

Research Article

V-set and Ig domain-containing 4 (VSIG4)-expressing hepatic F4/80⁺ cells regulate oral antigen-specific responses in mouse

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Oral tolerance can prevent unnecessary immune responses against dietary antigens. Members of the B7 protein family play critical roles in the positive and/or negative regulation of T cell responses to interactions between APCs and T cells. V-set and Ig domain-containing 4 (VSIG4), a B7-related co-signaling molecule, has been known to act as a co-inhibitory ligand and may be critical in establishing immune tolerance. Therefore, we investigated the regulation of VSIG4 signaling in a food allergy and experimental oral tolerance murine models. We analyzed the contributions of the two main sites involved in oral tolerance, the mesenteric lymph node (MLN) and the liver, in VSIG4-mediated oral tolerance induction. Through the comparative analysis of major APCs, dendritic cells (DCs) and macrophages, we found that Kupffer cells play a critical role in inducing regulatory T cells (Tregs) and establishing immune tolerance against oral antigens via VSIG4 signaling. Taken together, these results suggest the possibility of VSIG4 signaling-based regulation of orally administered antigens.

Keywords: F4/80⁺ cells · Kupffer cell · Oral tolerance · Regulatory T cell · V-set and Ig domain-containing 4



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Introduction

Oral tolerance induction is mediated by APCs with orally administered Ags. Ingested Ags are absorbed in the intestine via transcellular transport by M cells or epithelial cells, diffusion through

epithelial tight junctions, exosome formation by enterocytes, or capture by extended macrophages [1]. Several lines of evidence demonstrate the critical roles of the intestinal immune system, comprising the GALT, mesenteric lymph node (MLN), and lamina propria (LP). In CCR7-deficient mice, which showed impaired

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migration of intestinal dendritic cells (DCs) to the MLN, oral tolerance could not be induced, suggesting the importance of Ag delivery of intestinal DCs to the MLN as an oral tolerance inductive site [2]. The co-signaling molecules B7-H1 and B7-DC on MLN-DCs, rather than on systemic DCs, play a critical role in FoxP3⁺ iTreg generation induced by Ag feeding [3]. In addition, LP-DCs express the suppressive cytokine IL-10 and have the potential to induce hyporesponsiveness to orally ingested Ags [4].

Although the intestinal mucosa-related immune system is important in establishing oral tolerance, Ags disseminated via the portal vein pathway or lymphatic pathway also contribute to the development of immune tolerance against oral Ags. The liver is a possible site of oral tolerance induction. Parenchymal and non-parenchymal cells in the liver have immunomodulatory activities involved in oral tolerance, infection, inflammation, cancer, or transplantation [5, 6]. When Ags are administered orally, hepatic plasmacytoid DCs mediate oral tolerance by inducing Ag-specific T cell anergy or clonal deletion [7]. Liver sinusoidal endothelial cells (LSECs) contribute to CD8⁺ T cell tolerance against oral Ags via B7-H1 and PD1 interactions [8, 9]. Although there is evidence that liver-resident macrophages (Kupffer cells, KCs) play a critical role as tolerogenic APCs under steady-state conditions [10] and in portal venous tolerance [11], allergic reactions [12], and liver transplantation [13], concrete examples and mechanisms of the role of KCs in oral tolerance have not yet been reported.

In oral tolerance, the immune regulatory functions of tolerogenic APCs are based on several molecular mechanisms: production of suppressive cytokines such as IL-10 [14] and TGF- β [15], production of prostaglandin E2 [10], expansion or functional modulation of Tregs [16], and expression of inhibitory molecules including B7-H1 [17]. In the present study, we focused on the role of co-signaling molecules on tolerogenic APCs.

V-set and Ig domain-containing 4 (VSIG4) is a member of the B7 protein family, also known as a complement receptor of the immunoglobulin superfamily (CRIg), and functions as an inhibitory ligand on APCs for the regulation of T cell responses [18–20]. VSIG4-expressing macrophages mediate the inhibition of T cell proliferation and cytokine production [18]. In a concanavalin A-induced hepatitis model, VSIG4-expressing KCs were important in inducing and maintaining liver tolerance through T cell and natural killer T (NKT) cell suppression [19]. Negative regulatory activity of VSIG4 on helper T cells resulted in impaired isotype switching in Ab responses [20]. In the Lewis lung carcinoma tumor model, tumor growth was significantly inhibited in VSIG4 knockout (KO) mice compared with that in wild-type (WT) mice [21].

In the present study, we investigated the role of VSIG4 in the oral tolerance mechanism. In particular, it had been reported that VSIG4 expression was restricted to limited cell populations, including tissue-resident macrophages (especially in the liver), neutrophils, and activated DCs [18]. Using a VSIG4 KO mouse system, we determined which sites among

the gut, liver, and other organs were critical for establishing oral tolerance.

Results

The role of VSIG4 signaling in tolerance induced by a single high dose of oral Ag

First, we analyzed the role of VSIG4 signaling in a widely used experimental model to study oral tolerance (Fig. 1A) [3, 22]. A single high dose (25 mg/mouse) of orally administered ovalbumin (OVA) weakened the OVA-specific IgG1 response and OVA-specific T cell proliferation (Fig. 1B and D). Oral Ag-specific Ab and T cell responses were not reduced by Ag feeding in VSIG4KO mice (Fig. 1C and E). Collectively, this indicates that VSIG4 signaling is involved in the down-regulation of Ag-specific immune responses in oral tolerance.

We also performed the oral tolerance experiment on VSIG4KO mice with a C57BL/6 background. The results were consistent with those of the VSIG4KO mice with a BALB/c background (data not shown).

Oral Ags disseminated into the liver and MLN

To identify which cells were involved in VSIG4-mediated oral tolerance, we compared the distribution of oral Ags in WT and VSIG4KO mice. CFSE-labeled DO11.10 T cells were adoptively transferred into naïve BALB/c and VSIG4KO mice, and one day later, 25 mg/mouse OVA was orally administered. After 48 h, mice were sacrificed, and T cell proliferation was analyzed by CFSE dilution. Based on previous research [1], we hypothesized that oral Ags might be absorbed in the small intestine and distributed to the MLN and may also be disseminated systemically via the lymphatic and portal vein pathways. Therefore, we analyzed the MLN, which is the site of the gut-associated lymphatic pathway; the liver, which is the site of the portal vein pathway; the spleen, which represents the systemic immune response; and the ILN, which represents an irrelevant regional lymph node. As a result, we observed oral Ag-induced Ag-specific CD4⁺ T cell proliferation (Fig. 2). T cells proliferated vigorously in response to orally administered Ags in the MLN, while T cell division was minimally detected at other sites. Interestingly, T cell division in the liver was elevated in VSIG4KO mice compared with that in WT mice. In the MLN, a considerable number of T cells divided four times, and these cells were not detected in the liver. Therefore, T cell proliferation was induced at sites where naïve T cells met Ags, and newly divided T cells did not migrate into other tissues/organs, at least not into the liver, within 48 h. Collectively, this indicates that oral Ags were mainly distributed in the gut-draining lymph node, MLN and also reached the liver. Moreover, hepatic T cell proliferation induced by oral Ag was enhanced by the lack of VSIG4 signaling.

