

RESEARCH ARTICLE

Neuromyelitis optica (NMO)-IgG-driven organelle reorganization in human iPSC-derived astrocytes

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Abstract

Neuromyelitis optica (NMO) is an autoimmune disease that primarily targets astrocytes. Autoantibodies (NMO-IgG) against the water channel protein, aquaporin 4 (AQP4), are a serologic marker in NMO patients, and they are known to be responsible for the pathophysiology of the disease. In the brain, AQP4 is mainly expressed in astrocytes, especially at the end-feet, where they form the blood-brain barrier. Following the interaction between NMO-IgG and AQP4 in astrocytes, rapid AQP4 endocytosis initiates pathogenesis. However, the cellular and molecular mechanisms of astrocyte destruction by autoantibodies remain largely elusive. We established an in vitro human astrocyte model system using induced pluripotent stem cells (iPSCs) technology in combination with NMO patient-derived serum and IgG to elucidate the cellular and functional changes caused by NMO-IgG. Herein, we observed that NMO-IgG induces structural alterations in mitochondria and their association with the endoplasmic reticulum (ER) and lysosomes at the ultrastructural level, which potentially leads to impaired mitochondrial functions and dynamics. Indeed, human astrocytes display impaired mitochondrial bioenergetics and autophagy activity in the presence of NMO-IgG. We further demonstrated NMO-IgG-driven ER membrane deformation into a multilamellar structure in human astrocytes. Together, we show that NMO-IgG rearranges cellular organelles and alter their functions and that our in vitro system using human iPSCs offers previously unavailable experimental opportunities to study the pathophysiological mechanisms of NMO in human astrocytes or conduct large-scale screening for potential therapeutic compounds targeting astrocytic abnormalities in patients with NMO.

KEYWORDS

astrocytes, autophagy, endoplasmic reticulum, human iPSC, lysosome, metabolic flux, mitochondria, neuromyelitis optica (NMO)

Abbreviations: AQP4, aquaporin 4; CNS, central nervous system; ER, endoplasmic reticulum; IgG, immunoglobulin G; IP3R, inositol 1,4,5-triphosphate receptor; iPSC, induced pluripotent stem cell; NMO, neuromyelitis optica; NPC, neuronal progenitor cell; OMN, outer membrane of mitochondria; PLA, proximity ligation assay; VDAC1, voltage-dependent anion-selective channel protein1.

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1 | INTRODUCTION

Neuromyelitis optica (NMO) is a primary astrocytic disease associated with inflammation and secondary myelin loss in the central nervous system (CNS).¹ The presence of autoantibodies (NMO-IgG) in patient sera targeting aquaporin 4 (AQP4), a water channel, is an important clinical biomarker that plays a central role in disease pathogenesis.^{2,3} In the CNS, AQP4 is predominantly expressed in astrocytes, especially on their end-foot processes.⁴ Upon NMO-IgG binding to AQP4, endocytosis is initiated, which consequently reduces AQP4 membrane levels.^{5,6} The interaction between NMO-IgG and astrocytic AQP4 leads to the synthesis of components of the complement system and cytokines, which contribute to pathogenesis in other cell types such as microglia.^{7,8} While NMO-IgG plays a central role in disease pathogenesis, the cellular changes driven by NMO-IgG in astrocytes and their associated molecular mechanisms are largely unknown.

Astrocytes are key components of the CNS. They provide trophic support for synaptogenesis, maintain a homeostatic environment, regulate synaptic activity, and sustain the metabolic needs of neurons.^{9–11} Dysfunctional astrocytes are associated with multiple CNS disorders, including epilepsy, inflammatory demyelinating diseases, metabolic disorders, and neurodegenerative disorders.¹² Changes in astrocyte morphology, gene expression, and intracellular organelle networks are largely linked to their functions.^{13–16} Recent studies have suggested the importance of understanding the mechanisms of organelle remodeling and subsequent functional alterations in astrocytes under pathological conditions, such as neuroinflammation and neurodegeneration.^{16–19}

Fundamental biology of astrocytes and pathogenic changes in NMO has been uncovered by rodent systems, which provide the groundwork for human studies.^{5–8,20–23} To complement animal models and further our understanding of pathogenic mechanisms in a human context, here we establish an *in vitro* human model system for NMO. Using human induced pluripotent stem cells (iPSCs) technology and NMO patient-derived serum and IgG, we sought to elucidate the effect of NMO-IgG on astrocyte biology.

Our observations provide evidence that patient-derived NMO-IgGs alter cellular organelle architecture, including mitochondrial integrity, endoplasmic reticulum (ER) membranes, mitochondria-ER contact sites, and the extent of lysosomal interactions with both mitochondria and ER. In accordance with the structural modifications in organelles, the analysis of mitochondrial oxidative metabolism showed impaired spare and maximum respiratory capacity in astrocytes under NMO-IgG treatment.

Astrocytes also became disruptive in autophagic flux, particularly during the lysosomal degradation step after exposure to NMO-IgG.

2 | MATERIALS AND METHODS

2.1 | Ethical considerations

This study was approved by the internal review boards of Korea Brain Research Institute (KBRI-201905-BR-001-01) and DGIST (DGIST-190828-BR-070-01).

2.2 | Human sera and IgG purification

De-identified human sera were obtained from the Samsung hospital. Sera from 10 healthy individuals and 11 NMO patients were pooled, and NMO-IgG positivity was confirmed in all patients. Sera were heat-inactivated for 30 min at 65°C. Control and NMO IgG were isolated from sera by using HiTrap Protein G HP columns (GE Healthcare) according to the manufacturer's protocols. Serum or IgG was applied on astrocytes at 10% or 1 mg/ml concentration respectively.

