Intravenous Immunoglobulin Suppresses Abortion Relates to an Increase in the CD44bright NK Subset in Recurrent Pregnancy Loss Model Mice 1

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Intravenous Immunoglobulin Suppresses Abortion Relates to an Increase in the CD44^{bright} NK Subset in Recurrent Pregnancy Loss Model Mice[1]

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ABSTRACT

Recurrent pregnancy loss (RPL), which mostly is of unknown etiology (unexplained RPL, uRPL), is defined as three or more consecutive spontaneous abortions. Some women with uRPL display a higher fraction and cytotoxicity of natural killer (NK) cells in the periphery and endometrium. Therefore, some uRPL cases have been explained by autoimmune abnormalities. The efficacy of intravenous immunoglobulin (IVIg) for uRPL has been confirmed in several clinical trials; however, its mechanism remains unknown, mainly because the abortion mechanism remains to be elucidated. In the present study, we analyzed the mechanisms of both abortion and IVIg action using a uRPL mouse model in which abortion was induced by lipopolysaccharide injection. IVIg attenuated the abortion rate in the uRPL model mice. The suppressive effect of IVIg was maximized by high dose administration early after lipopolysaccharide injection. Specifically, we discovered the presence of two distinct uterine NK (uNK) subsets: CD44^{bright} and CD44^{mid}. In uRPL model mice, we observed an increase in the number of CD44^{bright} uNK cells, while the CD44^{mid} uNK subset remained unchanged. Furthermore, when abortion was reduced by IVIg administration, the cell number of the CD44^{bright} uNK subset did not increase, which might allow differentiating pathological from normal uNK cells based on CD44 expression. Based on these results, we propose not only an effective administration protocol of IVIg to the uRPL model mice, but also a novel mechanism of abortion related to the increase in the CD44^{bright} subset and of IVIg, which suppresses the increase of the CD44^{bright} subset.

abortion, IVIg, NK cells, recurrent pregnancy loss

INTRODUCTION

The vertebrate immune system is maintained in a state of equilibrium by highly evolved, complex, and structured mechanisms. In the tightly regulated system, maternal immunity can be said to be an exception: it does not reject the fetus and/or trophoblasts that form the maternofetal interface despite the latter expressing paternal alloantigens. It is considered that this phenomenon is because maternal immune effector cells against fetal antigen are selectively silenced by regulatory cells, especially, regulatory T cells [1, 2]. Thus, if the mechanism is not well regulated, maternal immunity can cause abortion. Unfortunately, the mechanisms of abortion-related fetomaternal tolerance disorder are not yet completely understood.

Recurrent pregnancy loss (RPL) is defined as the experience of three or more consecutive spontaneous abortions with the same partner and not more than one live birth with that partner. However, the causes of RPL are largely unknown [3–5]. Thus, the majority of RPLs is of unknown etiology, designated as unexplained RPL (uRPL). However, it is known that some women with uRPL have higher frequencies and cytotoxic activity of natural killer (NK) cells, both in the periphery and in the endometrium [6–8]. In humans, the uterine NK (uNK) population consists of CD56^{bright}CD16^{-} NK cells, which are a minority of the blood NK population, and CD56^{dim}CD16^{+} NK cells, which are more toxic and make up the majority of peripheral blood NK cells (hence, are termed peripheral NK cells). They are obviously present in maternal blood that perfuses the placenta. Some uRPL women, specifically those aborting chromosomally normal embryos, appear to have elevated peripheral NK cells. In mice, equivalent uNK populations exist: those that are associated with spontaneous abortion and those that are not. Although murine NK cells do not express CD56, it has been reported that murine peripheral NK and resident uNK cells can be characterized by *Dolichos biflorus* (DBA) lectin binding [9] and by flow cytometry (CD3^{-}CD122^{+}NK1.1^{-}CD49b^{-}) [10], respectively. Although the mechanism that triggers uNK activation remains unexplained, most uRPL abortions are likely immune-mediated and of inflammatory nature [11, 12].

Intravenous immunoglobulin (IVIg) has been primarily used in patients with antibody deficiencies [13], but nowadays, it is also being utilized for treatment of several autoimmune disorders such as Kawasaki disease and idiotypic immune thrombocytopenic purpura [14, 15]. The proposed mechanisms of IVIg action in autoimmune diseases involve the modulation of Fc receptors, interference with the cytokine network and complements, provision of anti-idiotypic antibodies, suppression of lymphocyte effector function [14, 16, 17], presentation of regulatory T cell-inducing epitope termed Tregitope [18], and sialylated immunoglobulin G (IgG) that indirectly upregulates the inhibitory IgG Fc receptor FcγRIIB on effector macrophages [15]. Clark et al. reported that in the case of abortion, soluble CD200 molecule containing IVIg suppresses the cytotoxic activity of peripheral NK cells [19]. Although these studies typified the increased research interest in the role of IVIg for autoimmune disease treatment during the last decade, the precise mechanism of action of IVIg remains unclear.

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There are several reports that IVIg exhibits therapeutic effects in the treatment of uRPL in clinical practice [7, 8, 20]; however, it has also been reported that IVIg did not show beneficial clinical effects [21]. Consequently, the conclusions drawn from these trials have been controversial. Because the exact mechanisms of IVIg and uRPL-related abortion are unknown, different IVIg administration protocols have been used in these studies, which may account for the discrepancies. Thus, it seems that there should be an optimum protocol of IVIg administration for uRPL. However, the optimum protocol is currently obscure because the precise mechanisms of abortion and IVIg action remain unclear. Therefore, clarification of the mechanisms of not only IVIg action but also abortion in uRPL is needed.

To determine the mechanism underlying IVIg action and of the pathogenesis of abortion in uRPL, in this study, we employed a female CBA/J × male DBA/2N mating model of uRPL [22], and maternal immunity was activated by lipopolysaccharide (LPS) injection [23]. LPS is the primary component in the cell wall of gram-negative bacteria and induces an acute inflammatory reaction. It is known that high (50–100 μg/mouse) as well as low doses (0.1–1 μg/mouse) of LPS can induce abortion in pregnant mice. However, the underlying mechanisms are distinctly different: the abortion mechanism of high-dose LPS resembles severe infection or systemic inflammation, while low-dose LPS induces immune activation-mediated abortion. The low-dose LPS-induced mouse abortion model is well documented and provides a means to study immune-activated abortion [24, 25]. Additionally, we previously observed that IVIg affects CD44 expression of some immune cells in autoimmune disease model mice (our unpublished data). However, no relationship between the CD44 expression level of NK cells and abortion has been reported until date. Therefore, in this study, we examined the preventive effect of IVIg administration on immune reproductive failure in the uRPL model mice, with a special focus on CD44 expression in NK cells.

MATERIALS AND METHODS

Mice and Mating

CBA/J female and DBA/2N male mice were purchased from Charles River Japan, Inc. The mice were maintained under specific pathogen-free conditions and a 12L:12D cycle, and were used at an age of 7 wk or older. Female CBA/J mice were mated with male DBA/2N mice (two females were housed with a single male from the evening to the next morning), and the presence of a vaginal plug was checked every morning. The day of plug detection was defined as Gestational Day (gd) 0.5. All animal procedures were approved by the Animal Care and Use Committee of Japan Blood Products organization. The study was performed in accordance with the guidelines of the Animal Care and Use Committee of Japan Blood Products organization, which conforms to the policies of the Japan Health Sciences Foundation.

