Identification of the Rheumatoid Arthritis Shared Epitope Binding Site on Calreticulin

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Abstract

Background: The rheumatoid arthritis (RA) shared epitope (SE), a major risk factor for severe disease, is a five amino acid motif in the third allelic hypervariable region of the HLA-DRβ chain. The molecular mechanisms by which the SE affects susceptibility to – and severity of - RA are unknown. We have recently demonstrated that the SE acts as a ligand that interacts with cell surface calreticulin (CRT) and activates innate immune signaling. In order to better understand the molecular basis of SE-RA association, here we have undertaken to map the SE binding site on CRT.

Principal Findings: Surface plasmon resonance (SPR) experiments with domain deletion mutants suggested that the SE binding site is located in the P-domain of CRT. The role of this domain as a SE-binding region was further confirmed by a sulfo-SBED photoactive cross-linking method. In silico analysis of docking interactions between a conformationally intact SE ligand and the CRT P-domain predicted the region within amino acid residues 217–224 as a potential SE binding site. Site-directed mutagenesis demonstrated involvement of residues Glu217 and Glu223 - and to a lesser extent residue Asp220 - in cell-free SPR-based binding and signal transduction assays.

Significance: We have characterized here the molecular basis of a novel ligand-receptor interaction between the SE and CRT. The interaction represents a structurally and functionally well-defined example of cross talk between the adaptive and innate immune systems that could advance our understanding of the pathogenesis of autoimmunity.

Introduction

The “shared epitope” (SE) is a five amino acid sequence motif in positions 70–74 of HLA-DRβ chains encoded by HLA-DRB1 alleles that are strongly associated with susceptibility to severe rheumatoid arthritis (RA). The mechanism underlying SE-RA association is unclear. Based on the known role of MHC class II molecules in presentation of antigenic peptides to helper T cells, it has been hypothesized over the past two decades that RA-SE association is due to presentation of arthritogenic self or foreign peptides [1,2]. However, this theory is difficult to reconcile with lack of conclusive evidence to support antigen-specific responses as the primary event in RA, the promiscuous association of the SE with other human diseases and various autoimmunity models in different species, plus the unexplained SE gene-dose effect on disease severity and penetrance [reviewed in [3]].

Based on our recent data [4,5,6], we have proposed an alternative hypothesis, postulating that the SE, analogous to certain domains of class I MHC-molecules [7,8], acts as an innate immune system ligand. We have demonstrated that the SE acts as a signaling ligand in its native conformation within cell surface-expressed HLA-DR molecules, as well as a cell-free HLA-DR tetrameric molecule. The activity could also be observed when the ligand was genetically engineered into non-HLA recombinant proteins, or as a short synthetic peptide. In all these configurations, the SE activated robust production of nitric oxide (NO) and reactive oxygen species (ROS) in other cells [4,5,6].

In previous studies [6] we have shown that SE-activated signaling depends on cell surface calreticulin (CRT). The affinity of SE-CRT interaction was calculated to be at a low-μM range, similar to many other receptor-ligand interactions in the immune system. CRT is critical for SE-triggered signaling, as anti-CRT antibodies and small interfering RNA oligonucleotides blocked SE-activated robust production of nitric oxide (NO) and reactive oxygen species (ROS) in other cells [4,5,6].

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has long been known to function as an innate immune system receptor. For example, CRT has been implicated as a receptor for C1q, mannose binding lectin and members of the collectin family [10,11]. Due to its critical importance for elimination of apoptotic cells [12], CRT is believed to play a pivotal role in the junction between tolerance and autoimmunity [13]. Aberrant activation of the CRT-mediated pathway can lead to autoimmunity, as exemplified by conditions that involve defective CRT-mediated clearance of apoptotic cells [14]. CRT has been previously hypothesized to play a role in autoimmune human diseases, including RA [15]. Thus, our findings that CRT serves as the signal transduction receptor for SE, a major factor in RA disease susceptibility and severity, could provide important new insights into the role of CRT in autoimmunity. To gain better understanding of SE-CRT interaction, here we have undertaken to map the SE binding site on CRT.

