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Chemiluminometric Immuno-Analysis of Innate Immune Response against Repetitive Bacterial Stimulations for the Same Mammalian Cells

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For monitoring of human cellular response to repetitive bacterial stimulations (e.g., *Pseudomonas aeruginosa* in a lysate form), we devised a chemiluminescent immuno-analytical system for toll-like receptor 1 (TLR1) as marker present on cell surfaces (e.g., A549). Upon stimulation, TLR1 recognizes pathogen-associated molecular patterns of the infectious agent and are then up-regulated via activation of the nuclear factor-κB (NF-κB) pathway. In this study, the receptor density was quantified by employing an antibody specific to the target receptor and by producing a chemiluminometric signal from an enzyme labeled to the binder. The activated status was then switched back to normal down-regulated stage, by changing the culture medium to one containing animal serum. The major factors affecting activation were the stimulation dose of the bacterial lysate, stimulation timing during starvation, and up- and down-regulation time intervals. Reiterative TLR regulation switching up to three times was not affected by either antibody remained after immunoassay or enzyme substrate (e.g., hydrogen peroxide) in solution. This immuno-analysis for TLRs could be unique to acquire accumulated response of the human cells to repeated stimulations and, therefore, can eventually apply to persistency testing of the cellular regulation in screening of anti-inflammatory substances.

Biosensing based on mammalian cells is beyond mere analysis through analyte-receptor reactions to determine the presence of an analyte or to quantify an unknown sample[1]. Because the analytical technique uses live cells as the sensing element, it enables us to measure the cellular response to external change (e.g., heat, chemicals, shock, and infection). Such responses can then be converted to useful information by combining them with conventional detection for changes in cellular metabolism, cytotoxic responses, and bioavailability of medicinal substances. Therefore, the biosensing technology has been applied as an essential tool to various bio-analytical sectors including drug discovery, toxicology, pharmacology, bio-assay, pathogen and toxin screening, environmental monitoring, and bio-security[2–5].

Mammalian cell-based analyses have been traditionally carried out by fixing cells on a solid substrate, which requires destroying cells and consequently limits the assay to one-time use[6]. The most frequent examples are microscopic observations after fluorescent staining of structural regions[7] and arrayed or microfluidic-channel biochip assays[8]. Although these techniques ensure direct measurement and high throughput analysis, information accumulated on the same cells such as time-dependent responses is not available. Alternatively, a continuous monitoring technique for identical cells could deliver cellular responses in real time, and save reagents and disposables[9]. Furthermore, as this novel approach mimics *in vivo* conditions, it would eventually provide a technological basis for the development of alternative means for *in vivo* testing using animal.

As a typical application, mammalian cells are highly responsive to pathogenic infection via its recognition by toll-like receptors (TLRs) present on the cell surfaces, which function as sentinels in the innate immune system[10]. The receptors bind the conserved regions of pathogenic microorganisms, i.e., pathogen-associated molecular patterns (PAMPs). This then activates signal transduction pathways mediated by transcription factors, i.e., nuclear factor-κB (NF-κB), which is a protein complex that controls the transcription of DNA[11]. Such responses include production of cytokines, and also expression of various surface receptors such as TLRs and bradykinin

receptors[12–14]. Since these are present on the cellular membrane surfaces or secreted out of the cells, they can be monitored via immunoassays using antibodies specific to each target, and such methods do not require destruction of the cells for activity monitoring.

Infection is the major cause of acute inflammation and developing to a chronic state can bring about various diseases including cancers[15]. Upon pathogenic invasion, the innate immune response is initiated by TLR-PAMP binding and the infection signal is delivered to activate NF-κB. This process eventually produces various inflammation mediators to serially induce complex precursory reactions of the inflammatory response, eventually leading to vasodilation[16]. Since the activation status of NF-κB is suitable to monitor inflammation reactions, TLRs-PAMP interactions may be proposed as a facile marker of inflammation relatively to other mediators. Furthermore, the inflammatory state can be regulated by adding an anti-inflammatory substance (e.g., aspirin) inhibiting a step in the NF-κB activation pathway[17,18]. In such a case, the TLRs level could decrease, which can also be monitored by using an antibody specific to a target receptor. The degree of decreased level would indicate the anti-inflammatory effect of the substance. TLRs might further be used as marker for monitoring the presence of inflamed tissue in the body, generating again inflammatory products in cyclic manner[19]. As the TLR activation occurs at an early stage of the cycle, it might be used as blocking point for the treatment of chronic inflammation.

In this study, we investigated a regulation model of the marker level to support the concept of repeated monitoring of the inflammatory status, which may also be suitable for testing of anti-inflammatory substances to the human cells. To this end, TLRs, interacting with PAMPs of pathogenic microorganisms, were employed as markers indicating the activated status of cellular response by infection[20]. To establish an experimental model, *Pseudomonas aeruginosa* (*P. aeruginosa*) in the lysate was used to infect human lung adenocarcinoma epithelial cell line (A549 cell line) as the host. The human cells will be needed to utilize the model eventually for human drug investigation. The receptors expressed on the cell membrane surfaces were analyzed via immunoassays using antibodies specific to each marker (without cell destruction). After the receptor density was returned to the background, the cyclic responses to repetitive bacterial stimulations were mimicked using the same cell cultures. Based on this method, we could measure the persistent effects of anti-inflammatory candidates regarding efficacy and toxicity.

Methods

Materials. All reagents used in this study were of analytical grade and are listed in Supplementary Information.

Preparation of bacterial lysate and mammalian cell. Bacterial lysates of *Pseudomonas aeruginosa* (PAK strain; *P. aeruginosa*), *Shigella sonnei* (*S. sonnei*), and *Vibrio parahaemolyticus* (*V. parahaemolyticus*) were prepared by means of sonication as described in a previous report[21]. The bacteria were separately grown in Luria-Bertani broth, and the harvested cells were killed by heat-shock and were then sonicated. Each bacterial protein solution (0.1 mg/mL protein) diluted in 10 mM phosphate buffer, pH 7.4, containing 140 mM NaCl (PBS) was stored at −80°C after snap-freezing.

