Nicolaus Copernicus University in Toruń
Faculty of Biological and Veterinary Sciences

NICOLAUS COPERNICUS
UNIVERSITY
IN TORUŃ
Faculty of Biological
and Veterinary Sciences

Mgr inż. Henryk Mikołaj Kozłowski

Hyperthermia and Immune Response: exploring the immunoregulatory potential of fever-range temperatures

Dissertation for a doctoral degree

Dissertation for a doctoral degree conducted at the Department of Immunology

Supervisor: dr hab. Sylwia Wrotek, prof. NCU

Toruń 2023

I would like to thank my supervisor dr hab. Sylwia Wrotek, prof. NCU, for your help, mentoring, and guiding me through the darkest moments and celebrating the smallest victories in my academic journey. Your invaluable insights, your passion, your expertise, wisdom, open-door policy, and constructive feedback have molded me into a better researcher and a more resilient individual. Your guidance has shaped not only my research but also my character, and I am forever grateful for the incredible opportunity to work under your supervision.

I would also like to extend my gratitude to all my colleagues from the Department of Immunology. Their insightful comments, unwavering support, collaborative brainstorming sessions, and the countless cups of coffee we shared during our discussions have been immensely valuable to me. I deeply appreciate your contributions and will forever be grateful for the friendship and enriching experiences we shared.

I extend my sincerest thanks to Dr. Alexander von Ardenne, from the Von Ardenne Institute of Applied Medical Research in Dresden, for exploding in me a deep passion for hyperthermia and for encouraging me to explore new avenues of knowledge in this field.

Finally, I would like to express my deepest gratitude to my family and friends, for your solid patience, understanding, and undeniable support throughout my academic journey. I am grateful for your constant encouragement and for embracing the challenges and sacrifices that came along the way. Your belief in me has been a source of strength, and I am truly thankful for your unwavering presence in my life.

Table of contents

List of abbreviations

FRH - *Fever-range hyperthermia*

TLRs - *Toll-like receptors*

COX-2 – *cyclooxygenase 2*

ILs - *Interleukins*

TNF-α – *Tumour necrosis factor alpha*

IFNs - *Interferons*

G-CSF – *Granulocyte colony stimulating factor*

MIF-1α – *Macrophage inhibitory factor alpha*

LPS - *Lipopolysaccharide*

ROS – *Reactive oxygen species*

NO – *Nitric oxide*

iNOS – *Inducible nitric oxide synthase*

Arg-1 – *Arginase 1*

CDs – *Cluster of differentiation*

ME – *Mistletoe extract*

NF-κB – *Nuclear factor kappa B*

IRF - *Interferon regulatory factors*

MAPK - *Mitogen-activated protein kinase*

PGE_2 - *Prostaglandin* E_2

WBH – *Whole body hyperthermia*

HSPs – *Heat shock proteins*

PBMCs – *Peripheral blood mononuclear cells*

dsRNA – *double-stranded RNA*

TGF-β – *Tumour growth factor β*

Tarbp2 - *TAR RNA-binding protein 2*

1. Introduction

Body temperature has been of great interest among physicians since ancient times. Generally, fever was regarded as a positive sign for infected individuals, with Greek philosopher Parmenides (540-470 BC) famously stating "Give me the power to produce fever and I will cure all diseases" [1]. Aulus Cornelius Celsus (25 BC-50 AD) further recognized the significance of fever as a cardinal sign of inflammation, alongside redness (*rubor*), swelling (*tumor*), pain (*dolor*), and loss of function (*functio laesa*) [2].

In modern times, the study of fever has continued to be of great interest to medical professionals, with notable contributions from German physicians Bush and Bruns, who observed that infectious fever may contribute to cancer remission [3]. Other interesting cases described William B. Coley, a surgeon from New York. His contribution to medicine includes the development of a treatment known as Coley's Toxins, which involved the injection of patients with a mixture of bacterial toxins to trigger a fever response and stimulate the immune system to attack cancer cells. The resulting treatment, was successful in treating some cases of cancer [3].

The beneficial effects of fever were not only limited to cancer treatment. Julius Wagner-Jauregg, was awarded by the Nobel prize in 1927 for his discovery of "the therapeutic value of malaria inoculation in the treatment of dementia paralytica". Further studies showed, that some of the patients suffering from progressive paralysis indicated signs of improvement after the induction of fever-related to malaria [4].

After therapies involving injecting bacteria or other pathogens were discontinued due to their potential risk, the observation of the beneficial effects of elevated body temperature led to the development of new methods for artificially raising body temperature. As a result, fever-range hyperthermia (FRH), which is often utilized as a therapeutic approach, has been increasingly used as a model to study the effects of fever on the immune system without the confounding effects of an active infection. Thus, this model allows for the study of the effects of elevated temperature itself on e.g., cell signaling and gene expression patterns. It has been found that these effects are crucial for proper immune function and cancer cell damage [5].

1.1. Fever vs hyperthermia

Although hyperthermia induced by external heat application and fever both are manifested with body temperature increase, there are significant differences between them. It is known that body temperature regulation is an important mechanism maintaining the homeostasis. Therefore, human body temperature is under control of the thermoregulatory center localized in the preoptic-anterior hypothalamus. The increase in body temperature during fever is related to the presence of molecules called pyrogens that, according to current concept, change the 'set-point' in hypothalamus [6]. In a consequence, internal heat is produced metabolically, especially by intense muscular activity [3]. It is important to note that there is a complex molecular mechanism that underlies fever.

To date, numerous studies have been conducted to elucidate the molecular basis of fever. One of the most well-known models for studying infectious fever is provoking temperature rise with lipopolysaccharide (LPS), a molecule found on the outer membrane of Gram-negative bacteria [7]. In experimental animals, LPS injection triggers a cascade of events that leads to the production of fever, that has at least 3 phases in rats and mice [8,9].

The initial stage of the molecular process that leads to fever involves the identification of LPS by immune cells like macrophages and dendritic cells. These cells play a key role in the innate immune response, which depends on a small number of germline-encoded pattern recognition receptors. These molecules can detect common structural patterns present in different microbial molecules, such as LPS. Toll-like receptors (TLRs) are the primary proteins that participate in this recognition process [10].

The TLRs are type I integral membrane receptors consist of three domains: an extracellular domain, a single transmembrane helix, and an intracellular signaling domain. The extracellular domain of TLRs has a solenoid structure shaped like a horseshoe and is responsible for recognizing common structural patterns found in different microbial molecules [11]. There are currently ten Toll-like receptors (TLRs) identified in humans, and one of them, TLR-4, recognizes lipopolysaccharide. In a model of endotoxic fever, TLR-4 detects the presence of LPS in the tissues and bloodstream, which triggers a pro-inflammatory response, resulting in

the development of fever [11,12]. TLR-4 is found in myeloid immune cells, including monocytes, macrophages, and dendritic cells. It activates two signaling pathways: MyD88-dependent signaling from plasma membrane-localized TLR-4 and TRIF-dependent signaling from TLR-4 internalized in endosomes [12]. Both of the proteins initiate signal transduction pathways that culminate in the activation of nuclear factor kappa B (NF-κB), interferon regulatory factors (IRFs), or mitogen-activated protein kinase (MAPK). All of these factors induce pro-inflammatory cytokine genes, among others, such as: interleukin (IL)1β, tumor necrosis factor (TNF) α, IL-6, IL-12, and type I interferons (IFNs) including IFN-α and IFN-β [10,13,14].

The onset of fever is initiated by the release of endogenous pyrogens from various types of immune cells, including macrophages. Cytokines, particularly IL-1β and IL-6, are among the most important endogenous mediators of fever [15]. These cytokines are synthesized and secreted after stimulation with LPS, due to the activation of NF-κB, and then released into the bloodstream to selectively induce the expression of cyclooxygenase-2 (COX-2)[16]. COX-2 is an early response enzyme that is activated by various physiological stimuli such as LPS, IL-1β or TNF-α. The overexpression of COX-2 leads to biosynthesis and an accumulation of prostaglandin E_2 (PGE_2) from arachidonic acid [17]. Research by Milton et al. (1971) revealed that PGE_2 is an essential mediator of fever, acting downstream of endogenous pyrogens and responsible for the upward resetting of the thermoregulatory center [18].

In the final step of fever induction, PGE_2 binds to EP3 prostaglandin receptors on thermoregulatory neurons in the median preoptic nucleus of the hypothalamus [19]. To elevate body temperature, endotherms release norepinephrine, which increases thermogenesis in brown adipose tissue and induces vasoconstriction to reduce heat loss. Acetylcholine signaling also converts stored chemical energy into thermal energy and increases metabolic rates [20].

Unlike fever, hyperthermic increase in the body is beyond the confines of the ‘set-point’ temperature and it is independent of the hypothalamus [6]. It is possible to induce hyperthermia with some drugs that act on the central nervous system [21] or a simpler way is by providing

an excess of heat energy that the body cannot dissipate. Thus, usually, hyperthermia is a forced overheating of the body and not an intentional reaction of the body itself.

1.2. Fever-range hyperthermia in a medical application

The medical application of heat has a long history [1]. In the 19th and 20th centuries, methods such as thermotherapy, in which heat is applied to a specific area of the body, were developed and refined. Based on the size of heated area, a few types of hyperthermia are recognized.

Local hyperthermia is a technique that targets a specific small area of the body, such as a joint or a tumor, with high temperatures without affecting the surrounding tissues [22]. The goal of local hyperthermia is to promote healing by increasing blood flow and oxygen supply to the affected area or to destroy cancer cells [23]. It is known that increased temperature induces significant changes in the motion of all macromolecular components of cells, thus leading to transitions in cellular structures, on the level of nucleic acids, proteins and lipids [24]. Local hyperthermia can be induced using different devices, such as radiofrequency, microwave, ultrasound, or infrared heaters [25].

During *regional hyperthermia*, large areas such as whole organs, limb or cavity are overheated. Regional hyperthermia is induced by the external applicators using electromagnetic fields with rapid field alternations [26]. In comparison to local hyperthermia, regional hyperthermia is more complicated due to physiological differences of various tissues which are overheated. This kind of hyperthermia is commonly used in combination with chemo- or radiotherapy, as it effectively sensitizes tumor cells for treatment [27]. Among multiple cancer types, this method is used for cervical cancer, malignant melanoma, recurrent breast cancer, soft tissue sarcoma and bladder cancer treatments [28]. In general, clinical outcomes after hyperthermia treatments are related to direct cytotoxicity against tumors [29,30]. This therapy is associated with the low number of systemic side effects such as burns, blisters, discomfort, or pain [30,31], however, hyperthermia-induced immune cell infiltration may be both beneficial or harmful [29,30].

Whole-body hyperthermia (WBH) is a medical procedure involving the increase of core temperature to the range of 37.5-41.5°C of the entire organism [32]. Among methods of WBH induction, hot water immersion, hot blankets, inductive loops, thermal chambers or infrared radiation are commonly used [30]. Among the different methods of WBH, the energy transfer with water-filtered infrared radiation infrared permits a rapid rise of body-core temperature and a high thermal constancy in the plateau phase [32]. The goal of WBH is to boost the immune system and activate the body's natural defenses against infections or cancer [33,34]. Its medical application was discussed in my review article [35].

1.3. Macrophages as key players in inflammation

Macrophages are a versatile and essential component of the immune system, playing a key role in both immune defense and tissue repair [36]. They are a crucial component of the mononuclear phagocyte system, which comprises a family of cells of bone marrow origin, including blood monocytes and tissue macrophages [37].

Monocytic cells have the ability to differentiate into macrophages with varying phenotypes and functions, depending on the cues in the microenvironment. These macrophages can be distributed to different tissues throughout the body [38]. In the context of inflammation, macrophages serve three primary functions: antigen presentation, phagocytosis, and immunomodulation through the production of various cytokines and growth factors [37].

It is well known, that macrophages play an important role in host defense against pathogens by producing reactive oxygen species (ROS) such as nitric oxide (NO) to eliminate phagocytosed bacteria. Toll-like receptors are expressed on macrophages allow them to detect and bind to pathogen components like LPS, double-stranded RNA (dsRNA), CpG DNA, and extracellular proteins such as heat shock proteins (HSPs) [36,39,40]. Furthermore, macrophages play a crucial role in the development of fever. The research conducted by members of my department revealed that *scid* mice lacking mature and functional B and T lymphocytes can still develop fever following injections with LPS. Interestingly, no significant difference in serum IL-6 levels was observed between the *scid* mice and the wild type. These

findings highlight the pivotal role of macrophages in both inflammation and fever development [41].

Research has shown that exposure to heat can affect both innate and adaptive immunity, which together constitute a complex system involving multiple physical and chemical components that operate at the molecular, cellular, and tissue levels. It is established that innate immunity, especially granulocytes, dendritic cells (DCs), macrophages and natural killer (NK) cells, act as the first line of defense against external insults [42] and play role in the tumor immune surveillance [43]. Interestingly, heat shock proteins released in response to FRH exhibit opposing roles in inflammatory response of macrophages, this issue was extensively discussed in the review article by Lee et al. (2012). These authors described that moderate hyperthermia was found to stimulate the production of TNF-α, IL-6, and NO by macrophages via a mechanism dependent on HSP70/72. On the other hand, moderate hyperthermia was seen to inhibit the production of pro-inflammatory cytokines by macrophages, possibly through a rise in heat shock transcription factor 1 (HSF-1) and HSP70 expression [44]. These findings indicate the important, but unclear, role of macrophages in response to heat treatment. The contradictory impact of HSPs on macrophage response implies that investigations should emphasize the global consequences of heat on cells, instead of solely analyzing the impacts of individual HSP proteins.

1.4. Macrophage polarization state

Macrophages, derived from blood monocytes, can differentiate into various subtypes depending on the tissue they reside in. This specialization is reflected in their morphology, pathogen recognition abilities, and cytokine production. In 2000, Mills and colleagues proposed a model for macrophage activation that classified two major opposing macrophage activities into subtypes: classical M1 and alternative M2 macrophages [45]. The balance between M1 and M2 macrophages is important for maintaining homeostasis and proper immune responses [46].

M1 macrophages are classically activated and induce a pro-inflammatory response. They arise due to the exposure to factors such as TLR-4 ligand (e.g., LPS) or in the presence of Th1 cytokines such as IFN-γ [47]. M1 macrophages are involved in the immune response against pathogens and cancer cells. Furthermore, they secrete cytokines such as IL-1β, IL-6, IL-12, and TNF-α, which promote inflammation and enhance the immune response [45]. In addition, M1 cells exhibit increased levels of intracellular markers such as ROS and NO, as well as inducible nitric oxide synthase (iNOS) [38], making them valuable indicators of M1 activation. Beside the production of pro-inflammatory factors, M1 cells also express a range of surface proteins such as CD80 and CD86 [48].

On the other hand, M2 macrophages are alternatively activated and have anti-inflammatory properties [45]. The M2 subset arises when exposed to tumor growth factor beta (TGF-β), IL-10, IL-4, and/or IL-13, leading to the secretion of anti-inflammatory cytokines like IL-4, IL-10, and IL-13 [45]. M2 cells mitigate inflammation, promote tissue repair, wound healing and are involved in immunosuppression. These macrophages are distinguished by elevated levels of intracellular markers like arginase-1 (Arg-1), as well as overexpression of surface molecules such as CD163 and CD206 [49].

Very quickly scientists realized that the M1/M2 system cannot reflect the complexity of the macrophage subsets, and intermediate phenotypes likely existed between them [50]. This led to the discovery of new, more intricate subclasses of M2 macrophages, which have been described in more recent review papers [38,40,50].

2. Aim of the study

Through close observation of patients with fevers, medical practitioners since ancient times have recognized the significant impact that heat can have on the body's natural healing processes. This understanding has been applied in a variety of medical treatments over the years. Despite this, there is still much to be learned about how fever-range temperatures specifically affects the immune system. Protocols for disease prevention are particularly desirable, so my research primarily aimed to investigate the potential utility of FRH in healthy organisms/cells. The objectives of this dissertation were as follows:

1) *to comprehensively investigate how FRH treatment affects hematological profile, and regulatory molecules such as cytokines, and miRNA at the level of entire organism* (**Article #1**).

   To achieve this goal, I have developed a rapid method of FRH in rats that allowed me to investigate the effect of FRH on a whole organism. I analyzed white blood cells count in a peripheral blood and, in key organs such as liver and spleen, the expression of granulocyte-colony stimulating factor (G-CSF) - that regulates granulocytopoesis and macrophage migration inhibitory factor (MIF) - that regulate cells migration. Moreover, in peripheral blood mononuclear cells (PBMCs), the expression of pro-inflammatory IFN-γ and anti-inflammatory IL-10 were analyzed. To check whether miRNA machinery may be involved in FRH triggered effects, the expression of Dicer1 and Tarbp2 in spleen, liver and PBMCs were measured. Finally, the expression of miRNA-155 in the plasma of rats was analyzed.

2) *to accurately identify the FRH-induced macrophage phenotype, including the diversity of macrophage subsets* (**Article #2**).

   In order to accomplish this objective, I analyzed, factors such as: cytokines (IL-1β, IL-6), COX-2 and TLR-4 that are involved in inflammation; reactive oxygen species, nitric oxide, inducible nitric oxide synthase – that affect redox balance, arginase-1 – that

controls availability of arginine. In these cells, I also analyzed cell cycle distribution and surface markers of macrophage polarization.

3) *to explore the potential of FRH to modify the effects of the TLR-4-dependent stimulators* (**Article #2 and Article #3**).

   To get this aim, I investigated effect of FRH on macrophages that are cultured in the presence of various TLR-4 ligands (LPS and Mistletoe Extract (ME)), and checked whether FRH modifies their response. I examined the potential of FRH to modify the ROS level, cell cycle distribution and expression of pro-inflammatory cytokines triggered by ME. Furthermore, I investigated macrophage polarization induced by LPS, and role of TLR-4 in this process.

I utilized several research methods, including *in vivo* studies in rats and *in vitro* experiments using mammalian cells lines (in accordance with 3R principle). In my research, I employed the following research techniques: flow cytometry (macrophage polarization, cell cycle, reactive oxygen species), colorimetric assay (nitric oxide level, MTT assay), Dot-blot and Western blot assay (protein expression), real-time PCR (gene expression, miRNA level), surgical procedures (anesthetizing and implantation of biotelemetry devices), deep body temperature measurement (biotelemetry), hematological analysis (impedance principle), whole body fever-range hyperthermia (induced by infrared lamp).

3. Publication list

This dissertation contains selected results detailed described in the following articles:

Article 1	**Kozłowski, H.M**.; Sobocińska, J.; Jędrzejewski T.; Maciejewski B.; Dzialuk A.; Wrotek S. *Fever-range whole body hyperthermia leads to changes in immune related genes and miRNA machinery in Wistar rats.*	Under review in International Journal of Hyperthermia (May 2023)
Article 2	**Kozłowski, H.M**.; Sobocińska, J.; Jędrzejewski T.; Maciejewski B.; Dzialuk A.; Wrotek S. *Fever-range hyperthermia can effectively switch macrophage polarization towards regulatory phenotype*	Sent to the Journal (May 2023)
Article 3	**Kozłowski, H.M**.; Pawlikowska, M.; Sobocińska, J.; Jędrzejewski, T.; Dzialuk, A.; Wrotek, S. *Distinct Modulatory Effects of Fever-Range Hyperthermia on the Response of Breast Cancer Cells and Macrophages to Mistletoe (Viscum album L.) Extract*. Pharmaceuticals 2021, 14, 551. https://doi.org/10.3390/ph14060551	5-year Impact Factor: 5,711 Ministry points: 100

3.1. Article 1

Kozłowski, H.M.; Sobocińska, J.; Jędrzejewski T.; Maciejewski B.; Dzialuk A.; Wrotek S. *Fever-range whole body hyperthermia leads to changes in immune related genes and miRNA machinery in Wistar rats*. Under review in International Journal of Hyperthermia (May 2023).

Title: Fever-range whole body hyperthermia leads to changes in immune related genes and miRNA machinery in Wistar rats

Henryk Mikołaj Kozłowski [1,2], Justyna Sobocińska [1], Tomasz Jędrzejewski [1], Bartosz Maciejewski [1], Artur Dzialuk [2], Sylwia Wrotek [1]

[1] Department of Immunology, Faculty of Veterinary and Biological Sciences, Nicolaus Copernicus University, 1 Lwowska Str., 87-100 Torun, Poland

[2] Department of Genetics, Faculty of Biological Sciences, Kazimierz Wielki University, 10 Powstańców Wielkopolskich Ave., 85-090 Bydgoszcz, Poland

Abstract

Fever is defined as a rise in body temperature upon disease. Fever-range whole body hyperthermia (FRH) is a simplified model of fever and a well-established medical procedure. Despite its beneficial effects, the molecular changes induced by FRH remain poorly characterized. The aim of this study was to investigate the influence of FRH on regulatory molecules such as cytokines and miRNAs involved in inflammatory processes. In this study, we developed a novel, fast rat model of infrared-induced FRH. The body temperature of animals was monitored using biotelemetry. FRH was induced by the infrared lamp and heating pad. White blood cell counts were monitored using Auto Haematology Analyzer. In peripheral blood mononuclear cells, spleen and liver expression of immune-related genes (IL-10, MIF, G-CSF, IFN-γ) and miRNA machinery (DICER1, TARBP2) was analysed with RT-qPCR. Furthermore, RT-qPCR was used to explore miRNA-155 levels in the plasma of rats. We observed a decrease in the total number of leukocytes due to lower number of lymphocytes, and an increase in the number of granulocytes. Furthermore, we observed elevated expressions of DICER1, TARBP2, and granulocyte colony-stimulating factor (G-CSF) in the spleen, liver, and PBMCs immediately following FRH. FRH treatment also had anti-inflammatory effects, evidenced by the downregulation of pro-inflammatory macrophage migration inhibitor factor (MIF) and miR-155, and the increased expression of anti-inflammatory IL-10. FRH affects the expression of molecules involved in inflammatory processes leading to alleviated inflammation. We suppose these effects may be miRNAs-dependent and FRH can be involved in therapies where anti-inflammatory action is needed.

Key words: Fever-range whole body hyperthermia, miRNA machinery, miRNA-155, cytokines, immunomodulation

1. Introduction

Fever is defined as a rise in body temperature (increase in body heat) upon disease. As part of the acute phase response, fever is almost invariably accompanied by uncomfortable sickness symptoms and behaviours, notably lethargy, depression and aches (1). Physiologists ascribe fever to an upward resetting of the thermoregulatory set point induced by either infectious agents or factors synthesized by the host. The purpose of the increase in body temperature that is associated with fever remains the most poorly understood aspect of the acute inflammatory response.

To investigate the significance of the thermal component itself, fever-range hyperthermia (FRH) has been employed. There are many *in vitro* research that presents the effect of high ambient temperature on various cell lines (2–4). In our previous *in vitro* study, we described the effect of hyperthermia on macrophages. We have found that hyperthermia may affect therapy in which mistletoe extract is involved. We showed that the treatment of macrophages (RAW 264.7 cells) with elevated temperatures (39°C and 41°C) stimulated the expression of pro-inflammatory cytokines, including interleukin (IL)-6 and IL-1β (5). Thus, we indicated its immunomodulatory effects. However, that *in vitro* model did not reflect the response of a whole body to FRH. Therefore, in this paper we employed rats to investigate molecular mechanisms triggered by the heat.

Among organs that are affected by FRH, the liver deserves special attention. This is an organ that displays temperature dependent metabolic rate changes (6), and releases various regulatory molecules, including cytokines and complement proteins (7). Similarly, the spleen plays an important role in the modulation of the immune system. This is a place for the differentiation and activation of T and B cells (8).

The count of white blood cells and the distribution of these cells in a body is regulated by many factors including G-CSF which regulates granulocytopoesis and MIF (macrophage migration inhibitory factor). MIF is a cytokine released by a number of cell types including, monocytes, macrophages and T cells. Research has shown its important role as a regulator of innate immunity and its pro-inflammatory properties (9). The expression of MIF may be induced by various pro-inflammatory factors including cytokines, microbial products and in response to stress (4,9).

Another important pro-inflammatory factor is interferon-γ (IFN- γ) which acts as the first line of defense against viral infection in mammals. IFN-γ can act directly on infected cells or is able to induce indirect anti-viral response through modulating the differentiation and maturation of T cells and B cells (10). IFN is well known as a mediator of adaptive immunity but it may also stimulate innate immunity through the activation of macrophages (11).

To counteract the damage caused by inflammatory processes anti-inflammatory agents such as IL-10 are released by organisms. Activation of IL-10 receptors leads to inhibition of pro-inflammatory mediators, decrease of phagocytosis and the releasing of anti-inflammatory molecules such as interleukin-1 receptor antagonist, soluble tumor necrosis factor α (TNFα) receptor, and IL-27 (3,12).

Although, the role of G-CSF, MIF, IFN-γ and IL-10 in inflammatory processes is established, it is not known whether FRH can affect these factors. Furthermore, it is still not known how the network of these cytokines is regulated. Recently, the role of miRNAs is postulated (13–16). Increasing evidence suggests that the liver can be a source of micro RNAs (miRNAs)(7). Although miRNAs are intracellular regulators of gene expression, they can be secreted into the extracellular matrix and body fluids, and delivered to distant cells to trigger systemic effects (17).

miRNAs are small (~22 nt), single-stranded, non-coding RNAs that regulate gene expression through base-specific pairing between the seed region of the mature miRNA and the 3'-untranslated regions (3'-UTR) of target mRNAs (18). The biogenesis of miRNAs begins in the nucleus, where long RNA transcripts termed primary miRNAs (pri-miRNAs) are excised. pri-miRNAs are then processed and final miRNAs are released. Dicer1 and Tarbp2 are key to this process (7), and regulate the expression and functioning of all miRNA molecules including miRNA-155 (19). miRNA-155 has been extensively investigated due to its role in immune defence. It may be expressed by a wide spectrum of cells and it regulates both innate and adaptive immunity, including monocytes, macrophages, dendritic cells, B cells and T cells (20).

In this study, we developed a fast rat model of fever-ranged hyperthermia (FRH) induced by infrared radiation. Our study aimed to investigate the effect of FRH on the expression of cytokines that regulate inflammation. We further analyzed the effects of FRH on the expression of miRNA machinery molecules (DICER1 and TARBP2) and miR-155.

2. Materials and methods

2.1 Experimental animals

Male Wistar Cmd:(WI)WU rats aged 4-5 weeks and weighing 120-150 g were purchased from the Mossakowski Medical Research Centre of the Polish Academy of Sciences (Warsaw, Poland). Rats were acclimatized for 14 days and housed at constant relative humidity (60 ± 5%) and temperature (22 ± 1°C), with a 12:12 h light-dark cycle (light at 7:00 am). Food and water were provided *ad libitum*. All procedures were approved by the local Bioethical Committee for Animal Care (permission no. 49/2020).

2.2 Temperature measurements

To monitor deep body temperature (Tb), animals were implanted intraabdominally with temperature-sensitive miniature biotelemeters (PhysioTels model TA10TA-F40; Data Sciences International, St. Paul, MN, USA) under sterile conditions according to manufacturer's recommendations. Prior to the implantation, rats were anaesthetized with a mixture of ketamine (Biowet, Puławy, Poland)/xylazine (ScanVet, Gniezno, Poland) (87 mg/kg and 13 mg/kg, respectively) injected intramuscularly. Following shaving and sterilization of a small surgical area, an incision was made in the skin and muscles of the abdomen. A miniature temperature-sensitive telemetry device was then placed into the peritoneal cavity and the abdomen and skin were independently sutured. All surgical procedures were performed a minimum of 10 days before experimentation.

2.3 Treatment of rats with fever range whole body hyperthermia

FRH was induced using an infrared lamp and heating pad (Data Science International). Prior to induction, rats were anaesthetized with a mixture of ketamine/xylazine injected intramuscularly. Animals were placed on their backs on a heating pad and temperature probes were introduced *per rectum* to monitor deep body temperatures during FRH. Following assessment of the initial body temperature, the infrared Sollux lamp Breuer (150W 230V, Solllux, Berlin, Germany) was switched on and heating was performed for 5 min with a lamp placed 30 cm from the body surface. Upon exceeding a body temperature equal to 38.1°C, the lamp was switched off. To maintain a constant temperature of 39°C for 1 h, a heating pad was employed. If the deep body temperature decreased to 38.7°C, the lamp was switched on for additional 30s. Following heating, during restorationto the physiological range, the deep body temperature was measured using a biotelemetry system (Data Science International).

2.4 Tissue sample collection from rats

Tissue samples were collected immediately after FRH treatment (FRH-t_0) or 24 h post-FRH (FRH-t_{24}). Liver and spleen samples (whole glands) were dissected from anaesthetised rats, rinsed twice with cold sterile PBS, dried and frozen in liquid nitrogen. Samples were stored at -80°C prior to analysis.

2.5 Blood sample collection and plasma isolation

Blood was collected into EDTA-treated tubes following cardiac puncture. Plasma separations were performed through centrifugation of whole blood samples for 10 min at 1000 x g. Samples were transferred into fresh tubes and stored at -80°C prior to assessment.

2.6 Evaluation of blood cell counts in rats

The total number of blood leukocytes, lymphocytes and granulocytes were investigated using an Auto Hematology Analyzer BC-2800Vet (Mindray, Shenzhen, China) according to the manufacturer's recommendations.

2.7 Peripheral blood mononuclear cells isolation

Density gradient centrifugation was used for the isolation of peripheral blood mononuclear cells (PBMCs). Following the collection of whole blood, samples were diluted 1:1 with sterile PBS at room temperature. Dilutions were layered onto Lymphocyte Separation Medium (BioWest, Nuaillé, France) in 15 mL tubes, centrifuged (35 min, 400 x g, room temperature) and the PBMCs were collected. To remove the separation medium, cells were washed with sterile PBS and centrifuged for 5 min at 400 x g. Cells were resuspended in Fetal Bovine Serum (Merck KGaA, Darmstadt, Germany) supplemented with 10% dimethyl sulfoxide (DMSO), frozen and stored in liquid nitrogen prior to their use.

2.8 Total RNA extraction

For RNA extraction, liver and spleen tissues (100 mg) or PBMCs were lysed in PureZOL™ RNA Isolation Reagent (Bio-Rad, Hercules, CA, USA) with mechanical disruption for tissue samples. mRNA extraction was performed according to the Chomczynsky and Sacchi method (21). The final concentration of total mRNA in the samples was measured using a Take3 Micro-

Volume Plate on a Synergy HT Multi-Mode Microplate Reader (BioTek Instruments, Winooski, VT, USA).

2.9 RT-qPCR

cDNA synthesis was performed on 1000 ng of total RNA using iScript™ Reverse Transcription Supermix for RT-qPCR. qRT-PCRs were performed using iTaq Universal SYBR® Green Supermix and PrimePCR™ SYBR® Green Assays with a final volume of 10 µL. Unique assay IDs of the primers are shown in Table 1. Test samples were run in triplicate using the CFX Connect Real-Time PCR Detection System. Melt curve analysis was performed as a control for specificity. Standard curves were prepared for both target and reference (*Actb*) genes. Calibrator-normalized quantifications were performed using CFX Manager Software 3.1. Reactions were repeated at least twice. All reagents were purchased from Bio-Rad.

Table 1. Unique Assay ID of primers used for RT-qPCR.

Gene name	Gene/PROTEIN symbol	Unique Assay ID	Cat. No
Actin	*Actb*/ACTB	qRnoCID0056984	Cat. No. 10025636
RISC-loading complex subunit TARBP2	*Tarbp2*/TARBP2	qRnoCED0004684	Cat. No. 10025636
Dicer1	*Dicer1/ DICER1*	qRnoCID0006103	Cat. No. 10025636
Macrophage migration inhibitory factor	*Mif*/ MIF	qRnoCED0007731	Cat. No. 10025636
Granulocyte colony-stimulating factor precursor	*Csf3*/ G-CSF	qRnoCED0001885	Cat. No. 10025636
Interferon gamma	*Ifng*/IFN-γ	qRnoCID0006848	Cat. No. 10025636
Interleukin-10	*IL10*/ IL-10	qRnoCID0005930	Cat. No. 10025636

2.10 miRNA extraction and expression

Circulating miRNA molecules were isolated from 200 µL of plasma using the miRNeasy Serum/Plasma Kit (Qiagen, Hilden, Germany) according to the manufacturer's

recommendations. TaqMan®microRNA RT Kits were used to reverse transcribe cDNA of TaqMan®MicroRNA Assays for mmu-miR-155 and U6 snRNA as an internal reference (Table 2)(22). qPCRs were performed in a final volume of 20 µL and included 1.33 µL of RT product, 1X TaqMan™ Universal PCR Master Mix II, no UNG, and probe mix (Thermo Fisher Scientific, Waltham, Massachusetts, USA). Samples were analysed in triplicate using the CFX Connect Real-Time PCR Detection System (Bio-Rad). Relative expression levels were obtained through normalisation to pre-FRH samples. Values were calculated using CFX Manager Software 3.1 (Bio-Rad).

Table 2. Unique primer IDs for miRNA analysis by RT-qPCR.

	ID No.	Cat. No
RT-U6 snRNA	001973	PN4427975
RT- mmu-miR-155-5p	002571	PN4427975
TM-U6 snRNA	001973	PN4427975
TM- mmu-miR-155-5p	002571	PN4427975

2.11 Statistical analysis

Relative miRNA expression level and gene expression levels were calculated and compared using the $2^{-\Delta\Delta CT}$ method. All the data analysis were performed three times with three technical repetitions for each one. Multiple group comparisons were performed using a one-way analysis of variance (ANOVA) followed by the Tukey test. For calculations, analysis, and results visualization GraphPad Prism version 8 (GraphPad Software Inc., La Jolla, CA, USA) statistical software was used. The statistical standard of significance was set at $p < 0.05$.

3. Results

3.1 Establishment of a rat model of fever-range whole body hyperthermia using infrared radiation

To investigate the effects of heat in rats, we developed a whole body FRH model. The initial average deep body temperature of the animals was 36.77 ± 0.57°C (mean ± S.D.). The average time of heating animals with an infrared lamp to the level of 38.10°C was 5 ± 2.55 minutes. Treatment of rats with an infrared lamp increased the body temperature to 39.00°C within next 3 ± 2.35 min since switching off the infrared lamp, which was maintained for 1 h. The body temperature did not fall below 38.94 ± 0.05°C and did not exceed 40.08 ± 0.30°C

(Figure 1). Upon cessation of heating, the average time required for normalisation to physiological levels was 35 min.

