

Identification of a porcine TLR2-targeting peptide ligands using a cell-based phage display combined with high-throughput sequencing

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Research Article

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Abstract

M cell targeting is one of the critical issues to develop efficient mucosal vaccine design. In this study, peptide ligands with high affinity to porcine TLR2, which is highly expressed in M cells and play an important role in mucosal immune responses in pigs, were identified through the cell-based phage display technique combined with high-throughput sequencing. A random phage-peptide library was applied to the porcine TLR2 overexpressing cell line and total 85,557 unique peptide sequences were identified from approximately 9.0×10^7 reads after three rounds of both subtractive and non-subtractive biopanning via high-throughput sequencing. Among the unique sequences, three candidate peptide sequences, NAGHLSQ, VPSKPGL, and RANLDGQ, were selected based on their abundance in the third round of biopanning. Consequently, NAGHLSQ showed the highest affinity exclusively to porcine TLR2 compared with other candidates and its binding mechanism was inferred to be directly associated with ligand binding site of the TLR2 through the *in vitro* competitive analysis. The peptide identified in this research could be used in development of effective porcine mucosal vaccine as an M cell targeting moiety to enhance the transport of antigens into the Peyer's patch via oral route.

Introduction

The outbreaks of many catastrophic infectious diseases, such as PED, PRRS, and FMD, have been threatening the swine industry and have caused a huge economic burden for a long time¹. The causative agents of these diseases mostly access the host through mucosal surfaces, which necessitates the development of a mucosal vaccine that confers a pathogen-specific protective mechanism against the pathogen invading mucosal surfaces. Orally administered vaccines could be encountered immune cells in the Peyer's patches of the small intestine to evoke acquired immune responses after sampling by M cells located in the follicle-associated epithelium (FAE) on the Peyer's patch². Activated immune cells then secrete antigen-specific sIgA into the intestinal lumen to protect pathogens. These oral vaccines, however, show limited effects due to the harsh environment of the gastrointestinal tract. Most of the vaccine molecules are flushed out with intestinal contents before they are processed by mucosal immune system, which accounts for the remarkably low efficacy of orally administered vaccines^{2,3}. In this context, targeting vaccine molecules to immune-associated cells, such as M cells or dendritic cells, is needed to increase mucosal vaccine efficiency. Many researchers, including our group, have investigated M cell-targeting strategies and confirmed their effectiveness²⁻⁴. However, research related to porcine M cell targeting remains to be established.

To target porcine M cells, the protein expression exclusive to porcine M cells should be characterized. In this regard, TLR2 is considered a target candidate. It is a type of toll-like receptor (TLR), a representative pattern recognition receptor that perceives various microbial molecules. TLR2 recognizes the cell wall components of pathogens such as peptidoglycan, microbial lipoprotein, lipoteichoic acid, and zymosan, and plays a key role in the intestinal immune response^{5,6}. In pigs, TLR2 is highly expressed in M cells⁵, suggesting that porcine TLR2 could be involved in ligand-specific transcytosis in M cells, which are

responsible for antigen uptake and initiating immune responses. Furthermore, TLR2 is also expressed in immune cells, such as B cells, T cells, and dendritic cells, which meet after M cell entry and are crucial for induction of the acquired immune response^{6,7}. Therefore, targeting porcine TLR2 is a critical way to improve the efficiency of vaccine delivery.

To find a targeting moiety against porcine TLR2, we performed an *in vitro* cell-based peptide phage display. A peptide phage display is one of the most powerful tools for identifying the targeting moiety against specific biological targets using enormously diverse peptide phage libraries. We performed peptide phage display on whole cells, which is referred to as cell-based phage display because selection using whole cells can maintain the native conformation of a receptor (TLR2 in this study) with natural post-translational modifications⁸. To overcome the possibility of selecting a non-target receptor due to an insufficient level of TLR2 expression, lentiviruses were used to maximize the expression levels of TLR2 in a stable manner. Although some TLR2-targeting materials such as lipopeptides have been discovered and used for vaccine delivery^{9,10}, short peptide ligands in peptide phage displays have several advantages. Targeting peptides can be expressed by recombinant bacteria and animal cells, indicating that the peptides can be produced with protein drugs or vaccines. Short peptide ligands can also be easily synthesized by chemical methods on a large scale and conjugated with vaccines, drugs, or their delivery carriers. Peptide ligands can be transported into cells through receptor-mediated endocytosis¹¹.

In this study, porcine TLR2-expressing IPEC J2 cells were established to identify porcine TLR2-targeting peptide ligands using lentiviruses carrying the TLR2-eGFP fusion protein. Subtractive and non-subtractive biopannings were performed on TLR2-overexpressing cells, and candidate peptides were selected using high-throughput sequencing. Final candidate peptides were validated using *in vitro* analysis. The porcine TLR2-targeting peptide ligand identified in this study contributes to the vaccine delivery field. Furthermore, this study offers details for cell-based phage display combined with high-throughput sequencing.

Results

Construction of the porcine TLR2-overexpressing cell line

We first constructed TLR2-overexpressing IPEC-J2 cells to screen the TLR2-targeting peptide using a cell-based phage display. For this, IPEC-J2 cells were transduced with a lentiviral vector carrying the TLR2-eGFP gene, and the heterologous expression of TLR2-eGFP was confirmed using western blotting and flow cytometry. eGFP was genetically conjugated to the C' terminus of TLR2 (TLR2-eGFP) for the successful detection of TLR2 expression and was designed to be located in the cytoplasm of the cell after expression to avoid hindering the biopanning of phage library to TLR2 portion. Western blot analysis showed that the IPEC-J2 cells transduced by only eGFP-carrying lentiviruses showed an eGFP band (27 kDa), while the cells transduced by TLR2-eGFP-carrying lentiviruses showed a TLR2-eGFP band with an expected molecular weight of 115 kDa (Fig. 2A). Flow cytometry also showed that the TLR2-overexpressing IPEC-J2 cells had a high eGFP-positive rate (82.9%) compared with wild-type IPEC-J2 cells (Supplementary Fig. S1).

To screen for a genuine peptide that binds to endogenous TLR2 in the swine intestinal lumen, transduced TLR2 should have a proper structure. Since zymosan, a natural TLR2 agonist, binds to properly expressed TLR2 and induces its internalization, we confirmed whether the transduced TLR2 has a normal structure by observing the internalization of TLR2-eGFP after zymosan treatment. The transduced cells expressed eGFP on the plasma membranes of the cells; however, after 1 h of treatment with Texas Red-labeled zymosan, receptors were internalized and the signal of eGFP and Texas Red were co-localized, indicating that the transduced TLR2 maintained a normal structure (Fig. 2B).

