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Supplementary Material

Aquaporin-9 Aggravates Lipopolysaccharides Induced Acute Lung Injury Via Facilitating M1-Like Macrophage Polarization

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MATERIALS AND METHODS

ALI mouse model

Male wild-type (WT) and *AQP9* KO mice aged 6 to 8 weeks were utilized (three to five mice per group), sourced from mating pairs of *AQP9* heterozygous parents, and housed in a specific pathogen-free (SPF) facility at Nanjing University of Chinese Medicine. The mice were maintained on standard chow and provided ad libitum access to water, unless otherwise specified, in an environment with a 12-hour light/dark cycle.

Cell culture

AMs were obtained from mouse alveolar lavage fluid, washed with PBS, and resuspended in RPMI 1640 medium (Solarbio, China) containing 10% FBS (Gibco, USA), supplemented with 1% penicillin/streptomycin (Gibco, USA). After 4–6 h of incubation at 37°C with 5% CO_2, nonadherent cells were removed, and the remaining adherent cells were cultured. Neutrophils were isolated from the peripheral blood of C57BL/6J mice (male) using the Mouse Peripheral Blood Neutrophil Cells Isolation Kit. Briefly, the blood samples were collected and centrifugated using neutrophils separation solution (800 g, 25 min) to get different layers of cells. Then the neutrophils were carefully removed from the tube and centrifuged at 250 g for 10 min. The sedimented cell pellets were washed with PBS and resuspended in RPMI 1640 medium containing 10% FBS and 1% penicillin/streptomycin.

Histological analysis

For histological examination, 3 μm thick slices were stained with hematoxylin and eosin (H&E). The histological features were visualized and documented using a light microscope (Leica, DM2500, Germany). For immunohistochemical (IHC) staining, sections were heated in 0.1 M citric acid buffer at 98°C for 10 min, followed by blocking with 3% BSA. Sections were then incubated with antibody against CD68 (Arigo, ARG10514, China). For immunofluorescence analysis, primary antibodies against iNOS (Arigo, ARG56509, China) and CD206 (Arigo, ARG22456, China) were applied, followed by incubation with fluorochrome-conjugated secondary antibodies. Nuclei were counterstained with DAPI Fluor mount-G (SouthernBiotech, USA). Images were captured using a Leica DM2500 fluorescence microscope and analyzed using ImagePro Plus software. Each experiment included at least three samples from each group.

RT-qPCR analysis

Primer sequences are provided in Supplementary Table SI.

Western blot analysis

The following antibodies were used for western blot: AQP9 (Santa Cruz, sc-74409, 1:600), NOX2 (Santa Cruz, sc-130543, 1:500), phospho-IKKα/β (Abmart, TP56290, 1:2000), IKKα/β (Abmart, T55660, 1:2000), IκBα (Abmart, T55026, 1:2000), phospho-p65 (Abmart,

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TP56372, 1:2000), p65 (Abmart, T55034, 1:2000), GAPDH (Proteintech, 10494-1-AP, 1:5000), Histone H3 (Proteintech, 17168-1-AP, 1:5000).

Supplementary Table SI. Primer sequences for RT-qPCR.

Gene	Primer Sequence (5′ → 3′)
Aqp9	F: GGGAGCCTTTGTCGGGGCTG
	R: ATGGGCTCCAGGCCTCTGGG
Il-1β	F: TGCCACCTTTTGACAGTGATG
	R: TGATGTGCTGCTGCGAGATT
Tnf-α	F: AAGCCTGTAGCCCACGTCGTA
	R: GGCACCACTAGTTGGTTGTCTTTG
Il-6	F: TAGTCCTTCCTACCCCAATTTCC
	R: TTGGTCCTTAGCCACTCCTTC
Il-10	F: CTTACTGACTGGCATGAGGATCA
	R: GCAGCTCTAGGAGCATGTGG
inos	F: CACCAAGCTGAACTTGAGCG
	R: CGTGGCTTTGGGCTCCTC
Cd206	F: CTCTGTTCAGCTATTGGACGC
	R: CGGAATTTCTGGGATTCAGCTTC
β-actin	F: CTGTGCCCATCTACGAGGGCTAT
	R: TTTGATGTCACGCACGATTTCC