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# Oral tolerance to food-induced systemic anaphylaxis mediated by the C-type lectin SIGNR1

Yufeng Zhou[1,7], Hirokazu Kawasaki[1,7], Shih-Chang Hsu[1], Reiko T Lee[2], Xu Yao[1,3], Beverly Plunkett[1], Jinrong Fu[1], Kuender Yang[4], Yuan C Lee[2] & Shau-Ku Huang[1,5,6]

**We propose that a C-type lectin receptor, SIGNR-1 (also called Cd209b), helps to condition dendritic cells (DCs) in the gastrointestinal lamina propria (LPDCs) for the induction of oral tolerance in a model of food-induced anaphylaxis. Oral delivery of BSA bearing 51 molecules of mannoside ($Man_{51}$-BSA) substantially reduced the BSA-induced anaphylactic response. $Man_{51}$-BSA selectively targeted LPDCs that expressed SIGNR1 and induced the expression of interleukin-10 (IL-10), but not IL-6 or IL-12 p70. We found the same effects in IL-10–GFP knock-in (*tiger*) mice treated with $Man_{51}$-BSA. The $Man_{51}$-BSA–SIGNR1 axis in LPDCs, both *in vitro* and *in vivo*, promoted the generation of $CD4^+$ type 1 regulatory T (Tr1)-like cells that expressed IL-10 and interferon-γ (IFN-γ), in a SIGNR-1– and IL-10–dependent manner, but not of $CD4^+CD25^+Foxp3^+$ regulatory T cells. The Tr1-like cells could transfer tolerance. These results suggest that sugar-modified antigens might be used to induce oral tolerance by targeting SIGNR1 and LPDCs.**

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Analyses of DCs in tissues of the gastrointestinal tract have identified several subtypes with diverse functions, depending, in part, on the nature of the subset, maturation state and the initial stimulus from the microenvironment[1]. Disturbances at different steps in homeostasis result in a defective tolerance state and gastrointestinal hypersensitivity, as seen in food allergy and anaphylaxis[2–4], but the underlying mechanism of this hypersensitivity remains unclear. At present, strict avoidance of allergen sources is the only option for alleviating potentially fatal food anaphylaxis, highlighting the need to understand the mechanism of gastrointestinal homeostasis and to develop strategies for inducing oral tolerance.

Mucosal DCs are essential for the induction of immunity and tolerance, in part because they generate regulatory cytokines and because the cytokine milieu controls the generation of functionally diverse T cells[5]. DCs with regulatory functions have been found in Peyer's patches, the lamina propria and mesenteric lymph nodes (the MLN). Whereas DCs from both Peyer's patches and the MLN are crucial for T cell priming, DCs from the lamina propria and MLN seem to be essential for the induction of oral tolerance[6–10]. DCs might induce oral tolerance by inducing the generation of T cells with regulatory activities, including naturally occurring $CD4^+CD25^+Foxp3^+$ regulatory T ($T_{reg}$) cells, type 3 helper T ($T_H3$) cells or Tr1 cells[11–13]. This has been highlighted by the recent observation that DCs in both the MLN and the lamina propria can convert $CD4^+$ T cells into $Foxp3^+$ $T_{reg}$ cells *in vitro*[14,15] and can induce IL-10–producing $CD4^+$ Tr1-like cells[16]. Although mucosal DCs are known to be crucial for the induction of immunity and tolerance, the 'decision-making mechanism' that generates this dichotomized response remains to be fully defined.

DCs use their innate pattern-recognition Toll-like receptors (TLRs) and C-type lectin receptors (CLRs) to generate innate immunity and influence the adaptive response[17,18]. CLRs recognize complex glycan structures on various pathogens through their C-type carbohydrate-recognition domains (CRDs) and have evolved to facilitate the endocytosis of pathogens[19,20], but the exact expression profiles of various CLRs in specific subsets of DCs remain to be defined. Among the mouse homologs of the human CLR DC-SIGN, it seems that SIGNR1, which has a binding specificity similar to those of human DC-SIGN and its homolog L-SIGN, is mainly expressed in the peritoneal macrophages and lymphoid endothelium[21–23], although its expression level and cell-type specificity remain unclear. Also, depending on their tissue localization and differentiation state, DC subsets with different sets of CLRs can recognize distinct classes of infectious agents to induce tolerance or activate immunity, a process in which complex glycan structures on pathogens might have a key role[18]. The efficiency and specificity of the ligand-CLR interaction may be major factors in determining the immune outcomes and could potentially be harnessed into a targeting strategy for the induction of tolerance. Here our goal was to test the hypothesis that targeting the intestinal DCs with neoglyco-antigen (sugar-modified antigen), mimicking highly mannosylated structures on pathogens, leads to the induction of oral tolerance by CLRs in a prophylactic model of food allergy. We show that oral delivery of $Man_{51}$-BSA selectively targets LPDCs through SIGNR1 and induces the expression of IL-10, leading to the induction of tolerance.

[1]Johns Hopkins Asthma and Allergy Center, Baltimore, Maryland, USA. [2]Department of Biology, Johns Hopkins University, Baltimore, Maryland, USA. [3]Institute of Dermatology, Chinese Academy of Medical Sciences, Nanjing, China. [4]Chang Gung Memorial Hospital at Kaohsiung and Chang Gung University, Taiwan. [5]Center of Excellence for Environmental Medicine, Kaohsiung Medical University, Kaohsiung, Taiwan. [6]Graduate Institute of Medicine, Kaohsiung Medical University, Kaohsiung, Taiwan. [7]These authors contributed equally to this work. Correspondence should be addressed to S.-K.H. (skhuang@jhmi.edu).

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## RESULTS

### Sugar-modified BSA protects mice from BSA-induced anaphylaxis

To investigate whether BSA coupled with mannoside mediates a differential immune response *in vivo*, we used a mouse model of food allergy[24] and treated mice with BSA plus cholera toxin (a commonly used adjuvant) or mannosylated BSA with 51 molecules of mannosides ($Man_{51}$-BSA; **Supplementary Fig. 1**; refs. 25,26) plus cholera toxin. C3H/HeJ mice that were sensitized orally once each week for 7 weeks with BSA (200 μg per mouse) and cholera toxin (10 μg per mouse) showed signs of hypersensitivity after challenge with BSA (mean symptom severity score 4 out of 5 (fatality)); mice sensitized with $Man_{51}$-BSA (200 μg per mouse plus 10 μg per mouse of cholera toxin) did not develop any signs of hypersensitivity after challenge with BSA (**Fig. 1a**) or with $Man_{51}$-BSA (data not shown). BSA-sensitized mice that were pretreated with $Man_{51}$-BSA (200 μg per mouse without cholera toxin), but not with BSA, showed a significant decrease in the severity of anaphylaxis after challenge with BSA ($P < 0.05$; **Fig. 1b**).

The reduced anaphylactic response in mice treated with $Man_{51}$-BSA was associated with significant decreases in plasma histamine ($P < 0.05$; **Fig. 1c**) and vascular permeability upon challenge (**Supplementary Fig. 2**). When we measured serum levels of BSA-specific antibodies, mice that had been both sensitized and challenged with BSA showed significant levels of IgE, IgG1 and IgG2a ($P < 0.05$ in all cases), whereas mice that had been pretreated with $Man_{51}$-BSA did not show any appreciable increase in IgE and IgG1 antibodies. Mice treated with $Man_{51}$-BSA showed a modest increase in IgG2a before sensitization with BSA (**Fig. 1d**), but no further increase after BSA sensitization.

