HOKKAIDO UNIVERSITY

Title	Structural analysis for glycolipid recognition by the C-type lectins Mincle and MCL
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Supplementary materials

Supplementary materials and methods

Plasmid construction

We amplified the nucleotide sequences corresponding to residues 74-219 of Mincle and reidues 61 - 215 of MCL, using the following primer sets: 5'-GAGATATACATATGGGTTCAGTCAAGAATT -3' and 5'-AGAGGATTCTTAAAGAGATTTTCCTTTGTT-3' (for mincle, restriction sites are underlined) and 5'-GAGATATACATATGCATGCAAAGCTCAAAT-3' and 5'-AGAGGATTCGTTCAATGTTGTTCCAGGTATTTT-3' (for MCL, restriction sites are underlined), and the Mincle and MCL expression plasmids in human embryonic kidney (HEK293T) cells. The PCR-amplified Mincle and MCL products were digested with *Bam*HI/*Nde*I and ligated into *Bam*HI/*Nde*I-digested pET22 (Novagen), to produce pET22-Mincle and pET22-MCL, respectively.

The construction of the plasmid to express the I99K mutant is described below. Initially, two fragments, T7 promoter to mutant point and mutant point to T7 terminator, were amplified by the primer sets 5'-TAATACGACTCACTATAG-3' and 5'-GGTGTCAGTAGAAAAGAAGTAGCAG-3', and 5'-AAATCCTGGGCGTTAAGTTTAAAGA -3' and 5'-CCGCTGAGCAATAACTAGC-3', using pET22-Mincle 5 amino acids delete, respectively (mutation site is underlined). The entire mutated Mincle fragment was amplified by PCR from the fragments of T7 promoter to mutant point and mutant point to T7 terminator, with the primer sets 5'-TAATACGACTCACTATAG-3' and 5'-CCGCTGAGCAATAACTAGC-3'. The amplified entire mutated Mincle PCR product was digested with *Bam*HI/*Nde*I and ligated into *Bam*HI/*Nde*I-digested pET22,

to produce pET22-hmincle I99K.

Table S1. Crystallographic statistics of Mincle and MCL.

Fig. S1. The elution profiles of MCL (**A**) and Mincle (**B**) from the size exclusion chromatography using the Superdex 75 10/300 GL column (GE Healthcare, Fairfield, USA) are shown. The eluted fractions of MCL (**C**) and Mincle (**D**) were separated and stained by Coomassie Brilliant Blue. (**E**) The superimposition of crystal (yellow) and solution (PDB:2LS8) (green) structures of MCL are shown. Spheres indicate Ca^{2+} ions.

Fig. S2. Crystal structures of MCL and Mincle. (A) The stick model of lysine mutated from isoleusine at residue 99 of Mincle is shown onto the cartoon model of Mincle. (B) The superimposed structures of MCL (yellow) and Mincle (cyan) are shown. Spheres are Ca^{2+} ions. (C) The amino acid substitutions on the hydrophobic patch between human and mouse Mincles are mapped onto the crystal structure together with their solvent accessible areas. The area sizes of solvent accessible surface of each residue in putative hydrophobic loops are shown in table. The sizes were calculated by AREAIMOL program in ccp4.

Fig. S3. Control experiments of Figs. 3 and 4. *In vitro* binding assay of Mincle and MCL mutants and reporter cell assay of Mincle mutants.
(**A**-**B**) Mincle-Ig, mutated Mincle-Igs or hIgG (**A**) and MCL-Ig, mutated MCL-Igs or hIgG (**B**) were incubated with plate-coated anti-IgG antibody. The bound proteins were detected by anti–hIgG-HRP. (**C**) Reporter cells expressing Mincle or its mutants were

stimulated with an anti-Mincle antibody, 13D10-H11, (13D10-H11 mAb), for 18 h. (D) Characterization of the 13D10-H11 mAb. 13D10-H11 was tested for cell surface staining of the reporter cells (top) and western blotting analysis for the lysate of the reporter cells (bottom). While the control anti-Mincle mAb, IMG-6782, detected Mincle protein in both assays, the 13D10-H11 mAb could only detect Mincle on the cell surface. This suggest that the 13D10-H11 mAb recognizes the conformational epitopes.