Phage display with the porcine TLR2-overexpressing cells

Before the phage display, a naive phage library (2×10^{11} pfu) was analyzed to investigate the quality of the library and to verify a sequencing method. We obtained 33,853,550 reads containing 20,539,490 unique sequences from the naive library. Supplementary Figure S2A and S2B show the theoretical amino acid composition for the Ph.D.-C7C naive phage library and the actual amino acid distribution of the naive library provided by the manufacturer, respectively. We obtained more than 20 million unique sequences for the naive library and analyzed the amino acid distribution. The observed amino acid distribution was approximately the same as the actual composition provided by the manufacturer (Supplementary Fig. S2C and S2D), suggesting that the sequencing method was reliable.

Phage display was carried out with non-subtractive and subtractive biopanning for the elimination of phage binding to wild-type IPEC-J2 cells only (Fig. 1). Phage titration data showed progressive enrichment of the phage population during the three rounds in both types of biopanning (Fig. 3A). In the case of subtractive biopanning, the phage titer increased approximately 8-fold over in the third round compared with the first round of biopanning. Similarly, a 6-fold increase in the phage titer was observed in non-subtractive biopanning. In addition, the high-throughput sequencing results showed that the number of unique sequences decreased during the 3 rounds for both types of biopanning (Fig. 3B). Both results indicated specific phages binding to TLR2 or IPEC-J2 cells were successfully enriched. Additionally, a higher phage titer and diversity were observed in non-subtractive biopanning compared to subtractive biopanning during the three rounds, suggesting that a large population of phages which associated with binding to wild-type IPEC-J2 cells could be removed through the subtraction process in subtractive biopanning.

Selection of candidate peptides targeting porcine TLR2

The peptide-encoding DNA of the phages collected from each round of two types of biopanning was sequenced using the Illumina sequencer. We obtained a total of 89,573,096 paired-reads after quality filtering and matching with each pair (an average of 14,928,849 reads per round; see Table 1). False positive sequences were screened by web-based softwares, which revealed that irrelevant peptide

sequences with target binding increased gradually during three rounds in both cases of subtractive and non-subtractive biopanning (Supplementary Fig. S3). All false positives identified in this step were excluded from further analysis.

We counted the number of peptides sorted by the most abundant sequence in each round (Fig. 3C). In subtractive biopanning, NAGHLSQ was the most abundant peptide across three rounds of biopanning, increasing 850-fold from the first round to the third round (Table 2). In case of non-subtractive biopanning, RANLDGQ showed the highest binding affinity, increasing 1,257-fold from the first round to the third round. Interestingly, VPSKPGL was the most common enriched peptide in both types of biopanning; this peptide was ranked third in subtractive biopanning and ranked second in non-subtractive biopanning. We selected three candidate peptides as porcine TLR2-targeting peptides (NAGHLSQ, RANLDGQ, and VPSKPGL) to validate their TLR2-binding ability (Table 2).

***In vitro* validation of the selected peptides**

The three candidate peptides and a peptide as a negative control were chemically synthesized for *in vitro* validation. The negative control peptide (CGLHPAFQC) is a transdermal tissue-targeting peptide that was previously identified in our laboratory and not associated with TLR2¹².

To test the ability of the candidate peptides to bind to TLR2, wild-type and TLR2-overexpressing IPEC-J2 cells were treated with various concentrations of candidate peptides conjugated with rhodamine B, and the fluorescence was measured by flow cytometry analysis (Fig. 4A). The control peptide did not show any significant binding affinity for the two types of cells at any concentration. In the case of TLR2-overexpressing IPEC-J2 cells, all three candidate peptides showed a dose-dependent effect. Among them, NAGHLSQ showed the highest binding affinity at 200 nM and 2 μ M treatment. RANLDGQ and VPSKPGL showed the second and third binding affinities, respectively. In wild-type IPEC-J2 cells, RANLDGQ and VPSKPGL showed a concentration-dependent affinity, while NAGHLSQ showed no significant binding affinity at any concentration, indicating that NAGHLSQ has an affinity to TLR2 itself irrelevantly with the host cell line, IPEC-J2, while RANLDGQ and VPSKPGL were assumed to be not associated with an affinity to TLR2 in spite of their significance in IPEC-J2 binding.

To confirm whether the binding was mediated by TLR2, a ligand competition assay was performed using zymosan. The wild-type and TLR2-overexpressing IPEC-J2 cells were treated with 2 μ M of rhodamine B conjugated candidate peptides together with various concentrations of zymosan, and the fluorescence was measured using flow cytometry (Fig. 4B). If the binding of peptides is mediated by TLR2, a high concentration of zymosan would compete with the peptide, subsequently weakening the fluorescence signal. In the case of wild-type IPEC-J2 cells, none of the three candidate peptides responded to zymosan treatment at any dose. In TLR2-overexpressing IPEC-J2 cells, however, NAGHLSQ showed an extensive attenuation of the fluorescence signal against zymosan treatment in a concentration-dependent manner. VPSKPGL also showed a moderate concentration-responsive interruption against zymosan treatment,

while RANLDGQ showed no significant results both in TLR2-overexpressing IPEC-J2 cell and wild-type IPEC-J2 cell.

Discussion

Phage display biopannings were performed to identify porcine TLR2-targeting peptide ligands. The porcine TLR2-overexpressing IPEC-J2 cell line was established by lentiviral transfection, and two types of biopanning were applied on TLR2-overexpressing IPEC-J2 cells. A progressive enrichment of the phage population and specific abundant peptides was observed across three rounds of biopanning. We identified three peptides in biopanning; NAGHLSQ and RANLDGQ were the most enriched peptides in subtractive biopanning and non-subtractive biopanning, respectively, and VPSKPGL was the most common peptide enriched in both types of biopanning. Through the peptide binding assay and the competition assay, NAGHLSQ was selected as a peptide ligand with the highest affinity to porcine TLR2 among the candidates.

For the screening of targeting peptide ligands to porcine TLR2, TLR2-overexpressing IPEC-J2 cell was adapted in cell-based phage display technique. As TLR2 is a transmembrane receptor, it is difficult to purify and maintain the natural conformation of the receptor. In this context, the host cell, IPEC-J2, could help maintain the native conformation of the receptor with normal post-translational modification. However, the cell-based phage display technique possesses a critical weak point about non-specific binding because host cell already expresses a variety of native surface receptors besides the overexpressed target receptor. To overcome this problem, subtractive biopanning step is generally included in cell-based phage display, in which phage-peptide library firstly applied to wild-type cells for the negative selection before the biopanning to target-overexpressing cells. In this research, the porcine jejunum epithelial cell, IPEC-J2 cell line, was used for the porcine TLR2 overexpression, but wild-type IPEC-J2 also expresses endogenous porcine TLR2¹³. Thus, non-subtractive and subtractive biopanning were compared to each other to identify true binders for porcine TLR2.