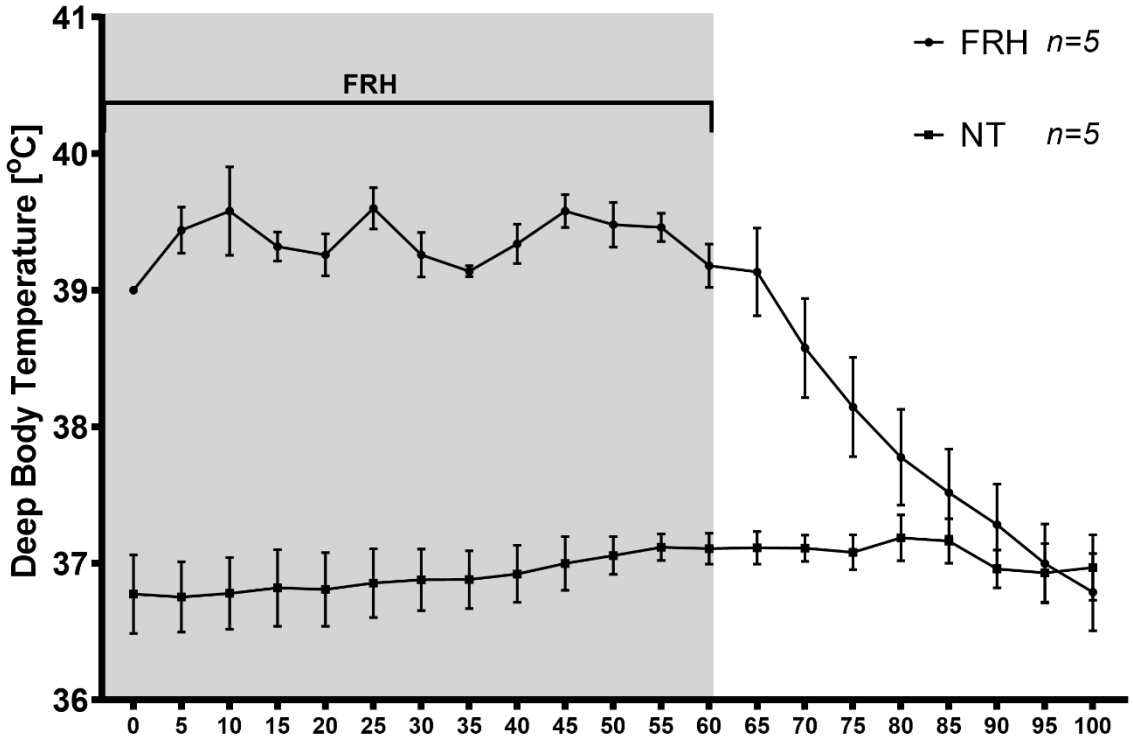

Figure 1. Changes in body temperature within 100 min of FRH. The grey background denotes the mean body temperature during 60 min of FRH. The additional 40 min represents the time required for the restoration of the body temperature to physiological levels. FRH: Fever Range Hyperthermia treatment; NT: control group, n: sample size per group.

3.2 Fever range hyperthermia decreases the number of white blood cells

We next evaluated the effects of FRH on WBC counts. We observed a significant decrease in total WBC numbers post-FRH in rats ($p < 0.05$; Figure 2A). After 24 h, the number of WBCs still decreased, though these changes were not statistically significant ($p = 0.44$).

Lymphocyte numbers were found to decrease in response to FRH (Figure 2B), which occurred directly following FRH treatment ($p < 0.05$) and continued for 24 h ($p < 0.01$). The percentage of lymphocytes in WBCs decreased after 24 h ($p < 0.01$), compared to the control group (Figure 2C). Our analyses revealed that this restoration of WBC numbers after 24 h was related to a rise in the number of granulocytes in comparison to the control group and rats directly post-FRH ($p < 0.01$ & $p < 0.05$, respectively; Figure 2D). Similarly, we observed substantial changes in the percentage of granulocytes only after 24 h ($p < 0.01$; Figure 2E).

Although the number of monocytes decreased following FRH, the decline was not statistically significant ($p = 0.47$; Figure 2F). Similar, differences in the percentage of monocytes in response to FRH were observed (Figure 2G). Collectively, these data suggest that

FRH leads to substantial growth in granulocyte numbers while the number of lymphocytes decreases.

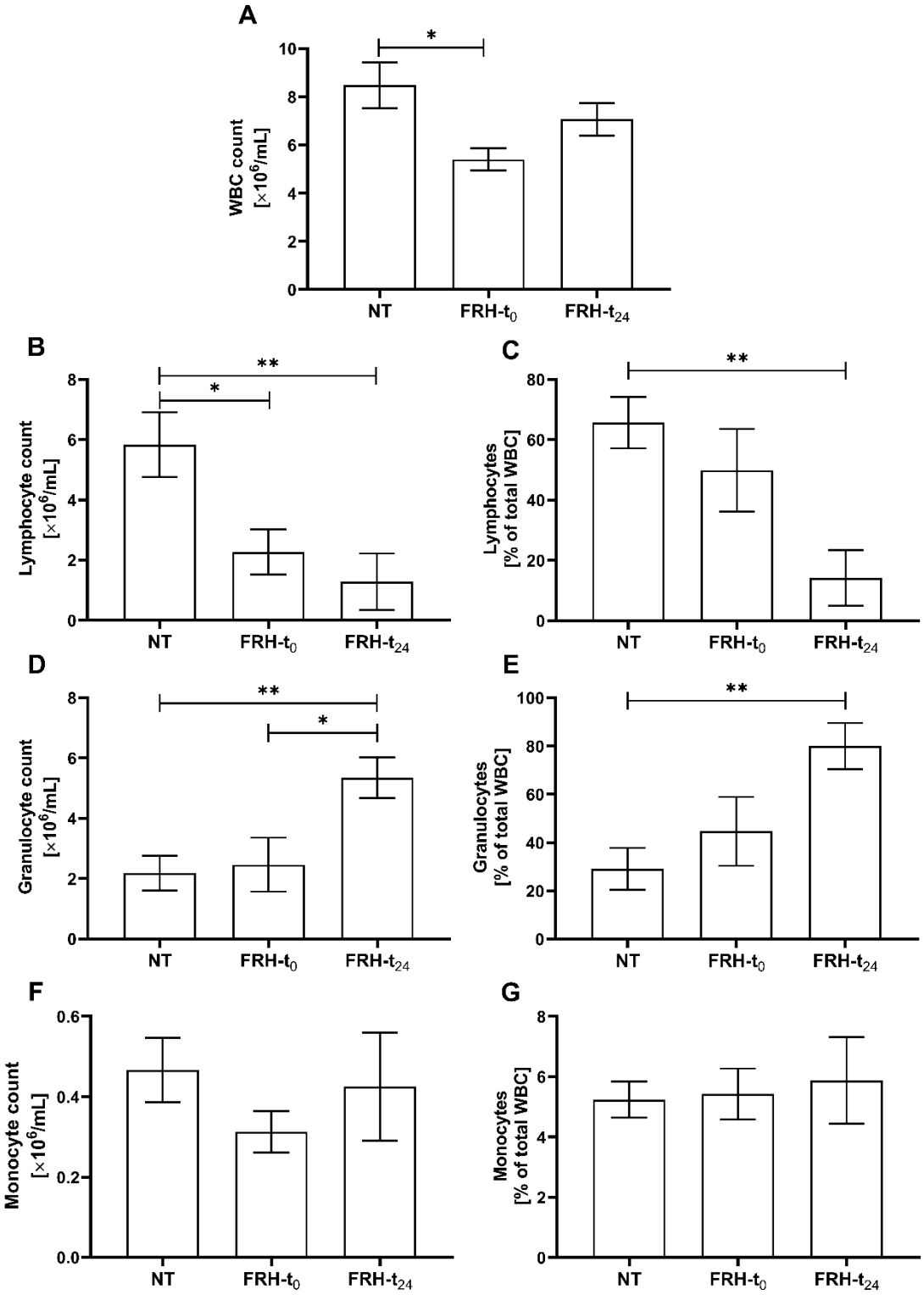

Figure 2. Effects of FRH on white blood cell (WBC) counts. White blood cell count (A), total number of lymphocytes (B); lymphocytes as a percentage of WBCs (C); total number of granulocytes (D); granulocytes as a percentage of WBCs, (E); total number of monocytes (F); and percentage of

monocytes (G) in the peripheral blood of FRH-treated rats. Data are the mean ± S.E.M. Asterisks indicate significant differences (** $p < 0.01$, * $p < 0.05$). NT: control animals (n = 6), FRH-t_0: samples collected directly after FRH treatment (n = 6), FRH-t_{24}: samples collected 24 h post-FRH treatment (n = 5). n indicates the sample size per group.

3.3 Fever range hyperthermia modulates immune related genes in various organs

We next investigated the ability of FRH to induce changes in immune-related gene expression. In livers from FRH-treated rats, we evaluated the expression of *Csf3*, *Mif*, and *IL10*. We observed an important and immediate increase in *Csf3* expression upon FRH treatment ($p < 0.01$; Figure 3A). This effect was significantly reversed after 24 h ($p < 0.01$). No changes in *Mif* expression were observed directly after FRH treatment in rat livers (Figure 3B), but a substantial decrease in its expression 24 h post-FRH treatment was observed. No significant changes in *IL10* mRNA expression were seen (Figure 3C). *Ifng* could not be detected (*data not shown*).

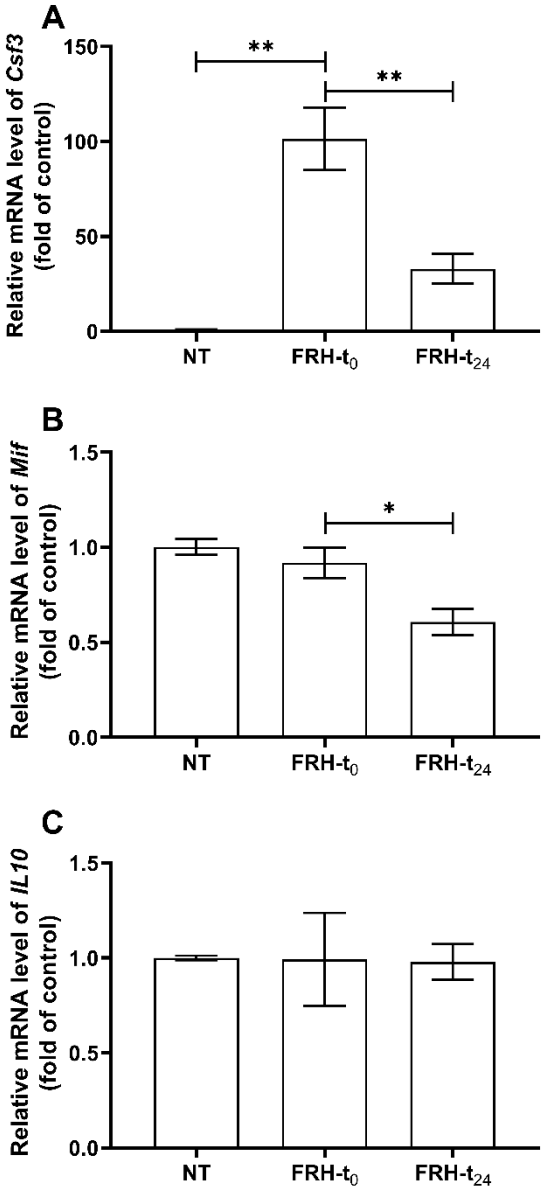

Figure 3. Effects of FRH on the expression of Csf3 (**A**), Mif (**B**) and IL10 (**C**) in rat livers. mRNA expression was determined by RT-qPCR. Data are shown as the mean ± S.E.M. of three independent experiments. Asterisks indicate significant difference between groups (** $p < 0.01$, * $p < 0.05$). NT: control animals (n = 6), FRH-t_0: samples collected directly following FRH treatment (n = 6), FRH-t_{24}: samples collected 24 h post-FRH treatment (n = 5), n: sample size per group

We next examined spleen tissues from rats treated with FRH. We observed a significant increase in *Csf3* expression following FRH treatment ($p < 0.01$; Figure 4A) that was abolished after 24 h ($p < 0.001$). This decrease was statistically significant compared to non-treated animals ($p < 0.001$). In contrast to *Csf3*, we observed a substantial decrease in *Mif* expression in the spleen immediately following FRH ($p < 0.01$; Figure 4B) which continued to decline after 24 h ($p < 0.001$). *Mif* expression 24 h post-FRH was indeed lower than in non-treated rats ($p < 0.001$). A modest rise in *IL10* expression directly after FRH treatment was also observed, but this effect was not statistically significant ($p = 0.67$; Figure 4C). After 24 h, we observed an increase in *IL10* expression in the spleen compared to non-treated animals ($p < 0.05$). No changes in *Ifng* expression occurred in the spleen in response to FRH (Figure 4D). A modest increase in *Ifng* expression was observed after 24h, but this was not significant ($p = 0.17$).

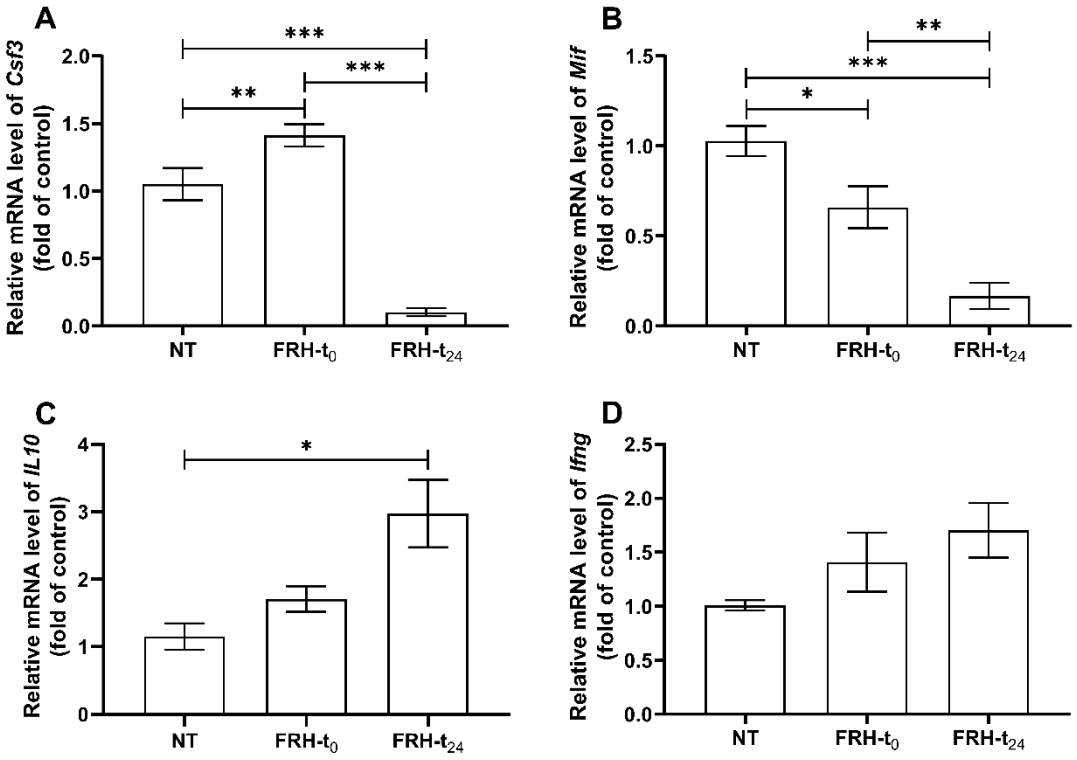

Figure 4. Effects of FRH on the expression of Csf3 (**A**), Mif (**B**), IL10 (**C**) and Ifng (**D**) in the spleen of rats. mRNA expression was determined by RT-qPCR. Data are shown as the mean ± S.E.M. of three independent experiments. Asterisks indicate significant differences between groups (*** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$). NT: control animals (n = 6), FRH-t_0: samples collected directly following FRH treatment (n = 6), FRH-t_{24}: samples collected 24 h post-FRH treatment (n = 5). n indicates the sample size per group.

Finally, we investigated changes in gene expression in the PBMCs isolated from rats. No significant differences in *Csf3* expression following FRH treatment were observed (Figure 5A). However, a reduction of *Csf3* expression was observed in PBMCs 24 h post-FRH treatment compared to the control group ($p < 0.05$). Furthermore, no changes in *Mif* expression post-FRH was observed ($p = 0.22$; Figure 5B), but after 24 h, its expression significantly declined ($p < 0.05$) in comparison to the FRH-t_0 group. We observed a substantial rise in *IL10* in PBMCs following FRH treatment ($p < 0.001$; Figure 5C), which decreased after 24 h ($p < 0.001$). Although *IL10* expression remained high after 24 h, no substantial change was observed in comparison to non-treated controls ($p = 0.10$). Looking at *Ifng,* a substantial growth in the PBMCs of FRH-treated rats was observed ($p < 0.01$; Figure 5D). This effect decreased after 24 h ($p < 0.001$), at which point its levels were comparable to the non-treated control group.

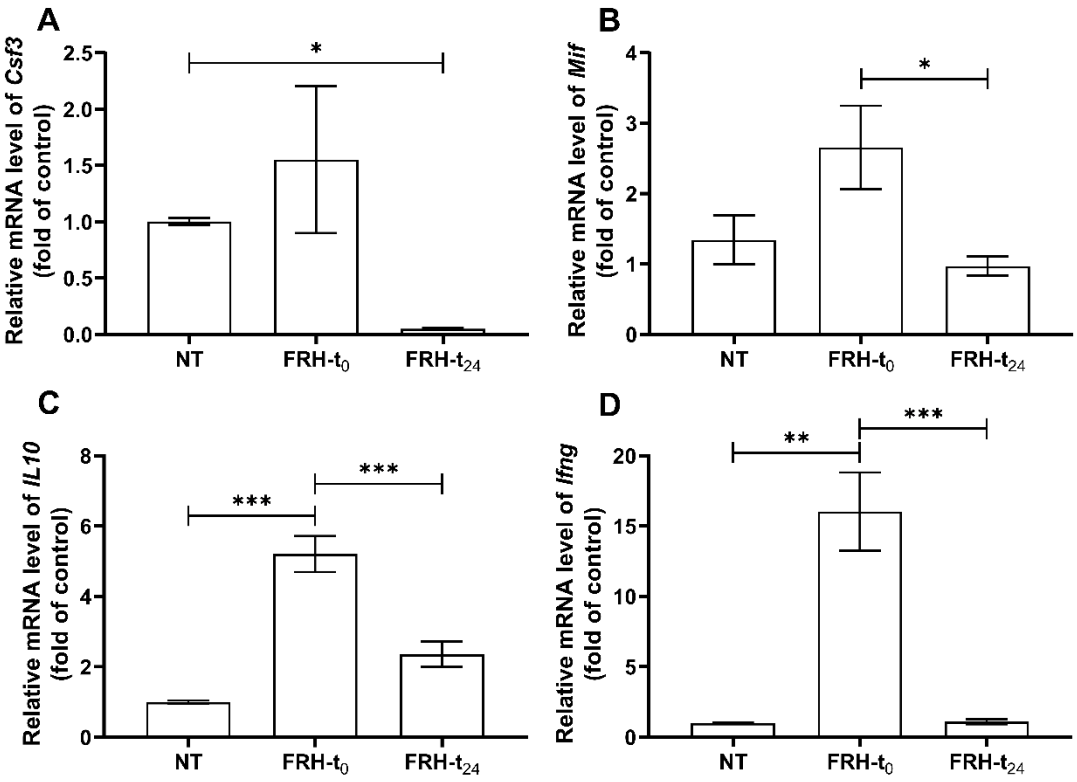

Figure 5. Effects of FRH on the expression of Csf3 (**A**), Mif (**B**) IL10 (**C**) and Ifng (**D**) in PBMCs of rats. mRNA expression was determined by RT-qPCR. Data are the mean ± S.E.M. of three independent experiments. Asterisks indicate significant differences between groups (*** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$). NT: control animals (n = 6), FRH-t_0: samples collected directly following FRH treatment (n=6), FRH-t_{24}: samples collected 24 h post-FRH treatment (n = 5). n indicates the sample size per group.

3.4 Fever range hyperthermia affects the expression of the miRNA machinery

To identify changes in miRNA expression in FRH-treated rats, levels of key members of the miRNA machinery, including *Dicer1* and *Tarbp2* were determined in PBMCs, liver and spleen tissues. In PBMCs, a significant rise in *Dicer1* mRNA expression was observed

immediately after FRH treatment ($p < 0.01$; Figure 6A), which was abolished after 24 h ($p < 0.001$). Similarly, we observed a substantial growth in *Tarbp2* expression in PBMCs following FRH treatment ($p < 0.001$; Figure 6B), with the return to physiological levels seen after 24 h ($p < 0.001$).

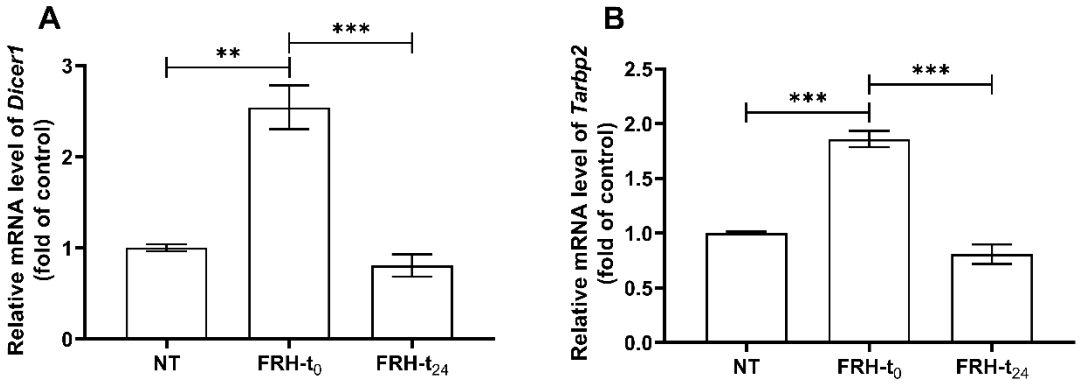

Figure 6. Effects of Fever Range Hyperthermia on the expression of Dicer1 (**A**) and Tarbp2 (**B**) in PBMCs of rats. mRNA expression was assessed via RT-qPCR. Data are shown as the mean ± S.E.M. of three independent experiments. Asterisks indicate significant difference between groups (*** $p < 0.001$, ** $p < 0.01$). NT: control animals (n = 6), FRH-t_0: samples collected directly after FRH treatment (n = 6), FRH-t_{24}: samples collected 24 h post-FRH treatment (n = 5), n: sample size per group.

We next measured the expression of *Dicer1* and *Tarbp2* in the liver as this is known to be a heat-sensitive organ. Directly following FRH treatment, we observed a significant growth in *Dicer1* mRNA expression ($p < 0.01$), which declined after 24 h ($p < 0.05$; Figure 7A). We observed a further substantial increase in *Tarbp2* expression directly following FRH treatment ($p < 0.05$; Figure 7B).

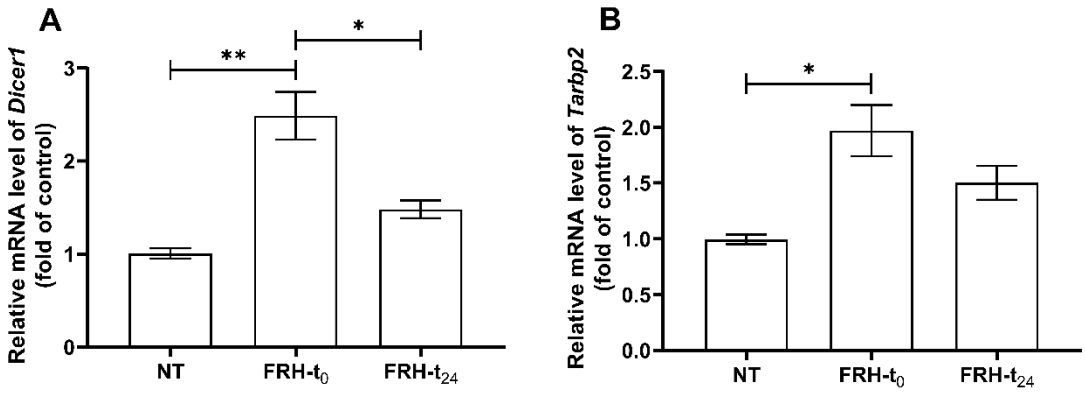

Figure 7. Effects of Fever Range Hyperthermia on the expression of Dicer1 (**A**) and Tarbp2 (**B**) in the liver of rats. mRNA expression was determined by RT-qPCR. Data are shown as the mean ± S.E.M. of three independent experiments. Asterisks indicate significant difference between groups (** $p < 0.01$,

* $p < 0.05$). NT: control animals (n = 6), FRH-t_0: samples collected directly following FRH treatment (n = 6), FRH-t_{24}: samples collected 24 h post-FRH treatment (n = 5). n indicates the sample size per group.

We next investigated *Dicer1* and *Tarbp2* expression in the spleen, given its key role in immune defences. Similarly, to both liver tissue and PBMCs, we observed an important increase in the expression of *Dicer1* immediately after FRH ($p < 0.05$), which returned to physiological levels after 24 h (Figure 8A). The levels of *Tarbp2* in the spleen also raised directly following FRH ($p < 0.05$; Figure 8B) and remained high after 24 h, though this effect was not statistically significant ($p = 0.16$).

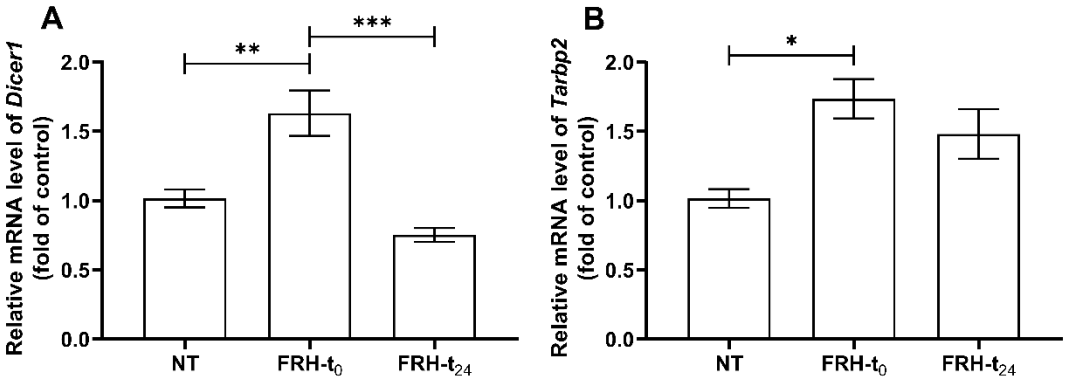

Figure 8. Effects of Fever Range Hyperthermia on the mRNA expression of Dicer1 (**A**) and Tarbp2 (**B**) in the spleen of rats. mRNA expression was determined by RT-qPCR. Data are shown as the mean ± S.E.M. of three independent experiments. Asterisks indicate significant difference between groups (*** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$). NT: control animals (n=6), FRH-t_0: samples collected directly following FRH treatment (n = 6), FRH-t_{24}: samples collected 24 h post-FRH treatment (n = 5). n indicates the sample size per group.

3.5 Whole body hyperthermia influences miRNA-155 expression in rats

To confirm whether the effects of FRH on the miRNA machinery leads to changes in miRNA expression, we analyzed miRNA-155 levels. This molecule is well known for promoting the pro-inflammatory response (23), thus we wanted to determine whether FRH may affect its expression. Interestingly, we found that FRH decreased the expression of miRNA-155 in rat plasma immediately following treatment ($p < 0.001$; Figure 9). This effect remained after 24 h ($p < 0.001$).

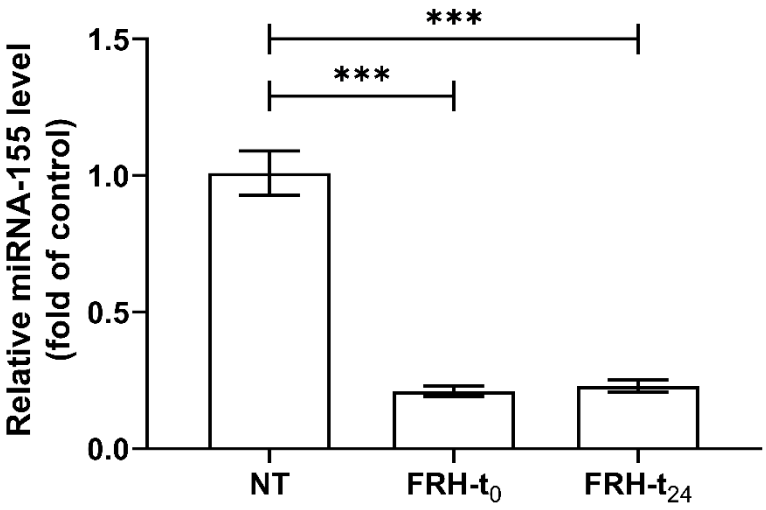

Figure 9. Effects of fever range hyperthermia on miRNA-155 expression in the plasma of rats. miRNA expression was determined by RT-qPCR. Data are shown as the mean ± S.E.M. Asterisks indicate significant differences between groups (*** $p < 0.001$). NT: control animals (n = 6), FRH-t_0: samples collected directly after FRH treatment (n = 6), FRH-t_{24}: samples collected 24 h post-FRH treatment (n = 5). n indicates the sample size per group.

4. Discussion

Fever is a part of the acute phase response that is manifested with an increase in body temperature and sickness symptoms such as lethargy or depression (1). Although fever is observed in many medical conditions, the role of the heat is often ignored and antipyretics are commonly used. Fever-range whole body hyperthermia (FRH) is a simplified model of fever and a well-established medical procedure that is used in medicine. While medical devices have been designed to apply whole body hyperthermia, its use has been stalled in modern medicine. This is due to the relatively smaller number of publications and clinical trials describing the benefits of whole body hyperthermia. The literature is dominated by observational studies, with a limited understanding of the molecular mechanism(s) induced by hyperthermia (24,25).

In humans, infrared radiation has been used to achieve reproducible ranges of fever. The duration of the heat-retention phase for FRH is mostly 60 minutes, however, it may be prolonged up to 90 minutes for treatments disorders such as ankylosing spondylitis, or in other fields of medicine such as rheumatology, dermatology, or psychiatry (26–28). In a particular case, such as supporting cancer treatment, even longer therapy is recommended (26).

In an effort to imitate infrared-triggered FRH, we developed a rapid method of FRH in rats. Since smaller animals are more sensitive to heat because of their higher surface-to-mass ratio and the presence of fur (29,30) we decided to maintain the rats' core body temperature

at 39°C for 60 minutes. Before this study, hyperthermia in animal models was usually achieved using convection models such as streams of heated air (31,32) water-baths (33–35), or a high ambient temperature in an incubator or chamber (36,37). Additionally, these models were used in research focused on application of FRH for cancer (34,35), or reumathic disorders treatment (37), showing therapeutic potential of FRH in these disorders.

There are a few differences between the types of hyperthermia models. In convection models of FRH skin and the surface of the body are heated first, and then, heat is transferred by blood to the whole body. This method of whole body hyperthermia induction requires more time than infrared-induced FRH due to the prolonged heat-retention phase. Unlike the convection model of FRH, infrared radiation penetrates the deep layers of skin and then heat is transferred to the core of the body through blood circulation. The skin heats up less therefore this method allows for limitation of the risk of skin burns (38,39). Importantly, in our model based on infrared radiation, we achieved fever range hyperthermia within minutes, while other methods need approximately twice a longer time (34,35).

The main limitation of various models of hyperthermia (including ours) is the necessity to anaesthetize rats, if stress prevention is needed. It is known that rats in response to various stressors (e.g. moving cages, noise, light) develop a stress-induced increase in body temperature (40). These kinds of stressors affect the secretion of neuroendocrine mediators, exposing immune cells to altered signals and interactions (40,41). On the other hand, it should be kept in mind that anaesthesia induces vasodilation that weakens thermoregulatory defense mechanisms (42,43). Therefore, before choosing an appropriate model of FRH-induction, a wide spectrum of potential risks should be considered, including the way of core temperature measurements.

Having refined a model replicating FRH in humans, we assessed changes in the number of leukocytes in the blood. Since other authors, who investigated different models of hyperthermia, observed changes in the immune system after 24 h including the number of white blood cells count and level of various cytokines (27,44), we wanted to check whether a similar effect is observed in our model. Indeed, we found that FRH provoked a temporary decrease in the total number of WBCs that returned to initial levels after 24 h. The observed changes in the number of WBCs were due to a decrease in the number of lymphocytes. In agreement with these data, a decreased number of lymphocytes directly following FRH treatment was observed in patients with advanced solid tumors (44). Since FRH is not cytotoxic for WBCs (5,45), hyperthermia likely enhances the diapedesis of lymphocytes and intensifies immune surveillance.

Unlike lymphocytes, we observed a significant increase in granulocyte number following FRH. This suggested that FRH may enhance granulocytopoesis. To verify this hypothesis, we examined the expression of G-CSF (encoded by the *Csf3* gene), a key growth factor that regulates granulocytopoesis, induces the proliferation of granulocytic precursors and promotes the release of mature cells from the bone marrow into the blood (46). The analysis of these genes revealed increased expression immediately after FRH in all examined organs. Of note, *Csf3* expression was most pronounced (100-fold increase *vs*. control group) in the liver. Since Piscaglia et al. (2007) highlighted the ability of G-CSF to induce hepatic

regeneration by increasing the migration of bone marrow progenitors to the liver (47) our results suggest that fever range whole body hyperthermia could be beneficial for the treatment of liver injuries by inducing G-CSF expression.

We further investigated the effects of FRH on the count of monocytes. Since G-CSF is a key regulator of granulocytopoesis and monocytopoiesis (48), we anticipated downstream effects of FRH on monocyte number. Surprisingly, we observed a modest but non-significant change in the number of monocytes in blood samples. We additionally evaluated the expression of *Mif*, and observed decreased levels in all examined organs after 24 h of FRH. Despite its obvious role in the regulation of the migration of macrophages, MIF regulates the expression of pro-inflammatory cytokines. Furthermore, it is a key regulator of the innate immune system, most notably the inflammatory response to microbial infection (9). These findings suggest that FRH acts as an immunomodulatory factor responsible for the maintenance of immune homeostasis. FRH has been shown to influence the expression of anti-inflammatory cytokines including IL-10 (12). Consistent with these studies, we observed that its expression in PBMCs and spleen significantly increased following FRH. Thus we found that FRH regulates both pro-inflammatory and anti-inflammatory factors.

Previous studies have highlighted an interplay between G-CSF and IL-10. Shaklee et al. (2004) found that G-CSF increases LPS-induced IL-10 expression in the spleen (49). Consistent with these studies, we observed a significant increase in spleen G-CSF mRNA levels directly after FRH treatment, followed by an increase in the expression of IL-10 after 24 h. Zauner et al. observed, that hyperthermia treatment induced exppression of anti-inflammatory IL-10 in patients with ankylosing spondylitis (AS) and the control group. However, this increase was earlier, higher and more sustained in AS patients (27). This increase is in line with our observations, thus FRH seems to be usefull for arthritis treatment. Borges et al. explained that increase in IL-10 level may result from HSP-70 treatment. They observed, that extracelluar HSP-70 decrease pro-inflammatory cytokines level due to increased IL-10 production in bone marrow-derived murine dendritic cells (50).

In our study, IFN-γ mRNA levels in rats also increased in PBMCs, but only modestly increased in the spleen. It is well known that IFN-γ is a molecule synthesized in response to viral attack to limit viral infection (10). In experiments designed to induce its production in lymphoid cells, the activity of mitogens or cytokines are needed (51). Until recently, the ability of hyperthermia to induce IFN-γ was restricted to simultaneous stimulation with other factors. Zhu et al. (2010) found that local hyperthermia induced temperature dependent increases in IFN-γ in HPV-infected tissues (2), while Downing et al. reported increased IFN-γ expression in PHA-stimulated lymphocytes isolated from patients treated with FRH at 39°C (52). Here, we observed that FRH alone could stimulate IFN-γ, which was in accordance with Mace et al. (2012), who showed that FRH induces temperature-dependent IFN-γ expression in murine CD8+ T cells *in vitro* (53).