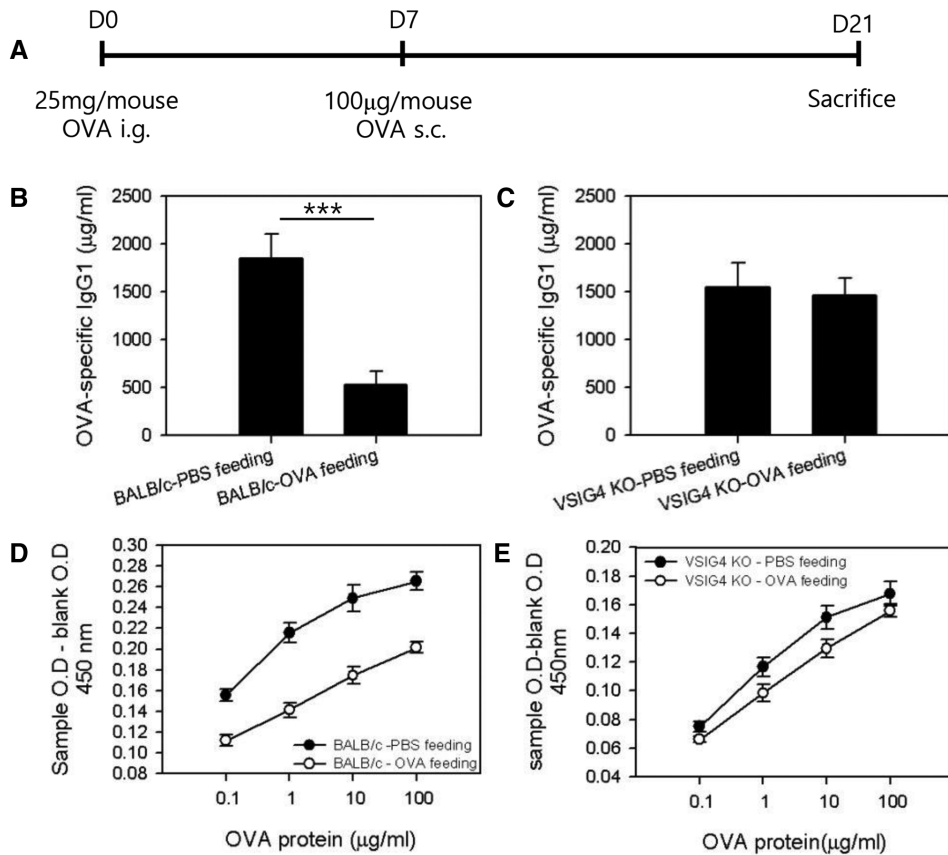


Figure 1. OVA-specific Ab production and T cell activation were not inhibited by oral Ag feeding in VSIG4KO mice. To induce oral tolerance, VSIG4KO and WT mice were fed with OVA. After 7 days, they were primed with OVA in CFA sc. Two weeks later, sera and splenocytes were obtained. (A) Experimental schedule for oral tolerance development. (B, C) OVA-specific IgG1 in serum was evaluated by ELISA. (D, E). Splenocytes were stimulated with the indicated OVA protein for 72 h. After incubation, WST assay was performed to assess live cells in antigen-stimulated splenocytes. Statistical analysis: t-test (paired). *** $p < 0.001$. Representative data from three independent experiments with 4 mice per condition are shown. Data are shown as means $\pm$ SEM.

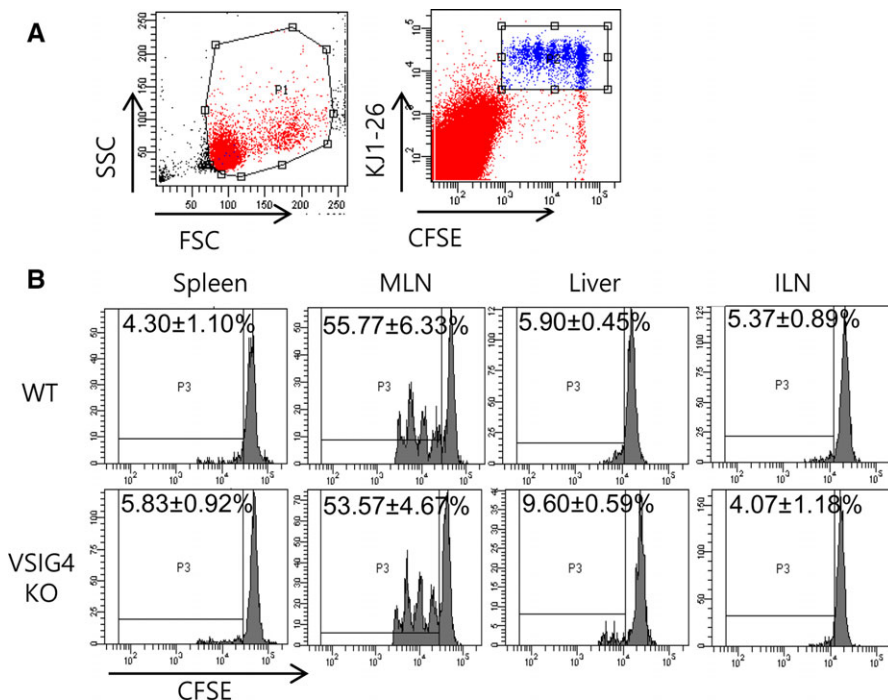


Figure 2. Orally administered OVA dispersed into the MLN and liver. CFSE-labeled DO11.10 cells were transferred into BALB/c and VSIG4KO mice. The next day, the recipient mice were given oral OVA. Two days later, the indicated lymphoid organs were removed and analyzed by flow cytometry. (A) Gating strategy: First, gate was set on FSC/SSC to gate lymphocytes. CFSE⁺KJ1-26⁺ population were gated on the transferred DO11.10 cells. (B) Data are shown as means $\pm$ SEM ($n = 3$ /group). Representative data from two independent experiments are shown.