2.3 | Human iPSC culture and astrocyte differentiation

Human iPSC lines from a healthy control were purchased from the Coriell Institute (#GM23720, female, Age 22) or obtained from Dr Bruce Yankner's laboratory,²⁴ in which stemness, pluripotency, and normal karyotypes were certified. iPSCs were cultured in mTeSR1 media (STEMCELL Technologies) in six-well culture plates (Corning) coated with hESC-qualified matrigel (Corning) until they reached ~100% confluence. Then cells were differentiated into neural progenitor cells (NPCs) by following previous protocols.²⁵ Once the neural rosette structures uniformly formed, NPCs were prepared for astrocyte differentiation as described previously.²⁶ After 28 days of differentiation, cells were subjected to fluorescence-activated cell sorting with an anti-GLAST antibody (Miltenyi Biotec) to increase the purity.

2.4 | Autophagy flux analysis

Tandem fluorescent-tagged LC3 from Addgene (mRFP-GFP-MAP1LC3B, 21074, deposited by Tamotsu Yoshimori) was transfected with Lipofectamin Stem Transfection reagent (Invitrogen) by following the manufacturer's

protocol. After 24 h of transient transfection, cells were subjected to HC-IgG or NMO-IgG for 6 h and fixed with 4% paraformaldehyde (PFA). Images were captured under a confocal microscope (Zeiss LSM800) and analyzed by quantifying numbers of RFP/GFP colocalization puncta and RFP puncta to monitor autophagy flux.

2.5 | Immunocytochemistry

Astrocytes were cultured on glass coverslips and washed with Dulbecco's phosphate-buffered saline (DPBS) before fixation with 4% PFA. Following 10 min fixation, cells were washed with PBS and incubated with blocking buffer (0.1% Triton X-100, 10% normal donkey serum, 2% BSA, and 1 M glycine in PBS) for 1 h at room temperature. The primary antibody was diluted in blocking buffer. After 1 h incubation at room temperature, cells were washed with 0.1% PBST (0.1% Triton X-100 in PBS) for 10 min three times. Following secondary antibody incubation and washing, coverslips were mounted with DAKO mounting media (Agilent). For surface labeling, cells were treated in non-permeabilizing conditions (without detergent) and follow the same procedure as described above.

2.6 | Transmission electron microscopy

Astrocytes were treated with HC IgG or NMO IgG for 6 h followed by fixation with 2.5% glutaraldehyde and 2% PFA in a 0.15 M sodium cacodylate buffer for 2 h at room temperature. After washing three times with cacodylate buffer, post-fixation with 2% osmium tetroxide was performed for 1 h at room temperature. Following washing three times with DH_2O , samples were dehydrated with a series of graded ethanol. Samples were then embedded in Epon. Ultrathin sections (60 nm) were prepared and stained with lead citrate and uranyl acetate. The sections were imaged with FEI Tecnai G2 F20 TWIN TMP at 200 Kv accelerating voltage using Gatan TEM CCD camera.

2.7 | Correlative light and electron microscopy

Astrocytes were plated on gridded glass-bottom dishes (MatTek Corp. #P35G-2-14-CGRD) and stained with LysoTracker (LysoTracker Red DND-99, Thermofisher; 250 nM) and ER-Tracker (ER-Tracker Blue-White DPX, Thermofisher; 500 nM) for 30 min after 6 h of IgG treatment. Cells were fixed with 4% PFA and transferred to a confocal microscope (Zeiss LSM 800) to acquire overview images. Samples then were processed with a standard

TEM sampling procedure as described above and the electron micrographs were fitted to fluorescence images to identify the multilamellar structure.

2.8 | Morphological analysis of mitochondria

To work from large photomontages, we reconstructed the whole astrocyte for unbiased quantitative analysis of mitochondrial morphology in the cell body area. The mitochondrial structure was analyzed using Fiji software. After manually tracing mitochondria on electron micrographs of astrocytes, mitochondrial perimeter, circularity ($(4\pi \times \text{area})/(\text{perimeter}^2)$), and form factor ($(\text{perimeter}^2)/(4\pi \times \text{area})$) were analyzed as to their shape descriptor. Using identical images used for the shape analysis, quantification of mitochondrial ultrastructural defects was measured. Abnormal mitochondria were defined according to one of the following criteria: mitochondria with swollen, irregular, or deficient cristae; mitochondria with the discontinuous or swollen outer membrane. To analyze mitochondria-ER contact, we defined the contact regions that are within a close apposition (within 20 nm) of the mitochondrial outer membrane and the ER membrane as described previously.²⁷ At least 255 mitochondria were analyzed from two astrocytes per condition and the quantification was performed blinded to conditions.

2.9 | Lactate dehydrogenase assay

Cellular toxicity was measured as the release of Lactate dehydrogenase (LDH). The cells were cultured in the conditions described above, and cells were treated with or without human complement in the presence of IgG. LDH activity in the supernatant was spectrophotometrically measured using sodium pyruvate (25 mmol/L) as a substrate in 50 mmol/L sodium phosphate buffer (pH 7.5).

2.10 | Western blot

Astrocytes were washed with cold DPBS then incubated with RIPA buffer (50 mM Tris pH 8, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, protease, and phosphatase inhibitor cocktails) for 30 min on ice. The homogenates were centrifuged at 13 000× g for 15 min at 4°C and protein concentrations were measured using the Bradford assay kit (Bio-Rad). The equal amount of protein from each sample was subjected to SDS-PAGE and transferred to nitrocellulose membranes.

The membranes were blocked with 5% non-fat dry milk in PBS with 0.1% Tween 20 for 1 h at RT and incubated overnight at 4°C with appropriate primary antibody diluted in 3% BSA and 0.1% Tween 20 in PBS. Following incubation with secondary antibody, protein bands were detected with FUSION FX7 and densitometric analysis was performed using Fiji.