Having observed the effect of FRH on cytokine production, we next investigated the potential role of miRNAs. miRNAs have emerged as key regulators of biological processes including cell development, differentiation and homeostasis (17,54). Importantly, various miRNA molecules regulate the expression of G-CSF (13), MIF (14), IFN-γ (15) and IL-10 (16). We

therefore investigated the influence of heat on the expression of the machinery required for miRNA processing. Mature miRNA duplexes are recruited for the RNA-induced silencing complex (RISC). The miRNA machinery includes e.g., DICER1 (RNase III family member) and transactivation response RNA Binding Protein (TARBP2 also known as TRBP)(55). In rats, we observed altered expression of *Dicer1* and *Tarbp2* in all organs examined. These data are consistent with *in vitro* studies highlighting the ability of heat to induce the expression of DICER1 (56,57). Increasing data suggest, that other molecules involved in miRNA machinery such as argonaute2 protein (Ago2), may be modulated by heat shock proteins (HSPs)(58,59). These results reveal that the regulation of miRNA machinery is even more complicated. Therefore further functional analysis are required. However, to the best of our knowledge, no study has considered heat-induced *Tarbp2* expression. Hence, our results provide new insight into the role of miRNA machinery in response to FRH.

We next investigated whether FRH can influence the expression of miRNA-155, a known regulator of both innate and adaptive immune responses (23). We found that FRH led to a significant decrease in miRNA-155 expression in rat plasma, further highlighting its ability to promote anti-inflammatory responses. This ability of FRH may be beneficial for a range of disease pathologies involving chronic inflammation, including rheumatoid arthritis. Indeed, the therapeutic benefits of FRH in controlling the progression of arthritis in clinically relevant mouse models are comparable to methotrexate. Heat treatment has been shown to increase IL-10 production in inflamed joints (37), though the molecular mechanisms of these effects have not been investigated. We observed an FRH-dependent decrease in MIF expression that regulates the expression of pro-inflammatory cytokines such as IL-6 and tumor necrosis factor (TNF)-α (60). Furthermore, elevated levels of miRNA-155 have been reported in the synovial tissue of rheumatoid arthritis patients, which preceeds with the release of pro-inflammatory cytokines by fibroblast-like synoviocytes (61). We observed that FRH induces a decrease in miRNA-155, downregulates MIF and upregulates IL-10, which may be beneficial for chronic disease. This requires further investigation, as *in vitro* findings by Li et al. suggest that the increased expression of miRNA-155 in heat-treated microglial cells increases the expression of pro-inflammatory molecules (62).

In summary, we show that FRH significantly influences WBC numbers and immune-related gene expression in an array of organs. Our data support FRH as an immunomodulator that influences key anti-inflammatory factors including IL-10, as well as pro-inflammatory molecules such as MIF and IFN-γ. Of note, upregulation of G-CSF, which is considered to display a dual role in inflammation (63), was also observed. Regarding changes in miRNA machinery expression and miRNA-155 itself, we hypothesize that all the changes described above may occur through the regulation of miRNAs.

5. Declarations

Ethics approval

The animal study protocol was approved by the local Bioethical Committee for Animal Care of Bydgoszcz University of Science and Technology at the Faculty of Animal Breeding and Biology (permission no. 49/2020, date of approval 24.01.2020).

Availability of data and materials

The authors confirm that the data supporting the findings of this study are available within the article. The datasets used and analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

Funding

This study was supported by Project no POWR.03.05.00-00-Z302/17 “Universitas Copernicana Thoruniensis in Futuro” (2018-2022) co-financed by the European Social Fund—the Operational Programme Knowledge Education Development. Module 5. Interdisciplinary PhD School “Academia Copernicana” and the Polish Minister of Science and Higher Education under the program “Regional Initiative of Excellence” in 2019–2023 (Grant No. 008/RID/2018/19).

Authors' contributions

Conceptualization, H.M.K. and S.W.; methodology, H.M.K., B.M., J.S. and T.J.; software, H.M.K. and B.M.; validation, T.J and J.S.; formal analysis, T.J. and J.S.; investigation, H.M.K. and B.M.; resources, H.M.K., S.W. and A.D.; data curation, H.M.K. and B.M.; writing—original draft prep-aration, H.M.K.; writing—review and editing, T.J. and S.W.; visualization, H.M.K. and J.S.; su-pervision, S.W. and A.D.; project administration, H.M.K. and S.W.; funding acquisition, S.W. and A.D.

6. References

1. Wrotek S, Legrand EK, Dzialuk A, Alcock J. Let fever do its job: The meaning of fever in the pandemic era. Evol Med Public Health [Internet]. 2021 Feb 26 [cited 2023 Jan 15];9(1):26–35. Available from: https://academic.oup.com/emph/article/9/1/26/5998648

2. Zhu L li, Gao XH, Qi R, Hong Y, Li X, Wang X, et al. Local hyperthermia could induce antiviral activity by endogenous interferon-dependent pathway in condyloma acuminata. Antiviral Res [Internet]. 2010 Nov [cited 2022 Jun 27];88(2):187–92. Available from: https://pubmed.ncbi.nlm.nih.gov/20797409/

3. Katschinski DM, Wiedemann GJ, Longo W, D'Oleire FR, Spriggs D, Robins HI. Whole body hyperthermia cytokine induction: A review, and unifying hypothesis for myeloprotection in the setting of cytotoxic therapy [Internet]. Vol. 10, Cytokine and Growth Factor Reviews. Cytokine Growth Factor Rev; 1999 [cited 2022 Jun 27]. p. 93–7. Available from: https://pubmed.ncbi.nlm.nih.gov/10743501/

4. Yoshihisa Y, Rehman MU, Kondo T, Shimizu T. Role of macrophage migration inhibitory factor in heat-induced apoptosis in keratinocytes. FASEB Journal [Internet]. 2016 Nov 1 [cited 2022 Jun 27];30(11):3870–7. Available from: https://pubmed.ncbi.nlm.nih.gov/27528627/

5. Kozłowski HM, Pawlikowska M, Sobocińska J, Jędrzejewski T, Dzialuk A, Wrotek S. Distinct modulatory effects of fever-range hyperthermia on the response of breast cancer cells and macrophages to mistletoe (Viscum album l.) extract. Pharmaceuticals. 2021 Jun 8;14(6):551.

6. Thorne AM, Ubbink R, Bruggenwirth IMA, Nijsten MW, Porte RJ, de Meijer VE. Hyperthermia-induced changes in liver physiology and metabolism: A rationale for hyperthermic machine perfusion [Internet]. Vol. 319, American Journal of Physiology - Gastrointestinal and Liver Physiology. American Physiological Society; 2020 [cited 2022 Jun 27]. p. G43–50. Available from: https://journals.physiology.org/doi/10.1152/ajpgi.00101.2020

7. Wang K, Zhang S, Marzolf B, Troisch P, Brightman A, Hu Z, et al. Circulating microRNAs, potential biomarkers for drug-induced liver injury. Proc Natl Acad Sci U S A [Internet]. 2009 Mar 3 [cited 2022 Jun 27];106(11):4402–7. Available from: /pmc/articles/PMC2657429/

8. Lewis SM, Williams A, Eisenbarth SC. Structure-function of the immune system in the spleen. Sci Immunol [Internet]. 2019 Mar 3 [cited 2023 Jan 15];4(33). Available from: /pmc/articles/PMC6495537/

9. Calandra T, Roger T. Macrophage migration inhibitory factor: A regulator of innate immunity. Nat Rev Immunol [Internet]. 2003 [cited 2022 Jun 27];3(10):791–800. Available from: https://www.nature.com/articles/nri1200

10. Kang S, Brown HM, Hwang S. Direct antiviral mechanisms of interferon-gamma. Immune Netw [Internet]. 2018 Oct 1 [cited 2022 Jul 8];18(5). Available from: /pmc/articles/PMC6215902/

11. Hu X, Chakravarty SD, Ivashkiv LB. Regulation of IFN and TLR Signaling During Macrophage Activation by Opposing Feedforward and Feedback Inhibition Mechanisms. Immunol Rev [Internet]. 2008 Dec [cited 2023 Jan 15];226(1):41. Available from: /pmc/articles/PMC2630590/

12. Iyer SS, Cheng G. Role of interleukin 10 transcriptional regulation in inflammation and autoimmune disease. Crit Rev Immunol [Internet]. 2012 [cited 2022 Jun 28];32(1):23–63. Available from: /pmc/articles/PMC3410706/

13. Worm J, Stenvang J, Petri A, Frederiksen KS, Obad S, Elmén J, et al. Silencing of microRNA-155 in mice during acute inflammatory response leads to derepression of c/ebp Beta and down-regulation of G-CSF. Nucleic Acids Res [Internet]. 2009 Sep 1 [cited 2022 Jul 8];37(17):5784–92. Available from: https://academic.oup.com/nar/article/37/17/5784/1079248

14. Tu Y, Guo R, Li J, Wang S, Leng L, Deng J, et al. MiRNA Regulation of MIF in SLE and Attenuation of Murine Lupus Nephritis With miR-654. Front Immunol. 2019 Sep 19;10:2229.

15. Ma F, Xu S, Liu X, Zhang Q, Xu X, Liu M, et al. The microRNA miR-29 controls innate and adaptive immune responses to intracellular bacterial infection by targeting interferon-γ. Nat Immunol [Internet]. 2011 Jul 24 [cited 2022 Jul 8];12(9):861–9. Available from: https://www.nature.com/articles/ni.2073

16. Castro-Leyva V, Arenas-Huertero F, Espejel-Núñez A, Giono Cerezo S, Flores-Pliego A, Espino y Sosa S, et al. miR-21 differentially regulates IL-1β and IL-10 expression in human decidual cells infected with streptococcus B. Reprod Biol. 2022 Mar 1;22(1):100604.

17. Martinez MA, Tural C, Franco S. Circulating MicroRNAs as a Tool for Diagnosis of Liver Disease Progression in People Living with HIV-1. Viruses [Internet]. 2022 May 24 [cited 2022 Jun 27];14(6):1118. Available from: https://www.mdpi.com/1999-4915/14/6/1118/htm

18. Huang JT, Wang J, Srivastava V, Sen S, Liu SM. MicroRNA machinery genes as novel biomarkers for cancer. Vol. 4 MAY, Frontiers in Oncology. Frontiers Research Foundation; 2014. p. 113.

19. Benoit MPMH, Imbert L, Palencia A, Pérard J, Ebel C, Boisbouvier J, et al. The RNA-binding region of human TRBP interacts with microRNA precursors through two independent domains. Nucleic Acids Res [Internet]. 2013 Apr [cited 2022 Jun 27];41(7):4241–52. Available from: https://pubmed.ncbi.nlm.nih.gov/23435228/

20. Chen M, Wang F, Xia H, Yao S. MicroRNA-155: Regulation of Immune Cells in Sepsis [Internet]. Vol. 2021, Mediators of Inflammation. Hindawi Limited; 2021 [cited 2022 Jun 27]. Available from: /pmc/articles/PMC7810547/

21. Chomczynski P, Sacchi N. The single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction: Twenty-something years on. Nat Protoc [Internet]. 2006 Jun 27 [cited 2022 Jun 27];1(2):581–5. Available from: https://www.nature.com/articles/nprot.2006.83

22. Peltier HJ, Latham GJ. Normalization of microRNA expression levels in quantitative RT-PCR assays: Identification of suitable reference RNA targets in normal and cancerous human solid tissues. RNA [Internet]. 2008 May [cited 2022 Jun 27];14(5):844–52. Available from: /pmc/articles/PMC2327352/

23. Wang W, Liu Z, Su J, Chen WS, Wang XW, Bai SX, et al. Macrophage micro-RNA-155 promotes lipopolysaccharide-induced acute lung injury in mice and rats. Am J Physiol Lung Cell Mol Physiol [Internet]. 2016 Aug 1 [cited 2022 Jul 4];311(2):494–506. Available from: https://journals.physiology.org/doi/10.1152/ajplung.00001.2016

24. Hanusch KU, Janssen CH, Billheimer D, Jenkins I, Spurgeon E, Lowry CA, et al. Whole-body hyperthermia for the treatment of major depression: Associations with thermoregulatory cooling [Internet]. Vol. 170, American Journal of Psychiatry. American Psychiatric Association; 2013 [cited 2022 Jul 7]. p. 802–4. Available from: https://ajp.psychiatryonline.org/doi/10.1176/appi.ajp.2013.12111395

25. Romeyke T, Stummer H. Multi-modal pain therapy of fibromyalgia syndrome with integration of systemic whole-body hyperthermia-effects on pain intensity and mental state: A non-randomised controlled study. J Musculoskelet Pain [Internet]. 2014 Dec 1 [cited 2022 Jul 7];22(4):341–55. Available from: https://www.tandfonline.com/doi/abs/10.3109/10582452.2014.949336

26. Heckel-Reusser S. Whole-Body Hyperthermia (WBH): Historical Aspects, Current Use, and Future Perspectives [Internet]. Water-filtered Infrared A (wIRA) Irradiation. Springer, Cham; 2022 [cited 2023 Mar 27]. 143–154 p. Available from: https://link.springer.com/chapter/10.1007/978-3-030-92880-3_11

27. Zauner D, Quehenberger F, Hermann J, Dejaco C, Stradner MH, Stojakovic T, et al. Whole body hyperthermia treatment increases interleukin 10 and toll-like receptor 4 expression in patients with ankylosing spondylitis: a pilot study. Int J Hyperthermia [Internet]. 2014 Sep 1 [cited 2023 Mar 26];30(6):393–401. Available from: https://pubmed.ncbi.nlm.nih.gov/25256892/

28. Tarner IH, Müller-Ladner U, Uhlemann C, Lange U. The effect of mild whole-body hyperthermia on systemic levels of TNF-alpha, IL-1beta, and IL-6 in patients with ankylosing spondylitis. Clin Rheumatol [Internet]. 2009 Dec 17 [cited 2023 Mar 26];28(4):397–402. Available from: https://link.springer.com/article/10.1007/s10067-008-1059-x

29. Wanner SP, Prímola-Gomes TN, Pires W, Guimarães JB, Hudson ASR, Kunstetter AC, et al. Thermoregulatory responses in exercising rats: methodological aspects and relevance to human physiology. Temperature: Multidisciplinary Biomedical Journal. 2015 Oct 2;2(4):457.

30. Havenith G. Human surface to mass ratio and body core temperature in exercise heat stress—a concept revisited. J Therm Biol. 2001 Sep 1;26(4–5):387–93.

31. Hill RW, Welsch CW. Suppression of growth of 7,12-dimethylbenzanthracene-induced mammary carcinomas in female rats by repeated whole-body hyperthermia (41-42°C). Int J Radiat Oncol Biol Phys. 1988 Feb 1;14(2):337–45.

32. Ito M, Takahashi H, Yano H, Shimizu YI, Yano Y, Ishizaki Y, et al. High mobility group box 1 enhances hyperthermia-induced seizures and secondary epilepsy associated with prolonged hyperthermia-induced seizures in developing rats. Metab Brain Dis. 2017 Dec 1;32(6):2095–104.

33. Bruguerolle B, Roucoules X, Attolini L, Lorec AM. Bupivacaine kinetics during hyperthermia in rats. Canadian Journal of Anaesthesia. 1994 Mar;41(3):253–6.

34. Rowe RW, Strebel FR, Proett JM, Deng W, Chan D, He G, et al. Fever-range whole body thermotherapy combined with oxaliplatin: A curative regimen in a pre-clinical breast cancer model. International Journal of Hyperthermia [Internet]. 2010 Sep [cited 2023 Mar 27];26(6):565–76. Available from: https://www.tandfonline.com/doi/abs/10.3109/02656736.2010.483635

35. Sumiyoshi K, Strebel FR, Rowe RW, Bull JMC. The effect of whole-body hyperthermia combined with “metronomic” chemotherapy on rat mammary adenocarcinoma metastases. International Journal of Hyperthermia [Internet]. 2009 Mar [cited 2023 Mar 27];19(2):103–18. Available from: https://www.tandfonline.com/doi/abs/10.1080/0265673021000017091

36. Capitano ML, Nemeth MJ, Mace TA, Salisbury-Ruf C, Segal BH, McCarthy PL, et al. Elevating body temperature enhances hematopoiesis and neutrophil recovery after total body irradiation in an IL-1-, IL-17-, and G-CSF-dependent manner. Blood. 2012;120(13):2600–9.

37. Lee CT, Kokolus KM, Leigh ND, Capitano M, Hylander BL, Repasky EA. Defining immunological impact and therapeutic benefit of mild heating in a murine model of arthritis. PLoS One [Internet]. 2015 Mar 20 [cited 2022 Jun 27];10(3):e0120327. Available from: https://journals.plos.org/plosone/article?id=10.1371/journal.pone.0120327

38. Wehner H, Von Ardenne A, Kaltofen S. Whole-body hyperthermia with water-filtered infrared radiation: technical-physical aspects and clinical experiences. Int J Hyperthermia [Internet]. 2001 [cited 2023 Mar 22];17(1):19–30. Available from: https://pubmed.ncbi.nlm.nih.gov/11212877/

39. Kok HP, Cressman ENK, Ceelen W, Brace CL, Ivkov R, Grüll H, et al. Heating technology for malignant tumors: a review. Int J Hyperthermia [Internet]. 2020 Jan 1 [cited 2023 Mar 27];37(1):711. Available from: /pmc/articles/PMC7781160/

40. Dallmann R, Steinlechner S, Von Hörsten S, Karl T. Stress-induced hyperthermia in the rat: comparison of classical and novel recording methods. Lab Anim [Internet]. 2006 Apr [cited 2023 Mar 22];40(2):186–93. Available from: https://pubmed.ncbi.nlm.nih.gov/16600078/

41. Scarola SJ, Trejo JRP, Granger ME, Gerecke KM, Bardi M. Immunomodulatory Effects of Stress and Environmental Enrichment in Long–Evans Rats (Rattus norvegicus). Comp Med [Internet]. 2019 Feb 1 [cited 2023 Mar 22];69(1):35. Available from: /pmc/articles/PMC6382044/

42. Lenhardt R. The effect of anesthesia on body temperature control. Front Biosci (Schol Ed) [Internet]. 2010 Jun 1 [cited 2023 Mar 27];2(3):1145–54. Available from: https://pubmed.ncbi.nlm.nih.gov/20515846/

43. Díaz M, Becker DE. Thermoregulation: Physiological and Clinical Considerations during Sedation and General Anesthesia. Anesth Prog [Internet]. 2010 Mar [cited 2023 Mar 27];57(1):25. Available from: /pmc/articles/PMC2844235/

44. Kraybill WG, Olenki T, Evans SS, Ostberg JR, O’Leary KA, Gibbs JF, et al. A phase I study of fever-range whole body hyperthermia (FR-WBH) in patients with advanced solid tumours: Correlation with mouse models. International Journal of Hyperthermia [Internet]. 2002 [cited 2022 Jun 30];18(3):253–66. Available from: https://www.tandfonline.com/action/journalInformation?journalCode=ihyt20

45. Huang YH, Haegerstrand A, Frostegård J. Effects of in vitro hyperthermia on proliferative responses and lymphocyte activity. Clin Exp Immunol. 1996;103(1):61–6.

46. Richards MK, Liu F, Iwasaki H, Akashi K, Link DC. Pivotal role of granulocyte colony-stimulating factor in the development of progenitors in the common myeloid pathway. Blood. 2003 Nov 15;102(10):3562–8.

47. Piscaglia AC, Shupe TD, Oh SH, Gasbarrini A, Petersen BE. Granulocyte-Colony Stimulating Factor Promotes Liver Repair and Induces Oval Cell Migration and Proliferation in Rats. Gastroenterology [Internet]. 2007 [cited 2022 Jun 27];133(2):619–31. Available from: /pmc/articles/PMC3130597/

48. Zhan Y, Lieschke GJ, Grail D, Dunn AR, Cheers C. Essential Roles for Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF) and G-CSF in the Sustained Hematopoietic Response ofListeria monocytogenes–Infected Mice. Blood. 1998 Feb 1;91(3):863–9.

49. Shaklee CL, Guo J, Faggioni R, Fantuzzi G, Senaldi G. Pretreatment with granulocyte-colony stimulating factor decreases lipopolysaccharide-induced interferon-γ production in mice in association with the production of interleukin-18. Cytokine. 2004 Feb 7;25(3):119–26.

50. Borges TJ, Lopes RL, Pinho NG, Machado FD, Souza APD, Bonorino C. Extracellular Hsp70 inhibits pro-inflammatory cytokine production by IL-10 driven down-regulation of C/EBPβ and C/EBPδ. International Journal of Hyperthermia [Internet]. 2013 [cited 2023 Apr 1];29(5):455–63. Available from: https://www.tandfonline.com/action/journalInformation?journalCode=ihyt20http://informahealthcare.com/hth

51. Chan SH, Perussia B, Gupta JW, Kobayashi M, Pospisil M, Young HA, et al. Induction of interferon γ production by natural killer cell stimulatory factor: Characterization of the responder cells and synergy with other inducers. Journal of Experimental Medicine. 1991;173(4):869–79.

52. Downing JF, Martinez-Valdez H, Taylor MW, Elizondo RS, Walker EB, Martinez-Valdez H. Hyperthermia in Humans Enhances Interferon-γ Synthesis and Alters the Peripheral Lymphocyte Population. J Interferon Res. 1988;8(2):143–50.

53. Mace TA, Zhong L, Kokolus KM, Repasky EA. Effector CD8 +T cell IFN-γ production and cytotoxicity are enhanced by mild hyperthermia. International Journal of Hyperthermia [Internet]. 2012 [cited 2022 Jun 30];28(1):9–18. Available from: https://www.tandfonline.com/action/journalInformation?journalCode=ihyt20

54. Gebert LFR, MacRae IJ. Regulation of microRNA function in animals [Internet]. Vol. 20, Nature Reviews Molecular Cell Biology. NIH Public Access; 2019 [cited 2022 Jul 8]. p. 21–37. Available from: /pmc/articles/PMC6546304/

55. Redfern AD, Colley SM, Beveridge DJ, Ikeda N, Epis MR, Li X, et al. RNA-induced silencing complex (RISC) Proteins PACT, TRBP, and Dicer are SRA binding nuclear receptor coregulators. Proc Natl Acad Sci U S A [Internet]. 2013 Apr 16 [cited 2022 Jun 30];110(16):6536–41. Available from: /pmc/articles/PMC3631673/

56. Devasthanam AS, Tomasi TB. Dicer protein levels elevated by mild hyperthermia promote a pro-survival phenotype. Oncotarget. 2017;8(40):67001–16.

57. Oshlag JZ, Devasthanam AS, Tomasi TB. Mild hyperthermia enhances the expression and induces oscillations in the Dicer protein. International Journal of Hyperthermia [Internet]. 2013 [cited 2022 Jun 30];29(1):51–61. Available from: /pmc/articles/PMC4745122/

58. Iwasaki S, Kobayashi M, Yoda M, Sakaguchi Y, Katsuma S, Suzuki T, et al. Hsc70/Hsp90 chaperone machinery mediates ATP-dependent RISC loading of small RNA duplexes. Mol Cell

[Internet]. 2010 Jul [cited 2022 Dec 12];39(2):292–9. Available from: https://pubmed.ncbi.nlm.nih.gov/20605501/

59. Liu X, Yang YY, Wang Y. HSP90 and Aha1 modulate microRNA maturation through promoting the folding of Dicer1. Nucleic Acids Res [Internet]. 2022 Jul 8 [cited 2022 Dec 12];50(12):6990–7001. Available from: https://academic.oup.com/nar/article/50/12/6990/6613913

60. Bilsborrow JB, Doherty E, Tilstam P v., Bucala R. Macrophage migration inhibitory factor (MIF) as a therapeutic target for rheumatoid arthritis and systemic lupus erythematosus [Internet]. Vol. 23, Expert Opinion on Therapeutic Targets. NIH Public Access; 2019 [cited 2022 Jun 29]. p. 733–44. Available from: /pmc/articles/PMC6800059/

61. Wang Y, Feng T, Duan S, Shi Y, Li S, Zhang X, et al. miR-155 promotes fibroblast-like synoviocyte proliferation and inflammatory cytokine secretion in rheumatoid arthritis by targeting FOXO3a. Exp Ther Med [Internet]. 2019 Feb 1 [cited 2022 Jul 4];19(2):1288–96. Available from: http://www.spandidos-publications.com/10.3892/etm.2019.8330/abstract

62. Li P, Wang G, Zhang XL, He GL, Luo X, Yang J, et al. MicroRNA-155 promotes heat stress-induced inflammation via targeting liver X receptor α in microglia. Front Cell Neurosci. 2019 Jan 29;13:12.

63. Bhattacharya P, Budnick I, Singh M, Thiruppathi M, Alharshawi K, Elshabrawy H, et al. Dual Role of GM-CSF as a Pro-Inflammatory and a Regulatory Cytokine: Implications for Immune Therapy [Internet]. Vol. 35, Journal of Interferon and Cytokine Research. Mary Ann Liebert, Inc.; 2015 [cited 2022 Jul 13]. p. 585–99. Available from: /pmc/articles/PMC4529096/

3.2. Article 2

Kozłowski, H.M.; Sobocińska, J.; Jędrzejewski T.; Maciejewski B.; Dzialuk A.; Wrotek S. *Fever-range hyperthermia can effectively switch macrophage polarization towards regulatory phenotype* Sent to the Journal (May 2023).

Title: *Fever-range hyperthermia can effectively switch macrophage polarization towards regulatory phenotype*

Henryk M. Kozłowski [1,2], Justyna Sobocińska [2], Tomasz Jędrzejewski [2], Bartosz Maciejewski [2], Artur Dzialuk[1], Sylwia Wrotek [2]

[1] Department of Genetics, Faculty of Biological Sciences, Kazimierz Wielki University, 10 Powstańców Wielkopolskich Ave., 85-090 Bydgoszcz, Poland

[2] Department of Immunology, Faculty of Veterinary and Biological Sciences, Nicolaus Copernicus University, 1 Lwowska Str., 87-100 Torun, Poland

Abstract:

Background: Fever-range hyperthermia (FRH) is a therapeutic intervention used in the treatment of some chronic diseases and in research it is a model to investigate a thermal compound of fever. Among cells that display an increased susceptibility to heat, macrophages arouse the most interest. It is known that their diverse functions depend on their polarization state, but it is not well recognized whether this process can be modulated by FRH.

Results: To address this, we used two different macrophage cell lines that were treated with FRH. Next, to define macrophage phenotype we examined their functional surface markers: CD80 and CD163, and intracellular markers such as inducible nitric oxide synthase (iNOS), arginase-1 (Arg-1). Additionally, in FRH-treated cells we analyzed an expression of Toll-like receptor 4 (TLR-4) and its role in macrophage polarization. We also checked whether FRH can switch the polarization of macrophages in pro-inflammatory condition triggered by lipopolysaccharide (LPS). Our findings demonstrate that FRH induces M2 polarization, as evidenced by significant increase in expression of CD163 and Arg-1. However, the increased expression of cyclooxygenase 2 (COX-2) and TLR-4 shows that these cells may still display pro-inflammatory properties, what suggest polarization towards M2b phenotype. Interestingly, FRH is able to shift lipopolysaccharide (LPS)-induced M1 polarization towards M2 phenotype and reduces the levels of anti-microbial molecules such as ROS and NO.

Conclusion: In summary, our results suggest that FRH is a strong modulator of macrophage polarization that favors M2 phenotype even in pro-inflammatory condition.

Keywords: Fever-range hyperthermia; macrophage polarization; fever; macrophages; TLR-4

1. Introduction

Fever range hyperthermia (FRH) is a condition used in research to investigate a thermal component of fever (1). FRH is also a medical procedure that increases the core body temperature to mimic fever. Despite reported benefits in a treatment of chronic diseases such as rheumatic diseases or cardiovascular disorders (2), the molecular changes induced by FRH remain poorly characterized. In our previous studies on mistletoe extract we observed that macrophages are heat-sensitive cells that respond to heat with increased expression of pro-inflammatory cytokines such as interleukin (IL)-1β and IL-6 (3). This observation is in accordance to Pritchard et al. 2005 (4).

It is well established that macrophages play a decisive role in the detection, recognition, and neutralization of pathogens. They are involved in antigen presentation and initiation of immune response by releasing cytokines and chemokines that activate other immune cells (5). Many antigens are sensed by macrophages by Toll-like receptors (TLRs) leading to release of pro-inflammatory mediators such as cytokines or reactive oxygen species (ROS) and nitrogen oxide (NO)(6). Among ten known human TLRs, TLR-4 is of great biological significance due to its role in initiation of immune response triggered not only by lipopolysaccharide (LPS) but also by damage-associated molecular patterns (DAMPs)(6,7). Importantly, heat-shock proteins (HSPs) such as HSP-70, which are released among others in hyperthermic conditions, have been also identified as molecules that interact with TLR-4 (8). Thus, it seems plausible that TLR-4 may be involved in heat-induced effects.

In response to various stimuli, macrophages can undergo polarization, which is a process that involves changes in their gene expression, morphology, and function (5). In general, the heterogeneity of macrophages includes two main populations: classically activated M1 cells and alternatively activated M2 cells (9). Classically activated M1 macrophages are commonly known as a pro-inflammatory cell, which arise due to the exposure to factors such as TLR-4 ligand (LPS) or in the presence of Th1 cytokines such as interferon gamma (IFN-γ) and granulocyte-macrophage colony-stimulating factor (GM-CSF)(9,10). In a response to these stimuli, M1 cells release high level of pro-inflammatory molecules such as tumor necrosis factor alpha (TNF-α), IL-6, IL-1β, IL-12 (10). Furthermore, the intracellular markers such as ROS and NO or inducible nitric oxide synthase (iNOS) are also elevated in M1 cells (5), and therefore are also considered as a useful M1 markers. Besides these intracellular markers and

production of pro-inflammatory factors, M1 cells express on their surface set of various proteins including CD80 and CD86 (10).

In contrast to classically activated macrophages, alternatively activated M2 cells reveal anti-inflammatory properties (11). In general, M2 subpopulation develops in the presence of transforming growth factor-beta (TGF-β), IL-10, IL-4 and/or IL-13, and releases anti-inflammatory molecules such as IL-4, IL-10 and IL-13 (9). M2-like cells are characterized by increased expression of intracellular markers such as arginase-1 (Arg-1), and overexpression of surface molecules including such as CD163, and CD206 (5,12). Studies have shown that M2 macrophages can be further subdivided into four subsets such as M2a induced by IL-4 and IL-13, M2b induced by immunocomplexes and ligands of TLRs, M2c induced by IL-10 and glucocorticoids, and M2d induced by IL-6 (12,13). To date, the role of FRH and interaction between HSPs and TLR-4 in modulation of macrophage response remain poorly understood (14). Furthermore, which phenotype of macrophages is induced by FRH, and whether TLR-4 is involved in this process has not been recognized so far. Since changes in macrophage polarization modify functional activity of these cells, the aim of this study was to identify which phenotype is induced in response to FRH.

We investigated two cell lines of murine macrophages treated with FRH, in which the expression of functional surface markers as well as intracellular markers was measured. Our results showed that although FRH-treated macrophages display M2 cell markers, their express proteins involved in pro-inflammatory response such as cyclooxygenase 2 (COX-2) and TLR-4. Thus, our data indicates M2b-like phenotype of heat-treated macrophages.

2. Materials and Methods

2.1 Cell culture

The murine macrophages RAW264.7 cell line were obtained from the European Collection of Cell Cultures (cat. No. 91062702; Salisbury, UK), and the murine macrophages J774A.1 cell line was a gift from the prof. Katarzyna Kwiatkowska PhD, DSc form the Nencki Institute of Experimental Biology of the Polish Academy of Sciences. The RAW264.7 and J774A.1 cells were cultured in high or low-glucose DMEM culture medium, respectively (Biowest; Nuaillé, France). Both culture media were supplemented with 10% heat-inactivated fetal bovine serum (FBS)(Sigma Aldrich, Darmstadt, Germany) and a mixture of antibiotics (100 µg/mL streptomycin and 100 IU/ml penicillin) (Sigma Aldrich). Cells were maintained under

controlled conditions at 37°C and in a humidified atmosphere with 5% of CO_2. Depending on cell confluence, the culture media were changed every 2 or 3 days. In the order to collect the cells, they were rinsed with fresh media, and cell scrapers were used to remove adherent cells from the culture flasks.

2.2 Measurement of intracellular reactive oxygen species (ROS)

The effect of co-treatment with heat and LPS derived from *Escherichia coli* 0111:B4 (Sigma-Aldrich) on ROS generation in TLR-4 inhibited and non-inhibited RAW264.7 and J774A.1 cells were measured by H_2DCF_DA (Sigma-Aldrich) staining followed by flow cytometry analysis. Cells were seeded in 6-well plates at the density of 1 x 10^6 per well. Following overnight pre-incubation, one-hour lasting pretreatment with TAK-242 (TLR-4 inhibitor purchased from Cayman Chemical, Ann Arbor, MI, USA) at a concentration of 0.1µM at 37°C was carried out. Next, the LPS at a concentration of 100 ng/mL was added, and cells were incubated for 24h at 37°C or 39°C. Then, cells were harvested, washed twice with PBS, and cultured in a serum-free transparent DMEM medium containing 10 µM H_2DCFH_DA for 30 min at 37 °C in the dark. After incubation with fluorescent dye, cells were washed twice with PBS and DCF fluorescence distribution was detected by flow cytometry using BriCyte E6 (Mindray, Shenzhen, China) at an excitation wavelength of 488 nm and an emission wavelength of 525 nm. Results are presented as a ratio of a geometric mean of treated/control cells.

2.3 Nitric Oxide determination

Nitric oxide evaluation was carried out with the Griess reagent (modified) (Sigma-Aldrich) according to manufacturer instruction. The detection solution was prepared with ultrapure distilled water, and the analysis was conducted in the presence of standard curve in a range of 0.5-65 µM of NO_2^-. RAW264.7 and J774A.1 cells were seeded in the DMEM media without phenol red (Biowest) at the density of 5 × 10^5/well in 24-well plate. Following one-hour lasting pretreatment with TAK-242 at a concentration of 0.1 µM at 37°C, cells were simultaneously treated with 100ng/mL LPS and exposed to 37°C or 39°C for 24h. After treatment, the supernatants were collected, centrifuged and mixed in equal volumes with Griess reagent. The absorbance was read at 540 nm after 15 minutes using a Synergy HT Multi-Mode microplate reader (BioTek Instruments, Winooski, VT, USA).

2.4 Western Blot analysis

To evaluate TLR-4, COX-2, iNOS, Arg-1 expression, western blot analysis were conducted. RAW264.7 and J774A.1 cells were seeded in 24-well plates at the density of 5 x 10^5 cells/well. Following overnight pre-incubation, and one hour-lasting pretreatment with TAK-242 at a concentration of 0.1 µM at 37°C, 100 ng/mL of LPS was added and cells were incubated for 2h or 24h at 37°C or 39°C. After incubation, the cells were rinsed with ice-cold PBS and lysed in 100 µL of a 1 x RIPA buffer supplemented with 1% SDS and 0.5% protease inhibitor cocktail (all reagent were purchased from Sigma Aldrich). To evaluate the protein concentration in the lysates, the Pierce™ BCA Protein Assay Kit (Thermo Fischer Scientific, Waltham, MA, USA) was used, according to the manufacturer's instruction. After dilution of lysates with sample buffer, SDS-PAGE electrophoresis was done using 4-20% precast polyacrylamide gels (Bio-Rad, Hercules, CA, USA). Following the transfer onto nitrocellulose, the membranes were immunoblotted with appropriate primary antibodies followed by incubation with secondary antibodies conjugated with horseradish peroxidase (HRP). All antibodies used in these studies are described in Table 1. Immunoreactive bands were visualized by chemiluminescence using SuperSignal West Pico substrate (Thermo Fisher Scientific). The densitometrical analysis was conducted using the ImageJ program (National Institute of Mental Health, Bethesda, MD, USA).