The negative selection of phage-peptide library against wild-type IPEC-J2 in the subtractive biopanning consequently conferred a huge impact on the results. Although three successive biopannings were common, there was no common peptide between the top five most abundant peptide lists of subtractive and non-subtractive biopanning, except for one peptide (Fig. 3C). The recovered phage titer after the first round of biopanning strongly supported the significance of negative selection again (Fig. 3A). Theoretically, around 1,483 pfu/ μ l of recovered phages after the first round of non-subtractive biopanning seems to be trimmed down by negative selection, resulting in only a few recovered phages around 340 pfu/ μ l. These phages are considered, so called as, 'elite few' which consists of a phage population having peptide ligands with potentially high affinity to TLR2 except non-specific binder to host cell, IPEC-J2. In cell-based phage display, the endogenous expression of target proteins in the host cell line should be carefully considered to avoid an ambiguous result because the certain phage population containing the peptide ligands with high affinity to target proteins could be removed during the subtraction process. Although TLR2 is already known to be expressed endogenously in IPEC-J2¹³, its expression level was

revealed too low to interrupt biopannings in our study. The wild-type IPEC-J2 cells seemed to express very low basal level of TLR2 on the cell surface because they exhibited just limited binding affinity with NAGHLSQ, a TLR2-specific binding peptide identified in this study, at any concentration (Fig. 4A).

Three candidate peptide ligands were selected by their abundance in the third round of biopanning after high-throughput sequencing analysis and subsequently validated by *in vitro* analysis. NAGHLSQ showed no significant binding response against wild-type IPEC-J2 cells at any concentration, whereas it showed a concentration-dependent binding response against TLR2-overexpressing IPEC-J2 cells. The other two peptide candidates, RANLDGQ and VPSKPGL, showed concentration-dependent binding affinity for both cell types (Fig. 4A). This suggests that NAGHLSQ could bind to TLR2 or other factors including changes derived from lentiviral transduction or eGFP, although it was intended to be expressed inside the cells. To exclude these possibilities, the ligand competition assay was performed using zymosan, a natural TLR2 agonist (Fig. 4B). The data revealed that NAGHLSQ strongly competes with zymosan against TLR2-overexpressing IPEC-J2 cells along with its concentration escalation, while RANLDGQ maintained its binding affinity regardless of zymosan concentration. The concentration of zymosan in our study did not seem to affect non-specific peptide binding affinity because no significant changes were detected for peptide binding affinity against wild-type IPEC-J2 cells upon concentration escalation. These data strongly suggest that NAGHLSQ is a true binder to porcine TLR2, especially at a site related to the ligand binding site. Interestingly, VPSKPGL also showed moderate competition upon zymosan treatment, suggesting that it could also have potential binding affinity to TLR2. Since VPSKPGL also had a binding affinity against wild-type IPEC-J2 cells (Fig. 4A), we inferred that VPSKPGL could bind the TLR2 as well as wild-type IPEC-J2 cells by the uncertain mechanism, resulting in its survival after negative selection and record third and second enrichment frequency after subtractive and non-subtractive biopanning, respectively.

In this study, the feasibility of high-throughput sequencing in phage displays was re-elucidated. In traditional phage display procedure, eluted phages should be grown on plates with bacterial hosts in general. Small portion of phage plaques were then selected randomly and each colony was analyzed separately by Sanger sequencing. This procedure has many disadvantages, such as being labor-intensive, time-consuming, and vulnerable to biased data collection because of the limited number of samples^{14,15}. In particular, the existence of false-positives such as plastic binders and sequences with propagation advantages often hinder to identify the true binder to target because the probability would be very low to find them from the limited number of phage plaque selection. Moreover, the false-positives could be accumulated along the round of biopanning (Supplementary Fig. S3). However, high-throughput sequencing enables us to overcome such limitations of traditional phage sequencing. It is possible to rapidly characterize and quantify a tremendous number of phage DNA sequences simultaneously without the phage propagation and plaque-selection process. Full investigation of clones enabled us to identify true binders without several selection rounds¹⁵. Actually, the peptide ligands with high ranks in their abundance having potentially high affinity to TLR2 have appeared apparently even in the first round of biopanning in this study. In the case of subtractive biopanning, the top ten peptide ligands in their

abundance was overlapped 60% between the first and the third rounds and 80% between the second and the third round. Similar tendency also has been found in non-subtractive biopanning (Supplementary Fig. S4). It suggests that the population of true binders would already be outlined in early round of biopanning. However, abundance of peptide ligands should be traced along the rounds of biopanning even combined with high-throughput sequencing, since the actual number of each individual phage in the original phage-peptide library is different and too low (theoretically 167 phage copies/peptide sequence on average), resulting in premature interpretations after first round of biopanning.

In this research, a small peptide with specific affinity to porcine TLR2 ligand binding site was identified. This peptide could be potentially used as an M cell-targeting ligand because the TLR2 is considered as one of the molecular markers on the M cells. Especially in swine industry, M cell-targeting ligand would have a great importance on the issue about development of effective mucosal vaccine delivery system. In this context, there has been a trial to target porcine M cells for mucosal vaccine development¹⁶. Du et al. modified vaccine delivery nanoparticles with *Ulex europaeus* agglutinin 1 (UEA-1), a well-known lectin that binds to α -L-fucose on M cells, to enhance the efficacy of an oral vaccine against PRRS through porcine M cell targeting. Although the conjugation of UEA-1 lectin augmented intestinal IgA levels in mice and piglets, the immune responses could be improved if sufficient levels of lectin receptors on M cells were guaranteed. However, the expression level of lectin receptor on M cells, which varies widely among species, is elusive in pigs^{2,17,18}. Utilization of TLR2 to target porcine M cells is promising because TLR2 is known to be highly expressed in M cells and located in both the cytoplasm and the apical membrane of porcine M cells⁵. The nature of short peptides is also a great advantage for the future applications. Peptides can be genetically conjugated with antigens and expressed easily by recombinant bacteria and animal cells. In addition, short peptides can also be easily synthesized by chemical methods on a large scale and utilized with delivery carriers. Moreover, NAGHLSQ is expected to have an immunological adjuvant function beyond M cell targeting ability because its binding mechanism against porcine TLR2 is believed to be associated with the very spot of ligand binding (Fig. 4B). TLRs are representative pattern-recognition receptors that detect foreign substances and activate innate immunity, which is a prerequisite for acquired immunity⁷. This feature enabled researchers to utilize TLR ligands as an adjuvant for vaccine development, including ligands responsible for TLR2¹⁹⁻²¹. Taken together, NAGHLSQ could be a promising peptide for porcine mucosal vaccine development. It could be used not only in vaccine design but also in targeted therapeutics. Validation of NAGHLSQ in target animals, however, is needed for further study. In addition, other peptide candidates after subtractive biopanning (TYLNSAK, NSHRHGA, and PTGLHHA), not tested in this study, should also be investigated.

To the best of our knowledge, this study is the first to identify a short peptide ligand targeting porcine M cells. Furthermore, our study provides a better understanding of cell-based phage display combined with high-throughput sequencing.

Methods

Cell culture

The porcine jejunum epithelial cell line IPEC-J2 (DSMZ, Braunschweig, Germany) was maintained in DMEM/Ham's F-12 medium mixture (Gibco Life Technologies, GrandIsland, NE, USA) supplemented with 5% heat-inactivated fetal bovine serum (Invitrogen, GrandIsland, NE, USA), 1% insulin-transferrin-selenium-X (Gibco, Waltham, MA, USA), and antibiotics (penicillin/streptomycin). The HEK 293 LTV cell line (Cell Biolabs, San Diego, CA, USA) was cultured according to the manufacturer's instructions. IPEC-J2 and HEK 293 LTV cells were grown at 37 °C in an atmosphere of 5% CO₂.

