

In Silico Structural Insights For Toll-like Receptor 4 (TLR4) Targeted Imaging Probe Design

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Background

- Neuroinflammation is an inflammatory response in the brain or spinal cord by factors including aging, cancer, neurodegeneration.
- Toll-like receptor 4 (TLR4) is a key mediator of innate immune activation and neuroinflammatory signaling, implicated in Alzheimer's disease, cancer, and infectious pathologies.
- In neurodegeneration, aggregated amyloid- β (A β) and hyperphosphorylated tau activate TLR4 on microglia, with A β and tau detectable up to 15-20 years before clinical symptom onset.^{1,2}
- Current probes lack the specificity and affinity for high-resolution imaging of neuroinflammation that could enable early detection and monitoring.
- This study aims to characterize the MD-2 LPS-binding pocket and identify key residues to inform the design of TLR4 antagonists and imaging probes.

Materials & Methods: Structure Preparation

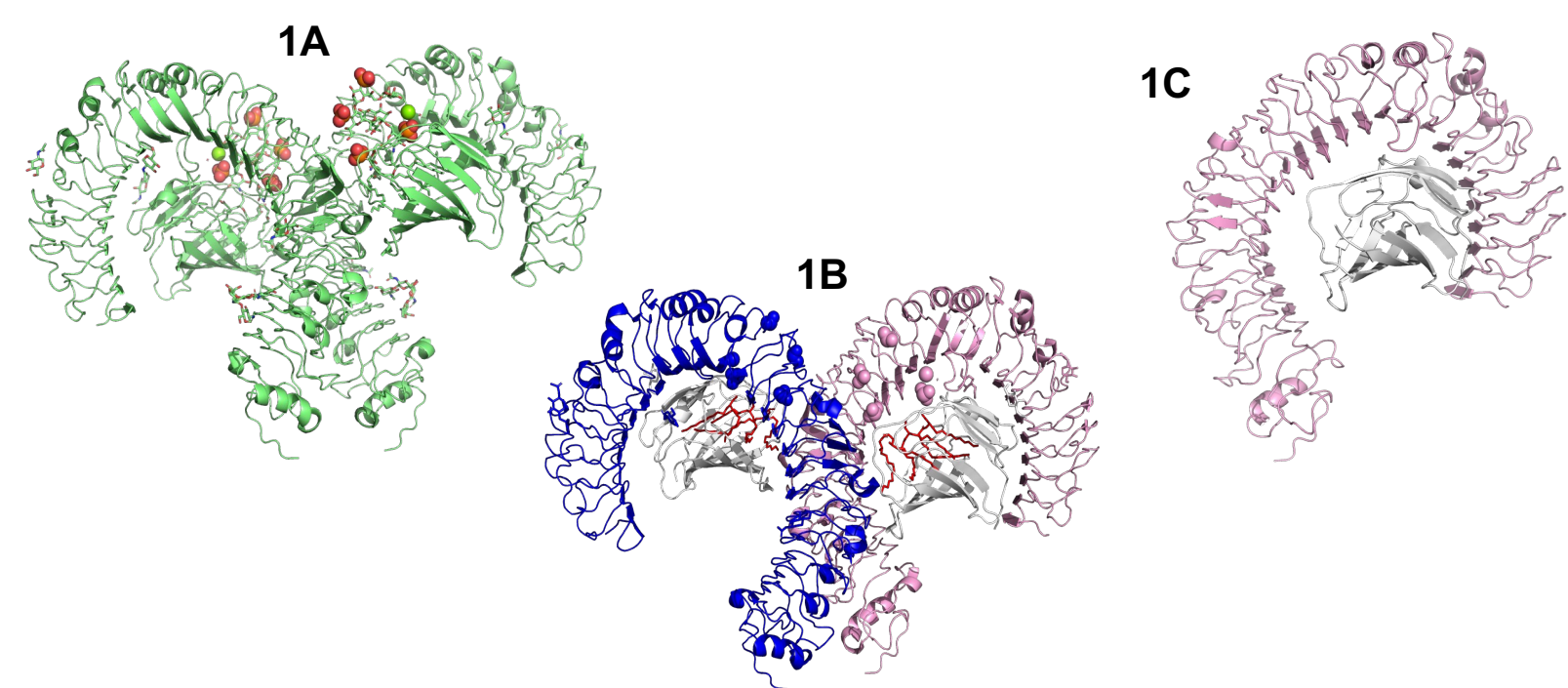


Figure 1. Structural representation of the human TLR4 complex (PDB 3FXI): **A)** Crystal structure of TLR4-MD-2-LPS, **B)** cleaned MD-2 (gray) with LPS (red) highlighting two TLR4 heterodimers (blue/pink), and **C)** induced-fit apo MD-2/TLR4 heterodimer.

- Human MD-2-LPS-TLR4 heterotetramer (PDB 3FXI) prepared in PyMOL; by removing crystallization artifacts and LPS to generate a holo-apo induced-fit conformation. (**Fig. 1A-B**).
- Structure validated via clash-check, to ensure a biological relevancy and structural integrity (**Fig.1C**).

