalin. Plates were then read at 485-nm excitation filter and 535-nm emission wavelengths, using a Fusion εHT Universal Microplate Analyzer (PerkinElmer Life Sciences).

Signal transduction assays
NO concentrations and production rates and pro-oxidative signaling were determined as we previously described (8, 9).

Results
As a first step to identifying candidate SE-binding molecules, M1 cell protein extracts were loaded onto peptide affinity columns prepared with synthetic peptides corresponding to the region 65–79.
encoded by SE-positive and -negative HLA-DRB1 alleles. As can be seen (Fig. 1A), the eluates from affinity columns prepared with SE-positive peptides (65–79*0401 or 65–79*0404) displayed multiple protein bands. In contrast, the eluates from column prepared with a SE-negative peptide (65–79*0402) contained a single band (∼39 kDa), which appeared to bind nonspecifically to all three columns.

The N termini of 11 protein bands were successfully sequenced (Table I). Of these, two are of special interest: CRT and HSP60, as both of them are found on the cell surface (13, 14) and have been previously implicated in the pathogenesis of RA (15, 16). The identities of these two bands as CRT and HSP60 were confirmed with a SE-negative peptide (65–79*0402) containing a single band (∼39 kDa), which appeared to bind nonspecifically to all three columns.

To better characterize the interaction between the SE and CRT and to determine the fine molecular specificity of this interaction, we used a SPR technique. rCRT was immobilized on a biosensor chip and different synthetic peptides were injected in the analyte. As can be seen (Fig. 3A), SE-positive peptides 65–79*0401 and 65–79*0404 bound to CRT in biologically significant affinities (∼108 M and ∼106 M, respectively), while the SE-negative peptide 65–79*0402 bound to CRT in significantly lower affinities (∼1010 M).

The specificity of SE/CRT binding was further corroborated by a TR-FRET-binding assay. Binding of 15-mer peptides to CRT was determined by labeling biotinylated peptides with Cy5-streptavidin as the acceptor unit. On the donor side, we used a CRT-GST fusion protein and a europium cryptate-labeled anti-GST Ab (Fig. 3B, upper panel). TR-FRET assays showed that CRT binding of the SE-positive peptide 65–79*0404 was much more robust than the SE-negative peptide 65–79*0402 (p < 0.0001, Fig. 3B, lower panel). Using a panel of 5-mer peptides M1 cells, which constitutively express CRT on their surface, and L cell transfectants expressing SE-positive or SE-negative surface HLA-DR molecules (Fig. 2). As can be seen, L565.5 transfectants (expressing on their surface the SE-positive DR molecule encoded by DRA1*0101/DRB1*0401) had a more robust interaction with M1 cells, compared with transfectants L514.3 (DRA1*0101/DRB1*0402) or L259.3 (DRA1*0101/DRB1*0403), which express SE-negative DR molecules on their surfaces. Anti-CRT Abs inhibited the interaction, confirming the identity of the SE-binding molecule as cell surface CRT (Fig. 2). The incomplete inhibitory effect suggests that the Abs produced only partial steric hindrance, although the possibility that cell surface molecules besides CRT may contribute to the binding deserves further consideration.

To determine whether the native DR molecule interacts with native surface CRT, cell-binding assays were performed between M1 cells, which constitutively express CRT on their surface, and L cell transfectants expressing SE-positive or SE-negative surface HLA-DR molecules (Fig. 2). As can be seen, L565.5 transfectants (expressing on their surface the SE-positive DR molecule encoded by DRA1*0101/DRB1*0401) had a more robust interaction with M1 cells, compared with transfectants L514.3 (DRA1*0101/DRB1*0402) or L259.3 (DRA1*0101/DRB1*0403), which express SE-negative DR molecules on their surfaces. Anti-CRT Abs inhibited the interaction, confirming the identity of the SE-binding molecule as cell surface CRT (Fig. 2). The incomplete inhibitory effect suggests that the Abs produced only partial steric hindrance, although the possibility that cell surface molecules besides CRT may contribute to the binding deserves further consideration.

