Ninjurin1 regulates lipopolysaccharide-induced inflammation through direct binding

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Abstract. Ninjurin1 is a transmembrane protein involved in macrophage migration and adhesion during inflammation. It was recently reported that repression of Ninjurin1 attenuated the lipopolysaccharide (LPS)-induced inflammatory response in macrophages; however, the precise mechanism by which Ninjurin1 modulates LPS-induced inflammation remains poorly understood. In the present study, we found that the interaction between Ninjurin1 and LPS contributed to the LPS-induced inflammatory response. Notably, pull-down assays using lysates from HEK293T cells transfected with human or mouse Ninjurin1 and biotinylated LPS (LPS-biotin) showed that LPS directly bound Ninjurin1. Subsequently, LPS binding assays with various truncated forms of Ninjurin1 protein revealed that amino acids (aa) 81-100 of Ninjurin1 were required for LPS binding. In addition, knockdown experiments using *Ninj1* siRNA resulted in decreased nitric oxide (NO) and tumor necrosis factor-α (TNFα) secretion upon LPS treatment in Raw264.7 cells. Collectively, our results suggest that Ninjurin1 regulates the LPS-induced inflammatory response through its direct binding to LPS, thus, identifying Ninjurin1 as a putative target for the treatment of inflammatory diseases, such as sepsis and inflammation-associated carcinogenesis.

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Introduction

Inflammation is involved with diverse pathological processes, including infection, diabetes, atherosclerosis, neurodegenerative disease, and cancer (1). In particular, inflammation plays a critical role in either inhibiting or promoting tumor progression, depending on the context, such as the stage and origin of the cancer. Inflammatory cells were initially thought to actively target tumor cells (2); however, recent research suggests that cancer could be provoked by the signaling crosstalk that occurs between inflammatory cells and tumor cells (3,4). For example, tumor-associated macrophages that express cathepsins B and S are known to promote cancer growth and invasion in a pancreatic tumor model (5). Therefore, further investigation of the connection between inflammation and cancer is important for a better understanding of tumor pathology and developing improved tumor-directed therapeutics.

Bacterial infection is a major cause of inflammation and is mediated by a variety of bacterial cell compartments (6). LPS in the outer membrane is a principal pathogenic molecule in the case of gram-negative bacteria, whereas plasma membrane lipoprotein plays a similar role in mycoplasma, which lacks a cell wall and outer membrane (7). Both LPS and lipoprotein contain lipid moieties, lipid A and a lipoylated amino-terminal cysteinyl residue, respectively, that are responsible for stimulating the host immune response (7,8). Ag 243-5 lipoprotein, originally isolated from *Mycoplasma arginini*, but also described as the P47 lipoprotein of *M. hyorhinis*, is reported to have a metastasis-promoting activity (9,10). Interestingly, Ag 243-5 shows significant sequence homology with macrophage-activating lipoprotein-404 (MALP-404) from *M. fermentans*, which is known to increase cytokine production in human monocytes (11).

Several LPS binding molecules facilitate the biological effect of LPS on host cells. For instance, LPS-binding protein

(LBP) binds aggregated LPS and then delivers monomeric LPS to CD14 (12). Membrane anchored protein CD14 functions as a critical toll-like receptor 4 (TLR4) co-receptor, and transfers LPS to the myeloid differentiation factor 2 (MD2)-TLR4 complex (13). Finally, LPS triggers the dimerization of TLR4, which subsequently initiates intracellular signaling cascades (14,15). Besides, several other proteins are reported to bind LPS, including high mobility group box 1 protein (HMGB1) (16), CXCR4 (17), and β_2-glycoprotein I (18). These proteins are actively studied as inflammatory regulators and therapeutic candidates in inflammatory diseases, but the current data are not sufficient to address any meaningful applications.

Ninjurin1 was originally identified as an upregulated protein in injured nerves and is comprised of two transmembrane domains (aa 72-100 and aa 118-139), N-terminal (aa 1-71) and C-terminal (aa 140-152) extracellular domains, and cytosolic region (aa 101-117) (19). Our group and others have reported on the various roles of Ninjurin1 in macrophages, such as increasing adhesion to endothelial cells and motility during early ocular development and experimental autoimmune encephalomyelitis (20,21). Moreover, it was recently revealed that Ninjurin1 modulates the TLR4-dependent inflammatory response triggered by LPS via p38 phosphorylation and activator protein-1 (AP-1) activation (22); however, the precise mechanism of its roles in the inflammatory response is enigmatic. In this study, we report that Ninjurin1 mediates LPS-induced inflammation by directly binding to LPS. This identification of Ninjurin1 and LPS binding likely provides an important insight into the regulation of macrophage-mediated inflammatory response and diseases.

Materials and methods

Cell culture. HEK293T and Raw264.7 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and the Korean Cell Line Bank (KCLB, Seoul, Korea), respectively. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM, GenDEPOT, Barker, TX, USA) supplemented with 10% fetal bovine serum (FBS, GenDEPOT), and 100 U/ml penicillin and 100 μg/ml streptomycin (GenDEPOT) at 37˚C in a humidified 5% CO_2 atmosphere.