2.11 | Metabolic flux analysis

Astrocytes were cultured on a Seahorse XFe24 well plate (Seahorse BioSciences) at a density of 75 000 cells per well two days before analysis. On the day of analysis, cells were subjected to HC-IgG or NMO-IgG treatment for 6 h, and media was changed to fresh DMEM with 25 mM glucose, 1 mM pyruvate, and 2 mM L-glutamine. After incubation in a non-CO₂ incubator for 30 min, oxygen consumption rate (OCR) was measured in the Seahorse Extracellular Flux (XF) metabolic analyzer (Agilent). Following baseline measurements for 20 min, OCR was continuously recorded with the sequential injection of oligomycin (1 μM), FCCP (carbonyl cyanide 4-(trifluoromethoxy)-phenylhydrazone) (1 μM), and rotenone (1 μM).

2.12 | Staining of fluorescent organelle dyes

Astrocytes were incubated with LysoTracker (LysoTracker Red DND-99, Thermofisher; 250 nM), MitoTracker (MitoTracker Green FM, Thermofisher; 250 nM), and ER-Tracker (ER-Tracker Blue-White DPX, Thermofisher; 500 nM) for 30 min at 37°C. After washing with DPBS three times, samples were fixed with 4% PFA for 10 min. Samples were immediately imaged with a confocal microscope (Zeiss LSM 800).

2.13 | IL-6 ELISA

The supernatants were collected from astrocytes treated with 10% sera or 1 mg/ml IgG from control or NMO patients for 6 h. The concentration of IL-6 was measured using a Human ELISA kit (R&D Systems) according to the manufacturer's protocol.

2.14 | Statistical analysis

GraphPad Prism 9.0 software was used to perform statistical analysis. Results are expressed as means with their standard errors. Differences among the means were tested

for statistical significance by two-tailed Student's *t*-test or one-way ANOVA and differences were considered significant at *p* < .05.

3 | RESULTS

3.1 | In vitro human model system to study astrocyte pathology in NMO

To establish an in vitro human model system for the pathophysiology of NMO, we first differentiated iPSCs originating from the fibroblasts of a healthy individual (female, age 22) into NPCs with SMAD inhibitors, as previously described.²⁸ NPCs were then cultured with astrocyte media for >1 month, followed by GLAST-positive sorting to increase astrocyte purity (Figure 1A).²⁶ The identity of human astrocytes was confirmed by the expression of astrocyte-specific markers, GFAP and S100β, and a distinct AQP4 expression level was observed in astrocytes compared to NPCs (Figure 1B,C). IgG was isolated from the sera of both NMO patients and healthy donors, and human iPSC-derived astrocytes were subjected to treatment with NMO-IgG or control IgG to investigate NMO-IgG-induced pathology (Figure 1A).

Consistent with previous studies using murine models and immortalized human astrocytes, we observed a significant reduction in AQP4 surface expression after 6 h of NMO-IgG treatment (Figure 1D). While there was no change in the total AQP4 levels with 6 h treatment (Figure 1E), a significant decrease was observed in astrocytes after 24 h NMO-IgG treatment (Figure 1E). We also found that NMO-serum alone is sufficient to increase IL-6 secretion and that NMO-IgG facilitates complement-dependent cytotoxicity in human iPSC-derived astrocytes (Figure S1A,B), which was verified in an additional iPSC-derived astrocytes from an independent healthy individual (Figure S2A,B). By combining human iPSC technologies with patient-derived IgG, we could recapitulate astrocyte pathologies defined by animal models and immortalized human astrocytes.

3.2 | NMO-IgG induces alterations in cellular organelles and their interactions in human astrocytes

Astrocytes play a broad range of roles in brain homeostasis and function. They metabolically cooperate with neurons to maintain their energy demand, modulate synaptic activity via the Ca²⁺ signaling network, and eliminate unwanted synapses or proteins.^{9–11,25,29} Many of these functions are governed by cellular organelles, such as the mitochondria, ER, and lysosomes.

