Our study is aimed at addressing the hypothesis that IL-10 regulates the immune response to the poly(I:C) that causes fetal demise. Our results support this hypothesis and demonstrate that IL-10 coupled with TLR3 activation plays a role in inflammatory induction of the activating receptor NKG2D. In particular, NKG2D is best characterized for virus and tumor elimination and recognizes ligands that are induced by cellular transformation, stress, or infection (31). Our results demonstrate that in response to poly(I:C) treatment of pregnant WT mice, a significant proportion of uNK cells acquires a fetus-damaging, TNF-α–producing phenotype marked by IL-10–dependent induction of NKG2D expression. Furthermore, induced expression of the cognate NKG2D ligand, Rae-1 (retinoic acid early inducible-1) (32), is predominantly detected on uterine F4/80$^+$ macrophages from poly(I:C)-treated WT mice. In IL-10$^{-/-}$ mice, poly(I:C) treatment amplifies TNF-α–producing uterine T cells. Our data suggest that although IL-10 is a cytokine that is compatible with pregnancy (7, 25), TLR3-mediated induction of inflammation at the maternal-fetal interface may alter the anti-inflammatory characteristics of this cytokine.

Materials and Methods

Mice

C57BL/6 WT or IL-10$^{-/-}$ mice were obtained from the Jackson Laboratory and housed in pathogen-free conditions in the Central Research Facility at Rhode Island Hospital. All protocols were approved by the Lifespan Committee for Animal Welfare. Syngeneic matings were used in this study, and visualization of a vaginal plug was designated as gestation day (GD) 0.

In vivo treatments

All reagents were injected i.p. in pregnant mice in a time-dependent fashion. Mice were injected on GD 6 with saline or varying doses of poly(I:C) (Invivogen) to establish a dose curve for maximum fetal resorption. Neutralization of TNF-α was accomplished by injecting 250 μg/mouse anti–TNF-α Ab (BD Biosciences) on GD 5 and GD 7 in combination with GD 6 injection of poly(I:C) or saline. An isotype-matched Ab was used as a control. Asialo-GM1 Ab (Wako) or isotype-matched control were injected at a dose of 100 μg/mouse on GD 4, 6, and 8 to deplete NK cells. Depletion of T cells was monitored with dual injection of 200 μg anti-CD4 and anti-CD8 or isotype control Abs (BD Bioscience) on GD 5 and GD 7 in conjunction with saline or poly(I:C) treatment. NK1.1 or NGK2D was blocked with injection of 250 μg/mouse of anti-NK1.1 Ab (BD Bioscience) or anti-NKG2D Ab (eBioscience), respectively, or corresponding isotype controls on GD 5 and GD 7. Recombinant IL-10 (eBioscience) was injected at a dose of 500 ng/mouse on GD 4, 6, and 8 to restore WT phenotype in IL-10$^{-/-}$ mice. When required, uterine horns were visualized and photographed to assess fetal resorption or healthy fetal units.

Tissue sampling

Spleen, implantation units, and serum were harvested from IL-10$^{-/-}$ or WT mice on GD 10. Blood was collected and centrifuged at 2000 rpm for 15 min and serum was stored at −80°C. Spleen and implantation units were immediately placed in RPMI 1640 supplemented with 5% FBS, and tissues were manually dissociated between frosted microslides. Cell preparations were placed on a Ficoll-Lite (Atlanta Biologicals) gradient and centrifuged at room temperature for 30 min. Lymphocytes and trophoblasts appearing at different densities were collected, washed, counted, and placed in 96-well plates at 1×10^6 cells/well for further analysis.

Flow cytometry

Analysis was performed as described previously (7, 23, 24). Surface staining with Abs for NK1.1, CD45, CD3, CD4, CD8 (BD Bioscience) and NKG2D, F4/80, CD11b, Rae-1 (eBioscience), or isotype-matched controls was performed by flow cytometry (FACS Canto, Becton Dickinson). Intracellular staining was done for TNF-α, IFN-γ, and IL-12 (eBioscience) as described previously (7, 23, 24). Cells were washed with PBS, stained for surface Ags for 30 min, washed, treated with Cytofix/Cytoperm (BD Biosciences), and stained intracellularly in PermWash (BD Bioscience) with Ab or isotype-matched control. Cells were washed and analyzed by FACS.

Cytochemistry and immunocytochemistry

Saline or poly(I:C)-treated uterine horns were collected on GD 10 and stored in 10% neutral buffered formalin (Protocol) and embedded into paraffin within 48 h after harvest and section prepared for immunohistochemical analysis as described (7, 23, 24). Serial sections were cut at 5–10-μm intervals. Apoptotic cells were probed using the ApopTag Fluorescence In Situ Apoptosis Detection Kit (Millipore) per manufacturer's instructions. Mouse monoclonal anti-cytokeratin 8 (TROMA-I; Developmental Hybridoma) and goat anti-mouse Rae-1 (Santa Cruz Biotechnology) were used to stain trophoblasts and Rae-1–positive decidual and placental cells, respectively, as detected with Streptavidin-FITC (Vector Laboratories). For uNK cell identification, *Dolichos biflorus* (DBA) lectin cytochemistry (14) and perforin (PRF) immunocytochemistry (rabbit-polyclonal anti-PRF Ab; Torrey Pines Biolabs) were performed. Analyses used a Nikon eclipse 80i with a SPOT advanced camera (version 4.1.2–Nikon Instruments) for fluorescence photomicroscopy.

ELISA

Serum samples were analyzed by ELISA to measure TNF-α, IFN-γ, IL-12 (R&D Systems) and IFN-β (IFN Source). Experiments were performed according to the manufacturer's instructions.

Statistics

Two groups were compared with two-tailed unpaired Student t test. Significance was determined as $p < 0.05$. Time course of multiple groups were compared with two-way ANOVA.

Results

Distinct uterine immune populations amplify in response to poly(I:C) treatment in pregnant WT and IL-10$^{-/-}$ mice

In our previous studies using LPS or CpG to induce adverse pregnancy outcomes, we demonstrated that IL-10$^{-/-}$ mice were highly sensitive to low doses of LPS and CpG for induction of fetal resorption or preterm birth (7, 23, 24). This prompted us to compare responses to viral infections as mimicked by poly(I:C), a TLR3 ligand, when administered i.p. on GD 6 of pregnancy. As shown in Fig. 1A, poly(I:C) treatment resulted in dose-dependent fetal resorption in both WT and IL-10$^{-/-}$ mice as assessed by evaluation of placental units on GD 10. WT and IL-10$^{-/-}$ mice experienced fetal resorption in both uterine horns at the same dose of 100 μg poly(I:C) per mouse with similar kinetics (Fig. 1B), suggesting that IL-10 is not protective against TLR-3–triggered fetal demise.

Since IL-10 has been shown in several viral models to directly suppress T cell function and is concomitantly produced by NK cells (33, 34), we profiled splenic and uterine CD3$^-$NK1.1$^+$ and CD3$^+$ lymphocytes by flow cytometry. Data are presented as representative flow cytometry plots (Fig. 1C) and average numbers from several experiments (Fig. 1D). There were no marked changes in splenic NK1.1$^+$ and CD3$^+$ cells between untreated and poly(I:C)-treated WT or IL-10$^{-/-}$ mice (Fig. 1D). Remarkably, poly(I:C) treatment induced amplification of NK1.1$^+$ cells in WT mice compared with saline-treated controls (22 ± 4% versus 10 ± 2%; Fig. 1C, 1D). However, in IL-10$^{-/-}$ mice, no significant changes in uNK1.1$^+$ cells were observed in response to poly(I:C) (Fig. 1C, 1D). In contrast, uterine CD3$^+$NK1.1$^-$ cells expanded markedly from 19 ± 2% to 30 ± 3% (Fig. 1C, 1D). These observations indicate that IL-10 contributes to uNK cell expansion in WT mice in response to poly(I:C) treatment.

TNF-α produced by uNK and T cells is required for fetal resorption in response to poly(I:C)

Because serum cytokines are altered during pregnancy and in response to inflammatory triggers, we first analyzed a panel of inflammatory cytokines that are associated with TLR3 activation (35). We observed no marked changes in serum IFN-γ, IL-12, or IFN-β as measured by ELISA (Fig. 2A). However, TNF-α was significantly increased in WT and IL-10$^{-/-}$ mice exposed to poly(I:C) (Fig. 2A).

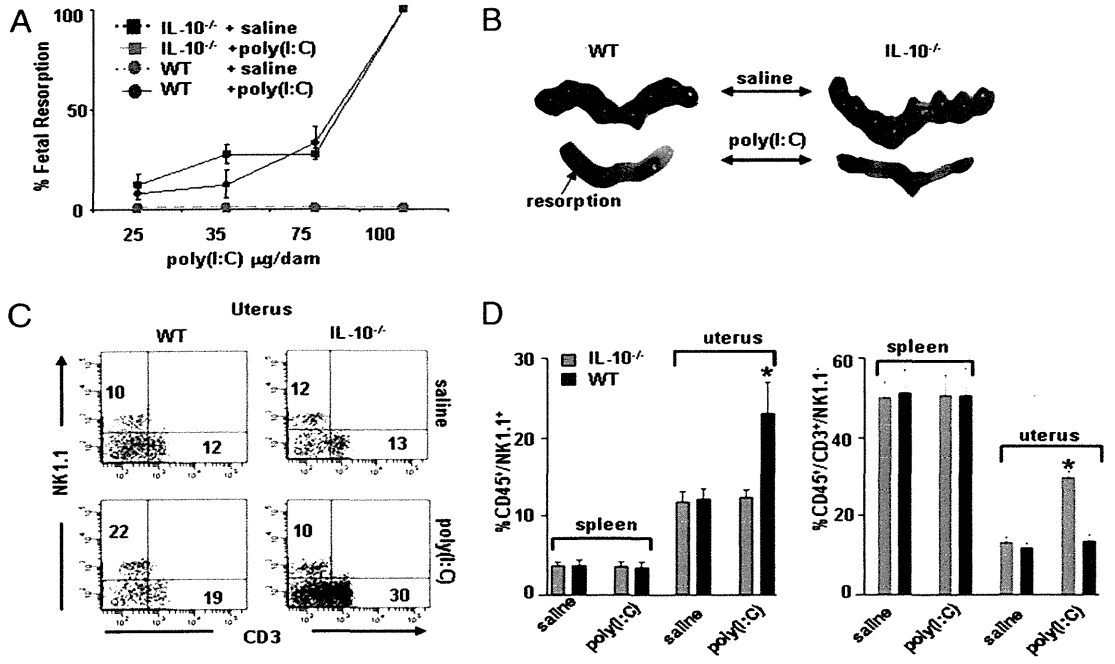


FIGURE 1. Fetal resorption and amplification of uterine NK and T cells in WT and IL-$10^{-/-}$ mice in response to poly(I:C) treatment. (**A**) poly(I:C)-injected i.p. on GD 6 was evaluated in a dose-dependent manner to induce fetal resorption as assessed by inspection of uterine placental units on GD 10. A dose of 100 μg/mouse induced 100% fetal resorption in both IL-$10^{-/-}$ and WT mice. A subset of these mice was allowed to deliver, and no pups were born. Data are plotted as mean ± SEM ($n = 6$ per treatment). (**B**) Representative GD 10 WT and IL-$10^{-/-}$ uterine horns from mice treated with saline or 100 μg/mouse poly(I:C) are shown. (**C**) Assessment of splenic and uterine immune cells from WT or IL-$10^{-/-}$ mice treated on GD 6 with saline or poly(I:C) (100 μg/mouse) and harvested on GD 10. Cellular populations were first gated on $CD45^+$ cells and then analyzed for NK1.1 versus CD3. Data from spleen and uterus are representative of eight mice per condition and numbers are averages of these data. (**D**) Graphs indicate statistical significance ($*p < 0.05$) of saline versus poly(I:C)-treated cellular populations as indicated.

Next, we identified the uterine cellular source of TNF-α in both WT and IL-$10^{-/-}$ mice as measured by intracellular staining and FACS analysis. Intracellular staining of $uNK1.1^+$, $uCD3/CD4^+$, and $uCD3/CD8^+$ cells showed that uNK cells were the main producer of TNF-α in WT mice in response to poly(I:C) (40 ± 4% versus 12 ± 2%; Fig. 2B). No such TNF-α increase occurred in WT mice from $CD4^+$ and $CD8^+$ T cells. In contrast, IL-$10^{-/-}$ mice showed both $CD4^+$ and $CD8^+$ T cells as the source of marked TNF-α production in response to poly(I:C) compared with saline treated controls ($CD4^+$: 23 ± 4% versus 8 ± 2%; $CD8^+$: 50 ± 4% versus 10 ± 2%; Fig. 2B).

To ensure that TNF-α was associated with fetal resorption, we neutralized TNF-α in vivo by injecting (i.p.) a neutralizing Ab on GD 5 and GD 7. As shown in Fig. 2C, normal fetal development was observed in poly(I:C)-treated WT and IL-$10^{-/-}$ mice upon TNF-α neutralization. In addition, in cases where mice with TNF-α neutralization were allowed to deliver, they gave birth to healthy litters of normal size at term (data not shown). Next, WT mice treated with NK cell–depleting Ab asialo-GM1 and exposed to saline or poly(I:C) exhibited no poly(I:C)-mediated fetal demise (Fig. 2D). Representative FACS data show that TNF-α production is abrogated and uNK cells are depleted in WT mice in response to poly(I:C) upon treatment with asialo-GM1 Ab (Fig. 2D). In contrast, similar NK cell depletion did not rescue pregnancy in IL-$10^{-/-}$ mice, and T cell subsets continued to produce TNF-α at high levels (data not shown). In this case, double depletion of both $CD4^+$ and $CD8^+$ T cells as assessed by flow cytometry significantly impaired TNF-α production and rescued pregnancy (Fig. 2D). These data provide evidence for the role of distinct uterine immune populations in inducing poly(I:C)-mediated fetal resorption in WT and IL-$10^{-/-}$ mice, respectively.

NKG2D is induced in $NK1.1^+$/TNF-α^+ uNK cells in WT mice in response to poly(I:C)

NKG2D is a molecule that enables NK cell activation and subsequent killing activity (36) and can be further induced in response to TLR3 activation (37). Next, we characterized the expression of NKG2D on splenic and uterine $NK1.1^+$ or $CD3^+$ cells from saline or poly(I:C)-treated WT or IL-$10^{-/-}$ mice. In agreement with the earlier data, we did not observe any changes in numbers of immune cells or NKG2D expression on splenic populations from either WT or IL-$10^{-/-}$ mice (Fig. 3A). WT mice treated with poly(I:C) exhibited significant upregulation of NKG2D on $uNK1.1^+$ cells compared with vehicle-treated mice (20 ± 4% versus 4 ± 2%; Fig. 3A). Importantly, IL-$10^{-/-}$ mice treated with poly(I:C) did not exhibit increased expression of NKG2D on $uNK1.1^+$ populations, nor was NKG2D induced in uterine $CD3^+$ population in WT or IL-$10^{-/-}$ mice (Fig. 3A, 3B).

