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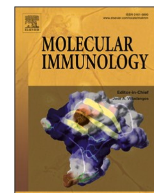

Escherichia coli-induced inflammatory responses are temperature-dependent in human whole blood *ex vivo*

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ABSTRACT

Systemic inflammatory conditions are often associated with hypothermia or hyperthermia. Therapeutic hypothermia is used in post-cardiac arrest and some other acute diseases. There is a need for more knowledge concerning the effect of various temperatures on the acute inflammatory response. The complement system plays a crucial role in initiating the inflammatory response. We hypothesized that temperatures above and below the physiologic 37 °C affect complement activation and cytokine production *ex vivo*. Lepirudin-anticoagulated human whole blood from 10 healthy donors was incubated in the presence or absence of *Escherichia coli* at different temperatures (4 °C, 12 °C, 20 °C, 33 °C, 37 °C, 39 °C, and 41 °C). Complement activation was assessed by the terminal C5b-9 complement complex (TCC) and the alternative convertase C3bBbP using ELISA. Cytokines were measured using a 27-plex assay. Granulocyte and monocyte activation was evaluated by CD11b surface expression using flow cytometry. A consistent increase in complement activation was observed with rising temperature, reaching a maximum at 41 °C, both in the absence (C3bBbP $p < 0.05$) and presence (C3bBbP $p < 0.05$ and TCC $p < 0.05$) of *E. coli*. Temperature alone did not affect cytokine production, whereas incubation with *E. coli* significantly increased cytokine levels of IL-1β, IL-2, IL-6, IL-8, IFN-γ, and TNF at temperatures > 20 °C. Maximum increase occurred at 39 °C. However, a consistent decrease was observed at 41 °C, significant for IL-1β ($p = 0.003$). Granulocyte CD11b displayed the same temperature-dependent pattern as cytokines, with a corresponding increase in endothelial cell apoptosis and necrosis. Thus, blood temperature differentially determines the degree of complement activation and cytokine release.

1. Introduction

An appropriate innate immune response is a delicate process relying on the interaction between various plasma proteins and the biochemical function of cells. These biochemical reactions are susceptible to physical environmental conditions such as temperature (Levi, 2018). Changes in body temperature can be caused by several factors, including infections, environmental factors, or targeted temperature management.

Hypothermia (body temperature below 37ºC) may occur in a patient during late sepsis, leading to adverse outcomes (Khodorkovsky et al., 2018). Hypothermia as a therapy has been evaluated over several decades. Animal studies have shown that preoperatively induced hypothermia has a neuroprotective effect (Yokobori et al., 2013). Therapeutic hypothermia is used to protect internal organs such as the heart, brain, and kidney against ischemia/reperfusion injury during heart surgery, transplantation, and after cardiac arrest (Niemann et al., 2015; Mild, 2002; Malinoski et al., 2019). The largest patient group evaluated with hypothermia are cardiac arrest patients. An initial clinical study demonstrated improved neurological outcomes after resuscitation ($author1$ et al., 2002). However, recent clinical studies have

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reported that hypothermic treatment after cardiac arrest did not reduce mortality (Dankiewicz et al., 2021). Hypothermia has several potential side effects, and prolonged hypothermia can diminish or even negate the potential benefits (Polderman, 2009).

Hyperthermia, commonly referred to as fever, is triggered by various infectious and non-infectious factors affecting the hypothalamic set point (Niven and Laupland, 2016). An increase of 1–4 °C in core body temperature may stimulate innate and adaptive immune responses (Evans et al., 2015). Several studies have shown the protective effect of fever and reported improved clinical outcomes (Holzer et al., 2005; Auer, 2001; Conrad et al., 2007), however, other investigations have shown opposite results (Dixon et al., 2002; Dallan et al., 2020). Consensus is lacking regarding the optimal target temperatures of the patient and when there is indication for treatment to reduce fever (Thomas-Rüddel et al., 2021).

While mild hypothermia is considered to have a protective effect by decreasing the uncontrolled inflammation process caused by severe ischemia/reperfusion injury (Bisschops et al., 2014a; Zhao et al., 2018; Wu et al., 2020), there are contradictory reports on the effect of hypothermia on complement activation (Polderman, 2009; Shah et al., 2014). Hypo- and hyperthermia may have different effects on our immune system in general, from beneficial to harmful. Changes in the body's core temperature affect homeostasis at various levels, including enzymatic kinetics and the dynamic equilibrium of different systems and cell functions.

The purpose of the present study was to study in whole blood ex vivo the effect of temperature on innate immune responses. This includes the complement system, the cytokine network, and activation of leukocytes. Furthermore, how this may influence the viability of granulocytes, monocytes and endothelial cells. In addition, we studied the effects of stimulation with *E. coli* at different temperatures.

2. Materials and methods

2.1. Cells, media, and reagents

Human umbilical vein endothelial Cells (HUVEC), Endothelial Cell Growth Medium (ECG), and Endothelial Cell Basal Medium (BM) were purchased from Cell Applications Inc. (San Diego, CA). Inactivated *Escherichia coli* (*E. coli*) were obtained from American Type Culture Collection (Manassas, VA). Gelatine type B (2%), NaCl, trypsin-EDTA (0.25%), Fixative-Free Lysing Solution, High-Yield Lyse, and antibodies conjugated with fluorochrome, *i.e.*, anti-CD45-PO (clone HI30), anti-CD15-eFluor® 450 (clone MMA) were purchased from Thermo Fisher Scientific (Oslo, Norway); anti-CD11b-PE (clone D12), anti-CD14-FITC (clone MϕP9), were purchased from BD Biosciences (San Jose, CA). Lepirudin (Refludan®) was purchased from Pharmion (Copenhagen, Denmark). All other reagents were purchased from Sigma-Aldrich (Oslo, Norway).

