Establishment of novel reporter cells stably maintaining transcription factor-driven human secreted alkaline phosphatase expression.

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Running title: Novel reporter constructs using EBNA1 and SEAP.

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Abbreviation used in this paper:

HTS, high-throughput screening; SEAP, human secreted embryonic alkaline phosphatase; EBV, Epstein-Barr virus; EBNA, Epstein-Barr virus nuclear antigen; OriP, origin of plasmid replication; NFkB, nuclear factor-kappa B; IRF, interferon regulatory factor; LPS, lipopolysaccharide; TLR, toll-like receptor; Damps, danger-associated molecular patterns; CIRP, Cold-inducible RNA-binding protein; PTMA, prothymosin-alpha; PRRs, pattern recognition receptors; ELAM, endothelial-leukocyte adhesion molecule; ISG, IFN stimulated gene; ISREs, interferon-stimulated regulatory elements;

Key words: EBNA1; SEAP; NFkB; IRF; Drug screening; Transcription activity

Abstract

Background: Transcriptional regulation is very important and pivotal function in myriad biological responses. Thus, methods to determine transcriptional activity are required in not only basic medical research but also drug discovery. We established novel reporter constructs using human secreted embryonic alkaline phosphatase (SEAP) and Epstein-Barr virus nuclear antigen (EBNA) 1, which can maintain constructs synchronized to host cell replication. **Methods**: We established nuclear factor-kappa B (NFkB) or interferon regulatory factor (IRF) driven SEAP expression constructs and then, introduced them into culture cells. **Results**: The cells maintain reporter constructs for a long period in the culture and produce SEAP into culture supernatant in response to each specific ligand such as lipopolysaccharide (LPS) and interferon-beta. Measuring SEAP with chemiluminescence makes it possible to get high standard dynamic range applying to high-throughput screening in drug discovery in both 96 and 384 well format. We can also use it to determine transcriptional activity in the cells transfected with expression plasmid or treated with various toll-like receptor (TLR) ligands in a concentration-dependent manner and time-dependent manner. Finally, we demonstrated drug screening using number of natural products library. **Conclusion**: We for the first time established the two novel reporter cells and validated their quality and accuracy enough to carry out drug screening.

1. Introduction

Transcription factors specifically regulate spatiotemporal gene expression in myriad biological phenomena [1]. Determining specific transcriptional activity is required and applied for not only basic research but also drug discovery, especially to perform high-throughput screening (HTS). In inflammatory responses, specific transcription factors such as nuclear factor kappa B (NFkB) [2] and interferon regulatory factor (IRF) [3] induce transcriptional complexes and then, regulate appropriate target gene expressions. Components of outer pathogens such as lipopolysaccharide (LPS) and flagellin [4] or danger-associated molecular patterns (Damps) including S100 proteins [5], cold-inducible RNA-binding protein (CIRP) [6] and prothymosin-alpha (PTMA) [7] induces activation of NFkB via Toll-like receptors or other pattern recognition receptors (PRRs). While, type I interferon (IFN) induces activation of IRF and its binding with STATs through a cognate receptor [8]. Recently, we identified TRIM39R as a novel inducer of type I IFN responses [9, 10]. PTMA also induces type I IFN response via IRF3 [7].

Epstein-Barr virus (EBV) nuclear antigen (EBNA) 1 is one of viral proteins formed as dimer in EBV and has multi-functions including gene regulation, extrachromosomal replication and maintenance of the viral genome in episomal fraction of infected cells [11]. EBNA1 maintains and replicates plasmids containing EBV origin, also known as origin of plasmid replication (OriP) [11]. The plasmids are stably expressing transgenes in the host cells for a long period.

In this study, we for the first time established two novel reporter constructs using human secreted embryonic alkaline phosphatase (SEAP) and EBNA1, which can maintain constructs synchronized to host cell replication. Then, we could measure the transcription activity with high-sensitive dynamic range using chemiluminescence enough to perform. We also demonstrated that various activators induce NFkB transcriptional activity in the cells and that ectopic expression of TRIM39R induces NFkB and IRF

transcriptional activity in the cells. Eventually, we demonstrated drug screening using natural products library in the two novel reporter cells.

2. Materials and methods

2.1. Materials

PrimeStar GXL polymerase, Great EscAPe SEAP Chemiluminescent kit and In-Fusion HD cloning kit reagent were obtained from TaKaRa Bio, Japan. pEB-Multi-Neo and G418 were obtained from Wako, Japan. Normocin, Blasticidine, Hygromycin B Gold, QUANTI-Blue, HEK293T-TLR4-MD2-CD14, pNiFty2-SEAP and pNiFty2-56K-SEAP were obtained from InvivoGen, San Diego, CA, USA. LPS were obtained from Sigma-Aldrich, St. Louis, MO, USA. Recombinant human IFN-beta was obtained from Peprotech, Rocky Hill, NJ, USA. Recombinant human CIRP and Flagellin (high purity) were obtained from Adipogen life sciences, San Diego, CA, USA. Mouse S100A13 and PTMA were generated from *E coli* and then, were subjected to endotoxin removal resin. In both of them the endotoxin concentration was below 1EU per microgram protein. Natural products library was kindly gifted from Dr. Yamada and Prof. Takeda.

