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The flexible stalk domain of sTREM2 modulates its interactions with phospholipids in the brain

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David Saeb, Emma E Lietzke, Daisy I Fuchs, Emma C Aldrich, Kimberley D Bruce, Kayla G Sprenger

Department of Chemical and Biological Engineering, University of Colorado Boulder, Boulder, CO, USA • Department of Endocrinology, Metabolism, and Diabetes, University of Colorado Anschutz, Medical Campus, Aurora, CO, USA

Abstract

The microglial surface protein Triggering Receptor Expressed on Myeloid Cells 2 (TREM2) plays a critical role in mediating brain homeostasis and inflammatory responses in Alzheimer's disease (AD). The soluble form of TREM2 (sTREM2) exhibits neuroprotective effects in AD, though the underlying mechanisms remain elusive. Moreover, differences in ligand binding between TREM2 and sTREM2, which have major implications for their roles in AD pathology, remain unexplained. To address these knowledge gaps, we conducted the most computationally intensive molecular dynamics simulations to date of (s)TREM2, exploring their interactions with key damage- and lipoprotein-associated phospholipids and the impact of the AD-risk mutation R47H. Our results demonstrate that the flexible stalk domain of sTREM2 serves as the molecular basis for differential ligand binding between sTREM2 and TREM2, facilitated by its role in stabilizing the Ig-like domain and altering the accessibility of canonical ligand binding sites. We identified a novel ligand binding site on sTREM2, termed the 'Expanded Surface 2', which emerges due to competitive binding of the stalk with the Ig-like domain. Additionally, we observed that the stalk domain itself functions as a site for ligand binding, with increased binding in the presence of R47H. This suggests that sTREM2's neuroprotective role in AD may, at least in part, arise from the stalk domain's ability to rescue dysfunctional ligand binding caused by AD-risk mutations. Lastly, our findings indicate that R47H-induced dysfunction in membrane-bound TREM2 may result from both diminished ligand binding due to restricted complementarity-determining region 2 loop motions and an impaired ability to differentiate between ligands, proposing a novel mechanism for loss-of-function. In summary, these results provide valuable insights into the role of sTREM2 in AD pathology, laying the groundwork for the design of new therapeutic approaches targeting (s)TREM2 in AD.

eLife Assessment

This **useful** manuscript addresses some key molecular mechanisms on the neuroprotective roles of soluble TREM2 in neurodegenerative diseases. Thw study will advance our understanding of TREM2 mutations, particularly on the damaging effect of known TREM2 mutations, and also explain why soluble TREM2 can antagonize Aβ aggregation. However, the primary experimental method, MD simulations, suffers from limited sampling, rendering the results **incomplete** for definite conclusions.

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Introduction

Alzheimer's disease (AD) is a fatal neurodegenerative condition marked by progressive memory loss, cognitive decline, and impaired daily functioning[1]. Despite extensive research, the root causes remain unknown and curative treatments remain elusive. AD is hallmarked by the presence of extracellular amyloid-β (Aβ) plaques, intracellular neurofibrillary tau tangles, lipid droplet accumulation, and neuroinflammation[2,3]. Microglia, the brain's macrophages, play a pivotal yet complex role in AD pathology. While their phagocytic activity toward Aβ is considered neuroprotective, they also release cytokines that increase neuroinflammation, ultimately harming neighboring neurons and glia[4,5]. Two newly-approved monoclonal antibodies targeting Aβ, while transformative, have shown limited efficacy, only modestly slowing disease progression[6]. This underscores the need for a deeper understanding of the intricate interactions between the neuroimmune system and AD-relevant proteins.

At the forefront of this investigation is Triggering Receptor Expressed on Myeloid Cells 2 (TREM2), a transmembrane receptor expressed on microglial surfaces. TREM2 propagates a downstream signal through interactions with its co-signaling partner, DNAX-activating protein 12 (DAP12). Recent research highlights its crucial role in modulating microglial responses and maintaining brain homeostasis[7]. TREM2 contains an extracellular immunoglobulin (Ig)-like domain (19-130 amino acid (aa)), a short extracellular stalk domain (131-174 aa), a helical transmembrane domain (175-195 aa), and an intracellular cytosolic tail domain (196-230 aa)[8]. The Ig-like domain contains several ligand binding sites. These include a hydrophobic tip, which has a highly positive electrostatic potential and contains several aromatic residues and three complementarity-determining regions (CDRs), the latter of which (CDR2) also span part of a positively-charged basic patch known as the putative ligand interacting region, or 'Surface 1' (**Fig. 1**)[8–11]. Mutations in TREM2 correlate with altered risks of developing AD, with the R47H mutation, found on Surface 1, standing out as a significant genetic risk factor[12–14]. *In vitro* and *in silico* studies have consistently revealed that mutations, including R47H, destabilize TREM2's CDRs[8,15–17], exposing once-buried negatively charged residues[8,16] and disrupting homeostatic TREM2-ligand binding behavior[9,10,16,18].

TREM2 binds diverse anionic and lipidic ligands, including Aβ species[10,19,20], lipoproteins[10,21–23], nucleic acids[24], carbohydrates[25], and phospholipids (PLs)[9,18,26]—the focus of our study. PLs play a crucial role in lipid metabolism and maintaining brain homeostasis[27]. TREM2 clears excess PLs during demyelination and interacts with PLs when bound to lipoproteins with a core of neutral lipids surrounded by a monolayer of PLs, free cholesterol, and apolipoproteins[28,29]. Many PLs bind to TREM2, including phosphatidyl-choline (PC), -serine (PS), -inositol (PI), -glycerol (PG), - ethanolamine (PE),

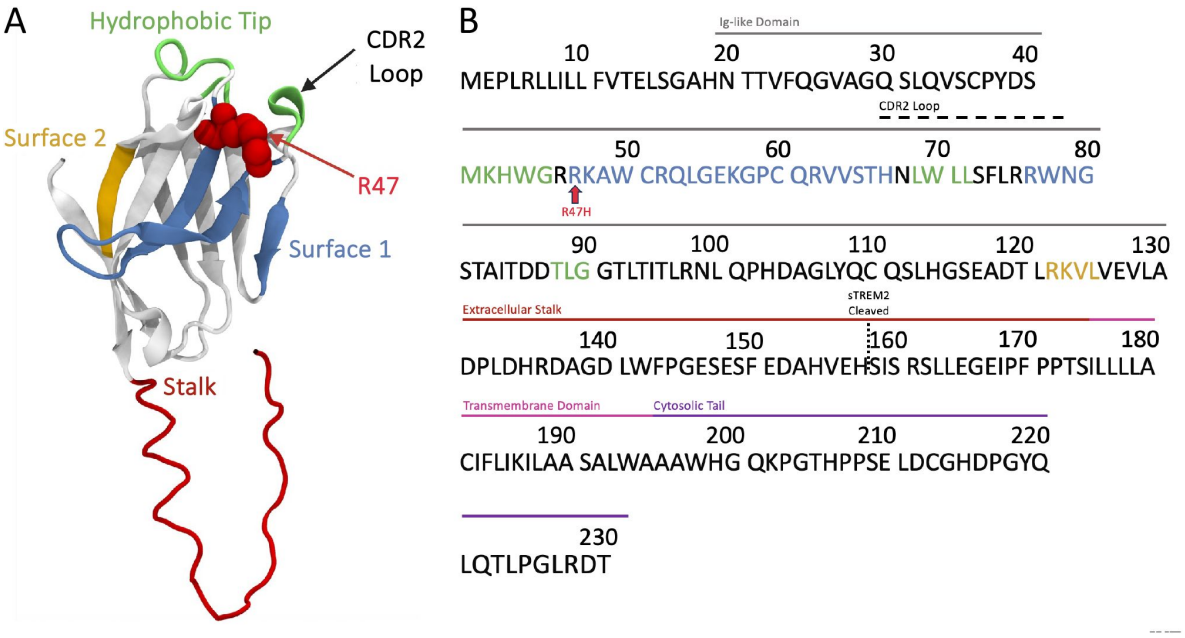

Figure 1.

Overview of structural domains in sTREM2.