2.2. Whole-blood model of inflammation

The study was performed with the approval (S-04114) of the regional ethical committee, South-Eastern Norway Regional Health Authority. A human whole-blood *ex vivo* model was used to study the effect of the temperature on the body's inflammatory network, including activation of the complement cascade, changes in leukocyte surface markers, the release of their granular proteins, and cytokine response (Mollnes, 2002). Lepirudin anticoagulated human whole blood from 10 healthy donors was incubated in the presence or absence of *E. coli* (1×10^7 bacteria/ml) with DPBS (as control) at the range of clinically relevant temperatures: hypothermia (4 °C, 12 °C, 20 °C, 33 °C), normothermia (37 °C) and hyperthermia (39 °C and 41 °C). We used an INCU-Line® digital incubator IL 23 and validated the built-in display temperature using an independent digital thermometer placed into the Nunc® CryoTubes® 1 ml with 200ul of PBS inside. Blood samples were incubated for 15, 30, and 120 min. EDTA plasma was collected by centrifuging the blood at 3000g for 15 min at 4°C. Plasma was stored at − 80°C until analyses.

2.3. Endothelial cells

HUVEC were seeded on 48-wells plates, coated with 1% gelatine at a concentration of 25000 cells/well. The cells were cultured in endothelial growth medium (EGM) and incubated at 37 °C in a 5% CO_2 and 95% humidified atmosphere. Cells were grown until 90–95% of confluence (2–3 days). All cells were used in passages 4–6. Confluent HUVEC layers were washed once with sterile, 37 °C DPBS before the addition of 500 µl basal medium (BM) and stored for 24 h in seven different incubators in room-air at 4 °C, 12 °C, 20 °C, 33 °C, 37 °C, 39 °C, and 41 °C. In a pilot experiment, cell media pH, pCO_2, pO_2, electrolytes, glucose and lactate were measured in all temperatures and showed comparable high pH, low pCO_2, high pO_2, and normal physiological range of electrolytes (Suppl. Table 1). Lactate, as a measure of glucose metabolism, increased in temperatures ≥ 33 °C confirming cellular functioning. After incubation, the endothelial cells were trypsinized and analyzed for viability by flow cytometry (Attune NxT Flow Cytometer 2019 from Invitrogen) using a Dead Cell Apoptosis Kit with Annexin V FITC and PI from Invitrogen™ (V13242). The viability of HUVEC cultured with EGM media at 37 °C in a 5% CO_2 and 95% humidified atmosphere served as a negative control. HUVEC stored at 37 °C with BM in room-air served as an experimental control to compare the effect of temperatures within the same condition. The viability analyses were performed according to the manufacturer's instructions. Briefly, the cells were suspended in 100 µl of annexin V binding buffer and incubated with 5 µl of annexin V and 1 µl of PI for 15 min at room temperature in the dark. Afterward, 400 µl of annexin V binding buffer was added, and samples were placed on ice. Immediately after staining, the cells were analyzed on a flow cytometer measuring the fluorescence emission at 488 nm excitation, a 530 nm bandpass filter for FITC, and a 620 nm filter for PI detection. The percentages of endothelial cells that went through apoptosis were detected by dual-color analysis. This staining allowed us to differentiate three groups of cells: viable cells (annexin V negative and PI negative), early apoptotic cells (annexin V positive and PI negative), and late apoptotic or necrotic cells (annexin V positive and PI-positive).

2.4. Analysis of complement activation

We measured complement activation products, C3bBbP of the alternative pathway, and sC5b-9 of the terminal pathway using in-house enzyme-linked immunosorbent assays (ELISA) reported previously (Bergseth et al., 2013). In short, C3bBbP was quantified by the capture mAb anti-factor P (clone #2, Quidel, San Diego, CA) and detected by a polyclonal anti-C3c antibody (Behring, Marburg GmbH, Germany). To quantify sC5b-9, we used the monoclonal antibody (aE11), which is specific for a neoepitope exposed on C9 when incorporated into TCC. The complement measured by C3bBbP and sC5b-9 is considered activated when the level is higher than 24 complement arbitrary units (CAU)/ml and 0.7 CAU/ml, respectively. The concentration is expressed in CAU defined by a serum standard activated with heat aggregated IgG and zymosan and defined to contain 1000 CAU/ml of the activation products (Bergseth et al., 2013).

2.5. Cytokine measurements

Cytokines in EDTA plasma collected after 120 min of whole blood incubation were analyzed using a commercial fluorescence magnetic bead-based immunoassay, with high-sensitivity detection range and precision (Bio-Plex Pro™ Human Cytokine 27-plex, Bio-Rad Laboratories Inc., Hercules, CA). The following cytokines were analyzed: interleukin (IL)− 1 beta (IL-1β), IL-1 receptor antagonist (IL-1ra), IL-2, IL-4, IL-5, IL-6, IL-7, IL-8 (C-X-C motif chemokine ligand 8 CXCL8), IL-

9, IL-10, IL-12, IL-13, IL-15, IL-17, eotaxin-1 (C-C motif chemokine ligand 11; CCL11), basic fibroblast growth factor (FGF-basic), granulocyte colony-stimulating factor (GCSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), interferon-gamma (IFN-γ), IFN-γ-inducing protein 10 (IP-10; CXCL10), monocyte chemoattractant protein 1 (MCP-1; CCL2), macrophage inflammatory protein-1-alpha (MIP-1α; CCL3), macrophage inflammatory protein-1-beta (MIP-1β; CCL4), platelet-derived growth factor-BB (PDGF-BB), RANTES (CCL5), tumor necrosis factor (TNF) and vascular endothelial growth factor (VEGF). The analyses were performed according to the manufacturer's instructions. Of the twenty-seven cytokines examined, none were induced by temperature alone, and six (IL-1β, IL-2, IL-6, IL-8, IFN-γ, and TNF) were induced by *E. coli* stimulation. These six cytokines fulfilled the criteria proposed earlier (Henno et al., 2017) and were detected in significant amounts in more than 75% of all study samples. The other 21 cytokines were below the detection limit and were, thus, not included in further analyses.