2.2. Plasmid construction

We cloned transcription factor response element and SEAP open reading frame using following primer set. The sense primer:5'-gttcgtccggggcggGGCCGCAATAAAATATCTTTATTTTCATTACATCTGTGTGTTGG-3' and the antisense primer: 5'-tgatctagagtcgcgGCCAGCTAGCTTCAATCCAGACGCTTGGAC-3'. The lowercase indicates homologous arms. We also cloned thymidine kinase promoter (pTK), EBNA1, origin of plasmid reprication (OriP) and Simian Virus 40 late polyadenylation signal (SV40 pAn) using following primer set. Sense primer: 5'-CGCGACTCTAGATCATAATCAGCCATACCACATTTGTAGAGG-3' and the

antisense primer:5'-CCGCCCCGGACGAACTAAACCTGACTACGACA-3' PCR reactions were performed using high-fidelity enzyme, Prime Star GXL polymerase with pEB-Multi-Neo, pNiFty2-SEAP which includes endothelial-leukocyte adhesion molecule (ELAM) minimal promoter containing five NFkB response elements or pNiFty2-56K-SEAP, which includes human IFN stimulated gene (ISG)-56 promoter containing two interferon-stimulated regulatory elements (ISREs) as a template. The condition was run for forty cycles using following protocol: 10 sec denaturation at 98 ºC; 15 sec annealing at 55 ºC; 5 min. Two DNA fragments containing pTK, EBNA1, OriP and SV40 pAn, and transcription factor response element (NFkB or IRF) and SEAP open reading frame were combined with homologous recombination using In-Fusion HD cloning kit reagent according to manufacturer's instruction. Then, reaction solutions were subjected to transformation with *E coli*. After that, we picked several colonies and extracted the plasmids. These were sequenced to confirm each sequence.

2.3. Cell culture and electroporation

HEK293 or HEK293/hTLR4-MD2-CD14 cells were cultured in DMEM (Invitrogen, CA, USA) containing 10% fetal bovine serum (FBS, Thermo Fisher Scientific, MA, USA) with or without Normocin (25 μg/ml), Blasticidine (5 μg/ml) and Hygromycin B Gold (50 μg/ml) at 37 ◦C with 5 % CO_2. Then, we introduced pEB-Multi-Neo, pEB- RO-NFkB or pEB- RO-IRF (10 μg) into 1x 10^6 cells using NEPA21 Transfection Electroporation (Nepa Gene Co., Ltd., Chiba, Japan). The condition is 115V, pulse width: 2.5 ms (second), pulse interval: 50 ms, times: 2 and attenuation rate: 10% in poring pulse, and 20V, pulse width: 50 ms, times: 5 and attenuation rate: 40% in transfer pulse. After that, the cells were cultured in DMEM containing 10% fetal bovine serum and G418 (25 μg/ml).

2.4. SEAP assay with colorimetric enzyme assay and chemiluminescence

The established reporter cells were seeded at 3x 10^4 or 3x 10^3 cells/well cultured onto 96 or 384 well

plate, respectively. 24 hours after the seeding, the cells were treated with or without LPS (0.001-10 μg/ml), IFN-beta (0.1-250 ng/ml), CIRP (0.0015-1.5 μg/ml), S100A13 (10 μg/ml) flagellin (0.01-100 ng/ml) or mouse PTMA (10 μg/ml) for 24 hours. The cells were also treated with 80 natural products in DMSO (the final concentraion is 0.5%) for 24 hours. Then, we collected supernatants from the treated cells. We also transfected pcDNA6 or pcDNA6-TRIM39R-V5 (3.175 – 50 ng) into 3x 10^4 cells in 96 well by Fugene HD as described [10]. 24 or 48 hours after the transfection, we collected the supernantants from the transfected cells. SEAP activity in the supernatant was determined by QUANTI-Blue or Great EscAPe SEAP Chemiluminescent kit 2.0 according to manufacturer's instruction. In brief, reagent was resolved in 50 mL endotoxin free water and then, put 20 μL reagent to 200 μL culture medium in 96 well. After that, we measured the absorbance at 630 nm using PHERAstar FS (BMG LABTECH JAPAN L.t.d., Saitama, Japna). If we performed chemiluminescence assay, added 4 volumes diluent buffer to supernatant and then, incubated at 65 degrees for 30 min in 96 or 384 well plate. After that, add 5 volumes SEAP substrate solution and then, kept at room temperature for at least 10 min. Finally we measured chemilminescence intensity by PHERAstar FS.

2.5. Statistics

Values are expressed as the mean ± standard error of the mean (SEM) from at indicated replicate samples in each experimental group; experiments were replicated to ensure consistency. Statistical significance was determined using Student's t-test. Values as *, **, ***, ****, *****, ******, *******, ********, ********* and ********** were considered to be statistically significant if their P values were $0.05 > P > 0.01$, $0.01 > P > 0.005$, $0.005 > P > 0.001$, $0.001 > P > 0.0005$, $0.0005 > P > 0.0001$, $0.0001 > P > 0.00005$, $0.00005 > P > 0.00001$, $0.00001 > P > 0.000005$, $0.000005 > P > 0.000001$ and $0.000001 > P$, respectively. Signal-to-Backgroud ratio (S/B), Signal-to-Noise ratio (S/N) and Z'-factor were calculated by formula as described below.