(A) Pre-MD structure of sTREM2^{WT} and (B) Full sequence of TREM2, indicating significant structural domains

phosphatidic acid (PA), sphingomyelin (SM), cholesterol, and sulfatide[9,16,18,29]. PLs primarily bind to TREM2's hydrophobic patch and Surface 1, with varying affinities observed in different contexts[9,16,18]. Direct binding assays show stronger TREM2 binding to anionic moieties (PS, PE, PA) and weaker binding to PC and SM. Conversely, TREM2-expressing reporter cells reveal high TREM2 stimulation from PC and SM, especially with the TREM2^{R47H} variant[18]. Collectively, however, these results emphasize TREM2's broad binding capabilities for PLs. Yet, one study suggested effective TREM2 stimulation by PLs may require co-presentation with other molecules, potentially reflecting the nature of lipoprotein endocytosis[30]. Another study observed minimal changes in TREM2-PL interactions despite TREM2 mutations (R47H, R62H, T96K)[9]. Ultimately, there remains a major gap in understanding the mechanism for how PLs differentially interact with TREM2 and how this interaction is altered in disease-associated variants.

The activation of TREM2, mediated by the binding of ligands such as PLs, shapes key microglial functions, including proliferation, phagocytosis, and lipid metabolism. Notably, in pathological conditions, ligand-induced TREM2 activation triggers microglial phenotype switching to Disease-Associated Microglia (DAM), characterized by the activation of inflammatory, phagocytic, and lipid metabolic pathways[31]. Additionally, TREM2's extracellular domain can undergo cleavage from ADAM 10/17 sheddase at residue H157, yielding soluble TREM2 (sTREM2). The role and relevance of sTREM2 in disease pathology has been heavily debated[32]. In the cerebrospinal fluid of individuals with early-stage AD, elevated sTREM2 levels have been detected and linked to slower AD progression[33–35]. Further, there is a strong correlation between the levels of sTREM2 in the cerebrospinal fluid and that of Tau, however correlation with Aβ is inclusive. These findings have established sTREM2 as a long-time biomarker for AD diagnosis and progression[36–38].

Many studies have indicated a neuroprotective role for sTREM2 in disease pathology[39]. It has been suggested, for instance, that sTREM2 may function as a "dummy receptor" in AD states, preventing disease-associated ligands from binding TREM2[32]. Moreover, *in vivo* AD mouse models evaluating the therapeutic potential of recombinant sTREM2 have observed the suppression of microglial apoptosis, reduced Aβ plaque load, and improved learning and memory abilities[40,41]. More recent studies have indicated that sTREM2 not only serves as an activator for microglial uptake of Aβ but also directly inhibits Aβ aggregation[10,11,42]. Specifically, the binding of Aβ to TREM2 has been shown to increase shedding of sTREM2[42], which can then bind to Aβ oligomers and fibrils to inhibit their secondary nucleation[11,42]. Interestingly, the effect of R47H on Aβ aggregation is unclear, highlighting the need to study mechanistic aspects of ligand binding[11,42].

Some anionic ligands, including Aβ, predominantly bind to Surface 1 on TREM2. Intriguingly, recent observations revealed that Aβ binds to an alternative binding region, termed 'Surface 2', on sTREM2, situated opposite Surface 1 (**Fig. 1**). Surface 2 features a group of positively charged residues surrounded by acidic residues, creating a variegated electrostatic potential[11]. Herein, we aimed to unravel the molecular basis behind this functionally significant distinction in ligand binding between soluble and membrane-bound TREM2, utilizing molecular dynamics (MD) simulations. We focused on (s)TREM2-PL interactions, establishing a controlled framework to assess the impacts of various PL chemistries on ligand binding, specifically comparing the binding behavior of anionic PS and neutral PC. We hypothesized that the oft-overlooked flexible stalk domain of sTREM2, minimally explored in previous *in silico* studies, may play a pivotal role in mediating the observed variations in binding. Furthermore, we sought to understand the impact of the AD-risk mutation R47H on ligand binding, thereby unraveling fundamental roles of (s)TREM2 in AD pathology. To our knowledge, this study represents the second-ever application of MD to investigate sTREM2, totaling an unprecedented 17.2 μs of simulation time. Ultimately, this research may unveil new insights into the mechanistic and therapeutic roles of sTREM2 in AD.

Methods

Preparation of simulated structures

We employed the AlphaFold[43,44] model (AF-Q9NZC2-F1) as the initial structure for wildtype (WT) sTREM2 in our simulations, chosen for its inclusion of the unstructured flexible stalk domain. The partial stalk domain spans residues 130 through 157, while the Ig-like domain consists of residues 19 through 130. For WT simulations, we constructed two protein systems: one with just the Ig-like domain ("Ig^{WT}") and another containing both the partial stalk and Ig-like domain ("$sTREM2^{WT}$"). Similarly, two protein systems were constructed for the variant simulations: one with just the Ig-like domain containing the R47H mutation ("Ig^{R47H}") and another containing both the partial stalk and mutant Ig-like domain ("$sTREM2^{R47H}$"). In contrast to the use of the AlphaFold model for WT, we utilized a crystal structure of $TREM2^{R47H}$ (Protein Data Bank (PDB) code 5UD8[16]), to which the unstructured stalk domain from the AlphaFold model was added using alignment tools in Visual Molecular Dynamics (VMD). Missing residues were incorporated into the $TREM2^{R47H}$ Ig domain using MODELLER[45]. The initial molecular structures for the PLs, stearoyl-oleoyl-PC (SOPC) and stearoyl-oleoyl-PS (SOPS), were obtained from CHARMM-GUI[46–50], with each considered as a singular PL. All eight protein-ligand systems (Ig^{WT}, $sTREM2^{WT}$, Ig^{R47H}, and $sTREM2^{R47H}$, each with SOPC or SOPS) and six pure-component systems were solvated with explicit water and with counterions added as needed to neutralize the charge.

Molecular dynamics simulations

All proteins, PLs, and counterions were parameterized using the CHARMM36 force field[51,52], while the TIP3P model was used to describe water[53]. Prior to subsequent docking studies, each pure-component system underwent steepest-descent energy minimization, initially in vacuum and then in a solvated state. This was followed by a multi-step equilibration protocol, which included a 1 ns NVT equilibration simulation at 310K using the Bussi-Donadio-Parrinello thermostat[53], followed by a 1 ns NPT equilibration simulation at 310K and 1 bar using the same thermostat and Berendsen barostat[54]. Finally, production simulations were carried out at 310 K and 1 bar, utilizing the same thermostat and Parrinello-Rahman barostat[55]. The duration of the production simulations was 150 ns for each pure-component PL system and 1 μs for each pure-component protein system.

All simulations were conducted using the GROMACS MD engine[56]. The LINCS algorithm[57] was used to constrain bonds involving hydrogen atoms, and particle-mesh Ewald (PME) summations[58] were employed for calculating long-range electrostatics with a cutoff of 1.2 nm. Lennard-Jones interactions were evaluated up to 1.2 nm and shifted to eliminate energy discontinuities. Neighbor lists were reconstructed in 10-step intervals with a 1.4 nm cutoff. A timestep of 2 fs was implemented in all simulations, and periodic boundary conditions were applied in the x, y, and z directions. Configurations from these production simulations were used as inputs in ensuing docking calculations (see next section). After the docking calculations, we conducted additional 150 ns production simulations on the combined post-docking models, employing the same parameters as described above for the pre-docking production simulations.

Molecular docking calculations

For each of the four pre-docking, pure-component protein systems, we clustered the 1 μs production simulation trajectory using the gromos method implemented in GROMACS. Representative structures from the top two clusters in each case were selected and prepared for subsequent docking calculations using AutoDock Tools[59]. Docking calculations were carried out with AutoDock Vina[60,61], treating the proteins as rigid receptors. Given that AutoDock Vina

employs a flexible ligand docking procedure, the final PL conformation from each pure-component simulation served as the ligand in the docking calculations. Grids with dimensions of 30 Å x 30 Å x 30 Å were constructed, redundantly covering the complete surface of each receptor protein. The exhaustiveness parameter for docking was set to eight. Docked complexes were analyzed based on the AutoDock score, the number of highly similar complexes, and biological relevance. Unique structures across grids and clusters for each ligand-receptor system were identified for post-docking MD simulations. From molecular docking, we obtained 7 unique SOPS/Ig^{WT} models, 5 SOPS/$sTREM2^{WT}$ models, 5 SOPC/Ig^{WT} models, 6 SOPC/$sTREM2^{WT}$ models, 4 SOPS/Ig^{R47H} models, 8 SOPS/$sTREM2^{R47H}$ models, 6 SOPC/Ig^{R47H} models, and 7 SOPC/$sTREM2^{R47H}$ models.