2.6. Leukocyte activation marker CD11b

The blood from six healthy volunteers was incubated with either DPBS (control) or *E. coli* (1×10^7 bacteria/ml) for 15 min at seven different temperatures. Afterward, the samples were lysed, centrifuged at $300 \times g$ for 5 min at 4 °C, and stained with fluorochromes: anti-CD11b-PE, anti-CD14-FITC, anti-human CD15, and anti-CD45-PO according to the manufactural instruction. The samples were washed with 0.1% PBSA, centrifuged, and fixed with 0.01% PFA. The analysis was performed on FACS Attune NxT Flow Acoustic Focusing Cytometer from Thermo Fisher Scientific (Waltham, MA). Data analysis was performed using FlowJo (Version10.7.1) software. First, the leukocyte population was identified with anti-CD45, and then monocytes (CD14 +) and granulocytes (CD15 +) were gated by the side scatter with CD11b expression. Results are given as median fluorescence intensity and cell count.

2.7. Statistical analyses

Values are presented as mean ± 95% CI for parametric data and median (± IQR) for non-parametric data. Complement activation product levels were determined at different temperatures and compared with those at 37 °C using a generalized linear mixed model with Sidak post-hoc correction for multiple testing. To analyze the trend of complement activation within each donor, time, group, and time-by-group interactions were addressed as fixed intercepts while the donor was assigned a random intercept. Differences in cytokine levels between the temperature treatment groups and 37 °C were assessed using one-way repeated measures ANOVA with Dunnett's pairwise multiple-comparison post-hoc test. Both CD11b expression and cell count of granulocytes and monocytes and cell viability were compared between 37 °C and the other temperature groups using repeated measurement Friedman test where each row represents donor-matched data. Differences between *E. coli*-induced and non-activated samples were analyzed separately at each temperature by the Wilcoxon test. Since cell viability was expected to decrease at higher temperatures, a one-sided test was used, whereas the other analyses were two-sided. No post-hoc adjustments were conducted for multiple testing in regard to the *in vitro* experiments with small sample sizes. Results with p-values < 0.05 were regarded as significant. Statistical analyses were performed in Statistical Package for the Social Sciences (SPSS) software (version 26, IBM, Armonk, NY) and GraphPad Prism (version 8, GraphPad Software, San Diego, CA).

3. Results

3.1. Effect of temperature on complement activation in human whole blood

Complement activation, represented by the alternative pathway convertase, C3bBbP, and the terminal complement complex, sC5b-9, was both time- and temperature-dependent (Fig. 1). *E-coli* induced a faster and stronger complement activation than temperature alone (Fig. 1). Both activation products increased consistently with increases in temperature, reaching maximum levels at 41 °C. Compared to 37 °C (green, bold, stippled line), both the hypo- and hyperthermic groups showed significant differences ($p < 0.05$), except for sC5b-9 when induced by temperature alone (Fig. 1C). Hypothermic conditions with *E. coli* (4 °C, 12 °C, and 20 °C) showed significantly lower levels of C3bBbP and sC5b-9 compared to the higher temperatures group (Fig. 1B and D).

3.2. Effect of temperature on cytokine release in human whole blood

Of the 27 cytokines analyzed, six (IL-1β, IL-2, IL-6, IL-8, IFN-γ, and TNF) were significantly influenced by temperature (Fig. 2). Notably, no increase in the cytokines were observed at any temperature without *E. coli.* In the presence of *E. coli* the six cytokines showed a common pattern with no increase below 33 °C, start of increase at 33 °C, continued increase to 37 °C, peaked at 39 °C, with a subsequent consistent decrease at 41 °C (Fig. 2). The *E-coli*-induced cytokine release at hypothermic condition (4 °C, 12 °C, 20 °C, and 33 °C) was significantly lower ($p \leq 0.03$) compared to 37 °C (Fig. 2). At 39 °C, IL-2, IL-6, and TNF levels were significantly higher compared to 37 °C ($p \leq 0.03$). However, all six cytokine levels were lower at 41 °C, compared to 37 °C; statistically significant for IL-1β ($p = 0.003$, Fig. 2), which was reduced to background levels.

3.3. Effect of temperature on granulocyte and monocyte activation in human whole blood

Histograms show one representative experiment of six expressing CD11b in the absence or presence of *E. coli* for granulocytes (Fig. 3A) and monocytes (Fig. 3B). The data are detailed for all six donors in Fig. 3C and D, respectively. In the absence of *E. coli*, both granulocytes (Fig. 3C) and monocytes (Fig. 3D) showed increased expression of CD11b at 41 °C compared to 37 °C ($p < 0.005$). In the presence of *E. coli*, the expression of CD11b was higher on granulocytes at all temperature groups as compared to the control without bacteria (Fig. 3C). Monocytes showed a similar pattern, reaching significance at 41 °C (Fig. 3D). The granulocyte population (count of cells) was significantly ($p < 0.05$) reduced at 41 °C in comparison to 37 °C (Fig. 3E). The monocyte population showed significantly higher numbers of cells at 37 °C in comparison to 33 °C, 39 °C, and 41 °C in the presence of *E. coli.* The count of granulocytes (Fig. 3E) and monocytes (Fig. 3F) did not differ between 39 °C and 41 °C.

