Video Article

Screening Bioactive Nanoparticles in Phagocytic Immune Cells for Inhibitors of Toll-like Receptor Signaling

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Abstract

Pharmacological regulation of Toll-like receptor (TLR) responses holds great promise in the treatment of many inflammatory diseases. However, there have been limited compounds available so far to attenuate TLR signaling and there have been no clinically approved TLR inhibitors (except the anti-malarial drug hydroxychloroquine) in clinical use. In light of rapid advances in nanotechnology, manipulation of immune responsiveness using nano-devices may provide a new strategy to treat these diseases. Herein, we present a high throughput screening method for quickly identifying novel bioactive nanoparticles that inhibit TLR signaling in phagocytic immune cells. This screening platform is built on THP-1 cell-based reporter cells with colorimetric and luciferase assays. The reporter cells are engineered from the human THP-1 monocytic cell line by stable integration of two inducible reporter constructs. One expresses a secreted embryonic alkaline phosphatase (SEAP) gene under the control of a promoter inducible by the transcription factors NF-κB and AP-1, and the other expresses a secreted luciferase reporter gene under the control of promoters inducible by interferon regulatory factors (IRFs).Upon TLR stimulation, the reporter cells activate transcription factors and subsequently produce SEAP and/or luciferase, which can be detected using their corresponding substrate reagents. Using a library of peptide-gold nanoparticle (GNP) hybrids established in our previous studies as an example, we identified one peptide-GNP hybrid that could effectively inhibit the two arms of TLR4 signaling cascade triggered by its prototypical ligand, lipopolysaccharide (LPS). The findings were validated by standard biochemical techniques including immunoblotting. Further analysis established that this lead hybrid had a broad inhibitory spectrum, acting on multiple TLR pathways, including TLR2, 3, 4, and 5. This experimental approach allows a rapid assessment of whether a nanoparticle (or other therapeutic compounds) can modulate specific TLR signaling in phagocytic immune cells.

Video Link

The video component of this article can be found at https://www.jove.com/video/56075/

Introduction

Toll-like receptors (TLRs) are one of the key elements in the innate immune system contributing to the first line of defense against infections. TLRs are responsible for sensing invading pathogens by recognizing a repertoire of pathogen-associated molecular patterns (or PAMPs) and mounting defense reactions through a cascade of signal transduction[1,2]. There are 10 human TLRs identified; except TLR10 for which the ligand(s) remain unclear, each TLR can recognized a distinct, conserved group of PAMPs. For example, TLR2 and TLR4, primarily located on the cell surface, can detect lipoproteins and glycolipids from Gram-positive and Gram-negative bacteria, respectively; while TLR3, TLR7/8 and TLR9, mainly present in the endosomal compartments, can sense RNA and DNA products from viruses and bacteria[3]. When stimulated by PAMPs, TLRs trigger essential immune responses by releasing pro-inflammatory mediators, recruiting and activating effector immune cells, and coordinating subsequent adaptive immune events[4].

The TLR signaling transduction can be simply categorized into two main pathways[5,6]. One is dependent upon the adaptor protein myeloid differentiation factor 88 (MyD88) — the MyD88-dependent pathway. All TLRs except TLR3 utilize this pathway to activate nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and mitogen-associated protein kinases (MAPKs), leading to the expression of pro-inflammatory mediators such as TNF-α, IL-6 and IL-8. The second pathway utilizes TIR-domain-containing adaptor-inducing interferon-β (TRIF) — the TRIF-dependent or MyD88-independent pathway — to activate interferon (IFN) regulatory factors (IRFs) and NF-κB, resulting in the production of type I IFNs. Intact TLR signaling is critical to our daily protection from microbial and viral infections; defects in TLR signaling pathways can lead to immunodeficiency and are often detrimental to human health.[7]

However, TLR signaling is a 'double-edged sword' and excessive, uncontrolled TLR activation is harmful. Overactive TLR responses contribute to the pathogenesis in many acute and chronic human inflammatory diseases[8,9]. For instance, sepsis which is characterized by systemic inflammation and multi-organ injury, is primarily due to acute, overwhelming immune responses toward infections, with TLR2 and TLR4 playing a crucial role in the sepsis pathophysiology[10,11,12]. In addition, TLR5 has been found to contribute to chronic lung inflammation of patients with cystic fibrosis[13,14]. Moreover, dysregulated endosomal TLR signaling (e.g., TLR7 and TLR9) is strongly associated with the development and

progression of several autoimmune diseases including systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA)[15,16]. These converging lines of evidence identify TLR signaling as a potential therapeutic target for many inflammatory diseases[17].

Although pharmacological regulation of TLR responses is anticipated to be beneficial in many inflammatory conditions, unfortunately, there are currently very few compounds clinically available to inhibit TLR signaling[9,17,18]. This is partly due to the complexity and redundancy of the TLR pathways involved in the immune homeostasis and disease pathology. Therefore, searching for novel, potent therapeutic agents to target multiple TLR signaling pathways could bridge a fundamental gap, and overcome the challenge of advancing TLR inhibitors into the clinic.