To confirm that the expression of NKG2D was specific to the TNF-α^+ uNK cell population, we assessed $NKG2D^+$ uNK cells for intracellular TNF-α^+ staining by flow cytometry (Fig. 3B). A significant proportion of $NK1.1^+$ cells from WT mice were found to be $NKG2D^+$ in response to poly(I:C) and produced significantly high levels of TNF-α compared with untreated mice (24 ± 3% versus 5 ± 2%; Fig. 3B). In contrast, the $NK1.1^+/NKG2D^+$ population did not amplify or exhibit production of TNF-α in IL-$10^{-/-}$ mice. However, uterine $CD3^+$ T cells showed significant TNF-α production in response to poly(I:C) treatment (28 ± 3% versus 6 ± 2%; Fig. 3B). A graphical representation of the data from three different experiments is presented in Fig. 3C. The activating receptor NKG2D is generally expressed on NK and $CD8^+$ T cells under pathologic conditions (38, 39) or in response to TLR3 triggering (37). Our results are suggestive of a critical role of IL-10

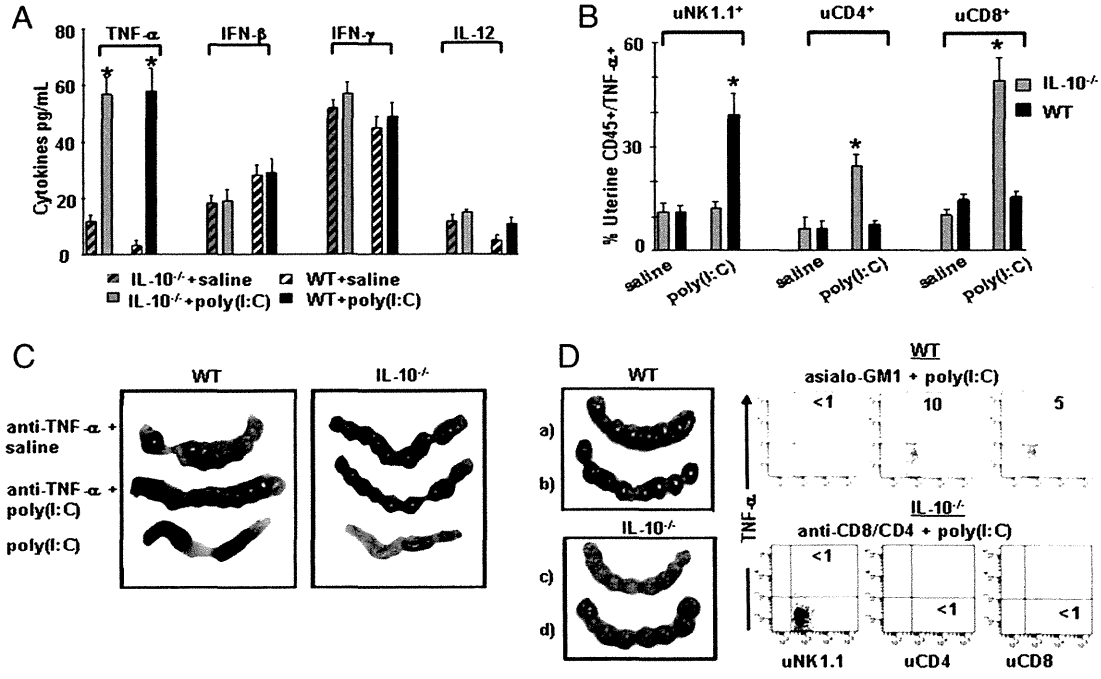


FIGURE 2. Measurement of cytokines and effect of in vivo TNF-α neutralization on fetal resorption in WT and IL-$10^{-/-}$ mice. (**A**) ELISA was performed using GD 10 serum samples from IL-$10^{-/-}$ or WT mice treated on GD 6 with saline or poly(I:C) (100 μg/mouse), and the analysis included TNF-α, IFN-β, IFN-γ, and IL-12. Bars represent mean ± SEM ($n = 8$ mice/group) and the asterisk ($*p < 0.05$) indicates significance between saline and poly(I:C) treatments. (**B**) FACS analysis of TNF-α–producing uterine NK1.1, $CD4^+$, and $CD8^+$ cells. Uterine mononuclear cells were isolated from GD 10 uteroplacental tissue and gated for CD45-positive staining and further analyzed for NK1.1, $CD4^+CD8^+$ subpopulations, and TNF-α expression. Graphic representation is provided from three independent experiments for intracellular staining of TNF-α produced by the indicated cellular populations in WT or IL-$10^{-/-}$ mice treated with saline or poly(I:C) on GD 6. Data are plotted as mean ± SEM ($n = 9$ mice/condition) and the asterisk indicates statistical significance ($*p < 0.05$) between saline- and poly(I:C)-treated samples. (**C**) Representative GD 10 uterine horns from WT (*left panel*) or IL-$10^{-/-}$ (*right panel*) mice treated with (from *top* to *bottom* of panels) saline plus anti–TNF-α Ab, poly(I:C) plus anti–TNF-α Ab, or poly(I:C) plus isotype Ab ($n = 4$ mice/condition). (**D**) Rescue of pregnancy in response to NK cell depletion by asialo-GM1 Ab and T cell depletion by anti-CD4 and anti-CD8 Abs: (**a**) GD 10 uterine horns from WT mice treated with asialo-GM1 Ab plus saline, (**b**) asialo-GM1 Ab plus poly(I:C), (**c**) GD 10 uterine horns from IL-$10^{-/-}$ mice treated with anti-CD4 Ab plus anti-CD8 Ab plus saline, and (**d**) anti-CD4 Ab plus anti-CD8 Ab plus poly(I:C). Corresponding dot plots represent data from four independent experiments in which TNF-α production was assessed from the cell populations indicated under depletion conditions featured in (b)–(d).

in induction of NKG2D on uNK cells when challenged by viral infections.

Direct evidence for IL-10–mediated upregulation of NKG2D

Our data thus far support the conclusion that the NKG2D receptor was induced in response to poly(I:C) only in WT, not IL-$10^{-/-}$, mice (Fig. 3). Thus, we aimed to provide direct evidence of whether IL-10 contributed to NKG2D induction on uNK cells. We first assessed the outcome of pregnancy in IL-$10^{-/-}$ mice supplemented with recombinant IL-10 (rIL-10) and treated with saline or poly(I:C). Uterine horns on GD 10 from IL-$10^{-/-}$ mice supplemented with rIL-10 showed resorbed embryo sites in response to poly(I:C) (Fig. 4A). Allowing a group of these mice to deliver confirmed these results as no pups were born (data not shown).

To determine whether rIL-10 directly induced $NKG2D^+$ uNK cell-expansion in IL-$10^{-/-}$ mice, we harvested uterine lymphocytes from IL-$10^{-/-}$ mice treated with rIL-10 and saline or poly(I:C). Uterine immune cell profile showed a significant increase in the $NK1.1^+/NKG2D^+$ population compared with saline-treated controls (26 ± 1% versus 10 ± 1%; Fig. 4B) and paralleled the uterine immune cell response observed in WT mice when treated with poly(I:C) (see Fig. 3). In contrast, $CD3^+$ uterine cells no longer amplified in response to poly(I:C) in IL-$10^{-/-}$ mice treated with rIL-10 (Fig. 4B). Furthermore, intracellular assessment of TNF-α production proved that $NK1.1^+/NKG2D^+$ cells now produced this cytokine (28 ± 3% versus 11 ± 1%; Fig. 4B). Analogous to WT mice, uterine $CD3^+$ T cells failed to amplify or produce TNF-α in response to poly(I:C) (Fig. 4B). Treatment of IL-10–deficient mice with rIL-10 showed similar NKG2D-mediated events as observed in WT mice, suggesting that pregnancy-compatible functions of IL-10 are compromised in the context of inflammatory challenges posed by poly(I:C)-like triggers (Fig. 7).

NKG2D is necessary for fetal loss induced by poly(I:C)

Based on the data presented in this study, we claim that induced expression of NKG2D on uNK cells is under the control of IL-10 and associated with fetal resorption in response to poly(I:C). Next, we aimed to determine whether $NKG2D^+$ uNK cells were the primary cause of fetal demise. We treated WT mice or IL-$10^{-/-}$ mice supplemented with rIL-10 with an NKG2D blocking Ab and injected either saline or poly(I:C) on GD 6 to assess fetal resorption on GD 10. Fig. 5A shows that an examination of uterine horns from WT and rIL-10–treated IL-$10^{-/-}$ mice did not reveal any evidence of fetal resorption, and when allowed, a subset of mice under these conditions gave birth to healthy litters at term (data not shown). Depletion of $NKG2D^+$ NK cells was confirmed by flow cytometry (Fig. 5B). No TNF-α–producing NK1.1/$NKG2D^+$ cells from the NKG2D depleted $uNK1.1^+$ population were observed in WT and rIL-10–supplemented IL-$10^{-/-}$ mice in response to poly(I:C) treatment (Fig. 5B).

Poly(I:C) treatment results in induction of NKG2D ligand Rae-1, predominantly in uterine macrophages

The NKG2D activating receptor interacts with the minor histocompatibility Ag ligands, the retinoic acid early inducible-1 (Rae-1) family members, and the heat shock 60 (H60) in mice (32, 40).

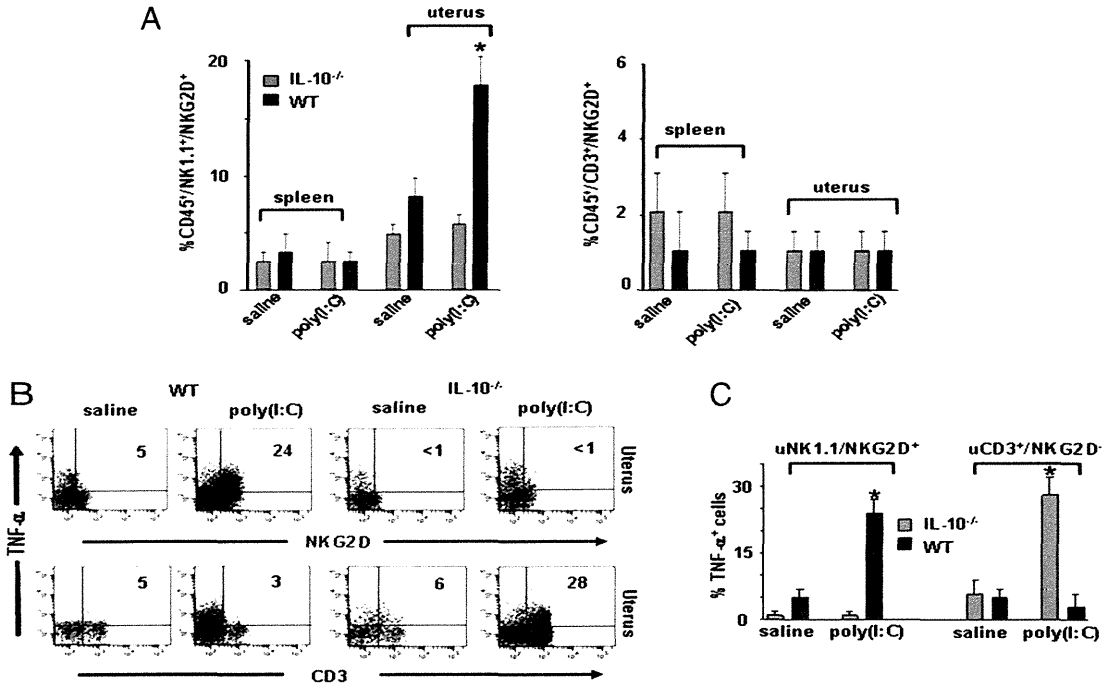


FIGURE 3. Induction of NKG2D in NK1.1$^+$/TNF- α^+ uNK cells in WT mice in response to poly(I:C). (**A**) Representative plots of splenic or uterine CD45$^+$NK1.1$^+$NKG2D$^+$ and CD45$^+$CD3$^+$NKG2D$^+$ cells as analyzed by FACS from GD 10 WT or IL-10$^{-/-}$ mice treated on GD 6 with saline or poly(I:C). Graphs are average of cell populations from eight mice per condition. The asterisk ($*p < 0.05$) indicates significance between saline and poly(I:C) treatment conditions. (**B**) Representative FACS analysis of intracellular TNF-α in uterine CD45$^+$ cells gated on the NKG2D$^+$ and CD3$^+$ subpopulations from GD 10 WT or IL-10$^{-/-}$ ice treated on GD 6 with saline or poly(I:C). (**C**) Summary graphs of TNF-α$^+$ cells gated NK1.1$^+$/NKG2D$^+$ and CD3$^+$/NKG3D$^-$ isolated from uterine tissue ($n = 8$ mice/condition). $*p < 0.05$ shows significance between saline and poly(I:C) treatment conditions.

In C57BL/6 mice, Rae-1 is thought to be the key induced NKG2D ligand in response to NK cell activation (41). Thus, the NKG2D–Rae-1 axis may further define the mechanism of poly(I:C)-induced fetal loss. Rae-1 has been shown to be expressed at the mRNA level in mouse embryonic tissues (32, 42). To assess its induced presence in the mouse placenta from untreated or poly(I:C)-treated WT mice, immunohistochemical analysis was performed using a Rae-1–specific Ab (Fig. 6A). Weak Rae-1–positive staining was observed throughout maternal and placental regions from saline-treated mice (original magnification ×4 and ×20), implying that decidual cells and trophoblasts express baseline levels of this NKG2D ligand. In contrast, Rae-1 immunostaining was notably induced in the decidual region from poly(I:C)-treated mice (original magnification ×20). To corroborate these data, we characterized decidual immune cells (T cells, NK cells, and macrophages) and trophoblast cells for Rae-1 expression by flow cytometry. Of the uteroplacental cell types (trophoblasts [CD45$^-$ CK7$^+$], T cells [CD3$^+$], macrophages [F4/80$^+$], and NK cells [NK1.1$^+$]) examined (data not shown), only macrophages (F4/80$^+$) showed significantly induced expression of Rae-1 in response to poly(I:C) when compared with saline-treated mice (24 ± 4% versus 5 ± 3%; Fig. 6B).