Trajectory analysis protocols

The Molecular Mechanics Poisson-Boltzmann Surface Area (MM-PBSA) approach was used to calculate PL-protein binding free energies, utilizing the gmx_MMPBSA implementation[62–64]. The analysis focused on temporal regions of the simulation trajectories where PL-protein complexes demonstrated stability, as indicated by relatively constant root-mean-square deviation (RMSD) values for at least 50 ns. Over the same temporal regions, we calculated Interaction Entropy, as implemented in gmx_MMPBSA, to estimate entropic trends in PL binding[63,65]. Fractional occupancy values, characterizing the frequency and location of PL binding on each protein surface, were determined by calculating the fraction of simulation trajectory frames in which a given protein residue was within 4 Å of the PL across all simulations. We calculated conformational changes of the CDR2 loop using Visual Molecular Dynamics (VMD)[66] by measuring the distance between residues 45 and 70 at the tops of the CDR1 and CDR2 loops, respectively.

Results and Discussion

The partial stalk domain of sTREM2 stabilizes its Ig-like domain via binding to ‘Surface 1’

Prior TREM2 research identified an open CDR2 loop in R47H models, disrupting ligand binding to Surface 1[8,15,16]. Our 1-μs simulations consistently showed open CDR2 loops in both Ig^{R47H} and $sTREM2^{R47H}$ (**Fig. 2A**, slate blue and teal lines). Contrary to past experiments[15,16], Ig^{WT} transitioned from a closed to an open CDR2 loop midway through our simulation (**Fig. 2A**, pink line). To understand this result, we examined whether variation in the protonation state of histidine residue H43 in the CDR2 loop of Ig^{WT} and Ig^{R47H} could explain the observed variations in CDR2 loop dynamics. Initial protonation states (HSE in Ig^{WT}, HDE in Ig^{R47H}) were identified by GROMACS’ pdb2gmx algorithm, based on optimal H-bonding conformations. Two additional 1 μs simulations of Ig^{WT} with the original protonation state (HSE) and three with the alternate state (HDE) were performed (**Fig. S1A**). The open CDR2 loop conformation was sampled in two out of the six trials, including the original Ig^{WT} simulation with the HSE protonation state for H43 and one trial with the HDE protonation state. This suggests that, while R47H consistently locks the CDR2 loop in an open state—as shown in past studies and **Fig. 2A**—the WT model’s loop opening may be more stochastic and does not depend on H43’s protonation state. We note that the CDR2 loop remained open in a 1 μs simulation of Ig^{R47H} with the alternate H43 protonation state (HSE).

Notably, $sTREM2^{WT}$ maintained a closed-loop conformation throughout the full 1 μs simulation (**Fig. 2A**, red line). This observation hints at a potential stabilizing effect on the CDR2 region, possibly attributed to the partial stalk domain present in $sTREM2^{WT}$ but which is absent in membrane-bound TREM2 (represented by Ig^{WT}). In the latter, stalk/Ig-like domain interactions are restricted by the connection of the stalk to TREM2’s transmembrane domain. Given the potential stochastic nature of CDR2 loop opening (in the absence of R47H), we aimed to scrutinize this

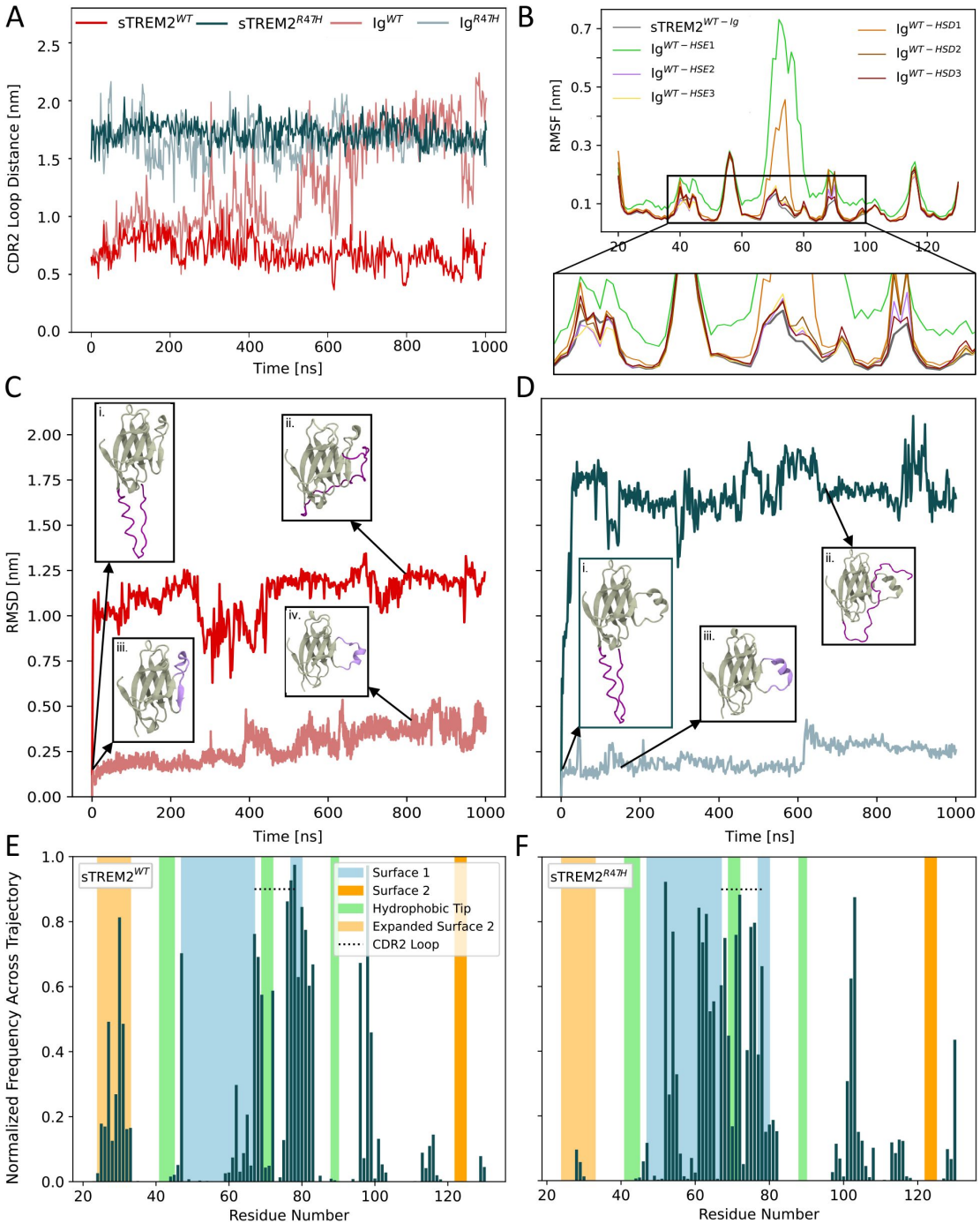

Figure 2.

The stalk of sTREM2^{WT} stabilizes the Ig-like domain via binding to Surface 1.

(A) Distance between CDR2 loop residues 45 and 70 vs. time for WT and R47H models of sTREM2 and TREM2 (Ig). (B) Temporally-averaged Cα-RMSF of the isolated Ig-like domain of WT sTREM2 (sTREM2$^{WT\text{-}Ig}$) vs. the Ig-like domain of TREM2 across six simulations (three trials each with two different protonation states for H43 (see main text for details)). (C-D) Cα-RMSD of (C) WT and (D) R47H models of sTREM2 and TREM2 vs. time, with corresponding snapshots shown for the initial and equilibrated structures in each case. The stalk domain of sTREM2 is shown in dark purple, while the CDR2 loop of TREM2 is shown in light purple. (E-F) Normalized fractional occupancy of residues in the Ig-like domain of (E) sTREM2^{WT} and (F) sTREM2^{R47H} by the stalk.

stabilization claim more rigorously through root-mean-square fluctuation (RMSF) calculations. These calculations involved the six Ig^{WT} simulations and the isolated Ig-like domain from the single $sTREM2^{WT}$ simulation ($sTREM2^{WT\text{-}Ig}$), assessed for each 1-μs trajectory (**Fig. 2B**). Compared to the six Ig^{WT} structures, $sTREM2^{WT\text{-}Ig}$ displayed reduced residue-level fluctuations across the entire Ig-like domain, suggesting broad stalk-induced stabilization. This effect was particularly evident around residues 66-77 and 84-90, encompassing portions of the hydrophobic tip and Surface 1, including the highly dynamic CDR2 loop region.