S/B = Average ligand/Average vehicle

S/N = (Average ligand - Average vehicle)/Standard deviation vehicle

Z'-factor = 1-(3x Standard deviation ligand+3x Standard deviation vehicle)/(Average ligand - Average vehicle)

3. Results

3.1. Establishment of novel reporter cells stably expressing transcription factor-driven SEAP expression.

In order to remarkably and stably determine a specific transcription activity, we established novel two constructs using combination of EBNA1, OriP, transcription factor response element, SEAP orf and SV40pAn (Fig. 1A). We named pEB-RO-NFkB and pEB-RO–IRF. pEB-RO-NFkB is composed of ELAM minimal promoter containing five NFkB response elements to monitor a transcription activity of NFkB, pEB-RO–IRF is composed of hISG-56 promoter containing two ISREs to monitor that of IRF (Fig. 1A). Because the two constructs are up to over 10 kbp, we performed electroporation as shown in Figure 1 B. We introduced them into HEK293T stably expressing human TLR4-MD2-CD14 and then, added 1 μg/mL LPS to culture medium containing colorimetric enzyme develop reagent changing to blue. We observed culture supernatant from the cells expressing NFkB-driven SEAP specifically changed to blue (Fig. 1C). While, in the cells introduced IRF-driven SEAP expression or empty vector, we did not observe changing to blue (Fig.1 C). To determine specific transcription activity in the cells, we performed colorimetric enzyme assay. Treatment of 10 μg/mL LPS for 24 hours induces significantly an absorbance at 630 nm (Fig. 1D). As shown in table 1, S/B and S/N, which are important in dynamic range and assay quality, and Z'-factor, which is a measure of statistical characteristic for HTS, were 11.025, 86.898 and 0.648, respectively. In preliminary experiment, we performed conventional method in the same cells treated with 10 μg/mL LPS. Its S/B, S/N and Z'-factor were less quality (table 1 upper). S/B, S/N and

Z'-factor should be required more than 3, 10 and 0.5, respectively to do HTS. Especially, the Z'-factor was below the requirement.

3.2. Measurement of SEAP with high standard dynamic range using chemiluminescence.

To obtain higher dynamic range in the reporter cells, we tried to applied chemiluminascence to SEAP assay. We succeeded in getting higher dynamic range in the cells stably expressing NFkB-driven SEAP at 24 hours after treatment of 10 μg/mL LPS (Fig. 2A left and table 1). The S/B, S/N and Z'-factor with chemiluminescence were 127.295, 313.773 and 0.675, respectively. While, we also could get high quality dynamic range in the cells stably expressing IRF-driven SEAP at 24 hours after treatment of 50 ng/mL IFN-beta (Fig. 2A right). The S/B, S/N and Z'-factor were 61.861, 716.293 and 0.522 although Z'-factor in an assay using absorbance was less than 0.5 (data not shown). IFN-beta also slightly induced NFkB-driven SEAP expression (Fig. 2A right).

To examine whether or not the two reporter cell systems can detect a ligand-induced transcription activity in a concentration dependent manner, we treated each cells treated with various concentration LPS or IFN-beta and 24 hours after that, determined SEAP activity. LPS ranging from 0.001 to 10 μg/mL significantly induced NFkB-driven SEAP activity compared to control cells (Fig. 2B left). Besides that, we could observe a significant concentration-dependency of the induction among 0.01, 0.1 and 1 μg/ mL (Fig. 2B left and data not shown). We also observed a significant concentration-dependency in the cells treated with IFN-beta ranging from 0.001 to 0.1 (Fig. 2B right). There were significant differences among 0.001, 0.01 and 0.1 μg/mL treatment (data not shown).

We also tried to examine whether or not we can observe changing of ligand-induced transcription activity in the supernatant from same culture cells, we collected the supernatant from each reporter cells treated with 50 ng/mL LPS or 100 ng/mL IFN-beta every 6 hours up to 24 hour after the treatment and then, measured the SEAP activity. In both the cells stably expressing NFkB- (Fig. 2C left) and IRF- (Fig.

2C right) driven SEAP, both of two ligands significantly induced transcription activity in a time-dependent manner.

Eventually, we could miniaturize the two novel reporter cell assays from 96- to 384-well format enough to perform HTS as shown in table 1. Even in 384-well format, we could obtain high-standard dynamic range and high-accuracy assay in independent measurements as shown in table 1.

3.3. Various ligand-induced transcription activity in the novel reporter cells.

We next tried to examine whether other inflammatory ligands induces transcription activity in the two reporter cells. Ten μg/mL S100A13, but not 10 μg/mL mouse PTMA, specifically and significantly induces NFkB-driven SEAP expression (Fig. 3A). The induction was 91-fold to control (Fig. 3A). Unexpectedly, mouse PTMA did not induce IRF-driven SEAP expression (Fig. 3B) although both of them were generated and purified from bacterial. Both S100A13 and PTMA induced a similar induction as S100 A 13 alone (85-fold to control) (data not shown). We also treated the cells stably expressing NFkB-driven SEAP with various concentrations of CIRP or Flagellin. One point five μg/mL CIRP induced significantly NFkB-driven SEAP expression (Fig. 3C). Flagellin ranging from 0.1 to 100 ng/mL significantly induces NFkB-driven SEAP expression in a concentration-dependent manner (Fig. 3D).

3.4. Exogenous gene expression-induced transcription activity in the novel reporter cells and screening using natural products library.

To examine whether or not the novel reporter cells can validate the effect of exogenous gene on specific transcription activities, we introduced pcDNA6-TRIM39R-V5 into the cells and then, quantified the SEAP activity. Twenty-four hours after the transfection of human TRIM39R induces NFkB-driven SEAP activity dependent on the amounts of DNA vectors (Fig. 4A). At 24 hours after the introduction, we could not observe any significant induction of IRF-driven SEAP expression by over-expression of human

TRIM39R (data not shown). However, 48 hours after the transfection, over-expression of TRIM39R significantly induced IRF-driven SEAP expression (Fig. 4B) although we could not observe any change dependent on the amounts of DNA vectors.