Fig. S4. Schematic representation of the MD chimera.
The hydrophobic loop in Mincle was mutated to the corresponding amino acid residues in Dectin-2.

Fig. S5. SPR analysis of Mincle and several lengths of acyl chains with trehalose were performed. (A-D) The sensorgrams for binding to C12(A), C10(B), C8(C) and trehalose (D) in Fig. 4G are shown.

Fig. S6. Schematic images of receptor recognition mechanisms of glycolipids.
(**A**) Mincle, (**B**) CD1d-β2m, (**C**) TLR4-MD2. (**D**) The modeling image of Mincle with a glycolipid (grey-shaded boxes and bue-shaded circles indicate sugar moieties and acyl chains, respectively).

Fig. S7. Competition of Mincle binding to glycolipids with citric acid. SPR (surface plasmon resonance) analysis of Mincle binding to C12 glycolipid with the citric acid (triangles) or the acetic acid (circles) was performed.

	Mincle-citrate	Mincle	MCL
Data collection			
Wavelength (Å)	1.000	1.000	1.000
Space group	$P3_1$	$P3_1$	I222
Unit-cell parameters (Å)	a = b = 49.213, c =43.55	a = b =49.074, c =43.042	a = 85.19, b =96.056, c =104.826
Resolution range (Å)	19.39 - 1.3 (1.346 - 1.3)	24.54 - 1.32 (1.367 - 1.32)	40.48 - 2.29 (2.372 - 2.29)
Total No. of observations	158,974	145,199	141,555
Unique reflections	29014 (2892)	27209 (2690)	19,569 (1784)
Redundancy	5.5(5.0)	5.3(3.5)	7.2 (7.1)
Completeness (%)	99.86 (99.31))	99.85 (98.53)	99.23(92.15)
Mean I/s (I)	23.0(3.1)	17.7(3.3)	8.65 (3.92)
R_{merge}	0.078(0.518)	0.070(0.298)	0.141 (0.496)
Refinement			
R_{work} / R_{free}	0.1279 /0.1560 (0.1646/0.2064)	0.1373/0.1711 (0.2980/0.3176)	0.1758/0.2180 (0.2170/0.2342)
No. atoms			
Protein	1090	1060	2,426
Ligand/ion	15	2	2
Water	175	165	231
B-factors			
Protein	8.60	12.60	34.70
Ligand/ion	14.70	13.50	30.10
Water	21.90	26.80	36.50
R.m.s. deviations			
Bond lengths (Å)	0.005	0.006	0.009
Bond angles (°)	1.12	1.13	1.170
Ramachandran Favored/outliers (%)	98/0	97/0	98/0

