Thermal cycling-hyperthermia down-regulates liposaccharide-induced inflammatory response in human whole-blood and helps clear herpes simplex virus type 1 by U-937 macrophages

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Abstract

When infection occurs, the temperature in the infected region or in the whole body will be elevated in order to weaken the infectious pathogens such as virus, or to promote the activity of immune systems. As a clinical alternative treatment, hyperthermia (HT) has been widely used in various diseases, which is also capable of regulating the release of pro-inflammatory cytokines and antiviral activity of immune systems. However, studies have also indicated that overheating impairs the health tissues as well as the immune cells. In this study, we provide a modified HT method–thermal cycling-hyperthermia (TC-HT) and investigate its effects on immunomodulation and cellular viabilities, showing that TC-HT could reduce the secretion of pro-inflammatory cytokines induced by lipopolysaccharide (LPS) *ex vivo*, and elevate the efficacy of U-937 macrophages for clearing herpes simplex virus type 1 (HSV-1) *in vitro*. Furthermore, the efficacy of U-937 macrophage for clearing HSV-1 could be further boosted up via optimizing the parameter of TC-HT. Collectively, we prove that TC-HT is a more promising and safer thermal treatment with stronger therapeutic effects in immunomodulation than HT, shedding light on the development of the therapy in immunological diseases.

Introduction

Viruses are ubiquitous infectious agents that can be pathogenic and hazardous to both animals and humans. There are various diseases which are caused by viruses, and some are lethal and devastating, such as influenza [1], acquired immunodeficiency syndrome (AIDS) [2], Ebola [3], and coronavirus disease 2019 (COVID-19) [4]. Some viruses can be controlled with antiviral agents such as ribavirin and oseltamivir, which regulates viral activity by interfering with the replication process [5] and restricting replicated viruses in cytoplasm [6], respectively. Besides, certain viral diseases such as smallpox [7] and hepatitis B [8] can be prevented by vaccine injection before infection, which trains the adaptive immune system to produce specific immunoglobulin and acquire the resistance against the virus. Although there are many antiviral therapies, the host immune system still plays the most important role in combating the infection.

It has been well known that the innate immune response is the first-line defense to the invasion of microbial pathogens such as viruses. The innate immunity firstly recognizes the pathogens via the receptors on the surface of innate immune cells, such

as macrophages, dendritic cells, and neutrophils [9]. The activated innate immune cells as well as the infected cells release chemokines and cytokines as chemical messengers. Chemokines are molecules producing chemotaxis to recruit immune cells to the infected site. Cytokines, on the other hand, modulate the innate immune responses via regulating the differentiation, proliferation and activation of immune cells or uninfected cells [10]. For example, tumor necrosis factor-alpha (TNF-α) is a common cytokine after infection that can activate nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and mitogen-activated protein kinase (MAPK) pathways, which are involved in cell differentiation, proliferation and apoptosis [11]. Interferon (IFN) is a cytokine the infected cells could secrete to warn uninfected cells and inhibit viral replication [12]. Besides, there is a subclass of cytokines interleukin (IL) that mediates the interactions between leukocytes, and plays an important role of bridging the innate and adaptive immune responses [13]. Cytokines first secreted after infection are proinflammatory and arouse inflammation. Blood flow and vascular permeability increase when inflammation occurs, and leukocytes and plasma proteins infiltrate into the infected sites. This results in the typical inflammatory symptoms such as topical redness, heat,

and swelling [14]. In the meanwhile, phagocytosis also occurs during inflammation. Phagocytosis is an important innate immune response to clear the pathogens. Infiltrated macrophages and dendritic cells at the infected sites will devour the pathogens and break them down into molecular patterns. These patterns are then represented to lymphocytes such as T cells, activating the adaptive immune response and hence generating host immunity [15].

Hyperthermia (HT), as a historical alternative therapy, has wide applications in clinical use on various diseases due to its pleiotropic effect on mammalian cells. For example, studies have demonstrated that HT could inhibit the cancer cell proliferation and induce apoptosis [16, 17]. The anticancer effects of some chemotherapy drugs and phytochemical molecules could be synergistically amplified by heat treatment. Besides, suitable heat stress could activate heat shock proteins and antioxidant pathways and perform neuroprotective effects [18]. Heat has also been shown to be beneficial for immunity. The enhancement of core body temperature has also been shown to improve the survival rate of several viral infections [19]. Besides, it has been shown that heat is able to up-regulate the immune function, such as the phagocytic potential of macrophages and dendritic cells [20, 21], and boosting the

adaptive immune response [19]. However, HT could also be adverse to produce damage to health tissues, and the thermal tolerance varies from different tissues. Skin cells could tolerate temperature to 47 °C [22], while temperature higher than 43 °C could demonstrate heat damage to central and peripheral nervous systems in both human [23, 24] and animal models [25, 26]. Temperatures higher than the febrile range have also been reported to potentially impair the function of natural-killer lymphocytes and T-lymphocytes [27]. Chronic heat exposure in the mouse model has also shown to suppress innate immunity by decreasing the number of macrophages and retarding the maturation of dendritic cells [28]. Overall, conventional continuous HT could produce a detrimental effect to the normal and healthy tissue and cells, and thus a modified method is necessary to further promote the clinical usage of HT.

In our previous research, we have developed a new heat treatment method named thermal cycling-hyperthermia (TC-HT). TC-HT was a repetitive heating process composed of a desired high temperature stage followed by a normothermic stage. Compared to HT, TC-HT performed a stronger inhibition effect than HT on pancreatic cancer cell lines along with anti-cancer agents, via modulating the live-death pathway such as apoptosis and cell cycle arrest [29-31]. In the meanwhile,

normal cells have been shown to be unaffected under the same treatment. Besides the therapeutic effect on cancers, TC-HT was also potential for neuroprotection. Our study has also shown that TC-HT was able to provide better neuroprotective effect than HT against the cytotoxicity of both hydrogen peroxide and beta-amyloid via regulating the ROS level and continually activating the neuroprotective pathways, as well as prevent the neural cells from the conventional damage of HT [32]. Therefore, TC-HT is a potential substitutive of HT, and could provide a stronger and safer immunomodulatory effect than continuous HT.