It is possible that interaction between NKG2D (uNK cells) and Rae-1 (macrophages and trophoblasts) activates NKG2D$^+$ NK cells to produce TNF-α, which in turn causes placental pathology and fetal resorption. We first attempted to assess placental tissue for trophoblast areas by staining with a cytokeratin 8-specific Ab (TROMA-I) to distinguish trophoblastic areas from maternal regions (Fig. 6C). These data demonstrate that trophoblasts in the saline-treated placental units remained flushed throughout the labyrinth and the junctional zone. In stark contrast, poly(I:C)-treated placental units showed excess trophoblast migration into the decidual region and beyond (Fig. 6C). We have shown previously that uNK cell–produced TNF-α caused placental cell death in response to LPS (23, 24). To elucidate whether uNK cells caused apoptosis in migrating trophoblasts as a result of direct interaction with Rae-1 ligand or TNF-α production, we stained uteroplacental tissue from saline or poly(I:C)-treated mice for TUNEL positive regions. Interestingly, TUNEL-positive cells were seen in significant numbers in the placental and decidual regions, particularly in invading trophoblasts from poly(I:C)-treated mice (Fig. 6C). Because TNF-α neutralization and NKG2D blockade abrogate TNF-α production and rescue pregnancy to term, our data in Fig. 6 suggest that NKG2D-Rae-1 interactions are critical for poly(I:C)-mediated fetal loss.

It has been suggested that trophoblast migration into the decidua is regulated by uNK cells and their product IFN-γ (43). It is then

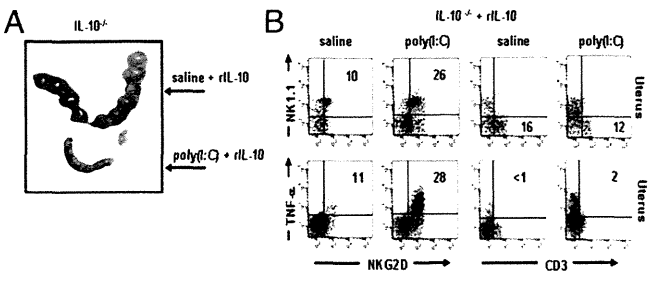


FIGURE 4. Recombinant IL-10 (rIL-10) induces NKG2D on uterine NK1.1 cells in IL-10$^{-/-}$ mice. (**A**) Representative GD 10 uterine horns from IL-10$^{-/-}$ mice treated with saline or poly(I:C) plus rIL-10 ($n = 4$ mice/condition). rIL-10 supplementation does not protect against fetal resorption. (**B**) Uterine populations were analyzed by FACS from IL-10$^{-/-}$ mice supplemented with rIL-10 and treated with saline or poly(I:C). Data represent mean ± SEM ($n = 4$ mice/condition). IL-10$^{-/-}$ mice supplemented with rIL-10 show NKG2D expression on NK1.1 cells that now produce TNF-α.

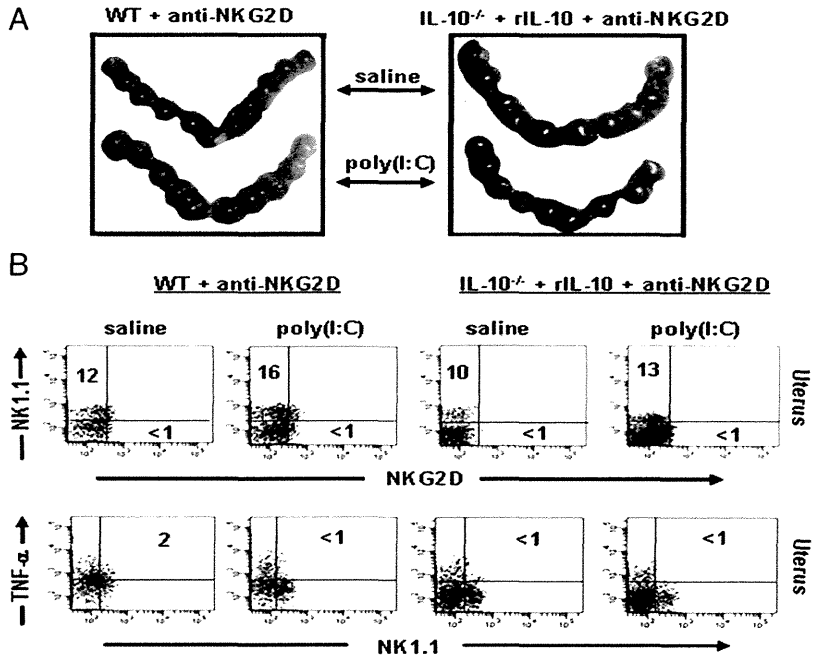


FIGURE 5. Depletion of NKG2D$^+$ NK cells rescues pregnancy and leads to loss of TNF-α production in WT and rIL-10 supplemented IL-10$^{-/-}$ mice in response to poly(I:C). (**A**) Representative uterine horns from GD 10 WT or rIL-10–supplemented IL-10$^{-/-}$ mice treated with anti-NKG2D Ab plus saline or anti-NKG2D Ab plus poly(I:C). Data represent mean ± SEM (n = 4 mice/condition). NKG2D depletion abrogated fetal resorption as demonstrated by normal fetal units. (**B**) Assessment of NKG2D depletion and loss of TNF-α production was performed by gating on CD45$^+$ NK1.1$^+$NKG2D$^+$ uterine cells from saline or poly(I:C)-treated GD 10 WT or rIL-10 plus IL-10$^{-/-}$ mice injected i.p. with anti-NKG2D Ab. Dot plots represent data from four or more animals per condition.

possible that uNK cells undergo proportional or functional changes, or both, in response to poly(I:C). Our data suggest that uNK cells, particularly NKG2D$^+$ cells, produce TNF-α and cause apoptosis in trophoblasts. To directly assess all uNK cells and their location in uteroplacental tissue from WT mice treated with poly(I:C), we performed DBA-lectin and PRF staining (14). Analyses of DBA-

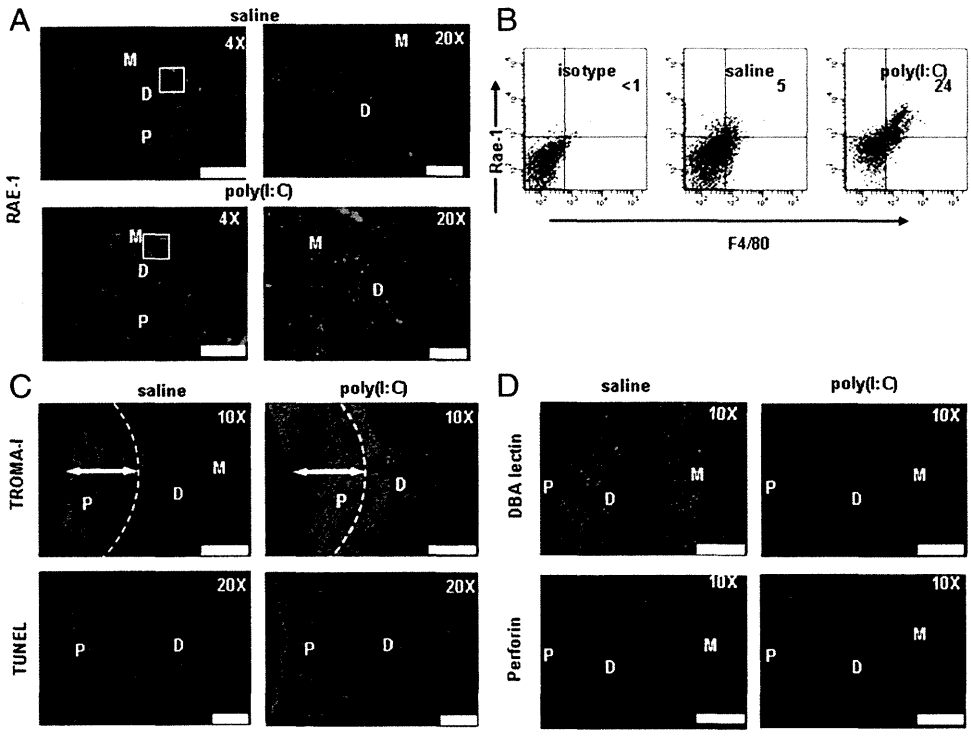


FIGURE 6. Exposure of pregnant WT mice to poly(I:C) results in induced expression of Rae-1 on macrophages and enhanced trophoblast invasion and cell death. (**A**) Representative images from uteroplacental units from GD 10 saline or poly(I:C)-treated WT mice were assessed by immunohistochemistry for RAE-1 expression. Weak staining for Rae-1 was detected in the decidua and placental regions (original magnification ×4; scale bar, 100 μm; and original magnification ×20; scale bar, 20 μm). (**B**) FACS analysis of Rae-1 on uterine immune cells. CD45$^+$ and CD45$^-$ cytokeratin7$^+$ cells were analyzed. Cells are gated from CD45$^+$ populations obtained from uterine populations. Data represent three independent experiments. (C) The *upper panel* illustrates immunohistochemical staining with TROMA-I Ab showing unique staining with increased invasion of trophoblast cells into the decidua in response to poly(I:C), but not saline (original magnification ×10; scale bar, 60 μm). The *lower panel* shows TUNEL staining (original magnification ×20, scale bar, 20 μm) demonstrating significantly increased cell death in the trophoblastic regions, including invading trophoblasts. Images are representative of three per condition. (**D**) DBA$^+$ and PRF$^+$ NK cell identification by immunofluorescence staining from uteroplacental unit from GD 10. In response to poly(I:C) treatment, DBA$^+$ intensity is diminished, but these cells maintain their PRF$^+$ phenotype. However, their distribution appears to be diffused through the placenta (P) region. Images are representative of results obtained from placental samples of three mice per condition (original magnification ×10; scale bar, 60 μm).

positive signal revealed greater numbers of DBA^+ cells in the mesometrial gland and the decidua basalis regions of uteroplacental tissue from saline-treated WT mice (Fig. 6D). In contrast, DBA staining intensity was poor in tissue from poly(I:C)-treated WT mice. Importantly, DBA^+ NK cells appeared to migrate to the placental zone. To rule out that poly(I:C) treatment does not lead to reduction in overall number of uNK cells, we performed PRF staining. As shown in Fig. 6D, PRF^+ uNK cells were predominant in the decidua basalis region in tissue from saline-treated animals and their presence is similar in intensity and number in tissue from poly(I:C)-treated mice. It is noteworthy that PRF^+ uNK cells in poly(I:C)-treated samples are present throughout the tissue and appear to migrate to the placental region, supporting the data for DBA staining. It is possible that $NKG2D^+$ uNK cells are DBA^{dim} but maintain their PRF^+ phenotype. The TUNEL-positive signal in the placental zone supports a possible role for DBA^{dim}/PRF^+ pathogenic uNK cells (Fig. 6C).

Discussion

In this report, we identify IL-10 as an integral cytokine that orchestrates TLR3-mediated expansion of effector uterine populations for fetal demise by contributing to induction of cytotoxic NK cell receptor NKG2D on uNK cells. $NKG2D^+$ uNK cells were identified as the source of TNF-α production, which led to fetal resorption in WT mice. This finding was supported by observations that blockade of the NKG2D receptor or neutralization of TNF-α rescued pregnancy to term. The relationship of IL-10 as an inducer of NKG2D was confirmed by direct upregulation of NKG2D on $uNK1.1^+TNF\text{-}\alpha^+$ cells in poly(I:C)-treated WT mice, but not $IL\text{-}10^{-/-}$ counterparts. Importantly, pregnant $IL\text{-}10^{-/-}$ mice supplemented with rIL-10 responded to poly(I:C) in the same manner as WT mice through the expansion of $NKG2D^+$ uNK cells. IL-10 is a pregnancy-compatible cytokine (4, 7, 25). Interestingly, our results indicate that TLR3-mediated induction of inflammation at the maternal-fetal interface could alter the anti-inflammatory characteristics of this cytokine. Rather, TLR3 activation and IL-10 together reverse the programming of a uterine immune response from T cell–mediated to NK cell–mediated. However, induced TNF-α production is still a key feature of both cell types in response to poly(I:C). Although not widely accepted a few years back, we proposed and demonstrated that uNK cells could be transformed into foes of pregnancy in response to LPS, and this pathway could be triggered in $IL\text{-}10^{-/-}$ mice even at a very low dose of LPS (0.5 μg/mouse) (23, 24, 44). In this study, we show that poly(I:C) can use an IL-10–rich environment to transform uNK cells into pregnancy-disrupting and TNF-α–producing $NKG2D^+$ NK cells. In the absence of IL-10, uterine T cells become the source of TNF-α and fetal loss. Our data strongly suggest that different inflammatory triggers are likely to exploit distinct immune cells and cytokine milieu at the maternal-fetal interface to cause pregnancy complications. This study also suggests that intrauterine viral infections alone as mimicked by poly(I:C) or in combination with other inflammatory triggers might transform the uterine immune milieu from tolerant to detrimental, resulting in adverse pregnancy outcomes.

