ONLINE METHODS

Expression and purification of sRP105–MD-1. Bovine, human, and mouse sRP105–MD-1 complexes were expressed extracellularly by a baculovirus expression system using a modified dual expression vector, pAcUW51 (BD Biosciences). The expression vector encodes MD-1 attached to C-terminal thrombin cleavage site and Strep-tag II, and sRP105 to C-terminal thrombin cleavage site and His_6-tag. To generate sRP105–MD-1 expressing baculovirus, SF9 insect cells were co-transfected with the expression vector DNA and linearized baculovirus DNA, ProFold-ER1 (AB vector). The sRP105 and MD-1 proteins were expressed for 2 days after infecting SF9 or Hi-5 cells with amplified recombinant virus. Secreted protein was purified by Ni-NTA (Qiagen) and Strep-Tactin (IBA) affinity chromatographies. After C-terminal tag cleavage by thrombin, the resulting protein was further purified by gel filtration chromatography. The purified proteins were screened for crystallization, but only bovine RP105–MD-1 generated diffraction quality crystals. As alternative expression to verify the sRP105–MD-1 homodimerization interaction, mouse sRP105 and mouse MD-1 (or its mutant, G52D or G71D) were simultaneously expressed by coinfection of Hi-5 insect cells with sRP105 and MD-1 expressing baculoviruses. The individual baculoviruses were generated using a modified pAcGP67 transfer vector that contains a C-terminal thrombin cleavage site and His_6-tag. The expressed sRP105 and MD-1 proteins were initially purified by Ni-NTA affinity chromatography and the resulting proteins were analyzed by gel filtration chromatography using a Superdex 200 HR 10/30 gel filtration column.

To improve the X-ray diffraction quality of bovine sRP105–MD-1 crystals beyond ~4Å, reductive methylation of lysine residues was applied to Strep-Tactin column-purified bovine sRP105–MD-1 complex (residues 24-626 and 22-159, respectively), as previously described[37].

The resultant dimethylated protein complex was further purified by gel filtration chromatography. The methylated complex protein was used to derive the final sRP105–MD-1 structure to 2.91 Å resolution.

Analytical gel filtration analysis showed that lysine methylation did not alter the oligomeric status of the sRP105–MD-1 complex. Consistently, the effect of lysine methylation on the RP105–MD-1 binding interfaces would be minimal since methyl groups are small and a lysine residue is able to make a H-bond even after methylation (pK_a of unmethylated lysine, 10.2; pK_a of dimethyl lysine, 10.2-10.4)[38]. However, there is a possibility that methylation may alter the H-bond status of two interface residues (Lys353 in primary interface-B and Lys113 in primary interface-A) from bi- or tri-furcated H-bonds to single H-bonds.

To obtain a different crystal lattice that could provide initial, additional phase information, 11 sRP105-VLR hybrids were designed by fusing the N-terminal region of bovine sRP105 to the C-terminal region of hagfish VLR B.61, in a design previously reported for other TLRs[5-6,8,39]. They were co-expressed and co-purified with bovine MD-1 (residues 22-159), as for the wild-type complex. One of sRP105-VLR hybrids [sRP105 residues 24-521 (LRRNT and LRR1-20) and VLR B.61 residues 125-200] provided a sufficient amount of protein for structural studies, and its complex with MD-1 was used to obtain experimental phases.

Crystallization and data collection. sRP105-VLR–MD-1 and methylated sRP105–MD-1 were crystallized at 23°C by sitting drop, vapor diffusion in spacegroups $P2_12_12_1$ and P1 with one and four 2:2 complexes, respectively, in the asymmetric unit. The sRP105-VLR–MD-1 crystals were obtained by mixing 0.5 µl of 10 mg ml^{-1} protein solution with 0.5 µl of well solution containing 20% w/v PEG MME 5000, 0.2 M NH_4Cl, and 100 mM sodium acetate pH 4.6. Heavy atom

derivative crystals were prepared for experimental phasing by soaking sRP105-VLR–MD-1 crystals in 1 mM $K_2Pt(NO_2)_4$ solution. X-ray diffraction data on the platinum derivative were collected at Pt peak wavelength (1.0718 Å) at 100 K at the Stanford Synchrotron Radiation Lightsource (SSRL) beamline 9-2. The methylated sRP105–MD-1 complex was crystallized in a drop containing 1 µl of 10 mg ml^{-1} protein solution and 1 µl of well solution containing 19% w/v PEG 8000 and 100 mM Tris pH 8.5. The sRP105–MD-1 diffraction data were collected at a wavelength of 1.0333 Å at 100 K at the Advanced Photon Source (APS) beamline 23ID-B. X-ray data were processed with *HKL2000* [40]. Data collection statistics are summarized in Table 1.

Structure determination and refinement. The sRP105-VLR–MD-1 structure was determined by a single-wavelength anomalous diffraction (SAD) phasing experiment performed on a platinum derivative crystal. 11 heavy atom sites were located by *SHELXD*[41] and phases were calculated by *SHELXE*[41]. The initial model of the 2:2 sRP105-VLR–MD-1 complex was built by docking and modifying the structures of chicken MD-1 (PDB ID code 3mtx)[33] and mouse sTLR4 (PDB ID code 2z64)[5] against electron density map obtained from *DM*[42]. The sRP105-VLR–MD-1 model was built with *COOT*[43] and refined with *REFMAC5*[44]. The sRP105–MD-1 structure was determined by molecular replacement with *PHASER*[45] using the 1:1 sRP105-VLR–MD-1 structure as a search model. Iterative model building and refinement were performed using *COOT*[43] and *REFMAC5*[44], respectively. The Ramachandran plot generated with *MolProbity* (http://molprobity.biochem.duke.edu) shows that 93.9% of residues are in favored regions with only 0.3% as outliers. Final refinement statistics are summarized in Table 1. All the molecular graphic figures were made using *Pymol* (http://www.pymol.org).

METHODS-ONLY REFERENCES

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