In this study we applied TC-HT treatment to the *ex vivo* model (human blood) with lipopolysaccharide (LPS) and the *in vitro* model with herpes simplex virus type 1 (HSV-1) to investigate its immunomodulatory effects and compare them with HT. In the *ex vivo* model, TC-HT reduced the LPS-induced secretion of pro-inflammatory cytokines TNF-α, interleukin-6 (IL-6), and interleukin-1 beta (IL-1β) than HT in both whole blood and isolated peripheral blood mononuclear cells (PBMCs), while preserving the cell viability of PBMCs more than HT. In the *in vitro* model, TC-HT also preserved the cell viability of U-937 macrophages more than HT, and activated U-937 macrophages to clear more HSV-1 than HT. Moreover, altering the

normothermic duration of TC-HT treatment to 2 min optimized the elevated efficacy of U-937 macrophages for clearing HSV-1. In conclusion, TC-HT has the potential to activate more efficient immune responses, and provide less damage and discomfort to patients.

Materials and Methods

HT and TC-HT applications

All HT and TC-HT treatments were administered by a modified polymerase chain reaction (PCR) machine (Applied Biosystems; Thermo Fisher Scientific, Inc., Waltham, MA, USA), as shown in Fig 1A. For HT treatment, the high temperature setting was 39, 42, or 45 °C continuously for 30 min. The TC-HT treatment was composed by a 10-cycle repeated procedure of a desired high temperature for a period of time and followed by a normothermic stage. In this study, the setting temperature of the hyperthermic stage was set at 42, or 45 °C for 3 min, and the normothermic stage was set at 37 °C for a desired duration (Fig 1B). The actual temperature of both HT and TC-HT treatment was measured via a type-K thermocouple and a

temperature/process controller (PZ900, RKC instrument Inc., Tokyo, Japan). The thermocouple was located at the bottom of the cell culture well, and the monitored actual temperature was recorded every 10 sec, as shown in Fig 1C.

Fig 1. Experimental setups for thermal treatments.

(A) TC-HT and HT treatments are applied by a PCR machine. (B) The schematic representation of the setting parameters of TC-HT and (C) the actual temperatures measured every 10 sec during thermal treatments by the thermocouple.

Whole-blood collection and stimulation

Venous blood samples were collected from the antecubital vein of six healthy volunteers using the VacutainerTM system and sterile endotoxin free blood-collection tubes (Becton Dickinson, Franklin Lakes, NJ, USA) containing 158 IU of sodium heparin. Informed consent was obtained from all the volunteers prior to the experiments. All volunteers did not receive any medication (antibiotics or anti-inflammatory agents) or any therapy. For whole-blood assay, 1:3 blood dilutions were made with sterile Roswell Park Memorial Institute (RPMI) 1640 medium

(HyClone; GE Healthcare Life Sciences, Chicago, IL, USA) supplemented with 1% penicillin-streptomycin (Gibco Life Technologies, Grand Island, NY, USA).

PBMCs isolation

The PBMCs in blood were isolated by Ficoll density gradient centrifugation. 1:1 blood dilution was made with sterile phosphate buffered saline (PBS) (HyClone). The dilutions were slowly layered onto Ficoll-1077 (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) and centrifuged at 400×g for 30 min at room temperature without brake. The PBMCs layer between the plasma and Ficoll was withdrawn carefully, and then rinsed and maintained with sterile RPMI-1640 medium supplemented with 1% penicillin-streptomycin. The cell density was counted and the PBMCs were cultured in a final concentration of 1.5×10^6 cells/mL in 24-well culture plates (Thermo Fisher Scientific, Inc.) for the following experiments.

Thermal treatments and inflammatory stimulation

To investigate the immunomodulatory effects of the HT and TC-HT treatment on blood and PBMCs, 50 ng/mL LPS (Sigma-Aldrich; Merck KGaA) was used to

stimulate inflammatory response. For the post-treatment group, LPS was treated 1 h prior to the HT or TC-HT treatment. For the pre-treatment group, LPS was treated 4 h after the HT or TC-HT treatment. Samples without LPS treatment were used as the control group. 6 h after the LPS treatment, the blood or PBMCs samples of each group were centrifuged at 2000×g for 5 min. The supernatants were collected and stored at -20 °C until the enzyme linked immunosorbent assay (ELISA) test.

ELISA test

Sandwich ELISA tests for LPS-induced TNF-α, IL-6, and IL-1β were carried out using ELISA Max Standard Set (Biolegend, San Diego, CA, USA). In brief, the detected cytokine was sandwiched by the corresponding capture antibody and detection antibody in accordance with the manufacturer's instructions in the microwell plates for ELISA (Corning Inc., Corning, NY, USA). After the targeted inflammatory cytokines were sandwiched, 100 μL TMB substrate (Biolegend) was used for color development in dark for 15-30 min. The color reaction was stopped by 100 μL sulfuric acid (Echo Chemical Co. Ltd., Miaoli, Taiwan) of concentration 1 M. The absorbance at 450 nm was analyzed by Multiskan GO microplate

Spectrophotometer (Thermo Fisher Scientific, Inc.), and the reference absorbance at 690 nm was subtracted. The concentration of the inflammatory cytokines was calculated according to the standard curve generated according to the manufacturer's instructions and independently in each assay.

Cell and virus culture

Human monocytic cell line U-937 and African green monkey kidney epithelial cell line Vero were purchased from Biosource Collection and Research Center (Hsinchu, Taiwan) and cultured at 37 °C in a humidified incubator with 5% CO2. U-937 cells were maintained in RPMI-1640 medium supplemented with 4.5 g/L glucose, 10 mM HEPES, 1.0 mM sodium pyruvate (Sigma-Aldrich; Merck KGaA), 10% fetal bovine serum (Hyclone) and 1% of penicillin-streptomycin (Gibco). Vero cells were maintained in MEM/EBSS medium (Hyclone) supplemented with 1.0 mM sodium pyruvate (Sigma-Aldrich; Merck KGaA), 10% fetal bovine serum (Hyclone), 0.1 mM non-essential amino acids and 1% penicillin-streptomycin (Gibco). The KOS strain of HSV-1 (ATCC VR-1493, American Type Culture Collection, Manassas, VA, USA) stocks were propagated in Vero cells, and were aliquoted and stored at -80 °C.

