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Intestinal mucus-derived nanoparticles mediate activation of Wnt/ β -catenin signaling plays a role in induction of liver NKT cell anergy

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Abstract

The Wnt/ β -catenin pathway has been known to play a role in induction of immune tolerance, but its role in the induction and maintenance of NKT cell anergy is unknown. We found that activation of the Wnt pathway(s) in the liver microenvironment is important for induction of NKT cell anergy. We identified a number of stimuli triggering Wnt/ β -catenin pathway activation, including exogenous NKT cell activator, glycolipid α -GalCer, and endogenous PGE₂. Glycolipid α -GalCer treatment of mice induced the expression of wnt3a and wnt5a in the liver, and subsequently resulted in a liver microenvironment that induced NKT cell anergy to α -GalCer restimulation. We also found that circulating PGE₂ carried by nanoparticles is stable, and that these nanoparticles are A33⁺. A33⁺ is a marker of intestinal epithelial cells, which suggests that the nanoparticles are derived from the intestine. Mice treated with PGE₂ associated with intestinal mucus-derived exosome-like nanoparticles (IDEN) induced NKT cell anergy. PGE₂ treatment leads to activation of the Wnt/ β -catenin pathway by inactivation of GSK-3 β of NKT cells. IDEN-associated PGE₂ also induces NKT cell anergy through modification of the ability of DCs to induce IL-12 and IFN- β in the context of both glycolipid presentation and TLR-mediated pathways. These findings demonstrate that IDEN associated PGE₂ serves as an endogenous immune modulator between the liver and intestines and maintains liver NKT cell homeostasis and this finding has implications for development of NKT cell-based immunotherapies.

Keywords

Intestinal mucus-derived nanoparticles; Exosomes; Wnt and PGE₂ signaling; liver NKT anergy

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Introduction

Unlike T cells, NKT cells respond to lipid-based antigens including self and foreign glycolipid and phospholipid antigens (1) presented by CD1d restricted APCs. Among these lipid-based antigens, alpha-galactosylceramide (α -GalCer) is a synthetic glycosphingolipid derived from the marine sponge, *Agelas mauritianus*, and is commonly used in mice and human NKT studies as a potent activator of NKT cells *in vivo* or *in vitro* (2). A single injection of the exogenous α -GalCer leads to NKT cell activation followed, by long-term anergy, thereby limiting its therapeutic use(3). A number of potential endogenous glycolipids derived from dietary metabolic products and lipids derived from some intestinal bacteria migrate constantly into the liver(4–6) and these lipids can activate liver NKT cells *in vitro*(7). It is, therefore, remarkable that liver NKT cells are normally quiescent even though they are constantly exposed to intestinal-derived products. The molecular mechanisms that underlie induction of liver NKT cell anergy regulated by either exogenous α -GalCer or endogenous lipids are largely unknown.

The gut communicates extensively with the liver(8) through a number of gut derived molecules that are constantly migrating into the liver. PGE₂ and Wnt ligands are enriched in the gut, and whether they migrate into the liver and subsequently contribute to induction of liver NKT anergy has not been fully investigated. Both PGE₂ (9) and Wnt (10) regulated pathways are known to play a crucial role in immune tolerance; however, a direct link between these two key pathways remains to be identified although recent studies have proposed involvement of the Wnt pathway in regulating T cells (11, 12) and DC(10) activation.

In this study, we demonstrate that preconditioning of the Wnt pathway for activation in the liver microenvironment is essential for induction of NKT cell anergy regardless of whether the NKT cells are naïve or educated. We further identified a previously undescribed mechanism in which the carriage of PGE₂ by intestinal mucus-derived exosome-like nanoparticles (IDEN) into the liver created an environment in which activation of the Wnt/ β -catenin pathway is induced.

Material and Methods

Adoptive transfer

NKT cells were enriched by negative magnetic sorting (Miltenyi Biotec) using anti-CD11b, B220, CD8 α , Gr-1, CD62L and CD11c antibodies. Enriched NKT cells (5×10^6 per mouse) were then injected *i.v.* into irradiated NOD-SCID mice. In some case, NK1.1⁺CD5⁺ surface stained cells (NKT) were sorted using a FACSVantage. Sorted NKT cells were 85–90% pure as determined by tetramer staining. To determine the effects of the liver microenvironment created by Wnt signaling on liver NKT cells, Tcf/LEF1-reporter mice as recipients were treated with α -GalCer (3 μ g, Avanti polar Lipids, Inc., Birmingham, AL) or lithium chloride (Sigma, 200 mg/kg) every three days for 12 days. Recipients were then irradiated (750 rads) before *i.v.* administering enriched NKT cells (10×10^6 per mouse) from C57BL/6 CD45.1⁺ mice. 24 h after cell transfer the mice were injected *i.v.* with α -GalCer (5 μ g/mouse).

