

Pentoxifylline Restores T Cell Viability in Hyper-inflammatory Conditions

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Research

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Abstract

Background: Immunity is the state of having sufficient biological defenses to avoid infection, trauma, or other unwanted biological invasions. T-cells and macrophages play important role in cell-mediated immunity. Pentoxifylline (PTX) is known to decrease pro-inflammatory cytokine and tumor necrosis factor (TNF- α). However, the effect on the immune system is not known well. This study aims to investigate the effect of PTX on inflammation.

Methods: THP-1 derived macrophages were incubated with lipopolysaccharide (LPS) and/or indicated concentration of PTX for 8hr and wash with PBS to eliminate the effect of LPS. In this media, Jurkat cells were plated into trans-well plate and co-culture was done at 12hr. The T cell viability was measured by MTT. Also, the expression of Interleukin-2 (IL-2) was analyzed by RT-PCR and western blots.

Results: PTX restored the increased concentration of MIF, TLR₄ protein level and mRNA expression of TLR₄ in LPS stimulated THP-1 derived macrophages. However, PTX did not restore the decreased T cell proliferation with PGE₂ in Jurkat cells. In the co-culture study, The T cell viability was decreased in the THP-1 derived macrophage cells stimulated with LPS. The additional PTX restored the T cell's viability. Besides, PTX restored the decrease in the IL-2 expression of Jurkat cells in the LPS stimulated THP-1 derived macrophages.

Conclusion: LPS stimulated THP-1 derived macrophages inhibited the T cell viability in hyper-inflammation conditions. However, PTX restored the T cells viability with increase IL-2. PTX influenced the cell-cell interaction, therefore, had its immunomodulatory effects.

Background

In the event of a shock caused by major trauma, hemorrhage, or infection, etc., many patients die despite the initial proper treatment. This is caused by excessive hyper-inflammatory conditions and the occurrence of immunoparalysis, i.e. the destruction of homeostasis, resulting in multiple organ failure, sepsis, etc. In general, immunity is the state of having sufficient biological defenses to avoid trauma, infection, or other unwanted biological invasions and preserve homeostasis in inflammatory syndromes. However, severe hyper-inflammatory conditions caused by various causes lead to systemic inflammatory response syndrome and immunoparalysis causing compensatory anti-inflammatory response syndrome, resulting in many complications. In the case of various injuries, monocytes and neutrophils react in the early stages of the injury. First, the monocytes in the blood vessels migrate to the damaged tissue and differentiate into macrophages. Antigens are captured by macrophages in peripheral tissues and processed to form major histocompatibility complex-peptide complexes. As a consequence of antigen deposition and inflammation, macrophages begin to mature, expressing molecules that will lead to bind and stimulate T cells in the T-cell areas of lymphoid tissues. After T blasts leave the blood at the original site of antigen deposition, recognition changes in the inflamed blood vessels. In brief, macrophage has been known to play a key role in the recognition of bacterial components during infection. Besides, toll-

like receptor (TLR) activation promotes the secretion of inflammatory cytokines, and chemokines and T-cells play important role in cell-mediated immunity [1, 2]. Yet, there were no studies on T lymphocytes stimulated by macrophages and the hyper-inflammation control effect of macrophages on T lymphocytes.

Pentoxifylline (PTX), 3,7-dimethyl-1-(5-oxohexyl)-3,7-dihydropurine-2,6-dione, a methylxanthine derivative and nonspecific phosphodiesterase inhibitor, is commonly used as a vasodilator and anticoagulant, which decrease the resistance of vessels, blood viscosity, platelet aggregation and augments blood flow in ischemic tissue. It also has been clinically used in the treatment of intermittent claudication in patients with atherosclerotic disease. Recently, a very important effect of PTX is inhibition of inflammatory response through blockage of extracellular signal-regulated kinase (ERK) phosphorylation, especially the downregulation of tumor necrosis factor (TNF- α) [3, 4]. Even though further study on the mechanism of the PTX was investigated, the effect of PTX on the immune system is still not known well. The aim of this study is to investigate the effects of PTX on lipopolysaccharide (LPS) induced TLR₄ expression in THP-1 derived macrophage and on the T-cell in hyper-inflammatory condition by using co-culture. In other words, in the occurrence of sepsis, multiple organ failure, a secondary complication in trauma, hemorrhage, and infection, we would like to check the intercellular mechanism of macrophages and T-lymphocytes and find out the usefulness of the phosphodiesterase inhibitor. In brief, we want to investigate the effect of PTX on inflammation reaction.

Methods

Cell culture, cell stimulation and co-culture

Human acute monocytic leukemic cell line (THP-1) cells (ATCC, Manassas, VA, USA) and lymphocytic cell line (Jurkat) cells (ATCC, Manassas, VA, USA) each were maintained in RPMI-1640 (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum, 2 mM glutamine, 10 mM HEPES, 100 U/mL penicillin/streptomycin at 37°C in 5% carbon dioxide incubator. Cells were cultured to a density of 5×10^5 cells/mL. Cell viability, as determined by tyropan blue dye exclusion, was > 99%. The THP-1 cells were incubated with Phorbol-12-myristate-13-acetate (PMA) for 3days for THP-1 cells differentiation. The cells were plated at a density of 1×10^4 cells/mL in 24-well plates and were stimulated with LPS (1 μ g/mL) (Sigma-Aldrich Co., St. Louis, MO, USA) in the absence or presence of PTX (0.1mM, 1mM, 2mM) (Sigma-Aldrich Co., St. Louis, MO, USA) for 8hr. The macrophage migration inhibitory factor (MIF) concentration was detected by ELISA. The TLR₄ protein level was detected by flow cytometry. The mRNA expression of TLR₄ was detected using a real-time polymerase chain reaction (RT-PCR). Jurkat cells (lymphocytic cell line) were plated into in 96-well plate and were stimulated with PGE₂ (1 μ g/mL) (Sigma-Aldrich Co., St. Louis, MO, USA) in the absence or presence of PTX (0.1mM, 1mM, 2mM) (Sigma-Aldrich Co., St. Louis, MO, USA) for 12hrs. The T cell viability was measured by MTT, expression of interleukin-2 (IL-2) was analyzed by RT-PCR and MIF concentration was detected by ELISA. For co-culture, the cells were plated at a density of 1×10^4 cells/mL \times 0.5ml in 24-well plates and were stimulated with LPS (1 μ g/mL) in the

presence or absence of PTX (2mM). After 8hr, the media was washed twice with PBS (Phosphate buffered saline), and add fresh media to eliminate the effect of LPS and PTX in media. Lymphocytic cell line (Jurkat) cells (ATCC, Manassas, VA, USA) were plated into 2×10^6 cells/ml \times 0.5ml in a trans-well plate and co-culture was done for 12hr. The T cell viability was measured by MTT and expression of interleukin 2 (IL-2) was analyzed by RT-PCR. Experiments were performed in triplicate using three consecutive wells of 96-well plates. Each experiment was repeated at least twelve times. This study was carried out with the approval of the Korea University Guro Hospital Institutional Review Board (approval number: 2018GR0155).

Enzyme-linked immunosorbent assay (ELISA) for MIF.

The MIF concentration in the culture supernatants was measured by sandwich Enzyme-linked immunosorbent assay (ELISA). Briefly, 2 μ g/ml of monoclonal capture antibody (R&D Systems, Minneapolis, MN, USA) was added to a 96-well plate and incubated for one day at room temperature. After incubation, the plates were incubated in a blocking solution of phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA) and 0.05% Tween 20 for 2hrs at room temperature. Test samples and standard recombinant MIF (R&D Systems) were added to the plates and incubated for 2 hrs at 4°C. Plates were washed three times with PBS containing Tween 20, and after the addition of 200ng/ml of biotinylated detection monoclonal goat-antihuman antibodies (R&D Systems), the plates were incubated for 2hrs at room temperature. The plates were washed, streptavidin-alkaline-phosphatase (1:2000; Sigma-Aldrich Co.) was added and the reaction was allowed to proceed for 20min at room temperature. The plates were washed three times and 1mg/ml of p-nitrophenylphosphate dissolved in diethanolamine (Sigma-Aldrich Co.) was added to induce a color reaction, which was stopped with 50 μ l of 1M NaOH. The optical density at 450nm was measured on an automated microplate reader (Bio-Rad Laboratories Inc., Hercules, CA, USA). A standard curve was generated by plotting the optical density vs. the log of the MIF concentration.

