European Journal of Immunology

Innate immunity

Basic

Research Article

α-Galactosylceramide-activated murine NK1.1$^+$ invariant-NKT cells in the myometrium induce miscarriages in mice

Tomoko Ichikawa[*1,2], *Yasuyuki Negishi*[*1,2], *Masumi Shimizu*[1], *Toshiyuki Takeshita*[2] *and Hidemi Takahashi*[1]

[1] Department of Microbiology and Immunology, Nippon Medical School, Tokyo, Japan
[2] Department of Obstetrics and Gynecology, Nippon Medical School, Tokyo, Japan

Innate immunity, which is unable to discriminate self from allo-antigens, is thought to be important players in the induction of miscarriages. Here, we show that the administration of IL-12 to syngeneic-mated C57BL/6 mice on gestation day 7.5 (Gd 7.5), drives significant miscarriages in pregnant females. Furthermore, the administration on Gd 7.5 of α-galactosylceramide (α-GalCer), which is known to activate invariant natural killer T (iNKT) cells, induced miscarriages in both syngeneic-mated C57BL/6 mice and allogeneic-mated mice (C57BL/6 (♀) × BALB/c (♂)). Surprisingly, the percentages of both DEC-205$^+$ DCs and CD1d-restricted NK1.1$^+$ iNKT cells were higher in the myometrium of pregnant mice treated i.p. with α-GalCer than in the decidua. IL-12 secreted from α-GalCer-activated DEC-205$^+$ DCs stimulated the secretion of cytokines, including IL-2, IL-4, IFN-γ, TNF-α, perforin, and granzyme B, from the NK1.1$^+$ iNKT cells in the myometrium, leading to fetal loss in pregnant mice. Finally, the i.p. administration of IL-12 and/or α-GalCer in iNKT-deficient Jα18(-/-) (Jα18 KO) mice did not induce miscarriages. This study provides a new perspective on the importance of the myometrium, rather than the decidua, in regulating pregnancy and a mechanism of miscarriage mediated by activated DEC-205$^+$ DCs and NK1.1$^+$ iNKT cells in the myometrium of pregnant mice.

Keywords: DC · DEC-205 · α-Galactosylceramide · Invariant NKT cell · Myometrium

Additional supporting information may be found in the online version of this article at the publisher's web-site

Introduction

Although the fetus contains paternal semialloantigen genes, it is protected from the maternal immune system such that the encoded antigens are tolerated during pregnancy [1]. From this point of view, the phenomenon of delivery can be considered a maternal rejection of the semialloantigeneic substances expressed by the fetus. In general, such rejected materials are typically broken into necrotic fragments; nevertheless, newborns are usually delivered complete and undamaged. Thus, delivery seems to not be initiated by immune rejection; rather, it is a naturally induced physiological phenomenon. Indeed, complete and undamaged deliveries are also observed in cases of murine syngeneic mating, in which the same genes are found in the fetuses and mothers and rejection cannot be initiated between them.

Pregnancy begins with implantation, followed by fetal growth under specific mechanisms for the maintenance of pregnancy and ending with a delivery. Using murine syngeneic mating, we

Correspondence: Dr. Hidemi Takahashi
e-mail: htkuhkai@nms.ac.jp

*These authors contributed equally to this work.

recently reported that these reproductive steps seem to be mainly regulated by two distinct types of innate DCs: 33D1 (DCIR2)$^+$ and DEC-205$^+$ [2]. The 33D1$^+$ DCs induce the maintenance of Th2 dominance, whereas the DEC-205$^+$ DCs establish Th1 polarization in the mouse. Interruption of Th2 dominance by the depletion of 33D1$^+$ DCs during pregnancy results in profound spontaneous abortion [2]. From these findings, we speculate that the balance between DC subsets during pregnancy may regulate DC-mediated innate immunity rather than acquired immunity, which initiates rejection.

Innate immunity, which is unable to discriminate self from alloantigens, and NK cells are thought to be important players in the induction of miscarriages [3]. Such miscarriages can be induced in pregnant mice via the i.p. injection of IL-12 [2]. Because IL-12 is normally produced by DEC-205$^+$ DCs and invariant natural killer T (iNKT) cells and NK cells in the innate arm express the IL-12 receptor [4], both NK cells and iNKT cells can be activated quickly by externally applied IL-12 [4, 5]. The activated innate effectors may elicit miscarriages even in a syngeneic mating combination.

These iNKT cells are mainly characterized by their TCRs, which comprise a Vα14Jα18 chain and a limited Vβ chain repertoire (Vβ2, Vβ7, Vβ8.1, Vβ8.2, or Vβ8.3) in mice [6] and that recognize glycosphingolipid antigens presented by the CD1d molecule, a nonpolymorphic MHC class I-like molecule [7–9]. iNKT cells rapidly produce a variety of cytokines, including IFN-γ, IL-4, and TNF-α [10, 11], upon stimulation with their specific ligand, α-galactosylceramide (α-GalCer) [7]. When activated, they exert their effector functions within hours. Numerous lines of evidence have indicated that miscarriages can be induced by any of the various triggers of iNKT cell activation [12]. Indeed, the exogenous and endogenous glycolipids presented by pathogenic bacteria such as *Streptococcus pneumoniae* and *Sphingomonas paucimobilis* are known to contain CD1d-restricted ligands that can bind iNKT cells [13]. Therefore, understanding the effects of iNKT cell activation by α-GalCer in vivo may aid in clarifying the mechanisms of miscarriage.

In this study, we examined the mechanisms of miscarriages induced by the i.p. administration of α-GalCer on Gd 7.5 to both syngeneic-mated pregnant C57BL/6 (B6) mice and allogeneic-mated pregnant mice (B6 (♀) × BALB/c (♂)). We found that the activation of DEC-205$^+$ DCs initiated the accumulation of NK1.1$^+$ iNKT cells in the myometrium, but not in the decidua or placenta, of pregnant mice. Moreover, when the pregnant mice were inoculated with NK1.1$^+$ iNKT cells obtained from the myometrium of pregnant mice pretreated with α-GalCer inoculation, the rate of miscarriage increased. Furthermore, we confirmed that in iNKT-deficient Jα18 KO mice, fetal loss was not induced by the i.p administration of IL-12 and/or α-GalCer. These findings indicate that the activation of DEC-205$^+$ DCs in the myometrium via mechanisms such as infection or immune disorders provokes fetal loss through the efficient induction of NK1.1$^+$ iNKT cells in pregnant mice. This study may offer a new perspective on the importance of the myometrium, rather than the decidua or placenta, of pregnant mice in the regulation of pregnancy as well as a mechanism of miscarriage mediated by innate immunity.

Results

I.p. IL-12 or α-GalCer administration induces fetal loss in mice

We recently reported that two i.p. injections of IL-12 (IL-12p70; 0.2 μg/mouse) on day 9.5 of gestation (Gd 9.5) and Gd 10.5 induced miscarriages in syngeneic (BALB/c (♀) × BALB/c (♂)) pregnant mice [2]. Based on these observations, the miscarriage rate in syngeneic (B6 (♀) × B6 (♂)) pregnant mice administered IL-12 (0.2 μg/mouse) was determined. A higher percentage of fetal loss was observed after treatment with a single i.p. injection of IL-12 (0.2 μg/mouse) on Gd 7.5 than on Gd 9.5 (Fig. 1A). A single injection of IL-12 on Gd 7.5 did not induce fetal loss in the syngeneic (BALB/c (♀) × BALB/c (♂)) pregnant mice (Y. Negishi & H. Takahashi, unpubl. obs.), suggesting that syngeneic-mated pregnant B6 mice were more vulnerable to IL-12 administration than syngeneic-mated pregnant BALB/c mice.

IL-12 is generally produced by innate DCs, and both iNKT cells and NK cells expressing the IL-12 receptor can be activated by externally added IL-12 [4, 5]. Moreover, it has been reported that 0.2–4 μg of α-GalCer can activate iNKT cells [4, 10], and these α-GalCer-activated iNKT cells provoked miscarriages in syngeneic-mated pregnant B6 mice [12, 14]. Based on these findings, we administered various amounts of α-GalCer i.p. to pregnant B6 mice on Gd 7.5 and compared the results with those after administering 0.2 μg of IL-12p70 (Fig. 1B). The rate of fetal loss in mice given 0.2 μg of α-GalCer was almost the same as in the vehicle control-treated group; however, the rate was significantly enhanced in mice injected with 2 or 20 μg of α-GalCer. These results suggest that α-GalCer caused fetal loss in a dose-dependent manner. Nevertheless, it was difficult to accurately evaluate the number of abortions following inoculation with 20 μg of α-GalCer because the abortuses appeared necrotic and self-digested. Thus, we used 2 μg of α-GalCer for further experiments to avoid miscalculating the number of abortions.