Mice that received adoptive transfers of splenocytes from $Man_{51}$-BSA–treated mice showed significant decreases in anaphylactic response ($P < 0.05$; **Fig. 1e**) and plasma histamine concentration ($P < 0.05$; **Fig. 1f**) after sensitization and challenge when compared to mice that received cells from BSA-treated mice. This effect was not due to the development of naturally occurring $CD4^+CD25^+Foxp3^+$ $T_{reg}$ cells in $Man_{51}$-BSA–treated mice, as we found no significant increase in $T_{reg}$ cells or Foxp3 expression after sensitization with BSA in Peyer's patches, the MLN (**Supplementary Fig. 3**) or the spleen (**Fig. 1g**) 5 d after the last oral administration of BSA or $Man_{51}$-BSA, when splenocytes can transfer tolerance.

### $Man_{51}$-BSA targeted LPDCs

To determine whether $Man_{51}$-BSA could target mucosal DCs *in vivo*, we treated naive mice orally with FITC-labeled $Man_{51}$-BSA or BSA and carried out flow cytometry analyses of the cells isolated 6 h later from the lamina propria, Peyer's patches, MLN and spleen. We found considerable staining of DCs co-expressing CD11c and CD11b in the lamina propria, with around 50% of those cells staining positive for FITC–$Man_{51}$-BSA (**Fig. 2a**). By contrast, we found few, if any, positively stained cells in other cell subsets, including $CD11c^-CD11b^+$ macrophages (**Fig. 2a**) in the lamina propria and in DCs from the MLN, Peyer's patches and spleen (data not shown). Immunocytochemical analyses of the small intestine also showed that most of the cells that took up FITC–$Man_{51}$-BSA were $CD11c^+$ DCs (**Fig. 2b**), as judged by marked dual-color staining of $CD11c^+$ cells in the lamina propria of mice that received $Man_{51}$-BSA. In contrast, the lamina propria of BSA-treated mice contained no apparent co-stained cells. At this time point, most of the labeled BSA was localized in the epithelium with diffuse staining patterns (**Fig. 2b**). Phenotypic analysis of this $CD11c^+CD11b^+$ LPDC subset revealed that it expressed the major histocompatibility complex class II molecule I-A, CD80, CD40, the chemokine receptor CCR7 and the α-integrin CD103, whereas it did not express CD8α, the B cell–associated marker B220, CD45RB or the macrophage marker F4/80 (**Supplementary Fig. 4**).

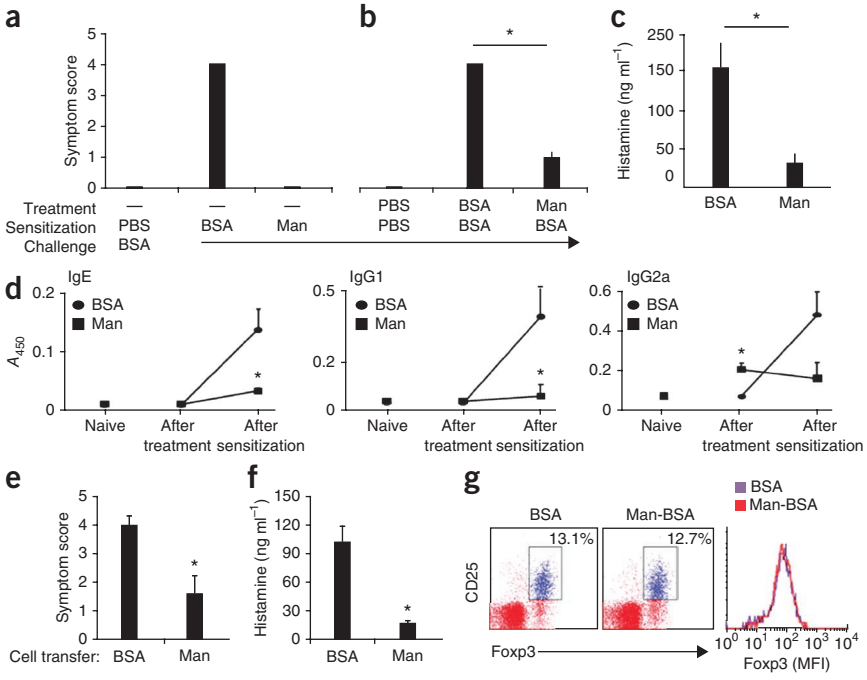


**Figure 1** Antigen-induced anaphylaxis in C3H/Hej mice. (**a**) The severity of anaphylactic responses, scored 30 min after antigen challenge by a scoring system[24] from 0 (no sign of shock) to 5 (death), in mice sensitized with PBS, BSA (200 μg per mouse) or $Man_{51}$-BSA (Man, 200 μg per mouse) plus cholera toxin (10 μg per mouse) and challenged with BSA (1 mg per mouse). (**b**) Mice were pretreated daily on 3 consecutive days with BSA or $Man_{51}$-BSA at 200 μg per mouse, followed by oral sensitization and challenge with BSA. $^*P < 0.05$. (**c**) Plasma histamine concentrations in the same groups of mice as in **b**. $^*P < 0.05$. (**d**) Relative amounts of specific IgE, IgG1 and IgG2a antibodies in naive mice and those after treatment (before BSA sensitization) with BSA or $Man_{51}$-BSA or after BSA sensitization. $^*P < 0.05$ versus BSA; $n = 8$–14 mice per group. (**e**,**f**) Symptom scores (**e**) and plasma histamine concentration (**f**) in sensitized and challenged mice that received spleen cell transfer from mice treated with BSA or $Man_{51}$-BSA. $^*P < 0.05$; $n = 6$–8 mice per group. (**g**) Representative flow cytometry analysis of splenic $Foxp3^+$ $T_{reg}$ cells in mice treated with BSA or $Man_{51}$-BSA. The percentage of $CD25^+Foxp3^+$ cells gated on $CD4^+$ cells and the relative intensity (MFI) of Foxp3 are shown. $n = 3$–5 mice per group. Results are representative of two independent experiments. Data are expressed as means ± s.e.m.

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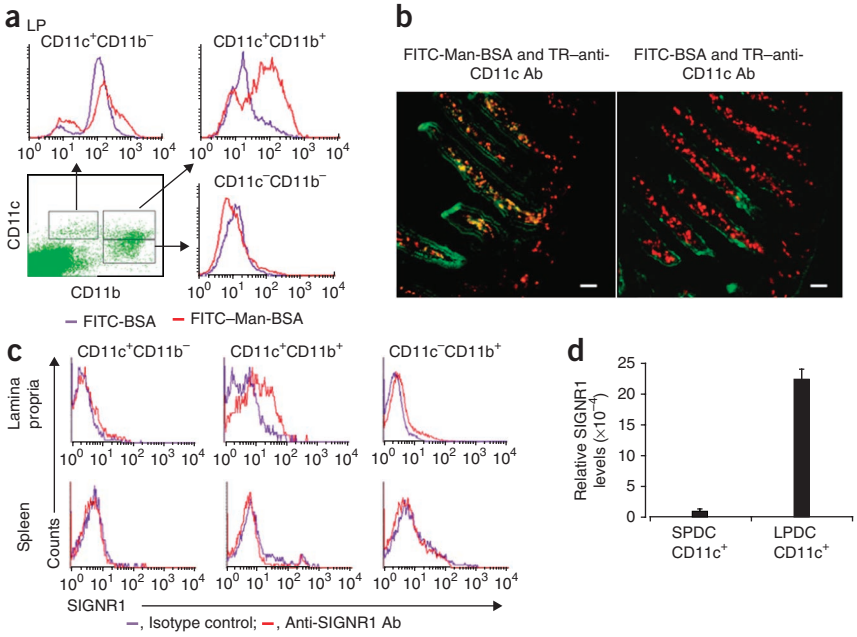


**Figure 2** $Man_{51}$-BSA targets the DC subset in the lamina propria. (**a**) A representative flow cytometry analysis of three main LPDC or macrophage subsets in mice that received oral administration of FITC-BSA or FITC-$Man_{51}$-BSA. (**b**) Immunocytochemical analysis of small intestine samples from the groups of mice in **a**. Two-color merge pictures; Ab, antibody; scale bar, 50 μm. (**c**) Flow cytometry analysis of SIGNR1 expression in three cell subsets from lamina propria and spleen. (**d**) The relative levels of SIGNR1 gene expression in LPDCs and splenic $CD11c^+$ DCs (SPDCs) measured by quantitative RT-PCR. Data are expressed as means ± s.e.m. of two experiments.