Construction of porcine TLR2-expressing IPEC-J2 cells

The porcine TLR2 gene was amplified from pUNO1-pTLR02 (InvivoGen, San Diego, CA, USA) by PCR using the designed primers (Supplementary Table S1). The primers included restriction enzyme sites, and additional sequences were inserted to remove the stop codon in the TLR2 sequence and to fit in the eGFP frame for the C-terminal eGFP-tagged TLR2 expression. PCR was initiated with a 4-min denaturation at 95 °C, followed by 5 cycles of 30 s at 95 °C, 30 s at 43 °C, and 2 min at 72 °C; 30 cycles of 30 s at 95 °C, 30 s at 62 °C, and 2 min at 72 °C. The PCR product was inserted into a lentiviral transfer plasmid pWPXL (Addgene, Watertown, MA, USA) by Pme/Mlu sites to generate pWPXL-pTLR2-eGFP.

To produce lentiviruses, three plasmids were used: the pWPXL lentiviral expression plasmid, the PsPAX2 packaging plasmid (Addgene), and the pLP/VSVG envelope plasmid (Invitrogen, Carlsbad, CA, USA). The porcine TLR2-expressing lentivirus was prepared as follows. Three hours before transfection, the cell medium was replaced with 25 µM chloroquine-containing medium. Then, 26.5 µg of pWPXL-pTLR2-eGFP, 9.2 µg of PsPAX2, and 5 µg of pLP/VSVG were transfected into 80% confluent HEK 293 LTV cells using the calcium phosphate precipitation method. After 12 h of transfection, transfected cells were treated with 15% glycerol solution and incubated for 24 h. Culture supernatants were harvested every 12 h and filtered using 0.45 µm pore filters (Sartorius, Republic of Korea). To precipitate lentiviruses, PEG 10000 and NaCl were added to the lentiviral supernatant and concentrated by centrifugation at 15,000 × g for 2 h at 4 °C. The virus pellet was dissolved in DMEM and stored at -70 °C.

For lentiviral transduction, IPEC-J2 cells were seeded at a density of 1×10^5 cells/well and grown to 60% confluence in a 35-mm culture dish before transduction. Cells were treated with lentiviruses in the presence of 8 µg/mL polybrene (Sigma-Aldrich, St. Louise, MO, USA). Forty-eight hours after transduction, the medium was replaced with fresh media, and transgene expression was observed by fluorescence microscopy.

Evaluation of lentiviral transduction

To evaluate eGFP expression in the transduced IPEC-J2 cells, flow cytometric assays and western blotting were performed. For flow cytometry, cells were detached using 0.25% trypsin and fixed with 4% paraformaldehyde. Fixed cells were analyzed using FACS Aria II (BD Biosciences, San Jose, CA, USA).

For western blotting, the transduced IPEC-J2 cells were lysed using RIPA buffer (Sigma-Aldrich) with protease inhibitor and phosphate inhibitor (Roche, Basel, Switzerland). Equivalent amounts of protein samples were electrophoretically separated, and the proteins were transferred to a nitrocellulose membrane. The membrane was blocked with 5% skim milk and 0.05% Tween-20 in TBS for 1 h at room temperature and incubated overnight at 4 °C with HRP-conjugated rabbit anti-GFP polyclonal antibody (Santa Cruz Biotechnology) (1:500). After washing three times with 0.05% Tween-20 in TBS, the membrane was reacted with ECL detection reagents (Santa Cruz Biotechnology) and analyzed using a luminescent image analyzer LAS-3000 (Fujifilm, Japan). Full-length blot image was presented in Figure 2A.

To evaluate porcine TLR2 expression in transduced IPEC-J2 cells, a ligand binding assay was performed. IPEC-J2 cells were seeded and grown to 60% confluence in 35 mm confocal glass bottom dishes (SPL, Republic of Korea). Cells were treated with pTLR2-eGFP carrying lentiviruses in the presence of 8 µg/ml polybrene (Sigma Aldrich) and incubated for 48 h. Then, 10 µg/mL of Texas Red-labeled Zymosan A from *Saccharomyces cerevisiae* (Molecular probes, Eugene, OR, USA) was incubated for 1 h at 37 °C and washed three times with PBS. Cells were then fixed in 4% PFA and analyzed using confocal microscopy (TCS SP8X, Leica, Wetzlar, Germany).

Cell-based phage display

The Ph.D.-C7C™ Phage Display Peptide Library Kit (New England Biolabs, Ipswich, MA, USA) was used for two types of biopanning: biopanning with and without a subtractive round (Fig. 1). In non-subtractive biopanning, after transduction with pTLR2-eGFP-carrying lentiviruses, IPEC-J2 cells were harvested with ice-cold PBS (without Ca²⁺ and Mg²⁺) containing 5 mM EDTA. The harvested cells were then washed with DMEM containing 1% BSA, and cell numbers were counted. Then, 1 × 10⁶ cells were prepared in DMEM containing 1% BSA and 2 × 10¹¹ pfu of the phage library (10 µL of the phage library) were added to the cell suspensions. Cells were incubated for 2 h on ice to prevent endocytosis of any bound phage. To remove unspecific phages, the cell pellet was washed three times with 500 µL of PBS containing 1% BSA and 0.05% Tween 20 by gentle pipetting. At the end of the final wash, 1 mL of 0.1 M glycine-HCl (pH 2.0) was added to the cell pellet to elute the cell-bound phage. After vortexing for 3 min, the tube was centrifuged for 3 min at 4°C. The supernatant containing the eluted phage was transferred into a new 1.5 ml tube and neutralized with 60 µL of 2 M Tris base. Eluted phages were quantified by titration using phage-plaque-forming assay after infection into *Escherichia coli* and also used for DNA sequencing to determine their peptide sequences according to the manufacturer's instructions (New England Biolabs).

The above steps were referred to as 'one round of biopanning', and an equal amount of phage was used for the consecutive another two rounds of biopanning after being amplified in *E. coli*.

The experimental procedures of biopanning with a subtractive round were the same as the above biopanning without a subtractive round except for one process of negative selection. In negative selection, 2×10^{11} pfu of phage was added to 1×10^6 wild-type IPEC-J2 cells for 30 min on ice. After centrifugation, cell pellets containing cell-bound phages were discarded to remove the wild-type IPEC-J2 cell-bound phage. The recovered supernatant was then directly added to 1×10^6 lentiviral-transduced IPEC-J2 cells, and three rounds of positive selection were performed.