Flow cytometry analysis for TLR₄

The expression of cell surface TLR₄ was determined by flow cytometry analysis. The macrophages were harvested by centrifugation. Cells were washed with 3ml ice-cold phosphate-buffered saline, then resuspended in 1ml flow cytometry staining buffer (eBioscience, San Diego, CA, USA) and blocked for 10 minutes on ice. The cells were then stained with 5 μ L (2 μ g) phycoerythrin-conjugated anti-TLR₄ monoclonal antibody (clone HTA125; eBioscience) or 5 μ L (0.5 μ g) isotype control (PE-conjugated mouse IgG2a K; eBioscience) per test in the dark for 100 min on ice following the manufacturer's recommended protocol. After washing, the stained cells were resuspended in 100 μ L staining buffer and analyzed by flow cytometry using cytomics FC 500 (Beckman Coulter, Brea, CA, USA) and CXP software (Beckman Coulter). In each case, 100,000 cells were acquired.

Real-time polymerase chain reaction for TLR₄, IL-2 expression

Expression of TLR₄ mRNA and IL-2 mRNA was analyzed by qRT-PCR. Total RNA was extracted from cells using an RNeasy Mini Kit (Qiagen, Hilden, Germany) and treated with DNase (Turbo DNA-free kit; Invitrogen, Carlsbad, CA, USA) prior to use. An amount of 200 ng total RNA from each sample was reverse transcribed into complementary DNA (cDNA) using a high-capacity cDNA reverse transcription kit. After completion of the first-strand cDNA synthesis, the TLR₄ and IL-2 probe were used to analyze 34 cycles of 50°C for 2 min, 95°C for 10 min, 95°C for 15 min and 60°C for 1 min, with an RT-PCR system (AB7300, Applied Biosystems, Foster City, CA, USA).

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) viability assay

The tetrazolium dye, MTT, is widely used to assess the viability or the metabolic state of the cells. The MTT-colorimetric monocyte mediated cytotoxicity assay is based on the ability of living cells that reduce MTT into formazan by mitochondrial succinate dehydrogenase in viable cells. After treatment at the different culture conditions, Jurkat cells were plated in 96-well flat-bottom tissue culture plates to attain a final concentration of 2×10^6 cells/mL. After incubation for 12 hours at 37°C, the resultant Jurkat cell viability was determined by the MTT viability assay (ATCC, Manassas, VA, USA)

Western blot analysis for IL-2 expression

The cells were washed 2 times in cold PBS and then centrifuged for 10 minutes. Cell pellets were suspended in 10 µL per 2×10^6 cell/mL pro-prepTM protein extraction buffer. Incubated on ice for 10 minutes, and then centrifuged at $3,000 \times g$ for 15 minutes at 4°C. The supernatant was then transferred to a new tube and used for the assay. The total protein concentration was determined by the Bradford method using a Bradford solution (Sigma Co.). The prepared protein was used for western blot analysis. The expression of IL-2 protein was quantified by western blot analysis. Proteins (20µg/sample) were fractionated on a 15% sodium dodecyl sulfate-polyacrylamide gel (Bio-Rad Laboratories Inc.) and transferred onto a nitrocellulose membrane. Membranes were blocked for 1 hour in 5% skim milk (Bio-rad Co.) and then incubated with a primary antibody, anti-human IL-2 (1:500; R&D systems). After washing, membranes were incubated with 1:1000 horseradish peroxidase-labeled anti-rabbit antibody (R&D systems) as the secondary antibody. The proteins were detected using ECL (Cyanagen) chemiluminescence kit.

Data and Statistical analysis

One-way ANOVA and the post hoc test were used for statistical analysis with the SPSS software package (version 13.0, IBM-SPSS Inc, Chicago, Illinois). A p-value < 0.05 was considered to indicate statistically significant differences. Paired t-test was used for statistical analysis with the SPSS software package (version 13.0, IBM-SPSS Inc, Chicago, Illinois). A p-value < 0.05 was considered to indicate statistically significant differences.

Results

The effect of PTX in the LPS-induced TLR₄ and MIF in differentiated THP-1 cells

TLR₄ mRNA expression in LPS-stimulated differentiated THP-1 cells was increased at 6hrs and 8hrs (Fig. 1). After 8hrs of incubation after adding PTX, the proportion of TLR₄ positive cells in LPS stimulated differentiated THP-1 cells decreased. The mean percentage (mean ± SD) of cells stained with anti TLR₄ monoclonal antibody was 0% in the control group, 49.3 ± 12.2 in the LPS stimulated group, 44.3 ± 11.8 at 0.1 mM PTX, 35.9 ± 11.3 at 1 mM PTX, 27.75 ± 11.6 at 2 mM PTX by flow cytometry. In the TLR₄ mRNA expression measured by RT-PCR, The TLR₄ mRNA expression was increased in the LPS stimulated differentiated THP-1 cells, however, PTX did not restore in LPS stimulated differentiated THP-1 cells (Fig. 2A)(Fig. 2B). While LPS increased the concentration of MIF in differentiated THP-1 cells, PTX restored LPS-induced concentration of MIF with the ELISA method (Fig. 3).

The effect of PTX in the PGE₂-induced T cell proliferation and MIF in Jurkat cells

PGE₂ decreased T cell proliferation in Jurkat cells. PTX did not restore in the T cell proliferation with PGE₂ by MTT assay (Fig. 4). PGE₂ also decreased the concentration of macrophage migration inhibitory factor (MIF) in Jurkat cells, however, PTX did not restore PGE₂-induced macrophage migration inhibitory factor with the ELISA method (Fig. 5).

The effect of PTX on the Jurkat cells in the hyper-inflammatory differentiated THP-1 cells

After conducting various combinations of co-culture, the ratio of differentiated THP-1 cells and Jurkat cells was determined as the ratio of differentiated THP-1 cells density of 1×10^4 cells/mL and Jurkat cells density of 2×10^6 cells/mL (Fig. 6). The percentage of the Jurkat cell viability decreased in the differentiated THP-1 cells stimulated with LPS. The additional PTX restored the Jurkat cell viability. The mean MTT value of Jurkat cell viability under non-stimulated differentiated THP-1 cells was 1.19 ± 0.08 in the control group, 1.05 ± 0.03 in the differentiated THP-1 cells stimulated with LPS, and the mean MTT

value of Jurkat cell viability were 1.16 ± 0.05 , 1.15 ± 0.04 , 1.27 ± 0.05 in the differentiated THP-1 cells stimulated with LPS and 0.1 mM, 1 mM, 2 mM PTX ($p < 0.05$) (Fig. 7). In the same manner, IL-2 was decreased in the differentiated THP-1 cells stimulated with LPS and was restored in the differentiated THP-1 cells stimulated with LPS and PTX. The mean of IL-2 mRNA expression ratio (IL-2/GAPDH ratio) was 6.11 ± 2.19 in the control group, 1.49 ± 1.50 in the differentiated THP-1 cells stimulated with LPS, and 6.15 ± 3.29 in the differentiated THP-1 cells stimulated with LPS and 2 mM PTX ($p < 0.05$) (Fig. 8). The results of IL-2 were similar in the western blot. The mean of IL-2 expression ratio (IL-2/Actin ratio) was 0.97 ± 0.52 in the control group, 0.88 ± 0.31 in the differentiated THP-1 cells stimulated with LPS, and 1.10 ± 0.42 in the differentiated THP-1 cells stimulated with LPS and 2 mM PTX ($p < 0.05$) (Fig. 9).

Discussion

Trauma patients account for about 25–35% of the patients visiting the emergency center, which takes a large proportion of deaths and disabilities among young age groups resulting in national losses due to the loss of economic ability.

In many countries, it is difficult to reduce on-site deaths even though trying to improve the level of primary care in hospitals and reduce complications that occur after primary treatment such as sepsis, multiple organ failure. It is important to maintain the homeostasis of trauma patients until surgical treatment in order to alleviate hyper-inflammatory and immune-paralysis conditions to prevent the occurrence of post-traumatic secondary complications, such as sepsis, multiple organ failure. This study was conducted because many patients died despite the proper treatment. The tendency of macrophages to differentiate THP-1 cells, which are human monocytic cell lines, is showing changes in macrophages that respond early in the event of various damage, such as trauma, hemorrhage, and infections. Also can identify changes in hyper-inflammatory conditions for early infection and find out the usefulness of PTX, which is of interest in our research. We used the Jurkat cell which plays the main role of immunity, the human T lymphocytic cell line, commercially purchased for the study of T lymphocytes, to find out the change in immune-paralysis conditions and to find out the effect of PTX. T lymphocytes were co-cultured into stimulated macrophages to find out the effect of hyper-inflammatory macrophages and the effect of PTX injection on T lymphocytes to identify the change of immunity.

