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**Ninjurin-1 Negatively Regulates Humoral and Cellular Immune Responses
Induced by the Saponin-Based Adjuvant Quil-A in Mice**

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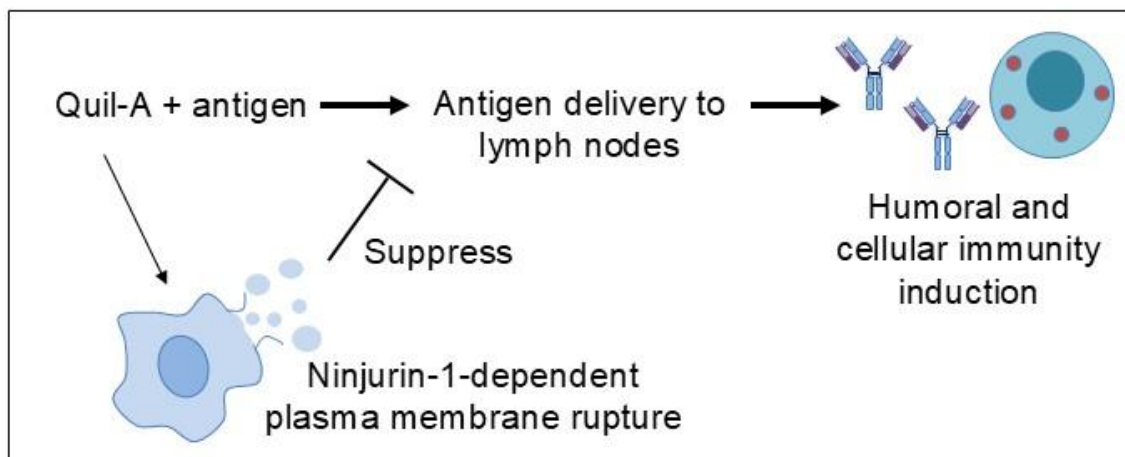
Pharmaceutical Sciences

Laboratory of Bioresponse Regulation

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23 GRAPHICAL ABSTRACT

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26 ABBREVIATIONS

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Abbreviation	Full name
APCs	Antigen-presenting cells
AS01	Adjuvant system 01
ATP	Adenosine triphosphate
BMDCs	Bone marrow-derived dendritic cells
DCs	Dendritic cells
FITC	Fluorescein isothiocyanate
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HMGB1	High-mobility group box 1
IFN- γ	Interferon-gamma
IL-1 β	Interleukin-1 beta
IL-17	Interleukin-17
IL-4	Interleukin-4
LDH	Lactate dehydrogenase
MPLA	Monophosphoryl lipid A
NINJ1	Ninjurin-1
OVA	Ovalbumin
PECs	Peritoneal exudate cells
PMR	Plasma membrane rupture
TLR4	Toll-like receptor 4

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ABSTRACT

Adjuvants are co-administered with antigens to improve vaccine protection. Saponins are plant-derived compounds with adjuvant properties, some of which are used in licensed vaccines. Macrophages and dendritic cells (DCs) exposed to saponin-based adjuvants have been reported to exhibit NLRP3-dependent interleukin-1 beta (IL-1 β) release, and NLRP3 signaling has been shown to limit their adjuvant activity. Saponin-based adjuvants also induce plasma membrane rupture (PMR) and the release of high-molecular-weight intracellular molecules; however, the molecular mechanisms that mediate PMR and its impact on adjuvant-induced immune responses remain unclear. We investigated the involvement of NINJ1, a key executor of PMR in responses to stimuli such as lipopolysaccharide, in saponin-based adjuvant-induced PMR and examined its role in regulating their adjuvant effects. Upon stimulation with the saponin mixture Quil-A, peritoneal macrophages and bone marrow-derived DCs (BMDCs) showed NLRP3-independent PMR but NLRP3-dependent IL-1 β release. Quil-A-induced PMR was almost completely suppressed in *Ninjl*^{-/-} peritoneal macrophages and BMDCs compared with wild-type cells, whereas IL-1 β release remained unaffected by NINJ1 deficiency. Immunization with Quil-A and ovalbumin (OVA) increased OVA-specific IgG, IgG2b, and IgG2c levels in *Ninjl*^{-/-} mice compared with wild-type mice. Splenocytes from *Ninjl*^{-/-} mice produced higher levels of interferon-gamma upon stimulation with class I- and class II-restricted OVA peptides than those from wild-type mice. *Ninjl*^{-/-} mice showed a higher frequency of OVA-bearing cells, particularly monocyte-derived DCs, in the draining lymph nodes than wild-type mice. Our results suggest that NINJ1 is critical for saponin-based adjuvant-induced PMR and negatively regulates adjuvant-induced immune activation by

53 restricting antigen delivery via antigen-presenting cells. These findings advance
54 understanding of saponin-based adjuvants' mechanisms and may inform improved
55 vaccine formulations.

56 **Keywords:** Saponin, Adjuvant, Quil-A, Ninjurin-1

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INTRODUCTION

Vaccination remains the most effective strategy for reducing the morbidity and mortality associated with infectious diseases. Many current vaccines use purified protein, polysaccharide, or recombinant antigens instead of whole pathogens. Although these vaccines are generally safe, they have weak intrinsic immunogenicity and often require additional immune stimulation to provide effective protection. This is typically accomplished by including adjuvants, which are substances that enhance immune responses to antigens.¹⁾ However, the mechanisms of many clinically approved adjuvants remain incompletely understood, limiting their rational design and optimized use in vaccination. A better understanding of their mechanisms could guide evidence-based vaccine development strategies.