TLR4 Signaling Pathway & Physiology

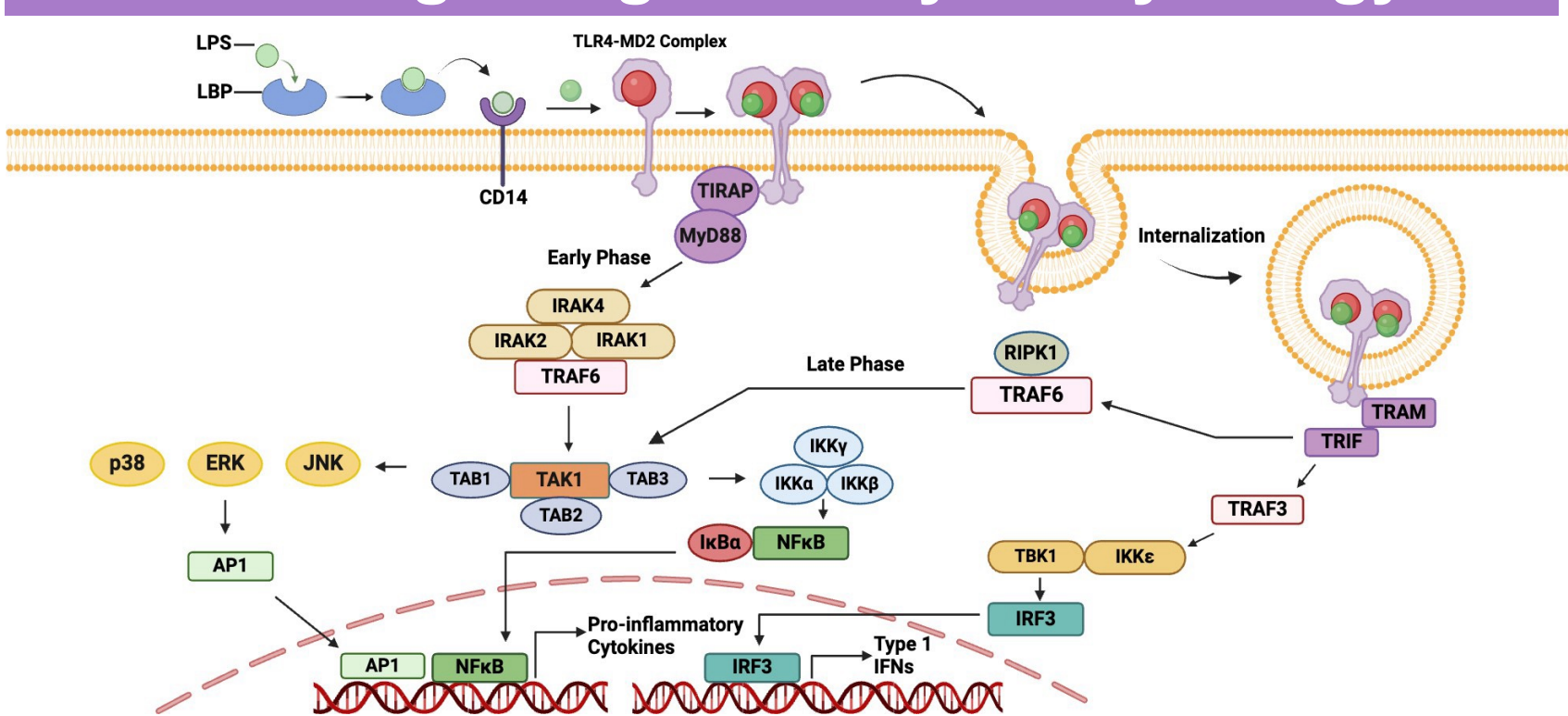


Figure 2. TLR4 Signaling Pathway³

- MyD88 Pathway (Immediate):** TLR4 dimerization recruits MyD88 at the TIR domain, rapidly activating NF- κ B and inducing pro-inflammatory cytokines (e.g., TNF- α , IL-6).
- TRIF Pathway (Delayed):** Endosomal internalization of the activated complex recruits TRIF, leading to IRF3 activation and production of Type I Interferons (e.g., IFN- β), supporting antiviral immune responses.

Materials & Methods: Computational Analysis

- The holo-apo structure of human TLR4-MD-2 (PDB ID: 3FXI) was analyzed *in silico* to characterize the Lipopolysaccharide binding site.
- Pocket detection and quantification were performed using the Fpocket (v3.1).
- Pockets were visualized with a custom gradient (**Fig. 3A**).