THP-1 cells were converted into differential THP-1 cells after 3 days of incubation with PMA injection to develop the propensity of macrophages for the experiment. The study was conducted on an eight-hour basis since TLR₄ increased after eight hours of injection of LPS. As a result, TLR₄, which is a bacterial infection receptor when injected with LPS, increased when measured with flow cytometry that directly measures the receptor in cells. It was meaningful that TLR₄ reduced as PTX capacity increased. The PCR that checks the mRNA expression in gene measurement showed that the mRNA expression of TLR₄ increased at LPS injection, but the injection of PTX did not show any change. The PTX reported anti-inflammatory tendencies by mitigating tumor necrosis factor- α (TNF- α) increases, nuclear factor- κ B (NF- κ B) activation, TLR₄ increases, MIF increases, etc. in stimulated cells [5]. As there is no change in the PCR

method, it is assumed that it does not affect the gene level. It is estimated the reduction of TLR₄ and MIF that react early in the stimulus decrease the phosphorylation of NF-κB indicating a decrease in TNF-α [5–7]. It was conducted using the Jurkat cell, a human acute lymphocytic leukemic cell, which is widely used for research on activation of T lymphocytes that play an important role in immunity. The injection of PGE₂, an immune-reducing substance, reduced the number of Jurkat cells by the MTT method. Unlike the restoration of reduced T lymphocyte

by hypertonic saline in our past studies [8], PTX did not restore the reduced T lymphocyte. PTX shows multiple beneficial effects on the inflammatory cascade according to various studies. PTX down-regulates the production of various pro-inflammatory cytokines (TNF-α, IL-6) and anti-inflammatory cytokines (IL-10) which increase in infection, hemorrhage, and shock. Also, inhibit the activation of NF-κB in various cells and inhibits the production of various reactive oxygen species (ROS), including superoxide anion (O₂⁻), hydrogen peroxide (H₂O₂) which is known to increase during ischemia-reperfusion [7, 9–11]. Despite the many studies on reports of increasing transforming growth factor (TGF)-β and an anti-inflammatory cytokine in coronary artery disease [12, 13], there have been no studies on T lymphocyte. Our research showed no effect on immunity increase by increased T lymphocytes.

The MIF has been shown to not only override the anti-inflammatory effects of glucocorticoid but also to induce TLR₄ expression on the surface of the cell, inhibit p53-mediated apoptosis and stimulate proliferation of cells [14]. Therefore, the MIF plays an important role in sepsis by controlling inflammatory reactions, including activations of various cytokines in macrophages, neutrophils, and T lymphocytes [15, 16]. In our study, as with the increase of TLR₄ during LPS injection, MIF was also increased to identify the relationship between TLR₄ and MIF, and PTX was effective by restoring TLR₄ and MIF. As the relationship between MIF and T cell proliferation was also known, the MIF was reduced when the T cell proliferation was reduced by PGE₂. This was the same as in our previous study that MIF was increased during the injection of LPS into macrophages, resulting in pro-inflammatory effects, and MIF was decreased in the injection of PGE₂ which indicates decreased immune function, along with the reduction of T cell proliferation, to indicate immune-paralysis [8]. In this study, PTX did not restore T cell proliferation and MIF. Moreover, PTX showed a decrease further than PGE₂ injection.

The two cells were co-cultured to find out the effects of macrophages on T lymphocytes, which play an important role in the immune system. Generally, the number of lymphocytes in normal healthy people was about 7 to 20 times that of monocyte, but in order to determine the appropriate number of cells for co-culture between two cells in the experiment, the Jurkat cells for MTT measurement evaluation were set at 2×10^6 /mL. The differentiated THP-1 cells were co-cultured in various numbers and differentiated THP-1 cells were selected to be 1×10^4 /mL by showing similar MTT as shown. The differential THP-1 cell stimulated by LPS was washed with PBS to remove the effects of LPS, placed at the bottom of the culture plate divided into two layers, and the Jurkat cell was placed upstairs to measure the MTT of the Jurkat cells. In the THP-1 cells group stimulated by the LPS, the Jurkat cells decreased compared to the non-stimulated THP-1 cells group showing macrophages in hyper-inflammatory conditions could induce

a decrease in T lymphocytes. Banchereau J et al. [1] reported that the Dendritic cell acts as an initiator and modulator for immune response. Munn DH, et al. [2] also reported that macrophage is known to suppress T cell proliferation due to the influence of macrophage colony-stimulating factor (MCSF), and MCSF-derived macrophages were capable of depleting the essential amino acid tryptophan from co-culture. Our study showed similar conclusions. Moreover, our research makes it easier to recognize immunity by measuring the Jurkat cell viability directly. The MTT (T cell proliferation) value of Jurkat cells group injected with PTX and LPS in the macrophages restored the MTT value of Jurkat cells group in the macrophages stimulated only by LPS to the normal level. Besides, as the capacity of the PTX increases, the MTT value of Jurkat cells increased. The IL-2, a cytokine that plays an important role in T cell proliferation, was tested using various methods (RT-PCR, western blots) to check the accuracy of MTT viability assay. The MTT viability assay showed a similar reduction in both groups, however, injection of LPS and PTX restored the control group. In other words, stimulated macrophages reduced T cell proliferation and IL-2. On the other hand, PTX restored the level of the control group [17–20]. Since IL-2 is known to increase immunity by inducing T cell proliferation, the injection of PTX into LPS stimulated macrophages could be determined to increase immunity by increasing T cell proliferation [11].

PTX starts with a substance that improves the microvascular blood flow and is reported to reduce the mortality of neonatal sepsis. Also reduces the activation of NF- κ B as well as downregulating CD66b and TNF- α expression as a mechanism to attenuate the various pro-inflammatory neutrophil functions [5]. There was no research on the T cells of PTX before us. In our study, PTX was not able to act directly on the T cells to enhance immunity, but the effect of restoring MIF and TLR4 expression increased in macrophages stimulated by LPS from co-culture. Moreover, PTX restored the reduced MTT value and IL-2 expression to the T cell control group. In conclusion, PTX was found to maintain homeostasis with the restoration of the T cells control group, which is clinically related to IL-2.

There are many limitations. First, studies of intercellular effects and relationships between macrophages and T lymphocytes, excluding studies of intercellular effects, such as neutrophil, should be conducted later. Second, in the co-culture of LPS-stimulated macrophages and T lymphocytes, PTX has restored the T cells proliferation to the level of the control group, but only IL-2 has been conducted in our study, so future research will be needed. However, we understand that MIF as well as IL-2 are involved in T cell proliferation in our previous research, but more research is expected to be needed. Third, for the usefulness of the PTX, further research will be needed, such as animal experiments or clinical trials.

Conclusions

Consequently, the occurrence of hyper-inflammatory and immune-paralysis conditions leads to death due to sepsis and multiple organ failure as a result of infection, trauma, and hemorrhagic shock, The usefulness of PTX was identified by using a similar cell, the Jurkat cell, for T cells proliferation, which is critical to the occurrence of sepsis. While PTX was found to restore excessive immuno-paralysis to the control group by coordinating hyper-inflammatory conditions in the culture of macrophages and T lymphocytes, it showed some problems with improving immuno-paralysis. The intercellular effects and

some other effects by coordinating hyper-inflammatory conditions somehow prevent immuno-paralysis. Therefore, PTX is effectively determined to maintain homeostasis, and further studies will be needed by either animal experiments or clinical trials.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

The data and materials used in the current study are all available from the corresponding author upon reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

SJP, SHC, and YDC conceived the idea. JYK, SHC, CSL, WYK designed the experiments and interpreted the data. SJP, HJC, KHK, and SHC performed the experiments. SJP and SHC wrote the manuscript. All authors read and approved the final manuscript.

