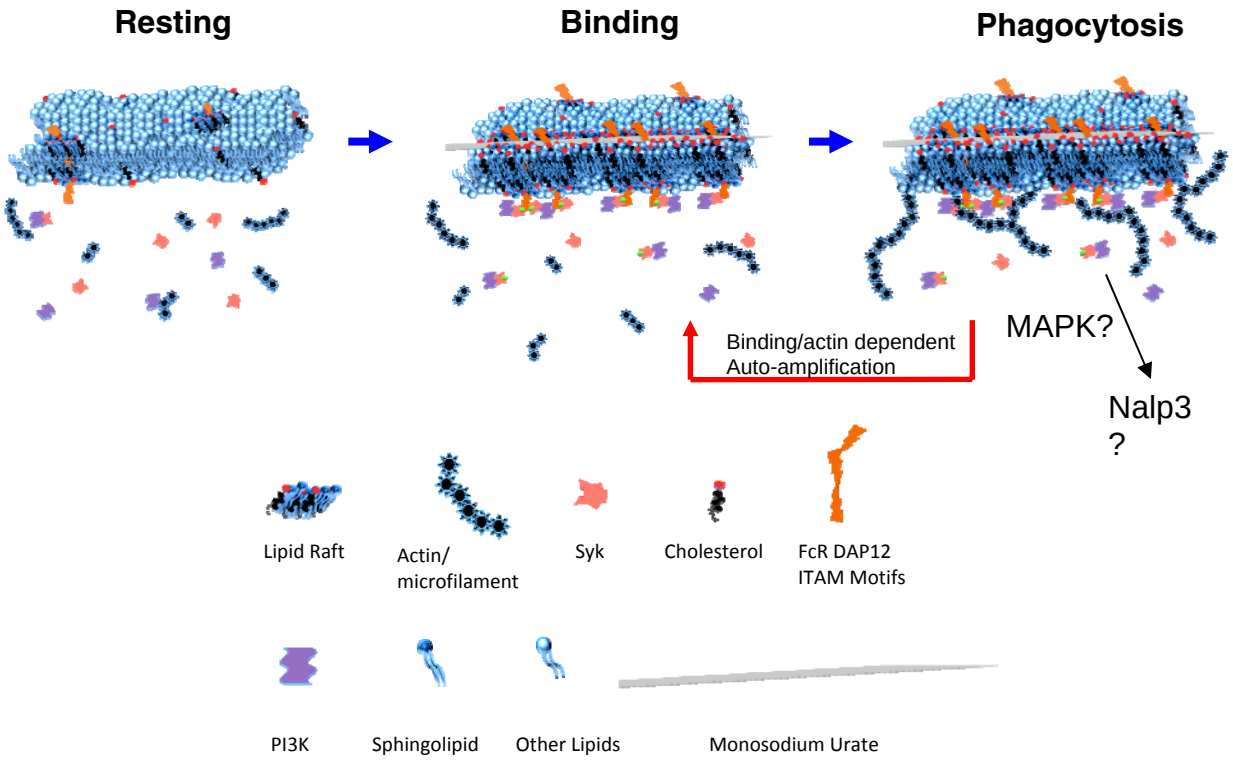
Supplemental Data

Receptor-Independent, Direct Membrane Binding Leads to Cell-Surface Lipid Sorting and Syk Kinase Activation in Dendritic Cells

Gilbert Ng, Karan Sharma, Sandra M. Ward, Melanie D. Desrosiers, Leslie A. Stephens, W. Michael Schoel, Tonglei Li, Clifford A. Lowell, Chang-Chun Ling, Matthias W. Amrein, and Yan Shi



One possible scheme of the receptor independent Syk kinase activation

Left: Resting state DC membrane where lipid rafts are dispersed, and Syk recruitment to the inner membrane is limited. Middle: upon MSU binding, a lipid sorting event occurs as a consequence of potential interaction between the crystal surface and cholesterol. Such a sorting aggregates raft associated ITAM containing signaling molecules, leading to the recruitment of Syk, which in turn recruits PI3K. Right: Syk/PI3K leads to actin/microfilament and topological

membrane changes that resemble phagocytosis, which further allows stronger and larger binding contacts in a manner of self-amplification. Since the binding strength increases without extracellular proteins (Fig. 3D), it remains unknown if the increased affinity is a result of more cholesterol binding or other lipid alterations that permit more intense membrane/solid surface interaction.