Activation of antigen-presenting cells (APCs), such as dendritic cells (DCs) and macrophages, is particularly critical for promoting adaptive immunity. Adjuvants achieve this through multiple modes of action. Some mimic pathogen-associated molecular patterns and directly engage pattern recognition receptors, thereby activating APCs.²⁾ Other adjuvants function through a distinct mechanism that involves the induction of cellular perturbations, leading to the release of intracellular molecules that modulate the activity of surrounding immune cells, including APCs.³⁾ Several clinically approved adjuvants belong to this latter category. For example, aluminum-based adjuvants induce the release of host deoxyribonucleic acid at the injection site, which enhances the recruitment of APCs.⁴⁾ The release of intracellular molecules induced by such adjuvants is an active process mediated by molecular mechanisms.^{5,6)} A detailed understanding of the molecules responsible for releasing intracellular molecules, as well as the effector molecules released during this process that modulate immune responses,

could enable the rational design and control of adjuvants that act through pathways that promote the release of intracellular mediators.

Saponins are plant-derived compounds, saponin-based adjuvant systems have been incorporated into several licensed vaccines, such as adjuvant system 01 used in Shingrix and Mosquirix, as well as the Matrix-M adjuvant used in NVX-CoV2373.^{7,8)}

Saponin-based adjuvants are capable of eliciting both strong cellular and humoral immunity,^{8,9)} a feature that cannot be achieved by conventional aluminum-based adjuvants, which primarily promote humoral immunity. Despite their substantial clinical potential, a more detailed understanding of the mechanisms underlying saponin-based adjuvants is required to enable their broader and more rational clinical application. Some saponin-based adjuvants have been shown to activate the NLRP3 inflammasome and trigger plasma membrane rupture (PMR).^{10,11)} Activation of the NLRP3 inflammasome is considered to induce pore formation in the plasma membrane, leading to the release of low-molecular-weight intracellular molecules such as interleukin-1 beta (IL-1 β).¹²⁾ Notably, *Nlrp3*^{-/-} mice immunized with QS-21, a saponin-based adjuvant used in multiple vaccines, showed significantly higher serum levels of antigen-specific IgG and increased production of interferon-gamma (IFN- γ) by T cells compared with wild-type controls, suggesting that molecules released from cells in an NLRP3-dependent manner may restrict the adjuvant effects of QS-21.¹⁰⁾ However, NLRP3-deficient cells still exhibited a loss of Calcein fluorescence upon stimulation with QS-21, suggesting that PMR occurs via an NLRP3-independent pathway.¹⁰⁾ PMR leads to the release of high-molecular-weight intracellular molecules that mediate inflammation.¹³⁾ Elucidating the molecular mechanisms of PMR induced by saponin-based adjuvants would help to better understand and modulate their adjuvant effects.

Recently, Ninjurin-1 (NINJ1) has been identified as a key executor of PMR in response to stimuli such as lipopolysaccharide.¹⁴⁻¹⁶⁾ NINJ1 is a small double-pass transmembrane protein broadly expressed across multiple tissues and hematopoietic lineages, with relatively high transcript levels in innate immune cells such as macrophages.¹⁷⁾ It is primarily localized to the plasma membrane, where it can oligomerize upon membrane-disruptive stimuli to form filamentous assemblies that disrupt the lipid bilayer and induce PMR.¹⁸⁾ Given that saponin-based adjuvants also induce PMR, it is plausible that NINJ1 mediates PMR induced by these adjuvants as well. To date, the role of NINJ1 in modulating adjuvant-induced immune responses has not been investigated. In this study, we aimed to determine whether NINJ1 mediates PMR induced by the saponin-based adjuvant Quil-A and to evaluate its impact on Quil-A-induced humoral and cellular immune responses.

MATERIALS AND METHODS

Reagents

Quil-A adjuvant, monophosphoryl lipid A (MPLA) and OVA_{323–339} peptide were purchased from InvivoGen (San Diego, CA, USA). OVA_{257–264} peptide was purchased from Selleck Chemicals (Houston, TX, USA). Ovalbumin, Low Endotoxin was purchased from Fujifilm Wako Pure Chemical Corporation (Osaka, Japan). Ovalbumin, Fluorescein Conjugate and recombinant mouse granulocyte-macrophage colony-stimulating factor (GM-CSF) were purchased from Thermo Fisher Scientific (Waltham, MA, USA). The Cytotoxicity LDH Assay Kit-WST was purchased from Dojindo Laboratories (Kumamoto, Japan). CellTiter-Glo 2.0 Assay was purchased from Promega (Madison, WI, USA). PE-conjugated anti-mouse F4/80 antibody (BM8), PE-Cy7-conjugated anti-mouse CD11b antibody (M1/70), APC-Cy7-conjugated anti-mouse CD11c antibody (N418), ELISA kits for mouse IL-1 β and IFN- γ were purchased from BioLegend (San Diego, CA, USA). Block Ace was purchased from KAC (Kyoto, Japan). Goat Anti-Mouse IgG, Human ads-HRP was purchased from SouthernBiotech (Birmingham, AL, USA). Goat Anti-Mouse IgG1 (HRP), Goat Anti-Mouse IgG2b heavy chain (HRP), and Goat Anti-Mouse IgG2c heavy chain (HRP) were purchased from Abcam (Cambridge, UK). Red Blood Cell Lysing Buffer Hybri-Max was purchased from Sigma-Aldrich (St. Louis, MO, USA). ELISA POD Substrate TMB Kit, RPMI 1640 medium, penicillin-streptomycin mixed solution, MEM non-essential amino acids solution, Dulbecco's Phosphate Buffered Saline, and 2-mercaptoethanol were purchased from Nacalai Tesque (Kyoto, Japan).