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Figures

TLR₄ expression in differentiated THP-1 cells

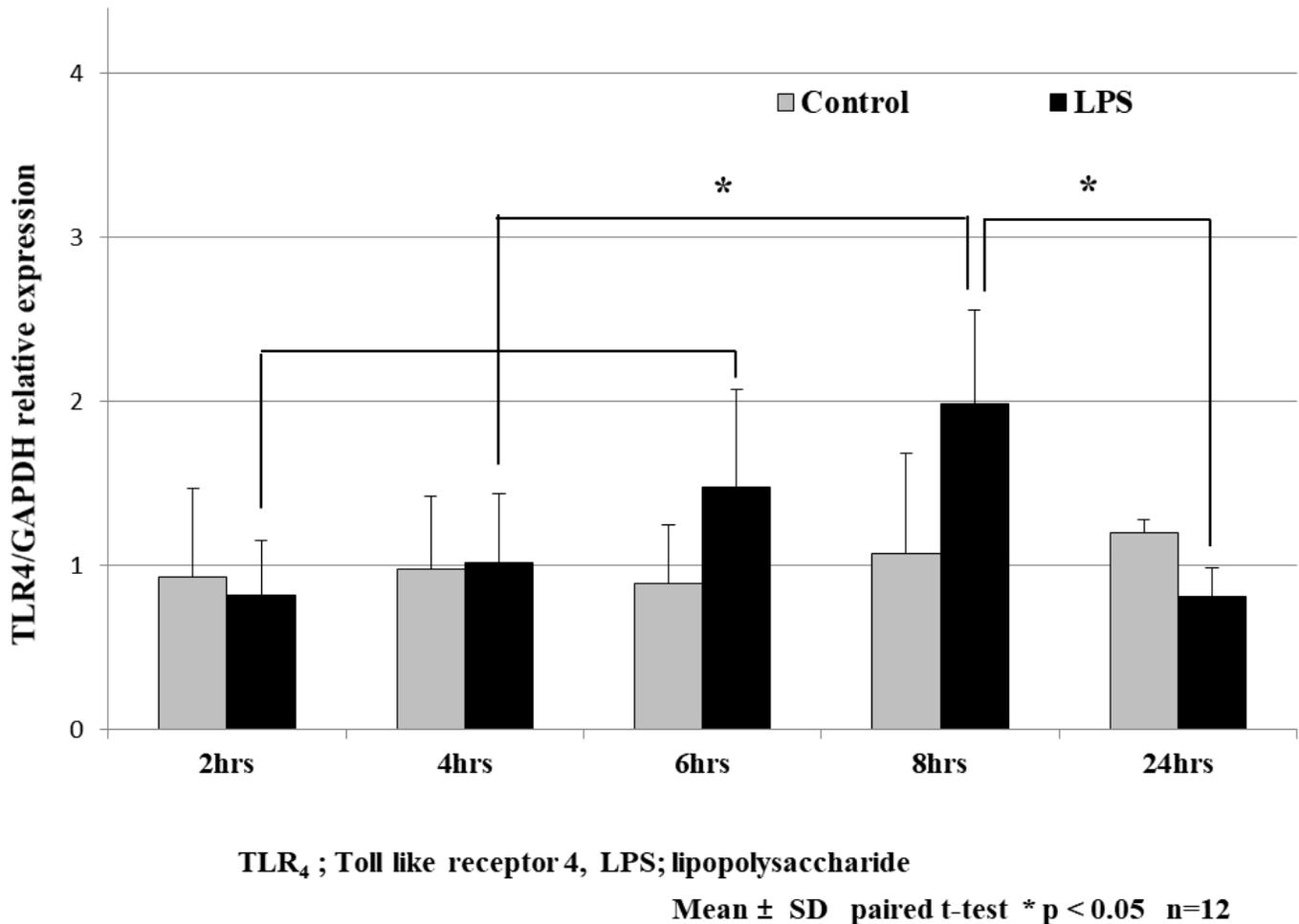


Figure 1

TLR4 expression in LPS-stimulated differentiated THP-1 cells was increased at 6, 8hrs by RT-PCR. The bar graph shows the TLR4 mRNA expression from repeated measurements (n=12). The mean of TLR4 mRNA expression ratio (TLR4/GAPDH ratio) were 0.93 ± 0.54 at 2hrs, 0.98 ± 0.45 at 4hrs 0.89 ± 0.35 at 6hrs, 1.07 ± 0.61 at 8hrs, 1.19 ± 0.09 at 24hrs in the control group and 0.87 ± 0.33 at 2hrs, 1.02 ± 0.42 at 4hrs, 1.47 ± 0.59 at 6hrs, 1.98 ± 0.58 at 8hrs, 0.81 ± 0.17 at 24hrs in the differentiated THP-1 cells stimulated with LPS. Data are presented as mean ± SD. *p < 0.05.

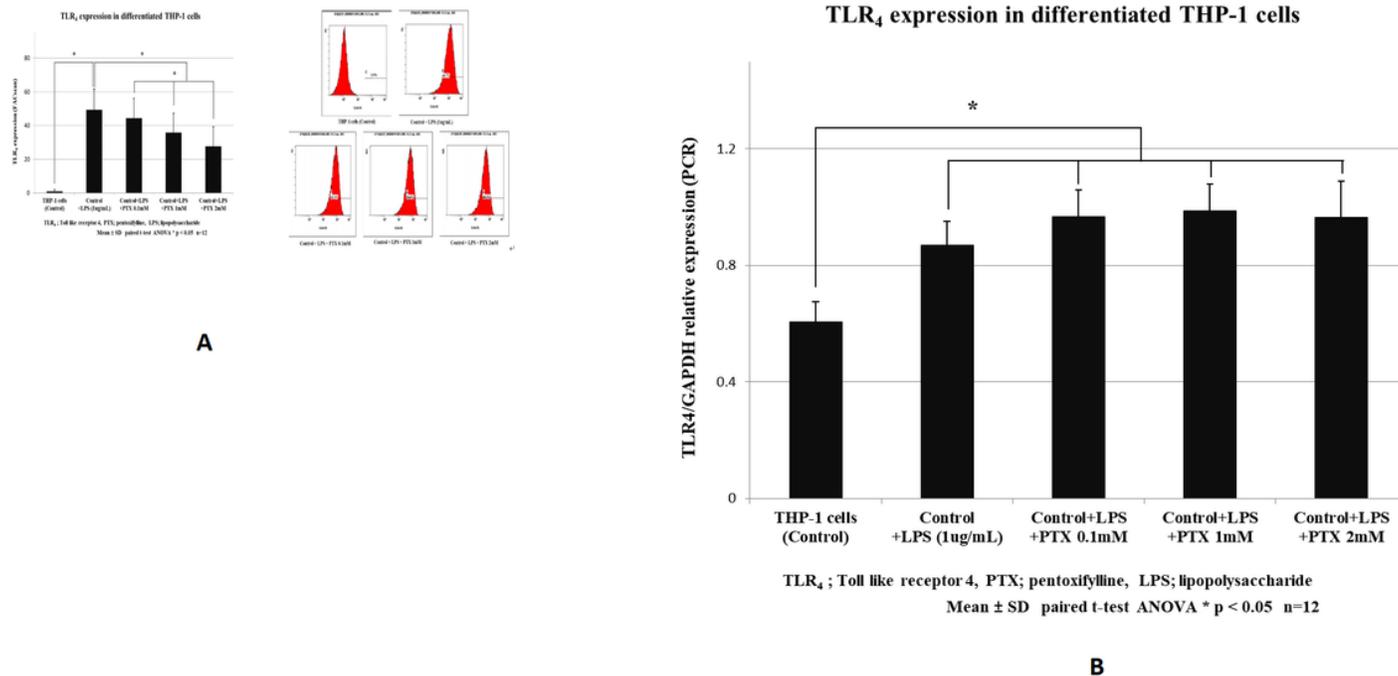
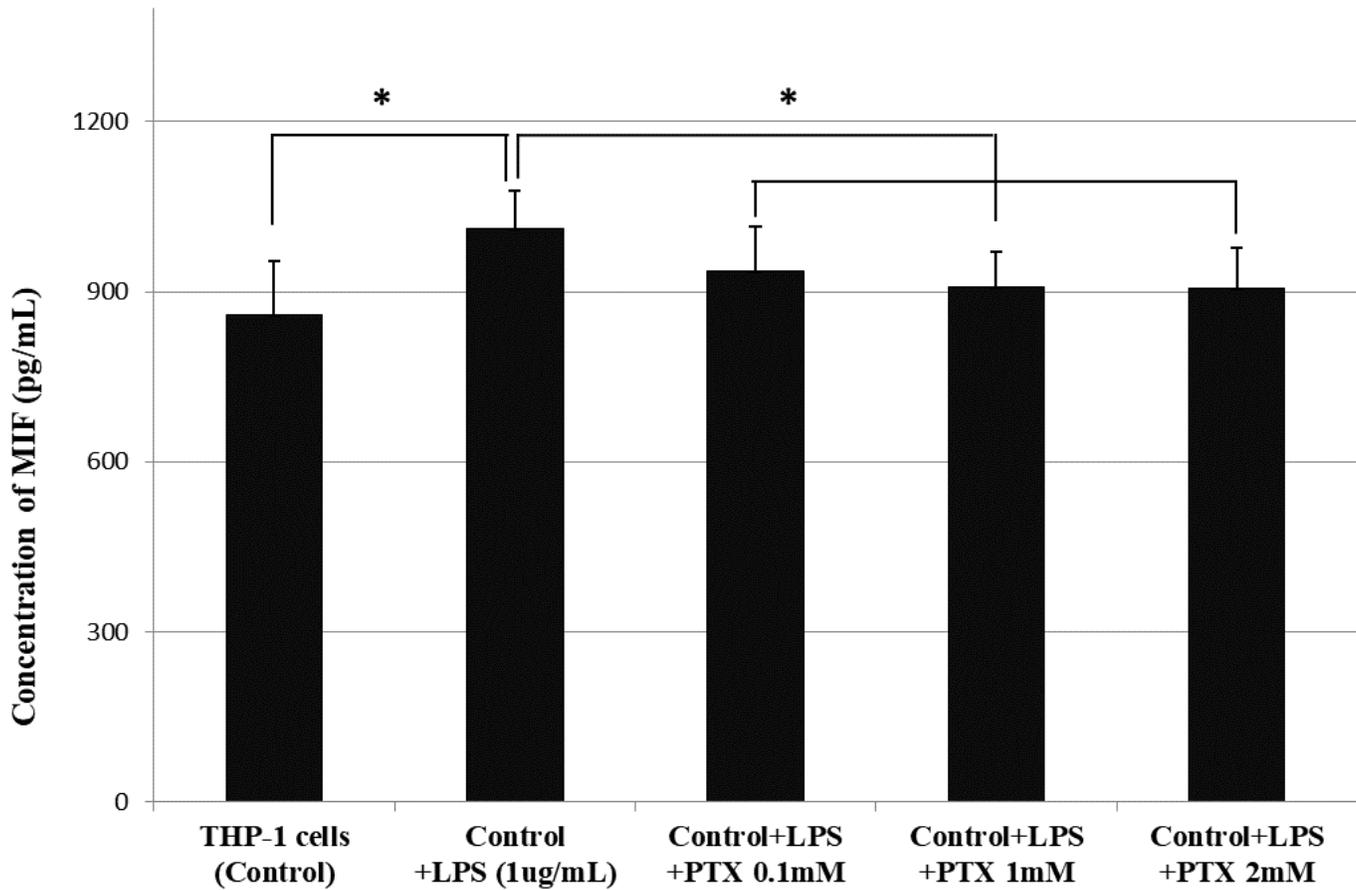


