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Neudesin, A Secretory Protein, Suppresses Cytokine Production in Bone Marrow-Derived Dendritic Cells Stimulated by Lipopolysaccharide

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Neudesin was identified as a secretory factor expressed in the nervous system. On the other hand, *neudesin* is expressed in various organs and cells, suggesting that it plays roles in tissues other than neural tissues. We found that *neudesin* was expressed in dendritic cells (DCs) in the mouse spleen, which play a crucial role in the initiation of adaptive immune responses. Therefore, considering the possibility that neudesin may affect the acquired immune response, we first investigated whether neudesin has an effect on DCs using bone marrow-derived dendritic cells (BMDCs). *Neudesin* expression levels increased during the differentiation of bone marrow cells to BMDCs, and its expression level in BMDCs was reduced by lipopolysaccharide (LPS) treatment. BMDCs from *neudesin* knockout mice showed increased production of various cytokines, such as IL-12p70 and TNF-α, under LPS-stimulated conditions, compared with BMDCs from wild-type mice. In addition, treatment with recombinant neudesin suppressed the expression of cytokine genes in LPS-stimulated BMDCs from *neudesin* knockout mice. T cell proliferation was more strongly induced by co-culture with BMDCs from *neudesin* knockout mice than by those from wild-type mice. BMDCs from *neudesin* knockout mice showed increased lactate production, glucose consumption, and expression levels of glycolysis-related factors, suggesting that neudesin inhibits glycolysis, which promotes DC activation. The increased cytokine production in BMDCs from *neudesin* knockout mice was suppressed by the glycolytic inhibitor, 2-deoxyglucose. These results suggested that neudesin is a novel suppressor of DC function through the inhibition of glycolysis.

Key words dendritic cell, neudesin, inhibitory factor, glycolysis, cytokine

INTRODUCTION

Dendritic cells (DCs) are distributed in various tissues throughout the body, including lymphoid tissues, skin, mucosal surfaces, and blood, and their primary role is to induce acquired immunity.[1,2] DCs in peripheral tissues, such as the skin and mucosal surfaces, are activated by foreign substances and migrate to the lymph nodes, where they present antigens to naïve T cells.[3] Resident DCs in the spleen are activated by foreign substances entering the blood and present antigens to naïve T cells in the spleen.[4] To eliminate foreign substances, DCs at both locations play key roles in initiating acquired immunity via antigen presentation to T cells.

Toll-like receptors (TLRs) recognize specific components of microorganisms and viruses that are involved in the induction of acquired immunity by DCs.[5,6] They then initiate signaling cascades that activate various downstream pathways. Subcellular localization and ligand specificity differ among TLRs, enabling them to recognize various substances and induce appropriate acquired immune responses against pathogens.[7] For example, lipopolysaccharide (LPS), an outer membrane component of gram-negative bacteria, strongly stimulates TLR4 on DC membranes and induces proinflammatory cytokines, such as tumor necrosis factor-α (TNF-α) and interleukin-12 (IL-12), the co-stimulatory molecule CD86, and major histocompatibility complex (MHC) class II molecules, which are critical for immune responses.[8]

We have been investigating the function of a protein named neudesin, which is a secretory factor with a heme/steroid-binding domain. We have previously shown that neudesin is strongly expressed in the brain and spinal cord of fetal and adult mice and that it has neurotrophic effects and promotes the differentiation of neural progenitor cells into neurons.[9,10] Neudesin also suppresses sympathetic nerve activity, and consequently, energy metabolism.[11] Additionally, we previously reported that neudesin directly acts on adipocytes and inhibits their differentiation[12] and that it is involved in testis development.[13] Thus, neudesin is a secretory factor implicated in various biological functions, suggesting that it may have other physiological roles that are yet to be elucidated.

We found that DCs are one type of *neudesin* expressing cells in the spleen, which led us to investigate whether endogenous neudesin acts on DCs. Bone marrow-derived DCs (BMDCs) from *neudesin* knockout (KO) mice showed enhanced LPS-induced cytokine production compared to those from wild-type mice. BMDCs from *neudesin* KO mice also promoted T cell proliferation in a co-culture system of DCs and T cells. The glycolytic pathway has been postulated to be

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involved in the enhanced function of *neudesin* KO BMDCs. These results suggested that neudesin is a novel suppressor of DC function through the inhibition of glycolysis.

MATERIALS AND METHODS

Mice Wild-type mice and *neudesin* KO mice were generated as previously described.[11] Six-week-old BALB/c mice were purchased from CLEA Japan (Tokyo, Japan). All animal studies were conducted in accordance with the International Guiding Principles for Biomedical Research Involving Animals and were approved by the Animal Research Committee of Kobe Pharmaceutical University.