It should be noted that the percentage of miscarriages induced by inoculation with 2 μg of α-GalCer was significantly higher than that induced by the administration of 0.2 μg of IL-12p70 (Fig. 1C). Additionally, we confirmed that Gd 7.5 was the optimal Gd for inducing miscarriages in pregnant B6 mice with 2 μg of α-GalCer (Fig. 1D). Moreover, very similar results were obtained in cases of allogeneic-mated pregnant mice (B6 (♀) x BALB/c (♂)) i.p. administered 2 μg of α-GalCer on Gd 7.5 (Fig. 1E). However, in the case of syngeneic-mated pregnant iNKT cell-deficient Jα18 KO mice [15], the miscarriages were hardly detected when the mice were i.p. inoculated with 2 μg of α-GalCer, 0.2 μg of IL-12p70, or α-GalCer plus IL-12p70 (Fig. 1F).

These results indicate that iNKT cells activated by the i.p. administration of either 2 μg of α-GalCer and/or 0.2 μg of IL-12 can cause miscarriages not only in syngeneic-mated but also in allogeneic-mated pregnant B6 mice.

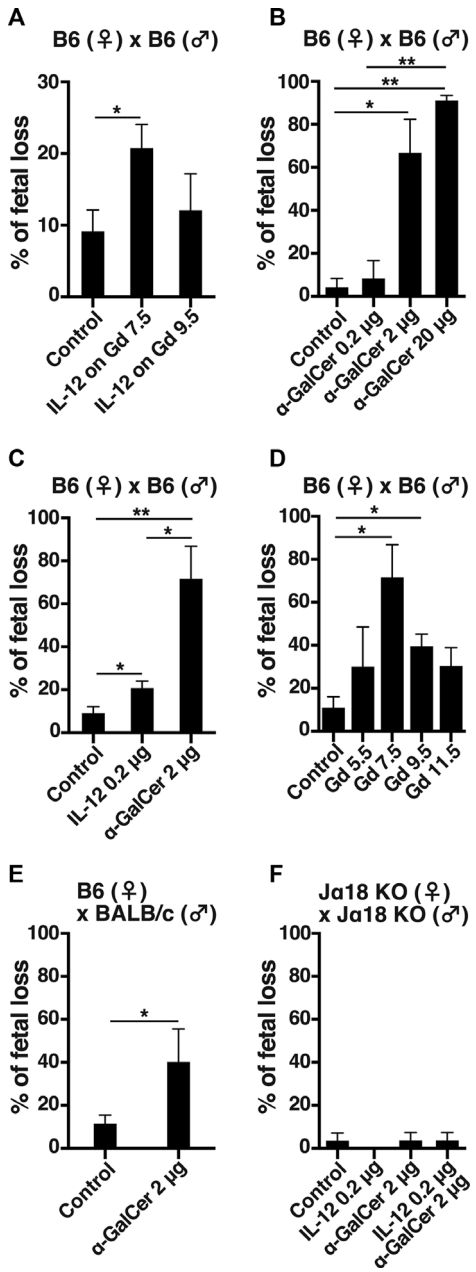

Figure 1. IL-12 or α-GalCer treatment induces fetal loss in pregnant mice. (A) Syngeneic-mated pregnant B6 (♀ × ♂) mice were administered recombinant IL-12p70 i.p. on Gd 7.5 or 9.5. Miscarriage was identified macroscopically as a dark, small fetus on Gd 10.5 (3 days after IL-12p70 or α-GalCer injection). (B) Induction of miscarriage in syngeneic-mated pregnant B6 mice by i.p. administration of various concentrations of α-GalCer on Gd 7.5. (C) Induction of miscarriage in syngeneic-mated pregnant B6 mice by i.p. administration of IL-12 (0.2 μg/mouse) or α-GalCer (2.0 μg/mouse) on Gd 7.5. (D) Induction of miscarriage in syngeneic-mated pregnant B6 mice by i.p. administration of α-GalCer (2.0 μg/mouse) on each Gd. (E) Induction of miscarriage in allogeneic-mated pregnant B6 (♀) X BALB/c (♂) mice by i.p. administration of α-GalCer (2.0 μg/mouse) on Gd 7.5. (F) Induction of miscarriage in syngeneic-mated pregnant Jα18 KO (♀ × ♂) mice by i.p. administration of α-GalCer (2.0 μg/mouse) and/or IL-12 (0.2 μg/mouse) on Gd 7.5. The control mice were i.p. inoculated with 100 μL of 0.025% polysolvate-20 in PBS. The data shown are the mean + SEM (six mice per group) and are pooled from three independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$; Mann–Whitney *U* test.

I.p. α-GalCer administration during pregnancy increases NK1.1$^+$ iNKT-cell percentages

To examine the distribution of iNKT cells activated by i.p. α-GalCer administration, cells were harvested from the spleen, myometrium, decidua, and placenta of pregnant mice on Gd 10.5 (3 days after α-GalCer injection). In general, iNKT cells can be divided into NK1.1$^+$ iNKT and NK1.1$^-$ iNKT cell subsets. Here, as indicated in Supporting Information Fig. 1, the percentage of live NK1.1$^+$ iNKT cells was determined by counting the α-GalCer-loaded CD1d tetramer$^+$ cells in the spleen, myometrium, decidua, and placenta. To avoid cells with nonspecific binding, empty CD1d-tetramer was used (Supporting Information Fig. 2).

To our surprise, the percentage of NK1.1$^+$ iNKT cells was significantly increased in the myometrium (25.8%) and was far higher than the percentage of those in the decidua (7.56%) and placenta (6.03%) (Fig. 2A). The percentage of α-GalCer-loaded CD1d tetramer$^+$ iNKT cells among the CD3$^+$ T cells was also determined in both syngeneic-mated pregnant mice (B6 (♀) x B6 (♂)) (Fig. 2B) and allogeneic-mated pregnant mice (B6 (♀) x BALB/c (♂)) i.p. administered 2 μg of α-GalCer on Gd 7.5 (Fig. 2C). In those cases, the percentages of iNKT cells were significantly increased in the spleen and myometrium.

The iNKT cell population contains two distinct subsets: one is an NK1.1$^+$ population that produces high levels of IFN-γ and IL-4 but a low level of IL-17 [16] and has the ability to reject tumors via IL-12 secretion [15]; the other is an NK1.1$^-$ population found in an immature state that predominantly produces IL-17 upon stimulation [16]. Thus, to determine which types of iNKT cells were present in the myometrium and spleen, the cells were stained with both α-GalCer-loaded CD1d-tetramer and anti-NK1.1 mAb and were analyzed by flow cytometry. After the administration of α-GalCer to pregnant mice, the percentage of NK1.1$^+$ iNKT cells was significantly increased in the myometrium but not in the spleen, whereas the percentage of NK1.1$^-$ iNKT cells was strongly increased in the spleen (Fig. 2A). Taken together, these findings indicate that NK1.1$^+$ iNKT cells with the capacity to eliminate foreign bodies via IFN-γ secretion were predominantly increased in the myometrium but not in the spleen of pregnant mice after α-GalCer inoculation.