Figure 2

2A. Flow cytometry was used to examine the changes in cell surface TLR4 protein levels on LPS stimulated differentiated THP-1 cells. To determine the percentage of TLR4 cells after 8 hours of incubation, the cells were then stained with 5 μ L (2 μ g) phycoerythrin-conjugated anti TLR4 monoclonal antibody and analyzed by flow cytometry. The mean percentage of cells stained with anti TLR4 monoclonal antibody was 0% in the control group, 49.3 \pm 12.2 in the LPS stimulated group, 44.3 \pm 11.8 at 0.1mM PTX, 35.9 \pm 11.3 at 1 mM PTX, 27.75 \pm 11.6 at 2mM PTX. Data are presented as mean \pm SD. *p < 0.05. 2B. In the TLR4 mRNA expression measured by RT-PCR, The TLR4 mRNA expression was increased in the LPS stimulated differentiated THP-1 cells, however, PTX did not restore in LPS stimulated differentiated THP-1 cells. The mean of TLR4 mRNA expression ratio (TLR4/GAPDH ratio) were 0.60 \pm 0.18 in the control group, 0.87 \pm 0.21 in the differentiated THP-1 cells stimulated with LPS, 0.97 \pm 0.24 in the differentiated THP-1 cells stimulated with LPS and 0.1mM PTX, 0.99 \pm 0.25 in the differentiated THP-1 cells stimulated with LPS and 1mM PTX, 0.97 \pm 0.33 in the differentiated THP-1 cells stimulated with LPS and 2mM PTX. Data are presented as mean \pm SD. *p < 0.05.

Concentration of MIF in differentiated THP-1 cells



MIF; macrophage migration inhibitory factor, PTX; pentoxifylline, LPS; lipopolysaccharide

Mean \pm SD paired t-test * p < 0.05 n=12

Figure 3

LPS increased the concentration of MIF in differentiated THP-1 cells, however, PTX restored LPS-induced concentration of MIF by using the ELISA method. The mean concentration of MIF was 859 ± 233 pg/mL in the control group, 1012 ± 163 pg/mL in the differentiated THP-1 cells stimulated with LPS, 935 ± 196 pg/mL in the differentiated THP-1 cells stimulated with LPS and 0.1mM PTX, 909 ± 152 pg/mL in the differentiated THP-1 cells stimulated with LPS and 1mM PTX, 906 ± 175 pg/mL in the differentiated THP-1 cells stimulated with LPS and 2mM PTX. The bar graph shows the concentration of MIF from repeated measurements (n=12). Data are presented as mean \pm SD. *p < 0.05.

