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ARTICLE

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Application of a TLR overexpression cell model in pyrogen detection

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Abstract

Pyrogens are components derived from microorganisms that induce complex inflammatory responses. Current approaches to detect pyrogens are complex and difficult to replicate, thus there is a need for new methods to detect pyrogens. We successfully constructed a pyrogen-sensitive cell model by overexpressing Toll-like receptor (TLR)2, TLR4, MD2, and CD14 in HEK293 cells. Since the cytokine IL-6 is specifically released upon stimulation of the TLR2 and TLR4 signaling pathways in response to pyrogen stimulation, we used it as a read out for our assay. Our results show that IL-6 is released in response to trace amounts of pyrogens in our cell model. Pyrogen incubation times and concentrations were explored to determine the sensitivity of our cell model, and was found to be sensitive to 0.05 EU/ml of LPS and 0.05 ug/ml of LTA after stimulation for 5 hr. Our TLR overexpressing cell model, with IL-6 as readout, could be a new method for in vitro testing of pyrogens and applicable for evaluating the safety of drugs.

KEYWORDS
LPS, LTA, pyrogen detection, toll-like receptor family

1 | INTRODUCTION

Pyrogens, which mainly originate from microorganisms, induce deleterious responses in humans, including chills, nausea, fever, septic shock, or even death, when they enter the blood stream (Dinarello, 2004). In 1875, Burdon-Sanderson isolated a substance, which he coined "pyrogen," from bacteria-free extracts of putrid meat. Studies by Seibert further strengthened the conclusion that pyrogens mainly originate from microorganisms. Today we know that most pyrogens are of microbial origin, being produced by bacteria, viruses and fungi, but also recognize that environmental particles can also be considered to be pyrogens (Braude, Mcconnell, & Douglas, 1960; Kozak, Hahn, Lennarz, & Wood, 1968; Monn & Becker, 1999; Stang et al., 2014).

The two major methods for testing for the presence of pyrogens are: (a) the rabbit pyrogen test (RPT), and (b) the Tachypleus amebocyte lysate (TAL), which is also called the bacterial endotoxin test (BET; Moesby, Jensen, Hansen, & Christensen, 1999; Suffredini, Hochstein, & Mcmahon, 1999). The seventh edition of the European Pharmacopoeia (2011 Edition) includes a new method, named the monocyte activation test (MAT) for pyrogens, which, in several studies, has been shown to correlate well with TAL, but is superior when samples contain high concentrations of protein (Eperon & Jungi, 1996; Stoddard, Pinto, Keiser, & Zollinger, 2010; Taktak et al., 1991). The RPT remains the gold-standard for in vivo pyrogen testing methods, because it was added to the USP in 1942 (Fennrich et al., 2016). However, the RPT is time-consuming, and cumbersome, with poor stability and not very qualitative or quantitative. TAL is simple and easy to operate and has a lower testing cost, and was added to the USP in 1980, but this test can be affected by the environment, yielding false-positive results, and can only detect LPS in Gram-negative bacteria, thus it is not a perfect method for overall pyrogen testing. MAT is based on human monocytes, which should fully mimic the exothermic reaction of humans, but it has been difficult to popularize as it requires fresh human mononuclear cells (Burger-Kentischer, Abele, Finkelmeier, Wiesmuller, & Rupp, 2010; Hasiwa et al., 2013; Martínez, Mitjans, & Vinardell, 2004; Nakagawa, Maeda, & Murai, 2002). Therefore, there is a demand for the development of additional new in vitro pyrogen testing methods.

Studies of the pyrogen-signaling pathway have demonstrated that toll-like receptors (TLR)4, MD2, and CD14 are the key players in the LPS-mediated pyrogen reaction, whereas TLR2 plays a key role in the LTA-mediated pyrogen reaction (Akira & Takeda, 2004; Casella & Mitchell, 2013; Lendemans et al., 2007; Liu, John, & Jarvis, 2010; Medzhitov, Preston-Hurlburt, & Janeway, 1997; Nagai et al., 2002; Tapping & Tobias, 1997). Here, we used TLR2, TLR4, MD2, and CD14 as starting points to build a new model for pyrogen testing. Our goal was to construct a cell model that expresses TLR2, TLR4, MD2, and CD14. The application of this successfully engineered a TLR2/TLR4/MD2/CD14 overexpressing cell model in pyrogen detection was studied. We confirmed that the cytokine IL-6 was specifically and sensitively secreted by our cell model in response to trace levels of a pyrogen, and thus could be used to detect the presence of pyrogens. We propose that our TLR overexpressing cell model, combined with the detection of IL-6, could be used as a new in vitro method for pyrogen testing that could be applied to evaluate the safety of drugs.

2 | MATERIALS & METHODS

2.1 | Reagents

Dulbecco's modified Eagle's medium (DMEM) was obtained from Life Technologies (California). Autophagy inducers rapamycin and 4′,6′-diamidino-2-phenylindole (DAPI) were purchased from Solarbio (Beijing, China). 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide and Thiazolyl Blue Tetrazolium Bromide were purchased from Promega (Wisconsin). Primary antibodies were from Cell Signaling Technology (MA) and Abcam Cambridge Science Park in Cambridge, UK). Potassium Aspartate and Magnesium Aspartate injections were from Richter Gedeon (Budapest, Hungary), Furosemide injection was from Tianjin Jin Yao Pharmaceutical Co., Ltd. (Tianjing, China) and Xinmailong was from Yunnan Tengyao Medicine Pharmaceutical Limited by Share Ltd (Yunnan, China).

2.2 | Cell culture and construction of overexpressing cell model

Human embryonic kidney (HEK) cell line 293T was obtained from the Shanghai cell bank of the Chinese Academy of Sciences. The cells was cultured in high glucose DMEM supplemented with 10% fetal bovine serum (HyClone), 10 mM HEPES (Amresco TX), 100 U/ml penicillin (Sigma, Missouri) and 100 μg/ml streptomycin (Sigma) and maintained at 37°C and 5% CO_2.