### Expression of SIGNR1 in mucosal DCs

To determine which CLR is responsible for taking up $Man_{51}$-BSA, we carried out flow cytometry analyses of intestinal DCs to profile the expression patterns of selected CLRs, including SIGNR1 and mannose receptor (CD206), both of which bind mannose-bearing antigens. Analyses of LPDCs showed that SIGNR1 was expressed selectively in the $CD11c^+CD11b^+$ DC subset (**Fig. 2c**), whereas DCs from spleen showed no expression of SIGNR1, as confirmed by quantitative PCR analyses (**Fig. 2d**). These analyses showed that CD205 was selectively expressed in the $CD11c^+CD11b^-$ DC subset, whereas dectin-1, the yeast zymosan receptor, seemed to be mainly expressed in the $CD11c^+CD11b^+$ subset, and DCs showed variable expression of CD206 and dectin-2 (**Supplementary Fig. 5**).

To test whether $Man_{51}$-BSA is a ligand for SIGNR1, we analyzed the ability of $Man_{51}$-BSA and other sugar-modified BSA derivatives, including galactosylated BSA ($Gal_{34}$-BSA), to bind SIGNR1. Mouse SIGNR1 showed considerable binding to $Man_{51}$-BSA but negligible binding to BSA and $Gal_{34}$-BSA (**Fig. 3a**). To confirm its binding, we performed a cell-based, competition analysis of SIGNR1-transfected cells, using FITC-labeled dextran, a known ligand for SIGNR1[23], and varying concentrations of neoglyco-antigens as competitors. $Man_{51}$-BSA inhibited, in a dose-dependent manner, the labeled dextran's binding to SIGNR1 but did not compete significantly with BSA (not shown), $Gal_{34}$-BSA or a mannosylated BSA derivative, $Man_8$-BSA, that has a much lower density of mannosides (**Fig. 3b**).

### $Man_{51}$-BSA selectively induces IL-10 expression in LPDCs

To investigate the functional effect of the $Man_{51}$-BSA–SIGNR1 axis, we examined the concentrations of cytokines in $CD11c^+$ DCs from the lamina propria stimulated with BSA or $Man_{51}$-BSA. As expected, CpG, a TLR-9 agonist, induced the expression of IL-10, IL-12 and IL-6 in all DC subsets (**Fig. 3c**,**d**). In $CD11c^+$ LPDCs, $Man_{51}$-BSA significantly induced the expression of IL-10 ($P < 0.05$; **Fig. 3c**), but not of IL-6, IL-12 p70 (**Fig. 3d**), IL-27 or transforming growth factor-β (TGF-β) (data not shown); in contrast, $CD11c^-CD11b^+$ cells from the lamina propria (**Fig. 3e**) and $CD11c^+$ spleen DCs (**Fig. 3f**) showed no detectable IL-10 expression after stimulation with $Man_{51}$-BSA.

The addition of a SIGNR1-specific blocking antibody or of mannan (a weak SIGNR1 antagonist) significantly decreased IL-10 in $Man_{51}$-BSA–treated LPDCs ($P < 0.05$; **Fig. 4a**). Moreover, the presence of a CD206-specific blocking antibody did not inhibit IL-10 expression induced by $Man_{51}$-BSA (**Fig. 4b**), nor did the addition of an inhibitor for MyD88, a TLR signaling molecule, as compared to that seen in medium control or in cultures with an irrelevant peptide (**Fig. 4c**). In contrast, the induction of IL-10 in LPDCs by CpG was significantly inhibited in the presence of a MyD88 peptide inhibitor (**Fig. 4c**). Furthermore, IL-10 expression in LPDCs induced by $Man_{51}$-BSA was associated with the activation

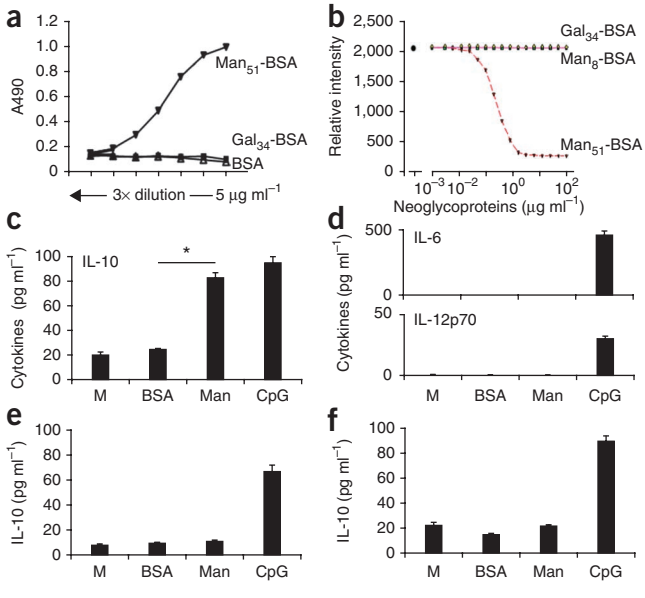


**Figure 3** Binding analysis of neoglyco-antigens. (**a**) Solid-phase binding analyses. The relative binding activity was expressed as absorbance at 490 nm after subtracting the background. (**b**) Representative competition analysis. The MFI of SIGNR1-transfectants stained with FITC-labeled dextran (10 μg $ml^{-1}$; dextran-FITC) in the presence or absence of varying concentrations of neoglyco-antigens as competitors as indicated, as determined by flow cytometry. (**c**,**d**) Concentrations of IL-10 (**c**) and IL-6 and IL-12p70 (**d**) in $CD11c^+$ DCs from lamina propria. (**e**,**f**) Concentrations of IL-10 in $CD11c^-CD11b^+$ cells from lamina propria (**e**) and $CD11c^+$ DCs from spleen (**f**). Cells in **c**–**f** were stimulated with medium alone (M), BSA (20 μg $ml^{-1}$), $Man_{51}$-BSA (Man, 20 μg $ml^{-1}$) or CpG (1 μM) for 24 h.*$P < 0.05$. Data are expressed as means ± s.e.m of three to five experiments in each panel.

