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Exosome-like Nanoparticles from Intestinal Mucosal Cells Carry Prostaglandin E2 and Suppress Activation of Liver NKT Cells

Zhong-Bin Deng,* Xiaoying Zhuang,* Songwen Ju,* Xiaoyu Xiang,* Jingyao Mu,* Yuelong Liu,† Hong Jiang,‡ Lifeng Zhang,** James Mobley,‡ Craig McClain,§ Wenke Feng,§ William Grizzle,† Jun Yan,* Donald Miller,* Mitchell Kronenberg,¶ and Huang-Ge Zhang*∗‖

Regulation and induction of anergy in NKT cells of the liver can inhibit autoimmune and antitumor responses by mechanisms that are poorly understood. We investigated the effects of PGE2, delivered by intestinal, mucus-derived, exosome-like nanoparticles (IDENs), on NKT cells in mice. In this study, we demonstrate that IDENs migrate to the liver where they induce NKT cell anergy. These effects were mediated by an IDENs’ PGE2. Blocking PGE2 synthesis attenuated IDENs inhibition of induction of IFN-γ and IL-4 by α-galactosylceramide (α-GalCer)–stimulated liver NKT cells in a PGE2 E-type prostano id 2/ε-type prostano id 4 receptor–mediated manner. Proinflammatory conditions enhanced the migration of IDENs to the liver where α-GalCer and PGE2 induced NKT anergy in response to subsequent α-GalCer stimulation. These findings demonstrate that IDENs carrying PGE2 can be transferred from the intestine to the liver, where they act as immune modulators, inducing an anergic-like state of NKT cells. These reagents might be developed as therapeutics for autoimmune liver diseases. The Journal of Immunology, 2013, 190: 3579–3589.

The gut-associated immune system is complex and involves extensive communication with the liver (1, 2). Glycolipids derived from dietary metabolic products and lipids derived from some intestinal bacteria (3, 4) migrate constantly into the liver, and these lipids can activate liver NKT cells through TCR engagement in vitro (5, 6). Liver NKT cells can also be activated in a TCR-independent manner (7, 8). It is remarkable that liver NKT cells are normally quiescent, even though they are constantly exposed to intestinal-derived products and are also thought to play an active role in systemic tumor surveillance and maintenance of tolerance (9).

Exosomes are microvesicles released naturally from a variety of cells (10). They carry a cargo of proteins, lipids, miRNAs, and/or microRNAs and can transfer their cargo to recipient cells, thus serving as extracellular messengers to mediate cell–cell communication. Numerous recent reports indicate that exosomes have a significant effect on physiological and pathological processes in recipient cells (11). Intestinal epithelial cells release exosomes (30–100 nm) (12) and have been shown to be capable of regulating the differentiation and activity of several different types of immune cells (12–14), which suggests the possibility that intestinal-derived exosome-like nanoparticles could play a key role in communication between the intestine and liver and in gut-related induction of immune tolerance or anergic-like state (12, 15).

In this study, we demonstrate that IDENs migrate into the liver and that inflammation promotes this migration. We also demonstrate that IDENs can induce an anergic-like state in liver NKT cells. Induction of a NKT cell anergic-like state by IDENs occurs through a previously undescribed mechanism where the transport of PGE2 by the IDENs (16) into the liver creates an environment that promotes the NKT cell anergic-like state.

Materials and Methods

Isolation and purification of IDENs

Mice were euthanized, and the small intestines were removed. The luminal contents of the intestines were removed by gently flushing the intestines with 10 ml ice-cold PBS. The intestines were then opened longitudinally, the mucus was collected by mild physical separation using round forceps, and the mucus was processed by differential centrifugation and sucrose gradient centrifugation to isolate and purify the exosome-like nanoparticles or microparticles (pellets after 10,000 × g centrifugation) using a previously described protocol (17). The identical protocol was also used for isolation and purification of human intestinal IDENs from surgically resected human intestines.
Enzymatic Assay Kit (Thermo Scientific). For histopathology, H&E-staining was performed on paraffin-embedded liver sections. For immunofluorescence analysis, OCT (Sakura Finetek)-embedded tissue cryosections (9-μm-thick) were fixed at −20°C in methanol/acetone (3:1). Slides were hydrated in PBS and blocked for 30 min at 25°C with Fc block (10 μg/ml) and 5% (v/v) normal horse serum in PBS. After blockade, slides were incubated for 30 min at 25°C with “mixtures” containing the following primary Abs in PBS: anti-A33 (Santa Cruz Biotechnology), anti-CD31 (MEC 13.3, BD Biosciences), and anti-Lyve-1 (R&D Systems). Primary Abs were detected with Alexa Fluor 647–donkey anti-goat, rabbit Ab to FITC–Alexa Fluor 488 (A11090) (S21381; all from Invitrogen Life Sciences), or streptavidin–allophycocyanin (BD Biosciences). Slides were mounted with Slow Fade Gold Antifade plus DAPI (S36938; Molecular Probes and Invitrogen Life Sciences). Tissues were assessed using a Zeiss LSM 510 confocal microscope equipped with a digital image analysis system (Pixeera). Apoptosis was evaluated using the TUNEL assay performed on frozen liver sections using the in situ cell death detection kit (Roche).