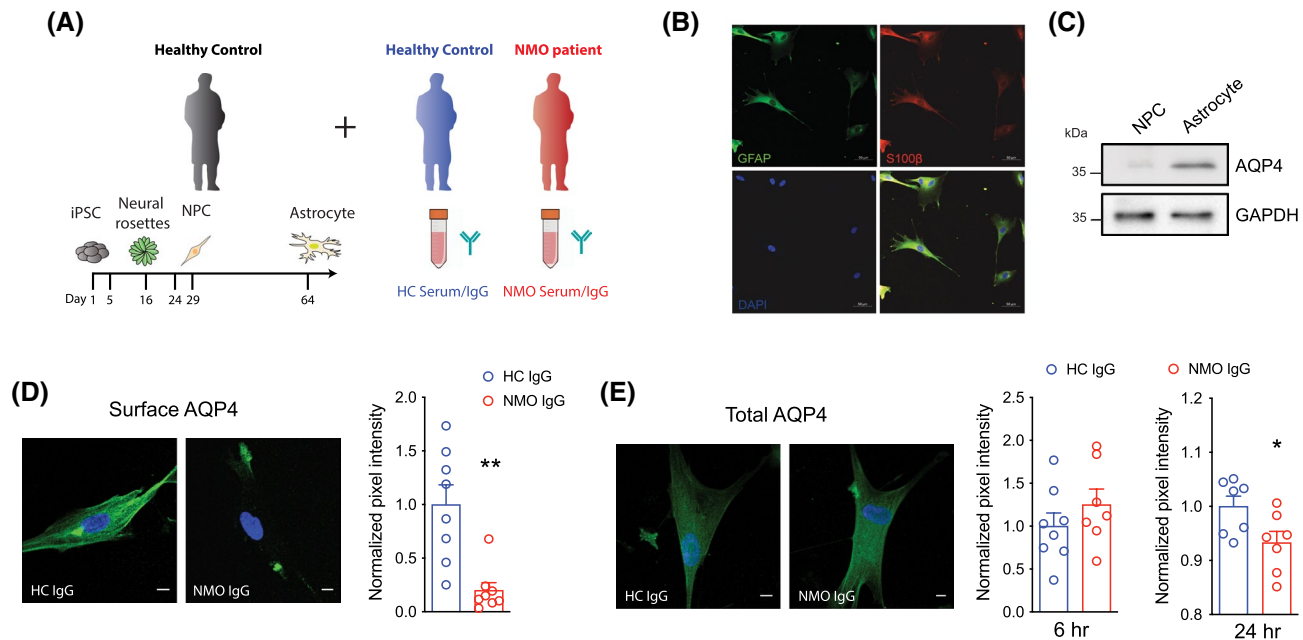


FIGURE 1 In vitro human model system to study astrocyte pathology in NMO. (A) Schematics of in vitro human model system to study NMO pathogenesis. (B) Validation of the identity of astrocytes with GFAP and S100β. Scale bar = 10 μm (C) Western blot image to show enriched expression of AQP4 in astrocytes. (D) Representative images of AQP4 level on the surface of astrocytes and their quantifications after treatment of HC-IgG (1 mg/ml) or NMO-IgG (1 mg/ml) for 6 h Scale bar = 10 μm. Error bars represent SEM. $n = 8$ images from 3 independent cultures. (E) Representative images of total AQP4 level in astrocytes and their quantifications after treatment of HC-IgG (1 mg/ml) or NMO-IgG (1 mg/ml) for 6 h Scale bar = 10 μm. $n = 7-8$ images from 3 independent cultures. Error bars represent SEM. $**p < .005$; Unpaired t -test

While NMO-IgG has been shown to induce AQP4 internalization and complement-dependent cytotoxicity in astrocytes, its effects on cellular organelles in astrocytes have not been precisely investigated. Thus, we utilized our models and used organelle trackers for lysosomes, mitochondria, and ER to address this point. Although we did not observe the difference in the size of cells between HC and NMO groups (Figure S1C), we calculated the percentage of area stained by each tracker in cells to take into account the size factor. We found that NMO-IgG significantly increased the areas of lysosomes and ER. There was a trend toward an increased mitochondrial area in NMO-IgG-treated astrocytes. Colocalized areas of lysosome-mitochondria, lysosome-ER, and mitochondria-ER were also shown to be increased in NMO-IgG-treated human astrocytes compared to those from control IgG-treated cells (Figure 2A,B). The effects of NMO-IgG on cellular organelles were verified in additional iPSC-derived astrocytes from an independent healthy individual (Figure S2C). We observed the same phenomenon by treating human iPSC-derived astrocytes with sera from NMO patients (Figure S1C), suggesting that IgGs from NMO patients are sufficient to recapitulate the effects from the patient's sera. To further validate whether the NMO patient serum-derived changes in astrocytic organelles are due to IgGs, we depleted the IgGs from serum

and applied them to human astrocytes, finding that IgG elimination abolished the effect of NMO patient serum on intracellular organelles (Figure S1D). This observation that astrocytes remodel cellular organelles in response to NMO-IgG implicates possible alterations to cellular signaling and function.

3.3 | Treatment with NMO-IgG alters mitochondrial integrity and metabolic profiles in human astrocytes

To complement the results of organelle tracker staining, we sought to analyze the structure and spatial relationships among organelles under electron microscopy, which provides powerful resolution with ultrastructural information. We took over 600-hundred microscopic images from each group to create large photomontages and reconstructed the whole astrocyte for unbiased quantitative analysis of mitochondrial morphology. We analyzed mitochondrial structure by evaluating shape descriptors and integrity. Increased mitochondrial perimeter and form factor along with decreased circularity were observed in NMO-IgG-treated astrocytes (Figure 3A,B). These results indicate that mitochondria became more elongated and increased branching in response to