Details of other methods used in this study are described in the supplemental experimental procedures.

Results

Wnt activation creates a microenvironment that causes NKT cell anergy to α -GalCer stimulation *in vitro* and *in vivo*

We first tested whether activation of Wnt/ β -catenin modulates the activity of liver NKT cells. Sorted liver NKT cells that were transfected with constitutively activated β -catenin (*Ctnnb1*) exhibited a reduction in α -GalCer tetramer-stimulated NKT-cell proliferation (Fig. 1A) and production of IFN- γ and IL-4 (Fig. 1B). Because of this result we tested whether the wnt/ β -catenin pathway was activated when mice are treated with α -GalCer. We found that a single injection of α -GalCer caused an increase in β -catenin/Tcf/LEF1 signaling throughout the liver of mice, as indicated by β -galactosidase activity. Multiple injections of α -GalCer resulted in much stronger β -catenin/Tcf/LEF1 signaling than a single injection (Fig. 2A). The α -GalCer treatment modulated the levels of expression of genes encoding multiple components of the Wnt pathway, including LEF1, Tcf7, Wnt5a, Wnt3a, Wnt7a, Wnt7b and Wnt10a, as determined by qRT-PCR (Supplementary Fig. 1). The results from both ELISA (Fig. 2B, left panel) and western blot analysis (Fig. 2B, right panel) indicate that a significant increase in the levels of Wnt5a and Wnt3a proteins and β -catenin protein was observed during liver recovery following α -GalCer restimulation. Since the α -GalCer stimulation or α -GalCer/ α -GalCer restimulation did not affect β -catenin transcription (Supplementary Fig. 1), the α -GalCer stimulation most likely influences the levels of β -catenin through non-transcriptional mechanisms. To determine whether activation of Wnt signaling occurs after α -GalCer stimulation *in vivo*, hepatocytes were isolated and cultured with liver leukocytes. Repeated addition of α -GalCer resulted in higher expression of Wnt5a and Wnt3a (Fig. 2C). In addition, treatment of a stably transfected, Tcf-driven-GFP NKT hybridoma with an α -GalCer tetramer led to an increase in the numbers of GFP⁺ cells, with restimulation leading to a further increase in the numbers of GFP⁺ cells (Fig. 2D). Consistent with the FACS analysis data, treatment of the NKT hybridoma with α -GalCer tetramer resulted in enhancement of phosphorylation of β -catenin and GSK3 β (Fig. 2E) as well as induction of expression of the genes encoding Wnt5a and Axin2 (Fig. 2F). Unlike the induction of expression of the genes encoding Wnt5a and Axin2, restimulation was required for induction of the genes encoding Cbl-b, Grail and Itch (Fig. 2F), which are known to be associated with the development of the anergic state after stable expression of β -catenin in T cells(13–15). Furthermore, knockdown of LEF1 in the NKT hybridoma led to a partial reversing of the α -GalCer restimulation did not elicit IL-2 production (supplemental Fig. 2), suggesting that LEF1 is a critical transcriptional factor that regulates α -GalCer mediated anergy of NKT cells.

To directly assess whether the liver microenvironment created by α -GalCer stimulation has functional significance in the induction of NKT cell anergy, we used an adoptive transfer approach in which NKT cells from naïve mice were transferred into γ irradiated Tcf/LEF1-reporter mice that had been preinjected with α -GalCer, LiCl, or vehicle (PBS) as a control. The reconstituted mice were then injected with α -GalCer. Staining of liver sections from the

