TLR4 and Caveolin-1 in Monocytes Are Associated With Inflammatory Conditions in Diabetic Neuropathy

T Zhu¹*, Q Meng¹,², J Ji¹, L Zhang¹ and X Lou³

The purpose of this study was to investigate the expression of TLR4 and caveolin-1 in monocytes among healthy volunteers as well as those with type-2 diabetes mellitus (T2DM) and diabetic peripheral neuropathy (DPN). Nineteen healthy control subjects, 18 patients with T2DM, and 20 patients with DPN were enrolled. Toll-like receptor (TLR)4, caveolin-1, MyD88, phosphorylated IκB, and plasma TNF-α and interleukin (IL)-6 were measured using real-time polymerase chain reaction, Western blotting, and enzyme-linked immunosorbent assay. Compared with the other two groups, the DPN group had higher expression of TLR4, MyD88, phosphorylated IκB, TNF-α, and IL-6, but significantly lower levels of caveolin-1 and total IκB in monocytes. Plasma concentrations of TNF-α and IL-6 were positively correlated with TLR4 and negatively correlated with caveolin-1 in patients with DPN. Plasma concentration of TLR4 was negatively correlated with caveolin-1 in patients with DPN. Reduced expression of caveolin-1 in monocytes could aggravate the TLR4-mediated inflammatory cascade.

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Study Highlights

WHAT IS THE CURRENT KNOWLEDGE ON THE TOPIC?
✔ Decreased caveolin-1 and increased TLR4 in monocytes are associated with inflammatory conditions in diabetic neuropathy.

WHAT QUESTION DID THIS STUDY ADDRESS?
✔ How caveolin-1 or TLR4 in monocytes are associated with inflammatory conditions in diabetic neuropathy.

WHAT THIS STUDY ADDS TO OUR KNOWLEDGE
✔ Caveolin-1 is decreased in diabetic neuropathy and is negatively associated with inflammatory cytokines (TLR4, TNF-α, and IL-6).

HOW THIS MIGHT CHANGE CLINICAL PHARMACOLOGY OR TRANSLATIONAL SCIENCE
✔ Caveolin-1 can act as a protective role in inflammation of diabetic neuropathy and might be a good therapy target in diabetic neuropathy.

DPN is one of the most common complications of T2DM, with 61.8% of patients with T2DM reporting mild-to-severe dysfunction in peripheral nerves. Pain, numbness, sensory loss, and stabbing/burning pains in hands and feet are typical signs and symptoms of DPN. The mechanism of hyperglycemia, dyslipidemia, inflammation, and insulin-resistance leading to DPN are not clear. By now, rigorous glycemic control has been the only treatment to ameliorate or prevent the neuropathy injury. Inflammation, which has been demonstrated to be a risk factor for T2DM, has also been proposed to be a major factor in the occurrence and progression of DPN. It has been indicated that pro-inflammatory cytokines were related to chronic inflammation in patients with T2DM with or without neuropathy. Moreover, patients with neuropathy have higher circulating levels of C-reactive protein (CRP) and pro-inflammatory cytokines. TLRs are transmembrane pattern-recognition receptors expressed on multiple cells, including monocytes. TLRs can trigger a signaling cascade that results in the release of pro-inflammatory cytokines after recognition of specific ligands. Activated TLR4 induces the release of pro-inflammatory cytokines and contributes to the inflammatory conditions in T2DM and its complications. However, whether TLR4 expression and downstream signaling pathways in monocytes are correlated to inflammatory conditions in patients with T2DM with neuropathy is not known. The membrane protein caveolin-1 is a 22-kDa protein that exerts pleiotropic cellular functions (e.g., cholesterol homeostasis, proliferation, and signal transduction) and has been postulated to be a modulator of innate immunity and inflammation. Upregulation of expression of caveolin-1 mRNA in adipose tissue obtained from obese humans and obesity-associated T2DM has been implicated in low-grade inflammation. Studies showed that effects of caveolin-1 on production of major pro-inflammatory cytokines, such as TNF-α, IL-6, IL-1, and granulocyte macrophage-colony stimulating factor could be mediated by direct interaction with TLR4 and regulation of TLR4 activation. Nevertheless, studies on caveolin-1 expression in monocytes with respect

¹Department of Anesthesiology, Songjiang Center Hospital, NanJing Medical University, Shanghai, China; ²Department of Anesthesiology, Nanjing Hospital, Nanjing Medical University & Nanjing First Hospital, Nanjing, China; ³Department of Central Laboratory, Songjiang Hospital, First People’s Hospital, Shanghai Jiao Tong University, Shanghai, China. *Correspondence: T Zhu (zt19192003@163.com)

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to chronic inflammation in patients with T2DM and DPN are lacking.