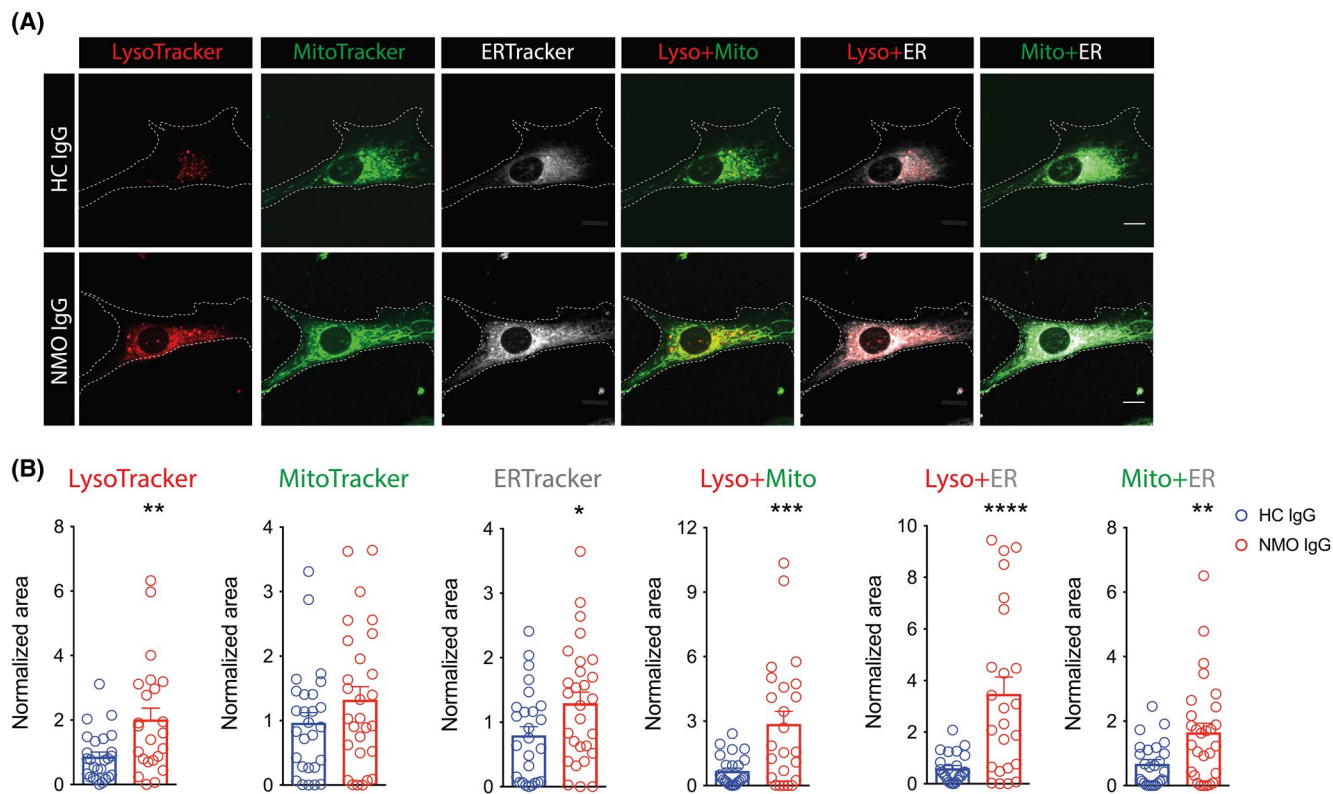


FIGURE 2 NMO-IgG induces alterations in cellular organelles and their interactions in human iPSC-derived astrocytes. (A) Representative image of astrocytes stained with organelle trackers after 6 h treatment of HC-IgG (1 mg/ml) or NMO-IgG (1 mg/ml). Scale bar = 10 μ m. (B) Quantifications of areas of each organelle trackers and colocalized areas of Lyso and MitoTracker, Lyso and ER-Tracker, and Mito and ER-Tracker in astrocytes with HC-IgG (1 mg/ml) or NMO-IgG (1 mg/ml) treatment. $n = 24$ images from 4 independent cultures. Error bars represent SEM. * $p < .05$, ** $p < .005$, *** $p < .001$, **** $p < .0001$; Unpaired t -test

NMO-IgG. Further, mitochondrial population with disturbed structure, including discontinuous or swollen outer membrane; swollen, irregular, or deficient cristae was increased in the NMO group (2.42% in healthy controls and 17.58% in NMO patients; Figure S3A). To examine the functional consequences of the disturbed ultrastructural integrity of the mitochondrial architecture, we evaluated the bioenergetic function of astrocytes after exposure to NMO-IgG. Cells were subjected to oxygen consumption rate measurements using Seahorse XF Analyzer (Figure 3C). While basal respiration and adenosine triphosphate (ATP) production remained unchanged, spare and maximal respiration were markedly reduced in the NMO-IgG-treated group (Figure 3D). To validate this observation, we utilized additional iPSCs from a healthy individual, differentiated them into astrocytes, and treated them with either HC-IgG or NMO-IgG. We observed more severe impairment of oxidative phosphorylation as such both basal and maximal respiration rates were completely abolished by NMO-IgG (Figure S2D) without affecting cell morphology and viability (Figure S2E). These data suggest that NMO-IgG perturbs metabolic profiles in human astrocytes.

3.4 | NMO-IgG treatment leads to alterations in organelle contacts

Mitochondria and ER are key organelles of cellular homeostasis, and physically and functionally form a network through dynamic contact sites known as mitochondria-associated membranes (MAMs). MAMs are important platforms for calcium transport, and they regulate mitochondrial energetics.^{30–32} The membranes of these two organelles are tethered by protein interactions. The voltage-dependent anion-selective channel protein1 (VDAC1) of the outer mitochondrial membrane and the inositol 1,4,5-triphosphate receptor (IP3R) on the ER membrane play crucial roles in Ca^{2+} transfer from the ER to the mitochondria.^{7,21} To determine whether the increased overlapping area between MitoTracker and ER-Tracker staining upon NMO-IgG treatment represents changes to their physical interactions, we determined MAM's interface in a quantitative manner by utilizing an in situ proximity ligation assay. By detecting VDAC1 and IP3R interactions, we demonstrated that astrocytes under NMO-IgG significantly upregulated mitochondria-ER coupling (Figure 4A). In addition, we observed