Results: TLR4/MD2 Binding Pocket Analysis

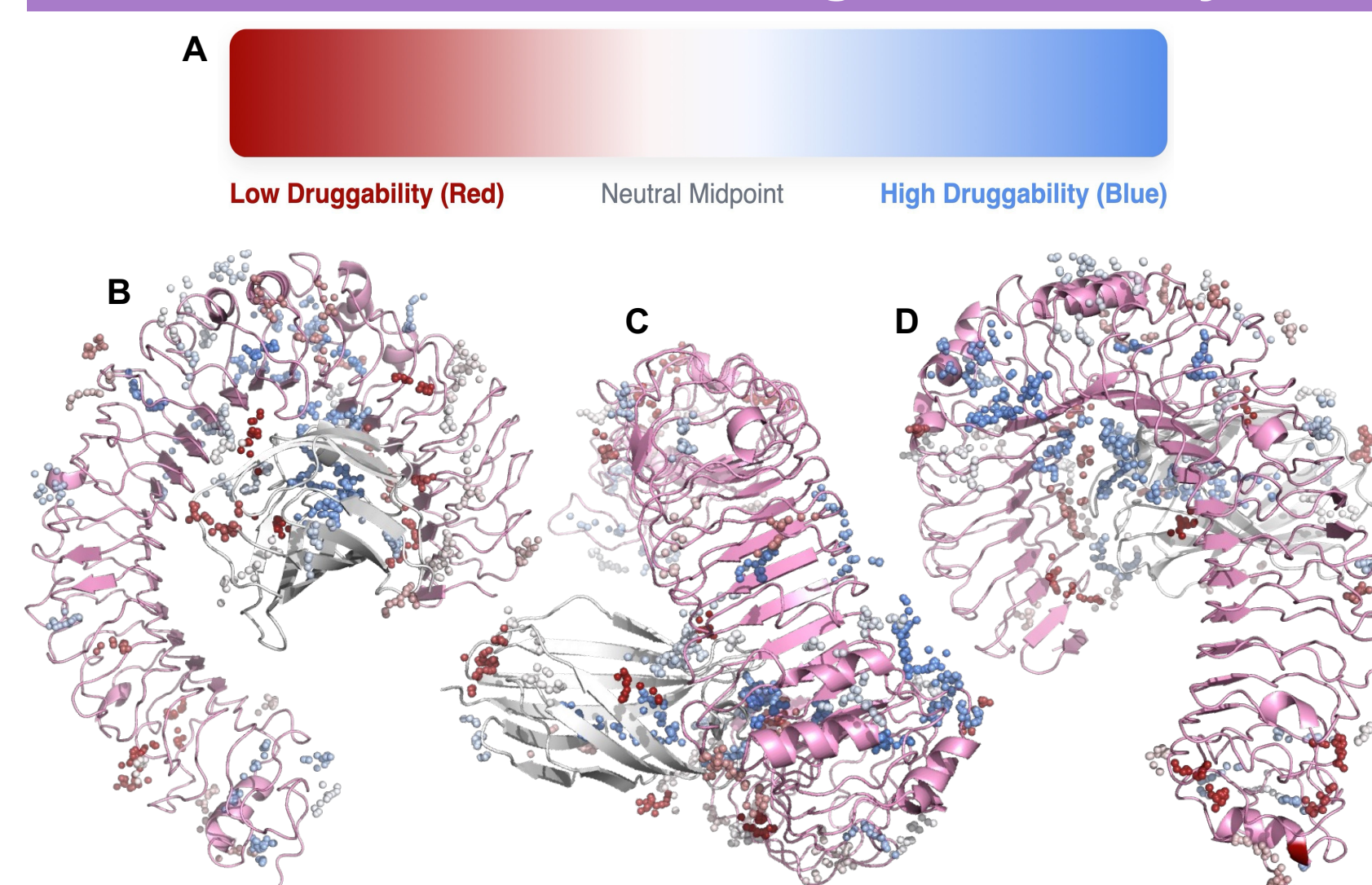


Figure 3. Fpocket analysis revealed 59 druggable pockets **A)** Druggability Gradient, **B)** Frontal, **C)** Top, and **D)** rear views of the identified pockets.

- 3A.** Custom 59-point color gradient for visualization, where red represents lowest (Pocket 59) and blue represents highest (Pocket 1) druggability scores.
- 3B-3D.** Fpocket identified 59 potential surface cavities within the TLR4-MD-2 protein complex ranked by druggability scores.
- Pocket 6 was selected due to alignment with the LPS-binding cavity and a high Druggability Score (0.984).
- The LPS-binding pocket is a deep cavity adapted to accommodate large amphipathic glycolipids such as LPS.