LPS Injection and Evaluation of Abortion

LPS from *Salmonella typhimurium* (Sigma-Aldrich), which has an abortifacient effect, was injected into pregnant mice with a single intraperitoneal (i.p.) injection of 0.8 μg/mouse on gd 7.5 [23]. Mice that received the same amount of saline served as controls. Abortion was evaluated on gd 13.5 by macroscopic examination. Abortion rates were calculated as follows: abortion rate (%) = (number of resorbed fetuses/[number of resorbed fetuses + viable fetuses]) × 100.

Administration of IVIg and IgG Fragments

IVIg (Japan Blood Products Organization) was administered on gd 7.5 at 1000, 500, 250, or 125 mg/kg (20 ml/kg) via single intravenous (i.v.) injection to pregnant CBA/J mice that had received LPS. Each fragment of IgG was dissolved in saline to the same molar concentration as IVIg, and same amounts of the fragments (20 ml/kg) were administered on gd 7.5 via single i.v. injection. In the experiment to determine the effective timing of IVIg administration to inhibit abortion, IVIg was administered as a single i.v. injection at 1000 mg/kg (20 ml/kg) at 1, 3, or 6 h after LPS injection. Pregnant mice receiving the same amount of saline (20 ml/kg) instead of IVIg served as controls.

Fragmentation of Human IgG

F $(ab')_2$, Fab, and Fc fragments used in this study were prepared as described previously [17]. Briefly, for preparation of the F $(ab')_2$ fragment, IVIg was dialyzed against 100 mM acetate buffer, pH 4.5. Pepsin (Calbiochem) was added to the solution, followed by incubation at 37°C for 24 h [26]. Then, the solution was dialyzed against PBS (TaKaRa) and purified by gel filtration using HiLoad 26/60 Superdex 200 prep grade (GE Healthcare). The collected F $(ab')_2$ fractions were further purified with HiTrap Protein G (GE Healthcare). For preparation of the Fab and Fc fragments, IVIg was dialyzed against 5 mM phosphate buffer, pH 8.0. Papain (MP Biomedicals) digestion was carried out using method of Snigurowicz and Powiertowska-Rezmer [27] with modifications. The dialyzed IgG solution in 5 mM phosphate buffer, pH 8.0, containing 0.1 mg/ml papain, 2 mM ethylenediaminetetraacetic acid, and 10 mM L-cysteine was incubated at 37°C for 24 h. Then, the solution was dialyzed against PBS and concentrated. To separate the Fab and Fc mixture from undigested IgG or smaller proteins, gel filtration was carried out. The fractions containing Fab and Fc were applied to a DEAE Sepharose Fast Flow (GE Healthcare) column. The flow-through fraction was further purified as Fab fragments with HiTrap Protein G (GE Healthcare). Fc fragments were collected from the column by increasing the NaCl concentration. The Fc fragment was crystallized as previously described [28]. The crystals were separated by centrifugation, and then dissolved in PBS containing 0.3 M NaCl. The collected Fc fractions were further purified by gel filtration. After preparation, each fragment was concentrated to 50 mg/ml and stored at −80°C until use. At the time of administration, fragments were diluted to the appropriate concentration in saline.

Sample Collection and Cell Preparations

Peripheral blood, uterine, and spleen samples were collected from different pregnant CBA/J mice that had received LPS with or without IVIg at various time points (peripheral blood: 3, 6, 12, 24, and 48 h after LPS injection; uterus: 1, 3, 6, 12, 18, and 24 h after LPS injection; and spleen: 1, 3, and 12 h after LPS injection). Animals were euthanized with carbon dioxide gas.

For analysis of peripheral immune cells, the collected peripheral blood was lysed with RBC lysis buffer (BD Pharm Lyse; BD Biosciences) for 15 min at 37°C, after which cells were twice washed, centrifuged at 200 × *g* for 5 min, and resuspended in PBS. For analysis of uNK cells, uterine horns were cleaned of surrounding fat and blood vessels and explanted by cutting just below the ovaries on each uterine horn and distal to the cervix. The uteri were carefully removed, excluding tissue from the fetus. Single-cell preparation was based on the method described by Takashima et al. [29]. Briefly, uterine tissues were cut into pieces of ~1 mm^3 and mechanically disrupted using low pressure. The disrupted tissues were digested with 0.15 mg/ml collagenase type D (Roche Applied Science) and 0.025 mg/ml DNase I (Roche Applied Science) at 37°C for 2 h, then passed through a 70-μm filter and centrifuged with PBS at 1700 × *g* for 5 min at 4°C.

Flow Cytometry

The following monoclonal antibodies were purchased from BioLegend: Brilliant Violet-conjugated anti-mouse CD3e (clone 145-2c11), PerCP/Cy5.5-conjugated anti-mouse CD14 (clone Sa14-2), and APC/Cy7-conjugated anti-mouse CD45 (clone 30-F11). PE-conjugated anti-mouse CD49b (clone HMα2), PE/Cy7-conjugated anti-mouse CD44 (clone IM7), and FITC-conjugated anti-mouse CD94 (clone 18d3) were purchased from eBioscience. FITC-conjugated anti-mouse CD69 (clone H1 2F3) was purchased from BD Biosciences. Isotype controls were used for the determination of negative cells. Each antibody was used at an appropriate dilution as determined under the experimental conditions according to the manufacturer's recommendation.

Prepared cells of each type were spun down, washed, and resuspended in PBS. For surface marker analysis, samples were stained with the appropriate antibodies. NK cells, natural killer T (NKT) cells, T cells, and monocytes in peripheral blood were identified by surface expression of CD45, CD14, CD3e, and CD49b: NK cells, $CD45^+CD14^-CD3e^-CD49b^+$; NKT cells, $CD45^+CD3e^+CD49b^+$; T cells, $CD45^+CD3e^+CD49b^-$; and monocytes, $CD45^+CD14^+CD3e^-CD49b^-$. NK cells in the uterus were determined as the $CD45^+CD3e^-CD49b^+$ cell population. The fraction of activated cells (the

percentage of CD69^{+} cells) and uNK cell subsets (CD44^{mid} or CD44^{bright}) were evaluated by flow cytometry (FACSVerse; BD Biosciences) and analyzed using the FlowJo software (Tree Star). The number of cells in each subset was calculated by multiplying the total cell number and the population of target cells in each sample.

Histological Analysis

Uterine and spleen samples were fixed in 10% neutral-buffered formalin solution, embedded in paraffin, and 5-μm thick tissue slices were prepared and stained with hematoxylin+eosin. For immunohistochemistry, sections were stained with an autostainer (Histostainer 36A; Nichirei Bioscience). Goat anti-human IgG polyclonal antibody (Abcam) was used for IVIg staining. Subsequently, the sections were incubated with peroxidase-labeled rabbit anti-goat IgG polyclonal antibody (Nichirei Bioscience). Human IgG was visualized with diaminobenzidine, and sections were counterstained with hematoxylin. Images were collected using a biological microscope (ECLIPSE Ni-U; Nikon) equipped with a digital camera (DXM 1200F; Nikon). The images were processed to enhance the contrast and decrease the brightness using FastStone Image Viewer 5.6 (FastStone Software). Two observers who blinded to the treatments performed all histological examinations.

Measurement of Plasma Progesterone Concentration

The plasma was collected by centrifugation at 1580 × *g* for 10 min and stored at −70°C. The plasma concentration of progesterone (P4) was measured using an enzyme immunoassay (EIA) kit (Enzo Life Sciences).