Effects of α-GalCer treatment on iNKT cells and CD11c$^+$ DCs in the myometrium and spleen

We previously reported that the depletion of 33D1$^+$ DCs in syngeneic-mated pregnant BALB/c mice significantly induced spontaneous abortion during the perinatal period, which was likely mediated by Th1 augmentation by DEC-205$^+$ DCs [2]. It was also recently reported that α-GalCer is selectively captured by CD8α$^+$ DEC-205$^+$ DCs [17]. Based on these findings, we examined the effects of i.p. α-GalCer administration on iNKT cells and CD11c$^+$ DCs both in syngeneic-mated pregnant B6 mice on Gd 7.5 and in nonpregnant mice. Three days after the inoculation, the numbers of iNKT cells in the myometrium and spleen of both

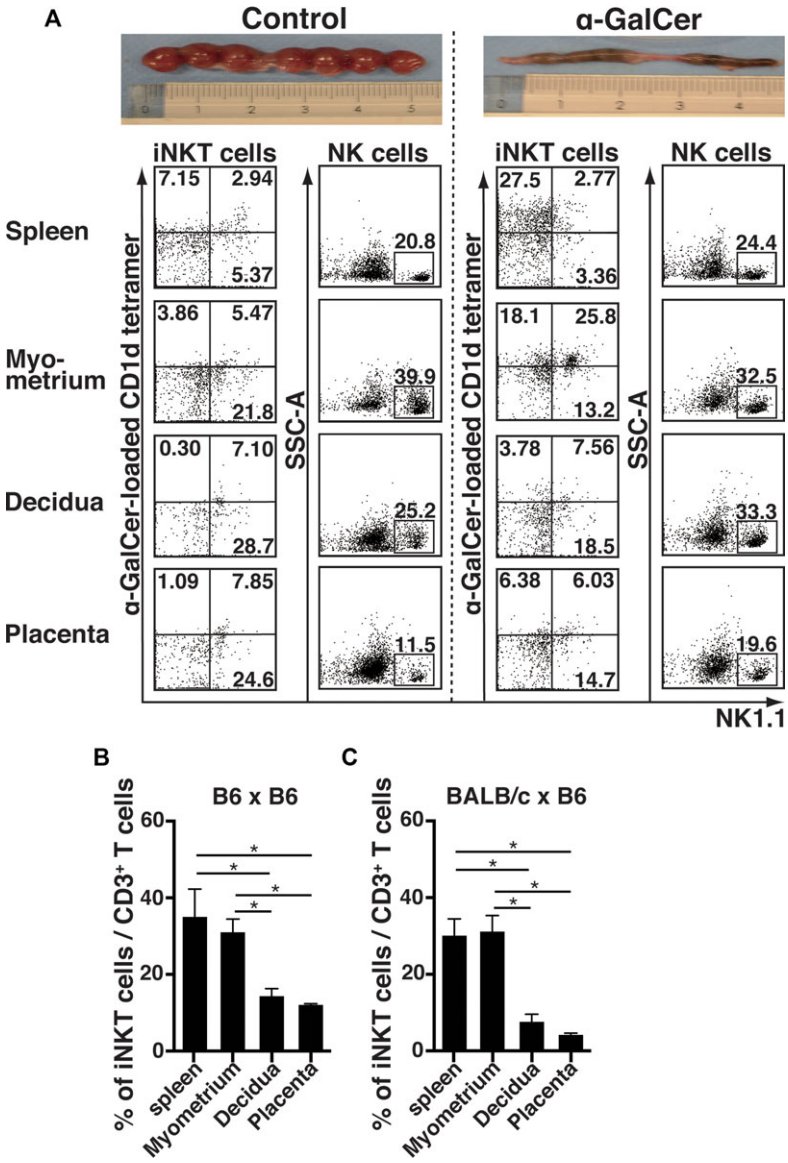

Figure 2. Distribution of iNKT cells in the spleen, myometrium, decidua, and placenta of pregnant mice administered with α-GalCer on Gd 7.5. (A) Typical microscopic appearance of a pregnant uterus of syngeneic-mated pregnant B6 (♀ × ♂) mice on Gd 10.5 (top). Flow cytometric analysis of iNKT cells and NK cells in the spleen, myometrium, decidua, and placenta of pregnant mice treated with α-GalCer or vehicle (bottom). The gating strategies are shown in Supporting Information Figs. 1 and 2. (B) Percentages of iNKT cells among $CD3^+$ T cells in the spleen, myometrium, decidua, and placenta of syngeneic-mated pregnant B6 (♀ × ♂) mice, and (C) of allogeneic-mated pregnant B6 (♀) × BALB/c (♂) mice, as determined by flow cytometry data. (B and C) The data shown are the mean + SEM (six mice per group) and are pooled from three independent experiments. $^*p < 0.05$; Mann–Whitney *U* test.

nonpregnant and pregnant mice had significantly increased (Fig. 3A, left side and center of each panel). Next, we examined the effects of α-GalCer inoculation on DEC-205^+ DCs and $33D1^+$ DCs in the myometrium and spleen. In nonpregnant B6 mice, the number of DEC-205^+ DCs was markedly increased in the myometrium and spleen (Fig. 3B, left side of each panel) after α-GalCer inoculation. Similarly, in pregnant B6 mice, the number of DEC-205^+ DCs increased in both the myometrium and the spleen (Fig. 3B, center of each panel). However, the number of $33D1^+$ DCs was almost unchanged in the myometrium and the spleen after the administration of α-GalCer to both nonpregnant and pregnant B6 mice (Fig. 3C). In addition, changes in the numbers of DCs and iNKT cells in pregnant Jα18 KO mice on a B6 background were examined. As expected, iNKT cells were not detected in the myometrium or the spleen of pregnant Jα18 KO mice (Fig. 3A, right side of each panel). Nonetheless, the number of DEC-205^+ DCs was slightly elevated in the myometrium but was almost unchanged in the spleen of pregnant Jα18 KO mice in response to α-GalCer (Fig. 3B, right side of each panel). Additionally, the number of $33D1^+$ DCs was not altered in response to α-GalCer in the myometrium or the spleen of pregnant Jα18 KO mice (Fig. 3C, right side of each panel).

Next, because the majority of DCs in the myometrium of syngeneic-mated pregnant B6 and pregnant Jα18 KO mice were DEC-205^+ DCs rather than $33D1^+$ DCs (Fig. 3B, C), changes in the expression of costimulation markers (CD40, CD80, and CD86) on DEC-205^+ DCs in the myometrium and spleen of the pregnant mice were examined. The expression levels of CD40, CD80, and CD86 increased significantly in the myometrium of both pregnant B6 and Jα18 KO mice (Fig. 3D, left panel). However, the expression of these costimulation markers on DEC-205^+ DCs in the spleen was also enhanced by the injection of α-GalCer in pregnant B6 mice, but not in Jα18 KO mice (Fig. 3D, right panel). These results indicated that DEC-205^+ DCs in the Jα18 KO mice