Quantitative RT-PCR Total RNA was extracted from tissues or cells using Sepasol-RNA I Super G (Nacalai Tesque, Kyoto, Japan), according to the manufacturer's instructions. cDNA was synthesized using the ReverTraAce qPCR RT kit (Toyobo, Osaka, Japan). Quantitative PCR was performed using Thunderbird SYBR qPCR mix (Toyobo). 18S ribosomal RNA was used as an internal control. The primers used in this study were as follows: *18S ribosomal RNA* (*18S*): F, 5′-CCG GAA TCG AAC CCT GAT T-3′ and R, 5-CGA ACG TCT GCC CTA TCA ACT T-3′; *neudesin*: F, 5′-ACT TGG CAG TGA AGG GAG TG-3′ and R, 5′-CCG TGA GAC CAG TAG TGT CG-3′; interleukin-12 subunit α (*IL-12p35)*: F, 5′-CTA GAC AAG GGC ATG CTG GT-3′ and R, 5′-GCT TCT CCC ACA GGA GGT TT-3′; interleukin-12 subunit β (*IL-12p40*): F, 5′-TGG TTT GCC ATC GTT TTG CTG-3′ and R, 5′-ACA GGT GAG GTT CAC TGT TTC T-3′*;* tumor necrosis factor-α (*TNF- α*): F, 5′- CCC TCA CAC TCA GAT CAT CTT CT-3′ and R, 5′-GCT ACG ACG TGG GCT ACA G-3′; interferon-β (*IFN-β*): F, 5′-CAG CTC CAA GAA AGG ACG AAC-3′ and R, 5′-GGC AGT GTA ACT CTT CTG CAT-3′; glucose transporter 1 (*Glut1*): F, 5′-GGA ATC GTC GTT GGC ATC CT-3′ and R, 5′-CGA AGC TTC TTC AGC ACA CTC-3′; lactate dehydrogenase A (*Ldha)*: F, 5′- TGT CTC CAG CAA AGA CTA CTG T -3′ and R, 5′-GAC TGT ACT TGA CAA TGT TGG GA-3′.

Cell Culture of BMDCs Bone marrow cells were collected from the femurs and tibiae of Six-week-old female wild-type mice and *neudesin* KO mice, and cultured in RPMI 1640 medium (Nissui Seiyaku, Tokyo, Japan) supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich, St. Louis, MO, USA), 10 ng/mL IL-4 (PeproTech, Cranbury, NJ, USA) and 20 ng/mL GM-CSF (PeproTech, Cranbury, NJ, USA). The cells were cultured in fresh medium and harvested on day 7 as BMDCs. BMDCs were treated with 10 ng/mL LPS (*Escherichia coli* serotype O111:B4; Sigma-Aldrich). In some experiments, the cells were cotreated with 200 ng/mL recombinant neudesin protein[9] or 2.5 mmol/L 2-deoxyglucose (Nacalai Tesque, Kyoto, Japan). Twenty-four hours after stimulation, culture supernatants were collected and stored at -80°C for further analysis. LPS-treated cells were lysed using Sepasol-RNA I Super G (Nacalai Tesque) for subsequent RNA extraction, harvested in RIPA buffer[14] for western blotting, or collected for flow cytometry.

Flow Cytometry BMDCs were incubated with fluorochrome-conjugated antibodies at 4°C for 20 min. Cells were fixed in 1% paraformaldehyde at 4°C and analyzed for surface marker expression by using a FACSAriaIII and FlowJo software (BD Biosciences, Franklin Lakes, NJ, USA). The following antibodies were used: anti-CD11c (N418; BioLegend, San Diego, CA, USA), anti-MHC II (M5/114.15.2; BioLegend), and anti-CD86 (GL1; BD Pharmingen, San Diego, CA, USA).

Cytokine Measurements Cytokine concentrations in the cell supernatants were determined using ELISA kits for IL-12p70 (Thermo Fisher Scientific, Waltham, MA, USA) and TNF-α (PeproTech) according to the manufacturer's instructions.

Measurement of Lactate and Glucose Lactate and glucose concentrations in the culture medium were measured using the Amplite Colorimetric L-Lactate Assay Kit (AAT Bioquest, Sunnyvale, CA, USA) and the Glucose Assay Kit (Cell Biolabs Inc., San Diego, CA, USA). Glucose consumption was calculated as the difference in glucose concentration before and after LPS stimulation.

Isolation of Splenic Immune Cells Splenocytes were prepared by applying pressure to the spleen using the head of a syringe and were collected through a 70-μm cell strainer. B cells ($B220^+$), helper T cells ($CD4^+$), cytotoxic T cells ($CD8^+$), dendritic cells ($CD11c^+$), macrophages ($F4/80^+$), granulocytes ($Gr\text{-}1^+$), and natural killer (NK) cells ($CD49b^+CD3^-$) were isolated from splenocytes by cell sorting using a FACSAria III. The antibodies used were anti-B220 (RA-6B2; BD Pharmingen), anti-CD4 (RM4-5; BD Pharmingen), anti-CD8 (53-6.7; BD Pharmingen), anti-F4/80 (BM8; BioLegend), anti-Gr-1 (1A8; BD Pharmingen), anti-CD49b (DX5; BioLegend), and anti-CD3 (145-2C11; BD Pharmingen).