Results: MD-2 Binding Pocket & Analysis

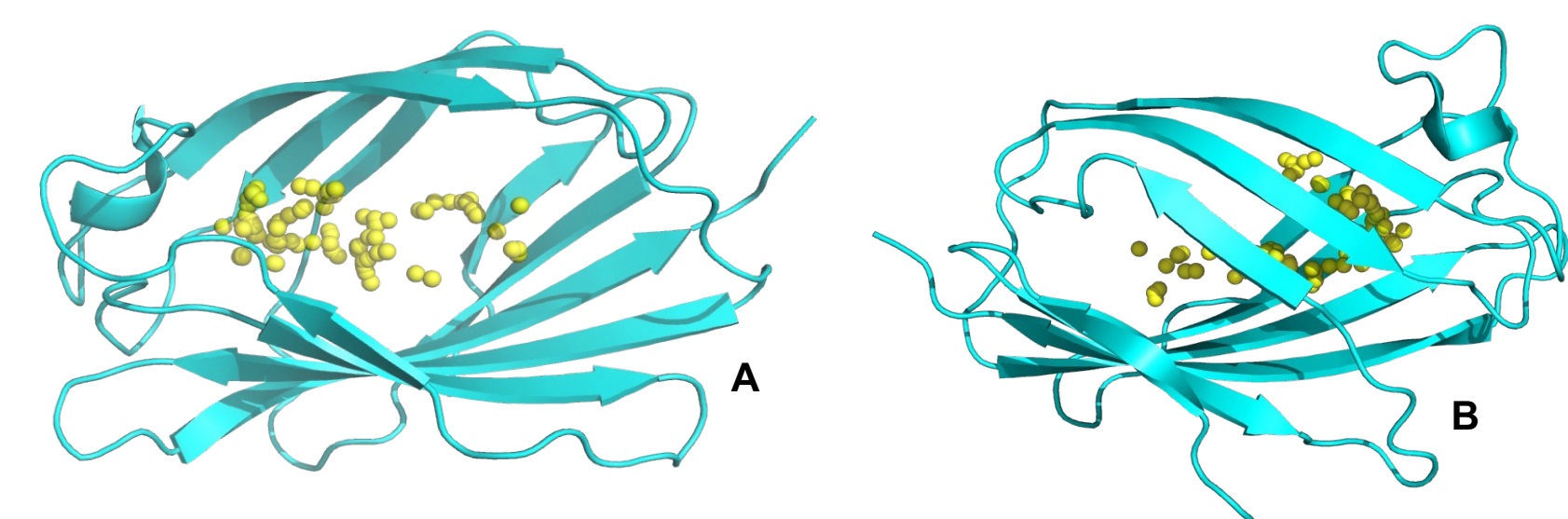


Figure 4. Front (A) and rear (B) views of pocket 6 in MD-2.

- Fpocket provided a detailed physicochemical characterization of Pocket 6. The pocket has a total binding volume of 941.353 Å³, a Druggability Score of 0.984, and a high Hydrophobicity Score of 88.333, reflecting its suitability for accommodating lipid chains.
- Together, these values highlight the pocket's defined chemical environment and high potential for ligand engagement.

Results: MD-2 Binding Pocket & Analysis

Metric	Value	Description
Druggability Score	0.98	Likelihood the pocket can bind drug-like molecules
Volume	941 Å ³	Total pocket size available for ligand binding
Hydrophobicity Score	88.33	Degree of hydrophobic character in the pocket
Apolar Alpha Sphere Proportion	1	Fraction of the pocket dominated by apolar regions
Total SASA	254 Å ²	Solvent-accessible surface area of the pocket
Mean Local Hydrophobic Density	71.15	Density of hydrophobic regions within the pocket
Structural Flexibility	0.15	Degree of conformational mobility in the pocket