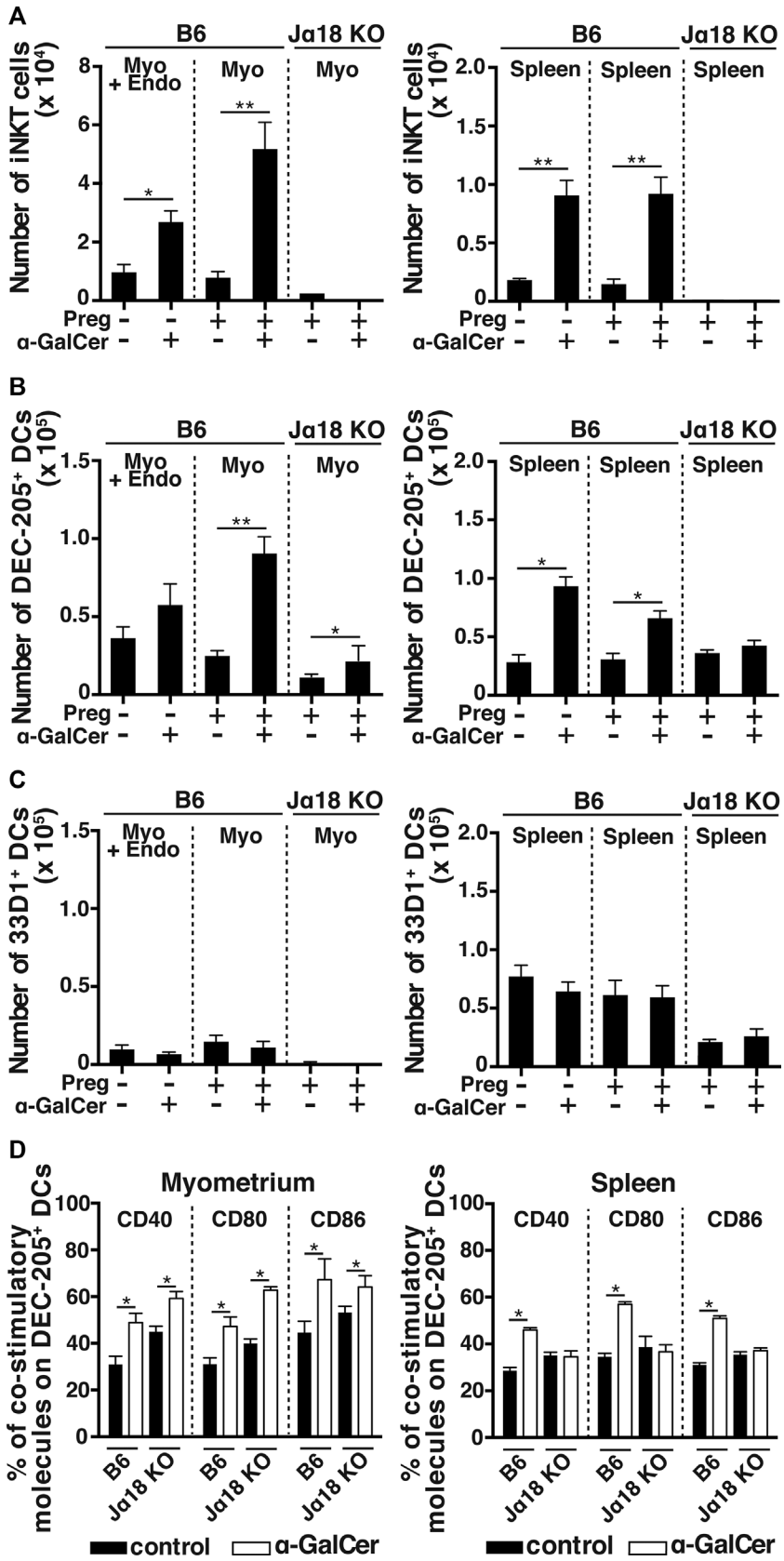

Figure 3. Kinetics of iNKT cells and DCs administered i.p. with α-GalCer as well as on Gd7.5 in nonpregnant B6, syngeneic-mated pregnant B6 (♀ × ♂), and pregnant Jα18 KO mice. (A) Numbers of iNKT cells in the myometrium (left) and spleen (right). (B) Numbers of DEC-205$^+$ DCs in the myometrium (left) and spleen (right). (C) Numbers of 33D1$^+$ DCs in the myometrium (left) and spleen (right). In the case of nonpregnant mice, the cells in the myometrium included endometrial cells since the myometrium and endometrium could not be separated. Also, because very few iNKT cells were found in the myometrium of nonpregnant mice, we examined cells from the pooled myometria of four nonpregnant mice. (D) Expression of costimulatory molecules (CD40, CD80, and CD86) on DEC-205$^+$ DCs in the myometrium (left panel) and spleen (right panel) of pregnant mice. The closed bars indicate the costimulatory molecules on unstimulated control DEC-205$^+$ DCs and the open bars indicate those on α-GalCer-stimulated DEC-205$^+$ DCs. (A, B, C) The data shown are the mean + SEM (eight mice per group) and are pooled from four independent experiments. $^*p < 0.05$, $^{**}p < 0.01$; Mann–Whitney *U* test. (D) The data shown are the mean + SEM (five mice per group) and are pooled from four independent experiments. $^*p < 0.05$; Mann–Whitney *U* test.

could be activated by α-GalCer without help from iNKT cells in the myometrium but not in the spleen.

In summary, the numbers of iNKT cells and DEC-205$^+$ DCs were significantly increased, particularly in the myometrium of pregnant mice, whereas 33D1$^+$ DCs were almost unaffected by α-GalCer administration. The increased numbers of iNKT cells and DEC-205$^+$ DCs in the myometrium seemed to initiate miscarriage.

α-GalCer selectively activates DEC-205$^+$ DCs and induces iNKT cells in the myometrium via IL-12

As shown in Fig. 3, the numbers of iNKT cells and DEC-205$^+$ DCs increased, whereas the number of 33D1$^+$ DCs tended to decrease following the injection of α-GalCer in vivo. A number of previous in vitro studies have demonstrated that the proliferation of iNKT cells is stimulated in the presence of α-GalCer [10, 18]. However, few articles have described the effects of α-GalCer on DEC-205$^+$ DCs and 33D1$^+$ DCs [17]. Thus, we attempted to confirm whether α-GalCer could stimulate the differentiation of DCs in vitro. Murine bone marrow-derived DCs (BMDCs) activated by GM-CSF were incubated for 3 days in the presence of various doses of α-GalCer and were analyzed for the surface expression of DEC-205 and 33D1 by flow cytometry.

When BMDCs obtained from either wild-type B6 or Jα18 KO mice were stimulated with 5 μg/mL α-GalCer for 3 days, a significant increase in the number of DEC-205$^+$ DCs (Fig. 4A, left panel) and an apparent decrease in the number of 33D1$^+$ DCs were observed (Fig. 4A, right panel). These findings indicate that DC differentiation can be regulated by α-GalCer. In addition, intracellular IL-12 (IL-12p40) production by α-GalCer-stimulated DEC-205$^+$ DCs and 33D1$^+$ DCs was examined. The levels of intracellular IL-12 production by DEC-205$^+$ BMDCs from B6 mice incubated for 24 h in the presence of 5 μg/mL α-GalCer was markedly elevated (Fig. 4B, left side of left panel), whereas the IL-12 levels did not increase in 33D1$^+$ DCs (Fig. 4B, left side of right panel).

In contrast, the level of intracellular IL-12 production by DEC-205$^+$ or 33D1$^+$ BMDCs from Jα18 KO mice did not increase following stimulation with α-GalCer in vitro (Fig. 4B, right side of both panels). Nevertheless, when DCs obtained from the myometrium of pregnant Jα18 KO mice were stimulated with α-GalCer, they produced a significant amount of IL-12 compared with unstimulated DCs, although splenic DCs from Jα18 KO mice did not produce increased amounts of IL-12 (Fig. 4C). These results strongly suggest that DCs in the myometrium during pregnancy may produce IL-12 in response to α-GalCer without iNKT cells. Therefore, although it was recently reported that DCs could not be activated by α-GalCer alone [17], DCs in the myometrium of pregnant mice could produce IL-12 by themselves through α-GalCer stimulation without a help of iNKT cells. Collectively, these results suggest that DEC-205$^+$ DCs can be selectively activated by treatment with α-GalCer and that IL-12 secreted from α-GalCer-activated DEC-205$^+$ DCs may stimulate iNKT cells,

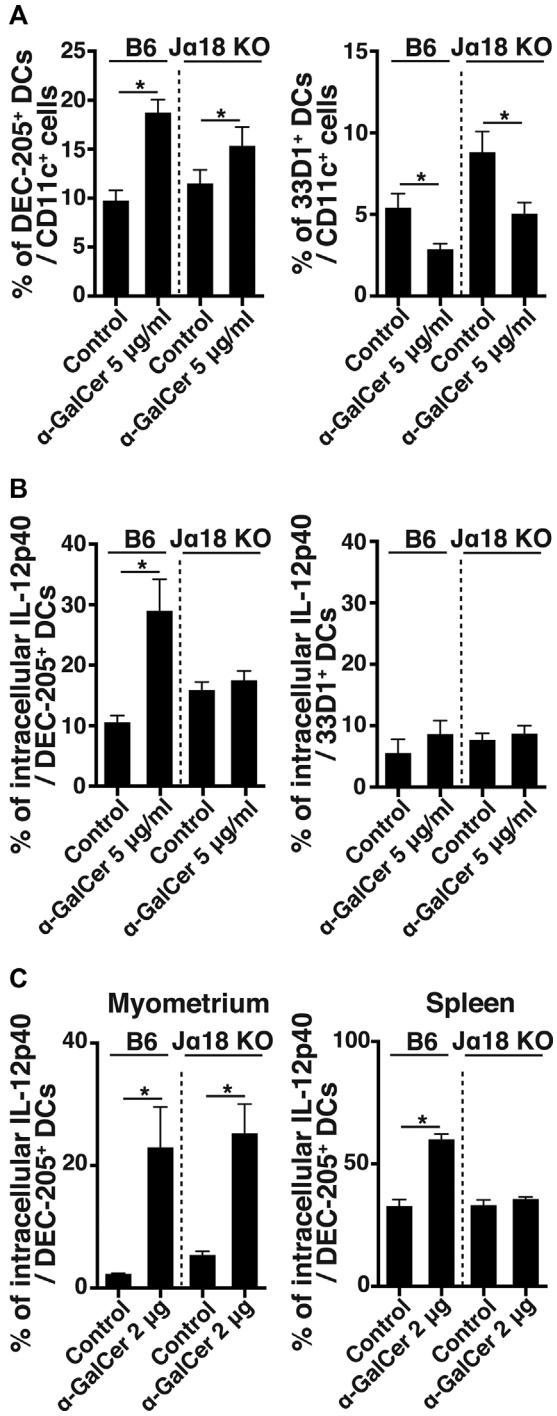

Figure 4. Differentiation and activation of DCs by α-GalCer stimulation in B6 and Jα18 KO mice. (A) Percentages of DEC-205$^+$ DCs (left) and 33D1$^+$ DCs (right) derived from bone marrow cells cocultured with α-GalCer or DMSO as a vehicle control evaluated by flow cytometry. (B) Intracellular IL-12 production in DEC-205$^+$ DCs (left) and 33D1$^+$ DCs (right) derived from bone marrow cells. (C) Intracellular IL-12 production in DEC-205$^+$ DCs in the myometrium (left) and spleen (right) of pregnant mice. The data shown are the mean + SEM (eight mice per group) and are pooled from four independent experiments. *$p < 0.05$; Mann–Whitney U test.

leading to fetal loss in pregnant mice. Moreover, the reduction in 33D1$^+$ DCs might establish Th1 dominance and provoke miscarriages.