In light of the rapid advances in nanoscience and nanotechnology, nanodevices are emerging as the next generation TLR modulators owing to their unique properties[19,20,23]. The nanoscale size allows these nano-therapeutics to have better bio-distribution and sustained circulation[24,25,26]. They can be further functionalized to meet the desired pharmacodynamic and pharmacokinetic profiles[27,28,29]. More excitingly, the bio-activity of these novel nanodevices arises from their intrinsic properties, which can be tailored for specific medical applications, rather than simply acting as a delivery vehicle for a therapeutic agent. For example, a high-density lipoprotein (HDL)-like nanoparticle was designed to inhibiting TLR4 signaling by scavenging the TLR4 ligand LPS[23]. In addition, we have developed a peptide-gold nanoparticle hybrid system, where the decorated peptides can alter the surface properties of the gold nanoparticles, and allow them to have various bio-activities[30,31,32,33]. This makes them a special class of drug (or "nano-drug") as the next generation nano-therapeutics.

In this protocol, we present an approach to identify a novel class of peptide-gold nanoparticle (peptide-GNP) hybrids that can potently inhibit multiple TLR signaling pathways in phagocytic immune cells[32,33]. The approach is based on commercially available THP-1 reporter cell lines. The reporter cells consist of two stable, inducible reporter constructs: one carries a secreted embryonic alkaline phosphatase (SEAP) gene under the control of a promoter inducible by the transcription factors NF-κB and activator protein 1 (AP-1); the other contains a secreted luciferase reporter gene under the control of promoters inducible by interferon regulatory factors (IRFs). Upon TLR stimulation, the signal transduction leads to the activation of NF-κB/AP-1 and/or IRFs, which turns on the reporter genes to secret SEAP and/or luciferase; such events can be easily detected using their corresponding substrate reagents with a spectrophotometer or luminometer. Using this approach to screen our previously established library of peptide-GNP hybrids, we identified lead candidates that can potently inhibit TLR4 signaling pathways. The inhibitory activity of the lead peptide-GNP hybrids was then validated using another biochemical approach of immunoblotting, and evaluated on other TLR pathways. This approach allows for fast, effective screening of novel agents targeting TLR signaling pathways.

Protocol

1. Preparation of Cell Culture Media and Reagents

1. **Prepare the complete cell culture medium R10 by adding the supplements of 10% fetal bovine serum (FBS), 2 mM L-glutamine and 1 mM sodium pyruvate into the RPMI-1640 medium.**
    1. Prepare the selection culture medium R10-Z by adding the antibiotics Zeocin (200 µg/mL) to R10 for maintaining the expression of SEAP under the control of NF-κB/AP-1 activation. To select for cells expressing both SEAP and luciferase reporter genes, add both Zeocin (100 µg/mL) and blasticidin (10 µg/mL) to R10 (as R10-ZB).

2. **Prepare the SEAP substrate solution by dissolving one pouch of the substrate powder (e.*g.*, QUANTI-Blue) into 100 mL ultrapure, endotoxin free water in a clean 125 mL glass flask.**
    1. Swirl the solution gently and incubate it at 37 °C for 1 h to ensure the complete dissolution of the substrates.
    2. Filter the solution using a 0.2 µm membrane to ensure its sterility (optional), and store it at 4 °C up to 2 weeks prior to use.

3. Prepare the luciferase substrate solution by dissolving one pouch of the substrate powder (*e.g.*, QUANTI-Luc) into 25 mL ultrapure, endotoxin free water in a sterile 50 mL centrifuge tube.
    1. After dissolving the powder completely, use the solution immediately. Alternatively, store the solution at 4 °C (up to a week) or at -20 °C (up to a month) prior to use.
    CAUTION: Both substrate solutions are light sensitive, and should avoid light exposure whenever is possible. Multiple freeze-thaw cycles of the solution can cause instability of the substrate and shorten its shelf-life.

4. Prepare the stock solution of phorbol 12-myristate 13-acetate (PMA) in molecular grade dimethyl sulfoxide (DMSO) to have a concentration of 500 µg/mL. Make aliquots of the stock solution (10 µL in a 500 µL tube) and store them at -20 °C.
5. Prepare LPS (E-coli K12) stock solution in sterile, endotoxin free water at a concentration of 5 mg/mL, and make aliquots for long-term storage at -20 °C. Prepare a working LPS solution by diluting the stock LPS into phosphate buffered saline (PBS) at a concentration of 100 µg/mL and store at -20 °C prior to use.
CAUTION: The reagent preparation for culture uses should be carried out in a biosafety cabinet, and all the containers should be sterilized prior to use. Repeated freeze-thaw cycles of PMA and LPS solutions should be avoided.

2. Culture of THP-1 reporter cell-derived macrophages

1. **Use two THP-1 reporter cell lines: THP-1-XBlue and THP-1-Dual cells. The former has a SEAP reporter gene controlled by NF-κB/AP-1, and the latter has a dual reporter gene system, the same SEAP reporter gene and a IRF controlled luciferase gene. Their culture procedures are identical except the selection culture media.**
    1. Thaw a working cell stock (~5 x 10^6 cells) in 10 mL of R10 medium, spin down the cells at 300 x g for 5 min. Resuspend cells in 10 mL of R10 medium, and transfer them into a T75 culture flask. Exchange the culture medium every 2-3 days until cells reach a density of 1 x 10^6 cells/mL.
    2. When cell growth reaches its capacity, passage cells to reduce the cell density to in the range of 2-5 x 10^5 cells/mL, so the cells can continue to grow.

