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Neuromyelitis Optica IgG Causes Placental Inflammation and Fetal Death

Samira Saadoun,* Patrick Waters,† M. Isabel Leite,‡ Jeffrey L. Bennett,†,§ Angela Vincent,† and Marios C. Papadopoulos*

Neuromyelitis optica (NMO) is an inflammatory demyelinating disease of the CNS and affects women of childbearing age. Most patients with NMO have circulating Abs, termed NMO-IgG, against the astrocytic water channel protein aquaporin-4. In the CNS, NMO-IgG causes complement-mediated astrocyte damage, inflammatory cell infiltration, and myelin loss. In this study, we show that aquaporin-4 is expressed in the syncytiotrophoblast of human and mouse placenta. Placental aquaporin-4 expression is high during mid-gestation and progressively decreases with advancing pregnancy. Intrapertoneally injected NMO-IgG binds mouse placental aquaporin-4, activates coinjected human complement, and causes inflammatory cell infiltration into the placenta and placental necrosis. There was no damage to maternal organs that express aquaporin-4, including the brain, spinal cord, kidneys, and skeletal muscle. In control experiments, no placentitis was found in mice injected with NMO-IgG without complement, non-NMO-IgG with human complement, or in aquaporin-4 null mice injected with NMO-IgG and human complement. The infiltrating cells were primarily neutrophils with a few scattered eosinophils and macrophages. NMO-IgG and human complement–induced placentitis caused fetal death, but some fetuses were born normal when lower amounts of NMO-IgG and human complement were injected. Sivelestat, a neutrophil elastase inhibitor, and aquaporinumab, a nonpathogenic IgG that competes with NMO-IgG for aquaporin-4 binding, significantly reduced NMO-IgG and human complement induced placentitis and fetal death. Our data suggest that NMO-IgG can cause miscarriage, thus challenging the concept that NMO affects only the CNS. These findings have implications for the management of NMO during pregnancy.

Materials and Methods

Mice

We used CD1 wild type (WT) and AQP4-null (KO) mice (21) that were 8–12 wk old. Protocols were approved by the British Home Office. Investigators analyzing the data were unaware of mouse genotype and type of IgG injected.

Mouse tissue

Anesthetized mice were perfused-fixed through the left cardiac ventricle with 0.9% saline followed by 4% formaldehyde. Tissues were removed and
postfixed in 4% formaldehyde, dehydrated, and processed into paraffin. We also purchased ready-to-use CD1 mouse embryonic day (E) 10 to E18 placenta tissue sections (AMS Biotechnology, Abingdon, U.K.). Sections were stained with H&E or immunostained as described.

**Human tissue**

We used normal human tissue (formalin fixed, paraffin embedded) including fetal brain and spinal cord (20 and 40 wk old; Abcam, Cambridge, U.K.), placenta (15–20 wk; AmsBio, Abingdon, U.K.; GeneTex/TebuBio, Peterborough, U.K.; Insight Biotechnology, Wembley, U.K.), ovaries, uterus, and cervix (Insight Biotechnology, Wembley, U.K.). Normal 40-wk-old placentas were obtained from the Department of Pathology at St. George’s Hospital. Tissue sections were stained with H&E or immunostained for AQP4.

**Quantification of staining**

We examined four sections for each human placenta and two sections for each mouse placenta.

**Baseline placental AQP4 immunoreactivity.** We quantified syncytiotrophoblast AQP4 expression as the percentage of 10 high-power fields that were immunopositive: 0, for 0–25%; +, for 25–50%; ++, for 50–75%; +++, for 75–100%.

**Placental inflammation (H&E).** We determined the placenta to be inflamed if it had at least one aggregate of extravascular inflammatory cells.

**Placental C5b-9 immunoreactivity.** We determined the placenta C5b-9 to be immunopositive if it had at least one immunolabeled area.