Human TLR2, TLR4, MD2, and CD14 coding sequence was obtained by PCR amplification of cDNA generated from human peripheral blood mononuclear cells (Primers are listed in Table 1). Standard conditions were used for PCR: Initial denaturation 95°C for 10 min, followed by 30 cycles 90°C for 60 s; 60°C for 60 s; 72°C for 30 s, and a final elongation was for 10 min at 72°C. Gel purified products were TA cloned into PMD-19T plasmid (TaKaRa, Japan), with sequences generated confirmed by sequencing. The TLR2 PCR product was digested with *Bam*HI and *Xho*I and subcloned into the corresponding sites of the expression plasmid pcDNA3-DUSP6-YFP and the resulting recombinant plasmid was named pcDNA3-TLR2-YFP, TLR4 product was digested with *Bam*HI and *Xho*I and subcloned into the corresponding sites of the expression plasmid pcDNA3-DUSP6-YFP, the product was digested with *Bam*HI and *Xho*I and subcloned into the corresponding sites of the expression plasmid pcDNA3--DUSP6-YFP. Human TLR4, MD2, and CD14 expressing plasmids were maintained by the laboratory. All plasmids were restriction enzyme digestion successfully (see Figure S1 for construct maps). To confirm the expression of the introduced coding sequence, 293T cells were transfected and TLR2 protein expression was detected by confocal immunofluoresence.

TABLE 1 Primers for amplification of introduced coding sequences

Gene	Primer sequence (5′-3′)
GAPDH	GCACCGTCAAGGCTGAGAAC TGGTGAAGACGCCAGTGGA
TLR2	AGCTCAGGATCTTTAAACTCCATTC AGGGAAGAAAAAGAATCTTCCTCTA
TLR4	GCTCGGTCAGACGGTGATAG AAGCTCTGGGTTTCATGCCA
MD2	GATTACTCTTTTTGCAGAGCTCTGA GAATTAGGTTGGTGTAGGATGACAA
CD14	TTGGTGCCAACAGATGAGGT TCGGCTGCCTCTTATATCCCA

A total of 293T cells were placed into six-well plates (Corning, NY) with a total number of 2×10^5 cells for each well and cultured overnight. Transfection was conducted using Neofect™ (Neofect Biotechnologies, Beijing, China). Transfection efficiency was tested via quantitative real-time polymerase chain reaction (RT-PCR) and western blotting of extracts from cells harvested 36 hr after treatment.

2.3 | RNA extraction and RT-PCR

RNA was extracted using TRIZOL and reverse-transcribed into cDNA with PrimerScript® 1st Strand cDNA Synthesis Kits (Takara, cat # D6110A). Quantitative detection of GAPDH, TLR2, TLR4, MD2, and CD14 messenger RNAs (mRNAs) was performed with StepOne™ System with the PowerUp™ SYBR® GreenMasterMix (Applied Biosystems, NY) according to the manufacturer's instructions. Primers for RT-PCR are listed in Table 1 and were synthesized by TSINGKE. PCR amplification was carried out in a total volume of 20 μl under the following conditions: 50°C for 120 s, 95°C for 120 s, 40 cycles at 95°C for 30 s, 58°C for 30 s, and extension at 72°C for 60 s. GAPDH was used as the internal control.

2.4 | Immunofluorescence

Before immunofuorescence, the cells were allowed to adhere to the glass bottom of confocal dishes for 3 hr. The cells were then washed three times (5 min each) with phosphate buffered saline (PBS, pH 7.2), fixed in 4% paraformaldehyde in PBS containing 0.1% (vol/vol) Triton X-100 (PBST) at room temperature for 15 min and then washed again in PBS three times (5 min each). The cells were blocked with 5% bovine serum albumin (Boehringer, Germany) in PBST for 30 min at 37°C. Primary antibodies (TLR4, ab89455, Abcam; TLR2, ab16894, Abcam; MD2, ab24182, Abcam; CD14, ab45870, Abcam) were diluted to 1:40 in blocking solution. Two-hundred microliter of the primary antibody solutions were added to each dish, and the cells were incubated overnight at 4°C. After incubation, the cells were washed three times (5 min each) with PBS. All subsequent steps were performed in the dark. Two-hundred microliter of secondary antibodies (TRITC goat anti-mouse IgG, ZF-0313, fluorescein isothiocyanate (FITC) goat anti-rabbit IgG, ZF-0311, FITC rabbit anti-goat IgG, ZF-0314, 1:50 dilution in blocking solution) was added to each dish and incubated for 60 min at room temperature. After incubation, excess secondary antibodies was removed and 200 μl of DAPI (C0065, Solarbio) was added to each dish and incubated for 10 min at room temperature in the dark. The cells were then washed three times (5 min each) with PBS and observed under a confocal microscope (Leica).

2.5 | Western blot analysis

Equal amounts of protein (100 μg/lane) from cell lysates or culture medium were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) or Tricine–SDS-PAGE and transferred to polyvinylidene difluoride membranes (Hybond-P; GE Healthcare, Pittsburgh). The blots were probed with the appropriate primary antibody, followed by HRP-conjugated anti-rabbit IgG (Cell Signaling Technology). Protein bands were visualized using an enhanced chemiluminescence (ECL) detection method (Bio-Rad, CA), and band intensity was analyzed with a densitometer (LAS-4000; GE Healthcare). Each experiment was repeated at least three times. GAPDH, measured quantitatively using a GAPDH antibody (ab181602), was used as control.

