Title: C-type lectin SIGNR1-mediated oral tolerance to food systemic anaphylaxis

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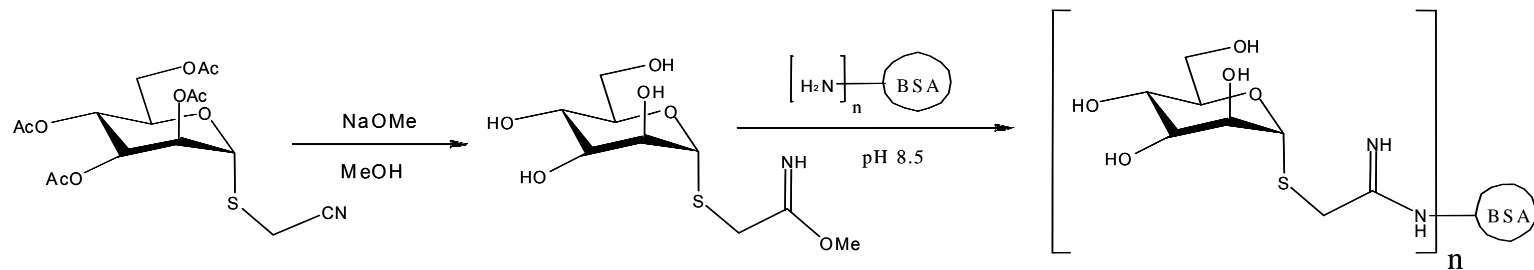
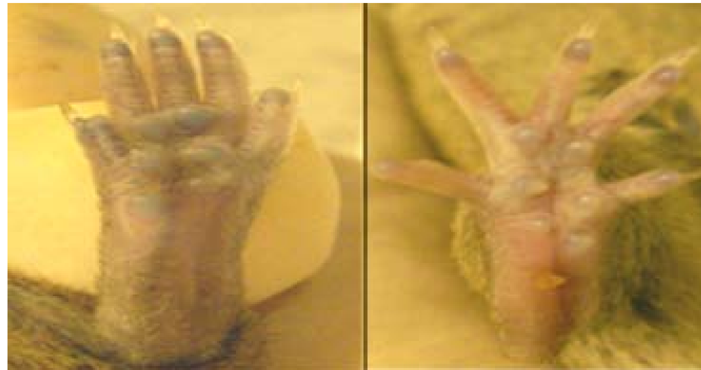
Supplemental Figure 1.