We examined expression of toll-like receptor (TLR)4, downstream signaling molecules, and caveolin-1 in monocytes isolated from patients with type 2 diabetes mellitus (T2DM) or diabetic peripheral neuropathy (DPN), as well as healthy controls. We explored the possible correlation between TLR4, caveolin-1, and different inflammation markers in T2DM and DPN.

MATERIALS AND METHODS

The study protocol was approved by the Medical Ethics Committee of the Central Hospital of Songjiang District (Shanghai, China). Written informed consent was obtained from all participants.

Study population

Healthy volunteers from a medical examination center and patients from the Department of Endocrinology of the Central Hospital of Songjiang District were enrolled between June and December 2013. All subjects were biologically unrelated, of Chinese Han ethnicity, and aged >18 years. The healthy control group (10 men and nine women) were recruited if they had: normal complete blood count; normal levels of electrolytes; no family history of diabetes mellitus; no hypertension; or other endocrine/metabolic disorders. Patients with T2DM and DPN were enrolled concomitantly from the Department of Endocrinology of our hospital. The T2DM group comprised nine men and nine women. The DPN group comprised 11 men and nine women. The diagnostic criteria of T2DM, as recommended by the World Health Organization in 1999, were adopted. Definitions of the minimum criteria for DPN meet the criteria of T2DM, as recommended by the World Health Organization.

Exclusion criteria were abnormal complete blood count, other types of diabetes mellitus, acute infections, cardiac, hepatic/renal or thyroid dysfunction, other types of diabetes mellitus, acute infections, cardiac, hepatic/renal or thyroid dysfunction, other types of acute and chronic complications of diabetes mellitus, complications with other connective-tissue diseases, or autoimmune diseases.

Clinical measurements

All clinical examinations and evaluations were conducted in the fasting state. Glycosylated hemoglobin % values was measured by a Variant II Turbo system (Bio Rad, Berkeley, CA). Lipid profiles were measured using the ISE900/D2400/P800 system (Roche, Basel, Switzerland). Quik Read go (Orion Diagnostica Oy, Espoo, Finland) was used to measure leukocyte count, monocyte count, and C-reactive protein (CRP) level. The body mass index (kg/m²) was also calculated. The mean of three measurements of systolic blood pressure and diastolic blood pressure was collected in anticoagulant tubes containing EDTA. Plasma was obtained and stored at −80°C for enzyme-linked immunosorbent assay. Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Hypaque density-gradient centrifugation (density, 1.077 g/mL; 400 × g for 40 min at room temperature). Freshly isolated PBMCs were washed twice with sterile phosphate-buffered saline to remove contaminating separation medium. They were then resuspended in RPMI-1640 (Gibco, Carlsbad, CA) containing 10% (v/v) heat-inactivated fetal calf serum, 50 U/mL penicillin, and 50 mg/mL streptomycin. PBMCs (1 × 10⁶) were cultured in six-well plates, and incubated for 1.5–2 h at 37°C in an atmosphere of humidified 5% CO₂ to obtain peripheral blood monocytes (1 × 10⁶). PBMCs or one well will be lysed by TRizol Reagent to isolate total RNA; 2 × 10⁶, PBMCs or two wells will be lysed to isolate total protein).