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containing single amino acid substitutions, we have previously identified a consensus amino acid motif of Q/R-K/R-x-x-A that is necessary and sufficient for triggering SE-dependent signaling (9). Consistent with those findings, we demonstrate here that 5-mer peptides expressing the Q/R-K/R-x-x-A motif bound specifically to CRT in SPR assays (Fig. 3). Thus, the same motif is required both for interaction and signal transduction.

To determine whether CRT plays a role in SE-triggered signal transduction, we first examined the effect of blocking Abs. Both anti-CRT Abs and Abs against the CRT surface-binding molecule CD91 (17) completely blocked SE-triggered signaling (Fig. 4A). Additionally, CRT antisense oligonucleotides suppressed CRT surface expression (Fig. 4B), inhibited SE-triggered NO production (Fig. 4C), and blocked the pro-oxidative effect of the SE ligand (Fig. 4A). Likewise, CRT-targeted siRNA oligonucleotides effectively silenced CRT mRNA expression (Fig. 5A) and inhibited SE-triggered signaling (Fig. 5B). Additionally, SE-positive peptides triggered NO signaling in a wild-type MEF, but no response was seen in MEF from a crt−/− (Fig. 6A) or a CD91-deficient mouse (Fig. 6B).

A recent study has demonstrated that exogenous rCRT added to culture medium attached to the surface of CRT-deficient cells and restored CRT-dependent immunogenic signaling events (18). To examine whether soluble CRT could restore SE signaling in CRT-deficient cells, we investigated SE-triggered signaling events in crt−/− MEF, which had been preincubated with the recombinant protein. Adding soluble CRT restored surface expression of CRT to a level comparable to that observed in the wild-type MEF (Fig. 7A). In contrast, CD91-deficient MEF (PEA-13) failed to bind soluble CRT on their surface, consistent with CD91 proposed role as CRT receptor (17). Importantly, soluble CRT restored the responsiveness of crt−/− MEF to SE-triggered signaling (Fig. 7, B–D), while it had no such effect on CD91-negative cells (data not shown). Thus, taken collectively, our data indicate that CRT is the cell surface protein that interacts with the SE and transduces its signaling.

**Discussion**

In this study, we demonstrate that cell surface CRT plays a critical role in SE-triggered signaling. The results reported here support four main conclusions: 1) SE-CRT interaction depends on a specific SE motif; 2) CRT is essential for SE-triggered signaling; 3) CD91 is necessary for CRT-mediated SE-triggered signal transduction; 4) soluble CRT can restore SE signaling in CRT-deficient cells.

We have previously identified a consensus Q/R-K/R-x-x-A motif, which is required for SE-triggered signaling (9). In the present study, we provide evidence that the same motif is required for
SE-CRT interaction. SE-positive, but not SE-negative, peptides, interacted with rCRT in SPR assays in biologically significant affinities. The calculated binding affinities between CRT and SE-expressing 15-mer peptides ($K_D = \sim 11 \times 10^{-6}$ M) were at the same range as binding affinities previously documented for many other immune system receptors, such as HLA class I molecules/NK receptors, class I MHC/TCR, CD2/CD58, as well as CD80/CD28 (reviewed in Ref. 19).

TR-FRET-based binding studies with 15-mer peptides that differ by only 3–aa residues corroborated SE-CRT-binding specificity (Fig. 3B). Furthermore, experiments with soluble CRT demonstrated that even a single amino acid difference (e.g., DRβ residue 74 alanine vs glutamic acid in alleles *0404 and *0403, respectively) was sufficient to prevent CRT-dependent SE-triggered signaling (Fig. 7D). Similarly, experiments with 5-mer peptides (Fig. 3C) demonstrated that the SE motif is necessary for the interaction in a cell-free system, in agreement with our previous signal transduction finding in intact cells (9). It is noteworthy that population-based immunogenetic analyses have shown that the Q/R-K/R-x-x-x-x-A sequence in residues 70–74 of the DRβ-chain correlates best with RA (20). Thus, the data shown here are consistent both with our previous findings and epidemiological analyses.