Tcf/LEF1-reporter mice for β -galactosidase showed that Wnt signaling in the liver is indeed activated by α -GalCer or LiCl treatment (Fig. 3A). The production of IFN- γ and IL-4 was significantly lower in the recipient mice that had been pretreated with α -GalCer or LiCl than in PBS-treated animals (Fig. 3B). The α -GalCer or LiCl treatments had a similar effect on the production of IL-12 (Fig. 3B), suggesting that anergy of IL-12-producing cells, including dendritic cells, is induced. To confirm whether the anergy of adoptive transferred NKT cells from naïve mice is induced, NKT cells were recovered from the liver of α -GalCer or LiCl treated recipient mice and cocultured with liver DCs from naïve mice. NKT cells re-isolated from the livers of recipient mice pretreated with α -GalCer or LiCl were not responsive to treatment with α -GalCer *ex vivo* (Fig. 3C); whereas, the NKT cells reisolated from the livers of recipient mice pretreated with vehicle control were responsive. To confirm these findings, we also adoptively transferred NKT cells recovered from vehicle, α -GalCer, or LiCl-injected mice to Rag1KO mice. The production of IFN- γ and IL-4 was significantly lower in the Rag1KO mice that received NKT cells from α -GalCer or LiCl-treated mice when compared to Rag1KO mice that received NKT cells from PBS-treated mice (Fig. 3D). Collectively, these results indicate that the liver microenvironment plays a key role in the induction of anergy in NKT cells because irrespective of whether the NKT cells had been pretreated with α -GalCer or not, they lost their responsiveness to α -GalCer as long as a Wnt-enriched liver environment was established.

PGE₂ plays a role in the induction of liver NKT cell anergy by activation of Wnt/ β -catenin

PGE₂ is known to modulate Wnt/ β -catenin activity in cancer cells. However, the role of PGE₂ in terms of regulating NKT cell activation is not known. Treatment of wild-type NKT cells with PGE₂ led to increased phosphorylation of β -catenin, as well as prompting phosphorylation of glycogen synthase kinase 3 β (GSK3 β) in a time dependent manner (Fig. 4A) and induction of the expression of the genes encoding the Wnt ligands, Wnt5a and Axin2 (Fig. 4B). Interestingly, treatment with PGE₂ together with α -GalCer had a synergistic effect on the induction of expression of the gene encoding Wnt 5a (Fig. 4B),

To further determine whether PGE₂ mediated inactivation of GSK-3 β plays an essential role in induction of NKT cell anergy, NKT cells were treated with two GSK-3 β specific inhibitors. Liver NKT cells were stimulated with the α -GalCer in thiadiazolidinone (TDZD), a non-ATP competitive inhibitor of GSK-3 β that binds to the active site of GSK-3 β . The addition of the non-ATP competitive GSK3 β inhibitor suppressed the production of IFN- γ and IL-4 in the α -GalCer-stimulated NKT cells (Fig. 4C). Similar results were obtained upon treatment of NKT cells with another non-ATP competitive GSK3 β inhibitor, lithium chloride (LiCl) (Fig. 4C). Treatment of the α -GalCer-stimulated NKT cells with Wnt3a or Wnt5a ligands resulted in effects similar with those obtained for treatment of NKT cells with GSK3 β inhibitors (Fig. 4D). Reduced IFN- γ production was not due to an intrinsic defect as there was no difference in the intracellular stained IFN- γ in the PMA and ionomycin stimulated NKT cells that had been treated with PGE₂ or PGE₂ vehicle (supplemental Fig. 3) Furthermore, blocking of the canonical Wnt activation pathways using IWR1 or IWP2 led to a partial reversal of PGE₂-mediated inhibition of production of IFN- γ and IL-4 (Fig. 4E). The results from real-time PCR analysis also indicate that the PGE₂ mediated reduction in IFN- γ and IL-4 correlated with the inhibition of expression of T-bet

and GATA-3 (Fig. 4F), key transcription factors regulating IFN- γ and IL-4 expression and maturation of NKT cells(16, 17). Furthermore, knockdown of LEF1 in the NKT hybridoma led to partial reversing of PGE₂ mediated inhibition of IL-2 production (supplemental Fig. 4), suggesting that LEF1 is a critical transcriptional factor that regulates PGE₂ mediated anergy of NKT cells. Collectively, PGE₂ stimulation led to activation of Wnt/ β -catenin and subsequently the induction of NKT cell anergy.

Intestinal mucus-derived exosome-like nanoparticle (IDEN)-associated PGE₂ plays a role in induction of liver NKT cell anergy

Exosome-like nanoparticles have a high capacity for binding PGE₂ (18) and maintaining its stability and thus activity. ELISA analysis of circulating exosome-like nanoparticles indicates that the circulating nanoparticles carry PGE₂ (Supplementary Fig. 5). FACS analysis of these circulating nanoparticles further indicates that they are also A33⁺ (Fig. 5A). A33⁺ is an intestinal epithelial marker, suggesting that these PGE₂⁺ nanoparticles are derived from the intestine.