Supplemental Experimental Procedures

Details of AFM operation

To be compatible with AFM (regular density DC cultures restrict AFM cantilever movement), BM DCs from all sources were cultured with one thirty second of regular density (one bone per 24 ml of culture in a 6 well plate) on poly-D-lysine coated or untreated 25 mm diameter round glass disk (VWR). On the day of reading, these cultures were set in a foam box, and the box was sealed for a brief transport from the incubator to the AFM to minimize the duration of suboptimal culture conditions. The AFM cantilever was lowered to be in contact with an individual DC by 0.5 to 2 um increments until the first force curve was generated. The cantilever was then set as the maximal depth and put into oscillating up/down motion every 2 second, with an additional 1 second pulse in the tip saver mode for the duration of measurement. When raw data were analyzed, they were normalized to the background reading. To subtract the background, a blank reading for each set of affinity measurement was collected immediately before the assay. The line of best fit for the background data was first generated. Then all the y-values (vertical deflection) in our standard reading were corrected with the background value with the following formula

Y = Y1 - MX + B, where Y1 = raw data Y value, M = Slope obtained from background information, X = Raw data X distance value, and B = the value on Y-axis to move the baseline reading to 0.

To obtain the minimum (the maximum attraction force shown as a negative value) Y value, Excel function @min was used to automatically select the lowest values, their

corresponding time points were obtained from calculating the time recorded for the file from the time the first data curve was collected.

Lipid/crystal binding and flow cytometry

MSU crystals in 0.1M boric acid was shaken, and allowed to settle for several minutes. The top layer of crystals in suspension (1 ml) was transferred to a different tube and mixed with 4 ul of 1 mg/ml NBD-PC or Bodipy-Cholesterol in ethanol or ethanol/chloroform 50:50 mix. The mixtures were incubated for 20 minutes with frequent stirring, and then washed with PBS 2 times. The flow cytometric analysis was identical to regular cell analysis except that MSU crystals demonstrated less forward scatter than cells.

For DC cellular lipid labeling, cells were first plated on glass disks in 6-well plates at low density over night. The media (10% FBS RPMI) was removed, and replaced with 100 ul of the same media. 4 ul of the NBD-PC, NBD-PE or Bodipy-Cholesterol in DMSO or ethanol (same concentration) were used to label the cells for 20 minutes followed by two washes with cell culture media. 100 ug of MSU crystals in 4 ml of cell culture media was added to the wells and the plates were allowed to sit for an additional 20 minutes. The plates were then washed again with media, and the disks were transferred to the AFM cell. An UV light source attached to the AFM was used to excite the lipid fluorescence and the fields were surveyed for fluorescing crystals.

Critical point dry and EM sample preparations

Cells on nickel grids described under “Surface protein removal and Nickel Grid cell trapping” and a cell attached to a cantilever described in “Affinity Force Reading between T cell

and DC, and between tip bound and crystal surfaces" were also prepared for inspection by SEM (Environmental Scanning Electron Microscope - XL30, FEI, Eindhoven, Holland). Samples were fixed by placing them in glutaraldehyde (1%) in PBS buffer for 5 hours. Specimens were then dehydrated by transferring through graded ethanols (30%, 40%, 50%, 60%, 70%, 80%, 90%, and 100%, at least 5 min each). Critical point drying was carried out in a SEEVAC Critical Point Dryer (SEEVAC Inc., FL, USA). Samples were then sputter coated with Gold-Palladium in a Techniques Hummer II Sputter Coater (Anatech, Hayward, CA).