Monocytes differentiation into macrophages

For differentiation, U-937 cells (2×10^5 cells/mL) were incubated in the culture medium containing 200 ng/mL of phorbol myristic acid (PMA) (Sigma-Aldrich; Merck KGaA) for 24 h to induce differentiation into macrophage cells. After the PMA treatment, the adherent U-937 macrophages were rinsed with PBS and resuspended in medium. Centrifugation at 200×g for 8 min was performed for cell collection. After counting the cell density, U-937 macrophages were cultured in 24-well culture plates (5×10^5 cells/well) and incubated at 37 °C in a humidified incubator with 5% CO_2 for 24 h prior to the HT and TC-HT treatments.

MTT assay

The viabilities of the PBMCs or U-937 macrophages were analyzed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma-Aldrich; Merck KGaA) assay. The MTT powder was dissolved in distilled water at a concentration of 5 mg/mL as the stock solution. Cells were incubated at 37 °C for 4 h

with a final MTT concentration 0.5 mg/mL in their culture medium 24 h after the HT or TC-HT treatment. Cells without HT or TC-HT treatment were used as the untreated control group. Formazan was crystalized after the MTT incubation, and equal volume of the solubilizing buffer of 10% sodium dodecyl sulfate (SDS) (Bioshop Canada Inc., Burlington, ON, Canada) solution in 0.01 N hydrochloric acid (HCl) (Echo Chemical Co. Ltd.) was used to dissolve the formazan crystals at 37 °C overnight in the dark. As the crystals in each well were fully dissolved, the absorbance of each well was detected by Multiskan GO microplate Spectrophotometer (Thermo Fisher Scientific). The quantity of formazan of each well was determined by the absorbance at 570 nm, and the reference absorbance at 690 nm was subtracted. The cell viabilities were expressed in percentage and the untreated control was set at 100%.

Plaque formation assay

To assess the effect of HT and TC-HT treatment on the viral clearance ability, U-937 macrophages were infected with HSV-1 at 0.1 multiplicity of infection (MOI) 1 h after the HT and TC-HT treatments. The infected U-937 macrophages without any

thermal treatment were used as the control group. 24 h after the HSV-1 infection, the maintenance medium was collected, and cells were then lysed in the maintenance medium of Vero cells for three freeze-thaw cycles. Vero cells for plaque reduction assay were cultured in 24-well culture plates (1.5×10^5 cells/well) 24 h prior to the assay. To analyze the plaque formation ability, Vero cells were incubated with the collected cell lysate samples at 37 °C for 1 h. Inoculums were removed and overlaid with the Vero maintenance medium containing 0.5% methylcellulose (Sigma-Aldrich; Merck KGaA) for 4 days. After the incubation, Vero cells were fixed with 4% paraformaldehyde (Sigma-Aldrich; Merck KGaA) for 10 min and stained with 0.1% crystal violet (Sigma-Aldrich; Merck KGaA). Total numbers of plaque in each well were counted, and the plaque formation of each group was expressed in percentage and the control group was set at 100%.

Statistical Analysis

The results were presented as the mean ± standard deviation. Statistical analysis using one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test was

performed by OriginPro 2015 software (OriginLab Corporation, Northampton, MA, USA). Results were considered to be statistically significant when P-value < 0.05.

Results

Moderate TC-HT post-treatment could down-regulate the LPS-induced inflammatory response *ex vivo*

HT treatment has been reported to be able to modulate the inflammatory response induced by LPS *ex vivo* [33]. In our results, the regulatory effects of HT post-treatment on the LPS-induced pro-inflammatory cytokines in blood were first assessed. As shown in Fig 2A-C, LPS treatment greatly elevated the concentration of TNF-α (47.76 ± 1.02 to 3390.61 ± 445.53 pg/mL), IL-6 (143.72 ± 8.42 to 3998.96 ± 312.03 pg/mL), and IL-1β (8.42 ± 0.02 to 89.24 ± 2.46 pg/mL) in blood. The elevations were further boosted up by 39 °C HT post-treatment (TNF-α from 3390.61 ± 445.53 to 4296.10 ± 158.27 pg/mL, IL-6 from 3998.96 ± 312.03 to 4103.02 ± 122.04 pg/mL, and IL-1β from 89.24 ± 2.46 to 212.9 ± 1.17 pg/mL), and were greatly inhibited by 45 °C HT post-treatment (TNF-α from 3390.61 ± 445.53 to 233.42 ±

7.59 pg/mL, 3998.96 ± 312.03 to 131.94 ± 2.14 pg/mL, and 89.24 ± 2.46 to 9.10 ± 0.04 pg/mL, respectively). On the other hand, 42 °C HT post-treatment showed down-regulatory effects on TNF-α (3390.61 ± 445.53 to 2498.09 ± 593.01 pg/mL) and IL-6 (3998.96 ± 312.03 to 3378.4 ± 92.2 pg/mL), but not on IL-1β (89.24 ± 2.46 to 140.33 ± 2.06 pg/mL). However, it was noted that the releases of all the three pro-inflammatory cytokines were significantly decreased as the temperature of HT post-treatment increased, thus suggesting that higher temperature was more favorable for anti-inflammatory treatment.

Although 45 °C HT post-treatment performed strong inhibitory effects on LPS-induced inflammatory responses *ex vivo*, studies have also indicated that overheating could impair the viability of immune cells or their immunological functions [27, 28], as a result of which the effect of high temperature on LPS-induce inflammatory response was further assessed by HT pre-treatment. Fig 2D-F showed that 42 °C HT pre-treatment did not alter LPS-induced secretion of TNF-α (3110.65 ± 450.34 and 3079.80 ± 466.87 pg/mL) and IL-6 (3486.53 ± 174.55 and 3610.29 ± 185.00 pg/mL), but promoting the secretion of IL-1β (91.52 ± 1.82 to 246.62 ± 2.72 pg/mL). This promotion by 42 °C HT pre-treatment on IL-1β was stronger than 42 °C

HT post-treatment (246.62 ± 2.72 and 141.77 ± 5.13 pg/mL), but there was no difference on TNF-α (3079.80 ± 466.87 and 3007.42 ± 302.09 pg/mL) and IL-6 (3610.29 ± 185.00 and 3527.33 ± 89.09 pg/mL) between 42 °C HT pre- and post-treatment. Noticeably, when blood was pre-treated by 45 °C HT, LPS was nearly unable to stimulate any inflammatory response in all the three cytokines, indicating that 45 °C HT treatment may dysregulate the inflammatory function of blood to LPS, and hence was not suitable for anti-inflammatory treatments. Thus, 42 °C was chosen as the setting temperature of the hyperthermic stage for the subsequent investigations.