Two distinct types of iNKT cells are established in the myometrium and spleen during pregnancy

As indicated in the right panel of Fig. 2A, two distinct types of iNKT cells were present during pregnancy following the administration of α-GalCer: NK1.1$^+$ iNKT cells, which were predominantly observed in the myometrium, and NK1.1$^-$ iNKT cells, which were predominantly present in the spleen. As previously reported, NK1.1$^+$ iNKT cells showed anti-tumor activity when stimulated with IL-12 [15]; thus, NK1.1$^+$ iNKT cells in the myometrium may have the capacity to initiate miscarriage. Additionally, these iNKT cells could secrete various cytokines, such as IFN-γ and TNF-α, upon stimulation with their glycosphingolipid ligands [11], and these cytokines might initiate fetal loss [19]. Thus, we compared the levels of various intracellular cytokines, such as IL-2, IL-4, IFN-γ, TNF-α, perforin, and granzyme B, in α-GalCer-loaded CD1d tetramer$^+$ iNKT cells from the myometrium and spleen. The levels of these intracellular cytokines produced in iNKT cells were significantly increased in the myometrium compared to the spleen (Fig. 5A). These differences indicate that iNKT cells in the myometrium were more strongly activated to secrete various cytokines than those in the spleen and those miscarriages were induced by the activated NK1.1$^+$ iNKT cells in the myometrium.

To confirm the functional differences between iNKT cells in the myometrium and the spleen regarding the induction of fetal loss, we performed adoptive transfer experiments in pregnant mice. The cells for transfer were obtained from either the myometrium or the spleen of pregnant mice administered α-GalCer on Gd 7.5. The obtained cells were administered i.v. to other pregnant mice on Gd 7.5. A majority of the transferred iNKT cells purified from the myometrium were NK1.1$^+$ iNKT cells, whereas those obtained from the spleen were NK1.1$^-$ iNKT cells (Fig. 5B). The rate of fetal loss in pregnant mice administered myometrium-derived iNKT cells was significantly higher than in those given splenic iNKT cells (Fig. 5C). These results indicate that fetal loss was induced more strongly by myometrium-derived NK1.1$^+$ iNKT cells than by splenic NK1.1$^-$ iNKT cells. Finally, we examined the possibility that the activated DEC-205$^+$ DCs produced NK1.1$^+$ iNKT cells. Using a nylon-wool column, we purified splenic T cells containing iNKT cell precursors (pre-iNKT cells) and incubated them with either DEC-205$^+$ DCs or 33D1$^+$ DCs in the presence of α-GalCer. As shown in Fig. 5D, a higher number of NK1.1$^+$ iNKT cells was observed in cultures of pre-iNKT cells incubated with DEC-205$^+$ DCs than with 33D1$^+$ DCs. Taken together, these results indicate that α-GalCer-activated DEC-205$^+$ DCs induced NK1.1$^+$ iNKT cells in the myometrium more efficiently and provoked fetal loss in pregnant mice.

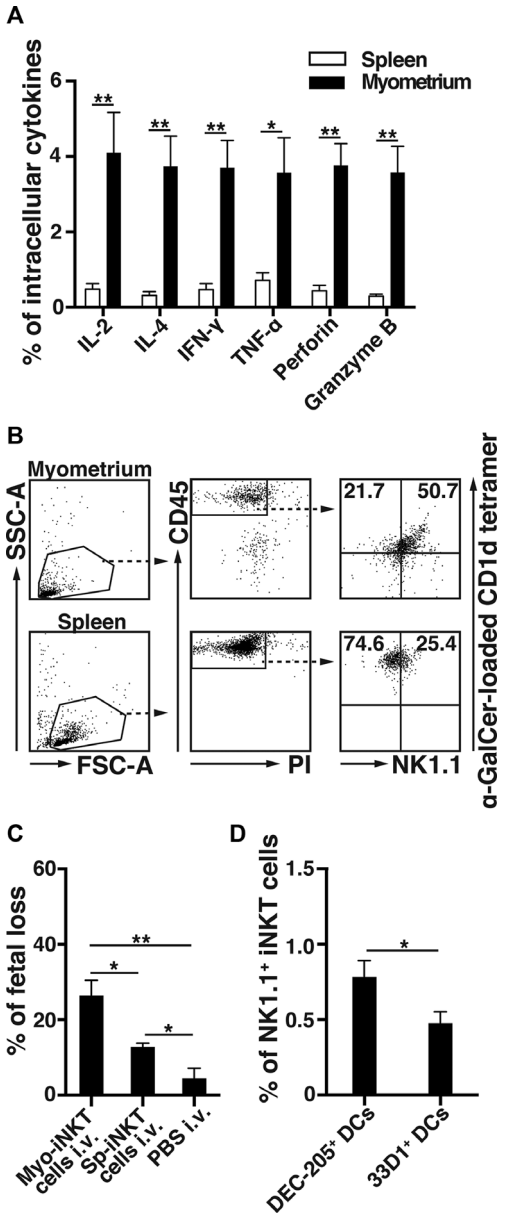

Figure 5. Functional differences to induce fetal loss between iNKT cells in the myometrium and those in the spleen of syngeneic-mated pregnant B6 mice preadministered α-GalCer. (A) Levels of intracellular cytokine production in iNKT cells from the myometrium (closed bars) and the spleen (open bars). α-GalCer was i.p. administered on Gd 7.5, and the mice were euthanized on Gd 10.5. The cells were collected and analyzed by flow cytometry. The data shown are the mean + SEM (six mice per group) and are pooled from three independent experiments. $^*p < 0.05$, $^{**}p < 0.01$; Mann–Whitney *U* test. (B) iNKT cells were obtained on Gd 7.5 from the myometrium or spleen of pregnant mice stimulated with α-GalCer using α-GalCer-loaded CD1d tetramer-conjugated MicroBeads and the cells were transferred to other pregnant mice on Gd 7.5. (C) The rate of fetal loss in pregnant mice on Gd 7.5 induced by adoptive cell transfer. (D) Percentages of NK1.1$^+$ iNKT cells induced by coculture with DEC-205$^+$ or 33D1$^+$ BMDCs and purified splenic T cells containing pre-iNKT cells in the presence of 5 μg/mL α-GalCer. The cells were collected and analyzed by flow cytometry. (C and D) The data shown are the mean + SEM (six mice per group) and are pooled from three independent experiments. $^*p < 0.05$, $^{**}p < 0.01$; Mann–Whitney *U* test.

Discussion

In this study, we found that stimulated NK1.1$^+$ iNKT cells in the myometrium of pregnant mice were selectively activated by α-GalCer in vivo and caused miscarriages via the secretion of various cytokines, such as IL-2, IL-4, IFN-γ, TNF-α, perforin, and granzyme B. The stimulation of NK1.1$^+$ iNKT cells appeared to be induced by the neighboring DEC-205$^+$ DCs, which were also activated by α-GalCer. This result was consistent with a recent report indicating that α-GalCer was captured by CD8α$^+$ DEC-205$^+$ DCs in vivo and that the activated DCs selectively stimulated iNKT cells [17].

Although the critical presence of NK1.1$^+$ iNKT cells in the myometrium for the induction of fetal loss was unexpected, some studies have shown that DCs largely exist in the myometrium rather than in the decidua in pregnant animals. Indeed, it was reported that the number of DEC-205$^+$ DCs in the human decidua during the first trimester was far smaller than the number of macrophages [20], and the major site of DC precursor (pre-DC) growth was the myometrium [21]. This finding may correlate with reported differences in CSF-1 expression levels: there were high levels in the myometrium and low levels in the decidua during pregnancy, and a high level of CSF-1-coupled maintenance of the relatively high DC density in the growing myometrium was reported [22]. In addition, uterine CSF-1 synthesis during gestation is under systemic control and involves ovarian sex steroids, such as progesterone and 17β-estradiol [23]. Additionally, because the decidua has fewer lymphatic vessels, the DCs near the extravillous trophoblasts seem to have difficulty homing to the uterine lymph nodes [24]. Moreover, the levels of CCL21 transcripts during DC migration were low in the decidua but high in the myometrium [25]. Taken together, these results show that the number of DCs is far higher in the myometrium than in the decidua of pregnant mice, and the myometrium appears to be the major site for controlling pregnancy.

