Investigating the role of thermoneutrality on malaria inflammatory response and disease progression.

Fiorella Vialard
Department of Microbiology and Immunology,
McGill University Montreal
July, 2021

A thesis submitted to McGill University in
partial fulfillment of the requirements of the
degree of Master's of Science.

Table of Contents

FIGURES

ABSTRACT

Malaria is a neglected tropical disease that continues to cause many fatalities in endemic regions. It is characterized by a fever-like illness caused by the interaction of parasitic agents (e.g. native hemozoin; nHZ) with innate immune cells during the blood stage in the vertebrate host. A robust mouse model of malaria disease is key to improving our understanding of the host-parasite interaction. However, compared to humans, who spend the majority of their time at thermoneutral temperatures (TT), most studies using murine disease models are conducted at sub-optimal housing temperature (ST). Experiments performed at TT on various disease models have revealed an altered immune response to pathogens. How such conditions affect the inflammatory response (IR) to *Plasmodium berghei* ANKA (PbA) infection and subsequent disease progression in malarial disease models is currently unknown.

We hypothesized (1) that the IR of bone-marrow derived macrophages (BMDM) to LPS would be different at TT and fever-like temperature (FT) compared to ST, and (2a) that the IR to native hemozoin (nHZ) injection in a peritonitis model and (2b) disease progression in PbA-infected mice would be different at TT compared to ST.

To test our first hypothesis, BMDMs were stimulated with LPS at FT (39ºC), TT (37ºC) or ST (30ºC) for 6, 12 and 24 hours. Nitric oxide (NO) and cytokine concentrations were determined from the culture supernatant. We observed that BMDMs released the highest concentrations of NO at FT followed by TT and ST and that these concentrations increased overtime. We also observed differential pro-inflammatory cytokine production at different temperatures.

To test our second hypothesis, C57BL6 mice were acclimatized for 3 weeks at TT (28 – 31ºC) or ST (20 – 22ºC). In one study, the acclimatized mice were injected intraperitoneally with nHZ or *Leishmania* at TT or ST, and immune cells were isolated from the peritoneal cavity (PEC) fluid, counted and sub-typed. Cytokine concentrations and extracellular vesicle (EV) profiles were also determined from the PEC. *Leishmania* injection resulted in decreased neutrophil recruitment in mice housed at TT compared to ST and a similar trend was observed for nHZ-injected mice. Phagocytosis of nHZ was higher in mice housed at TT and cytokine profiles differed at TT and ST. EV concentrations in mice injected with parasitic agents were lower at TT than ST, but there were no differences in the release of EVs by specific cell types at the two temperatures.

In another study, mice were injected with 10^4 luciferase-tagged PbA-infected RBCs. Symptoms were monitored until death, when blood and organs were collected. We observed trends in

increased survival and lower parasite organ sequestration, using a luciferase quantification assay, at TT compared to ST. Mice at TT experienced significantly less body weight loss and decreased body temperature than their ST-housed counterparts. The cytokine serum concentrations were similar at end-point for the two housing temperatures.

Our results suggest that thermoneutrality might change the progression of malarial disease by dampening the IR of the host to parasitic agents.

RÉSUMÉ

Le paludisme est une maladie tropicale négligée qui continue de causer de nombreux décès dans les régions endémiques. Cette maladie se caractérise par des symptômes similaires à de la fièvre causée par l'interaction d'agents parasitaires (par exemple, l'hémozoïne native ; nHZ) avec des cellules immunitaires innées pendant la phase sanguine chez l'hôte vertébré. Un modèle murin robuste de la maladie du paludisme est essentiel pour améliorer notre compréhension de l'interaction hôte-parasite. Cependant, par rapport aux humains, qui passent la majorité de leur temps à des températures thermoneutres (TT), la plupart des études utilisant des modèles de maladies murines sont menées à une température d'hébergement sous-optimale (ST). Les expériences réalisées aux TT des modèles de maladies variées ont révélées une réponse immunitaire altérée aux agents pathogènes. Comment ces conditions affectent la réponse inflammatoire (IR) à l'infection à *Plasmodium berghei* ANKA (PbA) et la progression ultérieure de la maladie dans les modèles de maladie paludéenne est actuellement inconnue.

Nous avons émis l'hypothèse (1) que l'IR des macrophages dérivés de la moelle osseuse (BMDM) a LPS serait différente à TT et à température fébrile (FT) par rapport à ST, et (2a) que l'IR à l'injection d'hémozoïne native (nHZ) dans un modèle de péritonite et (2b) la progression de la maladie chez les souris infectées par PbA seraient différentes à TT par rapport à ST.

Pour tester notre première hypothèse, les BMDM ont été stimulés avec du LPS à FT (39ºC), TT (37ºC) ou ST (30ºC) pendant 6, 12 et 24 heures. Les concentrations d'oxyde nitrique (NO) et de cytokines ont été déterminées à partir du surnageant de culture. Nous avons observé que les BMDM libéraient les concentrations les plus élevées de NO à FT suivi de TT et ST et que ces concentrations augmentaient avec le temps. Nous avons également observé une production différentielle de cytokines pro-inflammatoires à différentes températures.

Pour tester notre deuxième hypothèse, des souris C57BL6 ont été acclimatées pendant 3 semaines à TT (28 – 31ºC) ou ST (20 – 22ºC). Dans une première étude, les souris acclimatées ont reçu une injection intrapéritonéale de nHZ ou de *Leishmania* à TT ou ST, et des cellules immunitaires ont été isolées du liquide de la cavité péritonéale (PEC), comptées et sous-typées. Les concentrations de cytokines et les profils de vésicules extracellulaires (EV) ont également été déterminés à partir de la PEC. L'injection de *Leishmania* a entraîné une diminution du recrutement de neutrophiles chez les souris hébergées à TT par rapport à ST et une tendance similaire a été observée pour les souris ayant reçu une injection de nHZ. La phagocytose de nHZ

était plus élevée chez les souris logées à TT et les profils de cytokines différaient à TT et ST. Les concentrations d'EV chez les souris injectées avec des agents parasitaires étaient plus faibles à TT qu'à ST, mais il n'y avait aucune différence dans la libération d'EV par des types de cellules spécifiques aux deux températures.

Dans une autre étude, des souris ont reçu une injection de 10^4 globules rouges infectés par du PbA marqués à la luciférase. Leur symptômes ont été surveillés jusqu'à la mort, lorsque le sang et les organes ont été prélevés. Nous avons observé des tendances en termes de survie accrue et de séquestration plus faible des organes parasitaires, en utilisant un test de quantification de la luciférase, à TT par rapport à ST. Les souris à TT ont subi une perte de poids corporel et une diminution de la température corporelle significativement moindre comparé à leurs homologues hébergées à ST. Les concentrations sériques de cytokines étaient similaires au point final pour les deux températures de logement.

Nos résultats suggèrent que la thermoneutralité pourrait influencer la progression de la maladie palustre en atténuant l'IR de l'hôte aux agents parasitaires.

Acknowledgements

Firstly, I would like to thank my parents, Jorge Vialard and Laurence De Moerlooze, for instilling in me the love for research from a very young age and supporting me through all my endeavours in Canada despite the distance. I would not have made it without their help.
Secondly, to my supervisor, Dr. Martin Olivier, thank you for accepting me in your lab and trusting me with this original one-of-a kind research project, as well as giving me your trust to run my experiments independently and reinforcing the importance of hard work.
To my advisory committee members, Dr. Momar Ndao and Dr. Petra Rohrbach, thank you for your insight on this project and the important questions you raised during our meetings.
Next, I want to acknowledge all the people involved in the thermoneutrality and malaria project. Thank you Dr. David Labbé and Dr. Luciana Mirotti for answering all my questions about conducting experiments in thermoneutral housing. Thank you Dr. Éric Boilard and Isabelle Allaeys for conducting the high-sensitivity flow-cytometry experiments and helping me analyze and graph the interesting data we generated.
Thank you to all members of the Martin Olivier Lab, past and present, your companionship made my experience easier. To Caroline Martel, thank you for welcoming me to the lab and introducing me to how things work in the Olivier Lab. To Line Larivière, thank you for your help with my mice experiments. To George Dong, thank you for running the TEM and NTA experiments and our discussions on movies, the stock market and politics. Thank you Edouard Charlebois for the help with IP injections. Thank you Carlos, for being a great friend, giving me advice and letting me impart a bit of my knowledge on malaria research before leaving the lab. Thank you Nada Al-Emadi and Duha Al-Awad for your friendship and continued support during our MSc. Thank you to Laura Paez and Dr. Emmanuella Fajardo for giving me great advice and lessons from life experience. I would also like to mention Alonso Lira Filho, Andrea Lafleur, Aretha Chan, Selena Yu, Emilie Desnoyer and Mohammed Daoudi.
I want to thank my BSc. Honours project advisor, Dr. John Barta, for teaching me the importance of accurate scientific writing and supporting me through my application to this MSc. Program.
Finally, I would like to thank all the people that made my move to Montreal easier and lockdown more bearable. My sister for being the best roommate, my cousins, uncle and aunt for helping me get through lockdown, my friends in Montreal, Toronto and Brussels for conversations and music (especially Syd!) and my friends and family for all the love from a distance.

Preface

This thesis was written in accordance with McGill University's "Guidelines for Thesis Preparation". The candidate has chosen to present their thesis as a "manuscript-based thesis": "as an alternative to the traditional format, the thesis may be presented as a collection of scholarly papers of which the student is the first author or co-first author; that is, a manuscript based Master's thesis must include the text of one or more manuscripts published, submitted, or to be submitted for publication and re-formatted according to the described requirements. The thesis must contain additional text that will connect the manuscript in a logical progression, producing a cohesive, unitary focus, and documenting a single program of research."

All work towards this thesis was performed under the supervision of Dr. Martin Olivier. The candidate is the first author of the manuscript presented in section 2, which will be submitted for publication. The candidate is also the first author of a review on thermoneutrality which has been integrated in the literature review under section 1.4. Contributions to the content of this manuscript are as described: Overall project was designed and envisioned by Martin Olivier. *In vitro* experiments were performed by Fiorella Vialard. In vivo experiments were designed and performed by F. Vialard and M. Olivier with aid from George Dong, Edouard Charlebois and Line Larivière. David Labbé was consulted for *in vivo* experiments in thermoneutral housing. High-sensitivity flow cytometry experiments were performed by Isabelle Allaeys in the laboratory of Eric Boilard and data was analysed by I. Allaeys and F. Vialard. TEM imaging and NTA measurements were performed by G. Dong. Statistical analysis were performed by F. Vialard. F. Vialard wrote the manuscript with editorial guidance and direction from M Olivier.

List of Abbreviations

3×Tg-AD	triple-transgenic mouse model of Alzheimer's disease
AP	activator protein
AR	adrenergic receptor
BAT	brown adipose tissue
BBB	blood-brain barrier
BCT	body core temperature
BMDM	bone-marrow-derived macrophage
BSA	bovine serum albumin
CCL	C-C motif ligand
CDC	Centre for Disease Control
CM	cerebral malaria
CL	cutaneous leishmaniasis
CVD	cardiovascular disease
CXCL	C-X-C motif ligand
DC	dendritic cell
DMEM	Dulbecco's Modified Eagle Medium
EC	endothelial cell
ECM	experimental cerebral malaria
EV	extracellular vesicles
FBS	fetal bovine serum
FFA	free fatty acid
FSC-PMT	with forward scatter coupled to a photomultiplier tube small particles option
FT	fever-like temperature
G-CSF	granulocyte-colony stimulating factor
GFP	green fluorescent protein
GM-CSF	granulocyte-macrophage colony-stimulating factor
GPI	glycosylphosphatidylinositol
GVHD	graft vs host disease
HMGB	high mobility group box
HFD	high fat diet

IFN	interferon
Ig	immunoglobulin
IL	interleukin
IP-10	interferon g-induced protein 10
IP	intraperitoneal
IR	inflammatory response
iRBC	infected red blood cell
KC	keratinocyte chemoattractant
KO	knockout
LM	litter mate
LPS	lipopolysaccharide
MCP	monocyte chemoattractant protein
MDSC	myeloid-derived suppressor cells
MIG	monokine induced by interferon gamma
MIP	macrophage inflammatory protein
Mz	merozoites
NAFLD	non-alcoholic fatty liver disease
NE	norepinephrine
nHZ	native hemozoin
NO	nitric oxide
NTA	nanoparticle tracking analysis
NTD	neglected tropical disease
PbA	*Plasmodium berghei* ANKA
PBS	phosphate-buffered saline
PS	phosphotidylserine
RANTES	regulated on activation normal T cell expressed and secreted
RDT	rapid diagnostic test
ROS	reactive oxygen species
SA/V	surface area to volume ratio
SDM	Schneider’s Drosophila Medium
SSC-H	size and inner complexity

ST	sub-optimal temperature
Sz	Sporozoites
TEM	Transmission electron microscope
TG	triglyceride
Th	T helper cell
TH	thyroid hormone
Tlc	lower critical temperature
TLR	toll-like receptor
TN	thermoneutrality
TNZ	thermoneutral zone
TNF	tumour necrosis factor
TT	thermoneutral temperatures
UCP	uncoupling protein
VEGF	vascular endothelial growth factor
VSA	variant surface antigen
WHO	World Health Organization
WT	wild-type

1. Literature Review and Research Objectives

1.1. Malaria

1.1.1. Burden of disease

Malaria is an amniotic vertebrate disease characterized by febrile acute illness that is caused by the blood apicomplexan of the *Plasmodium* genus (1-3). It is transmitted by an insect vector, the female *Anopheles* spp. mosquito (see section 1.1.2) (3). Most of the cases in humans are caused by infection with one of four *Plasmodium* species: *P. falciparum*, *P. vivax, P. ovale* and *P. knowlesi* (2). In non-immune individuals, symptoms can progress into severe disease and, if not treated, can lead to death (3). Clinical presentation of malaria will be discussed in more detail in section 1.1.5. Malaria is considered a neglected tropical disease (NTD) by the World Health Organization (WHO). It estimated the global burden of the disease in 2019 to be 229 million cases and 409 thousand deaths in 87 endemic countries (Figure 1). Most of these cases and deaths (i.e. 94%) occurred in the African Region (4).

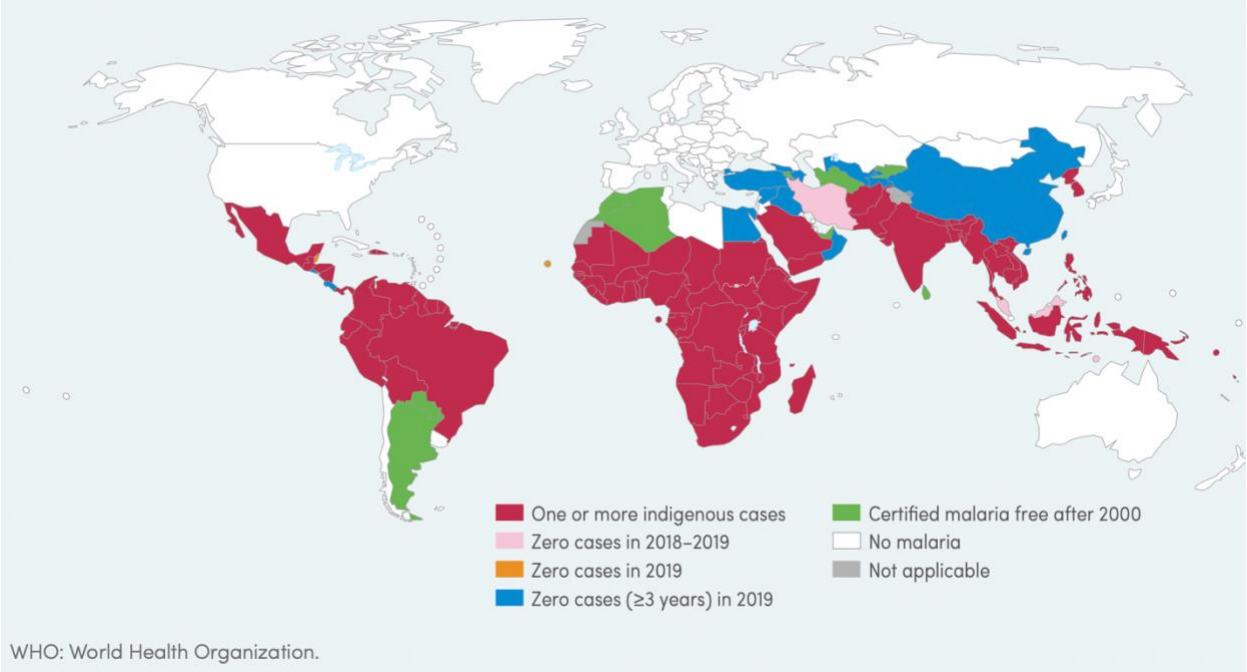

Figure 1. Malaria endemic regions from 2000 to 2019.
Colored countries regions represent regions where malaria was endemic or achieved elimination from 2000 to 2019. Countries in red represent countries where malaria is still a concern as of 2019 (4).

1.1.2. Transmission

Transmission in endemic regions is driven by three factors: 1) vector biology, 2) environmental conditions, and 3) human immunity (3). *Anopheles* spp. that have longer life-spans drive more transmission because the parasite has more time to complete its life-cycle in the insect vector (see section 1.1.4) (3). In addition, mosquito species that have a preference for humans over other mammals as a blood meal source increase transmission risk (3). Because female mosquitoes lay their eggs in water, temperature, humidity and rainfall patterns influence transmission (3). In many endemic tropical regions, peak transmission usually occurs immediately after the rainy season (3). Finally, human immunity is inversely correlated with transmission (3). In areas of high transmission, exposure to the parasite occurs more often and thus the population develops partial immunity over time (3). The immune response to malaria will be discussed in more detail in section 1.2.

1.1.3. Prevention

The WHO recently reported that despite advances in progression of elimination of malaria, the milestones to reduce the mortality cases to at least 40% by 2020 would not be achieved (4). They identified several reasons for this, including the rise of *Plasmodium* mutants undetectable by current rapid diagnostic tests (RDTs), the rise of resistance to antimalarial drugs, vector resistance to insecticides, and disruption of essential services to combat malaria due to restrictions linked to the COVID-19 epidemic. Climate change has also been implicated in the stagnation of disease burden by increasing the survival and reach of the anopheline vector in some endemic regions (5). In order to achieve elimination, the WHO has recommended investment into malaria disease research and development initiatives (4).

To date, the only vaccine that has been shown to reduce malaria and life-threatening severe malaria caused by *P. falciparum* in young children is RTS,S/AS01 (3). Vaccine introduction in selected endemic areas of Sub-Saharan Africa has been recommended by the WHO (3).

1.1.4. Parasitic life-cycle

The *Plasmodium* life cycle is complex, involving multiple stages in an insect vector and a vertebrate host. The cycle is initiated when an *Anopheles* mosquito ingests the male (i.e. microgametocytes) and female (i.e. macrogametocytes) parasitic forms during a blood meal of a

previously infected host. The gametocytes fuse in the mosquito's midgut in a process called sporogony that results in the production of zygotes (2) The zygotes acquire motile and elongated forms called ookinetes (2) that migrate through the midgut wall and become growing oocysts. They eventually rupture and release sporozoites (Sz) (2), which travel to the mosquito's salivary glands (2) The Szs are then transmitted to a vertebrate host during a subsequent blood meal. They are transported in the blood to the liver where they infect the hepatocytes and undergo a round of replication, generating merozoites (Mz). The merozoites are released from the hepatocytes and begin the blood stage by invading RBCs, developing asexually to trophozoites, and forming schizonts that eventually rupture the RBCs and release large numbers of Mz (Figure 2). Each Mz infects a naïve RBC and, after multiple rounds of asexual replication, forms gametocytes that can reproduce sexually in the mosquito host (Figure 2) (6). Most clinical symptoms occur at this stage of the life-cycle (2). Lysis of infected RBCs (iRBCs) results in release of parasites and iRBC cytosolic content (e.g. nHZ), both of which interact with innate immune cells (Figure 2) (6). In turn, the affected immune cells release pro-inflammatory pyrogenic cytokines that contribute to the fever response and chemokines that attract other immune cells to the blood circulation (1, 6). There is also evidence that the parasite induces release of extracellular vesicles (EVs) from iRBCs that contribute to the immune response (7-9). EVs are cell-membrane derived microparticles that act as messengers by transporting nucleic acids, proteins or lipids between cells. Their specific role in the IR to parasitic agents will be discussed in more detail in section 1.1.2 (10).

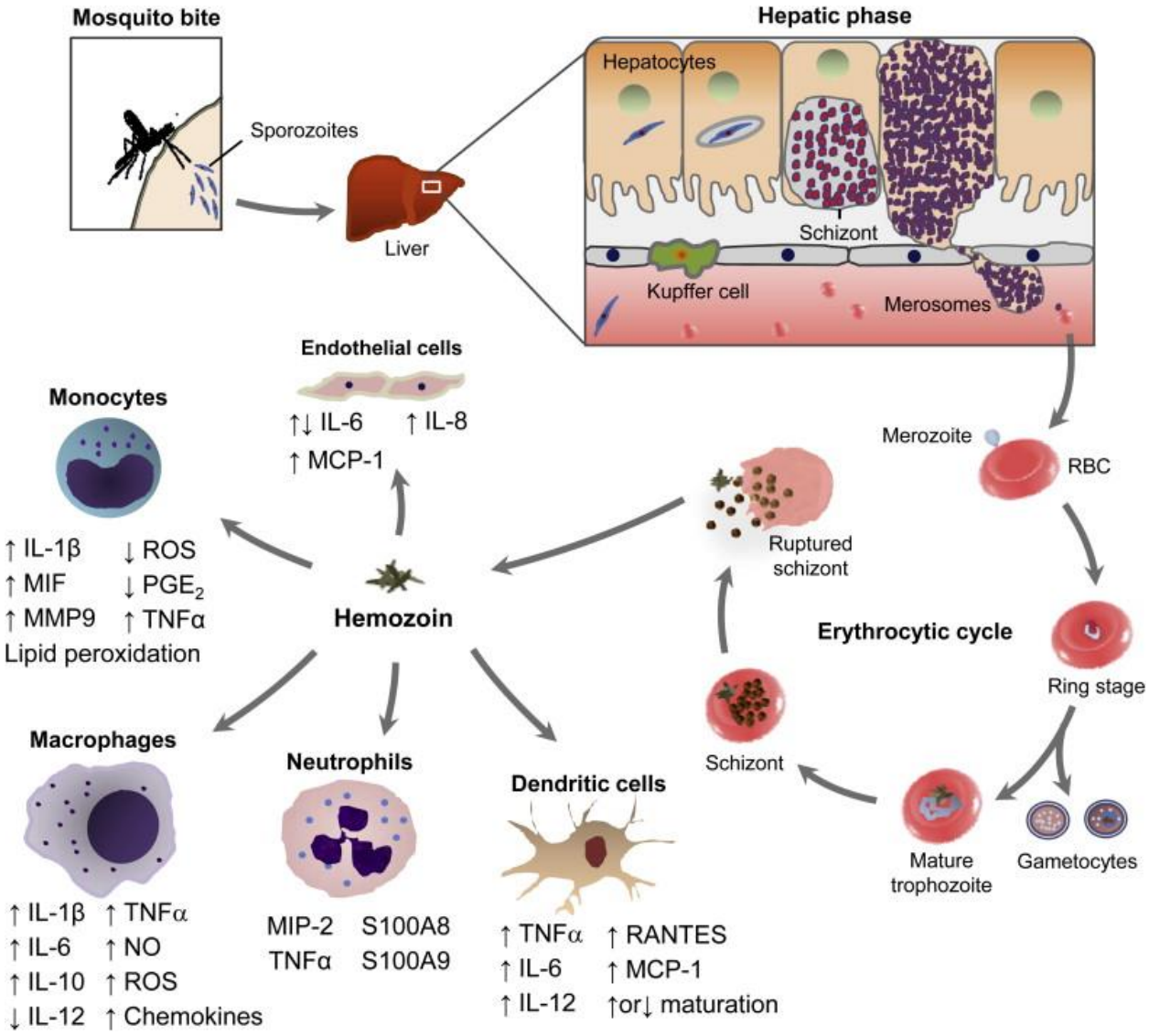

Figure 2. Intermediate host segment of the *Plasmodium* spp. parasite life-cycle.
The release of HZ that accompanies the rupture of iRBCs and the various innate immune cells it interacts with and the cytokines they produce are illustrated (6).

1.1.5. Clinical presentation

There are two forms of disease that can occur in malaria: uncomplicated and severe malaria (2). In the former, patients present with a short, fever-like illness that resolves after a few days (11). This form of the disease usually occurs in older individuals from high-transmission endemic regions that have been exposed to the parasite and are able to mount an adequate immune response to control subsequent infections (11, 12). Individuals with uncomplicated malaria are the main reservoir for continued transmission in endemic regions as they are often not diagnosed or treated (12). The severe form of malaria is associated with organ failure or abnormalities (2).