In our previous studies, TC-HT showed higher therapeutic efficacies than HT for cancer treatment and neuroprotection [29, 31, 32]. As for the anti-inflammatory effect against LPS, 42 °C TC-HT post-treatment also showed a better performance than 42 °C HT post-treatment (Fig 2G-I). Secretion of TNF-α and IL-6 stimulated by LPS was significantly down-regulated by 42 °C TC-HT post-treatment (3373.61 ± 474.96 to 1767.3 ± 350.90 pg/mL, and 3748.51 ± 304.59 to 2999.39 ± 255.25 pg/mL, respectively), though IL-1β level was elevated (88.74 ± 4.03 to 106.52 ± 2.05 pg/mL). Nevertheless, in LPS-induced TNF-α, IL-6 and IL-1β secretion, 42 °C TC-HT post-treatment had significantly less inflammatory responses compared to 42 °C HT

post-treatment, pointing out that TC-HT had more therapeutic potential than HT in anti-inflammatory treatment.

Fig 2. Effect of TC-HT and HT treatments on regulating the secretion of LPS-induced pro-inflammatory cytokines in whole-blood *ex vivo*.

The concentrations of LPS-induced TNF-α, IL-6, and IL-1β in whole-blood were analyzed via ELISA assay in order to compare the immunomodulatory effects of (A-C) HT post-treatments, (D-F) HT pre-treatment and post-treatment, and (G-I) TC-HT and HT post-treatment. Data were presented as the mean ± standard deviation (n = 3 in each group). One-way ANOVA with Tukey's post hoc test is used to determine statistical significance (*$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ between indicated groups).

Inflammatory cytokines in blood were mainly secreted by immune cells, as a result of which we further isolated PBMCs to assess the regulatory effects of TC-HT and HT on LPS-induced inflammatory response. As shown in Fig 3A-C, 42 °C HT post-treatment was unable to inhibit LPS-induced PBMCs on releasing TNF-α

(201.85 ± 10.29 and 239.57 ± 61.98 pg/mL) and IL-6 (305.00 ± 6.09 and 315.11 ± 10.63 pg/mL), though had a down-regulation in IL-1β (13.92 ± 0.26 to 10.10 ± 0.02 pg/mL). On the contrary, 42 °C TC-HT post-treatment significantly inhibited the secretion of TNF-α (201.85 ± 10.29 to 119.03 ± 3.50 pg/mL), IL-6 (305.00 ± 6.09 to 228.68 ± 4.49 pg/mL), and IL-1β (13.92 ± 0.26 to 10.30 ± 0.09 pg/mL) stimulated by LPS, and had a significant lower level than 42 °C HT post-treatment except IL-1β. Furthermore, the viabilities of PBMCs 24 h after 42 °C HT post-treatment were significantly lower than 42 °C TC-HT post-treatment (Fig 3D). Similar results were observed as the setting temperature of the hyperthermic stage was raised to 45 °C. Collectively, 42 °C TC-HT post-treatment was a safer and more effective anti-inflammatory treatment than 42 °C HT post-treatment against LPS *ex vivo*, and hence was chosen for the following immunomodulatory experiments.

Fig 3. Effect of TC-HT and HT treatments on regulating the secretion of LPS-induced pro-inflammatory cytokines in PBMCs and on cell viability *ex vivo*. (A-C) The concentrations of LPS-induced TNF-α, IL-6, and IL-1β in whole-blood were analyzed via ELISA assay in order to compare the immunomodulatory effects of

42 °C TC-HT and HT post-treatment, and (D) the cell viability of PBMCs 24 h after TC-HT and HT treatments was analyzed by MTT assay. Data were presented as the mean ± standard deviation (n = 3 in each group). One-way ANOVA with Tukey's post hoc test is used to determine statistical significance (*P < 0.05, **P < 0.01, and ***P < 0.001 between indicated groups).

TC-HT treatment could up-regulate macrophage's viral clearance ability but not affect its viabilities

TC-HT has been shown to be capable of down-regulating LPS-induced inflammatory response *ex vivo*, and we then further investigated its regulatory effect on the viral clearance by U-937 macrophages *in vitro*. The effects of TC-HT and HT treatment on U-937 macrophages viabilities were first analyzed via MTT assay 24 h after the thermal treatments. In Fig 4A, it was observed that 42 °C HT treatment decreased the viability of U-937 macrophages to 89.97%, while 42 °C TC-HT treatment preserved the viability at 110.03%. As the setting temperature of the hyperthermic stage was raised to 45 °C, HT treatment further reduced the viability of U-937 macrophages to 82.01%, while TC-HT treatment still remained the viability at 109.34%. Therefore,

TC-HT treatment was a safer thermal treatment than HT treatment, and 42 °C was chosen as the setting temperature of the hyperthermic stage for the following experiments.

Next, we compare the plaque formation by the heated and non-heated HSV-1 in Vero cells, in order to verify the effect of heat on the formation of HSV-1 plaques in Vero cells. Fig 4B showed that the plaque formation in Vero cells was greatly reduced as HSV-1 was heated by 42 °C HT for 30 min before infection, suggesting that heat could inhibit HSV-1 viability. Therefore, to exclude the interference of heat on the formation of HSV-1 plaques in Vero cells, both TC-HT and HT treatments were applied to U-937 macrophages before the HSV-1 infection. 24 h after HSV-1 infection, the cell lysates of U-937 macrophages were collected as described in Materials and Methods. The lysates of the TC-HT-treated U-937 macrophages formed significantly fewer plaques (78.64%) than that of the HT-treated U-937 macrophages (91.26%), as shown in Fig 4C. These results pointed out that 42 °C TC-HT treatment enhanced the efficacy of U-937 macrophages for clearing HSV-1 than 42 °C HT treatment *in vitro*.

Besides the setting temperature of the hyperthermic stage in TC-HT, the duration of the normothermic stage was also crucial for the neuroprotective effects in our previous study [32]. Similar results were also observed in the plaque formation assay, where the plaque formation was greatly inhibited down to 36.00% as the normothermic duration in TC-HT extended to 2 min (Fig 4D), suggesting a stronger HSV-1 clearance efficacy by U-937 macrophages. However, this boost in the HSV-1 clearance diminished as the normothermic duration was further extended beyond 3 min. These results indicated that 2 min was the optimal setting of normothermic duration for 42 °C TC-HT treatment in order to clear HSV-1 by U-937 macrophages. For longer or shorter normothermic durations, the efficacy for HSV-1 clearance by U-937 macrophages was unable to be fully established, hence showed more HSV-1 plaques in Vero cells.