3. After at least 1 passage, start to culture the cells in the selection culture medium: R10-Z for THP-1-XBlue, and R10-ZB for THP-1-Dual. After culturing the cells with selection culture medium for at least 1 passage, cells are ready for the experiment.
   CAUTION: Cell overgrowing can lead to significant cell death; the cell viability should maintain >98%. Keep a record of the passage number as the cells may behave differently after many passages (> 20 passages).
   NOTE: Cell culture flasks can be reused several times to save costs; however, this practice may increase the risk of general contamination and cross-contamination among different flasks (if handling different cell lines at the same time). During media exchange and cell passage, the centrifugation is set at 300 x g for 5 min.

2. **To perform reporter cell assay, seed cells into a 96-well flat-bottom cell culture plate and differentiate them into macrophages. The procedures are described in the following.**
   1. Transfer the cells from the flask to a centrifuge tube, spin down the cells at 300 x g for 5 min, and resuspend them in the R10 medium at a concentration of 1 x 10^6 cells/mL. Add aliquots of the PMA solution into the cell suspensions with a final concentration of 50 ng/mL.
   2. Transfer 100 µL of the cell suspensions into each well of the 96-well plate using a multi-channel pipette. Incubate the plate at 37 °C (in a cell culture incubator) for 24 h.
   3. After incubation, carefully remove the culture medium using a vacuum aspirator (or with a multi-channel pipette), and gently wash the cells with PBS (100 µL/well) twice; add 100 µL of fresh R10 medium in each well. Rest the cells for 2 days in an incubator before conducting the reporter assay.
      CAUTION: The resting step is very important to allow cells to calm down after PMA stimulation to their normal quiet status. This can significantly reduce the background signals of the reporter genes under unstimulated conditions.
      NOTE: After 24 stimulation with PMA, cells are differentiated into macrophage-like phenotype, with a characteristic of cell adhesion at the bottom of the well, and a morphological feature of pseudopodia.

3. Screening for Potential TLR4 Nano-inhibitors Using the Reporter Cells

NOTE: Since TLR4 signaling utilizes both MyD88-dependent and TRIF-dependent pathways, it is selected as the primary target to encompass a wide range of TLR signaling pathways. THP-1-XBlue reporter cells are used to mainly examine the NF-κB/AP-1 activation while THP-1-Dual cells are for IRF activation from the TRIF-dependent signal transduction.

1. **Identify the optimal LPS dose by generating a dose-response curve prior to the screening.**
   1. Dilute the working LPS solution to a final concentration of 0.01 - 100 ng/mL in R10 medium (make dilution in log10 scale). Layout the plate design and make dilution in a 96-well round-bottom culture plate. Make sure that each well has a minimum of 110 µL of solution after dilution.
   2. Gently remove the culture medium from the cell-seeded plate (from 2.2.3) using a vacuum aspirator.
   3. Transfer the LPS containing R10 medium prepared in the dilution plate (3.1.1) into the culture plate according to the layout of the samples. Incubate the plate at 37 °C (in a cell culture incubator) for 24 h.
   4. At 24 h, carefully transfer the supernatants (80 µL/well) into a new 96-well round-bottom plate. Conduct the colorimetric and/or luciferase luminescence assay on these solutions immediately or store them at 4 °C (several hours) or -20 °C (days) prior to the assay development.
      NOTE: The design of plate layout should be simple and clear for experiment and data analysis. For each condition, 2-4 replicates should be considered. Always include a negative control group (LPS null). It is recommended to use THP-1-XBlue cells for NF-κB/AP-1 activation as the Dual cells have a relatively high background of NF-κB/AP-1 activation after being differentiated into macrophages. However, it is feasible to use the Dual cells for reporting both NF-κB/AP-1 and IRF activation.

2. **Develop the colorimetric assay for the NF-κB/AP-1 activation and the luciferase luminescence assay for IRF activation.**
   1. To assess the NF-κB/AP-1 activation, transfer 20 µL of supernatant of each sample into a new 96-well flat-bottom culture plate; and add 180 µL of pre-warmed SEAP substrate solution into each well. Incubate the plate at 37 °C for 1 - 2 h to allow the color development (pink to dark blue). Collect the absorption at 655 nm on a plate reader.
      CAUTION: The incubation time can be varied based on the color development (30 min up to overnight incubation). It is recommended to wait until the optical density (O.D.) of the darkest color reaches above 1, while avoiding the saturation of the color development (O.D >3).
   2. For the analysis of IRF activation, transfer 10 µL of supernatant of each sample into a 96-well clear flat-bottom white plate. Add the luciferase solution (50 µL) and immediately collect the luminescence well by well.
      NOTE: It is highly recommended to use a luminescence plate reader with an auto-injection function to ensure the consistency in the luminescence reading from well to well and from plate to plate. If the substrate solutions are manually injected, please ensure a consistent incubation time and reading set-up for each well.

