Osteopontin regulates development and function of invariant natural killer T cells

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Invariant natural killer T (iNKT) cells belong to a subset of lymphocytes bridging innate and acquired immunity. We demonstrated that osteopontin (OPN) is involved in the activation of iNKT cells. In the present work, we examined whether OPN affects development and function of iNKT cells. We found that the number of peripheral iNKT cells was significantly reduced in OPN-deficient mice compared with wild-type mice. Although the number of thymic iNKT cells was not different between WT and OPN-deficient mice, intrathymic iNKT cell maturation was impaired in OPN-deficient mice. iNKT cell function was also significantly altered in OPN-deficient mice, as evidenced by (i) deficient down-regulation of iNKT cell receptor, (ii) reduction of IL-4 production while preserving production of IFN- γ , and (iii) reduction of Fas ligand (FasL) expression, leading to reduced Fas/FasL-dependent cytotoxicity against hepatocytes. Importantly, activation of the transcription factors NFAT2 (nuclear factor of activated T cells 2) and GATA-3 was impaired, whereas activation of T-bet was preserved in iNKT cells of OPN-deficient mice. These data collectively indicate that OPN plays a pivotal role not only in the development, but also in the function of iNKT cells.

nuclear factor of activated T cells (NFAT) | GATA-3 | Fas ligand (FasL)

Invariant natural killer T (iNKT) cells constitute a subset of T cells and specifically recognize glycolipid antigens presented by CD1d (1), regulating T helper (Th)1 and Th2 immune responses by producing various cytokines (1, 2). iNKT cells are involved in the development of several human diseases such as type I diabetes, multiple sclerosis, and systemic lupus erythematosus (3–5).

It has been shown that specific transcription factors are critical for the development of polarized Th1 and Th2 responses (6) in conventional T cells. The T-bet is a master coordinator of gene expression in T cells and is critical for the development of Th1 T cells (7). T-bet-deficient CD4⁺ T cells exhibit a marked decrease in IFN- γ production. Interestingly, mature iNKT cells cannot be formed in the absence of T-bet (8). GATA-3 is a key transcription factor that controls Th2 cell development and Th2 cytokine production in conventional T cells (9). CD4⁺ T cell numbers are markedly reduced in GATA-3-deficient mice, and *in vitro* differentiated CD4⁺ T cells from these animals have defects in Th2 cytokine production. Similarly, mature iNKT cells are drastically reduced in GATA-3-deficient mice. In addition, GATA-3 mRNA expression correlates with IL-4 expression in iNKT cells (10). STAT6 is critical for the development of Th2 responses in conventional T cells, yet iNKT cells do not require STAT6 for IL-4 production (11). The nuclear factor of activated T cells (NFAT) family is also involved in regulating the Th1/Th2 balance (6). NFAT activation leads to the production of a variety of cytokines in conventional T cells. In contrast, NFAT2 activation leads to the up-regulation of IL-4 expression, but not TNF- α or IFN- γ in NKT cells (12). Thus, T-bet, STAT6, GATA-3, and NFAT2 behave differently between iNKT and conventional T cells.

Osteopontin (OPN) skews T cell differentiation toward Th1 (13). OPN gene expression is regulated by T-bet, and, importantly, T-bet-dependent expression of OPN in T cells is essential for skewing of T cells toward Th1 cell differentiation (14). T-bet is also critical for OPN expression in plasmacytoid dendritic cells (pDC) (15). Intracellular OPN acts as an integral part of the signal transduction machinery in pDC. OPN has been implicated in the pathogenesis of various immunological disorders (16, 17). Therefore, it is important to examine whether OPN affects the development and function of immune cells beside T cells and pDC. In this regard, iNKT cells are involved in the pathogenesis of hepatic injury (18). We demonstrated that OPN-deficient mice were protected from Con A-induced hepatic injury and OPN is critically involved in iNKT cell activation (17). Therefore, we attempted to clarify how OPN affects the development and function of iNKT cells. Here, we show that iNKT cell number in the liver and spleen of OPN-deficient mice, compared with WT mice, is significantly reduced. Although the thymic iNKT cell number was not different between wild-type (WT) and OPN-deficient mice, intrathymic development of iNKT cells was blocked in OPN-deficient mice. iNKT cell function was also significantly altered in OPN-deficient mice, as evidenced by (i) deficient down-regulation of iNKT cell receptor upon activation; (ii) reduction of IL-4 secretion and production, while preserving production of IFN- γ ; and (iii) reduction of Fas ligand (FasL) expression, leading to reduced Fas/FasL-dependent cytotoxicity against hepatocytes. Importantly, activation of the transcription factors NFAT2 and GATA-3 was impaired, whereas activation of T-bet was preserved in iNKT cells of OPN-deficient mice. These data collectively indicate that OPN plays a pivotal role not only in the development but also in the function of iNKT cells.