Construction of expression plasmids and transfection. Expression plasmids for human (NM_004148) and mouse (NM_013610) Ninjurin1 were constructed as described previously (21). To construct expression plasmids for N-terminal MYC-tagged human and mouse Ninjurin1, cDNA were amplified by PCR and subcloned into pCS2$^+$-Myc. Truncated forms of mouse Ninjurin1, MYC-mNINJ1 (1-71), MYC-mNINJ1 (72-152), MYC-mNINJ1 (1-100), MYC-mNINJ1 (1-90), MYC-mNINJ1 (1-80), MYC-mNINJ1 (81-152), MYC-mNINJ1 (91-152), MYC-mNINJ1 (101-152), and MYC-mNINJ1 (71-110) plasmids were also constructed using pCS2$^+$-Myc as backbone. Designing and cloning of expression plasmid of non-tagged Ninjurin1 was described previously (23). Briefly, mouse Ninjurin1 cDNA was subcloned into pcDNA3.1$^+$ myc/his backbone without removing a stop codon.

HEK293T cells at $3x10^6$ cells/dish were cultured in 100-mm culture dishes for 24 h and then expression plasmids were transfected using polyethylenimine (PEI, Sigma-Aldrich, St. Louis, MO, USA). After 48 h of culture, cells were washed with PBS and collected.

RNA interference. Ninjurin1 downregulation was performed with RNA interference. siNinj1 targeted to mouse Ninjurin1 was purchased from Life Technologies (Grand Island, NY, USA). Negative control of RNA interference, siControl with the scrambled sequence was purchased from Bioneer (Daejeon, Korea). The following sequences were used: siControl: 5'-CCTACGCCACCAAUUUCGUdTdT-3'; siNinj1: 5'-ACCGGCCCAUCAAUGUAAACCAUUA-3'. Raw264.7 cells at $2x10^5$ cells/dish were cultured in 60-mm culture dishes for 12 h. siRNAs (20 nM) were transfected using Lipofectamine RNAiMAX transfection reagent (Life Technologies). After 24 h of transfection, media were changed with presence or absence of 1 μg/ml LPS (Sigma-Aldrich). After another 24 h, cultured supernatant and cells were collected.

Immunoblot analysis. Proteins were extracted in cell lysis buffer containing 50 mM Tris-Cl (pH 7.4), 300 mM NaCl, 5 mM EDTA, 0.02% (w/v) sodium azide, 1% (w/v) Triton X-100, 10 mM iodoacetamide, 1 mM phenylmethanesulfonyl fluoride, 2 μg/ml leupeptin, and protease inhibitor cocktail (Calbiochem, Billerica, MA, USA). Lysates were separated with SDS-PAGE and transferred to nitrocellulose membrane (GE Healthcare Life Sciences, Pittsburgh, PA, USA). The transferred membrane was probed with the specific antibodies. Antibodies to NOS2 purchased from BD Bioscience (San Diego, CA, USA), MYC and GAPDH from Santa Cruz Biotechnology (Dallas, TX, USA). Endogenous Ninjurin1 was detected by custom-made antibody that was raised in rabbit with aa 139-152 of mouse Ninjurin1 (Ninj1 $Ab_{139-152}$) (23). Image was acquired using LAS3000 machine (GE Healthcare Life Sciences).

Immunoprecipitation and silver staining. Protein lysates (1,000 μg) and 1 μg of MYC antibody were incubated overnight at 4˚C with gentle rotation. Protein A agarose (10 μl) (EMD Millipore, Billerica, MA, USA) was added to each sample and the mixture was incubated at 4˚C for 4 h with gentle rotation. After washing 5 times with washing buffer containing 50 mM Tris-Cl (pH 7.4), 300 mM NaCl, 5 mM EDTA, 0.02% (w/v) sodium azide, and 0.1% (w/v) Triton X-100, precipitated proteins were eluted by heating with SDS sample buffer at 95˚C for 10 min. Eluted sample was separated with SDS-PAGE followed by silver staining procedure using PlusOne Silver Staining kit (GE Healthcare Life Sciences) as recommended by the manufacturer.

Binding assay of Ninjurin1 with MALP-2. Macrophage-activating lipopeptide-2 (2 μg) (MALP-2, Enzo Life Sciences, Farmingdale, NY, USA) was conjugated to NHS-activated agarose beads (Life Technologies) as recommended by the manufacturer. Protein lysates (500 μg) and MALP-2 conjugated beads were incubated overnight at 4˚C with gentle rotation. After washing 5 times with washing buffer, binding proteins were eluted by heating with SDS sample buffer at 95˚C for 10 min. Eluted samples were further analyzed by immunoblot analysis.

Binding assay of Ninjurin1 with LPS. Binding assays were performed by pull-down with streptavidin sepharose beads (GE Healthcare Life Science) or immunoprecipitation with MYC antibody. For pull-down with streptavidin sepharose beads, 500 μg of protein lysates and 4 μg of LPS-biotin (InvivoGen, San Diego, CA, USA) were incubated overnight at 4˚C with gentle rotation. Streptavidin sepharose beads (10 μl) were added to each sample and incubated at 4˚C for 2 h with gentle rotation. After washing 5 times with washing buffer, the bound proteins were eluted by heating with SDS sample buffer at 95˚C for 10 min. The eluted samples were further analyzed by immunoblot analysis. In case of immunoprecipitation with MYC antibody, 1,000 μg of protein lysates, 1 μg of MYC antibody, and 10 μl of protein A agarose were incubated overnight at 4˚C with gentle rotation, after which the unbound molecules were removed using 5 washes with washing buffer. Washed protein A agarose was eluted for silver staining or incubated with 10 μg of LPS-biotin at 4˚C for 4 h. After washing 5 times with washing buffer, the bound molecules were eluted. Eluted samples were detected using streptavidin-HRP (Thermo Fisher Scientific, Waltham, MA, USA).