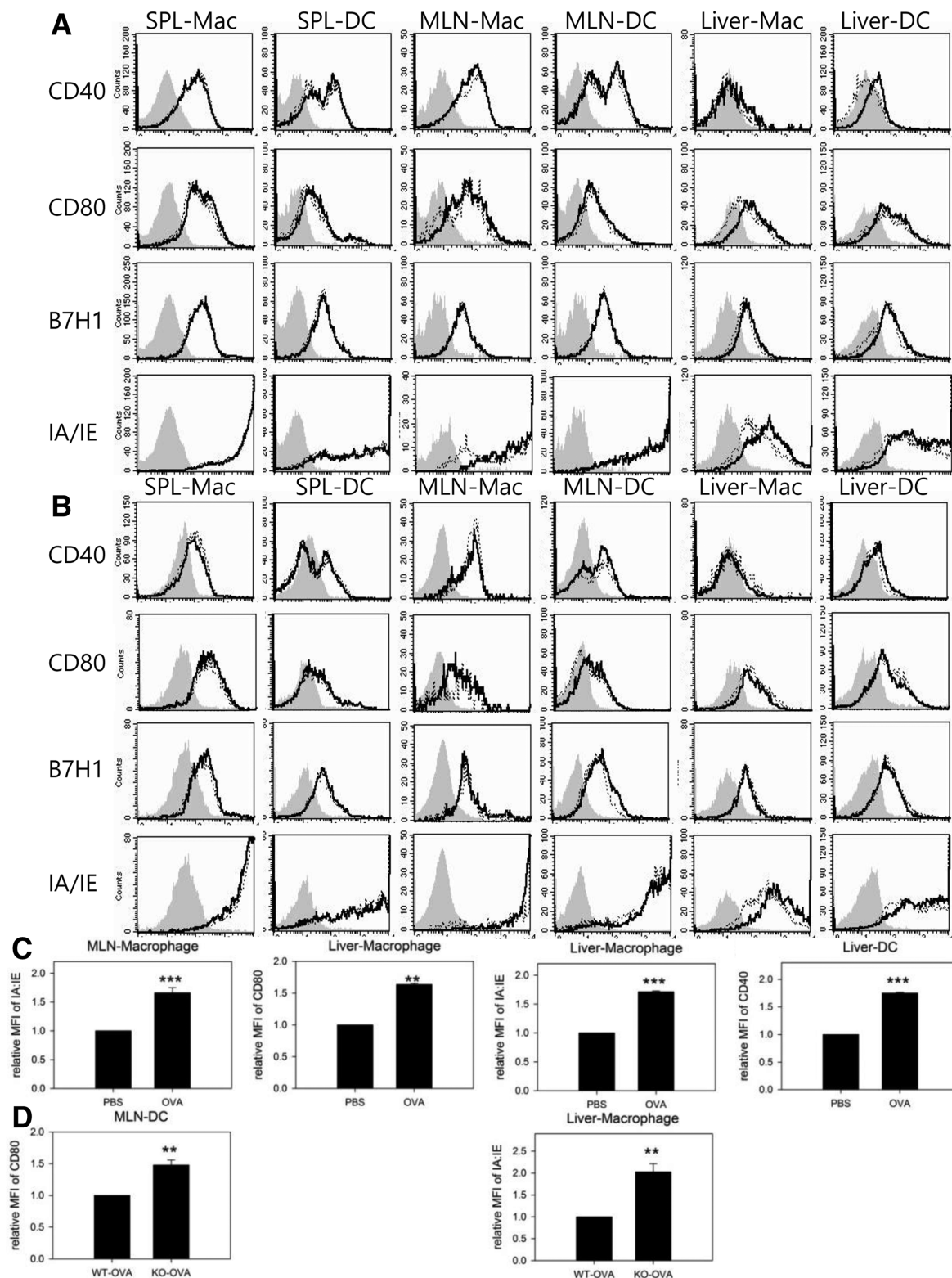


Figure 3. Oral Ag administration induced phenotypic changes in major APCs. (A) BALB/c mice ($n = 3/\text{group}$) were fed with OVA protein or PBS (as control). After 48 h, CD40, CD80, B7H1, and MHC class II expression on CD11c⁺ DCs and F4/80⁺ macrophages was analyzed by flow cytometry. Filled area: isotype control; dotted line: PBS feeding; bold line: OVA feeding. (B) BALB/c and VSIG4KO mice ($n = 3/\text{group}$) were fed 25 mg/mouse OVA protein. After 48 h, phenotypic analysis was performed. Filled area: isotype control; dotted line: BALB/c mice; bold line: VSIG4KO mice. (C,D) Significantly altered relative mean fluorescence intensities (MFIs) are shown as a bar graph. The relative MFI was calculated by dividing each sample's MFI by the control MFI. Statistical analysis: t-test (paired). ** $p < 0.01$; *** $p < 0.001$. Data are representative of three independent experiments. Data are shown as means $\pm$ SEM.

Oral Ag-induced phenotypic changes in APCs in the MLN and liver

Ag exposure renders APCs either immunogenic or tolerogenic. After oral OVA administration, we assessed the levels of the activation markers CD40 [23], CD80 [24], and MHC class II [25, 26] and the levels of the inhibitory marker B7H1 [27] on APCs in the MLN, liver, and spleen. In WT mice, OVA feeding resulted in upregulation of MHC class II molecules on F4/80⁺ macrophages in the MLN and increases in CD80 and MHC class II molecules on F4/80⁺ macrophages in the liver (Fig. 3A and C). In addition, levels of CD40 were increased by oral Ag administration in hepatic CD11c⁺ DCs. In contrast, expression of surface molecules on F4/80⁺ cells and CD11c⁺ cells in the spleen was not significantly altered by oral Ag.

In VSIG4KO mice, MHC class II upregulation of F4/80⁺ KCs resulting from oral Ag administration increased compared with that in WT mice (Fig. 3B and D). In addition, CD80 expression on MLN-DCs was slightly increased in VSIG4KO mice. Levels of other surface molecules on the APCs of VSIG4KO mice were similar to those of WT mice. Although the upregulation of activation markers on APCs does not ensure augmented immune responses, we found that the disseminated oral Ag influenced APCs in the liver as well as in the MLN.

The role of VSIG4 in regulatory T cell generation induced by oral Ag feeding

One of the mechanisms for inducing and/or maintaining oral tolerance is mediated by Tregs. To identify the mechanism of oral tolerance, we evaluated levels of Treg induced by oral Ag. OVA-specific transgenic T cells (DO11.10 CD4⁺ T cells) were adoptively transferred into WT and VSIG4KO mice, and the next day, mice were treated with a high dose (25 mg/ml) of OVA. Ten days later, mice were sacrificed, and FoxP3⁺ Treg cells were detected in the MLN, liver, and spleen (Fig. 4). In WT mice, the percentage of Tregs among DO11.10 T cells was higher in the MLN and liver than in the spleen. Interestingly, hepatic Treg cells were significantly reduced in VSIG4KO mice compared with levels in WT mice. These data suggest that the liver rather than the MLN may be an important site for exacerbating immune responses induced by oral Ag administration in VSIG4KO mice.