Cell sorting. For measuring cytokine induction, NK1.1+CD5+ NKT cells were sorted from liver leukocytes using a method as described (23). The purity of the NKT cells collected, typically >90%, was assessed using CD1d tetramers loaded with α-galactosylceramide (α-GalCer; provided by the National Institute of Allergy and Infectious Diseases Tetramer Facility, National Institutes of Health). For proliferation assays, NKT cells were also enriched from liver by negative magnetic bead sorting (Miltenyi Biotec) using anti-CD11b, B220, CD8α, Gr-1, L-selectin, and CD11c Abs using a standard protocol (24). Although CD4 is not depleted in this case, the specificity of NKT cell proliferation was determined by stimulation of α-GalCer, which specifically activates NKT cells but not CD4 T cells. The purity of NKT and CD11c cells was determined by FACS analysis as described previously (24).

Cell proliferation and CFSE dilution analysis
NKT cells enriched by negative selection (4 × 10^5) were cultured with liver dendritic cells (DCs) (1 × 10^5) isolated by CD11c magnetic bead sorting (Miltenyi Biotec). In vitro cultures were incubated for 72 h under the indicated culture conditions, and [3H]thymidine was added for the last 16 h. Incorporation of radioactivity was determined using a Betaplate liquid scintillation counter (Packard Bioscience). Results are expressed as mean cpm of triplicate wells ± SEM. For CFSE (Invitrogen) dilution analysis, splenocytes were labeled with 5 μM CFSE for 10 min at 37°C in PBS containing 0.1% BSA and washed twice with complete RPMI 1640 medium. Labeled NKT cells (4 × 10^4 cells/well) were cultured with liver DCs (5 × 10^3) under the indicated culture conditions for 96 h in complete RPMI 1640 medium and stained with TCR-β and α-GalCer/CD1d. CFSE dilution was examined on gated α-GalCer/CD1d tetramer+ NKT cells.

Western blot analysis
Western blots were carried out as described previously (19). In brief, cells or exosomes were lysed in protein lysis buffer containing 1% Triton X-100, 0.1% SDS, 150 mM NaCl, 50 mM Tris-HCl, 1 mM EDTA, 1 mEq EGTA, 5 mM sodium molybdate, and 20 mM phenylphosphate with protease and phosphatase inhibitors (1 mM PMSF, 10 μg/ml aprotinin, 20 μg/ml leupeptin, 20 μg/ml pepstatin A, 50 μM NaF, and 1 mM sodium orthovanadate). Proteins of lysed cells were separated on 10% polyacrylamide gels using SDS-PAGE and transferred to nitrocellulose membranes. The membranes were stained with Pierce Red to validate that all samples contained similar amounts of protein.

RNA extraction and PCR
Total RNA was isolated from livers or NKT hybridomas with TRIzol, according to the manufacturer’s specifications (Invitrogen), and the RNA repurified with an RNeasy mini kit (Qiagen). RNA (1 μg) was reverse transcribed with Superscript III and random primers (Invitrogen). For quantification of genes of interest, cDNA samples were amplified in a CFX96 Real-time System (Bio-Rad Laboratories, Hercules, CA) and SsoFast Evagreen supermix (Bio-Rad Laboratories), according to the manufacturer’s instructions. Fold changes in mRNA expression between treatments and controls were determined by the 6CT method as described previously (25). Differences between groups were determined using a two-sided Student t test and one-way ANOVA. Error bars on plots represent ± SE, unless otherwise noted. The data were normalized to a GAPDH reference. All primers were listed in the Supplemental Table I.