This presentation of the disease is primarily caused by *P.falciparum* in areas of high transmission, occurring in young children who are not yet immune to the parasite (13). The main symptoms include cerebral malaria (CM), which is characterized by neurological signs and coma response, severe anemia, hemoglobureniа, acute respiratory distress syndrome, abnormalities in blood coagulation, cardiovascular events, kidney injury, hyper-parasitemia, metabolic acidosis, hypoglycemia, and hepato-splenomegaly (13). Severe malaria also occurs in all age groups in low transmission countries or in travellers from non-endemic regions (3). Malarial infection in pregnant women is a particular cause for concern in endemic regions as it has been shown to lead to poor birth outcomes (14). The organ pathologies occur due to iRBC sequestration to the endothelial cells in the vasculature via variant surface antigens (VSAs) on the surface iRBC upregulated by the parasite (13, 14). The damage to the organs is caused by the iRBC directly (e.g. obstruction of blood flow) or through the cytotoxic action of the host immune cells at the site of sequestration (13, 14). The immune mechanisms involved in severe malaria will be discussed in more detail in Section 1.2.

1.2. Host innate immune response: focus on severe malaria

1.2.1. Introduction

Disease presentation in individuals infected with malaria depends on a tight equilibrium between the host immune response and parasite growth in the host (15). Severe disease occurs when: 1) the host immune response is insufficient and parasite growth is uncontrolled; 2) the host immune response is exacerbated, leading to severe tissue inflammation; or, 3) both the parasite growth is uncontrolled and the immune response exacerbated (15). Several mouse models used to study these different states will be described in Section 1.3. The progression of parasite growth and the immune response during infection depends on various factors such as host genetics, nutritional status and co-infection (15). For example, non-O blood type, nutrient deficiencies, and HIV infection have all been associated with increased disease severity (16-18). Since most of the disease symptoms occur during the erythrocytic stage of the malaria life-cycle (discussed in Section 1.2), this review will mainly focus on the innate immune response at this stage.

1.2.2. Immune-modulatory factors

During the malarial erythrocytic cycle, several parasite-associated immunomodulatory factors released from iRBCs interact with various innate immune cells to generate an inflammatory response (IR) (1, 6). For example, glycosylphosphatidylinositol (GPI), glycolipid anchors found on the *Plasmodium* Mz and Sz membranes, and within the schizont cytoplasm, are recognized by TLR2-bearing immune cells and act as endotoxins. Uric acid is a by-product of nucleic acid metabolism by the parasite inside the iRBC (15). Parasitic nucleic acids are recognized by macrophages and dendritic cells (DCs) (15). Heme, a by-product of hemoglobin digestion in the food vacuole of the parasite, has been associated with CM. It crosses the blood brain barrier (BBB) and recruit neutrophils and reactive oxygen species (ROS) (6, 9, 15). However, because heme is toxic to the parasite, it has developed mechanisms to detoxify it through oxidization and addition ferric elements that transform heme into hemozoin (HZ) (6, 15). HZ is an inorganic crystal that is also referred to as malarial pigment because it confers a characteristic brown colour to the blood and organs of patients. Its interaction with innate immune cells has been well documented and is summarized in Figure 2 (6). It creates an inflammatory response characterized by the release of inflammatory cytokines, chemokines and reactive species such as, TNF-α, IL-1β, IL-6, IL-10, IL-8, MCP-1, IL-12, RANTES, MIP-2, nitric oxide (NO) and ROS, by various innate immune cell sub-types (6).

HZ is readily taken up by phagocytic immune cells that are subsequently cleared from the circulation, resulting in its accumulation in various tissues such as the lung, spleen and the liver, thereby contributing to the hepato-splenomegaly observed in patients with severe malaria (6, 15, 19). Finally, an important element in the pathogenesis of malaria is the generation of parasite-derived extracellular vesicles (EVs). EVs are cell-membrane derived microparticles that normally function as intercellular messengers by transporting nucleic acids, lipids and proteins from cell to cell (7). In the context of malarial infection, they are released by iRBCs and have been shown to be enriched in parasitic RNA, proteins, and lipids (7, 8, 20, 21). They have immune-modulatory properties that can affect the severity of disease by stimulating macrophages, neutrophils and platelets, changing endothelial permeability in the BBB, promoting gametocytogenesis, and facilitating cyto-adherence (7, 8, 20, 21). EV release is also observed in immune cells responding to infectious agents (22-25). In the case of malaria and

other parasitic diseases, EVs released by host cells is also associated with increased severity of disease (23).

1.2.3. Innate immune cells

Multiple innate immune cell types are involved in the response to the presence of iRBCs and malarial parasitic agents during the erythrocytic stage. For example, macrophages and neutrophils recognize iRBCs and parasitic agents released by schizont lysis and clear them from circulation (6, 15, 19, 26). Macrophages also release NO and ROS in response to parasitic agents (6, 27). DCs phagocytize parasitic agents that may possess ligands that can bind to their TLR receptors (6, 26). Natural killer cells protect against malaria infection by releasing IFN-γ and exercising a cytotoxic role against iRBCs (6, 15, 26). Endothelial cells (ECs) participate in parasite cyto-adherence resulting in sequestering to various tissues (6, 15, 19). Finally, eosinophils and basophils release cytotoxic granules in response to malarial agents (6, 15, 19) and platelets have been shown to engulf malarial parasitic agents (15).

1.2.4. Inflammatory factors

Multiple pro-inflammatory factors have been shown to be involved in the inflammatory response to *Plasmodium* parasites, as illustrated in Figure 2 of Section 1.1.4. The first cytokines described to be involved in the malaria response *in vitro* and *in vivo* were TNF-α, IL-1β, and IL-6 (1, 28, 29). High levels of TNF-α are associated with severe symptoms in human infection and mice models of experimental CM, hyper-parasitemia, hypoglycemia, and anemia (1, 6, 15, 26, 29). TNF-α is produced by macrophages, monocytes, neutrophils, T cells, and NK cells (1, 26, 29-31). Its presence leads to the release of IL-1β by macrophages, which in turns induces IL-6 secretion by macrophages, endothelial cells and T helper cells (1, 6, 15, 29). IL-1β and IL-6 are pyrogens that contribute directly to elevated temperature in the host and have also been associated with disease severity (1, 15, 16). Another important cytokine involved in malaria is IFN-γ. It is released by natural killer T cells, γδT cells and T helper (TH) cells. It has been associated with disease protection (e.g. decreased parasitemia) and exacerbation (e.g. CM) (1, 6, 8, 15). IFN-γ also leads to the release of IP10/CXCL10 by monocytes, T cells, NK cells, and DCs (15). IL-12 released by macrophages has been show to contribute to parasite clearance (6, 15, 29).

Several chemokines are involved in the pathogenesis of malaria. IP10/CXCL10 has been shown to attract CD8+ T cells to the brain, thereby contributing to the establishment of CM (6, 9, 15, 29, 32). G-CSF secreted by macrophages and endothelial cells induces the release of granulocytes which contributes to anti-parasite immunity (15). RANTES released by DCs and T cells attracts basophils, leukocytes and eosinophils to the site of inflammation and have been associated with recovery of symptoms associated with *Plasmodium falciparum* infection (15). In addition, MIG/CXCL9, MIP-1α/CCL3, MIP-1β/CCL4, MIP-2/CXCL2 and MCP-1/CCL2 have also been implicated in CM (6, 15, 16). The levels of some of these chemokines are elevated in children with severe malaria (i.e. IP10, MIG/CXCL9, MIP-1α/CCL3 MCP-1/CCL2) (16). Eotaxin/CCL3 is an eosinophil-specific chemotactic protein whose levels have been shown to be reduced in the plasma of pregnant women exposed to malaria (33). Finally, high levels of the pro-inflammatory factor NO have been reported to be associated with positive (e.g. parasite growth suppression) and negative (e.g. CM) effects in malaria patients (27).

1.2.5. Parasite organ sequestration

Plasmodium spp. parasites evade the host immune response by sequestering the iRBCs in which they are contained to the vasculature of various organs. This is achieved through up-regulation of VSAs on the surface of the iRBCs that promote binding to receptors such as CD36, ICAM-1 EPCR and PECAM-1, present on endothelial cells (19). The site of sequestration depends on the VSAs upregulated, which is in turn dependent on the genetic strain of the parasite (15). Sequestration has been reported in the lung, bone-marrow, spleen, liver, gastro-intestinal tract, brain and placenta. It can lead to organ failure by obstructing blood-flow and increasing inflammation-mediated tissue damage (15, 19).

1.3. Malarial disease mouse models

Several mouse models that reproduce certain aspects of the clinical presentation of malaria have been described (9, 15). They rely on infection with rodent-adapted *Plasmodium* spp. in multiple mouse strain backgrounds. Table 2 summarizes the mouse strains used in these models. One of the models used to study severe malaria is based on infection of C57BL6 mice with *Plasmodium berghei* ANKA (PbA) (15, 34, 35). In this model, mice exhibit hyper-parasitemia, and develop hepato-splenomegaly and experimental cerebral malaria (ECM) due to the sequestration of iRBCs to the endothelial cells of blood vessels in the liver, spleen, and lungs (15, 35). This

mouse model differs from the CM observed in severe malaria patients infected with *P. falciparum* in that the iRBCs in the mice do not sequester at the brain (35, 36). Instead, ECM in this model is caused by the recruitment of cytotoxic CD8+ T cells to the brain leading to disruption of the blood-brain-barrier and damage to brain tissues (15, 26, 32, 35). Mice infected with PbA experience decreased body temperature and body weight loss during the course of infection (35). They tend to succumb to the disease 7 to10 days after infection with parasitemia levels ranging from 5 to 25% (34, 35). We have used the PbA infected C57BL6 mice as the main model to investigate the impact of housing temperature on the host response to parasitic infection.

1.3.1. Peritonitis mouse model for studying the inflammatory response

The inflammatory response to parasitic agents can be studied in a peritonitis model where mice are injected with parasitic agents (e.g. *Leishmania* promastigotes) intraperitoneally (IP) and inflammatory cells and factors are monitored in the peritoneal cavity (PEC) (37). The presence of parasitic agents recruits immune cells and increases the presence of inflammatory factors and EVs to the site of infection (38, 39). Their abundance induces a robust IR that can be readily analyzed (37). The peritonitis model has been well characterized for studying the IR to *Leishmania major* (38, 39), a protozoan parasite that causes cutaneous leishmaniasis (CL). CL is considered a NTD by the WHO which estimates that 600 000 to 1 million new cases occur each year (40). If left untreated, it causes ulcers that leave scars which can lead to considerable disability and stigma (40). Like *Plasmodium* spp. parasites, *L. major* is transmitted by the bite of an insect vector, the phlebotomine sandfly (40). The sandfly injects the promastigote form of the parasite to the vertebrate host, where it infects macrophages and neutrophils by exploiting the phagocytic activity of these cells (41). This part of the life-cycle leads to the disease symptoms by causing a pro-inflammatory response (41).

1.4. Thermoneutrality and mouse models of disease

1.4.1. Introduction

Thermoneutrality (TN) has been defined as the metabolic state of an organism in an environmental temperature at which it does not have to generate or eliminate heat (42). It is a relatively recent term in the scientific literature with the first reference dating to 1968 (43). Nevertheless, general

interest in this concept is still limited. However, TN is an important concept to consider when conducting research using one of the most common human disease models, the mouse (42, 44-48). Mice are frequently used in research because of ease in maintenance and genetic manipulation (44, 49). However, much of the research conducted on mice models does not translate well to important impact in human clinical trials due to differences in data reporting, study parameters, and mouse vs. human metabolism (50-52). A substantial improvement and refining of murine models is required in order to reduce the variability of findings due to metabolic differences (52). Some problems in metabolic reproducibility stem from circadian rhythm differences with humans, social stressors, and, facility-based differences in the gut microbiome. Another important factor is housing temperature (46, 47, 52-57). In the laboratory setting, mice are routinely housed at temperatures (20-22ºC) below their thermoneutral zone (TNZ) (29-34ºC) (46). In this review, we will refer to the standard temperature used in most research settings as sub-optimal temperature (ST) because it subjects mice to a constant metabolic stress (46). Rodents are particularly affected by exposure to ST because they rely heavily on non-shivering thermogenesis for heat generation and possess a greater thermogenic demand due to their greater surface area to volume (SA/V) ratio than other animals (42, 46). Consequently, mice lose heat more quickly and need more energy to maintain their body core temperature (BCT) (44). Behavioral signs of cold stress can be observed in traditional housing systems: mice tend to huddle together or use nesting material in order to prevent heat loss (58). Mouse pups also vocalize more to indicate distress when they are housed below their thermoneutral temperature (TT) (59). In addition, given the choice, mice preferentially choose an area in their housing that is closer to their TT, regardless of the availability of nesting material (42, 60).

1.4.2. Role of environmental temperature on mouse metabolism

The rodent response to cold stress through brown adipose tissue (BAT)-mediated non-shivering thermogenesis is well understood (52). The environmental ST sensed by the brain (Figure 3A) causes the release of norepinephrine (NE) from the sympathetic nervous system (Figure 3B) (52). The released NE interacts with β-adrenergic receptors (β-AR) on the surface of brown adipocytes (Figure 3C) (52), resulting in break-down of triglycerides (TG) within the BAT into free-fatty acids (FFA) (Figure 3D). The FFA interact with uncoupling protein-1 (UCP-1) present in the cytosol of BAT cells (Figure 3E) and stimulate the mitochondria to produce energy in the form of

heat (Figure 3F) (52). The thyroid hormone (TH) is thought to play a role in this metabolic process by stimulating the brain regions responsible for BAT activation (Figure 3G) (52).

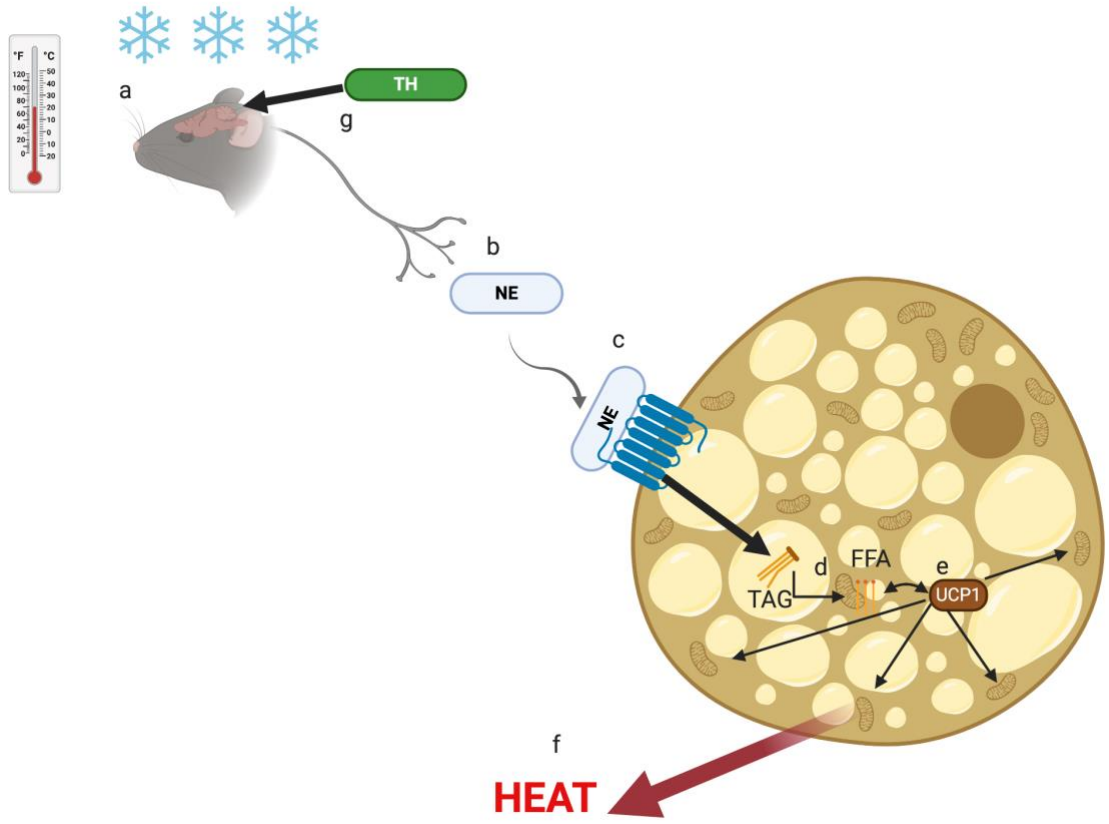

Figure 3. The non-shivering thermogenesis activation pathway.
Cold stress is sensed by the brain **(A)** and signals the sympathetic nervous system to release norepinephrine (NE) **(B)** that interact with b-adrenergic receptor (b-AR) on brown adipocytes **(C)**. As a result, triglycerides (TAG) are broken down to free fatty acids (FFA) **(D)** that interact with uncoupling protein-1 (UCP-1) **(E)** to induce the mitochondria to generate heat **(F).** The thyroid hormone (TH) plays a role in the cold stress sensor region of the brain **(G)** (61).

Various physiological and metabolic effects of housing mice at TT compared to ST have been documented in a number of publications (62-71). At TT, mice have lower energy expenditure in light and dark cycles, higher energy efficiency, and decreased nutrient mobilization and metabolization due to the removal of the cold stressor (63-66). Mice also spend less time awake at TT, suggesting that those housed at ST experience sleep deprivation (44, 64). Mice housed at TT exhibit less cancellous bone loss and are better adapted to deal with metabolic stressors such as

sleep deprivation or heat stress (62, 68, 69, 72). Most mouse models of longevity are more resistant to ST due to their lower BCT (44). In addition, the effects observed on mice housed at TT can often be mimicked at ST by treating the mice with β-AR antagonists, which suppresses the NE pathway for heat generation (69-71).

1.4.3. Role of environmental temperature on the immune system

A major consequence of housing mice at ST is reduced energy availability for the immune system. Energy needed to drive immune processes is instead redirected towards heat generation (49), resulting in clear differences in the immune response of mice at ST compared to TT . Some studies have reported an increase in thermogenesis-associated M2 macrophages at ST (73). These macrophages are associated with adipose tissue and provide the same function as NE during thermogenesis (73). Other studies have reported an increase in BAT inflammation with production of pro-inflammatory cytokines (MCP1, IFNγ, TNF-α, IL-1β, and IL-6) during exposure to ST (74). These alternatively activated macrophages have been shown to be associated with expression of IL-4 and IL-13 and are involved in the expression of catecholamines by BAT (75). T cell proliferation has been shown to be suppressed in part by myeloid-derived suppressor cells (MDSCs) that are generated in greater number via β-AR signalling under ST conditions (76, 77). Another study showed that BAT inflammation at ST is mediated by the type 2 immune response in mice (78). Treatment of mice at TT with IL-33, which triggers ILC2s that are involved in the type-2 response, resulted in an increase in BAT size, activity and energy expenditure (78). In addition, mice and rats housed at ST tend to experience latent hypothermia after immune challenge with LPS, while those at TT undergo a fever response (79, 80). This has also been documented in other animals (e.g. pigs and cattle) (81). In rats, the ST-hypothermic response is age-dependent, with a reduced effect observed in older animals (82, 83). At TT, a reduction in activity is generally sufficient to fight an immune challenge compared to the need for increases in processes such as oxygen consumption, blood glucose metabolism, and FA metabolism at ST (84). Some immune factors, such as TNF-α, Il-6, and Il-1β, have been implicated in the differential response to immune challenge at TT compared to ST due to their function as pyrogens in the fever response (69, 83, 85). In addition, high mobility group box 1 (HMGB1), the macrophage-derived factor involved in tissue repair and tissue fibrosis, was found at a higher level in circulation at TT and was associated with higher liver inflammation (86). Interestingly, immune-challenged rodents tend to prefer

environmental temperatures closer to their TT, indicating that TT is beneficial in the response to various pathogens (82). However, in this review we will outline cases in which housing at TT can exacerbate disease presentation (see Table 1). Figure 4 outlines the effect of thermoneutrality on different models of disease.

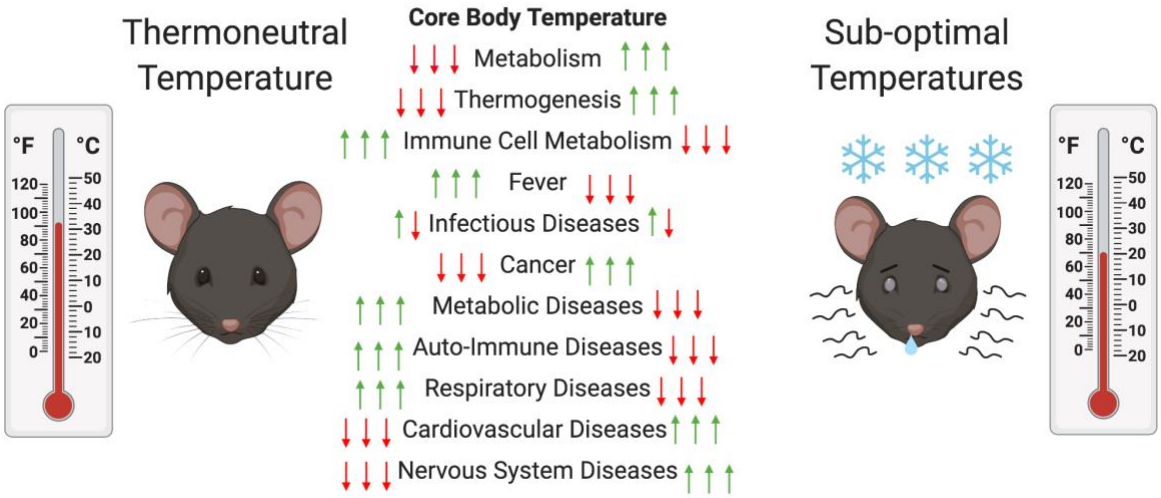

Figure 4. Effects of thermoneutral (TT) and suboptimal temperatures (ST) on mouse metabolism and disease.
In order to maintain core body temperature, mouse metabolism and thermogenesis are increased at ST and decreased at TT. This can have opposing effects on metabolic activity of immune cells and have alleviating (red arrow) or exacerbating (green arrow) effects on various disease models. In the case of infectious diseases, both consequences can occur, depending on the pathogen (61).

Table 1. Effects of sub-optimal vs thermoneutral environmental temperatures on the presentation of murine disease models.

Mouse model	ST (20-24ºC)	TT (27-36ºC)
Obesity	• Alleviated (87, 88) • Lower glucose intolerance (87, 88) • Resistance to treatment (89, 90)	• Exacerbated (87, 88) • Greater glucose intolerance (87, 88) • HFD, UCP-1 KO and T2D KO mice 87, 103, 104) • Better responses to treatments (89, 90)
Cardiovascular diseases (atherosclerosis)	Alleviated (86, 91)	Exacerbated • Increased markers (i.e. plaque build-up and immune cell infiltration) (86, 91)
Non-alcoholic fatty liver disease	Female resistance (92)	Sex-independent establishment (92)
Microbiome	Remodeling of the small intestine (93)	• Increased intestine permeability (92) • Increase in gram neg. bacterial populations (92)
Cancer	• Suppressed cytotoxic activity (94, 95) • Higher anti-apoptotic activity (67) • Resistance to treatment (45, 67, 96-99)	• Tumour resistance (76, 94, 95) • Improved response to treatment (45, 67, 96-99)
Viral infections	Exacerbated (62, 100, 101)	• Alleviated (Rabies, Influenza and Coxsackie virus) (62, 100, 101) • Improved immunization (Coxsackie and Foot and Mouth Disease virus) (101, 102)
Bacterial infections	Hypothermia response (103)	• Fever response (103) • Exacerbation of systemic inflammatory response (*E. coli*) (103, 104) • Lower mortality in *T. rickettsia* infection (105) • Improved immunization against *F. tularensis* (106)
Parasitic infections	• Increase in parasitemia and decrease in schizont size (*P. berghei*) (107) • (108) • Nodular presentation *L. mexicana* (109)	• Higher mortality rates of *P. berghei* infection (110) • Protective against *T. cruzi* infection (111, 112) • Cutaneous presentation *L. mexicana* (109)
Auto-immune diseases (Graft vs Host Disease)	Resistance (70)	Exacerbated response (70)
Respiratory diseases (Asthma)	Exacerbated (113)	Alleviated (113)
Nervous system diseases (Alzheimer's disease)	Increased sensitivity of 3×Tg-AD mouse model (114)	Improved memory function (114)

1.4.4. Respiratory diseases

The effect of TN on the respiratory system of mice has been investigated in the context of the ovalbumin-induced asthma disease model (113). Liao *et al.* observed lower inflammatory cell counts driven by a decrease in eosinophil populations in bronchoalveolar lavage from these mice (113). Airway hyper-responsiveness to asthma was also reduced in mice at TT compared to ST, characterized by lower IL-4, IL-10 and IgGE levels and higher IFN-γ levels (113). These results were attributed to an increase in regulatory T cell infiltration in the lungs at the higher temperature, which dampens the inflammatory response (113). In this context, TN mediates a protective immune response by promoting tight regulation of immune cells at the site of inflammation compared to ST. However, this phenomenon should be tested on other respiratory disease models.