Table 1 *Primary and secondary antibodies used for Western Blot analysis.*

Primary Antibodies				
Protein Name	**Protein symbol**	**Cat. No.**	**Source/Isotype**	**Company**
Cyclooxygenase 2	COX-2	#12282	Rabbit IgG	Cell Signalling Technology (Danvers, MA, US)
Inducible Nitric Oxide Synthase	iNOS	#2982	Rabbit IgG	Cell Signalling Technology
Toll-like Receptor 4	TLR-4	#14358	Rabbit IgG	Cell Signalling Technology
Arginase-1	Arg-1	#93668	Rabbit IgG	Cell Signalling Technology
Actin	Actb	612657	Mouse IgG	BD Bioscience (Franklin Lakes, NJ, US)
Secondary antibodies				
Target	**Origin**		**Type of conjugate**	**Company**
Anti-Rabbit	Goat IgG		Peroxidase-conjugated Anti-Rabbit	Sigma Aldrich

Anti-Mouse	Goat IgG	Peroxidase-conjugated Anti-Mouse	Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA, USA)

2.5 Analysis of macrophages polarization by flow cytometry

Murine macrophage cell lines RAW264.7 and J774A.1 were seeded in 12-well plates at a density of 1 x 10^6 cells/well. The surface expression of CD86 as a marker of M1-phenotype polarization, and CD163 as a marker of M2-phenotype polarization were analyzed. Following one hour-lasting pretreatment with 0.1 µM TAK-242 at 37°C, the cells were simultaneously exposed to LPS (100 ng/mL) and FRH for 24h. Then, macrophage polarization was investigated by flow cytometry analysis. Cells were collected into separated tubes and washed twice with washing buffer containing ice-cold PBS, 1% bovine serum albumin and 0.02% sodium azide (all reagents were purchased from Sigma-Aldrich). The blocking of Fc receptors was carried out with Mouse Seroblock FcR (Bio-Rad), for 10 minutes in dark at 4°C, according to the manufacturer's instruction. Then, the cells were incubated with fluorescently labeled antibodies (FITC anti-mouse CD80, APC anti-mouse CD163, both purchased from Sony Biotechnology Inc., San Jose, CA, USA) on ice for additional 30 min. Next, cells were washed and analyzed by BriCyte E6 flow cytometer. The data were evaluated in FlowJo software (Becton, Dickinson & Company, Franklin Lakes, NJ, USA) and presented as the % of marker positive cells and the ratio of $CD86^+/CD163^+$ of positive events in the population of 2 x 10^4 events.

2.6 Statistical analysis

All values are reported as mean ± standard error of the mean (SEM) of three independent experiments. Statistical significance was determined using analysis of variance (two-way ANOVA) followed by the Tukey test at a critical value of $p < 0.05$.

3. Results

3.1 Fever-Range Hyperthermia induces expression of M2-like surface markers

To investigate the ability of FRH to induce macrophage polarization we analyzed of two surface markers: CD80 and CD163. It is known, that seeding density significantly affect expression of surface markers and the releasing various cytokines in numerous macrophage cells due to regulation of intracellular signals that impact the inflammatory response (15,16). Therefore, in our experiments all cells were cultured at the same density, to limit this effect to spontaneous polarization of macrophages. We observed an increased expression of surface CD80 in comparison to CD163 marker in control RAW264.7 cells cultured at 37°C (Figure 1A)($p < 0.001$). In contrast, FRH treatment upregulated expression of CD163 compared to CD80 ($p < 0.001$). The expression levels of surface molecules in J774A.1 control cells (cultured at 37°C) were significantly lower than RAW264.7 macrophages, however, elevated expression of CD163 in comparison to CD80 was observed (Figure 1C)($p < 0.001$). This effect was potentiated after raising the temperature to the range of 39°C ($p < 0.001$). Thus, in both examined cell lines we observed a substantial decrease in a ratio of M1/M2 surface markers (Figure 1B and 1D; $p < 0.01$ for RAW264.7 cells, and $p < 0.05$ for J774.A cells, respectively). These results indicate the potential of FRH to induce macrophages polarization into M2-like cells.

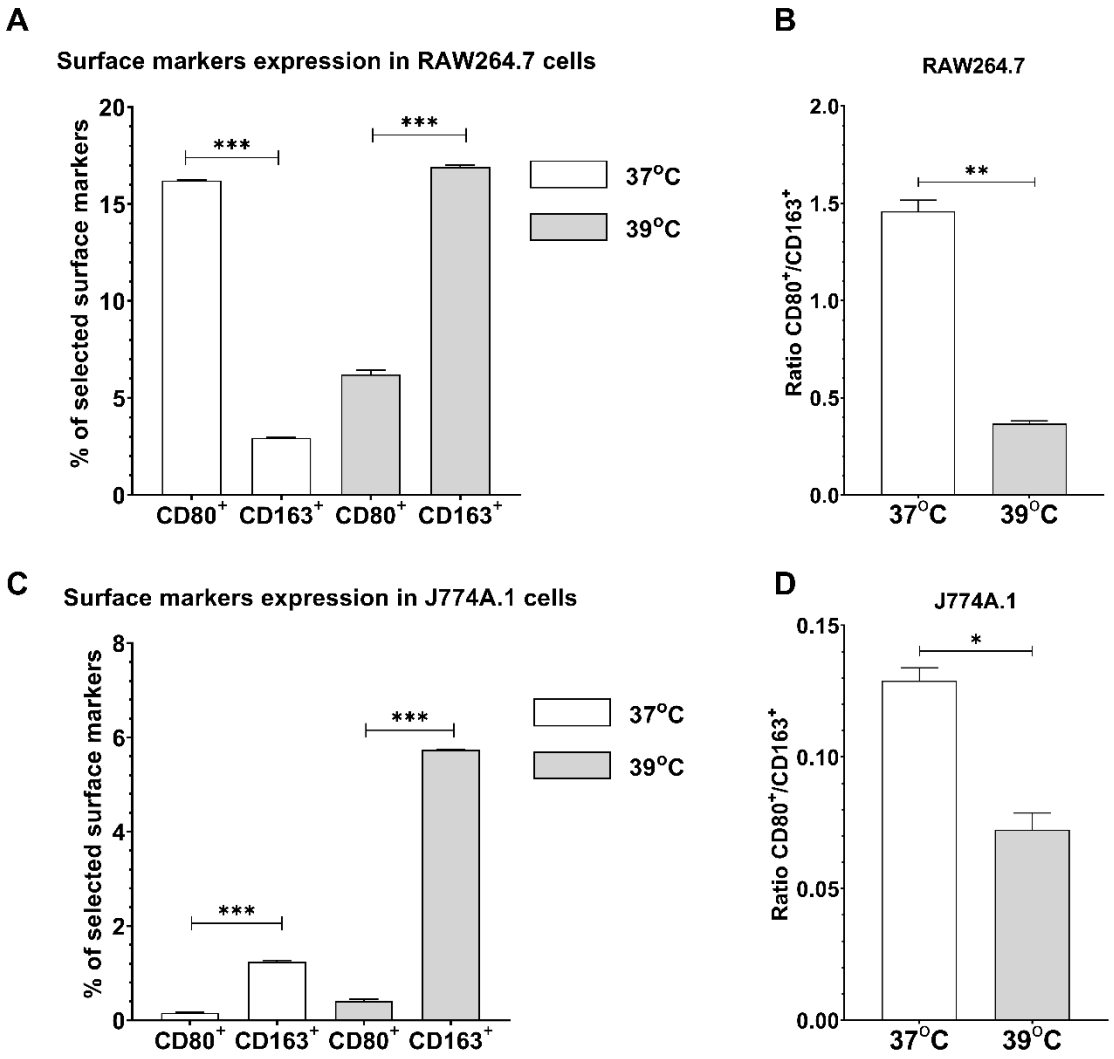

Figure 1 *FRH-induced macrophage polarization. RAW264.7 cells (A and B) and J774A.1 cells (C and D) were cultured at 37°C or 39°C. Shadowed bars indicate cells cultured at 39°C. The expression of surface markers CD80 and CD163 was assessed by flow cytometry. Anti-CD163 antibodies were conjugated with APC whereas anti-CD80 antibodies were conjugated with FITC. Bars represent the ratio of M1/M2 surface markers (B and D). Asterisks indicate the statistical significance (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).*

3.2 Fever-Range Hyperthermia modifies LPS-induced M1 polarization towards M2 phenotype

To verify the potential of FRH to shift macrophage phenotype in pro-inflammatory conditions we used LPS, which is also a well-known factor inducing M1 phenotype. As expected, in LPS-treated RAW264.7 cells and J774A.1 cells cultured at 37°C we observed an increased expression of CD80 (Figure 2A; $p < 0.001$ and Figure 2C; $p < 0.001$ respectively). Interestingly, we noticed an increased expression of CD163 and decreased expression of CD80

in both LPS-treated cell lines cultured at 39°C in comparison to cells cultivated at 37°C ($p < 0.001$ in all examined groups). The ratio of CD80/CD163 surface markers was diminished after FRH-treatment in the both cell lines (Figure 2B and 2D; $p < 0.001$ for RAW264.7 cells, and $p < 0.01$ for J774A.1 cells, respectively) what suggests that FRH is able to switch macrophage phenotype into M2-like cells even in pro-inflammatory conditions.

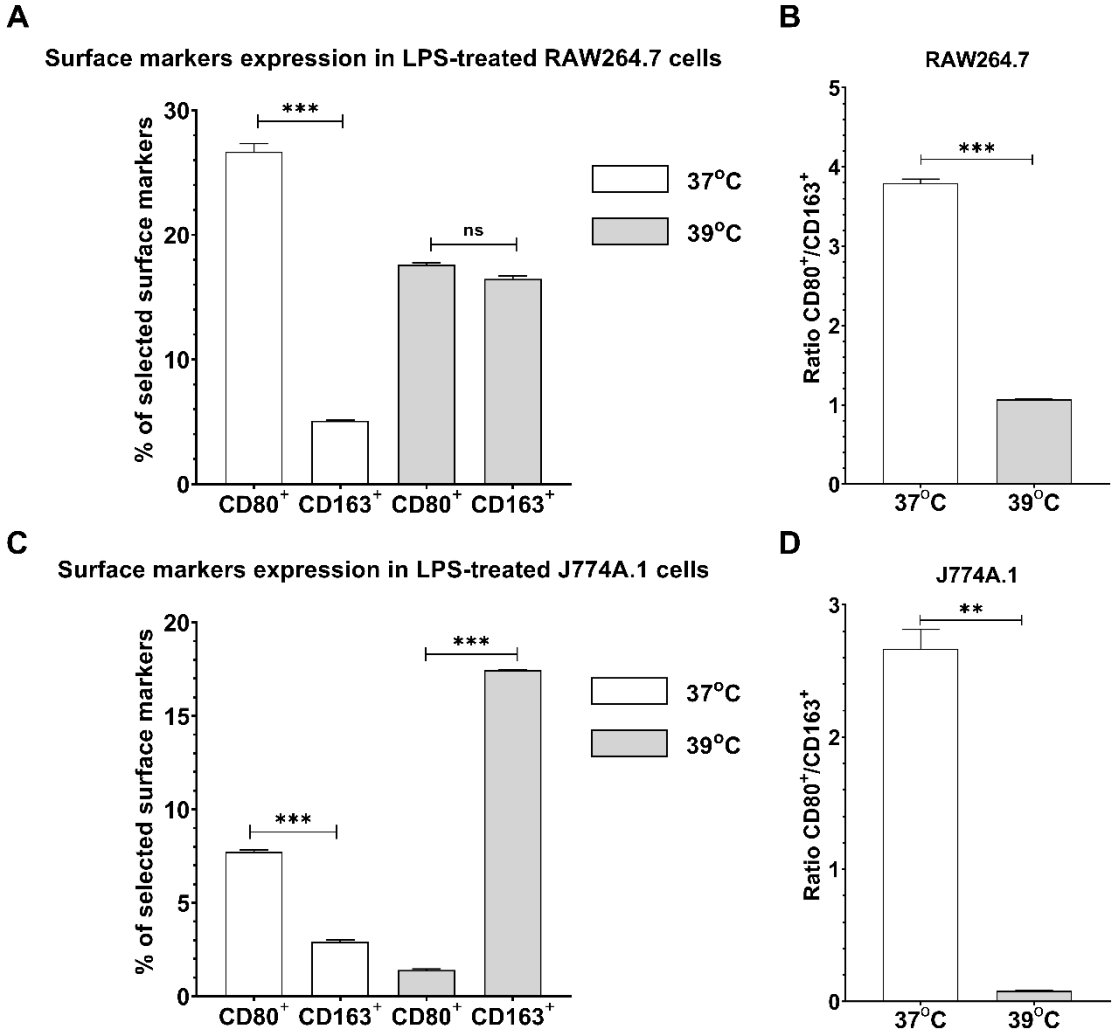

Figure 2 *LPS-induced macrophage polarization changes under hyperthermic conditions. RAW264.7 cells (A and B) and J774A.1 cells (C and D) were treated with LPS and cultured at 37°C or 39°C. Shadowed bars indicate cells cultured at 39°C. The expression of surface markers CD80 and CD163 was assessed by flow cytometry. Anti-CD163 antibodies were conjugated with APC whereas anti-CD80 antibodies were conjugated with FITC. Bars represents the ratio of M1/M2 surface markers (B and D). Asterisks indicate the statistical significance (** $p < 0.01$; *** $p < 0.001$).*

3.3 FRH-induced M2-like cells express pro-inflammatory proteins but reveal anti-inflammatory properties

Since we observed that FRH changes phenotype of macrophages into M2-like cells, we wanted to verify whether this may influence the expression of key molecules involved in inflammatory response such as TLR-4 and COX-2. As expected, we observed an increased expression of COX-2 and TLR-4 in RAW264.7 cells treated with LPS (Figure 3A and 3B; $p < 0.001$ and $p < 0.01$, respectively). Interestingly, FRH alone also induced upregulated expression of both COX-2 and TLR-4 in RAW264.7 cells in comparison to control cells ($p < 0.05$, and $p < 0.001$, respectively). Furthermore, we observed that the co-treatment with FRH and LPS had additive effect on the increased expression of both examined proteins in RAW264.7 cells ($p < 0.001$ for both COX-2 and TLR-4). Similarly, to RAW264.7 cells treatment, we observed increased expression of COX-2 in J774A.1 cells stimulated with LPS at 37°C ($p < 0.05$)(Figure 3C). Noteworthy, FRH alone induced upregulated COX-2 expression in J774A.1 cells ($p < 0.05$), and this effect was even higher in cells simultaneously treated with LPS and FRH ($p < 0.001$). We did not notice any changes in TLR-4 expression induced by LPS at 37°C or under the influence of FRH itself in J774A.1 cells (Figure 3D). However, co-treatment with LPS and FRH induced significant increase in TLR-4 expression in J774A.1 cells ($p < 0.001$).

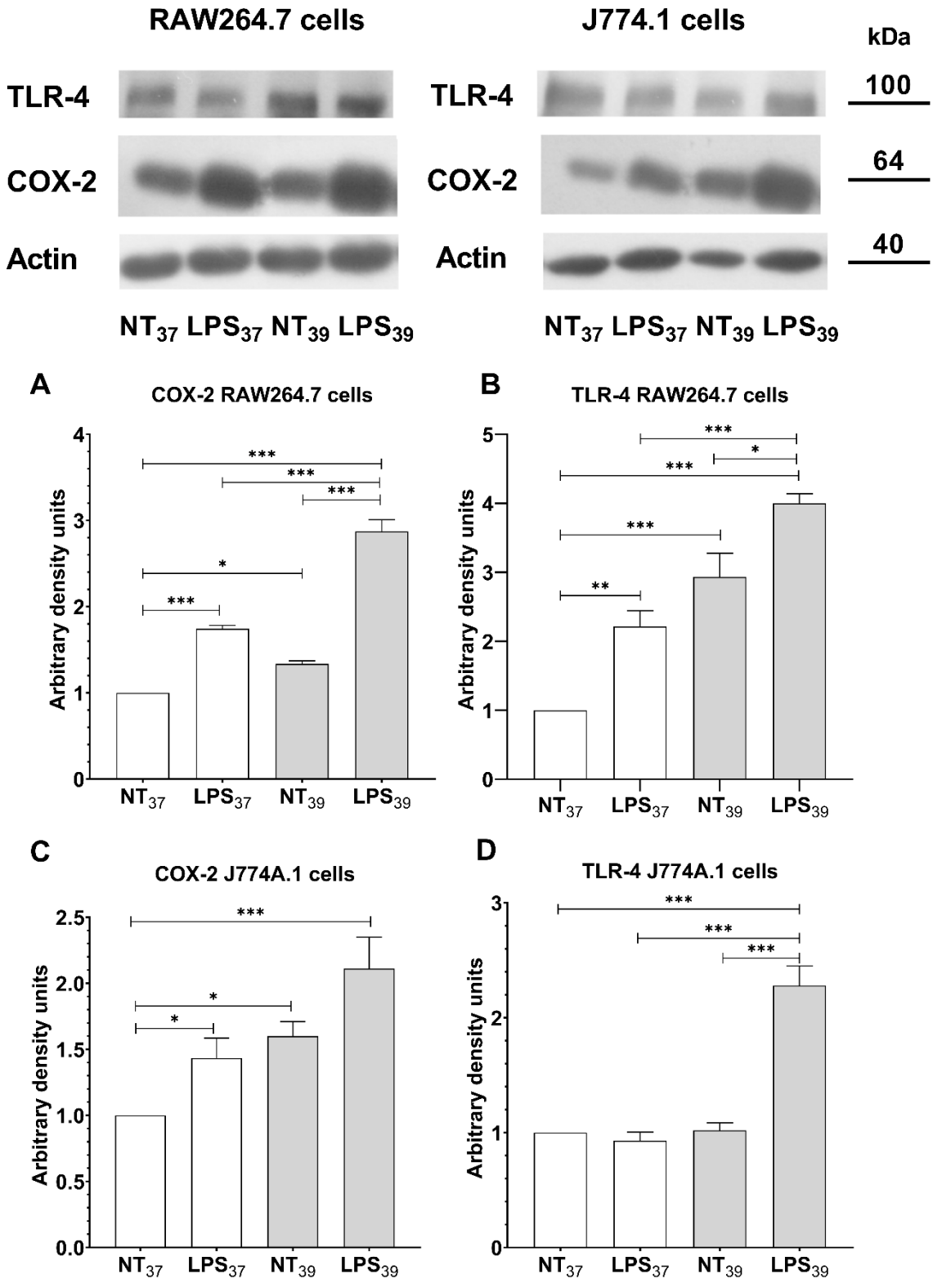

Figure 3 *Western Blot analysis of cyclooxygenase-2 (COX-2) and Toll-like Receptor 4 (TLR-4) expression in RAW264.7 (A and B) and J774A.1 (C and D) cells simultaneously treated with LPS and FRH. Actin was used as a protein loading control. Data represent the mean and standard error of the mean (SEM)*

obtained from three independent experiments. Asterisks indicate the statistical significance ($p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).*

Since we observed increased expression of surface markers specific to M2-like cells and increased expression of pro-inflammatory COX-2, we wanted to check the influence of FRH on the functional activity of macrophages. Therefore, to verify whether FRH may influence the response of cells to infection we examined the level of ROS and nitric oxides NO. We confirmed a commonly known fact that in RAW264.7 and J774.1 cells treated with LPS increased level of NO and ROS (Figure 4A-B; $p < 0.001$ and $p < 0.01$, respectively, and 4C-D; $p < 0.001$ and $p < 0.05$ respectively). This effect was abolished by additional treatment of RAW264.7 cells with FRH ($p < 0.001$ and $p < 0.05$, respectively). Furthermore, FRH alone did not induce production of NO in RAW264.7 cells. However, FRH induced slight increase in ROS level in comparison to control cells ($p < 0.05$).

In J774.1 cells we observed that FRH alone did not affect NO level whereas ROS production was increased ($p < 0.01$). Furthermore, similarly to RAW264.7 cells treatment, we observed significant decrease in NO and ROS level in J774A.1 cells simultaneously treated with LPS and FRH in comparison to LPS alone ($p < 0.001$ and $p < 0.05$ respectively).

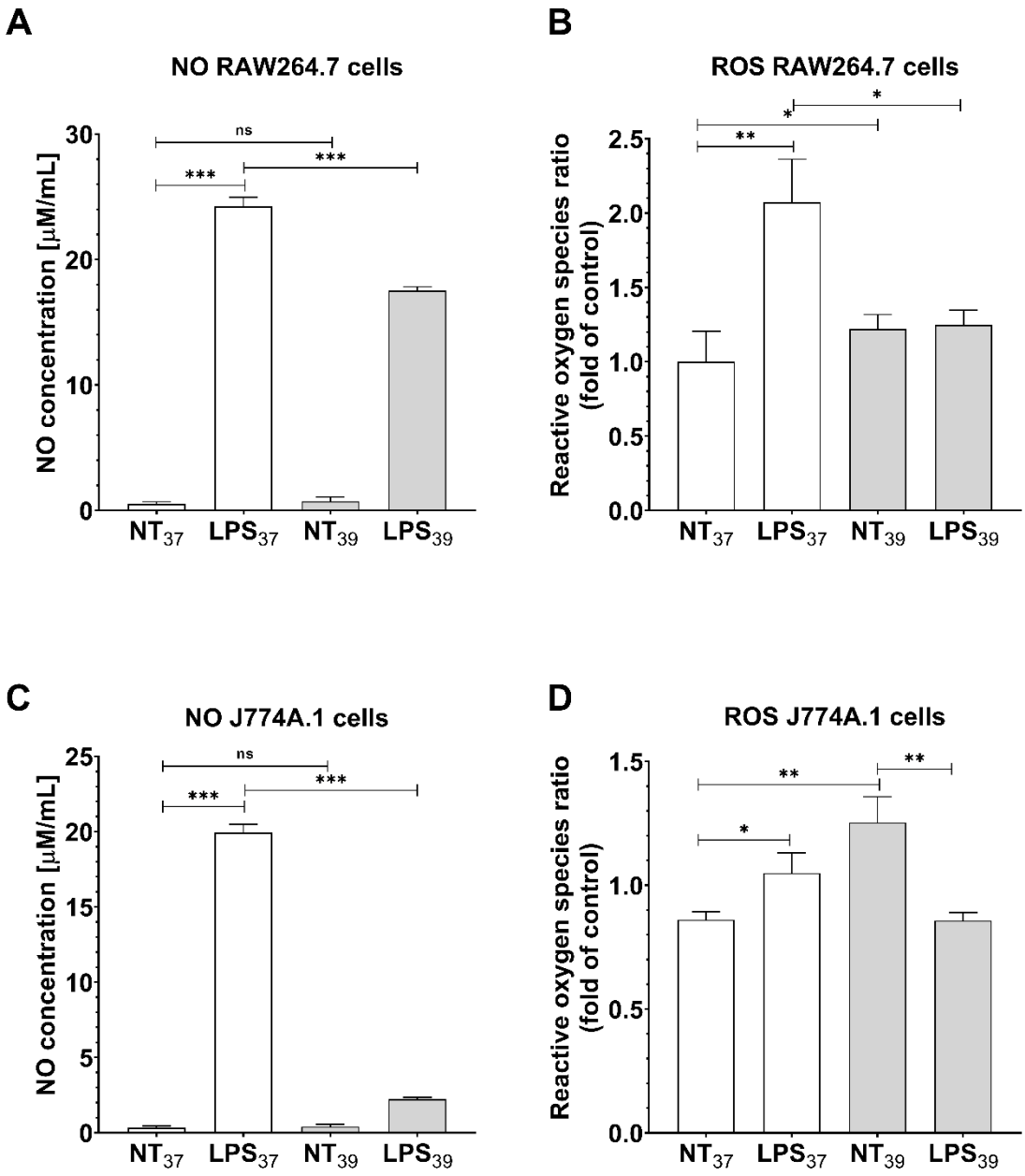

Figure 4 *Oxidative status of RAW264.7 (A and B) and J774A.1 (C and D) cells in response to simultaneous treatment with FRH and LPS. The effect was measured as NO concentration (colorimetric) and relative level of ROS (fluorescent) assessed by flow cytometry. Data represent mean and standard error of the mean (SEM) obtained from three independent experiments. Asterisks indicate the statistical significance (ns $p > 0.05$; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).*

3.4 Fever-Range Hyperthermia changes macrophages phenotype in TLR-4-independent way

It is well known, that in response to hyperthermia various heat shock proteins, including HSP-70, are released in RAW264.7 cells. Similarly, it has been proved that HSPs can activate

TLR-4 downstream signaling pathway (8). Since TLR-4 is important receptor involved in macrophage polarization into both phenotypes (5), we wondered whether inhibition of TLR-4 may affect FRH-induced macrophage polarization. We employed TAK-242, a small-molecule-specific inhibitor of TLR-4 signaling pathway (17). We observed, that inhibition of TLR-4 decreased the spontaneous polarization of non-treated RAW264.7 cells into M1 phenotype, which was presented in the Figure 1. Here, we detected that TLR-4 did not change the CD80 and CD163 expression in RAW264.7 cells cultured at 37°C (Figure 5A and 5B). Interestingly, after additional treatment with FRH we observed overexpression of surface marker CD163 ($p < 0.001$) in comparison to CD80 surface marker. In J774A.1 cells we observed just a slight increase in CD163 expression after TLR-4 inhibition at 37°C in comparison to CD80 (Figure 5C and 5D; $p < 0.01$). This effect was significantly stronger after additional treatment with FRH ($p < 0.01$). Thus, we hypothesized that FRH-induced polarization into M2-like cells was TLR-4 independent phenomenon.

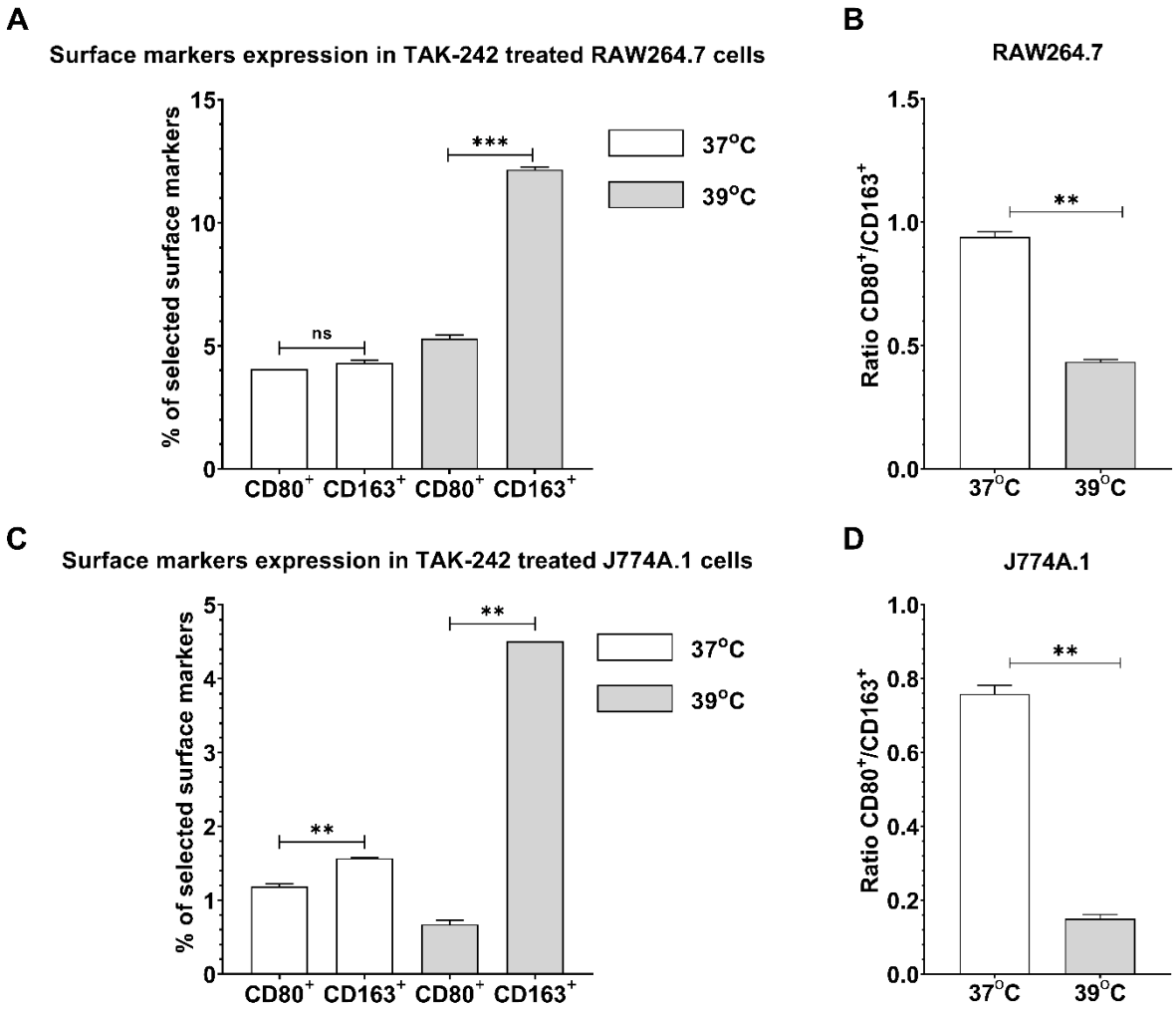

Figure 5 *Macrophages polarization surface markers after TLR-4 inhibition. RAW264.7 cells (A and B) and J774.A cells (C and D) were pre-treated with 0.1 µM TAK-242 for 1h at 37°C, and further cultured at 37°C and 39°C for 24h. Shadowed bars indicate cells cultured at 39°C. The expression of surface markers CD80 and CD163 was assessed by flow cytometry. Anti-CD163 antibodies were conjugated with APC whereas anti-CD80 antibodies were conjugated with FITC. Bars represent the ratio of M1/M2 surface markers (B and D). Asterisks indicate the statistical significance (ns $p > 0.05$; ** $p < 0.01$; *** $p < 0.001$).*

Next, to extend our research on FRH-induced macrophage polarization we analyzed intracellular markers such as inducible Nitric Oxide Synthase (iNOS) and Arginase-1 (Arg-1). As we expected, LPS increased expression of iNOS, (a marker of M1 cells) in RAW264.7 cells (Figure 6A; $p < 0.001$), and TLR-4 inhibition did not affect this expression in RAW264.7 cells in both thermal conditions ($p < 0.001$, and $p < 0.001$, respectively). We also detected slight increase in iNOS expression after FRH alone, however, this change was statistically

insignificant ($p = 0.94$). Interestingly, simultaneous treatment with LPS and FRH triggered upregulated iNOS expression in comparison to control cells ($p < 0.001$).

In J774A.1 cells we noticed overexpression of iNOS after LPS administration in both examined temperatures (Figure 6C; $p < 0.001$ and $p < 0.001$, respectively). Similarly, to RAW264.7 we did not observe changes in iNOS level after FRH alone. However, simultaneous treatment of J774A.1 cells with LPS and FRH reduced the level of iNOS in comparison to LPS alone ($p < 0.05$).

In the case of the measurement of Arg-1 (a marker of M2 cells), we noticed a statistically insignificant increase in Arg-1 expression in both examined cell lines RAW264.7 and J774A.1 cells after LPS treatment at 37°C (Figure 6B and 6D). Surprisingly, in RAW64.7 cells Arg-1 level was upregulated after inhibition of TLR-4 by TAK242 in both thermal conditions in comparison to control group ($p < 0.001$ and $p < 0.01$ respectively), whereas in J774A.1 cells, significant increase was observed only at 37°C ($p < 0.01$). In accordance to surface markers, we observed significant increase of Arg-1 after FRH treatment in both tested cell lines ($p < 0.001$ for RAW264.7 cells and $p < 0.01$ for J774A.1 cells). In both cell lines, this effect was abolished by additional treatment with LPS at 39°C ($p < 0.001$ for RAW264.7 and $p < 0.05$ for J774A.1). However, in RAW264.7 cells the level of Arg-1 was still upregulated in comparison to non-treated cells at 37°C ($p < 0.05$).

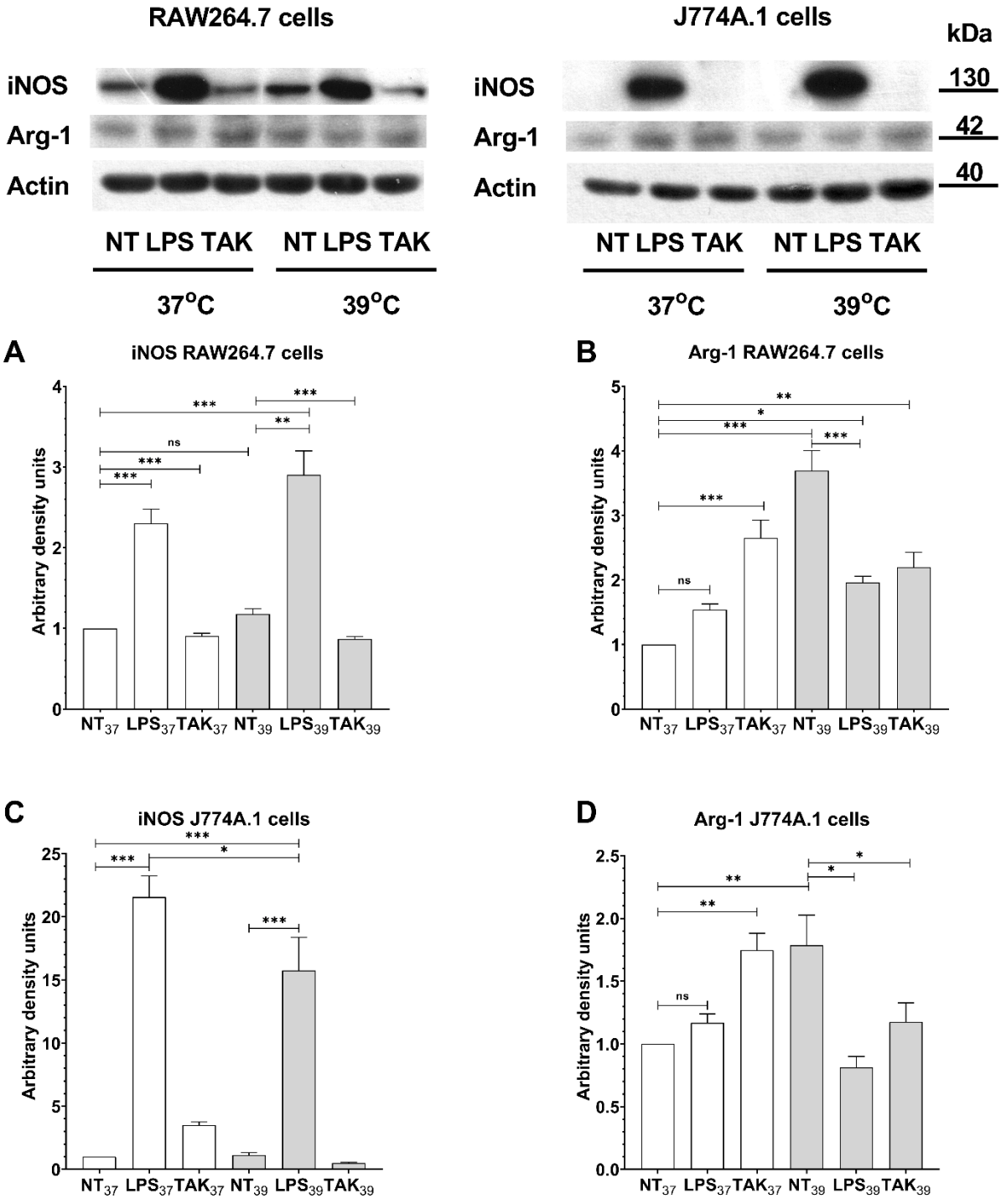

Figure 6 *Western blot analysis of inducible nitric oxide synthase (iNOS) and Arginase-1 (Arg-1) in RAW264.7 cells (A and B) and J774.A cells (C and D) pre-treated with TAK-242 for 1h at 37°C, and further cultured at 37°C and 39°C for 24h. Actin was used as a protein loading control. Data represent the mean and standard error of the mean (SEM) obtained from three independent experiments. Asterisks indicate the statistical significance (ns $p > 0.05$; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).*

4. Discussion

Fever range hyperthermia (FRH) is a condition used in research to investigate a thermal component of fever (1). FRH is also a medical procedure that may trigger benefits in patients who suffer from chronic diseases such as rheumatic diseases, and some cardiovascular disorders (2). Additionally, FRH can support standard cancer treatments, including chemotherapy and radiation (18). Although it is known that macrophages are sensitive to heat (3), relatively little attention has been given to the underlying molecular mechanism.