Phage sequencing and screening false positives

After the third round of biopanning in each non-subtractive and subtractive cell-based phage display, ssDNA from the eluted M13 phages was isolated using the NaI/EtOH precipitation method²². Then, 50 ng of isolated phage ssDNA was amplified using primers flanking the variable region at the N-terminus of the *pIII* gene of the phage. The PCR reaction contained 10 × PCR buffer, 25 mM MgCl₂, 2.5 mM dNTP, 1.5 U Ex-Taq polymerase (Takara, Kyoto, Japan), and 10 pmol of forward and reverse primers. Thirty-five cycles (10 s at 98 °C, 20 s at 58 °C, and 30 s at 72 °C) were performed. PCR products were purified from a 2.5% agarose gel using a QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany). Forty nanograms of DNA per sample was pooled together in a volume of 100 μL.

A phage ssDNA library for Illumina sequencing was constructed using the Nextera XT DNA Library Preparation Kit (Illumina, San Diego, CA, USA) according to the manufacturer's recommendations. The prepared libraries were then sequenced using an Illumina HiSeq 2000 for 100 bp paired-end reads. Adapter sequences of the reads were trimmed with Cutadapt 1.10²³, and the sequence reads were quality-filtered using in-house Perl scripts^{24,25}. In brief, when 95% of the nucleotide bases in a read were given a quality score of over 31 (Illumina 1.8+) and the read length was ≥ 70 bp, the read was used in the next step. The sequence reads that were precisely matched with the two nucleotide sequences consisting of the flanking region of the phage display variable region (upstream: 5'-TATTCTCACTCTGCTTGT-3' and downstream 5'-TGCGGTGGAGGT-3') were retained for further processing. Nucleotide sequences of phage display variable regions for each sample were extracted, and the variable regions encoding each unique peptide sequence were counted for each sample. The relative abundance of each peptide was calculated as the fraction of total reads in the library that encoded the peptides.

False positives were screened using two web-based programs, SAROTUP and PepBank, with default options. SAROTUP was screened for identification of 'non-target sequences' which could be frequently selected irrelevant peptide sequences with target binding during phage display, such as plastic binders and sequences having propagation advantages during amplification in *E. coli*²⁶. PepBank was searched for sequences already reported from previous researches²⁷.

Validation of identified peptide ligands

To evaluate the binding ability of the peptides to TLR2, peptide binding assays and ligand competition assays were performed. First, peptides for the assays were synthesized with a purity of > 95% (Peptron, Republic of Korea). The peptides included a consensus motif (CX7C, seven amino acids from each selected peptide with two cysteines back and forth, a total of nine amino acids with a single intra-disulfide bond), rhodamine B conjugated as the fluorescence label, and alanine and glycine residues at N-terminal and C-terminal of the peptide, respectively, to increase the stability of the synthetic peptides.

For the peptide binding assay, IPEC-J2 cells and lentiviral-transduced IPEC-J2 cells were incubated with 2 nM, 20 nM, 200 nM, and 2 μ M of each peptide. Cells were then washed three times with PBS for 3 min and fixed in 4% PFA. Fluorescence was measured using a FACS Aria II (BD Biosciences).

For the ligand competition assay, IPEC-J2 cells and transduced IPEC-J2 cells were pre-incubated with 2 μ M of each peptide for 30 min at 4 °C, and 0, 1, 2, 5, or 10 μ g of zymosan A (Thermo Scientific) was added. Cells were then washed three times with PBS for 3 min and fixed in 4% paraformaldehyde. Fluorescence was measured using a FACS Aria II (BD Biosciences).

Statistical analysis

Results are expressed as the mean \pm SD. Statistical significance was determined using Student's t-test and one-way ANOVA. All statistical significance is denoted by * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

Data Availability

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Abbreviations

ANOVA, analysis of variance; DMEM, Dulbecco's modified Eagle medium; ECL, enhanced chemiluminescence; eGFP, enhanced green fluorescent protein; EDTA, ethylenediaminetetraacetic acid; FACS, fluorescence-activated cell sorting; FAE, follicle-associated epithelium; FMD, foot-and-mouth disease; HEK 293, human embryonic kidney cells 293; HRP, horseradish peroxidase; IL, interleukin; IPEC-J2, intestinal porcine epithelial cell-J2; PBS, phosphate-buffered saline; PED, porcine epidemic diarrhea; PEG, polyethylene glycol; PRRS, porcine reproductive and respiratory syndrome; RIPA,

radioimmunoprecipitation assay; SD, standard deviation; ssDNA, single-strand DNA; TBS, Tris-buffered saline; TLR, toll-like receptor; UEA-1, *Ulex europaeus* agglutinin 1

Declarations

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Author contributions

S.B.A. designed and performed the experiments, analyzed the data and generated all the figures. S.H.O. performed vector construction, analyzed the data and wrote most of the manuscript. K.H.C. and C.K.L. helped and performed pTLR2-overexpressing cell line establishment through lentiviral transduction. J.Y.L. discussed the results and corrected the manuscript. Y.J.C. and S.K.K. supervised the project.

Additional Information

The authors declare no financial or non-financial conflict of interest.

References

- 1 VanderWaal, K. & Deen, J. Global trends in infectious diseases of swine. *Proceedings of the National Academy of Sciences* **115**, 11495-11500 (2018).
- 2 Kang, S. H., Hong, S. J., Lee, Y.-K. & Cho, S. Oral Vaccine Delivery for Intestinal Immunity—Biological Basis, Barriers, Delivery System, and M Cell Targeting. *Polymers* **10**, 948 (2018).
- 3 Kim, S.-H. & Jang, Y.-S. Antigen targeting to M cells for enhancing the efficacy of mucosal vaccines. *Experimental & molecular medicine* **46**, e85-e85 (2014).
- 4 Kang, S. K. *et al.* Identification of a peptide sequence that improves transport of macromolecules across the intestinal mucosal barrier targeting goblet cells. *Journal of biotechnology* **135**, 210-216 (2008).