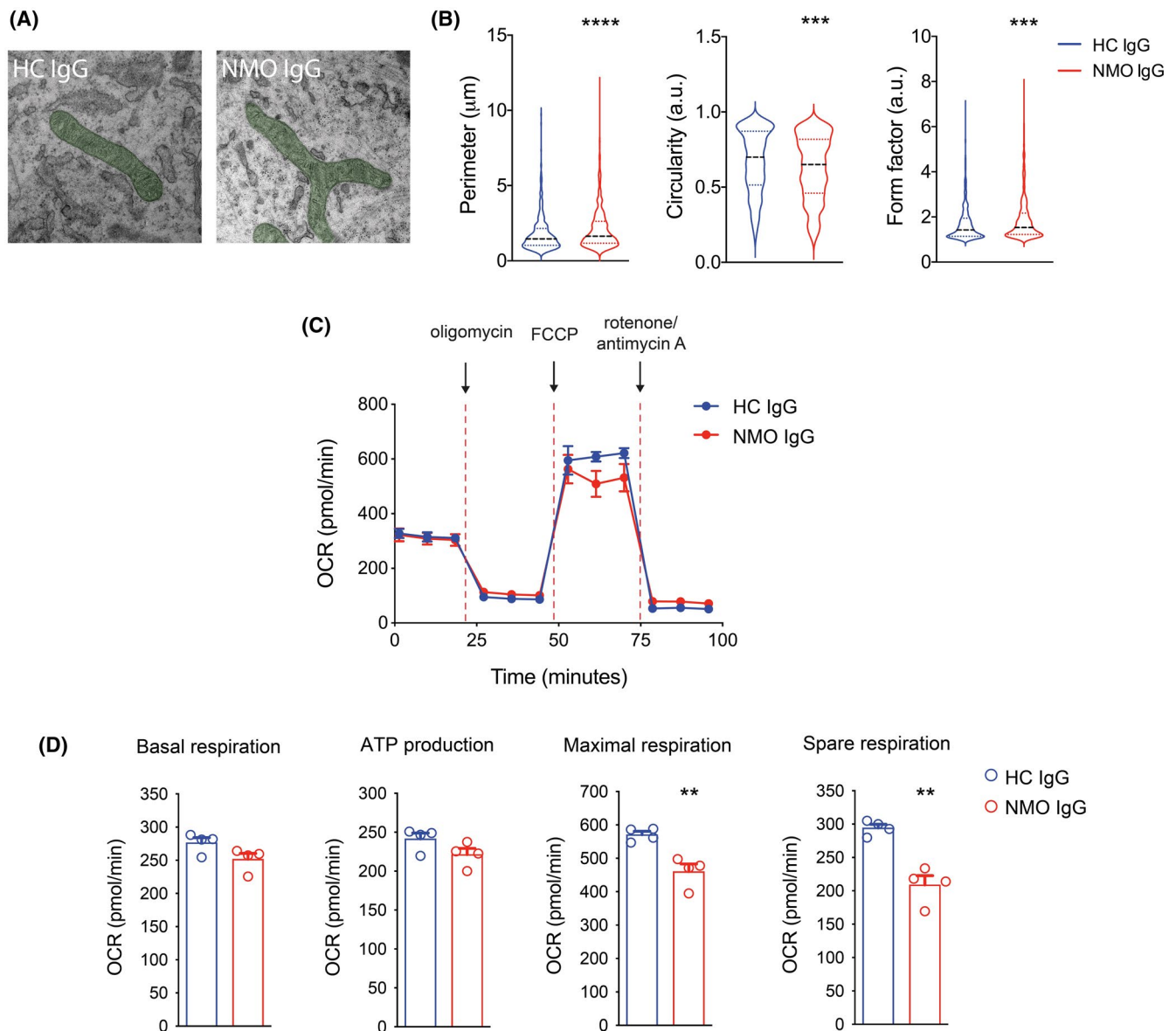


FIGURE 3 Treatment with NMO-IgG alters mitochondrial integrity and metabolic profiles in human astrocytes. (A) Ultrastructure of astrocytes. Mitochondria are pseudocolored in green. (B) Quantifications of mitochondrial perimeter, circularity, and form factor. 522 mitochondria in the HC-IgG-treated group and 675 mitochondria from the NMO-IgG-treated group were analyzed from two astrocytes each that were cultured independently. (C) Seahorse XF analysis trace measuring oxygen consumption rate in astrocytes. $n = 4$ independent cultures. (D) Quantifications of basal respiration, ATP production, maximal respiration, and spare respiration in astrocytes with HC-IgG (1 mg/ml) or NMO-IgG (1 mg/ml). Error bars represent SEM. ** $p < .005$, *** $p < .001$, **** $p < .0001$; Unpaired t -test

that astrocytes treated with NMO-IgG exhibited an increased mitochondrial population with ER associations under an electron microscope (Figure 4B,C). MAM properties were further examined at the ultrastructural level, finding that NMO-IgG increased the length of mitochondria-ER contacts (Figure 4D). Taken together, these findings provide evidence that NMO-IgG treatment led to increased MAM interfaces through VDAC1 and IP3R interactions.

Interestingly, we observed that both NMO-serum and IgG treatment in human astrocytes led to the accumulation

of a multilamellar structure (Figure S3B-E). To determine the origin of these structures, we performed correlative light and electron microscopy analysis. We first stained NMO-IgG-treated astrocytes with Lyso and ER-Tracker to fluorescently label organelles and observed them with confocal microscopy (Figure S3F). The cells were then subjected to electron microscopic analysis, and multilamellar structures were observed in Lyso and ER-Tracker positive puncta (Figure S3G,H). These results indicate that NMO-IgG induces ER membrane deformation into a multilamellar structure fused to lysosomes.

