Calreticulin (CRT) plays a role in the clearance of dying cells and has been implicated in autoimmunity. Recent evidence indicates that cell surface CRT (csCRT) acts as a signal transducing receptor for the rheumatoid arthritis (RA) shared epitope (SE). The SE binding site on CRT has been mapped to amino acid residues 217–223 in the P-domain. Upon interaction with dendritic cells (DCs), the SE activates potent immune regulatory events. In CD8α+ DCs, which express higher abundance of csCRT, the SE inhibits the tolerogenic enzyme indoleamine 2,3 dioxygenase with resultant inhibition of regulatory T (Treg) cell differentiation. In CD8α− DCs, the SE ligand increases secretion of IL-6 and IL-23 and facilitates generation of Th17 cells, a T cell subset known to play a role in autoimmunity. On the basis of these recent findings, we discuss the possibility that the csCRT may play a pathogenic role in RA by transducing SE-activated Th17-polarizing signals.

Keywords: calreticulin; rheumatoid arthritis; shared epitope; nitric oxide; Th17; Treg
Little is known about the role of CRT in rheumatoid arthritis (RA). Although the protein has long been known to be targeted by autoantibodies in RA, the pathogenic significance of that immune recognition is unclear. Moreover, the possibility that the innate receptor function of CRT could play a role has not been addressed. Below we discuss recent evidence strongly implicating csCRT as an innate immune system receptor that may play an important role in the pathogenesis of RA.

The rheumatoid arthritis shared epitope activates innate signaling through cell surface calreticulin

Susceptibility to RA and the severity of the disease are both closely associated with HLA-DRB1 alleles encoding a five amino acid sequence motif—commonly referred to as the “shared epitope” (SE)—in residues 70–74 of the DRβ chain. SE-encoding DRB1 alleles confer higher risk of developing RA in most ethnic groups. Interestingly, despite the strong influence of genetic factors on RA susceptibility, the concordance rate in monozygotic twins is only 12–15%, suggesting that stochastic environmental or epigenetic factors are required to precipitate disease onset in genetically susceptible individuals. Consistent with this idea, epidemiological studies in Europe and North America have demonstrated interaction between smoking and the SE: the presence of both factors resulted in a much higher RA risk than the risk conferred by either smoking or SE alone.

In addition to being a risk factor for RA, the SE has also been shown to associate with disease severity. The disease in SE-positive individuals is more erosive than in SE-negative individuals. In addition, the number of SE-encoding alleles has been found to directly correlate with disease severity.

The mechanism by which the SE increases susceptibility to—and severity of—RA is unknown. The known role of MHC class II molecules in antigen presentation prompted the prevailing paradigms, which postulate that either presentation of arthritogenic self-peptides, molecular mimicry with foreign antigens, or T cell repertoire selection are involved. Notwithstanding their plausibility, these hypotheses are difficult to reconcile with the fact that data-supporting antigen-specific responses as the primary event in RA are inconclusive. In addition, several other human diseases have also been shown to be associated with SE-encoding DRB1 alleles, including polymyalgia rheumatica, giant cell arteritis, type I diabetes, erosive bone changes in psoriatic arthritis, autoimmune hepatitis, and early-onset chronic lymphoid leukemia, among other conditions. The SE has been also shown to be associated with spontaneous RA-like disease in dogs and to facilitate collagen-induced arthritis, spontaneous diabetes, and experimental autoimmune encephalomyelitis in HLA-DRB1*0401 transgenic mice. Furthermore, the increasing incidence of RA with age and the positive correlations observed between the number of copies of SE alleles and RA severity and disease penetrance are all inconsistent with an antigen presentation-based mechanism. Thus, taken together, these inconsistencies suggest that the SE may have as yet unknown antigen nonspecific effects independent of the well documented role of class II MHC molecules in antigen presentation. Our recent data reveal that the SE may have a unique ability to activate innate signaling events, which could lead to immune dysregulation.

Given the inadequacies of antigen presentation-based theories discussed earlier, over the past few years we have examined whether the SE, similar to its spatially homologous class I MHC ligands, can trigger innate immune signaling. In all cases, our prior studies have demonstrated that the SE could activate nitric oxide (NO) signaling in a strictly allele-specific manner. The SE was able to do this, whether expressed in its native conformation on the cell surface, as a cell-free HLA-DR tetrameric molecule, engineered into large recombinant proteins, or as a short synthetic peptide. A consensuss motif comprising of the 70Q/R-K/R-x-x-A74 sequence was found to be necessary for triggering the signal.