Finally, we tried to demonstrate drug screening from small number of natural products library, which are derived from plants used for Chinese herbal medicine and marine bacteria, using the two novel reporter assays. Each reporter cells incubated with 80 natural products for 24 hours and then, SEAP activity in the supernatant was quantified. We could get high-quality signal compared to vehicle (0.5% DMSO) in both NFkB- (Fig. 4C) and IRF- (Fig. 4D) driven SEAP expression. The assay quality of both two screening is sufficient to perform HTS (data not shown). We set threshold as vehicle + 3SD and identified 5 products as 'Hit' in NFkB-driven SEAP expression (Fig. 4C red circle). All of them are extracts from marine bacterium. While, we could not identify any products inducing IRF-driven SEAP expression (Fig. 4D).

4. Discussion

In this study, we for the first time established novel two reporter cells stably expressing NFkB- and IRF-driven SEAP expression. The cells maintain reporter constructs for a long period by EBNA1 in the culture and produce SEAP into culture supernatant in response to each specific ligand such as LPS and IFN-beta. At first, we introduced them into HEK293T cells stably expressing TLR4, MD2 and CD14 and then, demonstrated that LPS- or IFN-beta-induced transcription activation with high sensitivity in a concentration-dependent manner. Besides that, we could validate the time-dependent change of the transcription activity using supernatant from same culture cells. It has much more benefit compared to conventional reporter assay based on transient transfection. Besides colorimetric assay, we succeeded in establishing high standard and accuracy assay enough to carry HTS using chemiluminescence. It's notably that only 2.5 μL supernatant was sufficient to do the assay in 384 well plates. In preliminary study,

we introduced them into HEK293 or THP1 cells and then, also imiquimod-induced IRF-driven SEAP expression (data not shown). These reporter constructs can be applied to another culture models.

We also validated S100A13, fragellin and CIRP-induced NFkB activation in this reporter system. S100A13 binds to its receptor for advanced glycation end products (RAGE), probably resulting in an activation of NFkB in various cells [12]. RAGE, but not TLR4, is expressed in HEK293T [13, 14]. Although S100A13 has not yet been cleared that it binds and activates TLR4 directly, S100A13 probably activates NFkB via RAGE and/or TLR4 complex. We also observed that flagellin induced NFkB activation in a concentration-dependent manner. In coincidence with previous report that TLR5, but not TLR2, TLR4 and MD2, is endogenously expressed in HEK293T cells [14]. These reporter systems can validate transcription activity in physiological responses to flagellin or IFN-beta. LPS also could not induce SEAP expression in HEK293T cells introduced with pEB-RO-NFkB (data not shown). Recent study identified that CIRP initiates inflammatory responses in patients with hemorrhagic and septic shock via TLR4/MD2 complex [6]. In this report, 1.5 μg/mL CIRP effectively induced inflammatory cytokine from macrophages via TLR4/MD2 complex, and thus our result seems to be similar.

We previously identified human TRIM39R as a novel susceptible gene from Behcet disease using genome association studies [9] and that ectopic-expression of TRIM39R induces inflammatory cytokine pathways and/or type I interferon responses using microarray analysis at 48 hours after the introduction of exogenous TRIM39R expression [10]. In agreement with that, we could confirmed that ectopic-expression of human TRIM39R induces NFkB- and IRF-driven SEAP expression at 24 hours and 48 hours after the introduction of them although we did not observe any induction of IRF transcriptional activities at 24 hours. Therefore, our results suggested that firstly NFkB activation is occurred directly and subsequently IRF pathway is activated in the reporter cells.

Finally, we demonstrated drug screenings from 80 extracts, which are derived from plants used for Chinese herbal medicine and marine bacteria, using the two novel reporter cells and then, identified 5

extracts potentially affecting NFkB activation although there was no extract induced IRF activation.

5. Conclusion

In conclusion, we for the first time established the two novel reporter cells and validated their quality and accuracy enough to carry out HTS. In future, we are going to identify compounds from bigger chemical compound library by the novel reporter cells expressing IRF-SEAP for therapeutic products to multiple sclerosis because PEGylated IFN-beta has already been used [15].