Fig 4. Effect of TC-HT and HT treatments on the viability of U-937 macrophages, HSV-1 infectivity and HSV-1 clearance efficacy of U-937 macrophages *in vitro*. (A) Cell viabilities of U-937 macrophages 24 h after TC-HT and HT treatments were analyzed by MTT assay. (B) Effects of heat on HSV-1 infectivity on Vero cells and

(C, D) promoting the efficacy of U-937 cells for clearing HSV-1 by HT and TC-HT treatments were assessed by plaque formation assay. Data were presented as the mean ± standard deviation (n = 3 in each group). One-way ANOVA with Tukey's post hoc test is used to determine statistical significance (*$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ between indicated groups).

Discussion

Being a part of the immunological mechanism, inflammation is a common response among various diseases [34]. As the immune cells recognize exogenous pathogens, proinflammatory cytokines are secreted and more immune cells are recruited to the infected region. LPS, a large molecule on the outer membrane of Gram-negative bacteria [35], is a classical activator of inflammatory responses in monocytes and macrophages [36], which stimulates the cells to secrete pro-inflammatory cytokines such as TNF-α, IL-6, and IL-1β via NF-κB pathways [37]. TNF-α is an early mediator of inflammation, and IL-1β is secreted from macrophages in infection [38, 39]. Besides, IL-6 is important in the acute-phase response of

inflammation [40]. These cytokines are important indicators to inflammatory responses, and thus are used to evaluate the degrees of inflammation in the study.

HT is a widely used alternative treatment in cancer, cardiological diseases and physiotherapy [41]. For immunomodulation, HT has also been reported to be capable of inhibiting the release of pro-inflammatory cytokines *ex vivo* and in clinical research [33, 42]. In the study, HT post-treatment decreased LPS-induced TNF-α, IL-6 and IL-1β levels in whole-blood as the setting temperature was over 42 °C (Fig 2A-C). However, excessively high setting temperature (> 42 °C) of HT would result in a dysfunctional inflammatory response to LPS (Fig 2D-F). Besides, the viability of isolated PBMCs was significantly reduced by both 42 and 45 °C HT post-treatments but not by TC-HT post-treatments (Fig 3D), suggesting that TC-HT post-treatment was less detrimental than HT post-treatment in immunomodulation *ex vivo*. Furthermore, 42 °C TC-HT post-treatment showed significant anti-inflammatory effects on suppressing LPS-induced TNF-α and IL-6 levels in both whole-blood (Fig 2G and 2H) and isolated PBMCs (Fig 3A and 3B). For LPS-induced IL-1β, although the level was not inhibited, the elevation after 42 °C TC-HT post-treatment was significantly less than after 42 °C HT post-treatment, while 42 °C TC-HT and HT

post-treatment both suppressed LPS-induced IL-1β in isolated PBMCs. Together, these results proved that TC-HT post-treatment was a more favorable anti-inflammatory regulation than HT post-treatment *ex vivo*.

In spite of regulating the inflammatory response against exogenous LPS, heat is also reported to help antiviral activities. Febrile temperature could not only reduce viral entry and replication [43], but also activate the innate and adaptive immune system to boost defensive activities [44]. As shown in Fig 4B, HSV-1 treated with 42 °C HT directly for 30 min formed less plaques in Vero cells than the untreated HSV-1, suggesting a reduction on HSV-1 infectivity on Vero cells by heat. Besides, the lysates of the 42 °C HT-treated U-937 macrophages formed less HSV-1 plaques in Vero cells than the lysates of untreated U-937 macrophages (Fig 4C). Moreover, the HSV-1 plaques formed by lysates of 42 °C TC-HT-treated U-937 macrophages were significantly less than that of 42 °C HT-treated U-937 macrophages, indicating that 42 °C TC-HT treatment provided U-937 macrophages a stronger efficacy for clearing HSV-1 *in vitro*. In addition to promoting the efficacy, TC-HT also showed a more favorable result for the viabilities of heat-treated U-937 macrophages than HT at setting temperatures 42 and 45 °C (Fig 4A). Together, these results proved that

TC-HT was a safer and more effective thermal treatment than HT in increasing the HSV-1 clearance efficacy of U-937 macrophages *in vitro*.

In TC-HT, normothermic stages are interspersed into the continuous hyperthermic process, and thus demonstrated stronger immunomodulatory effects than HT both *ex vivo* and *in vitro*. The results might be attributed to the suitable normothermic duration between hyperthermic stages, by which disrupted the adverse effects of high temperature. As a result, the duration of the normothermic stage might be critical for the immunomodulatory effects of TC-HT. In Fig 4D, we showed that the HSV-1 clearance efficacy of U-937 macrophages reached an optimal result as the duration of the normothermic stage in 42 °C TC-HT was extended to 2 min. As the duration extended beyond 2 min, the promoted HSV-1 clearance efficacy diminished, and even disappeared. The importance of a suitable normothermic duration for therapeutic effect was also observed in neuroprotection in our previous study [32]. Therefore, it was suggested that the duration of normothermic stage in TC-HT was critical to attain the optimal therapeutic efficacy.

In summary, this is the first study demonstrating the immunomodulatory effects of TC-HT both *ex vivo* and *in vitro*. In the whole-blood assay, LPS-stimulated TNF-α

and IL-6 levels were significantly decreased by 42 °C TC-HT post-treatment, but not by 42 °C HT post-treatment. Besides, although LPS-stimulated IL-1β level showed an increase after 42 °C TC-HT post-treatment, the elevation was significantly milder than that by 42 °C HT post-treatment. On the other hand, LPS-stimulated TNF-α, IL-6, and IL-1β levels were significantly reduced by 42 °C TC-HT post-treated in PBMCs, while 42 °C HT post-treatment has no inhibitory effect on LPS-stimulated TNF-α, IL-6, but on IL-1β. In addition, 42 °C TC-HT activated U-937 macrophages to clear more HSV-1 than the HT, and the optimized normothermic duration in 42 °C TC-HT treatment further boosted up the HSV-1 clearance efficacy of U-937 macrophages. Noteworthily, the cell viabilities of PBMCs and U-937 macrophages were both preserved more by TC-HT, indicating that TC-HT is a safer thermal treatment than HT both *ex vivo* and *in vitro*. Overall, the study showed the immunomodulatory potential of TC-HT on anti-inflammation and viral clearance, and further studies to investigate the modulatory mechanism of TC-HT are in progress.