3.4. Effect of temperature on endothelial cell viability

Cell viability, defined as the percentage of annexin V- and PI-negative cells, was above 95% at 37 °C in the negative control with EGM culture media and 5% CO_2, as shown in the representative scatter plots (Fig. 4A, upper left panel marked as "Ctrl"). The negative controls were used for gating purpose, which were applied to the cells stored at different temperatures. Cells in BM stored at normothermia (37 °C) were used for making statistical comparison with other temperatures (Fig. 4B, C and D). Hypothermia (12 °C and 20 °C) preserved a ($p < 0.04$) higher percentage of live cells compared with normothermia, whereas hyperthermia (41 °C) reduced ($p = 0.03$) the percentage of live cell (Fig. 4B). Hypothermia reduced the percentage of apoptosis (at 12 °C and 20 °C;

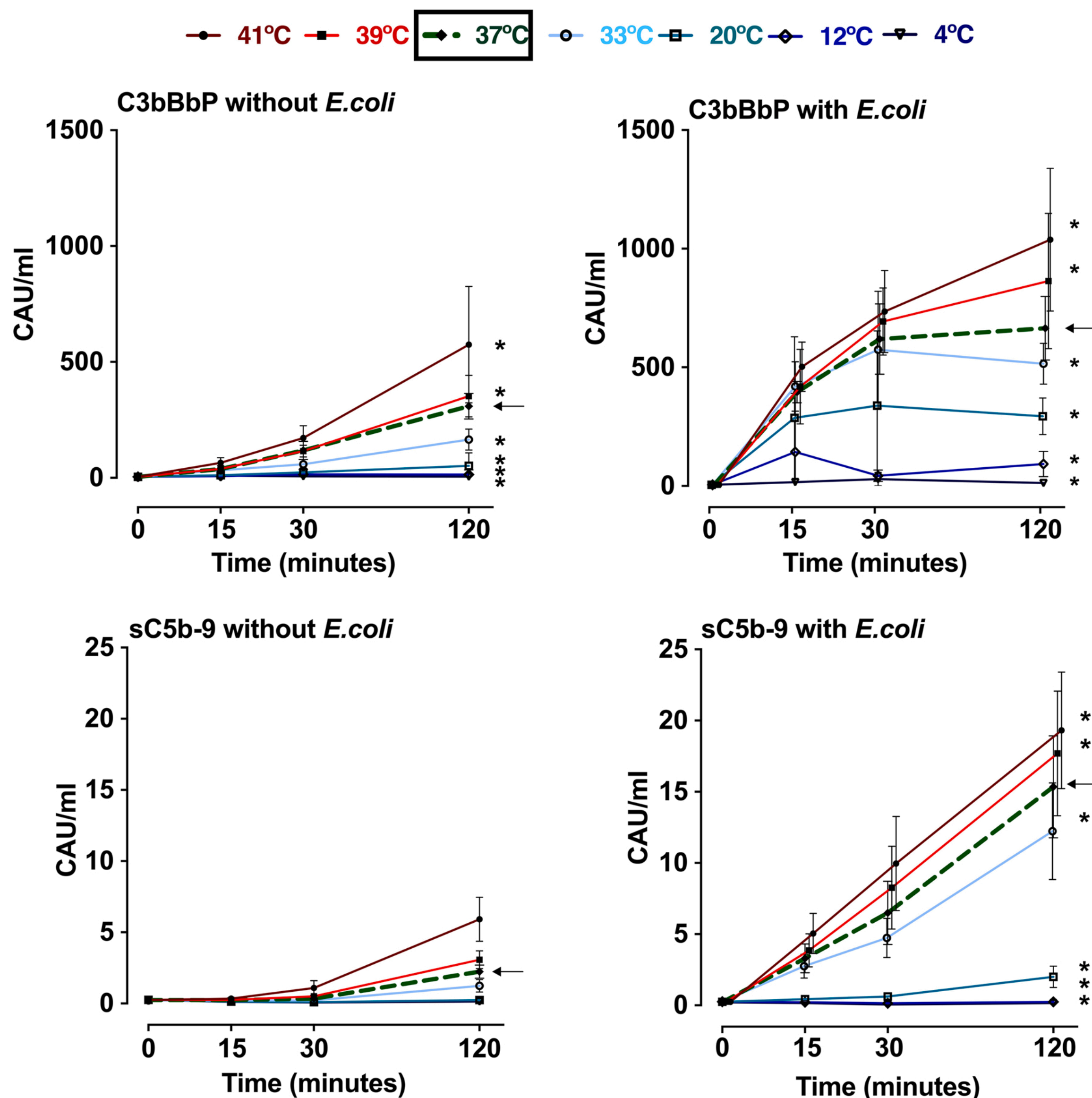

Fig. 1. Temperature-dependent complement activation in human whole blood measured by the formation of C3bBbP (A and B) and sC5b-9 (C and D) with and without the presence of E. coli. Results are expressed as CAU/ml (y-axis). Data are given as mean values ± 95% CI for n = 10 donors. Time 0 represents the baseline activation measured immediately after collecting the blood while times 15, 30, and 120 represent the incubation period in minutes. Statistical significance was estimated over the whole incubation time using a mixed model analysis. Each temperature was compared to the reference temperature of 37 °C, indicated by an arrow at the left margins of the panels. Multiple comparisons were post-hoc corrected with Sidak test. Significance levels are denoted as star (*), where $p \leq 0.05$.

$p < 0.05$) compared to the normothermia (Fig. 4C). Hyperthermia (41 °C) increased the apoptotic cell percentage identified by annexin V-positivity and PI-negativity (41 °C, 38% (25%–47%); $p = 0.03$) compared to 37 °C Fig. 4C). Among the hypothermic groups, 12 °C had lower percentage ($p = 0.03$) of necrotic cells *i.e.*, annexin V-negative and PI-positive cells, compared to 37 °C (Fig. 4D). Percentage of necrotic cells were increased at hyperthermia (41 °C) (45% (31%–63%); $p = 0.03$) (Fig. 4D).