Nanosized particles in the gut migrate into the liver (19, 20) where the majority of the NKT cells reside. We tested whether intestinal mucus derived exosome-like nanoparticles (IDEN) can induce liver NKT cell anergy. The results from electron microscopy examination showed that they are nanoparticles in size (Fig. 5B). The nanoparticles were enriched for PGE₂ (Supplemental Fig. 5). We then tested whether IDEN associated PGE₂ plays a role in the induction of NKT cell anergy. NKT cells were purified from the livers of mice that had been administered IDEN or vehicle intravenously. NKT cells were cocultured *in vitro* with dendritic cells from the livers of untreated mice in the presence of α -GalCer. The results show that the NKT cells purified from the mice that had been administered IDEN had significantly lower production of both IFN- γ and IL-4 of NKT cells to α -GalCer stimulation (Fig. 5C). Liver NKT cells pretreated with circulating exosomes also produce less IFN- γ and IL-4 in response to α -GalCer stimulation (Supplemental Fig. 6), suggesting that IDEN PGE₂ mediated induction of NKT cell anergy is physiological relevant.

To further determine whether the IDEN-associated PGE₂ played a role in the induction of NKT cell anergy, mice were treated with indomethacin, a cyclo-oxygenase 2 inhibitor that blocks the generation of PGE₂. The effects of IDEN isolated from indomethacin treated mice, on the induction of NKT cell anergy were then evaluated. Indomethacin treatment reduced significantly the amounts of PGE₂ associated with the IDEN (Supplementary Fig. 5), which ultimately led to the attenuation of the IDEN-mediated anergy induction in NKT cells to α -GalCer stimulation (Fig. 5D).

To further demonstrate the role of IDEN mediated anergy induction of liver NKT cells *in vivo*, we used a ConA-induced hepatitis mouse model. ConA-induced hepatitis is dependent on NKT cell activity (21). Using an adoptive transfer approach, we found, as expected, that SCID mice that received liver NKT cells from PBS-treated mice exhibited the typical liver necrosis (Fig. 6A) and elevation of serum ALT and AST (Fig. 6B). In marked contrast, the histologic evidence of necrosis in the liver (Fig. 6A) and the ConA-induced elevation of the levels of serum ALT and AST (Fig. 6B) were reduced significantly in the SCID mice that

received liver NKT cells from IDEN-treated mice, indicating that the IDEN has a direct effect on induction of anergy in liver NKT cells.

Intestinal mucus-derived exosome-like nanoparticles (IDEN) also induce NKT cell anergy in the context of both glycolipid presentation and TLR-mediated pathways

Antigen presenting cells play an essential role in liver NKT cell activation by presenting lipid related antigen on an APCs CD1d molecule dependent and independent manner(22). First, we tested whether DCs take up IDEN. Both CD11c⁺ DCs and F4/80⁺ macrophages from the livers of naïve mice took up IDEN rapidly (as early as 2 h) and continued to take up IDEN over 24 h (Fig. 7A, Supplementary Fig. 7). We then tested whether IDEN treatment has an effect on NKT cell activation of mice. FACS analysis of liver leukocytes suggested that the expression of MHCII and CD86 by the DCs (Fig. 7B) was reduced in mice that had been administered IDEN. On co-culture of DCs purified from the livers of mice that had been administered IDEN or vehicle with CFSE-labeled liver NKT cells isolated from mice that had been administered IDEN or vehicle in the presence of α -GalCer, DCs from the livers of mice that had been administered IDEN exhibited a reduced ability to stimulate the proliferation of NKT cells, regardless of the source of the NKT cells (Fig. 7C). RT-PCR analysis further indicated that the expression of IL-12 and TNF α , which are critical for activation of DCs and DC-mediated activation of NKT cells, was significantly lower in DCs sorted from the livers of α -GalCer-injected mice that had been administered IDEN (Fig. 7D). Additionally the levels of the immunosuppressive cytokine IL-10 were higher. DCs also can activate NKT cells in a CD1d-independent manner through Toll-like receptor (TLR)-induced release of soluble mediators, including IL-12 and type I interferons (IFNs) (23, 24). We found that IDEN suppressed the expression of IL-12 and IFN- β in TLR-stimulated DCs (Fig. 7E) and that IFN- γ release was reduced greatly when TLR ligand-treated DCs were co-cultured with liver NKT cells in the presence of IDEN (Fig. 7F). Thus, IDEN can also induce NKT cell anergy through modification of the ability of DCs to stimulate NKT cell anergy in the context of both glycolipid presentation and TLR-mediated pathways. To further determine whether IDEN-associated PGE₂ plays a role in the inhibition of production of IL-12, the effects of IDEN isolated from indomethacin treated mice on the production of TLR stimulated DCs was evaluated. ELISA results (Fig. 7G) indicate that IDEN isolated from indomethacin treated mice have a reduced potency to inhibit IL-12 production, suggesting that IDEN associated PGE₂ plays a role in inhibiting the production of IL-12 of TLR stimulated DCs.