Mass spectrometry. Candidate protein bands excised from silver stained gels were destained with destaining solution consisted of 30 mM potassium ferricyanide and 100 mM sodium thiosulfate for 5 min and then incubated with 200 mM ammonium bicarbonate for 20 min. The gels were dehydrated with acetonitrile and dried in a vacuum centrifuge. The dried gels were rehydrated with 50 mM ammonium bicarbonate containing 200 ng trypsin (Promega, Madison, WI, USA) for 45 min, replaced solution to 50 mM ammonium bicarbonate, and incubated overnight at 37˚C . Digested peptide was purified using a desalting column (GE loader tip, Eppendorf, Hamburg, Germany). Each peptide sample was applied to ESI-Q-TOF MS/MS spectrometer (ABSciex, Framingham, MA, USA). The deduced peptide sequence after MS/MS were analyzed by MASCOT search engine (http://www.matrixscience.com) against Swiss-Prot and NCBI databases.

Nitric oxide (NO) assay. Raw264.7 cells were plated into 60-mm culture dishes at $2x10^5$ cells/dish for 12 h and then transfected with *Ninj1* or negative control siRNA. After 24 h of culture, media were changed with or without LPS (1 μg/ml). After incubation for 24 h, the culture supernatant was collected and cells were removed by centrifugation at 500 g for 3 min. Culture supernatant (100 μl) was mixed with 100 μl of Griess reagent (1:1 mixture of 1% sulfanilamide in 30% acetate and 0.1% N-1-naphthylethylenediamine dihydrochloride in 60% acetate) at room temperature for 10 min. The absorbance of the incubated samples was measured by using microplate reader at 540 nm. A standard curve drawn with known concentrations of sodium nitrite was applied to calculate the concentration of nitrite, the stable end product of NO.

Measurement of TNFα secretion. Raw264.7 cells were cultured in 96-well culture plates at $1x10^4$ cells/well for 12 h. The Raw264.7 cells were transfected with *Ninj1* or negative control siRNA for 24 h and then media was removed and replaced with 200 μl of fresh media with or without LPS (1 μg/ml). After 24 h of incubation, the culture supernatant was collected. Amount of secreted TNFα was measured using Mouse TNFα ELISA MAX kit (BioLegend, San Diego, CA, USA) following the manufacturer's instructions.

Statistical analysis. The data are expressed as the mean ± SD. Differences between groups were analyzed by the unpaired two-tailed Student's t-test. $P<0.05$ denoted the presence of statistically significance.

Results

Ag 243-5 protein binds human and mouse Ninjurin1. At the start of this study, immunoprecipitation, gel separation, and mass spectrometry analysis were performed to identify novel Ninjurin1 binding partners. For this, HEK293T cells were transfected with MYC-tagged human Ninjurin1 (MYC-hNINJ1) or empty control (mock) plasmid. Total lysates from transfected HEK293T cells were immunoprecipitated with MYC antibody, separated by SDS-PAGE, and then stained with silver nitrate. Notably, we found a ~47-kDa protein that co-immunoprecipitated with ~36 kDa MYC-hNINJ1 protein, but was not observed in the control sample (Fig. 1). To examine whether this ~47-kDa protein bound mouse Ninjurin1, immunoprecipitations were repeated with lysates from HEK293T cells transfected with MYC-tagged mouse Ninjurin1 (MYC-mNINJ1). Significantly, both hNINJ1 and mNINJ1 were capable of pulling-down the ~47-kDa protein (Fig. 1). As a control, MYC-h/mNINJ1 expression was confirmed by immunoblot analysis with MYC antibody (Fig. 1).

The ~47-kDa band was excised and analyzed by mass spectrometry in order to identify the protein of interest. The resulting ESI-MS spectrum presented with m/z peaks ranging from 750 to 850 (Fig. 2A). The peak at 793.9 m/z was sequenced and identified as a 14-amino acid peptide (IFSPATVFFTSIEK) in further MS/MS analysis (Fig. 2B). Unexpectedly, queries in the Swiss-Prot and NCBI databases using the MASCOT search engine revealed that the peptide sequence was identical to aa 317-330 of Ag 243-5 (BAA04082) (Fig. 2C). To determine the reason for mycoplasma protein existence in our HEK293T cell lysates, we tested our cultures for mycoplasma and found that the HEK293T cells used in the analysis were positive for contamination. Subsequent analyses revealed that non-contaminated HEK293T cell lysates did not contain this ~47-kDa protein band (data not shown). Although unexpected, these observations were intriguing since several previous reports demonstrated a role for Ninjurin1 in the inflammatory response, but the precise mechanism was unknown. Thus, we hypothesized that Ninjurin1 could recognize microbial pathogens conjugated with lipid moieties, such as mycoplasma lipopeptide MALP-2, the MALP-404 N-terminus, and LPS.

MALP-2 and LPS bind human and mouse Ninjurin1. Both MALP-2 and LPS are bacterial endotoxins that induce inflammatory macrophage activation in a manner dependent on their lipid moieties. Therefore, we tested whether MALP-2 and LPS were also able to bind Ninjurin1 as observed with Ag 243-5. For this, MYC-h/mNINJ1 HEK293T cell lysates were incubated with MALP-2-conjugated beads, and the bound proteins were eluted and examined by immunoblot analysis. The same process was used for control samples with