Mixed Lymphocyte Reaction BMDCs generated from wild-type or *neudesin* KO C57BL/6 mice (H-2b) on C57BL/6 background were prepared as described above and stimulated with 10 ng/mL LPS for 24 h. They were then treated with 50 mg/mL mitomycin C for 1 h and collected. $CD4^+$ T cells were isolated from the spleens of BALB/c mice (H-2d) using $CD4^+$ T Cell Isolation Kit (Miltenyi Biotech, Bergisch Gladbach, Germany) according to the manufacturer's protocol. $CD4^+$ T cells (1×10^5 cells/well) were co-cultured with BMDCs (4×10^3 cells/well) in RPMI 1640 medium supplemented with 10% FBS for 3 days. The cells were collected and counted using CellDrop BF (DeNovix Wilmington, DE, USA).

Western Blotting Protein expression levels were quantified as described previously.[14] The primary antibodies used were rabbit antibodies against IκBα (1:1,000; Cell Signaling Technology, Danvers, MA, USA) and β-actin (1:1,000; Proteintech, Rosemont, IL, USA). The secondary antibody was a horseradish-peroxidase-conjugated goat anti-rabbit antibody (1:1,000; Cell Signaling Technology).

Statistical Analysis Data were analyzed using Prism software (GraphPad Software, San Diego, CA, USA). All presented data are expressed as the mean ± standard deviation. An unpaired Student's t-test was used to test for differences between two groups and a two-way analysis of variance followed by Tukey's post-hoc test was used to test for differences between more than two groups.

RESULTS

Tissue Distribution of *Neudesin* mRNA in Mice We collected various organs from female 6-week-old C57BL/6 mice and examined the distribution of *neudesin* mRNA using RT-PCR. The results revealed that the brain and heart had the highest expression levels among the organs tested (Fig. 1A). *Neudesin* was also found to be expressed at a certain level in