Mice

Wild-type C57BL/6N and C57BL/6J mice were purchased from CLEA Japan

(Shizuoka, Japan) and Japan SLC, Inc. (Shizuoka, Japan). *Nlrp3*^{-/-} mice (JAX stock #021302) were obtained from Jackson Laboratory (Bar Harbor, ME, USA).¹⁹⁾ To generate *Ninjl*^{-/-} mice, C57BL/6N-*Ninjl*^{tm1a(KOMP)Wtsi}/MbpMmucd allele sperm were imported from KOMP repository (MMRRC:048810-UCD, <http://www.komp.org>) and *in vitro* fertilized with eggs from wild-type C57BL/6N mice. Homozygous mice were generated by breeding the heterozygote mice. The mice were housed in standard cages in a temperature-controlled room under a 12-h light/dark cycle at the Animal Care Facility of the Graduate School of Pharmaceutical Sciences, The University of Osaka, during the experimental period. The mice were provided with standard laboratory mouse chow and drinking water ad libitum. All animal experiments were approved by the Animal Care and Use Committee of the Graduate School of Pharmaceutical Sciences at The University of Osaka.

Cell Preparation and Stimulation

Peritoneal exudate cells (PECs) were isolated from 12-week-old male mice 3 days after intraperitoneal injection with 2 mL of 4% thioglycolate and used as macrophages. To prepare DCs, mouse bone marrow cells were cultured in RPMI 1640 supplemented with 8% fetal calf serum, 10 ng/mL GM-CSF, and 50 μ M 2-mercaptoethanol. The culture medium was replaced every two days. On day 9, cells were harvested and used as bone marrow-derived DCs (BMDCs). PECs and BMDCs were seeded at a density of 1×10^5 cells per well in flat-bottom 96-well cell culture plates and cultured in RPMI 1640 supplemented with 8% fetal calf serum overnight. On the following day, cells were primed with MPLA (500 ng/mL) or left unprimed for 6 h, followed by stimulation with Quil-A (30 μ g/mL) for 2 h. Culture supernatants were collected after centrifugation at $440 \times g$ for 5 min at 4°C. Supernatants and cell samples

were analyzed as described below.

Measurement of Cytokine Levels in Culture Supernatants

The levels of IL-1 β and IFN- γ in the culture supernatants were measured using ELISA, in accordance with the manufacturer's instructions.

Measurement of LDH Release

Lactate dehydrogenase (LDH) release from mouse PECs and BMDCs was measured as a marker of PMR using the Cytotoxicity LDH Assay Kit-WST according to the manufacturer's instructions.

Measurement of Cellular Activity

The activity of mouse PECs and BMDCs was measured using CellTiter-Glo 2.0 Cell Viability Assay according to the manufacturer's instructions.

Immunization

8-week-old female mice were immunized subcutaneously at the base of the tail with 10 μ g Quil-A plus 20 μ g OVA on days 0, 28, and 42. Blood samples were collected from the retro-orbital vein before the first immunization and on day 28 and analyzed to measure the OVA-specific antibody levels in the serum as described below. Ten days after the final immunization, spleens were collected and subjected to T cell stimulation assay as described below.

Measurement of OVA-Specific Antibody Levels in the Serum

Clear Flat-Bottom Immuno Nonsterile 96-Well Plates (Thermo Fisher Scientific) were coated overnight with 10 μ g/mL of OVA in 100 mM bicarbonate buffer, pH 9.4. The plates were blocked with 2 $\times$ Block Ace buffer for 1 h at room temperature. The plates were incubated with mouse serum (diluted 1:256 in 1 $\times$ Block Ace buffer) for 2.5 h. Each HRP-conjugated secondary antibody (goat anti-mouse IgG,

IgG1, IgG2b heavy chain, and IgG2c heavy chain) was diluted 1:2000 in 1× Block Ace and added to the plates, which were then incubated at room temperature for 2 h. The wells were washed four times with phosphate-buffered saline plus 0.05% (v/v) Triton X-100 between each step. After adding TMB, the plates were developed at room temperature in the dark. Finally, 1 M HCl was added, and the absorbance of the solution was measured at 450 nm.

T Cell Stimulation Assay

Spleens were homogenized to obtain single-cell suspensions. Splenocytes were filtered through a 40-μm cell strainer, and red blood cells were lysed using Red Blood Cell Lysing Buffer Hybri-Max. Cells were seeded into flat-bottom 96-well cell culture plates at a density of 1×10^6 cells per well in 200 μL of RPMI 1640 supplemented with 8% fetal bovine serum, 1% penicillin-streptomycin, 1% non-essential amino acids, and 50 μM 2-mercaptoethanol. Antigen-specific stimulation was performed by adding OVA₃₂₃₋₃₃₉ and OVA₂₅₇₋₂₆₄ peptides (10 μg/mL each), while control wells contained medium alone. Cells were cultured at 37°C for 3 days, and the supernatants were analyzed by ELISA.