In light of these findings, we conducted a similar RMSF analysis to compare the dynamics of the isolated Ig-like domain from $sTREM2^{R47H}$ ($sTREM2^{R47H\text{-}Ig}$) against the two simulations of Ig^{R47H} with the varying H43 protonation states (**Fig. S1B**). Similar to the WT model, the presence of the stalk led to substantially reduced residue-level fluctuations across the entire Ig-like domain of $sTREM2^{R47H\text{-}Ig}$, especially around residues 38-45 and 60-77. Collectively, these findings strongly suggest a stabilizing role for the partial stalk domain in sTREM2 Ig-like domain dynamics. This effect also appears to be largely independent of the degree of openness of the CDR2 loop, given the differences in CDR2 loop dynamics between $sTREM2^{WT}$ and $sTREM2^{R47H}$ shown in **Fig. 2A**.

We next aimed to elucidate the molecular basis for the stabilization of the Ig-like domain by the partial stalk domain of sTREM2. To this end, we calculated the RMSD of the Cα atoms in Ig^{WT} and $sTREM2^{WT}$ (**Fig. 2C**), and in Ig^{R47H} and $sTREM2^{R47H}$ (**Fig. 2D**), tracking their temporal positions relative to the respective energy-minimized configuration. The RMSD of each structure converged relatively quickly during the simulations. The Ig^{R47H} and Ig^{WT} simulations show behavior that mimics the CDR2 loop distances depicted in **Fig. 2A**. We observe a consistently low temporal RMSD profile for Ig^{R47H}, indicative of a persistently open CDR2 loop (inset panel **iii** of **Fig. 2D**). Similarly, the RMSD profile for Ig^{WT} is consistently low but increases slightly around 400 ns, signaling a transition from a closed to an open CDR2 loop (inset panels **iii** and **iv** of **Fig. 2C**). The CDR2 loop remains open for the remainder of the simulation, yet exhibits considerably larger RMSD fluctuations compared to the open CDR2 loop in Ig^{R47H}, indicating the R47H mutation likely imparts some stability to the open CDR2 loop.

Sharp initial increases in RMSD are observed with both $sTREM2^{WT}$ and $sTREM2^{R47H}$. In both cases, the starting structures (inset panels **i** of **Figs. 2C-D**) exhibited a generally linear configuration of the stalk, potentially resembling its membrane-bound form. After the initial RMSD increases, the partial stalk domains were observed to consistently interact with the Ig-like domains of $sTREM2^{WT}$ and $sTREM2^{R47H}$ (inset panels **ii** of **Figs. 2C-D**). To identify the specific regions of the protein surface with which the partial stalk interacted during each simulation, we generated per-residue fractional occupancy maps characterizing the contact frequency (atom-atom distance within 4 Å) between the stalk and each residue in $sTREM2^{WT}$ (**Fig. 2E**) and $sTREM2^{R47H}$ (**Fig. 2F**). We observed a notable increase in the interaction frequency between residues on Surface 1 and the partial stalk domain of $sTREM2^{R47H}$ compared to $sTREM2^{WT}$. This difference is likely due to the more open CDR2 loop of $sTREM2^{R47H}$, providing greater accessibility of Surface 1 residues for interaction with the stalk.

Given the highly negative overall charge of the stalk domain (−8), its recurrent interactions with the positively-charged residues in Surface 1 are perhaps intuitive. In addition to these interactions, the stalk domain in $sTREM2^{WT}$ frequently interacted with residues 20-35, although with lower occupancy typically. While this region has not been directly noted in past experimental studies, it is located directly adjacent to residues highlighted in Surface 2 by Belsare et al[11]. Hence, we termed it the 'Expanded Surface 2'. Considering the diverse surface regions the stalk domain appears to interact with, and the overlap in many cases with known ligand binding sites on TREM2, these results may offer a potential mechanism for observed differences in ligand binding between sTREM2 and TREM2, which we explore in detail in the following section.

sTREM2's partial stalk domain modulates PL binding by promoting interactions with a new site on the Ig domain and creating an additional binding surface within the stalk itself

In vitro studies investigating the molecular interactions of (s)TREM2 with ligands have been limited. One recent study found that sTREM2 demonstrated lower affinity for Aβ (of length 42 aa) compared to TREM2[10], suggesting potential differences in ligand binding between the two proteins. Another study presented a crystal structure featuring a TREM2 trimer bound by three PS molecules. This structure revealed broad PS binding to residues in the CDR2 loop and hydrophobic tip of TREM2[16]. While this study did not specifically explore PL/sTREM2 interactions, we hypothesized that similar differences in (s)TREM2 binding might be observed with PLs as seen with Aβ.

To test our hypothesis, we conducted 150 ns MD simulations of both Ig^{WT} and $sTREM2^{WT}$ bound by the PLs stearoyl-oleoyl-PC (SOPC) and stearoyl-oleoyl-PS (SOPS) in various initial favorable docking configurations (see Methods for details). Subsequently, we analyzed the surface binding patterns of the PLs by determining the fractional occupancy of each protein residue, temporally averaged across all simulations for each protein/PL system. Across all four systems, we observed the highest occupancy by both PLs of residues located in the CDR2 loop and hydrophobic tip (**Figs. 3A-D**, primarily green regions), confirming earlier *in vitro* findings and validating our approach. Nevertheless, distinctions between the SOPS and SOPC models are evident, underscoring the influence of ligand charge on binding. Specifically, SOPS, being negatively charged, exhibited higher relative occupancy of the positively charged residues on Surface 1 compared to SOPC, which is neutrally charged (**Fig. 3A-B** vs. **3C-D**, resp., blue regions). Conversely, SOPC showed higher relative occupancy of residues in the hydrophobic tip than SOPS (**Fig. 3C-D** vs. **3A-B**, resp., green regions).

Upon comparing the fractional occupancy plots of the PL/$sTREM2^{WT}$ simulations (**Fig. 3B** and **3D**) with those of the PL/Ig^{WT} simulations (**Figs. 3A** and **3C**), we observed a noticeable decrease in the relative occupancy of Surface 1, the hydrophobic tip, and specifically CDR2 loop residues in the PL/$sTREM2^{WT}$ simulations compared to the corresponding Ig^{WT}/PL simulation. Instead, both PL/$sTREM2^{WT}$ simulations revealed ligand binding to the newly defined Expanded Surface 2 (**Fig. 3B** and **3D**, light orange regions), to which the $sTREM2^{WT}$ stalk was previously shown to bind, albeit with lower occupancy than Surface 1 residues (**Fig. 2E**). Significantly, there was an almost complete lack of PL binding to Expanded Surface 2 in the Ig^{WT} simulations. Collectively, these results suggest that Expanded Surface 2 serves as a secondary binding surface in sTREM2, becoming available for ligand binding when Surface 1 is occupied by the partial flexible stalk (and when Expanded Surface 2 is not *itself* bound by the stalk). Finally, we observed frequent interactions between both PLs and residues throughout the stalk domain of $sTREM2^{WT}$ during the simulations (**Fig. 3B** and **3D**, pink regions). This suggests that the stalk domain of sTREM2 may autonomously function as an additional binding site for diverse ligands.

We gained deeper insights into PL/(s)TREM2 interactions through visual analysis of our simulation trajectories using VMD and binding free energy calculations employing the MM-PBSA approach (see Methods). Representative snapshots from converged portions of the simulations for each protein/PL system (e.g., constant RMSD for at least 50 ns) are shown in **Fig. 3E-H**, alongside corresponding binding free energies. Among the WT systems, SOPS/Ig^{WT} exhibited the most favorable (lowest) binding free energy model, aligning with TREM2's known affinity for anionic ligands. Notably, SOPS and SOPC both directly interacted with Ig^{WT}'s CDR2 loop in the complexes with the lowest binding free energy (**Fig. 3E**, gray model and **Fig. 3G**, orange model).