3. **Conduct the screening assay on various peptide-GNP hybrids with 10 ng/mL LPS stimulation (based on 3.1). Follow the same procedure in 3.2 for the reporter assay development.**
   1. Concentrate the peptide-GNP hybrids to 200 nM in R10 medium using the centrifugation method. Centrifuge 20 volumes of the hybrid solution (10 nM) at 18,000 x g for 30 min, and carefully discard the supernatants. Collect the hybrids (at the bottom of the tube) into a tube, wash them with PBS twice, and re-suspend the hybrids in one volume of the R10 medium.
   2. Mix equal volumes of the concentrated hybrids and the LPS (20 ng/mL) containing R10 medium to have a final concentration of the hybrids and LPS to be 100 nM and 10 ng/mL, respectively.
   3. Remove the culture medium from the cultured plate (2.2.3) and add 100 µL of the mixed solution into each well (3 replicates for each condition); include a negative control (medium only) and a LPS control (10 ng/mL LPS without hybrids).
   4. After 24 h incubation at 37 °C, transfer the medium of each well into a tube and centrifuge the tubes at 18,000 x g, 4 °C for 30 min. Collect the supernatants (50-80 µL/tube) into a 96-well round-bottom plate, and perform the reporter assay as described in 3.2.
      CAUTION: When discarding the supernatants after centrifugation, do not agitate the hybrids at the bottom of the tube.

NOTE: The centrifugation in the step 3.3.4 is very important to remove the non-internalized nanoparticle hybrids from the culture medium, and can avoid the interference of the hybrids with the colorimetric/luminescence readings. This is because the gold nanoparticles can absorb wide wavelengths of light depending on their size and agglomeration.

4. Validating the Inhibitory Effect of the Potential Candidates

NOTE: To confirm the inhibitory effect of the potential candidates from the screening, two approaches are employed. One is to examine the dose responses of the stimulants (LPS) at a fixed hybrid concentration (or the other way around); the other is to directly look at the inhibition on the NF-κB/AP-1 and IRF3 signals via immunoblotting.

1. In approach one, perform the reporter assay with 100 nM of the lead hybrids and two LPS concentrations at 1 ng/mL and 10 ng/mL following the same procedures described in 3.3. Include an inactive hybrid (based on the screening results) as a hybrid control for comparison.
2. **For the immunoblotting approach, please follow the standard experimental procedures.**
    1. Culture THP-1 cells in R10 medium, seed the cells into a 12-well culture plate (2 x 10^6 cells/well), and differentiate them into macrophages by treating the cells with 50 ng/mL PMA for 24 h followed by resting for 2 days.
    2. After cell differentiation, stimulate cells with 10 ng/mL LPS with/without the hybrids (100 nM) over time (up to 4 h). At various time points (0, 5, 15, 30, 60, 120 and 240 min), prepare the cell lysates for immunoblotting. Include an inactive hybrid as a control.
    3. Probe the signals of IκBα, phosphorylated p65, and phosphorylated IRF3 to examine the inhibitory effect of the lead hybrids on the activation of NF-κB and IRF3. Probe the β-actin and total IRF3 signals as internal controls.
    NOTE: The signal transduction often occurs much faster (in a few hours) then the expression of the reporter enzymes SEAP and luciferase (24 h). It is highly recommended to also perform a viability assay of the tested hybrids at 24 h as another validation method.

5. Evaluating the TLR Specificity

NOTE: To investigate the TLR specificity of the lead peptide-GNP hybrid, other TLR signaling pathways are tested, including TLR2, TLR3 and TLR5. TLR7, 8 and 9 are excluded because the THP-1 derived macrophages do not respond well to the stimulation of these TLRs due to the lack of TLR7, 8 and 9 expression in macrophages[34].

1. Test various concentrations (1 ng/mL to 25 μg/mL) of the ligands specific to TLR2 (Pam3CSK4), TLR3 (poly I:C) and TLR5 (flagellin) on both reporter cells derived macrophages to obtain the optimal concentration following the same procedure in 3.1 and 3.2.
2. Treat the cells with the mixtures of the lead hybrid (100 nM) and each TLR ligand at the concentration obtained from 5.1 to evaluate the inhibitory specificity of the lead hybrid according to the experimental procedure described in 3.3. Include the inactive hybrid as a control for comparison.

Representative Results

The overall experimental approach is illustrated in **Figure 1**. The two THP-1 reporter cell lines, THP-1-XBlue and THP-1-Dual, are used to fast screen the TLR responses by probing the activation of NF-κB/AP-1 and IRFs, respectively. The activation of NF-κB/AP-1 can be detected by the SEAP colorimetric assay, whereas IRF activation is monitored by luciferase luminescence. The monocytic THP-1 cells can be easily derived into macrophages to screen the nanodevices for their immunomodulatory activity on the innate phagocytic immune cells. With the reporter system, the screening can be conducted in a high-throughput fashion; such an approach is versatile to the discovery of new immunotherapeutics, particularly targeting on innate immune signaling such as TLR signaling.