Real-time reverse transcription-polymerase chain reaction

Total RNA was isolated from peripheral blood monocytes with TRizol Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. RNA samples were treated with DNase I to remove traces of contaminating genomic DNA. First-strand cDNA was synthesized at 37°C for 60 min, 85°C for 5 min, and 4°C for 5 min in 25μL reaction mixture using Oligo (dT)18 primers and RevertAid M-MuLV (Thermo Scientific, Waltham, MA). Real-time reverse transcription-polymerase chain reaction was carried out according to the protocol in the SYBR Green polymerase chain reaction kit (Thermo Scientific). ABI Prism 7300 sodium dodecyl sulfate (Applied Biosystems, Foster City, CA) was used to continually monitor increases in fluorescence (which is positively correlated with polymerase chain reaction products). The sequences of primers (Generay Biotech, Shanghai, China) used were (forward and reverse, respectively) TLR4: 5′-CCGCTTTTCATCTTCTCA-C-3′ and 5′-CATCCTGGCACTA TCCTC-3′; caveolin-1: 5′-GGGTGCAACATGGTGTTC-3′ and 5′-TCTTTCTCTGGCGAAAAG-3′; and glycer-aldehyde 3-phosphate dehydrogenase (GAPDH): 5′-CACCACCTCCACACTTGTTG-3′ and 5′-CCACACACTTTGCTGTAG-3′. Amplification was carried out in a total reaction volume of 25 μL and done according to the protocol. To account for differences in the amount and quality of total RNA added to each reaction, GAPDH (endogenous control) was amplified for each sample. Data were calculated using the 2ΔΔCT method.

Western blot analyses

Monocytes were broken down in lysis buffer with a protease inhibitor cocktail (Keygen Biotech, Nanjing, China). Protein extraction was carried out according to directions in the ProteoExtract kit (Keygen Biotech). Supernatants of cell lysates were collected after centrifugation at 4°C for 10 min at 14,000 × g. Protein concentrations of extracts were measured using a modified Bicinchoninic AcidProtein Assay kit (SangonBiotech, Shanghai, China) according to the manufacturer's instructions. Supernatants were then boiled in sodium dodecyl sulfate sample buffer for 5 min. Equal amounts of total protein were loaded into 4–15% sodium dodecyl sulfate-polyacrylamide gels and transferred after
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Table 1   Clinical characteristics of studied population

|                          | Normal control | T2DM       | DPN        |
|--------------------------|----------------|------------|------------|
| Age, y                   | 57.68 ± 11.82  | 58.83 ± 10.43 | 56.4 ± 10.88 |
| Sex, male/female         | 10/9           | 9/9        | 11/9       |
| Duration, y              | 0              | 7.48 ± 4.07 | 10.75 ± 5.41 |
| BMI, kg/m²               | 22.8 ± 2.18    | 22.39 ± 2.32 | 23.16 ± 2.73 |
| SBP, mmHg                | 124.2 ± 13.18  | 126.5 ± 14.98 | 128.1 ± 15.37 |
| DBP, mmHg                | 76.47 ± 8.6    | 82.56 ± 9.36 | 82.25 ± 10.69 |
| HbA1c(%)                 | 5.31 ± 1.07    | 7.71 ± 0.93 | 8.36 ± 0.92 |
| TC, mmol/L               | 4.51 ± 0.78    | 4.46 ± 0.81 | 4.72 ± 1.02 |
| TG, mmol/L               | 1.79 ± 0.65    | 1.98 ± 0.8  | 1.86 ± 0.76 |
| LDL-C, mmol/L            | 2.7 ± 0.94     | 2.84 ± 1.4  | 3.11 ± 1.28 |
| HDL-C, mmol/L            | 1.521 ± 0.52   | 1.249 ± 0.49 | 1.277 ± 0.54 |
| CRP, mg/L                | 2.731 ± 1.64   | 4.769 ± 2.77 | 9.366 ± 6.82 |
| Leukocyte count, /nL     | 5.98 ± 1.03    | 6.16 ± 0.89 | 5.85 ± 0.98 |
| Monocyte count, /μL      | 298.97 ± 51.7  | 277.3 ± 40.21 | 302.53 ± 54.34 |

BMI, body mass index; CRP, C reactive protein; DBP, diastolic pressure; DPN, diabetic peripheral neuropathy; HbA1c(%), glycosylated hemoglobin; HDL-C, high density lipoprotein-cholesterol; LDL-C, low density lipoprotein-cholesterol; SBP, systolic pressure; T2DM, type 2 diabetes mellitus; TC, total cholesterol; TG, triglyceride.