Based on the *in vivo* Treg generation study, we hypothesized that APCs from the spleen, MLN, and liver would exhibit varying potential to generate Tregs via VSIG4 signaling. To identify the role of APCs from each source in Treg induction, we performed an *in vitro* Treg generation experiment. We isolated the spleen, MLN, and liver and purified their CD11c⁺ or F4/80⁺ cells using a MACS system. Naïve DO11.10 CD4⁺ T cells were incubated with APCs from each source under inducible conditions of Treg differentiation. DCs and macrophages isolated from the spleen, MLN, and liver were able to present OVA peptides to DO11.10 T cells and induce differentiation into Tregs (Fig. 5A and B). The

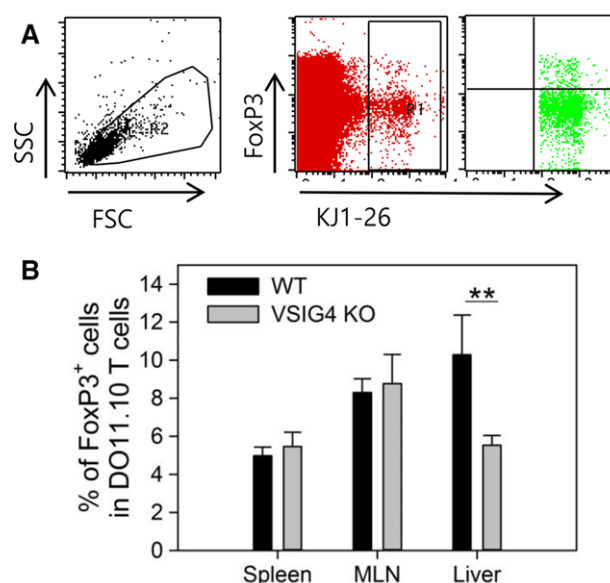


Figure 4. The percentage of hepatic Tregs induced by oral Ab feeding was reduced in VSIG4KO mice. CD11.10 CD4⁺ T cells were transferred into WT BALB/c and VSIG4KO mice. One day later, mice were fed with OVA protein. Ten days after oral feeding, splenocytes, MLN lymphocytes, and liver mononuclear cells were isolated from mice and analyzed by flow cytometry. (A) Gating strategy: Gate was set on FSC/SSC to gate lymphocytes. Among KJ1-26⁺ cells, FoxP3 positive population was gated. (B) Percentages of FoxP3⁺KJ1-26⁺ cells are shown as a bar graph. Statistical analysis: t-test (paired). ***p* < 0.01.

percentages of FoxP3⁺ Tregs induced by splenic APCs or MLN APCs were not significantly different between VSIG4KO mice and WT mice. Interestingly, hepatic F4/80⁺ macrophages, not hepatic CD11c⁺ DCs, dramatically lost their ability to differentiate naïve T cells into Tregs without VSIG4.

Next, we analyzed the direct effect of VSIG4 signaling in Treg induction using VSIG4.Ig. We observed an induction of *Foxp3* gene transcription in cultures of naïve CD4⁺ T cells stimulated with plate-bound anti-CD3 in the presence of the VSIG4.Ig fusion protein as compared to levels observed with control Ig, and this induction increased over time (Fig. 5C). These data suggest that the VSIG4 molecule may interact with a ligand on T cells directly and contribute to Treg induction.

Therefore, we concluded that hepatic macrophages (KCs) may play a critical role in oral tolerance via VSIG4 signaling. In VSIG4KO mice, oral Ag-induced Treg cells were reduced, resulted in the enhancement of Ag-specific immune responses.

Increased food allergies in VSIG4KO mice

Finally, we confirmed the contribution of VSIG4 signaling to a distinct model of food allergy. We established an experimental model of BALB/c mice with food allergies (Fig. 6A) based on previous research involving the development of IgE-dependent allergies against diet Ags [28, 29]. Repeated oral Ag administration induced Ag-specific Abs and inflammation of the small intestine in