**Results**

**Identification of the SE-binding CRT domain**

The primary sequence of CRT suggests that similar to calnexin it has three domains [16]. Because the tri-dimensional structure of CRT has not been fully resolved, it is a common practice in the literature to model it based on the known crystal structure of calnexin [17]. According to this model (Fig. 1A) the amino-terminal segment (N-domain) has a globular \( \beta \)-sheet structure. This domain is followed by a proline-rich sequence, called the P-domain that folds into a long hairpin-like structure with two pairs of short anti-parallel \( \beta \)-sheet sequences. The third region of CRT, called the C-domain, forms, together with the N-domain, a globular “head” to the molecule.

To determine to which of these three domains the SE binds, we first used domain deletion mutants that code truncated segments of CRT (Fig. 1B). The recombinant products of these mutants were immobilized on a CM5 biosensor chip and their binding to SE-expressing 15mer peptides was assayed as previously described [6]. As can be seen in the Figures 1C and 1D, consistent with our previous report [6], SE-expressing peptides 65-79*0401 and 65-79*0404 both interacted with the intact CRT molecule. Truncated proteins containing single domains (N, P or C-domains) failed to bind the SE. However, when truncated proteins containing the N-domain plus P-domain, or P-domain plus C-domain were tested, near-normal binding interactions could be seen. This pattern indirectly implicates the P-domain and suggests that a conformation-dependent binding site is involved.

To more directly identify the binding domain, we have used a photoactive cross-linking approach with sulfosuccinimidyl-2-[6-(biotinamido)-2-(\( \beta \)-azido-benzamido) hexanoamido] ethyl-1,3-dithiopropionate (sulfo-SBED) [18]. This cross-linker has 4 functional groups: a NHS-ester group, a UV-activatable aryl azide group, a cleavable disulfide bond, and a biotin group (Figure 2A). The NHS-ester group was chemically attached to the N-terminal amine group of SE-positive peptide 65-79*0404 and the compound was allowed to interact with CRT. Following UV cross-linking,
cleavage of the disulphide bond and trypsinization, the biotinylated CRT protein digest was captured by an avidin column, and the affinity-purified biotinylated fragments were analyzed by mass spectrometry (MS). A representative experiment, one of two repetitions, is shown in Fig. 2. Four major peaks were identified (Fig. 2B). Of these, peaks 1895 and 1582 were found to correspond to contaminating non-CRT peptides (avidin and the SE peptides, respectively, data not shown). However, peaks 1838 (Fig. 2C) and 2047.1 (Fig. 2D) were found to correspond, respectively, to the 262–275 and 196–211 regions in the CRT P-domain. It is worth noting that despite a 51 amino-acid distance between the two regions, NMR data [19] indicate that they are spatially adjacent – on opposite sides of the two antiparallel arms of the P-domain hairpin structure (Fig. 2E). Given the size of the sulfo-SBED compound (Fig. 2A), when taken together, these findings strongly suggest that the SE binding site is located in the CRT P-domain, within ~30 Å from regions 262–275 and 196–211.

Computer simulation of SE-CRT interaction

In order to identify candidate SE binding sites on CRT P-domain, we have used the BioMediCaché 6.1 docking software. To this end, the third allelic hypervariable region (aa 65–79) of the HLA-DR β chain, including the SE ligand region (aa 70–74) was modeled in its α helical conformation as predicted by HLA-DR1 [20] and HLA-DR4 [21] crystal structure data. The P-domain was maintained in its tri-dimensional conformation as previously predicted by NMR analysis [19]. The conformationally-rigid SE ligand and the CRT P-domain (PDB ID: 1HHN) were docked using the BioMediCaché Augmented MM3 software. As shown in Table 1, four docking models with significant levels of docking energy were identified. Intriguingly, all four models predicted the region 217–224 as the binding site.

The two most significant docking models are depicted graphically in Figure 3. As can be seen, in model A (the most significant one, with docking score of ~232 kcal/mole), CRT residues Glu217 and
Asp$^{220}$ interact with SE residue Gln$^{70}$, while CRT residues Glu$^{223}$ and His$^{224}$ interact with SE residue Arg$^{72}$. In model B (docking score of $-170$ kcal/mole), CRT residue Glu$^{223}$ is in close proximity to SE residues Lys$^{71}$ and Gln$^{70}$, while CRT Asp$^{220}$ is in close proximity to SE residue Gln$^{70}$ and Ala$^{74}$.