Our results provide a mechanistic explanation for poly(I:C)-mediated TLR3 activation at the uterine level and its convergence with IL-10 in regulating innate and adaptive immune responses that lead to fetal loss (Fig. 7). Unscheduled expansion of uterine $NKG2D^+$ inflammatory NK cells and TNF-α production in WT mice support this notion. Importantly, induced expression of the NKG2D ligand Rae-1 by uterine macrophages can trigger overproduction of TNF-α by $NKG2D^+$ NK cells. TNF-α alone or cell-cell-contact between $NKG2D^+$ uNK cells and invading $Rae\text{-}1^+$ trophoblasts can lead to cell death as demonstrated by TUNEL positive staining in these cells. In contrast, IL-10 deficiency is

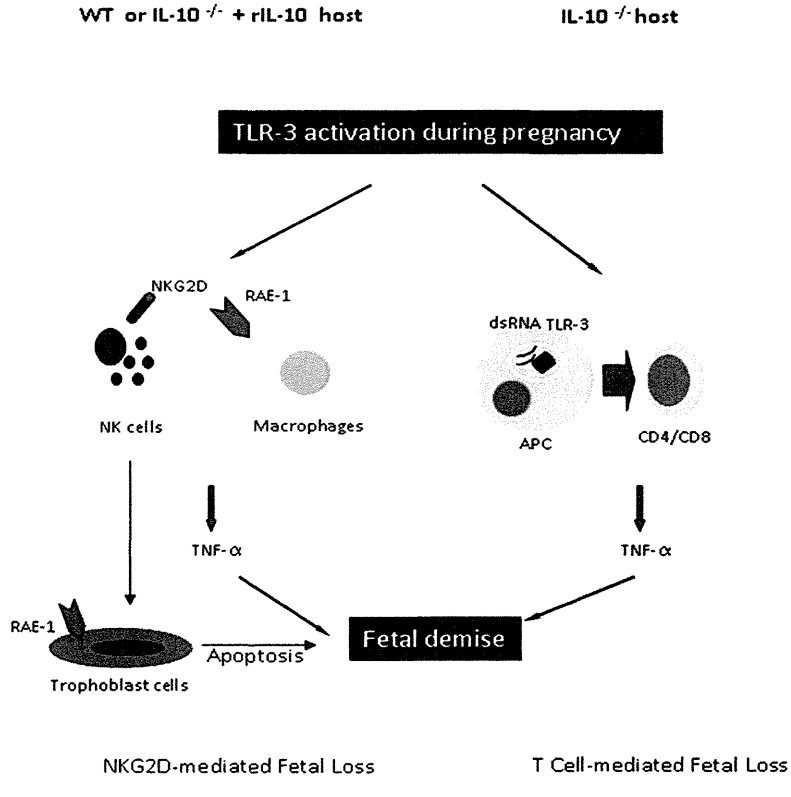


FIGURE 7. Schematic representation of the poly(I:C)-induced events in WT and $IL\text{-}10^{-/-}$ mice. The model recapitulates the pathways leading to uterine NK or T cell–mediated cytotoxicity and fetal resorption in WT and $IL\text{-}10^{-/-}$ mice, respectively. Rae-1–positive macrophages or trophoblasts can interact with $NKG2D^+$ NK cells and induce TNF-α production and trophoblast cell death in response to poly(I:C). In contrast, uterine T cells can be activated by poly(I:C) in the absence of IL-10 to produce TNF-α and to cause fetal demise.

likely to unleash T cell activation and cytokine storm that can restrain NK cell-mediated responses. Support for this notion comes from the results of Kim et al. (45), who suggested that a cytokine storm from adaptive immune cells could temper initial innate immune responses.

NKG2D is a well-characterized lectin-like activating receptor originally detected on NK cells (36). In humans, NKG2D has been shown to be expressed on $CD8^+$ T cells, γδ T cells (46), and intestinal epithelial cells in pathologic conditions or in response to treatment with poly(I:C) (37). Its blockade in NOD mice has been shown to prevent autoimmune diabetes (47). Surprisingly, we did not observe NKG2D expression on uterine T cells in either WT or IL-$10^{-/-}$ mice treated with poly(I:C) during pregnancy. Because uterine immune cells are specialized in their phenotypic and functional repertoire, it is possible that the response of uterine immune cells to poly(I:C) is equally unique.

Our data support the view that a significant proportion of $NK1.1^+$ NK cells acquires induced expression of NKG2D and produces TNF-α. In this regard, in vivo blockade of NKG2D alone rescued pregnancy in WT mice. It is intriguing that upon blockade of NKG2D, the integrity of the $NK1.1^+$ population was intact, and these cells remained pregnancy compatible. These results agree with a model of transplantation where NKG2D blockade allowed for increased graft survival, but the $NK1.1^+$ population remained unaffected and still migrated into the transplanted organ (38). These findings imply that it is important to identify requirements for molecular cascades that break immune tolerance at the maternal-fetal interface.

Our data in Fig. 6 provide important insights into the mechanisms underlying fetal demise in response to inflammatory triggers such as poly(I:C). We show enhanced trophoblast migration into the mesometrial decidual region in poly(I:C)-treated WT mice as demonstrated by TROMA-I positive trophoblast cells. These invading trophoblasts undergo cell death as demonstrated by TUNEL-positive signal in this region. Ain et al. (48, 49) have demonstrated that enhanced trophoblast migration occurs on GD 14 or thereafter in pregnant rats or mice. This timing is linked with reduction in uNK cells and their product IFN-γ in the mesometrial decidua. Because $NKG2D^+$ uNK cells are amplified in response to poly(I:C), it is possible that trophoblast migration occurs only as a result of reduced uNK cell population and their altered functional characteristics and localization pattern. Our data suggest that $NKG2D^+$ uNK cells produce TNF-α and maintain their PRF^+ phenotype. In the current study, excessive trophoblast invasion into the mesometrial decidua region occurs on GD 10 in response to poly(I:C). It is possible that induction of NKG2D on uNK cells alters their regulatory ability, which allows trophoblast invasion even on GD 10. However, their ability to produce TNF-α and to interact with trophoblasts via NKG2D–Rae-1 coupling also leads to cell death, resulting in defective hemochorial placentation. We reiterate that although uterine NK cells are beneficial in regulating normal pregnancy, they can be transformed into detrimental cells in response to bacterial and viral infections (23, 24, 44). Because $NK1.1^+$ cells represent only a subpopulation of uNK cells (15), it is possible that $NKG2D^+$ uNK cells could also belong to non-NK1.1 population and acquire the cytotoxic phenotype as a result of TNF-α production and different regulatory properties. Our data warrant a fresh look at the roles of IL-10, uterine NK cells, and T cells in adverse pregnancy outcomes.

Acknowledgments

We thank Paula Weston of the Molecular Pathology Core at Brown University for aiding in initial help with immunohistochemical analysis and Drs. Ann Hill, James Padbury, Sunil Shaw, and Angela Tatum for suggestions and critical reading of the manuscript.

Disclosures

The authors have no financial conflicts of interest.

References

1. Goldenberg, R. L., J. C. Hauth, and W. W. Andrews. 2000. Intrauterine infection and preterm delivery. *N. Engl. J. Med.* 342: 1500–1507.
2. Haun, L., N. Kwan, and L. M. Hollier. 2007. Viral infections in pregnancy. *Minerva Ginecol.* 59: 159–174.
3. Romero, R., J. Espinoza, T. Chaiworapongsa, and K. Kalache. 2002. Infection and prematurity and the role of preventive strategies. *Semin. Neonatol.* 7: 259–274.
4. Thaxton, J. E., T. A. Nevers, and S. Sharma. 2010. TLR-mediated preterm birth in response to pathogenic agents. *Infect. Dis. Obstet. Gynecol.* 2010: 1–8.
5. Moffet, A., and C. Loke. 2006. Immunology of placentation in eutherian mammals. *Nat. Rev. Immunol.* 8: 584–594.
6. Croy, B. A., J. A. Luross, M. J. Guimond, and J. S. Hunt. 1996-1997. Uterine natural killer cells: insights into lineage relationships and functions from studies of pregnancies in mutant and transgenic mice. *Nat. Immun.* 15: 22–33.
7. Thaxton, J. E., R. Romero, and S. Sharma. 2009. TLR9 activation coupled to IL-10 deficiency induces adverse pregnancy outcomes. *J. Immunol.* 183: 1144–1154.
8. Houser, B. L., T. Tilburgs, J. Hill, M. L. Nicotra, and J. L. Strominger. 2011. Two unique human decidual macrophage populations. *J. Immunol.* 186: 2633–2642.
9. Kopcow, H. D., D. S. Allan, X. Chen, B. Rybalov, M. M. Andzelm, B. Ge, and J. L. Strominger. 2005. Human decidual NK cells form immature activating synapses and are not cytotoxic. *Proc. Natl. Acad. Sci. USA* 102: 15563–15568.
10. Hanna, J., D. Goldman-Wohl, Y. Hamani, I. Avraham, C. Greenfield, S. Natanson-Yaron, D. Prus, L. Cohen-Daniel, T. I. Arnon, I. Manaster, et al. 2006. Decidual NK cells regulate key developmental processes at the human fetal-maternal interface. *Nat. Med.* 12: 1065–1074.
11. Kalkunte, S. S., T. F. Mselle, W. E. Norris, C. R. Wira, C. L. Sentman, and S. Sharma. 2009. Vascular endothelial growth factor C facilitates immune tolerance and endovascular activity of human uterine NK cells at the maternal-fetal interface. *J. Immunol.* 182: 4085–4092.
12. King, A., N. Balendran, P. Wooding, N. P. Carter, and Y. W. Loke. 1991. CD3-leukocytes present in the human uterus during early placentation: phenotypic and morphologic characterization of the $CD56^+$+ population. *Dev. Immunol.* 1: 169–190.
13. Lash, G. E., S. C. Robson, and J. N. Bulmer. 2010. Review: Functional role of uterine natural killer (uNK) cells in human early pregnancy decidua. *Placenta* 31 (Suppl): S87–S92.
14. Paffaro, V. A., Jr., M. C. Bizinotto, P. P. Joazeiro, and A. T. Yamada. 2003. Subset classification of mouse uterine natural killer cells by DBA lectin reactivity. *Placenta* 24: 479–488.
15. Yadi, H., S. Burke, Z. Madeja, M. Hemberger, A. Moffett, and F. Colucci. 2008. Unique receptor repertoire in mouse uterine NK cells. *J. Immunol.* 181: 6140–6147.
16. Piccinni, M. P. 2010. T cell tolerance towards the fetal allograft. *J. Reprod. Immunol.* 85: 71–75.
17. Aluvihare, V. R., M. Kallikourdis, and A. G. Betz. 2004. Regulatory T cells mediate maternal tolerance to the fetus. *Nat. Immunol.* 5: 266–271.
18. Shima, T., Y. Sasaki, M. Itoh, A. Nakashima, N. Ishii, K. Sugamura, and S. Saito. 2010. Regulatory T cells are necessary for implantation and maintenance of early pregnancy but not late pregnancy in allogeneic mice. *J. Reprod. Immunol.* 85: 121–129.
19. Kahn, D. A., and D. Baltimore. 2010. Pregnancy induces a fetal antigen-specific maternal T regulatory cell response that contributes to tolerance. *Proc. Natl. Acad. Sci. USA* 107: 9299–9304.
20. Nepomnaschy, P. A., E. Sheiner, G. Mastorakos, and P. C. Arck. 2007. Stress, immune function, and women's reproduction. *Ann. N. Y. Acad. Sci.* 1113: 350–364.
21. Lai, Y., A. Di Nardo, T. Nakatsuji, A. Leichtle, Y. Yang, A. L. Cogen, Z. R. Wu, L. V. Hooper, R. R. Schmidt, S. von Aulock, et al. 2009. Commensal bacteria regulate Toll-like receptor 3-dependent inflammation after skin injury. *Nat. Med.* 15: 1377–1382.
22. Cavassani, K. A., M. Ishii, H. Wen, M. A. Schaller, P. M. Lincoln, N. W. Lukacs, C. M. Hogaboam, and S. L. Kunkel. 2008. TLR3 is an endogenous sensor of tissue necrosis during acute inflammatory events. *J. Exp. Med.* 205: 2609–2621.
23. Murphy, S. P., L. D. Fast, N. N. Hanna, and S. Sharma. 2005. Uterine NK cells mediate inflammation-induced fetal demise in IL-10-null mice. *J. Immunol.* 175: 4084–4090.
24. Murphy, S. P., N. N. Hanna, L. D. Fast, S. K. Shaw, G. Berg, J. F. Padbury, R. Romero, and S. Sharma. 2009. Evidence for participation of uterine natural killer cells in the mechanisms responsible for spontaneous preterm labor and delivery. *Am. J. Obstet. Gynecol.* 200: 308.e1–e9.
25. Kalkunte, S., T. Nevers, W. E. Norris, and S. Sharma. 2011. Vascular IL-10: a protective role in preeclampsia. *J. Reprod. Immunol.* 88: 165–169.
26. Gallucci, S., M. Lolkema, and P. Matzinger. 1999. Natural adjuvants: endogenous activators of dendritic cells. *Nat. Med.* 5: 1249–1255.
27. Sauter, B., M. L. Albert, L. Francisco, M. Larsson, S. Somersan, and N. Bhardwaj. 2000. Consequences of cell death: exposure to necrotic tumor cells, but not primary tissue cells or apoptotic cells, induces the maturation of immunostimulatory dendritic cells. *J. Exp. Med.* 191: 423–434.
28. Marshak-Rothstein, A. 2006. Toll-like receptors in systemic autoimmune disease. *Nat. Rev. Immunol.* 6: 823–835.