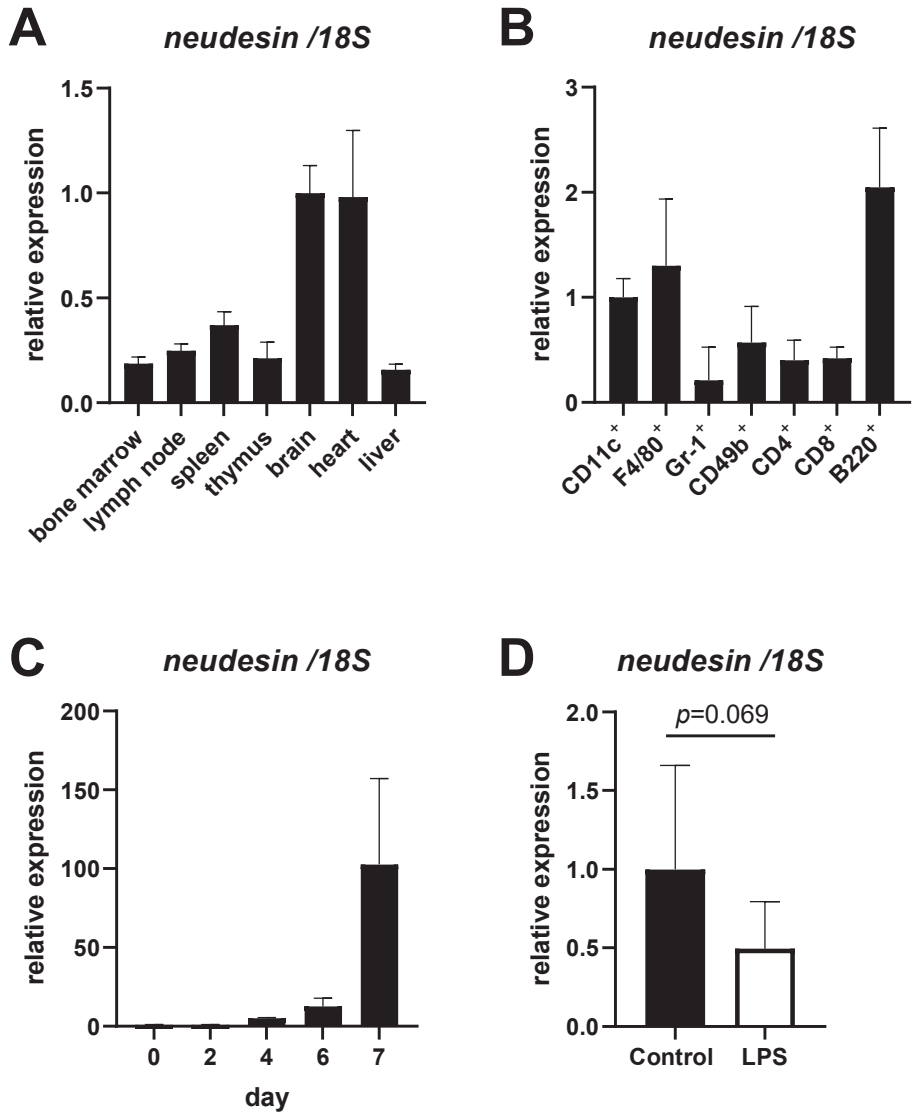

Fig. 1. *Neudesin* Is Expressed in Dendritic Cells

(A) Relative expression levels of *neudesin* in various tissues of 6-week-old mice were determined by RT-PCR (n = 4). The results are presented as the fold difference versus mRNA expression levels in the brain. (B) Relative expression levels of *neudesin* in CD11c$^+$ DCs, F4/80$^+$ macrophages, Gr-1$^+$ granulocytes, CD49b$^+$CD3$^-$ NK cells, CD4$^+$ T cells, CD8$^+$ T cells, and B220$^+$ B cells in the spleen are shown (n = 4). The results are presented as the fold difference versus mRNA expression levels in CD11c$^+$ DCs. (C) Mouse bone marrow cells were collected and treated with GM-CSF (20 ng/mL) and IL-4 (10 ng/mL) for 7 days to induce differentiation into BMDCs. During differentiation from bone marrow cells to BMDCs, the expression of *neudesin* was evaluated by RT-PCR (n = 4). The results are presented as the fold induction versus the expression level at day 0. (D) BMDCs were incubated with or without LPS (10 ng/mL) for 24 h. After incubation, *neudesin* expression in the LPS-treated (LPS) and untreated groups (control) was evaluated by RT-PCR (n = 8).

the spleen and lymph nodes, which are secondary immune organs.

Expression of *Neudesin* in Dendritic Cells Next, *neudesin*-producing cells were identified in secondary lymphoid organs, such as the spleen. Spleen cell subsets, such as B cells, CD4$^+$ and CD8$^+$ T cells, DCs, macrophages, granulocytes, and NK cells were sorted by flow cytometry. We found high expression levels of *neudesin* in three types of antigen-presenting cells (B220$^+$ B cells, CD11c$^+$ DCs, and F4/80$^+$ macrophages) in the spleen (Fig. 1B).

Next, we investigated the expression level of *neudesin* in DCs, which play key roles in initiating acquired immunity via antigen presentation to T cells. Since many immune cells, including DCs, differentiate from bone marrow cells, we confirmed the expression of *neudesin* during the differentiation process from bone marrow cells to DCs using BMDCs induced with GM-CSF and IL-4, which were used as culture models of DCs. When bone marrow cells obtained from mice were induced to differentiate into BMDCs, *neudesin* expression levels increased (Fig. 1C). Furthermore, when BMDCs were activated by LPS, *neudesin* expression levels tended to decrease (Fig. 1D). These results indicated that *neudesin* is highly expressed in mature DCs and that its production decreases under activated conditions.

BMDCs from *Neudesin* KO Mice As DCs are important in adaptive immune responses, we investigated the possibility that DC-derived neudesin affects the function of DCs. Therefore, we generated BMDCs from *neudesin* KO mice and analyzed them. The differentiation of bone marrow cells to CD11b$^+$ CD11c$^+$ BMDCs was comparable between *neudesin* KO mice and wild-type (WT) mice (data not shown). Under LPS-stimulated or unstimulated conditions, the levels of CD86 were almost unchanged between BMDCs derived from *neudesin* KO mice and those derived from WT mice (Fig. 2A).