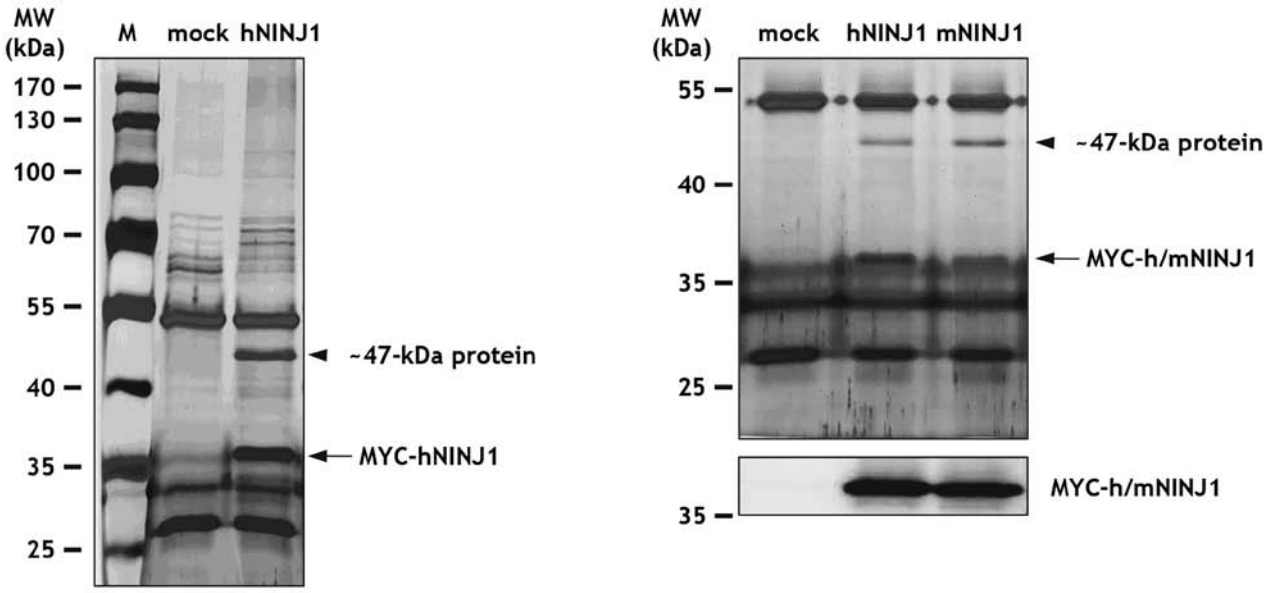

Figure 1. Binding of a ~47-kDa protein with Ninjurin1 in immunoprecipitation with MYC antibody. HEK293T cells were transfected with expression plasmid of MYC-tagged human or mouse Ninjurin1 (hNINJ1, mNINJ1) paralleling with empty control (mock) plasmid. Cell lysates (1,000 μg) were immunoprecipitated using MYC antibody and agarose A beads. Precipitated protein was separated by SDS-PAGE and stained by silver nitrate. Arrow indicates MYC-h/mNINJ1 and arrowhead indicates Ninjurin1 binding protein with ~47-kDa molecular weight. Expression of MYC-h/mNINJ1 was detected by immunoblot analysis with MYC antibody using 30 μg of lysates (right lower). M, molecular-weight marker.