The screening procedure and representative results are shown in **Figure 2**. Briefly, the reporter cells are seeded into a 96-well plate and derived into macrophages (**Figure 2A** and **2B**). Different concentrations of the TLR ligands (TLR4 as an example) are first tested to obtain the optimal concentration for the actual screening of the peptide-GNP hybrids. The TLR4 stimulation by LPS resulted in the activation of NF-κB/AP-1 and the production of SEAP. The released SEAP converted the substrate and changed its photophysical property, which could be monitored by the shifting in the light absorption, leading to the solution color change (**Figure 2C**). Such a change is proportional to the amount of SEAP released upon stimulation, and can be quantified by measuring the absorbance at 655 nm on a spectrophotometer (**Figure 2D**). Similarly, the activation of IRFs (triggered by LPS) led to the expression of luciferase, which catalyzed the substrate to produce luminescence (**Figure 2E**). Based on these dose responses, an optimal concentration of LPS (10 ng/mL) was used to screen a small previously established library of peptide-GNP hybrids (**Table 1**). The fabrication of the hybrids and their physicochemical characteristics were described in our previous publications[30,31,32]. From the screening, a group of hybrids (P12 and its derivatives) were identified for their potent inhibitory activity on both NF-κB/AP-1 and IRF activation triggered by LPS (**Figure 2F**); interestingly, the hybrid P13, just slightly different from P12 in the peptide coatings, did not have any inhibitory activity, which could be served as a hybrid control for comparison. The P13 derivatives showed various degree of mild inhibitory activity depending on the other decorated peptide on the surface.

After identifying the lead hybrid, it is important to validate the inhibitory activity. The inhibition was first confirmed by examining the different ratios of the hybrid to LPS to exclude potential false positive results due to technical artifacts. As the concentration of LPS increased, the inhibitory effect of the hybrid (at a fixed concentration) reduced as expected (**Figure 3A** and **3B**). To further ensure that the observed inhibition from the reporter assays was indeed a result of down-regulating the NF-κB and IRF signaling by the lead hybrid, the immunoblotting was conducted to directly assess the protein signal transduction over time. The activation of NF-κB and IRF3 was examined by probing the phosphorylation of the NF-κB subunit p65 and the degradation of the NF-κB inhibitor IκBα, and phosphorylation of IRF3, respectively. As shown in **Figure 3C**, the lead hybrid P12 could reduce p65 phosphorylation, inhibit IκBα degradation, and delayed IRF3 phosphorylation, while the inactive hybrid P13 could not (data not shown). All these results confirmed that the identified lead hybrid was able to inhibit LPS-mediated TLR4 signaling by down-regulating both NF-κB and IRF3 activation.

In addition to TLR4 signaling, the inhibitory activity of the lead hybrid was further evaluated on other TLR pathways including TLR2, TLR3 and TLR5 to address the TLR specificity. As shown in **Figure 4**, the lead hybrid P12 was able to reduce TLR2- and TLR5-mediated NF-κB/AP-1 signaling, as well as TLR3-mediated IRF activation; again, the inactive hybrid P13 did not show any inhibitory activity. These results suggested that the identified lead hybrid has a potent inhibitory activity on multiple TLR pathways.

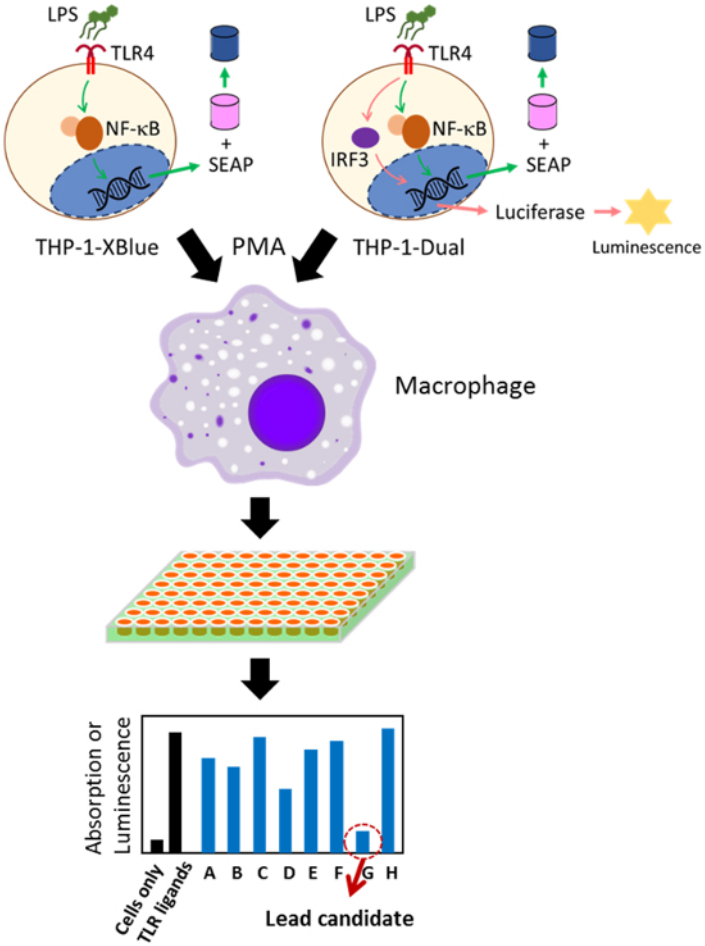

Figure 1: Overall experimental approach of high-throughput screening of TLR inhibitors using the reporter cell assay. Two reporter cell lines are used: THP-1-XBlue and THP-1-Dual. The former has a SEAP reporter gene under the control of NF-κB/AP-1 activation, whereas the Dual system has an additional luciferase reporter gene under the control of IRFs activation. These cells can be easily differentiated into macrophages for high-throughput screening of immune modulatory nanoparticles on innate immune signaling. Please click here to view a larger version of this figure.