**Mouse i.p. IgG and Chu injections**

To determine placental inflammation and fetal death, we injected pregnant mice with 0.8 ml polyclonal Ab (IgGNMO) or 2.5 mg recombinant mAb (NMO-IgG53, NMO-IgG58, CON-IgG2B4) plus 0.8 ml Chu. Pregnant mice were injected at E12, reinjected at E13, and killed at E14. One mouse was injected at E7, reinjected at E8, and killed at E9. To determine binding of NMO-IgG53[CY3] to the placenta, 25 μg was injected i.p., and mice were killed 6 h later. To determine the litter size at birth, mice were injected i.p. with 10 μg NMO-IgG53 (or CON-IgG2B4) plus 0.4 ml Chu at E12, E15, and E18.

**Figure 1.** AQP4 is expressed in human and mouse placenta. AQP4 immunoreactivity in (A) human ovary (H-OVARY), (B) human uterus (H-UTERUS), and (C) human placenta (top; H-PLACENTA; 20 and 40 wk gestation), AQP4 immunofluorescence in 20 wk human placenta (bottom left), and human placental AQP4 versus gestational age (bottom right). AQP4 immunoreactivity in (D) mouse ovary (M-OVARY), (E) mouse uterus (M-UTERUS), (F) mouse placenta (left; M-PLACENTA; E10, E13, E17, and E18). KO is AQP4 null mouse. Red arrowheads show AQP4. Mouse placental AQP4 versus gestational age is shown (right). (G) Binding of i.p. injected NMO-IgG53[Cy3] or CON-IgG2B4[Cy3] to placenta (WT or KO mouse). Tissue was double-stained (FITC) with commercial anti-AQP4: blue (DAPI), green (FITC), red (Cy3), and yellow (Merge). Scale bars, 20 μm (C, F), 50 μm (A, D, E, G), and 100 μm (B). Ce, Cervix; Cx, cortex; En, endometrium; Fo, follicle; My, myometrium; Pe, perimetrium.
Sivelestat and aquaporumab

Sivelestat (ONO-5046) was purchased from Tocris Bioscience (Bristol, U.K.). Point mutations were introduced into the IgG1 Fc sequence of the NMO-IgG53 H chain (L234A, L235A) to produce an aquaporumab that lacks effector functions (10, 22). We injected 3 mg sivelestat or 75 μg aquaporumab i.p. (plus 25 μg NMO-IgG53 and 0.8 ml Chu) at E12, re-injected at E13, and killed the mice at E14.

Tissue staining

Sections were incubated with primary Ab (1 h, at room temperature or overnight at 4˚C) followed by biotinylated secondary Ab (Vector Laboratories, Peterborough, UK) and avidin-linked HRP. Primary Abs were polyclonal rabbit anti-AQP4 (1:100; Millipore, Livingstone, U.K.), polyclonal rabbit anti–C5b-9 (1:100; Abcam, Cambridge, U.K.), polyclonal rat 1A8 anti Ly6G for neutrophils (1:100; BD Biosciences, Oxford, U.K.), polyclonal rat anti-macrophage (1:100; eBioscience, Hatfield, U.K.), polyclonal rabbit anti-CD3 (1:500; Dako Cytomation, Ely, U.K.). Immunostaining was visualized brown using DAB/H2O2. Counterstaining was performed hematoxylin. Some human placentas were immunostained for AQP4 followed by AlexaFluor-labeled secondary Ab. Eosinophils were visualized fluorescent red after tissue staining using the Eoprobe kit (SurModics, Edina, MN).

In vivo AQP4 labeling of mouse placenta

E12 pregnant mice were injected i.p. with 25 μg NMO-IgG3[Cy3] or CON-IgG2B4[Cy3] and killed after 6 h. The placentas were removed, fixed in paraformaldehyde for 1 h at room temperature, dehydrated in 30% sucrose overnight, and embedded in OCT. Tissue sections (7 μm) were incubated with polyclonal rabbit anti-AQP4 (1:100; Millipore) followed by FITC-anti-rabbit secondary Ab (1:200, Vector Labs, Peterborough, U.K.). Nuclei were labeled blue with DAPI.

Statistics

Two groups were compared with two-tailed Student t test using Microsoft Excel for Mac 2011 (version 14.3.2). Data in Fig. 4 (sivelestat, aquaporumab) were compared with one-way ANOVA and posthoc Tukey test at www.vassarstats.net.