Macrophages are cells that are recognized as the early warning system, swiftly sounding the alarm in response to infection (19). To protect the body against infections, macrophages produce various molecules including reactive oxygen species (ROS) and nitrogen oxide (NO) (20,21). Of note, ROS and NO are commonly known markers of M1 phenotype in macrophages (20). Both of these molecules induce oxidative stress and activate inflammation. Dysregulation of these molecules can lead to chronic inflammation and tissue damage and therefore it is important to identify factors that can modulate their level in a body (21). In our research we observed that FRH alone did not induce oxidative stress in macrophages what is in accordance to results published by others (4,22). Furthermore, in LPS-treated cells which produce increased level of ROS and NO (23,24), we observed that FRH was able to diminish this effect.

Macrophages represent a continuum of highly plastic effector cells, resembling a spectrum of diverse phenotype states. Depending on their phenotype, macrophages can play either pro-inflammatory or anti-inflammatory role (5). Whether the heat (e.g., produced during fever or during therapy with FRH) can affect this process, has been not recognized yet. We revealed that FRH alone induces M2 phenotype manifested by increased expression of CD163 and Arg-1. However, our previous experiments showed that FRH-treated macrophages can release pro-inflammatory cytokines such as IL-6 and IL-β (3). Similarly, other authors also noticed that FRH may induce overexpression of pro-inflammatory cytokines including TNF-α and IL-6 (22,25). Although research seemingly contradicted the general M1/M2 polarization, finally we managed to classify these cells using additional classification among M2-like cells (26,27). Thus, our data indicate, that FRH induces M2b polarization. M2b macrophages are considered as regulatory cells (26) because except for the pro-inflammatory cytokines such as IL-1β, TNF-α or IL-6, these macrophages produce a high amount of anti-inflammatory IL-10 (5). It is believed that M2b macrophages are effective at suppressing inflammation in a process that is IL-10 dependent (5,26).

The analysis of other pro-inflammatory factors in FRH treated macrophages showed an increased expression of COX-2, an enzyme that is involved in the development of fever (28,29) In accordance to our results, other authors observed that increased expression of COX-2 may induce polarization of macrophages into M2 phenotype through the PGE2-EP4 axis (30). It suggests that not only FRH but also COX-2 dependent fever are regulators of macrophage polarization.

Generally, it is believed that Toll-like receptors, particularly TLR-4 signaling pathway activated by LPS, polarizes macrophages towards the M1 cells (31)**.** Indeed, we observed that LPS alone induces a shift into M1 phenotype in macrophages cultured at 37°C. This was manifested by increased expression of M1 surface marker CD80, and increased level of intracellular markers such as ROS, NO and inducible nitric oxidase. However, we have found that FRH significantly affects triggered by LPS polarization of macrophages and in a consequence M1 macrophages start to express M2 markers. Comparatively to our findings, Ostberg et al. 2000 have shown, that FRH *in vitro* decreases the level of LPS-induced pro-inflammatory cytokines such as IL-6, TNF-α and IL-1β in cells isolated from peritoneal cavity (32).

There are studies showing that in the response to heat treatment, heat shock proteins are released (8,33). These proteins have been identified as molecules that interact with TLR-4 (8). Therefore, we wondered whether TLR-4 is involved in FRH-induced macrophage polarization. We used TAK-242, an inhibitor of TLR-4 (17) that prevents activation and downstream signaling, leading to the inhibition of the inflammatory response. Published data showed the potential of TAK-242 to induce M2 polarization in microglial cells (34), what stays in line with our results. Since, it is believed that FRH acts through the releasing of HSPs and activation of TLR-4 pathway (8,35) we wanted to check, whether this pathway is involved in macrophage polarization. Although we showed, that increased temperature induces M2 polarization, we found that this effect is TLR-4 independent.

In conclusion, our research showed that FRH may significantly diminish macrophage-induced acute inflammatory response, what could be used in therapies which require anti-inflammatory action. Furthermore, since FRH may be considered as a model to investigate thermal component of fever it is likely that febrile increase in body temperature regulates macrophage polarization by inducing M2b phenotype. It is known, that the differentiation of

macrophages into such regulatory phenotype contributes to the resolution of inflammation (36). Thus, it seems that fever-associated heat, may be an important regulator that shifts macrophages from pro-inflammatory M1 phenotype that develops at the beginning of infection towards regulatory M2b, to enhance tissue repair and regeneration. It is likely, that inhibition of fever keeps macrophages in pro-inflammatory M1 phenotype and may lead to harmful effects. This issue, however, needs further research.

5. Declarations

Availability of data and materials

The authors confirm that the data supporting the findings of this study are available within the article. The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

Funding

This study was supported by Project no POWR.03.05.00-00-Z302/17 “Universitas Copernicana Thoruniensis in Futuro” (2018-2022) co-financed by the European Social Fund—the Operational Programme Knowledge Education Development. Module 5. Interdisciplinary PhD School “Academia Copernicana” and the Polish Minister of Science and Higher Education under the program “Regional Initiative of Excellence” in 2019–2023 (Grant No. 008/RID/2018/19).

Authors' contributions

Conceptualization, H.M.K. and S.W.; methodology, H.M.K., B.M., J.S. and T.J.; software, H.M.K. and B.M.; validation, T.J and J.S.; formal analysis, T.J. and J.S.; investigation, H.M.K. and B.M.; resources, H.M.K., S.W. and A.D.; data curation, H.M.K. and B.M.; writing—original draft preparation, H.M.K.; writing—review and editing, T.J. and S.W.; visualization, H.M.K. and J.S.; supervision, S.W. and A.D.; project administration, H.M.K. and S.W.; funding acquisition, S.W. and A.D.

Consent for publication

All authors consent the publication of this manuscript in BMC Biology

6. References

1. Hasday JD, Shah N, Mackowiak PA, Tulapurkar M, Nagarsekar A, Singh I. Fever, Hyperthermia, and the Lung: It's All about Context and Timing. Trans Am Clin Climatol Assoc [Internet]. 2011 [cited 2023 Mar 14];122:34. Available from: /pmc/articles/PMC3116334/

2. Chicheł A, Skowronek J, Kubaszewska M, Kanikowski M. Hyperthermia - Description of a method and a review of clinical applications. Vol. 12, Reports of Practical Oncology and Radiotherapy. No longer published by Elsevier; 2007. p. 267–75.

3. Kozłowski HM, Pawlikowska M, Sobocińska J, Jędrzejewski T, Dzialuk A, Wrotek S. Distinct modulatory effects of fever-range hyperthermia on the response of breast cancer cells and macrophages to mistletoe (Viscum album l.) extract. Pharmaceuticals [Internet]. 2021 Jun 8 [cited 2022 Jul 18];14(6):551. Available from: https://www.mdpi.com/1424-8247/14/6/551/htm

4. Pritchard MT, Li Z, Repasky EA. Nitric oxide production is regulated by fever-range thermal stimulation of murine macrophages. J Leukoc Biol [Internet]. 2005 Jul 6 [cited 2023 Apr 2];78(3):630–8. Available from: https://pubmed.ncbi.nlm.nih.gov/16000392/

5. Martinez FO, Sica A, Mantovani A, Locati M. Macrophage activation and polarization. Front Biosci [Internet]. 2008 [cited 2023 Mar 2];13(2):453–61. Available from: https://pubmed.ncbi.nlm.nih.gov/17981560/

6. Molteni M, Gemma S, Rossetti C. The Role of Toll-Like Receptor 4 in Infectious and Noninfectious Inflammation. Mediators Inflamm [Internet]. 2016 [cited 2023 Mar 5];2016. Available from: /pmc/articles/PMC4887650/

7. Swanson L, Katkar GD, Tam J, Pranadinata RF, Chareddy Y, Coates J, et al. TLR4 signaling and macrophage inflammatory responses are dampened by GIV/Girdin. Proc Natl Acad Sci U S A [Internet]. 2020 Oct 27 [cited 2023 Mar 2];117(43):26895–906. Available from: https://www.pnas.org/doi/abs/10.1073/pnas.2011667117

8. Gupta A, Cooper ZA, Tulapurkar ME, Potla R, Maity T, Hasday JD, et al. Toll-like Receptor Agonists and Febrile Range Hyperthermia Synergize to Induce Heat Shock Protein 70 Expression and Extracellular Release. Journal of Biological Chemistry. 2013 Jan 25;288(4):2756–66.

9. Zheng XF, Hong YX, Feng GJ, Zhang GF, Rogers H. Lipopolysaccharide-Induced M2 to M1 Macrophage Transformation for IL-12p70 Production Is Blocked by Candida albicans Mediated Up-Regulation of EBI3 Expression. PLoS One [Internet]. 2013 [cited 2023 Mar 2];8(5):63967. Available from: www.plosone.org

10. Qin H, Holdbrooks AT, Liu Y, Reynolds SL, Yanagisawa LL, Benveniste EN. SOCS3 Deficiency Promotes M1 Macrophage Polarization and Inflammation. The Journal of Immunology [Internet]. 2012 Oct 1 [cited 2023 Mar 2];189(7):3439–48. Available from: https://journals.aai.org/jimmunol/article/189/7/3439/86210/SOCS3-Deficiency-Promotes-M1-Macrophage

11. Liu B, Zhang M, Zhao J, Zheng M, Yang H. Imbalance of M1/M2 macrophages is linked to severity level of knee osteoarthritis. Exp Ther Med [Internet]. 2018 Dec 1 [cited 2023 Mar 2];16(6):5009–14. Available from: http://www.spandidos-publications.com/10.3892/etm.2018.6852/abstract

12. Skytthe MK, Graversen JH, Moestrup SK. Targeting of CD163+ Macrophages in Inflammatory and Malignant Diseases. International Journal of Molecular Sciences 2020, Vol 21, Page 5497 [Internet]. 2020 Jul 31 [cited 2023 Mar 2];21(15):5497. Available from: https://www.mdpi.com/1422-0067/21/15/5497/htm

13. Ferrante CJ, Pinhal-Enfield G, Elson G, Cronstein BN, Hasko G, Outram S, et al. The adenosine-dependent angiogenic switch of macrophages to an M2-like phenotype is independent of Interleukin-4 receptor alpha (IL4Rα) signaling. Inflammation [Internet]. 2013 Aug [cited 2023 Mar 2];36(4):921. Available from: /pmc/articles/PMC3710311/

14. Lee CT, Repasky EA. Opposing roles for heat and heat shock proteins in macrophage functions during inflammation: A function of cell activation state? Front Immunol. 2012 Jun 1;3(JUN):140.

15. Vaughan-Jackson A, Stodolak S, Ebrahimi KH, Johnson E, Reardon PK, Dupont M, et al. Density dependent regulation of inflammatory responses in macrophages. Front Immunol. 2022 Dec 16;13:7607.

16. Lee CM, Hu J. Cell density during differentiation can alter the phenotype of bone marrow-derived macrophages. Cell Biosci [Internet]. 2013 Jul 29 [cited 2023 Apr 1];3(1):1–5. Available from: https://cellandbioscience.biomedcentral.com/articles/10.1186/2045-3701-3-30

17. Matsunaga N, Tsuchimori N, Matsumoto T, Ii M. TAK-242 (resatorvid), a small-molecule inhibitor of Toll-like receptor (TLR) 4 signaling, binds selectively to TLR4 and interferes with interactions between TLR4 and its adaptor molecules. Mol Pharmacol [Internet]. 2011 Jan [cited 2023 Mar 5];79(1):34–41. Available from: https://pubmed.ncbi.nlm.nih.gov/20881006/

18. Katschinski DM, Wiedemann GJ, Longo W, D'Oleire FR, Spriggs D, Robins HI. Whole body hyperthermia cytokine induction: A review, and unifying hypothesis for myeloprotection in the setting of cytotoxic therapy [Internet]. Vol. 10, Cytokine and Growth Factor Reviews. Cytokine Growth Factor Rev; 1999 [cited 2022 Jun 27]. p. 93–7. Available from: https://pubmed.ncbi.nlm.nih.gov/10743501/

19. Hirayama D, Iida T, Nakase H. The Phagocytic Function of Macrophage-Enforcing Innate Immunity and Tissue Homeostasis. Int J Mol Sci [Internet]. 2018 Jan 1 [cited 2023 Apr 1];19(1). Available from: /pmc/articles/PMC5796042/

20. Palmieri EM, McGinity C, Wink DA, McVicar DW. Nitric Oxide in Macrophage Immunometabolism: Hiding in Plain Sight. Metabolites [Internet]. 2020 Nov 1 [cited 2023 Apr 2];10(11):1–34. Available from: /pmc/articles/PMC7693038/

21. Canton M, Sánchez-Rodríguez R, Spera I, Venegas FC, Favia M, Viola A, et al. Reactive Oxygen Species in Macrophages: Sources and Targets. Front Immunol. 2021 Sep 30;12:4077.

22. Hop HT, Arayan LT, Reyes AWB, Huy TXN, Min WG, Lee HJ, et al. Heat-stress-modulated induction of NF-κB leads to brucellacidal pro-inflammatory defense against Brucella abortus infection in murine macrophages and in a mouse model. BMC Microbiol [Internet]. 2018 May 25 [cited 2023 Apr 2];18(1). Available from: /pmc/articles/PMC5970535/

23. Lin CY, Kao SH, Hung LC, Chien HJ, Wang WH, Chang YW, et al. Lipopolysaccharide-induced nitric oxide and prostaglandin e2 production is inhibited by tellimagrandin ii in mouse and human macrophages. Life [Internet]. 2021 May 1 [cited 2023 Apr 3];11(5). Available from: /pmc/articles/PMC8146495/

24. Ji SY, Cha HJ, Molagoda IMN, Kim MY, Kim SY, Hwangbo H, et al. Suppression of Lipopolysaccharide-Induced Inflammatory and Oxidative Response by 5-Aminolevulinic Acid in RAW 264.7 Macrophages and Zebrafish Larvae. Biomol Ther (Seoul) [Internet]. 2021 Nov 1 [cited 2023 Apr 3];29(6):685–96. Available from: https://pubmed.ncbi.nlm.nih.gov/33820881/

25. Lee CT, Zhong L, Mace TA, Repasky EA. Elevation in Body Temperature to Fever Range Enhances and Prolongs Subsequent Responsiveness of Macrophages to Endotoxin Challenge. PLoS One [Internet]. 2012 Jan 10 [cited 2023 Apr 1];7(1). Available from: /pmc/articles/PMC3254634/

26. Wang L xun, Zhang S xi, Wu H juan, Rong X lu, Guo J. M2b macrophage polarization and its roles in diseases. J Leukoc Biol [Internet]. 2019 Aug 1 [cited 2023 Apr 3];106(2):345–58. Available from: https://onlinelibrary.wiley.com/doi/full/10.1002/JLB.3RU1018-378RR

27. Gordon S, Martinez FO. Alternative activation of macrophages: Mechanism and functions. Immunity. 2010 May;32(5):593–604.

28. Zhao Y, Usatyuk P V., Gorshkova IA, He D, Wang T, Moreno-Vinasco L, et al. Regulation of COX-2 Expression and IL-6 Release by Particulate Matter in Airway Epithelial Cells. Am J Respir Cell Mol Biol [Internet]. 2009 Jan 1 [cited 2023 Apr 3];40(1):19. Available from: /pmc/articles/PMC5459547/

29. Cao C, Matsumura K, Yamagata K, Watanabe Y. Involvement of cyclooxygenase-2 in LPS-induced fever and regulation of its mRNA by LPS in the rat brain. https://doi.org/101152/ajpregu19972726R1712 [Internet]. 1997 [cited 2023 Apr 3];272(6 41-6). Available from: https://journals.physiology.org/doi/10.1152/ajpregu.1997.272.6.R1712

30. Tian F, Chen H, Zhang J, He W. Reprogramming Metabolism of Macrophages as a Target for Kidney Dysfunction Treatment in Autoimmune Diseases. Int J Mol Sci [Internet]. 2022 Jul 1 [cited 2023 Apr 14];23(14). Available from: /pmc/articles/PMC9316004/

31. Lee CH, Choi EY. Macrophages and Inflammation. Journal of Rheumatic Diseases [Internet]. 2018 Jan 1 [cited 2023 Apr 2];25(1):11–8. Available from: https://www.jrd.or.kr/journal/view.html?doi=10.4078/jrd.2018.25.1.11

32. Ostberg JR, Taylor SL, Baumann H, Repasky EA. Regulatory effects of fever-range whole-body hyperthermia on the LPS-induced acute inflammatory response. J Leukoc Biol [Internet]. 2000 Dec 1 [cited 2023 Apr 3];68(6):815–20. Available from: https://onlinelibrary.wiley.com/doi/full/10.1189/jlb.68.6.815

33. Hagiwara S, Iwasaka H, Matsumoto S, Noguchi T. Changes in cell culture temperature alter release of inflammatory mediators in murine macrophagic RAW264.7 cells. Inflammation Research [Internet]. 2007 Jul 1 [cited 2023 Apr 3];56(7):297–303. Available from: https://link.springer.com/article/10.1007/s00011-007-6161-z

34. Cui W, Sun C, Ma Y, Wang S, Wang X, Zhang Y. Inhibition of TLR4 Induces M2 Microglial Polarization and Provides Neuroprotection via the NLRP3 Inflammasome in Alzheimer's Disease. Front Neurosci. 2020 May 20;14:444.

35. Zhao W, An H, Zhou J, Xu H, Yu Y, Cao X. Hyperthermia differentially regulates TLR4 and TLR2-mediated innate immune response. Immunol Lett. 2007 Feb 15;108(2):137–42.

36. Filardy AA, Pires DR, Nunes MP, Takiya CM, Freire-de-Lima CG, Ribeiro-Gomes FL, et al. Proinflammatory clearance of apoptotic neutrophils induces an IL-12(low)IL-10(high) regulatory phenotype in macrophages. J Immunol [Internet]. 2010 Aug 15 [cited 2023 Apr 13];185(4):2044–50. Available from: https://pubmed.ncbi.nlm.nih.gov/20660352/

3.3. Article 3

Kozłowski, H.M.; Pawlikowska, M.; Sobocińska, J.; Jędrzejewski, T.; Dzialuk, A.; Wrotek, S. *Distinct Modulatory Effects of Fever-Range Hyperthermia on the Response of Breast Cancer Cells and Macrophages to Mistletoe (Viscum album L.) Extract*. Pharmaceuticals 2021, 14, 551. https://doi.org/10.3390/ph14060551

pharmaceuticals

MDPI

Article

Distinct Modulatory Effects of Fever-Range Hyperthermia on the Response of Breast Cancer Cells and Macrophages to Mistletoe (*Viscum album* L.) Extract

Henryk M. Kozłowski [1,*], Małgorzata Pawlikowska [1], Justyna Sobocińska [1], Tomasz Jędrzejewski [1], Artur Dzialuk [2,*] and Sylwia Wrotek [1]

[1] Department of Immunology, Faculty of Biological and Veterinary Sciences, Nicolaus Copernicus University, 1 Lwowska Str., 87-100 Torun, Poland; m_pawlikowska@umk.pl (M.P.); j.sobocinska@umk.pl (J.S.); tomaszj@umk.pl (T.J.); wrotek@umk.pl (S.W.)

[2] Department of Genetics, Faculty of Biological Sciences, Kazimierz Wielki University, 10 Powstańców Wielkopolskich Ave., 85-090 Bydgoszcz, Poland

* Correspondence: mikolajkozlowski@doktorant.umk.pl (H.M.K.); dzialuk@ukw.edu.pl (A.D.)

Abstract: Heat utility as a critical component of fever is often ignored, although the symptom is observed in many medical conditions. Mistletoe extract (ME) is an adjunctive medication prescribed to cancer patients. The increase in body temperature is frequently observed in patients following ME administration. Nevertheless, the impact of this fever on the effectiveness of therapy is unknown. Therefore, we aimed to investigate the effect of fever-range temperatures on ME-treated breast cancer cells and macrophages. The cells were simultaneously stimulated with ME and subjected to fever-range hyperthermia (FRH; 39 °C or 41 °C). After co-treatment, the cell viability, generation of reactive oxygen species (ROS), cell cycle distribution, and production of pro-inflammatory factors (interleukin (IL)-1β, IL-6, and cyclooxygenase (COX)-2) were evaluated. The results showed that the exposure of ME-treated breast cancer cells to FRH at 39 °C resulted in a slight decrease in their viability, whereas FRH of 41 °C enhanced this effect. Only FRH of 41 °C induced minor changes in ROS level in ME-treated breast cancer cell lines. In ME-treated macrophages, FRH stimulated cell proliferation. The cell cycle distribution analysis showed a difference between cells cultured at 39 °C and 41 °C in all examined cell lines. Moreover, hyperthermia at 41 °C completely inhibited the ME-induced increase in IL-1β and IL-6 expression in MCF-7 breast cancer cells, whereas this effect was not observed in 4T1 breast cancer cells. In contrast, in ME-treated macrophages, FRH of 41 °C strongly up-regulated expression of the pro-inflammatory factors. We conclude that fever is an important component of ME therapy that differentially affects cancer and immune cells.

Keywords: mistletoe extract; hyperthermia; cytokines; fever; inflammation; reactive oxygen species; cell cycle distribution

Citation: Kozłowski, H.M.; Pawlikowska, M.; Sobocińska, J.; Jędrzejewski, T.; Dzialuk, A.; Wrotek, S. Distinct Modulatory Effects of Fever-Range Hyperthermia on the Response of Breast Cancer Cells and Macrophages to Mistletoe (*Viscum album* L.) Extract. *Pharmaceuticals* **2021**, *14*, 551. https://doi.org/10.3390/ph14060551

Academic Editor: Jan-Pieter Konsman

Received: 5 May 2021
Accepted: 6 June 2021
Published: 8 June 2021

1. Introduction

Breast cancer is the most common cancer and also the primary cause of mortality due to cancer in females around the world. In spite of many improvements in the use of hormonal and adjuvant cytotoxic therapies for breast cancer patients, the reduction in mortality is still not satisfactory [1].

Mistletoe extract (ME) is the most frequently prescribed therapy as an adjunct to standard treatment regimens for various malignancies in Europe [2,3]. It is widely used based on its ability to reduce emotional stress. However, it is also an immunostimulant, and its anticancer properties have been confirmed in experimental studies as well as clinical trials [3,4].

ME is generally well tolerated, although some common side effects such as local reactions at the injection site (e.g., redness, swelling, itchiness) and mild flu-like symptoms

including fever have been described [5–10]. Although fever was observed in a large group of ME-treated patients [10], little is known about its significance in ME therapy [11], and the direct functional consequences of ME-induced body temperature increase are unclear. Thus, it is not known whether ME-induced fever is only a side effect, which can be inhibited, or is a pivotal factor that acts synergistically with ME.

According to published data, the increase in body temperature is induced by the binding of some ME compounds, such as lectins, to the pattern recognition receptors on immune cells [11,12]. Then, the signal is transmitted, and the expression of pyrogenic cytokines such as interleukin (IL)-1β, IL-6, and tumor necrosis factor α (TNF-α) is observed. These cytokines activate cyclooxygenease-2 (COX-2), which is an enzyme involved in prostaglandin E2 production (PGE2), which is a final mediator of fever [13,14]. Our previous research on mice with severe combined immunodeficiency (SCID) showed that in the process of fever induction, the involvement of the innate immune cells such as macrophages, rather than lymphocytes, is needed [15]. Since macrophages secrete many factors including cytokines and reactive oxygen species (ROS) that create a microenvironment for tumor cells, we wanted to investigate whether various thermal conditions may affect their response to ME. Additionally, due to the unresolved question as to whether fever may support anticancer ME therapy, we decided to explore the effects of heat on cancer cells directly. The aim of our study was to examine whether fever-range temperatures (above 37 °C) modify the effects triggered by ME therapy in macrophages and breast cancer cells. To resolve this problem, we utilized febrile-range hyperthermia (FRH), which is considered to be a surrogate for fever in in vitro experiments.

We found that FRH modulates cell viability, ROS generation, cell cycle arrest, and the expression of inflammatory markers (i.e., pro-inflammatory cytokines and COX-2) in breast cancer cells and in macrophages. The effect was dependent on the level of FRH, and FRH exerted different effects on ME-treated breast cancer cells and macrophages.

2. Results

2.1. Exposure to Heat at 41 °C Decreased Viability of ME-Treated Mcf-7 Cells

To determine whether thermal conditions affect cell viability in ME-treated breast cancer cells, the MTT assay was conducted. The observation of control MCF-7 cells that were not treated with ME revealed that exposure to heat alone at 41 °C significantly inhibits their proliferation ($p < 0.001$) (Figure 1A). We found that the exposure of MCF-7 cells cultured at 37 °C to ME alone decreased their viability compared to not-treated cells ($p < 0.01$), whereas the exposure of ME-treated MCF-7 cells to heat at 41 °C enhances this reduction in cell viability ($p < 0.001$).

2.2. Exposure to Heat at 41 °C Increased Reactive Oxygen Species Production in ME-Treated MCF-7 Cells

Having observed that both not-treated and treated with ME MCF-7 cells cultured at 41 °C display a decrease in cell viability (Figure 1A), we wanted to determine whether this effect is associated with an increase in ROS production. Indeed, we observed a significant increase in ROS generation in cells cultured at 41 °C, regardless of exposure to ME ($p < 0.01$) (Figure 1B). We did not notice any significant changes in the ROS level in ME-treated MCF-7 cells cultured at 37 °C and 39 °C in comparison to control cells (Figure 1B).

2.3. ME Combined with Heat Exposure to 39 °C Induced a Significant Cell Cycle Arrest at the G1 Phase in MCF-7 Cells

Since exposure of breast cancer cells to heat at 41 °C is able to decrease their viability (Figure 1A), we decided to check whether these culture conditions modulate cell cycle distribution in the MCF-7 cell line. In ME-treated cells, we did not notice any significant changes in the cell cycle of breast cancer cells cultured at 41 °C as well as cells cultured at 39 °C, in comparison to control, which was not-treated with ME cells (Figure 1C,D). Similarly, in ME-treated MCF-7 cells cultured at 37 °C, we did not observe any significant changes in cell cycle distribution in comparison to control cells. However, the elevation of

the culture temperature to 39 °C after ME treatment induced a cell cycle arrest at the G1 phase in comparison to control cells and ME-treated cells cultured at 37 °C ($p < 0.05$ and $p < 0.05$, respectively). Furthermore, in ME-treated cells cultured at 39 °C, we observed a decrease in the percentage of cells in the G2/M phase in comparison to ME-treated cells cultured at 37 °C ($p < 0.05$). Although ME-treated cells exposed to heat at 41 °C displayed a significant reduction in cell viability (Figure 1A), we did not observe any significant changes in cell cycle distribution.

2.4. Exposure to Fever-Range Hyperthermia Decreased the Viability of ME-Treated 4T1 Cells

To determine whether thermal conditions affect cell viability in ME-treated 4T1 breast cancer cells, we used the MTT assay. In our control groups, which were not treated with ME, we have observed that exposure to heat alone at 39 °C increases cell viability ($p < 0.01$) (Figure 2A). Nevertheless, the elevation of culture temperature to 41 °C revealed a significant decrease in cell viability ($p > 0.001$). In ME-treated 4T1 cells cultured at 37 °C as well as cultured at 39 °C, we have found a significant decrease in cell viability in comparison to not-treated cells ($p < 0.001$ and $p < 0.001$, respectively). Elevation of ambient temperature to a range of 41 °C potentiates in ME-treated 4T1 cells the effect of reduced cell viability compared to not-treated and ME-treated cells cultured at 37 °C ($p < 0.001$ and $p < 0.001$, respectively).

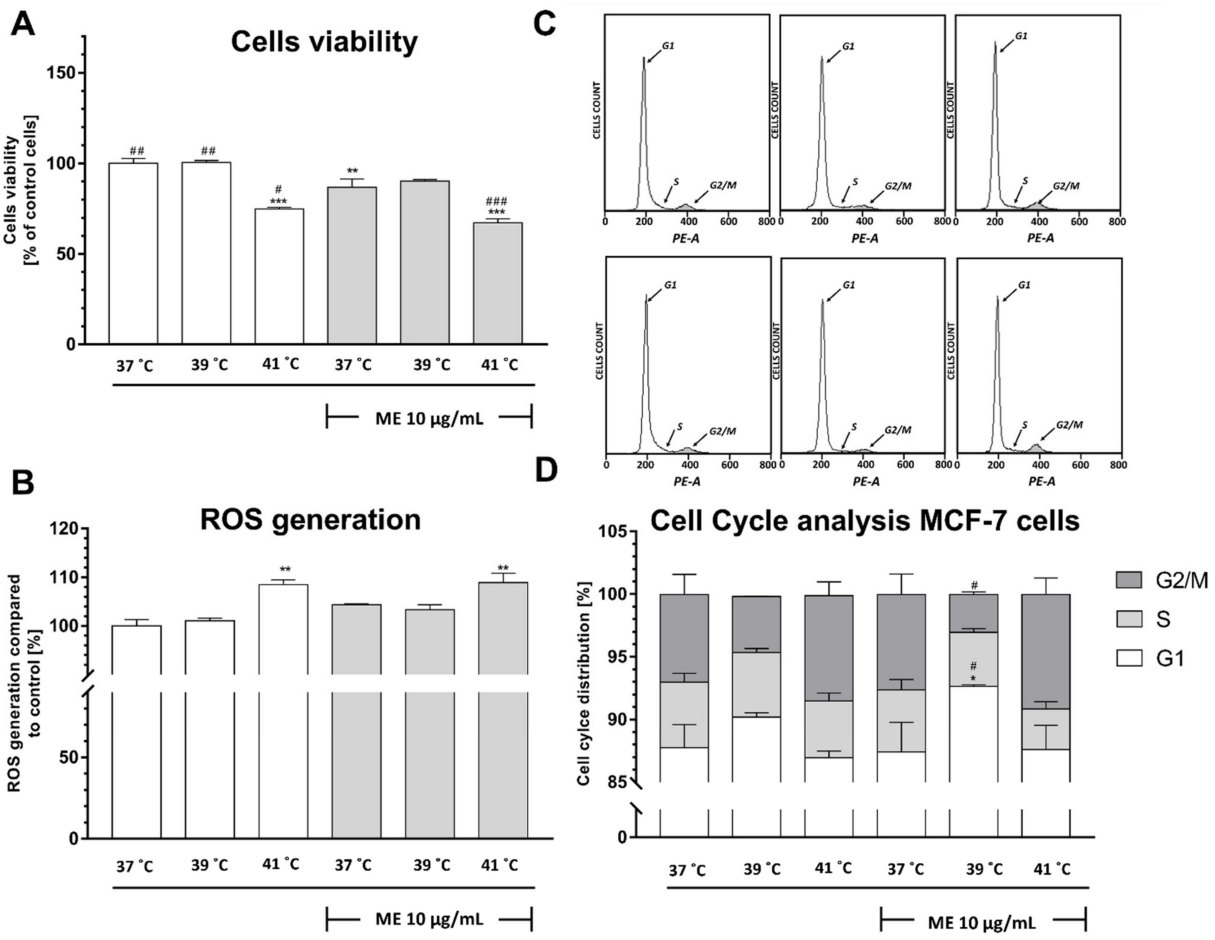

Figure 1. Effect of heat exposure on the viability, as measured using the MTT test (**A**), ROS production, as measured using carboxy–H_2DCFDA staining followed by flow cytometry analysis (**B**), and cell cycle distribution, as measured using propidium iodide staining followed by flow cytometry analysis (**C**,**D**), of ME-treated MCF-7 cells. Data are presented as the mean ± S.E.M. of three independent experiments. Asterisks indicate statistical significance in comparison to untreated control cells, and slashes indicate significance in comparison to ME-treated cells cultured at 37 °C (*** or ### $p < 0.001$, ** or ## $p < 0.01$, * or # $p < 0.05$). ME indicates mistletoe extract.

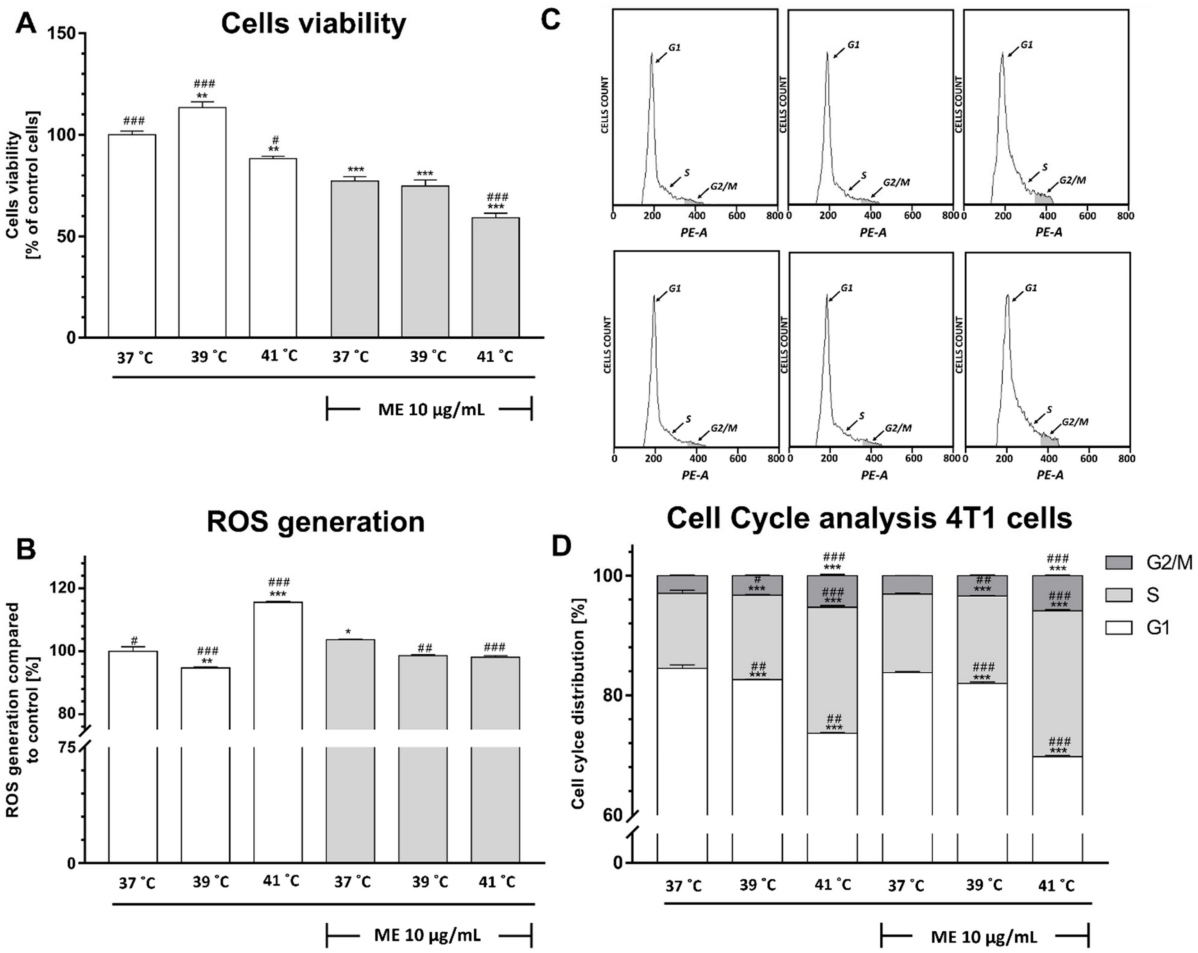

Figure 2. Effect of heat exposure on the viability, measured using MTT test (**A**), ROS production, measured using carboxy-H_2DCFDA staining followed by flow cytometry analysis (**B**), and cell cycle distribution, measured using propidium iodide staining followed by flow cytometry analysis (**C**,**D**) of ME-treated 4T1 cells. Data are presented as the mean ± S.E.M. of three independent experiments. Asterisks indicate statistical significance in comparison to untreated control cells, and slashes indicate significance in comparison to ME-treated cells cultured at 37 °C (*** or ### $p < 0.001$, ** or ## $p < 0.01$, * or # $p < 0.05$). ME indicates mistletoe extract.