Statistical Analysis

Data are expressed as the mean ± SEM. The results are representative of two or more independent experiments. Means were compared using Student *t*-test or Dunnett multiple comparison test. Regression analysis was performed to evaluate the possible dose dependency of the effects of IVIg. $P < 0.05$ was considered significant.

RESULTS

Suppressive Effect of IVIg on Abortion

As an approach to analyzing the mechanism of IVIg in uRPL-related abortion, we first examined the effect of IVIg on the abortion rate of uRPL model mice. We determined the abortion rate of pregnant CBA/J mice that had received LPS with or without various doses of IVIg. The abortion rate was increased by LPS injection and was significantly suppressed by simultaneously administered IVIg. The suppressive effect was dose-dependent, with 1000 mg/kg of IVIg being the most effective (Fig. 1A). We verified that IVIg did not suppress spontaneous abortion in pregnant CBA mice that did not receive LPS (Supplemental Fig. S1, Supplemental Data are available online at www.biolreprod.org). Moreover, we confirmed that IVIg suppressed the abortion rate in the CBA × BALB/c combination, a non-abortion-prone murine mating combination, administered with either saline, LPS alone, or LPS+IVIg (Supplemental Fig. S2).

Next, we aimed to identify the effective portion of IVIg for abortion suppression in uRPL model mice by testing the digested fragments F (ab′)$_2$, Fab, and Fc. F (ab′)$_2$ at 666 mg/kg and Fab and Fc at 333 mg/kg, which were doses equimolar to that of IVIg, failed to suppress LPS-induced abortion (Fig. 1B). These data suggested that the suppressive effect of IVIg on abortion of uRPL mice depends on the intact form of IgG. Similarly, the Fab fragment at 1000 mg/kg, which was same amount as that used for IVIg, showed no abortion-suppressive effect (Fig. 1B), suggesting that the effect of IVIg is not an artifact of high-dose injection of heterogeneous protein. In all following experiments, 1000 mg/kg IVIg was used.

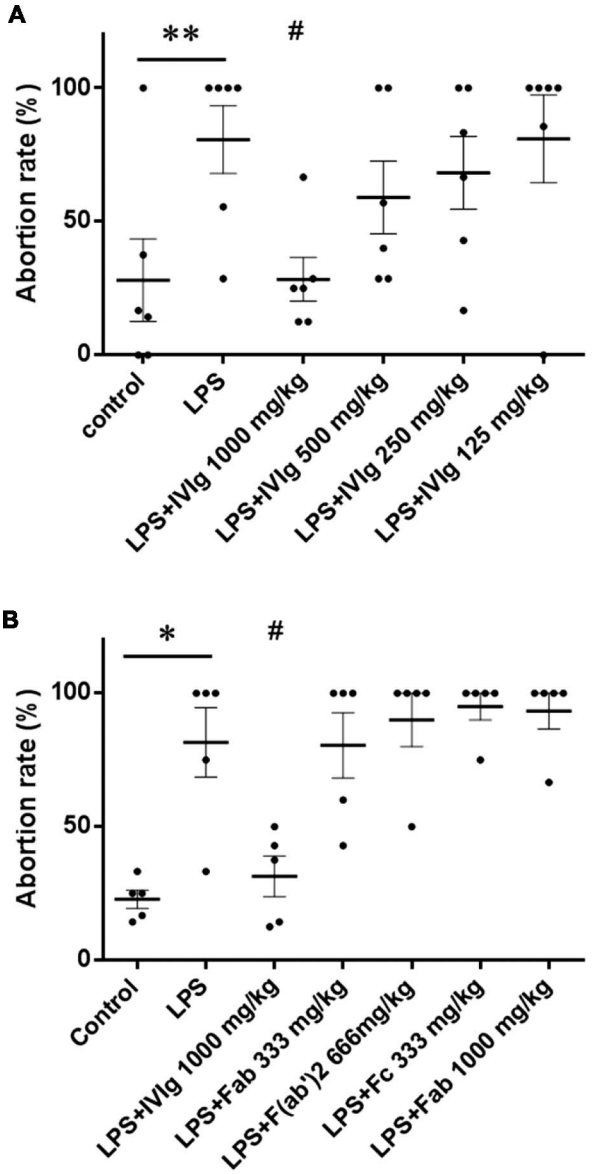

FIG. 1. Suppression of abortion by IVIg in uRPL model mice depends on the dose and intact form of IVIg. **A**) Abortion rate upon i.v. administration of IVIg (1000, 500, 250, or 125 mg/kg) following i.p. injection of LPS to pregnant CBA/J mice on gd 7.5. Data are the mean ± SEM (n = 6), $^{**}P < 0.01$ (Student *t*-test), $\#P < 0.05$ versus LPS group (Dunnett multiple comparison test). **B**) Abortion rate upon i.v. administration of IVIg, Fab, F (ab′)$_2$, or Fc at indicated concentrations following i.p. injection of LPS to pregnant CBA/J mice on gd 7.5. Data are the mean ± SEM (n = 5). $^{*}P < 0.05$ versus LPS group (Student *t*-test), $\#P < 0.05$ versus LPS group (Dunnett multiple comparison test). Evaluation of abortion was performed on gd 13.5 by macroscopic examination. Results are representative of two or more independent experiments.

IVIg Does Not Affect Activation of Peripheral Immune Cells and the Plasma P4 Level

To evaluate the effect of IVIg on the activation of peripheral immune cells of the uRPL model mice, we examined the activated (CD69^{+}) populations of NK cells, T cells, monocytes, and NKT cells in the peripheral blood. Unexpectedly, IVIg did not suppress LPS-induced activation of any of these immune cells (Fig. 2, A–D). These results suggested that IVIg does not affect activation of peripheral immune cells in the uRPL model mice, despite suppressing the abortion rate.