Agonists, antagonists, and inhibitors
IDEN lipids were extracted according to Bligh and Dyer (26) in chloroform/methanol/water (2:5:2.5:2:1, v/v/v/v). Lipids for treatment of cells were...
dried under nitrogen and sonicated in PBS at pH 7.4 and 0.05% (v/v) Tween-20 before use. Polyclonal anti-A33 Ab was purchased from Santa Cruz Biotechnology (Santa Cruz, CA), and cAMP analogs (dibutyryl cAMP and N6-Bnz-cAMP) and protein kinase A (PKA) inhibitors (H-89, Rp-8-CPT-cAMPS, and Rp-8-Br-cAMPS) were purchased from Sigma-Aldrich. PGE2, Butaprost, misoprostol, sulprostone, and PGE2 ELISA kits were obtained from Cayman Chemical. E-type prostanoid (EP) 4 agonists (ONO-AE1-329), EP1 antagonist (ONO-8713), EP3 antagonist (ONO-AE3-240), and EP4 antagonist (ONO-AE3-208) were gifts from Ono Pharmaceutical (Chuo-ku, Osaka, Japan).

Cell cultures
Bone marrow-derived dendritic cells (BMDCs) were cultured as described previously (27). To study the activity of IDENs or IDEN lipid, total liver mononuclear (4 × 10^7/well) or BMDCs cultured with FACS-sorted NKT cells (1 × 10^6 BMDCs + 4 × 10^4 NKTs/well) or in the presence of IDENs (30 μg/ml) for 3 h and then stimulated by α-GalCer (100 ng/ml) for 24–48 h in round bottom 96-well plates containing RPMI 1640 medium supplemented with 10% FCS. In some experiments, sorted NKT cells pretreated with IDENs were stimulated by α-GalCer–tetramer. In some cases, 100 μM PGE2, 10 μM butaprost (EP2 agonist), 40 μM misoprostol (EP4 agonist), 10 μM prostanoid (EP1/EP3 agonist), or 100 μM dibutyryl-cAMP 100 μM N6-Bnz-cAMP, 3 mM H-89, 100 mM Rp-8-Br-cAMPS, or 100 mM Rp-8-CPT-cAMPS were added to cocultures. Cells were assessed by flow cytometry, or supernatants were used for cytokine measurement by ELISA.

Liposomes assembled from IDENs
Liposomes were formed from IDENs used the protocol as previously described (28) with minor modification as described below: 1 ml IDENs in PBS were mixed together with 3.75 ml chloroform/methanol at a 1:2 ratio (v/v) in borosilicate glass test tubes, before adding 1.25 ml chloroform to the mixed sample and vortexing the mixture. Subsequently, 1.25 ml deionized water was added, and the mixture was vortexed again. The sample was centrifuged at 1000 rpm in a table-top microcentrifuge for 5 min at 22°C. The bottom-phased liquid that contained lipids was collected using a pasteur pipette. Solvent in the collected sample was completely removed via a constant flow of nitrogen gas at 60°C. Extracted IDEN lipids were hydrated with 800 μl Milli-Q water prior to bath sonication for 3 min and then sonicated for an additional 2 min after addition of HEPES-buffered saline (final concentration, 10 mM HEPES and 50 mM NaCl). This resulted in a clear dispersion being produced that was stored at 4°C for 48 h prior to further experimentation or examination for nanoparticle formation using a conventional procedure (29).

Coupling of IDENs or microparticles to latex beads and immunofluorescence analysis
IDENs or microparticle-coated beads were prepared using a conventional procedure (29) and washed twice in PBS containing 10% FCS, stained with Ab directed against Annexin V (eBioscience), and analyzed using a BD Biosciences FACSCalibur. A “bead-only” control and an isotype-matched Ab directed against Annexin V (32, 33). Annexin V predominantly detected in microparticles was demonstrated by FACS analysis of Annexin V (Fig. 1B, left panel). Electron micrographs (Fig. 1C, left panel) show that IDENs were also present in the small intestines of human and shared a number of exosomal protein markers identified on mouse IDENs (Fig. 1C, right panel).