Chemical syntheses of cholesterol and cholic derivatives

A. The control for SW.I.30 synthesis (supp Fig 1)

To a solution of 11-bromoundecanoic acid **1** (2.65 g, 9.99 mmol) in acetone (10 mL) was added potassium thioacetate (1.71 g, 14.97 mmol, 1.5 eq), and the reaction was stirred at room temperature for 3 hours. The mixture was diluted with EtOAc (100 mL) and washed with water, brine and 2N HCl. The organic solution was dried over anhydrous Na_2SO_4 and evaporated. Hexane was added to the residue and the precipitate was filtered off. The filtrate was concentrated and purified by column chromatography on silica gel using 1% MeOH – CH_2Cl_2 as eluent to afford the desired compound **2** (1.14 g, 44% yield). ^{1}H NMR ($CDCl_3$, 400 MHz): δ_H 2.87 (t, 2H, *J* 7.3 Hz, H-11), 2.35 (t, 2H, *J* 7.5 Hz, H-2), 2.33 (s, 3H, CH_3COS), 1.64 (m, 2H, H-10), 1.57 (m, 2H, H-3), 1.28 (m, 12H, 6 × CH_2). ^{13}C NMR ($CDCl_3$): δ_C 196. 08, 179.27, 33.96, 30.61, 29.45, 29.34, 29.28, 29.152, 29.13, 29.03, 29.01, 28.75, 24.67. ESI m/z calc'd for $C_{13}H_{24}O_3SNa$ ($M+Na^+$): 283; found: 283.

Acid **2** (86.0 mg, 0.33 mmol, 1.1 eq) and cholesterol **3** (110 mg, 0.30 mmol) were combined in a flame dried flask under argon and anhydrous CH_2Cl_2 (2 mL) was added followed

by the additions of DCC (68.0 mg, 0.33 mmol, 1.1 eq), triethylamine (100 μL) and DMAP (10 mg). After stirring overnight at room temperature, the reaction mixture was filtered and the filtrate was concentrated and purified by column chromatography on silica gel using 50% toluene – hexanes as eluent to afford the desired compound **4** (103 mg, 56% yield). ^{1}H NMR (400 MHz, $CDCl_3$): δ_H 5.38 (m, 1H, H-6), 4.62 (m, 1H, H-3α), 2.86 (t, *J* 7.31 Hz, 2H, H-11_chain), 2.25-2.37 (m, 7H, CH_3COS + 2 × H-2_chain + 2 × H-7), 0.86-2.00 (m, 52H, cholesterol + 8 × CH_2_chain), 0.68 (s, 3H, CH_3-18). ^{13}C NMR ($CDCl_3$, 100 MHz): δ_C 173.25, 139.70, 122.54, 73.66, 56.68, 56.13 50.02, 42.30, 39.73 39.50, 38.15, 36.99, 36.59, 36.17, 35.77, 34.69, 31.89, 29.47, 29.36, 29.07 28.78 28.21, 27.99, 27.81, 25.03, 24.27, 23.8, 22.79, 22.54, 21.02, 19.30, 18.70. ESI m/z calc'd for $C_{40}H_{67}O_3SNa$ ($M+Na^+$): 651; found: 651.

Thioacetate **4** (70 mg, 0.11 mmol) was dissolved in chloroform (350 μL) under argon, benzylamine (0.36 μL, 0.33 mmol, 3.0 eq) was added and the reaction was left stirring at room temperature overnight. The solvent was removed and the residue was purified by column chromatography on silica gel using 50% toluene – hexanes as eluent to afford the desired compound **5** (28.7 mg, 44.5% yield). ^{1}H NMR (400 MHz, $CDCl_3$): δ_H 5.38 (m, 1H, H-6), 4.62 (m, 1H, H-3α), 2.68 (t, 2H, *J* 7.5 Hz, H-11_chain), 2.27 (m, 4H, 2 × H-2_chain + 2 × H-7), 0.80-1.78 (m, 55H, cholesterol + 8 × CH_2_chain), 0.68 (s, 3H, CH_3-18). ^{13}C NMR ($CDCl_3$, 100 MHz): δ_C 173.24, 139.71, 122.55, 73.67, 56.75, 56.14, 50.030, 42.31, 41.23, 39.85, 39.73, 39.51, 39.19, 38.16, 37.00, 36.60, 36.18, 35.781, 34.70, 31.90, 31.86, 29.42, 29.36, 29.21, 29.09, 28.51, 28.22, 28.00 27.82, 25.04, 24.28, 23.82, 22.80, 22.54, 21.03, 19.312, 18.71, 11.85. Negative ESI m/z calc'd for $C_{40}H_{65}O_3S$ ($M-H^-$): 585; found: 585.