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Figure legends

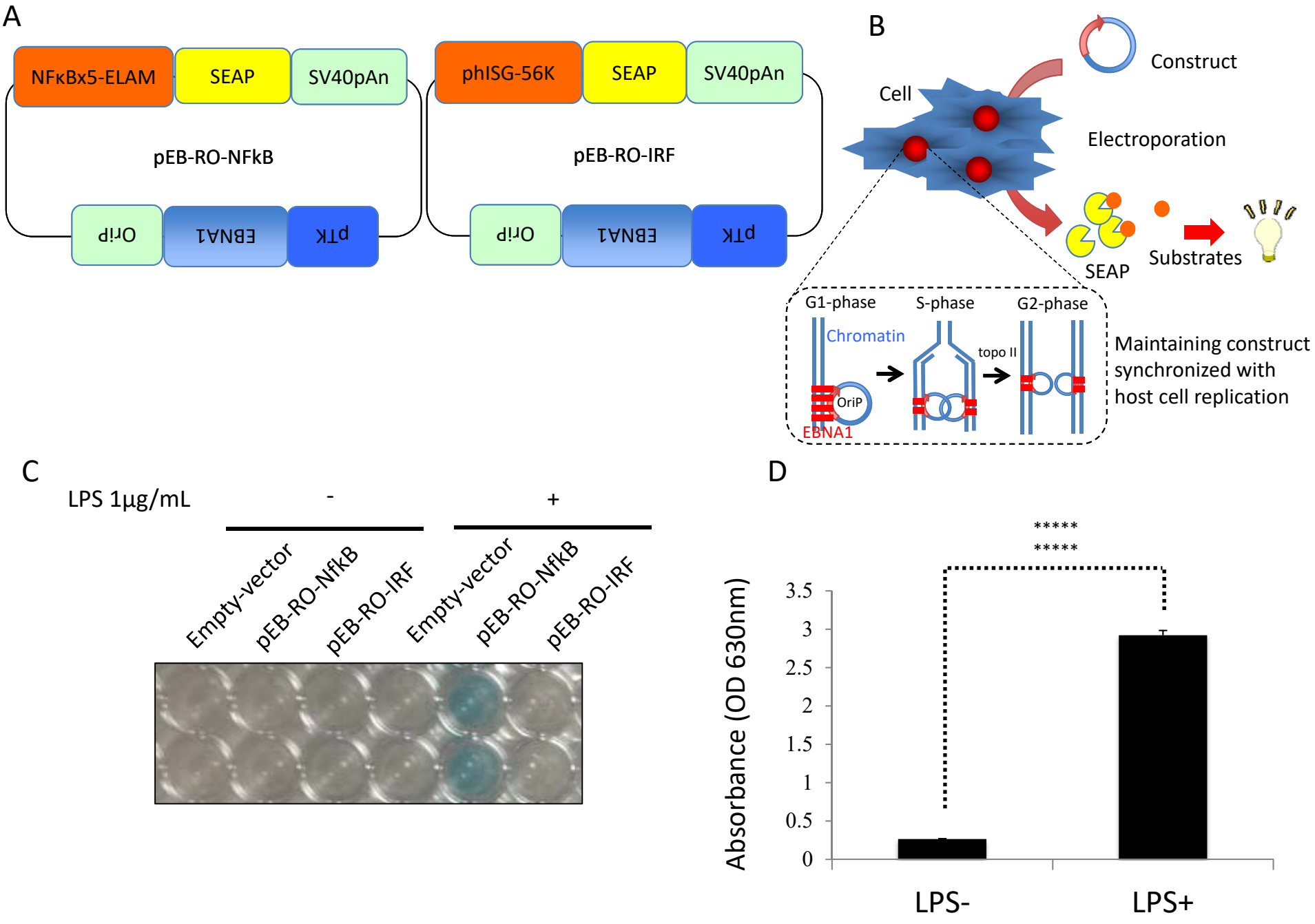
Figure 1. Establishment of novel reporter cells stably expressing transcription factor-driven SEAP expression. (A) The structures of novel two constructs stably expressing NFkB- (left, pEB-RO-NFkB) and IRF- (right, pEB-RO-IRF) driven SEAP. (B) Schimatic figure indicates procedures from electroporation of the constructs into culture cells to detection of SEAP activity and maintaining reporter constructs synchronized with host cell replication in culture supernatant. (C) The picture of LPS-induced specific SEAP expression in supernatant from HEK293T cells stably expressing human TLR4-MD2-CD14 introduced with empty vector, pEB-RO-NFkB or pEB-RO-IRF. One μg/mL LPS induced changing of medium color from colorless of DMEM phenol red minus to blue responded to SEAP proteins. (D) Quantification of SEAP activity in the supernatant from the cells treated with or without 10 μg/mL LPS using colorimetric assay. The data are expressed as means ± SEM (n= 20). *, **, ***, ****, *****, ******, *******, ********, ********* and ********** indicates P values were 0.05 > P > 0.01, 0.01 > P > 0.005, 0.005 > P > 0.001, 0.001 > P > 0.0005, 0.0005 > P > 0.0001, 0.0001 > P > 0.00005, 0.00005 > P > 0.00001, 0.00001 > P > 0.000005, 0.000005 > P > 0.000001 and 0.000001 > P, respectively (Student's t-test).

Figure 2. Measurement of SEAP with high standard dynamic range using chemiluminescence. (A) We quantified NFkB- (left) or IRF- (right) driven SEAP acitivity in the cells treated with or without each agonist such as 10 μg/mL LPS or 50 ng/mL IFN-beta using chemiluminescence. The data are expressed as means ± SEM (n= 4). (B) SEAP activity was quantified by chemiluminescence in the cells treated with various concentrations of LPS (left) or IFN-beta (right) ranging from 0.0001 to 10 μg/mL and 0.0001 to 0.1 μg/mL, respectively. The data are expressed as means ± SEM (n= 8). (C) LPS (left) or IFN-beta (right) -induced SEAP activity in the supernatant from same culture cells every 6 hours up to 24 hour after the treatment. The data are expressed as means ± SEM (n= 8). *, **, ***, ****, *****, ******, *******, ********, ********* and ********** indicates P values were 0.05 > P > 0.01, 0.01 > P > 0.005, 0.005 > P > 0.001, 0.001 > P > 0.0005, 0.0005 > P > 0.0001, 0.0001 > P > 0.00005, 0.00005 > P > 0.00001, 0.00001 > P > 0.000005, 0.000005 > P > 0.000001 and 0.000001 > P, respectively (Student's t-test).