Human lung adenocarcinoma epithelial cell line (A549) was maintained and stocked according to standard protocols[22]. The A549 cell was suspended in a supplemented RPMI1640 culture medium and was cultivated in an incubator until the solid surfaces were 90% confluent. The attached cells were recovered by adding Trypsin-EDTA solution, suspended in the supplemented medium, and dispensed again for further expansion or snap-freezing in liquid nitrogen prior to storage.

For the bacterial stimulation experiment, the mammalian cells (density: 3×10^5 cells/mL) were pre-cultivated as mentioned and were then dispensed into 96-well culture plates (200 μL per well). After stable attachment, the medium was exchanged with a serum-free one and cells incubated for a further 2 h for starvation. Experimental details are not mentioned in this section, but are described in Supplementary Information.

Conventional analytical procedures for TLR and for cell densities. TLRs expressed on A549 were quantified by employing the conventional protocol, accompanied by cell fixation, to measure the cellular response to bacterial stimulation. The bacterial lysate in the serum-free medium was added to the pre-cultivated mammalian cells, and TLRs were subsequently immunochemically analyzed. To this end, the cells were first fixed by adding formaldehyde solution, and after blocking with casein, an antibody specific to each TLR and anti-rabbit IgG labeled with HRP were sequentially reacted. A color signal from the enzyme was produced by adding a substrate solution containing TMB, stopped by adding sulfuric acid, and measured at an absorbance of 450 nm. The viable cell density in each culture was determined according to the modified Janus green B whole-cell staining protocol[23]. Each measurement was repeated twice under identical conditions.

The same procedure was also used to monitor repetitive bacterial stimulations in a cycle: starvation of the host cells for 2 h, bacterial stimulation for 4 h, and restoration in the serum-supplemented medium for 18 h. A negative control was additionally run without stimulation. The signal-to-background ratio was then calculated and plotted as a function of time. Experimental conditions and procedures are specified in Supplementary Information.

Optimization of TLR up- and down-regulation conditions. The receptor density responses of A549 to bacterial stimulation were optimized by selecting a TLR marker, and by controlling the stimulation dose and stimulation timing. To select a marker, the mammalian cell was infected with different doses of the bacterial lysate, and the density of TLR1, 2, and 4 on the cell surfaces was then measured by using the immuno-analytical procedure, as mentioned. The cell densities were also determined by the staining method.

To test the dose effect on down-regulation after stimulation of the host cells, the culture medium was replaced with one which was FBS-supplemented. At predetermined times, the cells were analyzed as described, and the signal-to-background and maximum recovery ratios were calculated for plotting. Detailed protocols are presented in Supplementary Information.

Analysis for TLR regulation switching without cellular damage. TLR1 expressed on the same cell culture was repeatedly monitored by investigating a non-destructive immuno-analytical scheme, biocompatible signal generation method, and tolerable repetitive stimulation conditions. First, an immuno-analytical procedure for the receptor, not requiring cell fixation, was devised. After pre-cultivation of A549, the cells were starved in serum-free medium and were then stimulated with the lysate for 4 h as described earlier. After washing, sequential immuno-reactions were carried out, and the color signal was produced from the enzyme and measured at an absorbance of 450 nm. The results were compared with those obtained by using conventional assay protocol with cell fixation.

Second, for the cell recycling in aliveness, we adopted a chemiluminometric substrate for HRP and its signal generation condition biocompatible with the animal cell culture. We adopted the same procedure as described above, except for the use of a chemiluminometric substrate for HRP and its signal generation condition. The luminescent signal was produced, and was detected after 3 min using a cooled charge-coupled device (CCD) camera (exposure time: 5 s; ProgRes MF cool, JENOPTIK; Jena, Germany) which was installed within a dark chamber. The focus of the CCD camera was initially adjusted to the well bottom for a clear capture of the image of the cells under cultivation. After adding the luminol-containing substrate, the luminescent signal was detected by using the CCD camera and the captured image was digitized by a Java-based image processing program, Image J (National Institutes of Health; Bethesda, MD). The optical densities were integrated in the vertical direction and were then plotted by using the Excel program (Microsoft; Seoul, Korea). Data were presented as mean ± standard deviation and statistical analyses were further performed using one-way analysis of variance followed by Tukey's post hoc multiple range tests[24] using the SPSS software package for Windows (SPSS Inc.; Chicago, IL, USA). A $P < 0.05$ was considered significant.

Third, the biocompatible TLR-based chemiluminometric assay without cell fixation was applied to the repetitive monitoring of TLR1 according to up- and down-regulation switching. After initially measuring the background level in the first cycle, the culture was starved in serum-free medium for 2 h, stimulated with bacterial lysate for 2 to 4 h, and the TLR1 density was then determined. The same cells were then restored in serum-containing medium for 18 h. For the second cycle, the identical procedure was repeated. The whole process was also negatively controlled using medium in the absence of an infectious agent. Experimental procedures and conditions are shown in detail in Supplementary Information.

Results and Discussion

Analytical model based on TLR regulation switching. The natural processes of TLRs regulation (refer to Supplementary Figure 1 for details) may be mimicked *in vitro* and used to test potential anti-inflammatory substances for efficacy and toxicity. This approach can also be applied to the periodic surveillance of high-risk groups, food safety, and environmental protection against infection. Since the receptors are expressed in response to an invading pathogen, an increase in TLR level can be used as an indicator of infection of host cells, which can be quantified by measuring TLR density using well-established immunoassays. After analysis of a test sample, the receptor density can be reset by switching the culture

conditions to the original growth medium. The down-regulation of TLRs could also be initiated by supplementing agents (e.g., animal serum) in the medium for protection and detoxication[25]. Such 'regulation switching' would eventually allow us to recycle the cell culture for the repetitive monitoring of infectious agents. On the other hand, the repetitive analysis number may be limited for the same cell culture by the fixed surface area of cell culture container and also by variable signal generation conditions out of incubator.