2.6 | Cell viability

Cytotoxicity was evaluated using the 3-(4,5-dime-thylthiazo-l-2-yl)-2,5-diphenyl-2H-tetrazolium bromide reagent (MTS) colorimetric assay. Injectable drugs, Potassium Aspartate and Magnesium Aspartate injection, Furosemide injection, and Xinmailong were diluted in culture medium and added to cells at 1:1-1:1000 dilutions. Treatment was for 6 hr. The cells were then treated with 0.5 mg/ml MTS (Promega) for 2–4 hr at 37°C. The cells were then lysed with lysis buffer (0.1 M HCl, 10% Triton X-100, dissolved in isopropanol), and absorbances at 490 nm measured on a microplate spectrophotometer.

2.7 | Test for interferences

LPS and LTA were used as standard pyrogens. 293T cells (2×10^4) in 96-well plates were transfected with the TRL2/TLR4/MD2/CD14 expression plasmids before pyrogen stimulation. Demonstrating the absence of interfering factors is required to guarantee reliable results for the assay. This test was conducted by incubating diluted three injections with spiked (LPS or LTA) sample dilutions and assayed in parallel with the corresponding unspiked dilution. Dilutions with endotoxin recovery within the 50–200% range were considered to be interference-free. All experiments were repeated three times and the results were either from a representative experiment or all experiments (with mean ± SD shown).

2.8 | Statistical analysis

All data are expressed as mean ± SD. Comparisons of the means among greater than three groups were done by one-way ANOVA, followed by post-hoc tests (PRISM, GraphPad software). p values ≤ 0.05 were considered to be significant.

3 | RESULTS

3.1 | Overexpression of human TLR2, TLR4, MD2, and CD14 in HEK 293T cells

Human TLR2, TLR4, MD2, and CD14 coding sequences for our expression constructs were amplified from RNA isolated from human peripheral blood mononuclear cells by PCR (Figure S1). To confirm that each of our constructs express the cloned genes, plasmids (pcDNA3-TLR2-YFP expressing TLR2; pMCS.

DTA-TLR4-GFP expressing TLR4; pCAG-MD2-CD14-RFP expressing both MD2 and CD14) were separately transfected into HEK 293T cells and assessed after 24 hr for fluorescence from their linked florescence genes (YFP, GFP, and RFP, respectively). All three constructs displayed expression of the linked florescence genes (Figure S2), suggesting the expression of TLR2, TLR4, MD2, and CD14 in these cells. To confirm the expression of the four introduced genes we performed RT-PCR, with β-actin used as an endogenous control. mRNA levels of all four introduced genes were higher in the transfected cells, compared with the parental strain (Figure 1a–d). Overexpression of the proteins was assessed using immunofluorescence, with TLR4 and TLR2 detected using