Intestinal mucus is a physically cross-linked hydrogel with mesh sizes on the order of 10–100 nm (34, 35). Penetration of this barrier is largely dependent on the size of the particles (34, 35). The size of the IDENs is ~30–100 nm, and we used gavage administration of IDENs labeled with an infrared fluorescent membrane dye (DiR) to track their migration patterns in vivo (17). Imaging revealed an accumulation of fluorescent signals in the gut over the first 24 h (Fig. 1D). At 48 h, signals were detectable in dissected liver and mesenteric lymph nodes but not the lung, spleen (Fig. 1E), or other organs (data not shown). The presence of IDENs in the liver was confirmed by immunohistochemistry and containing for A33 (Fig. 1F). The colocalization of the DiR and A33 signals with CD31, a marker of endothelial cells (Fig. 1G), and LYVE1, a marker of lymphatic capillaries (Fig. 1H), along the length of the intestinal endothelial and lymphatic vessels within 6 h of administration of IDENs suggests that the IDENs migrate into the liver from the gut primarily through the vascular and lymphatic vessels. The migration of A33-positive IDENs to the liver (Fig. 1I) was enhanced in mice treated with either Con A, a lectin that induces inflammatory liver injury in mice and is a model of autoimmune liver disease (36), or DSS, which induces colonic inflammation with characteristics of acute ulcerative colitis. Thus, IDENs can gain access and traffic within the vascular or lymphatic system of the liver. Inflammation in the liver or colon enhances this migration.

IDENs protect against inflammation-mediated liver damage
To explore the biological effects of the accumulation of IDENs in the liver, we tested the effects of pretreatment of mice with purified IDENs on Con A–induced liver injury (36). Mice that were pretreated with vehicle only succumbed rapidly (Fig. 2A) and exhibited symptoms characteristic of Con A–induced liver injury, including elevated levels of serum ALT and AST (Fig. 2B). In addition, there were histopathological changes in the liver (Fig. 2C) with extensive areas of confluent hepatocellular necrosis, marked sinusoidal hyperemia associated with hemorrhage, and mononuclear cell infiltrates scattered diffusely throughout the viable parenchyma. Pretreatment with IDENs markedly enhanced the survival of the mice (Fig. 2A), dampened the Con A–induced elevation in the levels of serum ALT and AST (Fig. 2B), and limited the liver histopathology to minor inflammatory infiltrates and small necrotic patches (Fig. 2C). Alleviation of liver injury appeared to be associated with a reduction in inflammatory responses because mice pretreated with IDENs had serum levels of the proinflammatory cytokines IFN-γ and TNF-α that were significantly lower than in the mice that were pretreated with vehicle only (Fig. 2D). Furthermore, the administration of IDENs reduced apoptosis of liver cells, as assayed by TUNEL staining (Fig. 2E).

IDENs induce hyporesponsiveness of liver NKT cells to α-GalCer
Con A–induced hepatitis is also dependent on NKT cell activity (37). Unlike CD4/CD8 T cells, NKT cells respond to glycolipid ligands. The glycolipid α-GalCer is a potent stimulator of NKT cell expansion when first administered but loses its effectiveness on repeated administration (9). To determine the effects of accumulation of IDENs in the liver on α-GalCer–driven NKT cell expansion, mice were injected i.v. with IDENs every 2 d for 2 wk and 3 h after the last injection the mice were injected i.v. with α-GalCer.
FIGURE 1. Characterization of IDENs isolated from intestinal mucus. Intestine from B6 mice (A) or human (C) was used for isolation of nanoparticles or microparticles (B) by differential centrifugation as described previously (27). Sucrose-purified nanoparticles were examined by electron microscopy [(A) and (C), left panel] (bar, 100 nm). FACS analysis was performed to determine expression of Annexin V (Figure legend continues).
FIGURE 2. IDENs protect mice against Con A–induced liver damage. (A–E) C57BL/6 mice were administered IDENs (100 μg/mouse in PBS, injected i.v. every 2 d for 14 d) prior to injection of Con A (37.5 mg/kg of body weight, injected i.v. 4 h after the last injection of exosomes) and assessed at specified times (n = 10 mice/group) for survival of mice after Con A injection (A) and levels of ALT and AST in the sera (B). *p < 0.05, **p < 0.01 as compared with mice injected with PBS (Student t test); (C) H&E-stained sections of the livers; original magnification ×20. (D) Levels of IFN-γ and TNF-α in the sera. *p < 0.05, **p < 0.01 as compared with mice injected with PBS (Student t test). (E) Apoptosis in the liver as assessed by TUNEL analysis. Stained with DAPI (blue) and TUNEL (green). Original magnification ×20.

