The peri-implantation uterus contains an expanded population of NK1.1+ $\text{V}14+$ TCR<sup>\text{in}+</sup> (NKT) lymphocytes. Although these cells bear the above features in common with other NKT cell populations in thymus, bone marrow, liver, and spleen, they differ from these other populations in terms of an altered V$\beta$ repertoire and absence of a CD4<sup>+</sup> component. In this study, we demonstrate that the uterine population also differs from other NKT cell populations because they recognize a class I/class I-like molecule other than CD1, whereas most previously described V$\alpha$14<sup>+</sup> NKT cells are CD1-restricted. Moreover, the class I/class I-like molecule leading to the uterine NKT cell expansion may be supplied by the fetus. These data demonstrate a novel mechanism whereby the fetus is capable of modulating the maternal immune system. The Journal of Immunology, 2001, 166: 3641–3644.

Yushe Dang* and Kent D. Heyborne<sup>2</sup>†

---

As the fetal trophoblasts invade the uterine wall at implantation, maternal immune cells, either circulating or resident within the uterine lining (decidua), may be exposed to fetal Ags. The ensuing maternal immune response and its potential positive or negative effects upon the developing fetus are of great interest in perinatal medicine. It has been hypothesized that the fetus may in fact modulate the nature and constituents of this immune response, and recently examples of this phenomenon have been documented. For example, human trophoblasts express HLA-G, an oligomorphic class Ia molecule that appears to regulate maternal macrophages, NK, and T cells at the maternal-fetal interface. (1–3) Additionally, in separate transgenic murine models, T cells expressing the transgenic TCR specific for either a paternally inherited (7) or a maternally inherited (K<sup>b</sup>) (5) Ag were both deleted andtolerized in an Ag-specific fashion during pregnancy, possibly via placentation expression of Fas ligand (5, 6).

Recently, we demonstrated a large increase in uterine NK1.1+ $\text{V}14+$ CD4<sup>+</sup> /CD8<sup>+</sup> (NKT) cells in the maternal decidua at peri-implantation. (7) Here, we demonstrate that a fetal Ag regulates this increase. This Ag is most likely a class I-like molecule since $\beta_2$-microglobulin-deficient (β<sub>2</sub>m<sup>−/−</sup>) mice do not have increased numbers of NKT cells at peri-implantation. However, the Ag does not appear to be CD1, the class I-like molecule recognized by NKT cells in other tissues, as CD1<sup>−/−</sup> mice have normal numbers of decidua NKT cells.

Materials and Methods

Mice

Pathogen-free CD1<sup>−/−</sup> C57BL/6 and BALB/c (8) mice were obtained from the laboratory of C.-R. Wang at the University of Chicago (Chicago, IL), bred in the animal care facility at Swedish Medical Center (Denver, CO), and screened, as described, by PCR to obtain CD1<sup>−/−</sup> and CD1<sup>+/−</sup> mice of each strain for use in additional experiments (8). Six- to 8-week-old β<sub>2</sub>m<sup>−/−</sup> C57BL/6 and BALB/c (9) mice were obtained commercially from The Jackson Laboratory (Bar Harbor, ME). All experiments were performed with agreement from the HealthONE Institutional Animal Care and Utilization Committee, which acts for the Swedish Medical Center.

Cell preparation

Placental/decidual tissues<sup>4</sup> were obtained from early pregnancies (days 6–8, day of plug detection = day 0), as previously described (7). In all cases, (C57BL/6 (female) $\times$ BALB/c (male)) matings were used, with the animals being wild-type (WT; CD1<sup>+/−</sup>, $\beta_2$m<sup>+/−</sup>), CD1<sup>−/−</sup> (CD1<sup>−/−</sup>, $\beta_2$m<sup>−/−</sup>) or $\beta_2$m<sup>−/−</sup> /CD1<sup>+/−</sup>, $\beta_2$m<sup>−/−</sup>). Timed pregnant female mice were sacrificed using CO<sub>2</sub> inhalation, and placental/decidual tissues were collected, placed in a Celllector tissue sieve (VMR Scientific Productions, Willard, OH), and mechanically dispersed into balanced salt solution (BSS). Spleens, livers, and thymi were obtained from the same pregnant mice and similarly prepared. Placental/decidual cells, splenocytes, hepatocytes, and lymphocytes were centrifuged at 1000 rpm ($250 \times g$) for 5 min, cell pellets were resuspended in 1 ml of BSS and 3 ml of Gey’s solution (0.155 M NH<sub>4</sub>Cl and 0.01 M KHCO<sub>3</sub>) for 5 min at room temperature to lyse RBC, washed with BSS, resuspended in 2 ml of BSS/5% FCS (Sigma, St. Louis, MO), and lymphocytes were enriched over nylon wool, as previously described (10). During each experiment, the phenotype of each $\beta_2$m<sup>−/−</sup> or CD1<sup>−/−</sup> mouse was verified by cytofluorographic analysis (data not shown).