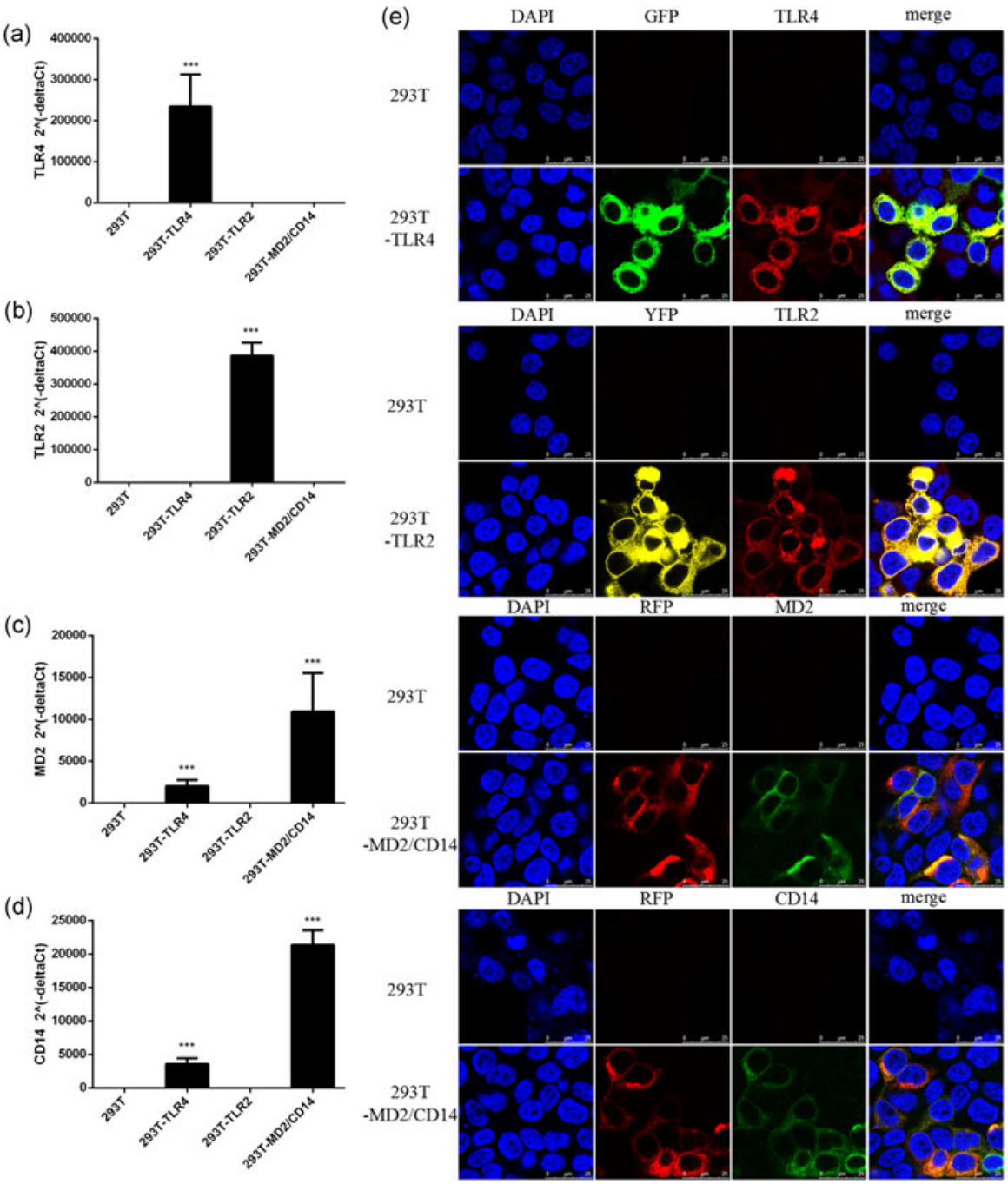

FIGURE 1 Expression of human TLR2, TLR4, MD2, and CD14 in transiently transfected overexpressing cells. HEK 293T cells were transiently transfected with pCAG-TLR4-GFP (293T-TLR4), pcDNA3-TLR2-YFP (293T-TLR2), or pCAG-MD2-CD14-RFP (293T-MD2/CD14). (a–d) The RT-PCR analysis of (a) TLR4, (b) TLR2, (c) MD2, and (d) CD14 mRNA expression in cells transfected with different expression constructs. β-actin was used as the endogenous control. Significance of the differences in mRNA levels compared with parental 293T cells are indicated by $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.0001$ versus 293T cells. (e) Immunofluorescent detection of overexpressed gene products in the different cell lines ($n = 3$). DAPI: 4′,6′-diamidino-2-phenylindole; mRNA: messenger RNA; RT-PCR: quantitative real-time polymerase chain reaction; TLR: toll-like receptors [Color figure can be viewed at wileyonlinelibrary.com]

anti-human TLR4 and TLR2 antibodies and a secondary antibody labeled with tetramethyl rhodamine isothiocynate (TRITC, red) and MD2 and CD14 detected with anti-human MD2 and CD14 antibodies and a secondary antibody labeled with FITC (green). As shown in Figure 1e, overexpression of all four proteins, compared with the parental cells, was detected by immunofluorescence. Thus, both RT-PCR and immunofluorescence indicate that the introduced constructs express TLR2, TLR4, MD2, and CD14 mRNAs and proteins in HEK 293T cells.

3.2 | Identification of a TLR overexpression cell model

To build a pyrogen-sensitive cell model, we transiently expressed combinations of the expression plasmids described above. The generated cells expressed TLR2 and TLR4 (293T-TLR4-TLR2 cells, expressing plasmids pMCS.DTA-TLR4-GFP and pcDNA3-TLR2-YFP), TLR4, MD2 and CD14 (293T-TLR4-MD2/CD14 cells, expressing pMCS.DTA-TLR4-GFP and pCAG-MD2-CD14-RFP), TLR2, MD2, and CD14 (293T-TLR2-MD2/CD14 cells, expressing pcDNA3-TLR2-YFP and pCAG-MD2-CD14-RFP), and TLR2, TLR4, MD2, and CD14 (293T-TLR4-TLR2-MD2/CD14 cells, expressing pMCS.DTA-TLR4-GFP, pcDNA3-TLR2-YFP and pCAG-MD2-CD14-RFP) in HEK293T cells (293T). Examination of the mRNA levels by RT-PCR (Figure 2a–d) and protein levels by western blot (Figure 2e) of the introduced genes indicated that we had successful transient transfection. Western blot analysis, using anti-human antibodies to the TLR4, TLR2, CD14, and MD2 proteins, detected bands of the expected sizes in lysates from the cells for TLR4 (94 kDa), TLR2 (89 kDa), CD14 (53 kDa), and MD2 (26 kDa; Figure 2e). These results also indicate that multiple proteins can be expressed in the same cells. TLR4, TLR2, CD14, and MD2 proteins were not detectable in the parental 293T cell line. The 293T-TLR4 cell line has TLR4 expression but no TLR2, CD14, or MD2. The 293T-TLR2 cell line has TLR2 expression but no TLR4, CD14, or MD2. The 293T-MD2/CD14 cell line has MD2 and CD14 expression but no TLR4 or TLR2. The 293T-TLR4-MD2/CD14 cell line has TLR4, MD2, and CD14 expression but no TLR2. The 293T-TLR2-MD2/CD14 cell line has TLR2, MD2, and CD14 expression but no TLR4. The 293T-TLR4-TLR2 cell line has TLR4 and TLR2 expression but no CD14 or MD2. The cell line with all three transfected plasmids, the 293T-TLR4-TLR2-MD2/CD14 cells, has the expression of all four genes, and thus should have an intact pyrogen sensing signaling pathway.

3.3 | Secretion of IL-6 in response to LPS and LTA in TRL2, TLR4, MD2, and CD14 overexpressing cells

Stimulation of intact TLR4 and TLR2 signaling pathways by LPS or LTA typically results in the secretion of the cytokine IL-6 (Perdomo-Morales, Pardo-Ruiz, Spreitzer, Lagarto, & Montag, 2011). To determine whether our eight cell models have intact TLR4 and TLR2 signaling pathways we measured the levels of IL-6 secreted into the supernatant by ELISA after pyrogen stimulation. Parental 293 and 293T cells release very low levels of this cytokine after 12 hr of stimulation by LPS (5 EU/ml) or LTA (5 μg/ml; Figure 3a and b). The expression of TLR2 (293T-TLR2), or MD2 and CD14 (293T-MD2/CD14) generated cells that still released only very low levels of IL-6 after LPS stimulation. The expression of TLR4 alone (293T-TLR4) or with TLR2 (293T-TLR4-TLR2), MD2 and CD14 (293T-TLR4-MD2/CD14), or all three additional genes (293T-TLR4-TLR2-MD2/CD14) yielded cells that released detectable amounts of IL-6 after LPS stimulation, with the 293T-TLR4-MD2/CD14 and 293T-TLR4-TLR2-MD2/CD14 cells releasing the highest amounts of IL-6 (Figure 3a). Similarly, parental 293T cells, and the cells expressing TLR4 (293T-TLR4) or MD2 and CD14 (293T-MD2/CD14) released very low levels of IL-6 after LTA stimulation. All of the other transfected cells (293T-TLR4-MD2/CD14, 293T-TLR2, 293T-TLR4-TLR2, 293T-TLR2-MD2/CD14 and 293T-TLR4-TLR2-MD2/CD14) released detectable amounts of IL-6 after LTA stimulation (Figure 3b). Thus, our overexpression strategy generated several types of cells that secrete IL-6 in response to pyrogens.