1.4.5. Graft vs host disease

TN housing has revealed a gap in the understanding of murine models is in the context of graft vs host disease (GVHD) (70, 77). This disease occurs when the immune system of an individual grafted with cells or tissue from a genetically-distinct individual recognizes it as foreign and induces a fulgurant immune response. It was previously thought that congenic mice were resistant to the establishment of GVHD but Leigh *et al.* (27) found that grafting mice with bone marrow-derived T cells caused severe GVHD characterized by lesions in the intestines and liver of mice housed at TT (70). They attributed the resistance in mice housed at ST to high NE levels that impaired their immune response. Resistance at ST was reversed when the mice were treated with a β-AR antagonist or the gene encoding β-AR was knocked-out (70). However, when mice were treated with a β-AR at TT agonist, GVHD was decreased (70). In another study, the same group demonstrated that mice with β-AR deficient T cells housed at ST experienced a Th1 response when implanted with an allograft, suggesting that β-AR signalling plays a direct role in the severity of GVHD (77). They also showed that treating mice with a β-AR agonist decreased the severity of GVHD (77). These two studies demonstrated a clear link between TN and a metabolically-activated immune system mediated by β-AR signalling.

1.4.6. Cancer

TN has been investigated more frequently in cancer than in other non-metabolic diseases. Housing at TT has been shown to confer tumour-growth resistance in mice with various types of cancers (76, 94, 95). For example, mice inoculated with skin melanoma, colon carcinoma, pancreatic carcinoma, or mammary gland adenoma tumour-cells all had lower tumour formation, growth, and metastasis at TT compared to ST (94). In another study, mice inoculated with mammary gland adenoma had smaller tumours size at TT compared to ST (95). This effect was determined to involve a change in number, activity and function of CD8+ T cells and dendritic cells at higher temperatures (94, 95). A ST environment increases the number of immature or incomplete phenotype DCs, while at TT, CD8+ T cells are more likely to be activated and express IFNγ (94, 95). Also, mice housed at ST exhibited cold stress accompanied by elevated levels of NE that decreased the energy available for their immune system and reduced their response to tumour cells (45, 96-99). Mohammadpour *et al.*, showed that mammary gland adenoma murine models, circulating NE released under ST conditions activated MDSCs through their β-AR, resulting in decreased T cells proliferation and increased tumour growth (76). This effect was reversed, in β-AR knockout mice at ST or with mice treated with β-AR agonists at TT (76). The beneficial effect of TN housing was further demonstrated by the preference for that temperature in tumour-bearing mice when given the choice (94). The effect of cold stress at ST is even stronger in individually ventilated cages (IVC), a system that is widely used in the current laboratory settings (115). Mice housed in IVCs had smaller tumours, lower tumour metabolism, larger adrenal weights and more signs of cold stress than those housed in a static system at the same temperature (115). This was somewhat reduced when the mice were given the opportunity to shelter with nesting material (115).

TN may have a positive influence on various cancer therapies. Mice with colon adenocarcinoma had lower responses to irradiation and chemotherapy at ST compared to TT (98). Mice bearing pancreatic tumours had higher sensitivity to cytotoxic treatments at TT compared to ST (67). Melanoma tumour-bearing mice had lower tumour growth after 3 weeks of aquatic exercise at TT compared to ST (116). This response was attributed to an enhanced cytotoxic-cell mediated immune response characterized by increases in populations of natural killer cells; natural killer T cells; γδT cells; CD8+ T cells; and, IFN-γ at the higher temperatures (116). In addition, resistance

to treatments at ST was mostly attributed to elevated levels of NE because treatment with beta-blockers resulted in responses similar to those observed at TT (45, 67, 96-99). Interestingly, higher levels of anti-apoptotic molecules (BAD, MCL-1, BCL-2 and BCL-xL) and CREB, a transcription factor involved in the survival of these molecules were seen at ST compared to TT, which may also contributed to lower treatment efficacy at ST (67).

An irradiation-induced cancer model displayed a higher propensity for hematopoietic stem cells to undergo apoptosis when the animal received total body irradiation at TT compared to ST (117). Therefore, investigation of tumorigenesis in mouse models only at ST is likely to limit our understanding of this process.

The studies described above were limited to certain types of cancers and do not exclude that some cancer types would react differently to a TT environment. Also, different acclimatization times for the cage temperatures in the studies were likely to affect mouse metabolism differently (67, 94, 95, 98, 99, 117).

1.4.7. Metabolic diseases

Most studies conducted at TT have been on obesity-related research. Obesity can be induced in mice by providing them with an *ad libitum* high fat diet (HFD) (118). As mentioned in the introduction, the mouse metabolism is less active at TT with evidence of lower fasting glucose levels, decreased food intake, lower energy expenditure and decreased BAT activation because the animal does not have to generate heat (63-66, 87). These diet-induced changes occur independently of the type of diet fed to mice (87). However, some diet-based differences occur at TT. In most HFD-obese mice models, mice housed at TT have lower glucose clearance, higher adiposity, increased hepatic fat accumulation, increased adipose inflammation and greater glucose intolerance than mice at ST fed the same diet (87, 88). This suggests that housing at TT exacerbates the establishment of obesity.

Diet-dependent metabolic changes are closely related to the reduction of the NE-mediated BAT activation pathway at TT. Disruption of the pathway by gene knock-out or treatment with exogenous disruptors in mice housed at ST leads to similar results (71, 74, 119-123). For example, when C57BL6 mice were treated with BMP7, an inducer of BAT differentiation, they displayed

increased food intake; increased BAT weights; increased expression of *ucp-1*, CD36 and lipase; increased energy expenditure; lower WAT weights, and higher lipolysis activity at ST, but not at TT (119). UCP-1 KO mice have been used to study the establishment of obesity because they were thought to be resistant to it when fed an HFD (123). However, it was shown that this resistance disappeared at TT because of a decrease in metabolic efficiency (121, 122). However, these KO mice did not exhibit adipose tissue inflammation at TT compared to ST, which suggests that BAT inflammation is due to exposure to cold and not to accumulation of fat (74). In addition, disruption of TH activity by knocking-out the type 2 deiodinase enzyme required for its activation had a similar effect as knocking-out UCP-1 (120). The obesity phenotype was decreased at ST, but increased at TT due to the inability of those mice to activate diet-induced thermogenesis compared to WT mice (120).

Thermoneutrality also has an effect on obesity-related disease presentations such as atherosclerosis and non-alcoholic fatty liver disease (NAFLD) (86, 91, 92, 124). Atherosclerosis is defined as plaque build-up coupled with immune cell infiltration in the blood vessel wall and is one of the leading causes of cardiovascular disease (CVD) (124). It occurs when there is an increase of lipids in the circulation as a result of changes in the metabolic environment (124). When housed at TT, mice display two metabolic markers of atherosclerosis (i.e. adipose and vasculature inflammation) faster than at ST, even if there is no change in insulin resistance (86, 91). Macrophage infiltration in the aorta and upregulation of pro-inflammatory cytokines, chemokines and other inflammatory mediators were observed at TT (91). In addition, the combination of a low metabolism at TN and a HFD initiated atherosclerosis in WT mice by altering blood lipid profiles and increasing aortic plaque size (124). In mice that are predisposed to atherosclerosis, the disease was exacerbated by the same combination (124). These results suggest that the link between adipose tissue inflammation and insulin-resistance only occur in cold stressed mice and thus are not applicable to our understanding of human atherosclerosis (91). Diet-induced NAFLD is the most common chronic liver disease and may lead to complications, the need for liver transplantation, and death (92). Female mice were thought to be resistant to the establishment of the disease due to hormonal differences (92). However, multiple strains of mice housed at TT and fed a HFD had lower corticosterone production; upregulated pro-inflammatory cytokines; and, exacerbated diet-induced NAFLD, independently of sex (92). Sex-independent increases in intestinal permeability and an

altered microbiome were also observed at TT. Depletion of gram-negative bacteria, TLR4 deletion, or IL-17 axis activation reversed all TT-associated effects (92). This strongly implicates a role for environmental temperature on the composition of the gut microbiome in the establishment of NAFLD because the skewing of gram-negative bacteria at TT increases intestinal permeability, which in turn causes more LPS to interact with TLR4 to increase inflammation (92). This is particularly relevant because the small intestine is remodeled by the microbiome during cold stress acclimatization (93). These results led some researchers to speculate that the mouse microbiome is different at TT compared to ST, but more studies should be conducted to establish the effect of TN on the microbiome (49). Housing temperature does not seem to influence joint replacement therapy failure models (125). Joint replacement is used to treat joint degenerative disease. Wear and tear on the joint as well as lower circulating levels of leptin in obese individuals are thought to play a role on osteolysis leading to joint replacement failure (125). However, obese (*ob/ob*) mouse models are resistant to the development of osteolysis at ST and TT suggesting that this effect is independent of TN (125).

Finally, some of the proposed obesity treatments have different outcomes at TT compared to ST (89, 90). Because obese mice treated with DNP, a weight loss drug, had no reduction in obesity phenotype at ST, the drug was initially thought to be ineffective (89). However, although treatment with DNP for 4 weeks at TT did not affect food intake, energy expenditure increased; body weight and fat mass decreased; glucose tolerance improved; steatosis was reduced; and decreases were observed in TH levels, UCP-1 expression and BAT thermogenesis (89). In addition, when mice underwent weight reduction surgery, they showed similar energy reduction at ST and TT, but those at TT showed lower energy basal demand at TT (90). They also exhibited less torpor, defined as a depression in all physiological functions at TT, suggesting that TN improved the outcomes of surgery by enabling mice to recover better (90).

The limitations of these studies include different temperatures; mice models; and, acclimatization times used in different experiments. These difference may explain why some contradictory effects where observed in different studies such as the presence or absence of temperature-dependent glucose tolerance at TT (87, 88).

1.4.8. Cardiovascular diseases

There are clear links in rodents between metabolic activity and changes in cardiovascular system activity that can predispose to CVD. Several studies showed that the metabolism of rodents at TT changes some of their cardiovascular parameters (64, 126-128). Mice and rats housed at temperatures oscillating between ST and TT, had lower mean blood pressure; heart rate; and, pulse pressure when the cage temperature was closer to TT as a consequence of lower thermogenic demands at TT (52, 126). The differences were more significant in mice than rats, most likely due to their greater metabolic demands (57). Similar results were also obtained for mice and rats housed at TT compared to ST (64, 128).These changes are clearly representative of a change in metabolism since they were observed with greater intensity when mice at TT were fasting (127). Oxygen consumption was unaffected at TT for mice but decreased for rats (127). These changes can predispose rodents to cardiovascular events like coronary artery disease or atherosclerosis, described in the 'metabolic diseases' section (86, 91). It is not yet clear whether TN has a protective effect on other CVDs.

1.4.9. Alzheimer's disease

The impact of TN on the central nervous system has been studied in the context of Alzheimer's disease (AD) (114). Vandal *et al.* found that the triple-transgenic mouse model of AD (3×Tg-AD) was more susceptible to a cold environment despite having a higher non-shivering thermogenesis activity than WT mice, thus suggesting that ST would play a greater impact on these mice (114). At TT, the memory function and neuropathology of 3xTg-AD mice was improved within a week compared to 3xTg-AD mice housed at ST (114).

1.4.10. Bacterial infection

In the context of the fever-response to bacterial agents, housing temperatures closer to TN may alleviate or exacerbate disease presentation. As mentioned previously, rodents challenged with LPS develop hypothermia when housed at ST and fever when housed at TT (103). This was also observed in rats treated with a dose of *Escherichia coli* which induced a systemic inflammatory response syndrome (103). Hypothermic rats housed at ST also had decreased neutrophil infiltration in their lungs, lower arterial pressure, decreased endotoxemia, increased liver bacterial load, lower

organ dysfunction, and lower mortality rates (103). This demonstrates that fever in rodents housed at TT can exacerbate disease, probably due to increased sensitivity to infectious agents and immune-cell mediated bacteriolysis followed by sepsis (103, 104). Interestingly, changes were not seen in levels of the major fever-associated cytokines (103).

It is important to note that an exacerbated response does not occur in all bacterial infections. Mice injected with *Typhus rickettsia*, which develops more favourably in a colder environment, had a 75% decrease in mortality rates at TT compared to ST (105). Importantly, increased immune sensitivity at TT can be beneficial in vaccination against bacterial disease. Balb/c mice housed at TT vaccinated with live attenuated *Francisella tularensis* had a higher IFNγ-mediated antigen-specific T-cell response when challenged with the pathogen (106). Unfortunately, information on the effect of TT on bacterial diseases in mice is limited. The studies cited above show variation in the bacterial species or strain, animal species, strain, age and sex, acclimatization time, type of cage system, and type of inoculum tested (103, 105, 106). This makes it difficult to draw solid conclusions pertaining to the specific role of TN in the context of bacterial infections.

1.4.11. Viral infection

A TN environment may also be protective for the murine host experiencing viral infection. Lower inflammation (i.e. lower leucopenia and lower cytokine induction) was reported in mice infected with influenza virus at TT compared to ST, despite the presence of equivalent viral titers in both groups (62). The animals at TT also exhibited a fever-response and better sleep recuperation (62). It has also been shown that mice infected with rabies virus at 37ºC had lower mortality rates and lower brain-viral burden than those at 24ºC (100). Similar results were obtained with mice infected with Coxsackie virus at 36ºC compared to 25ºC (101). In addition, mice housed at 36ºC responded better to a pre-challenge live-vaccine (101). Interestingly, the effect observed at TT was absent in animals treated with an immune-suppressor, suggesting that the change in survival and tissue-burden was mediated by increased immune cell competence at TT (101). In another study, mice at TT that received a foot-and-mouth DNA vaccine had a superior immune response, with increased IgG levels, T cell proliferation, IFNγ production in both CD4+ and CD8+ T cells, and IL-4 production in CD4+ T cells, compared to heat-stressed mice at 38ºC (102). However, this response was not tested at ST and the temperature defined by researchers as TT (24ºC) was lower than the

generally recognized TN range for mice (46, 102). There may be viral infections in which TN is detrimental for the host, but these have not been reported in the literature.

There are important limitations in the investigations of the TN effect on the response to viral infections. The first is the wide range of temperatures defined as being thermoneutral (62, 102). In addition, as with studies on bacterial infections, mouse strain, age, sex and acclimatization time should be taken into account in future studies. Use of additional viral disease models will be required to gain a better understanding of TN on the immune response to viruses.

1.4.12. Parasite infections

The effects of TN on the outcome of parasitic infections are still largely unknown. However, a few studies investigating the role of temperature on parasitized murine hosts offer insights into potential consequences of TN housing on parasitic disease models (107-112).

1.4.12.1. Malaria

Malaria-causing parasites belonging to the *Plasmodium* genus have a complex life cycle (129).The species that infect mammals incorporate temperature changes that occur during transmission between invertebrate to vertebrate hosts into their development (129). The blood-stage of these parasites causes the majority of malaria-associated symptoms, including fever, which also has consequences on the parasite (129). Thus, housing *Plasmodium*-infected mice at TT could have significantly different clinical presentation compared to those housed at ST. There is some evidence to support this possibility in the literature. For example, mice exposed to cold temperatures had an increase in parasitemia after 4 days, suggesting that parasitemia may diminish at TT (107). In addition, blood-stage parasite development may be affected by TT. Rats infected with two different strains of *Plasmodium berghei* had schizont-stage parasites of smaller sizes when exposed to colder temperatures (12ºC) compared to TT, highlighting a potential protective effect of lower temperatures (108). This difference was not observed in rats housed at 20ºC (108). Housing density also seems to be a factor in the outcome of *Plasmodium* infection. Mice housed in groups of five at TT (27ºC) had higher mortality rates than those housed individually. The latter had the same mortality rates as mice housed at ST (21ºC) in groups of five, but higher rates than those housed individually at 21ºC (110). The study investigators concluded that the group-housed

mice were probably more susceptible to disease at both temperatures because of their inability to dissipate body heat during the fever-response induced by the blood-stage of malaria (110). In this context, TT would be expected to exacerbate the malarial symptoms. In order to gain a better understanding of TN and malarial disease, other aspects of this disease such as its role on sequestration or spleen megalopathy should be investigated.

1.4.12.2. Trypanosomiasis

It is unclear whether malarial disease clinical signs would be diminished or exacerbated at TN. However, higher temperatures seem to have a protective effect against infections with *Trypanosoma cruzi*, the causative agent of Chagas disease. Mice infected with the parasite at 35ºC survived and overcame the infection, whereas those infected at 26ºC experienced chronic infections (111). Similar results were obtained in a different mouse strain, where a reduction of parasitemia and tissue pathology, and an increase in immune responsiveness was observed (112). In both studies, the protective effects of high temperatures were completely reversed by treatment with immunosuppressors or depletion of CD8+ immune cells, implicating the competence of immune cells at TT (111, 112).

1.4.12.3. Leishmaniasis

Finally, the environmental temperature of the host has been tested on an infection caused by a third type of parasite: *Leishmania mexicana* (109). Balb/C mice at 32ºC had inoculation site nodular inflammation with macrophage infiltration and phagocytosis presentation whereas those at 22ºC had a more cutaneous presentation with mast cell infiltration (109). Both showed visceralisation as confirmed by parasite culture from lesions, immunohistology and PCR (109). However, the mice were not conditioned to the different temperatures beforehand (109).

The parasitic infections described above only represent a minor fraction of all parasitic diseases that could be impacted by TT. For each of those diseases, a wide variety of models exist, constituted of various mouse strain and parasite species/strain combinations that can be investigated at TN. In addition, many of the studies described above did not acclimatize the animals to the temperature conditions, which could bias the results (108, 109, 112). Murine host

sex and age differences, as well as difference in acclimatization time and caging system will also have to be taken into consideration when conducting future experiments.

1.4.13. Opposing views to the thermoneutral model

Most of the current literature on TN advocates for conducting experiments on mice in housing at approximately 30ºC, which is within the TNZ of mice (42, 44-47, 49, 54, 96, 130, 131) . Thus results obtained at this temperature are more likely to be consistent with the metabolic state experienced by humans (42, 44-47, 49, 54, 96, 130, 131). However, some investigators have argued that the state of mice at this temperature is not representative of the average human metabolic state and experiments conducted at 30ºC cannot be extrapolated to humans (132). In their review, Speakman *et al.* state that most humans function in environments that are on average 3ºC below their lower critical temperature (Tlc) (132). This means that they occupy a space at a temperature that is below the lowest temperature within their TNZ (132). This metabolic state in humans corresponds to about 23-25ºC in mice (132). In addition, Speakman *et al.* argue that mice are routinely given the opportunity to shelter and huddle in group housing, which increases their BCT (132). In this context, the researchers claim that the Tlc of the mice is further reduced to 20-22ºC (132). One of the assumptions of this argument is that the murine response to change in environmental temperature is the same as the human response (132). However, as mentioned in the introductory section of this report, the metabolism of mice and humans is very different. Mice have a higher metabolic demand due to their smaller SA/V ratio and they rely more heavily on non-shivering thermogenesis than humans (42, 46). In addition, there is substantial evidence that humans mostly operate at a temperature within their TNZ (44, 54, 133).Thus, we are in agreement with the belief held by other investigators that more murine studies should be conducted at TT and the results compared to those obtained at ST. This will provide a better understanding of mouse disease models and refine their use for application to human health.

1.4.14. Summary

As outlined in the Introduction, very few results obtained in mouse studies translate to human trials (50, 53, 57). This is generally attributed to biological differences between mice and humans and has led to calls by the scientific community to improve the murine models used to study human diseases (50, 53). One major aspect of studies with murine models that has been overlooked is the housing environment. Besides the consequences due to social stressors and microbiome differences, housing temperature also plays a role in the response to immune challenge (53, 57). Sub-optimal housing temperatures routinely used in the laboratory setting induce metabolic activity for heat generation, reducing energy available for metabolism of other biological functions like the immune system (46). The differing metabolic states of mice housed at ST and TT are likely to complicate our understanding of disease in murine models (Figure 2) (46). In this review, we illustrated that housing at TN could either exacerbate or have protective effects in the context of bacterial, viral and parasitic infections, as well as in metabolic, cardiovascular and cancer diseases. We also showed the differential effect of TT compared to ST housing in Alzheimer's disease, respiratory disease and graft vs host disease. Taking into consideration the various original research articles and independent reviews done on the subject, we feel that it is safe to assume that housing temperature can influence the outcome of studies on murine models of disease and therefore affect our understanding of human diseases, in spite of confounding factors linked to sheltering material and group 'huddling' (58, 132). We conclude that there is an advantage to investigating the role of TN in various disease models and comparing results to those obtained at ST in an effort to improve our understanding of human diseases.

1.5. Objective of research and rationale

Malaria is one of the most important parasitic diseases with urgent need for elimination due to the high case mortality still occurring globally. Severe malaria causes most of these deaths and is characterized by a fever-like illness and organ pathologies. One of the targets for elimination is to increase our efforts in malaria disease research and development. The blood-stage of the *Plasmodium* parasite is an appropriate focus for studies as it constitute the part of the life-cycle where most of the symptoms occur due to parasitic immunomodulatory factors such as nHZ interacting with innate immune cells. This releases a series of inflammatory factors (e.g. cytokines, chemokines, NO and immune-cell derived EVs) that synergistically create an IR. To study the malaria severe disease, the malaria model of PbA-infected C57BL6 can be used. However, like in most mouse model of diseases, the mice used for research, unlike humans, are housed at a temperature below their TT. This ST has the effect of changing the IR and disease progression in a variety of disease models because the energy used by the mice to drive thermogenesis can no longer be used for immune cell metabolism. This difference in metabolism between laboratory mice and humans is one of the factors implicated in the lack of translatability to human clinical trials. Most studies investigating the role of thermoneutrality have done so in cancer, metabolic and bacterial diseases but there is very limited publications available on the role of thermoneutrality on the severe malaria disease model.

This led us to pose the research question: “How does thermoneutrality affect malaria inflammatory response and disease progression?”

We hypothesized (1) that the IR of bone-marrow derived macrophages (BMDM) to infectious agents (i.e. LPS) would be different at TT and fever-like temperature (FT) compared to ST, and (2a) that the IR to nHZ injection in a peritonitis model and (2b) disease progression in PbA-infected mice would be different at TT compared to ST.

The following research objectives were developed to test these hypotheses:

1. Determine the effect of nHZ on the IR using a murine macrophage *in vitro* model at FT vs TT vs ST.
2. Investigate the role of TT on malarial disease progression and associated IR *in vivo.*

2. Methods, results and discussion

2.1. Preface

The results of this project will be submitted in the form of a manuscript ("the paper") for publication. The paper focuses on the research question described in section 1.2 that was to investigate the effect of thermoneutrality on 1) the IR of BMDMs *in vitro* 2) the IR and disease progression of the severe malaria mouse model. The methods and results described in this paper were *in vitro* stimulation of BMDMs with infectious agents at ST, TT and FT, *in vivo* stimulation of mice at St and TT with parasitic agents (i.e. nHZ and *L. major*) in a peritonitis model and mice infections with PbA and disease monitoring at ST and TT.

2.2. Author contributions

Overall project was designed and envisioned by MO. *In vitro* experiments were performed by FV. *In vivo* IR study experiments were performed by FV and MO. High sensitivity flow-cytometry experiments were performed by IA in the laboratory of EB, data was analyzed by IA and FV. TEM sample preparation and imaging was performed by GD. NTA measurement was performed by GD and analysis was done by FV. Disease progression study experiments were performed by FV. Statistical analysis was performed by FV. DPL was consulted for questions about mice housing at thermoneutrality.

Thermoneutrality and severe malaria: investigating the effect of warmer environmental temperatures on the inflammatory response and disease progression.

Fiorella Vialard1, Isabelle Allaeys2, George Dong1, Eric Boilard, David P. Labbé1, Martin Olivier1.

1 Research Institute of the McGill University Health Centre, 1001 Boulevard Décarie, Montréal, QC, H4A 3J1, Canada.
2 Centre Hospitalier Universitaire de Québec, Université Laval, 2705 Boulevard Laurier, Québec, QC G1V 4G2, Canada

Correspondence : martin.olivier@mcgill.ca

Keywords : Thermoneutrality, malaria, mouse models, *Plasmodium berghei* ANKA, *Leishmania major*, inflammatory response, disease progression.