Flow Cytometric Analysis

Cells collected from the inguinal lymph nodes of 7–9-week-old male mice injected subcutaneously at the base of the tail with 10 μg Quil-A plus 100 μg fluorescein isothiocyanate (FITC)-labeled OVA were stained with antibodies against mouse F4/80, CD11b, and CD11c in accordance with the manufacturer's instructions. FITC-positive cells were identified as OVA-bearing cells. F4/80⁺ CD11c⁺ CD11b⁺ cells, F4/80⁻ CD11c⁺ cells, and F4/80⁺ CD11c⁻ CD11b⁺ cells were identified as monocyte-derived DCs (MoDCs), DCs, and macrophages, respectively. Data were acquired using

a flow cytometer (CytoFLEX; Beckman Coulter, Brea, CA, USA) and analyzed using FlowJo software (Tree Star, Ashland, OR, USA).

Statistical Analysis

Statistical analysis was performed with GraphPad Prism 8.0 (Boston, MA, USA). Unpaired two-tailed Student's T-tests were used for comparisons between two groups. A one-way analysis of variance, followed by Tukey-Kramer *post-hoc* test, was performed to compare multiple groups. Statistical significance was set at $p < 0.05$.

RESULTS

Quil-A-Induced PMR in Mouse Macrophages and DCs Depends on NINJ1

Quil-A, a crude saponin extract from *Quillaja saponaria*, is widely used as a model saponin-based adjuvant in experimental studies.²⁰⁾ It contains QS-21, a more highly purified fraction.¹⁰⁾ We first examined whether NINJ1 mediates PMR in PECs and BMDCs induced by Quil-A. To adequately induce NLRP3 inflammasome activation and IL-1 β release, cells were primed with the Toll-like receptor 4 (TLR4) agonist MPLA. MPLA is an attenuated derivative of lipopolysaccharide and is used as an immunostimulatory component in clinically approved saponin-based adjuvant formulations.⁷⁾ In wild-type PECs and BMDCs, Quil-A stimulation induced LDH release both with and without MPLA priming, indicating that PMR occurs independently of TLR4 activation (Fig. 1A and B). IL-1 β was markedly released only in MPLA-primed cells after Quil-A stimulation. Quil-A induced minimal IL-1 β release from *Nlrp3*^{-/-} cells, whereas LDH release was comparable to that in wild-type cells. Importantly, *Ninj1*^{-/-} cells exhibited a significant reduction in LDH release upon Quil-A stimulation, whereas IL-1 β release remained unaffected (Fig. 1C and D). These results suggest that Quil-A-induced PMR is dependent on NINJ1 but not on NLRP3, while NLRP3-dependent IL-1 β release occurs independently of NINJ1.

NINJ1 Negatively Regulates Quil-A-Induced Antigen-Specific Humoral and Cellular Immune Responses

To assess whether NINJ1 influences humoral immunity induced by saponin-based adjuvants, wild-type and *Ninj1*^{-/-} mice were immunized subcutaneously with Quil-A-adjuvanted OVA. Total IgG levels represent the overall strength of the antigen-specific antibody response, whereas IgG subclasses serve as important indicators of the

qualitative features of adjuvant-induced antibody responses. IgG1 is typically associated with T helper 2 responses driven by IL-4, whereas IgG2b and IgG2c reflect T helper 1 responses dominated by IFN- γ . Saponin-based adjuvants have been reported to induce multiple IgG subclasses, including IgG1, IgG2b, and IgG2c.¹⁰⁾ Immunization with Quil-A induced significantly higher serum levels of OVA-specific total IgG, IgG2b, and IgG2c in *Ninjl*^{-/-} mice compared with wild-type mice, whereas IgG1 levels were not significantly changed (Fig. 2). Because saponin-based adjuvants also elicit potent cellular immunity, we further examined whether NINJ1 modulates Quil-A-induced OVA-specific T cell responses. Splenocytes from *Ninjl*^{-/-} mice produced significantly higher levels of IFN- γ upon stimulation with OVA₃₂₃₋₃₃₉ and OVA₂₅₇₋₂₆₄ than those from wild-type mice (Fig. 3A and B), suggesting that both the Th1 and cytotoxic T lymphocyte responses were enhanced in the absence of NINJ1. IL-4 and IL-17 were also measured but were undetectable under the experimental conditions used (data not shown). Collectively, these results indicate that NINJ1 restrains Quil-A-induced antigen-specific humoral and cellular immune responses.

NINJ1 Restricts Antigen Delivery by MoDCs to Draining Lymph Nodes After Quil-A-Adjuvanted Immunization

Quil-A-induced PMR depends on NINJ1, and PMR is accompanied by the release of intracellular molecules, which may modulate the activity of surrounding APCs. We then investigated whether antigen delivery to draining lymph nodes by APCs is affected by NINJ1 deficiency in mice following Quil-A-adjuvanted immunization. Mice were immunized with FITC-labeled OVA and Quil-A, and OVA-bearing (FITC⁺) cells were analyzed among total cells as well as within MoDCs, conventional DCs, and macrophages (Fig. 4A). Compared with wild-type mice, *Ninjl*^{-/-} mice exhibited a higher

frequency of FITC⁺ cells in the draining lymph nodes (Fig. 4B). Within each cell type, the frequency of FITC⁺ cells was significantly higher in MoDCs from *Ninjl*^{-/-} mice, whereas frequencies in conventional DCs and macrophages did not differ significantly (Fig. 4C to E). The frequency of whole MoDCs, DCs, and macrophages among total cells was comparable between *Ninjl*^{-/-} and wild-type mice (data not shown). Thus, NINJ1 negatively regulates APC-mediated antigen delivery to the draining lymph nodes after Quil-A-adjuvanted immunization, particularly in MoDCs.