(5 μg/mouse). The rationale for choosing a 2-wk treatment schedule was based on data published by others (24). In naive mice, a single injection of α-GalCer caused TCR downregulation of NKT cells within 24 h and a decrease in the percentages of TCR+NKT cells recovered 3 d after the injection. This result is also consistent with what has been reported by others (24). In contrast, pretreatment with IDENs dampened the recovery of TCR+NKT cells after α-GalCer injection (Fig. 3A) and reduced the α-GalCer–induced expression of the markers of early activation of NKT cells (i.e., CD69 and its costimulator CD40L (Fig. 3B)). Moreover, the α-GalCer induced elevation of ALT and AST serum levels (Fig. 3C), and production of IFN-γ and IL-4 (Fig. 3D) by liver NKT cells was reduced substantially in mice that had been pretreated with IDENs, indicating that IDENs can induce an anergic-like state in NKT cells exposed to inflammatory signals in vivo.

To directly test whether IDENs can induce an anergic-like state in NKT cells to glycolipids, we purified NKT cells from the livers of mice that had been administered IDENs or vehicle i.v. and cocultured NKT cells in vitro with DCs from the livers of untreated mice in the presence of α-GalCer. The results confirmed that the NKT cells purified from the mice that had been administered IDENs were hyporesponsive to α-GalCer stimulation (Fig. 4A). Consistent with this, the addition of IDENs to liver NKT cells in culture suppressed the ex vivo–proliferative response of the NKT cells to α-GalCer (Fig. 4B). We also analyzed the responses of FACS-sorted cells in vitro. BMDCs were cocultured with naive liver NKT cells treated with IDENs or PBS for 3 h and then stimulated with α-GalCer for an additional 24 h (Fig. 4C), or FACS-sorted liver NKT cells were treated with IDENs or PBS for 3 h and subsequently stimulated with α-GalCer tetramer (Fig. 4D). Analysis of the supernatants indicated that the α-GalCer–induced production of IFN-γ and IL-4 by the NKT cells was significantly reduced when IDENs were added to either NKT cells cocultured with DCs pulsed with α-GalCer (Fig. 4C) or purified NKT cells stimulated with tetramer (Fig. 4D).

IDEN-associated PGE2 inhibits α-GalCer induction of NKT cell activation

We found that liver NKT cells stimulated in vitro with αGalCer in the presence of lipids extracted from IDENs produced much less IFN-γ and IL-4 (Fig. 5A), suggesting that the lipid content of the IDENs plays a role in the IDEN-induced effects on NKT cells. Liposomes assembled from IDENs also have a similar effect as IDENs and IDEN lipids (Supplemental Fig. 1), suggesting that the structure of liposomes assembled from IDEN lipids may not be essential for IDEN-mediated inhibition of activation of NKT cells on the surface of IDENs or microparticles derived from mouse intestinal mucus (B, left panel). The presence of intestinal epithelial–specific marker (A33 and villin-1) and exosome markers (CD63, CD9, CD81, RAB5, RAB11, and HSP70) in the sucrose gradient–purified exosomes from the supernatants of 36-h cultured TS/A mouse breast tumor cells (51), mouse intestinal nanoparticles (A), microparticles (B), or human intestinal nanoparticles (C) was determined by Western blot analysis (A–C). The presence of Annexin V was determined by FACS analysis (B, left panels). The imaging and Western blot are representative of at least three experiments. (D) In vivo imaging of and quantitation of signals in C57BL/6 mice gavage-administered IDENs labeled with the infrared dye DiR (100 μg/mouse in 100 μl PBS) or nonlabeled IDENs in PBS, and (E) images of the washed intestines (cut longitudinally) and other organs obtained 48 h after administration of IDENs. (F) Confocal image analysis of frozen sections of the livers of mice administered IDENs labeled with PKH26 red dye. Sections stained for A33, an intestinal epithelial cell–specific marker (green), and nuclei stained with DAPI. Distribution of IDENs in the intestines of mice 12 h after gavage administration of IDENs labeled with DiR dye (100 μg in 100 μl of PBS) assessed by (G) confocal image analysis of the frozen sections of intestine with immunofluorescent staining for A33 (green) and CD31 (red); original magnification ×40. (H) Confocal image analysis of the frozen sections of intestine with immunofluorescent staining for LYVE (green) (original magnification ×60 [left panel]) with enlargement of indicated area shown in the right panel. (I) Accumulation of DiR dye-labeled IDENs (100 μg/mouse in 100 μl PBS) administered by gavage 24 h after treatment with Con A, DSS, or PBS representative scan. Data are the mean ± SEM of three experiments (D) or representative of three experiments (E–I) (n = 10 mice/group).
stimulated by α-GalCer. Our data (38) and that of others (39) indicate that PGE$_2$ from various sources is enriched on exosomes, and we found that the IDENs carried significant amounts (>80 ng/μg) of PGE$_2$ (Fig. 5B). PGE$_2$ is known to regulate T cell activation through the modulation of expression of PGE$_2$ receptors on T cells (40). Whether the expression of PGE$_2$ receptors on NKT cells are regulated by α-GalCer stimulation is not known. RT-PCR analysis of the expression of the four known PGE$_2$ receptor subtypes indicated selective upregulation of the EP2 and EP4 receptors on α-GalCer–stimulated liver NKT cells (Supplemental Fig. 2). This suggested the possibility that the IDEN-associated PGE$_2$ could play a role in the induction of the NKT cell anergic-like state.