Table.S1

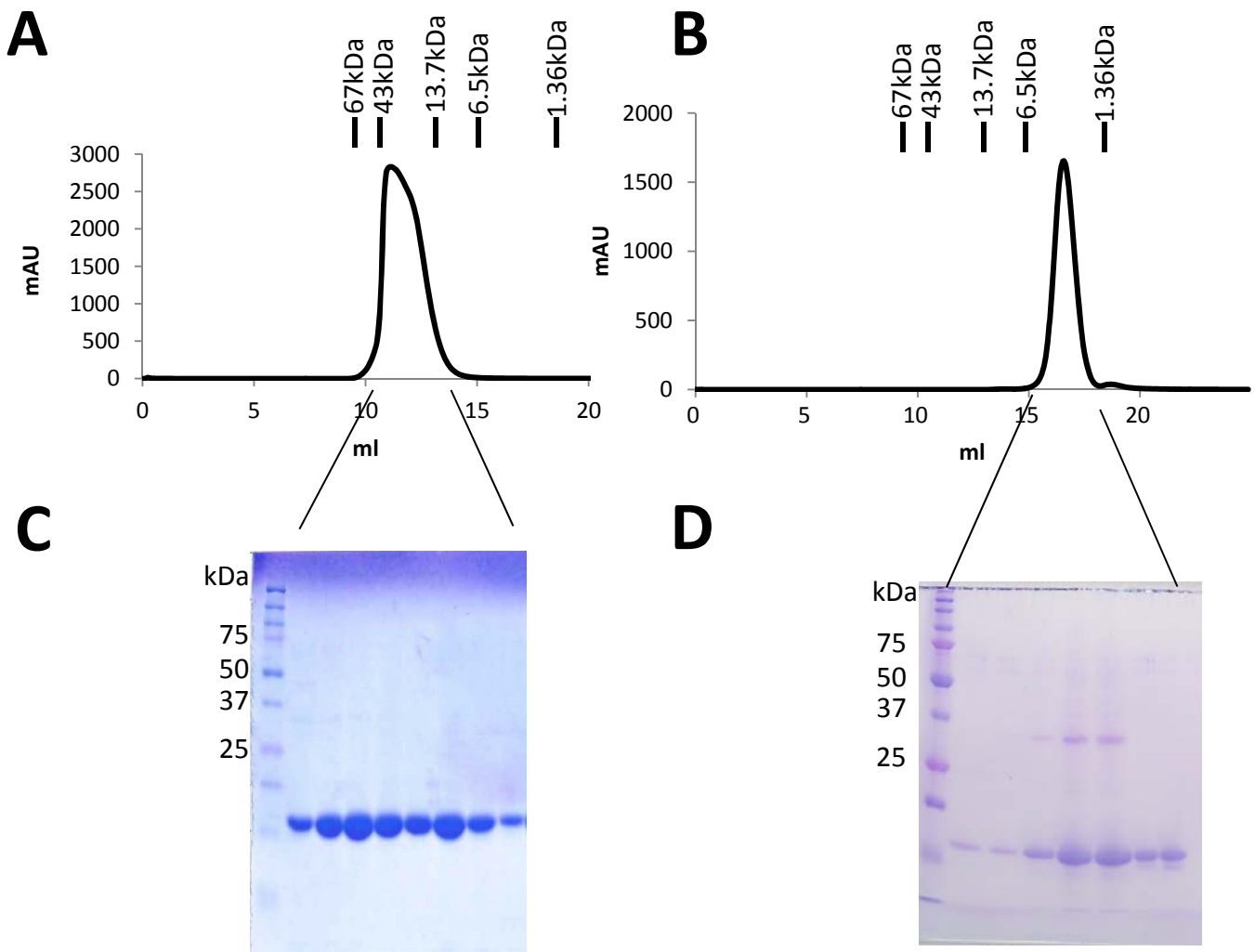
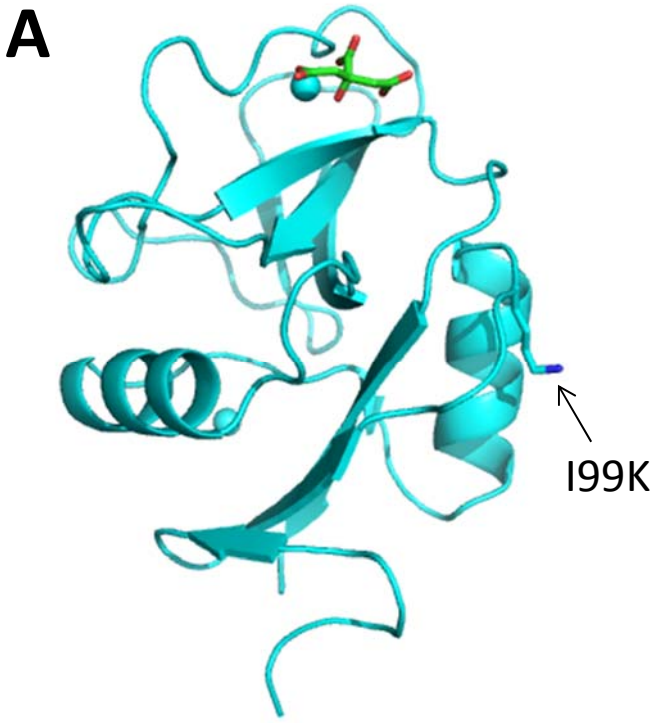