4. Discussion

We incubated *E.coli* under different temperatures in human whole blood, making all the present biological systems able to mutually interact. This holistic approach is in contrast to previous *in vitro* studies which investigated single plasma cascades or cells under reductionistic conditions. Thus, we argue that our data are more translational to a biologic or clinic situation than previous reports. We demonstrated that complement activation in human whole blood was dependent on temperature in a dose-dependent way and that the effect was amplified by *E. coli*. Notably, the change in temperature alone did not induce cytokine release, but the cytokines increased in a temperature-dependent manner when *E. coli* was added. The increase of cytokines peaked at 39 °C and consistently decreased at 41 °C, whereas complement activation increased until 41 °C. Monocytes and granulocytes were activated temperature-dependently by *E. coli.* Endothelial cells showed temperature-dependent viability with the highest viability at low temperatures.

Clinical and pre-clinical studies evaluating post-cardiac arrest care with hypothermia suggest that mild hypothermia inhibits complement activation (Gong et al., 2015; Bisschops et al., 2014b). Here, we got similar result in terms of complement activation *ex vivo* using human whole blood. Generally, the enzymatic activity increases with rising temperature as the kinetic energy increases. A study by Shah et al. reported a contradictory finding, describing increased classical

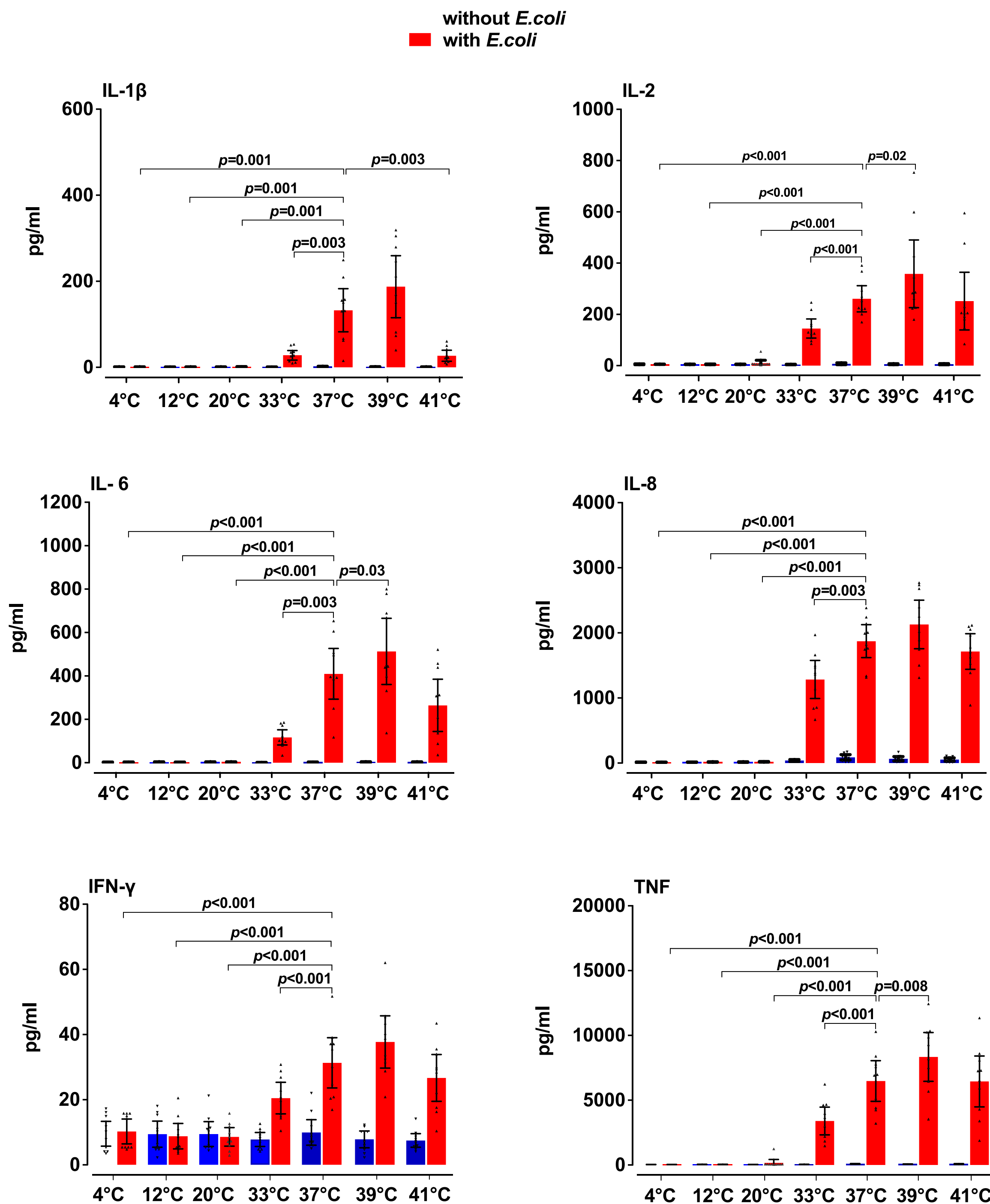

Fig. 2. Cytokine release in human whole blood after 120 min of incubation at defined temperatures with and without the presence of *E. coli*. *E. coli* incubated in whole blood shows cytokine expression indicated as red bars and whole blood without *E. coli* as blue bars. The release of IL-1β, IL-2, IL-6, IL-8, TNF, and INF-γ were measured. Cytokine levels were detected using a multiplex assay. Results are expressed as pg/ml (y-axis). Data are given as mean values ± 95% CI for n = 10 donors. Statistical significance between the effect of the temperature on cytokine release was estimated by using one-way repeated measurement ANOVA with reference to 37 °C with the Dunnett's pairwise multiple comparison post-hoc test.

complement activation upon hypothermia (Shah et al., 2014). However, in the aforementioned study, an antibody-initiated activation was used in low serum concentrations, factors which promote classical pathway activation without amplification *via* the alternative pathway. Here, we assessed complement activation after a broad and physiological stimulus and directly measured the presence of the soluble complement complexes, C3bBbP and sC5b-9, formed only upon complement activation.