To investigate the mechanism underlying the increased antigen delivery in *Ninjl*^{-/-} mice, we assessed intracellular adenosine triphosphate (ATP) levels in PECs and BMDCs after Quil-A stimulation. ATP levels may influence numerous cellular functions, including migration, antigen processing, and other metabolic processes, all of which could potentially contribute to the frequency of antigen-bearing APCs in draining lymph nodes. Quil-A reduced intracellular ATP levels in both wild-type and *Ninjl*^{-/-} cells to a comparable extent (Fig. 5A and B). Therefore, the increased frequency of antigen bearing APCs observed in *Ninjl*^{-/-} mice is unlikely to result from differences in ATP availability.

DISCUSSION

This study demonstrated that Quil-A induces NINJ1-dependent PMR, and that both humoral and cellular immune responses are significantly enhanced in *Ninjl*^{-/-} mice compared with wild-type controls following Quil-A-adjuvanted immunization. These findings suggest that NINJ1-mediated PMR negatively regulates the immune-potentiating effects of saponin-based adjuvants. Since saponin-based adjuvants other than Quil-A are also known to induce LDH release,^{10,11,21)} it is likely that saponin-based adjuvants generally trigger PMR. Therefore, NINJ1 may negatively regulate the activity of other saponin-based adjuvants as well, through its involvement in PMR induction. Interestingly, a previous study reported that NLRP3-dependent IL-1 β release induced by QS-21 also suppressed the ability of QS-21 to induce antigen-specific humoral and cellular immune responses.¹⁰⁾ Our findings suggest that saponin-based adjuvants activate two independent negative regulatory pathways: NINJ1-dependent PMR that releases high molecular-weight inhibitory factors, and NLRP3-dependent pathway that releases low molecular-weight ones. Although NINJ1 and NLRP3 are primarily recognized as pro inflammatory mediators, our findings reveal a paradoxical regulatory role in the context of saponin-based adjuvants, where both molecules function as negative regulators that limit vaccine-induced immunity. This represents a previously unrecognized regulatory mechanism that may have broader implications for vaccine design.

Importantly, our results imply that inhibition of NINJ1 activity could be an effective strategy to enhance the efficacy of vaccines formulated with saponin-based adjuvants. The activity of NINJ1 can be suppressed by neutralizing antibodies or by pharmacological agents such as glycine.^{22,23)} Furthermore, specific inhibitors of NLRP3

have already been developed for clinical use.²⁴⁾ Therefore, appropriately suppressing the activities of both NINJ1 and NLRP3 during administration of vaccines formulated with saponin-based adjuvants would effectively enhance the induction of antigen-specific humoral and cellular immune responses.

The enhanced adjuvant effect of Quil-A observed in *Ninj1*^{-/-} mice is likely attributable to the increased accumulation of antigen-bearing APCs, particularly MoDCs, in draining lymph nodes. MoDCs are known to be highly efficient at priming naive T cells and can bridge innate and adaptive immunity. The increased frequency of antigen-bearing MoDCs in draining lymph nodes likely promoted more efficient T cell priming, leading to enhanced antigen-specific cellular immune responses in the spleen and increased antibody production by plasma cells in secondary lymphoid organs. The comparable intracellular ATP levels after Quil-A stimulation in wild-type and *Ninj1*^{-/-} cells indicate that Quil-A-induced metabolic perturbation occurs independently of NINJ1-mediated PMR. This finding is consistent with a previous report showing that ATP release through small membrane pores formed by Gasdermin D does not require NINJ1, and suggests that the differential immune responses in *Ninj1*^{-/-} mice are not due to the bioavailability of intracellular ATP.²⁵⁾ Hence, we hypothesize that Quil-A-induced PMR in wild-type cells promotes the release of intracellular molecules that exert paracrine inhibitory effects on the trafficking or accumulation of antigen-bearing APCs in draining lymph nodes. In *Ninj1*^{-/-} cells, where PMR is substantially reduced, this negative regulatory mechanism is attenuated, resulting in increased antigen delivery in draining lymph nodes.

Quil-A induces the release of both low-molecular-weight molecules, such as mature IL-1 β (~17 kDa), and high-molecular-weight molecules, such as LDH (~140

kDa), by DCs and macrophages. The former are released in an NLRP3-dependent but NINJ1-independent manner, likely exit through small pores approximately 10–20 nm in diameter formed by Gasdermin D in the plasma membrane following NLRP3 activation.^{14,26)} In contrast, the latter are released in a NINJ1-dependent but NLRP3 independent manner.^{14,27)} High-mobility group box 1 (HMGB1) has been reported to be released extracellularly in a NINJ1-dependent manner during PMR.¹⁴⁾ HMGB1 is a nuclear protein ubiquitously expressed across tissues and released into the extracellular space during various forms of sterile inflammatory responses or tissue damage.^{28,29)} HMGB1 mediates inhibitory signaling via the CD24-siglec-10 axis in DCs.³⁰⁾ However, extracellular HMGB1 has also been reported to act as a pro-inflammatory mediator in some contexts.²⁸⁾ In mice immunized with QS-21, HMGB1 was reported to promote rather than suppress the adjuvant activity of QS-21.³¹⁾ Therefore, comprehensive proteomic analysis is needed to identify the full spectrum of high-molecular-weight molecules released through NINJ1-dependent PMR following saponin stimulation. Such studies should focus particularly on molecules with known immunosuppressive properties that could act on APCs to limit their migration, antigen presentation capacity, or T cell priming ability. Identifying these key inhibitory factors could reveal novel therapeutic targets for enhancing saponin-based vaccine efficacy.