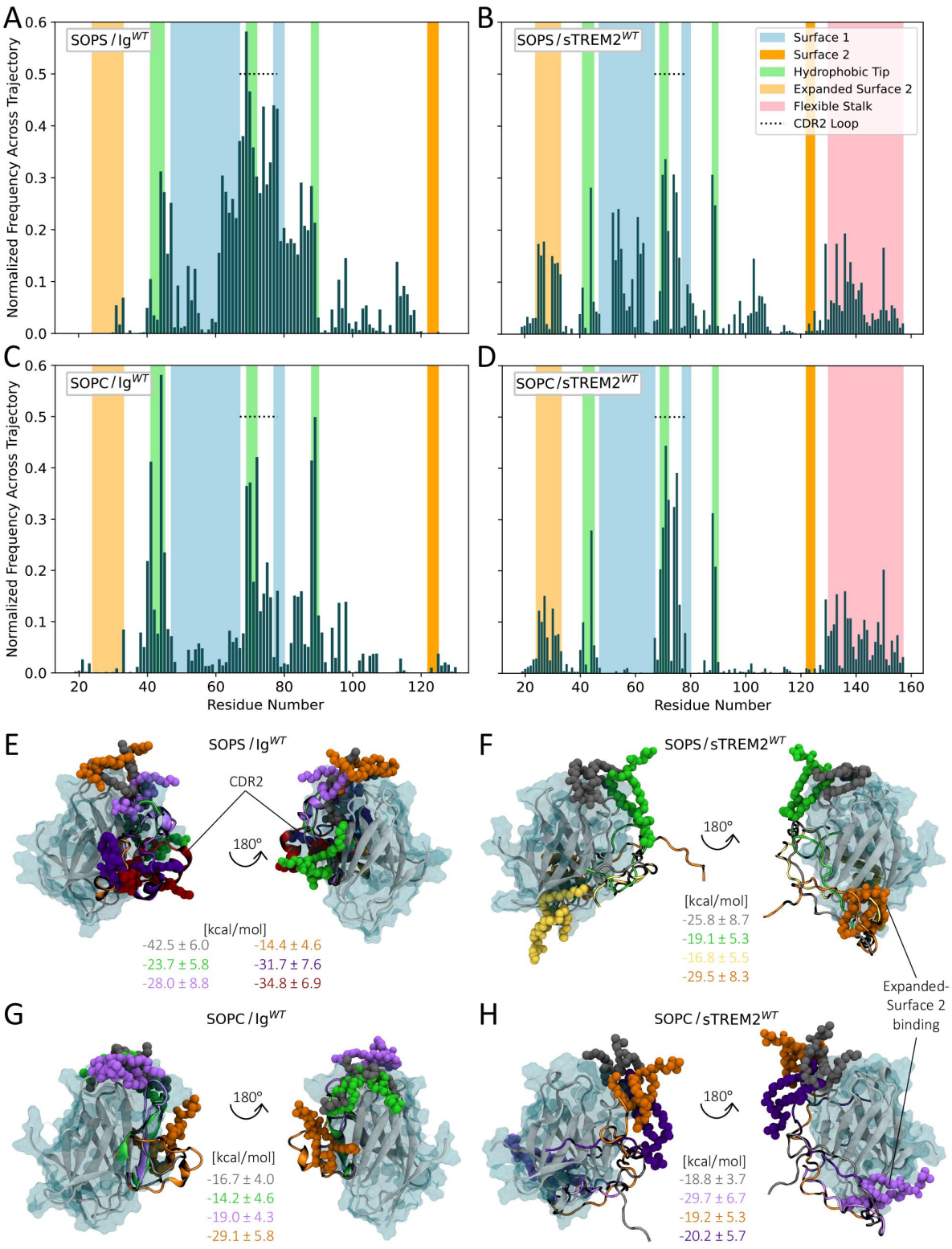

Figure 3.

The stalk of sTREM2^{WT} modulates phospholipid binding.

(A-D) Normalized fractional occupancy of residues in (A) Ig^{WT} by SOPS, (B) sTREM2^{WT} by SOPS, (C) Ig^{WT} by SOPC, and (D) sTREM2^{WT} by SOPC, averaged across seven, five, five, and six 150 ns simulations, respectively. (E-H) VMD snapshots of representative structures from the PL/(s)TREM2^{WT} simulations, with PLs and corresponding complex binding free energies shown in matching colors.

In contrast, both SOPS/sTREM2^{WT} and SOPC/sTREM2^{WT} bind most favorable at the newly coined 'Expanded Surface 2' (**Fig. 3F**, orange model and **Fig. 3H**, purple model). This is explained by RMSF calculations (**Fig. S2**) and visual trajectory analysis which reveal heightened dynamics in the stalk domain, indicating that SOPS and, to a lesser extent, SOPC binding disrupts homeostatic stalk-Ig domain interactions. This disruption is further supported by the observation that, in the absence of a ligand, the stalk most frequently interacts with Surface 1 (**Fig. 2E**), where SOPS and SOPC bind in the absence of the stalk (**Fig. 3A**). Cumulatively, this indicates competitive binding between the PLs and the stalk domain for the CDR2 loop and Surface 1 given their similar, negative/amphipathic-charged natures.

To gain deeper insights into the thermodynamics of (s)TREM2/PL interactions, we performed Interaction Entropy (IE) calculations (see Methods). Although many free energy calculations overlook entropy, recent studies have emphasized its importance in providing a complete understanding of a system's Gibbs free energy[67,68]. IE calculations take into account changes in the ligand, protein, and solvent, and they efficiently estimate entropies directly from MD simulations. These calculations are particularly useful for assessing relative entropies[65]. Our results broadly indicate that the entropic contributions (-TΔS) are positive, with higher values for SOPS models compared to SOPC models; however, this difference is not statistically significant **(Fig. S3)**. Entropic loss upon ligand binding is attributed to the restriction of conformational freedom in the ligand, protein, and solvent[65,67]. The greater entropic loss observed for SOPS models suggests greater 'snugness' of binding[69], implying that binding is more enthalpically driven, particularly for SOPS. However, the applicability of our IE results is constrained by high standard deviations, likely due to the dynamic nature of TREM2-PL binding[70].

Uniquely, some PL/TREM2 complexes were observed to undergo rapid conformational changes in the CDR2 loop during the simulations, seemingly occurring on much shorter timescales than in the ligand-free simulations. These changes included the dynamic opening and closing of the CDR2 loop and alterations to its α-helical character (**Fig. 3E-H**). This suggests a potential mechanism for a dynamically responsive CDR2 loop in the context of ligand binding. Nevertheless, further experiments are essential to better understand the functional significance of CDR2 loop remodeling in response to ligand binding and to identify the circumstances in which it may occur.

The AD-risk mutation R47H diminishes ligand discrimination by and binding to the Ig-like domain of (s)TREM2, while sTREM2's stalk domain partially restores overall ligand binding

To investigate the roles of (s)TREM2 in disease pathology, we examined PL binding in the presence of the AD-risk mutation R47H. Previous studies have suggested that the decreased ligand-binding capabilities of TREM2^{R47H} may underlie its observed loss-of-function[9,10,16,18]. Notably, one study showed reduced reporter cell activity of TREM2^{R47H} compared to TREM2^{WT} when binding anionic lipids such as PS, while no significant difference was observed in their binding to PC[18]. Separately, sTREM2^{R47H} and sTREM2^{WT} were shown to bind Aβ and inhibit fibrillization to a similar extent[11].

Upon examining the fractional occupancy plots of the PL/(s)TREM2^{R47H} simulations, we observed broadly similar patterns in binding to the Ig-like domain, regardless of the presence of the stalk or PL charge (**Fig. 4A-D**). Across all four simulations, PLs predominantly occupied the CDR2 loop and Surface 1 of the Ig-like domain. This uniformity in binding contrasts sharply with the previously discussed behavior observed in the PL/(s)TREM2^{WT} simulations (**Fig. 3A-D**), where notable differences in binding to SOPS and SOPC were observed for both Ig^{WT} and sTREM2^{WT}, as well as between the two proteins for a given PL. These results suggest that the presence of R47H diminishes the ability of Ig^{R47H} and sTREM2^{R47H} to distinguish between crucial brain-based ligands, proposing a novel mechanism for loss-of-function due to AD-risk mutations.