*P < 0.01
**P < 0.05, compared with normal controls
***P < 0.05, compared with T2DM.

RESULTS

Baseline characteristics of study subjects
The clinical characteristics of the study population are presented in Table 1. Duration of T2DM, glycosylated hemoglobin c level, and CRP level were significantly higher in patients with DPN compared with T2DM and control groups. Compared with healthy volunteers, patients with T2DM had T2DM for a longer time, and had higher levels of glycosylated hemoglobin c and CRP. Age, body mass index, sex, lipid profiles, systolic blood pressure, diastolic blood pressure, or quantities of leukocytes and monocytes were not significantly different among the three groups. CRP reference value is 0–8 mg/L.

Expression of toll-like receptor 4 and caveolin-1 mRNA in peripheral blood monocytes
To ascertain whether expression of TLR4 and caveolin-1 in monocytes is associated with DPN, we measured TLR4 and caveolin-1 mRNA levels by real-time polymerase chain reaction (Table 2). TLR4 expression in DPN was significantly higher than in patients with T2DM, but caveolin-1 mRNA was reduced compared with patients with T2DM. Results were normalized to mRNA levels of the housekeeping protein (GAPDH). Further analyses showed that TLR4 mRNA expression in monocytes was negatively correlated with caveolin-1 mRNA in DPN groups (Figure 2).

Toll-like receptor 4/nuclear factor-kappa B signaling pathway and caveolin-1 protein in monocytes
To further explore TLR4-mediated inflammation, we examined the MyD88-dependent signaling pathway using the Western blotting technique. Expression of TLR4, MyD88, and IκB protein in monocytes was higher in patients with DPN compared with patients with T2DM and...
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Table 2 Toll-like receptor 4 and caveolin-1 mRNA expression in monocytes and plasma tumor necrosis factor-α and interleukin-6 concentration of study groups

|                  | Normal control (n = 19) | T2DM (n = 18) | DPN (n = 20) |
|------------------|------------------------|---------------|--------------|
| TLR4             | 1.17 ± 0.54            | 1.86 ± 0.45a  | 2.9 ± 1.05ab |
| Caveolin-1       | 1.04 ± 0.3             | 0.679 ± 0.15a | 0.42 ± 0.1ab |
| TNF-α, pg/mL     | 3.01 ± 1.01            | 5.32 ± 1.86a  | 7.49 ± 1.54bc |
| IL-6, pg/mL      | 1.46 ± 0.7             | 2.59 ± 0.9a   | 3.19 ± 1.22bc |

DPN, diabetic peripheral neuropathy; IL, interleukin-6; T2DM, type 2 diabetes mellitus; TLR, toll-like receptor; TNF, tumor necrosis factor.

Data are given as mean ± SD.

aP < 0.01, compared with normal control

bP < 0.01

cP < 0.05, compared with T2DM.

Figure 1 Caveolin-1, toll-like receptor (TLR)4, and its downstream signaling proteins MyD88, IκB, and phosphorylated IκB expression in monocytes isolated from groups. (a) Protein expressions were determined using specific antibodies to the respective (phospho) proteins by Western blot analysis, as described in the Research Design and Methods section. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) served as the standard. Each blot is repeated three times. (b) Western blotting chart of proteins expression. Normal control (NC)1, NC2, NC3: normal healthy group; type 2 diabetes mellitus (T2DM1, T2DM2, T2DM3: T2DM group; diabetic peripheral neuropathy (DPN)1, DPN2, DPN3: DPN group. Values are mean ± SE (n = 9). *P < 0.01, #P < 0.05, compared with normal control; P < 0.05, compared with T2DM.

Lower levels of total IκB and caveolin-1 protein were found in patients with DPN compared with the T2DM group. Densitometry ratios corroborated the data in Figure 1b.

Plasma levels of the pro-inflammatory cytokines tumor necrosis factor-α and interleukin-6
There was a significant increase in levels of both pro-inflammatory mediators in patients with T2DM and DPN compared with control subjects (Table 2). IL-6 reference value is 0–7 pg/mL. Higher levels of TNF-α and IL-6 were noted in patients with DPN compared with patients with T2DM.