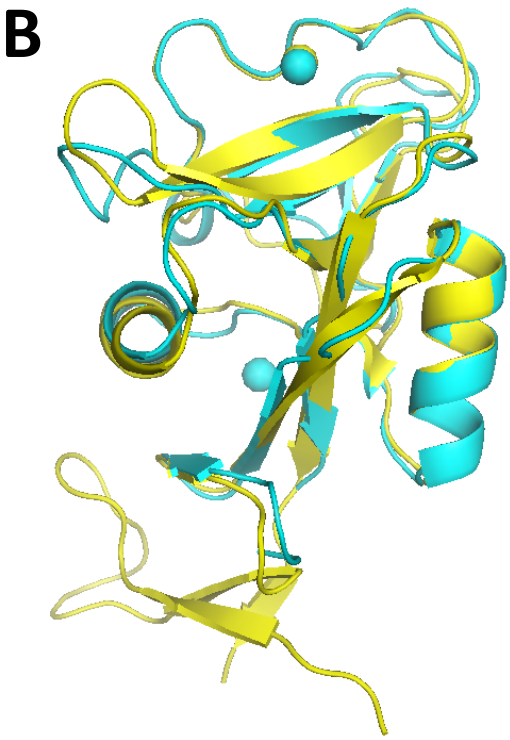
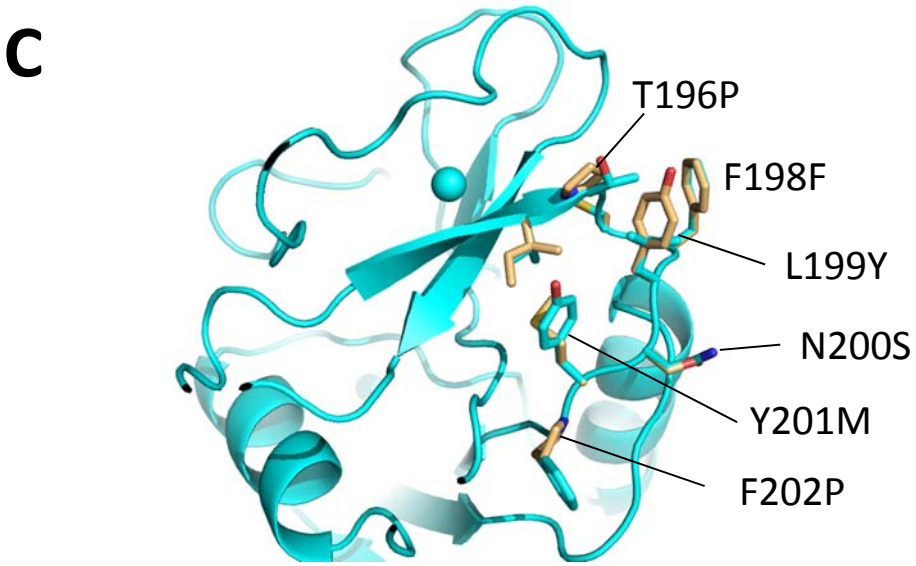