3.4 | Responsiveness of the TLRs overexpressing cells to pyrogens

To assess the responsiveness of the TLR overexpressing cells, we examined the relationship between time and cytokine release to 5 EU/ml of LPS and 5 μg/ml LTA. Our results show that peak levels of secreted IL-6 are detected at 5-6 hr after pyrogen stimulation (Figure 4a,b). We tested the sensitivity of our TLR overexpressing cells to differing amounts of LPA and LTA pyrogens. As shown in Figure 4c,d, and Figure S3, significant amounts of IL-6 was release and detected with as little as 0.05 EU/ml of LPS or 0.05 μg/ml LTA.

3.5 | The application of TLRs overexpressing cells in medicine

The samples that are to be tested for the presence of pyrogens can potentially kill cells, and thus prevent the production of IL-6 yielding a false negative signal for the presence of pyrogens, despite the presence of pyrogens in the sample. Thus, the cytotoxicity of samples needs to be considered for any pyrogen testing model. To examine the cytotoxicity and cell survival with our model we used the MTS colorimetric assay to quantify cell death. 293T-TLR4-TLR2-MD2/CD14 cells were cultured with several drugs (Potassium Aspartate and Magnesium Aspartate Injection, Furosemide Injection and Xinmailong Injection) at different concentrations in DMEM media for 6 hr. Cell death was then quantified with the addition of 0.5 mg/ml MTS (Promega), which was incubated for 2 hr at 37°C, and absorbance measured at 490 nm. To test for the presence of pyrogens, we choose drug concentrations that yielded greater then 90% survival (Figure 5). It was found that 1:50 dilutions of Potassium aspartate and Magnesium aspartate injection diluted by 1:50 (Figure 5a) and Furosemide injection (Figure 5b), and a 1:100 dilution of Xinmailong injection (Figure 5c) would be suitable for testing.

Demonstrating the absence of interfering factors is required to guarantee reliable results of the assay. We then examined our TLRs