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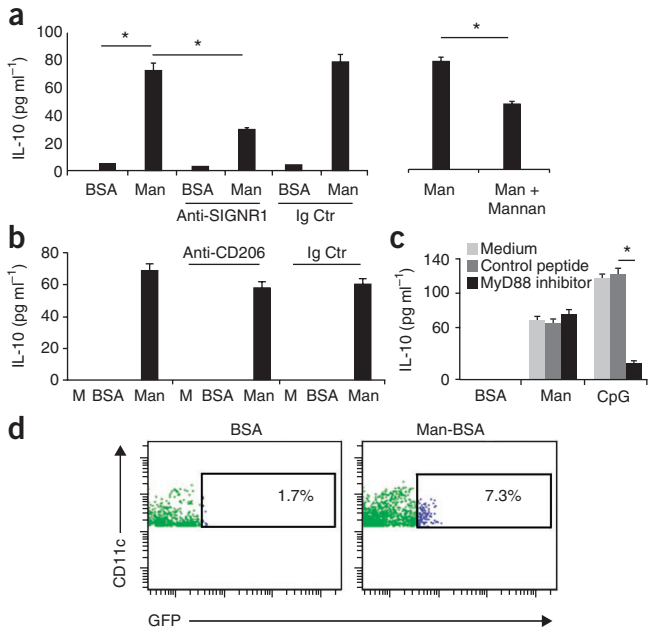


**Figure 4** Inhibition analyses of IL-10 induction by $Man_{51}$-BSA in LPDCs. (**a**–**c**) The concentrations of IL-10 in CD11c$^+$ LPDCs ($2 \times 10^5$ per condition). (**a**) Cells were stimulated with medium alone, BSA (20 μg ml$^{-1}$) or $Man_{51}$-BSA (20 μg ml$^{-1}$) with or without a blocking SIGNR1-specific antibody (50 μg ml$^{-1}$), isotype control (Ig Ctr) or, in separate assays, mannan (20 μg ml$^{-1}$). (**b**) Cells were stimulated as in **a** with or without an antibody specific to mannose receptor (CD206; 50 μg ml$^{-1}$) or isotype control. (**c**) Cells were stimulated as in **a** and pretreated with a MyD88 peptide inhibitor or a control peptide (200 μM). DCs were stimulated with 1 μM CpG in separate cultures for comparison. The concentrations of IL-10 in LPDCs after 24 h simulation were measured by ELISA. *$P < 0.05$. (**d**) Flow cytometry analysis of CD11c$^+$GFP$^+$ (IL-10$^+$) cells in lamina propria of IL-10-GFP *tiger* mice. Data are expressed as means ± s.e.m of three experiments in each panel.

of a member of the mitogen-activated protein kinase family, c-Jun N-terminal kinase, but not of p38 or extracellular signal–regulated kinase (**Supplementary Fig. 6**), consistent with those noted in a mouse RAW cell line overexpressing SIGNR1-Flag fusion proteins and activated with a Flag-specific antibody[27]. Moreover, analysis of intestinal DCs from IL-10–GFP *tiger* mice[28] corroborated the finding that LPDCs are the primary targets of $Man_{51}$-BSA, as the percentage of GFP$^+$CD11c$^+$ DCs increased from 1.7% to 7.3% in the lamina propria of mice that had received $Man_{51}$-BSA (**Fig. 4d**), whereas the spleen contained far fewer GFP$^+$ cells (0.5%) and there was no difference in the number of GFP$^+$ cells from the MLN and Peyer's patches at this time point (data not shown).

### $Man_{51}$-BSA-treated DCs promote the generation of Tr1-like T cells

Next we investigated effects of $Man_{51}$-BSA–treated LPDCs on the T cell differentiation pathway. We found significantly increased concentrations of IL-10 and IFN-γ in T cells cultured with $Man_{51}$-BSA–pulsed LPDCs ($P < 0.05$; **Fig. 5**) as compared to BSA-pulsed cells. This effect was ablated by a SIGNR1-blocking antibody (**Fig. 5a**) or an IL-10–neutralizing antibody (**Fig. 5b**), but not by a TGF-β–neutralizing antibody (data not shown). The frequency of Foxp3$^+$ T cells in the cocultures remained similar throughout the assay period (data not shown). When we used CpG as an additional stimulus, we found no significant difference in the amount of cytokines in T cells co-cultured with either BSA- or $Man_{51}$-BSA–pulsed DCs (data not shown).

We noted a similar pattern of T cell differentiation into IL-10– and IFN-γ–expressing cells *in vivo* in CD4$^+$ T cells from mice treated with $Man_{51}$-BSA, but not BSA, after stimulation (**Fig. 5c**). Mice that received adoptive transfers of CD4$^+$ T cells, but not of CD11c$^+$ splenic DCs, from mice treated with $Man_{51}$-BSA, but not with BSA, showed decreases in anaphylactic response ($P < 0.05$; **Fig. 5d**) and plasma histamine concentration ($P < 0.05$; **Fig. 5e**).

### SIGNR1 and IL-10 are necessary for tolerance induction

To determine whether SIGNR1 is required for the induction of tolerance by $Man_{51}$-BSA, we analyzed the generation of splenic Tr1-like T cells *in vivo* in SIGNR1-deficient and wild-type mice (C57BL/6 background). $Man_{51}$-BSA did not induce expression of IL-10 (**Fig. 6a**) and IFN-γ (**Fig. 6b**) in Tr1-like T cells from SIGNR1-deficient mice treated with $Man_{51}$-BSA when compared with wild-type mice treated with $Man_{51}$-BSA. Moreover, although C57BL/6 mice are known to be a poor IgE and $T_H2$ responder strain, and the severity of anaphylaxis in these mice

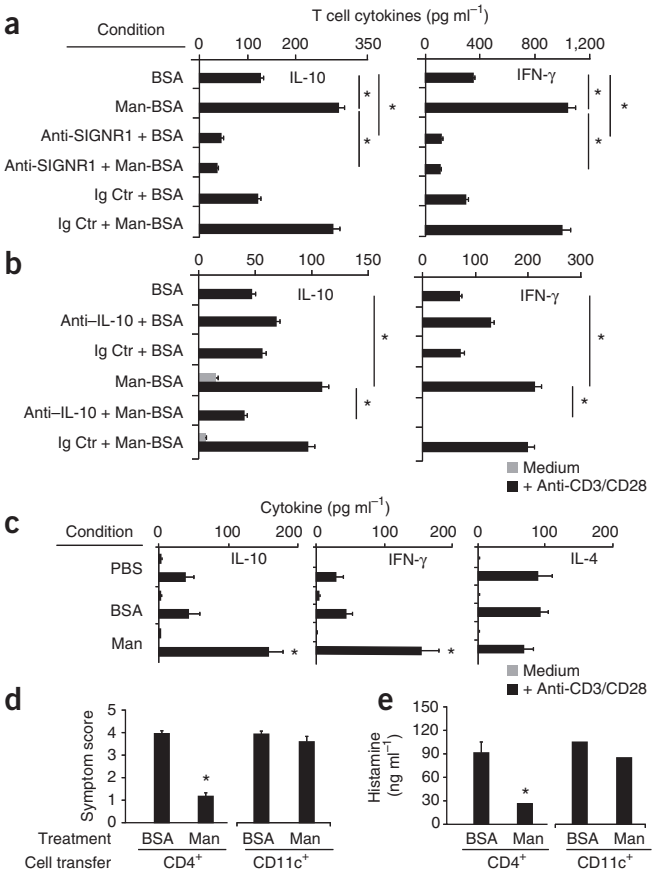


**Figure 5** Analysis of cytokine expression in T cells. (**a**,**b**) Expression of IL-10 and IFN-γ (as measured by ELISA) in T cells *in vitro* when cocultured with $Man_{51}$-BSA–pulsed LPDCs (CD11c$^+$), in the presence or absence of SIGNR1-specific or isotype control (Ig Ctr) antibody (**a**) or neutralizing IL-10–specific (5 μg ml$^{-1}$) or isotype control (Ig Ctr) antibody (**b**). (**c**) Expression of IL-10 and IFN-γ in T cells from mice receiving oral administration of PBS, BSA or $Man_{51}$-BSA (200 μg per mouse). *$P < 0.05$ versus PBS or BSA. (**d**,**e**) Symptom scores (**d**) and plasma histamine concentration (**e**) in sensitized and challenged mice receiving transfers of CD4$^+$ T cells or CD11c$^+$ DCs from mice treated with BSA or $Man_{51}$-BSA. *$P < 0.05$; $n = 6$–8 mice per group. Data are expressed as means ± s.e.m.