Next, we tested the effects the amount of indomethacin (a cyclooxygenase [COX] 2 inhibitor that blocks the generation of PGE$_2$) would have on the association of PGE$_2$ with IDENs. ELISA results indicated that indomethacin treatment reduced the amount of PGE$_2$ associated with the IDENs (Fig. 5B). When comparing the IDENs isolated from the intestines of naive mice pretreated with vehicle only to the IDENs isolated from the intestines of indomethacin-treated mice, the IDENs from indomethacin-treated mice were less potent at inducing the anergic-like state of NKT cells exposed to α-GalCer stimulation (Fig. 5C). Indomethacin treatment resulted in promoting Con A–induced mouse mortality (Fig. 5D), more severe hepatocellular necrosis (Fig. 5E), and higher sera levels of IFN-γ and ALT (Fig. 5F). Our data (Fig. 6A) and the data published by others suggest that there was a selective decrease in liver NKT cells in DSS-induced colitis (41). Whether IDENs isolated from DSS-induced colitis have less capacity to inhibit live NKT cell activation is unknown. We found that in comparison with IDENs isolated from the intestines of naive mice, the IDENs isolated from the intestines of mice pretreated with 3% DSS in drinking water are less potent in inducing the IDEN-mediated anergic-like state of NKT cells to α-GalCer stimulation (Fig. 6B). This reduction appears to be associated with a decreased amount of IDEN PGE$_2$ (Fig. 6C). PGE$_2$ is also known to modulate CD4 T cell activation, and our data indicated that IDENs or PGE$_2$ treatment leads to a significant reduction of both IFN-γ and IL-2 produced by Con A–induced CD4 T cells activation (Fig. 6D). suggesting that IDEN PGE$_2$ may play a role in the inhibition of CD4 T cell activation, as well as in the Con A–induced mouse liver inflammation model.

Although the immunomodulatory effects of PGE$_2$ are well known (42, 43), PGE$_2$ has not been implicated previously in modulation of NKT cell activity. We found that addition of exogenous PGE$_2$ to monocytes isolated from the livers of naive mice (Fig. 7A) or to cocultures of mature BMDCs with NKT cells purified from the livers of naive mice (Fig. 7B) led to inhibition...
of α-GalCer–induced production of both IFN-γ and IL-4 in a PGE₂ dose-dependent manner. Similar results were obtained using the NKT1.2 NKT hybridoma (Supplemental Fig. 3). Addition of either EP2- or EP4- selective agonists to cocultures of FACS-purified liver NKT cells with BMDCs in the presence of α-GalCer suppressed the secretion of IFN-γ and IL-4 (Fig. 7C), whereas agonists selective for EP1 and EP3 had no effect (data not shown). Furthermore, the addition of either the EP4 antagonist, ONO-AE3-208 (Fig. 7D), or the EP2 antagonist, AH6809 (Fig. 7E), partially reversed the PGE₂ or IDEN-mediated inhibition of IFN-γ and IL-4 production, whereas EP1 and EP3 antagonists had no effect (data not shown). These findings lead to the novel conclusion that PGE₂ can inhibit the production of IFN-γ and IL-4 in α-GalCer–stimulated NKT cells and that these effects are mediated by the EP2 and EP4 receptors.