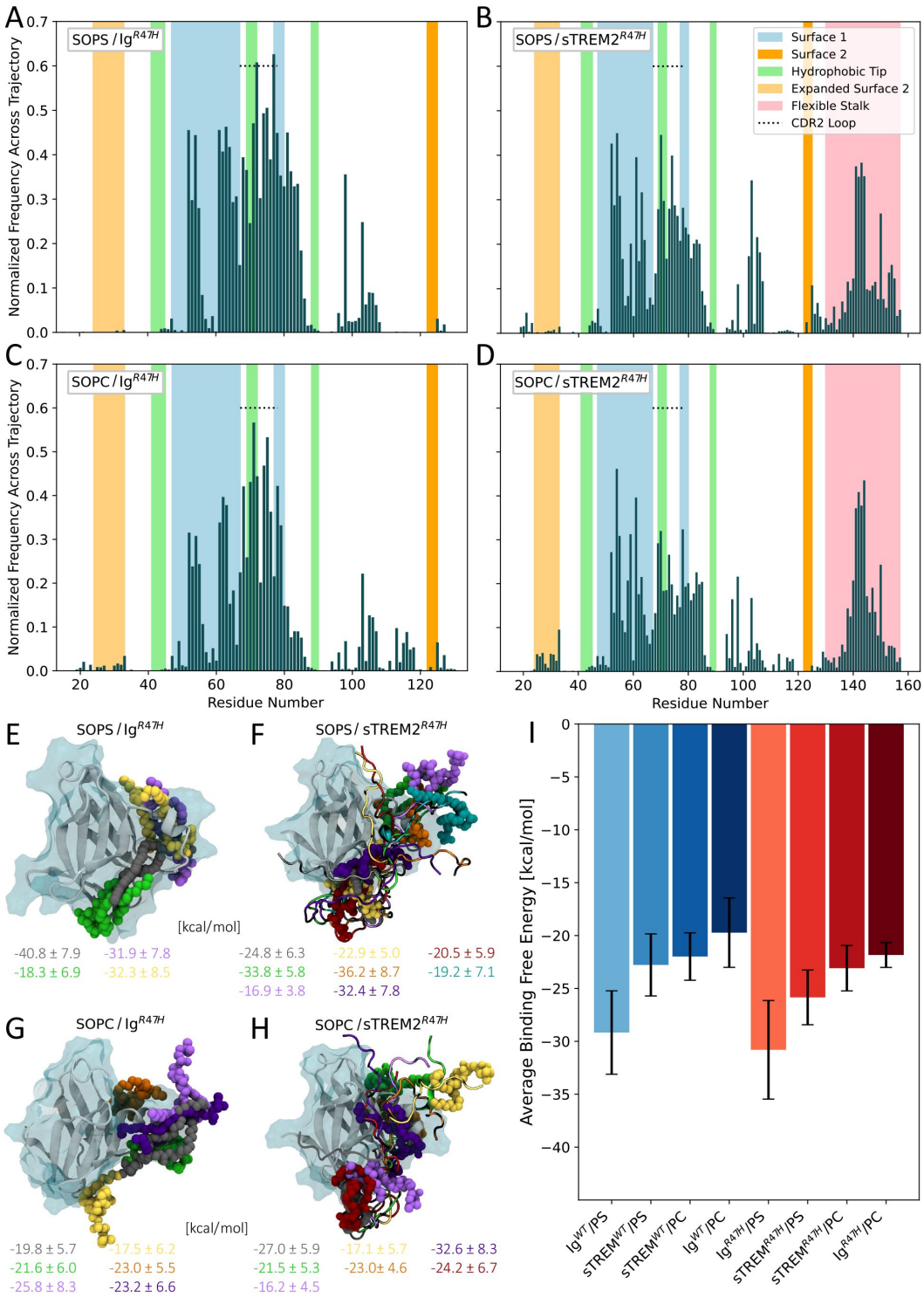

Figure 4.

R47H mutation decreases ligand discrimination but increases binding to the flexible stalk.

(A-D) Normalized fractional occupancy of residues in (A) Ig^{R47H} by SOPS, (B) sTREM2^{R47H} by SOPS, (C) Ig^{R47H} by SOPC, and (D) sTREM2^{R47H} by SOPC, averaged across four, seven, six, and eight 150 ns simulations, respectively. (E-H) VMD snapshots of representative structures from the PL/(s)TREM2^{R47H} simulations, with PLs and corresponding complex binding free energies shown in matching colors. (I) Comparison of the binding free energies averaged across all WT and R47H models for each PL/protein system. Errors bars represent the standard error of the mean.

Compared to the PL/(s)TREM2^{WT} simulations, all four PL/(s)TREM2^{R47H} simulations demonstrated marked reductions in PL binding to the hydrophobic tip. These differences likely arose from structural impacts on the hydrophobic tip region due to the more open and rigid nature of the nearby CDR2 loop in the presence of R47H, altering the ligand binding properties of the hydrophobic tip. Furthermore, variations in binding between the stalk and Ig-like domain due to R47H (**Fig. 2E-F**) also likely contributed to these PL binding changes. Additionally, diminished binding to Expanded Surface 2 was observed for PL binding to sTREM2^{R47H}.

Compensating for these reductions in binding, increased PL binding to Surface 1 was observed in all four R47H simulations, along with enhanced PL binding to the flexible stalk of sTREM2^{R47H}. These results provide further support for the notion that the sTREM2's stalk domain functions as an independent ligand binding site. Moreover, these results suggest the stalk domain may possess an inherent ability to 'rescue' sTREM2 deficiencies in ligand binding to the Ig-like domain due to disease-associated mutations, offering an explanation for sTREM2's neuroprotective role in AD. Indeed, this could explain why, as mentioned earlier, the presence of R47H does not significantly reduce sTREM2^{R47H}'s ability to bind Aβ and inhibit fibrillization to the same degree as sTREM2^{WT}.

Following the methodologies used to evaluate the PL/(s)TREM2^{WT} simulations, we conducted visual analysis of our trajectories paired with binding free energy calculations. Representative structures with corresponding binding free energies are shown in **Fig. 4E-H**. Broadly, binding free energy trends align closely with those observed for the WT complexes (**Fig. 3E-H**), with the SOPS/Ig^{R47H} complexes again exhibiting the lowest (most favorable) energy model (**Fig. 4E**). Unlike the WT complexes, however, these results do not indicate competitive binding between SOPS and the partial stalk region, evidenced by highly favorable binding in the CDR2/Surface 1 region for PL/(s)TREM2^{R47H} models. This may be a result of the persistently open CDR2 loop which alters surface accessibility and stalk dynamics.

The results show that across all models, sTREM2 and TREM2 bind PS more favorably than PC, affirming previous findings that TREM2 favors anionic ligands[30]. However, these differences are not statistically significant. Additionally, this analysis shows no significant difference or trend in binding free energies for PS/WT complexes compared to their PS/R47H equivalents, contrasting with previous experimental findings that the R47H mutation reduces TREM2's affinity for endogenous ligands[10,16,18] (**Fig. 4I**). Similar to WT models, we observe that for R47H models, SOPS binding poses exhibit higher average entropic contributions than their SOPC counterparts. These observations suggest that weaker ligand binding does not comprise the entire mechanism for signaling deficits in the R47H variant. Broadly, these results suggest that differences in binding across a range of ligands, (s)TREM2 variants, and between sTREM2 and TREM2 are governed by accessibility of protein surface residues and ligand binding patterns, factors which extend beyond mere differences in binding affinities.

Lastly, we noted an intriguing difference in the stability of the CDR2 loop between Ig^{R47H} and Ig^{WT}. Throughout the majority of the simulations, regardless of PL presence, the CDR2 loop in Ig^{R47H} remained stable in an open conformation, contrasting with the Ig^{WT} simulations where the CDR2 loop exhibited dynamic opening and closing in response to PL binding. Thus, in addition to directly impacting ligand affinities and impairing the ability to differentiate between ligands, the R47H mutation may inhibit the CDR2 loop's ability to dynamically respond to ligand binding, thereby preventing physiological TREM2 signaling. However, further studies of the TREM2-DAP12 complex are needed to understand how ligand binding to the Ig-like domain propagates signaling and how these mechanisms may be disrupted in the R47H variant.

Discussion and Conclusions

Main Conclusions

We utilized long-timescale MD simulations to investigate relevant structural domains of sTREM2 and TREM2, along with their interactions with key PLs in the brain. Through the analysis of RMSF, RMSD, and surface occupancy calculations, we established the flexible stalk domain of sTREM2 as the molecular basis for differential ligand binding between sTREM2 and TREM2. This difference in binding arises from the stalk's role in stabilizing the Ig-like domain and altering the ligand-accessibility of its surface residues. By integrating free energy, interaction entropy, and ligand occupancy calculations, we quantified the energetics of these interactions, confirming the presence of an alternate ligand binding site on $sTREM2^{WT}$, which we termed the 'Expanded Surface 2'. The binding of PLs to this site results from the competitive binding of the flexible stalk domain to Surface 1 on the Ig-like domain. These stalk-Ig-like domain interactions were disrupted in the presence of the AD-risk mutation R47H and entirely absent in the Ig (TREM2) models, indicating occupancy of Expanded Surface 2 occurs solely with $sTREM2^{WT}$. These observations underscore our conclusion that, rather than (or in addition to) its previously conceived role as a dummy receptor for TREM2, the flexible stalk domain confers sTREM2 with unique ligand binding preferences and patterns that facilitate distinct endogenous functions compared to TREM2.