Results

AQP4 expression in human female reproductive organs

No AQP4 was found in the human ovary including stroma, cortex, follicles (Fig. 1A) or uterus including endometrium, myometrium, perimetrium, and cervix (Fig. 1B). AQP4 was strongly expressed in human placental syncytiotrophoblast obtained from the second trimester of pregnancy, with little or no AQP4 expression in the third trimester (Fig. 1C). There was no AQP4 in the placental stroma or endothelium. Immunofluorescence staining suggested plasma membrane AQP4 expression in the syncytiotrophoblast.

AQP4 expression in mouse female reproductive organs

No AQP4 was found in the mouse ovary or uterus (Fig. 1D, 1E). Mouse placental syncytiotrophoblast began to express AQP4 at E11, reaching maximal level at E13, with progressively reduced AQP4 immunoreactivity until birth (Fig. 1F). AQP4 immunostaining was in a wire-loop pattern, characteristic of the syncytiotrophoblast plasma membrane. There was no AQP4 in the placenta of E13 KO mice. Therefore, AQP4 expression in the female mouse reproductive tract is comparable with human.

FIGURE 2. NMO-IgG causes complement-mediated placental inflammation in mice. WT pregnant mice were injected i.p. at E12 and reinjected at E13 with NMO-IgG58 plus Chu (n = 4), IgG58 plus Chu (n = 2), CON-IgG2B4 plus Chu (n = 5), IgG2B4 plus Chu (n = 2) or NMO-IgG58 (n = 3) and killed at E14. KO pregnant mice were similarly injected with NMO-IgG58 plus Chu (n = 3). (A) E14 placentas stained with H&E (top), and immunostained for C5b-9 (middle) and AQP4 (bottom). Neutrophils are indicated by black arrowheads; eosinophil is indicated by a purple arrowhead; AQP4 is indicated by red arrowheads. (B) Percent of placentas with (left) inflammation, (middle) C5b-9 immunoreactivity, and (right) normal AQP4 immunoreactivity. Each dot is a pregnant mouse. (C) E14 placentas (H&E). (D) Inflammatory cells within placental lesions; neutrophils (n, black arrowheads), eosinophils (e, purple arrowheads), macrophages (m, green arrowheads), and T lymphocytes (T). (E) A WT pregnant mouse was injected with NMO-IgG58 plus Chu at E7, reinjected at E8, and killed at E9. E9 placenta is stained with H&E. (F) Brain, spinal cord, kidney, and skeletal muscle from a pregnant mother. Insets show H&E and AQP4 immunostain. Scale bars, 10 μm (D), 20 μm (A), 50 μm [E, F, insets], 200 μm [C, F, sk. Muscle], 500 μm [(F), brain, sp. Cord, kidney]. fRBC, Fetal RBC; mRBC, maternal RBCs.*p < 0.01, ***p < 0.001.
NMO-IgG binds placental AQP4 in vivo

Cy3-tagged, AQP4-specific, recombinant monoclonal NMO-IgG (NMO-IgG58[Cy3]) or isotype recombinant IgG control (CON-IgG2B4[Cy3]) was injected i.p. in E12 pregnant mice. At 6 h, NMO-IgG58[Cy3] labeled the syncytiotrophoblast (Fig. 1G). In double labeling experiments, NMO-IgG58[Cy3] colocalized with commercial FITC-tagged anti-AQP4 Ab. There was no syncytiotrophoblast labeling when CON-IgG2B4[Cy3] was injected or in KO mice injected with NMO-IgG58[Cy3]. Therefore, circulating NMO-IgG enters the placenta and binds placental AQP4.