Pre-DCs can be stimulated by two distinct cytokines: CSF-1 (M-CSF) and CSF-2 (GM-CSF). CSF-1 stimulates pre-DC extravasation into the myometrium to promote the expansion of DCs and enhance innate immunity during pregnancy [22]. CSF-2 stimulates pre-DCs to become inflammatory DCs that mediate acquired T-cell immunity to particulate antigens [26]. Our current results suggest that CSF-1-activated DCs in the myometrium differentiated into DEC-205$^+$ DCs, which mainly activated NK1.1$^+$ iNKT cells to secrete a variety of cytokines that elicited fetal loss, whereas those in the spleen predominantly activated NK1.1$^-$ iNKT cells, which did not induce fetal loss in pregnant mice upon α-GalCer stimulation in vivo. This discrepancy may exist because myometrial DCs and splenic DCs in pregnant mice have distinct susceptibilities to α-GalCer; thus, the characteristics and activities of iNKT cells appear to differ between myometrial iNKT cells and splenic iNKT cells. However, the precise mechanisms by which these DC differences are evoked in the pregnant state and the iNKT cell differences that are induced by stimulation with α-GalCer remain to be elucidated. Nevertheless, using iNKT cell-deficient pregnant Jα18 KO mice, we confirmed here that myometrial DC precursors differentiated into DEC-205$^+$ DCs and produced IL-12 in vivo after α-GalCer stimulation without iNKT cell help.

It has been reported that activated maternal decidual iNKT cells secrete Th1-like cytokines through their interaction with CD1d-expressing fetal cells, such as trophoblasts, to play an immunoregulatory role at the maternal-fetal interface [27]. However, our current findings indicate that iNKT cells – in particular, NK1.1$^+$ iNKT cells – that can secrete various proinflammatory cytokines, including IL-2, IL-4, IFN-γ, TNF-α, perforin, and granzyme B, in the myometrium, have a potent ability to induce uterine contractions, hypoxia, and apoptosis followed by miscarriage in pregnant mice. Considering that CFS-1-stimulated DEC-205$^+$ DCs are predominantly expanded in the myometrium during pregnancy and that most pregnancies result in safe deliveries, the actual effectors that elicit miscarriages seem to be activated NK1.1$^+$ iNKT cells induced in the myometrium by DEC-205$^+$ DCs, which themselves may be triggered by the reduction in progesterone and 17β-estradiol levels during the final stage of pregnancy.

The uterus is the principal target organ of these sex hormones, and it undergoes significant changes in morphology and function during maturation and reproduction. In the uterus, the endometrium becomes the decidua, which forms the maternal part of the placenta during pregnancy. It has been thought that immune-mediated miscarriage is initiated at the maternal-fetal interface, i.e. the decidua. Nonetheless, our current findings demonstrate that the myometrium, rather than the decidua, seems to be the principal region that induces miscarriage through a mechanism involving an imbalance in innate immunity mediated mainly by DEC-205$^+$ DCs and NK1.1$^+$ iNKT cells. It has been reported that the major neutral glycolipids, such as globo-series glycolipids (Gb3), were identified in the mature myometrium and endometrium [28]. Among the forms of Gb3, isoglobotrihexosylceramide (iGb3) has been proposed as the natural iNKT cell-selecting ligand and has been shown to be involved in the peripheral activation of iNKT cells by DCs [8]. Thus, iNKT cells in the myometrium are ready to be activated into NK1.1$^+$ iNKT cells through IL-12 secretion by DEC-205$^+$ DCs together with glycolipids during pregnancy.

Moreover, it has been shown that monosialoganglioside (GM3) and disialoganglioside (GD3) within the myometrium and endometrium exhibited striking reciprocal patterns of expression during the menstrual cycle, pregnancy, and aging [28]. Therefore, although a relationship between the menstrual cycle and iNKT cells has not been previously reported, similar to pregnancy, menstruation may be regulated by innate DCs and iNKT cells at the myometrium.

Finally, we found that in iNKT-deficient pregnant Jα18 KO mice, miscarriages were never elicited by the administration of α-GalCer and/or IL-12. This finding suggests that innate iNKT cells, and particularly NK1.1$^+$ iNKT cells, in the myometrium of pregnant mice may terminate the birth of unfavorable offspring. Collectively, the discoveries shown in the present study may offer a new perspective on the importance of the myometrium and NK1.1$^+$ iNKT cells in the innate immunity-mediated regulation

of various reproductive processes, such as menstruation and pregnancy.

Materials and methods

Mice

Female and male B6 and BALB/c mice were purchased from Charles River Laboratories (Kanagawa, Japan), and female and male Jα18 KO mice on the B6 back-ground were kindly provided by M. Taniguchi (RIKEN Institute, Yokohama City, Japan) [15]. All the mice were maintained under specific-pathogen-free conditions. Virgin female (8–12 weeks) B6 mice were mated with syngeneic males or allogeneic BALB/c males. The day when a vaginal plug was detected in a mated female was defined as Gd 0.5. The pregnant mice were administered α-GalCer (Kirin Beer, Tokyo, Japan) or a vehicle control of 0.025% polysolvate-20 in PBS on Gd 5.5, 7.5, 9.5, or 11.5. Each mouse was euthanized 3 days after treatment. The myometrium, decidua, placenta, and spleen were obtained from the pregnant mice, and cells of interest were purified from these organs. All animal experiments were performed according to the guidelines for the care and use of laboratory animals of the National Institutes of Health (NIH, MD, USA) and were approved by the Review Board of Nippon Medical School (Tokyo, Japan).

Induction of fetal loss by injection of α-GalCer or recombinant murine IL-12p70

Pregnant mice were injected on Gd 5.5, 7.5, 9.5, or 11.5 and were euthanized 3 days after the injection. The pregnant females were injected i.p. with 100 μL of α-GalCer at a dose of 0.2, 2, or 20 μg/mouse or 0.025% Polysolvate-20 in PBS as a vehicle control. For IL-12 administration, the pregnant mice were injected i.p. with 100 μL of murine recombinant IL-12p70 (0.2 μg/mouse; R&D Systems, Minneapolis, MN) or PBS on Gd 7.5 or 9.5 and were euthanized 3 days later. The percentage of fetal loss was calculated as follows: (number of absorbed embryos/total number of embryos) × 100.

Cell preparation and flow cytometric analysis

In mice during early pregnancy (Gd 5.5), the embryo cannot be easily separated from other tissues; therefore, whole tissues were used. Because the embryo does not contain lymphoid cells at Gd 5.5, this preparation did not alter our results. At Gd 7.5, 9.5, and 11.5, the myometrium, decidua, and placenta were carefully separated from each other before being cut into small pieces with scissors, and treated with 1 mg/mL collagenase D (Roche, Basel, Switzerland) at 37°C for 20 min. Because there were very few myometrial cells in the nonpregnant mice, we collected the cells of interest from four female mice and then attempted to examine the number of iNKT cells in the myometrium. The obtained cells were finely mashed and passed through a nylon mesh. Mononuclear cells were purified with Lympholyte (Cedarlane, Ontario, Canada) by centrifugation at 1000 × *g* for 20 min at 22°C.