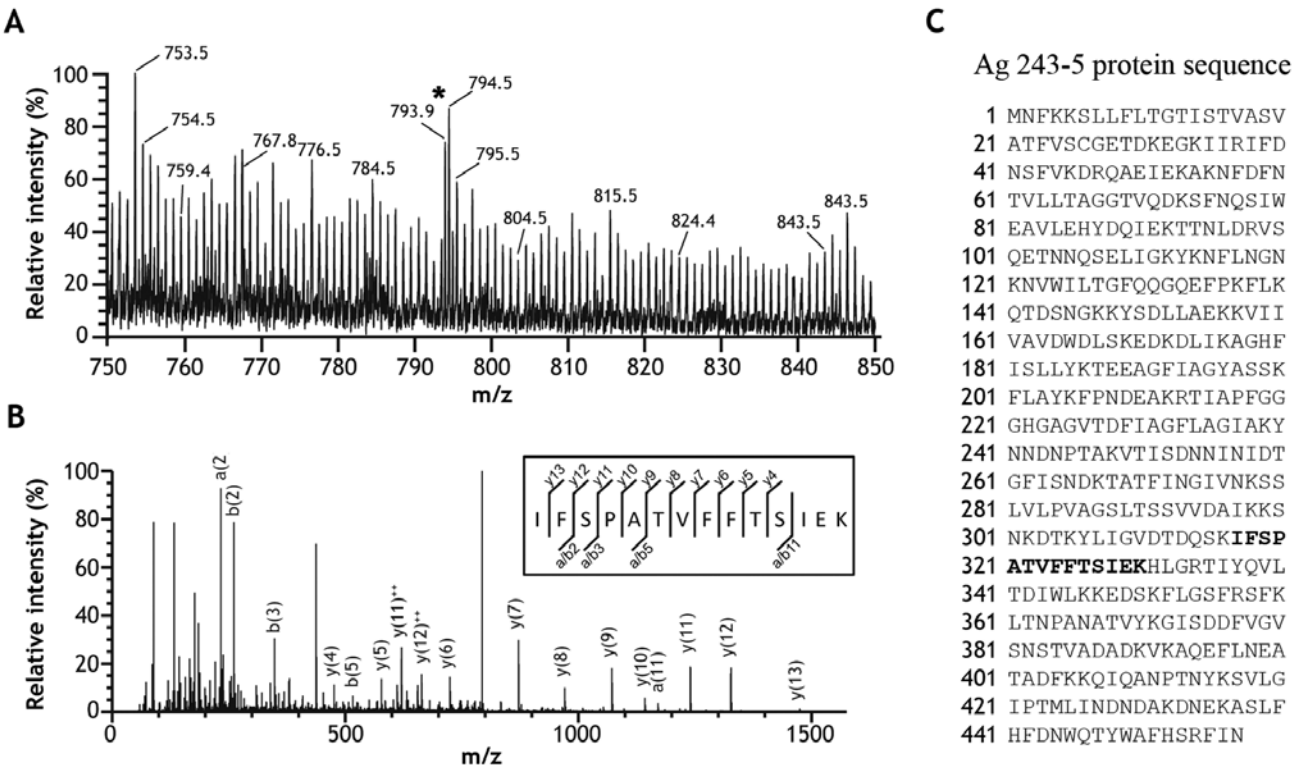

Figure 2. Identification of the ~47-kDa protein by ESI-MS/MS analysis. (A) ESI-MS spectrum of trypsin-digested of the ~47-kDa protein band. The peak at 793.9 m/z (marked with asterisks) was subjected in further MS/MS analysis. (B) The MS/MS spectrum was identified as the partial tryptic peptide IFSPATVFFTSIEK. (C) Database searching with Swiss-Prot and NCBI protein databases. Peptide sequence (bold) was matched with aa 317-330 of *Mycoplasma arginini* protein Ag 243-5.

unconjugated beads. Results showed that human and mouse Ninjurin1 was efficiently pulled down with MALP-2 beads (Fig. 3A), but not control samples. In the case of LPS-biotin, Ninjurin1-expressing cell lysates were incubated with or without LPS-biotin and streptavidin sepharose beads. Similar to that observed with MALP-2, human and mouse Ninjurin1