2.5. ME Enhances a Slight Increase in Reactive Oxygen Species Level

Since the exposure of 4T1 breast cancer cells to ME triggered a decrease in cell viability, we wonder whether this effect is associated with ROS production. The observation of our control, not-treated with ME cells, revealed that heat at 39 °C induces a slight, but statistically significant, decrease in reactive oxygen species level ($p < 0.01$) (Figure 2B). In contrast, heat at 41 °C gives rise to ROS levels compared to not-treated cells cultured at 37 °C ($p < 0.001$). ME induces a slight increase in ROS level in breast cancer cells cultured at 37 °C compared to control cells ($p < 0.05$); this effect can be abolished by introducing heat at both examined temperatures 39 °C and 41 °C in comparison to ME-treated cells ($p < 0.01$ and $p < 0.001$, respectively).

2.6. ME Combined with Heat Exposure to 41 °C Induced a Significant Cell Cycle Arrest at the S Phase in 4T1 Cells

Having observed that 4T1 cells treated with ME exhibited a significant reduction in cell viability at 37 °C (Figure 2A), we decided to assess whether this effect is associated with a change of cell cycle distribution (Figure 2C,D). Our control groups not-treated with ME have shown that the elevation of ambient temperature to 39 °C caused a slight decrease

in cell cycle arrest in the G1 phase associated with a slight increase in cell count in the S phase in comparison to control cells cultured at 37 °C ($p < 0.001$ and $p < 0.001$, respectively). Similarly, the elevation of ambient temperature to 41 °C reduced cells counts in the G1 phase compared to control cells ($p < 0.001$). At the same time, we have noticed an increased number of cells in the S phase as well as in the G2/M phase in comparison to control cells not-treated with ME and cultured at 37 °C ($p < 0.001$ and $p < 0.001$, respectively). Interestingly, we observed that ME reduces 4T1 cell viability at 37 °C, but we did not notice any significant changes in cell cycle distribution after ME treatment. Nevertheless, after the elevation of ambient temperature to 39 °C in ME-treated cells, there was a significant increase in the number of cells in the S phase and decreased count of cells in the G1 phase in comparison to ME-treated cells cultured at 37 °C ($p < <0.01$ and $p < 0.001$, respectively) as well as in comparison to not-treated control cells cultured at 37 °C ($p < 0.001$ and $p < 0.001$, respectively). Furthermore, cells treated with ME and cultured at 41 °C have shown an increased number of cells in S phase and G2/M phase in comparison to ME-treated cells cultured at 37 °C ($p < 0.001$ and $p < 0.001$, respectively) as well as in comparison to not-treated control cells cultured at 37 °C ($p < 0.001$ and $p < 0.001$, respectively). This finding was associated with a significant decrease of cells in G1 phase compared to ME-treated and not-treated cells cultured at 37 °C ($p < 0.001$ and $p < 0.001$, respectively).

2.7. Exposure to Temperatures of 39 °C or 41 °C Prevented the ME-Induced Decrease in RAW 264.7 Cell Viability

To determine whether thermal conditions affect the cell viability in ME-treated RAW 264.7 cells, we conducted an MTT assay. The control RAW 264.7 cells, which were not-treated with ME, revealed that exposure to heat at 41 °C stimulates the proliferation of macrophages ($p < 0.001$), whereas the exposure to heat at 39 °C did not induce any changes in the cell viability in comparison to control cells cultured at 37° C (Figure 3A). We found that the exposure of RAW 264.7 cells cultured at 37 °C to ME significantly inhibited their viability in comparison to untreated cells ($p < 0.001$). This effect can be abolished by elevation of the temperature to 39 °C or 41 °C ($p < 0.001$ and $p < 0.001$, respectively). The elevation of temperature to 39 °C and 41 °C not only abolished this effect, but it also stimulated cell proliferation ($p < 0.05$ and $p < 0.01$, respectively).

2.8. Exposure to Heat at 41 °C Increased Reactive Oxygen Species Generation in RAW 264.7 Cells

Since the production of ROS by macrophages as a mechanism to kill tumor cells is well established [16], in this experiment, we wanted to assess whether various thermal conditions affect ROS generation in ME-treated RAW 264.7 cells. The control, not-treated with ME cells, displayed a significant increase in ROS level ($p < 0.001$) after exposure to heat at 41 °C (Figure 3B). The treatment of RAW 264.7 cells with ME did not change ROS levels in cells cultured at 37 °C or at 39 °C. Interestingly, ME-treated cells exposed to 41 °C display two times higher ROS generation than control cells ($p < 0.001$).

2.9. ME Treated RAW 264.7 Cells Demonstrate a Significant Arrest in the G2/M Phase after Heat Exposure

Having observed that treatment of RAW 264.7 cells with ME induced a significant reduction in cell viability at 37 °C (Figure 3A), we decided to assess whether this effect is associated with a change of cell cycle distribution (Figure 3C,D). We did not observe any changes in cell cycle distribution in RAW264.7 cells not-treated with ME cultured at 41 °C. Interestingly, we noticed that the elevation of ambient temperature to 39 °C caused a significant increase in cell cycle arrest in the G1 phase associated with a decrease in cell count in the G2/M phase in comparison to control cells ($p < 0.001$ and $p < 0.001$, respectively). Similarly, the elevation of ambient temperature to 39 °C induced in ME-treated cells a cell cycle arrest in the G1 phase and resulted in a significant reduction of cells in the G2/M phase compared to ME-treated cells cultured at 37 °C ($p < 0.001$ and $p < 0.001$, respectively). In our study, we did not find any significant change in cell cycle distribution after ME-treatment at 37 °C. However, cells treated with ME and cultured

at 41 °C demonstrated an increase in the number of G2/M cells and a decreased number of cells in the S phase in comparison to not-treated with ME control cells ($p < 0.001$ and $p < 0.05$, respectively). Furthermore, there was a significant increase in the number of cells in the G2/M phase and decreased count of cells in the S phase in comparison to ME treated cells cultured in 37 °C ($p < 0.01$ and $p < 0.01$, respectively).

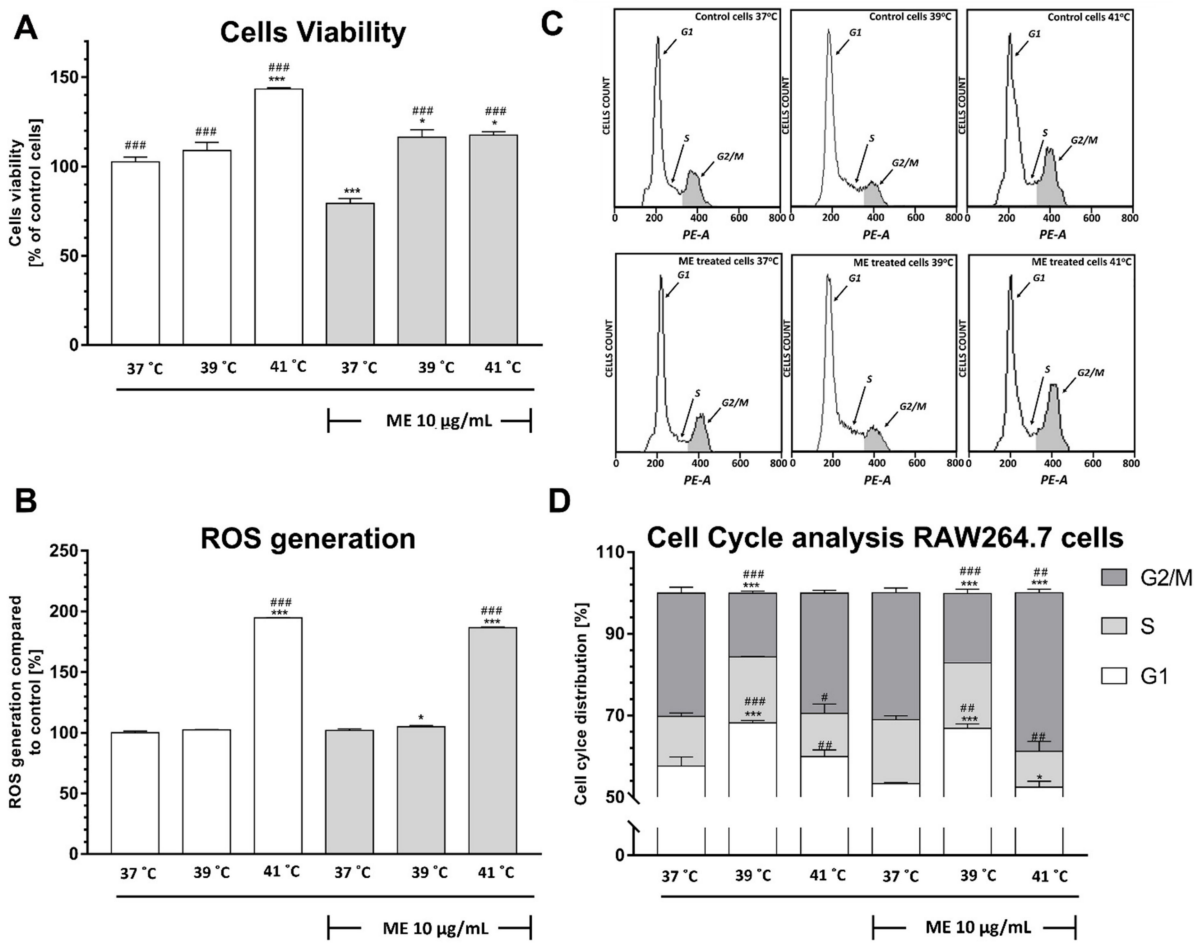

Figure 3. Effect of heat exposure on the viability, measured using MTT test (**A**), ROS production, measured using carboxy-H_2DCFDA staining followed by flow cytometry analysis (**B**), and cell cycle distribution, measured using propidium iodide staining followed by flow cytometry analysis (**C**,**D**) of ME-treated RAW 264.7 cells. Data are presented as the mean ± S.E.M. of three independent experiments. Asterisks indicate statistical significance in comparison to untreated control cells and slashes indicate significance in comparison to ME-treated cells cultured at 37 °C (*** or ### $p < 0.001$, ## $p < 0.01$, * or # $p < 0.05$). ME indicates mistletoe extract.

2.10. Exposure to Temperatures of 39 °C or 41 °C Inhibited ME-Induced mRNA Expression of IL-1β and IL-6 in MCF-7 Cells

Since we observed that thermal conditions significantly affect the cell viability, ROS generation, and cell cycle distribution of ME-treated MCF-7 cells, we next wanted to determine whether FRH affects the expression of pro-inflammatory cytokines. The control cells that were not treated with ME but were exposed to heat at 39 °C displayed an increase in mRNA expression of IL-1β in comparison to control cells ($p < 0.01$), whereas exposure to 41 °C did not trigger such an effect (Figure 4A). ME significantly enhanced mRNA expression of IL-1β in MCF-7 cells cultured at 37 °C ($p < 0.01$). Exposure of ME-treated MCF-7 cells to heat at either 39 °C or 41 °C significantly diminished this expression in comparison to the ME-treated cells cultured at 37 °C ($p < 0.001$ and $p < 0.001$, respectively).

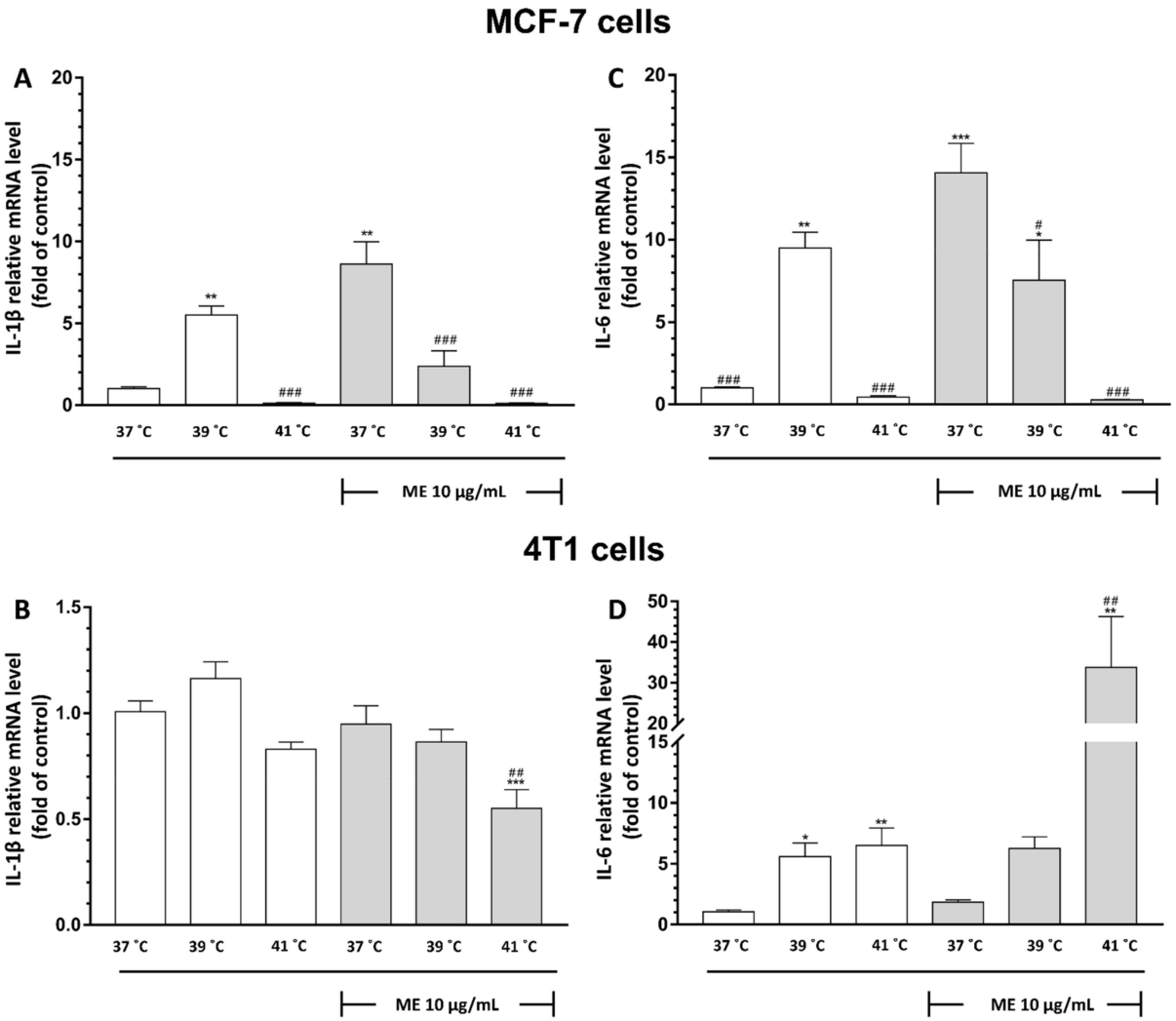

Figure 4. Effect of heat exposure on ME-induced mRNA expression of (**A**,**B**) IL-1β, (**C**,**D**) IL-6 in MCF-7 cells (**A**,**C**), and 4T1 cells (**B**,**D**), respectively. mRNA expression was determined by quantitative real time-PCR. Data are presented as the mean ± S.E.M. of three independent experiments. Asterisks indicate statistical significance in comparison to untreated control cells, and slashes indicate significance in comparison to ME+/37 °C (*** or ### $p < 0.001$, ** or ## $p < 0.01$, * or # $p < 0.05$). ME indicates mistletoe extract.

The observation of not-treated MCF-7 cells revealed the statistically significant increase in mRNA expression of IL-6 observed only in cells exposed to heat at 39 °C ($p < 0.01$) (Figure 4B). ME treatment enhanced mRNA expression of IL-6 in MCF-7 cells cultured at 37 °C in comparison to control cells ($p < 0.001$). It is noteworthy that we observed a temperature-dependent decrease in mRNA expression in ME-treated cells exposed to heat. The relative expression of IL-6 mRNA in ME-treated cells cultured at 39 °C was almost two times lower than cells treated with ME and cultured at 37 °C. ($p < 0.05$). In ME-treated MCF-7 cells, an exposure to 41 °C caused a complete inhibition of IL-6 mRNA expression compared to ME-treated cells cultured at 37 °C ($p < 0.001$).

2.11. Exposure of ME-Treated 4T1 Breast Cancer Cells to Heat at 41 °C Differentially Modulates mRNA Expression of IL-1β and IL-6

Since we observed that heat exposure could modify ME-induced cytotoxicity, ROS generation, and cell cycle distribution, we wonder about the influence of the combination of ME and heat on pro-inflammatory cytokines mRNA expression. Our results have shown that the elevation of ambient temperature to 39 °C and 41 °C alone is not able to change IL-1β mRNA expression in 4T1 cells in comparison to not-treated cells cultured at 37 °C

(Figure 4B). Interestingly, ME did not influence IL-1β mRNA expression at 37 °C, but the elevation of ambient temperature to 41 °C of ME-treated cells reduced IL-1β mRNA expression in comparison to ME-treated and not-treated cells cultured at 37 °C ($p < 0.01$ and $p < 0.001$, respectively).

The observation of not-treated with ME 4T1 breast cancer cells revealed a statistically significant increase in IL-6 mRNA expression after the exposure to heat at 39 °C and 41 °C in comparison to cells cultured at 37 °C ($p < 0.05$ and $p < 0.01$, respectively) (Figure 4D). Furthermore, ME-treated cells cultured at 37 °C did not show any significant changes in IL-6 mRNA expression compared to not-treated control cells. Nevertheless, the elevation of ambient temperature to 41 °C of ME-treated cells has shown a significant increase in IL-6 mRNA expression compared to ME-treated and not-treated cells cultured at 37 °C ($p < 0.01$ and $p < 0.01$, respectively).

2.12. Exposure to Heat at 41 °C Enhanced mRNA Expression of IL-1β and IL-6 in ME-Treated RAW 264.7 Cells

Having found a significant influence of heat on the expression of pro-inflammatory cytokines in MCF7 cells, we decided to determine whether similar effects are observed in RAW264.7 cells. We observed that cells exposed to heat at 41 °C revealed an increased expression of IL-1β in comparison to control cells ($p < 0.05$) (Figure 5A). The exposure of RAW 264.7 cells to ME did not change the mRNA expression of IL-1β in cells cultured at 37 °C in comparison to control cells ($p > 0.05$). Similarly, the exposure of ME-treated cells to heat at 39 °C did not affect this expression ($p > 0.05$). However, in cells cultured at 41 °C, we observed a significant increase in IL-1β mRNA expression ($p < 0.001$), which was about two times higher than in cells cultured at 41 °C without ME ($p < 0.01$).

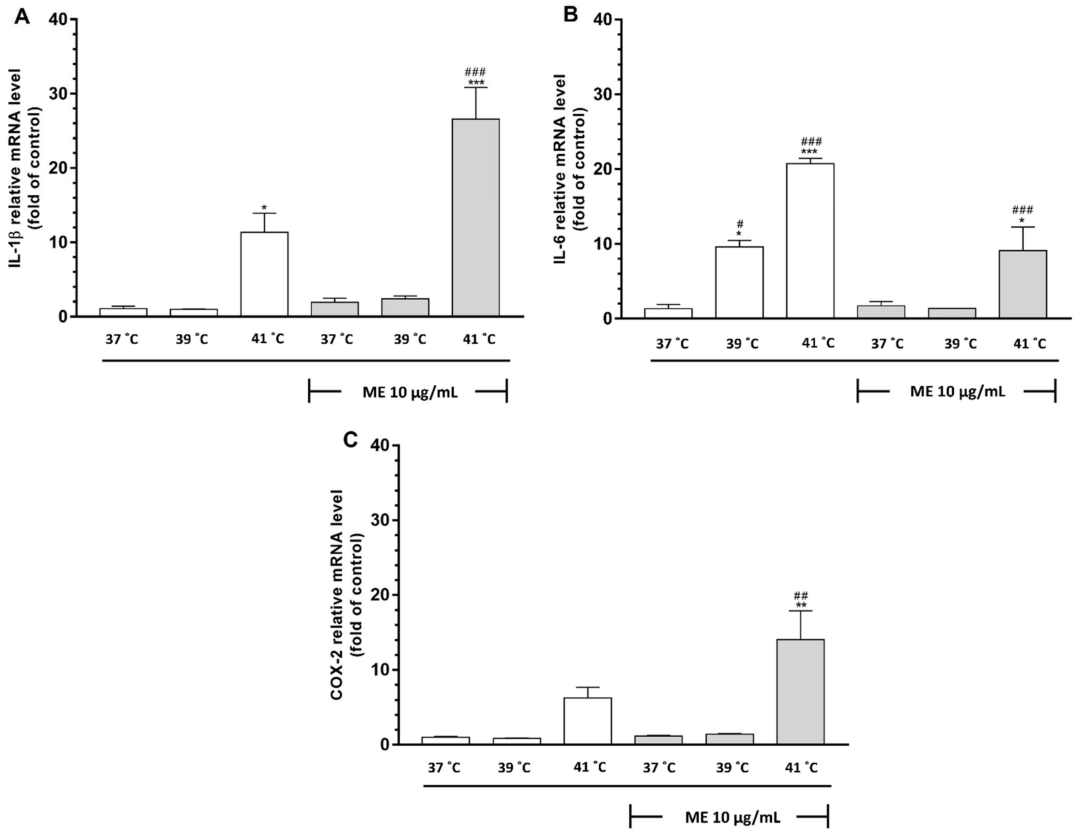

Figure 5. Effect of heat exposure on ME-induced mRNA expression in RAW 264.7 cells. (**A**) IL-1β, (**B**) IL-6, and (**C**) COX-2 mRNA expression was determined by quantitative real-time PCR. Data are presented as the mean ± S.E.M. of three independent experiments. Asterisks indicate statistical significance in comparison to untreated control cells and slashes indicate significance in comparison to ME-treated cells cultured at 37 °C (*** or ### $p < 0.001$, ** or ## $p < 0.01$, * or # $p < 0.05$). ME indicates mistletoe extract.

The observation of not-treated with ME cells revealed that exposure to heat alone enhances IL-6 mRNA expression at both 39 °C and 41 °C compared to cells cultured at 37 °C ($p < 0.05$ and $p < 0.001$, respectively) (Figure 5B). ME did not change mRNA expression of IL-6 in RAW 264.7 cells cultured at 37 °C in comparison to control cells ($p > 0.05$). Similarly, the simultaneous treatment of RAW 264.7 cells with ME and incubation at 39 °C did not affect this expression ($p > 0.05$). However, in ME-treated cells, incubation at 41 °C triggered a significant elevation of mRNA expression in comparison to ME-treated cells cultured at 37 °C and control cells ($p < 0.05$ and $p < 0.001$, respectively).

2.13. ME Combined with Incubation at 41 °C Caused a Significant Increase in Expression of Cyclooxygenase-2 in RAW 264.7 Cells

Having found an enhanced expression of pyrogenic cytokines in response to heat exposure in RAW 264.7 cells, we decided to check whether COX-2, another factor involved fever mechanism, is also stimulated. In RAW 264.7 cells that were not treated with ME, we observed that exposure to heat at 39 °C did not affect COX-2 expression (Figure 5C). After heat exposure at 41 °C, RAW264.7 cells revealed an increased expression of COX-2 in comparison to control cells, but this change was statistically insignificant ($p > 0.05$). ME at 37 °C did not affect COX-2 expression in RAW 264.7 cells compared to control cells. Surprisingly, exposing the ME-treated cells to heat at 41 °C significantly enhanced this expression, whereas culturing cells at 39 °C did not change it ($p < 0.01$, and $p > 0.999$, respectively).

3. Discussion

Mistletoe preparations (ME) are widely used in patient-centered integrative cancer care. They induce a significant increase in beta-endorphin plasma levels; therefore, they can be used to reduce patients' emotional stress [17]. In addition to these psychoactive properties, their immunomodulatory properties have been established [18,19]. In many patients, ME administration generates a dose-dependent fever-like reaction. Whether this reaction is important for ME therapy is unknown.

Fever is one of the most commonly recognized features of acute inflammation. There is much evidence that infectious fever acts on the immune system and may promote an anticancer response in patients [20–22]. However, the significance of fever following ME administration is not well understood, and what is known is based only on observational studies that do not explain the molecular consequence of the increase in body temperature. Therefore, to investigate this issue, we cultured ME-treated breast cancer cells (MCF-7, and 4T1) and immune cells (macrophages, RAW 264.7) in FRH. These conditions reflect in vitro fever-associated body temperature increases. Since it has not been established whether moderate (37.5–40.5 °C) or high (above 40.5 °C) hyperthermia is more beneficial for cancer treatment, we tested both. To the best of our knowledge, this is the first investigation examining the impact of febrile temperatures on ME-induced effects observed in normal and malignant cells.

In the current study, we investigated the cell viability, ROS generation, cell cycle distribution, and the expression of pro-inflammatory factors. We observed only slight differences between cell viability, ROS production, and cell cycle distribution in between both cancer cell lines. We found that ME in a dose of 10 μg/mL used as a monotherapy triggers only a slight decrease in the cell viability of breast cancer cells. These results are in accordance with results described by Weissenstein and colleagues [23], who found that ME only displays a dose-dependent anti-proliferative effect on breast cancer cells at concentrations $\geq$ 10 μg/mL. Similarly, Klingbeil and colleagues [24] found an increase in mortality of neck squamous cell carcinoma cell lines when ME was used in a dose of 300 μg/mL for 48 h. After we established that 10 μg/mL of ME is not sufficient to provoke a decrease in the viability of breast cancer cells, we utilized a surrogate of fever called fever-range hyperthermia (FRH) to assess whether heat may inhibit their proliferation. Indeed, we found that both ME-treated lines of cancer cells that were cultured at high FRH (41 °C) demonstrated a significant decrease in cell viability. In MCF-7 cells, this effect can result from excessive ROS generation, but in the 4T1 cells, this effect seems to be independent of

ROS generation. ME-treated MCF-7 breast cancer cells cultured at moderate FRH (39 °C) did not display any additional ROS generation. In contrast, in ME-treated 4T1 cells, we observed a minor decrease in ROS generation after moderate FRH treatment. Additionally, we have found only a slight decrease in cell viability in both cancer cell lines. Since our control of not-treated with ME breast cancer cell lines revealed that exposure to 41 °C significantly modulates cell viability and ROS production, we suppose that fever in a range of 41 °C may trigger similar effects in cancer cells.

That ME-treated breast cancer cells react differentially to various FRH was also observed in cell cycle distribution analysis. Although 41 °C inhibits cell proliferation in comparison to 37 °C, it did not change the distribution of the cell cycle in MCF-7 cell lines, whereas at 39 °C, it induced only a slight decrease in cell proliferation that was accompanied by an increase of cells in the G1 pool. Interestingly, since we observed a significant decrease in cell viability of ME-treated 4T1 breast cancer cells cultured at 41 °C, we observed a simultaneous decrease of cells in the G1 phase, whereas in cells cultured at 39 °C, we observed only a slight decrease in cell viability and a slight decrease in the number of cells in the G1 phase. Thus, although the effect of FRH on ME-treated cancer cell viability is similar in both examined cell lines, their response in ROS generation and cell cycle distribution is slightly different.

Therapy based on ME may affect not only cancer cells but also immune cells. Therefore, in the current research, apart from breast cancer cells, we also investigated macrophages treated with FRH and ME. Our investigation revealed that macrophages responded to ME and FRH differently than breast cancer cells. The incubation of macrophages with ME resulted in the cell viability decrease, although the production of ROS and distribution of cells throughout the cell cycle did not change in comparison to control cells. This decrease was prevented by additional treatment of cells with FRH at 39 °C as well as 41 °C. Although, both types of hyperthermia induced an increase in proliferation of ME-treated cells in comparison to ME-treated cells cultured at 37 °C, the distribution of cell cycle differed significantly and was temperature dependent. At 39 °C, a significant increase of G1 was observed, whereas at 41 °C, we observed an increase of cells in the G2/M pool. This observation clearly shows that FRH creates conditions that favor the proliferation of macrophages, although the cell cycle distribution of cells cultured at these two thermal conditions differed significantly. Moreover, we observed that both ME-treated and not-treated with ME macrophages that were exposed to 41 °C displayed a significant increase in ROS generation along with increased proliferation. It is known that macrophages produce intracellular ROS that are involved in the phagocytic process. There is also a notion that macrophage-generated ROS are essential for the uptake and clearance of apoptotic cells [25,26]. Furthermore, ROS can function as second messengers and modulate cell functions by activation of cell signaling pathways in response to stimulation with various agents [25].

In the next step of our research, we investigated whether FRH modifies mRNA expression of pro-inflammatory cytokines in both ME-treated cell types. Although we found that 10 μg/mL of ME triggered only a slight decrease in the cell viability of breast cancer cells, this dose of ME significantly affects IL-1β and IL-6 mRNA expression. Interestingly, the expression of cytokines in both types of breast cancer cell lines differed significantly e.g., ME significantly enhanced the expression of IL-1β and IL-6 genes in MCF-7 cells, whereas we did not observe such an effect in 4T1 cells. Furthermore, FRH at 41 °C decreases the IL-6 mRNA level in ME-treated MCF-7 cells, whereas in the ME-treated 4T1 cell line, a significantly increase in IL-6 mRNA level was observed. Additionally, we have found that a combination of ME and mild FRH (39 °C) reduced this expression, and the combination of ME and high FRH (41 °C) inhibited the expression of these cytokines completely in MCF-7 cells. Thus, FRH inhibits IL-1β and IL-6 mRNA expression in these breast cancer cells in a temperature-dependent manner. In ME-treated 4T1 cells, we did not observe an increase in the expression of IL-1β and IL-6 mRNA; even additional treatment with moderate FRH (39 °C) did not change it. Nevertheless, high FRH (41 °C) decreases the IL-1β mRNA

level, and it surprisingly increases the level of IL-6 mRNA in ME-treated 4T1 breast cancer cells. Furthermore, our control group, not-treated with ME, revealed an increase in IL-6 mRNA expression that was temperature-dependent. We suppose that these differences in cytokines expression can be associated with different aggressiveness and characteristic features of both examined breast cancer cell lines. 4T1 cells are considered to be highly aggressive, triple negative cells [27]. In contrast, MCF-7 cells are poorly invasive [28], with estrogen and progesterone receptors [29]. This issue needs further research, since the production of pro-inflammatory cytokines by cancer cells is considered harmful [30]. Our research revealed also that not-treated with ME breast cancer cells display an increased mRNA expression of IL-1β and IL-6 in response to heat at 39 °C alone but not to 41 °C.

Unlike cancer cells, the co-treatment of macrophages with ME and high FRH (41 °C) induced a strong up-regulation of IL-1β and IL-6 mRNA expression. These findings together with the observation of increased ROS production in ME-treated macrophages cultured at 41 °C suggests that there is a switch toward the M1 phenotype [26,31]. It is believed that this type of macrophage is implicated in the effective elimination of tumor cells [32]. Thus, the finding that FRH may orchestrate the switch toward M1 is of great significance. Najafi and colleagues [33] postulated that macrophage switching toward an anti-inflammatory M1 phenotype could be used as an adjuvant with other approaches, including radiotherapy and immune checkpoint blockades, such as anti-PD-L1/PD-1 strategies. Our findings show that this phenotype can be induced by the exposure of ME-treated macrophages to FRH at 41 °C.

Having found an enhanced expression of pro-inflammatory cytokines in ME-treated macrophages, we additionally measured the mRNA expression of COX-2, which is not only another factor involved in inflammation but is also an M1 macrophage marker [34]. We observed that only high FRH (41 °C) increased COX-2 mRNA expression. Since our research revealed that incubation at 41 °C induces an increase in the expression of pyrogenic cytokines and COX-2 in macrophages, we speculate that this FRH triggers signaling pathways that are similar to those induced by pathogens. Additionally, this finding suggests that heat in a range of 41 °C acts as an adjuvant of inflammatory response. These hypotheses are supported by observations demonstrating the role of heat shock proteins in inflammation. It is commonly known that intracellular HSP70 acts as a chaperone that exerts anti-apoptotic and cytoprotective actions [35], and other research has shown that extracellular HSP70 is also an immunomodulator, which, similarly to lipopolysaccharide, acts as a Toll-like receptor agonist [36,37]. Therefore, further research is needed to determine whether the increase in the expression of pro-inflammatory cytokines and COX-2 observed in our investigation is indeed a consequence of heat shock proteins released after FRH treatment.

Given that the expression of genes such as IL-1β, IL-6, and COX-2 is nuclear factor-κB (NF-κB) dependent, we hypothesize that in poorly invasive cancer cells, the heat is an inhibitor of NF-κB, which acts in a dose-dependent manner. Since we observed different effects in highly invasive breast cancer cells, the role of heat remains unclear. Nevertheless, in macrophages, the heat at 41 °C activates the NF-κB-dependent signaling pathway, leading to the production of IL-1β, IL-6, and COX-2. Our opinion is consistent with conclusions by Harper and colleagues [38], who showed that temperature regulates the inflammatory response through the modulation of NF-κB signaling and its downstream mediators.

4. Materials and Methods

4.1. Mistletoe Extract (ME)

Commercially available, standardized ME (brand name Iscador Qu; Weleda AG, Schwäbisch Gmünd, Germany; PZN 1386131) from mistletoe grown on oak trees was used at a dose of 10 μg/mL, which corresponds to doses used for intravenous applications [39].

4.2. MCF-7 Cell Line

The human breast cancer cell line MCF-7 was obtained from the European Collection of Cell Cultures (Lot. 13K023; Salisbury, UK). Cells were cultured in RPMI 1640 culture medium supplemented with 1% antibiotic mixture (100 IU/mL penicillin and 100 µg/mL streptomycin), 10% heat-inactivated fetal bovine serum, and 1× non-essential amino acids (all reagents were from Sigma Aldrich, Darmstadt, Germany). Cells were grown under stable thermal conditions (37 °C), in a humidified atmosphere of 5% CO_2. The culture medium was changed every second day, and cells were removed from culture flasks or plates using 0.25% trypsin–EDTA solution (Sigma Aldrich).

4T1 Cell Line

The murine breast cancer 4T1 cell line was purchased from the American Type Culture Collection (Manassas, VA, USA). Cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS), 100 µg/mL streptomycin, and 100 IU/mL penicillin (all reagents were from Sigma-Aldrich) at 37 °C in a humidified atmosphere with 5% CO_2. The culture medium was changed every 2–3 days. Cells were passaged and/or collected using 0.25% trypsin–EDTA solution (Sigma-Aldrich) when reaching 70–80% of confluency.

