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Neuromyelitis Optica IgG Causes Placental Inflammation and Fetal Death

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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 (AQP4). 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. Intrapititoneally injected NMO-IgG binds mouse placental aquaporin-4, activates co-injected 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 placenta 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.

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Abbreviations used in this article: AQP4, aquaporin-4; C5b-9, complement membrane attack complex; C100, human complement; CON-IgG1, measles virus–specific recombinant IgG1; CON-IgG3/Cy3, Cy3 labeled CON-IgG3; E, embryonic day; IgG53, serum IgG fraction from healthy volunteers; IgG53/AQP4 serum IgG fraction from NMO-IgG+ patients; KO, AQP4 null; NMO, neuromyelitis optica; NMO-IgG1, rAb-53 monoclonal recombinant anti-AQP4 IgG1; NMO-IgG3, rAb-58 monoclonal recombinant anti-AQP4 IgG3; WT, wild type.

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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.

**Placental AQP4 expression after i.p. injection.** AQP4 expression was determined to be normal if the grade was ++ or +++. Investigators were unaware of the experimental conditions when examining samples.

**NMO-IgG and control IgG**

Sera from two patients with NMO (with strong AQP4 autoantibody serum positivity), and two healthy subjects were processed to obtain the IgG fractions, termed IgGNMOM and IgGCON, respectively. IgG concentration was 6–38 mg/ml. C

Mouse i.p. IgG and C

To determine placental inflammation and fetal death, we injected pregnant mice with 0.8 ml polyclonal Ab (IgGNMO) or 25 μg 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] (and CON-IgG2B4[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 (Cy5), 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-IgG53[Cy3] or CON-IgG2B4[Cy3] and killed after. 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.

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.
**NMO-IgG binds placental AQP4 in vivo**

Cy3-tagged, AQP4-specific, recombinant monoclonal NMO-IgG (NMO-IgG53[Cy3]) or isotype recombinant IgG control (CON-IgG2B4[Cy3]) was injected i.p. in E12 pregnant mice. At 6 h, NMO-IgG53[Cy3] labeled the syncytiotrophoblast (Fig. 1G). In double labeling experiments, NMO-IgG53[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-IgG53[Cy3]. Therefore, circulating NMO-IgG enters the placenta and binds placental AQP4.

**NMO-IgG causes placental inflammation**

In these experiments, C$_{hu}$ 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 C$_{hu}$ or the IgG fraction from NMO patient serum (IgG$_{NMO}$) plus C$_{hu}$ (Fig. 2A, 2B). C$_{5b}$-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 C$_{5b}$-9 immunoreactivity, and no loss of AQP4 expression were found in placentas after injecting i.p. CON-IgG2B4 plus C$_{hu}$ or IgG$_{CON}$ plus C$_{hu}$ or NMO-IgG58 (without C$_{hu}$). There was no placental leukocyte infiltration and no C$_{5b}$-9 immunoreactivity after injecting i.p. NMO-IgG58 plus C$_{hu}$ in KO mice. There was no placental inflammation in an E9 pregnant mouse that had i.p. injections of NMO-IgG58 plus C$_{hu}$ at E7 and E8—that is, at gestational stages without placental AQP4 expression (Fig. 2E). The results of these experiments suggest that after binding the syncytiotrophoblast, NMO-IgG causes C$_{hu}$ 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 C$_{hu}$ 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 C$_{hu}$ or IgG$_{NMO}$ plus C$_{hu}$ or CON-IgG2B4 plus C$_{hu}$ or NMO-IgG58 (without C$_{hu}$) in WT mice or NMO-IgG58 plus C$_{hu}$ in KO mice (Fig. 3A, 3B). There were significantly more dead fetuses in WT mice after injecting NMO-IgG58 (or IgG$_{NMO}$) plus C$_{hu}$ versus CON-IgG2B4 (or IgG$_{CON}$) plus C$_{hu}$. There were no dead fetuses after injecting NMO-IgG58 (without C$_{hu}$) and only one dead fetus after injecting NMO-IgG58 plus C$_{hu}$ in KO mice. Pregnant mice, which received a low dose of another AQP4-specific, recombinant monoclonal NMO-IgG (NMO-IgG53) plus C$_{hu}$ every 2 d starting at E12, delivered significantly fewer pups than did pregnant mice similarly injected with CON-IgG2B4 plus C$_{hu}$ or noninjected mice (Fig. 3C). The pups from the mice injected with NMO-IgG53 plus C$_{hu}$ 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 frontal 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 C$_{hu}$ (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-IgG$_{53}$–induced C$_{hu}$ activation or loss of AQP4 expression in the placenta. Although C$_{hu}$-mediated damage to the syncytiotrophoblast was not inhibited, sivelestat markedly reduced placental neutrophil infiltration and fetal death. Aquaporumab inhibited the NMO-IgG$_{53}$–induced C$_{hu}$ 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). 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
AQP4 is one of several aquaporins expressed in the placenta (31). The function of AQP4 and other aquaporins in the placenta during normal pregnancy is unknown. We previously reported that KO×KO mouse matings produce normal pups and normal litter size with normal male:female ratio (32), which suggests that placental AQP4 has only a minor role in normal gestation in mice. We showed that AQP4 is expressed in human fetal CNS as early as 20 wk, consistent with an earlier report (33). AQP4 in the human fetus is found pervasively and in the glia limitans, as in human adults. Little or no AQP4 was seen in the brain or spinal cord of fetal mice (not shown), in agreement with rat studies (34). These observations are consistent with the fact that human brains are more developed in utero compared with rodents (35). For example, the brains of 20- and 40-wk-old human embryos correspond developmentally to the brains of mice aged 21 and 30 d after coitus, respectively (36). The lack of AQP4 expression in rodent fetal CNS suggests that fetal death after NMO-IgG and CμH4 injection is not a direct effect of NMO-IgG on the fetal CNS. However, the presence of AQP4 in human fetal CNS raises the possibility that maternal NMO-IgG might directly damage the human fetal CNS.

Systemically injected NMO-IgG binds AQP4 in peripheral organs (including kidney, skeletal muscle, and stomach), but not in the CNS apart from the area postrema (23). Mice injected i.p. with NMO-IgG and CμH4 have placental inflammation without CNS or peripheral organ inflammation. There is no CNS inflammation probably because the blood-brain barrier inhibits entry of circulating NMO-IgG and CμH4 into the CNS. Possible explanations for the lack of inflammation in peripheral organs (other than placenta) include low AQP4 expression, only little CμH4 reaching these organs, high complement regulator expression, and unique interstitial environments (e.g., high renal osmolality, gastric acidity) that might preclude CμH4 activation.

We provided proof-of-principle that sivelestat (24) and aquaporumab (22) reduce the risk of NMO-IgG–induced miscarriage. The therapeutic efficacy of sivelestat in mice suggests that, after injection with NMO-IgG plus CμH4, fetal death is caused by the placental neutrophil infiltration rather than the CμH4 activation. Trophoblast regeneration (37, 38) might explain why Chu activa-

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