Regarding clinical translation, several considerations should be noted. because the present study was conducted in mice, its direct relevance to human vaccination remains to be established. NINJ1 is broadly expressed across multiple hematopoietic lineages in humans and functions as a conserved executor of PMR,^{17,32)} therefore, it is plausible that NINJ1-dependent PMR is also engaged in human APCs exposed to saponin-based adjuvants and contributes to their immunomodulatory effects.

In addition, OVA was employed as a model antigen to facilitate tracking of antigen uptake and antigen-specific T cell responses; however, OVA has strong intrinsic immunogenicity and may differ from clinically used recombinant protein antigens formulated with saponin-based adjuvants in terms of immunogenicity,^{33–35} which could attenuate the magnitude of immune responses observed at the same Quil-A dose and thereby influence the extrapolation of the present findings. Accordingly, further validation of our findings under clinically relevant formulation conditions, including the use of human APCs, will be required. Finally, because the NINJ1-dependent PMR identified here is directly linked to local cell lysis and the release of intracellular immunoregulatory molecules at the vaccination site, future studies should also evaluate how modulation of the NINJ1-dependent PMR affects both immunogenicity and safety-related parameters, thereby enabling optimization of vaccine efficacy and tolerability.

In conclusion, this study reveals a previously unrecognized negative regulatory role of NINJ1 in immune responses, wherein NINJ1-mediated PMR limits immune activation by suppressing antigen delivery to draining lymph nodes.³⁾ These findings have several important implications. First, they identify NINJ1 as a potential therapeutic target for enhancing saponin-based vaccine efficacy. Second, because many clinically approved adjuvants, including aluminum-based formulations and emulsion-based adjuvants, also induce the release of intracellular molecules, NINJ1-dependent negative regulation may represent a general mechanism limiting adjuvant activity. Finally, understanding how NINJ1-mediated PMR suppresses APC function could enable the rational design of next-generation adjuvants that maximize immune activation while avoiding unwanted negative regulatory pathways. Future studies should focus on identifying the specific inhibitory molecules released through PMR and

384 developing strategies to selectively block their activity during vaccination.

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FIGURE CAPTIONS

Fig. 1. Antigen-Presenting Cells Stimulated with Quil-A Exhibit NINJ1-Dependent LDH Release and NLRP3-Dependent IL-1 β Release

(A) Peritoneal exudate cells (PECs) and (B) bone marrow-derived dendritic cells (BMDCs) were isolated from wild-type and *Nlrp3*^{-/-} mice. Cells were primed with 500 ng/mL MPLA or left unprimed for 6 h, followed by stimulation with 30 μ g/mL Quil-A for 2 h. Supernatants were collected and analyzed for IL-1 β and lactate dehydrogenase (LDH) release. (C) PECs and (D) BMDCs from wild-type and *Ninj1*^{-/-} mice were primed with MPLA or left unprimed, followed by stimulation with Quil-A. Supernatants were collected and analyzed for LDH and IL-1 β release. Data are shown as means $\pm$ standard deviation (SD) of values from triplicate wells. ****, $p < 0.0001$; ns, not significant. The experiment was independently repeated twice with similar results.

Fig. 2. Quil-A-Immunized *Ninj1*^{-/-} Mice Exhibit Enhanced Antigen-Specific IgG Production

Wild-type and *Ninj1*^{-/-} mice were immunized subcutaneously with 10 μ g Quil-A plus 20 μ g ovalbumin (OVA). Blood samples were collected before the first immunization and on day 28. OVA-specific total IgG, IgG1, IgG2b, and IgG2c antibodies were measured by ELISA. Data are presented as means $\pm$ SD (n = 4). *, $p < 0.05$; ****, $p < 0.0001$; ns, not significant. The experiment was independently repeated twice with similar results.

Fig. 3. Quil-A-Immunized *Nin1*^{-/-} Mice Exhibit Enhanced Antigen-Specific IFN- γ

Production from Splenocytes

Wild-type and *Nin1*^{-/-} mice were immunized subcutaneously on days 0, 28, and 42 with 10 μ g Quil-A plus 20 μ g ovalbumin (OVA), and splenocytes were isolated on day 52. Splenocytes were stimulated with (A) OVA₃₂₃₋₃₃₉ or (B) OVA₂₅₇₋₂₆₄ peptides (10 μ g/mL each) for 3 days. Supernatants were collected and analyzed for IFN- γ production. Data are presented as means $\pm$ SD (n = 4). ****, $p < 0.0001$. The experiment was independently repeated twice with similar results.

Fig. 4. Quil-A-Immunized *Nin1*^{-/-} Mice Exhibit Increased Frequencies of Antigen-

Bearing Cells in Draining Lymph Nodes

Wild-type and *Nin1*^{-/-} mice were immunized subcutaneously with 100 μ g of FITC-conjugated OVA plus 10 μ g of Quil-A. Leukocytes from the inguinal lymph nodes were harvested 18 h post-immunization. Single-cell suspensions were stained with mouse anti-F4/80, anti-CD11c, and anti-CD11b antibodies and analyzed by flow cytometry. (A) Gating strategy used to define monocyte-derived dendritic cells (MoDCs), dendritic cells (DCs), and macrophages. Leukocytes were gated based on forward scatter (FSC-A) versus side scatter (SSC-A) and then selected for singlets using FSC-A versus FSC-H. Within singlets, MoDCs (F4/80⁺ CD11c⁺ CD11b⁺), DCs (F4/80⁻ CD11c⁺), and macrophages (F4/80⁺ CD11c⁻ CD11b⁺) were identified. The frequency of OVA-bearing (FITC⁺) (B) leukocytes, (C) MoDCs, (D) DCs, and (E) macrophages among total leukocytes was analyzed. Data are presented as means $\pm$ SD (n = 7). *, $p <$

0.05; ns, not significant. The experiment was independently repeated twice with similar results.