Correlation between tumor necrosis factor 4/caveolin-1 and tumor necrosis factor-α/interleukin-6
TLR4 expression in monocytes was positively correlated with plasma levels of TNF-α and IL-6 (r = 0.7250 and 0.8527, respectively), but caveolin-1 exhibited a negative correlation with levels of TNF-α and IL-6 (r = -0.4544 and -0.648, respectively) in patients with DPN (Figure 2). In patients with T2DM, TLR4 mRNA was positively correlated with plasma levels of TNF-α and IL-6 (r = 0.5186 and 0.6174, respectively). Caveolin-1 mRNA exhibited a negative correlation with levels of TNF-α, but has no relationship with IL-6 (r = -0.4795 and -0.3504, respectively). The overall correlation across the three groups showed that TLR4 mRNA was positively correlated with plasma levels of TNF-α and IL-6 (r = 0.7469 and 0.7395, respectively). Caveolin-1 mRNA exhibited a negative correlation with levels of TNF-α and IL-6 (r = -0.6838 and -0.6555, respectively). Our results indicated that TLR4 and caveolin-1 had higher correlation with TNF-α and IL-6 in patients with DPN than in patients with T2DM without neuropathy. The correlation index between caveolin-1 and TLR4 are -0.6455, -0.5437, and -0.7391 of the overall, T2DM, and DPN groups, which indicated that caveolin-1 had higher correlation with TLR4 in the DPN group.

DISCUSSION
The present study found that patients with T2DM and DPN had increased TLR4 expression in monocytes compared with control subjects. Expression of TLR4 in monocytes was significantly higher in patients with DPN compared with the T2DM group. These results suggested that increased expression of TLR4 in monocytes could be linked to systemic...
inflammation in peripheral neuropathy in T2DM. Studies showed that the expression and activation of TLR4 in human monocytes (the predominant cells of the innate immune system) was significantly elevated under hyperglycemic conditions, whereas the activated pro-inflammatory pathway of peripheral blood monocytes was the “gateway” to exacerbated inflammation in many types of tissue. A higher level of TLR4 expression suggested that patients with peripheral neuropathy have further increased inflammation compared with those with T2DM. Further support was provided by a higher plasma level of CRP in patients with DPN compared with the T2DM group. Such findings were consistent with the results of Doupis et al. In the review by Donath and Shoelson, they demonstrated that systemic inflammation had a significant role in the etiology and comorbidities of T2DM, but the TLR4 level in DPN was not assayed. Furthermore, insulin resistance was an independent risk factor in DPN. Recent studies suggested that expression of TLR4 in monocytes or macrophages was critical for the pathogenesis of insulin resistance, T1DM, and T2DM. Yan et al. reported that TLR4 expression in the spinal cord of streptozotocin-induced diabetic rats with DPN was gradually upregulated and positively correlated with expression of pro-inflammatory cytokines. Experimental evidence suggested that systemic free-radical scavenger antioxidant coenzyme Q10 suppresses neuropathic pain in T2DM by downregulation of TLR4 expression. A single-center cross-sectional pilot study involving 246 patients with T1DM and 530 cases of T2DM reported that DPN in patients with T1DM was not associated with the single nucleotide polymorphisms of TLR4. In patients with T2DM, however, the TLR4 gene was involved in DPN. The present study showed that significant upregulation of TLR4 in monocytes may contribute to inflammation in DPN, we believe, for the first time.