29. de Fougerolles, A. R., and M. G. Baines. 1987. Modulation of the natural killer cell activity in pregnant mice alters the spontaneous abortion rate. *J. Reprod. Immunol.* 11: 147–153.
30. Koga, K., I. Cardenas, P. Aldo, V. M. Abrahams, B. Peng, S. Fill, R. Romero, and G. Mor. 2009. Activation of TLR3 in the trophoblast is associated with preterm delivery. *Am. J. Reprod. Immunol.* 61: 196–212.
31. Raulet, D. H. 2003. Roles of the NKG2D immunoreceptor and its ligands. *Nat. Rev. Immunol.* 3: 781–790.
32. Cerwenka, A., A. B. Bakker, T. McClanahan, J. Wagner, J. Wu, J. H. Phillips, and L. L. Lanier. 2000. Retinoic acid early inducible genes define a ligand family for the activating NKG2D receptor in mice. *Immunity* 12: 721–727.
33. Lee, S. H., K. S. Kim, N. Fodil-Cornu, S. M. Vidal, and C. A. Biron. 2009. Activating receptors promote NK cell expansion for maintenance, IL-10 production, and CD8 T cell regulation during viral infection. *J. Exp. Med.* 206: 2235–2251.
34. Brooks, D. G., K. B. Walsh, H. Elsaesser, and M. B. Oldstone. 2010. IL-10 directly suppresses CD4 but not CD8 T cell effector and memory responses following acute viral infection. *Proc. Natl. Acad. Sci. USA* 107: 3018–3023.
35. Ghosh, T. K., D. J. Mickelson, J. C. Solberg, K. E. Lipson, J. R. Inglefield, and S. S. Alkan. 2007. TLR-TLR cross talk in human PBMC resulting in synergistic and antagonistic regulation of type-1 and 2 interferons, IL-12 and TNF-alpha. *Int. Immunopharmacol.* 7: 1111–1121.
36. Jamieson, A. M., A. Diefenbach, C. W. McMahon, N. Xiong, J. R. Carlyle, and D. H. Raulet. 2002. The role of the NKG2D immunoreceptor in immune cell activation and natural killing. *Immunity* 17: 19–29.
37. Zhou, R., H. Wei, R. Sun, J. Zhang, and Z. Tian. 2007. NKG2D recognition mediates Toll-like receptor 3 signaling-induced breakdown of epithelial homeostasis in the small intestines of mice. *Proc. Natl. Acad. Sci. USA* 104: 7512–7515.
38. Kim, J., C. K. Chang, T. Hayden, F. C. Liu, J. Benjamin, J. A. Hamerman, L. L. Lanier, and S. M. Kang. 2007. The activating immunoreceptor NKG2D and its ligands are involved in allograft transplant rejection. *J. Immunol.* 179: 6416–6420.
39. Bauer, S., V. Groh, J. Wu, A. Steinle, J. H. Phillips, L. L. Lanier, and T. Spies. 1999. Activation of NK cells and T cells by NKG2D, a receptor for stress-inducible MICA. *Science* 285: 727–729.
40. Diefenbach, A., E. R. Jensen, A. M. Jamieson, and D. H. Raulet. 2001. Rae1 and H60 ligands of the NKG2D receptor stimulate tumour immunity. *Nature* 413: 165–171.
41. Hamerman, J. A., K. Ogasawara, and L. L. Lanier. 2004. Cutting edge: Toll-like receptor signaling in macrophages induces ligands for the NKG2D receptor. *J. Immunol.* 172: 2001–2005.
42. Xie, X., H. He, M. Colonna, T. Seya, T. Takai, and B. A. Croy. 2005. Pathways participating in activation of mouse uterine natural killer cells during pregnancy. *Biol. Reprod.* 73: 510–518.
43. Ain, R., L. N. Canham, and M. J. Soares. 2003. Gestation stage-dependent intrauterine trophoblast cell invasion in the rat and mouse: novel endocrine phenotype and regulation. *Dev. Biol.* 260: 176–190.
44. Kalkunte, S., C. O. Chichester, F. Gotsch, C. L. Sentman, R. Romero, and S. Sharma. 2008. Evolution of non-cytotoxic uterine natural killer cells. *Am. J. Reprod. Immunol.* 59: 425–432.
45. Kim, K. D., J. Zhao, S. Auh, X. Yang, P. Du, H. Tang, and Y. X. Fu. 2007. Adaptive immune cells temper initial innate responses. *Nat. Med.* 13: 1248–1252.
46. Stern-Ginossar, N., and O. Mandelboim. 2009. An integrated view of the regulation of NKG2D ligands. *Immunology* 128: 1–6.
47. Ogasawara, K. J. A., J. A. Hamerman, L. R. Ehrlich, H. Bour-Jordan, P. Santamaria, J. A. Bluestone, and L. L. Lanier. 2004. NKG2D blockade prevents autoimmune diabetes in NOD mice. *Immunity* 20: 757–767.
48. Ain, R., J. S. Tash, and M. J. Soares. 2003. Prolactin-like protein-A is a functional modulator of natural killer cells at the maternal-fetal interface. *Mol. Cell. Endocrinol.* 204: 65–74.
49. Ain, R., G. Dai, J. H. Dunmore, A. R. Godwin, and M. J. Soares. 2004. A prolactin family paralog regulates reproductive adaptations to a physiological stressor. *Proc. Natl. Acad. Sci. USA* 101: 16543–16548.

doi:10.1111/j.1447-0756.2012.01973.x J. Obstet. Gynaecol. Res. 2012

Frequency of recurrent spontaneous abortion and its influence on further marital relationship and illness: The Okazaki Cohort Study in Japan

Mayumi Sugiura-Ogasawara[1], Sadao Suzuki[2], Yasuhiko Ozaki[1], Kinue Katano[1], Nobuhiro Suzumori[1] and Tamao Kitaori[1]

Departments of [1]Obstetrics and Gynecology and [2]Public Health, Graduate School of Medical Sciences, Nagoya City University, Nagoya, Japan

Abstract

Aims: The aim of this study was to examine the influence of recurrent spontaneous abortion (RSA) on marital relationships, and the association between past/present illness and RSA.

Material and Methods: A total of 2733 Japanese women who underwent a medical examination responded to the questionnaire.

Results: The frequency of recurrent miscarriage and two or more consecutive RSA were 0.88% and 4.2%, respectively. Women with a history of miscarriages (hazard ratio: 1.596) and RSA (hazard ratio: 3.103) were at a higher risk of their relationships ending as compared with the women without a history of miscarriage. Existence of a relation was seen between a history of RSA and the occurrence of gastric ulcer, gastritis, fatty liver, and atopic dermatitis. Overall, 89.5% of the women with RSA experienced cumulative live births.

Conclusions: Miscarriage was found to be a severe life event with an influence on marital relationships, and to be associated with an elevated risk of gastric disease or atopic dermatitis.

Key words: atopic dermatitis, gastric ulcer, marital relationship, recurrent miscarriage, recurrent spontaneous abortion.

Introduction

Miscarriage is the most common complication during pregnancy, and a sporadic miscarriage is defined as a single or a maximum of two episodes of spontaneous pregnancy loss prior to the completion of 20 weeks' gestation. The estimated incidence is 12–16%, and mainly depends on the woman's age.[1] Recurrent miscarriage (RM) is conventionally defined as three or more miscarriages, and the estimated incidence is about 1%.[2] Some clinical researchers define two or more miscarriages as recurrent spontaneous abortion (RSA). Established causes of RSA are the presence of antiphospholipid antibodies (aPL), uterine anomalies, and abnormal chromosomes in either partner, particularly translocations;[3–5] chromosomal abnormalities in the embryo have also been reported as a causative factor of RSA.[6] However, in some cases, RSA remains unexplained.

Recently, a prospective population-based cohort study reported that women who experienced pregnancy loss are at a substantially higher risk of developing myocardial infarction.[7] The authors stated that RM and stillbirth should be considered as important factors in cardiovascular risk monitoring and in the adoption of preventive measures against cardiovascular diseases. It is speculated that recurrent pregnancy loss, which includes RM and/or stillbirth, is associated with

Received: January 10 2012.
Accepted: May 17 2012.
Reprint request to: Dr Mayumi Sugiura-Ogasawara, Department of Obstetrics and Gynecology, Nagoya City University, Graduate School of Medical Sciences, Kawasumi 1, Mizuho-ku, Nagoya 4678601, Japan. Email: og.mym@med.nagoya-cu.ac.jp

an elevated risk of cardiovascular disease mediated by aPL. Diagnostic criteria of the antiphospholipid syndrome include pregnancy complications, such as RM, intrauterine fetal death, and arterial/venous thrombophilia.[8] There is limited epidemiological information concerning the association between miscarriage and the history of past/present illness, although such population-based cohort studies have the potential to clarify the causes of miscarriage.

We conducted a population-based cohort study to examine the frequency of at least one miscarriage and RSA, and their influence on marital relationships, and also the association between miscarriage and the history of past/present illness in the Japanese population.

Methods

Participants

This study was conducted in Okazaki City, Aichi Prefecture, from April 2007 to May 2010. Study candidates were selected from female health checkup examinees ranging from 35 to 79 years old at the Okazaki Public Health Center during the period specified above. Invitation letter with study protocol and questionnaire were individually mailed before the examination. On the morning of the examination day, the study staff (medical doctors or nurses) provided a one-on-one explanation of the purpose and design of the study to the subjects. A total of 2733 women who agreed to participate in the study provided written informed consent, including answering the questionnaire described below. The percentage of the population in this age range in Okazaki City in 2009 was 2.7%.

Questionnaire

The questionnaire included questions to determine the following: sex, age, marital status, height, bodyweight at present and at 20 years old, and the menstrual and pregnancy history, including the age at menarche; the menstrual history was classified on a Likert scale of 1–4 as follows: 1, regular; 2, slightly irregular; 3, irregular; 4, no menses.

The dysmenorrhea severity was classified on a Likert scale of 1–4 as follows: 1, no dysmenorrhoea; 2, have pain sometimes; 3, often have (had) pain; 4, frequently have severe pain during menstruation.

The experience of pregnancy, no or yes; total number of pregnancies, including miscarriages and stillbirths.

Experience of miscarriage, no or yes; total number of consecutive miscarriages; total number of miscarriages.

RM was defined as three or more consecutive miscarriages. RSA was defined as two or more consecutive spontaneous abortions.

Sporadic miscarriage was defined as one or two miscarriages that were not consecutive and were interrupted by live births.

No miscarriage was defined as no history of miscarriage in women with a pregnancy.

Experience of birth, no or yes; total number of births, including stillbirths; age at the time of first birth.

Experience of breast-feeding, no or yes.

Self-score from 0–100 points to describe the physical health status.

Self-score from 0–100 points to describe the feeling of happiness.

History of past/present illness: gastric ulcer, duodenal ulcer, gastritis, large intestinal polyp, hepatitis B, hepatitis C, cirrhosis, fatty liver, tuberculosis, asthma, bronchitis, diabetes, hyperlipidemia, hypertension, atopic dermatitis, urinary calculi, mastopathy, myocardial infarction, stroke.

We examined the association between the experience of at least one miscarriage and RSA and the history of past /present illness.

The study was approved by the Research Ethics Committee at Nagoya City University Medical School.

Statistics

Data were analyzed by *t*-tests and χ^2-test using SAS software. $P < 0.05$ was considered to denote statistical significance.

Results

The total number of participants in the Okazaki Cohort Study was 6086 (female: 2733). The mean age was 56.8 ± 10.3 years. The mean (SD) age of the first marriage was 23.9 years (3.4).

The mean (SD) number of pregnancies in a total of 2503 women was 2.96 (1.21). In all, 38.3% (958/ 2503) of the women with a history of pregnancy had experienced at least one miscarriage. Among these, 22 women gave a history of three or more miscarriages and 105 gave a history of two or more miscarriages; 83 women had two, 20 had three, and two had four miscarriages. Thus, the frequency of RM and RSA were 0.88% and 4.2%, respectively. The mean age of the women with sporadic miscarriage and RSA was higher than that of the women who had never suffered a miscarriage (<0.0001, 0.057, Table 1).

Table 1 Characteristics of women with recurrent spontaneous abortion, sporadic miscarriage and no miscarriage

	No miscarriage		Sporadic miscarriage		*P*-value	RSA		*P*-value
Number	1542		853			105		
Age*	56.3	(10.0)	59.1	(9.8)	<0.0001	58.2	(10.5)	0.057
BMI at 20 years of age*	20.6	(2.2)	20.8	(2.4)	0.12	20.7	(2.3)	0.82
Menstrual cycle								
Regular	1125	73.0%	612	71.7%	0.35	68	64.8%	0.028
Slightly irregular	268	17.4%	147	17.2%		20	19.0%	
Irregular	148	9.6%	93	10.9%		17	16.2%	
No menses	1	0.1%	1	0.1%		0	0.0%	
Dysmenorrhoea								
Yes (at least 'sometimes')	991	64.3%	558	65.4%	0.6	76	72.4%	0.11
Not at all	549	35.6%	295	34.6%		29	27.6%	
Marital status								
Married	1476	97.0%	804	95.3%	0.043	93	91.2%	0.0043
Divorce	46	3.0%	40	4.7%		9	8.8%	
Cumulative birth								
Yes	1506	99.0%	820	97.0%	0.0007	100	95.2%	0.0033
No	15	1.0%	25	3.0%		5	4.8%	
Breast-feeding								
Yes	1450	95.5%	767	93.8%	0.095	94	94.0%	0.067
No	69	4.5%	51	6.2%		6	6.0%	
Feeling about physical health status*	74.4	(14.2)	75.2	(14.9)	0.41	75.7	(13.3)	0.32
Feeling of happiness*	78.4	(14.8)	79.8	(14.8)	0.52	79.4	(14.6)	0.48

*Average (SD). *P*-values: vs 'No miscarriage'. BMI, body mass index.

The divorce rate in women with a history of RSA or sporadic miscarriage was significantly higher than that in the women who had no experience of a miscarriage (8.8% vs 4.7% vs 3.0%). Women with a history of miscarriages (hazard ratio: 1.596; 95% confidence interval [CI], 1.036–2.460; $P = 0.043$) and RSA (hazard ratio: 3.103; 95%CI: 1.474–6.53; $P = 0.0043$) were at a higher risk of their relationships ending, as compared with the women who had never suffered a miscarriage.

Body mass index (BMI) and obesity at 20 years of age and a history of dysmenorrhoea were not associated with miscarriage. A history of irregular menstrual cycles was not found to be associated with miscarriage, but was associated with RSA ($P = 0.028$).

There was no correlation between feeling about physical health status and the experience of miscarriage, with scores for this item in the women with RSA, sporadic miscarriage and no miscarriage, of 74.4 (14.2), 75.2 (14.9) and 75.7 (13.3), respectively. There was also no correlation between the score for a feeling of happiness and the experience of miscarriage, because the scores for this item in the women with RSA, sporadic miscarriage and no miscarriage, were 78.4 (14.8), 79.8 (14.8) and 79.4 (14.6), respectively.

A relation was seen between a history of RSA and the occurrence of gastric ulcer ($P = 0.035$), gastritis (<0.0001), fatty liver (0.031), and atopic dermatitis (0.031, Table 2). No significant relation was noted between a history of miscarriage and the risk of development of myocardial infarction (0.065).

Of the 105 women with RSA, 100 (95.2%) experienced cumulative births, and furthermore, 94 (89.5%) described a history of breast-feeding, a surrogate marker for live birth. A discrepancy of numbers occurred because some participants did not respond to some questions.

Discussion

The frequency of RM was estimated to be 0.9%, and that of RSA to be 4.2% in Japan, and 38% of all women with a history of pregnancy had experienced at least one miscarriage.