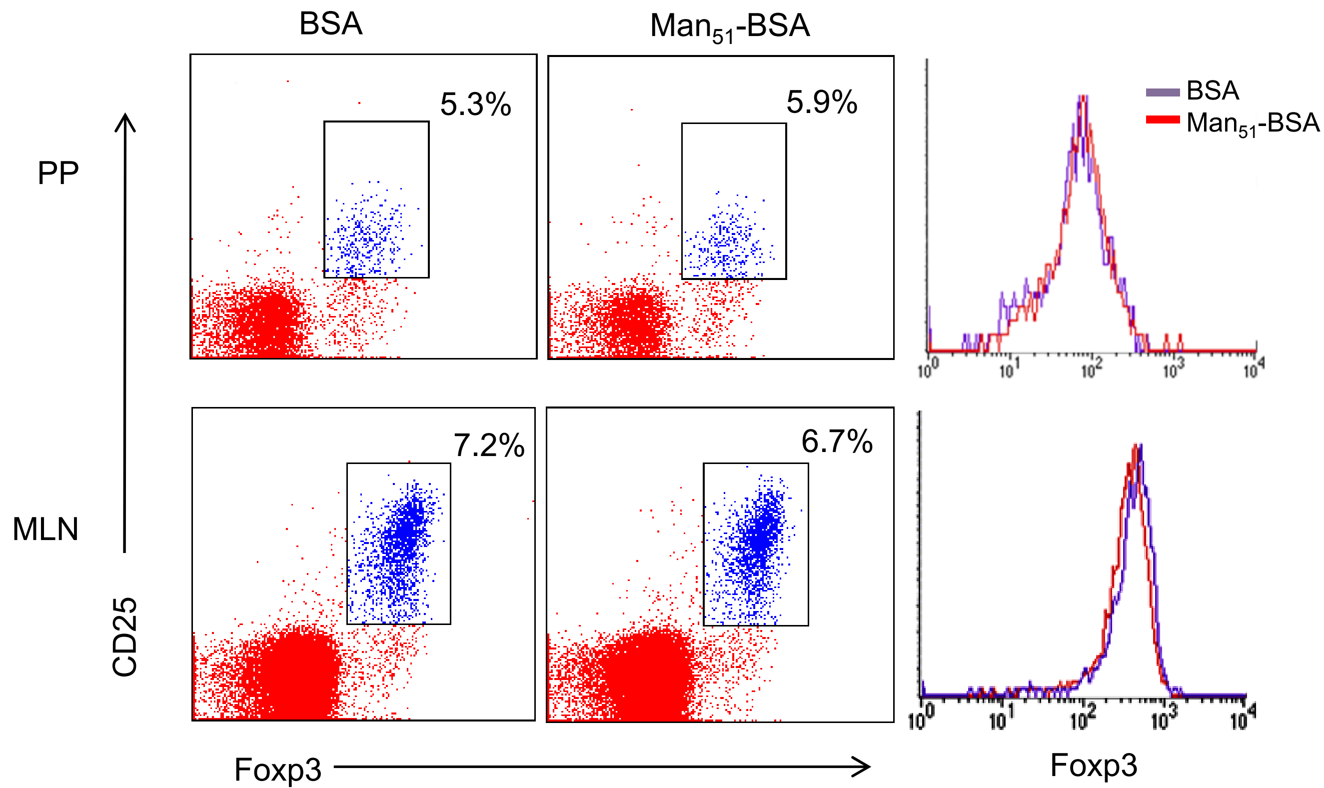
Supplemental Figure 1. An example of mannosylating BSA. Man-AI-BSA of the amidino type linkage is synthesized with imidate of α-thiomannoside, which in turn is prepared from cyanomethyl α-thiomannoside as shown above. Upon reacting the imidate with amino groups of protein (BSA, in this case), a neoglycoprotein modified with multiple Man residues is obtained. The ratio of the imidate to BSA determines the degree of modification at the ε-amino groups of lysyl residues. By this method, the modified amino groups still maintain positive charge (refs. 25 and 26).

Supplemental Figure 2.