We further tested whether IDEN mediated Wnt/ β -catenin activation also plays a role in TLR stimulated DC activation. BMDCs derived from B6.Cg-Tg(BAT-lacZ)3Picc/J mice have a remarkable increase in β -galactosidase activity, where the β -galactosidase gene expression is driven by a Tcf/LEF1 promoter in the presence of IDEN (Fig. 7H).

Paradoxically, IDEN treatment also led to a reduction in IL-12 from BMDCs stimulated with TLR ligands as listed in figure 7I. The addition of canonical Wnt inhibitors, IWR1 and IWP2, led to the partial reversing of IDEN mediated inhibition of IL-12 production (Fig. 7J). Collectively, these results suggest that IDEN mediated activation of the Wnt pathway in DCs also plays a role in induction of NKT cell anergy.

Discussion

The results presented in this study suggest a model (Fig. 8) in which PGE₂ or α -GalCer stimulation leads to induction and releasing Wnt ligands in the liver where NKT cells reside. NKT Wnt signaling activation mediated by PGE₂, α -GalCer induced Wnt ligands, or PGE₂ via inactivation of GSK-3 β (a β -catenin inactivator), eventually activate β -catenin/LEF1 mediated transcriptional machinery which causes induction of NKT cell anergy. Alternatively, PGE₂ mediated activation of the Wnt/ β -catenin pathway in DCs leads to prevention of TLR stimuli induced production of IL-12 that is required for CD1d independent NKT cell activation. In this study, we provide for the first time evidence that activation of the Wnt/ β -catenin pathway leads to anergy of NKT cells. We also provide evidence for PGE₂ cross-talk with the Wnt signaling pathway, which can occur through regulation of β -catenin/GSK3 β activity. The evidence for PGE₂ cross-talk with the Wnt signaling pathway is consistent with the literature in which a role for PGE₂ in regulation of Wnt signaling at the level of β -catenin stability has been demonstrated in zebrafish hematopoietic stem cells(25).

It has also been reported that some factors through ubiquitin-mediated proteasome degradation may induce NKT cell anergy(26). The inhibition of α -GalCer-induced phosphorylation of ERK tyrosine kinase in NKT cells plays a role in induction of NKT cell anergy(27). Lacking co-stimulatory signals and cytokines provided by dendritic cells may also lead to NKT cell anergy(28–30). Whether these factors also cross-talk with PGE₂/Wnt/ β -catenin we identified in this study and lead to NKT cell anergy, will require further investigation to discern.

Recent studies suggest that exosome-like nanoparticles play a critical role in cell-cell communication(31, 32). Intestinal epithelial cells are known to release nanosized microvesicles(33, 34), and the nanoparticles have been shown to migrate into liver(19, 35). Our study further demonstrated that the gastrointestinal tract communicates directly with the liver via IDEN that carry PGE₂. PGE₂ carried by IDEN has at least two unique characteristics in comparison with the free form of PGE₂. 1) The stability of PGE₂ carried by IDEN is increased significantly as shown by the fact that the half-life of PGE₂ is approximately 30 second in the circulator system (36, 37), and intravenous injection of chemically synthesized PGE₂ did not have any effect on the induction of IFN- γ and IL-4 of mice treated with α -GalCer (Data not shown) and therefore could have no effect on activation of Wnt signaling in NKT cells. Besides the stability of PGE₂ regulated by the local balance between the COX2-driven synthesis and 15-hydroxyprostaglandin dehydrogenase (15-PGDH)-mediated degradation of PGE₂ (38, 39), in this study, we demonstrated that amount of PGE₂ carried by IDEN is also associated with the potency of induction of liver NKT cell anergy as the data presented in supplementary Figs. 5, 6. It is conceivable that the factors regulating the amount available and the affinity of IDEN binding to PGE₂ may also contribute to PGE₂ mediated Wnt signaling. The role of ceramide (40) and others factors that affect COX2/15-PGDH mediated PGE₂ synthesis and degradation warrants further study. In addition, factors regulating gut permeability which are critical factors in regulating the amount of nanoparticle trafficking from the gut to the liver (41–43) needs further study to. Caution should be exercised when drawing conclusions