A previous study had indicated an increased risk of early miscarriage (odds ratio [OR] 1.2, 95%CI 1.01–1.46, $P = 0.04$) and RM (OR 3.5, 95%CI 1.03–12.01, $P = 0.04$) in obese women after natural conception, as compared with women within the normal weight range.[9] The problem of obesity is reported to be the greatest among non-Hispanic African-American women (48.8%), as compared with Mexican-American (38.9%) and non-Hispanic Caucasian women (31.3%).[9] There were only

Table 2 Influence of miscarriage on past/present illness

	No miscarriage		Sporadic miscarriage			RSA			*P* for
	Yes/no	OR	Yes/no	OR	(95%CI)	Yes/no	OR	(95%CI)	trend
Gastric ulcer	132/1402	Reference	95/760	1.31	(0.99–1.74)	13/90	1.50	(0.82–2.77)	0.035
Duodenal ulcer	82/1455	Reference	64/787	1.41	(1.01–1.99)	5/100	0.88	(0.35–2.21)	0.194
Gastritis	135/1391	Reference	124/719	1.71	(1.31–2.22)	28/76	3.70	(2.31–5.92)	<0.0001
Colon polyp	82/1448	Reference	65/781	1.29	(0.91–1.81)	6/98	0.97	(0.41–2.29)	0.317
Hepatitis B	10/1518	Reference	10/834	1.81	(0.74–4.39)	2/101	2.99	(0.65–13.86)	0.090
Hepatitis C	17/1513	Reference	9/835	0.91	(0.40–2.05)	1/104	0.82	(0.11–6.23)	0.774
Cirrhosis	6/1532	Reference	4/848	1.20	(0.33–4.32)	0/105	<0.001	<0.001-	0.882
Fatty liver	104/1415	Reference	67/768	1.14	(0.83–1.57)	15/89	2.23	(1.24–3.99)	0.031
Tuberculosis	35/1501	Reference	24/827	1.01	(0.59–1.72)	4/100	1.45	(0.50–4.22)	0.671
Asthma	103/1437	Reference	62/789	1.17	(0.84–1.63)	10/94	1.55	(0.78–3.06)	0.163
Bronchitis	46/1493	Reference	28/819	1.09	(0.68–1.77)	5/99	1.62	(0.63–4.18)	0.398
Diabetes	77/1462	Reference	48/803	0.94	(0.65–1.37)	3/102	0.48	(0.15–1.54)	0.336
Hyperlipidemia	399/1134	Reference	244/592	0.97	(0.79–1.18)	28/73	0.95	(0.59–1.52)	0.716
Hypertension	302/1234	Reference	201/648	1.06	(0.86–1.31)	29/76	1.38	(0.87–2.21)	0.243
Myocardial infarction	30/1510	Reference	32/818	1.49	(0.89–2.50)	5/99	2.05	(0.76–5.55)	0.065
Stroke	20/1521	Reference	19/832	1.53	(0.81–2.91)	3/102	2.03	(0.59–6.98)	0.121
Atopic dermatitis	102/1429	Reference	71/777	1.44	(1.05–1.99)	9/95	1.42	(0.69–2.92)	0.031
Urinary calculus	80/1457	Reference	62/787	1.35	(0.95–1.90)	7/97	1.26	(0.56–2.80)	0.125
Mastopathy	257/1265	Reference	160/679	1.16	(0.93–1.45)	18/87	1.02	(0.60–1.72)	0.312

CI, confidence interval; OR, odds ratio; RSA, recurrent spontaneous abortion.

seven women with a BMI of 30 or greater in the present study. Thus, there was no influence of BMI on the risk of miscarriage in the Japanese population.

Fatty liver is common in women with the polycystic ovary syndrome (PCOS).[10] PCOS is a well-known causative factor of RSA in Caucasian women.[11] It is unclear whether PCOS may also cause RSA in Japanese women, because our previous study, which was conducted to investigate this issue, failed to demonstrate any association; furthermore, the frequency of PCOS is relatively low in Japanese women.[12] In the present study, a direct association was noted between fatty liver and RSA.

Luteal phase defect, defined by the criterion of a mid-luteal serum progesterone level of <10 ng/mL, was seen in 23.4% of the patients with RSA.[13] The progesterone levels did not have a predictive value for further miscarriages. There is no evidence to support the routine use of progestogens to prevent miscarriages.[14] However, there seems to be some evidence of the beneficial effect of progestogen use in women with a history of RM.[14] Further study is warranted to confirm whether progesterone treatment might prevent further miscarriages in women with a history of irregular menstruation.

The present study is the first to provide evidence of the existence of an association between miscarriage and atopic dermatitis/gastric disease. Atopic dermatitis is a major public health problem worldwide, with a lifetime prevalence in children of 10–20% and a prevalence of 1–3% in adults.[15] The disease prevalence has increased by two- to threefold during the past 3 decades in industrialized countries, whereas it remains much lower in agricultural regions, such as China, Eastern Europe, and rural Africa. The risk factors include a small family size, increased income and education, migration from rural to urban environments, and increased use of antibiotics, all of which represent the so-called Western lifestyle.[16] Several genes have been identified that may explain the occurrence of the disease in some cases.[17] While allergens, such as house dust mites and foods, may be important in some cases, non-allergic factors, such as rough clothing, *Staphylococcus aureus* infections, exposure to microbes during infancy, exposure to excessive heat, and exposure to irritants that disrupt the function of the skin barrier, may also be important.[16]

Two hypotheses concerning the mechanism of atopic dermatitis have been proposed. One suggests that the primary defect resides in an immunologic disturbance that causes IgE-mediated sensitization, with epithelial-barrier dysfunction occurring as a consequence of the local inflammation. The other proposes that an intrinsic defect in the epithelial cells leads to dysfunction of the skin barrier; the immunologic aspects are considered to be epiphenomena.[18] A high seroprevalence of

autoantibodies, such as antiphospholipid antibody and anti-laminin 1 antibody, has been found in patients with RSA.[8,19] An immunologic disturbance of the semiallograft at the feto-maternal interface is one of the proposed hypotheses to explain the mechanism underlying unexplained RSA.

Helicobacter pylori infection-related diseases are known to include gastritis, gastric and duodenal ulcer, gastric cancer, idiopathic thrombocytopenic purpura, iron-deficient anemia, urticaria, reflux esophagitis, and some lifestyle-related diseases.[20] Existence of an association between *Helicobacter pylori* infection, reduced cobalamin absorption and the cobalamin status, and consequently, elevated homocysteine levels, has been suggested.[21] It has been shown that homocysteine involved in the pathogenesis of arteriosclerosis induces lifestyle-related diseases. Elevated levels of homocysteine may be among the causative factors of miscarriage.[22] Further study concerning the relation with *Helicobacter pylori* infection is needed, as there is no evidence in the published reports yet, to the best of our knowledge, of *Helicobacter pylori* infection as a causative factor of miscarriage.

A previous study reported that women who experienced miscarriages (hazard ratio: 1.22 [95%CI: 1.08–1.38]; $P = 0.001$) or stillbirths (hazard ratio: 1.40 [95%CI: 1.10–1.79]; $P = 0.007$) were at a significantly greater risk of their relationships ending, as compared with women whose pregnancies ended in live births.[1] In the present study, a higher risk of divorce was seen in women with a history of miscarriages (hazard ratio: 1.596 [95%CI: 1.036–2.460]; $P = 0.043$) and RSA (hazard ratio: 3.103 [95%CI: 1.474–6.53]; $P = 0.0043$). Miscarriage and its recurrence were found to have a more severe adverse influence on marital relationships in Japan than in the USA. The emotional impact of miscarriage is higher in women than in men, and this discrepancy might alter the spousal relationship.[23]

The most important limitation of this study was that the history of illness was not confirmed by medical records but by self-declaration, and stillbirth was not distinguished from miscarriage. However, it was found that at least 89.5% of the women with a history of RSA had subsequent live births, although our previous study reported that the cumulative live birth rate in women with RSA was 85.5%.[4] Therefore, in order to prevent decline of marital relationships related to this issue, it is necessary to inform women that miscarriage is a very common complication during pregnancy and that about 90% of women with RSA may be expected to have live births.

Acknowledgments

This study was supported by Grants-in-Aid for Scientific Research from the Ministry of Health, Labour and Welfare (to M.S-O) and the Ministry of Education, Culture, Sports, Science, and Technology of Japan (to S.S.).

Disclosure

None of the authors has anything to disclose. The corresponding author has the right to grant on behalf of all authors and does grant on behalf of all authors an exclusive license.

References

1. Gold KJ, Sen A, Hayward RA. Marriage and cohabitation outcomes after pregnancy loss. *Pediatrics* 2010; **125**: 1202–1207.
2. Herz-Picciotto I, Samuels SJ. Incidence of early pregnancy loss of pregnancy. *N Engl J Med* 1998; **319**: 1483–1484.
3. Farquharson RG, Pearson JF, John L. Lupus anticoagulant and pregnancy management. *Lancet* 1984; **28**: 228–229.
4. Sugiura-Ogasawara M, Ozaki Y, Kitaori T, Kumagai K, Suzuki S. Midline uterine defect size correlated with miscarriage of euploid embryos in recurrent cases. *Fertil Steril* 2010; **93**: 1983–1988.
5. Sugiura-Ogasawara M, Ozaki Y, Sato T, Suzumori N, Suzumori K. Poor prognosis of recurrent aborters with either maternal or paternal reciprocal translocation. *Fertil Steril* 2004; **81**: 367–373.
6. Ogasawara M, Aoki K, Okada S, Suzumori K. Embryonic karyotype of abortuses in relation to the number of previous miscarriages. *Fertil Steril* 2000; **73**: 300–304.
7. Kharazmi E, Dossus L, Rohrmann S, Kaaks R. Pregnancy loss and risk of cardiovascular disease: A prospective population-based cohort study (EPIC-Heidelberg). *Heart* 2010; **97**: 49–54.
8. Miyakis S, Lockshin MD, Atsumi T *et al.* International consensus statement of an update of the classification criteria for definite antiphospholipid syndrome (APS). *J Thromb Haemost* 2006; **4**: 295–306.
9. Catalano PM. Management of obesity in pregnancy. *Obstet Gynecol* 2007; **109**: 419–433.
10. Cerda C, Pérez-Ayuso RM, Riquelme A *et al.* Nonalcoholic fatty liver disease in women with polycystic ovary syndrome. *J Hepatol* 2007; **47**: 412–417.
11. Zolghadri J, Tavana Z, Kazerooni T, Soveid M, Taghieh M. Relationship between abnormal glucose tolerance test and history of previous recurrent miscarriages, and beneficial effect of metformin in these patients: A prospective clinical study. *Fertil Steril* 2008; **90**: 727–730.
12. Sugiura-Ogasawara M, Sato T, Suzumori N, Nakanishi T, Nozawa K, Ozaki Y. The polycystic ovary syndrome does not predict further miscarriage in Japanese couples experiencing recurrent miscarriages. *Am J Reprod Immunol* 2009; **61**: 62–67.

13. Ogasawara M, Katano K, Aoyama T, Kajiura S, Aoki K. Are serum progesterone levels predictive of recurrent miscarriage in future pregnancies? *Fertil Steril* 1997; **68**: 806–809.
14. Haas DM, Ramsey PS. Progestogen for preventing miscarriage. *Cochrane Database Syst Rev* 2008; (2): CD003511.
15. Leung DY, Bieber T. Atopic dermatitis. *Lancet* 2003; **361**: 151–160.
16. Williams HC. Atopic dermatitis. *N Engl J Med* 2005; **352**: 2314–2324.
17. Walley AJ, Chavanas S, Moffatt MF *et al.* Gene polymorphism in Netherton and common atopic disease. *Nat Genet* 2001; **29**: 175–178.
18. Bieber T. Atopic dermatitis. *N Engl J Med* 2008; **358**: 1483–1494.
19. Inagaki J, Matsuura E, Nomizu M *et al.* IgG anti-laminin-1 autoantibody and recurrent miscarriages. *Am J Reprod Immunol* 2001; **45**: 232–238.
20. Dooley CP, Cohen H, Fitzgibbons PL *et al.* Prevalence of Helicobacter pylori infection and histologic gastritis in asymptomatic persons. *N Engl J Med* 1989; **321**: 1562–1566.
21. Dierkes J, Ebert M, Malfertheiner P, Luley C. Helicobacter pylori infection, vitamin B12 and homocysteine. A review. *Dig Dis* 2003; **21**: 237–244.
22. Nelen WL, Blom HJ, Steegers EA, den Heijer M, Eskes TK. Hyperhomocysteinemia and recurrent early pregnancy loss: A meta-analysis. *Fertil Steril* 2000; **74**: 1196–1199.
23. Aoki K, Furukawa T, Ogasawara M, Hori S, Kitamura T. Psychosocial factors in recurrent miscarriages. *Acta Obstet Gynecol Scand* 1998; **77**: 572–573.

Peripheral natural killer cell activity as a predictor of recurrent pregnancy loss: a large cohort study

Kinue Katano, M.D.,[a] Sadao Suzuki, M.D.,[b] Yasuhiko Ozaki, M.D.,[a] Nobuhiro Suzumori, M.D.,[a] Tamao Kitaori, M.D.,[a] and Mayumi Sugiura-Ogasawara, M.D.[a]

Departments of [a] Obstetrics and Gynecology and [b] Public Health, Nagoya City University, Graduate School of Medical Sciences, Nagoya, Japan

Objective: To determine the predictive value of preconceptional peripheral blood natural killer (pNK) cell activity in patients with recurrent pregnancy loss (RPL).
Design: Cohort study.
Setting: University department.
Patient(s): A total of 552 patients with a history of two to six consecutive miscarriages.
Intervention(s): None.
Main Outcome Measure(s): The predictive value of preconceptional pNK cell activity for subsequent miscarriage was analyzed using multivariable logistic regression analysis, with age, number of previous miscarriages, and presence/absence of previous live births and bed rest as covariates.
Result(s): Age and number of previous miscarriages, but not high pNK cell activity, were found to be independent risk factors for a subsequent miscarriage. No effect of bed rest and previous live birth on the likelihood of live birth was observed (odds ratios 1.28 [95% confidence interval 0.81–2.02] and 0.91 [0.52–1.59], respectively).
Conclusion(s): Elevated pNK cell activity was found to not be an independent risk factor for subsequent miscarriage. Clinicians should not measure the plasma NK activity as a systematic recurrent pregnancy loss examination, because its clinical significance is yet to be established. (Fertil Steril® 2013;100:1629–34. ©2013 by American Society for Reproductive Medicine.)
Key Words: Recurrent pregnancy loss, natural killer cell activity, predictor, cohort study

Discuss: You can discuss this article with its authors and with other ASRM members at http://fertstertforum.com/katanok-natural-killer-cell-recurrent-pregnancy-loss/

Use your smartphone to scan this QR code and connect to the discussion forum for this article now.*

* Download a free QR code scanner by searching for "QR scanner" in your smartphone's app store or app marketplace

Established causes of recurrent pregnancy loss (RPL) include presence of antiphospholipid antibodies in the serum, presence of uterine anomalies, and presence of abnormal chromosomes, particularly translocations, in either partner (1–3). According to previous reports, in approximately half of the cases seen at research centers, the cause of RPL remains unexplained despite conventional examinations conducted to identify the cause (4–6).