mAbs and cytofluorographic analysis

H57.597 (anti-pan TCR-αβ, FITC conjugated) (11) and PK136 (anti-NK1.1, PE conjugated) (12) were purchased commercially from BD PharMingen (San Diego, CA). mAb S19.8 (anti-$\beta_2$m, FITC conjugated) (13) was generated as cell culture supernatant from an existing cell line, 3 Abbreviations used in this paper: $\beta_2$m, $\beta_2$-microglobulin; WT, wild type; BSS, balanced salt solution.

4 Because the embryo cannot easily be separated from the other tissues at this early gestation, it was included in the tissue preparations. Because the embryo does not contain lymphoid cells at this time, its inclusion will not alter the results. Due to the intimate apposition of the fetal trophoblasts and maternal tissues, they cannot be easily separated. For simplicity, we refer to the fetal and maternal tissues thus obtained as “placenta decidua.”
purified by affinity chromatography on protein G-Sepharose G25 (Sigma), concentrated, dialyzed, and conjugated with FITC (Sigma).

For cytofluorographic analysis, cells (0.1–1 × 10^6/well in 96-well plates) were preincubated with rat anti-mouse FcγR Ab (24G2, 1 μg/ml) (14) or undiluted normal mouse serum to block nonspecific binding and one- or two-color staining was performed. Cells were analyzed by flow cytometry using a Becton Dickinson FACSort cytometer (Becton Dickinson, San Jose, CA), and data plots were generated using the CellQuest version 1.2 software package supplied by the manufacturer. In all experiments, set up and calibration were performed with nylon wool prepared splenocytes, and these cells were also used to set gates for thymocytes and hepatic and placental/decidual lymphocytes. In all cases, appropriate negative control experiments were performed to verify staining specificity.

Results
Distribution of NKT cells
We analyzed percentages of NKT cells in placental/decidual tissues from (CD1^+/+ × CD1^+/+), (CD1^-/- × CD1^-/-) and (β_m^-/- × β_m^-/-) matings. For comparison, we also analyzed percentages for NKT cells in thymus, spleen, and liver from the same pregnant female mice. In the (CD1^-/- × CD1^-/-) mice, thymus, spleen, liver, and placenta/decidua contain ∼1% (1.3 ± 0.2, mean ± SD, n = 7), 2% (2.0 ± 0.4, mean ± SD, n = 6), and 18% (18.3 ± 3.9, mean ± SD, n = 5), and 4% (4.1 ± 1.2, mean ± SD, n = 7) NKT cells among all nylon wool-purified cells, respectively. Typically staining data are shown in Fig. 1a. Percentages of NKT cells are greatly reduced in the (CD1^-/- × CD1^-/-) females in thymus, liver, and spleen, as previously reported (8, 15), but are unchanged in placental/decidual tissues from these mice (Fig. 1b), suggesting that CD1 is not required for the expansion of uterine NKT cells. In (β_m^-/- × β_m^-/-) matings, percentages of NKT cells were also greatly reduced in thymus, liver, and spleen, as previously reported (16–19) and were also greatly reduced in placental/decidual tissues (Fig. 1c). Together with the data from Fig. 1b, these data suggest that a β_m-associated molecule other than CD1 regulates the expansion of uterine NKT cells.

Next, we analyzed percentages of NK1.1+ cells coexpressing αβ TCR and the percentages of αβ T cells coexpressing NK1.1 in these same organs. In (CD1^-/- × CD1^-/-) matings, it was found that nearly 80% of thymus and liver NK1.1+ cells, one-third of spleen NK1.1+ cells, and one-quarter of uterine NK1.1+ cells coexpress αβ-TCR on their surface (Fig. 2, left). Similarly, it was found that 5.2% of αβ-TCR+ cells in thymus, 28% in liver, 3.4% in spleen, and 8.6% in placenta/decidua coexpress NK1.1 (Fig. 2, right). By contrast, these percentages were reduced in the (CD1^-/- × CD1^-/-) matings in thymus, spleen, and liver, but not in placenta/decidua. Again, the percentages were reduced in all organs in the (β_m^-/- × β_m^-/-) matings, suggesting regulation of uterine NKT cells by a β_m-associated molecule other than CD1. The data from Fig. 2, c–f, suggest that there may be a small pool of CD1-independent β_m-dependent NKT cells in liver and spleen as well.