E. coli triggered the release of cytokines IL-1β, IL-2, IL-6, IL-8, INF-γ, and TNF, which is in accordance with previous findings (Evans et al., 2015; Brekke et al., 2008; Boneberg and Hartung, 2003; Qadan et al., 2009; Lundeland et al., 2012). All detected cytokines are commonly found to increase in a febrile patient with an infection. However, none of the above-mentioned cytokines were increased by temperature alone. Thus, cytokine expression may first need to be induced by a stimulus, here, *E-coli,* while temperature affects the level of production and release. Our observation of IL-1β reduction at 41 °C was in line with another study by Boneberg *et. al (*Qadan et al., 2009*)*.. However, TNF has been reported to increase in hypothermia (Qadan et al., 2009;

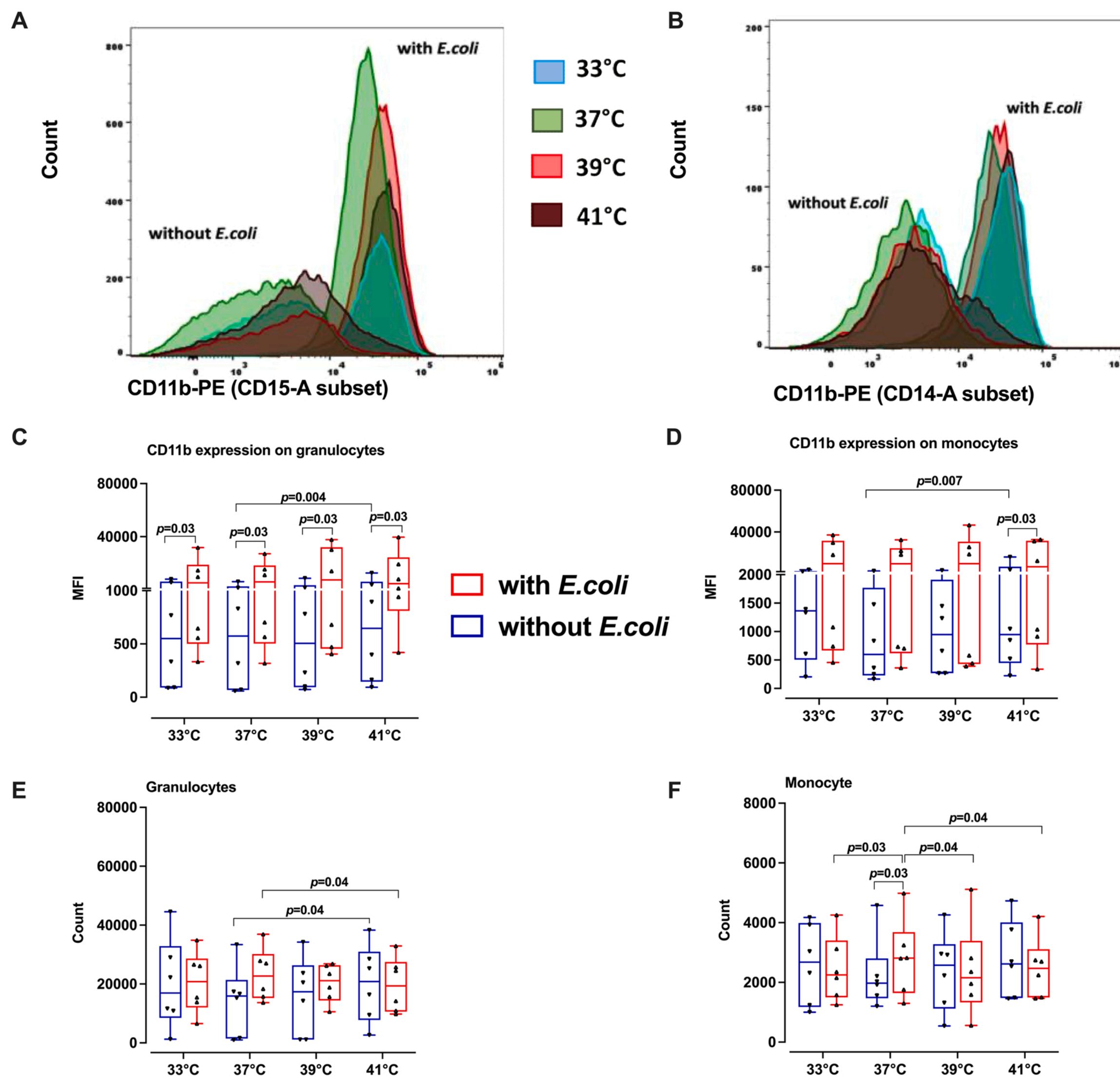

Fig. 3. Effect of temperatures 33 °C, 37 °C, 39 °C and 41 °C on granulocytes and monocytes in the presence or absence of *E. coli* after 15 min incubation. Histograms display the CD11b expression on granulocytes (A) and monocytes (B) with and without *E. coli* incubation from one of six representative experiments summarized in C-F.
Monocyte and granulocyte activation measured without *E. coli* are indicated in blue boxes. *E. coli*-incubated samples are indicated in red boxes. Results are expressed as mean fluorescence intensity (MFI) and cell count. Data are given as a box plot with the median as a line and box indicating the 25th – 75th percentile and whiskers representing from minimum to maximum of the data set for n = 6 donors. Statistical significance between the temperature groups was estimated by using the repeated measurement Friedman test with reference to 37 °C without post-hoc correction for multiple testing, as this is an exploratory *in vitro* study with small sample sizes. The paired Wilcoxon test was applied to compare the significant difference between stimulated with *E. coli* and un-stimulated samples within the same temperature.