Abstract

Most studies using murine disease models are conducted at sub-optimal housing temperature (ST). However, experiments performed at thermoneutral temperature (TT) have revealed an altered immune response to pathogens. How such conditions affect the inflammatory response (IR) to *Plasmodium berghei* ANKA (PbA) infection and subsequent disease progression is unknown.

We hypothesized (1) that the IR of bone-marrow derived macrophages (BMDM) to infectious agents (i.e. LPS) would be different at TT and fever-like temperature (FT) compared to ST, and (2a) that the IR to native hemozoin (nHZ) injection in a peritonitis model and (2b) disease progression in PbA-infected mice would be different at TT compared to ST.

To test our first hypothesis, BMDMs were stimulated with LPS at FT (39ºC), TT (37ºC) or ST (30ºC) for 6, 12 and 24 hours. Nitric oxide (NO) and cytokine concentrations were determined from the culture supernatant. We observed that BMDMs released increased concentrations of NO at TT compared to ST and that these concentrations increased overtime. We also observed differential NO and pro-inflammatory cytokine production at different temperature with FT resulting in the highest release of NO and most cytokines.

To test our second hypothesis, C57BL6 mice were acclimatized for 3 weeks at TT (28 – 31ºC) or ST (20 – 22ºC). In one study, mice were injected intraperitoneally with nHZ or *Leishmania* at both temperatures, and immune cells were isolated from the peritoneal cavity (PEC) fluid, counted and sub-typed using microscopy. Cytokine concentrations and extracellular vesicle (EV) profiles were also determined from the PEC. *Leishmania* injection resulted in decreased neutrophil recruitment in mice housed at TT compared to ST and a similar trend was observed for nHZ-injected mice. Phagocytosis of nHZ was higher in mice housed at TT and cytokine concentrations differed at TT and ST. EV concentrations in mice injected with parasitic agents were lower at TT. There was no difference in the release of EVs by specific cell types at both temperatures except for a trend of increased proteasome-derived EVs and decreased leukocyte-derived EVs at TT.

In another study, mice were injected with 10^4 luciferase-tagged PbA-infected RBCs. Symptoms were monitored until death, when blood and organs were collected. We observed trends in increased survivability and lower parasite organ sequestration, using a luciferase quantification

assay, at TT compared to ST. Mice at TT experienced a significantly lower loss of body weight and body temperature than their ST-housed counterparts. The cytokine serum concentrations were similar at end-point.

Our results suggest that thermoneutrality might change the progression of malarial disease by dampening the IR of the host to parasitic agents, but of interest it is clear that various metabolic-related issues are influenced.

2.3. Introduction

Malaria is a neglected tropical disease (NTD) caused by blood-apicomplexan parasites of the *Plasmodium* genus and transmitted by the bite of infected female *Anopheles* spp. mosquitos (4). The World Health Organization (WHO) attributed 229 million human cases and 409 thousand deaths to malaria in 2019 in endemic regions (4). These deaths typically occur in non-immune individuals (e.g. children or adults from low-transmission/non-endemic areas where exposure is low) in which the clinical presentation of the disease progresses to severe malaria (3). Severe malaria is most often caused by *Plasmodium falciparum* and is characterized by high parasitemia and organ pathologies (e.g. hepato-splenomegaly, respiratory distress and kidney failure) resulting from parasite sequestration to these organs followed by immune-mediated cytotoxicity (3, 13). One of the hallmarks of severe malaria due to *P. falciparum* infection is cerebral malaria (CM), during which patients experience coma-like symptoms following parasite-sequestration to the brain (13).

Despite advances in programs targeting malaria elimination, the milestone set by the WHO to reduce mortality by at least 40% by 2020 has not been achieved (4). Several factors have been identified for the stagnation in elimination efforts: the COVID-19 epidemic, parasite resistance to anti-malarial drugs, vector resistance to long-lasting insecticide-treated nets (LLINs), the rise of *Plasmodium* mutants undetectable with rapid diagnostic tests (RDTs), and climate change (4, 5). The WHO has recommended continued investment in malaria research and development initiatives in order to achieve elimination of the disease (4). Therefore, a more thorough understanding of the effect of the parasite on the immune system of the vertebrate host is critical to future control of the disease.

The erythrocytic cycle of *Plasmodium* spp. is key to understanding malarial disease because it is the stage of the parasitic life-cycle in which most symptoms occur (2). Briefly, after the vector transmits *Plasmodium* spp. sporozoites to the vertebrate host and the parasites undergo a round of replication in the liver, the resulting merozoites (Mz) initiate the blood stage by invading RBCs, developing asexually to trophozoites, and forming schizonts that eventually rupture the infected RBCs (iRBCs) and release additional Mz (6). Each Mz can then infect a naïve RBC and initiate multiple rounds of RBC infection and Mz release (6). A fraction of trophozoites differentiate and form gametocytes that can reproduce sexually in the mosquito host (6). The lysis of iRBCs results in release of parasites and iRBC cytosolic content, both of which can interact with and affect innate immune cell function (6). The engaged immune cells release inflammatory factors, such as pro-inflammatory cytokines/chemokines, nitric oxide (NO) and reactive oxygen species (ROS) that drive the

inflammatory response (IR) and lead to the fever-like symptoms observed in malaria patients (1, 6). One of the cytosolic iRBC contents released during schizont lysis is hemozoin (HZ), an inorganic crystal produced from the digestion of hemoglobin by the parasite within iRBCs (6). It is also referred to as malarial pigment because it confers a brown colour to blood and organs where it tends to accumulate in patients with severe malaria suffering from hyper-parasitemia (6). Its effect on the malarial IR of the host has been extensively characterized in our lab using *in vivo* models (6, 27).

Extracellular vesicles (EVs) are cell-membrane derived microparticles released by all living cells that can transport nucleic acids, proteins and lipids from cell to cell and elicit biological responses. In the context of malaria, there is evidence that EVs released by iRBCs and other host cells can contribute to the severity of disease by modulating the IR (7, 8, 20, 21, 23).

Mouse models of disease have been used effectively to study malaria IR and disease progression. *Plasmodium berghei* ANKA (PbA)-infected C57BL6 mice are used as a model for severe malaria as it mimics most of the symptoms occurring in human patients (15, 34, 35). In addition, a mouse peritonitis model has been used to study the IR to several parasitic agents. Our lab and others, have shown that injecting mice intraperitoneally (IP) with the *Leishmania* parasite causes the recruitment of inflammatory cells to the peritoneal cavity (PEC) (38, 39). *Leishmania* spp. are other apicomplexan parasites responsible for *Leishmaniasis*, a group of NTDs that is also transmitted by the bite of an infected arthropod vector (i.e. the sandfly) (40). However, the major metabolic limitations of this murine model and others should be considered as it has been implicated in the lack of translatability from mouse model studies to human clinical trials (61).

Almost all studies using mice are conducted at temperatures below their thermoneutral temperature (TT) (i.e. 30ºC). Housing at sub-optimal temperature (ST) (i.e. 20ºC) has an impact on the IR because energy required for immune cell metabolism is instead re-directed towards non-shivering thermogenesis (61). This effect has been studied in the context of cancer, metabolic diseases, respiratory diseases, graft-vs-host disease, cardiovascular diseases, Alzheimer's disease, bacterial and viral diseases. However, reports on parasitic infections at thermoneutrality are limited (61). The IR to parasitic infection and progression of disease might be exacerbated at TT if the immune cells are more performant and induce a fulgurant response. Alternatively, they may be reduced if the animal's immune cells are able to control the parasitic infection without causing severe pathologies. Therefore, we hypothesized (1) that the IR of bone-marrow derived macrophages (BMDM) to infectious agents (i.e. LPS) would be different at TT and fever-like temperature (FT) compared to

ST, and (2a) that the IR to nHZ injection in a peritonitis model and (2b) disease progression in PbA-infected mice would be different at TT compared to ST.

2.4. Material and methods

2.4.1. Cell cultures and conditions

The immortalized murine BMDM litter mate 1 (LM-1) cell line (generated in our lab(134)) was maintained in culture at 37°C in 5% CO2 in Dulbecco's Modified Eagle Medium (DMEM) (Wisent Inc., St-Bruno, QC) supplemented with 10% heat inactivated FBS (Invitrogen, Burlington, ON, Canada), 2mM L-glutamine, 100 U/ml penicillin and 100 µl/ml streptomycin (Wisent).

Cells were seeded in 6-well plates ($1x10^6$ cells/well) or 24-well plates ($5 x10^5$ cells/well), treated with LPS (100 ng/mL) or endotoxin-free PBS (Wisent) (i.e. negative control) in triplicate, and incubated at 30ºC (ST), 37ºC (TT) or 39ºC (FT) for 6, 12, or 24 hours. At the end of the incubation period, the cell culture medium was collected to determine nitic oxide and cytokine/chemokine levels.

2.4.2. Nitric oxide assay

Nitric Oxide (NO) quantification was conducted on cell culture medium using a NO quantification kit according to the manufacturer's protocol (Invitrogen), in conjunction with an Infinite 200 Pro plate reader (Tecan Trading AG, Switzerland).

2.4.3. Animals and ethics

All experiments using mice were carried out in pathogen-free housing under the Animal Use Protocol 7607 at McGill University according to CACC Guidelines and approved by the McGill University Animal Care Committee,

Female C57BL/6 wild-type mice (6-8 weeks) from our in-house colony were used for PbA infection to obtain nHZ.

For all other experiments, female C57/BL6 wild-type mice (3-4 week old) were purchased from Charles River Laboratory (Wilmington, MA, US) and acclimatized in standard housing at ST (19-22ºC) or in a temperature controlled cabinet at TT (27-31ºC) for three weeks before the start of experiments. Mice were housed individually to prevent warming by clustering in ST-housed mice (61). Cages were changed once a week and humidity level, ammonia level, cage temperatures, food/water intake, and mouse body weights were monitored twice a week.

2.4.4. Native hemozoin preparation

C57BL6 mice were treated with iron dextran before infection with PbA following a previously established protocol(32). This treatment enabled us to obtain blood samples with high parasitemia by preventing experimental cerebral malaria (ECM) that normally occurs in PbA-infected mice.(32) The spleens of two infected mice (parasitemia > 60%) were collected and homogenized for nHZ extraction following a previously established protocol (27). Briefly, the homogenates were treated with Tris-Hcl (100mM) and centrifuged at 3000 RPM for 10 min to release and separate the nHZ crystals from cells. The resulting pellet containing nHZ crystals was washed in PBS and centrifuged three times at 3000 RPM before drying on a heat plate at 95ºC overnight. The dried nHZ was weighed, resuspended in PBS, and sonicated on ice for 15 min at maximum amplitude using an Ultrasonic Liquid Processor s4000 (Misonix, Farmingdale, NY, USA).

2.4.5. Parasite cultures

Leishmania major strain NIH S (MHOM/SN/74/Seidman) clone A2, generously supplied by Dr Shaden Kamhawi of the NIH, was cultured at 25ºC in Schneider's Drosophila Medium (Gibco-BRL, Grand Island, NY) supplemented with 10% heat-inactivated FBS, 5mg/ml HEMIN, 2 mM L-glutamine, 100 U/ml penicillin, and 100 µl/ml streptomycin. Cultures of promastigotes growing at logarithmic phase (day 3-4 post passage) were passaged bi-weekly at least twice and grown to stationary phase (day 6-8 post passage) for use in mouse studies.(135)

2.4.6. Intraperitoneal inoculation

Frozen nHZ was thawed and diluted in 1X PBS to a final concentration of 1mg/mL. Mice at ST and TT were injected with 200ul of endotoxin-free PBS (negative control), nHZ or 10^8 *L. major*

promastigotes (positive control) intraperitoneally. After six hours, 5ml of cold PBS were injected into the peritoneal cavity (PEC) and immune cells were collected by lavage.

2.4.7. Inflammatory cells monitoring and bio-banking for further analysis

The number of live cells in the PEC lavage samples was counted with a hemocytometer as follows. An aliquot of 150 µL of the cell suspension was transferred to a Cytofunnel (ThermoFisher, Waltham, MA, USA) and centrifuged at 300 rpm for 5 minutes for deposition onto a glass slide. The slides were dried and stained using a Diff-Quick kit following the manufacturer's protocol (RAL Diagnostics, Martillac, France). Cells were phenotyped by microscopy at 100X with oil-immersion. The slides were incubated for 24 and 48 hours at 37ºC, dried and stained using a Diff-Quick kit to visualize phagocytosis of *L. major* by macrophages and neutrophils. The remaining volumes of the PEC lavage samples were centrifuged at 1800 RPM for 10 min to separate cells and supernatants. Supernatants were aliquoted and frozen at -80ºC for further analyses. Cell pellets were resuspended in 1mL Trizol (ThermoFisher) for 5 min and frozen at -80ºC for further analyses.

2.4.8. Extracellular vesicle extraction

Aliquots of approximately 2 mL of frozen supernatants from PEC lavage were thawed, pooled (3 mice per experimental group) and filtered using a 0.45 µm filter to exclude debris. The filtrate was transferred to 17 mL thin-wall polypropylene tubes (Beckman Coulter, Brea, CA, USA) and completed with exosome buffer (137mM NaCl, 20mM HEPES). The tubes were centrifuged at 100000xg (RCFavg) overnight (18 hours) at 4°C in an SW32.1 Ti swinging bucket rotor (Beckman Coulter). The supernatant was discarded, fresh exosome buffer was added to the tube to wash the pellet and centrifuged using the same conditions. This wash step was repeated and the pellet was re-suspended in 400ul exosome buffer Protein concentrations were determined using a microBCA assay following the manufacturer's protocol (Thermo Fisher). The results were read in a Tecan plate reader. The remainder of the extracted EVs were aliquoted and frozen at -80ºC for further analyses.

2.4.9. Transmission electron microscopy

EVs suspended in exosome buffer were deposited onto Fomvar carbon grids (Mecalab, Montreal, QC, Canada), fixed with 1% glutaraldehyde in 0.1M sodium cacodylate buffer, washed 3 times with autoclaved Milli-Q water, and stained with 1% uranyl acetate. Each step was performed for 1 minute in duration. Samples were visualized with a FEI Technai-12 120kV transmission electron microscope and AMT XR80C CCD Camera (Facility for Electron Microscopy Research, McGill University, Montreal, Canada).

2.4.10. Nanoparticle tracking analysis

Aliquots of extracted EV (80 ml) were diluted in 1000 ul of exosome buffer and injected into the sample chamber. EV size distribution was determined using nanoparticle tracking analysis (NTA) software and a NanoSight NS300 instrument (Malvern Panalytical, Malvern, Worcestershire, UK). Three videos were captured for 30 seconds each at 37ºC, using optimized camera settings that were kept consistent for all samples. Particle concentration, mean, median, and mode size were calculated and graphed using the NTA 3.4 Build 3.4.4 software.(136)

2.4.11. High sensitivity flow-cytometry analysis

Aliquots of supernatant of PEC lavage samples and serum from mock- and PbA-infected mice were analyzed through a method of high-sensitivity flow-cytometry to phenotype the EV released by immune cells in these experiments. These analyses were performed using a BD FACS Canto II instrument (BD Biosciences) with forward scatter coupled to a photomultiplier tube small particles option (FSC-PMT) as described previously (137, 138). Size gating was determined using silica beads (Fluorescent green plain silica particles, 3PSI-G, Kisker Biotech, Steinfurt, Germany) of 100 nm, 500 nm and 1000 nm diameter. Data were analyzed using Diva (version 6.1.3) or FlowJo software (version 9.9.6). For quantitative analysis a known concentration of fluorescent counting beads (2 µm Cy5-silica particles (Nanocs Inc,) was added to each tube. 20 µl of mouse peritoneal lavage were labeled with 250 nM LWA300 proteasome probe (gift from Dr Marie-Josée Hébert, in a total volume of 50 µL annexin buffer for 1 h at 30°C (139, 140). Samples were then incubated with BV421-Annexin V (BD Biosciences) and 2 µM of CellTracker™ DeepRed (ThermoFisher Scientific) for 30 min at RT and then diluted into 300 µL of annexin buffer and analyzed by flow cytometry.

2.4.12. EV labelling

20 µl of mouse peritoneal lavage were also labeled with V450 anti-mouse CD45 (clone 11F30, BD Biosciences), FITC anti-mouse CD41 (clone MWReg30, BD Biosciences), and BV421 anti-mouse Ly6G (clone 1A8, BD Biosciences) or MitoTrackerTM Deep Red FM for mitochondria (ThermoFisher Scientific) for 30 min at RT, diluted into 250 µL of PBS and analyzed by flow cytometry. Extracellular vesicles were first gated according to their fluorescence and then to their size and inner complexity (FSC PMT-H vs. SSC-H) to consider events between approximately 100 and 1000 nm. The staining specificity was confirmed by negative controls: 1) 0.2% Triton X-100 was used on positive samples for 20 min at RT, before labeling, 2) 20 mM EDTA were added to the annexin buffer prior to the Annexin V labeling.

2.4.13. Disease progression monitoring

Red blood cells infected with *Plasmodium berghei* ANKA parasites expressing a green fluorescent protein (GFP)-luciferase fusion protein (Malaria Research and Reference Reagent Resource Center, Manassas, BA, USA) were used for the disease progression study. Acclimatized mice were infected by IP inoculation of 10^4 infected iRBC or 200 ul of 1X PBS as a mock-control. Starting from 3 days-post infection, tail-vein blood was collected daily. Blood smears were stained with Diff-Quik, and parasitemia was determined by counting at least 500 cells. Mouse body weight, food and water intake, and body temperature were monitored until end-point, when mice developed ECM symptoms (between 7 and 10 days post-infection). Upon euthanasia, blood was collected by cardiac puncture and placed on ice. Spleens, livers and lungs were collected, weighed and frozen in liquid nitrogen. Between 200 and 500 ul of blood was centrifuged at 2000 RPM for 15 min. The serum was collected and frozen at -80ºC.

2.4.14. Parasite load luciferase assay

Organs of infected mice were thawed on ice and 10 to 40 ug was homogenized in 500ul of lysis buffer (1X Tris-NaCl-EDTA, 20% Glycerol and 1% Igepal in ddH2O) with a sterile plastic homogenizer. Blood was homogenized in 100ul lysis buffer. The homogenates were placed on a shaker in a cold room (4ºC) overnight and then centrifuged at 1500 RPM for 10 min. The supernatant was collected and protein levels were determined using a Bradford assay following the manufacturer's protocol (Bio-Rad, Mississauga, ON, Canada). Results were read in a Tecan

Plate Reader. Organ and blood parasite loads were determined using the Firefly Luciferase kit following the manufacturer's protocol (Biotum, Inc, Fremont, CA, USA). Results were read in a Synergy H4 luminescence reader. Parasite loads were determined by dividing the relative luminescence units (RLU) by protein amounts in the reading volume.

2.4.15. Cytokine/Chemokine analysis using multiplex array

Cytokine and chemokine levels were determined from 100ul of cell culture supernatant using a multiplex mouse cytokine array/chemokine array 10-plex assay (Eve Technologies, Calgary, AB, Canada) that provides detection for IFNγ, IL-1β, GM-CSF, IL-2, IL-3, IL-4, IL-6, IL-10, IL-12 (p70), MCP-1 and TNF-α.

Cytokine and chemokine levels were determined from 100 ul of PEC lavage supernatant and 100 ul of 1:2 diluted serum using a multiplex mouse cytokine/chemokine array 32-plex assay (Eve Technologies) that provides detection for Eotaxin, G-CSF, GM-CSF, IFNγ, IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-12 (p40), IL-12 (p70), IL-13, IL-15, IL-17, IP-10, KC, LIF, LIX, MCP-1, M-CSF, MIG, MIP-1α, MIP-1β, MIP-2, RANTES, TNF-α, and VEGF.

2.4.16. Statistical analysis

All statistical analyses were conducted using Prism Software for Mac version 8.00 (Graphpad PrismTM, San Diego, CA, USA). Unless stated otherwise in the figure legends, all p-values were determined using two-way ANOVA and Sidak's multiple comparison test.

2.5. Results

2.5.1. Macrophages in culture respond differently to bacterial agents depending on environmental temperature

In order to study the impact of environmental temperature on macrophages, we established a methodology for testing the IR of BMDMs at ST compared to TT by stimulating LM-1 BMDMs with LPS to induce the IR (see Section 2.4.1). Because experiments on mammalian cell lines are generally conducted at 37°C, we chose this as the TT and compared the results to those obtained from cells stimulated at 30°C as a ST. We stimulated LM-1 cells at 37°C and 30°C with LPS for 24 hours and assessed response by NO quantification. The cells were responsive at 30°C, albeit to a lower degree than at 37°C (Figure 5A). This is in agreement with the concept that immune

response of cells exposed to lower environmental temperatures is suppressed (141-143). In order to determine the response kinetics, we repeated the experiment and conducted NO quantification on supernatants collected at 6, 12 and 24 hours after stimulation. LPS increased the induction of NO production over time and the induction was greater at TT than ST at 12 and 24 hours (Figure 5B).

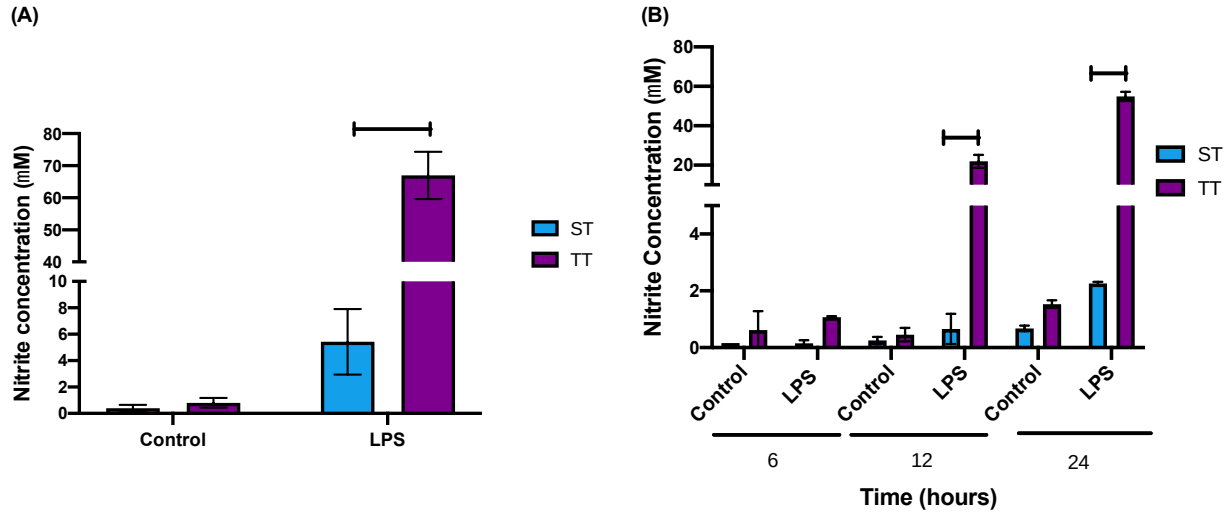

Figure 5. BMDMs stimulated with LPS show an increased inflammatory response at TT compared to ST.

Nitric oxide release after **(A)** 24 hours (n = 6) and **(B)** 6, 12 and 24 hours (n = 3) in supernatant of 10^6 BMDMs stimulated with LPS (100 ng/mL) at 30°C or 37°C (n = 6 or 3). The NO release increased over time at both temperatures but was always highest at ST. Bars represent mean +/- Standard Mean Error (SEM). **** indicates $p<0.0001$.

In addition, we tested LM1 stimulation at a third temperature (FT) corresponding to a fever-like response in the mammalian host (i.e. 38 – 40°C) (144). When stimulated with LPS, the cells showed increased NO release after 24 hours at ST compared to TT (Figure 6A). We also determined whether temperature affected production of specific cytokine and chemokines implicated in the macrophage IR to infectious agents (30, 31). We measured the concentrations of TNF-α, MCP-1, IL-6, and GM-CSF in the cell supernatants and observed that IL-6 and GM-CSF were present at higher concentrations at TT, and especially at FT compared to ST (Figure 6B). These results are consistent with reported increased activity of immune cells at febrile temperatures (145, 146). In accordance with our NO release results, the concentrations of MCP-1, IL-6 and GM-CSF were lower in cultures at ST compared to FT and TT (Figure 6B). However, the concentration of TNF-α was highest at ST (Figure 6B), possibly due to a

previously described role in the hypothermic response of cultured cell lines (147). Cells exposed to cold temperatures (≤ 32ºC) respond to stimuli (e.g. LPS) differently due to metabolic changes and they release pro-inflammatory cytokines specific to cold temperatures (147). In our study, TNF-α was released at lower concentrations at FT compared to TT, consistent with reports showing that macrophages switch off secretion of this pyrogen at higher temperature when the febrile temperature has been reached (146). Altogether, these results confirm that environmental temperature might play a role on the metabolism of immune cells and their response to infectious agents.