Figure 3. Various ligand-induced transcription activity in the novel reporter cells. SEAP activity was quantified using chemiluminescence in the two reporter cells stably expressing NFkB- (A) and IRF- (B) driven SEAP treated with 5 μg/mL LPS, 250 ng/mL IFN-beta, 10 μg/mL S100A13 or 10 μg/mL mouse PTMA. The data are expressed as means ± SEM (n= 3). SEAP activity was quantified by chemiluminescence in the cells treated with various concentrations of CIRP (C) or flagellin (D) ranging from 0.0015 to 1.5 μg/mL and 0.01 to 100 ng/mL, respectively. The data are expressed as means ± SEM (n= 8). *, **, ***, ****, *****, ******, *******, ********, ********* and ********** indicates P values were 0.05 > P > 0.01, 0.01 > P > 0.005, 0.005 > P > 0.001, 0.001 > P > 0.0005, 0.0005 > P > 0.0001, 0.0001 > P > 0.00005, 0.00005 > P > 0.00001, 0.00001 > P > 0.000005, 0.000005 > P > 0.000001 and 0.000001 > P, respectively (Student's t-test).

Figure 4. Exogenous gene expression-induced transcription activity in the novel reporter cells and screening using natural products library. (A) We transfected empty vector or pcDNA6-hTRIM39R-V5 ranging from 3.125 to 50 ng per well to the cells stably expressing NFkB-driven SEAP (3x 10^4 cells) and quantified the SEAP activity 24 hours after the transfection. We also transfected the vectors in same amount to the cells stably expressing IRF-driven SEAP (3x 10^4 cells) and quantified the SEAP activity 48 hours after the transfection. The results of drug screening in the cells treated with 80 natural products using NFkB- (C) or IRF- (D) driven SEAP activity. Closed circle indicates ligand such as 50 ng/ml LPS or 100 ng/ml IFN-beta-induced SEAP activity (n= 8). Yellow circle indicates SEAP activity in vehicle (0.5% DMSO) (n= 8). Dashed line indicates threshold as average of vehicle + that of 3SD and then, identified 5 products (red circle).

Fig. 1


A
NFκBx5-ELAM
SEAP
SV40pAn
pEB-RO-NFkB
OriP
EBNA1
pTK
phISG-56K
SEAP
SV40pAn
pEB-RO-IRF
OriP
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Substrates
G1-phase
S-phase
G2-phase
Chromatin
topo II
OriP
EBNA1
Maintaining construct
synchronized with
host cell replication
C
LPS 1µg/mL
-
+
Empty-vector
pEB-RO-NfkB
pEB-RO-IRF
Empty-vector
pEB-RO-NfkB
pEB-RO-IRF
D
*****
*****
Absorbance (OD 630nm)
3.5
3
2.5
2
1.5
1
0.5
0
LPS-
LPS+