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Figure 1

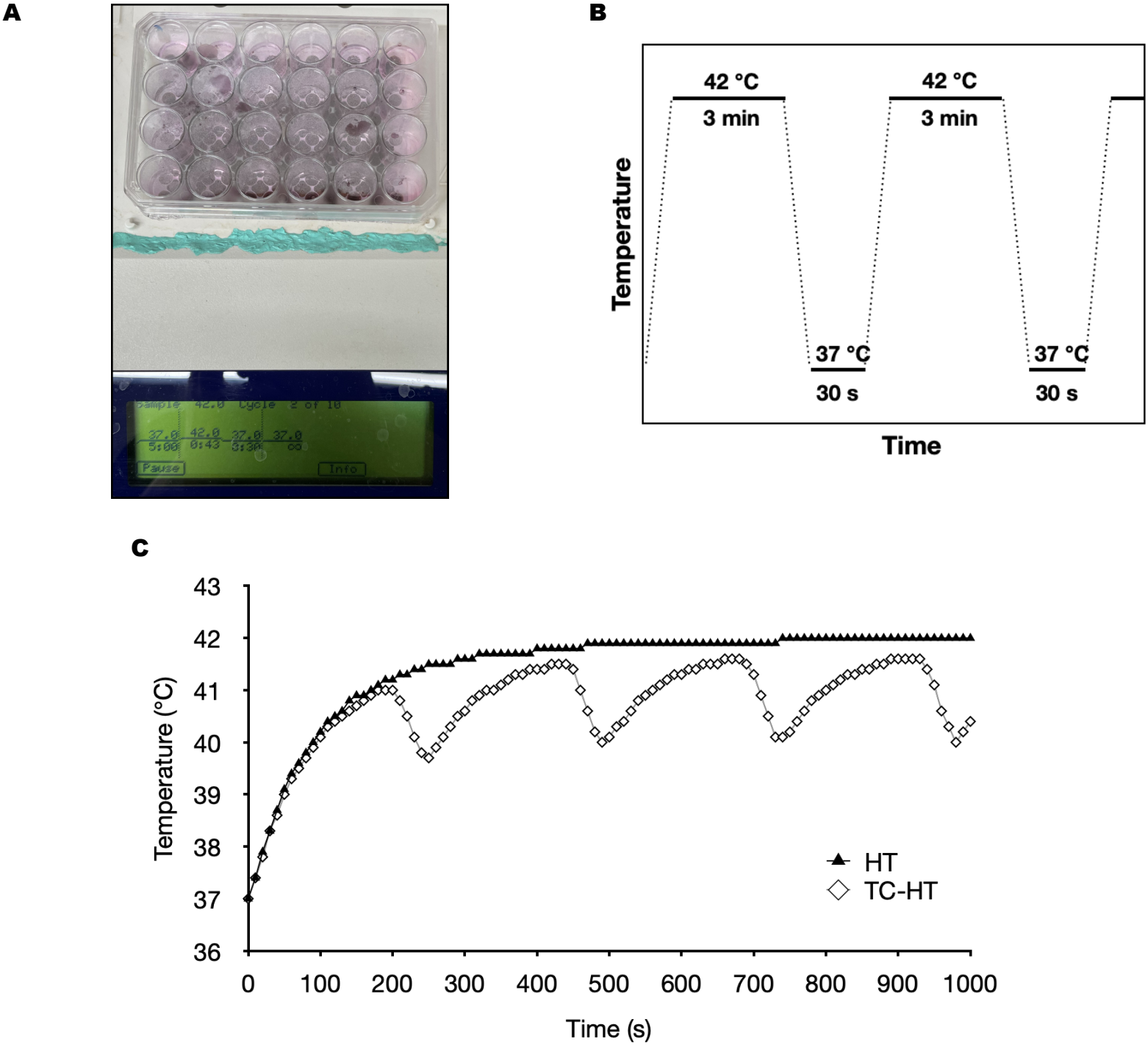

A
B
42 °C
3 min
42 °C
3 min
37 °C
30 s
37 °C
30 s
Temperature
Time
C
Temperature (°C)
43
42
41
40
39
38
37
36
0
100
200
300
400
500
600
700
800
900
1000
Time (s)
HT
TC-HT