**Site-directed mutational analysis**

Based on the docking models mentioned above, we next performed site-directed mutagenesis in residues predicted to interact with the SE. The resultant point-mutated recombinant proteins were analyzed by circular dichroism (CD) spectroscopy and showed only minor, insignificant alterations in the secondary structure (Supplemental Figure S1).

To determine the effect of individual substitutions on CRT-SE interaction, we first used a SPR assay. Wild-type (WT) or mutant CRT proteins were immobilized on a biosensor chip at about 500 RU using a standard primary amine coupling method. SE pepidic ligands 65-79 or 65-79*0401 were run as analyte. The results are summarized in Table 2. As can be seen, the CRT E217A and E223A mutants, but not mutants D220A, H224A, Y254A or N279A, showed significantly lower binding of both SE ligands. Notwithstanding the seemingly modest variations in SE-binding potency among the different CRT species, these differences were reproducible, statistically significant and, more importantly, correlated well with signaling potencies, as discussed below.

We [6] and others [22,23] have previously demonstrated that soluble CRT can attach to the cell surface and restore CRT receptor-mediated signaling in CRT-negative cells. Accordingly, to determine the effect of CRT point mutations on signal transduction, soluble WT or mutant CRT proteins were added to $\beta$-cell line K42 [24]. There was no difference in cell surface binding capacity between WT CRT and its mutants (data not shown). As can be seen in Fig. 4A, CRT mutant E217A failed to transduce SE-activated ROS signaling and mutant E223A transduced a significantly reduced signals compared to the WT protein. No significant signaling inhibition was caused by either the D220A or Y282A mutations. Representative time-course ROS production curves with WT CRT and mutant E217A are shown in Fig. 4B. As can be seen, the E217A mutation produced complete inhibition of SE-activated ROS production. Consistent with our previous data showing close correspondence between NO and ROS signaling [4,5,6], Figures 4C and 4D demonstrate that the inhibitory effect of mutated residues 217 and 223 affected both NO and ROS signaling. Importantly, Figure 4 demonstrates that the inhibitory effect on SE-activated signaling by mutated residues 217 and 223 could be seen when the SE was expressed in its natural tri-dimensional conformation in the form of a tetramer.

### Table 1. Docking models: Interactions between SE and CRT residues.

| Docking score (kcal/mole) | Gln$^{70}$ | Lys$^{71}$ | Arg$^{72}$ | Ala$^{73}$ | Ala$^{74}$ |
|---------------------------|-----------|-----------|------------|------------|-----------|
| Model A                   |           |           |            |            |           |
| A                         | $-232$    | Glu$^{217}$ | Asp$^{220}$ | Asp$^{220}$ |           |
| B                         | $-170$    | Glu$^{223}$ | Glu$^{223}$ | His$^{224}$ | Asp$^{220}$ |
| C                         | $-166$    | Glu$^{223}$ | His$^{224}$ | Asp$^{220}$ |           |
| D                         | $-92$     | Asp$^{220}$ | Glu$^{223}$ |           |           |

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**Figure 3. SE-CRT docking models.** Graphic representation of the two most significant docking models shown in Table 1: Model A (left) and Model B (right). Closer views of the interactions between CRT (black font) and SE (cyan font) residues are shown in the lower panels.

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(Figures 4A and 4B) or when expressed in its physiologic α-helical conformation in Hepatitis B core (HBc) particles (Figures 4C and 4D). Consistent with our prior studies [6], no measurable differences in cell survival were observed in the presence or absence of WT CRT or its mutants (not shown). Although the data clearly implicate residues Glu\(^{217}\) and Glu\(^{223}\), it should be pointed out that Fig 4C suggests that, albeit weakly, residue Asp\(^{220}\) may be involved as well. Taken together, our findings indicate that SE-activated signaling maps to the 217–223 region of the CRT P-domain and depends primarily on Glu\(^{217}\) and Glu\(^{223}\) and, to a lesser extent, on Asp\(^{220}\).