Fig. 2

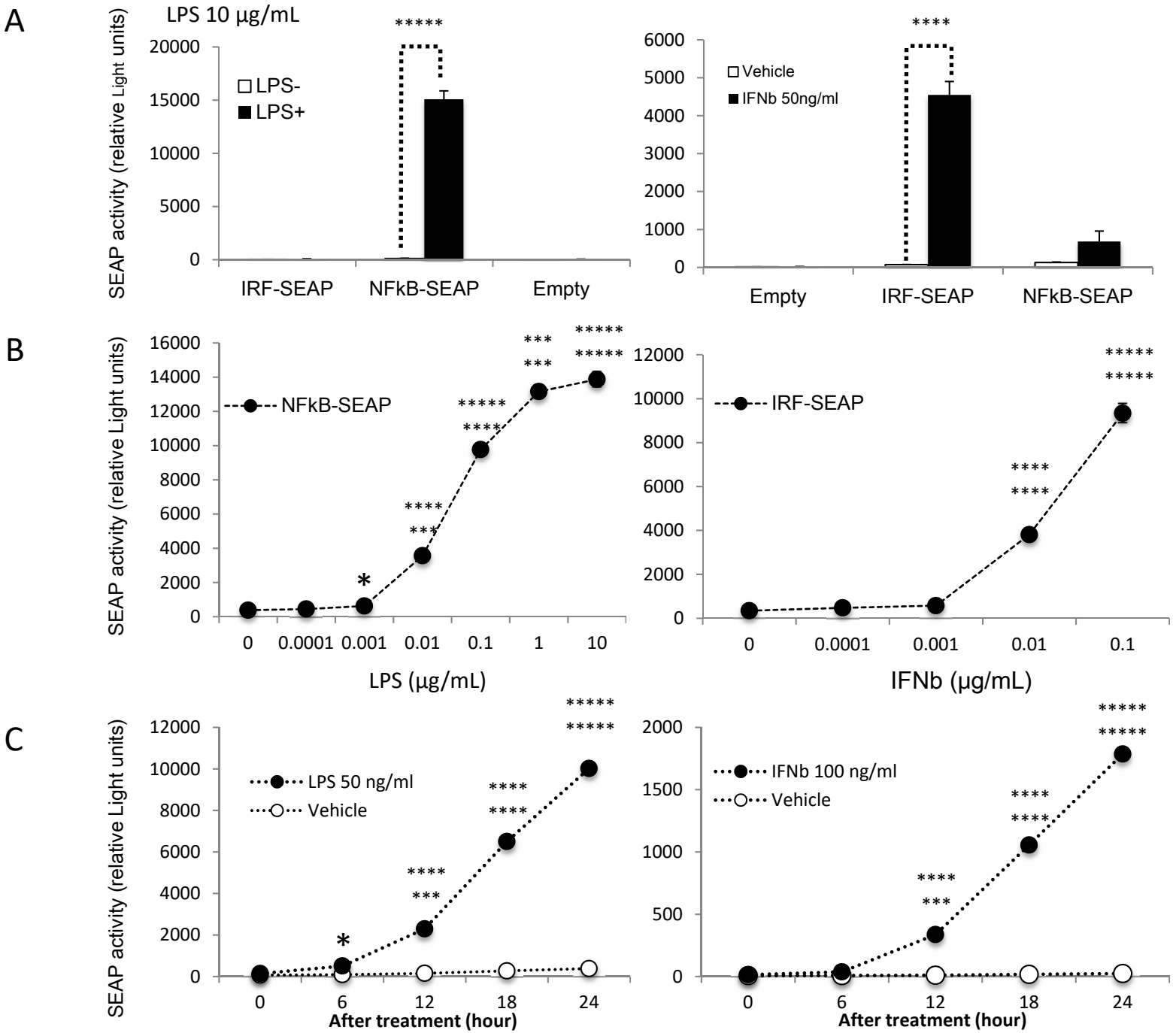

A
LPS 10 µg/mL
SEAP activity (relative Light units)
LPS-
LPS+
IRF-SEAP
NFkB-SEAP
Empty
Vehicle
IFNb 50ng/ml
B
NFkB-SEAP
IRF-SEAP
LPS (µg/mL)
IFNb (µg/mL)
C
LPS 50 ng/ml
Vehicle
IFNb 100 ng/ml
After treatment (hour)