regarding the biological effect of PGE₂ on IDENs. Effects on the Wnt signaling pathway may be different when comparing PGE₂ on IDENs to that of free form of PGE₂ since miRNAs and other lipids are packed in the IDEN and may also contribute to the PGE₂ mediated Wnt signaling pathway. Identifying whether IDEN miRNAs and/or lipids have a role in PGE₂ mediated Wnt signaling pathway needs further study. 2) PGE₂ carried by IDEN induces anergy of NKT cells not only through direct targeting of NKT cells but also through DCs activation via a TLR mediated pathway. The finding that IDENs can carry a number of therapeutic agents(44) and target antigen presenting cells may provide an avenue to pursue IDEN modulation of antigen presenting cell function and their role in gut immune tolerance.

These findings also open up a new avenue for investigating further the possible role of IDEN carrying other molecules released in gut that could induce both gut and liver immune tolerance. Furthermore, from therapeutic standard point, IDEN from intestines of other species may also be a useful vehicle for delivering therapeutic reagents(44, 45) to treat gastrointestinal diseases as well as diseases such as liver diseases treated by oral administration.

In this study, the finding that IDEN PGE₂ activated the Wnt pathway and suppressed cytokine expression via inactivation of the GSK3/β-catenin pathway raises a number of important questions that need to be addressed in future studies. PGE₂ binds and activates four G-protein-coupled E receptors (EP1–EP4). Unlike Ep1 and EP3, EP2 and EP4 have been shown to activate the GSK3/β-catenin pathway, as well as the adenylate cyclase-triggered cAMP/PKA/EPAC pathway(46–48). Whether IDEN PGE₂ also suppresses IFN-γ and IL-4 expression via cAMP/PKA/CREB dependent pathway is unknown. If IDEN PGE₂ does suppress cytokine production, also it needs to be determined if there is cross-talk with the cAMP/PKA/CREB pathway at unidentified points to ultimately regulate the production these cytokines.

Finally, the Wnt signaling pathway is known to play a crucial role in prevention of autoimmune responses and in promotion of tumor growth. PGE₂ is a potent signaling molecule that regulates immune tolerance and promotes tumor growth in addition to having a role in hematopoiesis, regulation of blood flow, renal filtration and blood pressure, regulation of mucosal integrity, and vascular permeability(49). Our findings provide a basis for further studies regarding the biological effects of PGE₂ cross-talk with the Wnt/β-catenin pathway on these systems as well.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Stable expression of β -catenin gene induces anergy in liver NKT cells

Sorted β -catenin-GFP⁺ or control vector-GFP⁺ retroviral transduced NKT cells were co-cultured with BMDCs in the presence of α -GalCer. (A) Cell proliferation measured by ³H-thymidine incorporation assay; (B) Levels of IFN- γ and IL-4 in the supernatant 24 h after α -GalCer treatment of cultures. **p < 0.01 (Student's t-test). Data are the mean SEM of three experiments.

Figure 2. α -GalCer simulation leads to the activation of Wnt signaling in the liver (A–C) TCF/LEF1-reporter mice were given a single-dose of α -GalCer (5 μ g, α -GC), multiple doses α -GalCer (5 μ g, every 3 d for 3 times, α -GC+ α -GC), or vehicle by intravenous injection and assessed 24 h after the last injection (n=7). (A) X-gal staining of sections of paraffin-embedded liver tissue. Magnification = $\times 20$. (B) Expression of Wnt 3a and Wnt 5a in whole liver extracts from treated mice as assessed using an ELISA (left two panels) and immunoblot analysis (right panel). (C) Immunoblot analysis of proteins of primary hepatocytes co-cultured with liver leucocytes in the presence of α -GalCer for 24 h. (D) FACS analysis of GFP expression on Tcf-GFP-NKT hybridoma 1.2 after stimulation with a single dose of α -GalCer-tetramer (α -GC-Tet) or after restimulation with α -GalCer-tetramer ($-\alpha$ -GC-Tet+ α -GC-Tet). (E) Immunoblot analysis of phosphorylated β -catenin ser (675) and GSK3 β ser (9) in NKT cell hybridoma 1.2 stimulated with α -GalCer tetramer. (F) Changes in mRNA abundance in NKT cell hybridoma 1.2 treated with α -GalCer tetramer for 24 h, washed with PBS, and then restimulated with α -GalCer tetramer (α -GC-Tet+ α -GC-Tet), or untreated NKT cell hybridoma 1.2 stimulated by α -GalCer (α -GC-Tet) or vehicle (vehicle) for 5h. Data are the mean $\pm$ SEM of three experiments (B,F) or representative of three experiments (A, B, right panel, C, D, E).