- 5 Tohno, M. *et al.* Toll-like receptor 2 is expressed on the intestinal M cells in swine. *Biochemical and biophysical research communications* **330**, 547-554 (2005).
- 6 Wetzler, L. M. The role of Toll-like receptor 2 in microbial disease and immunity. *Vaccine* **21**, S55-S60 (2003).
- 7 Iwasaki, A. & Medzhitov, R. Toll-like receptor control of the adaptive immune responses. *Nature immunology* **5**, 987-995 (2004).
- 8 Watters, J. M., Telleman, P. & Junghans, R. P. An optimized method for cell-based phage display panning. *Immunotechnology* **3**, 21-29 (1997).
- 9 Eriksson, E. M. & Jackson, D. C. Recent advances with TLR2-targeting lipopeptide-based vaccines. *Current Protein and Peptide Science* **8**, 412-417 (2007).
- 10 Chua, B. Y. *et al.* Hepatitis C VLPs delivered to dendritic cells by a TLR2 targeting lipopeptide results in enhanced antibody and cell-mediated responses. *PLoS One* **7**, e47492 (2012).
- 11 Park, T.-E. *et al.* Mucosal delivery of vaccine by M cell targeting strategies. *Current Drug Therapy* **9**, 9-20 (2014).
- 12 Lee, N. K. *et al.* Identification of a peptide sequence targeting mammary vasculature via RPLP0 during lactation. *Peptides* **31**, 2247-2254 (2010).
- 13 Wang, J. *et al.* Swine-Derived Probiotic *Lactobacillus plantarum* Modulates Porcine Intestinal Endogenous Host Defense Peptide Synthesis Through TLR2/MAPK/AP-1 Signaling Pathway. *Frontiers in immunology* **10**, 2691 (2019).
- 14 Petrenko, V. Evolution of phage display: from bioactive peptides to bioselective nanomaterials. *Expert opinion on drug delivery* **5**, 825-836 (2008).
- 15 AC't Hoen, P. *et al.* Phage display screening without repetitious selection rounds. *Analytical biochemistry* **421**, 622-631 (2012).
- 16 Du, L. *et al.* Targeted delivery of GP5 antigen of PRRSV to M cells enhances the antigen-specific systemic and mucosal immune responses. *Frontiers in cellular and infection microbiology* **8**, 7 (2018).
- 17 Giannasca, P. J., Giannasca, K. T., Leichtner, A. M. & Neutra, M. R. Human intestinal M cells display the sialyl Lewis A antigen. *Infection and immunity* **67**, 946-953 (1999).
- 18 Clark, M., Jepson, M. A., Simmons, N. L., Booth, T. A. & Hirst, B. H. Differential expression of lectin-binding sites defines mouse intestinal M-cells. *Journal of Histochemistry & Cytochemistry* **41**, 1679-1687 (1993).

- 19 Sieling, P. A., Chung, W., Duong, B. T., Godowski, P. J. & Modlin, R. L. Toll-like receptor 2 ligands as adjuvants for human Th1 responses. *The Journal of Immunology* **170**, 194-200 (2003).
- 20 Toussi, D. N., Liu, X. & Massari, P. The FomA porin from *Fusobacterium nucleatum* is a Toll-like receptor 2 agonist with immune adjuvant activity. *Clinical and Vaccine Immunology* **19**, 1093-1101 (2012).
- 21 Toussi, D. N. & Massari, P. Immune adjuvant effect of molecularly-defined toll-like receptor ligands. *Vaccines* **2**, 323-353 (2014).
- 22 Matochko, W. L. *et al.* Deep sequencing analysis of phage libraries using Illumina platform. *Methods* **58**, 47-55 (2012).
- 23 Martin, M. Cutadapt removes adapter sequences from high-throughput sequencing reads. *EMBnet journal* **17**, 10-12 (2011).
- 24 Kim, E. B. & Marco, M. L. Nonclinical and clinical *Enterococcus faecium* strains, but not *Enterococcus faecalis* strains, have distinct structural and functional genomic features. *Applied and environmental microbiology* **80**, 154-165 (2014).
- 25 Lee, J.-Y., Han, G. G., Kim, E. B. & Choi, Y.-J. Comparative genomics of *Lactobacillus salivarius* strains focusing on their host adaptation. *Microbiological research* **205**, 48-58 (2017).
- 26 Huang, J., Ru, B., Li, S., Lin, H. & Guo, F.-B. SAROTUP: scanner and reporter of target-unrelated peptides. *Journal of Biomedicine and Biotechnology* **2010** (2010).
- 27 Shtatland, T., Guettler, D., Kossodo, M., Pivovarov, M. & Weissleder, R. PepBank-a database of peptides based on sequence text mining and public peptide data sources. *BMC bioinformatics* **8**, 1-10 (2007).

Tables

Table 1. Number of total sequences and unique sequences along 3 rounds of selection in two types of biopanning.

Round	Number of sequences	Number of unique sequences
1 (with subtraction)	11,028,969	158,375
1 (without subtraction)	21,606,549	305,411
2 (with subtraction)	12,004,478	82,351
2 (without subtraction)	16,416,249	193,264
3 (with subtraction)	15,386,519	28,745
3 (without subtraction)	13,130,332	56,812
Total	89,573,096	-

Table 2. Candidate peptides selected from the phage display.

Peptide sequence	Originated from	Counts in 1st round	Counts in 2nd round	Counts in 3rd round
NAGHLSQ	Subtractive biopanning	8,546	1,016,557	7,268,172
RANLDGQ	Non-subtractive biopanning	4,896	226,754	6,156,589
VPSKPGL	Subtractive biopanning	4,326	22,146	809,923
	Non-subtractive biopanning	3,852	327,346	2,926,354

Figures

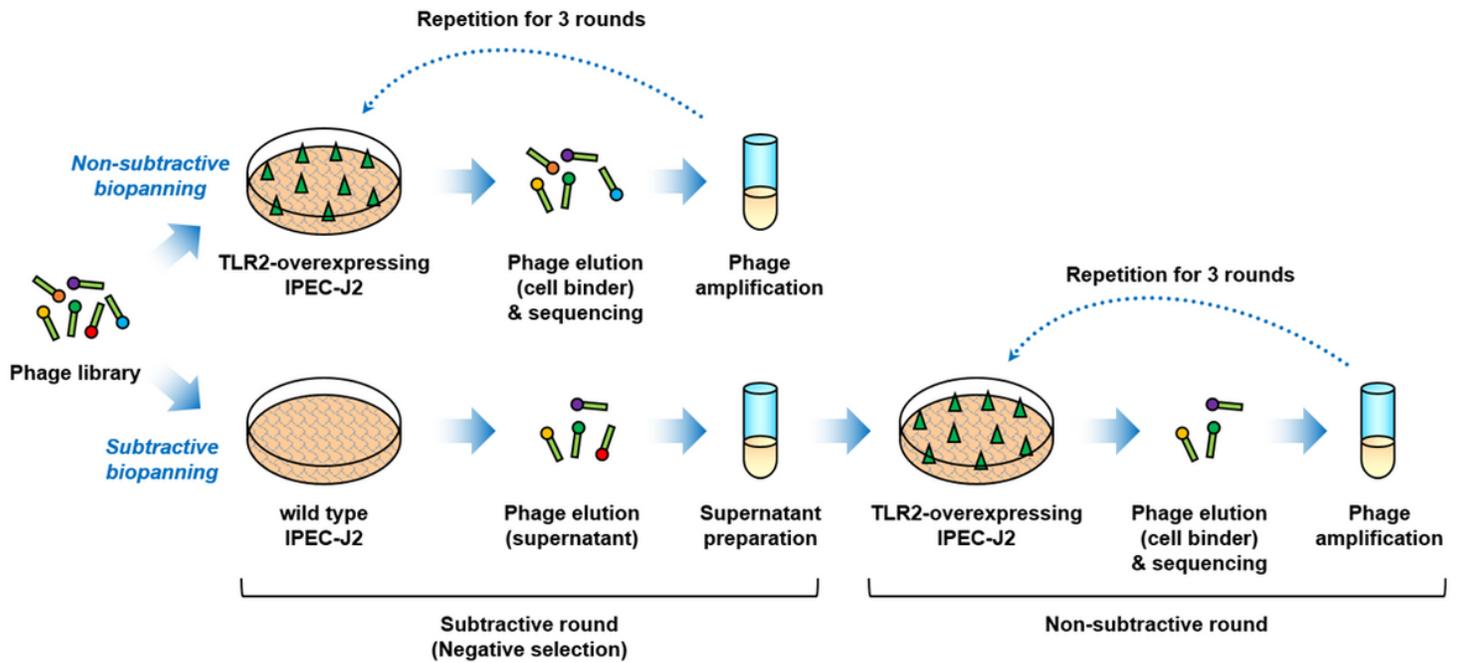


Figure 1

A scheme for the cell-based phage display procedure consisting of two types of biopanning.