The cAMP–PKA pathway plays an essential role in the IDEN-associated PGE₂-dependent induction of NKT cell anergy

Both EP2 and EP4 are Gs protein–coupled receptors, which upon binding PGE₂ act to increase the levels of intracellular cAMP (44). We found that addition of a dibutylryl cAMP analog (dB-cAMP), a PKA-specific agonist (N⁶-Bnz-cAMP), or forskolin, a downstream activator of the cAMP-mediated pathway, suppressed the in vitro production of IFN-γ and IL-4 by NKT cells (Fig. 8A). Furthermore, both the IDEN (Fig. 8B) and PGE₂ (Fig. 8C)–mediated suppression of IFN-γ and IL-4 production was inhibited by H-89, a PKA inhibitor, or Rp-8-Br-Camps and Rp-8-CPT-cAMPS, type I and type II PKA inhibitors, respectively, confirming that the IDEN-mediated inhibition of IFN-γ and IL-4 production by NKT cells requires PGE₂-EP2/EP4 signaling and is dependent on the cAMP/PKA pathway.
In this study, we demonstrate that IDENs migrate to the liver where they induce a NKT cell anergy-like state. We also establish for the first time, to our knowledge, that a PGE\(_2\)-mediated pathway plays a role in the induction of the anergic-like state in NKT cells, which can occur through a cAMP/PKA activity-dependent mechanism. The rapid enhancement of the activity occurs through a traditional positive feedback mechanism in which the PGE\(_2\)-induced enhancement of expression of the EP2 and EP4 receptors can amplify PGE\(_2\) signaling in NKT cells. Notably, the activation of the PGE\(_2\) pathway also appears to create in the liver a microenvironment in which naive NKT cells are resistant to activation by \(\alpha\)-GalCer stimulation, which is one of the major obstacles in the use of \(\alpha\)-GalCer for the treatment of certain types of cancer.

It is a reasonable assumption that control of the balance between the anergic-like state and activation of NKT cells is a complex process involving the integration of numerous signals. The current studies pinpoint a regulatory mechanism by which IDENs can influence this balance through mechanisms that involve considerable cross-talk between intestinal IDENs, PGE\(_2\), and NKT cells. For example, in the current studies we found that inflammation induced by either Con A injection or drinking DSS water can alter the amount of PGE\(_2\) carried by the IDENs. Specifically, our data suggest that although inflammation induced by Con A or DSS-induced colitis resulted in increasing amounts of IDENs accumulating in the liver and significant reduction of PGE\(_2\) carried by IDENs, which made them less potent for inhibiting \(\alpha\)-GalCer stimulated liver NKT cell activation. This suggests that different microenvironmental conditions may tailor the ability of the IDENs to either promote or suppress NKT cell activation. Although two acute inflammatory mouse models were used in this study, IDENs derived from chronic inflammatory driven disease models such as colon...
FIGURE 8. IDEN PGE2 induces an anergic-like state in NKT cells via EP2/EP4 mediating the cAMP–PKA pathway. Levels of IFN-γ and IL-4 in supernatants of sorted hepatic NKT cells cocultured with BMDCs that were pretreated for 3 h with the following agents and then stimulated with α-GalCer for 24 h. The agents were db-cAMP (100 μM), Rp-Br-cAMP (100 μM), forskolin (10 μM) (A), or in combination with IDENs (B) or PEG2 (C) with/without H-89 (3 μM), Rp-8-Br-cAMPS (100 μM), or Rp-8-CPT-cAMPS (100 μM). ELISA of IFN-γ and IL-4 in the cultured supernatants. Data (A–C) are the mean ± SEM of four experiments (n = 5). *p < 0.05, **p < 0.01 (one-tailed unpaired Student t test).

cancer may have different phenotypes, which we have not addressed in this study. In addition, although the regulator that controls the amounts of PGE2 carried by IDENs has not been identified, we are interested in determining whether IDENs prefer carrying PGE2 rather than other PGs regulated by Cox1 and Cox2. Furthermore, COX-1 is constitutively expressed in most tissues including intestine. In contrast, COX-2 is induced in inflammation by various stimuli including cytokines and growth factors. Whether IDENs have a higher capacity in preferentially binding PGs regulated by Cox1 than Cox2 needs to be addressed by comparing lipidomic profiles of IDENs isolated from Cox1 and Cox2 knockout mice.