A fetal β_m-associated molecule regulates expansion of placental/decidual NKT cells
The experiments performed indicate that the expansion of placental/decidual NKT cells largely relies on the expression of a β_m-associated molecule other than CD1. The next experiment was designed to assess whether paternal (placental) expression of the β_m-associated molecule is sufficient for expansion of placental/decidual NKT cells. To address this question, we bred C57BL/6 β_m^-/- females with WT BALB/c males and analyzed NKT cells from the resulting gestations. Although thymic, splenic, and hepatic NKT cell numbers remained at the very low levels seen in (β_m^-/- × β_m^-/-) gestations, the numbers of NKT cells from these (β_m^-/- × WT) pregnancies were restored to those of WT matings. Typical staining data are shown in Fig. 3. Thus, even in the absence of maternal β_m, fetal β_m derived from the paternal genome is sufficient to expand the placenta/decidual NKT cell population. These data are summarized in Fig. 4.

To determine whether paternal β_m expression is necessary for the expansion of placenta/decidual NKT cells, (WT × β_m^-/-) gestations were analyzed and normal numbers of NKT cells were found (data not shown). Thus, paternal β_m expression is sufficient, but not necessary, for NKT cells expansion.

Discussion
Multiple studies in recent years have characterized a unique cell population known as NKT cells. (16, 20, 21). These cells, found primarily in thymus, bone marrow, spleen, and liver, are characterized by several unique features. In addition to coexpression of NK1.1 and αβ-TCR, they uniformly express Vα14-Jα281 with a canonical VJ junction, have a limited Vβ repertoire (predominantly Vβ8.2 in addition to smaller proportions of Vβ2 and Vβ7), express TCR at an intermediate level, secrete large amounts of IL-4 upon engagement of the TCR (8, 22), and recognize the class I-like CD1 molecule. (15, 23). In a recent study, we added the IL-4 environment has been shown to be critical for reproductive success (24–27).

Ito et al. (28) have also recently confirmed the presence of NKT cells in the pregnant uterus. These authors speculated that NKT cells might mediate spontaneous abortion based upon experiments involving in vivo stimulation of NKT cells with α-galactosylceramide, a known ligand of Vα14+ NKT cells. The physiological significance of these findings remains to be demonstrated.
In our description of NKT cells in the pregnant uterus and their expansion at peri-implantation, we noted two features that suggested these cells might develop independently from NKT cells in other organs. First, unlike other populations of NKT cells that contain both CD4\(^+\) and CD4\(^-\)/CD8\(^-\) (DN) components, uterine NKT cells are entirely CD4\(^-\). Additionally, although uterine NKT cells are V\(\alpha\)14\(^+\), their V\(\beta\) repertoire is different from other organs, with an apparent predominance of V\(\beta\)3\(^+\) cells and few V\(\beta\)8\(^+\) cells.\(^5\)

We did note expression of CD1 within the pregnant uterus and speculated that these cells might nonetheless recognize CD1. Although experiments have not yet been done directly assessing cognate recognition of CD1 by uterine NKT cells, the experiments reported herein suggest that development of this uterine population is regulated by a class I/class I-like molecule other than CD1. Additionally, these findings indicate that the relevant \(\beta\)2m-associated molecule can be supplied by the paternal genome, presumably via placental expression. In the HLA-G experiments discussed in the introduction, HLA-G likely mediates its effects via placental expression, as its expression is highest in the trophoblasts (29). It seems likely that the relevant fetal Ags (H-Y, K\(^b\)) in the placenta.

\(^5\) K. Ito, M. Karasawa, T. Kawano, T. Akasaka, H. Koseki, Y. Akutsu, E. Kondo, S. Sekiya, K. Sekikawa, M. Harada, et al., who characterized NKT cells at a later gestation, found a predominance of V\(\beta\)7\(^+\) cells in their report. We did note a high degree of staining with anti-V\(\beta\)7 mAb in lymphocyte preparations from uterine tissues, but found this staining to be nonspecific.