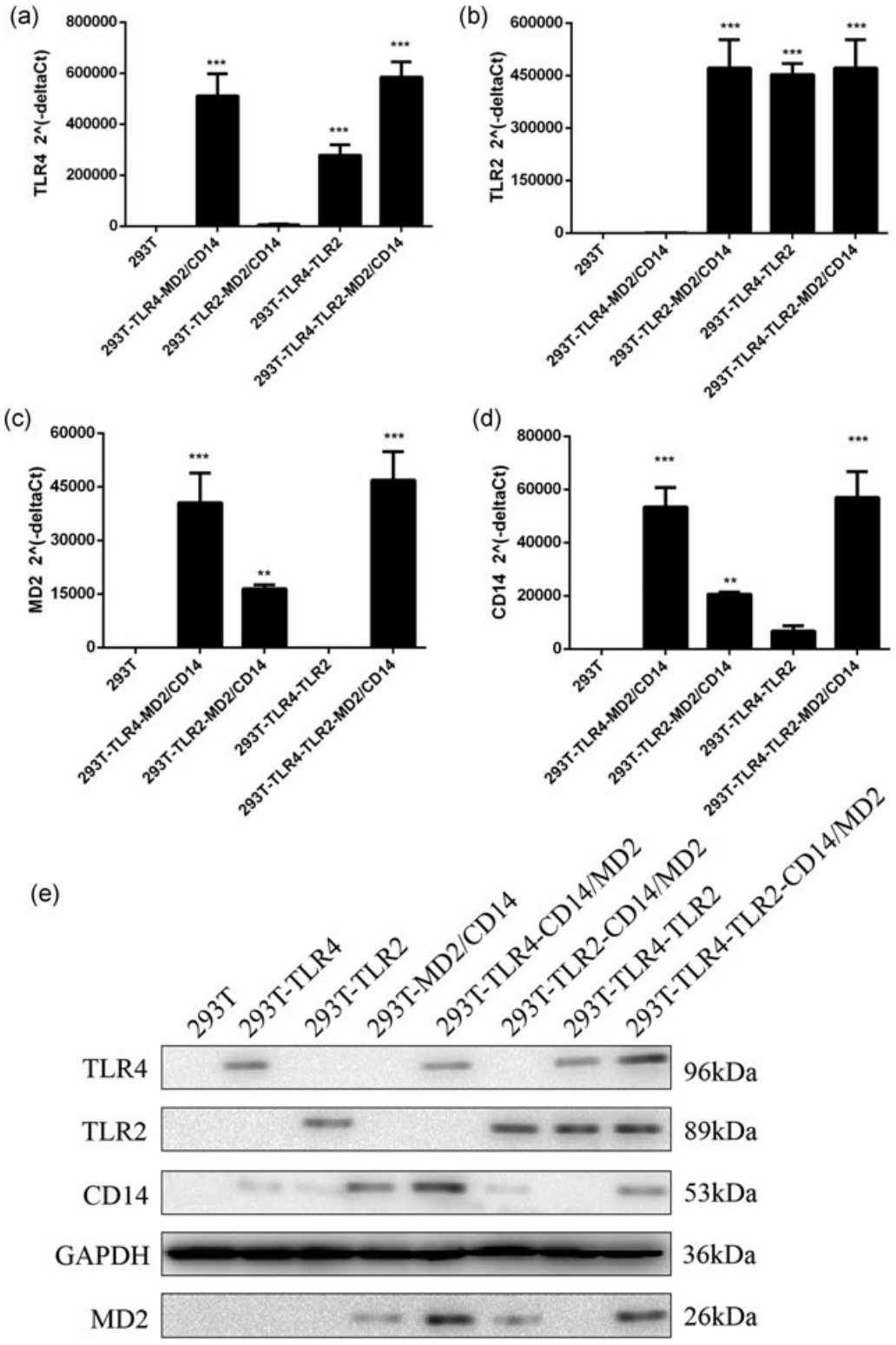

FIGURE 2 Characterization of TLR overexpressing cells. The expression plasmids singly and in combinations (pCAG-TLR4-GFP and pCAG-MD2-CD14-RFP (293T-TLR4-MD2/CD14), pCAG-TLR4-GFP and pcDNA3-TLR2-YFP (293T-TLR4-TLR2), pcDNA3-TLR2-YFP and pCAG-MD2-CD14-RFP (293T-TLR2-MD2/CD14), and pCAG-TLR4-GFP, pcDNA3-TLR2-YFP and pCAG-MD2-CD14-RFP (293T-TLR4-MD2/CD14)) were transiently transfected into HEK293T (293T) cells. (a-d) The RT-PCR analysis of (a) TLR4, (b) TLR2, (c) MD2, and (d) CD14 mRNA in different plasmid combination transfection models, with β-actin used as an endogenous control. Significance of the difference in expression versus 293T cells is indicated by $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.0001$. (e) Western blot analysis of TLR4, TLR2, CD14, MD2, and GAPDH expression in the single and combination transfection cell lines ($n = 3$). mRNA: messenger RNA; TLR: toll-like receptors

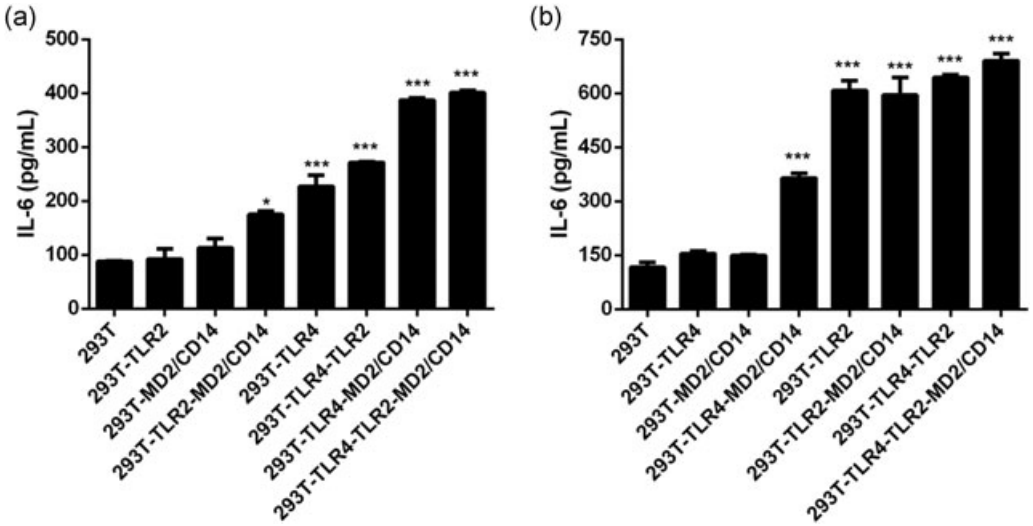

FIGURE 3 Secretion of IL-6 after stimulation of model cell lines with LPS and LTA pyrogens. Detection of secreted IL-6 in eight cell lines transfected with single or combinations of expression plasmids for TLR2, TLR4, MD2 and CD14 after stimulation with (a) 5 EU/ml of LPS and (b) 5 ug/ml of LTA ($n = 3$). Significant increases in IL-6 levels after pyrogen stimulation, compared with wild type 293T cells, is indicted by $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.0001$. TLR: toll-like receptors

overexpressing cell model/IL-6 pyrogen testing method with the interferences test [23], which determines the level of an endotoxin that can be recovered from positive control spikes. TLR over-expressing cells, cultured in the exponential phase, were divided into experimental and control groups. The control group received only cell-culture media. Experimental groups were incubated with different concentrations of pyrogen spiked dilutions of the tested drug and assayed in parallel with the corresponding unspiked dilution. Dilutions with pyrogen recovery between 50% and 200% are considered to be interference-free (Perdomo-Morales et al.,