4.3. RAW 264.7 Cell Line

The murine macrophage cell line RAW 264.7 was obtained from the European Collection of Cell Cultures (cat. No. 91062702). RAW 264.7 cells were cultured in DMEM culture medium (Sigma Aldrich) supplemented with 10% heat-inactivated FBS and a mixture of antibiotics (100 µg/mL streptomycin and 100 IU/mL penicillin). Cells were maintained under controlled conditions at 37 °C and in a humidified atmosphere with 5% CO_2. The culture medium was changed every second day. To collect the cells, cells were rinsed, and a cell scraper was used to remove adherent cells from the culture plates or culture flasks.

4.4. Heat Exposure and Treatment with Mistletoe Extract

After collection of the cells, the total number of viable cells was determined by trypan blue exclusion, using the LUNA^{TM} automated cell counter (Logos Biosystems, Annandale VA, USA). The cells were seeded in 96-well, 12-well, or 6-well plates at a density of 5×10^3/well, 2×10^5/well, or 3×10^5/well, respectively, depending on the experiments. Following an overnight preincubation at 37 °C, the cells were co-treated with ME (Iscador Qu, Weleda) at a concentration of 10 µg/mL and cultured at 37 °C, 39 °C, or 41 °C for 24 h. Cells cultured at 37 °C and not treated with ME were included as the control cells.

4.5. Assessment of Cell Viability Using the MTT Assay

The viability of the cells (MCF-7,4T1 and RAW 264.7) treated with ME and exposed to various heat conditions was determined using the MTT viability assay. In living cells, the absorbed yellow 3-(4,5-dimethyl thiazolyl)-2,5-diphenyl-tetrazolium bromide (MTT; Sigma Aldrich) is reduced by mitochondrial dehydrogenase to a blue formazan product. Briefly, the cell supernatants were removed, the MTT solution was added (100 µL/well; final concentration of 0.5 mg/mL), and plates were incubated for 3 h at 37 °C, 5% CO_2 in a humidified atmosphere. After the incubation period, the supernatants were removed, and 100 µL of dimethyl sulfoxide was added to dissolve the formazan crystals. The plates were mixed horizontally for 5 min, and the absorbance was measured at 570 nm (with a reference wavelength of 630 nm) using a Synergy HT Multi-Mode Microplate Reader (BioTek Instruments, Winooski, VT, USA). Cell viability was calculated as the percentage of the absorbance 570/630 nm ratio in experimental wells compared to the control wells.

4.6. Reactive Oxygen Species Determination Using Flow Cytometry

Cellular reactive oxygen species (ROS) level was measured by a technique that converts carboxy-H_2DCFDA (5(6)-carboxy-2′,7′-dichlorofluorescein diacetate, Sigma Aldrich)

into a green fluorescence dye 2′,7′–dichlorofluorescein (DCF). Briefly, cultured cells were counted, washed with phosphate-buffered saline (PBS), and resuspended in carboxy-H2DCFDA at a final concentration of 10 µM in regular culture medium with reduced serum (2%). Cells were incubated in the dark for 30 min in a conventional incubator at 37 °C. After the incubation period, cells were washed with PBS, seeded in six-well plates at the density of 3×10^5 cells/well, and treated with ME at a final concentration of 10 µg/mL in regular culture medium with reduced serum (2%). These cells were cultured for 24 h at different thermal conditions. Next, the cells were collected as described above, washed twice with PBS, and resuspended in PBS. ROS level was assessed by immediately analyzing cells using flow cytometry (FL1 channel/green fluorescence) BriCyte E6 flow cytometer (Mindray, Shenzhen, China). Results are presented as a percent change in comparison to control cells (cultured at 37 °C and not treated with ME).

4.7. Cell Cycle Analysis by Flow Cytometry

The cell cycle was determined by the quantitation of DNA content using the nucleic acid stain propidium iodide followed by flow cytometry analysis. Propidium iodide (PI) is a fluorescence dye that binds both types of nucleic acids, DNA and RNA, proportionally to the amount of material present in the nucleus. Cells were seeded at a density of 3 × 105 in six-well plates, as described above. Following overnight preincubation, cells were treated with ME and exposed to various temperature conditions for 24 h. After treatment, the culture media was removed, and cells were collected as described above. Cell cycle analysis was carried out using a CellCycleFlowEx® Kit (Exbio, Vestec, Czech Republic) according to the manufacturer's instructions. Cells were stained for 30 min with PI, and RNA was digested using RNAse. Next, flow cytometry analysis was conducted within 4 h using a BriCyte E6 flow cytometer (Mindray, Shenzhen, China). The cell cycle distribution was presented as the percentage of cells containing 2n (G1 phase), 4n (G2 and M phase), and between 2n and 4n (S phase), as determined by PI staining.

4.8. Quantification of Cytokines and COX-2 mRNA Expression

Following heat exposure and ME treatment, determination of IL-1β, IL-6, and COX-2 mRNA expression was conducted using two-step RT-qPCR. The extraction of total mRNA was conducted according to the Chomczynsky and Sacchi method [40]. PureZOL™ RNA Isolation Reagent (Bio-Rad, Hercules, CA, USA) was used according to the manufacturer's instructions. The final concentration of total mRNA in samples was measured using a Take3 Micro-Volume Plate for the Synergy HT Multi-Mode Microplate Reader. Synthesis of cDNA was carried out on 300 ng of total RNA using iScript™ Reverse Transcription Supermix for RT-qPCR (Bio-Rad), according to the manufacturer's instructions. Quantitative real-time PCR was performed according to the manufacturer's protocol in a final volume of 10 µL containing cDNA, iTaq Universal SYBR® Green Supermix (Bio-Rad) and PrimePCR™ SYBR® Green Assay to amplify IL-1β (Unique Assay ID: qMmuCID0005641 and qHsaCID0022272), IL-6 (Unique Assay ID: qMmuCID0005613 and qHsaCED0044677), COX-2 (Unique Assay ID: qHsaCED0042341), and GAPDH (Unique Assay ID: qMmuCED0027497 and qHsaCED0038674) as a reference gene using the CFX Connect Real-Time PCR Detection System (Bio-Rad). All assays were obtained from Bio-Rad. Test samples were run in triplicates. Each reaction was repeated at least two times. Melt curve analysis was performed on a Bio-Rad CFX96 as a control for specificity of the products. Standard curves were prepared for target (IL-1β, IL-6, and COX-2) and reference (GAPDH) genes. Calibrator-normalized relative quantification was carried out using CFX Manager Software 3.1 (Bio-Rad).

4.9. Statistical Analysis

All values are reported as mean ± standard error of the mean (SEM) of three independent experiments. Statistical significance was determined using analysis of variance (two-way ANOVA) followed by the Tukey test at a critical value of $p < 0.05$.

5. Conclusions

In conclusion, FRH significantly modulates ME-induced effects in both cancer cell lines, as well as in immune cells; therefore, we think that the increase in body temperature observed in some patients after treatment with immunomodulators may be of great significance not only for ME therapy, but for immunotherapies in general. Thus, if fever is not activated after ME administration, whole body FRH can be used as an alternative to fever [41]. However, this procedure needs careful consideration of all consequences, including those pointed in this article. We suggest that more attention should be directed to the role of temperature in the regulation of immune and cancer cells, especially when they are treated with immunomodulators.

Author Contributions: Conceptualization, S.W., H.M.K. and A.D.; methodology, H.M.K., M.P., J.S. and T.J.; validation, M.P., T.J. and J.S.; formal analysis, S.W. and A.D.; investigation, H.M.K., M.P., J.S. and T.J.; resources, A.D.; data curation, H.M.K.; writing—original draft preparation, H.M.K. and S.W.; writing—review and editing, M.P., J.S., T.J. and A.D.; visualization, H.M.K. and J.S.; supervision, S.W.; funding acquisition, H.M.K., S.W. and A.D. All authors have read and agreed to the published version of the manuscript.

Funding: This study was supported by Project nr POWR.03.05.00-00-Z302/17 "Universitas Copernicana Thoruniensis in Futuro" (2018-2022) co-financed from the European Social Fund—the Operational Programme Knowledge Education Development. Module 5. Interdisciplinary PhD School "Academia Copernicana" and the Polish Minister of Science and Higher Education under the program "Regional Initiative of Excellence" in 2019–2022 (Grant No. 008/RID/2018/19).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Data available in a publicly accessible repository (https://repod.icm.edu.pl/, accessed on 8 June 2021).

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Siegel, R.L.; Miller, K.D.; Jemal, A. Cancer statistics, 2019. *CA Cancer J. Clin.* **2019**, *69*, 7–34. [CrossRef] [PubMed]
2. Kienle, G.S.; Berrino, F.; Büssing, A.; Portalupi, E.; Rosenzweig, S.; Kiene, H. Mistletoe in cancer—A systematic review on controlled clinical trials. *Eur. J. Med. Res.* **2003**, *8*, 109–119.
3. Tröger, W.; Galun, D.; Reif, M.; Schumann, A.; Stanković, N.; Milićević, M. *Viscum album* [L.] extract therapy in patients with locally advanced or metastatic pancreatic cancer: A randomised clinical trial on overall survival. *Eur. J. Cancer* **2013**, *49*, 3788–3797. [CrossRef] [PubMed]
4. Marvibaigi, M.; Supriyanto, E.; Amini, N.; Abdul Majid, F.A.; Jaganathan, S.K. Preclinical and Clinical Effects of Mistletoe against Breast Cancer. *Biomed. Res. Int.* **2014**, *2014*, 785479. [CrossRef] [PubMed]
5. Schöffski, P.; Riggert, S.; Fumoleau, P.; Campone, M.; Bolte, O.; Marreaud, S.; Lacombe, D.; Baron, B.; Herold, M.; Zwierzina, H.; et al. Phase I trial of intravenous aviscumine (rViscumin) in patients with solid tumors: A study of the European Organization for Research and Treatment of Cancer New Drug Development Group. *Ann. Oncol.* **2004**, *15*, 1816–1824. [CrossRef]
6. Kienle, G.S.; Glockmann, A.; Schink, M.; Kiene, H. *Viscum album* L. extracts in breast and gynaecological cancers: A systematic review of clinical and preclinical research. *J. Exp. Clin. Cancer Res.* **2009**, *28*, 79. [CrossRef] [PubMed]
7. Kienle, G.S.; Kiene, H. Review article: Influence of *Viscum album* L. (European mistletoe) extracts on quality of life in cancer patients: A systematic review of controlled clinical studies. *Integr. Cancer Ther.* **2010**, *9*, 142–157. [CrossRef] [PubMed]
8. Legnani, W. Mistletoe in conventional oncological practice: Exemplary cases. *Integr. Cancer Ther.* **2008**, *7*, 162–171. [CrossRef] [PubMed]
9. Kienle, G.S.; Grugel, R.; Kiene, H. Safety of higher dosages of *Viscum album* L. in animals and humans–systematic review of immune changes and safety parameters. *BMC Complement. Altern. Med.* **2011**, *11*, 72. [CrossRef]
10. Hutt, N.; Kopferschmitt-Kubler, M.; Cabalion, J.; Purohit, A.; Alt, M.; Pauli, G. Anaphylactic reactions after therapeutic injection of mistletoe (*Viscum album* L.). *Allergol. Immunopathol.* **2001**, *29*, 201–203. [CrossRef]
11. Schläppi, M.; Ewald, C.; Kuehn, J.J.; Weinert, T.; Huber, R. Fever Therapy with Intravenously Applied Mistletoe Extracts for Cancer Patients: A Retrospective Study. *Integr. Cancer Ther.* **2017**, *16*, 479–484. [CrossRef]
12. Reuter, U.R.M.; Oettmeier, R.; Hobohm, U. Safety of Therapeutic Fever Induction in Cancer Patients Using Approved PAMP Drugs. *Transl. Oncol.* **2018**, *11*, 330–337. [CrossRef]
13. Blomqvist, A.; Engblom, D. Neural Mechanisms of Inflammation-Induced Fever. *Neuroscientist* **2018**, *24*, 381–399. [CrossRef]

14. Zampronio, A.R.; Soares, D.M.; Souza, G.E.P. Central mediators involved in the febrile response: Effects of antipyretic drugs. *Temperature* **2015**, *2*, 506–521. [CrossRef]
15. Kozak, W.; Wrotek, S.; Walentynowicz, K.; Waszak, P. Fever and symptoms of sickness are present in mice lacking functional B and T lyphocytes. *Acta Biol. Crac. Ser. Zool.* **2006**, *48*, 9–20. [CrossRef]
16. Liou, G.-Y.; Storz, P. Reactive oxygen species in cancer. *Free Radic. Res.* **2010**, *44*, 479–496. [CrossRef]
17. Heiny, B.M.; Beuth, J. Mistletoe extract standardized for the galactoside-specific lectin (ML-1) induces beta-endorphin release and immunopotentiation in breast cancer patients. *Anticancer Res.* **1994**, *14*, 1339–1342.
18. Büssing, A. Immune Modulation Using Mistletoe (*Viscum album* L.) Extracts Iscador. *Arzneimittelforschung* **2011**, *56*, 508–515. [CrossRef]
19. Oei, S.L.; Thronicke, A.; Schad, F. Mistletoe and Immunomodulation: Insights and Implications for Anticancer Therapies. *Evid. Based Complement. Alternat. Med.* **2019**, *2019*, 5893017. [CrossRef]
20. Kienle, G.S. Fever in Cancer Treatment: Coley's Therapy and Epidemiologic Observations. *Glob. Adv. Health Med.* **2012**, *1*, 92–100. [CrossRef]
21. Wrotek, S.; Brycht, Ł.; Wrotek, W.; Kozak, W. Fever as a factor contributing to long-term survival in a patient with metastatic melanoma: A case report. *Complement. Ther. Med.* **2018**, *38*, 7–10. [CrossRef] [PubMed]
22. Kozak, W.; Jedrzejewski, T.; Pawlikowska, M.; Piotrowski, J.; Wrotek, S. Toward Antitumor Immunity and Febrile Infections: Gamma/Delta (γδ) T Cells Hypothesis. *Q. Rev. Biol.* **2018**, *93*, 187–205. [CrossRef]
23. Weissenstein, U.; Kunz, M.; Urech, K.; Baumgartner, S. Interaction of standardized mistletoe (*Viscum album*) extracts with chemotherapeutic drugs regarding cytostatic and cytotoxic effects in vitro. *BMC Complement. Altern. Med.* **2014**, *14*, 6. [CrossRef] [PubMed]
24. Klingbeil, M.G.; Xavier, F.C.A.; Sardinha, L.R.; Severino, P.; Mathor, M.B.; Rodrigues, R.V.; Pinto, D.S. Cytotoxic effects of mistletoe (*Viscum album* L.) in head and neck squamous cell carcinoma cell lines. *Oncol. Rep.* **2013**, *30*, 2316–2322. [CrossRef]
25. Forman, H.J.; Torres, M. Redox signaling in macrophages. *Mol. Aspects Med.* **2001**, *22*, 189–216. [CrossRef]
26. Tan, H.-Y.; Wang, N.; Li, S.; Hong, M.; Wang, X.; Feng, Y. The Reactive Oxygen Species in Macrophage Polarization: Reflecting Its Dual Role in Progression and Treatment of Human Diseases. *Oxid. Med. Cell Longev.* **2016**, *2016*, 2795090. [CrossRef]
27. Lasso, P.; Llano Murcia, M.; Sandoval, T.A.; Urueña, C.; Barreto, A.; Fiorentino, S. Breast Tumor Cells Highly Resistant to Drugs Are Controlled Only by the Immune Response Induced in an Immunocompetent Mouse Model. *Integr. Cancer Ther.* **2019**. [CrossRef]
28. Gayan, S.; Teli, A.; Dey, T. Inherent aggressive character of invasive and non-invasive cells dictates the in vitro migration pattern of multicellular spheroid. *Sci. Rep.* **2017**, *7*, 11527. [CrossRef]
29. Comşa, S.; Cîmpean, A.M.; Raica, M. The Story of MCF-7 Breast Cancer Cell Line: 40 years of Experience in Research. *Anticancer Res.* **2015**, *35*, 3147–3154.
30. Lee, O.-Y.; Park, Y.; Seo, M.W.; Lee, D.-S. IL-1β induces IL-6 production and increases invasiveness and estrogen-independent growth in a TG2-dependent manner in human breast cancer cells. *BMC Cancer* **2016**, *16*, 724. [CrossRef]
31. Wang, L.; Zhang, S.; Wu, H.; Rong, X.; Guo, J. M2b macrophage polarization and its roles in diseases. *J. Leukoc. Biol.* **2019**, *106*, 345–358. [CrossRef]
32. Dhabekar, G.; Dandekar, R.; Kingaonkar, A. Role of macrophages in malignancy. *Ann. Maxillofac. Surg.* **2011**, *1*, 150. [CrossRef]
33. Najafi, M.; Hashemi Goradel, N.; Farhood, B.; Salehi, E.; Nashtaei, M.S.; Khanlarkhani, N.; Khezri, Z.; Majidpoor, J.; Abouzaripour, M.; Habibi, M.; et al. Macrophage polarity in cancer: A review. *J. Cell Biochem.* **2019**, *120*, 2756–2765. [CrossRef]
34. Veremeyko, T.; Yung, A.W.Y.; Anthony, D.C.; Strekalova, T.; Ponomarev, E.D. Early Growth Response Gene-2 Is Essential for M1 and M2 Macrophage Activation and Plasticity by Modulation of the Transcription Factor CEBPβ. *Front. Immunol.* **2018**, *9*, 2515. [CrossRef]
35. Guzhova, I.V.; Shevtsov, M.A.; Abkin, S.V.; Pankratova, K.M.; Margulis, B.A. Intracellular and extracellular Hsp70 chaperone as a target for cancer therapy. *Int. J. Hyperthermia* **2013**, *29*, 399–408. [CrossRef]
36. Asea, A.; Rehli, M.; Kabingu, E.; Boch, J.A.; Bare, O.; Auron, P.E.; Stevenson, M.A.; Calderwood, S.K. Novel signal transduction pathway utilized by extracellular HSP70: Role of toll-like receptor (TLR) 2 and TLR4. *J. Biol. Chem.* **2002**, *277*, 15028–15034. [CrossRef]
37. Fang, H.; Wu, Y.; Huang, X.; Wang, W.; Ang, B.; Cao, X.; Wan, T. Toll-like receptor 4 (TLR4) is essential for Hsp70-like protein 1 (HSP70L1) to activate dendritic cells and induce Th1 response. *J. Biol. Chem.* **2011**, *286*, 30393–30400. [CrossRef]
38. Harper, C.V.; Woodcock, D.J.; Lam, C.; Garcia-Albornoz, M.; Adamson, A.; Ashall, L.; Rowe, W.; Downton, P.; Schmidt, L.; West, S.; et al. Temperature regulates NF-κB dynamics and function through timing of A20 transcription. *Proc. Natl. Acad. Sci. USA* **2018**, *115*, E5243–E5249. [CrossRef]
39. Weissenstein, U.; Kunz, M.; Urech, K.; Regueiro, U.; Baumgartner, S. Interaction of a standardized mistletoe (*Viscum album*) preparation with antitumor effects of Trastuzumab in vitro. *BMC Complement. Altern. Med.* **2016**, *16*, 271. [CrossRef]
40. Chomczynski, P.; Sacchi, N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* **1987**, *162*, 156–159. [CrossRef]
41. Lassche, G.; Crezee, J.; Van Herpen, C.M.L. Whole-body hyperthermia in combination with systemic therapy in advanced solid malignancies. *Crit. Rev. Oncol. Hematol.* **2019**, *139*, 67–74. [CrossRef]

4. Discussion

The dysregulation of the immune system can arise from a range of factors, including both genetic and environmental influences such as pollutants, toxins, and infectious agents [51,52]. Moreover, chronic stress, inadequate nutrition, physical inactivity, and the natural aging process are linked to a diminished function of the immune system, resulting in heightened vulnerability to infections, increased risk of developing autoimmune disorders, and a greater susceptibility to cancer [53]. In addition, the emergence of novel infectious diseases, such as COVID-19, underscores the critical significance of preserving a robust immune system [54]. Consequently, there is a pressing need to advance the development of efficacious immunomodulatory therapies, that could precisely modulate the immune system's activity as per the specific needs by either stimulating or inhibiting it [55].

To suppress the immune system, drugs that are commonly known as immunosuppressants are used. Some common examples of immunosuppressants include corticosteroids (such as prednisone), calcineurin inhibitors (such as tacrolimus and cyclosporine), and biologic drugs (monoclonal antibodies such as rituximab and infliximab). Such immunosuppressants are used either for treating autoimmune diseases, or to prevent organ rejection [56]. On the other hands, drugs, such as interferons and interleukins, can be used to enhance the immune system's activity in immunodeficiencies of various sources such as primary immunodeficiency disorders or HIV infection [57,58]. These therapies can boost the immune system's ability against infections and prevent from side effects. Furthermore, immunomodulatory therapies can be used in cancer treatment to stimulate the immune system to recognize and attack cancer cells. In recent years, these therapies have shown great promise, and they are effectively used for previously difficult-to-treat cancers like melanoma, lung cancer, and bladder cancer [59].

Although the above-mentioned drugs are invaluable in the treatment of patients, they come with many side effects. Therefore, it is important to further explore other methods of modulating the immune system. Especially desirable are those that could be used in disease prevention.

Fever-range hyperthermia is a medical treatment that involves heating of the body to a temperature between 39°C to 41°C [32]. The use of FRH as an immunomodulatory therapy has

been studied extensively in clinics for recent years, and has shown promising results in the treatment of various medical conditions, including viral infections, autoimmune disorders and to support cancer treatment [5,30,60]. Although studies have shown that FRH can have a range of beneficial effects on the immune system, further research is still needed to fully understand the underlying mechanisms. Therefore, in my research I focused on three tasks that I discuss below.

#Aim 1 *to comprehensively investigate how FRH treatment affects hematological profile, and regulatory molecules such as cytokines, and miRNA at the level of entire organism*

Whole body FRH in addition to being a therapy, is also used as a surrogate of fever in experimental settings since it is the most similar to fever in terms of the physiological response. Published data showed that whole body FRH affects murine immune system, resulting in changes in leukocyte number and their activity that are dependent on time and temperature [61]. Hyperthermia treatment over 41°C, and lasting 20 minutes increases the total number of murine peripheral leukocytes shortly after the treatment. However, after 4 hours, the total number of peripheral blood leukocytes returns to a physiological range [61]. Despite this, whole body hyperthermia lasting more than 2 hours at range 39-40°C, shows total decrease of circulating leukocytes due to a decrease in circulating lymphocytes and an increased number of granulocytes. This shift in leukocyte population distribution may persist for up to one week [61] and is associated with increased G-CSF expression induced by hyperthermia [62]. In my research on rats, I found that directly after 1-hour lasting FRH treatment, a decrease in the number of circulating lymphocytes was observed (**Article #1**). Next, in **Article #3**, I presented that FRH is not cytotoxic and increases the viability of macrophages. These findings (decrease in the number of lymphocytes and lack of cytotoxic properties of FRH) are in accordance with Evans et al (2001) who have shown that FRH treatment can stimulate the adhesion of lymphocytes to high endothelial venules in lymph nodes through both L-selectin and α4β7 integrin pathways [63]. Thus, it is likely that FRH can lead to a temporary decrease in circulating lymphocytes due to increased trafficking and homing of lymphocytes to lymph nodes. This process can enhance immune surveillance and better protection of the body from potential threats [63].

Additionally, I observed an increase in the number of circulating granulocytes, which I found to be associated with changes in the expression of G-CSF (**Article #1**). It is noteworthy, that similar observations were made during my preliminary research in humans, where after a single FRH treatment I observed an increase in the level of G-CSF in human serum (*data not published*).

FRH treatment has been found to induce the release of heat shock proteins (HSPs) from cells [64,65]. HSPs are a group of highly conserved proteins that play a crucial role in cellular stress response and act as chaperones for other proteins to maintain their proper folding and function [66]. The release of HSPs during FRH treatment may contribute to immunomodulatory effects [67]. Studies have shown that various HSPs released during FRH treatment can stimulate the production of various set of cytokines (IL-6, TNF-α, or G-CSF) leading to an enhanced immune response [68]. Indeed, in my studies I found elevated expression of selected cytokines such as IL-10, G-CSF, IFN-γ, MIF-1α after FRH treatment *in vivo* as well as IL-6 and IL-1β *in vitro*. Interestingly, I have found, that FRH treatment triggers other regulatory molecules such as miRNAs that have not been extensively studied so far.

Although it has been shown that FRH modifies the expression of selected miRNA in selected tissues or cells [69,70], little attention has been paid whether FRH induces changes in serum miRNA, which play a distinct regulatory role in entire organism. Serum miRNAs are small, non-coding RNA molecules that are found in the blood. They are involved in the regulation of gene expression and play a key role in various physiological and pathological processes [71]. Serum miRNAs have been shown to be stable and resistant to degradation, making them attractive candidates for use as diagnostic and prognostic biomarkers for a range of diseases, including cancer, cardiovascular disease, and neurodegenerative disorders [71,72]. In my *in vivo* research I have shown, that FRH increases in all examined tissues' expression of key molecules involved in the processing and maturation of miRNAs i.e., Dicer1 and Tarbp2. Furthermore, I have found that FRH decreases the expression of serum miRNA-155 in rats. A similar observation was made by Oshlag et al. (2013), who observed increased Dicer expression in various cell lines following FRH treatment, which was linked to changes in miRNA expression. However, these data were obtained in *in vitro* studies [73]. The heat-induced

alterations in Dicer and Tarbp2 suggest that miRNA biogenesis may be heat-sensitive. Thus, these findings reveal potentially new regulatory molecules that are activated by FRH treatment.

In research by Sun et al. (2012) an increased expression of miRNA-155 was observed at early stages in breast cancer patients in comparison to healthy control. These authors believe that it can be used as diagnostic biomarker, as well as biomarker for treatment efficiency [74,75]. Since I showed, that whole body FRH decreases the level of miRNA-155 (**Article #1**), as well as I have noticed direct cytotoxic effect of FRH on breast cancer cell lines (**Article #3**), my data provides support for the efficacy of hyperthermia as a valid treatment option for at least breast cancer.

Based on the evidence I collected through my research; it can be concluded that whole body FRH treatment exerts a regulatory influence on the entire organism by altering the expression of various regulatory molecules. FRH affects gene expression at multiple levels, including mRNA transcription, post-transcriptional processing (miRNA), and protein synthesis. All these changes lead to global change in the number of circulating leukocytes. In addition, my findings suggest that FRH treatment can modify the expression of regulatory molecules such as cytokines, as well as miRNA molecules.

#**Aim 2** *to accurately identify the FRH-induced macrophage phenotype, including the diversity of macrophage subsets*

In my research, I have a particular interest in macrophages, which are a type of innate immune cell. These cells have the unique ability to work independently, as they are specialized to detect, engulf, and eliminate pathogens. Macrophages can also activate other cells and trigger inflammation by releasing cytokines and presenting antigens to T cells [46].

In rats, I observed that the FRH treatment induced a decrease in the number of circulating lymphocytes, which subsequently resulted in a predominance of monocytes in their PBMCs (**Article #1**). My further analysis of the expression of pro-inflammatory IFN-γ and anti-inflammatory IL-10 in these PBMCs showed a dynamic increase. It is well known that these

factors are involved in macrophage polarization into opposing phenotypes [36]. To resolve the issue of FRH-induced macrophage phenotype, I conducted additional *in vitro* research.

My *in vitro* study began by examining the effect of FRH on macrophage viability. In **Article #3**, I found that FRH has a significant impact on the cell cycle distribution of macrophages. The increase in the number of cells in the G1 phase at 39°C and the increase in the number of cells in the G2/M phase at 41°C suggest that hyperthermia stimulates macrophage proliferation (**Article #3**). Indeed, I observed a temperature-dependent increase in cell proliferation measured by MTT assay (**Article #3**). Therefore, I showed, that FRH creates conditions that favor the proliferation of macrophages *in vitro*.

Next, I have shown that FRH-treated macrophages display increased expression of surface CD163, and increased expression of arginase-1, both are markers of alternatively activated M2 macrophages (**Article #2**). Previously, other authors have found that M2 cells produce anti-inflammatory cytokines such as IL-10 and TGF-β [76]. My results, which showed an increased expression of IL-10 in PBMCs isolated from rats (**Article #1**), are consistent with this finding. Additionally, Zhang et al. (2020) demonstrated that miR-155 expression is upregulated in M1 microglia phenotype and downregulated in M2 cells, and suppressing miR-155 promotes the shift from a pro-inflammatory M1 polarization to an anti-inflammatory M2. Therefore, my findings showing a decrease in miRNA-155 after FRH treatment (**Article #1**) support the conclusion that FRH induces an M2 phenotype in macrophages. In addition to these findings, I observed significantly lower levels of M1 markers such as CD80, iNOS, and nitric oxide (**Article #2**).

There are also other factors that serve as markers of macrophage polarization. Studies have shown that macrophages with an M1 pro-inflammatory phenotype produce high levels of reactive oxygen species. It is believed, that M1 macrophages use ROS to kill invading microorganisms such as bacteria, viruses, and fungi [77]. Together with nitric oxide and other oxygen compounds, ROS are part of the respiratory burst [78]. Other authors have shown that hyperthermia enhances the respiratory burst of granulocytes and increases their antibacterial and phagocytic activity [79]. In addition to this, I found that FRH treatment increases the level

of ROS in macrophages (**Article #2 and Article #3**). Since I identified this effect, it has become clear that hyperthermia leads to one of the intermediate states of polarization. Thus, taking into consideration my finding:

- presented in **Article #3** that FRH increases the level of pro-inflammatory cytokines such as IL-1β and IL-6;

- presented in **Article #1** that directly after FRH treatment the expression of pro-inflammatory IFN-γ dynamically increases in PBMC's isolated from rats;

- presented in **Article #1** that FRH triggers overexpression of G-CSF in rats' liver, which is considered as a pro-inflammatory cytokine.

I concluded that the cells that I considered to be M2 belong to M2b subtype. M2b macrophages are a subset of alternatively activated macrophages that are induced by exposure to immune complexes and TLR ligands [80]. Based on my research, FRH can be classified as one of the factors that induce M2b macrophages.

Although M2b cells are classified as an anti-inflammatory cell due to the increased production of IL-10 [80], what is consistent with my finding described in **Article #1**, these cells are able to release pro-inflammatory cytokines such as IL-1β and IL-6 [80], what I observed and described in the **Article #3**.

In conclusion, my study has shown that FRH induces the expression of M2b polarization markers in macrophages. This intermediate state of polarization is characterized by the expression of both M1 and M2 markers and is considered as regulatory macrophages. Thus, FRH could be a promising therapeutic approach for treating chronic diseases that are linked to imbalances in macrophage phenotypes. Additionally, when interpreting the obtained results in the context of fever's mechanism, it appears that the elevation of body temperature during fever may play a role in resolving inflammation.

#Aim 3 *to explore the potential of FRH to modify the effects of the TLR-4-dependent stimulators*

In **Article #2**, I demonstrated that FRH is a potent modulator of macrophage polarization into the M2b phenotype. Additionally, FRH can stimulate the production of pro-inflammatory mediators such as ROS (as shown in **Article #2** and **Article #3**) and various cytokines (as demonstrated in **Article #1** and **Article #3**), as well as pro-inflammatory proteins such as COX-2 and TLR-4 (**Article #2**). All of these factors share a common reliance on the TLR-4 pathway. Consequently, it is of interest to investigate whether FRH can modify the effects of other immunomodulators, such as LPS or ME, that act via the TLR-4 pathway.

As reported in the literature, LPS triggers M1 polarization in macrophages [47]. In my study, I found that treatment of macrophages with LPS leads to elevated expression of all tested M1 markers, such as ROS, NO, iNOS and surface CD80 (**Article #2**). However, when FRH was administered in addition to LPS, the M1 polarization induced by LPS was reversed to an M2 phenotype. This was evidenced by the increased expression of CD163 and decreased expression of CD80 surface markers, as well as a reduction in ROS and NO levels. Notably, simultaneous treatment with LPS and FRH resulted in an increased expression of iNOS and Arg-1 (**Article #2**). Based on these results, it can be concluded that FRH is capable of inducing an M2b regulatory phenotype in macrophages that is more potent than the M1 polarization induced by LPS. These findings emphasize a new role for fever-associated heat in both the development of inflammation and the restoration of tissue homeostasis. FRH has the ability to shift macrophages from the pro-inflammatory M1 phenotype that develops early in infection to a regulatory M2b phenotype that enhances tissue repair and regeneration. This supports the opinion that fever plays a significant role in maintaining proper immune homeostasis.

Increase in body temperature may result from reactions to Mistletoe Extract administration [81], however little is known about significance of this effect for ME-induced immunomodulation. ME is derived from the European mistletoe (*Viscum album*), and has been used for centuries as a traditional medicine [82]. The extract contains a variety of biologically active compounds, including lectins, viscotoxins, and triterpene acids. Lectins are proteins that

can bind to specific sugar molecules on the surface of cells, and mistletoe lectins have been shown to have immunomodulatory and anti-tumor effects. Viscotoxins are peptide toxins that can affect cell membranes and cause cell death, and have been shown to have cytotoxic effects on cancer cells [83]. It is believed, that mistletoe lectin similarly to LPS exhibits potent immunomodulatory properties dependently on TLR-4 pathway [84].

I observed that ME did not affect the cell cycle distribution in macrophages, however it reduces macrophage viability (**Article #3**). Interestingly, this negative effect was mitigated by additional treatment with FRH. These findings indicate, that fever following ME administration may be of great significance for proper immune responses. Therefore, the application of FRH may be a potential treatment option to consider in cases where there is a lack of fever following ME administration. However, further research is necessary to fully elucidate the potential therapeutic applications of hyperthermia in the context of immune modulation.

The administration of ME did not generate ROS in macrophages; however, when FRH was applied at 41°C, a twofold increase in ROS levels was observed compared to FRH at 39°C, (as described in **Article #3**). Moreover, FRH at 41°C induced higher expression of pro-inflammatory cytokines (IL-6 and IL-1β) as well as COX-2 mRNA compared to FRH at 39°C (**Article #3**). These results suggest that FRH at 41°C may elicit a stronger pro-inflammatory response, thus pro-inflammatory properties of FRH seems to be temperature-dependent.

It is commonly accepted that FRH treatment induces the release of heat shock proteins (HSPs) from cells [64,65]. Since M2b macrophages express high levels of TLR-4 (**Article #2**), and selected HSPs may act as ligands for TLR-4 [67], it was important to determine whether M2b polarization was also TLR-4 dependent. I observed that inhibition of TLR-4 with TAK-242 small molecule inhibitor did not affect the expression of M2 surface markers in macrophages. Interestingly, TAK-242 alone induced Arg-1 expression in both examined cell lines, however, this effect was weaker than in FRH-treated cells. These findings stay in line with available literature, showing that TAK-242 may induce M2 polarization in microglial cells [85]. Additionally, there was no significant influence of TLR-4 inhibition on the iNOS level

(**Article #2**). Therefore, I concluded, that FRH induces M2b polarization in TLR-4 independent manner.

Research examining the effects of FRH on macrophages has demonstrated that it influences the TLR-4 pathway, leading to increased expression of TLR-4, pro-inflammatory cytokines (IL-1β, IL-6), and COX-2 (**Article #2** and **Article #3**). Although this suggests that FRH may act through the TLR-4 pathway to increase the pro-inflammatory response, analysis of macrophage polarization indicates, that this effect is TLR-4 independent (**Article #2**). Further studies are necessary to fully understand the mechanisms by which FRH affects macrophage function and to identify potential therapeutic applications of hyperthermia in the context of immune modulation.