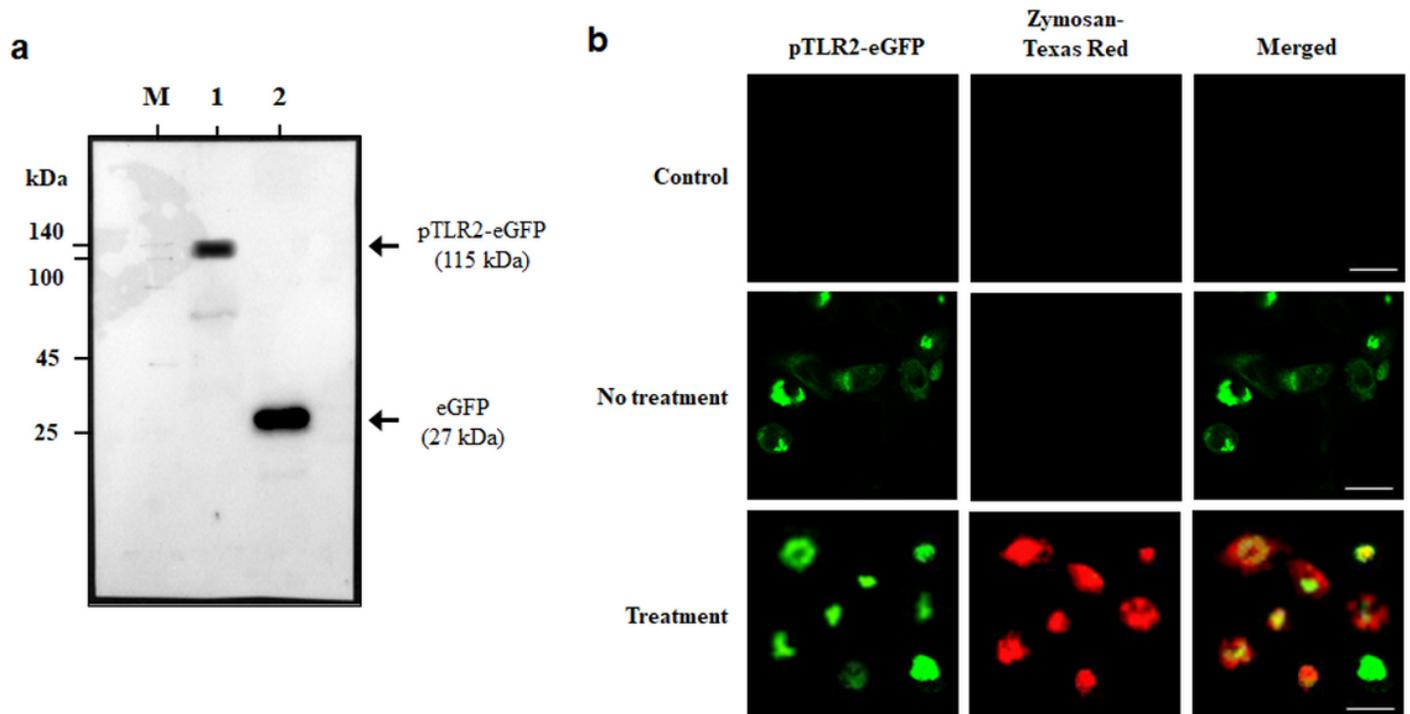


Figure 2

Construction of TLR2-overexpressing IPEC-J2 cells. (a) Western blot analysis of lentiviral transduced IPEC-J2 cells. M, protein marker; lane 1, IPEC-J2 cells transduced with pTLR2-eGFP carrying lentiviruses;

lane 2, IPEC-J2 cells transduced with only eGFP-carrying lentiviruses. Twenty micrograms of each sample was loaded and incubated with anti-eGFP antibody. Full-length blot image was presented. (b) Co-localization of pTLR2 and its ligand (zymosan). Control, wild-type IPEC-J2 cells; No treatment, IPEC-J2 cells transduced with pTLR2-eGFP carrying lentiviruses but not treated with zymosan; Treatment, treatment with 10 μ g/mL of Texas Red-conjugated zymosan to IPEC-J2 cells after transduction with pTLR2-eGFP-carrying lentiviruses. Green signals represent eGFP and red signals represent zymosan. Scale bar represents 100 μ m.