Results and Discussion

Involvement of OPN in iNKT Cell Development. We isolated iNKT cells from normal WT mice and studied OPN expression at the mRNA level. Resident intrahepatic iNKT cells clearly expressed OPN, whereas iNKT cells from OPN-deficient mice did not (Fig. 1A). OPN is involved in the survival of cells (16) and differentiation and development of dendritic cells (DCs) (19). Thus, we examined whether OPN deficiency impacted the development of iNKT cells. In our previous preliminary examination, we detected only a slight

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Fig. 2. Deficient TCR-mediated signaling in OPN-deficient mice. (A) OPN^{-/-} and WT mice were injected with Con A (10 mg/kg) or PBS, and percentages of NKT cells were analyzed at 2 h after injection. (Left) Cells from liver. (Right) Cells from spleen. Data represent mean $\pm$ SEM, $n = 5$ per group. (B) OPN^{-/-} and WT mice were injected with Con A (10 mg/kg) or PBS, and iNKT cell numbers in liver and spleen were analyzed at the indicated time points. Data represent mean $\pm$ SEM, $n = 5$ per group. (C) Serum cytokine levels were measured after α -GalCer injection at the indicated time points. Data represent mean $\pm$ SEM, $n = 8$ per group. (D) Production of cytokines by iNKT cells from WT or OPN^{-/-} mice. iNKT cells were stimulated for 3 days *in vitro* with α -GalCer. Cytokine production in the culture supernatant was measured by ELISA. Data represent mean $\pm$ SEM of three independent experiments. *, $P < 0.05$; **, $P < 0.005$; NS, not significant.

A leads to a rapid reduction in iNKT cell numbers, caused by profound down-regulation of iNKT cell receptors (18, 22). Therefore, we used α -GalCer-loaded CD1d-dimer or NK1.1 and TCR β to trace the response of iNKT cells to Con A and examined whether down-regulation of the iNKT cell receptor is impaired in OPN-deficient mice. As shown in Fig. 2A, injection of Con A led to a significant reduction of percentage of iNKT cells in the liver and spleen of WT mice at 2 h. In contrast, such a reduction of percentage of splenic iNKT cells was not observed in OPN-deficient mice. A significant reduction of percentage of hepatic iNKT cells was observed in OPN-deficient mice, but this was much milder compared with WT mice. To characterize the down-regulation of iNKT cells in the presence or absence of OPN further, we monitored the actual number of iNKT cells at various time points after Con A injection. After an initial reduction of iNKT cell number at 24 h, there was a significant increase in iNKT cell number at 48 and 72 h in spleen and liver of WT mice (Fig. 2B). In contrast, although iNKT cell number increased at 48 and 72 h in spleen and liver of OPN-deficient mice, this increase was substantially lower than in WT mice. It should be noted that at 24 h, iNKT cell numbers in OPN-deficient mice were much higher than in WT mice. This finding is explained by the lack of significant receptor down-regulation in OPN-deficient mice. Thus, our data demonstrated that iNKT cell function, as judged by TCR down-regulation, is impaired in OPN-deficient mice. These data led us to hypothesize that other iNKT cell functions might be impaired in the absence of OPN as well.

Impaired Secretion of IL-4 by OPN-Deficient iNKT Cells. One unique feature of iNKT cells is the rapid secretion of cytokines, including IFN- γ and IL-4, upon activation. Cytokine secretion by iNKT cells plays a critical role in the pathogenesis of various diseases (23, 24). iNKT cells produce high levels of cytokines, especially IFN- γ and

IL-4, in response to α -GalCer both *in vivo* and *in vitro* (1). To determine whether deficiency of OPN also affects cytokine secretion by iNKT cells *in vivo*, we injected i.v. α -GalCer into OPN-deficient and WT mice. IL-4 levels reached a peak in the serum of WT mice at 2 h after injection (Fig. 2C). In OPN-deficient mice, IL-4 levels were significantly lower than those in WT mice. In contrast, there was no significant difference in IFN- γ levels between WT and OPN-deficient mice. Nevertheless, neither IL-4 nor IFN- γ was detected in serum of α -GalCer-treated CD1d-deficient (thus, iNKT cell-deficient) mice, indicating that those cytokines are derived from iNKT cells. However, it is possible that other cells were activated by iNKT cell-derived cytokines, leading to the production of IL-4 or IFN- γ . Thus, purified iNKT cells were stimulated *in vitro* with α -GalCer. Consistent with our *in vivo* work, significantly lower amounts of IL-4 were secreted by iNKT cells from OPN-deficient mice than WT mice, but similar levels of IFN- γ were secreted (Fig. 2D). Thus, it appears that the lack of OPN results in reduced IL-4 secretion by iNKT cells. To confirm this finding further, purified iNKT cells were stimulated *in vitro* with α -GalCer in the presence or absence of exogenous recombinant OPN. We found that treatment of iNKT cells of OPN-deficient mice with exogenous OPN resulted in significant augmentation of IL-4, but not IFN- γ production (Fig. S5). Nevertheless, iNKT cells derived from WT mice secreted similar amounts of IL-4 in the absence or presence of exogenous OPN. We then tested whether the lack of OPN simply affected secretion or also production of IL-4. Therefore, purified iNKT cells were stimulated *in vitro* with α -GalCer, and cytokine production was examined by real-time PCR. Results showed impaired production of IL-4 but not IFN- γ in OPN-deficient mice (Fig. S6).