In order to demonstrate this analytical concept, a bacterium was eventually selected as an infectious agent[26], and a human cell line, A549, was selected as the host. Since bacteria can grow at a rapid rate even at ambient temperature, the bacterial lysate form was used as the infectious agent to precisely control the inoculum number. This consequently enabled us to reproduce the infectious effects on the human cell culture, caused by metabolic substances produced by the bacterium. In the next sections, we described the selection of the TLR to monitor the cellular response to bacterial stimulation, determined optimal conditions for the TLR regulations, and demonstrate the potential for repetitive TLR expression against repetitive stimulations.

Determination of optimal TLR regulation conditions. *Infectious agent and TLR marker selection*. Among the TLRs (e.g., TLR1, TLR2, TLR4, TLR5, and TLR6) present on the cell membrane surfaces, we tested TLR1, TLR2, and TLR4 as sensing elements for PAMPs present on three different species of pathogens (*P. aeruginos*, *S. sonnei*, and *V. parahaemolyticus*). Each bacterial lysate (100-fold dilution of the stock in 0.1 mg/mL protein) was used to stimulate the pre-cultured host cells (A549) to compare the cellular responses (Figure 1A). The TLR expression levels were detected using the respective anti-TLR antibodies (refer to the inset). Comparing to the backgrounds without stimulation, TLR1 stimulated with *P. aeruginos* and TLR4 with *S. sonnei* were shown to increase about 3.4-fold and 2.1-fold, respectively. On the other hand, the TLR2 expression was relatively insignificant for all infectious agents used. The elevated level of each marker could be determined by the PAMPs contained in each bacterium, and *P. aeruginosa* inducing the highest signal relative to background was consequently used as infectious agent of the host cells in the rest of this study.

The selected bacterium was then used to examine the cellular response to different lysate doses (500-, 100-, and 20-fold dilutions; Figure 1B). The respective TLRs responded variably, where TLR1 and TLR2 were expressed in proportion to the stimulation dose and TLR4 was not expressed in a structured pattern after invasion. Between the receptors that gave positive responses, the signal from TLR1 was much more intense than that from the other receptors tested. We also checked that the density of the cells remained constant using the Janus green B method to stain the mitochondria (the inset)[23]. The standard curve for this method was linear in the range of 1×10^4 to 1×10^5 cells/well (refer to Supplementary Figure 4, the right). Since TLR1 expression was most sensitive to stimulation with the *P. aeruginosa* lysate, this receptor was used as a marker. We next determined the optimal conditions for the up- and down-regulation of TLR1.

Optimal conditions for TLR1 expression. Based on bacterial stimulation of TLR1 expression, optimal up-regulation levels were then determined by applying different lysate concentrations and also by controlling stimulation timing. The TLR1 level, which was determined using the immuno-analytical procedure described above, was monitored against stimulation time (see Supplementary Figure 2) or its timing (Supplementary Figure 3). The activation level of receptors was proportional to the stimulation dose and was also increased as the time of stimulation was increased up to 4 h. As the stimulation time elongates, the cellular response may be regulated by its own program such as endocytosis, which could result in a down-regulation of the receptor density in a time-dependent manner. Regarding the timing, the stimulation after a delay for 2 h was determined to be optimal since under this condition the signal-to-background and recovery rate were high.

A constant amount of infectious agent applied to the cell culture up-regulated the TLR for a limited period of time. Since the binding between the receptor and PAMP is an equilibrium reaction[27,28], the receptor-mediated signal should be proportional to the ligand concentration. This increased receptor density on the cell surfaces by up-regulation reached a maximum level due to the presence of a limited amount of PAMP in the culture. At a certain point, the complexes could be transferred into the cytoplasmic space by endocytosis (see Supplementary Figure 1B)[29,30]. When the culture was maintained for an extended period (e.g., total 12 h), a gradual detachment of cells from the solid surfaces was observed (data not shown). This could reflect the development of a less-favorable environment for cell survival.