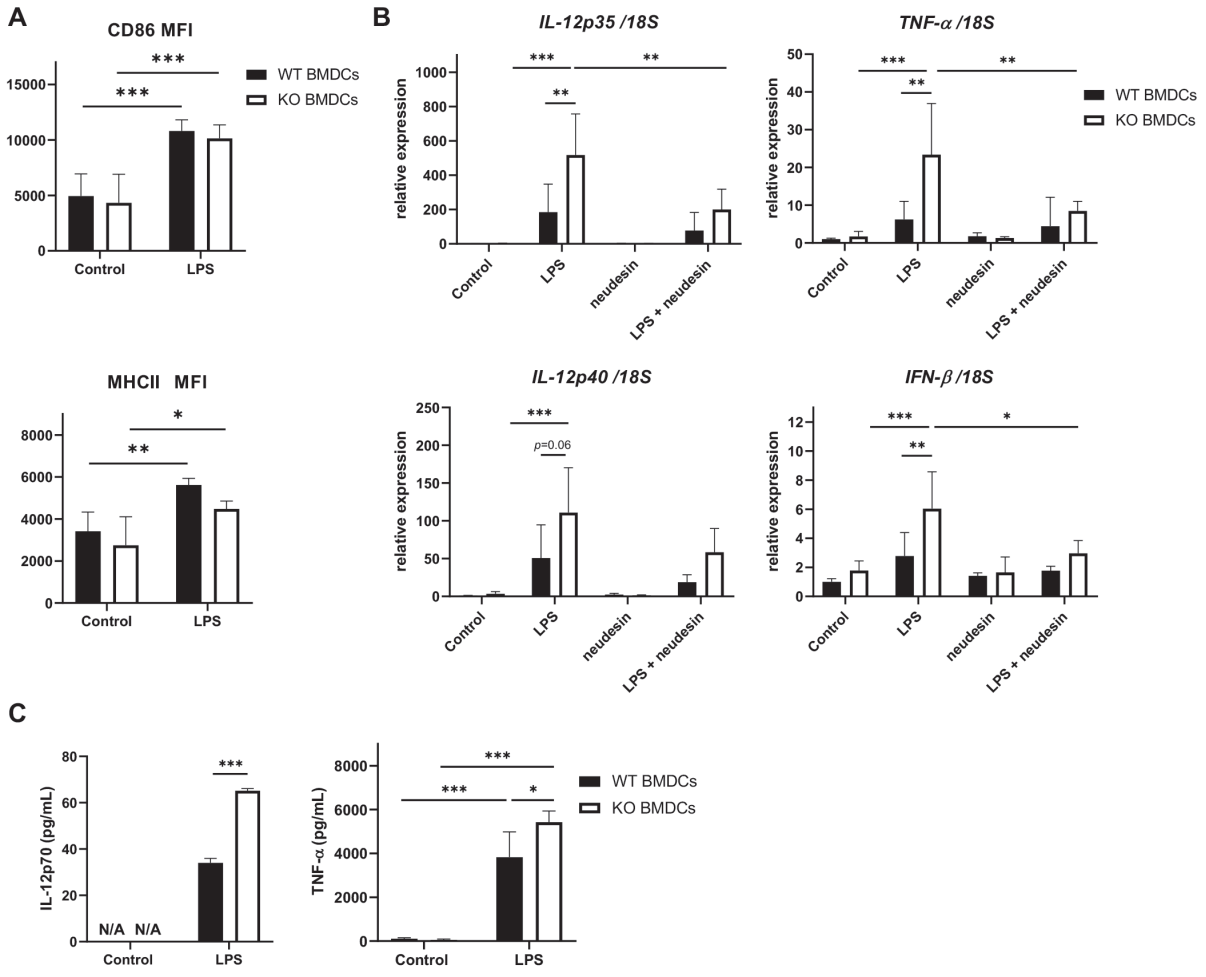

Fig. 2. Increased Cytokine Expression Levels in *Neudesin* KO BMDCs Treated with LPS

(A) The mean fluorescence intensity (MFI) of CD86 and MHC class II molecules on BMDCs from WT and *neudesin* KO mice treated with or without LPS (10 ng/mL) for 24 h was measured by flow cytometry (n = 6). (B) Expression of cytokines in BMDCs from WT and *neudesin* KO mice was evaluated by real-time RT-PCR (n = 3–5). Both BMDCs were treated with LPS (10 ng/mL) and/or recombinant neudesin protein (200 ng/mL) for 24 h. (C) TNF-α and IL-12p70 levels in the supernatant of BMDCs from WT and *neudesin* KO mice were measured by ELISA (n = 4). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. N/A, not applicable.

The levels of MHC class II were slightly decreased in *neudesin* KO BMDCs under LPS-stimulated condition, but this difference was not statistically significant ($p = 0.13$).

Activated DCs secrete various cytokines. Therefore, we investigated the mRNA expression of interleukin-12 subunit α (*IL-12p35*), interleukin-12 subunit β (*IL-12p40*), *TNF-α,* and *IFN-β*, the major cytokines derived from DCs. We found that the levels of all cytokines were significantly increased in BMDCs from *neudesin* KO mice compared to BMDCs from WT mice after LPS stimulation (Fig. 2B). In addition, cotreatment with recombinant neudesin and LPS suppressed the expression of cytokine genes in *neudesin* KO BMDCs (Fig. 2B). We also found that the protein levels of IL-12p70 and TNF-α in the culture supernatants were increased more in LPS-stimulated BMDCs from *neudesin* KO mice, than in those from WT mice (Fig. 2C).

CD4⁺ T Cell Response to BMDCs from *Neudesin* KO Mice BMDCs from *neudesin* KO mice showed increased expression levels of cytokine genes after LPS stimulation, suggesting that their ability to activate T cells may also be enhanced. Therefore, we investigated the effects of BMDCs from *neudesin* KO mice on allogeneic CD4⁺ T cells in a co-culture system. The number of cells increased more in co-cultures with BMDCs from *neudesin* KO mice on a C57BL/6 background than in co-cultures with BMDCs from C57BL/6 WT mice (Fig. 3). Considering that the proliferation of BMDCs is inhibited by pretreatment with mitomycin C, this difference of cell number is due to proliferation of CD4⁺ T

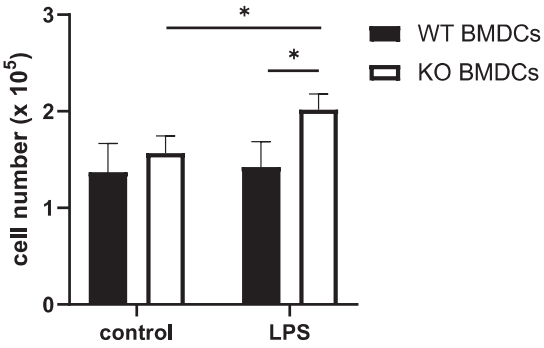

Fig. 3. Mixed Lymphocyte Reaction with BMDCs from *Neudesin* KO Mice

Mitomycin-treated BMDCs were cultured with CD4⁺ T cells at a ratio of 1:25. The total number of cells was determined after 3 days of mixed lymphocyte reaction (MLR; n = 6). *$p < 0.05$.