Figure 3. Activation of Wnt signaling in the liver led to NKT cell anergy

Recipient TCF/LEF1-reporter mice were treated with α -GalCer (3 μ g, i.v.), LiCl (200 mg/kg, i.p.) or vehicle as a control every three days for 12 days then irradiated (750 rads). After 5d, they were injected i.v. with enriched liver NKT cells (10×10^6 per mouse) from naïve C57BL/6 CD45.1⁺ mice. **(A)** X-gal staining of sections of paraffin-embedded liver tissue obtained 24 h after the last injection of LiCl. **(B)** Serum cytokine levels in the reconstituted mice at different time points after α -GalCer injection. **(C)** Proliferation of NKT cell recovered from recipient mice 3 days after cell transfer as described above and co-cultured with liver DCs from naïve mice in the presence of α -GalCer. **(D)** The recovered NKT cells were transferred into Rag1 KO mice for 9h, and the mice were then administered α -GalCer (intravenously); the serum cytokine levels assessed were examined at the indicated time points after the α -GalCer injection. * $P < 0.05$ and ** $P < 0.01$ (Student's t -test (B), one-tailed unpaired Student's t -test (A, C, D). Data are the mean $\pm$ SEM of at least 3 experiments ($n=5$) (A, C,D), or are representative of at least three independent experiments (D).

Figure 4. PGE₂ cross-talk with Wnt signaling leads to NKT cell anergy

(A) Immunoblot analysis of phosphorylated β-catenin ser (675) and GSK3β ser (9) in sorted liver NKT cells stimulated with PGE₂. (B) Changes in mRNA abundance in NKT cell hybridoma 1.2 stimulated with α-GalCer-tetramer (α-GC-Tet) and/or PGE₂ for 5 h. (C–E) Levels of IFN-γ and IL-4 in the supernatants of liver NKT cells co-cultured with BMDCs that had been pretreated with PGE₂ or the following inhibitors/ligands for 3h and then stimulated with α-GalCer or vehicle (DMSO) for 24 h. (C) TDZD (10 μM), LiCl (10 mM); (D) Wnt3a (0.5 μg/ml), wnt5a (0.5 μg/ml); and/or (E) IWR1 (5 μM), IWP2 (5 μM). (F) Changes in mRNA abundance in NKT cell hybridoma 1.2 stimulated with α-GalCer-tetramer (α-GC-Tet) and/or PGE₂ for 5 h. **P* < 0.05 and ***P* < 0.01 (one-tailed unpaired Student's *t*-test). Data (B–F) are the mean ± SEM of at least 4 experiments (n=5) or representative of three independent experiments (A).

Figure 5. PGE₂ associated with intestinal mucus derived exosome-like nanoparticles (IDEN) induces liver NKT cell anergy

(A) FACS analysis was performed to determine expression of A33 on the surface of exosomes derived from peripheral blood. (B) Characterization of nanosized nanoparticles isolated from intestinal mucus. Intestine from B6 mice was used for isolation of nanosized nanoparticles by differential centrifugation as described previously(44). Sucrose-purified nanosized nanoparticles were examined by electron microscopy (Bar = 200 nm). (C) Cytokine production by liver NKT cells co-cultured with BMDCs in the presence of α -GalCer (100ng/ml) for 24h. NKT cells isolated from mice were *i.v.* injected along with IDEN or PBS every 2 days for 14 days. (D) Cytokine production by liver NKT cells from naïve mice cultured with BMDCs in the presence of PBS-IDEN, Indo-IDEN or PBS for 3h, and then stimulated by α -GalCer (100ng/ml) for 24h. * $P < 0.05$ and ** $P < 0.01$ (Student's *t*-test). Data are representative of three experiments (A, B) or are the mean $\pm$ SEM of five independent experiments (C, D).