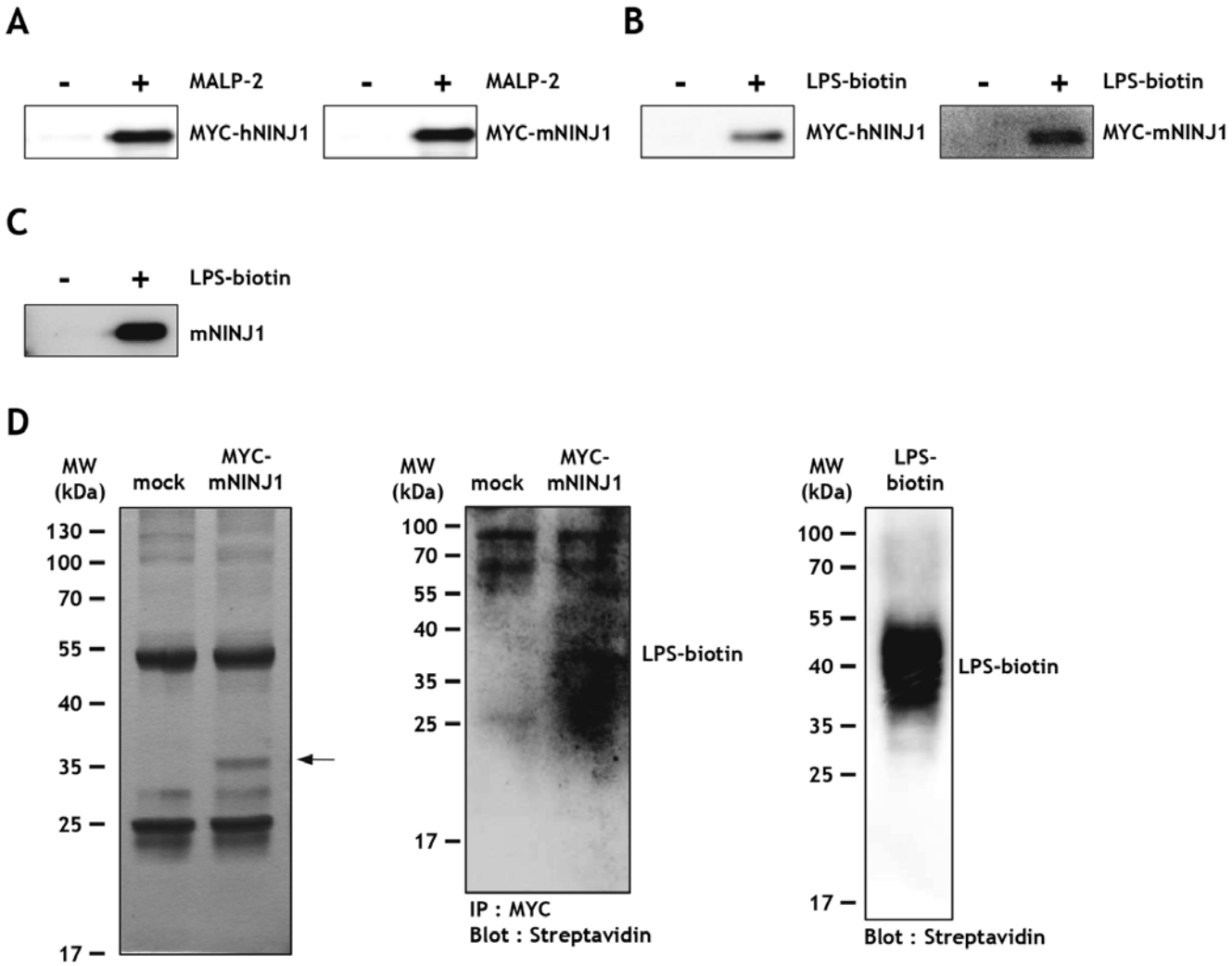

Figure 3. Binding assay between Ninjurin1 and LPS. MYC-h/mNINJ1 (A, B and D) or non-tagged mouse Ninjurin1 (C) was expressed in HEK293T cells. (A-C) Cell lysates (500 μg) and mycoplasma lipoprotein macrophage-activating lipopeptide-2 (MALP-2, 2 μg) or biotinylated LPS (LPS-biotin, 4 μg) were mixed and pulled down by conjugating with beads or streptavidin beads, respectively. Binding of the Ninjurin1 protein to MALP-2 or LPS was detected with MYC (A and B) or Ninj1 $Ab_{139-152}$ (C) antibodies. (D) Cell lysates (1,000 μg) were immunoprecipitated with MYC antibody, the unbound proteins were washed away, and LPS-biotin (10 μg) was then added. The protein was washed and immunoprecipitated, followed by staining with silver nitrate (left) and the binding of LPS to Ninjurin1 protein was detected using streptavidin-HRP (right). Arrow indicates MYC-mNINJ1.

were both able to bind LPS-biotin (Fig. 3B). To rule out the possibility of an interaction between the MYC peptide tag and LPS, LPS binding was assessed using lysates from HEK293T cells transfected with non-tagged mouse Ninjurin1 (mNINJ1) plasmid. As expected, non-tagged mouse Ninjurin1 also bound LPS-biotin (Fig. 3C). To exclude indirect binding through potential adaptor proteins, the unbound proteins were washed away from the immunoprecipitated MYC-mNINJ1 cell lysates, followed by addition of LPS-biotin. MYC-mNINJ1 protein was detected in the silver nitrate stained gel (Fig. 3D, arrow) and MYC-mNINJ1-bound LPS-biotin was detected by streptavidin-HRP, thus confirming a direct interaction between Ninjurin1 and LPS (Fig. 3D, right).

The aa 81-100 region of Ninjurin1 is responsible for LPS binding. To specify the region of Ninjurin1 responsible for LPS binding, truncated mouse Ninjurin1 expression plasmids were constructed. MYC-mNINJ1 (1-71) and MYC-mNINJ1 (72-152), encoding the extracellular N-terminal region and the two transmembrane, cytosolic, and extracellular C-terminus domains, respectively, were cloned into $pCS2^{+}$-Myc backbone plasmid. Full length MYC-mNINJ1, MYC-mNINJ1 (1-71), and MYC-mNINJ1 (72-152) were transfected to HEK293T cells and expression was confirmed by immunoblot analysis (Fig. 4A, lower). LPS binding assays were then performed using equal amounts of cell lysates and LPS-biotin (Fig. 4A, upper). Results showed that full-length MYC-mNINJ1 and MYC-mNINJ1 (72-152) bound LPS, whereas MYC-mNINJ1 (1-71) did not. To further delineate the binding region within Ninjurin1, additional expression plasmids of the truncated N- and C-terminals of mouse Ninjurin1 were constructed as follows: MYC-mNINJ1 (1-100), MYC-mNINJ1 (1-90), MYC-mNINJ1 (1-80), MYC-mNINJ1 (81-152), MYC-mNINJ1 (91-152), MYC-mNINJ1 (101-152), and MYC mNINJ1 (71-100). Ninjurin1 mutant expression was then examined by immunoblot analysis (Fig. 4B and 4C, lower). Notably, binding assays demonstrated that MYC-mNINJ1 (1-100), MYC-mNINJ1 (1-90), MYC-mNINJ1 (81-152), MYC-mNINJ1 (91-152), and MYC-mNINJ1 (71-110) bound