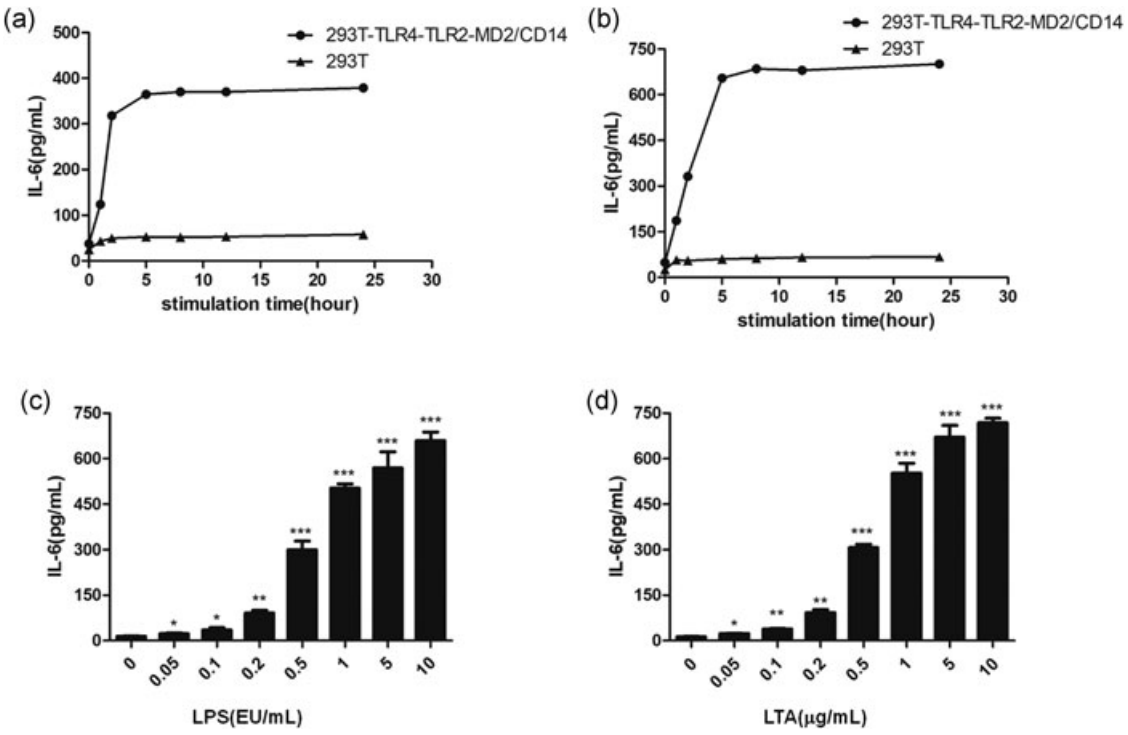

FIGURE 4 Time dependence and dose response of the secretion of IL-6 after stimulation with pyrogens in the 293T-TLR4-TLR2-MD2/CD14 cell line. (a,b) Time dependence of the secretion of IL-6 from 293T-TLR4-TLR2-MD2/CD14 cells after stimulation with (a) 5 EU/ml of LPS and (b) 5 ug/ml of LTA. (c,d) Dose responses of IL-6 secretion from 293T-TLR4-TLR2-MD2/CD14 cells after stimulation with (c) LPS ranging from 0.001 to 10 EU/ml and (d) LTA ranging from 0.001 to 10 ug/ml

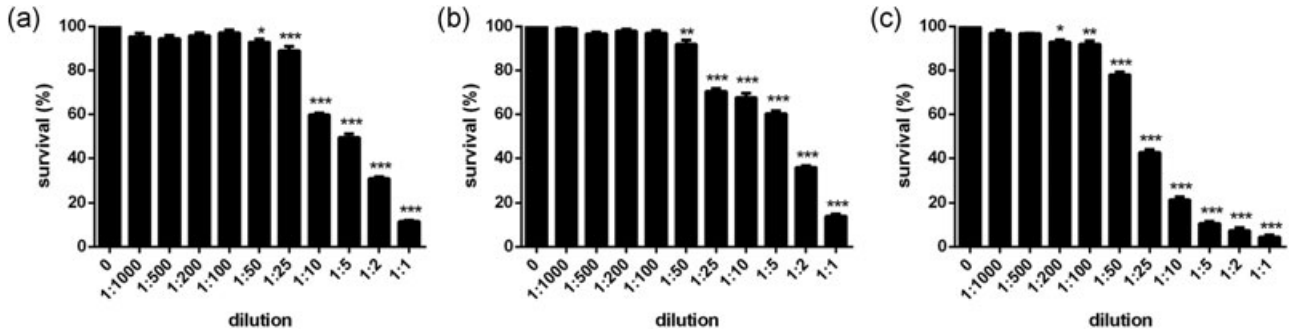

FIGURE 5 Cell survival rate in the presence of injectable drugs. Cytotoxicity of (a) Potassium aspartate and Magnesium aspartate injection, (b) Furosemide injection, and (c) Xinmailong injection injectable drugs evaluated using the MTS colorimetric assay. Compared with 293T-TLR4-TLR2-MD2/CD14 cells without drugs coculture, is indicted by $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.0001$. MTS: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide reagent

2011). (Table 2). Results of the interference test are depicted in Table 2. We found that pyrogen recovered from the spikes are in the accepted range, the TLRs overexpressing cells model is a suitable pyrogen test for the three injection.

4 | DISCUSSION

The gut of a single human contains 1-2 kg of bacteria, with about 50 g of endotoxin, which is enough endotoxin, if purified, to kill one million people or induce fever in one billion (Hartung, 2015). There are challenges in detecting pyrogens. Currently, the two most commonly-used pyrogen tests are the RPT and the TAL (Fennrich et al., 2016). The RPT is an in vivo test able to detect various kinds of pyrogens, but is cumbersome with poor repeatability and is easily affected by the presence of antibiotics, blood products, and other factors, and it requires the use of laboratory animals. TAL is simpler, and has high sensitivity, but is greatly affected by external environmental factors and is prone to false-positive results due to the chemical nature of the tested biological agents (Unger, Peters, Sartoris, Freese, & Kirkpatrick, 2014). BET (Suffredini et al., 1999) is often considered as a replacement for the animal test, but it is unable to detect pyrogens other than endotoxins (Dullah & Ongkudon, 2017). Toll-like receptors (TLRs) recognize specific patterns derived from invading microorganisms or damaged cells/tissues and are critical in provoking innate and adaptive immune responses (Lester & Li, 2014).

Here we established a TLRs overexpressing cell model, with IL-6 as a readout, and evaluated the reliability of this model for detecting pyrogens. On the basis of the existing knowledge, we targeted the TLR2 and TLR4 signal transduction pathways, and overexpressed important regulatory proteins of this pathway in HEK293T cells to reconstruct toll receptor signaling and generate a pyrogen-sensitive cell model that should allow the quantitative detection of pyrogens (Ingalls, Lien, & Golenbock, 2000).