Figure 6. Intestinal mucus derived exosome-like nanoparticles (IDEN)-protect mice against ConA induced hepatitis

Effects of adoptive transfer of NKT cells isolated from mice treated with IDEN (100 μ g/mouse) or vehicle control (PBS) given *i.v.* every 3 d for 15 d into irradiated NOD-SCID mice on ConA-induced liver damage (25 mg ConA/kg, by intravenous injection 2 d after the last injection of IDEN) (n = 10). (A) H&E-staining of liver sections. (B) Levels of serum ALT and AST.

Figure 7. Wnt signaling plays a role in Intestinal mucus-derived exosome-like microparticle (IDEN) induced NKT cell anergy in the context of both glycolipid presentation and TLR-mediated pathways

(A) CD11c⁺ DCs take up IDEN. Hepatic MNCs (5×10^5) were cultured with PKH26-labeled IDEN (10 μ g/ml) for 4-24 h at 37°C. IDEN positive cells are positive for: CD11c (green) and PKH26⁺ IDEN (red). At least 10^4 total events were collected for each sample. Data are representative of three independent experiments done in triplicate. (B–D) C57BL/6

mice were injected *i.v.* with PBS or IDEN (100 $\mu\text{g}/\text{mouse}$, every 2 d for 14 d. **(B)** Expression of MHC II and CD86 on CD11c⁺ DCs from the livers of mice pretreated with IDE (red) or PBS (blue) 24 h after being *i.v.* injected with α -GalCer (5 $\mu\text{g}/\text{ml}$). **(C)** Liver NKT cells and DCs were sorted from mice pretreated with IDEN or PBS. The division of CFSE labeled liver NKT cells co-cultured with liver DCs for 3 d in the presence of α -GalCer was FACS analyzed. **(D)** Analysis of IL-10, IL-12 and TNF- α mRNA in DCs sorted from the livers of PBS or IDEN-treated mice 5 h after injection of α -GalCer. **(E)** Levels of IL-12 and IFN- β in supernatants of BMDCs cultured for 24 h in the presence of IDEN (50 $\mu\text{g}/\text{ml}$) with pam3cy4, LPS, poly(I:C), CL097 or CpG. **(F)** Presence of IFN- γ in supernatants of BMDCs cultured with sorted hepatic NKT cells in the presence of IDEN for 3 h, and then stimulated for 24 h with different TLR ligands indicated in the figure. **(G)** Levels of IL-12 in supernatants of BDMCs cultured for 24 h in the presence of IDEN (50 $\mu\text{g}/\text{ml}$) with LPS or CL097. IDEN were derived from mice treated with PBS or indomethacin. **(H–J)** IDEN mediated Wnt/ β -catenin BMDC activation. **(H)** IDEN treatment leads to transactivation of TCF/LEF1 reporter. BMDCs from transgenic TCF/LEF1 reporter mice were treated by PBS or IDEN, and β -galactosidase activity was measured by flow cytometry with fluorescein di- β -D-galactosidase (FDG) as a substrate. Data are representative of three independent experiments. **(I)** Levels of IL-12 in supernatants of BCMCs cultured for 24 h in the presence of IWR1, IWP2 and/or IDEN (50 $\mu\text{g}/\text{ml}$) with LPS or CpG. **(J)** Presence of IFN- γ in supernatants of BMDCs co-cultured with sorted hepatic NKT cells in the presence of IWR1, IWP2 and/or IDEN for 3 h, and then stimulated by LPS or CpG for 24 h. * $P < 0.05$ and ** $P < 0.01$ (Student's *t*-test). Data are representative of three experiments (A, B, C, H) or are the mean SEM of three independent experiments (**D, E, F, G, I, J**).

Figure 8.

We propose three possible pathways that could lead to liver NKT cell anergy. **(A).** Repeated α -GalCer stimulation or PGE_2 carried by IDEN leads to induction of Wnt ligands in the liver. Released Wnt 3a and Wnt 5a subsequently bind to liver NKT cells and activate β -catenin mediated TCF/LEF1 activation, which results in induction of NKT cell anergy. **(B)** Up take of IDEN- PGE_2 by hepatic DCs results in activation of the β -catenin mediated pathway which then prevents the secretion of IL-12 induced by TLR stimuli. **(c)** IDEN- PGE_2 could also directly bind to PGE_2 receptors and subsequently activate the β -catenin mediated pathway by inactivation of GSK-3 β .