SPR-binding studies between 5-mer peptides and CRT (Fig. 3C) showed that peptide RRRAA produced a substantially higher RU reading compared with peptide QKRAA. The sequence RRRAA is the SE motif encoded by *DRB1*1001, a relatively rare allele that according to some studies is associated with more severe RA (21). It is therefore tempting to speculate that the higher RU values observed with peptide RRRAA is biologically meaningful. It should be cautioned, however, that RU values do not correlate well with affinities, as exemplified in Fig. 3A. Although substantial RU differences were found between SE-positive 15-mer peptides 65–79*0401 and 65–79*0404, the $K_D$ values of these two peptides were very similar.

In addition to characterizing its role as a specific SE-binding molecule, we demonstrate here that CRT is essential for SE-triggered signaling. Anti-CRT Abs, CRT gene disruption or mRNA silencing, were all found to interfere with SE-triggered signaling, indicating that CRT plays a critical role in SE-induced signaling activation. Because CRT lacks a transmembrane domain, it has been previously proposed that CD91 is serving as a CRT receptor (17). Consistent with a recent report (18), we demonstrated here that adding soluble CRT to CRT+/− MEF restored signaling. Furthermore, we also show that SE-triggered signaling depends on the expression of both CD91 (Figs. 4 and 6) and CRT (Figs. 4–6) and soluble CRT does not attach to the surface CD91-deficient cells. Thus, taken collectively, our findings suggest that CD91 may be functioning as a coreceptor for the SE by serving as a surface attachment molecule for CRT. This idea requires further experimental exploration.

CRT is a ubiquitous multifunctional calcium-binding protein. Although originally characterized as an ER molecular chaperone, more recently it has been shown to be present on the surface of many cell types and has been implicated in signal transduction events associated with innate immunity, cell adhesion, angiogenesis, and apoptosis (reviewed in Ref. 22). Cell surface CRT plays a pivotal role in the removal of apoptotic cells and, depending on the cosignaling context, ligation of CRT can lead to activation of either pro- or anti-inflammatory pathways (23). Impairments, which upset this multifactorial balance, could conceivably result in autoimmunity (24). Indeed, CRT has long been considered potential culprit in autoimmune diseases, including RA (13). Two previous reports proposed a direct role for CRT in RA pathogenesis (25, 26). Verreck et al. (25) immunoprecipitated HLA-DR/peptide complexes from DR4/DR5*0401/DRw53-positive EBV-transformed B cells and eluted from those complexes a 15-mer peptide corresponding to amino acids 278–292 of human CRT. Max et al. (26) eluted from similar immunoprecipitates a 16-mer peptide corresponding to the same region of the protein. Both groups hypothesized that the origin of those peptides was from the HLA-DR peptide-binding groove. However, in light of the data presented here, it is equally plausible that rather than representing bona fide antigenic groove peptides, those peptides could have been fragments of SE-bound CRT.

Consistent with our previous studies showing that the SE acts as a pro-inflammatory signal-triggering ligand (9), CRT has long been known to play important roles in cellular stress responses in the innate immune system (27–33). Binding of C1q to neuronal cell surface CRT triggers increased levels of cellular reactive oxygen species (29). In neutrophils, CRT engagement by C1q leads to production of superoxide (30). Similarly, in monocytes (31) and neutrophils (32), cell surface CRT binds the antibacterial peptide L5 and transduces superoxide-dependent bactericidal signaling. In addition, in monocytes, cell surface CRT binds the neutrophil secretory protein azurocidin, which results in increased production of IL-6 (33). Thus, cell surface CRT is an important innate immune system receptor.

The results presented here suggest that binding the SE ligand, CRT may function as a bridge, which allows an adaptive immune system molecule to aberrantly activate an innate system signal transduction pathway. We believe these data could help explaining some of the enigmas surrounding RA-SE associations (reviewed in 34). For example, an adaptive immune mechanism is difficult to reconcile with the fact that although presentation of antigenic peptides to CD4+ T cells is the best known function of class II MHC molecules, there is no conclusive evidence to indicate that Ag presentation is the mechanism underlying SE-RA association.