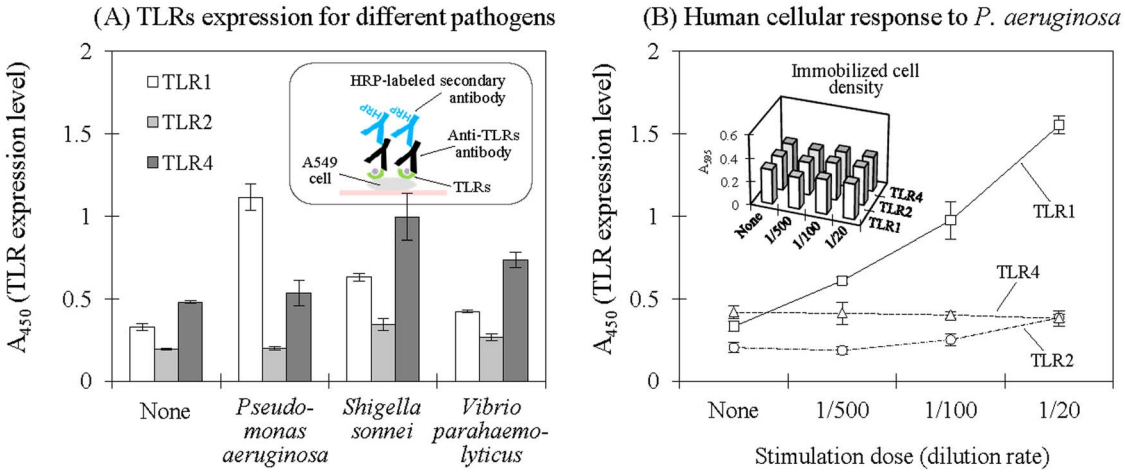

Figure 1 | Expression of TLRs on the cell surfaces of A549 in response to the stimulation with bacterial lysate. To select an infectious agent, three bacteria, *P. aeruginosa*, *S. sonnei*, and *V. parahaemolyticus*, were initially used in 100-fold dilution of each lysate stock (0.1 mg/mL protein) to compare their TLRs induction efficiencies (A). Among the TLRs, TLR1, TLR2, and TLR4 were selected and the expression levels were measured using antibodies specific to each marker. Since the TLR1 expression relative to background ((A), None) in response to the stimulation with *P. aeruginosa* was most sensitive, the cellular response was closely examined toward the lysate dose (B). The cell numbers in the individual cultures were monitored, using the Janus green B staining technique, to show quantitatively constant (the inset). Among the TLRs, TLR1 revealed the highest sensitivity to stimulation.

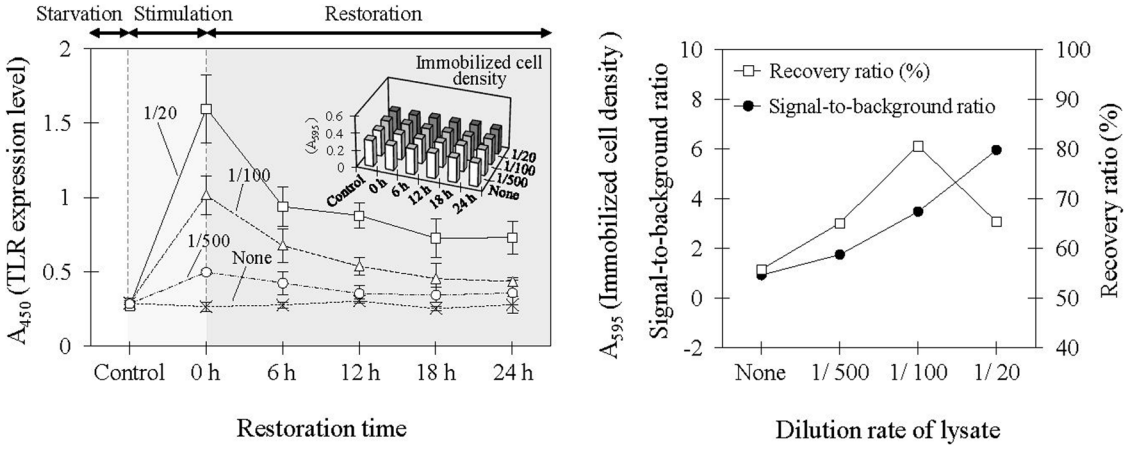

Figure 2 | Induction of down-regulation of TLR1 expression on the A549 cells. The cells were previously stimulated with different doses of *P. aeruginosa* lysate (see the text for details) and the TLR1 level was returned to the background by changing the medium to one containing animal serum. The recovery after 24 h was needed to restore the level to equilibria (the left), and the cell numbers in each culture were maintained constant (the inset). The maximum recovery ratio attained during this period was plotted against the stimulation dose of lysate (the right). The TLR signal relative to the background was also graphed. The stimulation dose compromising the two parameters was a 1/100 dilution of the lysate.

Acceptable stimulation doses and minimal recovery time. Although up-regulation of TLR on the cell surfaces was optimized, monitoring of repetitive stimulations for the same culture requires restoration of the receptor density to levels close to the background (Figure 2, the left). To this end, we investigated stimulation doses that allowed us to rapidly bring the expressed density back to the initial state. The TLR expression level was decreased by eliminating the stimulation source and supplementing the media with components which could protect and detoxify the cells. These conditions were simply achieved by changing the medium to one containing animal serum. The receptor density was then monitored at predetermined times using the immunological assay described above (line graph in the left). During the monitoring of down-regulation, the cell density of each culture was assured to be constant by employing the same staining method described above (bar graph).

When serum was present in the medium, the TLR1 density gradually decreased over 24 h regardless of the stimulation dose employed. The maximum recovery ratio was calculated by dividing the TLR signal by the background signal, which was elevated as the stimulus size was increased up to 1/100 dilution, and then diminished at a higher dose (Figure 2, the right). Under the conditions used, the maximum recovery of the background level was achieved (80%). An increase in the restoration time period did not significantly further decrease expression. On the other hand, the signal-to-background ratio measured during the stimulation period was directly dependent on the stimulus size (the right). Therefore, the optimal stimulation dose for repeated stimulations was determined to be a lysate dose of 1/100.

The TLR1 density on the cell surfaces could be up-regulated via bacterial stimulation under starvation conditions, and sequentially down-regulated by changing the medium to one containing animal serum. We next examined the potential of using this system to monitor cyclic stimulation with the same cell culture. This would allow us to establish an *in vitro* experimental model, offering a basis for the repetitive monitoring of human cellular response against bacterial stimulations.

Repetitive immuno-analysis of cellular response to stimulation. *Immunoassay procedure without cell fixation.* To monitor the cellular response in a repetitive mode, an immuno-analytical method which does not destroy reporter cells should first be devised. We have conventionally carried out the assay by following a protocol requiring the cell fixation on solid substrate by using an organic solvent and then the membrane permeabilization by using detergent[31]. This allowed for the development of the assay procedure, though the resultant cell death did not allow for cell recycling.