Figure.S1







	human	mouse	solvent accesibility surface(Å^2)
195	Val	Ile	5.7
196	Thr	Pro	35.3
197	Cys	Cys	3
198	Phe	Phe	155.8
199	Leu	Tyr	78.1
200	Asn	Ser	102.6
201	Tyr	Met	47
202	Phe	Pro	32.6
203	Arg	Trp	5.7

Figure.S2

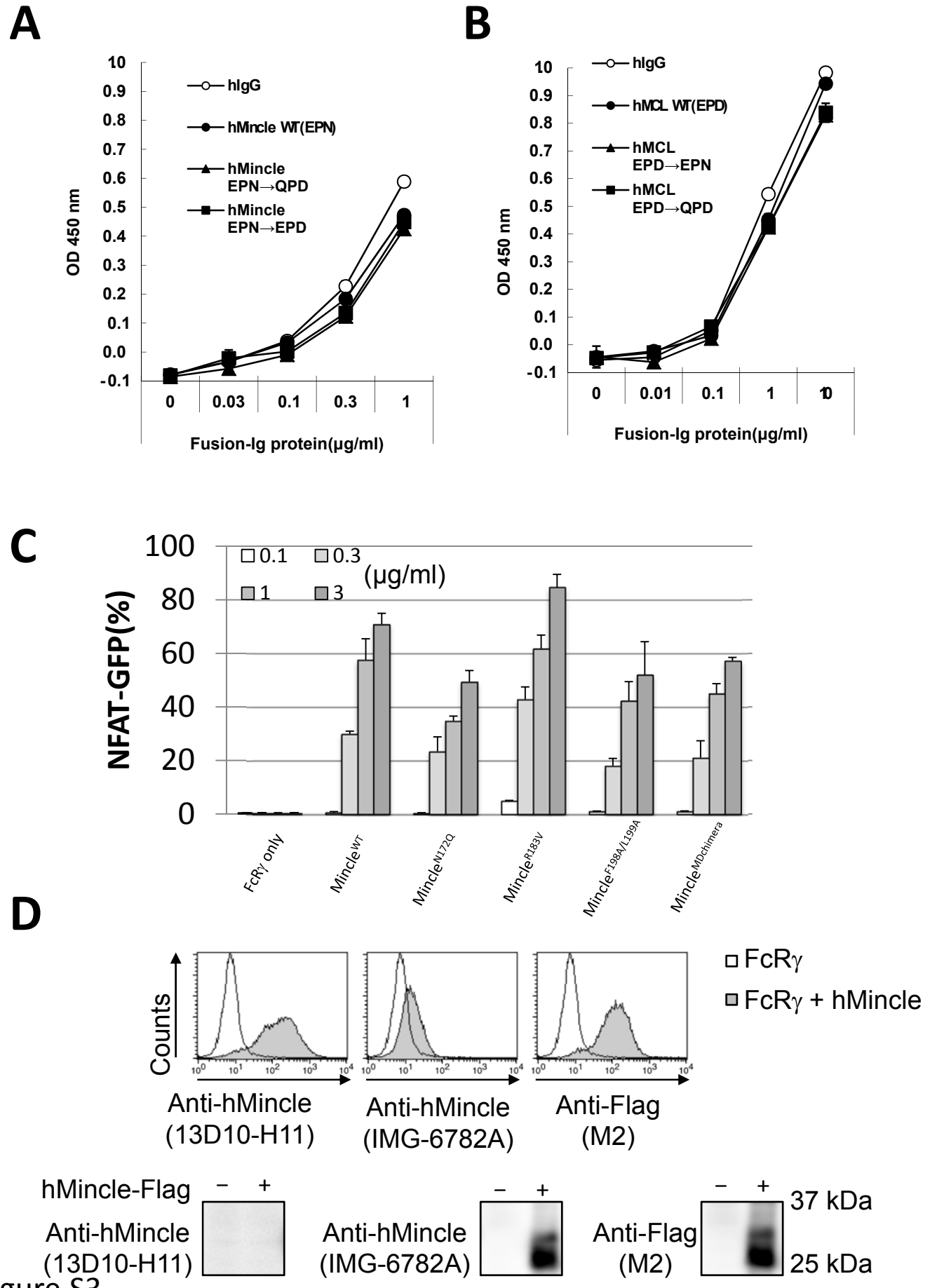

A
B
hIgG
hMincle WT(EPN)
hMincle EPN→QPD
hMincle EPN→EPD
hMCL WT(EPD)
hMCL EPD→EPN
hMCL EPD→QPD
OD 450 nm
Fusion-Ig protein(μg/ml)
C
NFAT-GFP(%)
0.1
0.3
1
3
(μg/ml)
FcRγ only
Mincle WT
Mincle N172Q
Mincle R183V
Mincle F198A/L199A
Mincle MDchimera
D
Counts
FcRγ
FcRγ + hMincle
Anti-hMincle (13D10-H11)
Anti-hMincle (IMG-6782A)
Anti-Flag (M2)
hMincle-Flag
37 kDa
25 kDa