**Discussion**

We have previously demonstrated that the SE interacts with cell surface CRT and activates innate immune signaling [4,5,6]. To better characterize this interaction, here we determined the SE binding site on CRT. By using a combination of SPR-based binding studies, photoactive cross-linking methods, an *in silico* docking simulation and mutational analysis, we have mapped the SE binding site to the 217–223 region of CRT P-domain and identified residues Glu\(^{217}\) and Glu\(^{223}\) as key players. The mature CRT protein contains three structurally and functionally distinct domains. The 180 N-terminal amino acid residues and the 115 C-terminal residues form the N-domain and a C-domain, respectively. Based on the known crystal structure of a homologous protein calnexin, these domains are predicted to fold into a composite globular domain. The intervening sequence (residues 181-284), is proline-rich and is therefore called the P-domain. This domain has been shown by NMR studies to form an arm-like hairpin structure, stabilized by two antiparallel β-sheets.
Consistent with our findings with domain-deleted mutants, prior studies have demonstrated that CRT tertiary conformation is critically important for its biologic activity [25].

The use of photoactive cross-linkers, followed by identification of cross-linked regions by MS analysis has proven useful in complex interactions, especially where tertiary conformational factors play a role [18,26,27]. Using that method, we identified two P-domain sequences located, respectively, in positions 196–211 and 262–275 of the CRT P-domain. It is worth mentioning that despite a 51 amino acid residues gap in their primary sequence, the 196–211 and 262–275 regions are spatially adjacent, on two anti-parallel segment of the P-domain hairpin. Moreover, these two regions are both within the reach of the cross-linking sulfo-SBED compound, 27.9 Å and 23.1 Å, respectively, from the SE actual binding site [19].

Our data with HLA-DR tetramers and HBc particles attest to the significance of the findings, since these physiologically-folded SE-expressing ligands closely mimic the natural conformation of the epitope. It should be mentioned that tetramers consist of four identical units of the HLA-DR molecule, each folded in its native tertiary conformation. Likewise, SE-expressing HBc particles have been engineered to allow the SE to be expressed outside of the HLA-DR context in its native α helical conformation [4]. Therefore, the significance of the point-mutant studies shown here is two-fold: First, they demonstrate high consistency between all the different mapping-approaches and, second, by using conformationally-intact reagents, these studies attest to the physiologic relevance of the data. Both SPR binding assay and signaling assay confirm that CRT Glu217 and Glu223 play critical role in binding the SE. However, we cannot rule out the possibility that other residues on other CRT domains might be involved in the binding as well. Because crystal structure data on the whole CRT molecule is unavailable, our computer docking model could only focus on the CRT P-domain.

The binding site identified here has several unique characteristics. Most of the previously reported interactions between CRT and various other proteins were found to involve its C- or N-domains, whereas little is known about the role the P-domain. Three notable exceptions in which a P-domain binding site has been implicated are ERp57 [28,29], C1q [30,31] and the 4-aminobutyrate type A receptor associated protein (GABARAP) [17,32].

Using a site-directed mutagenesis approach, Martin et al [29] have mapped the ERp57 binding site to tip of the P-domain (residues Glu239, Asp241, Glu243 and Trp244). That binding site is different from the one reported here. Additionally, while CRT-ERp57 interaction takes place inside cells, the interaction reported here occurs on the cell surface. Cell surface CRT has been long known as an innate immune system receptor that binds and transduces C1q signals [30,31]. The binding site of the collagen-like segment of C1q was assigned to an “S-domain”, a CRT fragment (residues 160–203), which spans both the N- and P-domains [33]. The precise site in this fragment that binds C1q has not been mapped to date and therefore its relevance to the SE binding site (residues 217–223) cannot be determined. The third known P-domain-binding ligand is GABARAP, a nervous system adaptor protein that plays a role in intracellular vesicle trafficking. Thielmann et al [17] have demonstrated that CRT residue Trp183 plays a critical role in GABARAP-CRT interaction. No site specific mutagenesis of the CRT protein was attempted in those studies. Given the location of Trp183 near the junction between the N-domain and P-domain, the study could not rule out participation of the N-domain in stabilizing the interaction between GABARAP and CRT [17].

It is worth mentioning that, different from GABARAP that interacts with CRT intracellularly, the SE represents an extracellular ligand that activates signaling through its interaction with cell surface CRT. Thus, the distance between Trp183 and the SE-binding site, the dissimilarity in the biologic effect of the two pathways and their distinct compartmentalization, all suggest that the SE and GABARAP binding sites are neither structurally nor biologically related. Thus, the SE binding site reported here is unique, both topologically and functionally.