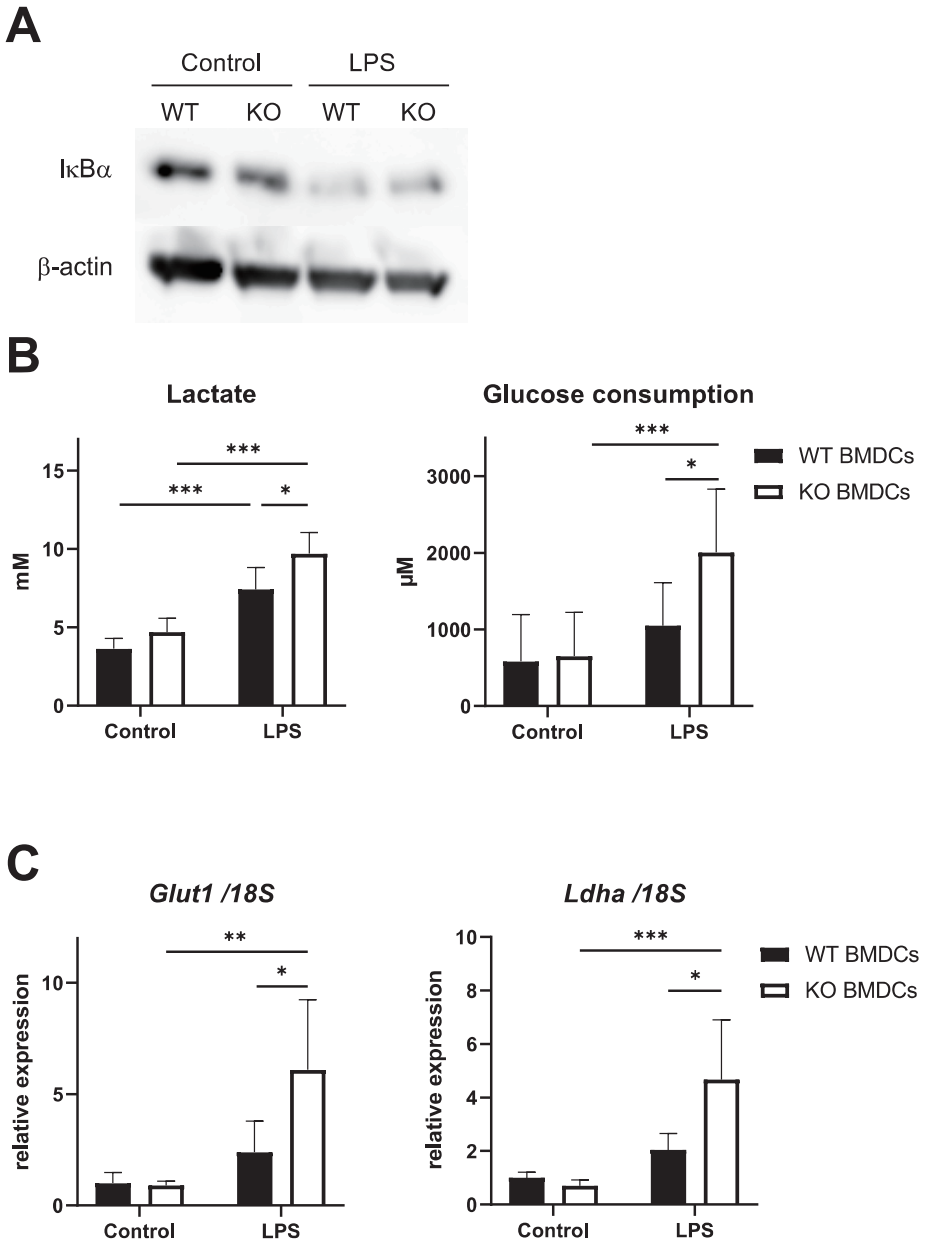

Fig. 4. Glycolysis Is Increased in BMDCs from *Neudesin* KO Mice

(A) Degradation of IκBα protein in BMDCs from WT mice or *neudesin* KO mice treated with LPS (10 ng/mL) for 60 min was measured by western blotting. The β-actin expression level was used as an internal control. (B) BMDCs from WT or *neudesin* KO mice were incubated with or without LPS (10 ng/mL). After incubation, the release of lactate into the culture supernatant (n = 5) and glucose consumption (n = 10) by BMDCs from WT mice or *neudesin* KO mice were assessed. (C) Expression levels of *Glut1* and *Ldha* in BMDCs from WT and *neudesin* KO mice were evaluated by RT-PCR (n = 5). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

cells. Therefore, this result suggests that neudesin produced by DCs suppresses their ability to activate allogeneic $CD4^+$ T cells.