Figure.S3

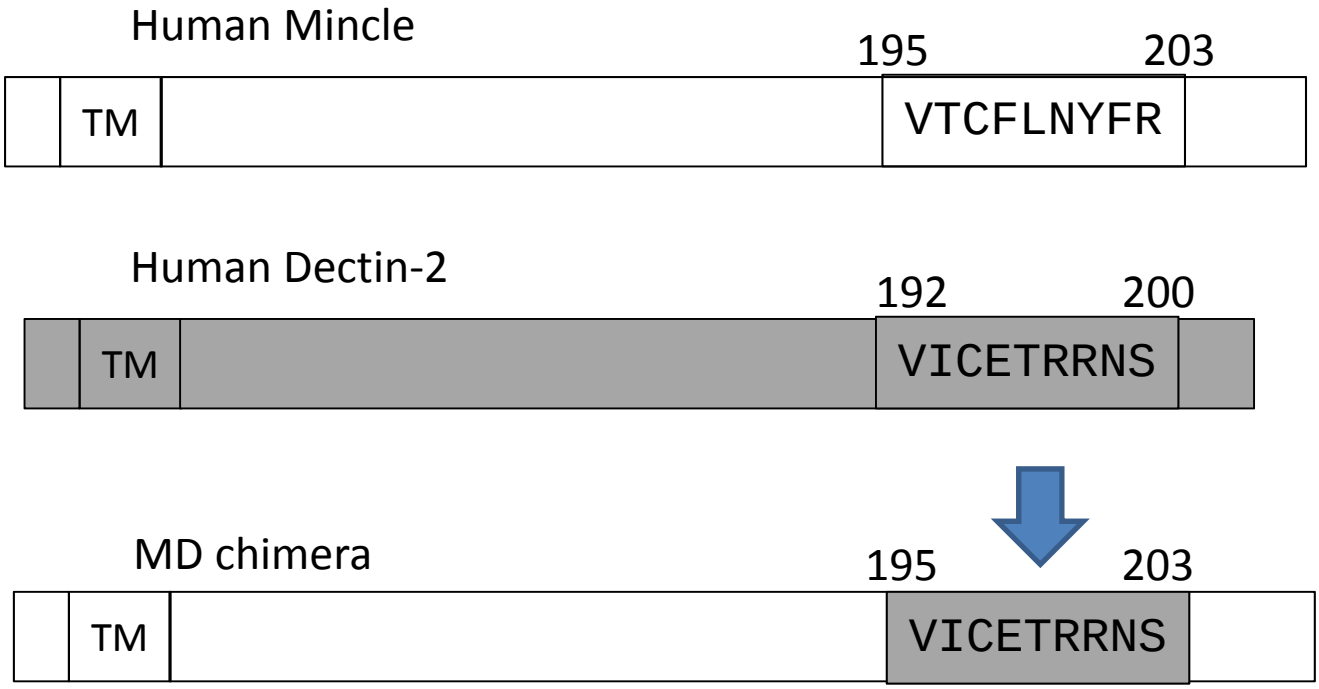

Human Mincle
195
203
TM
VTCFLNYFR
Human Dectin-2
192
200
TM
VICETRRNS
MD chimera
195
203
TM
VICETRRNS