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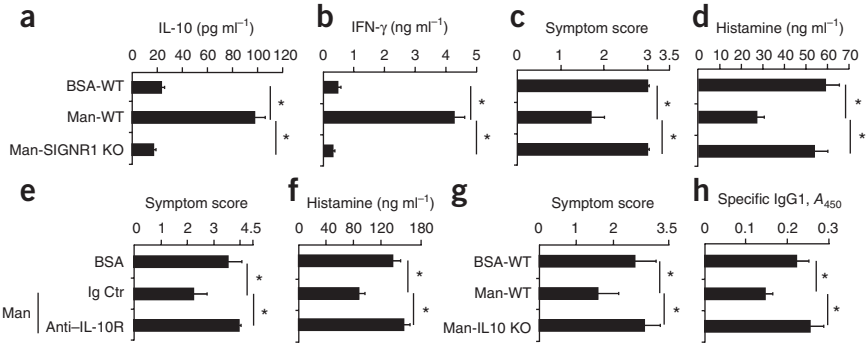


**Figure 6** Reversal of $Man_{51}$-BSA–mediated tolerance in SIGNR1- and IL-10–deficient mice. (**a**,**b**) IL-10 (**a**) and IFN-γ (**b**) abundance in splenic $CD4^+$ T cells from wild-type (WT) or SIGNR1-deficient (SIGNR1 KO) mice that were treated with oral administration of BSA or $Man_{51}$-BSA and stimulated. $^*P < 0.05$. (**c**,**d**) Symptom scores (**c**) and plasma histamine concentrations (**d**) in sensitized and challenged wild-type and SIGNR1-deficient mice. $^*P < 0.05$; $n = 6$–8 mice per group. (**e**,**f**) Symptom scores (**e**) and plasma histamine (**f**) in C3H/HeJ mice treated with BSA or $Man_{51}$-BSA that received IL-10R–specific or isotype control (Ig Ctr) antibodies. $^*P < 0.05$; $n = 4$–6 mice per group. (**g**,**h**) Antigen-induced symptom scores (**g**) and specific IgG1 response (**h**) in wild-type mice adoptively transferred with splenic $CD4^+$ T cells from IL-10–deficient (IL-10 KO) or wild-type (WT) mice treated with BSA or $Man_{51}$-BSA. $^*P < 0.05$; $n = 4$–6 mice per group. Data are expressed as means ± s.e.m.

tends to be milder than that seen in the C3H/HeJ strain, we found significantly more severe symptoms ($P < 0.05$; **Fig. 6c**) and plasma histamine concentrations ($P < 0.05$; **Fig. 6d**) in SIGNR1-deficient mice treated with $Man_{51}$-BSA compared with wild-type mice treated with $Man_{51}$-BSA after sensitization and challenge with antigen.

$Man_{51}$-BSA–mediated suppression of antigen-induced anaphylactic severity (**Fig. 6e**) and plasma histamine abundance (**Fig. 6f**) was no longer apparent when C3H/HeJ mice received IL-10 receptor (IL-10R)-specific, but not isotype control, antibodies 1 day before and 2 weeks after the initial antigen sensitization. The inability of $Man_{51}$-BSA to induce tolerance was also seen in mice receiving a transfer of splenic $CD4^+$ T cells from $Man_{51}$-BSA–treated, IL-10–deficient, mice, but not from wild-type mice, as we found no significant reduction in symptom scores (**Fig. 6g**) or specific IgG1 concentrations (**Fig. 6h**). Also, $Man_{51}$-BSA–mediated tolerance to the antigen-induced IgG1 response was lost in IL-10–deficient mice as compared with that of wild-type mice (data not shown). These results suggest, therefore, the crucial role of IL-10 from $Man_{51}$-BSA–modified LPDCs in generating Tr1-like T cells and in conferring the suppressive activity of Tr1-like T cells on the antigen-induced anaphylactic response.

## DISCUSSION

We have provided evidence that a sugar-modified antigen, $Man_{51}$-BSA, can induce oral tolerance in a model of food allergy by a sequential process originating from targeting of LPDCs by $Man_{51}$-BSA and leading to the development of $CD4^+$, Tr1-like T cells that express IL-10 and IFN-γ in the spleen. SIGNR1 and IL-10 seem to be crucial for this process. Although the expression of CCR7 and CD103 in the $CD11c^+CD11b^+$ LPDC subset seemed indistinguishable from that in the $CD11c^+CD11b^-$ DC subset, the pattern of CLR expression varied between subsets. SIGNR1 expression seemed to mark the $CD11c^+CD11b^+CD8^-$ subset and served as the primary CLR in mediating the effect of $Man_{51}$-BSA. Functionally, the $Man_{51}$-BSA–SIGNR1 axis generated tolerogenic DCs by inducing the expression of IL-10, and $Man_{51}$-BSA–mediated tolerance required SIGNR1 and IL-10.

The finding that spleen DCs from mice treated with $Man_{51}$-BSA could not transfer tolerance is consistent with their inability to respond to $Man_{51}$-BSA *in vitro*. This might be due to their lack of, or low level of, SIGNR1 expression, and also to the possibility that they are resident in the spleen and are not migratory. Our results suggest that the mucosal immune response, particularly in the villous lamina propria, which accounts for most protein uptake from the intestine[9], can be preprogrammed for the induction of tolerance through the CLR-ligand interaction. It is unclear where Tr1-like T cells were primed by $Man_{51}$-BSA–stimulated DCs. The $Man_{51}$-BSA–targeted $CD11c^+CD11b^+$ LPDC subset expressed CCR7, suggesting that they might migrate into the MLN with subsequent priming of Tr1-like T cells. This possibility is supported by the finding that CCR7-dependent migration of DCs from the lamina propria to the MLN is required for the induction of oral tolerance[29]. A detailed kinetic analysis is required to track the migratory patterns of DCs and the development of Tr1-like T cells *in situ*.

Spleen-derived DCs that have a plasmacytoid phenotype ($CD45RB^+B220^+$) can be modified by TGF-β–induced $T_{reg}$ cells, with subsequent generation of IL-27, which promotes the development of IL-10–expressing Tr1 cells[30]. By contrast, $Man_{51}$-BSA–targeted $CD11c^+CD11b^+$ LPDCs did not express CD45RB and B220, could not secrete IL-27 and TGF-β after activation with $Man_{51}$-BSA and had no effect on the generation of $T_{reg}$ cells. These phenotypic and functional features distinguish LPDCs from spleen-derived, $CD45RB^+B220^+$ plasmacytoid-like DCs and from mucosal DCs that can induce $T_{reg}$ cells through TGF-β[14]. Although $Man_{51}$-BSA could elicit IL-10 production in LPDCs with subsequent generation of Tr1-like T cells in the absence of TLR signaling, its selective effect on IL-10 expression in T cells was lost when a TLR9 agonist, CpG, was added to the culture. Therefore, it is likely that the nature of the ligand-receptor axis in DC priming can determine the DCs' function. The selective expression of SIGNR1 and its candidacy as a target for induction of IL-10 in LPDCs might therefore represent a previously undescribed role of tolerogenic DCs in the intestinal mucosa in maintaining the homeostatic state.