Results: Identification of Critical Binding Residues

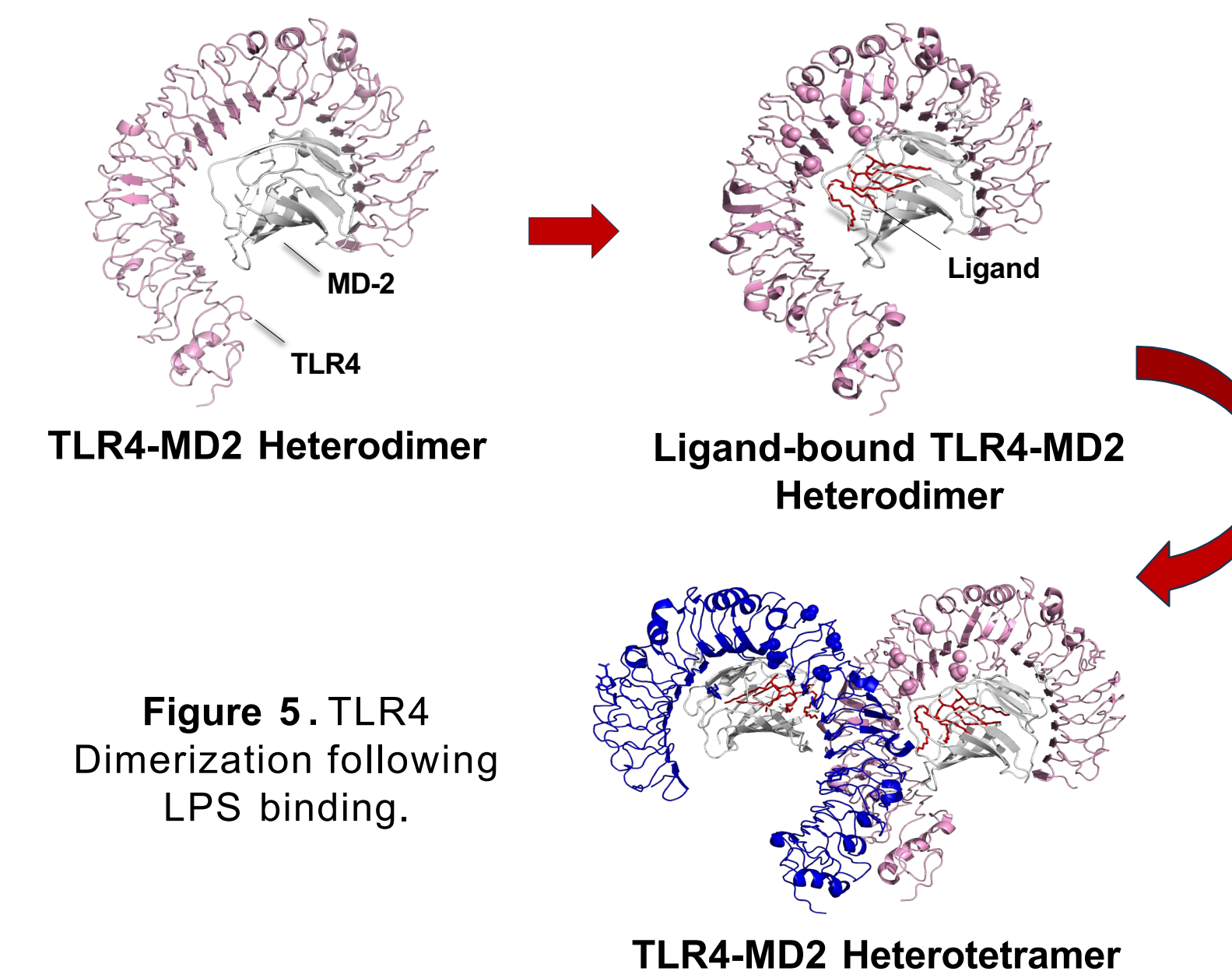


Figure 5. TLR4 Dimerization following LPS binding.

- An inter-heterodimer bridge forms via contact between critical TLR4 residues Phe⁴⁴⁰ and Phe⁴⁶³ on one heterodimer and the MD-2 of an adjacent heterodimer.⁵