In the TLR assay, the human cell line, A549, is anchorage-dependent, not needing an additional fixation, and the receptor marker is present on the outer membrane surfaces, already offering favorable accessibility to immuno-reagents. Under such conditions, we have established a new immunoassay protocol maintaining a viable state of the animal cells in consideration of: 1) minimization of shear stress development, 2) use of the cell culture medium as a diluent of assay reagents, and 3) application of serum proteins in the medium for blocking. To reduce shear force, the washing of excess reactants at each step was applied very gently and was limited to 3 washes. This can protect the mammalian cells, which do not have cell wall, from the mechanical stress-induced damage. The RPMI medium containing 10% FBS was used for dilution of the reagents, which could support the cell viability during the assay and also limit non-specific interactions of the reagents with the residual solid surfaces.

When this immunoassay protocol was applied to the quantification of TLR expression, the receptor level varied in a directly proportional manner to the stimulation dose, while the negative control with the non-stimulated cells showed only background signals (Figure 3A). The signal-to-background ratio was enhanced for all doses applied when compared to the conventional assay accompanied by cell fixation (3B). We further checked, after reaction with antibodies, the animal cells which were viable and stably attached, via microscopic observation and Janus green B staining, respectively (refer to Supplementary Figure 4, A and B). However, colorimetric signal generation from the enzyme in the final step damaged the cells due to toxic chemicals contained in the enzyme substrate solution (Supplementary Figure 4, D). The relatively low performance in the cell fixation-accompanied assay could result from the use of formaldehyde as a reagent under warm temperature for a long time period. Under such conditions, the substance may cross-link primary amino acids in proteins with other nearby nitrogen atoms in proteins or DNA molecules through a -CH_2- linkage[32], which transformed the protein residues. Therefore, the new assay protocol was revealed to be superior to the conventional format, in that the background was decreased and the TLR signal was increased.

Chemiluminescent TLR analysis without cell destruction. We have developed an alternative method for TLR analysis, where repetitive monitoring can be carried out by recycling the same cells. To this end, a chemiluminometric substrate for HRP, luminol, can substitute

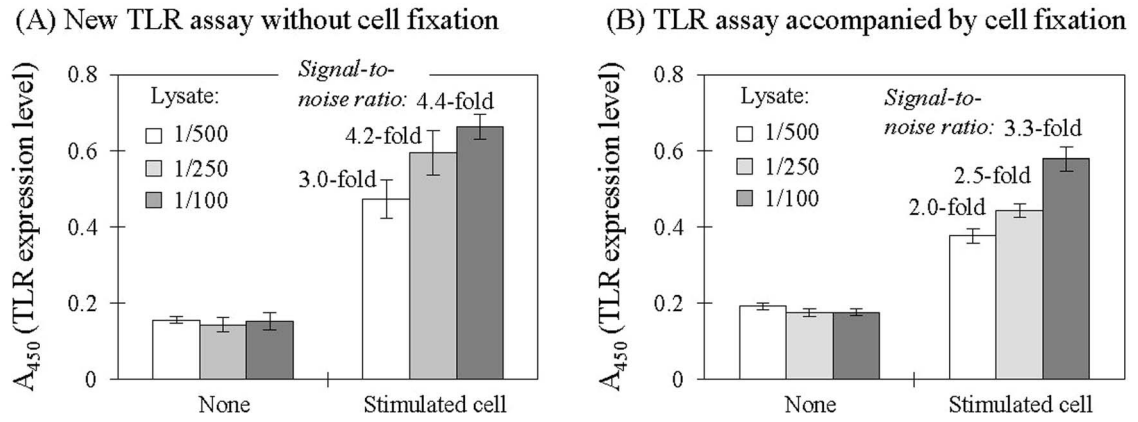

Figure 3 | Performance characterization of a new analytical protocol for TLR1 expressed on A549 cells without cell fixation (A) and comparison with that of the conventional cell fixation-based method (B). The new protocol enhanced the assay sensitivity, indicated by increased signal-to-noise ratios for all doses of lysate tested.

TMB, since it provided relatively favorable reaction conditions in at least two regards: use of mild pH for the substrate reaction and no need to accumulate the signal[33]. Upon catalytic oxidation at pH 8.5, luminol emitted a light signal measurable at a wavelength of 425 nm. Although the substrate can inhibit poly(ADP-ribose) polymerase activity[34], the luminol concentration used in the reaction was also extremely low, and so, the risk of damaging cells or causing deformation was low. Furthermore, the signal intensity was stably maintained and measured over a short time period (e.g., less than 3 min). The HRP substrate, hydrogen peroxide, could also be harmful, but the concentration used for the signal generation was far lower (0.002 mM[33];) than the minimal amount showing cytotoxicity (about 0.06% corresponding to 17.8 mM[35];). In this case, the cells may have undergone minimal oxidative stress, possibly caused by hydrogen peroxide, and thus, may experience limited cell damage. These postulations were supported by microscopic cell monitoring and staining-based viable cell quantification, showing that the cell number (total 5.5×10^4 cells in average; Supplementary Figure 4, C) under the test was only 10% higher than that (5.0×10^4 cells; A) of the control. Such increase could be insignificant when standard deviation (22.7%) is taken into consideration.

To measure the chemiluminescent intensity as a signal, a cooled-CCD camera was installed within a dark space, of which the floor was used as a stage for placing cell culture well plates (Figure 4A). The culture plate was selected as it was black in color and the well bottom was made of thin glass, which resolved a main problem in the luminescence detection, i.e., interference between the wells by light dispersion. When a signal image as an indication of bacterial stimulation was obtained, the bottom was first brought into focus by adjusting the lens under illumination (4B, a). After turning the lighting off, the chemiluminescent signal was measured as an image using the Image J program, and the signal of the non-stimulated negative control was also monitored (4B, b). The color intensity of the image was then increased using the software, in order to add contrast, which clearly showed the light signal converged on the edge of the culture well (4C, c). Each image was digitized by using the same program, and the optical densities were then transferred into a computer program (e.g., MS Excel) and vertically added to construct a graph (4C, d). The signal for the TLR expression on the cell culture was finally measured by integrating the optical densities after subtraction of those of the background. Since such a detected signal was proportional to the stimulation dose, the chemiluminescent immuno-anaysis method was applied to the cyclic TLR monitoring for the same cells in response to repetitive stimulations in the next section. It is notable that the signal detection system can be typically 10 times inexpensive comparing to the conventional device measuring fluorescent dye as label. This comparison was made based on the need of extra instrumentation for the fluorescent detection, comparing to that for the chemiluminescent measurement, such as light source for excitation of the dye and a filter of the emitted light[36].