**FIGURE 2.** Data from Fig. 1 were analyzed to obtain the percentage of NK1.1\(^+\) cells coexpressing \(\alpha\beta\)-TCR (a, c, e, and g) and the percentage of \(\alpha\beta\)-TCR\(^+\) cells coexpressing NK1.1 (b, d, f, and h) in thymus (a and b), liver (c and d), spleen (e and f), and placenta/decidua (g and h). Data (mean ± SEM) obtained from five to seven experiments.

**FIGURE 3.** Numbers of lymphocytes expressing NK1.1 and/or \(\alpha\beta\)-TCR in thymus, spleen, liver, and placenta/decidua of \((\beta\)2\(\text{m}^{-/-} \times \text{WT})\) matings. Nylon wool-prepared cells from each organ were stained with anti-NK1.1 and anti-\(\alpha\beta\)-TCR and analyzed cytofluorographically. Percentages indicate percentages of all nylon wool-nonadherent cells. Five to seven experiments were performed; representative data are shown.
Transgenic models also mediate the observed effects via placental expression, as the placental trophoblast are the cells most likely to be encountered by maternal immune cells. In this regard, it is interesting to note that the paternal genome is preferentially expressed in the placental tissues, presumably due to imprinting (30). Since we have not specifically identified the β2m-associated molecule that regulates the expansion of uterine NK T cells, we cannot yet assess whether it is imprinted or not. We did observe that maternal β2m expression is capable of regulating uterine NK T cell expansion. Whether this effect is mediated by placental expression of the relevant β2m-associated maternal allele or by expression by adjacent maternal tissues is unknown at present.

In these experiments, as in previous reports, thymus, liver, and spleen of β2m−/− females contain very few NKT cells (16–19, 23). Similarly, the uteri of pregnant β2m−/− females contain very few NKT cells when crossed with β2m−/− males. In contrast, when crossed with β2m−/+ males, normal numbers of uterine NKT cells are found, while numbers of NK T cells in the other organs remain very low. Current data support both thymic and extrathymic development of NK T cells (reviewed in Ref. 23). It is unclear from these data whether the local expansion of uterine NK T cells represents de novo development of uterine NK T cells from a resident progenitor or a local expansion of a small resident pool. The data do not support thymic development and migration. This model may provide an interesting system for the study of NK T cell development.

Interactions between the mother and fetus have long been known to occur via a variety of steroid and peptide hormones. These data document another mechanism whereby maternal-fetal interaction occurs via their immune systems. Unlike similar situations that occur in tumor and transplant models, for example, these phenomena take place within the physiologic setting of the hemochorial placenta and emphasize the specialized nature of maternal-fetal immunology.