There has been an intense interest in determining how DCs are programmed to generate immunity or induce tolerance, and the DC-expressed CLRs, with their diverse roles in antigen uptake and interactions between T cells and DCs, have been considered attractive targets. For example, injection of yeast zymosan plus ovalbumin in mice induces T cell tolerance through TLR2 and the CLR dectin-1 (ref. 31). Also, targeting of DEC-205 by a specific antibody has been shown to generate T cell tolerance[32]. A similar tolerogenic activity has also been found for human DC-SIGN[33]. Furthermore, DCs can be rendered tolerogenic when exposed to galectin-1, a glycan-binding protein, and this promotes IL-27–dependent and IL-10–mediated T cell tolerance[34]. These studies, combined with the finding that DC-specific immune receptor, a member of the CLR family, is a negative regulator of DC expansion[35], support the role of the CLR-ligand axis in immune modulation.

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There is evidence that pro- and prebiotics might be useful for alleviating allergic responses[36], but in most studies the detailed mechanisms are unclear, and the potential development of anaphylaxis upon delivery remains a major concern. Engineering, with relatively simple synthetic chemistry, sugar-modified antigens to target tolerogenic DCs could circumvent these limitations and offer safer and more efficacious designs. Notably, feeding a prebiotic formula with oligosaccharides to infants with a parental history of atopy has been associated with a lower incidence of allergic diseases[37]. The potential modulatory effect of oligosaccharides and, perhaps, microbials[38] might be mediated through the ligand-CLR axis, providing a conditioning effect on mucosal DCs. The integration by DCs of CLR signaling to control innate immunity and adaptive responses might thus represent a key mechanism for controlling mucosal immunity and could help in the design of effective strategies for alleviating the increasingly common and potentially fatal problem of food allergies.

## METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/naturemedicine/.

*Note: Supplementary information is available on the Nature Medicine website.*


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### AUTHOR CONTRIBUTIONS

Y.Z. conducted experiments involving characterizing mucosal DC subsets and their regulatory role, analyzed data and wrote the manuscript; H.K. performed *in vivo* experiments on antigen-induced anaphylactic responses, analyzed data and wrote the manuscript; S.-C.H. conducted experiments involving binding analyses of neoglyco-antigens; R.T.L. synthesized neoglyco-antigens and wrote the manuscript; X.Y. conducted *in vitro* experiments characterizing T cell responses; B.P. performed flow cytometric experiments and edited the manuscript; J.F. conducted *in vivo* cell transfer experiments; K.Y. contributed to the design and preparation of neoglyco-antigens; Y.C.L. designed and prepared neoglyco-antigens and supervised their synthesis; S.-K.H. planned, designed, supervised and coordinated the overall research efforts.

### COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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## ONLINE METHODS

**Mice, sensitization and challenge.** Four- to six-week-old male and female mice (C3H/Hej, C57BL/6, IL-10-deficient (B6.129P2-IL-10$^{tm1Cgn}$/J), IL-10–GFP knock-in (*tiger*) from the Jackson Laboratory and SIGNR1-deficient mice from the Consortium for Functional Glycomics) were maintained under specific pathogen–free conditions, and all experiments were approved by the Animal Care and Use Committee at Johns Hopkins University School of Medicine. Mice received seven weekly sensitizations with PBS or BSA (Sigma-Aldrich) (200 μg per mouse) plus cholera toxin (B subunit: 10 μg per mouse (List Biological Laboratories)) as adjuvant, and 1 week after the final sensitization they were challenged intraperitoneally with 1 mg of BSA. In some cases, C3H/HeJ mice were injected intraperitoneally with antibody to IL-10R receptor (0.25 mg per mouse; clone 1B1.3a, BD Biosciences) 1 d before and 2 weeks after the initial sensitization.

Coupling of BSA with mannosides or galactosides was performed as previously described[25,26]. Mice were orally treated with 200 μg per mouse of BSA or $Man_{51}$-BSA for three successive days before the first sensitization. Systemic anaphylaxis-like symptoms were evaluated in a blinded manner within 30 min after challenge using a previously described scoring system[24]. Plasma histamine concentrations were determined by enzyme immunoassays, and serum antibody concentrations were analyzed by ELISA[24] before and after sensitization.

**Binding analysis and analyses of immunological responses.** The binding activity of neoglyco-antigens to SIGNR1 was determined by solid-phase binding assays as previously described[39]. A cell-based competition analysis of SIGNR1-transfectant BaF3 cells was performed with varying concentrations of neoglyco-antigens as competitors in the presence of FITC-labeled dextran (2,000 kd, 10 μg ml$^{-1}$ (Sigma-Aldrich)), a known ligand for SIGNR1 (ref. 23).

Single-cell suspensions of intestinal lamina propria and Peyer's patches were enriched for CD11c$^+$ cells with anti-CD11c MACS beads, and CD11c$^-$CD11b$^+$ cells were positively selected from the flow-through cells. For flow cytometric analysis of the cell target of $Man_{51}$-BSA, cells were isolated from mice 6 h after oral administration of 200 μg of $Man_{51}$-BSA or BSA labeled with FITC and stained with biotinylated FITC-specific antibody and FITC-conjugated streptavidin. For analysis of SIGNR1 expression, the cells were stained with Alexafluor 488–conjugated antibody to SIGNR1 or isotype control and gated on the expression of CD11c (N418) and CD11b (M1/70). The relative levels of *Signr1* transcripts were evaluated by quantitative RT-PCR.

For analysis of $Man_{51}$-BSA target cells, frozen sections of small intestines of mice that had received FITC-conjugated BSA or $Man_{51}$-BSA were incubated with CD11c-specific antibody (hamster IgG, clone N418), Texas red–conjugated hamster-specific IgG, rabbit antibody to FITC, isotype control antibody and FITC-conjugated antibody to rabbit IgG. Total lamina propria cells were isolated from IL-10–GFP *tiger* mice on day 4 after oral administration of BSA or $Man_{51}$-BSA, and the percentage of GFP$^+$ cells was determined by gating on the CD11c$^+$ cells. For analysis of $Man_{51}$-BSA–induced cytokine production, CD11c$^+$ and CD11c$^-$CD11b$^+$ cells ($2 \times 10^5$ per condition) were stimulated with medium alone, BSA (20 μg ml$^{-1}$), $Man_{51}$-BSA (20 μg ml$^{-1}$) or 1 μM of CpG (ODN1668) in the presence or absence of antibody to SIGNR1 (50 μg ml$^{-1}$; ER-TR9), isotype control antibody, mannan (20 μg ml$^{-1}$), antibody to mannose receptor (50 μg ml$^{-1}$), 200 μM of a MyD88 peptide inhibitor or a control peptide, for 24 h, and cytokine levels were analyzed with ELISA. All antibody sources are in the **Supplementary Methods**.

For adoptive cell transfer experiments, total spleen cells, CD4$^+$ cells or CD11c$^+$ cells positively selected from pooled spleen cells of the same group were isolated from mice 5 d after three daily oral administrations of 200 μg of BSA or $Man_{51}$-BSA and were transferred into naive mice via the tail vein 1 d before the first sensitization, followed by sensitization once a week for 7 weeks and the challenge as described above. In all cases, the purity of isolated cells was >95%, as assessed by flow cytometry. The numbers of transferred cells were $1 \times 10^7$ for whole spleen cells, $2 \times 10^6$ for isolated CD4$^+$ cells and $1 \times 10^6$ for isolated CD11c$^+$ cells.