Analysis of cellular responses in repetitive mode. The chemiluminescent detection method was applied to monitoring of the regulation switching of TLR1 density in response to repetitive bacterial stimulations (Figure 5). The initial time protocol for each cyclic response was as follows, except pre-cultivation for cell attachment for 24 h (5A): a) basal or down-regulated level detection for 2 h, b) starvation for 2 h, c) stimulation for 4 h under starvation conditions, d) up-regulated level detection for 2 h, and restoration for 18 h using the serum-containing medium. This stimulation-restoration cycle using the same cell culture was repeated two times to repeatedly observe the cellular response regarding TLR expression (5B). Using this protocol, two separate, cyclic responses in the TLR level (the left axis) were observed: an increase upon stimulation under starvation conditions and then a decrease during the restoration stage. For the control, the TLR density of the non-stimulated cell culture was continuously elevated, even if at a negligible rate. The relative value of the two densities at each stage, which is the signal-to-noise ratio (the right axis), explicitly showed an up-and-down response pattern twice, although both the maximum and minimum values in each cycle were slightly increased. The increased rate was 16% for the maximum at up-regulated state by stimulation, and 23% for the minimum at down-regulated state by restoration.

The initial conditions (i.e., cell immobilization for 24 h and stimulation for 4 h), however, did not allow us to repeat analytical cycle more than two times due to cell death observed by floating in the aqueous phase at the next cycle. Such cell detachment could be caused by a high level of cell confluency via overgrowth. To extend the number of cycle, the scheme was further adjusted to half of the attachment and stimulation periods (i.e., for 12 h and 2 h, respectively), at which signal-to-noise ratio for the first stimulation was kept about 3.0 at an optimal stimulation dose (e.g., dilution rate: 1/100; Figure 6A). Under the new settings, we were able to finally obtain three complete cyclic responses to the repetitive lysate stimulations for the same cells (6B, Triplicate stimulations). Since gradual increases were observed in the sequential treatment cycles here and also in Figure 5B, the effect of cell expansion was examined by conducting two control experiments which were not treated for the first cycle or for the initial two cycles. Control without the first treatment revealed that the next two cyclic response levels were about the same as those of the first two cycles in the triplicate stimulations, and their patterns were also similar (6B, Control 1). This observation was further supported by statistic analysis as indicated 'no significance

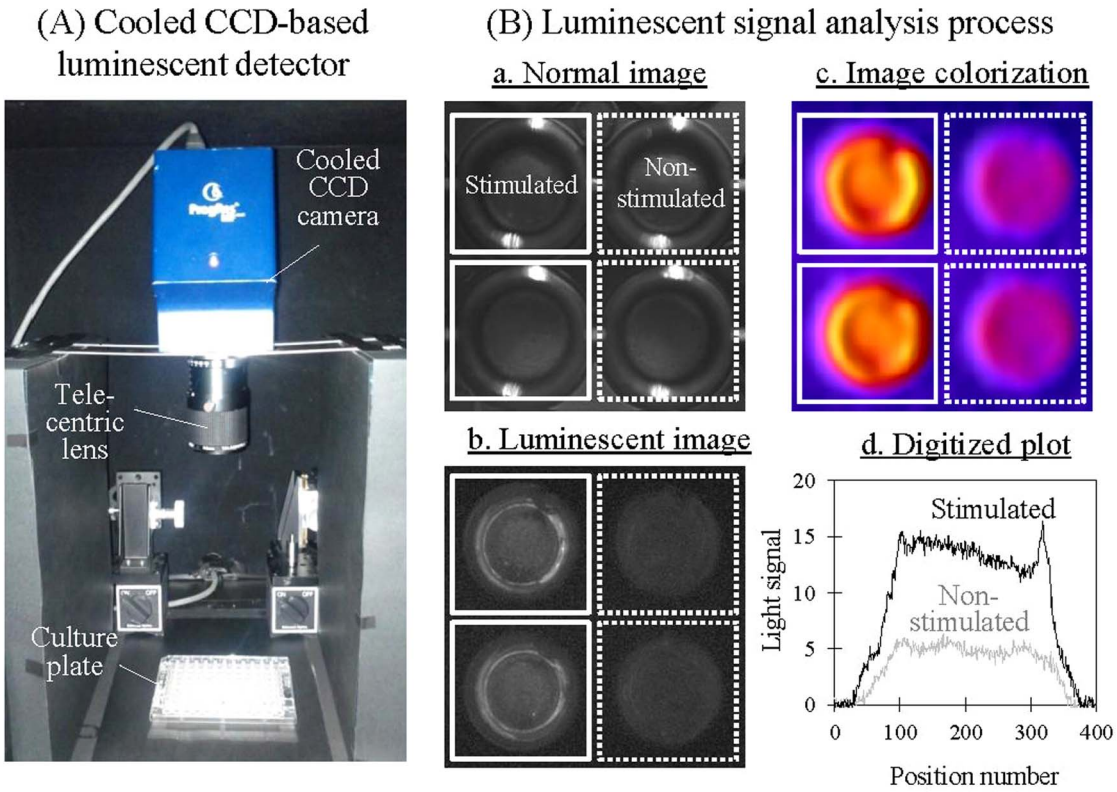

Figure 4 | Mammalian cell-based chemiluminescent detection system for bacterial stimulation (A) and the analytical procedure (B). The system was developed to quantitatively measure the TLR expression on the A549 cell surfaces with minimal cell damage. The measurement protocol was shown to be effective to clearly distinguish the stimulation signal from the background.

