Supplementary Material

Structure-Activity Correlations of Variant Forms of the B Pentamer of *Escherichia coli* Type II Heat-Labile Enterotoxin LT-IIb With Toll-like Receptor 2 Binding

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Methods: Production and Purification of Toll-like Receptors TLR1 and TLR2

In brief, the genes for TLR1 and TLR2 were supplied in the truncated transfer vector pVL1393 (Becton Dickinson, and Co, San Diego) as described (Jin et al., 2007). These plasmids were co-transfected separately with 0.5 □g of BaculoGold Baculovirus DNA following the manufacturer's protocol. A positive control (pVL1392-XylE) was co-transfected as well. The negative control contained only sf9 cells. Cultures of $2.0x10^6$ cells were plated in sterile Petri dishes for each co-transfection and were incubated overnight at 27°C. Cells reached >75% confluence. Cultures with the DNA insert were incubated overnight at 27°C for four days. On day 6, viral supernatant was harvested and labeled P1 virus and stored at -80°C. P2 and P3 viral supernatant was generated by amplification of the P1 stock. P2 amplification was made with $0.5x10^7$ sf9 cells that were infected with 500 □L of viral supernatant (P1) in 15 mL of BakPak Complete medium (Clonetech) in a vented T-75 flask. The amplification was allowed to incubate at 27°C for three days. Harvested supernatant (P2 viral stock) was determined to be $2.21x10^7$ pfu/mL via plaque assay using the manufacturer's recommendations. A second round of amplification (P3) was performed in a 75 mL suspension culture at an MOI of 0.1 for three days. Titer assay showed $2.45x10^8$ pfu/mL. P3 virus was used for all large scale infections/expression (6-8L of sf9 cells) studies that were carried out in oxygenated spinner flasks that were stirred at 80 rpm with oxygenation of 30%. Cells were infected with virus optimized at a MOI of 7.5 for TLR1 and at MOI of 11.0 for TLR2. Cells were incubated for 72 hours or until cell viability was between 68-75% via trypan blue exclusion. Protein expression levels were reduced to less than 1 mg/L if cells were harvested with viability higher than 75%. Typical yields were 2.9 mg/L for TLR1 and 1.8 mg/L for TLR2.

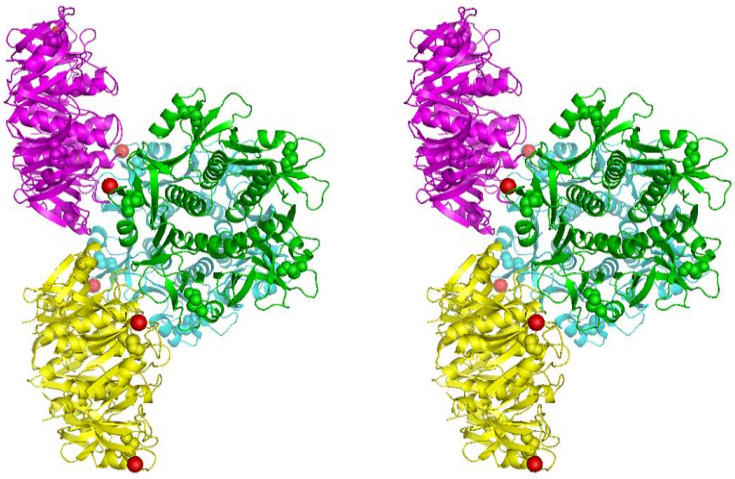
Purification of TLR1 and TLR2

Cells were resuspended in Buffer A (50mM Tris HCl, pH-7.4, 150 mM NaCl, and 0.2 mM TCEP) and then lysed using a Branson Sonifier set at 70% intensity with 3 cycles of 30s on followed by 30s off while on ice. Lysed cells were centrifuged in a Ti-45 rotor set at 45,000 rpm for 45 minutes at 4°C to pellet debris. The resulting supernatant was filtered through a 0.22 □M syringe