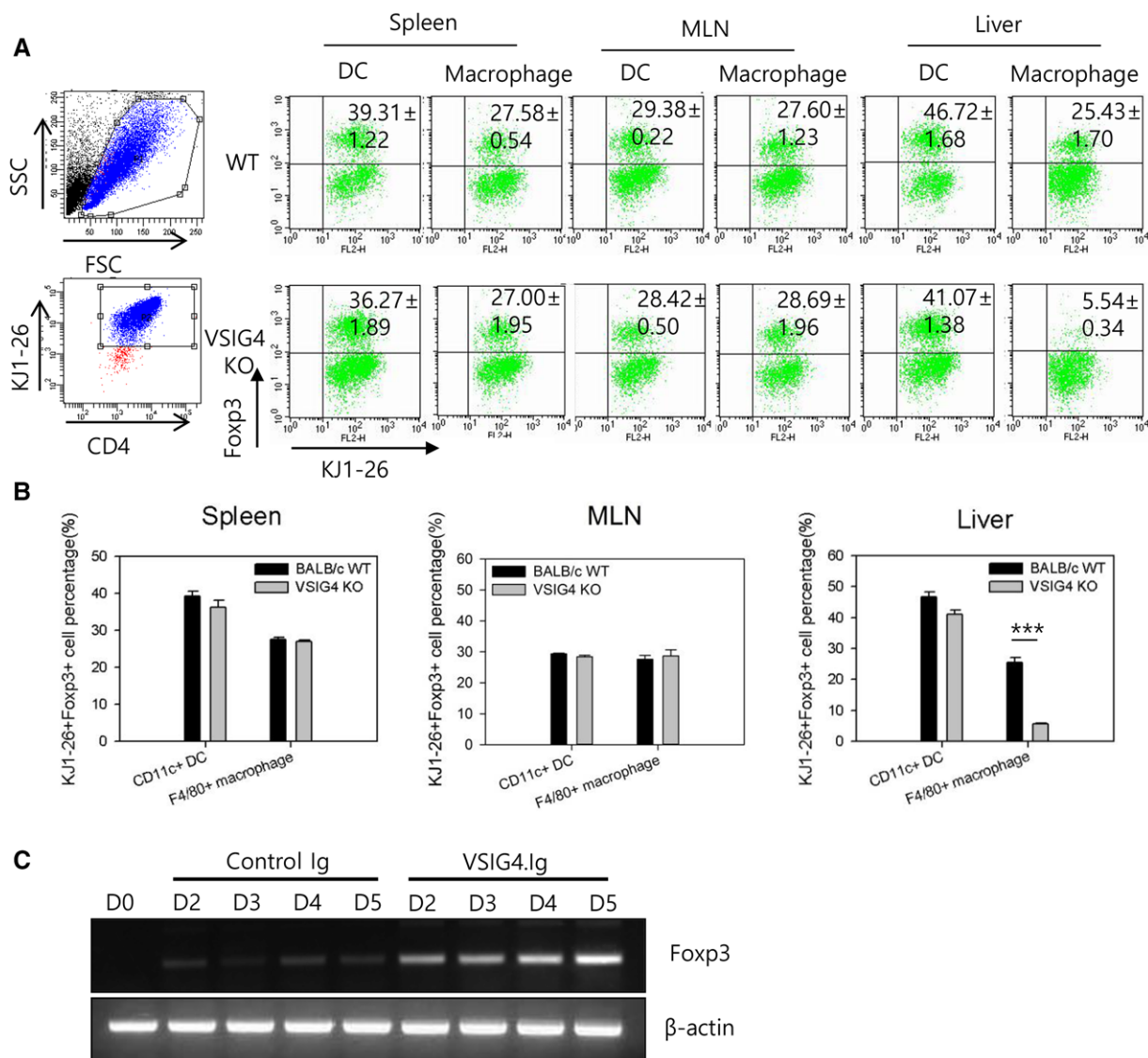


Figure 5. KC-mediated Treg induction via VSIG4 signaling. CD11c⁺ DCs and F4/80⁺ macrophages were isolated from the spleens, MLNs, and livers of BALB/c mice or VSIG4KO mice. DO11.10 CD4⁺ T cells were labeled with CFSE and cultured with each APC in the presence of OVA₃₂₃₋₃₃₉ for 96 h. To induce Tregs in vitro, TGF- β and IL-2 were added to CD4⁺ T cells. Samples were analyzed by flow cytometry. (A) Gating strategy: Gate was set on FSC/SSC to gate lymphocytes. CD4⁺KJ1-26⁺ DO11.10 T cells were gated. Representative data are shown as a dot plot, and numbers indicate percentages of Foxp3⁺ cells among KJ1-26⁺ T cells. (B) Percentages of Foxp3⁺KJ1-26⁺ cells are shown as a bar graph. Data are representative of 2 independent experiments with 3 mice per experiment. Data are shown as means $\pm$ SEM. Statistical analysis: *t*-test (paired). ****p* < 0.001. (C) BALB/c T cells were stimulated with plate-bound anti-CD3 and control Ig or VSIG4.Ig in the presence of anti-CD28 Ab for 2–5 days. Total RNA was purified and subjected to RT-PCR for *Fxp3* and β -actin. Data are representative of two independent experiments.

Ag-sensitized mice. Both OVA-specific and total IgE and IgG1 levels in sera were increased, while OVA-specific IgG2a and total IgG2a in sera were decreased by OVA feeding (Fig. 6B–D). These data suggest that our model was biased toward Th2-mediated allergy, as demonstrated by the IgG1- and IgE-dominant responses. Interestingly, serum IgE and IgG1 levels were higher in VSIG4KO mice than in WT mice.

In histological analysis, OVA-fed mice exhibited significant inflammation in the small intestine (Fig. 6E). Morphological changes in the villi in the proximal jejunum were induced by

prolonged Ag feeding. We performed quantitative evaluation of histological samples in three categories: villus length, shape, and expansion. We found that OVA feeding resulted in significant villus transformations in sensitized mice. Furthermore, morphological changes in the small intestines were exacerbated in VSIG4KO mice (Fig. 6F–H). In addition, the number of infiltrated mast cells increased following OVA feeding in VSIG4KO mice (Fig. 6I). Taken together, this suggests that a lack of VSIG4 signaling contributed to the exacerbation of food allergy induced by prolonged Ag feeding.