Additionally, SE disease association is not exclusive for RA, as several other human diseases have been shown to be significantly more common in individuals carrying SE-encoding DRB1 alleles (35–39). SE-positive DRB1 alleles can also aggravate collagen-induced arthritis (40, 41) and experimental autoimmune encephalomyelitis (42), and trigger spontaneous diabetes (43) in transgenic mice. Endogenous SE-encoding class II MHC genes are associated with spontaneous arthritis in dogs (44) and a multitude of induced or spontaneous autoimmune diseases in SJL mice (45–47). These pathogenically distinct models involve immune responses to many unrelated target Ags.Attributing the effect of the SE in all these conditions to Ag presentation is inconsistent with the structural stringency of the SE motif on the one hand, and the strict specificity in which antigenic epitope are being recognized, on the other.

Furthermore, several studies have shown that advancing age is a strong risk factor both in RA (48) and in several spontaneous SE-associated autoimmune diseases in mice (43, 46). Such aging-associated susceptibility is unlikely to be due to improved presentation of specific Ags, because adaptive immune responses are expected to decline with age. Finally, a strictly adaptive immunity based mechanism is inconsistent with the positive correlations observed between the number of copies of SE-encoding alleles and RA severity (49) and penetrance (50).

The mechanism proposed here provides plausible explanations to these inconsistencies: it could better explain why SE associations lack Ag, species, or disease specificity. A signal transduction-based paradigm is also more consistent with the dose-dependent nature of SE associations. Similarly, it is conceivable that due to its pro-inflammatory effect, the SE accelerates an aging-associated epigenetic drift or increases the frequency of stochastic events, thereby...
continuously increasing the risk of triggering disease-initiating aberrations (reviewed in Ref. 34).

It should be clarified, however, that the mechanism proposed above does not rule out the involvement of groove peptides in RA. It is possible that while class II MHC-restricted Ag presentation determines the specificity of the immune response, SE-triggered innate signaling determines its intensity, or its proinflammatory polarization. It is also possible that certain groove peptides may have a stabilizing effect on SE-expressing class II molecules, thereby allowing a more efficient interaction with CRT.

Activation of innate immune receptors by adaptive immune system ligands is not without precedence. Ligands encoded by class I MHC superfamily genes interact with a diverse repertoire of activating or inhibitory innate immune system receptors. For example, classical class I MHC molecules have been shown to bind to KIR and modulate NK cytolytic activity (2). Similarly, the human non-classical class I MHC molecule HLA-E interacts with NKG2D receptors and activates antitumor cytolytic responses (3). Similar to the SE, class I MHC ligand-triggered activation of innate immune signaling has been proposed to play a role in autoimmunity (51). Different from class I ligand-mediated effect, however, SE signaling is allele restricted.

Our data indicate that, similar to the engagement of NK receptors by class I MHC ligands, the interaction between the SE and CRT can take place in trans (i.e., between two opposite cells). Whether in-cis interactions (5, 6) are also possible is presently unknown. It is also unclear whether other cell surface receptors can affect the intensity of SE-CRT interaction or its functional consequences. As mentioned above, depending on the cosignaling context, CRT ligation can lead to activation of either pro- or anti-inflammatory pathways. We propose that investigating those signaling modulating pathways in RA could provide important insights into the role of the SE-CRT axis in the pathogenesis of this disease.

Acknowledgments

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Disclosures

J. Holoshitz and S. Ling are coinventors of a related technology owned by the Regents of the University of Michigan.

References


5. Ling, S., Z. Li, O. Borschukova, L. Xiao, and P. Holoshitz. 2007. The rheumatoid arthritis shared epitope increases cellular susceptibility to oxidi-


42. Ito, K., H. J. Bian, M. Molina, J. Han, J. Magram, E. Saar, C. Belunis, D. R. Bolin, R. Arceo, R. Campbell, et al. 1996. HLA-DR4-IE chimeric class II transgenic, murine class II-deficient mice are susceptible to experimental allergic encephalomyelitis. J. Exp. Med. 183: 2655–2644.