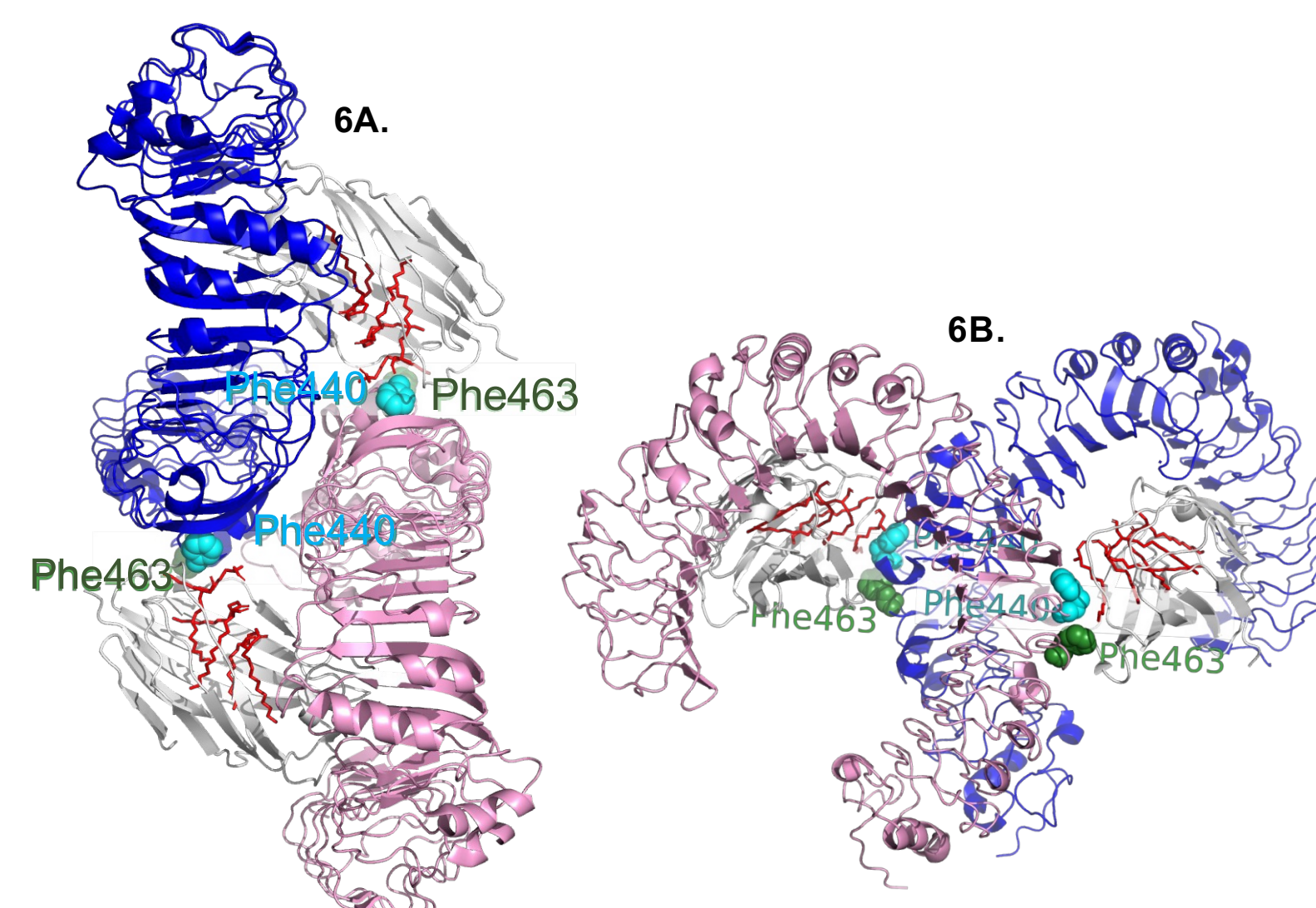


Figure 6. Top (A) and front (B) views of Phe⁴⁴⁰ and Phe⁴⁶³.

- Phe⁴⁴⁰ and Phe⁴⁶³ is positioned at the dimerized TLR4-MD-2 interface.
- Structural context demonstrates that Phe⁴⁴⁰ and Phe⁴⁶³ reinforce inter-chain alignment for TLR4 by aromatic packing at the [TLR4-MD-2]₂ interface, stabilizing its geometry.⁵ (**Fig. 5**)