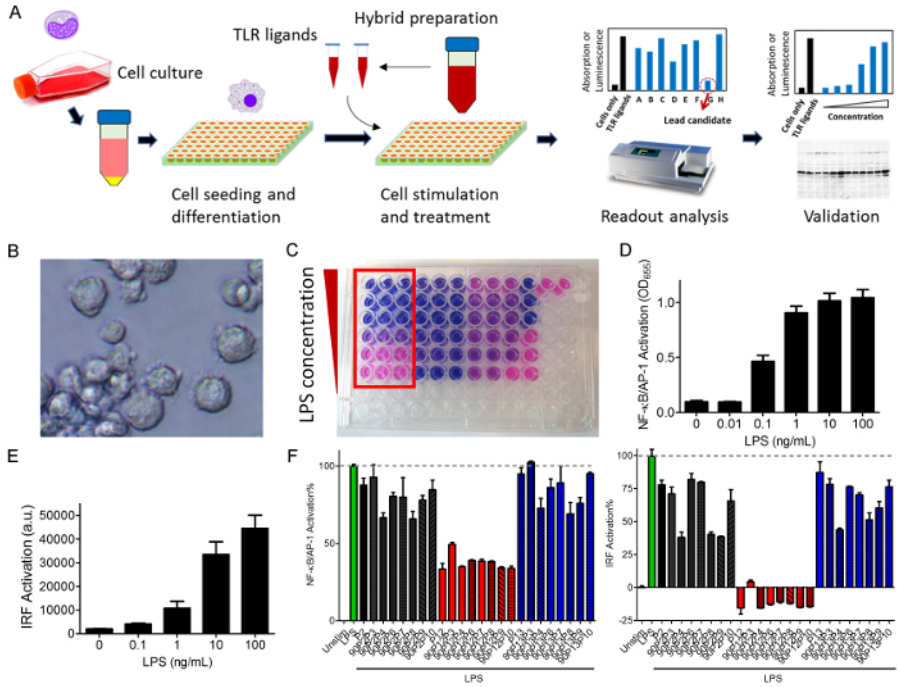

Figure 2: High-throughput screening of nano-inhibitors on TLR4 signaling.
(**A**) A scheme of experimental procedures. (**B**) An optical microscopic image of differentiated macrophages (200x magnification). (**C**) A representative image of the solution color change from the SEAP reporter assay; the color of the substrate solution turned into purple or dark blue (from original pink) depending on the SEAP expression in the culture medium. (**D**) Quantitative analysis of the SEAP substrate absorption at 655 nm in response to LPS stimulation. (**E**) The luciferase luminescence from the IRF reporter system in proportion to LPS stimulation. (**F**) High-throughput screening identifying the lead peptide-GNP hybrid P12 and its derivatives in inhibiting both NF-κB/AP-1 and IRF pathways of TLR4 signaling; the hybrid concentration = 100 nM; the LPS concentration = 10 ng/mL; the bar represents mean ± standard deviation; n = 2. Please click here to view a larger version of this figure.