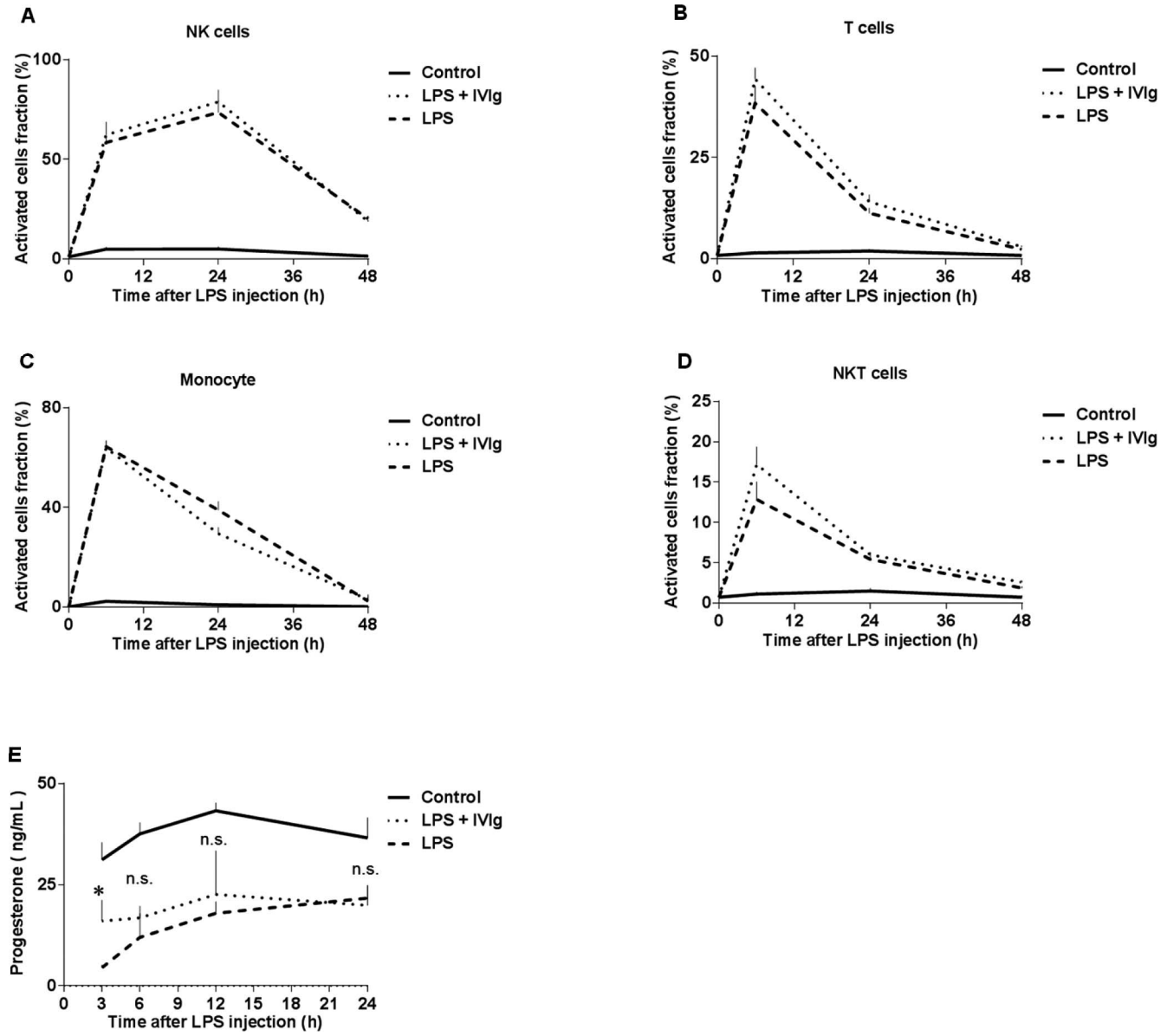

FIG. 2. IVIg does not affect activation of peripheral immune cells and plasma P4 concentration. IVIg (1000 mg/kg) was i.v. administered following i.p. injection of LPS to pregnant CBA/J mice on gd 7.5. Peripheral blood samples were collected at 6, 24, and 48 h after saline or LPS injection with or without IVIg, and were evaluated for activated ($CD69^+$) cell fractions of NK cells (**A**), T cells (**B**), monocytes (**C**), and NKT cells (**D**) by flow cytometry, or for the plasma concentration of P4 (**E**) by EIA. Data are the mean ± SEM (n = 3–5), $*P < 0.05$ (vs. same time control group by Student *t*-test). Results are representative of two or more independent experiments.

Next, we measured the plasma levels of the pregnancy hormone P4 [30–32] by EIA. The plasma P4 level was significantly decreased by LPS injection, regardless of IVIg administration. No difference in P4 level was found between the LPS and LPS+IVIg groups after 6 h, suggesting that IVIg does not affect plasma P4 levels, despite suppressing abortion (Fig. 2E). Additionally, we confirmed that concomitant administration of P4 and estradiol did not affect the LPS-induced abortion rate (data not shown). This result suggested that abortion in this model was not induced by a decrease in the plasma P4 concentration.

IVIg Suppresses the LPS-Induced Increase in uNK Cells

Because IVIg did not affect the activation of peripheral immune cells, we focused on evaluating the effect of IVIg on local immunity in the uterus. Lee et al. [23] reported that the number of uNK cells in the uterus (implantation site) was increased by LPS injection on gd 7.5 in pregnant C57BL/6 mice. Therefore, we evaluated the number of uNK cells at 24 h after LPS injection with or without IVIg. The results showed that uNK cells significantly increased upon LPS treatment, which was suppressed to the control level by IVIg (Fig. 3A). Shimada et al. [20] reported that the expression of CD94 on peripheral NK cells of uRPL patients (at that time, uRPL was termed recurrent spontaneous abortion) was increased by the administration of a high dose of IVIg. CD94 is a glycoprotein that binds to either an NKG2A or an NKG2C glycoprotein via a disulfide bond [33–35]. CD94/NKG2A inhibits NK cell activation [36]. On the other hand, CD94/NKG2C activates NK cells [37]. To allow comparison between clinical conditions and the mouse model, we measured the expression level of CD94 on uNK cells. The fraction of $CD94^+$ uNK cells