Figure 2

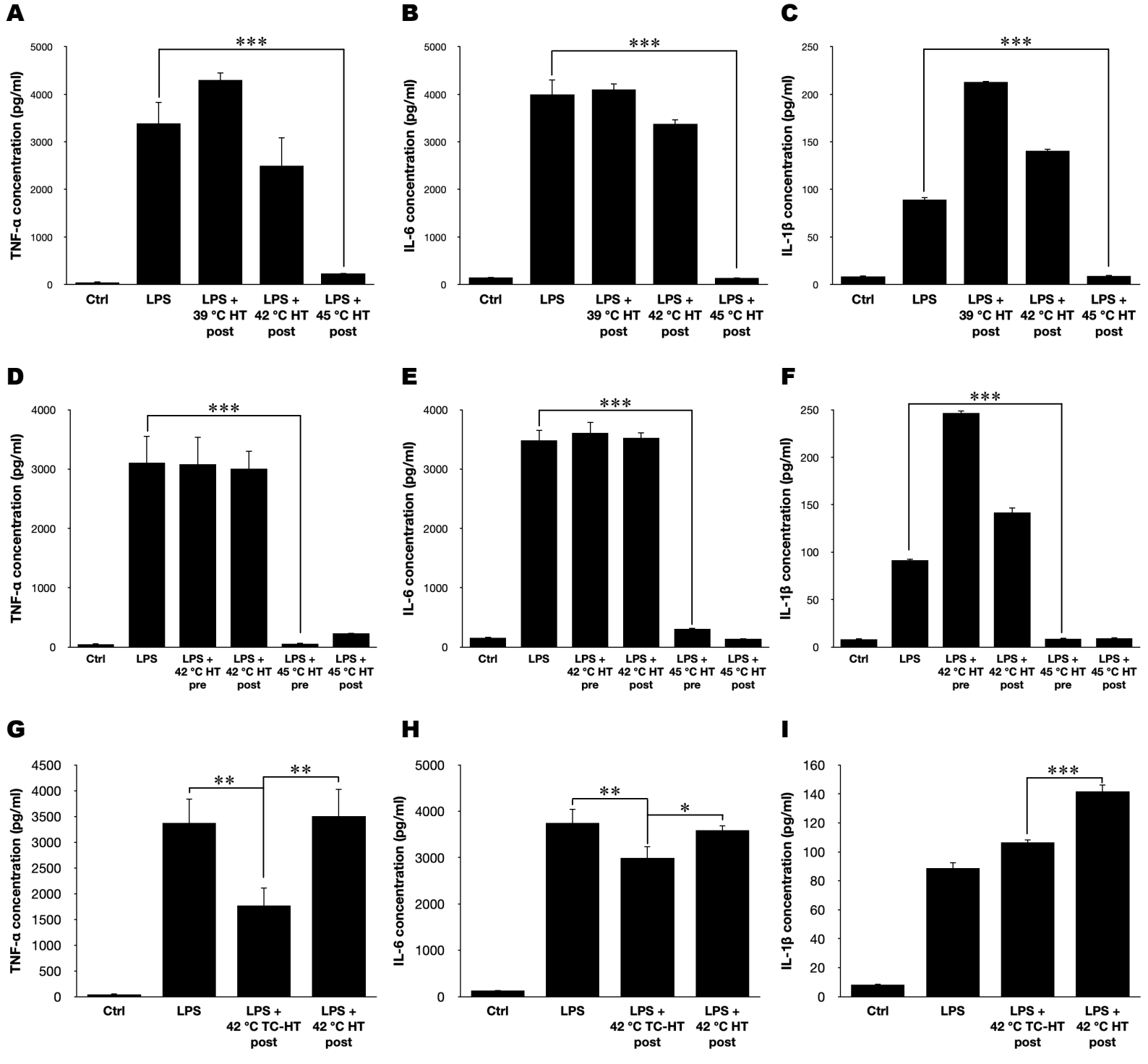

A
TNF-α concentration (pg/ml)
5000
4000
3000
2000
1000
0
***
Ctrl
LPS
LPS + 39 °C HT post
LPS + 42 °C HT post
LPS + 45 °C HT post
B
IL-6 concentration (pg/ml)
5000
4000
3000
2000
1000
0
***
Ctrl
LPS
LPS + 39 °C HT post
LPS + 42 °C HT post
LPS + 45 °C HT post
C
IL-1β concentration (pg/ml)
250
200
150
100
50
0
***
Ctrl
LPS
LPS + 39 °C HT post
LPS + 42 °C HT post
LPS + 45 °C HT post
D
TNF-α concentration (pg/ml)
4000
3000
2000
1000
0
***
Ctrl
LPS
LPS + 42 °C HT pre
LPS + 42 °C HT post
LPS + 45 °C HT pre
LPS + 45 °C HT post
E
IL-6 concentration (pg/ml)
4000
3000
2000
1000
0
***
Ctrl
LPS
LPS + 42 °C HT pre
LPS + 42 °C HT post
LPS + 45 °C HT pre
LPS + 45 °C HT post
F
IL-1β concentration (pg/ml)
250
200
150
100
50
0
***
Ctrl
LPS
LPS + 42 °C HT pre
LPS + 42 °C HT post
LPS + 45 °C HT pre
LPS + 45 °C HT post
G
TNF-α concentration (pg/ml)
4500
4000
3500
3000
2500
2000
1500
1000
500
0
**
**
Ctrl
LPS
LPS + 42 °C TC-HT post
LPS + 42 °C HT post
H
IL-6 concentration (pg/ml)
5000
4000
3000
2000
1000
0
**
*
Ctrl
LPS
LPS + 42 °C TC-HT post
LPS + 42 °C HT post
I
IL-1β concentration (pg/ml)
160
140
120
100
80
60
40
20
0
***
Ctrl
LPS
LPS + 42 °C TC-HT post
LPS + 42 °C HT post