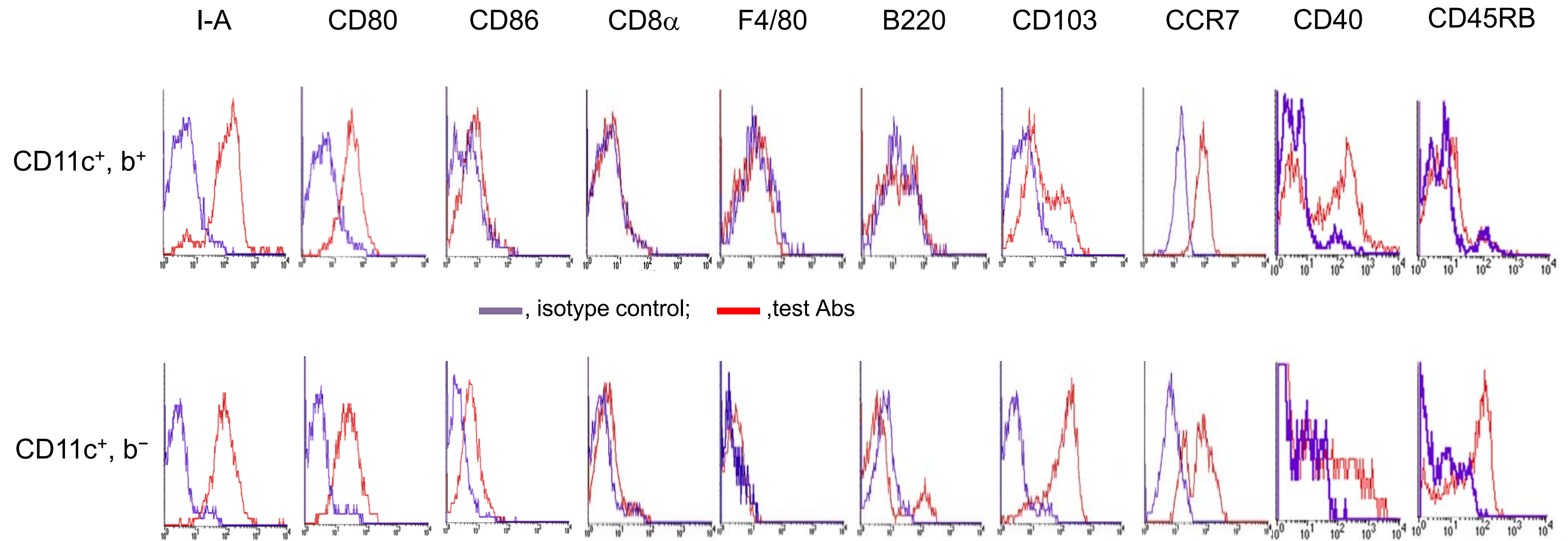
Supplemental Figure 2. Vascular leakage. BSA- (G1) and Man_{51}-BSA-treated (G2) mice were injected with Evan's blue dye (200 μl) into the tail vein immediately before i.p. challenge with BSA. After challenge, mice were monitored for 10 min and killed to record the effect of anaphylactic vascular leakage (blue color in the extremities). Photographs were taken using a Nikon camera, scanned, enlarged and adjusted for brightness and contrast using Adobe Photoshop. Blue color in the feet indicates dye leakage through the vascular bed because of increased capillary permeability.

Supplemental Figure 3.



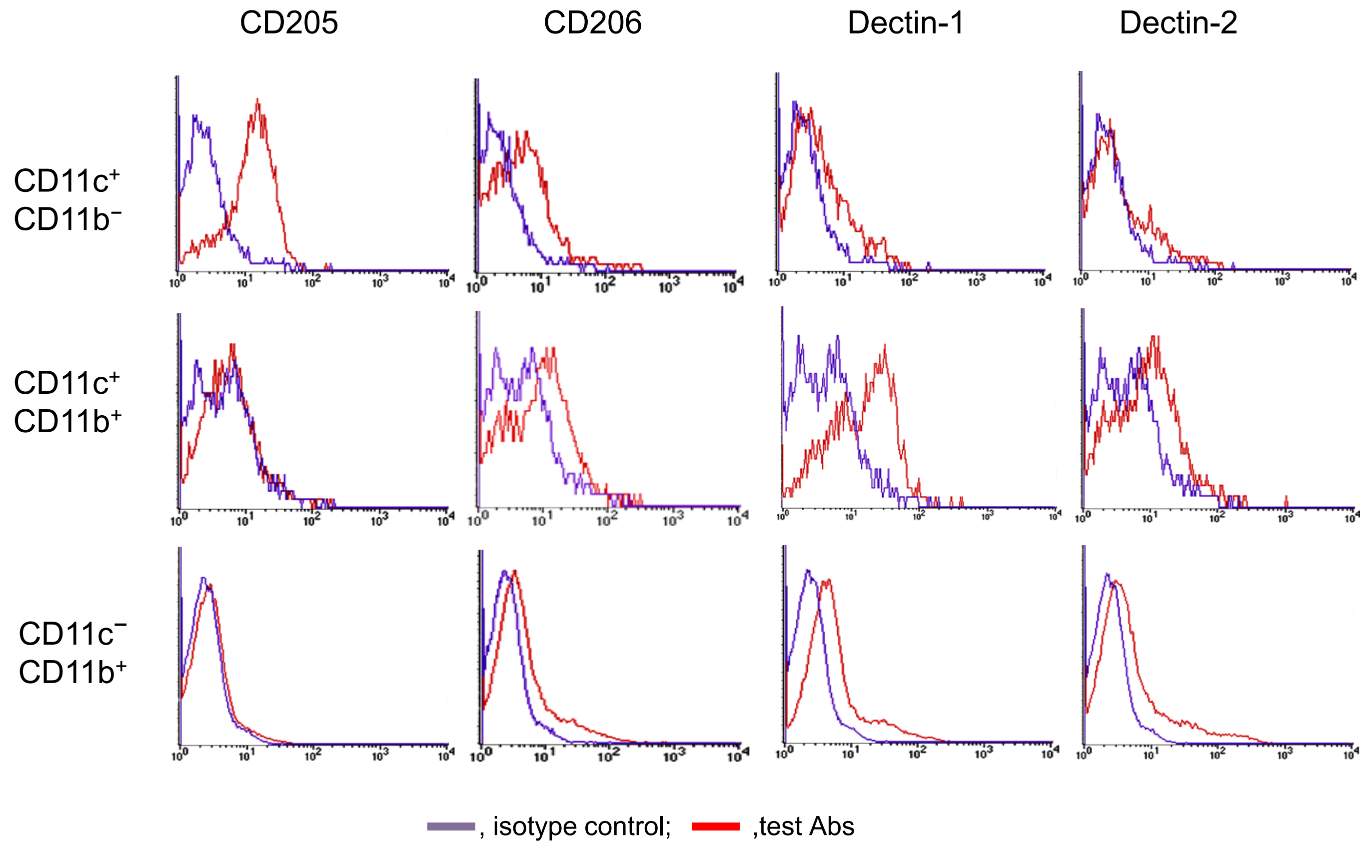
Supplemental Figure 3. Flow cytometric analysis of Tregs. $CD4^+$ T cells from PP and MLN were gated on $CD25^+$ cells, and intracellular Foxp3 staining was performed using APC-labeled anti-mouse Foxp3 (clone FJK-16s, eBioscience). Flow cytometric analysis was performed on a Becton Dickinson FACSCaliber Flow Cytometer. The % of $CD4^+CD25^+Foxp3^+$ cells in $CD4^+$ cells and the relative intensity of Foxp3 in $CD4^+CD25^+$ cells are shown.