NMO-IgG causes placental inflammation

In these experiments, Cnu was coinjected with NMO-IgG because NMO-IgG does not activate mouse complement (12). We observed inflammatory cell infiltration into E14 placenta, after i.p. injections at E12 and E13 of NMO-IgG58 plus Cnu or the IgG fraction from NMO patient serum (IgGNSMO) plus Cnu (Fig. 2A, 2B). C5b-9 was deposited widely, and AQP4 expression was lost in the inflamed placentas. Some placentas had marked leukocyte infiltration and necrotic areas (Fig. 2C). Most of the infiltrating leukocytes were neutrophils with a few scattered eosinophils and macrophages, but no T lymphocytes (Fig. 2D). In control experiments, no leukocyte infiltration, no C5b-9 immunoreactivity, and no loss of AQP4 expression were found in placentas after injecting i.p. CON-IgG2B4 plus Cnu IgG from healthy individuals (IgGCON) plus Cnu or NMO-IgG58 (without Cnu). There was no placental leukocyte infiltration and no C5b-9 immunoreactivity after injecting i.p. NMO-IgG58 plus Cnu, in KO mice. There was no placental inflammation in an E9 pregnant mouse that had i.p. injections of NMO-IgG58 plus Cnu at E7 and E8—that is, at gestational stages without placental AQP4 activation, loss of AQP4 expression, and placental leukocyte infiltration.

Although circulating NMO-IgG also binds AQP4 in other organs including kidney and skeletal muscle (23), there was no inflammatory cell infiltration or loss of AQP4 expression in the brains, spinal cords, kidneys, or skeletal muscles of the injected mice (Fig. 2F). Therefore, i.p. injected NMO-IgG and Cnu selectively damage the placenta sparing other AQP4 expressing organs.

NMO-IgG–induced placentalitis causes fetal death

We counted the number of dead fetuses (in utero and spontaneously aborted) at E14 after injecting (at E12 and E13) NMO-IgG58 plus Cnu, or IgGNSMO plus Cnu, or CON-IgG2B4 plus Cnu or NMO-IgG58 (without Cnu) in WT mice or NMO-IgG58 plus Cnu in KO mice (Fig. 3A, 3B). There were significantly more dead fetuses in WT mice after injecting NMO-IgG58 (or IgGNSMO) plus Cnu versus CON-IgG2B4 (or IgGCON) plus Cnu. There were no dead fetuses after injecting NMO-IgG58 plus Cnu and only one dead fetus after injecting NMO-IgG58 plus Cnu in KO mice. Pregnant mice, which received a low dose of another AQP4-specific, recombinant monoclonal NMO-IgG (NMO-IgG53) plus Cnu every 2 d starting at E12, delivered significantly fewer pups than did pregnant mice similarly injected with CON-IgG2B4 plus Cnu or noninjected mice (Fig. 3C). The pups from the mice injected with NMO-IgG53 plus Cnu appeared normal (Fig. 3D) and had histologically normal brains, spinal cords, kidneys, and skeletal muscles (Fig. 3E). Therefore, NMO-IgG-induced placentalitis causes fetal death, but some fetuses are born normal when NMO-IgG levels are lower.

AQP4 is expressed in human fetal CNS

There was strong AQP4 expression in the front lobes and spinal cords of two human fetuses aged 20 and 40 wk (Fig. 3F). As in adult CNS, the fetal AQP4 was located perivascularly and in the glia limiting membrane in the brain and spinal cord. No AQP4 was found in the brain or spinal cord of E14 and E18 fetal mice (not shown).

Sivelestat and aquaporumab reduce NMO-IgG-induced placentalitis

We tested whether two emerging NMO treatments, sivelestat and aquaporumab, reduce placentalitis and fetal death induced by NMO-IgG plus Cnu (Fig. 4). Sivelestat is a selective neutrophil elastase inhibitor that inhibits neutrophil infiltration into mouse brain NMO lesions (24). Aquaporumab is a recombinant monoclonal NMO-IgG that lacks effector functions and sterically hinders pathogenic NMO-IgG from binding AQP4, thus reducing brain NMO lesions in mice (22). Sivelestat did not inhibit NMO-IgG53–induced Cnu activation or loss of AQP4 expression in the placenta. Although Cnu-mediated damage to the syncytiotrophoblast was not inhibited, sivelestat markedly reduced placental neutrophil infiltration and fetal death. Aquaporumab inhibited the NMO-IgG53–induced Cnu activation, loss of placental AQP4 expression and the placental neutrophil infiltration and fetal death.
Discussion