Figure 3

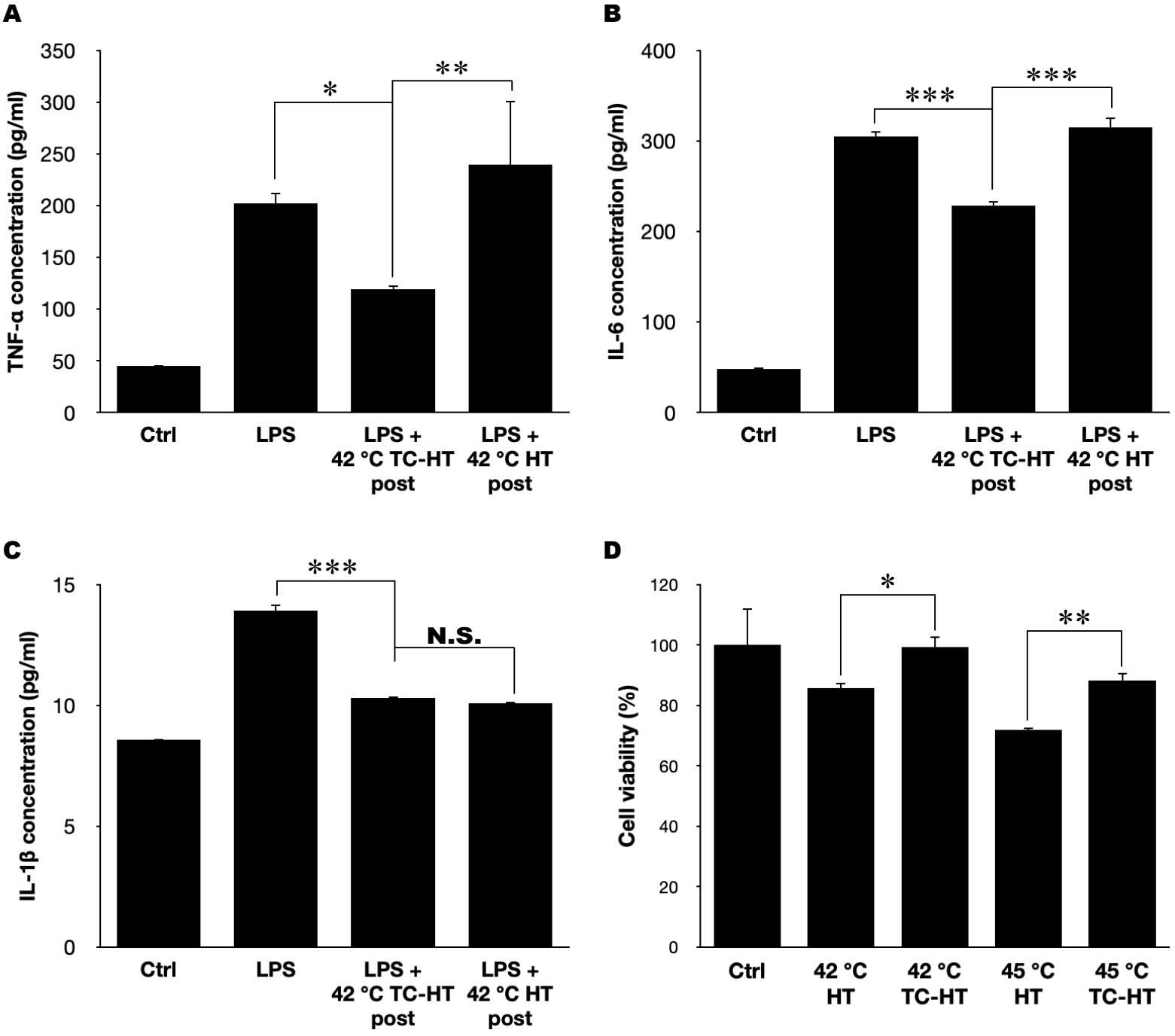
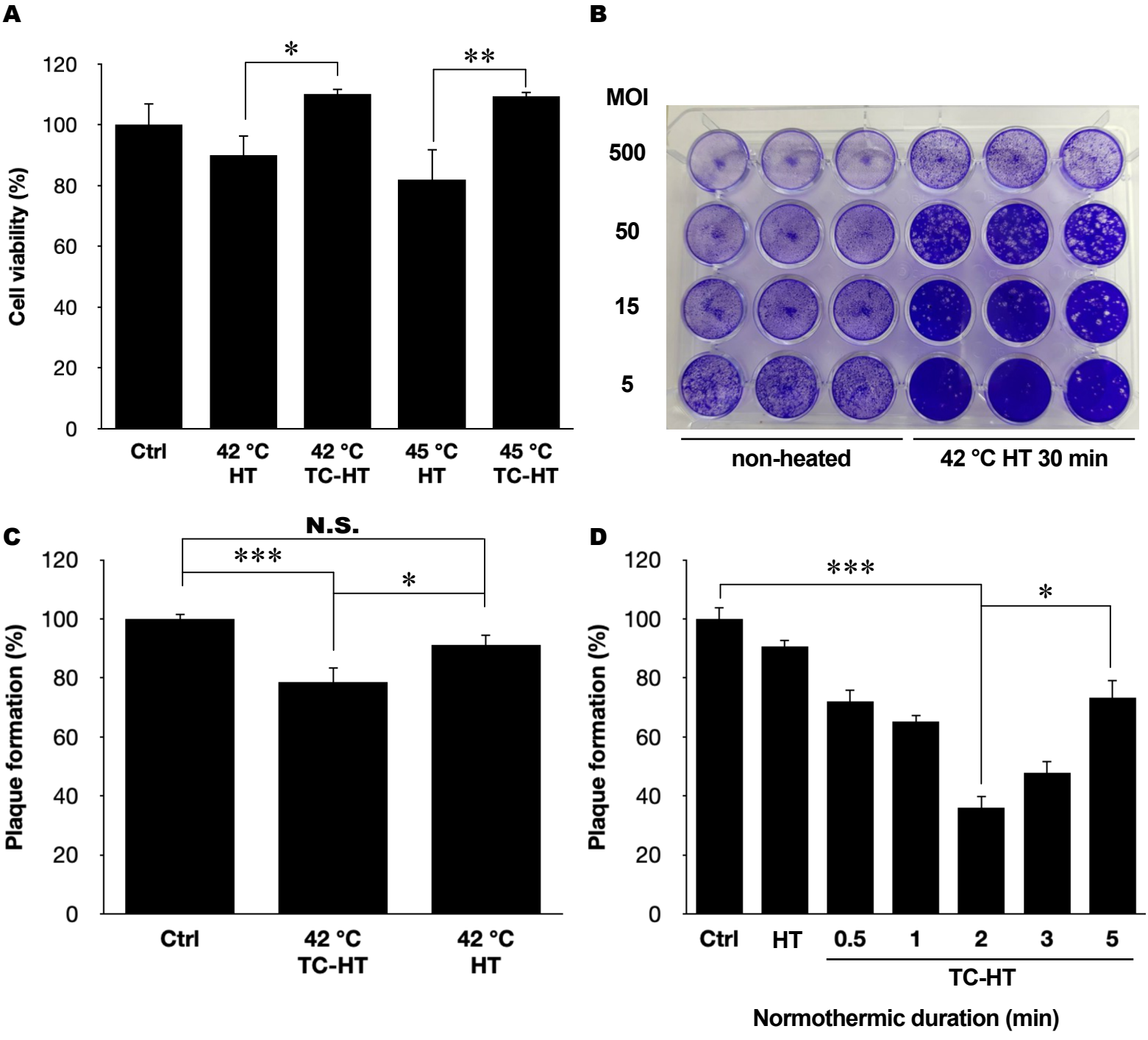

A
TNF-α concentration (pg/ml)
350
300
250
200
150
100
50
0
*
**
Ctrl
LPS
LPS + 42 °C TC-HT post
LPS + 42 °C HT post
B
IL-6 concentration (pg/ml)
400
300
200
100
0
***
***
Ctrl
LPS
LPS + 42 °C TC-HT post
LPS + 42 °C HT post
C
IL-1β concentration (pg/ml)
15
10
5
0
***
N.S.
Ctrl
LPS
LPS + 42 °C TC-HT post
LPS + 42 °C HT post
D
Cell viability (%)
120
100
80
60
40
20
0
*
**
Ctrl
42 °C HT
42 °C TC-HT
45 °C HT
45 °C TC-HT

Figure 4


A
Cell viability (%)
120
100
80
60
40
20
0
*
**
Ctrl
42 °C HT
42 °C TC-HT
45 °C HT
45 °C TC-HT
B
MOI
500
50
15
5
non-heated
42 °C HT 30 min
C
Plaque formation (%)
N.S.
***
*
Ctrl
42 °C TC-HT
42 °C HT
D
Plaque formation (%)
***
*
Ctrl
HT
0.5
1
2
3
5
TC-HT
Normothermic duration (min)