Cell viability in Jurkat cells

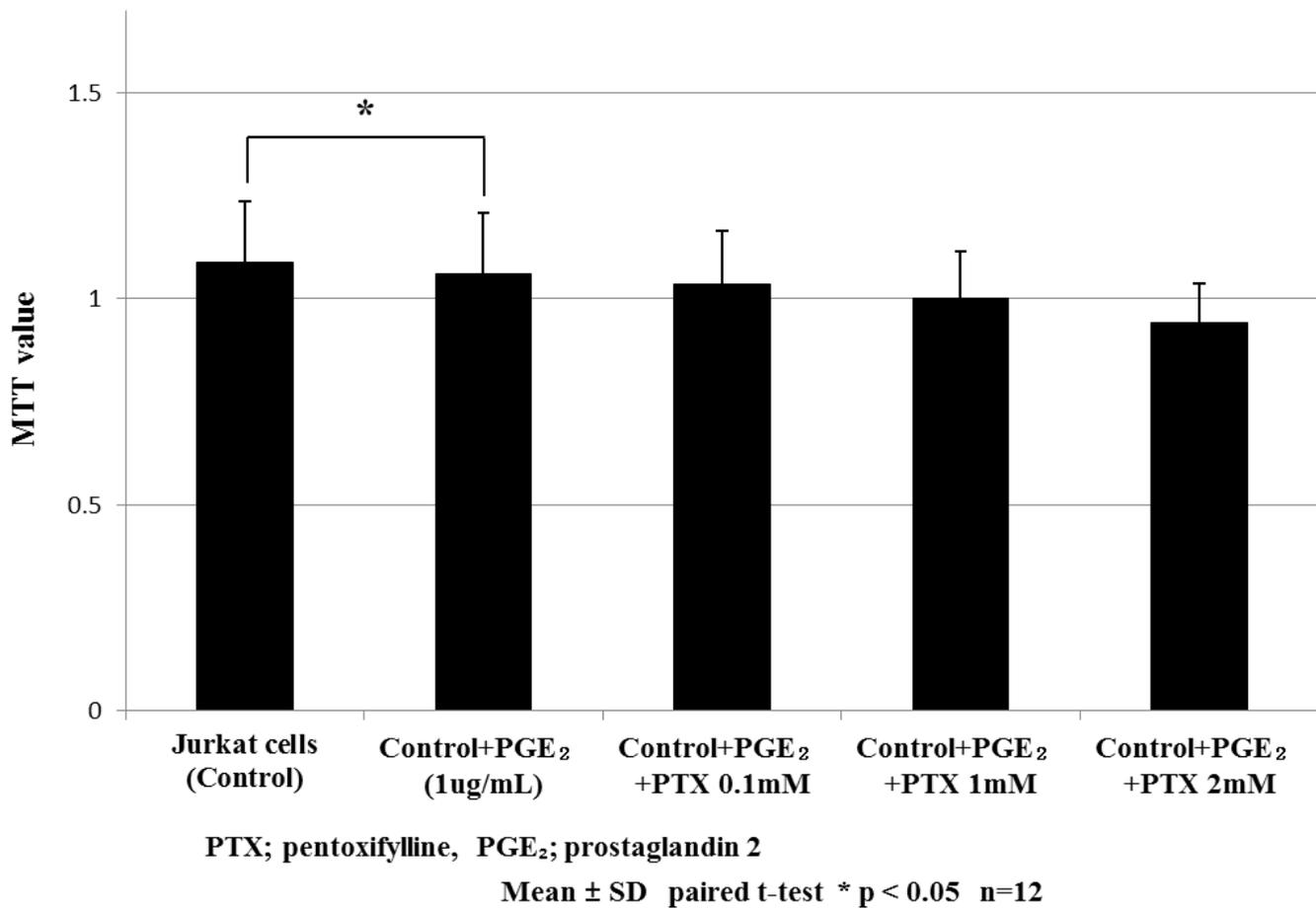
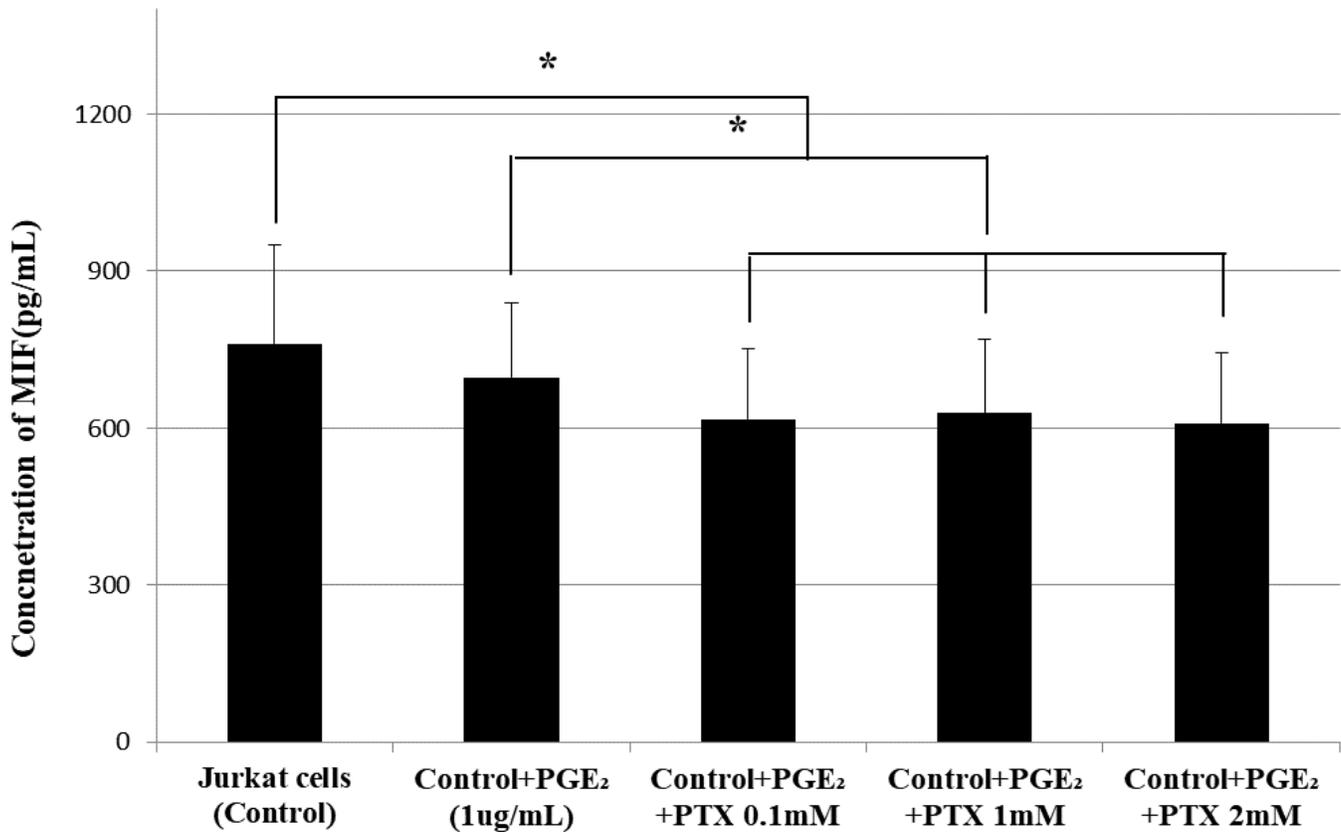


Figure 4

Prostaglandin 2 (PGE₂) decreased T cell proliferation in Jurkat cells. Pentoxifylline did not restore in the T cell proliferation with PGE₂ by MTT assay. The mean MTT value of Jurkat cell viability under non-stimulated differentiated THP-1 cells was 1.09 ± 0.68 in the control group, 1.06 ± 0.68 in the differentiated THP-1 cells stimulated with LPS, the mean MTT value of Jurkat cell viability were 1.03 ± 0.59 , 1.01 ± 0.52 , 0.95 ± 0.44 in the differentiated THP-1 cells stimulated with LPS and 0.1mM, 1mM, 2mM PTX. The bar graph shows the cell viability from repeated measurements (n=12). Data are presented as mean ± SD. *p < 0.05.

Concentration of MIF in Jurkat cells



MIF; macrophage migration inhibitory factor, PTX; pentoxifylline, PGE; prostaglandin 2
Mean ± SD paired t-test * p < 0.05 n=12

Figure 5

Prostaglandin 2 (PGE₂) decreased the concentration of macrophage migration inhibitory factor (MIF) in Jurkat cells, however, pentoxifylline did not restore PGE₂-induced MIF using the ELISA method. The mean concentration of MIF under non-stimulated differentiated THP-1 cells was 762 ± 187 pg/mL in the control group, 697 ± 142 pg/mL in the differentiated THP-1 cells stimulated with LPS, the mean MTT value of Jurkat cell viability was 617 ± 134 pg/mL, 629 ± 135 pg/mL, 665 ± 135 pg/mL in the differentiated THP-1 cells stimulated with LPS and 0.1mM, 1mM, 2mM PTX. The bar graph shows the concentration of MIF from repeated measurements (n=12). Data are presented as mean ± SD. *p < 0.05.