Cytotrophoblasts that express human leukocyte antigen G (HLA-G) come in direct contact with maternal lymphocytes. Many natural killer (NK)-like large granular lymphocytes have been detected in the human decidua of early pregnancy (7). Large numbers of NK cells appear in the mid-secretory phase. Natural killer cells have been thought to play a key role in the establishment of successful pregnancy by facilitating immunologic adaptation of the semiallogenic developing embryo. Recently, Fu et al. (8) reported that recruitment of TH17 cells and local inflammation can occur at the maternal–fetal interface during natural allogenic pregnancies, and that decidual NK cells promote immune tolerance and successful pregnancy

Received April 17, 2013; revised and accepted July 26, 2013; published online September 4, 2013.
K.K. has nothing to disclose. S.S. has nothing to disclose. Y.O. has nothing to disclose. N.S. has nothing to disclose. T.K. has received payment for manuscript preparation by the Japan Society of Thrombosis Haemostasis. M.S.-O. has received payment for lectures from Bayer, Sanofi-Aventis, Eizai Co., Kissei Pharmaceutical Co., Nippon Shinyaku Co., Dainippon Sumitomo Pharma, Takeda Pharmaceutical Company Limited, Fuji Pharma Co., and Mochida Pharmaceutical Co., payment for manuscript preparation by Igaku shoin Co. and Chunichi Shinbun; and payment for travel expenses by the Korean Society for Reproductive Medicine.
This study was supported by a Grant-in-Aid for Nagoya City University Hospital. N.S. and M.S.-O. are supported by a grant from the Japanese Ministry of Education, Science, and Technology. T.K. and M.S.-O. are supported by a grant from the Japanese Ministry of Health, Labor and Welfare.
Reprint requests: Mayumi Sugiura-Ogasawara, M.D., Department of Obstetrics and Gynecology, Nagoya City University, Graduate School of Medical Sciences, Mizuho-ku, Nagoya 4678601, Japan (E-mail: og.mym@med.nagoya-cu.ac.jp).

Fertility and Sterility® Vol. 100, No. 6, December 2013 0015-0282/$36.00

http://dx.doi.org/10.1016/j.fertnstert.2013.07.1996

by diminishing the recruitment of inflammatory TH17 cells via interferon-γ secreted by the $CD56^{bright}CD27^{-}$ NK cell subset. This NK cell-mediated regulatory response is lost in patients with recurrent spontaneous abortions, resulting in a prominent TH17 response and extensive local inflammation.

We previously reported (9) that elevated preconceptional peripheral blood NK (pNK) cell activity may be predictive of subsequent miscarriage in 68 patients with RPL. The 24 women with high pNK cell activity, defined as a value equal to or exceeding the mean pNK cell activity of the 47 controls + 1 SD, had a significantly higher miscarriage rate in the subsequent pregnancy than the 44 women with normal pNK cell activity (71% vs. 20%; relative risk 3.5; 95% confidence interval [CI] 1.8–6.5). However, some studies have shown no differences in pNK cell parameters between patients with RPL and controls (10–12). Whereas 90% of pNK cells are $CD56^{dim}$ and $CD16^{-}$, 80% of the uterine NK cells are $CD56^{bright}$ and $CD16^{-}$ (13, 14). Peripheral blood NK cells are phenotypically and functionally different from uterine NK cells. Decidual leukocytes have low cytotoxic activity as compared with peripheral lymphocytes (15).

The sample size of our previous study was too small. The prognostic value of measuring pNK cell parameters remains uncertain (16). Therefore, we conducted a cohort study of 552 patients with RPL to determine whether pNK might indeed be predictive of subsequent miscarriage.

MATERIALS AND METHODS

Patients

We studied 1,127 patients with a history of two or more (2 to 12) consecutive miscarriages, in whom the study examinations could be completed and subsequent pregnancies were established between January 1996 and May 2011 in Nagoya City University Hospital. Patients with identifiable causes and patients who received any kinds of treatment were excluded from the present cohort.

The conventional examinations were completed in all patients, including hysterosalpingography, transvaginal ultrasonography, chromosomal analysis of both partners, determination of the presence/absence of antiphospholipid antibodies, including lupus anticoagulant (LA), by diluted activated partial thromboplastin time (aPTT), diluted Russel viper venom time (RVVT), and $\beta 2$ glycoprotein I–dependent anticardiolipin antibody methods (17), and blood tests for hypothyroidism and diabetes mellitus, before the subsequent pregnancy.

Antiphospholipid antibody syndrome (APS) was diagnosed according to the criteria of the International Congress on Antiphospholipid Antibodies (18). Patients with APS were treated with low-dose aspirin plus heparin (19). Diabetes mellitus, hyper- or hypothyroidism, and hyperprolactinemia in the patients were controlled with medication before conception. Patients with three or more unexplained miscarriages received paternal mononuclear cell immunization from 1996 to 1999, a biologic response modifier from 1996 to 2004 (20), and low-dose aspirin and/or heparin from 2000 to 2007.

Gestational age was calculated from basal body temperature charts. A total of 654 patients were admitted for rest, and ultrasonography was performed twice per week from 4 to 8 weeks of gestation before 2004. The pregnancy outcome of 473 patients was followed once per week by ultrasonography without admission after 2004.

The study was conducted with the approval of the Research Ethics Committee at Nagoya City University Medical School.

Measurement

Preconceptional pNK cell activity was examined in the midsecretory phase. Peripheral blood NK cell activity was measured by a chromium-51 release cytotoxicity assay, with K562 human myeloid leukemia cells as the targets. A total of 3.7×10^3 Bq ^{51}Cr-labeled target cells (1×10^4 per well) were seeded with 2×10^5 effector cells per well (fresh peripheral blood mononuclear cells) in triplicate, in U-bottomed 96-well plates. After 4-hour incubation at 37°C, the activity in the supernatant from each well was measured in an autogramma scintillation counter. The percentage cytotoxicity was calculated as follows: ([test cpm−spontaneous cpm]/[maximum cpm−spontaneous cpm]) × 100, where cpm = counts per minute.

Lupus anticoagulant was detected using fivefold diluted aPTT methods, as previously described, with brain cephalin (Automated aPTT; Organon Teknica) as the phospholipid reagent (17). The 1:1 mixing test was performed at the same time. The clotting time was measured using an Option 4 Biomerieux calculator. Lupus anticoagulant was considered positive when prolonged clotting times (>mean + 3 SD of 10 healthy nonpregnant control plasma, 7.37 seconds) failed to correct when mixed 1:1 with standard plasma. Diluted Russel viper venom time for LA was performed as previously described (Gradipore). To detect $\beta 2$ glycoprotein I–dependent anticardiolipin antibody, we used a modified ELISA system (Yamasa).

Analysis

Patients with identifiable causes and patients who received any kinds of treatments were excluded from the present cohort. Biochemical pregnancy, ectopic pregnancy, and hydatidiform mole were excluded from the analysis of the subsequent pregnancy outcome.

Miscarriage rate was analyzed according to pNK cell activity, age, number of previous miscarriages, and presence/absence of previous live births and bed rest. Peripheral blood NK cells, age, and previous number of miscarriages were categorized into quartiles, because they showed normal distribution. The previous number of miscarriages was categorized into two, three, four, or five to six.

Crude logistic regression was performed to examine the predictive value of pNK cell activity for subsequent miscarriage. We also examined the influence of age, previous number of miscarriages, and presence/absence of previous live birth and bed rest on the likelihood of subsequent miscarriage.

Age is well known to influence the miscarriage rate. Age is also associated with number of previous miscarriages and number of previous live births. Thus, first, we chose

age-adjusted logistic regression. This analysis was applied to all the variables listed in the table except age.

Furthermore, multivariable logistic regression analysis was performed using pNK cell activity, age, number of previous miscarriages, and presence/absence of previous live births and bed rest as covariates. Linear multivariable logistic regression analysis was also performed using pNK cell activity, age, and number of previous miscarriages.

The analysis was carried out using SAS version 19.0 (SAS Institute), and $P<.05$ was considered to denote statistical significance.

RESULTS

In the subjected 1,127 patients, 4.4% (50) had an abnormal chromosome in either partner, 4.1% (46) of patients had a major uterine anomaly, 3.4% (38) had thyroid disease, 1.9% (21) had diabetes mellitus, and 2.9% (33) had APS (Fig. 1). In total, 180 patients were excluded from the cohort because several patients had two or three identifiable causes.

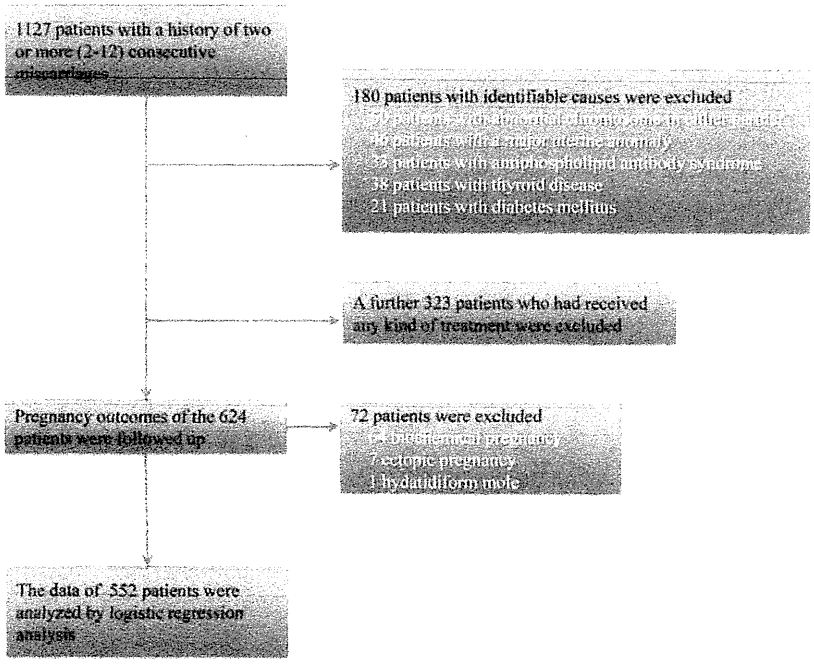
To eliminate the influence of the treatment, a further 323 patients who received any kind of treatment were excluded. A total of 72 patients—64 biochemical pregnancy, 7 ectopic pregnancy, and 1 hydatidiform mole—were excluded in the present study (Fig. 1).

The miscarriage rate of a total of 552 patients with unexplained RPL who received no medication is shown in Table 1. The subsequent miscarriage rate was 22.5% (124 of 552). The mean (SD) age and median (interquartile range) number of previous miscarriages were 31.9 (4.37) and 2 (2–3), respectively. The live birth rates of patients with previous two, three, four, five, and six miscarriages were 81.1% (309 of 381), 71.2% (99 of 139), 65.4% (17 of 26), 60.0% (3 of 5), and 0 (0 of 1), respectively.

Linear multivariable logistic regression showed that pNK was not an independent risk factor for subsequent miscarriage. However, in the crude analysis of the categorization of each variable, the miscarriage rate in the patients with 5%–24% pNK cell activity was significantly higher than that in the patients with 25%–34% pNK cell activity ($P=.046$). On the other hand, the miscarriage rate in the patients with 47%–78% pNK cell activity was similar to that in the patients with 25%–34% pNK cell activity. The plasma NK cell activity showed a weak inverse correlation with age in the 1,127 patients ($r=-0.068$).

Five variables, namely pNK cell activity, age, number of previous miscarriages, and absence of bed rest and previous live birth, were entered into the multiple logistic regression analysis for subsequent miscarriage detection in all 552 patients. The miscarriage rate in patients with 25%–34% pNK cell activity tended to be higher than that in patients with 5%–24% pNK cell activity (odds ratio [OR] 0.56, 95% confidence interval [CI] 0.31–1.00, $P=.051$). Crude, age-adjusted, and multivariable logistic regression analyses showed similar results in relation to pNK cell activity. Elevated pNK cell activity was confirmed to not be an independent risk factor for a subsequent miscarriage.

FIGURE 1



A total of 552 patients were analyzed in the present study. Of the 1,127 women initially enrolled, 180 patients with identifiable causes, 323 patients who received any kind of medication, and 72 patients whose pregnancy outcomes were biochemical or ectopic pregnancy were excluded.

Katano. Pregnancy loss and natural killer. Fertil Steril 2013.

TABLE 1

Miscarriage rate according to pNK cell activity, age, and number of previous miscarriages, and age-adjusted and multivariable logistic regression analysis to identify the risk factors for subsequent miscarriage.

		Crude analysis logistic regression		Age-adjusted logistic regression[a]		Multivariable logistic regression[b]		
Parameter	Miscarriage rate, % (n)	OR (95% CI)	*P* value	OR (95% CI)	*P* value	OR (95% CI)	*P* value	Trend *P* value
Peripheral NK cell activity (%)								.365
5–24	28.1 (41/146)	Reference		Reference		Reference		
25–34	17.9 (24/134)	0.56 (0.32–1.00)	.046	0.55 (0.31–0.98)	.042	0.56 (0.31–1.00)	.051	
35–46	22.6 (31/137)	0.75 (0.44–1.28)	.293	0.73 (0.42–1.26)	.261	0.78 (0.45–1.36)	.385	
47–74	20.7 (28/135)	0.67 (0.39–1.16)	.154	0.69 (0.39–1.20)	.186	0.73 (0.41–1.30)	.282	
Age (y)								.0002
19–29	13.2 (22/167)	Reference				Reference		
30–31	28.7 (33/115)	2.65 (1.45–4.85)	.0015			2.49 (1.35–4.59)	.0036	
32–35	20.9 (33/158)	1.74 (0.96–3.14)	.0658			1.46 (0.79–2.71)	.226	
36–45	32.1 (36/112)	3.12 (1.72–5.68)	.0002			2.54 (1.35–4.76)	.0037	
No. of previous miscarriages								.0014
2	18.9 (72/381)	Reference		Reference		Reference		
3	28.8 (40/139)	1.73 (1.11–2.71)	.012	1.57 (0.99–2.48)	.055	1.38 (0.85–2.26)	.198	
4	34.6 (9/26)	2.27 (0.97–5.30)	.052	1.84 (0.77–4.37)	.168	1.65 (0.67–4.10)	.280	
5–6	50.0 (3/6)	4.29 (0.85–21.70)	.080	3.56 (0.68–18.55)	.132	3.73 (0.69–20.10)	.126	

[a] The only covariate used was age for the age-adjusted logistic regression analysis. This analysis was applied to all the variables listed in the table except age.
[b] The covariates used for the multivariable logistic regression analysis were pNK activity, age, number of previous miscarriages, presence/absence of previous live births, and presence/absence of bed rest.

Katano. Pregnancy loss and natural killer. Fertil Steril 2013

The miscarriage rate in the patients without bed rest was significantly higher than that in the patients who were admitted for rest ($P=.016$; Table 2). However, there were differences in the mean [SD] age (30.9 [4.0] vs. 32.8 [4.5] years; $P<.0001$) and the median number of previous miscarriages (three vs. two) between the patients with and without bed rest.