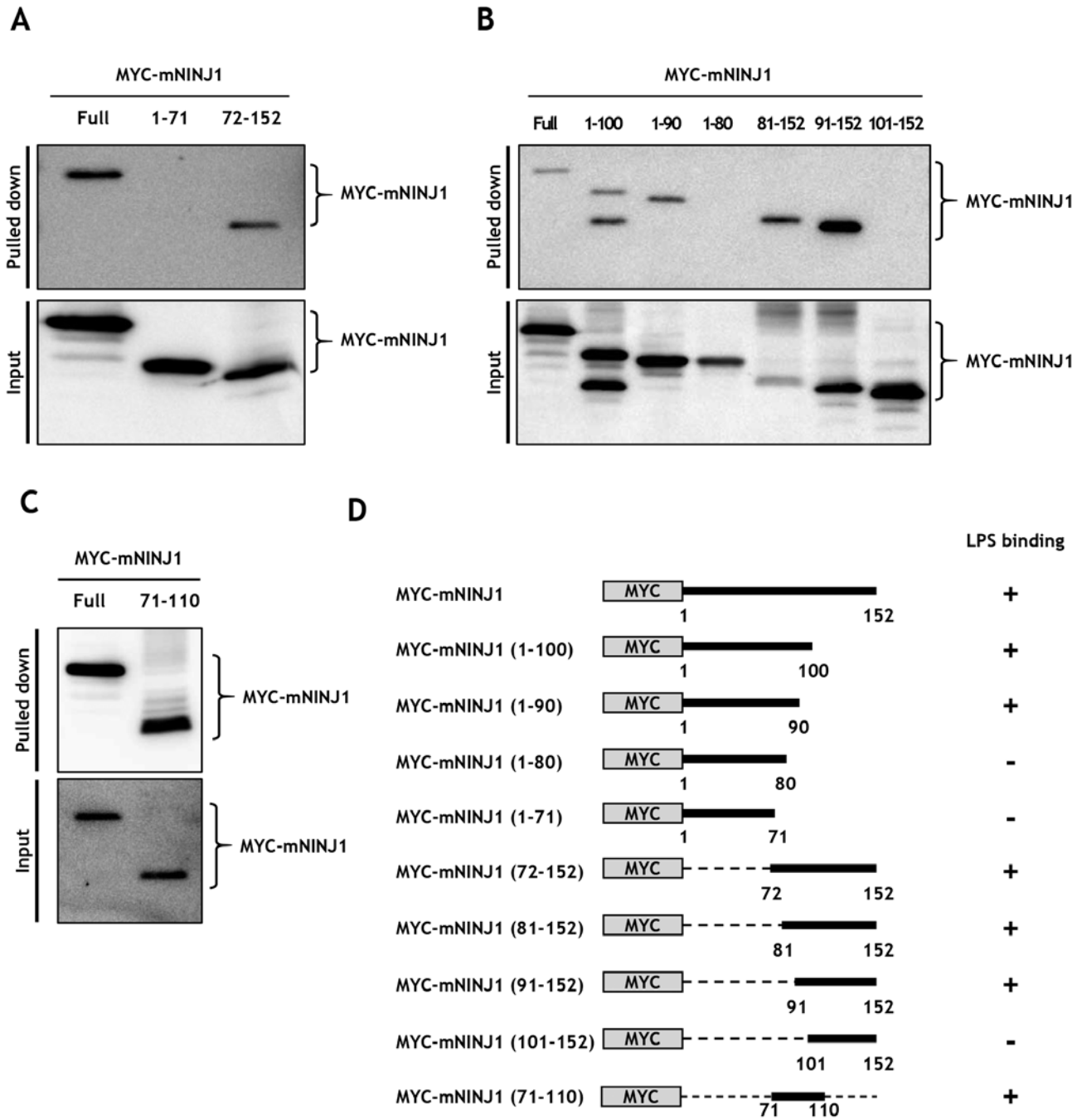

Figure 4. Determination of the LPS binding region of Ninjurin1. (A) Binding assay between LPS and MYC-mNINJ1 (1-71) or MYC-mNINJ1 (72-152). (B) Binding assay between LPS and MYC-mNINJ1 (1-100), MYC-mNINJ1 (1-90), MYC-mNINJ1 (1-80), MYC-mNINJ1 (81-152), MYC-mNINJ1 (91-152), and MYC-mNINJ1 (101-152). (C) Binding assay between LPS and MYC-mNINJ1 (71-110). Expression of transfected mNINJ1 constructs was detected by immunoblot analysis with MYC antibody (A-C, lower). Bindings between LPS and truncated Ninjurin1 were tested (A-C, upper). (D) A schematic diagram of MYC-tagged truncated mNINJ1 constructs.

LPS, whereas MYC-mNINJ1 (1-80) and MYC-mNINJ1 (101-152) did not (Fig. 4B and C, upper). The binding abilities of these recombinant Ninjurin1 mutant proteins are summarized in Fig. 4D, and indicate that the aa 72-152 region of Ninjurin1 is required for LPS binding. Probably an aa 81-100 region of Ninjurin1 is essential for LPS binding, but it requires further experiments to define the essential region.

Ninjurin1 downregulation inhibits LPS-induced NO and TNFα secretion in Raw264.7 macrophages. Next, we investigated the physiological basis of Ninjurin1 and LPS binding in the macrophage-mediated inflammatory response. Notably, LPS induced an increase of Ninjurin1 expression in Raw264.7 macrophages, consistent with a previous report (22). To elucidate the role of Ninjurin1 in the LPS-mediated inflammatory response, we silenced Ninjurin1 expression with specific or negative control siRNAs (siNinj1 and siControl, respectively) in Raw264.7 cells, and subsequently, analyzed the production of two well-known macrophage activation markers, NOS2 and TNFα. Interestingly, the induction of NOS2 protein expression