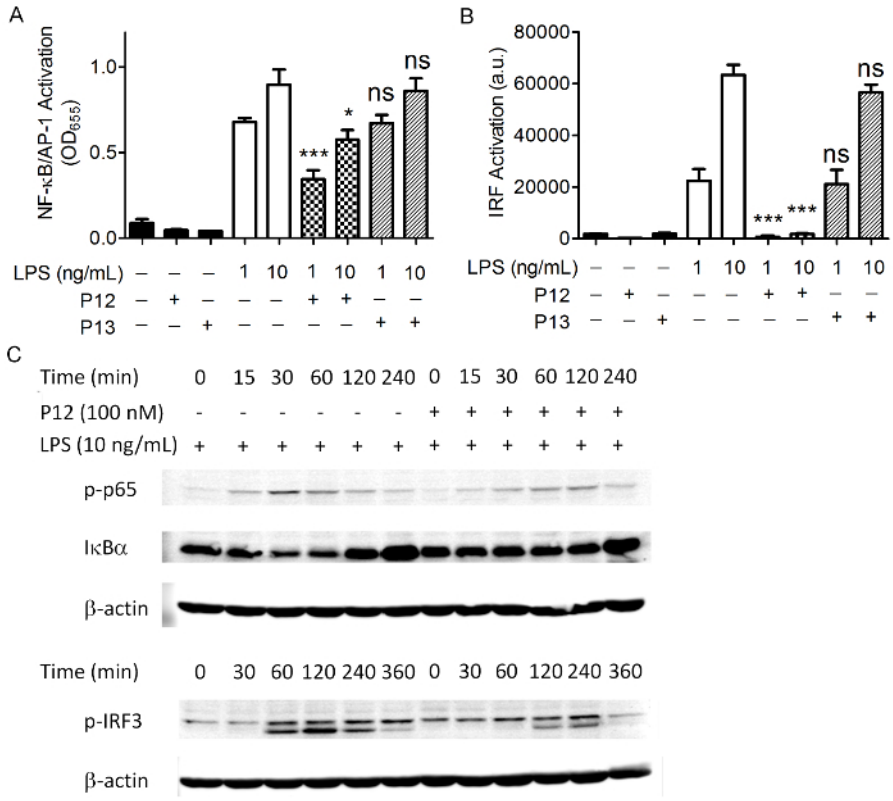

Figure 3: Validation of the inhibitory activity of the lead hybrid. Confirmation of the inhibitory effect of the lead hybrid with various concentrations of LPS on both (**A**) SEAP and (**B**) luciferase reporter systems. (**C**) Confirming the inhibition of the lead hybrid on the NF-κB and IRF3 signaling via immunoblotting. The inactive hybrid P13 was used as a hybrid control for comparison. The hybrid concentration = 100 nM; the LPS concentration = 10 ng/mL; the bar represents mean ± standard deviation; n = 3; * and *** denote $p < 0.05$ and $p < 0.001$, respectively, using one way ANOVA analysis. Please click here to view a larger version of this figure.

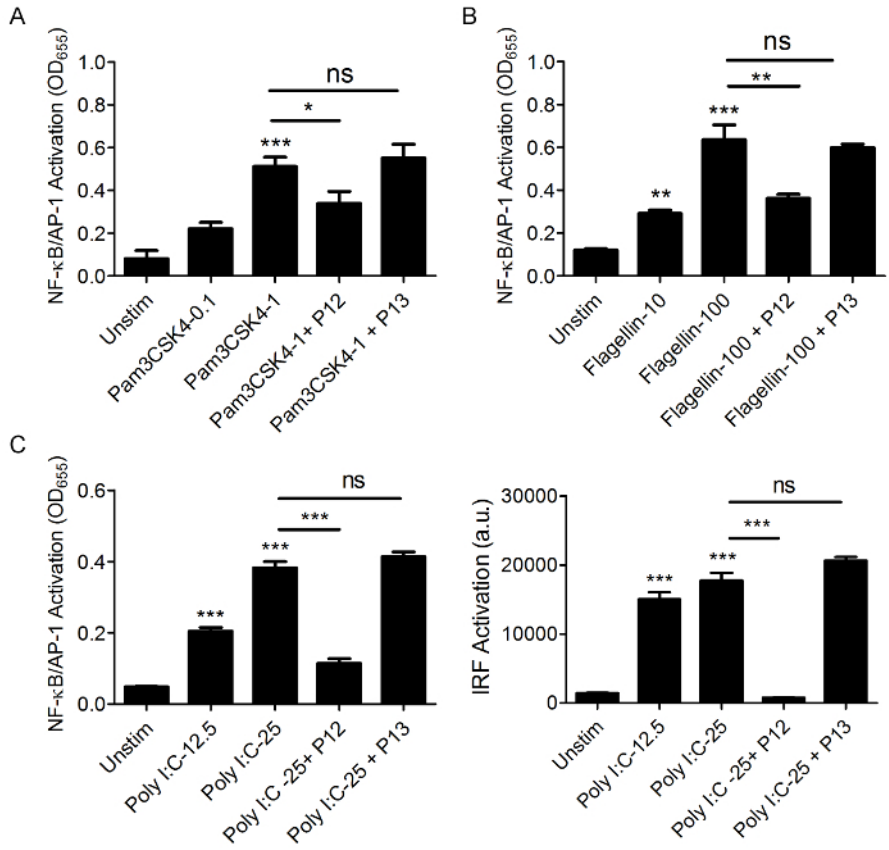

Figure 4: Inhibitory effect of the lead hybrid on other TLR signaling pathways.
The inhibition of NF-κB/AP-1 by the lead hybrid following the TLR2 stimulation (Pam3CSK4 = 1 ng/mL) (**A**) and TL5 stimulation (flagellin = 100 ng/mL) (**B**). (**C**) The reduction of both NF-κB/AP-1 and IRF signaling by the lead hybrid following the TLR3 stimulation (poly I:C = 25 µg/mL). The hybrid concentration = 100 nM; the bar represents mean ± standard deviation; n = 3; *, **, and *** denote p <0.05, p <0.01, and p <0.001, respectively, using one way ANOVA analysis; ns: non-significant. Please click here to view a larger version of this figure.