Cell viability of Jurkat cells in coculture condition

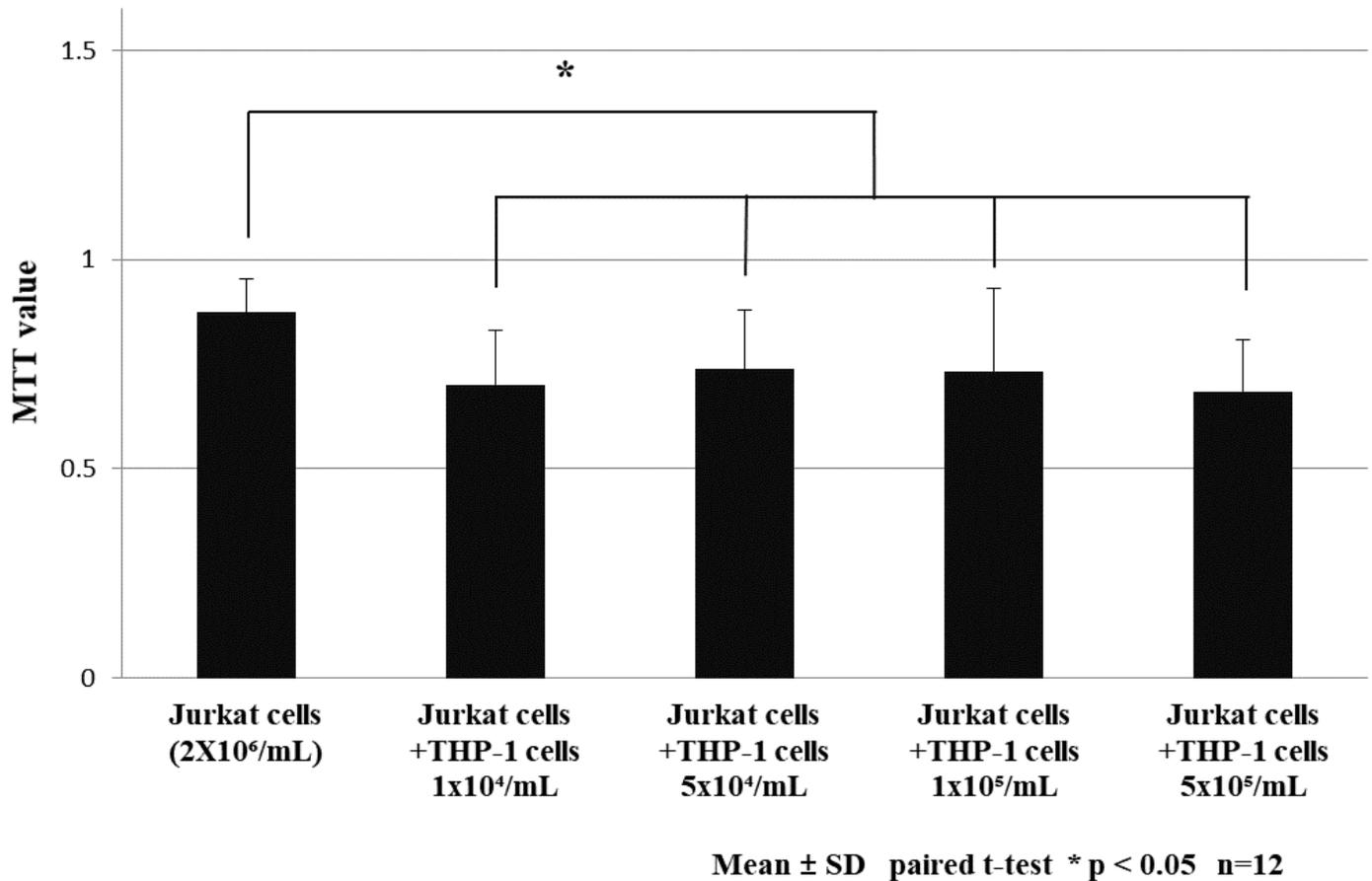
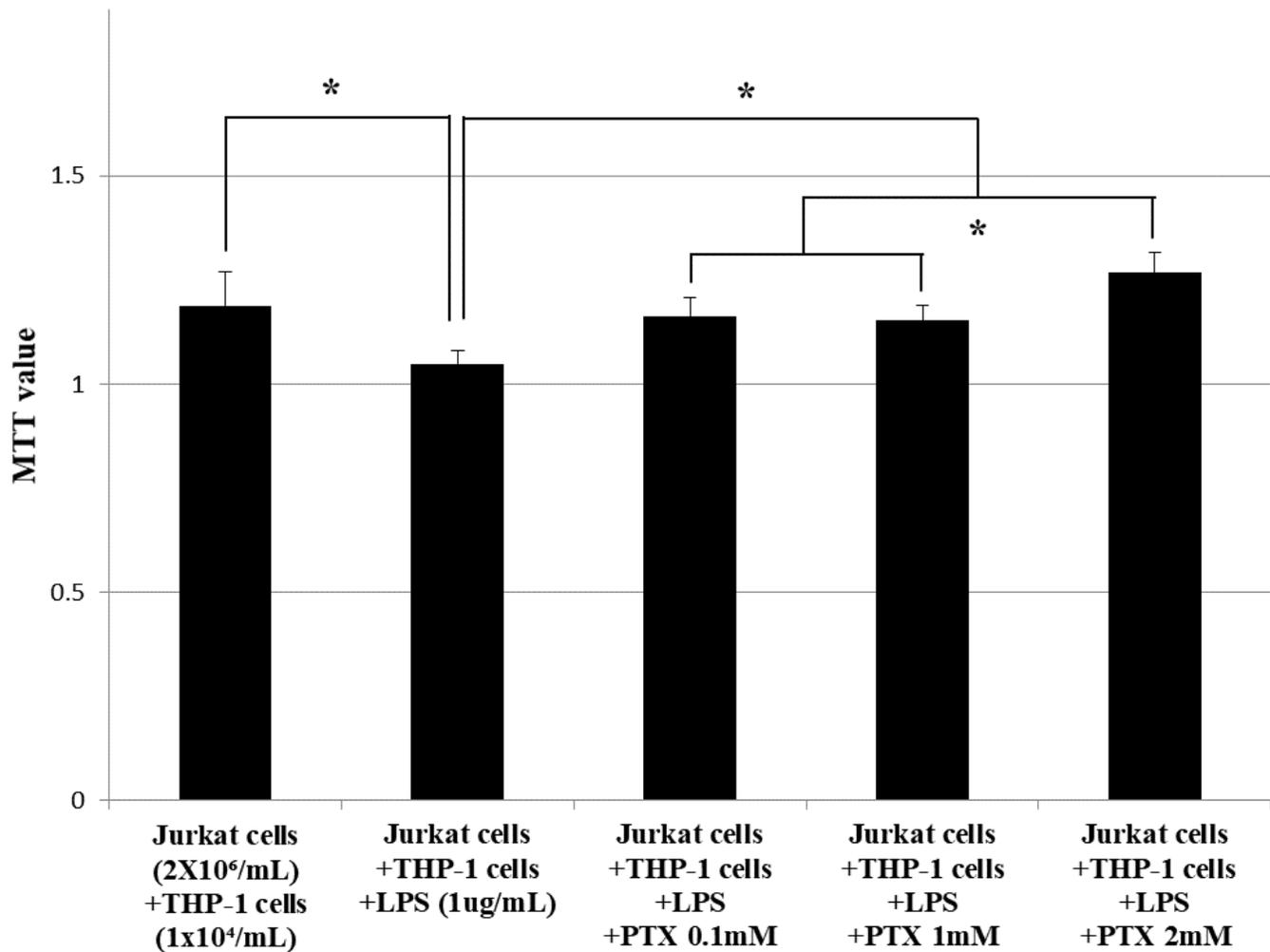


Figure 6

To know the appropriate ratio between the two cells for the co-culture of differentiated THP-1 cells and Jurkat cells, two cells of various concentrations were co-cultured. The mean MTT value in Jurkat cell density of 2x10⁶ cells/mL was 0.87 ± 0.08 in the control group, 0.70 ± 0.13 in the Jurkat cells co-cultured with differentiated THP-1 cells density of 1x10⁴ cells/mL, 0.74 ± 0.14 in the Jurkat cells co-cultured with differentiated THP-1 cells density of 5x10⁴ cells/mL, 0.73 ± 0.20 in the Jurkat cells co-cultured with differentiated THP-1 cells density of 1x10⁵ cells/mL, 0.69 ± 0.12 in the Jurkat cells co-cultured with differentiated THP-1 cells density of 5x10⁵ cells/mL. And we decided the ratio was determined as the ratio of differentiated THP-1 cells density of 1x10⁴ cells/mL and Jurkat cell density of 2x10⁶ cells/mL after conducting various combinations of co-culture. The bar graph shows the concentration of MTT value from repeated measurements (n=12). Data are presented as mean ± SD. *p < 0.05.

