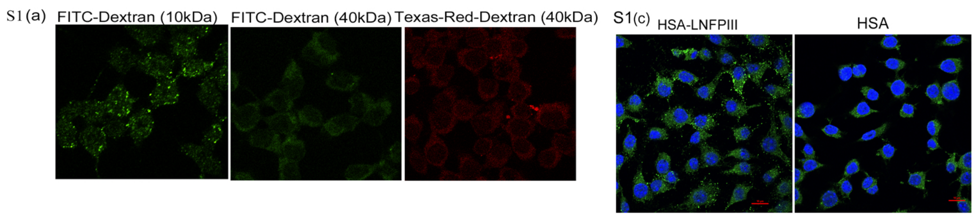
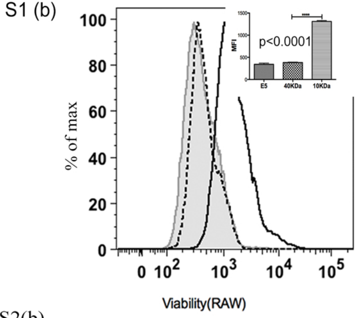
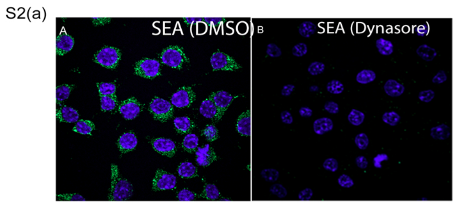
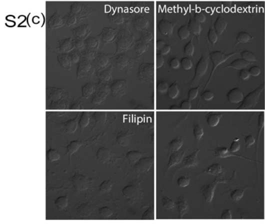
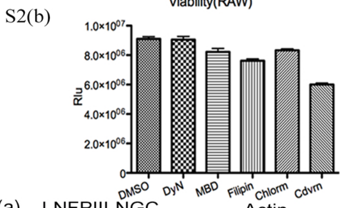
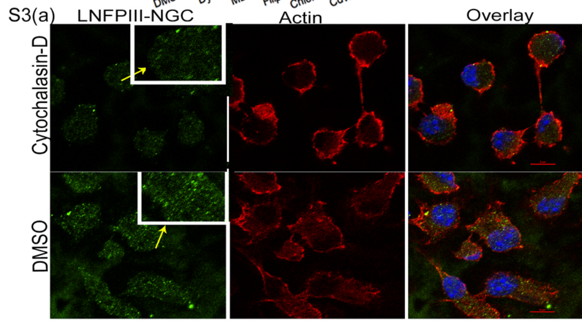

S1 (a)
FITC-Dextran (10kDa)
FITC-Dextran (40kDa)
Texas-Red-Dextran (40kDa)
S1(c)
HSA-LNFPIII
HSA

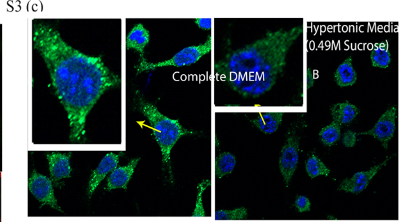

S1 (b)
100
80
60
40
20
0
% of max
p<0.0001
MFI
E5
40KDa
10KDa
Viability(RAW)

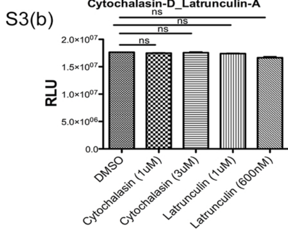

S2(a)
SEA (DMSO)
SEA (Dynasore)

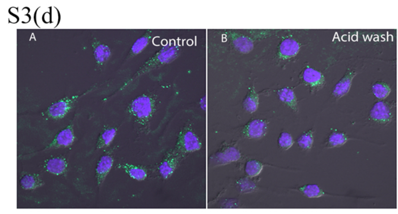

S2(c)
Dynasore
Methyl-b-cyclodextrin
Filipin

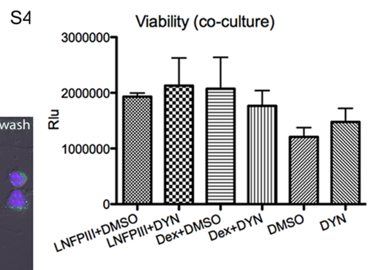

S2(b)
Rlu
DMSO
DyN
MBD
Filipin
Chlorm
Cdvrn

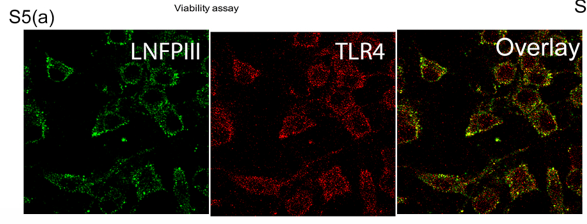

S3(a)
LNFPIII-NGC
Actin
Overlay
Cytochalasin-D
DMSO

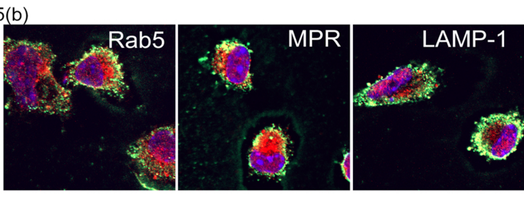

S3 (c)
Hypertonic Media (0.49M Sucrose)
Complete DMEM
B


S3(b)
Cytochalasin-D_Latrunculin-A
ns
ns
ns
ns
RLU
DMSO
Cytochalasin (1uM)
Cytochalasin (3uM)
Latrunculin (1uM)
Latrunculin (600nM)
Viability assay