The spleen tissue was cut into small pieces with ophthalmic scissors and digested with 1 mg/mL collagenase D at 37°C for 45 min. The obtained cells were finely mashed, passed through a nylon mesh, and washed with RPMI 1640 medium (Sigma-Aldrich, St. Louis, MO). As previously described [29], the cells were then resuspended in 0.1 × PBS (pH7.2, 0.15 M NaCl) for osmotic hemolysis, and an equal amount of 2 × PBS was added immediately to eliminate contaminating red blood cells. The cells were centrifuged (1200 rpm, 20°C, 8 min) and collected. After incubation with an Fc blocker (clone 24G2; American Type Culture Collection, Manassas, VA) for 15 min to reduce nonspecific antibody binding to Fc receptors, the cells were stained for 30 min at 4°C with 50 μL of diluted FITC-, PE-, allophycocyanin-, PE/Cy7-, and allophycocyanin/Cy7-labeled mAbs. FITC-conjugated anti-CD11c (N418), FITC-conjugated anti-CD3 (145-2C11), PE-conjugated anti-CD4 (RM4.5), and PE-conjugated anti-NK1.1 (PK136) antibodies were purchased from BD Biosciences (San Jose, CA). PE-conjugated anti-DEC-205 (NLDC-145), PE-conjugated anti-33D1 (33D1), allophycocyanin-conjugated anti-DEC-205 (NLDC-145), allophycocyanin-conjugated anti-33D1 (33D1), allophycocyanin-conjugated anti-CD40 (3/23), PE/Cy7-conjugated anti-DEC-205 (NLDC-145), PE/Cy7-conjugated anti-CD19 (6D5), and allophycocyanin/Cy7-conjugated anti-CD45 (30-F11) antibodies were purchased from BioLegend (San Diego, CA). Allophycocyanin-conjugated anti-CD80 (16-10A1) and allophycocyanin-conjugated anti-CD86 (GL1) antibodies were purchased from eBioscience (San Diego, CA). Additionally, allophycocyanin-conjugated α-GalCer-loaded CD1d tetramer and allophycocyanin-conjugated negative control CD1d tetramer were purchased from Proimmune (Oxford, UK). After the stained cells were washed twice with FACS buffer solution (PBS containing 2% heat-inactivated FCS (Hyclone, Logan, UT) and 0.1% sodium azide), the dead cells were identified using propidium iodide (PI; Sigma-Aldrich) and the stained cells were analyzed with a FACSCantoII flow cytometer (BD Biosciences) using FlowJo software (Tree Star, Ashland, OR). The gating strategy for iNKT cells is shown in Supporting Information Fig. 1 and 2. FITC-labeled rat IgG2a (R35-95; BD Biosciences), PE-labeled rat IgG2a (RKT4530; BioLegend), allophycocyanin-labeled Armenian hamster IgG (HTK888; BioLegend), PE/Cy7-labeled rat IgG2a (RTK2758; BioLegend), and allophycocyanin/Cy7-labeled goat IgG (poly 4053; BioLegend) were used as negative controls to set the gating thresholds for each analysis.

Subset of $CD11^+$ DCs and intracellular IL-12 induced by α-GalCer among bone marrow-derived DCs

Bone marrow cells were obtained from the femurs of virgin female B6 mice and Jα18 KO mice. The cells were suspended in RPMI

1640, centrifuged (1500 rpm, 20°C, 5 min) and collected. As described above, the contaminating red blood cells in the collected samples were eliminated, and the obtained bone marrow cells were cultured at a density of 1×10^6 cells in 1 mL of RPMI 1640-based complete culture medium [30] supplemented with 2 mM L-glutamine (Sigma-Aldrich), 1 mM sodium pyruvate (Invitrogen Life Technologies, Carlsbad, CA), 0.1 mM nonessential amino acids (Invitrogen Life Technologies), a mixture of vitamins (Invitrogen Life Technologies), 1 mM HEPES (Invitrogen Life Technologies), 100 U/mL penicillin (Invitrogen Life Technologies), 100 μg/mL streptomycin (Invitrogen Life Technologies), 50 mM 2ME (Sigma-Aldrich), and heat-inactivated 10% FCS containing 10 ng/mL GM-CSF (BioSource International, Camarillo, CA) and 10 ng/mL IL-4 (BioSource International) in 24-well flat-bottom multiwell plates (Corning, Corning, NY) at 37°C. The culture medium was replaced with fresh medium on day 3, and 5 μg/mL α-GalCer or control DMSO was added. On day 5, the cells that were floating or loosely adherent were collected, washed, and analyzed with a FACSCantoII flow cytometer.

Intracellular IL-12 production by the bone marrow-derived DCs was determined with the following procedure. For surface and intracellular staining, a Cytofix/Cytoperm™ kit (BD-Pharmingen, San Jose, CA) was used according to the manufacturers' recommendations. The fluorochrome used in this experiment was PE-conjugated anti-IL-12/IL23p40 (C17.8; eBioscience, San Diego, CA). α-GalCer (5 μg/mL) was added at 24 h, and Brefeldin-A (Sigma-Aldrich) was added 4 h before the FACS analysis.

In vivo analysis of DC production of IL-12 and iNKT cell production of IL-2, IL-4, IFN-γ, TNF-α, perforin, and granzyme B

Pregnant mice were treated with α-GalCer on Gd 7.5 and euthanized 3 days later. Four hours before euthanasia, the pregnant mice were i.p. injected with 250 ng of Brefeldin-A. DCs and iNKT cells in the myometrium and the spleen were immediately isolated and stained to examine their surface molecule expression. The cells were then fixed, permeabilized, and stained intracellularly with PE-conjugated anti-IL-2 (JES6-5H4), anti-IL-4 (11B11), anti-IFN-γ (XMG1.2), and anti-TNF-α (MP6-XT22) antibodies, all purchased from BioLegend, and with anti-perforin (eBioOMAK-D), anti-granzyme B (NGZB), and anti-IL-12/IL-23p40 antibodies from eBioscience.

Cell preparation for adoptive cell transfer

Cells for transfer were obtained from either the myometrium or the spleen of pregnant mice preadministered α-GalCer on Gd 7.5. CD1d tetramer$^+$ iNKT cells were isolated by positive selection with anti-allophycocyanin MicroBeads (Miltenyi Biotec, San Diego, CA) according to the manufacturer's instructions. α-GalCer-induced CD1d tetramer$^+$ iNKT cells obtained from either the myometrium or the spleen of pregnant mice were i.v. transferred into other pregnant mice on Gd 7.5 (3×10^4 cells for each mouse). Three days later (Gd 10.5), the fetal loss rate was analyzed.

Induction of iNKT cells

As indicated above, bone marrow cells were harvested from the femurs of virgin female B6 mice and were resuspended, and red blood cells were eliminated. The obtained bone marrow cells (8×10^6) were cultured in 4 mL of RPMI 1640 with 10 ng/mL IL-4 and 10 ng/mL GM-CSF in six-well flat-bottom multiwell plates (Corning) at 37°C. The culture medium was replaced with fresh medium, and on day 3, the floating cells were removed, and 5 μg/mL α-GalCer was added. On day 6, floating and loosely adherent cells were collected and washed. DEC-205$^+$ DCs or 33D1$^+$ DCs were isolated by positive selection with anti-allophycocyanin MicroBeads (Miltenyi Biotec) according to the manufacturer's instructions. To obtain purified splenic T cells, spleen cells were passed through a nylon wool column as previously described [29] and the purified splenic T cells were placed into a 96-well U–bottom multiwell plate (Corning). Then, bone marrow-derived DCs (2×10^4) were added to the splenic T cells (1×10^5) with 5 μg/mL α-GalCer and 20 μL of T-STIM (BD Biosciences). Five days later, the floating cells were collected, washed, and analyzed using a FACSCantoII flow cytometer.

Statistical analysis

Statistical analyses were performed with Prism (GraphPad Software, San Diego, CA). The results were analyzed using the Mann–Whitney U test and are presented as the mean + SEM. The data were considered significant at $p < 0.05$.

Acknowledgments: We thank Drs. Toshinori Nakayama and Kiyoshi Hirahara of Chiba University for helping provide the Jα18 KO mice and Dr. Ayako Wakabayashi of Nippon Medical School for helpful advice regarding experiments. This study was supported in part by grants from the Ministry of Education, Science, Sport, and Culture (25461715 to H.T.), from the Ministry of Health and Labor and Welfare, Japan, from the Japanese Health Sciences Foundation, from the Promotion and Mutual Aid Corporation for Private Schools of Japan and from the MEXT-Supported Program for the Strategic Research Foundation at Private Universities, Japan.

Conflict of interest: The authors declare no financial or commercial conflict of interest.

References

1 **Tafuri, A., Alferink, J., Moller, P., Hammerling, G. J. and Arnold, B.**, T cell awareness of paternal alloantigens during pregnancy. *Science* 1995. **270**: 630–633.

2 **Negishi, Y., Wakabayashi, A., Shimizu, M., Ichikawa, T., Kumagai, Y., Takeshita, T. and Takahashi, H.**, Disruption of maternal immune balance maintained by innate DC subsets results in spontaneous pregnancy loss in mice. *Immunobiology* 2012. **217**: 951–961.

3 **King, A., Burrows, T. and Loke, Y. W.**, Human uterine natural killer cells. *Nat. Immunity* 1996. **15**: 41–52.