Structural Insights for Binding and Activation

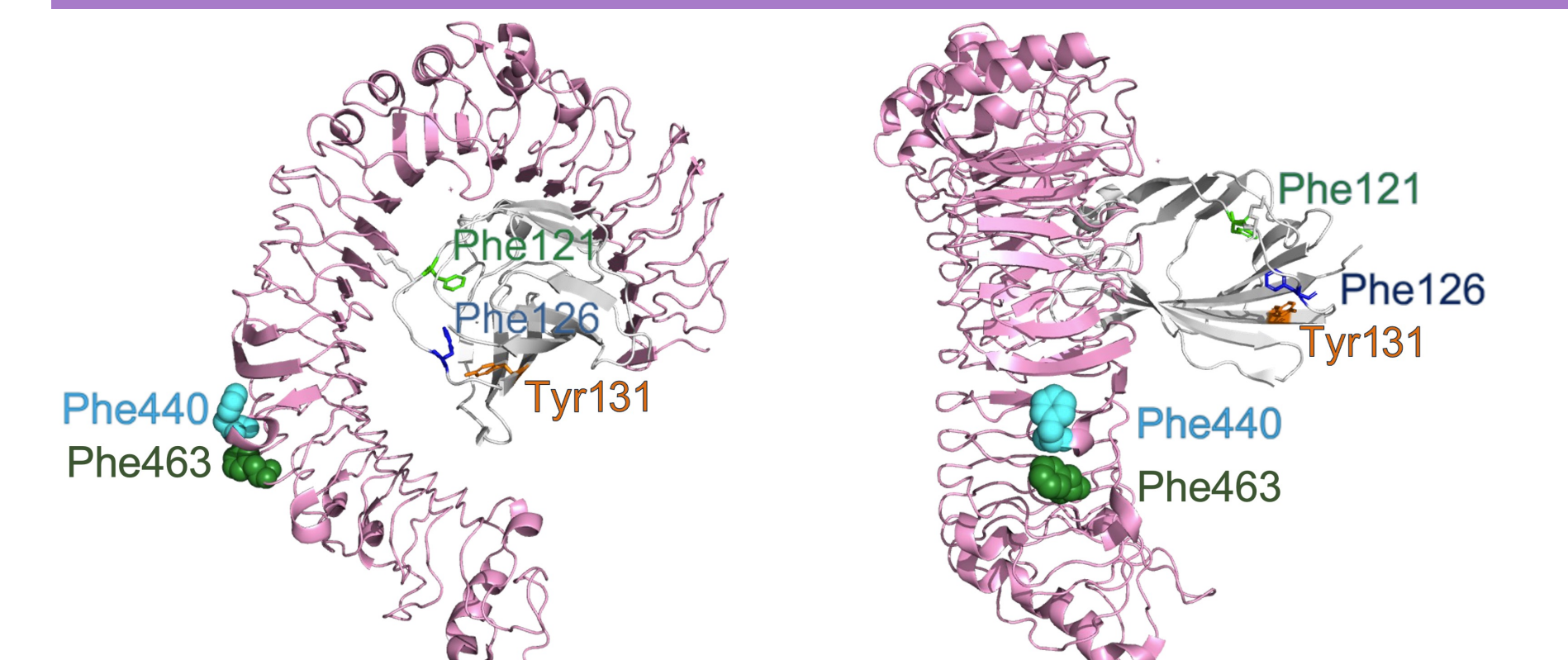


Figure 7A. Front View of key residues.

Figure 7B. Side View of key residues.

The front (**7A**) and side (**7B**) views of the human TLR4/MD-2 complex showing the spatial positioning of key aromatic residues within the MD-2 cavity and TLR4 extracellular domain.

- HEK293 mutagenesis identifies Phe¹²¹ and Tyr¹³¹ as critical for CD14-to-MD-2 endotoxin transfer and assembly of a signaling-competent TLR4 complex.⁴
- Phe¹²¹ lies near the MD-2 hydrophobic pocket entrance, supporting acyl-chain insertion.
- Tyr¹³¹ is positioned near the phosphate-binding region that orients lipid A headgroups. (**Fig. 7A-B**).^{4,5}
- The F126A mutation retains LPS binding but blocks receptor activation, transforming MD-2 towards an antagonist-like state.^{4,5}

Residues Critical for TLR4 Activation

- Phe¹²¹ and Tyr¹³¹ MD-2 stabilize LPS binding by anchoring acyl chains and orienting phosphate headgroups, enabling efficient CD14-to-MD-2 transfer and agonist competent loading.⁴
- Phe¹²⁶ functions as a conformational switch controlling the exposure of the sixth LPS acyl chain, regulating agonism and antagonism (F126A disrupts signaling).⁴
- Phe⁴⁴⁰ and Phe⁴⁶³ mediate inter-heterodimer packing and lock the [TLR4-MD-2]₂ complex into an active signaling state (F440A/F463A validated).⁵

Conclusions & Future Directions

- Validate binding-site interactions using site-directed mutagenesis (F121A, F126A, Y131A, F440A, F463A) combined with NF- κ B and IRF reporter assays.
- Design a high-affinity imaging probe by exploiting the Phe¹²¹ anchor site for enhanced affinity and stability for noninvasive molecular imaging.
- Design ligands that sterically or allosterically interfere with the Phe¹²⁶ switch or block the Phe⁴⁴⁰/Phe⁴⁶³ interface, to prevent signaling initiation.
- Perform X-ray crystallography or cryo-EM binding studies of MD-2 with lead ligands and pathological ligands (e.g., A β oligomers, tau fragments) to determine high-resolution co-structures.

References

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