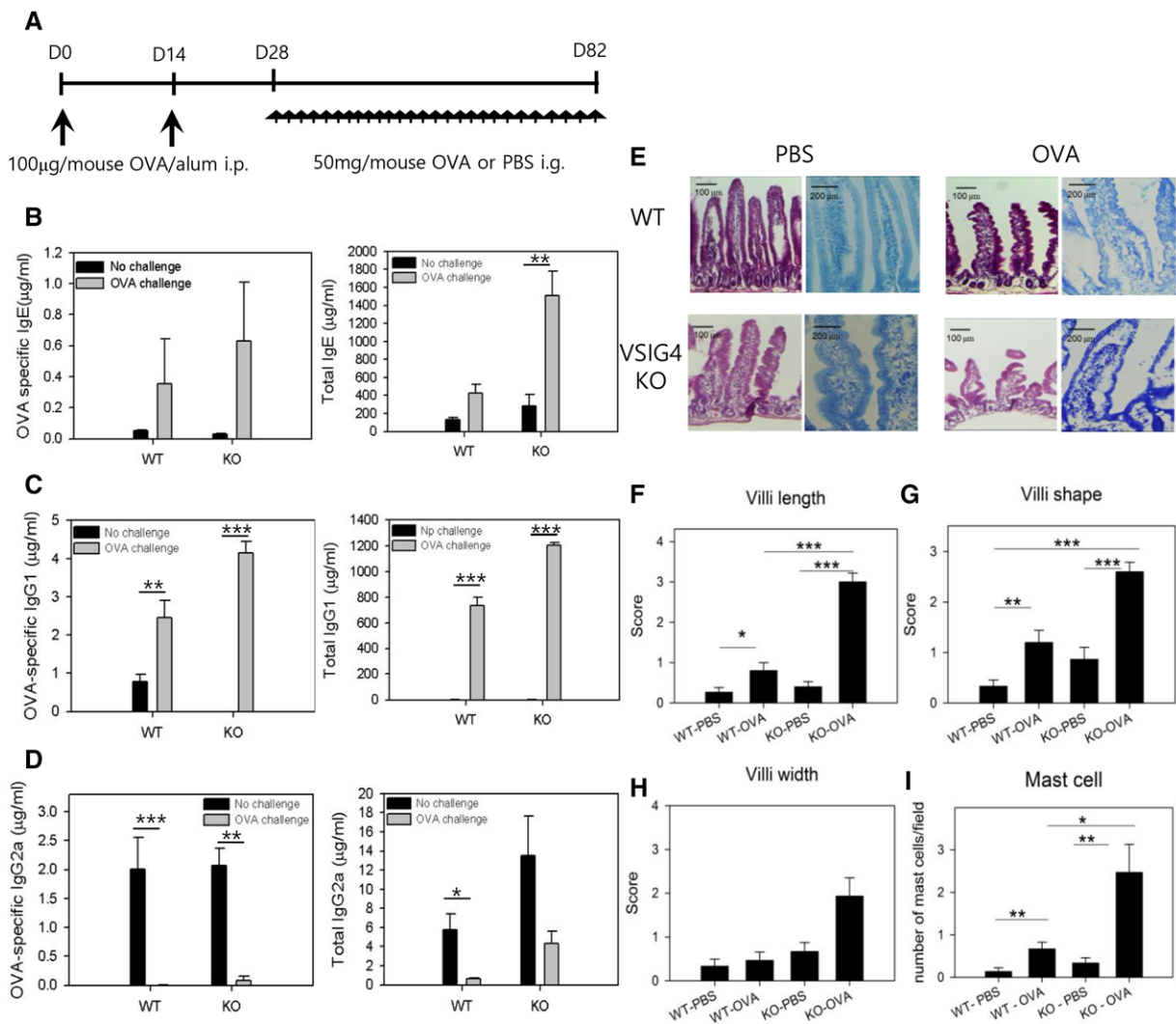


Figure 6. Food allergies were exacerbated by VSIG4 signaling. BALB/c and VSIG4KO mice were sensitized with OVA plus alum on day 0 and day 14. Two weeks after boosting, mice were orally administered OVA or PBS every other day from day 28 to day 82. (A) Experimental schedule for food allergy induction. (B) Anti-OVA-specific and total IgE concentrations in sera (ELISA). (C) OVA-specific and total IgG1 concentrations in sera (ELISA). (D) OVA-specific and total IgG2a concentrations in sera (ELISA). (E) The proximal jejunum was isolated for histological analysis on day 82. H&E and toluidine blue staining were performed according to standard protocols. (E, upper left) PBS feeding of BALB/c mice. (E, upper right) OVA feeding of BALB/c mice. (E, lower left) PBS feeding of VSIG4KO mice. (E, lower right) OVA feeding of VSIG4KO mice. In each panel, the image on the left is the H&E-stained sample, and the image on the right is the toluidine blue-stained sample. Scale bar: 100 or 200 µm as reported in Figure. (F) Bar graph of villus length changes. (G) Bar graph of villus shape changes. (H) Bar graph of villus expansion changes. (I) Bar graph of mast cell numbers. Data are shown as means ± SEM. Data are pooled from one single experiment with 6 mice per condition. Statistical analysis: t-test (paired). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Discussion

Recently, it has been reported that the B7-related protein VSIG4 has a regulatory function in T cell activation [18–20, 30]. In the present study, we first found that VSIG4-deficient mice showed severe allergic responses against food Ags compared with those of WT mice. To identify the role of VSIG4 signaling, we established a BALB/c food allergy model, and we confirmed the increased oral Ag-specific and total IgE and IgG1 levels, as well as levels of the reduced Th1-related isotype, IgG2a. This model showed the characteristic type I hypersensitivity that is mediated by Ag-specific IgE [31]. In addition, oral Ag-specific IgG1, which can cause type

II or type III hypersensitivity, was induced by long-term oral Ag feeding of OVA-sensitized mice. As a result, we observed significant histological changes in allergy-induced mice and confirmed the regulatory activity of VSIG4 in food allergy. Although oral Ag-induced IgE and IgG1 responses were enhanced under VSIG4-deficient conditions, the deterioration of intestinal inflammatory parameters in VSIG4KO mice demonstrated the regulatory activity of VSIG4 in food allergy more convincingly.

Although allergy is a typically Th2 cell-mediated disease, other CD4⁺ T cell subsets may be involved in immune responses to allergens [32]. The proinflammatory Th17 produces members of the IL-17 family, such as IL-17a and IL-17f, and contributes to the

pathogenicity inflammatory diseases, including Crohn's disease and asthma [32, 33]. Historically, it has been recognized that Th2 polarization results in the development of Th2 cytokine (IL-4, IL-5, and IL-13)-mediated allergic diseases. There are also several lines of evidence that decreased levels of IFN- γ -producing Th1 cells contribute to allergic diseases [34, 35]. Both naturally occurring CD4⁺CD25⁺FoxP3⁺ Tregs and iTregs are known as key regulators in immune tolerance-related diseases [32, 36, 37]. They inhibit effector T cells, such as Th2 and Th17 cells, and also eosinophils, basophils, and inflammatory APCs. To induce peripheral tolerance to oral Ags, APCs play a critical role in Treg generation [32]. In particular, specific DC subsets, such as plasmacytoid DCs [38] and IL-10-producing DCs [39], contribute to Treg differentiation in allergic conditions. As a result, allergen-specific Tregs suppress proliferative cellular and proinflammatory cytokine responses and control allergic inflammation.

In a previous study, we found that VSIG4⁺ KCs inhibited cytokine production in T cells and NKT cells during Ag presentation compared with that in VSIG4-deficient KCs [19]. Herein, we focused on the Treg population to elucidate the oral tolerance mechanism associated with VSIG4 signaling. In VSIG4-deficient mice, hepatic FoxP3⁺ Treg cells were reduced by oral Ag administration, suggesting that VSIG4 mediated Treg generation *in vivo*. In particular, the percentage of Tregs was highest in the liver compared to that in the spleen or MLN, though this difference was not statistically significant. However, we should also consider that the number of Tregs in each organ may be reversed because of the difference in the total cell numbers of each organ.

Previously, it had been reported that KCs could expand anti-CD3 Ab-stimulated Tregs [16]. However, it was also reported that KCs were not potent APCs for inducing Ag-specific T cell activation compared with DCs and that they inhibited DC-induced Ag-specific T cell activation [10]. Regulatory mechanisms of KCs have been known to involve the production of prostaglandins or the suppressive cytokines IL-10 and TGF- β [6]. In the present study, we found that *Foxp3* induction in T cells stimulated with anti-CD3 Abs and in CD25⁻CD4⁺ naïve T cells stimulated with Ag was mediated by VSIG4 signaling. In this setting, KCs acted as APCs to provide TCR stimulation and VSIG4 co-signaling for Treg induction.

It has been reported that most types of APCs can induce iTreg generation; in particular, tolerogenic DCs efficiently induce naïve T cells to develop into Tregs [40–42]. We found that DCs and macrophages isolated from the spleen, MLN, or liver induced Ag-specific iTreg generation *in vitro* and that splenic DCs and hepatic DCs were superior to splenic macrophages or KCs in inducing iTreg generation. Although the capacity of KCs to induce Treg differentiation was lower than those of splenic DCs and hepatic DCs, only KCs were significantly inhibited from inducing Treg generation by defective VSIG4 signaling. Our data showed that only the percentage of Tregs among total CD4⁺T cells and the count of CD4⁺ T cells differed depending on the APC type. We detected a reduced number of CD4⁺ T cells among cultured cells from hepatic APCs. Hepatic DCs or KCs may therefore be inferior to splenic APCs and MLN APCs in inducing the proliferation and/or maintenance of T cells.