TABLE 2 Interferences test of LPS and LTA in three injection

Drugs	LPS (EU/ml) LTA (ug/ml)	LPS spike recovery (%)	LTA spike recovery (%)
Potassium Aspartate and Magnesium Aspartate injection	0.05	59.51	58.08
	0.1	70.91	59.86
	0.2	81.34	68.98
	0.5	95.85	71.76
	1	97.33	79.36
Furosemide injection	0.05	67.48	54.29
	0.1	75.62	60.33
	0.2	85.21	77.36
	0.5	94.54	77.28
	1	97.69	80.45
Xinmailong injection	0.05	53.68	53.54
	0.1	57.02	52.22
	0.2	71.65	58.85
	0.5	83.15	66.73
	1	85.03	70.75

4.1 | MD2, CD14, and TLR4 enhance the abundance of each other

MD2 is an essential component of the TLR4/MD2 complex and has a hydrophobic pocket that interacts with LPS, binding of LPS to MD2 is the initial step leading to TLR4 activation (Koo, Park, Kim, & Lee, 2013). CD14 was initially discovered as a receptor for lipopolysaccharide-binding protein-bound LPS, and it is central to the mammalian response to endotoxin (Fitzgerald, Rowe, & Golenbock, 2004; Schumann et al., 1990). An interesting observation of our transient transfection experiments was that overexpression of MD2 and CD14 or just TLR4-yielded cells capable of responding to LPS (Figure 3a), albeit, with a response that was weaker than that generated when all three TLR4 signaling components were overexpressed. The RT-PCR analysis showed that with the overexpression of TLR4, the abundance of CD14 and MD2 increased. Similarly, with the overexpression of CD14-MD2, the abundance of TLR4 mRNA increased. In addition, the abundance of TLR4, MD2, and CD14 mRNAs in cell lines that overexpressed TLR4 and MD2/CD14 were significantly different from cell lines that overexpressed TLR4 or MD2/CD14 alone. (Figure 2a,c,d). These results suggest that expression of one component modulates the stability of other components of this pathway in these cells.

4.2 | Optimization of TLRs overexpression cell model/IL6 conditions

Increased levels of IL-6 found in the circulation after the injury correlates with increased body temperature (Kluger, 1991; Rothwell & Hopkins, 1995) and thus correlates to pyrogen exposure. Here, we used ELISA to quantify the release of IL-6 to assess the responsiveness of our TLR overexpressing cell model to pyrogens (Brown & Gordon, 2003; Dinarello, 2004; Fitzgerald et al., 2003; Perdomo-Morales et al., 2011). Our results show that wild type 293T cells have very little responsiveness to pyrogen stimulation as measured by IL-6 release. 293T-TLR4-TLR2-MD2/CD14 overexpressing cells had the strongest response to both LPS and LTA stimulation. 293T cells with TLR4 or MD2/CD14 overexpression had a response to LPS stimulation, which was weaker than when both TLR4 and MD2-CD14 were cotransfected into the 293T cell line. We found that $2 \times 10\text{^}5$ cells per well in 96-well plates initially cultured overnight before transfection with plasmids and allowed to recover for 24 hr after transfection yielded the greatest sensitivity. For pyrogen stimulation, a 6-hr exposure yielded the strongest responses. A detection limit of 0.05 EU/ml for LPS and 0.05 μg/ml for LTA was found for our IL-6 readout when 100 μl of supernatant was concentrated to a volume of 50 μl.

4.3 | Feasibility of using TLR overexpressing cells for pyrogen testing

We tested different cell densities and transfection conditions to determine the conditions with the highest transfection efficiencies for our pyrogen detection experiments. The expression of the various components was assessed by RT-PCR, expression of linked fluorescent reporter genes and immunofluorescent detection of the encoded proteins (Figures 1,2). While our transiently transfected overexpressing cells demonstrate that these cells can be used to test pyrogens, they are not suitable for routine testing at this time as they are not in a stable cell line. Several methods have been proposed for the rapid detection of pyrogens. Li et al. (2018) reported a new method by using the luciferase reporter assay and H. Jiang, D. Jiang, Shao, Sun, & Wang, (2016) used fluorescent protein reporter. These methods are basically based on the NF-kB signaling pathway, which could prove that our method is feasible. We constructed cell model by overexpressing TLR2, TLR4, MD2, and CD14 in HEK293 cells, which didn't express these genes. The release level of IL-6 was detected by double antibody enzyme-linked immunoassay. We detect the endogenous pyrogen IL-6 directly.

5 | CONCLUSIONS & FUTURE PERSPECTIVE

Pyrogens are components of microorganisms, such as bacteria, viruses or fungi, which induce complex inflammatory responses in the human body. LPS is the typical pyrogen produced by gram-negative bacteria whereas LTA is from gram-positive bacteria. As existing pyrogen tests, including the RPT, TAL, and the MAT have limitations, there is a need to develop new models for pyrogen testing. By overexpressing TLR4, TLR2, MD2, and CD14 components of the Toll-like signaling system in HEK293 cells we established a TLR2/TLR4/MD2/CD14 overexpressing cell model that produces IL-6 in response to pyrogen challenge. This model can be used to test injectable medicines, which cannot be tested using the TAL test approach. Our method can supplement classical pyrogen detection methods as it detects multiple types of pyrogens has high sensitivity and low cost. Our method is quantitative, with detection limits of 0.05 EU/ml for LPS and 0.05 ug/ml for LTA, and lays a foundation for the development and establishment of new and easier methods for the detection of pyrogens.

6 | SUMMARY POINTS

TLR4, MD2, and CD14 play key role in the LPS-mediated pyrogen reaction and TLR2 plays key role in the LTA-mediated pyrogen reaction.

Overexpression of TLR2/TLR4/MD2/CD14 in HEK 293 cells established Toll receptor signaling that lead to IL-6 secretion in response to a pyrogen challenge.

Our TLRs cell model is sensitive for pyrogens and can be used to test injectable drugs, which cannot be tested using the TAL test approach.

Our TLR cell model could be adapted for routine testing for pyrogens in a clinical setting.

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CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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