Figure.S4

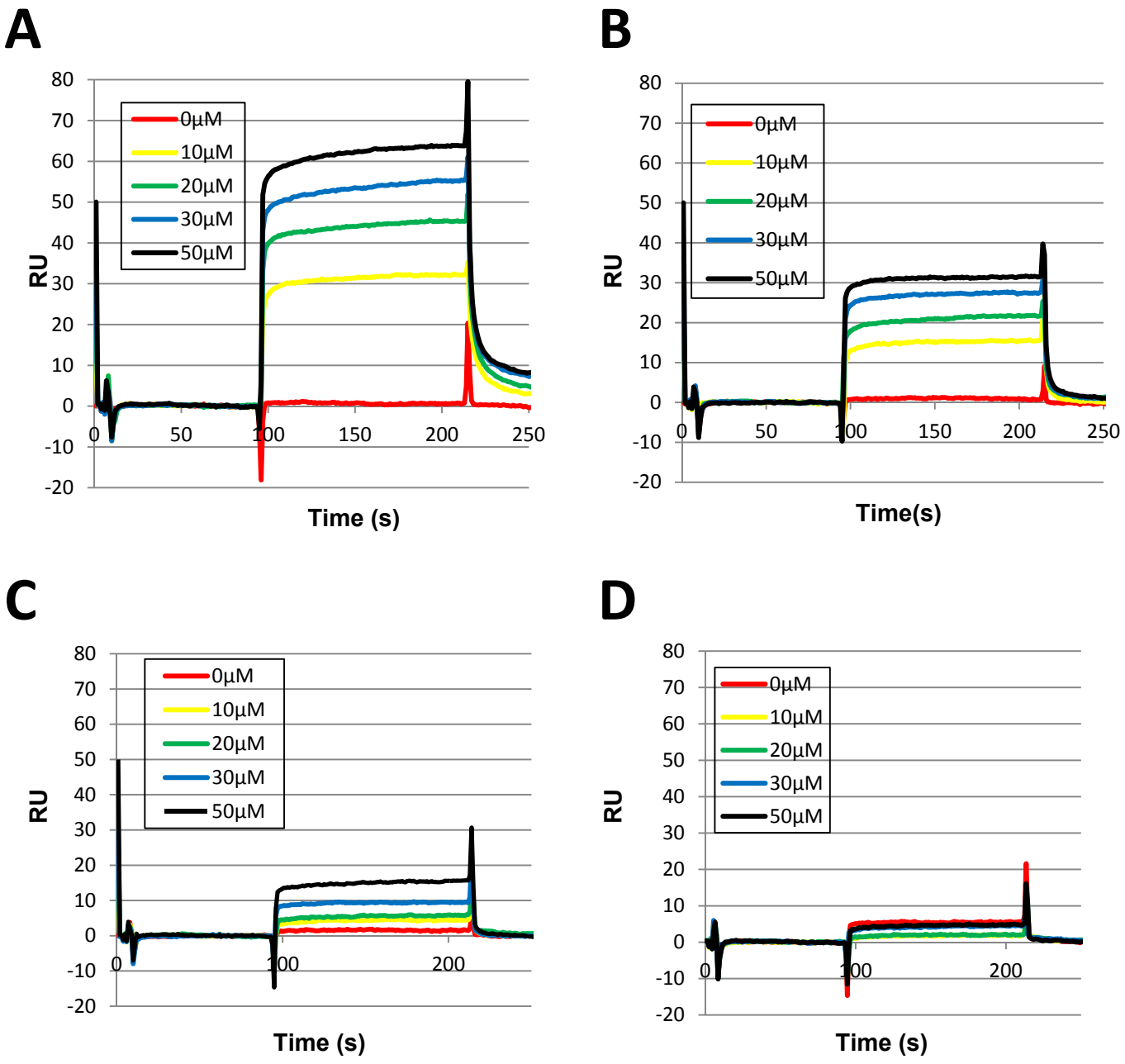


Figure.S5

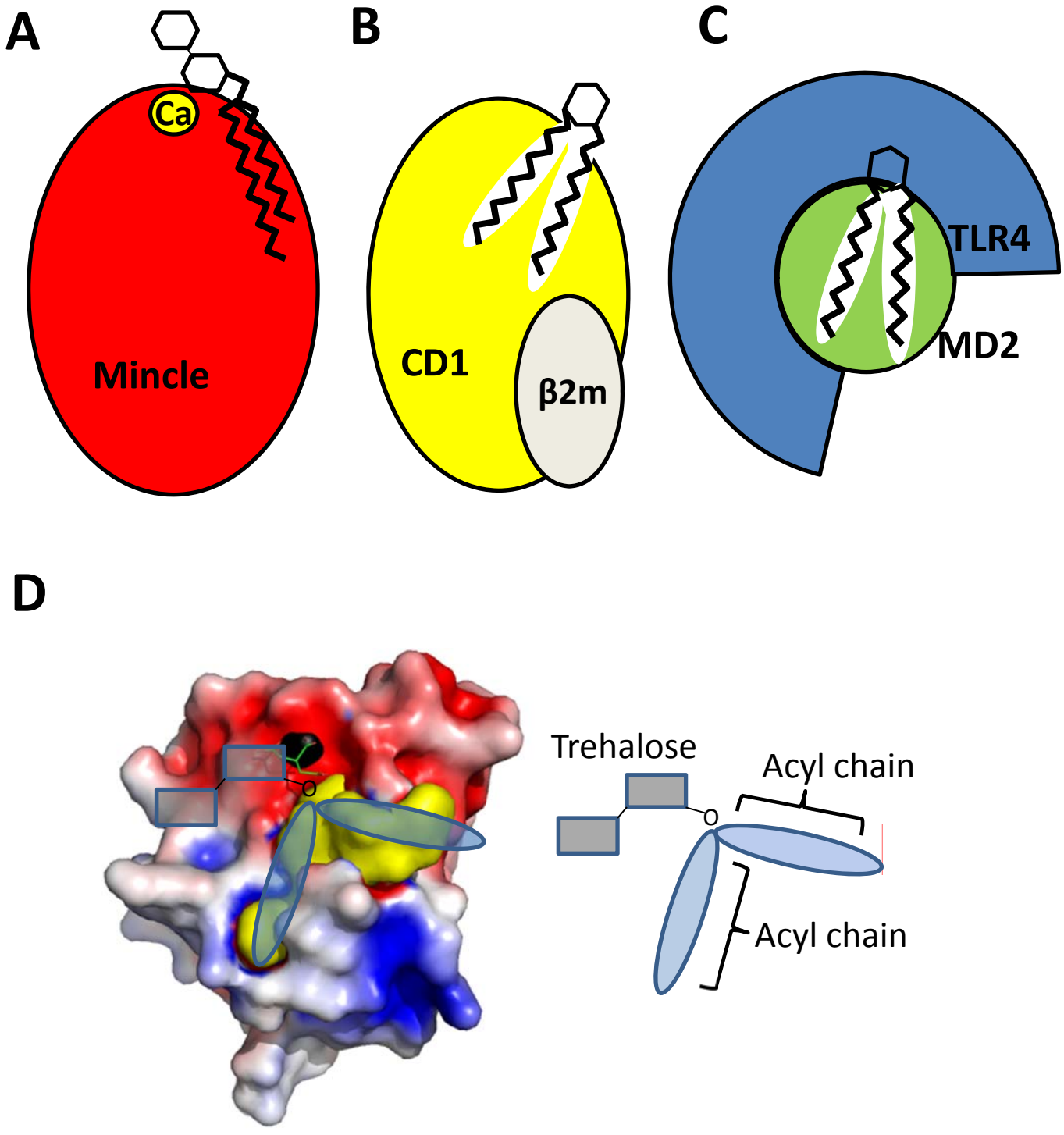

A
Ca
Mincle
B
CD1
β2m
C
TLR4
MD2
D
Trehalose
Acyl chain
Acyl chain