Supplemental Figure 4.



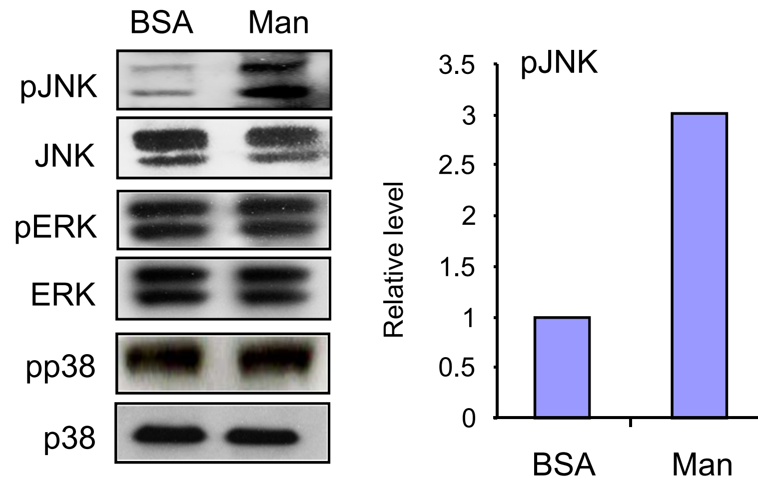
Supplemental Figure 4. Flow cytometric analysis of surface markers on LPDCs. Isolated small intestinal LPDC cells were re-suspended in PBS containing 3% FBS. After incubation for 15 min with blocking anti-CD16/CD32 Abs (2.4G2, BD Bioscience) , the cells were then stained with labeled Abs. The following Abs from BD Bioscience were used: anti-IA/IE (M5/114.15.2), anti-CD80 (16-10A1), anti-CD86 (GL1), CD8α (53-6.7); from eBioscience: anti-CD40 (HM40-3), anti-F4/80 (BM8), Anti-CD45RB (C363.16A), anti-B220 (RA3-6B2), anti-CD103 (2E7), anti-CCR7 (4B12).