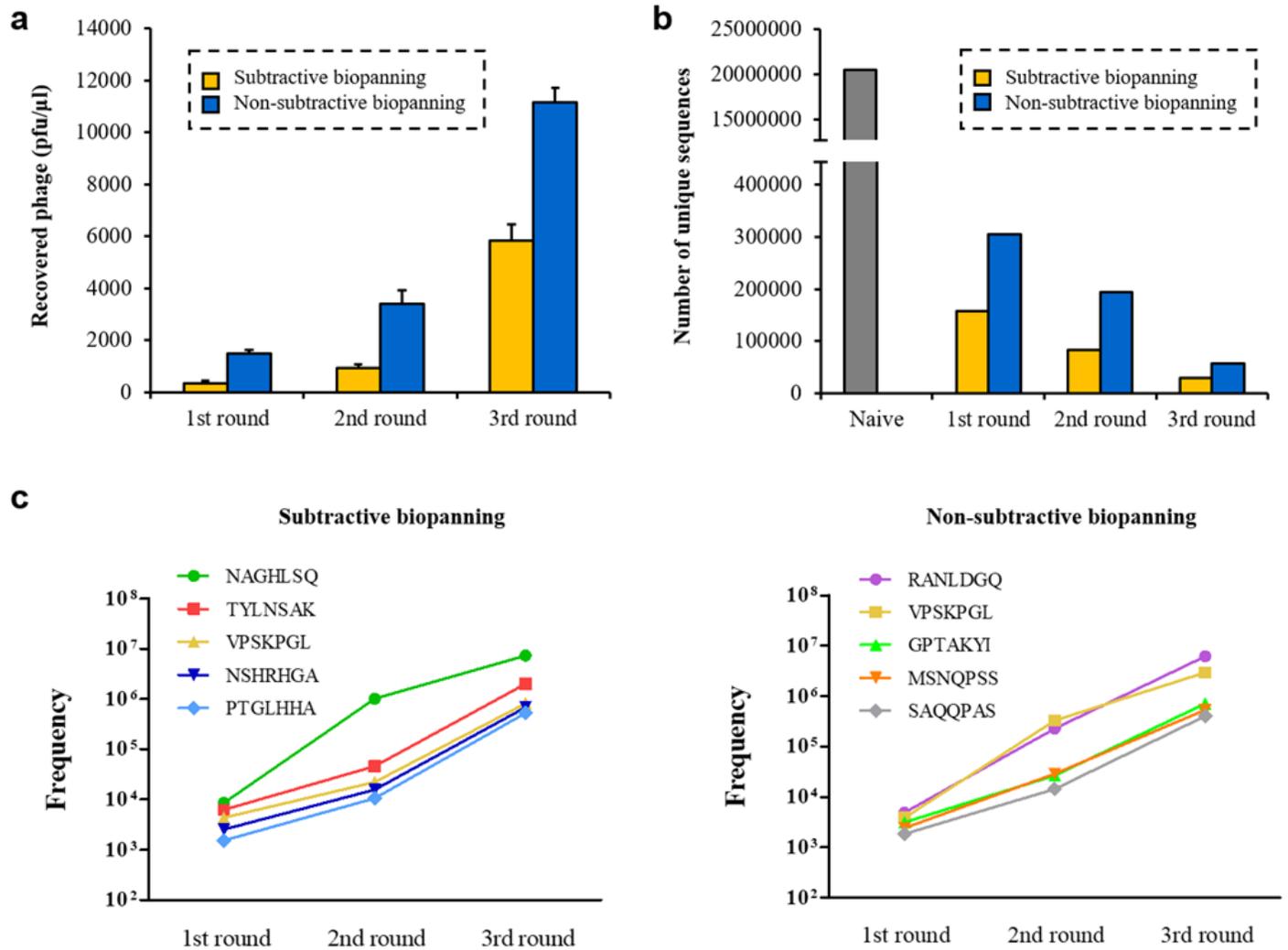


Figure 3

Results of the cell-based phage display. (a) A progressive enrichment of phages after the phage titration. (b) The progressive decrease in the number of unique sequences across 3 rounds of biopanning. (c) The counts of the most abundant peptides in two types of biopanning. False positives were excluded.

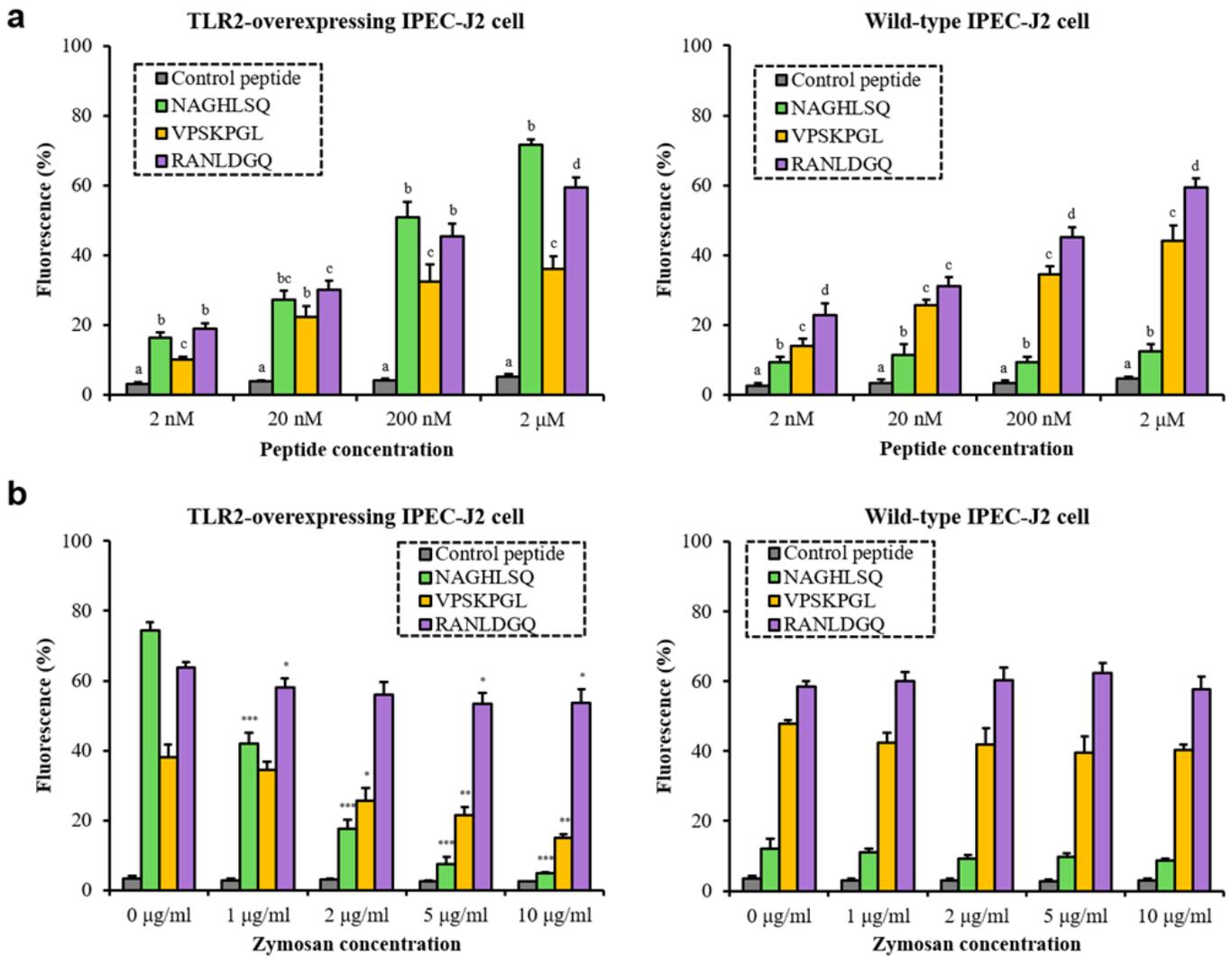


Figure 4

In vitro validation of the candidate peptides selected from the phage display. (a) Binding assay of rhodamine B-conjugated synthetic peptides. Left, TLR2-overexpressing IPEC-J2 cells were treated with different concentrations of candidate peptides and the control peptide. Fluorescence from rhodamine-conjugated synthetic peptides was measured by flow cytometry analysis. Right, wild-type IPEC-J2 cells were treated with different concentrations of candidate peptides and the control peptide. The data were analyzed using one-way ANOVA. Averages followed by the same letters in each column are not significantly different ($p < 0.05$) (b) Competition binding assay of rhodamine B-conjugated synthetic peptides and TLR2 ligands (zymosan). Left, TLR2-overexpressing IPEC-J2 cells were treated with 2 μ M of rhodamine B-conjugated synthetic peptides followed by different concentrations of zymosan; Right, wild-type IPEC-J2 cells were treated with 2 μ M of synthetic peptides and different concentrations of zymosan. Each peptide was compared between the untreated group (0 μ g/ml) and other groups using one-way ANOVA. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

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