For analysis of T cell cytokines, splenic CD4$^+$CD62L$^+$ T cells ($1 \times 10^6$ per condition) from naive mice were cocultured with CD11c$^+$ LPDCs ($1 \times 10^5$ per condition) pulsed with BSA (20 μg ml$^{-1}$) or $Man_{51}$-BSA (20 μg ml$^{-1}$) in the presence or absence of antibody to SIGNR1 (50 μg ml$^{-1}$; ER-TR9) or neutralizing antibodies to IL-10 (5 μg ml$^{-1}$; JES5-16E3) for 96 h, followed by stimulation with or without a combination of antibodies to CD3 and CD28 (1 μg ml$^{-1}$ each; 145-2C11 and 37.51, respectively) for 24 h, and the abundance of cytokines were measured by ELISA. All antibody sources are in the **Supplementary Methods**. In some cases, splenic CD4$^+$ T cells were isolated ($1 \times 10^6$ cells per condition) from mice on day 8 after oral administration of PBS, BSA or $Man_{51}$-BSA (200 μg per mouse) and stimulated with antibodies to CD3 and CD28 as described above for 24 h. Further details can be found in the **Supplementary Methods**.

**Statistical analysis.** Data are expressed as the means ± s.e.m. for each group. Differences in each of the quantitative parameters were analyzed by *t* test, and the Wilcoxon signed-rank test was used to analyze differences in the anaphylactic symptom scores. Differences among groups of mice were determined by analysis of variance (StatView). If differences between groups were significant ($P < 0.05$), Fisher's protected least significant difference test was used to distinguish between pairs of groups.

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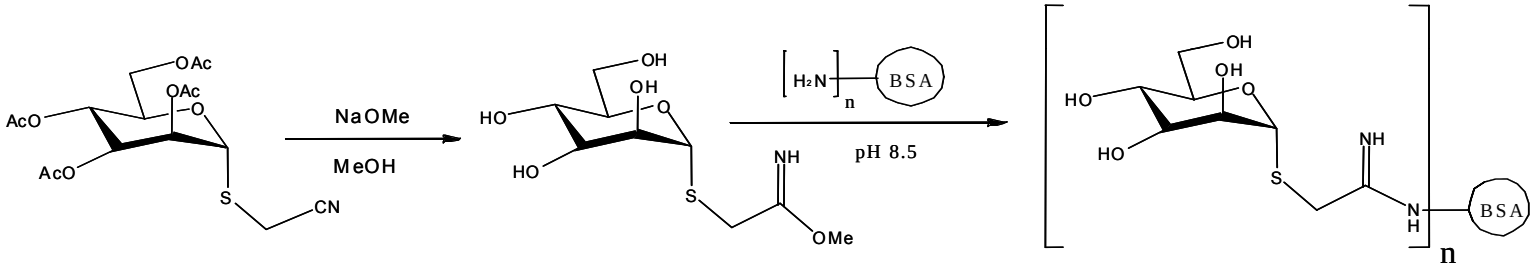
**Title: C-type lectin SIGNR1-mediated oral tolerance to food systemic anaphylaxis**

Authors: Yufeng Zhou, Hirokazu Kawasaki, Shih-Chang Hsu, Reiko T. Lee, Xu Yao, Beverly Plunkett, Jinrong Fu, Kuender Yang, Yuan C. Lee, and Shau-Ku Huang

**Supplemental Figure 1.**



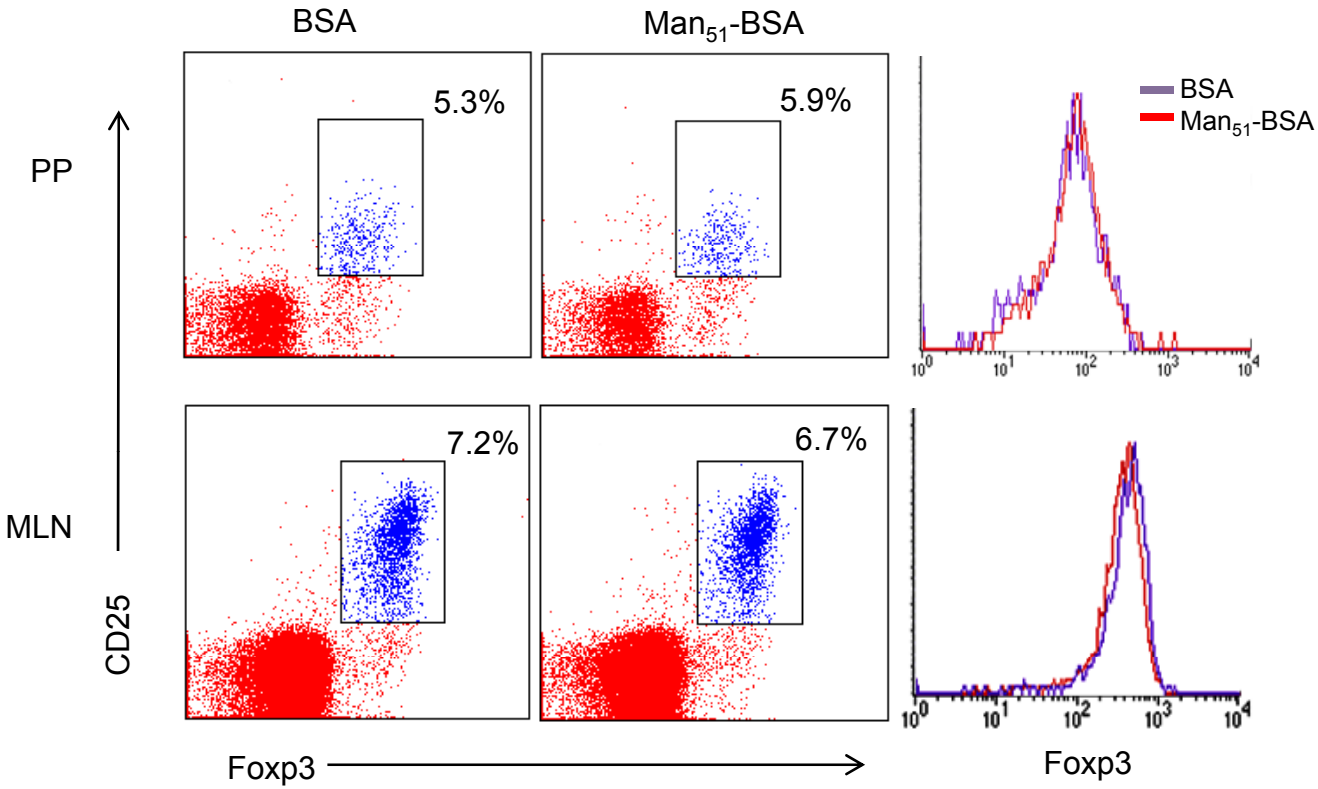

Supplemental Figure 1. An example of mannosylating BSA. Man-AI-BSA of the amidino type linkage is synthesized with imidate of α-thiomannoside, which in turn is prepared from cyanomethyl α-thiomannoside as shown above. Upon reacting the imidate with amino groups of protein (BSA, in this case), a neoglycoprotein 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.**

(G1) BSA (G2) $Man_{51}$-BSA

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.