Supplemental Figure 5.



Supplemental Figure 5. Flow cytometric analysis of CLR expression on LPDCs. Isolated small intestinal LPDC cells were analyzed for the expression of CLRs using the following Abs (AbD Serotec): anti-CD205 (NLDC-145), anti-CD206 (MR5D3), anti-Dectin-1 (2A11), anti-Dectin-2 (D2.11E4).

Supplemental Figure 6.



Supplemental Figure 6. Western blot analysis of Man_{51}-BSA-induced signaling. $CD11C^+$ LPDC isolated from C3H/hej mice were stimulated with 100 μg/ml of BSA or Man_{51}-BSA (Man) for 30 min. After stimulation, the total cellular extracts were subjected to gel electrophoresis and probed with Abs against for members of the MAPK family (Cell Signaling Technology and Millipore). The relative levels of phosphorylated proteins were quantified by densitometric analysis of the band intensities and normalized to those of the respective total kinases.

Supplemental Methods

Isolation and analysis of mucosal DCs: Small intestines were removed and flushed with ice-cold calcium- and magnesium-free HBSS (BioWhittaker). Peyer's patches were excised and the intestines were opened longitudinally and cut into small segments. Segments of the small intestines were treated for 30 min at 37 ^oC with HBSS containing 10% FCS, 2.5 mM EDTA for removal of epithelial cells, and then were washed extensively with PBS. Segments of the small intestines were digested with collagenase (0.5 mg/ml; StemCell Technologies) and DNase (100 μg/ml; Sigma) for 1 h at 37°C to yield a single cell suspension. In some experiments cell suspensions were enriched in $CD11c^+$ cells using anti-CD11c MACS beads (Miltenyi Biotec) and $CD11c^-CD11b^+$ cells were positively selected from the flow through cells ($CD11c^-$ cells) following the manufacturer's instructions, and the isolated cells were used directly for FACS staining or for in vitro culture. For immunofluorescence analysis of Man_{51}-BSA's cell target, cells were isolated from mice receiving oral administration of 200 μg of Man_{51}-BSA or BSA labeled with fluorophore (FITC) using FluoReporter®FITC protein labeling kit (Invitrogen), and 6 hrs later, flow cytometry analyses were performed, using biotinylated antibodies for FITC (Sigma, clone FL-D6) and FITC-conjugated streptavidin. Also, for flow analysis of SIGNR1 expression in LP and spleens, the cells were stained with Alexa-fluor®488-conjugated-antibody to SIGNR1 (ER-TR9; AbD Serotech) or an isotype control antibody, and gated on the surface expression of CD11c (N418, eBiosciences) and CD11b (M1/70, eBiosciences) markers. In some cases, the relative levels of SIGNR1 transcripts in LP or splenic DCs were evaluated by the use of quantitative RT-PCR (SYBR Green method, Applied Biosciences) and were calculated by the change in cycling threshold (ΔCT) method as $2^{-\Delta CT}$, where ΔCT is [CT (*Signr1*) – CT (*Gapdh*)]. The sequences for primers used are as follows: For *Signr1*, sense: 5'-

CGTCACAGCTTGCAAAGAAG-3’, antisense, 5’- CCATCCAGGTTGGTCCTTTA-3’; for *Gapdh*, sense, 5’-AACTTTGGCATTG TGGAAGG-3’, antisense, 5’-ACAC ATTGGGGGTAGGAACA-3’.