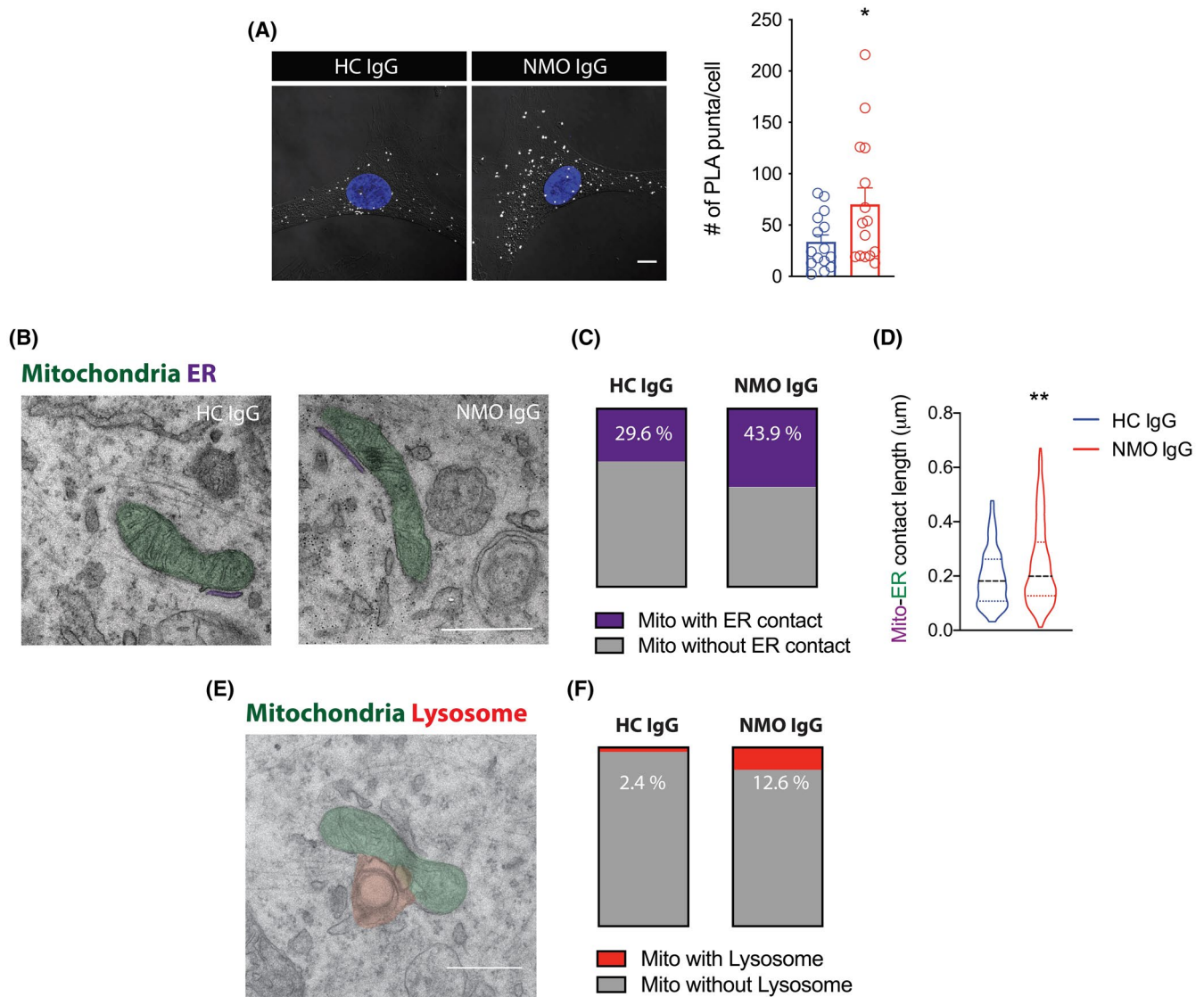


FIGURE 4 NMO-IgG treatment leads to alterations in organelle contacts. (A) Representative images and quantifications of in situ proximity ligation assay in astrocytes with HC-IgG (1 mg/ml) or NMO-IgG (1 mg/ml) treatment. Scale bar = 10 μ m. $n = 15$ images from 3 independent cultures. (B–F) Ultrastructure of mitochondria-associated with ER or lysosome. Mitochondria, ER and lysosome are pseudocolored in green, violet and red, respectively. Scale bar = 500 nm. (C and D) Quantification of mitochondria and ER contacts on electron micrographs of astrocytes, and Mito-ER contact length. 133 mitochondria-ER contacts from two HC-IgG-treated astrocytes and 197 mitochondria-ER contacts from two NMO-IgG-treated astrocytes were analyzed. (F) Quantifications of lysosome-associated mitochondria. Error bars represent SEM. * $p < .05$, ** $p < .005$; Unpaired t -test

3.5 | NMO-IgG impairs autophagic flux

Increased lysosomal associations with mitochondria and ER were observed in NMO-IgG-treated astrocytes both in staining of organelle trackers and ultrastructural studies (Figures 2A,B and 4E,F). To determine whether these changes implicate alterations in autophagy regulated by lysosomal degradation, we monitored autophagy fluxes by the tandem fluorescent protein mRFP-GFP-LC3. NMO-IgG significantly increased the percentage of autophagosomes detected as RFP-GFP colocalized puncta, whereas the percentage of RFP-only puncta, representing

autolysosomes, decreased in NMO-IgG-treated astrocytes, suggesting a decrease in autophagic flux by affecting lysosomal degradation (Figure 5A,B). Consistent with these findings, p62 accumulation, an autophagy substrate, was significantly induced in astrocytes by NMO-IgG and patient serum (Figures 5C and S4A,B). We further validated the accumulation of p62 and impaired autophagic flux by NMO-IgG in additional iPSC-derived astrocytes from an independent healthy individual (Figure S2F,G). Together, these results demonstrate that NMO-IgG application promotes deregulation of autophagy flux by perturbing lysosomal degradation.