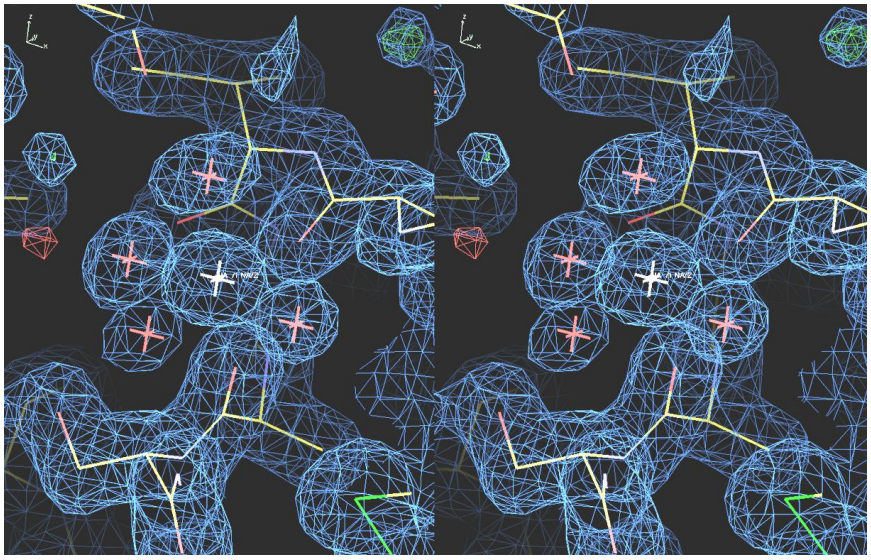
filter and applied to a Protein A column. TLR1-IgG1 (Fc) or TLR2-IgG (Fc) was eluted using Buffer B (100mM Na citrate, pH 4.5). The resulting peak containing the TLR of interest were quickly exchanged into Buffer A using a Centricon YM-10 filter due to instability at low pH conditions. The protein was added to a GF200 column pre-equilibrated with Buffer A. Peak fractions were verified via SDS-PAGE and flash frozen in liquid nitrogen and stored at -80^{o}C.

Packing arrangement in the LT-IIb-B_5(T13I) variant

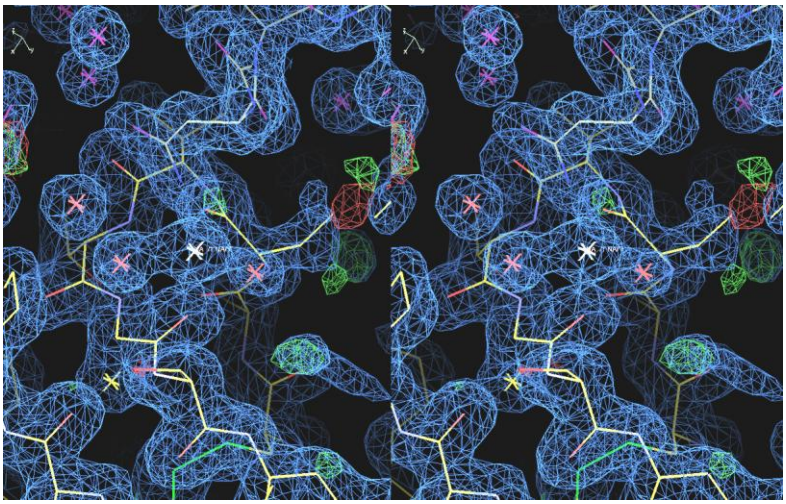
Structural results for the LT-IIb-B_5(T13I) variant revealed the presence of eight Na^+ ions present in the crystallization media. The Na^+ ions form octahedral binding interactions utilizing the backbone carbonyls of residues Arg12 and Ala15 and with water with contact distances between 2.3 – 2.5Å (Fig. S1). The Na^+ ions are coordinated with three types of interactions: Arg12, Ala15 and four waters (Fig. S2a); Arg12, Ala15, Ser16 and three waters; or Arg12, Ala15, Asn33 from a symmetry-related molecule (Fig. S2b). These interactions help stabilize the four unique pentamers in the asymmetric unit of the crystal lattice.



Supplementary Fig. S1. Packing arrangement of the four pentamers (green,cyan,violet and yellow) in the structure of the LT-IIb-B_5(T13I) variant (residue 13 as space filling representation) highlighting the interactions of the Na^+ ions (red spheres) present in the crystallization buffer.



(a)



(b)

Supplementary Fig. S2. (a) Example of the environment around one of the Na^+ ions (white X) in the structure of the LT-IIb-B_5(T13I) variant. The octahedral coordination uses the carbonyl oxygens from Arg12 and Ala15 and water (red X) molecules (2.3-2.5Å). (b) Example of the environment around one of the Na^+ ions (white X) in the structure of the LT-IIb-B_5(T13I) variant. The coordination uses the carbonyl oxygens from Arg12 and Ala15, the amine from Asn33 from a symmetry-related molecule, and water (red X) molecules (2.3-2.5Å).