S3(d)
A
Control
B
Acid wash


S4
Viability (co-culture)
3000000
2000000
1000000
0
Rlu
LNFPIII+DMSO
LNFPIII+DYN
Dex+DMSO
Dex+DYN
DMSO
DYN


S5(a)
LNFPIII
TLR4
Overlay


S5(b)
Rab5
MPR
LAMP-1

Supplementary

Fig. S.1 Endocytosis of 40 kDa Dextran and Human serum albumin (HSA) conjugated LNFPIII by antigen presenting cells. a) RAW 264.7 cells were incubated with FITC labeled 10 and 40 kDa (green) and Texas Red labeled 40kDa Dextran (red) for 10 min on ice, followed by incubation at 37°C for 30min. Cells were fixed, permeabilized and images were acquired at Nikon confocal microscope. b) RAW 264.7 cells were incubated with FITC labeled dextran (10 and 40 kDa) and later fixed, permeabilised and analyzed on flowcytometer to quantitate internalization as described in Fig. 1. FITC-dextran 10kDa, Black thick line; FITC-dextran 40kDa, filled gray; and Secondary antibody control, thick dotted line. Statistical significance was calculated using graph pad student's t test. c) RAW264.7 cells were incubated with HSA (human serum albumin) and HSA conjugated LNFPIII for 30 mins at 37°C, as described in methods. Cells were then treated with anti-HSA or anti-Lewis^{x} antibody (E.5) followed by an Alexa-488 conjugated secondary antibody (green). Cells were analyzed and images taken using a Nikon A1R confocal microscope at 60X objective. All the data represent 3 independent experiments performed.

Fig. S.2 Lewis^{x} in SEA is endocytosed by Dynamin mediated pathways. a) RAW 264.7 cells were pretreated with 80uM Dynasore (Dynamin inhibitor) for 40 mins, and then incubated with 25ug/ml SEA for 30min at 37°C. Cells were then fixed and stained for endocytosed SEA molecules expressing Lewis^{x} using E.5 monoclonal antibody (green). Cells were double stained with Hoechst dye (blue) to visualize nuclei. Panel A, cells treated with carrier solvent DMSO. Panel B, cells pretreated with 80uM Dynasore.

b) Cell viability assay after treatment with various inhibitors. Cells were pre-treated with different inhibitors for 40 min before the inducing endocytosis. Cells were harvested 30 mins post-endocytosis and cell viability assayed using CellTiter-Glo® Luminescent Cell Viability Assay kit from Promega. For each experiment samples were in quadruplicate and the data represents 4 independent experiments performed. Treatment with the indicated concentration of inhibitors did not result in cell death. c). Cells were also fixed and visualized under microscope to see any phenotypic effect.

Fig. S.3 Internalization of LNFPIII-NGC is actin dependent and receptor mediated. a) Cells were pretreated with 3uM of Cytochalasin-D for 15min on ice and endocytosis of LNFPIII-NGC was induced for 20min at 37°C. Cells were fixed, permeabilized and immunolabeled with anti-Lewis^x antibody followed by Alexa-488 conjugated secondary antibody. Cells were analyzed and pictures were taken on Nikon confocal microscope. Zoomed in image of a single cell has been put as an inset b) Cell viability assay for the actin inhibitors, Cytochalasin-D and Latrunculin-A was performed using CellTiter-Glo® Luminescent Cell Viability Assay kit from Promega. All the samples were in quadruplicate and the experiment was repeated 3 times. Result represents one of the 3 independent experiments performed. Statistical significance calculated by Graph pad Quick calc student's t-test. c) RAW 264.7 cells were pre-incubated for 15min in hypertonic media containing 0.45M sucrose, then stimulated with LNFPIII-NGC for 20 min. Panel A, cells stimulated in complete DMEM (control). Cells were harvested and immunolabeled for internalized LNFPIII as described in methods. d) RAW 264.7 cells were pretreated with mild acidic solution containing 20mM acetic acid (pH 2.8) +150mM

NaCl for 5 min at 37°C. Acid stripping was terminated by transferring the cells to pre-warmed, buffered DMEM. LNFPIII-NGC was added to the cells and endocytosis induced. After 30 mins, cells were stained for endocytosed LNFPIII-NGC using monoclonal antibody E.5 (green). We found significant reduction in the internalization of LNFPIII-NGC after acid pretreatment (panel B) compared to control in panel A.

Fig. S.4 Cell Viability assay for DC/CD4^{+}T cell co-culture experiment. A viability assay was performed to determine if dynasore treatment impacts cell viability. Treatment with 40uM dynasore showed no cell death. Cell viability was assayed using CellTiter-Glo® Luminescent Cell Viability Assay kit from Promega. All the samples were in quadruplicate and the data represents one out of 3 independent experiment performed.

Fig. S.5 LNFPIII-NGC co-localizes with TLR4. a) LNFPIII-NGC was incubated with RAW 264.7 macrophages for 20 min at 37°C. Cells were then fixed with 3% paraformaldehyde and immune-labeled with antibody to TLR4 (red) and anti-Lewis^{x} antibody E.5 (green). Co-localized image is shown as overlay in yellow. b) Internalization of LNFPIII-NGC in TLR-4 KO dendritic cell. Endocytosis of LNFPIII-NGC was induced for 30 min as described before. Cells were fixed, permeabilized and stained for internalized LNFPIII (green) and other endosomal markers (red).