Furthermore, we found that the stalk domain itself serves as an independent site for ligand binding, with heightened PL occupancy observed in the presence of R47H compared to the wildtype model. This suggests that the stalk domain may have the capacity to partially 'rescue' dysfunctional ligand binding to the Ig-like domain of sTREM2 caused by disease-associated mutations like R47H. Moreover, our observations for both sTREM2 and TREM2 indicate that R47H-induced dysfunction may result not only from diminished ligand binding but also an impaired ability to discriminate between different ligands in the brain, proposing a novel mechanism for loss-of-function. In summary, the findings of this study reveal the endogenous structural and dynamical mechanisms of (s)TREM2, a critical component in AD pathology. These insights offer new fundamental knowledge that can serve as guiding principles for the design of future therapeutics, paving the way for potential advancements in AD treatment strategies.

Ideas and Speculation

Our findings suggest that loss-of-function in sTREM2 and TREM2 occurs not only through experimentally observed loss of binding affinity, but also through a change in binding patterns and a loss of ligand discrimination capacity. Altered ligand binding may hinder the ability of TREM2 and its co-signaling partner DAP12 to transmit signals across the cell membrane. This deficiency implicates an impaired microglial response to ligand binding as a key mechanism for dysfunction in AD. Pathologically, this would lead to reduced lipid uptake[71], diminished Aβ plaque clearance[19], decreased microglial activation[71], and inhibited intracellular lipid metabolism[72]. Previous studies speculate that reduced microglial lipid metabolism can trigger neurotoxic activation states or loss of neuroprotective functions[73]. Furthermore, the inability to discriminate among PLs may result in an invariable response from microglia when presented with diverse ligands. While PC is the most abundant PL in cell membranes, PS expression on the outer membrane leaflet increases in apoptotic cells, acting as a damage-associated signal[74]. Failure to differentiate between these PLs may lead to chronic over-activation of microglia, or at the very least, loss of endogenous protective functions.

Recently, monoclonal antibodies targeting TREM2 have emerged[75–77], designed to bind to the stalk region, above its cleavage site[76,77]. Consequently, the primary binding epitope of at least one of these antibodies also resides on sTREM2. Notably, this antibody also reduces the shedding of sTREM2[77]. It is conceivable that treatment with this and similar antibodies may compromise the

endogenous function of sTREM2, given that the stalk, a domain our study has identified as functionally significant, may be sequestered by the antibody. This may ultimately result in off-target effects for these therapeutics and prompts a consideration of how to separately target sTREM2 and TREM2.

Limitations

As with all models, particularly computational ones, it is crucial to recognize their limitations. In our study, the initial positions of PLs bound to (s)TREM2 were determined exclusively by AutoDock Vina. To mitigate potential bias from the docking process, we conducted MD simulations on all unique structures observed in the top 20 docked models. Rigorous, yet computationally efficient free energy calculations remain a challenge for computational protein-ligand studies. Despite employing the latest MM-PBSA free energy calculation algorithms and novel interaction entropy calculations, achieving statistical significance for many of the trends we observed that align with experiments remained challenging. This difficulty was further compounded by our approach of averaging statistics across multiple models of PLs docked to different initial binding locations on (s)TREM2. While this approach was intended to holistically capture PL/(s)TREM2 interactions, it effectively diluted our sample size against any specific epitope, potentially impacting the statistical significance of our findings. Future studies could address these challenges by utilizing larger sample sizes of independent MD simulations and more intensive energy calculations as computational power and resources continue to advance.

Moreover, our study, like previous investigations of TREM2, assumed that the Ig-like domain is representative of membrane-bound TREM2. This assumption overlooks potential contributions from the membrane, membrane-bound flexible stalk domain, DAP12, or TREM2 glycosylation. Ultimately, more biologically representative simulations of membrane-bound TREM2 are warranted to test this assumption. Additionally, it is unlikely that PLs are presented as individual soluble ligands in the brain in an *in vivo* setting. Instead, they are more likely co-presented with other components in lipoproteins or in contexts such as demyelination, cell debris, or Aβ. This co-presentation would influence the charge and steric profiles of lipid presentation. While our study provides valuable insights and fills existing knowledge gaps, further research is necessary to fully understand TREM2 binding in more biologically relevant ligand-binding contexts.

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Editors

Reviewing Editor
Volker Dötsch
Goethe University Frankfurt, Frankfurt am Main, Germany

Senior Editor
Volker Dötsch
Goethe University Frankfurt, Frankfurt am Main, Germany

Reviewer #1 (Public review):

In this manuscript, Saeb et al reported the mechanistic roles of the flexible stalk domain in sTREM2 function using molecular dynamics simulations. They have reported some interesting molecular bases explaining why sTREM2 shows protective effects during AD, such as partial extracellular stalk domain promoting binding preference and stabilities of sTREM2 with its ligand even in the presence of known AD-risk mutation, R47H. Furthermore, they found that the stalk domain itself acts as the site for ligand binding by providing an "expanded surface", known as 'Expanded Surface 2' together with the Ig-like domain. Also, they observed no difference in the binding free energy of phosphatidyl-serine with wild TREM2-Ig and mutant TREM2-Ig, which is a bit inconsistent with the previous report with

experiment studies by Journal of Biological Chemistry 293, (2018), Alzheimer's and Dementia 17, 475-488 (2021), Cell 160, 1061-1071 (2015).

Perhaps the authors made significant efforts to run a number of simulations for multiple models, which is nearly 17 microseconds in total; none of the simulations has been repeated independently at least a couple of times, which makes me uncomfortable to consider this finding technically true. Most of the important conclusions that authors claimed, including the opposite results from previous research, have been made on the single run, which raises the question of whether this observation can be reproduced if the simulation has been repeated independently. Although the authors stated the sampling number and length of MD simulations in the current manuscript as a limitation of this study, it must be carefully considered before concluding rather than based on a single run.

sTREM2 shows a neuroprotective effect in AD, even with the mutations with R47H, as evidenced by authors based on their simulation. sTREM2 is known to bind Aβ within the AD and reduce Aβ aggregation, whereas R47H mutant increases Aβ aggregation. I wonder why the authors did not consider Aβ as a ligand for their simulation studies. As a reader in this field, I would prefer to know the protective mechanism of sTREM2 in Aβ aggregation influenced by the stalk domain.

In a similar manner, why only one mutation is considered "R47H" for the study? There are more server mutations reported to disrupt tethering between these CDRs, such as T66M. Although this "T66M" is not associated with AD, I guess the stalk domain protective mechanism would not be biased among different diseases. Therefore, it would be interesting to see whether the findings are true for this T66M.

In most previous studies, the mechanism for CDR destabilization by mutant was explored, like the change of secondary structures and residue-wise interloop interaction pattern. While this is not considered in this manuscript, neither detailed residue-wise interaction that changed by mutant or important for 'ligand binding" or "stalk domain".

The comparison between the wild and mutant and other different complex structures must be determined by particular statistical calculations to state the observed difference between different structures is significant. Since autocorrelation is one of the major concerns for MD simulation data for predicting statistical differences, authors can consider bootstrap calculations for predicting statistical significance.

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Reviewer #2 (Public review):

Significance:

TREM2 is an immunomodulatory receptor expressed on myeloid cells and microglia in the brain. TREM2 consists of a single immunoglobular (Ig) domain that leads into a flexible stalk, transmembrane helix, and short cytoplasmic tail. Extracellular proteases can cleave TREM2 in its stalk and produce a soluble TREM2 (sTREM2). TREM2 is genetically linked to Alzheimer's disease (AD), with the strongest association coming from an R47H variant in the Ig domain. Despite intense interest, the full TREM2 ligand repertoire remains elusive, and it is unclear what function sTREM2 may play in the brain. The central goal of this paper is to assess the ligand-binding role of the flexible stalk that is generated during the shedding of TREM2. To do this, the authors simulate the behavior of constructs with and without stalk. However, it is not clear why the authors chose to use the isolated Ig domain as a surrogate for full-length TREM2. Additionally, experimental binding evidence that is misrepresented by the authors contradicts the proposed role of the stalk.