Caveolin-1 regulated the receptor signaling in the membranes by direct binding to the receptor or downstream molecules, and was implicated as a modulator of innate immunity and inflammation. One study showed that glucose was associated with caveolin-1 expression. We showed, for the first time, that caveolin-1 expression in monocytes was significantly reduced in T2DM and DPN than in healthy controls. Hayashi et al. reported that elevated glucose concentrations diminished the number and size of caveolae, and suppressed the level of caveolin-1 expression in monocytes. It was well established that high glucose-induced downregulation of caveolin-1 was associated with the pathophysiological progression of DPN. Certainly, a compensatory mechanism for hyperglycemia-induced downregulation of caveolin-1 needs to be explored. In this study, the Pearson correlation coefficient suggested that reduced expression of caveolin-1 in monocytes was negatively correlated with expression of TLR4 and pro-inflammatory cytokines (TNF-α and IL-6) in DPN. Our results indicated that caveolin-1 had higher correlation with TLR4, TNF-α, and IL-6 in patients with DPN than in patients with T2DM without neuropathy, which mean that caveolin-1 could be a better predictive marker in patients with DPN than in...
patients without neuropathy. Evidence that is more recent suggested that caveolin-1 played an important part in suppressing inflammation. Wang et al.\textsuperscript{15} showed that upregulation of caveolin-1 in murine macrophages dramatically inhibited TNF-\(\alpha\) and IL-6 production. A further study showed that caveolin-1 conferred its anti-inflammatory effects through direct binding of TLR4 and functionally suppressing assembly of the TLR4 complex with MyD88 in murine macrophages. Furthermore, monocytes were much more sensitive to variation of caveolin-1 expression because the baseline level of caveolin-1 in monocytes was much lower than that in other cell types.\textsuperscript{16,17} These observations raised the possibility that the interplay between caveolin-1 and TLR4 in monocytes was involved in the inflammatory pathogenesis of T2DM and DPN.

The MyD88-dependent signaling pathway involved activation of nuclear factor-kappa B, and was essential to TLR4 signaling and production of TNF-\(\alpha\) and IL-6. Phosphorylated I\(\kappa\)B contributed to nuclear factor-kappa B activation and triggered an inflammatory cascade.\textsuperscript{18} Our research showed elevated levels of MyD88, phosphorylated I\(\kappa\)B, TNF-\(\alpha\), and IL-6, but significantly lower total I\(\kappa\)B in patients with DPN compared with the two other groups. TLR4 expression in monocytes was positively correlated with the plasma concentrations of TNF-\(\alpha\) and IL-6, whereas, for caveolin-1, an inverse association was found in the DPN group. These results inferred that the TLR4/nuclear factor-kappa B signaling pathway in monocytes could play an important part in the pathogenesis of DPN. Herder et al.\textsuperscript{19} demonstrated a strong relationship between IL-6 and DPN impairment. However, the association between TNF-\(\alpha\) and DPN seems to be stronger than with IL-6.\textsuperscript{4} Additionally, infliximab could be a safe and effective long-term approach for DPN treatment due to its suppression of increased serum levels of TNF-\(\alpha\).\textsuperscript{20} We also noted significantly higher expression of MyD88, phosphorylated I\(\kappa\)B, TNF-\(\alpha\), and IL-6, and reduced expression of total I\(\kappa\)B in T2DM, which were consistent with Dasu et al.\textsuperscript{7}

The present study had limitations. First, our study was cross-sectional, so we could not differentiate between causes and effects. It could be claimed that the observed discrepancies are secondary to DPN and the inflammation associated with DPN. Therefore, more detailed studies on the mechanisms between caveolin-1 and the TLR4/nuclear factor-kappa B signaling pathway in DPN need to be studied. Second, the T2DM and DPN groups were not matched in terms of disease duration, and the observed differences could correlate to this mismatch. However, Doupis et al.\textsuperscript{4} showed that expression of inflammatory cytokines tended to be higher in a subgroup of painful neuropathy, even though T2DM duration in this group was shorter than that in the painless neuropathy subgroup. Third, DPN did not have strict diagnostic criteria. Patients with T2DM with symptoms or signs of neuropathy and abnormal neural conduction were diagnosed as having DPN in the present study. The definition of DPN was confirmed by a recent statement by the European Association for the Study of Diabetes.\textsuperscript{21}

**CONCLUSION**

Our results provide proof of concept that inflammatory conditions in patients with DPN are associated with increased expression of TLR4 and activation of the downstream MyD88-dependent signaling pathway and reduced expression of caveolin-1 in monocytes. Furthermore, we hypothesize that the effect of downregulation of caveolin-1 expression on the TLR4-mediated inflammatory cascade may also be implicated in progression of the inflammatory state of DPN. Therefore, we plan further studies of the mechanism of action and hope that such information will aid in understanding the pathogenesis of DPN.

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**Conflict of Interest.** The authors declared no conflict of interest.
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