Lundeland et al., 2012). This may be explained by differences in the basic methodological design, including *E. coli* concentrations, incubation conditions, and time.

Our study demonstrated that hypothermic conditions invariably decreased all investigated cytokines. This effect might have beneficial effects described in, *e.g.*, acute ischemic stroke (Bisschops et al., 2014a; Kimura et al., 2002). In other conditions like sepsis, it has, however, been shown that temperatures below 36 °C were associated with higher mortality (Beverly et al., 2016; Young and Saxena, 2014). Hyperthermia increased the level of cytokines up to 39 °C. This is in agreement with findings that fever might exacerbate cytokine production and decreasing body temperature might be protective (Liu et al., 2012; Garami et al., 2018). Notably, all investigated cytokines decreased at 41 °C compared to 39 °C, highly significant for IL-1β. This is in accordance with *in vivo* observations that temperatures higher than 40 °C may compromise cellular function and viability. Previous studies showed that temperatures of 41.5 °C and higher could cause heat-induced cell death (Takahashi et al., 2004).

Different levels of cytokines might cause down-stream effects on cellular function. Thus, we investigated leukocytes and endothelial cells under different temperatures. Monocytes and granulocytes play an essential role in acute inflammation, *e.g.,* by releasing inflammatory cytokines like IL-1β, IL-2, IL-6, IL-8, and TNF (Altstaedt et al., 1996; Willis et al., 2003). Upon activation, leukocytes rapidly express CD11b on their surfaces. CD11b is a surface receptor essential for cell-cell interactions. And CD11b forms the phagocytosis receptor CR3 together with CD18, contributing to the thrombo-inflammatory response (Swoboda et al., 2014). CD11b expression on both cell populations remained low in the absence of *E. coli* but increased when incubated with *E. coli.* Each donor had a different response in CD11b expression in both populations, which can be explained as an interindividual response variability (Herold et al., 2021). The cell count remained stable at different