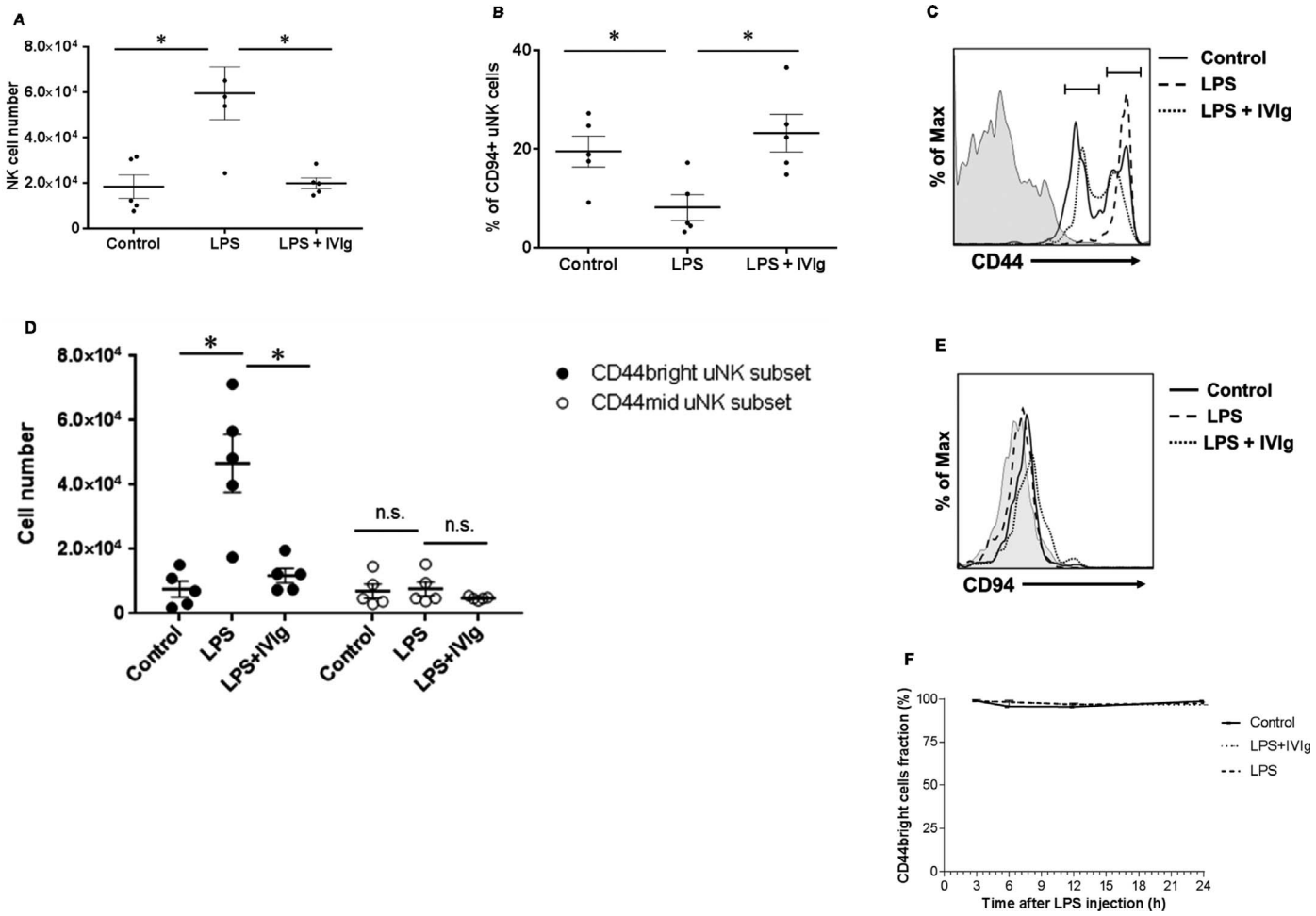

FIG. 3. IVIg specifically suppresses the increase in the cell number of the CD44^{bright} subset, which is CD94^{-}. IVIg (1000 mg/kg) was i.v. administered following i.p. injection of LPS to pregnant CBA/J mice on gd 7.5. Samples were collected at 24 h after saline or LPS injection with or without IVIg and were evaluated for total uNK, CD44^{bright} and CD44^{mid} uNK subset cell numbers, and expression of CD44 or CD94 by flow cytometry. **A**) Number of uNK cells calculated from the total number of cells in the sample and the population of uNK cells in the sample. Data are the mean ± SEM (n = 5). **B**) Population of CD94^{+} cells in the uNK cells was evaluated by flow cytometry. Data are the mean ± SEM (n = 5). **C**) Expression level of CD44 in the uNK cells of control (solid line), LPS (dotted line), and LPS+IVIg (dashed line) groups as determined by flow cytometry. The isotype control is shaded gray. **D**) Cell numbers of the CD44^{bright} and CD44^{mid} subsets of uNK cells calculated from the total number of cells in the sample and the population of each of the target cells in the sample. Data are the mean ± SEM (n = 5). **E**, **F**) Expression level of CD94 in the CD44^{bright} uNK subset (**E**) and of CD44 in peripheral NK cells (**F**) of control (solid line), LPS (dotted line), and LPS+IVIg (dashed line) groups as determined by flow cytometry. Results are representative of two or more independent experiments. *$P < 0.05$, n.s. > 0.05 versus same time in the control group (Student *t*-test).

was significantly decreased in the uRPL model mice and was normalized by IVIg administration (Fig. 3B).

From the seemingly conflicting results that LPS treatment increased the total number of uNK cells while it decreased the fraction of CD94^{+} uNK cells in the uRPL model mice, we hypothesized that two or more subsets of NK cells are present in the uterus. We used flow cytometry as a new approach to characterize uNK cells based on CD44 expression. We observed two distinct uNK subsets, which we designated as CD44^{bright} and CD44^{mid} (Fig. 3C). Interestingly, the number of CD44^{bright} uNK cells was significantly increased in uRPL mice, while the number of CD44^{mid} uNK cells remained unchanged. Furthermore, upon IVIg administration, the CD44^{bright} uNK subset did not increase (Fig. 3D). To confirm that this was because of a decrease in CD94^{+} uNK cells (Fig. 3B), we evaluated the expression of CD94 on the CD44^{bright} uNK subset. The CD44^{bright} uNK subset showed low expression of CD94 (Fig. 3E). Thus, we confirmed that the fraction of CD94^{+} uNK cells decreased with increasing number of CD94^{-}CD44^{bright} uNK cells. On the other hand, activated peripheral NK cells of the uRPL model mice consisted mostly of CD44^{bright} cells from 3 to 24 h after LPS injection, regardless of IVIg injection (Fig. 3F).

Time Course of the IVIg Effect in uRPL Model Mice

To evaluate the time course of the IVIg effect on abortion in the uRPL model mice, we evaluated the dynamics of total uNK and CD44^{bright} and CD44^{mid} uNK cells after LPS injection with or without IVIg administration. The numbers of uNK and CD44^{bright} uNK cells started to increase at 3 h after LPS injection and lowered after 12 h. Upon IVIg administration, the number of uNK cells decreased from 6 h to the level at 3 h (Fig. 4A). The CD44^{bright} subset showed a similar pattern (Fig. 4B), while the CD44^{mid} subset did not significantly change from 3 to 24 h after LPS with or without IVIg administration (Fig. 4C). These data indicated that the change in the total uNK cell number depended on the change in the number of CD44^{bright} cells.