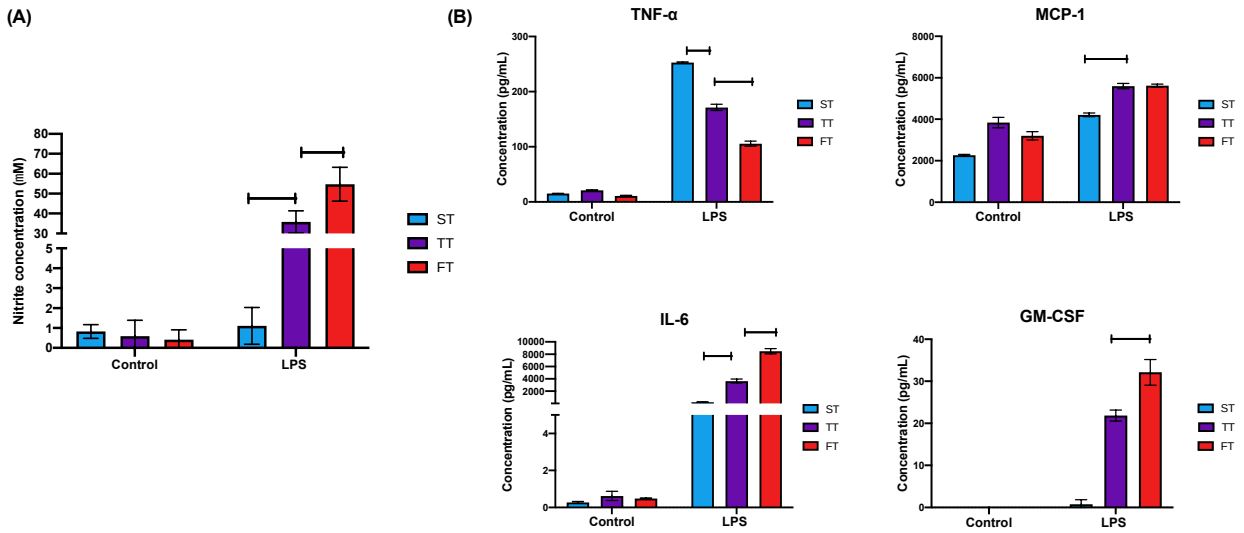

Figure 6. Environmental temperatures change the BMDM IR to infectious agents.
(A) nitric oxide release (n = 6) **(B)** pro-inflammatory factors release (n = 3) in supernatant of $2.5x10^6$ BMDMs stimulated with LPS (100 ng/mL) at 30ºC, 37ºC or 38.5ºC. Except for TNF-α, NO and cytokine/chemokine release was always proportional to temperature. Bars represent mean +/- SEM **** indicates $p<0.0001$, ** indicate $p<0.01$.

2.5.2. Recruitment of inflammatory cells to the site of injection is decreased at thermoneutral temperatures

The fever-like response observed in malarial patients occurs during the erythrocytic stage and is associated with the release of nHZ and other malarial agents from the iRBCs (6). To determine whether the mouse IR to malarial or leishmanial components differed at TT compared to ST, we injected groups of mice with nHZ or *L. major* promastigotes intraperitoneally and collected PEC lavages after six hours. *L. major* was used as a positive parasitic control as its role in the IR has been documented (38, 39). We observed a lower recruitment of total inflammatory cells in the PEC of mice injected with *L. major* (i.e. 4-fold increase) at TT compared to mice housed at ST

(i.e. 6-fold increase) relative to PBS-treated mice at the corresponding temperature (Figure 7A). This trend was also observed for mice treated with nHZ, but was not statistically significant (Figure 7A).

We examined the proportion of various inflammatory cell populations and observed that the most abundant cells present in the PEC at baseline (i.e. PBS injection) were macrophages (89.4% at ST and 79.6% at TT) as expected . In contrast, neutrophils which were present at low level at baseline: 1.9% at ST and 6.5% at TT, were the most abundant cell type recruited to the PEC after stimulation with nHZ (31.0% at ST and 27.9% at TT) (Figure 7B). Neutrophils were also the most abundant cells recruited to the PEC at ST and TT following *L. major* injection, but to a higher degree (64.4%) than with nHZ inoculation (Figure 7B). Eosinophils were also recruited following nHZ and *L. major* injection. They accounted for 6.3% at ST and 9.8% at TT of all cells following nHZ injection and 10.1% at ST and 11.5% at TT after *L. major* injection (Figure 7B). The percentage of macrophages was reduced to 54.4% and 50.4% with nHZ injection and 23.8% and 21.2% with *L. major* injection at ST and TT, respectively (see figure 7B).

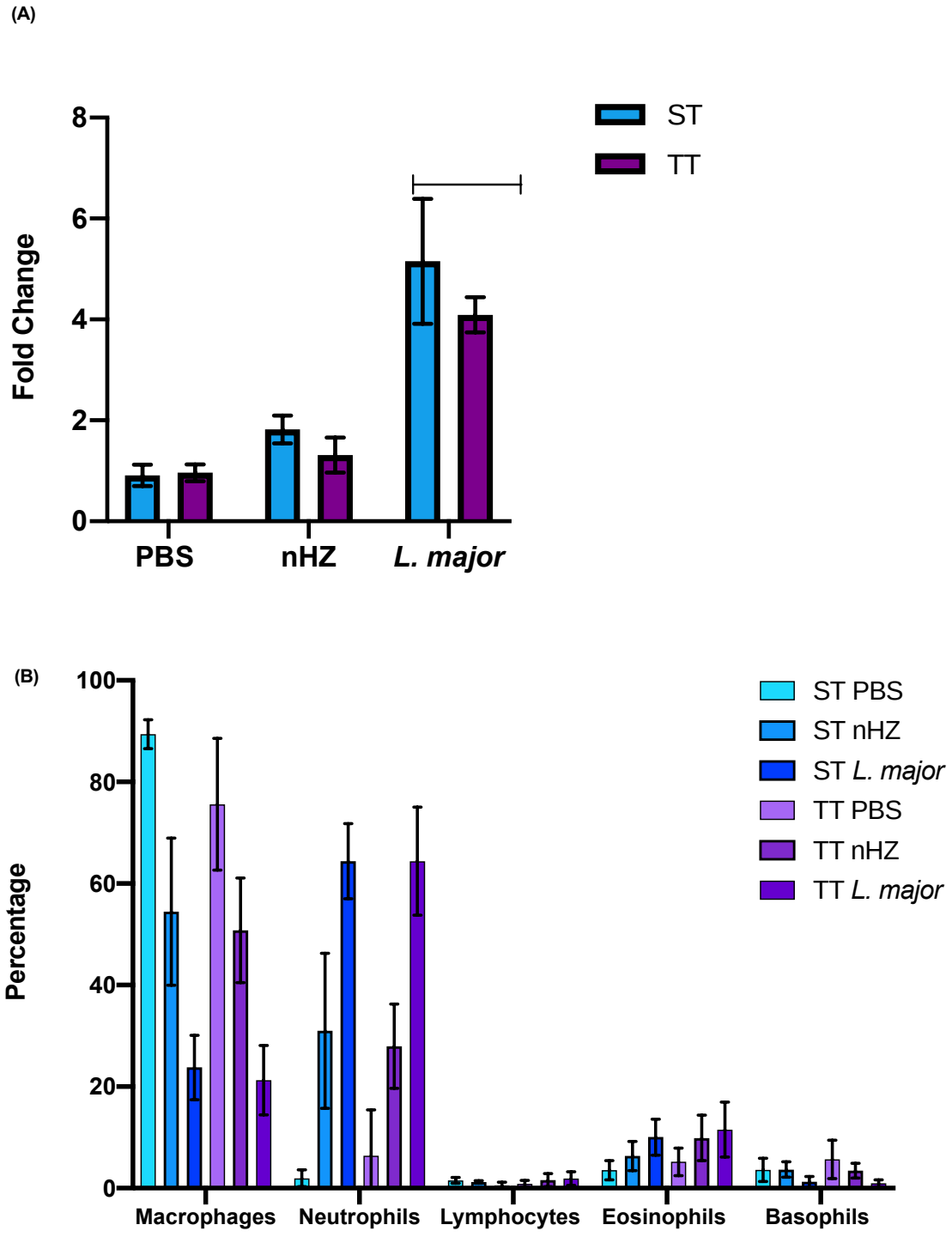

Figure 7. Inflammatory cells are recruited to a lower degree by parasitic agent injection at TT compared to ST.

Fold change of inflammatory cell recruitment relative to PBS-control and **(B)** proportions of immune cell subsets in the PEC of mice injected intraperitoneally with nHZ (1 mg/mL) or 10^8 *L. major* promastigotes housed at TT (n=5) or ST (n=6) was measured after 6 hours. The majority of recruited cells were neutrophils at both temperatures. Bars represent mean +/- SEM * indicates a p-value <0.05.

The levels of some inflammatory cytokines and chemokines also varied depending on housing temperature (Figure 8). Some pro-inflammatory cytokines (e.g. IL-5 and IL-6) and chemokines (MCP-1, MIP-2 and MIG) showed a trend lower concentrations in mice housed at TT compared to those at ST. This was consistent with lower inflammatory cell recruitment at TT in our study and with other studies that have reported higher production of certain pro-inflammatory cytokines (e.g. MCP1, IFNγ, TNF-α, IL-6, and IL-1ß) when exposed to ST (69, 74, 83, 85). However, most cytokine/chemokine levels remained unchanged (e.g. G-CSF, Eotaxin, MIP-1α, MIP-1ß) in accordance with results from previous studies in mice injected with LPS at ST and TT (103). Interestingly, concentrations of IP-10 appeared to be lower at TT in mice injected with nHZ but higher in mice injected with *L. major*. Altogether, these results suggest a trend towards a dampened inflammatory response in mice exposed to parasitic agents at thermoneutral temperatures.

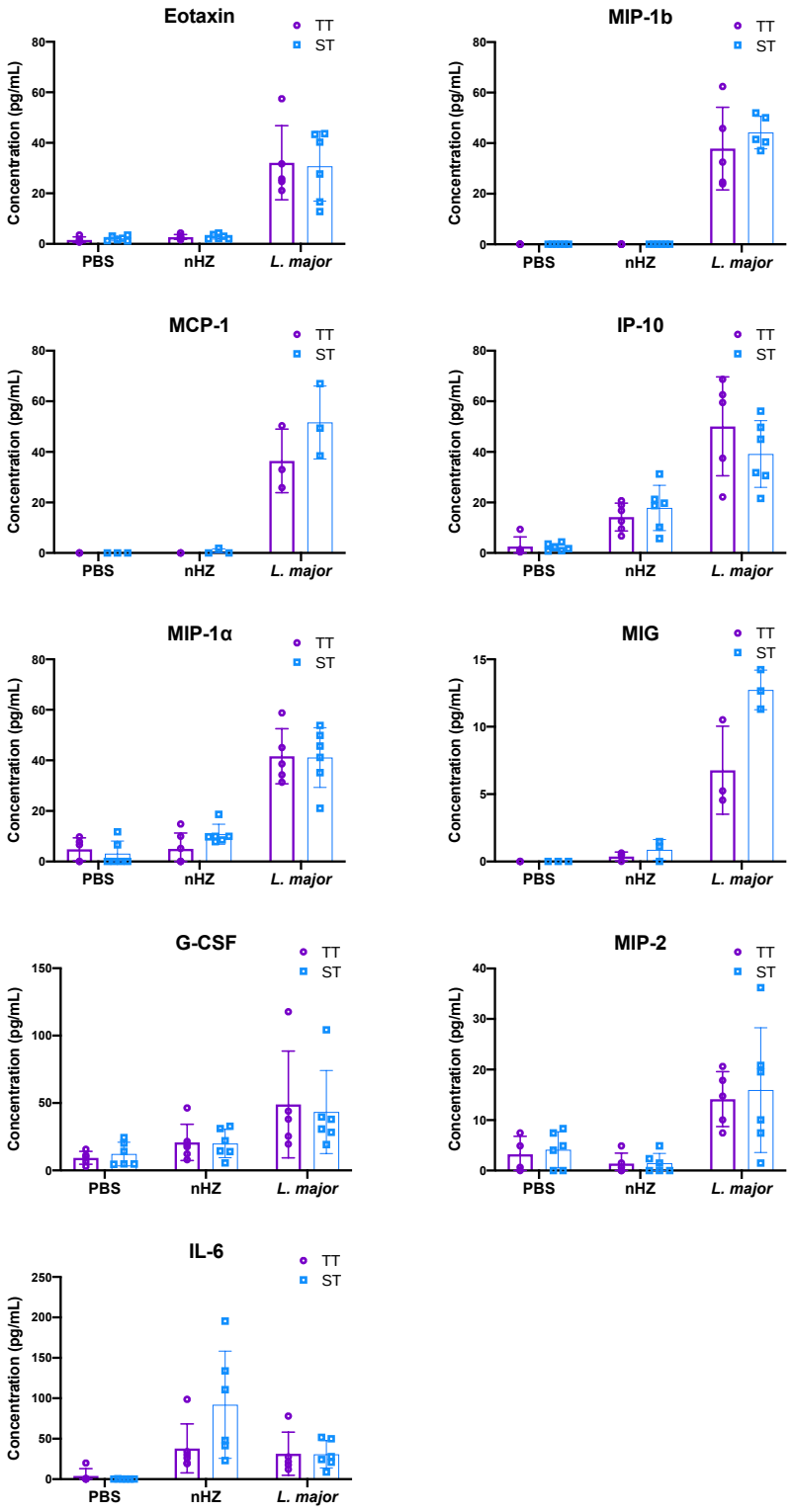

Figure 9. The concentrations of pro-inflammatory cytokines and chemokines differ at TT compared to ST. Pro-inflammatory cytokines and chemokines concentrations were monitored in the PEC of mice injected intraperitoneally with nHZ (1 mg/mL) or 10^8 *L. major* promastigotes at ST and TT (n = 3 or 6). Some pro-inflammatory cytokines and chemokines showed a trend of lower concentration at TT. Bars represent mean +/- SEM.

2.5.3. Thermoneutrality enhances macrophage phagocytic activity induced by nHZ, but not *L. major* promastigotes.

We evaluated the phagocytic activity of macrophages and neutrophils isolated from the PEC of mice injected with parasitic agents at ST and TT by determining the percentage of cells containing nHZ or *L. major* promastigotes. Macrophages and neutrophils were involved in the phagocytosis of nHZ crystals at both temperatures (Figure 9A). We did not observe a difference in the phagocytic activity of neutrophils at the two temperatures (Figure 9A). However, the phagocytic activity of macrophages from mice housed at thermoneutrality was higher (27.3%) compared to those housed at ST (17.9%) (Figure 9A). However, this difference was not associated with a change in phagocytosis activation-associated cytokine profiles (e.g. IFN-γ, IL-12 and TNF-α) (31). Also, we did not observe a difference in phagocytic activity in macrophages from mice treated with *L. major* (Figure 9B). Overall these data are inconclusive, but do not exclude that thermoneutrality may affect the macrophage phagocytic activity of malarial parasitic agents through a cytokine-independent mechanism.

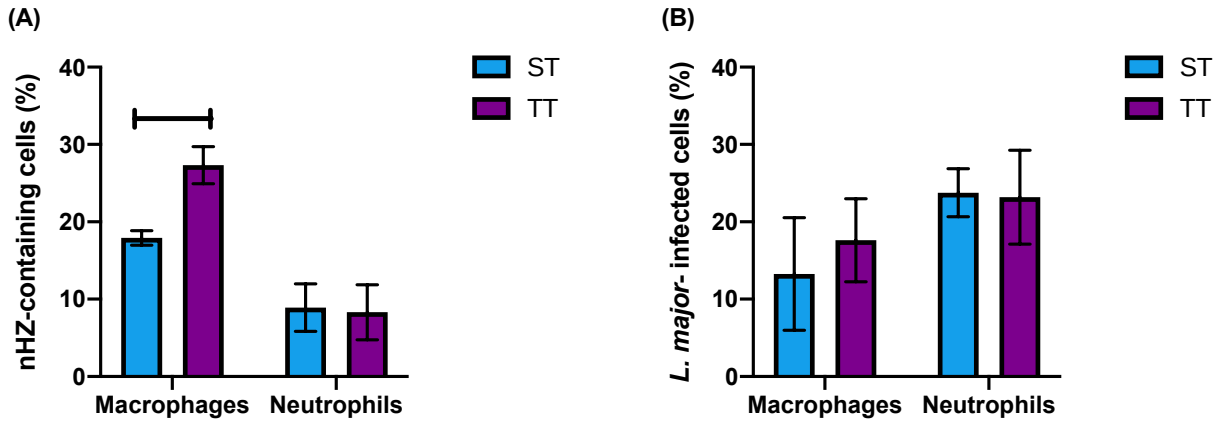

Figure 11. Thermoneutrality increases phagocytosis of nHZ but not L. major by macrophages. Percentages of macrophages and neutrophils containing **(A)** nHZ crystals or **(B)** *L. major* promastigotes were counted in the PEC of mice injected intraperitoneally with nHZ (1 mg/mL) or 10^8 *L. major* promastigotes at ST and TT (n=3). Neutrophil percentages did not change based on temperature. Bars represent mean +/- SEM. * indicates a p-value <0.05.

2.5.4. Effect of thermoneutrality on profile of EVs released following parasitic agent injection

Because EVs have been shown to play an immunomodulatory role in parasitic diseases, we sought to determine whether housing temperature influences EV release by immune cells after injection with parasitic agents (7, 8, 148-150). We confirmed the presence of EVs in biological

samples through a conjunction of Nanoparticle tracking analysis (NTA) and transmission electron microscopy (TEM) in concordance with the International Society for Extracellular Vesicles guidelines (see Sections 2.4.9-10) (151).

Transmission electron microscopy (TEM) images of the EVs isolated from the supernatant of peritoneal lavage fluid reveal that these EVs observed had similar morphology with their size up to 150nm in diameter. They are almost uniform in external shape, consisted of bilayer lipid membrane (Figure 10).

NTA analysis revealed that the EVs present in the samples ranged in size from 50 to 800 nm (Figure 10). Mice injected with PBS had a more heterogeneous EV profile, based on size distribution, than those injected with nHZ and *L. major*. A major peak at 100 to 180 nm was substantially more pronounced in the samples from mice injected with the parasitic agents (Figure 10).

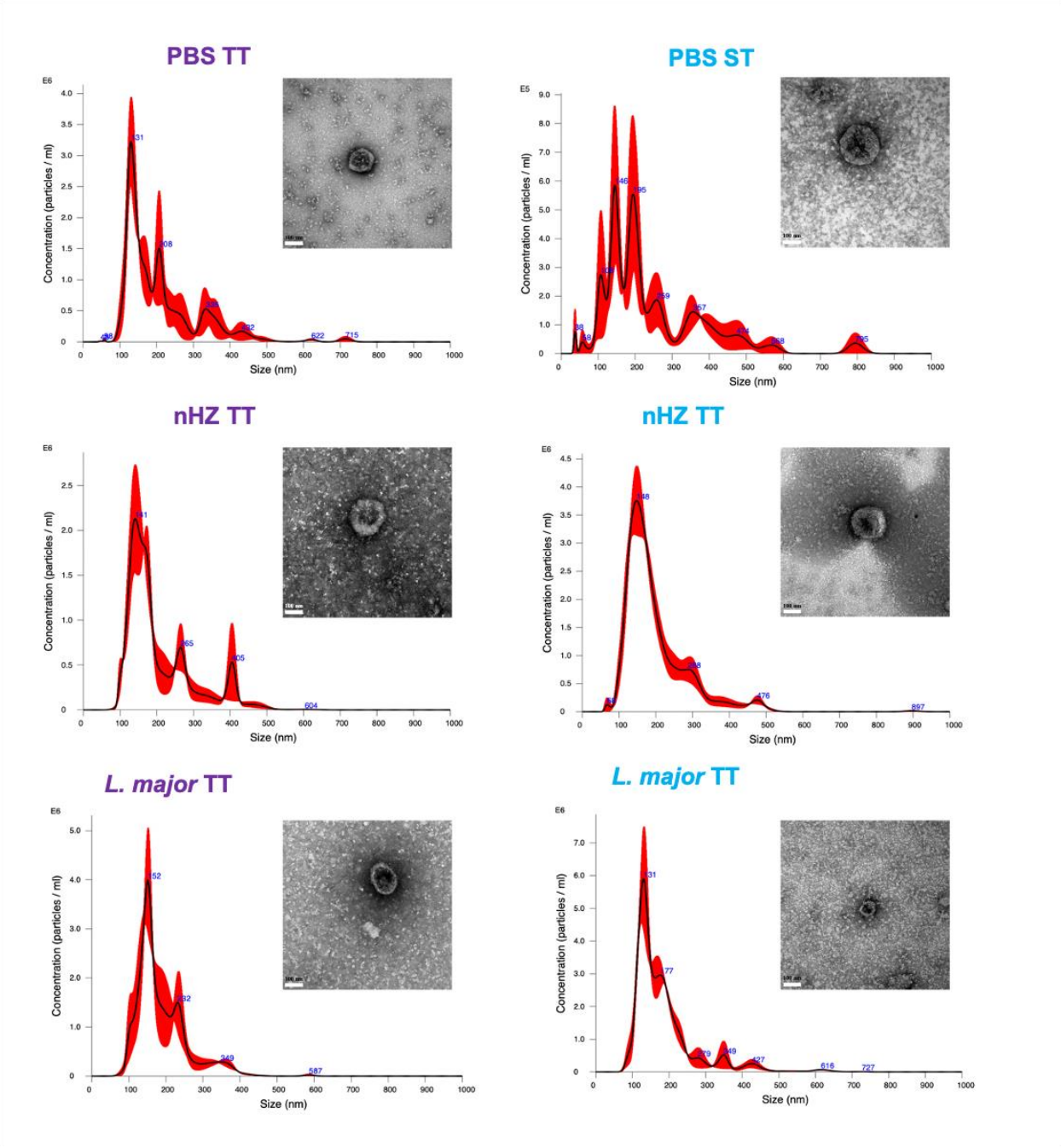

Figure 12. EV are released in the PEC of mice at TT and ST.
EV distribution and concentration was determined by NTA analysis and EV morphologies were visualized by TEM from representative PEC lavages of mice injected intraperitoneally with nHZ (1 mg/mL) or 10^8 *L. major* promastigotes. Photos were taken at 49000x; scale bar represents 100nm.

The EV profile differences did not appear to be housing-temperature dependent as mean size of EV were similar at TT and ST across injection groups (Figure 11A). However, the samples obtained from mice at TT showed a trend of lower concentrations of EVs compared to those obtained from mice at ST injected with nHZ or *L. major* (Figure 11B). These results are consistent with the overall decreased recruitment of inflammatory cells to the PEC.

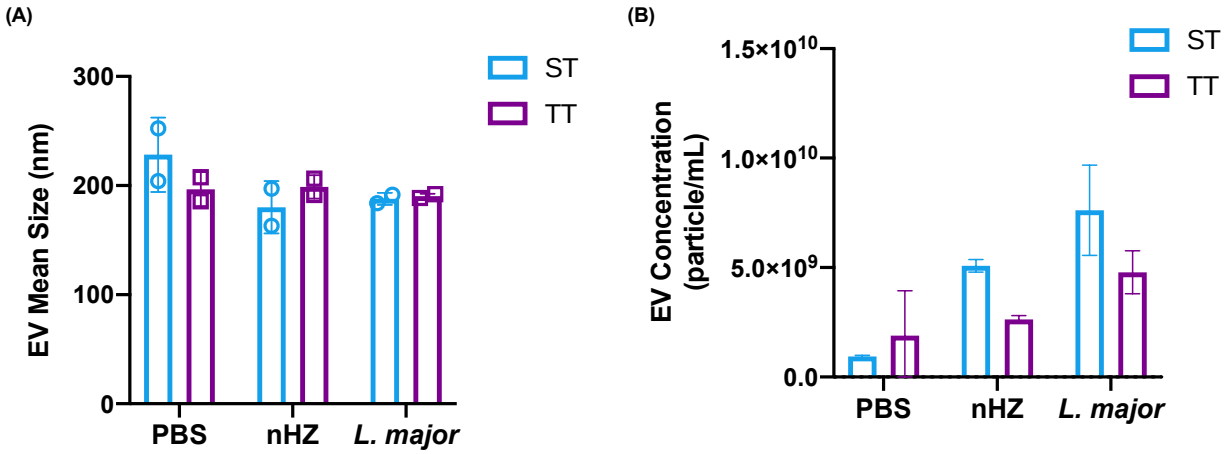

Figure 13. Thermoneutrality induces a trend of lower EV release for a similar mean size distribution in the presence of parasitic agents.
EV **(A)** mean size and **(B)** concentration were determined by NTA from the PEC of mice injected intraperitoneally with nHZ (1 mg/mL) or 10^8 *L. major* promastigotes. Bars represents mean +/- SEM, each point represents 2 or 3 mice pooled.