Summary and strengths:

The authors carry out MD simulations of WT and R47H TREM2 with and without the flexible stalk. Simulations are carried out for apo TREM2 and for TREM2 in complex with various lipids. They compare results using just the Ig domain to results including the flexible stalk that is retained following cleavage to generate sTREM2. The computational methods are well-described and should be reproducible. The long simulations are a strength, as exemplified in Figure 2A where a CDR2 transition happens at ~400-600 ns. The stalk has not been resolved in structural studies, but the simulations suggest the intriguing and readily testable hypothesis that the stalk interacts with the Ig domain and thereby contributes to the stability of the Ig domain and to ligand binding. I suspect biochemists interested in TREM2 will make testing this hypothesis a high priority.

Weaknesses:

Unfortunately, the work suffers from two fundamental flaws.

(1) The authors state that reported differences in ligand binding between the TREM2 and sTREM2 remain unexplained, and the authors cite two lines of evidence. The first line of evidence, which is true, is that there are differences between lipid binding assays and lipid signaling assays. However, signaling assays do not directly measure binding. Secondly, the authors cite Kober et al 2021 as evidence that sTREM2 and TREM2 showed different affinities for Abeta1-42 in a direct binding assay. Unfortunately, when Kober et al measured the binding of sTREM2 and Ig-TREM2 to Abeta they reported statistically identical affinities (Kd = 3.8 {plus minus} 2.9 μM vs 5.1 {plus minus} 3.7 μM) and concluded that the stalk did not contribute measurably to Abeta binding.

(2) The authors appear to take simulations of the Ig domain (without any stalk) as a surrogate for the full-length, membrane-bound TREM2. They compare the Ig domain to a sTREM2 model that includes the stalk. While it is fully plausible that the stalk could interact with and stabilize the Ig domain, the authors need to demonstrate why the full-length TREM2 could not interact with its own stalk and why the isolated Ig domain is a suitable surrogate for this state.

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Author response:

> ***Review #1:***
>
> *Also, they observed no difference in the binding free energy of phosphatidylserine with wild TREM2-Ig and mutant TREM2-Ig, which is a bit inconsistent with the previous report with experiment studies by Journal of Biological Chemistry 293, (2018), Alzheimer's and Dementia 17, 475-488 (2021), Cell 160, 1061-1071 (2015).*

We directly note this contrast with experimental findings in the body of our work, particularly given the known limitations of free energy calculations in MD simulations, as outlined in the Limitations section. Our claim is that the loss of function in the R47H variant extends beyond decreased binding affinities and also impacts binding patterns. As stated in our manuscript: ‘Our observations for both sTREM2 and TREM2 indicate that R47H-induced dysfunction may result not only from diminished ligand binding but also an impaired ability to discriminate between different ligands in the brain, proposing a novel mechanism for loss-of-function.’

> *Perhaps the authors made significant efforts to run a number of simulations for multiple models, which is nearly 17 microseconds in total; none of the simulations has been repeated independently at least a couple of times, which makes me uncomfortable to consider this finding technically true. Most of the important conclusions that authors claimed, including the opposite results from previous research, have been made on the single run, which raises the question of whether this observation can be reproduced if the simulation has been repeated independently. Although the authors stated the sampling number and length of MD simulations in the current manuscript as a limitation of this study, it must be carefully considered before concluding rather than based on a single run.*

The reviewer raises an interesting point regarding the repetition of individual simulations, a consideration we carefully evaluated during the design of this study. However, we believe our approach—running multiple independent models of the same system—offers a more rigorous methodology than simply repeating simulations of the same docked model. This strategy allows us to sample several distinct starting configurations, thereby minimizing biases introduced by docking algorithms and single-model reliance.

In our study, we demonstrate that within the 150 ns timescale of our protein/ligand (PL) simulations, the relatively small ligands are able to move from their initial docking positions to a specific binding site. While ideally, replicates of these independent models would further strengthen the findings, this was not computationally feasible given the unprecedented total duration of our simulations. Importantly, our conclusions are seldom based on the results of a single protein/PL simulation.

Moreover, the ergodic hypothesis suggests that over sufficiently long timescales, simulations will explore all accessible states. Additionally, we have performed several replicate simulations of our WT and R47H Ig-like domain models in solution, specifically to investigate CDR2 loop dynamics.

In this case, since the system involves only the protein and lacks the independent replicates seen in the protein/PL simulations, these runs were chosen to effectively capture the stochastic nature of CDR2 loop movement.

> *sTREM2 shows a neuroprotective effect in AD, even with the mutations with R47H, as evidenced by authors based on their simulation. sTREM2 is known to bind Aβ within the AD and reduce Aβ aggregation, whereas R47H mutant increases Aβ aggregation. I wonder why the authors did not consider Aβ as a ligand for their simulation studies. As a reader in this field, I would prefer to know the protective mechanism of sTREM2 in Aβ aggregation influenced by the stalk domain.*

Our initial approach for this study used Aβ as a ligand rather than phospholipids. However, we noted the difficulties in simulating Aβ, particularly in choosing relevant Aβ structures and oligomeric states (n-mers). We believe that phospholipids represent an equally pertinent ligand for TREM2, given its critical role in lipid sensing and metabolism. Furthermore, there is growing recognition in the AD research community of the need to move beyond Aβ and focus on other understudied pathological mechanisms.

> *In a similar manner, why only one mutation is considered "R47H" for the study? There are more server mutations reported to disrupt tethering between these CDRs, such as T66M. Although this "T66M" is not associated with AD, I guess the stalk domain protective mechanism would not be biased among different diseases. Therefore, it would be interesting to see whether the findings are true for this T66M.*

In most previous studies, the mechanism for CDR destabilization by mutant was explored, like the change of secondary structures and residue-wise interloop interaction pattern. While this is not considered in this manuscript, neither detailed residue-wise interaction that changed by mutant or important for 'ligand binding" or "stalk domain".

These are both excellent points that deserve extensive investigation. While R47H is the most common and prolific mutation in literature, an extensive catalog of other mutations is important to explore. We are currently preparing two separate publications that will delve into these gaps in more detail, as addressing them was beyond the scope of the present study.

> *The comparison between the wild and mutant and other different complex structures must be determined by particular statistical calculations to state the observed difference between different structures is significant. Since autocorrelation is one of the major concerns for MD simulation data for predicting statistical differences, authors can consider bootstrap calculations for predicting statistical significance.*

We are currently working to address this comment to strengthen the validity of our results and statistical conclusions in the revised manuscript.

> ***Review #2:***
>
> *The authors state that reported differences in ligand binding between the TREM2 and sTREM2 remain unexplained, and the authors cite two lines of evidence. The first line of evidence, which is true, is that there are differences between lipid binding assays and lipid signaling assays. However, signaling assays do not directly measure binding. Secondly, the authors cite Kober et al 2021 as evidence that sTREM2 and TREM2 showed different affinities for Abeta1-42 in a direct binding assay. Unfortunately, when Kober et al measured the binding of sTREM2 and Ig-TREM2 to Abeta they reported statistically identical affinities (Kd = 3.8 {plus minus} 2.9 μM vs 5.1 {plus minus} 3.7 μM) and concluded that the stalk did not contribute measurably to Abeta binding.*

We appreciate the reviewer's insight and acknowledge the need to clarify our interpretation of Kober et al. (2021). We will adjust and refocus how we reference this evidence from Kober et al. in our revised manuscript.

In line with these findings, our energy calculations reveal that sTREM2 exhibits weaker—but still not statistically significant—binding affinities for phospholipids compared to TREM2. These results suggest that while overall binding affinity might be similar, differences in binding patterns or specific lipid interactions could still contribute to functional differences observed between TREM2 and sTREM2.

> *The authors appear to take simulations of the Ig domain (without any stalk) as a surrogate for the full-length, membrane-bound TREM2. They compare the Ig domain to a sTREM2 model that includes the stalk. While it is fully plausible that the stalk could interact with and stabilize the Ig domain, the authors need to demonstrate why the full-length TREM2 could not interact with its own stalk and why the isolated Ig domain is a suitable surrogate for this state.*

We believe that this is a major limitation of all computational work of TREM2 to-date, and of experimental work which only presents the Ig-like domain. This is extensively discussed in the limitations section of our paper. Hence, we are currently working toward a manuscript that will be the first biologically relevant model of TREM2 in a membrane and will challenge the current paradigm of using the Ig-like domain as an experimental surrogate for TREM2.

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