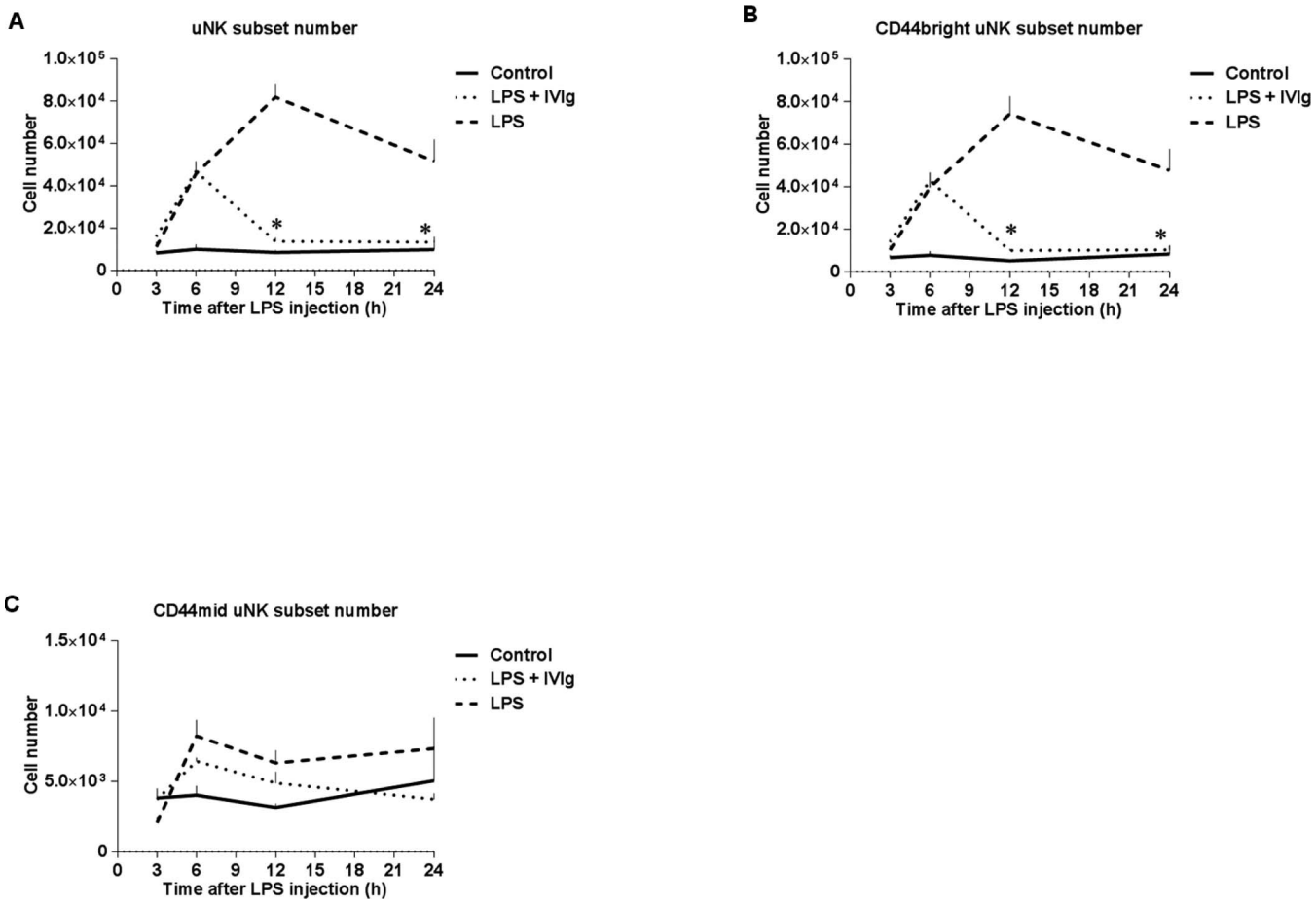

FIG. 4. IVIg suppresses the increase in the CD44^{bright} subset in uNK cells after 6 h. IVIg was i.v. administered following i.p. injection of LPS to pregnant CBA/J mice on gd 7.5. Samples were collected at 3, 6, 12, and 24 h after saline or LPS injection with or without IVIg (1000 mg/kg), and were evaluated for the number of uNK (**A**), CD44^{bright} uNK (**B**), and CD44^{mid} uNK (**C**) cells by flow cytometry. The number of cells was calculated from the total number of cell in each sample and the population of each of the target cells in the sample. Data are presented as mean ± SEM (n = 5). Results are representative of two or more independent experiments. *$P < 0.05$ versus same time in the LPS group (Student *t*-test).

Next, we examined the presence of IVIg in the uterus (Fig. 5A) and spleen (Fig. 5B), and the onset of abortion (Fig. 6, A–C) by pathological analysis. IVIg was observed in the uterus (Fig. 5AVII), but not in the spleen (Fig. 5BVII) in the LPS+IVIg group after 1 h. We did not confirm the IVIg (human IgG)-positive sites from 1–12 h in the control and LPS groups (Fig. 5, A and B). At 12 h after LPS injection, there were no or only minor changes in the configuration of the fetus (Fig. 6AIII). The control and LPS+IVIg groups showed normal phenotypes (Fig. 6A, I and V). At 18 h after LPS injection, the configuration, especially surrounding the embryo including the mesometrial and ante-mesometrial decidua, was dramatically disrupted (Fig. 6, AIV and B, and Supplemental Fig. S3); however, IVIg-administered uRPL mice showed normal fetuses (Fig. 6, AVI and C). These results suggested that IVIg was present the uterus within 1 h after administration and morphological changes related to the abortion started from 12 h after injection.

Effective Timing of IVIg Administration for Abortion Suppression

Because we observed no differences in the CD44^{bright} uNK subset between the LPS and LPS+IVIg groups until 6 h after injection (Fig. 4B), we determined the injection timing at which IVIg could suppress abortion in the uRPL model mice. To test this, IVIg was administered 1, 3, or 6 h after LPS injection. Unexpectedly, IVIg administered at 1 h after LPS injection caused a similar effect as simultaneous injection with LPS, while IVIg administered 3 or 6 h after LPS injection failed to lower the abortion rate (Fig. 7). This result suggested that administration of IVIg at 3 h after LPS injection is too late to suppress abortion in the uRPL model mice.

DISCUSSION

In the present study, we demonstrated a novel flow cytometry-based approach for characterizing uNK subsets in the uRPL model mice based on CD44 expression. Specifically, we discovered the presence of two distinct subsets: CD44^{bright} and CD44^{mid}. The CD44^{bright} uNK subset was increased in the uRPL model mice, while the CD44^{mid} uNK subset remained unchanged. Furthermore, the increase in the number of CD44^{bright} uNK cells was abolished when abortion was suppressed by IVIg administration. The correlation between abortion and increase in the CD44^{bright} subset suggests a role for the CD44^{bright} subset in abortion (but does not fully prove it). It is known that CD44 expression on mouse NK cells is upregulated by IL-2, IL-12, IL-15, and IL-18 [38]. If the CD44 expression level was changed from mid to bright in response to LPS injection, the CD44^{mid} subset should diminish. However, the number of CD44^{mid} cells did not change upon LPS

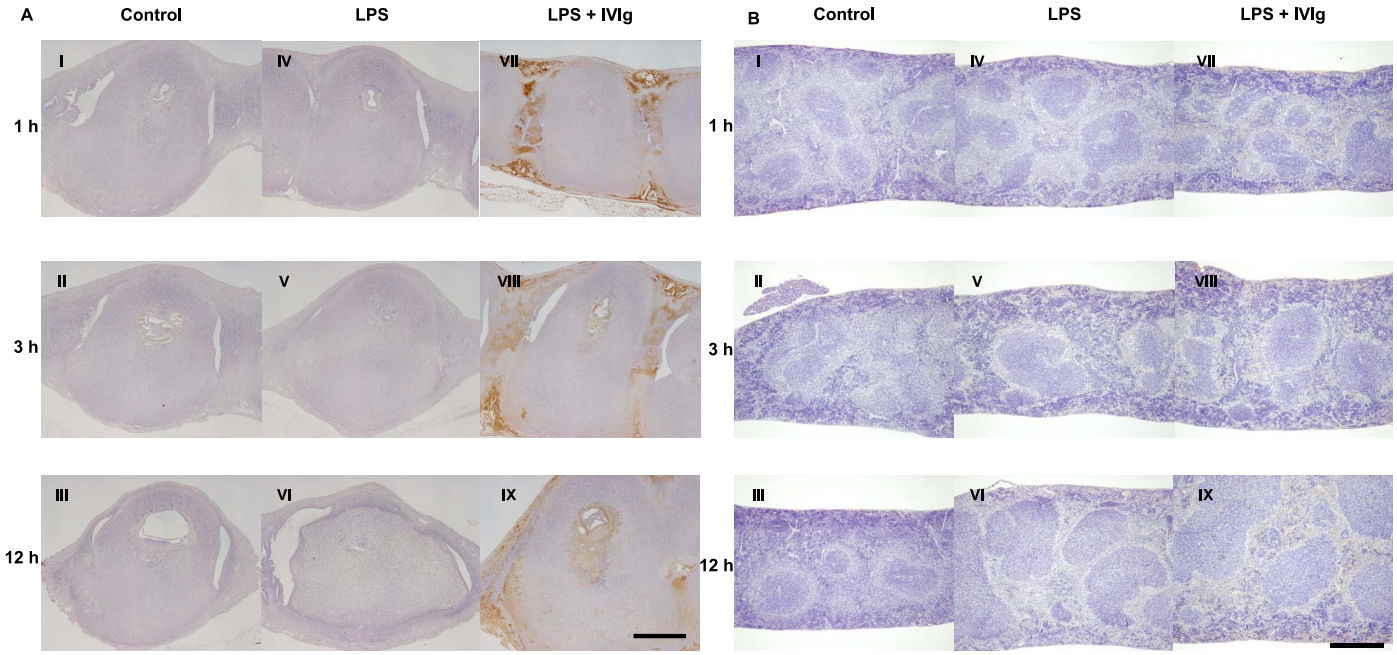

FIG. 5. IVIg existed in the uterus, but not in the spleen, after 1 h. IVIg (1000 mg/kg) was i.v. administered following i.p. injection of LPS into pregnant CBA/J mice on gd 7.5. Samples were collected at 1, 3, and 12 h after saline or LPS injection with or without IVIg and were analyzed for the presence of human IgG (hIgG) as the IVIg in the uterus (**A**) and spleen (**B**). Uteroplacental cross sections were stained with hIgG-specific antibody. The hIgG was stained the brown at 1, 3, and 12 h after saline or LPS injection with or without IVIg. Scale bars provided in **AIX** (1000 μm) and **BIX** (500 μm) serve all views in **A** and **B**, respectively. Original magnifications ×2 (**A**) and ×4 (**B**). Results are representative of two or more independent experiments.