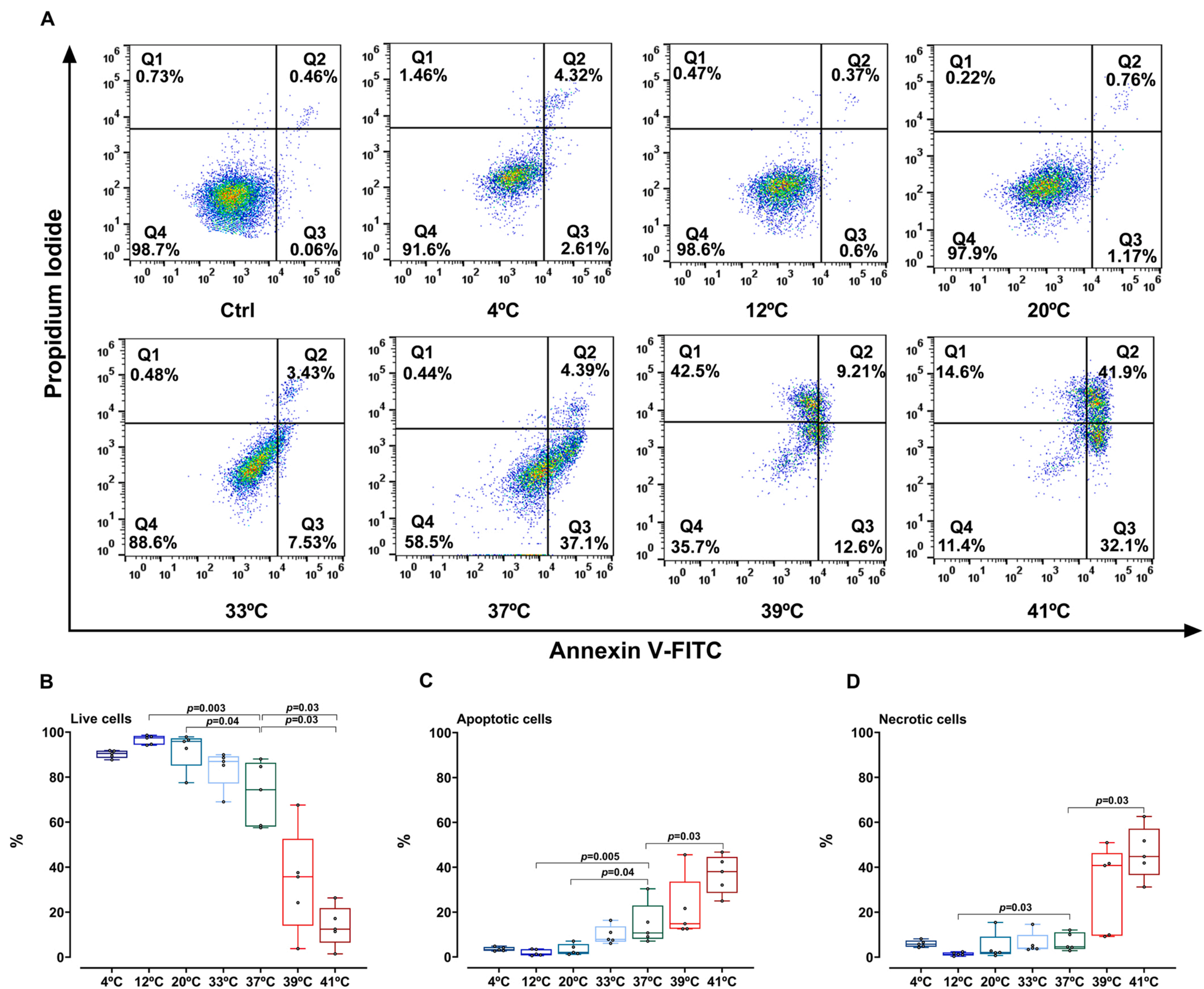

Fig. 4. Temperature affects the viability of HUVEC after 24hrs storage at 4 °C, 12 °C, 20 °C, 33 °C, 37 °C, 39 °C, and 41 °C. (A) Scatterplots representing one of five independent experiments for each temperature condition. HUVECs cultured in EGM at 37 °C with 5% CO_2 supply served as negative control and formed the basis for gating of apoptotic and necrotic cells (Ctrl). Cell distribution in four quadrants represents live cells (lower left quadrant), apoptotic cells (lower right quadrant, annexin V positive), necrotic cells (upper left quadrant, propidium iodide (PI) positive), and necrotic and apoptotic cells (upper right quadrant, PI- and annexin V-positive), which were attributed to the necrotic cell fraction. HUVECs stored in basal medium at increasing temperatures showed increasing apoptosis, followed by necrosis. The percentage of live cells are shown in (B), apoptotic cells in (C) and necrotic cells on (D). Data are given as box plot with median as line, box indicating 25th – 75th percentile, and whiskers showing the range with dots that denote individual experiments of total n = 6 experiments. Significance levels are denoted as star (*), where * $p \leq 0.05$, * * $p < 0.01$.

temperatures in the presence and absence of *E. coli*. However, the cells were in highly different phenotypes from normal until necrotic. The reduction of cytokine level is most likely explained by direct temperature effects on cytokine production and release.

Using primary HUVEC, we showed that temperature (after 24 h of exposure) influenced the viability of the endothelial cells. Hypothermia (12 °C and 20 °C) maintained higher viability than normothermia. These findings are in-line with reports on other cell types, such as epithelial cells, showing preserved viability at hypothermic temperatures, particularly between 12 °C and 16 °C (Islam et al., 2015, 2020; Pasovic et al., 2013). Hyperthermia (39 °C and 41 °C) reduced cell viability. The cell death in hyperthermia was due to apoptosis (37–39 °C) and necrosis (39–41 °C). These findings highlight the higher vulnerability of endothelial cells compared to cancer cells, which show apoptosis and necrosis at higher temperatures only (Leber et al., 2012).

Our study shows that the temperature affects both cell-dependent immune process, such as cytokines and plasma protein-based cascades, such as complement. As an essential part of the vascular system, we further studied temperature effects on endothelial cells. In our laboratory, we have previously demonstrated the critical role of endothelial cells in inflammation (Nymo et al., 2016). However, the findings of this study should be interpreted with caution with respect to the observed temperature effects on endothelial cells. The pH turned alkaline for all temperatures, which put cells in equal cultivating conditions. In addition, the HUVEC cells had reached 95% confluence prior to the experiment and growth was therefore not in the exponential phase. One of the limitations of our current study is that we lack information on monocyte viability after incubation in various temperatures.

E. coli is a pathogen that commonly induces fever, and its effect in the whole blood model at 37 °C is well documented. Sterile inflammation is difficult to induce, however, we have shown here that hyperthermia itself can lead to complement activation. In both scenarios developing high fever may turn complement from a defense system into an aggressor that drives immune and inflammatory diseases, as shown in

previous studies (Mollnes and Huber-Lang, 2020).

A limitation of our study is that it is not *in vivo*. Our experimental model is an *ex vivo* human lepirudin-based whole blood model combined with incubation with primary endothelial cells. This model may be physiologically as relevant as possible to obtain *ex vivo*. In *ex vivo*, we could precisely control the temperatures in whole blood and cells, whereas controlling temperature is difficult in mammals *in vivo*. The *in vivo* situation is more complex, and future studies should evaluate the temperature effects *in vivo*.

5. Conclusion

Hypothermia significantly reduced the inflammatory response in human whole blood. A gradual increase in complement activation was observed over the whole temperature range, reaching the top at the highest tested temperature, *i.e.*, 41 °C. The cytokines reached the maximum release at 39 °C, with a reduced release at 41 °C, particularly for the important inflammasome player IL-1β. Monocyte activation and endothelial cell survival decreased at temperature greater than 37 °C. Thus, this study provides mechanistic insight into the temperature-dependency of the innate immune system-driven inflammatory response, through complement activation and cytokine release, in the blood.

CRediT authorship contribution statement

Viktoriia Chaban: Conceptualization, Methodology, Investigation, Formal analysis, Visualization, Writing – original draft, Writing – review & editing. **Eline de Boer:** Formal analysis, Visualization, Writing – review & editing. **Karin E. McAdam:** Investigation, Writing – review & editing. **Jarle Vaage:** Writing – review & editing, Supervision. **Tom Eirik Mollnes:** Conceptualization, Methodology, Resources, Writing – review & editing, Supervision. **Per H. Nilsson:** Methodology, Writing – review & editing. **Søren Erik Pischke:** Conceptualization, Methodology, Formal analysis, Resources Writing – review & editing, Supervision. **Rakibul Islam:** Conceptualization, Methodology, Investigation, Writing – review & editing, Supervision.

Data Availability

Data will be made available on request.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.molimm.2023.03.006.

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