We showed that NMO-IgG can damage the mouse placenta and cause fetal death. Three factors (NMO-IgG, AQP4, and Chu) are required for the placental inflammation to occur. Excluding any one of these factors (using CON-IgG instead of NMO-IgG, using a KO mouse instead of WT, omitting Chu) does not produce placental inflammation. These findings might explain the placental inflammation, complement activation in the syncytiotrophoblast, loss of AQP4 expression, and miscarriage in an NMO-IgG+ pregnant patient, which occurred at 21 wk (when placental AQP4 expression is high) (19). Our data might also account for the increased risk of miscarriage in NMO-IgG+ women (Leite et al., manuscript in preparation). It would be interesting to investigate the risk of miscarriage in seronegative NMO patients and in NMO-IgG+ patients who do not meet all clinical criteria for NMO (25). Because NMO-IgG is essential for placental inflammation and fetal death to occur, we predict that the risk of miscarriage is not elevated in seronegative NMO patients, but is high in NMO-IgG+ patients. Our data suggest that, to prevent miscarriage, NMO-IgG levels should be monitored during pregnancy and kept low. However, in some patients with NMO, autoantibodies other than NMO-IgG (26, 27) might also have a role in pregnancy-related complications.

Based on our findings, we propose the following mechanism for NMO-IgG–mediated placental damage (summarized in Fig. 5).

**FIGURE 4.** Sivelestat and aquaporumab reduce NMO-IgG–mediated placental damage. (A) Placentas (H&E, C5b-9 immunostain, AQP4 immunostain) after i.p. injection of NMO-IgG53 plus Chu (n = 3), NMO-IgG53 plus Chu plus sivelestat (+S, n = 3) or NMO-IgG53 plus Chu plus aquaporumab (+A, n = 3). (B) Percentage of placentas with neutrophil infiltration and C5b-9 deposits and percentage of dead fetuses per pregnant mouse. Scale bars, 30 μm [(A) H&E right, C5b-9, AQP4], 300 μm [(A) H&E left]. *p < 0.01.

NMO-IgG binds the placental syncytiotrophoblast of fetal villi and activates the classical complement pathway. C5b-9 becomes deposited in the syncytiotrophoblast plasma membrane, thus damaging the syncytiotrophoblast and causing loss of AQP4 expression. Leukocytes (primarily neutrophils) then infiltrate into the placenta, releasing elastase and other proteases that cause further placental damage. Some histologic features of the placental NMO lesions (loss of AQP4 expression, C5b-9 deposition at sites of AQP4 expression, leukocyte infiltration) are analogous to the CNS NMO lesions. Severely inflamed placentas become necrotic, which causes fetal death or spontaneous miscarriage. Less inflamed placentas (as seen when less NMO-IgG and Chu is injected) are compatible with normal fetal survival and birth. The mechanism proposed in this study might explain why some NMO-IgG+ women miscarry, but others have successful, healthy pregnancies.

Little or no AQP4 expression was detected in the human and mouse female reproductive tracts, which is consistent with previous studies that reported little or no AQP4 protein in the human vagina (28) and ovary (29). There is also no AQP4 in human testes and sperm (not shown). The lack of AQP4 expression in the female reproductive tract and sperm suggests that NMO-IgG does not impair the early stages of conception (ovulation, sperm migration, fertilization, and implantation). Our finding of high AQP4 expression in human and mouse placental syncytiotrophoblast, with progressive downregulation throughout pregnancy, is consistent

**FIGURE 5.** Proposed mechanism of NMO-IgG–induced placental inflammation. (A) Normal fetus in uterus with a (B) magnified view of a normal fetal villus showing AQP4 within the syncytiotrophoblast plasma cell membrane. (C) NMO-IgG binds extracellular epitopes on AQP4 and (D) activates complement causing the deposition of membrane attack complexes (C5b-9) in the syncytiotrophoblast plasma membrane. (E) Leukocytes infiltrate the placenta, primarily neutrophils (NΦ) with some eosinophils (EΦ) and macrophages (MΦ). (F) Severe placental inflammation causes fetal death, but (G) mild placental inflammation allows normal fetal growth. Aquaporumab (A) inhibits NMO-IgG binding, and sivelestat (S) inhibits neutrophil-mediated damage.
NMO-IgG CAUSES PLACENTAL INFLAMMATION

Disclosures
The authors have no financial interests of conflict.

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