(ns)' in the figure. Control skipped in the first two treatments also showed an identical level in the third treatment cycle to that of the first response to the two or more repeated stimulations (6B, Control 2). This was also supported based on statistics to be 'ns'. These results indicated that the gradual increasing pattern was not associated with the elevating number of responsive cells. Therefore, the triplicate cyclic responses did not only enable us to adequately demonstrate the novel analytical concept proposed (refer to Supplementary Figure 1), but also, the repetitive monitoring of the human cell responses to external stimulations.

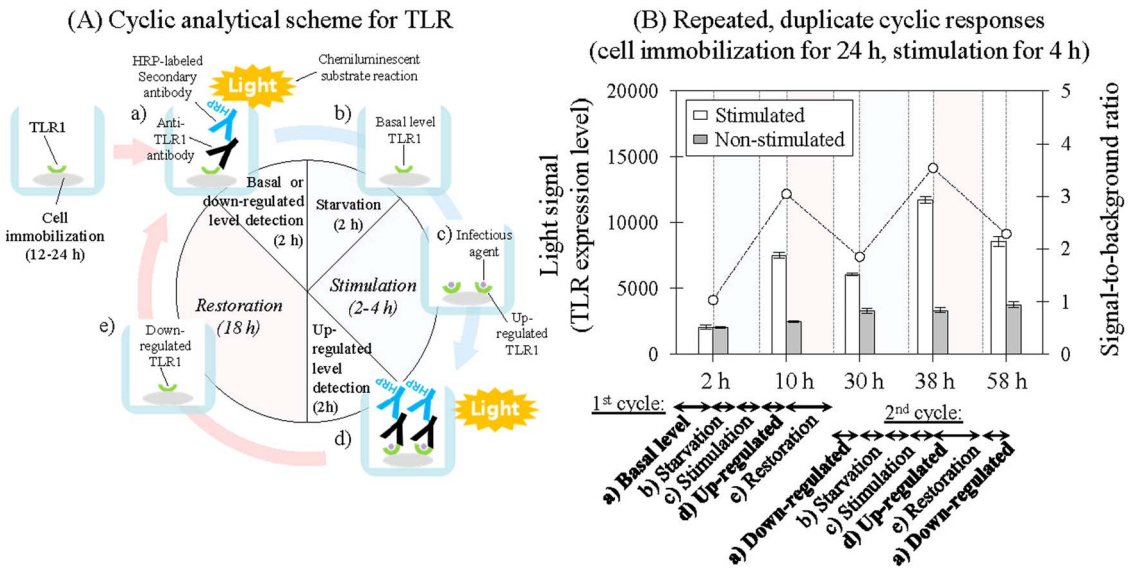

Figure 5 | Repetitive analysis for cyclic responses of the mammalian cells to repeated stimulations with bacterial lysate. According to the prepared scheme (A), the cellular response to the stimulation increased the TLR level ((B), the left axis) under starvation conditions and then brought the status closely back to the background under appropriate cultivation settings (refer to the stages labeled on the bottom of each graph). This cyclic response could be repeated twice for the same cell culture, where the cell immobilization was carried out for 24 h and stimulation for 4 h each cycle. The TLR level at each stage was converted to the ratio of the density of stimulated cells over that of non-stimulated cells, i.e., signal-to-noise ratio (the right axis), which clearly showed the increasing and decreasing response pattern.

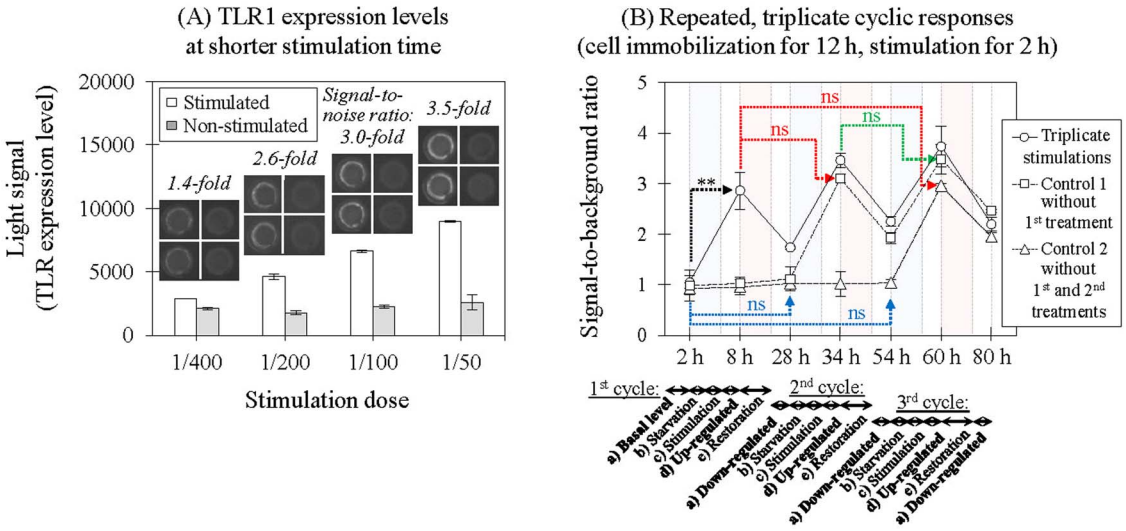

Figure 6 | Triplicate cyclic analysis for cellular responses using amended stimulation scheme. TLR1 expression levels were not significantly decreased by reduction to half (i.e., 2 h) of the stimulation time initially used (A). The scheme was further modified by shortening the cell immobilization time to the half (i.e., 12 h), finally enabling us to repeatedly monitor the three consecutive cycles ((B), Triplicate stimulations). Additional experiments without treatment for the first cycle ((B), Control 1) or initial two cycles ((B), Control 2) revealed that the extra cells grown in the culture did not affect the sequential response pattern. Each measurement was repeated twice and the results were then compared with each control. We marked as '**' for highly significant ($P < 0.01$) and as 'ns' for no significance ($P > 0.05$).