Based on the critical role of CRT residues Glu217 and Glu223 shown here, on the one hand and the previously determined functional role of individual SE residues on the other, Figure 5 depicts a proposed model of CRT-SE interaction. According to this model - and consistent with docking Model A discussed above - CRT Glu217 interacts with SE Glu70, while CRT Glu223 interacts with SE Arg72. This model is supported by several considerations: 1. Model A has been assigned the most significant docking score (−232 kcal/mol); 2. Different from the other docking models considered here, it implicates Glu223, a residue that shows the most significant impact on both binding and signaling; 3. It involves Glu70 on the SE side, a residue that has been previously shown to play a critical role in RA pathogenesis [34,35]; 4. It involves the SE Arg72, a residue that is common to all the SE variants (QKRAA, QRRAA and RRRAA). 5. This model allows for engagement of both the CRT P-domain and the groove peptide without spatial interference (Fig. 5). In order to examine this model, we are presently gearing towards experiments with

![Figure 5. Proposed CRT-SE interaction model. A. A SE-positive HLA-DR molecule (PDID: 25DE) interacting with the SE-binding site in the CRT P-domain. The HLA-DRα chain is shown in green, HLA-DRβ chain is in yellow, the SE is in cyan, CRT P-domain is in purple, and the groove peptide is shown in orange. B. A closer view of the interactions between CRT Glu217 and SE Glu70 and between CRT Glu223 and SE Arg72. doi:10.1371/journal.pone.0011703.g005](image)
HLA-DR tetramers carrying site-specific mutations in the SE (residues 70–74). It should be mentioned, however that while point mutation analyses on both sides will help clarifying the spatial confines of SE-CRT interaction, more definitive data might be obtained from co-crystallization studies. Unfortunately, such studies may be technically challenging, given that the crystal structure of CRT has not been resolved to this date.

In summary, we have characterized here a biologically consequential interaction site between the SE, a physiologically-folded novel extracellular ligand, and the innate immunity receptor CRT. The interaction represents a structurally and functionally well-defined example of cross talk between the adaptive and innate immune systems. In order to better understand the role of this interaction in disease pathogenesis, we are presently determining the functional consequences of SE-CRT activated pathway in the immune system. These studies could shed new light on the function of the SE and help to advance our understanding of its role in RA.

Materials and Methods

Cells and reagents

The mouse embryonic fibroblast (MEF) line K42, isolated from crt−/−, has been previously described [24], pBAD-cRT-His, pGEX-cRT, and its domain deletions pGEX-cRT-N[1-182], pGEX-cRT-P[139-273], pGEX-cRT-C[270-401], pGEX-cRT-N[1-290], and pGEX-cRT-P[139-401] plasmids were prepared as described [36,37]. Ni-NTA Superflow resin and other molecular biology reagents were purchased from Qiagen (Valencia, CA). Diaminomuorescein Diacetate (DAF-2 DA) was purchased from EMD-Calbiochem (Gibbstown, NJ), and 5-(and-6)-chloromethyl-2, 7' dichlorodihydrofluorescein (CM-H2DCFDA) was purchased from Invitrogen (Carlsbad, CA). SE ligands expressed as either 15mer synthetic peptides (65-79*0401 or 65-79*0404), engineered into hepatitis B core particles (HBc*0401) or expressed as either 15mer synthetic peptides (65-79*0401 or 65-79*0404), and the native CRT as a physiologically-folded novel extracellular ligand, and the innate immunity receptor CRT. The interaction represents a structurally and functionally well-defined example of cross talk between the adaptive and innate immune systems. In order to better understand the role of this interaction in disease pathogenesis, we are presently determining the functional consequences of SE-CRT activated pathway in the immune system. These studies could shed new light on the function of the SE and help to advance our understanding of its role in RA.

Surface plasmon resonance (SPR)

SPR experiments were performed with a BIAcore 2000 instrument (GE Healthcare, Piscataway, NJ) as we described [6]. All assays were performed at 25°C in a binding buffer containing 10 mM HEPES, pH 7.4, 50 mM KCl, 0.5 mM CaCl2, 100 μM ZnCl2, and 0.005% surfactant P-20. The analyte was injected at a flow rate of 10 μl/min.