Mapping of the shared epitope-binding site on calreticulin

We have previously identified csCRT as the cell surface receptor that transduces the SE signaling. In brief, affinity chromatography purification, cell-binding assays, surface plasmon resonance (SPR), and time-resolved fluorescence resonance energy transfer techniques identified csCRT as the SE-binding molecule. SE-triggered signaling could be blocked (by anti-CRT antibodies or antibodies against CD91) and by CRT-specific
anti-sense or small interfering RNA oligonucleotides. Murine embryonic fibroblasts from \( \text{Crt}^{-/-} \) or \( \text{cd91} \)-deficient mice failed to transduce SE-activated signals. Exogenously added soluble CRT attached to the cell surface and restored signaling responsiveness in \( \text{Crt}^{-/-} \) cells.48

More recently, we have mapped the SE-binding site on CRT.49 CRT has three domains: an N-terminal domain, a C-terminal domain, and a middle domain called “P-domain.” To identify the domain that binds the SE, we first determined SE-CRT binding by SPR, using domain-selective deletion mutants. These experiments strongly suggested that the SE binding site is located in the P-domain. The role of this domain as a SE-binding region was further confirmed by a photoactive cross-linking technique.49 To predict the interacting residues, the BioMedCAChe 6.1 (Fujitsu, Sunnyvale, CA) in silico docking software was used. The ligand and the receptor were modeled on the basis of the published crystal structure of the SE within the native HLA-DR molecule and published P-domain NMR-based structural data, respectively. Four potential docking scenarios with significant energy were identified. Intriguingly, region 217–224 of the CRT P-domain was invariably found to be the SE-binding site in all four docking models, with the most significant roles played by amino acid residues E217, D220, and E223 of the CRT P-domain.

To address the respective role of each one of these candidate SE-binding P-domain residues, point-mutants expressing single amino acid substitutions have been used. Cell-free SPR binding assays where the point-mutated CRT proteins were immobilized on a biosensor chip and the SE peptides were in the analyte, have shown a significantly diminished interaction between the SE and CRT receptors that express E217A or E223A substitutions. Experiments to determine the signal transduction efficiency of these mutant receptors in a cell culture system demonstrated that SE-expressing ligands activated significantly diminished signals when the E217A, D220A, or E223A CRT mutants were used as cell surface receptors, with the most significant role played by CRT P-domain residue E217.49 Taken collectively, these data indicates that the SE-binding site on CRT is located in the 217–223 region in the P-domain, with a critical role played by amino acid residue E217. A proposed receptor–ligand interaction model is shown in Figure 1.

**Figure 1.** CRT–SE interaction. A SE-positive HLA-DR4 molecule interacts with the SE-binding site in the CRT P-domain. The HLA-DRα chain is shown in green, the HLA-DRβ chain in yellow, the SE is in cyan, the CRT P-domain is in pink, and the groove peptide is shown in orange.

**Immune polarization by the shared epitope**

As mentioned earlier, one of the hallmarks of SE-induced csCRT-mediated signaling is the production of NO, a ubiquitous signaling molecule with versatile effects in the immune system. In RA, increased NO levels correlate significantly with inflammatory markers of the disease and anti-rheumatic agents have been shown to suppress NO production.50–52 NO has also been implicated in the pathogenesis of experimental autoimmune models in mice. For example, SJL mice are known for their NO overproduction.53 These mice are susceptible to many autoimmune diseases,54 and their autoimmune tendencies are attributed to their NO overproduction.55 Similar to human RA, SJL mice display aging-associated increase in disease incidence,56 excessive DNA damage,57 higher mutation rates,58 and a higher incidence of spontaneous lymphoma.59 Thus, there is ample evidence that excessive NO levels may facilitate autoimmunity.

Dendritic cells are professional antigen-presenting cells strategically positioned in the interface between the innate and adaptive immune systems. Relevant to this review, csCRT is expressed on both human and murine DCs.12 In human DCs, a maturation-dependent expression was found, with more abundant csCRT expression in immature DCs compared to mature cells.60 In addition to their role in potentiating adaptive immune responses, DCs also induce tolerance through a variety of mechanisms, including a direct cross talk
with regulatory T (Treg) cells. A growing body of evidence indicates that the tolerogenic effect of DCs is mediated partly by indoleamine 2,3 dioxygenase (IDO), an enzyme that catalyzes the catabolism of tryptophan.

DCs play a key role in immune regulation and are implicated in the pathogenesis of RA. Therefore, we carried out studies to examine whether the SE can activate innate signaling in murine DCs. Our data clearly demonstrated that, similar to its effect in all other cell lines previously studied, the SE activates NO signaling in murine DCs. Given the fact that NO has been previously found to inhibit IDO activity, we have examined whether SE-expressing ligands could affect IDO activity. Our data showed that cell-free SE-positive ligands effectively and specifically blocked conversion of tryptophan to kynurenine in DCs and fibroblasts. Moreover, murine L cells expressing SE-positive DR molecules on their surface through cDNA transfection produced significantly less kynurenine in response to IFNγ, compared to transfectants expressing SE-negative DRβ chains. Thus, the SE ligand effectively inhibits the activity of the tolerogenic enzyme IDO in both human and murine cells. We have confirmed published observations that IDO activity is found in DCs expressing the CD8α surface marker but not in CD8α− DCs and found that the SE effect on IDO activity was restricted to that DCs subset. While the mechanism of the dichotomy of SE activity in the two subsets is presently unknown, it is worth noting that the CD8α+ subset showed higher abundance of the SE signal transducing receptor, csCRT.