4 **Kitamura, H., Iwakabe, K., Yahata, T., Nishimura, S., Ohta, A., Ohmi, Y., Sato, M. et al.**, The natural killer T (NKT) cell ligand alpha-galactosylceramide demonstrates its immunopotentiating effect by inducing interleukin (IL)-12 production by dendritic cells and IL-12 receptor expression on NKT cells. *J. Exp. Med.* 1999. **189**: 1121–1128.

5 **Brigl, M., Tatituri, R. V., Watts, G. F., Bhowruth, V., Leadbetter, E. A., Barton, N., Cohen, N. R. et al.**, Innate and cytokine-driven signals, rather than microbial antigens, dominate in natural killer T cell activation during microbial infection. *J. Exp. Med.* 2011. **208**: 1163–1177.

6 **Koseki, H., Imai, K., Nakayama, F., Sado, T., Moriwaki, K. and Taniguchi, M.**, Homogenous junctional sequence of the V14$^+$ T-cell antigen receptor alpha chain expanded in unprimed mice. *Proc. Natl. Acad. Sci. USA* 1990. **87**: 5248–5252.

7 **Kawano, T., Cui, J., Koezuka, Y., Toura, I., Kaneko, Y., Sato, H., Kondo, E. et al.**, Natural killer-like nonspecific tumor cell lysis mediated by specific ligand-activated Valpha14 NKT cells. *Proc. Natl. Acad. Sci. USA* 1998. **95**: 5690–5693.

8 **Zhou, D., Mattner, J., Cantu, C., 3rd, Schrantz, N., Yin, N., Gao, Y., Sagiv, Y. et al.**, Lysosomal glycosphingolipid recognition by NKT cells. *Science* 2004. **306**: 1786–1789.

9 **Bendelac, A.**, Positive selection of mouse NK1$^+$ T cells by CD1-expressing cortical thymocytes. *J. Exp. Med.* 1995. **182**: 2091–2096.

10 **Kawano, T., Cui, J., Koezuka, Y., Toura, I., Kaneko, Y., Motoki, K., Ueno, H. et al.**, CD1d-restricted and TCR-mediated activation of valpha14 NKT cells by glycosylceramides. *Science* 1997. **278**: 1626–1629.

11 **Spada, F. M., Koezuka, Y. and Porcelli, S. A.**, CD1d-restricted recognition of synthetic glycolipid antigens by human natural killer T cells. *J. Exp. Med.* 1998. **188**: 1529–1534.

12 **Ito, K., Karasawa, M., Kawano, T., Akasaka, T., Koseki, H., Akutsu, Y., Kondo, E., Sekiya, S. et al.**, Involvement of decidual Valpha14 NKT cells in abortion. *Proc. Natl. Acad. Sci. USA* 2000. **97**: 740–744.

13 **Kinjo, Y., Illarionov, P., Vela, J. L., Pei, B., Girardi, E., Li, X., Li, Y. et al.**, Invariant natural killer T cells recognize glycolipids from pathogenic Gram-positive bacteria. *Nat. Immunol.* 2011. **12**: 966–974.

14 **Boyson, J. E., Nagarkatti, N., Nizam, L., Exley, M. A. and Strominger, J. L.**, Gestation stage-dependent mechanisms of invariant natural killer T cell-mediated pregnancy loss. *Proc. Natl. Acad. Sci. USA* 2002. **103**: 4580–4585.

15 **Cui, J., Shin, T., Kawano, T., Sato, H., Kondo, E., Toura, I., Kaneko, Y. et al.**, Requirement for Valpha14 NKT cells in IL-12-mediated rejection of tumors. *Science* 1997. **278**: 1623–1626.

16 **Michel, M. L., Keller, A. C., Paget, C., Fujio, M., Trottein, F., Savage, P. B., Wong, C. H. et al.**, Identification of an IL-17-producing NK1.1(neg) iNKT cell population involved in airway neutrophilia. *J. Exp. Med.* 2007. **204**: 995–1001.

17 **Arora, P., Baena, A., Yu, K. O., Saini, N. K., Kharkwal, S. S., Goldberg, M. F., Kunnath-Velayudhan, S. et al.**, A single subset of dendritic cells controls the cytokine bias of natural killer T cell responses to diverse glycolipid antigens. *Immunity* 2014. **40**: 105–116.

18 **Brennan, P. J., Brigl, M. and Brenner, M. B.**, Invariant natural killer T cells: an innate activation scheme linked to diverse effector functions. *Nat. Rev. Immunol.* 2013. **13**: 101–117.

19 **Chaouat, G., Menu, E., Clark, D. A., Dy, M., Minkowski, M. and Wegmann, T. G.**, Control of fetal survival in CBA × DBA/2 mice by lymphokine therapy. *J. Reproduct. Fertil.* 1990. **89**: 447–458.

20 **Rieger, L., Honig, A., Sutterlin, M., Kapp, M., Dietl, J., Ruck, P. and Kammerer, U.**, Antigen-presenting cells in human endometrium during the menstrual cycle compared to early pregnancy. *J. Soc. Gynecol. Invest.* 2004. **11**: 488–493.

21 **Erlebacher, A.**, Immunology of the maternal-fetal interface. *Annu. Rev. Immunol.* 2013. **31**: 387–411.

22 **Tagliani, E., Shi, C., Nancy, P., Tay, C. S., Pamer, E. G. and Erlebacher, A.**, Coordinate regulation of tissue macrophage and dendritic cell population dynamics by CSF-1. *J. Exp. Med.* 2011. **208**: 1901–1916.

23 **Pollard, J. W., Bartocci, A., Arceci, R., Orlofsky, A., Ladner, M. B. and Stanley, E. R.**, Apparent role of the macrophage growth factor, CSF-1, in placental development. *Nature* 1987. **330**: 484–486.

24 **Volchek, M., Girling, J. E., Lash, G. E., Cann, L., Kumar, B., Robson, S. C., Bulmer, J. N. and Rogers, P. A.**, Lymphatics in the human endometrium disappear during decidualization. *Hum. Reprod.* 2010. **25**: 2455–2464.

25 **Collins, M. K., Tay, C. S. and Erlebacher, A.**, Dendritic cell entrapment within the pregnant uterus inhibits immune surveillance of the maternal/fetal interface in mice. *J. Clin. Invest.* 2009. **119**: 2062–2073.

26 **Greter, M., Helft, J., Chow, A., Hashimoto, D., Mortha, A., Agudo-Cantero, J., Bogunovic, M. et al.**, GM-CSF controls nonlymphoid tissue dendritic cell homeostasis but is dispensable for the differentiation of inflammatory dendritic cells. *Immunity* 2012. **36**: 1031–1046.

27 **Boyson, J. E., Rybalov, B., Koopman, L. A., Exley, M., Balk, S. P., Racke, F. K., Schatz, F. et al.**, CD1d and invariant NKT cells at the human maternal-fetal interface. *Proc. Natl. Acad. Sci. USA* 2002. **99**: 13741–13746.

28 **Zhu, Z., Deng, H., Fenderson, B. A., Nudelman, E. D. and Tsui, Z.**, Glycosphingolipids of human myometrium and endometrium and their changes during the menstrual cycle, pregnancy and ageing. *J. Reprod. Fertil.* 1990. **88**: 71–79.

29 **Takahashi, H., Cease, K. B. and Berzofsky, J. A.**, Identification of proteases that process distinct epitopes on the same protein. *J. Immunol.* 1989. **142**: 2221–2229.

30 **Takahashi, H., Nakagawa, Y., Leggatt, G. R., Ishida, Y., Saito, T., Yokomuro, K. and Berzofsky, J. A.**, Inactivation of human immunodeficiency virus (HIV)-1 envelope-specific CD8$^+$ cytotoxic T lymphocytes by free antigenic peptide: a self-veto mechanism? *J. Exp. Med.* 1996. **183**: 879–889.

Abbreviations: **BMDC**: bone marrow-derived DC · **Gd**: days of gestation · **α-GalCer**: α-galactosylceramide · **iNKT**: invariant natural killer T

Full correspondence: Dr. Hidemi Takahashi, Department of Microbiology and Immunology, Nippon Medical School, 1-1-5 Sendagi, Bunkyo-ku, Tokyo 113–8602, Japan
Fax: +81-3-3316-1904
e-mail: htkuhkai@nms.ac.jp

Received: 14/7/2015
Revised: 22/4/2016
Accepted: 17/5/2016
Accepted article online: 20/5/2016