Photoactive cross-linking studies

The sulfosuccinimidyl-2-[6-(biotinamido)-2-(p-azido-benzamido) hexanoamido] ethyl-1,3-dithiopropionate (Sulfo-SBED) method was used according to the manufacturer's protocol. Briefly, the 15mer peptide 65-79*0401 or 65-79*0404, engineered into hepatitis B core particles (HBc*0401) or expressed as either 15mer synthetic peptides (65-79*0401 or 65-79*0404), were used according to the manufacture's instructions. Biotin-labeled peptide 65-79*0401 with purified rabbit CRT in the presence of DTT, the Sulfo-SBED-cross-linked peptide-protein interaction was performed by incubating Sulfo-SBED-labeled 65-79*0404 with purified rabbit CRT in the dark at room temperature for 1 hr. Cross linking was achieved by UV irradiation using a Philips UV lamp (360 nm, 40 W, 3 D system PCA) for 15 minutes on ice at a distance of 5 cm. After cleaving the disulfide bond by DTT, the Sulfo-SBED-cross-linked CRT was trypsinized overnight at 37°C. Biotin-labeled peptide fragments were then isolated by a monomeric avidin column.

Docking models

The BioMediCACHe 6.1 software (Fujitsu, Sunnyvale, CA) was used for in silico modeling of the interaction between the SE and the CRT P-domain. The β-chain third allelic hypervariable regions (residues 65–79) of HLA-DR4 (PDB ID: 1PDB ID: 2SEB) were used as ligands and the rat CRT P-domain (PDB ID: 1HHN) was used as the receptor. Both binding partners were modeled in their rigid conformation, using augmented MM3 parameters [38]. Docking scores were calculated using the manufacturer’s software. In all cases, the minimum potential energy was calculated for the most stable geometry.

Site-directed mutagenesis

CRT site-directed mutants were generated following the QuickChange protocol (Stratagene La Jolla, CA). The primers used in this study are listed in supplemental Table S1. Sequences were verified by the University of Michigan DNA Sequencing Core. Rabbit and mouse wild-type CRT and site-directed mutants were constructed in pBAD vector. Wild-type and mutant CRT plasmids were transformed into GC10 cells for protein expression. The 6xHis-tagged protein expression was induced by 0.002% L-arabinose for 4 hr and the protein was purified using a Ni-NTA resin, following the manufacturer’s protocol. Rabbit CRT domain-deletion mutants were constructed in a pGEX vector. GST-fusion CRT domain-deletion mutants were expressed and purified as described [6,39]. The GST segment was removed by Factor Xa.

Circular dichroism spectroscopy

Proteins were in a 5 mM HEPES (pH 7.4), 100 mM KF, 2 mM CaCl2 buffer. CD spectra were determined in 1 mm bandpass quartz cuvettes by far-UV circular dichroism on an Aviv 215 spectropolarimeter (Aviv Associates, Lakewood, NJ). Corresponding baseline levels were obtained with buffer only and subtracted from the sample spectrum.

Signal transduction assays

To measure NO production, crt−/− MEF, 30,000 cells per well, were seeded in a 96-well plate. Cultures were loaded with 1 μg/ml of recombinant WT or mutant CRT overnight. Cells were then...
labeled by 20 μM DAF-2 DA and stimulated with SE ligands. NO production rates were determined as we previously described [4]. ROS production was quantified similarly, with the exception that cells were labeled with 10 μM CM-H2DCFDA as we previously described [5].

Supporting Information

Figure S1 CD spectra of WT CRT and its mutants. Proteins, in a 5 mM HEPES (pH 7.4), 100 mM KF, 2 mM CaCl2 buffer were placed in 1 mm bandpass quartz cuvettes and analyzed by far-UV circular dichroism on an Aviv 215 spectropolarimeter (Aviv Associates, Lakewood, NJ). Corresponding baselines were obtained with buffer which were subtracted from the sample spectrum. No statistically significant conformational differences between WT CRT and its mutants were found.

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Author Contributions

Conceived and designed the experiments: SL, PP, MM, JH. Performed the experiments: SL, AC. Analyzed the data: SL, AC, JH. Contributed reagents/materials/analysis tools: PP, MM. Wrote the paper: SL, JH. Read the manuscript: AC, PP. Edited the manuscript: MM.

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