treatment with or without IVIg. Therefore, we hypothesized that the CD44^{bright} and CD44^{mid} subsets consist of distinct cells. Moreover, it seems that the CD44^{bright} subset is related to abortion, while the CD44^{mid} subset maintains the pregnancy. In future studies, we will verify the precise effects of CD44^{bright} and CD44^{mid} subsets. Moreover, we will compare the expression level of CD44 in decidual NK cells between uRPL patients and healthy, pregnant women.

CD44 is a transmembrane glycoprotein involved in cell-cell interactions, cell adhesion, and migration. It is expressed in most tissues, including white and red blood cells, the uterus, placenta, colon, stomach, and type-II pneumocytes [39–43]. However, no relationship between the CD44 expression level of NK cells and abortion has been reported to date. To the best of our knowledge, we are the first to show that the CD44^{bright} uNK subset is highly related to abortion in an uRPL mouse model and is a novel target of IVIg in these mice.

There are several types of NK cells, which were officially classified as prototypical members of the group 1 innate lymphoid cells [44, 45]. Conventional NK cells, also termed peripheral NK cells, can have cytotoxic activity in the peripheral blood. In the uterus, resident uNK cells form the main subset and are considered pregnancy protecting [9, 10]. In humans, CD56^{bright}—but not CD56^{dim}—NK cells that are poorly cytotoxic but are potent cytokine producers are considered the cells supporting the maintenance of pregnancy [46–49]. Although mouse NK cells resemble their human counterparts in many regards, including their cytotoxic ability and cytokine production, they do not express the murine homolog of CD56. Mouse resident uNK cells, but not peripheral NK cells, have been reported to react with DBA lectin, allowing their identification [9]. Moreover, Yadi et al. showed that the CD3^{-}CD122^{+}NK1.1^{-}CD49b^{-} subset was a distinct uNK subset [10]. Unfortunately, we could not evaluate the expression level of CD122 or DBA-binding capacity of the CD44^{bright} and CD44^{mid} uNK subsets by flow cytometry in this study, and thus, we cannot infer whether they correspond to peripheral NK and resident uNK cells, respectively. However, the CD44 may be used as a marker of abortion-related cells in the uterus, and the CD44^{bright} uNK subset represents a novel target of IVIg for the uRPL model mice.

At present, the precise mechanisms of IVIg action as well as abortion in uRPL are unknown. The Fab, F $(ab')_2$, or Fc fragments of IVIg proved inactive compared to intact IVIg (Fig. 1B), possibly because generating IgG fragments may not preserve noncovalently associated anti-abortive soluble CD200 [19]. Few reports on the chronology of abortion are available, although several studies reported on various events of abortion using the uRPL mouse model, such as time of onset [50, 51], decreased P4 levels [30], or increased uNK cell numbers [23]. In this study, we showed the time course of abortion in this model. First, peripheral immune cells are activated within 6 h after LPS injection. Second, the number of CD44^{bright} NK cells in the uterus increases between 3 to 12 h. Third, an inflammation-related morphological change of the fetus is noticeable between 12 to 18 h. Finally, abortion occurs after 24 h. When IVIg is administered, 1) IVIg exits the uterus, but not the spleen, within an hour, 2) IVIg suppresses the increase in the cell number of the CD44^{bright} NK subset in the uterus between 6 to 12 h, and 3) abortion is prevented. These time course data indicated that IVIg affects the early phase of abortion in the uRPL mice. Furthermore, we demonstrated that IVIg did not affect the activated immune cells in the peripheral blood and the decreased level of peripheral P4, despite suppressing the abortion rate. Thus, these changes might be irrelevant for abortion in the uRPL model mice. Our results demonstrated that expansion of the CD44^{bright} NK subset in the uterus is the most important for abortion in uRPL mice, and IVIg specifically and quickly suppresses this.

When IVIg was administered simultaneously with LPS, the effect of IVIg on uNK cells did not appear until 6 h after injection (Fig. 4), indicating that there is a time lag between the