A difference in VSIG4 expression on each type of APC may explain the exclusive contribution of KCs to VSIG4 signaling. A previous report showed that resting tissue-resident macrophages, especially in the liver, expressed surface VSIG4, while thioglycolate or LPS-activated macrophages lost surface VSIG4 expression in BALB/c mice [18]. This study also reported that naïve and activated DCs and other immune cells did not express surface VSIG4 protein and that there was minimal expression of VSIG4 mRNA in the spleen. In our study, we found that KCs, which were reported to be major VSIG4-expressing cells, contributed to Treg generation via the VSIG4 pathway, with VSIG4-deficient KCs contributing to the loss of oral tolerance in VSIG4KO mice. In contrast, APCs in the MLN may not be involved in the increased immune responses observed in oral Ag-treated VSIG4KO mice.

Tolerogenic APCs contribute to immune tolerance toward oral Ags via Ag presentation. We confirmed that orally administered OVA induced vigorous Ag-specific T cell proliferation in the MLN. Interestingly, we detected small but clear peaks of proliferated T cells in the livers of VSIG4KO mice. Previously, Chung et al. showed that orally administered Ag induced T cell proliferation, mainly in the MLN and Peyer's patches, but also slightly in the spleen, and induced oral Ag-specific systemic T cell tolerance [43]. Orally administered Ags might be absorbed and then recognized by T cells in Peyer's patches and/or the MLN [44]. Moreover, the liver receives a significant amount of intestinally derived Ags through the portal veins [44]. However, several studies have shown that immunologically relevant amounts of oral Ag could disseminate systemically and induce T cell proliferation in the spleen or peripheral LN [45, 46]. Collectively, many studies agree that Ag recognition occurs in the MLN within a few hours of Ag feeding; thus, the systemic dissemination of immunologically relevant amounts of oral Ag may be influenced by the feeding dose or experimental models used.

The dilution pattern of CFSE-labeled T cells in the liver was different from that in the MLN, suggesting that the dividing T cells detected in the liver had not migrated from the MLN but were responding to disseminated Ag in the liver. In conclusion, the induction of oral tolerance through Ag presentation to naïve T cells may proceed in sites other than the gut, such as in the liver.

Based on these distribution results, we analyzed changes in APCs resulting from oral Ag treatment. Co-signaling molecules and MHC class II molecules on splenic DCs or macrophages were minimally altered by oral Ag administration within 48 h; however, APCs in the MLN or liver responded to oral Ag treatment during the oral tolerance induction stage. This result also supports that the possibility that hepatic APCs, as well as MLN APCs, participate in Ag presentation for oral tolerance induction.

Another name for VSIG is the complement receptor of the immunoglobulin superfamily (CRIg), as it is known to bind the complement fragments C3b and iC3b [47]. VSIG4 expression on KCs is required for the binding and phagocytosis of C3-opsonized particles. In addition, VSIG4 signaling enhances the phagosome activity of KCs in a chloride intracellular channel 3-dependent manner [48] and also induces autophagosome formation [49]. In the present study, VSIG4-deficient macrophages exhibited more

MHC class II molecules on their surfaces than VSIG4-expressing macrophages in the oral tolerance model. These data suggest that VSIG4-mediated interactions may work bi-directionally and that VSIG4 signaling may play a role in the modulation of APC immunogenicity. However, intracellular signaling in KCs to induce immune tolerance has not yet been reported.

In our previous study, we showed that the adoptive transfer of VSIG4-expressing KCs could alleviate ConA-mediated liver injury [19]. To demonstrate the direct role of VSIG4⁺ KCs in the regulation of liver inflammation *in vivo*, we adoptively transferred WT or VSIG4 KO KCs into VSIG4KO mice 7 days before ConA injection. Levels of ALT and IFN- γ in the serum were significantly reduced in VSIG4KO mice that received WT KCs compared with those that received VSIG4KO KCs. Consistently, VSIG4KO mice were largely free of hepatic parenchymal necrosis after receiving WT KCs, unlike those given VSIG4KO KCs. Recently, we identified the physiological role of VSIG4 signaling in immune regulation in a mouse tumor model. We found that the infusion of VSIG4⁺, but not VSIG4⁻, tumor-associated macrophages significantly decreased the immune response in recipient mice (not published yet). Regrettably, we have not performed the adoptive transfer of KCs in the oral tolerance model. However, based on the data from the hepatitis model and tumor model, we carefully speculate that adoptively transferred VSIG4-expressing KCs may have the potential to regulate oral Ag-induced immune responses in recipient VSIG4KO mice.

Further studies using agonistic anti-VSIG4 Abs or soluble VSIG4 proteins are needed to elucidate the mechanism by which additional VSIG4 signaling can inhibit immune responses under normal conditions. In addition, blockers of VSIG4 signaling may have the potential to enhance immune responses induced by oral Ags during oral vaccination.

Materials and methods

Mice

Specific pathogen-free female BALB/c mice were purchased from Orient Bio (Korea). VSIG4 KO mice with a BALB/c background (VSIG4KO) were generously provided by Dr. Campagne (Genentech). TCR transgenic DO11.10 mice were kindly gifted by Dr. Kang Chang-Yuil from Seoul National University. All mice were kept in the Animal Resource Center of Inje University. The animal experiments in this study were approved by the Institutional Animal Care and Use Committee of Inje University (approval number: 2015-12/2016-005).

Oral tolerance induction

BALB/c and VSIG4KO mice were orally administered 25 mg OVA. Seven days later, mice were primed with 100 μ g/mouse OVA in

the presence of the same volume of CFA (Sigma-Aldrich, USA). Two weeks later, mice were sacrificed for analysis.

Water soluble tetrazolium (WST) salts assay

Splenocytes (2×10^5 cells/well) were stimulated with OVA protein at 100, 10, 1, or 0.1 μ g/mL for 72 h. After incubation, WST reagent (Daeillab, Korea) was added for viability testing. After 4 h of incubation, the OD at 450 nm was detected by ELISA reader (Sunrise, Tecan, Switzerland).

ELISA

We detected OVA-specific Abs in sera from orally challenged mice. MaxiSorp flat-bottom ELISA plates (Thermo Fisher Scientific, USA) were coated with 10 μ g/mL OVA. After blocking with BSA buffer (Amresco, USA), we added diluted serum or standard solution (OVA-specific IgE standard from AbD Serotec, UK; OVA-specific IgG1 standard from Biolegend, USA; OVA-specific IgG2a standard from Novus Biologicals, USA). After 2 h of incubation, HRP-conjugated detection Abs (IgE detection Abs from Biolegend, USA; IgG1 detection Abs and IgG2a detection Abs from Southern Biotech, USA) were added. TMB (3,3',5,5'-tetramethylbenzidine, Gendepot, USA) was used as a substrate. After stopping the enzyme reaction with stop buffer (Gendepot, USA), we analyzed the OD of each well at 450 nm using an ELISA reader (Sunrise, Tecan, Switzerland).