Fig. 5. ATP Decrease in Quil-A-Stimulated Antigen-Presenting Cells Is Unaffected by NINJ1 Deficiency

(A) Peritoneal exudate cells and (B) bone marrow-derived dendritic cells from wild-type and *Ninj1*^{-/-} mice were stimulated with 30 µg/mL Quil-A for 6 h. Cells were collected and analyzed for intracellular ATP levels. Data are shown as means ± SD of values from triplicate wells. ns, not significant. The experiment was independently repeated twice with similar results.

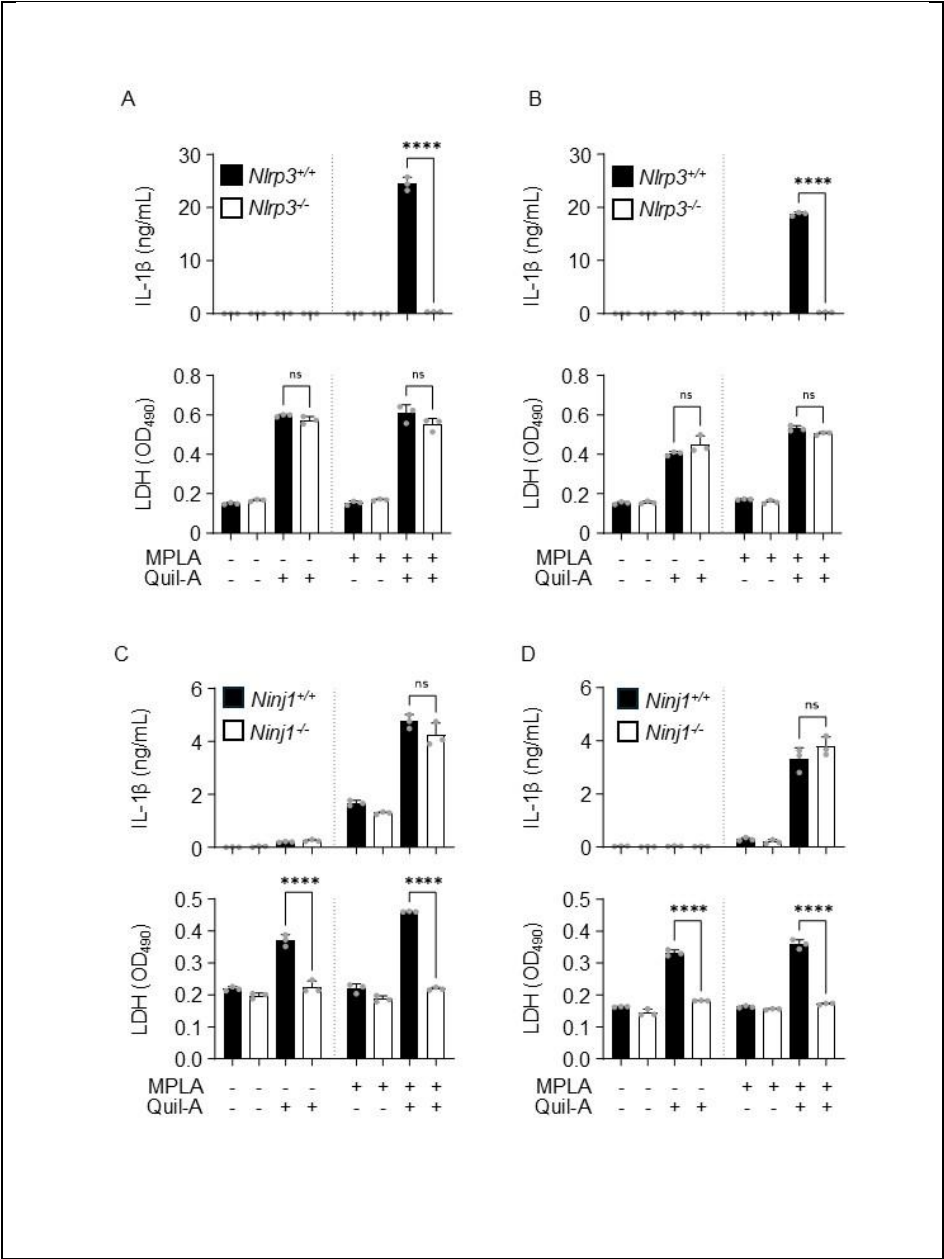


Fig. 1. Antigen-Presenting Cells Stimulated with Quil-A Exhibit NINJ1-Dependent LDH Release and NLRP3-Dependent IL-1β Release