Fig.3

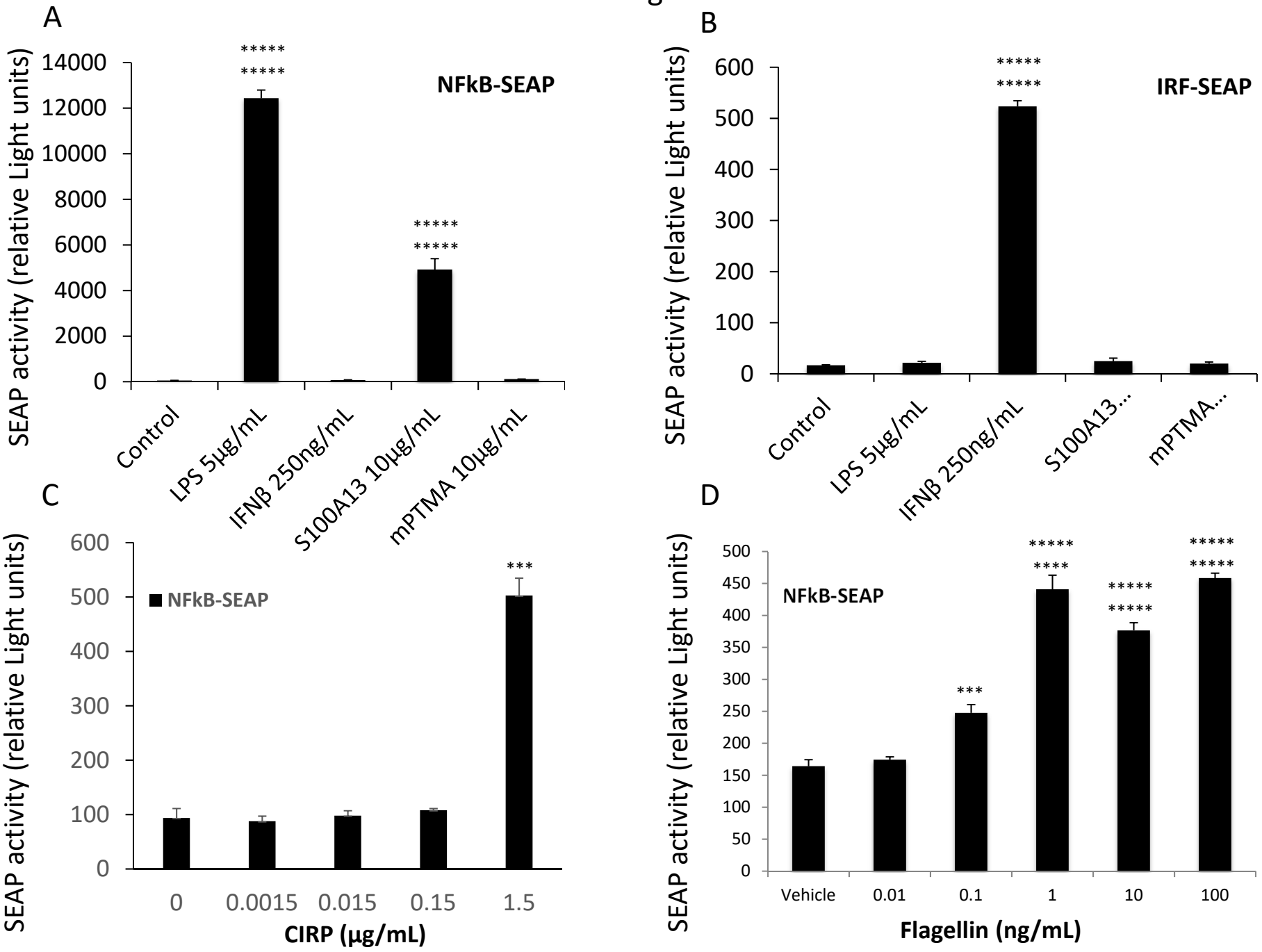
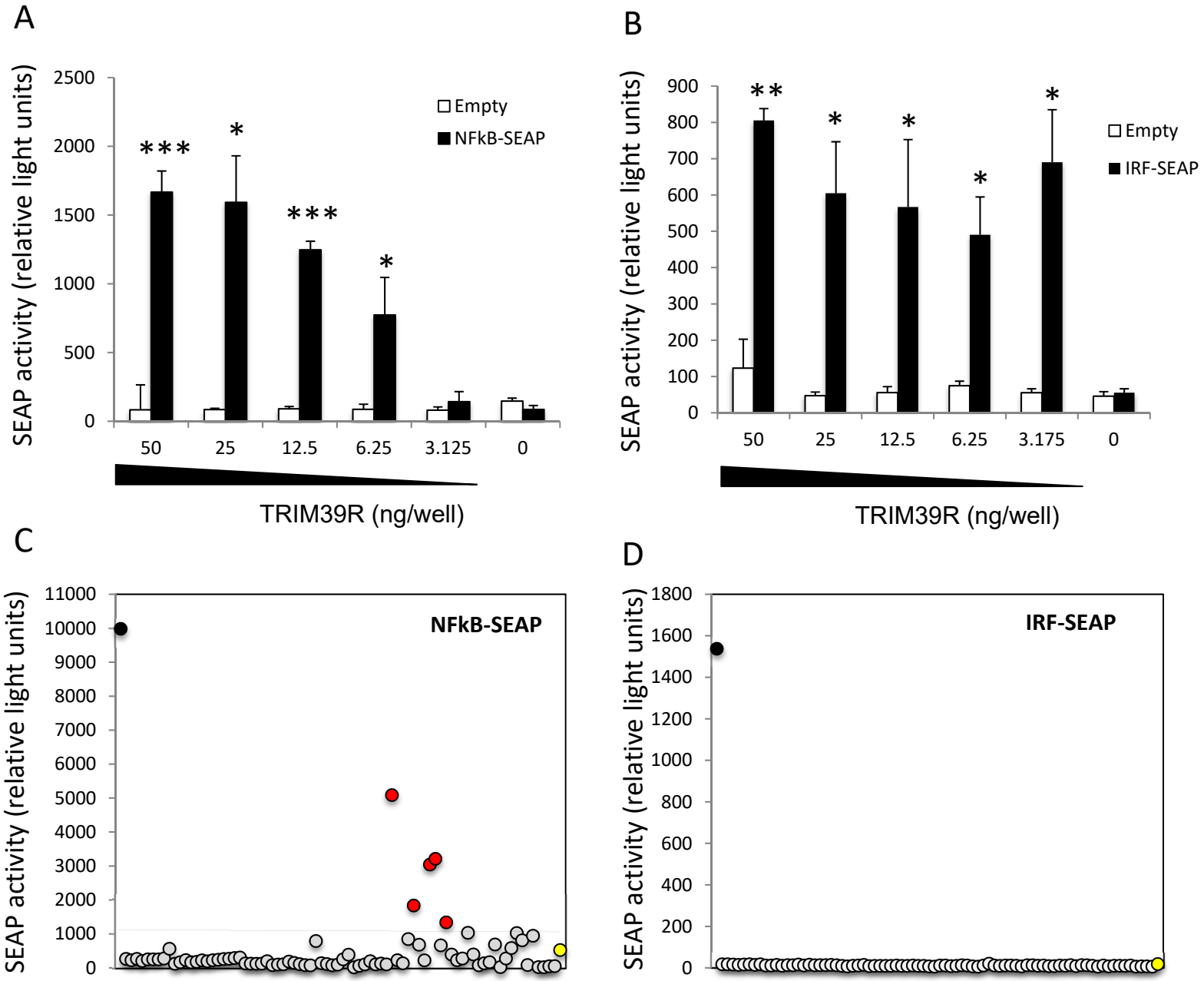

A
NFkB-SEAP
SEAP activity (relative Light units)
14000
12000
10000
8000
6000
4000
2000
0
*****
*****
*****
*****
Control
LPS 5µg/mL
IFNβ 250ng/mL
S100A13 10µg/mL
mPTMA 10µg/mL
B
IRF-SEAP
SEAP activity (relative Light units)
600
500
400
300
200
100
0
*****
*****
Control
LPS 5µg/mL
IFNβ 250ng/mL
S100A13...
mPTMA...
C
NFkB-SEAP
SEAP activity (relative Light units)
600
500
400
300
200
100
0
***
0
0.0015
0.015
0.15
1.5
CIRP (µg/mL)
D
NFkB-SEAP
SEAP activity (relative Light units)
500
450
400
350
300
250
200
150
100
50
0
***
*****
****
*****
*****
*****
*****
Vehicle
0.01
0.1
1
10
100
Flagellin (ng/mL)

Fig. 4


A
SEAP activity (relative light units)
2500
2000
1500
1000
500
0
***
*
***
*
Empty
NFkB-SEAP
50
25
12.5
6.25
3.125
0
TRIM39R (ng/well)
B
SEAP activity (relative light units)
900
800
700
600
500
400
300
200
100
0
**
*
*
*
*
Empty
IRF-SEAP
50
25
12.5
6.25
3.175
0
TRIM39R (ng/well)
C
SEAP activity (relative light units)
11000
10000
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
NFkB-SEAP
D
SEAP activity (relative light units)
1800
1600
1400
1200
1000
800
600
400
200
0
IRF-SEAP