In addition to IDO-mediated regulation, DCs can regulate immune responses by production of various cytokines that can activate or expand particular subsets of T cells, thereby polarizing the immune response. To determine whether SE-mediated signaling in DCs could induce cytokine production, we have studied supernatants of SE-stimulated DCs. Our data showed that in the CD8α− DCs, but not in the CD8α+ subset, the SE ligand activated a robust production of IL-6. Although IL-23 levels did not increase following stimulation with the SE alone, in the presence of suboptimal concentrations of LPS, the SE had a prolonged synergistic effect on the production of this cytokine. Other cytokines did not show any increased production, indicating the specificity of SE effect. Consistent with the reciprocal effects of IDO and IL-6 on Treg differentiation, we have demonstrated that the SE ligand potently inhibited Treg differentiation (Fig. 2). When DCs were pre-incubated with the SE-positive peptide 65−79*0401 Treg differentiation was significantly inhibited, while SE-negative control peptide 65−79*0402 had no effect. Similar results were obtained using SE-positive versus SE-negative HLA-DR tetramers. Thus, the SE ligand has a potent inhibitory effect on Treg.

As mentioned earlier, in CD8α− DCs, the SE-activated robust production of two Th17-activating cytokines: IL-6 and IL-23. Th17 cells produce several effector cytokines, which play important roles in inflammation and autoimmunity, including, IL-17A, IL-17F, IL-21, and IL-22. Over the past few years, the Th17 subset has emerged as a key player both in host defense and autoimmunity. There is compelling evidence that Th17 cells may play a direct role in the pathogenesis of RA. For example, in RA, both IL-17-producing cells and IL-17 are abundantly expressed in the RA joint compartment. In addition, IL-17 has been shown to induce many factors known to enhance autoimmune or pro-arthritogenic processes, such as angiogenesis, MMPs production, osteoclastogenesis, leucocyte recruitment, angiogenesis and inflammation, among other effects. Neutralization of IL-17 prevents disease development in experimental mouse models of autoimmunity.

Consistent with its effect on DCs, the SE was found to have a robust effect on Th17 differentiation and expansion (Fig. 2), as well as activation of proliferative responses and secretion of IL-17. Importantly, the SE effect on Th17 polarization could be seen both in vitro and in vivo. Taken together, these findings indicate that the SE ligand, particularly when presented in its native conformation, has a potent Th17-polarizing effect.

A proposed model

Figure 3 shows a proposed model of CRT-mediated SE effect in RA. In physiologic condition, DCs maintain a fine balance between immune stimulation and tolerance. They are programmed to prevent autoimmune reactions by responding to tolerance-enhancing signals delivered either by Th1-derived IFNγ or by CTLA4, expressed on Treg. These signals activate IDO that increases tryptophan metabolites,
Figure 2. T cell polarization by the SE. (A) DCs were incubated overnight without (medium) or with SE-positive (65-79*0401) or SE-negative (65-79*0402) 15mer peptides. Subsequently, naive CD4 T cells were added and cocultured in the presence of TGFβ and anti-CD3 antibodies. (B) Mean ± SEM values of triplicate flow cytometry determinations of the experiment shown in A. (C) Th17 polarization by the SE. DCs were cocultured with naive CD4 T cells in Th17-differentiating medium in the absence or presence of SE-positive (65-79*0401) or SE-negative (65-79*0402) peptides. (D) DCs were incubated overnight with peptides or recombinant hepatitis B core (HBc) particles containing the SE motif QKRAA (SE+) or a non-SE sequence DERAA (SE−) and then cocultured with naive CD4 T cells as above. IL-17A concentrations in supernatants were determined by ELISA.

which preserve Treg-mediated immune tolerance. The default setting of this intricate system assures a well-balanced immune response and the prevention of autoimmune reactions.

In individuals carrying SE-encoding HLA-DRBI alleles, the SE ligand, normally expressed on APC or on lymphocytes, interacts with csCRT. Our model states that in healthy SE-positive individuals SE-csCRT interaction activates low-grade signaling with moderate Th17 polarization, which could be advantageous against pathogens. However, upon exposure to critical levels of environmental pollutants (e.g., cigarette smoke), this beneficial polarization effect could become excessive owing to as yet unknown stochastic events. Such aberrant interaction may lead to excessive Th17 polarization that allows the development of RA. It should be pointed out that excessive Th17 polarization has been previously shown in mice following exposure to environmental aromatic hydrocarbons and the SE has been shown to synergize with cigarette smoking as a risk factor for RA. It is therefore tempting to entertain a scenario in which the SE and environmental pollutants may cooperatively push Th17 polarization beyond a threshold that allows RA development.
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Conflict of interest

The authors declare no conflict of interest.

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