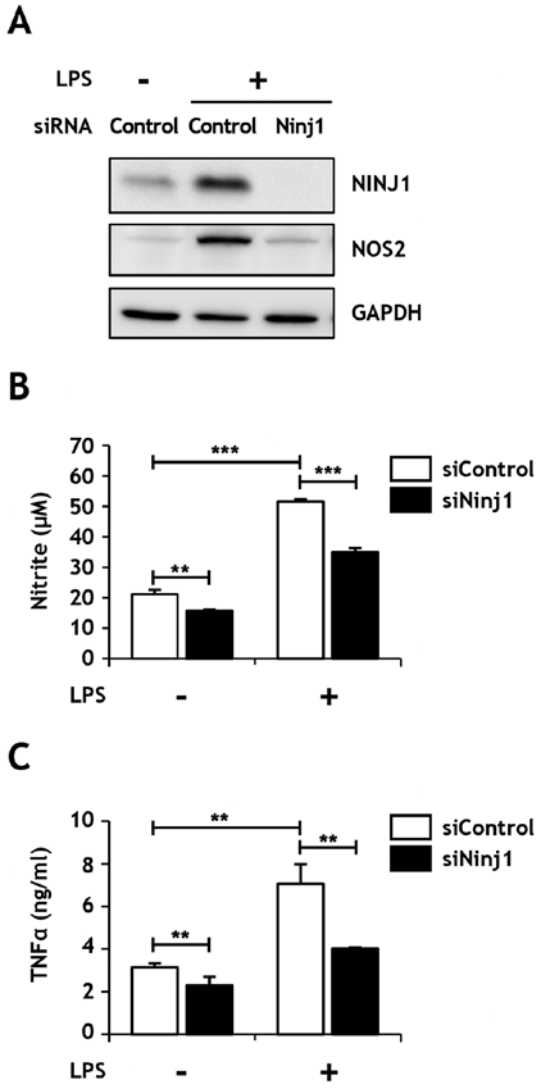

Figure 5. Effects of Ninjurin1 downregulation on LPS-induced Raw264.7 macrophage cell inflammation. The expression of Ninjurin1 was downregulated by transfecting Ninjurin1 targeted siRNA (siNinj1) or negative control siRNA (siControl) in Raw264.7 cells. After 24 h of incubation, media were changed to with or without 1 μg/ml of LPS. Cell and conditioned media at 24 h were collected after LPS stimulation. (A) Protein lysates were analyzed by immunoblot analysis with Ninj1 $Ab_{139-152}$, NOS2, and GAPDH antibodies. (B) NO release was determined using Griess reagent. Concentration of nitrite was calculated from sodium nitrite standard curve. (C) TNFα secretion was measured by ELISA assay. Conditioned media (100 μl) was applied to the assay. The data are mean ± SD. $^{**}p<0.01$, $^{***}p<0.001$.

by LPS treatment was inhibited in cells transfected siNinj1 (Fig. 5A), as was NO release (Fig. 5B). Similarly, TNFα secretion induced by LPS treatment was markedly inhibited in Ninjurin1-knockdown Raw264.7 cells (Fig. 5C). These results suggest that the LPS-induced inflammatory response was significantly inhibited by Ninjurin1-knockdown, likely due to the decreased direct interaction of Ninjurin1 and LPS.

Discussion

In this study, we identified Ninjurin1 as a novel LPS binding partner (Fig. 3). To determine the region of Ninjurin1 that conveyed its ability to bind LPS, binding assays were performed with Ninjurin1 mutant proteins. These results showed that LPS bound to Ninjurin1 aa 81-100, which belongs to first transmembrane domain (Fig. 4). Ninjurin1 has two transmembrane domains (aa 72-100 and 118-139), and both the regions are highly hydrophobic. In the binding assay using MYC-mNINJ1 (101-152), containing second transmembrane domain, LPS-biotin failed to bind to Ninjurin1 (Fig. 4B). Based on this observation, we proposed that LPS binds specifically to aa 81-100 of Ninjurin1. To address whether Ninjurin1-LPS binding affects cellular function, we repressed Ninjurin1 expression with siRNA in Raw264.7 cells. Notably, Ninjurin1 downregulation inhibited LPS-induced NOS2 enzyme induction, NO release, and TNFα secretion in Raw264.7 macrophages (Fig. 5), suggesting that the direct binding of LPS to Ninjurin1 was required for the inflammatory activation of macrophages by LPS.

The binding properties of Ninjurin1 were previously investigated mainly focussing on its homophilic binding domain (20,24). In addition, a heterophilic interaction with unknown molecules has also been suggested based on results that the basal adhesion of wild-type Jurkat cells was inhibited by treatment with peptides containing Ninjurin1 adhesion motif (25). Moreover, Ninjurin1 overexpression led to enhanced macrophage adhesion to umbilical vein endothelial cells and extracellular matrix proteins, such as fibronectin, type I collagen, vitronectin, and type IV collagen (21,26). In this study, we identified LPS as a novel heterophilic binding partner of Ninjurin1. Since the lipid moiety of LPS is crucial for its binding with Ninjurin1, it would be worthwhile to investigate the interaction between Ninjurin1 and other lipid-containing molecules.

Binding region of LPS-interacting partners would likely be a potent therapeutic target for inflammatory diseases. For example, synthetic peptides of the HMGB1 LPS-binding region, aa 3-15 or aa 80-96, inhibits the interaction between LPS to HMGB1 *in vitro*, and also decreases TNFα production in a subclinical endotoxemia mouse model (27). Thus, we also sought to determine the region of Ninjurin1 responsible for LPS binding. It is already known that Ninjurin1 contains a homophilic binding domain (aa 26-37) important for its role in immune cell aggregation and macrophage-endothelial cell adhesion. Moreover, the aggregation of Ninjurin1-expressing Jurkat cells is completely abolished by treatment with Ninjurin1 aa 26-37 peptide (25), and treatment with antibody directed towards this protein fragment blocks macrophage adhesion and transmigration to endothelial cells (20). Furthermore, LPS-induced *IL-6* and *TNFα* transcription is inhibited by treatment with this aa 26-37 peptide (22); however, according to our result, LPS specifically bound aa 81-100 of Ninjurin1, but not the N-terminus containing the homophilic binding domain. This result indicates that Ninjurin1 harbors an LPS-binding motif separated from its homophilic binding domain. Therefore, identification of the specific LPS binding region in Ninjurin1 could be valuable for the precise regulation of LPS-induced inflammation, as it would presumably not affect the protein's role in cell adhesion.

Besides macrophages, Ninjurin1 is expressed in various cell types, such as endothelial cells, pericytes, fibroblasts, and epithelial cells (26,28,29). Moreover, Ninjurin1 is implicated in several human diseases, including carcinogenesis of non-muscle-invasive urothelial bladder cancer (30) and hepa-

tocellular carcinoma (31). Likewise, LPS also affects various cell types and pathological conditions, which are not only restricted to immune cells. For example, human endothelial cells treated with LPS produce neutrophil chemotactic factor (32), whereas LPS stimulation in mouse CT26 colon cancer cells triggers NF-κB-DNA binding (33).

Collectively, the Ninjurin1-LPS interaction would likely affect various cellular functions beyond macrophage inflammation. Thus, the Ninjurin1 LPS-binding domain would be an attractive therapeutic target for inflammatory diseases.

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