B. SW.I.30 synthesis (supp Fig 1)

Cholic acid **7** (2.00 g, 4.90 mmol) and alcohol **6** (6.15 g, 24.75 mmol, 5 eq) were dissolved in anhydrous tetrahydrofuran (25 mL) under argon. *p*-Toluenesulfonic acid (0.28 g, 1.47 mmol, 0.30 eq) and sodium sulfate (3.48 g, 24.75 mmol, 5 eq) were added. The reaction was stirred overnight at 50^{o} C. The solution was neutralized with triethylamine (2.0 mL) and then concentrated under reduced pressure to dryness. Compound **8** was obtained by column chromatography using 90% acetone - dichloromethane to afford compound **8** (2.73 g, 86.9%). ^{1}H NMR (300 MHz, $CDCl_3$): δ_H 4.03 (t, *J* 6.72 Hz, 2H, 2 × H-1_chain), 3.96 (m, 1H, H-12β), 3.83 (m, 1H, H- 7β), 3.45 (m, 1H, H-3β), 3.39 (t, 3H, *J* 6.9 Hz, H-11_chain), 2.11-2.41 (m, 4H, H-4α + H-9α + 2 × H-23), 0.87-1.97 (m, 47H, cholic acid + 9× CH_2_chain), 0.66 (s, 3H, CH_3-18). ^{13}C NMR ($CDCl_3$, 100 MHz): δ_C 174.23, 136.94, 115.95, 72.80, 71.32, 64.43, 47.20, 46.50, 40.88, 37.60, 35.44, 34.53, 34.03, 33.11, 32.81, 31.40, 30.88, 30.85, 29.45, 29.42, 29.39, 29.21, 28.74, 28.64, 28.62, 28.16, 27.20, 25.93, 24.33, 22.21, 17.63, 12.87.