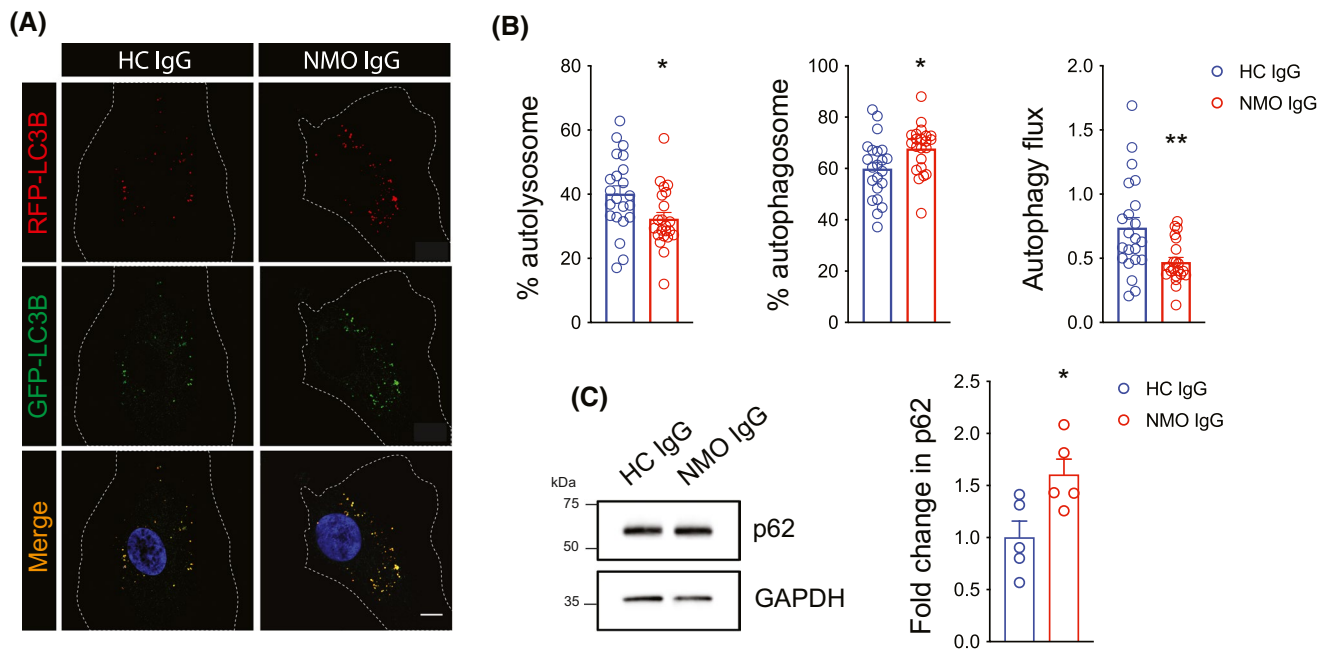


FIGURE 5 NMO-IgG impairs autophagic flux. (A) Representative images of autophagy flux assay with mRFP-GFP-MAP1LC3B transfected astrocytes. Scale bar = 10 μ m. (B) Quantifications of % autolysosome, % autophagosome, and autophagy flux in astrocytes with HC-IgG (1 mg/ml) or NMO-IgG (1 mg/ml) treatment. n = 22 images from 3 independent cultures. (C) Representative blot images and a quantification of p62 expression in astrocytes. n = 5 independent cultures. Error bars represent SEM. * p < .05, ** p < .005; Unpaired t -test

4 | DISCUSSION

NMO is distinct from multiple sclerosis due to the presence of circulating IgG autoantibodies against AQP4, which is predominantly expressed in astrocytes.^{7,22,23,33–35} Brain lesions in NMO patients are frequently observed in regions where AQP4 expression is relatively high, including the spinal cord and optic nerve.^{36,37} Consistently, NMO IgG-induced pathology was absent in AQP4 knockout mice.^{5,6,22,33,38,39} These studies suggest that AQP4 autoantibodies play an essential role in NMO pathogenesis.

Although AQP4 loss was observed in NMO patients, it is unclear whether NMO-IgG impairs AQP4 function as a water-permeable channel, or it is simply a consequence of astrocyte dysfunction and death. AQP4 internalization in NMO conditions, and whether it is the major mechanism underlying NMO pathology, are controversial. While complement-dependent cytotoxicity is generally observed and accepted in various models, whether NMO-IgG is sufficient or requires complement for pathogenesis is to be further elucidated.^{7,22,23,33–35}

Our in vitro human model system recapitulates NMO-related pathological features in human iPSC-derived astrocytes, including decreased surface AQP4 levels, increased IL-6 secretion, and complement-dependent cytotoxicity.^{5,6,22,33,38,39} This preclinical experimental model system provides a platform to mine preventative components to halt further CNS damage or therapeutics to restore

astrocyte abnormalities. Light and electron microscopic analysis revealed previously uncharacterized pathological changes in astrocytic organelles in response to NMO-IgG, including alterations in the extent of organelle interactions, ER membrane deformation, and disturbed mitochondrial structural integrity. Further, the metabolic profiles of mitochondria and autophagy flux were perturbed by NMO-IgG treatment. This work provides an understanding of astrocyte pathology at the cellular level and adds valuable new insights into the role of cellular organelles and their networks in NMO pathogenesis.

Although our study provides new insight into the disease pathogenesis by demonstrating NMO-IgG dependent changes in astrocyte organelles, additional questions emerge to be answered. Further studies are required to determine whether NMO-IgG-induced alterations in organelle architecture such as abnormal mitochondrial integrity are caused by loss of function for AQP4 or excessive NMO-IgG endocytosis. It is also important to address whether ER membrane deformation in NMO-IgG-treated astrocytes represents ER stress and affects protein synthesis, which might be responsible for the loss of AQP4 expression in NMO-affected brains. As women are most commonly affected, accounting for roughly 85% of cases,⁴⁰ we used iPSCs originating from females to generate astrocytes. In the future, it will be of interest to compare differences in the effect of NMO-IgG between astrocytes differentiated from male and female

iPSCs. Additional works also need to focus on unraveling the functional consequences of organellar changes in astrocytes in terms of brain homeostasis. To determine how changes in individual organelles and organelle networks shape astrocyte functions during NMO pathogenesis, further studies with functional assays including neurotransmitter uptake, calcium dynamics, and phagocytic activity will be crucial. It is also important to address whether NMO-IgG-experienced astrocytes would be more susceptible to subsequent injury including immune challenges. Moreover, the potential translation of findings to the interactions with other cell types in the CNS including neurons, microglia, endothelial cells, and oligodendrocytes is to be determined in future studies.

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DISCLOSURES

All authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

Sukhee Cho conceived and designed the research. Sukhee Cho and Hyein Lee performed biochemical experiments and analyzed data. Sukhee Cho and Minkyoo Jung performed transmission electron microscopy experiments. Kirim Hong assisted with TEM analysis. Seung-Hwa Woo and Young-Sam Lee performed XF analysis and analyzed data. Byoung Joon Kim and Mi Young Jeon diagnosed NMO patients and collected sera. Jinsoo Seo supervised iPSC maintenance and astrocyte differentiation to establish an in vitro model system. Jinsoo Seo and Ji Young Mun provided reagents and funding and coordinated the project. Sukhee Cho and Jinsoo Seo wrote the manuscript.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the Supporting Information section.

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