We employed a hsFC method to determine the level of EVs released and the cells from which they were released in the PEC of mice treated with nHZ or *L. major*. The identity of the cells from which EVs were released in the PEC was determined through detection of cell subtype-specific cell surface markers (see Sections 2.4.11-12) (137, 138). The total levels of EVs released in the PEC, as indicated by the AnnexinV marker, which is a phosphotidylserine (PS)-binding molecule that can be used to detect eukaryotic cell-derived EVs, did not vary between the different housing temperatures (Figure 12A) (152). There was a slight decrease in leukocyte-derived EV release at TT compared to ST (Figure 12B). The main population of cells recruited to the PEC by nHZ and *L. major* injection were neutrophils (see Section 2.3.3). We therefore evaluated release of neutrophil-derived EVs bearing the Ly6G receptor in the PEC of mice (Figure 12C). Here again, we did not observe housing temperature-based differences (Figure 12C). We investigated whether other cell populations such as platelets were releasing EVs in the PEC, but found no temperature-based differences (Figure 12D). However, we found a trend

towards higher release of proteasome-derived EVs that were positive for the LWA+ proteasome marker (Figure 12E}. Our experiments provide evidence for proteasomal-derived EVs released in)the PEC of mice injected with parasitic agents. In addition, our results show a trend of higher proteasomal-derived EV release at TT compared to ST (Figure 12E).

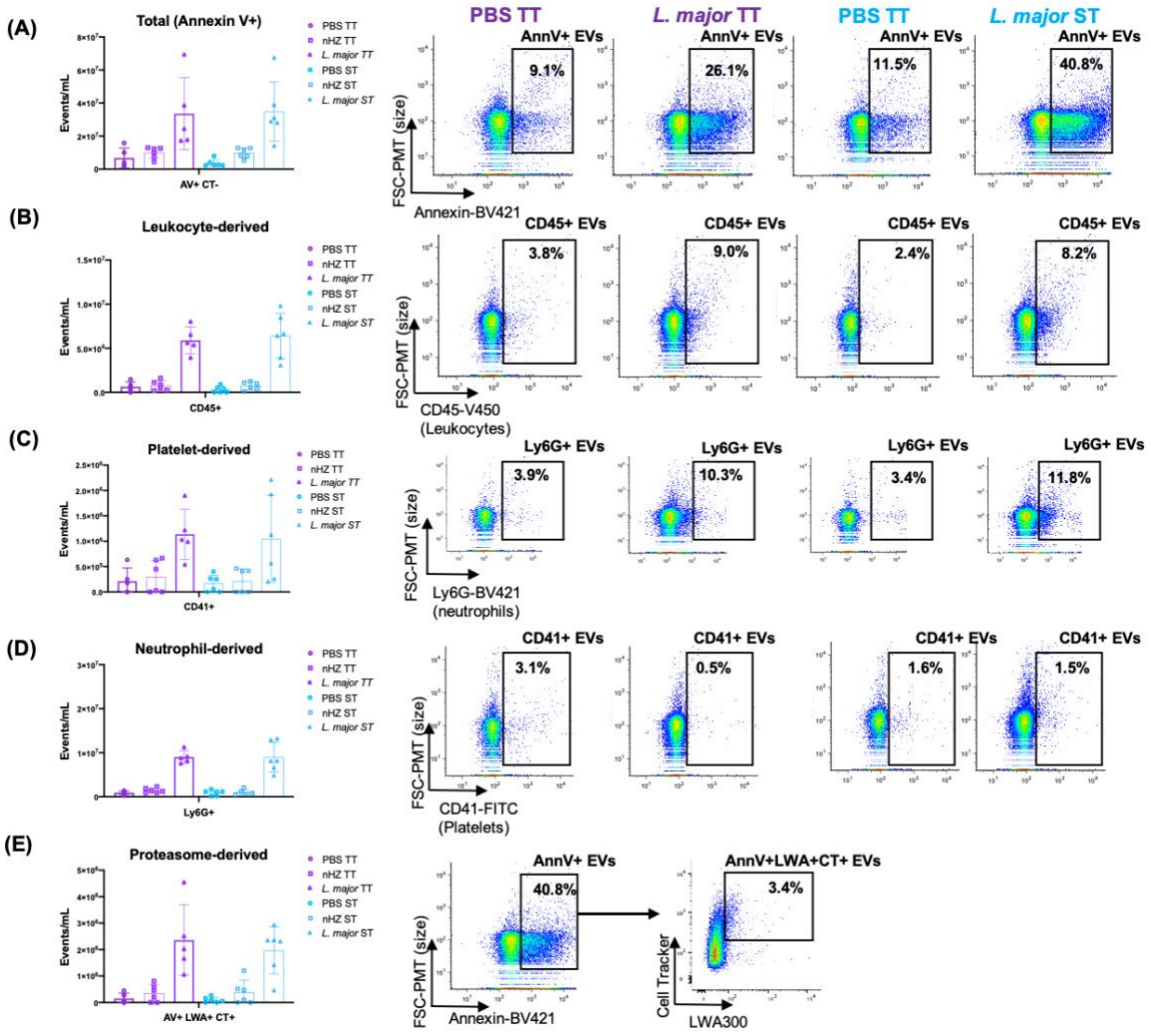

Figure 14. Thermoneutrality may influence the profiles of host cell EVs release by parasitic agent injections.

(A) Total (AV+), **(B)** leukocyte-derived (CD45+), **(C)** Neutrophil-derived (Ly6G+) **(D)** Platelet-derived (CD41+), **(E)** Proteasome-derived (AV+LWA+CT+) EVs in the PEC of mice injected intraperitoneally with nHZ (1 mg/mL) or 10^8 *L. major* promastigotes were monitored by hsFC at TT (n= 5 or 6) or ST (n = 5 or 6). Bars represent mean +/- SEM. Scatter plots illustrate results for single representative mice injected with PBS and *L. major*. Only leukocyte-derived and proteosome-derived EVs showed a trend of different EV concentration based on temperature.

To further confirm that the small particles detected by hsFC were EVs, we treated representative aliquots of the PEC samples that had been previously stained with antibody-dyes with X-100 Triton and EDTA, which disrupt EV-membranes and surface PS, respectively (see Sections 2.4.11-12). The treatments reduced EV detection in all samples, confirming that the particles detected by hsFC were EVs (Figure 13). iRBC-derived EVs containing proteasomal factors have been shown to promote the growth of *P. falciparum* parasites previously but the exact role of the proteasomal derived host cell EVs has not been elucidated in parasitic diseases (153).

Overall, we did not observe, any meaningful housing temperature-based difference in EVs released in the PEC of parasitic-agent treated mice.

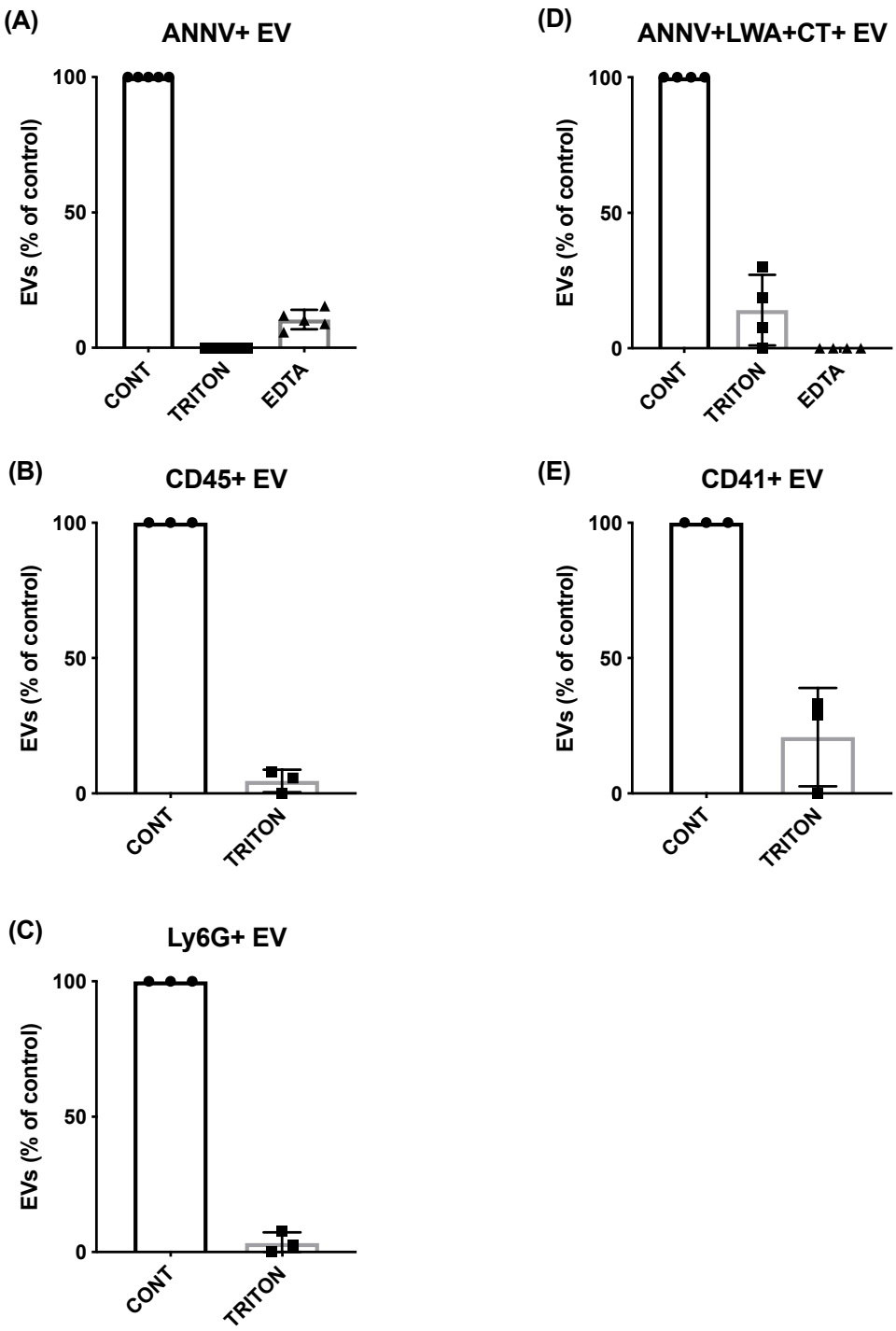

Figure 15.Negative controls for EV detection by hsFC of representative samples.
(A) Total (AV+), **(B)** leukocyte-derived (CD45+), **(C)** Neutrophil-derived (Ly6G+) **(D)** Platelet-derived (CD41+), **(E)** Proteosome-derived (AV+LWA+CT+) EVs percentages in PEC samples with or without detergent treatment. Markers were incubated with aliquots of PEC lavages of mice injected intraperitoneally with 10^8 *L. major* promastigotes and treated with Triton X-100 to lyse EV membrane **(A-F)** and EDTA **(A, D)** to lyse Annexin V (n=3). The treatment with detergent dramatically decreased the percentage of detected EVs. Bars represent mean +/- SEM.

2.5.5. PbA infected mice at TT show a trend of later onset of disease and decreased symptoms.

To determine the effect of thermoneutrality on the clinical progression of severe malaria, mice were acclimatized at TT and ST, infected with luciferase-tagged PbA and parasitemia was monitored for 7 to 10 days, the time for fatality to occur in this model (35). Blood and organs were collected at death to assess parasite load as quantified by luciferase levels. The mice housed at TT appeared to have slightly increased survival (Figure 14A). Although the difference between TT and ST house mice was non-significant ($p = 0.1585$), it seemed to be most pronounced between days 8 and 9 (Figure 14A). The mice housed at TT had a similar level of parasitemia as their ST-housed counterparts and clinical progression was indistinguishable (Figure 14 B-C). However, for a similar organ to body-weight ratio upon euthanasia, the mice housed at TT showed a trend of lower sequestration of parasite iRBCs in the lung, liver, spleen, and blood (Figure 14D-E).

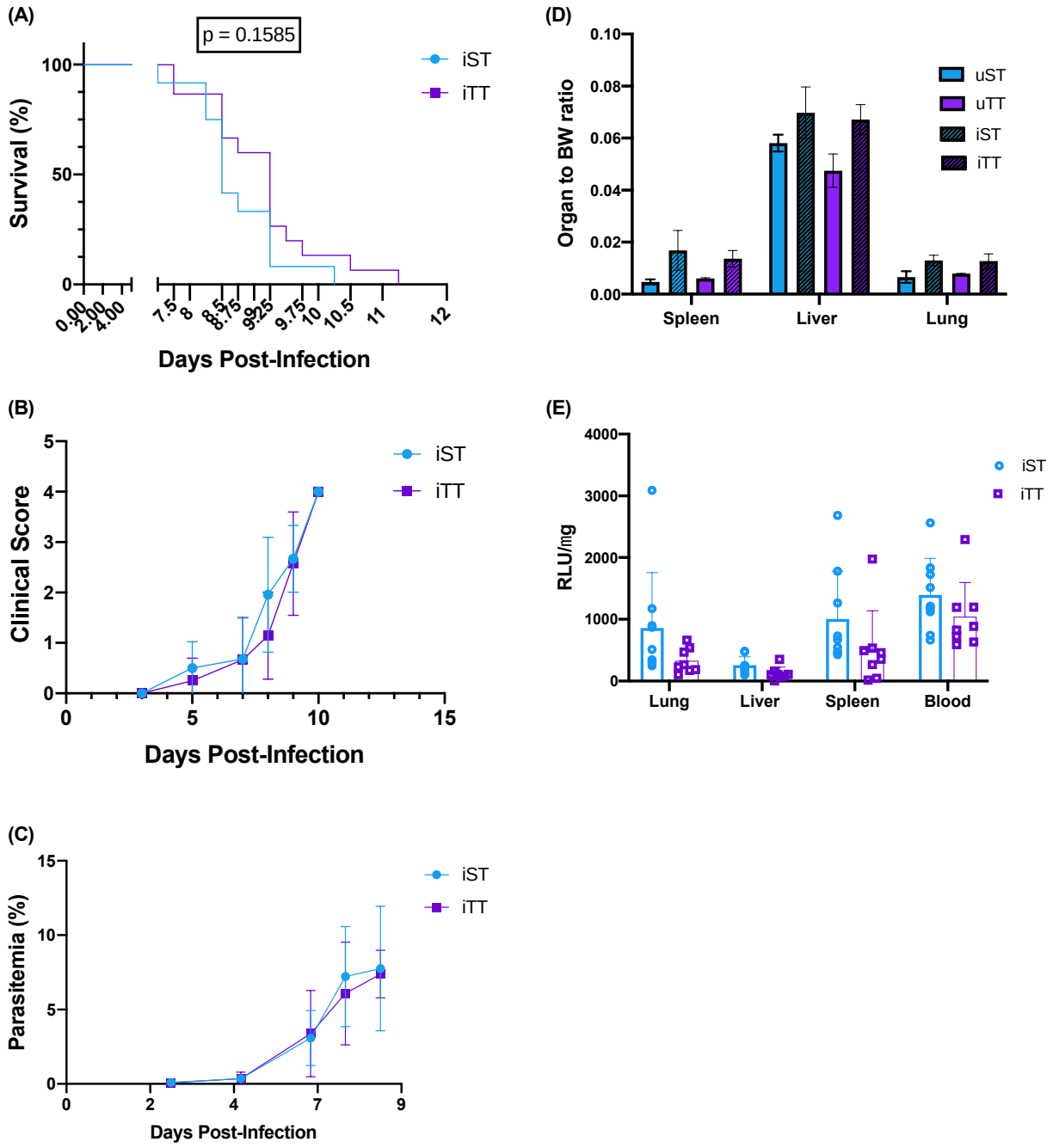

Figure 16. Mice at TT showed a trend in increased survivability and decrease in some symptoms of malaria compared to ST-housed counterparts.

(A) Kaplan-Meier survival curve, (B) clinical score, (C) parasitemia, (D) organ to body weight-ratios and (E) organ and blood parasite load (n= 8 or 9) were measured in mice were infected with 10^4 luciferase-tagged PbA iRBCs at TT (n=13) or ST (n=14) or PBS as a mock control at TT (n=3) or ST (n=3). Bars represent Mean +/-SEM. For (A) statistical test is a log-rank (Mantel-Cox) test, For (B-E) statistical test Is a two-way ANOVA followed by Sidak's multiple comparison test.

Finally, losses in body weight and temperature are metabolic hallmarks of mice infected with PbA (35). We confirmed a progressive loss of body weight in mice housed at ST after day 27 post-infection (Figures 15A and B). However, the weight of the infected mice housed at TT did not differ from their uninfected counterparts. Similarly, the body temperature of the infected mice housed at ST decreased dramatically after day 8 post-infection (Figure 15C). The body temperature of the mice housed at TT decreased minimally in the same time frame. Altogether, these results suggest that malarial symptom presentation, particularly those linked to metabolism, is diminished in mice housed at thermoneutrality, which might have led to a slight delay in disease progression. These results are in line with studies conducted on other parasitic disease models where thermoneutrality was protective for disease presentation in *Leishmania* and *Trypanosoma* infections (109, 111, 112).

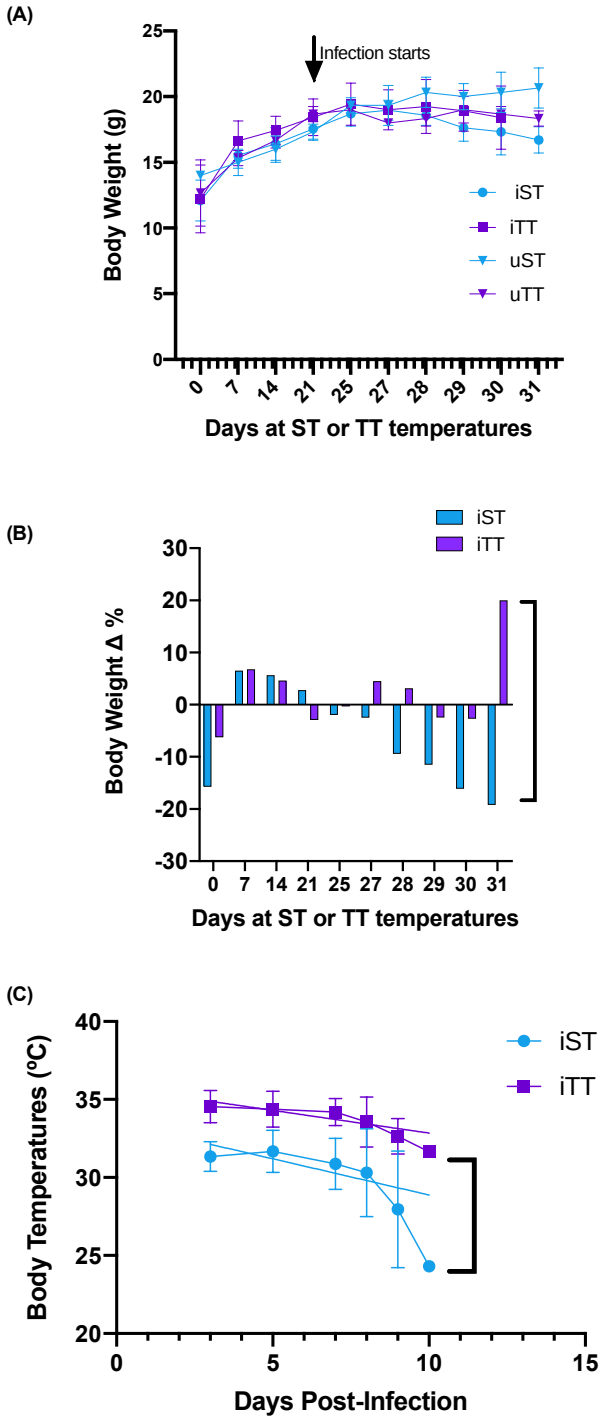

Figure 17. Mice at TT showed a decrease in metabolism-associated symptoms of malaria compared to ST-housed counterparts.
(A) body weights, **(B)** body weight change, **(C)** body temperature evolution of mice infected with 10^4 luciferase-tagged PbA iRBCs at TT (n=13) or ST (n=14) or PBS as a mock control at TT (n=3) or ST (n=3) were measured. Bars represent Mean+/- SEM. For (B) statistical test was an unpaired t-test ** indicated a p-value of <0.01. * indicated a p-value of <0.05.

2.5.6. Effect on housing temperature on the life-cycle of *Plasmodium berghei* ANKA

In order to investigate whether the life-cycle of the parasite is affected by thermoneutrality, we monitored the various blood life-cycle stages of parasites (i.e. gametocytes, schizonts, ring forms and trophozoites) in the blood smears of mice housed at ST and TT over the course of infection with PbA. We found that the distributions of the parasitic forms in the different stages were similar from day 7 to 10 after infection at both temperatures (Figure 16), suggesting that thermoneutrality did not have a direct effect on the life-cycle of the *Plasmodium* parasite. This agrees with results from previous studies where the schizont size did not differ in animals housed at 28°C compared to 21°C (108).

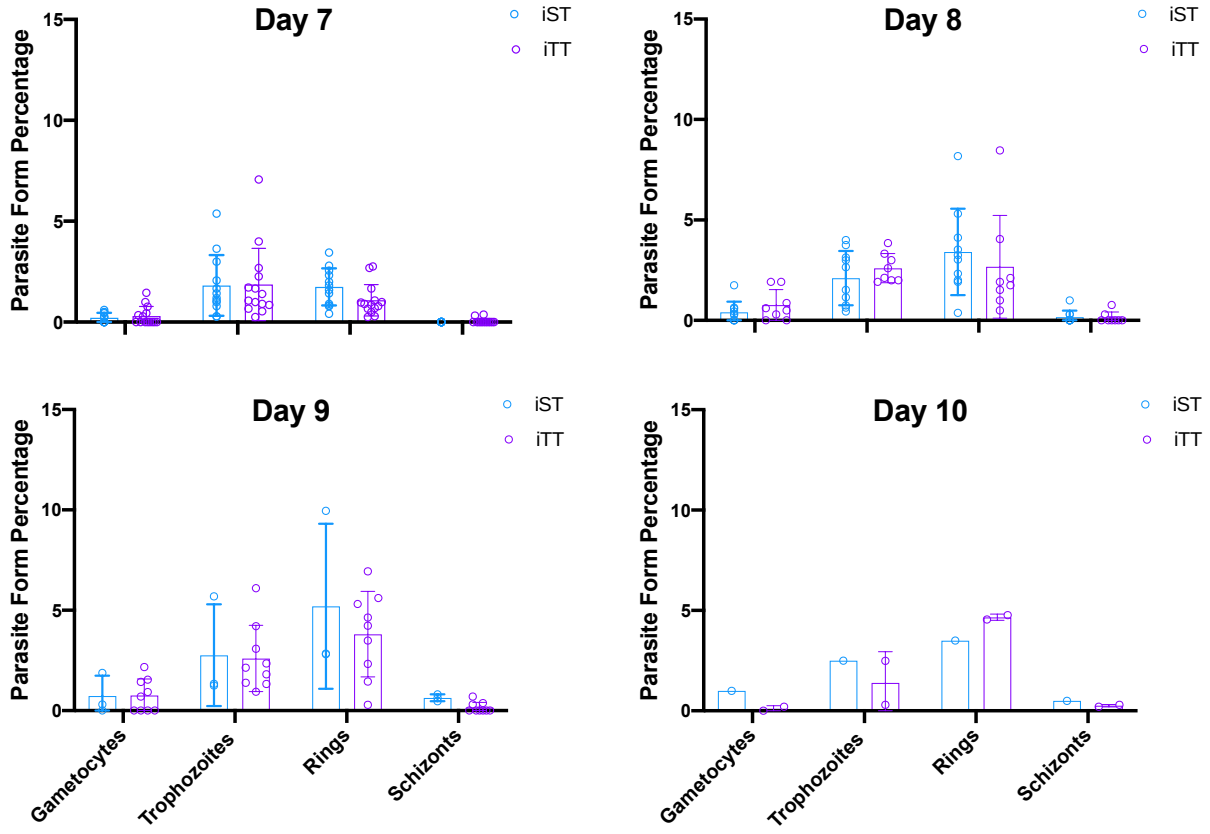

Figure 18. Thermoneutrality impact on the parasite life-cycle stages distribution.

The percentage of gametocytes, trophozoites, ring-stage and schizonts in the smears of mice infected with 10^4 luciferase-tagged PbA iRBCs at TT (n=13) or ST (n=14) on day 7, 8, 9 and 10 after infection. Bars represent SEM. No temperature-based statistical difference was observed. Life-cycle stage distribution did not seem to differ at TT compared to ST.