**References**

1. Carosella, E. D., N. Rouas-Freiss, P. Paul, and J. Dausset. 1999. HLA-G: a tolerance molecule from the major histocompatibility complex. Immunol. Today 20:560.
2. Carosella, E., P. Paul, P. Moreau, and N. Rouas-Freiss. 2000. HLA-G and HLA-E: fundamental and pathophysiological aspects. Immunol. Today 21:532.
3. Brand, Y. M., D. S. Allan, and A. J. McMichael. 1999. Functions of nonclassical MHC and -MHC-encoded class I molecules. Curr. Opin. Immunol. 11:110.
4. Jiang, S. P., and M. S. Vacchis. 1998. Multiple mechanisms of peripheral T cell tolerance to the fetal “alloagraft.” J. Immunol. 160:3086.
5. Tafuri, A., J. Alferink, P. Moller, G. J. Hammerling, and B. Arnold. 1995. T cell awareness of paternal alloantigens during pregnancy. Science 270:630.
6. Hunt, J. S., D. Vassmer, T. A. Ferguson, and L. Muller. 1997. Fas ligand is positioned in mouse uterus and placenta to prevent trafficking of activated leukocytes between the mother and the conceptus. J. Immunol. 158:4122.
7. Dang, Y., J. Beckers, C.-R. Wang, and K. Heyborne. 2000. Natural killer 1.1 αβ T cells in the perimammatation uterus. Immunology 101:484.
8. Chen, Y. H., N. M. Chiu, M. Mandal, N. Wang, and C. R. Wang. 1997. Impaired NK T cell development and early IL-4 production in CD1-deficient mice. Immunity 6:459.
9. Koller, B. H., and O. Smithies. 1989. Inactivating the β2-microglobulin locus in mouse embryonic stem cells by homologous recombination. Proc. Natl. Acad. Sci. USA 86:8932.
10. Julius, M. H., E. Simpson, and L. A. Herzenberg. 1973. A rapid method for the isolation of functional thymus-derived murine lymphocytes. Eur. J. Immunol. 3:645.
11. Kubo, R. T., W. Born, J. W. Kappler, P. Marrack, and M. Pigeon. 1989. Characterization of a monoclonal antibody which detects all murine αβ T cell receptors. J. Immunol. 142:2726.
12. Koo, G. C., and J. R. Peppard. 1984. Establishment of monoclonal anti-NK-1.1 antibody. Hybridoma 3:301.
13. Tada, N., S. Kimura, A. Hatzfeld, and U. Hammerling. 1980. Ly-11 the H-3 region of mouse chromosome 2 controls a new surface alloantigen. Immunogenetics 11:441.
14. Unkeless, J. C. 1979. Characterization of a monoclonal antibody directed against mouse macrophage and lymphocyte Fc receptors. J. Exp. Med. 150:580.
15. Bendelac, A., O. Lantz, M. E. Quimby, J. W. Yewdell, J. R. Bennink, and R. R. Brutkiewicz. 1995. CD1 recognition by mouse NK1.1 T lymphocytes. Science 268:863.
16. Ohle, T., and H. R. MacDonald. 1994. Major histocompatibility complex class I related molecules control the development of CD4+8− and CD4−8+ subsets of natural killer 1.1 β T cell receptor-αβ+ cells in the liver of mice. J. Exp. Med. 180:699.
17. Bendelac, A., N. Killeen, D. R. Littman, and R. H. Schwartz. 1994. A subset of CD4+8− thymocytes selected by MHC class I molecules. Science 263:1774.
18. Bix, M., M. Coles, and D. Raulet. 1993. Positive selection of Vα8+ CD4−8+ thymocytes by class I molecules expressed by hematopoietic cells. J. Exp. Med. 178:901.
19. Coles, M. C., and D. H. Raulet. 1994. Class I dependence of the development of CD4+8− NK1.1+ thymocytes. J. Exp. Med. 180:395.
20. Sykes, M. 1990. Unusual T cell populations in adult murine bone marrow: prevalence of CD3+ CD4− CD8− and αβ TCR NK1.1+ cells. J. Immunol. 145:3209.
21. Lantz, O., and A. Bendelac. 1994. An invariant T cell receptor a chain is used by a unique subset of major histocompatibility complex class I-specific CD4+ and CD4−8+ T cells in mice and humans. J. Exp. Med. 180:1097.
22. Yoshimoto, T., A. Bendelac, C. Watson, J. Hu-Li, and W. E. Paul. 1995. Role of NK1.1+ T cells in a TH2 response and in immunoglobulin E production. Science 270:1845.
23. Bendelac, A. 1995. Mouse NK1+ T cells. Curr. Opin. Immunol. 7:367.
24. Hill, J. A., K. Polgar, and D. J. Anderson. 1995. T-helper 1-type immunity to Leishmania major in pregnant C57BL/6 mice increases implantation failure and fetal resorptions: correlation with increased IFN-γ and TNF and reduced IL-10 production by placental cells. J. Immunol. 156:653.
25. Lin, H., T. R. Mosmann, L. Guibert, S. Tuntipopipat, and T. G. Wegmann. 1993. Synthesis of T helper 2-type cytokines at the maternal-fetal interface. J. Immunol. 151:4552.
26. Raghupathy, R. 1997. Th1-type immunity is incompatible with successful pregnancy. Immunol. Today 18:478.
27. Ito, K., M. Karasawa, T. Kawano, T. Akasaka, H. Koseki, Y. Akutsu, E. Kondo, S. Sekiya, K. Sekikawa, M. Harada, et al. 2000. Involvement of decidual Vα14 NKT cells in abortion. Proc. Natl. Acad. Sci. USA 97:740.
28. Kovats, S., E. K. Main, C. Librach, M. Stubblebine, S. J. Fisher, and R. DeMars. 1990. A class I antigen, HLA-G, expressed in human trophoblasts. Science 248:420.
29. Telghman, S. M. 1999. The sins of the fathers and mothers: genomic imprinting in mammalian development. Cell 96:185.