Mechanisms Underlying the Effects of Neudesin on DCs TLR4 signaling activates nuclear transcription factor kappa B (NF-κB), which induces the expression of various cytokines. The activation of NF-κB requires phosphorylation and subsequent degradation of inhibitor of NF-κB α (IκBα). The protein expression level of IκBα in BMDCs from *neudesin* KO mice after LPS stimulation was not different from its expression level in BMDCs from WT mice (Fig. 4A), suggesting that neudesin is unlikely to affect NF-κB signaling, which is important for the activation of DCs. It has also been reported that glycolysis is initiated by a pathway downstream of TLRs in DCs and promotes DC activation, including cytokine production.[15] Therefore, we next focused on glycolysis. When activated by LPS, the lactate concentration and glucose consumption in the culture supernatant were higher in BMDCs from *neudesin* KO mice than in those from WT mice (Fig. 4B). The expression levels of *Glut1* and *Ldha*, which are involved in glucose metabolism, were increased in BMDCs from *neudesin* KO mice (Fig. 4C). These results suggested that the glycolytic system was activated in BMDCs from *neudesin* KO mice.

To investigate the contribution of glycolysis to the effects of neudesin, BMDCs were treated with 2-deoxyglucose (2-DG), a glycolytic inhibitor. The elevation in cytokine gene

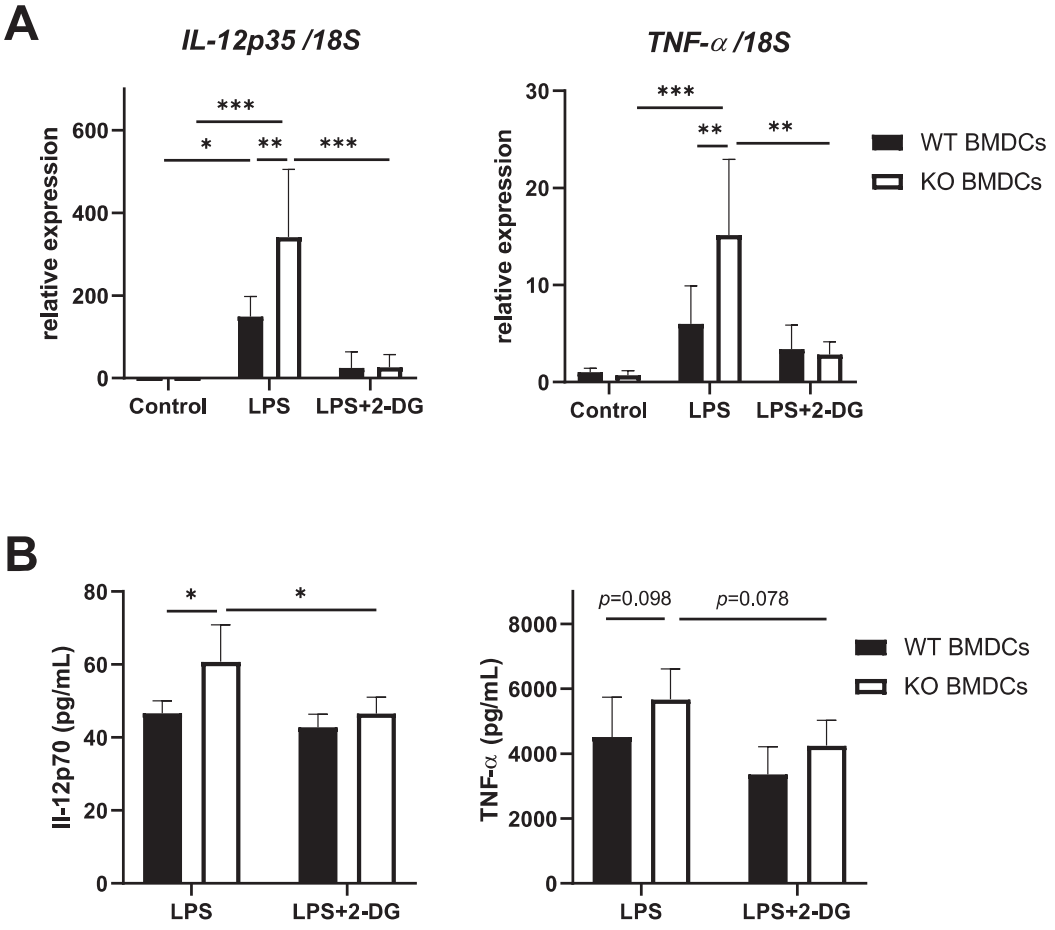

Fig. 5. Increased Glycolysis Is Required for Increased Cytokine Expression in BMDCs from *Neudesin* KO Mice

(A) BMDCs from WT or *neudesin* KO mice were treated with LPS (10 ng/mL) and/or 2-deoxyglucose (2.5 mM) for 24 h. After incubation, *IL-12p35* and *TNF-α* levels were evaluated by RT-PCR (n = 5). (B) TNF-α and IL-12p70 levels in the supernatant were measured by ELISA (n = 4–9). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

expression levels and the increase in IL-12p70 protein levels in BMDCs from *neudesin* KO mice after LPS treatment were abolished by 2-DG treatment (Fig. 5A, B). In addition, the concentration of TNF-α in the supernatant tended to increase in BMDCs from *neudesin* KO mice and this effect disappeared after 2-DG treatment. (Fig. 5B). Therefore, neudesin may suppress the cytokine production in DCs through inhibition of glycolysis.