2.5.7. Cytokine levels were similar in the serum of mice housed at TT compared to ST.

Finally, we monitored the serum cytokine levels of mice housed at TT and ST upon euthanasia. The choice of cytokines and chemokines measured was based on their reported implication in severe malaria (i.e. IFNγ, TNF-α, IL6, IL-1ß, RANTES, MIP-1, MCP-1, IP10) (9, 15, 129). The levels of cytokines were similar in infected and uninfected animals at ST and TT (Figure 17). However, we cannot exclude that cytokine levels were affected differentially during progression of the infection.

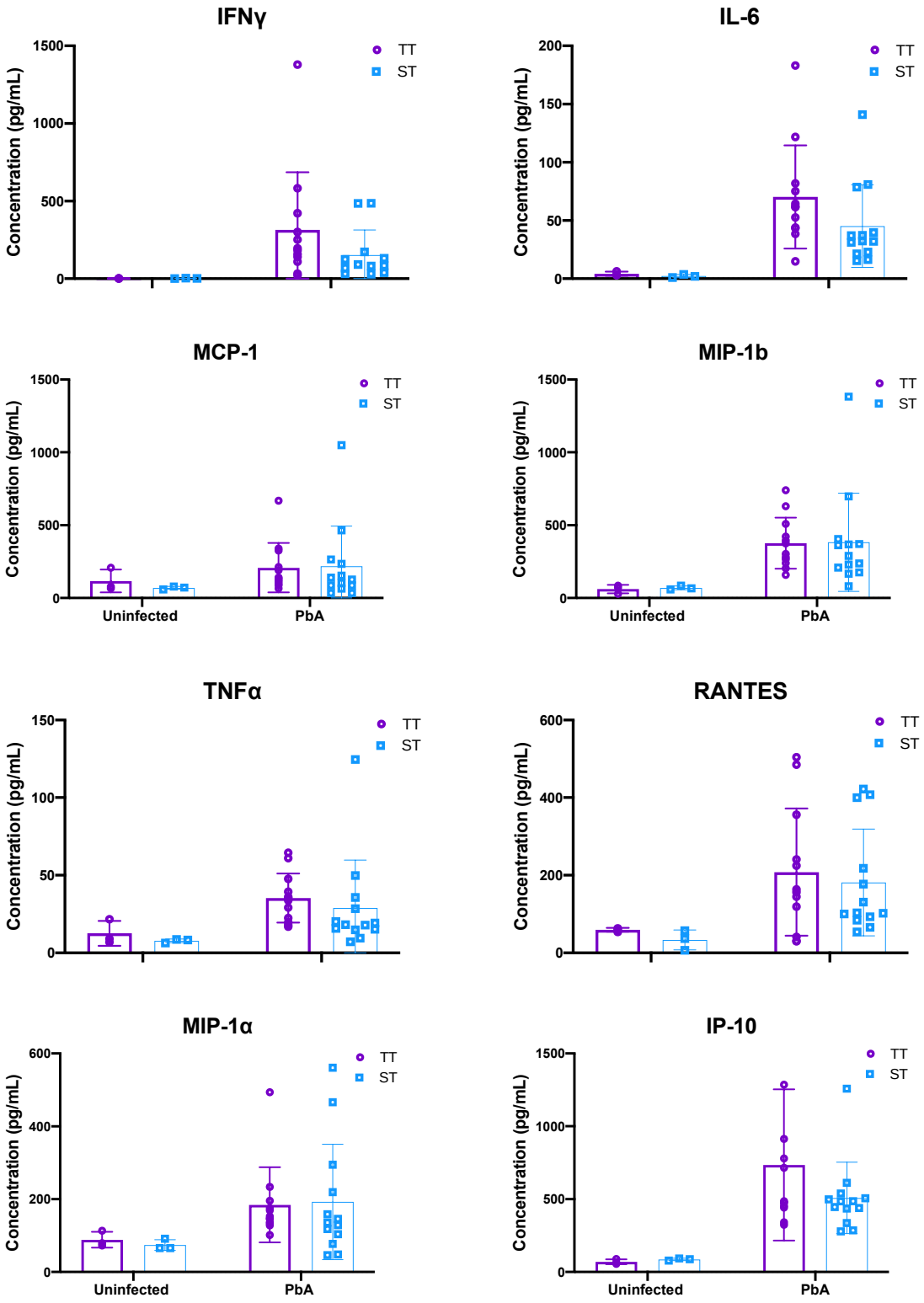

Figure 19. Cytokine levels upon euthanasia at TT compared to ST.

Cytokine and chemokine serum concentrations were measured from mice infected with 10^4 luciferase-tagged PbA iRBCs at TT (n=13) or ST (n=14) or PBS as a mock control at TT (n=3) or ST (n=3). Levels did not seem to change at TT compared to ST

2.6. Discussion

Malaria remains a global disease associated with significant mortality (4). In addition, new challenges like global warming and potential wider spread of vector mosquitoes, the COVID-19 pandemic, hurdles in Malaria vaccination implementation and drug/insecticide resistance are stalling efforts to control this disease if novel solutions are not found(4). A promising avenue for disease control is new immune-based targets for therapy and/or prophylaxis. However, effective immune targeting requires the use of appropriate animal models that are representative of the human disease to ensure predictability and applicability to human trials (53). For this reason, we believe our model using a housing temperature that reflects the normal metabolic condition of the host might provide the scientific community with a better tool to understand malaria and find effective disease control and/or therapy targets.

2.6.1. Macrophage response to infectious agents at different temperatures *in vitro*

Sub-optimal temperatures have been shown to impact the host immune response to diseases by decreasing the amount of energy allocated to drive the immune cell metabolism and use it for heat generation instead (61). There is also evidence that NE released by ST-housed mice may act directly on immune cells bearing the β-AR and modulate their response. For example, MDSCs proliferate in greater number at ST in response to β-AR activation and change the outcome of cancer and host vs graft disease progression by suppressing T cell proliferation (76, 77). Murine macrophages have been shown to be impacted by ST in various ways. Studies have shown that ST is associated with M2 type macrophage differentiation and others have shown pro-inflammatory macrophage response (i.e. MCP-1, IFN-γ, TNFα, IL-1β, IL-6, IL-4, IL-13) (73-75). By contrast, in animals at TT, macrophages produced lower amounts of these pro-inflammatory cytokines and were producing tissue-repair associated immune factors (i.e.HMGB1) (86).

Based on these *in vivo* observations, we decided to conduct an *in vitro* macrophage stimulation study and measured the IR of macrophages to infectious agents at FT, TT and ST by stimulating them with LPS in incubators set at three different temperatures and measuring their nitric oxide and cytokine release. We showed that in general, the nitric oxide and cytokine concentrations were highest at FT, followed by TT and then ST after stimuli with the bacterial agent LPS. These data are in line with studies conducted on immune cells in culture where reduced activities were

observed at lower temperatures and with *in vivo* models where the inflammatory response to LPS was higher at TT (103, 104, 141-143). One exception was TNF-α which had the highest concentration at ST and lowest concentration at FT. This might be explained by the fact thar this cytokine is typically released by cells when they are cold-stressed and their release is switched off once febrile temperatures are reached (146, 147).

One limitation of this study with regard to its applicability to Malaria is that we did not test the response of macrophages to malarial agents at ST, TT and FT. Because cultured macrophages respond differently to malarial vs bacterial endotoxin agents, our results might have diverged if we stimulated them with nHZ (28). Thus, a future direction would be to test the response of these macrophages at different environmental temperatures by stimulating them with nHZ. Based on the results we obtained with LPS, stimulated cells would likely release more pro-inflammatory cytokines and NO at FT and TT than ST but TNF-α may also act differently at TT.

In addition, the exact mechanism by which temperature influences the macrophage metabolism is not known. Two approaches could be pursued to determine the metabolic response to viral, bacterial and parasitic agents: a metabolomics approach would allow to understand the effect of environmental temperature on the metabolism of these cells. A proteomics analysis to investigate which proteins are down or up-regulated at these three different temperatures could also provide further insights on the exact mechanisms implicated in the response at ST, TT and FT.

Finally, TTs have a more complex effect in a multicellular organism like mice than cell-lines since it acts on multiple cell types at once. This might also explain why the results we obtained from our *in vitro* experiments showed an increased IR at TT while our *in vivo* results study showed a suppressed IR at TT.

2.6.2. In vivo IR to parasitic agents at thermoneutrality

Thermoneutral temperatures have shown to change the IR to infectious agents and parasitic diseases (61). In rodents injected IP with bacteria or bacterial agents, thermoneutrality increased the IR with more neutrophil infiltration at TT without major changes in the release of pro-inflammatory cytokines (103, 104). In viral infections, TT has been shown to reduce pro-inflammatory cytokine levels and leukocyte infiltration (62, 100, 101). In parasitic diseases, TT may be protective by changing the IR of the host to parasitic agents. Some cells are present in

greater number at the site of infection with *T. cruzi* and *L. mexicanum* (109, 111, 112). However, results describing IR to nHZ and/or *L. major* using a peritonitis model at thermoneutrality were never reported to our knowledge. In our IR study, we showed that lower amounts of inflammatory cells were recruited and that some pro-inflammatory cytokines showed a trend of lower concentration in the PEC after injection with parasitic agents. Those results differ from results obtained at TT in mice injected with LPS in a peritonitis model but aligns with viral and some parasitic infections models obtained at TT (62, 100, 103, 104, 109, 111, 112). Although many of our results are not statistically significant, they show trends on the impact of thermoneutrality on the IR to parasitic diseases aligned with other relevant mice models and provide direction for further investigations.

One possible future direction would be using flow-cytometry to phenotype the infiltrating cells inside the PEC to unravel the roles of other cell population that might have be involved in dampening the host IR at TT. For example, regulatory cells like γδT cells, MDSCs and Tregs which have all been shown to play a role in dampening the immune response to malaria might have been recruited to the PEC in different proportions during our injections at TT vs ST (15, 26, 154). This would also help identify if any early adaptive immunity mechanisms are involved in the differential response to parasitic agents at TT compared to ST. Finally, analyzing the transcriptomics of inflammatory cells in the PEC of mice would help determine which genes are upregulated or downregulated at different environmental temperatures.

In addition, this study is the first one to our knowledge where the release of EVs upon parasitic IP injection is reported at TT. Traditional means of EV detection (i.e. TEM and NTA) were used as well as a novel method of EV phenotyping (i.e. hsFC) that allows for differentiation of EVs derived from specific cell types through detection of unique markers (137, 138). We showed an overall trend of lower concentration of EVs in the PEC of mice at TT vs ST which aligns with our results of lower recruitment of inflammatory cells at the site of injection. Although we did not observe any significant temperature-based difference for most of the EV populations we surveyed (i.e. Leukocyte-derived, Proteasome-derived, Neutrophil-derived, Platelet-derived), we were able to show for the first time that they were released in the PEC of mice at both temperatures, upon injection with parasitic agents. Platelets are not amongst the cells recruited to the PEC during inflammation, yet we detected the presence of platelet-derived EVs. This

infiltration of platelet EVs to the site of inflammation has been shown before in rheumatoid arthritis but never int the case of malaria (155). These results provide further evidence that EVs are important modulatory factors in the IR to parasitic agents. In the future, analyzing the protein cargo of these EVs by proteomics would give further insight on the potential roles of EVs in the IR of malaria and other parasitic diseases at thermoneutrality. These methods would allow us to determine if there are any unique proteins involved in biological processes that are carried by EVs of mice housed at TT compared to ST, or if some proteins are present in greater or lower amounts at either temperatures.

2.6.3. In vivo disease progression of severe malaria at thermoneutrality

Thermoneutral temperatures have been shown to influence the disease progression and outcome in a variety of disease models (61). For some viral diseases and parasitic infections, thermoneutrality has been shown to be protective (62, 100, 101, 111, 112). Particularly, mice infected with *T. cruzi* had an increased survivability and decreased symptoms (111). There are no studies reported on the impact of thermoneutrality on severe malaria. However, early experiments investigating the role of temperature on malaria disease evolution can give us an insight on how thermoneutrality might impact the severe malaria model (107, 108, 110). In our disease progression study, we showed that mice infected with PbA showed a trend in increased survivability which is different from a study where PbA-infected mice housed at different temperatures including 27C had higher mortality rates including when housed individually (110). However, in this study, mice weren't acclimatized for three weeks as in our study. During our acclimatization period mice at TT gained slightly more body weight than those at ST which might have played a protective role during the disease progression as the mice did not experience as dramatic loss of a body weight and body temperature compared to their ST counterparts. In addition, the trend of decreased parasite sequestration in the organs of mice at TT might also explain why the disease seemed less severe.

When we investigated the cytokine levels in serum of mice at end-point at TT and ST we did not observe a difference between housing temperatures. However, regular blood sampling throughout the course of the disease should be considered in future experiments to determine whether they could play a role during the disease progression.

Although we did not observe any effect of thermoneutrality on the parasite blood stage life-cycle by microscopy, we cannot exclude that housing temperature might have an effect on the *Plasmodium* parasite itself. A transcriptomics analysis of PbA-infected mice blood should be considered in the future to determine if certain parasite-specific genes associated with parasite growth would be up- or down-regulated at TT.

In addition, since our PbA-infected mice succumbed to ECM, future experiments conducted at thermoneutrality should consider analysing the brain pathology of these mice to determine if there is any difference in BBB breakdown and/or immune cell infiltration at TT compared to ST. Finally, flow-cytometry could be used to survey the immune cell populations present in various organs, specifically the spleen to elucidate whether they are influenced by housing temperature.

2.6.4. The case for a thermoneutral mouse model of malaria

Altogether, our results give us insight on how thermoneutrality might impact the disease progression of a severe mouse malaria model and directions for future more targeted investigations. Since the thermoneutral mouse model more closely resembles the metabolic state of humans, having an idea of how malaria develops in those mice might improve the accuracy of our mouse model to reflect how the disease progresses in humans. And as such investigating vaccine candidates or targets for therapeutics in mice housed at TT might offer better translatability to human trials which is important in our continued fight against this disease.

3. Concluding remarks

Malaria continues to cause significant mortality in endemic regions. Despite efforts to eliminate the disease, the 2020 targets set by the WHO for its eradication were not reached due to several factors, including the COVID-19 epidemic and rise of control-resistant *Plasmodium* parasites and anopheline vectors. To continue the battle against this neglected disease, a better understanding is needed of the host immune response, particularly during the parasitic blood-stage when the majority of symptoms occur. To this effect, mouse models of malarial disease can be employed to study the immune response as well as overall disease progression. One major limitation of studies in mouse models is that they commonly use animals housed in conditions below their thermoneutral temperature. Sub-optimal housing temperatures influence mouse metabolism, which can change the outcome of disease. This can have an impact on clinical translatability because humans generally exist at TT. This thesis summarizes the relevant literature on thermoneutrality in the context of various diseases and particularly in the context of malaria and its associated IR. In existing literature, TT has been shown to either suppress or exacerbate disease symptoms in different models. Experiments conducted for this project demonstrate that TT can exacerbate the IR of BMDMs stimulated with infectious agents by increasing their cytokine and NO release. However, the IR of mice injected with parasitic agents in a peritonitis model was suppressed, as evidenced by a decrease in inflammatory cell recruitment to the PEC. We also conducted experiments with PbA infection, which suggested that TT may affect disease progression in the severe malaria mouse model by suppressing some symptoms without having an effect on parasite biology itself. Overall, our studies show that TT has an effect on the metabolism of immune cells and may suppress malaria disease presentation by lowering the IR to parasitic agents. However, many of our results were not statistically significant, possibly due to limited experimental group sizes. Confirmation of the trends observed in our studies should be carried out by repeating the experiments with a greater number of animals. This would also allow us to investigate the role of thermoneutrality in the context of malaria using additional types of analyses such as transcriptomics, metabolomics, and proteomics of various tissues from infected animals. Such analyses could lead to the identification of pathways that are modulated in the IR or disease progression of malaria at TT. Improving the translatability of mouse models of severe malaria disease constitutes a first step in finding therapeutic targets for the control and, ultimately, elimination of this disease.

REFERENCES

1. Oakley MS, Kumar S, Anantharaman V, Zheng H, Mahajan B, Haynes JD, et al. Molecular factors and biochemical pathways induced by febrile temperature in intraerythrocytic *Plasmodium falciparum* parasites. Infect Immun. 2007;75(4):2012-25.
2. Malaria: Center for Disease Control and Prevention; 2021 [updated November 2019. Available from: https://www.cdc.gov/parasites/malaria/index.html.
3. Malaria factsheet: World Health Organization; 2021 [Available from: https://www.who.int/news-room/fact-sheets/detail/malaria.
4. World malaria report 2020. World Health Organization; 2020.
5. Endo N, Yamana T, Eltahir EAB. Impact of climate change on malaria in Africa: a combined modelling and observational study. The Lancet. 2017;389.
6. Shio MT, Kassa FA, Bellemare MJ, Olivier M. Innate inflammatory response to the malarial pigment hemozoin. Microbes Infect. 2010;12(12-13):889-99.
7. Sampaio NG, Cheng L, Eriksson EM. The role of extracellular vesicles in malaria biology and pathogenesis. Malaria Journal. 2017;16(1):245.
8. Mantel P-Y, Marti M. The role of extracellular vesicles in *Plasmodium* and other protozoan parasites. Cellular Microbiology. 2014;16(3):344-54.
9. Coban C, Lee MSJ, Ishii KJ. Tissue-specific immunopathology during malaria infection. Nat Rev Immunol. 2018;18(4):266-78.
10. Robbins PD, Morelli AE. Regulation of immune responses by extracellular vesicles. Nature Reviews Immunology. 2014;14(3):195-208.
11. Deen JL, Von Seidlein L, Dondorp A. Therapy of uncomplicated malaria in children: a review of treatment principles, essential drugs and current recommendations. Tropical Medicine & International Health. 2008;13(9):1111-30.
12. Abdul-Ghani R, Beier JC. Strategic use of antimalarial drugs that block falciparum malaria parasite transmission to mosquitoes to achieve local malaria elimination. Parasitology Research. 2014;113(10):3535-46.
13. Severe malaria. Tropical Medicine & International Health. 2014;19(s1):7-131.
14. Rogerson SJ, Hviid L, Duffy PE, Leke RFG, Taylor DW. Malaria in pregnancy: pathogenesis and immunity. The Lancet Infectious Diseases. 2007;7(2):105-17.
15. Deroost K, Pham T-T, Opdenakker G, Van den Steen PE. The immunological balance between host and parasite in malaria. FEMS Microbiology Reviews. 2015;40(2):208-57.
16. Farrington L, Vance H, Rek J, Prahl M, Jagannathan P, Katureebe A, et al. Both inflammatory and regulatory cytokine responses to malaria are blunted with increasing age in highly exposed children. Malaria Journal. 2017;16(1):499.
17. Degarege A, Gebrezgi MT, Ibanez G, Wahlgren M, Madhivanan P. Effect of the ABO blood group on susceptibility to severe malaria: A systematic review and meta-analysis. Blood Reviews. 2019;33:53-62.
18. Herrero MD, Rivas P, Rallón NI, Ramírez-Olivencia G, Puente S. HIV and malaria. AIDS reviews. 2007;9(2):88-98.
19. Coban C, Lee MSJ, Ishii KJ. Tissue-specific immunopathology during malaria infection. Nature Reviews Immunology. 2018;18(4):266-78.
20. Mantel P-Y, Hjelmqvist D, Walch M, Kharoubi-Hess S, Nilsson S, Ravel D, et al. Infected erythrocyte-derived extracellular vesicles alter vascular function via regulatory Ago2-miRNA complexes in malaria. Nature Communications. 2016;7(1):1-15.

21. Shrivastava SK, Dalko E, Delcroix-Genete D, Herbert F, Cazenave P-A, Pied S. Uptake of parasite-derived vesicles by astrocytes and microglial phagocytosis of infected erythrocytes may drive neuroinflammation in cerebral malaria. Glia. 2017;65(1):75-92.
22. Mehaffy C, Kruh-Garcia Nicole A, Graham B, Jarlsberg Leah G, Willyerd Charis E, Borisov A, et al. Identification of *Mycobacterium tuberculosis* peptides in serum extracellular vesicles from persons with latent tuberculosis infection. Journal of Clinical Microbiology.58(6):393-20.
23. Schorey JS, Cheng Y, Singh PP, Smith VL. Exosomes and other extracellular vesicles in host–pathogen interactions. EMBO reports. 2015;16(1):24-43.
24. Madeira RP, Dal'Mas Romera LM, de Cássia Buck P, Mady C, Ianni BM, Torrecilhas AC. New biomarker in Chagas disease: extracellular vesicles isolated from peripheral blood in chronic Chagas disease patients modulate the human immune response. Journal of Immunology Research. 2021;2021:1-14.
25. Driedonks TAP, van der Grein SG, Ariyurek Y, Buermans HPJ, Jekel H, Chow FWN, et al. Immune stimuli shape the small non-coding transcriptome of extracellular vesicles released by dendritic cells. Cellular and Molecular Life Sciences. 2018;75(20):3857-75.
26. Stevenson MM, Riley EM. Innate immunity to malaria. Nature Reviews Immunology. 2004;4(3):169-80.
27. Jaramillo M, Gowda DC, Radzioch D, Olivier M. Hemozoin increases IFN-gamma-inducible macrophage nitric oxide generation through extracellular signal-regulated kinase- and NF-kappa B-dependent pathways. J Immunol. 2003;171(8):4243-53.
28. Scragg IG, Hensmann M, Bate CAW, Kwiatkowski D. Early cytokine induction by *Plasmodium falciparum* is not a classical endotoxin-like process. European Journal of Immunology. 1999;29(8):2636-44.
29. Jakobsen PH, Bate CAW, Taverne J, Playfair JHL. Malaria: toxins, cytokines and disease. Parasite Immunology. 1995;17(5):223-31.
30. Dinarello CA. Cytokines as endogenous pyrogens. J Infect Dis. 1999;179 Suppl 2:S294-304.
31. Arango Duque G, Descoteaux A. Macrophage cytokines: involvement in immunity and infectious diseases. Frontiers in immunology. 2014;5:491-.
32. Van Den Ham KM, Shio MT, Rainone A, Fournier S, Krawczyk CM, Olivier M. Iron prevents the development of experimental cerebral malaria by attenuating CXCR3-mediated T cell chemotaxis. PloS one. 2015;10(3):e0118451.
33. Requena P, Campo JJ, Umbers AJ, Ome M, Wangnapi R, Barrios D, et al. Pregnancy and malaria exposure are associated with changes in the B cell pool and in plasma eotaxin levels. The Journal of Immunology. 2014;193(6):2971.
34. Amani V, Boubou MI, Pied S, Marussig M, Walliker D, Mazier D, et al. Cloned lines of *Plasmodium berghei* ANKA differ in their abilities to induce experimental cerebral malaria. Infect Immun. 1998;66(9):4093-9.
35. de Oca MM, Engwerda C, Haque A. *Plasmodium berghei* ANKA (PbA) infection of C57BL/6J mice: a model of severe malaria. Methods Mol Biol. 2013;1031:203-13.
36. Stevenson MM, Gros P, Olivier M, Fortin A, Serghides L. Cerebral malaria: human versus mouse studies. Trends in Parasitology. 2010;26(6):274-5.
37. Fernandez JU, Millrine D, Jones SA. Tracking competent host defence to chronic inflammation: an *in vivo* model of peritonitis. In: Jenkins BJ, editor. Inflammation and Cancer: Methods and Protocols. New York, NY: Springer New York; 2018. p. 65-75.