For immunocytochemistry analysis of Man_{51}-BSA target cells, specimens of intestines from mice receiving FITC-conjugated BSA or Man_{51}-BSA were fixed in 4% paraformaldehyde, embedded in OCT compound and frozen at -80°C. After blocking, the tissue sections were incubated with antibody to mouse CD11c (Hamster IgG, clone N418), Texas red conjugated antibody to hamster IgG (Vector Laboratories) and rabbit antibody to FITC and FITC-conjugated antibody to Rabbit IgG Abs (Invitrogen). Rabbit IgG (BD Bioscience Pharmingen) was used for isotype control. In some cases, total LP cells were isolated from IL-10-GFP tiger mice (Jackson Laboratory) on day 4 after oral administration of BSA or Man_{51}-BSA, and analyzed by flow cytometry. The percentage of GFP^+ cells was determined by gating on the $CD11c^+$ cell population. For analysis of Man_{51}-BSA-induced cytokine response in LPDCs, $CD11c^+$ and $CD11c^-CD11b^+$ cells ($2x10^5$/condition) from LPs and spleens of C3H/Hej mice were stimulated with medium alone, BSA (20 μg/ml), Man_{51}-BSA (20 μg/ml), or 1 μM of CpG (ODN1668, InvivoGen) for 24 hrs. IL-6, IL-10 and IL-12p70 in the supernatants were measured with ELISA (for IL-10 and IL-12p70, R&D; for IL-6, eBioscience). In some cases, the cells were stimulated in the presence or absence of a blocking antibody to SIGNR1 (50 μg/ml; ER-TR9), isotype control antibody, mannan (20 μg/ml; V-Lab), antibody to mannose receptor (50 μg/ml; clone 310301, R&D Systems) or 200 μM of a MyD88 peptide inhibitor or a control peptide (IMGENEX). Also, in some cases, LPDCs were isolated from B6 and SIGNR1-deficient mice and analyzed following the same protocol.

Spleen cells, $CD4^+$ cells and $CD11c^+$ cells and adoptive cell transfer: C3H/Hej, B6, SIGNR1- or IL-10-deficient mice were orally administered 200 μg of BSA or Man_{51}-BSA for 3

successive days. Five days after the last treatment, spleens from individual mice from the same group were pooled. $CD4^+$ or $CD11c^+$ cells were positively selected using anti-mouse CD4- or CD11c-microbeads (MACS; Miltenyi Biotec) according to the manufacturer's instructions. In all cases, the purity of isolated cells was >95%, as assessed by flow cytometry. For adoptive cell transfer experiments, cells were transferred into naive mice via the tail veins 1 day before the first sensitization followed by 7 times weekly sensitization and the challenge as described above. The numbers of transferred cells were 10^7 for whole spleen cells, $2x10^6$ for isolated $CD4^+$ cells or 10^6 for isolated $CD11c^+$ cells.

For in vitro analysis of T-cell cytokine response, splenic $CD4^+CD62L^+$ T cells ($1x10^6$/condition) isolated from naïve mice, using $CD4^+CD62L^+$ T-cell isolation kit (Miltenyi Biotec), were co-cultured with isolated $CD11c^+$ LPDCs ($1x10^5$/condition), pulsed with BSA (20 μg/ml) or Man_{51}-BSA (20 μg/ml), in the presence or absence of antibody to SIGNR1 (50 μg/ml; ER-TR9), neutralizing antibody to IL-10 (5 μg/ml; JES5-16E3, eBiosciences) or antibody to TGF-β (5 μg/ml; 1D11, R&D Systems) for 96 hrs, followed by washing and stimulation with or without a combination of antibodies to CD3/CD28 (1 μg/ml each; 145-2C11 and 37.51, respectively; BD Biosciences) for 24 hrs. T-cell cytokines, IL-10, IL-4 and IFN-γ, were measured by ELISA (eBioscience and R & D Systems). In some cases, LPDCs were stimulated with CpG oligonucleotides (1 μM) in conjunction with BSA or Man_{51}-BSA as above. For analysis of in vivo-generated T-cell response, mice (C3H/hej, B6, SIGNR1- or IL-10-deficient) received oral administration of PBS, BSA or Man_{51}-BSA (200 μg/mouse) daily for 3 days, and five days after last treatment, splenic $CD4^+$ T cells were isolated ($1x10^6$ cells/condition) and stimulated with anti-CD3/CD28 Abs as above for 24 hrs. T-cell cytokines, IL-4, IL-10 and IFN-γ, were measured by ELISA as above. For flow cytometric analysis of splenic Tregs, $CD4^+$ T cells were gated on

$CD25^+$ cells and intracellular Foxp3 staining was performed using APC-labeled anti-mouse Foxp3 (clone FJK-16s; eBioscience).