In conclusion, studies on the effects of FRH on macrophages have shown that it can stimulate macrophage proliferation and increase the expression of pro-inflammatory cytokines and COX-2. Although the effect was initially thought to be TLR-4 dependent, additional investigation revealed that it is actually not influenced by TLR-4. Collectively, these findings suggest that fever may play a significant role in maintaining proper immune homeostasis and have potential therapeutic applications in immune modulation.

5. Conclusions and future perspectives

The studies presented in this dissertation explored the potential of fever-range hyperthermia to modify immune system. Despite the fact that hyperthermia has been used in medicine for a long time, it is still not fully understood in terms of all its effects on cells or the body. The results presented in this dissertation provided evidences to support the conclusion that:

1) FRH as a medical treatment has potential for achieving various therapeutic effects at the level of gene expression, proteins, or whole organisms. Furthermore, FRH may also be beneficial in promoting recovery, as it has been shown to maintain immune homeostasis.
2) Febrile increase in body temperature is a crucial factor in the resolution of inflammation, as it may help to prevent chronic inflammation by inducing a regulatory macrophage phenotype.
3) Fever-range hyperthermia is a potent immunomodulator that can change the effects of other stimulators. Therefore, the application of FRH as a complementary therapy during treatment with immunomodulators can be considered. This approach may have the potential to enhance therapeutic efficacy and provide additional benefits.

In summary, my research has revealed that the biological effects of fever-range hyperthermia are more complex than previously believed. FRH has a multifaceted influence on biological processes, spanning from molecular and cellular to organismal levels. The complexity of FRH-induced changes extends beyond the heat shock proteins and their immunomodulatory effects observed via the TLR-4 pathway. My findings suggest that FRH may also act through other molecules, such as cytokines and miRNA leading to alterations in leukocyte populations and macrophage functional activity. Overall, these results indicate that FRH has significant potential as a powerful immunomodulator of the immune system. In addition, my research highlights the essential contribution of heat associated with fever to the inflammatory response.

6. Acknowledgments

6.1. Founding

Research included in this dissertation were founded by:

Project no POWR.03.05.00-00-Z302/17 “Universitas Copernicana Thoruniensis in Futuro” (2018-2022) co-financed by the European Social Fund—the Operational Programme Knowledge Education Development. Module 5. Interdisciplinary PhD School “Academia Copernicana”

6.2. Ethics approval

The animal study protocol was approved by the local Bioethical Committee for Animal Care of Bydgoszcz University of Science and Technology at the Faculty of Animal Breeding and Biology (permission no. 49/2020, date of approval 24.01.2020).

The human samples collection, used in preliminary studies was approved by the Bioethics Committee of the Nicolaus Copernicus University in Toruń at the Ludwik Rydygier Collegium Medicum in Bydgoszcz (permission no KB735/2018, date of approval 30.10.2018)

7. References

1. Gas, P. Essential Facts on the History of Hyperthermia and Their Connections with Electromedicine. *Przeglad Elektrotechniczny* 2011, *87*, 37–40.
2. Cavaillon, J.M. Once upon a Time, Inflammation. *J Venom Anim Toxins Incl Trop Dis* **2021**, *27*, doi:10.1590/1678-9199-JVATITD-2020-0147.
3. Seegenschmiedt M.H.; Fessenden P.; Vernon C.C. *Thermoradiotherapy and Thermochemotherapy*; 1st ed.; Springer Berlin, Heidelberg: Berlin, 1995;
4. Gartlehner, G.; Stepper, K. Julius Wagner-Jauregg: Pyrotherapy, Simultanmethode, and 'Racial Hygiene.' *J R Soc Med* **2012**, *105*, 357, doi:10.1258/JRSM.2012.12K0049.
5. Hasday, J.D.; Shah, N.; Mackowiak, P.A.; Tulapurkar, M.; Nagarsekar, A.; Singh, I. Fever, Hyperthermia, and the Lung: It's All about Context and Timing. *Trans Am Clin Climatol Assoc* **2011**, *122*, 34.
6. Balli, S.; Shumway, K.R.; Sharan, S. Physiology, Fever. *StatPearls* **2022**.
7. Steiner, A.A.; Chakravarty, S.; Rudaya, A.Y.; Herkenham, M.; Romanovsky, A.A. Bacterial Lipopolysaccharide Fever Is Initiated via Toll-like Receptor 4 on Hematopoietic Cells. *Blood* **2006**, *107*, 4000, doi:10.1182/BLOOD-2005-11-4743.
8. Romanovsky, A.A.; Kulchitsky, V.A.; Simons, C.T.; Sugimoto, N. Methodology of Fever Research: Why Are Polyphasic Fevers Often Thought to Be Biphasic? *Am J Physiol* **1998**, *275*, doi:10.1152/AJPREGU.1998.275.1.R332.
9. Rudaya, A.Y.; Steiner, A.A.; Robbins, J.R.; Dragic, A.S.; Romanovsky, A.A. Thermoregulatory Responses to Lipopolysaccharide in the Mouse: Dependence on the Dose and Ambient Temperature. *Am J Physiol Regul Integr Comp Physiol* **2005**, *289*, doi:10.1152/AJPREGU.00370.2005.
10. Janeway, C.A.; Medzhitov, R. Innate Immune Recognition. *Annu Rev Immunol* **2002**, *20*, 197–216, doi:10.1146/ANNUREV.IMMUNOL.20.083001.084359.
11. Park, B.S.; Lee, J.O. Recognition of Lipopolysaccharide Pattern by TLR4 Complexes. *Experimental & Molecular Medicine 2013 45:12* **2013**, *45*, e66–e66, doi:10.1038/emm.2013.97.
12. Ciesielska, A.; Matyjek, M.; Kwiatkowska, K. TLR4 and CD14 Trafficking and Its Influence on LPS-Induced pro-Inflammatory Signaling. *Cellular and Molecular Life Sciences* **2021**, *78*, 1233, doi:10.1007/S00018-020-03656-Y.
13. Kawasaki, T.; Kawai, T. Toll-like Receptor Signaling Pathways. *Front Immunol* **2014**, *5*, 461, doi:10.3389/FIMMU.2014.00461/BIBTEX.

14. Akira, S.; Takeda, K. Toll-like Receptor Signalling. *Nature Reviews Immunology 2004 4:7* **2004**, *4*, 499–511, doi:10.1038/nri1391.

15. Kozak, W.; Kluger, M.J.; Tesfaigzi, J.; Kozak, A.; Mayfield, K.P.; Wachulec, M.; Dokladny, K. Molecular Mechanisms of Fever and Endogenous Antipyresis. *Ann N Y Acad Sci* **2000**, *917*, 121–134, doi:10.1111/J.1749-6632.2000.TB05376.X.

16. Blatteis, C.M.; Li, S.; Li, Z.; Feleder, C.; Perlik, V. Cytokines, PGE2 and Endotoxic Fever: A Re-Assessment. *Prostaglandins Other Lipid Mediat* **2005**, *76*, 1–18, doi:10.1016/J.PROSTAGLANDINS.2005.01.001.

17. Gandhi, J.; Khera, L.; Gaur, N.; Paul, C.; Kaul, R. Role of Modulator of Inflammation Cyclooxygenase-2 in Gammaherpesvirus Mediated Tumorigenesis. *Front Microbiol* **2017**, *8*, doi:10.3389/FMICB.2017.00538.

18. Milton, A.S.; Wendlandt, S. Effects on Body Temperature of Prostaglandins of the A, E and F Series on Injection into the Third Ventricle of Unanaesthetized Cats and Rabbits. *J Physiol* **1971**, *218*, 325–336, doi:10.1113/JPHYSIOL.1971.SP009620.

19. Nakamura, K.; Morrison, S.F. A Thermosensory Pathway That Controls Body Temperature. *Nat Neurosci* **2008**, *11*, 62, doi:10.1038/NN2027.

20. Evans, S.S.; Repasky, E.A.; Fisher, D.T. Fever and the Thermal Regulation of Immunity: The Immune System Feels the Heat. *Nat Rev Immunol* **2015**, *15*, 335, doi:10.1038/NRI3843.

21. Jamshidi, N.; Dawson, A. The Hot Patient: Acute Drug-Induced Hyperthermia. *Aust Prescr* **2019**, *42*, 24, doi:10.18773/AUSTPRESCR.2019.006.

22. Georgitis, J.W. Nasal Hyperthermia and Simple Irrigation for Perennial Rhinitis: Changes in Inflammatory Mediators. *Chest* **1994**, *106*, 1487–1492, doi:10.1378/chest.106.5.1487.

23. Weinberger, A.; Fadilah, R.; Lev, A.; Shohami, E.; Pinkhas, J. Treatment of Articular Effusions with Local Deep Microwave Hyperthermia. *Clin Rheumatol* **1989**, *8*, 461–466, doi:10.1007/BF02032097.

24. Lepock, J.R. Cellular Effects of Hyperthermia: Relevance to the Minimum Dose for Thermal Damage. *Int J Hyperthermia* **2003**, *19*, 252–266, doi:10.1080/0265673031000065042.

25. Toraya-Brown, S.; Fiering, S. Local Tumour Hyperthermia as Immunotherapy for Metastatic Cancer. *International Journal of Hyperthermia* **2014**, *30*, 531–539, doi:10.3109/02656736.2014.968640.

26. Fiorentini, G.; Sarti, D.; Gadaleta, C.D.; Ballerini, M.; Fiorentini, C.; Garfagno, T.; Ranieri, G.; Guadagni, S. A Narrative Review of Regional Hyperthermia: Updates From 2010 to 2019. *Integr Cancer Ther* **2020**, *19*, doi:10.1177/1534735420932648.

27. Yi, G.Y.; Kim, M.J.; Kim, H.I.; Park, J.; Baek, S.H. Hyperthermia Treatment as a Promising Anti-Cancer Strategy: Therapeutic Targets, Perspective Mechanisms and Synergistic Combinations in Experimental Approaches. *Antioxidants* **2022**, *11*, doi:10.3390/ANTIOX11040625.

28. Kok, H.P.; Wust, P.; Stauffer, P.R.; Bardati, F.; van Rhoon, G.C.; Crezee, J. Current State of the Art of Regional Hyperthermia Treatment Planning: A Review. *Radiation Oncology 2015 10:1* **2015**, *10*, 1–14, doi:10.1186/S13014-015-0503-8.

29. Datta, N.R.; Kok, H.P.; Crezee, H.; Gaipl, U.S.; Bodis, S. Integrating Loco-Regional Hyperthermia Into the Current Oncology Practice: SWOT and TOWS Analyses. *Front Oncol* **2020**, *10*, 819, doi:10.3389/FONC.2020.00819/BIBTEX.

30. Behrouzkia, Z.; Joveini, Z.; Keshavarzi, B.; Eyvazzadeh, N.; Aghdam, R.Z. Hyperthermia: How Can It Be Used? *Oman Med J* **2016**, *31*, 89, doi:10.5001/OMJ.2016.19.

31. Wust, P.; Hildebrandt, B.; Sreenivasa, G.; Rau, B.; Gellermann, J.; Riess, H.; Felix, R.; Schlag, P. Hyperthermia in Combined Treatment of Cancer. *Lancet Oncology* **2002**, *3*, 487–497, doi:10.1016/S1470-2045(02)00818-5.

32. Wehner, H.; Von Ardenne, A.; Kaltofen, S. Whole-Body Hyperthermia with Water-Filtered Infrared Radiation: Technical-Physical Aspects and Clinical Experiences. *Int J Hyperthermia* **2001**, *17*, 19–30, doi:10.1080/02656730150201561.

33. Steinhart, C.R.; Ash, S.R.; Gingrich, C.; Sapir, D.; Keeling, G.N.; Yatvin, M.B. Effect of Whole-Body Hyperthermia on AIDS Patients with Kaposi's Sarcoma: A Pilot Study. *Journal of Acquired Immune Deficiency Syndromes and Human Retrovirology* **1996**, *11*, 271–281, doi:10.1097/00042560-199603010-00007.

34. Ostrow, S.; van Echo, D.; Whitacre, M.; Aisner, J.; Simon, R.; Wiernik, P.H. Physiologic Response and Toxicity in Patients Undergoing Whole-Body Hyperthermia for the Treatment of Cancer Available online: https://www.ncbi.nlm.nih.gov/pubmed/7237454 (accessed on 28 February 2020).

35. Ponikowska, I.; Kozłowski, H.M.; Adamczyk, P.; Wrotek, S. Hipertermoterapia Ogólnoustrojowa – Zastosowanie w Medycynie Whole Body Hyperthermotherapy – Application in Medicine. *Acta Balneologica* **2019**, *LXI*, 269–273.

36. Gordon, S.; Martinez, F.O. Alternative Activation of Macrophages: Mechanism and Functions. *Immunity* **2010**, *32*, 593–604, doi:10.1016/J.IMMUNI.2010.05.007.

37. Fujiwara, N.; Kobayashi, K. Macrophages in Inflammation. *Curr Drug Targets Inflamm Allergy* **2005**, *4*, 281–286, doi:10.2174/1568010054022024.

38. Martinez, F.O.; Sica, A.; Mantovani, A.; Locati, M. Macrophage Activation and Polarization. *Front Biosci* **2008**, *13*, 453–461, doi:10.2741/2692.

39. Hop, H.T.; Arayan, L.T.; Reyes, A.W.B.; Huy, T.X.N.; Min, W.G.; Lee, H.J.; Rhee, M.H.; Chang, H.H.; Kim, S. Heat-Stress-Modulated Induction of NF-KB Leads to Brucellacidal pro-Inflammatory Defense against Brucella Abortus Infection in Murine Macrophages and in a Mouse Model. *BMC Microbiol* **2018**, *18*, doi:10.1186/S12866-018-1185-9.

40. Lee, C.-H.; Choi, E.Y. Macrophages and Inflammation. *Journal of Rheumatic Diseases* **2018**, *25*, 11–18, doi:10.4078/JRD.2018.25.1.11.

41. Kozak, W.; Wrotek, S.; Walentynowicz, K.; Waszak, P. Fever and Symptoms of Sickness Are Present in Mice Lacking Functional B and T Lyphocytes. *ACTA BIOLOGICA CRACOVIENSIA Series Zoologia* **2006**, *48*, 9–20, doi:10.13140/2.1.3640.8006.

42. Cantet, J.M.; Yu, Z.; Ríus, A.G. Heat Stress-Mediated Activation of Immune–Inflammatory Pathways. *Antibiotics* **2021**, *10*, doi:10.3390/ANTIBIOTICS10111285.

43. Zhang, H.G.; Mehta, K.; Cohen, P.; Guha, C. Hyperthermia on Immune Regulation: A Temperature's Story. *Cancer Lett* **2008**, *271*, 191–204, doi:10.1016/J.CANLET.2008.05.026.

44. Lee, C.T.; Repasky, E.A. Opposing Roles for Heat and Heat Shock Proteins in Macrophage Functions during Inflammation: A Function of Cell Activation State? *Front Immunol* **2012**, *3*, 140, doi:10.3389/FIMMU.2012.00140/BIBTEX.

45. Mills, C.D.; Kincaid, K.; Alt, J.M.; Heilman, M.J.; Hill, A.M. M-1/M-2 Macrophages and the Th1/Th2 Paradigm. *J Immunol* **2000**, *164*, 6166–6173, doi:10.4049/JIMMUNOL.164.12.6166.

46. Mosser, D.M.; Hamidzadeh, K.; Goncalves, R. Macrophages and the Maintenance of Homeostasis. *Cellular & Molecular Immunology 2020 18:3* **2020**, *18*, 579–587, doi:10.1038/s41423-020-00541-3.

47. Zheng, X.-F.; Hong, Y.-X.; Feng, G.-J.; Zhang, G.-F.; Rogers, H. Lipopolysaccharide-Induced M2 to M1 Macrophage Transformation for IL-12p70 Production Is Blocked by Candida Albicans Mediated Up-Regulation of EBI3 Expression. *PLoS One* **2013**, *8*, 63967, doi:10.1371/journal.pone.0063967.

48. Qin, H.; Holdbrooks, A.T.; Liu, Y.; Reynolds, S.L.; Yanagisawa, L.L.; Benveniste, E.N. SOCS3 Deficiency Promotes M1 Macrophage Polarization and Inflammation. *The Journal of Immunology* **2012**, *189*, 3439–3448, doi:10.4049/JIMMUNOL.1201168.

49. Skytthe, M.K.; Graversen, J.H.; Moestrup, S.K. Targeting of CD163+ Macrophages in Inflammatory and Malignant Diseases. *International Journal of Molecular Sciences 2020, Vol. 21, Page 5497* **2020**, *21*, 5497, doi:10.3390/IJMS21155497.

50. Mosser, D.M.; Edwards, J.P. Exploring the Full Spectrum of Macrophage Activation. *Nature Publishing Group* **2008**, *8*, doi:10.1038/nri2448.

51. Maeda, M.; Nishimura, Y.; Kumagai, N.; Hayashi, H.; Hatayama, T.; Katoh, M.; Miyahara, N.; Yamamoto, S.; Hirastuka, J.; Otsuki, T. Dysregulation of the Immune System Caused by Silica and Asbestos. *http://dx.doi.org/10.3109/1547691X.2010.512579* **2010**, *7*, 268–278, doi:10.3109/1547691X.2010.512579.

52. Illouz, T.; Biragyn, A.; Iulita, M.F.; Flores-Aguilar, L.; Dierssen, M.; De Toma, I.; Antonarakis, S.E.; Yu, E.; Herault, Y.; Potier, M.C.; et al. Immune Dysregulation and the Increased Risk of Complications and Mortality Following Respiratory Tract Infections in Adults With Down Syndrome. *Front Immunol* **2021**, *12*, 2375, doi:10.3389/FIMMU.2021.621440/BIBTEX.

53. Gouin, J.P.; Hantsoo, L.; Kiecolt-Glaser, J.K. Immune Dysregulation and Chronic Stress Among Older Adults: A Review. *Neuroimmunomodulation* **2008**, *15*, 251, doi:10.1159/000156468.

54. Davitt, E.; Davitt, C.; Mazer, M.B.; Areti, S.S.; Hotchkiss, R.S.; Remy, K.E. COVID-19 Disease and Immune Dysregulation. *Best Pract Res Clin Haematol* **2022**, *35*, 101401, doi:10.1016/J.BEHA.2022.101401.

55. Razmi, M.; Hashemi, F.; Gheytanchi, E.; Dehghan Manshadi, M.; Ghods, R.; Madjd, Z. Immunomodulatory-Based Therapy as a Potential Promising Treatment Strategy against Severe COVID-19 Patients: A Systematic Review. *Int Immunopharmacol* **2020**, *88*, 106942, doi:10.1016/J.INTIMP.2020.106942.

56. Bascones-Martinez, A.; Mattila, R.; Gomez-Font, R.; Meurman, J.H. Immunomodulatory Drugs: Oral and Systemic Adverse Effects. *Med Oral Patol Oral Cir Bucal* **2014**, *19*, e24, doi:10.4317/MEDORAL.19087.

57. Roy-Ghanta, S.; Orange, J.S. Use of Cytokine Therapy in Primary Immunodeficiency. *Clin Rev Allergy Immunol* **2010**, *38*, 39–53, doi:10.1007/S12016-009-8131-4.

58. Kedzierska, K.; Crowe, S.M. Cytokines and HIV-1: Interactions and Clinical Implications. *Antivir Chem Chemother* **2001**, *12*, 133–150, doi:10.1177/095632020101200301.

59. Khalil, D.N.; Smith, E.L.; Brentjens, R.J.; Wolchok, J.D. The Future of Cancer Treatment: Immunomodulation, CARs and Combination Immunotherapy. *Nat Rev Clin Oncol* **2016**, *13*, 273, doi:10.1038/NRCLINONC.2016.25.

60. Cihoric, N.; Tsikkinis, A.; Van Rhoon, G.; Crezee, H.; Aebersold, D.M.; Bodis, S.; Beck, M.; Nadobny, J.; Budach, V.; Wust, P.; et al. Hyperthermia-Related Clinical Trials on Cancer Treatment within the ClinicalTrials.Gov Registry. *International Journal of Hyperthermia* **2015**, *31*, 609–614, doi:10.3109/02656736.2015.1040471.

61. Ostberg, J.R.; Repasky, E.A. Comparison of the Effects of Two Different Whole Body Hyperthermia Protocols on the Distribution of Murine Leukocyte Populations. *Int J Hyperthermia* **2000**, *16*, 29–43, doi:10.1080/026567300285402.

62. Hasday, J.D.; Garrison, A.; Singh, I.S.; Standiford, T.; Ellis, G.S.; Rao, S.; He, J.R.; Rice, P.; Frank, M.; Goldblum, S.E.; et al. Febrile-Range Hyperthermia Augments Pulmonary Neutrophil Recruitment and Amplifies Pulmonary Oxygen Toxicity. *Am J Pathol* **2003**, *162*, 2005–2017, doi:10.1016/S0002-9440(10)64333-7.

63. Evans, S.S.; Wang, W.C.; Bain, M.D.; Burd, R.; Ostberg, J.R.; Repasky, E.A. Fever-Range Hyperthermia Dynamically Regulates Lymphocyte Delivery to High Endothelial Venules. *Blood* **2001**, *97*, 2727–2733, doi:10.1182/BLOOD.V97.9.2727.

64. Lanneau, D.; de Thonel, A.; Maurel, S.; Didelot, C.; Garrido, C. Apoptosis versus Cell Differentiation: Role of Heat Shock Proteins HSP90, HSP70 and HSP27. *Prion* **2007**, *1*, 53–60, doi:10.4161/pri.1.1.4059.

65. Mayer, M.P.; Bukau, B. Hsp70 Chaperones: Cellular Functions and Molecular Mechanism. *Cellular and Molecular Life Sciences* **2005**, *62*, 670–684, doi:10.1007/s00018-004-4464-6.

66. Borges, T.J.; Lopes, R.L.; Pinho, N.G.; Machado, F.D.; Souza, A.P.D.; Bonorino, C. Extracellular Hsp70 Inhibits Pro-Inflammatory Cytokine Production by IL-10 Driven down-Regulation of C/EBPβ and C/EBPδ. *International Journal of Hyperthermia* **2013**, *29*, 455–463, doi:10.3109/02656736.2013.798037.

67. Gupta, A.; Cooper, Z.A.; Tulapurkar, M.E.; Potla, R.; Maity, T.; Hasday, J.D.; Singh, I.S. Toll-like Receptor Agonists and Febrile Range Hyperthermia Synergize to Induce Heat Shock Protein 70 Expression and Extracellular Release. *Journal of Biological Chemistry* **2013**, *288*, 2756–2766, doi:10.1074/JBC.M112.427336.

68. Wischmeyer, P.E.; Mintz-Cole, R.A.; Baird, C.H.; Easley, K.A.; May, A.K.; Sax, H.C.; Kudsk, K.A.; Hao, L.; Tran, P.H.; Jones, D.P.; et al. Role of Heat Shock Protein and Cytokine Expression as Markers of Clinical Outcomes in GLN-Supplemented Parenteral Nutrition (GLND) Trial in Surgical ICU Patients. *Clin Nutr* **2020**, *39*, 563, doi:10.1016/J.CLNU.2019.02.045.

69. Erbes, T.; Hirschfeld, M.; Waldeck, S.; Rücker, G.; Jäger, M.; Willmann, L.; Kammerer, B.; Mayer, S.; Gitsch, G.; Stickeler, E. Hyperthermia-Driven Aberrations of Secreted MicroRNAs in Breast Cancer in Vitro. *International Journal of Hyperthermia* **2016**, *32*, 630–642, doi:10.3109/02656736.2016.1161832/SUPPL_FILE/IHYT_A_1161832_SM6631.DOCX.

70. Potla, R.; Singh, I.S.; Atamas, S.P.; Hasday, J.D. Shifts in Temperature within the Physiologic Range Modify Strand-Specific Expression of Select Human MicroRNAs. *RNA* **2015**, *21*, 1261, doi:10.1261/RNA.049122.114.

71. Kupec, T.; Bleilevens, A.; Iborra, S.; Najjari, L.; Wittenborn, J.; Maurer, J.; Stickeler, E. Stability of Circulating MicroRNAs in Serum. *PLoS One* **2022**, *17*, e0268958, doi:10.1371/JOURNAL.PONE.0268958.

72. Glinge, C.; Clauss, S.; Boddum, K.; Jabbari, R.; Jabbari, J.; Risgaard, B.; Tomsits, P.; Hildebrand, B.; Kaèaèb, S.; Wakili, R.; et al. Stability of Circulating Blood-Based MicroRNAs – Pre-Analytic Methodological Considerations. *PLoS One* **2017**, *12*, doi:10.1371/JOURNAL.PONE.0167969.

73. Oshlag, J.Z.; Devasthanam, A.S.; Tomasi, T.B. Mild Hyperthermia Enhances the Expression and Induces Oscillations in the Dicer Protein. *http://dx.doi.org/10.3109/02656736.2012.753471* **2013**, *29*, 51–61, doi:10.3109/02656736.2012.753471.

74. Sun, Y.; Wang, M.; Lin, G.; Sun, S.; Li, X.; Qi, J.; Li, J. Serum MicroRNA-155 as a Potential Biomarker to Track Disease in Breast Cancer. *PLoS One* **2012**, *7*, doi:10.1371/JOURNAL.PONE.0047003.

75. Su-Ying, Z.; Qian, W.; Fen, G.; Chun-Bing, Z.; Xue-Wen, Y. Increased Expression of MicroRNA-155 in the Serum of Women with Early-Stage Breast Cancer. *Lab Med* **2012**, *43*, 177–180, doi:10.1309/LMD71DRHD9SJPWBR.

76. Bi, Y.; Chen, J.; Hu, F.; Liu, J.; Li, M.; Zhao, L. M2 Macrophages as a Potential Target for Antiatherosclerosis Treatment. *Neural Plast* **2019**, *2019*, doi:10.1155/2019/6724903.

77. Herb, M.; Schramm, M. Functions of Ros in Macrophages and Antimicrobial Immunity. *Antioxidants* **2021**, *10*, 1–39, doi:10.3390/antiox10020313.

78. Palmieri, E.M.; McGinity, C.; Wink, D.A.; McVicar, D.W. Nitric Oxide in Macrophage Immunometabolism: Hiding in Plain Sight. *Metabolites* **2020**, *10*, 1–34, doi:10.3390/METABO10110429.

79. Ostberg, J.R.; Ertel, B.R.; Lanphere, J.A. AN IMPORTANT ROLE FOR GRANULOCYTES IN THE THERMAL REGULATION OF COLON TUMOR GROWTH. *Immunol Invest* **2005**, *34*, 259, doi:10.1081/IMM-200064477.

80. Wang, L. xun; Zhang, S. xi; Wu, H. juan; Rong, X. lu; Guo, J. M2b Macrophage Polarization and Its Roles in Diseases. *J Leukoc Biol* **2019**, *106*, 345–358, doi:10.1002/JLB.3RU1018-378RR.

81. Hutt, N.; Kopferschmitt-Kubler, M.C.; Cabalion, J.; Purohit, A.; Alt, M.; Pauli, G. Anaphylactic Reactions after Therapeutic Injection of Mistletoe (Viscum Album L.). *Allergol Immunopathol (Madr)* **2001**, *29*, 201–203, doi:10.1016/S0301-0546(01)79056-8.

82. Horneber, M.A.; Bueschel, G.; Huber, R.; Linde, K.; Rostock, M. Mistletoe Therapy in Oncology. *Cochrane Database of Systematic Reviews* **2008**, doi:10.1002/14651858.CD003297.pub2.

83. Szurpnicka, A.; Kowalczuk, A.; Szterk, A. Biological Activity of Mistletoe: In Vitro and in Vivo Studies and Mechanisms of Action. *Arch Pharm Res* **2020**, *43*, 593, doi:10.1007/S12272-020-01247-W.

84. Kim, J.J.; Hwang, Y.H.; Kang, K.Y.; Kim, I.; Kim, J.B.; Park, J.H.; Yoo, Y.C.; Yee, S.T. Enhanced Dendritic Cell Maturation by the B-Chain of Korean Mistletoe Lectin (KML-B), a Novel TLR4 Agonist. *Int Immunopharmacol* **2014**, *21*, 309–319, doi:10.1016/J.INTIMP.2014.05.010.

85. Cui, W.; Sun, C.; Ma, Y.; Wang, S.; Wang, X.; Zhang, Y. Inhibition of TLR4 Induces M2 Microglial Polarization and Provides Neuroprotection via the NLRP3 Inflammasome in Alzheimer's Disease. *Front Neurosci* **2020**, *14*, 444, doi:10.3389/FNINS.2020.00444/BIBTEX.

8. Abstract in English

For centuries, medical practitioners have recognized the beneficial effects of fever, leading to the development of techniques aimed at intentionally inducing elevated body temperatures for therapeutic purposes. While this type of treatment called fever-range hyperthermia (FRH) is currently in use, its full effects are not yet well understood.

In my studies I employed *in viv*o and *in vitro* methods to investigate the potential of FRH to affect immune system. I developed a new model of FRH in rats to investigate the impact of FRH on hematology profile and the expression of regulatory molecules, including cytokines and miRNA. I observed that FRH significantly influences lymphocytes and granulocytes what was associated with changed expression of regulatory molecules, including macrophage inhibitory factor (MIF-1α) and granulocyte colony-stimulating factor (G-CSF). I also found that hyperthermia affected the miRNA machinery, resulting in changes in miRNA-155 expression in the serum.

Since it is known that macrophages are heat sensitive cells, I decided to identify the phenotype of these cells. My initial in vitro studies showed, that FRH induced expression of CD163 and arginase-1 (Arg-1), markers specific to M2 macrophages. Furthermore, FRH inhibited the expression of inducible nitric oxide synthase (iNOS), a marker for M1 macrophages. Interestingly, FRH also increased the expression of pro-inflammatory markers such as IL-1β, IL-6, reactive oxygen species (ROS), cyclooxygenase 2 (COX-2), and Toll-like receptor 4 (TLR-4), indicating that FRH induces an M2b phenotype. Thus, FRH treated cells exhibit both pro-inflammatory and anti-inflammatory properties and therefore are considered regulatory cells.

As TLR-4 pathway is essential for inflammation, I investigated the effect of FRH on immunomodulators such as Mistletoe Extract (ME) and LPS that are TLR-4 dependent. I found that FRH could prevent ME-induced cell death, improve macrophage viability, and activate the NF-κB pathway, leading to the development of a pro-inflammatory response. Although LPS is widely recognized for its ability to induce the polarization of macrophages into the M1 phenotype, I found that FRH can effectively reverse this effect and instead promote the

induction of a regulatory M2b phenotype. However, I also observed that this mechanism was not solely TLR-4 dependent.

In conclusion, my research on FRH has revealed that the biological effects of hyperthermia are complex and multifaceted, extending beyond the previously known immunomodulatory effects observed via the TLR-4 pathway. Hyperthermia causes changes at the gene, protein, cell, and organism levels, affecting leukocyte populations, cytokines, and miRNA expression. My findings suggest that macrophages play a critical role in the response to FRH, exhibiting an M2b phenotype that has both pro-inflammatory and anti-inflammatory properties. Based on my results, far-reaching conclusions can be drawn that fever-associated increase in body temperature may be a switch for macrophages, that change M1 macrophages towards M2 phenotype to gradually finish inflammation.

9. Streszczenie

Prozdrowotne efekty gorączki były obserwowane przez lekarzy od wieków, co stało się podstawą do opracowania metod sztucznego podnoszenia temperatury ciała. Chociaż współczesna medycyna z powodzeniem stosuje hipertermię z zakresu gorączki (FRH), nadal mechanizm jej działanie nie jest do końca znany.

W moich badaniach wykorzystałem modele *in vivo* i *in vitro*, aby ocenić wpływ FRH na układ immunologiczny. Opracowałem, nowy, szybki i bezpieczny model indukowania hipertermii u szczurów, celem określenia wpływu FRH na profil hematologiczny oraz ekspresję cząsteczek regulatorowych takich jak cytokiny czy miRNA. Zaobserwowałem, że FRH znacząco modyfikuje pulę krążących we krwi obwodowej limfocytów i granulocytów. Zmiany w tych populacjach leukocytów były powiązane ze zmianą ekspresji cząsteczek regulatorowych takich jak czynnik stymulujący wzrost kolonii granulocytów (G-CSF) czy czynnik hamujący makrofagi (MIF-1α). Ponadto, stwierdziłem, że hipertermia wpływa na maszynerię miRNA, co prowadzi do zmiany ekspresji miRNA-155 w surowicy szczurów poddanych zabiegowi hipertermii.

Powszechnie wiadomo, że makrofagi są komórkami wrażliwymi na zmiany temperatury, dlatego zdecydowałem się, na analizę ich fenotypu w odpowiedzi na hipertermię. Moje badania *in vitro*, wykazały, że hipertermia indukuje ekspresję markerów specyficznych dla fenotypu M2, tj. CD163 i arginazy-1 (Arg-1). Ponadto, hipertermia hamowała ekspresję markerów polaryzacji makrofagów w fenotyp M1 CD80 i indukowanej syntetazy tlenku azotu (iNOS). Co ciekawe, FRH indukowała ekspresję markerów stanu zapalnego takich jak interleukiny IL-1β, IL-6, reaktywne formy tlenu (ROS), cykooksygenza-2 (COX-2) czy receptor Toll-like-4 (TLR-4). Wyniki te wskazują, że FRH indukuje polaryzację makrofagów w kierunku fenotypu M2b, który posiada zarówno właściwości pro- jak i przeciwzapalne i stanowi ważną pulę regulatorowych makrofagów.

Ścieżka sygnalizacyjna TLR-4 jest kluczowa dla rozwoju stanu zapalnego, dlatego zbadałem wpływ FRH na odpowiedź makrofagów traktowanych immunomodulatorami zależnymi od szlaku TLR-4 tj. lipopolisacharydem (LPS) i ekstraktem z jemioły (ME). Na

podstawie uzyskanych wyników stwierdziłem, że FRH zabezpiecza komórki przed indukowaną ekstraktem z jemioły śmiercią komórek, poprawiając ich żywotność i aktywując jądrowy czynnik transkrypcyjny NF-κB, co w konsekwencji prowadziło do rozwoju odpowiedzi prozapalnej. Ponadto, wykazałem, że FRH jest zdolna indukować polaryzację makrofagów w regulatorowy fenotyp M2b, nawet w obecności silnego induktora fenotypu M1 jakim jest LPS. Jednakże, polaryzacja makrofagów w fenotyp M2b okazała się niezależna od szlaku TLR-4.

Podsumowując, wyniki moich badań wskazują, że działanie hipertermii jest złożone i wieloaspektowe, wykraczające poza dotychczas opisane efekty wynikające z aktywacji szlaku TLR-4. FRH działa na poziomie genów, białek, pojedynczych komórek jak i na poziomie całego organizmu prowadząc do zmian w populacji leukocytów, ekspresji cytokin i miRNA. Uzyskane przeze mnie wyniki, sugerują, że makrofagi odgrywają kluczową rolę w odpowiedzi na wzrost temperatury i przyjmując regulatorowy fenotyp M2b, są zdolne do jednoczesnej odpowiedzi o charakterze pro- i przeciwzapalnym. Na podstawie uzyskanych wyników można przypuszczać, że wzrost temperatury ciała związany z gorączką, może być istotnym czynnikiem prowadzącym do zmiany fenotypu makrofagów, a w konsekwencji do stopniowego wyciszania stanu zapalnego.