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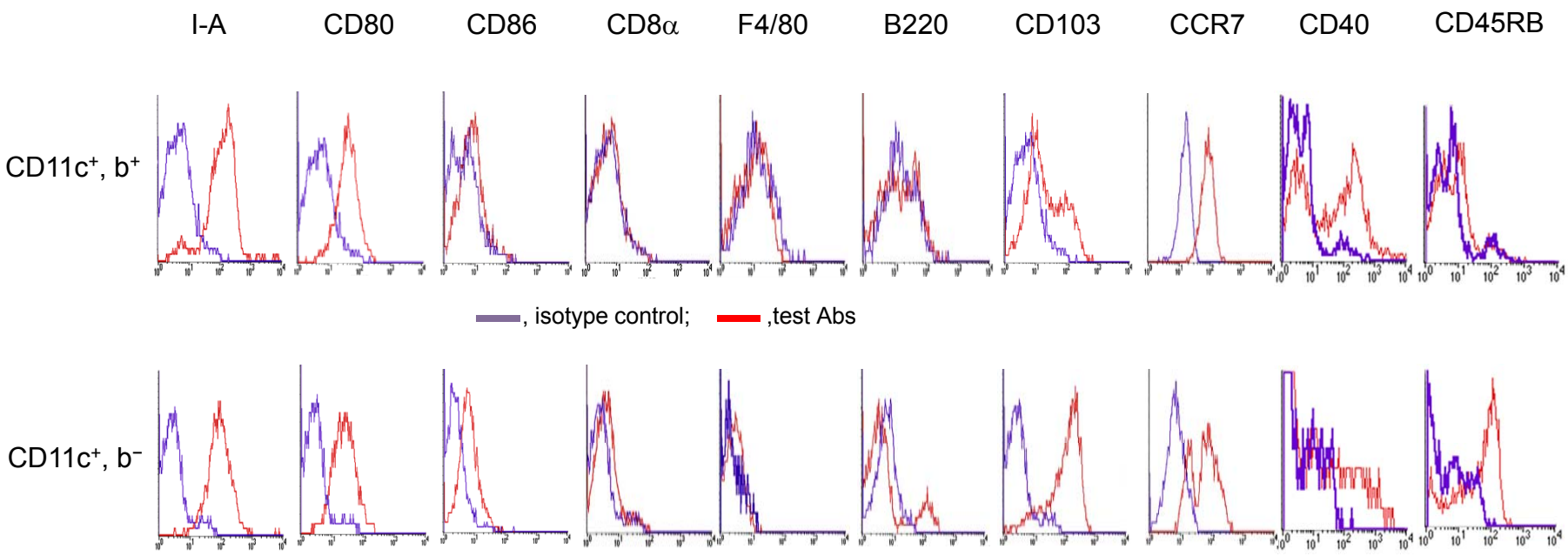
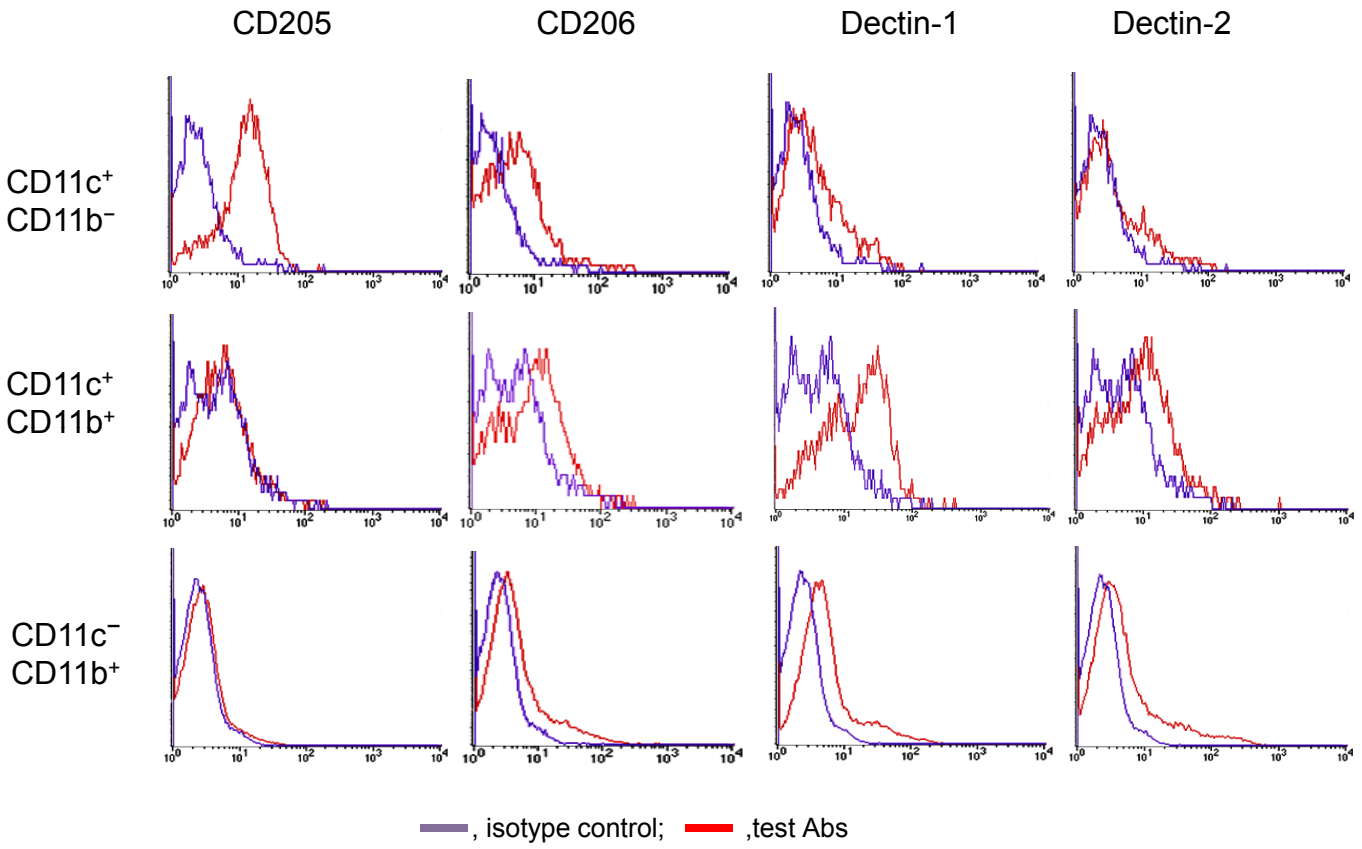
**Supplemental Figure 4.**



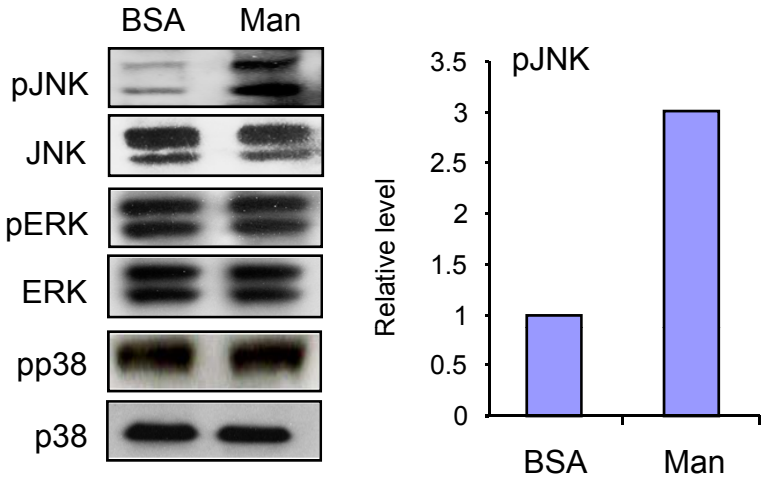

Supplemental Figure 4. Flow cytometric analysis of surface markers on LPDCs. Isolated small intestinal LPDC cells were re-suspended in PBS containing 3% FBS. After incubation for 15 min with blocking anti-CD16/CD32 Abs (2.4G2, BD Bioscience) , the cells were then stained with labeled Abs. The following 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 $^{o}$C 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$^{o}$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).