Reduced Cytotoxicity of OPN-Deficient iNKT Cells Against Hepatocytes. iNKT cells exhibit cytotoxicity against hepatocytes in a Fas/FasL-dependent manner in a Con A-induced hepatic injury

Fig. 4. Deficient activation of NFAT2 by iNKT cells in OPN-deficient mice. (A) Western blot analysis of splenic nuclear extracts. Nuclear extracts were prepared from WT and OPN-deficient mice 30 min after α -GalCer injection. Data are representative of three independent experiments. (B and C) Quantitative real-time PCR analyses of gene expression of NFAT2, GATA-3, and T-bet. The relative value of gene expression was normalized against the gene expression levels of G3PDH. Data represent mean $\pm$ SEM of three independent experiments. (D) NFAT2 expression by 1B6 hybridoma cells. 1B6 hybridoma cells were treated with OPN-siRNA and stimulated with α -GalCer and irradiated CD1d-transfectants. IL-4 and IFN- γ production in the culture supernatant was measured by ELISA. (Upper) Data are representative of three independent experiments. (Lower) Data represent mean $\pm$ SEM of three independent experiments. *, $P < 0.05$; **, $P < 0.005$; NS, not significant.

cells is illustrated in Fig. 5. The absence of OPN results in impaired iNKT cell development and defective peripheral iNKT cell activation, as evidenced by deficient receptor down-regulation and IL-4 production. iNKT cell antigen receptor-mediated signaling pathways, such as activation of NFAT2, was also impaired. Reduced IL-4 production caused by defective activation of NFAT2 led to reduced FasL expression on iNKT cells, which resulted in attenuated cytotoxic activity of iNKT cells against Fas-positive hepatocytes. Collectively, our findings have revealed a critical contribution of OPN in the development and function of iNKT cells, which might be exploited for treatment of diseases that are controlled by OPN or iNKT cells.

Materials and Methods

Animals. Specific pathogen-free female C57BL/6 (B6) mice were purchased from Japan SLC. OPN-deficient (OPN^{-/-}) mice (29), backcrossed 11 times to B6 mice at the Institute for Genetic Medicine, Hokkaido University, were used. All mice were maintained under specific pathogen-free conditions and used according to institutional guidelines.

Flow Cytometric Analysis. Hepatic lymphocytes were isolated as described in ref. 17. Liver, spleen, and thymus cells were first incubated with anti-Fc γ R (2.4G2; BD

Biosciences). Cells were stained with a primary antibody (α -GalCer-loaded mouse CD1d-IgG1 fusion protein DimerX) for 60 min at 4°C, followed by a secondary anti-mouse IgG1-phycoerythrin (PE) and anti-TCR β -antigen-presenting cell (H57-597) antibody for 30 min at 4°C. iNKT cells were defined as α -GalCer-loaded CD1d dimer⁺ TCR β ⁺ cells. Apoptosis was assayed by annexinV-FITC apoptosis detection kit I. Other antibodies used for staining were anti-NK1.1-PerCP-Cy5.5 (PK136), anti-FasL-PE (MFL3), anti-Fas-PE (Jo2), anti-CD69-FITC (H1.2F3), and anti-CD44-FITC (IM7) (all from BD Biosciences). All analyses were performed on a FACSCalibur (BD Biosciences) with CellQuest software.

In Vitro and in Vivo iNKT Cell Stimulation. iNKT cells (gated as α -GalCer-loaded CD1d-dimer⁺ TCR β ⁺) were isolated by a FACS Vantage instrument (Becton Dickinson). The sorted iNKT cells were cultured overnight with recombinant IL-2. NKT cells were further stimulated with 100 ng/ml α -GalCer (Pharmaceutical Research Laboratories, Kirin Brewery) in the presence of 100-Gy-irradiated CD11c⁺ DCs. In some experiments, mice were injected i.v. with 100 μ g/kg α -GalCer or vehicle. CD1d-transfected cells were provided by Albert Bendelac (University of Chicago, Chicago, IL).

Analysis of mRNA Expression. Total RNA was isolated by using TRIzol (Invitrogen). The specific primers used were as follows: glyceraldehyde-3-phosphate dehydrogenase (G3PDH), 5'-ACCACAGTCCATGCCATCAC-3' (sense), 5'-TCCACCACCCCT-GTTGCTGA-3' (antisense); Fas, 5'-GAGAATTGCTGAAGACATGACAATCC-3'