We also speculate that exosome or exosome-like nanoparticle recipient cells may also interact in a coordinated manner with the exosome producing cells. For an example, when the host microenvironment is under "fire" such as in infection or when there is an accumulation of damaged cells, inflammation is required for removing these cells. In this case, immune suppressors such as PGE2 carried by exosomes or exosome-like nanoparticles must be reduced for the recipient cells to be activated. Coordinated interactions of exosome or exosome-like nanoparticle producing cells with recipient cells may cause a subsequent release of particles predominantly packed with proinflammatory molecules that further amplify inflammatory responses via paracrine and autocrine pathways until the infected or damaged cells are removed. As a result, the newly achieved balance between proinflammatory and anti-inflammatory factors may be more related to the ratio of proinflammatory and anti-inflammatory factors to each other than absolute quantities of each. Therefore, it is conceivable that the data presented in Fig. 2D, where the sera levels of TNF-α and IFN-γ are higher than those from naive mice, that the overall balance or ratio of proinflammatory and anti-inflammatory factors is achieved such that liver damage because of the Con A injection was prevented. Dysregulation of this balance may contribute to the development of disease. This assumption is supported by recent results reported by another groups. Kornek et al. (45) showed that activated T cells released microparticles and that the levels of released microparticles correlated with the histological severity of patients with active hepatitis C. Another study (46) indicated that microparticles derived from CD14+ monocytes and NKT cells are critical to adipose liver inflammation of patients with NAFL/NASH. Further research is required to determine whether IDEN-mediated inhibition of liver NKT activation also has an inhibiting effect on the releasing of proinflammatory microparticles from NKT.

A large number of glycolipids that can activate NKT cells have been studied, whereas only a few of the NKT inhibitory lipids have been studied. Earlier studies demonstrated that neutral glycolipid, Gg3Cer shed from a tumor cell line can inhibit CD1d1-mediated Ag presentation to NKT cells (47). Di-palmitoyl-phosphatidyl-ethanolamine (48) covalently attached to polyethylene glycol acts as an antagonist to inhibit the α-GalCer-induced phosphorylation of ERK tyrosine kinase in NKT cells, thus blocking NKT TCR signaling and activation of NKT cells. However, under physiological conditions these lipid complex are not detected in vivo, and mechanism(s) underlying how the anergic-like state in liver NKT cells is maintained is not known. Data presented in this study suggest that intestinal mucus derived PGE2 carried by exosome-like nanoparticle-mediated can induce the NKT cell anergy-like state in mouse models. This finding opens up a new avenue to further study whether other intestinal-related Ags carried by intestinal mucus derived exosome-like nanoparticles play a role in induction of immune tolerance.

Although the role of IDEN associated PGE2 in the induction of the NKT cell anergic-like state are addressed in this study, we speculate that this finding may also be applied to other types of cells that express PGE2 receptors. A large body of evidence suggests that PGE2 receptors are expressed on not only NKT, but also CD4 T cells (49) and other types of cells (50) as well. However, if those cells do not have the in vivo capacity of taking up IDENs, PGE2 carried by IDENs may not have an effect on these cells even if these cells are PGE2 receptor positive.

In summary, to our knowledge, this is the first study to demonstrate a PGE2 role in inducing NKT cell anergy. Our data support a model in which the PGE2 carried by IDENs is taken up by Ag presenting cells or NKT cells of the liver and then initiates a unique suppressive mechanism. This mechanism does not require recognition of lipid Ags. In addition, the IDENs work as a functional bridge between the intestinal and liver immune systems. We propose that this mechanism of suppression is a major pathway that counterbalances intestinal lipid-mediated activation of NKT cells in the liver.

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Disclosures
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References


Corrections


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Supplementary figure legends

Supplementary Figure 1 Quantities of IFN-γ and IL-4 in supernatants from sorted liver NKT cells cultured for 3 h with BMDCs in the presence of IDENs or total liposomes isolated from IDENs and then stimulated by α-GalCer (100ng/mL) for 24 h. *P < 0.05 and **P < 0.01 (one-tailed unpaired Student’s t-test). Data are the mean ± SEM. of 3 experiments (n=5).

Supplementary Figure 2 Real-time RT-PCR analysis of expression of genes encoding EP1, EP2, EP3, and EP4 on sorted liver NKT cells stimulated with α-GalCer tetramer for 5 h. **P < 0.01 (Student’s t-test). Data are the mean ± SEM of at least 3 experiments (n=5).
**Supplementary Figure 3** Release of IL-2 from cells of the Vα14i NKT cell hybridoma 1.2 stimulated with α-GalCer tetramer for 24h in the presence of serially diluted PGE2. *P < 0.05 and **P < 0.01 (Student’s t-test). Data are the mean ± SEM of at least 4 experiments (n=5).
### Supplemental table 1. Primers used for Real-time PCR

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<th>Forward primer</th>
<th>Reverse primer</th>
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