DISCUSSION

Whereas neudesin has been shown to contribute to the survival and development of neurons and to the metabolism of adipose tissues as a secretory factor,[10,11] there have been few reports on the role of neudesin in the immune system. In this study, we found that neudesin was expressed in DCs and suppressed cytokine production in BMDCs. Indeed, the number of allogeneic T cells increased when they were co-cultured with *neudesin* KO BMDCs compared with wild-type BMDCs, suggesting that neudesin may affect the activation of T cells through cytokine production by DCs. Considering that the expression level of *neudesin* in BMDCs was reduced after LPS treatment, it is expected that neudesin prevents cytokine production in steady-state DCs and suppresses the excessive activation of T cells.

DCs can initiate adaptive immune responses by presenting antigens to naïve T cells and producing cytokines. The function of DCs is suppressed by various factors. For example, CTLA-4, which is expressed on the surface of regulatory T cells, binds to CD80 and CD86 on DCs more strongly than to CD28 on T cells, thereby inhibiting the ability of DCs to activate T cells.[16,17] Additionally, IL-10 and TGF-β produced by regulatory T cells bind to receptors on the surface of DC membranes, thereby reducing DC function.[16] In the tumor microenvironment, Wnt5a and prostaglandin E_2 derived from cancer cells have been reported to suppress DC function.[18,19] Among the suppressors of DC function mentioned above, IL-10 and TGF-β are known to be produced by DCs and they play crucial roles in the immune response, in part by modulating DC function.[20,21] In this study, we characterized neudesin produced by DCs as a suppresser of DC function, suggesting that neudesin plays an important role in the process of the acquired immune system, as do IL-10 and TGF-β.

Glycolysis is responsible for supporting metabolic and functional changes in DCs activated by LPS or other stimuli.[15] In DCs and macrophages, cytokine production is controlled at the transcriptional[22,23] and translational levels[24] via LPS-induced glycolysis. Our experiments showed that lactate pro-

duction and glucose consumption were higher in *neudesin* KO BMDCs than in WT BMDCs, and pharmacological inhibition of glycolysis suppressed cytokine expression in *neudesin* KO BMDCs, suggesting that neudesin exerts its effects by inhibiting glycolysis. The expression levels of proteins involved in glucose uptake and glycolysis, such as GLUT1 and LDH, increase after LPS stimulation, through the transcription factor HIF-1α.[25,26] In DCs, LPS enhances the transcriptional activity of HIF-1 α protein, mainly through its stabilization by post-translational modification.[27] In the present study, we found that *Glut1* and *Ldha* levels increased in *neudesin* KO BMDCs after LPS treatment, suggesting that neudesin may regulate HIF-1α-mediated pathways. The significance of HIF-1α in the suppression of DC function by neudesin needs to be explored further.

Identification of the receptors and downstream signals of neudesin remains a topic of interest. Currently, neudesin receptors are unknown. However, it has been reported that neudesin activates intracellular signaling pathways, such as the PI3K-Akt and Erk pathways, in neuronal cells.[10] Conflicting reports exist regarding the role of the PI3K-Akt pathway in the function of DCs[28,29] Additionally, one report suggested that the Erk pathway inhibits the activation of DCs.[30] Therefore, it is necessary to identify the receptors for neudesin and investigate the significance of the PI3K-Akt and Erk pathways to elucidate the mechanism underlying the suppression of DC function by neudesin.

In recent years, the mechanisms of immune suppression have been actively explored for their application in clinical treatments. For example, in the context of cancer, therapies targeting immune inhibitory mechanisms within the tumor microenvironment have been developed. The membrane protein PD-L1, expressed on cancer cells, inhibits T cell function by binding to PD-1 on T cells. Drugs targeting PD-1 and PD-L1 are used in clinical practice. However, the response rates of these approaches are not entirely satisfactory, and new strategies are required.[31-33] Although we showed that neudesin is derived from DCs, it has also been reported that neudesin is expressed in certain cancer cells and that it promotes tumor growth.[34] Therefore, neudesin may act as a secreted suppressor of DC function within the cancer microenvironment, potentially facilitating tumor growth. The immunosuppressive role of neudesin in tumor growth is clinically important and should be further investigated.

In summary, we report here that neudesin is produced by DCs and inhibits cytokine expression in BMDCs through the inhibition of glycolysis. Our data suggest that neudesin is involved in adaptive immune responses and is a potential target for cancer therapy or immune-related diseases.

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Conflict of interest NK is an employee of Nippon Shinyaku Co. Ltd. The other authors declare no conflicts of interest.

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