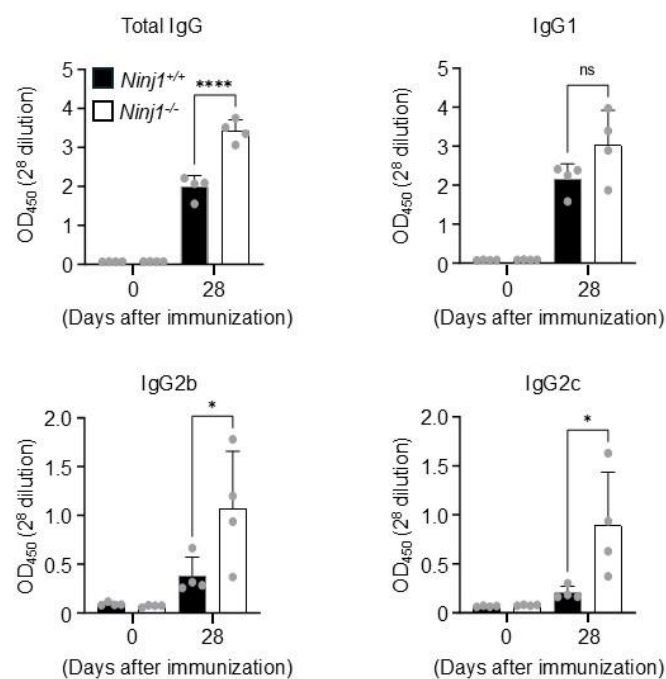


Fig. 2. Quil-A-Immunized *Ninj1*^{-/-} Mice Exhibit Enhanced Antigen-Specific IgG Production

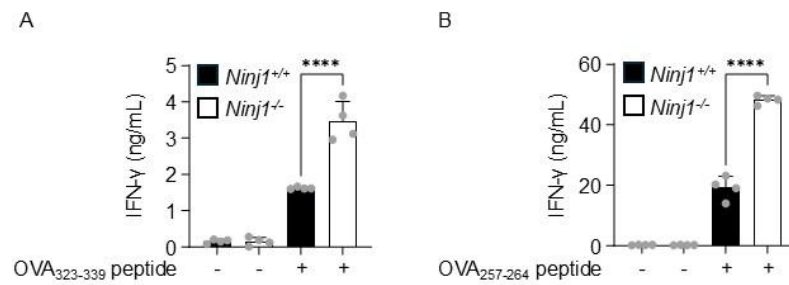


Fig. 3. Quil-A-Immunized *Ninj1*^{-/-} Mice Exhibit Enhanced Antigen-Specific IFN- γ Production from Splenocytes

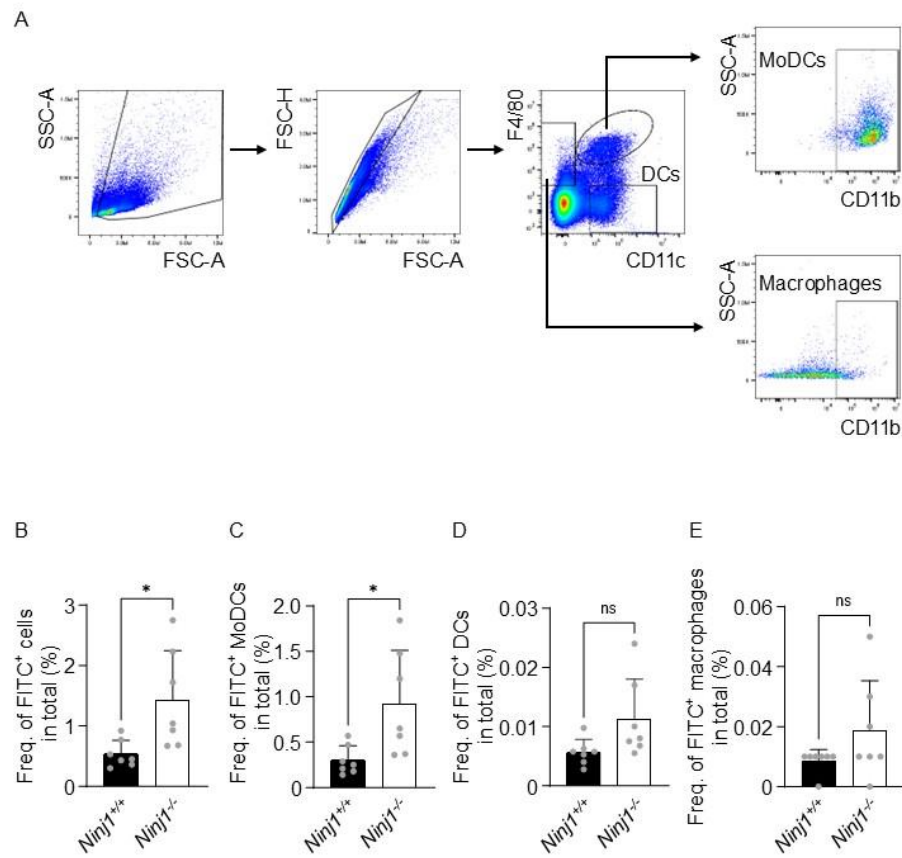


Fig. 4. Quil-A-Immunized *Ninj1*^{-/-} Mice Exhibit Increased Frequencies of Antigen-Bearing Cells in Draining Lymph Nodes

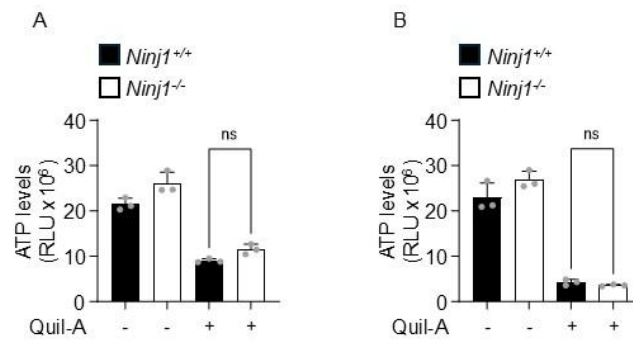


Fig. 5. ATP Decrease in Quil-A-Stimulated Antigen-Presenting Cells Is Unaffected by NINJ1 Deficiency