To detect total IgG1 or IgG2a, the plate was coated with anti-mouse IgG capture Abs (Biolegend, USA). For capturing total IgE Abs, anti-mouse IgE Abs (Biolegend, USA) were used. Total IgG standard and total IgE standard (both from Biolegend, USA) were added for each standard curve. After 2 h of incubation, HRP-conjugated anti-IgG1 Abs, anti-IgG2a Abs, or anti-IgE Abs were added. After removal of unbound detection Abs, we added TMB as a substrate and stop buffer, and OD values were evaluated by ELISA reader.

Cell isolation

For isolation of APCs, the spleen, MLN, and liver were isolated from sacrificed mice. Using digestion buffer containing collagenase D (Roche, Swiss) and DNase I (Roche, Swiss), the spleen and MLN were dissociated into single cells. Hepatic mononuclear cells were prepared using GentleMACS dissociator (Miltenyi Biotec, Germany), according to the manufacturer's recommendation. Red blood cells were removed by ACK lysis buffer (Gibco, USA) treatment, and the remaining cells were stained with microbeads for MACS. Anti-CD11c microbeads, anti-PE microbeads (both from Miltenyi Biotec, Germany), and PE-labeled anti-F4/80 Abs (Biolegend, USA) were used. Microbead-bound cells were positively selected using LS column (Miltenyi Biotec, Germany).

For isolation of splenic CD4⁺ T cells, splenocytes were stained with CD4⁺ T cell biotin antibody cocktail and anti-biotin

microbeads (Miltenyi Biotec, Germany) and separated using LS column.

Adoptive T cell transfer

CD4⁺ T cells were purified from splenocytes of DO11.10 mice and labeled with 10 μ M CFSE (Invitrogen, USA). Labeled CD4⁺ T cells (3×10^6 cells/mouse) were transferred into syngeneic BALB/c or VSIG4KO mice. One day later, recipient mice were administered 25 mg/mouse OVA orally. After 48 h, mice were sacrificed, and OVA-specific CD4⁺ T cell division was analyzed in the spleen, MLN, liver, and inguinal lymph node (ILN) by flow cytometry.

Abs for flow cytometry

For analysis of the activation status of APCs, we used PE-labeled anti-CD11c Abs and FITC-labeled anti-F4/80 Abs for gating DCs and macrophages. We additionally stained cells with allophycocyanin-labeled anti-CD40 Abs, anti-CD80 Abs, anti-B7H1 Abs, or anti-IA/IE Abs (all from Biolegend, USA). Allophycocyanin-labeled hamster IgG Abs, rat IgG2a Abs, and rat IgG2b Abs (all from Biolegend, USA) were used as isotype control Abs.

For analysis of Tregs, PE-conjugated KJ1-26 Abs (Biolegend, USA) and allophycocyanin-conjugated anti-FoxP3 Abs (eBioscience, USA) were used. Fluorescent Ab-bound cells were detected with FACSCalibur (BD Biosciences, USA).

In vivo Treg induction

BALB/c mice and VSIG4KO mice were adoptively transferred with 5×10^6 cells/mouse CD4⁺ T cells isolated from naïve DO11.10 mice. The next day, mice were orally administered 25 mg/mouse OVA. Ten days after feeding, mice were sacrificed, and the spleen, MLN, and liver were isolated. Total splenocytes, MLN lymphocytes, and liver mononuclear cells were stained with PE-conjugated KJ1-26 Abs and allophycocyanin-conjugated anti-FoxP3 Abs using a FoxP3 staining buffer set (eBioscience, USA) and were analyzed by flow cytometry.

In vitro Treg induction

CD11c⁺ cells or F4/80⁺ cells from the spleen, MLN, or liver were purified from BALB/c and VSIG4KO mice. Naïve DO11.10 CD4⁺ T cells were stimulated with 10 μ g/mL OVA₃₂₃₋₃₃₉ peptide (Sigma, USA) in the presence of rhTGF- β (10 ng/mL), IL-2 (0.5 ng/mL) (both from R&D Systems, USA), anti-IFN- γ Abs (10 μ g/mL), and anti-IL-4 Abs (10 μ g/mL) (both from BD Biosciences, USA). After 96 h of incubation, T cells were stained with PE-conjugated KJ1-26 Abs and allophycocyanin-conjugated anti-FoxP3 Abs using a FoxP3 staining buffer set.

To analyze the role of VSIG4 signaling in Treg induction, we prepared VSIG4-human Fc fusion protein (VSIG4.Ig), as described previously [19]. Naïve CD4⁺CD25⁻ T cells were isolated from BALB/c mice using a Treg isolation kit (Miltenyi Biotec, Germany) and plated in 96-well flat-bottom plates that were pre-coated with indicated concentrations of mouse anti-CD3e antibody (145-2C11) together with VSIG4.Ig or control Ig (20 μ g/mL). CD4⁺FoxP3⁺ Tregs were induced by the addition of anti-CD28 Ab and rhTGF- β 1 (10 ng/mL). Total RNA was purified and subjected to RT-PCR for *Foxp3* (sense: 5'-TTCATGCATCAGCTCTCCAC-3'; anti-sense: 5'-CTGGACACCCATTCCAGACT-3') and β -actin (sense: 5'-CGCTCG TTGCAATAGTGAT-3'; anti-sense: 5'-TCTGGTCGTACCACAGGCAT-3').

Food allergy model

As reported previously [50], BALB/c mice were administered OVA (Sigma-Aldrich, USA) at 50 μ g/mouse and alum adjuvant at 2 mg/mouse via i.p. injection on day 0 and day 14. On day 28, they were challenged with 50 mg/mouse OVA every other day. After 27 feedings, allergic responses were analyzed.

Histological analysis

Isolated jejunums were fixed with 10% formaldehyde (Sigma-Aldrich, USA) and dehydrated with sucrose buffer. They were embedded in OCT compound (Sakura, Netherlands) and frozen for cryosection. Tissues were spliced at 10 μ m and stained with H&E or toluidine blue (Abion CRO, Korea). Samples were analyzed using digital optical microscope (ZIESS, Germany). For quantitative analysis of histological samples, we scored samples with references from previous reports [51, 52]. The severity of inflammation was evaluated in three categories (Supporting Information Table): villus length, villus expansion, and villus shape were analyzed in each field of view at 10 $\times$ magnification (15 fields/mouse, 3 mice/group). Mast cells were counted in each field of view at 20 $\times$ magnification (16 fields/mouse, 3 mice/group).

Statistical analysis

The results of statistical analyses are shown as the average $\pm$ standard deviation. Student's *t*-test was performed to compare differences between groups.

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