The miscarriage rate in patients without previous live births tended to be lower than that in the patients with previous live births ($P=.096$). There were differences in the mean age (33.9 [3.7] vs. 31.6 [4.4] years; $P<.0001$) and the median number of previous miscarriages (three vs. two) between the patients with and without previous live births.

No effect of bed rest and previous live birth on the likelihood of live birth was observed (OR 1.28, 95% CI 0.81–2.02 and OR 0.91, 95% CI 0.52–1.59, respectively).

Age and number of previous miscarriages were determined to be risk factors for subsequent miscarriage according to both crude and linear multivariable logistic regression.

Age and numbers of previous miscarriages were confirmed to be independent risk factors. However, number of previous miscarriage, but not age, was found to be influenced by the other factors in the present study.

DISCUSSION

The results of this study suggest that elevated pNK cell activity is not a reliable predictor of subsequent miscarriage. The miscarriage rate was higher in patients with lower pNK cell activity.

TABLE 2

Miscarriage rate according to the presence/absence of previous live births and bed rest, and age-adjusted and multivariable logistic regression analysis to identify the risk factors for subsequent miscarriage.

				Crude analysis logistic regression		Age-adjusted logistic regression[a]		Multivariable logistic regression[b]	
Parameter	Miscarriage rate, % (n)	Mean (SD) age (y)	Median no. of previous miscarriage	OR (95% CI)	*P* value	OR (95% CI)	*P* value	OR (95% CI)	*P* value
Absence of previous live birth									
Presence	28.4 (25/88)*	33.9 (3.7)*	3*	Reference		Reference		Reference	
Absence	21.3 (99/464)*	31.6 (4.4)*	2*	0.68 (0.41–1.14)	.096	0.58 (0.41–1.14)	.146	0.91 (0.52–1.59)	.736
Absence of bed rest									
Presence	18.2 (47/258)*	30.9 (4.0)*	3*	Reference		Reference		Reference	
Absence	26.2 (77/294)*	32.8 (4.5)*	2*	1.59 (1.06–2.40)	.016	1.59 (1.06–2.40)	.026	1.28 (0.81–2.02)	.288

[a] The only covariate used was age for the age-adjusted logistic regression analysis. This analysis was applied to all the variables listed in the table except age.
[b] The covariates used for the multivariable logistic regression analysis were pNK activity, age, number of previous miscarriages, presence/absence of previous live births, and presence/absence of bed rest.
* $P<.05$ was considered to denote statistical significance.

Katano. Pregnancy loss and natural killer. Fertil Steril 2013

Uterine endometrial NK (uNK) cell activity is known to be strongly involved in the maintenance of normal pregnancy. Lachapelle et al. (21) proved that the proportion of uNK cells was identical in recurrent miscarriage (RM) patients and normal controls, but the $CD56^{bright}$ and $CD16^{-}$ NK cell subset, which is predominant in normal decidua and endometrium, was significantly decreased in favor of an important contingent of $CD56^{dim}$ and $CD16^{-}$ NK cells in all patients. Quenby et al. (22) demonstrated that prednisolone therapy during the first trimester of pregnancy reduced the risk of miscarriages and improved the live birth rate in patients with idiopathic RM and increased the numbers of uNK cells in the endometrium. Measurement of pNK cell activity has been performed to determine whether it might be predictive of a successful subsequent pregnancy (23, 24). We have reported for the first time that elevated pNK cell activity might be predictive of subsequent miscarriage in patients with RM (9). Some have affirmed, whereas others have denied, the predictive value of pNK for the subsequent pregnancy outcome. However, none of these reports were based on studies of large cohorts, and there is no clear evidence yet (16, 25).

Patients with unexplained RM have been treated empirically with expensive immunoglobulin, on the basis of the conjecture that the functions of uNK cells and pNK cells are similar and that, therefore, measurement of pNK cell activity would reflect uNK cell activity. Tang et al. (16) reported a systematic review and came to the conclusion that there is no association between the subsequent pregnancy outcome and either pNK or uNK cell activity in women with RM and infertility. In the present study the correlation between the subsequent pregnancy outcome and pNK cell activity was not linear. The miscarriage rate in patients with low pNK cell activity tended to be higher than that in patients with 25%–74% pNK cell activity. Age, number of previous miscarriages, bed rest, and number of previous live births were found to exert no significant influence on pNK cell activity.

It is well known that stress and exercise increase pNK cell activity; therefore, these factors should be borne in mind while drawing blood for testing. Abnormal data pertaining to the number or activity of pNK cells may reflect transient stress reactions in daily life. It is not clear whether uNK cells may have the same significance. Peripheral blood NK and uNK cells are different types of cells, and both the models and functions of these cells are entirely different. It has been reported that measurement of pNK cell activity does not provide any information on the condition of the endometrial membrane (7, 14, 25). There is also no evidence of treatment using the data on pNK cell activity. We do not recommend measurement of pNK cell activity as part of the systematic examination in patients with RPL.

Mentally depressed patients with RPL need tender loving care (26). However, there is no evidence that subsequent miscarriage can be prevented by hospitalization. Klebanoff et al. (27) concluded that there was no difference in the miscarriage rate between women who had a heavy workload and long working hours and wives of male residents who had many kinds of jobs. Duckitt et al. (28) found no direct evidence from randomized, controlled trials regarding the influence of bed rest in women with unexplained RM. In the present study the live birth rate in patients without bed rest was significantly lower than that in the patients who were admitted for rest. However, there were significant differences in the mean age and median number of previous miscarriages between the patients with and without bed rest, because the average age of women at pregnancy is increasing year by year in Japan. Neither age-adjusted nor multivariable logistic regression analysis showed any effect of bed rest on the live birth rate. We concluded that there is no necessity to advise preventive bed rest for pregnant women, in the absence of symptoms of threatened abortion.

This study revealed that previous live birth was not predictive of a subsequent live birth, although there have been a few reports suggesting a favorable influence of a previous live birth in secondary RM patients (29, 30). Nielsen (31) reported that secondary RM is more common after the birth of a boy and that the subsequent live birth rate is reduced in secondary RM patients with a firstborn boy, owing to the pathogenic role of the aberrant maternal H-Y immune response. Both our previous study and the crude analysis in the present study indicated that the live birth rate increased as the number of previous miscarriages increased (32). However, the significant difference disappeared after adjustment for age, because age also increased with increasing number of previous miscarriages.

In more than half of the cases, the cause of RPL remains unexplained despite conventional examinations (4, 5). Recently we found that an abnormal embryonic karyotype was the most frequent cause, accounting for as much as 41% of the cases, and the percentage of truly unexplained was limited to 24.5% (33). Associations have been reported between many kinds of polymorphisms, such as those of annexin A5 and NLRP7, and RPL (34, 35). The influence of one single-nucleotide polymorphism associated with RPL might be speculated to be very small, because the OR of each gene mutation is relatively small (34). Even though it would be highly desirable, it might be difficult to identify clinically useful predictors of the outcome of a subsequent pregnancy.

We previously reported that elevated pNK cell activity may be predictive of subsequent miscarriage in patients with RPL (9). However, we wish to correct our initial conclusion, because in this study, high pNK cell activity was confirmed to not be an independent risk factor for subsequent miscarriage. Clinicians should not measure pNK activity as a systematic RPL examination, because the clinical significance or treatment method is yet to be established. Patients need not give up working, because no effect of bed rest on the likelihood of live birth was observed.

REFERENCES

1 Farquharson RG, Pearson JF, John L. Lupus anticoagulant and pregnancy management. Lancet 1984;28:228–9.
2 Sugiura-Ogasawara M, Ozaki Y, Sato T, Suzumori N, Suzumori K. Poor prognosis of recurrent aborters with either maternal or paternal reciprocal translocation. Fertil Steril 2004;81:367–73.

3 Sugiura-Ogasawara M, Ozaki Y, Kitaori T, Kumagai K, Suzuki S. Midline uterine defect size correlated with miscarriage of euploid embryos in recurrent cases. Fertil Steril 2010;93:1983–8.

4 Clifford K, Rai R, Watson H, Regan L. An informative protocol for the investigation of recurrent miscarriage: preliminary experience of 500 consecutive cases. Hum Reprod 1994;9:1328–32.

5 Stephenson MD. Frequency of factors associated with habitual abortion in 197 couples. Fertil Steril 1996;66:24–9.

6 Jaslow CR, Carney JL, Kutteh WH. Diagnostic factors identified in 1020 women with two versus three or more recurrent pregnancy losses. Fertil Steril 2010;93:1234–43.

7 Bulmer JN, Morrison L, Longfellow M, Ritson A, Pace D. Granulated lymphocytes in human endometrium: histochemical and immunohistochemical studies. Hum Reprod 1991;6:791–8.

8 Fu B, Li X, Sun R, Tong X, Ling B, Tian Z, et al. Natural killer cells promote immune tolerance by regulating inflammatory TH17 cells at the human maternal-fetal interface. Proc Natl Acad Sci U S A 2013;110:E231–40.

9 Aoki K, Kajura S, Matsumoto Y, Ogasawara M, Okada S, Yagami Y, et al. Preconceptional natural-killer cell activity as a predictor of miscarriage. Lancet 1995;345:1340–2.

10 Emmer PM, Nelen WL, Steegers EA, Hendriks JC, Veenhoek M, Joosten I. Peripheral natural killer cytotoxicity and CD56(pos)CD16(pos) cells increase during early pregnancy in women with a history of recurrent spontaneous abortion. Hum Reprod 2000;15:1163–9.

11 Souza SS, Ferriani RA, Santos CM, Voltarelli JC. Immunological evaluation of patients with recurrent abortion. J Reprod Immunol 2002;56:111–21.

12 Wang Q, Li TC, Wu YP, Cocksedge KA, Fu YS, Kong QY, et al. Reappraisal of peripheral NK cells in women with recurrent miscarriage. Reprod Biomed Online 2008;17:814–9.

13 Nagler A, Lanier LL, Cwirla S, Phillips JH. Comparative studies of human FcRIII-positive and negative natural killer cells. J Immunol 1989;143:3183–91.

14 King A, Balendran N, Wooding P, Carter NP, Loke YW. CD3-leukocytes present in the human uterus during early placentation: phenotypic and morphologic characterization of the CD56++ population. Dev Immunol 1991;1:169–90.

15 Deniz G, Christmas SE, Brew R, Johnson PM. Phenotypic and functional cellular differences between human CD3- decidual and peripheral blood leukocytes. J Immunol 1994;152:4255–61.

16 Tang AW, Alfirevic Z, Quenby S. Natural killer cells and pregnancy outcomes in women with recurrent miscarriage and infertility: a systematic review. Hum Reprod 2011;26:1971–80.

17 Ogasawara M, Aoki K, Matsuura E, Sasa H, Yagami Y. Anti-β2glycoprotein I antibodies and lupus anticoagulant in patients with recurrent pregnancy loss: prevalence and clinical significance. Lupus 1996;5:587–92.

18 Miyakis S, Lockshin MD, Atsumi T, Branch DW, Brey RL, Cervera R, et al. International consensus statement of an update of the classification criteria for definite antiphospholipid syndrome (APS). J Thromb Haemost 2006;4:295–306.

19 Couchock FS, Reece EA, Balaban D, Branch DW, Plouffe L. Repeated fetal losses associated with antiphospholipid antibodies: a collaborative randomized trial comparing prednisone with low-dose heparin treatment. Am J Obstet Gynecol 1992;166:1318–23.

20 Katano K, Ogasawara M, Aoyama T, Ozaki Y, Kajiura S, Aoki K. Clinical trial of immunostimulation with a biological response modifier in unexplained recurrent spontaneous abortion patients. J Clin Immunol 1997;17:472–7.

21 Lachapelle MH, Miron P, Hemmings R, Roy DC. Endometrial T, B, and NK cells in patients with recurrent spontaneous abortion. Altered profile and pregnancy outcome. J Immunol 1996;156:4027–34.

22 Quenby S, Kalumbi C, Bates M, Farquharson R, Vince G. Prednisolone reduces preconceptual endometrial natural killer cells in women with recurrent miscarriage. Fertil Steril 2005;84:980–4.

23 King K, Smith S, Chapman M, Sacks G. Detailed analysis of peripheral blood natural killer (NK) cells in women with recurrent miscarriage. Hum Reprod 2010;25:52–8.

24 Moffett A, Regan L, Braude P. Natural killer cells, miscarriage, and infertility. BMJ 2004;329:1283–5.

25 Dosiou C, Giudice LC. Natural killer cells in pregnancy and recurrent pregnancy loss: endocrine and immunologic perspectives. Endocr Rev 2005;26:44–62.

26 Sugiura-Ogasawara M, Furukawa TA, Nakano Y, Hori S, Aoki K, Kitamura T. Depression influences subsequent miscarriage in recurrent spontaneous aborters. Hum Reprod 2002;17:2580–4.

27 Klebanoff MA, Shiono PH, Rhoads GG. Outcomes of pregnancy in a national sample of resident physicians. N Engl J Med 1990;323:1040–5.

28 Duckitt K, Qureshi A. Recurrent miscarriage. Clin Evid (Online) 2011;2011:1409.

29 Nielsen HS, Steffensen R, Lund M, Egestad L, Mortensen LH, Andersen AN, et al. Frequency and impact of obstetric complications prior and subsequent to unexplained secondary recurrent miscarriage. Hum Reprod 2010;25:1543–52.

30 Stephenson MD, Kutteh WH, Purkiss S, Librach C, Schultz P, Houlihan E, et al. Intravenous immunoglobulin and idiopathic secondary recurrent miscarriage: a multicentered randomized placebo-controlled trial. Hum Reprod 2010;25:2203–9.

31 Nielsen HS. Secondary recurrent miscarriage and H-Y immunity. Hum Reprod Update 2011;17:558–74.

32 Ogasawara M, Aoki K, Okada S, Suzumori K. Embryonic karyotype of abortuses in relation to the number of previous miscarriages. Fertil Steril 2000;73:300–4.

33 Sugiura-Ogasawara M, Ozaki Y, Katano K, Suzumori N, Kitaori T, Mizutani E. Abnormal embryonic karyotype is the most frequent cause of recurrent miscarriage. Hum Reprod 2012;27:2297–303.

34 Hayashi Y, Sasaki H, Suzuki S, Nishiyama T, Kitaori T, Mizutani E, et al. Genotyping analyses for polymorphisms of *ANXA5* gene in patients with recurrent pregnancy loss. Fertil Steril 2013;100:1018–24.

35 Huang JY, Su TM, Lin SH, Kuo PL. A genetic association study of NLRP2 and NLRP7 genes in idiopathic recurrent miscarriage. Hum Reprod 2013;28:1127–34.