For comparison, we also carried out the same experiments by employing the conventional method. The method was accompanied by cell fixation on the surfaces of a solid matrix and consequent cell destruction (refer to Supplementary Information). In this case, the cellular responses to serial stimulations can only be obtained by starting the animal cell culture and then destroying the cells for TLR analysis at each stage. Such methodology allowed us to only discretely monitoring the TLR expression status, which, nevertheless, showed the same pattern of cyclic TLR responses twice (Supplementary Figure 5A). A similar response pattern was also observed up to three times (5B). These results supported that the cyclic TLR responses to repeated bacterial stimulation were reproducible and detectable in a consistent manner. Such experimental results indicated that the bound antibody remained after each immunoassay for the TLR in repetitive monitoring did not leave any other effect in the stimulation-restoration process. The antibody-TLR complex could concurrently undergo endocytosis as the receptor was internalized[37] or, otherwise, the antibody was dissociated away into the bulk solution. It is notable that the background measured by using the conventional method was maintained during the time course, while those determined in the repetitive mode were persistently increased. Such increase in the noise level seemed to be caused by accumulation of cellular stresses imposed by chemiluminescence signaling, such as pH change, the presence of hydrogen peroxide and luminol, and temperature fluctuation.

The repetitive monitoring technique for human cells could be unique in the analytical aspect comparing to other conventional methods. For examples, TLR mRNA detection and NF-κB translocation assay required cell disruption. Although transcriptional assay (e.g., luciferase reporter assay[38];) has been reported for NF-κB activation monitoring of live cells, the cellular response was limited to just single stimulation because the genetic property was not conveyed to their subcultures. In case of cytokine (e.g., TNF-α) assay, the cells may also not be destroyed and, however, the concentration (<10 pg/mL[39];) secreted from the human cell line was too low to be assayed using the commercial ELISA kit (Supplementary Figure 6). The production of cytokines was induced in mouse macrophages, but not in human macrophages and epithelial cells as previously reported[39]. The apparent insensitivity of human cells is most likely attributable to species differences in the regulation of cytokine production. We further tested the secreted enzyme (e.g., alkaline phosphatase) from an engineered, commercially available reporter cell line, RAW-Blue (data not shown). All of the secreted markers for inflammation were shown to weaken as the same cells were repeatedly stimulated. Therefore, the engineered cell line did not support consistent monitoring of the repetitive innate immune responses.

Based on collected information, the cellular response against bacterial stimulation can be repeatedly monitored using the following TLR on A549. This human cell utilized *in vitro* model could be suitable for the screening of human drugs for, for examples, anti-inflammation and anti-cancer, and may eventually substitute experimental animal for persistency testing of drug candidate substances[40]. Although the stimulation-restoration chart in Figure 6B showed an elevating cyclic response, this will not be a matter since each response to test substances is compared with the standards for inflammation and anti-inflammation regarding the relative position. The future of medicinal substance examination could be estimated from the fact that testing of cosmetic ingredients using animal has been totally prohibited by law in European countries[40]. Human drugs should be investigated regarding efficacy and toxicity in human cells[41]. The repetitive monitoring for the cellular response may offer extra information regarding test substances such as dilatory cytotoxic effect. Although they initially revealed an efficacy, the cytotoxic effect can be caused in the second or third stage by accumulation if a test drug was repetitively applied to the same cells. To our knowledge, repetitive monitoring of the TLR level as a reproducible indicator of cellular response was first shown in this study. It is pointed out that most previous reports on the monitoring of inflammatory mediators have been limited to cellular responses only to a single stimulation[42–44].

Conclusions

The regulation switching of mammalian cells regarding TLR expression on the membrane surfaces was developed *in vitro*, which enabled the cyclic detection of repetitive bacterial stimulations in the same culture. The TLR expression was induced upon bacterial stimulation under starvation conditions and the initial background level was then restored by providing favorable culture conditions. Such repeated

TLR regulation switching on human cells such as A549 can be utilized as an *in vitro* experimental model for persistency testing of anti-inflammatory substances. For example, in the case of human medicinal substances that inhibit the NF-κB pathway[17,18], the transcription for TLR production would be suppressed. Thus, this would cause a decrease in the up-regulation of the receptor density when compared to that of the control and the persistent effect of the drug can then be tested in regards to the efficacy and toxicity in the next cycles. It should be noted that the repeated monitoring technology currently had limitations that the maximum number of repetition was three due to potential cellular stress imposed by constant surface area and signal generation from an enzyme label. While the former could be alleviated by employing 3-dimensional cell culture[45], the latter depended on optimization for the chemical, physical conditions. In the next study, by using the up- and down-regulated TLR levels as the stimulation and background control standards, respectively, an analytical method for screening of anti-inflammatory substances will be investigated. For high-throughput automation of the testing procedure, we are investigating an analytical methodology coupled with micro-fluidic chip, which should allow for the development of a miniaturized cell chip. Such supplementation would further provide *in vivo*-like environments for TLR regulation that reflect the clinical demands of the body.

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Author contributions

J.W.J. designed and carried out experiments. I.H.C., U.H.H. and S.K.S. contributed to the discussion for planning the experiment related to cell surface receptor monitoring and the results. J.W.J. and S.H.P. wrote the paper. All authors discussed the results and commented on the manuscript.

Additional information

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