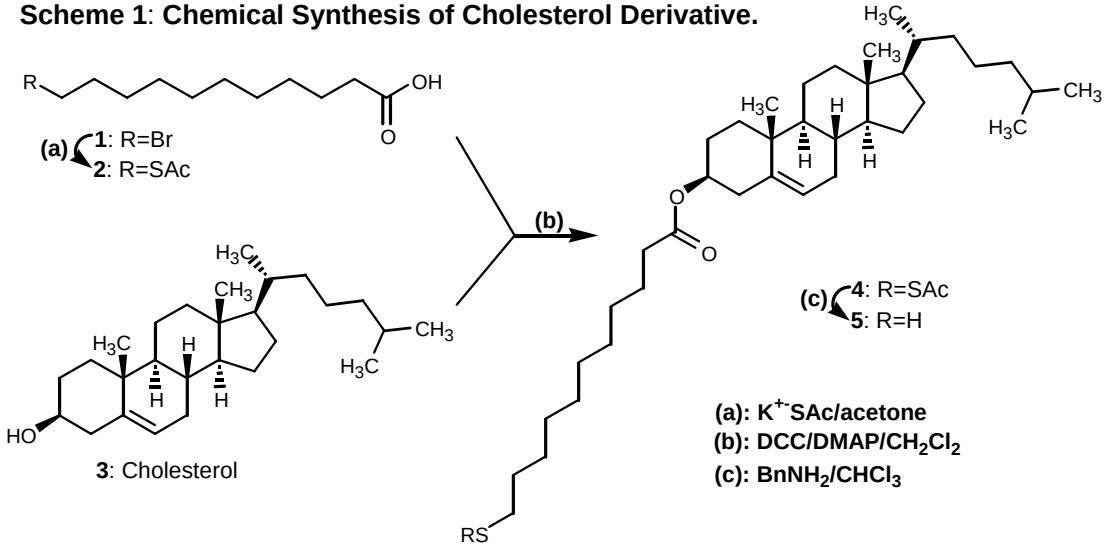
The above cholic acid derivative **8** (1.00 g, 1.56 mmol) and potassium thioacetate (0.27 g, 2.35 mmol, 1.5 eq) were dissolved in acetone (10.0 mL), and the reaction was left stirring overnight at room temperature. EtOAc (50 mL) was added and the mixture was washed with water, brine and 2N HCl. The organic solution was dried over anhydrous Na_2SO_4 and evaporated. The product was purified by column chromatography using 5% MeOH – CH_2Cl_2 as eluent to afford thioacetate **9** (0.71 g, 71.0 %). ^{1}H NMR (400 MHz, $CDCl_3$): δ_H 4.05 (t, 2H, *J* 6.7 Hz, 2 × H-1_chain), 3.97 (m, 1H, H-12β), 3.84 (m, 1H, H-7β), 3.45 (m, 1H, H-3β), 2.86 (t, 2H, *J* 7.3 Hz, H-11_chain), 2.30-2.44 (m, 7H, H-4α + H-9α + 2 × H-23 + CH_3COS), 0.86-1.96 (m, 47H, cholic acid + 9× CH_2_chain), 0.68 (s, 3H, CH_3-18). ^{13}C NMR ($CDCl_3$, 100 MHz): δ_C 71.9, 68.4, 64.4, 47.1, 46.5, 41.7, 41.5, 39.5, 39.5, 35.2, 35.2, 34.7, 34.6, 31.3, 30.9, 30.6, 30.4, 29.5, 29.4, 29.4, 29.2, 29.1, 29.1, 28.8, 28.6, 28.2, 27.4, 26.4, 25.9, 23.2, 22.5, 17.3, 12.5. ESI m/z calc'd for

$C_{37}H_{64}O_6SNa$ (M+Na^+): 659; found: 659. ESI m/z calc'd for $C_{37}H_{64}O_6SK$ (M+K^+): 675; found: 675.

Cholic acid derivative **9** (100 mg, 0.16 mmol) was dissolved in chloroform (1.0 mL) and *n*-butylamine (46 μL, 0.47 mmol, 3 eq) was added. After stirring overnight at room temperature, the solution was evaporated to dryness and the product was purified by column chromatography using 50% acetone - dichloromethane as eluent to afford **10 (**65.0 mg, 70.0%). ^{1}H NMR (400 MHz, $CDCl_3$): δ_H 4.03 (t, 2H, *J* 6.7 Hz, 2 × H-1_chain), 3.95 (m, 1H, H-12β), 3.83 (m, 1H, H-7β), 3.42 (m, 1H, H-3β), 2.84 (t, 2H, *J* 7.31 Hz, H-11_chain), 2.16-2.34 (m, 4H, H-4α + H-9α + 2 × H-23), 0.85-1.98 (m, 47H, cholic acid + 9× CH_2_chain), 0.66 (s, 3H, CH_3-18). ^{13}C NMR ($CDCl_3$, 100 MHz): δ_C 196.06, 174.42, 73.03, 71.87, 68.42, 64.40, 53.82, 47.04, 46.42, 41.65, 41.48, 39.52, 39.50, 39.38, 35.28, 35.23, 34.75, 34.68, 31.72, 31.64, 31.35, 30.93, 30.62, 30.42, 29.46, 29.45, 29.41, 29.21, 29.12, 29.07, 28.78, 28.63, 28.17, 27.45, 26.36, 23.21, 22.45, 20.04, 17.29, 13.72, 12.46. Negative ESI m/z calc'd for $C_{35}H_{61}O_5S$ (M-H^-): 593; found 593.

Figure S1.

Scheme 1: **Chemical Synthesis of Cholesterol Derivative.**



Scheme 2: **Chemical Synthesis of Cholic Acid Derivative.**