38. Kiessling S, Dubeau-Laramée G, Ohm H, Labrecque N, Olivier M, Cermakian N. The circadian clock in immune cells controls the magnitude of *Leishmania* parasite infection. Sci Rep. 2017;7(1):10892.
39. Loiseau PM, Mbongo N, Bories C, Boulard Y, Craciunescu DG. In vivo antileishmanial action of Ir-(COD)-pentamidine tetraphenylborate on *Leishmania donovani* and *Leishmania major* mouse models. Parasite [Internet]. 2000; 7(2):[103-8 pp.].
40. Leishmaniasis factsheet: World Health Organization; 2021 [Available from: https://www.who.int/news-room/fact-sheets/detail/leishmaniasis.
41. Leishmaniasis: Center for Disease Control and Prevention; 2020 [Available from: https://www.cdc.gov/parasites/leishmaniasis/biology.html.
42. Gordon CJ. Thermal physiology of laboratory mice: defining thermoneutrality. Journal of Thermal Biology. 2012;37(8):654-85.
43. Poczopko P. Studies on the effect of environmental thermal conditions on geese: deep and external body temperature in goslings at thermoneutrality and during short exposures to cold. Acta Physiol Pol. 1968;19(5):719-35.
44. Maloney SK, Fuller A, Mitchell D, Gordon C, Overton JM. Translating animal model research: does it matter that our rodents are cold? Physiology (Bethesda). 2014;29(6):413-20.
45. Hylander BL, Repasky EA. Thermoneutrality, mice, and cancer: a heated opinion. Trends Cancer. 2016;2(4):166-75.
46. Gordon CJ. The mouse thermoregulatory system: its impact on translating biomedical data to humans. Physiol Behav. 2017;179:55-66.
47. Overton JM. Phenotyping small animals as models for the human metabolic syndrome: thermoneutrality matters. Int J Obes (Lond). 2010;34 Suppl 2:S53-8.
48. Kingma B, Frijns A, van Marken Lichtenbelt W. The thermoneutral zone: implications for metabolic studies. Front Biosci (Elite Ed). 2012;4:1975-85.
49. Ganeshan K, Chawla A. Warming the mouse to model human diseases. Nat Rev Endocrinol. 2017;13(8):458-65.
50. Landis SC, Amara SG, Asadullah K, Austin CP, Blumenstein R, Bradley EW, et al. A call for transparent reporting to optimize the predictive value of preclinical research. Nature. 2012;490(7419):187-91.
51. Richter SH, Garner JP, Würbel H. Environmental standardization: cure or cause of poor reproducibility in animal experiments? Nat Methods. 2009;6(4):257-61.
52. Cannon B, Nedergaard J. Nonshivering thermogenesis and its adequate measurement in metabolic studies. The Journal of Experimental Biology. 2011;214(2):242-53.
53. Troublesome variability in mouse studies. Nat Neurosci. 2009;12(9):1075.
54. Karp CL. Unstressing intemperate models: how cold stress undermines mouse modeling. J Exp Med. 2012;209(6):1069-74.
55. Bass J, Lazar MA. Circadian time signatures of fitness and disease. Science. 2016;354(6315):994-9.
56. Longo VD, Panda S. Fasting, circadian rhythms, and time-restricted feeding in healthy lifespan. Cell metabolism. 2016;23(6):1048-59.
57. Turner PV. The role of the gut microbiota on animal model reproducibility. Animal Models and Experimental Medicine. 2018;1(2):109-15.

58. Gordon CJ, Aydin C, Repasky EA, Kokolus KM, Dheyongera G, Johnstone AFM. Behaviorally mediated, warm adaptation: a physiological strategy when mice behaviorally thermoregulate. Journal of Thermal Biology. 2014;44:41-6.
59. Okon EE. The effect of environmental temperature on the production of ultrasounds by isolated non-handled albino mouse pups. Journal of Zoology. 1970;162(1):71-83.
60. Gaskill BN, Rohr SA, Pajor EA, Lucas JR, Garner JP. Some like it hot: mouse temperature preferences in laboratory housing. Applied Animal Behaviour Science. 2009;116(2):279-85.
61. Vialard F, Olivier M. Thermoneutrality and immunity: how does cold stress affect disease? Frontiers in Immunology. 2020;11(3031).
62. Jhaveri KA, Trammell RA, Toth LA. Effect of environmental temperature on sleep, locomotor activity, core body temperature and immune responses of C57BL/6J mice. Brain Behav Immun. 2007;21(7):975-87.
63. Kaiyala KJ, Morton GJ, Thaler JP, Meek TH, Tylee T, Ogimoto K, et al. Acutely decreased thermoregulatory energy expenditure or decreased activity energy expenditure both acutely reduce food intake in mice. PLoS One. 2012;7(8):e41473.
64. Lo Martire V, Silvani A, Bastianini S, Berteotti C, Zoccoli G. Effects of ambient temperature on sleep and cardiovascular regulation in mice: the role of hypocretin/orexin neurons. PLoS One. 2012;7(10):e47032.
65. David JM, Chatziioannou AF, Taschereau R, Wang H, Stout DB. The hidden cost of housing practices: using noninvasive imaging to quantify the metabolic demands of chronic cold stress of laboratory mice. Comp Med. 2013;63(5):386-91.
66. Jun JC, Shin MK, Yao Q, Devera R, Fonti-Bevans S, Polotsky VY. Thermoneutrality modifies the impact of hypoxia on lipid metabolism. Am J Physiol Endocrinol Metab. 2013;304(4):E424-35.
67. Eng JW, Reed CB, Kokolus KM, Pitoniak R, Utley A, Bucsek MJ, et al. Housing temperature-induced stress drives therapeutic resistance in murine tumour models through beta2-adrenergic receptor activation. Nat Commun. 2015;6:6426.
68. Iwaniec UT, Philbrick KA, Wong CP, Gordon JL, Kahler-Quesada AM, Olson DA, et al. Room temperature housing results in premature cancellous bone loss in growing female mice: implications for the mouse as a preclinical model for age-related bone loss. Osteoporosis International. 2016;27(10):3091-101.
69. Szentirmai E, Kapas L. Sleep and body temperature in TNFalpha knockout mice: the effects of sleep deprivation, beta3-AR stimulation and exogenous TNFalpha. Brain Behav Immun. 2019;81:260-71.
70. Leigh ND, Kokolus KM, O'Neill RE, Du W, Eng JW, Qiu J, et al. Housing temperature-induced stress is suppressing murine graft-versus-host disease through beta2-adrenergic receptor signaling. J Immunol. 2015;195(10):5045-54.
71. Xiao C, Goldgof M, Gavrilova O, Reitman ML. Anti-obesity and metabolic efficacy of the beta3-adrenergic agonist, CL316243, in mice at thermoneutrality compared to 22 degrees C. Obesity (Silver Spring). 2015;23(7):1450-9.
72. Eng JW, Reed CB, Kokolus KM, Repasky EA. Housing temperature influences the pattern of heat shock protein induction in mice following mild whole body hyperthermia. Int J Hyperthermia. 2014;30(8):540-6.
73. Ruiz de Azua I, Mancini G, Srivastava RK, Rey AA, Cardinal P, Tedesco L, et al. Adipocyte cannabinoid receptor CB1 regulates energy homeostasis and alternatively activated macrophages. J Clin Invest. 2017;127(11):4148-62.

74. Bond LM, Burhans MS, Ntambi JM. Uncoupling protein-1 deficiency promotes brown adipose tissue inflammation and ER stress. PLoS One. 2018;13(11):e0205726.
75. Nguyen KD, Qiu Y, Cui X, Goh YP, Mwangi J, David T, et al. Alternatively activated macrophages produce catecholamines to sustain adaptive thermogenesis. Nature. 2011;480(7375):104-8.
76. Mohammadpour H, MacDonald CR, Qiao G, Chen M, Dong B, Hylander BL, et al. β2 adrenergic receptor–mediated signaling regulates the immunosuppressive potential of myeloid-derived suppressor cells. The Journal of Clinical Investigation. 2019;129(12):5537-52.
77. Mohammadpour H, Sarow JL, Chen GL, MacDonald CR, Sharma U, Herr MM, et al. β2-adrenergic signaling regulates graft versus host disease after allogenic transplantation while preserving graft versus leukemia effect. Blood. 2019;134(Supplement_1):1915-.
78. Lee MW, Odegaard JI, Mukundan L, Qiu Y, Molofsky AB, Nussbaum JC, et al. Activated type 2 innate lymphoid cells regulate beige fat biogenesis. Cell. 2015;160(1-2):74-87.
79. Rudaya AY, Steiner AA, Robbins JR, Dragic AS, Romanovsky AA. Thermoregulatory responses to lipopolysaccharide in the mouse: dependence on the dose and ambient temperature. Am J Physiol Regul Integr Comp Physiol. 2005;289(5):R1244-52.
80. Wanner SP, Yoshida K, Kulchitsky VA, Ivanov AI, Kanosue K, Romanovsky AA. Lipopolysaccharide-induced neuronal activation in the paraventricular and dorsomedial hypothalamus depends on ambient temperature. PLoS One. 2013;8(9):e75733.
81. Carroll JA, Burdick NC, Chase CC, Jr., Coleman SW, Spiers DE. Influence of environmental temperature on the physiological, endocrine, and immune responses in livestock exposed to a provocative immune challenge. Domest Anim Endocrinol. 2012;43(2):146-53.
82. Peloso ED, Florez-Duquet M, Buchanan JB, Satinoff E. LPS fever in old rats depends on the ambient temperature. Physiol Behav. 2003;78(4-5):651-4.
83. Buchanan JB, Peloso E, Satinoff E. A warmer ambient temperature increases the passage of interleukin-1beta into the brains of old rats. Am J Physiol Regul Integr Comp Physiol. 2008;295(1):R361-8.
84. Ganeshan K, Nikkanen J, Man K, Leong YA, Sogawa Y, Maschek JA, et al. Energetic trade-offs and hypometabolic states promote disease tolerance. Cell. 2019;177(2):399-413.e12.
85. Hamzic N, Blomqvist A, Nilsberth C. Immune-induced expression of lipocalin-2 in brain endothelial cells: relationship with interleukin-6, cyclooxygenase-2 and the febrile response. J Neuroendocrinol. 2013;25(3):271-80.
86. Personnaz J, Piccolo E, Branchereau M, Filliol A, Paccoud R, Moreau E, et al. Macrophage-derived HMGB1 is dispensable for tissue fibrogenesis. FASEB Bioadv. 2019;1(4):227-45.
87. Clayton ZS, McCurdy CE. Short-term thermoneutral housing alters glucose metabolism and markers of adipose tissue browning in response to a high-fat diet in lean mice. Am J Physiol Regul Integr Comp Physiol. 2018;315(4):R627-r37.
88. Stemmer K, Kotzbeck P, Zani F, Bauer M, Neff C, Muller TD, et al. Thermoneutral housing is a critical factor for immune function and diet-induced obesity in C57BL/6 nude mice. Int J Obes (Lond). 2015;39(5):791-7.
89. Goldgof M, Xiao C, Chanturiya T, Jou W, Gavrilova O, Reitman ML. The chemical uncoupler 2,4-dinitrophenol (DNP) protects against diet-induced obesity and improves energy homeostasis in mice at thermoneutrality. J Biol Chem. 2014;289(28):19341-50.

90. Ravussin Y, LeDuc CA, Watanabe K, Leibel RL. Effects of ambient temperature on adaptive thermogenesis during maintenance of reduced body weight in mice. Am J Physiol Regul Integr Comp Physiol. 2012;303(4):R438-48.
91. Tian XY, Ganeshan K, Hong C, Nguyen KD, Qiu Y, Kim J, et al. Thermoneutral housing accelerates metabolic inflammation to potentiate atherosclerosis but not insulin resistance. Cell Metab. 2016;23(1):165-78.
92. Giles DA, Moreno-Fernandez ME, Stankiewicz TE, Graspeuntner S, Cappelletti M, Wu D, et al. Thermoneutral housing exacerbates nonalcoholic fatty liver disease in mice and allows for sex-independent disease modeling. Nat Med. 2017;23(7):829-38.
93. Chevalier C, Stojanović O, Colin DJ, Suarez-Zamorano N, Tarallo V, Veyrat-Durebex C, et al. Gut microbiota orchestrates energy homeostasis during cold. Cell. 2015;163(6):1360-74.
94. Kokolus KM, Capitano ML, Lee CT, Eng JW, Waight JD, Hylander BL, et al. Baseline tumor growth and immune control in laboratory mice are significantly influenced by subthermoneutral housing temperature. Proc Natl Acad Sci U S A. 2013;110(50):20176-81.
95. Kokolus KM, Spangler HM, Povinelli BJ, Farren MR, Lee KP, Repasky EA. Stressful presentations: mild cold stress in laboratory mice influences phenotype of dendritic cells in naive and tumor-bearing mice. Front Immunol. 2014;5:23.
96. Hylander BL, Eng JW, Repasky EA. The impact of housing temperature-induced chronic stress on preclinical mouse tumor models and therapeutic responses: an important role for the nervous system. Adv Exp Med Biol. 2017;1036:173-89.
97. Hylander BL, Gordon CJ, Repasky EA. Manipulation of ambient housing temperature to study the impact of chronic stress on immunity and cancer in mice. J Immunol. 2019;202(3):631-6.
98. MacDonald CR, Bucsek MJ, Qiao G, Chen M, Evans L, Greenberg DJ, et al. Adrenergic receptor signaling regulates the response of tumors to ionizing radiation. Radiat Res. 2019;191(6):585-9.
99. Qiao G, Chen M, Bucsek MJ, Repasky EA, Hylander BL. Adrenergic signaling: a targetable checkpoint limiting development of the antitumor immune response. Front Immunol. 2018;9:164.
100. Bell JF, Moore GJ. Effects of high ambient temperature on various stages of rabies virus infection in mice. Infect Immun. 1974;10(3):510-5.
101. Underwood GE, Baker CA, Weed SD. Protective effect of elevated temperature on mice infected with Coe virus. J Immunol. 1966;96(6):1006-12.
102. Hu Y, Jin H, Du X, Xiao C, Luo D, Wang B, et al. Effects of chronic heat stress on immune responses of the foot-and-mouth disease DNA vaccination. DNA Cell Biol. 2007;26(8):619-26.
103. Liu E, Lewis K, Al-Saffar H, Krall CM, Singh A, Kulchitsky VA, et al. Naturally occurring hypothermia is more advantageous than fever in severe forms of lipopolysaccharide- and *Escherichia coli*-induced systemic inflammation. American Journal of Physiology-Regulatory, Integrative and Comparative Physiology. 2012;302(12):R1372-R83.
104. Previte JJ, Berry LJ. The effect of environmental temperature on the host-parasite relationship in mice. The Journal of Infectious Diseases. 1962;110(3):201-9.
105. Moragues V, Pinkerton H. Variation in morbidity and mortality of murine typhus infection in mice with changes in the environmental temperature. J Exp Med. 1944;79(1):41-3.
106. Rubin RL. Mice housed at elevated vivarium temperatures display enhanced T-cell response and survival to *Francisella tularensis*. Comp Med. 2017;67(6):491-7.

107. McQuisition TE. Effect of temperature and clofibrate on *Plasmodium berghei* infection in mice. Am J Trop MedHyg. 1979;28(1):12-4.
108. Yoeli M, Young C, Jadin JB. Effects of lowered environmental temperature on the growth of exoerythrocytic stages of *Plasmodium berghei*. Am J Trop Med Hyg. 1975;24(5):769-75.
109. Quinonez-Diaz L, Mancilla-Ramirez J, Avila-Garcia M, Ortiz-Avalos J, Berron A, Gonzalez S, et al. Effect of ambient temperature on the clinical manifestations of experimental diffuse cutaneous leishmaniasis in a rodent model. Vector Borne Zoonotic Dis. 2012;12(10):851-60.
110. Plaut SM, Ader R, Friedman SB, Ritterson AL. Social factors and resistance to malaria in the mouse: effects of group vs individual housing on resistance to *Plasmodium berghei* infection. Psychosom Med. 1969;31(6):536-52.
111. Amrein YU. Effects of environmental temperature on *Trypanosoma cruzi* infection in mice. The Journal of Parasitology. 1967;53(6):1160-.
112. Ming Z, Davis CD. CD8+ T lymphocytes required for enhanced survival of *Trypanosoma cruzi*-infected mice at elevated environmental temperature. J Parasitol. 2003;89(3):630-2.
113. Liao W, Zhou L, Zhao X, Song L, Lu Y, Zhong N, et al. Thermoneutral housing temperature regulates T-regulatory cell function and inhibits ovabumin-induced asthma development in mice. Sci Rep. 2017;7(1):7123.
114. Vandal M, White PJ, Tournissac M, Tremblay C, St-Amour I, Drouin-Ouellet J, et al. Impaired thermoregulation and beneficial effects of thermoneutrality in the 3xTg-AD model of Alzheimer's disease. Neurobiol Aging. 2016;43:47-57.
115. David JM, Knowles S, Lamkin DM, Stout DB. Individually ventilated cages impose cold stress on laboratory mice: a source of systemic experimental variability. J Am Assoc Lab Anim Sci. 2013;52(6):738-44.
116. Lee B, Kim G, Jo Y, Lee B, Shin YI, Hong C. Aquatic exercise at thermoneutral water temperature enhances antitumor immune responses. Immune Netw. 2019;19(2):e10.
117. Povinelli BJ, Kokolus KM, Eng JW, Dougher CW, Curtin L, Capitano ML, et al. Standard sub-thermoneutral caging temperature influences radiosensitivity of hematopoietic stem and progenitor cells. PLoS One. 2015;10(3):e0120078.
118. Bell RR. Exercise and Diet-Induced Obesity in Mice. In: Owen JB, Treasure JL, Collier DA, editors. Animal models — disorders of eating behaviour and body composition. Dordrecht: Springer Netherlands; 2001. p. 97-116.
119. Boon MR, van den Berg SA, Wang Y, van den Bossche J, Karkampouna S, Bauwens M, et al. BMP7 activates brown adipose tissue and reduces diet-induced obesity only at subthermoneutrality. PLoS One. 2013;8(9):e74083.
120. Castillo M, Hall JA, Correa-Medina M, Ueta C, Kang HW, Cohen DE, et al. Disruption of thyroid hormone activation in type 2 deiodinase knockout mice causes obesity with glucose intolerance and liver steatosis only at thermoneutrality. Diabetes. 2011;60(4):1082-9.
121. Feldmann HM, Golozoubova V, Cannon B, Nedergaard J. UCP1 ablation induces obesity and abolishes diet-induced thermogenesis in mice exempt from thermal stress by living at thermoneutrality. Cell Metab. 2009;9(2):203-9.
122. Klaus S, Munzberg H, Truloff C, Heldmaier G. Physiology of transgenic mice with brown fat ablation: obesity is due to lowered body temperature. Am J Physiol. 1998;274(2):R287-93.
123. Liu X, Rossmeisl M, McClaine J, Riachi M, Harper M-E, Kozak LP. Paradoxical resistance to diet-induced obesity in UCP1-deficient mice. The Journal of clinical investigation. 2003;111(3):399-407.

124. Giles DA, Ramkhelawon B, Donelan EM, Stankiewicz TE, Hutchison SB, Mukherjee R, et al. Modulation of ambient temperature promotes inflammation and initiates atherosclerosis in wild type C57BL/6 mice. Mol Metab. 2016;5(11):1121-30.
125. Philbrick KA, Branscum AJ, Wong CP, Turner RT, Iwaniec UT. Leptin increases particle-induced osteolysis in female *ob/ob* mice. Sci Rep. 2018;8(1):14790.
126. Swoap SJ, Overton JM, Garber G. Effect of ambient temperature on cardiovascular parameters in rats and mice: a comparative approach. Am J Physiol Regul Integr Comp Physiol. 2004;287(2):R391-6.
127. Williams TD, Chambers JB, Henderson RP, Rashotte ME, Overton JM. Cardiovascular responses to caloric restriction and thermoneutrality in C57BL/6J mice. Am J Physiol Regul Integr Comp Physiol. 2002;282(5):R1459-67.
128. Overton JM, Williams TD, Chambers JB, Rashotte ME. Cardiovascular and metabolic responses to fasting and thermoneutrality are conserved in obese Zucker rats. Am J Physiol Regul Integr Comp Physiol. 2001;280(4):R1007-15.
129. Oakley MS, Gerald N, McCutchan TF, Aravind L, Kumar S. Clinical and molecular aspects of malaria fever. Trends Parasitol. 2011;27(10):442-9.
130. Hankenson FC, Marx JO, Gordon CJ, David JM. Effects of rodent thermoregulation on animal models in the research environment. Comp Med. 2018;68(6):425-38.
131. Messmer MN, Kokolus KM, Eng JW, Abrams SI, Repasky EA. Mild cold-stress depresses immune responses: implications for cancer models involving laboratory mice. Bioessays. 2014;36(9):884-91.
132. Speakman JR, Keijer J. Not so hot: optimal housing temperatures for mice to mimic the thermal environment of humans. Mol Metab. 2012;2(1):5-9.
133. Cannon B, Nedergaard J. Nonshivering thermogenesis and its adequate measurement in metabolic studies. The Journal of Experimental Biology. 2011;214(2):242.
134. Forget G, Siminovitch KA, Brochu S, Rivest S, Radzioch D, Olivier M. Role of host phosphotyrosine phosphatase SHP-1 in the development of murine leishmaniasis. Eur J Immunol. 2001;31(11):3185-96.
135. da Silva R, Sacks DL. Metacyclogenesis is a major determinant of *Leishmania* promastigote virulence and attenuation. Infect Immun. 1987;55(11):2802-6.
136. Soo CY, Song Y, Zheng Y, Campbell EC, Riches AC, Gunn-Moore F, et al. Nanoparticle tracking analysis monitors microvesicle and exosome secretion from immune cells. Immunology. 2012;136(2):192-7.
137. Rousseau M, Belleannee C, Duchez AC, Cloutier N, Levesque T, Jacques F, et al. Detection and quantification of microparticles from different cellular lineages using flow cytometry. Evaluation of the impact of secreted phospholipase A2 on microparticle assessment. PLoS One. 2015;10(1):e0116812.
138. Marcoux G, Duchez AC, Cloutier N, Provost P, Nigrovic PA, Boilard E. Revealing the diversity of extracellular vesicles using high-dimensional flow cytometry analyses. Sci Rep. 2016;6:35928.
139. Verdoes M, Florea BI, Menendez-Benito V, Maynard CJ, Witte MD, van der Linden WA, et al. A fluorescent broad-spectrum proteasome inhibitor for labeling proteasomes in vitro and in vivo. Chem Biol. 2006;13(11):1217-26.
140. Raz V, Raz Y, Paniagua-Soriano G, Roorda JC, Olie C, Riaz M, et al. Proteasomal activity-based probes mark protein homeostasis in muscles. J Cachexia Sarcopenia Muscle. 2017;8(5):798-807.

141. Akriotis V, Biggar WD. The effects of hypothermia on neutrophil function *in vitro*. Journal of Leukocyte Biology. 1985;37(1):51-61.
142. Broug-Holub E, Persoons JHA, Schornagel K, Mastbergen SC, Kraal G. Effects of stress on alveolar macrophages: a role for the sympathetic nervous system. American Journal of Respiratory Cell and Molecular Biology. 1998;19(5):842-8.
143. Salman H, Bergman M, Bessler H, Alexandrova S, Djaldetti M. Ultrastructure and phagocytic activity of rat peritoneal macrophages exposed to low temperatures *in vitro*. Cryobiology. 2000;41(1):66-71.
144. Banet M. Fever in mammals: is it beneficial? Yale J Biol Med. 1986;59(2):17–124.
145. Murphy PA, Hanson DF, Guo YN, Angster DE. The effects of variations in pH and temperature on the activation of mouse thymocytes by both forms of rabbit interleukin-1. Yale J Biol Med. 1985;58(2):115-23.
146. Hanson DF. Fever, temperature, and the immune response. Annals of the New York Academy of Sciences. 1997;813(1):453-64.
147. Mitchell HM, White DM, Domowicz MS, Kraig RP. Cold pre-conditioning neuroprotection depends on TNF-α and is enhanced by blockade of interleukin-11. J Neurochem. 2011;117(2):187-96.
148. Torrecilhas AC, Soares RP, Schenkman S, Fernández-Prada C, Olivier M. Extracellular vesicles in trypanosomatids: host cell communication. Frontiers in Cellular and Infection Microbiology. 2020:NA.
149. Olivier M, Minguez-Menendez A, Fernandez-Prada C. *Leishmania Viannia guyanensis*. Trends Parasitol. 2019;35(12):1018-9.
150. Douanne N, Dong G, Douanne M, Olivier M, Fernandez-Prada C. Unravelling the proteomic signature of extracellular vesicles released by drug-resistant *Leishmania infantum* parasites. PLoS Negl Trop Dis. 2020;14(7):e0008439.
151. Théry C, Witwer KW, Aikawa E, Alcaraz MJ, Anderson JD, Andriantsitohaina R, et al. Minimal information for studies of extracellular vesicles 2018 (MISEV2018): a position statement of the International Society for Extracellular Vesicles and update of the MISEV2014 guidelines. Journal of Extracellular Vesicles. 2018;7(1):1535750.
152. Kastelowitz N, Yin H. Exosomes and microvesicles: identification and targeting by particle size and lipid chemical probes. Chembiochem. 2014;15(7):923-8.
153. Dekel E, Yaffe D, Rosenhek-Goldian I, Ben-Nissan G, Ofir-Birin Y, Morandi MI, et al. 20S proteasomes secreted by the malaria parasite promote its growth. Nature communications. 2021;12(1):1172-.
154. Lamsfus Calle C, Fendel R, Singh A, Richie TL, Hoffman SL, Kremsner PG, et al. Expansion of functional myeloid-derived suppressor cells in controlled human malaria infection. Frontiers in immunology. 2021;12:625712.
155. Boilard E, Blanco P, Nigrovic PA. Platelets: active players in the pathogenesis of arthritis and SLE. Nat Rev Rheumatol. 2012;8(9):534-42.