Figure.S6

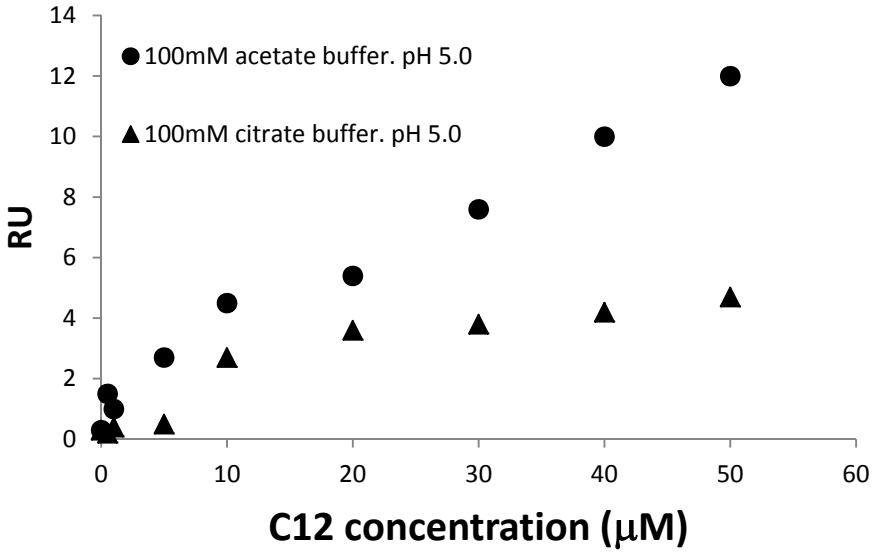

14
12
10
8
6
4
2
0
RU
100mM acetate buffer. pH 5.0
100mM citrate buffer. pH 5.0
0
10
20
30
40
50
60
C12 concentration (μM)

Figure.S7