Cell viability of Jurkat cells in coculture condition



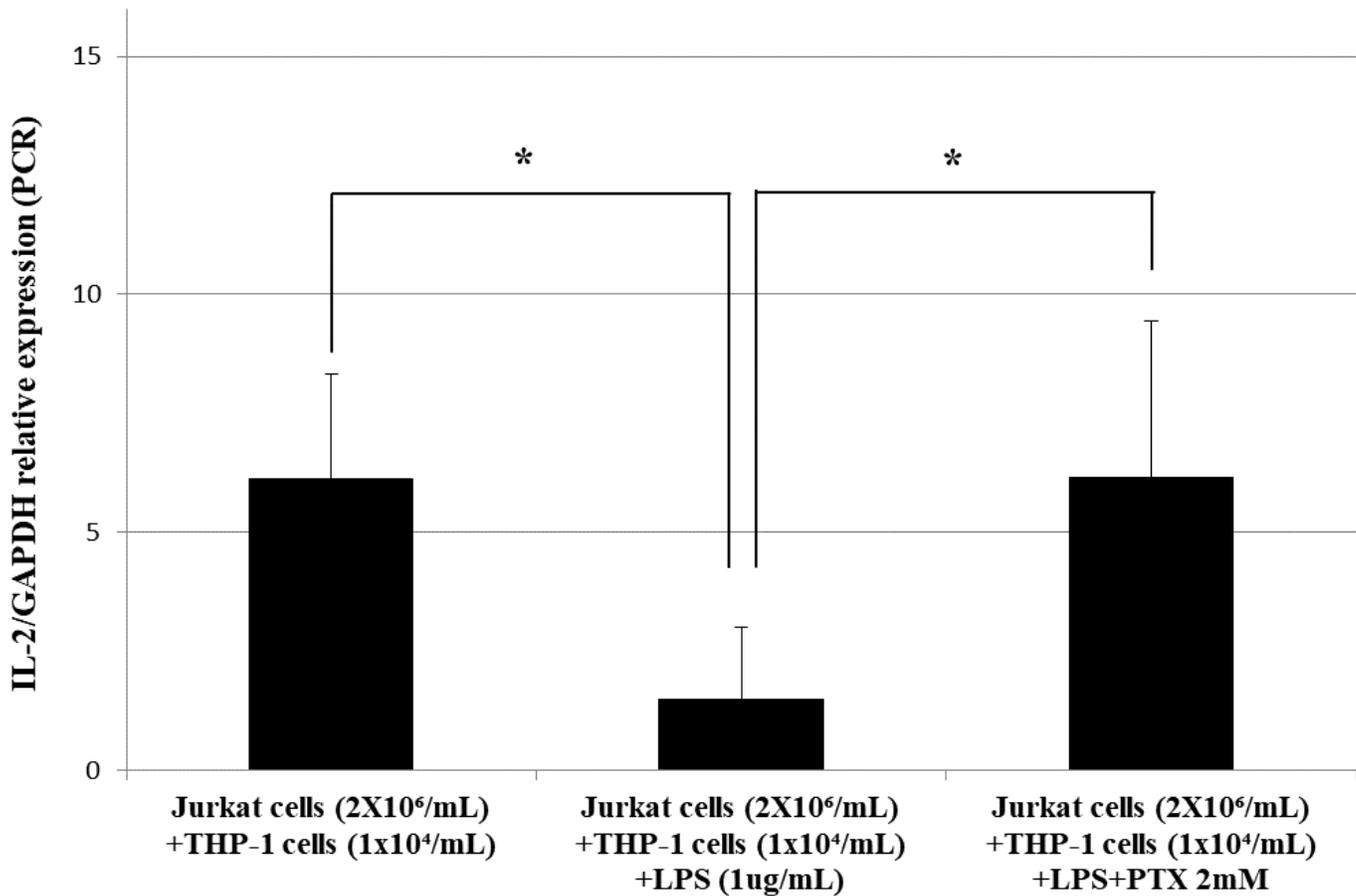
PTX; pentoxifylline, LPS; lipopolysaccharide

Mean ± SD paired t-test * p < 0.05 n=12

Figure 7

The mean MTT value of Jurkat cell viability under non-stimulated differentiated THP-1 cells was 1.19 ± 0.08 in the control group, 1.05 ± 0.03 in the differentiated THP-1 cells stimulated with LPS, the mean MTT value of Jurkat cell viability was 1.16 ± 0.05 , 1.15 ± 0.04 , 1.27 ± 0.05 in the differentiated THP-1 cells stimulated with LPS and 0.1mM, 1mM, 2mM PTX. Therefore, the percentage of the Jurkat cell viability decreased in the differentiated THP-1 cells stimulated with LPS. The additional PTX restored the Jurkat cell viability. Experiments were performed in triplicate using three consecutive wells. Data are presented as mean \pm SD *p<0.05

IL-2 expression of Jurkat cells in coculture condition



IL-2; Interleukin 2, PTX; pentoxifylline, LPS; lipopolysaccharide

Mean ± SD paired t-test * p < 0.05 n=12

Figure 8

The IL-2/GAPDH-relative mRNA expression was measured by quantitative real-time polymerase chain reaction. The mean of IL-2 mRNA expression ratio (IL-2/GAPDH ratio) was 6.11 ± 2.19 in the control group, 1.49 ± 1.50 in the differentiated THP-1 cells stimulated with LPS, 6.15 ± 3.29 in the differentiated THP-1 cells stimulated with LPS and 2mM PTX, Therefore, IL-2 mRNA expression was decreased in the differentiated THP-1 cells stimulated with LPS, and was restored in the differentiated THP-1 cells stimulated with LPS and PTX. The bar graph shows the IL-2 mRNA expression from repeated measurements. Data are presented as mean ± SD *p<0.05

IL-2 expression of Jurkat cells in coculture condition

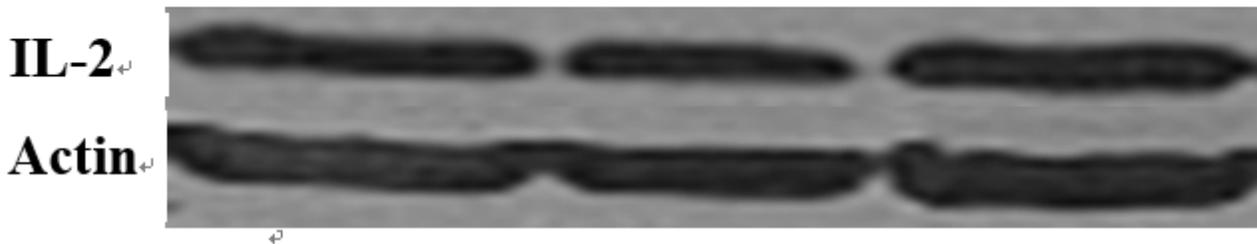
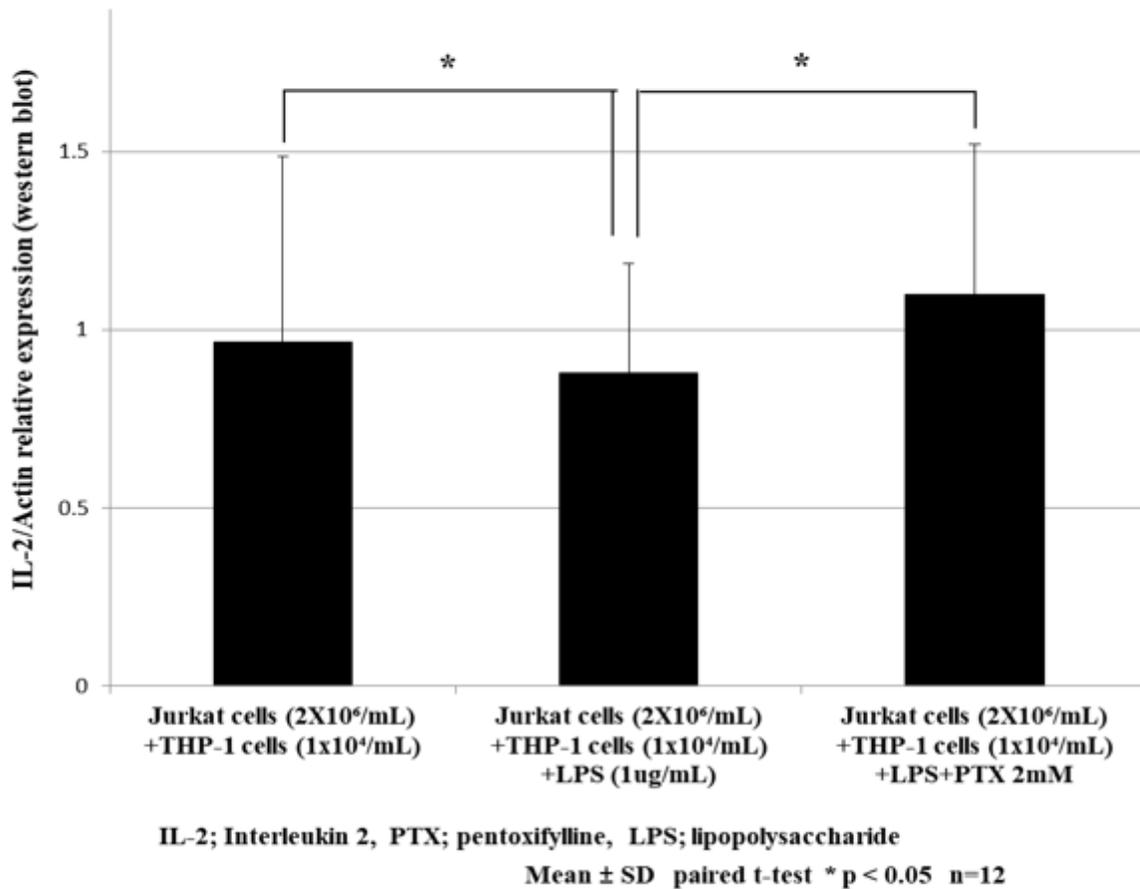


Figure 9

In the western blots, The mean of IL-2 expression ratio (IL-2/Actin ratio) was 0.97 ± 0.52 in the control group, 0.88 ± 0.31 in the differentiated THP-1 cells stimulated with LPS, 1.10 ± 0.42 in the differentiated THP-1 cells stimulated with LPS and 2mM PTX. The bar graph shows the IL-2 expression from repeated measurements. Data are presented as mean \pm SD *p<0.05