Hybrid Name	Peptide Coatings		Sequence of the Decorated Peptides
P2	100% P2		P2: n-CAAAAE-c
90P2P3	90% P2	10% P3	P3: n-CAAAAS-c
90P2P4	90% P2	10% P4	P4: n-CAAAAW-c
90P2P6	90% P2	10% P6	P6: n-CAAAAL-c
90P2P7	90% P2	10% P7	P7: n-CAAAAI-c
90P2P8	90% P2	10% P8	P8: n-CAAAAF-c
90P2P9	90% P2	10% P9	P9: n-CAAAAY-c
90P2P10	90% P2	10% P10	P10: n-CAAAAV-c
P12	100% P12		P12: n-CLPFFD-c
90P12P3	90% P12	10% P3	P13: n-CLPAAD-c
90P12P4	90% P12	10% P4	
90P12P6	90% P12	10% P6	
90P12P7	90% P12	10% P7	
90P12P8	90% P12	10% P8	
90P12P9	90% P12	10% P9	
90P12P10	90% P12	10% P10	
P13	100% P13		
90P13P3	90% P13	10% P3	
90P13P4	90% P13	10% P4	
90P13P6	90% P13	10% P6	
90P13P7	90% P13	10% P7	
90P13P8	90% P13	10% P8	
90P13P9	90% P13	10% P9	
90P13P10	90% P13	10% P10	

Note: Red and blue colors indicate the amino acid variations in the sequence among the same peptide groups

Table 1: A small library of peptide-GNP hybrids established in our early studies.
The hybrids are made of a gold nanoparticle core (~13 nm in diameter) and various hexapeptide coatings on the surface. Please click here to view a larger version of this figure.

Discussion

Since TLRs are involved in the pathogenesis of many inflammatory diseases, they have emerged as therapeutic targets for the modulation of immune responses and inflammatory conditions. However, the clinical development of therapeutics to inhibit TLR signaling pathways has had limited success to date. The antimalarial drug hydroxychloroquine which inhibits TLR7 and TLR9 is in clinical use[35,36]. Similarly, only a limited number of compounds have progressed to clinical trials including eritoran, a TLR4 antagonist, that exhibited potent inhibitory effects on LPS-mediated inflammatory responses in pre-clinical studies[37,38], showed positive results in the phase I/II clinical trials[39,40,41], but ultimately the phase III trial failed to reduce the mortality of patients with severe sepsis[42]. This failure has many possible reasons, one being that sepsis-associated inflammatory responses are often triggered through multiple TLR pathways, and thus blocking only TLR4 may not be sufficient to reduce the overwhelming inflammation. Therefore, developing novel, potent poly-TLR inhibitors could overcome such clinical challenges and become the next generation anti-inflammatory therapeutics. The screening strategy and protocol described here are expected to serve as an efficient experimental tool in studying TLR signaling, and more importantly as a drug discovery platform to accelerate the search for the next generation TLR inhibitors.

This screening approach provides several advantages in searching for new TLR inhibitors. First, the screening can be achieved in a high-throughput fashion using the reporter cell systems that are fast, sensitive and quantitative. Second, with both reporter cell lines, the screening can be done to cover a wide range of TLR signaling cascades, including the NF-κB/AP-1 pathway and the type I interferon signaling (via IRFs); thus, they are ideal for the screening of multiple TLR pathways. Third, these reporter cells are genetically engineered from the human monocytic THP-1 cell line, which is a good model system to study interventions targeting the innate immune response. Fourth, the cell line is easily maintained, and particularly, can be differentiated into macrophages; and since macrophages play a key role in many disease-associated inflammatory conditions, they serve as an ideal target for the screening of immunotherapeutics targeting TLR signaling. Fifth, monocytes and macrophages have impressive phagocytic capacity, which allows for high cellular uptake of the nanoparticles, making them especially suitable for studying nanoscale therapeutic agents. Furthermore, this screening protocol can be applied to search for not only the nano-based therapeutic agents, but also other types of bio-active compounds.

Although this screening platform is very versatile and robust, some caution must be applied to avoid false discovery. The screening is primarily based on the reporter assay, which relies on the expression of the reporter gene under control of specific signaling events. Ideally, the reporter gene expression (SEAP and luciferase) is proportional to the intensity of the signal transduction pathway of interest, and the impact of the drug candidates is reflected in the assay readouts. However, in reality, any biological events occurring upstream of the reporter gene expression could affect the result, sometimes leading to a false positive discovery. For instance, low expression of SEAP could result from the inhibition of the protein synthesis process rather than from the down regulation of TLR signaling[43]. To avoid such a false discovery, the inhibitory activity of the identified candidates should always be validated, and the gold standard method is to directly look at the signaling pathways via immunoblotting. Another important aspect in screening nanoparticle-based therapeutic agents is the surface properties of these nanodevices. Based on the surface modifiers, the nanodevices can have various biological activity. However, they may also have non-specific binding capability to certain biomolecules. In the case of non-specific binding to SEAP or luciferase, the catalytic activity to the substrates could be compromised by these nanodevices, leading to potential false discovery. Including extra control groups (*e.g.*, nanodevice only) in the screening reduces the risk of false discovery. Last but not the least, the cytotoxicity of the identified lead candidates must be examined to exclude cytotoxicity as a contributing factor to false discovery. This can be done simultaneously during the screening process using a standard viability assay (*e.g.*, MTS or MTT), or in a separate experiment.

Disclosures

The authors have nothing to disclose.

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