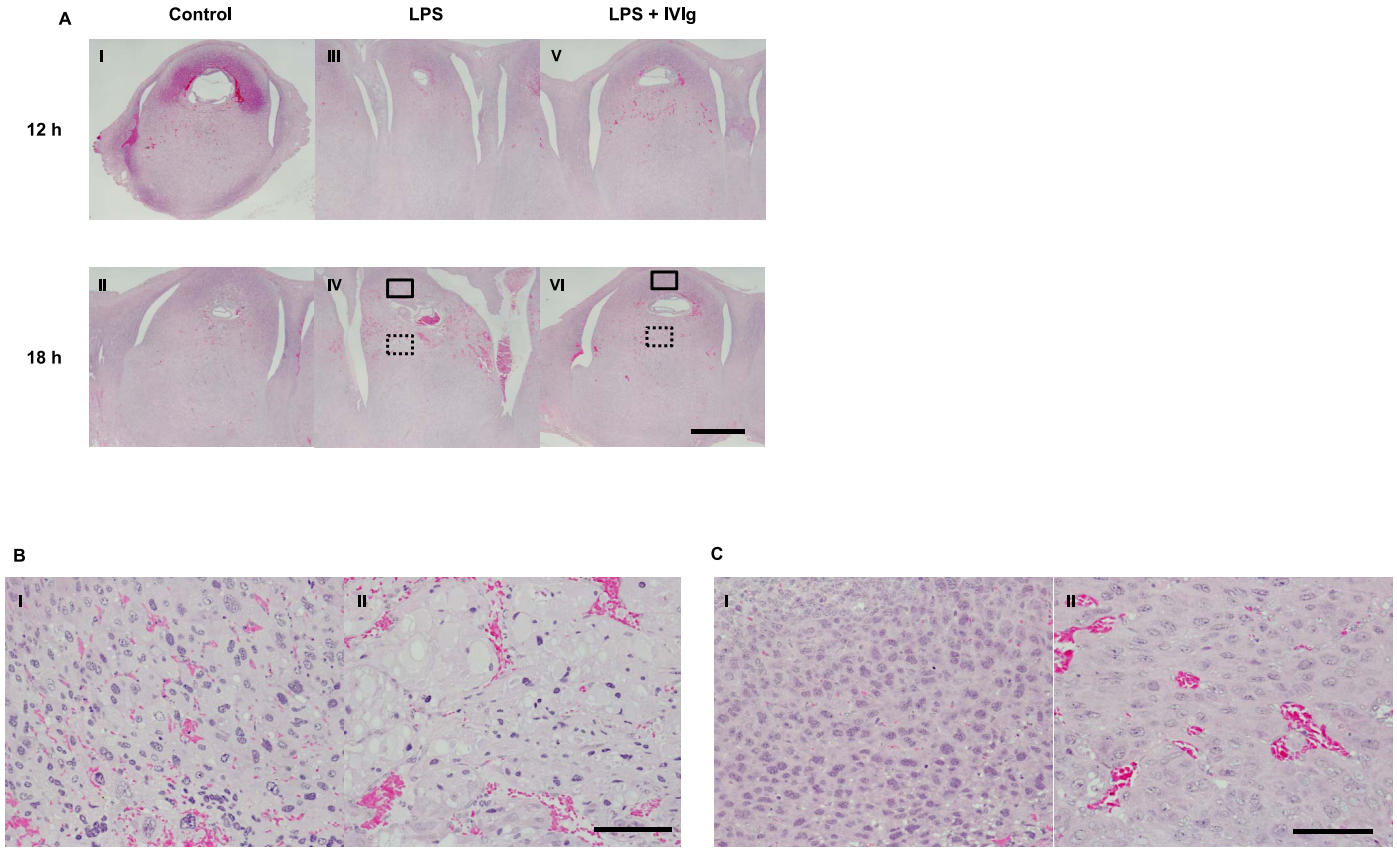

FIG. 6. IVIg suppresses morphological change of the fetus starting from 12 h. IVIg (1000 mg/kg) was i.v. administered following i.p. injection of LPS into pregnant CBA/J mice on gd 7.5. Samples were collected at 12 and 18 h after saline or LPS injection with or without IVIg and were analyzed for morphological changes (**A**–**C**). **A**) Photomicrographs of hematoxylin+eosin-stained uteroplacental cross sections at 12 and 18 h after saline or LPS injection with or without IVIg. Scale bar = 1000 μm; the scale bar provided in **A**VI serves all of **A**. **B**) Atypical location of morphological change in the LPS group at 18 h; magnification of the solid line square (**BI**) or dashed line square (**BII**) indicated in **AIV**; scale bar = 100 μm. **C**) Normal morphology of LPS+IVIg group at 18 h; magnification of the solid line square (**CI**) or dashed line square (**CII**) indicated in **AVI**; scale bar = 100 μm. Original magnifications ×2 (**C**) and ×20 (**B**, **C**). Results are representative of two or more independent experiments.

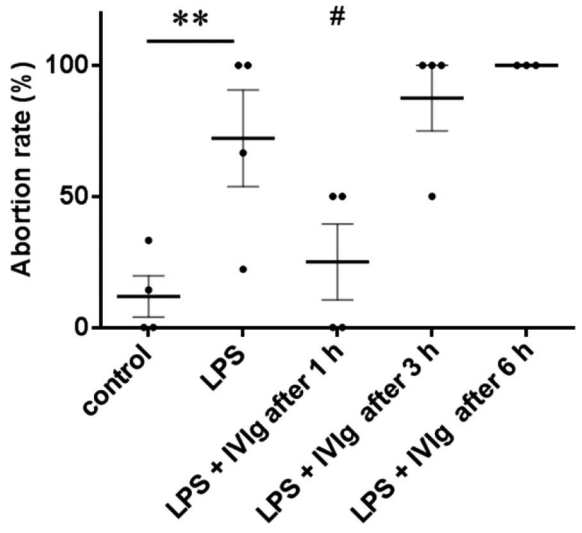

FIG. 7. IVIg administered 1, but not 3 or 6 h, after LPS injection suppresses the abortion rate of uRPL model mice. Pregnant CBA/J mice of each group were injected with LPS on gd 7.5 and with IVIg (1000 mg/kg) at 1, 3, or 6 h after LPS injection. Evaluation of abortion was performed on gd 13.5 by macroscopic examination. Data are the mean ± SEM (n = 5). Results are representative of two independent experiments. $^{**}P < 0.01$ (Student *t*-test). $\#P < 0.05$ versus LPS group (Dunnett-type multiple comparison method).

injection and the onset of the effect. On the one hand, IVIg administered at 3 h after LPS injection did not suppress abortion (Fig. 7). To explain these seemingly inconsistent observations, we hypothesize that IVIg does not affect NK cells but does suppress chemokine secretion by chemokine-producing cells in the uterus. Our group previously reported that IVIg improved the disease course in model mice of both systemic sclerosis [52] and chronic inflammatory demyelinating polyneuropathy [53] by suppressing chemokines. In this study, the time lag between IVIg administration and onset of effect can be explained as follows: upon intraperitoneal injection of LPS, some chemokine-producing cells in the uterus start to secrete chemokines, and after LPS injection, the concentration of chemokines rapidly reaches a level sufficient to induce $CD44^{bright}$ or pathogenic NK cells to migrate, resulting in the increase in these cells in the uterus. On the other hand, it takes about 6 h for intravenously administered IVIg to affect chemokine-producing cells in the uterus and downregulate chemokine secretion. We speculate that IVIg administration after 3 h of LPS injection is not effective because the total amount of secreted chemokines at 3 h after LPS injection would be sufficient to induce migration of pathogenic NK cells. Therefore, delayed IVIg administration cannot suppress abortion despite stopping chemokine secretion. In this study, we could not identify the specific chemokines involved; candidates are macrophage chemotactic protein-1, monocyte inflammatory protein-1 alpha, or

RANTES (regulated on activation, normal T cell expressed and secreted) because these receptors are expressed by peripheral NK cells [54, 55]. Evaluation of chemokine dynamics in the uterus remains to be conducted.

In the clinical setting, the fact that no optimum protocol for administration of IVIg to uRPL patients is available poses a major problem. There are two major strategies of IVIg therapy for uRPL patients. One is to administrate a high amount of IVIg at the early phase of pregnancy. The other is to maintain the concentration of IVIg in the peripheral blood at a certain level throughout the pregnancy, and even before pregnancy by preconceptional administration. In the present study, we evaluated dose- and time-dependent effects of IVIg administration in the uRPL model mice. We confirmed that a high dose of IVIg suppressed abortion in uRPL model mice. However, a high dose of IVIg administered at 3 h after LPS injection did not. On the other hand, we showed the presence of IVIg in the uterus, but not the spleen, at 1 h after IVIg administration. These results suggested that IVIg specifically affects the uterus at the early phase and a high dose is required for it to be effective in this model. In agreement with our findings, Yamada et al. reported a high live birth rate among uRPL patients who underwent high-dose IVIg therapy at the early phase of pregnancy (400 mg/kg for 5 days at Gestational Weeks 4–7) [6, 56], but not in patients who received medium-dose IVIg therapy (20 g/day for 3 days from gestational sac detection) [57]. Thus, it highly recommended that IVIg be administered to uRPL patients as early and as highly dosed as possible.

In the present study, we presented an effective protocol of IVIg administration to the uRPL model mice. Concomitantly, our findings revealed the suppression of the increase in the number of CD44^{bright} subset in uterine tissues of uRPL mice as a novel mechanism of action of IVIg. Because the high dose of IVIg specifically suppressed the increase in the pathogenic cell subset, our data indicate that IVIg should be administered to uRPL patients as early and as highly dosed as possible. Further, we propose CD44 as a marker of pathogenic uNK cells, with the CD44^{bright} uNK subset acting as a novel target for uRPL. Finally, our findings suggest the CD44^{bright} uNK subset as a potential therapeutic target for the treatment of uRPL.

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